

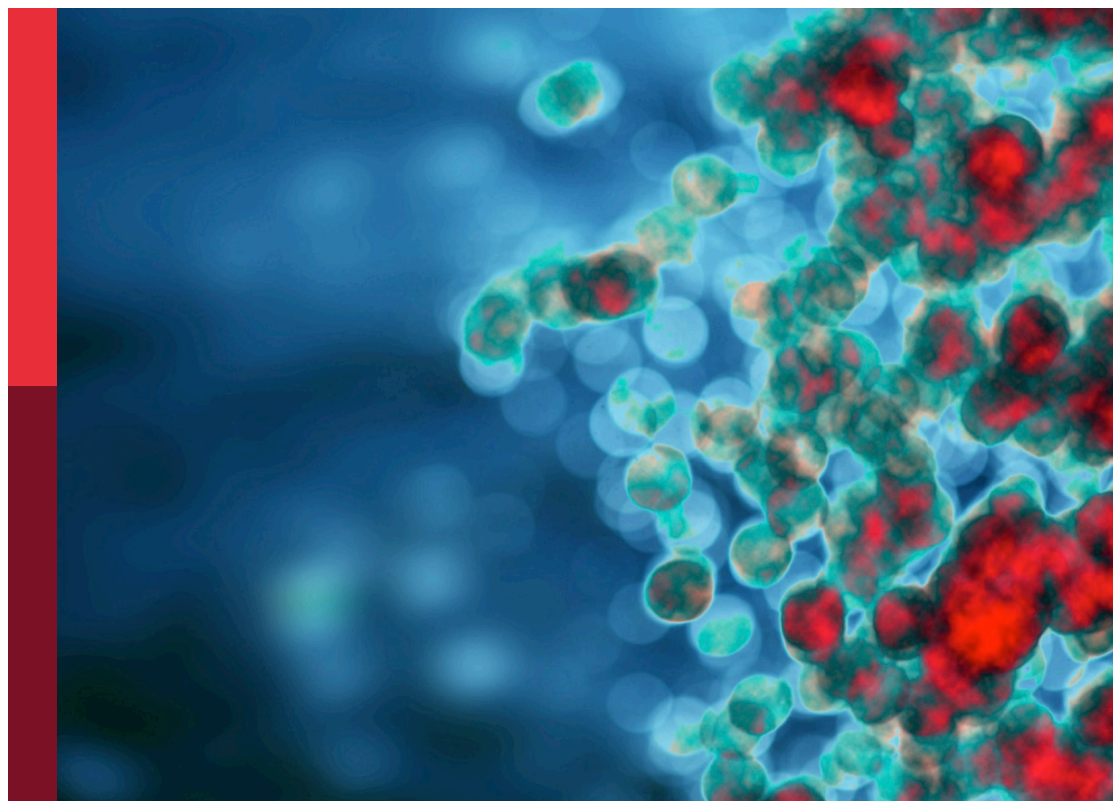
RNA modifications in cancer: unraveling roles and therapeutic potential in immunity and immunotherapy

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Published in

Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-8325-6848-4
DOI 10.3389/978-2-8325-6848-4

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RNA modifications in cancer: unraveling roles and therapeutic potential in immunity and immunotherapy

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Citation

Wang, Z., Liu, W., Dong, H., He, X., eds. (2025). *RNA modifications in cancer: unraveling roles and therapeutic potential in immunity and immunotherapy*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6848-4

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OPEN ACCESS

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RECEIVED 28 May 2024

ACCEPTED 05 August 2024

PUBLISHED 20 August 2024

CITATION

Liu J-X, Zhang X, Xu W-H and Hao X-D
(2024) The role of RNA modifications in
hepatocellular carcinoma: functional
mechanism and potential applications.
Front. Immunol. 15:1439485.
doi: 10.3389/fimmu.2024.1439485

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The role of RNA modifications in hepatocellular carcinoma: functional mechanism and potential applications

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Hepatocellular carcinoma (HCC) is a highly aggressive cancer with a poor prognosis. The molecular mechanisms underlying its development remain unclear. Recent studies have highlighted the crucial role of RNA modifications in HCC progression, which indicates their potential as therapeutic targets and biomarkers for managing HCC. In this review, we discuss the functional role and molecular mechanisms of RNA modifications in HCC through a review and summary of relevant literature, to explore the potential therapeutic agents and biomarkers for diagnostic and prognostic of HCC. This review indicates that specific RNA modification pathways, such as N6-methyladenosine, 5-methylcytosine, N7-methylguanosine, and N1-methyladenosine, are erroneously regulated and are involved in the proliferation, autophagy, innate immunity, invasion, metastasis, immune cell infiltration, and drug resistance of HCC. These findings provide a new perspective for understanding the molecular mechanisms of HCC, as well as potential targets for the diagnosis and treatment of HCC by targeting specific RNA-modifying enzymes or recognition proteins. More than ten RNA-modifying regulators showed the potential for use for the diagnosis, prognosis and treatment decision utility biomarkers of HCC. Their application value for HCC biomarkers necessitates extensive multi-center sample validation in the future. A growing number of RNA modifier inhibitors are being developed, but the lack of preclinical experiments and clinical studies targeting RNA modification in HCC poses a significant obstacle, and further research is needed to evaluate their application value in HCC treatment. In conclusion, this review provides an in-depth understanding of the complex interplay between RNA modifications and HCC while emphasizing the promising potential of RNA modifications as therapeutic targets and biomarkers for managing HCC.

KEYWORDS

hepatocellular carcinoma, N6-methyladenosine, 5-methylcytosine, N1-methyladenosine, N7-methylguanosine, biomarkers, therapeutic targets

1 Introduction

Liver cancer as one of the most prevalent malignant tumors worldwide, ranks as the sixth most frequently diagnosed cancer and the third leading cause of cancer-related deaths globally, with 865,000 new cases and 758,000 deaths in 2022, accounting for 4.3% and 7.8% of all malignant tumor morbidities and deaths respectively (1, 2). Hepatocellular carcinoma (HCC), as the most common liver cancer (75%–85% of cases) with the higher incidence and mortality rates, is the top three causes of cancer-related death in many countries (3, 4). HCC development is linked to multiple factors such as hepatitis virus infection, alcohol consumption, non-alcoholic fatty liver disease (NAFLD), and cirrhosis (2, 5). The primary risk factors for the development of HCC are infection with hepatitis B virus (HBV) and hepatitis C virus, with non-alcoholic steatohepatitis linked to metabolic syndrome or diabetes mellitus emerging as a more common risk factor in Western countries (5). HBV is a DNA virus that undergoes a complex life cycle involving reverse transcription. Chronic infection with this virus is a leading cause of liver cancer and cirrhosis on a global scale (6). In addition to environmental factors, certain genetic factors, such as gene mutations of TERT promoter, TP53, CTNNB1, amplifications of VEGFA, etc. also play a role in the occurrence and development of HCC (7, 8). The molecular mechanisms underlying HCC differ based on the specific genotoxic factors and causes (5). Despite advancements in our comprehension of the disease's pathophysiology and triggers, this information has not yet been sufficient implemented in clinical settings.

Surgical therapies for HCC, including surgical resection and liver transplantation represent potentially curative options for appropriate candidates with tumors detected at earlier stages (4, 9). However, only a minority of patients are eligible for this treatment because of factors such as cirrhosis (10), and local ablation is the preferred method for patients diagnosed with HCC in its early stages who are not candidates for surgery or transplantation (4). For patients with intermediate-stage HCC, chemoembolization is the main treatment strategy. Due to the suboptimal sensitivity of existing HCC surveillance tools and their underutilization in clinical practice, most patients with HCC are diagnosed at an advanced stage, which leaves minimal options for effective treatment (4, 11). Despite the advancements in immune-checkpoint inhibitor-based therapies, the objective response rate for patients with advanced-stage HCC is only around 30%, and the 3-year overall survival rate is still below 50% (4). Challenges faced in the management of HCC include difficulties in early diagnosis, high rates of recurrence, poor prognosis, limited effective treatment options and drug resistance (12).

RNA modification refers to the process of chemically modifying RNA, which can impact RNA stability, translation efficiency, and function (13). Recently, there has been increasing interest in studying RNA modifications in HCC. RNA modification can promote HCC progression by regulating other risk factors for HCC, such as fat metabolism and virus life cycle. RNA modifications have the ability to control viral replication by either modifying the viral genome or altering the expression of genes crucial for viral replication (6). NAFLD stands as a significant risk

factor for the development of HCC. Researchers have revealed the impact of RNA modifications on crucial aspects including steatosis, inflammation, fibrosis, and tumorigenesis. RNA modification induces NAFLD by regulating lipid metabolism, ultimately leading to HCC transformation (14). More and more evidences suggest that aberrant modifications of specific RNAs have been correlated with the occurrence, development, metastasis, and prognosis of HCC (15). One common RNA methylation is N6-methyladenine (m6A), which has been found to be associated with tumor proliferation, metastasis, and drug resistance in HCC (16). Additionally, other RNA modifications, such as 5-methylcytosine (m5C), N1-methyladenosine (m1A), and N7-methyladenosine (m7G), have also been reported to play a role in the development and progression of HCC (17). Currently, research on the role of RNA modification in HCC mainly focuses on m6A, m1A, m5C, and m7G. Recent studies have highlighted the crucial role of these RNA modifications in HCC progression, which indicates their potential as therapeutic targets and biomarkers for managing HCC.

In this review, we comprehensively summarize the functional roles, molecular mechanisms, and potential clinical applications of various RNA modifications in HCC. Clarifying the functional mechanism of RNA modifications and identifying new therapeutic targets in HCC will provide novel strategies for treatment. Additionally, RNA modifications also exhibit potential as biomarkers for the early diagnosis and prognostic evaluation of HCC, thereby improving diagnostic accuracy and patient survival rates. Overall, this review enhances our understanding of the role of RNA modifications in HCC and offers new perspectives on its diagnosis, prognosis, and treatment.

2 RNA modifications

As early as the 1950s, scientists discovered that there were some special chemical modifications in RNA molecules (18–22). With advancements in technology and in-depth research, various RNA modification types have been discovered (23). Methylations on RNA nucleotides, such as m6A, were the earliest modification type identified. In 1955, scientists first discovered methylations in RNA molecules (18). Subsequently, there has been continuous identification of new RNA modifications, including m5C, m1A, m7G, and pseudouridine (23). Increasing evidence shows that RNA modifications play complex regulatory roles in the cell and exert important effects on gene expression and cellular functions (24, 25). Recent studies indicate that specific RNA modifications, such as m6A, m5C, m1A, and m7G, through a series of modification regulatory proteins, affect the fate of RNA molecules such as precursor RNA processing, RNA splicing, stability, transport processes and translation, thereby regulating the expression of HCC-associated genes, and are involved in the progression of HCC.

2.1 N6-methyladenosine

N6-methyladenosine, also referred to as m6A, is an RNA methylation that involves the transfer of a methyl group to

adenosine (A) at N6 and is catalyzed by an RNA methyltransferase (Figure 1). It is the most prevalent, abundant, and evolutionarily conserved RNA methylation in eukaryotes (26). The regulation of RNA m6A methylation involves three groups of proteins: m6A methyltransferases (“writers”), m6A demethylases (“erasers”), and m6A methylation recognition proteins (“readers”) (Figure 2) (27). Accumulating evidence suggests that m6A is essential for the progression of HCC. The expression of m6A regulators, including “writer”, “eraser” and “reader” proteins, changed significantly in HCC (Table 1).

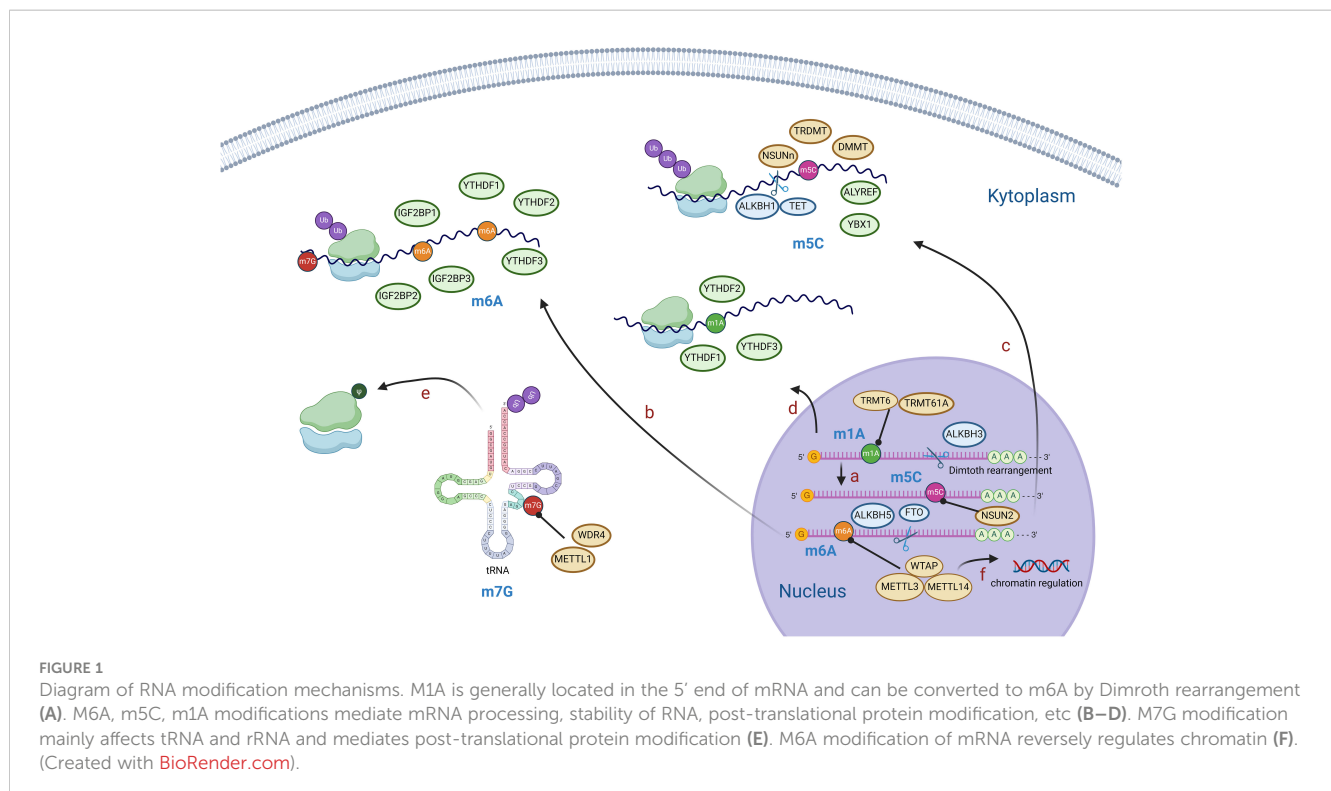
The “writers” refer to the m6A methyltransferases, which include methyltransferase-like protein (METTL) 3, METTL14, Wilms’ tumor 1-associating protein (WTAP), and vir like m6A methyltransferase associated (KIAA1429). These enzymes are responsible for catalyzing m6A modification (Figure 2) (27). The methylation of RNA to m6A is primarily carried out by the METTL3/METTL14 complex. Within this complex, METTL3 acts as the catalytic agent, while METTL14 serves as an allosteric activator that aids in binding to target RNA (46, 66).

The m6A modification of RNA is reversible, as it can be removed by “eraser” enzymes such as the m6A demethylases AlkB homolog 5 (ALKBH5) and fat mass and obesity-associated protein (FTO). These enzymes can convert m6A to A and rapidly remove m6A in a dynamic manner (Figure 2) (67). FTO belongs to the Alkb dioxygenase family and is associated with obesity (26). Knockdown of FTO significantly increases the level of RNA m6A modification (26). Another important demethylase, ALKBH5, is responsible for demethylating mRNAs within the nucleus. Knockout of ALKBH5 results in a significant increase in the level of RNA m6A modification (26, 67).

m6A “readers,” such as embryonic tumor-associated RNA-binding protein (IGF2BPs, insulin like growth factor 2 mRNA binding proteins), proteins containing YTH domains (YTH family proteins), and leucine-rich PPR-motif-containing protein (LRPPRC), have the ability to recognize and bind to m6A-modified RNA. Subsequently, they regulate the expression of related genes through various processes (Figure 2) (68). Proteins with YTH domains (YTHDF), such as YTHDF1, YTHDF2, and YTHDF3, can directly bind to m6A and then regulate translation or RNA decay. Specifically, YTHDF1 primarily enhances the translation efficiency of m6A-modified mRNAs, while YTHDF2 accelerates the degradation of m6A-modified mRNAs by recruiting several complexes to promote their degradation (61). The function of YTHDF3 is relatively complex, as it interacts with both YTHDF1 to promote protein synthesis and with YTHDF2 to facilitate mRNA degradation (69). IGF2BPs increase the stability and translational efficiency of m6A-modified mRNAs by recognizing m6A (70). Different members of the IGF2BP family may exhibit differences in regulating mRNA translation and stability; for example, IGF2BP1 and IGF2BP2 may have a wider range of substrate mRNAs in some cell types, whereas IGF2BP3 may focus more on specific mRNA molecules (71).

2.2 5-methylcytosine

5-Methylcytosine, also known as m5C, plays a pivotal role in the modification of RNA. It is produced during RNA synthesis by converting cytosine to 5-methylcytosine through the action of cytosine deaminase (Figure 1) (72). The methylation of RNA cytosines at the C5 position is facilitated by members of the



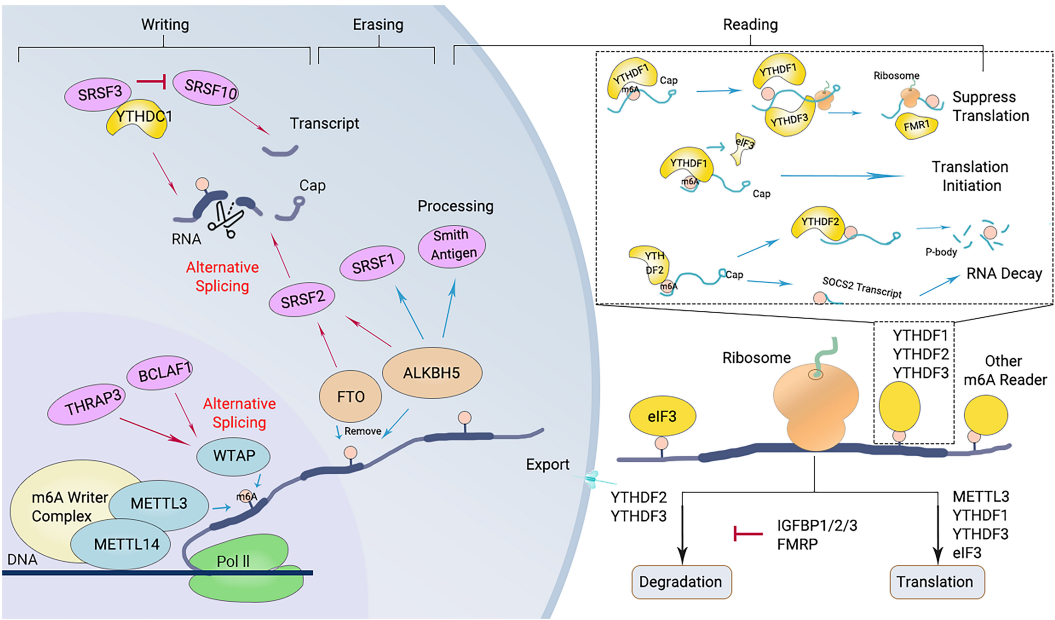


FIGURE 2
Diagram of the m6A modification mechanism. M6A “writers”, including METTL3/14 and WTAP, catalyze the m6A modification of adenosine on RNA. Removing the methylation of RNA needs the functions of m6A “erasers” that mainly consist of FTO and ALKBH5. M6A “readers” (such as YTHDF1/2/3 and others) recognize m6A modification sites and exert corresponding functions.

TABLE 1 The roles of m6A regulators in hepatocellular carcinoma.

M6A regulator	Change (regulator)	Target	Change (target)	Molecular Mechanism	Function	REF
Writer						
METTL3	Up	circ-CCT3	Up	circ-CCT3/miR-378a-3p/FLT1	Promotes HCC cell proliferation, invasion, and migration	(8)
	Up	circ-ARL3	Up	circ-ARL3/miR-1305 ceRNA	Promotes HCC progression	(28)
	Up	lncRNA GBAP1	Up	lncRNA GBAP1/miR-22-3p/BMP1A/SMAD	Promotes the migration, invasion and proliferation of HCC cells	(29)
	Up	LINC00958	Up	LINC00958/miR-3619-5p/HDGF	Promotes the proliferation, migration and invasion of HCC	(30)
	Up	Lnc-CTHCC	Up	Lnc-CTHCC/hnRNPK/YAP1	Promotes the growth and metastasis of HCC	(31)
	Up	LncRNA MAAS	Up	c-Myc/cyclins	Promotes the proliferation of HCC cells	(32)
	Up	PTEN	Up	HBV/METTL3/PTEN/IRF-3; PTEN/PI3K/AKT	Affects innate immunity and the development of HCC	(33)
	Up	ASPM	Up	Up-regulated ASPM expression	Promotes the proliferation, migration and invasion of HCC	(34)
	Up	EGFR	–	EGFR-pak2-erk5	Promotes drug resistance in HCC cells	(35)
	Up	MTF1	Up	Up-regulated MTF1 expression	Promotes tumor growth and migration of liver cancer	(36)
	Up	–	–	UBC9/SUMOylated METTL3/Snail	Promotes the growth and metastasis of HCC cells	(37)

(Continued)

TABLE 1 Continued

M6A regulator	Change (regulator)	Target	Change (target)	Molecular Mechanism	Function	REF
Writer						
METTL14	Down	circSTX6	Up	circSTX6/HNRNP/ATF3	Accelerates HCC proliferation and tumorigenicity and strengthens tumor metastasis	(38)
	Down	USP48	Down	USP48/SIRT6	Stimulates HCC exacerbation	(39)
	Down	HNF3 γ	Down	HNF3 γ /OATP1B1/OATP1B3	Weakens the sorafenib response and promotes HCC progression	(40)
	Down	EGFR	Up	EGFR/PI3K/AKT	Promotes the migration, invasion, and EMT of HCC cells	(41)
METTL3/14	Down	ACLY; SCD1	Up	FA synthesis and lipid production	Leads to decreased HCC cell death and cell survival	(42)
WTAP	Up	ETS1	Up	WTAP/ETS1-p21/p27	Promotes the proliferative ability and tumor growth of HCC cells	(43)
	Up	LKB1	Down	WTAP/LKB1/AMPK	Resists autophagy and promotes cell proliferation	(44)
	Up	lncRNA AC115619	Down	lncRNA AC115619/WTAP	Promotes HCC progression	(45)
KIAA1429	Up	–	–	E-Ca/slug/snail	Promotes invasion, migration, and EMT of HCC	(46)
	Up	GATA3	Down	KIAA1429/GATA3	Promotes tumor growth and metastasis	(47)
Eraser						
ALKBH5	Down	LYPD1	Up	ALKBH5/LYPD1	Stimulates HCC exacerbation	(48)
	Down	LINC02551	Up	The ALKBH5/lnc C02551/DDX24 axis	Promotes HCC growth and metastasis	(49)
	Up	MAP3K8	Up	ALKBH5/MAP3K8; ERK/JNK/IL-8	Promote HCC cell proliferation and metastasis	(50)
	Up	HBX	Up	HBX/wdr5/h3k4me3	Inhibits the growth and migration of HBV-driven tumor cells	(51)
FTO	Up	GLUT1 and PKM2	Up	FTO-it1/FTO/c-Myc	Promotes HCC progression	(52)
	Down	GNAO1	Down	SIRT1/FTO/GNAO1	Enhances HCC proliferation and <i>in vitro</i> invasion	(53)
RALYL	Up	TGF- β 2	Up	PI3K/AKT; STAT3 pathways	Promotes HCC tumorigenicity, self-renewal, chemoresistance, and metastasis	(54)
ZC3H13	Down	m6A modifications	Down	miR-362-3p/miR-425-5p-ZC3H13	Correlates with poor prognosis and poor outcome in HCC	(55)
Reader						
IGF2BP1	Up	circMDK	Up	miR-346/874-3p-ATG16L1; PI3K/AKT/mTOR	Promotes cancer cell proliferation, migration and invasion	(56)
	Up	circMAP3K4	Up	circMAP3K4-455aa/AIF	Prevents cisplatin-induced apoptosis of HCC cells	(57)
	Up	lncRNA MIR4435-2HG	Up	MIR44352HG/NOP58	Enhances the stem cell properties of HCC cells and promote tumorigenesis <i>in vitro</i> and <i>in vivo</i>	(58)
	Up	MGAT5	Up	Stability of MGAT5 mRNA	Promotes CSC liver phenotype and tumor metastasis	(59)

(Continued)

TABLE 1 Continued

M6A regulator	Change (regulator)	Target	Change (target)	Molecular Mechanism	Function	REF
Reader						
YTHDF1	Up	ATG2A and ATG14	Up	HIF-1α/YTHDF1/ATG2A/ATG14	Promotes hypoxia-induced autophagy of HCC and autophagy-related malignancies	(60)
YTHDF2	Up	OCT4	Up	m6A methylation of OCT4 mRNA	Promotes CSC liver phenotype and tumor metastasis	(61)
YTHDF3	Up	circ_KIAA1429	Up	TYHDF3/Zeb1/KIAA1429	Facilitates the migration, invasion, and EMT process of HCC	(62)
	Up	EGFR	Up	YTHDF3/m6A-EGFR/STAT3 and EMT axis	Promotes the proliferation, invasion and migration of HCC cells	(63)
	Up	PFKL	Up	YTHDF3/m6A- PFKL	Promotes proliferation, migration and invasion of HCC cells	(64)
LRPPRC	Up	PD-L1	Up	Up-regulated PD-L1 expression	Promotes tumor growth, improve tumor immunity and immune infiltration	(65)

HCC, hepatocellular carcinoma; REF, references; EMT, epithelial-mesenchymal transition; CSC, cancer stem cell.

NOL1/NOP2/SUN structural domain (NSUN) protein family and DNA (cytosine-5)-methyltransferase-like protein 2 (DNMT2). Additionally, this modification can be reversed by the AlkB homolog 1 (ALKBH1) and ten-eleven translocation (TET) demethylases (73). Furthermore, proteins such as Aly/REF export factor (ALYREF) and Y-Box binding protein 1 (YBX1) can recognize and bind to RNA m5C sites, resulting in downstream biological effects (74).m5C modification has been reported to regulate a broad variety of RNA functions, including increasing the stability of RNA molecules and increasing their stability in cells; affecting the translation efficiency of RNA; regulating the expression level of proteins by affecting the translation of mRNA and affecting the splicing process of RNA; and processing and trimming the precursor RNA, thus affecting its maturation and function (72, 75, 76). Via abnormal expression of regulators, m5C modification regulates the expression of HCC-associated genes, and is involved in the progression of HCC (Table 2).

2.3 N1-methyladenosine

N1-methyladenosine is a prevalent RNA modification primarily found on the adenylate residues of mRNAs. This modification is facilitated by an enzyme known as adenylate methyltransferase, which adds a methyl group to the guanine ribose ring of adenylate, resulting in m1A modification (89). The RNA m1A methyltransferases tRNA methyltransferase 10C (TRMT10C), 61B (TRMT61B), 6 (TRMT6), and 61A (TRMT61A) can be reversed by the ALKBH1 and AlkB homolog 3 (ALKBH3) demethylases (90). Additionally, YTHDF1, YTHDF2, YTHDF3, and YTH N6-methyladenosine RNA binding protein C1 (YTHDC1) serve as binding proteins that specifically recognize the m1A site and induce downstream effects (90). m1A modification plays a critical role in enhancing RNA stability, reducing the degradation rate (91), and protecting RNA molecules from damage in the external

environment. This modification also extends their lifespan within the cell (92). Furthermore, m1A is involved in the regulation of RNA translation. Research has indicated that it can impact the efficiency and accuracy of RNA translation, thereby controlling protein synthesis levels (89). This may be achieved by influencing the assembly and recognition of translation initiation complexes (93). The abnormal expression of m1A regulators, including “writer”, “eraser” and “reader” proteins, were also found in HCC indicating a pivotal role of m1A in HCC (Table 2).

2.4 N7-methylguanosine

N7-methylguanosine, also referred to as m7G, is an RNA modification that involves the addition of a methyl group (-CH3) to the nitrogen atom at position 7 of the guanine nucleotide within the RNA molecule (88). This process is carried out by methyltransferase enzymes, such as METTL1 and WDR4 (WD repeat domain 4) (71). M7G modifications are primarily found in eukaryotic mRNAs and certain noncoding RNAs, including ribosomal RNA (rRNA) and transfer RNA (tRNA) (Figure 1) (94).

Similar to other modifications, m7G modification is also crucial for regulating gene expression. m7G modifications in RNA can help to stabilize mRNA molecules and reduce their degradation rate, thus extending the lifespan of mRNAs (95). In addition, m7G modification is involved in the translational regulation of mRNAs. The formation of a cap structure and m7G modification can affect the formation and recognition of translation initiation complexes, which in turn affects the rate and precision of protein synthesis (96). Furthermore, m7G modification also affects the transcriptional regulation of mRNAs, including steps such as splicing and translocation (94, 96). The upregulated expressions of m7G “writer” proteins WDR4 and METTL1, are associated with the progression of HCC via regulating the HCC-associated gene expressions (Table 2).

TABLE 2 The role of other RNA modification regulators in hepatocellular carcinoma.

Category	Regulator	Change (regulator)	Target	Change (target)	Molecular Mechanism	Functions	REF
m5C							
Writer	USUN2	Up	LncRNA H19	Up	USUN2/H19RBA/G3BP1	Promotes the proliferation, migration and invasion of HCC cells	(77)
	USUN2	Up	GRB2	Up	Ras and p-Erk pathways	Promotes HCC progression and resistance to sorafenib	(78)
	NOP2	Down	XPD	Down	NOP2/XPD	Promotes the proliferation, migration, and invasion of HCC cells	(79)
Reader	ALYREF	Up	EGFR	Up	STAT3 signaling pathway	Promotes the progress of liver hepatocellular carcinoma	(80)
m1A							
Writer	TRMT6/TRMT61A	Up	tRNA	Up	Increases PPAR δ translation	Triggers cholesterol synthesis, activates Hedgehog signaling, and drives self-renewal and tumorigenesis of hepatic CSC	(81)
Eraser	ALKBH3	Up	–	–	p21/p27-mediated cell-cycle arrest	Promotes HCC tumor cell proliferation and tumor formation	(82)
Reader	YTHDF1 YTHDF2 YTHDF3	Up	–	–	–	Regulates immune cell infiltration in HCC tissues	(83)
m7G							
Writer	WDR4	Up	CCNB1	Up	MYC/WDR4/CCNB1; PI3K/AKT; P53	Promotes the proliferation, metastasis, and sorafenib resistance of HCC	(84)
	WDR4	Up	TRIM28	Up	TRIM28/Target genes (IRF2, OCT4...)	Increases cell-acquired stemness and lenvatinib resistance	(85)
	METTL1	Up	tRNA	Up	m7G dependent translation control	Promotes hepatocarcinogenesis	(86)
	METTL1/WDR4	Up	tRNA	Up	EGFR pathway	Induces lenvatinib resistance in HCC cells	(87)
	METTL1/WDR4	Up	tRNA	Up	SLUG/SNAIL	Promotes the recurrence and metastasis of HCC after IRFA	(88)

HCC, hepatocellular carcinoma; REF, references; CSC, cancer stem cell.

3 Functional mechanism of RNA modifications in HCC

3.1 m6A in HCC

By regulating the RNA m6A modification of HCC-associated genes, m6A regulators are involved in HCC cell proliferation, invasion, migration, epithelial–mesenchymal transition (EMT), autophagy, immune evasion, and drug resistance and play important roles in the progression of HCC (Table 2, Figure 3).

3.1.1 The “writers” and HCC

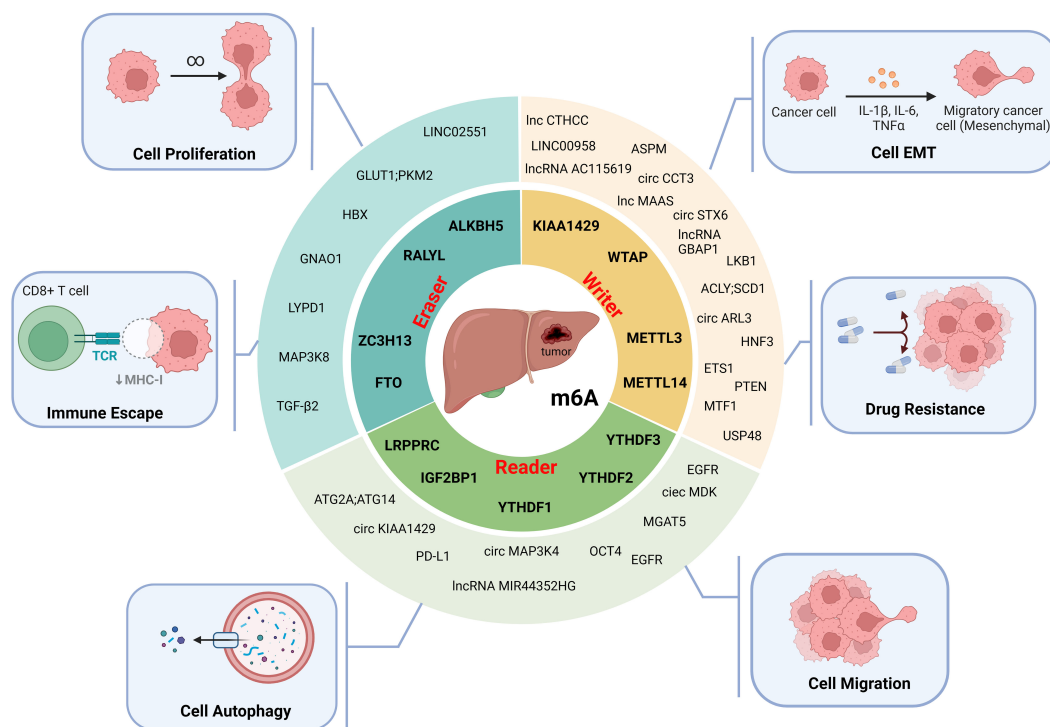
3.1.1.1 METTL3

METTL3, the first methyltransferase found to be involved in m6A modification, is significantly upregulated in HCC (29). By modifying the methylation of circular RNA (circRNA) (28, 66), long noncoding RNA (lncRNA) (29–32), and transcripts of other

cancer-associated genes (33–37), METTL3 is involved in the progression of HCC by regulating the stability of related RNAs (Figure 3, Table 1).

Circ-CCT3 and circ-ARL3 play oncogenic roles in HCC. High expression of circ-CCT3 has been reported to be associated with poor prognosis in HCC patients (66). METTL3 can increase the level of m6A modification of circ-CCT3; promote HCC cell proliferation, invasion, and migration through the circ-CCT3/miR-378a-3p/FLT1 axis; and subsequently promote HCC progression (66). The upregulated expression of METTL3 caused by HBx (an X protein encoded by hepatitis B virus) increases the m6A modification level of circ-ARL3 and then leads to increased stability and enhanced expression of circ-ARL3, causing dysregulation of the circ-ARL3/miR-1305 axis and ultimately facilitating HCC progression (28).

METTL3 regulates m6A modifications of the oncogenes lncRNA GBAP1, LINC00958, lnc-CTHCC, and MAPKAPK5_AS1 (MAAS) and subsequently promotes the progression of HCC (29–31). The expression of the lncRNA GBAP1 is significantly increased in



Phosphatase and tensin homolog (PTEN) is a tumor suppressor that reduces carcinogenesis by inhibiting the PI3K/AKT pathway (33). HBV increases the m6A methylation of PTEN RNA through the regulation of METTL3, which leads to a decrease in its protein level and promotes HCC (33). Abnormal spindle-like microcephaly (ASPM) has been shown to be involved in tumor progression, and its high expression in HCC predicts a poor prognosis (34). METTL3-mediated m6A modification promotes the expression of

The function of METTL3 is closely associated with its acetylation. When METTL3 undergoes substantial acetylation, its binding to metal-responsive transcription factor 1 (MTF1) mRNA, METTL14, and WTAP weakens, resulting in a decrease in m6A modification induced by the METTL3-METTL14-WTAP methyltransferase complex (36). In HCC, reduced m6A modification of MTF1 mediated by METTL3 acetylation leads to enhanced MTF1 expression, thereby promoting cell proliferation and tumor progression (36). Furthermore, mitogen stimulation leads to an increase in the small ubiquitin-related modifier (SUMO)ylation of METTL3, which is correlated with the upregulation of ubiquitin-conjugating enzyme 9 (UBC9) and is positively associated with the high metastatic potential of liver cancer (37). The UBC9/SUMOylated METTL3/Snai axis represents a novel pathway for SUMO involvement in HCC progression (37).

As a homolog of METTL3, METTL14 shares some similarities, but METTL14 is downregulated in HCC, is closely associated with tumor metastasis, and plays a regulatory role in the process of HCC tumor metastasis (Figure 3, Table 1) (39).

METTL14 can inhibit circSTX6 expression via m6A modification (38). Downregulation of METTL14 dysregulates the

circSTX6/HNRNPD/ATF3 axis, accelerates HCC proliferation and tumorigenicity, and enhances tumor metastasis (38). Notably, the circSTX6-encoded protein circSTX6-144aa also independently promoted HCC progression and is expected to be a potential biomarker and therapeutic target for HCC (38).

Ubiquitin-specific peptidase 48 (USP48) is a member of the ubiquitin-specific protease family and has been identified as an inhibitor of HCC tumorigenesis by stabilizing sirtuin 6 (SIRT6) (39). The downregulation of METTL14 in HCC leads to decreased methylation levels of USP48 mRNA, resulting in reduced expression of USP48. This dysregulation consequently affects glycolysis through the USP48-SIRT6 axis and contributes to the deterioration of HCC (39). These findings indicate that specifically targeting hepatocyte USP48 or the USP48-SIRT6 axis may be a potential therapeutic strategy for future HCC treatment.

Hepatocyte nuclear factor 3 γ (HNF3 γ) is a hepatocyte nuclear factor that can inhibit HCC growth by transactivating organic anion transporting polypeptide 1B1 (OATP1B1) and 1B3 (OATP1B3) expression, which sensitizes HCC cells to sorafenib-induced growth inhibition and apoptosis (40). METTL14-mediated m6A modification plays an important role in maintaining high HNF3 γ expression, and downregulation of METTL14 in HCC cells decreases HNF3 γ expression and promotes HCC progression (40). Another study showed that high levels of METTL3/14 enhanced the expression of ATP citrate lyase (ACLY) and stearoyl-CoA desaturase 1 (SCD1) by regulating their mRNA stability, which accelerated fatty acid synthesis and lipogenesis, ultimately leading to lipid peroxidation or endoplasmic reticulum stress, resulting in HCC cell death and a decrease in HCC cell viability (42). Targeting METTL3/14 has proven to be a promising anticancer therapeutic strategy.

3.1.1.3 Other “writers” and HCC

The m6A methylases WTAP and KIAA1429, also known as tumor-associated proteins, are significantly upregulated in HCC (43). WTAP can cause posttranscriptional repression of ETS proto-oncogene 1 (ETS1) through m6A modification and promote the proliferative capacity and tumor growth of HCC cells through the WTAP/ETS1-p21/p27 axis (43). The micropeptide encoded by lncRNA AC115619 inhibited the growth of HCC tumors by binding to WTAP and hindering the assembly of the m6A methyltransferase complex, resulting in a reduction in the overall methylation level (45). In addition, when researchers knocked out the WTAP gene in HCC, the m6A level of liver kinase B1 (LKB1) mRNA decreased, and its stability increased, which in turn promoted autophagy in HCC cells via the WTAP/LKB1/AMPK axis, suggesting that it is a promising target for HCC therapy (44). KIAA1429 was found to be significantly upregulated in HCC tissues. It has been demonstrated to promote the invasion, migration, and EMT of sorafenib-resistant HCC cells (46). Additionally, through its mediation of m6A methylation, KIAA1429 was observed to decrease the expression of the E-Ca protein while increasing the expression of the slug and snail proteins (46).

3.1.2 The “erasers” and HCC

3.1.2.1 ALKBH5

ALKBH5 is a demethylating enzyme with extremely complex regulatory mechanisms *in vivo* (67). The expression of ALKBH5 was reported to be downregulated in HCC, causing elevated m6A levels and increased stability of Ly6/Plaur domain-containing 1 (LYPD1), leading to dysregulation of the ALKBH5/LYPD1 axis and promoting the progression of HCC (48). DEAD-box helicase 24 (DDX24) is a tumor-associated gene that is often used as a marker to assess tumor risk and treatment efficacy (49). Downregulation of ALKBH5 promotes the expression of LINC02551, which acts as a molecular junction to block the binding of DDX24 to the E3 ligase tripartite motif 27, thereby reducing the ubiquitination and subsequent degradation of DDX24 (49). Moreover, stabilized DDX24 promotes EMT in HCC (49). However, some researchers have reported conflicting results. In contrast to the aforementioned study, they proposed that ALKBH5 is actually upregulated in HCC. They suggested that this upregulation occurs through the activation of the ERK/JNK pathway and the regulation of interleukin-8 (IL-8) expression via the ALKBH5/MAP3K8 axis. Ultimately, this promotes HCC development, metastasis, and macrophage recruitment (50). According to reports, there is a strong positive correlation between HBx and ALKBH5 in HBV-HCC tissues (51). It has been discovered that HBV increases the expression of ALKBH5 through the HBx/wdr5/h3k4me3 pathway, while ALKBH5, in turn, enhances the stability of HBx mRNA by reducing m6A modification (51). Additionally, studies have indicated that the depletion of ALKBH5 significantly suppresses the proliferation and migration of HBV-induced tumor cells both *in vitro* and *in vivo* (51).

3.1.2.2 Other “erasers” and HCC

FTO plays key roles in the regulation of adiposity, lipogenesis, and body weight (97). The expression level of FTO is elevated in HCC, which is promoted by the lncRNA FTO-IT1 recruiting interleukin enhancer binding factor 2 (ILF2) and 3 (ILF3) (52). FTO overexpression increases the mRNA expression of the glycolysis-associated genes glucose transporter type 1 (GLUT1), pyruvate kinase M2 (PKM2), and c-Myc by decreasing the expression of m6A modifications, which subsequently promotes HCC progression in a glycolysis-dependent manner (52). Sirtuin 1 (SIRT1) is a known deacetylase silencing information regulator that destabilizes FTO, and its presence is positively correlated with malignancy and metastasis (53). A reduction in FTO by SIRT1 can increase the m6A modification level of guanine nucleotide-binding protein G (o) subunit α (GNAO1) and cause downregulation of its mRNA expression (53). Deletion of GNAO1 significantly enhanced HCC proliferation and invasion *in vitro* (53).

RALY RNA Binding Protein Like (RALYL) is a recently discovered demethylase that can increase the stability of TGF- β 2 mRNA by reducing its m6A modification (54). RALYL promotes the tumorigenicity, self-renewal, chemoresistance, and metastasis of HCC through the TGF- β 2/PI3K/AKT and STAT3 pathways (54).

Zinc finger CCCH-type containing 13 (ZC3H13) is a methyltransferase whose expression is downregulated in HCC (55). The miR-362-3p/miR-425-5p-ZC3H13 axis leads to downregulation of ZC3H13 and thus a reduction in m6A modifications, which correlates with poor prognosis and poor outcome in HCC patients (55).

3.1.3 The “readers” and HCC

3.1.3.1 IGF2BP1

IGF2BP1, a common m6A methylation recognition protein that recognizes a wide range of m6A-modified RNAs, is highly expressed in HCC (59). The expression of autophagy related 16 like 1 (ATG16L1), a member of the autophagy family, is increased in both HCC cells and tissues. This finding suggested that autophagy may play a role in the progression of HCC (56). IGF2BP1 can recognize circMDK via m6A sites, activate the PI3K/AKT/mTOR signaling pathway through the miR-346/874-3p-ATG16L1 axis, and ultimately promote the proliferation, migration and invasion of hepatoma cells (56). IGF2BP1 recognizes the m6A modification of circMAP3K4 and promotes its translation, thereby preventing cisplatin-induced apoptosis in HCC cells by increasing the interaction of circMAP3K4-455 aa with apoptosis inducing factor (AIF) (57). High levels of circMAP3K4 serve as an independent prognostic factor for poor overall survival and disease-free survival in HCC patients (57). Furthermore, IGF2BP1 is capable of recognizing the m6A-modified lncRNA MIR4435-2HG and enhancing its expression in HCC (58). Moreover, overexpression of MIR4435-2HG significantly reduces cell sensitivity to lenvatinib, enhances the stem cell properties of HCC cells, and promotes tumorigenesis *in vitro* and *in vivo* (58). Additionally, IGF2BP1 enhances alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase V (MGAT5) mRNA stability by increasing m6A modification, which contributes to the promotion of the HCC stem cell phenotype, self-renewal, chemotherapy resistance, and tumorigenesis in mice (59). In conclusion, IGF2BP1 is highly expressed in HCC and enhances the translational stability of corresponding HCC-associated RNAs by recognizing the m6A site. This finding suggests that it may be an important target for anticancer therapy.

3.1.3.2 YTHDF family

The YTHDF family is classified as m6A recognition proteins and consists of YTHDF1, YTHDF2, and YTHDF3. These proteins have been linked to the progression of HCC (60–63). Under hypoxic conditions, hypoxia-inducible factor-1 α (HIF-1 α) promotes the expression of YTHDF1 by directly binding to its promoter region. This mechanism facilitates hypoxia-induced autophagy in HCC and autophagy-related malignancies through the HIF-1 α /YTHDF1/ATG2A/ATG14 axis (60). Additionally, YTHDF2 regulates the m6A methylation of octamer-binding transcription factor 4 (OCT4) mRNA, thereby promoting a cancer stem cell (CSC) liver phenotype and tumor metastasis (61). Targeting YTHDF2 to eliminate CSCs is a key focus in the development of novel anticancer therapies (61). The m6A modification mediated by YTHDF3 has been shown to promote HCC migration, invasion, and EMT processes, underscoring its

potential as a promising therapeutic target for HCC (62, 63). Additionally, Circ_KIAA1429 has been identified as an oncogene that targets zinc finger E-box binding homeobox 1 (Zeb1) downstream of HCC. Its high expression level has been associated with a lower overall survival rate in HCC patients (62). Through M6A-dependent mechanisms, YTHDF3 enhances Zeb1 mRNA stability and promotes HCC progression through the YTHDF3/Zeb1/KIAA1429 axis (62). Furthermore, YTHDF3 plays a role in enhancing the translation and stability of m6A-modified epidermal growth factor receptor (EGFR) mRNA. This stimulation further drives HCC progression through the YTHDF3/m6A EGFR/STAT 3 and EMT pathways (63).

3.1.3.3 LRPPRC

Programmed cell death ligand 1 (PD-L1) is a transmembrane protein expressed on the surface of activated T cells, B cells, and natural killer cells, and acts as a ligand for programmed cell death protein 1 (PD-1) and is commonly found on the surface of tumor cells (98). The interaction between these two proteins can effectively suppress the activation and proliferation of T cells, diminish the cytotoxic capabilities of T-cells towards tumor cells, and promote immune evasion and tumor cell growth (65). PD-L1 plays a crucial role as a coinhibitory immune checkpoint, and the PD1/PD-L1 signaling pathway functions to dampen the cytotoxic T-cell-mediated killing effect within the tumor microenvironment, thereby aiding in immune evasion by tumors (65). Research has indicated that LRPPRC is upregulated in HCC and enhances PD-L1 expression through a m6A-mediated mechanism. This process leads to increased stability of PD-L1 mRNA in cancer cells, ultimately promoting tumor growth and facilitating immune evasion and invasion by tumor cells (65).

3.2 m5C in HCC

NSUNs are a class of m5C methyltransferases in which NSUN2 and NSUN4 are upregulated and NOP2 is downregulated in HCC tissues and cells (77, 78, 99). The expression of NSUN4 varies across different survival rates and grade distributions, indicating its potential as an independent prognostic factor for HCC (99).

NSUN2-mediated gene mRNA and lncRNA H19 m5C modifications regulate the progression of HCC (77, 78). In HCC tissues, the m5C modification level of mRNA is significantly greater than that in normal tissue, and growth factor receptor-bound protein 2 (GRB2), ring finger protein 115 (RNF115) and apoptosis antagonizing transcription factor (AATF) are the top NSUN2-related m5C hypermethylated genes (78). GRB2 is a growth factor receptor-binding protein that is expressed at significantly higher levels in HCC tissues than in normal tissues and is considered a potential therapeutic target for HCC (78). NSUN2 interacts with lin-28B, a protein capable of recognizing m5c, to facilitate the m5c modification of GRB2 and enhance the stability of GRB2 mRNA. This process ultimately contributes to promoting resistance in HCC cells to sorafenib by activating the Ras and p-Erk pathways (78). H19, an important tumor-related lncRNA, is targeted by NSUN2 in HCC

(77). The NSUN2-mediated lncRNA H19 m5C modification enhances its stability and increases its ability to bind specifically to Ras-GTPase activating SH3 domain-binding protein 1 (G3BP1) to promote the proliferation, migration and invasion of HCC cells (77).

NOP2, also known as NSUN1, is a member of the NSUN family whose expression is downregulated in HCC (79). NOP2 overexpression enhances the expression of the antioncogene xeroderma pigmentosum D (XPD) via m5C methylation of XPD, thereby inhibiting the proliferation, migration and invasion of HCC cells (79). These findings suggest that XPD may be a potential target for HCC treatment.

ALYREF is a protein that recognizes m5C and is upregulated in HCC (100). The EGFR signaling pathway plays a crucial role in various cellular processes, and abnormal activation of EGFR is observed in a wide range of tumors (100). ALYREF stabilizes EGFR by binding to the m5C site on EGFR mRNA, thereby activating STAT3 signaling and promoting HCC progression (80).

3.3 m1A in HCC

m1A modification is involved in the progression and treatment of HCC (Table 2). In HCC tissues, the m1A modification levels of RNA are aberrantly elevated (81). The expression levels of m1A methyltransferases, such as TRMT6 and TRMT61A, are significantly elevated in HCC tissues and are negatively correlated with patient prognosis (81). The PPAR δ protein, also known as peroxisome proliferator-activated receptor, is associated with cholesterol synthesis (81). TRMT6/TRMT61A enhances m1A methylation in tRNA, leading to an increase in PPAR δ translation. This subsequently promotes cholesterol synthesis and activates Hedgehog signaling, ultimately driving self-renewal and tumorigenesis of liver CSCs (81).

In addition, the m1A demethylase ALKBH3 is significantly upregulated in HCC tissues compared to nontumor tissues. Furthermore, high expression of ALKBH3 has been associated with poor prognosis (43). The overexpression of ALKBH3 has been found to stimulate the proliferation and tumorigenesis of HCC tumor cells, indicating a functional role for m1A modification in promoting the cell cycle (82).

For binding proteins that specifically recognize the m1A site, members of the YTH domain family, such as YTHDF1, YTHDF2, and YTHDF3, have been reported to be upregulated in HCC tissues compared to normal tissues (83). Additionally, the expression levels of YTHDF1, YTHDF2, and YTHDF3 are positively correlated with immune cell infiltration in HCC tissues. This finding indicates a functional role for m1A regulators in regulating immune cell infiltration (83).

In addition, there have been reports suggesting that patients with smaller tumors and good liver function may benefit from a combined regimen of mitoxantrone, 5-fluorouracil, and cisplatin (90). The clinical use of doxorubicin in patients with elevated levels of m1A modification is more scientifically supported. These findings contribute to mitigating the risk of doxorubicin-induced cardiotoxicity in patients and reducing unnecessary overtreatment (90).

3.4 m7G in HCC

Methylation of m7G has been found to be associated with the progression of HCC and resistance to drugs (Table 2). Both METTL1 and WDR4, which are essential components of the m7G methyltransferase complex, have been reported to be upregulated in HCC tissues and cells (84, 86). In HCC cells, MYC activates the transcription of WDR4, which subsequently enhances the stability and translation of cyclin B1 (CCNB1) mRNA by facilitating binding of eukaryotic initiation factor 2A (EIF2A) to CCNB1 mRNA. This process ultimately promotes proliferation, metastasis, and resistance to sorafenib (84, 86). Furthermore, METTL1-mediated modification of tRNA m7G contributes to hepatocarcinogenesis through translational control of target mRNAs (86).

Long-term drug resistance is a significant challenge in the treatment of HCC. Studies have indicated that METTL1 and WDR4 are upregulated in lenvatinib-resistant cells (87). METTL1/WDR4-mediated tRNA m7G modification enhances the translation of genes within the EGFR pathway, ultimately leading to lenvatinib resistance in HCC cells. This suggests a potential strategy for overcoming drug resistance (87). In glioblastoma, tripartite motif containing 28 (TRIM28) has been identified as a specific marker of stem-like cells, contributing to their invasion (85). Additionally, WDR4 amplifies TRIM28 expression, subsequently affecting the expression of target genes and promoting cell-acquired stemness as well as lenvatinib resistance (85).

Insufficient radiofrequency ablation (IRFA) is a major contributing factor to the high recurrence rate of HCC treatment (88). Studies have shown an association between m7G-tRNA modification and HCC recurrence following IRFA treatment (88). Specifically, after IRFA therapy, there was a significant increase in the level of m7G tRNA modification and its associated methyltransferase complex component METTL1/WDR4. This increase facilitated the translation of SLUG/SNAIL during sublethal heat stress in a manner dependent on codon frequency, ultimately leading to elevated HCC recurrence and metastasis (88).

In summary, RNA modifications, such as m6A, m5C, m1A, and m7G, through a series of modification regulatory proteins regulate the expression of HCC-associated genes and are involved in the progression of HCC via different signal axes. It is important to emphasize that the alterations in the expression of RNA modification regulatory proteins with similar functions in HCC are somewhat inconsistent. Methyltransferases, such as METTL3 and METTL14, as well as USUN2 and NOP2, exhibit opposite expression changes in HCC. Their opposite expression changes play a similar role in the progression of HCC by regulating the expression of different target genes and subsequent signaling pathways through RNA modification. There is no consensus on the expression changes of some RNA modification regulatory proteins, such as ALKBH5 and FTO, in HCC. This suggests that the expression of RNA modification regulatory proteins in HCC may be dynamic and have different mechanisms of action in various stages and types of the HCC. Therefore, more in-depth and detailed classification research is needed to clarify this.

At present, research on the role of RNA modification in HCC mainly focuses on m6A, m1A, m5C, m7G, etc. Their combined interactions form an intricate RNA modification network that significantly influences the physiological and pathological processes of HCC cells. However, whether these RNA modifications interact with each other in HCC has not been studied yet. The interactions between different types of RNA modifications are complex and diverse, and they may affect each other through synergistic effects, competitive relationships, cascade effects (101). Both m6A and inosine occur at the N6 position of the adenosine ring, but they do not compete for the same adenosine (102). m5C and m6A work together to regulate the export of mRNA to the cytoplasm through interactions with ALYREF and ALKBH5 (101). The m6A and m1A modifications on mRNA are recognized by the same reader proteins YTHDF1-3. In addition, m7G plays a role in facilitating the N6, 2-O-dimethyladenosine modification within the cap structure of the extended RNA polymerase II transcript (103). Despite the specific interplay between RNA modifications not being fully elucidated, further research is anticipated to unveil more about the mechanism and function of these modifications, offering new insights and approaches for the treatment and diagnosis of diseases.

4 Potential applications of RNA modifications in HCC

4.1 Potential for use as diagnosis and prognosis biomarkers

Proteins and genes associated with RNA modifications have the potential to serve as biomarkers for the diagnosis and prognostic evaluation of HCC (Table 3). This capability has the potential to improve diagnostic accuracy and ultimately enhance the survival rate of patients.

4.1.1 Role of m6A regulators in HCC diagnosis, prognosis and drug resistance

The expression levels of KIAA1429, ALKBH5 and FTO were reported to have the diagnostic potential in HCC (Table 3). A study found that KIAA1429 expression had up to 0.85 of the area under the curve (AUC) in the receiver operating characteristic curve (ROC) analysis, which indicated that it has a relatively high diagnostic value in HCC (47). Although studies have suggested the potential diagnostic potential of ALKBH5 and FTO, their potential and value as diagnostic markers have been reduced due to inconsistent expression in different studies (52, 104, 105) and a lack of ROC analysis data for diagnostic markers (48).

For the upregulated m6A methyltransferases in HCC cells, the higher expression levels of KIAA1429, WTAP, and METTL3 are associated with poorer overall survival outcomes and indicate a poor prognosis and increased likelihood and severity of patients (35, 37, 43, 46). The micropeptide AC115619-22aa encoded by lncRNA AC115619, as an inhibitor of WTAP, is also a potential prognostic indicator for HCC (36). Additionally, the elevated expression of ASPM facilitated by METTL3 via m6A modification is also strongly

associated with a poor prognosis of HCC (34). The methyltransferase METTL14 is downregulated in HCC, and lower expression levels of METTL14 are associated with increased likelihood and severity of HCC patients who underwent a poorer overall survival (41). Besides, there are many pathways downstream of METTL14 that increase the likelihood and severity of HCC (107). Reduced HNF3 γ expression is associated with the malignant features of HCC and is correlated with poor patient survival (40). CircSTX6 and its encoded proteins are expected to have the potential to serve as biomarkers for the diagnosis, prognosis, and treatment of HCC (38).

Low expressions of demethylases ALKBH5 and ZC3H13 are associated with poor prognosis in HCC patients (48, 50, 55). Additionally, the overexpression of ALKBH5 has been found to cause a decrease in the level of the lncRNA LINC02551, which has been utilized as a prognostic biomarker for HCC (49). Furthermore, the overexpression of the demethylase RALYL has been associated with a poorer prognosis, lower levels of differentiation, and an increased likelihood of metastasis in clinical HCC patients (54). The expression of FTO varies in different studies, with one study showing high expression and another showing low expression, both of which were linked to poorer survival in HCC patients (52, 104, 105). These conflicting findings indicate the multifaceted and functionally complex nature of FTO in HCC, highlighting the need for further research to elucidate its molecular mechanism. The deacetylase SIRT1 reduces the expression of the m6A demethylase FTO, thereby increasing the m6A levels of its downstream target GNAO1 and downregulating its mRNA expression during HCC tumorigenesis (53). The discovery of potential diagnostic biomarkers such as SIRT1, FTO, and GNAO1 offers a promising direction for future research. This area holds significant promise for further investigation and exploration (53).

The significant overexpression of the m6A reading proteins IGF2BP1, YTHDF1, YTHDF2, YTHDF3, and LRPPRC in HCC tissues has been associated with a poorer prognosis for patients (Table 3). IGF2BP1 can promote the progression of HCC through the m6A-mediated upregulation of circMDK and circMAP3K4, which are associated with poor survival in HCC patients and serve as potential tumor biomarkers (56, 57). Furthermore, the results of multivariate Cox regression analysis indicated that the expression of YTHDF1 served as an independent prognostic factor for patients with HCC (60). In addition, scientists have shown a negative correlation between YTHDF2 expression and patient survival (61). Therefore, YTHDF2 plays a significant role in the oncogenesis of HCC and can serve as a valuable biomarker for HCC patients. Additionally, the expression level of YTHDF3 in HCC tissues was found to be significantly greater than that in adjacent liver tissues (62). YTHDF3 plays a crucial role in regulating the progression of HCC, providing a promising new target for HCC treatment (63). In conclusion, the YTHDF family is closely associated with the prognosis of HCC patients and may represent a potential target for the future treatment of HCC. Additionally, LRPPRC is frequently upregulated in HCC tumors, which is linked to advanced disease stages and poor prognosis (65).

Overall, about thirteen m6A regulators are reported to be closely related to HCC prognosis and have a potential for use as

TABLE 3 Potential for use as diagnosis and prognosis biomarkers of HCC.

Biomarker	Expression	Diagnosis (AUC)	Prognosis (HR)	Treatment decision utility	REF
KIAA1429	Up	HCC diagnosis (TCGA: 0.85)	Poor prognosis (>1)	Promote sorafenib resistance	(46, 47)
WTAP	Up	–	Poor prognosis (>3)	–	(43)
METTL14	Down	–	Increased likelihood and severity (>2)	Weaken sorafenib response	(39–41)
METTL3	Up	–	Increased likelihood and severity (>1)	Promote lenvatinib resistance	(35, 37)
ALKBH5	Down	HCC diagnosis (-)	Poor prognosis (>2)	–	(48)
FTO	Up	HCC diagnosis (-)	Poor survival (>1)	–	(52, 104)
	Down	–	Poor survival (>1)	–	(105)
RALYL	Up	–	Poor survival (>4)	Enhance cisplatin and 5-fluorouracil resistance	(54)
ZC3H13	Down	–	Poor survival(>1)	–	(55)
IGF2BP1	Up	–	Poor survival (>1)	Promote lenvatinib resistance	
YTHDF1	Up	–	Poor prognosis (>1)	–	(60)
YTHDF2	Up	–	Poor prognosis (>1)	–	(61)
YTHDF3	Up	–	Poor prognosis (>5)	–	(62, 64)
LRPPRC	Up	–	Poor prognosis (>1)	–	(65)
NSUN2	Up	–	Increased likelihood and severity (>1)	–	(78)
ALYREF	Up	HCC diagnosis (-)	Poor prognosis (>2)	–	(80, 106)
METTL1	Up	–	Increased likelihood and severity (>1)	Promote lenvatinib resistance	(87)
WDR4	Up	–	Increased likelihood and severity (>1)	Promote sorafenib resistance	(84)

HCC, hepatocellular carcinoma; REF, references; AUC, area under receiver operating characteristic curve; HR; hazard ratio of overall survival; TCGA, the cancer genome atlas.

prognosis biomarkers, including four methyltransferases (KIAA1429, WTAP, METTL14 and METTL3), four demethylases (ALKBH5, FTO, RALYL and ZC3H13), and five reading proteins (IGF2BP1, YTHDF1-3 and LRPPRC (Table 3). Among them, the high expression of WTAP, RALYL, YTHDF3, etc. showed a higher hazard ratio of overall survival (>3) in survival analysis, indicating a more significant correlation and impact on poor survival rates, suggesting that these markers have relatively higher prognostic value (Table 3). It should be noted that the expression changes of ALKBH5, and FTO in HCC vary in different studies, and these contradictory results need further research and clarification. In addition, the differences in the patient source, quantity, and calculation method of the survival curve analysis of these regulatory proteins indicate strong heterogeneity. Therefore, the confirmation of the prognostic value of each factor still requires multi-center large-scale sample validation. Confirming the prognostic performance of these potential markers through further ROC curve analysis of large sample sizes is an important research direction for the future. In addition, most previous studies

have analyzed these factors as independent prognostic factors for patients with HCC, attempting to conduct a joint analysis of these factors for prognosis, which may provide a more comprehensive display of the relationship between m6A regulators and HCC prognosis.

In addition to patient survival rates, some m6A regulators also exhibit a correlation with targeted anti-cancer drugs or chemotherapy drugs, promoting cancer cell drug resistance (Table 3). High expression of KIAA1429 and low expression of METTL14 can promote sorafenib resistance in HCC cells. KIAA1429 is involved in promoting invasion, migration, and EMT in sorafenib-resistant HCC by mediating m6A methylation (21). THNF3γ reduction caused by METTL14 knockdown upregulates the expression of the sorafenib influx transporters OATP1B1 and OATP1B3, thereby rendering sorafenib resistance in HCC, and enforced HNF3γ expression enhances the cellular response to sorafenib in HCC (40). Similarly, high expression of METTL3 and IGF2BP1 can promote lenvatinib resistance in HCC cells, while high expression of RALYL enhances cisplatin and 5-fluorouracil resistance. Targeting METTL3 with the

specific inhibitor STM2457 improved sensitivity to lenvatinib in both *in vitro* and *in vivo* studies (35). This discovery suggested that METTL3 might serve as a potential therapeutic target for overcoming lenvatinib resistance in HCC. There have been reports suggesting that patients with smaller tumors and good liver function may benefit from a combined regimen of mitoxantrone, 5-fluorouracil, and cisplatin (108). Inhibiting RALYL could potentially improve the effectiveness of a combination therapy involving mitoxantrone, 5-fluorouracil, and cisplatin, providing new strategies for treating HCC. These results not only provide a theoretical basis for personalized medication for patients but also offer new directions for increasing drug sensitivity research. The combination of specific inhibitors or downstream target genes that modify related proteins with chemotherapy drugs may provide new strategies for the treatment of HCC.

4.1.2 Role of m5C regulators in HCC diagnosis, prognosis and drug resistance

Regulators of RNA m5C modification and their target HCC-related oncogenes are potential diagnostic and prognostic biomarkers for HCC (Table 3). A significant increase in the expression of lncRNA H19 was reported to be associated with the development of various types of tumors (77). The RNA m5C methyltransferase NSUN2 regulates the stability of lncRNA H19 through m5C modification and may serve as a potential new target and biomarker for the treatment of HCC (77). In addition, NSUN2 has been found to impact the sensitivity of HCC cells to sorafenib by regulating the activity of the Ras pathway (78). The sensitivity of NSUN2 knockout cell lines to sorafenib was significantly greater than that of control cells (78). ALYREF, functioning as a “reader” for the m5C site in RNA, is a protein that shuttles between the nucleus and cytoplasm, and plays a critical role in facilitating the transportation of RNA from the nucleus to the cytoplasm. The upregulation of ALYREF has been associated with poor prognosis in HCC patients, indicating its potential as a valuable target for diagnosing and predicting prognosis in HCC patients (80, 100). The higher HR value of ALYREF than NSUN2 suggests a priority prognostic value of ALYREF. However, due to the lack of necessary ROC analysis, further analysis of large sample sizes is needed to confirm the prognostic or diagnostic values of NSUN2 and ALYREF.

4.1.3 Role of m7G regulators in HCC prognosis and drug resistance

The upregulation of the m7G methyltransferases METTL1 and WDR4 has been reported to be correlated with advanced tumor stage and unfavorable patient survival outcomes in the literature (86). Investigators have shown that METTL1-mediated tRNA m7G modification plays a critical role in lenvatinib resistance in HCC cells by enhancing the translation of EGFR pathway genes (87). WDR4 overexpression significantly increased the half-maximal inhibitory concentration value of sorafenib in HCC cells (77). Some researchers propose that both METTL1 and WDR4 have the potential to serve as biomarkers for predicting the efficacy of lenvatinib and sorafenib (87). The potential application of m7G

regulators in HCC is still in the early stages of research. Further exploration of new m7G regulators involved in the occurrence and progression of HCC may provide new targets for the prognosis and treatment of HCC.

4.1.4 Potential applications of non-coding RNAs regulated by RNA modification

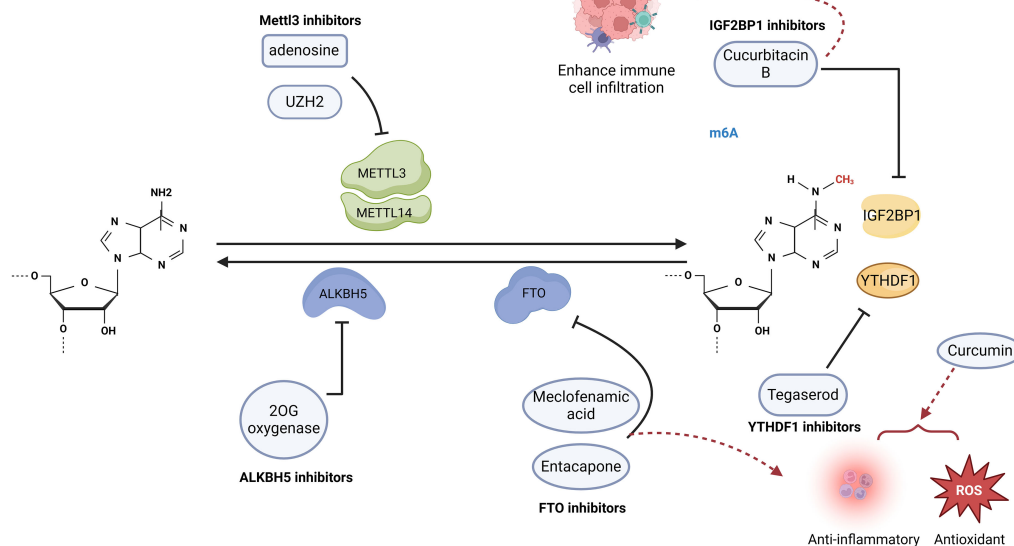
Non-coding RNAs, as important target sequences regulated by RNA modifications, play a crucial role in the pathogenesis of HCC (Figure 3). Upregulated circRNAs dependent on M6A modification, including circ-CCT3, circSTX6, circ-ARL3, circMDK, circMAP3K4, and circ_KIAA1429, are involved in the proliferation, invasion, migration, and apoptosis of HCC cells. Upregulated lncRNAs dependent on m6A modification, such as GBAP1, lnc-CTHCC, LINC00958, MIR4435-2HG, etc., play similar functional roles in the progression of HCC. Certain non-coding RNAs regulated by RNA modification showed significant application potential in the diagnosis, prognosis, and treatment of HCC. The elevated expression of circSTX6 has an AUC of up to 0.8565 in the diagnostic ROC analysis, suggesting a considerable diagnostic significance in HCC (38). The high expression of LINC00958, lnc-CTHCC showed a higher hazard ratio of overall survival (>2) in survival analysis, indicating a more significant correlation and impact on poor survival rates, suggesting that these markers have relatively higher prognostic value (30, 31). Overexpression of MIR4435-2HG significantly reduced cell susceptibility to lenvatinib, suggesting its role in cell resistance to lenvatinib (58). Nevertheless, most research has focused solely on investigating the impact of partial non-coding RNA in m6A modification, leaving a gap in understanding the connection between miRNA and RNA modification, along with the correlation between m5C, m1A, m7G, and RNA modification. This presents a promising avenue for further investigation.

4.2 Potential agents targeting RNA modification

4.2.1 Potential agents targeting m6A

As the link between RNA modifications and cancer continues to be discovered, the demand for inhibitors of the associated proteins is increasing. The role of the m6A methyltransferase METTL3 in various diseases is continuously expanding, and there is also a growing focus on the development of METTL3 inhibitors. This has garnered increasing attention in academic research and scholarly discourse (Figure 4). Adenosine was the first reported METTL3 inhibitor that acts in a SAM-competitive manner to reduce the level of m6A modification (109). UZH2 is a small molecule inhibitor that selectively targets METTL3 and reduces the m6A levels of polyadenylated RNA in the MOLM-13 (acute myeloid leukemia) and PC-3 (prostate cancer) cell lines and has great potential as a therapeutic agent for cancer (110, 111). Curcumin, a polyphenolic pigment derived from turmeric, is yellow in color and has been reported to exhibit anti-inflammatory, anticancer, antioxidant, and antibacterial activities (112). Curcumin can increase the level of m6A modification in piglet liver by affecting the mRNA expression

m6A-related medications



other-related medications

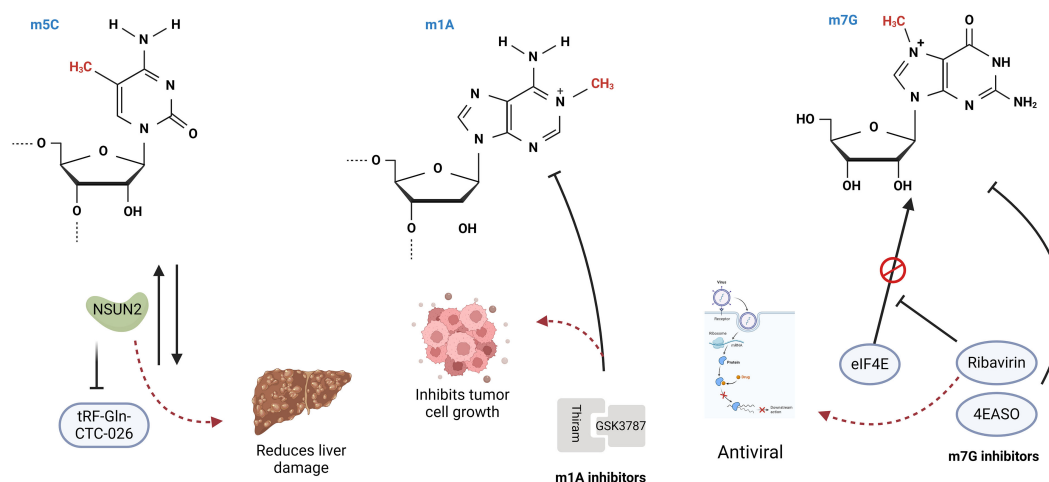


FIGURE 4

RNA modification-related agents. Research has found that some agents act on RNA modified regulators, and can trigger a series of downstream effects. (Created with BioRender.com).

of METTL3, METTL14, ALKBH5, FTO, and YTHDF2 and subsequently attenuate polysaccharide-induced disorders of hepatic lipid metabolism (113).

FTO, a m6A demethylase, has been reported to play important roles in various diseases, including HCC and diabetes mellitus, and is a potential therapeutic for related diseases (114). Entacapone and meclofenamic acid (MA) are reported to be inhibitors of the m6A demethylase FTO (Figure 4) (114–116). As a chemical inhibitor, entacapone inhibits FTO-mediated metabolic regulation through forkhead box O1 (FOXO1) (114). MA, a nonsteroidal anti-inflammatory drug, has been identified as a highly selective FTO inhibitor that competitively binds to FTO, thereby enhancing overall m6A modification levels (97). In the case of inhibitors

targeting another m6A demethylase, ALKBH5, only a small number of nonselective compounds have been discovered (Figure 4). The majority of these compounds are 2OG oxygenase inhibitors, with only a limited few characterized as ALKBH5 inhibitors. Currently, there is a lack of significant selective inhibitors for ALKBH5 (117).

In addition, Hong et al. conducted a structure-based drug screening and identified tegaserod as a potential inhibitor of YTHDF1 (Figure 4) (118). Tegaserod functions by blocking the binding of YTHDF1 to m6A-modified mRNA, thereby inhibiting the YTHDF1-mediated translation of Cyclin E2. This suggests that tegaserod may serve as a promising antitumor agent (119). Modulation of the tumor immune microenvironment has

emerged as a novel approach for cancer immunotherapy in HCC, with m6A modification being an ideal target due to its role in the immune response (120). The expression of IGF2BP1, which recognizes m6A modifications, is significantly upregulated in HCC and is associated with immune cell infiltration (121). Cucurbitacin B has been demonstrated to inhibit the binding of IGF2BP1 to m6A-modified mRNA and increase immune cell infiltration, suggesting that it is a potential anti-HCC agent (Figure 4) (121).

4.2.2 Potential agents targeting m5C

The m5C writing protein is a promising target for the treatment of HCC. The upregulation of the m5C methyltransferase NSUN2 in HCC has been observed (77). Knocking down NSUN2 leads to reduced levels of m5C modification, and some unmodified tRNAs undergo complex changes to become tRNAs (tRF-Gln-CTC-026), which effectively alleviate liver injury by inhibiting global protein synthesis (GPS) (122). Efforts to deplete NSUN2 and provide protective tRNAs are potential treatments for liver injury and have significant implications for reducing the risk of HCC occurrence.

4.2.3 Potential agents targeting m1A

Thiram, a potent inhibitor of the m1A methyltransferase complex TRMT6/TRMT61A, has been shown to significantly inhibit the self-renewal of hepatic CSCs and hepatic tumor growth (Figure 4) (81). Furthermore, when combined with the PPARS antagonist GSK3787, thiram synergistically inhibits the development of HCC and the growth of tumors that are hypermethylated with m1A (81).

4.2.4 Potential agents targeting m7G

Ribavirin is a widely used antiviral medication that has recently been shown to possess antitumor effects as a m7G cap analog that inhibits cell proliferation (Figure 4) (123). Additionally, the eIF4E antisense oligonucleotide drug (4EASO) is a well-established medication that competitively inhibits eIF4E binding to the m7G cap (124). These medications have shown promising results in halting cancer progression and improving prognosis, offering hopeful prospects for the development of additional m7G-targeted drugs.

5 Challenges and opportunities for RNA modification

Currently, research on the role of RNA modification in HCC mainly focuses on m6A, m1A, m5C, and m7G. The expression of RNA modification regulators, including “writer”, “eraser” and “reader” proteins, changed significantly in HCC, and are involved in the proliferation, autophagy, innate immunity, invasion, metastasis, immune cell infiltration, and drug resistance of HCC via different signal axes. The varying expression of RNA modification regulatory proteins with similar functions in HCC, along with the lack of consensus on the expression changes of some

RNA modification regulatory proteins, indicates that the expression of these proteins in HCC may be dynamic and have different mechanisms of action in various stages and types of the disease. Consequently, further comprehensive and detailed classification research is necessary to elucidate this matter. Additionally, additional investigation is required to ascertain the relationships between these various forms of RNA modifications. In addition to the aforementioned modifications, there are still multiple types of RNA modifications involved in regulating post-transcriptional gene expression, including 5-hydroxymethylcytosine, pseudouridine, and 2'-O-methylation. Exploring whether these recently discovered RNA modifications are also involved in the occurrence and development of HCC may be a new research hotspot in the future. As high-throughput sequencing technology and novel biomarkers continue to advance, we will be able to delve deeper into the intricate regulatory network of RNA modifications in HCC, ultimately providing more precise and effective approaches for clinical diagnosis and treatment.

Studying RNA modifications in HCC will contribute to an in-depth understanding of the pathogenesis of HCC, the search for new diagnostic and prognostic markers, the development of new therapeutic strategies, and the assessment of treatment efficacy and the monitoring of recurrence. These findings provide a new perspective for the diagnosis and treatment of HCC by targeting specific RNA-modifying enzymes, recognition proteins, or related RNAs. More than ten RNA-modifying regulators showed the potential for use for the diagnosis, prognosis, and treatment decision utility biomarkers of HCC. KIAA1429 has a relatively high diagnostic value in HCC. For other potential diagnostic markers, further research is needed because of inconsistent expression in different studies and a lack of ROC analysis data for diagnostic markers. WTAP, RALYL, and YTHDF3 exhibit significantly higher prognostic significance in HCC. However, the validation of their prognostic value necessitates extensive multi-center sample validation and ROC curve analysis in the future. On the other hand, prior research has focused on examining these factors separately as prognostic indicators for HCC patients. Conducting a combined analysis of these factors could offer a more comprehensive understanding of the association between RNA-modifying regulators and HCC prognosis. Some RNA-modifying regulators also exhibit an association with targeted anti-cancer drugs or chemotherapy drugs, which provide a theoretical basis for personalized medication for patients. The concurrent administration of drugs and inhibitors of regulatory proteins based on the relationship between regulatory proteins and drug resistance may enhance the sensitivity of drugs and offer novel approaches for managing HCC.

A growing number of RNA modifier inhibitors are being developed, but the lack of preclinical experiments and clinical studies targeting RNA modification in HCC poses a significant obstacle, and further research is needed to evaluate their application value in HCC treatment. Studying RNA modifications in liver cancer necessitates careful consideration of the potential and challenges associated with its translational application. Although RNA modification may present a novel target for HCC treatment, the lack of specific drugs targeting RNA modification poses a

significant obstacle. Many RNA modifications occur at low levels, making it difficult to selectively target and manipulate modified RNA molecules without affecting unmodified ones. Additionally, our understanding of the structural domains of certain RNA modifiers, such as those in the NSUN and TRMT families, remains limited. This lack of knowledge makes it even more challenging to design inhibitors that effectively target these proteins. Therefore, further investigation into the mechanism of action of RNA modification is imperative to identify effective strategies for regulating this process and to evaluate its safety and efficacy. The development of effective tools and techniques for targeting and manipulating specific RNA modifications is an ongoing challenge. Overcoming these obstacles will pave the way for the development of targeted therapies and interventions that can harness the potential of RNA modifications in various biological and disease settings.

6 Conclusion

RNA modifications play a crucial role in HCC progression. Specific RNA modification pathways, such as m6A, m5C, m1A, and m7G, are erroneously regulated by a series of modification regulatory proteins, thereby regulating the expression of HCC-associated genes, and are involved in the proliferation, autophagy, innate immunity, invasion, metastasis, immune cell infiltration, and drug resistance of HCC. These functional roles and molecular mechanism advancements of RNA modification offer new perspectives on the pathogenesis, as well as potential new diagnostic and prognostic markers and therapeutic strategies of HCC. At present, over ten RNA-modifying regulators have displayed promise as biomarkers for the diagnosis, prognosis, and treatment decisions related to HCC. However, the practical application of these biomarkers in HCC necessitates thorough validation across multiple centers in the future. Agents targeting RNA modification are potential therapeutic strategies for HCC. The concurrent administration with the inhibitors of RNA modification may enhance the sensitivity of drugs and offer novel approaches for

managing HCC. While a growing number of RNA modifier inhibitors are being developed, the absence of preclinical and clinical studies focusing on targeting RNA modification in HCC presents a significant challenge. Further investigation is crucial to assess the efficacy of these inhibitors in the treatment of HCC.

Author contributions

J-XL: Writing – original draft, Writing – review & editing. XZ: Writing – review & editing. W-HX: Funding acquisition, Writing – review & editing. X-DH: Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by the Shandong Provincial Natural Science Foundation, China (ZR2020MC059 and RH2200000157).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

- Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* (2024) 74:229–63. doi: 10.3322/caac.21834
- Huang DQ, Mathurin P, Cortez-Pinto H, Loomba R. Global epidemiology of alcohol-associated cirrhosis and hcc: trends, projections and risk factors. *Nat Rev Gastroenterol Hepatol.* (2023) 20:37–49. doi: 10.1038/s41575-022-00688-6
- Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: Cancer J Clin.* (2021) 71:209–49. doi: 10.3322/caac.21660
- Singal AG, Kanwal F, Llovet JM. Global trends in hepatocellular carcinoma epidemiology: implications for screening, prevention and therapy. *Nat Rev Clin Oncol.* (2023) 20:864–84. doi: 10.1038/s41571-023-00825-3
- Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, et al. Hepatocellular carcinoma. *Nat Rev Dis Primers.* (2021) 7:6. doi: 10.1038/s41572-021-00245-6
- Kostyusheva A, Brezgin S, Glebe D, Kostyushev D, Chulanov V. Host-cell interactions in hcv infection and pathogenesis: the emerging role of M6a modification. *Emerg Microbes infect.* (2021) 10:2264–75. doi: 10.1080/22221751.2021.2006580
- Wang Y, Deng B. Hepatocellular carcinoma: molecular mechanism, targeted therapy, and biomarkers. *Cancer metastasis Rev.* (2023) 42:629–52. doi: 10.1007/s10555-023-10084-4
- Chidambaramathan-Reghupaty S, Fisher PB, Sarkar D. Hepatocellular carcinoma (Hcc): epidemiology, etiology and molecular classification. *Adv Cancer Res.* (2021) 149:1–61. doi: 10.1016/bs.acr.2020.10.001
- Yegin EG, Oymaci E, Karatay E, Coker A. Progress in surgical and nonsurgical approaches for hepatocellular carcinoma treatment. *Hepatobil Pancreat Dis Int.* (2016) 15:234–56. doi: 10.1016/s1499-3872(16)60097-8
- Delis SG, Dervenis C. Selection criteria for liver resection in patients with hepatocellular carcinoma and chronic liver disease. *World J Gastroenterol.* (2008) 14:3452–60. doi: 10.3748/wjg.14.3452

11. Jiang C, Cai YQ, Yang JJ, Ma CY, Chen JX, Huang L, et al. Radiomics in the diagnosis and treatment of hepatocellular carcinoma. *Hepatobil Pancreat Dis Int.* (2023) 22:346–51. doi: 10.1016/j.hbpd.2023.03.010
12. Vogel A, Meyer T, Sapisochin G, Salem R, Saborowski A. Hepatocellular carcinoma. *Lancet (London England).* (2022) 400:1345–62. doi: 10.1016/s0140-6736(22)01200-4
13. Barbieri I, Kouzarides T. Role of rna modifications in cancer. *Nat Rev Cancer.* (2020) 20:303–22. doi: 10.1038/s41568-020-0253-2
14. Li S, Mehal WZ, Ouyang X. Rna modifications in the progression of liver diseases: from fatty liver to cancer. *Sci China Life Sci.* (2024). doi: 10.1007/s11427-023-2494-x
15. Feng Q, Wang D, Xue T, Lin C, Gao Y, Sun L, et al. The role of rna modification in hepatocellular carcinoma. *Front Pharmacol.* (2022) 13:984453. doi: 10.3389/fphar.2022.984453
16. Chen L, Gao Y, Xu S, Yuan J, Wang M, Li T, et al. N6-methyladenosine reader ythdf family in biological processes: structures, roles, and mechanisms. *Front Immunol.* (2023) 14:1162607. doi: 10.3389/fimmu.2023.1162607
17. Li D, Li K, Zhang W, Yang KW, Mu DA, Jiang GJ, et al. The M6a/M5c/M1a regulated gene signature predicts the prognosis and correlates with the immune status of hepatocellular carcinoma. *Front Immunol.* (2022) 13:918140. doi: 10.3389/fimmu.2022.918140
18. Dunn DB, Smith JD. Occurrence of a new base in the deoxyribonucleic acid of a strain of bacterium coli. *Nature.* (1955) 175:336–7. doi: 10.1038/175336a0
19. Desrosiers R, Friderici K, Rottman F. Identification of methylated nucleosides in messenger rna from novikoff hepatoma cells. *Proc Natl Acad Sci United States America.* (1974) 71:3971–5. doi: 10.1073/pnas.71.10.3971
20. Dubin DT, Taylor RH. The methylation state of poly a-containing messenger rna from cultured hamster cells. *Nucleic Acids Res.* (1975) 2:1653–68. doi: 10.1093/nar/2.10.1653
21. Adams JM, Cory S. Modified nucleosides and bizarre 5'-termini in mouse myeloma mrna. *Nature.* (1975) 255:28–33. doi: 10.1038/255028a0
22. Perry RP, Kelley DE, Friderici K, Rottman F. The methylated constituents of L cell messenger rna: evidence for an unusual cluster at the 5' Terminus. *Cell.* (1975) 4:387–94. doi: 10.1016/0092-8674(75)90159-2
23. Ontiveros RJ, Stoute J, Liu KF. The chemical diversity of rna modifications. *Biochem J.* (2019) 476:1227–45. doi: 10.1042/bcj20180445
24. Tang Q, Li L, Wang Y, Wu P, Hou X, Ouyang J, et al. Rna modifications in cancer. *Br J Cancer.* (2023) 129:204–21. doi: 10.1038/s41416-023-02275-1
25. Yang Z, Zhang S, Xia T, Fan Y, Shan Y, Zhang K, et al. Rna modifications meet tumors. *Cancer Manage Res.* (2022) 14:3223–43. doi: 10.2147/cmar.S391067
26. Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, et al. N6-methyladenosine in nuclear rna is a major substrate of the obesity-associated fto. *Nat Chem Biol.* (2011) 7:885–7. doi: 10.1038/nchembio.687
27. Zhu ZM, Huo FC, Pei DS. Function and evolution of rna N6-methyladenosine modification. *Int J Biol Sci.* (2020) 16:1929–40. doi: 10.7150/ijbs.45231
28. Rao X, Lai L, Li X, Wang L, Li A, Yang Q. N(6) -methyladenosine modification of circular rna circ-arl3 facilitates hepatitis B virus-associated hepatocellular carcinoma via sponging mir-1305. *IUBMB Life.* (2021) 73:408–17. doi: 10.1002/iub.2438
29. Liu R, Yin G, Tuo H, Guo Y, Zhu Y, Zhang L, et al. Mettl3-induced lncrna gbp1 promotes hepatocellular carcinoma progression by activating bmp/smad pathway. *Biol direct.* (2023) 18:53. doi: 10.1186/s13062-023-00409-2
30. Zuo X, Chen Z, Gao W, Zhang Y, Wang J, Wang J, et al. M6a-mediated upregulation of linc00958 increases lipogenesis and acts as a nanotherapeutic target in hepatocellular carcinoma. *J Hematol Oncol.* (2020) 13:5. doi: 10.1186/s13045-019-0839-x
31. Xia A, Yuan W, Wang Q, Xu J, Gu Y, Zhang L, et al. The cancer-testis lncrna lnc-thcc promotes hepatocellular carcinogenesis by binding hnnp K and activating yap1 transcription. *Nat Cancer.* (2022) 3:203–18. doi: 10.1038/s43018-021-00315-4
32. Tao L, Li D, Mu S, Tian G, Yan G. Lncrna mapkapk5_As1 facilitates cell proliferation in hepatitis B virus -related hepatocellular carcinoma. *Lab investigation; J Tech Methods Pathol.* (2022) 102:494–504. doi: 10.1038/s41374-022-00731-9
33. Kim GW, Imam H, Khan M, Mir SA, Kim SJ, Yoon SK, et al. Hbv-induced increased N6 methyladenosine modification of pten rna affects innate immunity and contributes to hcc. *Hepatology (Baltimore Md).* (2021) 73:533–47. doi: 10.1002/hep.31313
34. Wang A, Chen X, Li D, Yang L, Jiang J. Mettl3-mediated M6a methylation of aspm drives hepatocellular carcinoma cells growth and metastasis. *J Clin Lab Anal.* (2021) 35:e23931. doi: 10.1002/jcla.23931
35. Wang L, Yang Q, Zhou Q, Fang F, Lei K, Liu Z, et al. Mettl3-M(6)a-egfr-axis drives lenvatinib resistance in hepatocellular carcinoma. *Cancer Lett.* (2023) 559:216122. doi: 10.1016/j.canlet.2023.216122
36. Yang Y, Qian Cai Q, Sheng Fu L, Wei Dong Y, Fan F, Zhong Wu X. Reduced N6-methyladenosine mediated by mettl3 acetylation promotes mtf1 expression and hepatocellular carcinoma cell growth. *Chem biodivers.* (2022) 19:e202200333. doi: 10.1002/cbdv.202200333
37. Xu H, Wang H, Zhao W, Fu S, Li Y, Ni W, et al. Sumo1 modification of methyltransferase-like 3 promotes tumor progression via regulating snail mrna homeostasis in hepatocellular carcinoma. *Theranostics.* (2020) 10:5671–86. doi: 10.7150/thno.42539
38. Lu J, Ru J, Chen Y, Ling Z, Liu H, Ding B, et al. N(6) -methyladenosine-modified circstx6 promotes hepatocellular carcinoma progression by regulating the hnmpd/atf3 axis and encoding a 144 amino acid polypeptide. *Clin Trans Med.* (2023) 13:e1451. doi: 10.1002/ctm2.1451
39. Du L, Li Y, Kang M, Feng M, Ren Y, Dai H, et al. Usp48 is upregulated by mettl14 to attenuate hepatocellular carcinoma via regulating sirt6 stabilization. *Cancer Res.* (2021) 81:3822–34. doi: 10.1158/0008-5472.Can-20-4163
40. Zhou T, Li S, Xiang D, Liu J, Sun W, Cui X, et al. M6a rna methylation-mediated hnf3γ Reduction renders hepatocellular carcinoma dedifferentiation and sorafenib resistance. *Signal transduct target Ther.* (2020) 5:296. doi: 10.1038/s41392-020-00299-0
41. Shi Y, Zhuang Y, Zhang J, Chen M, Wu S. Mettl14 inhibits hepatocellular carcinoma metastasis through regulating egfr/pi3k/akt signaling pathway in an M6a-dependent manner. *Cancer Manage Res.* (2020) 12:13173–84. doi: 10.2147/CMAR.S286275
42. Yang Y, Cai J, Yang X, Wang K, Sun K, Yang Z, et al. Dysregulated M6a modification promotes lipogenesis and development of non-alcoholic fatty liver disease and hepatocellular carcinoma. *Mol therapy: J Am Soc Gene Ther.* (2022) 30:2342–53. doi: 10.1016/j.jymthe.2022.02.021
43. Chen Y, Peng C, Chen J, Chen D, Yang B, He B, et al. Wtap facilitates progression of hepatocellular carcinoma via M6a-hur-dependent epigenetic silencing of ets1. *Mol Cancer.* (2019) 18:127. doi: 10.1186/s12943-019-1053-8
44. Li G, Deng L, Huang N, Cui Z, Wu Q, Ma J, et al. M(6)a rna methylation regulates lkb1 to promote autophagy of hepatoblastoma cells through upregulated phosphorylation of ampk. *Genes.* (2021) 12:1747. doi: 10.3390/genes12111747
45. Zhang Q, Wei T, Yan L, Zhu S, Jin W, Bai Y, et al. Hypoxia-responsive lncrna ac115619 encodes a micropeptide that suppresses M6a modifications and hepatocellular carcinoma progression. *Cancer Res.* (2023) 83:2496–512. doi: 10.1158/0008-5472.Can-23-0337
46. Kuang Y, Cheng Y, Wang J, Li H, Cao X, Wang Y. Kiaa1429 mediates epithelial mesenchymal transition in sorafenib-resistant hepatocellular carcinoma through M6a methylation modification. *Cancer Med.* (2023) 12:7222–33. doi: 10.1002/cam4.5432
47. Lan T, Li H, Zhang D, Xu L, Liu H, Hao X, et al. Kiaa1429 contributes to liver cancer progression through N6-methyladenosine-dependent post-transcriptional modification of gata3. *Mol Cancer.* (2019) 18:186. doi: 10.1186/s12943-019-1106-z
48. Chen Y, Zhao Y, Chen J, Peng C, Zhang Y, Tong R, et al. Alkbh5 suppresses malignancy of hepatocellular carcinoma via M(6)a-guided epigenetic inhibition of lypd1. *Mol Cancer.* (2020) 19:123. doi: 10.1186/s12943-020-01239-w
49. Zhang H, Liu Y, Wang W, Liu F, Wang W, Su C, et al. Alkbh5-mediated M(6)a modification of lincrna linc02551 enhances the stability of ddx24 to promote hepatocellular carcinoma growth and metastasis. *Cell Death Dis.* (2022) 13:926. doi: 10.1038/s41419-022-05386-4
50. You Y, Wen D, Zeng L, Lu J, Xiao X, Chen Y, et al. Alkbh5/map3k8 axis regulates pd-L1+ Macrophage infiltration and promotes hepatocellular carcinoma progression. *Int J Biol Sci.* (2022) 18:5001–18. doi: 10.7150/ijbs.70149
51. Qu S, Jin L, Huang H, Lin J, Gao W, Zeng Z. A positive-feedback loop between hbx and alkbh5 promotes hepatocellular carcinogenesis. *BMC Cancer.* (2021) 21:686. doi: 10.1186/s12885-021-08449-5
52. Wang F, Hu Y, Wang H, Hu P, Xiong H, Zeng Z, et al. Lncrna fto-it1 promotes glycolysis and progression of hepatocellular carcinoma through modulating fto-mediated N6-methyladenosine modification on glut1 and pkm2. *J Exp Clin Cancer res: CR.* (2023) 42:267. doi: 10.1186/s13046-023-02847-2
53. Liu X, Liu J, Xiao W, Zeng Q, Bo H, Zhu Y, et al. Sirt1 regulates N(6) -methyladenosine rna modification in hepatocarcinogenesis by inducing ranbp2-dependent fto sumoylation. *Hepatology (Baltimore Md).* (2020) 72:2029–50. doi: 10.1002/hep.31222
54. Wang X, Wang J, Tsui YM, Shi C, Wang Y, Zhang X, et al. Ralyl increases hepatocellular carcinoma stemness by sustaining the mrna stability of tgfb-β2. *Nat Commun.* (2021) 12:1518. doi: 10.1038/s41467-021-21828-7
55. Wu S, Liu S, Cao Y, Chao G, Wang P, Pan H. Downregulation of zc3h13 by mir-362-3p/mir-425-5p is associated with a poor prognosis and adverse outcomes in hepatocellular carcinoma. *Aging.* (2022) 14:2304–19. doi: 10.18632/aging.203939
56. Du A, Li S, Zhou Y, Disoma C, Liao Y, Zhang Y, et al. M6a-mediated upregulation of circmdk promotes tumorigenesis and acts as a nanotherapeutic target in hepatocellular carcinoma. *Mol Cancer.* (2022) 21:109. doi: 10.1186/s12943-022-01575-z
57. Duan JL, Chen W, Xie JJ, Zhang ML, Nie RC, Liang H, et al. A novel peptide encoded by N6-methyladenosine modified circmap3k4 prevents apoptosis in hepatocellular carcinoma. *Mol Cancer.* (2022) 21:93. doi: 10.1186/s12943-022-01537-5
58. Zhu Y, Xiao B, Liu M, Chen M, Xia N, Guo H, et al. N6-methyladenosine-modified oncofetal lncrna mir4435-2hg contributed to stemness features of hepatocellular carcinoma cells by regulating rna 2'-O methylation. *Cell Mol Biol Lett.* (2023) 28:89. doi: 10.1186/s11658-023-00493-2
59. Yang Y, Wu J, Liu F, He J, Wu F, Chen J, et al. Igf2bp1 promotes the liver cancer stem cell phenotype by regulating mgat5 mrna stability by M6a rna methylation. *Stem Cells Dev.* (2021) 30:1115–25. doi: 10.1089/scd.2021.0153

60. Li Q, Ni Y, Zhang L, Jiang R, Xu J, Yang H, et al. Hif-1 α -induced expression of M6a reader ythdf1 drives hypoxia-induced autophagy and Malignancy of hepatocellular carcinoma by promoting atg2a and atg14 translation. *Signal transduct target Ther.* (2021) 6:76. doi: 10.1038/s41392-020-00453-8
61. Zhang C, Huang S, Zhuang H, Ruan S, Zhou Z, Huang K, et al. Ythdf2 promotes the liver cancer stem cell phenotype and cancer metastasis by regulating oct4 expression via M6a rna methylation. *Oncogene.* (2020) 39:4507–18. doi: 10.1038/s41388-020-1303-7
62. Wang M, Yang Y, Yang J, Yang J, Han S. Circ-Kiaa1429 accelerates hepatocellular carcinoma advancement through the mechanism of M(6)a-ythdf3-zeb1. *Life Sci.* (2020) 257:118082. doi: 10.1016/j.lfs.2020.118082
63. Hu B, Gao J, Shi J, Wen P, Guo W, Zhang S. M(6) a Reader Ythdf3 Triggers the Progression of Hepatocellular Carcinoma through the Ythdf3/M(6) a-Egfr/Stat3 Axis and Emt. *Mol carcinogene.* (2023) 62:1599–614. doi: 10.1002/mc.23602
64. Zhou R, Ni W, Qin C, Zhou Y, Li Y, Huo J, et al. A functional loop between yth domain family protein ythdf3 mediated M(6)a modification and phosphofructokinase pfkl in glycolysis of hepatocellular carcinoma. *J Exp Clin Cancer Res.* (2022) 41:334. doi: 10.1186/s13046-022-02538-4
65. Wang H, Tang A, Cui Y, Gong H, Li H. Lrp1 facilitates tumor progression and immune evasion through upregulation of M(6)a modification of pd-L1 rna in hepatocellular carcinoma. *Front Immunol.* (2023) 14:1144774. doi: 10.3389/fimmu.2023.1144774
66. Liu H, Jiang Y, Lu J, Peng C, Ling Z, Chen Y, et al. M(6)a-modification regulated circ-ctc3 acts as the sponge of mir-378a-3p to promote hepatocellular carcinoma progression. *Epigenetics.* (2023) 18:2204772. doi: 10.1080/15592294.2023.2204772
67. Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang CM, Li CJ, et al. Alkbh5 is a mammalian rna demethylase that impacts rna metabolism and mouse fertility. *Mol Cell.* (2013) 49:18–29. doi: 10.1016/j.molcel.2012.10.015
68. Lence T, Paolantoni C, Worpenberg L, Roignant JY. Mechanistic insights into M(6)a rna enzymes. *Biochim Biophys Acta Gene Regul Mech.* (2019) 1862:222–9. doi: 10.1016/j.bbargm.2018.10.014
69. Xiong M, Ou C, Yu C, Qiu J, Lu J, Fu C, et al. Qi-shen-tang alleviates retinitis pigmentosa by inhibiting ferroptotic features via the nrf2/gpx4 signaling pathway. *Heliyon.* (2023) 9:e22443. doi: 10.1016/j.heliyon.2023.e22443
70. Huang H, Weng H, Sun W, Qin X, Shi H, Wu H, et al. Recognition of rna N(6)-methyladenosine by igf2bp proteins enhances mrna stability and translation. *Nat Cell Biol.* (2018) 20:285–95. doi: 10.1038/s41556-018-0045-z
71. Ramesh-Kumar D, Guil S. The igf2bp family of rna binding proteins links epitranscriptomics to cancer. *Semin Cancer Biol.* (2022) 86:18–31. doi: 10.1016/j.semcancer.2022.05.009
72. Courtney DG, Tsai K, Bogerd HP, Kennedy EM, Law BA, Emery A, et al. Epitranscriptomic addition of M(5)C to hiv-1 transcripts regulates viral gene expression. *Cell Host Microbe.* (2019) 26:217–27.e6. doi: 10.1016/j.chom.2019.07.005
73. Reid R, Greene PJ, Santi DV. Exposition of a family of rna M(5)C methyltransferases from searching genomic and proteomic sequences. *Nucleic Acids Res.* (1999) 27:3138–45. doi: 10.1093/nar/27.15.3138
74. Song H, Zhang J, Liu B, Xu J, Cai B, Yang H, et al. Biological roles of rna M(5)C modification and its implications in cancer immunotherapy. *biomark Res.* (2022) 10:15. doi: 10.1186/s40364-022-00362-8
75. Kong W, Biswas A, Zhou D, Fiches G, Fujinaga K, Santoso N, et al. Nucleolar protein nop2/nsun1 suppresses hiv-1 transcription and promotes viral latency by competing with tat for tar binding and methylation. *PloS Pathog.* (2020) 16:e1008430. doi: 10.1371/journal.ppat.1008430
76. Zhou Y, Kong Y, Fan W, Tao T, Xiao Q, Li N, et al. Principles of rna methylation and their implications for biology and medicine. *Biomed pharmacother = Biomed pharmacother.* (2020) 131:110731. doi: 10.1016/j.biopha.2020.110731
77. Sun Z, Xue S, Zhang M, Xu H, Hu X, Chen S, et al. Aberrant nsun2-mediated M(5)C modification of H19 lncrna is associated with poor differentiation of hepatocellular carcinoma. *Oncogene.* (2020) 39:6906–19. doi: 10.1038/s41388-020-01475-w
78. Song D, An K, Zhai W, Feng L, Xu Y, Sun R, et al. Nsun2-mediated mrna M(5)C modification regulates the progression of hepatocellular carcinoma. *Genom Proteomics Bioinf.* (2023) 21:823–33. doi: 10.1016/j.gpb.2022.09.007
79. Sun GF, Ding H. Nop2-mediated M5c methylation of xpd is associated with hepatocellular carcinoma progression. *Neoplasma.* (2023) 70:340–9. doi: 10.4149/neo_2023_230110N17
80. Nulali J, Zhang K, Long M, Wan Y, Liu Y, Zhang Q, et al. Alyref-mediated rna 5-methylcytosine modification promotes hepatocellular carcinoma progression via stabilizing egfr mrna and pstat3 activation. *Int J Biol Sci.* (2024) 20:331–46. doi: 10.7150/ijbs.82316
81. Wang Y, Wang J, Li X, Xiong X, Wang J, Zhou Z, et al. N(1)-methyladenosine methylation in trna drives liver tumorigenesis by regulating cholesterol metabolism. *Nat Commun.* (2021) 12:6314. doi: 10.1038/s41467-021-26718-6
82. Wang Q, Wang G, Wang Y, Liu C, He X. Association of alkb homolog 3 expression with tumor recurrence and unfavorable prognosis in hepatocellular carcinoma. *J Gastroenterol Hepatol.* (2018) 33:1617–25. doi: 10.1111/jgh.14117
83. Liu M, Zhao Z, Cai Y, Bi P, Liang Q, Yan Y, et al. Yth domain family: potential prognostic targets and immune-associated biomarkers in hepatocellular carcinoma. *Aging.* (2021) 13:24205–18. doi: 10.18632/aging.203674
84. Xia P, Zhang H, Xu K, Jiang X, Gao M, Wang G, et al. Myc-targeted wdr4 promotes proliferation, metastasis, and sorafenib resistance by inducing ccnb1 translation in hepatocellular carcinoma. *Cell Death Dis.* (2021) 12:691. doi: 10.1038/s41419-021-03973-5
85. Han WY, Wang J, Zhao J, Zheng YM, Chai XQ, Gao C, et al. Wdr4/trim28 is a novel molecular target linked to lenvatinib resistance that helps retain the stem characteristics in hepatocellular carcinomas. *Cancer Lett.* (2023) 568:216259. doi: 10.1016/j.canlet.2023.216259
86. Chen Z, Zhu W, Zhu S, Sun K, Liao J, Liu H, et al. Mettl1 promotes hepatocarcinogenesis via M(7) G trna modification-dependent translation control. *Clin Trans Med.* (2021) 11:e661. doi: 10.1002/ctm2.661
87. Huang M, Long J, Yao Z, Zhao Y, Zhao Y, Liao J, et al. Mettl1-mediated M7g trna modification promotes lenvatinib resistance in hepatocellular carcinoma. *Cancer Res.* (2023) 83:89–102. doi: 10.1158/0008-5472.Can-22-0963
88. Zhu S, Wu Y, Zhang X, Peng S, Xiao H, Chen S, et al. Targeting N(7)-methylguanosine trna modification blocks hepatocellular carcinoma metastasis after insufficient radiofrequency ablation. *Mol therapy: J Am Soc Gene Ther.* (2023) 31:1596–614. doi: 10.1016/j.ymthe.2022.08.004
89. Li J, Zhang H, Wang H. N(1)-methyladenosine modification in cancer biology: current status and future perspectives. *Comput Struct Biotechnol J.* (2022) 20:6578–85. doi: 10.1016/j.csbj.2022.11.045
90. Tong C, Wang W, He C. M1a methylation modification patterns and metabolic characteristics in hepatocellular carcinoma. *BMC Gastroenterol.* (2022) 22:93. doi: 10.1186/s12876-022-02160-w
91. Saikia M, Fu Y, Pavon-Eternod M, He C, Pan T. Genome-wide analysis of N1-methyl-adenosine modification in human trnas. *RNA (New York NY).* (2010) 16:1317–27. doi: 10.1261/rna.2057810
92. Helm M, Brulé H, Degoul F, Cepanec C, Leroux JP, Giegé R, et al. The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial trna. *Nucleic Acids Res.* (1998) 26:1636–43. doi: 10.1093/nar/26.7.1636
93. Kawarada L, Suzuki T, Ohira T, Hirata S, Miyauchi K, Suzuki T. Alkbh1 is an rna dioxigenase responsible for cytoplasmic and mitochondrial trna modifications. *Nucleic Acids Res.* (2017) 45:7401–15. doi: 10.1093/nar/gkx354
94. Roundtree IA, Evans ME, Pan T, He C. Dynamic rna modifications in gene expression regulation. *Cell.* (2017) 169:1187–200. doi: 10.1016/j.cell.2017.05.045
95. Zhang LS, Liu C, Ma H, Dai Q, Sun HL, Luo G, et al. Transcriptome-wide mapping of internal N(7)-methylguanosine methylome in mammalian mrna. *Mol Cell.* (2019) 74:1304–16.e8. doi: 10.1016/j.molcel.2019.03.036
96. Malbec L, Zhang T, Chen YS, Zhang Y, Sun BF, Shi BY, et al. Dynamic methylome of internal mrna N(7)-methylguanosine and its regulatory role in translation. *Cell Res.* (2019) 29:927–41. doi: 10.1038/s41422-019-0230-z
97. Azzam SK, Alsafar H, Sajini AA. Fto M6a demethylase in obesity and cancer: implications and underlying molecular mechanisms. *Int J Mol Sci.* (2022) 23:3800. doi: 10.3390/ijms23073800
98. Han Y, Liu D, Li L. Pd-1/pd-L1 pathway: current researches in cancer. *Am J Cancer Res.* (2020) 10:727–42.
99. Cui M, Qu F, Wang L, Liu X, Yu J, Tang Z, et al. M5c rna methyltransferase-related gene nsun4 stimulates Malignant progression of hepatocellular carcinoma and can be a prognostic marker. *Cancer biomark: sect A Dis Markers.* (2022) 33:389–400. doi: 10.3233/cbm-210154
100. Xue C, Gu X, Zheng Q, Shi Q, Yuan X, Su Y, et al. Alyref mediates rna M(5)C modification to promote hepatocellular carcinoma progression. *Signal transduct target Ther.* (2023) 8:130. doi: 10.1038/s41392-023-01395-7
101. Rengaraj P, Obrdlik A, Vukic D, Varadarajan NM, Keegan LP, Vanacova S, et al. Interplays of different types of epitranscriptomic mrna modifications. *RNA Biol.* (2021) 18:19–30. doi: 10.1080/15476286.2021.1969113
102. Fu Y, Dominissini D, Rechavi G, He C. Gene expression regulation mediated through reversible M(6)a rna methylation. *Nat Rev Genet.* (2014) 15:293–306. doi: 10.1038/nrg3724
103. Akichika S, Hirano S, Shichino Y, Suzuki T, Nishimasu H, Ishitani R, et al. Cap-specific terminal N(6)-methylation of rna by an rna polymerase ii-associated methyltransferase. *Science.* (2019) 363:eaav0080. doi: 10.1126/science.aav0080
104. Li J, Zhu L, Shi Y, Liu J, Lin L, Chen X. M6a demethylase fto promotes hepatocellular carcinoma tumorigenesis via mediating pkm2 demethylation. *Am J Transl Res.* (2019) 11:6084–92.
105. Liu L, Gu M, Ma J, Wang Y, Li M, Wang H, et al. Circgpr137b/mir-4739/fto feedback loop suppresses tumorigenesis and metastasis of hepatocellular carcinoma. *Mol Cancer.* (2022) 21:149. doi: 10.1186/s12943-022-01619-4
106. Xue C, Zhao Y, Li G, Li L. Multi-omic analyses of the M(5)C regulator alyref reveal its essential roles in hepatocellular carcinoma. *Front Oncol.* (2021) 11:633415. doi: 10.3389/fonc.2021.633415
107. Liu X, Qin J, Gao T, Li C, Chen X, Zeng K, et al. Analysis of mettl3 and mettl14 in hepatocellular carcinoma. *Aging.* (2020) 12:21638–59. doi: 10.18632/aging.103959
108. Yang TS, Chang HK, Chen JS, Lin YC, Liao CT, Chang WC. Chemotherapy using 5-fluorouracil, mitoxantrone, and cisplatin for patients with advanced hepatocellular carcinoma: an analysis of 63 cases. *J Gastroenterol.* (2004) 39:362–9. doi: 10.1007/s00535-003-1303-8

109. Fiorentino F, Menna M, Rotili D, Valente S, Mai A. Mettl3 from target validation to the first small-molecule inhibitors: A medicinal chemistry journey. *J medicinal Chem.* (2023) 66:1654–77. doi: 10.1021/acs.jmedchem.2c01601
110. Dolbois A, Bedi RK, Bochenkova E, Müller A, Moroz-Omori EV, Huang D, et al. 1,4,9-triazaspiro[5.5]undecan-2-one derivatives as potent and selective mettl3 inhibitors. *J medicinal Chem.* (2021) 64:12738–60. doi: 10.1021/acs.jmedchem.1c00773
111. Xu P, Ge R. Roles and drug development of mettl3 (Methyltransferase-like 3) in anti-tumor therapy. *Eur J medicinal Chem.* (2022) 230:114118. doi: 10.1016/j.ejmech.2022.114118
112. Giordano A, Tommonaro G. Curcumin and cancer. *Nutrients.* (2019) 11:2376. doi: 10.3390/nu11102376
113. Lu N, Li X, Yu J, Li Y, Wang C, Zhang L, et al. Curcumin attenuates lipopolysaccharide-induced hepatic lipid metabolism disorder by modification of M (6) a rna methylation in piglets. *Lipids.* (2018) 53:53–63. doi: 10.1002/lipd.12023
114. Peng S, Xiao W, Ju D, Sun B, Hou N, Liu Q, et al. Identification of entacapone as a chemical inhibitor of fto mediating metabolic regulation through foxo1. *Sci Trans Med.* (2019) 11:eau7116. doi: 10.1126/scitranslmed.aau7116
115. Huang Y, Yan J, Li Q, Li J, Gong S, Zhou H, et al. Meclofenamic acid selectively inhibits fto demethylation of M6a over alkhh5. *Nucleic Acids Res.* (2015) 43:373–84. doi: 10.1093/nar/gku1276
116. Su R, Dong L, Li Y, Gao M, Han L, Wunderlich M, et al. Targeting fto suppresses cancer stem cell maintenance and immune evasion. *Cancer Cell.* (2020) 38:79–96.e11. doi: 10.1016/j.ccell.2020.04.017
117. Shen D, Wang B, Gao Y, Zhao L, Bi Y, Zhang J, et al. Detailed resume of rna M(6) a demethylases. *Acta Pharm Sin B.* (2022) 12:2193–205. doi: 10.1016/j.apsb.2022.01.003
118. Hong YG, Yang Z, Chen Y, Liu T, Zheng Y, Zhou C, et al. The rna M6a reader ythdf1 is required for acute myeloid leukemia progression. *Cancer Res.* (2023) 83:845–60. doi: 10.1158/0008-5472.Can-21-4249
119. Micaelli M, Dalle Vedove A, Cerofolini L, Vigna J, Sighel D, Zaccara S, et al. Small-molecule ebsele binds to ythdf proteins interfering with the recognition of N (6)-methyladenosine-modified rnas. *ACS Pharmacol Trans Sci.* (2022) 5:872–91. doi: 10.1021/acspsci.2c00008
120. Cao X, Geng Q, Fan D, Wang Q, Wang X, Zhang M, et al. M(6)a methylation: A process reshaping the tumour immune microenvironment and regulating immune evasion. *Mol Cancer.* (2023) 22:42. doi: 10.1186/s12943-022-01704-8
121. Liu Y, Guo Q, Yang H, Zhang XW, Feng N, Wang JK, et al. Allosteric regulation of igf2bp1 as a novel strategy for the activation of tumor immune microenvironment. *ACS Cent Sci.* (2022) 8:1102–15. doi: 10.1021/acscentsci.2c00107
122. Ying S, Li P, Wang J, Chen K, Zou Y, Dai M, et al. Trf-gln-ctg-026 ameliorates liver injury by alleviating global protein synthesis. *Signal transduct target Ther.* (2023) 8:144. doi: 10.1038/s41392-023-01351-5
123. Kentsis A, Volpon L, Topisirovic I, Soll CE, Culjkovic B, Shao L, et al. Further evidence that ribavirin interacts with eif4e. *RNA (New York NY).* (2005) 11:1762–6. doi: 10.1261/rna.2238705
124. Cai M, Yang C, Wang Z. N7-methylguanosine modification: from regulatory roles to therapeutic implications in cancer. *Am J Cancer Res.* (2023) 13:1640–55.



OPEN ACCESS

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RECEIVED 12 July 2024

ACCEPTED 02 September 2024

PUBLISHED 20 September 2024

CITATION

Qin X, Liu H, Zhang Q, Che Y, Lei T, Tang F
and Hu Q (2024) RNA modifications in
cancer immune therapy: regulators of
immune cells and immune checkpoints.
Front. Immunol. 15:1463847.
doi: 10.3389/fimmu.2024.1463847

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RNA modifications in cancer immune therapy: regulators of immune cells and immune checkpoints

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RNA modifications are epigenetic changes that alter the structure and function of RNA molecules, playing a crucial role in the onset, progression, and treatment of cancer. Immune checkpoint inhibitor (ICI) therapies, particularly PD-1 blockade and anti-CTLA-4 treatments, have changed the treatment landscape of various cancers, showing great potential in the treatment of different cancer patients, but sensitivity to these therapies is limited to certain individuals. This review offers a comprehensive survey of the functions and therapeutic implications of the four principal RNA modifications, particularly highlighting the significance of m6A in the realms of immune cells in tumor and immunotherapy. This review starts by providing a foundational summary of the roles RNA modifications assume within the immune cell community, focusing on T cells, NK cells, macrophages, and dendritic cells. We then discuss how RNA modifications influence the intricate regulatory mechanisms governing immune checkpoint expression, modulation of ICI efficacy, and prediction of ICI treatment outcomes, and review drug therapies targeting genes regulated by RNA modifications. Finally, we explore the role of RNA modifications in gene editing, cancer vaccines, and adoptive T cell therapies, offering valuable insights into the use of RNA modifications in cancer immunotherapy.

KEYWORDS

RNA modification, cancer, immune checkpoint, immune cell, immune therapy

Introduction

RNA modification is an epigenetic change that alters the structure and function of RNA molecules by inserting, deleting, or substituting nucleotides at specific locations, playing a key role in cellular physiology and pathology. Currently, the roster of RNA modifications that have been pinpointed has extended to over 170 distinct subtypes (1), including methylation, acetylation, pseudouridination, among others.

RNA methylation modifications are regulated by specific proteins, including methyltransferase “writers” that add marks, demethylase “erasers” that remove them, and “readers” that recognize these modifications. Among RNA modifications, N1-methyladenosine (m1A) (2), 5-methylcytosine (m5C) (3), N6-methyladenosine (m6A) (4), and 7-methylguanosine (m7G) have been the focus of comprehensive studies regarding their oncogenic properties and are viewed as key determinants in the occurrence (5), development, and treatment of tumors.

Immune cells within tumors play a key role in clearing the tumor. Activating immune cells, especially the tumor-clearing functions of T cells, can suppress tumor growth. However, immune cells in the tumor microenvironment are typically in a state of suppression, leading to immune evasion. We have found that RNA modifications are involved in the regulation of immune cell suppression in tumors. Immunotherapy, such as anti-PD-1/PD-L1 therapy, has been widely applied in clinical practice, but only a subset of patients is sensitive to immunotherapy. The efficacy of cancer immunotherapy is contingent upon a multitude of factors, including the type of tumor, tumor’s mutational burden, the stability of microsatellites, and the combined use of chemotherapy drugs. Notably, the presence of immune cell infiltration within the tumor microenvironment and the expression of immune checkpoints on the tumor’s surface play pivotal roles in the therapy’s success. We have found that RNA modifications, primarily the m6A modification, have a complex regulatory effect on the tumor infiltration of immune cells and the expression of PD-L1. By targeting the m6A regulators in tumors or immune cells, it is possible to increase immune cell infiltration, regulate the expression of PD-L1, sensitize patients to immunotherapy, inhibit tumor growth, and ultimately improve patient prognosis.

We have compiled a summary of the regulatory roles of four types of RNA methylation modifications, mainly m6A modification, in immune cells, as well as their regulatory effects on the expression of PD-L1 in tumor cells and the research progress on m6A regulators as therapeutic targets. We also reported on the predictive role of methylation modification-related genes in the efficacy of immunotherapy.

Overview of RNA modifications

m6A, representing N6-adenosine methylation, is recognized as the most frequent and plentiful type of post-transcriptional RNA modification, predominantly occurring within mRNA in the nucleus (6). This modification contributes to essential life processes such as hematopoiesis, central nervous system development, and germ cell differentiation (4). Common regulatory enzymes include “writers” METTL3, METTL14, METTL16, “readers” YTHDF1, YTHDF2, YTHDF3, LRPPRC, IGF2BP1 and IGF2BP3, among others, and “erasers” FTO, ALKBH5. METTL3 is the most critical ‘writer’ enzyme for m6A modification, serving as the catalytic subunit of the methyltransferase complex that mediates N6-methyladenosine (m6A) methylation on mRNA. It commonly forms a heterodimer with METTL14, facilitating the process (7). Wilms Tumor 1

Associated Protein (Wtap), a regulatory subunit of the complex, enhances the binding affinity of METTL3 for mRNA (8). METTL3 and METTL14 are primarily located in the nucleus, while METTL16 is mainly found in the cytoplasm. Although METTL16 also mediates the m6A modification, its role in promoting tumorigenesis in various cancers is more noteworthy, which is related to the acceleration of mRNA translation by METTL16 binding to eIF3a/b in the cytoplasm (9). YTHDFs recognize mRNAs modified with m6A; YTHDF1 promotes the translation of these mRNAs, while YTHDF2 accelerates their degradation (10). YTHDF3 interacts with YTHDF1 to augment translation of m6A-modified mRNA and influences the RNA binding activity of YTHDF2 (11). And IGF2BPs enhance the stability of mRNA after recognizing m6A modification (12). FTO and ALKBH5 are the only two demethylases identified for m6A demethylation, which remove the m6A modification from RNA (Figure 1). The role of m6A regulatory enzymes in cancer has been widely reported. Several preclinical experiments have demonstrated that the use of drugs targeting m6A regulators can inhibit tumor growth or enhance the therapeutic efficacy of immunotherapy (Table 1).

m1A and m5C modifications predominantly occur in mRNA, tRNA, and rRNA. The m1A modification is mainly found in tRNA and shares some similarities with m6A modification in mRNA, although it occurs at a much lower frequency. The similarities between m1A and m6A modifications are also reflected in their shared binding proteins YTHDF1, YTHDF2, YTHDF3, and the demethylase FTO (2). m5C plays an important role in maintaining the structure and stability of tRNA and rRNA. m5C modification can occur in mRNA and can regulate the stability of mRNA, but the research on m5C in mRNA is not sufficiently in-depth and comprehensive (3). m7G is widely present in mRNA and is crucial in the translation process and is involved in many important physiological processes (5).

The emergence of new sequencing technologies and bioinformatics tools has facilitated the detection and in-depth understanding of RNA modifications. However, there are still many RNA modifications that have not yet been discovered, and research on many that have been confirmed is far from adequate. We have primarily focused on the function of m6A modification, as it is the most prevalent and well-studied modification in mRNA, but it is important to recognize that other RNA modifications also hold significant potential. The relationship between RNA modifications and the occurrence and development of tumors, as well as their therapy, is currently a hotbed of research. Multiple regulators of RNA modifications are considered oncogenes and have shown potential therapeutic value. The exploration of gene editing technology to enhance RNA modifications that combat tumors and reduce those that promote tumor growth also presents a valuable research avenue.

RNA modifications in immune cells

T cells

T cells have been pivotal in the ongoing oversight and extermination of tumors (33), and their function within tumors is

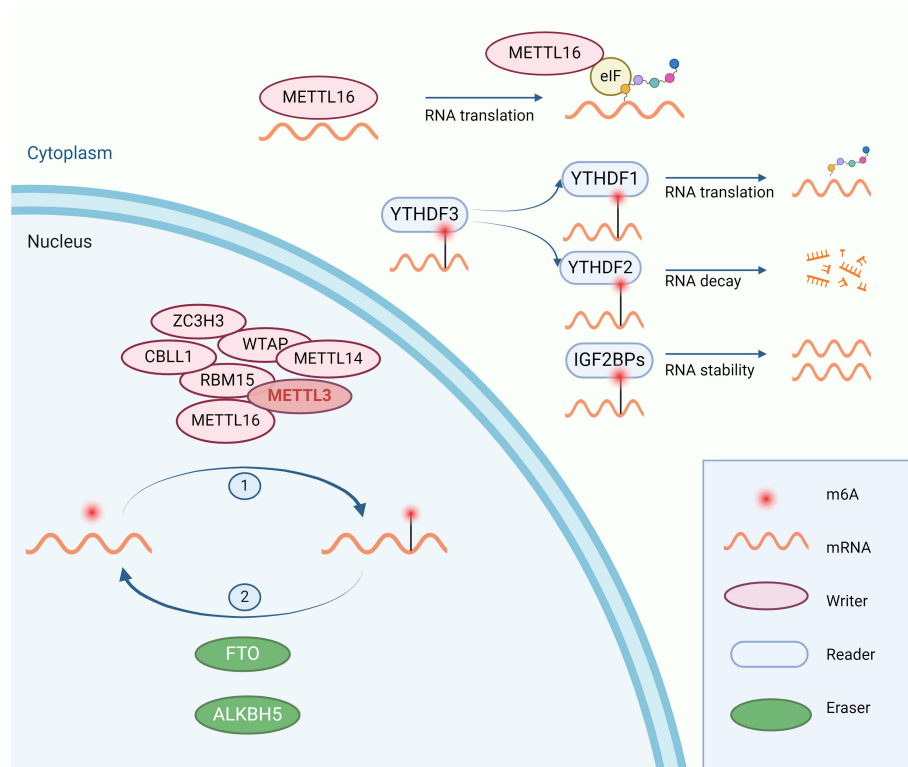


FIGURE 1

The process of m6A modification: m6A modification is catalyzed by “writers” in the nucleus and regulates the stability, translation, and degradation of RNA after being recognized by “readers” in the cytoplasm. The m6A modification is removed by “erasers”.

regulated by RNA modifications (Figure 2). The m6A methylation catalyzed by METTL3 is essential for preserving T cell stability and directing their differentiation, with influence over the growth and specialization of naive T cells through the IL-7/STAT5/SOCS axis. Removal of METTL3 from T cells leads to a contraction of the Th17 and Th1 lineages and an escalation in the Th2 cell presence among naive T cells, contrasting with the METTL14 knockout, which impedes T cell maturation beyond the naive stage (34). The m6A/ALKBH5 mechanism is instrumental in controlling the equilibrium of $\gamma\delta$ T cell development. ALKBH5 modulates the signaling of Jagged1/Notch2 by removing m6A modifications on them, thereby imposing a restraint on the developmental progression and the lineage specification of $\gamma\delta$ T cells (35). Moreover, METTL3 dictates the progression and role of iNKT cells through the METTL3/m6A/Creb1 axis. Deletion of METTL3 impairs the proliferation, differentiation, and cytokine secretion of iNKT cells, leading to a deficiency in tumor resistance (36).

In CD4⁺ T cells, the m1A modification (tRNA-m1A58) at the 58th nucleotide position within the tRNA sequence, mediated by the “writer” protein TRMT61A, ensures the efficient translation of Myc mRNA, thereby supporting the propagation and the functional diversification of CD4⁺ T cells (37). The expression levels of m5C “writer” NSUN3 and NSUN4 are positively correlated with CD8⁺ T cell infiltration, and NSUN4 expression levels are positively associated with the presence of CD4⁺ T cells (38). The expression level of NSUN2 is also related to CD4⁺ T cells and may be involved in the regulation of CD4 memory T cells (39). Regulatory T cells

(Treg), a specialized subset of CD4⁺ T lymphocytes, are pivotal in sustaining immune tolerance and averting autoimmune responses. In tumors, Treg cells can suppress immune responses within the tumor, leading to immune evasion (40). Mettl3 contributes to the upregulation of the IL-2/STAT5 pathway via its capacity to catalyze m6A modifications, regulating members of the SOCS family, thereby maintaining the function and stability of Treg cells and promoting T cell suppression (41). YTHDF1 reads the m6A-modified c-Myc mRNA, thereby regulating the translation and expression of c-Myc in Treg cells, and thus coordinating Treg homeostasis (42). YTHDF2 is involved in the regulation of Treg cells in the tumor microenvironment (TME), maintaining the survival and function of Treg cells by controlling the TNF-NF- κ B signaling pathway within Treg cells. The absence of YTHDF2 in Treg cells of the tumor microenvironment leads to an increase in CD8⁺ T cell infiltration and the expansion of the antitumor CD4⁺ TH1 subset (43). In squamous cell carcinoma of the head and neck, METTL1 deletion leads to a substantial reduction in Treg cells and the amelioration of CD4⁺ T cell exhaustion. This indicates that METTL1-mediated m7G modulates immune infiltration by regulating the levels of Treg cells (44, 45). Follicular helper T (Tfh) cells, a specialized subset of CD4⁺ T cells, are instrumental in the orchestration of humoral immune responses. METTL3 regulates the Tcf7 transcript through m6A modification, ensuring proper differentiation and development of TFH (46). As an indispensable element of the heterodimeric methyltransferase complex, Wtap also participates in the regulation of T cells. The

TABLE 1 Drugs targeting m6A and their mechanisms.

Drugs	Targeting Regulators	Cancer type	Function	References
STM2457	METTL3	myeloid leukemia	decrease acute myeloid leukemia growth, increase its differentiation and apoptosis	(13)
miR-4429		gastric cancer	inhibit GC cells proliferation and induce apoptosis	(14)
Metformin		breast cancer	inhibit the miR-483-3p/METTL3/m6A/p21 pathway to suppress proliferation of breast cancer cells	(15)
BTYNB	IGF2BP1	melanoma and ovarian cancer	inhibit the binding of IMP1 to c-Myc mRNA suppresses the proliferation of ovarian cancer and melanoma cells	(16)
Triptonide		nasopharyngeal carcinoma	disrupt Lnc-THOR-IGF2BP1 signaling and inhibit tumor growth	(17)
Ucubitacin B		HCC	induce tumor cell apoptosis and increase immune cell infiltration by allosterically blocking the interaction between IGF2BP1 and m6A through the KH1–2 domain	(18)
CWI1-2	IGF2BP2	acute myeloid leukemia(AML)	inhibited glutamine absorption and impair mitochondrial function in AML cells, suppress AML progression	(19)
ABCF1 mRNA	IGF2BP3	Ewing's sarcoma	bind and inhibit IGF2BP3, suppress the growth of Ewing's sarcoma cells	(20)
Berberine		colorectal cancer	downregulate IGF2BP3 and inhibit PI3K/AKT pathway to inhibit the proliferation of colorectal cancer cells	(21)
LNP-iYthdfl	YTHDF1	nonalcoholic steatohepatitis-related hepatocellular carcinoma	enhance the efficacy of PD-1 blockade therapy	(22)
DF-A7	YTHDF2	colon adenocarcinoma and melanoma	promote the infiltration and M2 polarization of CX3CR1 ⁺ macrophages, inhibit glycolysis in tumor cells, and enhance the effector functions of CD8 ⁺ T cells	(23)
JX5		T-cell acute lymphoblastic leukemia	bind to the IGF2BP2 KH3-4 domain and inhibit proliferation of tumor cells	(24)
CS1 and CS2	FTO	AML	inhibit FTO activity and signaling thereby suppresses the viability of AML cells	(25)
Saikosaponin D			inhibit proliferation of AML cells, promote cells apoptosis and cycle arrest	(26)
R-2HG			target the FTO/m6A/MYC/CEBPA signaling to inhibit proliferation of AML cells	(27)
FB23-2			inhibit proliferation of AML cells	(28)
Dac51		melanoma	increase infiltration and cytotoxicity of CD4 ⁺ T cells, inhibit tumor growth, and have a synergistic effect with anti-PD-L1 blockade	(29)
MV1035	ALKBH5	Glioblastoma	inhibit ALKBH5, thereby suppress the migration and invasiveness of tumor cells	(30)
ALK-04		melanoma	inhibit Mct4/Slc16a3 expression, lactate content, Treg cells MDSC in TME and enhance the efficacy of GVAX/anti-PD-1 therapy	(31)
Hiram	TRMT6/TRMT61A	HCC	inhibit the growth of HCC	(32)

knockout of WTAP leads to a reduction in the abundance and spontaneous activation of peripheral CD4⁺ and CD8⁺ T cells and eliminates the expansion effect induced by the T cell receptor (TCR). Additionally, Wtap is essential for the regulatory control of apoptosis in CD4⁺ T cells, impacting their survival upon TCR engagement (47).

The absence of YTHDF2 in tumors impairs tumor glycolytic metabolism, therefore enhancing the mitochondrial respiration of

CD8⁺ T cells to strengthen antitumor capabilities (23). A pan-cancer examination has shown that METTL1, an enzyme responsible for m7G methylation, is positively correlated with Treg cell numbers in diverse cancer subtypes. The m7G methyltransferase WBSCR22 has been shown to regulate the Zac1/p53 pathway to exert a pro-tumorigenic effect and is highly expressed in activated CD8⁺ T cells, indicating that WBSCR22 may participate in the regulation of CD8⁺ T cells (48, 49). The m5C

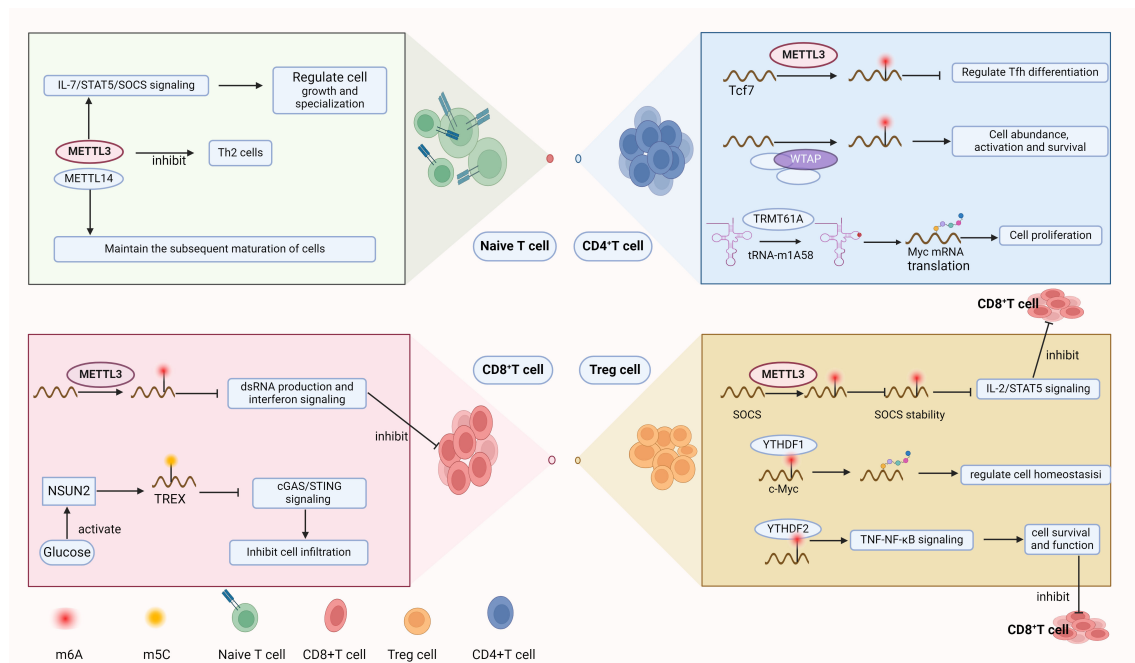


FIGURE 2

RNA modification regulates the development of Naive T cells and can exert regulatory effects in various T cell subsets.

methylation reader YBX1 is positively correlated with CD4⁺ memory T cells, CD8⁺ T cells, type 1 and type 2 T helper cells in pancreatic ductal adenocarcinoma (50). The m5C methyltransferase NSUN2 mediates m5C modification of TREX2 transcripts after being activated by glucose, inhibiting the activation of cGAS/STING, thus inhibiting the infiltration of CD8⁺ T cells (51). Inhibition of METTL3 leads to an increase in dsRNA formation, which in turn enhances interferon signaling and augments the capacity of T cells to eliminate cancer cells (52). Similarly, the knockout of Mettl14 leads to a significant reduction in iNKT cells mediated by the p53 apoptosis pathway (53). Additionally, m6A modification mediated by Mettl3 in T cells regulates the migration of T cells in an acidic tumor microenvironment. By inducing Mettl3-mediated m6A modification in T cells, the expression of integrin α subunit ITGB1 can be upregulated, thereby enhancing T cell tumor infiltration and antitumor activity, and relieving the suppression of T cells by the acidic microenvironment (54).

Natural killer cells

As part of the innate immune response, NK cells are essential for monitoring and eliminating cancerous cells, and they represent a key target for cancer immunotherapy (55). The m6A modification plays an indispensable role in maintaining the tumor infiltration and cytotoxicity of NK cells, primarily regulated by METTL3, METTL14, and YTHDF2. METTL3-mediated m6A methylation promotes the maturation, expansion, and functionality of NK cells through the modulation of IL-15 signaling within the AKT-mTOR/MAPK-ERK pathway. Similarly, YTHDF2 in NK cells regulates the

proliferation or survival of NK cells after reading the m6A modification of Tardbp, and modulates the expression of cytotoxicity-related molecules through the STAT5-YTHDF2 positive feedback axis, which participates in the survival, proliferation, and terminal maturation of IL-15-mediated NK cells (56). When METTL3 is knocked out in NK cells, the tumor infiltration and the ability to secrete immune factors such as GzmB and INF- γ are significantly decreased, cytotoxicity is markedly reduced, and an increase in expression levels of the inhibitory receptor TIGIT is observed in the TME (57). m6A contributes to mRNA stability and promotes the early activation and effector functions of NK cells by directly modifying important mRNAs such as *Prf1* and *Gzmb*. The mTORC1 supports m6A methylation in NK cells through the c-MYC-MAT2A axis to promote SAM synthesis (58). The expression level of the m5C methyltransferase NSUN2 is associated with the level of resting NK cells and may take part in the regulation of NK cells (39). The m5C methylation reader YBX1 is found to be positively related to the presence of NK cells in cases of pancreatic ductal adenocarcinoma (50).

Macrophages

Macrophages are innate immune cells that play a crucial and complex role in tumor immunity, with the antitumor M1 polarization and the pro-tumor M2 polarization being two common differentiations of macrophages in tumor immunity. Tumor-infiltrating macrophages (TAMs) typically acquire the immunosuppressive M2 polarization (59, 60). RNA modifications participate in the regulation of macrophages in various aspects. Tumor-associated macrophages constitute a major part of the

cellular composition within the TME. METTL3 promotes the degradation of Irakm mRNA by adding m6A modification, enhancing TLR4 signaling, activating macrophages, and inducing M1 polarization in TAMs, thereby increasing their tumor-killing ability (61). The METTL3 in macrophages increases the stability of STAT1 mRNA transcripts by adding m6A modifications, promoting the M1 polarization of macrophages (62). In myeloid cells, METTL3 activates the NF- κ B pathway and STAT3 signaling, leading to M1 and M2-like polarization of macrophages, and fosters the proliferation and spread of cancer cells, contingent upon the infiltration of M1 and M2 phenotype-like TAMs (63), and maintains YTHDF1-mediated SPRED2. Additionally, the FTO demethylase influences the NF- κ B pathway, stabilizing STAT1 and PPAR- γ mRNAs, crucial for activating M1 and M2 macrophages. YTHDF2 is involved in this process and antagonizes FTO in regulating the stability of PPAR- γ mRNA (64). IL-4 stimulates an increase in IGF2BP2, which then binds to the m6A-modified TSC1. This binding modulates the signaling through the TSC1/mTORC1 and TSC1/2/MEK/ERK axes, orchestrating the balance between M2 and M1 macrophage states, and driving a transition toward the M2 phenotype within macrophage populations (65). In the tumor lactoacidotic environment, H3K178ac induces the acetylation and upregulation of METTL3 expression. METTL3 mediates m6A modification of the Jack1 mRNA transcript in TIM, which is then read by YTHDF1 to enhance the translation efficiency of JAK1 and the phosphorylation of STAT3, thereby enhancing the immunosuppressive function of TIM (66). The high-risk score of m5C-lncRNA is associated with the high expression of M0 and M2 phenotype macrophages in pancreatic cancer, suggesting that m5C-related lncRNAs may regulate the polarization of macrophages in pancreatic cancer (67). The m7G methyltransferase METTL1 is negatively correlated with M2 and M0 macrophages in tenosynovial giant cell tumors, suggesting that METTL1 may induce M1 polarization of macrophages in tenosynovial giant cell tumors. However, in prostate cancer, METTL1-mediated m7G modification induces M2 polarization of macrophages, indicating the heterogeneity of METTL1 functions in different tumors (44, 68). Additionally, tumor-associated macrophages regulate the expression of CD8⁺ T cells through m6A-associated mechanisms. C1q⁺TAMs specifically express METTL14 and YTHDF2, and maintain the level of tumor infiltration and cytotoxicity of CD8⁺ T cells in a METTL14-dependent manner (69).

Dendritic cells

Dendritic cells, pivotal in the immune response, function as essential antigen-presenting entities that engage in the acquisition, modification, and conveyance of tumor antigens, as well as in the stimulation of T cell responses (70). In tumor immunity, RNA modifications regulate the antigen cross-presentation of dendritic cells and their subsequent function in activating T cells, as well as the migration of dendritic cells. YTHDF1 boosts the synthesis of lysosomal proteases by recognizing the m6A mark on their mRNA,

potentially accelerating the breakdown of tumor antigens internalized by dendritic cells. This action may consequently suppress the dendritic cells' capacity to initiate a cross-priming response in T cells (61, 71). In studies on gastric cancer, YTHDF1 was shown to not only suppress the recruitment of mature DC cells and T cell activation but also inhibit the expression of MHC II and IL-12 (72). METTL3 facilitates the development and maturation of dendritic cells, as well as the subsequent activation of T cells, through its role in m6A methylation. It boosts the translation efficiency of mRNAs encoding CD40, CD80, and the TLR4-associated signaling molecule Tirap. Additionally, METTL3 amplifies the activity of the TLR4/NF- κ B signaling cascade and stimulates the synthesis of cytokines that drive an inflammatory response (73). m6A modification is also involved in the migration of dendritic cells; after being read by YTHDF2, it reduces the expression level of lnc-Dpf3, whose expression negatively regulates the induction of CC-chemokine receptor 7 (CCR7) and the migration of dendritic cells to the draining lymph nodes. Moreover, lnc-Dpf3 forms a complex with HIF-1 α , impeding the expression of glycolysis-driven genes under HIF-1 α 's control, like *Ldha*. This interaction curtails the glycolytic activity and the movement potential of DCs (74). The m5C methylation reader YBX1 is related to activated dendritic cells in pancreatic ductal adenocarcinoma (50).

Research on the specific regulators of m1A/m5C/m7G and their relationship with immune cells is limited, but growing evidence suggests that these RNA modifications are involved in the regulation of immune cells in various types of cancer. For instance, in clear cell renal cell carcinoma (ccRCC), the score of m7G-related genes is positively correlated with CD4⁺, CD8⁺ T cells, and Treg cells, M0 macrophages, and negatively correlated with dendritic cells, M2 macrophages, and other immune cells (75). In diffuse large B-cell lymphoma, m5C-related genes regulate the infiltration of eosinophils, Treg cells, and M2 macrophages, and control the activation of T cells by modulating immune checkpoints such as PD-L1 and CTLA-4 (76). In prostate cancer, there are significant expression differences in CD8⁺ T cells, M1 macrophages, and M2 macrophages among the two m5C immune subtypes. In colon cancer, a low m1A score is related to the proliferation of CD8⁺ T effector cells (77). In lung adenocarcinoma, the m1A score is related to all immune cells (78). We have summarized the effects of m6A modification on immune cells that have not been previously mentioned (Table 2).

We have reviewed the important role of RNA modifications, especially m6A modification, in regulating immune cells. m6A modification has a profound impact on tumor immune surveillance and the response to immunotherapy by affecting the maturation, differentiation, and function of T cells, NK cells, macrophages, and dendritic cells. These findings not only reveal new regulatory mechanisms of immune cells in the tumor microenvironment but also provide potential targets for the development of new cancer treatment strategies.

Despite significant progress in existing research, there are still some limitations. For example, most studies focus on specific types of cancer, and the universality of RNA modifications across different cancer types and their roles at various stages of tumor development are not yet fully understood. Future research needs to

TABLE 2 Regulation of immune cells by m6A modification.

	Regulators	Cancer type	Function	References
Writers	METTL3	colorectal carcinoma	reduce infiltration of CD8 ⁺ T cells	(79)
			increase recruitment of M2 macrophages	(80)
		colorectal cancer	inhibit CD4 ⁺ T cells and CD8 ⁺ T cells by promoting the accumulation of MDSCs	(81)
			facilitate M2 macrophage polarization	(82)
		HCC	reduce infiltration of GZMB ⁺ IFN- γ ⁺ CD8 ⁺ T cells	(83)
			promote macrophages recruitment and M2 polarization	(84)
		melanoma	reduce infiltration of CD8 ⁺ T cells	(79)
		NSCLC	reduce infiltration of CD8 ⁺ T cells	(85)
		thyroid cancer	reduce M2 macrophages and infiltration of Tregs	(86)
	METTL14	colorectal carcinoma	reduce infiltration of CD8 ⁺ T cells	(79)
		melanoma	reduce infiltration of CD8 ⁺ T cells	(79)
		colorectal cancer	maintain the function of CD8 ⁺ T cells	(69)
		cervical cancer	increase the proportion of PD-1 ⁺ TAMs, inhibit the phagocytic function of macrophages	(87)
		lung cancer	inhibit activation and infiltration of CD8 ⁺ T cells	(88)
	METTL16	HCC	promote macrophages recruitment and M2 polarization	(84)
		pancreatic ductal adenocarcinoma	increase infiltration of CD8 ⁺ T cells and B cells	(89)
Readers	YTHDF1	HCC	inhibit CD8 ⁺ T cells by promoting the accumulation of MDSCs	(22)
		prostate cancer	inhibit the cytotoxicity of CD8 ⁺ T cells	(90)
		colorectal cancer	inhibit CD8 ⁺ T cells by promoting the migration of MDSCs	(91)
	YTHDF2	bladder cancer	inhibit recruitment of CD8 ⁺ T cells	(92)
		melanoma	reduce infiltration of CD8 ⁺ T cells and reduce CD4 ⁺ Th1 subset	(43)
		colon carcinoma		
		triple-negative breast cancer	promote the pro-tumoral phenotype polarization of macrophages	(93)
	YTHDF3	ccRCC	increase infiltration of CD8 ⁺ T cells	(94)
		melanoma	inhibit recruitment and antitumoral polarization of macrophages and activation and cytotoxicity of CD8 ⁺ T cells	(23)
		colon adenocarcinoma		
		thyroid cancer	increase infiltration of CD4 ⁺ T cells and macrophages	(95)
	IGF2BP1	colon cancer	inhibit the cytotoxicity of CD8 ⁺ T cells	(96)
		HCC	increase infiltration of CD4 ⁺ , CD8 ⁺ T cells, CD56 ⁺ NK cells and F4/80 ⁺ macrophages	(18)
	IGF2BP3	HCC	promote infiltration and M2 polarization of macrophages, inhibit activation of CD8 ⁺ T cells	(97)
	LRPPRC	HCC	reduce infiltration of CD4 ⁺ and CD8 ⁺ T cells as well as CD56 ⁺ NK cells and F4/80 ⁺ macrophages	(18, 98)
Erasers	FTO	melanoma	restrict activation and function of CD8 ⁺ T cells	(29)
		HCC	inhibit recruitment and activation of CD8 ⁺ T cells	(99)
	ALKBH5	colorectal cancer		(100)

(Continued)

TABLE 2 Continued

	Regulators	Cancer type	Function	References
			inhibit CD8 ⁺ T cells and NK cells by promoting the accumulation of MDSCs	
			promote M2 macrophage polarization	(101)
		intrahepatic cholangiocarcinoma	inhibit the expansion and cytotoxicity of CD8 ⁺ T cells	(102)
		HCC	increase recruitment of PD-L1 ⁺ macrophages	(103)
		glioblastoma multiforme	increase recruitment of tumor-associated macrophages, reduce infiltration of CD3 ⁺ , CD4 ⁺ , CD8 ⁺ T cells	(104, 105)
		ovarian cancer	promote M2 polarization of macrophages	(106)
		NSCLC	recruit PD-L1 ⁺ TAMs, promote M2 macrophage polarization	(107)
		lung adenocarcinoma	regulate the polarization of M1/M2 macrophages	(108)

more comprehensively and deeply explore the regulatory effects of RNA modifications on immune cells in different cancer types and assess their potential as biomarkers and therapeutic targets.

RNA modifications and immune checkpoint

RNA modifications have been shown to be associated with the expression of various immune checkpoints, such as the upregulation of PDCD1 and KIR3DL1 and the downregulation of TIGIT, IDO1, and BTLA in the high-risk group established based on m6A scoring in diffuse large B-cell lymphoma (109). In breast cancer, the groups differentiated by m1A scoring exhibit differential expression of immune checkpoints, for example, TIGIT, IDO1, LAG3, and ICOS (110). We mainly summarize and introduce the research findings related to RNA modifications and PD-1/PD-L1 and CTLA-4.

PD-L1

PD-L1 is mainly expressed on the surface of tumor cells and suppresses the functions of cytotoxic T cells by binding to PD-1 on the surface of T cells. PD-1/PD-L1 blockade therapy has improved the prognosis of many cancers, yet only a subset of patients are sensitive to anti-PD-1 or PD-L1 treatment, and resistance may occur (111). Across a range of malignancies, the metrics derived from the analysis of m6A, m1A, m5C, and m7G regulatory factors exhibit a substantial association with the levels of PD-L1 protein expression (50, 75, 77, 78, 112–121). Firstly, regulators of m6A modification can directly or indirectly regulate the stability and activation of PD-L1 mRNA. In Non-Small Cell Lung Carcinoma (NSCLC), METTL3-mediated m6A modification destabilizes PD-L1 mRNA, resulting in a reduction of PD-L1 expression (122). Furthermore, METTL3 can also regulate the ubiquitination of PD-L1 by controlling LINC02418 in NSCLC, thereby downregulating

the expression of PD-L1 (85). Additionally, METTL3 mediates m6A modification of circIGF2BP3, upregulating the expression of PKP3, which enhances the stability of OTUB1 mRNA and increases the abundance of PD-L1 by reducing the ubiquitination of PD-L1 (123). Conversely, within the context of breast cancer, the m6A modification catalyzed by METTL3 not only bolsters the longevity of PD-L1 mRNA transcripts but also facilitates their transcriptional activation, a process that hinges on the recognition of the m6A mark by the IGF2BP3 protein (124). YTHDF3 reads and destroys m6A-modified CBX1 mRNA in nasopharyngeal carcinoma, inhibiting the upregulation of PD-L1 mediated by the IFN- γ /STAT1 signal (125). In HNSCC, TRMT61A-mediated tRNA-m1A modification upregulates the expression of PD-L1, which may be accomplished by regulating INF γ (126). m5C methylation reader YBX1 is related to PD-L1 expression levels in pancreatic ductal adenocarcinoma (50). In colorectal cancer, m6A-modified IFIT1 upregulates the expression of PD-L1 by reducing the ubiquitination and degradation of PD-L1 (127). Similarly, in cholangiocarcinoma, METTL14 mediates m6A modification of Siah2 and promotes the degradation of Siah2 upon reading by YTHDF2, inhibiting the ubiquitination of PD-L1 mediated by Siah2, ultimately leading to an increase in PD-L1 expression levels (128). It is worth mentioning that in colorectal cancer, the metabolite S-adenosylmethionine of methionine promotes the occurrence of m6A modification in cancer cells and enhances the translation of PD-L1 (129). m6A-regulated lncRNA has been proven to be associated with PD-L1 expression in various tumors (130–133). In pancreatic cancer, METTL3 increases the expression of PD-L1 by upregulating the expression of lncRNA MALAT1 in cancer cells (134). In hepatocellular carcinoma(HCC), intestinal bacterial lipopolysaccharide regulates the expression of PD-L1 on the surface of cancer cells by upregulating METTL14 and METTL14-mediated m6A modification of MIR155HG, modulating the miR-223/STAT1 axis (135). We present a summary of the effects of m6A modifications on PD-1/PD-L1 that have not been previously mentioned in this review (Table 3).

TABLE 3 Regulation of PD-L1 by m6A modification.

Regulators	Cancer type	Function	References
METTL16	colorectal cancer	reduce PD-L1 expression	(136)
	pancreatic ductal adenocarcinoma	reduce PD-L1 expression	(89)
METTL3	ccRCC	increase PD-L1 expression	(137)
IGF2BP1	colon cancer	increase PD-L1 expression	(96)
	HCC	increase PD-L1 expression	(18)
IGF2BP3	bladder cancer	increase PD-L1 expression	(138)
YTHDF1	NSCLC	reduce PD-L1 expression	(139)
YTHDF2	NSCLC	reduce PD-L1 expression	(139)
	lower-grade glioma	increase PD-1 expression	(140)
ALKBH5	glioblastoma multiforme	increase PD-L1 expression	(105)
	intrahepatic cholangiocarcinoma	promote the expression of PD-L1 on monocytes/macrophages	(141)
	HCC	recruit PD-L1 + macrophages	(103)
FTO	OSCC	increase PD-L1 expression	(142)

CTLA-4

CTLA-4, like PD-1, is a non-redundant checkpoint that inhibits the proliferation and activation of T cells. Therapy targeting the CTLA-4 pathway has been implemented in the field of immunotherapy and may be synergistically combined with PD-1 inhibitory therapy for specific cancer types (111). There are no definitive research results regarding how RNA modifications regulate CTLA-4, but we have found that RNA modifications are associated with the expression levels of CTLA-4 and can predict the outcomes of CTLA-4 blockade immunotherapy. The m6A-modified reader YTHDF2 has been proven to be positively correlated with the expression of CTLA-4 in low-grade glioma (140). In the high-risk group related to m5C-lncRNA, CTLA-4 is highly expressed (143). In tumors such as pancreatic adenocarcinoma, hepatocellular carcinoma, and pancreatic cancer, the scores of m6A-modified regulatory genes are significantly correlated with the expression of CTLA-4 and can be used to predict prognosis and response to immunotherapy (144–146). A study of m5C in diffuse large B-cell lymphoma has identified a correlation between m5C-affected genes and the modulation of

immune checkpoint genes, specifically CTLA-4 and PD-L1. In prostate cancer, CTLA-4 is also found to be differentially expressed in two m5C immune subtypes and is related to the degree of immune cell infiltration (76). Similarly, in the immunological model calculated based on the regulatory genes of m7G such as CDK1, ANO1, and PDGFRA, CTLA-4 is highly expressed in the high-risk group (147). Another similar study shows that m7G is not only related to immune checkpoints such as CTLA-4 but also can predict the effects of immunotherapy (148). In addition, a low score based on the methylation enzyme scoring established by m6A/m5C/m1A/m7G regulatory genes is related to the positive expression of CTLA4 (149).

In this section, we have summarized the regulatory effects of RNA modifications on the immune checkpoint PD-L1, as well as the correlation with CTLA-4 expression and its predictive role in therapeutic efficacy. It provides new insights into the understanding of tumor immune evasion mechanisms. The research indicates that RNA modifications influence the expression of PD-L1 and CTLA-4 through various mechanisms, including the regulation of mRNA stability, transcriptional activation, and interactions with specific proteins. These findings offer new perspectives for anti-PD-1/PD-L1 therapy and reveal the research value of RNA modifications in anti-CTLA-4 therapy.

The regulatory role of RNA modification in ICI therapy

RNA modifications exhibit differential impacts on PD-1 blockade therapy across various tumors. Targeting specific RNA modification regulators can enhance the efficacy of PD-1 blockade therapy (Figure 3). In NSCLC, METTL3-driven m6A methylation leads to the destabilization of PD-L1 mRNA, which reduces the therapeutic efficacy of anti-PD-1/PD-L1 interventions; however, the knockout of METTL3 increases immune cell infiltration and enhances the therapeutic efficacy of anti-PD-1/PD-L1 (122). Similarly, the deletion of METTL3 or METTL14 in immune-resistant melanoma tumor cells makes the tumor sensitive to immunotherapy (79). Targeting METTL3 in NAFLD-HCC and NSCLC can improve the effectiveness of PD-1 therapy. In the NAFLD-HCC mouse model, the knockout of METTL3 in conjunction with anti-PD-1 therapy synergistically suppressed tumor growth, resulting in a reduction of over 90% in both tumor volume and weight (83). In NSCLC, the suppression of METTL3 can enhance the sensitivity of tumor-bearing mice to anti-PD-1 treatment, and patients with NSCLC exhibiting low METTL3 expression have a more favorable prognosis with immunotherapy (122). In thyroid cancer, high expression of METTL3 in tumor cells inhibits the demethylation of CD70mRNA, maintains the degradation of transcripts mediated by YTHDF2, thereby releasing T cells from suppression and enhancing the efficacy of PD-1 blockade (86). Similar observations have been made in melanoma and lung cancer, where high expression of METTL3 in macrophages is beneficial for immunotherapy (63). Targeting the YY1-CDK9 transcription elongation complex in glioblastoma results in lowered METTL3 expression, which in turn,

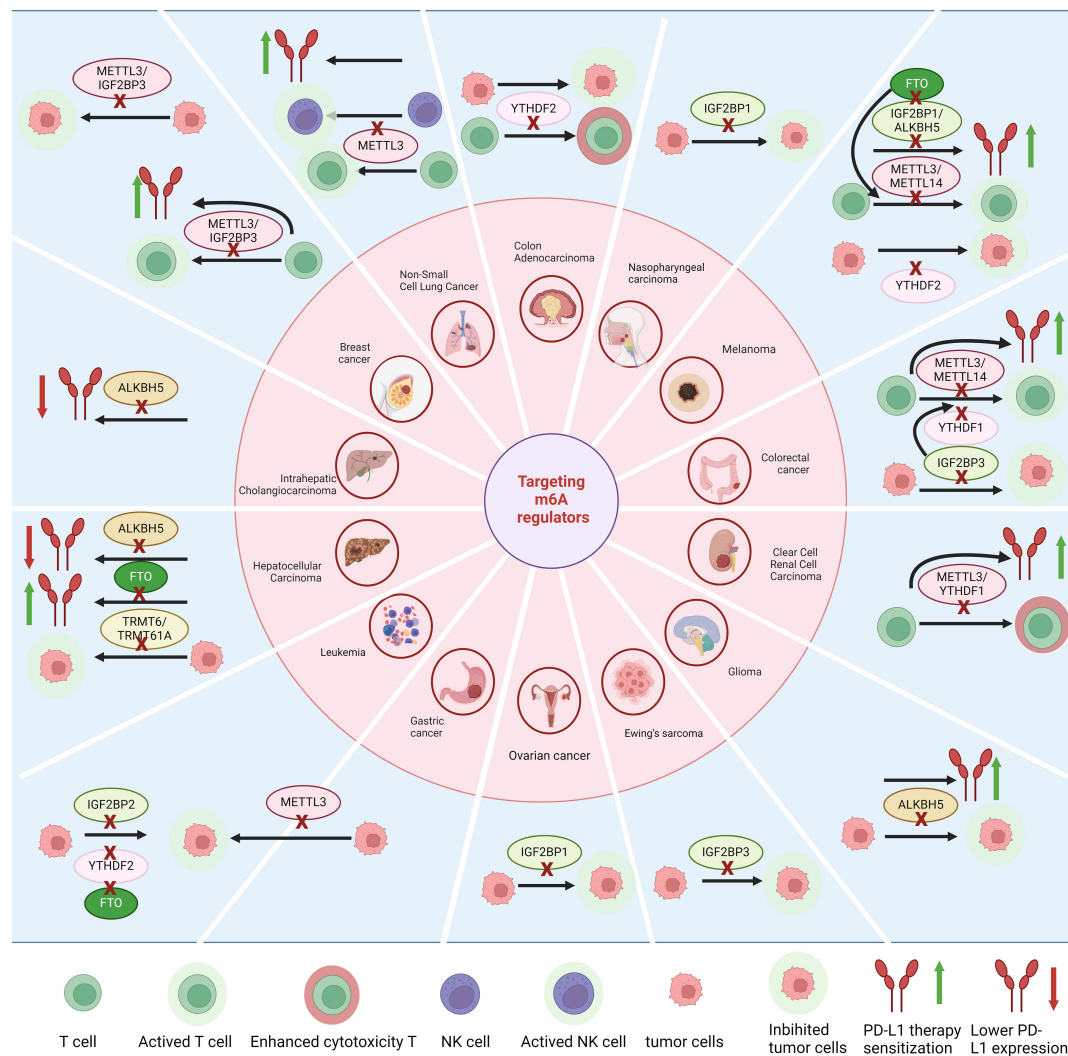


FIGURE 3

Targeting regulators with m6A modifications can enhance T cell infiltration and tumor-killing effects in various types of cancer. It also regulates the expression of PD-L1 and the therapeutic effects of anti-PD-L1 treatment, while inhibiting tumor growth.

enhances the therapeutic impact of PD-1 blockade (150). Abirine treatment inhibits IFN- γ -induced m6A modification, thereby regulating JAK1/STAT1 and suppressing the expression of PD-L1. Combined therapy with PD-1 blockade can inhibit tumor growth (151). Targeting YTHDF1 in colorectal cancer can relieve the inhibition of CD8⁺ T cells and enhance the efficacy of anti-PD-1. Targeting YTHDF1 significantly reduced the resistance to anti-PD-1 therapy in the MC38 tumor model, leading to better prognosis for tumor-bearing mice. Similar observations were made in mice with the CT26 tumor model, which is insensitive to PD-1 therapy; the deletion of YTHDF1 in CT26 cells followed by anti-PD-1 treatment markedly inhibited tumor growth (91). Knocking out ALKBH5 in glioma or targeting it with IOX1 reduces the expression of the PD-L1 protein, inhibits tumor growth, extends the survival of mice, and enhances these effects of PD-1 blockade therapy (105). Similarly, inhibiting ALKBH5 in melanoma enhances the efficacy of PD-1 blockade, patients with low expression of ALKBH5 are more likely to benefit

from PD-1 blockade therapy (31). The use of FTO inhibitors in HCC and melanoma can enhance immune activation and sensitivity to anti-PD-1 treatment (99, 152). Targeting circular RNA circRHBD1 can block its m6A-dependent mediated rapid translation of PIK3R1 and improve the efficacy of anti-PD-1 therapy (153). NSUN2-mediated m5C methylation modulates TREX2 expression, thereby suppressing the cGAS/STING pathway and contributing to resistance against PD-1 checkpoint blockade (51).

In this part, we have explored the regulatory role of RNA modifications on PD-1 blockade therapy across different types of tumors. By affecting the expression of genes involved in immune activation and suppression, RNA modifications have significantly impacted the therapeutic efficacy of immune checkpoint blockade and have provided ideas for specific treatment plans: by targeting specific RNA modification regulators, we may be able to increase the response rate to immunotherapy and overcome patient resistance to existing treatments.

RNA modification and prediction of immunotherapy efficacy

Genes related to the RNA modifications m6A, m1A, m5C, and m7G can predict tumor sensitivity to immunotherapy, either individually or in combination. By conducting bioinformatics analysis on many samples in databases, key genes are screened, and corresponding scores are established. For instance, in HCC, an m6A score derived from m6A-associated feature genes categorizes patients into distinct risk groups. The high-risk cohort demonstrates increased immune evasion and immune system dysregulation, correlating with heightened responsiveness to anti-CTLA-4 and anti-PD-1 therapies (154). The m6A score has been studied in multiple tumors but varies depending on the analysis method and selected genes. Patients with oral squamous cell carcinoma exhibiting a high m6A score are more likely to experience enhanced efficacy from treatments targeting the PD-1 and CTLA-4 pathways (155), lung squamous cell carcinoma (156), soft tissue sarcoma (157), gastric cancer (102), and hepatocellular carcinoma (158), while a low m6A score suggests greater sensitivity to immunotherapy in thyroid cancer (159), NSCLC (160), follicular lymphoma (161), breast cancer (162), and head and neck squamous cell carcinoma (163). Additionally, the score of m6A methyltransferase regulators can effectively predict the efficacy of immunotherapy in urothelial cancer patients (164).

A prognostic model established using m6A-related lncRNA suggests that esophageal cancer in the low-risk group responds better to immunotherapy (165). Similarly, a model established using m5C-related genes can also evaluate prognosis and immune therapy efficacy, with liver and pancreatic cancers with lower m5C scores being more sensitive to anti-CTLA-4 therapy, and pancreatic cancer also being sensitive to anti-PD-1 therapy (166). A score based on m7G indicates that colorectal cancer and rectal cancer in the low-scoring group are more sensitive to anti-PD-1 therapy (119, 121). Lung adenocarcinoma with a low score has a higher immune prognostic score (120). Interestingly, in low-grade glioma, the high m7G score group is sensitive to anti-PD-1 treatment, while the low m7G score group is more sensitive to anti-PD-L1 (167). In colorectal cancer, a low m1A score suggests a better prognosis with anti-PD-L1 treatment (77). Lung adenocarcinoma with a low m1A score has a lower TIDE score, indicating greater sensitivity to immunotherapy (78).

In addition to establishing models based on the scores of single RNA modifications, analyzing multiple RNA modification genes simultaneously can also establish effective predictive models. In cervical cancer, a prognostic model established using m6A/m5C/m1A indicates that the high-risk group is more sensitive to anti-CTLA-4 treatment (168). In colon cancer, the low-risk group is more sensitive to anti-CTLA-4 and anti-PD-1 treatments (169). In HCC, a methylation score composed of m1A/m5C/m3C/m6A suggests that a low score is sensitive to anti-PD-L1 therapy (170). The Writer-Score established according to m1A and m6A RNA modification enzymes indicates that a low score is associated with better outcomes in immunotherapy (171).

Through bioinformatics analysis of large-sample databases, we have been able to screen for key genes and establish corresponding scoring systems. These scoring systems have demonstrated the potential to predict responses to immunotherapy across various types of tumors, offering new tools for personalized medicine. However, many scoring systems still require further validation and research to explore their applicability in oncology and immunotherapy strategies.

RNA modification regulators as therapeutic targets

Therapeutic interventions targeting RNA modification-related genes or proteins have been extensively studied, and numerous effective drugs have been developed. For instance, metformin can specifically inhibit FTO and block its demethylation effect on m6A modification (172). Targeting specific regulatory genes or proteins of RNA modification can enhance the therapeutic efficacy of immunotherapy in certain types of cancer. For example, targeting ALKBH5 in melanoma can enhance the effectiveness of PD-1 blockade (31). Lower levels of METTL3 in NSCLC are also associated with better outcomes from anti-PD-1 therapy (122). Moreover, targeting specific RNA modification regulators' genes or proteins can directly exert tumor-suppressive effects. For example, miR-4429, which targets METTL3 in gastric cancer, can inhibit the proliferation of GC cells and induce apoptosis (14). Although the vast majority of drug treatments target m6A regulatory genes or proteins, we found that targeting the m1A methyltransferase complex TRMT6/TRMT61A in HCC with Hiram can effectively inhibit the progression of HCC (32), indicating that targeting other RNA modification regulatory genes or proteins also has therapeutic significance. We have summarized the specific targeted drugs and their mechanisms of action (Table 1).

This section of our study extensively explores therapeutic interventions targeting genes or proteins associated with RNA modifications and outlines the development of a series of effective drugs. Targeting regulators such as METTL3, METTL14, IGF2BP3, or YTHDF1 can alleviate T-cell suppression in melanoma, colorectal cancer, non-small cell lung cancer (NSCLC), and breast cancer. Targeting regulators like METTL3, METTL14, the IGF2BP family, ALKBH5, or FTO can enhance the efficacy of PD-1 blockade therapy in renal clear cell carcinoma, colorectal cancer, melanoma, NSCLC, breast cancer, hepatocellular carcinoma, and intrahepatic cholangiocarcinoma. Inhibition of tumor cell proliferation can be achieved by blocking METTL3, TRMT6/TRMT61A, the IGF2BP family, YTHDF2, or ALKBH5. Targeting "writers" such as METTL3 or METTL14 blocks the formation of m6A and thus can play a role in multiple tumor-suppressing or immune therapy-enhancing effects; however, this may also greatly impact normal physiological functions and lead to severe side effects. RNA modifications have a considerable number of "readers," so targeting specific "readers" in specific tumors may achieve precise therapeutic effects with lower side effects, but it may need to be specific to certain

tumors and may not be universally applicable. Targeting “erasers,” mainly FTO and ALKBH5, seems to primarily enhance PD-1 blockade therapy and inhibit tumor cells, being key to combined immunotherapy. However, targeting FTO and ALKBH5 results in the inability to remove m6A, which may also lead to severe side effects.

Discussion

In this review, we primarily discuss the relationship between m6A, m1A, m5C, and m7G modifications and tumor immunity and immunotherapy, and summarized the regulation of immune cells and immune checkpoints, as well as drug treatment targeting RNA modification regulators and immune prediction based on the four RNA modifications. Research related to RNA modifications has the following limitations: m6A is the most abundant modification in mRNA and has been extensively studied; however, the physiological processes in which m6A is involved are not fully understood, and there may be undiscovered regulatory proteins related to m6A modification. The specific roles of m1A, m5C, and m7G in tumors have been insufficiently studied, possibly due to variations in modification sites and abundance. There are also limitations and challenges in tumor therapy related to RNA modifications: some proteins among RNA modification-related proteins have dual identities, and the same regulatory factor may have multiple roles, possessing both oncogenic and tumor-suppressive effects, requiring specific research to provide solutions. For example, knocking out METTL3 in melanoma can inhibit tumor development and increase the infiltration of CD8⁺ T cells (79), but another study has shown that the absence of METTL3 expression in macrophages can promote the growth and metastasis of melanoma and weaken the efficacy of PD-1 blockade (63). Additionally, RNA modifications are widely involved in physiological activities, and it is challenging to target RNA modifications in the tumor microenvironment without affecting normal physiological processes. Researchers have obtained drugs with high specificity through complex drug design and optimization, but this issue has not been resolved. Moreover, although many RNA modification regulators have been identified, only a few can be used as therapeutic targets for cancer treatment. Finally, current research is mainly focused on mouse models, and there may be differences in the physiological activities and tumor therapy based on mRNA between mice and humans, requiring more research to clarify the specific situation.

Despite certain achievements of drugs targeting RNA modifications in preclinical models, no related drugs have yet entered the clinical research phase. Future research in the following areas may be helpful: designing drugs to specifically target RNA modification regulators, especially in the tumor microenvironment, to reduce the impact on normal tissues; exploring appropriate drug dosages to balance efficacy and side effects; continuing drug safety research to assess the side effects of different targeted drugs; developing new therapeutic targets or drugs to improve treatment safety; and exploring the combined application of RNA modification-targeting drugs with other drugs in tumor therapy. In addition, tumor vaccines are also related to RNA modifications such as m6A and have therapeutic potential. Introducing encoded mRNA molecules into the bodies of tumor patients, translating them into proteins with anti-

tumor effects in the body to trigger anti-tumor immune responses is a new treatment method (173). RNA modifications play a key role in cancer vaccines. Through RNA modifications, the immunogenicity of RNA vaccines can be eliminated, and the rapid translation of anti-tumor proteins may be promoted, improving anti-tumor effects. This direction has considerable potential for development. Furthermore, lifting the immune suppression caused by RNA modifications may help improve the efficacy of adoptive immunotherapy. By lifting the toxicity suppression and infiltration suppression of T and NK cells caused by RNA modifications, the corresponding tumor-killing ability can be restored, which can be used for ex vivo expansion and then re-introduced into the patient's body to play an anti-tumor role. Lifting the immune suppression of the tumor microenvironment is beneficial for adoptive immune cells to clear the tumor. This could greatly enhance adoptive immunotherapy and holds value for research.

Author contributions

XQ: Writing – original draft. HL: Writing – original draft, Writing – review & editing. QZ: Writing – original draft. YC: Writing – review & editing. TL: Writing – review & editing. FT: Writing – review & editing, Conceptualization. QH: Writing – review & editing, Project administration, Resources.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This article was funded by the Special Project of Science and Technology Cooperation between Hubei Province and Chinese Academy of Sciences (Grant No. 42000021817T300000050) and The National Natural Science Foundation of China (No. 32200706).

Acknowledgments

Figures 1–3 created with biorender.com.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

- Cappannini A, Ray A, Purta E, Mukherjee S, Boccaletto P, Moafinejad SN, et al. MODOmics: a database of RNA modifications and related information. 2023 update. *Nucleic Acids Res.* (2024) 52:D239–d244. doi: 10.1093/nar/gkad1083
- Li J, Zhang H, Wang H. N(1)-methyladenosine modification in cancer biology: Current status and future perspectives. *Comput Struct Biotechnol J.* (2022) 20:6578–85. doi: 10.1016/j.csbj.2022.11.045
- Song H, Zhang J, Liu B, Xu J, Cai B, Yang H, et al. Biological roles of RNA m(5)C modification and its implications in Cancer immunotherapy. *biomark Res.* (2022) 10:15. doi: 10.1186/s40364-022-00362-8
- Jiang X, Liu B, Nie Z, Duan L, Xiong Q, Jin Z, et al. The role of m6A modification in the biological functions and diseases. *Signal transduction targeted Ther.* (2021) 6:74. doi: 10.1038/s41392-020-00450-x
- Luo Y, Yao Y, Wu P, Zi X, Sun N, He J. The potential role of N(7)-methylguanosine (m7G) in cancer. *J Hematol Oncol.* (2022) 15:63. doi: 10.1186/s13045-022-01285-5
- Ke S, Pandya-Jones A, Saito Y, Fak JJ, Vågbo CB, Geula S, et al. m(6)A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover. *Genes Dev.* (2017) 31:990–1006. doi: 10.1101/gad.301036.117
- Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L, et al. A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nat Chem Biol.* (2014) 10:93–5. doi: 10.1038/nchembio.1432
- Ping XL, Sun BF, Wang L, Xiao W, Yang X, Wang WJ, et al. Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase. *Cell Res.* (2014) 24:177–89. doi: 10.1038/cr.2014.3
- Su R, Dong L, Li Y, Gao M, He PC, Liu W, et al. METTL16 exerts an m(6)A-independent function to facilitate translation and tumorigenesis. *Nat Cell Biol.* (2022) 24:205–16. doi: 10.1038/s41556-021-00835-2
- Wang X, Zhao BS, Roundtree IA, Lu Z, Han D, Ma H, et al. N(6)-methyladenosine modulates messenger RNA translation efficiency. *Cell.* (2015) 161:1388–99. doi: 10.1016/j.cell.2015.05.014
- Shi H, Wang X, Lu Z, Zhao BS, Ma H, Hsu PJ, et al. YTHDF3 facilitates translation and decay of N(6)-methyladenosine-modified RNA. *Cell Res.* (2017) 27:315–28. doi: 10.1038/cr.2017.15
- Huang H, Weng H, Sun W, Qin X, Shi H, Wu H, et al. Recognition of RNA N(6)-methyladenosine by IGF2BP proteins enhances mRNA stability and translation. *Nat Cell Biol.* (2018) 20:285–95. doi: 10.1038/s41556-018-0045-z
- Yankova E, Blackaby W, Albertella M, Rak J, De Braekeleer E, Tsagkogeorga G, et al. Small-molecule inhibition of METTL3 as a strategy against myeloid leukaemia. *Nature.* (2021) 593:597–601. doi: 10.1038/s41586-021-03536-w
- He H, Wu W, Sun Z, Chai L. MiR-4429 prevented gastric cancer progression through targeting METTL3 to inhibit m(6)A-caused stabilization of SEC62. *Biochem Biophys Res Commun.* (2019) 517:581–7. doi: 10.1016/j.bbrc.2019.07.058
- Cheng L, Zhang X, Huang YZ, Zhu YL, Xu LY, Li Z, et al. Metformin exhibits antiproliferation activity in breast cancer via miR-483-3p/METTL3/m(6)A/p21 pathway. *Oncogenesis.* (2021) 10:7. doi: 10.1038/s41389-020-00290-y
- Mahapatra L, Andruska N, Mao C, Le J, Shapiro DJ. A novel IMP1 inhibitor, BTYNB, targets c-myc and inhibits melanoma and ovarian cancer cell proliferation. *Trans Oncol.* (2017) 10:818–27. doi: 10.1016/j.tranon.2017.07.008
- Wang SS, Lv Y, Xu XC, Zuo Y, Song Y, Wu GP, et al. Triptonide inhibits human nasopharyngeal carcinoma cell growth via disrupting Lnc-RNA THOR-IGF2BP1 signaling. *Cancer Lett.* (2019) 443:13–24. doi: 10.1016/j.canlet.2018.11.028
- Liu Y, Guo Q, Yang H, Zhang X-W, Feng N, Wang J-K, et al. Allosteric regulation of IGF2BP1 as a novel strategy for the activation of tumor immune microenvironment. *ACS Cent Sci.* (2022) 8:1102–15. doi: 10.1021/acscentsci.2c00107
- Weng H, Huang F, Yu Z, Chen Z, Prince E, Kang Y, et al. The m(6)A reader IGF2BP2 regulates glutamine metabolism and represents a therapeutic target in acute myeloid leukemia. *Cancer Cell.* (2022) 40:1566–1582.e10. doi: 10.1016/j.ccell.2022.10.004
- Mancarella C, Pasello M, Ventura S, Grilli A, Calzolari L, Toracchio L, et al. Insulin-like growth factor 2 mRNA-binding protein 3 is a novel post-transcriptional regulator of ewing sarcoma Malignancy. *Clin Cancer research: an Off J Am Assoc Cancer Res.* (2018) 24:3704–16. doi: 10.1158/1078-0432.CCR-17-2602
- Zhang Y, Liu X, Yu M, Xu M, Xiao Y, Ma W, et al. Berberine inhibits proliferation and induces G0/G1 phase arrest in colorectal cancer cells by downregulating IGF2BP3. *Life Sci.* (2020) 260:118413. doi: 10.1016/j.lfs.2020.118413
- Wang L, Zhu L, Liang C, Huang X, Liu Z, Huo J, et al. Targeting N6-methyladenosine reader YTHDF1 with siRNA boosts antitumor immunity in NASH-HCC by inhibiting EZH2-IL-6 axis. *J Hepatol.* (2023) 79:1185–200. doi: 10.1016/j.jhep.2023.06.021
- Xiao S, Ma S, Sun B, Pu W, Duan S, Han J, et al. The tumor-intrinsic role of the m6A reader YTHDF2 in regulating immune evasion. *Sci Immunol.* (2024) 9:eadi2171. doi: 10.1126/sciimmunol.adl2171
- Feng P, Chen D, Wang X, Li Y, Li Z, Li B, et al. Inhibition of the m(6)A reader IGF2BP2 as a strategy against T-cell acute lymphoblastic leukemia. *Leukemia.* (2022) 36:2180–8. doi: 10.1038/s41375-022-01651-9
- Su R, Dong L, Li Y, Gao M, Han L, Wunderlich M, et al. Targeting FTO suppresses cancer stem cell maintenance and immune evasion. *Cancer Cell.* (2020) 38:79–96.e11. doi: 10.1016/j.ccell.2020.04.017
- Sun K, Du Y, Hou Y, Zhao M, Li J, Du Y, et al. Saikosaponin D exhibits anti-leukemic activity by targeting FTO/m6A signaling. *Theranostics.* (2021) 11:5831–46. doi: 10.7150/thno.55574
- Su R, Dong L, Li C, Nachtergaele S, Wunderlich M, Qing Y, et al. R-2HG exhibits anti-tumor activity by targeting FTO/m(6)A/MYC/CEBPA signaling. *Cell.* (2018) 172:90–105.e23. doi: 10.1016/j.cell.2017.11.031
- Huang Y, Su R, Sheng Y, Dong L, Dong Z, Xu H, et al. Small-molecule targeting of oncogenic FTO demethylase in acute myeloid leukemia. *Cancer Cell.* (2019) 35:677–691.e10. doi: 10.1016/j.ccell.2019.03.006
- Liu Y, Liang G, Xu H, Dong W, Dong Z, Qiu Z, et al. Tumors exploit FTO-mediated regulation of glycolytic metabolism to evade immune surveillance. *Cell Metab.* (2021) 33:1221–1233.e11. doi: 10.1016/j.cmet.2021.04.001
- Malacrida A, Rivara M, Di Domizio A, Cislighi G, Miloso M, Zuliani V, et al. 3D proteome-wide scale screening and activity evaluation of a new ALKBH5 inhibitor in U87 glioblastoma cell line. *Bioorganic medicinal Chem.* (2020) 28:115300. doi: 10.1016/j.bmc.2019.115300
- Li N, Kang Y, Wang L, Huff S, Tang R, Hui H, et al. ALKBH5 regulates anti-PD-1 therapy response by modulating lactate and activity evaluation of a new ALKBH5 inhibitor in tumor microenvironment. *Proc Natl Acad Sci United States America.* (2020) 117:20159–70. doi: 10.1073/pnas.1918986117
- Wang Y, Wang J, Li X, Xiong X, Wang J, Zhou Z, et al. N(1)-methyladenosine methylation in tRNA drives liver tumorigenesis by regulating cholesterol metabolism. *Nat Commun.* (2021) 12:6314. doi: 10.1038/s41467-021-26718-6
- Zeng Z, Chew HY, Cruz JG, Leggatt GR, Wells JW. Investigating T Cell Immunity in Cancer: Achievements and Prospects, International journal of molecular sciences. *Int J Mol Sci* (2021) 22(6):2907. doi: 10.3390/ijms22062907
- Li HB, Tong J, Zhu S, Batista PJ, Duffy EE, Zhao J, et al. m(6)A mRNA methylation controls T cell homeostasis by targeting the IL-7/STAT5/SOCS pathways. *Nature.* (2017) 548:338–42. doi: 10.1038/nature23450
- Ding C, Xu H, Yu Z, Roulis M, Qu R, Zhou J, et al. RNA m(6)A demethylase ALKBH5 regulates the development of $\gamma\delta$ T cells. *Proc Natl Acad Sci United States America.* (2022) 119:e2203318119. doi: 10.1073/pnas.2203318119
- You M, Liu J, Li J, Ji C, Ni H, Guo W, et al. Mettl3-m6A-Creb1 forms an intrinsic regulatory axis in maintaining iNKT cell pool and functional differentiation. *Cell Rep.* (2023) 42:112584. doi: 10.1016/j.celrep.2023.112584
- Liu Y, Zhou J, Li X, Zhang X, Shi J, Wang X, et al. tRNA-m(1)A modification promotes T cell expansion via efficient MYC protein synthesis. *Nat Immunol.* (2022) 23:1433–44. doi: 10.1038/s41590-022-01301-3
- Jin S, Li J, Shen Y, Wu Y, Zhang Z, Ma H. RNA 5-Methylcytosine Regulator NSUN3 promotes tumor progression through regulating immune infiltration in head and neck squamous cell carcinoma. *Oral Dis.* (2024) 30:313–28. doi: 10.1111/odi.14357
- Sun G, Ma S, Zheng Z, Wang X, Chen S, Chang T, et al. Multi-omics analysis of expression and prognostic value of NSUN members in prostate cancer. *Front Oncol.* (2022) 12:965571. doi: 10.3389/fonc.2022.965571
- Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res.* (2017) 27:109–18. doi: 10.1038/cr.2016.151
- Tong J, Cao G, Zhang T, Sefik E, Amezcuea Vesely MC, Broughton JP, et al. m6A mRNA methylation sustains Treg suppressive functions. *Cell Res.* (2018) 28:253–6. doi: 10.1038/cr.2018.7
- Wang A, Huang H, Shi JH, Yu X, Ding R, Zhang Y, et al. USP47 inhibits m6A-dependent c-Myc translation to maintain regulatory T cell metabolic and functional homeostasis. The Journal of clinical investigation. *J Clin Invest* (2023) 133(23):e169365. doi: 10.1172/JCI169365
- Zhang L, Dou X, Zheng Z, Ye C, Lu TX, Liang HL, et al. YTHDF2/m6A/NF- κ B axis controls anti-tumor immunity by regulating intratumoral Tregs. *EMBO J.* (2023) 42:e113126. doi: 10.15252/embj.2022113126
- Gao Z, Xu J, Zhang Z, Fan Y, Xue H, Guo X, et al. A comprehensive analysis of METTL1 to immunity and stemness in pan-cancer. *Front Immunol.* (2022) 13:795240. doi: 10.3389/fimmu.2022.795240
- Chen J, Li K, Chen J, Wang X, Ling R, Cheng M, et al. Aberrant translation regulated by METTL1/WDR4-mediated tRNA N7-methylguanosine modification drives head and neck squamous cell carcinoma progression. *Cancer Commun (Lond).* (2022) 42:223–44. doi: 10.1002/cac2.12273
- Yao Y, Yang Y, Guo W, Xu L, You M, Zhang YC, et al. METTL3-dependent m(6)A modification programs T follicular helper cell differentiation. *Nat Commun.* (2021) 12:1333. doi: 10.1038/s41467-021-21594-6
- Ito-Kureha T, Leoni C, Borland K, Cantini G, Bataclan M, Metzger RN, et al. The function of Wtap in N(6)-adenosine methylation of mRNAs controls T cell receptor signaling and survival of T cells. *Nat Immunol.* (2022) 23:1208–21. doi: 10.1038/s41590-022-01268-1
- Jangani M, Poolman TM, Matthews L, Yang N, Farrow SN, Berry A, et al. The methyltransferase WBSR22/Merm1 enhances glucocorticoid receptor function and is

regulated in lung inflammation and cancer. *J Biol Chem.* (2014) 289:8931–46. doi: 10.1074/jbc.M113.540906

49. Nakazawa Y, Arai H, Fujita N. The novel metastasis promoter Merm1/Wbscr22 enhances tumor cell survival in the vasculature by suppressing Zaci/p53-dependent apoptosis. *Cancer Res.* (2011) 71:1146–55. doi: 10.1158/0008-5472.CAN-10-2695

50. Yun D, Yang Z, Zhang S, Yang H, Liu D, Grützmann R, et al. An m5C methylation regulator-associated signature predicts prognosis and therapy response in pancreatic cancer. *Front Cell Dev Biol.* (2022) 10:975684. doi: 10.3389/fcell.2022.975684

51. Chen T, Xu Z-G, Luo J, Manne RK, Wang Z, Hsu C-C, et al. NSUN2 is a glucose sensor suppressing cGAS/STING to maintain tumorigenesis and immunotherapy resistance. *Cell Metab.* (2023) 35(10):1782–98.e8. doi: 10.1016/j.cmet.2023.07.009

52. Guirguis AA, Ofir-Rosenfeld Y, Knezevic K, Blackaby W, Hardick D, Chan Y-C, et al. Inhibition of METTL3 results in a cell-intrinsic interferon response that enhances antitumor immunity. *Cancer Discovery.* (2023) 13:2228–47. doi: 10.1158/2159-8290.CD-23-0007

53. Cao L, Morgun E, Genardi S, Visvabharathy L, Cui Y, Huang H, et al. METTL14-dependent m(6)A modification controls iNKT cell development and function. *Cell Rep.* (2022) 40:111156. doi: 10.1016/j.celrep.2022.111156

54. Wang Z, Shang J, Qiu Y, Cheng H, Tao M, Xie E, et al. Suppression of the METTL3-m6A-integrin β 1 axis by extracellular acidification impairs T cell infiltration and antitumor activity. *Cell Rep.* (2024) 43:113796. doi: 10.1016/j.celrep.2024.113796

55. Hodgins JJ, Khan ST, Park MM, Auer RC, Ardolino M. Killers 2.0: NK cell therapies at the forefront of cancer control. *J Clin Invest.* (2019) 129:3499–510. doi: 10.1172/JCI129338

56. Ma S, Yan J, Barr T, Zhang J, Chen Z, Wang L-S, et al. The RNA m6A reader YTHDF2 controls NK cell antitumor and antiviral immunity. *The Journal of experimental medicine.* (2021) 218(8):e20210279. doi: 10.1084/jem.20210279

57. Song H, Song J, Cheng M, Zheng M, Wang T, Tian S, et al. METTL3-mediated m6A RNA methylation promotes the anti-tumour immunity of natural killer cells. *Nat Commun.* (2021) 12:5522. doi: 10.1038/s41467-021-25803-0

58. Meng M, Zhong Z, Song L, Zhang Z, Yin X, Xie X, et al. mTOR signaling promotes rapid m6A mRNA methylation to regulate NK-cell activation and effector functions. *Cancer Immunol Res.* (2024). 12(8):1039–57. doi: 10.1158/2326-6066.c7380107

59. Christofides A, Strauss L, Yeo A, Cao C, Charest A, Boussiotis VA. The complex role of tumor-infiltrating macrophages. *Nat Immunol.* (2022) 23:1148–56. doi: 10.1038/s41590-022-01267-2

60. Pathria P, Louis TL, Varner JA. Targeting tumor-associated macrophages in cancer. *Trends Immunol.* (2019) 40:310–27. doi: 10.1016/j.it.2019.02.003

61. Han D, Liu J, Chen C, Dong L, Liu Y, Chang R, et al. Anti-tumour immunity controlled through mRNA m(6)A methylation and YTHDF1 in dendritic cells. *Nature.* (2019) 566:270–4. doi: 10.1038/s41586-019-0916-x

62. Liu Y, Liu Z, Tang H, Shen Y, Gong Z, Xie N, et al. The N6-methyladenosine (m6A)-forming enzyme METTL3 facilitates M1 macrophage polarization through the methylation of STAT1 mRNA. *American journal of physiology. Cell Physiol.* (2019) 317:C762–75. doi: 10.1152/ajpcell.00212.2019

63. Yin H, Zhang X, Yang P, Zhang X, Peng Y, Li D, et al. RNA m6A methylation orchestrates cancer growth and metastasis via macrophage reprogramming. *Nat Commun.* (2021) 12:1394. doi: 10.1038/s41467-021-21514-8

64. Gu X, Zhang Y, Li D, Cai H, Cai L, Xu Q. N6-methyladenosine demethylase FTO promotes M1 and M2 macrophage activation. *Cell Signal.* (2020) 69:109553. doi: 10.1016/j.cellsig.2020.109553

65. Wang X, Ji Y, Feng P, Liu R, Li G, Zheng J, et al. The m6A reader IGF2BP2 regulates macrophage phenotypic activation and inflammatory diseases by stabilizing TSC1 and PPAR γ . *Adv Sci (Weinh).* (2021) 8:2100209. doi: 10.1002/advs.202100209

66. Xiong J, He J, Zhu J, Pan J, Liao W, Ye H, et al. Lactylation-driven METTL3-mediated RNA m6A modification promotes immunosuppression of tumor-infiltrating myeloid cells. *Mol Cell.* (2022) 82:1660–77. doi: 10.1016/j.molcel.2022.02.033

67. Yuan H, Liu J, Zhao L, Wu P, Chen G, Chen Q, et al. Prognostic risk model and tumor immune environment modulation of m5C-related lncRNAs in pancreatic ductal adenocarcinoma. *Front Immunol.* (2021) 12:800268. doi: 10.3389/fimmu.2021.800268

68. García-Vilchez R, Añazco-Guenkova AM, Dietmann S, López J, Morón-Calvente V, D'Ambrosi S, et al. METTL1 promotes tumorigenesis through tRNA-derived fragment biogenesis in prostate cancer. *Mol Cancer.* (2023) 22:119. doi: 10.1186/s12943-023-01809-8

69. Dong L, Chen C, Zhang Y, Guo P, Wang Z, Li J, et al. The loss of RNA N6-adenosine methyltransferase Mettl14 in tumor-associated macrophages promotes CD8 + T cell dysfunction and tumor growth. *Cancer Cell* (2021) 39(7):945–57.e10. doi: 10.1016/j.ccell.2021.04.016

70. Wculek SK, Cueto FJ, Mujal AM, Melero I, Krummel MF, Sancho D. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol.* (2020) 20:7–24. doi: 10.1038/s41577-019-0210-z

71. Accapezzato D, Visco V, Francavilla V, Molette C, Donato T, Paroli M, et al. Chloroquine enhances human CD8+ T cell responses against soluble antigens *in vivo*. *J Exp Med.* (2005) 202:817–28. doi: 10.1084/jem.20051106

72. Bai X, Wong CC, Pan Y, Chen H, Liu W, Zhai J, et al. Loss of YTHDF1 in gastric tumors restores sensitivity to antitumor immunity by recruiting mature dendritic cells. *J immunotherapy Cancer.* (2022) 10(2):e003663. doi: 10.1136/jitc-2021-003663

73. Wang H, Hu X, Huang M, Liu J, Gu Y, Ma L, et al. Mettl3-mediated mRNA m(6) A methylation promotes dendritic cell activation. *Nat Commun.* (2019) 10:1898. doi: 10.1038/s41467-019-09903-6

74. Liu J, Zhang X, Chen K, Cheng Y, Liu S, Xia M, et al. CCR7 chemokine receptor-inducible lnc-dpf3 restrains dendritic cell migration by inhibiting HIF-1 α -mediated glycolysis. *Immunity.* (2019) 50:600–615.e15. doi: 10.1016/j.immuni.2019.01.021

75. Xiao Y, Yang J, Yang M, Len J, Yu Y. Comprehensive analysis of 7-methylguanosine and immune microenvironment characteristics in clear cell renal cell carcinomas. *Front Genet.* (2022) 13:866819. doi: 10.3389/fgene.2022.866819

76. Cui W, Luo C, Zhou L, Yu T, Meng Y, Yu Q, et al. Roles of RNA m5C modification patterns in prognosis and tumor microenvironment infiltration of diffuse large B-cell lymphoma. *Am J Cancer Res.* (2024) 14:1768–83. doi: 10.62347/NXDR1826

77. Gao Y, Wang H, Li H, Ye X, Xia Y, Yuan S, et al. Integrated analyses of m(1)A regulator-mediated modification patterns in tumor microenvironment-infiltrating immune cells in colon cancer. *Oncoimmunology.* (2021) 10:1936758. doi: 10.1080/2162402X.2021.1936758

78. Bao G, Li T, Guan X, Yao Y, Liang J, Xiang Y, et al. Comprehensive analysis of the function, immune profiles, and clinical implication of m1A regulators in lung adenocarcinoma. *Front Oncol.* (2022) 12:882292. doi: 10.3389/fonc.2022.882292

79. Wang L, Hui H, Agrawal K, Kang Y, Li N, Tang R, et al. m(6) A RNA methyltransferases METTL3/14 regulate immune responses to anti-PD-1 therapy. *EMBO J.* (2020) 39:e104514. doi: 10.15252/emboj.2020104514

80. Ouyang P, Li K, Xu W, Chen C, Shi Y, Tian Y, et al. METTL3 recruiting M2-type immunosuppressed macrophages by targeting m6A-SNALC-CXCL2 axis to promote colorectal cancer pulmonary metastasis. *J Exp Clin Cancer Res.* (2024) 43:111. doi: 10.1186/s13046-024-03035-6

81. Chen H, Pan Y, Zhou Q, Liang C, Wong CC, Zhou Y, et al. METTL3 inhibits antitumor immunity by targeting m(6)A-BHLHE41-CXCL1/CXCR2 axis to promote colorectal cancer. *Gastroenterology.* (2022) 163:891–907. doi: 10.1053/j.gastro.2022.06.024

82. Chen Z, Zhou J, Wu Y, Chen F, Li J, Tao L, et al. METTL3 promotes cellular senescence of colorectal cancer via modulation of CDKN2B transcription and mRNA stability. *Oncogene.* (2024) 43:976–91. doi: 10.1038/s41388-024-02956-y

83. Pan Y, Chen H, Zhang X, Liu W, Ding Y, Huang D, et al. METTL3 drives NAFLD-related hepatocellular carcinoma and is a therapeutic target for boosting immunotherapy. *Cell Rep Med.* (2023) 4:101144. doi: 10.1016/j.xcrim.2023.101144

84. Wei H, Li W, Yang M, Fang Q, Nian J, Huang Y, et al. METTL3/16-mediated m6A modification of ZNNT1 promotes hepatocellular carcinoma progression by activating ZNNT1/osteopontin/S100A9 positive feedback loop-mediated crosstalk between macrophages and tumour cells. *Clin Immunol.* (2024) 261:109924. doi: 10.1016/j.clim.2024.109924

85. Sun Z, Mai H, Xue C, Fan Z, Li J, Chen H, et al. Hsa-LINC02418/immu-4930573107Rik regulated by METTL3 dictates anti-PD-L1 immunotherapeutic efficacy via enhancement of Trim21-mediated PD-L1 ubiquitination. *J immunotherapy Cancer.* (2023) 11(12):e007415. doi: 10.1136/jitc-2023-007415

86. Ning J, Hou X, Hao J, Zhang W, Shi Y, Huang Y, et al. METTL3 inhibition induced by M2 macrophage-derived extracellular vesicles drives anti-PD-1 therapy resistance via M6A-CD70-mediated immune suppression in thyroid cancer. *Cell Death Differ.* (2023) 30:2265–79. doi: 10.1038/s41418-023-01217-x

87. Wang B, Mao Z, Ye J, Jiao X, Zhang T, Wang Q, et al. Glycolysis induced by METTL14 is essential for macrophage phagocytosis and phenotype in cervical cancer. *J Immunol.* (2024) 212:723–36. doi: 10.4049/jimmunol.2300339

88. Sun C, Wang J, Li H, Liu L, Lin Y, Zhang L, et al. METTL14 regulates CD8+T-cell activation and immune responses to anti-PD-1 therapy in lung cancer. *World J Surg Oncol.* (2024) 22:128. doi: 10.1186/s12957-024-03402-9

89. Lu L, Zheng D, Qu J, Zhuang Y, Peng J, Lan S, et al. METTL16 predicts a favorable outcome and primes antitumor immunity in pancreatic ductal adenocarcinoma. *Front Cell Dev Biol.* (2022) 10:759020. doi: 10.3389/fcell.2022.759020

90. Wang Y, Jin P, Wang X. N6-methyladenosine regulator YTHDF1 represses the CD8 + T cell-mediated antitumor immunity and ferroptosis in prostate cancer via m6A/PD-L1 manner. *Apoptosis.* (2024) 29:142–53. doi: 10.1007/s10495-023-01885-7

91. Bao Y, Zhai J, Chen H, Wong CC, Liang C, Ding Y, et al. Targeting m6A reader YTHDF1 augments antitumor immunity and boosts anti-PD-1 efficacy in colorectal cancer. *Gut.* (2023) 72:1497–509. doi: 10.1136/gutjnl-2022-328845

92. Zhang L, Li Y, Zhou L, Zhou H, Ye L, Ou T, et al. The m6A reader YTHDF2 promotes bladder cancer progression by suppressing RIG-I-mediated immune response. *Cancer Res.* (2023) 83:1834–50. doi: 10.1158/0008-5472.CAN-22-2485

93. Jin H, Chen Y, Zhang D, Lin J, Huang S, Wu X, et al. YTHDF2 favors protumoral macrophage polarization and implies poor survival outcomes in triple negative breast cancer. *iScience.* (2024) 27:109902. doi: 10.1016/j.isci.2024.109902

94. Dai C, Cao J, Tang Y, Jiang Y, Luo C, Zheng J. YTHDF3 phase separation regulates HSPA13-dependent clear cell renal cell carcinoma development and immune evasion. *Cancer Sci.* (2024) 115(8):2588–601. doi: 10.1111/cas.16228

95. Zhang Y, Chen Y, Chen R, Zhou H, Lin Y, Li B, et al. YTHDF3 as a prognostic predictive biomarker of thyroid cancer and its correlation with immune infiltration. *BMC Cancer*. (2023) 23(1):882. doi: 10.1186/s12885-023-11361-9
96. Peng Y, Zhang Z, Yang G, Dai Z, Cai X, Liu Z, et al. N6-methyladenosine reader protein IGF2BP1 suppresses CD8+ T cells-mediated tumor cytotoxicity and apoptosis in colon cancer. *Apoptosis*. (2024) 29:331–43. doi: 10.1007/s10495-023-01893-7
97. Ma L, Jiang J, Si Q, Chen C, Duan Z. IGF2BP3 enhances the growth of hepatocellular carcinoma tumors by regulating the properties of macrophages and CD8(+) T cells in the tumor microenvironment. *J Clin Trans Hepatol*. (2023) 11:1308–20. doi: 10.14218/JCTH.2023.00184
98. Wang H, Tang A, Cui Y, Gong H, Li H. LRPPRC facilitates tumor progression and immune evasion through upregulation of m6A modification of PD-L1 mRNA in hepatocellular carcinoma. *Front Immunol*. (2023) 14:1144774. doi: 10.3389/fimmu.2023.1144774
99. Chen A, Zhang VX, Zhang Q, Sze KM-F, Tian L, Huang H, et al. Targeting the oncogenic m6A demethylase FTO suppresses tumorigenesis and potentiates immune response in hepatocellular carcinoma. *Gut*. (2024). doi: 10.1136/gutjnl-2024-331903
100. Zhai J, Chen H, Wong CC, Peng Y, Gou H, Zhang J, et al. ALKBH5 drives immune suppression via targeting AXIN2 to promote colorectal cancer and is a target for boosting immunotherapy. *Gastroenterology*. (2023) 165:445–62. doi: 10.1053/j.gastro.2023.04.032
101. Sun M, Yue Y, Wang X, Feng H, Qin Y, Chen M, et al. ALKBH5-mediated upregulation of CPT1A promotes macrophage fatty acid metabolism and M2 macrophage polarization, facilitating Malignant progression of colorectal cancer. *Exp Cell Res*. (2024) 437:113994. doi: 10.1016/j.yexcr.2024.113994
102. Zhang B, Wu Q, Li B, Wang D, Wang L, Zhou YL. m(6)A regulator-mediated methylation modification patterns and tumor microenvironment infiltration characterization in gastric cancer. *Mol Cancer*. (2020) 19:53. doi: 10.1186/s12943-020-01170-0
103. You Y, Wen D, Zeng L, Lu J, Xiao X, Chen Y, et al. ALKBH5/MAP3K8 axis regulates PD-L1+ macrophage infiltration and promotes hepatocellular carcinoma progression. *Int J Biol Sci*. (2022) 18:5001–18. doi: 10.7150/ijbs.70149
104. Dong F, Qin X, Wang B, Li Q, Hu J, Cheng X, et al. ALKBH5 facilitates hypoxia-induced paraspeckle assembly and IL8 secretion to generate an immunosuppressive tumor microenvironment. *Cancer Res*. (2021) 81:5876–88. doi: 10.1158/0008-5472.CAN-21-1456
105. Tang W, Xu N, Zhou J, He Z, Lenahan C, Wang C, et al. ALKBH5 promotes PD-L1-mediated immune escape through m6A modification of ZDHHC3 in glioma. *Cell Death Discovery*. (2022) 8:497. doi: 10.1038/s41420-022-01286-w
106. An Y, Duan H. ALKBH5 modulates macrophages polarization in tumor microenvironment of ovarian cancer. *J Ovarian Res*. (2024) 17:84. doi: 10.1186/s13048-024-01394-4
107. Hua X, Xu Q, Wu R, Sun W, Gu Y, Zhu S, et al. ALKBH5 promotes non-small cell lung cancer progression and susceptibility to anti-PD-L1 therapy by modulating interactions between tumor and macrophages. *J Exp Clin Cancer Res*. (2024) 43:164. doi: 10.1186/s13046-024-03073-0
108. Tan J, Chen F, Wang J, Li J, Ouyang B, Li X, et al. ALKBH5 promotes the development of lung adenocarcinoma by regulating the polarization of M2 macrophages through CDCA4. *Gene*. (2024) 895:147975. doi: 10.1016/j.gene.2023.147975
109. Xie Z, Li M, Hong H, Xu Q, He Z, Peng Z. Expression of N(6)-methyladenosine (m(6)A) regulators correlates with immune microenvironment characteristics and predicts prognosis in diffuse large cell lymphoma (DLBCL). *Bioengineered*. (2021) 12:6115–33. doi: 10.1080/21655979.2021.1972644
110. Li L, Yang W, Jia D, Zheng S, Gao Y, Wang G. Establishment of a N1-methyladenosine-related risk signature for breast carcinoma by bioinformatics analysis and experimental validation. *Breast Cancer (Tokyo Japan)*. (2023) 30:666–84. doi: 10.1007/s12282-023-01458-1
111. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Sci (New York N.Y.)*. (2018) 359:1350–5. doi: 10.1126/science.aar4060
112. Yi L, Wu G, Guo L, Zou X, Huang P. Comprehensive analysis of the PD-L1 and immune infiltrates of m6A RNA methylation regulators in head and neck squamous cell carcinoma. *Mol Ther Nucleic Acids*. (2020) 21:299–314. doi: 10.1016/j.omtn.2020.06.001
113. Ji H, Zhang J-A, Liu H, Li K, Wang Z-W, Zhu X. Comprehensive characterization of tumor microenvironment and m6A RNA methylation regulators and its effects on PD-L1 and immune infiltrates in cervical cancer. *Front Immunol*. (2022) 13:976107. doi: 10.3389/fimmu.2022.976107
114. Xu Y, He X, Deng J, Xiong L, Li Y, Zhang X, et al. Comprehensive analysis of the immune infiltrates and PD-L1 of m6A RNA methylation regulators in hepatocellular carcinoma. *Front Cell Dev Biol*. (2021) 9:681745. doi: 10.3389/fcell.2021.681745
115. Xu Z, Chen Q, Shu L, Zhang C, Liu W, Wang P. Expression profiles of m6A RNA methylation regulators, PD-L1 and immune infiltrates in gastric cancer. *Front Oncol*. (2022) 12:970367. doi: 10.3389/fonc.2022.970367
116. Wu X, Sheng H, Wang L, Xia P, Wang Y, Yu L, et al. A five-m6A regulatory gene signature is a prognostic biomarker in lung adenocarcinoma patients. *Aging (Albany NY)*. (2021) 13:10034–57. doi: 10.18632/aging.v13i7
117. Chen Z, Li Q, Lin Y, Lin S, Gao J, Chen S. m5C regulator-mediated methylation modification phenotypes characterized by distinct tumor microenvironment immune heterogeneity in colorectal cancer. *Sci Rep*. (2023) 13:11950. doi: 10.1038/s41598-023-37300-z
118. Dong Y, Li Y, Yao Y, Song Q. A novel defined m7G regulator signature to investigate the association between molecular characterization and clinical significance in lung adenocarcinoma. *Front Oncol*. (2022) 12:897323. doi: 10.3389/fonc.2022.897323
119. Chen J, Song Y-W, Liang G-Z, Zhang Z-J, Wen X-F, Li R-B, et al. A novel m7G-related gene signature predicts the prognosis of colon cancer. *Cancers*. (2022) 14(22):5527. doi: 10.3390/cancers14225527
120. Li Z, Wang W, Wu J, Ye X. Identification of N7-methylguanosine related signature for prognosis and immunotherapy efficacy prediction in lung adenocarcinoma. *Front Med (Lausanne)*. (2022) 9:962972. doi: 10.3389/fmed.2022.962972
121. Li K, Wang W. Establishment of m7G-related gene pair signature to predict overall survival in colorectal cancer. *Front Genet*. (2022) 13:981392. doi: 10.3389/fgene.2022.981392
122. Yu H, Liu J, Bu X, Ma Z, Yao Y, Li J, et al. Targeting METTL3 reprograms the tumor microenvironment to improve cancer immunotherapy. *Cell Chem Biol*. (2024) 31(4):776–791.e7. doi: 10.1016/j.chembiol.2023.09.001
123. Liu Z, Wang T, She Y, Wu K, Gu S, Li L, et al. N6-methyladenosine-modified circIGF2BP3 inhibits CD8+ T-cell responses to facilitate tumor immune evasion by promoting the deubiquitination of PD-L1 in non-small cell lung cancer. *Mol Cancer*. (2021) 20:105. doi: 10.1186/s12943-021-01398-4
124. Wan W, Ao X, Chen Q, Yu Y, Ao L, Xing W, et al. METTL3/IGF2BP3 axis inhibits tumor immune surveillance by upregulating N6-methyladenosine modification of PD-L1 mRNA in breast cancer. *Mol Cancer*. (2022) 21:60. doi: 10.1186/s12943-021-01447-y
125. Zhao Y, Huang S, Tan X, Long L, He Q, Liang X, et al. N6-methyladenosine-modified CBX1 regulates nasopharyngeal carcinoma progression through heterochromatin formation and STAT1 activation. *Adv Sci (Weinh)*. (2022) 9:e2205091. doi: 10.1002/advs.202205091
126. Li S, Feng T, Liu Y, Yang Q, Song A, Wang S, et al. m1A inhibition fuels oncolytic virus-elicited antitumor immunity via downregulating MYC/PD-L1 signaling. *Int J Oral Sci*. (2024) 16:36. doi: 10.1038/s41368-024-00304-0
127. Gao Y, Zou T, Xu P, Wang Y, Jiang Y, Chen Y-X, et al. Fusobacterium nucleatum stimulates cell proliferation and promotes PD-L1 expression via IFT1-related signal in colorectal cancer. *Neoplasia*. (2023) 35:100850. doi: 10.1016/j.neo.2022.100850
128. Zheng H, Zheng W-J, Wang Z-G, Tao Y-P, Huang Z-P, Yang L, et al. Decreased expression of programmed death ligand-L1 by seven in absentia homolog 2 in cholangiocarcinoma enhances T-cell-mediated antitumor activity. *Front Immunol*. (2022) 13:845193. doi: 10.3389/fimmu.2022.845193
129. Li T, Tan Y-T, Chen Y-X, Zheng X-J, Wang W, Liao K, et al. Methionine deficiency facilitates antitumor immunity by altering m6A methylation of immune checkpoint transcripts. *Gut*. (2023) 72:501–11. doi: 10.1136/gutjnl-2022-326928
130. Lei L, Li N, Yuan P, Liu D. A new risk model based on a 11-m6A-related lncRNA signature for predicting prognosis and monitoring immunotherapy for gastric cancer. *BMC Cancer*. (2022) 22:365. doi: 10.1186/s12885-021-09062-2
131. Chen K, Zhu S, Yu W, Xia Y, Xing J, Geng J, et al. Comprehensive analysis of N6-methyladenosine-related long non-coding RNAs signature in prognosis and tumor microenvironment of bladder cancer. *Front Oncol*. (2022) 12:774307. doi: 10.3389/fonc.2022.774307
132. Huang Y, Li X, Chen W, He Y, Wu S, Li X, et al. Analysis of the prognostic significance and potential mechanisms of lncRNAs associated with m6A methylation in papillary thyroid carcinoma. *Int Immunopharmacol*. (2021) 101:108286. doi: 10.1016/j.intimp.2021.108286
133. Song W, Ren J, Yuan W, Xiang R, Ge Y, Fu T. N6-methyladenosine-related lncRNA signature predicts the overall survival of colorectal cancer patients. *Genes (Basel)*. (2021) 12(9):1375. doi: 10.3390/genes12091375
134. Song Z, Wang X, Chen F, Chen Q, Liu W, Yang X, et al. lncRNA MALAT1 regulates METTL3-mediated PD-L1 expression and immune infiltrates in pancreatic cancer. *Front Oncol*. (2022) 12:1004212. doi: 10.3389/fonc.2022.1004212
135. Peng L, Pan B, Zhang X, Wang Z, Qiu J, Wang X, et al. Lipopolysaccharide facilitates immune escape of hepatocellular carcinoma cells via m6A modification of lncRNA MIR155HG to upregulate PD-L1 expression. *Cell Biol Toxicol*. (2022) 38:1159–73. doi: 10.1007/s10565-022-09718-0
136. Wang A, Sun Y, Wang X, Yan Z, Wang D, Zeng L, et al. m6A methyltransferase METTL16 mediates immune evasion of colorectal cancer cells via epigenetically regulating PD-L1 expression. *Aging (Albany NY)*. (2023) 15:8444–57. doi: 10.18632/aging.v15i16
137. Chen Z, Ruan W, Guo C, Chen K, Li L, Tian J, et al. Non-SMC condensin I complex subunit H participates in anti-programmed cell death-1 resistance of clear cell renal cell carcinomas. *Cell Prolif*. (2023) 56:e13400. doi: 10.1111/cpr.13400
138. Cui J, Zhu Y, Liu X, Wang W, Jiang X, Xia Y, et al. Comprehensive analysis of N6-methyladenosine regulators with the tumor immune landscape and correlation between the insulin-like growth factor 2 mRNA-binding protein 3 and programmed

death ligand 1 in bladder cancer. *Cancer Cell Int.* (2022) 22:72. doi: 10.1186/s12935-022-02456-7

139. Tsuchiya K, Yoshimura K, Inoue Y, Iwashita Y, Yamada H, Kawase A, et al. YTHDF1 and YTHDF2 are associated with better patient survival and an inflamed tumor-immune microenvironment in non-small-cell lung cancer. *Oncoimmunology.* (2021) 10:1962656. doi: 10.1080/2162402X.2021.1962656

140. Lin X, Wang Z, Yang G, Wen G, Zhang H. YTHDF2 correlates with tumor immune infiltrates in lower-grade glioma. *Aging (Albany NY).* (2020) 12:18476–500. doi: 10.18632/aging.v12i18

141. Qiu X, Yang S, Wang S, Wu J, Zheng B, Wang K, et al. M6A demethylase ALKBH5 regulates PD-L1 expression and tumor immunoenvironment in intrahepatic cholangiocarcinoma. *Cancer Res.* (2021) 81:4778–93. doi: 10.1158/0008-5472.CAN-21-0468

142. Li X, Chen W, Gao Y, Song J, Gu Y, Zhang J, et al. Fat mass and obesity-associated protein regulates arecoline-exposed oral cancer immune response through programmed cell death-ligand 1. *Cancer Sci.* (2022) 113:2962–73. doi: 10.1111/cas.15332

143. Zhou H, Meng M, Wang Z, Zhang H, Yang L, Li C, et al. The Role of m5C-Related lncRNAs in Predicting Overall Prognosis and Regulating the Lower Grade Glioma Microenvironment. *Front Oncol.* (2022) 12:814742. doi: 10.3389/fonc.2022.814742

144. Du Y, Ma Y, Zhu Q, Liu T, Jiao Y, Yuan P, et al. An m6A-related prognostic biomarker associated with the hepatocellular carcinoma immune microenvironment. *Front Pharmacol.* (2021) 12:707930. doi: 10.3389/fphar.2021.707930

145. Wang L, Zhang S, Li H, Xu Y, Wu Q, Shen J, et al. Quantification of m6A RNA methylation modulators pattern was a potential biomarker for prognosis and associated with tumor immune microenvironment of pancreatic adenocarcinoma. *BMC Cancer.* (2021) 21:876. doi: 10.1186/s12885-021-08550-9

146. Zou T, Shi D, Wang W, Chen G, Zhang X, Tian Y, et al. Identification of a new m6A regulator-related methylation signature for predicting the prognosis and immune microenvironment of patients with pancreatic cancer. *Mediators Inflammation.* (2023) 2023:5565054. doi: 10.1155/2023/5565054

147. Regmi P, He Z-Q, Lia T, Paudyal A, Li F-Y. N7-methylguanosine genes related prognostic biomarker in hepatocellular carcinoma. *Front Genet.* (2022) 13:918983. doi: 10.3389/fgene.2022.918983

148. Li DX, Feng DC, Wang XM, Wu RC, Zhu WZ, Chen K, et al. M7G-related molecular subtypes can predict the prognosis and correlate with immunotherapy and chemotherapy responses in bladder cancer patients. *Eur J Med Res.* (2023) 28:55. doi: 10.1186/s40001-023-01012-x

149. Wang W, Zhang D, Chang D, Li Y, Ren L. Identification of methyltransferase modification genes associated with prognosis and immune features of pancreatic adenocarcinoma. *Mol Cell Probes.* (2023) 67:101897. doi: 10.1016/j.mcp.2023.101897

150. Qiu Z, Zhao L, Shen JZ, Liang Z, Wu Q, Yang K, et al. Transcription elongation machinery is a druggable dependency and potentiates immunotherapy in glioblastoma stem cells. *Cancer Discovery.* (2022) 12:502–21. doi: 10.1158/2159-8290.CD-20-1848

151. Liang X, Gao H, Xiao J, Han S, He J, Yuan R, et al. Abrine, an IDO1 inhibitor, suppresses the immune escape and enhances the immunotherapy of anti-PD-1 antibody in hepatocellular carcinoma. *Front Immunol.* (2023) 14:1185985. doi: 10.3389/fimmu.2023.1185985

152. Yang S, Wei J, Cui Y-H, Park G, Shah P, Deng Y, et al. m6A mRNA demethylase FTO regulates melanoma tumorigenicity and response to anti-PD-1 blockade. *Nat Commun.* (2019) 10:2782. doi: 10.1038/s41467-019-10669-0

153. Cai J, Chen Z, Zhang Y, Wang J, Zhang Z, Wu J, et al. CircRHBDD1 augments metabolic rewiring and restricts immunotherapy efficacy via m6A modification in hepatocellular carcinoma. *Mol Ther oncolytics.* (2022) 24:755–71. doi: 10.1016/j.omto.2022.02.021

154. Chen S, Liu J, Zhang S, Zhao L, Zhang J, Han P, et al. Deciphering m6A signatures in hepatocellular carcinoma: Single-cell insights, immune landscape, and the protective role of IGFBP3. *Environ Toxicol.* (2024). doi: 10.1002/tox.24177

155. Pan L, She H, Wang K, Xia W, Tang H, Fan Y, et al. Characterization of the m6A regulator-mediated methylation modification patterns in oral squamous cell carcinoma. *Sci Rep.* (2023) 13:6617. doi: 10.1038/s41598-023-33891-9

156. Li P, Xiong P, Li X, Zhang X, Chen X, Zhang W, et al. Tumor microenvironment characteristics and prognostic role of m6A modification in lung squamous cell carcinoma. *Heliyon.* (2024) 10:e26851. doi: 10.1016/j.heliyon.2024.e26851

157. Huang ZD, Lin LL, Liu ZZ, Hu C, Gu HY, Wei RX. m6A modification patterns with distinct immunity, metabolism, and stemness characteristics in soft tissue sarcoma. *Front Immunol.* (2021) 12:765723. doi: 10.3389/fimmu.2021.765723

158. Huang X, Qiu Z, Li L, Chen B, Huang P. m6A regulator-mediated methylation modification patterns and tumor microenvironment infiltration characterization in hepatocellular carcinoma. *Aging (Albany NY).* (2021) 13:20698–715. doi: 10.18632/aging.v13i16

159. Zhou W, Lin J, Liu J, Zhang R, Fan A, Xie Q, et al. Thyroid cancer risk prediction model using m6A RNA methylation regulators: integrated bioinformatics analysis and histological validation. *Aging (Albany NY).* (2023) 15:846–65. doi: 10.18632/aging.v15i3

160. Zhang Z, Zhang C, Luo Y, Wu P, Zhang G, Zeng Q, et al. m(6)A regulator expression profile predicts the prognosis, benefit of adjuvant chemotherapy, and response to anti-PD-1 immunotherapy in patients with small-cell lung cancer. *BMC Med.* (2021) 19:284. doi: 10.1186/s12916-021-02148-5

161. Zhang T, Liu H, Gao F, Gong W, Cui Y, He J, et al. m6A-regulator expression signatures identify a subset of follicular lymphoma harboring an exhausted tumor microenvironment. *Front Immunol.* (2022) 13:922471. doi: 10.3389/fimmu.2022.922471

162. Dong M, Shen W, Yang G, Yang Z, Li X. Analysis of m6A methylation modification patterns and tumor immune microenvironment in breast cancer. *Front Cell Dev Biol.* (2022) 10:785058. doi: 10.3389/fcell.2022.785058

163. Yang Z, Ming X, Huang S, Yang M, Zhou X, Fang J. Comprehensive analysis of m(6)A regulators characterized by the immune cell infiltration in head and neck squamous cell carcinoma to aid immunotherapy and chemotherapy. *Front Oncol.* (2021) 11:764798. doi: 10.3389/fonc.2021.764798

164. Kong J, Lu S, Zhang L, Yao Y, Zhang J, Shen Z, et al. m6A methylation regulators as predictors for treatment of advanced urothelial carcinoma with anti-PDL1 agent. *Front Immunol.* (2022) 13:1014861. doi: 10.3389/fimmu.2022.1014861

165. Wang W, Dong D, Yu P, Chen T, Gao R, Wei J, et al. Prognostic model based on m6A-associated lncRNAs in esophageal cancer. *Front Endocrinol (Lausanne).* (2022) 13:947708. doi: 10.3389/fendo.2022.947708

166. Zhan M, Song H, Tian D, Wen Q, Shi X, Wang Y, et al. Molecular features, biological behaviors and clinical implications of m5C RNA methylation modification regulators in gastrointestinal cancers. *Cancer Biol Ther.* (2023) 24:2223382. doi: 10.1080/15384047.2023.2223382

167. Maimaiti A, Feng Z, Liu Y, Turhon M, Xie Z, Baihetiyaer Y, et al. N7-methylguanosine regulators-mediated methylation modification patterns and characterization of the immune microenvironment in lower-grade glioma. *Eur J Med Res.* (2023) 28:144. doi: 10.1186/s40001-023-01108-4

168. Wang Y, Mao Y, Wang C, Jiang X, Tang Q, Wang L, et al. RNA methylation-related genes of m6A, m5C, and m1A predict prognosis and immunotherapy response in cervical cancer. *Ann Med.* (2023) 55:2190618. doi: 10.1080/07853890.2023.2190618

169. Liu J, Dou M, Liu X, Lu Y, Lu W. A novel m6A/m5C/m1A score signature to evaluate prognosis and its immunotherapy value in colon cancer patients. *J Cancer Res Clin Oncol.* (2023) 149:11995–2012. doi: 10.1007/s00432-023-05033-1

170. Zhang J, Gao J, Hu M, Xu S, Cheng C, Zheng W, et al. Integrated investigation of the clinical implications and targeted landscape for RNA methylation modifications in hepatocellular carcinoma. *Eur J Med Res.* (2023) 28:46. doi: 10.1186/s40001-023-01016-7

171. Zhou B, Bie F, Zang R, Zhang M, Song P, Liu L, et al. RNA modification writer expression profiles predict clinical outcomes and guide neoadjuvant immunotherapy in non-small cell lung cancer. *EBioMedicine.* (2022) 84:104268. doi: 10.1016/j.ebiom.2022.104268

172. Huang Y, Yan J, Li Q, Li J, Gong S, Zhou H, et al. Meclofenamic acid selectively inhibits FTO demethylation of m6A over ALKBH5. *Nucleic Acids Res.* (2015) 43:373–84. doi: 10.1093/nar/gku1276

173. Mei Y, Wang X. RNA modification in mRNA cancer vaccines. *Clin Exp Med.* (2023) 23:1917–31. doi: 10.1007/s10238-023-01020-5



OPEN ACCESS

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RECEIVED 23 July 2024

ACCEPTED 07 October 2024

PUBLISHED 24 October 2024

CITATION

Ding Q, Liu M, Pan Y, Wu Z, Wang J, Li Y,
Liu X, Lai J, Hu D and Qiu S (2024)
Tumor-related IGF2BP1-derived molecular
subtypes to predict prognosis and immune
microenvironment in head and neck
squamous cell carcinoma.
Front. Immunol. 15:1469435.
doi: 10.3389/fimmu.2024.1469435

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Tumor-related IGF2BP1-derived molecular subtypes to predict prognosis and immune microenvironment in head and neck squamous cell carcinoma

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Background: Recent studies have underscored the biological significance of RNA modifications in tumorigenicity and progression. However, the potential roles of RNA modifications in immune regulation and the formation of the tumor microenvironment (TME) in head and neck squamous carcinoma (HNSC) remain unclear.

Methods: We collected 199 untreated HNSC samples and clinicopathological data from Fujian Provincial Cancer Hospital. MeRIP-seq and RNA-seq were performed to generate methylation and gene expression profiles, respectively. Consensus molecular subtyping was employed to identify prognosis-related genes and RNA modification patterns in HNSC. Experiments confirmed the potential oncogenic behavior influenced by key genes. Molecular subtypes were identified through consensus clustering and validated using external cohort validation sets.

Results: Among the RNA modification-related genes, IGF2BP1 emerged as the most prognostic. HNSC patients were categorized into high and low IGF2BP1 expression groups. High-expressing patients exhibited poorer survival and reduced chemosensitivity, coupled with increased tumor mutational burden, low PD-L1 expression, and limited immune cell infiltration, indicative of aggressive disease. Analysis revealed two distinct RNA modification patterns associated with IGF2BP1 expression: biosynthetically intense type (BIT) and oncogenically active type (OAT), each characterized by distinct clinical features, outcomes, and biological pathways. In an independent immunotherapy cohort, BIT patients displayed enhanced immune responses and sustained clinical benefits.

Conclusions: This study highlights the crucial link between RNA modification and TME diversity. Evaluating RNA modification in tumors improves our understanding of TME features and supports the development of effective immunotherapy strategies.

KEYWORDS

tumor microenvironment, IGF2BP1, molecular subtypes, head and neck squamous carcinoma, RNA modification

Introduction

RNA modification, involving chemical group addition to RNA nucleotides, governs RNA functions (1). Modifications like m1A, m6A, m6Am, m5C, m7G, ac4C, m3C, and Ψ regulate gene expression, affecting mRNA stability, splicing, translation, and localization (2, 3). RNA modification related genes (RMGs), including writers, erasers, and readers, orchestrate these processes, crucial for cellular function (1, 4). Dysregulation of RMGs may lead to aberrant cell growth and survival, particularly in cancer (5). RMGs impact tumor development by disrupting gene expression, presenting potential therapeutic targets (6, 7). Understanding RNA modification mechanisms is pivotal for comprehending cancer pathogenesis and devising novel therapeutic strategies.

Head and neck squamous cell carcinoma (HNSC) is a highly lethal malignancy with significant mortality rates despite treatment advancements (8–10). Extensive studies on mRNAs, lncRNAs, and EVs have identified numerous biomarkers and therapeutic targets; however, precise prognostic markers remain critically lacking (11–14). Genetic aberrations drive HNSC pathogenesis, influencing tumor initiation, progression, and therapy resistance (15, 16). Research has unveiled mutations in oncogenes and tumor suppressor genes, disrupting signaling pathways and fostering uncontrolled cell growth (17, 18). Yet, the comprehensive genetic landscape, notably in RNA modification regulators, and their impact on HNSC progression remain incompletely elucidated. Bridging this gap is imperative for tailored therapeutics and enhanced patient outcomes in HNSC.

This study endeavored to (i) characterize genetic variations and identify prognostic RMGs, (ii) investigate their functional roles in HNSC biology and treatment responses, and (iii) delineate novel molecular subtypes to refine HNSC classification and assess clinicopathological features.

Methods and materials

Cell culture

SCC7 and CAL27 cell lines were obtained from the Fujian Cancer Hospital Cell Bank and cultured under optimal conditions to ensure robust growth and viability. The cells were grown in

Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS). 1% penicillin-streptomycin was included in the medium. The cells were maintained in an incubator set to 37°C with a humidified atmosphere of 5% CO₂, closely mimicking physiological conditions. Transfections used siNC and siIGF2BP1 RNA sequences (19, 20).

Collection of clinical samples

This study included two cohorts. The first cohort comprised 3 cases of HNSC tumor tissue and 3 cases of normal tissue for merIP-seq analysis. The second cohort was utilized for RNA-seq analysis. Fresh tumor biopsy specimens were obtained from 193 head and neck cancer patients at Fujian Cancer Hospital (January 2015 - January 2018, Table 1). Tumor classification and staging followed the TNM system. The research was approved by the Ethics Committee of Fujian Cancer Hospital (Fujian Branch of Fudan University Shanghai Cancer Center, approval number K2022-084-01). The written consent of all participants was obtained in advance. External validation data from The Cancer Genome Atlas (TCGA) included 504 tumor samples and 44 normal samples.

Total RNA isolation, construction, and sequencing of mRNA library

Total RNA was extracted from the tumor tissues using the TRIzol reagent kit, following the manufacturer protocol to ensure optimal yield and purity. To enrich the mRNA, oligo(dT)-attached magnetic beads were employed, selectively binding to the poly(A) tails of mRNA molecules. The enriched mRNA was then fragmented using the Optimal Dual-mode mRNA Library Prep Kit (BGI-Shenzhen, China) to facilitate subsequent cDNA synthesis. Reverse transcription of the fragmented mRNA into complementary DNA (cDNA) was performed, creating a double-stranded cDNA library. In addition to repairing the ends, these purified double-stranded cDNA fragments had their 3' ends modified by adding an adenine nucleotide. This A-tailing step is crucial for the subsequent adapter ligation. Adapters, containing sequences necessary for amplification and

TABLE 1 Clinical features profile of in-house cohort patients.

Characteristics	Male	Female
N	136 (70.5%)	57 (29.5%)
Age, Mean ± SD	48.824 ± 10.827	47.421 ± 9.8488
T, n (%)		
1	24 (12.4%)	16 (8.3%)
2	31 (16.1%)	12 (6.2%)
3	44 (22.8%)	18 (9.3%)
4	37 (19.2%)	11 (5.7%)
N, n (%)		
0	13 (6.7%)	2 (1%)
1	45 (23.3%)	22 (11.4%)
2	53 (27.5%)	25 (13%)
3	25 (13%)	8 (4.1%)
M, n (%)		
0	128 (66.3%)	54 (28%)
1	8 (4.1%)	3 (1.6%)
stage, n (%)		
I	4 (2.1%)	0 (0%)
II	24 (12.4%)	15 (7.8%)
III	50 (25.9%)	23 (11.9%)
IV	58 (30.1%)	19 (9.8%)

sequencing, were ligated to the cDNA. The adapter-ligated cDNA was then amplified through PCR to ensure sufficient quantities for sequencing. Afterwards, BGI Technology Services Co. Ltd. sequenced the cDNA library, utilizing advanced sequencing platforms to generate high-quality data for further analysis. Detailed experimental procedures are available in the [Supplementary Materials](#).

MeRIP-seq and bioinformatic analysis

Total RNA was isolated and fragmented into ~100 nt pieces using a fragmentation buffer. The RNA was split into two parts: one for input and the other enriched with an m6A-specific antibody. Enriched RNA was transcribed into cDNA using random primers, end-repaired, and ligated to Illumina adaptors, creating a library sequenced on an Illumina NovaSeq™ 6000. Fastp (v0.20.0) filtered the sequencing data to obtain high-quality reads by eliminating adaptor-containing, high-N content, poly-A, and low-quality reads. ExomePeak2 (v1.0.0) was used for peak calling, identifying read-enriched regions ($p < 0.05$) as peaks. Peak-associated genes were validated using genomic position and gene annotation data. Peak distribution in 3'UTR, 5'UTR, and CDS regions was assessed. MEME suite and DREME were used for motif analysis in peak-associated transcript sequences.

Transwell assay for cell migration and invasion

For the invasion assay, transwell inserts were coated with 50 μ L of Matrigel diluted 1:8 in serum-free medium (SFM) and incubated at 37°C for 30 minutes to solidify. After solidification, 1×10^5 cells suspended in 200 μ L of SFM were seeded into the upper chamber of each insert. The lower chamber was filled with 600 μ L of complete medium containing 10% fetal bovine serum (FBS) as a chemoattractant.

For the migration assay, Matrigel was not applied, but all other procedures remained consistent. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 hours for migration and 48 hours for invasion assays. After incubation, non-migratory cells on the upper surface of the membrane were removed with a cotton swab. Migratory cells on the lower surface were fixed with 4% paraformaldehyde for 15 minutes, stained with 0.1% crystal violet for 10 minutes, and washed with PBS. Cells were counted under a microscope in three randomly selected fields per insert.

Cell counting kit 8 assay

After seeding 1000 cells per well in 96-well plates for uniform growth, overnight incubation was followed by addition of 10 μ L CCK8 reagent to each well to evaluate cell viability and proliferation via colorimetric changes. Cells were then cultured for specified durations (3h, 6h, 9h, and 12h), and absorbance was measured at 450 nm using a multifunctional microplate reader at these intervals. This methodology provides a quantitative assessment of cell proliferation and viability over time.

5-ethynyl-2-deoxyuridine assay

EdU cell proliferation assays were conducted using the BeyoClick™ EdU Cell Proliferation Assay Kit. All subsequent procedures were conducted according to the manufacturer instructions. For each experimental group, cells were subjected to treatment with EdU at a concentration of 10 μ mol/L for 2 hours. Fluorescence microscopy was employed for visualizing and recording fluorescent signals.

Single-cell data acquisition and processing

Single-cell RNA sequencing data were obtained from the Gene Expression Omnibus (GEO) database under the accession number GSE103322. For data processing, we employed the Seurat R package to perform quality control, normalization, and scaling of the single-cell data. We conducted dimensionality reduction using principal component analysis (PCA) and Uniform Manifold Approximation and Projection (UMAP) to visualize the data in lower-dimensional space. Subsequently, cell clustering was performed to identify distinct cell populations, and cell types

were annotated based on canonical marker genes using the SingleR algorithm and manual validation against known cell type signatures. To investigate the intercellular communication dynamics between immune cells and RNA-modified tumor cells, we employed the CellChat R package. This analysis was performed to identify and visualize the ligand-receptor interactions among different cell population.

Identification and analysis of prognostic RMG

To identify RMGs with significant prognostic value for PFS, we employed the least absolute shrinkage and selection operator (LASSO) Cox regression model. Differentially expressed genes (DEGs) from sequencing data of tumor samples in the in-house cohort were visualized using the R package “ggplot2”. DEGs were selected based on a fold-change >1.3 and a p-value < 0.05. The mutational landscape of these RMGs and signatures from TCGA genomic data were analyzed using the “maftools” package.

Predictive power assessment of IGF2BP1 and identified classification pattern

To evaluate the predictive power of IGF2BP1 and identified classification pattern, receiver operating characteristic (ROC) curves for 3-, 4-, and 5-year survival were plotted using the “timeROC” package in the internal cohort. High- and low-IGF2BP1 groups were stratified based on the optimal cut-off value determined by the “survival” package. Survival curves were compared using Kaplan-Meier analysis and log-rank test. Univariate Cox regression models determined the prognostic value of the IGF2BP1 expression.

Chemotherapy sensitivity assessment

The NCI-60 is a well-characterized panel of 60 human cancer cell lines developed by the National Cancer Institute (NCI, https://dtp.cancer.gov/discovery_development/nci-60/cell_list.htm). This panel includes cell lines derived from nine different types of cancer: leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney. The NCI-60 panel is widely used for drug discovery and cancer research because it provides a comprehensive representation of cancer diversity. By analyzing the NCI-60 tumor cell line panel, we explored the involvement of IGF2BP1 in drug sensitivity. Drug sensitivity data, quantified by half-maximal inhibitory concentration (IC50) values, were retrieved from the CellMiner database (<https://discover.nci.nih.gov/cellminer/>), a publicly accessible resource that integrates data on the molecular profiles and drug responses of the NCI-60 cell lines. Further analysis involved 218 FDA approved drugs and 574 drugs or compounds from clinical trials. The R packages “impute” and “limma” were utilized to evaluate the impact

of IGF2BP1 on drug sensitivity. The impute knn function was employed to estimate missing data for certain medications.

Immune cell type fractions analysis

The TIMER, CIBERSORT, and MCP-counter algorithms were utilized to calculate the infiltration levels of various immune cell types residing within each HNSC sample. These immune cell type fractions analyses employ deconvolution algorithms to test the presence of immune cells and their percentage. The ESTIMATE algorithm infers tumor cellularity and purity from transcriptional profiles. Using ESTIMATE, we calculated immune scores to assess infiltrating immune cells, finding that higher immune cell infiltration correlates with higher immune scores.

Quantification of immune response predictors using IPS, and TIDE

The Immunophenoscore (IPS) predicts response to anti-CTLA-4 and anti-PD-1 therapies by measuring tumor immune proximity and intratumoral immune profile (21). This study assesses differences in CTLA-4-negative and PD-1-negative percentages across different subgroups. The Tumor Immune Dysfunction and Exclusion (TIDE) algorithm, which mimics the mechanisms of tumor immune evasion, predicts response to immunotherapy (22); higher TIDE scores indicate more severe immune evasion and lower response rates to immune checkpoint inhibitors.

Consensus clustering

Consensus clustering was performed using the “Consensus ClusterPlus” tool in R to identify molecular subtypes. The optimal number of clusters (k) was evaluated by evaluating values between 2 and 10, with the clustering process repeated 1000 times to ensure reproducibility and robustness.

Gene set variation analysis

Gene Set Variation Analysis (GSVA) was conducted on HNSC samples using the “GSVA” package in R. Enrichment scores, representing gene set activity, were calculated from transcriptome data. The Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets were utilized to determine the variations in functional signatures across samples.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.4.1. Student’s t-test or Wilcoxon rank sum test was used for continuous variables and chi-square test for categorical variables.

For more than two groups, the Kruskal-Wallis test was used. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results

Identification of key prognostic genes IGF2BP1 in 59 RMGs and predictive ability

In total, 59 RMGs for RNA modifications (m1A, m6A, m6Am, m5C, m7G, ac4C, m3C, and Ψ) were obtained from a comprehensive review of previously published studies (Supplementary Table S1) (1, 2, 23–25). A chromosomal localization map of the 59 genes is displayed in Supplementary Figure S1A. The 59 aforementioned genes exhibited significant differential expression between tumor and normal tissues in the TCGA-HNSC dataset (Supplementary Figure S1B). We subsequently determined the prevalence of somatic mutations in 20 m6A and 16 m5C regulatory genes in HNSC. Among the 20 m6A regulators, WTAP and YTHDC2 exhibited the highest

mutation frequency at 10.9%, followed by YTHDC1 at 9.4% (Supplementary Figure S1C). Within the 16 m5C regulators, TET1, DNMT3B, and DNMT3BA showed the highest mutation frequency at 13.8%, followed by TET3 at 12.1%, and NSUN2 and DNMT1 both at 10.3% (Supplementary Figure S1D). Further analysis of the 59 RMGs revealed a high prevalence of CNV mutations. RBM15B, ZC3H13, YTHDF2, and PUS3 exhibited widespread CNV amplifications. Conversely, KIAA1429, IGF2BP2, YTHDF3, DNMT3BA, NSUN2, NSUN3, TRMT10C, and NUDT16 predominantly showed CNV deletions (Supplementary Figure S1E).

Given the ubiquity, abundance, and conservation of m6A as an endogenous modification in eukaryotic RNA, we conducted meRIP-seq analysis targeting m6A RNA methylation. Methylation of m6A in mammalian mRNAs predominantly occurs in the coding sequences (CDS) and 3' untranslated regions (3' UTR). Here, the m6A peaks were observed to be enriched in the CDS and 3' UTR regions (Figure 1A), and only the consensus GGAC motif was detected (Figure 1B), indicating the successful enrichment of m6A-modified mRNAs. Considering that a gene may possess multiple m6A binding

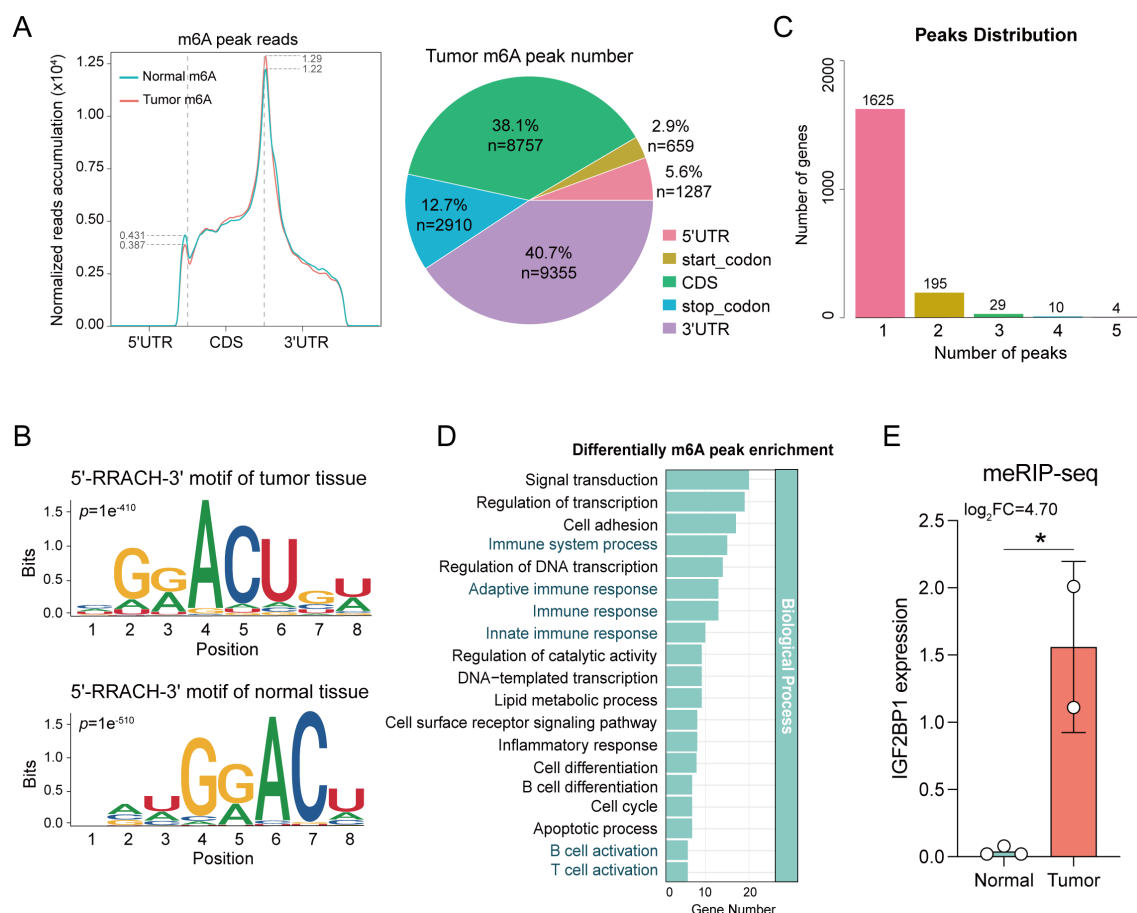


FIGURE 1

Comparative analysis of m6A levels between tumor and normal tissue. (A) The distribution of m6A peak reads and proportions in the 5'UTR, start codon, CDS, stop codon and 3'UTR in mRNA transcripts. (B) The m6A motif detected by MEME motif analysis. The RRACH (R=A/G, H=A/C/U) conserved sequence motifs for m6A-containing peak regions. (C) Genes with different number of peaks. (D) Enrichment analysis elucidates the functions of different m6A methylation modification genes. (E) IGF2BP1 exhibits differential expression in meRIP-seq analysis. * $p < 0.05$.

sites, we enumerated genes with varying numbers of peaks (Figure 1C). To elucidate the functions of genes with differential m6A methylation modifications, a Gene Ontology enrichment analysis was conducted, revealing that these genes were significantly enriched in various immune-related pathways, including Immune system process, Immune response, B cell activation, and T cell activation (Figure 1D). These findings suggest a potential correlation between m6A methylation modification and immune function.

To identify the genes most predictive of progression-free survival (PFS) in HNSC patients, we conducted LASSO regression and multivariate Cox regression analyses on 59 RMGs. This analysis identified IGF2BP1 as having the highest prognostic value for HNSC patients (Supplementary Figures S2A, B), and it also exhibited differential expression between tumor and normal tissues in meRIP-seq analysis (Figure 1E). In our in-house cohort, which comprised fresh tumor biopsy specimens obtained from 193 head and neck cancer patients at Fujian Cancer Hospital (from January 2015 to January 2018), a comparison of DEGs between cancer and paraneoplastic tissues revealed a significant upregulation of IGF2BP1 in cancer tissues (Figure 2A). Data from the TCGA-HNSC cohort supported this observation (Figure 2B). The best threshold value derived from the PFS analysis distinguished high- and low-IGF2BP1 expression levels. Chemokine families showed increased expression in the low-IGF2BP1 group in both the internal dataset and the external validation cohort. This upregulation was associated with a lower incidence of disease progression (Figure 2C; Supplementary Figure S2C). Patients in the high-IGF2BP1 group exhibited poorer tumor progression and worse PFS and OS prognosis (Figures 2D, E; Supplementary Figure S2D). The area under the ROC curve (AUC) demonstrated high predictive value, with scores of 0.69 at 3 years, 0.75 at 4 years, and 0.77 at 5 years (Figure 2F), and reached 0.930 in the TCGA-HNSC dataset (Supplementary Figure S2E). From the results of the univariate Cox analysis (Figure 2G), IGF2BP1 and age demonstrated strong survival predictive ability compared with other clinical features. Furthermore, IGF2BP1 expression was elevated in HPV-negative patients (Supplementary Figure S2F), those with lymphovascular invasion (Supplementary Figure S2G), and individuals with higher clinical T classifications (Supplementary Figure S2H). Additionally, IGF2BP1 expression exhibited a negative correlation with PD-L1 expression (Supplementary Figure S2I). Collectively, these findings suggest that high IGF2BP1 expression is associated with poorer prognosis and suboptimal treatment outcomes.

High IGF2BP1 expression correlates with active cancer-related pathways and chemotherapy insensitivity

Next, to further elucidate the role of IGF2BP1 in cancer progression, we examined its association with cancer-related pathways and its impact on the sensitivity to common chemotherapeutic agents. We found that IGF2BP1 expression was positively correlated with scores in common cancer-related pathways, including the Hippo signaling pathway (Figure 3A) and the Wnt signaling pathway (Figure 3B). In our cohort, low levels of

IGF2BP1 expression were associated with a high enrichment of cytokine-related HALLMARK pathways, such as complement signaling, IL2_STAT5_signaling, and inflammatory response signaling (Figure 3C). Additionally, chemotherapeutic agents used to treat HNSC, including 5-fluorodeoxyuridine, carboplatin, gefitinib, and gemcitabine, face resistance issues in patients with high IGF2BP1 expression, rendering these treatments less effective (Figures 3D, E).

Evaluation of the TME and immunotherapeutic response

Given the insensitivity to chemotherapy, we shifted our focus to the immunotherapy response in patients with high IGF2BP1 expression. We analyzed immune microenvironment differences between high- and low-IGF2BP1 expression groups, assessing immune scores and cell infiltration. The low-IGF2BP1 group had higher immune scores (Figure 4A). The immune cell occupancy of each sample in the HNSC-TCGA cohort is illustrated in Supplementary Figure S3A, providing a visual representation of the infiltration of various immune cell types within each sample. From a quantitative point of view, TIMER algorithm revealed significant differences in six immune cell types between groups (Figure 4B), with T cells, CD8+ cells, B cells, and NK cells more active in the low IGF2BP1 group (Figure 4C). At the single-cell level, IGF2BP1 was predominantly expressed in malignant cells (cluster 0), with negligible expression observed in other cell types (Figures 4D–F); consequently, we designated cluster 0 as RNA-modified tumor cells. Notably, the immune cell type exhibiting the most significant interaction was CD8 Tex cells (Figure 4G), with the most active ligand-receptor pair identified as MIF - (CD74+CXCR4). The composition of the immune microenvironment critically modulates the efficacy of immunotherapy. We found that IGF2BP1 expression was negatively correlated with immune checkpoint expression validated in our in-house cohort (Figure 4I). Notably, PDCD1 showed significant differences between the high- and low- IGF2BP1 subgroups (Figure 4J). The low-IGF2BP1 group had a lower TIDE score, indicating a stronger immune response (Figure 4K). Patients in the low-IGF2BP1 group exhibited higher immune responses in the HNSC patient cohort at Fujian Cancer Hospital (Figure 4L).

IGF2BP1 promotes malignant biological behavior of HNSC cells

To further validate the function of IGF2BP1 as an oncogene, we constructed IGF2BP1 knockdown cell lines in the human-derived CAL27 and murine-derived SCC7 cell lines. High IGF2BP1 expression enhanced the proliferative capacity of HNSC cells, with more cells in the proliferative phase (Figures 5A, B). Additionally, high IGF2BP1 levels correlated with increased self-cloning ability (Figure 5C). IGF2BP1 downregulation reduced both migratory (Figures 5D, E) and invasive abilities (Figure 5F). Overall, these results indicated that IGF2BP1 plays a significant part in promoting the malignant biological behavior of HNSC tumor cells.

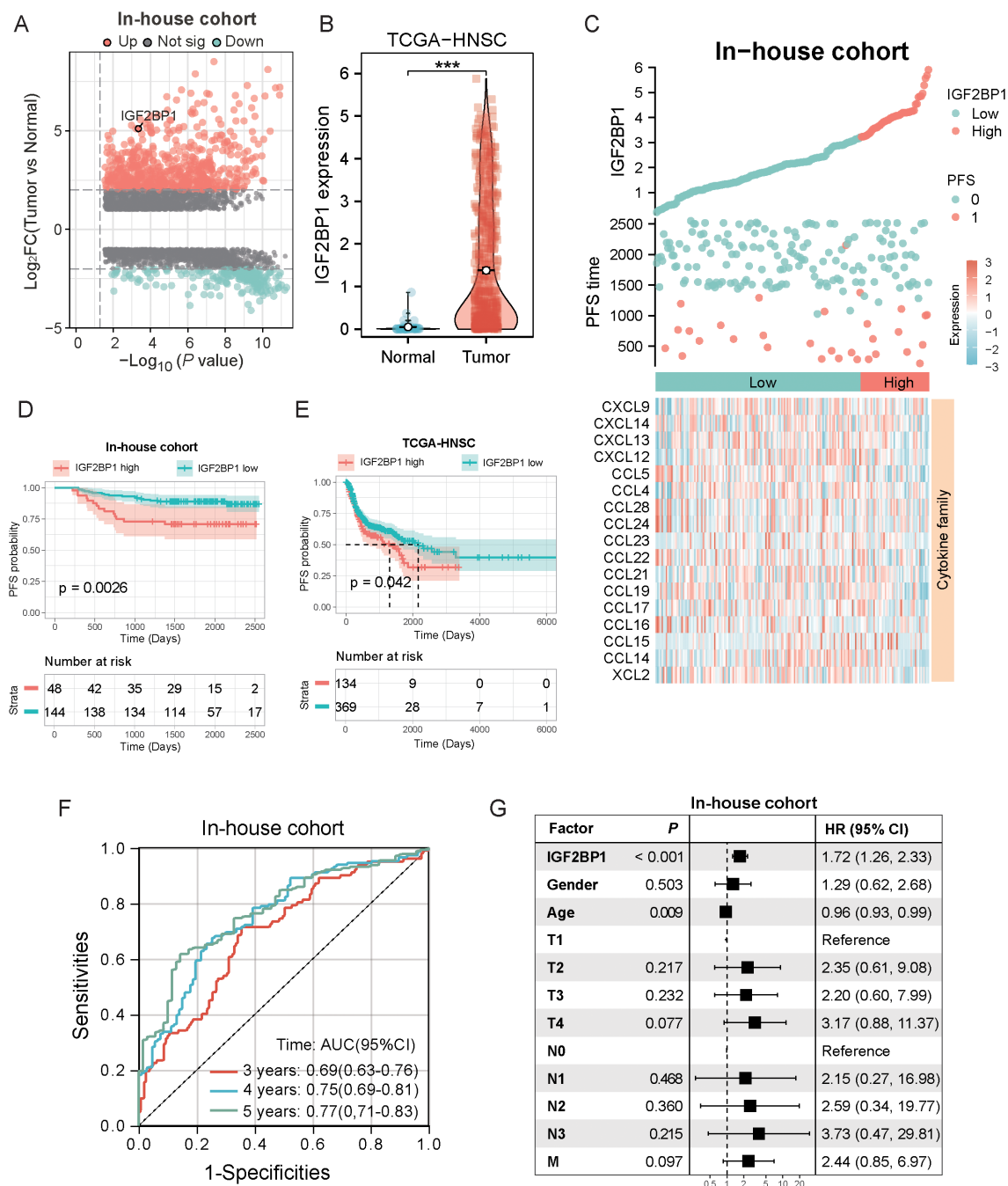


FIGURE 2 Screening of prognostically critical genes and the association with clinical features and prognostic predictive ability. **(A)** The volcano plot illustrates the differential gene expression between tumor and normal samples in the in-house cohort, highlighting the upregulation of IGF2BP1 in tumors (n=193). **(B)** The boxplot demonstrated that IGF2BP1 expression was significantly upregulated in the tumor samples of the TCGA-HNSC cohort. **(C)** Correlation of IGF2BP1 expression with that of the chemokine family. **(D, E)** In both the internal cohort **(D)** and the TCGA-HNSC cohort **(E)**, patients with high IGF2BP1 expression had shorter progression-free survival (PFS) and a worse prognosis. **(F)** ROC curve showing the predictive value of IGF2BP1 expression for 3-, 4-, and 5-year survival rates. **(G)** Univariate Cox analysis evaluate the prognostic value of IGF2BP1 expression in terms of PFS. *** $p < 0.001$.

HNSC patients were clustered into two subtypes with distinct clinical characteristics

Although HNSC patients were categorized into two groups based on PFS prognosis-related IGF2BP1 expression levels, the

underlying genetic changes remain unknown. To address this, we investigated the potential transcriptional expression changes of IGF2BP1 alteration in RNA modification patterns. Using the limma method, we identified 15 DEGs associated with high- and low-IGF2BP1 expression, which are considered characteristic genes related to RNA modification (Figure 6A). The expression of these

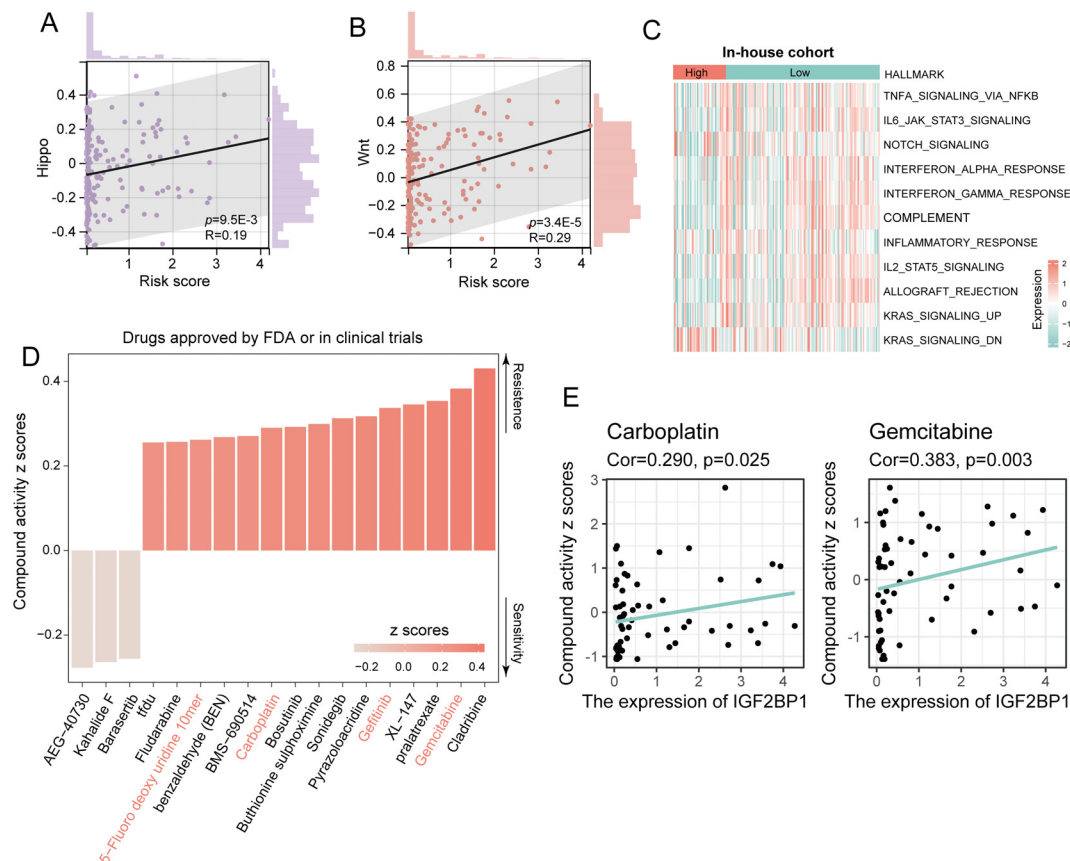


FIGURE 3

Assessment of the correlation between IGF2BP1 expression and cancer-related pathways, along with the prediction of chemotherapeutic drug sensitivity. (A, B) IGF2BP1 expression showed a positive correlation with the activity of cancer-related pathways. (C) Assessment of HALLMARK scores in patients with high and low IGF2BP1 expression. (D) The relationship between IGF2BP1 expression and chemotherapy drug sensitivity was evaluated. (E) The correlation between IGF2BP1 expression and the activity of carboplatin and gemcitabine compounds was assessed.

genes and their correlation with clinical features are shown in [Figure 6B](#). These genes are enriched in several immune-related pathways, including immune response-regulating signaling, B cell activation, and T cell activation regulation ([Figure 6C](#)).

Precise and detailed clinical typing is essential for individualized treatment and the optimization of medical resources. To achieve this, we mapped the pathway characteristics of HNSC samples using the KEGG database. Through consensus clustering with the k-means technique, we identified two distinct clusters, each characterized by unique pathway activity patterns ([Figure 6D](#)). Specifically, cluster C1 actively participated in the biosynthetic pathways like glycerolipid metabolism, arachidonic metabolism, and fatty acid metabolism. In contrast, cluster C2 showed low metabolic pathway activity but high oncogenic activation, including MAPK signaling, ERBB signaling, cell cycle, mTOR signaling, and WNT signaling pathways. Thus, C1 was defined as the biosynthetic intense type (BIT), and C2 as the oncogenic active type (OAT) ([Figure 6E](#)). PCA revealed distinct transcriptional profiles and heterogeneity, showing strong separation between samples from the two clusters ([Figure 6F](#)). Similarly, in TCGA-HNSC samples, two distinct groups were identified based on the aforementioned clustering ([Supplementary Figures S4A, B](#)).

The role of identified classification in clinical relevance and immunotherapeutic benefits

To evaluate the clinical application value of this classification, we assessed its prognostic significance and predictive efficacy for immunotherapy outcomes. In both the in-house cohort and TCGA-HNSC dataset, patients with BIT had longer progression-free survival and a significantly better prognosis ([Figures 7A, B](#)). Univariate Cox analysis indicated that patients in the OAT group had a hazard ratio of 2.28, predicting worse PFS ([Figure 7C](#)). Moreover, the chemokine family was significantly enriched in the BIT subtype, indicating more active cytokine chemotactic activity ([Figure 7D](#)). Consequently, we explored the infiltration of immune cells in the TME. In the in-house cohort, B cells and CD4+ T cells were significantly elevated in the BIT subtype ([Figure 7E](#)), consistent with findings from the TCGA-HNSC cohort ([Supplementary Figure S5A](#)). Overall, the BIT subtype exhibited higher levels of immune cell infiltration, suggesting a more active immune cytotoxic function ([Figure 7E](#); [Supplementary Figure S5A, B](#)).

Given the observed immune cell infiltration patterns, it is unsurprising that the BIT subtype exhibited a higher immune

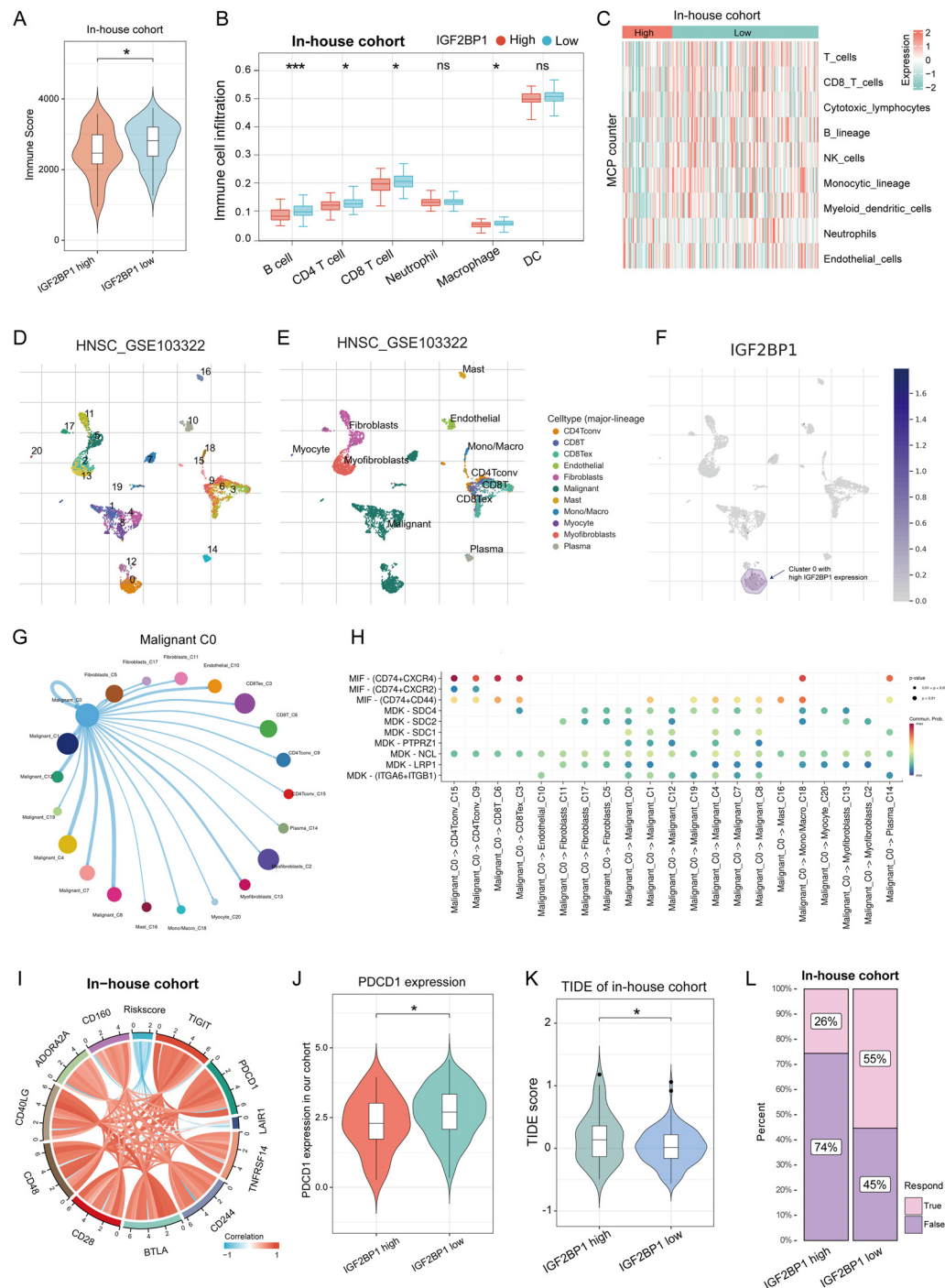


FIGURE 4

IGF2BP1 expression patterns correlate with the immune microenvironment and predict immunotherapy response. **(A)** Immune score significantly differed between high and low IGF2BP1 expression subgroups. **(B, C)** Significant differences in immune cell infiltration were observed between high and low IGF2BP1 subgroups using TIMER **(B)** and MCP counter **(C)** algorithms. **(D, E)** Standard single-cell analysis process of HNSC samples, including dimensionality reduction **(D)** and annotation of cell types **(E)**. **(F)** Expression levels of IGF2BP1 across various cell subpopulations. **(G)** A circular plot illustrating the intensity of intercellular communication between malignant cluster 0 and other subpopulations, where line thickness indicates the strength of communication and the size of the bubbles reflects the number of interactions. **(H)** A bubble plot demonstrating the activity of ligand-receptor pairs during communication between malignant cluster 0 and other subpopulations. **(I)** IGF2BP1 expression correlated with immune checkpoint expression. **(J)** PDCD1 expression varied between high and low IGF2BP1 subgroups. **(K)** High IGF2BP1 expression was associated with higher TIDE scores. **(L)** More patients with low IGF2BP1 expression responded to immunotherapy. * $p < 0.05$; *** $p < 0.001$.

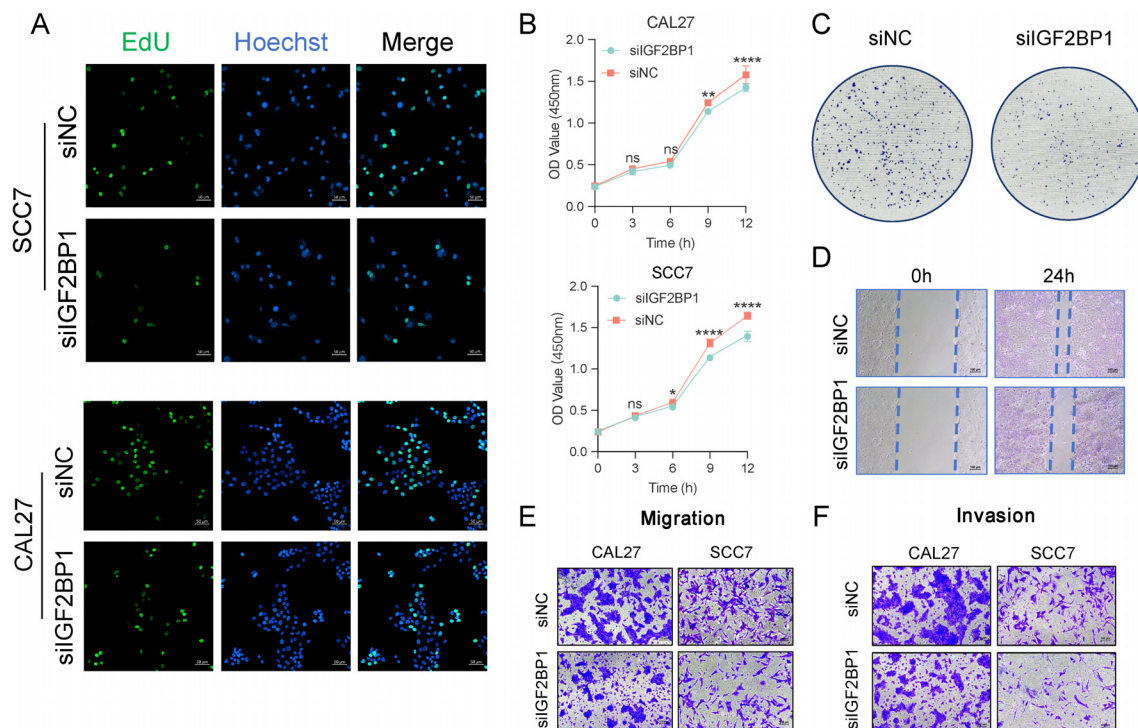


FIGURE 5

The malignant behavior related to IGF2BP1 expression. (A) The EdU assay assessed the proliferative capacity of cells; scale bar: 50 μ m. (B) The Cell Counting Kit 8 experiment assessed overall proliferative capacity. (C) A clone formation assay was performed to assess the impact of altered IGF2BP1 expression on the self-cloning ability of cells. (D) A scratch healing assay was conducted to evaluate the impact of altered IGF2BP1 expression on cell migration ability. (E, F) The Transwell assay was used to evaluate migration ability (E) of cells after 24 h and invasive capacity (F) after 48 h. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

score compared to the OAT subtype in the in-house cohort (Figure 7F). Moreover, the IPS score for CTLA4-neg PD1-neg in TCGA-HNSC was higher in the OAT subtype compared to the BIT subtype, suggesting that the OAT subtype has lower immune checkpoint expression and, consequently, a reduced likelihood of response to checkpoint inhibitor therapy (Figure 7G). Further analysis revealed that the OAT subtype had a significantly higher TIDE score than the BIT subtype in both the internal cohort (Figure 7H) and TCGA-HNSC (Supplementary Figure S5C), reinforcing the notion of a poorer immunotherapy response in the OAT subtype. Additionally, IFN-gamma expression was markedly lower in the OAT subtype compared to the BIT subtype (Supplementary Figure S5D). The response rate to immunotherapy decreased in the OAT subtype, as illustrated in Figure 7I. Collectively, these findings indicate that patients with the OAT subtype are less likely to derive benefit from immunotherapy.

Discussion

In cancer biology, RMGs are pivotal due to their influence on tumor development (26). This study analyzed genetic variations among RMGs in HNSC, identifying prognostic genes like IGF2BP1, strongly linked to tumor progression. Elevated IGF2BP1 expression correlated with aggressive tumor behavior, chemotherapy resistance, and immune microenvironment alterations, indicating its central role in HNSC

malignancy. We also introduced a new molecular classification, BIT and OAT, revealing unique clinical characteristics. BIT subtype exhibited better prognosis, heightened immune activity, and enhanced response to immunotherapy, promising for HNSC management.

RNA modifications and their regulatory mechanisms are closely intertwined with the TME in HNSC and other tumor types, impacting immune molecules, cells, and signal pathways (27, 28). Recent studies highlight the role of RNA modifications in regulating immune cell activation, infiltration, and subsequent immunotherapy outcomes, making them valuable targets for tumor immunotherapy (29–31). For instance, ALKBH5, an m6A demethylase, impacts T cell function and tumor growth (32). METTL3-mediated m6A modification influences NK cell homeostasis and function, affecting tumor growth and survival (33). Additionally, circIGF2BP3 overexpression in non-small cell lung cancer suppresses CD8⁺ T cell infiltration, compromising antitumor immunity (34). In this study, identification of key prognostic genes like IGF2BP1 enables its potential as a valuable biomarker, aiding in stratifying HNSC patients according to their risk of disease progression. The current study demonstrated that the MIF - (CD74+CXCR4) ligand-receptor pair is significantly active in tumor cells with elevated IGF2BP1 expression. MIF (Macrophage Migration Inhibitory Factor), a pivotal pro-inflammatory cytokine, orchestrates various immune responses and promotes the recruitment of immunosuppressive cells via its interaction with CD74 and CXCR4 receptors (35). This interaction is crucial for enhancing tumor immune evasion mechanisms within the TME (36). The activation of the MIF -

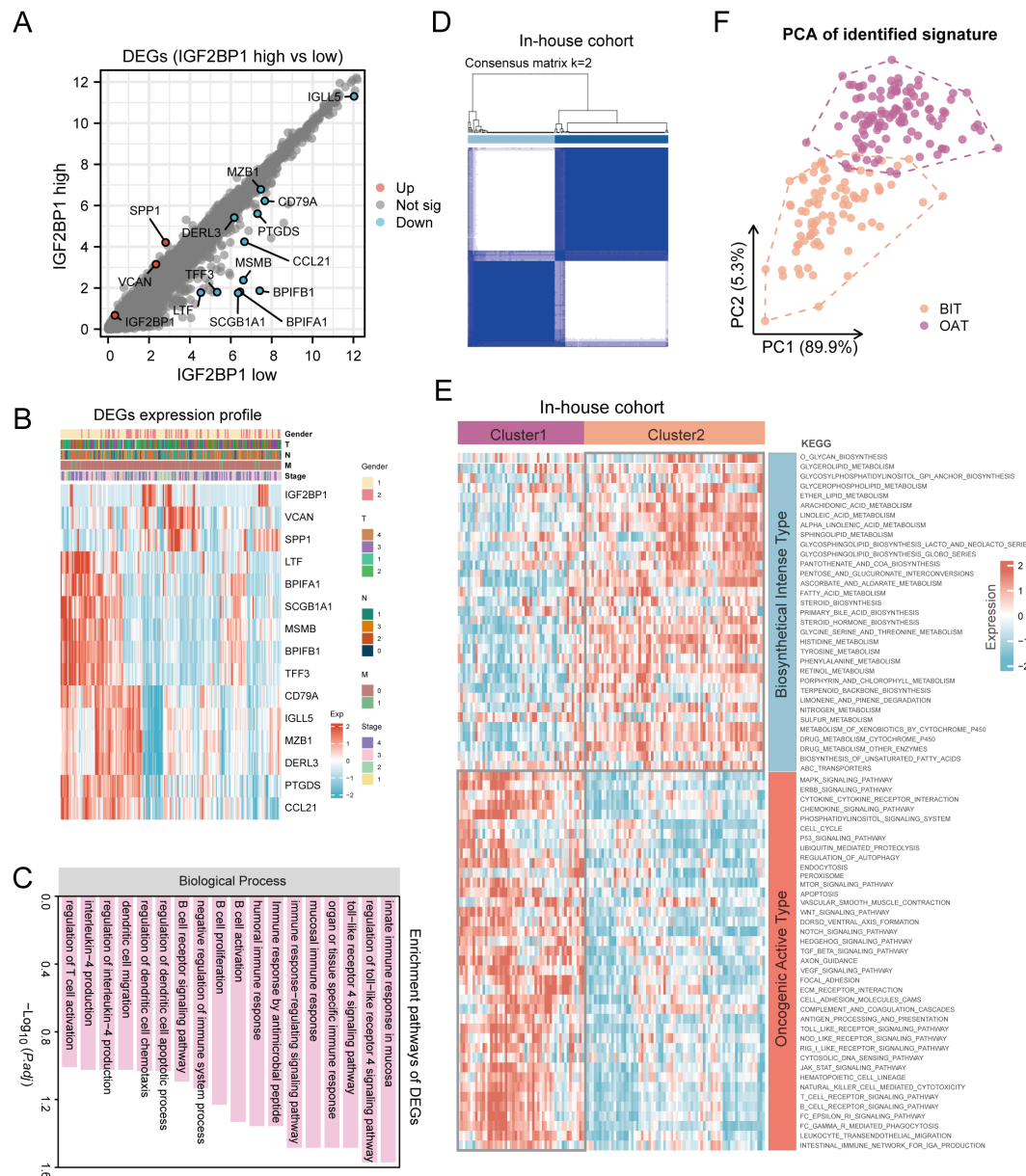


FIGURE 6

Consensus clustering identified two molecular subtypes in patients with HNSC. (A) Identification of DEGs between IGF2BP1 high- and low-expression groups in 193 HNSC samples; (B) Expression patterns of these DEGs; (C) Enriched pathways associated with identified DEGs; (D) Heatmap illustrating consensus clustering ($k = 2$) in 193 HNSC samples. (E) Heatmap depicting pathway scores for BIT and OAT molecular subtypes. (F) Principal component analysis plot demonstrating distinct expression patterns between BIT and OAT subtypes, with orange dots representing BIT and purple dots representing OAT.

(CD74+CXCR4) axis indicates that IGF2BP1-overexpressing tumor cells may facilitate immune evasion through this pathway, consequently undermining anti-tumor immune responses and adversely impacting patient prognosis in immunotherapy contexts.

HNSC exhibits diverse treatment responses and prognoses despite similar histologic types or TNM stages (37–39). The rapid advancements in precision medicine have significantly augmented our comprehension of tumor heterogeneity, offering deeper insights into the complex nature of cancer. Molecular subtyping of HNSC is advancing, with genomic studies identifying genetic alterations, including PI3KCA mutations, Kras activation, SMAD4 mutations,

and activation of PI3K/Akt/mTOR and Wnt pathways (40–44). An HPV-related classification has been established, correlating subtypes with smoking behavior and tumor immune response, though immune cell components in the TME are overlooked (45). HNSC subtypes, like atypical, basal, classical, and mesenchymal, feature distinct characteristics, with the mesenchymal subtype displaying heightened epithelial-mesenchymal transition (EMT) and inferior survival outcomes, yet the unique role of immunotherapy in HNSC remains unexplored (46). Our study compiled 193 RNA expression profiles, categorizing samples into BIT or OAT subtypes based on pathway activity. The system demonstrated reproducibility,

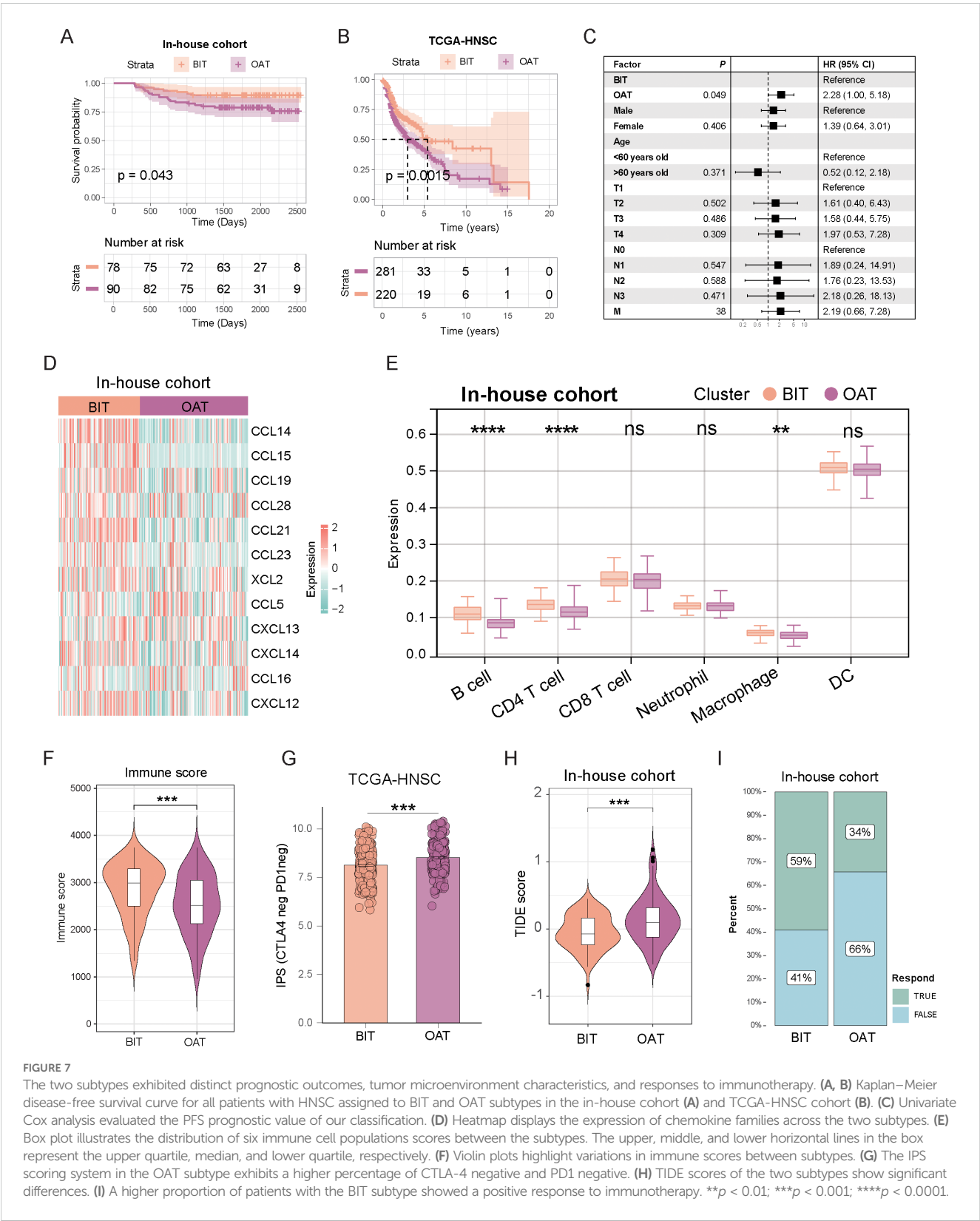


FIGURE 7 The two subtypes exhibited distinct prognostic outcomes, tumor microenvironment characteristics, and responses to immunotherapy. **(A, B)** Kaplan–Meier disease-free survival curve for all patients with HNSC assigned to BIT and OAT subtypes in the in-house cohort **(A)** and TCGA-HNSC cohort **(B)**. **(C)** Univariate Cox analysis evaluated the PFS prognostic value of our classification. **(D)** Heatmap displays the expression of chemokine families across the two subtypes. **(E)** Box plot illustrates the distribution of six immune cell populations scores between the subtypes. The upper, middle, and lower horizontal lines in the box represent the upper quartile, median, and lower quartile, respectively. **(F)** Violin plots highlight variations in immune scores between subtypes. **(G)** The IPS scoring system in the OAT subtype exhibits a higher percentage of CTLA-4 negative and PD1 negative. **(H)** TIDE scores of the two subtypes show significant differences. **(I)** A higher proportion of patients with the BIT subtype showed a positive response to immunotherapy. $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

predictability, and substantial prognostic value, although internal cohort validation is warranted.

In recent years, the evaluation of immunotherapy efficacy and prognosis in specific tumor types has gained considerable attention in modern medical practice (47). Tumor-microenvironment interactions classify tumors into hot spots (abundant immune cells, responsive to immunotherapy) and cold spots (limited immune cells, less responsive) (48). Our study developed a predictive model for immune cell infiltration, also estimating chemotherapeutic drug sensitivity and immune checkpoint treatment response. Patients

with high immune scores and immune cell infiltration, indicative of hot-spot tumors and robust immune responses, are likely to benefit from immunotherapy with improved prognosis.

While our study provides valuable insights, several limitations need to be acknowledged. The sample size, although substantial, may still limit the generalizability of our findings. Moreover, potential biases in the TCGA-HNSC dataset and our in-house cohort could influence the results. Further validation in larger, independent cohorts is necessary to confirm the prognostic value of the identified subtypes.

In conclusion, our study enhances comprehension of RNA modification regulators in HNSC by identifying key prognostic genes and elucidating the functional roles in cancer progression and treatment responses. We also introduce a novel HNSC classification based on transcriptomics, demonstrating significant predictive value for patient survival. These findings promise to advance personalized medicine in HNSC management through novel prognostic biomarkers and targeted therapies.

Data availability statement

The data presented in this study are available in the GEO repository under accession number GSE103322 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE103322>), as well as from TCGA-HNSC (<https://portal.gdc.cancer.gov/projects/TCGA-HNSC>), the NCI repository (https://dtp.cancer.gov/discovery_development/nci-60/cell_list.htm), and the CellMiner database (<https://discover.nci.nih.gov/cellminer/>). All original contributions discussed in this study are included in the article and its **Supplementary Material**. Additional information will be provided by the authors upon request, without any undue restrictions.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Fujian Cancer Hospital (Fujian Branch of Fudan University Shanghai Cancer Center, approval number K2022-084-01). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

QD: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. ML: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. YP: Methodology, Writing – original draft, Writing – review & editing. ZW: Methodology, Writing – original draft, Writing – review & editing. JW: Investigation, Writing – original draft, Writing – review

& editing. YL: Investigation, Writing – original draft, Writing – review & editing. XL: Writing – original draft, Writing – review & editing. JL: Writing – original draft, Writing – review & editing. DH: Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. SQ: Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the grants of Science and Technology Program of Fujian Province, China (2018Y2003); Fujian Provincial Clinical Research Center for Cancer Radiotherapy and Immunotherapy (2020Y2012); Supported by the National Clinical Key Specialty Construction Program (2021); Fujian Clinical Research Center for Radiation and Therapy of Digestive, Respiratory and Genitourinary Malignancies (2021Y2014); National Natural Science Foundation of China (82072986); Major Scientific Research Program for Young and Middle-aged Health Professionals of Fujian Province, China (Grant No. 2021ZQNZD010); Science and Technology Pilot Program of Fujian Province, China (2021Y0053); Wu Jieping Medical Foundation (320.6750.2021-01-27); Joint Funds for the Innovation of Science and Technology, Fujian province (2021Y9196, 2023Y9436); High-level Talent Training Program of Fujian Cancer Hospital (2022YNG07, 2022YNG16); Innovative Medicine Subject of Fujian Provincial Health Commission, China (2021CXA029); and Natural Science Foundation of Fujian province (2023J011267).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1469435/full#supplementary-material>

References

- Barbieri I, Kouzarides T. Role of RNA modifications in cancer. *Nat Rev Cancer*. (2020) 20:303–22. doi: 10.1038/s41568-020-0253-2
- Nombela P, Miguel-López B, Blanco S. The role of m6A, m5C and Ψ RNA modifications in cancer: Novel therapeutic opportunities. *Mol Cancer*. (2021) 20:18. doi: 10.1186/s12943-020-01263-w
- Boccalletto P, Machnicka MA, Purta E, Piatkowski P, Baginski B, Wirecki TK, et al. MODOMICS: a database of RNA modification pathways. 2017 update. *Nucleic Acids Res*. (2018) 46:D303–7. doi: 10.1093/nar/gkx1030
- Dor Y, Cedar H. Principles of DNA methylation and their implications for biology and medicine. *Lancet*. (2018) 392:777–86. doi: 10.1016/S0140-6736(18)31268-6
- Orsolic I, Carrier A, Esteller M. Genetic and epigenetic defects of the RNA modification machinery in cancer. *Trends Genet*. (2023) 39:74–88. doi: 10.1016/j.tig.2022.10.004
- Guan A, Wong JLL. Targeting RNA modifications with pharmacological agents: New frontiers in cancer therapy. *Cancer Med*. (2024) 13:e6989. doi: 10.1002/cam4.v13.7
- Wang Z, Zhou J, Zhang H, Ge L, Li J, Wang H. RNA m6A methylation in cancer. *Mol Oncol*. (2023) 17:195–229. doi: 10.1002/1878-0261.13326
- Sacco AG, Cohen EE. Current treatment options for recurrent or metastatic head and neck squamous cell carcinoma. *J Clin Oncol*. (2015) 33:3305–13. doi: 10.1200/JCO.2015.62.0963
- Rached L, Laparra A, Sakkal M, Danlos FX, Barlesi F, Carbonnel F, et al. Toxicity of immunotherapy combinations with chemotherapy across tumor indications: Current knowledge and practical recommendations. *Cancer Treat Rev*. (2024) 127:102751. doi: 10.1016/j.ctrv.2024.102751
- Jiménez-Labaig P, Rullan A, Hernando-Calvo A, Llop S, Bhide S, O'Leary B, et al. A systematic review of antibody-drug conjugates and bispecific antibodies in head and neck squamous cell carcinoma and nasopharyngeal carcinoma: Charting the course of future therapies. *Cancer Treat Rev*. (2024) 128:102772. doi: 10.1016/j.ctrv.2024.102772
- Passaro A, Al Bakir M, Hamilton EG, Diehn M, André F, Roy-Chowdhuri S, et al. Cancer biomarkers: Emerging trends and clinical implications for personalized treatment. *Cell*. (2024) 187:1617–35. doi: 10.1016/j.cell.2024.02.041
- Mandal R, Chan TA. Personalized oncology meets immunology: the path toward precision immunotherapy. *Cancer Discov*. (2016) 6:703–13. doi: 10.1158/2159-8290.CD-16-0146
- Asao T, Tobias GC, Lucotti S, Jones DR, Matei I, Lyden D. Extracellular vesicles and particles as mediators of long-range communication in cancer: connecting biological function to clinical applications. *Extracell Vesicles Circ Nucl Acids*. (2023) 4:461–85. doi: 10.20517/evcna.2023.37
- Raposo G, Stahl PD. Extracellular vesicles - on the cusp of a new language in the biological sciences. *Extracell Vesicles Circ Nucl Acids*. (2023) 4:240–54. doi: 10.20517/evcna.2023.18
- Huo XX, Wang SJ, Song H, de L, Yu H, Wang M, et al. Roles of major RNA adenosine modifications in head and neck squamous cell carcinoma. *Front Pharmacol*. (2021) 12:779779. doi: 10.3389/fphar.2021.779779
- Sun X, Fu S, Yuan X, Pu X, Wang R, Wang X, et al. RNA N6-methyladenosine (m6A) modification in HNSCC: molecular mechanism and therapeutic potential. *Cancer Gene Ther*. (2023) 30:1209–14. doi: 10.1038/s41417-023-00628-9
- Jing FY, Zhou LM, Ning YJ, Wang XJ, Zhu YM. The biological function, mechanism, and clinical significance of m6A RNA modifications in head and neck carcinoma: A systematic review. *Front Cell Dev Biol*. (2021) 9:683254. doi: 10.3389/fcell.2021.683254
- Wu K, Chang F, Li W, Su T, Lei D. Role of IGF2BPs in head and neck squamous cell carcinoma. *Front Oncol*. (2022) 12:1003808. doi: 10.3389/fonc.2022.1003808
- Zhang X, Yao Y, Han J, Yang Y, Chen Y, Tang Z, et al. Longitudinal epitranscriptome profiling reveals the crucial role of N6-methyladenosine methylation in porcine prenatal skeletal muscle development. *J Genet Genomics*. (2020) 47:466–76. doi: 10.1016/j.jgg.2020.07.003
- Zhou X, Zhang CZ, Lu SX, Chen GG, Li LZ, Liu LL, et al. miR-625 suppresses tumour migration and invasion by targeting IGF2BP1 in hepatocellular carcinoma. *Oncogene*. (2015) 34:965–77. doi: 10.1038/ncr.2014.35
- Charoentong P, Finotello F, Angelova M, Mayer C, Efremova M, Rieder D, et al. Pan-cancer immunogenomic analyses reveal genotype-immunophenotype relationships and predictors of response to checkpoint blockade. *Cell Rep*. (2017) 18:248–62. doi: 10.1016/j.celrep.2016.12.019
- Jiang P, Gu S, Pan D, Fu J, Sahu A, Hu X, et al. Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nat Med*. (2018) 24:1550–8. doi: 10.1038/s41591-018-0136-1
- Cui L, Ma R, Cai J, Guo C, Chen Z, Yao L, et al. RNA modifications: importance in immune cell biology and related diseases. *Signal Transduct Target Ther*. (2022) 7:334. doi: 10.1038/s41392-022-01175-9
- Frye M, Harada BT, Behm M, He C. RNA modifications modulate gene expression during development. *Science*. (2018) 361:1346–9. doi: 10.1126/science.aau1646
- Roundtree IA, Evans ME, Pan T, He C. Dynamic RNA modifications in gene expression regulation. *Cell*. (2017) 169:1187–200. doi: 10.1016/j.cell.2017.05.045
- Gilbert WV, Bell TA, Schaening C. Messenger RNA modifications: Form, distribution, and function. *Science*. (2016) 352:1408–12. doi: 10.1126/science.aad8711
- Li G, Yao Q, Liu P, Zhang H, Liu Y, Li S, et al. Critical roles and clinical perspectives of RNA methylation in cancer. *MedComm*. (2020). (2024) 5:e559. doi: 10.1002/mco2.v5.5
- Acharya P, Parkins S, Tranter M. RNA binding proteins as mediators of pathological cardiac remodeling. *Front Cell Dev Biol*. (2024) 12:1368097. doi: 10.3389/fcell.2024.1368097
- Deng LJ, Deng WQ, Fan SR, Chen MF, Qi M, Lyu WY, et al. m6A modification: recent advances, anticancer targeted drug discovery and beyond. *Mol Cancer*. (2022) 21:52. doi: 10.1186/s12943-022-01510-2
- An Y, Duan H. The role of m6A RNA methylation in cancer metabolism. *Mol Cancer*. (2022) 21:14. doi: 10.1186/s12943-022-01500-4
- Wu C, Li L, Tang Q, Liao Q, Chen P, Guo C, et al. Role of m6A modifications in immune evasion and immunotherapy. *Med Oncol*. (2024) 41:159. doi: 10.1007/s12032-024-02402-9
- Qiu X, Yang S, Wang S, Wu J, Zheng B, Wang K, et al. M6A demethylase ALKBH5 regulates PD-L1 expression and tumor immunoenvironment in intrahepatic cholangiocarcinoma. *Cancer Res*. (2021) 81:4778–93. doi: 10.1158/0008-5472.CAN-21-0468
- Song H, Song J, Cheng M, Zheng M, Wang T, Tian S, et al. METTL3-mediated m6A RNA methylation promotes the anti-tumour immunity of natural killer cells. *Nat Commun*. (2021) 12:5522. doi: 10.1038/s41467-021-25803-0
- Liu Z, Wang T, She Y, Wu K, Gu S, Li L, et al. N6-methyladenosine-modified circIGF2BP3 inhibits CD8+ T-cell responses to facilitate tumor immune evasion by promoting the deubiquitination of PD-L1 in non-small cell lung cancer. *Mol Cancer*. (2021) 20:105. doi: 10.1186/s12943-021-01398-4
- Thavayogarahaj T, Sinitski D, El Bounkari O, Torres-Garcia L, Lewinsky H, Harjung A, et al. CXCR4 and CD74 together enhance cell survival in response to macrophage migration-inhibitory factor in chronic lymphocytic leukemia. *Exp Hematol*. (2022) 115:30–43. doi: 10.1016/j.exphem.2022.08.005
- Lue H, Dewor M, Leng L, Bucala R, Bernhagen J. Activation of the JNK signalling pathway by macrophage migration inhibitory factor (MIF) and dependence on CXCR4 and CD74. *Cell Signal*. (2011) 23:135–44. doi: 10.1016/j.cellsig.2010.08.013
- Canning M, Guo G, Yu M, Myint C, Groves MW, Byrd JK, et al. Heterogeneity of the head and neck squamous cell carcinoma immune landscape and its impact on immunotherapy. *Front Cell Dev Biol*. (2019) 7:52. doi: 10.3389/fcell.2019.00052
- Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. *Nat Rev Cancer*. (2018) 18:269–82. doi: 10.1038/nrc.2018.11
- Cohen EEW, Bell RB, Bifulco CB, Burtress B, Gillison ML, Harrington KJ, et al. The Society for Immunotherapy of Cancer consensus statement on immunotherapy for the treatment of squamous cell carcinoma of the head and neck (HNSCC). *J Immunother Cancer*. (2019) 7:184. doi: 10.1186/s40425-019-0662-5
- Kim DH, Lim ST, Kim HR, Kang EJ, Ahn HK, Lee YG, et al. Impact of PIK3CA and cell cycle pathway genetic alterations on durvalumab efficacy in patients with head and neck squamous cell carcinoma: Post hoc analysis of TRIUMPH study. *Oral Oncol*. (2024) 151:106739. doi: 10.1016/j.oraloncology.2024.106739
- Deng M, Guo J, Ling Z, Zhang C, He L, Fan Z, et al. KRAS mutations upregulate Runx1 to promote occurrence of head and neck squamous cell carcinoma. *Mol Carcinog*. (2023) 62:1284–94. doi: 10.1002/mc.23563
- Hernandez AL, Young CD, Bian L, Weigel K, Nolan K, Frederick B, et al. PARP inhibition enhances radiotherapy of SMAD4-deficient human head and neck squamous cell carcinomas in experimental models. *Clin Cancer Res*. (2020) 26:3058–70. doi: 10.1158/1078-0432.CCR-19-0514
- Bernard M, Cardin GB, Cahuzac M, Ayad T, Bissada E, Guertin L, et al. Dual inhibition of autophagy and PI3K/AKT/MTOR pathway as a therapeutic strategy in head and neck squamous cell carcinoma. *Cancers (Basel)*. (2020) 12:2371. doi: 10.3390/cancers12092371
- Nguyen PT, Shimojukkoku Y, Kajiya Y, Oku Y, Tomishima A, Shima K, et al. Gene alterations in the nuclear transport receptor superfamily: A study of head and neck cancer. *PloS One*. (2024) 19:e0300446. doi: 10.1371/journal.pone.0300446
- Chuang YH, Lin CY, Lee JC, Lee CH, Liu CL, Huang SH, et al. Identification of the HNSC88 molecular signature for predicting subtypes of head and neck cancer. *Int J Mol Sci*. (2023) 24:13068. doi: 10.3390/ijms241713068
- Li J, Wu T, Song K, Zhu L, Wang Y, Chen T, et al. Integrative network analysis reveals subtype-specific long non-coding RNA regulatory mechanisms in head and neck squamous cell carcinoma. *Comput Struct Biotechnol J*. (2023) 21:535–49. doi: 10.1016/j.csbj.2022.12.030
- Goliwas KF, Deshane JS, Elmetts CA, Athar M. Moving immune therapy forward targeting TME. *Physiol Rev*. (2021) 101:417–25. doi: 10.1152/physrev.00008.2020
- Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discov*. (2019) 18:197–218. doi: 10.1038/s41573-018-0007-y



OPEN ACCESS

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RECEIVED 26 October 2024

ACCEPTED 18 November 2024

PUBLISHED 02 December 2024

CITATION

Wang X, Ma X, Chen S, Fan M, Jin C, Chen Y,
Wang S, Wang Z, Meng F, Zhang C and
Yang L (2024) Harnessing m1A modification: a
new frontier in cancer immunotherapy.
Front. Immunol. 15:1517604.
doi: 10.3389/fimmu.2024.1517604

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Harnessing m1A modification: a new frontier in cancer immunotherapy

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N1-methyladenosine (m1A) modification is an epigenetic change that occurs on RNA molecules, regulated by a suite of enzymes including methyltransferases (writers), demethylases (erasers), and m1A-recognizing proteins (readers). This modification significantly impacts the function of RNA and various biological processes by affecting the structure, stability, translation, metabolism, and gene expression of RNA. Thereby, m1A modification is closely associated with the occurrence and progression of cancer. This review aims to explore the role of m1A modification in tumor immunity. m1A affects tumor immune responses by directly regulating immune cells and indirectly modulating tumor microenvironment. Besides, we also discuss the implications of m1A-mediated metabolic reprogramming and its nexus with immune checkpoint inhibitors, unveiling promising avenues for immunotherapeutic intervention. Additionally, the m1AScore, established based on the expression patterns of m1A modification, can be used to predict tumor prognosis and guide personalized therapy. Our review underscores the significance of m1A modification as a burgeoning frontier in cancer biology and immuno-oncology, with the potential to revolutionize cancer treatment strategies.

KEYWORDS

m1A modification, cancer immunotherapy, tumor microenvironment, m1AScore, immune checkpoint inhibitor

1 Introduction

Epigenetic modifications of RNA refer to chemical modifications that occur on RNA molecules without altering their basic sequence, yet they significantly affect the stability, localization, translation efficiency, and other biological functions of RNA (1, 2). Since the first discovery of RNA modification as a gene expression control mechanism beyond DNA sequence in the 1950s (3), it has become a prominent focus in life science. Researchers have gradually elucidated its regulatory mechanisms and its crucial role in regulating gene expression, cellular differentiation, tissue development, and the onset and progression of diseases. Up to now, more than 170 chemical modifications of RNA have been identified (4).

Common RNA modifications encompass N6-methyladenosine (m6A), N5-methylcytosine (m5C), N1-methyladenosine (m1A), N7-methylguanine (m7G), N4-acetylcytosine (ac4C), pseudouridine (Ψ), uridylation, and adenosine-to-inosine editing (A-to-I), among which m6A is the cutting-edge research domain (5). These modifications are added to RNA by specific “writers” enzymes, removed by “erasers” enzymes, and can be recognized by “readers” proteins, thereby participating in diverse biological processes of RNA (6). In recent years, among the myriad of RNA modifications, the m1A modification has attracted increasing attention. It is a methylation

modification of the first nitrogen atom of adenosine. Apart from m6A methylation, m1A methylation is the most prevalent, abundant, and evolutionarily conserved internal post-transcriptional modifications in eukaryotic RNA (7). Furthermore, m1A and m6A have a close relationship—m1A can not only be converted into m6A under alkaline conditions through the Dimroth rearrangement, but also they share some common regulatory factors, like YTHDF1-3 and FTO (8).

First discovered in the 1960s (9), m1A modification has been the subject of research for over half a century (Figure 1). With the recent advancements in high-throughput sequencing technology, it has been revealed that m1A modification is ubiquitously present in various types of RNA, such as tRNA, rRNA, lncRNA, and mRNA (10). The detection technologies for m1A modification have been continuously evolving over time, providing critical insights into its biological functions in transcription and translation (11). Especially, single-base resolution detection methods, referring to technologies that precisely identify and quantify specific methylation modifications in RNA molecules at the base level, provides detailed information on gene expression regulation, epigenetics, and disease-related variations (12–14). The Yi research group, leveraging the mismatch caused by m1A during reverse transcription, has developed a high-resolution “m1A-MAP” single-base resolution technology. This technique first enriches

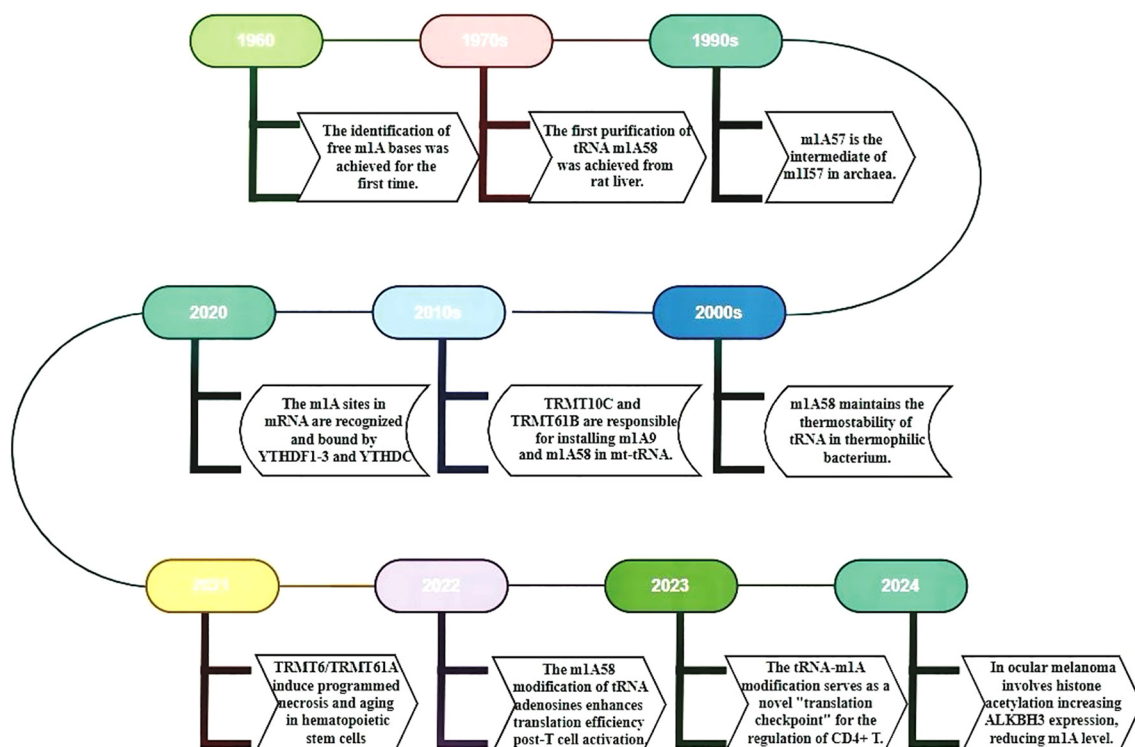


FIGURE 1

The timeline summarizes key m1A RNA research milestones from 1960 to 2024. It uses different colored boxes to represent key research milestones and discoveries. The timeline provides a detailed account of the evolution of m1A RNA from its initial discovery to a more profound comprehension of its functions. Additionally, it illustrates recent research advancements concerning the functions, regulatory mechanisms, and roles of m1A in diseases.

RNA containing m1A modifications using m1A antibodies, then employs reverse transcriptase to generate an A-C mismatch when encountering m1A, resulting in a G-A to A-C transition in cDNA. By comparing the mismatch rates of demethylated and untreated samples, m1A modification sites can be precisely located, thus revealing the distribution and abundance of m1A in the transcriptome (15). The Yi group has also developed “m1A-ID-seq,” a novel m1A RNA methylation sequencing technology that combines antibody enrichment with specific enzymatic reactions (7). Both technologies hold significant positions in the detection of m1A modifications. For example, the most commonly used technique is MeRIP-seq/m1A-seq, a methylated RNA immunoprecipitation sequencing method based on antibody enrichment (16). It employs m1A-specific antibodies to enrich RNA fragments with m1A modifications, followed by a high-throughput sequencing to map the precise location and quantify the abundance information of m1A modifications on RNA (17). Recently, Xie et al. has developed m¹A demethylation editing tool (termed AI-dm¹A) as well as an m¹A methylation editing tool (termed AI-m¹A) by combining the CRISPR/dCas13b system with Chemically Induced Proximity (CIP) technology, enabling the precise and reversible regulation of m1A modification. This tool offers a real-time controllable and reversible means to study m1A dynamics, offering invaluable insights into m1A's biological functions (18).

Previous reviews on m1A modification have mainly focused on the role of m1A modification in cancer (19), understanding the function of m1A modification in different RNAs and its role in diverse spectrum of malignancies (20). Researches focusing on m1A in the field of cancer immunotherapy are relatively scarce. Cancer immunotherapy has been a significant breakthrough in the context of cancer treatment. It works by activating or enhancing the patient's own immune system to attack cancer cells and has achieved certain clinical results. However, due to differences in the immune systems and tumor characteristics of different patients, Some patients may not respond or develop tolerance (21). Moreover, the current clinical research evaluation system lacks corresponding methods to assess the durability and special clinical course of immunotherapy (22). Therefore, it is necessary to searching for new targets for cancer immunotherapy continuously (23). The modification of RNA has emerged as a promising direction due to its significant influence on multiple facets of immunotherapy.

This review delves into the significant role and potential of m1A modification in cancer immunotherapy. By revealing how m1A modification affects immune cell function, the tumor microenvironment (TME), and responses to immune checkpoint inhibitor therapy, we provide a scientific basis for developing novel cancer treatment strategies. Furthermore, the concept of m1AScore elaborated in this review may help predict the prognosis of tumor patients and guide clinical treatment decisions, auspiciously improving patients' treatment outcomes and quality of life. Overall, we emphasize m1A modification as a cutting-edge frontier in the field of cancer biology and immuno-oncology, with the potential to improve approaches to cancer treatment.

2 m1a regulators and their biological roles

The modification of m1A is typically enriched in the 5' UTR region of mRNAs (7), particularly at the first and second positions of the transcripts, as well as near the translation initiation site (17). Additionally, m1A modification is present within the coding sequences, where it is positively correlated with protein synthesis. In some organisms, such as dinoflagellates, m1A modification is predominantly enriched in the 3' UTR region, where it is negatively correlated with translation efficiency (24). Besides, m1A is also commonly found at conserved sites in tRNA (especially at positions 9, 14, 16, 22, 57, and 58 of tRNA), rRNA and lncRNA (20). Thereby, m1A modification plays an important role in maintaining RNA stability, promoting protein synthesis, and regulating gene expression (1, 2, 25). m1A carries a positive charge under physiological conditions, which may alter the charge distribution of the RNA molecule, thereby affecting its interactions with proteins (10). Additionally, m1A modification disrupts the normal Watson-Crick base pairing, leading to unstable mismatches with other nucleotides. These alterations could potentially impact the secondary structure of RNA and RNA-protein interactions, thereby affecting RNA metabolism processes, including splicing, transport, degradation, and translation (26, 27). The process of m1A methylation involves three types of molecules: “writer”, “eraser” and “reader”, collectively referred to as RNA modification proteins (Figure 2). “Writers” are responsible for the methylation of RNA, “erasers” play a role in removing the m1A from RNA, and “readers” can recognize and bind to the m1A-modified transcript and participate in the regulation of downstream biological processes (15, 28).

2.1 Writers

Thus far, human cells have been identified six m1A methyltransferases: TRMT6/TRMT61A, TRMT61B, TRMT10C, NML (including RRP8 and RRAM-1 homologues), BMT2, and MTR1 (27, 29). Both TRMT61B and TRMT10C function within the mitochondria (30). TRMT61B is essential for sustaining mitochondrial function and cellular responses to stress, by regulating the methylation of mitochondrial tRNA, thus influencing mitochondrial protein synthesis and overall mitochondrial activity. A reduction in TRMT61B levels can diminish expression of various mitochondrial-encoded proteins, thereby constraining mitochondrial capability, leading to decrease in ATP production, and disruption in oxidative phosphorylation and energy metabolism (31). Additionally, the absence of TRMT61B can lead to senescence in melanoma cell with low levels of aneuploidy, while in melanoma cell with high levels of aneuploidy, it can lead to apoptosis. This may serve as a potential biomarker and therapeutic target for highly aneuploid tumors (32). TRMT10C primarily functions in the methylation of adenosine and guanosine nucleotides at the 9th position of tRNA. Due to the lower GC content of mitochondrial tRNA compared to cytoplasmic tRNA, and the fact that their D-, T-, and variable loops are either absent or of different lengths in supporting the folding of cytoplasmic tRNA,

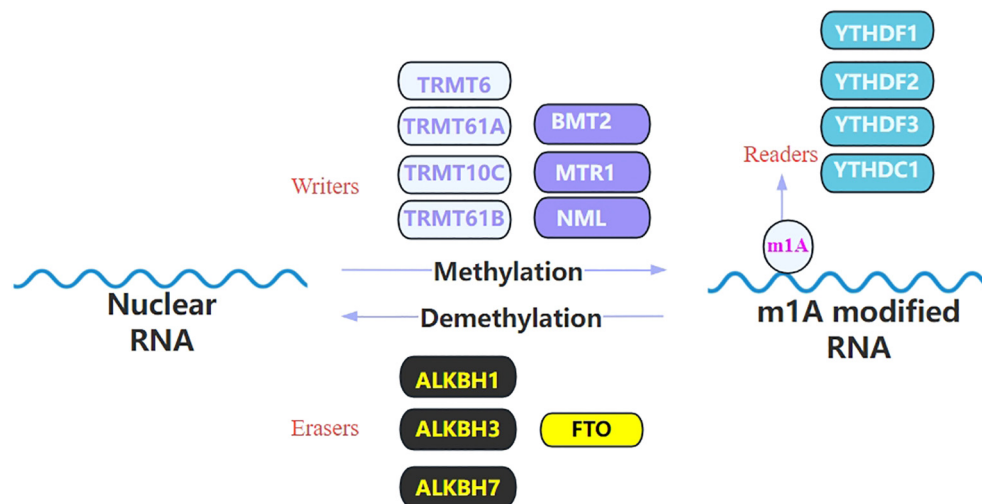


FIGURE 2

List the identified regulators of m1A modification. m1A RNA modification is catalyzed by the writer and removed by the eraser and it can be recognized by its reader proteins. This image illustrates the m1A modification process of RNA, which is a dynamic regulatory mechanism involving methylation and demethylation. During the methylation process, enzymes known as “writers,” including TRMT6, TRMT61A, TRMT10C, TRMT61B, BMT2, MTR1, and NML, aim to add the m6A modification to RNA molecules. In contrast, “eraser” enzymes such as ALKBH1, ALKBH3, ALKBH7, and FTO are responsible for removing the m1A modification from RNA, thereby achieving demethylation. The modified RNA can be recognized by “reader” proteins, which include YTHDF1, YTHDF2, YTHDF3, and YTHDC1. These proteins participate in the regulation of RNA stability, translation efficiency, and degradation by recognizing the m1A modification.

TRMT61C is crucial for ensuring the functional folding and stability of these structurally distinct tRNAs (33).

TRMT61A works together with TRMT6 to form a complex responsible for the m1A modification of mRNA and mitochondrial tRNA, thereby regulating multiple biological processes. Research by He HQ et al. has shown that overexpression of the TRMT6-TRMT61A complex promotes astrocyte senescence through tRNA-m1A58 modification. This modification also induces necroptosis in hematopoietic stem cells (HSCs) by generating 3'-tiRNA-Leu-CAG and activating the RIPK1-RIPK3-MLKL cascade (34), a programmed cell death process mediated by TNF-stimulated signaling (35). Tumor cell-induced necroptosis in endothelial cells facilitates tumor cell extravasation and metastasis (36). Moreover, the specific deletion of TRMT6 in HSCs leads to abnormal expansion and significantly reduced self-renewal capacity in the short term. The tRNA-m1A58 modification also regulates mTORC1 signaling in HSCs to meet their rapid translational demands (37). The overactivation of the mTORC1 pathway in various cancers is widely recognized and is closely associated with cancer cell proliferation, survival, and metabolism (38, 39). Given the critical roles of TRMT6 and tRNA-m1A modification in HSC function, they may serve as potential therapeutic targets for certain hematological malignancies, especially those related to abnormal HSC functions, such as leukemia (40).

2.2 Erasers

The erasers of m1A include ALKBH1, ALKBH3, ALKBH7 from the AlkB family, as well as FTO. Among these, ALKBH3 and FTO are the most prominent m1A erasers, making this modification

reversible (41). ALKBH3 removes methyl groups from m1A and other alkylated bases (42, 43), modulating key cellular processes like cell cycle regulation and key factors (vascular endothelial growth factor (VEGF), tRNA-derived small RNAs (tDRs) etc.) in the TME. By knocking down ALKBH3, the expression of p21WAF1/Cip1 and p27Kip1, leading to cellcycle arrest at the G1 phase, cellular senescence, and a robust inhibition of cell growth *in vitro* (44). Furthermore, in human urothelial carcinoma cells, ALKBH3 enhances tumor survival, invasiveness, and angiogenesis by modulating the production of reactive oxygen species and the expression of several critical factors like VEGF (45). Additionally, ALKBH3 elevates the sensitivity of tRNA to angiogenin-mediated cleavage, leading to the formation of tDRs. This triggers ribosome assembly and interacts with cytochrome c to prevent apoptosis, thereby promoting cancer progression (46). As for FTO, it can directly inhibit translation by catalyzing m1A tRNA demethylation in both the nucleus and the cytoplasm, thereby suppressing the survival and proliferation of tumor cells (47). This will be further discussed in the following text.

2.3 Readers

m1A readers include YTHDF1, YTHDF2, YTHDF3, YTHDC1, all of which belong to YTH family. These proteins can directly interact with m1A-modified RNA molecules through their characteristic YTH domains (48). Compared to the researches on m1A's “writers” and “erasers”, the study of “readers” has been relatively scarce. Currently, YTHDF3 has been recognized as being able to negatively regulate the invasion and migration of cells. By binding to IGF1R mRNA with m1A modification, YTHDF3

enhances the degradation of IGF1R mRNA, and subsequently reducing the expression of matrix metalloproteinase 9, an enzyme involved in extracellular matrix remodeling and tumor cell invasion (49). With respect to YTHDC1, in addition to its known binding to m6A-RNA, it also binds to m1A-containing RNA after alkylation. YTHDC1, together with the THO complex, prevents DNA breaks induced by nuclear RNA m1A methyltransferases (43). YTHDF2 facilitates the transport of the modified RNA to the P-body via its N-terminal domain, thereby hastening the degradation of the m1A-modified RNA (50). YTHDF1 primarily participates in the metabolism of ATP5D to regulate glycolysis (51).

3 Application of m1A RNA modification in tumor immunity

Over the past decade or so, cancer treatment has undergone revolutionary changes. It is no longer limited to traditional therapies that target tumors, such as chemotherapy and radiotherapy (52). With the rapid development and continuous innovation of cancer immunotherapy, more precise and personalized treatments have provided patients with novel therapeutic options and better survival prognoses (53). The main driving force behind this shift is a deeper understanding of the TME. The TME is a complex ecosystem composed of cancer cells, non-cancer cells (including fibroblasts, immune cells, endothelial cells, and vascular cells), as well as extracellular matrix, blood vessels, and nerve fibers, among other non-cellular components (54, 55). The TME not only provides physical support and nutrients for tumor cells but also participates in regulating tumor growth, invasion, metastasis, and response to treatment (56). Additionally, the development of new immunotherapeutic drugs has made a significant contribution to cancer treatment. In particular, the first generation of immune checkpoint inhibitors (ICIs), such as anti-programmed death-1 (PD-1)/programmed cell death 1 ligand 1 (PD-L1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies, can restore the antitumor activity of T cells by blocking immune-inhibitory signaling pathways (57, 58).

As cancer immunotherapy has become a frontier in oncology, understanding how m1A contributes to immune modulation offers new possibilities for treatment strategies. Currently, m1A modification has been recognized as a crucial player in directly affecting the behavior of immune cells, and indirectly regulating TME. Additionally, evaluating the expression patterns of multiple m1A regulators in tumor samples can predict tumor prognosis and the state of the TME.

3.1 m1A modification and immune cells

3.1.1 m1A modification and T cell

T lymphocytes are the primary effector cells in cellular immunity, producing cytokines to mediate inflammation and regulate other types of immune cells in immune responses (59).

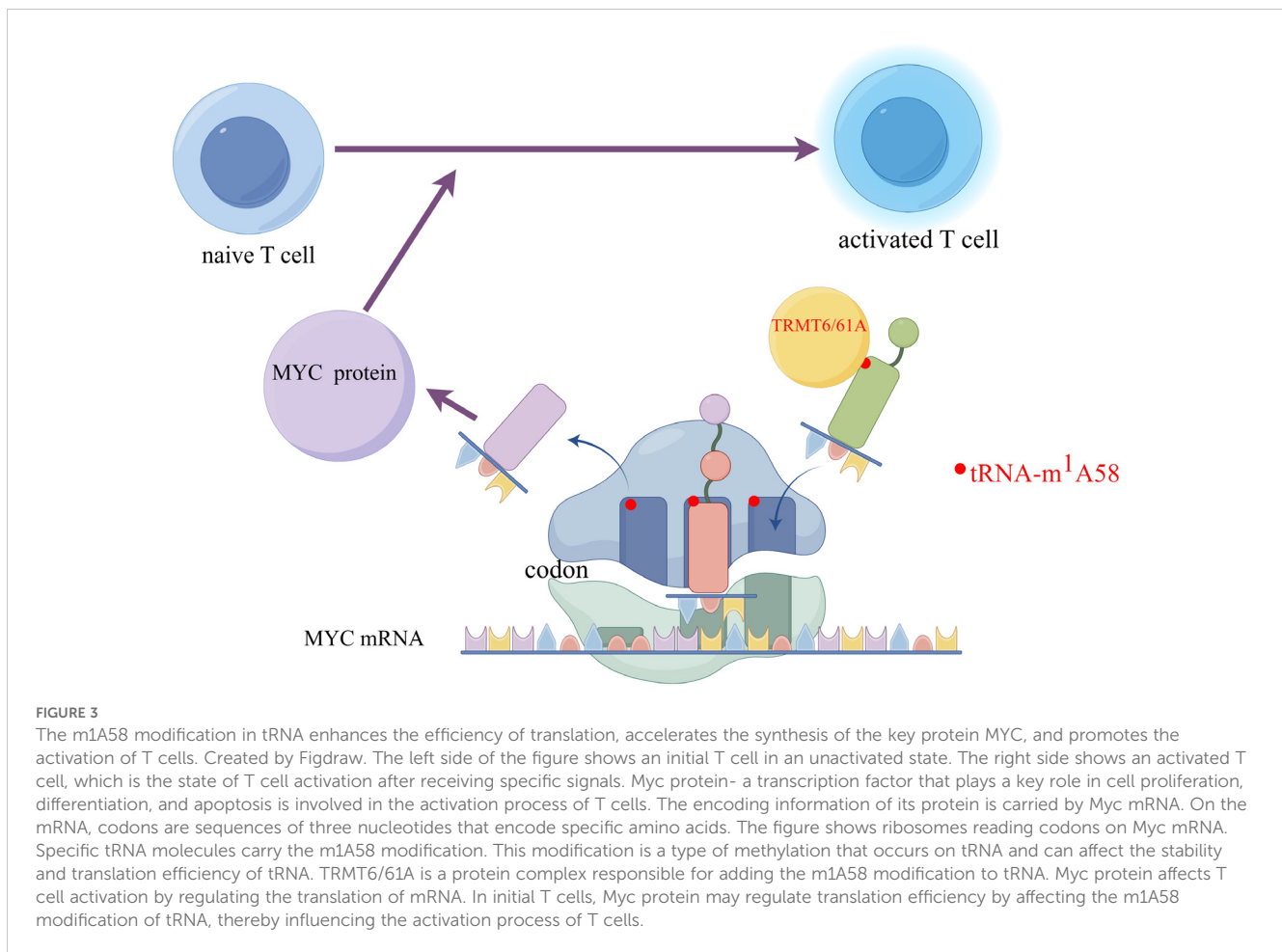
Among them, CD4⁺T cells primarily recognize foreign antigens presented by antigen-presenting cells and mount a response. This response can modulate the activity of other immune cells, such as B cells or CD8⁺ T cells, and can also initiate new immune responses (60). Upon encountering specific antigens, CD4⁺T cells rapidly transition from a resting state to an active state, and begin to proliferate and differentiate rapidly (61). This process requires the prompt synthesis of a large amount of functional proteins to meet the demands of bioenergetics and biosynthesis (62, 63). Furthermore, Liu et al. have discovered that the catalytic action of the TRMT6/61A complex at the 58th site of cytoplasmic tRNA can enhance translation initiation and elongation (64).

On this established foundation, Li Huabing's team has uncovered that the m1A modification on tRNA increases translation efficiency, leading to rapid synthesis of key functional proteins such as MYC (65). MYC can regulate the clonal expansion of CD4⁺T cells by affecting metabolic reprogramming and cell cycle control (66). Consequently, the MYC protein directs naive T cells to transition from a quiescent state to a proliferative one and promotes the swift expansion of activated T cells. Li et al. first observed that during T cell activation, protein translation-related pathways are upregulated, and various tRNAs also exhibit dynamic expression patterns that are upregulated. The tRNA-m1A58 modification enzymes TRMT6 and TRMT61A are also upregulated during the activation process (65). Then they used TRMT6A conditionally knockout mice and found in both *in vivo* and *in vitro* experiments that T cell activation and immune function were impaired, and their proliferative capacity was reduced. It was also discovered that after T cell activation, the translation of various key proteins was hindered, particularly the transcription factor MYC (67). This study suggests that TRMT61A-mediated tRNA-m1A58 modification could serve as a novel "translational checkpoint" for the regulation of CD4⁺T cell proliferation (Figure 3), offering a new RNA epigenetic regulatory strategy for the clinical modification of CD4⁺T cell functions to treat cancer (67).

3.1.2 m1A modification and macrophages

The impact of m1A modification on immune cells is primarily focused on T cells, with relatively fewer studies on other immune cells. While, still some progress has been made in macrophages. Macrophages can produce a range of cytokines that are crucial for modulating immune reactions, both promoting inflammatory responses and maintaining anti-inflammatory balance. They can also polarize into different phenotypes based on the changing signals from the surrounding microenvironment, adapting to diverse immune demands (68). Besides, macrophages recognize specific molecular patterns of pathogens through their pattern recognition receptors, thereby activating immune responses (69). The following discusses the association between m1A modification and macrophages from two perspectives: cytokines and macrophage polarization.

Research by Woo & Chambers has found that ALKBH3 can enhance the stability of Colony-Stimulating Factor 1 (CSF-1) mRNA. CSF-1 is a cytokine mainly responsible for regulating the



generation, survival, differentiation, and function of macrophages (70, 71). Then, CSF-1 activates its receptor CSF-1R to affect the survival, proliferation, migration and invasiveness of cancer cells like breast and ovarian cancer cells. Moreover, increased expression of CSF-1 in breast and ovarian cancer cells has been associated with poor prognosis (72). Therefore, it is possible to explore inhibitors targeting ALKBH3, block the CSF-1/CSF-1R signaling pathway, and develop epigenetic therapies targeting m1A modification to control tumor progression (70). However, further research and clinical trials are needed to translate these findings into clinical applications.

The study of m1A involved in macrophage has also been applied in abdominal aortic aneurysms (AAA). AAA is characterized by the pathological dilation of the abdominal aorta and the continuous weakening of the aortic wall (73). Currently, effective drug treatments are scarce, and surgical repairs pose risks and limitations (74). Infiltration of inflammatory immune cells in the adventitia of the artery is a key characteristic of AAA (75). Strikingly, the transformation of M0 macrophages into pro-inflammatory M1 type or anti-inflammatory M2 type macrophages has a regulatory effect on the vascular inflammation process in AAA (76–78). Moreover, various epigenetic mechanisms are associated with macrophage polarization inspires the exploration and utilization of

m1A to modulate macrophage polarization in AAA (79, 80). Research by Wu et al. has provided new insights into the pathogenesis of AAA from the perspective of m1A epigenetic regulation and macrophage polarization (74). The varying expression levels of YTHDF3 acting as “readers” are associated with the infiltration of different immune cells in AAA (80). Using IF double staining analysis, co-expression of YTHDF3 and the macrophage surface marker CD68 was observed in a cell from the adventitia of AAA. Further experiments showed that knockdown of YTHDF3 in M0 macrophages inhibits macrophage M1 polarization but promotes macrophage M2 polarization. Specifically, knockdown of YTHDF3 significantly impaired LPS/IFN- γ -induced macrophage M1 polarization and attenuated the secretion of the inflammatory cytokine IL12, significantly reversing the M0 to M1 polarization of macrophages. Besides, the specific inhibitor of YTHDF3 expression may act as a modulator of macrophage M2 polarization adaptation, which would reduce the secretion of matrix metalloproteinases, promote the repair process of the aortic wall, and alleviate vascular inflammation by downregulating the expression levels of pro-inflammatory cytokines such as IL1 β and TNF, and upregulating the secretion of anti-inflammatory cytokines and chemokines such as IL10 and TGF β , suggesting that YTHDF3 is a potential therapeutic target for AAA (74, 81).

3.2 m1AScore in tumor prognosis and immunotherapy

More application of m1A modification in cancer research focuses on analyzing the expression patterns of m1A-related genes to establish m1AScore, which is used to assess prognosis and risk, and guide personalized treatment. Specifically, high-throughput sequencing technologies, such as RNA-seq, are typically employed to collect gene expression data from tumor samples. Genes associated with m1A, including “writers”, “erasers”, and “readers”, are then identified from this data. Subsequent analysis focuses on the expression patterns of these related genes, examining their levels of expression and variations. Statistical methods, such as linear and logistic regression, are utilized to construct a scoring model that predicts the prognosis of cancer patients based on the expression patterns of m1A-related genes. This scoring model is then validated and optimized using independent datasets. Next, by inputting a patient’s gene expression data into the scoring model, an m1AScore is calculated for each individual. Notably, the specific calculation method for the m1AScore may vary across studies, with different research potentially employing distinct sets of genes, statistical approaches, and model-building strategies (82–86). Different scoring systems are employed in various tumor models, which are often also related to immunity, such as the function of immune cells, the response to immunotherapy, and the characteristics of immune cell infiltration in the TME. Therefore, m1A is an indicative biomarker to predict the effectiveness of immunotherapy.

3.2.1 Ovarian cancer (OC)

In the study of ovarian cancer, by comprehensively assessing the m1A modification patterns in 474 OC patients based on 10 m1A regulators and linked them to the immune infiltration characteristics of the TME, Liu et al. found a high m1A score is usually associated with better survival benefits and a lower mutational burden. Moreover, m1A modification affects the TME of ovarian cancer, including the infiltration and composition of immune cells. Researchers identify three distinct m1A modification patterns corresponding to three tumor immune phenotypes: immune desert, immune-inflammatory, and immune-exclusion phenotypes. Tumor patients with an immune-inflammatory phenotype may have a good response to ICIs, while those with an immune-desert phenotype may require other treatment methods to enhance their sensitivity to immunotherapy (85, 87).

3.2.2 Colon cancer

Gao et al. employed m1AScore, which is generated by using profile of expression of the 71 m¹A-related genes to further demonstrate the m1A patterns in colon cancer. They found a low m1AScore is accompanied by enhanced proliferative capacity of CD8⁺ T cells, increasing the tumor-killing ability of immune cells. Additionally, a low m1AScore is correlated with high microsatellite instability (86), rendering patients have a better response to immune checkpoint inhibitor therapy (88). Moreover, it is also associated with a higher tumor neoantigen burden, which can be

recognized by the immune system and elicit an immune response (89). Furthermore, it is related to the expression levels of PD-L1. Therefore, it can be predicted that patients with a low m1AScore will exhibit longer survival times and better treatment responses when undergoing antitumor immunotherapy (86).

3.2.3 Head and neck squamous cell carcinoma (HNSCC)

Wang et al. shed light on the correlation between lncRNAs that harbor modifications of m6A, m5C, and m1A with the survival outcomes, immune contexture, and tumor mutational burden in patients with HNSCC (90). They found m1A modification may affect the stability and function of lncRNAs, which may be involved in the regulation of immune-related gene expression, such as immune checkpoint molecules (91). Moreover, modified RNA influences the composition of immune cells in the TME. The high-risk subgroup may contain a higher number of immunosuppressive cells, such as Regulatory T cells (Tregs) and M2 macrophages, while the low-risk subgroup may have a higher number of immunoactivating cells, such as NK cells and Th1 cells. Thus, by modulating the expression or function of these lncRNAs, it might be possible to enhance the antitumor immune response, thereby improving therapeutic outcomes (90).

3.2.4 Lung cancer

Zhou et al. established a Writer-Score system based on the expression levels of RNA modification writers, such as enzymes related to m1A, m6A, A-to-I, and APA modifications to quantify RNA modification patterns and predict the clinical outcomes of patients with non-small cell lung cancer (NSCLC). These groups of RNA modification patterns show a strong association with various TME characteristics and biological processes. The Writer-Score is also used to predict the prognosis of NSCLC patients receiving neoadjuvant immunotherapy. The study found that patients with a low Writer-Score had a better disease-free survival ($p=0.021$) and were associated with a better pathological response. Different RNA modification patterns are related to different levels of immune cell infiltration. For example, certain RNA modification patterns are associated with a high level of T helper cells, Tregs, or other immune cells, and the presence of these cells may affect the effectiveness of immunotherapy (92).

3.2.5 Oral squamous cell carcinoma (OSCC)

Three distinct m1A modification patterns were identified in OSCC based on the expression levels of 10 m1A regulators from 502 patients’ samples. These patterns were found to be significantly associated with patient prognosis and the TME characteristics. The cluster with high expression of m1A regulators correlated with lower immune cell infiltration, lower single-sample gene set enrichment analysis (ssGSEA) scores, and higher tumor purity, indicating that m1A modification may influence the formation of TME in OSCC. The expression levels of immune checkpoint molecules such as CTLA-4, PD-1, T cell immunoglobulin and so on, were positively associated with the expression of m1A regulators, immune cell infiltration, and ssGSEA scores (93).

m1Ascore also contributes substantially to pancreatic cancer (94), hepatocellular carcinoma (83), low-grade glioma (84) and other types of cancers. In summary, it shows potential in prognostic research across different cancers and has a certain correlation with immune responses. By combining other clinical parameters, such as tumor mutational burden, m1Ascore can provide more accurate information for personalized treatment and prognostic assessment of cancer patients.

3.3 m1A and metabolism regulation

Emerging researches highlight the role of metabolite regulation in enhancing tumor immunotherapy, particularly through modifications like m6A. For example, inhibiting RNA demethylase ALKBH5, has been shown to boost tumor sensitivity to immunotherapy, by downregulating the expression of MCT4/SLC16A3, a lactate transporter, thereby reducing lactate levels in the TME. This metabolic change reduces the presence of immunosuppressive cells like myeloid-derived suppressor cells (MDSCs) and Tregs, ultimately enhancing the tumor's response to immunotherapy. This discovery highlights ALKBH5 as a potential target for new immunotherapies (95). Although research on the impact of m1A modification on the TME and immune responses is still limited, the findings regarding m6A may provide insights into the effects of m1A modification on metabolic reprogramming and its influence on immune responses (8).

Wu et al. found that the m1A demethylase ALKBH3 can regulate cancer cell glycolysis through modulating ATP5D, a key subunit of adenosine 5'-triphosphate synthase in two manners (51).

On the one hand, the m1A modification at A71 in exon 1 of ATP5D negatively regulates its translation elongation by increasing its binding to the YTHDF1/eRF1 complex, thereby promoting the release of mRNA from the ribosome complex. On the other hand, m1A also regulates the mRNA stability of E2F1, which directly binds to the ATP5D promoter to initiate transcription (96). Overall, ALKBH3 enhances transcriptional and translational efficiency of ATP5D. Targeted demethylation of ATP5D m1A via the dm1ACRISPR system has been shown significantly increase the expression of ATP5D and the glycolysis of cancer cells (Figure 4). Other regulatory factors of RNA modification, such as ALKBH5, YTHDF2, and FTO, are also involved in the regulation of glucose metabolism (97).

Wang et al. found that m1A modification mediated by the TRMT6/TRMT61A complex enhances the translation of peroxisome proliferator-activated receptor delta (PPAR δ) protein. The activation of PPAR δ can promote the expression of genes related to fatty acid oxidation, such as ATP citrate lyase (ACLY) and stearoyl-CoA desaturase 1 (SCD1). It also activates the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) in the cholesterol synthesis pathway to increase cholesterol production. Additionally, PPAR δ can affect the uptake and excretion of cholesterol, thereby regulating the levels of cholesterol within the cell (98).

Key enzymes in glycolysis and fatty acid synthesis, such as hexokinase and enolase, as well as fatty acid synthase and acetyl-CoA carboxylase, are targets of m1A modification (97). Tumor cells, by enhancing glycolysis and cholesterol synthesis, may deprive immune cells of the metabolic materials they need, thereby suppressing the function of immune cells. It also alters the

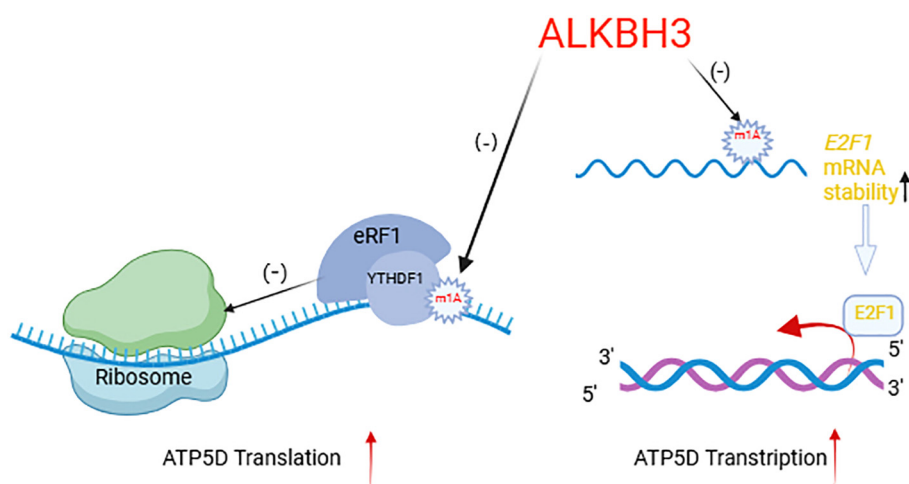


FIGURE 4

ALKBH3 regulates ATP5D transcription and translation mechanism. Created with BioRender.com. ALKBH3, as a demethylase, specifically targets the m1A modification on mRNA. The arrow pointing to the m1A mark on the mRNA indicates the demethylation process. By removing the m1A, ALKBH3 can affect the stability and translation of mRNA. When the m1A mark is removed, the stability of mRNA increases, as shown by the upward arrow next to "E2F1 mRNA stability," which may consequently increase the levels of the corresponding protein. The diagram also illustrates the impact of ALKBH3 on translation efficiency. When the m1A mark is bound by the writer YTHDF1 and eRF1 complex, it inhibits translation, as indicated by the negative sign (-). In contrast, after ALKBH3 removes the m1A mark, it allows for more efficient translation, as shown by the positive sign (+) and the upward arrow next to "ATP5D Translation." In summary, this diagram provides a visual representation of how ALKBH3, through its demethylation activity, can regulate mRNA stability and translation, ultimately influencing protein synthesis.

metabolic state of the TME, leading to the accumulation of immune-suppressive cells, thus promoting tumor immune evasion. In addition, changes in cholesterol levels affect the expression of immune checkpoint molecules (such as PD-L1) (16, 99, 100).

4 m1A modification and ICI treatments

ICIs are a class of cancer immunotherapies that enhance anti-tumor immune responses by targeting immune checkpoint molecules on the surface of T cells. By blocking the PD-1/PD-L1 and CTLA-4/CD80/86 signaling pathways, they enhance effective immune responses against cancer cells, restore tumor antigen recognition, and ultimately lead to the death of cancer cells (101–103). Although ICIs have achieved significant therapeutic effects in some patients, most patients still experience disease progression after initial treatment. To improve the effectiveness of ICIs, it is crucial to search for new, effective targets and to address issues of resistance (104). A growing number of research highlights the connection between m1A modification and the efficacy of ICIs, such as anti-PD-1 and anti-CTLA-4 therapies.

4.1 m1A and PD-L1

Overexpression of MYC protein is closely associated with the occurrence and development of various tumors. However, due to the lack of an enzyme active site pocket suitable for direct action by small molecule drugs, MYC protein is considered an “undruggable” target (105). Recently, Wang et al. reported TRMT61A-mediated tRNA-m1A modification provides a new mechanism and potential therapeutic strategy for the regulation of MYC protein in two ways. First, inhibition of TRMT61A can directly inhibit the proliferation of tumor cells by reducing the synthesis of MYC protein. Furthermore, in tumors treated with oncolytic herpes simplex virus (oHSV), the level of m1A modification increases, leading to reactive upregulation of PD-L1 (106, 107). Therapeutic TRMT61A inhibitors reduce m1A modification, thereby decreasing the *de novo* synthesis of PD-L1, which weakens the immune escape ability of tumor cells and makes them more susceptible to immune system attacks (107). In summary, inhibition of TRMT61A, as a new therapeutic strategy, may improve the sensitivity of tumors to immunotherapy and OV therapy by simultaneously affecting MYC and PD-L1, making it a promising therapeutic target. Future research needs to evaluate the mechanism, efficacy, and safety of TRMT61A inhibitors, in order to provide more effective treatment options for cancer patients.

Moreover, it has been discovered that METTL3, a dual regulator of m1A and m6A, has a close relationship with PD-L1. Ai et al. found that METTL3 can regulate the m6A modification level of PD-L1 in the model of OSCC (108). METTL3 may regulate the transcription or mRNA stability of PD-L1 through m6A modification, thereby affecting the protein level of PD-L1.

Knocking down METTL3 reduces the invasion, migration, and proliferation abilities of OSCC cells, and weakens the activation of CD8+ T cells. METTL3 intensifies the metastasis and proliferation of OSCC by regulating the m6A amount of PD-L1, indicating that METTL3 may be a therapeutic target for OSCC patients.

4.2 m1A and PD-1

Bao et al. reported that targeting m6A reader YTHDF1 promotes the translation of p65 to upregulate CXCL1, thereby facilitating the migration of MDSCs through the CXCL1-CXCR2 axis (109). The increased MDSCs, in turn, antagonize functional CD8+ T cells in the tumor TME (110). Additionally, depletion of YTHDF1 can reduce tumor growth and enhance anti-colorectal cancer immunity by restoring the infiltration of CD8+ T cells and synergizes with PD-1 blockade to better control tumors (109). Since research has indicated that proteins within the YTH domain family could interact with RNAs that have m1A modifications, possibly serving the role of an m1A reader (48). This opens up research directions for understanding the relationship between m1A modification and the binding of PD-1.

FTO, another regulatory factor shared between m1A and m6A, has been also shown to have a close relationship with PD-1. Yang et al. (111) found that FTO gene expression is upregulated in response to metabolic stress, particularly through the activation of autophagy and the NF- κ B signaling pathway. When FTO is knocked down, the methylation level of m6A in key genes that promotes melanoma development, such as PD-1, is increased. This elevated m6A methylation enhances RNA degradation through the action of the m6A reader protein YTHDF2. The reduction of FTO also makes melanoma cells more responsive to interferon gamma (IFN γ) and improves the effectiveness of anti-PD-1 therapy in mice. These findings highlighted the significant role of FTO as an m6A demethylase in the development of melanoma and its resistance to anti-PD-1 treatment. They also suggest that combining FTO inhibitors with anti-PD-1 therapy could potentially overcome resistance to immunotherapy in melanoma patients. Although there is no clear literature showing a connection between m1A modification and PD-1, there are studies have shown that FTO can directly inhibit translation by catalyzing the demethylation of m1A in tRNA (47), therefore, providing a direction for future research.

ICIs therapy has achieved certain successes in cancer treatment. However, primary and acquired resistance limit its clinical application, making it particularly important to explore new treatment strategies to enhance the antitumor effects of immunotherapy (112–114). m1A modification, as a potential mechanism for regulating the expression of immune checkpoints, may become a new target to improve the efficacy of ICI therapy. Currently, the combined application of m1A modification and ICI therapy is still in the research phase. Future research needs to further explore the specific mechanisms of RNA methylation in tumor immunity and develop more RNA methylation regulators, with the hope of achieving breakthroughs in clinical applications.

5 Conclusions and prospects

This review initially elucidates the regulatory mechanisms of m1A modification, involving three categories of key enzymes: methyltransferases (writers), demethylases (erasers), and recognition proteins (readers) (15, 28). These enzymes add, remove, or recognize m1A modifications on RNA molecules, participating in the regulation of RNA metabolism and translation processes (24, 25). m1A modification is closely related to the occurrence and development of tumors (Figure 5). m1A regulates specific molecules and signaling pathways in various types of cancer, affecting cellular behaviors such as proliferation, migration, invasion, apoptosis, and senescence. Among them, ALKBH3 primarily influences various signaling pathways to regulate the cell cycle and invasiveness of tumor cells (42, 44–46, 70). METTL3 mainly affects RNA stability and regulates the transcription process (108, 115, 116). TRMT6/TRMT61A affects all RNAs, influencing the proliferation and apoptosis processes in tumor cells (31, 32, 34). In the context of tumor immunotherapy, the article emphasizes m1A modification can directly impact immune cell functions (65), such as the proliferation of T cells (60, 65, 67) and the maturation of macrophages (68, 76, 117, 118), and can also indirectly affect immune responses by altering the TME. Furthermore, m1A modification is associated with the responsiveness of tumor cells to immune checkpoint inhibitors (ICIs) (65, 95, 104, 111), such as regulating PD-L1 expression to influence tumor cell immune evasion. This review further introduces the concept of m1AScore, a scoring system based on the expression patterns of m1A modification regulators, used to predict tumor patient prognosis and guide personalized therapy.

The m1AScore reflects the overall level of m1A modification in tumor tissues and is closely related to the TME, immune cell infiltration, and patient responsiveness to immunotherapy (83, 85, 86, 94). Additionally, we conclude the role of m1A modification in tumor metabolic reprogramming, indicating that m1A modification may affect immune cell function and tumor microenvironmental metabolic competition by influencing metabolic pathways in tumor cells, such as glucose metabolism and lipid metabolism (51, 98).

Compared with m6A modification, m1A modification still has many areas that have not been fully explored. First, this review has briefly summarized the effects on T cells and macrophages, but there are currently no research results on the role of m1A modification in other immune cells. There is already clear literature explaining the role and mechanism of m6A in immune cells such as NK cells (116), dendritic cells (119), CD8+T cell (115). Therefore, the exploration of its application in immune cells has a certain level of feasibility. In addition, the role of m1A modification in tumor immune escape has not been as specifically reported in dedicated articles as m6A (120).

Although some roles of m1A modification in tumor immunotherapy have been revealed, there are still many potential research directions worth further exploration. With further research and based on the successful cases of m6A, m1A modification may provide new strategies and targets for tumor immunotherapy. Further research is needed to clarify the functions of regulatory factors m1A in gene and protein regulation, especially shared with m6A, and to confirm the clinical utility of m1A modification.

In the research on m6A, it has been reported that there are two main challenges: the scarcity of novel modifications and the promiscuous substrate specificity of many mRNA modifiers.

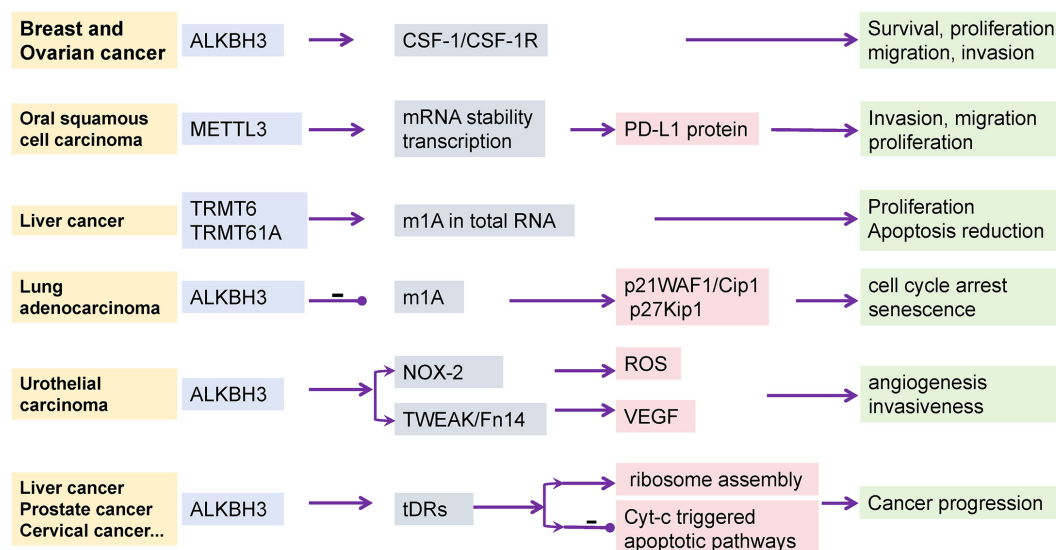


FIGURE 5

The effect of tumor occurrence and progression by m1A regulators. In different types of cancer, distinct m1A modifications regulate the behavior of tumor cells by affecting specific molecules and signaling pathways. For instance, in breast and ovarian cancers, m1A modifications exert their effects through the CSF-1 signaling pathway; whereas in oral squamous cell carcinoma, m1A modifications influence tumor immune evasion through the expression of PD-L1 protein. The role of ALKBH3 modifications in cancer progression involves multiple levels, including cell cycle regulation, oxidative stress response, and apoptotic pathways, demonstrating the complexity of cancer progression. This figure provides an overview of the role of m1A modifications in different types of cancer and emphasizes its diversity and complexity in tumor biology.

Research is hindered by high error rates, low specificity, and low reproducibility, leading to overestimation or underestimation of modification occurrence (121). There is currently no specific research on drug formulations for m1A modification, but it is likely to face similar challenges. These could all lead to off-target phenomena, such as modifications on tRNA becoming modifications on mRNA. Additionally, the specificity of m1A agents may face other challenges—the selectivity of modification enzymes, as well as subcellular localization. Off-target effects may also lead to some toxic side effects. For example, Zhang et al. explored m1A modifications in mRNA, lncRNA, and circRNA in normal and oxygen-glucose deprivation/reoxygenation-treated mouse neurons, and analyzed the impact of m1A on different RNAs. It was found that m1A may affect the regulatory mechanisms of non-coding RNAs, such as the interaction between lncRNA and RNA-binding proteins, and the translation of circRNA. m1A modification also mediates the competing endogenous RNA (ceRNA) mechanism of circRNA/lncRNA-miRNA-mRNA, and modification in the 3'UTR of mRNA can hinder the binding of miRNA to mRNA. As a result, m1A modification affects the formation and function of synapses, thereby affecting neural transmission and communication between neurons, and subsequently altering neuronal survival, apoptosis, and autophagy (122, 123). Fortunately, the application of computer-aided design and gene editing technologies may help improve this issue. For example, studies have shown that using genome editing technologies such as CRISPR/Cas9 (124) or CRISPR-Cas12a (125) can precisely knock out or knock in specific m1A modification sites to study their function and the specificity of drugs.

With a deeper understanding of the role of m1A modification in cancer immunotherapy, it is anticipated to become a new target for cancer treatment, providing a scientific basis for the development of new immunotherapeutic strategies. Future research will continue to explore the mechanisms and clinical applications of m1A modification, aiming to achieve more precise and effective cancer treatments.

Author contributions

LY: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. XW: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing –

review & editing. XM: Writing – original draft. SC: Visualization, Writing – original draft. MF: Data curation, Writing – original draft. CJ: Writing – original draft. YC: Writing – original draft. SW: Writing – original draft. ZW: Writing – original draft. FM: Conceptualization, Writing – original draft. CZ: Conceptualization, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Natural Science Foundation of China (No. 82273959), the Fundamental Research Funds for the Central Universities (No. 2632023TD10).

Acknowledgments

The authors regret that this paper could not cite all studies owing to space limitations.

Conflict of interest

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References

1. Roundtree IA, Evans ME, Pan T, He C. Dynamic RNA modifications in gene expression regulation. *Cell*. (2017) 169:1187–200. doi: 10.1016/j.cell.2017.05.045
2. Zhao LY, Song J, Liu Y, Song CX, Yi C. Mapping the epigenetic modifications of DNA and RNA. *Protein Cell*. (2020) 11:792–808. doi: 10.1007/s13238-020-00733-7
3. Davis FF, Allen FW. Ribonucleic acids from yeast which contain a fifth nucleotide. *J Biol Chem*. (1957) 227:907–15. doi: 10.1016/s0021-9258(18)70770-9
4. Boccaletto P, Stefaniak F, Ray A, Cappannini A, Mukherjee S, Purta E, et al. Modomics: A database of RNA modification pathways. 2021 update. *Nucleic Acids Res*. (2022) 50:D231–d5. doi: 10.1093/nar/gkab1083
5. Qiu L, Jing Q, Li Y, Han J. RNA modification: mechanisms and therapeutic targets. *Mol BioMed*. (2023) 4:25. doi: 10.1186/s43556-023-00139-x
6. Flamand MN, Tegowski M, Meyer KD. The proteins of mrna modification: writers, readers, and erasers. *Annu Rev Biochem*. (2023) 92:145–73. doi: 10.1146/annurev-biochem-052521-035330

7. Li X, Xiong X, Wang K, Wang L, Shu X, Ma S, et al. Transcriptome-wide mapping reveals reversible and dynamic N(1)-methyladenosine methylome. *Nat Chem Biol.* (2016) 12:311–6. doi: 10.1038/nchembio.2040
8. Liu H, Zeng T, He C, Rawal VH, Zhou H, Dickinson BC. Development of mild chemical catalysis conditions for M(1)a-to-M(6)a rearrangement on RNA. *ACS Chem Biol.* (2022) 17:1334–42. doi: 10.1021/acscchembio.2c00178
9. Dunn DB. The occurrence of 1-methyladenine in ribonucleic acid. *Biochim Biophys Acta.* (1961) 46:198–200. doi: 10.1016/0006-3002(61)90668-0
10. Jin H, Huo C, Zhou T, Xie S. M(1)a RNA modification in gene expression regulation. *Genes (Basel).* (2022) 13:910. doi: 10.3390/genes13050910
11. Li K, Peng J, Yi C. Sequencing methods and functional decoding of mrna modifications. *Fundam Res.* (2023) 3:738–48. doi: 10.1016/j.fmre.2023.05.010
12. Sun Q, Li H, Lin Z, Cao G, Yang D, Tang D, et al. Mass-spectrometry-based assay at single-base resolution for simultaneously detecting M(6)a and M(6)Am in RNA. *Anal Chem.* (2024) 96:11126–36. doi: 10.1021/acs.analchem.3c04003
13. Liu Y, Hu Z, Cheng J, Siejka-Zielińska P, Chen J, Inoue M, et al. Subtraction-free and bisulfite-free specific sequencing of 5-methylcytosine and its oxidized derivatives at base resolution. *Nat Commun.* (2021) 12:618. doi: 10.1038/s41467-021-20920-2
14. Safra M, Sas-Chen A, Nir R, Winkler R, Nachshon A, Bar-Yaacov D, et al. The M1a landscape on cytosolic and mitochondrial mrna at single-base resolution. *Nature.* (2017) 551:251–5. doi: 10.1038/nature24456
15. Li X, Xiong X, Zhang M, Wang K, Chen Y, Zhou J, et al. Base-resolution mapping reveals distinct M(1)a methylome in nuclear- and mitochondrial-encoded transcripts. *Mol Cell.* (2017) 68:993–1005.e9. doi: 10.1016/j.molcel.2017.10.019
16. Bauman DR, Bitmansour AD, McDonald JG, Thompson BM, Liang G, Russell DW. 25-hydroxycholesterol secreted by macrophages in response to toll-like receptor activation suppresses immunoglobulin a production. *Proc Natl Acad Sci United States America.* (2009) 106:16764–9. doi: 10.1073/pnas.0909142106
17. Dominissini D, Nachtergaele S, Moshitch-Moshkovitz S, Peer E, Kol N, Ben-Haim MS, et al. The dynamic N(1)-methyladenosine methylome in eukaryotic messenger RNA. *Nature.* (2016) 530:441–6. doi: 10.1038/nature16998
18. Xie G, Lu Y, He J, Yang X, Zhou J, Yi C, et al. Small molecule-inducible and photoactivatable cellular RNA N1-methyladenosine editing. *Angew Chem Int Ed Engl.* (2024) 63:e202320029. doi: 10.1002/anie.202320029
19. Liu Y, Zhang S, Gao X, Ru Y, Gu X, Hu X. Research progress of N1-methyladenosine RNA modification in cancer. *Cell Commun Signal.* (2024) 22:79. doi: 10.1186/s12964-023-01401-z
20. Smoczynski J, Yared MJ, Meynier V, Barraud P, Tisne C. Advances in the structural and functional understanding of M(1)a RNA modification. *Acc Chem Res.* (2024) 57:429–38. doi: 10.1021/acs.accounts.3c00568
21. Damo M, Hornick NI, Venkat A, William I, Clulo K, Venkatesan S, et al. Pd-1 maintains cd8 T cell tolerance towards cutaneous neoantigens. *Nature.* (2023) 619:151–9. doi: 10.1038/s41586-023-06217-y
22. Lang D, Wahl G, Poier N, Graf S, Kiesel D, Lamprecht B, et al. Impact of pet/ct for assessing response to immunotherapy—a clinical perspective. *J Clin Med.* (2020) 9:3483. doi: 10.3390/jcm9113483
23. Li H, Wei W, Xu H. Drug discovery is an eternal challenge for the biomedical sciences. *Acta Materia Med.* (2022) 1:1–3. doi: 10.15212/amm-2022-1001
24. Li C, Li Y, Guo J, Wang Y, Shi X, Zhang Y, et al. Abundant mrna M(1)a modification in dinoflagellates: A new layer of gene regulation. *EMBO Rep.* (2024) 25:4655–73. doi: 10.1038/s44319-024-00234-2
25. Shima H, Igarashi K. N1-methyladenosine (M1a) RNA modification: the key to ribosome control. *J Biochem.* (2020) 167:535–9. doi: 10.1093/jb/mvaa026
26. Lu L, Yi C, Jian X, Zheng G, He C. Structure determination of DNA methylation lesions N1-mea and N3-mec in duplex DNA using a cross-linked protein-DNA system. *Nucleic Acids Res.* (2010) 38:4415–25. doi: 10.1093/nar/gkq129
27. Xiong W, Zhao Y, Wei Z, Li C, Zhao R, Ge J, et al. N1-methyladenosine formation, gene regulation, biological functions, and clinical relevance. *Mol Ther.* (2023) 31:308–30. doi: 10.1016/j.jymthe.2022.10.015
28. Li J, Zhang H, Wang H. N(1)-methyladenosine modification in cancer biology: current status and future perspectives. *Comput Struct Biotechnol J.* (2022) 20:6578–85. doi: 10.1016/j.csbj.2022.11.045
29. Yokoyama W, Hirota K, Wan H, Sumi N, Miyata M, Araoi S, et al. rRNA adenine methylation requires T07a9.8 gene as rram-1 in *Caenorhabditis elegans*. *J Biochem.* (2018) 163:465–74. doi: 10.1093/jb/mvy018
30. Bar-Yaacov D, Frumkin I, Yashiro Y, Chujo T, Ishigami Y, Chemla Y, et al. Mitochondrial 16S rRNA is methylated by tRNA methyltransferase trmt61b in all vertebrates. *PLoS Biol.* (2016) 14:e1002557. doi: 10.1371/journal.pbio.1002557
31. Chujo T, Suzuki T. Trmt61b is a methyltransferase responsible for 1-methyladenosine at position 58 of human mitochondrial tRNAs. *Rna.* (2012) 18:2269–76. doi: 10.1261/rna.035600.112
32. Martín A, Epifano C, Vilaplana-Martí B, Hernández I, Macías RIR, Martínez-Ramírez Á, et al. Mitochondrial RNA methyltransferase trmt61b is a new, potential biomarker and therapeutic target for highly aneuploid cancers. *Cell Death Differentiation.* (2023) 30:37–53. doi: 10.1038/s41418-022-01044-6
33. Meynier V, Hardwick SW, Catala M, Roske JJ, Oerum S, Chirgadze DY, et al. Structural basis for human mitochondrial tRNA maturation. *Nat Commun.* (2024) 15:4683. doi: 10.1038/s41467-024-49132-0
34. He H, Wang Y, Zhang X, Li X, Liu C, Yan D, et al. Age-related noncanonical trmt6-trmt61a signaling impairs hematopoietic stem cells. *Nat Aging.* (2024) 4:213–30. doi: 10.1038/s43587-023-00556-1
35. Shan B, Pan H, Najafov A, Yuan J. Necroptosis in development and diseases. *Genes Dev.* (2018) 32:327–40. doi: 10.1101/gad.312561.118
36. Strlic B, Yang L, Albarrán-Juárez J, Wachsmuth L, Han K, Müller UC, et al. Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. *Nature.* (2016) 536:215–8. doi: 10.1038/nature19076
37. Zuo H, Wu A, Wang M, Hong L, Wang H. tRNA M(1)a modification regulate hsc maintenance and self-renewal via mtorcl1 signaling. *Nat Commun.* (2024) 15:5706. doi: 10.1038/s41467-024-50110-9
38. Gremke N, Polo P, Dort A, Schneikert J, Elmsäuser S, Brehm C, et al. Mtor-mediated cancer drug resistance suppresses autophagy and generates a druggable metabolic vulnerability. *Nat Commun.* (2020) 11:4684. doi: 10.1038/s41467-020-18504-7
39. Zou Z, Tao T, Li H, Zhu X. Mtor signaling pathway and mtor inhibitors in cancer: progress and challenges. *Cell Bioscience.* (2020) 10:31. doi: 10.1186/s13578-020-00396-1
40. Wu F, Chen Z, Liu J, Hou Y. The akt-mtor network at the interface of hematopoietic stem cell homeostasis. *Exp Hematol.* (2021) 103:15–23. doi: 10.1016/j.exphem.2021.08.009
41. Zhang C, Jia G. Reversible RNA modification n1-methyladenosine (M1a) in mrna and tRNA. *Genomics Proteomics Bioinf.* (2018) 16:155–61. doi: 10.1016/j.gpb.2018.03.003
42. Kuang W, Jin H, Yang F, Chen X, Liu J, Li T, et al. Alkbh3-dependent M1a demethylation of aurora a mrna inhibits ciliogenesis. *Cell Discov.* (2022) 8:25. doi: 10.1038/s41421-022-00385-3
43. Aas PA, Otterlei M, Falnes PO, Vågbo CB, Skorpén F, Akbari M, et al. Human alkb oxidative demethylases repair alkylation damage in both RNA and DNA. *Nature.* (2003) 421:859–63. doi: 10.1038/nature01363
44. Tasaki M, Shimada K, Kimura H, Tsujikawa K, Konishi N. Alkbh3, a human alkb homologue, contributes to cell survival in human non-small-cell lung cancer. *Br J Cancer.* (2011) 104:700–6. doi: 10.1038/sj.bjc.6606012
45. Shimada K, Fujii T, Tsujikawa K, Anai S, Fujimoto K, Konishi N. Alkbh3 contributes to survival and angiogenesis of human urothelial carcinoma cells through nadph oxidase and tweak/fn14/vegfr signals. *Clin Cancer Res.* (2012) 18:5247–55. doi: 10.1158/1078-0432.Ccr-12-0955
46. Chen Z, Qi M, Shen B, Luo G, Wu Y, Li J, et al. Transfer RNA demethylase alkbh3 promotes cancer progression via induction of tRNA-derived small RNAs. *Nucleic Acids Res.* (2019) 47:2533–45. doi: 10.1093/nar/gky1250
47. Wei J, Liu F, Lu Z, Fei Q, Ai Y, He PC, et al. Differential M(6)a, M(6)a(M), and M(1)a demethylation mediated by fto in the cell nucleus and cytoplasm. *Mol Cell.* (2018) 71:973–85.e5. doi: 10.1016/j.molcel.2018.08.011
48. Dai X, Wang T, Gonzalez G, Wang Y. Identification of yth domain-containing proteins as the readers for N1-methyladenosine in RNA. *Anal Chem.* (2018) 90:6380–4. doi: 10.1021/acs.analchem.8b01703
49. Zheng Q, Gan H, Yang F, Yao Y, Hao F, Hong L, et al. Cytoplasmic M(1)a reader ythdf3 inhibits trophoblast invasion by downregulation of M(1)a-methylated igf1r. *Cell Discovery.* (2020) 6:12. doi: 10.1038/s41421-020-0144-4
50. Seo KW, Kleiner RE. Ythdf2 recognition of N(1)-methyladenosine (M(1)a)-modified RNA is associated with transcript destabilization. *ACS Chem Biol.* (2020) 15:132–9. doi: 10.1021/acscchembio.9b00655
51. Wu Y, Chen Z, Xie G, Zhang H, Wang Z, Zhou J, et al. Rna M(1)a methylation regulates glycolysis of cancer cells through modulating atp5d. *Proc Natl Acad Sci United States America.* (2022) 119:e2119038119. doi: 10.1073/pnas.2119038119
52. Chabner BA, Roberts TG Jr. Timeline: chemotherapy and the war on cancer. *Nat Rev Cancer.* (2005) 5:65–72. doi: 10.1038/nrc1529
53. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: A common denominator approach to cancer therapy. *Cancer Cell.* (2015) 27:450–61. doi: 10.1016/j.ccr.2015.03.001
54. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell.* (2012) 21:309–22. doi: 10.1016/j.ccr.2012.02.022
55. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med.* (2013) 19:1423–37. doi: 10.1038/nm.3394
56. Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med.* (2011) 17:320–9. doi: 10.1038/nm.2328
57. Sharma P, Allison JP. The future of immune checkpoint therapy. *Sci (New York NY).* (2015) 348:56–61. doi: 10.1126/science.aaa8172
58. Choi C, Yoo GS, Cho WK, Park HC. Optimizing radiotherapy with immune checkpoint blockade in hepatocellular carcinoma. *World J Gastroenterol.* (2019) 25:2416–29. doi: 10.3748/wjg.v25.i20.2416

59. Dong C. Cytokine regulation and function in T cells. *Annu Rev Immunol.* (2021) 39:51–76. doi: 10.1146/annurev-immunol-061020-053702
60. Montauti E, Oh DY, Fong L. Cd4(+) T cells in antitumor immunity. *Trends Cancer.* (2024) 10:969–85. doi: 10.1016/j.trecan.2024.07.009
61. Buck MD, O'Sullivan D, Pearce EL. T cell metabolism drives immunity. *J Exp Med.* (2015) 212:1345–60. doi: 10.1084/jem.20151159
62. Saravia J, Chapman NM, Chi H. Helper T cell differentiation. *Cell Mol Immunol.* (2019) 16:634–43. doi: 10.1038/s41423-019-0220-6
63. Tan H, Yang K, Li Y, Shaw TI, Wang Y, Blanco DB, et al. Integrative proteomics and phosphoproteomics profiling reveals dynamic signaling networks and bioenergetics pathways underlying T cell activation. *Immunity.* (2017) 46:488–503. doi: 10.1016/j.immuni.2017.02.010
64. Liu F, Clark W, Luo G, Wang X, Fu Y, Wei J, et al. Alkbh1-mediated tRNA demethylation regulates translation. *Cell.* (2016) 167:816–28.e16. doi: 10.1016/j.cell.2016.09.038
65. Liu Y, Zhou J, Li X, Zhang X, Shi J, Wang X, et al. tRNA-M(1)a modification promotes T cell expansion via efficient myc protein synthesis. *Nat Immunol.* (2022) 23:1433–44. doi: 10.1038/s41590-022-01301-3
66. Marchingo JM, Sinclair LV, Howden AJ, Cantrell DA. Quantitative analysis of how myc controls T cell proteomes and metabolic pathways during T cell activation. *eLife.* (2020) 9:e53725. doi: 10.7554/eLife.53725
67. Lin P, Li G, Wu M. RNA Methylation into M(1)a Era: A New Regulation over T-Cell Function. *Signal Transduct Target Ther.* (2023) 8:78. doi: 10.1038/s41392-023-01360-4
68. Chen S, Saeed A, Liu Q, Jiang Q, Xu H, Xiao GG, et al. Macrophages in immunoregulation and therapeutics. *Signal Transduct Target Ther.* (2023) 8:207. doi: 10.1038/s41392-023-01452-1
69. Wu C, Xu Y, Zhao Y. Two kinds of macrophage memory: innate and adaptive immune-like macrophage memory. *Cell Mol Immunol.* (2022) 19:852–4. doi: 10.1038/s41423-022-00885-y
70. Woo HH, Chambers SK. Human alkbh3-induced M(1)a demethylation increases the csf-1 mrna stability in breast and ovarian cancer cells. *Biochim Biophys Acta Gene Regul Mech.* (2019) 1862:35–46. doi: 10.1016/j.bbagr.2018.10.008
71. Otero K, Turnbull IR, Poliani PL, Vermi W, Cerutti E, Aoshi T, et al. Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving dap12 and β -catenin. *Nat Immunol.* (2009) 10:734–43. doi: 10.1038/ni.1744
72. Toy EP, Azodi M, Folk NL, Zito CM, Zeiss CJ, Chambers SK. Enhanced ovarian cancer tumorigenesis and metastasis by the macrophage colony-stimulating factor. *Neoplasia.* (2009) 11:136–44. doi: 10.1593/neo.81150
73. Thompson RW, Geraghty PJ, Lee JK. Abdominal aortic aneurysms: basic mechanisms and clinical implications. *Curr problems Surg.* (2002) 39:110–230. doi: 10.1067/msg.2002.121421
74. Wu Y, Jiang D, Zhang H, Yin F, Guo P, Zhang X, et al. N1-methyladenosine (M1a) regulation associated with the pathogenesis of abdominal aortic aneurysm through ythdf3 modulating macrophage polarization. *Front Cardiovasc Med.* (2022) 9:883155. doi: 10.3389/fcvm.2022.883155
75. Poteaux S, Tedgui A. Monocytes, macrophages and other inflammatory mediators of abdominal aortic aneurysm. *Curr Pharm design.* (2015) 21:4007–15. doi: 10.2174/1381612821666150826093855
76. Raffort J, Lareyre F, Clément M, Hassen-Khodja R, Chinetti G, Mallat Z. Monocytes and macrophages in abdominal aortic aneurysm. *Nat Rev Cardiol.* (2017) 14:457–71. doi: 10.1038/nrcardio.2017.52
77. Kawamura Y, Murtada SI, Gao F, Liu X, Tellides G, Humphrey JD. Adventitial remodeling protects against aortic rupture following late smooth muscle-specific disruption of tgfb signaling. *J mechanical Behav Biomed materials.* (2021) 116:104264. doi: 10.1016/j.jmbm.2020.104264
78. Gao F, Chambon P, Offermanns S, Tellides G, Kong W, Zhang X, et al. Disruption of tgfb signaling in smooth muscle cell prevents elastase-induced abdominal aortic aneurysm. *Biochem Biophys Res Commun.* (2014) 454:137–43. doi: 10.1016/j.bbrc.2014.10.053
79. . (!!! INVALID CITATION!!! (81)).
80. Davis FM, Gallagher KA. Epigenetic mechanisms in monocytes/macrophages regulate inflammation in cardiometabolic and vascular disease. *Arteriosclerosis thrombosis Vasc Biol.* (2019) 39:623–34. doi: 10.1161/atvbaha.118.312135
81. Dale MA, Xiong W, Carson JS, Suh MK, Karpisek AD, Meisinger TM, et al. Elastin-derived peptides promote abdominal aortic aneurysm formation by modulating M1/M2 macrophage polarization. *J Immunol (Baltimore Md: 1950).* (2016) 196:4536–43. doi: 10.4049/jimmunol.1502454
82. Bao G, Li T, Guan X, Yao Y, Liang J, Xiang Y, et al. Comprehensive analysis of the function, immune profiles, and clinical implication of M1a regulators in lung adenocarcinoma. *Front Oncol.* (2022) 12:882292. doi: 10.3389/fonc.2022.882292
83. Zhao M, Shen S, Xue C. A novel M1a-score model correlated with the immune microenvironment predicts prognosis in hepatocellular carcinoma. *Front Immunol.* (2022) 13:805967. doi: 10.3389/fimmu.2022.805967
84. Lei K, Sheng Y, Luo M, Liu J, Gong C, Lv S, et al. Comprehensive analyses of M1a regulator-mediated modification patterns determining prognosis in lower-grade glioma (Running title: M1a in lgg). *Heliyon.* (2024) 10:e27510. doi: 10.1016/j.heliyon.2024.e27510
85. Liu J, Chen C, Wang Y, Qian C, Wei J, Xing Y, et al. Comprehensive of N1-methyladenosine modifications patterns and immunological characteristics in ovarian cancer. *Front Immunol.* (2021) 12:746647. doi: 10.3389/fimmu.2021.746647
86. Gao Y, Wang H, Li H, Ye X, Xia Y, Yuan S, et al. Integrated analyses of M(1)a regulator-mediated modification patterns in tumor microenvironment-infiltrating immune cells in colon cancer. *Oncoimmunology.* (2021) 10:1936758. doi: 10.1080/2162402x.2021.1936758
87. Zheng S, Wang W, Shen L, Yao Y, Xia W, Ni C. Tumor battlefield within inflamed, excluded or desert immune phenotypes: the mechanisms and strategies. *Exp Hematol Oncol.* (2024) 13:80. doi: 10.1186/s40164-024-00543-1
88. Rizzo A, Ricci AD, Gadaleta-Caldarola G. Msi-H/dmmr and cancer immunotherapy: current state and future implications. *Expert Rev Precis Med Drug Dev.* (2021) 6:345–7. doi: 10.1080/23808993.2021.1946391
89. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Sci (New York NY).* (2015) 348:69–74. doi: 10.1126/science.aaa4971
90. Wang E, Li Y, Ming R, Wei J, Du P, Zhou P, et al. The prognostic value and immune landscapes of a M(6)a/M(5)C/M(1)a-related lncrnas signature in head and neck squamous cell carcinoma. *Front Cell Dev Biol.* (2021) 9:718974. doi: 10.3389/fcell.2021.718974
91. Chen YG, Satpathy AT, Chang HY. Gene regulation in the immune system by long noncoding RNAs. *Nat Immunol.* (2017) 18:962–72. doi: 10.1038/ni.3771
92. Zhou B, Bie F, Zang R, Zhang M, Song P, Liu L, et al. RNA modification writer expression profiles predict clinical outcomes and guide neoadjuvant immunotherapy in non-small cell lung cancer. *eBioMedicine.* (2022) 84:104268. doi: 10.1016/j.ebiom.2022.104268
93. Gao L, Chen R, Sugimoto M, Mizuta M, Kishimoto Y, Omori K. The impact of M1a methylation modification patterns on tumor immune microenvironment and prognosis in oral squamous cell carcinoma. *Int J Mol Sci.* (2021) 22:10302. doi: 10.3390/ijms221910302
94. Huang Y, Zhang W, Li Q, Wang Z, Yang X. Identification of M6a/M5c/M1a-associated lncrnas for prognostic assessment and immunotherapy in pancreatic cancer. *Sci Rep.* (2023) 13:3661. doi: 10.1038/s41598-023-30865-9
95. Li N, Kang Y, Wang L, Huff S, Tang R, Hui H, et al. Alkbh5 regulates anti-pd-1 therapy response by modulating lactate and suppressive immune cell accumulation in tumor microenvironment. *Proc Natl Acad Sci United States America.* (2020) 117:20159–70. doi: 10.1073/pnas.1918986117
96. Denechaud PD, Fajas L, Giral A. E2f1, a novel regulator of metabolism. *Front Endocrinol.* (2017) 8:311. doi: 10.3389/fendo.2017.00311
97. Liu WW, Zheng SQ, Li T, Fei YF, Wang C, Zhang S, et al. RNA modifications in cellular metabolism: implications for metabolism-targeted therapy and immunotherapy. *Signal Transduct Target Ther.* (2024) 9:70. doi: 10.1038/s41392-024-01777-5
98. Wang Y, Wang J, Li X, Xiong X, Wang J, Zhou Z, et al. N(1)-methyladenosine methylation in tRNA drives liver tumorigenesis by regulating cholesterol metabolism. *Nat Commun.* (2021) 12:6314. doi: 10.1038/s41467-021-26718-6
99. Hannedouche S, Zhang J, Yi T, Shen W, Nguyen D, Pereira JP, et al. Oxysterols direct immune cell migration via ebi2. *Nature.* (2011) 475:524–7. doi: 10.1038/nature10280
100. Xu Y, He L, Fu Q, Hu J. Metabolic reprogramming in the tumor microenvironment with immunocytes and immune checkpoints. *Front Oncol.* (2021) 11:759015. doi: 10.3389/fonc.2021.759015
101. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-pd-1 antibody in cancer. *New Engl J Med.* (2012) 366:2443–54. doi: 10.1056/NEJMoa1200690
102. Okazaki T, Honjo T. Pd-1 and pd-1 ligands: from discovery to clinical application. *Int Immunol.* (2007) 19:813–24. doi: 10.1093/intimm/dxm057
103. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. Pd-1 and its ligands in tolerance and immunity. *Annu Rev Immunol.* (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
104. Marei HE, Hasan A, Pozzoli G, Cenciarelli C. Cancer immunotherapy with immune checkpoint inhibitors (Icis): potential, mechanisms of resistance, and strategies for reinvigorating T cell responsiveness when resistance is acquired. *Cancer Cell Int.* (2023) 23:64. doi: 10.1186/s12935-023-02902-0
105. Dhanasekaran R, Deutzmann A, Mahauad-Fernandez WD, Hansen AS, Gouw AM, Felsner DW. The myc oncogene - the grand orchestrator of cancer growth and immune evasion. *Nat Rev Clin Oncol.* (2022) 19:23–36. doi: 10.1038/s41571-021-00549-2
106. Wang S, Song A, Xie J, Wang YY, Wang WD, Zhang MJ, et al. Fn-omv potentiates zbp1-mediated panoptosis triggered by oncolytic hsv-1 to fuel antitumor immunity. *Nat Commun.* (2024) 15:3669. doi: 10.1038/s41467-024-48032-7
107. Li S, Feng T, Liu Y, Yang Q, Song A, Wang S, et al. M(1)a inhibition fuels oncolytic virus-elicited antitumor immunity via downregulating myc/pd-L1 signaling. *Int J Oral Sci.* (2024) 16:36. doi: 10.1038/s41368-024-00304-0
108. Ai Y, Liu S, Luo H, Wu S, Wei H, Tang Z, et al. Mettl3 intensifies the progress of oral squamous cell carcinoma via modulating the M6a amount of prmt5 and pd-L1. *J Immunol Res.* (2021) 2021:6149558. doi: 10.1155/2021/6149558

109. Bao Y, Zhai J, Chen H, Wong CC, Liang C, Ding Y, et al. Targeting M(6) a reader ythdf1 augments antitumour immunity and boosts anti-pd-1 efficacy in colorectal cancer. *Gut*. (2023) 72:1497–509. doi: 10.1136/gutjnl-2022-328845
110. Limagne E, Euvrard R, Thibaudin M, Rêbê C, Derangère V, Chevriaux A, et al. Accumulation of mdsc and th17 cells in patients with metastatic colorectal cancer predicts the efficacy of a folfox-bevacizumab drug treatment regimen. *Cancer Res*. (2016) 76:5241–52. doi: 10.1158/0008-5472.CAN-15-3164
111. Yang S, Wei J, Cui Y-H, Park G, Shah P, Deng Y, et al. M6a mrna demethylase fto regulates melanoma tumorigenicity and response to anti-pd-1 blockade. *Nat Commun*. (2019) 10:2782. doi: 10.1038/s41467-019-10669-0
112. Liu M, Dong Q, Chen B, Liu K, Zhao Z, Wang Y, et al. Synthetic viability induces resistance to immune checkpoint inhibitors in cancer cells. *Br J Cancer*. (2023) 129:1339–49. doi: 10.1038/s41416-023-02404-w
113. Bai R, Lv Z, Xu D, Cui J. Predictive biomarkers for cancer immunotherapy with immune checkpoint inhibitors. *biomark Res*. (2020) 8:34. doi: 10.1186/s40364-020-00209-0
114. Karasarides M, Cogdill AP, Robbins PB, Bowden M, Burton EM, Butterfield LH, et al. Hallmarks of resistance to immune-checkpoint inhibitors. *Cancer Immunol Res*. (2022) 10:372–83. doi: 10.1158/2326-6066.Cir-20-0586
115. Guo W, Wang Z, Zhang Y, Li Y, Du Q, Zhang T, et al. Mettl3-dependent M(6)a modification is essential for effector differentiation and memory formation of cd8(+) T cells. *Sci Bull*. (2024) 69:82–96. doi: 10.1016/j.scib.2023.11.029
116. Song H, Song J, Cheng M, Zheng M, Wang T, Tian S, et al. Mettl3-mediated M(6)a RNA methylation promotes the anti-tumour immunity of natural killer cells. *Nat Commun*. (2021) 12:5522. doi: 10.1038/s41467-021-25803-0
117. Chambers SK, Wang Y, Gertz RE, Kacinski BM. Macrophage colony-stimulating factor mediates invasion of ovarian cancer cells through urokinase. *Cancer Res*. (1995) 55:1578–85.
118. Mesaros O, Onciul M, Matei E, Joldes C, Jimbu L, Neaga A, et al. Macrophages as potential therapeutic targets in acute myeloid leukemia. *Biomedicines*. (2024) 12:2306. doi: 10.3390/biomedicines12102306
119. Han D, Liu J, Chen C, Dong L, Liu Y, Chang R, et al. Anti-tumour immunity controlled through mrna M(6)a methylation and ythdf1 in dendritic cells. *Nature*. (2019) 566:270–4. doi: 10.1038/s41586-019-0916-x
120. Wang Y, Li L, Li J, Zhao B, Huang G, Li X, et al. The emerging role of M6a modification in regulating the immune system and autoimmune diseases. *Front Cell Dev Biol*. (2021) 9:755691. doi: 10.3389/fcell.2021.755691
121. Penning A, Jeschke J, Fuks F. Why novel mrna modifications are so challenging and what we can do about it. *Nat Rev Mol Cell Biol*. (2022) 23:385–6. doi: 10.1038/s41580-022-00485-8
122. Qi Z, Zhang C, Jian H, Hou M, Lou Y, Kang Y, et al. N(1)-methyladenosine modification of mrna regulates neuronal gene expression and oxygen glucose deprivation/reoxygenation induction. *Cell Death Discovery*. (2023) 9:159. doi: 10.1038/s41420-023-01458-2
123. Zhang C, Yi X, Hou M, Li Q, Li X, Lu L, et al. The landscape of M(1)a modification and its posttranscriptional regulatory functions in primary neurons. *eLife*. (2023) 12:e85324. doi: 10.7554/eLife.85324
124. Zhu M, Xu R, Yuan J, Wang J, Ren X, Cong T, et al. Tracking-seq reveals the heterogeneity of off-target effects in crispr-cas9-mediated genome editing. *Nat Biotechnol*. (2024). doi: 10.1038/s41587-024-02307-y
125. Hu Z, Sun A, Yang J, Naz G, Sun G, Li Z, et al. Regulation of the crispr-cas12a system by methylation and demethylation of guide RNA. *Chem Sci*. (2023) 14:5945–55. doi: 10.1039/d3sc00629h



OPEN ACCESS

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RECEIVED 07 September 2024

ACCEPTED 21 November 2024

PUBLISHED 12 December 2024

CITATION

Chen X, Xu W, Pan J, Yang H, Li Y, Chen X, Sun Y, Liu Q and Qiu S (2024) m6A methylation profiling as a prognostic marker in nasopharyngeal carcinoma: insights from MeRIP-Seq and RNA-Seq. *Front. Immunol.* 15:1492648. doi: 10.3389/fimmu.2024.1492648

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m6A methylation profiling as a prognostic marker in nasopharyngeal carcinoma: insights from MeRIP-Seq and RNA-Seq

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Background: Nasopharyngeal carcinoma (NPC) is a type of malignant tumors commonly found in Southeast Asia and China, with insidious onset and clinical symptoms. N6-methyladenosine (m6A) modification significantly contributes to tumorigenesis and progression by altering RNA secondary structure and influencing RNA-protein binding at the transcriptome level. However, the mechanism and role of abnormal m6A modification in nasopharyngeal carcinoma remain unclear.

Methods: Nasopharyngeal Carcinoma tissues from 3 patients and non-cancerous nasopharyngeal tissues from 3 individuals, all from Fujian Cancer Hospital, were sequenced for m6A methylation. These were combined with transcriptome sequencing data from 192 nasopharyngeal cancer tissues. Genes linked to prognosis were discovered using differential analysis and univariate Cox regression. Subsequently, a prognostic model associated with m6A was developed through the application of LASSO regression analysis. The model's accuracy was verified using both internal transcriptome databases and external databases. An extensive evaluation of the tumor's immune microenvironment and signaling pathways was performed, analyzing both transcriptomic and single-cell data.

Results: The m6A methylation sequencing analysis revealed 194 genes with varying expression levels, many of which are predominantly associated with immune pathways. By integrating transcriptome sequencing data, 19 m6A-modified genes were found to be upregulated in tumor tissues, leading to the development of a three-gene (EME1, WNT4, SHISA2) risk prognosis model. The group with lower risk exhibited notable enrichment in pathways related to immunity, displaying traits like enhanced survival rates, stronger immune profiles, and increased responsiveness to immunotherapy when compared to the higher-risk group. Single-cell analysis revealed that malignant cells exhibited the highest risk score levels compared to immune cells, with a high-risk score indicating worse biological behavior. The three hub genes demonstrated

significant correlation with m6A modification regulators, and MeRIP-RT-PCR confirmed the occurrence of m6A methylation in these genes within nasopharyngeal carcinoma cells.

Conclusions: A prognostic model for nasopharyngeal carcinoma risk based on m6A modification genes was developed, and its prognostic value was confirmed through self-assessment data. The study highlighted the crucial impact of m6A modification on the immune landscape of nasopharyngeal cancer.

KEYWORDS

nasopharyngeal carcinoma, m6A modification, tumor immune microenvironment, prognosis, transcriptome sequencing

1 Introduction

Nasopharyngeal carcinoma is a malignant tumor originating from the mucosa of the nasopharynx, with a notably high occurrence in specific areas, especially in Southeast Asia and South China. The causes of nasopharyngeal cancer are not completely known, but they are linked to multiple elements such as genetics, environmental influences, and viral infections. Due to its insidious early symptoms, it is often detected at a middle to late stage, posing a great challenge to treatment (1). Thus, identifying the new marker is crucial for the early diagnosis and treatment of NPC. Over the past few years, advancements in genomics and transcriptomics have led scientists to increasingly recognize the significant impact of epigenetic changes on cancer progression. N6-methyladenosine (m6A) modification, a prevalent RNA alteration, significantly influences gene expression, RNA processing, and protein synthesis by modifying RNA structure and function (2).

m6A modifications are added to RNA by m6A methyltransferase (“Writer”) and removed by m6A demethylase (“Eraser”) removal, and recognition and decoding by m6A recognition proteins (“Readers”). This modification system forms a dynamic equilibrium that regulates multiple biological processes such as RNA stability, transcription, translation, splicing, etc (3). Growing amounts of evidence suggest that m6A modification plays a crucial role in controlling tumor development, resistance to chemotherapy, response to immunotherapy, and prognosis (4–7). It has been demonstrated that m6A modification is significantly linked to the onset, spread, and progression of tumors (8, 9). Additionally, m6A modification is essential in the complexity and diversity of the tumor microenvironment (TME) (10, 11). The interaction between m6A modification and the TME influences the biological activities of cancer cells, immune cells, and stromal cells, affecting tumor initiation, progression, and treatment responses (12–14). Grasping the relationship between m6A modification and the tumor microenvironment is crucial for creating effective treatments and predicting outcomes. While certain studies have highlighted the involvement of m6A modifications in cancer

development, advancement, and therapy response, the majority of contemporary research is mainly centered on m6A regulatory proteins. The comprehensive study of how m6A-modified genes interact in nasopharyngeal carcinoma (NPC) and their effects on prognosis and the immune environment is still not well understood.

This research sought to combine m6A methylation histology with transcriptome data to pinpoint genes experiencing m6A methylation changes in nasopharyngeal carcinoma. The aim was to develop a predictive risk model utilizing m6A modification-associated genes to support treatment decisions for nasopharyngeal carcinoma patients and to investigate the model’s influence on the immune microenvironment.

2 Materials and methods

2.1 Patient samples

For transcriptome sequencing, tumor tissues from 192 nasopharyngeal cancer patients and normal tissues from 19 healthy individuals were collected from those diagnosed and treated at Fujian Provincial Cancer Hospital between January 9, 2015, and June 2, 2016 (in-house cohort). Additionally, tumor tissues from 3 nasopharyngeal cancer patients and non-tumor tissues from 3 healthy individuals were collected in 2023 for m6A methylation modification sequencing. Eligible participants included those newly diagnosed with nasopharyngeal carcinoma, undergoing standard radiotherapy, aged 18 or older, possessing normal blood, kidney, and liver functions, and free from other malignancies. Every patient gave their written consent after being informed. The Ethics Committees of both Fujian Cancer Hospital and Fujian Medical University Cancer Hospital granted approval for the research (approval code SQ2019-035-01). For future RNA extraction, tissue specimens were preserved in liquid nitrogen.

To confirm the reliability and relevance of the data in this study, NPC RNA-seq data from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>, GSE102349) were chosen as an

external validation cohort. To assess the predictive effect of the risk model on immunotherapy efficacy, we downloaded the NSCLC-GSE126044 immunotherapy dataset from the GEO database. The model's precision at the single-cell level was confirmed using the GSE150430 dataset, which also facilitated the investigation of cell-ligand receptor interactions within the NPC immune microenvironment.

2.2 m6A sequencing and processing of sequencing results

Hangzhou Lianchuan Biological Information Technology Co. handled the RNA extraction and the creation of sequencing libraries. The broken RNA was split into two sections. Initially, the sample was incubated for two hours at 4°C with an m6A-specific antibody (202003, Synaptic Systems, Germany) in immunoprecipitation buffer (50 mM Tris-HCl, 750 mM NaCl, and 0.5% isobaric acid). Tris-HCl, 750 mM sodium chloride, and 0.5% IGEPAL CA-630. The latter section functioned as a control to directly build a standard transcriptome sequencing library. The m6A-seq Library (IP) and RNA-seq Library (input) were individually processed for high-throughput sequencing on the Illumina NovaSeq™ 6000 platform in 150 PE mode. For superior read quality, the sequences underwent additional filtering with fastp (version fastp-0.19.4, available at <https://github.com/OpenGene/fastp>). To align the reads of all samples with the reference genome, we used the HISAT2 software package (<https://daehwankimlab.github.io/hisat2/>, version: hisat2-2.2.1). To analyze m6A and transcriptome samples, peak detection software along with the R package exomePeak 1.8 were employed, identifying peak positions on the genome, measuring peak lengths, and calculating differences between groups. ChIPseeker 1.0 was employed for further analysis.

2.3 Prognosis-related model construction and validation

Screening for differential m6A modifier genes between healthy population and nasopharyngeal carcinoma tissues by exomepeak2 analysis (15). The threshold criteria met these two conditions: a fold-change greater than 2 and a p-value less than 0.05. To delve deeper into the pathways enriched by DEGs, we utilized Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. A false discovery rate of <0.05 was set as a critical value. Subsequently, these genes were compared with those showing variations between healthy individuals and nasopharyngeal carcinoma patients in the in-house cohort. This comparison was used to develop a prognostic model for m6A risk through univariate Cox analysis and LASSO Cox regression. The R package 'glmnet' was employed to pinpoint genes with the most valuable prognostic biomarkers. A predictive risk score was formulated by linearly integrating the equation: $Risk\ score = \sum_{i=1}^N (exp \times coef)$, where 'exp' represents the gene expression value and 'coef' denotes the gene's coefficient in the LASSO analysis.

To assess the predictive accuracy of our risk prediction model, we categorized the sample into high-risk and low-risk groups based

on the median risk score. Survival analysis was conducted using the R package 'survival'. This approach facilitates a comprehensive understanding of the complex regulatory network associated with m6A modifications and provides valuable insights for identifying promising targets in the development of novel immunotherapeutic strategies. Survival curves were compared using the Kaplan-Meier technique. Later, the R package 'timeROC' was utilized to analyze the receiver operating characteristic (ROC) for individuals who survived 1, 3, and 5 years in both the self-assessment data cohort and the validation cohort GSE102349.

2.4 Multidimensional immunity- and carcinogenesis-related estimates

To assess immune cell infiltration in various ways, we used several immunoscore methods, such as TIMER and ssGSEA algorithms (16, 17). The Immunophenotyping score was estimated by the IOBR-R package (18). From earlier studies, we retrieved a set of 10 suppressive immune checkpoints with immunotherapeutic efficacy (19). A set of genes for tertiary lymphoid structure (TLS) was also obtained (20, 21).

2.5 Single-cell RNA-seq analysis

Additionally, this research employed Seurat (version 4.0.4) for the purposes of quality assurance, data reduction, and grouping of single-cell RNA sequencing data (22). The data were quality controlled, downscaled and clustered using Seurat (v4.0.4). To maintain data integrity, genes identified in less than three cells and cells with under 250 detected genes were omitted, and the proportion of mitochondrial genes was restricted to below 35%. Data were normalized using the logNormalize method. TISCH (<http://tisch.comp-genomics.org/>) offers comprehensive single-cell level cell type annotations. Subsequently, the 'FindAllMarkers' function was employed to detect marker genes within each cluster, utilizing a threshold of absolute log2-fold change (FC) ≥ 0.3 and requiring a minimum cluster fraction of 0.25.

2.6 Calculation of risk scores and analysis of intercellular communication in single-cell samples

For each individual cell sample from GSE150430, risk scores were determined using the Single Sample Gene Set Enrichment Analysis (ssGSEA) technique, utilizing the 'GSVA' and 'GSEABase' libraries in R. Similarly, the risk scores for each tumor in the GEO validation group were computed with the same 'GSVA' and 'GSEABase' packages. Leveraging single-cell data as a benchmark, we utilized a novel deconvolution method (CIBERSORTx) on bulk transcriptome datasets to quantitatively determine the cell type proportions within tumors in both the self-assessment and GEO validation cohorts. CellChat version 1.1.3 software was employed to deduce communication between cells through ligand-receptor

interactions. Cell groups containing fewer than 10 cells were excluded from the intercellular communication analysis. Pairwise tests of communication probability values were performed to assess statistical significance.

2.7 Statistical analyses

Data analysis was conducted with R (version 3.6.1) and SPSS (version 25.0) software. For continuous variables, the Wilcoxon rank-sum test was utilized, while the chi-square test was applied to categorical variables. In every analysis, pairs of two-by-two reveal significant statistical differences. Symbols *, **, ***, and **** denote significance levels of less than 0.05, 0.01, 0.001, and 0.0001, respectively.

3 Results

3.1 m6A modifier genes are differentiated in nasopharyngeal carcinomas

Analysis of m6A modification in three nasopharyngeal carcinoma samples and three normal nasopharyngeal tissue samples from Fujian Cancer Hospital revealed that m6A methylation predominantly took place in the coding sequences (CDS) and the 3' untranslated regions (3'UTR) of both cancerous and non-cancerous tissues (Figures 1A–C). Compared with normal nasopharyngeal tissues, the levels of m6A methylation modification genes were higher in tumor patients (Figure 1D). Motif analysis revealed that RRACH methylation modification sites were present in both normal nasopharyngeal tissues and nasopharyngeal carcinoma tissues (Figures 1E, F). A total of 194 differential m6A methylation modification sites were identified in tumor and non-tumor tissues (Figure 1G), and the quadrant plot indicated that 65 differential m6A methylation modification genes were upregulated in nasopharyngeal carcinoma (Figure 1H). GO enrichment analysis indicated that the molecular roles of m6A modification genes were predominantly concentrated in signaling and immune response pathways, including B cell activation, T cell activation, and the inhibition of calcium-mediated signaling. Pathway analysis enriched by KEGG indicated that m6A modifier genes were predominantly involved in homologous recombination, cell adhesion molecules, and the B cell receptor signaling pathway (Figures 1I, J). The results indicate that m6A modification levels vary between cancerous and normal tissues and are intimately connected to the tumor immune microenvironment.

3.2 Risk modeling and validation

Differential genes in normal nasopharyngeal epithelial tissues and nasopharyngeal carcinoma tissues in the in-house cohort were further intersected with upregulated m6A methylation modifier genes in tumor tissues to identify 19 differential genes (Figure 2A); a one-way Cox analysis of progression-free survival (PFS) was

performed using the survival R package to identify m6A modifier genes with prognostic significance (p -value < 0.05). This study identified 19 m6A modifier genes, including TFAP2A, TMEM178B, JPH1, EME1, POU6F2, DST, CSAG3, KCTD1, TCERG1L, INSM1, WNT4, GLS2, ICAM5, CNTNAP2, IQGAP3, BEX3, SYNPO2, SHISA2, and FZD7. Among these, three genes (EME1, WNT4, and SHISA2) had high prognostic significance (Figure 2B).

Based on these three central genes, the prognostic risk model (MRS) was established using the LASSO Cox regression model (Figure 2C). The dataset was split into high-risk and low-risk categories according to the median risk score. The in-house cohort confirmed that the high-risk category had a worse prognosis (Figure 2D). The high-risk group suggested a poorer prognosis, as was the case in the GEO validation cohort (Figure 2E). The MRS demonstrated strong predictive accuracy, achieving a 3-year ROC AUC of 0.77 (Figure 2F). Although the 3-year AUC of the validation cohort was only 0.63, it suggested the model's stability (Figure 2G). Additionally, in comparison to gender, age, stage, and EBV-DNA, the model demonstrated a superior AUC (Figure 2H), suggesting that MRS serves as an independent prognostic indicator for predicting the survival of nasopharyngeal carcinoma patients and tailoring individualized treatment plans.

3.3 Enrichment pathways for risk model

The pathways of gene enrichment suggested that the genes played roles in physiological processes. Gene Ontology (GO) enrichment analysis indicated that the genes in the low-risk category were predominantly associated with pathways related to cell growth, immune complex removal, and the modulation of T-cell co-stimulation, all of which play roles in B cell immune responses (Figure 3A). The heat map of the hallmark pathway and the KEGG enrichment analysis revealed that the high-risk group was predominantly enriched in pathways like homologous recombination, P53 signaling, glycolysis, and others. The low-risk category predominantly featured primary immunodeficiency, natural killer cell cytotoxicity, B-cell receptor signaling, and T-cell receptor signaling pathways (Figures 3B–D). To sum up, the immune microenvironment could be influenced by the low-risk group.

3.4 Assessment of the immune microenvironment

We assessed the variations in immune cell infiltration levels between groups at high and low risk. Using the ssGSEA technique, the makeup of the 28 immune cell types showed notable differences between the high- and low-risk groups. Nearly all immune cell infiltration levels were elevated in the low-risk group compared to the high-risk group, particularly for B cells and CD8⁺ T cells (Figure 4A). TIME analysis similarly validated these results (Figure 4B). Further analysis of marker genes for B cells and CD8⁺ T cells indicated a notable increase in their expression within the low-risk group (Figures 4C, D).

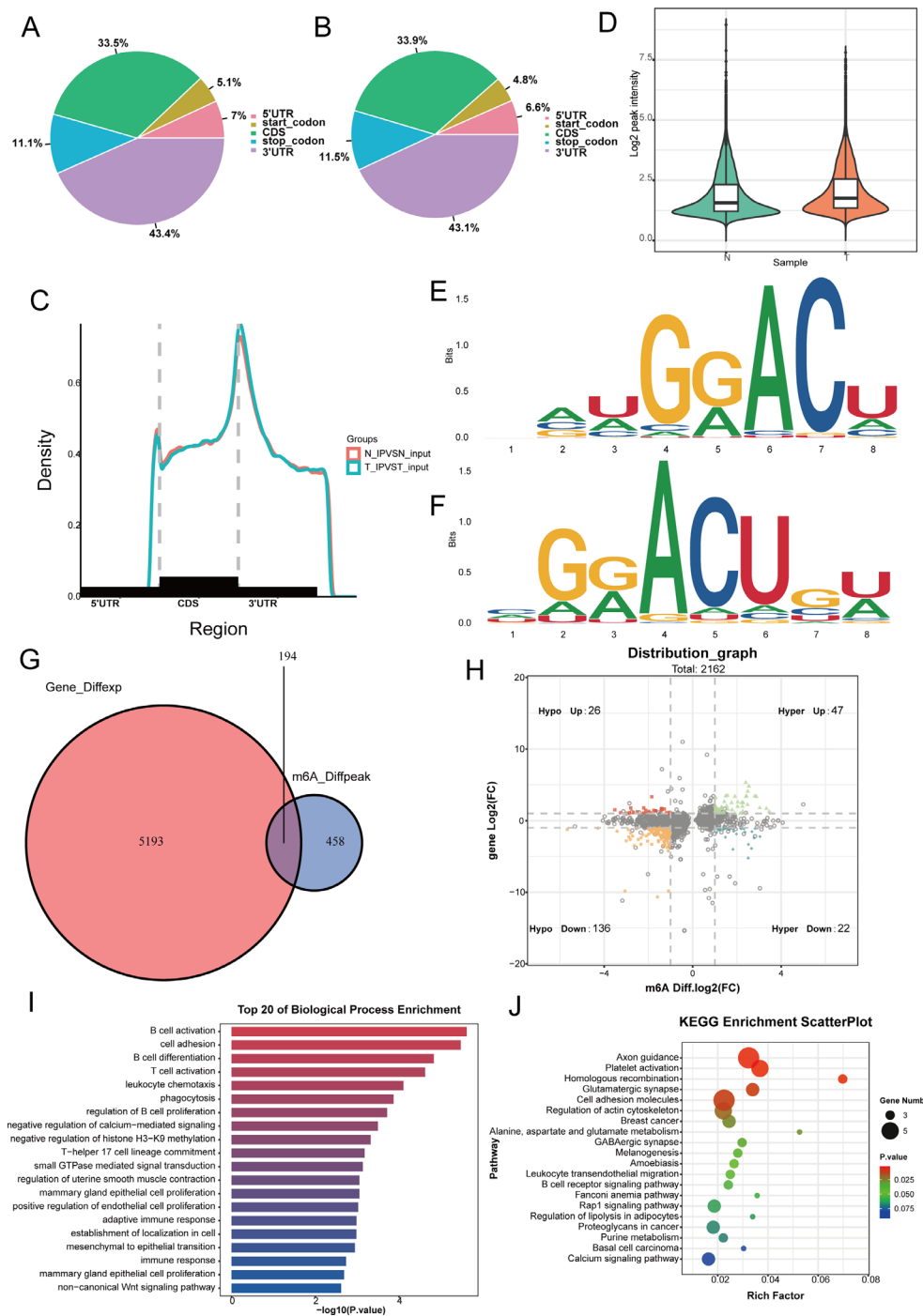


FIGURE 1

Analysis of m6A modifier profiles and identification of differentially expressed genes in nasopharyngeal carcinoma. (A, B) We use pie charts to count the distribution of peaks on gene functional elements between non-cancerous (A) and cancerous tissues (B). (C) Density of differential m6A peaks along transcripts. Each transcript is divided into three sections: 5'UTR, CDS, and 3'UTR. (D) Levels of m6A methylation modification in tumor and non-tumor tissues. (E, F) Differential of the most conserved sequence motif in the m6A peak region. (G) Venn diagram showing differentially expressed genes undergoing m6A methylation modification between non-cancerous and cancerous tissues. (H) The four-quadrant diagram shows the changes in differentially methylated peaks. (I, J) The KEGG and GO enrichment pathway analysis of differential m6A methylated genes.

3.5 Predictive power of immunotherapy efficacy

Moreover, a notable statistical disparity was observed in immune checkpoint inhibitors (CD86, PDCD1, TIGIT, CTLA-4, LAIR1, and

HAVCR2) between the high-risk and low-risk categories (Figure 5A). Research indicates that B cells infiltrating tumors and tertiary lymphoid structures associated with tumors enhance the effectiveness of immunotherapy. We subsequently evaluated TLS scores and found that low-risk patients had higher TLS scores

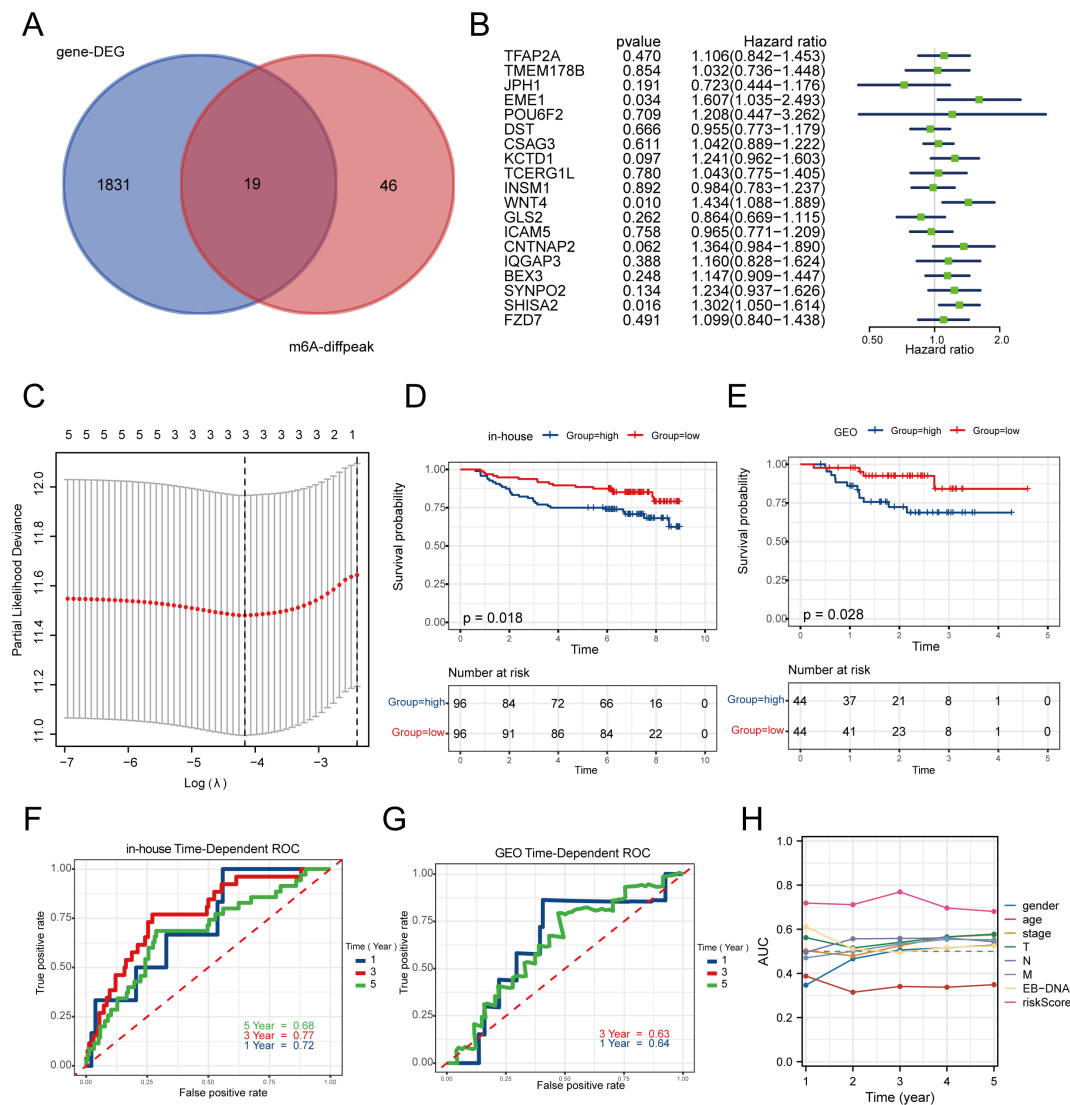


FIGURE 2

Construction and validation of a risk prognosis model for m6A related genes. **(A)** The intersection of m6A sequencing genes and 192 transcriptome data was used to screen for 19 m6A methylated genes upregulated in tumors. **(B)** Univariate Cox analysis was performed on these 19 genes with PFS. **(C)** Establishing prognostic biomarkers for three features (EME1, WNT4, SHISA2) identified in the in-house dataset using LASSO regression model. **(D, E)** In the in-house and GEO cohorts, low-risk group patients had a favorable PFS rate as opposed to those in the high-risk group formula. **(F, G)** The Receiver Operating Characteristic (ROC) curve for the 1-year and 3-year survival rates of in-house and GEO cohorts. **(H)** The ROC curve of clinical factors such as gender, age, stage, and risk score suggests that risk score has higher accuracy.

(Figure 5B), similar results were observed in many immune-related indices. In the low-risk patient group, the tumor enhanced immune cell activation and robust ligand-receptor interactions, providing the biological foundation for their favorable response to immunotherapy. There were notable differences in chemokine receptors and MHC molecules between the high- and low-risk groups. Specifically, receptors like CCR9, CCR3, and CXCR6 showed increased expression in the low-risk group, while the majority of MHC class II molecules exhibited decreased expression in the high-risk group, indicating a diminished capacity for antigen presentation and processing (Figures 5C, D). Figure 5E illustrates that, using the TIDE algorithm to evaluate nasopharyngeal cancer patients' responsiveness to immunotherapy, the low-risk group experienced greater benefits from the treatment. Likewise, a uniform trend was

seen in the group of patients undergoing immunotherapy for non-small cell lung cancer, with those in the low-risk category showing a stronger immune response (Figure 5F). The ips score also suggests this result (Figure 5G). To sum up, individuals classified as high-risk showed fewer advantages from immunotherapy and faced a poorer prognosis than those categorized as low-risk.

3.6 Single-cell analysis of immune environment and cell interactions

In order to clarify the function of MRS within the immune microenvironment, we employed the single-sample gene set enrichment analysis (ssGSEA) technique to determine the risk

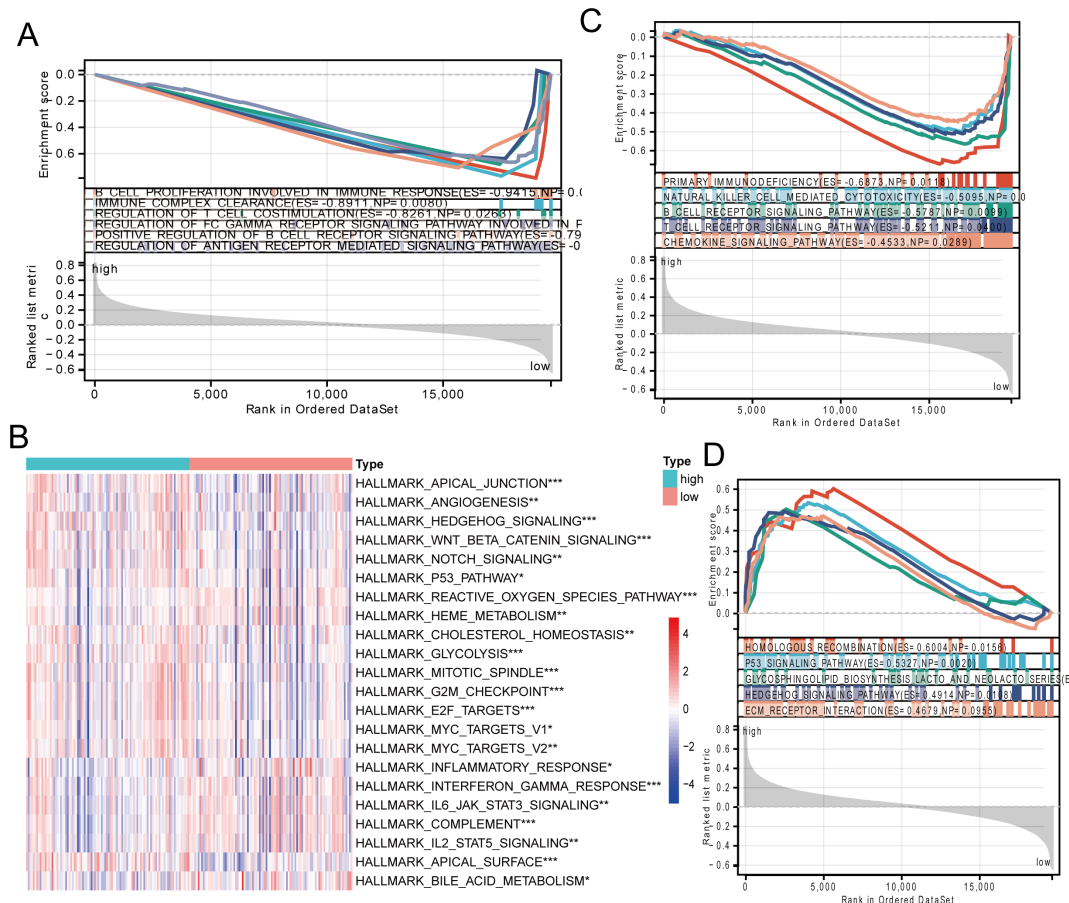


FIGURE 3

Signaling pathway enrichment analysis of risk models. (A) GO enrichment analysis of the low-risk group. (B) Heatmap showing HALLMARK pathway differences between high-risk and low-risk groups. (C, D) KEGG enrichment analysis in the low-risk group and high-risk group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

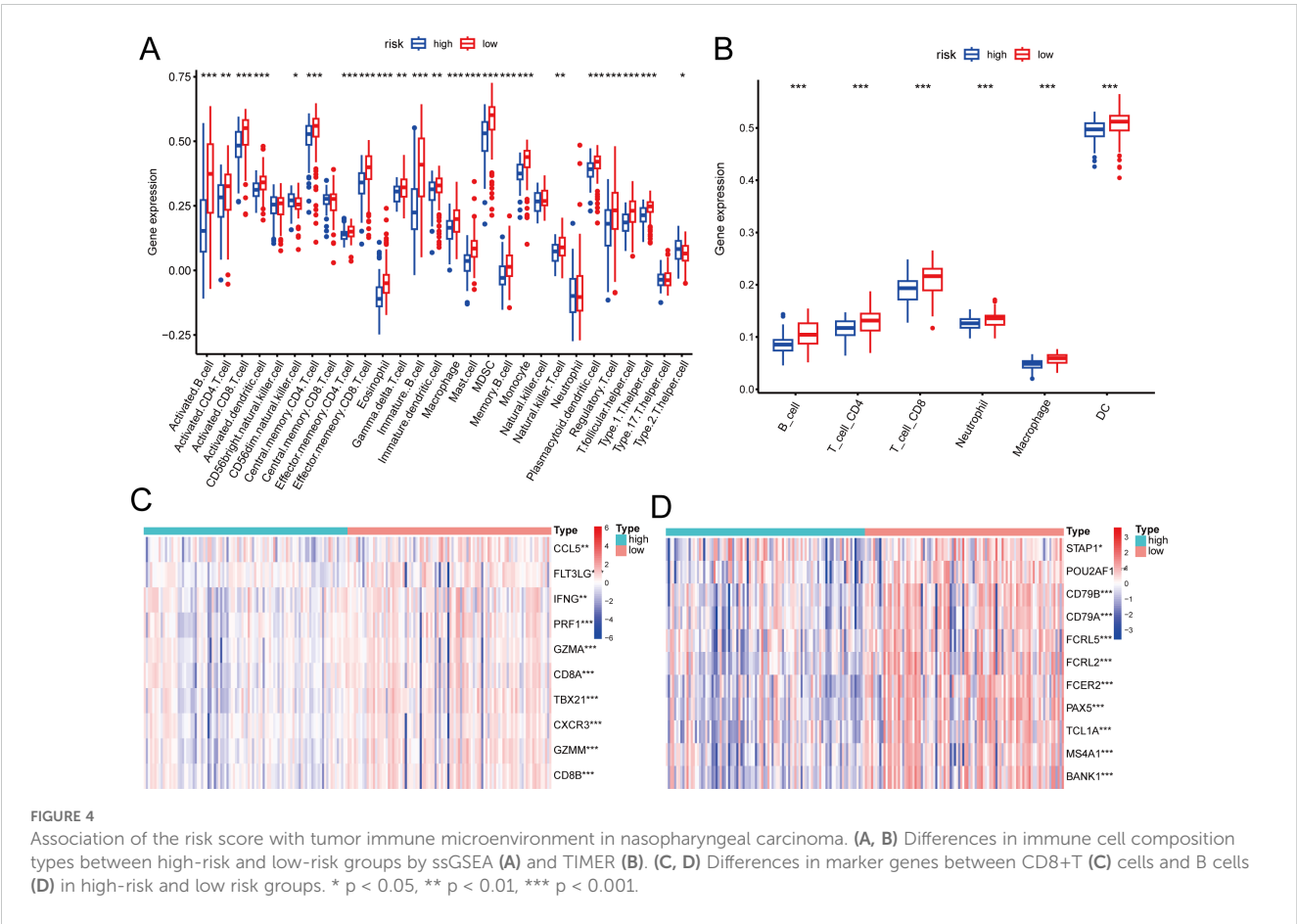
score for each cell from GSE150430 (23). The findings indicated that in cancerous tissues, cells with greater malignancy exhibited elevated risk scores (Figure 6A). Based on median risk values, the samples were divided into high and low-risk categories. Low-risk samples exhibited a notably higher proportion of B cells and CD8 T cells compared to high-risk samples, which had a significantly greater percentage of malignant cells (Figure 6B). We then mapped the cell types of the single-cell dataset to in-house cohort and the GSE102349 cohort by the CIBERSORTX method. Predictably, cancerous cells showed elevated scores in the high-risk category in both the GEO database and transcriptome sequencing results, whereas CD8+ T cells and B cells were more abundant in the low-risk category (Figures 6C, D). These findings are consistent with previous studies indicating that higher risk scores predict poorer biological behaviors, and that low-risk scores correlate with a greater abundance of immune cells.

Subsequently, we conducted a functional analysis. The primary routes enriched with differential genes in both high-risk and low-risk categories were associated with cell adhesion and immune cell activation, indicating variations in response and immune resistance to distant metastasis between these groups (Figure 6E).

Furthermore, the cellular signaling varied between the high-risk and low-risk groups. In the high-risk group, pathways such as CD70, SEMA3, FGF, KIT, BAG, and SPP1 were active, whereas in the low-risk group, pathways like LT, TNF, GRN, CSF, ncWNT, CHEMERIN, and CALCR were active (Figures 6F, G). Figure 6H illustrated the SPP1 in the high-risk category and the LT pathways in the low-risk category.

3.7 m6A methylation gene-related regulatory proteins

Correlation analysis of the three hub genes with m6A regulatory proteins in the GEO database and 192 cases of transcriptome sequencing revealed that EME1, WNT4, and SHISA2 were strongly correlated with most of the m6A modification regulators (Figures 7A, B). Subsequently, to verify whether the hub genes were methylated in nasopharyngeal carcinoma, we performed m6A methylation PCR on the three genes, and the results suggested that all three hub genes had high methylation levels in HK1 nasopharyngeal carcinoma cells (Figure 7C).



4 Discussion

This research underscores the crucial influence of m6A modifications on NPC tumor outcomes and the immune microenvironment, laying the groundwork for possible treatment approaches. Utilizing m6A and transcriptome sequencing, we identified three key prognostic genes (EME1, WNT4, SHISA2) with notable correlations, and developed an immune-related risk model for NPC. This model effectively forecasted progression-free survival in NPC and showed a strong connection with immune infiltration at both the transcriptome and single-cell levels.

In recent years, the exploration of methylation changes and the tumor immune microenvironment has become a prominent research area. RNA methylation is essential for maintaining internal balance and altering the metabolic landscape of the tumor microenvironment (TME), thereby influencing immune cell activity. One of the most prevalent RNA modifications is m6A methylation. m6A RNA methylation has been found to have multiple biological regulatory functions in cancer development and progression by regulating tumor immunity (7, 24, 25). Our research revealed that the m6A-based prognostic model for nasopharyngeal carcinoma risk showed a notable disparity in the immune microenvironment between high-risk and low-risk categories. The low-risk group exhibited a significant enrichment in various immune-regulatory pathways and demonstrated greater immune cell infiltration, particularly with B-cells and CD8+ T-cells, compared to the high-risk group. This indicates that individuals

with low-risk ratings exhibit a heightened immune activity within the tumor's surroundings, potentially leading to improved prognosis and therapeutic results.

Drugs that focus on PD-L1 and CTLA-4 are becoming more crucial in cancer therapy. The therapeutic impact of immune checkpoint inhibitors is directly influenced by the expression levels of PD-L1 or other immune checkpoints, thereby informing their clinical use. TLS is a lymphoid-like formation that typically develops in inflamed tissues. Recent research has indicated that tumor-infiltrating B cells and tumor-associated tertiary lymphoid structures are strongly linked to the effectiveness of immune checkpoint inhibitor treatments, offering new biomarkers for clinical decisions in immunotherapy. Our findings revealed that the low-risk group exhibited a higher count of memory B lymphocytes and elevated immune checkpoint expression, suggesting a higher likelihood of benefiting from immunotherapy. The precision of risk model forecasts was likewise confirmed across various immunotherapy groups. Beyond the topics covered here, further research is needed to explore the role of m6A methylation in various immune and immune-related cells, as well as its regulation in diverse biological processes and functions, such as metabolism, within immune cells, cancer cells, other stromal cells, and non-cellular components of the tumor microenvironment. This will help to fully understand the intricate regulatory network of m6A modifications and offer valuable insights for developing new immunotherapy approaches (26).

Three hub genes (EME1, WNT4, SHISA2) show strong correlation with m6A regulators and elevated levels of

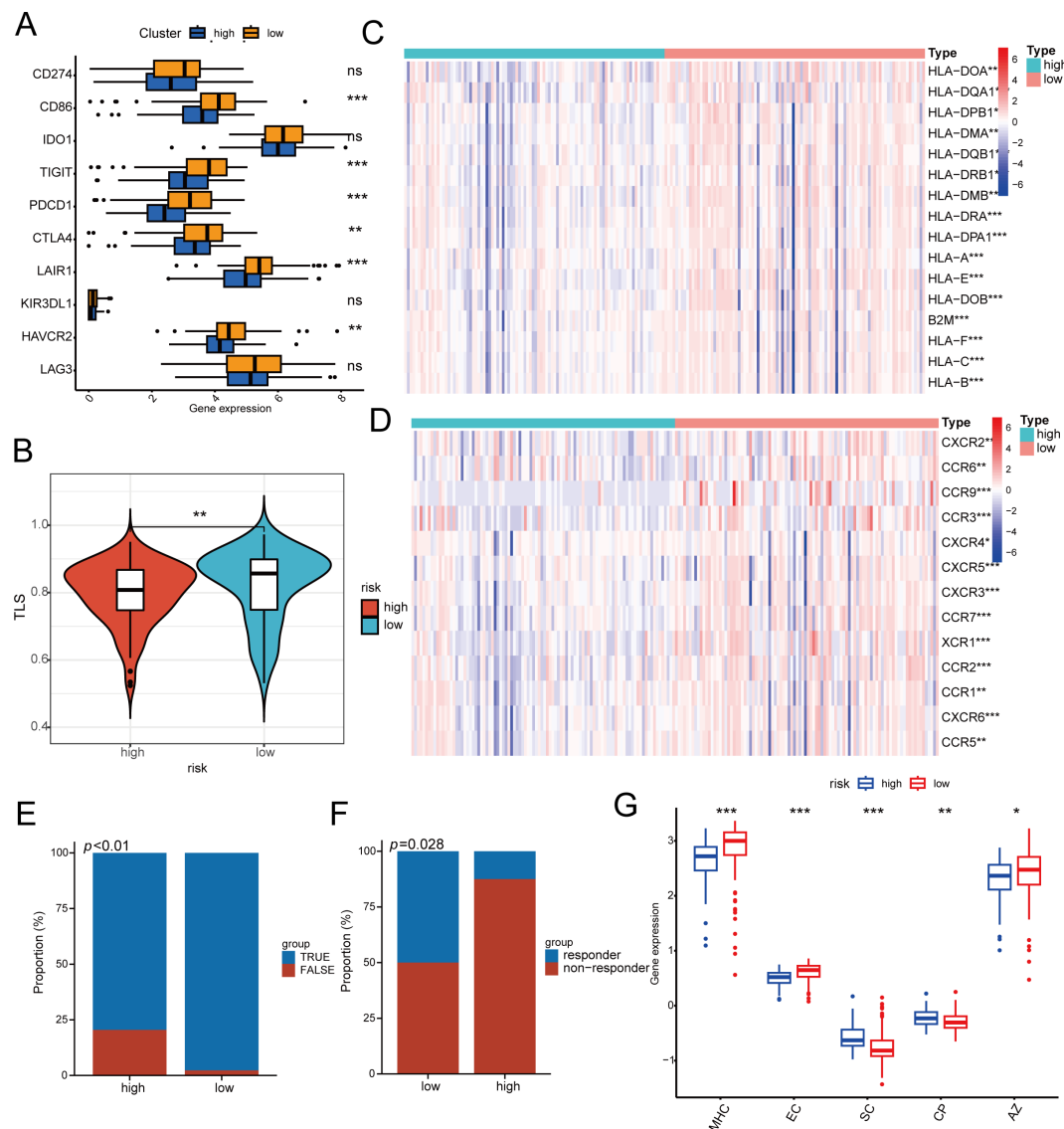


FIGURE 5

The response of immunotherapy of low- and high-risk groups. **(A)** The relationship between risk score and 10 inhibitory immune checkpoints. **(B)** Differences in TLS between high- and high-risk groups. **(C, D)** Differential Expression of Immune Cell Regulators and MHC in High and Low Risk Groups. **(E, F)** Patients in the low-risk group had higher immune responses in the cohorts of patients with nasopharyngeal carcinoma **(E)** and non-small cell lung cancer **(F)**. **(G)** Difference between low- and high-risk groups at ips score. MHC MHC molecules, EC effector cells, SC suppressor cells, CP immune checkpoints, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

methylation modifications in nasopharyngeal carcinoma tissues. These genes play important roles in a variety of cancers, such as EME1 interacts with Mus81 to form a structure-specific nucleic acid endonuclease that maintains genome stability in mammalian cells (27) and is involved in regulating the development of cancers such as gastric cancer and breast cancer (28, 29), Wnt family member 4 (WNT4) is involved in regulating the progression of cancers such as gastric cancer and germline tumors (30, 31), SHISA2 is highly expressed in high-grade prostate (32). Nonetheless, the potential of these three genes with m6A modifications and their regulatory elements as biomarkers for diagnosing and predicting nasopharyngeal carcinoma, along with their specificity and sensitivity, still requires investigation (33).

Although we constructed a prognostic model for MRGs and provided novel insights to improve nasopharyngeal carcinoma management, this study has several limitations. Initially, additional research is required to confirm these results in broader and more varied patient groups, as well as to investigate the interplay between m6A modifications and other epigenetic elements. Understanding how m6A modifications interact with genetic, environmental, and viral factors in NPC could provide a more comprehensive picture of the disease and inform more effective prevention and treatment strategies. Moreover, additional immunological studies are required to investigate the possible mechanisms of the three key genes within the immune microenvironment of NPC.

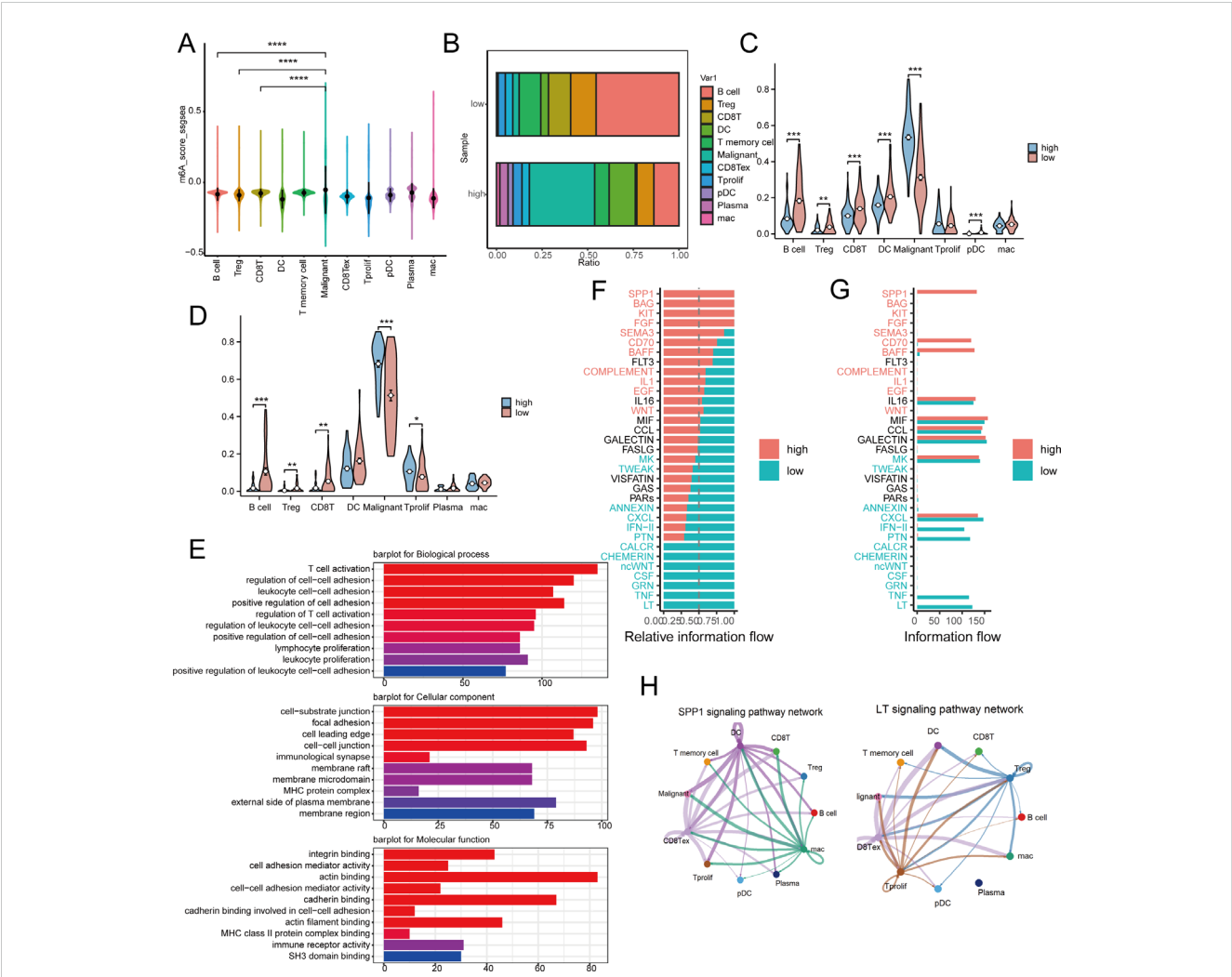


FIGURE 6 Risk model differences in immune landscapes and cellular communication at the single-cell level. (A) Risk scores for 11 different cell subgroup samples in the GSE150430 dataset. (B) The proportion of immune cell composition between high-risk and low-risk groups. (C, D) Detect immune cell infiltration in high-risk and low-risk groups in inhouse (C) and GEO (D) cohorts by CIBERSORTx tool. (E) The main pathways for accumulating differentially expressed genes between high-risk and low-risk populations. (F, G) Observing differences in active pathways between high-risk and low-risk groups. (H) SPP1 and LT signaling pathways in high-risk and low-risk groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

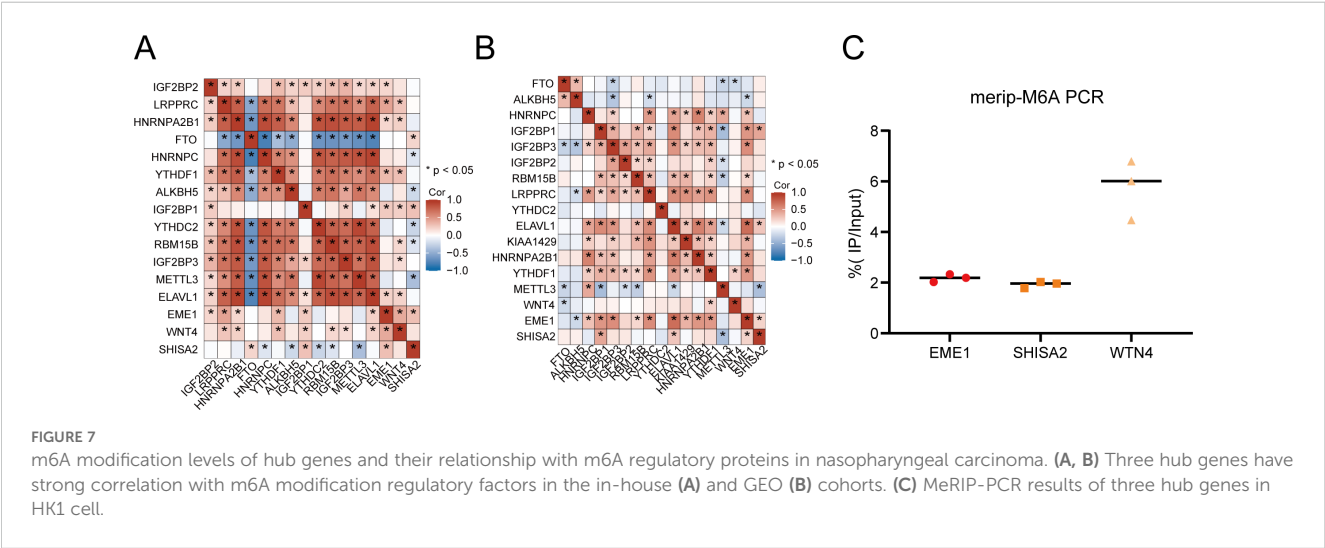


FIGURE 7 m6A modification levels of hub genes and their relationship with m6A regulatory proteins in nasopharyngeal carcinoma. (A, B) Three hub genes have strong correlation with m6A modification regulatory factors in the in-house (A) and GEO (B) cohorts. (C) MerIP-PCR results of three hub genes in HK1 cell.

5 Conclusions

In summary, this research underscores the crucial impact of m6A alterations on the prognosis of nasopharyngeal carcinoma and the immune environment. By establishing a risk-based prognostic model based on m6A modification genes, the study provides a valuable tool for predicting patient prognosis and tailoring therapeutic strategies. The distinct immune landscapes and pathway enrichments between high- and low-risk groups underscore the critical role of m6A modifications in NPC progression and treatment efficacy. These insights enhance our comprehension of NPC and open avenues for future studies and innovative therapies.

Data availability statement

The Partial datasets generated and analyzed during the current study are available in the GEO repository under accession number GSE102349 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102349>), GSE126044 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126044>) and GSE150430 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150430>). All original contributions discussed in this study are included in the article and its Supplementary Material. Additional information will be provided by the authors upon request, without any undue restrictions.

Ethics statement

The studies involving humans were approved by The Ethics Committees of both Fujian Cancer Hospital and Fujian Medical University Cancer Hospital granted approval for the research (approval code SQ2019-035-01). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

XCC: Conceptualization, Writing – original draft. WX: Writing – original draft. JP: Data curation, Writing – review & editing. HY: Visualization, Writing – review & editing. YL: Validation, Writing – review & editing. XC: Formal analysis, Writing – review & editing. YS: Project administration, Writing – review & editing. QL: Project

administration, Writing – review & editing. SQ: Funding acquisition, Project administration, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by Fujian Provincial Clinical Research Center for Cancer Radiotherapy and Immunotherapy (2020Y2012); Supported by the National Clinical Key Specialty Construction Program (2021); Fujian Clinical Research Center for Radiation and Therapy of Digestive, Respiratory and Genitourinary Malignancies; National Natural Science Foundation of China (82072986); Major Research Projects for Young and Middle-aged Researchers of Fujian Provincial Health Commission (2021ZQNZD010); Joint Funds for the innovation of science and Technology, Fujian province (2021Y9196); the Qihang Funds of Fujian Medical University (2022QH2049, 2023QH1159).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1492648/full#supplementary-material>

References

- Chua MLK, Wee JTS, Hui EP, Chan ATC. Nasopharyngeal carcinoma. *Lancet*. (2016) 387:1012–24. doi: 10.1016/S0140-6736(15)00055-0
- Li Y, Xiao J, Bai J, Tian Y, Qu Y, Chen X, et al. Molecular characterization and clinical relevance of m6A regulators across 33 cancer types. *Mol Cancer*. (2019) 18:137. doi: 10.1186/s12943-019-1066-3
- Han D, Liu J, Chen C, Dong L, Liu Y, Chang R, et al. Anti-tumour immunity controlled through mRNA m6A methylation and YTHDF1 in dendritic cells. *Nature*. (2019) 566:270–4. doi: 10.1038/s41586-019-0916-x
- Lin Z, Niu Y, Wan A, Chen D, Liang H, Chen X, et al. RNA m(6) A methylation regulates sorafenib resistance in liver cancer through FOXO3-mediated autophagy. *EMBO J*. (2020) 39:e103181. doi: 10.15252/embj.2019103181
- Wang L, Hui H, Agrawal K, Kang Y, Li N, Tang R, et al. m(6) A RNA methyltransferases METTL3/14 regulate immune responses to anti-PD-1 therapy. *EMBO J*. (2020) 39:e104514. doi: 10.15252/embj.2020104514
- Xu S, Tang L, Dai G, Luo C, Liu Z. Expression of m6A regulators correlated with immune microenvironment predicts therapeutic efficacy and prognosis in gliomas. *Front Cell Dev Biol*. (2020) 8:594112. doi: 10.3389/fcell.2020.594112

7. Li N, Kang Y, Wang L, Huff S, Tang R, Hui H, et al. ALKBH5 regulates anti-PD-1 therapy response by modulating lactate and suppressive immune cell accumulation in tumor microenvironment. *Proc Natl Acad Sci U.S.A.* (2020) 117:20159–70. doi: 10.1073/pnas.1918986117
8. Zheng ZQ, Li ZX, Zhou GQ, Lin L, Zhang LL, Lv JW, et al. Long Noncoding RNA FAM225A Promotes Nasopharyngeal Carcinoma Tumorigenesis and Metastasis by Acting as ceRNA to Sponge miR-590-3p/miR-1275 and Upregulate ITGB3. *Cancer Res.* (2019) 79:4612–26. doi: 10.1158/0008-5472.Can-19-0799
9. Li ZX, Zheng ZQ, Yang PY, Lin L, Zhou GQ, Lv JW, et al. WTAP-mediated m(6) A modification of lncRNA DIAPH1-AS1 enhances its stability to facilitate nasopharyngeal carcinoma growth and metastasis. *Cell Death Differ.* (2022) 29:1137–51. doi: 10.1038/s41418-021-00905-w
10. Zhao BS, Roundtree IA, He C. Post-transcriptional gene regulation by mRNA modifications. *Nat Rev Mol Cell Biol.* (2017) 18:31–42. doi: 10.1038/nrm.2016.132
11. Zhu H, Jia X, Wang Y, Song Z, Wang N, Yang Y, et al. M6A classification combined with tumor microenvironment immune characteristics analysis of bladder cancer. *Front Oncol.* (2021) 11:714267. doi: 10.3389/fonc.2021.714267
12. Zhang B, Wu Q, Li B, Wang D, Wang L, Zhou YL. m(6)A regulator-mediated methylation modification patterns and tumor microenvironment infiltration characterization in gastric cancer. *Mol Cancer.* (2020) 19:53. doi: 10.1186/s12943-020-01170-0
13. Li M, Zha X, Wang S. The role of N6-methyladenosine mRNA in the tumor microenvironment. *Biochim Biophys Acta Rev Cancer.* (2021) 1875:188522. doi: 10.1016/j.bbcan.2021.188522
14. Quan C, Belaydi O, Hu J, Li H, Yu A, Liu P, et al. N(6)-methyladenosine in cancer immunotherapy: an undervalued therapeutic target. *Front Immunol.* (2021) 12:697026. doi: 10.3389/fimmu.2021.697026
15. Singer J, Irmisch A, Ruscheweyh HJ, Singer F, Toussaint NC, Levesque MP, et al. Bioinformatics for precision oncology. *Brief Bioinform.* (2019) 20:778–88. doi: 10.1093/bib/bbx143
16. Li T, Fu J, Zeng Z, Cohen D, Li J, Chen Q, et al. TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic Acids Res.* (2020) 48:W509–w514. doi: 10.1093/nar/gkaa407
17. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods.* (2015) 12:453–7. doi: 10.1038/nmeth.3337
18. Yoshihara K, Shahmoradgoli M, Martínez E, Vegesna R, Kim H, Torres-Garcia W, et al. Inferring tumour purity and stromal and immune cell admixture from expression data. *Nat Commun.* (2013) 4:2612. doi: 10.1038/ncomms3612
19. Hu J, Yu A, Othmane B, Qiu D, Li H, Li C, et al. Siglec15 shapes a non-inflamed tumor microenvironment and predicts the molecular subtype in bladder cancer. *Theranostics.* (2021) 11:3089–108. doi: 10.7150/thno.53649
20. Ott PA, Bang YJ, Piha-Paul SA, Razak ARA, Bennouna J, Soria JC, et al. T-cell-inflamed gene-expression profile, programmed death ligand 1 expression, and tumor mutational burden predict efficacy in patients treated with pembrolizumab across 20 cancers: KEYNOTE-028. *J Clin Oncol.* (2019) 37:318–27. doi: 10.1200/jco.2018.78.2276
21. Bergomas F, Grizzi F, Doni A, Pesce S, Laghi L, Allavena P, et al. Tertiary intratumor lymphoid tissue in colo-rectal cancer. *Cancers (Basel).* (2011) 4:1–10. doi: 10.3390/cancers4010001
22. Lin WW, Xu LT, Chen YS, Go K, Sun C, Zhu YJ. Single-cell transcriptomics-based study of transcriptional regulatory features in the mouse brain vasculature. *BioMed Res Int.* (2021) 2021:7643209. doi: 10.1155/2021/7643209
23. Chen X, Ding Q, Lin T, Sun Y, Huang Z, Li Y, et al. An immune-related prognostic model predicts neoplasm-immunity interactions for metastatic nasopharyngeal carcinoma. *Front Immunol.* (2023) 14:1109503. doi: 10.3389/fimmu.2023.1109503
24. Galon J, Bruni D. Tumor immunology and tumor evolution: intertwined histories. *Immunity.* (2020) 52:55–81. doi: 10.1016/j.immuni.2019.12.018
25. Tang L, Wei X, Li T, Chen Y, Dai Z, Lu C, et al. Emerging perspectives of RNA N (6)-methyladenosine (m(6)A) modification on immunity and autoimmune diseases. *Front Immunol.* (2021) 12:630358. doi: 10.3389/fimmu.2021.630358
26. Guo L, Yang H, Zhou C, Shi Y, Huang L, Zhang J. N6-methyladenosine RNA modification in the tumor immune microenvironment: novel implications for immunotherapy. *Front Immunol.* (2021) 12. doi: 10.3389/fimmu.2021.773570
27. Xu Y, Li J, Zhu K, Zeng Y, Chen J, Dong X, et al. FIBP interacts with transcription factor STAT3 to induce EME1 expression and drive radioresistance in lung adenocarcinoma. *Int J Biol Sci.* (2023) 19:3816–29. doi: 10.7150/ijbs.83134
28. Guo Z, Liang E, Li W, Jiang L, Zhi F. Essential meiotic structure-specific endonuclease1 (EME1) promotes Malignant features in gastric cancer cells via the Akt/GSK3B/CCND1 pathway. *Bioengineered.* (2022) 12:9869–84. doi: 10.1080/21655979.2021.1999371
29. Vajen B, Bhowmick R, Greiwe L, Schäffer V, Eilers M, Reinkens T, et al. MicroRNA-449a inhibits triple negative breast cancer by disturbing DNA repair and chromatid separation. *Int J Mol Sci.* (2022) 23:5131. doi: 10.3390/ijms23095131
30. Pavličev M, McDonough-Goldstein CE, Zupan AM, Muglia L, Hu YC, Kong F, et al. A common allele increases endometrial Wnt4 expression, with antagonistic implications for pregnancy, reproductive cancers, and endometriosis. *Nat Commun.* (2024) 15:1152. doi: 10.1038/s41467-024-45338-4
31. Qian Z, Lin W, Cai X, Wu J, Ke K, Ye Z, et al. WYC-209 inhibited GC Malignant progression by down-regulating WNT4 through RARα. *Cancer Biol Ther.* (2024) 25:2299288. doi: 10.1080/15384047.2023.2299288
32. Tamura K, Furihata M, Satake H, Hashida H, Kawada C, Osakabe H, et al. SHISA2 enhances the aggressive phenotype in prostate cancer through the regulation of WNT5A expression. *Oncol Lett.* (2017) 14:6650–8. doi: 10.3892/ol.2017.7099
33. Liu ZX, Li LM, Sun HL, Liu SM. Link between m6A modification and cancers. *Front Bioeng Biotechnol.* (2018) 6:89. doi: 10.3389/fbioe.2018.00089



OPEN ACCESS

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RECEIVED 02 September 2024

ACCEPTED 25 November 2024

PUBLISHED 07 January 2025

CITATION

Tzaban S, Stern O, Zisman E, Eisenberg G,
Klein S, Frankenburg S and Lotem M (2025)
Alternative splicing of modulatory immune
receptors in T lymphocytes: a newly identified
and targetable mechanism for anticancer
immunotherapy.
Front. Immunol. 15:1490035.
doi: 10.3389/fimmu.2024.1490035

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Alternative splicing of modulatory immune receptors in T lymphocytes: a newly identified and targetable mechanism for anticancer immunotherapy

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Alternative splicing (AS) is a mechanism that generates translational diversity within a genome. Equally important is the dynamic adaptability of the splicing machinery, which can give preference to one isoform over others encoded by a single gene. These isoform preferences change in response to the cell's state and function. Particularly significant is the impact of physiological alternative splicing in T lymphocytes, where specific isoforms can enhance or reduce the cells' reactivity to stimuli. This process makes splicing isoforms defining features of cell states, exemplified by CD45 splice isoforms, which characterize the transition from naïve to memory states. Two developments have accelerated the use of AS dynamics for therapeutic interventions: advancements in long-read RNA sequencing and progress in nucleic acid chemical modifications. Improved oligonucleotide stability has enabled their use in directing splicing to specific sites or modifying sequences to enhance or silence particular splicing events. This review highlights immune regulatory splicing patterns with potential significance for enhancing anticancer immunotherapy.

KEYWORDS

alternative splicing, cancer, immunotherapy, T lymphocytes, immune receptors

Introduction

Biologists have long been puzzling how the human genome, which bears considerable similarity to lower eukaryotes, is responsible for the complex, sophisticated organisms it creates. Following Sharp and Roberts' description of RNA splicing, Gilbert, in 1978, hypothesized that alternative splicing (AS) might be the missing layer that leads to the immense protein diversity despite the only 23000-gene human genome.

RNA splicing is a “cut and paste” process, removing introns and rejoining exons from the primary gene transcript, the pre-mRNA. The process relies on the biochemical uniqueness of RNA, which DNA lacks, of extensive flexibility and intrinsic catalytic activity. Small nuclear RNAs that assemble sequentially are directed to conserved sequences in the 5'(GT) and 3'(AG) splice sites on the primary transcript in an orderly manner. Together, the small RNAs and numerous proteins form the spliceosome. An adenosine in the intronic segment performs a nucleophilic attack on the 5' end, cleaving the 5' nucleotide (generally the “G” in a GT); a loop is then formed and removed. Following, the exon upstream of the removed intron is ligated to the 5' end catalyzed by the spliceosomal RNAs and the ribonuclear proteins (RNPs).

Alternative splicing produces variants that differ from the constitutive RNA transcript. It occurs parallel to the transcription process and produces several isoforms from one gene. Each isoform may lack an exon or part of an exon from either side of the constitutive exon. This pattern, called ‘cassette-type alternative exon’ or ‘exon skipping’, is the most common. Intron retention, uncommon in humans, occurs mainly in untranslated regions. See [Figure 1](#) for common splicing patterns.

AS involves 95% of the genes (1). With deep RNA sequencing becoming a more common read-out in experimental systems and longer RNA reads being produced, it is now clear that the pattern of RNA splicing is dynamically regulated and constantly changes (2).

The ratio between a constitutive transcript and its alternatively spliced isoforms depends on splice site recognition, its occupancy by spliceosomal RNPs, and regulatory RNA binding proteins (RBPs) (3). These RBPs bind or complement *cis*-sequences on the pre-mRNA transcript on the intron or the exon. Sequences that promote spliceosome assembly at a splice site are called enhancers, and their RBPs are usually serine arginine-rich proteins. Sequences that reduce splice site recognition (silencers) attract heterologous nuclear RNPs (hnRNPs) (4). It is thought that enhancers usually act to generate constitutive mRNA, while the silencers yield AS

Exon skipping



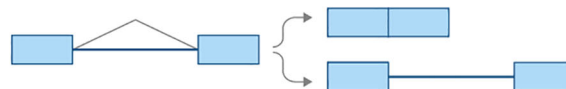
Alternative 5'ss



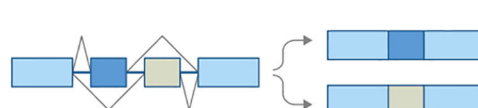
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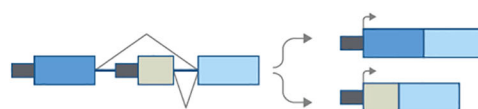
Intron retention



Mutually exclusive exons



Alternative promoters



Alternative polyadenylation

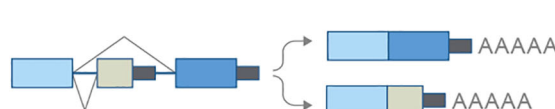


FIGURE 1
Alternative splicing patterns.

isoforms. However, how the basic mechanism of AS varies from cell to cell and what determines it remains to be further elucidated.

Here, we focus on the role of AS in the T-cell immune response, particularly on anticancer immunity. This review aims to draw attention to new therapeutic opportunities in the functional distinctions between a constitutive protein/receptor and its splice isoforms. The motivation to unveil the intriguing mechanisms by which AS amplifies and regulates immune functions relies on reports from our group and others that RNA transcripts of the same immune gene can act in different directions or magnitudes in the immune context (5, 6). Thus, AS is an essential layer of immune regulation and a potential therapeutic target.

Alternative splicing is a mechanism of dynamic adaptability

Splicing event regulation

Although the prime outcome of AS is the fold increase in functionally distinct proteins compared to the number of genes, AS also plays a significant role in the most fundamental biological processes: evolution, differentiation, and adaptation. AS is a source of evolutionary development, a determinant of organ, tissue, and cell characteristics, and part of cellular adaptation to a changing environment (2).

The concerted manner by which protein production is shifted from one isoform to another yields the regulatory characteristics of AS. Its preferences differ among tissues and developmental states and respond to extracellular signals in a dynamic manner that precedes or synchronizes with gene transcription. In parallel to the dependency of intracellular processes on transcriptional activation, cellular events emerge from the shift in protein isoform ratios. How splicing events are concerted and what network cascades occur is a field of active research emphasizing health disorders and malignancies (7). The regulation of splicing events depends on both cis-acting regulatory sequences, located in introns or exons, and trans-acting splicing factor proteins that can strengthen or weaken the spliceosome's recognition of the splice sites (8). These regulatory proteins belong to families of RNA-binding proteins, such as arginine-serine-rich (SR), heterogeneous nuclear ribonucleoprotein (hnRNP), and RNA-binding motif (RBM) proteins (9). They recognize specific regulatory sequences and enhance or inhibit the recognition of neighboring splice sites by the core splicing machinery (7). The expression level of the regulatory proteins is tissue- and state-specific (10), and they are subjected to regulatory splicing themselves (11).

From the evolutionary point of view, alternative splicing varies significantly among species. The insertion of multiple introns that separate exons has derived from ancestral genes and predated AS in eukaryote development. The option to skip exons was enabled by DNA mutations that may have resulted in splice sites with weaker binding affinity for spliceosomal components such as U1 small nuclear ribonucleoprotein (snRNP) (12). While the emergence of alternative splice sites contributed to protein diversity and is partly unique to a species, particularly when changing the reading frame,

comparative genomics indicates that sequences that regulate RNA binding proteins are conserved and shared (13, 14).

Over 200,000 identified isoforms are reported in genome databases, and the majority of them lack functional annotation. Some well-studied examples show how events of retention or exclusion of specific domains may change protein cell localization, membrane anchorage, shedding of ectodomains, mRNA stability, and translational efficiency. The molecular alterations that emerge from AS may occur without any change in the level of the general gene's transcript or before a change (15). Furthermore, the translational changes in reading frames may produce diverse translation outputs (13) and even insert poison exons, resulting in nonsense-mediated mRNA decay (NMD) and diminished protein levels (16). We can conclude that alternative splicing is timed and regulated in a manner that is not necessarily dependent on active simultaneous gene transcription.

Alternative splicing in T lymphocytes

T-cell states are associated with preferential expression of specific splicing isoforms. A unique characteristic of T lymphocytes is that they transform within minutes from a stationary naïve or inactive state to intense activity. In their effector state, T cells must adapt to synthesize large amounts of cytokines, migrate, proliferate, lyse target cells, and address accelerated metabolic needs. Already in 2006, it was found that memory T-cells respond to antigenic stimuli faster than naïve cells by omitting exons 4, 5, and 6 from the extracellular part of the membrane phosphatase CD45. CD45 is expressed in T and B cells and, in its constitutive, full-exon inclusive state, is referred to as CD45RA. The CD45RO variant shows variable exclusion of exons 4, 5, and 6. CD45 dephosphorylates both inhibitory and costimulatory tyrosines of the Src-family kinases (17). Oberdoerffer et al. showed that the transition from the RA to the RO form depends on the activity of the splicing factor hnRNPL (heterogeneous ribonucleoprotein L-like) (18). HnRNAPLL was suggested to be a master regulator in activated T cells, affecting not only CD45.

Before and in parallel to CD45, specific gene isoforms impacting T-cell function were discovered. Interesting events recorded in activated T-cells included the short isoform of CD28, which induces faster activation (19), splice variants of CD44 and CTLA4, which correlated with a higher risk of autoimmune disease (20, 21), and MALT1A, a paraprotease that integrates TCR activation with the downstream IKK/NF- κ B pathway. Reminiscent of CD45, naïve T-cells express MALT1B, a splice isoform missing exon 7, while activated T-cells express MALT1A, which includes exon 7 and is associated with rapid NF- κ B signaling and improved lymphocyte function (22, 23).

A landscape view of AS in immune cells was offered by Lynch et al. in 2004, preceding a complete landscape of the comprehensive gene involvement in this phenomenon (22). Although the list of spliced genes described to regulate lymphocyte activation was restricted, the diverse array of functions governed by splicing suggested the substantial ubiquity of this process (23).

In the last few years, analyses have focused on gene families and activation cascades as it becomes clear that AS affects most genes. An example is the production of anti-apoptotic splice isoforms of members of the BCL2 gene family in activated T-cells. Adding costimulation via CD28 increased the ratio of anti-apoptotic splice variants and augmented T-cell proliferation. Interestingly, the genes that displayed significant changes in their splice isoform ratios did not have the highest expression levels (24).

The concept of AS-induced effector transition is not limited to activated T lymphocytes but also plays a critical role in B cell affinity maturation. In these processes, poly-pyrimidine tract binding proteins PTBP1 and PTB3 are splicing factors that drive the appropriate expression of gene sets required to adapt B lymphocytes to antibody-producing cells (25).

Splicing events that generate soluble isoforms of immune receptors

A prevalent splicing pattern observed in immune receptors gives rise to soluble isoforms that lack membrane anchorage and are secreted into the extracellular space. These soluble receptors may regulate signaling cascades, which differ from those initiated by their parental receptor (Table 1). Most prominently, the soluble receptors can function as a decoy of their corresponding ligands and

compete with their constitutive, membrane-bound forms (26–35). The ratio between the membranal and the soluble isoforms of a receptor can remain fixed (26, 36). However, it might change depending on the cell’s metabolic, functional, or differentiation state (37, 38). Diverting the pre-mRNA splicing towards the soluble isoforms results in reduced expression of the membrane-bound receptor and can even negate its cellular effect. In a different context, the soluble receptors may have agonistic effects (28, 39) and initiate reverse signaling by binding to other receptors (40–42). For example, glucocorticoid-induced tumor necrosis factor receptor (GITR) ligand that is expressed on plasmacytoid dendritic cells prompts a reverse signal that initiates noncanonical NF-kappaB-dependent induction of indoleamine 2,3-dioxygenase upon binding to soluble GITR. This leads to the tryptophan catabolism immunoregulatory pathway (43). In addition, soluble isoforms have been documented to bind with their ligand to distinct membranal partners, activating trans-signaling pathways (44, 45). Furthermore, some soluble receptors stabilize their ligand configuration or alter their biodistribution (31, 45–47). Another typical example of important alternative splicing of immune cells is the removal of the hydrophobic transmembranal segment of the B-cell receptor to form a secreted immunoglobulin (48). Interestingly, soluble receptors may exert different functions (i.e., agonistic and antagonistic) depending on their concentration (45, 46, 49) (Figure 2).

TABLE 1 Soluble T-cell immune receptors due to alternative splicing.

Superfamily	Name	Splicing event	Suggested mechanism	Function	References
Immunoglobulin superfamily	BTLA	TMD skipping	Unknown	Increases cellular proliferation	(95, 96)
	CD28	TMD skipping and premature stop codon	Unknown	Inhibits T-cell proliferation induced by anti-CD3 antibodies or by mitogens	(97, 98)
	CD83	TMD skipping	Binds MD2 on monocytes	Inhibits DC-mediated T-cell stimulation, proliferation, and IL-2 secretion	(40, 42, 50, 99)
	CTLA-4	TMD skipping and premature stop codon	Binds B7 on APCs	CTLA-4 agonist, inhibits the immune response	(28, 39, 58)
	ICOS	ICD skipping	Binds ICOSL	Inhibits T-cell proliferation	(29)
	LAG-3	Alternative 5' splice site and premature stop codon	Unknown	Controversial	(47, 100, 101)
	PD-1	TMD skipping	Binds PD-L1 and/or PDL2	Enhances immune cell response: (a) Block PD-1/PD-L1 interaction. (b) Reverse signaling into DC.	(26, 41, 69)

(Continued)

TABLE 1 Continued

Superfamily	Name	Splicing event	Suggested mechanism	Function	References
Interleukin receptors	Common γ chain	TMD skipping and premature stop codon	Binds IL-2R β and IL-7R α	Antagonizes IL-2 and IL-7 signaling	(27)
	IL-1RAcP (co-receptor)	Exon skipping and premature stop codon	Binds IL-1RII and increases its affinity for IL-1 α and IL-1 β	Negative regulation of IL-1 signaling	(32, 38, 102)
	IL-4R α	Exon inclusion and premature stop codon	Binds IL-4	Both neutralizing and stabilizing IL-4	(31, 57, 103)
	IL-6R α	TMD skipping	Binds IL-6	(a) Stabilizes IL-6 (b) sIL-6R α /IL-6 trans-signaling via membranal IL-6ST	(44, 47, 54, 94, 104, 105)
	IL-6ST, gp130 (co-receptor)	Exon skipping and premature stop codon	Bind sIL-6R α /IL-6	Prevents sIL-6R α /IL-6 trans-signaling	(106)
	IL-7R α	TMD skipping	Binds IL-7	(a) Competes with membranal IL7R. (b) Decreases IL-7 early consumption and results in prolonged availability and increased IL-7 bioactivity	(33, 45)
TNFR superfamily	TNFR2	TMD skipping and premature stop codon	Binds TNF	High concentrations inhibit TNF signaling. Low concentrations stabilize TNF trimeric form	(46, 49, 59)
	TNFRSF6, FAS	TMD skipping	Competes with mFAS for FASL binding	Prevents cell death	(30, 52, 53)
	TNFRSF9, 4-1BB	TMD skipping, alternative 3' splice site, and premature stop codon	Competes with mCD137 for CD137L binding	Reduced T-cell proliferation and IL-2 secretion	(34, 35)
	TNFRSF18, GITR	Exon skipping and premature stop codon	Binds GITRL	Reverses signaling via membranal GITRL and proinflammatory effect	(56, 107)
TGF beta receptors	TGF- β Type II Receptor	Alternative 3' and 5' splice site - premature stop codon	Binds TGF- β	Inhibits the canonical TGF- β signaling pathways	(93, 108)

The common splicing patterns that lead to the generation of soluble isoforms of membranal receptors include (1) *Transmembrane domain (TMD) skipping*: In the process of alternative splicing, skipping of the transmembrane encoding exon results in the creation of a soluble product encompassing both the intracellular and extracellular domains (26, 33, 50–55); (2) *Alternative terminator*: a shortened soluble isoform is encoded by a sequence that includes a mutually exclusive exon containing an alternative polyadenylation site, or by alternative splicing that results in frameshift and premature stop codon (48, 56, 57). As a result, the translated proteins include only the extracellular domain, lose their membrane anchorage and become soluble (27, 58, 59).

It should be noted that alternative splicing is not the sole mechanism that creates soluble receptors. These segments can also

be made by proteolytic cleavage of extracellular domains by proteases in the extracellular matrix (Figure 3). However, unlike AS, the intracellular domain (ICD) of a cleaved receptor remains anchored and theoretically may retain its effect. The impact of a truncated signaling domain is diverse or unknown. Typical examples of receptors that utilize both mechanisms to produce their soluble formats are cytokine receptors, including the TNF and TNFR superfamily (59, 60). In addition, some immune receptor genes lack the transmembrane domain and are, therefore, constitutively expressed as soluble receptors with linked intracellular and extracellular domains. They mainly function as decoy receptors (61). For example, decoy receptor 3 (DcR3, TNFRSF6B) is a secreted TNFR superfamily member that lacks a transmembrane domain. DcR3 can interrupt FAS-FASL

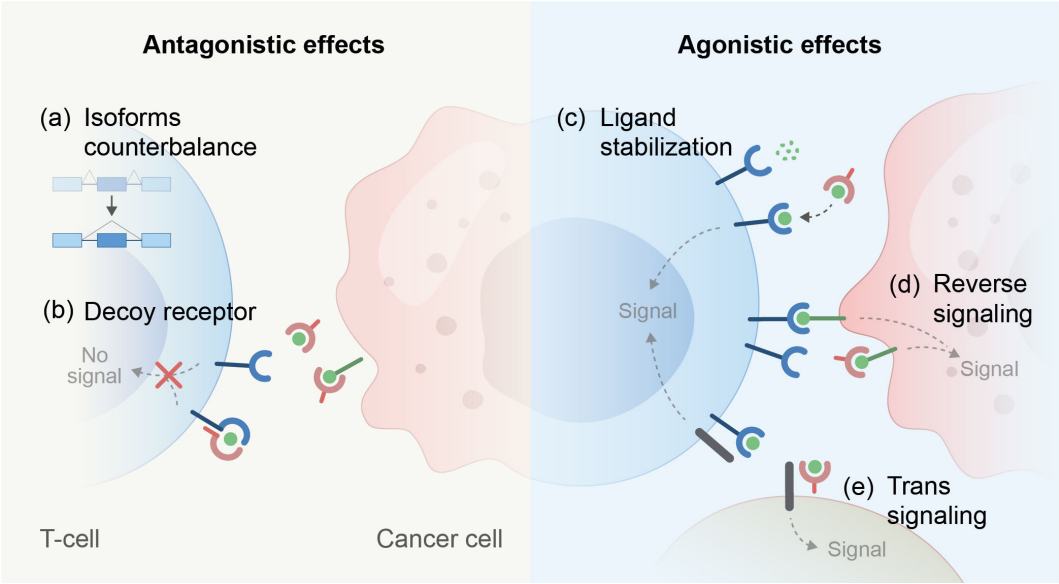


FIGURE 2
Mechanisms that lead to antagonistic or agonistic effects of soluble ectodomains derived from immune regulatory receptors. (A–E, mechanisms depicted).

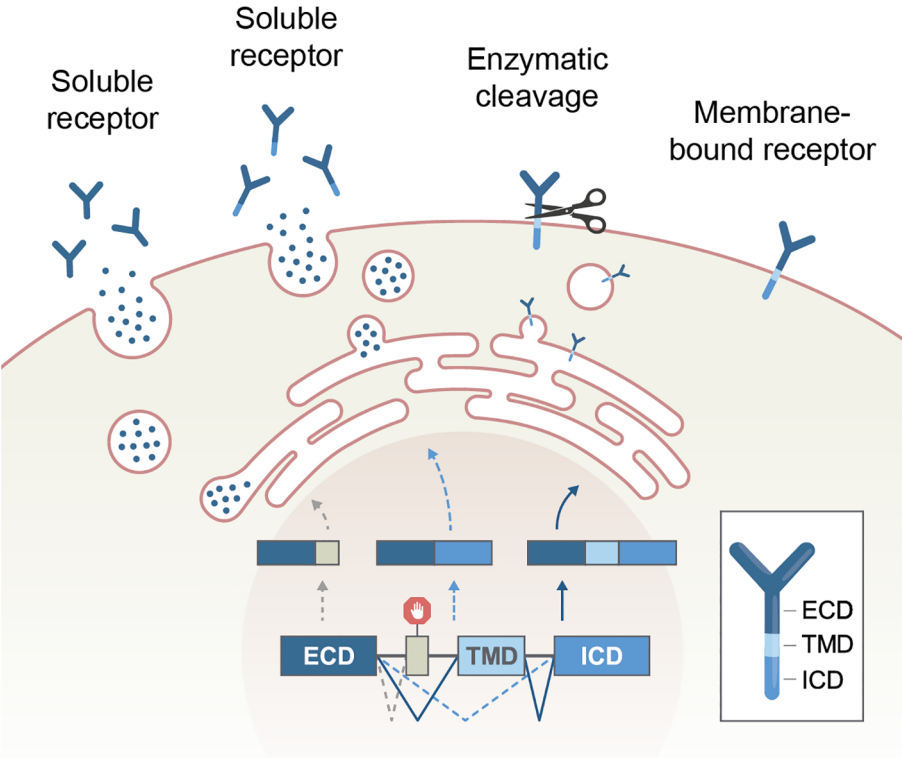


FIGURE 3
Production of soluble receptors by alternative splicing or enzymatic cleavage. Of note, the intracellular part of a receptor remains only in the cleavage process. ECD, extracellular domain, TMD, transmembrane domain, ICD, intracellular domain.

interaction by binding FASL and inhibiting FASL-induced apoptosis (62).

Alternative splicing of the immunoglobulin superfamily

The immunoglobulin (Ig) superfamily is a large group of proteins with a common Ig domain. The Ig superfamily is critical in the immune response networks (63). Some Ig superfamily receptors can be translated to a soluble form by an alternative splicing process (64, 65). Among these are CTLA4 (39, 58), CD83 (42, 66), and LAG3 (66). Here, we will focus on two specific examples: The programmed cell death receptor PD-1 and the B cell receptors (BCRs) that convert, after splicing, into immunoglobulins (antibodies).

PD-1

Following stimulation, PD-1 is expressed on T-cells in a membrane-bound form (mPD-1). When it binds to its ligand (PD-L1), mPD-1 inhibits the effector functions of T-cells, promotes apoptosis, and restricts proliferation (67, 68). PD-1 exon 3, which encodes the transmembrane domain, can be skipped by alternative splicing, generating a soluble receptor form (sPD-1). The ratio between the two isoforms is consistent during T-cell activation (36). sPD-1 can act as a decoy receptor and compete with the PD-1 receptor on the interaction with the ligands PD-L1 and PD-L2, and block the interaction of PD-L1 with B7-1 (69). The shedded PD-1 ectodomain exerts a similar effect, suppressing the PD-1 inhibitory function (70, 71). It has been speculated that sPD-1 has a reverse signaling effect when binding to PD-L1 on dendritic cells (41).

Immunoglobulins can be membrane-bound or secreted as antibodies. Naïve B cells express membrane-bound receptors, usually from the IgM class. Following stimulation, the B cell receptors undergo alternative splicing via an alternative terminator mechanism. As a result, the carboxy terminus no longer contains the hydrophobic transmembrane domain but, instead, has a hydrophilic secretory tail. The secreted antibodies play a crucial independent role during the immune response (48).

Alternative splicing of the TNF-receptor superfamily

The TNFRSF comprises trimeric receptors made of three homologous molecules that initiate signaling pathways involved in inflammation, proliferation, differentiation, cell migration, and induction of cell death (72). Like the Ig superfamily, TNFRSF members share similar splicing patterns that result in soluble isoforms.

FAS (CD95, TNFRSF6) is one of the best-known members of the TNF receptors superfamily. It is abundantly expressed in many

tissues, including the gastrointestinal tract, the respiratory system, and lymphoid tissues (73–75). FAS is mainly known for its pro-apoptotic pathway activation following FAS ligand (FASL) binding (76). However, it also has other functions, e.g., it takes part in the differentiation of naïve T cells to memory cells (77). FAS is robustly expressed on T-cells and has an apoptosis-inducing role during T-cell development (78) and activation (79–81). A specific alternative splicing event is the skipping of exon 6, which encodes the transmembrane domain of FAS, resulting in a soluble form of the FAS receptor (52, 53). The FAS exon 6 skipping mechanism has been studied extensively. It has been shown that many splicing factors can regulate this event, among them TIA-1 (82), PTB (82), HuR (83), hnRNP A1 (84), SRSF4 (85), SRSF7 (86), and SRSF6 (87). Similarly to PD-1, the soluble FAS receptor competes with membrane-bound FAS for FASL ligation, thereby limiting FAS signaling (30, 52). Bajgain et al. described the ability of secreted FAS extracellular domain to enhance CAR T-cell antitumor activity against a FAS-ligand-expressing tumor (88).

TGF β (transforming growth factor beta receptor) TGF β is of special interest because it controls immunity via a rich network of cells and mediators, with the end result being immune evasion of the cancer tissue. The biological functions of TGF β are mostly mediated by the monomeric, soluble form of the protein. The monomer is cleaved by proteases in the Golgi complex and later released from glycoproteins that ligate it in a non-covalent manner (89). TGF β enhances the expansion of regulatory T cells (Tregs), the inhibition of NK and effector T cells, and the induction of immune suppressive cytokines including IL-4 and IL-10. Active TGF β exerts its effect via receptors that activate SMAD transcription factors, a family with hundreds of regulatory elements. Tumors exploit TGF β to induce a supportive stroma that weakens the immune response by acting as a mechanical barrier and expressing inhibitory membranal ligands, such as PD-L1 (90, 91). In a series of patients with gynecological cancers who received immune checkpoint inhibitors, a high TGF β expression score correlated with treatment failure and reduced survival (92). The type II receptor for TGF β has a splicing variant which lacks the transmembrane domain, and exert a higher binding affinity to the three sub-types of TGF β . By doing so, it competes with the natural ligands and reduces fibrotic pathology (93).

Type I cytokine receptors

In addition to the Ig and TNFR superfamilies, members of other immune receptor families can generate soluble forms through alternative splicing, including type I cytokine receptors IL6R α (54, 94), IL-4R α (57), and IL-7R α (33). Another example is the common γ chain IL-2, 7, and 15 cytokine receptors, for which skipping exon 6 encoding the transmembrane domain results in a frameshift and a premature stop codon. The resultant proteins contain only the extracellular domain. The soluble IL-2 and IL-7 receptors were reported to impair T-cell signaling and function (27).

Pathology of splicing and alternative splicing

Splicing is an imperative regulator of most cellular functions. Therefore, disrupted splicing regulation can lead to different pathologies, depending on the involved tissue and the protein products of the aberrant transcript. The most investigated pathologies that result from erroneous splicing events include neurodegenerative disorders and cancers. The first arise from germline mutations, while the latter arise from somatic genome aberrations. However, splicing-related mutations can cause many other disorders, such as dilated cardiomyopathy and Marfan syndrome (109, 110).

It remains a mystery why germline splicing-related mutations primarily affect the brain. One theory holds that alternative splicing is crucial in determining the neural cell state (19) and that neural tissue is rich in tissue-specific splicing events. However, not all splicing-related mutations in neural cells lead to a change in alternative splicing. An example of this is Duchenne muscular dystrophy (DMD), where a deletion of an exon leads to the production of a truncated protein via the process of nonsense-mediated decay (NMD) rather than a new isoform of the original protein (111).

Some argue that 15–50% of pathological mutations affect gene splicing (9–11). Nevertheless, these diseases are not regarded as splicing-related disorders since mutations that do not change the coding sequence are typically misclassified as allelic variations (112–114). In addition, the wide use of exome sequencing, which filters out most intronic parts, introduces an inherent bias underscoring splicing mutations (115–118).

Dis-regulated splicing leading to neurodegenerative disorders

Neurodegenerative diseases are a group of disorders caused by the gradual loss of neuronal cell function or structure. Strikingly, splicing-related mutations are one of the leading causes of many neurodegenerative diseases (119). The most investigated neuronal disorder instigated by splicing is spinal muscular atrophy (SMA). Nonetheless, most neural pathologies, such as early-onset Parkinson's (119–121), Alzheimer's disease (122), familial dysautonomia (123), and Amyotrophic Lateral Sclerosis (ALS), could evolve from splicing mutations (124, 125).

Given the significant number of neurodegenerative disorders caused by mutations impacting RNA splicing, it is not surprising that there have been numerous efforts to investigate the use of splicing-editing techniques as a treatment option for these conditions. For example, antisense oligonucleotides (ASOs) are being widely researched for their potential use in treating SMA, DMD, and ALS (126). The use of ASOs to manipulate alternative splicing is further discussed elsewhere in this review. The use of CRISPR-Cas9 to affect alternative splicing has been suggested for Duchenne muscular dystrophy (127), and spliceosome-mediated RNA trans-splicing (SMaRT) (128) has been tested in Huntington's disease (129), DMD (130), and Alzheimer's disease (131).

Dis-regulated splicing causing cancer and immune evasion

Splicing-related mutations in cancer can be grouped into three categories: 1-those affecting the core spliceosome complex, resulting in new isoforms; 2-those impacting splicing factors, affecting the expression levels of multiple isoforms; and 3-those affecting splicing recognition sites, altering the expression level of a single gene or creating new isoforms (Table 2; Figure 4).

Splicing factors mutations are particularly prevalent in myeloid neoplasms; for example, SF3B1, that increases anti-apoptotic isoforms, enhances tumor proliferation and progression, and is associated with poor survival of patients (134, 140, 141, 180, 181). U2AF1 is another splicing factor mutated in myeloid malignancies that drives altered splicing preferences. Intronic mutations are more frequent than exonic, and a third of somatic mutations in the exon-intron boundary are associated with splicing changes. If a mutation occurs in a 5' or 3' splicing site, there is a greater than 50% chance of it leading to a splicing shift (182).

Splicing can be employed as a cancer treatment approach in various forms: using single-stranded oligonucleotides to change the splicing of specific genes and switch between oncogenic and tumor-suppressing forms, as has been demonstrated for the BCL gene (67); regulating specific splicing factors through drugs that directly impact them, such as blocking SF3B1 (68); or by attacking the pathway which the mutant splicing factor exploits. Thus, tumors with driver mutations in SF3B1 or U2AF1 may be vulnerable to NMD inhibition (68–72). Some widely used therapies, such as camptothecin and cisplatin, have been found to impact RNA splicing, potentially contributing to their efficacy (73–75).

As discussed in the following paragraph, recent attention has focused on the generation of neo-antigens by including erroneous transcripts. However, altered splicing and the emergence of usually unexpressed isoforms independently impact tumor immunogenicity. These effects often hinder the anticancer immune response. For example, HLA tumor-enriched alternative splicing events occur in 10–30% of lung and breast cancers, affecting MHC expression. When HLA expression is inconsistent, the ability of tumor epitopes to be presented and recognized is diminished or completely lost (183). In ovarian cancer, certain splicing factors, such as BUD31, SF3B4, and CTNNB1, may indirectly support immune evasion (184). This immune escape may involve increased PD-L1 expression and primary resistance to PD-1 inhibitors. Such mechanisms are seen in clear renal cell carcinoma, where an exon-including splicing event in the chromatin remodeling gene PBRM1 contributes to immune evasion (185).

Generation of cancer neo-antigens by mutations in splicing factors

While reports indicate that altered splicing isoforms contribute to tumor immune evasion, splicing alterations are now attracting significant interest as a source of cancer antigenicity. This interest stems from the potential of AS to drive isoforms that include

TABLE 2 Specific mutations associated with splicing dis-regulation in human cancers.

Mutation type	Mutation	Description	Cancer	References
Mutation in the core of spliceosome complex	U1	Characterized by different binding to the 5' splicing site	Over 30 types of malignancies, including hepatocellular carcinoma, chronic lymphocytic leukemia, medulloblastoma	(132, 133)
	U2AF1	Leads mostly to exon skipping and 3' alternative splicing in specific genes.	Hematological, pancreatic cancer, and lung adenocarcinoma	(134–139)
Changes in splicing factors	SF3B1	Alternative branch point selection leads to aberrant/cryptic 3' splicing sites.	The most common splicing factor mutation in cancer. Common in hematological malignancies, uveal melanoma, breast cancer	(140–147)
	hnRNP A1	Overexpression of hnRNP A1 leads to miss-regulated splicing and increases oncogenic isoforms.	Many types of cancers, including lung, breast, and gastric cancers.	(148–152)
	SRSF1	Overexpression of SRSF1 in tumor cells increases a wide range of genes. Overexpression can be caused by copy number variation or changes in the mRNA level.	Breast, lung, colon, and other tumors.	(153–159)
	SRSF6	SRSF6 is a proto-oncogene that, when overexpressed, leads to an increase in tumor-promoting isoforms.	Skin, colon, lung, and other cancers.	(160–162)
Mutation in splicing recognition sites	MET	Exon 14 skipping mutations in the gene MET leads to a protein missing the phosphorylation site, which impairs protein degradation.	Non-small cell lung cancer (NSCLC)	(163–168)
	MLH1	Some of the mutations associated with HNPCC are missense/ nonsense splicing-related mutations.	Colorectal cancer (CRC)	(169–174)
	TP53	2–4% of the mutations in TP53 are mutations in intronic splicing sites, which can lead to a truncated protein or a shift towards oncogenic splice isoforms. In addition, many other mutations in the gene can effect specific isoforms of TP53.	All tumors bearing TP53 mutations	(175–179)

retained intronic sequences. These intronic transcripts, in turn, may form neoantigens—peptide sequences that have not had the opportunity to tolerize the immune system. Such newly transcribed sequences hold the potential for generating protective immunity and improving clinical responses to immune checkpoint inhibitors (61–64).

Several pharmacological compounds have been used that either degrade splicing factors, disrupt spliceosome assembly, or inhibit nonsense-mediated decay (186, 187). One example is indisulam, an anticancer sulfonamide that generates aberrant transcripts. Interestingly, indisulam does not directly inhibit cancer growth; instead, it triggers a T-cell response against cryptic sequences from abnormal RNA, which impedes tumor progression. Other splicing-disruptive compounds, such as pladienolide B and H3B-8800, are

currently being evaluated in experimental systems and clinical trials for myeloid neoplasms. Predicting the effect of splice manipulation on the tumor microenvironment is challenging, but as will be discussed, induction of soluble ectodomains from immune-modulatory receptors may interfere with immune checkpoint inhibitors. Soluble PD1, for instance, may saturate PD-1 blocking antibodies and reduce their availability to rescue exhausted antitumor T cells (188).

RNA sequencing for splicing analysis

The technology developed to sequence RNA and obtain long transcript reads that capture added or missing nucleotides was crucial to assessing AS in health and disease.

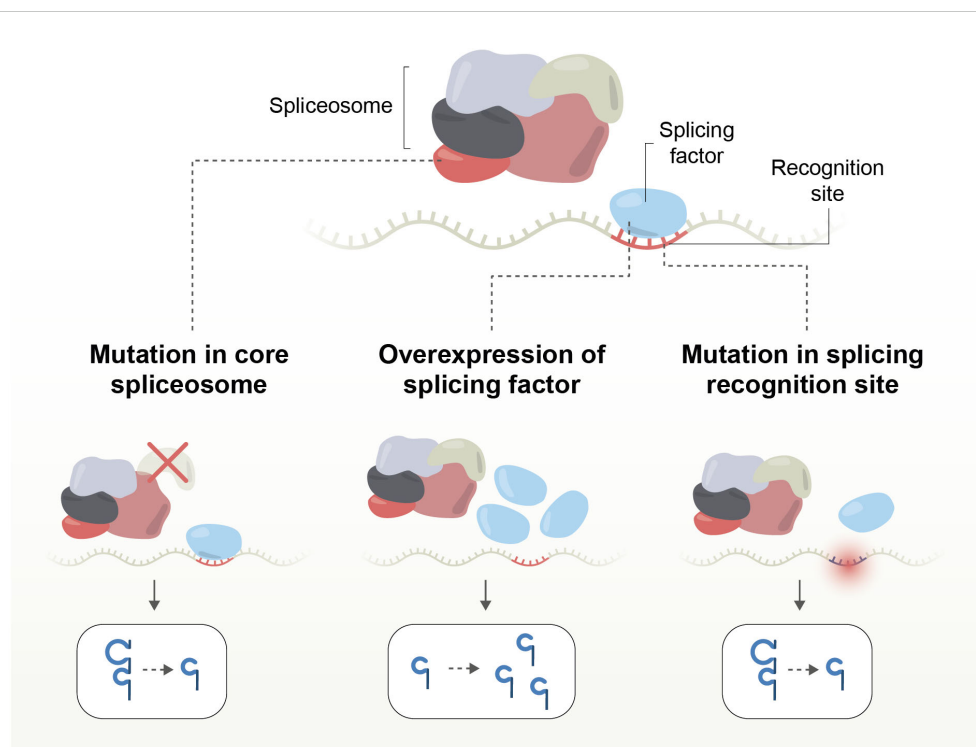


FIGURE 4

Mechanisms of splicing disruption by mutations affecting the core spliceosome complex; splicing factors, or splicing recognition sites, altering the expression level of a single gene or creating new isoforms.

Bulk RNA sequencing (RNA-seq) is mainly performed using two methodologies (Figure 5). The first is short-read sequencing, which can sequence RNA molecules in reads of up to 301 base pairs (bp), for which the Illumina platform is commonly used. The second is long-read sequencing, known as “third-generation sequencing.” This method can sequence up to 26,000 bp RNA molecules in the Nanopore™ platform (189). Long-read sequencing has the advantage of identifying full-length transcripts derived from each gene. However, this sequencing method had an accuracy of only 90% and is, therefore, error-prone. Erroneous sequencing interferes with the alignment of the reads to a reference genome and thus can miss spliced exons and their splice junction, an important feature required to determine the splicing pattern (190). However, recently, Nanopore announced that its sequencing accuracy has increased to 99.9%.

Since Illumina sequencing is well-established and widely used, most splicing analysis tools are designed for short reads. Analyzing bulk RNA-seq from Illumina data can be done in three ways. The first is determining the exon expression level and comparing its expression in varying biological settings or states. This method is called “exon-based”. The second method aims to deduce isoform expression from the short reads sequencing. This method is called “isoform-based”. The third approach, called “event-based,” computes the relative inclusion of an exon between two exons. This approach utilizes reads of splice junctions that overlap at least two exons.

A comparison of the main computational tools based on these three methods concluded that the event-based and exon-based tools

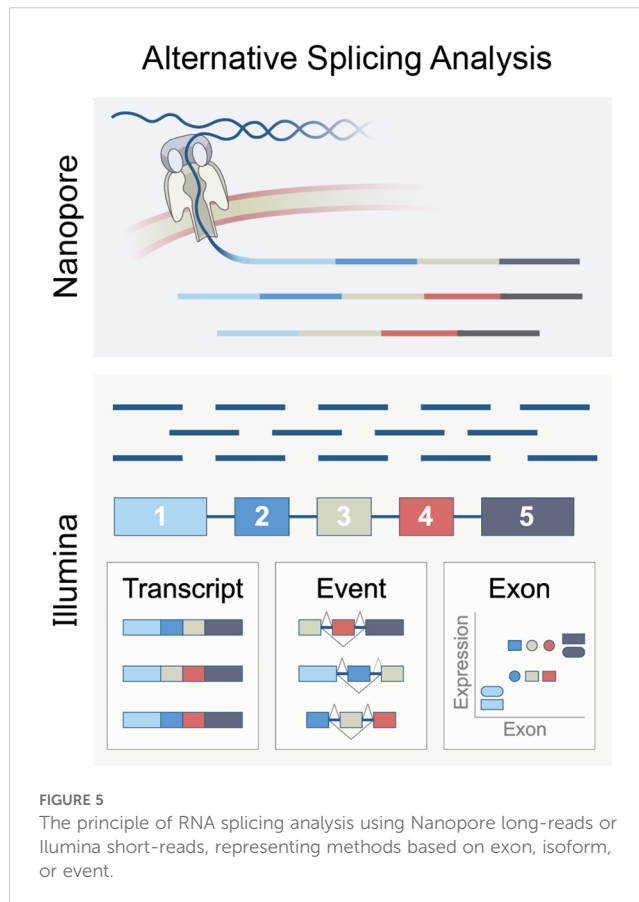


FIGURE 5

The principle of RNA splicing analysis using Nanopore long-reads or Illumina short-reads, representing methods based on exon, isoform, or event.

while having a relatively low overlap, seem to work the best. It is suggested that concurrent use of the two methods yields the optimal splicing map of a given cellular population (191).

Another critical parameter to consider when performing splicing analysis is the quality of the RNA-seq data. In this regard, two features need to be accounted for: the depth of the sequencing and the length of the reads. Mehmood et al. (191) have noted that a depth between 40–60 million reads per sample will be sufficient for a robust splicing analysis. When considering reading length, 100 bp reads were the threshold for thoroughly detecting splicing junctions (192). It is also advised to sequence the data using paired-end sequencing to increase the read length.

Extracting splicing data from single-cell RNA-seq is even more complex. In general, to apply splicing analysis tools, the samples must be produced to capture the full transcript. However, most single-cell RNA-seq technologies are based on a 3' or 5' capturing of the RNA molecule. As a result, while preparing the sequencing libraries, only the transcript's end is included; thus, there are limited options for splicing analysis (193). The main exception to these technologies is Smart-seq sequencing, which captures reads from all over the transcript. This technology enables splicing analysis with the limitation of read depth and length. This was demonstrated with the single-cell splicing analysis tool 'Expedition.' In their study, Yan Song et al. (194) used Smart-seq2 sequencing with a mean of 25 million reads per cell and a 100 bp read length. In comparison, 10XGenomics™ recommends a sequencing depth of 20,000–50,000 reads per cell and a read length of 28 bp (195); this is shallow sequencing compared to Yan Song's analysis. To overcome these problems, a new single-cell long-read RNA sequencing technology based on Pacific Bioscience's sequencing, called MAS-ISO-seq, was recently introduced. This technology is still new and needs further investigation. Furthermore, a joint project of Nanopore and 10x Genomics produced long-reads in single-cell RNA sequencing (196).

Splicing modification using antisense oligonucleotides

During the 70s, evidence accumulated for the promising ability of small RNA molecules to control translation processes (197–199). Paterson et al. were the first to generate a translation-inhibiting system based on a complementary mRNA–DNA hybrid, resulting in reversibly arrested β globin translation (199). In 1978, Paul Zamecnik and Mary Stephenson used synthetic DNA against RSV (200). Their 13-nucleotide product hybridized with the viral mRNA and prevented viral replication (201). Later, it was shown that the mechanism of action of the short, single-stranded oligonucleotides included RNase-H1 assembly to the DNA–mRNA hybrid, cleavage, and mRNA degradation (202). Despite the promising results, the research in this field was paused for a decade, mainly due to technical issues related to nucleotide synthesis (203, 204), skepticism about the ability of nucleic acids to enter target cells, and restricted knowledge of the human genome (205). The progress in these aspects re-ignited the research in nucleic acids-based manipulation. The chemically modified, short, single-stranded

antisense oligonucleotides (ASOs) improved durability, cellular uptake, delivery, and post-transcriptional effects.

Chemical modifications

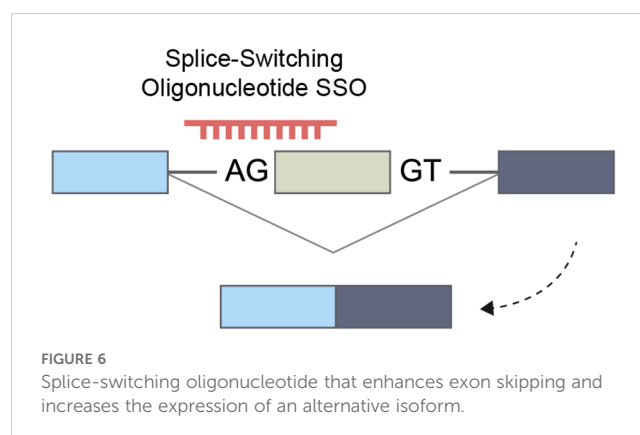
The advancement of chemical modifications of nucleic acids marked a significant milestone in the clinical application of this compound class. A key outcome was the development of splice-switching antisense oligonucleotides (SSOs), designed to modify alternative splicing patterns and enhance exon skipping. Specifically, chemical modifications that reduce RNaseH activity form a stable DNA–mRNA hybrid, preventing subsequent RNA degradation (206). These SSOs can be directed towards splice-site sequences, hindering and redirecting the spliceosome to an alternative splice site in the subsequent exon (207, 208) (Figure 6).

In addition to splicing alterations via complementation to splice sites, SSO can modify splicing by targeting splicing enhancers (ESE, ISE) or silencers (ESS, ISS) (209, 210). These interventions may interrupt splicing by inhibiting linkage to splicing factors, leading to exon exclusion or inclusion. In addition to whole exon skipping, the pre-mRNA splicing modulation can result in intron retention, alternative 5' and 3' splice sites, alternative promoter, or alternative polyadenylation sites (209).

Finding SSO-targetable splicing motifs is not trivial. A systematic scan of the exon of interest is necessary to spot the precise sequence, which the SSO should complement to alter the wild-type splicing pattern.

Two types of chemical modification are currently used for FDA-approved drugs: 2'-O-methoxyethyl (2'-MOE) nucleosides with phosphorothioate (PS) backbone and phosphorodiamidate morpholino oligomers (PMO) with a N, N-dimethylamino phosphorodiamidate backbone (Figure 7).

2'-MOE belongs to a group of modifications in the 2'O of the furanose ring of the nucleic acid. Other prevalent modifications are 2'-O-methyl (2'-OMe), locked nucleic acid (LNA), and SSOs containing 2'-constrained ethyl (2'-cEt). Alongside the RNaseH1 resistance, the 2'O modifications increase the SSO affinity (211). High affinity is attributed to higher potency, longer half-life, and less immune-provoking properties (212, 213). 2'MOE modifications are usually accompanied by switching Oxygen in the backbone to



Sulfur (PS). This switch decreases the SSO affinity but improves the resistance to nuclease activity (214, 215) and molecular binding to proteins – resulting in reduced kidney clearing (216) and improved uptake by target cells (217–219).

In PMO (220), a morpholino ring replaces the furanose ring. In addition, the negatively charged backbone is replaced by a N, N-dimethylamino phosphorodiamidate backbone. As a result of these changes, the SSOs have higher *in vivo* tolerance but faster kidney clearance, which requires a higher dosage (221, 222).

Although these are the main modifications currently used for SSO drugs, recent publications have shown how additional chemical modifications can further improve splicing modulation. For example, Langner et al. synthesized a hybrid that combines PMO modification with a PS backbone, which exhibits higher efficiency than 2'-MOE modification with the same backbone (223).

SSO-based drugs and clinical trials

Eighteen RNA-targeted oligonucleotide drugs have been approved, including five SSOs (206). The most advanced examples of clinical use of SSOs are in the field of genetic neuromuscular diseases.

The first SSO that the FDA approved is used for spinal muscular atrophy (SMA) treatment. The drug nusinersen, approved by the FDA in 2016, is an SSO with a 2'-MOE modification and a PS backbone. Nusinersen targets the splicing silencer located in *SMN2* intron 7 pre-mRNA, and by blocking the binding of hnRNPA1 and A2, it promotes higher exon 7 inclusion, increasing the *SMN2* protein synthesis (224, 225). The treatment results in prolonged survival and a dramatic improvement in motor development.

Other approved SSO drugs are used for the treatment of Duchenne muscular dystrophy (DMD). This severe, progressive muscle-wasting disease causes difficulty in movement and breathing and, eventually, early death. It is caused by mutations in the *DMD* gene, leading to impaired dystrophin protein production (226). In recent years, the FDA has approved four drugs based on a mechanism of SSO with PMO modification. The first drug approved, eteplirsen, was approved in 2016 and causes mutated exon 51 skipping (227). Three additional drugs that work in a similar mechanism have been approved in recent years for different

mutations that lead to DMD: golodirsen, which causes exon 53 skipping, was approved in 2019 (228); viltolarsen, approved in 2020, also causes exon 53 skipping (229); and casimersen, approved in 2021, induces exon 45 skipping (230). To date, DMD is the only disease for which even modest, consistent clinical benefit has been shown using PMOs. Thus, PMO SSOs have demonstrated minimal and doubtful applicability in mammalian systems (227, 231).

Considering the achievements of SSOs in DMD and SMA, several groups have recently published promising data demonstrating the potential of ASO in other diseases. For example, Yang et al. (232) show the use of SSO to prevent a splicing pattern that arises from an alternative 3' splice site between *SYNGAP1* exon 10 and exon 11. This splicing pattern leads to nonsense-mediated decay (NMD). Mutations in this gene are a common cause of autism and intellectual disability. Using SSO with 2'-MOE modification increased the expression of the active protein in an *in vitro* system. Promising results for the use of SSO can also be seen in the treatment of Dravet syndrome (233), Huntington's disease (234), and fragile X syndrome (235). Similarly, in cystic fibrosis, Oren et al. (236) and Michaels et al. (237) demonstrate the use of SSO that leads to mutated exon 23 skipping, increasing the expression of the CFTR protein.

SSO and cancer treatment

The use of SSO in cancer treatment is still in its early stages. There is currently no approved drug, but there are ongoing research studies. The primary approach for anticancer SSO is modulating the alternative splicing of oncogenes toward NMD, nonfunctional dominant negative isoforms, or isoforms with the opposite function.

For example, Dewaele et al. (238) used PMO-modified SSO for MDM4 exon 6 skipping, resulting in nonsense-mediated decay and rescue of MDM4's target - the tumor suppressor protein p53. The SSO administration reduces diffuse large B cell lymphoma growth both *in vitro* and *in vivo*.

Using SSOs for translatable alternative splicing isoforms was shown in the human epidermal growth factor receptor 2 (HER2) case. HER2 is an oncogene and established therapeutic target in a large subset of women with breast cancer (239). Wan et al. (240) and Pankratova et al. (241) used SSOs to skip HER2 exons 15 and 19,

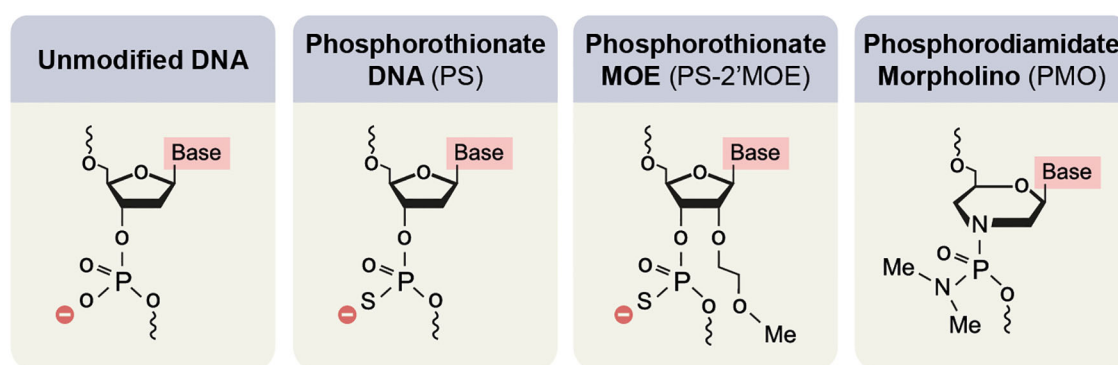


FIGURE 7

Main chemical modifications of nucleic acids to improve the clinical applicability of oligonucleotides.

respectively. The manipulations resulted in the upregulation of $\Delta 15\text{HER2}$, a HER2 inhibitor isoform, and $\Delta 19\text{HER2}$, a dominant negative isoform, leading to apoptosis and inhibition of proliferation.

Khurshid et al. (242) recently proposed using SSO for patients with rhabdomyosarcoma (RMS). In their article, the group describes the modification of the insulin receptor splicing pattern by targeting the binding site of the splicing factor CELF1. This prevents the skipping of exon 11, leading to an increase in the expression of the receptor in its full form (IR-B). The use of SSO in an RMS cell line system led to a decrease in proliferation, migration, and angiogenesis.

Manipulating cancer-associated metabolic programs using SSO was demonstrated by Wang et al. (243). The group found that elements in exon 10 of the pyruvate kinase M (PKM) gene influence the choice between the inclusion of exon 10 and exon 9. Exon 10 inclusion, the M2 isoform, is common in cancer tumors and is associated with their ability to switch to aerobic glycolysis (Warburg effect). The group demonstrated the possibility of using SSO for splicing modulation in favor of exon 9 inclusion and showed that the manipulation could lead to apoptosis of glioblastoma cell lines. Recently, the group showed a similar effect in a hepatocellular carcinoma mouse system (244). In summary, the use of SSOs to manipulate the immune system is still in its early stages. While there is significant progress in understanding the immune system at the molecular landscape, many complexities regarding the manipulation of T cells are yet to be unraveled.

Author contributions

ST: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. OS: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. EZ: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. GE: Conceptualization, Supervision, Writing – review & editing. SK: Conceptualization,

Investigation, Writing – review & editing. SF: Writing – review & editing. ML: Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The preparation of this review, part of the work performed by the Lotem lab in Jerusalem and reported here, as well as publication expenses, are supported by Dr. Miriam and Sheldon G. Adelson Medical Research Foundation and the European Union's Horizon 2021 Research and Innovation Programme under grant agreement no. 101057250.

Acknowledgments

Figures were created with HandMed by Dr. Yitzchak Yadegari.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Nilsen TW, Graveley BR. Expansion of the eukaryotic proteome by alternative splicing. *Nature*. (2010) 463:457–63. doi: 10.1038/nature08909
2. Baralle FE, Giudice J. Alternative splicing as a regulator of development and tissue identity. *Nat Rev Mol Cell Biol*. (2017) 18:437–51. doi: 10.1038/nrm.2017.27
3. Barash Y, Calarco JA, Gao W, Pan Q, Wang X, Shai O, et al. Deciphering the splicing code. *Nature*. (2010) 465:53–9. doi: 10.1038/nature09000
4. Holmes ME, Hertel KJ. Interdependent regulation of alternative splicing by SR and hnRNP proteins. *bioRxiv*. (2024). doi: 10.1101/2024.08.19.608666
5. Hajaj E, Zisman E, Tzaban S, Merims S, Cohen J, Klein S, et al. Alternative splicing of the inhibitory immune checkpoint receptor SLAMF6 generates a dominant positive form, boosting T-cell effector functions. *Cancer Immunol Res*. (2021) 9:637–50. doi: 10.1158/2326-6066.CIR-20-0800
6. Hanawa H, Ma Y, Mikolajczak SA, Charles ML, Yoshida T, Yoshida R, et al. A novel costimulatory signaling in human T lymphocytes by a splice variant of CD28. *Blood*. (2002) 99:2138–45. doi: 10.1182/blood.V99.6.2138
7. Bonnal SC, Lopez-Oreja I, Valcarcel J. Roles and mechanisms of alternative splicing in cancer - implications for care. *Nat Rev Clin Oncol*. (2020) 17:457–74. doi: 10.1038/s41571-020-0350-x
8. Lee Y, Rio DC. Mechanisms and regulation of alternative pre-mRNA splicing. *Annu Rev Biochem*. (2015) 84:291–323. doi: 10.1146/annurev-biochem-060614-034316
9. Fu XD, Ares M Jr. Context-dependent control of alternative splicing by RNA-binding proteins. *Nat Rev Genet*. (2014) 15:689–701. doi: 10.1038/nrg3778
10. Badr E, ElHefnawi M, Heath LS. Computational identification of tissue-specific splicing regulatory elements in human genes from RNA-seq data. *PLoS One*. (2016) 11: e0166978. doi: 10.1371/journal.pone.0166978
11. Lareau LF, Brenner SE. Regulation of splicing factors by alternative splicing and NMD is conserved between kingdoms yet evolutionarily flexible. *Mol Biol Evol*. (2015) 32:1072–9. doi: 10.1093/molbev/msv002
12. Carmel I, Tal S, Vig I, Ast G. Comparative analysis detects dependencies among the 5' splice-site positions. *RNA*. (2004) 10:828–40. doi: 10.1261/rna.5196404
13. Magen A, Ast G. The importance of being divisible by three in alternative splicing. *Nucleic Acids Res*. (2005) 33:5574–82. doi: 10.1093/nar/gki858
14. Brooks AN, Yang L, Duff MO, Hansen KD, Park JW, Dudoit S, et al. Conservation of an RNA regulatory map between *Drosophila* and mammals. *Genome Res*. (2011) 21:193–202. doi: 10.1101/gr.108662.110
15. Dillman AA, Hauser DN, Gibbs JR, Nalls MA, McCoy MK, Rudenko IN, et al. mRNA expression, splicing and editing in the embryonic and adult mouse cerebral cortex. *Nat Neurosci*. (2013) 16:499–506. doi: 10.1038/nn.3332
16. de Oliveira Freitas MaChado C, Schafranek M, Bruggemann M, Hernandez Canas MC, Keller M, Di Liddo A, et al. Poison cassette exon splicing of SRSF6 regulates

nuclear speckle dispersal and the response to hypoxia. *Nucleic Acids Res.* (2023) 51:870–90. doi: 10.1093/nar/gkac1225

17. Salmond RJ, McNeill L, Holmes N, Alexander DR. CD4+ T cell hyper-responsiveness in CD45 transgenic mice is independent of isoform. *Int Immunol.* (2008) 20:819–27. doi: 10.1093/intimm/dxn040

18. Oberdoerffer S, Moita LF, Neems D, Freitas RP, Hacohen N, Rao A. Regulation of CD45 alternative splicing by heterogeneous ribonucleoprotein, hnRNPL. *Science.* (2008) 321:686–91. doi: 10.1126/science.1157610

19. Zhang X, Chen MH, Wu X, Kodani A, Fan J, Doan R, et al. Cell-type-specific alternative splicing governs cell fate in the developing cerebral cortex. *Cell.* (2016) 166:1147–62 e15. doi: 10.1016/j.cell.2016.07.025

20. Latini A, Novelli L, Ceccarelli F, Barbati C, Perricone C, De Benedittis G, et al. mRNA expression analysis confirms CD44 splicing impairment in systemic lupus erythematosus patients. *Lupus.* (2021) 30:1086–93. doi: 10.1177/09612033211004725

21. AlFadhli S. Overexpression and secretion of the soluble CTLA-4 splice variant in various autoimmune diseases and in cases with overlapping autoimmunity. *Genet Test Mol Biomarkers.* (2013) 17:336–41. doi: 10.1089/gtmb.2012.0391

22. Ruefli-Brasse AA, French DM, Dixit VM. Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase. *Science.* (2003) 302:1581–4. doi: 10.1126/science.1090769

23. Meininger I, Griesbach RA, Hu D, Gehring T, Seeholzer T, Bertossi A, et al. Alternative splicing of MALT1 controls signalling and activation of CD4(+) T cells. *Nat Commun.* (2016) 7:11292. doi: 10.1038/ncomms11292

24. Blake D, Radens CM, Ferretti MB, Gazzara MR, Lynch KW. Alternative splicing of apoptosis genes promotes human T cell survival. *Elife.* (2022) 11. doi: 10.7554/eLife.80953.sa2

25. Monzon-Casanova E, Screen M, Diaz-Munoz MD, Coulson RMR, Bell SE, Lamers G, et al. The RNA-binding protein PTBP1 is necessary for B cell selection in germinal centers. *Nat Immunol.* (2018) 19:267–78. doi: 10.1038/s41590-017-0035-5

26. Khan M, Zhao Z, Arooj S, Fu Y, Liao G. Soluble PD-1: predictive, prognostic, and therapeutic value for cancer immunotherapy. *Front Immunol.* (2020) 11:587460. doi: 10.3389/fimmu.2020.587460

27. Hong C, Luckey MA, Ligons DL, Waickman AT, Park JY, Kim GY, et al. Activated T cells secrete an alternatively spliced form of common gamma-chain that inhibits cytokine signaling and exacerbates inflammation. *Immunity.* (2014) 40:910–23. doi: 10.1016/j.immuni.2014.04.020

28. Ward FJ, Dahal LN, Wijesekera SK, Abdul-Jawad SK, Kaewarpai T, Xu H, et al. The soluble isoform of CTLA-4 as a regulator of T-cell responses. *Eur J Immunol.* (2013) 43:1274–85. doi: 10.1002/eji.201242529

29. Li X, Li J, Zheng Y, Lee SJ, Zhou J, Giobbie-Hurder A, et al. Granulocyte-macrophage colony-stimulating factor influence on soluble and membrane-bound ICOS in combination with immune checkpoint blockade. *Cancer Immunol Res.* (2023) 11:1100–13. doi: 10.1158/2326-6066.CIR-22-0702

30. Papoff G, Cascino I, Eramo A, Starace G, Lynch DH, Ruberti G. An N-terminal domain shared by Fas/Apo-1 (CD95) soluble variants prevents cell death *in vitro*. *J Immunol.* (1996) 156:4622–30. doi: 10.4049/jimmunol.156.12.4622

31. Sato TA, Widmer MB, Finkelman FD, Madani H, Jacobs CA, Grabstein KH, et al. Recombinant soluble murine IL-4 receptor can inhibit or enhance IgE responses *in vivo*. *J Immunol.* (1993) 150:2717–23. doi: 10.4049/jimmunol.150.7.2717

32. Smith DE, Hanna R, Della F, Moore H, Chen H, Farese AM, et al. The soluble form of IL-1 receptor accessory protein enhances the ability of soluble type II IL-1 receptor to inhibit IL-1 action. *Immunity.* (2003) 18:87–96. doi: 10.1016/S1074-7613(02)00514-9

33. Crawley AM, Faucher S, Angel JB. Soluble IL-7R alpha (sCD127) inhibits IL-7 activity and is increased in HIV infection. *J Immunol.* (2010) 184:4679–87. doi: 10.4049/jimmunol.0903758

34. Luu K, Shao Z, Schwarz H. The relevance of soluble CD137 in the regulation of immune responses and for immunotherapeutic intervention. *J Leukoc Biol.* (2020) 107:731–8. doi: 10.1002/JLB.2MR1119-224R

35. Thum E, Shao Z, Schwarz H. CD137, implications in immunity and potential for therapy. *Front Biosci (Landmark Ed).* (2009) 14:4173–88. doi: 10.2741/3521

36. Nielsen C, Ohm-Laursen L, Barington T, Husby S, Lillevang ST. Alternative splice variants of the human PD-1 gene. *Cell Immunol.* (2005) 235:109–16. doi: 10.1016/j.cellimm.2005.07.007

37. Blake D, Lynch KW. The three as: Alternative splicing, alternative polyadenylation and their impact on apoptosis in immune function. *Immunol Rev.* (2021) 304:30–50. doi: 10.1111/imr.v304.1

38. Jensen LE, Whitehead AS. Expression of alternatively spliced interleukin-1 receptor accessory protein mRNAs is differentially regulated during inflammation and apoptosis. *Cell Signal.* (2003) 15:793–802. doi: 10.1016/S0898-6568(03)00039-1

39. Oaks MK, Hallett KM. Cutting edge: a soluble form of CTLA-4 in patients with autoimmune thyroid disease. *J Immunol.* (2000) 164:5015–8. doi: 10.4049/jimmunol.164.10.5015

40. Grosche L, Knippertz I, König C, Royzman D, Wild AB, Zinser E, et al. The CD83 molecule - an important immune checkpoint. *Front Immunol.* (2020) 11:721. doi: 10.3389/fimmu.2020.00721

41. Kuipers H, Muskens F, Willart M, Hijdra D, van Assema FB, Coyle AJ, et al. Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic cell-mediated CD4+ T cell activation. *Eur J Immunol.* (2006) 36:2472–82. doi: 10.1002/eji.200635978

42. Horvatinovich JM, Grogan EW, Norris M, Steinkasserer A, Lemos H, Mellor AL, et al. Soluble CD83 inhibits T cell activation by binding to the TLR4/MD-2 complex on CD14(+) monocytes. *J Immunol.* (2017) 198:2286–301. doi: 10.4049/jimmunol.1600802

43. Grohmann U, Volpi C, Fallarino F, Bozza S, Bianchi R, Vacca C, et al. Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy. *Nat Med.* (2007) 13:579–86. doi: 10.1038/nm1563

44. Vardam TD, Zhou L, Appenheimer MM, Chen Q, Wang WC, Baumann H, et al. Regulation of a lymphocyte-endothelial-IL-6 trans-signaling axis by fever-range thermal stress: hot spot of immune surveillance. *Cytokine.* (2007) 39:84–96. doi: 10.1016/j.cyto.2007.07.184

45. Lundstrom W, Highfill S, Walsh ST, Beq S, Morse E, Kockum I, et al. Soluble IL7Ralpha potentiates IL-7 bioactivity and promotes autoimmunity. *Proc Natl Acad Sci United States America.* (2013) 110:E1761–70. doi: 10.1073/pnas.1222303110

46. Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. *J Exp Med.* (1992) 175:323–9. doi: 10.1084/jem.175.2.323

47. Peters M, Jacobs S, Ehlers M, Vollmer P, Mullberg J, Wolf E, et al. The function of the soluble interleukin 6 (IL-6) receptor *in vivo*: sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of the plasma half-life of IL-6. *J Exp Med.* (1996) 183:1399–406. doi: 10.1084/jem.183.4.1399

48. Murphy KM, Weaver C. *Janeway's Immunobiology*. New York and London: Garland Science/Taylor & Francis Group L (2017) p. 196–7.

49. Kefaloyianni E. Soluble forms of cytokine and growth factor receptors: mechanisms of generation and modes of action in the regulation of local and systemic inflammation. *FEBS letters.* (2022) 596:589–606. doi: 10.1002/1873-3468.14305

50. Dudziak D, Nimmerjahn F, Bornkamm GW, Laux G. Alternative splicing generates putative soluble CD83 proteins that inhibit T cell proliferation. *J Immunol.* (2005) 174:6672–6. doi: 10.4049/jimmunol.174.11.6672

51. Goodwin RG, Friend D, Ziegler SF, Jerzy R, Falk BA, Gimpel S, et al. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell.* (1990) 60:941–51. doi: 10.1016/0092-8674(90)90342-C

52. Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, et al. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science.* (1994) 263:1759–62. doi: 10.1126/science.7510905

53. Cascino I, Ficuci G, Papoff G, Ruberti G. Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. *J Immunol.* (1995) 154:2706–13. doi: 10.4049/jimmunol.154.6.2706

54. Horiuchi S, Koyanagi Y, Zhou Y, Miyamoto H, Tanaka Y, Waki M, et al. Soluble interleukin-6 receptors released from T cell or granulocyte/macrophage cell lines and human peripheral blood mononuclear cells are generated through an alternative splicing mechanism. *Eur J Immunol.* (1994) 24:1945–8. doi: 10.1002/eji.1830240837

55. Monaghan SF, Banerjee D, Chung CS, Lomas-Neira J, Cygan KJ, Rhine CL, et al. Changes in the process of alternative RNA splicing results in soluble B and T lymphocyte attenuator with biological and clinical implications in critical illness. *Mol Med.* (2018) 24:32. doi: 10.1186/s10020-018-0036-3

56. Nocentini G, Ronchetti S, Bartoli A, Spinicelli S, Delfino D, Brunetti L, et al. Identification of three novel mRNA splice variants of GITR. *Cell Death Differ.* (2000) 7:408–10. doi: 10.1038/sj.cdd.4400670

57. Kruse S, Forster J, Kuehr J, Deichmann KA. Characterization of the membrane-bound and a soluble form of human IL-4 receptor alpha produced by alternative splicing. *Int Immunol.* (1999) 11:1965–70. doi: 10.1093/intimm/11.12.1965

58. Magistrelli G, Jeannin P, Herbault N, Benoit De Coignac A, Gauchat JF, Bonnefoy JY, et al. A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. *Eur J Immunol.* (1999) 29:3596–602. doi: 10.1002/(SICI)1521-4141(199911)29:11<3596::AID-IMMU3596>3.0.CO;2-Y

59. Lainez B, Fernandez-Real JM, Romero X, Esplugues E, Canete JD, Ricart W, et al. Identification and characterization of a novel spliced variant that encodes human soluble tumor necrosis factor receptor 2. *Int Immunol.* (2004) 16:169–77. doi: 10.1093/intimm/dxh014

60. Philippe C, Roux-Lombard P, Fouqueray B, Perez J, Dayer JM, Baud L. Membrane expression and shedding of tumour necrosis factor receptors during activation of human blood monocytes: regulation by desferrioxamine. *Immunology.* (1993) 80:300–5.

61. Levine SJ. Molecular mechanisms of soluble cytokine receptor generation. *J Biol Chem.* (2008) 283:14177–81. doi: 10.1074/jbc.R700052200

62. Sung HH, Juang JH, Lin YC, Kuo CH, Hung JT, Chen A, et al. Transgenic expression of decoy receptor 3 protects islets from spontaneous and chemical-induced autoimmune destruction in nonobese diabetic mice. *J Exp Med.* (2004) 199:1143–51. doi: 10.1084/jem.20031939

63. Odales J, Guzman Valle J, Martinez-Cortes F, Manoutcharian K. Immunogenic properties of immunoglobulin superfamily members within complex biological networks. *Cell Immunol.* (2020) 358:104235. doi: 10.1016/j.cellimm.2020.104235

64. Gu D, Ao X, Yang Y, Chen Z, Xu X. Soluble immune checkpoints in cancer: production, function and biological significance. *J Immunother Cancer*. (2018) 6:132. doi: 10.1186/s40425-018-0449-0
65. Chakrabarti R, Kapse B, Mukherjee G. Soluble immune checkpoint molecules: Serum markers for cancer diagnosis and prognosis. *Cancer Rep (Hoboken)*. (2019) 2:e1160. doi: 10.1002/cnr.2.1160
66. Triebel F. LAG-3: a regulator of T-cell and DC responses and its use in therapeutic vaccination. *Trends Immunol*. (2003) 24:619–22. doi: 10.1016/j.it.2003.10.001
67. Sharpe AH, Pauken KE. The diverse functions of the PD1 inhibitory pathway. *Nat Rev*. (2018) 18:153–67. doi: 10.1038/nri.2017.108
68. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. (2008) 26:677–704. doi: 10.1146/annurev.immunol.26.021607.090331
69. Song MY, Park SH, Nam HJ, Choi DH, Sung YC. Enhancement of vaccine-induced primary and memory CD8(+) T-cell responses by soluble PD-1. *J Immunother*. (2011) 34:297–306. doi: 10.1097/CJ1.0b013e318210ed0e
70. He L, Zhang G, He Y, Zhu H, Zhang H, Feng Z. Blockade of B7-H1 with sPD-1 improves immunity against murine hepatocarcinoma. *Anticancer Res*. (2005) 25:3309–13.
71. He YF, Zhang GM, Wang XH, Zhang H, Yuan Y, Li D, et al. Blocking programmed death-1 ligand-PD-1 interactions by local gene therapy results in enhancement of antitumor effect of secondary lymphoid tissue chemokine. *J Immunol*. (2004) 173:4919–28. doi: 10.4049/jimmunol.173.8.4919
72. Dostert C, Grusdat M, Letellier E, Brenner D. The TNF family of ligands and receptors: communication modules in the immune system and beyond. *Physiol Rev*. (2019) 99:115–60. doi: 10.1152/physrev.00045.2017
73. Fine A, Anderson NL, Rothstein TL, Williams MC, Gochoico BR. Fas expression in pulmonary alveolar type II cells. *Am J Physiol*. (1997) 273:L64–71. doi: 10.1152/ajplung.1997.273.1.L64
74. Pinkoski MJ, Brunner T, Green DR, Lin T. Fas and Fas ligand in gut and liver. *Am J Physiol Gastrointest Liver Physiol*. (2000) 278:G354–66. doi: 10.1152/ajpgi.2000.278.3.G354
75. Krammer PH. CD95's deadly mission in the immune system. *Nature*. (2000) 407:789–95. doi: 10.1038/35037728
76. Golstein P, Griffiths GM. An early history of T cell-mediated cytotoxicity. *Nat Rev*. (2018) 18:527–35. doi: 10.1038/s41577-018-0009-3
77. Klebanoff CA, Scott CD, Leonardi AJ, Yamamoto TN, Cruz AC, Ouyang C, et al. Memory T cell-driven differentiation of naive cells impairs adoptive immunotherapy. *J Clin Invest*. (2016) 126:318–34. doi: 10.1172/JCI81217
78. Sprent J, Kishimoto H. The thymus and negative selection. *Immunol Rev*. (2002) 185:126–35. doi: 10.1034/j.1600-065X.2002.18512.x
79. Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, et al. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med*. (1995) 181:71–7. doi: 10.1084/jem.181.1.71
80. Kunkele A, Johnson AJ, Rolczynski LS, Chang CA, Hoglund V, Kelly-Spratt KS, et al. Functional Tuning of CARs Reveals Signaling Threshold above Which CD8+ CTL Antitumor Potency Is Attenuated due to Cell Fas-FasL-Dependent AICD. *Cancer Immunol Res*. (2015) 3:368–79. doi: 10.1158/2326-6066.CIR-14-0200
81. Horton BL, Williams JB, Cabanov A, Spranger S, Gajewski TF. Intratumoral CD8(+) T-cell apoptosis is a major component of T-cell dysfunction and impedes antitumor immunity. *Cancer Immunol Res*. (2018) 6:14–24. doi: 10.1158/2326-6066.CIR-17-0249
82. Izquierdo JM, Majos N, Bonnal S, Martinez C, Castelo R, Guigo R, et al. Regulation of Fas alternative splicing by antagonistic effects of TIA-1 and PTB on exon definition. *Mol Cell*. (2005) 19:475–84. doi: 10.1016/j.molcel.2005.06.015
83. Izquierdo JM, Hu antigen R (HuR) functions as an alternative pre-mRNA splicing regulator of Fas apoptosis-promoting receptor on exon definition. *J Biol Chem*. (2008) 283:19077–84. doi: 10.1074/jbc.M800017200
84. Oh H, Lee E, Jang HN, Lee J, Moon H, Sheng Z, et al. hnRNP A1 contacts exon 5 to promote exon 6 inclusion of apoptotic Fas gene. *Apoptosis*. (2013) 18:825–35. doi: 10.1007/s10495-013-0824-8
85. Jang HN, Liu Y, Choi N, Oh J, Ha J, Zheng X, et al. Binding of SRSF4 to a novel enhancer modulates splicing of exon 6 of Fas pre-mRNA. *Biochem Biophys Res Commun*. (2018) 506:703–8. doi: 10.1016/j.bbrc.2018.10.123
86. Tejedor JR, Papasaikas P, Valcarcel J. Genome-wide identification of Fas/CD95 alternative splicing regulators reveals links with iron homeostasis. *Mol Cell*. (2015) 57:23–38. doi: 10.1016/j.molcel.2014.10.029
87. Choi N, Jang HN, Oh J, Ha J, Park H, Zheng X, et al. SRSF6 regulates the alternative splicing of the apoptotic fas gene by targeting a novel RNA sequence. *Cancers (Basel)*. (2022) 14(8):1990. doi: 10.3390/cancers14081990
88. Bajgain P, Torres Chavez AG, Balasubramanian K, Fleckenstein L, Lulla P, Heslop HE, et al. Secreted fas decoys enhance the antitumor activity of engineered and bystander T cells in fas ligand-expressing solid tumors. *Cancer Immunol Res*. (2022) 10:1370–85. doi: 10.1158/2326-6066.CIR-22-0115
89. Battle E, Massague J. Transforming growth factor-beta signaling in immunity and cancer. *Immunity*. (2019) 50:924–40. doi: 10.1016/j.immuni.2019.03.024
90. Chakravarthy A, Khan L, Bensler NP, Bose P, De Carvalho DD. TGF-beta-associated extracellular matrix genes link cancer-associated fibroblasts to immune evasion and immunotherapy failure. *Nat Commun*. (2018) 9:4692. doi: 10.1038/s41467-018-06654-8
91. Tauriello DVF, Palomo-Ponce S, Stork D, Berenguer-Llgero A, Badia-Ramentol J, Iglesias M, et al. TGFbeta drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature*. (2018) 554:538–43. doi: 10.1038/nature25492
92. Ni Y, Soliman A, Joehlin-Price A, Rose PG, Vlad A, Edwards RP, et al. High TGF-beta signature predicts immunotherapy resistance in gynecologic cancer patients treated with immune checkpoint inhibition. *NPJ Precis Oncol*. (2021) 5:101. doi: 10.1038/s41698-021-00242-8
93. Bertolio MS, La Colla A, Carrea A, Romo A, Canziani G, Echarte SM, et al. A novel splice variant of human TGF-beta type II receptor encodes a soluble protein and its fc-tagged version prevents liver fibrosis *in vivo*. *Front Cell Dev Biol*. (2021) 9:690397. doi: 10.3389/fcell.2021.690397
94. Lust JA, Donovan KA, Kline MP, Greipp PR, Kyle RA, Maihle NJ. Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine*. (1992) 4:96–100. doi: 10.1016/1043-4666(92)90043-Q
95. Garapati VP, Lefranc MP. IMGT Colliers de Perles and IgSF domain standardization for T cell costimulatory activatory (CD28, ICOS) and inhibitory (CTLA4, PDCD1 and BTLA) receptors. *Dev Comp Immunol*. (2007) 31:1050–72. doi: 10.1016/j.dci.2007.01.008
96. Andrzejczak A, Karabon L. BTLA biology in cancer: from bench discoveries to clinical potentials. *biomark Res*. (2024) 12:8. doi: 10.1186/s40364-024-00556-2
97. Magistrelli G, Jeannin P, Elson G, Gauchat JF, Nguyen TN, Bonnefoy JY, et al. Identification of three alternatively spliced variants of human CD28 mRNA. *Biochem Biophys Res Commun*. (1999) 259:34–7. doi: 10.1006/bbrc.1999.0725
98. Hebbard M, Jeannin P, Magistrelli G, Hatron PY, Hachulla E, Devulder B, et al. Detection of circulating soluble CD28 in patients with systemic lupus erythematosus, primary Sjogren's syndrome and systemic sclerosis. *Clin Exp Immunol*. (2004) 136:388–92. doi: 10.1111/j.1365-2249.2004.02427.x
99. Lechmann M, Krooshoop DJ, Dudziak D, Kremmer E, Kuhn C, Figdor CG, et al. The extracellular domain of CD83 inhibits dendritic cell-mediated T cell stimulation and binds to a ligand on dendritic cells. *J Exp Med*. (2001) 194:1813–21. doi: 10.1084/jem.194.12.1813
100. Burnell SEA, Capitani L, MacLachlan BJ, Mason GH, Gallimore AM, Godkin A. Seven mysteries of LAG-3: a multi-faceted immune receptor of increasing complexity. *Immunother Adv*. (2022) 2:ltab025. doi: 10.1093/immadv/ltab025
101. Gorgulho J, Roderburg C, Beier F, Bokemeyer C, Brummendorf TH, Loosen SH, et al. Soluble lymphocyte activation gene-3 (sLAG3) and CD4/CD8 ratio dynamics as predictive biomarkers in patients undergoing immune checkpoint blockade for solid malignancies. *Br J cancer*. (2024) 130:1013–22. doi: 10.1038/s41416-023-02558-7
102. Frenay J, Bellaye PS, Oudot A, Helbling A, Petitot C, Ferrand C, et al. IL-1RAP, a key therapeutic target in cancer. *Int J Mol Sci*. (2022) 23(23):14918. doi: 10.3390/ijms232314918
103. Blum H, Wolf M, Enssle K, Rollinghoff M, Gessner A. Two distinct stimulus-dependent pathways lead to production of soluble murine interleukin-4 receptor. *J Immunol*. (1996) 157:1846–53. doi: 10.4049/jimmunol.157.5.1846
104. Schumertl T, Lokau J, Rose-John S, Garbers C. Function and proteolytic generation of the soluble interleukin-6 receptor in health and disease. *Biochim Biophys Acta Mol Cell Res*. (2022) 1869:119143. doi: 10.1016/j.bbamcr.2021.119143
105. Hurst SM, Wilkinson TS, McLoughlin RM, Jones S, Horiuchi S, Yamamoto N, et al. IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. *Immunity*. (2001) 14:705–14. doi: 10.1016/S1074-7613(01)00151-0
106. Wolf J, Waetzig GH, Chalaris A, Reinheimer TM, Wege H, Rose-John S, et al. Different soluble forms of the interleukin-6 family signal transducer gp130 fine-tune the blockade of interleukin-6 trans-signaling. *J Biol Chem*. (2016) 291:16186–96. doi: 10.1074/jbc.M116.718551
107. Tian J, Zhang B, Rui K, Wang S. The role of GITR/GITRL interaction in autoimmune diseases. *Front Immunol*. (2020) 11:588682. doi: 10.3389/fimmu.2020.588682
108. Romo A, Rodriguez TM, Yu G, Dewey RA. Chimeric TbetarII-SE/Fc overexpression by a lentiviral vector exerts strong antitumoral activity on colorectal cancer-derived cell lines in vitro and on xenografts. *Cancer Gene Ther*. (2024) 31:174–85. doi: 10.1038/s41417-023-00694-z
109. Fenix AM, Miyaoka Y, Bertero A, Blue SM, Spindler MJ, Tan KKB, et al. Gain-of-function cardiomyopathic mutations in RBM20 rewire splicing regulation and redistribute ribonucleoprotein granules within processing bodies. *Nat Commun*. (2021) 12:6324. doi: 10.1038/s41467-021-26623-y
110. Wells QS, Becker JR, Su YR, Mosley JD, Weeke P, D'Aoust L, et al. Whole exome sequencing identifies a causal RBM20 mutation in a large pedigree with familial dilated cardiomyopathy. *Circ Cardiovasc Genet*. (2013) 6:317–26. doi: 10.1161/CIRCGENETICS.113.000011
111. Aartsma-Rus A, Van Deutekom JC, Fokkema IF, Van Ommen GJ, Den Dunnen JT. Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle Nerve*. (2006) 34:135–44. doi: 10.1002/mus.20586

112. Pagani F, Stuani C, Tzetis M, Kanavakis E, Efthymiadou A, Doudounakis S, et al. New type of disease causing mutations: the example of the composite exonic regulatory elements of splicing in CFTR exon 12. *Hum Mol Genet.* (2003) 12:1111–20. doi: 10.1093/hmg/ddg131
113. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet.* (2002) 3:285–98. doi: 10.1038/nrg775
114. Pagani F, Baralle FE. Genomic variants in exons and introns: identifying the splicing spoilers. *Nat Rev Genet.* (2004) 5:389–96. doi: 10.1038/nrg1327
115. Bergsma AJ, van der Wal E, Broeders M, van der Ploeg AT, Pim Pijnappel WWM. Alternative splicing in genetic diseases: improved diagnosis and novel treatment options. *Int Rev Cell Mol Biol.* (2018) 335:85–141. doi: 10.1016/bs.irmb.2017.07.008
116. Chou J, Ohsumi TK, Geha RS. Use of whole exome and genome sequencing in the identification of genetic causes of primary immunodeficiencies. *Curr Opin Allergy Clin Immunol.* (2012) 12:623–8. doi: 10.1097/ACI.0b013e3283588ca6
117. Ars E, Krueyer H, Gaona A, Serra E, Lazaro C, Estivill X. Prenatal diagnosis of sporadic neurofibromatosis type 1 (NF1) by RNA and DNA analysis of a splicing mutation. *Prenat Diagn.* (1999) 19:739–42. doi: 10.1002/(SICI)1097-0223(199908)19:8<739::AID-PD626>3.0.CO;2-A
118. Baralle D, Baralle M. Splicing in action: assessing disease causing sequence changes. *J Med Genet.* (2005) 42:737–48. doi: 10.1136/jmg.2004.029538
119. Nik S, Bowman TV. Splicing and neurodegeneration: Insights and mechanisms. *Wiley Interdiscip Rev RNA.* (2019) 10:e1532. doi: 10.1002/wrna.2019.10.issue-4
120. Samaranch L, Lorenzo-Betancor O, Arbelo JM, Ferrer I, Lorenzo E, Irigoyen J, et al. PINK1-linked parkinsonism is associated with Lewy body pathology. *Brain.* (2010) 133:1128–42. doi: 10.1093/brain/awq051
121. Fu RH, Liu SP, Huang SJ, Chen HJ, Chen PR, Lin YH, et al. Aberrant alternative splicing events in Parkinson's disease. *Cell Transplant.* (2013) 22:653–61. doi: 10.3727/096368912X655154
122. Tollervey JR, Wang Z, Hortobagyi T, Witten JT, Zarnack K, Kayikci M, et al. Analysis of alternative splicing associated with aging and neurodegeneration in the human brain. *Genome Res.* (2011) 21:1572–82. doi: 10.1101/gr.122226.111
123. Anderson SL, Coli R, Daly IW, Kichula EA, Rork MJ, Volpi SA, et al. Familial dysautonomia is caused by mutations of the IKAP gene. *Am J Hum Genet.* (2001) 68:753–8. doi: 10.1086/318808
124. Licatalosi DD, Darnell RB. Splicing regulation in neurologic disease. *Neuron.* (2006) 52:93–101. doi: 10.1016/j.neuron.2006.09.017
125. Li D, McIntosh CS, Mastaglia FL, Wilton SD, Aung-Htut MT. Neurodegenerative diseases: a hotbed for splicing defects and the potential therapies. *Transl Neurodegener.* (2021) 10:16. doi: 10.1186/s40035-021-00240-7
126. Holm A, Hansen SN, Klitgaard H, Kauppinen S. Clinical advances of RNA therapeutics for treatment of neurological and neuromuscular diseases. *RNA Biol.* (2022) 19:594–608. doi: 10.1080/15476286.2022.2066334
127. Amosii L, Long C, Li H, Mireault AA, Shelton JM, Sanchez-Ortiz E, et al. Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. *Sci Transl Med.* (2017) 9(418):eaan8081. doi: 10.1126/scitranslmed.aan8081
128. Puttaraju M, Jamison SF, Mansfield SG, Garcia-Blanco MA, Mitchell LG. Spliceosome-mediated RNA trans-splicing as a tool for gene therapy. *Nat Biotechnol.* (1999) 17:246–52. doi: 10.1038/6986
129. Rindt H, Yen PF, Thebeau CN, Peterson TS, Weisman GA, Lorson CL. Replacement of huntingtin exon 1 by trans-splicing. *Cell Mol Life Sci.* (2012) 69:4191–204. doi: 10.1007/s00018-012-1083-5
130. Lorain S, Peccate C, Le Hir M, Griffith G, Philippi S, Precigout G, et al. Dystrophin rescue by trans-splicing: a strategy for DMD genotypes not eligible for exon skipping approaches. *Nucleic Acids Res.* (2013) 41:8391–402. doi: 10.1093/nar/gkt621
131. Avale ME, Rodriguez-Martin T, Gallo JM. Trans-splicing correction of tau isoform imbalance in a mouse model of tau mis-splicing. *Hum Mol Genet.* (2013) 22:2603–11. doi: 10.1093/hmg/ddt108
132. Shuai S, Suzuki H, Diaz-Navarro A, Nadeu F, Kumar SA, Gutierrez-Fernandez A, et al. The U1 spliceosomal RNA is recurrently mutated in multiple cancers. *Nature.* (2019) 574:712–6. doi: 10.1038/s41586-019-1651-z
133. Suzuki H, Kumar SA, Shuai S, Diaz-Navarro A, Gutierrez-Fernandez A, De Antonellis P, et al. Recurrent noncoding U1 snRNA mutations drive cryptic splicing in SHH medulloblastoma. *Nature.* (2019) 574:707–11. doi: 10.1038/s41586-019-1650-0
134. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature.* (2011) 478:64–9. doi: 10.1038/nature10496
135. Graubert TA, Shen D, Ding L, Okeyo-Owuor T, Lunn CL, Shao J, et al. Recurrent mutations in the U2AF1 splicing factor in myelodysplastic syndromes. *Nat Genet.* (2011) 44:53–7. doi: 10.1038/ng.1031
136. Corrigendum. Comprehensive molecular profiling of lung carcinoma. *Can Genome Atlas Res Netw. Nat.* (2014) 511(7511):543–50. doi: 10.1038/nature13385
137. Bailey P, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, et al. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature.* (2016) 531:47–52. doi: 10.1038/nature16965
138. Brooks AN, Choi PS, de Waal L, Sharifnia T, Imielinski M, Saksena G, et al. A pan-cancer analysis of transcriptome changes associated with somatic mutations in U2AF1 reveals commonly altered splicing events. *PLoS One.* (2014) 9:e87361. doi: 10.1371/journal.pone.0087361
139. Przychodzen B, Jerez A, Guinta K, Sekeres MA, Padgett R, Maciejewski JP, et al. Patterns of missplicing due to somatic U2AF1 mutations in myeloid neoplasms. *Blood.* (2013) 122:999–1006. doi: 10.1182/blood-2013-01-480970
140. Lopez-Canovas JL, Del Rio-Moreno M, Garcia-Fernandez H, Jimenez-Vacas JM, Moreno-Montilla MT, Sanchez-Frias ME, et al. Splicing factor SF3B1 is overexpressed and implicated in the aggressiveness and survival of hepatocellular carcinoma. *Cancer Lett.* (2021) 496:72–83. doi: 10.1016/j.canlet.2020.10.010
141. Papaemmanuil E, Cazzola M, Boultonwood J, Malcovati L, Vyas P, Bowen D, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. *New Engl J Med.* (2011) 365:1384–95. doi: 10.1056/NEJMoa1103283
142. Furney SJ, Pedersen M, Gentien D, Dumont AG, Rapinat A, Desjardins L, et al. SF3B1 mutations are associated with alternative splicing in uveal melanoma. *Cancer Discovery.* (2013) 3:1122–9. doi: 10.1158/2159-8290.CD-13-0330
143. Maguire SL, Leonidou A, Wai P, Marchio C, Ng CK, Sapino A, et al. SF3B1 mutations constitute a novel therapeutic target in breast cancer. *J Pathol.* (2015) 235:571–80. doi: 10.1002/path.2015.235.issue-4
144. Gentien D, Kosmider O, Nguyen-Khac F, Albad B, Rapinat A, Dumont AG, et al. A common alternative splicing signature is associated with SF3B1 mutations in Malignancies from different cell lineages. *Leukemia.* (2014) 28:1355–7. doi: 10.1038/leu.2014.28
145. Obeng EA, Chappell RJ, Seiler M, Chen MC, Campagna DR, Schmidt PJ, et al. Physiologic expression of sf3b1(K700E) causes impaired erythropoiesis, aberrant splicing, and sensitivity to therapeutic spliceosome modulation. *Cancer Cell.* (2016) 30:404–17. doi: 10.1016/j.ccell.2016.08.006
146. Zhou Z, Gong Q, Wang Y, Li M, Wang L, Ding H, et al. The biological function and clinical significance of SF3B1 mutations in cancer. *biomark Res.* (2020) 8:38. doi: 10.1186/s40364-020-00220-5
147. Alsafadi S, Houy A, Battistella A, Popova T, Wassef M, Henry E, et al. Cancer-associated SF3B1 mutations affect alternative splicing by promoting alternative branchpoint usage. *Nat Commun.* (2016) 7:10615. doi: 10.1038/ncomms10615
148. Sun G, Zhou H, Chen K, Zeng J, Zhang Y, Yan L, et al. Retraction Note: HnRNP A1 - mediated alternative splicing of CCDC50 contributes to cancer progression of clear cell renal cell carcinoma via ZNF395. *J Exp Clin Cancer Res.* (2023) 42:43. doi: 10.1186/s13046-023-02613-4
149. Cogoi S, Rapozzi V, Cauci S, Xodo LE. Critical role of hnRNP A1 in activating KRAS transcription in pancreatic cancer cells: A molecular mechanism involving G4 DNA. *Biochim Biophys Acta Gen Subj.* (2017) 1861:1389–98. doi: 10.1016/j.bbagen.2016.11.031
150. Chen Y, Liu J, Wang W, Xiang L, Wang J, Liu S, et al. High expression of hnRNP A1 promotes cell invasion by inducing EMT in gastric cancer. *Oncol Rep.* (2018) 39:1693–701. doi: 10.3892/or.2018.6273
151. Ryu HG, Jung Y, Lee N, Seo JY, Kim SW, Lee KH, et al. HNRNP A1 promotes lung cancer cell proliferation by modulating VRK1 translation. *Int J Mol Sci.* (2021) 22(11):5506. doi: 10.3390/ijms22115506
152. Loh TJ, Moon H, Cho S, Jang H, Liu YC, Tai H, et al. CD44 alternative splicing and hnRNP A1 expression are associated with the metastasis of breast cancer. *Oncol Rep.* (2015) 34:1231–8. doi: 10.3892/or.2015.4110
153. Lv Y, Zhang W, Zhao J, Sun B, Qi Y, Ji H, et al. SRSF1 inhibits autophagy through regulating Bcl-x splicing and interacting with PIK3C3 in lung cancer. *Signal Transduct Target Ther.* (2021) 6:108. doi: 10.1038/s41392-021-00495-6
154. Karni R, de StanChina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat Struct Mol Biol.* (2007) 14:185–93. doi: 10.1038/nsmb1209
155. Comiskey DF Jr, Montes M, Khurshid S, Singh RK, Chandler PS. SRSF2 regulation of MDM2 reveals splicing as a therapeutic vulnerability of the p53 pathway. *Mol Cancer Res.* (2020) 18:194–203. doi: 10.1158/1541-7786.MCR-19-0541
156. Lei S, Zhang B, Huang L, Zheng Z, Xie S, Shen L, et al. SRSF1 promotes the inclusion of exon 3 of SRA1 and the invasion of hepatocellular carcinoma cells by interacting with exon 3 of SRA1pre-mRNA. *Cell Death Discovery.* (2021) 7:117. doi: 10.1038/s41420-021-00498-w
157. Liu H, Gong Z, Li K, Zhang Q, Xu Z, Xu Y. SRPK1/2 and PP1alpha exert opposite functions by modulating SRSF1-guided MKNK2 alternative splicing in colon adenocarcinoma. *J Exp Clin Cancer Res.* (2021) 40:75. doi: 10.1186/s13046-021-01877-y
158. Jiang L, Huang J, Higgs BW, Hu Z, Xiao Z, Yao X, et al. Genomic landscape survey identifies SRSF1 as a key oncogene in small cell lung cancer. *PLoS Genet.* (2016) 12:e1005895. doi: 10.1371/journal.pgen.1005895
159. Anczukow O, Akerman M, Clery A, Wu J, Shen C, Shirole NH, et al. SRSF1-regulated alternative splicing in breast cancer. *Mol Cell.* (2015) 60:105–17. doi: 10.1016/j.molcel.2015.09.005
160. Jensen MA, Wilkinson JE, Krainer AR. Splicing factor SRSF6 promotes hyperplasia of sensitized skin. *Nat Struct Mol Biol.* (2014) 21:189–97. doi: 10.1038/nsmb.2756
161. Wan L, Yu W, Shen E, Sun W, Liu Y, Kong J, et al. SRSF6-regulated alternative splicing that promotes tumour progression offers a therapy target for colorectal cancer. *Gut.* (2019) 68:118–29. doi: 10.1136/gutjnl-2017-314983

162. Cohen-Eliav M, Golan-Gerstl R, Siegfried Z, Andersen CL, Thorsen K, Orntoft TF, et al. The splicing factor SRSF6 is amplified and is an oncoprotein in lung and colon cancers. *J pathol.* (2013) 229:630–9. doi: 10.1002/path.4129
163. Kong-Beltran M, Seshagiri S, Zha J, Zhu W, Bhawe K, Mendoza N, et al. Somatic mutations lead to an oncogenic deletion of met in lung cancer. *Cancer Res.* (2006) 66:283–9. doi: 10.1158/0008-5472.CAN-05-2749
164. Onozato R, Kosaka T, Kuwano H, Sekido Y, Yatabe Y, Mitsudomi T. Activation of MET by gene amplification or by splice mutations deleting the juxtamembrane domain in primary resected lung cancers. *J Thorac Oncol.* (2009) 4:5–11. doi: 10.1097/JTO.0b013e3181913e0e
165. Schrock AB, Frampton GM, Suh J, Chalmers ZR, Rosenzweig M, Erlich RL, et al. Characterization of 298 patients with lung cancer harboring MET exon 14 skipping alterations. *J Thorac Oncol.* (2016) 11:1493–502. doi: 10.1016/j.jtho.2016.06.004
166. Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Langdon WY, et al. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol Cell.* (2001) 8:995–1004. doi: 10.1016/S1097-2765(01)00378-1
167. Awad MM, Lee JK, Madison R, Classon A, Kmak J, Frampton GM, et al. Characterization of 1,387 NSCLCs with MET exon 14 (METex14) skipping alterations (SA) and potential acquired resistance (AR) mechanisms. *J Clin Oncol.* (2020) 38. doi: 10.1200/JCO.2020.38.15_suppl.9511
168. Heist RS, Shim HS, Gingipally S, Mino-Kenudson M, Le L, Gainor JF, et al. MET exon 14 skipping in non-small cell lung cancer. *Oncologist.* (2016) 21. doi: 10.1634/theoncologist.2015-0510
169. Gorlov IP, Gorlova OY, Frazier ML, Amos CI. Missense mutations in hMLH1 and hMSH2 are associated with exonic splicing enhancers. *Am J Hum Genet.* (2003) 73:1157–61. doi: 10.1086/378819
170. Liu T, Tannergard P, Hackman P, Rubio C, Kressner U, Lindmark G, et al. Missense mutations in hMLH1 associated with colorectal cancer. *Hum Genet.* (1999) 105:437–41. doi: 10.1007/s004399900160
171. Wijnen J, Khan PM, Vasen H, Menko F, van der Klift H, van den Broek M, et al. Majority of hMLH1 mutations responsible for hereditary nonpolyposis colorectal cancer cluster at the exonic region 15–16. *Am J Hum Genet.* (1996) 58:300–7.
172. Stella A, Wagner A, Shito K, Lipkin SM, Watson P, Guanti G, et al. A nonsense mutation in MLH1 causes exon skipping in three unrelated HNPCC families. *Cancer Res.* (2001) 61:7020–4.
173. Clarke LA, Veiga I, Isidro G, Jordan P, Ramos JS, Castedo S, et al. Pathological exon skipping in an HNPCC proband with MLH1 splice acceptor site mutation. *Genes Chromosomes Cancer.* (2000) 29:367–70. doi: 10.1002/1098-2264(2000)9999:9999::AID-GCC1051>3.0.CO;2-V
174. Tanko Q, Franklin B, Lynch H, Knezetic J. A hMLH1 genomic mutation and associated novel mRNA defects in a hereditary non-polyposis colorectal cancer family. *Mutat Res.* (2002) 503:37–42. doi: 10.1016/S0027-5107(02)00031-3
175. Smeby J, Sveen A, Eilertsen IA, Danielsen SA, Hoff AM, Eide PW, et al. Transcriptional and functional consequences of TP53 splice mutations in colorectal cancer. *Oncogenesis.* (2019) 8:35. doi: 10.1038/s41389-019-0141-3
176. Bodner SM, Minna JD, Jensen SM, D'Amico D, Carbone D, Mitsudomi T, et al. Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. *Oncogene.* (1992) 7:743–9.
177. Surget S, Khoury MP, Bourdon JC. Uncovering the role of p53 splice variants in human Malignancy: a clinical perspective. *Onco Targets Ther.* (2013) 7:57–68. doi: 10.2147/OTT.S53876
178. Bourdon JC. p53 and its isoforms in cancer. *Br J cancer.* (2007) 97:277–82. doi: 10.1038/sj.bjc.6603886
179. Leroy B, Anderson M, Soussi T. TP53 mutations in human cancer: database reassessment and prospects for the next decade. *Hum Mutat.* (2014) 35:672–88. doi: 10.1002/humu.2014.35.issue-6
180. Bamopoulos SA, Batcha AMN, Jurinovic V, Rothenberg-Thurley M, Janke H, Ksienzyk B, et al. Clinical presentation and differential splicing of SRSF2, U2AF1 and SF3B1 mutations in patients with acute myeloid leukemia. *Leukemia.* (2020) 34:2621–34. doi: 10.1038/s41375-020-0839-4
181. Martin M, Masshofer L, Temming P, Rahmann S, Metz C, Bornfeld N, et al. Exome sequencing identifies recurrent somatic mutations in EIF1AX and SF3B1 in uveal melanoma with disomy 3. *Nat Genet.* (2013) 45:933–6. doi: 10.1038/ng.2674
182. Group PTC, Calabrese C, Davidson NR, Demircioglu D, Fonseca NA, He Y, et al. Genomic basis for RNA alterations in cancer. *Nature.* (2020) 578:129–36. doi: 10.1038/s41586-020-1970-0
183. Puttick C, Jones TP, Leung MM, Galvez-Cancino F, Liu J, Varas-Godoy M, et al. MHC Hammer reveals genetic and non-genetic HLA disruption in cancer evolution. *Nat Genet.* (2024) 56:2121–31. doi: 10.1038/s41588-024-01883-8
184. Wei L, Li Y, Chen J, Wang Y, Wu J, Yang H, et al. Alternative splicing in ovarian cancer. *Cell Commun Signal.* (2024) 22:507. doi: 10.1186/s12964-024-01880-8
185. Cho N, Kim SY, Lee SG, Park C, Choi S, Kim EM, et al. Alternative splicing of PBRM1 mediates resistance to PD-1 blockade therapy in renal cancer. *EMBO J.* (2024) 43(22):5421–44. doi: 10.1038/s44318-024-00262-7
186. Lu SX, De Neef E, Thomas JD, Sabio E, Rousseau B, Gigoux M, et al. Pharmacologic modulation of RNA splicing enhances anti-tumor immunity. *Cell.* (2021) 184:4032–47 e31. doi: 10.1016/j.cell.2021.05.038
187. Carvalho T, Martins S, Rino J, Marinho S, Carmo-Fonseca M. Pharmacological inhibition of the spliceosome subunit SF3b triggers exon junction complex-independent nonsense-mediated decay. *J Cell sci.* (2017) 130:1519–31. doi: 10.1242/jcs.202200
188. Mahoney KM, Shukla SA, Patsoukis N, Chaudhri A, Browne EP, Arazi A, et al. A secreted PD-L1 splice variant that covalently dimerizes and mediates immunosuppression. *Cancer Immunol Immunother.* (2019) 68:421–32. doi: 10.1007/s00262-018-2282-1
189. Jain M, Abu-Shumays R, Olsen HE, Akeson M. Advances in nanopore direct RNA sequencing. *Nat Methods.* (2022) 19:1160–4. doi: 10.1038/s41592-022-01633-w
190. Krizanovic K, Echchiki A, Roux J, Sikic M. Evaluation of tools for long read RNA-seq splice-aware alignment. *Bioinformatics.* (2018) 34:748–54. doi: 10.1093/bioinformatics/btx668
191. Mehmood A, Laiho A, Venalainen MS, McGlinchey AJ, Wang N, Elo LL. Systematic evaluation of differential splicing tools for RNA-seq studies. *Brief Bioinform.* (2020) 21:2052–65. doi: 10.1093/bib/bbz126
192. Chhangawala S, Rudy G, Mason CE, Rosenfeld JA. The impact of read length on quantification of differentially expressed genes and splice junction detection. *Genome Biol.* (2015) 16:131. doi: 10.1186/s13059-015-0697-y
193. Ziegenhain C, Vieth B, Parekh S, Reinus B, Guillaumet-Adkins A, Smets M, et al. Comparative analysis of single-cell RNA sequencing methods. *Mol Cell.* (2017) 65:631–43 e4. doi: 10.1016/j.molcel.2017.01.023
194. Song Y, Botvinnik OB, Lovci MT, Kakaradov B, Liu P, Xu JL, et al. Single-cell alternative splicing analysis with expedition reveals splicing dynamics during neuron differentiation. *Mol Cell.* (2017) 67:148–61 e5. doi: 10.1016/j.molcel.2017.06.003
195. Illumina. Available online at: <https://emea.support.illumina.com/bulletins/2020/04/maximum-read-length-for-illumina-sequencing-platforms.html>.
196. Available online at: <https://nanoporetech.com/resource-centre/application-note/isoform-detection-single-cell-and-spatial-resolution>.
197. Heywood SM, Kennedy DS. Purification of myosin translational control RNA and its interaction with myosin messenger RNA. *Biochemistry.* (1976) 15:3314–9. doi: 10.1021/bi00660a023
198. Bester AJ, Kennedy DS, Heywood SM. Two classes of translational control RNA: their role in the regulation of protein synthesis. *Proc Natl Acad Sci U S A.* (1975) 72:1523–7. doi: 10.1073/pnas.72.4.1523
199. Paterson BM, Roberts BE, Kuff EL. Structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. *Proc Natl Acad Sci U S A.* (1977) 74:4370–4. doi: 10.1073/pnas.74.10.4370
200. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci U S A.* (1978) 75:280–4. doi: 10.1073/pnas.75.1.280
201. Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci U S A.* (1978) 75:285–8. doi: 10.1073/pnas.75.1.285
202. Donis-Keller H. Site specific enzymatic cleavage of RNA. *Nucleic Acids Res.* (1979) 7:179–92. doi: 10.1093/nar/7.1.179
203. Sinha ND, Biernat J, McManus J, Koster H. Polymer support oligonucleotide synthesis XVIII: use of beta-cyanoethyl-N,N-dialkylamino-/N-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. *Nucleic Acids Res.* (1984) 12:4539–57. doi: 10.1093/nar/12.11.4539
204. Huang Y, Knouse KW, Qiu S, Hao W, Padial NM, Vantourout JC, et al. A P(V) platform for oligonucleotide synthesis. *Science.* (2021) 373(6560):1265–70. doi: 10.1126/science.abi9727
205. Tamm I, Dorken B, Hartmann G. Antisense therapy in oncology: new hope for an old idea? *Lancet.* (2001) 358:489–97. doi: 10.1016/S0140-6736(01)05629-X
206. Martin Egli MM. Chemistry, structure and function of approved oligonucleotide therapeutics. *Nucleic Acids Res.* (2023) 51:2529–73. doi: 10.1093/nar/gkad067
207. Mourich DV, Oda SK, Schnell FJ, Crumley SL, Hauck LL, Moentenich CA, et al. Alternative splice forms of CTLA-4 induced by antisense mediated splice-switching influences autoimmune diabetes susceptibility in NOD mice. *Nucleic Acid Ther.* (2014) 24:114–26. doi: 10.1089/nat.2013.0449
208. Graziewicz MA, Tarrant TK, Buckley B, Roberts J, Fulton L, Hansen H, et al. An endogenous TNF-alpha antagonist induced by splice-switching oligonucleotides reduces inflammation in hepatitis and arthritis mouse models. *Mol Ther.* (2008) 16:1316–22. doi: 10.1038/mt.2008.85
209. Bauman J, Jearawiriyapaisarn N, Kole R. Therapeutic potential of splice-switching oligonucleotides. *Oligonucleotides.* (2009) 19:1–13. doi: 10.1089/oli.2008.0161
210. Mendell JR, Goemans N, Lowes LP, Alfano LN, Berry K, Shao J, et al. Longitudinal effect of eteplirsen versus historical control on ambulation in Duchenne muscular dystrophy. *Ann Neurol.* (2016) 79:257–71. doi: 10.1002/ana.24555

211. Crooke ST, Vickers TA, Lima WF, Wu H-J. *Antisense Drug Technology: Principles, Strategies, and Applications*. Crooke ST, editor. Boca Raton, Fla., United States: CRC Press (2008) p. 3–46.
212. Swayze EE, Bhat B. *Antisense Drug Technology: Principles, Strategies, and Applications*. Crooke ST, editor. Boca Raton, Fla., United States: CRC Press (2008) p. 143–82.
213. Henry S. *Antisense Drug Technology-Principles, Strategies, and Applications*. Crooke ST, editor. Boca Raton, Fla., United States: CRC Press (2008) p. 327–64.
214. Gleave ME, Monia BP. Antisense therapy for cancer. *Nat Rev Cancer*. (2005) 5:468–79. doi: 10.1038/nrc1631
215. Eckstein F. Phosphorothioates, essential components of therapeutic oligonucleotides. *Nucleic Acid Ther*. (2014) 24:374–87. doi: 10.1089/nat.2014.0506
216. Sands H, Gorey-Feret LJ, Cocuzza AJ, Hobbs FW, Chidester D, Trainor GL. Biodistribution and metabolism of internally 3H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate. *Mol Pharmacol*. (1994) 45:932–43.
217. Bennett CF, Swayze EE. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu Rev Pharmacol Toxicol*. (2010) 50:259–93. doi: 10.1146/annurev.pharmtox.010909.105654
218. Wang S, Allen N, Vickers TA, Revenko AS, Sun H, Liang XH, et al. Cellular uptake mediated by epidermal growth factor receptor facilitates the intracellular activity of phosphorothioate-modified antisense oligonucleotides. *Nucleic Acids Res*. (2018) 46:3579–94. doi: 10.1093/nar/gky145
219. Brown DA, Kang SH, Gryaznov SM, DeDionisio L, Heidenreich O, Sullivan S, et al. Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. *J Biol Chem*. (1994) 269:26801–5. doi: 10.1016/S0021-9258(18)47090-1
220. Iversen PL. *Antisense Drug Technology*. Crooke ST, editor. Boca Raton, FL: CRC Press (2008) p. 565–82.
221. Summerton J. Morpholino antisense oligomers: the case for an RNase H-independent structural type. *Biochim Biophys Acta*. (1999) 1489:141–58. doi: 10.1016/S0167-4781(99)00150-5
222. Geary RS. Antisense oligonucleotide pharmacokinetics and metabolism. *Expert Opin Drug Metab Toxicol*. (2009) 5:381–91. doi: 10.1517/17425250902877680
223. Langner HK, Jastrzebska K, Caruthers MH. Synthesis and characterization of thiophosphoramidate morpholino oligonucleotides and chimeras. *J Am Chem Soc*. (2020) 142:16240–53. doi: 10.1021/jacs.0c04335
224. Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kirschner J, et al. Nusinersen versus sham control in infantile-onset spinal muscular atrophy. *N Engl J Med*. (2017) 377:1723–32. doi: 10.1056/NEJMoa1702752
225. De Vivo DC, Hwu W-L, Reyna SP, Farwell W, Gheuens S, Sun P, et al. Interim efficacy and safety results from the phase 2 NURTURE study evaluating nusinersen in presymptomatic infants with spinal muscular atrophy. *Neurology*. (2017) 2017: S46.003.
226. Duan D, Goemans N, Takeda S, Mercuri E, Aartsma-Rus A. Duchenne muscular dystrophy. *Nat Rev Dis Primers*. (2021) 7:13. doi: 10.1038/s41572-021-00248-3
227. Lim KR, Maruyama R, Yokota T. Eteplirsen in the treatment of Duchenne muscular dystrophy. *Drug Des Devel Ther*. (2017) 11:533–45. doi: 10.2147/DDDT.S97635
228. Heo YA. Golodirsen: first approval. *Drugs*. (2020) 80:329–33. doi: 10.1007/s40265-020-01267-2
229. Dhillon S. Viltolarsen: first approval. *Drugs*. (2020) 80:1027–31. doi: 10.1007/s40265-020-01339-3
230. Shirley M. Casimersen: first approval. *Drugs*. (2021) 81:875–9. doi: 10.1007/s40265-021-01512-2
231. Crooke ST, Liang XH, Baker BF, Crooke RM. Antisense technology: A review. *J Biol Chem*. (2021) 296:100416. doi: 10.1016/j.jbc.2021.100416
232. Yang R, Feng X, Arias-Cavieres A, Mitchell RM, Polo A, Hu K, et al. Upregulation of SYNGAP1 expression in mice and human neurons by redirecting alternative splicing. *Neuron*. (2023) 111:1637–50 e5. doi: 10.1016/j.neuron.2023.02.021
233. Yuan Y, Lopez-Santiago L, Denomme N, Chen C, O'Malley HA, Hodges SL, et al. ASO restores excitability, GABA signalling and sodium current density in a model of Dravet syndrome. *Brain*. (2024) 147(4):1231–46. doi: 10.1093/brain/awad349
234. Kim H, Lenoir S, Helfricht A, Jung T, Karneva ZK, Lee Y, et al. A pathogenic proteolysis-resistant huntingtin isoform induced by an antisense oligonucleotide maintains huntingtin function. *JCI Insight*. (2022) 7(17): e154108. doi: 10.1172/jci.insight.154108
235. Shah S, Sharp KJ, Raju Ponny S, Lee J, Watts JK, Berry-Kravis E, et al. Antisense oligonucleotide rescue of CGG expansion-dependent FMR1 mis-splicing in fragile X syndrome restores FMRP. *Proc Natl Acad Sci U S A*. (2023) 120:e2302534120. doi: 10.1073/pnas.2302534120
236. Oren YS, Avizur-BarChad O, Ozeri-Galai E, Elgrabli R, Schirelman MR, Blinder T, et al. Antisense oligonucleotide splicing modulation as a novel Cystic Fibrosis therapeutic approach for the W1282X nonsense mutation. *J cystic fibrosis: Off J Eur Cystic Fibrosis Society*. (2022) 21:630–6. doi: 10.1016/j.jcf.2021.12.012
237. Michaels WE, Pena-Rasgado C, Kotaria R, Bridges RJ, Hastings ML. Open reading frame correction using splice-switching antisense oligonucleotides for the treatment of cystic fibrosis. *Proc Natl Acad Sci U S A*. (2022) 119(3):e2114886119. doi: 10.1073/pnas.2114886119
238. Dewaele M, Tabaglio T, Willekens K, Bezzi M, Teo SX, Low DH, et al. Antisense oligonucleotide-mediated MDM4 exon 6 skipping impairs tumor growth. *J Clin Invest*. (2016) 126:68–84. doi: 10.1172/JCI82534
239. Oh DY, Bang YJ. HER2-targeted therapies - a role beyond breast cancer. *Nat Rev Clin Oncol*. (2020) 17:33–48. doi: 10.1038/s41571-019-0268-3
240. Wan J, Sazani P, Kole R. Modification of HER2 pre-mRNA alternative splicing and its effects on breast cancer cells. *Int J Cancer*. (2009) 124:772–7. doi: 10.1002/ijc.v124:4
241. Pankratova S, Nielsen BN, Shiraishi T, Nielsen PE. PNA-mediated modulation and redirection of Her-2 pre-mRNA splicing: specific skipping of erbB-2 exon 19 coding for the ATP catalytic domain. *Int J Oncol*. (2010) 36:29–38.
242. Khurshid S, Montes M, Comiskey DF Jr., Shane B, Matsa E, Jung F, et al. Splice-switching of the insulin receptor pre-mRNA alleviates tumorigenic hallmarks in rhabdomyosarcoma. *NPJ Precis Oncol*. (2022) 6:1. doi: 10.1038/s41698-021-00245-5
243. Wang Z, Jeon HY, Rigo F, Bennett CF, Krainer AR. Manipulation of PK-M mutually exclusive alternative splicing by antisense oligonucleotides. *Open Biol*. (2012) 2:120133. doi: 10.1098/rsob.120133
244. (2023). Available online at: <https://nanoporetech.com/about-us/news/oxford-nanopore-announces-breakthrough-performance-simplex-single-molecule-accuracy-~:text=They%20detailed%20breakthrough%20performance%20in,enzyme%20engineering%20and%20improved%20models>.



OPEN ACCESS

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RECEIVED 24 March 2025

ACCEPTED 11 April 2025

PUBLISHED 30 April 2025

CITATION

Chen X, Yuan Y, Zhou F, Huang X, Li L, Pu J, Zeng Y and Jiang X (2025) RNA m5C modification: from physiology to pathology and its biological significance.
Front. Immunol. 16:1599305.
doi: 10.3389/fimmu.2025.1599305

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RNA m5C modification: from physiology to pathology and its biological significance

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RNA 5-methylcytosine (m5C) modification is a crucial epitranscriptomic mark that regulates RNA stability, processing, and translation. Emerging evidence highlights its essential role in various physiological processes, including cellular differentiation, stem cell maintenance, and immune responses. Dysregulation of m5C modification has been implicated in multiple pathological conditions, particularly in cancer, neurodegenerative disorders, and metabolic diseases. This review provides a comprehensive overview of the molecular mechanisms governing m5C deposition, its functional consequences in normal physiology, and its contributions to disease pathogenesis. Furthermore, we discuss the potential of m5C as a biomarker and therapeutic target, offering new insights into its biological significance and clinical relevance.

KEYWORDS

5-methylcytosine, methods, physiology, pathology, biological significance, cancer immunotherapy

1 Introduction

To date, over 170 types of methylation modifications have been identified in RNA, including N6-methyladenosine (m6A) (1), 5-methylcytosine (m5C) (2), and 7-methylguanylate (m7G) (3). These modifications increase RNA complexity by affecting RNA tertiary structure, biogenesis, localization, and function, which are critical for cellular biological processes and cancer development. m5C methylation refers to the addition of a methyl group to the 5th carbon of the cytosine ring in DNA or RNA, which is a highly concentrated and reversible epigenetic modification (4). This modification was first discovered in DNA and later in RNA. RNA m5C modifications have widespread target sites, including messenger RNA (mRNA) and non-coding RNA (ncRNA), such as transfer RNA (tRNA), ribosomal RNA (rRNA), micro RNA (miRNA), small nuclear RNA (snRNA), and enhancer RNA (eRNA) (5).

With the continuous improvement of methylated RNA immunoprecipitation sequencing and liquid chromatography-mass spectrometry techniques, m5C modifications in mRNA have been found to affect various biological processes, such as mRNA stability, translation, splicing, and nucleocytoplasmic transport; DNA damage repair; cell proliferation and migration; and stem cell development, differentiation, and reprogramming (6–8). Previous research primarily focused on DNA, while studies on the function and regulatory mechanisms of m5C modifications in RNA are still in the early stages. In recent years, the development of methylation sequencing technologies has confirmed the presence of m5C methylation modifications in both coding and non-coding RNAs. RNA m5C methylation modifications rely mainly on methyltransferases (writers), demethylases (erasers), and binding proteins (readers) (9). Aberrant mRNA m5C modifications are associated with cancer, autoimmune diseases, and atherosclerosis (10).

In summary, 5-methylcytosine modification plays a crucial role in regulating gene expression, maintaining genomic stability, and influencing cellular differentiation. Its dynamic regulation, mediated by DNA methyltransferases and demethylases, ensures proper cellular function under physiological conditions. However, aberrant 5mC patterns are frequently associated with various pathological states, including cancer, neurological disorders, and autoimmune diseases. Understanding the mechanisms governing 5mC modification and its biological significance not only provides fundamental insights into epigenetic regulation but also offers potential therapeutic strategies for disease intervention. Future research should focus on deciphering the context-specific roles of 5mC and developing targeted approaches to modulate its function in disease treatment.

2 Regulatory mechanisms of RNA m5C methylation

RNA m5C methylation is a dynamic and reversible process, primarily regulated by three factors: m5C methyltransferases, demethylases, and m5C methylation binding proteins. RNA m5C methyltransferases mainly include NOL1/NOP2/sun (NSUN) methyltransferases and DNA methyltransferase-like DNMT2, which catalyze the formation of 5-methylcytosine (11). m5C methylation binding proteins function by recognizing and binding to m5C methylation sites, while demethylases catalyze the demethylation of RNA m5C.

2.1 m5C methyltransferases

m5C methyltransferases use S-adenosyl-L-methionine (SAM) as the methyl donor to transfer the methyl group to cytosine, forming 5-methylcytosine. Over ten RNA m5C methyltransferases have been identified, including the NSUN family, DNMT2, and the tRNA-specific methyltransferase TRDMT family (12, 13). The NSUN family proteins contain a Rossmann fold catalytic domain and a SAM

binding site. Members of the NSUN family include NSUN1–NSUN7 (14). NSUN1 directly binds to the 60S ribosomal precursor and catalyzes the m5C modification of human 28S rRNA. NSUN2 is the most extensively studied NSUN family member (15). It can catalyze the m5C methylation modification of various RNAs, including rRNA, tRNA, mRNA, mitochondrial RNA, and viral RNA. NSUN2-mediated m5C mRNA is widely distributed across all coding regions. NSUN2 performs various biological functions, such as regulating epithelial cell differentiation, HIV-1 transcription, and EB virus degradation (16). NSUN2 is highly expressed in several tumors, mediating tumorigenesis and progression. For instance, in gallbladder cancer, silencing NSUN2 inhibits the proliferation and tumor formation of gallbladder cancer cells (17). In liver cancer, the long non-coding RNA (lncRNA) H19 is a specific target of NSUN2. m5C-modified H19 promotes liver cancer development by recruiting Ras-GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) (18). NSUN3 is mainly localized to the mitochondria, catalyzing the methylation of the anticodon loop C34 site of mitochondrially encoded tRNA methionine (mt-tRNAMet) (19). NSUN4 is an rRNA-specific methyltransferase transported to the mitochondria in an N-terminal 26 amino acid motif-dependent manner (20). NSUN4 interacts with mitochondrial regulatory factor MTERF4, recruiting the mitochondrial large ribosomal subunit to promote mitochondrial ribosome assembly by methylating the 12S rRNA C911 site. NSUN5 is localized to the nucleolus and is also an rRNA-specific methyltransferase, catalyzing the methylation of the C2278 site in the IV domain of 25S rRNA (21). In colorectal cancer, highly expressed NSUN5 promotes tumor cell proliferation by regulating the cell cycle (22). NSUN6 is partially localized to the Golgi apparatus and centrosome and is a tRNA methylation regulator, catalyzing the methylation of C72 site tRNACys and tRNAThr, affecting tRNA biogenesis (23). NSUN6 expression is downregulated in tumors, and high NSUN6 expression is associated with better prognosis in some cancers (24). NSUN7 mediates the m5C methylation modification of enhancer RNA (eRNA) (25). DNMT2 possesses the sequence and structural characteristics of DNA methyltransferases and can catalyze cytosine DNA methylation (26). Additionally, DNMT2 catalyzes the methylation of C38 site tRNAAsp. DNMT2-catalyzed tRNA methylation plays important roles in tRNA processing, maintaining translation accuracy, stability, and differentiation, and protects against ribonuclease cleavage (27). Two other methyltransferases, TRM4A and TRM4B, specifically catalyze tRNA m5C methylation. In summary, methyltransferases are key regulatory factors of RNA m5C methylation, catalyzing the methylation of various RNAs (28). Although some studies have confirmed the crucial roles of methyltransferases in certain tumors, their roles and mechanisms in different tumor types remain to be elucidated.

2.2 m5C demethylases

The ten-eleven translocation (TET) family of demethylases is Fe (II) and α -ketoglutaric acid (α KG)-dependent dioxygenases,

including TET1, TET2, and TET3 (29, 30). TET3 is distributed in both the nucleus and cytoplasm, while TET1 and TET2 are mainly localized to the nucleus (31). The TET enzyme family can catalyze the oxidation of DNA 5-methyl-2'-deoxycytidine (5mdC) to form 5-hydroxymethyl-2'-deoxycytidine (5hmdC), 5-formyl-2'-deoxycytidine (5fdC), and 5-carboxyl-2'-deoxycytidine (5cadC) (32). TET enzymes also act as RNA demethylases, exhibiting activity on 5-methylcytidine (5mC) and its oxidative derivatives in coding and non-coding RNAs, including 5-hydroxymethylcytidine (5hmC), 5-formylcytidine (5fC), and 5-carboxycytidine (5carC) (33, 34). The TET family can catalyze various nucleic acid substrates, including dsDNA, ssDNA, ssRNA, and DNA-RNA hybrids (35). However, further research is needed to understand the structure and biological functions of TET enzymes and how to enhance the specificity and selectivity of TET-mediated oxidation.

2.3 m5C methylation binding proteins

The biological functions of RNA modifications are primarily associated with their binding proteins. The main m5C methylation binding proteins are ALYREF (Aly/REF export factor) and YBX1 (Y-box binding protein 1). ALYREF is a key component of the mRNA transport protein complex TREX (36). During mRNA nuclear export, ALYREF is first recruited to bind to the 5' end of mRNA mediated by CBP80 and to the 3' end mediated by PABPN1 (37). ALYREF further strengthens its binding to mRNA through direct interaction with the 3' end processing factor CstF64. In human HeLa cells and mouse tissues, ALYREF directly binds to mRNA m5C sites, promoting mRNA nucleocytoplasmic shuttling, with the binding affinity and nuclear export process mediated by NSUN2 (38). YBX1 is a newly discovered m5C binding protein that regulates mRNA stability in the cytoplasm. In bladder cancer, YBX1 recognizes and binds to m5C-modified mRNA through the indole ring of W65 in its cold-shock domain (CDS), stabilizing m5C-modified mRNA, thereby regulating mRNA metabolism (39–41). In lung cancer, YBX1 promotes tumor cell invasion, migration, and angiogenesis by directly binding to lncRNA LINC00312 (42). Recently, a notable study identified another novel RNA m5C methylation binding protein, SRSF2, and revealed its association with leukemia development. Further research found that the SRSF2P95H mutation in leukemia inhibits SRSF2 recognition of m5C, affecting mRNA alternative splicing mediated by SRSF2, and leukemia patients with impaired SRSF2-m5C binding have poor prognosis (43). YBX2 has recently been reported as a novel mammalian m5C-binding protein capable of undergoing liquid-liquid phase separation (LLPS) both *in vivo* and *in vitro* (44, 45). Other methylation binding proteins remain to be discovered and validated, and their regulatory mechanisms on RNA m5C modifications require further investigation.

3 The impact of m5C methylation on RNA

3.1 Impact of m5C methylation on mRNA

Extensive m5C methylation is present in mRNA, and its influence on mRNA function has become a research focus in recent years (46, 47). (1) Impact on mRNA translation: recent studies have shown a functional interdependence between m5C modifications and mRNA translation. However, m5C appears to have different effects depending on its location - specifically, it generally has negative effects in coding regions but can have positive effects in untranslated regions like the 3'-UTR. For example, in HeLa cells, m5C sites within coding regions are negatively correlated with translation efficiency (48). Another study demonstrated that NSUN2-induced m5C methylation, in collaboration with METTL3/METTL14-induced m6A methylation, mediates the methylation of the 3' -UTR of p21 mRNA, enhancing its translation efficiency (49). (2) Impact on mRNA Transport: Research has shown that m5C modifications are enriched in CG-rich regions and downstream of the start codon, playing a critical role in mRNA nuclear export (50). (3) Impact on mRNA Stability: In bladder cancer, YBX1 enhances the stability of m5C-modified mRNA by recruiting ELAVL1 (2). However, other studies have found no correlation or a negative correlation between m5C modification levels and mRNA stability (Figure 1). Thus, the effect of m5C methylation on mRNA stability remains to be further investigated.

3.2 Impact of m5C methylation on tRNA

m5C methylation regulates tRNA stability, cellular metabolism, and stress response. Studies have shown that m5C modifications mediated by NSUN2 and DNMT2 maintain tRNA stability and regulate cellular metabolism (14). In humans and mice, TRM4/NSUN2-mediated m5C methylation prevents tRNA degradation due to oxidative stress. DNMT2-mediated tRNA methylation protects tRNA from nucleases and regulates the stability of tRNA^{Asp}-GTC and tRNA^{Gly}-GCC (51, 52) (Figure 1).

3.3 Impact of m5C methylation on rRNA

m5C methylation regulates rRNA stability and ribosome synthesis. In the small subunit 12S rRNA, the m5C methyltransferase NSUN4 methylates cytosine 911 (m5C911) and forms a complex with MTERF4, ensuring the assembly of mature large and small subunit complexes (53). Loss of m5C2278 and G2288 methylation results in structural changes in 25S rRNA. When cells are exposed to hydrogen peroxide, the absence of

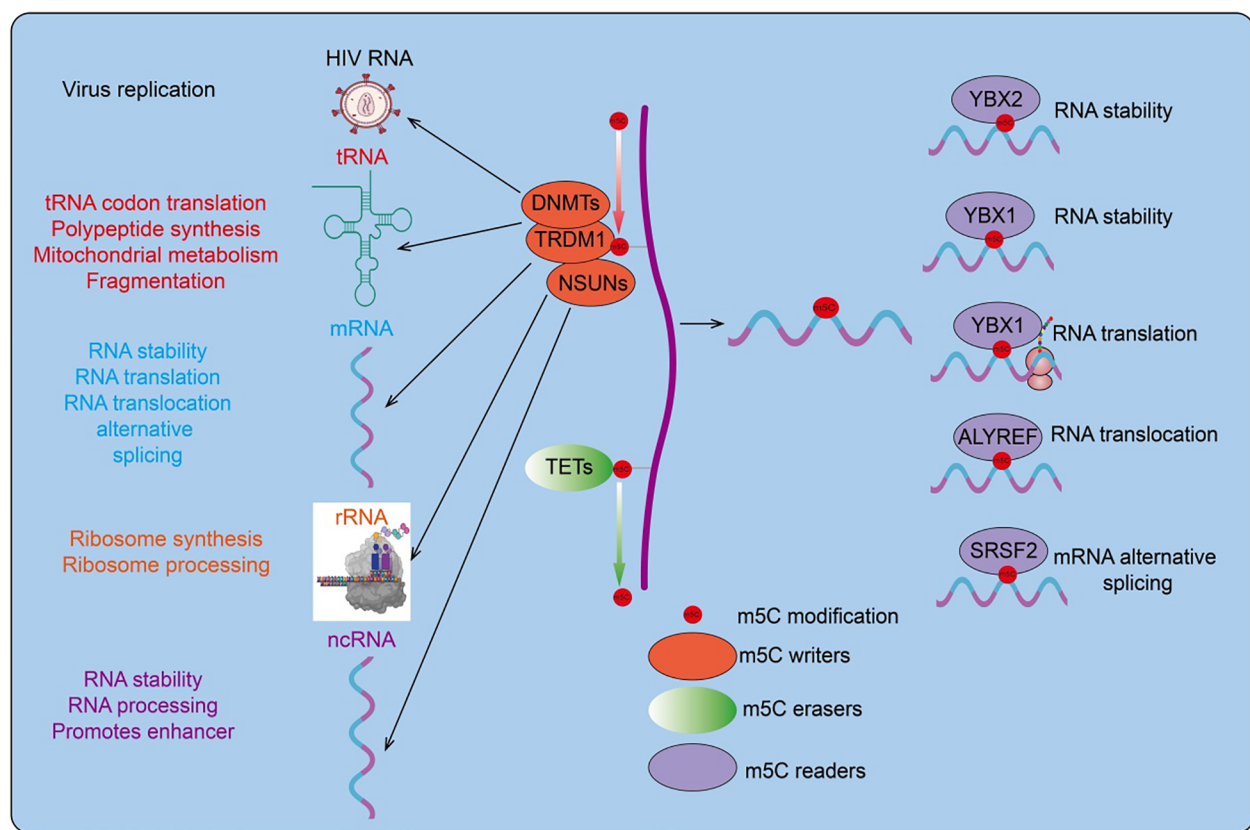


FIGURE 1

RNA m5C modification is a dynamic process. RNA m5C modification (5-methylcytosine modification) refers to the chemical modification where the cytosine residues in RNA molecules are methylated at the carbon 5 position. This modification is widely present in various types of RNA, including messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), and non-coding RNA (ncRNA). RNA m5C modification plays a crucial role in various biological processes.

Rcm1/NSUN5 leads to a more relaxed folding of sequences near 25S rRNA C2278, indicating that Rcm1/NSUN5 is crucial for maintaining rRNA stability under oxidative stress conditions (54) (Figure 1).

3.4 Impact of m5C methylation on other RNAs

m5C methylation also plays significant roles in viral RNA and lncRNA. For example, Recent studies have found that RNA cytosine-C(5)-methyltransferase (NSUN2) is upregulated in gastric cancer. NSUN2 enhances the expression of the long non-coding RNA NR_033928 through methylation modification. NR_033928, in turn, interacts with the IGF2BP3/HUR complex to upregulate the expression of glutaminase (GLS), thereby increasing the stability of GLS mRNA and promoting the progression of gastric cancer (55). m5C methylation in the interaction regions of lncRNA HOTAIR and XIST with chromatin-modifying complexes can affect XIST function by influencing its binding to the PRC2 complex (56). Viral RNAs exhibit extensive m5C methylation. Studies have shown that nucleolar protein NOP2/NSUN1 has been identified as an HIV-1

restriction factor. Functional studies confirm that NOP2 restricts HIV-1 replication. Depletion of NOP2 promotes the reactivation of latent HIV-1 proviruses in various cell lines and primary CD4+ T cells. Mechanistic studies show that NOP2 binds to the HIV-1 5' LTR and competes with HIV-1 Tat protein for interaction with HIV-1 TAR RNA, facilitating the m5C methylation of TAR (57) (Figure 1). In summary, m5C methylation is widespread across various RNA types and may play crucial roles in their function. Current research on the impact of m5C methylation on RNA is still limited and contentious, necessitating further investigation.

4 Methods for detecting RNA m5C methylation

Current methods for detecting RNA m5C modification primarily include the following: (1) physicochemical methods, such as chromatography, mass spectrometry (MS), high-performance liquid chromatography (HPLC), and liquid chromatography-tandem mass spectrometry (LC-MS/MS); (2) chemical conversion methods combined with next-generation sequencing (NGS) technologies, such as RNA bisulfite sequencing (RNA-BisSeq) and Tet-assisted oxidation sequencing using

tungsten acid (Tawo-seq); (3) immunoprecipitation combined with NGS technologies, such as 5-aza-seq (miCLIP) with m5C-specific single-nucleotide crosslinking and immunoprecipitation; and (4) third-generation sequencing (TGS) based on differential electric signal, such as Nanopore-seq (58). In practice, the most commonly used methods in research are the three described below, which we will primarily focus on, discussing their advantages, disadvantages, and other relevant aspects.

4.1 m5C MeRIP-seq

This method allows the examination of gene m5C methylation levels across the entire transcriptome, as well as at the tRNA level. The technical principle is as follows: m5C-specific antibodies are incubated with randomly fragmented RNA, capturing the methylated fragments for sequencing. A parallel sequencing of a control (Input) sample is also performed. The control sample consists of RNA fragments that have not undergone immunoprecipitation (IP). This control helps eliminate background noise from non-specific binding of methylated fragments. By comparing the sequencing fragments from the immunoprecipitation (IP) sample and the Input sample, m5C RNA methylation sites can be mapped to the transcriptome, allowing the calculation of m5C methylation levels in the sample.

4.2 m5C BS-seq

Earlier RNA m5C modification detection primarily relied on bisulfite sequencing (BS-seq). In BS-seq, unmodified cytosine (C) is converted into uracil (U), whereas m5C remains unchanged. Therefore, m5C modifications can be identified by detecting the unconverted C. Although BS-seq is straightforward and convenient, and can achieve single-base resolution of m5C modification quantification, there are three main drawbacks: 1) It detects m5C indirectly, relying on efficient conversion of unmodified C. Incomplete conversion can lead to false positives; 2) Harsh reaction conditions may cause RNA degradation, limiting detection in samples with low starting amounts or low-abundance RNA; 3) The conversion of C to U reduces sequence complexity, which affects alignment accuracy and limits m5C detection in low-complexity RNA sequences (59).

4.3 m5C-TAC-seq (m5C detection strategy enabled by TET-assisted chemical labeling)

The core principle of this technique is to combine enzymatic reactions with chemical labeling. The optimized TET enzyme reaction oxidizes RNA m5C to f5C, which is then specifically labeled using azidophenylfluorone (AI). This labeling product not only results in a C-to-T transition but also allows for enrichment through click chemistry, enabling direct detection of m5C modification at single-base resolution. m5C-TAC-seq can be

applied to various types of RNA, including low-abundance, low-sequence complexity, and low-modification m5C sites. Additionally, it allows for the dynamic detection of m5C modifications in multiple biological processes, thus contributing to the understanding and exploration of the biological functions of RNA m5C modifications (60).

These methods each have distinct advantages and limitations, with m5C MeRIP-seq being widely used for its comprehensiveness, BS-seq providing straightforward quantification, and m5C-TAC-seq enabling high sensitivity for low-abundance RNA modifications.

5 The role of m5C in normal physiological processes

5-methylcytosine (m5C) plays a crucial role in normal physiological processes by regulating various aspects of RNA metabolism and gene expression. Here, we summarize the current understanding of the functions of m5C modification in neurodevelopment, autoimmune diseases, spermatogenesis, and embryonic development.

5.1 The role of m5C in neurodevelopment

Mutations in the m5C methyltransferase NSUN2 result in microcephaly and other neurological abnormalities in mice and humans, such as behavioral defects, speech delay, gait abnormalities, growth retardation, unusual appearance, and skin anomalies (61). In mice, the absence of NSUN2 leads to impaired neurodevelopment, inhibition of neuronal migration, and disrupted neural stem cell differentiation, causing the accumulation of intermediate progenitors and the loss of upper-layer neurons in the developing cortex (62). In *Drosophila*, loss of the NSUN2 homolog results in severe short-term memory deficits. Studies have found that angiogenin binds with higher affinity to tRNA lacking site-specific NSUN2-mediated methylation. The loss of m5C methylation increases angiogenin-mediated tRNA nuclear cleavage, leading to the accumulation of 5'tRNA-derived fragments, reducing protein translation rates, activating stress pathways, and causing reduced cell volume and increased apoptosis in cortical, hippocampal, and striatal neurons (63). In addition, inhibition of angiopoietin during embryogenesis can rescue the increased sensitivity of NSun2-deficient brains to oxidative stress (63). Studies have shown that loss of NSun2 function caused by autosomal recessive mutations is associated with human neurological abnormalities. Specifically, reduced NSun2 protein expression and an increased pTau/NSun2 ratio have been observed in the brains of Alzheimer's disease (AD) patients (64). Conditional knockout of NSun2 in mouse brains promotes a decrease in m6A levels of miR-125b and excessive phosphorylation of tau. Moreover, neuronal NSun2 levels are reduced by amyloid- β oligomers (A β O). Interestingly, A β O-induced tau phosphorylation and cytotoxicity in human neurons can be rescued by NSun2 overexpression (64).

5.2 The roles of m5C methylation in spermatogenesis

The m5C modifications mediated by the NSUN family play a significant role in various aspects of testicular differentiation and embryonic development (65, 66). For example, the absence of NSUN2 can lead to multiple mitotic disorders and multipolar spindles, resulting in cell death (67). Studies have shown that NSUN2 deficiency leads to reduced testis size, decreased spermatogonia count, and lack of mature sperm in mice (68). Further research has revealed that NSUN2 deficiency blocks the first meiotic division and induces apoptosis in pachytene spermatocytes (69). Another member of the NSUN family, NSUN7, is also highly expressed in the testes. Its absence results in decreased sperm motility and abnormal movement, ultimately causing infertility in mice (70). Additionally, mutations in NSUN7 have been found in patients with asthenozoospermia, leading to infertility (71). The m5C modification can be inherited by offspring and is crucial for mediating acquired traits (72). Research indicates that elevated levels of m5C and m2G modifications in tRNA-derived small RNAs (tsRNAs) in sperm from high-fat diet-fed male mice affect the formation of these tsRNAs, enabling the offspring to inherit the paternal high-fat phenotype. However, the mechanism by which m5C modifications regulate tsRNA formation in sperm from high-fat diet-fed male mice remains unclear (72). Studies have demonstrated that m5C modifications mediated by DNMT2 are involved in regulating the acquired high-fat phenotype in the offspring of high-fat diet-fed mice (73). These findings suggest that abnormal m5C modifications can be inherited by offspring, leading to phenotypic changes. However, the exact mechanism of this inheritance is not yet understood, necessitating further experiments to elucidate the role of m5C in spermatogenesis regulation and epigenetics.

5.3 The roles of m5C methylation in embryonic development

The m5C methylation modifications mediated by NSUN family proteins have been extensively studied in the regulation of embryonic formation. Initially, researchers demonstrated the presence of NSUN2 to NSUN7 in early mouse embryos and analyzed their roles and expression patterns in embryonic development (65). They found that the m5C levels in six different animals (mice, humans, zebrafish, fruit flies, *Xenopus tropicalis*, and *Xenopus laevis*) were high during the early embryonic stages but sharply declined after the maternal-to-zygotic transition (MZT), remaining at low levels during subsequent developmental stages (74). The absence of m5C methylation modifications in early embryos leads to delayed cell cycles, preventing the timely initiation of the MZT process (74). DNMT2, another methyltransferase for m5C modifications, when singly deficient, results in neonatal mice phenotypes similar to those with dual deficiencies in NSUN2 and DNMT2, exhibiting immature hematopoietic systems, reduced numbers of hematopoietic stem cells

and progenitor cells, and defects in cell-autonomous differentiation (75). However, its regulatory role in other animal embryonic developments has not yet been reported. The loss of the m5C recognition protein YBX1 disrupts zebrafish embryo cleavage and MZT processes, resulting in zygotic death post-fertilization (76). Further studies revealed that YBX1 influences normal embryonic development by inhibiting maternal mRNA translation (77). During the MZT process in zebrafish, YBX1 preferentially recognizes m5C-modified mRNA, maintaining its stability and inhibiting the translation of the maternal mRNA pool (76). The loss of YBX1 affects transcriptional activity during zygotic genome activation (ZGA) in goats and mice, with abnormal expression of splicing factors and mRNA decay genes in embryos, indicating that YBX1 impacts maternal mRNA decay, selective splicing, and transcriptional activity necessary for early embryonic development (78). This, in turn, affects early embryonic development. In *Drosophila melanogaster*, the *Drosophila Tet* homolog gene *cg43444* (*dtet*) is positively correlated with hm5C levels. *Dtet*-deficient flies survive the larval stage but die during pupation, suggesting that *dtet*-mediated hm5C plays a regulatory role in embryonic development (79). This also indirectly highlights the importance of m5C methylation modifications in embryonic development.

6 The role of m5C in cancer

Studies have shown that RNA m5C modification plays an important role in cancer progression and remodeling of the tumor immune microenvironment by influencing RNA stability and translation efficiency. Therefore, in this manuscript, we systematically summarized the expression and function of RNA m5C modification in tumors, which will help us understand the occurrence and development of tumors and provide new potential targets for cancer therapy (Tables 1, 2).

6.1 Nervous system tumors

In gliomas, the expression of m5C methyltransferases varies with different clinical and pathological tumor characteristics. A risk prediction model constructed using five m5C methyltransferase genes can predict patient survival and clinical features in gliomas. Cox regression analysis has shown that the model's risk score is an independent prognostic factor for gliomas (80). Additionally, in glioblastoma multiforme (GBM), most miRNAs exhibit m5C modification, with methylation of miRNA-181a-5p correlating with poor prognosis in GBM patients. Mechanistically, the m5C modification of miR-181a-5p, mediated by a complex containing DNMT3a and AGO4, inhibits the formation of miRNA-181a-5p/mRNA duplexes, resulting in the loss of its tumor-suppressive effects (81). In the U87 human glioma cell line, NSUN2 regulates tumor cell migration by modulating the autocrine chemokine (ATX)-lysophosphatidic acid (LPA) axis. NSUN2 methylates the cytosine at position 2756 in the 3'-UTR of ATX mRNA, enhancing ATX mRNA translation. The ATX-LPA pathway mediates cancer

TABLE 1 The functional roles and mechanisms of m5C modification regulators in different cancer types: a systematic summary.

Cancer type	m5C regulators	expression	Target genes	Molecular functions	Potential mechanisms	Ref
Glioma	NSUN2	up	ATX	Enhances glioma cells proliferation	Enhancing ATX mRNA translation	(82)
LC	NSUN6	up	NH23-H1	Inhibits cell proliferation, migration and EMT in LC	Controls NM23-H1 expression by modifying the 3'-UTR of NM23-H1 mRNA using m5C.	(89)
LC	NSUN2	up	NRF2	Governs NRF2-induced ferroptosis resistance in NSCLC	Maintains the expression of NRF2 via YBX1 in NSCLC cells	(87)
LC	NSUN2	up	QSOX1	Causes gefitinib resistance and cancer recurrence in NSCLC	Regulates YBX1 and QSOX1 in NSCLC	(2)
LC	ALYREF	up	YAP1	Enhances tumor progression in NSCLC	Interacts with LINC02159; increase the stability of YAP1 mRNA; activates Hippo and beta-catenin	(37)
ESCC	NSUN2	up	GBR2	Enhances oncogenesis and progression in ESCC	Enhances m5C modification of GRB2 mRNA and its stability; activates ERK/MAPK, PI3K/AKT	(90)
GC	NSUN2	up	LINC00324	Facilitates tumor angiogenesis in GEC	Induces LINC00324 stability through m5C modification; decreases CBX3 mRNA degradation; increases VEGFR2 transcription	(134)
GC	NSUN2	up	ERK1/2	Promotes chemosensitivity in GC	Increases ERK1/2 phosphorylation; regulates Bcl-2 and Bax	(135)
GC	NSUN2	up	FOXC2	Promotes proliferation, migration, and invasion of GC cells	FOXC2-AS1 facilitates NSUN2 recruitment to FOXC2 mRNA, enhancing its m5C modification and interaction with YBX1	(96)
GC	NSUN2	up	NTN1	Promotes neural invasion in GC	DIAPH2-AS1 stabilizes NSUN2 and enhances the m5C modification of NTN1	(94)
CRC	NSUN2	up	SKIL	Promotes tumorigenesis and progression of CRC	Increases SKIL mRNA stability	(136)
HCC	NSUN5	up	ZEED3	Promotes proliferation of HCC cells	Activates Wnt/ β -catenin signaling pathway	(137)
HCC	ALYREF	up	EGFR	Facilitates cell proliferation, invasion, and EMT in HCC	Induces m5C modification and increases the stabilization of EGFR mRNA and pSTAT3 activation.	(138)
HCC	NSUN2	up	SARS2	promotes the proliferation, colony formation, migration, and invasion of HCC cells	Mediates m5C of the SARS2 and activates the Wnt signaling pathway	(105)
AML	NSUN2	up	SRSF2	Increases the development of leukemia	Reduces NSUN2 expression lowers mRNA m5C levels, diminishes SRSF2 binding, and affects RNA splicing.	(43)
AML	YBX1	up	MYC and BCL2	Sustains the function of leukemia cells	Enhancing the stability of MYC and BCL2	(39)
AML	TET2		TSPAN13	Promotes leukemia development, leukemia stem cell migration/homing, and leukemia stem cell self-renewal	Increases the stability and expression of TSPAN13 transcripts	(110)
BLCA	NSUN2	up	HDGF	Promotes the proliferation and invasion of BLCA cells	Stabilizes HDGF mRNA	(139)
EC	NSUN2		SLC7A11	Promotes EC cell proliferation	Increased mRNA stability of SLC7A11	(113)
HNSC	NSUN2	up	TEAD1	Enhancing tumor cell proliferation and invasion of HNSC	Increased mRNA stability of TEAD1	(117)

Up, up-regulated in cancer.

cell migration. Moreover, ALYREF interacts with methylated ATX mRNA, facilitating its export from the nucleus to the cytoplasm. NSUN2 knockout inhibits U87 cell migration, which can be restored by the addition of LPA (82). In *in vivo* glioma models, NSUN5 exhibits high methylation in CpG island promoter regions, leading to reduced transcript levels and epigenetic silencing. Silencing of NSUN5 induces a loss of methylation at the C3782 site of 28S rRNA. Under stress conditions, the unmethylated state

TABLE 2 A summary of abbreviations and full names of different cancer types.

Abbreviation	Full Name	Abbreviation	Full Name
ACC	Adrenocortical Carcinoma	LUAD	Lung Adenocarcinoma
BLCA	Bladder Urothelial Carcinoma	LUSC	Lung Squamous Cell Carcinoma
BRCA	Breast Invasive Carcinoma	MESO	Malignant Mesothelioma
CESC	Cervical Squamous Cell Carcinoma	OV	Ovarian Serous Cystadenocarcinoma
CHOL	Cholangiocarcinoma	PAAD	Pancreatic Adenocarcinoma
COAD	Colon Adenocarcinoma	PCPG	Pheochromocytoma and Paraganglioma
ESCA	Esophageal Carcinoma	PRAD	Prostate Adenocarcinoma
GBM	Glioblastoma Multiforme	READ	Rectum Adenocarcinoma
HNSC	Head and Neck Squamous Cell Carcinoma	SARC	Sarcoma
KICH	Kidney Chromophobe	SKCM	Skin Cutaneous Melanoma
KIRC	Kidney Renal Clear Cell Carcinoma	STAD	Stomach Adenocarcinoma
KIRP	Kidney Renal Papillary Cell Carcinoma	TGCT	Testicular Germ Cell Tumors
LAML	Acute Myeloid Leukemia	THCA	Thyroid Carcinoma
LGG	Lower Grade Glioma	THYM	Thymoma
LIHC	Liver Hepatocellular Carcinoma	UCEC	Uterine Corpus Endometrial Carcinoma

results in a global depletion of protein synthesis while activating specific mRNA translation programs, leading to upregulation of NAD(P)H-quinone oxidoreductase 1 (NQO1) (83). NQO1 overexpression enhances sensitivity to NQO1-targeted drugs. Therefore, NSUN5 epigenetic silencing is considered a protective factor in gliomas and is associated with better prognosis.

6.2 Respiratory system tumors

6.2.1 Lung cancer

In lung adenocarcinoma, two distinct m5C methylation modification patterns based on 11 m5C regulatory factors have been identified, each characterized by different tumor microenvironment immune cell infiltration profiles. A scoring system for m5C methylation modifications indicates that patients in the high-score group have better prognosis compared to those in the low-score group. A prognostic model constructed from 14 m5C-related lncRNAs shows that high-risk patients have poorer outcomes than low-risk patients, with high sensitivity and specificity. In lung squamous cell carcinoma (LUSC), the m5C regulatory factors NSUN3 and NSUN4 are highly expressed compared to normal lung tissue and are associated with poor prognosis (84). NSUN3 and NSUN4 expression is upregulated and correlates with adverse outcomes, and these factors are used to construct prognostic risk signatures. Additionally, NSUN3 and NSUN4 are related to the infiltration of six major immune cell types. In lung adenocarcinoma, *in vitro* experiments show that high expression of NOP2 or heterogeneous nuclear ribonucleoproteins

(hnRNP) is more likely associated with poor differentiation. NSUN3 genomic deletions are common in non-smokers with lung adenocarcinoma, occurring at a rate of 15% (85). Research indicates that high expression of NSUN2 leads to resistance to gefitinib and promotes recurrence of lung cancer tumors. Knockdown of NSUN2 can overcome the intrinsic resistance of lung cancer cells to gefitinib. Mechanistic studies show that NSUN2 regulates the m5C modification of QSOX1, and YBX1 enhances QSOX1 translation in an m5C-dependent manner, thereby promoting resistance to EGFR-mutant lung cancer (2). THOC3 is highly expressed in lung squamous cell carcinoma (LUSC) and significantly promotes the growth, migration, and glycolysis of LUSC cells. Mechanistic studies have shown that THOC3 can form a complex with YBX1 to promote the transcription of PFKFB4. Additionally, THOC3 facilitates the export of PFKFB4 mRNA to the cytoplasm, while YBX1 maintains the stability of PFKFB4 mRNA (86). NSUN2 upregulates the m5C modification of NRF2, with YBX1 binding to the m5C-modified NRF2 to maintain its transcript stability, thereby promoting the proliferation, migration, and ferroptosis resistance of NSCLC cells (87).The expression level of NOP2 is abnormally elevated in lung cancer, and its increased expression enhances the migratory and invasive abilities of lung cancer cells, as well as the growth and metastasis of transplanted tumors. This effect is achieved by regulating the m5C modification level of EZH2 mRNA, which in turn stabilizes EZH2 mRNA through ALYREF mediation (88).Conversely, NSUN6 is downregulated in lung cancer, and overexpression of NSUN6 inhibits the proliferation, migration, and EMT of lung cancer cells. This is attributed to NSUN6 regulating the expression of

NM23-H1 by m5C modification of NM23-H1 mRNA's 3'-UTR (89). NC02159 is reported to be upregulated in the tumor tissues and serum of NSCLC patients, and knocking down LINC02159 significantly inhibits the proliferation, migration, and invasion of NSCLC cells, induces apoptosis and cell cycle arrest, and slows tumor growth *in vivo*. The primary mechanism involves interaction with Aly/REF export factor (ALYREF), thereby upregulating the stability of YAP1 mRNA in an m5C-dependent manner, activating the Hippo and β -catenin signaling pathways, and promoting NSCLC progression (37).

6.3 Digestive system tumors

6.3.1 Esophageal cancer

Esophageal cancer is highly aggressive with early metastatic potential and poor prognosis. Its two major histological subtypes are squamous cell carcinoma and adenocarcinoma. NSUN2-methylated lncRNA (NMR) is significantly upregulated in esophageal cancer tissues and is associated with reduced overall survival (90). Screening for genes with reduced m5C levels and sequencing analysis reveal that the m5C levels of migration and invasion-related genes PLOD3, COL4A5, LAMB1, and HSPG2 decrease following NMR overexpression. This reduction may be due to the competitive inhibition of mRNA m5C levels by the upregulated NSUN2 lncRNA. NSUN2 expression is positively regulated by E2F transcription factor 1 (E2F1), which enhances NSUN2 expression by binding to its promoter (91). This, in turn, increases the m5C levels of growth factor receptor-bound protein 2 (GRB2). The RNA-binding protein lin-28 homolog B (LIN28B) preferentially binds to m5C-modified GRB2 mRNA, stabilizing it. Subsequently, increased GRB2 levels activate phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and extracellular regulated protein kinases (ERK)/mitogen-activated protein kinases (MAPK) signaling pathways, promoting the progression of esophageal squamous cell carcinoma (90). In esophageal cancer, RNA m5C methylation is primarily mediated by NSUN2 and participates in the disease process by affecting cancer-related genes and pathways. Recent studies have found that YBX1 is aberrantly overexpressed in esophageal squamous cell carcinoma (ESCC), with a significant correlation between high YBX1 levels and poor patient survival. YBX1 enhances the stability of spermine oxidase (SMOX) mRNA through an m5C-dependent mechanism mediated by NSUN2, promoting ESCC cell proliferation and metastasis (92).

6.3.2 Gastric cancer

Studies indicate that NSUN2 expression is upregulated in gastric cancer compared to adjacent non-cancerous tissues. *In vitro* and *in vivo* experiments confirm that NSUN2 promotes gastric cancer cell proliferation and tumor development. RNA sequencing has identified p57KIP2 as a downstream target of NSUN2 regulation. Mechanistically, the methyltransferase activity of NSUN2 and m5C modification in the 3'-UTR region of p57KIP2 mRNA disrupts its stability, thereby facilitating gastric cancer progression (93). Research indicates that NSUN2 is upregulated

in gastric cancer and is significantly associated with lower survival rates in patients. Functional studies reveal that NSUN2 methylates lncRNA-NR_033928, resulting in the upregulation of NR_033928. This lncRNA promotes the formation of the IGF2BP3/HUR complex, which subsequently maintains the stability of the downstream target gene GLS mRNA, leading to increased expression of glutaminase (GLS). This upregulation promotes gastric cancer (GC) cell proliferation and progression. NSUN2 has been reported to interact with lncRNAs to regulate the stability of target genes. In gastric cancer (GC) tissues, lncRNA-DIAPH2-AS1 is abnormally upregulated and is associated with poor prognosis in GC patients. Overexpression of DIAPH2-AS1 enhances the migration, invasion, and neural invasion potential of GC cells. Mechanistic studies have confirmed that DIAPH2-AS1 interacts with NSUN2, protecting NSUN2 from ubiquitin-proteasome degradation. This interaction further increases the stability of the downstream target gene NTN1 mRNA through m5C modification, ultimately inducing neural invasion in GC (94). Upregulation of m5C methyltransferases and binding proteins is observed in gastrointestinal cancers, and their high expression is significantly associated with poor patient survival (95). Bioinformatics analysis reveals that m5C regulatory proteins are closely related to the ErbB/PI3K-Akt signaling pathway, with GSK3B being a crucial target. FOXC2 antisense RNA 1 (FOXC2-AS1), a newly identified functional lncRNA, is highly expressed in gastric cancer tissues and cells, promoting cell proliferation, migration, and invasion, and correlates with poor prognosis. FOXC2-AS1 recruits NSUN2 to FOXC2 mRNA, increasing its m5C levels, and subsequently enhances FOXC2 mRNA stability through binding with m5C-binding protein YBX1 (96). Previous studies have shown that YBX1 is highly expressed in advanced gastric cancer tissues and is associated with shorter disease-free survival, though the exact mechanisms by which YBX1 promotes cancer progression through binding to RNA m5C methylation regions remain to be elucidated (96).

6.3.3 Gallbladder cancer

In gallbladder carcinoma (GBC), NSUN2 expression is upregulated in both cells and tissues. Silencing of NSUN2 inhibits GBC cell proliferation and tumorigenesis, whereas overexpression of NSUN2 promotes gallbladder cancer cell growth. RPL6 contributes to carcinogenesis by regulating the translation of NSUN2 mRNA. In RPL6-silenced cells, NSUN2 protein levels are reduced, leading to the accumulation of NSUN2 mRNA (17).

6.3.4 Colorectal cancer

Detecting tumor prognostic markers is crucial for identifying colorectal cancer patients with low survival rates and high mortality. Research has shown that in colorectal cancer patients and mouse models, the mRNA levels of NSUN5 and YBX1, as well as the total RNA m5C levels, are elevated (97). Co-culture experiments indicate that colorectal cancer cells promote the expression of NSUN5 and YBX1 in immune cells, leading to increased m5C levels in these cells. This suggests that m5C levels in peripheral blood immune cells may serve as potential biomarkers for distinguishing colorectal

cancer patients. In colorectal cancer, NSUN2 suppresses miR-125b expression and enhances the expression of Grb-associated binding protein 2 (Gab2), thereby promoting cell migration (98). Additionally, NSUN5 is upregulated in colorectal cancer tissues and cells, and NSUN5 knockout mice exhibit significantly reduced cell proliferation and cell cycle arrest. NSUN5 may regulate colorectal cancer cell proliferation through the Retinoblastoma (Rb)-cyclin-dependent kinase (CDK) pathway (22). Bioinformatics analysis of differentially expressed genes between colon cancer and adjacent tissues has identified DNMT2, NSUN6, and ALKBH1 as prognostic genes for colorectal cancer, with all three involved in MAPK and P53 signaling pathways, suggesting their potential oncogenic roles (99). Elevated NSUN2 levels in colorectal cancer are associated with poor patient survival. Silencing NSUN2 inhibits tumorigenesis and progression in NSUN2 knockout mouse models, with mechanistic studies showing that NSUN2 induces m5C modification of SKIL and mediates SKIL mRNA stability through YBX1. Increased SKIL levels activate transcriptional coactivators with PDZ-binding motifs (TAZ), promoting colorectal cancer progression (100).

6.3.5 Liver cancer

Comparative studies between hepatocellular carcinoma (HCC) and adjacent non-cancerous tissues have revealed that HCC exhibits significantly higher levels of m5C peaks in mRNA, with a broader distribution (101). In addition to coding RNAs, the frequency of m5C methylation and the number of methylated genes are also significantly higher in circRNA and lncRNA within HCC tissues compared to adjacent non-cancerous tissues (101). The presence of RNA m5C modifications promotes HCC progression, with elevated levels of m5C regulators NSUN4 and ALYREF correlating negatively with poor prognosis in HCC patients (102). Recent studies have shown that NSUN2 deficiency suppresses proliferation and migration in HepG2 liver cancer cells. Transcriptomic sequencing and bisulfite sequencing (Bis-Seq) have demonstrated a significant reduction in m5C methylation and gene expression of lncRNA H19 following NSUN2 loss. Mechanistically, lncRNA H19 is a specific target of the NSUN2 RNA methyltransferase, with m5C modification affecting H19 half-life and stability. The m5C-modified H19 can promote tumorigenesis through specific binding to the tumor protein G3BP1 (18). Elevated expression of NSUN5 is associated with reduced relapse-free and overall survival rates and predicts poor prognosis in hepatocellular carcinoma. NSUN5 mRNA and protein levels are upregulated in HCC tissues, and NSUN5 overexpression promotes HCC cell proliferation and migration. Bioinformatics analysis indicates a positive correlation between NSUN5 and ribosomal and translation-related genes in HCC (103). However, research on whether NSUN5 acts as a methyltransferase affecting RNA m5C levels in liver cancer remains unexplored. ALYREF is highly expressed in HCC cell lines, and its loss inhibits HCC cell proliferation. Gene knockout studies reveal that genes with differential methylation following ALYREF knockout bind to ALYREF protein, with their biological functions enriched in cell cycle and HCC pathways, suggesting that ALYREF may regulate

HCC development through influencing target gene methylation levels (104). A recent significant study found that NSUN2 is significantly upregulated in hepatocellular carcinoma (HCC), and its high expression is closely associated with poor prognosis in HCC patients (105). Functional studies showed that knockdown of NSUN2 significantly inhibited the proliferation, colony formation, migration, and invasion of HCC cells. Further molecular mechanism analysis revealed that NSUN2 mediates m5C RNA modification of the SARS2 gene, which in turn activates the Wnt signaling pathway, promoting liver cancer progression (105). These findings provide new insights into the role of NSUN2 in HCC and highlight its potential as a therapeutic target.

6.3.6 Pancreatic cancer

Pancreatic cancer is highly malignant, with an incidence rate nearly equal to its mortality rate and a poor prognosis. NSUN2 plays an enzymatic role in mediating m5C methylation enrichment in RNA within pancreatic cancer cells. Knockdown of NSUN2 in pancreatic cancer cells significantly downregulates m5C methylation levels (106, 107). In pancreatic cancer mouse models, NSUN2 expression is upregulated in cancer cells, and its knockdown slows the growth of pancreatic cancer spheroids. In contrast to normal pancreatic tissues, the protein level of NSUN6 is reduced in pancreatic cancer tissues (108). Overexpression of NSUN6 in pancreatic cancer cells inhibits cell proliferation, and low NSUN6 expression is associated with poor patient survival, indicating its potential as an independent prognostic factor for predicting recurrence and survival in pancreatic cancer (24). Contrary to findings in other cancers where m5C methyltransferases are often overexpressed, the reduced expression of NSUN6 in pancreatic cancer suggests it may act as a protective factor, though the role of NSUN6 in mediating RNA m5C modifications warrants further investigation.

6.4 Hematologic tumors

6.4.1 Leukemia

In leukemia, NSUN1 specifically interacts with BRD4 and directly binds to the CTD-S2P of RNA polymerase II (RNA-pol II). In 5-azacytidine (5-AZA)-resistant leukemia cells, a unique NSUN1/BRD4/RNA-pol II CTD-S2P complex is formed, mediating the development of 5-AZA-resistant chromatin structures and contributing to 5-AZA resistance in leukemia. Conversely, NSUN3 and DNMT2 exhibit opposing effects on 5-AZA-sensitive leukemia cells. Mechanistically, the RNA-binding protein hnRNPK directly interacts with m5C methyltransferases NSUN3 and DNMT2, lineage-determining transcription factors GATA1 and SPI1/PU.1, and CDK9/PTEFb, forming a unique complex at nascent RNA sites, which ultimately results in a 5-AZA-sensitive chromatin structure (109). Comparative analysis of bone marrow samples from 5-AZA-resistant and -sensitive leukemia patients reveals significantly higher levels of m5C mRNA in the 5-AZA-resistant samples. The expression levels of hnRNPK, NSUN1, and BRD4 are associated with leukemia progression and contribute to 5-AZA resistance and tumor development (109). Research reports

indicate that YBX1 maintains the survival of myeloid leukemia cells in an m6A-dependent manner, while having no effect on normal hematopoiesis. YBX1 interacts with m6A readers IGF2BPs through its conserved Cold Shock Domain (CSD) to indirectly bind m6A-modified mRNA, thereby enhancing the stability of apoptosis-related genes MYC and BCL2, which in turn sustains the function of leukemia cells (39). Recent research indicates that TET2 regulates the accumulation of 5-methylcytosine (m5C) modifications in TSPAN13 mRNA. These m5C modifications are specifically recognized by YBX1, which increases the stability and expression of TSPAN13 transcripts. This process promotes leukemia development, leukemia stem cell migration/homing, and leukemia stem cell self-renewal (110).

6.5 Genitourinary system tumors

6.5.1 Bladder cancer

In bladder cancer, RNA bisulfite sequencing (Bis-Seq) has identified frequent m5C methylation in cancerous tissues compared to adjacent non-cancerous tissues. Most m5C methylation sites are located in mRNA, with high-methylation mRNA significantly enriched in carcinogenic pathways (111). Further research shows that NSUN2 and YBX1 are aberrantly elevated in bladder cancer tissues. The proto-oncogene heparin-binding growth factor (HDGF) mRNA is methylated by NSUN2, and YBX1 stabilizes HDGF mRNA by binding to m5C methylation sites and recruiting ELAVL1, thereby promoting tumor development (112). Results demonstrate that ALYREF regulates the splicing and stabilization of hypermethylated RABL6 and TK1 mRNAs in an m5C-dependent manner to enhance the proliferation and invasion of UCB cells (112).

6.5.2 Endometrial cancer

Epigenetic enhancement mediated by H3K4me3 levels leads to significant upregulation of NSUN2 in endometrial cancer (EC). Upregulation of NSUN2 promotes EC cell proliferation, while NSUN2 knockdown significantly increases lipid peroxides and lipid ROS levels in EC cells, thereby enhancing sensitivity to ferroptosis. Mechanistically, NSUN2 enhances m5C modification of SLC7A11 mRNA and directly binds to m5C sites on SLC7A11 mRNA through YBX1, leading to increased mRNA stability and elevated SLC7A11 levels. Targeting the NSUN2/SLC7A11 axis can inhibit *in vivo* and *in vitro* tumor growth in EC cells by promoting lipid peroxidation and ferroptosis (113).

6.6 Breast cancer

In triple-negative breast cancer, overexpression of NSUN2 promotes cancer cell proliferation, migration, and invasion through Myc. NSUN6 regulates the mammalian sterile 20-like kinase 1 (MST1) target gene of Yes-associated protein 1 (YAP1), leading to osteoclast differentiation and breast cancer bone metastasis (114). In

breast cancer cells and tissues, hypomethylation of NSUN2 DNA results in overexpression of NSUN2 mRNA and protein. Upregulation of NSUN2 promotes proliferation, migration, and invasion of breast cancer cells, while NSUN2 knockout inhibits these processes. In triple-negative breast cancer (TNBC), upregulated NSUN2 acts as an oncogenic factor, while downregulated NSUN6 functions as a tumor suppressor. NSUN2 and NSUN6 influence tumorigenesis and the tumor immune microenvironment (TIM) in breast cancer. NSUN2/YBX1 synergistically upregulate HGH1 mRNA stability and promote breast cancer progression (115).

6.7 Neck squamous cell carcinoma

In head and neck squamous cell carcinoma (HNSCC), NSUN2 expression is significantly upregulated, which may be associated with mitochondrial function and cell cycle checkpoint-related genes. Additionally, DNA cytosine-5-methyltransferase 1 (DNMT1) is downregulated in HNSCC, potentially related to peptide cross-linking and humoral immunity. There is a negative correlation between NSUN2 expression and T cell activation scores (116). Furthermore, in hypopharyngeal squamous cell carcinoma (HPSCC), increased levels of NSUN3 enhance tumor proliferation and invasion. In HPSCC, both NSUN2 mRNA and protein levels are elevated. NSUN2 modifies the 3'-UTR of TEA domain transcription factor 1 (TEAD1) mRNA through m5C, promoting TEAD1 expression and thereby enhancing tumor cell proliferation and invasion (117). TEAD1 coordinates and integrates multiple signaling pathways, and its downregulation affects the expression of various oncogenes involved in tumor cell progression, metastasis, and resistance to chemotherapy.

7 The role of m5C in the tumor immune microenvironment and cancer immunotherapy

7.1 The role of m5C in the tumor immune microenvironment

The tumor immune microenvironment (TME) is closely associated with tumor progression and responses to immunotherapy. Recent studies have revealed that m5C modification regulates immune cell infiltration within tumors. TET2 and ten-eleven translocation 3 (TET3) play crucial roles in Treg cell immune homeostasis (118). Additionally, several m5C regulatory proteins within TME can serve as prognostic and diagnostic biomarkers for cancer. In lung adenocarcinoma, patients with high m5C scores have better prognoses, and different m5C modification patterns indicate varying immune infiltration profiles (119). Research indicates that m5C risk scores positively correlate with neutrophils, resting CD4+ memory T cells, and M2 macrophages in lung squamous cell carcinoma, while negatively correlating with follicular helper T cells, CD8+ T cells,

and activated NK cells (120). The impact of m5C on TME is also increasingly recognized in other cancers. Multiple studies have demonstrated that m5C modifications are involved in regulating TME in HNSCC (121). Knockdown of NSUN3 has been shown to regulate M1/M2 polarization of macrophages in HNSCC, increasing M1 macrophage infiltration and inhibiting HNSCC growth both *in vitro* and *in vivo*. Moreover, 28S rRNA methyltransferase NSUN5 downregulates β -catenin by promoting CTNNB1 mRNA degradation, thereby enhancing the phagocytic activity of tumor-associated macrophages (TAMs). Interestingly, NSUN5 directly interacts with CTNNB1 chromatin-associated RNA (caRNA) and deposits m5C (21). Findings reveal that the content of resting NK cells, M2 macrophages, and neutrophils in the low-risk group is significantly lower than in the high-risk group. Additionally, m6A/m5C/m1A-related lncRNAs are associated with the immune microenvironment and tumor mutation burden in HNSCC, providing potential prognostic markers for immunotherapy in this cancer (116).

7.2 The role of m5C in cancer immunotherapy

Significant advancements have been made in the basic and clinical research of m5C-related cancer immunotherapy. On one hand, study successfully induced apoptosis and immunogenic cell death in cancer cells by combining m5C inhibitors with immune checkpoint inhibitors (122). These effects are associated with endogenous antitumor immune responses and the conversion of cold immune tumors to hot ones. On the other hand, mechanisms of m5C methylation modification have been employed to enhance the efficacy of mRNA-based immunotherapy. Research has shown that m5C methylation reduces RNA antigenicity and suppresses immune responses. Following methylation modification, the immunogenicity of RNA diminishes or disappears, thus avoiding activation of the innate immune system. This represents a novel breakthrough in RNA-based immunotherapy. Accordingly, m5C/m1C combinatorial modifications have been utilized to enhance the ability of exogenous mRNA to evade Toll-like receptor activation and downstream innate immune signaling, thereby improving protein expression from mRNA. Biotechnological teams have designed materials to deliver m5C-modified mRNA for reprogramming tumor-associated macrophages or anticancer T cells, thereby inducing antitumor immunity and promoting tumor regression.

In head and neck squamous cell carcinoma (HNSCC), NSUN2 is negatively correlated with M2 macrophage polarization and T cell activation. Consequently, NSUN2 is considered a potential target for immune checkpoint blockade in HNSCC (123). Furthermore, NSUN2 negatively regulates immune cell infiltration in the nasopharyngeal carcinoma (NPC) tumor microenvironment, suggesting that NSUN2 may be inversely related to sensitivity to immunotherapy and chemotherapy. NSUN2 could be a significant oncogene involved in NPC progression. Recent research indicates that glucose, acting as a signaling molecule, directly binds to

NSUN2 at its amino acid residues 1-28, causing NSUN2 oligomerization and activation, and sustaining m5C RNA methylation independent of glucose metabolism (16). Glucose, as a standalone signaling molecule, can directly bind and activate NSUN2, leading to tumorigenesis and immune therapy resistance by inhibiting the cGAS/STING pathway (12). The glucose/NSUN2/TREX2 axis drives tumorigenesis and resistance to PD-L1 immune therapy in immune-competent syngeneic tumor mouse models by suppressing the cGAS/STING pathway, apoptosis, and CD8+ T cell infiltration. Notably, gene targeting of the glucose/NSUN2/TREX2 axis reduces tumorigenesis and overcomes resistance to PD-L1 immune therapy by promoting the cGAS/STING pathway, apoptosis, and CD8+ T cell infiltration (Figure 2). This research provides foundational evidence that targeting the glucose/NSUN2/TREX2 axis is a promising strategy for overcoming resistance to PD-1/PD-L1 immune therapies in cold tumors, offering a basis for converting prostate cancer and other cold tumors into hot tumors that respond to PD-1/PD-L1 immune therapy (16).

Luo et al. discovered that NSUN2 enhances the expression of ICAM-1 by upregulating m5C methylation in ICAM-1 mRNA, which improves the adhesion between leukocytes and endothelial cells, and inhibits M2 macrophage polarization and suppresses tumor metastasis (124) (Figure 2). Additionally, the absence of donor NSUN2 impedes the development of atherosclerosis in a rat model of allogeneic aortic transplantation, suggesting that the NSUN2-ICAM-1 regulatory axis is involved in endothelial cell inflammation. Beyond ICAM-1 mRNA, NSUN2 can also catalyze the methylation of other mRNAs and non-coding RNAs. Therefore, further research is needed to elucidate the mechanisms by which NSUN2 regulates vascular inflammation and the development of atherosclerosis (124) (Figure 2). Studies indicate that chemotherapy induces an immunosuppressive microenvironment within tumors and promotes immune evasion through YBX1-mediated upregulation of PD-L1 (programmed death-ligand 1) (125). Knocking out YBX1 reverses chemotherapy resistance by blocking PD-L1 expression and activating T cells in the tumor microenvironment. The upregulation of functional cytotoxic CD8+ T cells, and the downregulation of myeloid-derived suppressor cells and regulatory T cells, are associated with overcoming tumor immunosuppressive environments and immune evasion (126). Additionally, YBX1 knockout can reverse hepatocellular carcinoma (HCC) resistance by blocking PD-L1 expression and activating T cells in the tumor microenvironment (127). CDKL1 is highly expressed in lung cancer and promotes the growth and proliferation of lung cancer cells, while also enhancing their radiosensitivity. Further studies have discovered that CDKL1 interacts with YBX1, thereby inhibiting YBX1-mediated transcription of the PD-L1 gene and suppressing PD-L1 expression. This ultimately leads to the activation of CD8+ T cells and the inhibition of lung cancer immune evasion. Increased expression of CDKL1, combined with radiotherapy and anti-PD-L1 antibody therapy, can significantly improve the therapeutic outcomes for lung cancer (128) (Figure 2).

The development of m5C regulatory protein and lncRNA-related risk models also provides new insights for cancer

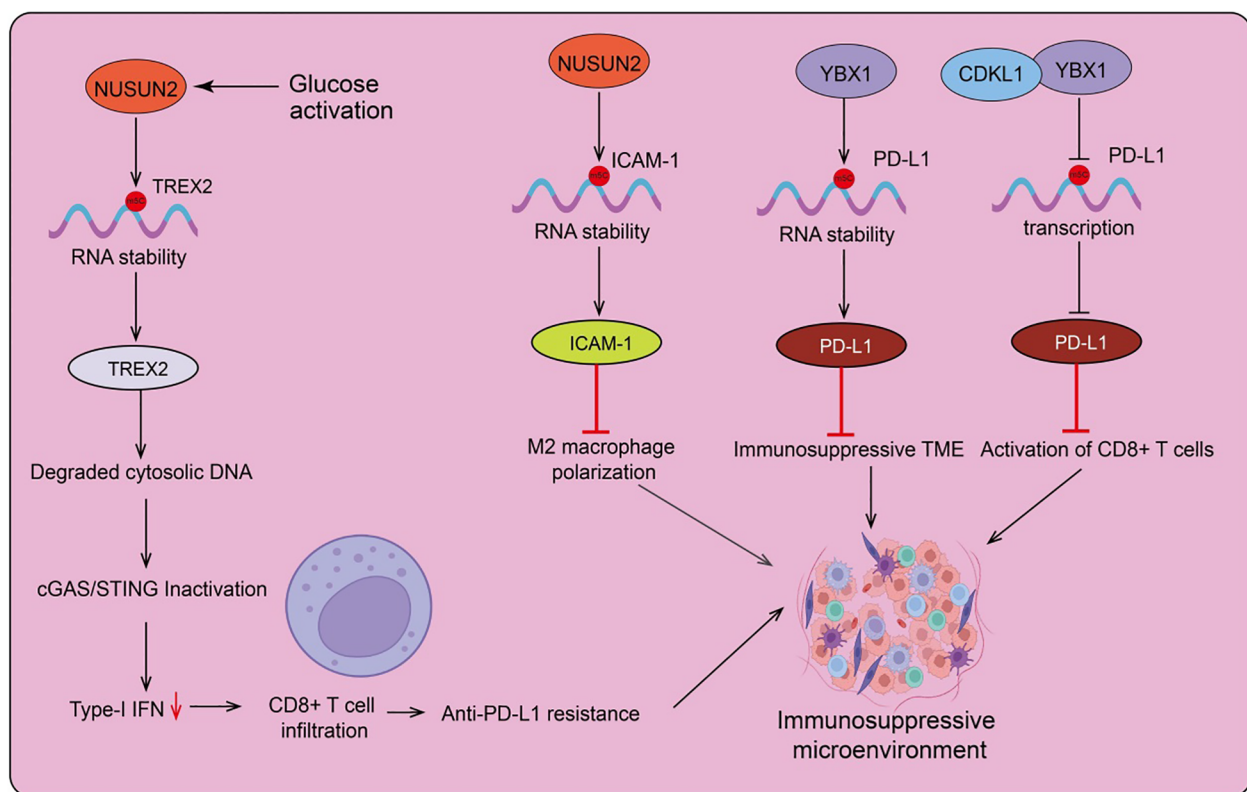


FIGURE 2

The role and mechanisms of RNA m5C modification in the regulation of the tumor microenvironment. NUSUN2 mediates m5C modifications of various downstream target genes, recruits the YBX1 reader protein, and subsequently regulates the RNA stability of these target genes, upregulating their expression. This plays a key role in tumor immune microenvironment remodeling, including M2 macrophage polarization, the formation of an immunosuppressive microenvironment, and CD8+ cell activation.

treatment and efficacy prediction, enabling more accurate and personalized immunotherapy regimens. The m5C risk score serves as an independent prognostic factor for colon cancer patients, with lower scores indicating greater sensitivity to immunotherapy and higher scores indicating greater sensitivity to chemotherapy (129). This score can predict colon cancer prognosis, immunotherapy response, and drug sensitivity. These immunotherapy prediction methods are also applicable to other cancers. In triple-negative breast cancer (TNBC), changes in the expression of m5C RNA methylation regulators, with upregulation of NSUN2 and downregulation of NSUN6, can significantly predict clinical prognosis risk in TNBC patients. Therefore, it may serve as a new prognostic marker for TNBC and provide insights into RNA epigenetic modifications in TNBC (130). Related studies have also confirmed that NSUN3 and NSUN4 can predict the prognosis of lung squamous cell carcinoma and regulate the immune microenvironment. In lung adenocarcinoma patients, different m5C patterns correlate with variations in TME immune cell infiltration, with high m5C scores associated with better prognosis. Additionally, m5C-regulated lncRNAs can predict overall survival in lung adenocarcinoma patients and impact the tumor immune microenvironment (131). In pancreatic cancer

patients, three m5C-related lncRNAs show prognostic value. The TIDE (Tumor Immune Dysfunction and Exclusion) algorithm indicates that patients with high m5C-lncRNA scores respond better to immunotherapy. In another study on pancreatic ductal adenocarcinoma (PDAC) (107), researchers evaluated the relationship between m5C-related lncRNAs and PDAC-infiltrating immune cells. Naïve B cells, CD8+ T cells, Treg cells, and resting NK cells were more highly expressed in the low-risk group, while M0 and M2 macrophage phenotypes were more highly expressed in the high-risk group, suggesting that m5C-related lncRNAs may regulate pancreatic cancer progression by promoting M2 macrophage polarization or infiltration in PDAC (107).

8 Summary and future directions

Current research has provided preliminary insights into the distribution characteristics of m5C methylation across various RNAs and the biological functions of m5C modifications. Future research efforts should primarily focus on elucidating the roles of specific m5C methylation sites, discovering new recognition

proteins, and understanding the precise roles of m5C modifications in diseases such as cancer. Significant progress has already been made in elucidating the protein crystal structures of certain m5C methylation enzymes and recognition proteins, as well as their RNA-binding domains. The development of inhibitors targeting m5C methylation-related enzymes has become a focal point of research. Furthermore, several studies have suggested that m5C-related modification enzymes could serve as diagnostic biomarkers for cancer. However, the use of specific m5C modification sites as cancer biomarkers still requires further investigation. Overall, the regulatory role of m5C methylation in tumorigenesis is gradually being uncovered, offering new perspectives for cancer diagnosis and personalized treatment.

Notably, RNA m5C methylation modifications have shown significant potential in cancer immunotherapy. Research indicates that modulating m5C methylation levels can enhance antitumor immune responses and improve the efficacy of immune checkpoint inhibitors. For instance, inhibiting the function of m5C-related proteins such as NSUN2 or ALYREF can restore T cell antitumor activity and enhance the effects of immunotherapy (16, 36). Therefore, in-depth studies on the mechanisms of RNA m5C methylation modifications and their applications in cancer immunotherapy are of substantial clinical significance. In summary, the functions and mechanisms of RNA m5C methylation modifications in neurodevelopment, autoimmune diseases, and cancer progression hold significant research value and application potential. Future research aiming to further elucidate the mechanisms of m5C modifications and their regulatory pathways is expected to reveal additional biological processes and advance their application in disease diagnosis and treatment.

To improve and develop techniques for detecting m5C (5-methylcytosine) modifications in RNA, various innovative sequencing technologies have been explored, such as Nanopore-seq and single-molecule real-time (SMART) sequencing (132, 133). These technologies aim to overcome limitations of traditional methods, offering advantages in terms of sensitivity, real-time analysis, and the ability to detect modifications at single-molecule resolution. Nanopore sequencing is an emerging technology that can directly sequence nucleic acids by passing them through a protein nanopore, which detects changes in the ionic current as the nucleotides translocate through the pore. However, challenges still exist in the high error rates associated with Nanopore sequencing, particularly for short sequences, and distinguishing between m5C and other modifications or sequence-context effects can be difficult. Efforts to improve base-calling algorithms and modify the sequencing technology to improve its accuracy are ongoing. SMART sequencing, pioneered by Pacific Biosciences (PacBio), is another promising technique for detecting RNA modifications like m5C (133). SMART sequencing relies on real-time observation of the DNA polymerase activity during the sequencing process. In summary, Nanopore-seq and SMART sequencing represent exciting advances in the detection of RNA modifications like m5C, offering significant advantages over traditional sequencing

technologies. As these techniques continue to evolve, they hold the potential to provide a more comprehensive, accurate, and real-time understanding of RNA modification dynamics, furthering our understanding of RNA biology and its implications in health and disease.

The application of Nanopore-seq and single-molecule real-time (SMART) sequencing in detecting m5C modifications holds significant promise for advancing our understanding of RNA epigenetics. These cutting-edge technologies offer unprecedented sensitivity and resolution for identifying m5C modifications at a single-base level, enabling researchers to explore m5C's dynamic role in gene regulation and disease processes. Future research should focus on optimizing these sequencing techniques for high-throughput, cost-effective detection of m5C across different cell types and tissues, particularly in the context of cancer and other diseases. Additionally, integrating Nanopore-seq and SMART with other omics technologies, such as transcriptomics and proteomics, could provide a comprehensive view of how m5C modifications interact with other epigenetic marks to regulate cellular functions. This integrated approach could pave the way for discovering novel biomarkers and therapeutic targets, ultimately improving our ability to diagnose and treat diseases driven by aberrant m5C regulation.

The targeting of m5C modification in the tumor immune microenvironment presents a promising avenue for future cancer research. As recent studies have shown, m5C modifications play a critical role in regulating gene expression and immune responses within tumors, potentially influencing tumor progression and immune evasion. Understanding the mechanisms by which m5C modification regulates immune cells, such as T cells, macrophages, and dendritic cells, could open up new strategies for enhancing anti-tumor immunity. Future research should focus on identifying specific m5C-modifying enzymes, exploring their interactions with immune checkpoints, and investigating how m5C modification can be harnessed to modulate the immune microenvironment. Additionally, combining m5C-based therapies with immune checkpoint inhibitors may offer synergistic effects, improving therapeutic outcomes. This emerging field holds great potential for developing novel cancer immunotherapies, offering hope for more effective and personalized treatments.

Author contributions

XC: Investigation, Writing – original draft, Data curation, Methodology, Conceptualization, Resources, Validation, Visualization, Formal Analysis, Project administration. YY: Software, Resources, Visualization, Investigation, Writing – original draft, Project administration, Methodology, Validation. FZ: Software, Visualization, Writing – original draft, Validation, Resources. XH: Investigation, Writing – original draft, Funding acquisition, Methodology. LL: Writing – original draft, Resources, Project administration, Software. JP: Writing – original draft, Investigation, Methodology. YZ: Formal Analysis, Supervision,

Conceptualization, Software, Project administration, Writing – original draft, Writing – review & editing, Validation, Resources, Data curation. XJ: Writing – original draft, Visualization, Formal Analysis, Resources, Software, Validation, Conceptualization, Methodology, Supervision, Project administration, Data curation, Investigation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This study was supported by NHC Key Laboratory of Drug Addiction Medicine (KN202417) and First-Class Discipline Team of Kunming Medical University (2024XKTDYS06).

Acknowledgments

We thank the BioRender platform (www.biorender.com) for assisting us in creating the figure.

References

- An Y, Duan H. The role of m6A RNA methylation in cancer metabolism. *Mol Cancer*. (2022) 21:14. doi: 10.1186/s12943-022-01500-4
- Wang Y, Wei J, Feng L, Li O, Huang L, Zhou S, et al. Aberrant m5C hypermethylation mediates intrinsic resistance to gefitinib through NSUN2/YBX1/QSOX1 axis in EGFR-mutant non-small-cell lung cancer. *Mol Cancer*. (2023) 22:81. doi: 10.1186/s12943-023-01780-4
- Luo Y, Yao Y, Wu P, Zi X, Sun N, He J. The potential role of N(7)-methylguanosine (m7G) in cancer. *J Hematol Oncol*. (2022) 15:63. doi: 10.1186/s13045-022-01285-5
- Bohnsack KE, Höbartner C, Bohnsack MT. Eukaryotic 5-methylcytosine (m⁵C) RNA methyltransferases: mechanisms, cellular functions, and links to disease. *Genes (Basel)*. (2019) 10. doi: 10.3390/genes10020102
- Xiong Y, Li Y, Qian W, Zhang Q. RNA m5C methylation modification: a potential therapeutic target for SARS-CoV-2-associated myocarditis. *Front Immunol*. (2024) 15:1380697. doi: 10.3389/fimmu.2024.1380697
- Balachander K, Priyadarsini JV, Roy A, Paramasivam A. Emerging role of RNA m5C modification in cardiovascular diseases. *J Cardiovasc Transl Res*. (2023) 16:598–605. doi: 10.1007/s12265-022-10336-8
- Liang Z, Riaz A, Chachar S, Ding Y, Du H, Gu X. Epigenetic modifications of mRNA and DNA in plants. *Mol Plant*. (2020) 13:14–30. doi: 10.1016/j.molp.2019.12.007
- Shi Q, Chu Q, Zeng Y, Yuan X, Wang J, Zhang Y, et al. Non-coding RNA methylation modifications in hepatocellular carcinoma: interactions and potential implications. *Cell Commun Signal*. (2023) 21:359. doi: 10.1186/s12964-023-01357-0
- Han X, Wang M, Zhao YL, Yang Y, Yang YG. RNA methylations in human cancers. *Semin Cancer Biol*. (2021) 75:97–115. doi: 10.1016/j.semcancer.2020.11.007
- Song P, Tayier S, Cai Z, Jia G. RNA methylation in mammalian development and cancer. *Cell Biol Toxicol*. (2021) 37:811–31. doi: 10.1007/s10565-021-09627-8
- Li M, Tao Z, Zhao Y, Li L, Zheng J, Li Z, et al. 5-methylcytosine RNA methyltransferases and their potential roles in cancer. *J Transl Med*. (2022) 20:214. doi: 10.1186/s12967-022-03427-2
- Song H, Zhang J, Liu B, Xu J, Cai B, Yang H, et al. Biological roles of RNA m(5)C modification and its implications in Cancer immunotherapy. *Biomark Res*. (2022) 10:15. doi: 10.1186/s40364-022-00362-8
- Sun H, Li K, Liu C, Yi C. Regulation and functions of non-m(6)A mRNA modifications. *Nat Rev Mol Cell Biol*. (2023) 24:714–31. doi: 10.1038/s41580-023-00622-x
- Chellamuthu A, Gray SG. The RNA methyltransferase NSUN2 and its potential roles in cancer. *Cells*. (2020) 9. doi: 10.3390/cells9081758
- Liao H, Gaur A, McConie H, Shekar A, Wang K, Chang JT, et al. Human NOP2/NSUN1 regulates ribosome biogenesis through non-catalytic complex formation with box C/D snoRNPs. *Nucleic Acids Res*. (2022) 50:10695–716. doi: 10.1093/nar/gkac817
- Chen T, Xu ZG, Luo J, Manne RK, Wang Z, Hsu CC, et al. NSUN2 is a glucose sensor suppressing cGAS/STING to maintain tumorigenesis and immunotherapy resistance. *Cell Metab*. (2023) 35:1782–1798.e1788.
- Gao Y, Wang Z, Zhu Y, Zhu Q, Yang Y, Jin Y, et al. NOP2/Sun RNA methyltransferase 2 promotes tumor progression via its interacting partner RPL6 in gallbladder carcinoma. *Cancer Sci*. (2019) 110:3510–9. doi: 10.1111/cas.v110.11
- Sun Z, Xue S, Zhang M, Xu H, Hu X, Chen S, et al. Aberrant NSUN2-mediated m(5)C modification of H19 lncRNA is associated with poor differentiation of hepatocellular carcinoma. *Oncogene*. (2020) 39:6906–19. doi: 10.1038/s41388-020-01475-w
- Murakami Y, Wei FY, Kawamura Y, Horiguchi H, Kadomatsu T, Miyata K, et al. NSUN3-mediated mitochondrial tRNA 5-formylcytidine modification is essential for embryonic development and respiratory complexes in mice. *Commun Biol*. (2023) 6:307. doi: 10.1038/s42003-023-04680-x
- Yang L, Ren Z, Yan S, Zhao L, Liu J, Zhao L, et al. Nsun4 and Mettl3 mediated translational reprogramming of Sox9 promotes BMSC chondrogenic differentiation. *Commun Biol*. (2022) 5:495. doi: 10.1038/s42003-022-03420-x
- Wu R, Sun C, Chen X, Yang R, Luan Y, Zhao X, et al. NSUN5/TET2-directed chromatin-associated RNA modification of 5-methylcytosine to 5-hydroxymethylcytosine governs glioma immune evasion. *Proc Natl Acad Sci U S A*. (2024) 121:e2321611121. doi: 10.1073/pnas.2321611121
- Jiang Z, Li S, Han MJ, Hu GM, Cheng P. High expression of NSUN5 promotes cell proliferation via cell cycle regulation in colorectal cancer. *Am J Transl Res*. (2020) 12:3858–70.
- Selmi T, Hussain S, Dietmann S, Heiß M, Borland K, Flad S, et al. Sequence- and structure-specific cytosine-5 mRNA methylation by NSUN6. *Nucleic Acids Res*. (2021) 49:1006–22. doi: 10.1093/nar/gkaa1193
- Yang R, Liang X, Wang H, Guo M, Shen H, Shi Y, et al. The RNA methyltransferase NSUN6 suppresses pancreatic cancer development by regulating cell proliferation. *EBioMedicine*. (2021) 63:103195. doi: 10.1016/j.ebiom.2020.103195
- Ortiz-Barahona V, Soler M, Davalos V, García-Prieto CA, Janin M, Setien F, et al. Epigenetic inactivation of the 5-methylcytosine RNA methyltransferase NSUN7 is associated with clinical outcome and therapeutic vulnerability in liver cancer. *Mol Cancer*. (2023) 22:83. doi: 10.1186/s12943-023-01785-z
- Adamczyk-Grochala J, Bloniarz D, Zielinska K, Lewinska A, Wnuk M. DNMT2/TRDMT1 gene knockout compromises doxorubicin-induced unfolded protein response and sensitizes cancer cells to ER stress-induced apoptosis. *Apoptosis*. (2023) 28:166–85. doi: 10.1007/s10495-022-01779-0

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27. Ehrenhofer-Murray AE. Cross-talk between dnmt2-dependent tRNA methylation and queuosine modification. *Biomolecules*. (2017) 7. doi: 10.3390/biom7010014
28. Ashapkin VV, Kutueva LI, Vanyushin BF. Dnmt2 is the most evolutionary conserved and enigmatic cytosine DNA methyltransferase in eukaryotes. *Genetika*. (2016) 52:269–82.
29. Huang Y, Tian C, Li Q, Xu Q. TET1 Knockdown Inhibits Porphyromonas gingivalis LPS/IFN- γ -Induced M1 Macrophage Polarization through the NF- κ B Pathway in THP-1 Cells. *Int J Mol Sci*. (2019) 20. doi: 10.3390/ijms20082023
30. Wu BK, Mei SC, Chen EH, Zheng Y, Pan D. YAP induces an oncogenic transcriptional program through TET1-mediated epigenetic remodeling in liver growth and tumorigenesis. *Nat Genet*. (2022) 54:1202–13. doi: 10.1038/s41588-022-01119-7
31. Chen B, Du YR, Zhu H, Sun ML, Wang C, Cheng Y, et al. Maternal inheritance of glucose intolerance via oocyte TET3 insufficiency. *Nature*. (2022) 605:761–6. doi: 10.1038/s41586-022-04756-4
32. Fahrner JA, Edited by Adam MP, Feldman J, GM M, RA P, SE W, et al. TET3-related beck-fahrner syndrome. In: *GeneReviews*[®]. University of Washington, Seattle Copyright © 1993–2024, University of Washington, Seattle (WA) (1993).
33. Melamed P, Yosefzon Y, David C, Tsukerman A, Pnueli L. Tet enzymes, variants, and differential effects on function. *Front Cell Dev Biol*. (2018) 6:22. doi: 10.3389/fcell.2018.00022
34. Li D, Guo B, Wu H, Tan L, Lu Q. TET family of dioxygenases: crucial roles and underlying mechanisms. *Cytogenet Genome Res*. (2015) 146:171–80. doi: 10.1159/000438853
35. Tsagaratou A, Lio CJ, Yue X, Rao A. TET methylcytosine oxidases in T cell and B cell development and function. *Front Immunol*. (2017) 8:220. doi: 10.3389/fimmu.2017.00220
36. Yang X, Yang Y, Sun BF, Chen YS, Xu JW, Lai WY, et al. 5-methylcytosine promotes mRNA export - NSUN2 as the methyltransferase and ALYREF as an m(5)C reader. *Cell Res*. (2017) 27:606–25. doi: 10.1038/cr.2017.55
37. Yang Q, Wang M, Xu J, Yu D, Li Y, Chen Y, et al. LINC02159 promotes non-small cell lung cancer progression via ALYREF/YAP1 signaling. *Mol Cancer*. (2023) 22:122. doi: 10.1186/s12943-023-01814-x
38. Xue C, Gu X, Zheng Q, Shi Q, Yuan X, Su Y, et al. ALYREF mediates RNA m(5)C modification to promote hepatocellular carcinoma progression. *Signal Transduct Target Ther*. (2023) 8:130. doi: 10.1038/s41392-023-01395-7
39. Feng M, Xie X, Han G, Zhang T, Li Y, Li Y, et al. YBX1 is required for maintaining myeloid leukemia cell survival by regulating BCL2 stability in an m6A-dependent manner. *Blood*. (2021) 138:71–85. doi: 10.1182/blood.2020090676
40. Wu R, Feng S, Li F, Shu G, Wang L, Gao P, et al. Transcriptional and post-transcriptional control of autophagy and adipogenesis by YBX1. *Cell Death Dis*. (2023) 14:29. doi: 10.1038/s41419-023-05564-y
41. Xu L, Li H, Wu L, Huang S. YBX1 promotes tumor growth by elevating glycolysis in human bladder cancer. *Oncotarget*. (2017) 8:65946–56. doi: 10.18632/oncotarget.19583
42. Peng Z, Wang J, Shan B, Li B, Peng W, Dong Y, et al. The long noncoding RNA LINC00312 induces lung adenocarcinoma migration and vasculogenic mimicry through directly binding YBX1. *Mol Cancer*. (2018) 17:167. doi: 10.1186/s12943-018-0920-z
43. Ma HL, Bizet M, Soares Da Costa C, Murisier F, de Bony EJ, Wang MK, et al. SRSF2 plays an unexpected role as reader of m(5)C on mRNA, linking epitranscriptomics to cancer. *Mol Cell*. (2023) 83:4239–4254.e4210. doi: 10.1016/j.molcel.2023.11.003
44. Wang X, Wang M, Dai X, Han X, Zhou Y, Lai W, et al. RNA 5-methylcytosine regulates YBX2-dependent liquid-liquid phase separation. *Fundam Res*. (2022) 2:48–55. doi: 10.1016/j.fmr.2021.10.008
45. Liu Y, Yang Y, Wu R, Gao CC, Liao X, Han X, et al. mRNA m(5)C inhibits adipogenesis and promotes myogenesis by respectively facilitating YBX2 and SMO mRNA export in ALYREF-m(5)C manner. *Cell Mol Life Sci*. (2022) 79:481. doi: 10.1007/s00018-022-04474-0
46. Li W, Li X, Ma X, Xiao W, Zhang J. Mapping the m1A, m5C, m6A and m7G methylation atlas in zebrafish brain under hypoxic conditions by MeRIP-seq. *BMC Genomics*. (2022) 23:105. doi: 10.1186/s12864-022-08350-w
47. Chen C, Chen LY, Zhang JX, Xu HG. 5-methylcytosine (m5C) modification patterns and tumor immune infiltration characteristics in clear cell renal cell carcinoma. *Curr Oncol*. (2022) 30:559–74. doi: 10.3390/curroncol30010044
48. Du X, Cheng C, Yang Y, Fan B, Wang P, Xia H, et al. NSUN2 promotes lung adenocarcinoma progression through stabilizing PIK3R2 mRNA in an m(5)C-dependent manner. *Mol Carcinog*. (2024) 63:962–76. doi: 10.1002/mc.23701
49. Li Q, Li X, Tang H, Jiang B, Dou Y, Gorospe M, et al. NSUN2-Mediated m5C Methylation and METTL3/METTL14-Mediated m6A Methylation Cooperatively Enhance p21 Translation. *J Cell Biochem*. (2017) 118:2587–98. doi: 10.1002/jcb.v118.9
50. Hu Y, Chen C, Tong X, Chen S, Hu X, Pan B, et al. NSUN2 modified by SUMO-2/3 promotes gastric cancer progression and regulates mRNA m5C methylation. *Cell Death Dis*. (2021) 12:842. doi: 10.1038/s41419-021-04127-3
51. Tuorto F, Liebers R, Musch T, Schaefer M, Hofmann S, Kellner S, et al. RNA cytosine methylation by Dnmt2 and NSun2 promotes tRNA stability and protein synthesis. *Nat Struct Mol Biol*. (2012) 19:900–5. doi: 10.1038/nsmb.2357
52. Schaefer M, Pollex T, Hanna K, Tuorto F, Meusburger M, Helm M, et al. RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. *Genes Dev*. (2010) 24:1590–5. doi: 10.1101/gad.586710
53. Metodieff MD, Spähr H, Loguericio Polosa P, Meharg C, Becker C, Altmueller J, et al. NSUN4 is a dual function mitochondrial protein required for both methylation of 12S rRNA and coordination of mitoribosomal assembly. *PLoS Genet*. (2014) 10: e1004110. doi: 10.1371/journal.pgen.1004110
54. Wagner A, Schosserer M. The epitranscriptome in ageing and stress resistance: A systematic review. *Ageing Res Rev*. (2022) 81:101700. doi: 10.1016/j.arr.2022.101700
55. Fang L, Huang H, Lv J, Chen Z, Lu C, Jiang T, et al. m5C-methylated lncRNA NR_033928 promotes gastric cancer proliferation by stabilizing GLS mRNA to promote glutamine metabolism reprogramming. *Cell Death Dis*. (2023) 14:520. doi: 10.1038/s41419-023-06049-8
56. Dinescu S, Ignat S, Lazar AD, Constantin C, Neagu M, Costache M. Epitranscriptomic signatures in lncRNAs and their possible roles in cancer. *Genes (Basel)*. (2019) 10. doi: 10.3390/genes10010052
57. Kong W, Biswas A, Zhou D, Fiches G, Fujinaga K, Santos N, et al. Nucleolar protein NOP2/NSUN1 suppresses HIV-1 transcription and promotes viral latency by competing with Tat for TAR binding and methylation. *PLoS Pathog*. (2020) 16: e1008430. doi: 10.1371/journal.ppat.1008430
58. Guo G, Pan K, Fang S, Ye L, Tong X, Wang Z, et al. Advances in mRNA 5-methylcytosine modifications: Detection, effectors, biological functions, and clinical relevance. *Mol Ther Nucleic Acids*. (2021) 26:575–93. doi: 10.1016/j.omtn.2021.08.020
59. Dai Q, Ye C, Irklyenko I, Wang Y, Sun HL, Gao Y, et al. Ultrafast bisulfite sequencing detection of 5-methylcytosine in DNA and RNA. *Nat Biotechnol*. (2024) 42:1559–70. doi: 10.1038/s41587-023-02034-w
60. Lu L, Zhang X, Zhou Y, Shi Z, Xie X, Zhang X, et al. Base-resolution m(5)C profiling across the mammalian transcriptome by bisulfite-free enzyme-assisted chemical labeling approach. *Mol Cell*. (2024) 84:2984–3000.e2988. doi: 10.1016/j.molcel.2024.06.021
61. Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Wu M. Epigenetics in neurodevelopment: emerging role of circular RNA. *Front Cell Neurosci*. (2019) 13:327. doi: 10.3389/fncel.2019.00327
62. Flores JV, Cordero-Espinoza L, Oeztuerk-Winder F, Andersson-Rolf A, Selmi T, Blanco S, et al. Cytosine-5 RNA methylation regulates neural stem cell differentiation and motility. *Stem Cell Rep*. (2017) 8:112–24. doi: 10.1016/j.stemcr.2016.11.014
63. Blanco S, Dietmann S, Flores JV, Hussain S, Kutter C, Humphreys P, et al. Aberrant methylation of tRNAs links cellular stress to neuro-developmental disorders. *EMBO J*. (2014) 33:2020–39. doi: 10.15252/embj.201489282
64. Kim YA, Siddiqui T, Blaze J, Cosacak MI, Winters T, Kumar A, et al. RNA methyltransferase NSun2 deficiency promotes neurodegeneration through epitranscriptomic regulation of tau phosphorylation. *Acta Neuropathol*. (2023) 145:29–48. doi: 10.1007/s00401-022-02511-7
65. Chi L, Delgado-Olguin P. Expression of NOL1/NOP2/sun domain (Nsun) RNA methyltransferase family genes in early mouse embryogenesis. *Gene Expr Patterns*. (2013) 13:319–27. doi: 10.1016/j.gexp.2013.06.003
66. Lu J, Zhang M, Liu Z, Guo L, Huang P, Xia W, et al. NSUN2-mediated m(5)C methylation impairs endometrial receptivity. *Lab Invest*. (2024) 104:100327. doi: 10.1016/j.labinv.2024.100327
67. Hussain S, Benavente SB, Nascimento E, Dragoni I, Kurowski A, Gillich A, et al. The nucleolar RNA methyltransferase Misu (NSun2) is required for mitotic spindle stability. *J Cell Biol*. (2009) 186:27–40. doi: 10.1083/jcb.200810180
68. Hussain S. The emerging roles of cytosine-5 methylation in mRNAs. *Trends Genet*. (2021) 37:498–500. doi: 10.1016/j.tig.2021.02.001
69. Trixl L, Lusser A. The dynamic RNA modification 5-methylcytosine and its emerging role as an epitranscriptomic mark. *Wiley Interdiscip Rev RNA*. (2019) 10: e1510. doi: 10.1002/wrna.2019.10.issue-1
70. Harris T, Marquez B, Suarez S, Schimenti J. Sperm motility defects and infertility in male mice with a mutation in Nsun7, a member of the Sun domain-containing family of putative RNA methyltransferases. *Biol Reprod*. (2007) 77:376–82. doi: 10.1095/biolreprod.106.058669
71. Khosronezhad N, Hosseinzadeh Colagar A, Mortazavi SM. The Nsun7 (A11337)-deletion mutation, causes reduction of its protein rate and associated with sperm motility defect in infertile men. *J Assist Reprod Genet*. (2015) 32:807–15. doi: 10.1007/s10815-015-0443-0
72. Chen Q, Yan M, Cao Z, Li X, Zhang Y, Shi J, et al. Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science*. (2016) 351:397–400. doi: 10.1126/science.aad7977
73. Zhang Y, Zhang X, Shi J, Tuorto F, Li X, Liu Y, et al. Dnmt2 mediates intergenerational transmission of paternally acquired metabolic disorders through sperm small non-coding RNAs. *Nat Cell Biol*. (2018) 20:535–40. doi: 10.1038/s41556-018-0087-2
74. Liu J, Huang T, Chen W, Ding C, Zhao T, Zhao X, et al. Developmental mRNA m(5)C landscape and regulatory innovations of massive m(5)C modification of maternal mRNAs in animals. *Nat Commun*. (2022) 13:2484. doi: 10.1038/s41467-022-30210-0

75. Tuorto F, Herbst F, Alerasool N, Bender S, Popp O, Federico G, et al. The tRNA methyltransferase Dnm2 is required for accurate polypeptide synthesis during hematopoiesis. *EMBO J.* (2015) 34:2350–62. doi: 10.15252/embj.201591382
76. Yang Y, Wang L, Han X, Yang WL, Zhang M, Ma HL, et al. RNA 5-methylcytosine facilitates the maternal-to-zygotic transition by preventing maternal mRNA decay. *Mol Cell.* (2019) 75:1188–1202.e1111. doi: 10.1016/j.molcel.2019.06.033
77. Sun J, Yan L, Shen W, Meng A. Maternal Ybx1 safeguards zebrafish oocyte maturation and maternal-to-zygotic transition by repressing global translation. *Development.* (2018) 145. doi: 10.1242/dev.166587
78. Deng M, Chen B, Liu Z, Wan Y, Li D, Yang Y, et al. YBX1 mediates alternative splicing and maternal mRNA decay during pre-implantation development. *Cell Biosci.* (2022) 12:12. doi: 10.1186/s13578-022-00743-4
79. Delatte B, Wang F, Ngoc LV, Collignon E, Bonvin E, Deplus R, et al. RNA biochemistry. Transcriptome-wide distribution and function of RNA hydroxymethylcytosine. *Science.* (2016) 351:282–5. doi: 10.1126/science.aac5253
80. Xiao Z, Li J, Liang C, Liu Y, Zhang Y, Zhang Y, et al. Identification of M5c regulator-mediated methylation modification patterns for prognosis and immune microenvironment in glioma. *Aging (Albany NY).* (2023) 15:12275–95. doi: 10.18632/aging.205179
81. Wen X, Li S, Guo M, Liao H, Chen Y, Kuang X, et al. miR-181a-5p inhibits the proliferation and invasion of drug-resistant glioblastoma cells by targeting F-box protein 11 expression. *Oncol Lett.* (2020) 20:235. doi: 10.3892/ol.2020.12098
82. Xu X, Zhang Y, Zhang J, Zhang X. NSUN2 promotes cell migration through methylating autotaxin mRNA. *J Biol Chem.* (2020) 295:18134–47. doi: 10.1074/jbc.RA119.012009
83. Janin M, Ortiz-Barahona V, de Moura MC, Martínez-Cardús A, Llinàs-Arias P, Soler M, et al. Epigenetic loss of RNA-methyltransferase NSUN5 in glioma targets ribosomes to drive a stress adaptive translational program. *Acta Neuropathol.* (2019) 138:1053–74. doi: 10.1007/s00401-019-02062-4
84. Pan J, Huang Z, Xu Y. m5C RNA methylation regulators predict prognosis and regulate the immune microenvironment in lung squamous cell carcinoma. *Front Oncol.* (2021) 11:657466. doi: 10.3389/fonc.2021.657466
85. Liu T, Hu X, Lin C, Shi X, He Y, Zhang J, et al. 5-methylcytosine RNA methylation regulators affect prognosis and tumor microenvironment in lung adenocarcinoma. *Ann Transl Med.* (2022) 10:259. doi: 10.21037/atm-22-500
86. Yu T, Zhang Q, Yu SK, Nie FQ, Zhang ML, Wang Q, et al. THOC3 interacts with YBX1 to promote lung squamous cell carcinoma progression through PFKFB4 mRNA modification. *Cell Death Dis.* (2023) 14:475. doi: 10.1038/s41419-023-06008-3
87. Chen Y, Jiang Z, Zhang C, Zhang L, Chen H, Xiao N, et al. 5-Methylcytosine transferase NSUN2 drives NRF2-mediated ferroptosis resistance in non-small cell lung cancer. *J Biol Chem.* (2024) 300:106793. doi: 10.1016/j.jbc.2024.106793
88. Yang Y, Fan H, Liu H, Lou X, Xiao N, Zhang C, et al. NOP2 facilitates EZH2-mediated epithelial-mesenchymal transition by enhancing EZH2 mRNA stability via m5C methylation in lung cancer progression. *Cell Death Dis.* (2024) 15:506. doi: 10.1038/s41419-024-06899-w
89. Lu Z, Liu B, Kong D, Zhou X, Pei D, Liu D. NSUN6 regulates NM23-H1 expression in an m5C manner to affect epithelial-mesenchymal transition in lung cancer. *Med Princ Pract.* (2024) 33:56–65. doi: 10.1159/000535479
90. Su J, Wu G, Ye Y, Zhang J, Zeng L, Huang X, et al. NSUN2-mediated RNA 5-methylcytosine promotes esophageal squamous cell carcinoma progression via LIN28B-dependent GRB2 mRNA stabilization. *Oncogene.* (2021) 40:5814–28. doi: 10.1038/s41388-021-01978-0
91. Niu X, Peng L, Liu W, Miao C, Chen X, Chu J, et al. A cis-eQTL in NSUN2 promotes esophageal squamous cell carcinoma progression and radiochemotherapy resistance by mRNA-m(5)C methylation. *Signal Transduct Target Ther.* (2022) 7:267. doi: 10.1038/s41392-022-01063-2
92. Liu L, Chen Y, Zhang T, Cui G, Wang W, Zhang G, et al. YBX1 promotes esophageal squamous cell carcinoma progression via m5C-dependent SMOX mRNA stabilization. *Adv Sci (Weinh).* (2024) 11:e2302379. doi: 10.1002/adv.202302379
93. Mei L, Shen C, Miao R, Wang JZ, Cao MD, Zhang YS, et al. RNA methyltransferase NSUN2 promotes gastric cancer cell proliferation by repressing p57(Kip2) by an m(5)C-dependent manner. *Cell Death Dis.* (2020) 11:270. doi: 10.1038/s41419-020-2487-z
94. Li Y, Xia Y, Jiang T, Chen Z, Shen Y, Lin J, et al. Long noncoding RNA DIAPH2-AS1 promotes neural invasion of gastric cancer via stabilizing NSUN2 to enhance the m5C modification of NTN1. *Cell Death Dis.* (2023) 14:260. doi: 10.1038/s41419-023-05781-5
95. Xiang S, Ma Y, Shen J, Zhao Y, Wu X, Li M, et al. m(5)C RNA Methylation Primarily Affects the ErbB and PI3K-Akt Signaling Pathways in Gastrointestinal Cancer. *Front Mol Biosci.* (2020) 7:599340. doi: 10.3389/fmolb.2020.599340
96. Yan J, Liu J, Huang Z, Huang W, Lv J. FOXC2-AS1 stabilizes FOXC2 mRNA via association with NSUN2 in gastric cancer cells. *Hum Cell.* (2021) 34:1755–64. doi: 10.1007/s13577-021-00583-3
97. Chen Z, Li Q, Lin Y, Lin S, Gao J, Chen S. m5C regulator-mediated methylation modification phenotypes characterized by distinct tumor microenvironment immune heterogeneity in colorectal cancer. *Sci Rep.* (2023) 13:11950. doi: 10.1038/s41598-023-37300-z
98. Yuan S, Tang H, Xing J, Fan X, Cai X, Li Q, et al. Methylation by NSUN2 represses the levels and function of microRNA 125b. *Mol Cell Biol.* (2014) 34:3630–41. doi: 10.1128/MCB.00243-14
99. Geng Q, Wei Q, Shen Z, Zheng Y, Wang L, Xue W, et al. Comprehensive analysis of the prognostic value and immune infiltrates of the three-m5C signature in colon carcinoma. *Cancer Manag Res.* (2021) 13:7989–8002. doi: 10.2147/CMAR.S331549
100. Zou S, Huang Y, Yang Z, Zhang J, Meng M, Zhang Y, et al. NSUN2 promotes colorectal cancer progression by enhancing SKIL mRNA stabilization. *Clin Transl Med.* (2024) 14:e1621. doi: 10.1002/ctm2.v14.3
101. Li D, Li K, Zhang W, Yang KW, Mu DA, Jiang GJ, et al. The m6A/m5C/m1A regulated gene signature predicts the prognosis and correlates with the immune status of hepatocellular carcinoma. *Front Immunol.* (2022) 13:918140. doi: 10.3389/fimmu.2022.918140
102. Zhang H, Zhai X, Liu Y, Xia Z, Xia T, Du G, et al. NOP2-mediated m5C Modification of c-Myc in an EIF3A-Dependent Manner to Reprogram Glucose Metabolism and Promote Hepatocellular Carcinoma Progression. *Res (Wash D C).* (2023) 6:0184. doi: 10.34133/research.0184
103. Zhang XW, Wu LY, Liu HR, Huang Y, Qi Q, Zhong R, et al. NSUN5 promotes progression and predicts poor prognosis in hepatocellular carcinoma. *Oncol Lett.* (2022) 24:439. doi: 10.3892/ol.2022.13559
104. Lu J, Wang H, Cao W, Chen D, He Z, Xu J. Construction of a m5C-related long non-coding RNA signature for the prognosis of hepatocellular carcinoma. *Hum Cell.* (2023) 36:712–24. doi: 10.1007/s13577-022-00845-8
105. Xing H, Gu X, Liu Y, Xu L, He Y, Xue C. NSUN2 regulates Wnt signaling pathway depending on the m5C RNA modification to promote the progression of hepatocellular carcinoma. *Oncogene.* (2024) 43:3469–82. doi: 10.1038/s41388-024-03184-0
106. Yun D, Yang Z, Zhang S, Yang H, Liu D, Grützmann R, et al. An m5C methylation regulator-associated signature predicts prognosis and therapy response in pancreatic cancer. *Front Cell Dev Biol.* (2022) 10:975684. doi: 10.3389/fcell.2022.975684
107. Huang Y, Zhang W, Li Q, Wang Z, Yang X. Identification of m6A/m5C/m1A-associated lncRNAs for prognostic assessment and immunotherapy in pancreatic cancer. *Sci Rep.* (2023) 13:3661. doi: 10.1038/s41598-023-30865-9
108. Zhang G, Liu L, Li J, Chen Y, Wang Y, Zhang Y, et al. NSUN2 stimulates tumor progression via enhancing TIAM2 mRNA stability in pancreatic cancer. *Cell Death Discov.* (2023) 9:219. doi: 10.1038/s41420-023-01521-y
109. Cheng JX, Chen L, Li Y, Cloe A, Yue M, Wei J, et al. RNA cytosine methylation and methyltransferases mediate chromatin organization and 5-azacytidine response and resistance in leukemia. *Nat Commun.* (2018) 9:1163. doi: 10.1038/s41467-018-03513-4
110. Li Y, Xue M, Deng X, Dong L, Nguyen LXT, Ren L, et al. TET2-mediated mRNA demethylation regulates leukemia stem cell homing and self-renewal. *Cell Stem Cell.* (2023) 30:1072–1090.e1010. doi: 10.1016/j.stem.2023.07.001
111. Chen X, Li A, Sun BF, Yang Y, Han YN, Yuan X, et al. 5-methylcytosine promotes pathogenesis of bladder cancer through stabilizing mRNAs. *Nat Cell Biol.* (2019) 21:978–90. doi: 10.1038/s41556-019-0361-y
112. Wang N, Chen RX, Deng MH, Wei WS, Zhou ZH, Ning K, et al. m(5)C-dependent cross-regulation between nuclear reader ALYREF and writer NSUN2 promotes urothelial bladder cancer malignancy through facilitating RABL6/TK1 mRNAs splicing and stabilization. *Cell Death Dis.* (2023) 14:139. doi: 10.1038/s41419-023-05661-y
113. Chen SJ, Zhang J, Zhou T, Rao SS, Li Q, Xiao LY, et al. Epigenetically upregulated NSUN2 confers ferroptosis resistance in endometrial cancer via m(5)C modification of SLC7A11 mRNA. *Redox Biol.* (2024) 69:102975. doi: 10.1016/j.redox.2023.102975
114. Li C, Wang S, Xing Z, Lin A, Liang K, Song J, et al. A ROR1-HER3-lncRNA signaling axis modulates the Hippo-YAP pathway to regulate bone metastasis. *Nat Cell Biol.* (2017) 19:106–19. doi: 10.1038/ncb3464
115. Zhang X, An K, Ge X, Sun Y, Wei J, Ren W, et al. NSUN2/YBX1 promotes the progression of breast cancer by enhancing HGH1 mRNA stability through m(5)C methylation. *Breast Cancer Res.* (2024) 26:94. doi: 10.1186/s13058-024-01847-0
116. Zheng L, Li M, Wei J, Chen S, Xue C, Duan Y, et al. NOP2/Sun RNA methyltransferase 2 is a potential pan-cancer prognostic biomarker and is related to immunity. *PLoS One.* (2023) 18:e0292212. doi: 10.1371/journal.pone.0292212
117. Chen L, Ding J, Wang B, Chen X, Ying X, Yu Z, et al. RNA methyltransferase NSUN2 promotes hypopharyngeal squamous cell carcinoma proliferation and migration by enhancing TEAD1 expression in an m(5)C-dependent manner. *Exp Cell Res.* (2021) 404:112664. doi: 10.1016/j.yexcr.2021.112664
118. Bai L, Hao X, Keith J, Feng Y. DNA methylation in regulatory T cell differentiation and function: challenges and opportunities. *Biomolecules.* (2022) 12. doi: 10.3390/biom12091282
119. Yue X, Lio CJ, Samaniego-Castruita D, Li X, Rao A. Loss of TET2 and TET3 in regulatory T cells unleashes effector function. *Nat Commun.* (2019) 10:2011. doi: 10.1038/s41467-019-09541-y

120. Tian L, Wang Y, Tian J, Song W, Li L, Che G. Prognostic value and genome signature of m6A/m5C regulated genes in early-stage lung adenocarcinoma. *Int J Mol Sci.* (2023) 24. doi: 10.3390/ijms24076520
121. Wu X, Tang J, Cheng B. Oral squamous cell carcinoma gene patterns connected with RNA methylation for prognostic prediction. *Dis.* (2024) 30:408–21.
122. Karikó K, Buckstein M, Ni H, Weissman D. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity.* (2005) 23:165–75. doi: 10.1016/j.immuni.2005.06.008
123. Lu L, Zhu G, Zeng H, Xu Q, Holzmann K. High tRNA transferase NSUN2 gene expression is associated with poor prognosis in head and neck squamous carcinoma. *Cancer Invest.* (2018) 36:246–53. doi: 10.1080/07357907.2018.1466896
124. Luo Y, Feng J, Xu Q, Wang W, Wang X. NSun2 Deficiency Protects Endothelium From Inflammation via mRNA Methylation of ICAM-1. *Circ Res.* (2016) 118:944–56. doi: 10.1161/CIRCRESAHA.115.307674
125. Tao Z, Ruan H, Sun L, Kuang D, Song Y, Wang Q, et al. Targeting the YB-1/PD-L1 axis to enhance chemotherapy and antitumor immunity. *Cancer Immunol Res.* (2019) 7:1135–47. doi: 10.1158/2326-6066.CIR-18-0648
126. Li R, Zhang Y, Wang A, Feng Y, Zhang T, Wang H, et al. The recruitment of CD8(+) T cells through YBX1 stabilization abrogates tumor intrinsic oncogenic role of MIR155HG in lung adenocarcinoma. *Cell Death Discov.* (2024) 10:334. doi: 10.1038/s41420-024-02102-3
127. Ru J, Lu J, Ge J, Ding B, Su R, Jiang Y, et al. IRGM is a novel regulator of PD-L1 via promoting S6K1-mediated phosphorylation of YBX1 in hepatocellular carcinoma. *Cancer Lett.* (2024) 581:216495. doi: 10.1016/j.canlet.2023.216495
128. Li Z, Xue H, Li J, Zheng Z, Liu Z, Dong X, et al. CDKL1 potentiates the antitumor efficacy of radioimmunotherapy by binding to transcription factor YBX1 and blocking PD-L1 expression in lung cancer. *J Exp Clin Cancer Res.* (2024) 43:89. doi: 10.1186/s13046-024-03007-w
129. Huang Y, Huang C, Jiang X, Yan Y, Zhuang K, Liu F, et al. Exploration of potential roles of m5C-related regulators in colon adenocarcinoma prognosis. *Front Genet.* (2022) 13:816173. doi: 10.3389/fgene.2022.816173
130. Huang Z, Pan J, Wang H, Du X, Xu Y, Wang Z, et al. Prognostic significance and tumor immune microenvironment heterogeneity of m5C RNA methylation regulators in triple-negative breast cancer. *Front Cell Dev Biol.* (2021) 9:657547. doi: 10.3389/fcell.2021.657547
131. Ouyang W, Jiang Y, Bu S, Tang T, Huang L, Chen M, et al. A prognostic risk score based on hypoxia-, immunity-, and epithelial-to-mesenchymal transition-related genes for the prognosis and immunotherapy response of lung adenocarcinoma. *Front Cell Dev Biol.* (2021) 9:758777. doi: 10.3389/fcell.2021.758777
132. Wang Y, Zhao Y, Bollas A, Wang Y, Au KF. Nanopore sequencing technology, bioinformatics and applications. *Nat Biotechnol.* (2021) 39:1348–65. doi: 10.1038/s41587-021-01108-x
133. Reed BD, Meyer MJ, Abramzon V, Ad O, Ad O, Adcock P, et al. Real-time dynamic single-molecule protein sequencing on an integrated semiconductor device. *Science.* (2022) 378:186–92. doi: 10.1126/science.abo7651
134. Pan A, Xue Y, Ruan X, Dong W, Wang D, Liu Y, et al. m5C modification of LINC00324 promotes angiogenesis in glioma through CBX3/VEGFR2 pathway. *Int J Biol Macromol.* (2024) 257:128409. doi: 10.1016/j.ijbiomac.2023.128409
135. Shen X, Sun H, Shu S, Tang W, Yuan Y, Su H, et al. Suppression of NSUN2 enhances the sensitivity to chemosensitivity and inhibits proliferation by mediating cell apoptosis in gastric cancer. *Pathol Res Pract.* (2024) 253:154986. doi: 10.1016/j.prp.2023.154986
136. Hu Y, Chen C, Lin K, Tong X, Huang T, Qiu T, et al. NSUN2 promotes colorectal cancer progression and increases lapatinib sensitivity by enhancing CUL4B/ErbB-STAT3 signaling in a non-m5C manner. *Clin Transl Med.* (2025) 15:e70282.
137. Gu X, Li P, Gao X, Ru Y, Xue C, Zhang S, et al. RNA 5-methylcytosine writer NSUN5 promotes hepatocellular carcinoma cell proliferation via a ZBED3-dependent mechanism. *Oncogene.* (2024) 43:624–35. doi: 10.1038/s41388-023-02931-z
138. Nulali J, Zhang K, Long M, Wan Y, Liu Y, Zhang Q, et al. ALYREF-mediated RNA 5-Methylcytosine modification Promotes Hepatocellular Carcinoma Progression Via Stabilizing EGFR mRNA and pSTAT3 activation. *Int J Biol Sci.* (2024) 20:331–46. doi: 10.7150/ijbs.82316
139. Chen B, Deng Y, Hong Y, Fan L, Zhai X, Hu H, et al. Metabolic Recoding of NSUN2-Mediated m(5)C Modification Promotes the Progression of Colorectal Cancer via the NSUN2/YBX1/m(5)C-ENO1 Positive Feedback Loop. *Adv Sci (Weinh).* (2024) 11:e2309840. doi: 10.1002/advs.202309840



OPEN ACCESS

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RECEIVED 23 October 2024

ACCEPTED 07 April 2025

PUBLISHED 30 April 2025

CITATION

Wang C, Chen M, Chen P, Han J, Hu H, Chen J, Wu Q, Zhao D, Wang T, Zhou J, Li Q, Zhou R, Wen Y, Yang J, Shi M and Wang Y (2025) RBM15-mediated metabolic reprogramming boosts immune response in colorectal cancer. *Front. Immunol.* 16:1515568. doi: 10.3389/fimmu.2025.1515568

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RBM15-mediated metabolic reprogramming boosts immune response in colorectal cancer

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Introduction: Immune checkpoint blockade (ICB) therapy has shown promise in treating advanced colorectal cancer, particularly in patients with microsatellite instability-high (MSI-H) tumors. However, only a subset of these patients responds favorably, highlighting the need for strategies to improve immunotherapy efficacy.

Methods: To identify potential regulators of immunotherapy response, we conducted a comprehensive analysis of colorectal cancer datasets from The Cancer Genome Atlas (TCGA). We performed multi-omics analyses and functional assays in both human and murine colorectal cancer cell lines. Additionally, we evaluated tumor growth and immune cell infiltration using syngeneic mouse models.

Results: Our analysis revealed that RNA binding motif protein 15 (RBM15) is highly expressed in colorectal cancer and correlates with poor patient prognosis. Functional studies demonstrated that RBM15 loss led to increased expression of fumarate hydratase (FH). This led to decreased levels of fumarate, a metabolite known to suppress anti-tumor immune responses. *In vivo*, RBM15 depletion significantly delayed tumor progression and enhanced CD8⁺ T cell infiltration and activation in the tumor microenvironment.

Discussion: These findings identify RBM15 as a negative regulator of anti-tumor immunity in colorectal cancer. Targeting RBM15 may represent a novel strategy to boost immune responsiveness and improve outcomes for patients undergoing immunotherapy.

KEYWORDS

colorectal cancer, RBM15, microenvironment, RNA modification, m⁶A

Introduction

Colorectal cancer (CRC) poses a significant clinical challenge worldwide, with a 5-year survival rate of less than 13% for patients with advanced diseases (1, 2). While immunotherapies open up therapeutic opportunities to advanced CRC, their effectiveness remains limited (3). Only 10–15% patients with microsatellite instability-high (MSI-H) tumors respond to immune checkpoint inhibitors (ICIs) such as anti-PD-1 antibodies (4). Moreover, clinical responses to immunotherapies are generally incomplete and not durable, due in part to high tumor heterogeneity and an immunosuppressive tumor microenvironment (3, 5). Therefore, there is an urgent need to identify novel therapeutic strategies to sensitize immunotherapy.

In recent years, the role of RBM15 in various cancers has attracted significant attention. Studies have shown that inhibition of RBM15 can promote macrophage infiltration and enhance its phagocytic activity toward pancreatic cancer cells. Moreover, RBM15 collaborates with methyltransferase 3 (METTL3) to upregulate N⁶-methyladenosine (m⁶A) modification of long non-coding RNAs, facilitating bladder cancer initiation and progression². Additionally, RBM15 regulates m⁶A methylation to upregulate integrin subunit beta like 1 (ITGBL1) expression, promoting the progression of colorectal adenocarcinoma. RBM15 also modulates procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 (PLOD3), enhancing tumor-infiltrating CD4⁺ T cell abundance in esophageal squamous cell carcinoma (ESCC), correlating with favorable prognosis in ESCC⁴. Furthermore, RBM15 may promote malignant progression and immune escape in breast cancer cells by regulating the stability of karyopherin subunit alpha 2 (KPNA2) mRNA⁵.

N⁶-Methyladenosine (m⁶A) is the most abundant and conserved modification of eukaryotic mRNAs, and its role in tumor immunomodulation has become a focus of extensive research (6, 7). For example, the m⁶A reader protein YTHDF1 drives immune evasion and resistance to immunotherapies by promoting the degradation of major histocompatibility complex class I (MHC-I) (8). RNA binding motif protein 15 (RBM15) is a crucial regulator of m⁶A modification (9). RBM15 interacts with the m⁶A writer complex and positively regulates m⁶A levels, influencing on alternative splicing and mRNA stability (10). While ample evidence supports the role of RBM15 in oncogenesis, these studies primarily focus on alterations in cancer cells within immunocompromised environments (11–13). Little is known about whether or how RBM15 regulates tumor immune surveillance in cancers.

Accumulating studies indicates that metabolic rewiring in malignant cells impairs both innate and adaptive immune functions, thus promoting tumor progression (14). Cancer cell-intrinsic and cancer cell-extrinsic mechanisms both play crucial roles in tumor immune evasion and responses. For example, cancer cells compete with CD8⁺ cytotoxic T lymphocytes (CTLs) for glucose uptake to meet their increased proliferative demands, which compromises CTL function (15). In addition, the

degradation of extracellular ATP by the ectonucleotidases CD39 and CD73 generates adenosine, which induces an immunosuppressive tumor microenvironment by reducing dendritic cell (DC) recruitment (16). However, whether RBM15 plays a role in metabolic reprogramming that could affect anti-tumor immunomodulation remains poorly understood.

In this study, we reveal a cancer cell-intrinsic function of RBM15 in driving immune evasion in colorectal cancer. We found that RBM15 is overexpressed in colorectal cancer and is associated with poor prognosis. RBM15 deficiency restrains tumor growth by enhancing immune cell infiltration. Mechanistically, RBM15 depletion increases the expression of fumarate hydratase (FH), which in turn decreases the level of fumarate, a known suppressor of anti-tumor immunity. Overall, our study identifies RBM15 as a potent suppressor of anti-tumor immunity and highlights RBM15 as a promising therapeutic target for restoring immune surveillance in colorectal cancer.

Results

RBM15 overexpression correlates with reduced immune cell infiltration in colorectal cancer

To unravel the role of m⁶A modification in tumor immunomodulation of colorectal cancer, we analyzed immune scores based on the expression level of 141 genes reflecting immune signatures using the ESTIMATE platform (17). We selected a total of 19 m⁶A regulators, including writers, readers, and erasers, to access the correlation between their expression levels and immune scores (Figure 1a). In the Cancer Genome Atlas (TCGA)-Colon adenocarcinoma (COAD) dataset, *RBM15* RNA showed the second strongest inverse correlation with immune cell infiltration levels (Figures 1b, c). The immunomodulatory role of the top-ranked gene, *YTHDC1*, has been extensively investigated in other cancer types (18). Consistently, *RBM15* expression was negatively correlated with tumor purity, suggesting *RBM15* may suppress the recruitment of immune cells (Figures 1b, d).

We further explored whether *RBM15* plays a role in immunomodulation in other digestive system cancers. Strikingly, *RBM15* RNA did not show a negative correlation with immune cell infiltration in liver hepatocellular carcinoma (LIHC), pancreatic adenocarcinoma (PAAD), or stomach adenocarcinoma (STAD) (Supplementary Figures S1A–F). Moreover, *RBM15* expression was not correlated with tumor purity in LIHC or PAAD, but was negatively correlated with that in STAD (Supplementary Figures S1G–I). These findings indicate a colon tissue-specific oncogenic role of *RBM15*. Furthermore, *RBM15* expression was significantly higher in colorectal cancer compared to adjacent normal tissues (Figure 1E).

Microsatellite instability (MSI) is a key predictor of responses to immunotherapy, in part because high MSI (MSI-H) tumors present foreign surface markers that are more easily recognized by immune

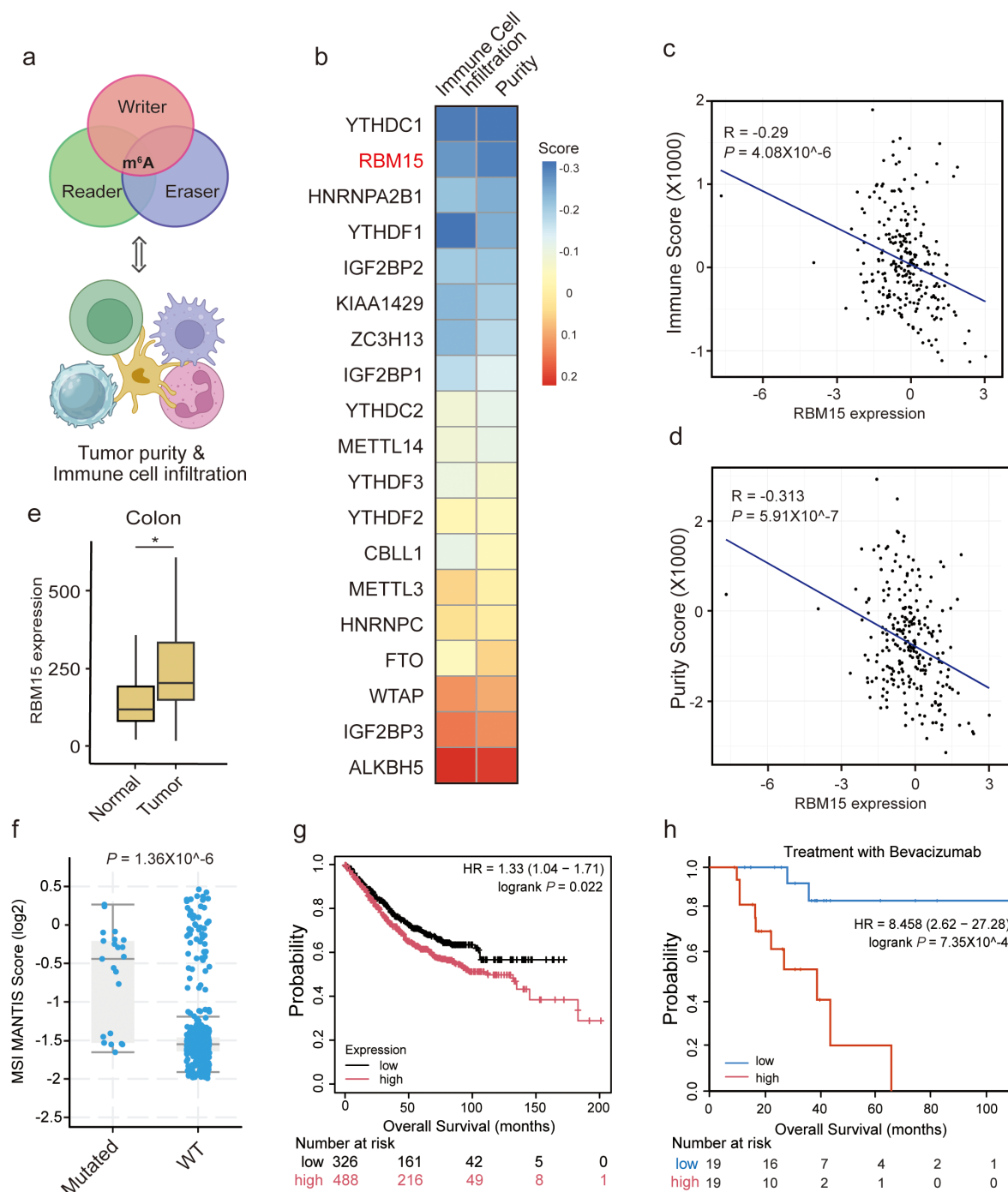


FIGURE 1

RBM15 overexpression is negatively correlated with immune cell infiltration in colorectal cancer. **(a)** A conceptual diagram illustrating the strategy to identify potential correlation between the expression levels of m⁶A regulators and tumor purity, as well as immune cell infiltration. **(b)** Heatmap of the correlation between m⁶A regulators and tumor purity, as well as immune cell infiltration in the Cancer Genome Atlas (TCGA)-Colon adenocarcinoma (COAD) dataset. **(c, d)** Scatter plot showing a negative correlation between RBM15 expression and the Immune Score **(c)** as well as tumor purity **(d)**. **(e)** Box plot representing RBM15 expression levels in adjacent normal and tumor colon tissues. **(f)** Comparison of MSI MANTIS Scores between mutant RBM15 (Mutated) and Wild-Type (WT) colorectal cancer, showing a significant difference between the two groups. **(g)** Kaplan-Meier survival curves displays the survival probability over time (months) for two groups of patients: high RBM15 expression (red curve) and low RBM15 expression (black curve). The number of patients at risk at various time points is indicated below the plot. High RBM15 expression is associated with worse survival outcomes compared to low RBM15 expression. **(h)** Kaplan-Meier survival analysis of overall survival (OS) for advanced colorectal cancer patients treated with Bevacizumab, stratified by RBM15 expression into low (blue) and high (red) groups. The number of patients at risk at various time points is indicated below the plot.

cells (19). Indeed, colorectal cancer patients with MSI-H status tend to have better outcomes and often achieve a strong response to ICIs (5). We found that mutant RBM15 significantly linked to higher MSI MANTIS scores in colorectal cancer, supporting the role of RBM15 in regulating tumor immunomodulation (Figure 1f). Furthermore, high expression of RBM15 was associated with poor prognosis in colorectal cancer patients (Figure 1g). Notably, this association was more pronounced in advanced colorectal cancer patients receiving Bevacizumab treatment (Figure 1h). Overall, these findings suggest that RBM15 overexpression is negatively correlated with immune cell infiltration in colorectal cancer, presenting an immunosuppressive function of RBM15 in colorectal tumor microenvironment.

RBM15 deficiency induces metabolic alterations in colorectal cancer

Recent studies have highlighted the cancer cell-intrinsic mechanisms involved in modulating tumor immunity (20, 21). To reveal the tumor-intrinsic functional role of RBM15 in colorectal cancer, we initially utilized the CRISPR/Cas9 gene editing system to knock out (KO) RBM15 in the human colorectal cancer cell line HCT15. We designed two sgRNA fragments targeting the *RBM15* gene to minimize potential off-target effects. Western blotting analysis showed that RBM15 protein was significantly depleted (Figure 2a). Consistent with previous findings, we observed a marked reduction in m⁶A modification in the mRNA of RBM15 KO cells, further suggesting the sufficient KO efficiency and a shared functional consequence by the two distinct sgRNAs (Figure 2b).

To unravel the gene expression alterations induced by RBM15, we performed bulk RNA sequencing (RNA-seq) in RBM15 KO and wild-type (WT) colorectal cancer HCT15 cells. RBM15 KO significantly reduced alternative splicing events at both the 5' and 3' end of RNAs (Figures 2c, d). This was consistent with the previous finding that m⁶A modification regulates the alternative splicing of precursor RNAs (22, 23). Further analysis of differential gene expression revealed that a total of 994 genes were significantly altered (Figure 2e). Strikingly, genes associated with metabolic pathways were among the top differentially expressed genes, including the fucosyltransferase 1 (FUT1), the phosphoglycerate dehydrogenase (PHGDH), and the cystine transporter solute carrier family 7 member 11 (SLC7A11) (Figure 2f). Indeed, gene set enrichment analysis (GSEA) confirmed the enrichment of multiple pathways associated with metabolism (Figure 2g).

We further performed cell mitochondrial test to establish the mitochondrial function of the human and murine cells with and without RBM15. RBM15 knockout significantly decreased the maximal mitochondrial respiration in both human and murine colorectal cancer cells (Supplementary Figure S2). These findings suggest that RBM15 may regulate mitochondrial metabolism to affect cellular energy supply. In addition, RBM15 knockdown caused a slight reduction of cell proliferation in either human or murine colorectal cancer cells (Supplementary Figure S3).

RBM15 depletion alters carbon metabolism and upregulates the expression of fumarate hydratase

To identify the fundamental metabolic pathways interfered by RBM15, we tracked the altered metabolites by RBM15 knockdown using untargeted high-resolution metabolic profiling. Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) revealed good reproducibility and discrimination for RBM15 knockdown and WT cells (Supplementary Figures S4A, B) (24). Through combining differential metabolites from both positive and negative ion modes, we identified a total of 860 significantly altered metabolites by RBM15 knockdown. We then categorized these altered metabolites based on their chemical taxonomy and showed that organic acids, lipids, and organ heterocyclic compounds accounted for the majority of the differentially expressed metabolites (Supplementary Figures S4C, S5A-B).

We next sought to determine the enriched differential metabolic pathway affected by RBM15 knockdown. Differential abundance analysis showed that carbon metabolism presented as one of the top downregulated metabolic pathways among all the enriched pathways matched from Kyoto Encyclopedia of Genes and Genomes (KEGG) (Figure 3a). We further examined the specific dysregulated metabolites associated with carbon metabolism by RBM15 knockdown. Metabolites including fumarate, glutamic acid, malic acid, alanine, citrate, and isocitric acid were significantly downregulated (Figure 3b). Accordingly, the expression levels of metabolic enzymes involved in carbon metabolism were altered due to RBM15 knockout, indicating a systematic reprogramming of cellular carbon metabolism caused by RBM15 depletion (Figure 3c). Furthermore, we confirmed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) that the expression of key catalytic enzymes involved in carbon metabolism was significantly changed (Figures 3d-f).

Fumarate is produced through the carbon metabolism, and its accumulation in tumor interstitial fluid has been shown to suppress CD8⁺ T cell activation and anti-tumor immune responses (20). Conversely, fumarate depletion by increasing the expression of fumarate hydratase (FH) in tumor cells dramatically enhances the anti-tumor cytotoxicity of chimeric antigen receptor (CAR) T cells (20). Strikingly, we found that FH expression was significantly upregulated in human and murine colorectal cancer cells by RBM15 KO, which in turn led to a reduction in fumarate levels (Figures 3b, c, f, Supplementary Figures S6A-C). Collectively, these findings suggest that RBM15 depletion significantly affects carbon metabolism and upregulates the expression level of FH, which in turn downregulates fumarate in colorectal cancer.

RBM15 deficiency delays tumor growth through enhanced immune infiltration

Given that RBM15 depletion reduces fumarate levels, which could potentially enhance CD8⁺ T cell activation and increase anti-tumor immune responses, we next determine whether RBM15

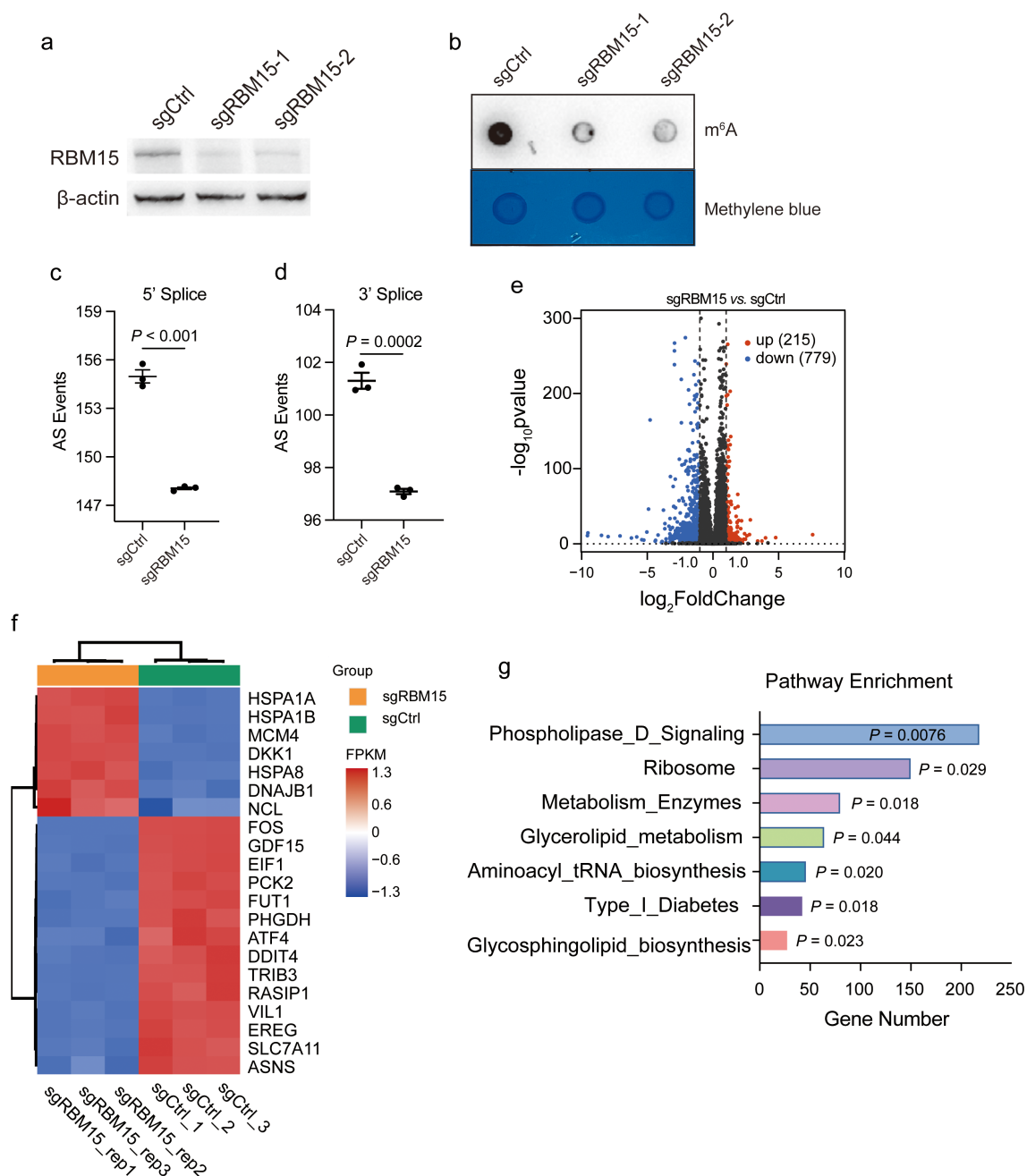


FIGURE 2

RBM15 deficiency induces metabolic alterations in colorectal cancer. **(a)** Western blot analysis showing RBM15 protein levels in the human colorectal cancer cell line HCT15 treated with control sgRNA (sgCtrl) and two different sgRNAs targeting RBM15 (sgRBM15-1 and sgRBM15-2). **(b)** Dot blot analysis showing m⁶A RNA methylation levels in HCT15 treated with control sgRNA (sgCtrl) and sgRNAs targeting RBM15 (sgRBM15-1 and sgRBM15-2). The methylene blue staining ensures equal loading across the samples. **(c, d)** Quantification of alternative splicing (AS) events at the 5' and 3' splice sites in control (sgCtrl) and RBM15 knockout (sgRBM15) of HCT15 cells. **(e)** Volcano plot illustrating differential gene expression between RBM15 KO (sgRBM15) and control (sgCtrl) HCT15 cells. Upregulated genes are marked in red (215 genes), while downregulated genes are marked in blue (779 genes). Non-significant genes are shown in black. **(f)** Heatmap representing top genes with differential expression between RBM15 KO (sgRBM15) and control (sgCtrl) HCT15 cells. The expression levels of selected genes (listed on the right) are shown as log-transformed Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values. **(g)** Pathway enrichment analysis of differentially expressed genes, comparing the RBM15 KO (sgRBM15) and control (sgCtrl) group.

deficiency could inhibit tumorigenesis via enhanced immune surveillance. We utilized the CRISPR/Cas9 gene editing system to deplete *Rbm15* gene in a synergetic mouse cell line MC38 (Supplementary Figure S7A). Then, immunocompetent C57BL/6J

mice were subcutaneously injected with either *Rbm15*-KO cells or WT cells. Notably, *Rbm15* knockout significantly prohibited tumorigenesis in immunocompetent mice, as evidenced by reductions in both tumor volume and weight, with no changes

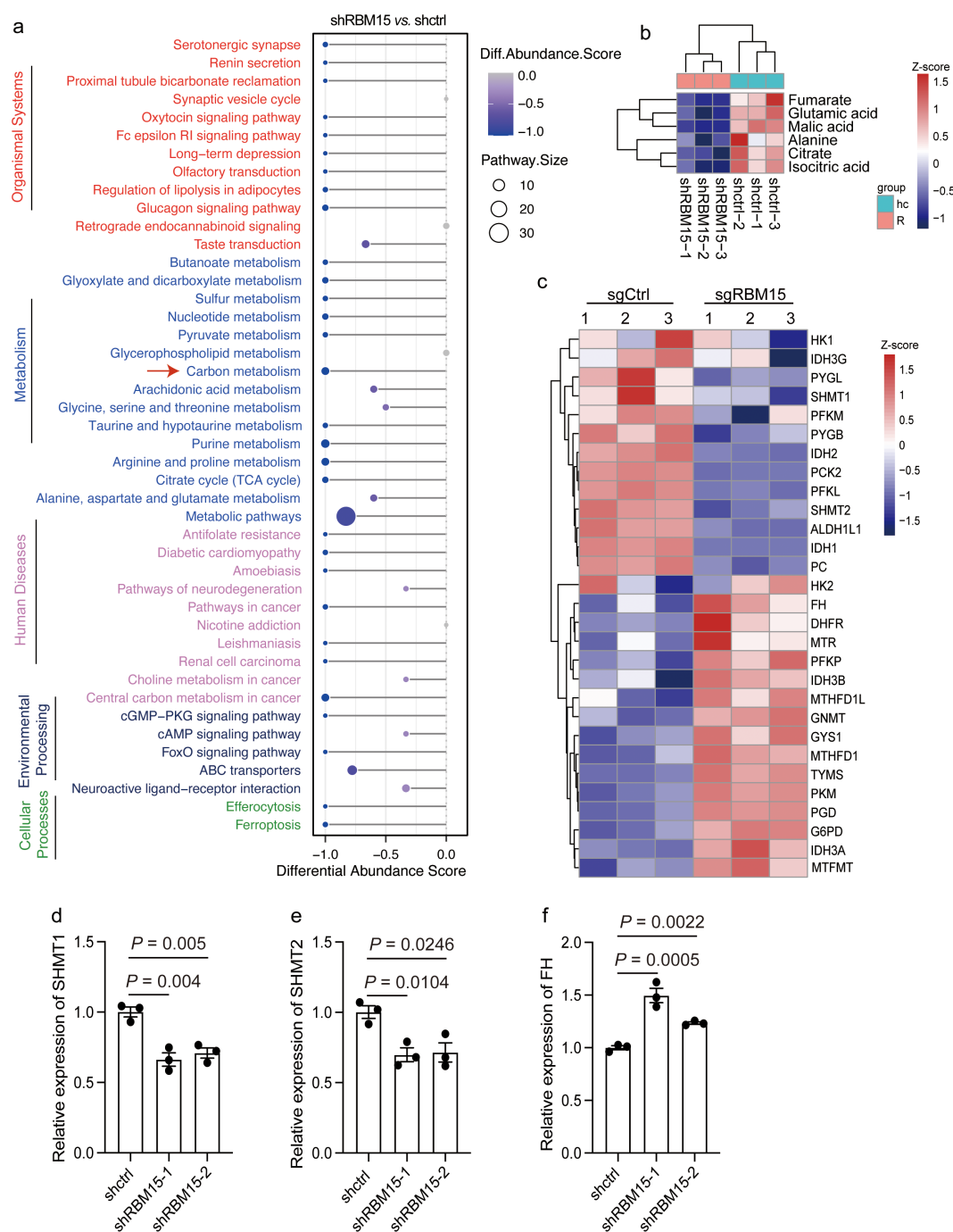


FIGURE 3

RBM15 depletion alters carbon metabolism and upregulates the expression of fumarate hydratase (FH). **(a)** Differential abundance score representing differentially downregulated pathways in cells with RBM15 knockdown (shRBM15) compared to control (shctrl) cells. The plot on the left displays the pathways grouped by functional categories. **(b)** Heatmap showing the Z-score normalized levels of specific metabolites associated with carbon metabolism including fumarate, glutamic acid, malic acid, alanine, citrate and isocitric acid, in cells with RBM15 knockdown (shRBM15) and control (shctrl) cells. **(c)** Heatmap depicting the Z-score normalized expression levels of metabolic enzymes involved in carbon metabolism in RBM15-knockout (sgRBM15) and control (sgCtrl) cells. **(d-f)** Bar graphs showing the relative expression levels of key catalytic enzymes involved in carbon metabolism, including SHMT1 **(d)**, SHMT2 **(e)**, and FH **(f)**, in RBM15-knockdown (shRBM15) and control (shctrl) cells. Data are represented as mean $\pm$ SEM. $n = 3$.

detected in body weights (Figures 4a, b, Supplementary Figures S7B-C). In contrast, there were limited differences in tumor weight between *Rbm15*-KO cells and WT cells in immunodeficient nude mice, suggesting that the reduced tumor growth caused by *Rbm15*

deficiency mainly attributed to the induction of anti-tumor immunity. (Figures 4c, Supplementary Figure S8A).

To investigate the alterations in the tumor immune microenvironment induced by RBM15, we performed multicolor

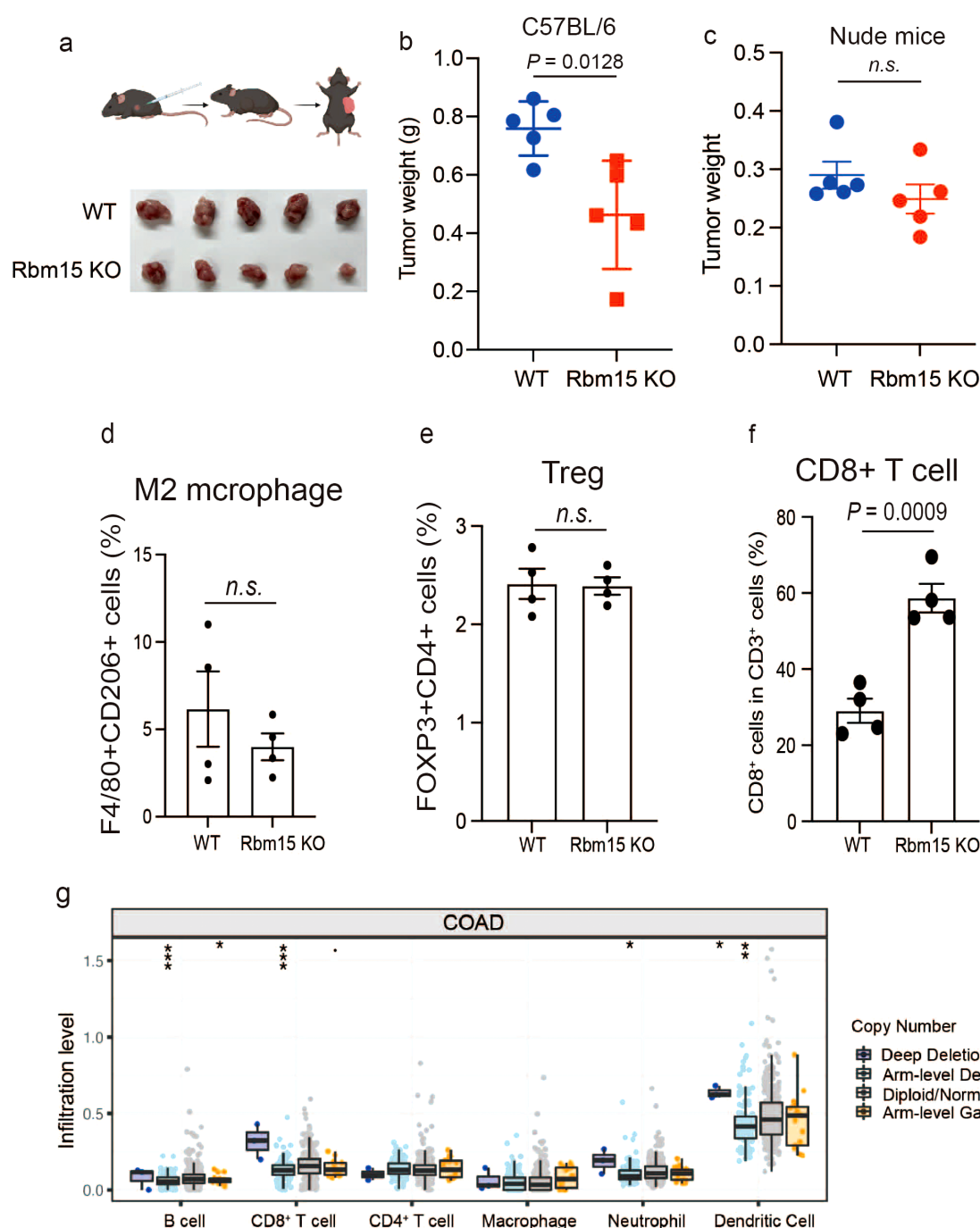


FIGURE 4

RBM15 deficiency delays tumor growth through enhanced immune infiltration. **(a)** Schematic illustration of subcutaneous injection of mouse syngeneic MC38 cells into immunocompetent C57BL/6J mice (upper panel) and tumor growth comparison between wild-type (WT) and Rbm15-knockout (Rbm15 KO) groups (lower panel). **(b)** Tumor weight in C57BL/6J mice comparing WT and Rbm15 KO groups. $n=5$ for each group. **(c)** Tumor weight in nude mice comparing WT and Rbm15 KO groups. $n=5$ for each group. **(d)** Representative flow cytometry analysis showing the expression of immune cell markers from the WT and Rbm15 KO tumors in C57BL/6J mice. **(d-f)** Quantitative flow cytometry analysis of immune cell populations within the immunocompetent tumor microenvironment of WT and Rbm15 KO mice. Analysis including the percentage of M2 macrophages (F4/80+CD206+ cells) **(d)**, Tregs (FOXP3+CD4+ cells) **(e)**, and CD8+ T cells **(f)**. **(g)** Infiltration levels of various immune cell types in colorectal adenocarcinoma (COAD) based on copy number alterations. Box plots show the infiltration levels of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells across groups with distinct copy number alterations, including deep deletion, arm-level deletion, diploid/normal, and arm-level gain. Statistical significance between groups is indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). $n.s.$, non-significant.

flow cytometry analysis along with multiplex immunofluorescence (mIHC) to assess immune cell infiltration in mouse tumors (Supplementary Figures S9, S10). The results showed that Rbm15 knockout did not significantly alter the proportion of M2 macrophages (F4/80+/CD206+), Treg cells (FOXP3+/CD4+), or IFN γ production (Figures 4d, e, Supplementary Figures S11A-B, S12A-C). However, there were a significant increased proportion of CD8 $^{+}$ T cells, DCs (CD11C+), and TNF- α^{+} production (Figures 4f, Supplementary Figures S11E-F, S12D, S13). Consistently, the infiltration levels of CD8 $^{+}$ T cells and DCs were markedly higher in human colorectal cancer tumors with genetic alterations leading to deep depletion of RBM15 (Figure 4g). Furthermore, analysis of publicly available single-cell RNA datasets further demonstrated that there was a negative correlation between RBM15 expression and the CD8 T cell infiltration in tumors of colorectal cancer patients (Supplementary Figure S14). In addition, Rbm15 knockout significantly exhibited enhanced FH expression in MC38-derived xenograft tumors, demonstrating that RBM15 may regulate anti-tumor immune responses via FH modulation (Supplementary Figure S15). Overall, these findings suggest that RBM15 deficiency prohibits tumorigenesis, potentially through increased CD8 $^{+}$ T cell infiltration and activation.

Discussion

Here we identify a cancer cell-intrinsic mechanism by which RBM15 suppresses the tumor immune response in colorectal cancer. Specifically, RBM15 deficiency significantly delayed colorectal tumor growth by enhancing immune cell infiltration, potentially due to reduced fumarate levels within the tumor microenvironment. This decrease in fumarate was linked to increased expression of fumarate hydratase (FH) induced by RBM15 depletion. Previous studies have highlighted the oncogenic role of RBM15 in various cancer types, such as breast and cervical cancer (12, 13). However, they did not address tumor immunity by focusing on immunocompromised environments. Our findings thus are significant because they connect the tumor-intrinsic functional role of RBM15 to the anti-tumor immune responses. Furthermore, we demonstrate that the increased infiltration of immune cells, such as CD8 $^{+}$ T cells, neutrophils, and dendritic cells, contributed to the enhanced anti-tumor immune responses. Nevertheless, whether CD8 $^{+}$ T cell infiltration is the dominant contributor to this response or whether it is part of a collective infiltration effect requires further investigation.

Tumor cells undergo metabolic rewiring to evade immune surveillance (25–27). Inhibitors targeting the cancer cell-intrinsic metabolic dysregulation have been shown to restore immunosurveillance, with several currently under development in clinical trials (21). However, increasing intrinsic fumarate levels through FH inhibition suppresses CD8 $^{+}$ T cell anti-tumor functions, making FH inhibitors unsuitable for exploring anti-tumor efficacy (20). Our study identifies RBM15 as an upstream regulator of FH, as RBM15 deficiency significantly increases FH expression while decreasing associated fumarate levels. Therefore,

our findings suggest that RBM15 is a promising therapeutic target for enhancing anti-tumor immune responses through metabolic reprogramming. Currently, no specific inhibitors for RBM15 have been identified. However, with the recent elucidation of the crystal structure for human RBM15, it offers great potential to design, screen, or optimize inhibitors for future translational applications (10).

Accumulating evidences have bridged RNA modification, particularly m 6 A modification, to anti-tumor immunity (28–31). RBM15 is a key component of the m 6 A writer complex that specifically interacts with WTAP and VIRMA, both of which positively regulate m 6 A levels (10). Alteration in m 6 A levels of mRNAs broadly influence post-transcriptional regulation, such as mRNA stability and degradation (32). Beyond this, RBM15 contains phosphoserine binding modules that recognizes the C-terminal domain (CTD) of RNA polymerase II (Pol II), thereby synergizing with the m 6 A methyltransferase complex to mediate co-transcription (10). In our study, we revealed that RBM15 negatively regulated the expression of FH. However, further exploration is needed to determine whether this regulation occurs at the transcriptional level or through post-transcriptional mechanisms. Nevertheless, the connection between RBM15 and FH adds another layer to the understanding of anti-tumor immune responses, by which the m 6 A writer component suppresses the anti-tumor immunity through regulating key enzymes in carbon metabolism, leading to the release of immunosuppressive metabolites.

In summary, this study identifies a cancer cell-intrinsic mechanism by which RBM15 acts as a suppressor of anti-tumor immune responses through metabolic rewiring. This study also provides a compelling rationale for establishing RBM15 as a promising therapeutic target for colorectal cancer.

Materials and methods

Cell culture and reagents

The human colorectal cancer cell line HCT15 (Fuheng Bio, FH0026) and the mouse colorectal cancer cell line MC38 (LYNJUNE, LYN-0573) were cultured in RPMI-1640 medium (KGL1505-500) supplemented with 1% penicillin/streptomycin and 10% FBS (Gibco, A5669402) at 37°C in a 5% CO $_2$ atmosphere. All cell lines were authenticated using short tandem repeat DNA profiling. Mycoplasma detection was performed monthly using a one-step mycoplasma detection kit (Vazyme, D201-01). CRISPR plasmids for inducing RBM15 knockout were purchased from Tsingke and utilized the pLentiCRISPR V2-puro backbone (Addgene #98290) with specific sgRNAs as insert fragments. The following sgRNAs were designed: human sgRBM15-1 (CCAGCTTAGTGACGAAGCGG), human sgRBM15-2 (GTGAAGGCCAAACGCTCCCG), and mouse sgRBM15-1 (GCGGCGCCGGCTCACGTACA). Additionally, lentiviral plasmids for RBM15 genetic knockdown were designed as follows with pLKO-puro as vector: human shRBM15-1 (GACGCCTTAGAGTAGACTTTG) and human shRBM15-2 (ATTACCTGGTCATGATCATTG).

Lentivirus infection and selection

Lentiviral particles were produced for CRISPR knockout and genetic knockdown. Briefly, HEK293T cells (Fuheng, FH0244) were co-transfected with lentiviral plasmids and packaging plasmids pMD2.G and psPAX2 (Addgene #12260 and Addgene #12259) using the cationic polymer polyethylenimine (PEI, MW25000) at a ratio of 1:3. After 48 to 72 hours of transfection, the lentiviral particles were harvested from the culture supernatant by filtering through a 0.45 μm filter (Sangon, F513144). The harvested lentivirus was aliquoted and stored at -80°C until further use. HCT15 and MC38 colorectal cancer cells were infected with the lentivirus in the presence of 8 $\mu\text{g}/\text{ml}$ polybrene (Biosharp, BL628A). Following transduction, 2 $\mu\text{g}/\text{ml}$ puromycin (Meilun, MA0318) was used for 48 to 72 hours to select for knockout or knockdown cell lines.

Western blot

Total protein was extracted from the indicated samples using RIPA buffer (50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mmol/L phenylmethylsulfonyl fluoride) on ice for 20 minutes. The total protein concentration was measured using the BCA Protein Quantification Kit (Beyotime, P0012). Equal amounts of protein lysates were mixed with 1 $\times$ loading buffer and heated at 95°C for 10 minutes. The samples were then loaded onto precast gels (GenScript, M00944) for electrophoresis. Following electrophoresis, proteins were transferred to a PVDF membrane (Millipore, ISEQ00010). The membrane was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature, followed by overnight incubation at 4°C with the following primary antibodies: m⁶A mAb (Proteintech, 68055-1-Ig, 1:1000), RBM15 polyclonal antibody (Proteintech, 10587-1-Ab, 1:1000), and β -actin mAb (STARTER, S0B0005-100 μg , 1:2000). After washing with TBST buffer, the membrane was incubated with horseradish peroxidase (HRP)-coupled secondary antibodies (CST, anti-rabbit #7074 and anti-mouse #7076, 1:3000) for 1 hour at room temperature. After washing three times with TBST buffer for 8 minutes each, protein bands were visualized using enhanced chemiluminescence (ECL) detection reagents (Millipore, WBKLS0500), and signals were detected using an Automatic ChemiDoc Imaging System (Tanon 5200).

Quantitative real-time polymerase chain reaction

Total RNA was isolated from the samples using the FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, RC112-01) according to the manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized

from 1 μg of total RNA using HiScript II Q RT SuperMix for qPCR (Vazyme, R223-01). Quantitative PCR (qPCR) was performed using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Q712) on an QuantStudio Real-Time PCR machine with specific primers. The cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing at 60°C for 40 seconds. The specificity of the PCR products was confirmed by melting curve analysis. The relative transcriptional expression of target genes was normalized to the geometric mean of reference gene (B2M) and were evaluated by the comparative Ct ($\Delta\Delta\text{Ct}$) method. Fold changes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. The following primers were used: human RBM15 For (ACGACCCGCAACAATGAAG), human RBM15 Rev (GGAAGTCGAGTCCTCACCAC), human B2M For (GAGGCTATCCAGCGTACTCCA), human B2M Rev (CGGCAGGCAT ACTCATCTTTT), human SHMT1 For (CTGGCACAACCCCTCAAAGA), human SHMT1 Rev (AGGCAATCAGCTCCAATC CAA), human SHMT2 For (CCCTTCTGCAACCTCAGCAG), human SHMT2 Rev (TGAGCTTATAGGGCATAGACTCG), human FH For (GGAGGTGTGACAGAACGCAT), human FH Rev (CATCTGCTGCCTTCATTATTGC).

Dot blot

Total RNA from the indicated samples was extracted using the FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, RC112-01). A final concentration of 1 $\mu\text{g}/\mu\text{l}$ RNA was dotted onto a nitrocellulose (NC) membrane (SIMUWU, SD0045). The membrane was air-dried for 10 minutes and subsequently blocked with a 1% BSA (in PBST) solution for one hour. Methylene blue staining (0.2%, Yuanye Bio-Technology, R20768) was applied as a loading control. The membrane was then incubated with primary antibodies (m⁶A mAb, Proteintech, 68055-1-Ig, 1:1000) diluted in 1% BSA (in PBST) at room temperature for one hour. After washing four times with PBST buffer for 5 minutes each, the membrane was incubated with secondary antibodies (CST, #7076) diluted in 1% BSA in PBST at room temperature for one hour. Following four additional washes with PBST buffer for 5 minutes each, the membrane was treated with enhanced chemiluminescence (ECL) detection reagents (Millipore, WBKLS0500) and imaged using an Automatic ChemiDoc Imaging System (Tanon 5200).

Cell proliferation assay

Cell proliferation was measured using the CellTiter-Lumi Cell Viability Assay (beyotime, #C0056). Cells were seeded in 96-well plates for 72 hours at indicated conditions. CellTiter-Lumi reagent was added to each well based on the manufacturers' manual and luminescence was measured on a white microplate (beyotime, #FCP968) using a microplate reader (PerkinElmer). Luminescence values presented as mean $\pm$ SEM. Statistical significance was determined using Student's t-test.

RNA-sequencing and analysis

Total RNA from control HCT15 and RBM15-knockout HCT15 cell lines was extracted using TRIzol according to the manufacturer's instructions (TSP401) across three independent groups. RNA sequencing (RNA-seq) libraries were constructed using the VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina (NR604-02) and subjected to a paired-end 150 bp sequencing run on the Illumina NovaSeq 6000. Raw data were aligned using HISAT2 v2.2.1 against the hg38 version of the human genome, and read counts and fragments per kilobase million (FPKM) values for each sample were calculated using StringTie v2.0.4. The R package DESeq2 v1.26.0 was utilized to assess the significance of differential expression between group pairs and to calculate normalized counts. Gene expression changes were considered significant if they met the threshold of $P < 0.05$. Enrichment analysis was conducted using Gene Set Enrichment Analysis (GSEA v4.1.0), with results deemed significant at $P < 0.05$. Alternative splicing events in RNA-seq data were analyzed using ASprofile v1.0.4. Raw RNA-seq reads were aligned to the human genome (hg38) using HISAT2 (v2.2.1). ASprofile was used to detect and quantify alternative splicing events including 5' splice site changes and 3' splice site changes. Statistical analysis was performed using the Chi-square test (χ^2 test) with events considered significant if $P < 0.05$.

Metabolic profiling

The cells were incubated and vortexed with a chilled extraction solution (2:2:1 v/v methanol/acetonitrile/water) for a minimum of 20 minutes. Subsequently, each sample was centrifuged at maximum speed at 4°C for 20 minutes, and the resulting supernatant was used for untargeted metabolomics analysis. The analysis was conducted using ultra-performance liquid chromatography coupled with a tandem quadrupole time-of-flight mass spectrometer (UHPLC-Q-TOF/MS). Chromatographic separation was achieved on an Agilent 1290 Infinity UHPLC system, with the column temperature maintained at 25°C, a flow rate of 0.3 mL/min, and an injection volume of 2 μ L. The mobile phase consisted of solvent A: water + 25 mM ammonium acetate + 25 mM ammonia, and solvent B: acetonitrile. The gradient elution program was as follows: 0–1.5 min, 98% B; 1.5–12 min, B linearly decreased from 98% to 2%; 12–14 min, B was maintained at 2%; 14–14.1 min, B linearly increased from 2% to 98%; 14.1–17 min, B was maintained at 98%. Throughout the analysis, samples were kept in a 4°C autosampler. High-resolution tandem mass spectrometry was performed using Triple TOF 6600 spectrometers (AB SCIEX) under the following conditions: nebulizer gas (Gas1) at 60 psi, auxiliary gas (Gas2) at 60 psi, curtain gas (CUR) at 30 psi, ion source temperature at 600°C, and spray voltage (ISVF) at ± 5500 V (for both positive and negative modes). The mass-to-charge ratio (m/z) range for the first stage was 80–1200 Da, with a resolution of 60,000 and a scan accumulation time of 100 ms. The second stage employed a segmented acquisition method, with a scan range of 70–1200 Da, a resolution of 30,000, a scan accumulation

time of 50 ms, and a dynamic exclusion time of 4 seconds. Raw data were transformed into the “.mzXML” format using ProteoWizard. Finally, annotation and quantification of metabolites were performed using XCMS software version 3.7.1.

Seahorse XF cell metabolism assay

To evaluate mitochondrial respiration, cells at indicated conditions were seeded in Seahorse XF 96-well plates (5×10^4 cells per well) and incubated overnight. After the incubation in XF RPMI1640 base medium, the following metabolic modulators were sequentially injected: oligomycin, trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP), and rotenone/antimycin A using XF Cell Mito Stress Test Kit (#103015-100). Oxygen consumption rate (OCR) was measured using the Seahorse XF Analyzer (Agilent Technologies) and was used to calculate maximal respiration. Each sample was normalized to protein quantity and presented as mean $\pm$ SEM.

Flow cytometry analysis

For immune infiltration analysis, subcutaneous tumors were excised, minced into small pieces (1 to 2 mm), and digested using digestion buffer (abs9482). The cells were then filtered through 40 μ m cell strainers. Analysis of tumor-infiltrating immune cells involved Live/Dead staining (BD, #564406), followed by Mouse Fc-blocking (BD, #553141), and surface staining in FACS buffer (BD, #554656) with fluorochrome-conjugated antibodies. The antibodies used included mouse CD45 (BD, #557659), mouse CD3 (BD, #553061), mouse CD8 (BD, #566985), mouse CD4 (BD, #550954), mouse CD11b (BD, #563015), and mouse F4/80 (BD, #565411), mouse FOXP3 (BD, #560408), mouse CD206 (BD, #568809), mouse TNF α (BD, #563943), and mouse IFN γ (BD, #561040). All FACS analyses were performed on a BD FACSCelesta, and the data were analyzed using FlowJo software.

Animal experiments

Animal studies were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University. Six- to eight-week-old mice were purchased from Hangzhou Ziyuan Experimental Animal Technology Co., Ltd. A total of 1×10^6 MC38 cells, with or without Rbm15 knockout, were subcutaneously injected into the right flank of male C57BL/6 or nude mice ($n = 5$ mice per group). Tumor volume, tumor weight, and body weight of the mice were measured at specified time points.

Data mining

The immune score was calculated using the ESTIMATE platform, an approach that provides with scores for tumor purity,

and the infiltration level of immune cells in tumor tissues based on expression data from The Cancer Genome Atlas (TCGA) tumors (17). Pearson correlation was used for calculating P and R values. Differential gene expression analysis in colorectal tumor vs normal tissues were performed by the TNMplot platform (33). MSI MANTIS score was calculated using the cBioPortal platform (34). The correlation between the expression of RBM15 and survival in colorectal patients were performed using Kaplan-Meier Plotter with probe datasets as1555760_a_at and cBioPortal (Colorectal Adenocarcinoma, TCGA, PanCancer Atlas). The abundances of six immune infiltrates (B cells, CD4+ T cells, CD8+ T cells, Neutrophils, Macrophages, and Dendritic cells) are calculated from the TIMER platform (35).

Statistics and reproducibility

For statistical analysis, experiments were conducted at least three times, unless otherwise specified. Statistical analyses were performed using GraphPad Prism 7 software. The significance of differences between groups was assessed using a two-tailed unpaired Student's t -test. Quantitative data are presented as mean $\pm$ SEM. A significance threshold of $P < 0.05$ was used for all statistical analyses.

Data availability statement

The bulk-RNA sequencing data will be available on the Gene Expression Omnibus (GSE279678).

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by the Animal Care and Use Committee of Shanghai Jiao Tong University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CW: Conceptualization, Data curation, Formal Analysis, Investigation, Resources, Validation, Writing – original draft, Writing – review & editing. MC: Data curation, Formal Analysis, Investigation, Validation, Writing – review & editing. PC: Data curation, Formal Analysis, Investigation, Validation, Writing – review & editing. JH: Data curation, Investigation, Writing – review & editing. HH: Methodology, Writing – review & editing. JC: Formal Analysis, Investigation, Methodology, Writing – review & editing. QW: Formal Analysis, Investigation, Validation, Writing – review & editing. DZ: Formal Analysis, Resources,

Conceptualization, Writing – review & editing. TW: Resources, Data curation, Formal Analysis, Writing – review & editing. JZ: Methodology, Writing – review & editing. QL: Methodology, Writing – review & editing. RZ: Methodology, Writing – review & editing. YWe: Methodology, Writing – review & editing. JY: Conceptualization, Resources, Supervision, Methodology, Writing – review & editing. MS: Conceptualization, Resources, Supervision, Validation, Writing – review & editing. YWa: Conceptualization, Methodology, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by Pujiang Project of Shanghai Magnolia Talent plan (Grant No. 24PJD098), the National Natural Science Foundation of China (Grant No. 82102802; No. 82170638; No. 82102049); Doctoral innovation talent base project for diagnosis and treatment of chronic liver diseases (RCJD2021B02); the Natural Science Foundation of the Science and Technology Commission of Shanghai Municipality (23ZR1458300); Key laboratory for Translational Research and Innovative Therapeutics of Gastrointestinal Oncology (ZDSYS-2023-02; ZDSYS-2023-05; ZDSYS-2021-03).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1515568/full#supplementary-material>

References

- Roshandel G, Ghasemi-Kebria F, Malekzadeh R. Colorectal cancer: epidemiology, risk factors, and prevention. *Cancers (Basel)*. (2024) 16. doi: 10.3390/cancers16081530
- Siegel RL, Wagle NS, Cercek A, Smith RA, Jemal A. Colorectal cancer statistics, 2023. *CA Cancer J Clin*. (2023) 73:233–54. doi: 10.3322/caac.21772
- Golshani G, Zhang Y. Advances in immunotherapy for colorectal cancer: a review. *Therap Adv Gastroenterol*. (2020) 13:1756284820917527. doi: 10.1177/1756284820917527
- Ros J, Baraibar I, Saoudi N, Rodriguez M, Salva F, Tabernero J, et al. Immunotherapy for colorectal cancer with high microsatellite instability: the ongoing search for biomarkers. *Cancers (Basel)*. (2023) 15. doi: 10.3390/cancers15174245
- Ganesh K, Stadler ZK, Cercek A, Mendelsohn RB, Shia J, Segal NH, et al. Immunotherapy in colorectal cancer: rationale, challenges and potential. *Nat Rev Gastroenterol Hepatol*. (2019) 16:361–75. doi: 10.1038/s41575-019-0126-x
- Winkler R, Gillis E, Lasman L, Safra M, Geula S, Soyris C, et al. m(6)A modification controls the innate immune response to infection by targeting type I interferons. *Nat Immunol*. (2019) 20:173–82. doi: 10.1038/s41590-018-0275-z
- Han D, Liu J, Chen C, Dong L, Liu Y, Chang R, et al. Anti-tumour immunity controlled through mRNA m(6)A methylation and YTHDF1 in dendritic cells. *Nature*. (2019) 566:270–4. doi: 10.1038/s41586-019-0916-x
- Lin W, Chen L, Zhang H, Qiu X, Huang Q, Wan F, et al. Tumor-intrinsic YTHDF1 drives immune evasion and resistance to immune checkpoint inhibitors via promoting MHC-I degradation. *Nat Commun*. (2023) 14:265. doi: 10.1038/s41467-022-35710-7
- Cao Y, Qiu G, Dong Y, Zhao W, Wang Y, et al. Exploring the role of m(6)A writer RBM15 in cancer: a systematic review. *Front Oncol*. (2024) 14:1375942. doi: 10.3389/fonc.2024.1375942
- Appel LM, Franke V, Benedum J, Grishkovskaya I, Strobl X, Polyansky A, et al. The SPOC domain is a phosphoserine binding module that bridges transcription machinery with co- and post-transcriptional regulators. *Nat Commun*. (2023) 14:166. doi: 10.1038/s41467-023-35853-1
- Liang Y, Zhong H, Zhao Y, Tang X, Pan C, Sun J, et al. Epigenetic mechanism of RBM15 in affecting cisplatin resistance in laryngeal carcinoma cells by regulating ferroptosis. *Biol Direct*. (2024) 19:57. doi: 10.1186/s13062-024-00499-6
- Park SH, Ju JS, Woo H, Yun HJ, Lee SB, Kim SH, et al. The m(6)A writer RBM15 drives the growth of triple-negative breast cancer cells through the stimulation of serine and glycine metabolism. *Exp Mol Med*. (2024) 56:1373–87. doi: 10.1038/s12276-024-01235-w
- Wang R, Tan W. RBM15-mediated N6-methyl adenosine (m⁶A) modification of EZH2 drives the epithelial-mesenchymal transition of cervical cancer. *Crit Rev Eukaryot Gene Expr*. (2024) 34:15–29. doi: 10.1615/CritRevEukaryotGeneExpr.2024052205
- Xia L, Oyang L, Lin J, Tan S, Han Y, Wu N, et al. The cancer metabolic reprogramming and immune response. *Mol Cancer*. (2021) 20:28. doi: 10.1186/s12943-021-01316-8
- Chang CH, Qiu J, D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell*. (2015) 162:1229–41. doi: 10.1016/j.cell.2015.08.016
- Thompson EA, Powell JD. Inhibition of the adenosine pathway to potentiate cancer immunotherapy: potential for combinatorial approaches. *Annu Rev Med*. (2021) 72:331–48. doi: 10.1146/annurev-med-060619-023155
- Yoshihara K, Shahmoradgoli M, Martinez E, Vegesna R, Kim H, Torres-Garcia W, et al. Inferring tumour purity and stromal and immune cell admixture from expression data. *Nat Commun*. (2013) 4:2612. doi: 10.1038/ncomms3612
- Yan H, Zhang L, Cui X, Zheng S, Li R. Roles and mechanisms of the m(6)A reader YTHDC1 in biological processes and diseases. *Cell Death Discovery*. (2022) 8:237. doi: 10.1038/s41420-022-01040-2
- Boland CR, Goel A. Microsatellite instability in colorectal cancer. *Gastroenterology*. (2010) 138:2073–2087 e3. doi: 10.1053/j.gastro.2009.12.064
- Cheng J, Yan J, Liu Y, Shi J, Wang H, Zhou H, et al. Cancer-cell-derived fumarate suppresses the anti-tumor capacity of CD8(+) T cells in the tumor microenvironment. *Cell Metab*. (2023) 35:961–978 e10. doi: 10.1016/j.cmet.2023.04.017
- De Martino M, Rathmell JC, Galluzzi L, Vanpouille-Box C. Cancer cell metabolism and antitumour immunity. *Nat Rev Immunol*. (2024) 24:654–69. doi: 10.1038/s41577-024-01026-4
- Rong S, Dai B, Yang C, Lan Z, Wang L, Xu L, et al. HNRNPC modulates PKM alternative splicing via m⁶A methylation, upregulating PKM2 expression to promote aerobic glycolysis in papillary thyroid carcinoma and drive Malignant progression. *J Transl Med*. (2024) 22:914. doi: 10.1186/s12967-024-05668-9
- Zhu ZM, Huo FC, Zhang J, Shan HJ, Pei DS. Crosstalk between m⁶A modification and alternative splicing during cancer progression. *Clin Transl Med*. (2023) 13:e1460. doi: 10.1002/ctm2.v13.10
- Worley B, Powers R. PCA as a practical indicator of OPLS-DA model reliability. *Curr Metabolomics*. (2016) 4:97–103. doi: 10.2174/2213235X04666160613122429
- Nicolini A, Ferrari P. Involvement of tumor immune microenvironment metabolic reprogramming in colorectal cancer progression, immune escape, and response to immunotherapy. *Front Immunol*. (2024) 15:1353787. doi: 10.3389/fimmu.2024.1353787
- Agarwala Y, Brauns TA, Sluder AE, Poznansky MC, Gemechu Y. Targeting metabolic pathways to counter cancer immunotherapy resistance. *Trends Immunol*. (2024) 45:486–94. doi: 10.1016/j.it.2024.05.006
- Zhou W, Liu H, Yuan Z, Zundell J, Towers M, Lin J, et al. Targeting the mevalonate pathway suppresses ARID1A-inactivated cancers by promoting pyroptosis. *Cancer Cell*. (2023) 41:740–756 e10. doi: 10.1016/j.ccell.2023.03.002
- Shi JX, Zhang ZC, Yin HZ, Piao XJ, Liu CH, Liu QJ, et al. RNA m⁶A modification in ferroptosis: implications for advancing tumor immunotherapy. *Mol Cancer*. (2024) 23:213. doi: 10.1186/s12943-024-02132-6
- Guo L, Yang H, Zhou C, Shi Y, Huang L, Zhang J. N6-methyladenosine RNA modification in the tumor immune microenvironment: novel implications for immunotherapy. *Front Immunol*. (2021) 12:773570. doi: 10.3389/fimmu.2021.773570
- Li X, Ma S, Deng Y, Yi P, Yu J. Targeting the RNA m(6)A modification for cancer immunotherapy. *Mol Cancer*. (2022) 21:76. doi: 10.1186/s12943-022-01558-0
- Qu Y, Gao N, Zhang S, Gao L, He B, Wang C, et al. Role of N6-methyladenosine RNA modification in cancer. *MedComm*. (2020) 5:e715. doi: 10.1002/mco.2715
- Jiang X, Liu B, Nie Z, Duan L, Xiong Q, Jin Z, et al. The role of m⁶A modification in the biological functions and diseases. *Signal Transduction Targeted Ther*. (2021) 6. doi: 10.1038/s41392-020-00450-x
- Bartha A, Gyorffy B. TNMplot.com: A web tool for the comparison of gene expression in normal, tumor and metastatic tissues. *Int J Mol Sci*. (2021) 22. doi: 10.3390/ijms22052622
- Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discovery*. (2012) 2:401–4. doi: 10.1158/2159-8290.CD-12-0095
- Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, et al. TIMER: A web server for comprehensive analysis of tumor-infiltrating immune cells. *Cancer Res*. (2017) 77:e108–10. doi: 10.1158/1538-7445.AM2017-108



OPEN ACCESS

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RECEIVED 17 October 2024

ACCEPTED 14 April 2025

PUBLISHED 08 May 2025

CITATION

Jiang Y, Liang X, Sun H, Yin P, Zhou J and Yu C (2025) Immunomodulatory role of RNA modifications in sex hormone-dependent cancers.
Front. Immunol. 16:1513037.
doi: 10.3389/fimmu.2025.1513037

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Immunomodulatory role of RNA modifications in sex hormone-dependent cancers

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Recent studies have identified that RNA epigenetic modifications, including m6A, m1A, m5C, etc., play pivotal roles in tumor progression. These modifications influence mRNA stability, RNA processing, translational efficiency, and decoding precision. However, comprehensive reviews detailing the connection between m6A RNA modifications and hormone-dependent cancers in both male and female populations remain scarce (breast cancer, ovarian cancer, and endometrial cancer, prostate cancer). In this article, we explore the cellular and molecular roles of various RNA modifications alongside the key elements of the tumor microenvironment. We examine how these RNA modifications influence the development of hormone-dependent cancers through their impact on immune mechanisms. By enhancing our understanding of the function of RNA modifications within the immune systems of four specific tumors, we offer fresh insights for their potential applications in diagnosis and treatment.

KEYWORDS

RNA modification, sex hormone-dependent cancer, tumor microenvironment, tumor-immunology, epigenetics

1 Introduction

Tumor development is influenced by multiple factors, among which a subset closely associated with hormones, known as hormone-dependent cancers, among the most typical ones are breast cancer, ovarian cancer, endometrial cancer, and prostate cancer (BC, OC, EC, and PC). Their development and treatment are intrinsically linked to hormones, mainly including progesterone receptor (PR), estrogen receptor (ER), androgen receptor (AR) and human epidermal growth factor receptor 2 (HER2). In women, BC is the first malignant tumor with the highest incidence rates and is also the most typical hormone-dependent intermediate utilized (1). In addition, complex molecular bidirectional interactions between hormone receptors (HRs), including ER, PR, and HER2 are present in BC (2, 3). Endocrine therapy (aromatase inhibitors and anti-estrogen therapy or anti-estrogen therapy alone) is the standardized method and is the backbone of adjuvant therapy that significantly reduces the risk of recurrence and mortality (4). Endometrial

cancer, on the other hand, is the female malignant tumor with the fastest growing mortality rate, which tends to show elevated ER α levels and promote PR expression (1, 5, 6). Patients are positive for both ER α levels and PR expression tend to have well-differentiated tumors and may be responsive to hormone therapy, resulting in a relatively good prognosis (7). In addition, ovarian cancer is an essential branch of female malignant tumors, and postoperative hormone replacement therapy is necessary to achieve a better quality of living for patients (8). Similar to women, the most prevalent malignant tumor for men, prostate cancer, is significantly affected by androgens. Hence, the basis of prostate cancer treatment is anti-androgen therapy (9, 10). However, their mechanisms of occurrence remain unspecified, and treatment outcomes remain unsatisfactory. This review emphasizes the importance of discovering alternative and targetable molecular pathways that could provide novel therapeutic opportunities.

RNA modifications refer to chemical alterations of RNA nucleobases or ribose molecules. Presently, over 150 distinct modifications have been documented. Pseudouridine Ψ was discovered in the 1950s as the first recognized RNA modification (11). Among the most prevalent mRNA modifications, N6-methyladenosine (m6A) was identified in 2011 (12–14). Other RNA modifications, such as m1A and m5C, have been identified and extensively studied in recent decades (15, 16). RNA methylation has an impact on almost the entire mRNA life cycle - starting from mRNA transcription, mRNA splicing, specific structure, stability and subsequent translation and finally degradation (17–21). Although much of the research has focused on the role of m6A in hormone-dependent cancers, this review also examines the impact of other RNA modifications, such as m1A, m5C, m7G, mcm5s2U, A-to-I, and Ψ . These modifications can be investigated using emerging techniques like RNA immunoprecipitation (RIP), chromatin immunoprecipitation (ChIP), and single-cell omics (22–25).

The role of the immune system in cancer development has attracted increasing attention, particularly concerning the complex immune components within the tumor microenvironment and the adaptable mechanisms of immune evasion. Consequently, immunotherapy has emerged as a novel approach in cancer treatment, aimed at remodeling the immune system and reactivate anti-tumor immune responses to avoid tumor escape (26). Various immunotherapeutic strategies have shown substantial promise in treating a wide range of cancers, predominantly involving immune checkpoint inhibitors like PD1, PD-L1, and CTLA4, antibody-drug couplings, and cancer vaccines (27). However, fewer immunotherapeutic agents have been approved for clinical use in hormone-dependent cancers (28–31). As a distinct regulatory mechanism, RNA modifications, exemplified by m6A, has garnered increasing attention. Tumor-derived intrinsic signals and environmental stimuli can drive aberrant expression and activity-modifying regulators of many RNAs, leading to abnormal RNA modifications, which are essential for shaping the tumor microenvironment and immune escape (32, 33).

Although a substantial body of literature has accumulated on RNA modifications in cancer, the research focus has predominantly centered on m6A modification and its associated enzymatic

machinery, with relatively limited exploration of other RNA modification types and a notable paucity of systematic reviews. While tumor immunology remains a prominent research frontier, investigations that integrate RNA modifications with tumor immunity to elucidate their epigenetic regulatory mechanisms remain relatively scarce. Notably, for hormone-dependent tumors, there is a conspicuous lack of comprehensive discussion regarding the potential shared immune regulatory mechanisms and epigenetic modification patterns that may arise from their similar endocrine microenvironment (34). This paper will focus on how RNA modifications play an immunomodulatory role in hormone-dependent cancers, including breast, ovarian, endometrial and prostate cancers (BC, OC, EC, and PC). The mechanisms and implications of prevalent RNA modifications will be explored. Specifically, we aim to elucidate the effects of RNA modifications in diverse immune cell types within hormone-dependent cancers.

2 RNA modification in sex hormone synthesis

2.1 Concepts of different RNA modifications

2.1.1 m6A

N6-methyladenosine (m6A) is defined as the methylation of adenine at the N6 position within RNA molecules. It represents the most prevalent modification in eukaryotic lncRNAs and mRNAs, and has also been detected in rRNAs, snRNAs, and tRNAs (Figure 1, 35). The m6A modification is conserved across yeast, mouse, and human mRNA, and is enriched in the RRACH (R = G or A; H = A, C, or U) consensus sequence (36–38). The m6A modification is primarily facilitated by the methyltransferase termed the “writer”, the demethylase known as the “eraser”, and recognition proteins referred to as “reader”. The writer assembles the m6A methyltransferase complex (MTC), which catalyzes site-specific methylation that can be reversed by the eraser. The reader proteins bind to methylated m6A sites and transmit downstream signals, thereby acting as post-transcriptional gene regulators. M6A writers mainly include METTL3, nephroblastoma 1-associated protein (WTAP), KIAA1429 (VIRMA), RBM15, METTL16, METTL14, HAKAI, and ZC3H13 (KIAA0853) (39). To date, only two types of erasers, FTO and ALKBH5, have been recognized (40). The readers involve the YTH family, the HNRNP family, and the IGF2BPs family (39). As an important component of epigenetics, m6A modification and these regulatory proteins are involved in various biological activities in which they play a regulatory role (40–43).

As the predominant “writer,” METTL3 serves as the core catalytic component of MTC, yet it remains inactive without METTL14. Although METTL14 lacks intrinsic methyltransferase activity due to the absence of an S-adenosylmethionine (SAM) binding domain, it aids in substrate RNA recognition and forms methyltransferase structural domains (MTDs) with METTL3 as a heterodimer (44). The MTD structural domains in isolation do not

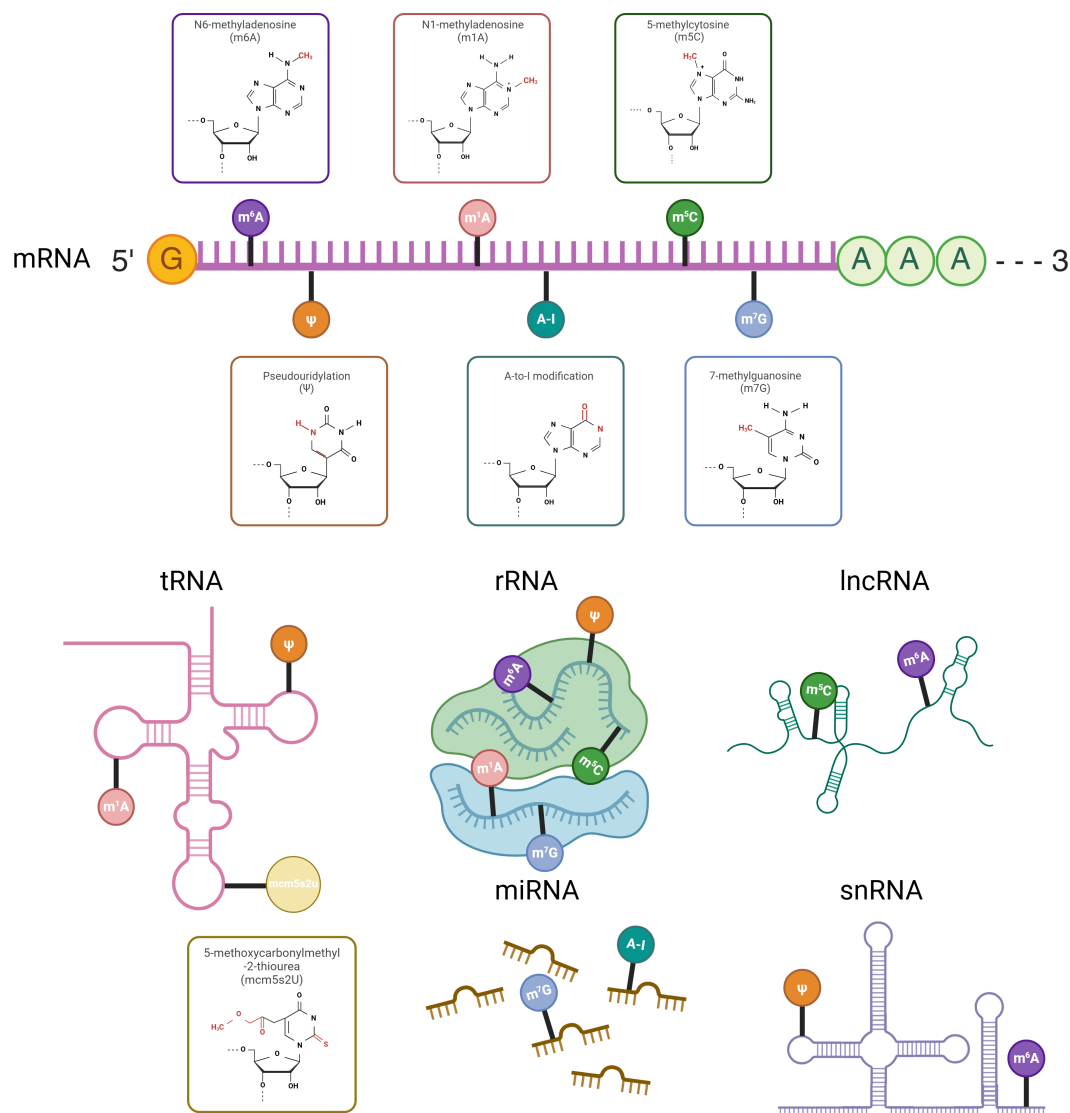


FIGURE 1

RNA modifications across different RNA types with chemical structures highlighted on the ribose moiety. Different RNA types undergo distinct chemical modifications that influence their stability, processing, and function. mRNA is primarily modified by m⁶A, m¹A, m⁵C, m⁷G, A-to-I, and Ψ, with m⁶A being the most prevalent. tRNA mainly carries m¹A, Ψ, and mcm5s2U, while rRNA undergoes m⁶A, m¹A, m⁵C, m⁷G, and Ψ modifications. lncRNA is modified by m⁶A and m⁵C, snRNA by m⁶A and Ψ, and miRNA by m⁷G and A-to-I. The chemical structures of these modifications are marked on the ribose moiety.

possess methyltransferase activity and necessitate the zinc-finger domain (ZFD) of METTL3 to become enzymatically active (45, 46). Wilms tumor 1-associating protein (WTAP), though not enzymatically active, assists in mRNA methylation by interacting with and recruiting the METTL3-METTL14 complex to target mRNA sites (47, 48). Further studies have identified VIRMA (49), ZC3H13 (50), RBM15/15B (51), and HAKAI (52) as additional cofactors of the METTL3-METTL14 complex. Besides METTL3, three distinct enzymes—METTL16, METTL5, and ZCCHC4—have been recognized as eukaryotic m⁶A methyltransferases, each responsible for incorporating m⁶A into U6 small nuclear RNAs (snRNAs) (53), 18S ribosomal RNAs (rRNAs) (54), and 28S rRNAs (55), respectively.

FTO and ALKBH5, known as m⁶A erasers, act as demethylases that catalyze the conversion of m⁶A to adenosine. FTO was the first m⁶A eraser identified, exhibiting specific oxidative demethylation activity against abundant m⁶A residues on RNA (13), while ALKBH5 was the second eraser discovered (40). FTO demethylates internal m⁶A residues on mRNAs and U6 RNA, as well as N6,2-O-dimethyladenosine (m⁶Am) on mRNAs and snRNAs, N1-methyladenosine (m¹A) on tRNAs, 3-methylthymine (m³T) on single-stranded DNA (ssDNA), and 3-methyluracil (m³U) on single-stranded RNA (ssRNA) (56, 57). ALKBH5 localizes within nuclear speckles and aids in the assembly of mRNA processing factors, primarily acting on substrates like nuclear nascent RNAs (ssRNAs) (40).

The primary readers of m6A are the YTH family proteins, including YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2, which contain an m6A-binding pocket within their YTH structural domains (58). YTHDF2, the first to be identified, promotes the degradation of cytoplasmic targets by recruiting CCR4-NOT complexes, with its m6A-binding affinity significantly enhanced by SUMOylation (19, 59). Additionally, YTHDF1 and YTHDF3, both cytoplasmic m6A readers, enhance the translation efficiency of target mRNAs and, in some instances, promote their degradation (19, 60, 61). As for nuclear m6A readers, YTHDC1 regulates mRNA fate through multiple mechanisms, such as mRNA splicing (17), nuclear body formation (62), and retrotransposon silencing (63). YTHDC2, localized in both the nucleus and cytoplasm (64), modulates deconjugase activity and influences mRNA decay and translation during spermatogenesis (65–67). Apart from the YTH family, IGF2BPs constitute a distinct group of m6A readers, recognizing m6A through their KH structural domains (20). The IGF2BP proteins (IGF2BP1/2/3) share similar structures, and their binding affinities for various target RNAs may be governed by their KH3 and KH4 structural domains (68).

2.1.2 m1A

The m1A modification, similar to m6A, entails the methylation of the first nitrogen on adenosine and is governed by specialized writers, erasers, and readers. This modification predominantly occurs in tRNAs and rRNAs, particularly within GC-rich RNA sequences, impacting ribosomal tertiary structure, RNA stability, and translation efficiency (Figure 1, 69–71). tRNA methyltransferases 6 and 61A (TRMT6/61A) form a complex that exerts MTC-like effects by catalyzing the addition of m1A to t-loop-like RNA structures (72). tRNA methyltransferases 10C and 61B (TRMT10C/61B) respectively catalyze the m1A modification at positions 9 and 58 in mitochondrial tRNAs (73). Moreover, TRMT61B has a similar recognition mechanism for rRNA and tRNA (74). Additionally, NML, also known as RRP8, localizes to the nucleus where it methylates m1A on 28S rRNAs (75). AlkB homologs 1, 3 and 7 act as erasers in charge of m1A demethylation (76–78). YTHDF1, YTHDF2, YTHDF3, and YTHDC1 act as m1A-modified readers to fulfill their biological roles (79).

2.1.3 m5C

m5C is a methylation modification at the 5th carbon atom of cytosine, found in mRNA and lncRNA, and enriched in cytoplasmic and mitochondrial rRNAs and tRNAs (Figure 1, 80, 81). To date, NSUN2 and NSUN6 are the only known m5C methyltransferases within the NSUN family that facilitate mRNA methylation by incorporating m5C, functioning as transcriptional modifiers. NSUN2 regulates the nucleoplasmic transport and RNA-binding affinity of the mRNA export adapter protein ALYREF, which specifically recognizes m5C, thereby influencing mRNA export (81). NSUN6 predominantly targets the 3 untranslated regions (3 UTRs) on the hairpin-like structural loops conserved sequence motif CTCCA, potentially participating in the quality control of translation termination fidelity (82). Two mechanisms for m5C “erasure” have been identified: first, oxidation by the TET

family on RNA to produce 5-hydroxymethylcytosine (hm5C); and second, the conversion of 5-formylcytosine (f5C) in mitochondrial tRNA by α -ketoglutarate and iron(II)-dependent dioxygenases ALKBH1 and ABH1 (83–85). BX-1 and the Aly-REF export factor (ALYREF) act as m5C readers, affecting the stability, translation, and transcription of the RNAs they target (81, 86).

2.1.4 m7G

The m7G modification involves the methylation of the 7th nitrogen atom in guanosine and primarily occurs at internal sites of rRNAs, tRNAs, miRNAs, as well as the 5 cap of mRNAs (Figure 1, 87, 88). Although no confirmed erasers or readers have been introduced for m7G yet, the m7G cap can undergo hypermethylation by trimethylguanosine synthase 1 (TGS1) to produce m2,2,7G or may be recognized by the eukaryotic translation initiation factor eIF4E, subsequently affecting RNA maturation, nuclear export, and translation (89, 90).

2.1.5 mcm5s2U

The 5-methoxycarbonylmethyl-2-thiurea modification (mcm⁵s²U), initiated by cm⁵U and mcm⁵U modifications on wobble uridines, is facilitated by human tRNA methyltransferase 9-like protein (TRM9L) and AlkB homologue 8 (ALKBH8) (Figure 1, 91). Wobble uridines, located at the first nucleotide position of the anticodon stem loop in tRNA, are essential for accurate mRNA translation and efficient protein synthesis (92, 93).

2.1.6 A-to-I modification

The A-to-I modification involves the selective hydrolytic deamination of adenosine to inosine (A-to-I editing), a process primarily regulated by the family of double-stranded RNA-specific adenosine deaminases, notably ADAR1, ADAR2, and ADAR3 (Figure 1, 94). ADAR1 and ADAR2 both mediate A-to-I editing in cellular RNA (95).

2.1.7 Pseudouridylation Ψ

The C5-glycosidic isoform of uridine, pseudouridine Ψ, is the most prevalent RNA modification, primarily found in tRNAs and rRNAs (Figure 1, 70, 96). Pseudouridylation occurs via two distinct pathways: RNA-independent pseudouridylation, catalyzed by pseudouridine synthases (PUSs) without a template strand, and RNA-dependent pseudouridylation, which requires the box H/ACA small nuclear ribonucleoprotein RNA-protein complex (82, 97).

2.1.8 Crosstalk between different RNA modification

Multiple RNA modifications are not isolated; rather, they are often interlinked, collaboratively regulating physiological and pathological states in the body. The vast majority of RNA modifications share similar regulatory mechanism, especially writers, allowing different modifications to be controlled by the same class of writers (Table 1), thus forming an interconnected regulatory network. Writers regulating m6A, m1A, and A-to-I modifications are not independent but exhibit significant cross-

TABLE 1 The various “writers”, “readers” and “erasers” associated with RNA modifications.

Key protein	m6A	m1A	m5C	m7G	mcm5s2U	A to I	Ψ
Writers	METTL3 METTL14 METTL16 WTAP VIRMA RBM15 HAKAI ZC3H13	TRMT6 TRMT61A TRMT10C TRMT61B RRP8	NSUN2 NSUN6	METTL1/WDR4	TRM9L ALKBH8	ADAR1 ADAR2 ADAR3	PUSs
Erasers	FTO ALKBH5	ALKBH1 ALKBH3 ALKBH7	TETs ALKBH1 ABH1	unknown	unknown	unknown	unknown
Readers	YTHs HNRNPs IGF2BPs	YTHDF1 YTHDF2 YTHDF3 YTHDC1	YBX-1 ALYREF	unknown	unknown	unknown	unknown

linking, which is closely associated with colorectal carcinogenesis, the tumor microenvironment (TME), drug sensitivity, and immunotherapy (98). Multiple RNA modifications can act on the same signaling pathway or target RNA to exert either synergistic or antagonistic effects. For example, m6A and m5C both modify FOXC2 mRNA, promoting gastric cancer cell growth (99, 100). Both m6A and A-to-I editing can alter c-MYC mRNA, contributing to the progression of hepatocellular carcinoma (101, 102). Similarly, m6A and Ψ modifications within the RAS pathway have been recognized for their oncogenic impact in colorectal cancer (103, 104). In pancreatic cancer, m6A stimulates the PI3K/Akt/mTOR pathway, driving cancer cell proliferation (105, 106), whereas m1A and m5C are linked with activation of the mTOR pathway and unfavorable prognoses (107, 108). Additionally, interactions between different RNA modifications have been observed. In breast cancer, METTL3-mediated m6A modification is regulated by ADAR1, which subsequently promotes breast cancer progression (109). Interactions among m6A, m5C, m1A, and m7G are also vital for TME regulation, immune infiltration, and immunotherapy in soft tissue sarcoma (STS) (110). The human body is a complex system, where crosstalk among multiple RNA modifications plays an essential role in disease development. Expanding research on RNA modification interactions holds significant clinical promise.

2.2 RNA modification with hormone receptors

Sex hormones in the human body, primarily estrogen, progesterone, and androgens, are regulated by gonadotropins, including gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Additionally, prolactin (PRL) can reflect the secretion levels of sex hormones in the body. RNA modifications specifically regulate the synthesis, secretion, and ligand-receptor interactions of sex hormones in organisms, thereby influencing physiological and pathological processes.

As the most common m6A methyltransferase, the specific knockdown of METTL3 can alter various biological processes, with

diverse and sometimes opposing effects in different cells and molecules. In testicular mesenchymal cells, ambient PM2.5 promotes METTL3-induced m6A modification of SIRT1 mRNA, leading to aberrant cellular autophagy, which inhibits testosterone synthesis and results in impaired spermatogenesis and infertility (111). Knockdown of METTL3 has been observed to significantly promote autophagic flow and increase testosterone production in testicular mesenchymal cells (111). Specific knockdown of the METTL3 gene in the endometrium stabilizes several mRNAs of estrogen-responsive genes, such as *Elf3* and *Celsr2*, while significantly reducing the expression levels of the progesterone receptor (PR) and its target gene *Myc* (112). Multiple m6A regulatory proteins can act synergistically to regulate hormone levels.

METTL3 suppresses the expression of androgen receptors in cardiac fibroblasts by introducing m6A modifications to AR mRNA, which are subsequently recognized by YTHDF2, leading to the degradation of AR-associated mRNA. This m6A modification by METTL3 enhances the binding of YTHDF2 at the modified sites, thereby reducing AR expression. This reduction rescues the inhibitory effects exerted by AR on glycolysis and cardiomyocyte proliferation, ultimately facilitating myocardial fibrogenesis (113). Researchers also found that applying antisense oligonucleotides (ASOs) to target METTL3 can restore Enzalutamide (an effective AR inhibitor) resistance *in vitro* and *in vivo* (114). In endometrial cells, METTL3-mediated m6A modification directly influences the mRNA of PR. Specifically, m6A modification in the 5' untranslated region (5'-UTR) of PR mRNA enhances the translational efficiency of PR proteins in a YTHDF1-dependent manner, a process that is conserved between mice and humans (112). M6A-related proteins such as METTL3 and METTL14 have been reported to increase follicle-stimulating hormone (FSH) levels while decreasing luteinizing hormone (LH) and testosterone (T) levels in PCOS rats, thereby reducing apoptosis and autophagy in ovarian tissue and improving ovarian morphology (115). Additionally, hormones can regulate biological processes by influencing m6A modifications. For instance, FSH can enhance the transcriptional activity of the METTL3 promoter in osteoclasts by inducing the phosphorylation of cyclic AMP response element-binding protein (CREB), which increases the m6A methylation of cathepsin K (CTSK). This methylation enhances the stability of CTSK and promotes osteoclast migration (116).

Other RNA modifications are also associated with hormone receptors, including A-to-I editing and m5C modification. In prostate cancer cells, numerous nucleotide transitions within AR gene transcripts have been identified as mutations that coincide with potential A-to-I, U-to-C, C-to-U, and G-to-A RNA editing sites (117). Furthermore, NSUN2 stabilizes AR mRNA through m5C modification, creating a positive feedback loop that promotes prostate carcinogenesis (118). Research has shown that inhibiting AR leads to the rearrangement of the alternative polyadenylation (APA) subcomplex and disrupts the interaction between the cleavage stimulation factor (CSTF) complex and the cleavage and polyadenylation specificity factor (CPSF) complex (119). In breast cancer, the luminal androgen receptor influences APA subtypes in patients with triple-negative breast cancer (120). In estrogen receptor-positive (ER⁺) breast cancer, estradiol (E2), a potent proliferative agent, induces APA and 3'-UTR shortening, subsequently activating proto-oncogenes (121).

3 RNA modification in hormone-dependent cancer

3.1 Tumor microenvironment in hormone-dependent cancer

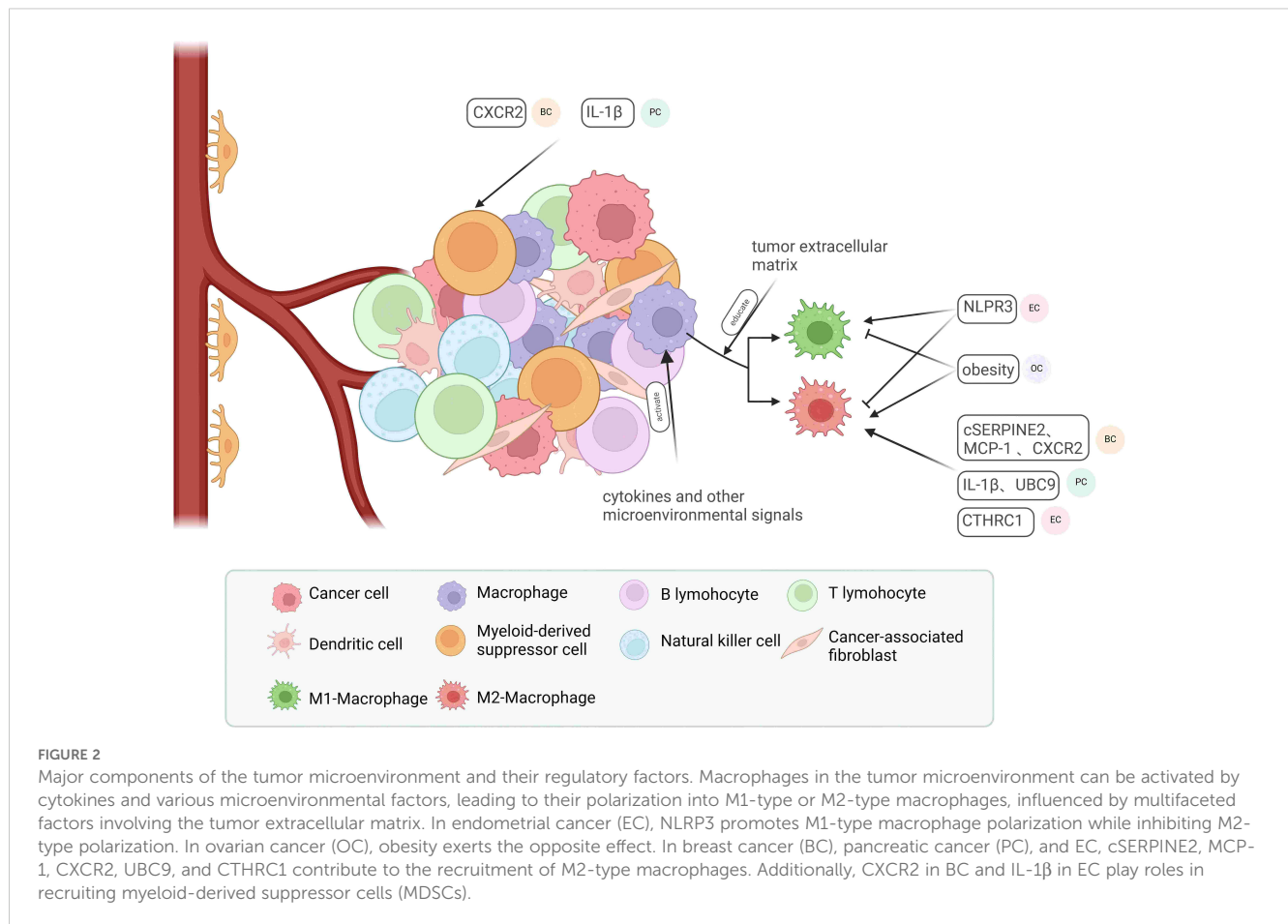
Research indicates that the tumor microenvironment (TME), which consists of infiltrating immune cells such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), along with stromal cells like cancer-associated fibroblasts (CAFs) and endothelial cells (122), plays a pivotal role in tumor development. It fosters tumor progression through complex interactions between cells and the extracellular matrix (ECM) (Figure 2) (123, 124).

Tumor-associated macrophages (TAMs) are the most prevalent immune cells in the tumor microenvironment, crucially supporting tumor progression and immune modulation (125). With their phagocytic and cytotoxic capabilities, macrophages are recognized as immunoreactive cells that can polarize into anti-tumor M1 macrophages or pro-tumor M2 macrophages in response to microenvironmental signals. TAMs closely resemble M2 macrophages and are associated with the Th2 immune response, characterized by high levels of IL-10 and TGF- β production, and they secrete pro-tumorigenic cytokines that promote tumor progression (126–128). Furthermore, TAMs influence angiogenesis and enhance cell proliferation and metastasis by inhibiting CD8⁺ T cell activity (129, 130). Consequently, various factors can affect tumor development and metastasis by modifying the polarization and recruitment of TAMs (131). In EC, NLRP3 deficiency leads to macrophage polarization into pro-inflammatory M2-type macrophages (132). The tumor exosome cSERPINE2 (133), the chemokines MCP-1 (134) and IL-1 β (135), and the secreted protein CTHRC1 (136) facilitate the progression of BC, EC, and pancreatic cancer (PC) by recruiting TAMs. An *in vitro* study demonstrated that the tumor extracellular matrix (ECM) can directly regulate macrophage populations in ovarian cancer tissues (137). MDSCs are also critical immunosuppressive components in the tumor

microenvironment. Two main classes of MDSCs, granulocytic/polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs), can be identified in humans and mice based on their origin, and both significantly suppress immune responses following prolonged exposure to cytokines released during chronic infections, inflammation, autoimmune diseases, and cancer (138). For instance, chronic psychological stress can recruit splenic MDSCs via CXCR2, promoting the formation of a metastatic pre-metastatic niche (PMN) in BC (139). Due to their significant immunosuppressive properties, TAMs and MDSCs are frequently studied as potential targets for tumor therapy. Approaches such as gene knockdown (140), blockade of key molecules (141–143), and remodeling of drug structure (144) aim to inhibit TAMs and MDSCs to achieve clinical benefits.

In most cancers, stromal cells are major components of the TME, playing critical roles in tumor metabolism, growth, and metastasis (145). Cancer-associated fibroblasts (CAFs), key constituents of the stroma, can be activated by various tumor-derived factors (146). CAFs exhibit enhanced expression of several markers, including α -smooth muscle actin (α -SMA), fibroblast activation protein (FAP), fibroblast-specific protein 1 (FSP1), platelet-derived growth factor receptor (PDGFR)- α/β , and poikilodulin (147), and the vast majority display pro-cancer effects (148). CAFs are highly heterogeneous, comprising multiple influential subgroups. In breast cancer, CD26⁺ and CD26-normal fibroblast populations are transformed into inflammatory CAFs (iCAFs) and myofibroblast CAFs (myCAFs), respectively (149). CD26⁺ normal fibroblasts (NFs) are converted into pro-tumorigenic iCAFs, which recruit myeloid cells via a CXCL12-dependent mechanism and promote tumor cell invasion through matrix metalloproteinase (MMP) activity (149). MyCAFs, located close to the tumor, are a subtype of CAFs. The molecular and functional diversity of myCAFs arises from diverse sources and activation mechanisms, among which TSPAN8⁺SIRT6^{low} myCAFs are linked to unfavorable outcomes in breast cancer patients (150). Similarly, in prostate cancer, androgen deprivation therapy (ADT) induces SPP1⁺ myCAFs, which are critical stromal components driving the progression of castration-resistant prostate cancer (CRPC) (151). Other non-classical subgroups include CD146⁺ CAFs, which promote endometrial cancer progression by inducing angiogenesis and vasculogenic mimicry (152), and α SMA⁺VIM⁺PDGFR β ⁺CAF, which are correlated with lower tumor immune infiltration and shorter survival in ovarian cancer patients (153).

Additionally, recent studies have found that solid tumors are hypoxic and acidic, with the physiochemical aspects of the TME sustained by chaotic tumor perfusion, resulting in tumor progression and resistance to immunotherapy (154). Apart from recruiting immunosuppressive cells like MDSCs, tumor cells can evade the immune system in various ways. For instance, they modulate T cell responses by altering the levels of immune checkpoint molecules, particularly through the upregulation of PD-L1 (155). Moreover, tumor cells evade recognition and destruction by cytotoxic T cells by reducing MHC-I expression and impairing antigen presentation (156). They may also inhibit the production of CXCL9 and CXCL10, obstructing the infiltration of CXCR3⁺ effector cells into the tumor, thus facilitating immune evasion and limiting T cell infiltration (157).



3.2 RNA modification in immune system of hormone-dependent cancers

A growing number of studies have demonstrated that human malignancies are correlated with epigenetic alterations in RNA (158, 159). Previous studies have identified RNA modifications, particularly m6A, as playing a pivotal role in hormone-dependent cancers (160–163). These modifications are essential in regulating tumor growth and metastasis (33, 164). In BC, m7G has been linked to immune cell infiltration, including initial B cells, CD4⁺ memory resting and activated T cells, CD8⁺ T cells, regulatory T cells, resting and activated natural killer (NK) cells, M1 macrophages, and resting mast cells, with NCBP1 mRNA identified as the most prominent target of m7G (165). The related regulatory enzyme, RBM15B, along with its associated genes TCP1 and ANKRD36, and the RNA demethylase ALKBH family, particularly ALKBH7, are also associated with immune infiltration in breast cancer and are positively correlated with tumor development (166, 167). Furthermore, both m6A and m5C can disrupt DNA replication and affect the tumor immune microenvironment in PC (168, 169). As more relevant studies emerge, the understanding of how RNA modifications govern the immune system in hormone-dependent cancers has been progressively refined at the cellular and molecular levels (Table 2).

3.2.1 T cells

RNA modification promotes tumor immune escape by regulating immune checkpoint molecule expression on T cells. Researchers identified a 4-DERRG signature based on 59 RNA modification-associated regulatory genes (ALYREF, ZC3H13, WTAP, and METTL1) and accordingly categorized the OC patients into two distinct groups, showing significant differences in the immune checkpoint molecule CD276. The regulation of immune checkpoint molecules by m6A is primarily mediated through PD-L1. METTL3-mediated m6A modification occurs in the 3'-UTR of PD-L1 mRNA, and circATAD2 can bind to it, enhancing the level of m6A modification (170, 171). The m6A reader IGF2BP3 recognizes this modification, thereby increasing PD-L1 mRNA stability and expression (171). In OC, IGF2BP1/2/3 also recognize m6A modifications, positively regulating circNFIX expression, which activates downstream JAK/STAT3 signaling and enhances PD-L1 expression (174). Thus, m6A-modified PD-L1 may serve as a potential therapeutic target. In BC, ADAR1 synergizes with DEAD-box RNA helicase 3X (DDX3X) to activate the cytoplasmic dsRNA pathway, increasing tumor infiltration of CD8⁺ T cells and DC cells (181). In PC, the reader YTHDF1 promotes the progression by regulating androgen function-related gene TRIM68 (172). For enhanced photothermal immunotherapy of PC, cyclodextrin-functionalized gold nanorods can deliver the

TABLE 2 The molecules, cells and mechanisms associated with the immunomodulatory role of RNA modifications in hormone-dependent cancers.

RNA modification	Related molecule	Immune cell	Key mechanism	Tumor	References
m6A	circATAD2 IGF2BP3	T cell	Enhancing PD-L1 mRNA stability and expression	BC	(170)
	METTL3 IGF2BP3	T cell	Upregulating PD-L1 expression and promoting stabilization of PD-L1 mRNA	BC	(171)
	YTHDF1	T cell	Enhancing PD-L1 transcriptional stability	PC	(172)
	m6A RNA demethylase inhibitor meclofenamic acid	T cell	Decreasing the stability of PD-L1 transcripts	PC	(173)
	circNFIIX IGF2BP1/2/3	T cell	Activating IL-6R/JAK/STAT3 signaling and enhancing PD-L1 expression	OC	(174)
	HNRNPC	Treg cell CD8 ⁺ T cell	Characteristic m6A gene profiles were associated with immune responses, in which HNRNPC as a marker protein enhances treg cell activation and suppresses effector CD8 ⁺ T cells.	PC	(175)
		CD4 ⁺ T cell Macrophage Mast cell	The levels of macrophages, mast cells and CD4 ⁺ T cells were significantly correlated with m6A-related genes.	PC	(176)
	KIAA1429	B cell	Inhibition of memory B cell infiltration	BC	(177)
		B cell Dendritic cell	Elevated m6A levels were accompanied by elevated dendritic cell and B cell levels.	EC	(178)
	ALKBHs (especially ALKBH8)	All tumor-related immune cells	Associated with immune infiltration and promotes tumor development	BC	(167)
m7G	NCBP1 mRNA	All Tumor-related Immune cells	Associated with low immune status and poorer prognosis	BC	(165)
	METTL1	CD8 ⁺ T cell Macrophage	The depletion of METTL1 promotes the biogenesis of 5'tRNA-derived small RNA, which correlates with increased pro-inflammatory immune cell polarization and CD8 ⁺ T cell inflation.	PC	(179)
m5C	TETs, NUSNs, etc	CD8 ⁺ T cell Macrophage B cell	m5C regulatory genes were associated with immune cell levels and tumor prognosis.	PC	(180)
A to I	ADAR1 DDX3X	CD8 ⁺ T cell Dendritic cell	Activation of the cytoplasmic dsRNA pathway increases tumor infiltration by CD8 ⁺ T cell and DC.	BC	(181)
RNA modification	RBM15B	All tumor-related immune cells	Associated with low immune status and poorer prognosis.	BC	(166)
	ALYREF ZC3H13 WTAP METTL1	CD4 ⁺ T cell Macrophage B cell	The risk scores based on the four-DERRG signature showed a positive correlation with CD4 ⁺ memory resting T cells, while demonstrating a negative correlation with M1 macrophages and plasma cells	OC	(182)
	Related writers	All tumor-related immune cells	Correlated with high expression of tumor infiltration-associated cells and B-cell receptor signaling pathways.	OC	(183)

m6A RNA demethylase inhibitor meclofenamic acid, thereby enhancing m6A methylation of mRNAs and decreasing the stability of PD-L1 transcripts (173). Besides, investigators have discovered that the m7G transferase METTL1 is highly expressed in both primary and advanced prostate tumors. Simultaneously, upon METTL1 deletion, the absence of m7G tRNA methylation promotes the generation of a new class of non-coding small RNAs originating from 5 tRNA fragments (179). These small RNAs regulate translation and support the production of key regulators essential for antitumor immune responses (179). These regulators are crucial for promoting CD8⁺ T cell infiltration and enhancing antitumor effects (179). Similarly, after clustering according to the regulatory genes of m5C (TET1, TET3, DNMT3B, YBX1, NSUN2, NSUN6, NOP2) in patients with PC, significant differences in CD8⁺ T cell infiltration were observed between the two clusters, with a strong negative correlation to patient prognosis (180).

3.2.2 Macrophages

RNA modification regulates macrophages mainly by altering the number or proportion of M1 and M2 type macrophages. For instance, circITGB6 specifically interacts with the KH1–2 domain of IGF2BP2, leading to increased mRNA stability of FGF9, leading to increased mRNA stability of FGF9 (184). This interaction further encourages the polarization of TAM towards the M2 phenotype, thereby inducing cisplatin resistance in OC (184). In PC, the removal of METTL1 results in the downregulation of anti-inflammatory cytokines, such as macrophage colony-stimulating factor (M-CSF), IL-10, and IL-13, which also promote M2 macrophage polarization (179, 185). In OC and BC, ALKBH3 enhances the half-life of CSF-1 mRNA by removing m1A from the GC-rich region of the 5' UTR of CSF-1 mRNA, facilitating macrophage recruitment and tumor invasion (186).

3.2.3 Other cells

In OC, based on the six lncRNA subgroups of RNA modification-associated writers (m6A, m1A, APA, and A-I), tumor-infiltrating cells such as mast cells, neutrophils, and B-cell receptor signaling pathways were highly expressed in the high-risk group (14). Moreover, m6A writer KIAA1429 was positively correlated with various advanced tumors such as BC, and negatively correlated with memory B-cell infiltration (177). In addition, in EC clusters classified by hypoxia genes, elevated m6A levels were observed alongside increased infiltration of B cells and dendritic cells (DCs) in the high-risk group (178).

3.3 Targeting regulators of RNA modification to treat hormone-dependent cancer

Despite being a relatively new field, drugs targeting RNA modifications are gradually transitioning from the laboratory to the public eye. However, fewer studies have been specifically conducted

on the four hormone-dependent cancers, and the following is only a list of drugs of general interest that can potentially be used on hormone-dependent cancers. For m6A, Several inhibitors targeting FTO and ALKBH5 have been developed to impede the progression of various cancers, such as R-2-hydroxyglutarate (R-2HG), FB23-2, IOX1, IOX3, Rhein, Entacapone, and meclofenamic acid (80, 97). Regarding m5C, NSUN2 is upregulated in both BC and PC, and its expression can be reduced by inhibiting sphingosine kinase (SPHK), which maintains sphingolipid balance during cell growth (187–189). Consequently, the SPHK1 inhibitor SK1 emerges as a potential agent for cancer treatment by targeting NSUN2 expression (187–189). Moreover, research on pseudouridine identifies pyrazoline and 5-fluorouracil as common DKC1 inhibitors, employed clinically as anticancer agents (97, 190). In the context of A-to-I editing, 8-azaadenosine and 8-chloroadenosine function as ADAR1 inhibitors, but their limited specificity temporarily precludes clinical application (191, 192).

Furthermore, while no clinical trials have been conducted to date, emerging mechanistic studies suggest that specific RNA modifications may exert dual therapeutic effects in hormone-dependent tumors: either enhancing treatment efficacy or paradoxically promoting drug resistance. In OC, RNA modifications can remodel the tumor microenvironment by upregulating immunogenic RNAs, thereby reversing tumor immune evasion phenotypes and potentially restoring clinical responsiveness to immunotherapy in previously non-responding patients (193). For instance, Y-box binding protein 1 (YBX1) has been shown to enhance homologous recombination proficiency and resistance to platinum-induced stress in OC through m5C modification (194). In breast cancer (BC), METTL3 knockdown significantly increases chemosensitivity to doxorubicin via modulation of the EGF/RAD51 signaling axis (195). Intriguingly, METTL3 depletion has also been found to activate the CDKN1A/EMT pathway and m6A-BAX/caspase-9/-3/-8 cascade, thereby promoting proliferation, migration, and drug resistance in hormone receptor-positive HER2-negative breast cancer (HR⁺HER2-BC) (196). These findings underscore the complex regulatory networks of RNA modifications in cancer therapeutics, necessitating comprehensive mechanistic elucidation and systematic clinical validation to delineate their therapeutic potential versus risk profiles.

4 Conclusion and perspectives

This review underscores the crucial role of RNA modifications in regulating the progression and immune landscape of hormone-dependent cancers, including breast, ovarian, endometrial, and prostate malignancies. These modifications facilitate tumor growth and metastasis by modulating key immunoregulatory pathways, such as PD-L1 expression, immune cell infiltration, and cytokine signaling, revealing their potential to improve cancer diagnosis and therapy.

Despite increasing recognition of RNA modifications in cancer, the precise molecular mechanisms—especially how these modifications integrate with hormone receptor signaling and shape the immune microenvironment—remain only partially understood. Future studies should elucidate the specific pathways by which RNA modifications influence immune regulation and hormone receptor activity. As RNA modifications affect both hormone receptor function and immunogenic pathways (e.g., PD-L1), there is a compelling rationale for combining hormone therapies (e.g., anti-estrogen, anti-androgen) with immunotherapies or RNA modification inhibitors. Such combination strategies may enhance tumor susceptibility to immune-mediated destruction and mitigate therapeutic resistance.

Although therapeutic applications remain challenging, mounting evidence highlights the significant role of RNA modifications in orchestrating immune regulation and driving hormone-dependent tumor progression. Further investigation into the detailed mechanisms underlying these modifications holds promise for developing more effective and precisely targeted interventions against hormone-dependent cancers.

Author contributions

YJ: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. XL: Supervision, Visualization, Writing – review & editing. HS: Conceptualization, Investigation, Supervision, Validation, Writing – review & editing. PY: Supervision, Writing – review & editing, Software, Funding acquisition. JZ: Funding acquisition, Supervision, Writing – review & editing, Validation, Visualization, Writing – original draft. CY: Supervision, Writing – review & editing, Conceptualization, Funding acquisition, Resources.

References

1. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. *CA Cancer J Clin*. (2024) 74:12–49. doi: 10.3322/caac.21820
2. Ouyang D, Su J, Huang P, Li M, Li Q, Zhao P, et al. Identification of lncRNAs via microarray analysis for predicting HER2-negative breast cancer response to neoadjuvant chemotherapy. *Int J Clin Exp Pathol*. (2018) 11:2621–8.
3. Giuliano M, Trivedi MV, Schiff R. Bidirectional crosstalk between the estrogen receptor and human epidermal growth factor receptor 2 signaling pathways in breast cancer: molecular basis and clinical implications. *Breast Care Basel Switz*. (2013) 8:256–62. doi: 10.1159/000354253
4. Gradishar WJ, Moran MS, Abraham J, Abramson V, Aft R, Agnese D, et al. NCCN guidelines® Insights: breast cancer, version 4.2023. *J Natl Compr Cancer Netw JNCCN*. (2023) 21:594–608. doi: 10.6004/jnccn.2023.0031
5. Rodriguez AC, Blanchard Z, Maurer KA, Gertz J. Estrogen signaling in endometrial cancer: a key oncogenic pathway with several open questions. *Horm Cancer*. (2019) 10:51–63. doi: 10.1007/s12672-019-0358-9
6. Cancer Genome Atlas Research Network, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, et al. Integrated genomic characterization of endometrial carcinoma. *Nature*. (2013) 497:67–73. doi: 10.1038/nature12113
7. Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol*. (1983) 15:10–7. doi: 10.1016/0090-8258(83)90111-7
8. Jayson GC, Kohn EC, Kitchener HC, Ledermann JA. Ovarian cancer. *Lancet*. (2014) 384:1376–88. doi: 10.1016/S0140-6736(13)62146-7
9. Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA Cancer J Clin*. (1972) 22:232–40. doi: 10.3322/canjclin.22.4.232
10. Albertsen P. Androgen deprivation in prostate cancer—step by step. *N Engl J Med*. (2009) 360:2572–4. doi: 10.1056/NEJMe0901737
11. Cohn WE, Volkin E. Nucleoside-5'-phosphates from ribonucleic acid. *Nature*. (1951) 167:483–4. doi: 10.1038/167483a0
12. *Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons*. (Accessed May 8, 2024).
13. Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, et al. N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nat Chem Biol*. (2011) 7:885–7. doi: 10.1038/nchembio.687
14. *Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq*. (Accessed May 8, 2024).
15. Wiener D, Schwartz S. The epitranscriptome beyond m6A. *Nat Rev Genet*. (2021) 22:119–31. doi: 10.1038/s41576-020-00295-8
16. Roundtree IA, Evans ME, Pan T, He C. Dynamic RNA modifications in gene expression regulation. *Cell*. (2017) 169:1187–200. doi: 10.1016/j.cell.2017.05.045
17. Xiao W, Adhikari S, Dahal U, Chen Y-S, Hao Y-J, Sun B-F, et al. Nuclear m(6)A Reader YTHDC1 Regulates mRNA Splicing. *Mol Cell*. (2016) 61:507–19. doi: 10.1016/j.molcel.2016.01.012
18. Liu N, Dai Q, Zheng G, He C, Parisien M, Pan T. N(6)-methyladenosine-dependent RNA structural switches regulate RNA-protein interactions. *Nature*. (2015) 518:560–4. doi: 10.1038/nature14234
19. Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, et al. N6-methyladenosine-dependent regulation of messenger RNA stability. *Nature*. (2014) 505:117–20. doi: 10.1038/nature12730

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by the National Natural Science Foundation of China (82205256, 82104555); Shanghai Famous Elderly Chinese Medicine Doctor Academic Experience Studio (SHGZS-202240); Scientific Mission Statement of the army Logistics Research Project (BHJ23C011).

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20. Huang H, Weng H, Sun W, Qin X, Shi H, Wu H, et al. Recognition of RNA N6-methyladenosine by IGF2BP proteins enhances mRNA stability and translation. *Nat Cell Biol.* (2018) 20:285–95. doi: 10.1038/s41556-018-0045-z
21. Wang X, Zhao BS, Roundtree IA, Lu Z, Han D, Ma H, et al. N(6)-methyladenosine modulates messenger RNA translation efficiency. *Cell.* (2015) 161:1388–99. doi: 10.1016/j.cell.2015.05.014
22. Xu W, Lai Y, Pan Y, Tan M, Ma Y, Sheng H, et al. m6A RNA methylation-mediated NDUFA4 promotes cell proliferation and metabolism in gastric cancer. *Cell Death Dis.* (2022) 13:715. doi: 10.1038/s41419-022-05132-w
23. Chen Y, Peng C, Chen J, Chen D, Yang B, He B, et al. WTAP facilitates progression of hepatocellular carcinoma via m6A-HuR-dependent epigenetic silencing of ETS1. *Mol Cancer.* (2019) 18:127. doi: 10.1186/s12943-019-1053-8
24. Hamashima K, Wong KW, Sam TW, Teo JHJ, Taneja R, Le MTN, et al. Single-nucleus multiomic mapping of m6A methylomes and transcriptomes in native populations of cells with sn-m6A-CT. *Mol Cell.* (2023) 25:S1097-2765(23)00649-4. doi: 10.1016/j.molcel.2023.08.010. S1097-2765(23)00649-4
25. Nie W, Wang S, He R, Xu Q, Wang P, Wu Y, et al. A-to-I RNA editing in bacteria increases pathogenicity and tolerance to oxidative stress. *PLoS Pathog.* (2020) 16:e1008740. doi: 10.1371/journal.ppat.1008740
26. Yao Z, Lin Z, Wu W. Global research trends on immunotherapy in cancer: A bibliometric analysis. *Hum Vaccines Immunother.* (2023) 19:2219191. doi: 10.1080/21645515.2023.2219191
27. Kundu M, Butti R, Panda VK, Malhotra D, Das S, Mitra T, et al. Modulation of the tumor microenvironment and mechanism of immunotherapy-based drug resistance in breast cancer. *Mol Cancer.* (2024) 23:92. doi: 10.1186/s12943-024-01990-4
28. Xin Q, Chen Y, Sun X, Li R, Wu Y, Huang X. CAR-T therapy for ovarian cancer: Recent advances and future directions. *Biochem Pharmacol.* (2024) 226:116349. doi: 10.1016/j.bcp.2024.116349
29. He Y, Xu W, Xiao Y-T, Huang H, Gu D, Ren S. Targeting signaling pathways in prostate cancer: mechanisms and clinical trials. *Signal Transduct Target Ther.* (2022) 7:198. doi: 10.1038/s41392-022-01042-7
30. Bogani G, Monk BJ, Powell MA, Westin SN, Slomovitz B, Moore KN, et al. Adding immunotherapy to first-line treatment of advanced and metastatic endometrial cancer. *Ann Oncol Off J Eur Soc Med Oncol.* (2024) 35:414–28. doi: 10.1016/j.jannonc.2024.02.006
31. Lan H-R, Chen M, Yao S-Y, Chen J-X, Jin K-T. Novel immunotherapies for breast cancer: Focus on 2023 findings. *Int Immunopharmacol.* (2024) 128:111549. doi: 10.1016/j.intimp.2024.111549
32. Luo Y, Tian W, Kang D, Wu L, Tang H, Wang S, et al. RNA modification gene WDR4 facilitates tumor progression and immunotherapy resistance in breast cancer. *J Adv Res.* (2024) 25:2090-1232(24)00266-2. doi: 10.1016/j.jare.2024.06.029. S2090-1232(24)00266-2
33. Han M, Huang Q, Li X, Chen X, Zhu H, Pan Y, et al. M7G-related tumor immunity: novel insights of RNA modification and potential therapeutic targets. *Int J Biol Sci.* (2024) 20:1238–55. doi: 10.7150/ijbs.90382
34. Chimento A, De Luca A, Avena P, De Amicis F, Casaburi I, Sirianni R, et al. Estrogen receptors-mediated apoptosis in hormone-dependent cancers. *Int J Mol Sci.* (2022) 23:1242. doi: 10.3390/ijms23031242
35. Fu Y, Dominissini D, Rechavi G, He C. Gene expression regulation mediated through reversible m⁶A RNA methylation. *Nat Rev Genet.* (2014) 15:293–306. doi: 10.1038/nrg3724
36. Bodi Z, Button JD, Grierson D, Fray RG. Yeast targets for mRNA methylation. *Nucleic Acids Res.* (2010) 38:5327–35. doi: 10.1093/nar/gkq266
37. Dominissini D, Moshitch-Moshkovitz S, Schwartz S, Salmon-Divon M, Ungar L, Osenberg S, et al. Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. *Nature.* (2012) 485:201–6. doi: 10.1038/nature11112
38. Meyer KD, Saletore Y, Zumbo P, Elemento O, Mason CE, Jaffrey SR. Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. *Cell.* (2012) 149:1635–46. doi: 10.1016/j.cell.2012.05.003
39. Zhang C, Liu N. N6-methyladenosine (m6A) modification in gynecological malignancies. *J Cell Physiol.* (2022) 237:3465–79. doi: 10.1002/jcp.30828
40. Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang C-M, Li CJ, et al. ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol Cell.* (2013) 49:18–29. doi: 10.1016/j.molcel.2012.10.015
41. Huang J, Jiang B, Li G-W, Zheng D, Li M, Xie X, et al. m6A-modified lincRNA DUBR is required for neuronal development by stabilizing YTHDF1/3 and facilitating mRNA translation. *Cell Rep.* (2022) 41:111693. doi: 10.1016/j.celrep.2022.111693
42. m6A-dependent maternal mRNA clearance facilitates zebrafish maternal-to-zygotic transition. (Accessed May 8, 2024).
43. Vu LP, Cheng Y, Kharas MG. The biology of m6A RNA methylation in normal and Malignant hematopoiesis. *Cancer Discov.* (2019) 9:25–33. doi: 10.1158/2159-8290.CD-18-0959
44. Wang X, Feng J, Xue Y, Guan Z, Zhang D, Liu Z, et al. Structural basis of N(6)-adenosine methylation by the METTL3-METTL14 complex. *Nature.* (2016) 534:575–8. doi: 10.1038/nature18298
45. Huang J, Dong X, Gong Z, Qin L-Y, Yang S, Zhu Y-L, et al. Solution structure of the RNA recognition domain of METTL3-METTL14 N6-methyladenosine methyltransferase. *Protein Cell.* (2019) 10:272–84. doi: 10.1007/s12328-018-0518-7
46. Šledz P, Jinek M. Structural insights into the molecular mechanism of the m(6)A writer complex. *eLife.* (2016) 5:e18434. doi: 10.7554/eLife.18434
47. Ping X-L, Sun B-F, Wang L, Xiao W, Yang X, Wang W-J, et al. Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase. *Cell Res.* (2014) 24:177–89. doi: 10.1038/cr.2014.3
48. Huang Q, Mo J, Liao Z, Chen X, Zhang B. The RNA m6A writer WTAP in diseases: structure, roles, and mechanisms. *Cell Death Dis.* (2022) 13:852. doi: 10.1038/s41419-022-05268-9
49. VIRMA mediates preferential m6A mRNA methylation in 3'UTR and near stop codon and associates with alternative polyadenylation. (Accessed May 8, 2024).
50. Zc3h13 Regulates Nuclear RNA m6A Methylation and Mouse Embryonic Stem Cell Self-Renewal. (Accessed May 8, 2024).
51. Patil DP, Chen C-K, Pickering BF, Chow A, Jackson C, Guttman M, et al. m(6)A RNA methylation promotes XIST-mediated transcriptional repression. *Nature.* (2016) 537:369–73. doi: 10.1038/nature19342
52. Růžicka K, Zhang M, Campilho A, Bodi Z, Kashif M, Saleh M, et al. Identification of factors required for m6 A mRNA methylation in Arabidopsis reveals a role for the conserved E3 ubiquitin ligase HAKAI. *New Phytol.* (2017) 215:157–72. doi: 10.1111/nph.14586
53. Pendleton KE, Chen B, Liu K, Hunter OV, Xie Y, Tu BP, et al. The U6 snRNA m6A Methyltransferase METTL16 Regulates SAM Synthetase Intron Retention. *Cell.* (2017) 169:824–835.e14. doi: 10.1016/j.cell.2017.05.003
54. van Tran N, Ernst FGM, Hawley BR, Zorbas C, Ulryck N, Hackert P, et al. The human 18S rRNA m6A methyltransferase METTL5 is stabilized by TRMT112. *Nucleic Acids Res.* (2019) 47:7719–33. doi: 10.1093/nar/gkz619
55. Pinto R, Vågbo CB, Jakobsson ME, Kim Y, Baltissen MP, O'Donohue M-F, et al. The human methyltransferase ZCCHC4 catalyzes N6-methyladenosine modification of 28S ribosomal RNA. *Nucleic Acids Res.* (2020) 48:830–46. doi: 10.1093/nar/gkz1147
56. Wei J, Liu F, Lu Z, Fei Q, Ai Y, He PC, et al. Differential m6A, m6Am, and m1A Demethylation Mediated by FTO in the Cell Nucleus and Cytoplasm. *Mol Cell.* (2018) 71:973–985.e5. doi: 10.1016/j.molcel.2018.08.011
57. Jia G, Yang C-G, Yang S, Jian X, Yi C, Zhou Z, et al. Oxidative demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA by mouse and human FTO. *FEBS Lett.* (2008) 582:3313–9. doi: 10.1016/j.febslet.2008.08.019
58. Xu C, Liu K, Ahmed H, Loppnau P, Schapira M, Min J. Structural basis for the discriminative recognition of N6-methyladenosine RNA by the human YTS21-B homology domain family of proteins. *J Biol Chem.* (2015) 290:24902–13. doi: 10.1074/jbc.M115.680389
59. Hou G, Zhao X, Li L, Yang Q, Liu X, Huang C, et al. SUMOylation of YTHDF2 promotes mRNA degradation and cancer progression by increasing its binding affinity with m6A-modified mRNAs. *Nucleic Acids Res.* (2021) 49:2859–77. doi: 10.1093/nar/gkab065
60. Shi H, Wang X, Lu Z, Zhao BS, Ma H, Hsu PJ, et al. YTHDF3 facilitates translation and decay of N6-methyladenosine-modified RNA. *Cell Res.* (2017) 27:315–28. doi: 10.1038/cr.2017.15
61. Li J, Chen K, Dong X, Xu Y, Sun Q, Wang H, et al. YTHDF1 promotes mRNA degradation via YTHDF1-AGO2 interaction and phase separation. *Cell Prolif.* (2022) 55:e13157. doi: 10.1111/cpr.13157
62. Cheng Y, Xie W, Pickering BF, Chu KL, Savino AM, Yang X, et al. N6-Methyladenosine on mRNA facilitates a phase-separated nuclear body that suppresses myeloid leukemic differentiation. *Cancer Cell.* (2021) 39:958–972.e8. doi: 10.1016/j.ccell.2021.04.017
63. Liu J, Gao M, He J, Wu K, Lin S, Jin L, et al. The RNA m6A reader YTHDC1 silences retrotransposons and guards ES cell identity. *Nature.* (2021) 591:322–6. doi: 10.1038/s41586-021-03313-9
64. Wojtas MN, Pandey RR, Mendel M, Homolka D, Sachidanandam R, Pillai RS. Regulation of m6A transcripts by the 3'→5' RNA helicase YTHDC2 is essential for a successful meiotic program in the mammalian germline. *Mol Cell.* (2017) 68:374–387.e12. doi: 10.1016/j.molcel.2017.09.021
65. Saito Y, Hawley BR, Puno MR, Sarathy SN, Lima CD, Jaffrey SR, et al. YTHDC2 control of gametogenesis requires helicase activity but not m6A binding. *Genes Dev.* (2022) 36:180–94. doi: 10.1101/gad.349190.121
66. Hsu PJ, Zhu Y, Ma H, Guo Y, Shi X, Liu Y, et al. Ythdc2 is an N6-methyladenosine binding protein that regulates mammalian spermatogenesis. *Cell Res.* (2017) 27:1115–27. doi: 10.1038/cr.2017.99
67. Li L, Krasnykov K, Homolka D, Gos P, Mendel M, Fish RJ, et al. The XRN1-regulated RNA helicase activity of YTHDC2 ensures mouse fertility independently of m6A recognition. *Mol Cell.* (2022) 82:1678–1690.e12. doi: 10.1016/j.molcel.2022.02.034
68. Ramesh-Kumar D, Guil S. The IGF2BP family of RNA binding proteins links epitranscriptomics to cancer. *Semin Cancer Biol.* (2022) 86:18–31. doi: 10.1016/j.semcancer.2022.05.009

69. Oerum S, Dégut C, Barraud P, Tisné C. m1A post-transcriptional modification in tRNAs. *Biomolecules*. (2017) 7:20. doi: 10.3390/biom7010020
70. Shi H, Chai P, Jia R, Fan X. Novel insight into the regulatory roles of diverse RNA modifications: Re-defining the bridge between transcription and translation. *Mol Cancer*. (2020) 19:78. doi: 10.1186/s12943-020-01194-6
71. Dominissini D, Nachtergaele S, Moshitch-Moshkovitz S, Peer E, Kol N, Ben-Haim MS, et al. The dynamic N(1)-methyladenosine methylome in eukaryotic messenger RNA. *Nature*. (2016) 530:441–6. doi: 10.1038/nature16998
72. Safra M, Sas-Chen A, Nir R, Winkler R, Nachshon A, Bar-Yaacov D, et al. The m1A landscape on cytosolic and mitochondrial mRNA at single-base resolution. *Nature*. (2017) 551:251–5. doi: 10.1038/nature24456
73. Li J, Zhang H, Wang H. N1-methyladenosine modification in cancer biology: Current status and future perspectives. *Comput Struct Biotechnol J*. (2022) 20:6578–85. doi: 10.1016/j.csbj.2022.11.045
74. Bar-Yaacov D, Frumkin I, Yashiro Y, Chujo T, Ishigami Y, Chemla Y, et al. Mitochondrial 16S rRNA Is Methylated by tRNA Methyltransferase TRMT61B in All Vertebrates. *PLoS Biol*. (2016) 14:e1002557. doi: 10.1371/journal.pbio.1002557
75. Sharma S, Hartmann JD, Watzinger P, Klepper A, Peifer C, Köttler P, et al. A single N1-methyladenosine on the large ribosomal subunit rRNA impacts locally its structure and the translation of key metabolic enzymes. *Sci Rep*. (2018) 8:11904. doi: 10.1038/s41598-018-30383-z
76. Zhang L-S, Xiong Q-P, Peña Perez S, Liu C, Wei J, Le C, et al. ALKBH7-mediated demethylation regulates mitochondrial polycistronic RNA processing. *Nat Cell Biol*. (2021) 23:684–91. doi: 10.1038/s41556-021-00709-7
77. Liu F, Clark W, Luo G, Wang X, Fu Y, Wei J, et al. ALKBH1-mediated tRNA demethylation regulates translation. *Cell*. (2016) 167:1897. doi: 10.1016/j.cell.2016.11.045
78. Chen Z, Zhu W, Zhu S, Sun K, Liao J, Liu H, et al. METTL1 promotes hepatocarcinogenesis via m7 G tRNA modification-dependent translation control. *Clin Transl Med*. (2021) 11:e661. doi: 10.1002/ctm.2661
79. Dai X, Wang T, Gonzalez G, Wang Y. Identification of YTH domain-containing proteins as the readers for N1-methyladenosine in RNA. *Anal Chem*. (2018) 90:6380–4. doi: 10.1021/acs.analchem.8b01703
80. Han X, Wang M, Zhao Y-L, Yang Y, Yang Y-G. RNA methylations in human cancers. *Semin Cancer Biol*. (2021) 75:97–115. doi: 10.1016/j.semcancer.2020.11.007
81. Yang X, Yang Y, Sun B-F, Chen Y-S, Xu J-W, Lai W-Y, et al. 5-methylcytosine promotes mRNA export - NSUN2 as the methyltransferase and ALYREF as an m5C reader. *Cell Res*. (2017) 27:606–25. doi: 10.1038/cr.2017.55
82. Selmi T, Hussain S, Dietmann S, Heiß M, Borland K, Flad S, et al. Sequence- and structure-specific cytosine-5 mRNA methylation by NSUN6. *Nucleic Acids Res*. (2021) 49:1006–22. doi: 10.1093/nar/gkaa1193
83. Nakano S, Suzuki T, Kawarada I, Iwata H, Asano K, Suzuki T. NSUN3 methylase initiates 5-formylcytidine biogenesis in human mitochondrial tRNA(Met). *Nat Chem Biol*. (2016) 12:546–51. doi: 10.1038/nchembio.2099
84. Fu L, Guerrero CR, Zhong N, Amato NJ, Liu Y, Liu S, et al. Tet-mediated formation of 5-hydroxymethylcytosine in RNA. *J Am Chem Soc*. (2014) 136:11582–5. doi: 10.1021/ja505305z
85. Haag S, Sloan KE, Ranjan N, Warda AS, Kretschmer J, Blessing C, et al. NSUN3 and ABH1 modify the wobble position of mt-tRNA^{Met} to expand codon recognition in mitochondrial translation. *EMBO J*. (2016) 35:2104–19. doi: 10.15252/embj.201694885
86. Yang R, Liang X, Wang H, Guo M, Shen H, Shi Y, et al. The RNA methyltransferase NSUN6 suppresses pancreatic cancer development by regulating cell proliferation. *EBioMedicine*. (2021) 63:103195. doi: 10.1016/j.ebiom.2020.103195
87. Zhang L-S, Liu C, Ma H, Dai Q, Sun H-L, Luo G, et al. Transcriptome-wide mapping of internal N7-methylguanosine methylome in mammalian mRNA. *Mol Cell*. (2019) 74:1304–1316.e8. doi: 10.1016/j.molcel.2019.03.036
88. Enroth C, Poulsen LD, Iversen S, Kirpekar F, Albrechtsen A, Vinther J. Detection of internal N7-methylguanosine (m7G) RNA modifications by mutational profiling sequencing. *Nucleic Acids Res*. (2019) 47:e126. doi: 10.1093/nar/gkz736
89. Monecke T, Dickmanns A, Ficner R. Structural basis for m7G-cap hypermethylation of small nuclear, small nucleolar and telomerase RNA by the dimethyltransferase TGS1. *Nucleic Acids Res*. (2009) 37:3865–77. doi: 10.1093/nar/gkp249
90. Culjkovic-Kraljic B, Skrabanek L, Revuelta MV, Gasiorek J, Cowling VH, Cerchietti L, et al. The eukaryotic translation initiation factor eIF4E elevates steady-state m7G capping of coding and noncoding transcripts. *Proc Natl Acad Sci U S A*. (2020) 117:26773–83. doi: 10.1073/pnas.2002360117
91. Leihne V, Kirpekar F, Vågbo CB, van den Born E, Krokan HE, Grini PE, et al. Roles of Trm9- and ALKBH8-like proteins in the formation of modified wobble uridines in Arabidopsis tRNA. *Nucleic Acids Res*. (2011) 39:7688–701. doi: 10.1093/nar/gkr406
92. Lentini JM, Ramos J, Fu D. Monitoring the 5-methoxycarbonylmethyl-2-thiouridine (mcm5s2U) modification in eukaryotic tRNAs via the γ -toxin endonuclease. *RNA N Y N*. (2018) 24:749–58. doi: 10.1261/rna.065581.118
93. Monies D, Vågbo CB, Al-Owain M, Alhomaidi S, Alkuraya FS. Recessive truncating mutations in ALKBH8 cause intellectual disability and severe impairment of wobble uridine modification. *Am J Hum Genet*. (2019) 104:1202–9. doi: 10.1016/j.ajhg.2019.03.026
94. Galeano F, Tomaselli S, Locatelli F, Gallo A. A-to-I RNA editing: the “ADAR” side of human cancer. *Semin Cell Dev Biol*. (2012) 23:244–50. doi: 10.1016/j.semcdb.2011.09.003
95. Erdmann EA, Mahapatra A, Mukherjee P, Yang B, Hundley HA. To protect and modify double-stranded RNA - the critical roles of ADARs in development, immunity and oncogenesis. *Crit Rev Biochem Mol Biol*. (2021) 56:54–87. doi: 10.1080/10409238.2020.1856768
96. Guzzi N, Cieřla M, Ngoc PCT, Lang S, Arora S, Dimitriou M, et al. Pseudouridylation of tRNA-derived fragments steers translational control in stem cells. *Cell*. (2018) 173:1204–1216.e26. doi: 10.1016/j.cell.2018.03.008
97. Nombela P, Miguel-López B, Blanco S. The role of m6A, m5C and Ψ RNA modifications in cancer: Novel therapeutic opportunities. *Mol Cancer*. (2021) 20:18. doi: 10.1186/s12943-020-01263-w
98. Chen H, Yao J, Bao R, Dong Y, Zhang T, Du Y, et al. Cross-talk of four types of RNA modification writers defines tumor microenvironment and pharmacogenomic landscape in colorectal cancer. *Mol Cancer*. (2021) 20:29. doi: 10.1186/s12943-021-01322-w
99. Shen X, Zhao K, Xu L, Cheng G, Zhu J, Gan L, et al. YTHDF2 inhibits gastric cancer cell growth by regulating FOXC2 signaling pathway. *Front Genet*. (2020) 11:592042. doi: 10.3389/fgene.2020.592042
100. Yan J, Liu J, Huang Z, Huang W, Lv J. FOXC2-AS1 stabilizes FOXC2 mRNA via association with NSUN2 in gastric cancer cells. *Hum Cell*. (2021) 34:1755–64. doi: 10.1007/s13577-021-00583-3
101. Zeng Z, Wang J, Xu F, Hu P, Hu Y, Zhuo W, et al. The m6A modification-mediated positive feedback between glycolytic lncRNA SLC2A1-DT and c-Myc promotes tumorigenesis of hepatocellular carcinoma. *Int J Biol Sci*. (2024) 20:1744–62. doi: 10.7150/ijbs.86658
102. Sun Y, Fan J, Wang B, Meng Z, Ren D, Zhao J, et al. The aberrant expression of ADAR1 promotes resistance to BET inhibitors in pancreatic cancer by stabilizing c-Myc. *Am J Cancer Res*. (2020) 10:148–63.
103. Kan G, Wang Z, Sheng C, Chen G, Yao C, Mao Y, et al. Dual inhibition of DKC1 and MEK1/2 synergistically restrains the growth of colorectal cancer cells. *Adv Sci Weinh Baden-Wuertt Ger*. (2021) 8:2004344. doi: 10.1002/adv.202004344
104. Liu T, Li H, Du G, Ma J, Shen J, Zhao H, et al. Comprehensive analysis of m6A regulator-based methylation modification patterns characterized by distinct immune profiles in colon adenocarcinomas. *Gene*. (2022) 821:146250. doi: 10.1016/j.gene.2022.146250
105. Chen J, Ye M, Bai J, Gong Z, Yan L, Gu D, et al. ALKBH5 enhances lipid metabolism reprogramming by increasing stability of FABP5 to promote pancreatic neuroendocrine neoplasms progression in an m6A-IGF2BP2-dependent manner. *J Transl Med*. (2023) 21:741. doi: 10.1186/s12967-023-04578-6
106. Zhang Y, Liu X, Wang Y, Lai S, Wang Z, Yang Y, et al. The m6A demethylase ALKBH5-mediated upregulation of DDIT4-AS1 maintains pancreatic cancer stemness and suppresses chemosensitivity by activating the mTOR pathway. *Mol Cancer*. (2022) 21:174. doi: 10.1186/s12943-022-01647-0
107. Yun D, Yang Z, Zhang S, Yang H, Liu D, Grützmann R, et al. An m5C methylation regulator-associated signature predicts prognosis and therapy response in pancreatic cancer. *Front Cell Dev Biol*. (2022) 10:975684. doi: 10.3389/fcell.2022.975684
108. Zheng Q, Yu X, Zhang Q, He Y, Guo W. Genetic characteristics and prognostic implications of m1A regulators in pancreatic cancer. *Biosci Rep*. (2021) 41:BSR20210337. doi: 10.1042/BSR20210337
109. Li Y, Wang N-X, Yin C, Jiang S-S, Li J-C, Yang S-Y. RNA editing enzyme ADAR1 regulates METTL3 in an editing dependent manner to promote breast cancer progression via METTL3/ARHGAP5/YTHDF1 axis. *Int J Mol Sci*. (2022) 23:9656. doi: 10.3390/ijms23179656
110. Qi L, Zhang W, Ren X, Xu R, Yang Z, Chen R, et al. Cross-talk of multiple types of RNA modification regulators uncovers the tumor microenvironment and immune infiltrates in soft tissue sarcoma. *Front Immunol*. (2022) 13:921223. doi: 10.3389/fimmu.2022.921223
111. Jiang L, Lin X, Jiang J, Qiu C, Zheng S, Zhao N, et al. METTL3-m6A-SIRT1 axis affects autophagic flux contributing to PM2.5-induced inhibition of testosterone production in Leydig cells. *Sci Total Environ*. (2024) 918:170701. doi: 10.1016/j.scitotenv.2024.170701
112. Wan S, Sun Y, Zong J, Meng W, Yan J, Chen K, et al. METTL3-dependent m6A methylation facilitates uterine receptivity and female fertility via balancing estrogen and progesterone signaling. *Cell Death Dis*. (2023) 14:349. doi: 10.1038/s41419-023-05866-1
113. Zhou Y, Song K, Tu B, Sun H, Ding J-F, Luo Y, et al. METTL3 boosts glycolysis and cardiac fibroblast proliferation by increasing AR methylation. *Int J Biol Macromol*. (2022) 223:899–915. doi: 10.1016/j.ijbiomac.2022.11.042
114. Li Y, Zhu S, Chen Y, Ma Q, Kan D, Yu W, et al. Post-transcriptional modification of m6A methylase METTL3 regulates ERK-induced androgen-deprived treatment resistance prostate cancer. *Cell Death Dis*. (2023) 14:289. doi: 10.1038/s41419-023-05773-5
115. Zhang Y, Zhou H, Ding C. The ameliorative effect of CangFu Daotan Decoction on polycystic ovary syndrome of rodent model is associated with m6A methylation and

- Wnt/ β -catenin pathway. *Gynecol Endocrinol.* (2023) 39:2181637. doi: 10.1080/09513590.2023.2181637
116. Li X, Fan C, Wang J, Li P, Xu X, Guo R, et al. Follicle-stimulating hormone accelerates osteoclast migration by enhancing methyltransferase-like 3-mediated m6A methylation of cathepsin K. *J Mol Endocrinol.* (2024) 72:e230130. doi: 10.1530/JME-23-0130
117. Martinez HD, Jasavala RJ, Hinkson I, Fitzgerald LD, Trimmer JS, Kung H-J, et al. RNA editing of androgen receptor gene transcripts in prostate cancer cells. *J Biol Chem.* (2008) 283:29938–49. doi: 10.1074/jbc.M800534200
118. Zhu W, Wan F, Xu W, Liu Z, Wang J, Zhang H, et al. Positive epigenetic regulation loop between AR and NSUN2 promotes prostate cancer progression. *Clin Transl Med.* (2022) 12:e1028. doi: 10.1002/ctm2.1028
119. Caggiano C, Pieraccioni M, Pitolli C, Babini G, Zheng D, Tian B, et al. The androgen receptor couples promoter recruitment of RNA processing factors to regulation of alternative polyadenylation at the 3' end of transcripts. *Nucleic Acids Res.* (2022) 50:9780–96. doi: 10.1093/nar/gkac737
120. Wang L, Lang G-T, Xue M-Z, Yang L, Chen L, Yao L, et al. Dissecting the heterogeneity of the alternative polyadenylation profiles in triple-negative breast cancers. *Theranostics.* (2020) 10:10531–47. doi: 10.7150/thno.40944
121. Akman BH, Can T, Erson-Bensan AE. Estrogen-induced upregulation and 3'-UTR shortening of CDC6. *Nucleic Acids Res.* (2012) 40:10679–88. doi: 10.1093/nar/gks855
122. Bader JE, Voss K, Rathmell JC. Targeting metabolism to improve the tumor microenvironment for cancer immunotherapy. *Mol Cell.* (2020) 78:1019–33. doi: 10.1016/j.molcel.2020.05.034
123. Baghban R, Roshangar L, Jahanban-Esfahlan R, Seidi K, Ebrahimi-Kalan A, Jaymand M, et al. Tumor microenvironment complexity and therapeutic implications at a glance. *Cell Commun Signal CCS.* (2020) 18:59. doi: 10.1186/s12964-020-0530-4
124. Mao X, Xu J, Wang W, Liang C, Hua J, Liu J, et al. Crosstalk between cancer-associated fibroblasts and immune cells in the tumor microenvironment: new findings and future perspectives. *Mol Cancer.* (2021) 20:131. doi: 10.1186/s12943-021-01428-1
125. Qiu S-Q, Waaijer SJH, Zwager MC, de Vries EGE, van der Vegt B, Schröder CP. Tumor-associated macrophages in breast cancer: Innocent bystander or important player? *Cancer Treat Rev.* (2018) 70:178–89. doi: 10.1016/j.ctrv.2018.08.010
126. Su S, Liu Q, Chen J, Chen J, Chen F, He C, et al. A positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis. *Cancer Cell.* (2014) 25:605–20. doi: 10.1016/j.ccr.2014.03.021
127. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* (2002) 23:549–55. doi: 10.1016/s1471-4906(02)02302-5
128. Sousa S, Brion R, Lintunen M, Kronqvist P, Sandholm J, Mönkkönen J, et al. Human breast cancer cells educate macrophages toward the M2 activation status. *Breast Cancer Res BCR.* (2015) 17:101. doi: 10.1186/s13058-015-0621-0
129. Xu M, Liu M, Du X, Li S, Li H, Li X, et al. Intratumoral delivery of IL-21 overcomes anti-HER2/neu resistance through shifting tumor-associated macrophages from M2 to M1 phenotype. *J Immunol Baltim Md.* (2015) 194:4997–5006. doi: 10.4049/jimmunol.1402603
130. Ruffell B, Coussens LM. Macrophages and therapeutic resistance in cancer. *Cancer Cell.* (2015) 27:462–72. doi: 10.1016/j.ccell.2015.02.015
131. Liu Y, Yang J, Hilliard TS, Wang Z, Johnson J, Wang W, et al. Host obesity alters the ovarian tumor immune microenvironment and impacts response to standard of care chemotherapy. *J Exp Clin Cancer Res CR.* (2023) 42:165. doi: 10.1186/s13046-023-02740-y
132. Zhu X, Xu Y, Wang J, Xue Z, Qiu T, Chen J. Loss of NLRP3 reduces oxidative stress and polarizes intratumor macrophages to attenuate immune attack on endometrial cancer. *Front Immunol.* (2023) 14:1165602. doi: 10.3389/fimmu.2023.1165602
133. Zhou B, Mo Z, Lai G, Chen X, Li R, Wu R, et al. Targeting tumor exosomal circular RNA cSERPINE2 suppresses breast cancer progression by modulating MALT1-NF- κ B-IL-6 axis of tumor-associated macrophages. *J Exp Clin Cancer Res CR.* (2023) 42:48. doi: 10.1186/s13046-023-02620-5
134. Yoshimura T, Li C, Wang Y, Matsukawa A. The chemokine monocyte chemoattractant protein-1/CCL2 is a promoter of breast cancer metastasis. *Cell Mol Immunol.* (2023) 20:714–38. doi: 10.1038/s41423-023-01013-0
135. Wang D, Cheng C, Chen X, Wang J, Liu K, Jing N, et al. IL-1 β is an androgen-responsive target in macrophages for immunotherapy of prostate cancer. *Adv Sci Weinh Baden-Wuert Ger.* (2023) 10:e2206889. doi: 10.1002/advs.202206889
136. Li L-Y, Yin K-M, Bai Y-H, Zhang Z-G, Di W, Zhang S. CTHRC1 promotes M2-like macrophage recruitment and myometrial invasion in endometrial carcinoma by integrin-Akt signaling pathway. *Clin Exp Metastasis.* (2019) 36:351–63. doi: 10.1007/s10585-019-09971-4
137. Puttock EH, Tyler EJ, Manni M, Maniati E, Butterworth C, Burger Ramos M, et al. Extracellular matrix educates an immunoregulatory tumor macrophage phenotype found in ovarian cancer metastasis. *Nat Commun.* (2023) 14:2514. doi: 10.1038/s41467-023-38093-5
138. Veglia F, Sanseviero E, Gabilovich DI. Myeloid-derived suppressor cells in the era of increasing myeloid cell diversity. *Nat Rev Immunol.* (2021) 21:485–98. doi: 10.1038/s41577-020-00490-y
139. Zheng Y, Wang N, Wang S, Zhang J, Yang B, Wang Z. Chronic psychological stress promotes breast cancer pre-metastatic niche formation by mobilizing splenic MDSCs via TAM/CXCL1 signaling. *J Exp Clin Cancer Res CR.* (2023) 42:129. doi: 10.1186/s13046-023-02696-z
140. Xiao J, Sun F, Wang Y-N, Liu B, Zhou P, Wang F-X, et al. UBC9 deficiency enhances immunostimulatory macrophage activation and subsequent antitumor T cell response in prostate cancer. *J Clin Invest.* (2023) 133:e158352. doi: 10.1172/JCI158352
141. Chung H, Gyu-Mi P, Na YR, Lee Y-S, Choi H, Seok SH. Comprehensive characterization of early-programmed tumor microenvironment by tumor-associated macrophages reveals galectin-1 as an immune modulatory target in breast cancer. *Theranostics.* (2024) 14:843–60. doi: 10.7150/thno.88917
142. Cen Y, Chen Y, Li X, Chen X, Yu B, Yan M, et al. Optical controlled and nuclear targeted CECR2 competitor to downregulate CSF-1 for metastatic breast cancer immunotherapy. *Biomaterials.* (2024) 308:122568. doi: 10.1016/j.biomaterials.2024.122568
143. Ducarouge B, Redavid A-R, Victor C, Chira R, Fonseca A, Hervieu M, et al. Netrin-1 blockade inhibits tumor associated Myeloid-derived suppressor cells, cancer stemness and alleviates resistance to chemotherapy and immune checkpoint inhibitor. *Cell Death Differ.* (2023) 30:2201–12. doi: 10.1038/s41418-023-01209-x
144. Huang T, Zhang Q, Yi J, Wang R, Zhang Z, Luo P, et al. PEG-sheddable nanodrug remodels tumor microenvironment to promote effector T cell infiltration and revise their exhaustion for breast cancer immunotherapy. *Small Weinh Bergstr Ger.* (2023) 19:e2301749. doi: 10.1002/smll.202301749
145. Andersson P, Yang Y, Hosaka K, Zhang Y, Fischer C, Braun H, et al. Molecular mechanisms of IL-33-mediated stromal interactions in cancer metastasis. *JCI Insight.* (2018) 3:e122375. doi: 10.1172/jci.insight.122375
146. Butti R, Nimma R, Kundu G, Bulbule A, Kumar TVS, Gunasekaran VP, et al. Tumor-derived osteopontin drives the resident fibroblast to myofibroblast differentiation through Twist1 to promote breast cancer progression. *Oncogene.* (2021) 40:2002–17. doi: 10.1038/s41388-021-01663-2
147. Chen X, Song E. Turning foes to friends: targeting cancer-associated fibroblasts. *Nat Rev Drug Discov.* (2019) 18:99–115. doi: 10.1038/s41573-018-0004-1
148. Mizutani Y, Kobayashi H, Iida T, Asai N, Masamune A, Hara A, et al. Meflin-positive cancer-associated fibroblasts inhibit pancreatic carcinogenesis. *Cancer Res.* (2019) 79:5367–81. doi: 10.1158/0008-5472.CAN-19-0454
149. Houthuijzen JM, de Bruijn R, van der Burg E, Drenth AP, Wientjens E, Filipovic T, et al. CD26-negative and CD26-positive tissue-resident fibroblasts contribute to functionally distinct CAF subpopulations in breast cancer. *Nat Commun.* (2023) 14:183. doi: 10.1038/s41467-023-35793-w
150. Fan G, Yu B, Tang L, Zhu R, Chen J, Zhu Y, et al. TSPAN8+ myofibroblastic cancer-associated fibroblasts promote chemoresistance in patients with breast cancer. *Sci Transl Med.* (2024) 16:eadj5705. doi: 10.1126/scitranslmed.adj5705
151. Wang H, Li N, Liu Q, Guo J, Pan Q, Cheng B, et al. Antiandrogen treatment induces stromal cell reprogramming to promote castration resistance in prostate cancer. *Cancer Cell.* (2023) 41:1345–1362.e9. doi: 10.1016/j.ccell.2023.05.016
152. Yu Z, Zhang Q, Wei S, Zhang Y, Zhou T, Zhang Q, et al. CD146+CAFs promote progression of endometrial cancer by inducing angiogenesis and vasculogenic mimicry via IL-10/JAK1/STAT3 pathway. *Cell Commun Signal CCS.* (2024) 22:170. doi: 10.1186/s12964-024-01550-9
153. Xu AM, Haro M, Walts AE, Hu Y, John J, Karlan BY, et al. Spatiotemporal architecture of immune cells and cancer-associated fibroblasts in high-grade serous ovarian carcinoma. *Sci Adv.* (2024) 10:eadk8805. doi: 10.1126/sciadv.adk8805
154. Kopecka J, Salaroglio IC, Perez-Ruiz E, Sarmiento-Ribeiro AB, Saponara S, De Las Rivas J, et al. Hypoxia as a driver of resistance to immunotherapy. *Drug Resist Update Rev Comment Antimicrob Anticancer Chemother.* (2021) 59:100787. doi: 10.1016/j.drup.2021.100787
155. de Boniface J, Mao Y, Schmidt-Mende J, Kiessling R, Poschke I. Expression patterns of the immunomodulatory enzyme arginase 1 in blood, lymph nodes and tumor tissue of early-stage breast cancer patients. *Oncoimmunology.* (2012) 1:1305–12. doi: 10.4161/onci.21678
156. van de Weijer ML, Samanta K, Sergejevs N, Jiang L, Dueñas ME, Heunis T, et al. Tapasin assembly surveillance by the RNF185/Membralin ubiquitin ligase complex regulates MHC-I surface expression. *Nat Commun.* (2024) 15:8508. doi: 10.1038/s41467-024-52772-x
157. Santoni M, Romagnoli E, Saladino T, Foghini I, Guarino S, Capponi M, et al. Triple negative breast cancer: Key role of Tumor-Associated Macrophages in regulating the activity of anti-PD-1/PD-L1 agents. *Biochim Biophys Acta Rev Cancer.* (2018) 1869:78–84. doi: 10.1016/j.bbcan.2017.10.007
158. Deng X, Qing Y, Horne D, Huang H, Chen J. The roles and implications of RNA m6A modification in cancer. *Nat Rev Clin Oncol.* (2023) 20:507–26. doi: 10.1038/s41571-023-00774-x
159. Zhuang H, Yu B, Tao D, Xu X, Xu Y, Wang J, et al. The role of m6A methylation in therapy resistance in cancer. *Mol Cancer.* (2023) 22:91. doi: 10.1186/s12943-023-01782-2
160. Ying Y, Wu Y, Zhang F, Tang Y, Yi J, Ma X, et al. Co-transcriptional R-loops-mediated epigenetic regulation drives growth retardation and docetaxel chemosensitivity enhancement in advanced prostate cancer. *Mol Cancer.* (2024) 23:79. doi: 10.1186/s12943-024-01994-0

161. Xu X, Zhuang X, Yu H, Li P, Li X, Lin H, et al. FSH induces EMT in ovarian cancer via ALKBH5-regulated Snail m6A demethylation. *Theranostics*. (2024) 14:2151–66. doi: 10.7150/thno.94161
162. Luo F, Zhang M, Sun B, Xu C, Yang Y, Zhang Y, et al. LINC00115 promotes chemoresistant breast cancer stem-like cell stemness and metastasis through SETDB1/PLK3/HIF1 α signaling. *Mol Cancer*. (2024) 23:60. doi: 10.1186/s12943-024-01975-3
163. Chen S-J, Zhang J, Zhou T, Rao S-S, Li Q, Xiao L-Y, et al. Epigenetically upregulated NSUN2 confers ferroptosis resistance in endometrial cancer via m5C modification of SLC7A11 mRNA. *Redox Biol*. (2024) 69:102975. doi: 10.1016/j.redox.2023.102975
164. Gu J, Cao H, Chen X, Zhang XD, Thorne RF, Liu X. RNA m6A modifications regulate crosstalk between tumor metabolism and immunity. *Wiley Interdiscip Rev RNA*. (2024) 15:e1829. doi: 10.1002/wrna.1829
165. Li J, Zheng L, Song L, Dong Z, Bai W, Qi L. Identification and validation of N7-methylguanosine-associated gene NCBP1 as prognostic and immune-associated biomarkers in breast cancer patients. *J Cell Mol Med*. (2024) 28:e18067. doi: 10.1111/jcmm.18067
166. Zhang X, Shen L, Zhu Y, Zhai C, Zeng H, Liu X, et al. Crosstalk of RNA methylation writers defines tumor microenvironment and alisertib resistance in breast cancer. *Front Endocrinol*. (2023) 14:1166939. doi: 10.3389/fendo.2023.1166939
167. Chen H, Zhou L, Li J, Hu K. ALKBH family members as novel biomarkers and prognostic factors in human breast cancer. *Aging*. (2022) 14:6579–93. doi: 10.18632/aging.204231
168. Xu Z, Chen S, Zhang Y, Liu R, Chen M. Roles of m5C RNA modification patterns in biochemical recurrence and tumor microenvironment characterization of prostate adenocarcinoma. *Front Immunol*. (2022) 13:869759. doi: 10.3389/fimmu.2022.869759
169. Liu Z, Zhong J, Zeng J, Duan X, Lu J, Sun X, et al. Characterization of the m6A-associated tumor immune microenvironment in prostate cancer to aid immunotherapy. *Front Immunol*. (2021) 12:735170. doi: 10.3389/fimmu.2021.735170
170. Zhang Z, Huo W, Li J. circATAD2 mitigates CD8+ T cells antitumor immune surveillance in breast cancer via IGF2BP3/m6A/PD-L1 manner. *Cancer Immunol Immunother*. (2024) 73:130. doi: 10.1007/s00262-024-03705-6
171. Wan W, Ao X, Chen Q, Yu Y, Ao L, Xing W, et al. METTL3/IGF2BP3 axis inhibits tumor immune surveillance by upregulating N6-methyladenosine modification of PD-L1 mRNA in breast cancer. *Mol Cancer*. (2022) 21:60. doi: 10.1186/s12943-021-01447-y
172. Wang Y, Jin P, Wang X. N6-methyladenosine regulator YTHDF1 represses the CD8+ T cell-mediated antitumor immunity and ferroptosis in prostate cancer via m6A/PD-L1 manner. *Apoptosis*. (2024) 29:142–53. doi: 10.1007/s10495-023-01885-7
173. Liu J, Song Y, Wang Y, Han M, Wang C, Yan F. Cyclodextrin-functionalized gold nanorods loaded with meclofenamic acid for improving N6-methyladenosine-mediated second near-infrared photothermal immunotherapy. *ACS Appl Mater Interfaces*. (2022) 14:40612–23. doi: 10.1021/acsami.2c09978
174. Wang R, Ye H, Yang B, Ao M, Yu X, Wu Y, et al. m6A-modified circNFIX promotes ovarian cancer progression and immune escape via activating IL-6R/JAK1/STAT3 signaling by sponging miR-647. *Int Immunopharmacol*. (2023) 124:110879. doi: 10.1016/j.intimp.2023.110879
175. Cheng Y, Li L, Wei X, Xu F, Huang X, Qi F, et al. HNRNPC suppresses tumor immune microenvironment by activating Treg cells promoting the progression of prostate cancer. *Cancer Sci*. (2023) 114:1830–45. doi: 10.1111/cas.15745
176. Li C, Peng D, Gan Y, Zhou L, Hou W, Wang B, et al. The m6A methylation landscape, molecular characterization and clinical relevance in prostate adenocarcinoma. *Front Immunol*. (2023) 14:1086907. doi: 10.3389/fimmu.2023.1086907
177. Ma C, Zheng Q, Wang Y, Li G, Zhao M, Sun Z. Pan-cancer analysis and experimental validation revealed the m6A methyltransferase KIAA1429 as a potential biomarker for diagnosis, prognosis, and immunotherapy. *Aging*. (2023) 15:8664–91. doi: 10.18632/aging.204968
178. Jiao Y, Geng R, Zhong Z, Ni S, Liu W, He Z, et al. A hypoxia molecular signature-based prognostic model for endometrial cancer patients. *Int J Mol Sci*. (2023) 24:1675. doi: 10.3390/ijms24021675
179. García-Vílchez R, Añazco-Guenkova AM, Dietmann S, López J, Morón-Calvente V, D'Ambrosi S, et al. METTL1 promotes tumorigenesis through tRNA-derived fragment biogenesis in prostate cancer. *Mol Cancer*. (2023) 22:119. doi: 10.1186/s12943-023-01809-8
180. Yu G, Bao J, Zhan M, Wang J, Li X, Gu X, et al. Comprehensive analysis of m5C methylation regulatory genes and tumor microenvironment in prostate cancer. *Front Immunol*. (2022) 13:914577. doi: 10.3389/fimmu.2022.914577
181. Choi H, Kwon J, Cho MS, Sun Y, Zheng X, Wang J, et al. Targeting DDX3X Triggers Antitumor Immunity via a dsRNA-Mediated Tumor-Intrinsic Type I Interferon Response. *Cancer Res*. (2021) 81:3607–20. doi: 10.1158/0008-5472.CAN-20-3790
182. Zheng P, Li N, Zhan X. Ovarian cancer subtypes based on the regulatory genes of RNA modifications: Novel prediction model of prognosis. *Front Endocrinol*. (2022) 13:863484. doi: 10.3389/fendo.2022.972341
183. Ye L, Pan K, Fang S, Wu S-N, Chen S, Tang S, et al. Four types of RNA modification writer-related lncRNAs are effective predictors of prognosis and immunotherapy response in serous ovarian carcinoma. *Front Immunol*. (2022) 13:863484. doi: 10.3389/fimmu.2022.863484
184. Li H, Luo F, Jiang X, Zhang W, Xiang T, Pan Q, et al. CircITGB6 promotes ovarian cancer cisplatin resistance by resetting tumor-associated macrophage polarization toward the M2 phenotype. *J Immunother Cancer*. (2022) 10:e004029. doi: 10.1136/jitc-2021-004029
185. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol*. (2019) 19:369–82. doi: 10.1038/s41577-019-0127-6
186. Woo H-H, Chambers SK. Human ALKBH3-induced m1A demethylation increases the CSF-1 mRNA stability in breast and ovarian cancer cells. *Biochim Biophys Acta Gene Regul Mech*. (2019) 1862:35–46. doi: 10.1016/j.bbagr.2018.10.008
187. Alshaker H, Thrower H, Pchejetski D. Sphingosine kinase 1 in breast cancer-A new molecular marker and a therapy target. *Front Oncol*. (2020) 10:289. doi: 10.3389/fonc.2020.00289
188. Zheng X, Li W, Ren L, Liu J, Pang X, Chen X, et al. The sphingosine kinase-1/sphingosine-1-phosphate axis in cancer: Potential target for anticancer therapy. *Pharmacol Ther*. (2019) 195:85–99. doi: 10.1016/j.pharmthera.2018.10.011
189. Alshaker H, Wang Q, Brewer D, Pchejetski D. Transcriptome-wide effects of sphingosine kinases knockdown in metastatic prostate and breast cancer cells: implications for therapeutic targeting. *Front Pharmacol*. (2019) 10:303. doi: 10.3389/fphar.2019.00303
190. Xue C, Chu Q, Zheng Q, Jiang S, Bao Z, Su Y, et al. Role of main RNA modifications in cancer: N6-methyladenosine, 5-methylcytosine, and pseudouridine. *Signal Transduct Target Ther*. (2022) 7:142. doi: 10.1038/s41392-022-01003-0
191. Cottrell KA, Torres LS, Dizon MG, Weber JD. 8-azaadenosine and 8-chloroadenosine are not selective inhibitors of ADAR. *Cancer Res Commun*. (2021) 1:56–64. doi: 10.1158/2767-9764.crc-21-0027
192. Goncharov AO, Shender VO, Kuznetsova KG, Kliuchnikova AA, Moshkovskii SA. Interplay between A-to-I editing and splicing of RNA: A potential point of application for cancer therapy. *Int J Mol Sci*. (2022) 23:5240. doi: 10.3390/ijms23095240
193. Gomez S, Cox OL, Walker RR, Rentia U, Hadley M, Arthofer E, et al. Inhibiting DNA methylation and RNA editing upregulates immunogenic RNA to transform the tumor microenvironment and prolong survival in ovarian cancer. *J Immunother Cancer*. (2022) 10:e004974. doi: 10.1136/jitc-2022-004974
194. Meng H, Miao H, Zhang Y, Chen T, Yuan L, Wan Y, et al. YBX1 promotes homologous recombination and resistance to platinum-induced stress in ovarian cancer by recognizing m5C modification. *Cancer Lett*. (2024) 597:217064. doi: 10.1016/j.canlet.2024.217064
195. Li E, Xia M, Du Y, Long K, Ji F, Pan F, et al. METTL3 promotes homologous recombination repair and modulates chemotherapeutic response in breast cancer by regulating the EGF/RAD51 axis. *eLife*. (2022) 11:e75231. doi: 10.7554/eLife.75231
196. Ouyang D, Hong T, Fu M, Li Y, Zeng L, Chen Q, et al. METTL3 depletion contributes to tumour progression and drug resistance via N6 methyladenosine-dependent mechanism in HR+HER2-breast cancer. *Breast Cancer Res*. (2023) 25:19. doi: 10.1186/s13058-022-01598-w



OPEN ACCESS

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RECEIVED 12 February 2025

ACCEPTED 28 April 2025

PUBLISHED 15 May 2025

CITATION

Xia L and Yin P (2025) Upregulated m7G methyltransferase METTL1 is a potential biomarker and tumor promoter in skin cutaneous melanoma.
Front. Immunol. 16:1575219.
doi: 10.3389/fimmu.2025.1575219

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Upregulated m7G methyltransferase METTL1 is a potential biomarker and tumor promoter in skin cutaneous melanoma

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The m7G methyltransferase METTL1 has been implicated in the occurrence and progression of several cancers. However, its clinical significance in cutaneous melanoma (SKCM) remains poorly understood. To address this gap, we conducted comprehensive data mining using publicly available datasets and two single-cell datasets. Additionally, we employed CCK8 assays, clone formation assays, and cell migration and invasion experiments to validate our findings from the data mining. Our results revealed that METTL1 is significantly upregulated in SKCM and is associated with a stem cell-like phenotype. Patients with high METTL1 expression exhibited worse prognosis. Furthermore, we identified that the high expression of METTL1 in SKCM is driven by copy number amplification and regulated by the transcription factor MYC. *In vitro* cellular studies confirmed that METTL1 knockdown significantly inhibited SKCM cell proliferation, clone formation, migration, and invasion. Notably, we observed a strong negative correlation between METTL1 expression and CD8+ T-cell infiltration in SKCM tissues. Moreover, our analysis revealed a significant negative correlation between METTL1 expression levels and the response to immunotherapy in SKCM patients, suggesting that METTL1 may serve as a potential biomarker for predicting immunotherapy response in SKCM. In summary, this study enhances our understanding of the role of m7G RNA modification in tumor progression and highlights METTL1 as a novel therapeutic target and biomarker for SKCM immunotherapy.

KEYWORDS

METTL1, SKCM, biomarker, immunotherapy, prognosis

Introduction

METTL1 (Methyltransferase-like 1), located on chromosome 12q13, is a protein-coding gene that has methyltransferase activity in cells (1). Normally, METTL1 is expressed in kidney, thyroid, skin, and 25 other tissues (2). METTL1 functions primarily in the nucleus and is involved in epigenetic modification of RNA. m7G tRNA modification

mediated by METTL1/WDR4 is essential for common RNA translation, regulation of cellular self-renewal, and differentiation (3). m7G is one of the most common RNA epitope modifications, often located in the 5' cap and internal positions of eukaryotic mRNAs, or within rRNAs and tRNAs in all species (4). m7G This modification helps to maintain the stability, proper folding, and functioning of tRNAs, and affects the process of protein synthesis (5). In addition, METTL1 activity may also have some effect on other RNA molecules, such as rRNAs and microRNAs (6). However, the function and regulatory mechanisms of METTL1 still require further studies to fully understand its role in cell biology and disease development.

Previous studies have shown that aberrant expression of METTL1 is closely associated with tumor development and patient survival. For example, METTL1 is associated with poor prognosis in bladder cancer, and it regulates the translation of EGFR/EFEMP1 by modifying certain tRNAs to inhibit the proliferation, migration and invasion of bladder cancer cells (7). In prostate cancer, METTL1 promotes tumorigenesis through tRNA-derived fragment biogenesis, and inhibition of METTL1 activity leads to favorable changes within the tumor, such as an increase in anti-tumor cytokines and infiltration of cytotoxic immune cells, including M1 macrophages and CD8+ T cells (8). METTL1 deficiency leads to reduced abundance and cell cycle alterations of m7G-modified tRNAs, particularly Arg-TCT-4-1, and inhibits oncogenicity (9). In addition, METTL1-mediated tRNA m7G modifications promote the translation of mTOR pathway components, thereby facilitating mTOR activation and progression of esophageal squamous cell carcinoma (10). These studies imply an important role for METTL1 in cancer development. However, its specific role and regulatory mechanism in the progression of cutaneous melanoma have not been clearly reported.

This study is based on public data mining in The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases, and systematically analyzes the expression, regulation, and clinical value of METTL1 in SKCM, providing reference for prognosis judging and personalized therapy of SKCM.

Materials and methods

Databases and data mining and analysis

The databases used in this study include, Kaplan-Meier Plotter, GEPIA2 database (<http://gepia2.cancer-pku.cn/#index>), BEST database, The Human Protein Atlas database, ASSISTANT for Clinical Bioinformatics tool, cBioportal database, TISCH database, UCSC genomics, and two single-cell datasets (GSE72056 and GSE174401). The Kaplan-Meier Plotter, GEPIA2 database, BEST database, Human Protein Atlas database, ASSISTANT for Clinical

Bioinformatics tool, and the cBioportal database were used to analyze the correlation between METTL1 expression and clinicopathological characteristics of SKCM patients. Two single-cell sequencing datasets, GSE72056 and GSE174401, were used to analyze the relationship between METTL1 and tumor cell trajectory by the algorithm of monocle, and the differentiation potential of cells by the CytoTRACE algorithm. ASSISTANT for Clinical Bioinformatics tool analyzed the correlation between METTL1 and stem cell-like phenotype of SKCM and with immune cells. cBioportal database analyzed the correlation between METTL1 copy number amplification and clinical characteristics. The spatial transcriptome data of formalin fixation and paraffin embedding samples on 10x Genomics (<https://www.10xgenomics.com/cn/datasets/human-melanoma-if-stained-ffpe-2-standard>) were used to analyze the colocalization of METTL1 with SKCM stem cell markers SOX10 and ABCB5. All analyses were conducted by simply selecting the disease type (SKCM in this study) in the database, followed by filtering and extracting relevant data according to the default parameters of the database.

Cell culture and treatment

The cell lines A875 and A375 were gifts from the Department of Dermatology, Xiangya Hospital, Central South University (Changsha, China), and were cultured in 1640 complete medium with 10% Fetal Bovine Serum (cat no. A2720801, Gibco, USA) at 37°C, 5% CO₂ incubator.

siRNA transfection

To knock down METTL1, A875 and A375 cells were transfected 20 nM siRNA (siRNA sequences: METTL1 #1: GATGACCCAAA GGATAAGAAA; METTL1 #2: GGATGTGCACTCATTTTCGA or empty plasmids (control) that mixed with Lip3000 for 48 hours. The transfected cells were used for further analysis.

Quantitative real time PCR

The transfected cells were collected and added TRIzol reagent to extract the total RNA. Ultra Micro Nucleic Acid Analyzer (NANODrop2000, Shimadzu, Japan) was used to determine the concentration and purity of RNA to meet the requirement of A260/A280 in the range of 1.8-2.0. The extracted RNA was used as the template for reverse transcription to synthesize the cDNA. The real-time fluorescent quantitative PCR (7300 plus, ABI, USA) was used to perform real-time quantitative amplification. The reaction conditions were as follows: 95°C for 30 s, 95°C for 5 s, 55°C for 30 s, 72°C for 30 s, 40 cycles; 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. The specificity of the primers was analyzed according to the melting curves, and the relative expression of the target genes was calculated by using the $2^{-\Delta\Delta C_t}$ method with GAPDH as the internal reference. The primer sequences were as follows:

Abbreviations: SKCM, skin cutaneous melanoma; CNV, Copy number variation; METTL1, Methyltransferase-like 1; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus.

METTL1-F: AAAGGGGACATGAAAGGGCAA,
 METTL1-R: CACCAGACAGACCAAGATGGAA;
 SOX10-F: GAGGCTGCTGAACGAAAGTG,
 SOX10-R: GCTCTTGTAGTGGGCCTGGA;
 CD4-F: GGGATACAGTGGAACTGACCTG,
 CD4-R: CAGAGTTGGCAGTCAATCCGAA;
 CD8A-F: ATGGCCTTACCAGTGACCG,
 CD8A-R: AGGTTCCAGGTCCGATCCAG.
 GAPDH-F: AATGGGCAGCCGTTAGGAAA,
 GAPDH -R: GCGCCCAATACGACCAAATC.

Clone formation

A875 and A375 cells were inoculated in 35 mm cell culture dishes (300 cells/dish) and incubated in an incubator for 2 weeks, with medium changes every 2 day. The supernatant was washed 3 times with PBS and fixed with methanol for 15 min, incubated with 2 mL of crystal violet staining solution for 30 min, washed 3 times with PBS, and air dried. When the diameter of cell clone was >0.75 mm, it was recognized as positive and manually counted.

RNA-seq data analysis

The METTL1 siRNA transfected A875 cells and control cells were collected and used for sequencing in BGISEQ-500 platform (Beijing Genomics institution, Shenzhen, China). mRNA was enriched using oligo(dT) magnetic beads and denatured to open its secondary structure. The mRNA was fragmented and used to synthesize double-strand cDNA to amplify. After denaturing the PCR product into single-stranded, the cyclization reaction system was performed to obtain the single-stranded cyclic product, and digest the linear DNA molecules that have not been cyclized. The single-stranded cyclic DNA molecules were used for rolling circle replication to form a DNA nanoball (DNB) containing multiple copies, which were sequenced by co-probe anchored polymerization (cPAS). The raw data obtained from sequencing was filtered using SOAPnuke (v1.5.6) and clean data was compared to the reference gene set using Bowtie2 (v2.3.4.3). Gene expression was quantified using RSEM (v1.3.1) software and differential gene detection was performed using DESeq2 (v1.4.5), and the heatmap was displayed. Gene function analysis was based on GO.

CCK8

METTL1 siRNA and negative control were transfected into A875 and A375 cells respectively for 24 h. After transfection, the cells were seeded into 96-well plates at 5×10^3 cells per well, and 75 μ M photosan was added into each well after the corresponding time points (0, 1, 2, 3, 4 day), and the cells were subjected to a 630 nm laser treatment for 4 h. 10 μ l CCK8 was added into each well after 24 h. The OD value of CCK8 was determined using a 450 nm wavelength and the growth curve was plotted.

Cell migration and invasion

The transfected cells were removed from the complete culture medium, replaced with serum-free medium for 24 h. The cells were digested and collected into centrifuge tubes, washed twice with PBS, and the cells were suspended in basal medium and precipitated. 5×10^4 cells were added to the upper chambers, and then supplemented with serum-free medium, and fresh complete medium was added to the lower chambers of the chambers. The chambers with matrix gel were used to detect cell invasion, and the chambers without matrix gel were used to detect cell migration. The cells were put into the incubator for 48 h. The liquid in the chambers was wiped out by cotton swabs and put into paraformaldehyde solution for 30 min; the chambers were clamped out, the liquid in the chambers was poured out, and the chambers were stained with crystal violet staining solution for 20 min; the chambers were put into ultrapure water for rinsing, and the liquid in the chambers was discarded, and the liquid was dried out upside down; the chambers were photographed under a microscope for cell counting and analyzing.

Cell cycle detection by flow cytometry

A875 cells were transfected with METTL1 siRNA and negative control respectively, and then cultured for 72 h after transfection, and then the cells were collected and counted. 1×10^5 cells were obtained and washed by pre-cooled PBS, and then were resuspended by adding 70% ethanol solution to the cell precipitates, fixed for 30 min, added with PI staining solution, and incubated for 30 min away from light, and then detected by flow cytometry.

Statistical analysis

All experiments were repeated three times, and the data are expressed as the mean $\pm$ standard deviation. Statistical analysis was performed using GraphPad Prism 8 software. A t test was used for comparisons between two groups. For multiple comparisons involving more than two groups, Tukey's *post hoc* test and one-way analysis of variance (ANOVA) were used. $P < 0.05$ was regarded as statistically significant difference.

Results

Upregulated METTL1 is an independent prognostic indicator of SKCM

Using the GEPIA2 online database (11), we first analyzed the expression of METTL1 in the TCGA SKCM cohort and found that METTL1 was significantly upregulated in SKCM (Figure 1A). Differential analysis of SKCM genomic variants according to their type into four subclasses, BRAF mutated, NF1 mutated, RAS

mutated, and wild-type without any of the three mutations, revealed that METTL1 was significantly upregulated in all four types of SKCM samples (Figure 1B). However, there was no significant difference in METTL1 expression in these mutant types of SKCM (Supplementary Figure S1A). METTL1 expression was also similarly markedly upregulated metastasis and recurrence tumor compared with primary and no-recurrence tumor in GSE46517, another independent SKCM cohort dataset (Figure 1C). In another independent SKCM dataset GSE98394, the expression of METTL1 increased with the increase of T grade, N grade, and clinical stage (Figure 1D). Subsequently, we also validated that the mRNA level of METTL1 was higher in SKCM tissues (N=19) compared with normal tissues (N=7) by qRT-PCR (Figure 1E), and similar results were observed in immunohistochemical staining from The Human Protein Atlas similarly (Figure 1F).

Furthermore, we analyzed the correlation between METTL1 and the clinicopathological features of SKCM patients. Survival analysis showed that patients with high METTL1 expression in the TCGA_SKCM cohort had shorter overall survival (Figure 1G), and consistent results were also obtained in the data from the three other SKCM cohorts, GSE46517, GSE98394, GSE190113, and GSE19234 (Figures 1H–J, Supplementary Figure S1B). In the following, we found that patients with high METTL1 expression in the GSE133713 dataset had lower recurrence-free survival (Supplementary Figure S1C). According to these survival curve results, METTL1 may function as an independent prognostic indicator for SKCM patients. This evidence implies that METTL1 may play a critical role in the development and progression of SKCM.

METTL1 is expressed in stem-like SKCM cells at early stage of differentiation

To further determine the spatiotemporal expression pattern of METTL1, we analyzed the relationship between METTL1 and the developmental chronology of tumor cells using monocle algorithm with two sets of single-cell sequencing data, GSE72056 and GSE174401. The analysis revealed that METTL1 was highly expressed in cluster6 in GSE72056, cluster 6 was located at the beginning of the trajectories (Figures 2A–C), and cluster 6 was significantly enriched for stem cell-associated MYC, Hedgehog, and WNT- β -catenin signaling pathways (Supplementary Figures S2A, B). Similarly, METTL1 was highly expressed in cluster 0 in GSE174401, which was also located at the beginning of the trajectories (Figures 2D–F). In addition, the differentiation potential of the cells was analyzed using the CytoTRACE algorithm, and it was found that cluster 0 had the highest differentiation potential and highly expressed ABCB5, an SKCM stem cell marker (Figures 2G, H). Analysis of the relationship between METTL1 expression and cell stemness scores using the ASSISTANT for Clinical Bioinformatics tool found that the mRNA_{si} score was higher in the METTL1 high expression group (Figure 3A), and the SKCM stem cell marker genes SOX10 and ABCB5 were highly expressed (Figures 3B, C), and METTL1

expression was significantly and positively correlated with these two marker genes (Figure 3D). In addition, spatial transcriptome data analysis revealed that METTL1 expression co-localized with stem cell marker genes SOX10 and ABCB5 (Figure 3E).

We further confirmed the function of METTL1 by *in vitro* cellular experiments. Knockdown of METTL1 in SKCM cells A875 and A375 by siRNA transfection and siRNA1 was selected for further analysis (Figure 3F). After METTL1 siRNA transfection, the protein expression of METTL1 was significantly knocked down (Figure 3G). Downregulation of METTL1 significantly inhibited the clone formation ability of SKCM cells (Figure 3H) and decreased the expression of stem cell marker gene SOX10 (Figure 3I). These results suggest that the function of METTL1 is closely related with stem cell-like SKCM cells.

Knockdown of METTL1 inhibits migration and invasion of SKCM cells

TISCH database analysis showed that METTL1 expression was higher in metastatic malignant tumor cells (Figure 4A). CCK8 assay showed that knockdown of METTL1 significantly inhibited the cell viability of A875 and A375 cells (Figure 4B), and significantly inhibited the migration (Figures 4C, D) and invasive ability of A875 and A375 cells (Figures 4E, F), and also had slight alterations on cell cycle (Supplementary Figure S3).

METTL1 functions as a marker of immunotherapy response in SKCM

Further, we carried out RNA-seq analysis after silencing METTL1. Principal component analysis showed that samples after silencing METTL1 and samples without silencing METTL1 could be significantly distinguished and were closer to each other in the same group (Figure 5A). 103 genes were considerably differentially expressed according to differential analysis, of which 32 had significant up-regulation and 71 had significant down-regulation (Figure 5B). These differentially expressed genes were mainly enriched in several immune-related signaling pathways, such as T cell migration, epithelial-mesenchymal transition, and inflammatory response (Figure 5C). We hypothesized that METTL1 might be related to immune cell infiltration and the response to immunotherapy since T cell migration and the inflammatory response are crucial for anti-tumor immunity. Then, we analyzed the association of METTL1 with B-cell, CD4+ T-cell, and CD8+ T-cell infiltration based on the TCGA SKCM cohort data. Interestingly, we found that the higher the METTL1 expression, the lower the degree of CD4+ T cell and CD8+ T cell infiltration in SKCM patients (Figure 5D). To confirm this phenomenon, we performed qRT-PCR experiments using SKCM samples. We found that the expression of METTL1 was not significantly correlated with the expression of CD4, while it was significantly negatively correlated with the expression of CD8A, a marker for CD8⁺ T cells (Figure 5E). This evidence suggested that

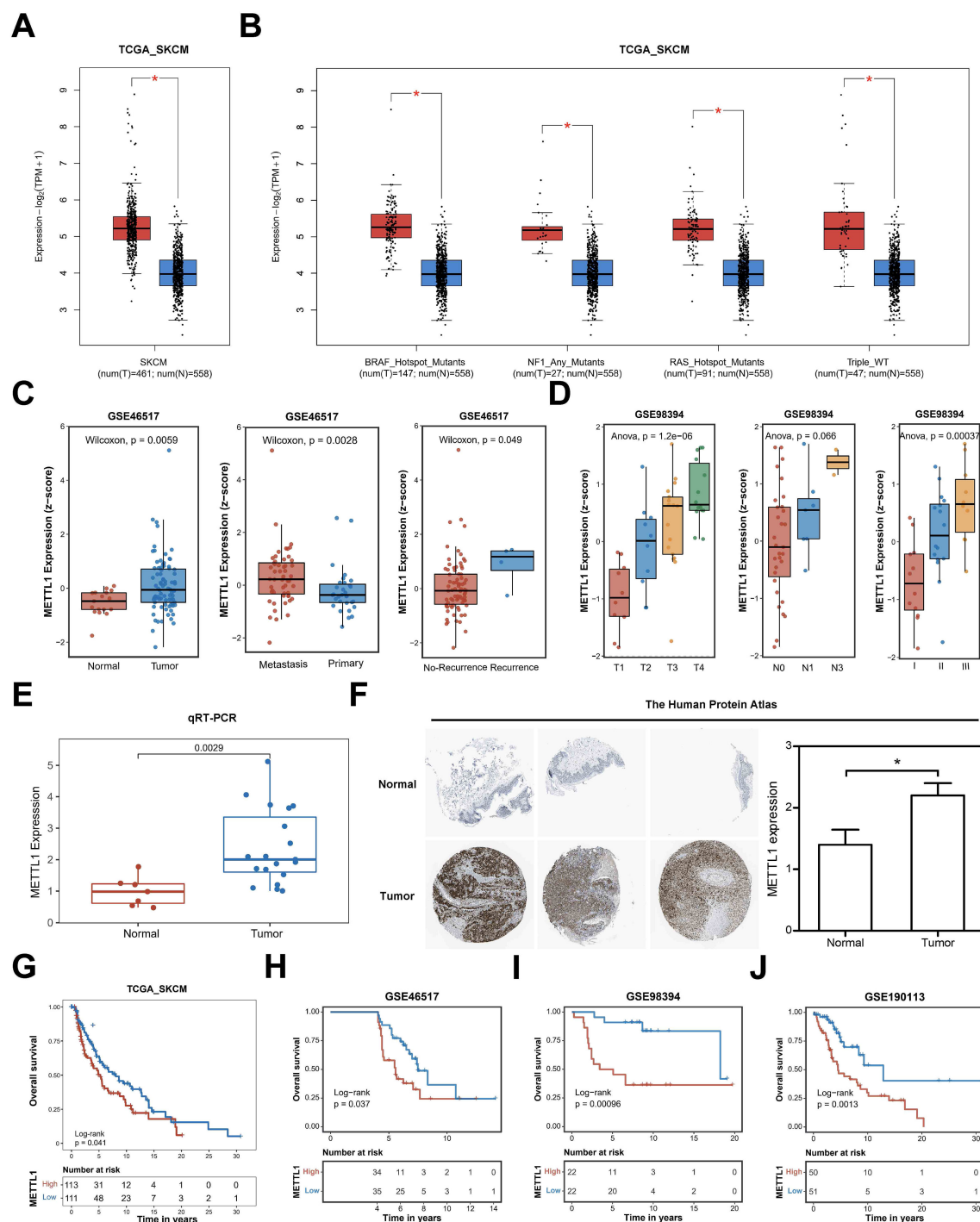
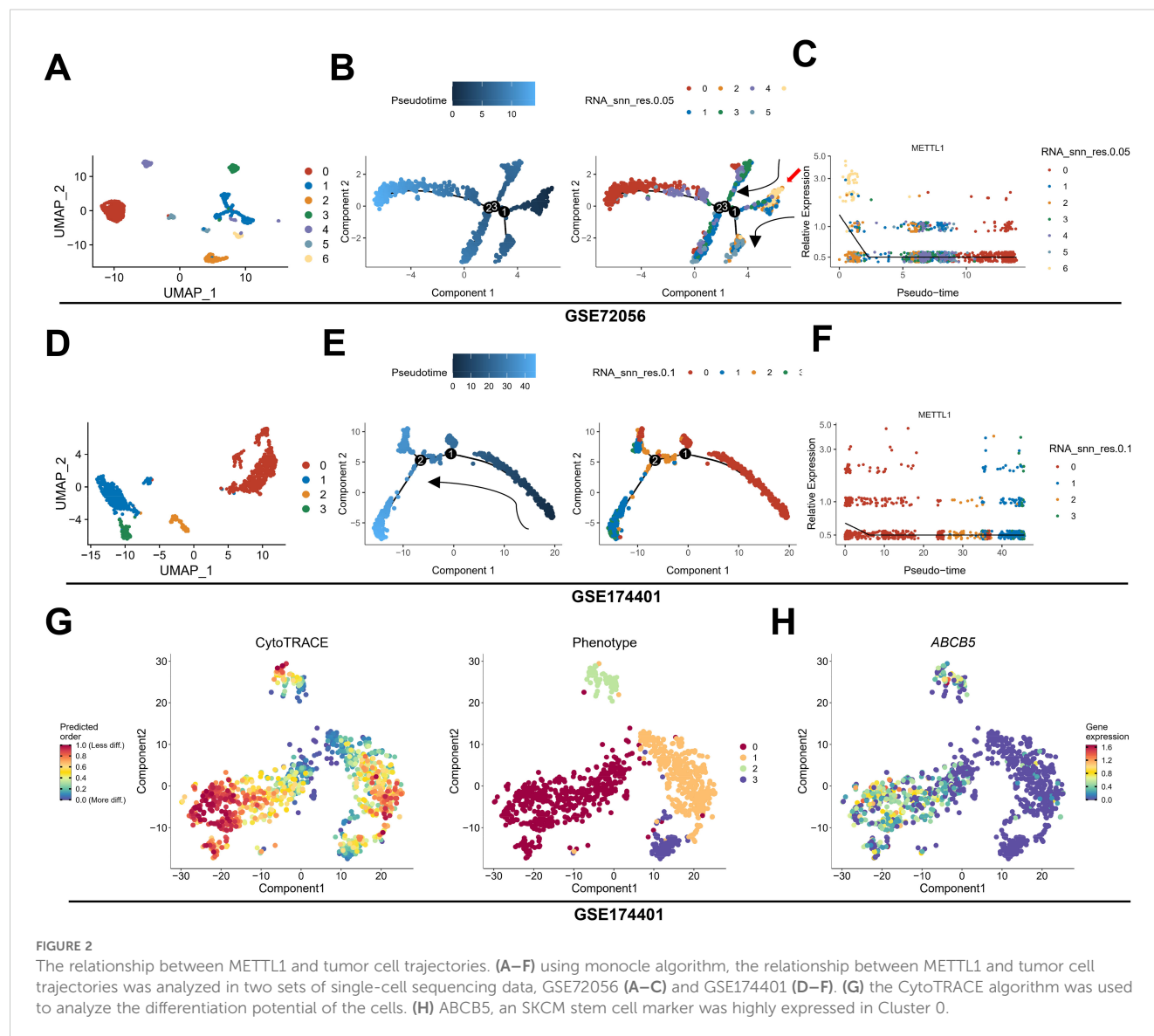


FIGURE 1

The association of high expression of METTL1 with clinicopathologic features of the SKCM patients. (A), the GEPIA2 online database analyzed the expression of METTL1 in the TCGA SKCM cohort. (B) Differential analysis of SKCM genomic variants according to their type into four subclasses, BRAF mutated, NF1 mutated, RAS mutated, and wild-type without any of the three mutations. (C) METTL1 expression was analyzed in GSE46517, another independent SKCM cohort dataset, including metastasis, recurrence tumor, primary, and no-recurrence tumor. (D) METTL1 expression was analyzed in another independent SKCM dataset GSE98394 with T grade, N grade, and clinical stage. (E) the mRNA level of METTL1 was validated in SKCM tissues (N=19) and normal tissues (N=7) by qRT-PCR. (F) Immunohistochemical staining of METTL1 in melanoma and normal tissues (N=5) from The Human Protein Atlas (METTL1 antibody, 1:400, cat no. HPA020914, Sigma-Aldrich) and quantification. (G) Survival analysis showed the overall survival of patients with high or low METTL1 expression in the TCGA_SKCM cohort. (H–J) Survival analysis showed the overall survival of patients with high or low METTL1 expression in the three other SKCM cohorts, GSE46517, GSE98394, GSE190113. * $P < 0.05$.



METTL1 might reduce the chance of cytotoxic T cells killing the tumor by inhibiting the infiltration of CD8⁺ T into the tumor, thereby promoting tumor progression.

Current immune checkpoint therapies for antitumor immunotherapy are precisely those targeting CD8⁺ T cells, and thus we hypothesized that the expression level of METTL1 might be correlated with patients' response to immune checkpoint therapy. Using the BEST data included a cohort analysis on immune checkpoint therapy, we found that METTL1 expression serves as a reliable predictor for response to anti-PD1 treatment (Figure 5F). Furthermore, utilizing the Kaplan-Meier Plotter online analysis tool to analyze data from a cohort of SKCM patients treated with anti-PD1 revealed that those with high levels of METTL1 had significantly worse prognosis (Figure 5G). These findings suggest that METTL1 may be a pan-cancer immunotherapeutic response marker, and marker studies targeting METTL1 with expanded sample size and tumor type are important for monitoring the clinical efficacy of anti-PD1 immunotherapy.

High expression of METTL1 may be driven by copy number amplification

Copy number variation directly affects the expression level of the genes it covers, and there are a large number of copy number variation events in SKCM. Therefore, we conjectured that the high expression of METTL1 is probably driven by an increase in its genomic copy number amplification. Based on this, we analyzed the genome sequencing data of several SKCM cohorts included in the cBioportal database. We found that METTL1 was amplified to varying degrees in all five cohorts (Figure 6A), and the mRNA level of METTL1 increased with the increase in the degree of METTL1 genomic copy number amplification (Figure 6B), and the copy number value of METTL1 was also significantly and positively correlated with the mRNA level of METTL1 (Figure 6C), which strongly suggests that the copy number amplification of METTL1 is what drives its expression. In addition, more the copy number value of METTL1 was altered in high SKCM stage (Figures 6D, E).

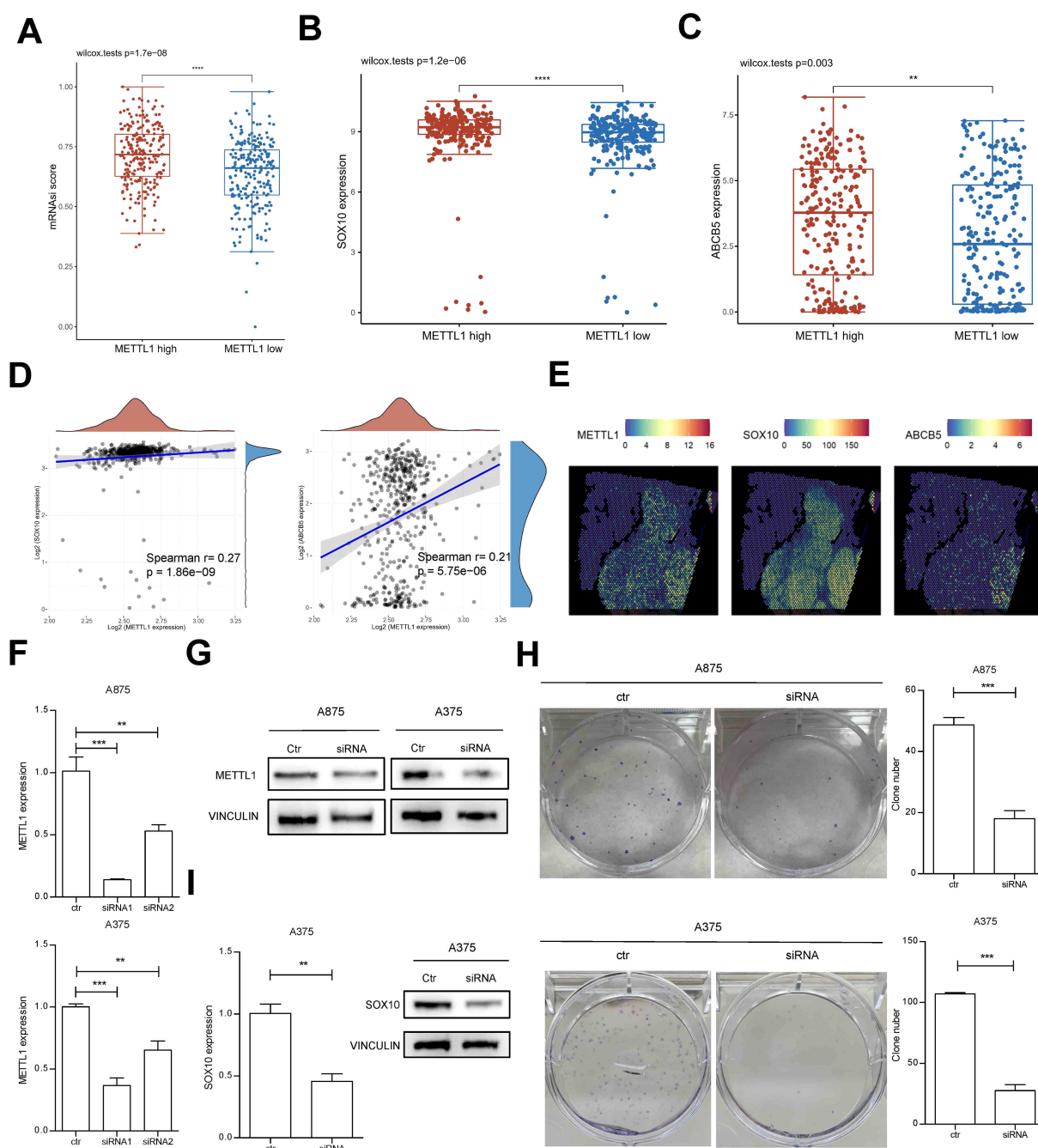


FIGURE 3

the relationship between METTL1 expression and cell stemness. **(A)** The ASSISTANT for Clinical Bioinformatics tool was used to analyze the relationship between METTL1 expression and cell stemness mRNAi score. **(B, C)** The SKCM stem cell marker genes SOX10 and ABCB5 were highly expressed in high METTL1 group. **(D)** METTL1 expression was significantly and positively correlated with SOX10 and ABCB5. **(E)** spatial transcriptome data was used to analyze the co-localization of METTL1 and stem cell marker genes SOX10 and ABCB5. **(F, G)** A875 and A375 SKCM cells were transfected with METTL1 siRNA and qPCR and western blot were performed to detect METTL1 expression. **(H)** the clone formation assay was performed to evaluate the functions of METTL1 on clone formation ability. **(I)** qPCR and western blot were performed to detect SOX10 expression after METTL1 knockdown. ** $P < 0.01$, *** $P < 0.001$, ns, no significant.

Previous work has identified elevated tumor aneuploidy as a marker of low overall survival and can be used as a biomarker for clinical outcomes of immunotherapy (12), and we found that the copy number value of METTL1 altered samples had higher aneuploidy

score (Figure 6F). In addition, We found that patients with altered METTL1 copy numbers also had worse overall survival (Figure 6G), suggesting that METTL1 be used as a prognostic indication for SKCM.

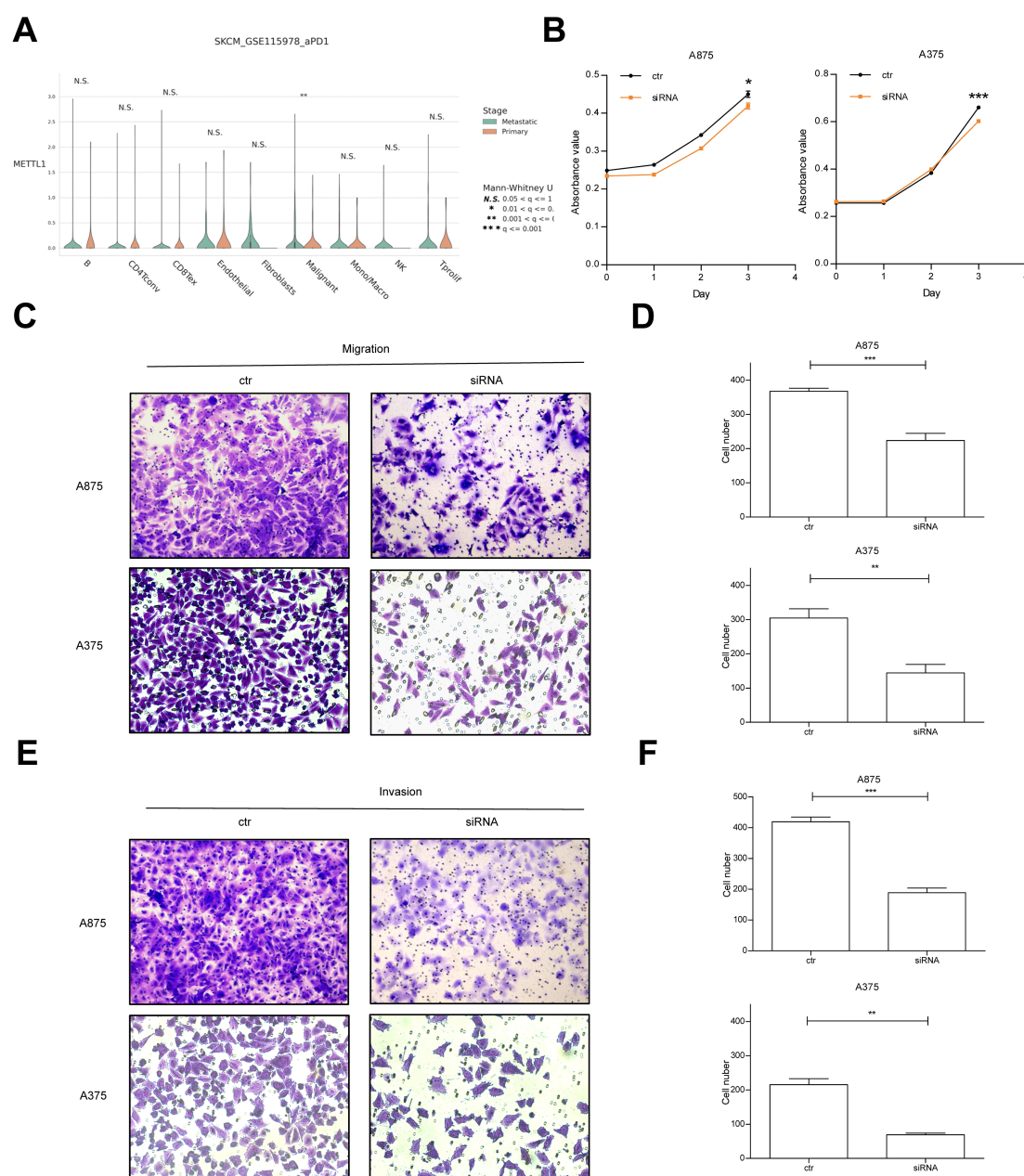


FIGURE 4

Knockdown of METTL1 inhibits migration and invasion of SKCM cells. **(A)** TISCH database was used to analyze the METTL1 expression in various cells of primary and metastatic tumor. **(B)** CCK8 assay was performed to evaluate the cell viability of A875 and A375 cells after METTL1 knockdown. **(C, D)** Transwell assay was performed to evaluate the cell migration of A875 and A375 cells after METTL1 knockdown. **(E, F)** Transwell assay was performed to evaluate the cell invasion of A875 and A375 cells after METTL1 knockdown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

MYC was a potential transcription factor of METTL1 in SKCM

Disturbance in transcriptional regulatory networks is a common feature of multiple tumors, and activation of oncogenic transcription factors leads to a wide range of downstream effects. We predicted that METTL1 should also be activated by some specific transcription factors. To explore the transcriptional regulatory mechanism of METTL1, we downloaded the single-cell sequencing data of GSE72056, a group of SKCM, from the TISCH

online database for analysis. After cell type annotation using this tool (Figures 7A, B), transcriptional regulator analysis was performed on the subpopulations that specifically highly expressed METTL1, and we found that MYC transcriptional regulators were significantly enriched (Figure 7C), MYC transcriptional regulators are also expressed at higher levels in this cell subpopulation (Figure 7D, Supplementary Figure S4). Therefore, we hypothesized that MYC may be the most important transcription factor for METTL1. Further analysis of the TCGA SKCM cohort data and SKCM spatial transcriptome data

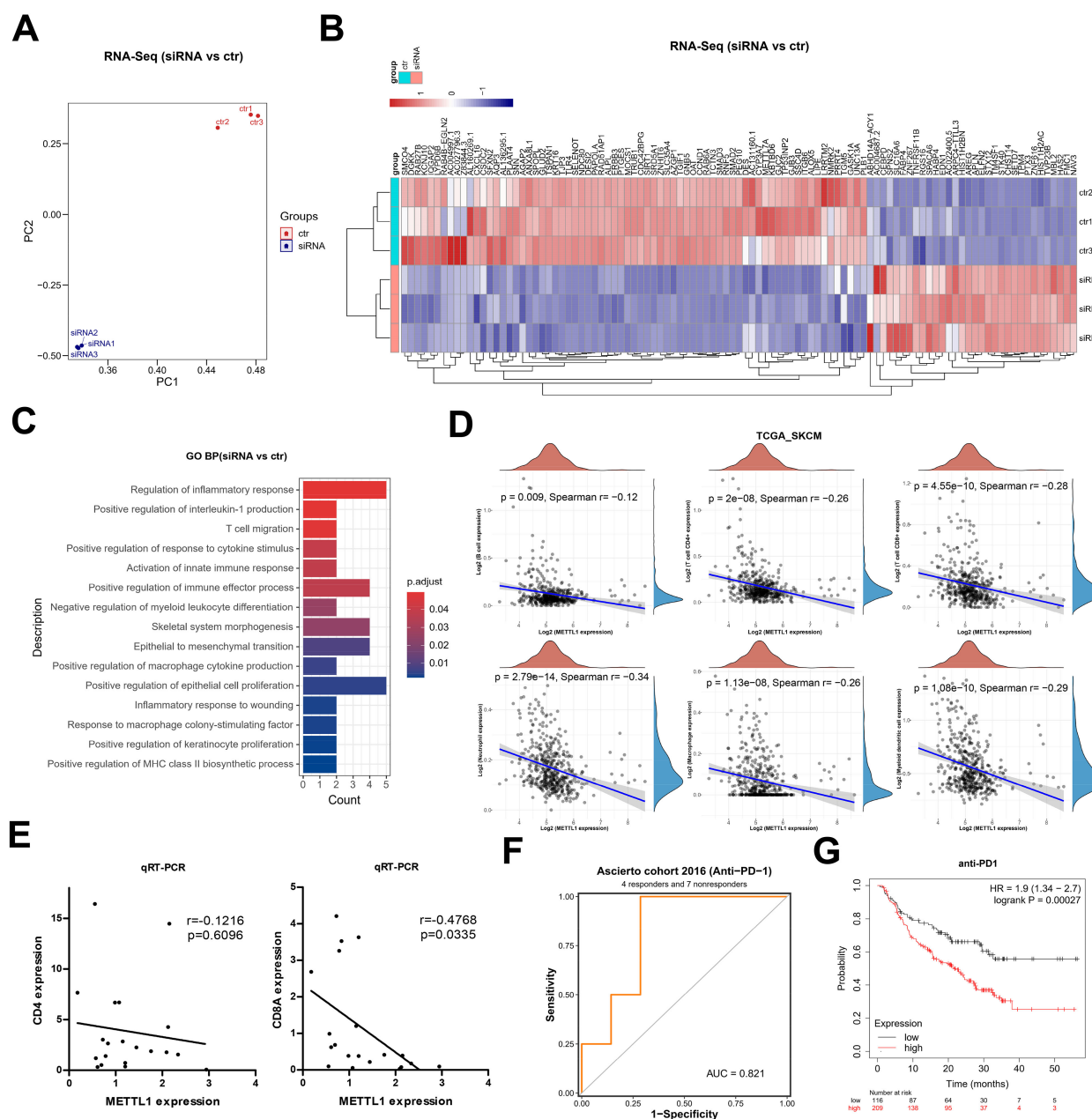


FIGURE 5

METTL1 as a marker of immunotherapy response in SKCM. (A) Principal component analysis of RNA-seq after silencing METTL1. (B) Heatmap showed the 103 differentially expressed genes, including 32 up-regulated genes and 71 down-regulated genes. (C) GO enriched analysis for these differentially expressed genes. (D) The association of METTL1 with B-cell, CD4+ T-cell, and CD8+ T-cell infiltration was analyzed based on the TCGA SKCM cohort data. (E) qRT-PCR experiments were performed using SKCM samples to detect the expression of CD4 and CD8A, the relationship of the expression of CD4 and CD8A, and METTL1 was analyzed. (F) the BEST data included a cohort analysis on immune checkpoint therapy was used to analyze the association of SKCM patients and response to anti-PD1 treatment. (G) the Kaplan-Meier Plotter online analysis tool was used to analyze the survival of SKCM patients treated with anti-PD1 with high or low levels of METTL1.

revealed that the expression levels of MYC and METTL1 were significantly positively correlated (Figures 7E, F), consistent with the regulatory relationship between transcription factors and target genes. Analysis of Chip-seq data from human epidermal keratinocytes also showed that MYC peak was significantly enriched near the METTL1 promoter (Figure 7G). These data suggest that MYC may be a potential transcription factor of METTL1.

Discussion

RNA modification is a type of epigenetic modification that can play an important role in regulating biological processes and tumor pathology by enhancing the stability and expression of oncogenic transcripts (13). The RNA modifications include N6-methyladenosine (m6A) modification, N1-methyladenosine (m1A) modification, 5-methylcytosine (m5C) modification, 7-methylguanosine (m7G)

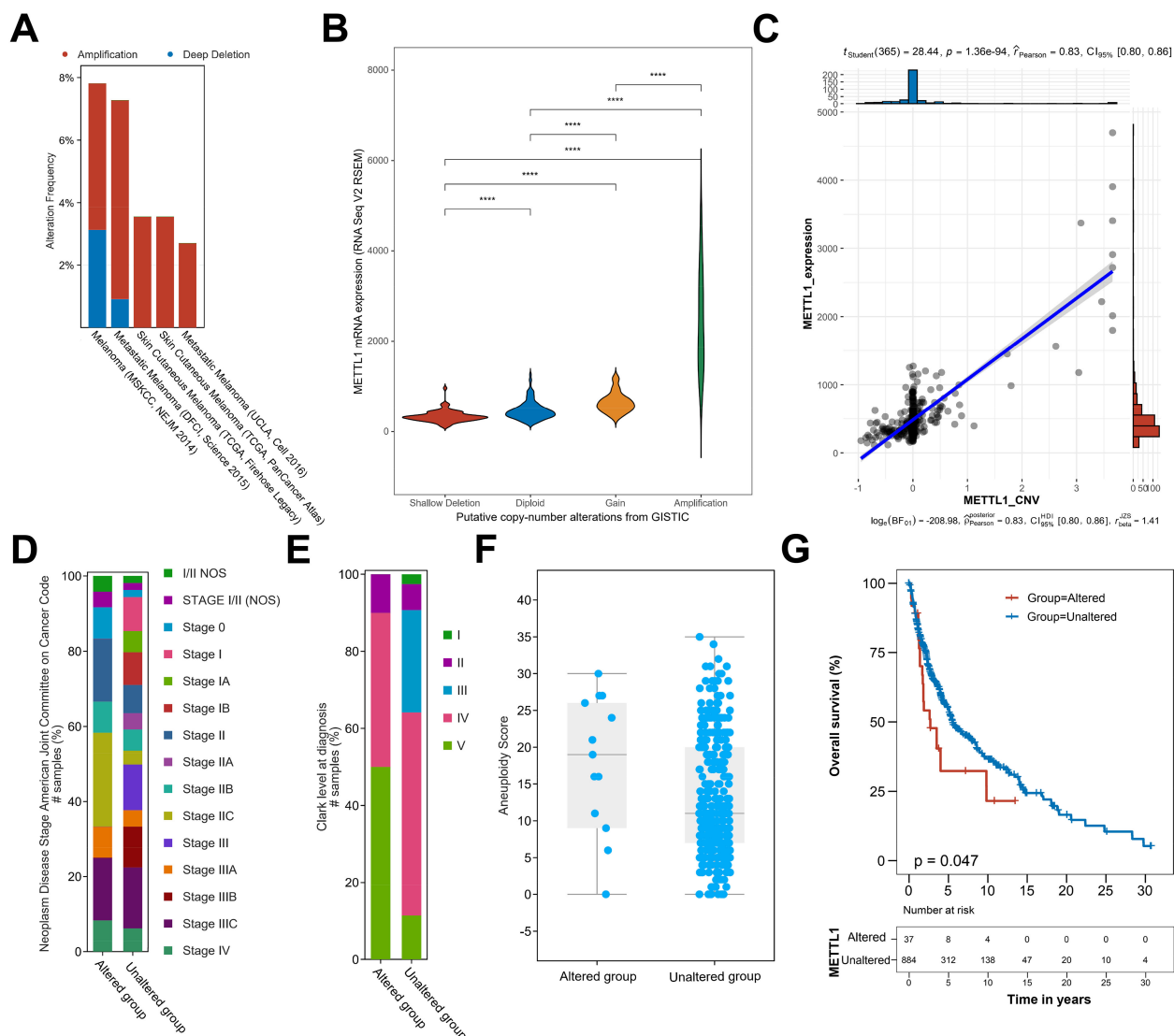
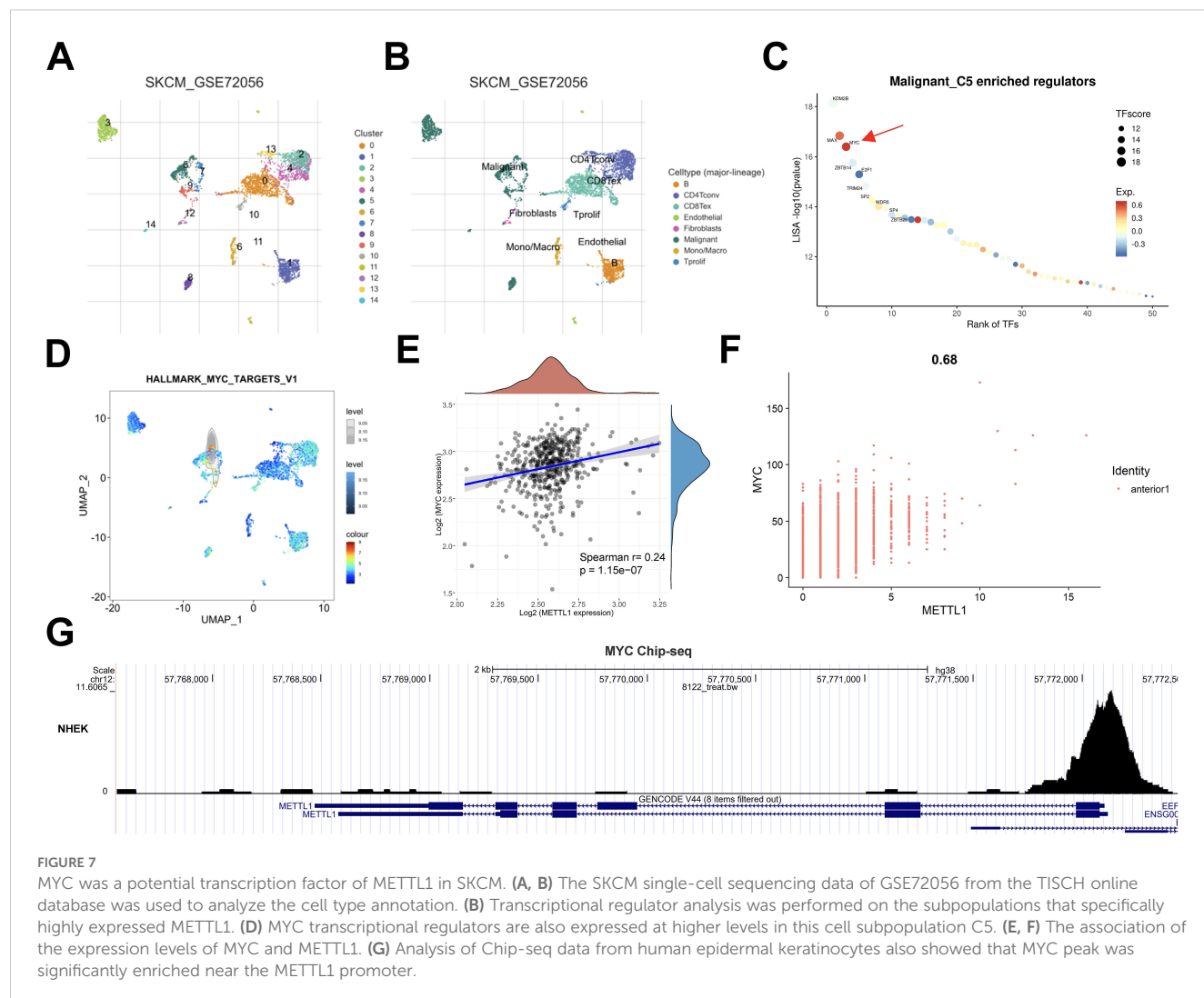


FIGURE 6

Copy number amplification induces METTL1 expression. (A) The genome sequencing data of SKCM cohorts included in the cBioportal database was used to analyze the copy number amplification of METTL1. (B) The association of genomic copy number amplification and the mRNA level of METTL1. (C) The association of the copy number value of METTL1 and the mRNA level of METTL1. (D, E) The percentage of samples with various stage in altered or unaltered METTL1 copy number group. (F) The aneuploidy score in altered or unaltered METTL1 copy number group. (G) The overall survival in altered or unaltered METTL1 copy numbers group. **** $P < 0.00001$.

modification, etc. (14) RNA modification can be mediated by methyltransferases, such as METTL1, METTL3, METTL16, and demethylating enzymes, such as FTO, ALKBH5. RNA modifications can be catalyzed, erased and recognized by methyltransferases such as METTL1, METTL3, METTL16, and accurately regulate the process of methylation, which plays an important role in the proliferation, metastasis, invasion, apoptosis, autophagy, and drug-resistance of tumor cells (15). m7G modification has been found firstly in the initial site of mRNA, and then in rRNAs and tRNAs (16). The m7G modification is a common 5'-modification of mRNAs and an internal modification of various non-coding RNAs. m7G modification is mediated in tRNAs by the METTL1 and WD repeat domain 4 (WDR4) complex, which is significantly involved in various tumorigenesis (6).

METTL1 is often aberrantly expressed and catalyzes m7G modification in tRNAs or miRNAs, which ultimately affects the expression of target genes and regulates tumor-related biological functions (17). It has been shown that the oncogenic function of METTL1 can promote tumor cell proliferation and migration by inhibiting the PTEN-related signaling pathway, and that inhibition of METTL1/WDR4 activity reduces m7G tRNA modification and slows down the progression of hepatocellular carcinoma (18). In lung adenocarcinoma and squamous carcinoma, the expression levels of METTL1 and WDR4 were significantly elevated compared with those in normal lung tissues, and were closely associated with poor prognosis of lung cancer patients (19). In this study, data mining of public databases revealed that METTL1 was abnormally highly expressed in SKCM and was a poor



prognostic factor. The high expression of METTL1 may be associated with abnormal copy number amplification and positive MYC regulation.

We further analyzed and found that METTL1 was co-expressed with stem cell markers in SKCM with positive correlation; knockdown of METTL1 significantly inhibited the clone formation ability of SKCM, suggesting that METTL1 has the function of regulating SKCM stem cells. METTL1 has been shown to have an important role in embryonic stem cell self-differentiation and neural differentiation (20, 21). METTL1 silencing leads to alteration of the entire m7G profile in human induced pluripotent stem cells (hiPSCs) and reduces the translation efficiency of stem cell marker genes. hiPSCs with METTL1-knockdown exhibited reduced totipotency and a slower cell cycle (22). In addition, METTL1 silencing accelerated the differentiation of hiPSC to the embryoid body and promoted the expression of mesoderm-related genes. Similarly, METTL1 knockdown enhanced teratoma formation and mesodermal differentiation *in vivo* by promoting cell proliferation and angiogenesis in nude mice (22).

These results suggest that METTL1 plays an important role in the malignant phenotype of SKCM stem cell-like tumor cells.

By mining the RNA-sequencing data after interfering with METTL1 as well as public data, we found that METTL1 functions as a marker of immunotherapy response in SKCM. Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) were enriched in advanced intrahepatic cholangiocarcinoma and significantly correlated with METTL1 (23). Zeng et al. found that liver-specific overexpression or knockdown of METTL1 significantly affected the accumulation of PMN-MDSCs and subsequently affected CD8 + T cell infiltration (24). It was found that the lower infiltrating levels of CD8+ T cells was found in clinical adrenocortical carcinoma samples with high METTL1 expression (25). In this study, we found that the expression of METTL1 was significantly negatively correlated with the expression of CD8A, a marker for CD8+ T cells. These findings suggested that METTL1 might reduce the chance of cytotoxic T cells killing the tumor by inhibiting the infiltration of CD8+T into the tumor, thereby promoting tumor progression. In addition, METTL1 expression serves as a reliable predictor for response to anti-PD1 treatment. Gao

et al. found that tumor cell lines with higher METTL1 expression were more sensitive to drugs targeting chromatin histone methylation, ERK-MAPK and WNT signaling pathways (26). *CXCL8* in human and *Cxcl5* in mouse are key translational targets of METTL1 that facilitate its function in promoting PMN-MDSC accumulation in tumor immune microenvironment of intrahepatic cholangiocarcinoma. Co-blockade of METTL1 and its downstream chemokine pathway enhances the anti-PD-1 efficacy in ICC preclinical mouse models (23). METTL1 mediates m⁷G methylation of PKM mRNA and enhances the expression of its encoded PKM2, while increased PKM2 dimer expression and nuclear translocation activated CD155 expression and induced colorectal cancer immune evasion (27). RNA methylation contributes to revealing the underlying mechanisms of many aspects of tumors, involving initiation, development, invasion, infiltration, and so on. The excessive m⁷G modification of certain genes leads to the acceleration of tumor development. METTL1-mediated m⁷G acts on different RNA targets, affecting the processes of tumorigenesis and immune response. These findings suggest that METTL1 may be a pan-cancer immunotherapeutic response marker, and marker studies targeting METTL1 with expanded sample size and tumor type are important for monitoring the clinical efficacy of anti-PD1 immunotherapy.

Our study relies heavily on data mining of publicly available databases. Although we preliminarily confirmed the oncogenic function of METTL1 in SKCM through cell biology experiments, the results inevitably exhibit some bias due to the small sample size and heterogeneity among samples. We hypothesized through public databases that METTL1 is positively regulated by MYC, but this hypothesis has not been supported by experimental data and requires further clarification. Additionally, we found that SKCM patients with high METTL1 expression had a worse prognosis after anti-PD1 immunotherapy. In light of the existing report that co-blockade of METTL1 and its downstream chemokine pathway enhances the anti-PD-1 efficacy in ICC preclinical mouse models (23). Thus, our inference is highly likely to be reliable, but still requires further confirmation through *in vivo* experiments. The specific mechanisms downstream of METTL1 also need to be validated through single-cell multi-omics analysis of human tissue samples and preclinical animal models.

In conclusion

The expression of METTL1 was markedly up-regulated in SKCM, and high expression of METTL1 was linked to poor prognosis for SKCM patients, which could serve as an independent prognostic indicator of METTL1. In addition, METTL1 promotes the malignant phenotypes of proliferation, migration, and invasion in SKCM, and may also impede the infiltration of CD8⁺ T cells into the interior of the tumor by enhancing the communication between tumor cells and fibroblasts and thus forming a physical barrier. Most interestingly, SKCM patients with high METTL1 expression had a worse prognosis after anti-PD1 immunotherapy; hence, it may be a potential biomarker for anti-PD1 immunotherapy in SKCM patients.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: TCGA: TCGA-SKCM; GEO: GSE46517, GSE98394, GSE190113, GSE72056, GSE174401 and GSE115978. cBioPortal: Metastatic Melanoma (UCLA, Cell 2016), Skin Cutaneous Melanoma (TCGA, Firehose Legacy), Skin Cutaneous Melanoma (TCGA, PanCancer Atlas), Melanoma (MSK, NEJM 2014), Metastatic Melanoma (DFCI, Science 2015).

Author contributions

LX: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. PY: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research and/or publication of this article.

Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1575219/full#supplementary-material>

References

- Cheng W, Gao A, Lin H, Zhang W. Novel roles of METTL1/WD4 in tumor via m(7)G methylation. *Mol Ther Oncolytics*. (2022) 26:27–34. doi: 10.1016/j.omto.2022.05.009
- Qi YN, Liu Z, Hong LL, Li P, Ling ZQ. Methyltransferase-like proteins in cancer biology and potential therapeutic targeting. *J Hematol Oncol*. (2023) 16:89. doi: 10.1186/s13045-023-01477-7
- Cai M, Yang C, Wang Z. N7-methylguanosine modification: from regulatory roles to therapeutic implications in cancer. *Am J Cancer Res*. (2023) 13:1640–55.
- Luo Y, Yao Y, Wu P, Zi X, Sun N, He J. The potential role of N(7)-methylguanosine (m7G) in cancer. *J Hematol Oncol*. (2022) 15:63. doi: 10.1186/s13045-022-01285-5
- Han M, Huang Q, Li X, Chen X, Zhu H, Pan Y, et al. M7G-related tumor immunity: novel insights of RNA modification and potential therapeutic targets. *Int J Biol Sci*. (2024) 20:1238–55. doi: 10.7150/ijbs.90382
- Du D, He J, Ju C, Wang C, Li H, He F, et al. When N(7)-methyladenosine modification meets cancer: Emerging frontiers and promising therapeutic opportunities. *Cancer Lett*. (2023) 562:216165. doi: 10.1016/j.canlet.2023.216165
- Ying X, Liu B, Yuan Z, Huang Y, Chen C, Jiang X, et al. METTL1-m(7) G-EGFR/EFEMP1 axis promotes the bladder cancer development. *Clin Transl Med*. (2021) 11: e675. doi: 10.1002/ctm2.v11.12
- Garcia-Vilchez R, Anazco-Guenkova AM, Dietmann S, Lopez J, Moron-Calvente V, D'Ambrosi S, et al. METTL1 promotes tumorigenesis through tRNA-derived fragment biogenesis in prostate cancer. *Mol Cancer*. (2023) 22:119. doi: 10.1186/s12943-023-01809-8
- Orellana EA, Liu Q, Yankova E, Pirouz M, De Braekeleer E, Zhang W, et al. METTL1-mediated m(7)G modification of Arg-TCT tRNA drives oncogenic transformation. *Mol Cell*. (2021) 81:3323–38.e14. doi: 10.1016/j.molcel.2021.06.031
- Han H, Yang C, Ma J, Zhang S, Zheng S, Ling R, et al. N(7)-methylguanosine tRNA modification promotes esophageal squamous cell carcinoma tumorigenesis via the RPTOR/ULK1/autophagy axis. *Nat Commun*. (2022) 13:1478. doi: 10.1038/s41467-022-29125-7
- Tang Z, Kang B, Li C, Chen T, Zhang Z. GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. *Nucleic Acids Res*. (2019) 47: W556–W60. doi: 10.1093/nar/gkz430
- Spurr LF, Weichselbaum RR, Pitroda SP. Tumor aneuploidy predicts survival following immunotherapy across multiple cancers. *Nat Genet*. (2022) 54:1782–5. doi: 10.1038/s41588-022-01235-4
- Tang Q, Li L, Wang Y, Wu P, Hou X, Ouyang J, et al. RNA modifications in cancer. *Br J Cancer*. (2023) 129:204–21. doi: 10.1038/s41416-023-02275-1
- Delaunay S, Helm M, Frye M. RNA modifications in physiology and disease: towards clinical applications. *Nat Rev Genet*. (2024) 25:104–22. doi: 10.1038/s41576-023-00645-2
- Liu Y, Zhu J, Ding L. Involvement of RNA methylation modification patterns mediated by m7G, m6A, m5C and m1A regulators in immune microenvironment regulation of Sjogren's syndrome. *Cell Signal*. (2023) 106:110650. doi: 10.1016/j.cellsig.2023.110650
- Zhao Z, Qing Y, Dong L, Han L, Wu D, Li Y, et al. QKI shuttles internal m(7) G-modified transcripts into stress granules and modulates mRNA metabolism. *Cell*. (2023) 186:3208–26.e27. doi: 10.1016/j.cell.2023.05.047
- Huang M, Long J, Yao Z, Zhao Y, Zhao Y, Liao J, et al. METTL1-Mediated m7G tRNA Modification Promotes Lenvatinib Resistance in Hepatocellular Carcinoma. *Cancer Res*. (2023) 83:89–102. doi: 10.1158/0008-5472.CAN-22-0963
- Tian QH, Zhang MF, Zeng JS, Luo RG, Wen Y, Chen J, et al. METTL1 overexpression is correlated with poor prognosis and promotes hepatocellular carcinoma via PTEN. *J Mol Med (Berl)*. (2019) 97:1535–45. doi: 10.1007/s00109-019-01830-9
- Ma J, Han H, Huang Y, Yang C, Zheng S, Cai T, et al. METTL1/WD4-mediated m(7)G tRNA modifications and m(7)G codon usage promote mRNA translation and lung cancer progression. *Mol Ther*. (2021) 29:3422–35. doi: 10.1016/j.jymthe.2021.08.005
- Lin S, Liu Q, Lelyveld VS, Choe J, Szostak JW, Gregory RI. Mettl1/Wdr4-Mediated m(7)G tRNA Methylome Is Required for Normal mRNA Translation and Embryonic Stem Cell Self-Renewal and Differentiation. *Mol Cell*. (2018) 71:244–55.e5. doi: 10.1016/j.molcel.2018.06.001
- Tooley JG, Catlin JP, Tooley CES. METTLing in stem cell and cancer biology. *Stem Cell Rev Rep*. (2023) 19:76–91. doi: 10.1007/s12015-022-10444-7
- Deng Y, Zhou Z, Ji W, Lin S, Wang M. METTL1-mediated m(7)G methylation maintains pluripotency in human stem cells and limits mesoderm differentiation and vascular development. *Stem Cell Res Ther*. (2020) 11:306. doi: 10.1186/s13287-020-01814-4
- Liu H, Zeng X, Ren X, Zhang Y, Huang M, Tan L, et al. Targeting tumour-intrinsic N(7)-methylguanosine tRNA modification inhibits MDSC recruitment and improves anti-PD-1 efficacy. *Gut*. (2023) 72:1555–67. doi: 10.1136/gutjnl-2022-327230
- Zeng X, Liao G, Li S, Liu H, Zhao X, Li S, et al. Eliminating METTL1-mediated accumulation of PMN-MDSCs prevents hepatocellular carcinoma recurrence after radiofrequency ablation. *Hepatology*. (2023) 77:1122–38. doi: 10.1002/hep.32585
- Xu F, Cai D, Liu S, He K, Chen J, Qu L, et al. N7-methylguanosine regulatory genes well represented by METTL1 define vastly different prognostic, immune and therapy landscapes in adrenocortical carcinoma. *Am J Cancer Res*. (2023) 13:538–68.
- Gao Z, Xu J, Zhang Z, Fan Y, Xue H, Guo X, et al. A comprehensive analysis of METTL1 to immunity and stemness in pan-cancer. *Front Immunol*. (2022) 13:795240. doi: 10.3389/fimmu.2022.795240
- Wang F, Yang C, Zheng F, Yan Y, Li G, Feng Y, et al. METTL1 mediates PKM m7G modification to regulate CD155 expression and promote immune evasion in colorectal cancer. *J Transl Med*. (2024) 22:1161. doi: 10.1186/s12967-024-05991-1



OPEN ACCESS

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RECEIVED 07 February 2025

ACCEPTED 09 June 2025

PUBLISHED 07 July 2025

CITATION

Song P, Ye J, Zhang H, Li Y, Cao R, Feng Y, Zhang L and Sun M (2025) Cross-talk of m⁶A methylation modification and the tumor microenvironment composition in esophageal cancer. *Front. Immunol.* 16:1572810. doi: 10.3389/fimmu.2025.1572810

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Cross-talk of m⁶A methylation modification and the tumor microenvironment composition in esophageal cancer

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Background: Esophageal cancer (EC) remains a significant clinical challenge, characterized by its aggressive nature and poor prognosis. Current therapeutic strategies, including targeted therapies, have limitations due to the complex interplay between tumor heterogeneity and the tumor microenvironment (TME). However, the specific contributions of N6-methyladenosine (m⁶A) methylation to the TME in EC are yet to be fully elucidated.

Methods: Through comprehensive bioinformatics analyses, a detailed examination of m⁶A regulators were conducted in EC using datasets from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). Single-cell RNA sequencing (scRNA-seq) and a consensus clustering algorithm was employed to classify m⁶A modification patterns and analyze their relationships with immune cell infiltration and clinical outcomes. Additionally, an m⁶A scoring system was developed based on principal component analysis to assess the prognostic value of identified m⁶A modification patterns.

Results: The findings revealed two distinct m⁶A modification clusters associated with divergent TME characteristics and immune infiltration profiles. Patients exhibiting the immune-inflamed phenotype (m⁶A cluster B) demonstrated significantly improved survival compared to those with the immune-excluded phenotype (m⁶A cluster A). Notably, m⁶A scores correlated positively with immune cell presence and related with adverse prognostic outcomes, indicating their potential as predictive biomarkers for immunotherapy responses. A low m⁶A score indicated a better response to immunotherapy.

Conclusion: This study highlights the critical role of m⁶A methylation in shaping the TME and influencing immune dynamics in EC. The m⁶A score developed

herein provides a novel quantitative tool for predicting tumor behavior and treatment efficacy, paving the way for more personalized immunotherapeutic strategies in clinical practice. This scoring system illustrates a strong correlation of EC with TME immune cell composition, suggesting potential as a biomarker for targeted therapeutic strategies for EC.

KEYWORDS

esophagus cancer, m⁶A, tumor microenvironment, immunotherapy, prognosis, immune infiltration

1 Introduction

N⁶-methyladenosine (m⁶A) has been recognized as a crucial RNA modification. m⁶A modifications are dynamically regulated by various regulators, including methyltransferase complex writers such as METTL3, METTL14, METTL16, RBM15, RBM15B, VIRMA, WTAP and ZC3H13, and several binding proteins, such as FMR1, HNRNPC, HNRNPA2B1, IGFBP1/2/3, LRPPRC, RBMX, YTHDC1/2, and YTHDF1/2/3, have been identified as readers, as well as demethylases erasers such as FTO and ALKBH5. Numerous studies have demonstrated that aberrant expression of m⁶A core modification and reading proteins is implicated in diverse physiological and pathological processes, including biological growth and development, DNA damage repair, biological rhythms, angiogenesis, and various types of tumors (1). In recent years, substantial progress has been achieved in m⁶A epitranscriptomics, underscoring its pivotal roles in cancer initiation and progression by modulating RNA stability, mRNA splicing, microRNA processing, and mRNA translation (2). Unlike genetic events, m⁶A modifications are reversible, making epigenetic regulation particularly interesting for the development of new therapeutic technologies for cancer treatment.

Esophageal cancer has the sixth highest cancer-related mortality rate, but research data on this disease are limited compared to other cancers (3–5). Esophageal cancer is characterized by its aggressive nature and dismal 5-year survival rate, which stands at only 18% (6). Recent advances in the identification of molecular markers specific to

esophageal cancer have led to the development of novel targeted therapy approaches by targeting these markers (7–12). However, inhibitors have the potential to cause primary or acquired resistance in patients who receive these treatments (13–16). Furthermore, in esophageal cancer, a diverse array soluble immunosuppressive factors and cells with immunosuppressive properties can interfere with immune effector cells, thereby creating a distinct immunosuppressive microenvironment.

Multiple factors influence the outcome of multi-modality treatments. An individual tumor's intrinsic features are crucial to shaping its immune microenvironment and affecting the effectiveness of immunotherapy in esophageal cancer (17). As our understanding of the tumor microenvironment deepens, we increasingly recognize the significance of immune cell subsets in tumor development and the identification of potential therapeutic targets. The microenvironment in esophageal cancer is complex, comprising of various components such as NK cells, tumor-associated macrophages, dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), neutrophils, mast cells (MCs), eosinophils, endothelial cells, tumor angiogenesis, and cancer-associated fibroblasts (CAFs) (18, 19). Extensive exploration has been conducted on the utilization of clinical immunotherapy approaches that target innate immune cells as adjuvant therapies in conjunction with surgical resection and chemoradiotherapy for the treatment of diverse cancers. The strategies encompass the utilization of immune checkpoint inhibitors and Chimeric Antigen Receptor T-Cell Immunotherapy (20, 21). Analyzing the characteristics of cells within the tumor microenvironment to predict immune infiltration is crucial for exploring new immunization strategies and studying responses to existing immune checkpoint inhibitors (22, 23). Recent research has categorized the microenvironments of tumors in cancer patients into three fundamental immune profiles: tumors that are immune inflamed ("hot"), immune excluded, and immune desert ("cold"). These profiles suggest different treatment options, excluding esophageal cancer, and provide valuable insights for effective therapeutic interventions (24, 25). To conclude, a meticulous and all-encompassing examination of the esophageal cancer tumor microenvironment, coupled with the determination of the corresponding tumor immunophenotype, can prove to be a valuable approach in directing immunotherapy and forecasting its effectiveness (20, 21, 23).

Abbreviations: m⁶A, N⁶-methyladenosine; TME, tumor microenvironment; DCs, dendritic cells; MDSCs, myeloid-derived suppressor cells; MCs, mast cells; CAR, chimeric antigen receptor; BMDMs, bone marrow-derived macrophages; DNMTs, drugs targeting DNA methyltransferases; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; ESCA, esophageal cancer; TPM, transcripts per kilobase million; CNV, copy number variation; ssGSEA, Single-sample gene set enrichment analysis; DEGs, differentially expressed genes; MF, molecular function; BP, biological process; CC, cellular component; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; PCA, principal component analysis; ROC, receiver operating characteristic; TMB, tumor mutation burden; TIDE, Tumor Immune Dysfunction and Exclusion; ICI, immune checkpoint inhibitor; mUC, metastatic urothelial cancer; EMT, Epithelial–mesenchymal transition.

Several studies have substantiated the significant involvement of m⁶A modification in the development of tumor microenvironment (TME) diversity and complexity, a phenomenon that cannot be entirely elucidated by the RNA degradation mechanism (26). m⁶A modulators affected inflammation infiltrates and neovascularization of tumor tissues in human abdominal aortic aneurysm samples, where the markers METT14, FTO, and YTHDF3 are strongly colocalized with CD45+ leukocytes and CD3+ T cells, as well as CD68+ macrophages (27). Similarly, a METTL3/FTO-m⁶A methylation-mediated generation of M1/M2 macrophages from murine bone marrow-derived macrophages (BMDMs) has been described (28, 29). A new class of drugs targeting DNA methyltransferases (DNMTs) has been shown to successfully restore coordinated immune responses in solid tumors by triggering MHC 1 and interferon (IFN)-triggered immune-related signaling (30, 31). However, most studies, which are constrained by the state of technology, focus on just one or two m⁶A regulators, which is insufficient to describe the intricate functions of regulators in tumors. These research were made feasible by the ongoing development and collection of transcriptomics and genomic data, which offer a wealth of tools and resources for the study of m⁶A regulators and immune modulation (32).

In current study, we conducted a comprehensive analysis of m⁶A modifications and identified two distinct patterns of modifications, termed m⁶A clusters. These patterns were associated with different survival advantages and exhibited characteristics relevant to the TME, immune cell infiltration, and transcriptome (33). The observation that the TME characteristics linked with each m⁶A modification pattern closely corresponded with the manifestations and features of immune exclusion and immune inflammation, respectively, was of significant interest. This indicates a significant influence of m⁶A modification on individual tumor microenvironments (34, 35). Furthermore, a scoring system was devised to evaluate singular m⁶A modifications, facilitating the prediction of prognosis and the efficacy of immunosuppressive therapy. The strong correlation between m⁶A modifications and TME immune cell infiltration suggests that these modifications could serve as important prognostic markers and guide immunotherapy decisions in esophageal cancer.

2 Materials and methods

2.1 Esophageal cancer data acquisition and preprocessing

The Supplementary Figure S1 depicts the workflow employed in this study. The esophageal cancer samples' transcriptional and clinical feature data were obtained from Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) databases and The Cancer Genome Atlas (TCGA, 2022.12.01, <https://portal.gdc.cancer.gov/>). Two distinct cohorts of esophageal cancer (ESCA), namely TCGA-ESCA and GSE13898, were used for further analysis. The RNA-Seq data obtained from the TCGA cohort underwent additional processing, resulting in the conversion of the data into transcripts

per kilobase (TPM). Retrieve the normalization matrix file from the GEO database and employ R's SVA package to address batch effects across distinct datasets. Obtain the survival duration and survival outcome data of two cohorts, with the exclusion of samples with survival periods less than 31 days and incomplete survival information. The Cancer Genome Atlas database was utilized to obtain somatic mutations, and copy number variation data for esophageal cancer were obtained from the UCSC Xena database (<http://xena.ucsc.edu/>) (36).

2.2 Classification according to 23 m⁶A regulators

These regulators include eight writers (METTL3, METTL14, METTL16, RBM15, RBM15B, VIRMA, WTAP and ZC3H13), 13 readers (FMR1, HNRNPC, HNRNPA2B1, IGFBP1/2/3, LRPPRC, RBMX, YTHDC1/2 and YTHDF1/2/3), and two erasers (FTO and ALKBH5). These modulators have been reported to affect or modulate the performance of RNA (Supplementary Figure S2). The expression levels of these 23 m⁶A regulators were utilized for unsupervised clustering analysis to identify distinct subtypes of m⁶A methylation modifications and classify patients for further analysis. The consensus clustering technique, implemented with the R package ConsensusClusterPlus, was utilized to calculate the number of clusters and assess their stability (37, 38).

2.3 scRNA-seq data processing

We analyzed the dataset GSE196756 about Esophageal Squamous Cell Carcinoma (ESCC) cells from the GEO repository (39). The data were sourced from Homo sapiens, with the data platform being GPL24676. We picked specific ESCC samples (GSM5900215, GSM5900216, GSM5900217, GSM5900218, GSM5900219, GSM5900220) for analysis. The R package: "Seurat" was used to analyze the transcript count matrix for quality control and preliminary data exploration (40). The filtering threshold was set as follows: Excluding genes detected in less than 3 cells, excluding cells with < 200 genes detected, Excluding cells with > 20% mitochondrial gene expression. We addressed batch differences using LogNormalize, Harmony and Principal Component analysis (PCA) helped us cluster cells based on variable genes via Seurat's "FindNeighbors" and "FindClusters" functions. Uniform t-distributed Stochastic Neighbor Embedding (t-SNE) helped visualize this.

2.4 Estimation of immune infiltrating cells in TME

The R software package GSVA was utilized to conduct GSVA enrichment analysis to look at variations in m⁶A modification patterns in biological processes. The GSVA technique is a

nonparametric and unsupervised approach that is predominantly employed to assess alterations in the activity of biological processes and pathways within samples (41). The gene sets from “c2.cp.kegg.v7.2.symbols” were downloaded from the MSigDB database for performing GSVA analysis. The present study utilized Single Sample Gene Set Enrichment Analysis (ssGSEA) in the R software package GSVA to assess the infiltration rates of 24 immune cells across various m⁶A regulator clusters. The differences between different m⁶A regulator clusters were assessed using the Wilcox test, and survival analysis was conducted to examine their association with patient outcomes.

2.5 Gene expression differences among phenotypes modified with m⁶A

Using a consensus clustering algorithm, we were able to divide esophageal cancer patients into two distinct subtypes according to m⁶A regulator expression. We revealed that the relationship between the two m⁶A clusters and immune landscape by CIBERSORT, EPIC, MCPOUNTER, QUANTISEQ, TIMER, and XCELL algorithms (42). The differentially expressed genes (DEGs) between these two m⁶A-modified clusters were subsequently identified using the Limma package. The significance criterion for determining differential genes was set at a p-value < 0.05.

2.6 Differentially expressed genes enriched in functional pathways and functions

An important bioinformatics tool for gene annotation and analysis is the Gene Ontology (GO). It encompasses three categories: cellular component (CC), biological process (BP), and molecular function (MF). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database serves as an integrative platform for genomic, chemical, and system function data, enabling the correlation of gene catalogs with higher-level system functions across various levels, including the cell, species, and ecosystem. To annotate the DEGs and gain insights into their biological functions, we utilized the clusterProfiler package, a widely used R package for functional enrichment analysis. The clusterProfiler package offers convenient functions to perform GO and KEGG enrichment analyses. For the study to be meaningful, the p-value must be less than 0.05 and the q-value must be less than 0.05.

2.7 The construction of the m⁶A score

We created a scoring system for m⁶A based on PCA to measure the patterns of m⁶A change in specific esophageal cancer patients. Genes demonstrating significant prognostic effects were selected from the different m⁶A modification clusters, based on which clustering of samples and construction of m⁶A scores were performed using a univariate Cox regression model. We

determined the number of gene clusters and ensured stability using the consensus clustering algorithm. Marker scores for m⁶A-related genes were generated using PCA, and the first and second principal components were extracted as the marker scores. The method emphasizes the scores based on the collective behavior of highly correlated or inversely correlated genes within significant gene clusters, while minimizing the impact of genes that do not align with other members of the cluster. PCA is a dimensionality reduction method typically used to reduce the dimensionality of a dataset by transforming a large number of variables into fewer variables that still contain most of the information in the set (43, 44). We used the following method to define m⁶A scores: m⁶Ascore = $\Sigma(\text{PCA1}_i + \text{PCA2}_i)$, the variable “i” denotes the final gene expression linked to the m⁶A phenotype (45, 46).

2.8 Evaluate the m⁶A scoring model

To evaluate the clinical applicability and reliability of the m⁶A score, receiver operating characteristic (ROC) curves were utilized to predict the outcomes at 1 year, 3 years, and 5 years. Initially, the ROC curve was constructed using all samples, followed by a separate analysis focusing on the TCGA-ESCA cohort to compare the prognostic predictive performance of the m⁶A score against other clinical variables. Correlations between the m⁶A score, clinical variables, and prognosis were examined using both univariate and multivariate Cox regression analyses. The purpose of the study was to examine the potential of the m⁶A score as a standalone predictive marker for esophageal cancer. Significance at the p < 0.05 level is usually used to determine statistical significance in a forest plot diagram. Furthermore, a nomogram was constructed using eight indicators (age, gender, tumor grade, stage T, N, M, pathologic stage, and m⁶A score) to anticipate the patient's 1-, 3-, and 5-year survival rates. The predictive performance of the nomogram was evaluated using ROC curves. The R packages timeROC, rms, survival and survminer were employed for the necessary calculations and graphical representation.

2.9 Data research on genome mutations

The frequency of copy number variation (CNV) for the 23 m⁶A regulators in the TCGA-ESCA cohort was computed to assess the occurrence of copy number increases or losses. Copy number variation plots were generated using the R package Circos to visualize CNV patterns of m⁶A regulators of human chromosomes. The Tumor Mutation Burden (TMB) was computed by quantifying the aggregate count of nonsynonymous mutations present in the TCGA-ESCA cohort. The R package maftools was employed to create an oncoprint, which visually represents the gene mutation landscape. Using these approaches, the copy number variation map and oncoprint provide insights into the copy number alterations and mutation profiles of the m⁶A regulators in esophageal cancer based on the TCGA-ESCA cohort.

2.10 Tumor immune dysfunction and exclusion prediction and IC50 estimation

The Tumor Immune Dysfunction and Exclusion (TIDE) model, developed by researchers (47, 48), is used to evaluate the clinical efficacy of immune checkpoint inhibition therapy. The TIDE model provides prediction scores that reflect the likelihood of a patient's response to immune checkpoint inhibition. Higher TIDE prediction scores are associated with a poorer response to immune checkpoint inhibition therapy. This model helps clinicians and researchers assess the potential effectiveness of immune checkpoint inhibition in individual patients.

2.11 Collect critical information for ICI-based cohorts

A systematic search was conducted to identify publicly available gene expression profiles of patients undergoing immune checkpoint inhibitor (ICI) therapy. The search aimed to identify datasets that included detailed clinical and pathological information. Ultimately, three immunotherapeutic cohorts were included in our study: metastatic melanoma patients treated with nivolumab (anti-PD-1 monoclonal antibody) (49) or ipilimumab (anti-CTLA-4 monoclonal antibody) (50), and patients who have been diagnosed with metastatic urothelial carcinoma (mUC) and have received treatment with the anti-PD-L1-targeting drug Atezolizumab (51). The ESCA-specific immunotherapy-treated cohort GSE165252 was found (n=45 ESCA patients treated with anti-PD-1 monoclonal antibody Atezolizumab), which contains the binary information on immune therapy response (response and non-response groups). We curated gene expression profiles from pre-therapy biopsy samples and transformed them into TPM (Transcripts Per Million) format. These datasets provide valuable information for our study on the response to ICI therapy and associated gene expression patterns.

2.12 Sensitivity analysis of anticancer drugs

For the study of molecular therapies for cancer and gene mutations, relevant data from the Genomics of Cancer Drug Sensitivity (GDSC) database were downloaded (52). This database offers a valuable resource for studying drug sensitivity in various cancer types. We utilized the pRRophetic package to obtain cell line gene mutation data and IC50 values associated with various anticancer drugs from GDSC, allowing us to analyze the correlation between patients with high- and low-risk scores and their sensitivity to different anticancer drugs. Through this analysis, we were able to examine the correlation between patients exhibiting high- and low-risk m⁶A scores and their responsiveness to a diverse array of anticancer medications (53). By leveraging these resources, we aimed to gain insights into the association between m⁶A modification patterns and the response to specific anticancer therapies, further enhancing our understanding of personalized cancer treatment approaches.

2.13 Cell transfection and cell line establishment

Esophageal carcinoma cell lines KYSE510 and TE-1 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at a temperature of 37 degrees Celsius and under a 5% CO₂ atmosphere. In preparation for cell transfection, these cell lines were seeded into 6-well culture plates and incubated overnight to allow for attachment and initial growth. On the subsequent day, once the cells had reached a confluence of 20%-30%, transfection was performed with siRNAs at a final concentration of 50 nmol/L using Lipofectamine 2000 (Invitrogen), following the protocol provided by the manufacturer.

2.14 Explore and validate potential oncogenic functions of RBMX In ESCA

Supplemental Experimental Procedures include the following information: Western blot for protein expression, Plate clone formation assay, EdU assay for cell proliferation detection, Wound healing assay for assessing cellular migration, Transwell migration/invasion assay ([Supplementary Data Sheet S1: Supplemental Experimental Procedures](#)).

2.15 Statistical analyses

Statistical analyses and graphical representations were conducted using R version 4.3.1. The Wilcoxon rank sum test, a statistical method, is useful for assessing and contrasting dissimilarities between two groups. The correlation between m⁶A regulators and prognosis was assessed with univariate Cox regression models and Kaplan-Meier survival analysis. The selection of cutoff points for the m⁶A score was performed by repeatedly testing all possible cutoffs using the survminer package in R, aiming to identify the maximum rank statistic. Partition the samples into groups based on their m⁶A scores, with one group consisting of high scores and the other of low scores. Prognosis was assessed using the Kaplan-Meier method, and the log-rank test was assessed for both cohorts. At the same time, there are other statistical methods for targeted analysis. Heatmaps were generated using the pheatmap package in R. All statistical tests were two-tailed, and a p-value less than 0.05 was considered statistically significant.

3 Results

3.1 Mutation of m⁶A regulators, immune infiltration, and construction of the prognostic landscape

Our study included 23 m⁶A regulators. Firstly, we calculated the frequency of mutations in the 23 regulators in ESCA. The 23 m⁶A regulators exhibited low mutation frequencies, with only 23 (12.5%) out of 184 ESCA samples from the TCGA database showing genetic

alterations. Mutation information for each gene in each sample was presented in the waterfall plot, with different colors and specific annotations at the bottom representing the different mutation types. Interestingly, the Oncoplot analysis revealed that ZC3H13 displayed the highest mutation rate, predominantly characterized by nonsense mutations, while YTHDC2 had a mutation frequency of 2% (Figure 1A).

Moreover, our analysis of copy number variations (CNVs) in the 23 m⁶A regulators highlighted the prevalence of CNV mutations in ESCA. Notably, YTHDC1, FMR1, VIRMA, YTHDF1, METTL3, WTAP, HNRNPA2B1, RBMX, HNRNPC, and IGFBP1 exhibited a high frequency of CNV amplification, while HNRNPC, RBM15, YTHDF2, IGFBP2, and RBM15B displayed extensive CNV deletions (Figure 1B). The chromosomal alterations associated with these CNVs are visually depicted in Figure 1C. In order to evaluate the influence of m⁶A regulators on patient prognosis, a Kaplan-Meier survival analysis was conducted. The findings demonstrated significant correlations between the prognosis of ESCA patients and 8 m⁶A regulators (Supplementary Figure S3). Additionally, seventeen modifiers with prognostic value in ESCA patients were identified using univariate Cox regression analysis (Supplementary Table S1). Furthermore, our analysis of the m⁶A network revealed the intricate interactions, connectivity, and prognostic significance of m⁶A regulators in ESCA (Figure 1D). Our findings indicate noteworthy correlations between the expression levels of m⁶A regulatory factors within the same functional class, as well as significant associations among the three distinct types of regulatory factors. This interplay is likely to contribute to the generation of distinct m⁶A modification patterns, which play a crucial role in the initiation and progression of cancer.

As well, we identified CNV alterations as a potential underlying cause of disrupted expression of m⁶A regulatory factors. To further investigate this, we compared the gene expression levels of the 23 m⁶A regulators between normal and tumor tissues (54). In ESCA tissues, m⁶A regulatory factors' expression exhibiting CNV amplification (such as METTL3, WTAP, VIRMA, YTHDC1, YTHDF1, HNRNPC, FMR1, and HNRNPA2B1) was significantly higher than in normal esophageal tissues, while the expression of IGFBP2 was lower (Figure 1E). Collectively, these analyses underscore the noteworthy diversity in the genetic and expression profiles of m⁶A regulators detected between normal and ESCA specimens. These findings emphasize the critical role of dysregulated expression of m⁶A regulators in the development and progression of ESCA.

3.2 scRNA-seq analysis

Single-cell RNA sequencing (scRNA-seq) of 25,796 immune and 8,197 non-immune cells from three primary tumor and paired normal samples in ESCA patients was generated by using 10x Genomics platform. Before filtration, there were 33993 cells in the 6 ESCA samples. For GSE196756, counts were normalized and technical covariates (mitochondrial percentage) were regressed out using the LogNormalize method (default settings), and batch effects

across samples (6 ESCA patients) were corrected for using Harmony with theta = 2 to preserve biological variance (55). We then performed data normalization and quality control, and finally selected the top 2000 highly expressed and variable genes for further analysis. PCA used to reduce the dimensionality of the data showed no clear tendency for cells to separate. Nonlinear dimensionality reduction was performed using the t-SNE algorithm, which successfully clustered the cells into 13 clusters (Figure 2A). We then annotated all clusters and identified 9 cell types (Figure 2B). Furthermore, the expression levels of 23 m⁶A modulators were most abundant in B cells and T cells (Figure 2C). WTAP, ZC3H13, YTHDC1, HNRNPC, HNRNPA2B1 and RBMX are expressed in most cell types.

3.3 Twenty-three regulator-mediated isoforms of m⁶A methylation

Using the ConsensusClusterPlus R software package, we performed patient classification based on the expression of the 23 m⁶A regulators to delineate distinct m⁶A-modified subtypes. Our analysis revealed two subtypes: subtype A consisting of 111 cases and subtype B consisting of 75 cases (Figures 3A–D and Supplementary Table S2). Notably, patients belonging to m⁶A regulator group B exhibited significantly longer survival compared to those in m⁶A regulator group A ($P = 0.019$, Figure 3E). We generated a heatmap to visualize the expression patterns of the 23 m⁶A regulators in the two m⁶A-modified subtypes (Figure 3F).

3.4 TME cell infiltration characteristics in distinct m⁶A modification patterns

To gain insights into the underlying biomolecular signatures associated with the different m⁶A-modified phenotypes, we integrated the expression profiling data of both TCGA-ESCA and GSE13898 cohorts and performed differential expression analysis using the Limma R software package. This analysis identified 2599 DEGs, which were subsequently annotated using the clusterProfiler R package. The DEGs were found to be enriched in several important biological processes, including T cell activation, regulation of immune effector process, neutrophil-mediated immunity, mesenchyme development, mesenchymal cell differentiation, leukocyte transendothelial migration, chemokine signaling pathway, and VEGF signaling pathway (Figures 4A, B, Supplementary Tables S3, S4).

In order to examine the biological alterations linked to diverse m⁶A modification patterns, a comparative analysis of immune cell composition in the TME was performed. The findings indicate that the A subcluster exhibited a higher degree of infiltration by memory B cells, immature B cells, T helper 1 (Th1) cells, activated memory CD4+ T cells, and regulatory T cells (Treg). On the other hand, m⁶A cluster B exhibited significantly increased infiltration of natural killer cells and neutrophils (Figure 4C).

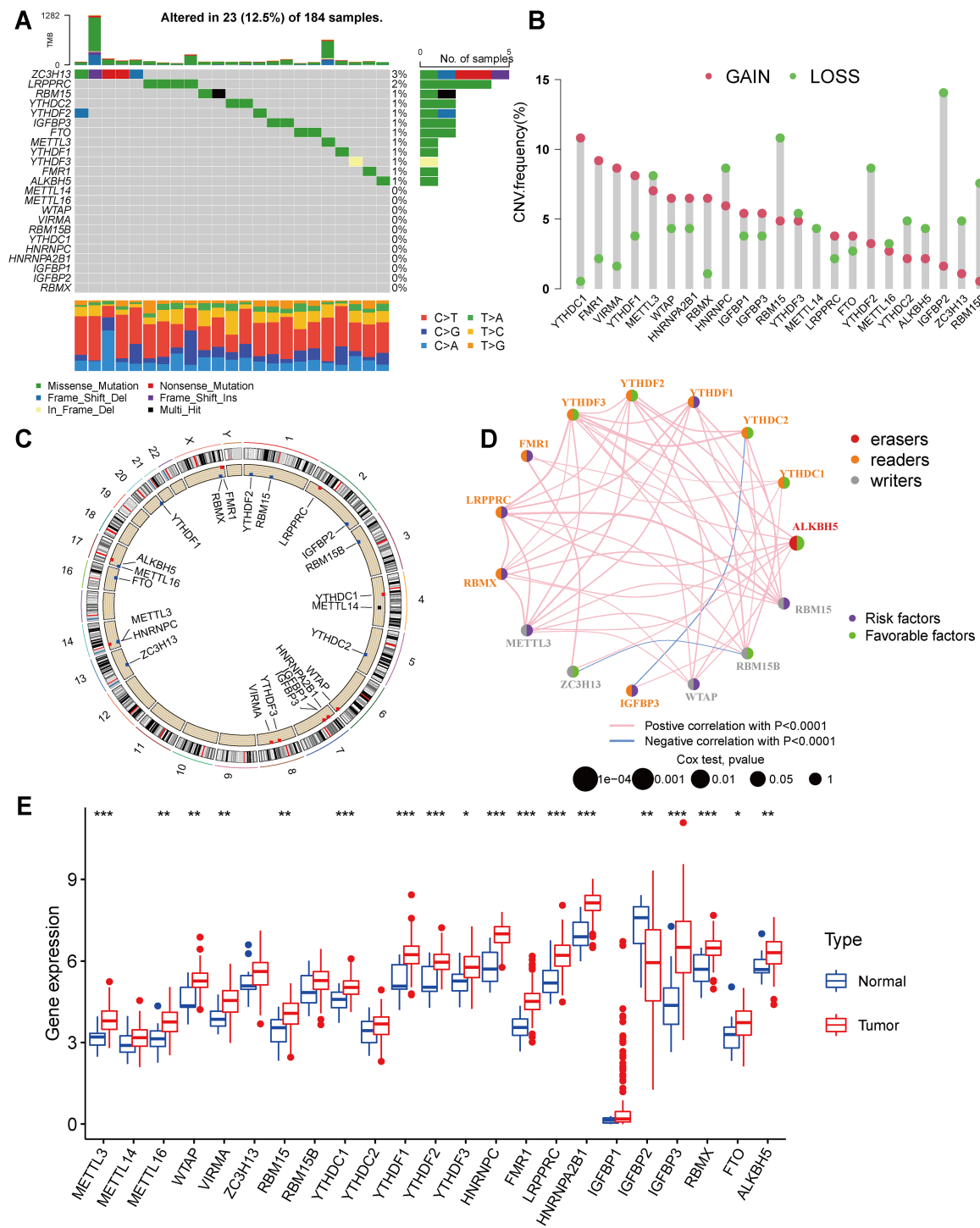


FIGURE 1
Landscape of genetic and expression variation of m⁶A regulators in esophagus cancer **(A)** Mutations of 23 m⁶A regulators in the TCGA-ESCA cohort. Each column represented individual patients. The upper barplot showed TMB, the number on the right indicated the mutation frequency in each regulator. The right barplot showed the proportion of each variant type. The stacked barplot below showed fraction of conversions in each sample. **(B)** In an TCGA-ESCA cohort, we looked at the CNV mutation rates of 23 m⁶A regulators. The findings were represented by red and green dots to represent increased and absent frequencies, respectively. **(C)** The precise chromosomal locations of CNVs in m⁶A regulators across all 23 chromosomes. **(D)** Interactions and prognostic implications of 23 m⁶A regulators in ESCA. The three types of m⁶A regulatory modifiers are represented by different colors: eraser in red, reader in orange, and writer in gray. The size of the circles corresponds to the prognostic relevance of each m⁶A modulator. The lines connecting the regulators indicate their interactions, with positive correlations in pink and negative correlations in blue. Prognostic risk factors are highlighted in purple, while prognostic protective factors are shown in green. **(E)** The expression of 23 m⁶A regulators between normal tissues and gastric tissues. Tumor, red; Normal, blue. The upper and lower ends of the boxes represented interquartile range of values. The lines in the boxes represented median value, and red and blue dots showed outliers. The asterisks represented the statistical p value (*P < 0.05; **P < 0.01; ***P < 0.001).

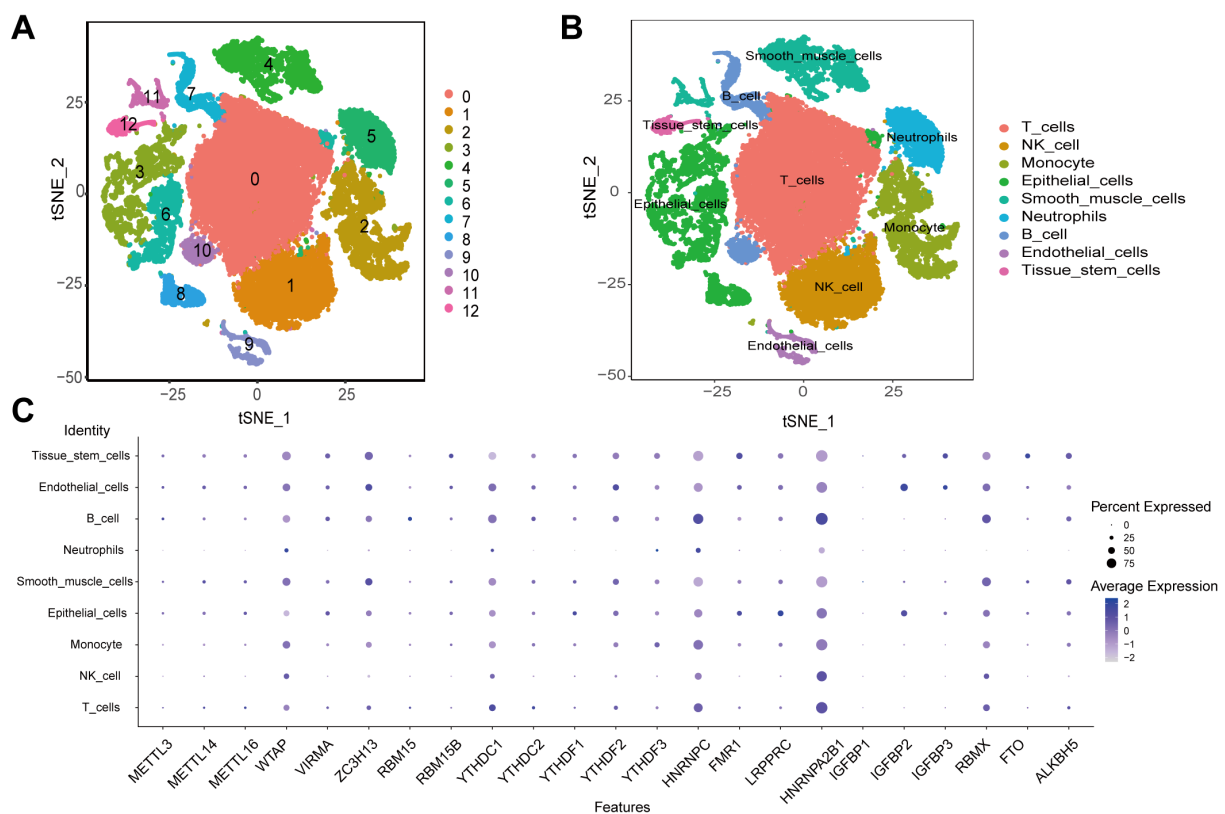


FIGURE 2
scRNA-seq data analysis. (A) The t-SNE algorithm divided the cells into 13 clusters by principal components. (B) The tSNE plot revealing 13 clusters was annotated into 9 different cell types. (C) The expression of 23 m⁶A regulators in 9 cell types.

We further employed GSVA enrichment analysis to gain insights into the biological activity associated with these distinct m⁶A modification patterns. The results of our observations indicate that m⁶A cluster A exhibits noteworthy enrichment in pathways linked to stroma and cancer metastasis, including ECM-receptor interaction, focal adhesion, and others. On the other hand, m⁶A cluster B showed enrichment in metabolic pathways such as histidine metabolism, fatty acid metabolism, propanoate metabolism, glycolysis, fructose, and mannose metabolism (Figure 4D).

Interestingly, GSVA enrichment analysis revealed that m⁶A cluster A exhibited significant enrichment in adaptive immune cell infiltration, encompassing memory B cells, activated memory CD4⁺ T cells, immature B cells, Th1 cells, regulatory T cells (Treg), and stromal activation (Figures 4C–G, 5E). Surprisingly, despite the higher immune cell infiltration, patients with this m⁶A modification pattern did not demonstrate a survival advantage (Figure 4F). Prior research has detected an immune-excluded phenotype within tumors, wherein immune cells exist in the stroma encircling nests of tumor cells, yet are unable to penetrate the tumor parenchyma. T-cell suppression is known to occur when the stroma in the TME is activated. Hence, our speculation is that the stromal activation observed in cluster A suppresses the antitumor effect of immune cells in patients with ESCA. The aforementioned conjecture was subsequently substantiated through analyses that demonstrated a marked increase in stromal activity within cluster A, which

encompassed the activation of epithelial-mesenchymal transition (EMT), transforming growth factor beta (TGF- β), and WNT pathways, all of which were found to be statistically significant (Figure 4G).

We have integrated immune deconvolution tools such as CIBERSORT, EPIC, MCP_COUNTER, QUANTISEQ, TIMER and XCELL to distinct immune microenvironments characterize two m⁶A clusters (Supplementary Figure S4). Comparative analysis of immune infiltration patterns between the two m⁶A clusters revealed significant heterogeneity. Cluster A exhibited higher infiltration of immunosuppressive regulatory T cells (Tregs, $P < 0.01$ by CIBERSORT/QUANTISEQ) and exhausted CD8⁺ T cells (PD-1⁺Tim-3⁺, $P < 0.05$), whereas cluster B showed elevated cytotoxic CD8⁺ T cells (Granzyme B⁺, $P < 0.001$ by TIMER). Pro-tumor M2 macrophages were enriched in cluster A ($P < 0.001$ across CIBERSORT/QUANTISEQ/XCELL), while cluster B had higher M1 macrophages ($P < 0.05$), suggesting divergent macrophage polarization states. CAFs were markedly increased in cluster A ($P < 0.001$ by EPIC/MCP-counter), correlating with elevated ECM remodeling scores (e.g., collagen cross-linking, $P = 0.002$). These findings were robust across multiple deconvolution algorithms (CIBERSORT, EPIC, XCELL).

Based on the comprehensive analyses conducted, it is intriguing to note that the two m⁶A modification patterns exhibit distinct characteristics in terms of TME cell infiltration. Cluster A is

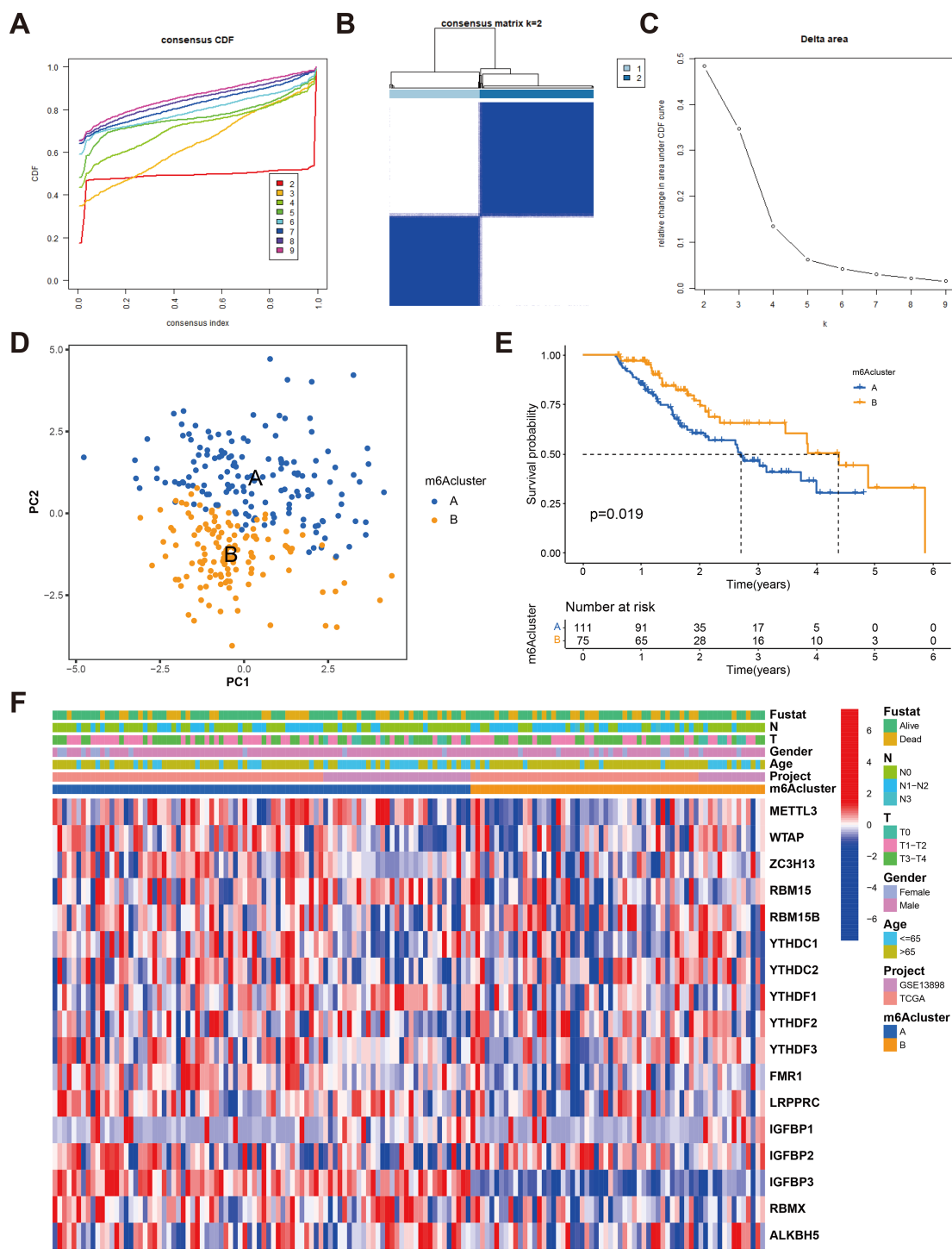
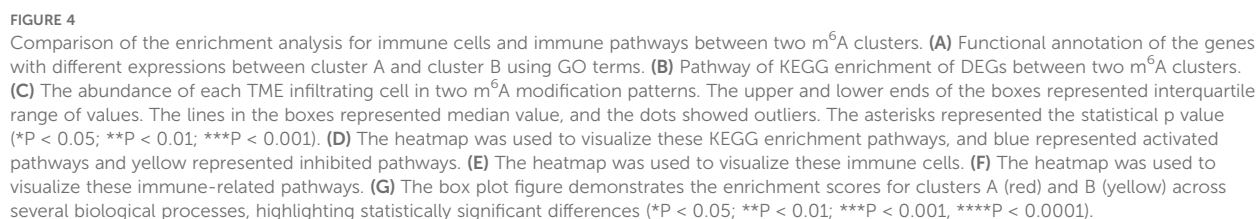


FIGURE 3 Identification of m⁶A methylation modification subtypes. **(A)** Consensus clustering and generated a CDF with the number of subtypes ranging from k = 2 to 9. **(B)** The heat map of sample clustering under k = 2 in 2 independent ESCA cohorts. **(C)** The relative change in the area under the CDF curve for values of k ranging from 2 to 9. **(D)** Principal component analysis of transcriptome profiles of two m⁶A modification patterns **(E)** Survival analyses for the two m⁶A modification patterns based on 186 patients with esophagus cancer from TCGA-ESCA and GEO cohorts (GSE13898) including 111 cases in m⁶Acluster-A, 75 cases in m⁶Acluster-B, Kaplan-Meier curves with Log-rank p value 0.019 showed a significant survival difference among two m⁶A modification patterns. The overall survival rate of cluster B in m⁶A cluster A and B subclusters is better. **(F)** Unsupervised clustering of 23 m⁶A regulators in two cohorts with heatmap analysis of m⁶A cluster, tumor stage, survival status, and age. Red is high expression, blue is low expression.

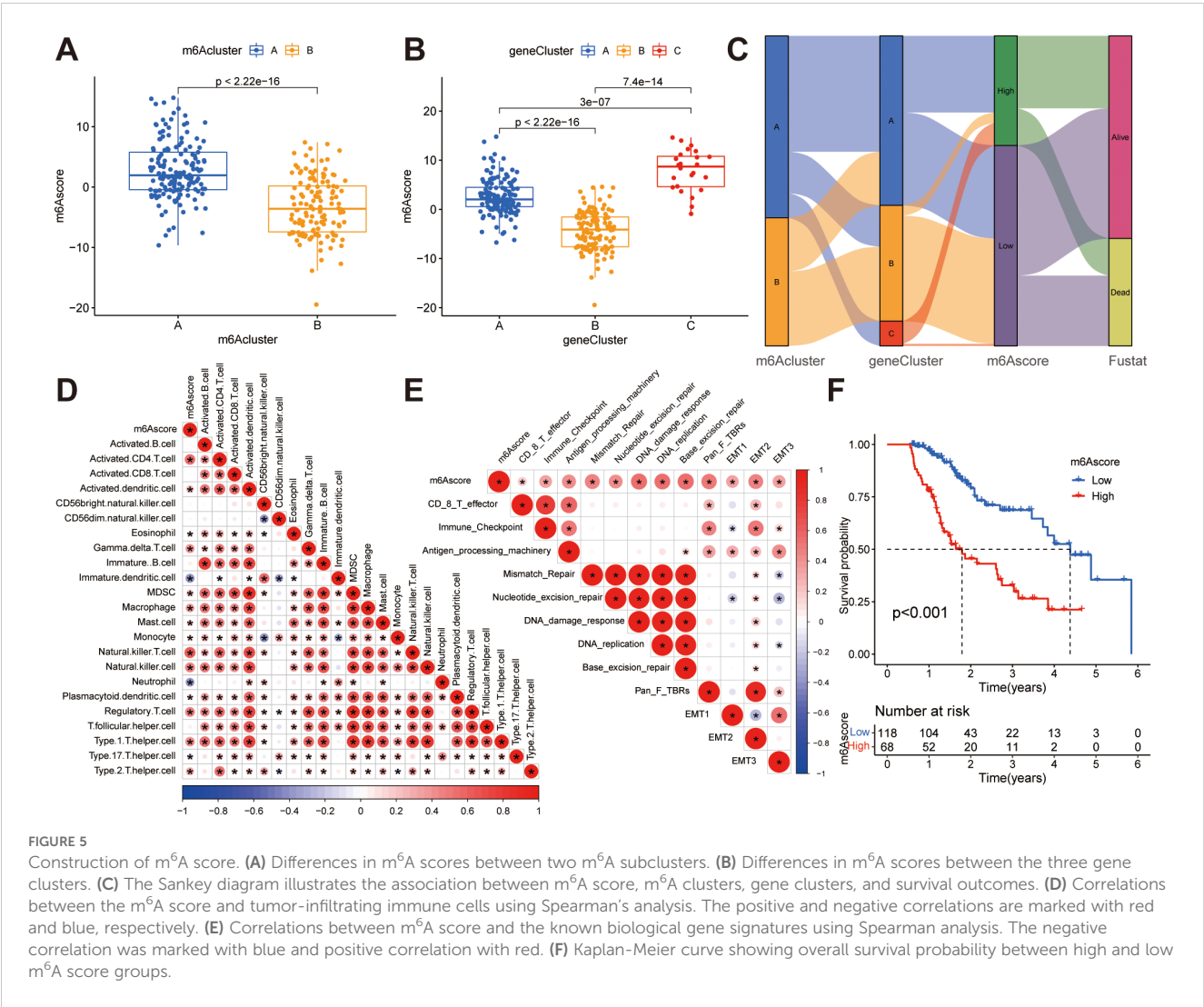


associated with an immune-excluded phenotype, characterized by the infiltration of adaptive immune cells and stromal activation. On the other hand, cluster B corresponds to an immune-inflamed phenotype, characterized by the infiltration of innate immune cells and metabolic reprogramming (Figures 4C–G). These findings suggest that m⁶A methylation modifications may be involved in tumor metabolism, EMT, immune regulation, and have close associations with tumor initiation and progression.

3.5 Characteristics of clinical and transcriptome traits in m⁶A-related phenotypes

Despite the successful categorization of ESCA patients into two subtypes through a consistent clustering algorithm utilizing m⁶A regulator expression, the genetic alterations responsible for these phenotypes and their prognostic implications remain inadequately comprehended. To gain deeper insights, we conducted univariate

Cox regression analysis on the 2599 DEGs identified between the previously established m⁶A clusters. A total of 80 survival-related genes were identified through this analysis, which we referred to as the m⁶A-related signature genes (Supplementary Table S5). Through unsupervised clustering analysis using representative m⁶A-associated marker genes, we identified three stable transcriptomic phenotypes, denoted as gene clusters A, B, and C (Figures 6A–C; Supplementary Table S6). The predictive importance of these gene subgroups was then investigated by fusing transcriptome data with survival data. Based on Kaplan-Meier analysis and log-rank test, it was observed that patients assigned to gene cluster B displayed a favorable prognosis (Figure 6D). A heat map was generated to visually depict the clinical characteristics of 80 m⁶A-related signature genes and the expression of m⁶A subgroups in the three gene clusters (Figure 6E). Notably, the three m⁶A gene clusters exhibited significant differential expression of m⁶A regulatory factors, which aligns with the methylation modification process and supports the predicted effects of m⁶A (Figure 6F).



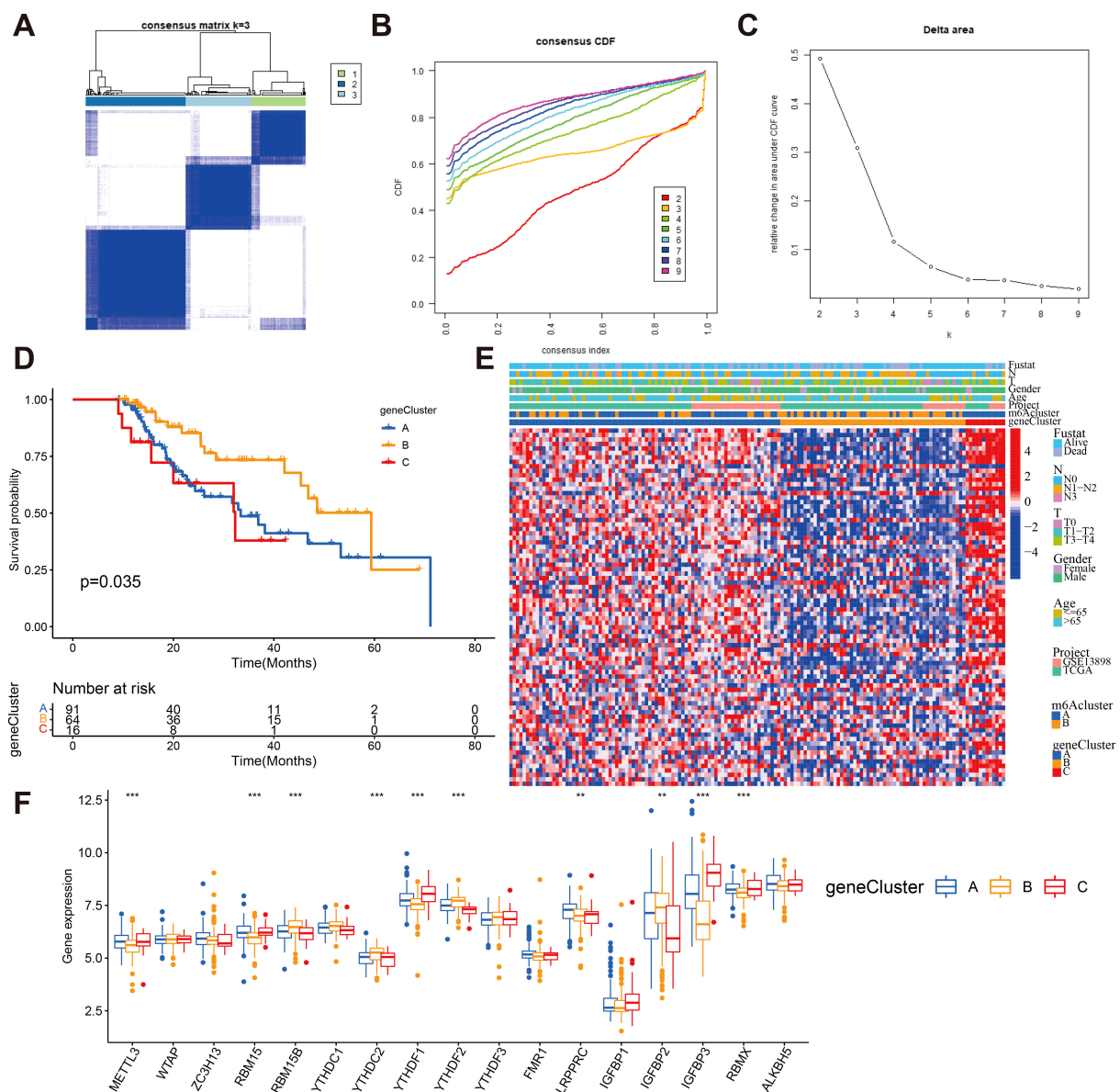


FIGURE 6

Consensus clustering of m⁶A-related gene subtypes (A) Consensus matrices of 80 m⁶A phenotype-related genes according to TCGA and GEO cohort for k = 3. (B) Consensus clustering CDF with the number of subtypes k = 2 to 9. (C) Consensus clustering cumulative distribution function k = 2 to 9. (D) Kaplan-Meier overall survival curves for patients in three m⁶A-related gene clusters. (E) Heatmap showing the correlation between the expression levels of the DEGs derived from 3 m⁶A clusters and sex, age, m⁶A clusters, tumor stage, survival status and gene clusters. Red is high expression, blue is low expression. (F) The expression of 23 m⁶A regulators in three gene cluster. The upper and lower ends of the boxes represented interquartile range of values (**P < 0.01; ***P < 0.001).

3.6 Establishment of m⁶A score and its association with tumor microenvironmental features

While previous analyzes have yielded valuable insights into the impact of m⁶A methylation on immune cell infiltration status and tumor prognosis, accurate prediction of m⁶A methylation patterns in individual patients remains a challenging task. To address this challenge, the PCA score was employed to compute the m⁶A score, which also provides a quantitative assessment of the modified m⁶A landscape in patients with ESCA. Figure 5A illustrates that patients

in m⁶A cluster B exhibit lower m⁶A scores compared to those in m⁶A cluster A, and Figure 5B demonstrates that patients in gene cluster B have lower m⁶A scores than those in gene clusters A and C. We have depicted the process of m⁶A score construction in a Sankey diagram (Figure 5C). In order to evaluate the association between the m⁶A score and tumor-infiltrating immune cells, a Spearman's analysis was conducted and the outcomes were presented in a heatmap (Figure 5D), revealing a positive correlation between the m⁶A score and the presence of immune cells within the tumor microenvironment. Additionally, we examined the correlation between the m⁶A score and known

signal pathway signatures. The resulting correlation matrix heatmap demonstrated that the m⁶A score exhibited significant positive associations with signatures related to EMT, stromal activity, DNA repair, antigen processing machinery, and the TGF- β pathway (Figure 5E). In addition, we conducted an evaluation of the prognostic relevance of the m⁶A score. Through implementation of the Kaplan-Meier survival analysis, it was determined that patients exhibiting low m⁶A scores experienced a more favorable prognosis in contrast to those with high m⁶A scores (Figure 5F). This indicates that the implementation of the m⁶A-score-based computation proficiently delineates the prognosis of patients.

In addition, we also found that in T0, T1-2, or T3-4 stage, N0, N1-2, or N3 stage, M0 or M1 stage, male or female, young or old patients, and patients, lower m⁶A score showed more significant survival advantage, which means that m⁶A score can also be used to access various clinical features of patients, such as age, gender, or clinical stage subgroup (Supplementary Figure S5).

3.7 Verification and clinical evaluation of m⁶A score

To validate the m⁶A score, we conducted ROC curve analysis for 1-year, 3-year, and 5-year intervals and calculated the corresponding area under the curve (AUC) values. The results showed that all three ROC curves in the total sample cohort (Figure 7A) and the separate TCGA-ESCA cohort (Figure 7B) showed AUC values exceeding 0.67. Furthermore, when comparing the m⁶A score with other clinical features, the AUC value of the m⁶A score was found to be the highest (Figure 7C).

The findings of the univariate Cox regression analysis indicate that the stage, stage M, stage N, and m⁶A score possess prognostic potential (Figure 7D). Additionally, the multivariate Cox regression analysis reveals that both the stage and m⁶A score exhibit independent prognostic value (Figure 7E). To quantitatively assess individual risks in the clinical setting, the integration of multiple clinical indicators can be achieved through a nomogram. In this study, we developed a nomogram for predicting patients' overall survival (OS) at 1-year, 3-year, and 5-year intervals (Figure 7F). The predictive performance of the nomogram was evaluated using ROC curve analysis. The present study determined the AUC values of the ROC curves for 1-year, 3-year, and 5-year intervals to be 0.784, 0.831, and 0.801, respectively (Figure 7G). These results indicate that the m⁶A score may serve as a promising clinical predictor and, when integrated with other clinical factors, could potentially improve the prognostic precision and clinical outcomes for patients diagnosed with ESCA.

3.8 Somatic variation correlates with m⁶A score

The potential of TMB as a tumor marker for immune checkpoint therapy in patients has been demonstrated. Given the

clinical significance of TMB, an analysis was conducted to investigate the genetic characteristics within each subgroup, as defined by the m⁶A score, and their association with TMB. Patients were divided into two subgroups based on TMB.

Based on the results depicted in Figures 8A, B, it was observed that both TP53 (86% vs. 71%) and TTN (44% vs. 32%) exhibited a higher rate of somatic mutation in the group with a high m⁶A score, suggesting a potential association with the poorer prognosis observed in this group (Figure 8C). Subsequently, we assessed the combined prognostic value of these scores in stratifying ESCA patients. Survival analysis revealed that the TMB status did not influence predictions based on the m⁶A score, consistently demonstrating a survival advantage in the low m⁶A score group (Figure 8D). The results of this study contribute to a more thorough comprehension of the impact of the m⁶A score on genomic variability, presenting innovative perspectives for investigating potential associations between m⁶A methylation modification and somatic mutations. These findings demonstrated that distinct m⁶A modification patterns significantly influenced tumor immune phenotypes and may serve as predictive biomarkers for anti-PD-1/PD-L1 immunotherapy response efficacy. It has also been revealed that the m⁶A score is indirectly used to predict the success of immunotherapy.

3.9 M⁶A score predicts the possibility of benefit from immunotherapy

Subsequently, the differences in the levels of other immune checkpoints between the high and low m⁶A score groups were compared. The high m⁶A score group had higher expression of CTLA4, CD70, TNFSF14, ICOS, CD80, TNFRSF9, HAVCR2, CD200, NRP1, TNFSF15, TNFSF4, CD40, TNFRSF14, LGALS9, CD86, ADORA2A, and CD28, while the low m⁶A score group had higher expression of BTLA (Figure 8E).

The use of ICI therapy, specifically CTLA-4/PD-1 inhibitors, has resulted in a significant advancement in antitumor treatment. Alongside established predictors such as TMB, PD-L1, and MSI (56, 57), newly discovered indicators such as TIDE are extensively utilized and highly recommended for assessing immune response. Our analysis further demonstrated a noteworthy reduction in TIDE within the low m⁶A score group, as evidenced by the TIDE distribution in TCGA-ESCA and GSE13898 (both $P < 0.01$) (Figures 8F, G). As a result of these findings, it is inferred that tumor m⁶A modification patterns play an important role in mediating immune responses in tumors.

Based on the significant correlation between m⁶A scores and immune responses, our subsequent investigation aimed to assess whether m⁶A modification signatures could serve as predictive markers for patient response to ICI therapy in three separate immunotherapy cohorts. Firstly, a high m⁶A score exhibited significantly shorter survival time (HR, 1.845 [95% CI, 1.254 to 2.714], $P = 0.013$, Figure 9A) and a markedly clinical response in an anti-PD-L1 therapy in a cohort of metastatic urothelial carcinoma (response rate, low vs. high m⁶A score, 53% vs. 19%, Figure 9B)

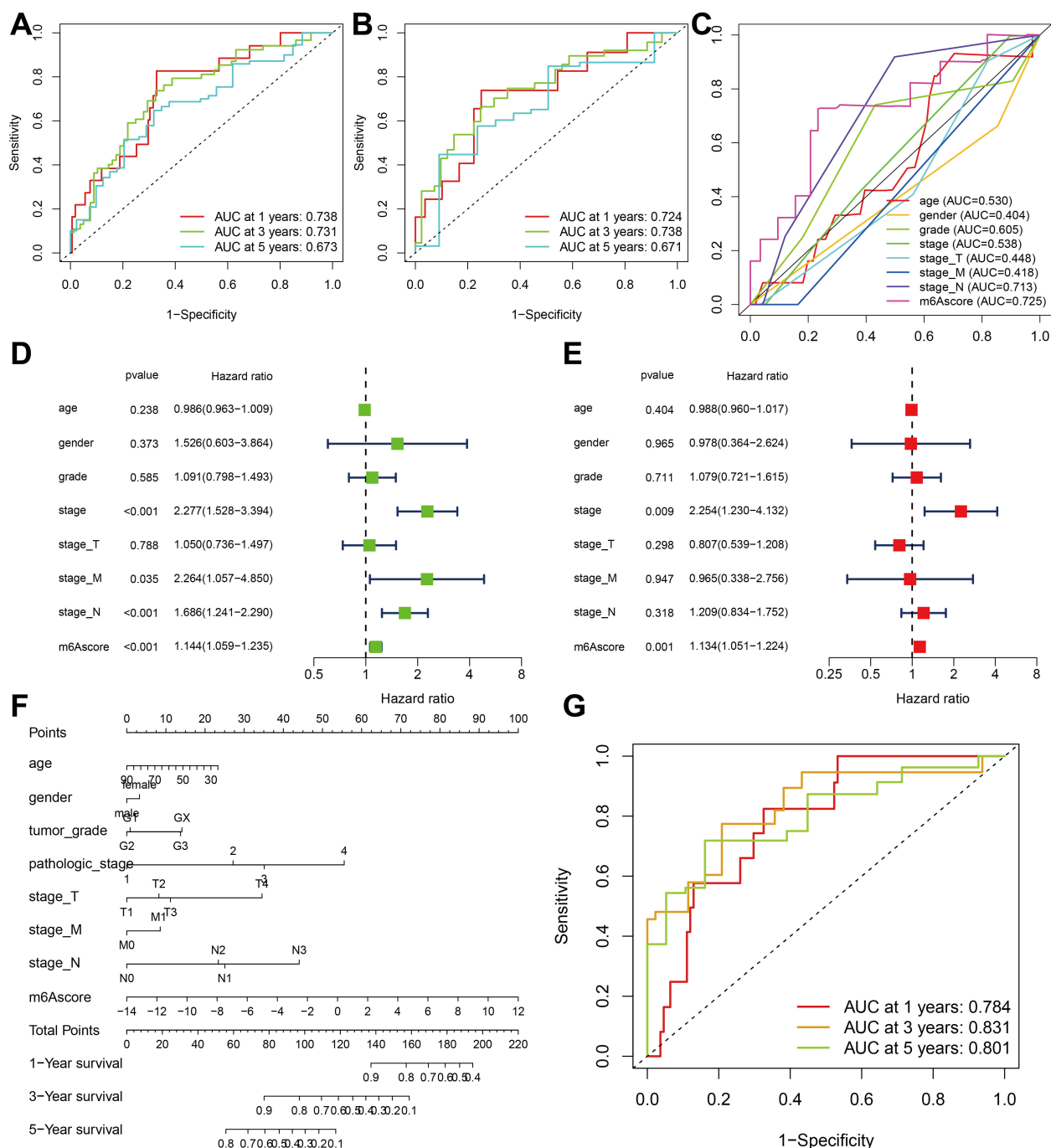


FIGURE 7

Verification and clinical evaluation of m^6A score. (A) For all samples, the area under the curve (AUC) values of 1-, 3-, and 5-year ROC curves for m^6A scores have been calculated. (B) For TCGA-ESCA cohort, the area under the curve (AUC) values of 1-, 3-, and 5-year ROC curves for m^6A scores have been calculated. (C) Comparison of 1-year ROC curves of the m^6A score model with other clinical features. (D) Univariate COX regression analysis of clinicopathological parameter and m^6A score. (E) Multivariate COX regression analysis of clinicopathological parameter and m^6A score. (F) The nomogram is used to forecast the probabilities of 1-year, 3-year, and 5-year survival rates. (G) The AUC values of the nomogram's 1-year, 3-year, and 5-year ROC curves.

(51). This result was also identified in both the anti-PD-1 cohort (49) and anti-CTLA-4 cohort (50). Patients belonging to the high m^6A score group demonstrated noteworthy clinical drawbacks and a considerably reduced lifespan (anti-PD-1, HR, 2.886 [95% CI, 1.002 to 8.314], $P = 0.018$. (Figure 9C) anti-CTLA-4, HR, 2.141

[95% CI, 1.018 to 4.503], $P = 0.035$, Figure 9E). The significant therapeutic benefits and immune response to ICI treatment were confirmed in patients with a low m^6A score compared to those with a high m^6A score (anti-PD-1, response rate, low vs. high m^6A score, 33% vs. 18%, Figure 9D; anti-CTLA-4, response rate, low vs. high

m⁶A score, 40% vs. 32%, **Figure 9F**). The m⁶A score of the GSE165252 cohort was further validated, the significant therapeutic benefits and immune response to anti-PD-1 treatment were confirmed in patients with a low m⁶A score compared to those with a high m⁶A score (anti-PD-1, response rate, low vs. high m⁶A

score, 39% vs. 18%) (**Supplementary Figure S6**). The m⁶A score is also associated with patient response to immunotherapy and can be used to predict patients' prognoses. In conclusion, the m⁶A score serves as a promising prognostic indicator in ESCA and may also provide guidance for ICI treatment in clinical practice.

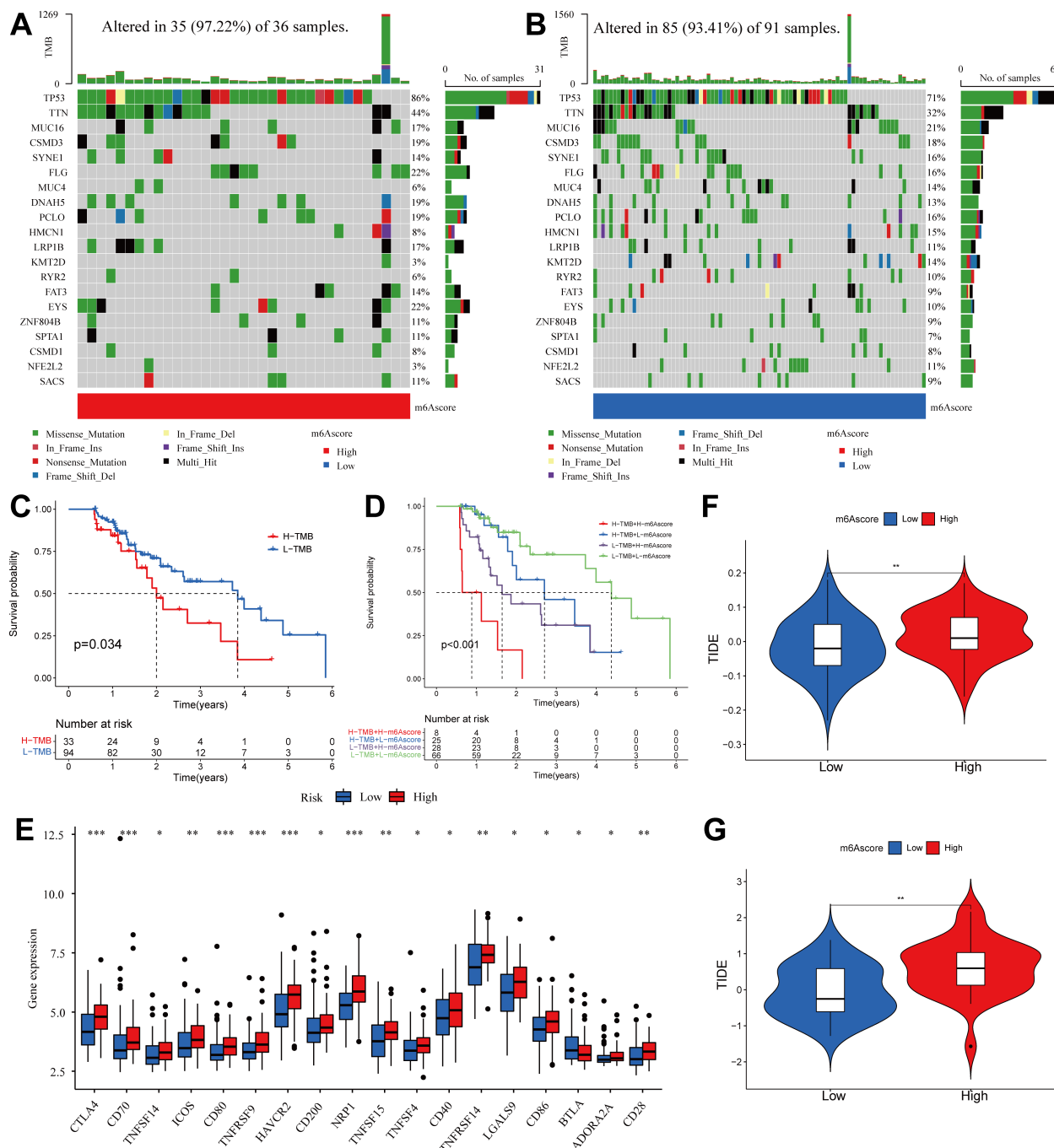


FIGURE 8

Correlation between m⁶A score and TMB and TIDE in the ESCA cohort. **(A, B)** OncoPrint for gene mutations in high and low m⁶A score groups. in the high m⁶A score group. **(C)** Kaplan-Meier curve showing overall survival probability between high TMB group and low TMB group (P < 0.05). **(D)** TMB and m⁶A scores were used in a stratified survival analysis. **(E)** Violin plot of differential expression of other immune checkpoints between groups with high and low m⁶A scores. (*P < 0.05, **P < 0.01, ***P < 0.001) **(F)** TIDE differences in the TCGA cohort between high and low m⁶A score groups. **(G)** TIDE differences in the GSE13898 cohort between high and low m⁶A score groups.

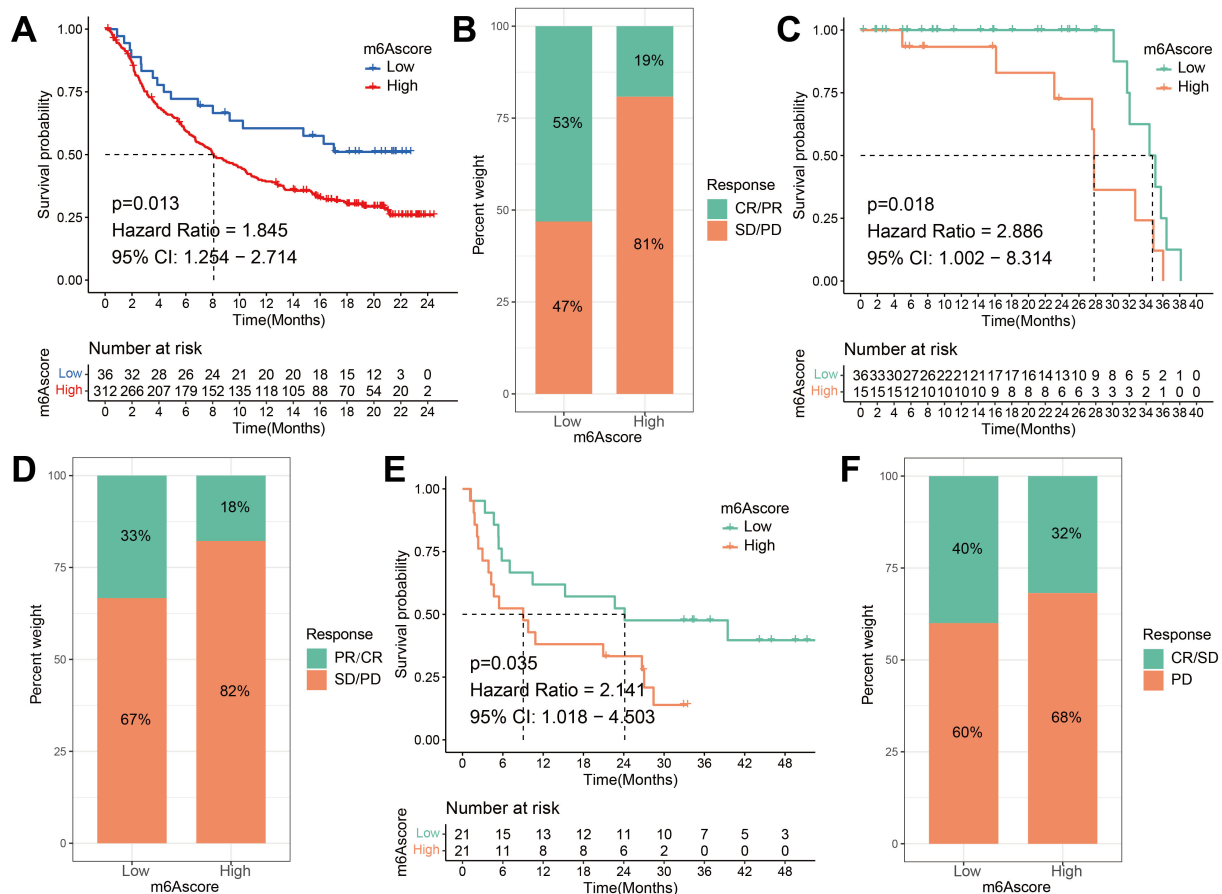


FIGURE 9

The m⁶A risk score predicts immunotherapeutic benefits. (A) Survival difference analysis of patients with high and low m⁶A risk score in the IMvigor210 cohort. $P = 0.013$. (B) Rate of clinical response to anti-PD-L1 immunotherapy in high or low m⁶A risk score groups in the IMvigor210 cohort. (C) Kaplan-Meier curves for high and low m⁶A risk score patient groups in the Riaz et al. cohort. Log-rank test, $P = 0.018$. (D) The fraction of patients with clinical response to anti-PD-1 immunotherapy (Riaz et al. cohort) in low or high m⁶A risk score groups. CR/PR vs. SD/PD: 33% vs. 67% in the low m⁶A risk score groups, 18% vs. 82% in the high m⁶A risk score groups. (E) Kaplan-Meier curves for high and low m⁶A risk score patient groups in the Vanallen et al. cohort. Log-rank test, $P = 0.035$. (F) The fraction of patients with clinical response to anti-CTLA-4 immunotherapy in low or high m⁶A risk score groups of Vanallen et al. cohort. CR/SD vs. PD: 40% vs. 60% in the low m⁶A risk score groups and 32% vs. 68% in the high m⁶A risk score groups. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

3.10 Sensitivity analysis of patients with ESCA to different small molecule drugs based on m⁶A risk score

We performed an estimation of IC₅₀ values and assessed the drug sensitivities of chemotherapeutic drugs for a cohort of 186 ESCA patients, utilizing data from the TCGA and GEO databases. The estimation process employed the “pRRophetic” R package, which utilized the expression profiles of the patients. Then, IC₅₀ values were compared between the groups with high and low m⁶A scores. The IC₅₀ values are utilized to assess the cellular response of various cell lines to a total of 138 distinct chemotherapeutic and small molecule anticancer drugs. The research found statistically significant differences ($P < 0.05$) between patients with high and low m⁶A risk scores in the IC₅₀ values of several chemotherapeutic drugs and small molecule anticancer medicines. Notably, Bortezomib, Camptothecin, Cytarabine, Erlotinib, Gefitinib, Gemcitabine, Metformin, Methotrexate, and Paclitaxel exhibited

particularly noteworthy differences (Figures 10A–I; Supplementary Figure S7).

3.11 RBMX's impact on ESCC cell proliferation and migration

To establish the mechanistic link between m⁶A modification and malignant progression in ESCC, we prioritized RBMX for functional interrogation based on its central position in the m⁶A regulatory network. Bioinformatics analysis identified RBMX as a hub gene in protein-protein-interaction network and co-expressed with key m⁶A regulators (METTL3, FTO, YTHDF2). RBMX expression levels were quantified in the ESCC cell lines KYSE510 and TE-1, revealing a notable reduction in protein expression following RBMX knockdown (Figure 11A). The clone formation assay demonstrated that the knockdown of RBMX significantly impeded the proliferative capacity of ESCC cells (Figure 11B). EDU staining corroborated

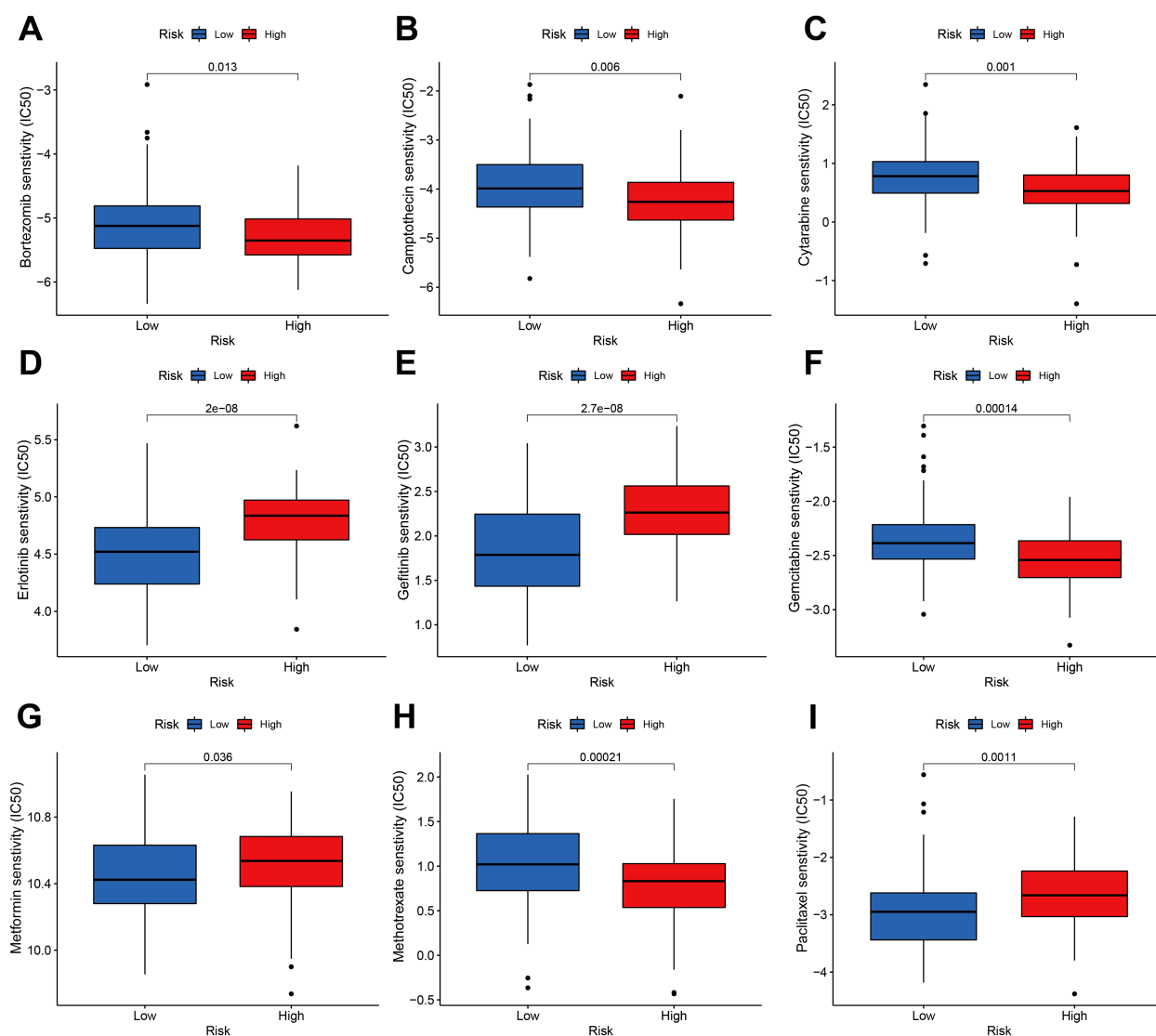


FIGURE 10

Sensitivity of the m⁶A risk score to different chemotherapy drugs and small molecule anticancer drugs was analyzed based on the GDSC database.

(A) Bortezomib. (B) Camptothecin. (C) Cytarabine. (D) Erlotinib. (E) Gefitinib. (F) Gemcitabine. (G) Metformin. (H) Methotrexate. (I) Paclitaxel.

these findings, indicating a significant decrease in the proliferative activity of si-RBMX-transfected KYSE510 and TE-1 cells (Figures 11C, D). The wound healing assay further illustrated that, after 48 hours, the wound closure ability of si-RBMX-transfected KYSE510 and TE-1 cells was markedly diminished compared to the Si-NC control group (Figure 11E). Additionally, migration and invasion assays were conducted to evaluate the impact of RBMX on ESCC cell motility. The knockdown of RBMX in KYSE510 and TE-1 cells led to a significant reduction in both the invasive and migratory capabilities of the cells (Figures 11F, G).

4 Discussion

Accumulating evidence from various studies emphasizes the important role of m⁶A methylation modification in the immune

process of organisms. Further investigation is necessary to achieve a thorough comprehension of the immune cell infiltration within the TME in ESCA that is mediated by multiple m⁶A regulators. Therefore, it is crucial to clarify the characteristics of immune cell infiltration in relation to diverse m⁶A modification patterns. This will enhance our understanding of the TME and antitumor immune responses within it, and offer approaches for risk stratification and clinical management of patients with esophageal cancer. This study identified two distinct modification patterns with the assistance of 23 m⁶A regulators. The mRNA transcriptome differences observed between these patterns were found to be significantly associated with T cell activation, regulation of immune effector processes, neutrophil-mediated immunity, mesenchyme development, mesenchymal cell differentiation, leukocyte transendothelial migration, the Chemokine signaling pathway, and the VEGF signaling pathway (Figures 4A, B). The two patterns exhibited

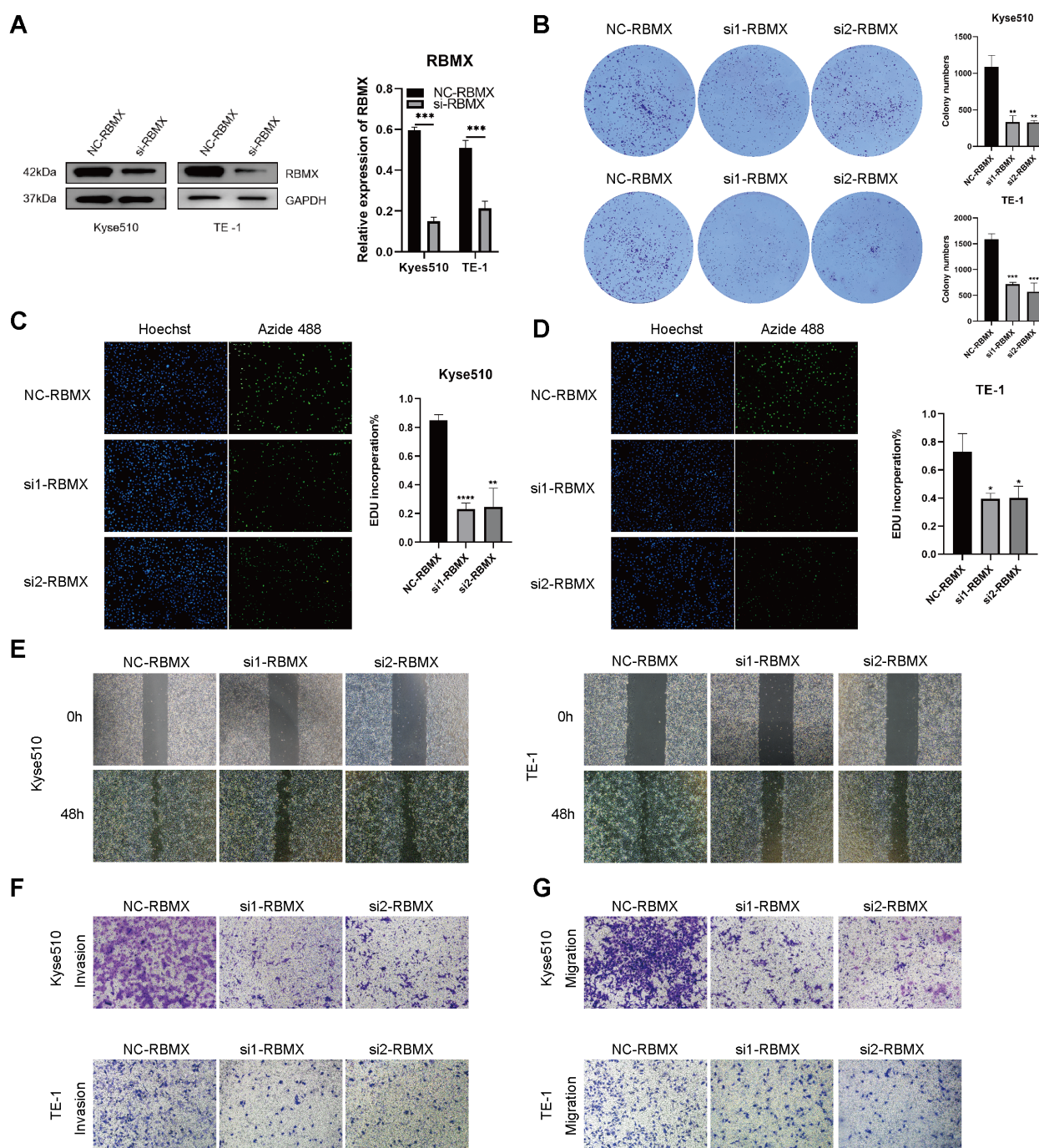


FIGURE 11

Impact of altered RBMX expression on proliferation and invasion of esophageal cancer cells. (A) RBMX protein expression levels in Kyse510 and TE-1 cells. (B) plate clone formation experiments assays in transfected Kyse510 cells and TE-1 cells. (C) EDU assays in transfected Kyse510 cells (D) and TE-1 cells. (E) Wound healing assays in transfected Kyse510 cells and TE-1 cells. (F) Invasion assay in transfected Kyse510 cells and TE-1 cells. (G) migration assay in transfected Kyse510 cells and TE-1 cells (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, **** $P < 0.0001$).

markedly different TME cell-infiltrating characteristics. Cluster A was categorized as an immune-excluded phenotype, marked by the infiltration of adaptive immune cells and stromal activation. On the other hand, cluster B was classified as an immune-inflamed phenotype, characterized by the infiltration of innate immune cells and metabolic reprogramming. The immune-inflamed phenotype, also known as “hot tumors,” is distinguished by

substantial immune cell infiltration within the TME (24, 58, 59). Despite the presence of a significant number of immune cells in the immune-excluded phenotype, their distribution is limited to the stromal compartment surrounding the tumor cell nests, rather than infiltrating the tumor parenchyma. The stromal compartment may be localized to the tumor periphery or may extend into the tumor, potentially leading to the misinterpretation that immune cells are

present within the tumor (60, 61). Consistent with the established definitions, our findings revealed that cluster A exhibited a pronounced stromal activation status, including elevated expression of EMT and TGF- β pathways (Figures 4D, E, G), which are associated with T-cell suppression. The observed TME cell-infiltrating characteristics in each cluster reinforce the validity of our immune phenotype classification based on distinct m⁶A modification patterns. Consequently, after comprehensively exploring the TME cell-infiltrating characteristics induced by distinct m⁶A modification patterns, it is not surprising that cluster A, despite having activated innate immunity, exhibited poorer prognosis.

The stromal activation in cluster A (e.g., TGF- β , EMT) and its link to immune exclusion are supported by recent studies on CAF subtypes (60–62). Cluster A exhibits an immunosuppressive stromal microenvironment owing to the enrichment of TGF- β and multiple EMT-related pathways and (Figures 4D, G). The tumor stroma, particularly CAFs and their remodeled extracellular matrix (ECM), plays a pivotal role in shaping the immunosuppressive TME by regulating T cell infiltration and function through both physical barrier and molecular mechanisms (60). The physical barrier prevents cytotoxic T cells from contacting cancer cells, creating an “immune-excluded” microenvironment (60). In addition to structural constraints, CAF heterogeneity further exacerbates immune evasion. Distinct CAF subpopulations may drive divergent stromal remodeling patterns: certain subsets promote the formation of rigid, cross-linked stroma that impedes T cell migration, while others secrete immunosuppressive factors (60, 61). Three functional subtypes of CAFs have been identified in non-small cell lung cancer (NSCLC) based on their heterogeneity (63). These functional disparities among CAFs are driven by their intrinsic TGF- β signaling. This CAF functional classification correlates with patients’ clinical responses to targeted therapies and is also associated with the tumor immune microenvironment (63). Notably, RNA modification “writers” (e.g., m⁶A/m¹A regulators) appear to influence CAF activation states, as evidenced by the association between high “Writers-Score”, poor prognosis, and suppressive immune infiltration (e.g., M2 macrophages, EMT) (62). These findings suggest that epigenetic reprogramming of CAFs may reinforce immune exclusion by coupling matrix stiffness with broader immunosuppressive signals, such as PD-L1 upregulation. Thus, stromal activation drives CAF heterogeneity and immune exclusion via coordinated ECM remodeling (e.g., collagen cross-linking, fibronectin deposition) and epigenetic reprogramming (e.g., m⁶A-mediated RNA stabilization of TGF- β signaling components). Therapeutically targeting these matrix-driven immunosuppressive mechanisms—such as through ECM-degrading enzymes (e.g., collagenase) or epigenetic inhibitors—could dismantle the stromal-T cell barrier, thereby enhancing the efficacy of T cell-mediated antitumor immunity. The study by Du et al. elucidates that RBMX stabilizes IL-33 mRNA through a liquid-liquid phase separation mechanism, thereby activating the TGF- β signaling pathway. This process orchestrates the bidirectional regulation of tumor plasticity and the immunosuppressive

microenvironment, providing a theoretical foundation for developing precision therapeutic strategies targeting the RBMX/TGF- β axis (64). We hypothesize that RBMX, as an m⁶A reader, regulates CAF crosstalk by enhancing RNA stability of the IL-33/TGF- β axis and increasing stromal stiffness, while simultaneously suppressing immune-activating signals (e.g., CXCL10-STAT1) to impair T cell function (61). This dual mechanism aligns with clinical observations in cluster A patients, where despite high immune cell infiltration, T cells are predominantly confined to stromal regions and exhibit significantly reduced survival rates. Such an “immune-excluded” phenotype closely mirrors the stroma-mediated immune privilege phenomenon proposed by Joyce et al. (60).

The immune landscape analysis underscores how m⁶A modification patterns shape tumor-immune interactions (Supplementary Figure S4). M⁶A cluster A (immune-excluded): Dominated by Tregs, M2 macrophages, and CAFs, this phenotype aligns with TGF- β -driven stromal activation. The concomitant suppression of cytotoxic lymphocytes (evidenced by low CD8+/NK cell ratios, $P < 0.001$) may explain poorer immunotherapy responses observed in this subgroup. M⁶A cluster B (immune-inflamed): Enriched for cytotoxic T/NK cells and immunostimulatory dendritic cells, this cluster demonstrates the potential of m⁶A modulation to overcome immune desertification. Notably, the M1/M2 macrophage balance ($P < 0.001$) mirrors metabolic reprogramming linked to m⁶A-regulated pathways.

The expression and function of m⁶A modulator genes in these cells may play an important role in regulating the tumor microenvironment. Especially in immune cells, the expression of m⁶A modulator genes may affect the function and activity of immune cells, thereby regulating tumor immune responses. Most of the 23 m⁶A modulator genes are distributed in epithelial cells, B cells, and T cells. As important components of the immune system, B cells and T cells play an important role in the tumor microenvironment. The expression level and functional status of m⁶A modulator genes may affect the activity, proliferation, migration and cell fate decisions of these immune cells, thereby affecting the efficacy of tumor immunotherapy. In addition, the expression of m⁶A modulator genes in tumor cells and epithelial cells may also directly affect tumor development and treatment response. Epithelial cells are often the cells of origin of tumors, and the expression of m⁶A modulator genes in these cells may regulate the proliferation, invasion, and metastasis capabilities of tumor cells. Recent advancements such as spatial transcriptomics and proteomics, exemplified by works utilizing techniques like spatial CITE-seq (65), multimodal tri-omics (66), and spatially resolved CRISPR screens (67), offer powerful methodologies for dissecting complex interactions within the tumor microenvironment. These technologies could provide novel insights into the spatial and functional dynamics of m⁶A methylation modifications and their impact on immune infiltration and cancer progression, potentially unveiling new therapeutic avenues.

M⁶A related characteristic genes were identified as DEGs associated with the prognosis of ESCA. By employing the m⁶A signature genes, we have successfully categorized the samples into

three distinct subtypes of m⁶A -related genes, which exhibit significant associations with stromal and immune activations. Therefore, it is imperative to conduct a comprehensive evaluation of m⁶A modification patterns to enhance our understanding of TME cell infiltration characterization. To mitigate inter-individual variations, quantification of the m⁶A modification pattern among m⁶A -modified tumors is necessary. To this end, we have devised a set of scoring systems, referred to as the m⁶A gene signature, to evaluate the m⁶A modification pattern. The m⁶A modification pattern associated with the immune-excluded phenotype demonstrated a higher m⁶A score, whereas the immune-inflamed phenotype exhibited a lower m⁶A score.

Our findings align with previous studies on the TME, supporting the notion that m⁶A methylation modifications play a vital role in influencing distinct immune properties within the TME. Scoring models constructed using specific biomarkers modified by m⁶A have been successfully used in gastric cancer and colorectal cancer, providing improved clinical treatment selection and prognosis assessment for cancer patients (62, 68, 69). The findings suggest that the m⁶A score possesses the capacity to serve as a comprehensive metric for assessing the m⁶A modification pattern of individual tumors, and may be employed in the investigation of tumor immunophenotype and TME immune cell infiltration. Additionally, the validation of the m⁶A score through the TCGA-ESCA cohort highlights its considerable potential as a prognostic indicator for patients afflicted with ESCA. The nomogram, incorporating the m⁶A score along with other clinical variables, demonstrated effective predictive capabilities for patient prognosis.

Furthermore, our m⁶A score demonstrated a superior predictive capability in the context of immunotherapy for esophageal cancer. These findings were robustly corroborated in the IMvigro210 cohort, as well as in cohorts receiving anti-PD-1 and anti-CTLA-4 treatments, where the immune phenotype had been established (49, 50, 56). We could also predict the efficacy of adjuvant chemotherapy and the patients' clinical response to anti-PD-1/PD-L1 immunotherapy through the m⁶A score.

The evaluation of genes that may drive mutations in tumors is an essential method for exploring the fundamental mechanisms of tumorigenesis and progression. Furthermore, it contributes to the rational selection of cancer diagnosis and treatment strategies. In our study, we observed a significant increase in the mutation rates of TP53 and TTN in the high m⁶A score group. TP53 mutations are prevalent in various cancer types and play a critical role in cancer progression. Loss or mutation of TP53 in cancer cells can disrupt T cell recruitment and impair T cell activity, aiding immune evasion and accelerating cancer growth in the process. Research on esophageal cancer has revealed that the absence of TP53, which encodes the P53 protein, Consequently, there is an augmentation of regulatory T cell (Treg) infiltration in both paracancerous and intratumoral tissues (70). On the other hand, TTN mutations have been associated with poor immune infiltration and worse prognosis in liver hepatocellular carcinoma, colorectal cancer, and ovarian serous cystadenocarcinoma (71–73). Notably, TTN

mutations are frequently detected in solid tumors and have been correlated with increased TMB. Moreover, TTN mutations have been found to be associated with the objective response to immune checkpoint blockade (ICB) therapy (74). These findings highlight the potential impact of TP53 and TTN mutations in modulating the immune response within the tumor microenvironment and their relevance to clinical outcomes. Understanding the role of these mutations in tumor biology can provide valuable insights for the development of targeted therapies and immunotherapeutic strategies in cancer treatment.

The study elucidates the role of RBMX in ESCC, focusing specifically on its impact on cell proliferation and migration. The findings suggest that the expression levels of RBMX are critical for the malignant behavior of ESCC cells. In the KYSE510 and TE-1 ESCC cell lines, significant reductions in RBMX protein expression were observed following knockdown. This indicates that RBMX may play a crucial role in maintaining the cancerous state of these cells. The plate colony formation assay revealed that RBMX knockdown significantly impaired the proliferative capacity of ESCC cells, highlighting its potential as a therapeutic target. EDU staining, which assesses DNA synthesis during the S phase, confirmed the reduced proliferative activity in cells with lower RBMX expression. These findings support the hypothesis that RBMX is a key regulator of cell cycle progression in ESCC. The wound healing assay demonstrated that RBMX knockdown significantly diminished the wound closure ability of ESCC cells, underscoring its role in cell migration, which is crucial for cancer invasion and metastasis. Migration and invasion assays further indicated significant reductions in both the invasive and migratory capabilities of ESCC cells following RBMX knockdown. These observations suggest that RBMX is central to ESCC cell motility, a key factor in the metastatic spread of cancer. In summary, this study provides evidence that RBMX has multiple influences on ESCC, impacting both cell proliferation and migration. These findings indicate that RBMX may serve as a promising target for therapeutic intervention in ESCC. Additional research is needed to elucidate the molecular mechanisms through which RBMX exerts its effects and to investigate the potential of RBMX-targeted therapies for treating ESCC.

The role of RBMX in tumors is highly tissue-specific. In hepatocellular carcinoma (HCC) and T-cell lymphoma, elevated RBMX expression enhances tumor progression and chemoresistance by stabilizing oncogenic long non-coding RNAs (lncRNAs), such as BLACAT1, or modulating RNA metabolism (75, 76). In contrast, in bladder cancer, RBMX exhibits an oncogenic effect by inhibiting hnRNP A1-mediated PKM splicing (77). This paradox indicates that the function of RBMX may rely on the tissue-specific expression of its interacting partners, such as hnRNP A1 and specific lncRNAs. RBMX has been linked to chemoresistance in both T-cell lymphoma and small-cell lung cancer (75, 78), suggesting that it may affect treatment responses in esophageal cancer, particularly in platinum-resistant ESCC, by modulating DNA damage repair and apoptotic pathways, such as those involving the BCL2 family.

Research by Tuersun and Bei has emphasized that RBMX is a significant prognostic biomarker in various cancers, including esophageal cancer, where its expression correlates with tumor progression and poor clinical outcomes (79, 80). Investigating how RBMX influences alternative splicing and m⁶A methylation, particularly in relation to other RNA-binding proteins such as TRA2A, may reveal new insights into the biology of esophageal cancer and resistance to therapies like sorafenib (80). The interaction of RBMX with splicing factors such as TRA2A and hnRNP A1 offers deeper insights into the regulatory networks governing esophageal cancer progression. RBMX's role in m⁶A methylation may contribute to the dynamic regulation of oncogenic lncRNAs, thereby influencing tumor biology. Future investigations should examine the mechanistic pathways by which RBMX influences alternative splicing and m⁶A modification across a broader range of cancers. Longitudinal studies are needed to assess its prognostic value over extended periods.

Our research has several limitations that should be acknowledged. Firstly, although we included 23 well-known m⁶A regulators reported in the literature, the significance of incorporating recently identified regulators to enhance the precision of m⁶A methylation pattern identification is incontrovertible. Incorporating additional regulators into the model can potentially improve the comprehensive understanding of m⁶A modifications. Secondly, while immunotherapy has shown significant benefits for some patients with low m⁶A scores, it is important to recognize that not all patients with low scores derive equal benefit. To enhance the predictive accuracy, it would be valuable to integrate additional clinicopathological features into the analysis. By incorporating these features, we can better identify patients who are more likely to respond favorably to immunotherapy. Thirdly, although we obtained a relatively large sample size of 186 ESCA patients from various cohorts, it is important to acknowledge that a larger and independent prospective cohort of ESCA patients who have undergone immunotherapy is required to validate our findings. Prospective trials with a substantial patient cohort are required to provide a more definitive demonstration of the prognostic value of the m⁶A score in relation to the response to immunotherapy. Furthermore, our study focused on a holistic analysis of the tumor microenvironment without further distinguishing between tumor, immune, and stromal components. This lack of component-specific analysis may mask certain subtype-specific information, which is a limitation of our study. Future investigations should consider dissecting the tumor microenvironment into its individual components to gain deeper insights into the interactions and contributions of different cell types. Lastly, we primarily aimed to propose molecular subtypes associated with m⁶A methylation across the tumor microenvironment and subsequently develop a scoring system. Furthermore, clinical analysis revealed that the m⁶A score, when combined with other clinical indicators, can serve as a

valuable adjunct to existing variables and effectively predict patient prognosis. Addressing these limitations through further research and validation will enhance the scientific significance and clinical applicability of our findings.

This study offers novel insights into the clinical application of immunotherapy, presenting potential implications for its use in the field. One potential avenue for the development of novel immunotherapy drugs or treatment strategies involves the modulation of m⁶A modification patterns through the targeting of m⁶A regulators or m⁶A -related marker genes. This approach may serve to reverse unfavorable immune cell infiltration in the tumor microenvironment, thereby converting immune cold tumors into hot tumors (81). These findings aid in the identification of distinct immune phenotypes, thereby enhancing our understanding of patient response to immunotherapy. This information may help with the clinical use of customized immunotherapy for the treatment of cancer (82). We also demonstrated that patients with high m⁶A scores had increased resistance to immunotherapy, which may lead to different treatment effects of classical chemotherapeutics in different patients.

5 Conclusions

We assessed the landscape of m⁶A methylation modifications mediated by 23 regulators based on 186 ESCA samples. The variety and complexity of immune infiltration in the TME are closely connected to m⁶A methylation modifications. An m⁶A score has been developed to offer a comprehensive evaluation of the m⁶A modification pattern and immune infiltration features within a singular tumor. This score also helps determine the tumor's immune phenotype, providing new insights and directions for identifying potential therapeutic targets.

Data availability statement

All code and data related to this study are available via GitHub: <https://github.com/mintsun0035/m6aTMEESCARBMX>.

Ethics statement

The studies involving humans were approved by the Research Ethics Committee of Hubei University of Medicine (Shiyan, Hubei, China). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Written informed consent was obtained from the

individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

MS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. PS: Conceptualization, Data curation, Formal analysis, Writing – original draft. JY: Project administration, Resources, Software, Supervision, Writing – review & editing. HZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Software, Writing – review & editing. YL: Investigation, Software, Validation, Visualization, Writing – review & editing. RC: Conceptualization, Resources, Validation, Visualization, Writing – review & editing. YF: Software, Supervision, Validation, Visualization, Writing – review & editing. LZ: Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This research was supported by the National Natural Science Foundation of China (81902498), the Natural Science Foundation of Hubei Province of China (2019CFB177, 2023AFB1008), Natural Science Foundation of Hubei Provincial Department of Education (Q20182105), Chen Xiao-Ping Foundation for the Development of Science and Technology of Hubei Provincial (CXPJJH11800001-2018333), The Foundation of Health and Family Planning Commission of Hubei Province (WJ2021Q007), Innovation and Entrepreneurship Training Program (201810929005, 201810929009, 20181092 9068, 201813249010, S201910929009, S201910929045, S202013249005, S202013249008 and 202010929009) and The Scientific and Technological Project of Taihe Hospital (2021JJXM009), and joint supported by Hubei Provincial Natural Science Foundation and Shiyan of China (2025AFD178).

Acknowledgments

We thank the Cancer Genome Atlas and the Gene Expression Omnibus for providing the data for this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1572810/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Integrated analysis and study design flowchart.

SUPPLEMENTARY FIGURE 2

The Metascape enrichment network is visually represented through a visualization that highlights similarities both within and between clusters of terms. The clustering of terms is indicated by consistent color coding.

SUPPLEMENTARY FIGURE 3

Kaplan-Meier survival curves of overall survival in ESCA cohort according to the expression value of YTHDF2, YTHDF1, RBMX, LRPPRC, IGFBP3, IGFBP1, FMR1 or ALKBH5 mRNA level in each tumor sample, the optimal value in each cohort was chosen as the cut-off point.

SUPPLEMENTARY FIGURE 4

Comparative immune landscape analysis of m⁶A modification clusters in esophageal cancer. Heatmap depicting immune cell infiltration patterns between m⁶A cluster A (left) and cluster B (right) as quantified by four deconvolution algorithms (CIBERSORT, EPIC, MCP_COUNTER, QUANTISEQ, TIMER and XCELL). Rows represent immune cell subsets grouped by lineage (T cells, B cells, myeloid cells, stromal cells), while columns represent individual samples. Color scale indicates relative abundance (z-score normalized).

SUPPLEMENTARY FIGURE 5

Relationship between the m⁶A score and different clinical characteristics. Kaplan-Meier curves showing the differences in survival depending on the m⁶A score and different clinical characteristics. (A) T0; (B) T1–2; (C) T 3–4; (D) N0; (E) N1–2; (F) N3; (G) M0; (H) M1; (I) male; (J) female; (K) age less than or equal to 65 years; (L) age above 65 years.

SUPPLEMENTARY FIGURE 6

The m⁶A risk score predicts immunotherapeutic benefits in the GSE165252 cohort. The fraction of patients with clinical response to anti-PD-1 immunotherapy in low or high m⁶A risk score groups.

SUPPLEMENTARY FIGURE 7

Sensitivity of the m⁶A risk score to different chemotherapy drugs and small molecule anticancer drugs was analyzed based on the GDSC database. (A) Axitinib, (B) Bexarotene, (C) Bicalutamide, (D) Vinblastine, (E) Bosutinib, (F) Bryostatins.1, (G) Lapatinib, (H) Imatinib, (I) Elesclomol.

References

- Miao YQ, Chen W, Zhou J, Shen Q, Sun Y, Li T, et al. N(6)-adenosine-methyltransferase-14 promotes glioma tumorigenesis by repressing argininosuccinate synthase 1 expression in an m6A-dependent manner. *Bioengineered*. (2022) 13:1858–71. doi: 10.1080/21655979.2021.2018386
- Taketo K, Konno M, Asai A, Koseki J, Toratani M, Satoh T, et al. The epitranscriptome m6A writer METTL3 promotes chemo- and radioresistance in pancreatic cancer cells. *Int J Oncol*. (2018) 52:621–9. doi: 10.3892/ijo.2017.4219
- Enzinger PC, Mayer RJ. Esophageal cancer. *New Engl J Med*. (2003) 349:2241–52. doi: 10.1056/NEJMra035010
- Zhang Y. Epidemiology of esophageal cancer. *World J Gastroenterol*. (2013) 19:5598–606. doi: 10.3748/wjg.v19.i34.5598
- Oh TK, Kim K, Jheon SH, Do SH, Hwang JW, Jeon YT, et al. Long-term oncologic outcomes, opioid use, and complications after esophageal cancer surgery. *J Clin Med*. (2018) 7:33. doi: 10.3390/jcm7020033
- Xi Y, Shen Y, Wu D, Zhang J, Lin C, Wang L, et al. CircBCAR3 accelerates esophageal cancer tumorigenesis and metastasis via sponging miR-27a-3p. *Mol Cancer*. (2022) 21:145. doi: 10.1186/s12943-022-01615-8
- Altio K, Mezzadra H, Jagannath S, Tsotles N, Rudek MA, Abdallah N, et al. A novel pharmacodynamic approach to assess and predict tumor response to the epidermal growth factor receptor inhibitor gefitinib in patients with esophageal cancer. *Int J Oncol*. (2010) 36:19–27.
- Kang X, Chen K, Li Y, Li J, D'Amico TA, Chen X. Personalized targeted therapy for esophageal squamous cell carcinoma. *World J Gastroenterol*. (2015) 21:7648–58. doi: 10.3748/wjg.v21.i25.7648
- Shah MA. Update on metastatic gastric and esophageal cancers. *J Clin Oncol*. (2015) 33:1760–9. doi: 10.1200/jco.2014.60.1799
- Doi T, Shitara K, Naito Y, Shimomura A, Fujiwara Y, Yonemori K, et al. Safety, pharmacokinetics, and antitumor activity of trastuzumab deruxtecan (DS-8201), a HER2-targeting antibody-drug conjugate, in patients with advanced breast and gastric or gastro-oesophageal tumours: a phase 1 dose-escalation study. *Lancet Oncol*. (2017) 18:1512–22. doi: 10.1016/s1473-2045(17)30604-6
- Flynn M, Young K, Cunningham D, Starling N. The evolving immunotherapeutic landscape in advanced oesophagogastric cancer. *Ther Adv Med Oncol*. (2018) 10:1758835918786228. doi: 10.1177/1758835918786228
- Babar L, Kosovec JE, Jahangiri V, Chowdhury N, Zheng P, Omstead AN, et al. Prognostic immune markers for recurrence and survival in locally advanced esophageal adenocarcinoma. *Oncotarget*. (2019) 10:4546–55. doi: 10.18632/oncotarget.27052
- Zhu ZJ, Pang Y, Jin G, Zhang HY, Wang WH, Liu JW, et al. Hypoxia induces chemoresistance of esophageal cancer cells to cisplatin through regulating the lncRNA-EMS/miR-758-3p/WTAP axis. *Aging*. (2021) 13:17155–76. doi: 10.18632/aging.203062
- Goltsov AA, Fang B, Pandita TK, Maru DM, Swisher SG, Hofstetter WL. HER2 confers resistance to foretinib inhibition of MET-amplified esophageal adenocarcinoma cells. *Ann Thorac surgery*. (2018) 105:363–70. doi: 10.1016/j.athoracsur.2017.09.003
- Marcone S, Buckley A, Ryan CJ, McCabe M, Lynam-Lennon N, Matallanas D, et al. Proteomic signatures of radioresistance: Alteration of inflammation, angiogenesis and metabolism-related factors in radioresistant oesophageal adenocarcinoma. *Cancer Treat Res Commun*. (2021) 27:100376. doi: 10.1016/j.ctarc.2021.100376
- Macedo-Silva C, Miranda-Gonçalves V, Lameirinhas A, Lencart J, Pereira A, Lobo J, et al. JmjC-KDMs KDM3A and KDM6B modulate radioresistance under hypoxic conditions in esophageal squamous cell carcinoma. *Cell Death disease*. (2020) 11:1068. doi: 10.1038/s41419-020-03279-y
- Fang P, Zhou J, Liang Z, Yang Y, Luan S, Xiao X, et al. Immunotherapy resistance in esophageal cancer: Possible mechanisms and clinical implications. *Front Immunol*. (2022) 13:975986. doi: 10.3389/fimmu.2022.975986
- Schiffmann LM, Plum PS, Fuchs HF, Babic B, Bruns CJ, Schmidt T. Tumor microenvironment of esophageal cancer. *Cancers (Basel)*. (2021) 13:4678. doi: 10.3390/cancers13184678
- Cui K, Hu S, Mei X, Cheng M. Innate immune cells in the esophageal tumor microenvironment. *Front Immunol*. (2021) 12:654731. doi: 10.3389/fimmu.2021.654731
- Sun H, Sun C. The rise of NK cell checkpoints as promising therapeutic targets in cancer immunotherapy. *Front Immunol*. (2019) 10:2354. doi: 10.3389/fimmu.2019.02354
- Demaria O, Cornen S, Daéron M, Morel Y, Medzhitov R, Vivier E. Harnessing innate immunity in cancer therapy. *Nature*. (2019) 574:45–56. doi: 10.1038/s41586-019-1593-5
- Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med*. (2018) 24:541–50. doi: 10.1038/s41591-018-0014-x
- Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug discovery*. (2019) 18:197–218. doi: 10.1038/s41573-018-0007-y
- Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. *Nature*. (2017) 541:321–30. doi: 10.1038/nature21349
- Abou Khouzam R, Goutham HV, Zaarour RF, Chamseddine AN, Francis A, Buart S, et al. Integrating tumor hypoxic stress in novel and more adaptable strategies for cancer immunotherapy. *Semin Cancer Biol*. (2020) 65:140–54. doi: 10.1016/j.semcancer.2020.01.003
- Li M, Zha X, Wang S. The role of N6-methyladenosine mRNA in the tumor microenvironment. *Biochim Biophys Acta Rev cancer*. (2021) 1875:188522. doi: 10.1016/j.bbcan.2021.188522
- He Y, Xing J, Wang S, Xin S, Han Y, Zhang J. Increased m6A methylation level is associated with the progression of human abdominal aortic aneurysm. *Ann Trans Med*. (2019) 7:797. doi: 10.21037/atm.2019.12.65
- Gu X, Zhang Y, Li D, Cai H, Cai L, Xu Q. N6-methyladenosine demethylase FTO promotes M1 and M2 macrophage activation. *Cell signalling*. (2020) 69:109553. doi: 10.1016/j.cellsig.2020.109553
- Liu Y, Liu Z, Tang H, Shen Y, Gong Z, Xie N, et al. The N(6)-methyladenosine (m6A)-forming enzyme METTL3 facilitates M1 macrophage polarization through the methylation of STAT1 mRNA. *Am J Physiol Cell Physiol*. (2019) 317:C762–c775. doi: 10.1152/ajpcell.00212.2019
- Li H, Chiappinelli KB, Guzzetta AA, Easwaran H, Yen RW, Vatawalli R, et al. Immune regulation by low doses of the DNA methyltransferase inhibitor 5-azacytidine in common human epithelial cancers. *Oncotarget*. (2014) 5:587–98. doi: 10.18632/oncotarget.1782
- Wrangle J, Wang W, Koch A, Easwaran H, Mohammad HP, Vendetti F, et al. Alterations of immune response of Non-Small Cell Lung Cancer with Azacytidine. *Oncotarget*. (2013) 4:2067–79. doi: 10.18632/oncotarget.1542
- Chong W, Shang L, Liu J, Fang Z, Du F, Wu H, et al. m(6)A regulator-based methylation modification patterns characterized by distinct tumor microenvironment immune profiles in colon cancer. *Theranostics*. (2021) 11:2201–17. doi: 10.7150/thno.52717
- Zhang B, Wu Q, Li B, Wang D, Wang L, Zhou Y. mA regulator-mediated methylation modification patterns and tumor microenvironment infiltration characterization in gastric cancer. *J Mol Cancer*. (2020) 19:53. doi: 10.1186/s12943-020-01170-0
- Li X, Ma S, Deng Y, Yi P, Yu J. Targeting the RNA mA modification for cancer immunotherapy. *J Mol Cancer*. (2022) 21:76. doi: 10.1186/s12943-022-01558-0
- Chong W, Shang L, Liu J, Fang Z, Du F, Wu H, et al. mA regulator-based methylation modification patterns characterized by distinct tumor microenvironment immune profiles in colon cancer. *J Theranostics*. (2021) 11:2201–17. doi: 10.7150/thno.52717
- Şenbabaoglu Y, Michailidis G, Li J. Critical limitations of consensus clustering in class discovery. *J Sci Rep*. (2014) 4:6207. doi: 10.1038/srep06207
- Wilkerson M, Hayes D. ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking. *J Bioinf*. (2010) 26:1572–3. doi: 10.1093/bioinformatics/btq170
- Yu Z, Wong H, Wang H. Graph-based consensus clustering for class discovery from gene expression data. *J Bioinf*. (2007) 23:2888–96. doi: 10.1093/bioinformatics/btm463
- Shi K, Li Y, Yang L, Zhang Z, Guo D, Zhang J, et al. Profiling transcriptional heterogeneity of epithelium, fibroblasts, and immune cells in esophageal squamous cell carcinoma by single-cell RNA sequencing. *FASEB J*. (2022) 36:e22620. doi: 10.1096/fj.202200898R
- Hao Y, Hao S, Andersen-Nissen E, Mauck WM 3rd, Zheng S, Butler A, et al. Integrated analysis of multimodal single-cell data. *Cell*. (2021) 184:3573–87. doi: 10.1016/j.cell.2021.04.048
- Wang X, Park J, Susztak K, Zhang N, Li M. Bulk tissue cell type deconvolution with multi-subject single-cell expression reference. *J Nat Commun*. (2019) 10:380. doi: 10.1038/s41467-018-08023-x
- Sturm G, Finotello F, Petitprez F, Zhang JD, Baumbach J, Fridman WH, et al. Comprehensive evaluation of transcriptome-based cell-type quantification methods for immuno-oncology. *Bioinf (Oxford England)*. (2019) 35:i436–45. doi: 10.1093/bioinformatics/btz363
- Huang L, Zhu J, Kong W, Li P, Zhu S. Expression and prognostic characteristics of m6A RNA methylation regulators in colon cancer. *Int J Mol Sci*. (2021) 22:2134. doi: 10.3390/ijms22042134
- Zhu M, Cui Y, Mo Q, Zhang J, Zhao T, Xu Y, et al. Characterization of m(6)A RNA methylation regulators predicts survival and immunotherapy in lung adenocarcinoma. *Front Immunol*. (2021) 12:782551. doi: 10.3389/fimmu.2021.782551
- Zeng D, Li M, Zhou R, Zhang J, Sun H, Shi M, et al. Tumor microenvironment characterization in gastric cancer identifies prognostic and immunotherapeutically relevant gene signatures. *Cancer Immunol Res*. (2019) 7:737–50. doi: 10.1158/2326-6066.Cir-18-0436

46. Sotiriou C, Wirapati P, Loi S, Harris A, Fox S, Smeds J, et al. Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. *J Natl Cancer Inst.* (2006) 98:262–72. doi: 10.1093/jnci/djj052
47. Wu Z, Li S, Zhu X. The mechanism of stimulating and mobilizing the immune system enhancing the anti-tumor immunity. *Front Immunol.* (2021) 12:682435. doi: 10.3389/fimmu.2021.682435
48. Jiang P, Gu S, Pan D, Fu J, Sahu A, Hu X, et al. Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *J Nat Med.* (2018) 24:1550–8. doi: 10.1038/s41591-018-0136-1
49. Riaz N, Havel JJ, Makarov V, Desrichard A, Urba WJ, Sims JS, et al. Tumor and microenvironment evolution during immunotherapy with nivolumab. *Cell.* (2017) 171:934–949 e16. doi: 10.1016/j.cell.2017.09.028
50. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science.* (2015) 350:207–11. doi: 10.1126/science.aad0095
51. Mariathasan S, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y, et al. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature.* (2018) 554:544–8. doi: 10.1038/nature25501
52. Yang W, Soares J, Greninger P, Edelman EJ, Lightfoot H, Forbes S, et al. Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. *Nucleic Acids Res.* (2013) 41:D955–61. doi: 10.1093/nar/gks1111
53. Geeleher P, Cox N, Huang RS. pRRophetic: an R package for prediction of clinical chemotherapeutic response from tumor gene expression levels. *PLoS One.* (2014) 9:e107468. doi: 10.1371/journal.pone.0107468
54. Lu X, Li R, Ying Y, Zhang W, Wang W. Gene signatures, immune infiltration, and drug sensitivity based on a comprehensive analysis of m6a RNA methylation regulators in cervical cancer. *J Trans Med.* (2022) 20:385. doi: 10.1186/s12967-022-03600-7
55. Korsunsky I, Millard N, Fan J, Slowikowski K, Zhang F, Wei K, et al. Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat Methods.* (2019) 16:1289–96. doi: 10.1038/s41592-019-0619-0
56. Balar AV, Galsky MD, Rosenberg JE, Powles T, Petrylak DP, Bellmunt J, et al. Atezolizumab as first-line treatment in cisplatin-ineligible patients with locally advanced and metastatic urothelial carcinoma: a single-arm, multicentre, phase 2 trial. *Lancet.* (2017) 389:67–76. doi: 10.1016/s0140-6736(16)32455-2
57. Chen H, Yang M, Wang Q, Song F, Li X, Chen K. The new identified biomarkers determine sensitivity to immune check-point blockade therapies in melanoma. *Oncotarget.* (2019) 8:1608132. doi: 10.1080/2162402X.2019.1608132
58. Turley SJ, Cremasco V, Astarita JL. Immunological hallmarks of stromal cells in the tumour microenvironment. *Nat Rev Immunol.* (2015) 15:669–82. doi: 10.1038/nri3902
59. Gajewski TF, Woo SR, Zha Y, Spaepen R, Zheng Y, Corrales L, et al. Cancer immunotherapy strategies based on overcoming barriers within the tumor microenvironment. *Curr Opin Immunol.* (2013) 25:268–76. doi: 10.1016/j.coi.2013.02.009
60. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. *Science.* (2015) 348:74–80. doi: 10.1126/science.aaa6204
61. Salmon H, Franciszkiewicz K, Damotte D, Dieu-Nosjean MC, Validire P, Trautmann A, et al. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. *J Clin Invest.* (2012) 122:899–910. doi: 10.1172/jci45817
62. Chen H, Yao J, Bao R, Dong Y, Zhang T, Du Y, et al. Cross-talk of four types of RNA modification writers defines tumor microenvironment and pharmacogenomic landscape in colorectal cancer. *Mol Cancer.* (2021) 20:29. doi: 10.1186/s12943-021-01322-w
63. Hu H, Piotrowska Z, Hare PJ, Chen H, Mulvey HE, Mayfield A, et al. Three subtypes of lung cancer fibroblasts define distinct therapeutic paradigms. *Cancer Cell.* (2021) 39:1531–1547 e10. doi: 10.1016/j.ccell.2021.09.003
64. Du Y, Zhan J, Chen S, Liu Q, Song J, Li J, et al. IDDF2024-ABS-0311 RBMX promotes tumor plasticity and immune suppression by activating IL-33/TGF-β signaling niche in colorectal cancer. *Gut.* (2024) 73:A193–8. doi: 10.1136/gutjnl-2024-IDDF.123
65. Liu Y, DiStasio M, Su G, Asashima H, Enninfu A, Qin X, et al. High-plex protein and whole transcriptome co-mapping at cellular resolution with spatial CITE-seq. *Nat Biotechnol.* (2023) 41:1405–9. doi: 10.1038/s41587-023-01676-0
66. Fan R, Zhang D, Rodriguez-Kirby L, Lin Y, Song M, Wang L, et al. Spatial dynamics of mammalian brain development and neuroinflammation by multimodal tri-omics mapping. *Res square.* (2024). doi: 10.21203/rs.3.rs-4814866/v1
67. Baysoy A, Tian X, Zhang F, Renauer P, Bai Z, Shi H, et al. Spatially Resolved *in vivo* CRISPR Screen Sequencing via Perturb-DBiT. *bioRxiv: preprint server Biol.* (2024). doi: 10.1101/2024.11.18.624106
68. Zhang B, Wu Q, Li B, Wang D, Wang L, Zhou YL. m(6A) regulator-mediated methylation modification patterns and tumor microenvironment infiltration characterization in gastric cancer. *Mol Cancer.* (2020) 19:53. doi: 10.1186/s12943-020-01170-0
69. Sun M, Xie M, Zhang T, Wang Y, Huang W, Xia L. m(6A) methylation modification patterns and tumor microenvironment infiltration characterization in pancreatic cancer. *Front Immunol.* (2021) 12:739768. doi: 10.3389/fimmu.2021.739768
70. Mariathasan S, Turley S, Nickles D, Castiglioni A, Yuen K, Wang Y, et al. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *J Nat.* (2018) 554:544–8. doi: 10.1038/nature25501
71. Wang R, Hu X, Liu X, Bai L, Gu J, Li Q. Construction of liver hepatocellular carcinoma-specific lncRNA-miRNA-mRNA network based on bioinformatics analysis. *PLoS One.* (2021) 16:e0249881. doi: 10.1371/journal.pone.0249881
72. Gomes FC, Figueiredo ERL. Social, genetics and histopathological factors related to titin (TTN) gene mutation and survival in women with ovarian serous cystadenocarcinoma: bioinformatics analysis. *Genes (Basel).* (2023) 14:1092. doi: 10.3390/genes14051092
73. Zhao L, Fan W. Construction of a TTN mutation-based prognostic model for evaluating immune microenvironment. *Cancer Stemness Outcomes Colorectal Cancer Patients.* (2023) 2023:6079957. doi: 10.1155/2023/6079957
74. Jia Q, Wang J, He N, He J, Zhu B. Titin mutation associated with responsiveness to checkpoint blockades in solid tumors. *JCI Insight.* (2019) 4:e127901. doi: 10.1172/jci.insight.127901
75. Schumann FL, Bauer M, Gross E, Terziev D, Wienke A, Wickenhauser C, et al. RBMX protein expression in T-cell lymphomas predicts chemotherapy response and prognosis. *Cancers (Basel).* (2021) 13:4788. doi: 10.3390/cancers13194788
76. Song Y, He S, Ma X, Zhang M, Zhuang J, Wang G, et al. RBMX contributes to hepatocellular carcinoma progression and sorafenib resistance by specifically binding and stabilizing BLACAT1. *Am J Cancer Res.* (2020) 10:3644–65.
77. Yan Q, Zeng P, Zhou X, Zhao X, Chen R, Qiao J, et al. RBMX suppresses tumorigenicity and progression of bladder cancer by interacting with the hnRNP A1 protein to regulate PKM alternative splicing. *Oncogene.* (2021) 40:2635–50. doi: 10.1038/s41388-021-01666-z
78. Zhang Z, Zhang C, Yang Z, Zhang G, Wu P, Luo Y, et al. m(6A) regulators as predictive biomarkers for chemotherapy benefit and potential therapeutic targets for overcoming chemotherapy resistance in small-cell lung cancer. *J Hematol Oncol.* (2021) 14:190. doi: 10.1186/s13045-021-01173-4
79. Tuersun H, Liu L, Zhang J, Maimaitizunong R, Tang X, Li H. m6A reading protein RBMX as a biomarker for prognosis and tumor progression in esophageal cancer. *Transl Cancer Res.* (2023) 12:2319–35. doi: 10.21037/tcr-23-84
80. Bei M, Hao S, Lin K, Chen Q, Cai Y, Zhao X, et al. Splicing factor TRA2A contributes to esophageal cancer progression via a noncanonical role in lncRNA m(6A) methylation. *Cancer Sci.* (2023) 114:3216–29. doi: 10.1111/cas.15870
81. Zhao B, Roundtree I, He C. Post-transcriptional gene regulation by mRNA modifications. *J Nat Rev Mol Cell Biol.* (2017) 18:31–42. doi: 10.1038/nrm.2016.132
82. Yoshihara K, Shahmoradgoli M, Martínez E, Vegesna R, Kim H, Torres-Garcia W, et al. Inferring tumour purity and stromal and immune cell admixture from expression data. *J Nat Commun.* (2013) 4:2612. doi: 10.1038/ncomms3612

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