ROLE OF EPIGENETIC REGULATORS IN THE INITIATION, PROGRESSION, AND METASTASIS OF CANCER

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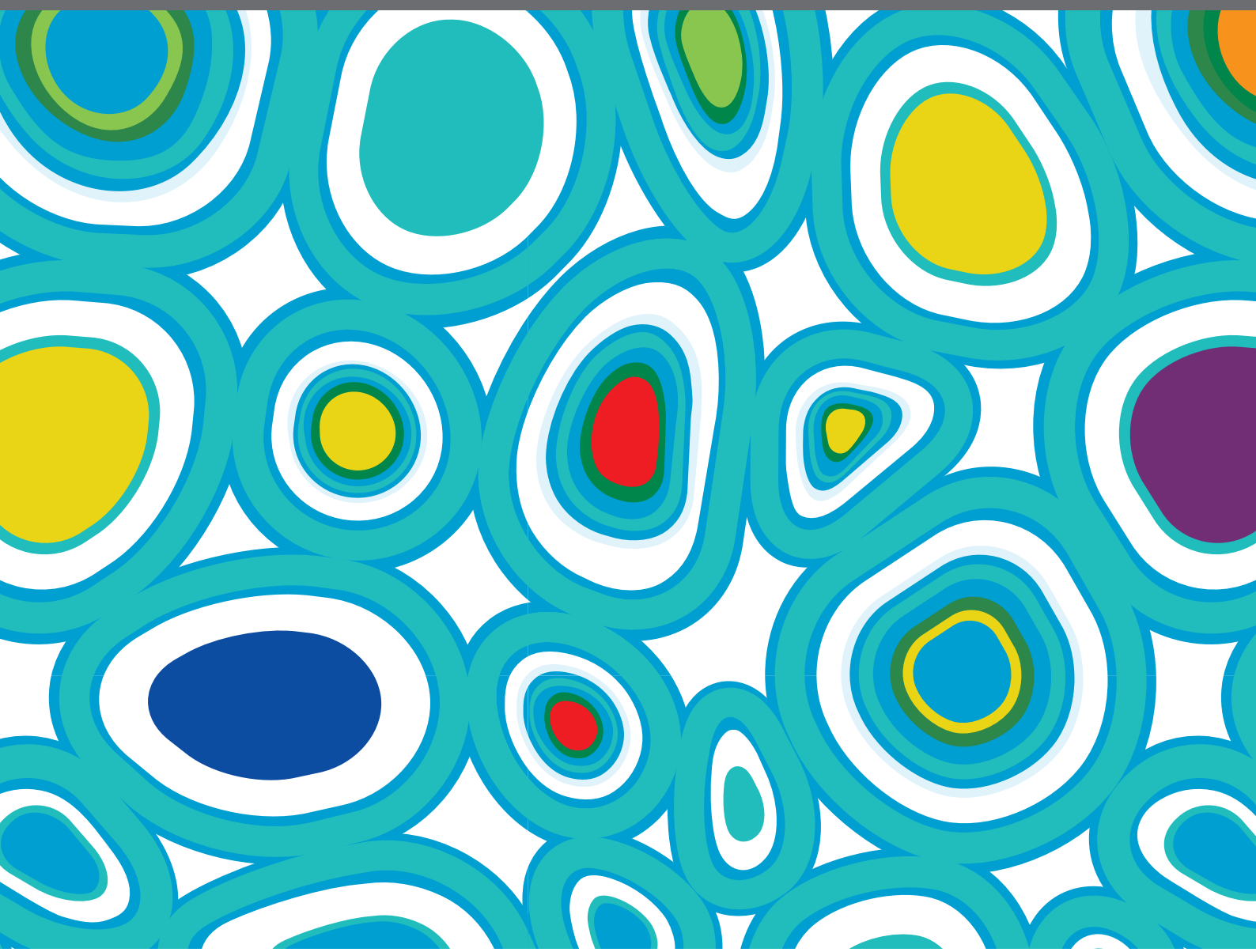

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ROLE OF EPIGENETIC REGULATORS IN THE INITIATION, PROGRESSION, AND METASTASIS OF CANCER

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Editorial: Role of epigenetic regulators in the initiation, progression, and metastasis of cancer

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Editorial on the Research Topic
Role of epigenetic regulators in the initiation, progression, and metastasis of cancer

Epigenetic alterations have emerged as major drivers of tumorigenesis and metastasis (Chatterjee et al., 2017). We present a series of new articles bringing together novel epigenetic players in cancer, ranging from those associated with DNA/RNA modifications to histone modifications, non-coding RNA signatures, as well as alternative polyadenylation that influence cancer cell function and treatment.

Aberrant DNA methylation leads to deregulated gene expression and plays a role in cancer initiation, progression, metastasis, and drug response (Banerjee et al., 2022). Four independent studies link DNA/histone methylation to the regulation of gene expression and highlight its relevance in disease pathogenesis or prognosis. Cai et al. have shown that a subset of the differentially expressed genes in lung cancer adenocarcinoma (LUAD) is regulated epigenetically by the DNA methylation or miRNAs and may hold prognostic/therapeutic significance. Zhu and Guo demonstrate the diagnostic significance of differential methylated and expressed genes in programmed cell death protein 1 (PD-1) negative hepatocellular carcinoma patients that are known to be resistant to immune checkpoint inhibitors. Global hypomethylation and large transcriptomic changes associated with PD-L1 (ligand of PD-1) have been previously reported (Chatterjee et al., 2018; Ahn et al., 2021). Sumei et al. showed a strong correlation of *DHRS3* (Dehydrogenase/Reductase 3) promoter hypermethylation with the histological type and poor tumour differentiation in gastric cancer patients. Similarly, Meng et al. focus on Transmembrane proteaseserine-2 (*TMPRSS2*), a gene involved in promoting the SARS-CoV-2 virus entry into the host cells, to examine how it is altered in various cancers and is significantly correlated with the promoter methylation status in a subset of these cancers. Interestingly, the authors further suggest that cancer patients with high *TMPRSS2* levels

may be more susceptible to SARS-CoV-2 infection and thus must exercise extra precautions, however this needs functional and clinical validation.

Besides DNA methylation, histone methylation also impacts genome regulation by affecting the binding of transcription factors and other expression machinery. Punnia-Moorthy et al. review the significance of lysine demethylases (KDMs) in melanoma pathobiology and elaborate on the interactive networks, potential inhibitors and their newly recognized role in immune response in cancer as well as in X-inactivation.

RNA modifications emerged as an important player in cancer. A review by Xu et al. summarizes the various RNA modifications (m6A, m5C, and m1A) involved in the regulation of transcript stability, export or translational efficiency in hepatocellular carcinoma pathophysiology. Liu et al. studied N6-Methyladenosine modification patterns in Pancreatic Adenocarcinoma (PAAD) patients and the impact it has on the tumor immune microenvironment and patient prognosis. Huang et al. performed correlations between the expression levels of m5C-related regulators and clinicopathological features in Colon Adenocarcinoma (COAD) patients. A study by Hu et al. demonstrates specific alternative polyadenylation events of transcripts as predictors of overall survival as well as progression-free survival of sarcoma patients with high accuracy. Considering that altered APA events may have a strong impact on the miRNA-mediated transcript regulation through its 3′UTR, the cross-talk of APA and regulatory non-coding RNAs must be investigated in future studies.

The long non-coding RNAs (lncRNAs) play a vital role in gene regulation through their interaction with DNA, RNA, or proteins. A review by Ti et al. sheds light on how lncRNAs act as the mediators of the cross-talk between cancer-associated fibroblasts (CAFs) and tumor cells in the lung cancer microenvironment leading to the promotion of a malignant phenotype and drug resistance. Two articles also highlight the importance of specific lncRNAs, PCAT6, and LINC00707 in cancer. Tan et al. identify a novel PCAT6/miR-143-3p/TAK1 axis as playing a crucial role in ovarian cancer by promoting proliferation, migration, and invasion. Yao et al. showcase LINC00707 as a promising biomarker and therapeutic target in various cancers and diseases. The review assesses various studies showing that LINC00707 by sponging various miRNAs affects the expression of downstream genes strongly associated with cancer progression/metastasis. In a review by Xue et al. the role of non-coding RNAs (miRNAs, lncRNAs, or circRNAs) in the regulation of Eukaryotic initiation factor 4A (eIF4A) family members is demonstrated along with the role of eIF4A family in the regulation of cancer proliferation, invasion, and metastasis. A review by Sharma et al. focuses on the role of the Wnt signaling pathway in development and cancer. It further delves into its interactions with the other pathways, epigenetic regulators, and non-coding RNAs leading to the fine-tuning of the cellular processes involved in normal development and cancer.

Overall, the large breadth and depth of the articles will facilitate the understanding of key roles played by epigenetic regulators in the initiation, progression, and aggressiveness of cancer. The series of articles will contribute to our understanding of the crucial role and varied mechanisms of epigenetic regulation in cancer and realise the immense potential of targeting these epigenetic players for the prediction and treatment of cancer.

Author contributions

RK drafted the manuscript and submitted it in its final form. AC contributed to writing and editing the manuscript draft. All authors contributed to the article and approved the submitted version.

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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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ORIGINAL RESEARCH
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Hypermethylation of *DHRS3* as a Novel Tumor Suppressor Involved in Tumor Growth and Prognosis in Gastric Cancer

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Background/Aims: The role of DHRS3 in human cancer remains unclear. Our study explored the role of *DHRS3* in gastric cancer (GC) and its clinicopathological significance and associated mechanisms.

Materials: Bisulfite-assisted genomic sequencing PCR and a Mass-Array system were used to evaluate and quantify the methylation levels of the promoter. The expression levels and biological function of *DHRS3* was examined by both *in vitro* and *in vivo* assays. A two-way hierarchical cluster analysis was used to classify the methylation profiles, and the correlation between the methylation status of the *DHRS3* promoter and the clinicopathological characteristics of GC were then assessed.

Results: The *DHRS3* promoter was hypermethylated in GC samples, while the mRNA and protein levels of *DHRS3* were significantly downregulated. Ectopic expression of *DHRS3* in GC cells inhibited cell proliferation and migration *in vitro*, decreased tumor growth *in vivo*. *DHRS3* methylation was correlated with histological type and poor differentiation of tumors. GC patients with high degrees of CpG 9.10 methylation had shorter survival times than those with lower methylation.

Conclusion: *DHRS3* was hypermethylated and downregulated in GC patients. Reduced expression of *DHRS3* is implicated in gastric carcinogenesis, which suggests *DHRS3* is a tumor suppressor.

Keywords: gastric cancer, DNA methylation, *DHRS3*, Mass-Array, carcinogenesis

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INTRODUCTION

The methylation patterns of DNA are highly variable among cell types and developmental stages and are influenced by environment and lifestyle (Verma and Manne, 2006; Horvath, 2013). DNA methylation is mainly found at the 5′-position of cytosine residues (5 mC or 5-methyl cytosine) followed by a guanine dinucleotide sequence (CpG). Regions in the genome that are

characterized by a particularly high CpG content are termed "CpG islands," and are present in approximately 60% of human gene promoters (Portela and Esteller, 2010). Aberrant DNA methylation has been linked to a variety of common diseases, especially cancer (Bock, 2009; Zhang and Jeltsch, 2010; Klutstein et al., 2016). It is now known that DNA methylation often occurs in gene promoters and is associated with transcriptional silencing of tumor suppressors or other genes important for normal cellular function (Corvalan, 2013; Huang et al., 2015; Gyorffy et al., 2016). Moreover, these alterations are usually among the earliest and most frequent molecular events known to occur during tumorigenesis (Akhavan-Niaki and Samadani, 2013). Great efforts have been made to identify novel methylation-based markers that can be used not only for the early detection of cancer, but also for determining risk, monitoring tumor progression, evaluating therapy response, and potentially for designing target specific epigenetic drugs (Beltran et al., 2008; Madu and Lu, 2010; Marzese and Hoon, 2015). Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related mortality worldwide (Torre et al., 2012). During the past few decades, extensive studies have been carried out to understand the molecular biology of GC. However, its molecular mechanisms are still not fully understood. Genetic and epigenetic alterations of tumor related genes have been studied widely (Tahara and Arisawa, 2015; Yoda et al., 2015). It was recently reported that the methylation of some molecules, such as cadherin 4, protocadherin 10, and Runt-related transcription factor 3 (Satoh et al., 2004; Yu et al., 2009; Llorca-Cardenosa et al., 2016), predisposes patients to GC, suggesting that DNA methylation may play an important role in GC tumorigenesis.

The human short-chain dehydrogenase/reductase (SDR) protein gene *DHRS3* was first identified in 1998 (Haeseleer et al., 1998), and is ubiquitously expressed in tissues and catalyzes the oxidation/reduction of a wide range of substrates including retinoids and steroids (Lundova et al., 2015). Deletion of *DHRS3* results in loss of local retinol storage, leading to shortage of vitamin A active metabolites and thus contributing to cancer development and progression (Cerignoli et al., 2002). Some studies have found monoallelic deletion of *DHRS3* in some neuroblastoma cell lines, but others have shown that *DHRS3* is constitutively expressed in breast cancer cell lines and highly expressed in papillary thyroid carcinoma (Haeseleer et al., 1998; Oler et al., 2008). Nevertheless, the differential expression patterns of *DHRS3* in different tumors indicate the context-dependent functions of this gene. Further study is needed to clarify the actual physiological roles of *DHRS3* in tumorigenesis in different types of tumors.

Recently, we utilized *in silico* sequence analysis to identify a long CpG island with high CpG density in the 5′ leader region of the *DHRS3* gene, spanning the gene promoter and the first exon. Here, we further evaluated the epigenetic regulation, expression profile, biological function, and clinical association of *DHRS3* in GC.

MATERIALS AND METHODS

Clinical Samples and Cell Lines

To detect methylation, paired primary cancer tissues and corresponding non-tumor tissues were obtained from 60 patients [10 for bisulfite-assisted genomic sequencing PCR (BSP), 50 for Mass-Array] with primal GC who had not received chemotherapy or radiotherapy before the operation. Clinical and pathological data were obtained from the surgical pathology records. The tissues were frozen in liquid nitrogen immediately after resection, and stored at −80°C. All patients provided informed consent before collection of the specimens in accordance with the guidelines of our institution. Our institution preserved four GC cell lines (SGC7901, MKN28, MKN45, and AGS) and one gastric epithelial cell line (GES). MKN28 cells were transfected with the *DHRS3* gene or empty vector using a lentiviral expression system (Genechem, Shanghai, China), cultured in 6-well plates, and continuously selected with puromycin to generate stable cell lines. The expression of *DHRS3* in cultured cells was confirmed by western blot via an anti-*DHRS3* antibody. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 80 units/mL penicillin and 100 mg/mL streptomycin at 37°C under 5% CO_2.

DNA Extraction and Bisulfite Modification

Genomic DNA was extracted from gastric tissues and cell lines using a QIAamp Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and analyzed by electrophoresis on 1.0% Tris/Borate/EDTA agarose gel containing 1% ethidium bromide. The DNA samples were modified by sodium bisulfite using a Zymo DNA Methylation-Gold kit (Zymo Research, Orange, CA) according to the manufacturer's instructions.

Bisulfite-Assisted Genomic Sequencing Analysis

Ten paired tissue specimens (also used for quantitative real-time PCR) were used for BSP. Briefly, genomic DNA treated with 2.5 μL of bisulfite was used as the template for amplification. The primer for the *DHRS3* promoter was designed using a web-based program (MethPrimer[1]) by the input of a 1 kb sequence upstream of the transcription start site (TSS) of *DHRS3* (Li and Dahiya, 2002); the following primer was used: *DHRS3* BSP: TTTTGTTTTTTTTAATTTGGAGAGG, ATCAAACTTTTAAAAATCCACTCTAC, 623 bp. The PCR products were cloned into a pMD 18-T Vector (TaKaRa, Dalian, China). Six clones were randomly chosen, and plasmids were extracted using a TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China) and sequenced using an ABI 3730 analyzer (Applied Biosystems, Foster City, CA). The most representative sequence for each

[1]http://www.urogene.org/methprimer/index1.html

sample was selected for analysis using a BiQ analyzer (Bock et al., 2005).

Mass-Array Analysis

To quantitatively determine the methylation status of the CpG islands of *DHRS3* in 50 paired tumor samples, the high-throughput quantitative methylation analysis platform Mass-Array (Sequenom, San Diego, CA) was carried out as described previously (Radpour et al., 2008, 2009). This system uses matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in combination with RNA base-specific cleavage. Briefly, a bisulfite-treated template was amplified using the same primers as those for bisulfite sequencing. The products were purified and then spotted on a 384-pad SpectroCHIP, followed by spectral acquisition on a Mass-Array Compact System. These signal pairs were used to estimate the ratio of methylated to unmethylated DNA. Methylation data of individual units (1–5 CpG sites per unit) were generated and analyzed using EpiTyper software (Agena Bioscience, Inc., San Diego, CA).

Demethylation With the DNA Demethylating Agent 5-Aza-2′-Deoxycytidine in GC Cells

SGC-7901, MKN28, MKN45, and AGS cells at 20% confluence were treated with 2 μmol/L of the DNA demethylating agent 5-Aza-2′-deoxycytidine (5-Aza, Sigma-Aldrich, St. Louis, MO) for 72 h. Cells were then harvested for RNA extractions.

Reverse-Transcription PCR and Quantitative Real-Time PCR

Total RNAs were extracted with Trizol reagent (Invitrogen, Carlsbad, CA). The mRNA expression level of *DHRS3* was measured by quantitative real-time PCR, using the following primer: *DHRS3*: CATGGGAAGAGCCTAATGGA, GACGCTTTGGATGTGCAGTA; 200 bp. The program was performed using a Light-Cycler 480 system (Roche Diagnostics, Risch-Rotkreuz, Switzerland) with the SYBR Premix Ex Taq II (TaKaRa) reagent. Amplification of each sample was repeated three times, and the specific product was analyzed by melting curve at the end of the program. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Gene expression level was quantified by the comparative CT method.

Immunohistochemical Staining

Immunohistochemical staining of *DHRS3* was performed on parallel histopathological sections from paraffin-embedded tumor sections and corresponding adjacent normal tissue using an anti-*DHRS3* antibody (Biosynthesis Biotechnology, Beijing, China) diluted at 1:300. All sections were examined microscopically and scored by three independent pathologists blinded to the clinical and pathological information. The expression of *DHRS3* was evaluated according to the ratio of positive cells per specimen and staining intensity. The ratio of positive cells per specimen was evaluated quantitatively and scored 0 for staining of 0–1%, 1 for staining of 2–25%, 2 for staining of 26–50%, 3 for staining of 51–75%, and 4 for staining >75% of the cells examined. Intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong staining. A total score of 0–12 was calculated using the following formula: total score = ratio of positively stained cells (score) × intensity of immunoreactivity (score) and graded as negative (I; score: 0–1), weak (II; 2–4), moderate (III; 5–8), or strong (IV; 9–12).

Cell Proliferation Assay

Stable transfected cells were seeded in 96-well plates at a density of 3,000 cells per well and grown under normal conditions. Viable cell counts were determined in triplicate on days 1–7 using the MTT assay.

Colony Formation Assay

Cells were infected with *DHRS3* or the empty vector using a lentiviral expression system and plated in 6-well plates at 300 cells/well. After 2 weeks, colonies stained with Giemsa solution were counted. The assay was performed in triplicate.

Wound-Healing Assays

Cells were seeded into 24-well plates and cultured until 80–90% confluence. A 10-μL pipette tip was used to make a vertical wound. Then, cells were washed three times with culture medium to remove cell debris. The extent of wound closure was monitored at designated time points in the same position.

Transwell *in vitro* Migration Assays

Cells in serum-free medium (200 μL containing 5×10^4 cells) were added to the top chambers of transwell chambers. The bottom chamber contained medium with 20% FBS as a chemoattractant. The cells were incubated for 24 h at 37°C. Then, cells that had migrated through the membrane and attached to the lower surface were stained using a fixative/staining solution (0.1% crystal violet, 1% formalin, and 20% ethanol) and quantified under a microscope. The tests were repeated three times.

Flow Cytometry Analysis

MKN28 cells were infected with *DHRS3* or empty vector using a lentiviral expression system. Cells were harvested and fixed in 70% ice-cold ethanol for 1 h, washed with phosphate-buffered saline (PBS), and stained with propidium iodide (PI) in PBS supplemented with RNase for 20 min. Cells were sorted by FACScan (BD Biosciences, Franklin Lakes, NJ). Cell apoptosis assays were performed using the annexin V/PI kit by flow cytometry analysis. The stained cells were finally analyzed by FACScan.

Tumorigenicity Assay in Nude Mice

MKN28 cells infected with *DHRS3* or control lentivirus were injected subcutaneously into the lateral root of one posterior limb of nude mice (2×10^6 cells/mouse; five mice in each experimental group). Tumor length was measured every week for 5 weeks. The care of the experimental animals

was in accordance with the institutional animal care and use committee guidelines.

Statistical Analysis

Data analysis was performed using SPSS version 17.0 (Chicago, IL). The Shapiro-Wilk test was used to analyze normality. The mRNA expression dataset was not normally distributed. The Wilcoxon signed ranks test was used to analyze the difference in expression levels between paired tumor specimens and the corresponding adjacent non-tumor tissue specimens. Independent-samples *t*-test was used for two groups of data and one-way analysis of variance was used for three groups of data to compare methylation levels between the sample groups. Using two-way hierarchical cluster analysis, we examined the relationships between methylation levels in CpG sites and tissues (Radpour et al., 2008). Overall survival in relation to methylation status was evaluated using the Kaplan-Meier survival curve. Two-sided values of $P < 0.05$ were considered statistically significant.

RESULTS

DHRS3 Promoter Was Hypermethylated in GC Patients

Using *in silico* sequence analysis, we identified a long CpG island with high CpG density in the 5′ leader region of *DHRS3* gene

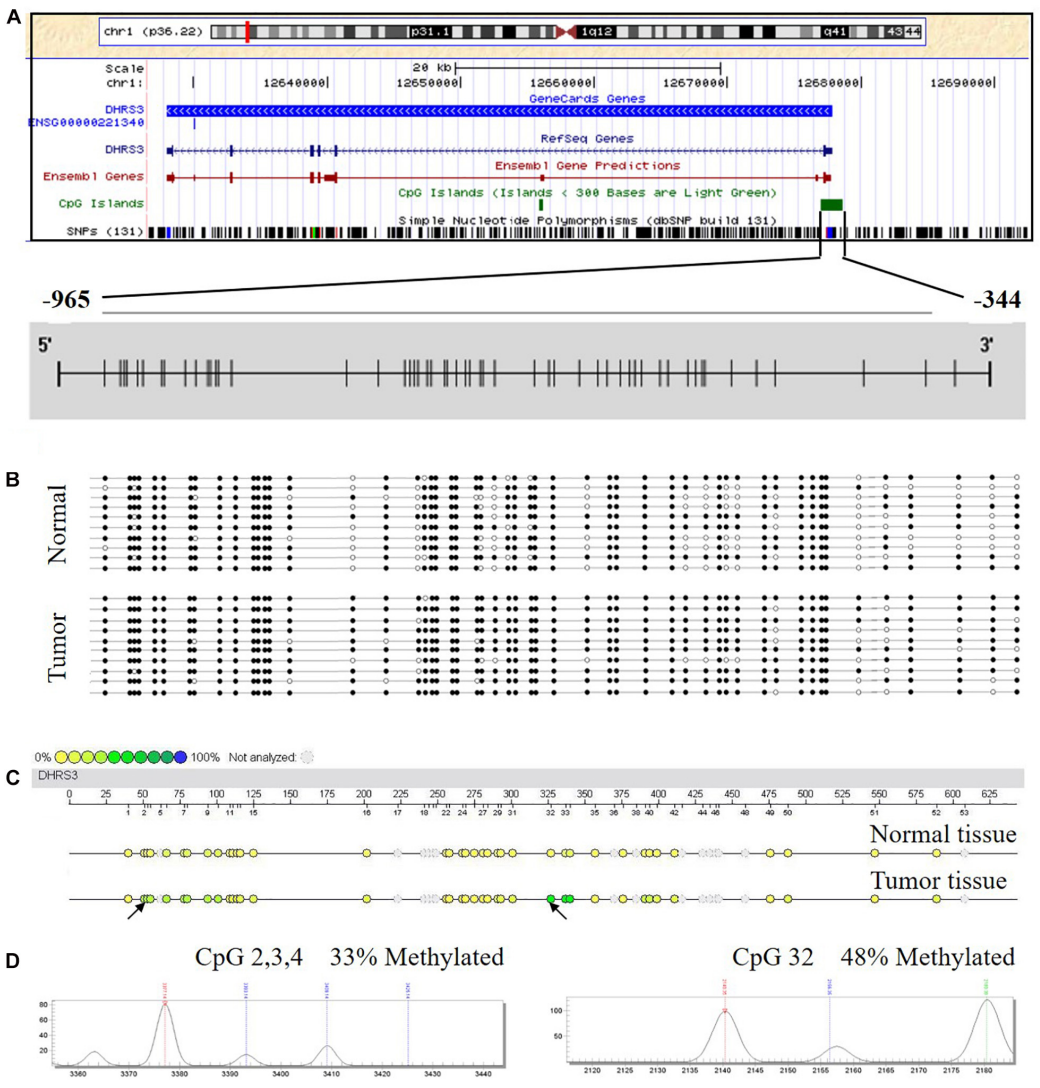


FIGURE 1 | The DHRS3 promoter in human GC tumors and cell lines is hypermethylated. **(A)** Gene location, amplicon, and CpG sites (short vertical lines) in relation to the promoter of DHRS3. **(B)** A simplified illustration of BSP reads for a tumor sample and its adjacent normal sample. Open and filled circles represent unmethylated and methylated CpG sites, respectively. **(C)** Methylation profile of CpG sites of the DHRS3 gene. The colors of the circles are related to the percentage methylated in each CpG site. Arrows indicate different methylation patterns between cancerous tissues and adjacent paired normal tissues. **(D)** Double dendrogram of the DHRS3 gene. Peaks show the methylation rates of the two CpG sites in the DHRS3 gene, which indicate differences between cancerous and paired normal tissues.

that spanned the gene promoter and the first exon, implying the potential for epigenetic regulation via DNA methylation. To test this hypothesis, we examined the methylation status of *DHRS3* promoters in 10 human GC tissues and 10 corresponding normal mucosa samples by BSP. The region we examined started from 344 bp upstream of the TSS, extended for another 621 nt upward, and contained 53 CpGs (**Figure 1A**). We found different methylation patterns of the *DHRS3* promoter between the tumor and normal tissues. *DHRS3* promoters in tumor samples were generally hypermethylated (**Figure 1B**). Moreover, the differently methylated regions were unevenly distributed along the promoter, with some sites more pronounced than others, such as CpG 16, 18, 25, 26, 32, 37, 38, 40, 41, 48, and 52. These results indicated the presence of primary sites of methylation, which may be critical for the maintenance of gene repression, and candidate sites for the initiation of inactivation (Park and Chapman, 1994).

Quantitative Methylation Analysis and Identification of Hypermethylated CpG Sites

We analyzed the methylation status of *DHRS3* in more detail in 100 primary tumor tissues and corresponding non-tumor tissues from 50 patients with GC by Mass-Array. We analyzed 53 CpG sites per sample (a total of 5,300 sites in 100 samples) and found that more than 71% of the CpG sites were in the amplicons (**Table 1**). These Mass-Array data demonstrated that the CpG sites were consistent with the BSP results (**Figures 1C,D**). Moreover, using Mass-Array, we detected a high degree of methylation in cancerous tissues compared with corresponding normal tissues ($P < 0.01$, **Table 2**). The mean methylation levels of the tumor group were increased by about 57% over the normal group ($P < 0.01$, **Figure 2A**). Among the CpG islands in tumors, 42% had significant changes in methylation. A two-way hierarchical cluster analysis was performed using CpGs with high discriminatory significance. For improved reliability, we included only the samples with at most one missing measure of CpG unit. Two major groups were identified: one was dominated by measurements from tumor cells and the other by normal cells (**Figure 2B**), although there were a few instances of crossover. Interestingly, further analysis showed that two tumor samples, which were clustered into the normal group, were actually in an early stage of tumor development (T_2). For the 10 normal samples clustered into the tumor group, 7 were from the normal group, among which the paired tumors were in an advanced stage (T_3 or T_4). These results suggest that some normal tissues adjacent to the advanced tumor may have already undergone epigenetic change toward tumor formation. On the other hand, some early stage tumors may still retain the epigenetic characteristics of normal tissue.

TABLE 2 | Characteristics of tumor group and adjacent normal group.

	Number of patients (%)	Methylation level of tumor sample (mean ± SD)	Methylation level of normal sample (mean ± SD)	*P**
Gender				
Male	39 (78%)	0.11 ± 0.11	0.07 ± 0.10	<0.001
Female	11 (22%)	0.10 ± 0.11	0.07 ± 0.08	0.005
Age (year)				
<65	36 (72%)	0.12 ± 0.12	0.07 ± 0.08	<0.001
≥65	14 (28%)	0.10 ± 0.11	0.08 ± 0.12	0.135
TNMstage				
T1, T2	13 (26%)	0.11 ± 0.10	0.09 ± 0.12	0.1
T3, T4	37 (74%)	0.10 ± 012	0.07 ± 0.09	<0.001
Vessel invasion				
Negative	41 (82%)	0.10 ± 0.11	0.08 ± 0.10	<0.001
Positive	9 (18%)	0.11 ± 0.14	0.06 ± 0.07	0.002
Lauren type				
Intestinal	33 (66%)	0.10 ± 0.10	0.07 ± 0.10	<0.001
Diffuse	7 (14%)	0.16 ± 0.22	0.09 ± 0.09	0.148
Mix type	10 (20%)	0.12 ± 0.12	0.08 ± 0.09	0.002
Differentiation				
Poor	27 (54%)	0.11 ± 0.11	0.08 ± 0.08	<0.001
Moderate	17 (34%)	0.09 ± 0.10	0.07 ± 0.13	0.048
Well	6 (12%)	0.08 ± 0.14	0.05 ± 0.06	<0.001
Total	50 (100%)	0.11 ± 0.11	0.07 ± 0.10	<0.001

**Calculated using independent samples t-test for two groups of data.*

DHRS3 Transcription Decreased in GC Tissues and Cell Lines

To explore the correlation between gene transcription and promoter methylation, we next examined the mRNA levels of *DHRS3* gene in the same set of samples as above. We found that *DHRS3* mRNA levels were significantly lower in tumor tissues than in normal tissues ($P < 0.05$, **Figure 3A**), supporting the notion that DNA methylation of the *DHRS3* gene promoter correlates inversely with the expression of the gene. To extend this observation, we treated gastric tumor cells (SGC7901, AGS, MKN45, and MKN28) with a demethylating agent (5′-Aza) and quantified the *DHRS3* mRNA. In comparison with normal gastric GES cells, all four GC cell lines showed significantly reduced levels of mRNA. Treatment with 5′-Aza for up to 72 h resulted in increased mRNA levels in the four GC cell lines (**Figure 3B**). The levels of *DHRS3* mRNA were significantly increased in well-differentiated MKN28 cells treated with 5′-Aza

TABLE 1 | High-throughput methylation analysis of informative CpG sites in amplicons for DHRS3.

Gene	Amplicon size (bp)	Total no. of CpG sites in amplicon	No. of analyzed CpG sites in amplicon	No. of analyzed CpG sites per amplicons	
				Single sites	Composite sites
DHRS3	622	53	38	10	28

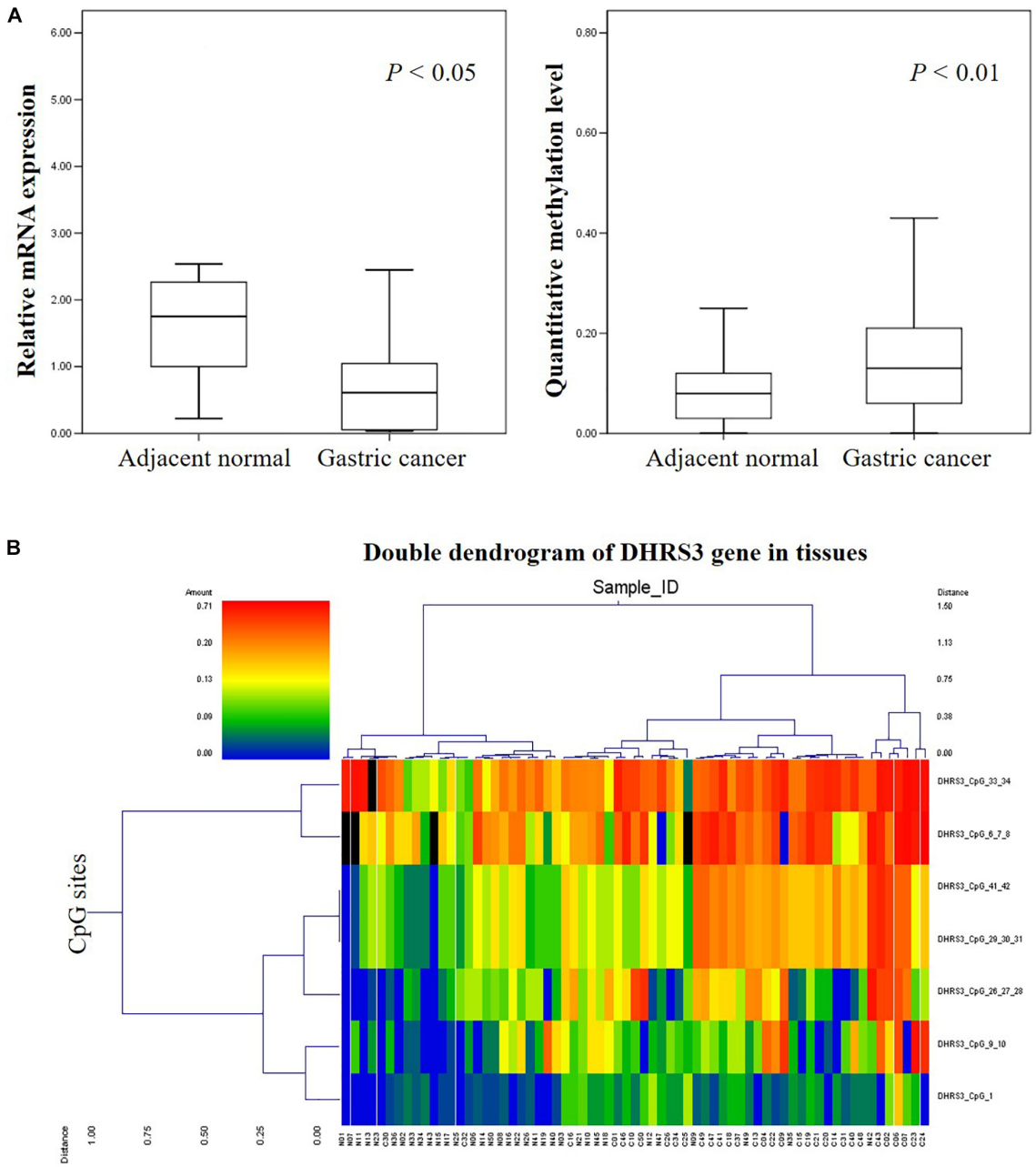


FIGURE 2 | High-throughput analysis of the informative CpG sites for the DHRS3 gene. **(A)** DHRS3 was hypermethylated and lacked mRNA expression in GC. (1) Box plots indicating the relative expression levels of DHRS3 mRNA in 21 paired GC tissues. Sample one served as a calibrator. *P*-values were estimated using a paired two-sample *t*-test. (2) Box plots indicating the quantitative DNA methylation analysis by Mass-Array. *P*-values were estimated using an independent samples *t*-test. **(B)** Two-way hierarchical cluster analysis of tumor tissues and adjacent normal tissues (red clusters are 71% methylated, green clusters are 0% methylated). The color gradient between red and green indicates methylation ranging from 0 to 71%, and black clusters indicate CpG sites that were not analyzed.

($P < 0.05$). In contrast, in the poorly differentiated MKN45 cells, 5′-Aza increased the mRNA level by about half that in MKN28, while the other two cell lines, AGS and SGC-7901, displayed marginal increases in *DHRS3* mRNA following 5′-Aza treatment. The response to 5′-Aza was cell-line dependent, which may also reflect differences in the initial methylation status in cultured cells.

Downregulation of *DHRS3* Protein in Primary GC Tissues

To further support the notion that hypermethylation of the *DHRS3* gene promoter results in decreased gene expression, we next determined *DHRS3* protein levels in GC and normal tissue by immunohistochemical staining in 70 paired samples (**Figure 3C**). Of the normal tissues, 82.9% were strongly stained

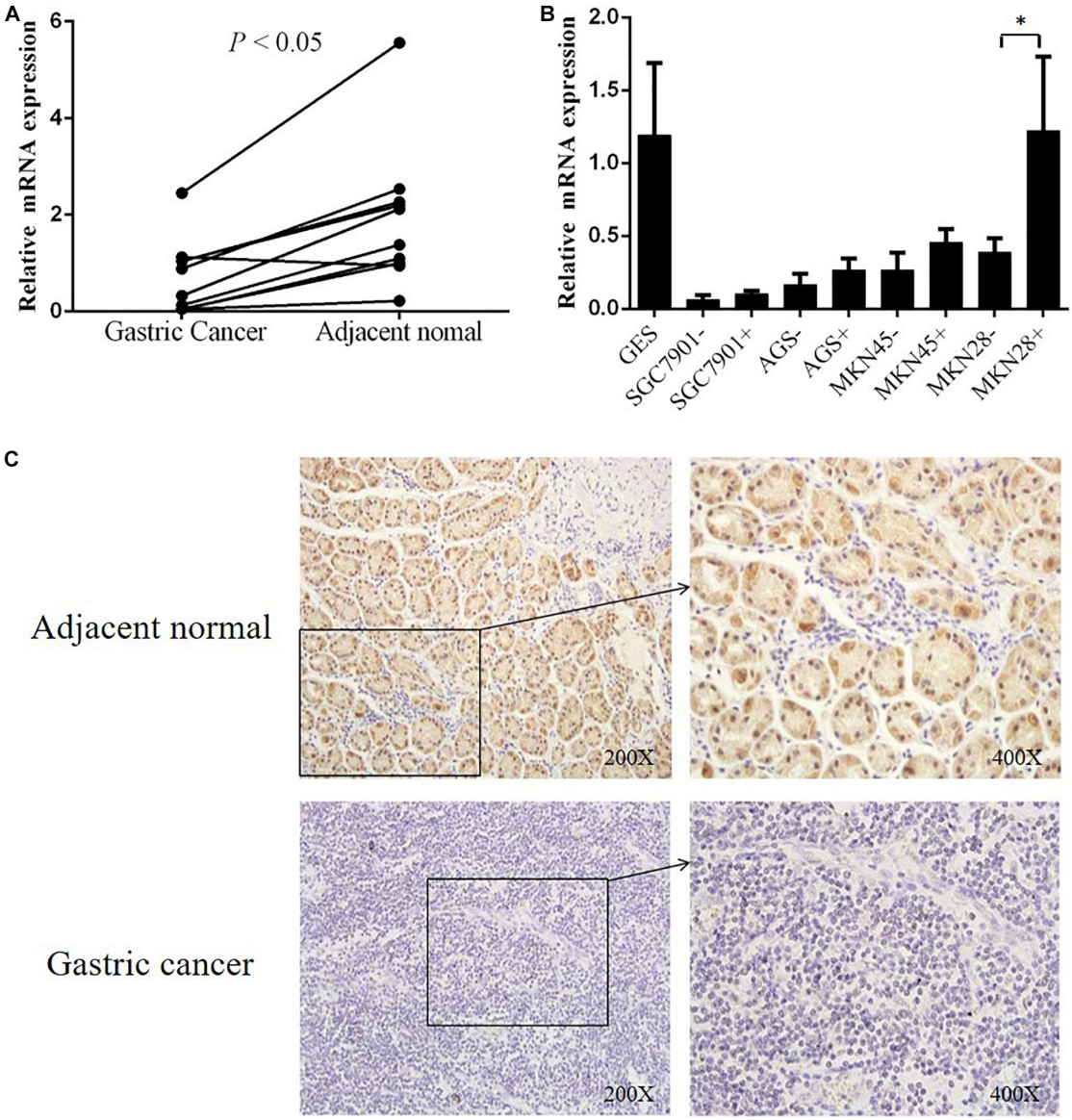


FIGURE 3 | DHRS3 gene expression was significantly down regulated in primary GC tumors. **(A)** Expression levels of DHRS3 mRNA in 10 paired human gastric specimens. The *P*-value was calculated using the paired two-sample *t*-test. **(B)** mRNA expression of DHRS3 in five cell lines (GES, SGC7901, MKN45, MKN28, and AGS) before and after 5′-Aza treatment. **(C)** Representative immunohistochemical staining of DHRS3 protein of primary GC tumors and their adjacent non-tumor tissues. $^*P < 0.05$.

with anti-*DHRS3* antibody, while most GC samples were weakly stained (38.6%) or negative (41.4%), demonstrating that *DHRS3* protein was significantly downregulated in GC tissues compared with normal tissues ($P < 0.01$, **Table 3**).

Overexpression of *DHRS3* Inhibited Cell Proliferation and Migration of GC Cells *in vitro* and *in vivo*

The silencing of *DHRS3* in primary GC cells suggests that *DHRS3* may function as a tumor suppressor. We infected MKN28 cells with a lentiviral expression system to enhance the expression of *DHRS3* and confirmed it by western blot analysis (**Figure 4A**). Ectopic expression of *DHRS3* significantly inhibited cell proliferation of MKN28 cells as determined by MTT assay ($P < 0.05$, **Figure 4B**). Colonies formed by cells over-expressing *DHRS3* were fewer in number and smaller in size than those formed by control cells ($P < 0.05$; **Figure 4C**). To further investigate the tumor suppressive effect of *DHRS3* in GC cells, we investigated whether *DHRS3* could suppress the growth of GC cells in nude mice. The mean longitudinal tumor length was assessed every week after injection with MKN28 cells infected with either *DHRS3* or vector lentivirus. The tumor growth curve showed that the mean longitudinal lengths of

TABLE 3 | DHRS3 expression in GC tissues and matched adjacent normal tissues.

	Negative (–)	Weak (+)	Moderate (++)	Strong (+++)	*P*
Normal	2 of 70 (2.8%)	4 of 70 (5.7%)	6 of 70 (8.6%)	58 of 70 (82.9%)	<0.01
GC	29 of 70 (41.4%)	27 of 70 (38.6%)	11 of 70 (15.7%)	3 of 70 (4.3%)	

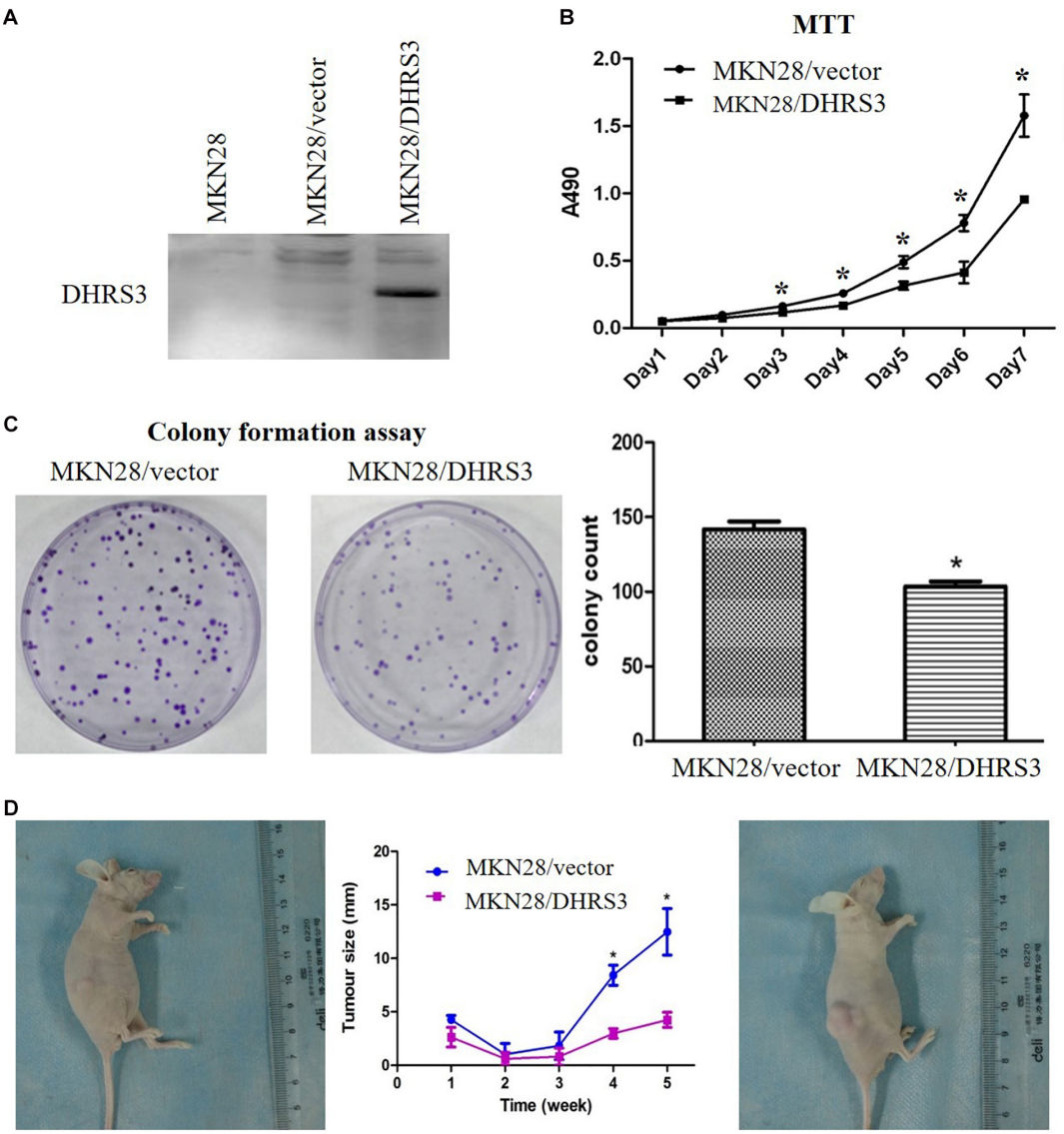


FIGURE 4 | Over-expression of DHRS3 inhibited GC cell growth in cultured cells and in nude mice. **(A)** Infection of MKN28 cells with DHRS3 and control lentivirus. After selection with puromycin for 3 weeks, the expression of DHRS3 was confirmed by western blotting. **(B)** Cell growth was measured using the MTT assay. Proliferation was significantly less in MKN28/DHRS3 cells compared with MKN28/vector cells. **(C)** The colony formation assay was used to test the effect of DHRS3 on cancer cell growth. Cells were plated in 6-well plates at 300 cells/well. After 2 weeks of incubation, cells were stained. The left panel shows representative dishes after transfection with empty vector or DHRS3. Quantitative analyses of colony numbers are shown in the right panel. Values are the mean ± standard deviation (SD) of at least three individual experiments. **(D)** DHRS3 inhibited tumor growth in nude mice. A representative picture of tumor growth in nude mice subcutaneously inoculated with MKN28/DHRS3 (left) or MKN28/vector (right) cells at the end of the observation. Tumor longitudinal length (mean ± SD) was assessed every week. Tumor growth curves were plotted against days after treatment. Statistical analysis indicated significantly slower tumor growth in the MKN28/DHRS3 group. *$P < 0.05$.

tumors over-expressing *DHRS3* were significantly smaller than tumors infected with vector ($P < 0.05$, **Figure 4D**). In addition, over-expression of *DHRS3* significantly inhibited the migration of MKN28 cells (**Figures 5A,B**). These results demonstrated that *DHRS3* inhibited cell growth and migration, indicating that *DHRS3* is a potential tumor suppressor, at least for GC.

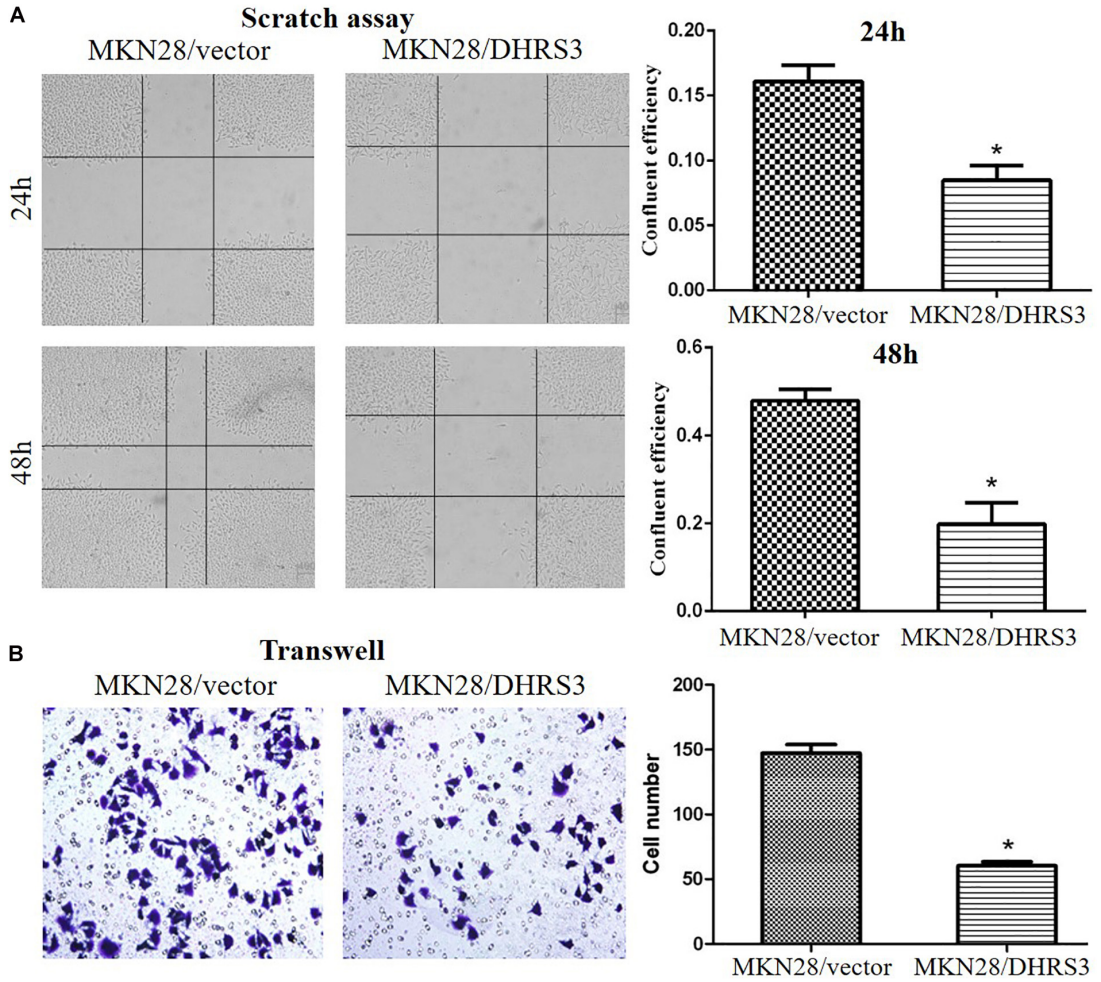


FIGURE 5 | Over-expression of DHRS3 inhibited GC cell migration. **(A)** MKN28 cells were infected with DHRS3 or the empty vector. When cells were confluent, a scratch wound healing assay was performed. The scratch gap was periodically monitored and recorded. Ectopic expression of DHRS3 suppressed tumor cell migration. **(B)** DHRS3 impeded tumor cell migration in MKN28/DHRS3 cells. The results are expressed as mean ± SD of three independent experiments. *$P < 0.05$.

Ectopic Expression of *DHRS3* Induced G1 Phase Arrest and Apoptosis in GC Cells

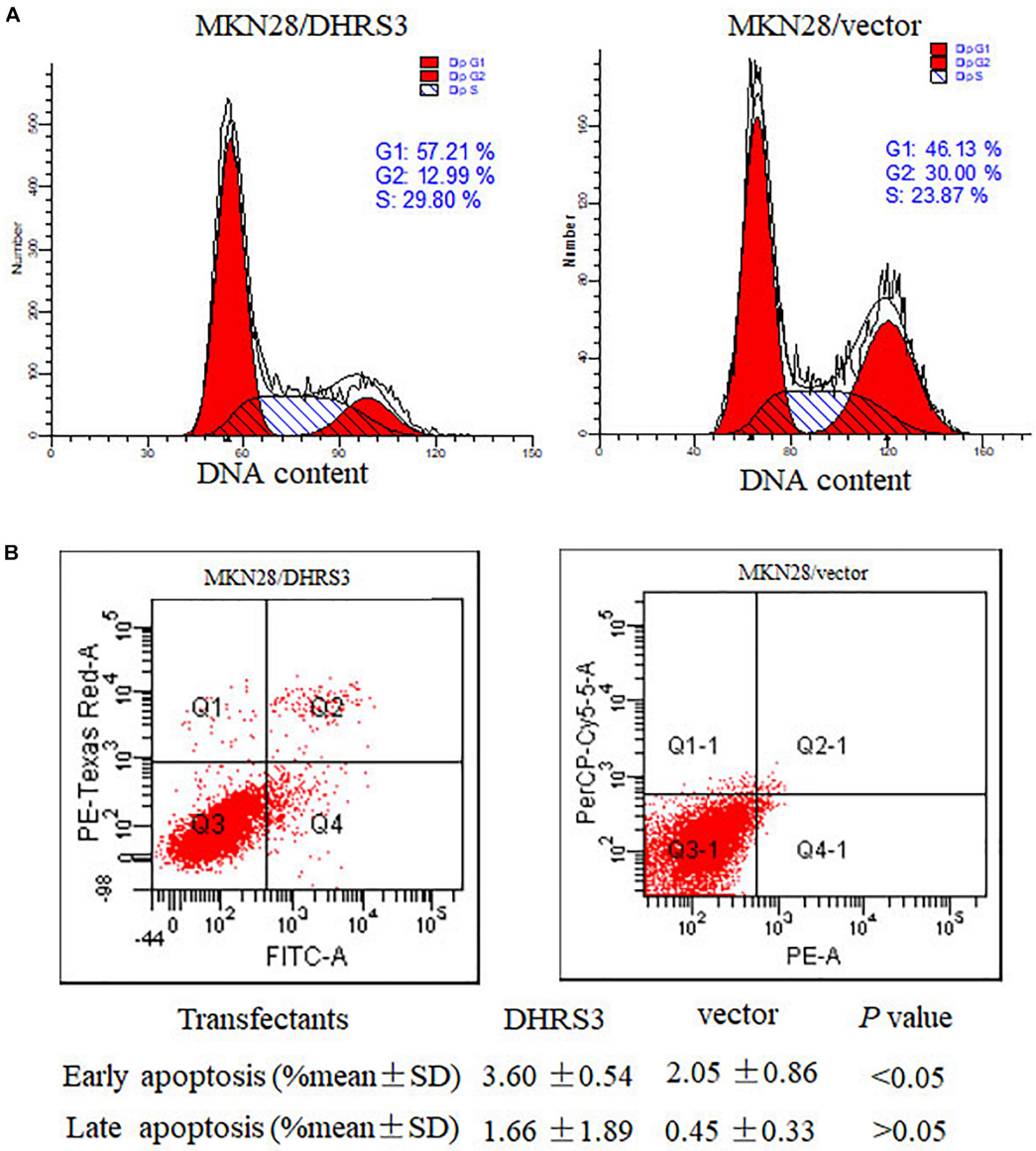
To explore the mechanism by which *DHRS3* suppressed cell growth and proliferation, we used flow cytometry to evaluate the effects of *DHRS3* on cell cycle progression and apoptosis in MKN28 cells. Ectopic expression of *DHRS3* resulted in apparent G1 phase arrest (**Figure 6A**) and induced early apoptosis ($P < 0.05$, **Figure 6B**). These data support the inhibitory effect of *DHRS3* on cell proliferation in GC cells.

Hypermethylation of *DHRS3* Was Associated With Histological Type and Poor Differentiation

We next extensively analyzed the correlation between the quantitative methylation based on Mass-Array data and the clinicopathological characteristics of patients including age, sex, tumor stage, histological type, differentiation, vessel invasion, and lymph node metastases (**Table 4**). First,we found a trend toward a positive association between hypermethylation of the *DHRS3* promoter and histological type ($P < 0.05$) and poor differentiation ($P < 0.05$). Second, because methylation of certain genes can increase with age, we analyzed the ages of patients at the time of surgery and the methylation level of the *DHRS3* gene promoter. No significant correlation was observed with regard to patient age, tumor stage, vessel invasion, or sex.

Low Methylation Levels of Composite CpG 9 and CpG 10 Was Associated With Longer Survival

In an attempt to assess the prognosis of GC patients based on the methylation levels of *DHRS3* after surgery, we further analyzed the Mass-Array data and found that composite CpG sites 9 and CpG 10 were associated with an increased risk of cancer-related death. GC samples were categorized into two groups according to the methylation level of CpG 9 and CpG 10 in the GC samples. Among them, 65.7% had below average methylation levels and



Transfectants	DHRS3	vector	*P* value
Early apoptosis (%mean ± SD)	3.60 ± 0.54	2.05 ± 0.86	<0.05
Late apoptosis (%mean ± SD)	1.66 ± 1.89	0.45 ± 0.33	>0.05

FIGURE 6 | DHRS3 induced cell-cycle arrest in G1 phase and cell apoptosis. **(A)** Analysis of the cell cycle was performed by flow cytometry in the MKN28 cells stably transfected with DHRS3 or empty vector. Overexpression of DHRS3 resulted in an increase in the percentage of cells in G1 phase (57.21 vs. 46.13% in control cells). **(B)** The rate of cell apoptosis was determined by flow cytometry. Q4 indicates early apoptotic cells, Q2 shows late apoptotic cells. Results were obtained from four independent experiments.

34.3% of GC samples showed above average methylation of CpG 9 and CpG 10. Kaplan–Meier survival curves demonstrated that GC patients with low methylation levels of CpG 9 and CpG 10 had significantly longer survival times (median, 20 months) than those with high methylation levels (median, 16 months; $P < 0.05$; **Figure 7A**).

DISCUSSION

Tumorigenesis is a multistep process with gradual accumulation of genetic and epigenetic alterations. An increasing number of tumor suppressor genes have been found to harbor CpG islands in their gene promoters and the first exon where the epigenetic regulation predominately occurs, indicating an alternative mechanism to genetic inactivation of tumor suppressor genes. In this study, we found that the *DHRS3* gene was hypermethylated in GC patients and correlated with decreased *DHRS3* mRNA and protein levels in primary tumors. Demethylation by treatment with 5′-Aza increased the expression of *DHRS3* in cultured GC cells. Moreover, high degree of *DHRS3* methylation was associated with unfavorable clinicopathological features and shorter survival times in GC patients. These results proved that *DHRS3* downregulation may

TABLE 4 | Methylation levels in GC tissues.

	Methylation level of tumor sample (mean ± SD)	*P**	Methylation level of normal sample (mean ± SD)	*P**
Gender				
Male	0.11 ± 0.11	0.936	0.07 ± 0.10	0.857
Female	0.10 ± 0.11		0.07 ± 0.08	
Age (year)				
<65	0.12 ± 0.12	0.688	0.07 ± 0.08	0.109
≥65	0.10 ± 0.11		0.08 ± 0.12	
TNMstage				
T1, T2	0.11 ± 0.10	0.473	0.09 ± 0.12	0.034
T3, T4	0.10 ± 0.12		0.07 ± 0.09	
Vessel invasion				
Negative	0.10 ± 0.11	0.811	0.08 ± 0.10	0.147
Positive	0.11 ± 0.14		0.06 ± 0.07	
Lauren type				
Intestinal	0.10 ± 0.10	0.011 (For intestinal versus diffuse $P = 0.009$)**	0.07 ± 0.10	0.154
Diffuse	0.16 ± 0.22		0.09 ± 0.09	
Mix type	0.12 ± 0.12		0.08 ± 0.09	
Differentiation				
Poor	0.11 ± 0.11	0.0164 (For poor versus well $P = 0.023$)**	0.08 ± 0.08	0.073
Moderate	0.09 ± 0.10		0.07 ± 0.13	
Well	0.08 ± 0.14		0.05 ± 0.06	

**Calculated by using independent samples t-test for two groups of data and using one-way ANOVA for three groups of data.*
***Compared by using lsd method for two of three groups.*

play an important role in the development of GC and may carry tumor suppressor potential.

As a member of the SDR family, *DHRS3*, encodes an enzyme involved in the metabolism of retinol (vitamin A). Retinol, apart from its unique role in the production of visual pigments, also has potential preventive effects on malignant neoplasms (Morriss-Kay and Ward, 1999; Chandrasekaran et al., 2000). It has been noted that vitamin A may influence gastric carcinogenesis (Larsson et al., 2005; Wang, 2005) and that it is inversely associated with GC (Stahelin et al., 1984; Stehr et al., 1985; De Stefani et al., 2000). *DHRS3*, which is frequently deleted or rearranged in neuroblastomas, is critical for the generation of a storage form of retinol. Further study has elucidated that deletion of *DHRS3* might contribute to cancer development and progression by reducing the production of vitamin A (Cerignoli et al., 2002). Thus, exploring the biofunction of *DHRS3* in GC will help to enable an understanding of the mechanisms underlying GC.

The GENT database indicates that *DHRS3* mRNA is down-regulated in cancers of the bladder, lung, ovary, skin, stomach, and vagina compared with corresponding normal tissues, but is upregulated in cancers of the brain, cervix, and uterus (**Figure 7B**). The distinct expression status of *DHRS3* among different tumor types needs further investigation to gain an understanding of the physiological function of the gene during tumorigenesis. We demonstrated that the protein expression of *DHRS3* was downregulated in a large percentage of gastric tumor specimens compared with corresponding non-tumor tissues. This dynamic change in the expression of *DHRS3* between primary tumors and corresponding normal samples implied that *DHRS3* may have important effects in the development and progression of GC.

To further study the supposed tumor suppressor function of *DHRS3* in GC, we performed both *in vitro* and *in vivo* assays. Re-expression of *DHRS3* significantly inhibited both growth and clone formation of cultured MKN28 cells and reduced tumor size in nude mice. Furthermore, ectopic expression of *DHRS3* induced G1 phase arrest and apoptosis. Taken together, it is implied that *DHRS3* plays the role of a tumor suppressor in GC.

Hypermethylation of CpG islands in gene promoter regions is an important mechanism of gene inactivation during tumorigenesis. By using BSP and Mass-Array, we found that the promoter region of *DHRS3* gene was frequently methylated in GC patients. Furthermore, our findings showed that some CpG sites are more frequently methylated in the promoter region. This result suggested that these candidate sites may play a more important role in *DHRS3* transcription, and this finding was consistent with those from a recent study showing that not all CpG sites in the promoter region have equal function (Zou et al., 2006).

Although promoter methylation has been widely studied in GC patients, most reports lack quantitative analysis. The Mass-Array system allows robust analysis of the methylation status of primary tumors compared with normal tissues for further use in molecular detection. In this study, we analyzed the majority of the cases by Mass-Array and found that *DHRS3* promoter methylation was tumor specific ($P < 0.01$). Tissues located 3–5 cm away from the tumor are currently considered adjacent normal tissues. However, in our two-way hierarchical cluster analysis, 10 morphologically normal samples clustered into the tumor group. For 7 of these 10 samples, their paired tumor samples belonged to T_3 or T_4 stage. This result suggested that the morphologically normal tissues may have already undergone epigenetic change, and this may provide a new insight for resection standards in the future. Most importantly, we found that high levels of *DHRS3* methylation correlated significantly with shorter survival time in GC patients. Thus, the inactivation of *DHRS3* by methylation of the promoter would favor tumor progression and lead to a worse outcome for patients. To our knowledge, this is the first report to demonstrate that *DHRS3* functions as a tumor suppressor gene in GC patients. *DHRS3* promoter hypermethylation in GC patients can be used as an epigenetic biomarker not only for making a diagnosis but also for predicting the prognosis.

In conclusion, we identified *DHRS3* as a specific target for aberrant CpG island hypermethylation in GC patients and its

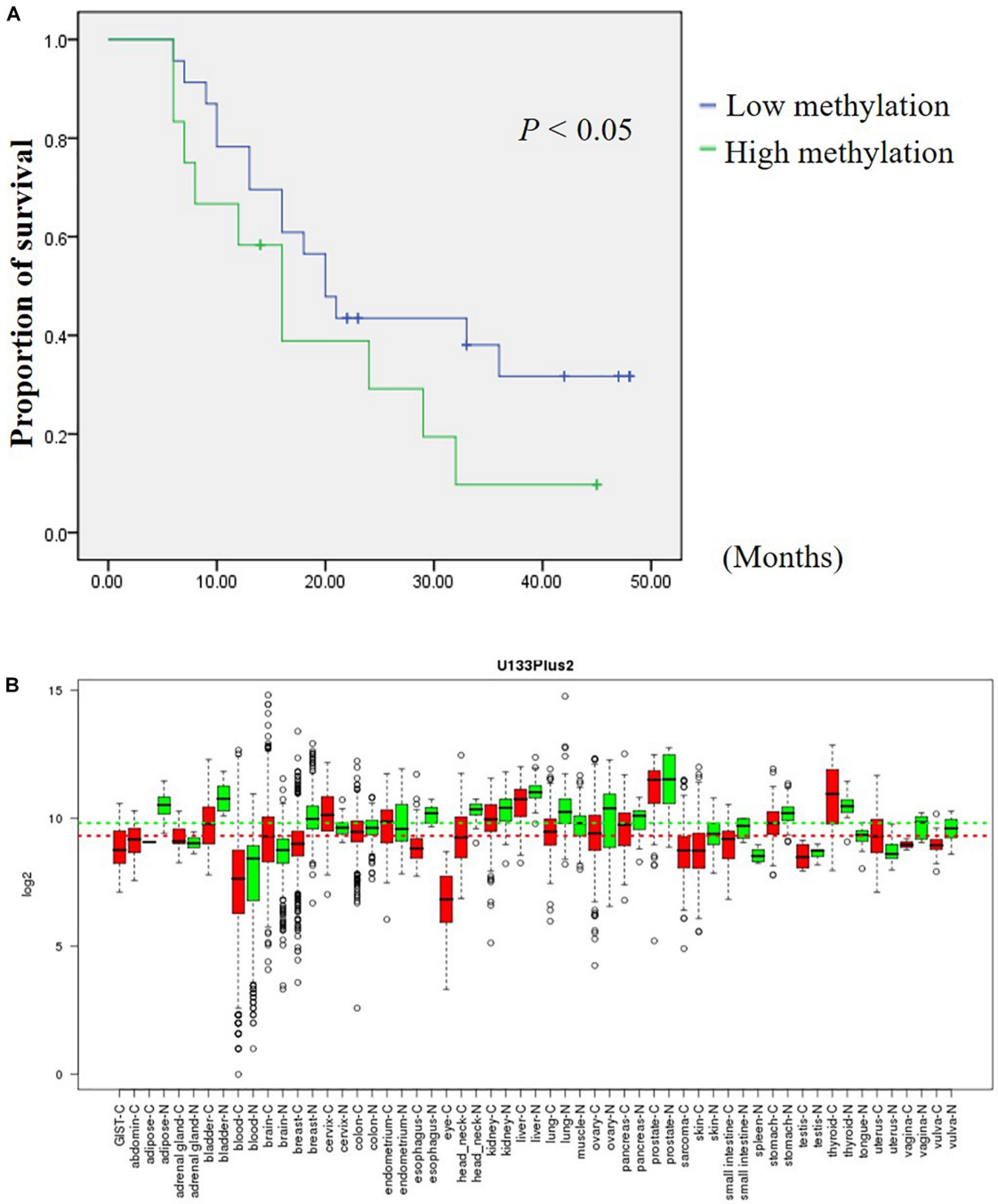


FIGURE 7 | DHRS3 is down regulated in various tumors and high methylation levels of DHRS3 was associated with poor survival. **(A)** Survival curves were plotted for Kaplan-Meier survival analysis. The methylation status of CpG 9 and CpG 10 of the GC samples was used as the variable to separate two groups. Patients with high levels of methylation had poorer survival times than those with low levels of methylation ($P < 0.05$). **(B)** Expression pattern of DHRS3 mRNA in normal and tumor tissues. DHRS3 mRNA in various types of cancer was searched for in the GENT database (http://medical-genomics.kribb.re.kr/GENT/). Boxes represent the median and 25th and 75th percentiles; dots represent outliers. Red boxes represent tumor tissues; green boxes represent normal tissues. Red and green dashed lines represent the average values for tumor and normal tissues, respectively.

inactivation may contribute to the malignant behavior of GC cells. These findings provide a basis for further investigation into *DHRS3* as a tumor-suppressor gene and the role of its inactivation in the pathogenic development of GC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Xijing Hospital of the Air Force Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Xijing Hospital of the Air Force Medical University.

AUTHOR CONTRIBUTIONS

SS, KX, and XB: molecular biology experiment. XB, SS, TY, and CF: article writing. WK, ZQ, and NY: experimental design. BB and SX: animal experiment. XB and SX: epigenetics experiment. All authors contributed to the article and approved the submitted version.

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The Cancer-Testis Long Non-coding RNA PCAT6 Facilitates the Malignant Phenotype of Ovarian Cancer by Sponging miR-143-3p

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Background: It has been reported that long non-coding RNAs (lncRNAs) play critical roles in tumorigenesis. However, their roles in ovarian cancer (OC) remain to be elucidated. The aim of this study was to uncover the function and underlying mechanisms of PCAT6 in OC.

Methods: The expression pattern of PCAT6 in OC was analyzed in the GSE137238, GSE143897 and Gene Expression Profile Interactive Analysis (GEPIA) datasets. Kaplan–Meier Plotter online software was used for survival analysis. Loss-of-function assays and gain-of-function assays were used to assess the function of PCAT6 in OC development. Moreover, small-RNA sequencing, bioinformatic analysis, luciferase assays and rescue experiments were carried out to clarify the potential mechanism of PCAT6 in OC.

Results: PCAT6 expression was significantly increased in OC tissues and positively correlated with advanced stages and with poor overall survival, progression-free survival and post-progression survival. Knockdown of PCAT6 in A2780 and SKOV3 cells inhibited OC cell proliferation, migration and invasion. In contrast, Overexpression of PCAT6 exerted the opposite effects on OC cells. Notably, PCAT6 bound to miR-143-3p and affected the expression of transforming growth factor (TGF)-β-activated kinase 1 (TAK1). Subsequent rescue assays confirmed that upregulation of miR-143-3p decreased the PCAT6 overexpression-induced promotion of proliferation, migration and invasion. Moreover, downregulation of miR-143-3p reversed the PCAT6 knockdown-induced inhibition of proliferation, migration, and invasion.

Conclusions: Our findings demonstrate that PCAT6 plays an oncogenic role in OC and may be useful as a therapeutic target for OC.

Keywords: PCAT6, ovarian cancer, miR-143-3p, TAK1, ceRNA

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INTRODUCTION

Ovarian cancer ranks as the seventh most common malignancy and the second most common cause of gynaecologic cancer death in women, with 239,000 new cases of ovarian cancer and 152,000 deaths annually worldwide (Bray et al., 2018). The early symptoms of ovarian cancer are not specific or pathognomonic, and the majority of cases are diagnosed at an advanced stage with widespread peritoneal dissemination throughout the abdomen and pelvis (Lheureux et al., 2019). Although cytoreductive surgery and platinum-taxane chemotherapy have increased the survival times of ovarian cancer patients, the 5-year survival rate for patients with metastatic epithelial ovarian cancer is <30% (Reavis and Drapkin, 2020). Given the high metastasis and recurrence rates of ovarian cancer, considerable effort should be made to find novel effective targets for early diagnosis and therapy of this disease.

In recent years, the number of scientific studies related to cancer-associated long non-coding RNAs (lncRNAs) has exponentially increased (Tripathi et al., 2018). Similar to coding genes, various lncRNAs can be classified as tumor suppressor genes and oncogenes according to their expression patterns and functions in cancer biology (Shi X. et al., 2018; Yeh et al., 2018; Hu et al., 2019; Yang et al., 2019). Previous studies have demonstrated that cancer-associated lncRNAs can act as competitive endogenous RNAs (ceRNAs) by binding to miRNAs, thus modulating the derepression of miRNA targets (Liang et al., 2018; Dong et al., 2019; Gokulnath et al., 2019; Wu et al., 2019). For example, MAGI2-AS3 has been reported to play a tumor-suppressive role in high-grade serous carcinoma (HGSC) by sponging miR-15-5p, miR-374a-5p, and miR-374b-5p (Gokulnath et al., 2019). Dong et al. found that HOXD-AS1 promotes ovarian cancer cell migration and invasion through the HOXD-AS1/miR-186-5p/PIK3R3 pathway (Dong et al., 2019). Findings by Liang et al. demonstrate that overexpression of PTAR can facilitate metastasis by regulating miR-101 in ovarian cancer cells, whereas downregulation of PTAR attenuates TGF-β1-induced carcinogenesis (Liang et al., 2018). Overall, the evidence indicates that it is essential to elucidate the molecular mechanisms of dysregulated lncRNAs in ovarian cancer. Knowledge of these mechanisms will contribute to understanding of ovarian cancer pathogenesis and exploration of therapeutic targets for the disease.

The intergenic lncRNA prostate cancer-associated transcript 6 (PCAT6), located at 1q32.1, has been reported to play oncogenic roles in multiple cancers. Wan et al. demonstrated that the expression level of PCAT6 is significantly increased in lung cancer tissues and cells and that PCAT6 knockdown inhibits cellular proliferation and metastasis (Wan et al., 2016). Moreover, PCAT6 can facilitate the proliferation and metastasis of cervical cancer cells but suppress apoptosis via the PCAT6/miR-543/ZEB1 axis (Ma et al., 2020). Additionally, Dan et al. determined that the PCAT6 expression levels in extracellular vesicles are significantly increased in the peripheral blood of lung cancer patients (Bai et al., 2019). One recent study has shown that PCAT6 might promote the malignancy of ovarian cancer cells by inhibiting PTEN (Kong et al., 2019). However, the detailed regulatory mechanism of PCAT6 remains to be fully elucidated. Furthermore, other underlying mechanisms of PCAT6 in ovarian cancer development should be explored.

In our research, we found that PCAT6 expression is upregulated in ovarian cancer tissues and is associated with clinical stage and patient survival. Loss-of-function and gain-of-function assays showed that PCAT6 induces cell proliferation, invasion and migration in ovarian cancer cells. We further revealed that PCAT6 acts as a ceRNA to regulate transforming growth factor (TGF)-β-activated kinase 1 (TAK1) expression by binding to miR-143-3p. Together, our findings indicate that PCAT6 is a potential ovarian cancer diagnostic and prognostic biomarker and that the PCAT6-miR-143-3p-TAK1 axis participates in proliferation, migration and invasion in ovarian cancer. Our results may provide novel insight that will aid in the development of therapeutic targets for ovarian cancer.

MATERIALS AND METHODS

PCAT6 Expression in Ovarian Cancer From Public Online Datasets and Survival Analysis

PCAT6 expression and clinical characteristics in ovarian cancer from the GSE137238 and GSE143897 datasets were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database. GSE137238 consisted of data for eight pairs of primary ovarian tumors and matched normal fallopian tubes. GSE143897 included data for 11 benign tissues, 79 serous ovarian cancer tumors and 32 ascites. PCAT6 expression was log2-transformed for further analysis. The expression levels of PCAT6 in ovarian cancer and normal ovarian tissues were obtained with the Gene Expression Profile Interactive Analysis (GEPIA) web server (http://gepia.cancer-pku.cn/index.html). Moreover, the associations between PCAT6 and overall survival (OS), progression-free survival (PFS) and post-progression survival (PPS) in ovarian cancer patients were analyzed with Kaplan–Meier Plotter (http://kmplot.com/analysis/index.php?p=service&cancer=ovar). The patients were grouped by the median PCAT6 expression, and the hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated.

Cell Culture

The human ovarian cancer cell lines SKOV3 and A2780 were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. The HEK293 human embryonic kidney cell line (HEK293T) was a kind gift from Professor Chenbo Ji (Nanjing Medical University, Nanjing, China). The SKOV3 cells were cultured in McCoy's 5a medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS). The A2780 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, KeyGEN BioTECH, Nanjing, China) supplemented with 10% FBS. All cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Transfection

LncRNA PCAT6 Smart Silencer, miR-143-3p mimics and a negative control (NC) sequence were designed and synthesized

by RiboBio, Inc. (Guangzhou, China). PCAT6 plasmids were synthesized by Genecreate Biological Engineering Co., Ltd. (Wuhan, China) and verified by sequencing. Cells were seeded into six-well plates (Costar, USA), and Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) was utilized to conduct transfection according to the manufacturer's protocols. After 48 h of culture, cells were harvested for RNA extraction.

Cell Proliferation

Cells (1×10^3/well) were seeded into 96-well plates 24 h after transfection. Proliferation was tested using a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Shanghai, China) in accordance with the manufacturer's protocol. CCK-8 reagent was added immediately after cell adhesion (marked as 0 h) and at 12, 24, 36, 48, and 72 h after cell adhesion. The absorbance was measured 1.5 h after CCK-8 was added, and the cell proliferation curves were plotted according to the results above.

Cell Migration and Invasion Assay

Cell invasion and migration were analyzed using Transwell chambers (Costar) coated or not coated with Matrigel (BD Biosciences, CA). After this step, 5×10^4 cells in 200 μl of serum-free medium were seeded into the upper chamber (for the cell invasion assay, the chamber was coated with Matrigel diluted 1:7 in McCoy's 5a Medium for SKOV3 and DMEM for A2780 cells). To the lower chamber, 600 ul of McCoy's 5a medium for SKOV3 cells or 600 ul of DMEM for A2780 cells supplemented with 20% FBS, 100 ug/ml streptomycin and 100 U/ml penicillin was added. The cells were incubated for 48 h for migration and 72 h for invasion at 37°C with 5% CO_2, after which the cells on the upper surface were removed. The cells on the lower surface were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 15 min. After removing the cells from the upper surface of the chamber, images were obtained under a microscope.

Wound Healing Assays

Cells were seeded into 6-well plates and allowed to grow to 90–95% confluence. Six hours after transfection, a single scratch wound was generated in each well. The cells were washed with PBS supplemented with serum-free medium to remove cell fragments and then monitored. Images were captured by phase-contrast microscopy at 0, 24, and 48 h after wounding.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocols. cDNA was synthesized from one microgram of total RNA by using a Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific). qRT-PCR was performed using PowerUp™ SYBR Green Master Mix (Thermo Scientific, Waltham, USA). The relative expression of PCAT6 and TAK1 was normalized to β-actin expression. The primer sequences are listed in **Supplementary Table 1**. For analysis of the expression of miR-143-3p, Bulge-loop™ miRNA qRT-PCR Primer Sets specific for miR-143-3p were designed by RiboBio (Guangzhou, China). miR-143-3p was quantified with a stem-loop real-time PCR miRNA kit (RiboBio, Guangzhou, China). The mRNA levels were normalized to U6 snRNA levels.

Small-RNA Sequencing and Bioinformatic Analysis

To explore miRNAs that bind to PCAT6, we performed small-RNA sequencing in A2780 cells after transfection with PCAT6-specific siRNAs (Si-PCAT6) and an NC siRNA (Si-NC). Briefly, total RNA was extracted with TRIzol, and RNA molecules in a size range of 18–30 nt were enriched by polyacrylamide gel electrophoresis (PAGE). Then, 3′ adapters were added, the 36–44 nt RNAs were enriched, and 5′ adapters were ligated to the RNAs. The ligation products were reverse transcribed by PCR amplification, and the 140–160 bp PCR products were enriched to generate a cDNA library, which was sequenced using an Illumina HiSeq™ 2500 (Zeng et al., 2019). Based on the expression in each sample, the miRNA expression level was calculated and normalized to the transcripts per million (TPM) value. Differentially expressed (DE) miRNAs were identified by the edgeR package in the R program. We considered miRNAs with a fold change values ≥ 1 and P-values < 0.05 as significant DE miRNAs. Then, the Encyclopedia of RNA Interactomes (ENCORI, http://starbase.sysu.edu.cn/) was used to explore the miRNAs that target PCAT6. Furthermore, we used the GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) tool to analyse the expression patterns of the candidate miRNAs in GEO miRNA datasets of ovarian cancer patients, namely, GSE83693, GSE119055, and GSE131790.

Luciferase Assay

The wild-type (WT) or mutant (MUT) PCAT6-binding sites were subcloned into the pmiR-RB-Report™ vector (RiboBio, Guangzhou, China). HEK293T cells were seeded onto 96-well plates. miR-143-3p mimics or the NC sequence (RiboBio, Guangzhou, China) were co-transfected with pmiR-RB-Report-WT-PCAT6 or pmiR-RB-Report-MUT-PCAT6. Two days after transfection, the cells were collected, and a luciferase activity was performed using a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blot Assay

After transient transfection in 6-well plates for 48 h, 80–100 μl/well of protein lysate (RIPA: PMSF = 100:1) was added to the 6-well plates and incubated on ice for a few minutes. After the cells were fully lysed, the proteins were scraped off using a spatula, transferred to 1.5 ml tubes, and centrifuged at 4°C and 12,000 rpm for 15–20 min. The supernatant was aspirated. Protein-loading buffer (G2013, Servicebio, Wuhan, China) was added, and the samples were incubated for 10 min at 95°C. The protein molecules were separated by SDS-PAGE, which was stopped when the target bands migrated to the appropriate positions for wet transfer. The proteins were transferred to a film in an ice bath at 350–400 mA for 90 min. The PVDF membrane was placed in 5% skimmed milk and blocked at room temperature for 2 h in a horizontal shaker. The blots were probed

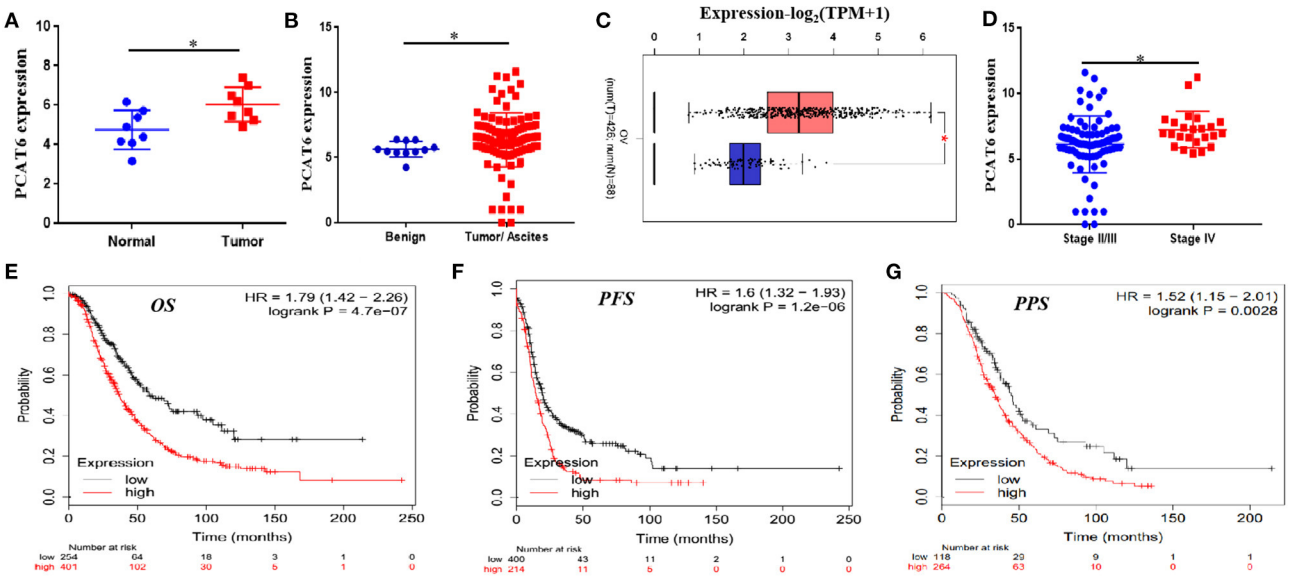

FIGURE 1 | PCAT6 is overexpressed in ovarian cancer tissues and correlates with poor prognosis of ovarian cancer patients. **(A)** Analysis of PCAT6 expression in primary ovarian cancer patient samples and matched normal fallopian tube samples based on GSE137238 data. **(B)** Analysis of PCAT6 expression in serous ovarian cancer tissues or ascites and benign tissues based on GSE143897 data. **(C)** Analysis of PCAT6 expression in The Cancer Genome Atlas (TCGA) ovarian cancer tissues and Genotype-Tissue Expression (GTEx) normal ovary tissues using the GEPIA web server. **(D)** Levels of PCAT6 expression in patients with different stages of ovarian cancer based on the GSE143897 dataset. **(E–G)** Kaplan–Meier Plotter results for OS, PFS, and PPS of ovarian cancer patients with low and high PCAT6 expression. *$P < 0.05$.

overnight at 4°C with primary antibodies: a rabbit anti-TAK1 antibody (Abcam, Cambridge, MA, USA, 1:1,000, ab109526) and a mouse anti-β-actin antibody (Proteintech, Wuhan, China, 1:1,000, 66009-I-Ig). After incubation with primary antibodies, the PVDF membrane was washed 3 times with TBST and incubated with goat anti-rabbit IgG (Biosharp, Anhui, China, 1: 20,000, BL003A) and goat anti-mouse IgG (Biosharp, Anhui, China, 1: 20,000, BL001A) secondary antibodies for 2 h. Images were obtained using a FluorChem M Multicolor fluorescence and chemiluminescence gel imaging system (ProteinSimple, Silicon Valley, USA).

Statistical Analysis

All data are presented as the mean ± standard deviation. Unless otherwise noted, the significance of differences between groups was estimated using Student's *t*-test. A value of $P < 0.05$ indicated a significant difference. All statistical analyses were performed using SPSS 20.0 software (IBM, Chicago, IL, USA).

RESULTS

PCAT6 Is Significantly Upregulated in Ovarian Cancer Tissues and Predicts Poor Prognosis of Ovarian Cancer Patients

In the GSE137238 dataset, we first observed significant upregulation of PCAT6 expression in primary ovarian cancer patient samples compared with matched normal fallopian tube samples ($P = 0.015$, **Figure 1A**). Then, we found that PCAT6 levels were higher in serous ovarian cancer tissues and ascites than in benign tissues based on the GSE143897 dataset ($P = 0.009$, **Figure 1B**). We verified the expression of PCAT6 in ovarian cancer and normal ovarian tissues using GEPIA and confirmed that PCAT6 was significantly overexpressed in ovarian cancer tissues ($P < 0.05$, **Figure 1C**). Furthermore, among patients with different stages of ovarian cancer based on the GSE143897 dataset, the expression of PCAT6 significantly increased from stage II/III to stage IV ($P = 0.02$, **Figure 1D**). The prognostic value of PCAT6 in ovarian cancer patients was assessed, and Kaplan–Meier Plotter analysis demonstrated that high expression of PCAT6 was correlated with poor OS, PFS and PPS in ovarian cancer patients (**Figures 1E–G**, all $P < 0.05$). The above findings indicate that PCAT6 may play oncogenic roles in the development and progression of ovarian cancer.

PCAT6 Promotes Malignant Phenotypes of Ovarian Cancer *in vitro*

To better understand the function of PCAT6 in ovarian cancer development, we then examined whether knockdown or overexpression of PCAT6 affects the proliferation, migration and invasion of ovarian cancer cells. We first used Si-PCAT6 to inhibit endogenous PCAT6 expression in SKOV3 and A2780 cells. In both cell types, PCAT6 knockdown resulted in lower PCAT6 expression than that in the control group (**Figure 2A**). Then, we found that PCAT6 knockdown significantly suppressed the viability of A2780 and SKOV3 cells by CCK-8 assay (**Figure 2B**). In migration and invasion assays, the numbers of migrated and invaded A2780 and SKOV3 cells in the Si-PCAT6 group were markedly lower than those in the NC group (*P*

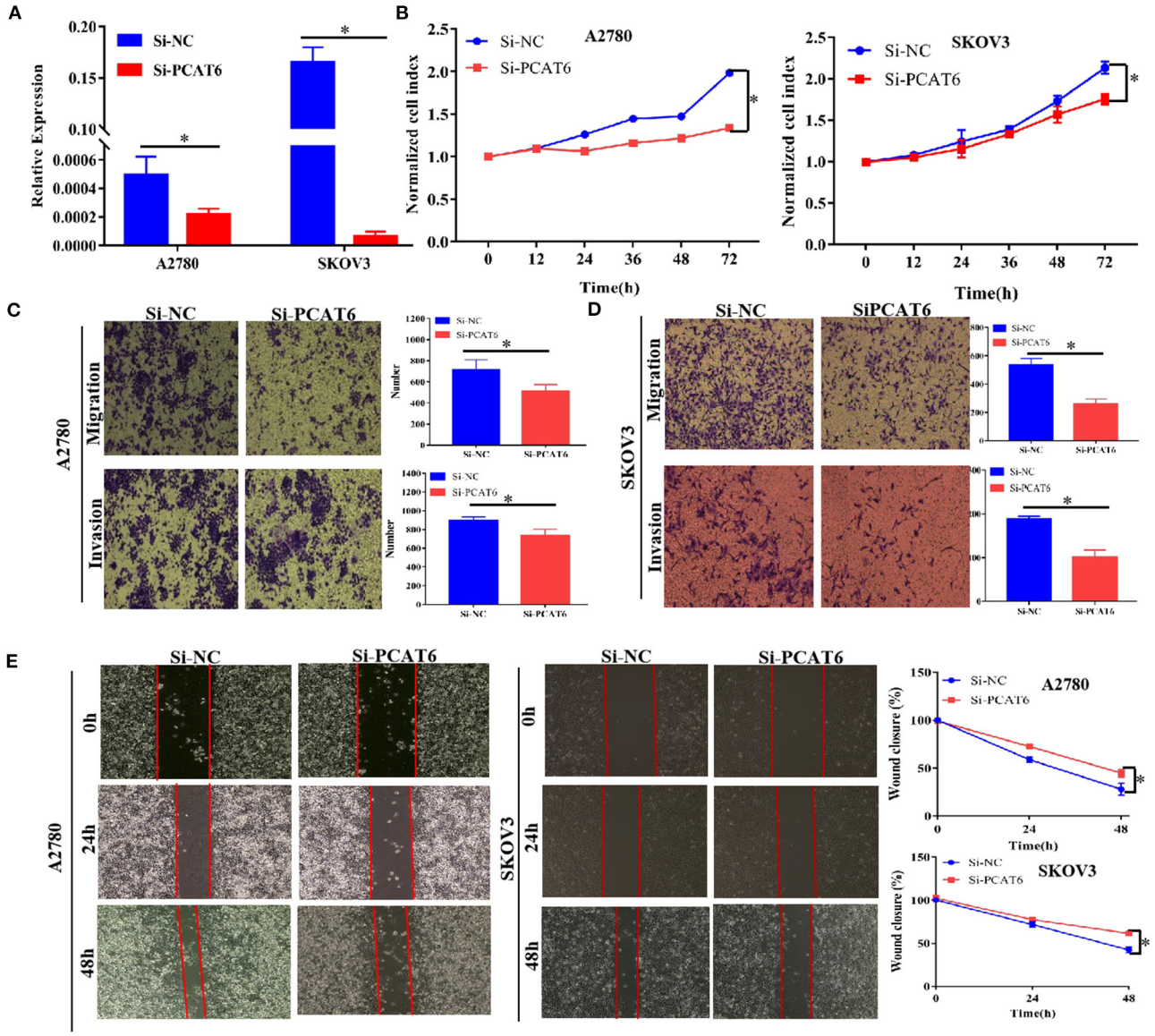

FIGURE 2 | Knockdown of PCAT6 in A2780 and SKOV3 cells inhibits ovarian cancer cell proliferation, migration and invasion. **(A)** The expression of PCAT6 was significantly lower in Si-PCAT6-transfected A2780 and SKOV3 cells than in Si-NC-transfected (control) cells. **(B)** CCK-8 assays showed that knockdown of PCAT6 inhibited A2780 (left) and SKOV3 (right) cell proliferation. **(C)** Representative images of the results of Transwell migration and invasion assays in A2780 cells (left). The mean numbers of migrated and invaded cells were lower in the Si-PCAT6 group than in the Si-NC group (right). **(D)** Representative images of the results of Transwell migration and invasion assays in SKOV3 cells (left). The mean numbers of migrated and invaded cells were lower in the Si-PCAT6 group than in the Si-NC group (right). **(E)** Representative images of wound healing assays in A2780 and SKOV3 cells (left). The motility of ovarian cancer cells was lower in the Si-PCAT6 group than in the Si-NC group (right). *$P < 0.05$.

< 0.05, **Figures 2C,D**). The wound healing assay also showed that PCAT6 knockdown suppressed the migration of A2780 and SKOV3 cells (**Figure 2E**). Additionally, we overexpressed PCAT6 by transfecting pcDNA3.1-PCAT6 into A2780 and SKOV3 cells. The level of PCAT6 in the overexpression group was markedly higher than that in the control group in both cell lines (**Supplementary Figure 1A**). Although overexpression of PCAT6 induced cell proliferation (**Supplementary Figure 1B**), the differences between the PCAT6-overexpressing cells and the control cells were small. Consistently, overexpression of PCAT6 increased the numbers of migrated and invaded cells (**Supplementary Figures 1C,D**) and promoted migration (**Supplementary Figure 1E**).

The lncRNA PCAT6 Acts as a ceRNA to Regulate TAK1 Expression by Binding to miR-143-3p

To identify the potential miRNA targets of lncRNA PCAT6, we performed small-RNA sequencing in A2780 cells transfected with siRNA or an NC sequence. We identified 390 DE miRNAs

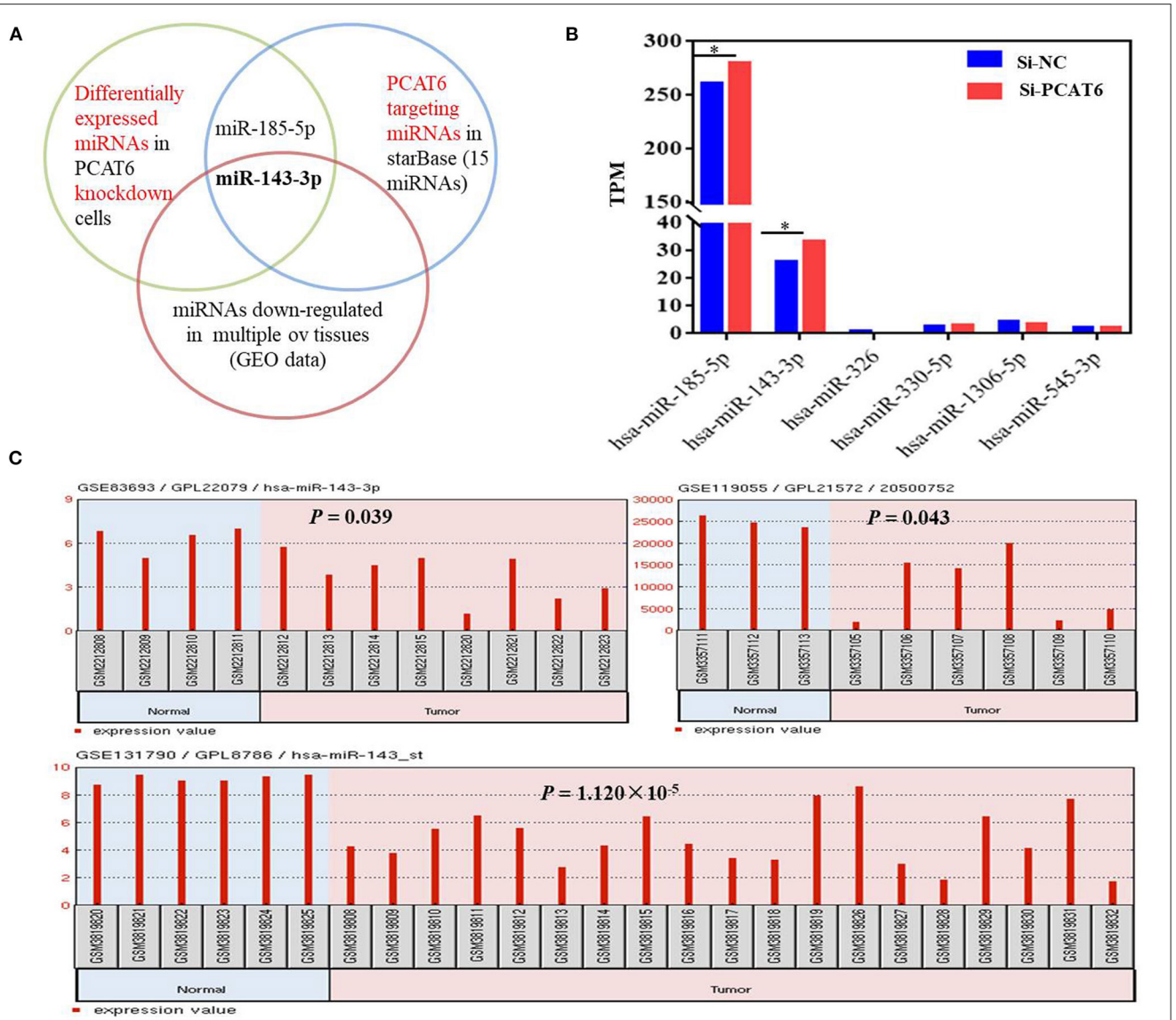

FIGURE 3 | miR-143-3p is a potential miRNA target of PCAT6 in ovarian cancer. **(A)** Diagram showing the intersection among DE miRNAs in Si-PCAT6-transfected A2780 cells compared with Si-NC-transfected A2780 cells (determined using small-RNA sequencing); PCAT6-targeting miRNAs from the ENCORI database; and miRNAs that were significantly downregulated in multiple ovarian cancer tissues based on the GSE83693, GSE119055, and GSE131790 datasets. **(B)** The expression levels of six miRNAs cataloged in the ENCORI database were detected in our small-RNA sequencing data. **(C)** miR-143-3p was consistently and significantly downregulated in tumor tissues compared to normal tissues based on the GSE83693, GSE119055, and GSE131790 datasets. *$P < 0.05$.

(**Supplementary Table 2**). Then, *in silico* analysis was performed by using ENCORI databases, which predicted that 15 miRNAs bind to PCAT6 (**Supplementary Table 3**, **Figure 3A**). The expression levels of six miRNAs were detected in our small-RNA sequencing data (**Figure 3B**), but only two miRNAs (miR-143-3p and miR-185-5p) were upregulated after PCAT6 was inhibited. miR-143-3p expression was consistently downregulated in ovarian cancer samples compared to normal tissues in the GSE83693, GSE119055, and GSE131790 datasets (**Figure 3C**), while miR-185-5p expression was upregulated in the GSE83693 and GSE119055 datasets but downregulated in the GSE131790 dataset (**Supplementary Figure 2**). Additionally, the expression of miR-143-3p was increased after PCAT6 was inhibited, while it was decreased after PCAT6 was overexpressed in both SKOV3 and A2780 ovarian cancer cells (**Figure 4A**). We further validated the binding of miR-143-3p with PCAT6 (**Figure 4B**) by using a luciferase reporter assay. As shown in **Figure 4C**, the miR-143-3p mimic markedly decreased the luciferase activity of PCAT6-WT but not PCAT6-MUT in HEK293T cells, indicating that miR-143-3p is a target of the lncRNA PCAT6. Based on these findings, we searched published studies and found that TAK1 has been identified as a direct target of miR-143-3p

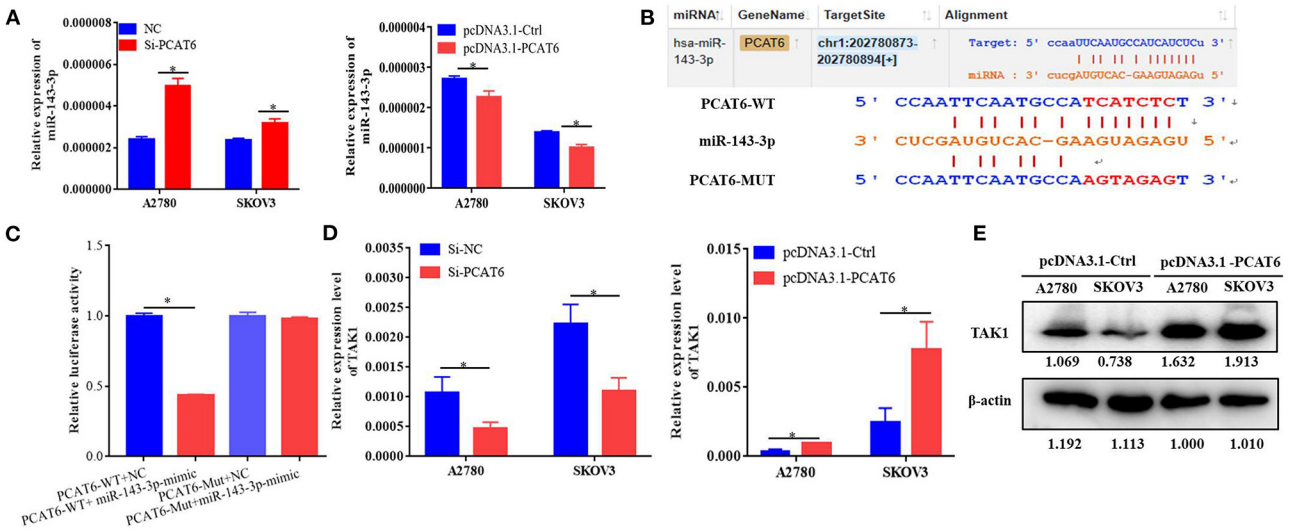

FIGURE 4 | PCAT6 competitively binds to miR-143-3p and regulates TAK1 expression. **(A)** Expression of miR-143-3p in A2780 and SKOV3 cells treated with Si-PCAT6 or PCDNA3.1-PCAT6. **(B)** Binding sites of PCAT6 and miR-143-3p identified by the online website ENCORI. **(C)** The binding of PCAT6 and miR-143-3p was verified by dual-luciferase reporter assay. The relative luciferase reporter activity was reduced in cells co-transfected with miR-143-3p and a WT PCAT6 reporter construct. **(D)** Expression of TAK1 in A2780 and SKOV3 cells treated with Si-PCAT6 or PCDNA3.1-PCAT6. **(E)** Protein expression of TAK1 in A2780 and SKOV3 cells treated with PCDNA3.1-PCAT6. *$P < 0.05$.

in ovarian cancer (Shi H. et al., 2018). In our study, TAK1 mRNA expression was significantly decreased when PCAT6 was inhibited but increased when PCAT6 was overexpressed in both cell lines (**Figure 4D**). We confirmed that TAK1 protein expression was upregulated in PCAT6-overexpressing cells (**Figure 4E**). Furthermore, we explored the role of TAK1 in PCAT6-overexpressing cells. Cell proliferation and invasion assays illustrated that silencing TAK1 in A2780 and SKOV3 cells inhibited the malignant phenotype of PCAT6 overexpression (**Supplementary Figure 3**). All these findings indicate that PCAT6 might affect the behaviors of ovarian cancer cells by regulating TAK1.

miR-143-3p Reverses the Function of PCAT6

Considering that PCAT6 acts as a ceRNA to regulate TAK1 expression by binding to miR-143-3p, we performed rescue assays to validate whether miR-143-3p is involved in the PCAT6-mediated promotion of proliferation, migration, and invasion in ovarian cancer cells. In SKOV3 cells, we observed that downregulation of miR-143-3p mitigated PCAT6 knockdown-induced suppression of proliferation, migration, and invasion (**Figures 5A–C**). On the other hand, upregulation of miR-143-3p reversed the PCAT6 overexpression-induced enhancement of proliferation, migration, and invasion (**Figures 5D–F**). Moreover, we observed that miR-143-3p rescued the expression of TAK1 in SKOV3 cells co-transfected with Si-PCAT6 and a miR-143-3p inhibitor and in PCAT6-overexpressing SKOV3 cells transfected with miR-143-3p mimics (**Figure 5G**).

DISCUSSION

Since lncRNAs play vital regulatory roles in tumorigenesis and progression, much attention has been given to the functions and mechanisms of lncRNAs in recent years. In the present study, we found that PCAT6 was upregulated in ovarian cancer tissues and that elevated expression of PCAT6 was correlated with reduced OS, PFS and PPS in ovarian cancer patients. Functional experiments demonstrated that PCAT6 promoted ovarian cancer cell proliferation, migration and invasion. Moreover, PCAT6 promoted malignancy of ovarian cancer via its interaction with miR-143-3p and effects on TAK1 expression. Taken together, these findings indicate that the lncRNA PCAT6 plays an oncogenic role in the development of ovarian cancer.

The lncRNA PCAT6 is a cancer/testis (CT) lncRNA that is normally silenced in healthy tissues except for the testes but is highly expressed in malignancies (Wang et al., 2016; Chen et al., 2019). Emerging evidence has shown that PCAT6 expression is upregulated in various cancers and plays oncogenic roles in cancer development. For instance, Chen et al. demonstrated that PCAT6 is significantly upregulated in hepatocellular carcinoma (HCC) tissues and that knockdown of PCAT6 in HCC cells can inhibit cell growth and migration (Chen et al., 2019). Luo et al. further confirmed that PCAT6 can predict poor prognosis in HCC and promote proliferation through regulation of cell cycle arrest and apoptosis (Luo et al., 2020). Based on pathway crosstalk analysis, PCAT6 has been identified as a hub lncRNA that is closely related to the clinical features of lung adenocarcinoma (Qi et al., 2019). Several studies have consistently demonstrated that aberrant upregulation of

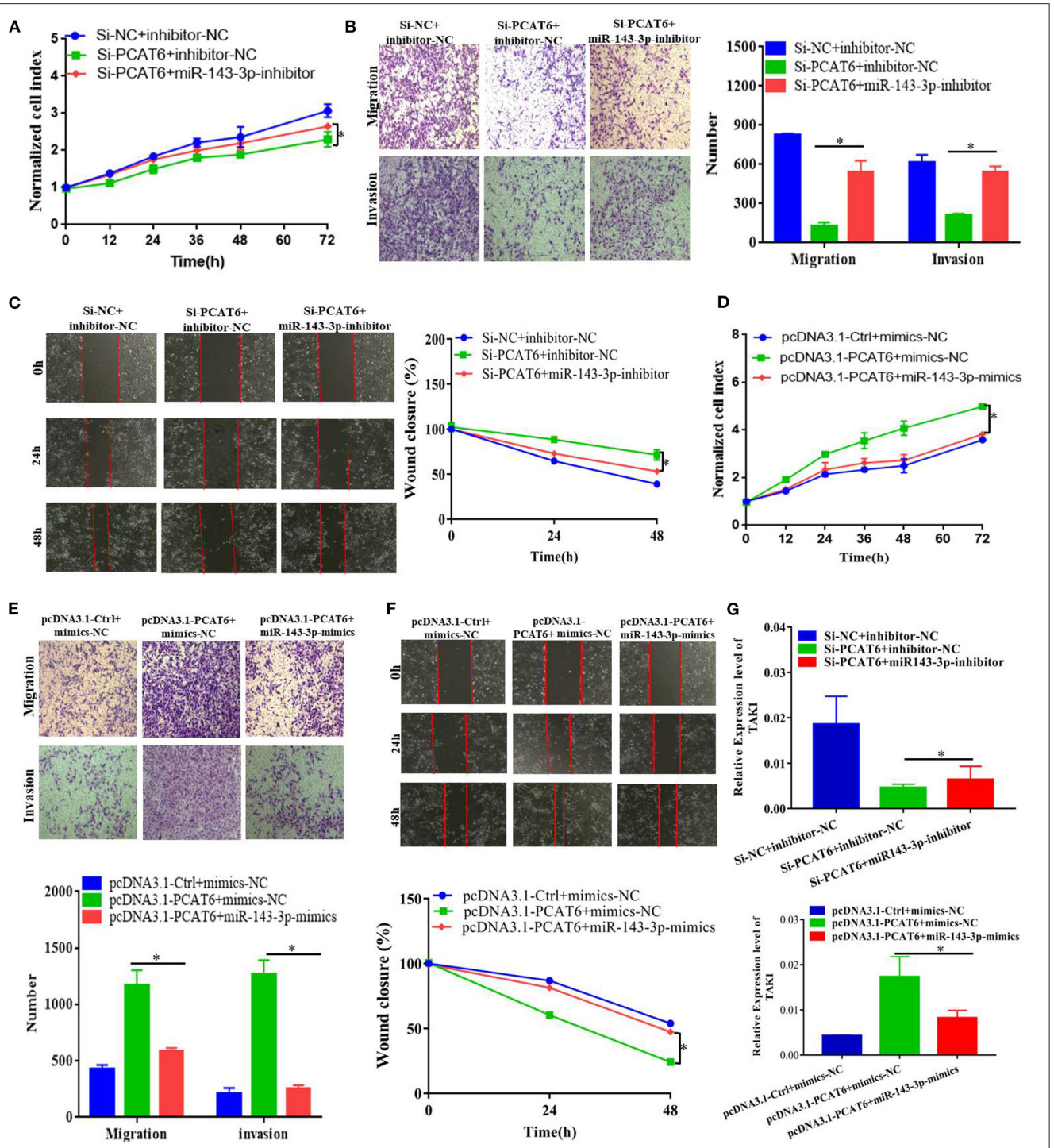

FIGURE 5 | PCAT6 affects ovarian cancer cell proliferation, migration, and invasion by modulating miR-143-3p. **(A)** Downregulation of miR-143-3p rescued the proliferation of SKOV3 cells with knockdown of PCAT6. **(B)** Downregulation of miR-143-3p rescued the migration and invasion abilities of SKOV3 cells with knockdown of PCAT6. **(C)** Downregulation of miR-143-3p rescued the migration of SKOV3 cells with knockdown of PCAT6, as demonstrated by wound healing assay. **(D)** Upregulation of miR-143-3p rescued the proliferation of SKOV3 cells overexpressing PCAT6. **(E)** Upregulation of miR-143-3p rescued the migration and invasion abilities of SKOV3 cells overexpressing PCAT6. **(F)** Upregulation of miR-143-3p rescued the migration of SKOV3 cells overexpressing PCAT6, as demonstrated by wound healing assay. **(G)** miR-143-3p rescued the expression of TAK1 in SKOV3 cells co-transfected with Si-PCAT6 and a miR-143-3p inhibitor and in PCAT6-overexpressing SKOV3 cells transfected with miR-143-3p mimics. $^*P < 0.05$.

PCAT6 is common in lung cancer tissues and promotes the proliferation, migration, and invasion of lung cancer cells (Wan et al., 2016; Cui et al., 2018; Shi X. et al., 2018). PCAT6 has also been reported to be overexpressed in gastric cancer tissues and to promote the development of gastric cancer (Xu et al., 2018). Ma et al. revealed that the levels of PCAT6 are enhanced in cervical cancer (CC) tissues and that PCAT6 accelerates the proliferation and metastasis of CC cells while suppressing apoptosis (Ma et al., 2020). More recently, findings by Kong et al. have indicated that PCAT6 is highly expressed in ovarian cancer tissues and cell lines and that knockdown of PCAT6 inhibits cell proliferation, invasion and migration in the SKOV3 and CAOV3 cell lines (Kong et al., 2019). Despite these findings, little is known about the underlying mechanisms of PCAT6 in ovarian cancer carcinogenesis. The key findings of our present study are that PCAT6 is significantly elevated in ovarian cancer tissues and that this elevation is associated with advanced TNM stage and poor prognosis, indicating that PCAT6 is a potential diagnostic and prognostic marker for ovarian cancer. Further functional experiments in two cell lines demonstrated that knockdown of PCAT6 suppresses cell proliferation, migration and invasion, while overexpression of PCAT6 produces the opposite results, indicating that PCAT6 exerts an oncogenic function in ovarian cancer development.

Accumulating evidence has shown that lncRNAs function as ceRNAs for specific miRNAs to regulate the target genes of these miRNAs (Liang et al., 2018; Gokulnath et al., 2019; Qi et al., 2020). Until now, the details of the underlying mechanism of PCAT6 in ovarian cancer have remained elusive. To explore whether PCAT6 can act as a ceRNA in ovarian cancer, we combined small-RNA sequencing, bioinformatic analysis and dual-luciferase reporter assays and verified that miR-143-3p was a direct target of PCAT6. miR-143-3p was negatively regulated by PCAT6 in ovarian cancer cells. The PCAT6 overexpression-induced increases in cell growth and metastasis were attenuated by the miR-143-3p mimic, while PCAT6 knockdown-induced suppression was partly rescued. Therefore, we propose that PCAT6 may play a major role in ovarian cancer cells by sponging miR-143-3p during ovarian cancer progression. Recent reports have demonstrated that miR-143-3p acts as a tumor suppressor in multiple kinds of malignant cancers, such as HCC, esophageal squamous cell carcinoma, bladder cancer and ovarian cancer (Noguchi et al., 2011; He et al., 2016; Shi H. et al., 2018; Peng et al., 2020). Interestingly, it has been reported that miR-143-3p regulates the expression of TAK1 in ovarian cancer cells and exerts an inhibitory effect on the proliferation, migration, and invasion of ovarian cancer cells (Shi H. et al., 2018). A direct interaction between TAK1 and miR-143 has been further demonstrated in pancreatic ductal adenocarcinoma (Huang et al., 2017). In our study, we observed that the expression of PCAT6 was significantly repressed in PCAT6-knockdown cells and upregulated in PCAT6-overexpressing cells. According to previous studies, TAK1 is a serine/threonine kinase of the MAP3K family (MAP3K7) that is a critical regulator of TGF-β signaling and participates in the activation of p38MAPK and JNK in various cellular systems (Safina et al., 2008; Rincón and Davis, 2009). Importantly, it has been reported that elevated TAK1 levels promote ovarian cancer cell growth and metastatic capacity through activation of NF-κB signaling (Cai et al., 2014). Based on these results, we infer that PCAT6 plays oncogenic roles in the malignancy of ovarian cancer by sponging miR-143-3p and reducing its activity, thus increasing TAK1 expression.

Notably, there are several limitations of our study. First, the SKOV3 and A2780 cell lines were used for both PCAT6 overexpression and knockdown experiments to evaluate the malignant phenotype of ovarian cancer. More representative cell lines, particularly stably lentivirus-transfected cell lines, should be used to validate our results. Second, we did not study the function or mechanism of PCAT6 *in vivo*. In future studies, we will evaluate the roles of PCAT6 *in vivo*. In summary, our study establishes the clinical and oncogenic functions of PCAT6 in ovarian tumorigenesis and progression. Additionally, we demonstrate that the oncogenic activity of PCAT6 is attributable to the ceRNA regulatory network of the PCAT6-miR-143-3p-TAK1 axis, which is a potential therapeutic target for ovarian cancer.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KX, HX, and JH designed the study. XT, YS, YT, SL, and WL performed the experiments. LX, YC, CS, JZ, and JH analyzed the public database and discussed the results. KX, XT, and YT prepared the manuscript, and all authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.593677/full#supplementary-material

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
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Construction of Two Alternative Polyadenylation Signatures to Predict the Prognosis of Sarcoma Patients

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Background: Increasing evidence indicates that alternative polyadenylation (APA) is associated with the prognosis of cancers.

Methods: We obtained gene expression and APA profiles of 259 sarcoma patients from the TCGA dataportal and TC3A database, respectively. The prognostic signatures, clinical nomograms, and regulatory networks were studied by integrated bioinformatics analyses. Then, the immune cell infiltration profile was obtained from the ImmuCellAI. The association between APA-based signature and immune cells was studied.

Results: A total of 61 and 38 APA events were identified as overall survival (OS)- and progress free-survival (PFS)-related biomarkers, respectively. Two signatures were generated. The area under the curves (AUC) values of OS signature were 0.900, 0.928, and 0.963 over 2-, 4-, and 6-years, respectively. And the AUC values of PFS signature at 2-, 4-, and 6-years were 0.826, 0.840, and 0.847, respectively. Overall and subgroup analyses indicated that high-risk patients had a worse prognosis than low-risk patients (all p-values < 0.05). In addition, immunomics analyses indicated that there are different patterns of immune cell infiltration between low- and high-risk patients. Furthermore, two clinical-APA nomograms were established and the C-indexes were 0.813 and 0.809 for OS nomogram and PFS nomogram, respectively. Finally, two APA regulatory networks were constructed. FIP1L1-VPS26B was identified as a key regulating relationship and validated in the pan-cancer analyses.

Conclusion: In this study, we identified prognostic predictors based on APA events with high accuracy for risk stratification in sarcoma patients and uncovered interesting regulatory networks in sarcoma that could be underlying mechanisms. This study not only provides novel potential prognostic biomarkers but promote precision medicine and provide potential novel research interests for immunotherapy.

Keywords: alternative polyadenylation, sarcoma, overall survival, progress free-survival, nomogram

INTRODUCTION

Sarcomas are a heterogeneous group of mesenchymal malignancies that can develop at any age, comprising approximately 1% of all adult malignancies and 15% of pediatric malignancies (Von Mehren et al., 2018). Although the incidence of sarcoma is relatively rare, more than 10,000 patients are diagnosed with soft tissue sarcomas in the United States and 40,000 in China each year (Yang et al., 2019). Surgery, radiotherapy, and chemotherapy are three mainstream treatments for sarcoma patients that have shown progressive effects (Nussbaum et al., 2016; Albertsmeier et al., 2018; Thanindratarn et al., 2019). Unfortunately, the prognosis of sarcoma patients is still unsatisfactory due to local recurrence and distant metastases (Cipriano et al., 2020). Therefore, it is urgent to develop a reliable prognostic predictor for guiding clinical practice. Based on either clinical data, gene expression profile, or tumor-infiltrating immune cell, several prognostic models have been developed for sarcoma patients before (Callegaro et al., 2019; Huang et al., 2019; Gu et al., 2020). Nevertheless, there have not been any reliable models due to the complexity and heterogeneity of sarcoma.

Alternative polyadenylation (APA) is an important post-transcriptional regulation mechanism, which occurs in >70% of human genes (Mayr and Bartel, 2009; Hoque et al., 2013). It was found that APA plays an essential role in protein diversification, mRNA stability, mRNA nuclear export and repression of gene expression by producing mRNAs with different 3′ untranslated regions (3′ UTRs) and/or encoding variable protein isoforms (Edwalds-Gilbert et al., 1997; Tian and Manley, 2017). Therefore, from the perspective of epigenetics, once APA is dysregulated, it will cause diverse pathological processes, such as cancer, viral infection, amyotrophic lateral sclerosis, and so on (Chen et al., 2017).

Nowadays, the deregulation of APA has caused widespread interest in cancer research, because APA generates mRNA 3' UTR isoforms with potentially different stabilities, subcellular localizations, translation efficiencies, and functions. In recent years, with the rapid development of high-throughput sequencing technology, genome-wide profiling for APA events has become a reality. In 2018, Li et al., (Xiang et al., 2018) have completed an analysis of pan-cancer analysis that helps us to understand the regulatory mechanisms and functional consequences of APA alterations in tumorigenesis. In total, 17 tumor types were studied and a series of important roles of APA in the tumor were discovered, such as gene expression regulation and cellular pathway remodeling (Xiang et al., 2018). Recently, Venkat et al. (2020) firstly performed a cancer-specific analysis and found that APA was an independent prognostic biomarker for pancreatic ductal adenocarcinoma patients. Generally, as with other well-researched post-transcriptional regulation mechanisms like alternative splicing and mRNA m6A methylation, APA also plays a vital role in the genesis, progress, and prognosis of cancers. Despite the effect of APA was preliminarily confirmed in this research, there were few attempts to study the role of APA for sarcoma, and no APA-based signature was constructed for such patients.

In the present study, a comprehensive analysis of APA events was performed based on a large cohort from the TCGA-SARC dataset. The prognostic value of APA events for sarcoma patients was uncovered, and two APA-based signatures were constructed. We further explored the potential relationship between APA signatures and clinicopathological data and developed two clinical-APA nomograms. Finally, we established the regulatory network between APA events and APA factors to elucidate the underlying mechanisms.

MATERIALS AND METHODS

Data Acquisition

The gene expression files and clinical data of TCGA-SARC were obtained from the UCSC Xena browser[1]. Additionally, the Percentage of Distal polyA site Usage Index (PDUI) value was used to quantify each APA event, and the data of PDUI of TCGA-SARC was downloaded from the TC3A database (Feng et al., 2018). According to the definition in published research, the PDUI score quantifies the relative poly (A) site usage for that gene in the sample by computing the abundances of 3'-UTR long and short forms (Venkat et al., 2020). Genes favoring distal PAS usage (long 3' UTRs) have PDUI scores near 1, whereas genes favoring proximal PAS usage (short 3' UTRs) have PDUI scores near 0 (Venkat et al., 2020). To construct as credible a set of APA events as possible, only APA events that met the following criteria were included in this study: (Von Mehren et al., 2018) percentage of samples with PDUI value ≥75%; (Yang et al., 2019) average PDUI value ≥0.05 (Li et al., 2019; Zhang et al., 2020b). All data were downloaded from the public databases hence it was not required to obtain additional ethical approval for our study.

Survival Analysis and Enrichment Analysis

To comprehensively understand the role of APA event in sarcoma, two primary endpoints were studied, including overall survival (OS) and progress-free survival (PFS). According to the PDUI value, all patients were stratified into low- and high-PDUI groups by the median cut. Then, the univariate Cox analysis was performed to identify prognostic APA events for sarcoma patients, including OS- and PFS-related APA events. To obtain the robust prognostic APA events, only APA events with a p-value < 0.01 in the univariate Cox analysis were selected for further analyses (Zhu et al., 2018). To further understand the mechanisms involved in APA events affecting the prognosis of patients, the parent genes of identified APA events were then incorporated into Gene Ontology (GO) enrichment analysis. The enrichment analyses were performed in the Metascape[2].

Establishment and Evaluation of APA-Based Signatures

To avoid overfitting among prognostic APA events, a machine learning algorism called Least absolute shrinkage and

[1]https://xenabrowser.net/

[2]http://metascape.org

selection operator (LASSO) was used to filter significant APA events. Finally, the multivariate Cox analysis was performed to identify independent prognostic APA events. A robust prognostic signature is valuable for prognostic prediction, clinical management, and accurate clinical trial. Previous research indicated that APA events can serve as effective prognostic biomarkers for survival prediction and that the power of APA events exceeded clinical covariates (Venkat et al., 2020; Zhang et al., 2020b). Therefore, based on corresponding independent prognostic APA events, two prognostic signatures were established, including OS and PFS signatures. To show the discrimination of signatures, a time-dependent receiver operating characteristic (ROC) curve with area under the curve (AUC) was generated (Heagerty et al., 2000). In addition, according to the median of risk score, all patients were stratified into low- and high-risk groups, and the survival curve with a log-rank test was used to show the distinct prognosis between low- and high-risk groups.

To confirm the stability of APA-based signatures in different subgroups, subgroup analyses were performed. Kaplan-Meier survival curves of low- and high-risk patients in several clinical subgroups, including age, sex, histological type, metastatic status, tumor site, surgical margin status, and multifocal status, were constructed. A log-rank test was used to compare the prognostic difference between patients in low- and high-risk groups.

GSVA Analysis and Immune Cell Infiltration

Gene set variation analysis (GSVA) is a non-parametric and unsupervised method that is commonly used to estimate the variation of the pathway and biological process activity in expression cohort samples (Hänzelmann et al., 2013). The gene set of "c2.cp.kegg.v7.1.symbols" was downloaded from MSigDB database for running GSVA analysis. Adjusted *P* with value less than 0.05 was considered as statistically significance. Previous studies reported that post-transcriptional regulation mechanism plays an important role in the formation of the tumor microenvironment (Li et al., 2019; Yi et al., 2020; Zhang et al., 2020a). Therefore, we further elucidated the association between immune cell infiltration and APA signatures. The immune cell infiltration profile was obtained from the ImmuCellAI (Miao et al., 2020). The difference between low- and high-risk groups were evaluated by Wilcoxon tests.

Development of Clinical-APA Nomogram for Sarcoma Patients

Nomogram is one of the effective tools for clinical practice, which was widely used as a prognostic device for cancer

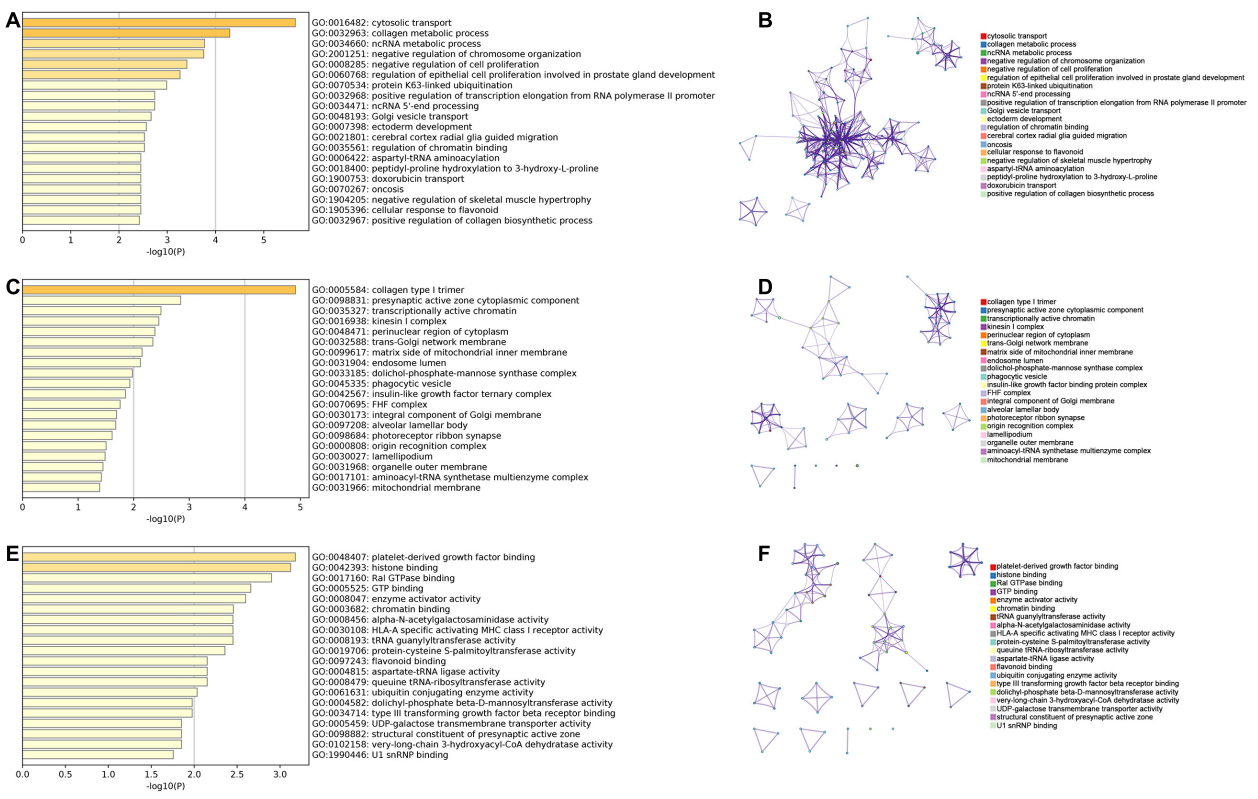

FIGURE 1 | GO analysis of parent genes for prognostic APA events. **(A,B)** The result of biological process of in the GO analysis. **(C,D)** The result of cellular component of in the GO analysis. **(E,F)** The result of molecular function of in the GO analysis. GO, Gene Ontology; APA, alternative polyadenylation.

patients (Iasonos et al., 2008). Therefore, to confirm that APA-based signatures are an independent prognostic predictor for sarcoma and to develop two clinical-APA nomograms, the clinical data, including age, sex, race, tumor site, histological type, distant metastatic status, postoperative radiotherapy, pharmacotherapy, multifocal status, and surgical margin resection status were extracted for further analyses. The univariate Cox analysis was used to identify prognostic variables, and variables with a $p < 0.05$ were incorporated into the multivariate Cox analysis. Then, two nomograms were developed by incorporating several independent predictors. A concordance index (C-index) was used to show the discrimination of nomograms, and the calibration curve was selected to show the calibration of nomograms (Iasonos et al., 2008).

Correlation Network Between APA and APA Factors

The 3′ end-processing machinery is composed of multiple protein factors and several APA factors were confirmed as vital regulatory factors for APA. Based on a published articles, 28 core APA factors were included (Tang et al., 2016; Qiu et al., 2017; Xiang et al., 2018; Chatrikhi et al., 2019; Jia et al., 2019; Zhang et al., 2019b). The expression of 28 APA factors was downloaded from the UCSC Xena browser (see text footnote 1). The prognostic role of all APA factors was investigated, and OS- and PFS-related APA factors were used for further correlation analyses. Pearson correlation analysis was used to determine the correlation coefficient between the PDUI of APA events and the expression of APA factors and to identify the potential regulatory networks between them ($|r| > 0.2$ and $p < 0.05$). The regulatory networks was visualized by Cytoscape 3.7.2 (Shannon et al., 2003).

Statistical Analyses

In the present study, all statistical analyses were performed with R software (version 3.6.1). Except for the special instructions, a p-value < 0.05 (two sides) was considered as statistically significant. Univariate, LASSO, and multivariate Cox analyses were used to select independent prognostic APA events by "survival" and "glmnet" packages. The "survivalROC" package was used to develop time-dependent ROC curves, and corresponding AUC values were generated simultaneously. The survival curve was generated by a "survminer" package. In addition, Pearson correlation analysis was used to identify the

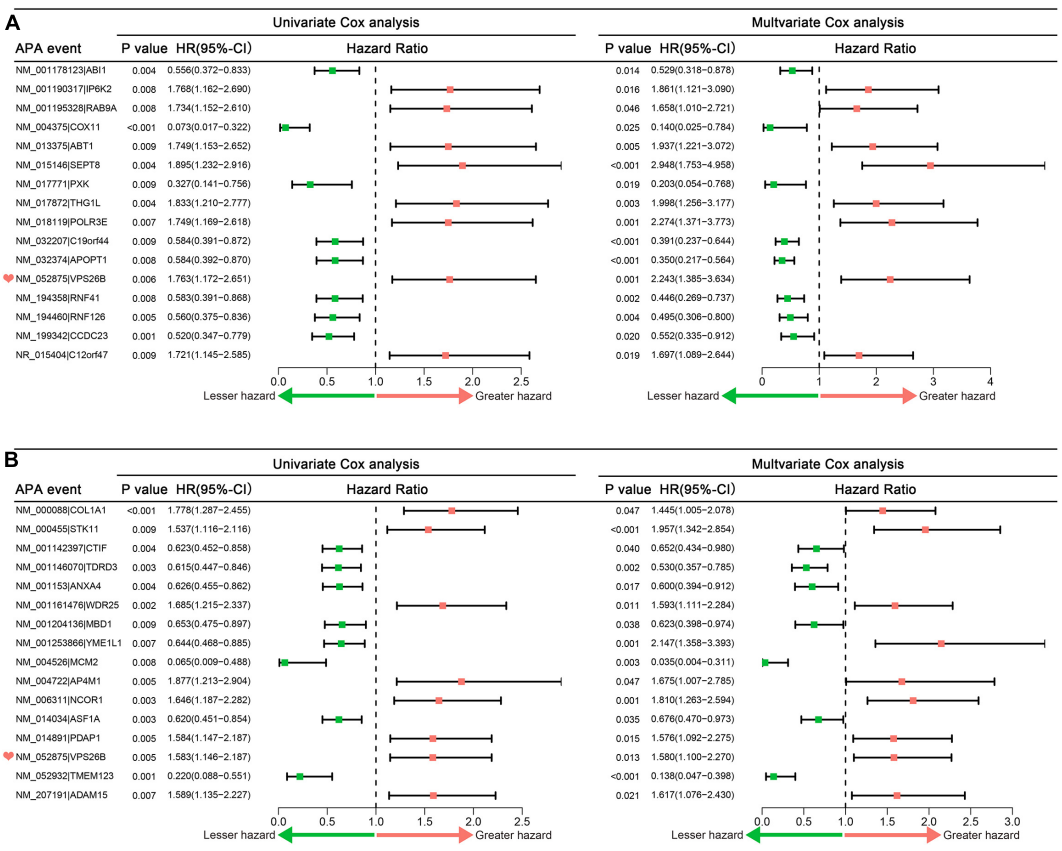

FIGURE 2 | Forrest plots of hazard ratios of prognosis associated APA events in sarcoma patients. **(A)** Univariate and multivariate Cox analysis results of independent OS-related alternative polyadenylation events. **(B)** Univariate and multivariate Cox analysis results of independent PFS-related alternative polyadenylation events. APA, alternative polyadenylation; OS, overall survival; PFS, progress free-survival.

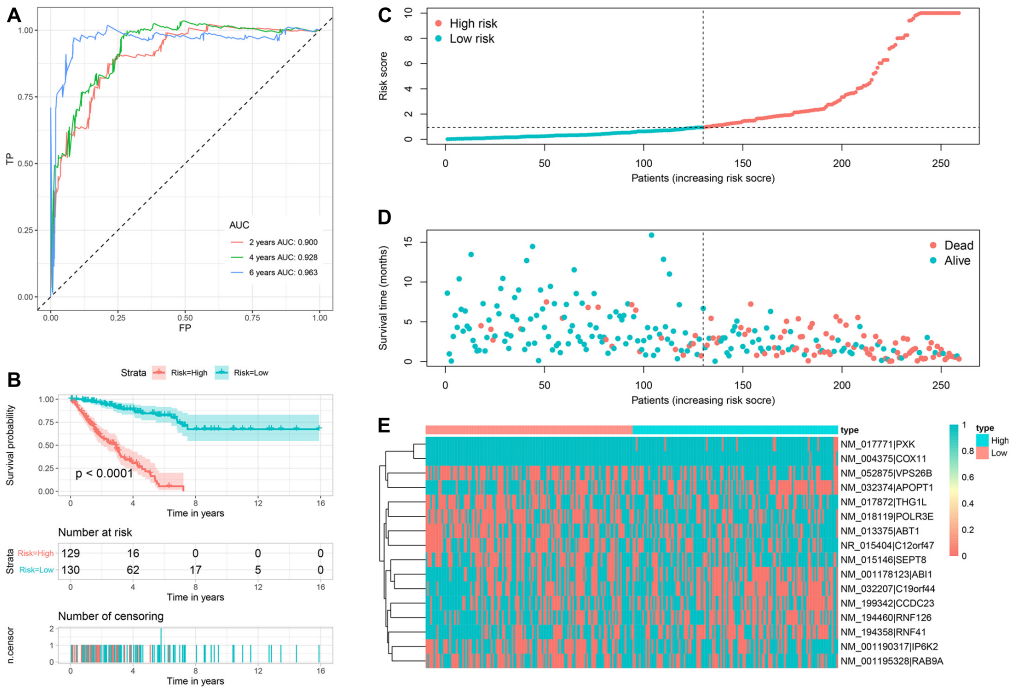

FIGURE 3 | Prognostic signature to predict OS of sarcoma patients. **(A)** ROC curves of OS signature. **(B)** Survival curve showed that high-risk patients were worse OS than low-risk patients. **(C)** Risk score distribution of 259 sarcoma patients. **(D)** OS status of 259 sarcoma patients. **(E)** Heatmap showed the distribution of PDUI of 16 OS-related APA events in low- and high-risk patients. OS, overall survival; ROC, receiver operating characteristic; APA, alternative polyadenylation.

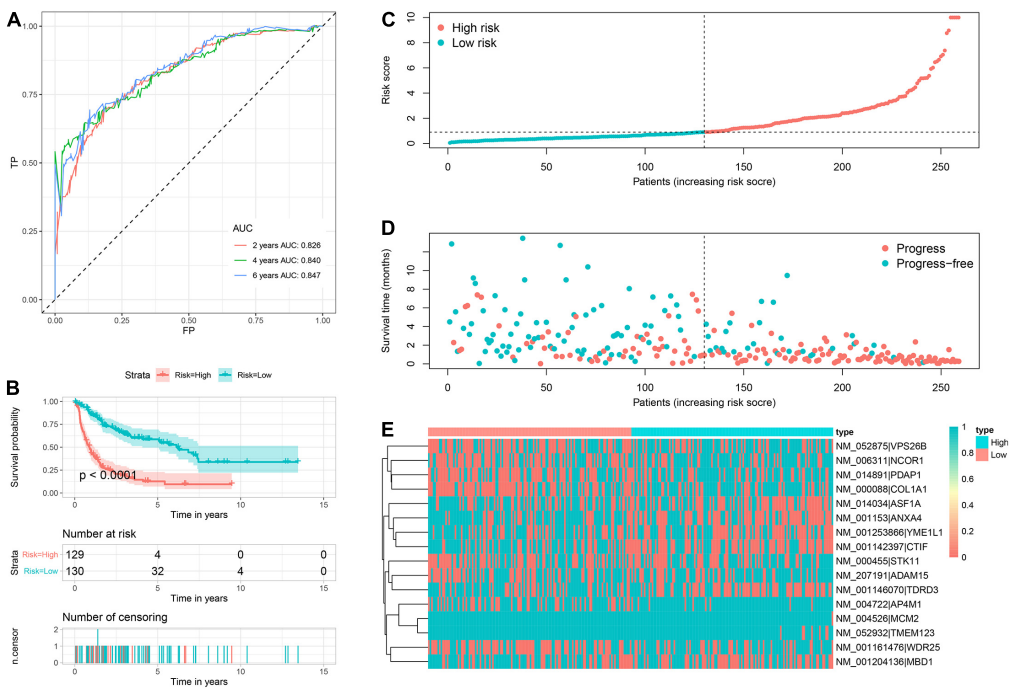

FIGURE 4 | Prognostic signature to predict PFS of sarcoma patients. **(A)** ROC curves of PFS signature. **(B)** Survival curve showed that high-risk patients were worse PFS than low-risk patients. **(C)** Risk score distribution of 259 sarcoma patients. **(D)** PFS status of 259 sarcoma patients. **(E)** Heatmap showed the distribution of PDUI of 16 PFS-related events in low- and high-risk patients. PFS, progress free-survival; ROC, receiver operating characteristic; APA, alternative polyadenylation.

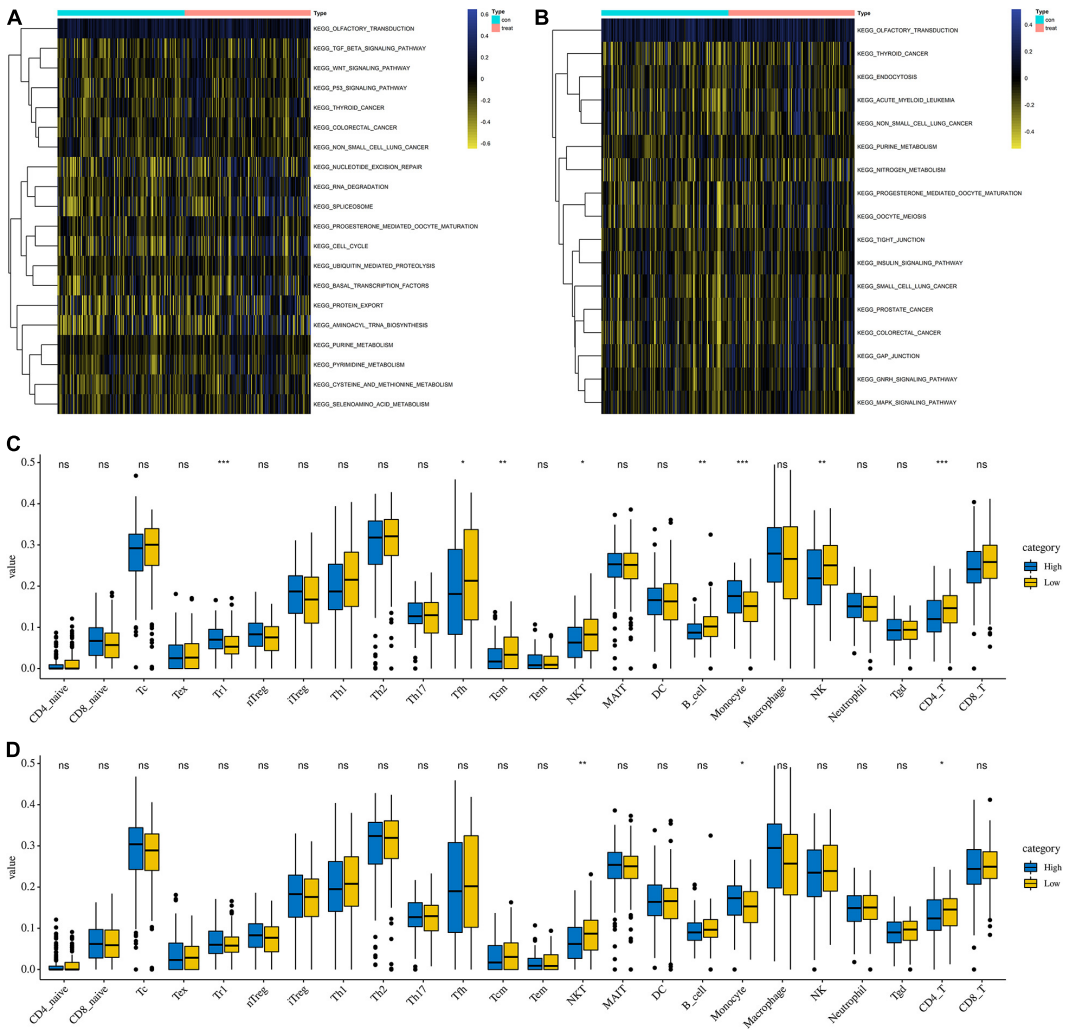

FIGURE 5 | GSVA analysis and immune feature between low- and high-risk groups. **(A)** GSVA analysis between low- and high-risk groups based on overall survival signature. **(B)** GSVA analysis between low- and high-risk groups based on progress free-survival survival signature. **(C)** Comparison of immune cell infiltration between low- and high-risk groups based on overall survival signature. **(D)** Comparison of immune cell infiltration between low- and high-risk groups based on progress free-survival signature.

potential regulatory network between APA events and APA factors ($|r| > 0.2$ and $p < 0.05$).

RESULTS

Overview of APA Events Profiling in Sarcoma

According to the aforementioned criterion, a total of 259 primary sarcoma patients were included in our study. The mean age was 60.71 ± 14.59, and the average follow up time was 3.26 years (range: 0.04–15.56 years). In total, 98 patients died during the follow-up duration and 153 patients progressed. For APA events, 8864 APA events were detected for the TCGA-SARC cohort in TC3A database. A total of 2179 APA events were excluded because more than 25% of patients lacked PDUI of these APA events or the average of PDUI value < 0.05. Finally, 6685 APA events were used for further analyses.

Identification of Prognosis-Related APA Events and Enrichment Analysis

According to the median of PDUI, all patients were stratified as low- and high-groups for each APA event. The univariate Cox analysis indicated that 61 and 38 APA events were OS- and PFS-related biomarkers, respectively ($p < 0.01$) (**Supplementary Tables 1**, **2**). The enrichment of the GO analysis is illustrated in **Figure 1**, which showed that specific GO categories were significantly related to sarcomas, like cytosolic transport, collagen metabolic process and negative regulation of chromosome organization.

Furthermore, LASSO analysis excluded 22 OS-related APA events and 7 PFS-related APA events

(**Supplementary Figures 1A–D**). Finally, 16 and 16 APA events were confirmed as independent OS- and PFS-related biomarkers, respectively (**Figures 2A,B**). Interestingly, we found that the APA event of VPS26B was the overlapping independent prognostic APA event between OS and PFS.

Construction of APA-Based Signatures

Based on corresponding independent prognostic APA events, two prognostic signatures were constructed to predict the OS and PFS, respectively. The AUC values of OS signature for 2-, 4-, and 6-years were 0.900, 0.928, and 0.963, respectively (**Figure 3A**). All AUC values were up to 0.900, indicating the great discrimination of this signature. In addition, the AUC values of PFS signature for 2-, 4-, and 6-years were 0.826, 0.840, and 0.847, respectively, which also suggested favorable discrimination (**Figure 4A**). According to the median of the risk score, all patients were stratified into low- and high-risk groups. Log-rank tests showed that the patients in the high-risk group has worse OS and PFS than the low-risk patients (**Figures 3B, 4B**). The distribution of risk score (**Figures 3C, 4C**), prognostic status (**Figures 3D, 4D**), and PDUI of each sample (**Figures 3E, 4E**) were visualized to facilitate the understanding of prognostic signatures. Furthermore, we compared the distribution of clinical covariates across high- and low- risk groups (**Supplementary Figure 2**). For the OS signature, the distribution of multifocal indicator, margin status, and metastasis were significantly different between two risk groups (**Supplementary Figure 2A**). Additionally, for the PFS signature, the distributions of age, margin status, and metastasis were significantly different between two risk groups (**Supplementary Figure 2B**).

The Differences of GSVA Analysis and Immune Cells Between High- and Low-Risk Groups

To explore the functions of differentially expressed parental genes in APA, GSVA analysis was conducted to predict the possible functions of APA events ($p < 0.05$). Two heatmaps were used to show differences between high- and low-risk groups (**Figures 5A,B**). The wnt signaling pathway, p53 signaling pathway, RNA degradation, and nucleotide excision repair were enriched in the high-risk group, which played a vital role in tumorigenesis. Meanwhile, we found that other malignant tumors were also enriched in the high-risk group, including colorectal cancer, prostate cancer, and small cell lung cancer, which suggested that the APA signature was

TABLE 1 | Overall survival analysis of APA signature and clinical data for sarcoma.

	Univariate Cox analysis			Multivariate Cox analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Risk (Low)	0.093	0.054–0.159	0.000	0.091	0.038–0.217	0.000
Age	1.020	1.005–1.036	0.010	1.025	1.002–1.049	0.032
Sex (Male)	0.855	0.572–1.277	0.443			
Race						
Asian						
Black	1.085	0.132–8.924	0.939			
White	0.791	0.108–5.773	0.817			
Histological						
DLP						
LMS	0.842	0.512–1.386	0.500			
MFS	0.703	0.328–1.507	0.364			
Other	0.739	0.319–1.710	0.479			
UPS	0.901	0.481–1.691	0.746			
Tumor site (Other)	1.229	0.792–1.906	0.357			
Metastasis (Yes)	3.014	1.834–4.954	0.000	2.410	1.335–4.349	0.004
Margin status (R1/2)	2.554	1.668–3.910	0.000	1.416	0.786–2.550	0.247
Multifocal indicator (Yes)	2.404	1.502–3.847	0.000	1.085	0.517–2.278	0.830
Radiotherapy (Yes)	0.988	0.619–1.579	0.961			
Pharmacotherapy (Yes)	1.382	0.815–2.341	0.230			

APA, alternative polyadenylation; HR, hazard ratio; CI, confidence interval; DLP, dedifferentiated liposarcoma; LMS, leiomyosarcoma; MFS, myxofibrosarcoma; UPS, undifferentiated pleomorphic sarcoma.

TABLE 2 | Progress free-survival analysis of APA signature and clinical data for sarcoma.

	Univariate Cox analysis			Multivariate Cox analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Risk (Low)	0.251	0.178−0.356	0.000	0.221	0.138−0.354	0.000
Age	1.006	0.995−1.018	0.267			
Sex (Male)	1.096	0.797−1.506	0.573			
Race						
Asian						
Black	3.146	0.402−24.609	0.275			
White	2.954	0.412−21.170	0.281			
Histological						
DLP						
LMS	0.926	0.619−1.386	0.708			
MFS	0.730	0.395−1.349	0.315			
Other	0.807	0.421−1.547	0.519			
UPS	0.870	0.528−1.433	0.584			
Tumor site (Other)	0.990	0.706−1.388	0.952			
Metastasis (Yes)	6.001	3.948−9.122	0.000	6.412	4.012−10.247	0.000
Margin status (R1/2)	2.202	1.565−3.099	0.000	2.589	1.634−4.100	0.000
Multifocal indicator (Yes)	1.972	1.307−2.975	0.001	1.567	0.850−2.887	0.150
Radiotherapy (Yes)	1.108	0.769−1.597	0.581			
Pharmacotherapy (Yes)	1.437	0.929−2.222	0.103			

APA, alternative polyadenylation; HR, hazard ratio; CI, confidence interval; DLP, dedifferentiated liposarcoma; LMS, leiomyosarcoma; MFS, myxofibrosarcoma; UPS, undifferentiated pleomorphic sarcoma.

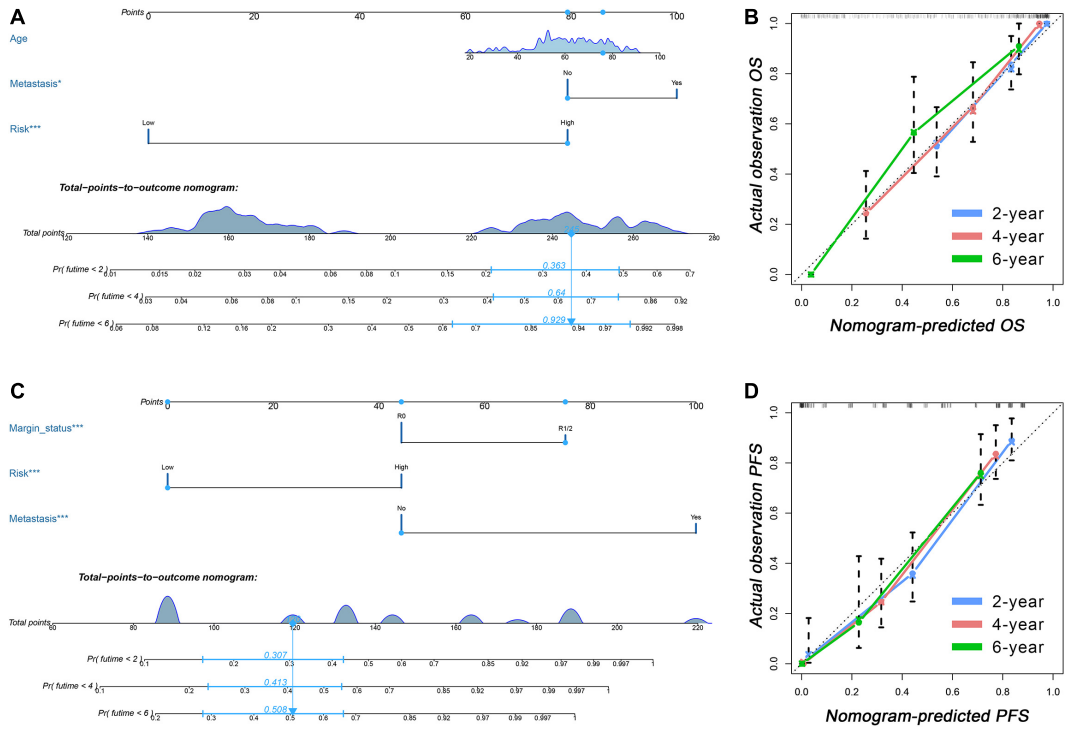

FIGURE 6 | Clinical-APA nomograms for sarcoma. **(A)** A nomogram incorporated APA signature and prognostic clinical data was established to predict OS of sarcoma patients. **(B)** Calibration curves showed that nomogram-predicted OS were highly consistent with actual OS. **(C)** A nomogram incorporated APA signature and prognostic clinical data was established to predict PFS of sarcoma patients. **(D)** Calibration curves showed that nomogram-predicted PFS were highly consistent with actual PFS. APA, alternative polyadenylation; OS, overall survival; PFS, progress free-survival.

positively related to the process of these kinds of tumors. Additionally, the difference in immune cell infiltration were also observed. Among 24 types of immune cells, the NKT cell, monocyte, and CD4 T cell were significantly different between low- and high-risk groups no matter if they were the in OS signature and PFS signature (**Figures 5C,D**). Moreover, the filtration of Tr1 cells, Tfh cells, Tcm cells, B cells, and NK cells was distinct between low OS group and high OS group (**Figure 5C**).

Subgroup Analyses of Signatures in Different Subgroups

To study the ability of prognostic signatures in different clinical subgroups, the Kaplan-Meier survival curves in several subgroups were constructed (**Supplementary Figures 3**, **4**). For the OS signature, in all 16 subgroups, survival analyses showed that patients in the low-risk group have a favorable OS than high-risk patients (**Supplementary Figures 3A–H**). Log-rank tests in all subgroups were statistically significant (all p-values < 0.05). Similarly, for the PFS signature, subgroup analyses indicated that low-risk patients had a better prognosis than high-risk patients (all p-values < 0.05) (**Supplementary Figures 4A–H**). Generally, subgroup analyses showed the stability and robustness of APA-based signatures, which further verified that APA events can serve as satisfactory prognostic predictors.

Development of Two Clinical-APA Nomograms

The results of the univariate Cox analysis indicated that APA signature, age, metastasis status, margin status, and multifocal indicator were OS-related variables ($p < 0.05$) (**Table 1**), while APA signature, metastasis status, margin status, and multifocal indicator were PFS-related variables (**Table 2**). We incorporated the above significant variables into the multivariate Cox analyses. The results indicated that both APA-based OS signature and APA-based PFS signature were robust prognostic predictors and were independent of clinical data (**Tables 1**, **2**). Meanwhile, age and metastasis status were confirmed as independent OS-related variables (**Table 1**), while metastasis and margin status were independent PFS-related variables (**Table 2**). Two nomograms were developed to predict the OS and PFS, respectively (**Figures 6A,C**). In the nomogram, values for individual patients are located along the variable axes, and a line is drawn upward to the point axis to determine the number of points assigned for each variable. There is a total point line at the bottom of the nomogram, and each variable score is summed to give the total points. Then, a vertical line is drawn from the total point scale to the bottom three lines to obtain the prognosis at 2-, 4-, and 6-years. For example, in the nomogram of PFS, a patient with low risk, no distant metastasis, and a margin status of R1/2 would have a total score of 120, and his 2-year, 4-year, and 6-year PFS rates would be 30.7, 41.3, and 50.8%, respectively. The

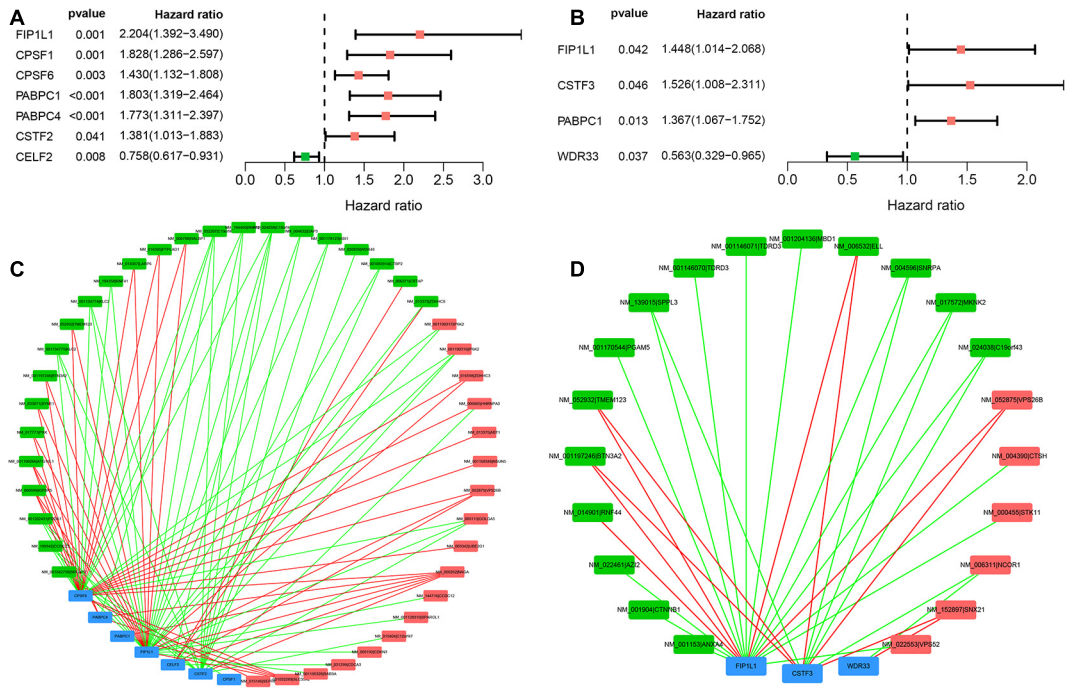

FIGURE 7 | Survival-associated APA factors and APA events correlation network in sarcoma. **(A)** Seven OS-related APA factors identified by univariate Cox analysis. **(B)** Four PFS-related APA factors identified by univariate Cox analysis. **(C)** Regulatory network of OS-related APA factors and APA events. Blue rectangles means APA factors, green rectangles means protective APA events, and red rectangles means risk APA events. Green line means negative correlation between the PDUI of APA events and the expression of APA factors. **(D)** Regulatory network of PFS-related APA factors and APA events. Blue rectangles means APA factors, green rectangles means protective APA events, and red rectangles means risk APA events. Green line means negative correlation between the PDUI of APA events and the expression of APA factors. APA, alternative polyadenylation; OS, overall survival; PFS, progress free-survival.

C-index for OS nomogram was 0.813 and 0.809 for the PFS nomogram, which means that both nomograms have favorable discrimination. The calibration curves for 2-, 4-, and 6-years indicated that the nomogram-predicted outcomes were highly consistent with actual observation outcomes, no matter whether in OS or PFS nomogram (**Figures 6B,D**).

A Network of Prognostic APA Events and APA Factors

According to the univariate Cox analysis, 61 and 38 APA events were OS- and PFS-related biomarkers, respectively. In addition, the expression of 28 core APA factors was extracted. In total, seven APA factors were identified as OS-related APA factors, and four APA factors were identified as PFS-related APA factors (**Figures 7A,B**). The correlation between APA events and APA factors was studied. Finally, two APA-APA factors regulatory networks were established (**Figures 7C,D**). The regulatory between APA events and APA factors were initially elucidated. The first network contained seven APA factors and 42 APA events to show the regulatory mechanism of OS-related APA events (**Figure 7C**). Another network including three APA factors and 21 APA events was established to show the regulatory mechanism of PFS-related APA events (**Figure 7D**). Throughout the two networks, we can find that one APA factor can regulatory more than one APA event. Additionally, one APA event can be regulated by more than one APA factor even two opposite regulations.

Pan-Cancer Analyses of FIP1L1-VPS26B Regulating Relationship

In two regulatory networks, one interesting regulating relationship presented itself for our attention. According to the survival analyses, APA events of VPS26B were identified as overlapping independent prognostic APA events between OS and PFS. In addition, FIP1L1 was shown to be significantly associated with both OS and PFS. More importantly, in the regulatory network, the expression of FIP1L1 was significantly related to the PDUI of VPS26B. Hence, we speculated this regulating relationship may have an important role in malignancy to comprehensively understand this regulating relationship in malignancy. We performed a pan-cancer analysis based on the UCSC Xena browser and TC3A database. A total of 9087 patients were included, including 30 cancer types. The Pearson correlation analysis indicated that the expression of FIP1L1 is significantly associated with the PDUI of VPS26B (**Figure 8A**). Furthermore, we analyzed the correlation of FIP1L1-VPS26B in specific cancers. Among 29 types (sarcoma was excluded), only four tumor cohorts showed that there was no significant correlation between FIP1L1 and VPS26B. The correlation coefficient is the highest in the TCGA-THYM

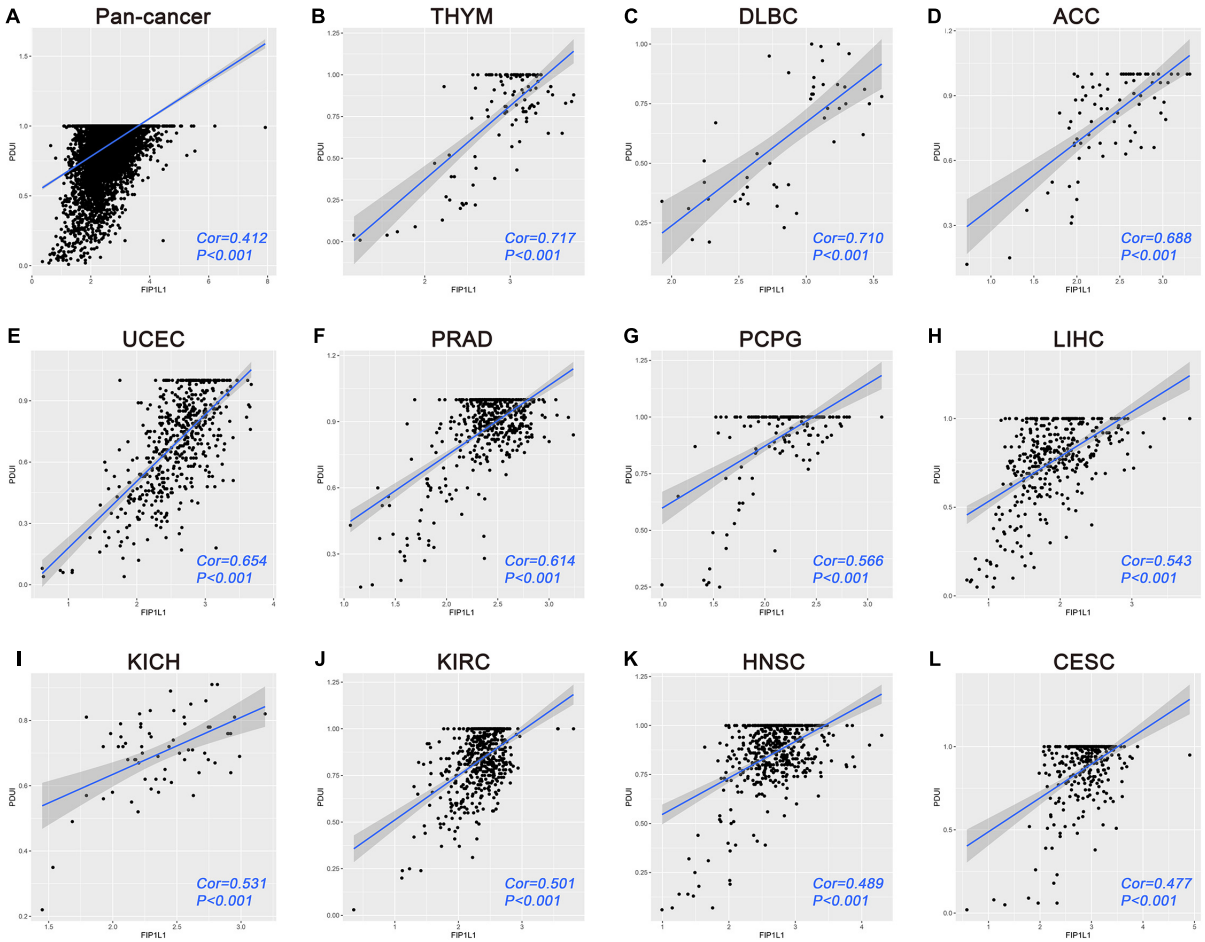

FIGURE 8 | Pan-cancer analyses of FIP1L1-VPS26B regulating relationship. **(A)** Correlation analysis of FIP1L1-VPS26B in 9087 patients indicated that the expression of FIP1L1 is significantly associated with the PDUI of VPS26B. **(B–L)** Correlation results of top 11 cancer in 30 cancer types.

cohort (cor = 0.717), followed by TCGA-DLBC (cor = 0.710) (**Figures 8B–L**).

DISCUSSION

Alternative polyadenylation is a RNA-processing mechanism that generates distinct 3' termini on mRNAs and other RNA polymerase II transcripts (Tian and Manley, 2017). It is widespread across all eukaryotic species and is recognized as a major mechanism of gene regulation (Tian and Manley, 2017). The role of APA in human cancers is only beginning to be appreciated, which shows the potentially robust predictive value for tumor patients. More importantly, compared with genomic data only, APA signature may have a better predictive ability in patients' prognosis (Xia et al., 2014; Feng et al., 2018).

In the present study, we mainly focused on the profiling of the prognostic value of APA events to explore the utilization of APA signatures in predicting the outcome of sarcoma patients. In total, 259 primary sarcoma patients from TC3A database and TCGA dataportal were included. A total of 61 and 38 APA events were determined as OS- and PFS-related biomarkers, respectively. Two 16 APA-based signatures were built and showed favorable prognostic predictors, with all AUC values above 0.900 for OS signature and 0.820 for the PFS signature. Combined with independent prognostic clinical variables, two APA-clinical nomograms were developed and showed satisfactory discrimination and calibration. Finally, combined survival and correlation network analyses between APA events and APA factors, our research uncovered the underlying mechanism of APA events involved in patient prognosis. To our knowledge, the present study is the first study to establish APA signatures for predicting the survival of sarcoma patients.

Identifying effective biomarker and constructing an ideal prognostic signature or nomogram has long been the focus of oncologists, which can individually predict the specific outcomes to guide the management of tumor patients. At present, a great number of biomarkers have been reported, and several prognostic models for sarcoma were constructed (Benassi et al., 2015; Callegaro et al., 2017, 2019; Yang et al., 2017; Huang et al., 2019; Raut et al., 2019; Zhang et al., 2019a; Gu et al., 2020; Hu et al., 2020; Zhu et al., 2020). For example, MS

Benassi et al. (2015) reported that the expression of IGFBP7, considered a tumor stroma marker in mesenchymal-derived cells, was highly prognostic in poor metastasis-free survival for soft tissue sarcoma. Additionally, clinicopathological variables, ncRNA data, or immune cells were also confirmed as predictors and used to develop prognostic models (Callegaro et al., 2017, 2019; Yang et al., 2017; Huang et al., 2019; Raut et al., 2019; Zhang et al., 2019a; Gu et al., 2020; Hu et al., 2020; Zhu et al., 2020). Nevertheless, clinical practice is not optimistic. Current research mainly focused on the prognostic role of clinical data or gene level, overlooking the transcriptional level. As one of the important post-transcriptional regulatory mechanisms, APA events have shown a potentially robust predictive value for tumor patients (Venkat et al., 2020; Zhang et al., 2020b). Our study confirmed that APA events are valuable predictors for sarcoma patients, no matter whether in general patients or several subgroups. In the mechanism, APA play a vital role in gene regulation and diverse cellular processes, including mRNA metabolism, protein diversity, and protein localization (Tian and Manley, 2017).

In total, 31 APA events, including 15 APA events for OS signature only, 15 APA events for PFS signature only, and 1 APA event for both OS and PFS signatures, were used to construct two signatures. Among the corresponding genes of these APA events, most of which were associated with tumorigenesis and progression. For example, by activating the EMT and non-canonical WNT signaling, ABI1 can drive the tumorigenesis of prostate cancer (Nath et al., 2019). Additionally, dysregulation of ABI1 was confirmed associated with the prognosis of gastric cancer, epithelial ovarian cancer, and breast cancer (Cui et al., 2010; Wang et al., 2011; Zhang et al., 2015). Another widely studied gene is MCM2, which is a vital initiation factor for DNA replication in humans. It also presents in the nucleus and is overexpressed in proliferating cells. The prognostic value of MCM2 was confirmed in pancreatic cancer, lung cancer, multiple myeloma, and oral cancer (Ramnath et al., 2001; Torres-Rendon et al., 2009; Deng et al., 2020; Quan et al., 2020). Significantly, the APA event of VPS26B was determined as an overlapping independent prognostic biomarker for sarcoma patients. Nevertheless, few studies reported the role of VPS26B or related regulation in cancers. Future studies may focus on its prognostic value in other tumors and their regulatory role in tumors.

In our research, APA and APA factor regulatory networks were also constructed. Various of regulating relationships between them were identified. Interestingly, one core regulating relationship was confirmed, and the same trend was also confirmed in the pan-cancer analysis. As a novel regulator for APA events, FIP1L1 can regulate the 3'UTR lengthening of leukemia-associated genes, including NRAS, BAALC, and MAPKAPK3 (Davis et al., 2018). Interestingly, both overexpression and knockdown of FIP1L1 are harmful to leukemia cells, demonstrating that mild alteration of gene expression may dramatically impact on cell fitness (Davis et al., 2018). In addition to FIP1L1, eight APA factors were included incorporated into regulatory networks. The mechanism of part of factors has been preliminarily elucidated. As a vital cleavage/polyadenylation factor, CSTF2 can shorten the length of 3'UTR RAC1 in human urothelial carcinoma of the bladder by mediating slow transcriptional elongation at RAC1 (Chen et al., 2018). Moreover, F6 was considered as a vulnerability target for breast cancer patients (Binothman et al., 2017). Despite this research, the regulatory mechanism between APA and APA factors in sarcoma remains unclear. More than 100 potential regulating relationships were detected in our research, which pointed out the directions for future research.

Although the strict bioinformatic and statistical methods were used and the prognostic value of APA events in sarcoma patients have been discovered in our research, there are some limitations. First, due to the relative rarity of sarcoma, only bioinformatic analyses were used in this study, and no further experimental analysis based on clinical samples was performed to validate our results. Second, external validation is vital for clinical application of prognostic signatures or nomograms. Unfortunately, no available independent cohort can be obtained from other databases. Thirdly, no available normal sample can be incorporated into our study. Therefore, the diagnostic value and the potential carcinogenic effect of APA events cannot be studied in this research. Finally, the data used in the study were obtained from public datasets from which the available clinical data is limited and incomplete. Therefore, some prognostic variables, such as tumor grade and size, were not available and were not analyzed in the present study.

CONCLUSION

Our data revealed the prognostic value of survival−related APA events for sarcoma. Some key APA factors might play essential roles in tumor initiation and progression by regulating the corresponding APA events. Our findings might offer a new prospect for effective therapies targeted at APA events for sarcoma.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://xenabrowser.net/ and tc3a.org.

AUTHOR CONTRIBUTIONS

CH, YX, and CL performed the data analysis and wrote the manuscript. JL, CL, JD, and XT contributed to the data analysis and manuscript revision. CH, TY, BC, and YD contributed to literature search and data extraction. CH and YX conceived and designed the study. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.595331/full#supplementary-material

Supplementary Figure 1 | LASSO analysis of OS- and PFS-related APA events. **(A,C)** Dotted vertical lines were drawn at the optimal values by using the minimum criteria. **(B,D)** LASSO coefficient profiles of the candidate OS **(B)** and PFS **(D)**-related alternative polyadenylation events. A coefficient profile plot was produced against the log λ sequence. LASSO, least absolute shrinkage and selection operator; APA, alternative polyadenylation; OS, overall survival; PFS, progress free-survival.

Supplementary Figure 2 | The distribution of clinical covariates across low- and high-risk groups. **(A)** Overall survival signature; **(B)** Progress free-survival signature. DLP, dedifferentiated liposarcoma; LMS, leiomyosarcoma; MFS, myxofibrosarcoma; UPS, undifferentiated pleomorphic sarcoma.

Supplementary Figure 3 | Subgroup analyses of OS signature. Survival curves showed that high-risk patients were significant worse OS than low-risk patients in subgroups of age **(A)**, sex **(B)**, cancer histological type **(C,D)**, metastatic status **(E)**, tumor site **(F)**, margin status **(G)**, and multifocal indicators **(H)**. OS, overall survival.

Supplementary Figure 4 | Subgroup analyses of PFS signature. Survival curves showed that high-risk patients were significant worse PFS than low-risk patients in subgroups of age **(A)**, sex **(B)**, cancer histological type **(C,D)**, metastatic status **(E)**, tumor site **(F)**, margin status **(G)**, and multifocal indicators **(H)**. PFS, progress-free survival.

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REVIEW
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Lysine Demethylases: Promising Drug Targets in Melanoma and Other Cancers

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Epigenetic dysregulation has been implicated in a variety of pathological processes including carcinogenesis. A major group of enzymes that influence epigenetic modifications are lysine demethylases (KDMs) also known as "erasers" which remove methyl groups on lysine (K) amino acids of histones. Numerous studies have implicated aberrant lysine demethylase activity in a variety of cancers, including melanoma. This review will focus on the structure, classification and functions of KDMs in normal biology and the current knowledge of how KDMs are deregulated in cancer pathogenesis, emphasizing our interest in melanoma. We highlight the current knowledge gaps of KDMs in melanoma pathobiology and describe opportunities to increases our understanding of their importance in this disease. We summarize the progress of several pre-clinical compounds that inhibit KDMs and represent promising candidates for further investigation in oncology.

Keywords: epigenetics, histones, lysine demethylases, small molecule inhibitors, cancer, melanoma

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INTRODUCTION

There are numerous genetic alterations which promote carcinogenesis which include mutations of certain genes and chromosomes. Epigenetics is defined as heritable functional changes in the genome which do not involve a change in the DNA sequence (Dupont et al., 2009). The Greek prefix "epi" denotes "over, outside of, or around" implying additional factors that may influence traditional genetic inheritance patterns. Epigenetics is essential in normal development and biology but dysregulation has been implicated as a key impetus of carcinogenesis and resistance. All cells contain genetic information in the form of DNA which is wound around proteins called histones (Kornberg, 1974). The DNA is assembled into units called nucleosomes which form a complex consisting of histones and DNA known as chromatin (Kornberg, 1974). Chromatin is organized into compact, transcriptionally inactive regions called heterochromatin, usually around the periphery of the nucleus and loosely arranged, transcriptionally active chromatin called euchromatin (Elgin, 1996). This formation has an important role in the regulation of gene expression as well as controlling the transcription, replication, recombination and repairing of DNA (Elgin, 1996). Various epigenetic changes can affect chromatin structure and hence gene expression. One of the most well studied epigenetic changes involves methylation which may occur at the DNA or histone level (Tost, 2010). DNA methylation involves the transfer of methyl groups by DNA methyltransferases (DNMTs) to individual nucleotide bases, altering gene expression

(Bilian Jin and Robertson, 2011). The family of DNMT enzymes adds methyl groups while removal is mediated by the ten eleven translocation (TET) family (Zhang et al., 2010).

The other major class of methylation is histone methylation which is a post-translational modification (PTM) of histone tails. Histone methylation involves the addition of (*via* writer enzymes) or removal of (*via* eraser enzymes) methyl groups, typically on lysine (K) or arginine (R) amino acids of histone type 1–4 tails (**Figure 1**). Lysine methyltransferases (KMTs) drive the addition of methyl groups, whereas lysine demethylases (KDMs) are responsible for the removal of methyl groups. Depending on the type of histone modifications, the consequence might induce either an open or closed chromatin state which regulates gene expression. KDMs rarely act in isolation as enzymatic erasers but are typically members of large epigenetic complexes, consisting of other enzymes and transcription factors. This is suggestive of a scaffolding protein role for KDMs in addition to a catalytic one. The most well investigated histone lysine methylation sites include H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20 (**Figure 1**) that will either compact or open chromatin depending on lysine position and the number of methyl groups. Hence this review will focus on what is currently known about KDMs in normal biology and cancers including melanoma. A summary of the progress of current KDM inhibitors is also discussed.

THE FUNCTION OF KDMs IN NORMAL DEVELOPMENT AND DEREGULATION IN CANCER

Lysine demethylases are critical for normal development but numerous studies have implicated the dysregulation of several KDMs in cancers. This is likely due to the ability of KDMs to govern major changes in the transcriptional networks of hundreds of genes with potential roles in all major hallmarks of cancer. The roles of each KDM in normal biology and cancer is described below and **Table 1** summarizes studies in cancer.

Amine Oxidase KDMs

Lysine demethylases are classified into two groups according to the catalytic mechanisms of demethylation- amine oxidase or jumonji C domain containing KDMs. Class I is the amine-oxidase lysine specific demethylases 1 and 2 (LSD1 and 2), also known as KDM1A and KDM1B. KDM1A and KDM1B use Flavin adenine dinucleotide (FAD) as a substrate to generate an imine intermediate which is hydrolyzed to produce the demethylated lysine residue (Walport et al., 2012). The amines-oxidase like (AOL) catalytic domain at the C-terminal in KDM1A and KDM1B consists of two folded subdomains. The FAD and substrate binding regions are structurally related to the superfamily of monoamine oxidases (MAO). In particular MAO-A and MAO-B- enzymes which catalyze the oxidation of monoamine (contain one amino group) neurotransmitters which include serotonin and dopamine (Shi et al., 2004; Willmann et al., 2012). The N-terminal of KDM1A contains the SWIRM domain which is named after the SWI3, RSC8 and MOIRA proteins and is essential for protein stability and interactions with histone tails.

KDM1A was the first histone demethylase identified (Shi et al., 2004). Functionally, KDM1A predominantly catalyzes the removal of methyl groups from mono and di-methylated lysine residues at H3K4 inducing gene repression (Walport et al., 2012). An early *in vivo* study showed that KDM1A demethylation of K1096 in the DNA methyl transferase enzyme DNMT1 was essential for the gastrulation stage of murine embryogenesis (Wang et al., 2009). Genetic ablation of KDM1A in a knockout mouse model was embryonic lethal (Wang et al., 2007, 2009).

Interestingly, KDM1A can demethylate non-histone proteins in osteosarcoma cells such as the tumor suppressor p53 which has an important role in regulation of pro-apoptotic genes. Another example showed KDM1A can demethylate K185 of transcription factor E2F1 which has an important role in regulating the cell cycle and tumor suppressor genes in lung cancer cells (Huang et al., 2007; Wang et al., 2009; Kontaki and Talianidis, 2010; Xie et al., 2011). A study found that overexpression of KDM1A induces E2F1 signaling *via* histone demethylation and promotes cell proliferation in oral cancer and that inhibition of KDM1A reduces E2F1 signaling, implying an oncogenic role of KDM1A in oral cancer (Narayanan et al., 2015).

The other KDM1 family member, KDM1B has been reported to catalyze the removal of methyl groups at H3K4 and this enzyme is essential for oocyte development (Ciccone et al., 2009). KDM1B knockout mice exhibit embryonic lethality (Ciccone et al., 2009), highlighting its importance in normal biology.

Jumonji C Demethylases

Class II KDMs are the demethylases that contain a Jumonji C domain (JMjC KDMs "Jumonji" meaning cruciform in Japanese). This includes the KDM2-6 subfamilies which consists of 20 enzymes that are grouped into five subfamilies: KDM2, KDM3, KDM4, KDM5, and KDM6 (Klose et al., 2006). The JMjC KDMs catalyze the removal of methyl groups from mono, di and trimethylated lysines at various sites and use dioxygen and 2-oxoglutarate (2OG) as substrates with Fe (II) as an essential cofactor (Walport et al., 2012). Unlike KDM1A/1B the JMjC KDMs are able to remove methyl groups from trimethylated lysine sites since the mechanism doesn't require a formation of an imine. The catalytic domain of the JMjC KDMs is structurally related to the superfamily of 2OG- dependent oxygenases which play an important role in fatty acid metabolism, protein biosynthesis and nucleic acid repair/modification (Loenarz and Schofield, 2008).

KDM2/3 Demethylases

The KDM2 subfamily consists of two demethylases KDM2A and KDM2B. Both KDM2A and KDM2B have been reported to be oncogenic (Pedersen and Helin, 2010). KDM2A has been reported to be upregulated and induces proliferation in lung, gastric and breast cancer. KDM2A overexpression was found to increase cell proliferation and invasion through activation of ERK1/2 signaling in lung cancer as well as being associated with poor prognosis in lung cancer patients (Wagner et al., 2013). KDM2A overexpression also promoted cell growth and migration in gastric cancer by downregulation of tumor suppressor gene programmed cell death 4 (PDCD4)

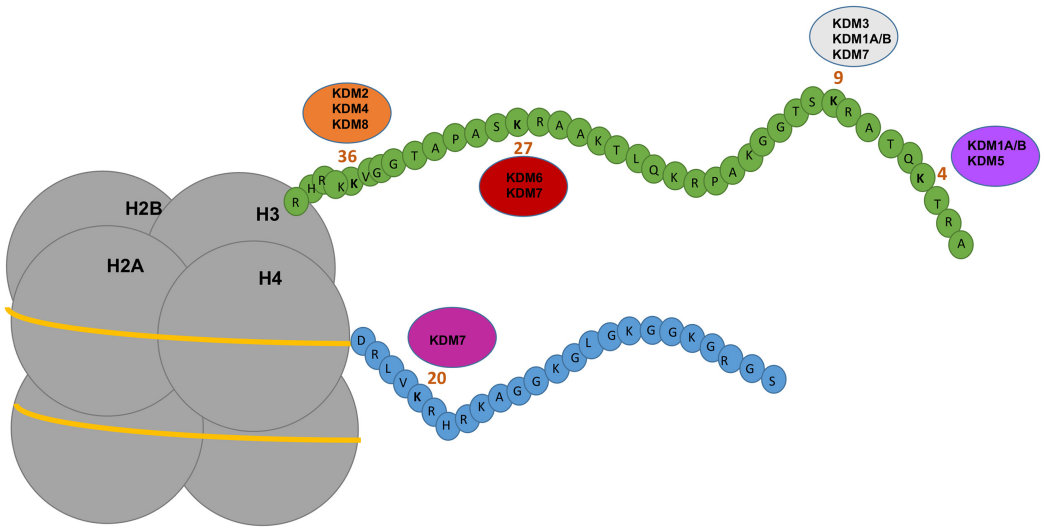


FIGURE 1 | Depicting the mechanism of action of KDMs on histones. Nucleosomes consist of four subunits of histones which contain amino acid tails that are modified by epigenetic regulators including KDMs. KDM1A/B demethylates H3K9me1/H3K9me2 and H3K4me1/me2 inducing gene repression or activation. KDM2 and KDM4 demethylases target H3K36me1/me2/me3 inducing gene activation. KDM3 demethylases target H3K9me1/me2 inducing gene activation. KDM5 demethylases targets H3K4me2/me3 inducing gene repression. KDM6 and KDM7 demethylases target H3K27me2/me3 inducing gene activation. KDM7 demethylases target H4K20me1 and H3K9me2/me3. KDM8 demethylase target H3K36me3 inducing gene activation. Figure is adapted from Verde et al. (2017).

(Huang et al., 2015). In breast cancer, KDM2A is highly expressed in myoepithelial cells which have been reported to have anti-tumor properties. KDM2A ablation in these cells induced increased invasion and migration *via* downregulation of MMP proteins and repression of EF21 signaling (Rizwani et al., 2014).

KDM2B is overexpressed in numerous cancers. Knockdown of KDM2B reduced cell growth in gastric cancer *in vitro* and *in vivo* and induced autophagy- a process in which cells remove damaged cell components (Zhao et al., 2017). KDM2B overexpression induces transformation of hematopoietic progenitor cells in acute myeloid leukemia whereas reduction of KDM2B inhibited Hox9/Meis1 induced leukemic transformation (He et al., 2011). Overexpression of KDM2B is observed in ovarian cancer and when knocked down *in vitro* and *in vivo* reduced cell proliferation, migration and tumor growth (Kuang et al., 2017). Pancreatic cancers with increased KDM2B promoted tumor formation in cooperation with the oncogene Kras in an *in vivo* model (Tzatsos et al., 2013). Another study showed that KDM2B overexpression was associated with poor prognosis in glioma and KDM2B knockdown inhibited cell proliferation and induced cell cycle arrest (Wang Y. et al., 2018).

The KDM3 subfamily consists of three demethylases; KDM3A, KDM3B, and JMJD1C. It has been reported that KDM3A is important in spermatogenesis and male KDM3A knockout mice are infertile (Pedersen and Helin, 2010). The function of KDM3B and JMJD1C is largely unknown.

KDM4/5 Demethylases

The KDM4 subfamily consists of five demethylases- KDM4A, KDM4B, KDM4C, KDM4D, and KDM4E. KDM4A, KDM4B, KDM4C, and KDM4D have been reported to be important in oncogenesis, but the function of KDM4E is unknown (Pedersen and Helin, 2010). KDM4A overexpression was found to stimulate the AR, inducing the expression of prostate specific antigen, implicated in the progression of prostate cancer (Kim et al., 2016). KDM4A is overexpressed in breast cancer and KDM4A knockdown inhibited cell proliferation, migration and invasion (Li et al., 2012). Lung cancers with increased KDM4A are associated with poor prognosis (Xu et al., 2016).

KDM4B overexpression promoted DNA damage in breast cancer cells which was significantly reduced upon pharmacological inhibition of KDM4B, by induction of apoptosis in triple negative breast cancers deficient in the tumor suppressor gene, PTEN (Wang W. et al., 2018; Xiang et al., 2019). KDM4B overexpression also promoted proliferation, growth and glucose uptake in colorectal cancer cells whereas KDM4B knockdown inhibited tumor growth significantly in an *in vivo* model (Li et al., 2020).

KDM4C overexpression is associated with poor prognosis in prostate cancer and can co-regulate transcriptional activation of the AR. KDM4C knockdown significantly reduced proliferation, colony formation, AR transcriptional activity in prostate cancer cells and inhibited tumor growth of a prostate cancer model in zebrafish (Lin et al., 2019).

The KDM5 subfamily consists of four demethylases- KDM5A, KDM5B, KDM5C, and KDM5D. This group of demethylases can remove methyl groups from di and trimethylated lysines on H3K4. The KDM5 subfamily has been reported to play an important role in development, identified in *drosophila melanogaster* as Lid (Little Imaginal Disks) protein due to the phenotype visible in mutant larvae (Gildea et al., 2000). This protein was classified as a H3K4 histone demethylase which had all the domains of the human JARID1 family. The Lid protein binds to *drosophila* Myc (dMyC)—a transcription factor

TABLE 1 | Summary of KDMs reported to be significant in cancer.

KDM	Alias	Target Histone demethylation site	Gene activation or repression	Cancer study implicated in
KDM1A	LSD1, AOF2, BHC110, KDM1	H3K4me1 H3K4me2	Repression Repression	Leukemia (Harris et al., 2012), prostate cancer (Willmann et al., 2012), breast cancer (Perillo et al., 2008), neuroblastoma (Schulte et al., 2009; Amente et al., 2015)
KDM1B	LSD2, AOF1	H3K4me1 H3K4me2	Repression Repression	Breast cancer (Katz et al., 2014; Chen et al., 2017)
KDM2A	JHDM1A, FBXL11	H3K36me1 H3K36me2	Activation Activation	Leukemia (Dong et al., 2013), non-small cell lung cancer (NSCLC) (Wagner et al., 2013), gastric cancer (Huang et al., 2015; Kong et al., 2016), breast cancer (Rizwani et al., 2014)
KDM2B	JHDM1B, FBXL10	H3K36me1 H3K36me2 H3K4me3	Activation Activation Repression	Leukemia (He et al., 2011), pancreatic cancer (Tzatsos et al., 2013), ovarian cancer (Kuang et al., 2017), gastric cancer (Zhao et al., 2017), glioma (Wang Y. et al., 2018)
KDM3A	JHDM2A, JMJD1A, JMJD1	H3K9me1 H3K9me2	Activation Activation	Breast cancer (Ramadoss et al., 2017a), ovarian cancer (Ramadoss et al., 2017b), Ewing sarcoma (Sechler et al., 2017), prostate cancer (Wilson et al., 2017)
KDM3B	JHDM2B, JMJD1B	H3K9me1 H3K9me2	Activation Activation	Leukemia (Kim et al., 2012)
KDM3C	JHDM2C	H3K9me1 H3K9me2	Activation Activation	unknown
KDM4A	JMDM3A, JMJD2A	H3K9me2 H3K9me3 H3K36me2 H3K36me3	Activation Activation Activation Activation	Endometrial cancer (Qiu et al., 2015), breast cancer (Berry et al., 2012)
KDM4B	JMDM3B, JMJD2B	H3K9me2 H3K9me3 H3K36me2 H3K36me3	Activation Activation Activation Activation	Breast cancer (Kawazu et al., 2011) Colorectal cancer (Li et al., 2020)
KDM4C	JMDM3C, JMJD2C	H3K9me2 H3K9me3 H3K36me2 H3K36me3	Activation Activation Activation Activation	Prostate cancer (Wissmann et al., 2007)
KDM4D	JMDM3D, JMJD2D	H3K9me3	Activation	Prostate cancer (Shin and Janknecht, 2007)
KDM4E	KDM4DL, JMJD2E	H3K9me3	Activation	Unknown
KDM5A	JARID1A RBBP2	H3K4me2 H3K4me3	Repression Repression	Leukemia (van Zutven et al., 2006),breast cancer (Hou et al., 2012), ovarian cancer (Feng et al., 2017), melanoma (Roesch et al., 2005)
KDM5B	JARID1B PLU1	H3K4me2 H3K4me3	Repression Repression	Breast cancer (Catchpole et al., 2011), prostate cancer (Xiang et al., 2007), melanoma (Roesch et al., 2010)
KDM5C	JARID1C SMCX	H3K4me2 H3K4me3	Repression Repression	Cervical cancer (Smith et al., 2010), renal cell carcinoma (Yan et al., 2007; Niu et al., 2012)
KDM5D	JARID1D	H3K4me2 H3K4me3	Repression Repression	Prostate cancer (Komura et al., 2016, 2018; Li et al., 2016)
KDM6A	UTX	H3K27me2 H3K27me3	Activation Activation	Bladder cancer (Ler et al., 2017), cervical cancer (Soto et al., 2017), breast cancer (Taube et al., 2017), multiple myeloma (Ezponda et al., 2017), lung cancer (Terashima et al., 2017), pancreatic cancer (Andricovich et al., 2018)
KDM6B	JMJD3	H3K27me2 H3K27me3	Activation Activation	Colon cancer (Pereira et al., 2011; Tokunaga et al., 2016), pancreatic cancer (Yamamoto et al., 2014), Prostate cancer (Daures et al., 2016), diffuse large B-cell lymphoma (Mathur et al., 2017), non-small cell lung cancer (Ma et al., 2015), clear cell renal carcinoma (Li et al., 2015), multiple myeloma (Ohguchi et al., 2017), acute myeloid leukemia (Li et al., 2018), melanoma (Park et al., 2016), ovarian cancer (Pinton et al., 2018)
KDM7A	JHDM1D	H3K27me1 H3K27me2 H3K9me1 H3K9me2 H4K20me2	Activation Activation Activation Activation Activation	Melanoma and cervical cancer (Osawa et al., 2011), Prostate cancer (Lee et al., 2018)
KDM8	JMJD5	H3K36me3	Activation	Breast cancer (Hsia et al., 2010) Prostate cancer (Wang et al., 2019)

that is important in cell cycle progression, cell proliferation and apoptosis, and is frequently dysregulated in cancer (Secombe and Eisenman, 2007; Li et al., 2010).

KDM5A was initially identified as a binding partner of retinoblastoma protein (pRB) (Defeo-Jones et al., 1991). pRB is a tumor suppressor that inhibits cell cycle progression, preventing cell growth and promoting senescence. Embryonic fibroblasts isolated from KDM5A knockout mouse revealed an important role for KDM5A in mitochondrial function (Varaljai et al., 2015) and cell differentiation (Benevolenskaya et al., 2005; Lopez-Bigas et al., 2008).

KDM5B/JARID1B/PLU1 was initially identified in a study targeting genes regulated by the tyrosine kinase HER2 (Lu et al., 1999). A later study found that KDM5B had H3K4 histone demethylase activity and inhibited the expression of tumor suppressor genes BRAC1 and CAV1 (Yamane et al., 2007). In addition, KDM5B has been reported to be overexpressed in numerous cancers and has been identified as a potential oncogene.

KDM5D/JARID1D is the least well investigated demethylases from the KDM5 subfamily, but has been implicated in prostate cancer progression (Perinchery et al., 2000).

KDM6/7 Demethylases

The KDM6 subfamily consists of three demethylases- KDM6A, KDM6B, and UTY. KDM6A also known as UTX can remove methyl groups from di and trimethylated lysines on H3K27- a mark associated with suppression of gene transcription (Pedersen and Helin, 2010). KDM6A was initially identified as playing an important role in embryonic development and cell differentiation (Hong et al., 2007). KDM6A together with methyltransferases MLL2 (KMT2D) and MLL3 (KMT2C) is an important component of the COMPASS complex also known as the ASCOM complex which mediates the transcriptional activation of genes *via* H3K4 trimethylation and H3K27me2/3 demethylation (Shilatifard, 2008; Van der Meulen et al., 2014; Ford and Dingwall, 2015). The COMPASS complex can also promote histone *acetylation* a demethylation independent activity, by interaction with histone acetyltransferase p300 (CBP) as well as chromatin remodeling *via* the SWI/SNF complex and transcriptional elongation by interacting with transcription elongation factors (**Figure 2**; Wang et al., 2017; Schulz et al., 2019). KDM6A therefore has dual roles in activation of gene expression. Not only does it remove suppressive marks on H3K27 but also activates genes by H3K4 trimethylation and H3K27 acetylation, highlighting the complex interplay between histone erasers and writers to remodel chromatin in a highly orchestrated fashion.

KDM6A exhibits another methylation independent role by directly interacting with DNA binding transcription factors, including nuclear receptors such as estrogen and retinoic acid receptors (Cho et al., 2007; Rocha-Viegas et al., 2014; Xie et al., 2017). KDM6A has been found to interact with retinoblastoma binding proteins including RBBP5 *in vivo*, potentially having an influence on the regulation of cell cycle and cell differentiation by RB family proteins (Shpargel et al., 2012; Van der Meulen et al., 2014).

KDM6A has been reported to be inactivated by mutations in 70% of non-invasive bladder cancer causing a loss of KDM6A expression (Ler et al., 2017). This is suggestive of a tumor suppressor role in this cancer. Studies have suggested loss of KDM6A may amplify PRC2 complex mediated gene repression and dependency in bladder cancer cells that can be sensitized to EZH2 inhibitors (Atala, 2017). Loss of KDM6A expression has also been associated with other cancers such as multiple myeloma (MM) in which KDM6A mutations lead to low KDM6A expression, resulting in increased proliferation, adhesion and tumorigenicity. Loss of KDM6A also sensitized MM cells to EZH2 inhibitors GSK343 and GSK126, inducing cell death and decreased proliferation (Ezponda et al., 2017). Squamous cell, metastatic pancreatic cancer in females was also associated with loss of KDM6A expression in a knockout mouse model. This was attributed to deregulation of the COMPASS complex and activation of oncogenes MYC and RUNX3 (Zhu et al., 2014). The cells also had increased sensitivity to bromodomains and extra-terminal (BET) inhibitors that target a type of epigenetic "reader" protein. Collectively this data suggests opportunities to indirectly target KDMs by studying the rich network of histone eraser or reader proteins that KDMs interact with.

In contrast to loss of KDM6A its presence may also be associated with other cancer types such as cervical cancer, where it appeared necessary for HPVE7 expressing cells to survive and de-repress the cell cycle DNA replication inhibitor p21 (Soto et al., 2017). In addition, KDM6A has been shown to support the oncogenic function of the estrogen receptor in breast cancer (Kim et al., 2017; Taube et al., 2017; Xie et al., 2017). This suggests KDM6A may operate in a cell type dependant manner and further investigation is required to resolve the tumor suppressive vs oncogenic role in different types of cancer.

KDM6B also known as JMJD3 has been reported to induce expression of oncogenes of the RAS/RAF MAP kinase signaling pathway and is expressed on activated macrophages, purported to play a role in inflammation (Agger et al., 2009).

Initial studies showed that KDM6B expression was important in the progression and prognosis of colon cancer (Pereira et al., 2011; Tokunaga et al., 2016). KDM6B has been reported to be overexpressed in prostate cancer with its expression increasing incrementally as the diseases progresses (Daures et al., 2016). In addition, KDM6B was found to induce epithelial to mesenchymal transition (EMT) and metastasis in clear cell renal carcinoma *via* activation of EMT factor Slug (Li et al., 2015). KDM6B has been reported to be overexpressed in diffuse large B-cell lymphoma (DLBCL) and is associated with poor survival. When DLBCL cells were treated with a small molecule KDM6 inhibitor (GSK-J4) not only was KDM6B expression inhibited, the cells were sensitized to chemotherapy agents (Mathur et al., 2017). KDM6B is highly expressed in multiple myeloma cells and when KDM6B was knocked down, growth and survival of these cells was inhibited (Ohguchi et al., 2017). Recent studies found that KDM6B overexpression promoted ovarian cancer cell migration and invasion *via* modulation of transforming growth factor-β1 (TGF-β1) (Pinton et al., 2018; Liang et al., 2019). In contrast, KDM6B overexpression induced cell apoptosis in non-small cell lung cancer (NSCLC) *via* translocation of FOXO1

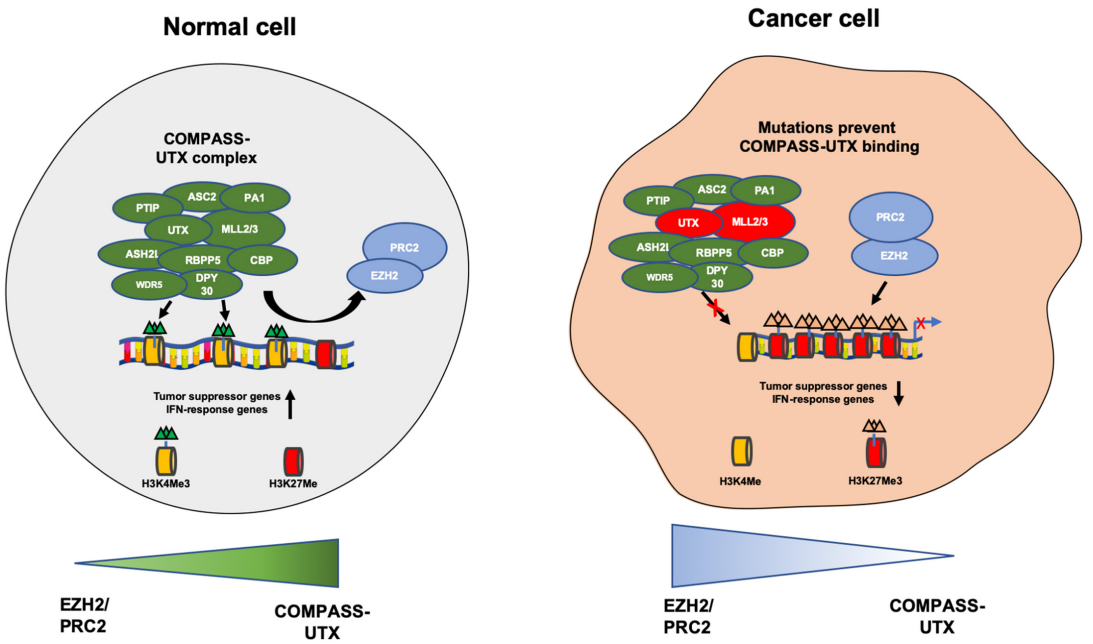


FIGURE 2 | Depicting the COMPASS complex and PCR2 complex that contribute to the open and closed chromatin states. In a normal healthy cell the COMPASS complex removes methyl groups from H3K27me3 and adds methyl groups to H3K4me and prevents PRC2 complex from adding methyl groups to H3K27me. This induces transcriptional activation of tumor suppressive and IFN-response genes. In the context of cancer UTX (KDM6A) and MLL2/3 are frequently mutated in the COMPASS complex, causing a loss of expression. This prevents UTX from binding to the compass complex and no H3K27me3 demethylation occurs, enabling the PRC2 complex to add methyl groups on H3K27me and remove methyl groups from H3K4me, inducing transcriptional repression of tumor suppressor and IFN response genes, promoting cancer cell growth.

(Ma et al., 2015) indicating that the role of KDM6B may be cell type dependent.

KDM7A/JHDM1D can remove methyl groups from di and trimethylated lysines on histone H3K4 as well as methyl groups from H3K9 (Klose et al., 2006; Wen et al., 2010). KDM7A has been reported to play an important role in the regulation of neural differentiation in particular the regions of the brain (Huang et al., 2010; Tsukada et al., 2010) but is not well studied in cancer.

KDM8 Demethylase

The most recently identified KDM is KDM8 also known as JMJD5 and demethylates H3K36me2 inducing gene activation. KDM8 was initially identified to play an important role in embryogenesis and stem cell renewal (Oh and Janknecht, 2012; Zhu et al., 2014) and can promote carcinogenesis. A study showed KDM8 overexpression induced the expression of cell cycle promoter gene cyclin D1, promoting cell proliferation in a breast cancer cells model *in vitro* and conversely, KDM8 knockdown inhibited cell growth (Hsia et al., 2010). In addition, KDM8 overexpression induces activation of AR transcriptional activity and promotes cell growth in prostate cancer *in vitro* and *in vivo* (Wang et al., 2019).

Lysine demethylases have been implicated in a variety of cancers (summarized in **Table 1**) however, very few KDMs have been investigated in melanoma. We analyzed the percentage of mutation rates and types found in KDMs in the melanoma cohort obtained from the cancer genome atlas (TCGA) (**Figure 3**) and in a second independent dataset known as the Australian Melanoma Genome Project (AMGP) (**Figure 4**). We found that KDM1B and KDM5B exhibited the highest mutation rates in melanoma in the TCGA dataset. The majority of KDMs with the exception of KDM6C are upregulated in this dataset further emphasizing their importance in melanogenesis (**Figure 3**). KDM2B and KDM4C contained the highest number of alterations in the AMGP, however, this dataset does not include mRNA expression. The current knowledge and relevance of KDMs in melanoma will be discussed in the following section.

Current Knowledge and Importance of KDMs in Melanogenesis

Melanoma is the most deadly type of skin cancer and is the most commonly diagnosed cancer in young Australians (AIHW, 2017). Analysis of two independent cohorts showed that KDM alterations are frequent in melanoma (**Figures 3**, **4**) and thus warrant further investigation into their mechanistic role.

The most extensively studied KDM in melanoma is KDM5B. Studies found that KDM5B expression is higher in melanocytic nevi compared to advanced and metastatic melanomas (Roesch et al., 2005, 2010). High KDM5B expression was associated with a slow cycling population of melanoma cells that prolonged growth and self-renewal. An effect was observed on Notch signaling in that KDM5B suppressed the Notch ligand Jagged 1, causing less notch cleavage and a decrease in the expression of Notch target genes. The study suggests that KDM5B may have an important

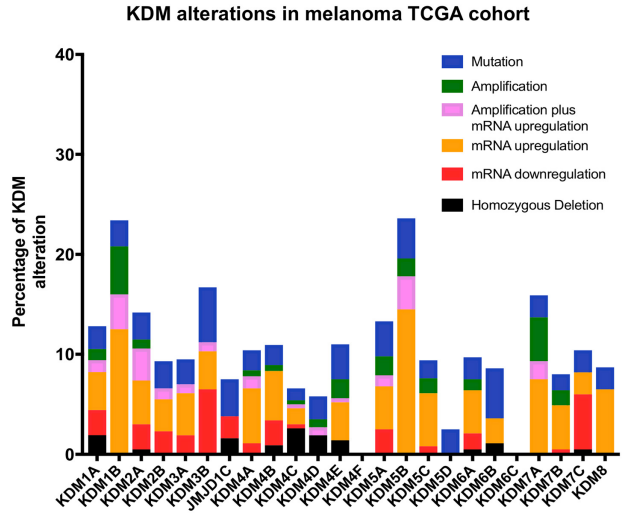


FIGURE 3 | Bar graph showing the percentage and type of each KDM mutations obtained from the cancer genome atlas (TCGA) database. The data was obtained from a total of 472 patients with skin cutaneous melanoma (SKCM).

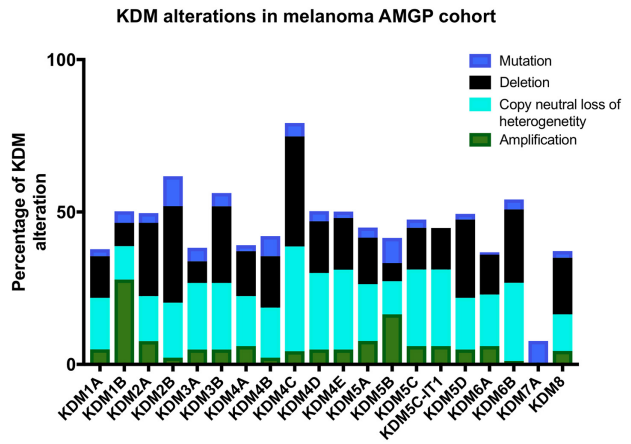


FIGURE 4 | Bar graph showing the percentage of each and type of KDM alterations in 183 melanoma patients from the Australian melanoma genome project (AMGP).

role in the maintenance of stem like progenitors that seed tumor progression and metastasis in melanoma.

KDM6B was found to be essential for melanoma tumor growth and metastasis (Park et al., 2016). KDM6B could activate NF-kB and bone morphogenic protein signaling promoting melanoma growth and progression.

Another recent study showed that H3K9 demethylases (which include KDM3B) can disable senescence, allowing *ras/braf* mutant melanoma development and progression. This was reversed when treated with H3K9 inhibitors *in vitro* and *in vivo* (Yu et al., 2018). The main role of KDM6A/B may be as an antagonist of EZH2 which has been implicated in the growth and progression in melanoma. Early studies found that elevated EZH2 expression was associated with poor survival (Bachmann et al., 2006; McHugh et al., 2007). A melanoma EZH2 mouse model showed that melanocyte specific loss of EZH2, or treatment with an EZH2 inhibitor, abolished the spread of metastatic melanoma (Zingg et al., 2015). EZH2 was able to induce resistance to immunotherapy treatments anti-CTLA-4 and IL-2 in a melanoma mouse model and when inactivated, reversed this resistance (Zingg et al., 2017). A recent study also found that EZH2 induced loss of primary cilia, enhanced Wnt signaling and promoted melanoma metastasis (Zingg et al., 2018). Collectively these studies show that EZH2 has an important role in the progression of melanoma, therefore its only known antagonist, KDM6A/B, is likely to be of equal importance and warrants exploration. It is also unclear what role the COMPASS complex which contains KDM6A may have in melanoma progression. Importantly it is not clear what role these complexes may have in determining the sensitivity or resistance to inhibitors of EZH2 and KDM6A/B.

Potential KDM Sex Specific Roles in Melanoma?

There is a striking and unexplained predominance for males to be diagnosed and die from cancer, including melanoma, compared to females (Joosse et al., 2013; Clocchiatti et al., 2016; Enninga et al., 2017). It is postulated that this is due to particular X-linked genes that escape X-inactivation also known as "escape from X-inactivation tumor suppressor" (EXITS) genes (Dunford et al., 2017). This means that females express two copies of EXITS genes compared to males, effectively doubling the amount of tumor suppressive function. One of these KDMS identified in this category was KDM6A. Our recent study showed that KDM6A had a significant prognostic effect in female melanoma patients inducing better overall survival (Emran et al., 2020). Our TCGA gene set enrichment analysis (GSEA) suggests that high KDM6A level associated with upregulation of several immune related pathways like IFN-gamma which may help anti-tumor immunity and survival advantage in female melanoma patients compared to male (Emran et al., 2020).

Other studies support KDM6A in having sex-specific roles in normal biology and cancer. An early study showed that KDM6A expression was significantly higher in the brains and organs of female mice compared to male mice (Xu et al., 2008). In the context of cancer, a study showed that KDM6A had a gender-specific, tumor suppressive effect in T-cell acute lymphoblastic leukemia (T-ALL). The study showed that KDM6A is frequently mutated in male T-ALL patients and KDM6A expression is significantly lower compared to females T-ALL patients (Van der Meulen et al., 2015). In addition, loss of KDM6A expression in female mice induced poorer survival by downregulation of T-ALL associated tumor suppressor genes and upregulation of T-ALL associated oncogenes (Van der Meulen et al., 2015). Loss of KDM6A expression in female mice exhibited a squamous-like, malignant phenotype *via* activation of oncogenes MYC and RUNX3 in a pancreatic cancer mouse model (Andricovich et al., 2018).

UTY also known as KDM6C is the male equivalent of KDM6A/UTX and is expressed on the Y chromosome but importantly, has significantly reduced H3K27me3

demethylation activity compared to KDM6A, due to the substitution of important amino acids in the Jumonji C domain (Walport et al., 2014).

A study in pancreatic cancer found that concurrent loss of UTY and KDM6A in male patients was associated with a more malignant phenotype and poorer and prognosis (Andricovich et al., 2018). In addition, male mice that expressed UTY and female mice with heterozygous KDM6A expression exhibited a less aggressive pancreatic cancer phenotype, while male and female mice with no UTY or KDM6A exhibited a more aggressive malignant phenotype (Andricovich et al., 2018). Hence this study suggests that KDM6A and UTY have tumor-suppressive roles in pancreatic cancer and that sex-specific mechanism should be investigated in melanoma and other cancers.

KDM INHIBITORS

Numerous KDM inhibitors are showing promise in targeting KDMs in certain types of cancers in both preclinical and clinical studies. A summary of inhibitors is provided below and in **Table 2**, grouped by KDM family.

KDM1

There have been numerous irreversible and reversible inhibitors that have been developed and tested preclinically and in clinical trials that target the KDM1 or LSD family. These include tranylcypromine derived KDM1A inhibitors that have comprehensively described elsewhere (Fang et al., 2019) and summarized below.

ORY-1001

ORY-1001 has been reported to induce expression of differentiation markers in mixed lineage leukemia (MLL) cells as well as reducing tumor growth in an acute myeloid leukemia (AML) mouse model and possesses good oral bioavailability (Harris et al., 2012; Maes et al., 2013, 2015). ORY-1001 has also been shown to suppress growth in lung cancer *in vitro* (Lu et al., 2018). ORY-1001 is currently being tested in phase I and II clinical trials in patients with relapsed and/or refractory AML and small cell lung cancer (SCLC) (Register ECT, 2018).

GSK2879552

Another selective KDM1A inhibitor is GSK2879552, tested in phase I clinical trials in SCLC. A study found that GSK2879552 promotes differentiation in AML cells and when SCLC cells were treated, proliferation was reduced *in vitro*. In mouse models of AML and SCLC, GSK2879552 prolonged survival (Dhanak and Jackson, 2014; Mohammed et al., 2014). However, clinical trials in patients with AML and SCLC and were terminated due to adverse side effects (**Table 2**).

INCB059872

Initially, the KDM1A inhibitor known as INCB059872 treatment was shown to induce cell differentiation in progenitor cells in AML (Johnston et al., 2020), prostate cancer and Ewing sarcoma (Fang et al., 2019). However, INCB059872 was tested in a clinical trial in patients with Ewing sarcoma and terminated due to recruitment issues, this inhibitor in conjunction with an anti-PD-1 checkpoint inhibitor is currently being tested in patients with colorectal and lung cancer (**Table 2**).

4SC-202

Another strategy involves dual inhibition of both histone deacetylases (HDAC) and KDM1A with 4SC-202 which targets HDAC1, 2, 3, and KDM1A and has been shown to inhibit the stem-related properties of cancer cells reducing their viability (Henning et al., 2010). A phase I clinical study has been conducted for 4SC-202 in patients with advanced leukemia, found to possess anti-cancer activity and to be well-tolerated in patients (von Tresckow et al., 2014, 2019). Another study found that treatment with 4SC-202 significantly increased the survival of mice that had AML tumors without any toxicity effects (Fiskus et al., 2014). Currently, there are clinical trials being undertaken that are testing the effect of 4SC-202 in patients with melanoma, merkel cell carcinoma and gastrointestinal cancer (**Table 2**).

SP-2577

SP-2577 reversibly inhibits KDM1A demethylation and has been recently found to promote anti tumor immunity in mutated ovarian cancer cells *in vitro* and has also been found to inhibit growth in Ewing Sarcoma xenografts (Salarius Pharmaceuticals, Inc., 2020; Soldi et al., 2020). Currently SP-2577 is being tested in patients with Ewing sarcoma, ovarian and endometrial cancers (**Table 2**).

CC-90011

The reversible KDM1A inhibitor CC-90011 has been found to induce cellular differentiation exhibits anti-tumor efficacy *in vitro* and *in vivo* in AML and SCLC (Kanouni et al., 2020). CC-90011 was also tested in patients with hematological cancers, showing robust anti-cancer properties and good tolerability in patients (Hollebecque et al., 2021). Currently, CC-90011 is being tested in clinical trials in patients with prostate and SCLC (**Table 2**).

KDM4

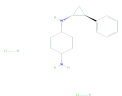
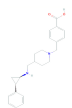
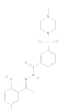
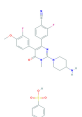
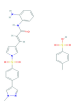
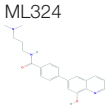
Caffeic Acid

The most prominent KDM4 inhibitor is caffeic acid, a naturally occurring compound found in various sources including eucalyptus globus (Santos et al., 2011). Caffeic acid has been reported to mainly target KDM4C (Nielsen et al., 2012) and displays potent anti-cancer activity against esophageal cancer *in vitro* and *in vivo* (Hirose et al., 1998). Currently, caffeic acid is being tested in a clinical trial in patients with esophageal cancer (**Table 2**).

JIB-04

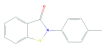
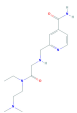
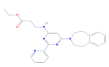
The most advanced preclinical KDM4 inhibitor is JIB-04 which targets KDM4A, KDM4B, and KDM4E and can inhibit growth and reduce tumor burden in non-small cell lung cancer (NSCLC) and breast cancer *in vitro* and *in vivo* (Wang et al., 2013). In addition, JIB-04 treatment reduced colony formation, growth and migration *in vitro* and reduced tumorigenic activity in a colorectal cancer model *in vivo*. The mechanism has been attributed to downregulating genes of the Wnt signaling pathway which are essential for promoting carcinogenesis (Kim et al., 2018). JIB-04 is yet to be tested in clinical trials.

TABLE 2 | Current KDM inhibitors in clinical and preclinical trials.

KDM inhibitor	Target	Pharmaceutical company	Clinical trial number	Cancer	Phase	Status
ORY-1001 	KDM1A	Oryzon Genomics S.A.	CL02-ORY 1001AML CL03-ORY-1001SCLC CL01-ORY-1001	Acute myeloid leukemia Small cell lung cancer Acute leukemia	Phase I Phase I Phase I	Ongoing Ongoing Completed
GSK2879552 	KDM1A	GlaxoSmithKline	NCT02177812 NCT02034123	Acute myeloid leukemia Small lung cell cancer	Phase I Phase I	Terminated Terminated
INCBO59872	KDM1A	Incyte corporation	NCT03514407	Refractory Ewing Sarcoma	Phase I	Terminated
SP-2577 (Seclidemstat) 	KDM1A	Salarius Pharmaceuticals	NCT03895684 NCT03600649 NCT04611139	Advanced solid tumors Ewing sarcoma Ovarian cancer, endometrial cancer	Phase I Phase I Phase I	Recruiting Recruiting Not yet Recruiting
CC-90011 	KDM1A	Celgene	NCT04628988 NCT03850067	Prostate cancer Small cell lung cancer	Phase I Phase I	Not yet recruiting Recruiting
Caffeic acid 	KDM4C	N/A (naturally occurring)	NCT03070262 NCT04648917	Esophageal cancer Squamous esophageal cell cancer	Phase III Phase III	Active, not recruiting Recruiting
Combination						
4SC-202 (Domatinostat) 	HDAC + KDM1A (dual)	4SC-AG	NCT01344707 NCT03278665 NCT03812796 NCT04393753	Leukemia Melanoma Gastrointestinal cancer Merkel cell carcinoma	Phase I Phase II Phase II Phase I	Completed Recruiting Recruiting Recruiting
INCBO59872 + embrolizumab + epacadostat	KDM1A + PD-1 + IDO1	Incyte corporation	NCT02959437	Non-small cell lung cancer and colorectal cancer	Phase I	Completed
CC-90011 + nivolumab	KDM1A + PD-1	Celgene	NCT04350463	Non-small and small cell lung cancer	Phase II	Recruiting
CC-90011 + cisplatin + etoposide	KDM1A + DNA damage	Celgene	NCT03850067	Small cell lung cancer	Phase I	Recruiting
Preclinical KDM inhibitors						
JIB-04	KDM4A/B, KDM4E	Sigma	N/A	Non-small cell lung cancer, breast cancer, colorectal cancer	N/A	N/A
ML324 	KDM4B, KDM4E	Sigma	N/A	Breast cancer, prostate cancer	N/A	N/A

(Continued)

TABLE 2 | Continued

KDM inhibitor	Target	Pharmaceutical company	Clinical trial number	Cancer	Phase	Status
EPT-103182	KDM5B	Unknown	N/A	AML, breast cancer, prostate cancer	N/A	N/A
PBIT 	KDM5B	Sigma	N/A	Breast cancer	N/A	N/A
KDOAM-25 	KDM5A-C	Sigma	N/A	Breast cancer, multiple myeloma	N/A	N/A
GSK-J4 	KDM6A/B	GlaxoSmithKline	N/A	T-ALL, AML, non-small cell lung cancer, breast cancer, ovarian cancer, B-lymphoma, neuroblastoma	N/A	N/A

ML324

Another KDM4 inhibitor in the toolbox is ML324 which targets KDM4B and KDM4E (Rai et al., 2010). A study reported that ML324 treatment reduced tumor volume and growth in a triple negative breast cancer mouse model (Wang W. et al., 2018) and also inhibits proliferation in prostate cancer *in vitro* and *in vivo* (Carter et al., 2017). ML324 is yet to be tested in clinical trials.

KDM5

EPT-103182

The most advanced KDM5 inhibitor is EPT-103182. This small molecule compound targets KDM5B which has been shown to have an anti-proliferative effect in hematological and solid cancer cell lines as well as inhibiting tumor growth in a dose-dependent manner in xenograft models (Hancock et al., 2015; Maes et al., 2015). This inhibitor has yet to be tested in clinical trials.

PBIT

Another recently identified KDM5 inhibitor is PBIT, shown to specially target and inhibit KDM5B (Sayegh et al., 2013). In the context of cancer, PBIT treatment inhibited proliferation of breast cancer by derepression and upregulation of the tumor suppressor HEXIMI *in vitro* (Montano et al., 2019). PBIT is yet to be tested in clinical trials.

KDOAM-25

KDOAM-25 has been shown to target and inhibit KDM5A-C demethylases (a pan-KDM5 family inhibitor), but especially KDM5B (Tumber et al., 2017). KDOAM-25 treatment has been shown to reduce proliferation and growth in breast cancer and multiple myeloma which highly expressed KDM5B (Tumber et al., 2017; Montano et al., 2019). KDOAM-25 is yet to be tested in clinical trials.

KDM6

GSK-J4

Other inhibitors which target JMJC domain containing KDMs are known as GSK-J4 that targets KDM6A and KDM6B, shown to reduce the production of pro-inflammatory cytokines by human macrophages, although there have been questions in regards to its specificity (Kruidenier et al., 2012; Heinemann et al., 2014). Over the last few years, numerous preclinical studies have demonstrated that GSK-J4 could be a potential therapeutic for certain cancers. GSK-J4 treatment was initially shown to be effective in inhibiting cell growth and inducing cell cycle arrest and apoptosis in primary human T-cell acute lymphoblastic leukemia (T-ALL) lines (Ntziachristos et al., 2014). A recent study found that GSK-J4 treatment inhibited cell proliferation and colony forming ability of acute myeloid leukemia (AML) cell lines and inhibited tumor growth in an AML xenograft mouse model (Li et al., 2018).

Other studies have shown that GSK-J4 treatment inhibited proliferation of castration-resistant prostate cancer cells by inhibiting AR–driven transcription and can also inhibit proliferation in glioma cells in a dose-dependent manner *in vitro* (Morozov et al., 2017; Sui et al., 2017). A study also showed that GSK-J4 suppressed the ability of breast cancer and ovarian cancer stem cells to proliferate and grow (Sakaki et al., 2015; Yan et al., 2017). GSK-J4 in combination with an anti-diabetic drug metformin induced cell death and inhibited cell growth in non-small cell lung cancer (NSCLC) cell lines (Watarai et al., 2016) and another study found GSK-J4 treatment sensitized diffuse large B-lymphoma cells to chemotherapy drugs (Mathur et al., 2017). GSK-J4 inhibited cell growth and upregulated apoptosis markers in neuroblastoma cell lines and inhibited tumor growth in an *in vivo* neuroblastoma model (Lochmann et al., 2018). GSK-J4 is yet to be tested in clinical trials.

CONCLUDING REMARKS

Dysregulation of lysine methyl demethylases due to genetic changes or aberrant signaling are associated with a number of different cancers. The amine oxidase group of

KDMs appear to mediate their effects by action on the H3K4 and H3K9 histone marks but in addition by interactions with the P53 tumor suppressor and E2F transcription factors regulating cell division.

The 2-oxoglutarate dependent oxygenases are the largest group of KDMs and mediate demethylation of histones at several important activating or repressive marks. Their effects in cancers range from tumor suppression to promotion and growth of cancers. Additional roles in immune responses against cancer have been revealed for KDM5C and KDM6A in survival studies on human melanoma patients and may be linked to higher expression from X linked chromosomes. Several KDMs are part of protein complexes like the COMPASS complex that contains KDM6A as well as methyl transferases MLL2 and MLL3 that regulate gene transcription in certain cancers.

The role of KDMs in cancer have identified them as potential therapeutic targets and a wide range of pharmacological agents have been developed. Given the complex interactions of KDMs with other epigenetic regulators it is not surprising that drugs targeting KDMs are yet to enter clinical practice and this remains a focus of future research.

AUTHOR CONTRIBUTIONS

GP-M, AE, and JT: design and structure of review. GP-M and AE: figures. GP-M: tables. GP-M, AE, PH, and JT: editing the manuscript. All authors contributed to the article and approved the submitted version.

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Combined DNA Methylation and Transcriptomic Assessments to Determine a Prognostic Model for PD-1-Negative Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) has the highest incidence and mortality of any malignancy in the world. Immunotherapy has been a major breakthrough for HCC treatment, but immune checkpoint inhibitors (ICIs) are effective in only a small percentage of HCC patients. In the present study, we screened programmed cell death protein 1 (PD-1) -negative HCC samples, which are frequently resistant to ICIs, and identified their methylation and transcription characteristics through the assessment of differential gene methylation and gene expression. We also screened for potential targeted therapeutic drugs using the DrugBank database. Finally, we used a LASSO (least absolute shrinkage and selection operator) regression analysis to construct a prognostic model based on three differentially methylated and expressed genes (DMEGs). The results showed that ESTIMATE (Estimation of Stromal and Immune Cells in Malignant Tumors using Expression Data) scores for the tumor samples were significantly lower compared to normal sample ESTIMATE scores. In addition, we identified 31 DMEGs that were able to distinguish PD-1-negative samples from normal samples. A functional enrichment analysis showed that these genes were involved in a variety of tumor-related pathways and immune-related pathways, and the DrugBank screening identified potential therapeutic drugs. Finally, the prognostic model based on three DMEGs (*UBD*, *CD5L,* and *CD213A2*) demonstrated good predictive power for HCC prognosis and was verified using an independent cohort. The present study demonstrated the methylation characteristics of PD-1-negative HCC samples, identified several potential therapeutic drugs, and proposed a prognostic model based on *UBD*, *CD5L,* and *CD213A2* methylation expression. In conclusion, this work provides an in-depth understanding of methylation in HCC samples that are not sensitive to ICIs.

Keywords: hepatocellular carcinoma, PD-1, methylation, ESTIMATE, prognosis

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the malignancy with the highest incidence and fatality rate in the world (especially in East Asia and Southern Africa), resulting in approximately 800,000 deaths per year (Forner et al., 2018). Although a variety of treatment methods (e.g., radiotherapy, chemotherapy, surgical treatment, and liver transplantation) have made great progress, the prognoses of patients with HCC have not improved significantly, mainly due to continued difficulty with early diagnosis, a high rate of recurrence, and limited indicators (El-Serag et al., 2008; Kawamura et al., 2020; Kudo, 2020b; Schoenberg et al., 2020). In recent years, immunotherapy has been a major breakthrough for HCC treatment (Li et al., 2019; Zhang Q. et al., 2020). With the development and application of immune checkpoint inhibitors (ICIs) targeting programmed cell death protein 1 (PD-1) and programmed death-ligand 1, the prognoses for some HCC patients have significantly improved (Kalathil et al., 2016; Mahn et al., 2020). However, immune escape and other related mechanisms within the tumor microenvironment have not been adequately studied, and only a small proportion of patients respond to immunotherapy (Sakuishi et al., 2010; Ng et al., 2020). Therefore, it is both urgent and necessary to further explore immune mechanisms related to HCC occurrence and development to identify new targets for immunotherapy.

A well-known epigenetic modification, DNA methylation occurs mainly in mammalian CpG islands and can regulate gene transcription to ensure cell-specific programmed gene expression (Bird, 2002; Oe et al., 2021; Singh and Edwards, 2021). Many studies have shown that abnormal DNA methylation is related to the occurrence of various diseases, including cancers (Meng et al., 2018; Zhang M. et al., 2020). The latest research has also confirmed that DNA methylation is closely related to tissue immune status (Lee et al., 2001; Thomas et al., 2012; Ghoneim et al., 2017). Delacher et al. found that an important feature of differentiated regulatory T-cell populations and lymphoid T cells in different tissues was the gain (or loss) of DNA methylation (Delacher et al., 2017). In addition, the blocking of DNA methylation has also been reported to maintain the effector functions of $CD8^+$ T cells during chronic infections (Ghoneim et al., 2017). Considering this close connection between DNA methylation and immune function, we reasoned that this epigenetic modification may be involved in the ICI process against HCC. However, few studies have extensively analyzed the relationship between DNA methylation and the effects of ICIs on HCC.

Previous studies have shown that the expression of PD-1 is closely related to ICI treatment (El-Khoueiry et al., 2017; Zhu et al., 2018). There have been many studies based on the expression of PD-1 in the immune microenvironment of liver cancer to explore its role, but these studies mainly focus on samples with high PD-1 expression (Tan et al., 2019; Voutsadakis, 2019). Here, we have demonstrated the DNA-methylation characteristics of PD-1-negative HCC samples and identified 31 differentially methylated and expressed genes (DMEGs) using combined analyses of methylation, transcriptomes, and prognostic information in concert with a functional-enrichment analysis to determine the potential functions of these genes. Furthermore, we identified potential HCC therapeutic drugs based on the DrugBank database. Based on these findings, we used a LASSO (least absolute shrinkage and selection operator) regression analysis to determine a prognostic model, based on three of these DMEGs, with good predictive ability. This research provides new insights for in-depth studies of methylation in PD-1-negative HCC.

Abbreviations: HCC, hepatocellular carcinoma; ICIs, immune checkpoint inhibitors; PD-1, programmed cell death protein 1; LASSO, least absolute shrinkage and selection operator; DMEGs, differentially methylated and expressed genes; ESTIMATE, Estimation of Stromal and Immune Cells in Malignant Tumors using Expression Data; DMGs, differentially methylated genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; TCGA, Cancer Genome Atlas; LIHC, liver hepatocellular carcinoma; UBD, Ubiquitin D; TNF-α, tumor necrosis factor alpha; NF-κB, nuclear factor kappa B; IL-13, interleukin-13.

MATERIALS AND METHODS

Data Acquisition and Processing

Data from TCGA was obtained through the TCGA Genomic Data Commons application programming interface. We obtained the most current (October 2, 2020) TCGA-LIHC expression profile data, DNA methylation data, and clinical follow-up information. Both normal samples ($n = 50$) and tumor samples ($n = 371$) were represented in TCGA data set, and 50 normal samples and 177 tumor samples were also represented in the DNA-methylation data set. HCC samples with PD-1 expression levels lower than the average found in normal samples were regarded as PD-1-negative samples. Using this criteria, 177 PD-1-negative samples were identified.

Processing of Gene-Expression Data and DNA-Methylation Data

Gene expression data from the PD-1-negative samples was log2-converted and then analyzed for differential expression using the "limma" package in R software (Phipson et al., 2016). The *p*-values were converted to FDR values based on the Benjamini and Hochberg method. FDR > 0.01 and log2FC > 1 were considered to be up-regulated gene expression; FDR > 0.01 and log2FC < 1 were considered to be down-regulated gene expression.

The same R software package was applied to the DNA methylation data set from TCGA-LIHC to identify differentially methylated CpG genes (DMGs). Methylation intensities were represented by β values, and the threshold for DMG recognition was FDR < 0.05 and an absolute delta β-value > 0.3. We subsequently calculated average β-values for different regions, including the 5′- untranslated region (5′-UTR), first exon, gene body, 3′-UTR, TSS1500, and TSS200.

Immune Infiltration Analysis

The "ESTIMATE" R software package was used to determine ESTIMATE scores, stromal scores, and immune scores for both

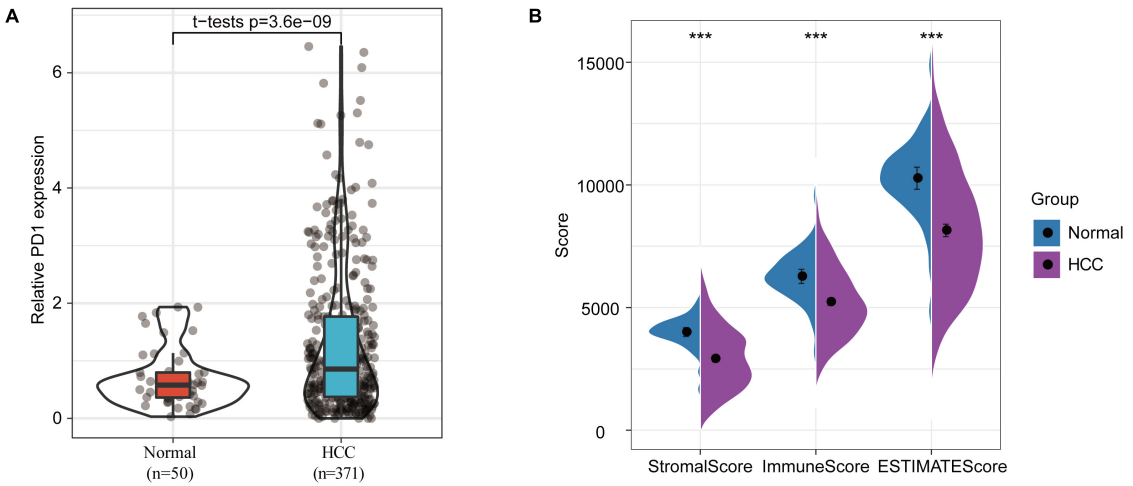

FIGURE 1 | Differences in immune infiltration between normal and HCC samples. **(A)** PD-1 expression differences between normal and HCC samples. **(B)** Differences in stromal scores, immune scores, and ESTIMATE scores between normal and HCC samples, ***$p < 0.001$.

the HCC and normal samples (Verhaak et al., 2010). These scores were used to describe the overall immune-cell infiltration of the microenvironment.

Identification of DEGs and DMGs

All gene identifications based on differences in FDR < 0.01 were considered to be credible. We then performed a joint analysis of both DEGs and DMGs and divided the resulting DMEGs genes into four groups: HypoUp (β-value < -0.3 and log2FC > 1); HypoDown (β-value < -0.3 and log2FC < 1); HyperUp (β-value > -0.3 and log2FC > 1); and HyperDown (β-value > -0.3 and log2FC < 1).

Functional Enrichment Analysis

We used the "clusterProfiler" package in R software to perform a gene ontology functional enrichment analysis and a KEGG pathway-annotation analysis of the DMGs, DEGs, and DMEGs to identify the important biological processes and pathways related to these differentially expressed genes (Yu et al., 2012).

Screening for Potential Target Drugs

We screened the DrugBank database[1] to identify potential drugs capable of up-regulating DMEGs. NetworkAnalyst 3.0[2], a web-based visual analysis platform for analyzing and interpreting systems-level gene expression data, was used to analyze protein-drug interactions from the DMEGs based on the DrugBank database (Zhou et al., 2019). The HCC-drug proximity was calculated using the following formula:

$$d(S, T) = 1 \sum t \in Tmins \in S(d(s, t) + \omega)$$

where S represents the DMEGs; D represents the degree of BPH-related gene-set nodes in the PPIs; T represents the drug-target gene set; the distance d (s,t) represents the shortest path between the s node and the t node; and ω is the weight of the target gene. If the target gene was a gene in the BPH-related gene set, the calculation method was ω = -ln(D + 1), otherwise ω = 0.

Analysis of DMEG-Related Prognostic Signature Genes

For DMEGs, we used the Principal Component Analysis method to distinguish between HCC and adjacent samples. Linear Discriminant Analysis was used to classify the samples using DMEG expression-profile data and methylation data, and the leave-one-out cross-validation method was used for verification.

To determine relationships between DMEG expressions and prognoses, we first randomly divided the PD-1-negative samples into two groups: a training set (n = 88) and a validation set (n = 89). For the DMEG-expression and clinical-survival data, we performed 1000 LASSO regression analyses, using 10-fold cross-validation, summarized the dimensionality reduction results each time, and then counted the number of times each probe appeared per 100 times.

RESULTS

PD-1 Expression in HCC and Normal Tissue Samples, and Their Microenvironment Characteristics

A differential-expression analysis indicated that the expression of PD-1 in HCC samples was significantly higher compared to its expression in normal samples (**Figure 1A**). As expected, stromal and immune scores based on the ESTIMATE (Estimation of Stromal and Immune Cells in Malignant Tumors using Expression Data) analysis were both significantly reduced in HCC samples compared to normal samples (**Figure 1B**). These results indicate that stromal and immune infiltrations in the

[1]https://www.drugbank.com/
[2]http://www.networkanalyst.ca/

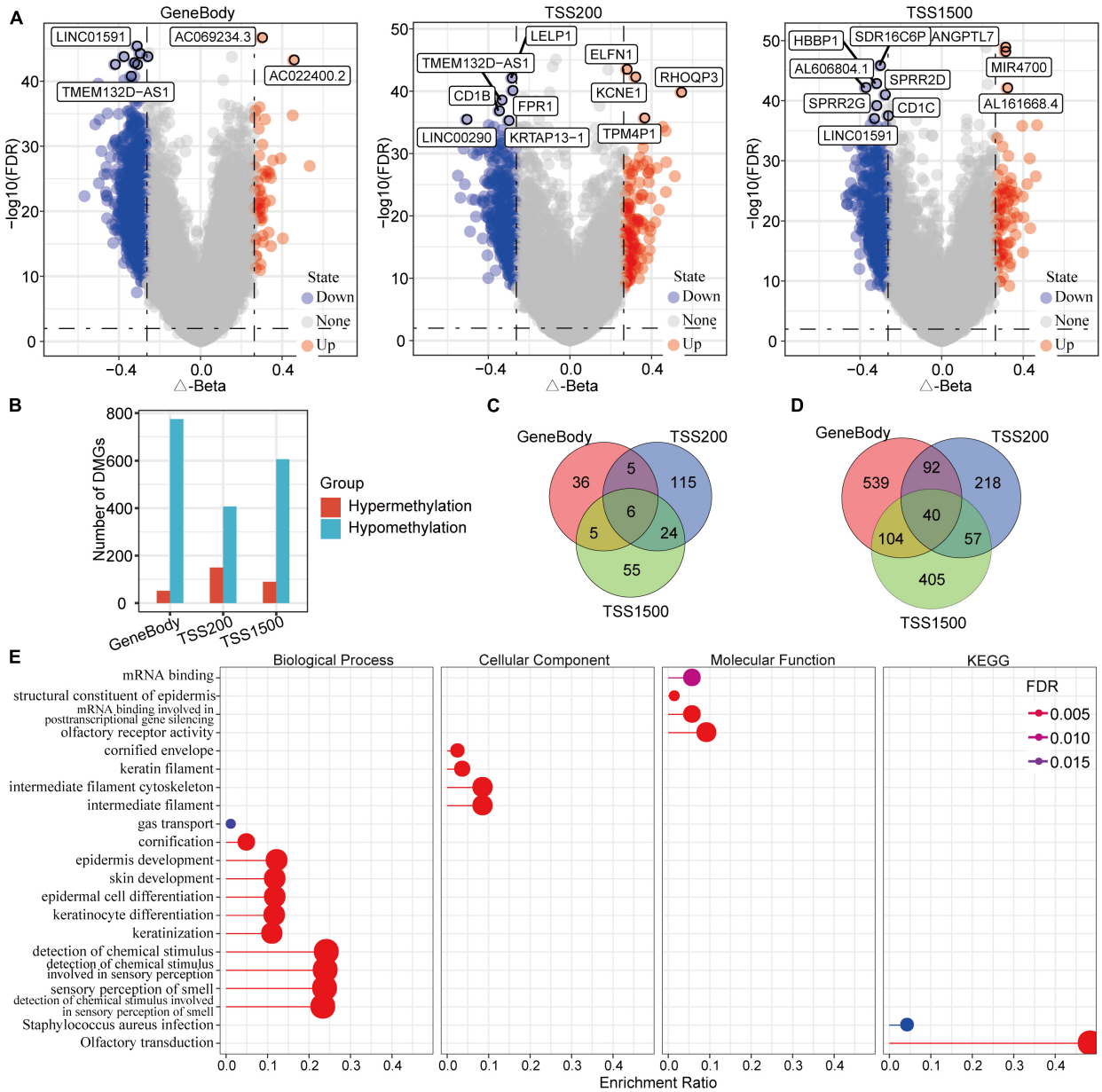

FIGURE 2 | Identification and characteristics of genes with differential DNA methylation in PD-1 negative HCC samples. **(A)** A volcano map of methylation differences of the gene body, TSS200, and TSS1500 regions in PD-1 negative HCC samples. **(B)** The differentially methylated genes in these three regions were mostly hypomethylated. **(C)** A Venn diagram of hypermethylated genes in the three regions. **(D)** A Venn diagram of hypomethylated genes in the three regions. **(E)** Gene ontology functional-enrichment analysis and KEGG annotation analysis of differentially methylated genes. The most-intense red color represents the smallest FDR, and dot size is proportional to enrichment number.

tumor microenvironment were significantly inhibited, which is consistent with previous studies (Binnewies et al., 2018; Ruf et al., 2021).

Gene Analysis for Differential DNA Methylation

To identify differentially methylated genes, we analyzed TSS200 [transcription start site (TSS) to 200 nucleotides upstream of the TSS], TSS1500 (200 to 1500 nucleotides upstream of the TSS), and gene body methylation levels in both the PD-1-negative HCC and normal samples. The results indicated a total of 1,700 differentially methylated genes in the HCC samples. Specifically, 52 hypermethylated and 775 hypomethylated genes were identified in gene body regions, 150 hypermethylated and 407 hypomethylated genes were identified in TSS200 regions, and 90 hypermethylated and 606 hypomethylated genes were identified in TSS1500 regions (**Figure 2A**). The number of hypomethylated

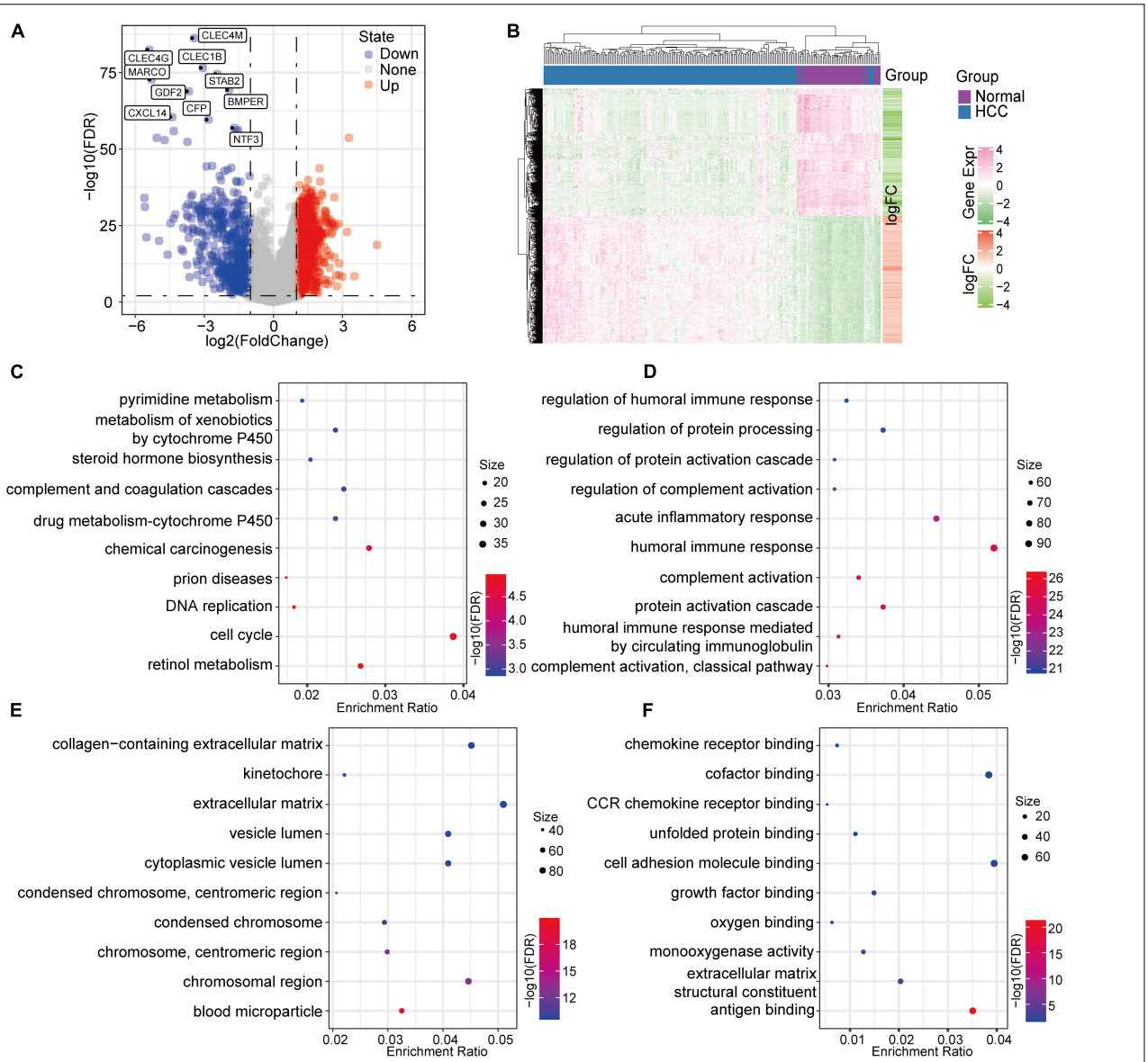

FIGURE 3 | Identification and characteristics of the DEGs based on PD-1-negative HCC samples. **(A)** A volcano map of the DEGs. **(B)** A differential-expression heat map of the normal and HCC samples. **(C)** KEGG annotation analysis of the DEGs. **(D)** Gene ontology biological-function enrichment analysis of the DEGs. **(E)** DEG analysis for gene ontology cell-component enrichment. **(F)** Molecular function enrichment analysis of the DEGs.

genes in these regions was far greater than the number of hypermethylated genes, especially for gene body regions (**Figure 2B**). A set-distribution analysis of the results showed that six hypermethylated genes were represented in all three of the regions, with 206 genes represented in only one of the regions, and that 40 hypomethylated genes were represented in all three of the regions, with 1162 genes represented in only one of the regions (**Figures 2C,D**), suggesting that DNA methylation levels are region-specific. The functional enrichment analysis showed that these differentially methylated genes (DMGs) were mainly enriched in 11 biological processes, four cellular components, four molecular functions, and two Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (including mRNA binding, structural constituents of the epidermis, and mRNA binding involved in posttranscriptional gene silencing) (**Figure 2E**).

Functional Enrichment Analysis of Differentially Expressed Genes (DEGs)

Gene-expression profile data from 177 PD-1-negative tumor samples and 50 normal samples were used to determine differential gene expression. The screening criteria were false discovery rate (FDR) < 0.01 and log2FC (fold change) > 1. A total of 2249 differentially expressed genes (DEGs) were identified, of which 1659 were up-regulated in tumors and 590 were down-regulated (**Figure 3A**). A unsupervised hierarchical

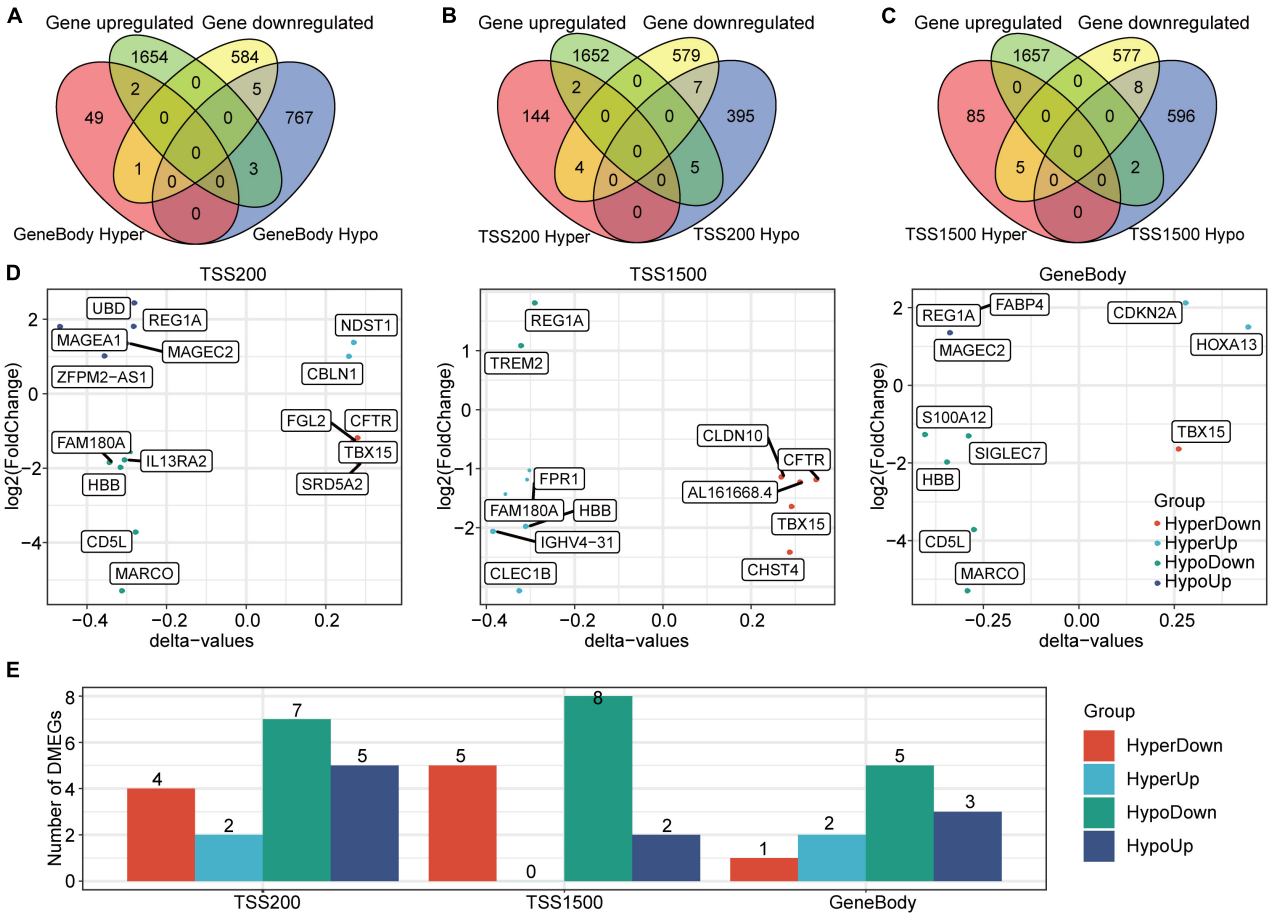

FIGURE 4 | Identification and expression patterns of DMEGs. **(A)** A Venn diagram of the DEGs and the DMGs in gene body regions. **(B)** A Venn diagram of the DEGs and the DMGs in TSS200 regions. **(C)** A Venn diagram of the DEGs and the DMGs in TSS1500 regions. **(D)** Distribution of the four DMEG expression modes in the three regions. **(E)** A histogram of the four DMEG regulatory modes in the three regions.

cluster analysis showed that these DEGs could distinguish between tumor and normal samples (**Figure 3B**). We performed a functional-enrichment analysis of these genes, and the results showed that a variety of tumor and immune-related pathways were enriched (e.g., cell cycle, DNA replication, complement and coagulation cascades, complement activation, and humoral immune responses) (**Figures 3C–F**).

Identification of DMEGs

Given the importance of both methylation and transcription in HCC occurrence and development, we jointly analyzed DMGs and DEGs to more fully explore the relationship between these two processes with the idea that genes demonstrating both differential DNA methylation and expression may play crucial HCC roles (Hu et al., 2020). A set-distribution analysis showed that 11 DMEGs were identified in gene body regions, 18 DMEGs were identified in TSS200 regions, and 15 DMEGs were identified in TSS1500 regions (**Figures 4A–C**). In addition, the extent to which these genes showed both differential methylation and expression is shown in **Figure 4D**. The observation that most of these DMEGs were hypomethylated in tumors is consistent with previous reports (Fabianowska-Majewska et al., 2021). Interestingly, several DMEGs (e.g., *TBX15*, *REG1A*, and *HBB*) were found in all three regions, indicating that these genes may have transcriptional differences caused by differential methylation. Previous reports have shown that *TBX15* expression can be used as a prognostic marker for HCC (Morine et al., 2020), and *HBB* has been reported to play a key role in prostate cancer differentiation and in a variety of important biological pathways (e.g., iron metabolism) (Chen and Sun, 2021; Lin et al., 2021). Based on the present expression data, we identified four different regulatory gene sets in the three DNA regions, for a total of 31 DMEGs (**Figure 4E**).

To better understand the potential functions of these genes in HCC, an in-depth chromosome (chr) distribution analysis was carried out (**Figure 5A**). We found that chr7, chr18, and chrX had the most DMEGs. Interestingly, the methylation patterns of the DMEGs in adjacent chromosomal gene regions were roughly consistent, suggesting that these genes may have cooperative expression patterns and functions. In order to verify gene effectiveness, we used the expression profiles of these 31 DMEGs and the methylation data from

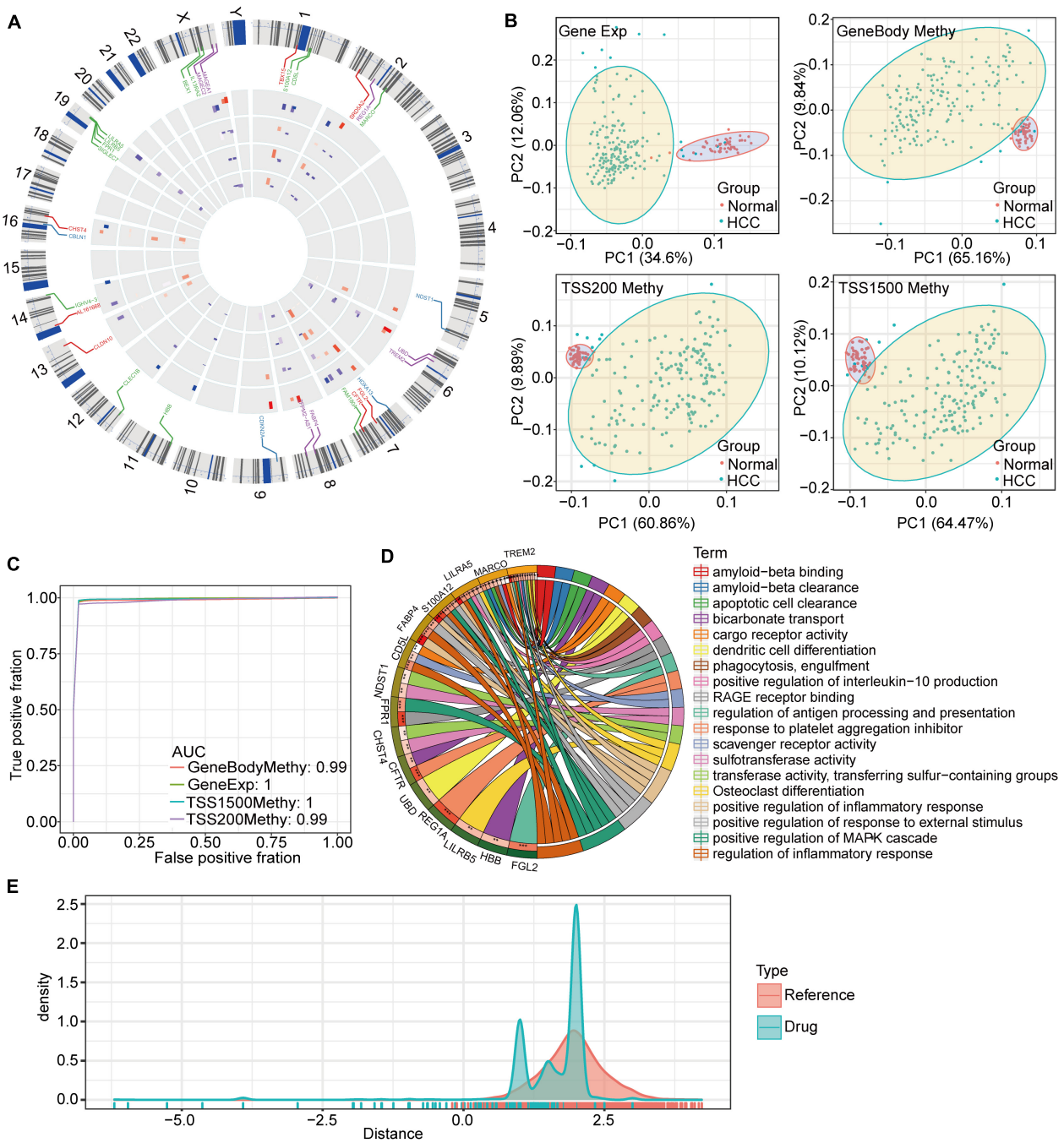

FIGURE 5 | Characteristics of DMEGs and drug distance. **(A)** DMEG distributions on chromosomes. **(B)** Principal component analysis of DMEG expression and methylation in the three DNA regions. **(C)** Construction of the linear discriminant model based on the expression profiles and methylation levels of DMEGs for predicting the ROC curves for HCC and normal samples. **(D)** The results of KEGG pathway-annotation and gene ontology enrichment analyses for the DMEGs, with different colors representing different pathways, and the lines indicating relationships between genes and pathways. **(E)** A drug distance-density plot for the differentially methylated and expressed gene set.

the DMEGs in each of the three regions to construct a linear judgment classification model. The results showed that these DMEGs could effectively distinguish PD-1-negative HCC samples from normal samples (**Figure 5B**). The corresponding receiver operating characteristic (ROC) curve analysis showed an area-under-the-curve (AUC) value ≥0.99 (**Figure 5C**). The functional enrichment analysis showed that DMEGs were mainly enriched in amyloid and apoptotic-cell clearance, as well as in the activation of signaling pathways, such as mitogen-activated protein kinase pathways (**Figure 5D**).

Identification of Potential Target Drugs Based on PD-1-Negative DMEGs

A DMEG protein-drug interaction analysis was performed using the NetworkAnalyst 3.0 tool based on the DrugBank database, and 6 genes were found to interact with drugs (**Table 1**). Among them, cystic fibrosis transmembrane conductance regulator (CFTR) and hemoglobin subunit beta (HBB) proteins had the most drug interactions: 12 with HBB (e.g., pentaerythritol tetranitrate, and 4-carboxycinnamic acid) and 7 with CFTR (e.g., colforsin, crofelemer, and lonidamine). Some of these identified drugs may be effective against HCC.

Based on these drug-target pairs from DrugBank, as well as the string key protein-protein interaction (PPI) network (threshold score was set at 600), we calculated drug-HCC proximities. For both our DMEGs as samples, and for randomly selected genes as samples, we found that the number of drugs was significantly reduced when drug distance was less than 0.8 (**Figure 5E**). This suggests that when HCC-drug proximity is less than 0.8, the drug may have a targeted impact on the disease.

Molecular Docking Analysis Verifies the Affinity of Candidate Drugs

Considering the accuracy of molecular docking, we chose SRD5A2 with a moderate molecular weight as a representative to perform molecular docking analysis in order to clarify the binding model between drug candidates and gene targets. We first downloaded the 3D model of SRD5A2 protein (PDB ID: 7BW1) from the PDB database for molecular docking experiments (**Figure 6A**). Autodock Vina molecular docking results show that the compound can bind tightly to the active site of the SRD5A2 protein, with a molecular docking score of -6.5kcal/mol (**Figure 6B**). In addition, we found that the compound could generate favorable hydrogen bonds with the important amino acid residues GLU57, GLN56 and TYR91 in the SRD5A2 protein, as shown in **Figure 6C**. The above results suggest that the drug Azelaic acid can interact closely with the SRD5A2 protein, thereby affecting the activity of the SRD5A2 protein. Meanwhile, we used molecular dynamics simulation to further evaluate the stability of the protein model combined with the drug, and used the RMSD method to estimate the stability of the protein model (**Figure 6D**). During the 100ns molecular dynamics simulation, we can find that the protein-backbone is maintained in a relatively stable state as a whole, indicating that the protein is relatively stable during the molecular dynamics simulation.

Prognostic Genetic Signature of DMEGs in PD-1-Negative Samples

We used a LASSO regression analysis to reduce the dimensionality of the expression and prognostic data for these DMEGs, and obtained a combined maximum frequency for three probe genes (**Figure 7A**; ENSG00000073754, ENSG00000123496, and ENSG00000213886). The trajectories for these three genes with different lambdas are shown in **Figure 7B**, and the standard-deviation distribution of the different lambdas is shown in **Figure 7C**. The survival-model results demonstrated that, with a median cutoff, the high-expression group was significantly different from the low-expression group using these three genes, indicating a highly effective model (**Figures 7D–F**). According to the LASSO analysis, the determination formula was:

$$RiskScore = -0.253 \times ENSG00000073754 + 0.111 \times ENSG00000213886 - 0.843 \times ENSG00000123496$$

TABLE 1 | The interaction between DMEGs and drugs.

Gene	Gene type	Drug count	Drug example
CFTR	HyperDown	7	Colforsin; Crofelemer; Lonidamine
HBB	HypoDown	12	4-Carboxycinnamic Acid; Pentaerythritol tetranitrate; 2-[(2-methoxy-5-methylphenoxy) methyl] pyridine
IL13RA2	HypoDown	1	AER001
MARCO	HypoDown	2	Titanium dioxide; Silicon dioxide
S100A12	HypoDown	1	Amlexanox
SRD5A2	HyperDown	3	Azelaic acid; Dutasteride; Finasteride

Detailed information about these three genes is presented in **Table 2**. Both *CD5L* and *CD213A2* were determined to be HCC protective factors, and *UBD* was determined to be a risk factor.

According to the above formula, we calculated the risk score for each sample and determined corresponding survival status and expression changes for the three different signature genes as risk values increased (**Figure 8A**). We found that most of the training-set samples had higher risk scores, and that samples with high-risk scores had worse prognoses. In addition, *UBD* expression was found to increase with increasing risk-score values, while *CD5L* and *CD213A2* expressions decreased with increasing risk scores. Furthermore, we conducted ROC analyses for the prognostic classification of risk scores, and the prognostic prediction classification efficiencies for 1 year, 3 years, and 5 years were 0.64, 0.74, and 0.94, respectively (**Figure 8A**). These data indicate that this model was highly predictive for long-term survival. In addition, based on risk-score value z-scores, we divided the samples into high- and low-risk groups. The survival curves showed very significant differences between these groups (log rank $P = 0.0011$), in which 57 of the samples were classified as high-risk and 19 samples were classified as low-risk. We also conducted an analysis of the validation set using the same model and coefficients as for the training-set analysis. The validation results showed that the expression trends of these three signature genes were consistent with the training set (**Figure 8B**). Compared to the low-risk group, the overall survival rate in the high-risk group was worse, but this difference was not statistically significant.

In order to further verify the effectiveness of this gene-signature model, we applied it to all PD-1 low-expression samples in the Cancer Genome Atlas (TCGA) and GSE10141 HCC data set. Similar to the previous results, most of these samples had higher risk scores that related to lower survival status (**Figure 8C**). The ROC analysis showed that the AUC values at 1,

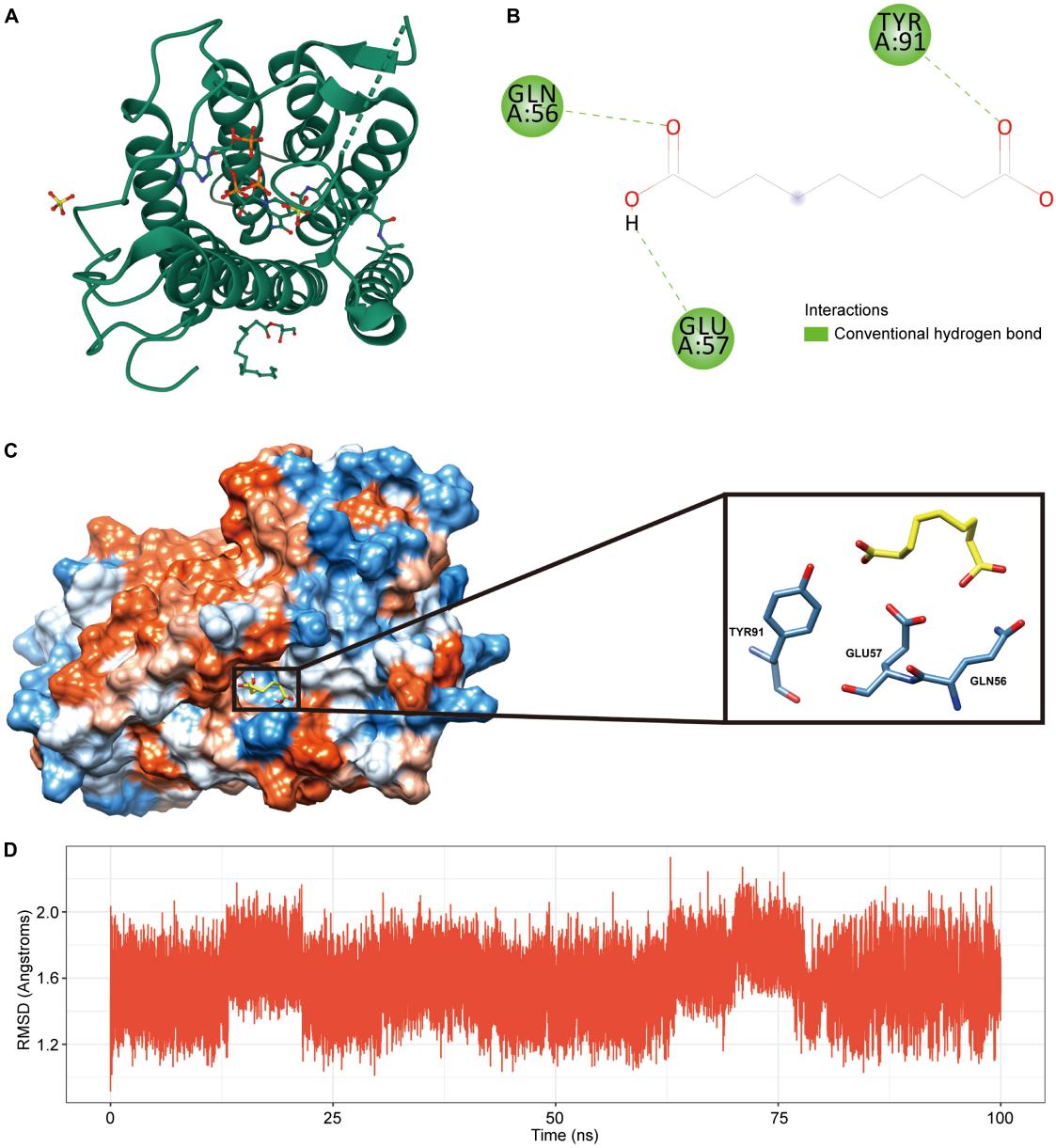

FIGURE 6 | SRD5A2- Azelaic acid molecular docking analysis. **(A)** 3D model of SRD5A2 protein structure. **(B)** 2D interaction diagram of Azelaic acid and SRD5A2 protein. **(C)** The binding domain of Azelaic acid and SRD5A2 protein, in which hydrogen bonds are shown in green and hydrophobic interactions are shown in pink. Amino acid residues are displayed as steel blue. **(D)** The curve of the RMSD value of the protein backbone during molecular dynamics in 100ns.

3, and 5 years were 0.63, 0.65, and 0.82, respectively (**Figure 8C**). At the same time, the overall survival rates for the high-risk group and the low-risk group were also significantly different (P = 0.0016, **Figure 8C**). The GSE10141 data set results showed that more samples were identified as having lower-risk scores, and that the 1-year, 3-year, and 5-year AUC values were 0.78, 0.78, and 0.67, respectively (**Figure 8D**). In addition, the prognoses for the high-risk group and the low-risk group were significantly different (P = 0.00099). These differences in prognostic scores and expression levels may have been due to the batch effect on the different platforms.

DISCUSSION

With the development of ICIs, immunotherapy has become the new focus of attention in the field of tumor treatments (Bersanelli et al., 2021). However, only a small proportion of HCC patients actually respond to ICIs, and one of the important reasons for this may be HCC expression of PD-1 and the infiltration of $CD4^+$ and $CD8^+$ T cells (Sia et al., 2017; Kurebayashi et al., 2018; Kudo, 2020a). Previous studies have shown that DNA methylation can affect the immune status of the tumor microenvironment and tumor responses to ICIs, and that a lack of DNA methylation

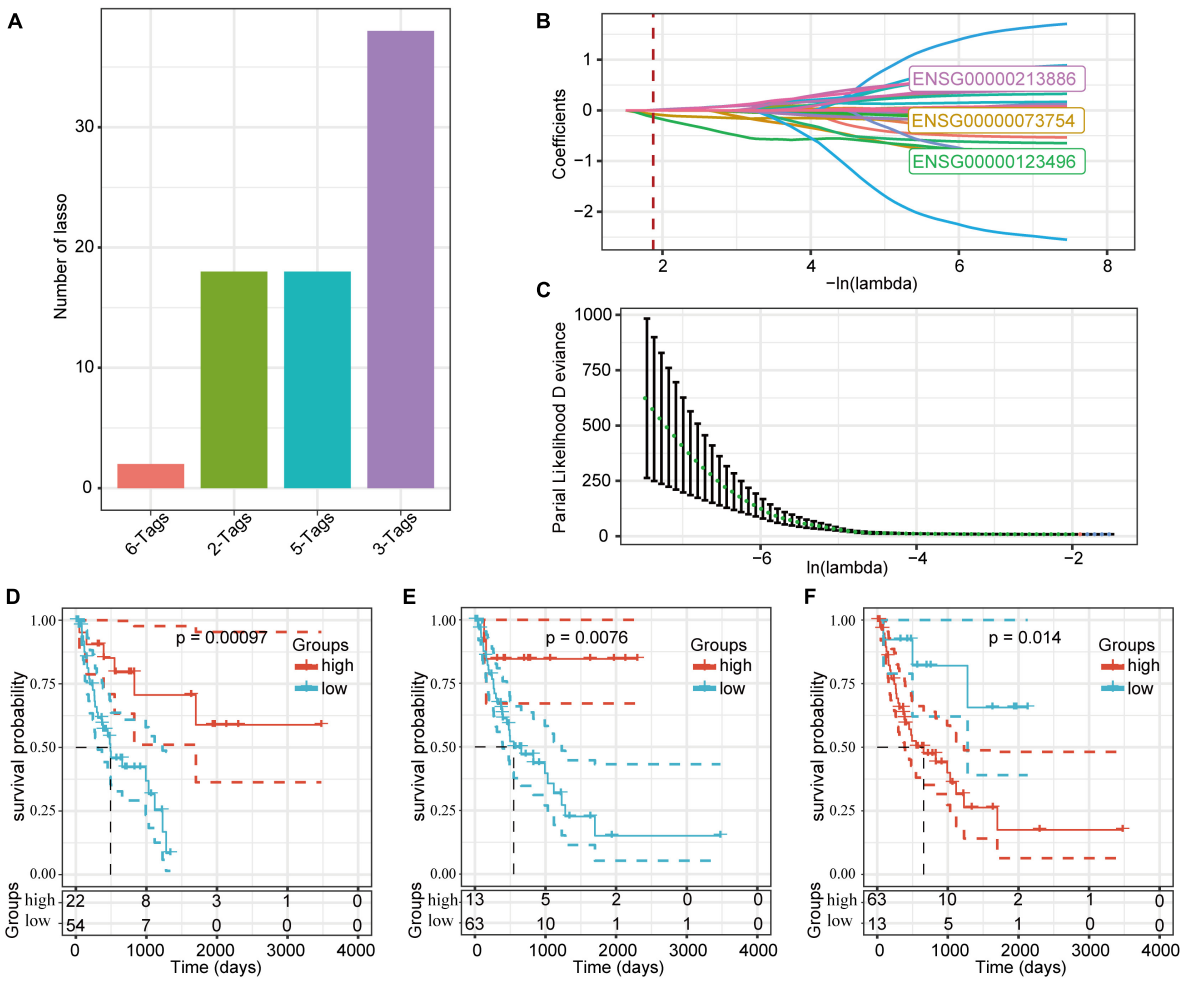

FIGURE 7 | Prognostic gene signatures related to DMEGs. **(A)** LASSO-regression frequencies for each combination of genes. **(B)** Change-coefficient trajectories for each of the three probe genes using different lambdas. **(C)** The distribution of standard deviations in this model under different lambda conditions. **(D)** Kaplan-Meier curves for the *CD5L* high- and low-expression groups. **(E)** Kaplan-Meier curves for the *CD213A2* high- and low-expression groups. **(F)** Kaplan-Meier curves for the *UBD* high- and low-expression groups.

TABLE 2 | LASSO identifies 3 prognostic-related DMEGs.

ENSG id	Gene symbol	P value	HR	Low 95% CI	High 95% CI
ENSG00000073754	CD5L	0.005001056	0.7130075	0.5630112	0.9029655
ENSG00000123496	CD213A2	0.019657250	0.3175769	0.1211487	0.8324900
ENSG00000213886	UBD	0.021742632	1.2143576	1.0287460	1.4334583

is related to immune-evasion characteristics (Duruisseaux et al., 2018; Jung et al., 2019). Therefore, it is of instructive significance to investigate the changes in DNA methylation in PD1-negative HCC samples on the loss of the anti-tumor effect of ICIs and to further search for other therapeutic targets.

Here, using the Liver Hepatocellular Carcinoma (LIHC) cohort from TGCA, we found that stromal-score, immune-score, and ESTIMATE-score values for tumor samples were significantly lower compared to normal samples, and that PD-1 expression was higher compared to normal samples. These differences highlight the immunosuppressive state in these tumors. Using PD-1-negative samples for screening, we identified DEGs and DMGs in three DNA regions, and a functional enrichment analysis showed that they were related to tumor immunity and cancer-related cell pathways (Hu et al., 2020). As there is evidence that DMEGs play a key role in tumorigenesis, we conducted a joint DEG-DMG analysis and identified 31 gene candidates. We then divided these DMEGs into four groups based on modes of expression. Interestingly, most DMEGs demonstrated hypomethylation. Consistent with these results, studies have shown that overall demethylation and hypomethylation of both oncogenes and metastasis-promoting

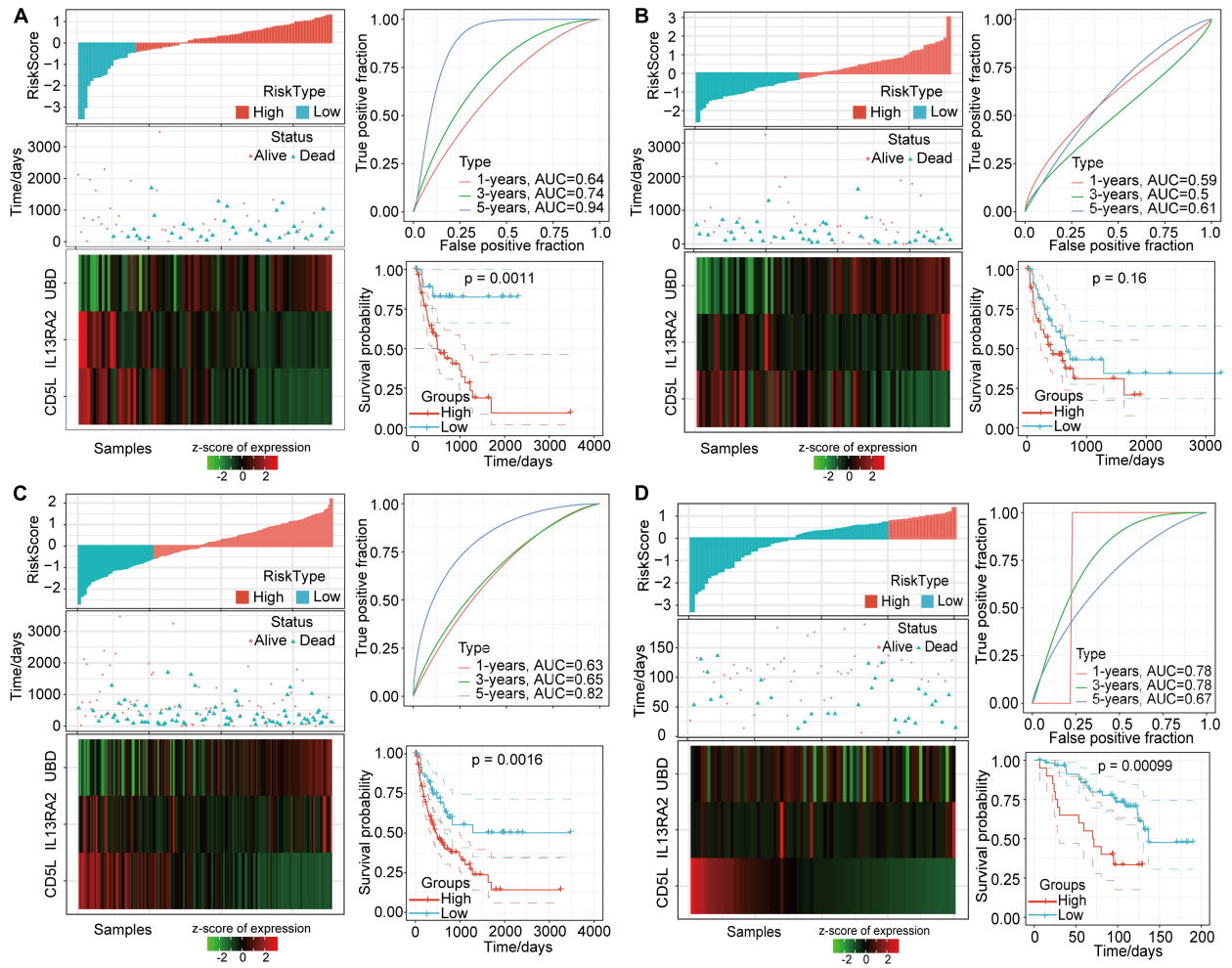

FIGURE 8 | Characteristics of prognostic gene signatures based on 3 DMEGs. **(A)** Training-set relationships between risk scores, survival status, and gene expressions for the three DMEGs and prognostic-signature ROC analysis and Kaplan-Meier curves. **(B)** Validation-set relationships between risk scores, survival status, and gene expressions for the three DMEGs and prognostic-signature ROC analysis and Kaplan-Meier curves. **(C)** The total TCGA-LIHC-set PD-1 negative samples relationships between risk scores, survival status, and gene expressions of the three DMEGs and prognostic-signature ROC analysis and Kaplan-Meier curves. **(D)** The GSE10141-set PD-1 negative samples relationships between risk scores, survival status, and gene expressions of the three DMEGs and prognostic-signature ROC analysis and Kaplan-Meier curves.

genes are characteristics common to almost all cancers, including HCC (Zhao et al., 2020; Fabianowska-Majewska et al., 2021). The chromosomal distribution of these genes showed that DMEGs in adjacent gene regions had similar expression patterns, suggesting that adjacent regions may be regulated by the same methylases/demethylases. In addition, these 31 DMEGs could distinguish between tumor and normal samples based on methylation and expression levels, indicating their potential importance.

The protein-drug interaction analysis provided another perspective for evaluating the potential therapeutic effects of DMEGs on HCC. Conductance regulator and *HBB* were identified as the DMEGs having the most drug interactions. Conductance regulator encodes chloride ion and bicarbonate ion channels and has been implicated in a variety of cancers. It has also been identified as a molecular biomarker for early HCC diagnosis (Hogan, 1999; Moribe et al., 2009). Hemoglobin subunit beta has also been reported to be a diagnostic biomarker in cancers (Shi et al., 2018). Here, we found that these genes interacted with a variety of drugs, including the commonly used anti-tumor drug Lonidamine, a mitochondrial hexokinase inhibitor, which can inhibit the glycolysis of tumor cells. The drugs identified using the analysis above may provide alternative ways to treat HCC. In addition, we found that when the drug-DMEG distance was less than 0.8, drug interactions were significantly reduced, suggesting that 0.8 may represent an important threshold. With distances <0.8, the corresponding drugs may have more precise targeting effects for HCC treatment.

In order to evaluate the predictive power of these DMEGs for HCC prognosis, we used a LASSO regression to determine a prognostic model for HCC based on three genes (*UBD*, *CD5L*, and *CD213A2*) that can modulate immune responses.

Ubiquitin D (UBD), a ubiquitin-like protein modifier, binds to target proteins by covalent bonding and then causes them to be degraded by the 26s proteasome (Guarascio et al., 2020). Studies have shown that UBD can regulate the activation of the tumor necrosis factor alpha (TNF-α) -induced, and lipopolysaccharide-mediated, innate immune response central mediator nuclear factor kappa B (NF-κB), by promoting TNF-α-mediated ubiquitinated-I-κB-α proteasome degradation (Kawamoto et al., 2019). We speculate that increased *UBD* expression may promote an immune response in the tumor microenvironment, which suppresses tumor growth. The cysteine-rich inflammatory regulator CD5L has been shown to promote proliferation and activate autophagy in HCC by binding heat shock-A5 proteins (Armengol et al., 2013; Sanjurjo et al., 2015; Aran et al., 2018). Consistent with previous studies, the present results show that *CD5L* expression is a risk factor for HCC and may help researchers to reinterpret its role from the new perspective of methylation and immunity. CD213A2 has been shown to bind to interleukin-13 (IL-13) and activate its immunomodulatory function (Tabata and Khurana Hershey, 2007); however, no direct tumor-related role has been found, so further study of *CD213A2* and its effects on HCC, including through methylation and immunity, is warranted.

The tumor's response to ICIs largely depends on the state of the tumor microenvironment. As mentioned above, the lack of DNA methylation is related to the immune evasion characteristics of the tumor microenvironment. Here, we explored the DNA methylation characteristics and potential functional pathways of PD1-negative HCC patients, and identified the genes that play a key role in this process. These genes participate in the tumor immune microenvironment through possible DNA methylation regulation and further affect the anti-tumor effect of ICIs. We have proposed a model for determining PD-1-negative HCC prognoses based on these three genes. This model was clearly able to divide PD-1-negative HCC samples into high- and low-risk groups, with clear trends for DMEG expressions, and significantly different prognoses between these two groups. The use of an independent verification queue also confirmed its effectiveness. Therefore, further exploring the role of these hub genes in this process will help guide researchers to have a deeper understanding of the PD1-negative tumor microenvironment. These hub genes are also expected to become potential targets for enhancing the efficacy of ICIs.

CONCLUSION

The present research has revealed the methylation/transcription characteristics of PD-1-negative HCC samples and identified potential therapeutic targets and drugs. Most importantly, we have demonstrated the effectiveness of a prognostic model for HCC based on three DMEGs. These results provide insights into potential treatment strategies for HCC that are not sensitive to PD-1 inhibitors and into mechanisms by which methylation may affect HCC.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

WG designed the study, wrote this manuscript, and collected samples. LZ searched the articles and made figures. Both authors read and approved the final manuscript.

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REVIEW
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Epigenetic Regulation of the Wnt/β-Catenin Signaling Pathway in Cancer

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Studies over the past four decades have elucidated the role of Wnt/β-catenin mediated regulation in cell proliferation, differentiation and migration. These processes are fundamental to embryonic development, regeneration potential of tissues, as well as cancer initiation and progression. In this review, we focus on the epigenetic players which influence the Wnt/β-catenin pathway via modulation of its components and coordinated regulation of the Wnt target genes. The role played by crosstalk with other signaling pathways mediating tumorigenesis is also elaborated. The Hippo/YAP pathway is particularly emphasized due to its extensive crosstalk via the Wnt destruction complex. Further, we highlight the recent advances in developing potential therapeutic interventions targeting the epigenetic machinery based on the characterization of these regulatory networks for effective treatment of various cancers and also for regenerative therapies.

Keywords: Wnt signaling, β-catenin, YAP, epigenetics, cancer, therapeutics

INTRODUCTION

The Wnt signaling pathway remains one of the most extensively studied signaling pathways to date and yet there is a long way to fully understand its functionality. The Wnt pathway was discovered in 1982 with the identification of the *int1* (*wnt1*) gene responsible for tumor growth (Nusse and Varmus, 1982). The hallmark Wnt pathway gene *wnt1* (*int1* at the time of identification) was discovered as the first proto-oncogene in mice mammary tumors using the pro-viral tagging screening method in 1982 (Nusse and Varmus, 1982). The Wnt pathway is associated with the widest array of biological processes, including cell proliferation, differentiation, organogenesis, regeneration and diseases such as neurodevelopmental diseases and cancer (Nusse and Clevers, 2017). Although the mammalian *wnt1* gene was discovered as a part of screening for proto-oncogenes, its *Drosophila* homolog *wingless* was already known to play a role in segmentation during development (Nüsslein-Volhard and Wieschaus, 1980). Other genes in this pathway (including, *armadillo, arrow, disheveled, zeste-white 3, porcupine*) were also identified due to segmentation polarity defects during *Drosophila* development (Peifer et al., 1991; Siegfried et al., 1992; Noordermeer et al., 1994; Kadowaki et al., 1996; Bafico et al., 2001). Overexpression studies of Wnt1 and GSK3β in *Xenopus* showed axis duplication (McCrea et al., 1993; Dominguez et al., 1995). Studies in planaria and *Hydra* have established the role of the Wnt pathway in regeneration as well (Hobmayer et al., 2000; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008;

Reddy et al., 2020). Due to the important roles of the Wnt pathway in the processes of development and regeneration, the interest in investigating its role in human cancers climbed steadily. *APC* was found to be the most frequently mutated gene in inherited forms of human cancer, causing multiple polyps in intestines (Familial Adenomatous Polyposis or FAP) (Su et al., 1992). Sporadic cases of cancers, on the other hand, didn't show alterations in the *APC* gene. Rather, other components of the Wnt pathway, such as *CTNNB1* and *Axin* showed mutations. Apart from genetic alterations in the Wnt pathway components, there are several epigenetic changes associated with tumor initiation and progression in Wnt driven cancers. Additionally, there are epigenetic changes which are a result of Wnt activation. There are three modes of mechanism for the epigenetic machinery to work. It can change the status of DNA methylation, histone modification profiles and can also work through a plethora of non-coding RNAs (Dai et al., 2020). Even though these do not change the DNA sequence but these are heritable changes and thus can contribute extensively toward tumorigenesis. Very recently, drugs targeting epigenetic machinery have been adopted into the chemotherapeutic regime for cancers reflecting the importance of epigenetic modulation in cancers (Topper et al., 2020).

This review is specifically aimed at providing an overview of all well characterized epigenetic mechanisms which modulate the expression and function of the canonical Wnt pathway components and therefore affect Wnt target genes in various cancer types. In addition to this, the review will also cover advances made in the therapeutic interventions targeting the Wnt signaling pathway, especially through the epigenetic players.

Wnt SIGNALING NETWORK – THE RECENT MOST SNAPSHOT

Wnt signaling is broadly classified into canonical and non-canonical Wnt signaling based on β-catenin -dependent and independent responses, respectively (Semenov et al., 2007). Canonical Wnt signaling is an intricate pathway involving 19 Wnt ligands, 10 Frizzled (FZD) receptors and 3 Disheveled (DVL) proteins. The selective combination of Wnt-FZD generates a wealth of information, modulating the signaling outcome crucial for normal development, multitude of cellular processes and development of various diseases (Semenov et al., 2007; Dijksterhuis et al., 2015; Voloshanenko et al., 2017). In unstimulated cells, the transcriptional co-activator β-catenin is engaged by a large cytoplasmic destruction complex composed of Adenomatous polyposis coli (APC), Axis inhibition protein (AXIN), Glycogen synthase kinase 3 (GSK3) and Casein kinase 1 (CK1), inducing sequential phosphorylation of β-catenin at Serine 33, Serine 37, Serine 45, and Threonine 41 by CK1 and GSK3β. The phosphorylated β-catenin is then ubiquitinated by βTrCP, an E3 ubiquitin ligase, followed by its degradation, preventing its nuclear transport (MacDonald et al., 2009). The stability of the destruction complex is very critical for continuous degradation of β-catenin. In canonical signaling, Wnt ligands bind to Frizzled and low-density lipoprotein receptor related proteins (LRP5/6) to stimulate Wnt signaling by recruiting the DVL proteins to the plasma membrane. Binding of DVLs to the membrane and sequential phosphorylation on the cytoplasmic domain of LRP5/6 promotes its interaction with AXIN, thereby destabilizing the destruction complex and inducing dis-engagement of β-catenin from the destruction complex. Moreover, the inactivation of ubiquitination and proteasomal degradation of β-catenin in the intact destruction complex have been shown to saturate the destruction complex, preventing further engagement and thereby degradation of β-catenin (Li et al., 2012). Disengaged β-catenin evades phosphorylation due to the multivesicular sequestration of GSK3β which prevents recognition by βTrCP E3 ubiquitin ligase and hence the degradation by the proteasome pathway (Taelman et al., 2010). Dephosphorylated β-catenin stabilizes and accumulates in the cytoplasm with subsequent translocation into the nucleus (MacDonald et al., 2009). Nuclear β-catenin interacts with the transcription factors of the TCF/LEF (T cell factor/lymphoid enhancer-binding factor) family by replacing corepressor, Groucho, to induce expression of Wnt/β-catenin target genes (Daniels and Weis, 2005).

Multiple factors have been shown to regulate the precise stimulation and inhibition of Wnt signaling. For instance, the R-spondin/LGR/RNF43 module is the key signaling paradigm that potentiates Wnt signaling. R-spondin family proteins regulate Wnt signaling via a common mechanism involving interaction with the Leucine-rich repeat-containing G-protein coupled (LGR) family of receptors (Kim et al., 2008). In the absence of R-spondins, two homologous ubiquitin E3 ligases, ZNRF3/RNF43 bind to the FZD receptors and target it for degradation. However, in the presence of R-spondins, ZNRF3/RNF43 interact with LGR4-6, leading to the inhibition of ZNRF3/RNF43 and dickkopf glycoproteins family member, DKK1 activity allowing subsequent accumulation of FZD receptors, thereby potentiating Wnt signaling (Kim et al., 2008; Wang et al., 2013; Xie et al., 2013; **Figure 1**). A recent study suggests that LGR5 potentiates Wnt signaling without sequestering ZNRF3/RNF43 by directly enhancing the Wnt signalosome mediated LRP6 phosphorylation (Park et al., 2020). This finding establishes that LGR4 and LGR5 have non-equivalent functions adopting different routes to stringently potentiate Wnt signaling.

Non-canonical Wnt signaling, consisting of Planar cell polarity (PCP) and Ca^{+} signaling pathways, has been shown to converge with multiple pathways and regulate a diverse number of cellular processes required for development and organogenesis. The combination of receptor/co-receptor at the plasma membrane binding to Wnts acts as a regulatory switch to activate either β-catenin-dependent or β-catenin-independent pathways. In the PCP pathway, Wnt ligands, Wnt5a and Wnt11 bind to receptors FZD3 or FZD6 along with tyrosine kinase co-receptors ROR1, ROR2 or RYK to orient toward Wnt/PCP signaling (Oishi et al., 2003; Martinez et al., 2015). Subsequently, this leads to recruitment of DVLs, leading to the activation of small GTPases of the Rho family [Cdc42, Rac1, RhoA and DVL-associated activator of morphogenesis1 (DAAM1)] (Habas et al., 2001; Liu et al., 2008). This cascade leads to the activation of

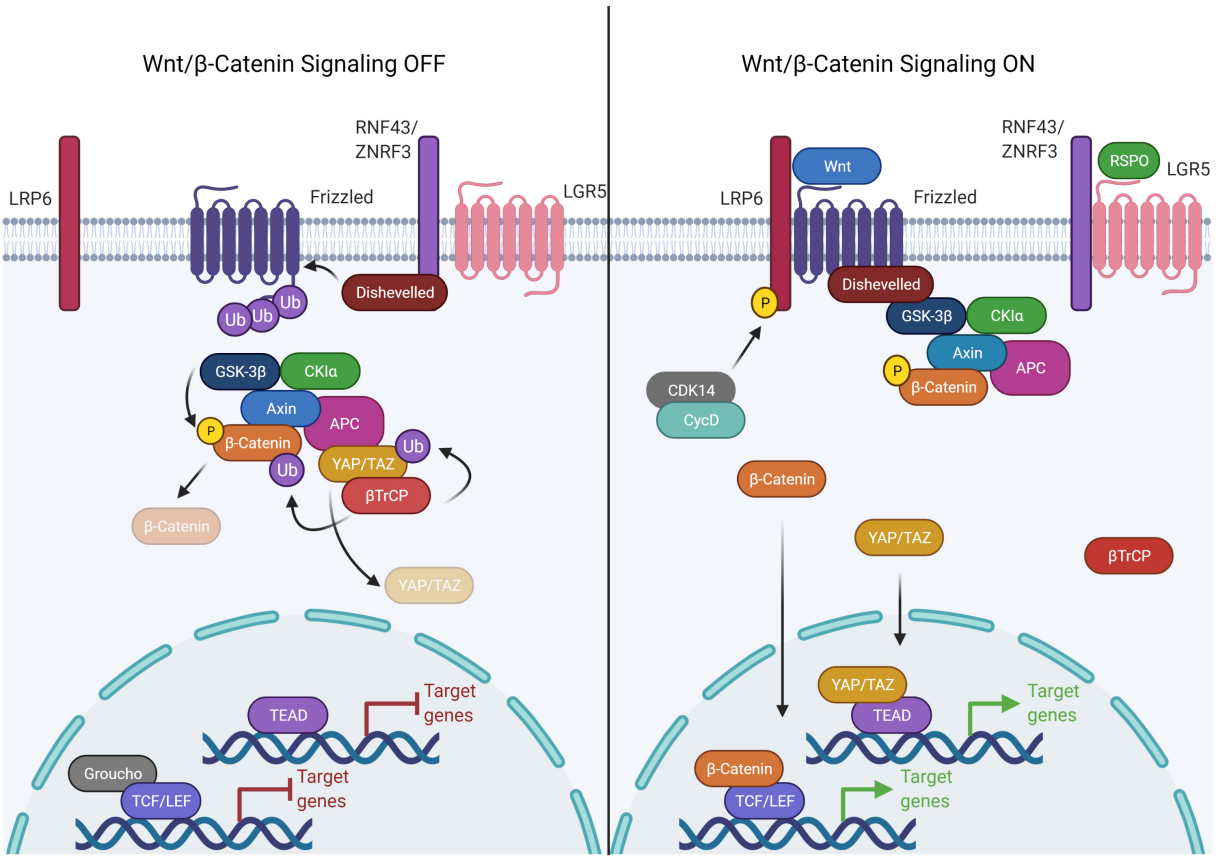

FIGURE 1 | Components of the canonical Wnt signaling pathway. The canonical Wnt pathway is primarily dependent on the transcriptional activity of β-catenin. In the Wnt OFF state, the Wnt destruction complex is actively engaged in the phosphorylation and subsequent ubiquitination mediated degradation of β-catenin. This is achieved by sequestering β-catenin to the Wnt destruction complex, consisting of APC, AXIN (scaffold proteins), GSK3β, and CK1α (kinases). Upon phosphorylation by the kinases, β-catenin is ubiquitinated by βTrCP which is recruited to the destruction complex by YAP/TAZ. The DNA binding partner of β-catenin, TCF/LEF, is bound to the DNA in a repressive complex with Groucho/TLE. Wnt signaling is activated upon binding of Wnt ligand causing complex formation between LRP6 and frizzled which allows Disheveled to bind to LRP. This activated form of Disheveled is able to bind and recruit the Wnt destruction complex thereby disrupting the binding of βTrCP. The receptor bound Wnt destruction complex is ubiquitination incompetent, hence β-catenin can't be targeted for proteasomal degradation, therefore leading to saturation of the destruction complex with phospho-β-catenin. The new pool of β-catenin is free to translocate into the nucleus and activate the transcription of Wnt responsive genes by binding the TCF/LEF family of transcription factors. In the same process, YAP/TAZ is unable to recruit β-TrCP to the destruction complex and escape degradation. YAP/TAZ translocates into the nucleus as well and binds to the TEAD family of transcription factors.

downstream c-Jun kinase and Rho associated kinase required for actin cytoskeletal remodeling and cell contractility (Sebbagh and Borg, 2014; Martinez et al., 2015). The Wnt/PCP pathway is also activated independent of DVLs and the cascade of signaling is transduced to activate Nemo like kinase (NLK) which is required for cell fate determination and cell movement (Thorpe and Moon, 2004). In the Wnt/Ca^{+} pathway, Wnt ligands bind to FZD with concomitant recruitment of DVLs leading to the production of intracellular signaling molecules, inositol 1,4,5-triphosphate (IP3), 1,2 diacylglycerol (DAG), and Ca^{2+} from the membrane-bound phospholipid phosphatidylinositol 4,5-bisphosphate via the action of membrane-bound enzyme phospholipase C (De, 2011). The network of this signaling paradigm leads to the activation of downstream signaling proteins such as protein kinase C, calcineurin and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), thereby regulating cell adhesion and cell migration (Malbon et al., 2000; **Figure 2**).

Wnt SIGNALING IN CANCERS

Tumorigenesis involves progressive accumulation of genetic, epigenetic and molecular changes altering the cellular phenotype. Wnt signaling is a complex network of proteins and regulates molecular processes in a regulatory manner. Any imbalance in its regulation induces untoward cellular changes responsible for the development of various diseases and tumorigenesis. Dysregulation of Wnt signaling has been described in multiple cancers and plays a dramatic role in the progression of cancers (Bugter et al., 2021). Colorectal cancer is most commonly caused due to dysregulation of the Wnt pathway. It is sporadic although 30% of them are genetic in nature (Byrne and Tsikitis, 2018). Despite the role of the genetic mutations linked to colorectal cancer being well studied, the precise mechanism of the disease is not clearly understood. Mutations in the critical regulatory switches of the Wnt signaling pathway have been implicated in

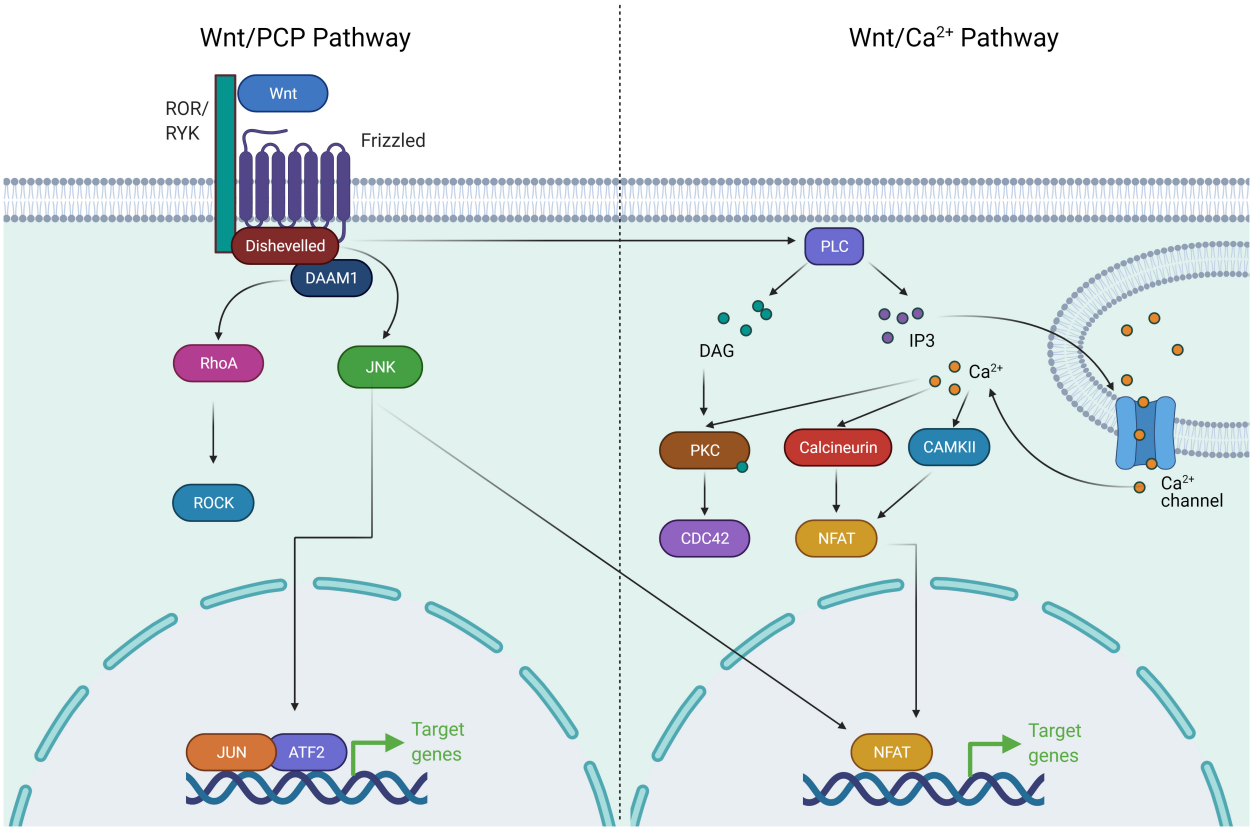

FIGURE 2 | Components of non-canonical Wnt signaling pathway. The non-canonical Wnt pathway is independent of β-catenin. Wnt activation leads to complex formation between ROR and Frizzled which allows activation of Disheveled. Disheveled is then able to activate DAAM1 and RHOA GTPase. RHOA in turn activates ROCK. Activation of ROCK and JNK leads to cytoskeletal remodeling. Wnt/Ca^{+} signaling involves activation of Disheveled upon Wnt activation, followed by activation of PLC. PLC activates the downstream effectors PKC, Calcineurin, and CAMKII via release of Ca^{+}. These factors drive NFAT mediated transcriptional program and cytoskeletal changes.

multiple cancers and loss of APC is a hallmark of colorectal cancer initiation and progression (Zhang and Shay, 2017). In most colorectal cancers, both the alleles of *APC* gene are mutated, especially in the region required for interaction with the armadillo (arm) repeats of β-catenin (Rubinfeld et al., 1997). Such mutations prevent the degradation of β-catenin and induce cellular changes critical for colorectal cancer initiation. Mutations in the *APC* gene are responsible for approximately 1% of all colorectal cancer cases (Bienz and Clevers, 2000; Half et al., 2009). The main feature of Wnt signaling driven cancers is the constitutive nuclear localization of β-catenin. Numerous studies have identified mutations in the serine and threonine residues of β-catenin causing abrogation of phosphorylation by GSK3β and CK1 kinases, ultimately leading to the hyperactivation of Wnt signaling (Sparks et al., 1998). Mutation in the serine/threonine regulatory domain of β-catenin has been found in 48% of colorectal cancers lacking *APC* mutations (Sparks et al., 1998). High throughput cancer genomics studies have cataloged mutations in the genes encoding multiple components of the Wnt signaling pathway, including the ligands, receptors and intracellular components. Besides *APC* and *CTNNB1*, mutations have been found in the regions of *AXIN1* gene coding for domains which are critical for its interaction with APC, GSK3β and β-catenin, resulting in destabilization of the destruction complex with concomitant increase in cytoplasmic as well as nuclear levels of β-catenin (Jin et al., 2003; Salahshor and Woodgett, 2005). Similarly, a mutation in *AXIN2* identified in colorectal cancers promotes GSK3β inhibition and stabilization of Snail, a positive regulator of Wnt signaling (Mazzoni et al., 2015). The increased expression of LRPs have also been linked with hyperactivation of Wnt signaling and development of colorectal cancers (Rismani et al., 2017; Boulagnon-Rombi et al., 2018; Yao et al., 2020). The R-spondin/LGR5/RNF43 module has also been implicated in Wnt signaling driven cancers. A deleterious mutation in *RNF43* has been reported in 19% of colorectal cancers lacking *APC* mutations (Giannakis et al., 2014). Further, a high number of R-spondin mutations and fusion proteins causing hyperactivation of Wnt signaling has been described in 10% of colon cancers lacking the *APC* mutations (Seshagiri et al., 2012; Giannakis et al., 2014).

Among gastric cancers, 30% exhibit nuclear β-catenin as a prominent acquired change (Clements et al., 2002). Around 18% of gastric cancers have mutations in *APC* and *RNF43* as the primary cause of the disease (Clements et al., 2002; Fang et al.,

2002; Wang K. et al., 2014; Flanagan et al., 2019). In addition to the genetic mutations, epigenetic changes (such as promoter methylation) have also been identified in Wnt antagonists such as the secreted frizzled-related proteins (sFRPs) and Wnt negative-modulators, the dickkopf family of glycoproteins (DKK1–3), leading to activation of Wnt signaling (Nojima et al., 2007; Yu et al., 2009; Flanagan et al., 2017). These will be elaborated in the following sections.

In cancers, heterogenous levels of activation determine the adversity and fate of different cancers. For example, 37% of *APC*-mutant gastric cancers have mutation in *RNF43*, suggesting a compound activation of Wnt signaling in the same tumor. However, only 5.5% of colon cancers have mutations both in *APC* and *RNF43*, suggesting a differential mechanism of optimal activation of Wnt signaling in gastric and colon cancers for tumor growth and progression (Albuquerque et al., 2002). The role of Wnt signaling in hematopoietic stem cells and leukemia progression has also been described wherein β-catenin appears to be essential for leukemia initiating cells and their self-renewal (Albuquerque et al., 2002; Luis et al., 2012; Lento et al., 2013). Chronic lymphocytic leukemia shows higher expression of multiple Wnt proteins with enhanced β-catenin-dependent Wnt signaling (Lu et al., 2004). Further, epigenetic silencing of Wnt signaling inhibitors DKK1/2 and somatic mutations in *FZD5* is associated with the development of chronic lymphocytic leukemia in 14% of the cases (Moskalev et al., 2012; Wang L. et al., 2014). **Table 1** summarizes the mutations in different Wnt components reported in various cancers. Recently, the synergistic interplay of the canonical and non-canonical Wnt signaling playing a major role in the development of cancers has also been established (Katoh, 2017; Flores-Hernández et al., 2020).

CONVERGENCE OF Wnt PATHWAY WITH OTHER SIGNALING NETWORKS IN CANCER

Signaling pathways do not operate linearly nor exclusively. Rather, there are multiple points of crosstalk between all the pathways which ultimately fine tune target gene expression. The Wnt pathway is known to work in conjunction with other pathways to regulate the fundamental cellular processes critical for normal development and cancer.

Wnt and Hippo Signaling Pathways: Partners in Action

Like the Wnt pathway, the Hippo signaling pathway was also identified in *Drosophila*. The pathway gets its name owing to the phenotype it induces in flies. Flies with mutations in the Hippo pathway components have abnormal growth due to loss of organ size control (Justice et al., 1995; Xu et al., 1995; Jia et al., 2003; Udan et al., 2003). Studying crosstalk between the Wnt and Hippo pathways has been of particular interest to researchers. It is due to the fact that the effector proteins of these pathways, β-catenin and YAP/TAZ, directly interact with each other and their stability is dependent on the Wnt destruction complex (Azzolin et al., 2012, 2014). The Hippo pathway does not consist of a defined set of receptors and has diverse upstream regulators (Pan, 2010; Totaro et al., 2018). Unlike other pathways, the activation of the Hippo pathway leads to inactivation of its effector molecules, YAP and TAZ. These regulators can either be components of other signaling pathways or, often, are part of the cell's mechanosensory machinery (Zhao et al., 2007; Lei et al., 2008). The mechanosensory proteins are the ones that are involved in the maintenance of cell-cell adhesion, apico-basal polarity and cell junctions, for example, *E*-cadherin, KIBRA, NF2, α-catenin and the Crumbs complex (Genevet et al., 2010; Varelas et al., 2010b; Zhang et al., 2010; Silvis et al., 2011; Benham-Pyle et al., 2015). Proteins, such as NF2, act as a scaffold for the assembly of Hippo kinases (Lallemand et al., 2003). Activation of Hippo signaling leads to the activation of a cascade of kinases, namely, Macrophage-stimulating protein (MST1/2), Salvador (SAV1), Large tumor suppressor (LATS1/2), and Mps one binder kinase (MOB1). MST1/2 is the upstream kinase. It gets activated upon phosphorylation and forms an active complex with SAV1. This complex further activates LATS1/2 via phosphorylation. LATS1/2, along with MOB1 leads to the phosphorylation of YAP/TAZ (Chan et al., 2005; Callus et al., 2006; Zhao et al., 2007; Praskova et al., 2008; Varelas et al., 2010b). The phosphorylated form of YAP/TAZ is sequestered in the cytoplasm and subsequently degraded due to ubiquitination (Zhao et al., 2007, 2010; Lei et al., 2008). Absence of Hippo signaling allows nuclear accumulation of YAP/TAZ which then bind to the TEAD (transcriptional enhanced associate domain) family of DNA binding proteins, leading to transcription of its downstream targets (Vassilev et al., 2001; Mahoney et al., 2005). YAP/TAZ can have other DNA binding partners as well, such as p75, RUNXs and SMADs (Yagi et al., 1999; Strano et al., 2001; Varelas et al., 2008). Due to its involvement in regulating organ size control and cell adhesion properties, its role in multiple cancer types is not surprising. It has been reported to be involved in cases of breast cancer, lung cancer, liver cancer, prostate cancer, etc. (Chan et al., 2008; Zhou et al., 2011; Chen Q. et al., 2014; Ma et al., 2014; Chen H.-Y. et al., 2015; Seo et al., 2017). Similar to the Wnt signaling pathway, the Hippo pathway is involved in the regulation of intestinal regeneration and is therefore frequently dysregulated in colorectal cancers as well (Cai et al., 2010; Gregorieff et al., 2015). Interestingly, even though the Hippo pathway is frequently perturbed in various cancer types, mutations in its components are uncommon (Harvey et al., 2013).

The first link between the Wnt and Hippo pathways was established in a study which showed TAZ as the negative regulator of the Wnt pathway. It was shown that TAZ inhibits interaction of DVL with CK1δ/ε thereby preventing its phosphorylation upon Wnt activation leading to inhibition of Wnt signaling (Varelas et al., 2010a). Recently, LATS1/2 have been shown to maintain the intestinal stem cell niche by sustaining Wnt signaling (Li Q. et al., 2020). The study shows that when the Hippo pathway is inactive, nuclear YAP/TAZ interacts with Groucho and prevents the Wnt mediated transcriptional program (**Figure 3**). Interestingly, LATS1/2 depletion was able to silence the expression of the Wnt target gene, *MYC*,

TABLE 1 | Mutations in Wnt signaling components in cancers.

Gene	Alteration	Cancer type	Outcome	References
APC	Exon 15	CRC	Loss of interaction with β-catenin	Rubinfeld et al., 1997; Bienz and Clevers, 2000; Half et al., 2009
APC	Exon 15	GC	Loss of interaction with β-catenin	Fang et al., 2002; Flanagan et al., 2019
β-catenin	Codons 33, 37, 41, and 45 in exon 3	CRC	Loss of phosphorylation by GSK3β and Ck1α	Sparks et al., 1998
AXIN1	Exon 1 and 5	CRC	Loss of interaction with APC, GSK3β and β-catenin	Jin et al., 2003; Salahshor and Woodgett, 2005
AXIN2	Stop codon at codon 663 in exon 7	CRC	Inhibition of GSK3β and stabilization of Snail	Mazzoni et al., 2015
RNF43	Truncation mutations	CRC	Inactivation of RNF43 and increased cell surface abundance of FZD5	Giannakis et al., 2014
RNF43	Truncation mutations	GC	Inactivation of RNF43 and increased cell surface abundance of FZD5	Wang K. et al., 2014
R-spondins	Gene fusions	CRC	Gene fusion of R-spondins (RSPO2 and RSPO3) with PTPRK and/or EIF3E causing elevated expression of R-spondins	Seshagiri et al., 2012

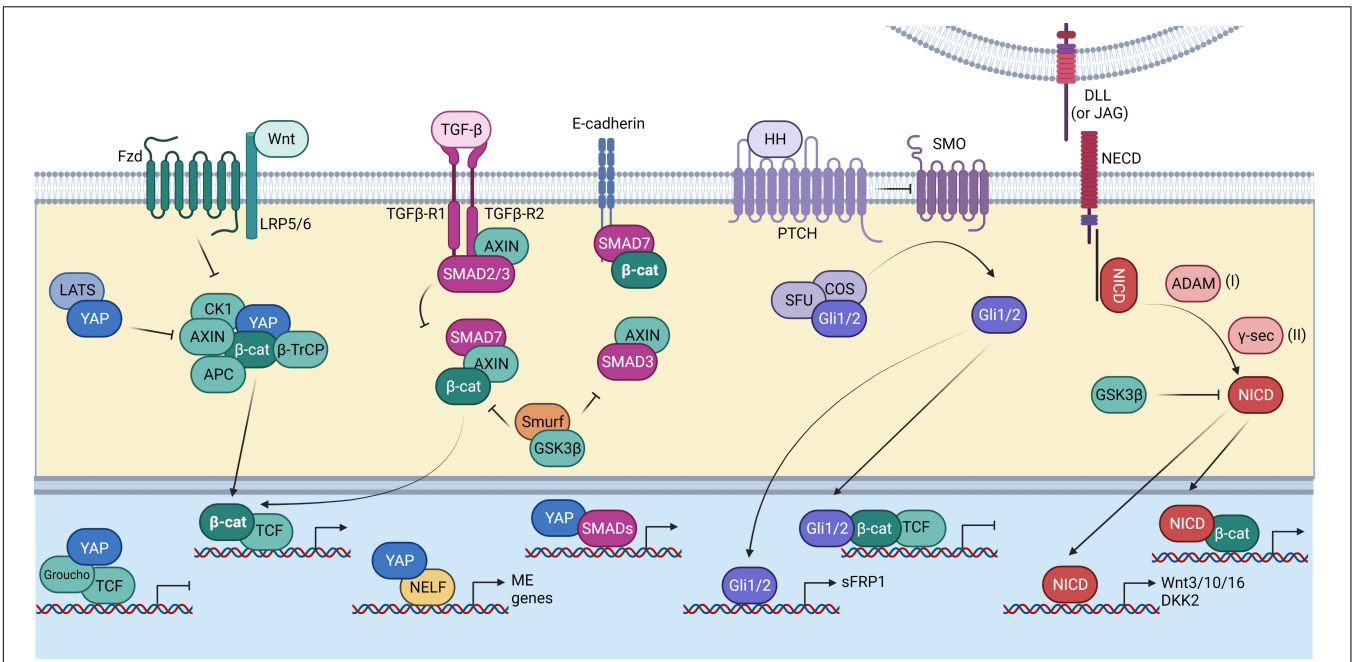

FIGURE 3 | Interplay of the Wnt pathway with other pathways. Summary of the interaction of the Wnt pathway components (depicted in green) with other signaling pathways, including the Hippo pathway (depicted in blue), TGF-β pathway (depicted in magenta), Hedgehog pathway (depicted in purple), and Notch pathway (depicted in red). The Hippo pathway helps in the stabilization of β-catenin by inhibiting YAP's interaction with the Wnt destruction complex, thereby preventing the recruitment of βTrCP. TGF-β signaling has synergistic effects with Wnt signaling. SMAD7 can help in preventing the GSK-3β mediated β-catenin degradation. The Hedgehog effector Gli1/2 can inhibit Wnt target gene expression by directly binding to the β-catenin/TCF complex. It can also drive the expression of the Wnt inhibitor sFRP1. The Notch signaling transcription factor, NICD, interacts with β-catenin. NICD can promote Wnt signaling by promoting the expression of the Wnt ligands. However, the Wnt inhibitor DKK2 is also a target of NICD. NICD itself is a substrate of GSK-β.

even in the *APC* mutant intestinal cells, making it a good candidate for intestinal tumor therapy. Other evidence of positive regulation by the Hippo pathway showed that S127D mutant YAP (phosphomimetic form and hence constitutively cytoplasmic) inhibited the nuclear accumulation of β-catenin (Imajo et al., 2015). In contrast, few reports have suggested a negative role of the Hippo pathway in the regulation of Wnt signaling. A study showed that *Taz* knockout in mice kidney led to a minimal increase in nuclear β-catenin (Makita et al., 2008). An increased expression of β-catenin was observed in the case of cardiomyocytes from *Salvador* (*Sav1*) knockout mice (Heallen et al., 2011). Additionally, it was shown that YAP/TEAD and β-catenin/TCF complexes bind cooperatively to a few of their target genes, for e.g., *snail2* and *sox2* (Heallen et al., 2011). Most importantly, all the three effectors, β-catenin, YAP and TAZ, are involved in regulating βTrCP mediated ubiquitination and

subsequent degradation of each other. YAP and TAZ themselves are part of the Wnt destruction complex (Azzolin et al., 2012). In Wnt off conditions, YAP/TAZ binds to the destruction complex and recruits βTrCP, thereby promoting degradation of β-catenin as well as YAP/TAZ (Azzolin et al., 2014). Similarly, β-catenin phosphorylated by GSK3β can bind to TAZ and initiate its ubiquitination (Azzolin et al., 2012). A recent report showed that the balance between β-catenin and YAP is crucial for the maintenance of the intestinal stem cell niche. YAP overexpression or LATS depletion resulted in the inhibition of $Lgr5^{+}$ intestinal stem cell growth by re-programming these cells into a state of low Wnt and high Klf6, driving the cells toward a 'wound-healing state'. The same study showed a similar effect in cases of organoids, patient derived xenografts as well as colorectal cancer cell lines (Cheung et al., 2020).

TGF-β Signaling Pathway

The interplay between TGF-β and Wnt pathways is context-dependent and can result in different outcomes. For example, Wnt3a stimulation increases TGF-β expression as well as SMAD2 phosphorylation and promotes differentiation in the case of myofibroblasts (Carthy et al., 2011). Wnt and TGF-β activation have synergistic effects during EMT in alveolar epithelial cells (Zhou et al., 2012). TGF-β and Wnt activation promotes expression of mesendodermal (ME) related genes in human embryonic stem cells (hESCs). YAP recruits Negative elongation factor (NELF) and inhibits the expression of ME related genes (Estarás et al., 2015). Inhibitory SMAD7 promotes β-catenin binding to plasma membrane through *E*-cadherin and prevents its degradation in cancer epithelial cells and helps in regulation of cell–cell adhesion. Complex formation between SMAD7 and AXIN prevents interaction of β-catenin with GSK3β and E3 ligase Smurf2 via AXIN. This prevents degradation of β-catenin but increases its localization on the membrane instead of nucleus (Tang et al., 2008). On the other hand, an earlier study showed that SMAD7 can promote the nuclear translocation of β-catenin in prostate cancer cells (Edlund et al., 2005). AXIN proteins have been shown to play a contrasting role in the regulation of TGF-β signaling. Earlier studies showed that AXIN acts as a positive regulator of TGF-β signaling by promoting phosphorylation of SMAD3 upon TGF-β activation (Furuhashi et al., 2001; Dao et al., 2007). However, another report showed that AXIN is able to mediate binding of SMAD3 (not SMAD2) to GSK3β and promotes SMAD3 phosphorylation and degradation in the absence of TGF-β signaling (**Figure 3**). Depletion of AXIN increased the transcriptional activity of SMAD3 even in the absence of TGF-β activation (Guo et al., 2008). As mentioned earlier, SMADs are known DNA-binding partners of YAP/TAZ, apart from the TEAD proteins. YAP and TAZ interact with activated SMAD2/4 and SMAD3/4 complexes and increase their nuclear localization (Hiemer et al., 2014). TGF-β activation also leads to an increase in TAZ mRNA and not YAP, in a SMAD3-independent manner (Miranda et al., 2017). TAZ has been shown to enhance the metastatic properties of breast cancer cells via expression of BMP4 and subsequent activation of BMP signaling (Lai and Yang, 2013). However, when the Hippo pathway is activated and YAP/TAZ accumulates in the cytoplasm, the nuclear translocation of SMADs is blocked (Barrios-Rodiles, 2005).

Hedgehog Signaling

The crosstalk between Hedgehog and Wnt signaling is stage-specific. The inhibitory feedback between the two pathways is responsible for driving developmental processes. Canonical Hedgehog signaling is an inhibitor of Wnt signaling especially in the context of intestinal homeostasis. Hedgehog signaling suppresses Wnt signaling by regulating the expression of sFRP1 via its effector proteins, Gli1 and Gli2 (He et al., 2006; **Figure 3**). Activation of Hedgehog signaling prevents the nuclear accumulation of β-catenin in liver cancers as well (He et al., 2006). However, the non-canonical Hedgehog signaling, which is Patched1 (PTCH1)-dependent and Smoothened (SMO) and Gli-independent, has been shown to positively regulate Wnt signaling in the intestinal tumors by promoting the undifferentiated state of cancer stem cells (Regan et al., 2017). Interestingly, one study showed that the advanced stages of colon cancer have a higher activation level of Hedgehog and not Wnt signaling. Activation of Hedgehog signaling also led to the expression of embryonic genes such as, POU5F1 and NANOG as well as inhibition of β-catenin/TCF transcriptional activity via Gli1. Activation of embryonic genes therefore might be responsible for increasing cancer stemness and metastatic properties in Hedgehog driven cases (Varnat et al., 2010). In contrast, Gli2 was shown to promote nuclear localization of β-catenin and together these factors drive the proliferation of osteosarcoma cells (Ma J. et al., 2019; Xu et al., 2019). Sonic hedgehog has been reported to positively regulate YAP in medulloblastoma. It not only drives the expression of YAP but also promotes its nuclear accumulation (Fernandez-L et al., 2012). However, in case of the intestinal mesenchyme, YAP/TAZ promote proliferation and self-renewal whereas Hedgehog signaling drives differentiation into smooth muscle (Cotton et al., 2017).

FGF Signaling

The Wnt-FGF gradient is a paradigm which has been very well established in the context of development. It plays an extensive role during axis formation in development (Dyer et al., 2014; Oginuma et al., 2017). Due to similar reasons, the interplay between the two pathways is important in cancer as well. Wnt1 and Fgf3 expression is upregulated in cases of breast cancer. Breast cancer cell lines overexpressing both these ligands and therefore with high Wnt and Fgf activity, exhibited higher mitochondrial biogenesis which correlated with enhanced mammosphere forming capacity and stemness (Lamb et al., 2015). An extensive axis between Wnt and FGF regulates the stem cell niche in the lungs. In a study by Volckaert et al. (2017), it was shown that YAP expression promotes the basal stem cell niche in the lung epithelium. Activation of the Hippo pathway keeps the differentiated epithelium in a quiescent state. However, injury leads to the activation of YAP caused due to the degradation of Merlin, an upstream regulator of the Hippo pathway. Yap in turn causes induction of Wnt7b which in turn drives the expression of FGF10 which is required for the maintenance of basal stem cells (Volckaert et al., 2017).

Notch Signaling

Notch activity is Wnt-dependent and it cooperates with Wnt in development as well as cancer. Cancer stem cells isolated from clear cell renal cell cancer specifically showed upregulation of the Wnt and Notch related genes including *TCF7L2*, *TCF3*, *LGR4*, *AXIN2*, *EP300RBPJ*, *NOTCH3*, *HES1*, and *JAG1* (Fendler et al., 2020). At a mechanistic level, it has been shown that the Notch intracellular domain (NICD) is a substrate for GSK3 kinases (Espinosa et al., 2003). The activity of GSK3-β is inturn dependent on the Wnt activation status. In Wnt inactive state, GSK3-β-dependent phosphorylation primes Notch for proteasomal degradation, thereby preventing its transcriptional activity (**Figure 3**; Espinosa et al., 2003). β-catenin can directly bind to NICD and enhance its transactivation. However, overexpression of LEF1 hinders this process by competing with NICD to interact with β-catenin (Jin et al., 2009). In another study, it was shown that Notch signaling is required for maintaining the stemness of mammary associated stem cells. Notch activation through stem cells in macrophages eventually led to expression of multiple Wnt ligands- Wnt3, 10, and 16 which ultimately promoted self-renewal in stem cells (Chakrabarti et al., 2018). Wnt signaling has been shown to prevent differentiation of hESCs into Medial Ganglionic Eminence (MGE)-like cells and promoted expansion of progenitors by driving the expression of the Notch ligand, JAG1 (Ma L. et al., 2019). However, it has also been shown that *DKK2* is a target of Notch signaling in the intestinal stem cells, indicating a negative feedback for Wnt by Notch signaling (Katoh and Katoh, 2007).

Transcription Factors

Wnt signaling pathway partners with various transcriptional factors and chromatin remodelers, mainly via interaction with β-catenin, presumably assisting the expression of target genes for these transcription factors in a Wnt-dependent manner. A few instances have been reported in which these transcription factors can act as negative regulators of Wnt signaling as well. Some of these include SOX17, CBP, FOXO, MLL1, TBP, and HIF1a. A number of these factors are reviewed in Valenta et al. (2012) and Söderholm and Cantù (2020). In this review, we have focused on the two protein families that have been recently identified to work alongside the Wnt pathway.

The Homeodomain (HOX) group of proteins are involved in embryogenesis. In adults, these are required for maintenance and homeostasis. HOXA5 has been shown to coordinate with the Wnt pathway in driving stem cell differentiation. HOXA5 acts as a tumor suppressor and promotes intestinal cell differentiation by inhibiting the Wnt/β-catenin pathway. Wnt pathway, on the other hand, inhibits HOXA5 via MYC which is a direct target of the Wnt signaling (Ordóñez-Morán et al., 2015). Another study using cervical cancer cells showed that HOXA5 can inhibit Wnt pathway genes by transactivating TP53. TP53 regulates the expression of the miR-200 family of microRNAs which target multiple Wnt pathway components, as mentioned in the proceeding sections (Ma et al., 2020). In contrast, HOXA13 exerts an opposite effect. It binds to β-catenin and helps in the nuclear translocation and subsequent activation of β-catenin/TCF/LEF mediated transcription (Gu et al., 2020). HOXB4 is another HOX gene family member which appears to have both tumor suppressive and oncogenic potential depending on the cancer type. In a recent study, it was shown to inhibit cancer cell proliferation and further tumorigenesis by inhibiting β-catenin at the transcriptional level by binding to its promoter in cervical cancer cells. Interestingly, several other Wnt related genes were found to be downregulated in cells overexpressing HOXB4, including *MYC*, whereas genes such as *FZD8*, *DKK1,* and *AXIN2* were upregulated (Lei et al., 2021).

The Specificity Protein (SP) family of zinc finger proteins have been shown to regulate Wnt signaling as well. SP1, one of the earliest transcription factors to be identified, has been shown to regulate the stability of β-catenin by modulating its interaction with the Wnt destruction complex. Interestingly, SP1 depletion led to an increased phosphorylation and ubiquitination of β-catenin in colorectal cancer cell lines (Mir et al., 2018). SP5, on the other hand, acts as a feedback inhibitor of Wnt signaling. Its expression is induced upon Wnt activation, especially in cells with stemness, including embryonic carcinoma cells (Huggins et al., 2017). Further, Huggins et al. (2017) hypothesized that SP5 might compete with SP1 for binding to a limited set of loci upon Wnt activation in case of human pluripotent stem cells. These loci are mainly Wnt target genes which are downregulated by SP5 (Huggins et al., 2017). Interestingly, SP5 is also strongly expressed in $Lgr5^{+}$ intestinal stem cells (Barker et al., 2010). Another study showed that SP5 and SP8 both assist the binding of β-catenin to LEF1 upon Wnt activation. This study also pointed out that SP5 and SP8 are responsible for regulating only a subset of Wnt target genes (Kennedy et al., 2016).

Wnt SIGNALING AND EPIGENETIC REGULATION

Modes of Epigenetic Regulation

Cells in multicellular organisms are homogenous at the genetic level but their phenotypic heterogeneity is a key hallmark at the structural and functional level. This heterogeneity is considered crucial in the regulation of gene expression and such heritable alterations that do not affect the DNA sequence are referred to as epigenetic changes. The epigenetic pathways respond to various signaling cues such as DNA methylation, histone variants, histone modifications, chromatin structure, nucleosome remodeling, and epigenetic interactions (Allis and Jenuwein, 2016). DNA methylation is one of the major epigenetic modifications regulating gene expression by altering DNA conformation, chromosome structure and by recruiting other epigenetic regulators (Denis et al., 2011; Hervouet et al., 2018; Laisné et al., 2018). DNA methylation is catalyzed by three methyltransferases DNMT1, DNMT3a and DNMT3b. DNMT3a and DNMT3b catalyzes *de novo* methylation while DNMT1 is required for maintenance and is critical for the inheritance of DNA methylation (Moore et al., 2013; Greenberg and Bourc'his, 2019). Around 1.5% of the human genome is methylated and the majority of methylation occurs at CpG sites on gene promoters.

Aberrant hypermethylation at CpG sites is associated with diverse cancers (Robertson, 2001).

The selective incorporation of histone modifications at gene promoters and enhancers acts as a regulatory switch to determine the fate of gene expression. Trimethylation of histone H3 at lysine 4 (H3K4me3) is enriched at the active promoters whereas trimethylation of histone H3 lysine 27 (H3K27me3) and histone H3 lysine 9 (H3K9me3) are typically seen at inactive promoters. The enhancers are enriched with histone H3 lysine 4 monomethylated (H3K4me) and acetylated histone H3 lysine 27 (H3K27Ac) modifications. The histone methyltransferases (KMTs) and histone demethylases (KDMs) add and remove methyl groups to/from histones respectively. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) add and remove acetyl groups to/from histones, respectively (Hon et al., 2009; Hawkins et al., 2011; Albini et al., 2019). The incorporation of these modifications involves dynamic regulation and any deviation from the regulation is associated with developmental defects and diseases (Hon et al., 2009; Hawkins et al., 2011).

MicroRNAs (miRs) are small non-coding RNAs typically consisting of 20–21 nucleotides and are essential for various developmental processes and diseases. They regulate gene expression post-transcriptionally either by inhibiting translation or inducing mRNA degradation (Filipowicz et al., 2008). Another group of non-coding RNAs called the long non-coding RNAs (lncRNAs) with less protein coding potential (also referred as transcription noise) are 200 nucleotide long and have been shown to be essential for regulating transcription, translation, splicing, chromatin modification and structure (Jarroux et al., 2017; Kopp and Mendell, 2018).

Epigenetic Regulation of Wnt Signaling Components

Wnt signaling is a complex pathway modulated by various cellular and environmental cues to precisely regulate the myriad of cellular processes during development and disease. Wnt driven cancers have either hyperactivation of positive regulators or hypoactivation of negative regulators. In this section, we provide the details on the epigenetic modulations undergone by the components of the Wnt pathway. **Table 2** summarizes the epigenetic regulation of Wnt components discussed in the following section.

Ligands

Several tumor types are dependent on the hyperactivation of Wnt ligands because it provides an advantage to the tumor cells. Wnt secretion not only activates autocrine signaling but can also act on neighboring cells via paracrine signaling (Carbone et al., 2018; Eyre et al., 2019). Cancer cells seem to utilize multiple mechanisms to upregulate the expression of Wnt ligands. For example, Wnt2 expression can be upregulated in colorectal cancer via loss of repressive histone mark H3K27me3 by EZH2 (Jung et al., 2015). Wnt2 expression is also enhanced by downregulation of its regulatory miRNAs. In case of colorectal cancer, miR-548b overexpression can suppress cell proliferation by targeting Wnt2 (Xu et al., 2020). Several upstream regulators can also be targeted to achieve higher expression of the Wnt ligands. miR-600 targets SCD1 (stearoyl desaturase 1), an enzyme required for the lipid modification of the Wnts. Downregulation of miR-600 has been shown to promote the self-renewal of breast cancer stem cells whereas overexpression promotes their differentiation (El Helou et al., 2017). However, contrasting reports of promoter hypermethylation in case of multiple Wnt genes, including *WNT5A*, *WNT9A*, and *WNT10B*, suggest a potential tumor suppressive role (Yoshikawa et al., 2007; Ying et al., 2008; Galamb et al., 2016). The role of Wnt ligands in colorectal cancer has been reviewed in Nie et al. (2020). Promoter hypermethylation is also involved in silencing the Wnt negative-modulators, the DKK family, leading to hyperactivation of Wnt signaling in cancers (Nojima et al., 2007; Yu et al., 2009; Moskalev et al., 2012; Flanagan et al., 2017). Interestingly, *DKK1* promoter methylation has been reported in the later stages of cancer (Felipe de Sousa et al., 2011). Therefore, it might be assumed that it plays an important role in cancer progression instead of initiation. Promoter hypermethylation of *DKK3* has been reported in both colorectal cancer and breast cancer. However, its effect is not via suppression of LRP5/6 activity. Rather, it promotes membrane localization of β-catenin from the nucleus (Xiang et al., 2013). Promoter hypermethylation of *DKK2* has been reported but no correlation was observed with its expression (Silva et al., 2014). Apart from promoter methylation, *DKK1* promoter is also targeted by removal of activatory histone marks, such as H3Ac and H3K4me3 due to loss of p300 and recruitment of HDACs to its promoter, leading to reduced expression (Kim H.-Y. et al., 2015; Bao et al., 2017; Huang et al., 2013). DKK1 expression is also inhibited due to the upregulation of certain miRNAs, such as miR-375 and miR-376 (Taube et al., 2013; Cui et al., 2016). Silencing the Wnt antagonistic protein sFRPs by promoter hypermethylation resulting in the aberrant activation of Wnt signaling has been identified in diverse cancers with increased tumorigenic risk (Yu et al., 2019). Promoter hypermethylation of S*FRP1, 2, 4,* and *5* has been reported which correlates with the expression and cancer stage in case of breast cancer and colorectal cancer (Suzuki et al., 2002; Fujikane et al., 2010; Li et al., 2018). However, S*FRP3* does not harbor a CpG island in the promoter region (Suzuki et al., 2002). sFRP1 is also downregulated via multiple miRNAs in breast cancer (Yang et al., 2015). Downregulation of sFRP1 by hypermethylation has been shown to be essential for self-renewal of colorectal cancer stem cells (Li et al., 2018). S*FRP2* promoter hypermethylation occurs in HBV-associated hepatocellular carcinoma and breast cancer (Veeck et al., 2008; Yu et al., 2019; Xiang et al., 2021). Downregulation of WIF1 occurs by promoter hypermethylation in colorectal cancer, breast cancer, ovarian cancer and several other cancer types (Ai et al., 2006; Paluszczak et al., 2015; Hu et al., 2018). miR-603 is known to target WIF1 in glioma (Guo et al., 2014).

Receptors

Almost all members of the Wnt receptor family, such as FZD4, 6, 7, 8, and 10 have been reported to be regulated by different miRNAs in multiple cancer types including colorectal cancer, prostate cancer, breast cancer (Gong et al., 2014; Kim B.-K. et al., 2015; Jiang et al., 2016; Song et al., 2017; Li Z.-T. et al., 2020). In a few cases, upregulation of miRNAs regulating the negative

TABLE 2 | Epigenetic mechanisms regulating the Wnt components in different types of cancer.

Wnt component	Epigenetic alteration	Cancer type	Outcome	References
Wnt2	'Loss of H3K27me3	CRC	Upregulation	Jung et al., 2015
	miR-548b downregulation	CRC	Upregulation	Xu et al., 2020
Wnt5a	Promoter hypermethylation	CRC	Downregulation	Ying et al., 2008
Wnt9a	Promoter hypermethylation	CRC	Downregulation	Galamb et al., 2016
Wnt10b	Promoter hypermethylation	CRC	Downregulation	Yoshikawa et al., 2007
DKK1	Promoter hypermethylation	CRC, CLL, BC	Downregulation	Nojima et al., 2007; Yu et al., 2009; Felipe de Sousa et al., 2011; Moskalev et al., 2012; Flanagan et al., 2017
	loss of H3Ac	Glioma, BC	Downregulation	Kim H.-Y. et al., 2015
	loss of H3K4me3	BC	Downregulation	Huang et al., 2013
	miR-375 upregulation	BC	Downregulation	Taube et al., 2013
	miR-376 upregulation	BC	Downregulation	Cui et al., 2016
DKK3	Promoter hypermethylation	CRC, BC	Downregulation	Xiang et al., 2013
sFRP1	Promoter hypermethylation	CRC	Downregulation	Suzuki et al., 2002; Fujikane et al., 2010; Li et al., 2018
	miR-139-5p and miR-9 upregulation	BC	Downregulation	Yang et al., 2015
sFRP2	Promoter hypermethylation	CRC, HCC, BC	Downregulation	Suzuki et al., 2002; Veeck et al., 2008; Yu et al., 2019; Xiang et al., 2021
sFRP4	Promoter hypermethylation	CRC	Downregulation	Suzuki et al., 2002
sFRP5	Promoter hypermethylation	CRC	Downregulation	Suzuki et al., 2002
WIF1	Promoter hypermethylation	CRC, BC, OC	Downregulation	Ai et al., 2006; Paluszczak et al., 2015
	miR-603 downregulation	Glioma	Downregulation	Hu et al., 2018 Guo et al., 2014
Fzd4	miR-505 downregulation	CC	Upregulation	Ma et al., 2017
	loss of H3K27me3	GC	Upregulation	Li Z.-T. et al., 2020
Fzd6	miR-199a-5p downregulation	CRC	Upregulation	Kim B.-K. et al., 2015
	miR-130-30p downregulation	BC	Upregulation	Poodineh et al., 2020
Fzd7	miR-613 downregulation	PC	Upregulation	Song et al., 2017
Fzd8	miR-100 downregulation	BC	Upregulation	Jiang et al., 2016
Fzd10	Gain of H3K9Ac	BC	Upregulation	Gong et al., 2014
LRP6	miR-34 downregulation	CRC, BC	Upregulation	Kim et al., 2011
	miR-130-30p downregulation	BC	Upregulation	Poodineh et al., 2020
	miR-487b downregulation	CRC	Upregulation	Hata et al., 2017
	miR-181 upregulation	OC	Upregulation	Ruan et al., 2020
LGR5	Promoter hypermethylation	CRC	Downregulation	Su et al., 2015
	miR-363 downregulation	CRC	Upregulation	Tsuji et al., 2014
	miR-34 downregulation	CRC	Upregulation	Hahn et al., 2013
APC	Promoter hypermethylation	CRC	Downregulation	Su et al., 1992, 1993; Arnold et al., 2004
	miR-135 upregulation	CRC	Downregulation	Segditsas et al., 2008; Liang et al., 2017
	miR-142 upregulation	BC	Downregulation	Nagel et al., 2008; Isobe et al., 2014
AXIN2	Promoter hypermethylation	CRC	Downregulation	Koinuma et al., 2006
	miR-34 upregulation	CRC	Downregulation	Kim et al., 2013
	miR-103/107 upregulation	CRC	Downregulation	Kim et al., 2013; Chen H.-Y. et al., 2019
GSK3-β	miR-224 upregulation	CRC	Downregulation	Li T. et al., 2016
	miR-1229 upregulation	BC	Downregulation	Tan et al., 2016
βTrCP	miR-182 upregulation	CRC	Downregulation	Wang S. et al., 2016
	miR-135b upregulation	OS	Downregulation	Jin et al., 2017
CK1α	miR-155 upregulation	CRC, BC	Downregulation	Zhang et al., 2012
	miR 135b upregulation	OS	Downregulation	Jin et al., 2017
YAP1	miR-506 downregulation	BC	Upregulation	Hua et al., 2015
	miR-506 upregulation	CRC	Downregulation	Krawczyk et al., 2017

(Continued)

TABLE 2 | Continued

Wnt component	Epigenetic alteration	Cancer type	Outcome	References
	miR-1224/CREB promoter	HCC	Downregulation	Yang et al., 2021
	hypermethylation	BC	Downregulation	Real et al., 2018
TAZ1	LINC00174 upregulation	CRC	Upregulation	Shen et al., 2018
	miR-125a downregulation	CRC	Upregulation	Yang M. et al., 2019
LATS1/2	Promoter hypermethylation	CRC, HNSCC, BC, RCC	Downregulation	Okami et al., 2005; Steinmann et al., 2009; Chen et al., 2013; Wierzbicki et al., 2013; Chen K.-H. et al., 2014
MST1/2	Promoter hypermethylation	HNSCC, Mesothelioma	Downregulation	Steinmann et al., 2009; Maille et al., 2019
β-catenin	miR-200a downregulation	CRC	Upregulation	Tian et al., 2014
	miR-141 downregulation	BC	Upregulation	Abedi et al., 2015
	miR-340 downregulation	BC	Upregulation	Abedi et al., 2015
	miR-520f-3p downregulation	GC	Upregulation	Chen et al., 2020
TCFL1/2	Promoter hypomethylation	CRC	Upregulation	Guo et al., 2015
TCF7	miR-29 downregulation	CRC	Upregulation	Subramanian et al., 2014
LEF1 (transcript from P2)	miR-34	BC	Upregulation	Kim et al., 2011
	gain of H3K9me3	BC	Downregulation	Yokoyama et al., 2010
TCF7	LOC728196 upregulation	Glioma	Upregulation	Wang O. et al., 2018

regulators of FZD proteins can also contribute toward the severity of cancer. For example, miR-106b is upregulated in breast cancer which suppresses BRMS1L. BRMS1L is responsible for regulating the expression of FZD10. It recruits HDAC1 to *FZD10* which leads to promoter H3K9Ac (Gong et al., 2014). miR-130-30p, which is downregulated in triple negative breast cancer, acts as a Wnt antagonist by targeting multiple Wnt components, including FZD6 and LRP6 (Poodineh et al., 2020). Another lncRNA, GATA4-AS1 inhibits Wnt signaling in gastric cancer by reducing FZD4 expression. It recruits EZH2 and increases H3K27me3 occupancy at the promoter region (Ma et al., 2017; Li Z.-T. et al., 2020). LRP6 is also under the regulation of multiple miRNAs which are downregulated in colorectal cancer and breast cancer (Kim et al., 2011; Hata et al., 2017; Poodineh et al., 2020). MEST (mesoderm specific transcript) is a known regulator of LRP6 (Ruan et al., 2020). It blocks LRP6 maturation via glycosylation. Inhibition of MEST, therefore, enhances Wnt signaling (Ruan et al., 2020). MEST itself is targeted by miR-181 in ovarian cancer cells thereby promoting Wnt signaling. However, in another study, MEST was shown to be a positive regulator of the Wnt pathway. It was found to be under the regulation of zinc finger protein ZFP57, a transcription factor involved in maintaining DNA methylation in ESCs via its interaction with DNMTs. Promoter hypermethylation at *MEST* locus by ZFP57 was shown to be lost in breast cancer which led to enhanced Wnt signaling (Chen L. et al., 2019). Upregulation of the upstream positive regulators for LGR5 have been reported to serve toward cancer progression. For instance, loss of miR-363 promotes GATA6 expression and loss of miR-34 promotes ZNF281 expression, both of which are required for the expression of LGR5 in colorectal cancer (Hahn et al., 2013; Tsuji et al., 2014). However, promoter hypermethylation in case of LGR5 suggests a tumor suppressive role instead. Interestingly, the degree of methylation correlated with the grade of tumor (Schuebel et al., 2005; Felipe de Sousa et al., 2011; Su et al., 2015).

Destruction Complex Components

Adenomatous polyposis coli is the most frequently mutated gene in Wnt driven cancer cases, especially in the case of familial adenomatous polyposis. APC is also downregulated due to promoter hypermethylation, which can occur with or without mutations in the gene itself (Su et al., 1992, 1993; Arnold et al., 2004; Segditsas et al., 2008; Liang et al., 2017). It is also regulated by multiple miR-RNAs, such as miR-135 in colorectal cancer and miR-142 in breast cancer (Nagel et al., 2008; Isobe et al., 2014). miR-135b suppresses APC and is itself part of a positive feedback loop with TAZ (Shen S. et al., 2017). AXIN2 is often induced after Wnt activation and is a part of a negative feedback loop. It limits the duration of Wnt signaling. It is primarily found to be expressed in colon cells (Lustig et al., 2002). AXIN2 repression helps in prolonging the duration of Wnt signaling by blocking the negative feedback loop as well as increasing the duration of Wnt target genes expression (Lustig et al., 2002). Therefore, *AXIN2* is frequently targeted in colorectal cancer by promoter hypermethylation (Koinuma et al., 2006). Interestingly, AXIN2 is also downregulated by miR-34 in colorectal cancer which should inhibit Wnt signaling but miR-34 targets a number of Wnt activators as well, thus limiting the action of AXIN2 (Kim et al., 2013). Recently, miR-103/107 were also shown to promote colorectal cancer stemness by targeting AXIN2. miR-103/107 also promotes colorectal cancer by targeting LATS2. AXIN2 overexpression also led to nuclear accumulation of GSK3-β which would ultimately promote Wnt signaling (Kim et al., 2013; Chen H.-Y. et al., 2019). As expected, GSK3-β, βTrCP and CK1α, are downregulated in Wnt driven cancers, thereby promoting β-catenin stabilization. **Table 2** summarizes the miRNAs reported to be responsible for negative regulation of these proteins in multiple cancers (Zhang et al., 2012; Li T. et al., 2016; Tan et al., 2016; Wang S. et al., 2016; Jin et al., 2017). DVL1, 2, and 3 are under the positive regulation by SIRT1 (a member of histone deacetylase family) in case of breast cancer (Holloway et al., 2010;

Simmons et al., 2014). DACT (Disheveled Binding Antagonist of β-catenin) family of proteins are known negative regulators of Wnt signaling. DACT3 undergoes transcriptional repression due to the deposition of bivalent histone modifications H3K27me3 (repressive) and H3K4me3 (activatory) (Jiang et al., 2008).

Due to the dual role of YAP/TAZ in regulating the Wnt signaling pathway, there have been contrasting reports showing both tumor suppressive as well as oncogenic roles of YAP and TAZ. For example, miR-506, which targets YAP, is downregulated in colorectal cancer but upregulated in breast cancer (Hua et al., 2015; Krawczyk et al., 2017). The *YAP* promoter also shows aberrant hypermethylation in breast cancer (Real et al., 2018). However, the majority of studies point toward an oncogenic role of YAP and TAZ. Some of these have been listed in **Table 2** (Shen et al., 2018; Yang M. et al., 2019; Yang et al., 2021). Hippo kinases and their upstream regulators are not a part of the Wnt signaling but it is important to mention them since these regulate the nuclear levels of YAP/TAZ and as a result, β-catenin. LATS has been reported as a tumor suppressor in colorectal cancer, head and neck squamous cell carcinoma, breast cancer and renal cell carcinoma. The promoter of *LATS1/2* undergoes hypermethylation (Okami et al., 2005; Steinmann et al., 2009; Chen et al., 2013; Chen K.-H. et al., 2014; Wierzbicki et al., 2013). Several miRNAs have also been reported to control the levels of LATS1/2 (Lee et al., 2009). Promoter hypermethylation has also been reported for *MST1/2* (Steinmann et al., 2009; Maille et al., 2019). MORC2 (microrchidia) acts as a negative regulator of Hippo signaling. MORC proteins are epigenetic readers as well as chromatin remodelers which help in DNA methylation (Pastor et al., 2014; Li S. et al., 2016). It forms a complex with DNMT3A and causes hypermethylation at the promoter regions of *NF2* and *KIBRA* which are upstream regulators of the Hippo pathway. Depletion of NF2 and KIBRA in turn increases the nuclear levels of YAP/TAZ and promotes stemness and oncogenicity in hepatocellular carcinoma (Wang T. et al., 2018).

Transcriptional Regulators

Promoter hypermethylation at *CTNNB1* gene is not observed frequently in colorectal cancer (Galamb et al., 2016). miR-34, which itself is a target of p53, regulates β-catenin. Loss of p53 in colorectal cancer and breast cancer in turn leads to upregulation of β-catenin (Kim et al., 2011). Some miRNAs responsible for regulating β-catenin levels are mentioned in **Table 2** (Tian et al., 2014; Abedi et al., 2015). Interestingly, miR-520f-3p attenuates nuclear localization of β-catenin by targeting degradation of SOX9 mRNA in gastric cancer (Chen et al., 2020). JMJD1A, a histone demethylase, is responsible for upregulation of β-catenin expression in colorectal cancer. JMJD1A can also directly interact with β-catenin leading to its transactivation and upregulation of expression of multiple Wnt target genes, such as *MYC*, *CCND1* and *MMP9* (Peng et al., 2018). *TCF7L1* and *TCF7L2* promoters have hypomethylation in colorectal cancer (Guo et al., 2015). Several miRNAs regulating various members of the TCF/LEF family are downregulated in cancers (**Table 2**; Kim et al., 2011; Subramanian et al., 2014; Wang O. et al., 2018). Interestingly, the LEF1 gene has two promoters, P1 and P2. P1 is under the regulation of Wnt signaling, therefore upregulation of Wnt leads to increased expression of LEF1. Transcription from P2, on the other hand, produces a dominant negative form of LEF1. Transcription from P2 promoter is lost in case of breast cancer due to the repressive mark H3K9me3 mediated by YY1 (Yokoyama et al., 2010). Chromatin modification-induced alterations in Groucho expression are not frequent. However, downregulation of Groucho has been reported in colorectal cancer via the action of HDAC3 (Godman et al., 2008).

Epigenetic Regulation Downstream of Wnt Signaling

Recent advances in cancer biology have identified the role of epigenetic regulation in cancers and numerous studies have identified mutations in the genes encoding both, the components and the regulators of Wnt signaling to aberrantly activate the Wnt signaling pathway in various cancers. In this section, we will elaborate upon the epigenetic modulations introduced by the Wnt pathway that promote cancer initiation as well as progression.

DNA Methylation

Multiple pathways and molecular players influence the outcome of the Wnt pathway. A study by Song et al. (2015) has shown that β-catenin interacts with DNMT1 and their interaction is required for mutual stabilization. Further, β-catenin complex with DNMT1 is a key requirement for DNMT1 dependent promoter methylation and Wnt/β-catenin signaling dependent target gene expression (Song et al., 2015). In another study, tumor suppressor- Na^+/H^+ exchanger regulatory factor 1 (NHERF1/EBP50), an adaptor molecule known to suppress Wnt signaling has been observed to be downregulated in colon cancer cells and is associated with decreased survival and increased intestinal tumor burden (Georgescu et al., 2016). Promoter methylation by DNMT1 has been implicated in reducing the expression of NHERF1/EBP50. Furthermore, Wnt signaling dependent upregulation of DNMT1 has been shown to be required to trigger hypermethylation of *NHERF1* promoter in colon cancer (Guo et al., 2018). Mechanistically, whether β-catenin recruits DNMT1 differentially on tumor suppressor genes and oncogenes, and what other molecular partners may be required to decide DNA methylation dependent and independent roles has not been fully explored. A recent study adds another layer of complexity by the Wnt signaling via crosstalk with various chromatin modifiers. The study shows that EZH2 dependent protein stability of LSD1, HDAC1, DNMT1, β-catenin, or SMAD2/4, via recruitment of deubiquitinase USP7, is key in suppressing neuronal genes and sustaining the expression of Wnt and TGFβ target genes in cancer cells (Lei et al., 2019). Understanding the role of β-catenin in DNMT1 regulation and recruitment would be crucial in developing future therapeutics.

Histone Modifications

The Wnt pathway employs diverse factors to regulate target gene expression and converges with epigenetic signaling at Wnt response elements through recruitment of epigenetic modulators. Upon Wnt stimulation, β-catenin translocates

to the nucleus and interacts with the TCF family proteins by replacing co-repressors with subsequent recruitment of CBP/p300 HATs, thereby promoting H3 and H4 lysine acetylation. This reverses the HDAC-dependent chromatin compaction and induces molecular events to promote Wnt target gene expression (Parker et al., 2008). Although this study has emphasized on the role of β-catenin as an transcriptional activator, dependent on the recruitment of CBP/p300 HATs, it has been demonstrated that CBP/p300 can act as a bimodal regulator, regulating TCF/β-catenin interaction and also facilitating transactivation of β-catenin (Li et al., 2007). The ability of β-catenin to recruit multiple factors on promoters and the regulatory role of Wnt/β-catenin signaling in determining heterogeneity at the cellular level suggest that β-catenin could facilitate a poised chromatin state during developmental programs. Furthermore, information on how β-catenin regulated gene loci are selected as poised chromatin loci during the unfolding of developmental programs is still incomplete. Blythe et al. (2010) found that in *Xenopus,* prior to the midblastula transition during embryogenesis, Wnt/β-catenin signaling uncouples the activation of dorsal specific genes by establishing a poised chromatin state through recruitment of arginine methyltransferase PRMT2 at target gene loci. The PRMT2 catalyzes histone H3 arginine 8 (H3R8me2) asymmetric methylation. The advantage of such a poised nature of chromatin is to enable synchronous and rapid activation of tissue specific genes (Blythe et al., 2010). Another study showed that the C-terminal transactivation of β-catenin domain interacts with TRIPP/TIP60 and mixed-lineage leukemia (MLLI/MLL2) SET1-chromatin modifying complexes and recruits H3K4me3 at the promoters of target genes (Sierra et al., 2006; Zhu et al., 2019). These studies provide a mechanistic clue that during embryogenesis, pattern and combinations of these modifications could serve as critical molecular cues deciding tissue specific gene expression. Also, PRMT2-dependent poised state during early developmental stages could subsequently be followed by MLL1/MML2 driven gene activation in later stages. A recent study has shown that the dual pattern of histone modifications dependent on Spindlin1, a multivalent epigenetic reader, which potentiates Wnt/β-catenin signaling by recognizing the dual histone modification pattern 'H3K4me3-H3R8me2' (Su et al., 2014). Recently, Spindlin1 has been shown to drive the growth of colorectal carcinoma which have high levels of β-catenin. Spindlin1 is expressed in $Lgr5^{+}$ intestinal stem cells and is required for self-renewal. It also promotes cancer stemness in a β-catenin-dependent manner (Su et al., 2014; Grinat et al., 2020). Previously, a report had shown that the MLL1/CBP/β-catenin complex promotes tumor initiation and metastasis in the head and neck squamous cell carcinoma by increasing the activation associated mark H3K4me3 at the promoters of target genes (Qiang et al., 2016). Interestingly, SETD7, a histone lysine methyltransferase, has been shown to be the part of the destruction complex itself. SETD7 mediated methylation of YAP aids in Wnt induced nuclear localization of β-catenin and thus is essential for intestinal regeneration and also plays a role in promoting tumorigenesis (Oudhoff et al., 2016). The studies demonstrating crosstalk of β-catenin transactivation with the epigenetic code provides new avenues to be tested in future for therapeutic intervention.

Wnt signaling and burst of chromatin reorganization

The genetic information on DNA is organized into the structural and regulatory framework of chromatin thereby allowing different machineries and signaling networks to switch off or switch on the gene expression. The major component of eukaryotic chromatin is the nucleosome comprising of 146 bp DNA wrapped around an octamer of core histones H2A, H2B, H3, and H4. The different machineries of transcription gain access to this complex structural framework by posttranslational modification of histones (Shilatifard, 2006; Berger, 2007; Fu et al., 2016). Depending upon the type of modifications and recruitment of modifiers, mediators and suppressors, gene activation or silencing is promoted (Kouzarides, 2007).

Wnt signaling drives molecular changes through the nuclear function of a prominent member of the ARM repeats containing protein, β-catenin. The central ARM repeats bound to TCF are critical for its function and their deletion completely abrogates β-catenin function (Xing et al., 2008). In the absence of nuclear β-catenin, TCF is bound to the co-repressor complex to stimulate compression of chromatin and suppress Wnt target gene expression (Cavallo et al., 1998; Brantjes et al., 2001; Courey and Jia, 2001). The N-terminal and C-terminal regions of β-catenin are transactivation domains known to interact with multiple cofactors to regulate Wnt target gene expression. The C-terminal domain (CTD) of β-catenin interacts with histone acetyltransferases (CBP or p300) resulting in chromatin modifications promoting the activation of Wnt target genes (Hecht, 2000; Takemaru and Moon, 2000; Courey and Jia, 2001). Upon Wnt stimulation, in CBP-dependent manner, nucleosomes are acetylated up to 30 kilobases with enhanced rate and saturated within 5.5 h, inducing a widespread wave of chromatin acetylation and re-organization (**Figure 4A**; Parker et al., 2008). The widespread use of acetylation and rapid activation of target genes is suggested to be dependent on the nature of TCF binding. TCF belongs to the high mobility group of proteins which are known to induce strong DNA bending upon binding. TCF induced bending facilitates the architectural framework for large chromatin regions to be organized together to stimulate a burst of chromatin reorganization dependent on β-catenin bound HAT (CBP) activity (Heintzman et al., 2007; Parker et al., 2008). This architectural framework of chromatin by the TCF/β-catenin/HAT complex provides a robust mechanism for rapid activation of Wnt target genes. The β-catenin CTD acts as a scaffold to recruit multiple diverse factors, such as, the SWI/SNF factors BRG1 and ISWI, HMTs, the Mediator component MED12, and the polymerase-associated factor 1 PAF1 (Waltzer et al., 2001; Kim et al., 2006; Mosimann et al., 2006; Sierra et al., 2006). These factors rearrange histone positions, modify the histones post-translationally and orient RNA Pol II, thereby providing the sequential exchange platform for remodeling chromatin architecture to induce rapid gene expression (**Figure 4C**; Mosimann et al., 2009). β-catenin has been shown to pre-pattern chromatin signatures during differentiation in an OCT4-dependent manner (Ying et al., 2015).

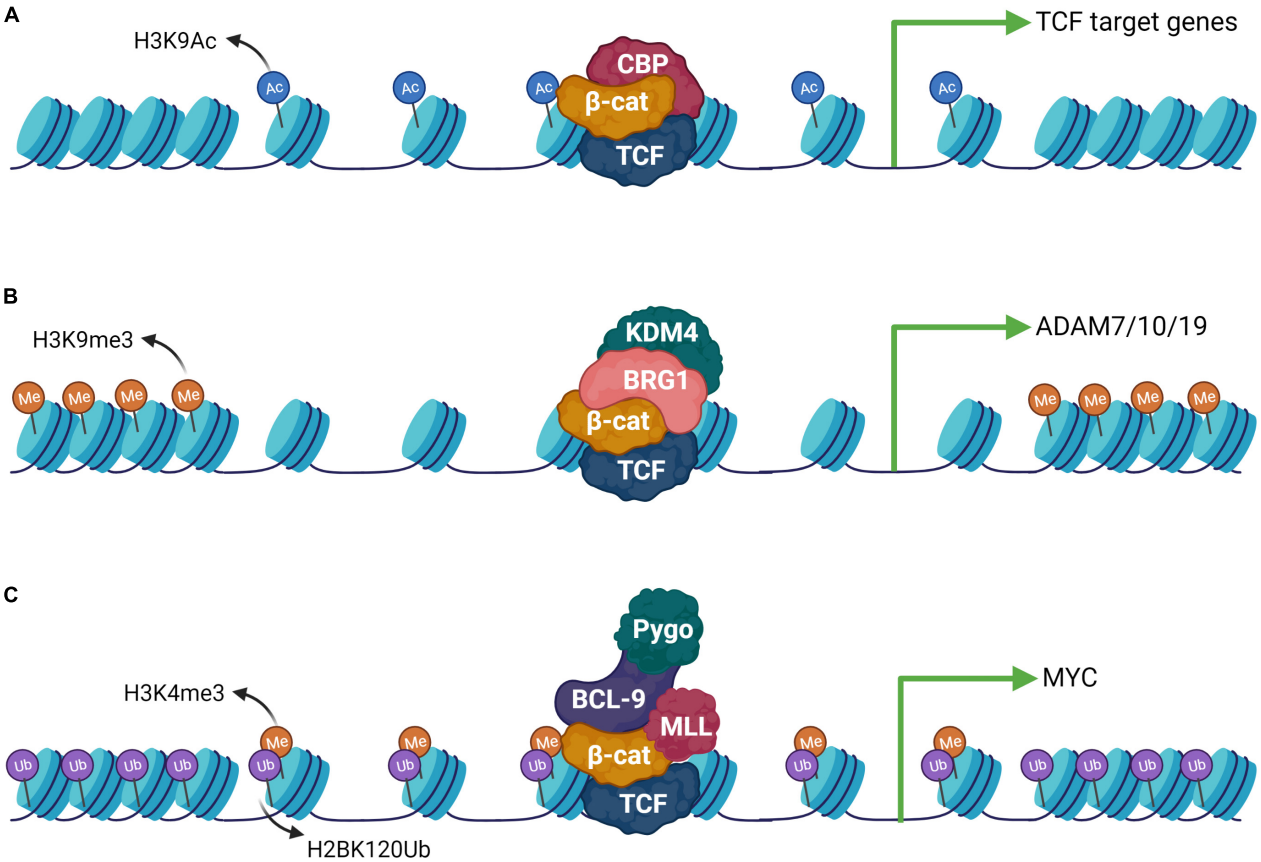

FIGURE 4 | Epigenetic machinery downstream to the Wnt signaling pathway. **(A)** β-catenin can form complexes with different epigenetic modulators and activate gene expression upon Wnt activation. CBP is a well-known interaction partner of β-catenin which helps in opening up of the chromatin by catalyzing H3K9 acetylation. **(B)** Histone demethylase KDM4 is recruited via BRG1, an interactor of β-catenin which stimulates the expression of the ADAM group of genes. **(C)** β-catenin can also form an elaborate complex with the chromatin remodelers composed of the MLL complex, Pygo and Bcl-9 leading to H3K4me3 modification. This activity requires prior ubiquitination of H2BK120.

Wnt signaling driven β-catenin acts as a molecular switch for the chromatin-associated high mobility group protein (HMG-17) to de-repress the inhibitory complex HMG-17/PITX2. The inhibitory complex HMG-17/PITX2 binds to specific chromatin regions primed for transcriptional activation and β-catenin forms a ternary complex with HMG-17/PITX2, thereby switching the inhibitory complex, modulating chromatin structure and inducing spatiotemporal expression of genes during embryogenesis (Amen et al., 2008; Ying et al., 2015). The histone methyltransferase and demethylase work in concert to stringently regulate gene expression. Protein lysine demethylases (KDMs) modify chromatin by demethylation of histones, especially repressive mark histone H3 lysine 9 trimethylation (Shi, 2007). The transactivation potential of β-catenin is very crucial for its nuclear function to target gene expression. Several studies have shown that β-catenin interacts with KDMs to epigenetically modulate target gene expression. For instance, β-catenin has been shown to interact with KDM3 on Wnt target genes inducing histone H3 lysine 9 (H3K9me2) demethylations. The β-catenin/KDM3 complex further drives MLL1-dependent H3K4 methylation to promote recruitment of BCL9 and Pygo2 to chromatin, thus promoting Wnt/β-catenin-dependent target gene expression (Li et al., 2019). In another study, KDM4 has been shown to physically interact with β-catenin and demethylated H3K9me3 on the promoters of Wnt target genes to promote target gene expression (**Figure 4B**; Peng et al., 2019). The complex nature of the cellular context requires multiple factors to work in concert for precise activation of target genes. For example, β-catenin recruits BRG1-dependent KDM4 isoforms on the promoters of Disintegrin and Metalloproteinase (ADAM) to regulate their expression in colorectal cancer cells (Sun et al., 2020). Thus, these findings establish that β-catenin recruits KDM to remove repressor marks and facilitate concomitant recruitment of activator complexes. However, the context-dependent and temporal nature of these β-catenin driven modulations require further investigation. The Wnt signaling-dependent activation of β-catenin and nuclear interaction with several transcription factors and chromatin acts as a molecular switch to regulate global gene activation. For example, β-catenin interacts with the chromatin organizer special AT-rich binding protein 1 (SATB1) and mediates the expression of specific set of genes during T helper-cell differentiation (Notani et al., 2010)

and colorectal cancer progression (Mir et al., 2016), presumably via alterations in chromatin domain organization (Galande et al., 2007). The precise spatiotemporal activation of gene expression during development is a critical event dependent on chromatin organization and Wnt signaling directs the chromatin modifying machinery to establish a poised state at promoters (Shahbazian and Grunstein, 2007; Blythe et al., 2010). Wnt signaling has been shown to regulate differential developmental outcomes; however, it is not clear which mechanism governs this. Recently, Esmaeili et al. (2020) established that loss of chromatin architecture at Wnt target gene promoters is responsible for differential outcome of β-catenin bound chromatin without changing the inductive signal. The studies so far have established that Wnt signaling driven β-catenin modulates multiple aspects of gene regulation by acting as a signaling switch to regulate diverse cellular processes during development and tissue homeostasis.

Non-coding RNAs

Wnt signaling and microRNAs play essential roles in embryonic development and tissue homeostasis. MicroRNAs are involved in tumorigenic pathways. They play differential roles either regulating oncogenes or tumor suppressors to promote cancer progression (Xu and Mo, 2012). A recent study by Roman et al., has shown in cell-based experiments that microRNAs can modulate Wnt signaling by either regulating positive regulators or negative regulators of the Wnt pathway (Anton et al., 2011). Aberrant activation of Wnt signaling in various cancers regulate a wide range of gene expression at transcript level including microRNAs (**Table 3**). Oncogenic miRs-miR-182, miR30e, miR122, and miR21 are directly regulated by Wnt signaling in tumorigenesis (Liao and Lönnerdal, 2010; Lan et al., 2012; Chiang et al., 2013). In another study, miR215 and miR137 are repressed and miR708, miR135b, and miR3 are upregulated in *APC* mutant tumors (Necela et al., 2011). In gastric cancers, the expression of miR4739, miR210, miR135-5P, and miR12334-3P are significantly increased and the expression of miR20a-3p, miR-23b-5p, miR335-3p, miR423-5p, and miR455-3p is significantly reduced upon knockdown of β-catenin (Wang et al., 2009; Dong et al., 2015). In hepatocellular carcinoma, Wnt/β-catenin signaling upregulates miR770, miR183, miR96, and miR182 (Leung et al., 2015; Wu et al., 2016) and downregulates miR375 (Ladeiro et al., 2008). In breast cancer, miR182 and miR125b are upregulated by Wnt signaling and miR Let-7 is downregulated (Cai et al., 2013; Chiang et al., 2013; Liu et al., 2013). Aberrant activation of Wnt/β-catenin and microRNAs also involve autoregulatory feedback loop in cancers, for example, miR218 expression is induced by Wnt signaling and in turn, it augments the Wnt signaling pathway by targeting Wnt signaling inhibitors (SOST, DKK2, and sFRP2) (Hassan et al., 2012). Similarly, miR146a expression is induced by Wnt signaling in CRC, and in turn, it hyperactivates Wnt signaling by reducing the expression of Numb (Hwang et al., 2014). In summary, crosstalk

TABLE 3 | miRNAs and lncRNAs regulated by Wnt pathway in various cancer types.

miRNAs			
Cancer type	**Upregulated**	**Downregulated**	**References**
CRC	miR-708, miR-31, miR-135b, miR-21, miR-182, miR574-3p, miR-30e	miR-215, miR-137	Liao and Lönnerdal, 2010; Lan et al., 2012; Chiang et al., 2013; Necela et al., 2011
GC	miR-20a-3p, miR-23b-5p, miR-335-3p, miR-423-5p, miR-455-3p	miR-1234-3p, miR-135b-5p, miR-210, miR4739, miR-122a	Wang et al., 2009; Dong et al., 2015
HCC	miR-770, miR-183, miR-96, miR-182	miR-375	Ladeiro et al., 2008; Leung et al., 2015; Wu et al., 2016
BC	miR-182, miR-125b	Let-7	Cai et al., 2013; Chiang et al., 2013; Liu et al., 2013
lncRNAs			
Cancer Type	**lncRNAs**	**Outcome**	**References**
CRC	KIAA0125	Downregulated	Yang Y. et al., 2019
CC	CALML3-AS1	Upregulated	Liu et al., 2019
BC	LUCAT1	Upregulated	Zheng et al., 2019
LC	LINC00673-v4	Upregulated	Lin et al., 2020
LC	AK126698	Downregulated	Fu et al., 2016
Pituitary adenoma	CLRN1-AS1	Downregulated	Wang et al., 2019
Glioma	SNHG17, MIR22HG, HOXC13-AS, LSINCT5, H19, BLACAT1, SNHG5, LINC01503, AGAP2-AS1, OIP5-AS1, DANCR, SNHG7, NEAT1, AB073614, MIR155HG, CCND2-AS1, CCAT2 and MALAT1	Upregulated	Han et al., 2020
Glioma	CASC7, Linc00320, TUNAR, MEG3, CASC2 and PTCSC3	Downregulated	Han et al., 2020
Colon cancer	CCAT2	Upregulated	Ling et al., 2013
LC	LINC00673-v4	Upregulated	Guan et al., 2019
Liver cancer	TCF7	Upregulated	Wang et al., 2015

CRC, colorectal cancer; CLL, chronic lymphocytic leukemia; BC, breast cancer; HCC, hepatocellular carcinoma; OC, ovarian cancer; CC, cervical cancer; GC, gastric cancer; HNSCC, head and neck squamous cell carcinoma; RCC, renal cell carcinoma; LC, lung cancer; PC, pancreatic cancer; OS, osteosarcoma.

between Wnt/β-catenin and miRs is crucial for the development of cancers and further understanding of this intricate signaling paradigm will provide insights for future therapeutics.

Numerous studies have shed light on the Wnt signaling-dependent role of lncRNAs in diverse cancers (**Table 3**) such as colorectal cancer (Yang Y. et al., 2019), cervical cancer (Liu et al., 2019; Yang Y. et al., 2019), breast cancer (Zheng et al., 2019), non-small cell lung cancer (Lin et al., 2020), and brain (Wang et al., 2019). Multiple mechanisms are operational via long non-coding RNAs to differentially regulate Wnt signaling. For instance, a gene desert located upstream of the *MYC* gene is known to express Wnt related lncRNAs such as CCAT1-L, CCAT1-S, CCAT2, and CASC11 (Shen P. et al., 2017). CCAT2 (Colon cancer associated transcript 2) is known to enhance Wnt/β-catenin signaling activity by binding to TCF and increasing its transcriptional activity subsequently activating the cascade of downstream gene expression crucial for cancer progression and invasion (Ling et al., 2013; Shen P. et al., 2017). lncRNA LINC00673-v4 enhances TCF/LEF activity and increases the binding of DEAD-box helicase 3 X-linked (DDX3) and casein kinase 1 (CK1), thereby potentiating Wnt signaling (Guan et al., 2019). Apart from acting as transcription factors, lncRNAs are also shown to interact with chromatin modifying enzymes to promote spatiotemporal expression of target genes (Marchese and Huarte, 2014; Guan et al., 2019). For instance, the lncRNA 34a regulates miR-34a by recruiting repressor epigenetic machinery DNMT3a/PHB2 and HDAC1 at the promoter of miR-34a, thereby inducing DNA methylation and histone deacetylation to repress its expression (Wang L. et al., 2016). miR-34a is a key component in the Wnt signaling pathway and it regulates TCF7 expression (Chen W.-Y. et al., 2015).

In summary, lncRNAs act as regulatory switches to modulate the signaling outcome by targeting various crucial components of the pathway. The lncRNA-Wnt signaling module employs several mechanisms to regulate the expression of Wnt target genes. LncRNAs provide signaling scaffolds and act as transcription factors, chromatin co-modifiers and mediators. The significant involvement of lncRNAs in Wnt signaling driven cancers makes them attractive targets for drug discovery and cancer therapy.

THERAPEUTICS

Epigenetic regulation plays a dynamic role in Wnt signaling driven cancers. Epigenetic alterations are reversible by using pharmacological inhibitors. Multiple approaches have been employed for epigenetic therapy in cancers. These include DNA methylating inhibitors, histone methyltransferase inhibitors and HDAC inhibitors. To reverse aberrant DNA methylation acquired in cancers, DNMT inhibitors azacitidine and decitabine were developed and approved for acute myeloid leukemia (AML) (Derissen et al., 2013). Aberrant methylation of histone tails is linked with gene silencing in cancers. Several methyltransferases such as EZH2, SETD2 and Dot1L have been implicated in cancers (Albert and Helin, 2010; Hamamoto and Nakamura, 2016). Currently, tazemetostat is the most advanced therapeutic drug against EZH2 whereas valemetostat, CPI-1205, and CPI-0209 are in clinical phase 2 trials (Gulati et al., 2018). Inhibition of EZH2 reverses the silencing of genes induced by EZH2 hyperactivation. Another tested mode of epigenetic therapy is the reversal of histone deacetylation promoted by HDACs. Four HDAC inhibitors- vorinostat, romidepsin, belinostat, and panobinostat have been approved by the FDA (Bates, 2020). Inhibition of HDACs causes concomitant increase in HAT activity resulting in global increase in acetylation, subsequently relaxing chromatin and inducing transcription of genes responsible for promoting cell death (Zhao et al., 2020). The mechanism of HDAC inhibitors inducing cell death is not clearly understood and needs to be addressed in future to have a deeper understanding of their mechanism of action for therapeutic use. The epigenetic therapies have been shown to reduce cell proliferation, induce cell death in hematological malignancies (Altucci and Minucci, 2009), but they have not been seen to be effective in solid tumors (Graham et al., 2009). The HDAC inhibitors are mostly non-isoform selective and with recent advances in drug discovery, isoform specific and dual capability HDAC inhibitors have been extensively developed for cancer treatment (Peng et al., 2020). Current knowledge and complexity of how HDAC inhibitors work in the cellular context and combination therapies targeting the epigenome could be useful for designing future therapeutic interventions.

Targeting the Wnt pathway is an attractive model for therapeutics and cancer therapy. Aberrant activation of Wnt signaling is associated with poor cancer outcomes and recent strategies involve targeting the pathway in diverse cancers (**Figure 5**). Inhibiting the stimulation of the Wnt pathway by using monoclonal antibodies against Wnt1 has been tested in many cancer cells and has resulted in reduction of downstream components and apoptosis (He et al., 2004; Esmaeili et al., 2020). Similarly, an anti-FZD antibody (Vantictumab) has been used to target the frizzled receptors and shown to reduce the tumor burden. This antibody was in clinical trials with patients of breast cancer and pancreatic cancer but eventually discontinued because of bone toxicity observed in these patients (Gurney et al., 2012; Smith et al., 2013). Ipafricept, a recombinant fusion protein consisting of FZD8 extracellular domain and human IgG1 FC fragment blocks Wnt ligands and xenograft assays have shown efficacy of this drug (Wall and Arend, 2020). It is in the first phase of clinical trials and is administered after chemotherapeutic agents (Fischer et al., 2017). Porcupine inhibitors, such as WNT974, inhibit Wnt secretion and are effective in reducing tumor growth. Porcupine catalyzes palmitoylation on Wnts required for their secretion. Currently, WNT974 is in phase 1 clinical trial (Koo et al., 2015; Agarwal et al., 2017). Another group of inhibitors such as IWR-1 and XAV939 target the stability of the β-catenin destruction complex, including inhibitors of tankyrase 1 and tankyrase 2; these induce AXIN protein stability. These inhibitors reduce Wnt signaling, EMT and angiogenesis (Arqués et al., 2016; Shetti et al., 2019).

The downstream signaling network of Wnt signaling pathway involves β-catenin nuclear signaling through interaction with various nuclear factors to drive target gene expression and tumorigenic phenotypic changes. Several attempts have been made to target this β-catenin and TCF interface to modulate

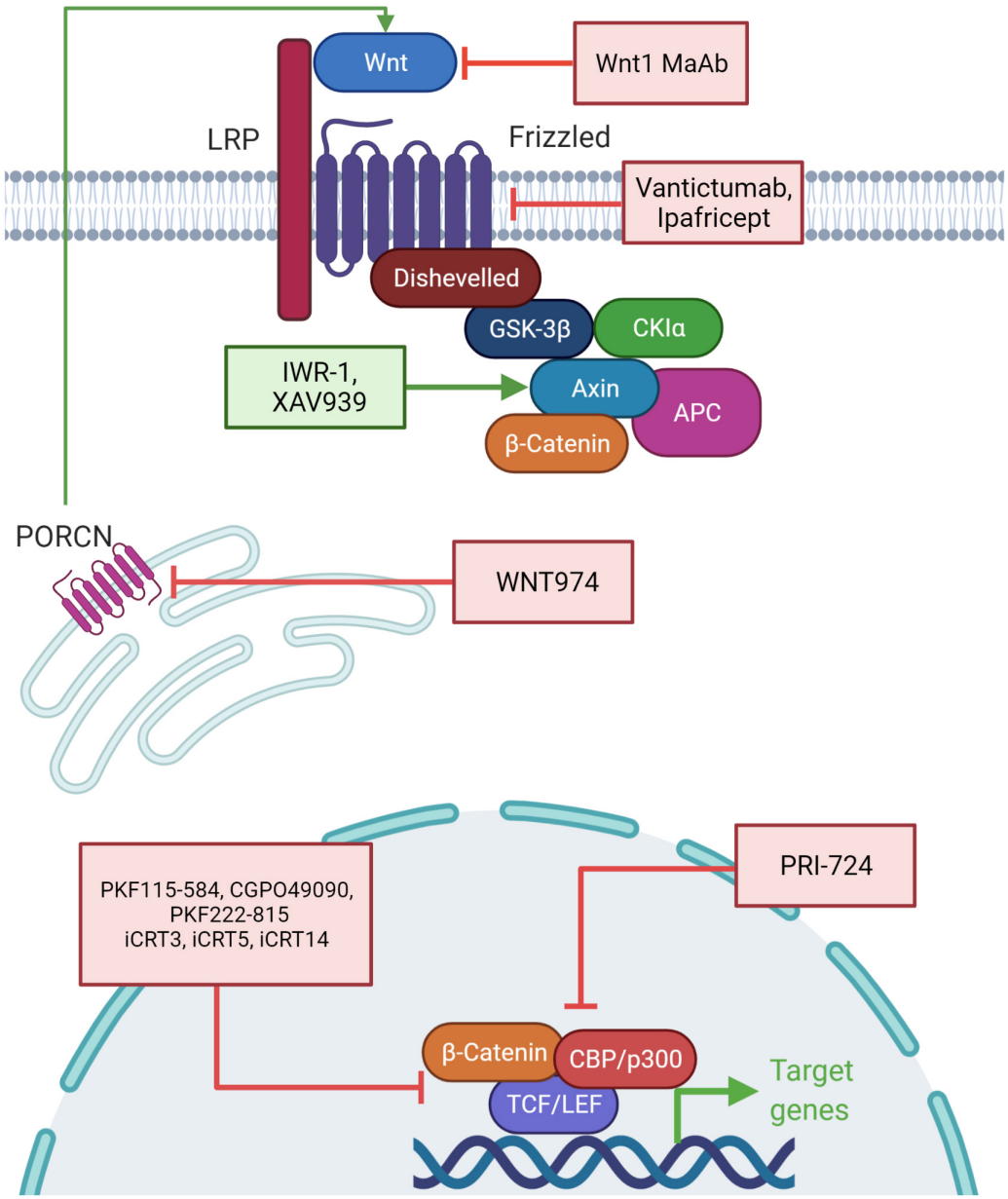

FIGURE 5 | Chemotherapeutic interventions targeting the components of the Wnt signaling pathway. Drugs leading to the activation of the Wnt response inhibitors and repression of the Wnt response activators are being used as therapeutic agents for cancer therapy (or are under clinical trials). Wnt1 monoclonal antibody (MAb), Vantictumab and Ipafricept target the upstream regulators of the Wnt pathway. WNT974 blocks PORCN which prevents processing of Wnt ligands. IWR-1 and XAV939 act by activating AXIN, thereby stabilizing the Wnt destruction complex. PRI-724 acts by inhibiting the interaction of β-catenin with CBP/p300. PKF115-584, CGPO49090, PKF222-815, iCRT3, iCRT5, and iCRT14 act by inhibiting interaction of β-catenin with TCF/LEF.

target gene expression and induce regression of tumors. For instance, PKF115-584, CGPO49090, and PKF222-815 are fungal derived drugs and inhibit β-catenin's interaction with TCF. These are currently in phase I and phase II of clinical trials (Lepourcelet et al., 2004). High throughput screening has identified three inhibitors, β-catenin responsive transcription inhibitor 3 (iCRT3), iCRT5, and iCRT14 specifically known to inhibit β-catenin and TCF interaction (Lepourcelet et al., 2004; Gonsalves et al., 2011). But clinical trials for these are not yet complete and potential side effects remain unknown.

The interaction of β-catenin with various co-factors is crucial for potentiating the Wnt pathway driven gene expression and associated cellular consequences, especially when the pathway is hyperactivated, has also been exploited to design drugs for cancer treatment. For instance, PRI-724 disrupts the interaction between β-catenin and its co-transcriptional activator CBP (Ko et al., 2016). Several natural compounds have also been used

to treat Wnt signaling driven cancers such as resveratrol, curcumin, and Genistein (Cheng et al., 2019). The combinatorial use of epigenome modulating drugs along with conventional chemotherapeutic drugs will be required to treat epigenomic alteration driven cancers.

DISCUSSION

Summary

In this review, we have provided an overview of the regulation of the Wnt signaling pathway in various cancer types, with a special emphasis on the crosstalk with the epigenetic machinery. Apart from undergoing alterations at the genetic level, the Wnt signaling pathway components are dysregulated due to various ncRNAs and chromatin modifiers. In turn, the Wnt effector β-catenin is also involved in various epigenetic processes due to its interaction with a multitude of epigenetic players. We have also provided an overview of the signaling pathways that work in conjunction or restrict the activity of the Wnt signaling pathway.

Therapeutic Potential

Aberrant Wnt pathway activation seems to be the root cause for the development and progression of multiple cancers. However, even four decades after the discovery of the Wnt signaling, therapeutic intervention of this key pathway remains far from achieving its potential. Several factors seem to be responsible such as: the pathway is intricate and regulated by multiple combinations of Wnt ligands and their receptors, crosstalk of canonical and non-canonical pathways, and crosstalk with other signaling pathways. Further, multiple mutations of the Wnt signaling components cause constitutive activation of β-catenin, making it difficult to target the upstream players. Additionally, the physiological roles of the Wnt/β-catenin signaling are important for normal cellular functions. It is the combination of various signaling pathways that results in an imbalance in the rate of activation. Hence, future therapeutics will be aimed at optimizing the rate of activation and directing it toward maintaining the cellular homeostasis.

Using certain synthetic biochemical tags that could regulate levels of crucial components of the Wnt signaling pathway will be useful to develop future therapeutic intervention. Proteolysis-targeting chimeras (PROTACs) have emerged as an efficient technology to degrade proteins of interest *in vivo* (Sakamoto et al., 2001). One such strategy was employed recently using PROTAC chemistry to degrade β-catenin in Wnt signaling driven colorectal cancers. Membrane permeable PROTACs were designed to tether β-catenin with the ubiquitin system to induce controlled degradation of β-catenin to regress the tumor burden in *Apc* mutant mice (Liao et al., 2020). Similarly, PROTACs using biochemical inhibitors were designed to tag SGK3 kinase with VHL ubiquitin ligases to facilitate selective and efficient degradation of SGK3 in breast cancers compared to conventional inhibitors because of their sub-stoichiometric catalytic mode of action (Tovell et al., 2019). Recently characterized roles of the lncRNAs and chromatin in Wnt signaling regulation make them an attractive target for therapeutics in the future. Using a combination of PROTAC chemistry for crucial components of the Wnt pathway and chromatin, and nanoparticle based targeting of non-coding RNAs could enhance the chances of better therapies in the future. The precise and balanced stimulation of Wnt/β-catenin signaling governs cellular outcomes, tissue homeostasis and developmental paradigms and disruption of this balance may lead to a multitude of processes either in their favor or disfavor. Therefore, future therapeutics will depend on understanding this complexity employing multiple ways to curb the signaling imbalance.

Future Perspectives: Open Questions

Wnt signaling crosstalks with several signaling and regulatory networks including epigenetic networks, mechano-sensing and environmental cues to modulate the expression of target genes crucial for developmental paradigms and tumorigenesis. Hence, holistic understanding of the molecular changes acquired during the initiation of Wnt signaling driven cancers is critical for disease etiology and future therapeutics. Identifying the pioneering molecular cues will be useful for developing better cancer therapies. The role of nuclear factors driving the Wnt/β-catenin signaling response raises hope of using drugs targeting these effectors. Obtaining a better picture of protein-protein interaction complexes regulating Wnt/β-catenin signaling will be helpful in identifying future therapeutic targets. Understanding the comprehensive picture of nuclear regulators and/or missing partner(s) crucial for convergence with other pathways will be essential for the treatment of Wnt signaling related diseases.

AUTHOR CONTRIBUTIONS

All the authors contributed to design the concept and structure of the review article, and approved the submitted version. AS and RM collected and analyzed the literature, and wrote bulk of the manuscript. AS prepared the figures. RM and SG provided critical inputs as the corresponding authors.

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REVIEW
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Expression and Functional Roles of Eukaryotic Initiation Factor 4A Family Proteins in Human Cancers

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The dysregulation of mRNA translation is common in malignancies and may lead to tumorigenesis and progression. Eukaryotic initiation factor 4A (eIF4A) proteins are essential for translation, exhibit bidirectional RNA helicase function, and act as RNA-dependent ATPases. In this review, we explored the predicted structures of the three eIF4A isoforms (eIF4A1, eIF4A2, and eIF4A3), and discussed possible explanations for which function during different translation stages (initiation, mRNA localization, export, and mRNA splicing). These proteins also frequently served as targets of microRNAs (miRNAs) or long noncoding RNAs (lncRNAs) to mediate epithelial-mesenchymal transition (EMT), which was associated with tumor cell invasion and metastasis. To define the differential expression of eIF4A family members, we applied the Tumor Immune Estimation Resource website. We figured out that the eIF4A family genes were differently expressed in specific cancer types. We also found that the level of the eIF4A family genes were associated with abundant immune cells infiltration and tumor purity. The associations between eIF4A proteins and cancer patient clinicopathological features suggested that eIF4A proteins might serve as biomarkers for early tumor diagnosis, histological classification, and clinical grading/staging, providing new tools for precise and individualized cancer treatment.

Keywords: eIF4A family, human cancer, clinicopathological features, biomarkers, immune infiltrations

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BACKGROUND

Cancer is one of the most common diseases affecting human health and has imposed a heavy economic burden on society worldwide (Bray et al., 2018). As a frequent characteristic of malignancy, the dysregulation of messenger RNA (mRNA) translation may lead to tumorigenesis and progression (Bhat et al., 2015; Vadivel Gnanasundram and Fåhraeus, 2018). The translation of mRNA is a complex process that includes the steps of initiation, elongation, and termination (Dever and Green, 2012). The initiation phase is the rate-limiting step (Sonenberg and Hinnebusch, 2009; Jackson et al., 2010). The majority of evidence has confirmed that various eukaryotic initiation factors are closely associated with the genesis and prognosis of many types of human cancers (Hsieh et al., 2010; Bhat et al., 2015; Pelletier et al., 2015).

In eukaryotes, members of the eukaryotic initiation factor 4A (eIF4A) family are essential factors for translation (Linder, 2003; Linder, 2006), and they also serve as prototypes of DEAD-box family members (Parsyan et al., 2011; Rogers et al., 2002; Rogers et al., 2001). The different eIF4A isoforms have been

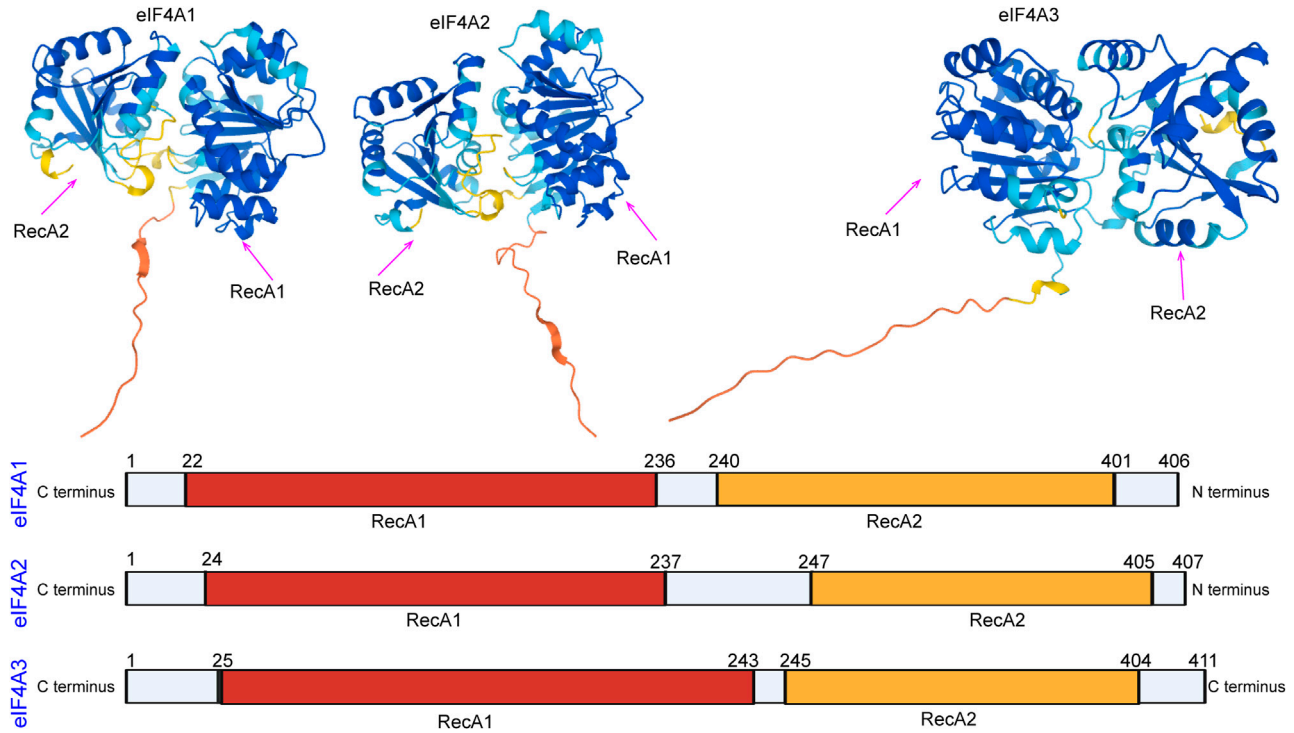

FIGURE 1 | Domain organization of eIF4A1, eIF4A2, and eIF4A3. The N-terminal RecA-like domain is depicted in yellow, and the C-terminus is depicted in red. The domain organization of the eIF4A family was downloaded from the alphafold protein structure database (https://alphafold.ebi.ac.uk/).

named as follows: eIF4A1 (DDX2A), eIF4A2 (DDX2B), and eIF4A3 (DDX48) (Iwatani-Yoshihara et al., 2017). eIF4A domains are the first determined DEAD-box protein structures that exhibit RecA-like folds (the nucleotide-binding site) and interactions between conserved motifs within the domains (**Figure 1**). Generally, eIF4A1 is more abundant in the cytoplasm than eIF4A2, while eIF4A3 is mainly localized in the nucleus (Lu et al., 2014). Both eIF4A1 and eIF4A2 participate in the initiation of translation. The eIF4A3 protein functions in RNA metabolism, including mRNA localization, export, and the coupling of mRNA splicing to translation (Mazloomian et al., 2019).

The ATP-dependent RNA helicase, eIF4A, plays important roles in human cancers (Wolf and Hatzfeld, 2010; Fukao et al., 2014; Sridharan et al., 2019). Alterations in the expression levels of eIF4A1, eIF4A2, and eIF4A3 have been observed in different types of malignancies and are closely associated with the clinicopathological characteristics of tumors (Ji et al., 2003) (Lin et al., 2018). With advances in the understanding of the regulation of the eIF4A family, several studies have suggested that eIF4A biomarkers could be used for human cancer diagnostics and therapies (Wang et al., 2002; Shaoyan et al., 2013a). Herein, we summarize the regulatory mechanisms and biological functions of eIF4A proteins during the process of mRNA translation. Additionally, we discuss the roles of each eIF4A isoform in tumorigenesis and cancer progression, and we propose their use as biomarkers for cancer prognosis, diagnostics, and treatment.

REGULATORY MECHANISMS OF THE EIF4A FAMILY

Role of eIF4A1 in Translation Initiation

eIF4A1 is a necessary component of eIF4F, which is a protein complex consisting of eIF4A1, eIF4E, and eIF4G (Jackson et al., 2010) (Merrick, 2015) (Topisirovic et al., 2011). Translational control usually occurs at the translation initiation step, in which ribosomes are recruited to the 5′ cap of the mRNA. First, eIF4E, as part of the eIF4F complex, promotes the recruitment of the 40S ribosomal subunit by interacting with the 5′ terminus of the mRNA (Siddiqui and Sonenberg, 2015). eIF4G plays a scaffolding role by interacting with both eIF4E and eIF4A1 (Lamphear et al., 1995; Mader et al., 1995). The recruitment of the 40S ribosomal subunit is induced by the interactions among eIF3, eIF4G, and the 40S subunit in mammals (except in yeast) (Jivotovskaya et al., 2006). Then, the 40S complex scans the 5′-untranslated region (UTR) for the AUG initiation codon. The elongation-competent 60S subunit is then recruited, and an elongation-competent 80S ribosome is formed. Notably, ribosomes have a weak capacity to unwind mRNA secondary structures (Takyar et al., 2005), while eIF4A1 has the ability to unwind stable secondary structures in the 5′-UTR during scanning (Sonenberg, 1988; Svitkin et al., 2001; Pestova and Kolupaeva, 2002) (**Figure 2**).

Prior studies have shown that the dysregulation of translation is an essential step in tumorigenesis and progression for the direct control of the selective translation and protein synthesis of oncogenic mRNA

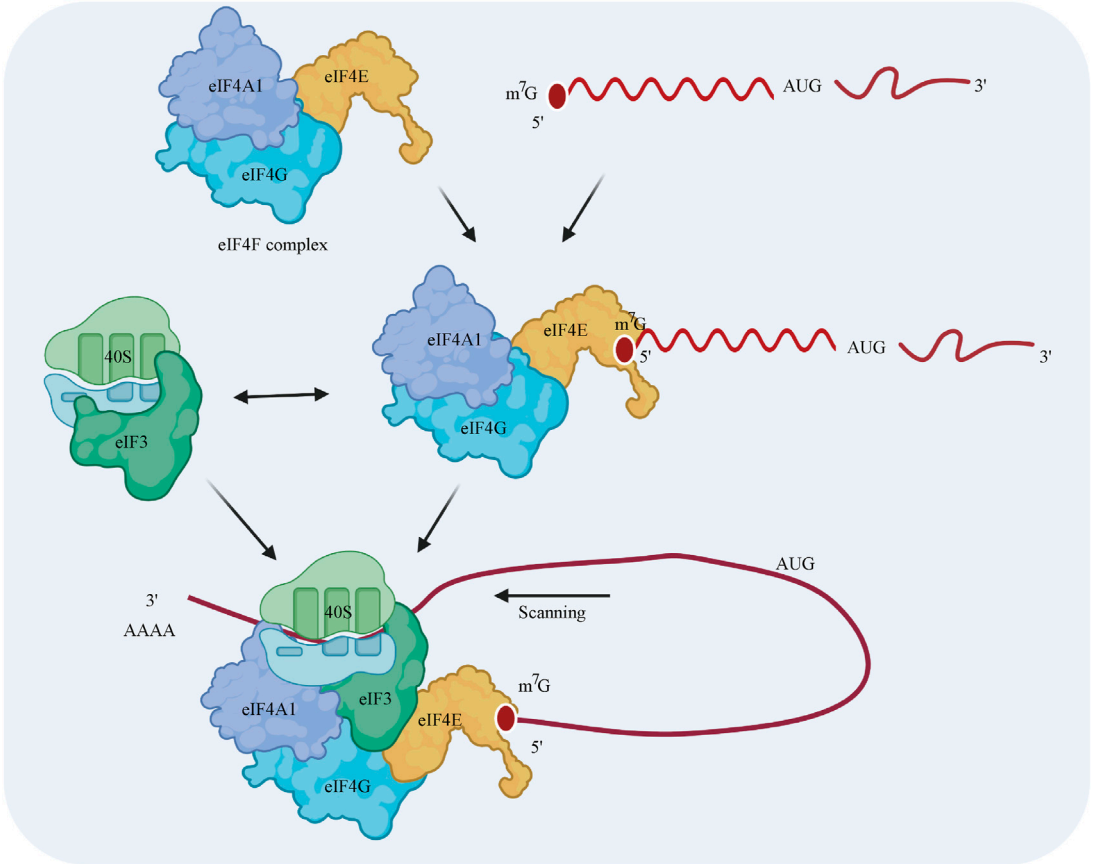

FIGURE 2 | Model of the regulatory mechanism by which eIF4A1 initiates cap-dependent translation. The eIF4F cap-binding complex is composed of the eIF4A1 translation initiation factor, the eIF4G scaffolding protein, and the eIF4E m^7G cap-binding protein. The 40S ribosomal subunit is recruited by interacting with eIF4F and eIF3 prior to the binding of eIF4F to the 5′ cap of the mRNA. This 43S preinitiation complex scans the 5′-UTR for the AUG initiation codon. During this process, eIF4A1 unwinds the stable secondary structures in the 5′-UTR of the mRNA.

(Silvera et al., 2010; Waldron et al., 2019). The eIF4F translation initiation complex controls the translation initiation rates of many pro-oncogenic mRNAs and serves as a critical node under the regulation of the PI3K/Akt/mTOR signaling pathway (Lin et al., 2008), the mitogen-activated protein kinase signal transduction pathway, and the caspase-dependent apoptotic pathway (Blagden and Willis, 2011). As an important component of eIF4F, eIF4A1 plays a vital role in malignant transformation and progression, and recent evidence has shown that eIF4A1 is dysregulated in gastric cancer (GC) (Gao et al., 2020), colorectal cancer (Li W. et al., 2017), cervical cancer (Liang et al., 2014), hepatocellular carcinoma (Zhang et al., 2020), ovarian cancer (Zhang et al., 2018), and other cancers.

Differences Between eIF4A2 and eIF4A1

eIF4A2 and eIF4A1 are approximately 90% identical at the amino acid level (Schütz et al., 2010) (**Figure 1**). Although both proteins have indistinguishable functions during translation initiation, eIF4A1 is essential for initiation, whereas eIF4A2 is not essential for initiation (Galicia-Vázquez et al., 2015). Inhibition of eIF4A1 leads to increased eIF4A2 transcription. However, eIF4A2 does not rescue the translation or cell proliferation inhibition caused by eIF4A1 inhibition (Galicia-Vázquez et al., 2012). Recent studies have found that mutations in eIF4A1 result in the repression of translation, whereas the expression of eIF4A2 mutants does not repress translation (Wilczynska et al., 2019). The amount of free functional eIF4A1 is regulated by programmed cell death 4 (PDCD4), and the abundance of eIF4A1 itself is regulated by mTOR and the carcinogen, miR-21. However, it is not clear whether inhibition of PDCD4 also affects eIF4A2 (Dorrello et al., 2006; Asangani et al., 2008).

Regulatory Molecule of eIF4A3

eIF4A3 exhibits 65% amino acid identity with human eIF4A1 (**Figure 1**) and functions differently from eIF4A1 and eIF4A2 (Li et al., 1999). eIF4A3 has the same ATPase activity, but eIF4A3 on its own does not show helicase activity and is not involved in the initiation of translation (Noble and Song, 2007; Rozovsky et al., 2008). eIF4A3 is well known to be a component of the exon junction complex (EJC) (Le Hir and Séraphin, 2008) and serves as a nucleation center to recruit other EJC components (i.e., MLN51 and Magoh/Y14) (Andersen et al., 2006; Ballut et al., 2005; Bono et al., 2006). The EJC is a group of proteins that deposits on and

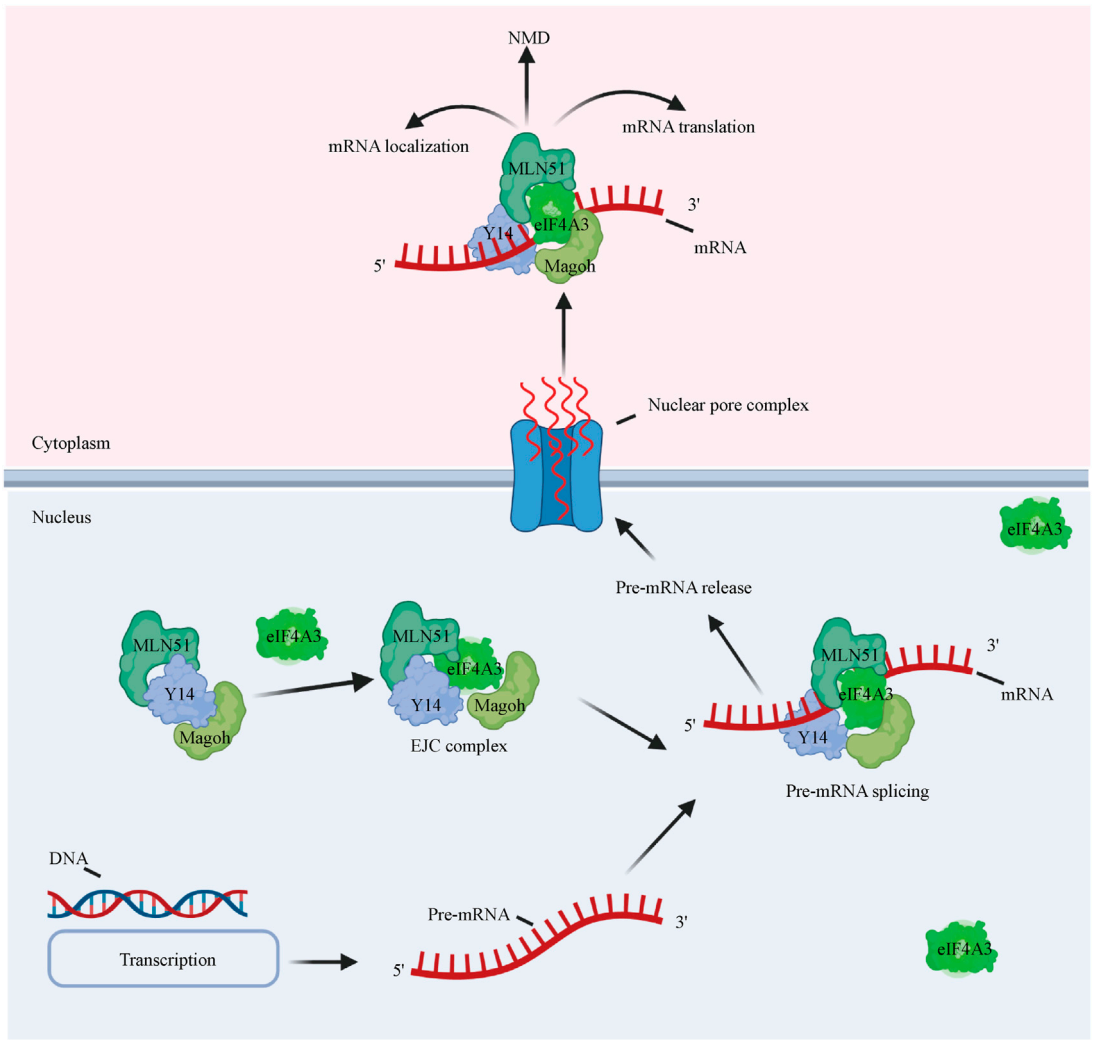

FIGURE 3 | eIF4A3 affects premRNA splicing and mRNA metabolism. eIF4A3 is an essential component of the EJC and serves as a nucleation center to recruit other EJC components (i.e., the Y14/Magoh heterodimer and MLN51). The complex binds the mRNA to facilitate its translocation to the cytoplasm from the nucleus to facilitate downstream processes, such as the NMD pathway, mRNA localization, and translation.

accompanies mRNAs from the nucleus to the cytoplasm and coordinates premRNA splicing with downstream processes, such as nonsense-mediated decay (NMD), mRNA localization, and translation (**Figure 3**) (Andreou and Klostermeier, 2013) (Blazquez et al., 2018). Although the mechanism by which the EJC is positioned on the mRNA is not clear, it is well established that the EJC stably binds the mRNA during premRNA splicing (Reed and Hurt, 2002; Ferraiuolo et al., 2004; Shibuya et al., 2004).

EIF4A EXPRESSION PATTERNS IN CANCER

The dysregulation and aberrant expression of eIF4A isoforms have been found in various tumor tissues (Raza et al., 2015; Lin et al., 2018; Wang et al., 2018). Although the exact roles of these members in tumorigenesis are not yet clear, they may be related to the dysfunction of the RNA helicase and lead to the expression of proteins formed by abnormal RNA translation (Polunovsky and Bitterman, 2006; Loh et al., 2009). We have summarized the data about eIF4A family members in various types of cancer in **Table 1**.

Expression Patterns of eIF4A1 and eIF4A2 in Gastric Cancer

Gao et al. examined the mRNA expression levels of eIF4A1 in GC by employing the Gene Expression Omnibus (GEO) and showed that eIF4A1 mRNA is significantly upregulated in GC tissues compared to adjacent normal tissues (Gao et al., 2020). Similarly, immunohistochemical staining of the eIF4A1 protein in patients with GC showed that eIF4A1 protein levels are generally increased in tumor tissues (Gao et al., 2020). Wei et al. demonstrated that the expression levels of

TABLE 1 | Expression patterns and clinical significance of the eIF4A family in human malignancies.

Isoform	References	Cancer type	Expression in tumor	Clinical significance	Prognosis
eIF4A1	22415234	Endometrial cancer	High	Not studied	Not studied
	32147684	Gastric cancer	High	Poor tumor differentiation, late T stage, lymph node metastasis, advanced TNM stage	Poor prognosis
	25611378	Breast cancer	Not studied	High histological grade	Poor prognosis
	31807078	Oral squamous cell carcinoma	Not studied	Poor differentiation	Poor prognosis
	24844222	Cervical cancer	High	Advanced stage, squamous cell histology, lymph node metastasis, deep stromal invasion	Poor prognosis
	12970751	Non-small cell lung cancer	Not studied	Metastasis	Poor prognosis
eIF4A2	23867391	Non-small-cell lung cancer	Low	Low histopathological classification, early tumor grade	Favorable prognosis
	31088567	Colorectal cancer	High	distant metastasis, TNM stage IV	Poor prognosis
	31308851	Triple-negative breast cancer	High	Not studied	Not studied
	32934744	Esophageal squamous cell carcinoma	High	Not studied	Not studied
eIF4A3	29571014	Ovarian cancer	High	Not studied	Not studied
	31975383	Hepatocellular carcinoma	High	Not studied	Not studied
	32307743	Gastric cancer	Low	Not studied	Not studied

eIF4A1 protein were upregulated in 74 clinical GC samples (Wei et al., 2019), similar to the results obtained by other research teams (Li et al., 2020a). Additionally, the overexpression of eIF4A1 has been positively associated with advanced tumor-node-metastasis (TNM) stage, poor tumor differentiation, and a poor prognosis in patients with GC (Gao et al., 2020).

Expression Patterns of eIF4A1 and eIF4A2 in Lung Cancer

Shaoyan et al. found that the mRNA expression of eIF4A2 was increased in 87.6% (148/170) of patients with nonsmall-cell lung carcinoma (NSCLC), and they observed elevated levels of eIF4A2 in tumor tissues (45.29%; 77/170) using immunohistochemistry (Shaoyan et al., 2013b). Contrary to these findings, eIF4A2 expression has been found to be low in tumor tissues but significantly related to three different clinicopathological features, namely, pathologic type, tumor grade, and overall survival (Shaoyan et al., 2013b). Furthermore, univariate and multivariate analyses have suggested that eIF4A2 is an independent prognostic factor in patients with NSCLC (Shaoyan et al., 2013b).

Expression Patterns of eIF4A1 and eIF4A2 in Colorectal Cancer

In colorectal cancer, eIF4A1 is overexpressed in 86% (44/51) of primary colorectal tumors compared to adjacent normal tissues according to immunohistochemical staining (Li W. et al., 2017). Yang et al. reported that eIF4A1 is recruited by the long noncoding RNA, MAPKAPK5-AS1, to promote the translation of MAPK-activated protein kinase 5 (Yang et al., 2020). In addition, either eIF4A2 knockdown or inhibition by silvestrol significantly suppresses colorectal cancer invasion and migration as well as enhances sensitivity to oxaliplatin treatment both *in vitro* and *in vivo* (Chen et al., 2019).

Expression Patterns of eIF4A1 and eIF4A2 in Cervical Cancer

eIF4A1 overexpression has been detected in 83.9% of cervical cancer tissues and is significantly related to advanced tumor stage, lymph node metastasis, squamous cell histology, deep stromal invasion, and poor survival in patients with cervical cancer (Liang et al., 2014).

Expression Patterns of eIF4A1 and eIF4A2 in Breast Cancer

Modelska et al. reported that eIF4A1 upregulation is associated with a higher histological grade in estrogen receptor-negative breast cancer tumors, and the combination of eIF4A1 with eIF4B and eIF4E might serve as an independent predictor of prognosis in patients with breast cancer (Modelska et al., 2015b). Liu et al. found that eIF4A2 mRNA expression levels in paclitaxel-resistant breast cancer tissues are dramatically enhanced compared to those in paclitaxel-sensitive tissues (Liu et al., 2019). Functional experiments have further suggested that eIF4A2 knockdown significantly inhibits triple-negative breast cancer cell proliferation and induces apoptosis (Liu et al., 2019).

Expression Patterns of eIF4A1 and eIF4A2 in Various Other Cancers

Zhao et al. reported that low levels of programmed cell death 4 and high levels of eIF4A1 predict poorer differentiation and a

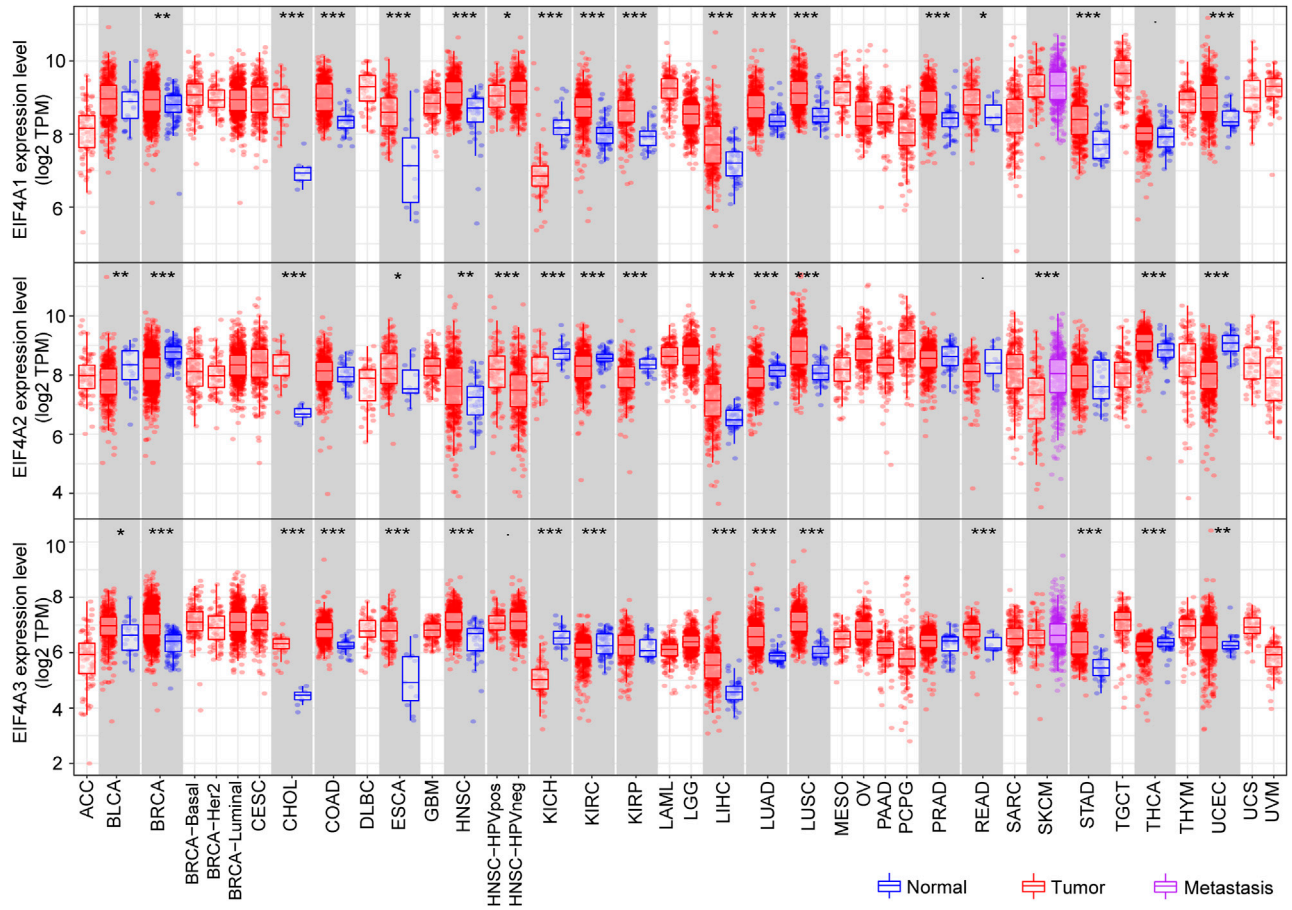

FIGURE 4 | eIF4A family gene mRNA expression levels. We adopted the TIMER website to detect the expression levels of eIF4A family genes. The results showed eIF4A family gene (eIF4A1, eIF4A2, and eIF4A3) mRNA expression levels in 32 tumor tissues compared to normal tissues across multiple cancers. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: Kidney Renal Clear Cell Carcinoma (KIRC); Kidney Renal Papillary Cell Carcinoma (KIRP); Kidney Chromophobe (KICH); Brain Lower Grade Glioma (LGG); Glioblastoma Multiforme (GBM); Breast Invasive Carcinoma (BRCA); Lung Squamous Cell Carcinoma (LUSC); Lung Adenocarcinoma (LUAD); Rectum Adenocarcinoma (READ); Colon Adenocarcinoma (COAD); Uterine Carcinosarcoma (UCS); Uterine Corpus Endometrial Carcinoma (UCEC); Ovarian Serous Cystadenocarcinoma (OV); Head and Neck Squamous Carcinoma (HNSC); Thyroid Carcinoma (THCA); Prostate Adenocarcinoma (PRAD); Stomach Adenocarcinoma (STAD); Skin Cutaneous Melanoma (SKCM); Bladder Urothelial Carcinoma (BLCA); Liver Hepatocellular Carcinoma (LIHC); Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma (CESC); Adrenocortical Carcinoma (ACC); Pheochromocytoma and Paraganglioma (PCPG); Sarcoma (SARC); Acute Myeloid Leukemia (LAML); Pancreatic Adenocarcinoma (PAAD); Esophageal Carcinoma (ESCA); Testicular Germ Cell Tumors (TGCT); Thymoma (THYM); Mesothelioma (MESO); Uveal Melanoma (UVM); Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC); Cholangiocarcinoma (CHOL).

higher postoperative recurrence rate in early oral squamous cell carcinoma than in normal tissues, suggesting the roles of these proteins as independent risk factors for this type of cancer (Jiang et al., 2019). Other studies in melanoma (Eberle et al., 1997; Eberle et al., 2002), B-cell malignancies (Thompson et al., 2021), hypopharynx cancer (Xu et al., 2013), pancreatic cancer (Ma et al., 2019), and endometrioid endometrial cancer (Lomnytska et al., 2012) have indicated tumor promoter roles for the eIF4A1 protein.

In esophageal squamous cell carcinoma, eIF4A2 has been found to be more highly expressed in neoplastic tissues than in normal tissues, and patients with high expression levels of eIF4A2 tend to have a poorer prognosis (Lyu et al., 2020). Furthermore, the univariate and multivariate analyses have suggested that eIF4A2 is an independent prognostic factor in esophageal squamous cell carcinoma (Lyu et al., 2020).

Expression Patterns of eIF4A3 in Cancers

Zhou et al. reported that the long noncoding RNA (lncRNA), HOXC-AS1, inhibits GC cell apoptosis by binding eIF4A3 in the Wnt/β-catenin signaling pathway (Zhou et al., 2020). Another study has revealed that eIF4A3 may bind the circular RNA, PVRL3 (Sun et al., 2018). Han et al. confirmed that when eIF4A3 binds lncRNA H19, the recruitment of eIF4A3 to cell cycle gene-related mRNAs is decreased (Han et al., 2016). In epithelial ovarian cancer, eIF4A3 is highly expressed in cancer tissues compared to adjacent normal tissues. Notably, eIF4A3 has been identified as a binding protein of lncRNA CASC2, thereby affecting epithelial ovarian cancer development (Zhang et al., 2018). Zhang et al. demonstrated that eIF4A3 is overexpressed in hepatocellular carcinoma. Functionally, eIF4A3 promotes cell proliferation, migration, and epithelial-mesenchymal

transition (EMT) by binding WD (Trp-Asp) repeat domain 66 and miR-2113 (Zhang et al., 2020).

MRNA EXPRESSION LEVELS OF THE EIF4A FAMILY BASED ON PUBLIC DATABASE ANALYSIS

The Tumor Immune Estimation Resource (TIMER) (http://cistrome.dfci.harvard.edu/TIMER/) is a user-friendly website that provides comprehensive investigation of molecular characterization of tumor-immune interactions (Li et al., 2016; Li T. et al., 2017). To determine eIF4A family gene expression in normal tissues versus corresponding tumor tissues, we adopted the TIMER database and explored the eIF4A family gene mRNA expression level among multiple cancers. The eIF4A family genes associated with the RNA-seq landscape of multiple malignancies in The Cancer Genome Atlas (TCGA) are illustrated in **Figure 4.** The results revealed that eIF4A family genes were significantly differentially expressed in various cancers compared to adjacent normal tissues (**Supplementary Table S1**). The patient information was similar to that in a previous study (He et al., 2021). As shown in **Figure 3**, eIF4A1 was significantly overexpressed in tumor tissues compared to normal control tissues, including breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD), and uterine corpus endometrial carcinoma (UCEC). eIF4A1 mRNA was downregulated in kidney chromophobe (KICH) tissues compared to normal tissues. Similarly, eIF4A2 was significantly upregulated in CHOL, COAD, ESCA, HNSC, HNSC-HPV pos, LIHC, LUAD, LUSC, and thyroid carcinoma (THCA) tissues. However, eIF4A2 was expressed at lower levels in bladder urothelial carcinoma (BLCA), BRCA, kidney chromophobe (KICH), KIRC, KIRP, skin cutaneous melanoma (SKCM), and UCEC tumor tissues compared to corresponding normal tissues. In addition, the eIF4A3 expression level in BLCA, BRCA, CHOL, COAD, ESCA, HNSC, LIHC, LUAD, LUSC, READ, STAD, THCA, and UCEC tumor tissues was increased compared to that in adjacent normal tissues. Furthermore, the eIF4A3 mRNA expression level was lower in KICH- and KIRC-related tumor tissues than in adjacent normal tissues. These studies indicated that eIF4A family genes are differentially expressed in various tumors and may function as tumor indicators in some specific types of cancers.

EIF4A FAMILY GENES HAVE A CLOSE RELATIONSHIP WITH IMMUNE CELL INFILTRATION ACROSS CANCERS

To further investigate the interactions between eIF4A family genes and the immune cell infiltration landscape and tumor purity (Gong et al., 2020; Zhang et al., 2017) in various cancer types, we employed TIMER and investigated the correlations between eIF4A family gene transcription levels and tumor infiltrating immune cells (Li et al., 2016; Li T. et al., 2017), such as B cells, $CD8^+$ T cells, $CD4^+$ T cells macrophages, neutrophils, and dendritic cells, as well as the tumor purity among 32 types of cancers (**Supplementary Table S2**), using methods described in our previous study (Xue et al., 2021). The results demonstrated that eIF4A family genes were closely correlated with immune cell infiltration in cancers. Notably, eIF4A1 had significant positive correlations with the infiltration levels of B cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in THCA. eIF4A1 also showed significant positive correlations with dendritic cells, $CD8^+$ T cells, and neutrophils in BLCA, KICH, KIRC, LIHC, pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), and thymoma (THYM). Tumor immune cell infiltration, which was positively correlated with the eIF4A2 mRNA expression level, was higher in KICH, THYM, COAD, PAAD, PCPG, LIHC, KICH BLCA, KIRC, and LGG than in other cancers. Dendritic cells, $CD8^+$ T cells, and neutrophils had higher infiltration levels in cancers with higher eIF4A2 mRNA expression. eIF4A2 was negatively correlated with the infiltration of $CD4^+$ T cells, macrophages, and dendritic cells in LGG and significantly positively correlated with the infiltration of B cells, $CD4^+$ T cells, macrophages, and dendritic cells in LIHC and PRAD. In general, THCA, LGG, LIHC, PRAD, and SKCM, which exhibited significant upregulation of eIF4A2, had higher immune cell infiltration. In addition, the eIF4A3 expression level was significantly positively correlated with the infiltration of B cells, $CD4^+$ T cells, macrophages, dendrites, and neutrophils in LGG, LIHC, and THCA. In THCA, eIF4A3 mRNA expression was associated with significantly higher B cell, $CD4^+$ T cell, $CD8^+$ T cell macrophage, and neutrophil infiltration levels. In PRAD, LIHC, LGG, and PAAD, eIF4A3 mRNA expression was associated with higher infiltration levels of B cells, $CD8^+$ T cells, dendritic cells, and neutrophils (**Figure 5**). Our studies strongly indicated that eIF4A family genes may play specific roles in immune infiltration and tumor purity, suggesting that they may function as valuable immune evaluation indicators.

BIOLOGICAL FUNCTIONS OF EIF4A PROTEIN IN CANCER

Most studies have demonstrated that eIF4A proteins possess protumor functions (Oblinger et al., 2016). Genome-wide studies of the eIF4A-associated translatome have revealed that eIF4A-dependent mRNAs include those that promote cell proliferation, cell survival, cell cycle progression, and angiogenesis (Rubio et al., 2014; Wolfe et al., 2014). Most studies have reported that high expression levels of eIF4A significantly promote a cancer cell malignant phenotype (proliferation, invasion, migration, and EMT) and inhibit apoptosis (Modelska et al., 2015a; Li W. et al., 2017; Liang et al., 2017; Li et al., 2020b; Gao et al., 2020).

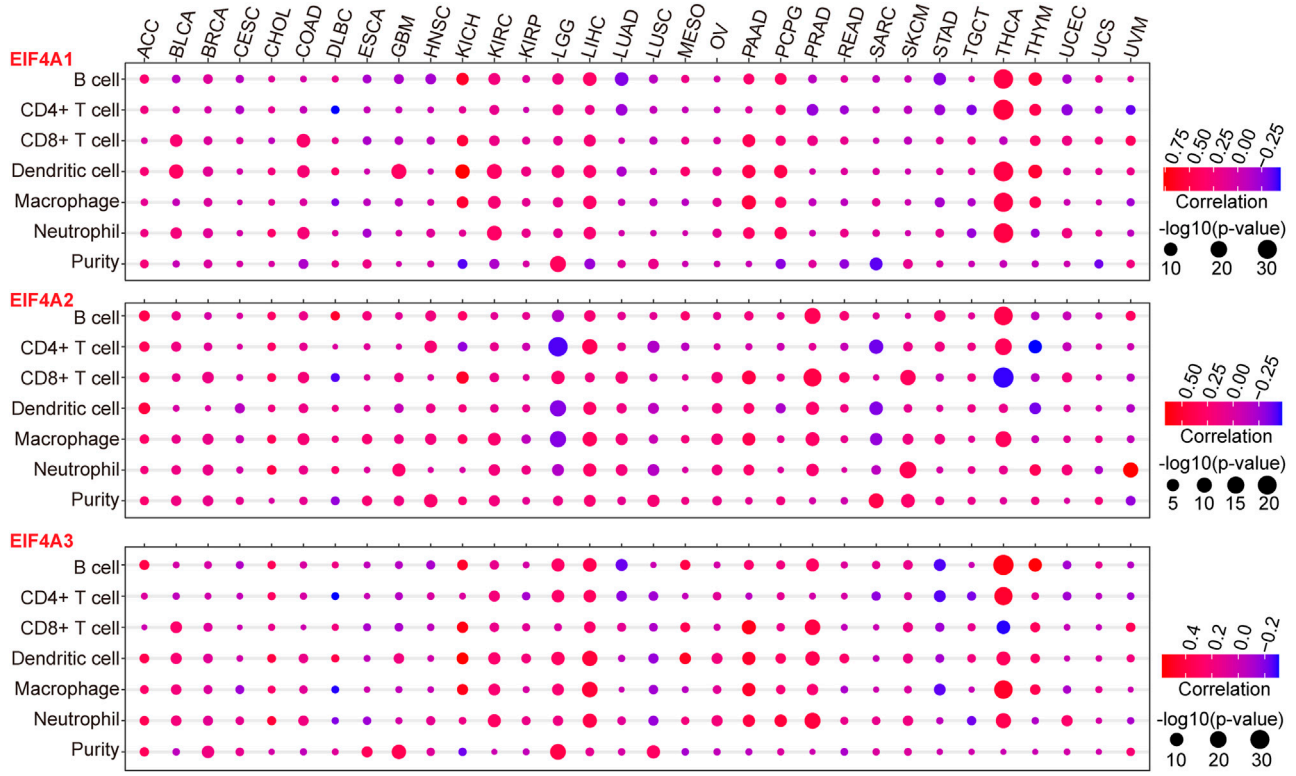

FIGURE 5 | Correlation of eIF4A family gene expression with immune infiltration level and tumor purity. We adopted the TIMER website to detect the association of eIF4A family gene expression and immune infiltration levels. The mRNA expression levels of eIF4A family genes (eIF4A1, eIF4A2, and eIF4A3) were significantly correlated with the infiltration levels of various immune cells. The color of the bubble of the graph indicates the difference in each of the various types of cancers, and the bubble size indicates the statistical significance of the correlation. A correlation >0 indicates a positive association of eIF4A family genes and immune cells in various cancers. A two-tailed $p < 0.05$ was considered significant. Abbreviations: Kidney Renal Clear Cell Carcinoma (KIRC); Kidney Renal Papillary Cell Carcinoma (KIRP); Kidney Chromophobe (KICH); Brain Lower Grade Glioma (LGG); Glioblastoma Multiforme (GBM); Breast Invasive Carcinoma (BRCA); Lung Squamous Cell Carcinoma (LUSC); Lung Adenocarcinoma (LUAD); Rectum Adenocarcinoma (READ); Colon Adenocarcinoma (COAD); Uterine Carcinosarcoma (UCS); Uterine Corpus Endometrial Carcinoma (UCEC); Ovarian Serous Cystadenocarcinoma (OV); Head and Neck Squamous Carcinoma (HNSC); Thyroid Carcinoma (THCA); Prostate Adenocarcinoma (PRAD); Stomach Adenocarcinoma (STAD); Skin Cutaneous Melanoma (SKCM); Bladder Urothelial Carcinoma (BLCA); Liver Hepatocellular Carcinoma (LIHC); Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma (CESC); Adrenocortical Carcinoma (ACC); Pheochromocytoma and Paraganglioma (PCPG); Sarcoma (SARC); Acute Myeloid Leukemia (LAML); Pancreatic Adenocarcinoma (PAAD); Esophageal Carcinoma (ESCA); Testicular Germ Cell Tumors (TGCT); Thymoma (THYM); Mesothelioma (MESO); Uveal Melanoma (UVM); Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC); Cholangiocarcinoma (CHOL).

eIF4A1 expression is regulated by circ-008035 via miR-599 binding, which ameliorates the effects of circ-008035 knockdown on GC cell proliferation and suppresses apoptosis (Li et al., 2020a). Li et al. reported that eIF4A1 is the direct target of miR-133a, which promotes colon cancer cell progression by inhibiting eIF4A1 expression (Wang et al., 2017). Similarly, the silencing of eIF4A1 in WM858 cells significantly decreases melanoma proliferation and invasion (Joyce et al., 2017). eIF4A1 has also been shown to promote the tumor cell malignant phenotype in breast (Modelska et al., 2015b), oral squamous cell (Zhao et al., 2019), and cervical (Liang et al., 2017) cancers.

Chen et al. reported that eIF4A2 dysfunction, induced by genetic knockdown or inhibition, suppresses colorectal cancer cell invasion, cell migration, and sphere formation as well as increases tissue sensitivity to oxaliplatin both *in vivo* and *in vitro* (Chen et al., 2019). In triple-negative breast cancer, miR-5195-3p upregulation increases the sensitivity of cancer cells to paclitaxel; the silencing of eIF4A2 mimics this effect, and the restoration of eIF4A2 blocks this effect (Long et al., 2019).

Han et al. reported that eIF4A3 is the binding protein of lncRNA H19, as shown by RNA-binding protein immunoprecipitation experiments, and that it participates in colorectal cancer cell proliferation via lncRNA H19 binding (Han et al., 2016). Xu et al. found that circ_cse1l is downregulated in colorectal cancer and that downregulated circ_cse1l inhibits PCNA expression by binding to eIF4A3 to inhibit the proliferation of colorectal cells (Xu et al., 2020). In epithelial ovarian cancer cells, eIF4A3 binds CASC2 and enhances cell viability, apoptosis, migration, and invasion (Zhang et al., 2018). Knockdown of eIF4A3 increases apoptosis (Zhang et al., 2018). In hepatocellular carcinoma, loss-of-function assays have shown that the silencing of eIF4A3 inhibits cell proliferation, migration, and EMT (Zhang et al., 2020). In GC tissues, eIF4A3 is downregulated compared to adjacent normal tissues, and the silencing of eIF4A3 increases

lncRNA HOXC-AS1 expression, which promotes GC cell proliferation and EMT but represses apoptosis (Zhou et al., 2020). In cervical cancer, Sui et al. reported that hsa_circ_0101119 promotes cell proliferation, migration, and invasion but suppresses apoptosis in cervical cancer via an interaction with eIF4A3 to inhibit TCEAL6 expression (Sui et al., 2021).

CONCLUSION

All the Members of the eIF4A family frequently serve as targets of microRNAs (miRNAs) or lncRNAs play key roles in tumor cell proliferation, invasion, and metastasis. Given the importance of mRNA translation in the development of cancer (Gingold et al., 2014), several small molecules have been shown to possess antitumor activities by targeting or inhibiting eIF4A1 (Stoneley and Willis, 2015; Howard et al., 2019; Howard et al., 2020). Previous studies have shown that the natural marine product, elatol, inhibits eIF4A1, providing a highly promising target for cancer therapy (Peters et al., 2018). Furthermore, hippuristanol, silvestrol, pateamine A, and oxo-aglaiastatin all target eIF4A1 (Itoua Maïga et al., 2019; Naineni et al., 2020; Steinberger et al., 2020). Rocaglates have been shown to possess potent antineoplastic activity both *in vivo* and *in vitro* by enhancing mRNA binding to both eIF4A1 and eIF4A2 (Chu et al., 2019). Some selective eIF4A3 inhibitors have also been identified as ATPase activation inhibitors (Ito et al., 2017a; Ito et al., 2017b). At present, inhibitors of the eIF4A family have stalled at a preclinical stage, and clinical evaluations are still lacking.

The present review presented that the eIF4A family genes were differently expressed in specific cancer types based on TIMER website, and we discussed the association between the eIF4A family genes and abundant immune cells infiltration and tumor purity, which could provide a clue for next study in the future. In addition, our findings posited functional roles of the eukaryotic initiation factor 4A family proteins in human cancer.

AUTHOR CONTRIBUTIONS

LL defined the review theme. CX and XG drafted the article and analyzed the data. GL and ZB helped with reference collection. All authors contributed to the writing and revision of the article, are aware of its content, and approved its submission.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Role of Main RNA Methylation in Hepatocellular Carcinoma: N6-Methyladenosine, 5-Methylcytosine, and N1-Methyladenosine

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RNA methylation is considered a significant epigenetic modification, a process that does not alter gene sequence but may play a necessary role in multiple biological processes, such as gene expression, genome editing, and cellular differentiation. With advances in RNA detection, various forms of RNA methylation can be found, including N6-methyladenosine (m6A), N1-methyladenosine (m1A), and 5-methylcytosine (m5C). Emerging reports confirm that dysregulation of RNA methylation gives rise to a variety of human diseases, particularly hepatocellular carcinoma. We will summarize essential regulators of RNA methylation and biological functions of these modifications in coding and noncoding RNAs. In conclusion, we highlight complex molecular mechanisms of m6A, m5C, and m1A associated with hepatocellular carcinoma and hope this review might provide therapeutic potent of RNA methylation to clinical research.

Keywords: hepatocellular carcinoma, RNA methylation, M6A, m5C, m1A

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common global disease. It has a poor prognosis and has become the third cause of cancer death (Couri and Pillai, 2019; Hua et al., 2019; Huang et al., 2021). Although medical technology has significantly improved in recent years, 5-years survival rates of patients remain low (8.5%) (Cai et al., 2019; Zhou et al., 2020). Mortality due to HCC remains high for several reasons. On one hand, clinical symptoms in the early stage are generally displayed uncharacteristic and the lack of effective diagnostic biomarkers, so many patients are easily misdiagnosed. On the other hand, as a result of affluent blood supply in the liver, tumor cells frequently proliferate at a growing rate and distant metastasis tends to appear in the early stage of cancer. Therefore, HCC seriously threatens human health and well-being and is seen as a tough challenge in clinical study. Emerging research is exploring the understanding of pathogenesis in HCC to prevent the dilemma of poor prognosis. Previous reports have showed that the leading pathogenic factor is chronic infection with virus, such as hepatitis B virus and hepatitis C virus

(Koshiol et al., 2021; Teng et al., 2021). Nevertheless, other molecular mechanisms involved in proliferation, invasion, metastasis, and chemoresistance in HCC remain unknown. Consequently, it is crucial to further investigate the complex mechanisms of tumorigenesis and tumor progression to discover novel makers and identify therapeutic targets.

RNA methylation is commonly regarded as posttranscriptional modification with multiple forms (Chen et al., 2019; Kagra et al., 2021). Although epigenetic modification of RNA has been documented over several decades (Cohn, 1960; Dubin and Taylor, 1975; Perry et al., 1975), our understanding of its biological functions is still limited. Recent research demonstrates that RNA modification may impact RNA metabolism, splicing, stability, and translation (Xue et al., 2020; Nombela et al., 2021), which distinctly influence gene expression. Thus, the effect of RNA methylation is gradually attracting broad attention in a broad array of specialties. For example, numerous investigations verified that m5C methylation in the 3′-UTR of mRNA increases translation efficiency (Schumann et al., 2020). Occurrence of RNA methylation ordinarily requires the participation of a large number of specific proteins called RNA-modifying proteins (RMPs), containing "writers," "erasers," and "readers" (Frye et al., 2018; Patil et al., 2018). "Writers" are a group of enzymes that catalyze methylation. In contrast, "erasers" are able to remove the decorate of methylation in RNA (Torres and Fujimori, 2015; Shi et al., 2019). "Readers" are a variety of proteins that recognize methylation sites catalyzed by "writers" and bind these sites to form complexes to affect the functions of RNA (Pozner et al., 2018; Grimanelli and Ingouff, 2020; Guo et al., 2020).

A prior survey revealed that abnormal regulation of these RMPs would give rise to incidence of various malignant tumors (Pan et al., 2018; Lan Q. et al., 2019; Zhuang et al., 2020; Li Y. et al., 2021). For instance, catalysis of m6A modification is mediated by methyltransferase-like 3 (METTL3), which is expressed at a high level in colon cancer. Previous evidence showed that METTL3 promoted miRNA-1246 upregulation and induced metastasis in colon cancer (Peng et al., 2019). In bladder cancer, YBX1 obviously emerged the appearance of overexpression than normal tissue. YBX1 is an RMP "reader" in m5A modification, and might advance the expression of the multidrug resistance-1 (MDR-1) gene to decrease sensitivity to chemotherapy drugs (Yamashita et al., 2017). Additionally, downregulation of DKC1 was found in breast cancer and gave rise to the impairment result of hTR stabilization (Montanaro et al., 2006). Similarly, overexpression of ALKBH3, methylating affluent m1A modification, ordinarily predicts a dismal prognosis in Hodgkin lymphoma (Yin et al., 2020). Other reports showed that PTR can lengthen survival time during stage M1a of non-small cell lung carcinoma (Li et al., 2019; Li H. et al., 2020), and was expected to be incorporated into promising therapeutic strategies for diagnosing patients with evolving ipsilateral pleural dissemination. Various experiments were testified that regulators of RNA methylation, like m6A, m5C, and m1A, participated in essential biological process for diverse cancers (Li C.-L. et al., 2020). However, discussions that effect of these regulatory factors in RNA methylation related to pathogenesis of HCC, are constricted in clinical study. Accordingly, in this review we illustrate functional consequences of m6A, m5C, and m1A in diverse RNAs. Cooperatively, we focus on targeting RMPs for clinical treatment in HCC in anticipation of providing patients with more promising overall survival and brighter futures.

RNA METHYLATION

Methylation refers to epigenetic transformations to influence gene expression but does not alter gene sequence, which can be mainly found in DNA, RNA, and protein (Wang X. et al., 2020; Hop et al., 2020; Anton and Roberts, 2021). As approaches to detect RNA modifications improve, RNA methylation began to broadly draw public notice. Multiple functional effects of modification of RNAs were further discussed, and the role of RNA methylation correlative with a variety of cancers also gradually become clearer. We briefly generalize these forms of methylating modification as follows.

N6-Methyladenosine

m6A is methylated adenosine at the nitrogen-6 position and was identified as a posttranscriptional modification in 1974 (Desrosiers et al., 1974). Previous survey has considered that m6A modifications are one of the most extensive methods of RNA methylation in mammals. It was estimated that m6A methylation was approximately present on a quarter of mRNAs (Meyer and Jaffrey, 2017; Chen et al., 2018). As approaches of detecting m6A is distinctly preferred, the utilization of ultraviolet crosslinking step realized the new outcome, identifying m6A positions at single-nucleotide resolution. Specific locations of m6A modification are detected, such as 3′ untranslated regions (3′-UTRs) (Dominguez et al., 2018), long internal exons, intergenic regions, introns, and 5' UTRs. METTL3 was first identified in the occurrence of m6A modification. METTL3 functions as the regulator mediating the export of mRNA by interacting with Per2 and Arntl. In addition, METTL3 can recruit eukaryotic translation initiation factor eIF3 to directly regulate translation flexibly (Lin et al., 2016; Choe et al., 2018). The other "writers" of m6A include METTL14, Wilms tumor 1-associated protein (WTAP), and RNA-binding motif protein 15 (RBM15) (Liu et al., 2014; Wen et al., 2018). Accordingly, "readers" of m6A are primarily proteins in the YT521-B homology (YTH) domain family and include YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2. Accumulating evidence demonstrates that promotion of translation can positively modulate the effect of YTHDF1. YTHDF3 accelerates protein synthesis by binding YTHDF1 to mediate ribosomal proteins (Shi H. et al., 2017; Li et al., 2017). In m6A methylation, fat mass and obesity-associated protein (FTO) and alkB homologue 5 (ALKBH5) are considered "erasers". FTO binds to introns of nascent mRNA molecules to modulate the biological process of splicing in mRNA (Bartosovic et al., 2017). Similarly, multiple reports also confirmed that ALKBH5 is a pivotal factor to participate in mRNA splicing (Zheng et al., 2013).

5-Methylcytosine

m5C is defined as the accession of methyl group on the fifth carbon atom of cytosine (Motorin et al., 2010; Huang et al., 2019). Abundant m5C occurs in a variety of RNAs, including mRNA, tRNA, rRNA, viral RNA, vault RNA, and lncRNA. In humans, m5C is introduced by NSUN family members and DNA methyltransferase 2 (DNMT2). NSUN2 methylates primarily tRNA and mRNA. The defined regions of tRNA are the variable loop and leucine at the wobble position (Hussain et al., 2013; Khoddami and Cairns, 2013). In mRNA, the specific sites of catalysis by NSUN2 are the region near the start codon and the noncoding 3 UTR. Distribution of NSUN2 is unique, because of converting altogether with different alteration of cell division cycle. NSUN2 can be found at the nucleolus in G1 phase, whereas it is located in the region between the nucleolus and nucleoplasm in S phrase. NSUN2 starts to gradually appear in the cytoplasm in G2 and M phase (Motorin et al., 2010). It was reported that centrioles could be detected abundant depositions of NSUN2 during M phrase. Previous study declared that NSUN2 played an indispensable role in phosphorylation, protein synthesis, cell cycle progression, and epidermal differentiation and tumorigenesis. NSUN4 and NSUN5 primarily catalyze methylation modification in 25s rRNA. NSUN4 protein is frequently found in mitochondria, but NSUN5 is distributed in the nucleolus. Overexpression of NSUN5 promotes synthesis of survival protein to enhance the response to oxidative stress (Schosserer et al., 2015). Most NSUN1 factors are detected in the nucleolus, although a few are detected in the cytoplasm. NSUN1 was found to participate in malignant invasion, cell cycle progression, and formation of chromatin (Sharma et al., 2013). NSUN3 and DNMT2 methylate tRNA and are distributed in mitochondria and cytoplasm, respectively. Numerous experiments indicated that DNMT2 has a critical influence in tumorigenesis, protein synthesis, cell differentiation, and HIV-1 RNA replication (Dev et al., 2017). The "erasers" of m5C methylation are primarily TET family members. TET1 catalyzes the removal of methylation in coding and non-coding RNAs. In addition, several reports suggested ALYREF recognizes and binds the methyl group catalyzed by NSUN2 in mRNA. ALYREF and NSUN2 together promote the transport of mRNA and increase the efficiency of nuclear-cytoplasmic shuttling (Shi M. et al., 2017). Upregulated YBX1 was observed in the cytoplasm and exerted a positive effect on mRNA stabilization, embryogenesis, and tumorigenesis (Yang et al., 2019).

N1-Methyladenosine

m1A is methylation at the N1 position of adenosine and is capable of altering RNA secondary structure. A previous study identified m1A in tRNA, rRNA, mRNA, and mitochondrial RNA. Affluent m1A modification is observed in tRNA and rRNA, while the level of m1A remains low in mRNA. The occurrence of m1A methylation in mRNA is represents a six-fold reduction compared to that of m6A methylation (Dominissini et al., 2016); however, m1A can be found in the coding sequence (CDS), 5′-UTR (Li et al., 2016), and 3′-UTRof mRNA. Emerging survey suggested method of m1A modification involving in protein synthesis, which improved the efficiency of translation by inhibiting binding of the releasing factor. In contrast, when m1A methylation occurs in the region of mRNA CDS, translation is suppressed to some degree. TRMT10C and TRMT61B serve as "writers" to participating in catalyzing m1A at position 9 and 58 of tRNA (Chujo and Suzuki, 2012). ALKBH3 and ALKBH1 not only demethylate the reversible modification of m6A, but are found to remove m1A (Liu F. et al., 2016). ALKBH3 can function as a repair enzyme to restore N-methylated bases. Recent investigations clarified that demethylation by ALKBH3 might improve the efficiency of translation. Therefore, silencing of ALKBH3 may have the effect of impeding protein synthesis by enhancing the level of m1A in tRNA. Moreover, ALKBH3 was regarded as prostate cancer antigen-1 (PCA-1) (Shimada et al., 2009; Yamato et al., 2012). Upregulation of ALKBH3 was observed in a variety of cancers, which stimulated angiogenesis and inhibited apoptosis in prostate cancer and pancreatic cancer patients. The present study suggested the function of m1A58 may result in decreased translation initiation. When ALKBH1 demethylates m1A, the elongation phase of translation might be impacted through reduced tRNA usage in protein synthesis (Haag et al., 2016; Kawarada et al., 2017).

FUNCTIONAL CONSEQUENCES OF RNA METHYLATION

RNA methylation takes place in various RNAs, which give rise to different outcomes to influence RNA function (Gilbert et al., 2016). The detailed functional consequences associated with modifications m6A, m5C, and m1A in RNAs are presented in **Table 1**.

Role of m6A in RNA

The stability of mRNA is mainly regulated by modification m6A. YTHDF2, an m6A "reader", might recruit mRNA into processing bodies and participate in the process of degradation to stabilize mRNA (Wang et al., 2014; Huang et al., 2018). Numerous studies reported that YTHDC1 is involved in triggering the SRSF3 pathway to mediate dynamic splicing of precursor mRNA (Molinie et al., 2016). Furthermore, YTHDC1 promotes the export of mRNA (Roundtree et al., 2017; Lesbirel et al., 2018), recruits nuclear transport receptors, and interacts with TREX mRNA adducts. In contrast, depletion of ALKBH5 accelerates export of mRNA (Zheng et al., 2013). Nuclear export is indispensable for translation of mRNA to protein. Several reports corroborated that METTL3 and METTL14 catalyze the modification m6A in the region of the 3′-UTR in p21 mRNA and positively increase the efficiency of translation. The recruitment of DCGR8 is mediated by METTL14 in pri-miRNA to encode and regulate the level of miR-126a (Wang et al., 2014). METTL14 is also important for transcriptional elongation of chromatin, which brings about the outcome of recruiting the microprocessor complex (Nombela et al., 2021).

Role of m5C in RNA

In mRNA, modification m5C can have a significant impact on metabolism. ALYREF was considered to enhance the efficiency of

TABLE 1 | The modification results of m6A, m5C, and m1A methylation in various RNA.

RNA type	Regulators	Modification type	Functional consequences	PMID
mRNA	YTHDF2	m6A	Enhance stability	29,476,152
mRNA	YTHDC1	m6A	Promote export	30,218,090
mRNA	ALKBH5	m6A	Promote export	23,177,736
mRNA	METTL3	m6A	Elevate translational efficiency	27,117,702
mRNA	METTL14	m6A	Elevate translational efficiency	24,284,625
mRNA	ALYREF	m5C	Promote export	28,418,038
mRNA	NSUN2	m5C	Promote transport and affect protein synthesis (promote, and inhibit)	25,063,673
tRNA	NSUN2	m5C	Enhance stability and promote survival proteins synthesis to repose stress	28,062,751
rRNA	NSUN5	m5C	Enhance stability	27,167,997
mRNA	—	m1A	Enhance stability and affect translation efficiency (promote, and inhibit)	28,230,814
tRNA	—	m1A	Promote HIV replication	29,908,293
tRNA	ALKBH1	m1A	Enhance stability	27,984,735

nuclear-cytoplasmic export by forming a complex with mRNA. Previous experiments confirmed that NSUN2 meditates mRNA transport, which facilitates ALYREF binding to mRNA (Yang et al., 2017). Therefore, m5C affects protein synthesis to a degree. For instance, modification m5C appears in CDS in mRNA, which impairs translation and reduces its efficiency. On the contrary, when m5C is located at the 3′-UTR, the productivity of protein synthesis is distinctly improved. Diverse locations of m5C might lead to different functional results. Moreover, the modification m5C might maintain the stability of mRNA and facilitate plant development. NSUN2 might mediate root-development–related transcripts to suppress root decay. The present survey found that NSUN2 and DNMT2 mediate m5C methylation and commonly play an essential role in stabilizing tRNA. When cells were exposed to hydrogen peroxide, NSUN2 generated survival proteins to respond to the stress (Blanco et al., 2014; David et al., 2017). DNMT maintains the stability of tRNA Asp-GTC and tRNA Gly-GCC and increases the efficiency of polypeptide synthesis (Tuorto et al., 2015). Several evidences suggest NSUN5 modulates rRNA stability under conditions of oxidative stress (Schosserer et al., 2016). NSUN4 impacts regulation of the last step of ribosomal biogenesis (Metodiev et al., 2014).

Role of m1A in RNA

Modification m1A is found predominantly in structured regions of the 5′-UTR and near alternative start codons, indicating that m1A is significantly involved in stabilizing mRNA structure. The accomplishment of m1A methylation also exerts an indispensable effect on translation efficiency. For instance, m1A in the CDS region of mRNA has been considered to block the productivity of protein synthesis because it disrupts Watson-Crick base pairing. The presence of m1A might be vital to regulate the structural thermostability of tRNAs. It was reported that m1A together with other post-transcriptional modifications is capable of enhancing the melting temperature of tRNAs. ALKBH1 deficiency improves the cellular level of tRNA-Met to maintain the functional effect of m1A, stabilizing tRNA-Met (Liu F. et al., 2016). On the contrary, deficiency of enzymes catalyzing the achievement of m1A, have the possibility of induce thermosensitivity (Oerum et al., 2017). Moreover, m1A in tRNA-Lys was found to play an important role in reverse transcription fidelity and participate in the process of HIV replication.

MECHANISM OF RNA METHYLATION IN HCC

Recently emerging evidence has demonstrated that RNA methylation plays a dramatic role in tumorigenesis, invasion, and migration of HCC and elucidated complex mechanisms. We present the evidence for regulators and the effect of m6A, m5C, and m1A related to initiation and progression of HCC in **Table 2**.

m6A Links to HCC

Numerous studies have recently probed the relationships between m6A methylation and HCC pathogenesis. Wang et al. demonstrated that circ-KIAA1429 is expressed at a higher level in HCC cells than in normal cells, and the patients generally have shorter survival times (Wang M. et al., 2020). In addition, upregulated circ-KIAA1429 can be found in node metastasis status. These results indicate the fact is that KIAA1429 serves as an oncogene to further HCC invasion and migration by altering the methylation of m6A in *ID2* and *GATA3* mRNA (Lan T. et al., 2019; Cheng et al., 2019). Previous evidence revealed that Zeb1 was considered to be the downstream target of KIAA1429. Meanwhile, YTHDF3 is able to increase the stability of *Zeb1* mRNA, which participates in HCC tumorigenesis. The lifetime of Zeb1 gain improved via the effect of m6A modification (Wang M. et al., 2020). It was reported that circ-KIAA1429 contributed to the growing of invasion and metastasis process in HCC together with the mechanism of m6A-YTHDF3-Zeb1. Chen et al. demonstrated that elevated expression of ALKBH5 can be seen as a critical suppressor to impede proliferation and invasion of HCC by regulating the downstream target LYPD1. In HCC, LYPD1 is considered the oncogene that triggers the physiological process. Silencing of LYPD1 impairs growth and invasion of HCC. ALKBH5 is capable of modulating m6A modification and is involved in the IGF2BP1-associated pattern to regulate target LYPD1 (Chen et al., 2020).

Previous survey unraveled HBXIP and METTL3 maintained high level in HCC patients. HBXIP could stimulate the occurrence of HCC cell malignant behaviors through the upregulation of METTL3 (Yang et al., 2021), catalyzing m6A methylation. METTL3 boosts HCC progression via post-transcriptional silencing of SOCS2 (Chen et al., 2018), whereas METTL3 knockdown reversed these effects by reducing m6A

TABLE 2 | The association of m6A, m5C, and m1A methylation in HCC.

Modification type	Regulators	Expression	Clinical characters	Function in HCC	Target	PMID
m6A	ALKBH5	Down	Favorable prognosis	Inhibit proliferation and invasion	LYPD1	32,772,918
m6A	METTL3	Up	Poor prognosis	Promote vascular invasion, and metastasis	HBXIP	33,305,825
m6A	YTHDF3	Up	Poor prognosis	promote invasion, migration, and EMT	Zeb1	32,653,519
m6A	METTL14	Down	Favorable prognosis	Inhibit invasion, migration, and EMT	EGFR/ PI3K/Akt	33,380,825
m6A	YTHDF1	Up	Poor prognosis	Promote proliferation, migration, and invasion	PI3K/Akt/ mTOR	34,088,349
m6A	FTO	Up	Poor prognosis	Promote initiation, metastasis, and chemoresistance	AMD1	33,783,988
m5C	NSUN2	Up	Poor prognosis and advanced TNM stage	Promote metastasis	H19	32,978,516
m5C	NSUN4	Up	Poor prognosis	—	—	32,269,723
m5C	ALYREF	Up	Poor prognosis	—	—	32,944,246
m1A	TRMT6	Up	Poor prognosis	—	PI3K/Akt	32,934,298

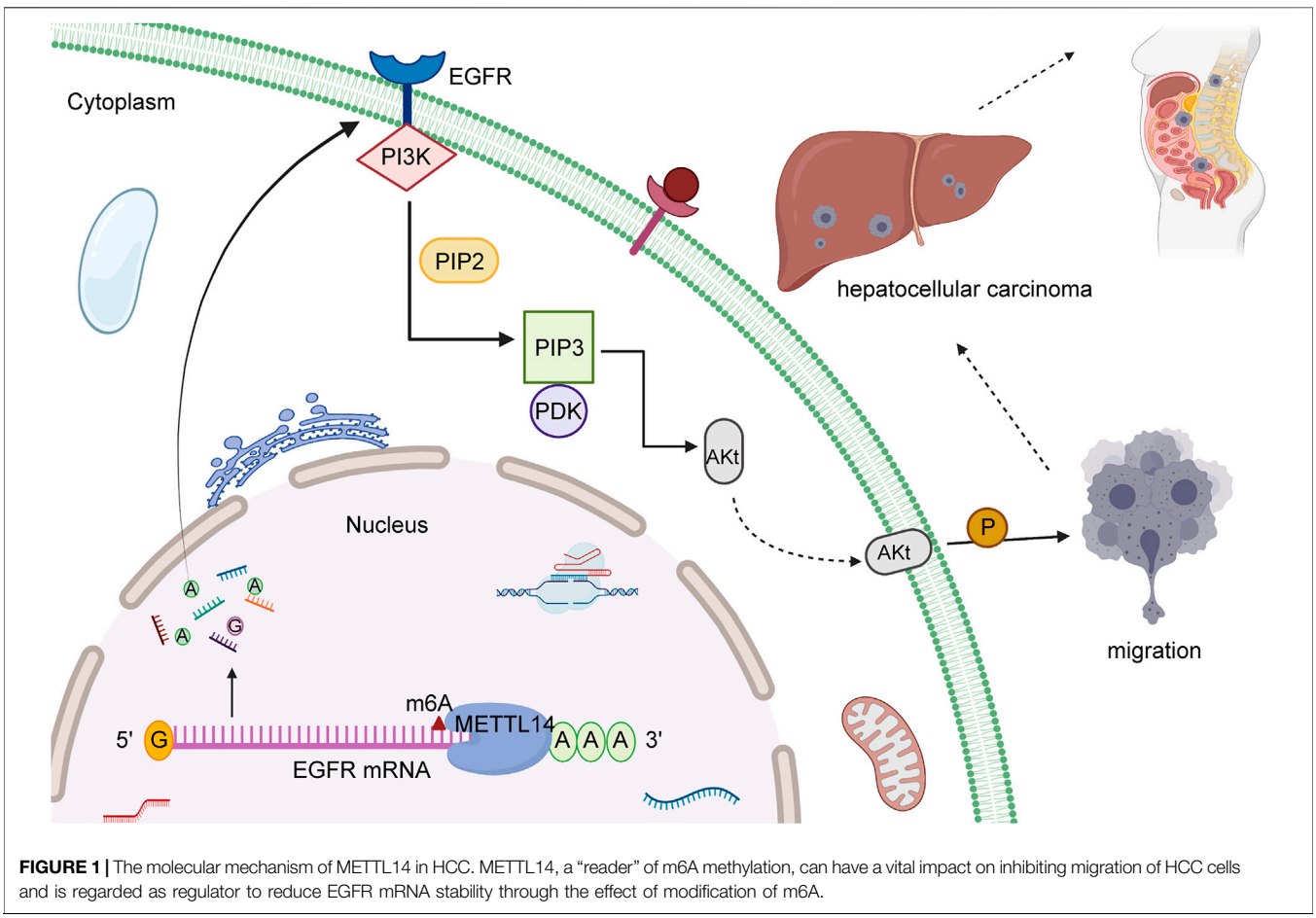

FIGURE 1 | The molecular mechanism of METTL14 in HCC. METTL14, a "reader" of m6A methylation, can have a vital impact on inhibiting migration of HCC cells and is regarded as regulator to reduce EGFR mRNA stability through the effect of modification of m6A.

methylation. In contrast, METTL14 has been found to block the metastasis program of HCC, which decreases the stability of *EGFR* mRNA via posttranscriptional modification of m6A in **Figure 1**. EGFR was reported to play a critical role in the pathogenesis of various malignant tumors, such as in breast, pancreatic, prostate, colorectal, and liver cancer. In HCC, EGFR has been confirmed to stimulate the PI3K-AKT signaling pathway and foster the invasive and metastatic capacity of cells. These evidences suggested that EGFR might have the potential to become the promising target for treatment of

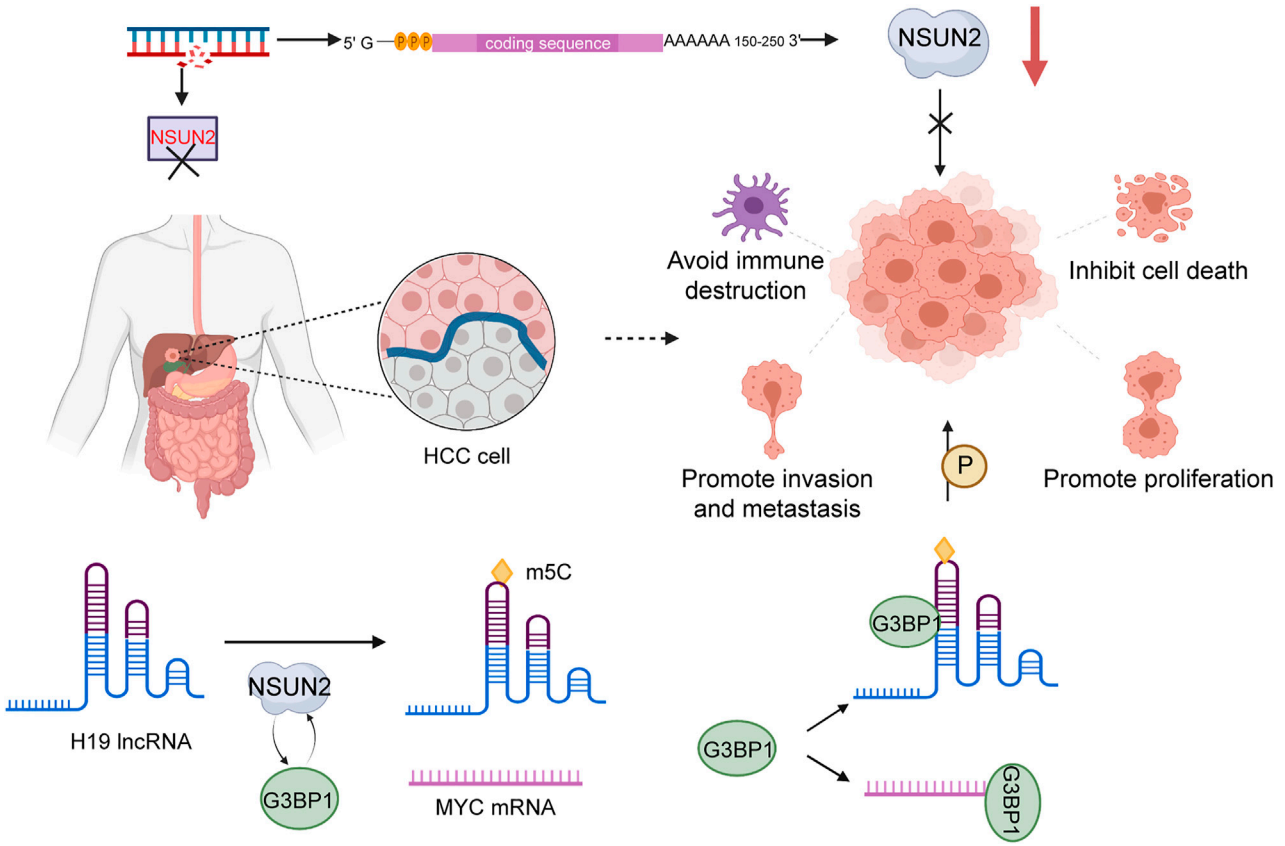

FIGURE 2 | The role of NSUN2 in HCC. NSUN2 catalyzes m5C methylation in H19 lncRNA and enhances the malignant potential of HCC by promoting the binding of G3BP1 and H19.

HCC. Consequently, upregulated METTL14 effectively prevents migration of HCC cells, and is associated with positive prognostic outcome in a majority of patients. The suppressive property of METTL14 was revealed in a number of experiments (Shi Y. et al., 2020). Li et al. displayed that YTHDF1, a "reader" of m6A methylation, is upregulated in patients related to HCC and ordinarily is associated with dismal prognosis. HIF-1α interacts with YTHDF1 promoters, and upregulation of YTHDF1 was observed in a HIF-1α dependent manner. HIF-1α has been widely identified to trigger the transcriptional target gene to respond to hypoxic stress. HIF-1α avoids enzymatic degradation during hypoxic stress (Li Q. et al., 2021). YTHDF1 expression, mediated by HIF-1α, supports that hypoxic stress might lead to the alteration of cancer epigenetics, such as the translation of m6A-modified oncogenic mRNAs, to facilitate HCC malignancy. A recent study corroborated that AMD1 expression is the independent factor for overall survival (OS) and disease-free survival (DFS) in HCC (Bian et al., 2021). Several investigations found high expression of AMD1 in HCC tissue and showed that AMD1 regulates the expression of NANOG, SOX2, and KLF4, which are involved in HCC initiation, metastasis, and chemoresistance. Nevertheless, knockdown of AMD1 might increase the sensitivity of HCC cells to sorafenib. Previous report illustrated that FTO can promote the transcription of gene through the effect of removing numerous m6A modifications in the positions of 5′-UTR and CDs. Upregulated FTO could restrain the effect of down-expressed AMD1. While FTO presents the condition of silencing, the effect of AMD1 overexpression will be reversed. As a result, FTO severe as the downstream target of AMD1, and avails the therapeutic advancement for HCC.

m5C Related to HCC

A recent survey demonstrated that m5C modification has effects on distribution in HCC tissues and normal tissues. Compared with adjacent non-tumor tissue, high expression of m5C was shown in HCC tissue, which indicated that m5C methylation is closely associated with HCC pathogenesis (He et al., 2020a). NSUN2, a methyltransferase mediating the modification m5C, was confirmed to be upregulated in a variety of tumors in a previous study. Sun et al. showed that NSUN2 is clearly upregulated in HCC tissue have obvious upregulation of NSUN2 than normal tissues, and NSUN2 is capable of promoting the appearance of phenomenon about poor differentiation in HCC. Consistently, NSUN2 knockdown blocked the proliferation, invasion, and migration of HCC cells. Furthermore, NSUN2 have the property of stabilizing H19 by methylating H19 lncRNA. Overexpression of H19 is

similarly found in HCC tissue with poor prognosis, and H19 is commonly seen as an important feature of poor differentiation in malignancy. Depletion of NSUN2 might give rise to cell inhibition in the G2 phase and prevent the increasing growth of HepG2 cells. Accumulating evidence demonstrates that the distribution of NSUN2 is variable during cell division; expression level is highest in S phase and lowest in G1. These results demonstrate that dynamic expression of NSUN2 has a profound impact on modulating cell division. NSUN2 catalyzed m5C methylation of H19 lncRNA to significantly affect malignant development of HCC. Consequently, H19 has the possibility of becoming a novel target of NSUN2. It was demonstrated that NSUN2 regulates m5C methylation of H19 lncRNA via interaction of Ras-GTPase–activating protein SH3 domain-binding protein 1 (G3BP1). G3BP1, a known oncoprotein that is generally expressed at a high level in multiple cancers that participate in diverse carcinogenesis-associated pathways containing Ras/MAPK (Liu S.-Y. et al., 2016), Wnt/β-catenin, PI3K/AKT (Zhang et al., 2019), and NF-κB/Her2 signaling pathways. These pathways could be regulated by NSUN2 through involvement in G3BP1 binding to H19 lncRNA (Sun et al., 2020), playing an essential role in malignant progression of HCC. In addition, G3BP1 also binds *MYC* mRNA to advance the effect of degradation (Tourrière et al., 2001). H19 lncRNA promotes tumor proliferation by binding G3BP1 and competing with *MYC* mRNA. When H19 lncRNA is poorly methylated, binding to G3BP1 will be further attenuated. Interestingly, MYC was found to accelerate H19 lncRNA transcription (Barsyte-Lovejoy et al., 2006). Therefore, the MYC-NSUN2-H19-G3BP1 axis was revealed to be associated with malignant behaviors of HCC (**Figure 2**). Moreover, the methylation modification could bring the decline number of circRNA, resulting in lack of suppression from crucial proteins and inducing the initiation of tumors (He et al., 2020b).

m1A Associated With HCC

As a burgeoning discussion hotspot, research on m1A modification links to multiple cancers is also gradually becoming the basis of extensive concern. The understanding of m1A function related to HCC still requires further exploration. Shi et al. illustrated that TP53 mutations were primarily correlated with regulators mediating m1A methylation (Shi Q. et al., 2020). TP53 is a suppressor of various malignancies. However, the occurrence of TP53 mutations rapidly promotes tumorigenesis; for example, TP53 mutations serve as prognostic indicators of short survival time in HCC. Additionally, m1A-associated regulators expression actively has the impact on promoting progression of high TNM stage, including expression of RMT6, TRMT61A, TRMT10C, and TRMT6. It was reported YTHDF1 is valuable in predicting prognosis due to improving TRMT6 expression. Mounting survey unravel that m1A methylation might be regulated by the PI3K/Akt signaling pathway in HCC. The PI3K/Akt pathway plays a key role in proliferation and inhibition of apoptosis in HCC (Fu et al., 2019; Zheng et al., 2019). Nevertheless, how the PI3K/Akt pathway is involved in m1A and induces the development of HCC still needs further study. These findings suggest m1A has the potential to become a valuable biomarker in HCC.

CONCLUSION

RNA methylation has emerged as the post-transcriptional modification to significantly affect a variety of genes expression processes, which not only has a broad influence on RNA metabolism but alters the function of various RNAs. Numerous proteins regulate methylation, demethylation, and specifically bind to diverse RNAs to promote or inhibit the biological functions, and are referred to, respectively, as “writers,” “erasers,” and “readers”. Prior research found aberrant expression of these regulators might lead to increasing disease. We summarize the distribution and functional consequences of m6A, m5C, and m1A modifications to further understand the role of RNA methylation and corresponding physiological mechanisms in HCC. For instance, overexpression of NSUN2 could promote malignant behaviors of HCC. METTL14, the “writer” of m6A, was proved to prevent metastasis of HCC. In this review, we found that RNA methylation may potentially serve as a novel marker and make valuable contributions to diagnosis and treatment in HCC, providing a promising future for a great many patients. Simultaneously, many studies are necessary to further explore and testify for clinical application.

AUTHOR CONTRIBUTIONS

Xu YT, Zhang MG, and Zhang QY drafted manuscript. Yu X and Sun ZZ drew the mechanism diagrams. He YT, and Guo WZ conceived of the study and guided the analysis. He YT, and Xu YT edited and reviewed the manuscript. All authors read and approved the final manuscript.

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Analysis of N6-Methyladenosine Modification Patterns and Tumor Immune Microenvironment in Pancreatic Adenocarcinoma

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Background: Pancreatic adenocarcinoma (PAAD) is a rare cancer with a poor prognosis. N6-methyladenosine (m6A) is the most common mRNA modification. However, little is known about the relationship between m6A modification and the tumor immune microenvironment (TIME) in PAAD.

Methods: Based on 22 m6A regulators, m6A modification patterns of PAAD samples extracted from public databases were systematically evaluated and correlated with the tumor immune and prognosis characteristics. An integrated model called the "m6Ascore" was constructed, and its prognostic role was evaluated.

Results: Three different m6Aclusters and gene clusters were successively identified; these clusters were characterized by differences in prognosis, immune cell infiltration, and pathway signatures. The m6Ascore was constructed to quantify the m6A modifications of individual patients. Subsequent analysis revealed that m6Ascore was an independent prognostic factor of PAAD and could be a potential indicator to predict the response to immunotherapy.

Conclusion: This study comprehensively evaluated the features of m6A modification patterns in PAAD. m6A modification patterns play a non-negligible role in the TIME of PAAD. m6Ascore provides a more holistic understanding of m6A modification in PAAD, and will help clinicians predict the prognosis and response to immunotherapy.

Keywords: N6-methyladenosine, pancreatic adenocarcinoma, tumor immune microenvironment, m6Ascore, prognosis

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INTRODUCTION

Pancreatic adenocarcinoma (PAAD) is a rare cancer with an incidence of 12.9 cases per 100,000 person-years. Although its incidence is low, PAAD is the third and fifth most common cause of cancer death in the United States and the United Kingdom, respectively (O'Reilly et al., 2018; Owens et al., 2019). Surgical intervention is the only way to improve the chance of long-term survival (Bilimoria et al., 2007); however, most PAAD cases present with unresectable disease, which is due to either locally advanced or metastatic disease (Singhi et al., 2019). Despite the use of different therapeutic measures, the median survival time is only 6–12 months (Warshaw and Castillo, 1992;

Mizrahi et al., 2020). According to recent data from the National Cancer Institute (NCI), the five-year survival rate for patients with localized PAAD is 37.4%. When distant metastases occur, the five-year survival rate drops to 2.9% (Owens et al., 2019). Since the prognosis is such poor, elucidating the genetic feature of PAAD is vital for developing valid treatments and predicting the prognosis.

In recent years, epigenetic modifications have been confirmed to be implicated in a variety of biological processes and disease progression. They mainly involved chromatin remodeling, DNA methylation, RNA modification, and histone modification (McGee and Hargreaves, 2019). More than 100 different types of post-transcriptional modifications have been confirmed in RNA (Pinello et al., 2018). Among them, N6-methyladenosine (m6A) is the most abundant mRNA modification in mammals (Dai et al., 2018). It is a reversible and complex RNA epigenetic process regulated by the interactions among m6A regulators, including "writers" (methyltransferases), "readers" (binding proteins), and "erasers" (demethylases) (Zaccara et al., 2019). m6A is involved in a variety of biological and disease processes by regulating target gene expression (Chen et al., 2019; Lan et al., 2019). Previous studies have shown that m6A is involved in cancer development and progression, including acute myeloid leukemia, breast cancer, glioblastoma, lung cancer, and hepatocellular carcinoma (Zhang et al., 2016; Li et al., 2017; Xiang et al., 2017; Dai et al., 2018). Recently, Zhou et al. (2021) constructed a model including 9 m6A regulators and found it could predict tumor aggressiveness and immune evasion in PAAD. However, the model is limited to the number of m6A regulators, while the role of them in the development and progression of PAAD depends on the interaction among multiple m6A regulators.

In this study, we systematically evaluated the features of m6A modification pattern and tumor immune microenvironment (TIME) in PAAD patients. Based on the m6A regulators and related genes, a model (termed "m6Ascore") was constructed and then proposed as a potential molecular classification method of PAAD. The study also demonstrated that the m6Ascore could serve as a potential tool to predict the prognosis and response to immunotherapy.

MATERIALS AND METHODS

Data Extraction and Processing

The RNA sequencing (RNA-seq) transcriptome data and corresponding clinicopathological features of PAAD samples were obtained from The Cancer Genome Atlas (TCGA) database in April 2021. Gene expression data (measured in fragments per kilobase of exon per million fragments mapped or FPKM) was transformed into transcripts per kilobase million (TPM). Simple nucleotide variation data was extracted from the TCGA database, while the copy number variation (CNV) data was obtained from the UCSC Xena Website (https://xena.ucsc.edu/). Sample differences in the tumor microenvironment (TME) were measured using Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data (ESTIMATE) analysis with the "estimate" R package (Yoshihara et al., 2013). In addition, an eligible PAAD cohort (GSE21501) was downloaded from the Gene Expression Omnibus (GEO) database. In subsequent analysis, TCGA and GEO datasets were selected as training and validation sets, respectively.

Acquirement of m6A Regulators and Survival Analysis

A total of 22 m6A regulators were collected from relevant studies (Li et al., 2020a; Yi et al., 2020; Zheng et al., 2021); the regulators included 7 "writers" (WTAP, METTL16, VIRMA, RBM15B, METTL3, RBM15, and ZC3H13), 13 "readers" (YTHDC1, YTHDF1, YTHDC2, YTHDF3, IGF2BP2, LRPPRC, YTHDF2, HNRNPA2B1, HNRNPC, RBMX, EIF3A, G3BP1 and FXR1), and 2 "erasers" (ALKBH5 and FTO). The prognostic role of the m6A regulators was assessed using the Kaplan-Meier (KM) diagrams and Cox proportional hazards model.

Consensus Clustering of m6A Regulators

Based on the expression matrix of 22 m6A regulators, patients in the TCGA cohort were classified into distinct clusters according to the best cutoff using the "ConsensusClusterPlus" R package (Wilkerson and Hayes, 2010). The number of clusters and their stability were confirmed by the consensus clustering algorithm. Survival analysis between distinct clusters was measured using the KM method. Differences in the biological processes between the distinct clusters were investigated through gene set variation analysis (GSVA) (Hänzelmann et al., 2013). The "c2.cp.kegg.v7.4.symbols" gene set was obtained from the Molecular Signatures Database (MSigDB). Adjusted p-value<0.05 was considered statistically significant.

Comparison of the TIME Between Distinct m6Aclusters

The single-sample Gene Set Enrichment Analysis (ssGSEA) algorithm was used to quantify the relative abundance of various immune cell subtypes in PAAD samples (Charoentong et al., 2017). Through enrichment score calculated by ssGSEA, the relative abundance of each immune cell type was represented in each sample. ESTIMATE analysis was performed to compare the differences in the TME with the "estimate" R package (Yoshihara et al., 2013). Furthermore, differences in the TIME and the expression of targeted immune checkpoint molecules between the distinct clusters were compared using the "limma" R package.

Identification of Prognosis-Related DEGs Between m6Aclusters

Principal component analysis (PCA) was used to test whether m6A regulators could separate distinct m6A modification patterns. Differentially expressed genes (DEGs) among the m6Aclusters were identified using the empirical Bayesian approach with the "limma" R package. The significance criterion of DEGs was set as p-value < 0.0001. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

analysis and Gene Ontology (GO) biological processes analysis were performed to investigate the pathway signatures of the DEGs. A critical value of adjusted p-value = 0.05 was selected as the filter criteria. After identifying the DEGs, prognosis-related genes were filtrated from the DEGs by univariate Cox regression analysis. The significance criterion was set as p-value < 0.001.

Consensus Clustering of Prognosis-Related DEGs

Based on the expression of prognosis-related DEGs, samples in the TCGA cohort were classified into different subtypes according to the best cutoff using the "ConsensusClusterPlus" R package. The KM method was used to perform survival analysis between different subtypes. A heatmap revealed the expression of prognosis-related DEGs between different subtypes using the "pheatmap" R package. Furthermore, differences in immune cell infiltration, ESTIMATE score and the expression of targeted immune checkpoint molecules were compared with the "limma" R package.

Construction of the m6Ascore Model

Based on the expression of prognosis-related DEGs, PCA was used to score the samples in the TCGA and GEO cohorts. Principal components 1 and 2 were used to act as signature scores. The m6Ascore was defined using a method similar to Genomic Grade Index (GGI) (Sotiriou et al., 2006; Zeng et al., 2019):

$$\text{m6Ascore} = \sum (\text{PC1 i} + \text{PC2 i})$$

Where i is the expression of overlapping genes with significant prognosis-related DEGs the m6Aclusters.

According to the score, samples were divided into low- and high-m6Ascore groups. We then compared the biological differences between the low- and high-m6Ascore groups, including (O'Reilly et al., 2018) survival analysis (Owens et al., 2019), immuno-correlation analysis (Bilimoria et al., 2007), clinical-correlation analysis (Singhi et al., 2019), tumor mutation burden (TMB) (Mizrahi et al., 2020), targeted immune checkpoint molecules.

Statistical Analysis

All statistical analyses in the study were performed using the R software (version 4.0.5). The Kruskal-Wallis test was used to perform difference comparison on three or more groups (Hazra and Gogtay, 2016). Continuous variables were dichotomized for patient survival using the optimal cutoff values determined by "survminer" R package. The KM and log-rank tests were used to evaluate the survival difference among different clusters. The CNV landscape of 22 m6A regulators in 23 pairs of chromosomes was plotted using the "RCircos" R package. The receiver operating characteristic (ROC) curves (R package "timeROC") and the area under the curve (AUC) values were used to evaluate the prognostic ability of the m6Ascore for 1-, 2-, 3-, and 4-year overall survival (OS) (Blanche et al., 2013). Univariate and multivariate independent prognostic analyses were performed to assess whether the model was an independent prognostic factor of PAAD. All statistical p values were two-sided, with $p < 0.05$ deemed statistically significant.

RESULTS

Expression Variation of the m6A Regulators in PAAD

A total of 22 m6A regulators (7 "writers," 13 "readers," and 2 "erasers") were collected in this study. CNVs and somatic mutations were integrated to explore the prevalence of m6A regulator variations in PAAD. The CNV incidence of 22 m6A regulators are shown in **Figure 1A**. Most regulators focused on the deletion of copy number, while VIRMA, G3BP1, and other five m6A regulators had a prevalent frequency of CNV amplification. The CNV landscape of m6A regulators in 23 pairs of chromosomes are shown in **Figure 1B**. The overall average mutation frequency of m6A regulators was low, with only 6 (3.8%) of 158 samples having m6A regulator mutation (**Figure 1C**).

The prognostic value of the m6A regulators was evaluated by KM method and univariate Cox regression analysis (**Supplementary Figure S1**; **Supplementary Table S1**). The results showed that most m6A regulators were associated with survival. The network of m6A regulators comprehensively demonstrated the m6A regulators' interactions, connection, and prognostic significance for PAAD patients (**Figure 1D**). The results showed that there was a distinct positive correlation between each other. Most regulators, such as IGF2BP2 and HNRNPC, presented tumorigenic characteristics, with higher gene expression levels correlating with poor prognosis. Conversely, several m6A regulators, such as ALKBH5 and METTL16, presented tumor-suppressing characteristics, with higher gene expression levels relating to favorable prognosis. These results suggested that the interrelations among regulators may have important effects on the development and progression of PAAD.

m6A Modification Patterns Mediated by m6A Regulators

Based on the expression of 22 m6A regulators, model-based clustering was performed to classify PAAD patients using the "ConsensusClusterPlus" R package. Through unsupervised clustering, three different m6A modification patterns were uncovered ultimately (identified as m6Aclusters A-C), including 30 samples in cluster A, 42 samples in cluster B, and 105 samples in cluster C (**Figure 2A**). Prognostic analysis showed there was a survival disadvantage in m6Acluster B (**Figure 2B**). The heatmap showed m6Acluster A presented significantly low expression levels of all m6A regulators, while m6Acluster B was characterized by the high expression levels of all m6A regulators (**Figure 2C**). Moreover, GSVA showed different biological behaviors between the m6Aclusters (**Figures 2D–F**). The results suggested that different m6A modifications had significant correlation with biological behaviors and prognosis of PAAD.

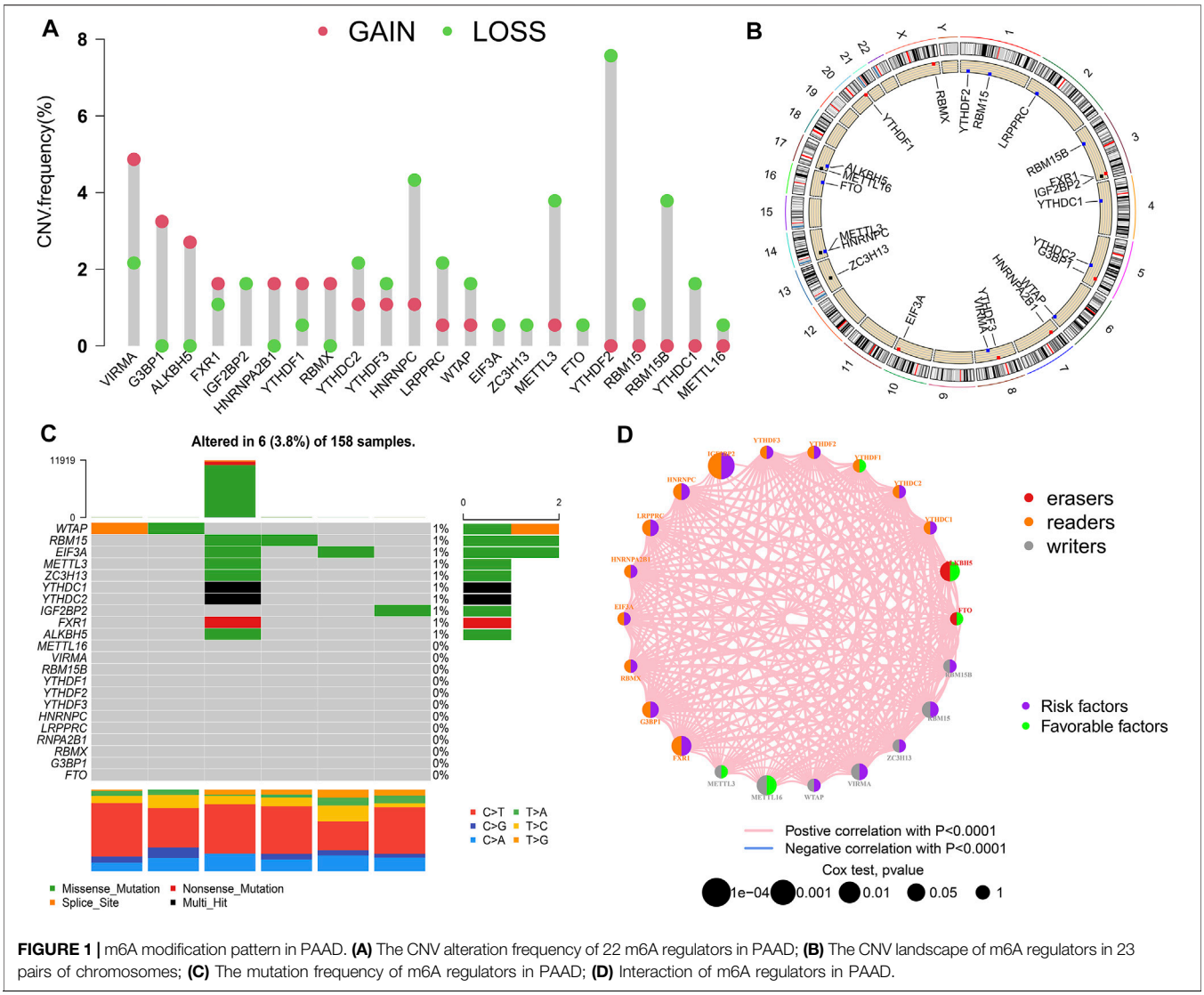

FIGURE 1 | m6A modification pattern in PAAD. **(A)** The CNV alteration frequency of 22 m6A regulators in PAAD; **(B)** The CNV landscape of m6A regulators in 23 pairs of chromosomes; **(C)** The mutation frequency of m6A regulators in PAAD; **(D)** Interaction of m6A regulators in PAAD.

Tumor Immune Landscape in Distinct m6Aclusters

Using ssGSEA, the study analyzed 23 different immune cell types in the m6Aclusters. The result revealed that m6Acluster B, which had a poor prognosis, was enriched in activated NK cells, mast cells, and T helper type 2 (Th2) cells. However, the abundance of CD56dim NK cells was enriched in m6Aclusters A and C (**Figure 3A**). These results indicated that m6A modification was associated with the infiltration of specific immune cell types and influenced the response to immunotherapy. In addition, the results of the ESTIMATE algorithm revealed that the stromal and ESTIMATE scores ($p < 0.05$) were higher in cluster B than in clusters A and C (**Figures 3B–D**). Combined with the heatmap, the study found that the expression level of m6A regulators showed a similar trend with the ESTIMATE score. Characterized by the higher expression levels of m6A regulators, m6Acluster B also had a higher ESTIMATE score. The results suggested that m6A regulators may play an important role in the regulation of the TME, thus affecting tumor progression and survival. Furthermore, the expression of targeted immune checkpoint molecules was different between the distinct clusters. As shown in the boxplots, the expression of the CTLA-4 gene was markedly high in m6Acluster B and the expression levels of the PD-1 and PD-L1 genes were markedly low in m6Acluster C (**Figures 3E–G**).

Generation of m6A Gene Clusters

PCA showed that m6A regulators could separate distinct m6A modification patterns perfectly (**Figure 4A**). To further investigate the potential biological behavior of each m6Acluster, a total of 2457 DEGs among three m6Aclusters were extracted eventually (**Figure 4B**). Similarly, the "clusterProfiler" R package was used to implement GO enrichment analysis and KEGG pathway analysis for the DEGs. The results showed that the DEGs were enriched in biological processes related to tumorigenesis and tumor progression, such as FoxO signaling pathway and ErbB

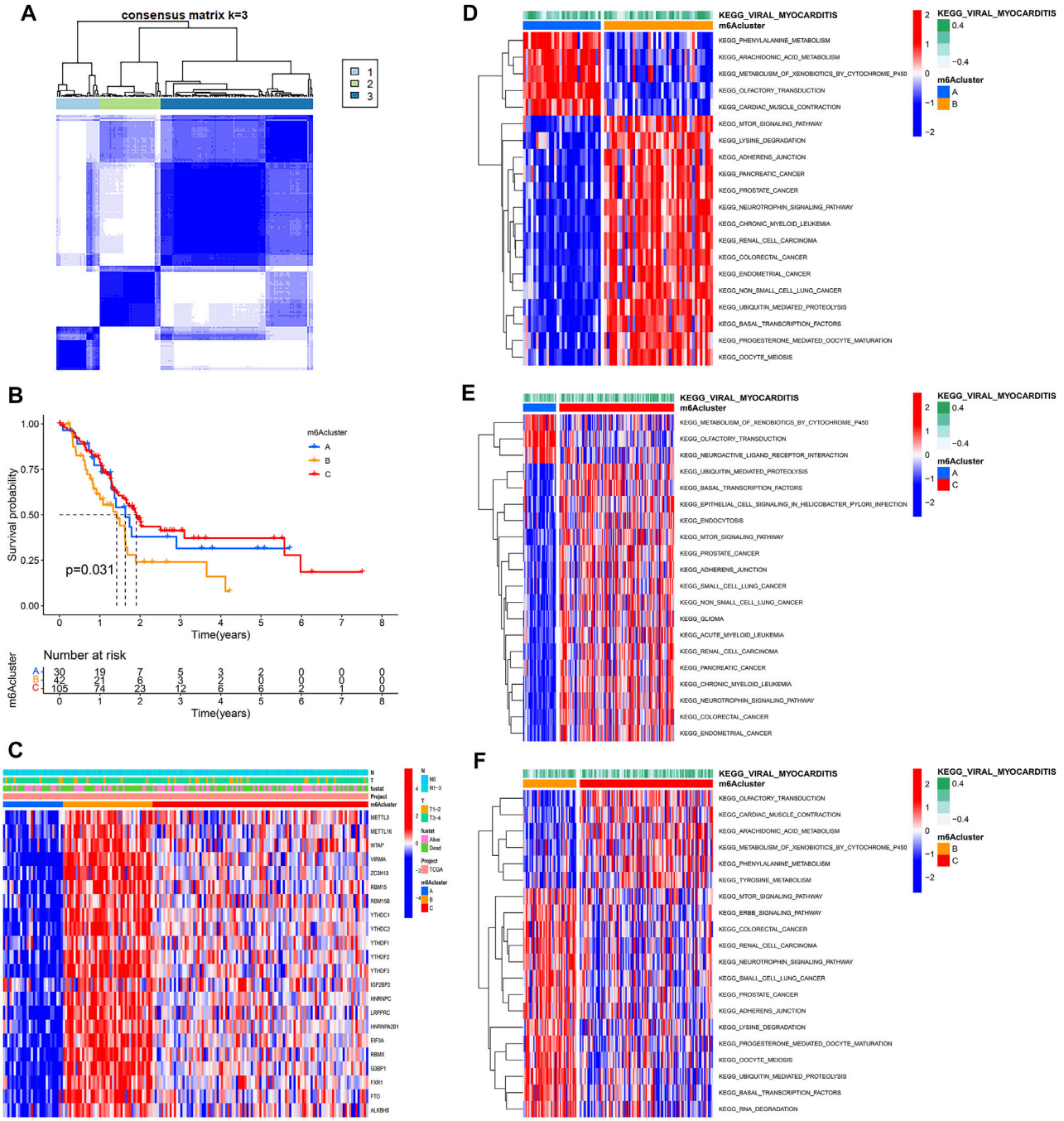

FIGURE 2 | Molecular characteristics of m6Aclusters. **(A)** Consensus clustering matrix for k = 3; **(B)** KM analysis of patients in distinct m6Aclusters; **(C)** The heatmap depicted the expression levels of m6A regulators in distinct m6A clusters; **(D)** GSVA enrichment analysis between m6Acluster A and m6Acluster B; **(E)** GSVA enrichment analysis between m6Acluster A and m6Acluster C; **(F)** GSVA enrichment analysis between m6Acluster B and m6Acluster C.

signaling pathway (**Figures 4C,D**). The results revealed that m6A modification played a significant role in the tumorigenesis and tumor progression of PAAD.

To further investigate this regulation mechanism, univariate Cox regression analysis was performed to extract prognosis-related genes among the DEGs (**Supplementary Table S2**). Based on the 53 prognosis-related DEGs obtained, patients were divided into three genomic subtypes through unsupervised clustering analyses (gene clusters A-C) (**Figure 5A**). Survival analysis showed that patients in gene cluster B had a worse outcome, while these in gene cluster C showed a prominent survival advantage (**Figure 5B**). The heatmap revealed that gene clusters A-C were characterized by different signature genes (**Figure 5C**). Prognosis-related DEGs

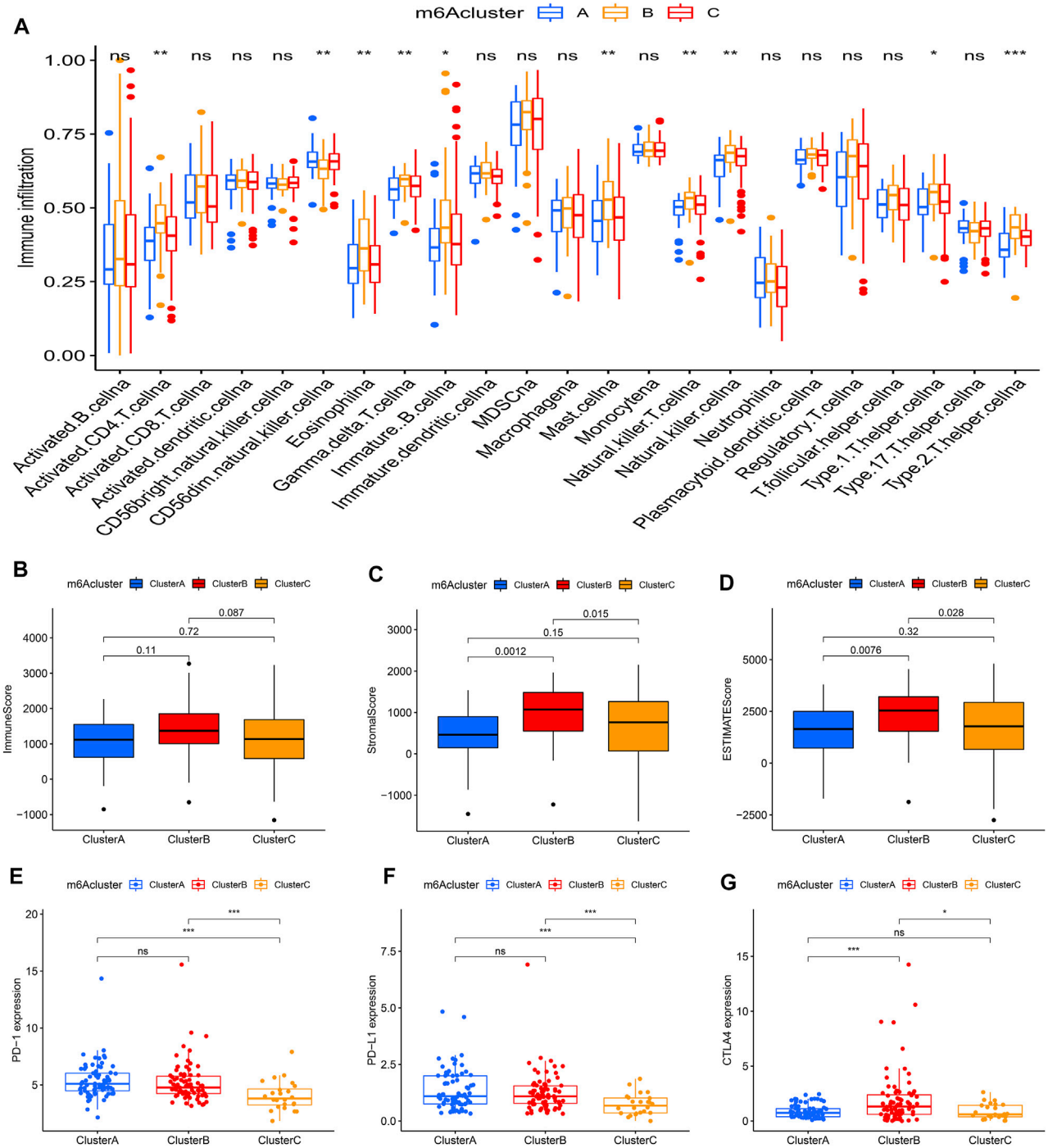

FIGURE 3 | Tumor immune landscapes in distinct m6Aclusters. **(A)** ssGSEA of patients in distinct m6Aclusters, the asterisks represented the statistical *p* value (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); **(B)** Immune score of patients in distinct m6Aclusters; **(C)** Stromal score of patients in distinct m6Aclusters; **(D)** ESTIMATE score of patients in distinct m6Aclusters; **(E)** PD-1 expression of patients in distinct m6Aclusters (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); **(F)** PD-L1 expression of patients in distinct m6Aclusters (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); **(G)** CTLA4 expression of patients in distinct m6Aclusters (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

were overexpressed in gene cluster B, and under-expressed in gene cluster C. Moreover, gene cluster B showed higher expression levels of all m6A regulators, while gene cluster C had lower expression levels of all m6A regulators (**Figures 5D**; **Supplementary Figure S2A**). Similarly, GSVA showed different biological behaviors between three gene clusters (**Supplementary Figures S2B–D**). In addition, ssGSVA showed gene cluster B had a high abundance of Th2 cells and NK cells (**Figure 6A**). The

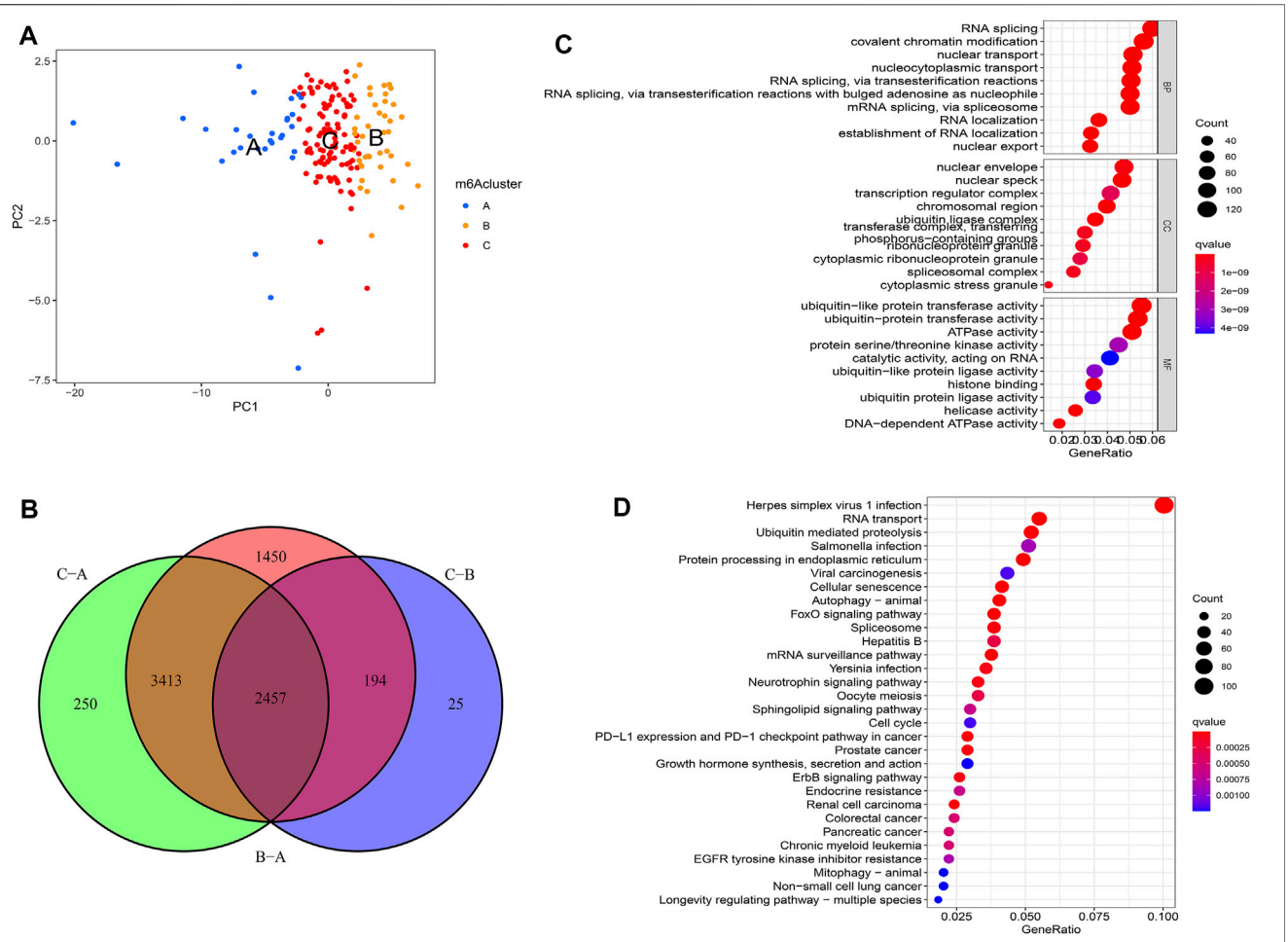

FIGURE 4 | Molecular characteristics of differentially expressed genes (DEGs) among m6Aclusters. **(A)** PCA among distinct m6Aclusters; **(B)** The DEGs extracted between three m6A clusters; **(C)** GO enrichment analysis for the DEGs; **(D)** KEGG pathway analysis for the DEGs.

stromal and ESTIMATE scores ($p < 0.05$) were higher in cluster B compared to cluster C (**Figures 6B–D**). The results further demonstrated that m6A modification patterns were tightly associated with the TME of PAAD. Similarly, the expression of targeted immune checkpoint molecules was different between the distinct gene clusters (**Figures 6E–G**).

Construction of the m6Ascore Model

Considering the individual heterogeneity and complexity of m6A modification in PAAD, the study used PCA to quantify the m6A modification pattern of samples based on the prognosis-related DEGs and then divided them into low- and high-m6Ascore groups. The alluvial diagram was used to visualize the attribute changes of individual samples, which showed that m6Acluster B had a high proportion of gene cluster B and was linked to a low m6Ascore (**Figure 7A**). Furthermore, Kruskal-Wallis test indicated a difference in m6Ascore among the m6Aclusters. The results showed that m6Acluster B had the lowest median score, while m6Acluster A had the highest median score (**Figure 7B**). The similar results were obtained when analyzing the correlation between m6Ascore and gene clusters. Gene cluster B had the lowest median score, while gene cluster C had the highest median score (**Figure 7C**).

Patients in the high m6Ascore group demonstrated a prominent survival benefit in both the TCGA cohort and GEO cohort (GSE21501) (**Figures 7D,E**). Moreover, there was a negative correlation between m6Ascore and survival state. The low m6Ascore group had a high proportion of patients in dead (**Figures 7F,G**). As shown in **Figure 7H**, m6Ascore was markedly related to OS (hazard ratio (HR): 0.920, 95% confidence interval (CI): 0.881–0.961, $p < 0.001$). In addition, a multivariate Cox regression model including age, gender, tumor grade, m6Ascore, and tumor stage confirmed that m6Ascore was an independent prognostic factor of PAAD (HR: 0.927, 95% CI: 0.885–0.970, $p = 0.001$) (**Figure 7G**). ROC analysis revealed that m6Ascore had an acceptable prognostic value for PAAD patients (1-year AUC = 0.6671, 2-year AUC = 0.6657, 3-year AUC = 0.7171, 4-year AUC = 0.7708; respectively) (**Figure 8A**). These results indicated that m6Ascore had a robust and stable OS-predictive ability for PAAD.

As shown in **Figures 8B,C**, there was a negative correlation between the m6Ascore and the TMB. Patients with high TMB had a low m6Ascore. Survival analysis revealed that patients with high

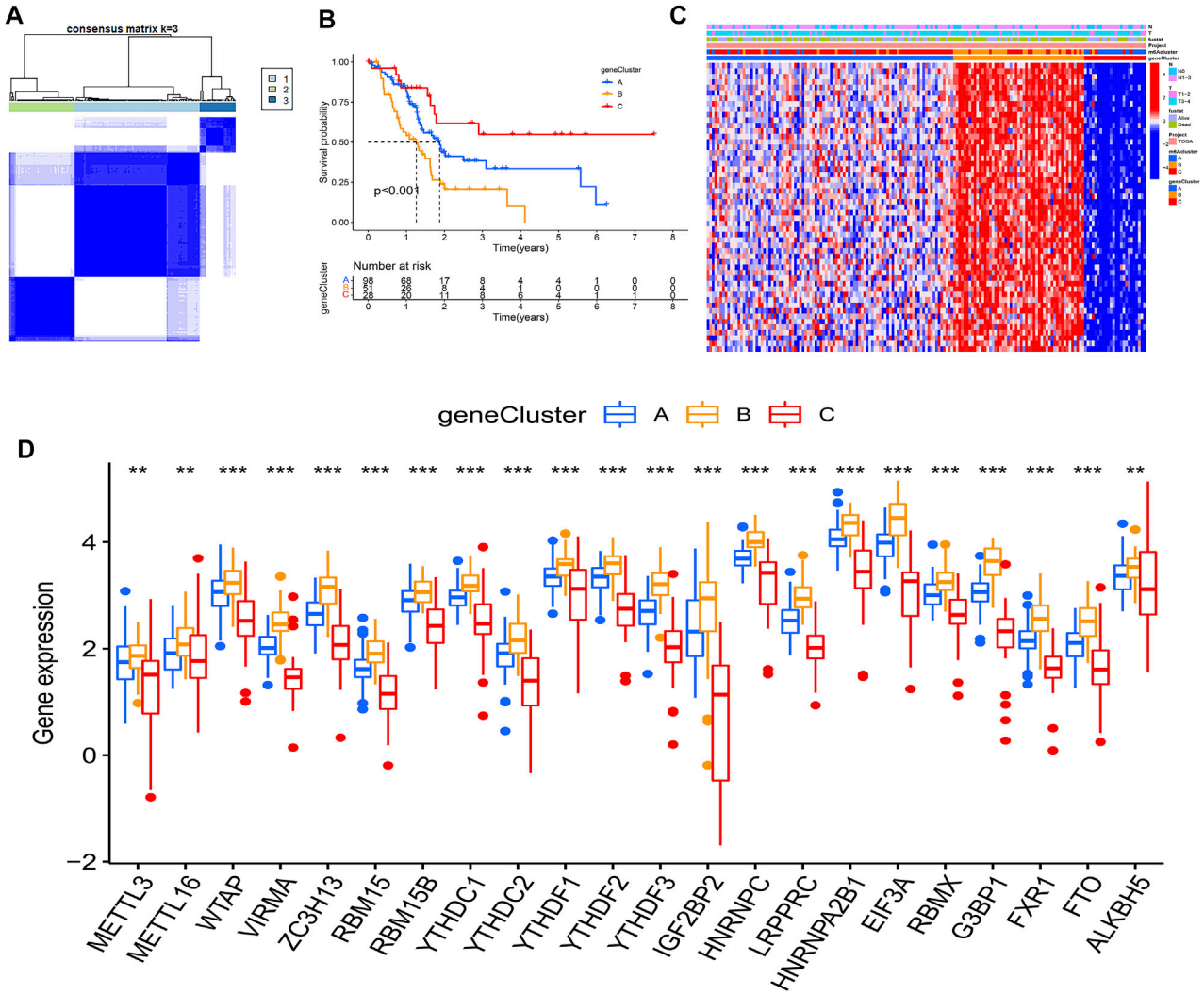

FIGURE 5 | Molecular characteristics of gene clusters. **(A)** Consensus clustering matrix for k = 3; **(B)** KM analysis of patients in distinct gene clusters; **(C)** The heatmap depicted the expression levels of prognosis-related DEGs in distinct gene clusters; **(D)** Expression of m6A regulators between distinct gene clusters, the asterisks represented the statistical *p* value (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

TMB had a poor prognosis; and the similar result was obtained from patients who had high TMB and low m6Ascore (**Figures 8D,E**). Of note, there were negative correlations between m6Ascore and some infiltrated immune cells, including activated Th2 cells, regulatory T cells, and NK cells et al. In addition, the expression levels of the PD-1and PD-L1 genes were higher in patients with low m6Ascore, while there was no difference in the CTLA4 gene expression (**Figures 8G–I**).

DISCUSSION

PAAD is a highly lethal malignancy, and its therapy remains a formidable challenge (Leinwand and Miller, 2020). Clinical efforts to use immune therapy have been shown to be largely ineffective for PAAD patients (Feng et al., 2017). With such a poor outcome, it is urgent to investigate the genetic features of PAAD and identify novel therapeutic strategies to improve its prognosis. m6A is regarded as the most pervasive, abundant, and conserved internal modification in RNAs, including mRNA, non-coding RNA, and ribosomal RNA (Fazi and Fatica, 2019). Considerable evidence indicated that the collaboration between m6A regulators played an important role in tumorigenesis, tumor progression, and immune response (Chang et al., 2019; Li et al., 2020b; Zhang et al., 2020). Previous studies have demonstrated that m6A modification was associated with the occurrence and development of PAAD. WTAP could promote tumor metastasis via stabilizing Fak mRNA and would result in a poor prognosis (Li et al., 2021a). Meanwhile, Xia et al. (2019) revealed that METTL3 promoted tumor cell proliferation and invasion, and could be a treatment target. In addition, it was found that ALKBH5 could prevent tumor progression by regulating the

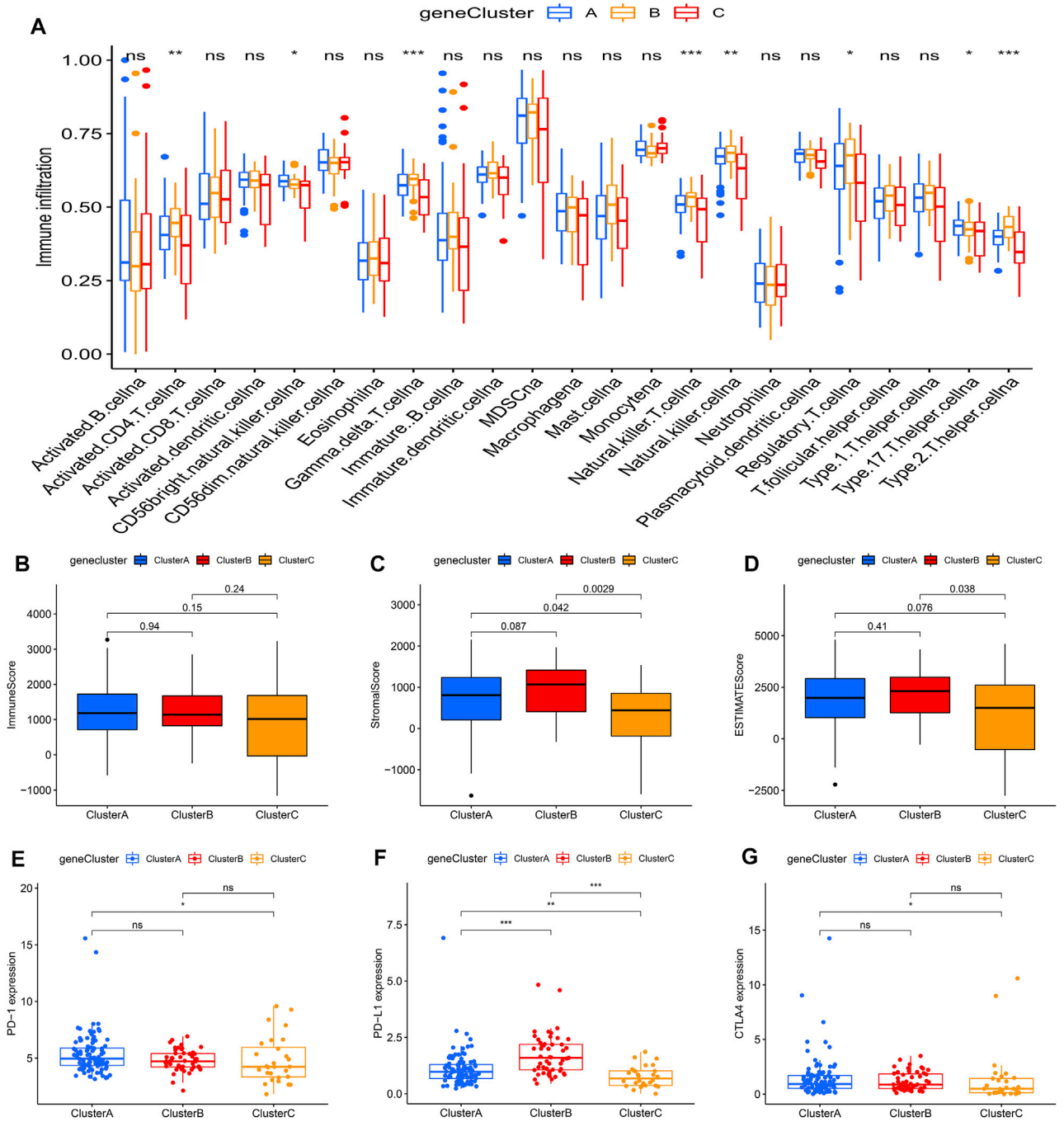

FIGURE 6 | Tumor immune landscapes in distinct gene clusters. **(A)** ssGSEA of patients in distinct gene clusters, the asterisks represented the statistical *p* value (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); **(B)** Immune score of patients in distinct gene clusters; **(C)** Stromal score of patients in distinct gene clusters; **(D)** ESTIMATE score of patients in distinct gene clusters; **(E)** PD-1 expression of patients in distinct gene clusters (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); **(F)** PD-L1 expression of patients in distinct gene clusters (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); **(G)** CTLA4 expression of patients in distinct gene clusters (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

posttranscriptional activation of PER1 (Guo et al., 2020). However, almost all studies have focused on single m6A regulator only. How m6A modification pattern mediate the TIME and tumor survival in PAAD remains unknown.

In this study, based on data from public databases, we comprehensively and systematically profiled the m6A modification patterns in PAAD patients. Using unsupervised clustering analyses, three m6Aclusters and gene clusters have been successively identified. A series of biological analyses were performed to explore the relationship between the m6A-related genes and the TIME in PAAD. In addition, a model called "m6Ascore" was constructed to quantify the m6A

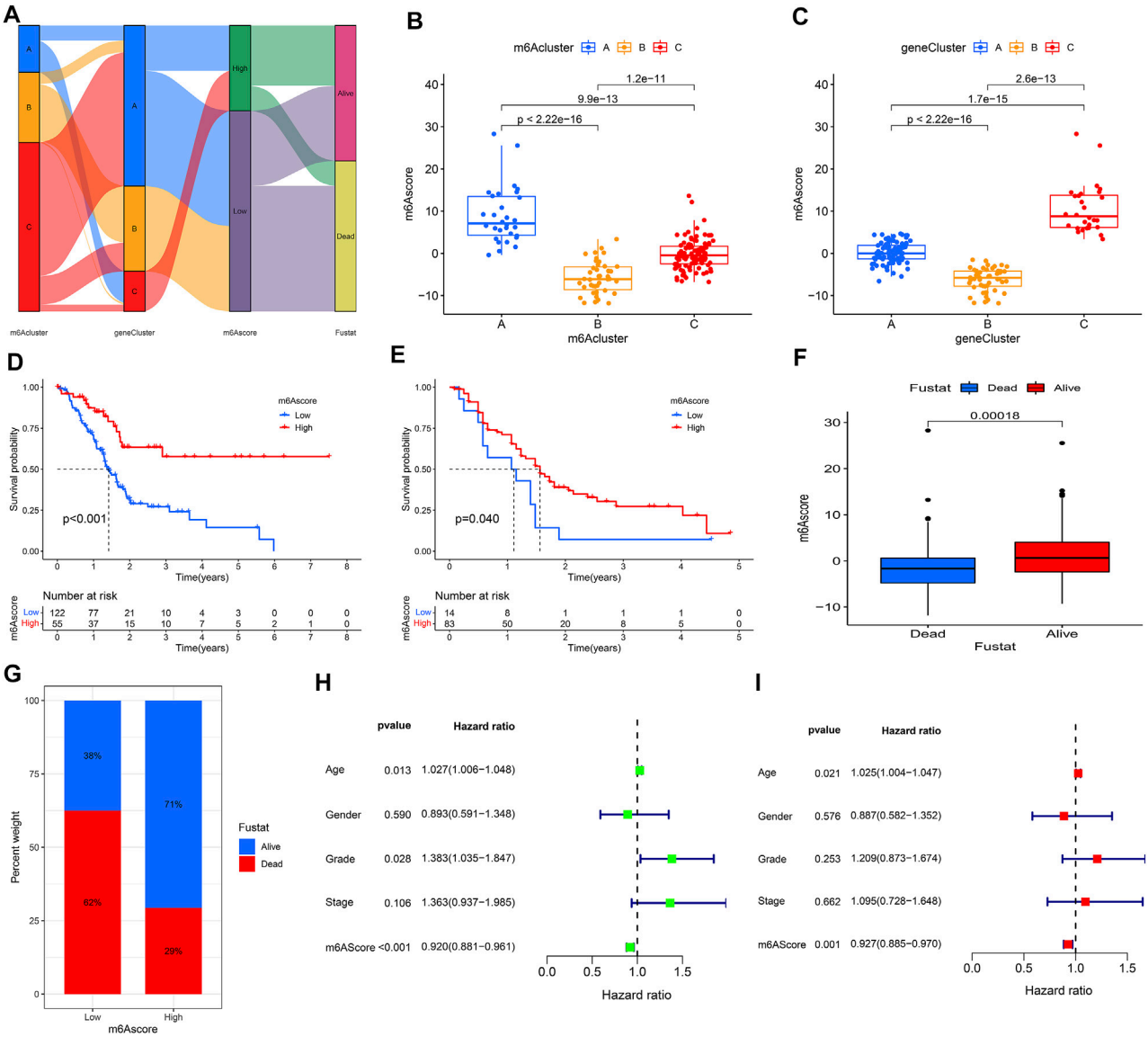

FIGURE 7 | Construction of m6Ascore model. **(A)** Alluvial diagram showed the changes of m6Aclusters, gene clusters, and m6Ascore; **(B)** Differences in m6Ascore between three m6Aclusters; **(C)** Differences in m6Ascore between three gene clusters; **(D)** KM analysis of patients in the high- and low-m6Ascore groups (TCGA cohort); **(E)** KM analysis of patients in the high- and low-m6Ascore groups (GEO cohort); **(F,G)** Clinical correlation analysis; **(H)** Univariate independent prognostic analysis; **(I)** Multivariate independent prognostic analysis.

modifications of individual patients. Subsequent analysis revealed that m6Ascore was an independent prognostic factor of PAAD and could be a potential indicator to predict response to immunotherapy.

As discovered by the Human Genome Project, many genetic regions display a variation in the number of copies. These genetic variants are termed CNVs and are defined as a DNA segment that is 1 kb or larger and present at variable copy number in comparison with a reference genome (Feuk et al., 2006). A CNV can be simple in structure, such as tandem duplication, or may involve complex gains or losses of homologous sequences at multiple sites in the genome (Redon et al., 2006). CNVs influence gene expression and phenotypic variation by disrupting genes and altering gene dosage (McCarroll et al., 2006). Previous studies have found the presence of CNVs in the human genome and their associations with cancers (Cheng et al., 2016; Verma and Sharma, 2018; Santarpia et al., 2016). The larger a CNV, the more likely it is to be associated with disease; however, the phenotypic effects are often unclear and unpredictable. In our study, the CNV incidence of YTHDF2 was higher than those of YTHDF1 and YTHDF3. However, there was no difference in the expression of these genes between tumor and normal tissues (**Supplementary Figure S3**). In addition, the prognostic value of YTHDF1-3 was evaluated by KM method and

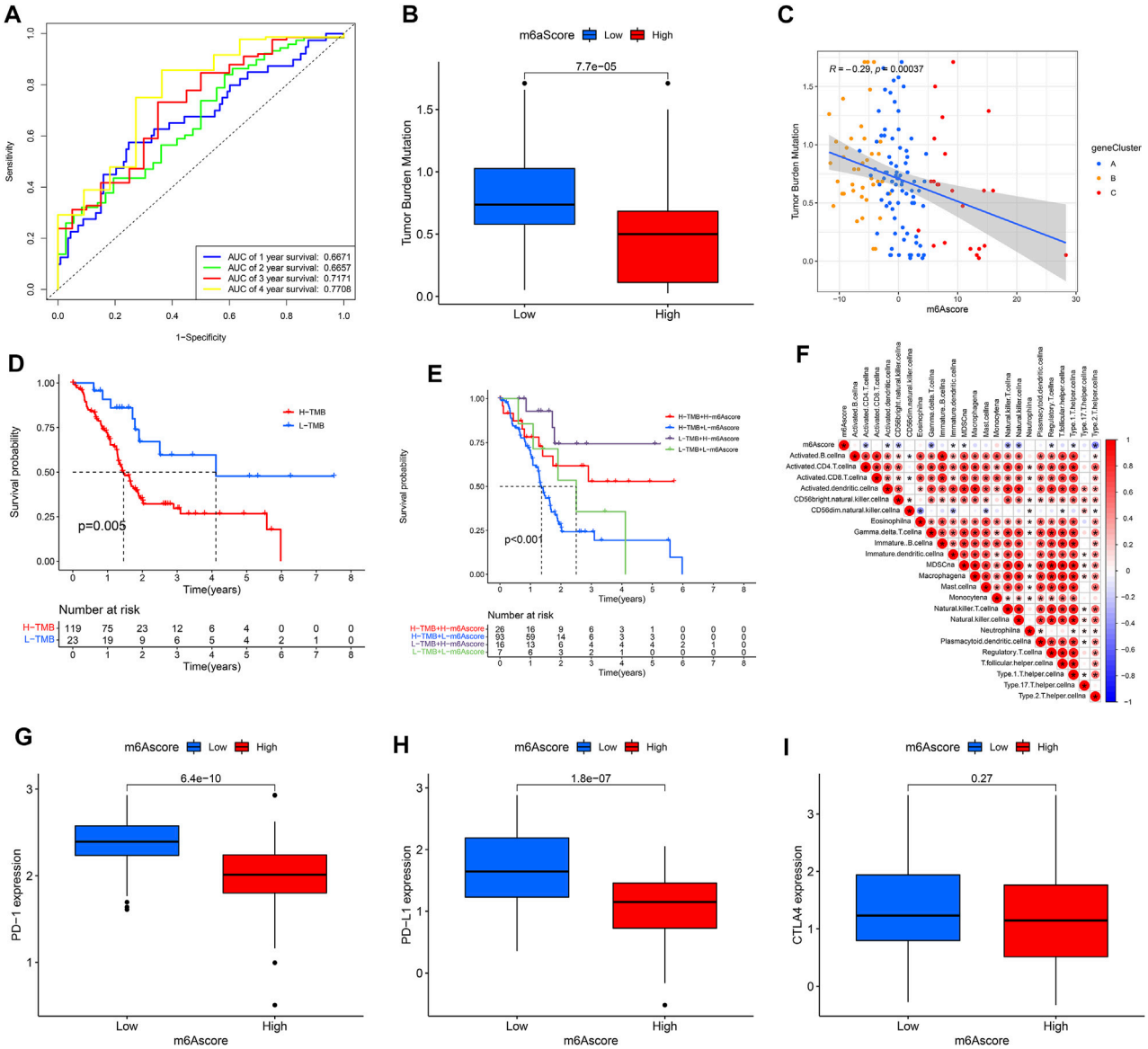

FIGURE 8 | Clinical value of m6Ascore model. **(A)** Receiver operating characteristic (ROC) curves of m6Ascore for predicting the 1/2/3/4-year survival; **(B,C)** tumor mutation burden (TMB) correlation analysis; **(D)** KM analysis of patients with distinct TMB; **(E)** KM analysis of patients with distinct TMB and m6Ascore; **(F)** Immuno-correlation analysis; **(G)** PD1 expression of patients in distinct m6Ascore groups; **(H)** PD-L1 expression of patients in distinct m6Ascore groups; **(I)** CTLA4 expression of patients in distinct m6Ascore groups.

univariate Cox regression analysis. The results showed that all of them were not associated with survival. Of note, Chen et al. (2017) found that YTHDF2 is up-regulated in PAAD and associated with the poor stage of patients. The reason for the different results may be that in our study, there were only a few normal samples and high-stage tumor samples. Furthermore, the tumor samples in our study contained different clinicopathological features, including age, gender, tumor grade. In addition, YTH-family genes play different role in PAAD. For instance, YTHDF2 orchestrated two cellular processes via TGF-β/Smad signaling pathway: promoted proliferation and inhibited migration and invasion in pancreatic cancer cells (Chen et al., 2017). Thus, further research with more samples was needed to explore this issue.

Since there were distinct correlations between the m6A regulators, patients were stratified into three m6Aclusters, which were different in prognosis, immune cell infiltration, and pathway signatures. The study found that the expression levels of m6A regulators were associated with the prognosis of PAAD. Patients with the high expression levels of m6A regulators had a poor prognosis. Of note, there was a positive correlation between m6A regulators and the ESTIMATE score. m6Acluster B, which had a high ESTIMATE score, was characterized by the high expression levels of m6A regulators. ESTIMATE score was

used to assess the level of infiltrating stromal and immune cells and infer tumor purity in tumor tissue, with a high ESTIMATE score indicating low tumor purity. Relevant studies have indicated that low tumor purity was related to an unfavorable prognosis in glioma and colon cancer, which was similar to our finding (Zhang et al., 2017; Mao et al., 2018). Moreover, there was significant difference in immune cell infiltration between the distinct clusters. m6Acluster B and gene cluster B, which had a poor prognosis, were characterized by the high infiltration level of NK cells and Th2 cells. Relevant research showed that a high number of NK cells was correlated with a poor prognosis in PAAD. This may be due to tumor cells affected the activation of NK cells by inhibiting IL-2, IFN-γ, and TNF-α secretion, thus rendering them inept (Yang et al., 2018). In addition, Th2 cells were correlated with cancer-associated fibroblast thymic stromal lymphopoietin and a high abundance of them could reduce survival in PAAD (De Monte et al., 2011).

TME is a complex system with multiple components, including immune cells and non-immune cells, that plays a crucial role in cancer development and progression (Ocaña et al., 2019). Accumulating studies have suggested that m6A modification played an important role in TME. For instance, large abnormalities of m6A mRNA were found in immune cells such as Dendritic cells (DCs). In this context, the altered m6A mRNA powerfully contributed to immune disorders and tumor escape, partially through the inhibition of immune cell function and migration (Li et al., 2021b). YTHDF1 was confirmed to induce the expression of lysosomal proteases by recognizing their m6A-marked mRNAs and increasing translation efficiency, which caused DCs to be unable constantly cross-present engulfed tumor neoantigens and then impeded the antigen-specific activation of CD8 + T cells (Han et al., 2019). Moreover, METTL14 and WTAP were confirmed to participate in the regulation of vascular endothelial cells (VECs) functions (He et al., 2019; Wang et al., 2020). In addition, increasing evidence has revealed that m6A methylation regulated TME remodeling in tumor metastasis, including gastric cancer, lung cancer and ovarian cancer (Hua et al., 2018; Yue et al., 2019; Wanna-Udom et al., 2020). For instance, METTL3-mediated m6A controlled TGF-β-induced epithelial-mesenehymal transition (EMT) in cancer cells, and obviously suppressed lung metastasis *in vivo* in response to METTL3 deficiency (Yue et al., 2019).

Considering the complex reciprocal regulatory relation between the m6A-related genes, it is necessary to accurately evaluate the m6A modification patterns of individual PAAD patients. In this study, a model (called "m6Ascore") was constructed. Based on m6Ascore, patients were divided into low- and high-m6Ascore groups. Patients in the low m6Ascore group demonstrated a poor prognosis. Integrated analyses demonstrated that m6Ascore was a robust and independent prognostic factor of PAAD. Meanwhile, m6Ascore was negatively correlated with the TMB and patients with a high TMB had a low m6Ascore. TMB represents the somatic coding errors such as base substitutions, insertions or deletion (Chan et al., 2019). A high TMB was found to promote immune cell infiltration and antigen formation, which could strengthen the immune response and improve immunotherapy efficacy in multiple cancers (Miao et al., 2018; Chan et al., 2019). In addition, the expression of targeted immune checkpoint molecules PD-1 and PD-L1 was high in the low m6Ascore group. Tumors often up-regulate immune checkpoints to avoid being detected and killed by the host immune system. Activation of checkpoint cascades such as those controlled by PD-1 or PD-L1 will result in inactivation of tumor-specific T cells and immune evasion (Iwai et al., 2002; Dunn et al., 2004; Brown et al., 2010). Treatment with anti-PD-1 and anti-PD-L1 could reinvigorate T cells and allow the adaptive immune system to target tumor cells (Pardoll, 2012). Previous studies have shown that the expression of PD-1 and PD-L1 could be as predictive biomarkers for immunotherapy response (Ferris et al., 2016; Herbst et al., 2016; Muro et al., 2016; Ott et al., 2017). Therefore, based on the close relationship between m6Ascore, TMB and the significant difference in the expression of targeted immune checkpoint molecules, m6Ascore could be identified as a potential and effective indicator to predict the response to immunotherapy.

Some limitations of this study have been observed. First, immune cell infiltration was assessed based on algorithms owing to technical limitations. Second, the regulatory mechanism of m6A regulators in TIME was not explored exhaustively, which needed further investigation. Last, there was no clinical cohort to verify the predictive value of m6Ascore in PAAD, thus, further research based on large cohort prospective clinical trials was needed.

In conclusion, this study comprehensively identified and systematically profiled the genetic features of m6A-related regulators in PAAD. Distinct m6A modification patterns contacted with different prognoses, immune cell infiltrations, and pathway signatures. The study also constructed a m6Ascore model, which was a potential therapeutic signature for PAAD. This study will help clinicians identify potential indicators of PAAD to improve the poor prognosis of this disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

YL and JL contributed to the conception and design of the study. YL and GL extracted data. YL, YY, and GL analyzed the data. YL, TW, and ZL drafted the manuscript. ZL, TW, XW, and YL contributed with a critical revision of the manuscript. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.752025/full#supplementary-material

Supplementary Figure 1 | Survival analysis of m6A regulators. **(A)** KM analysis of patients with distinct ALKBH5 expression; **(B)** KM analysis of patients with distinct EIF3A expression; **(C)** KM analysis of patients with distinct FTO expression; **(D)** KM analysis of patients with distinct FXR1 expression; **(E)** KM analysis of patients with distinct G3BP1 expression; **(F)** KM analysis of patients with distinct HNRNPA2B1 expression; **(G)** KM analysis of patients with distinct HNRNPC expression; **(H)** KM analysis of patients with distinct IGF2BP2 expression; **(I)** KM analysis of patients with distinct LRPPRC expression; **(J)** KM analysis of patients with distinct METTL3 expression; **(K)** KM analysis of patients with distinct METTL16 expression; **(L)** KM analysis of patients with distinct RBM15 expression; **(M)** KM analysis of patients with distinct VIRMA expression; **(N)** KM analysis of patients with distinct YTHDF3 expression; **(O)** KM analysis of patients with distinct ZC3H13 expression.

Supplementary Figure 2 | Molecular characteristics of gene clusters. **(A)** The heatmap depicted the expression levels of m6A regulators in distinct gene clusters; **(B)** GSVA enrichment analysis between gene cluster A and gene cluster B; **(C)** GSVA enrichment analysis between gene cluster A and gene cluster C; **(D)** GSVA enrichment analysis between gene cluster B and gene cluster C.

Supplementary Figure 3 | Molecular characteristics of YTH-family genes. **(A–C)** The expression differences of YTHDF1-3 between tumor and normal tissues; **(D–F)** The expression differences of YTHDF1-3 between the different tumor stage; **(G)** KM analysis of patients with distinct YTHDF1-3 expression.

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REVIEW
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The Interaction Between Long Non-Coding RNAs and Cancer-Associated Fibroblasts in Lung Cancer

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Despite great advances in research and treatment, lung cancer is still one of the most leading causes of cancer-related deaths worldwide. Evidence is mounting that dynamic communication network in the tumor microenvironment (TME) play an integral role in tumor initiation and development. Cancer-associated fibroblasts (CAFs), which promote tumor growth and metastasis, are the most important stroma component in the tumor microenvironment. Consequently, in-depth identification of relevant molecular mechanisms and biomarkers related to CAFs will increase understanding of tumor development process, which is of great significance for precise treatment of lung cancer. With the development of sequencing technologies such as microarray and next-generation sequencing, lncRNAs without protein-coding ability have been found to act as communicators between tumor cells and CAFs. LncRNAs participate in the activation of normal fibroblasts (NFs) to CAFs. Moreover, activated CAFs can influence the gene expression and secretion characteristics of cells through lncRNAs, enhancing the malignant biological process in tumor cells. In addition, lncRNA-loaded exosomes are considered to be another important form of crosstalk between tumor cells and CAFs. In this review, we focus on the interaction between tumor cells and CAFs mediated by lncRNAs in the lung cancer microenvironment, and discuss the analysis of biological function and molecular mechanism. Furthermore, it contributes to paving a novel direction for the clinical treatment of lung cancer.

Keywords: cancer-associated fibroblasts (CAFs), long non-coding RNAs (lncRNAs), tumor microenvironment, exosomes, lung cancer, targeted therapy

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INTRODUCTION

According to global statistics, lung cancer probably accounts for one-fifth of all cancer deaths and is one of the most deadly malignancies (Bray et al., 2020). In recent years, despite a large number of novel discoveries that have been achieved in treatment, the prognosis of patients with advanced lung cancer is still poor due to the delayed diagnosis of the disease, with the 5-year survival rate less than 5% (Hirsch et al., 2017). Therefore, it is an urgent problem to dig into the pathogenesis of lung cancer and improve the therapeutic effect of patients.

Tumor cells do not exist independently, but are surrounded by tumor microenvironment rich in stromal components and vascular networks. Microenvironment-mediated tumor initiation, progression, metastasis, and even drug resistance are the result of continuous interactions

between tumor cells and the surrounding stroma. Cancer-associated fibroblasts, a type of permanently activated fibroblasts, are the most prominent stromal components and have been proved to have profound effects on tumor regulation. CAFs are the most dominant cells that synthesize and reshape extracellular matrix (ECM) in the tumor microenvironment, which contribute to increasing the hardness of tumor tissue and thus increase the ability of local invasion and metastasis. Meanwhile, CAFs are also important sources of many growth factors, chemokines, cytokines, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), interleukin-6 (IL-6) and stromal cell-derived factor-1 (SDF-1). CAFs play multiple carcinogenic roles by regulating these factors and participating in various signaling pathways. A better understanding of the contribution of CAFs to tumor progression will lead to promising therapeutic approach interventions for stroma.

Ultimately, tumor is a genetic disease that alters the flow of information in cells to change cell homeostasis and promote its proliferation. The Cancer Genome Atlas (TCGA) and the Cancer LncRNA Census (CLC) have provided cancer researchers with useful datasets and annotated data that accelerate our understanding of molecular level of tumor, including the role of lncRNAs in tumor development (Taniue and Akimitsu, 2021). A growing body of evidence shows that lncRNAs participate in crosstalk between tumor cells and microenvironment. Here, we summarize the relationship between lncRNAs and CAFs, as well as specific role and mechanism of lncRNAs in CAFs' promotion of lung cancer invasion, immunosuppression and therapeutic resistance, finally explore the diagnostic, prognostic and therapeutic value of lncRNAs in the TME.

THE CURRENT UNDERSTANDING OF CANCER-ASSOCIATED FIBROBLAST BIOLOGY

Tumor is a complex ecosystem, including not only tumor cells, but also many stromal components. The stroma is made up of many distinct cell types, including fibroblasts, adipocytes, myeloid-derived suppressor cells (MDSCs), macrophages, lymphocytes, smooth muscle, blood vessels, and extracellular matrix (ECM). Together, they compose the microenvironment in which the tumor is located, known as the tumor microenvironment (TME) (Li et al., 2007; Zhang H. et al., 2020). Although the "seed and soil" theory was first proposed in the 1880s, the role of TME in affecting cancer initiation and development has not received widespread concern until recent decades (Paget, 1989; Chen and Song, 2019). There are differences between TME and normal tissue environment in terms of tissue structure, pH level, cell nutritional status, metabolism, and so on. Although TME may have some anticancer actions, it generally provides the best conditions for the growth, invasion and metastasis of various types of tumor cells. It can be said that TME is the essential "soil" for the breeding of tumor "seeds." Cancer-associated fibroblasts (CAFs), as one of the most important and active components in the tumor microenvironment, regulate tumorigenesis and therapeutic response by synthesizing ECM and secreting varieties of soluble factors (Yeh et al., 2015; Sahai et al., 2020). CAFs isolated from human lung cancer tissue induce EMT and enhance the metastatic potential of cancer cells by activating the IL-6/STAT3 signaling pathway (Wang et al., 2017). Fibroblasts in normal tissues, as the main producers of ECM, are activated during tissue injury, inflammation, and fibrosis, and thus play key roles in tissue repair and regeneration. NFs that are continuously activated in the tumor stroma are referred to CAFs (Chen and Song, 2019; Fang et al., 2020). Therefore, we hypothesized that CAFs could also be used for anticancer therapy in tumors long known as "wounds that do not heal." Compared to NFs, CAFs have increased proliferation and migration characteristics due to their differential gene and protein expression characteristics (De Wever et al., 2008; Saadi et al., 2010). In addition, CAFs secrete elevated levels of ECM proteins, such as fibronectin and type I collagen, to provide physical scaffolds for tumor tissue (Chan et al., 2017).

The Origin and Heterogeneity of Cancer-Associated Fibroblasts

There is growing evidence that CAFs are recognized as heterogeneous population of cells. This heterogeneity may be caused by different origins of CAFs (Kalluri, 2016). In-depth research into the origin and activation of CAFs in human malignancies has also expanded our understanding of the phenotypic heterogeneity and functional diversity of CAFs. Although the origin of CAFs has been further clarified by the use of lineage tracing mouse models, it is difficult to draw definitive conclusions about the origin of CAFs when markers of both normal fibroblasts and CAFs are not quite clear (Alcolea and Jones, 2013; LeBleu et al., 2013). Despite several CAFs markers have been identified by immunohistochemistry, including α-smooth muscle actin (α-SMA), fibroblast activation protein-α (FAP-α), vimentin, and fibroblast specific protein-1 (FSP-1) (Kraman et al., 2010). However, the expression of common fibroblast markers is extremely uneven and varies considerably among different CAF subsets. This leads to a limitation in understanding the activation of CAFs. The current consensus is that most CAFs are probably recruited and activated by local tissue fibroblasts, but there are clear examples of other sources (Sahai et al., 2020). CAFs can originate from normal resident tissue fibroblasts. Transforming growth factor-β (TGF-β), fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hypoxia, reactive oxygen species (ROS) and non-coding RNAs are key regulators of fibroblasts activation (Tape et al., 2016; Deng et al., 2020). As a strong inducer of proliferation and fibrosis, TGF-β activates fibroblasts in pancreatic adenocarcinoma (Löhr et al., 2001). Activation of hepatic stellate cells (HSCs) by PDGF results in myofibroid phenotypes, including features such as α-SMA expression, that are transformed into CAFs (Yin et al., 2013). Hypoxia induces

epigenetic reprogramming of normal breast fibroblasts, resulting in the pro-glycolytic phenotype of CAFs (Becker et al., 2020).

In addition, other cell types in TME (e.g., epithelial cells, endothelial cells, adipocytes and pericyte) may also participate in CAFs' differentiation. In breast cancer, kidney cancer, lung cancer and liver cancer, epithelial cells and endothelial cells adjacent to the cancer cells can differentiate into CAFs through epithelial-mesenchymal transition (EMT) and endothelial-mesenchymal transition (EndMT) (Kalluri and Neilson, 2003; LeBleu and Kalluri, 2018). Although conversion of adipocytes to CAFs is not a universal phenomenon in tumors, it has been partially reported in breast cancer. Human adipose tissue-derived stem cells (hASCs) adjacent to breast cancer cells are also one of the sources of CAFs and play an important role in tumor aggression (Jotzu et al., 2011). Evidence of pericyte transformation of CAF is relatively rare (Dulauroy et al., 2012; Bartoschek et al., 2018). Pericytes can be transferred into CAFs in a PDGF—dependent manner (Hosaka et al., 2016). In recent years there has been evidence that cancer stem cells (CSCs), which are thought to be the origin of cancer, can also differentiate into CAFs. It provides a new dimension for CAFs heterogeneity (Osman et al., 2020).

Distant cells outside the TME can also be transformed into CAFs. When mesenchymal stem cells (MSCs) are recruited by colorectal carcinoma microenvrionment, CXCR4/TGF-β1 signaling signaling pathway in this environment can mediate MSCs differentiation into CAFs (Tan et al., 2020). Cancer progression requires stromal support to maintain tumor growth. CAFs, as the main producer of ECM and paracrine signals, play key roles in tumorigenesis, angiogenesis, metastasis, tumor stem cell maintenance and metabolic reprogramming, immunosuppression, and drug resistance. Hence, it's key to reprogram normal fibroblasts into tumorigenic CAFs.

Cancer-Associated Fibroblasts: Functional Overview in Lung Cancer

Due to their heterogeneity, CAFs have been shown to promote lung cancer development through a variety of unique mechanisms. CAFs were first shown to promote tumor progression in a prostate cancer model (Olumi et al., 1999). Navab et al. (2016) found that CAFs can enhance the interstitial collagen hardness through overexpression of integrin α11β1, thereby promoting tumor progression in NSCLC. It is known that CAFs are large producers of IL-6 in the cancer microenvironment, inducing EMT through the IL-6/STAT3 signaling pathway and enhancing the metastatic potential of lung cancer cells (Wang et al., 2017). CAFs are also involved in maintaining the immunosuppressive and angiogenic environment that promotes tumor growth and evades immune surveillance. For example, CAFs can induce immunosuppression by recruiting immunosuppressive cells to a tumor site. CAFs can also recruit endothelial progenitor cells into carcinomas by secreting stromal cell derived factor 1 (SDF1), thereby stimulating tumor angiogenesis (Orimo et al., 2005). In addition, CAFs mediated drug resistance was observed in lung cancer. For example, IL-6 from CAFs significantly increases TGF-β1-induced EMT in cancer cells, thereby promoting cisplatin resistance in NSCLC (Shintani et al., 2016). Hepatocyte growth factor (HGF) from CAFs activated Met/PI3K/Akt, up-regulated the expression of GRP78, promoting the resistance of A549 cells to paclitaxel (Ying et al., 2015). Many of these aspects have been reviewed in the past. We pay particular attention to lncRNAs as relatively new biomolecules participated in the interactions with CAFs. Through lncRNAs, the metabolic characteristics of NFs were changed, leading to the activation of CAFs. Activated CAFs enhance malignant biological processes in tumor cells by interacting with lncRNAs, which can promote tumor progression more effectively. LncRNAs also participate in bidirectional communication between tumor cells and CAFs via exosomes, resulting in both types of cells being reprogrammed to maintain malignancy.

LONG NON-CODING RNA: CLASSIFICATION AND FUNCTIONAL CHARACTERISTICS

Based on the Encyclopedia of DNA Elements (ENCODE) project, 93% of the genome is transcribed into RNA, of which only 2% is translated to protein. Those RNAs that lack the ability of coding proteins are known as non-coding RNAs (ncRNAs). There are many types of non-coding RNAs, which can be divided by size into short non-coding RNAs with length less than 200 bp and long non-coding RNAs with more than 200 bp (Brosnan and Voinnet, 2009; Clark et al., 2011; Iyer et al., 2015). Initially, it was believed that lncRNA was a by-product of RNA polymerase II transcription, which was the result of transcriptional junk in the process of genome evolution and had no biological function (Kopp and Mendell, 2018). The development of whole genome and transcriptome sequencing technology has allowed in-depth examination of the noncoding genome. In recent years, the functional roles of lncRNAs have become clearer than initially anticipated.

Multiple mechanisms have been involved in the lncRNA-mediated gene regulation in many diseases, including tumors. This is probably due to their interactions with DNA, RNA or protein at three levels of transcriptional regulation, post-transcriptional regulation and epigenetic regulation. Most studies have demonstrated that the ability of lncRNAs to interact with different biomolecules extensively is of great significance in tumor development, such as tumor proliferation, metabolism, differentiation, apoptosis, migration and drug resistance. A recent series of experimental evidence indicates that their roles in TME are being recognized (Fang et al., 2020). In addition, tumor cells and CAFs can communicate more directly through lncRNA-loaded exosomes. Exosomes are a class of extracellular vesicles (evs) with a diameter of 30–100 nm, carrying microRNAs, lncRNAs, proteins, metabolites, and other bioactive substances. Exosomes secreted by CAFs can influence tumor progression. While exosomes released by cancer cells can also promote the transformation and activation of CAFs. Although the study of lncRNAs in TME

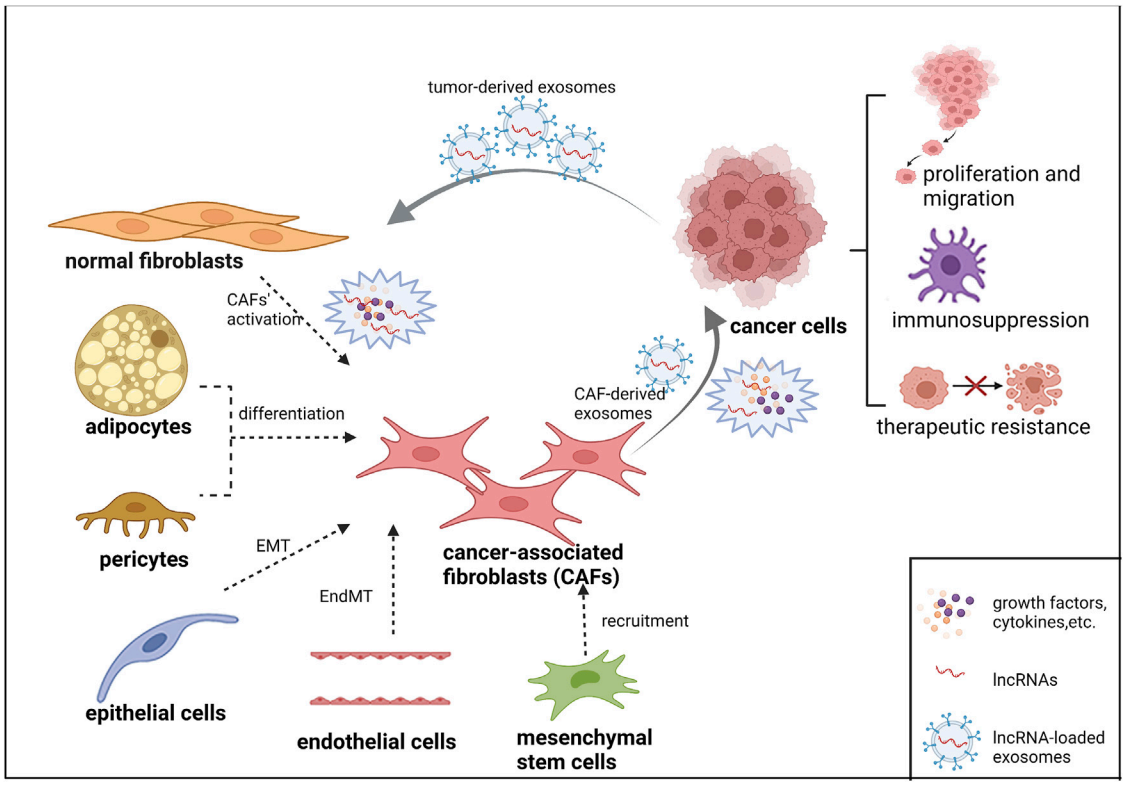

FIGURE 1 | The heterogeneous origin of CAFs and their interactions with tumor cells via lncRNAs.

has just begun, it can be predicted that significant progress can be made in the study of lncRNAs interaction with CAFs. The heterogeneous origin of CAFs and their interactions with tumor cells via lncRNAs are illustrated in **Figure 1**. Figure created with BioRender (https://biorender.com).

Normal fibroblasts mainly transformed into CAFs through coordination of cytokines, growth factors, lncRNAs, etc. Epithelial cells, endothelial cells, adipocytes, pericytes and mesenchymal stem cells may also participate in the differentiation of CAFs through different mechanisms. CAFs interact with lncRNAs to maintain tumor proliferation, migration, immunosuppression and therapeutic resistance. LncRNA-loaded exosomes are also widely involved in crosstalk between tumor cells and CAFs.

THE DYNAMIC CROSSTALK BETWEEN TUMOR CELLS AND CANCER-ASSOCIATED FIBROBLASTS MEDIATED BY LONG NON-CODING RNAS

The crosstalk between tumor cells and the tumor microenvironment is deemed to be necessary for tumor progression (Gascard and Tlsty, 2016). CAFs are major participants in TME, promoting tumor progression and metastasis through communication with adjacent tumor cells directly or indirectly. Now accumulating evidence has revealed that lncRNAs, as relatively novel regulatory factors, play critical roles not only in tumor cells but also in the tumor microenvironment. The dysregulation of lncRNAs and exosomal lncRNAs are involved in the dynamic crosstalk between CAFs and tumor cells. By exploring the relationship between lncRNAs, CAFs and cancer cells, using lncRNAs as biomarkers or targets may be a potential way to predict the prognosis of patients and improve the therapeutic effect of lung cancer in the future (Wang et al., 2018). The following details describe the CAFs-related lncRNAs and their potential mechanisms in lung cancer (**Table 1**).

Role in the Formation and Activation of Cancer-Associated Fibroblasts

Compared with resting fibroblasts, hyperactivated fibroblasts (i.e., CAFs) have stronger tumor-promoting ability in NSCLC (Navab et al., 2011). In addition to the well-known miRNAs, some studies have confirmed that lncRNAs are also one of the regulatory factors that contribute to the formation and activation of CAFs. By integrating the gene expression profiles of 32 cancer types and clustering the associated lncRNAs, 16 lncRNA modules were obtained. Twelve lncRNAs in one of these were associated with cancer fibroblasts activation. Differentiation of quiescent fibroblasts into cancer-associated phenotypes was reduced by siRNA knockdown experiment (Walters et al., 2019). Snail1

TABLE 1 | CAFs-related lncRNAs and their potential mechanisms in lung cancer.

LncRNA	Expression	Target molecules or pathways	Cell lines	Principal functions	References
PCAT-1	Upregulation	miR-182/miR-217 signaling	H1975, A549	Activate CAFs, Immunosuppression, Chemoresistance	Domvri et al. (2020)
TBILA	Upregulation	S100A7/JAB1	A549	promoting progression	Lu et al. (2018)
ANCR	Downregulaton	TGF-β signaling	NCI-H23, NCI-H522	inhibit migration and invasion	Wang et al. (2018)
Snail-1	Upregulation	Snail1 signaling	A549	promote EMT of epithelial lung cancer cells	You et al. (2019)
HOTAIR	Upregulation	caspase-3/BCL-2 signaling	A549	promote cisplatin resistance	Sun and Chen (2021)
ANRIL	Upregulation	caspase-3/BCL-2 signaling	A549	Promote cisplatin resistance	Zhang et al. (2017)
KCNQ1OT1	Upregulation	G1/S transformation	A549	Radiotherapy resistance	Mao (2019)

activates cancer-associated fibroblasts (CAF), which require signals from tumor cells such as TGF-β (Alba-Castellón et al., 2016). It was found that lncRNA FLJ22447 promoted the reprogramming of NFs into CAFs by up-regulating IL-33 level in oral squamous cell carcinoma (Ding et al., 2018). LncRNA LINC00092 interactes with 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) to sustain the CAFs-like features of fibroblasts within tumor microenvironment and promote ovarian cancer metastasis (Zhao et al., 2017). The transfer of bioactive molecules from malignant cells to stromal cells via exosomes is also a key regulator of CAF differentiation. To date, at least two dozen miRNAs have been identified in various cancer types mediate CAF differentiation through exosomes (Shoucair et al., 2020). Some studies have confirmed that the expression of lncRNA-containing exosomes released by some tumor cells upregulates in the stroma and also participate in the activation of CAFs, but researches hitherto remain sparse. A recent study found that exosomal lncRNA PCAT-1 involved in tumor stroma remodeling in lung cancer. High expression of PCAT-1 can trigger the differentiation of fibroblasts into CAFs. Rather, PCAT-1 knockdown impaired CAF-mediated stromal activation and tumor growth *in vivo* (Domvri et al., 2020).

Tumor Proliferation and Migration

Perhaps the best-known function of CAFs is to promote tumor progression. Microarray gene expression analysis of CAF and NF cell lines showed that differentially expressed genes were mainly regulated by the TGF-β signaling pathway. CAFs can promote EMT and tumor cell metastasis in a variety of ways through TGF-β-dependent mechanisms (Calon et al., 2014). TGF-β1, which is partially secreted by CAFs, is a critical medium for CAFs to induce EMT and metastasis of lung cancer cells, and an important medium for the interaction between stroma and cancer cells (Kalluri, 2016). It has been previously reported that CAF-derived TGF-β11 promotes EMT and invasion of bladder cancer cells through lncRNA-ZEB2NAT (Zhuang et al., 2015). In breast cancer, CAFs activate HOTAIR transcription by secreting TGF-β1, thereby promoting the metastasis activity tumor cells (Ren et al., 2018). Similarly, TGF-β1 secreted by CAFs leads to overexpression of lncRNA (TBILA) in NSCLC tissues, which can be induced by the classical TGF-β1 /Smad2/3 signaling pathway, cis-regulating HGAL (a TGF-β-induced gene), and activating the S100A7/JAB1 signaling pathway, thereby promoting the progress of NSCLC (Lu et al., 2018). As a tumor suppressor gene in NSCLC, lncRNA ANCR inhibits the migration and invasion of NSCLC cells by down-regulating TGF-β1 expression (Wang et al., 2018). Evidence shows that CAF-derived exosome lncRNAs also play an increased role in tumor proliferation. Exosome-lncRNA SNAI1 secreted by CAFs induces EMT in lung cancer cells. However, treatment of CAFs with exosome release inhibitor GW4869 significantly inhibited the induction of EMT in recipient cancer cells (You et al., 2019).

Tumor Immunosuppression

In recent years, immunotherapy has become a hot topic in cancer treatment. Nevertheless, only a small percentage of cancer patients benefit from tumor immunotherapy, and a number of patients develop resistance to the therapy (Cristescu et al., 2018; Horvath et al., 2020). There is growing evidence that TME is one of the vital factors in the regulation of tumor immune response, not only tumor cells themselves. CAFs interact with a variety of immune cells, both innate immune cells (macrophages, neutrophils, dendritic cells, natural killer cells, and bone marrow cells) and adaptive immune cells (T and B lymphocytes), to modulate tumor immune response (i.e., immunosuppression) and promote tumor progression (Piersma et al., 2020). CAFs-mediated effects can directly increase the amount of inhibitory T lymphocytes and counteract effector T cell function. In addition, CAFs produce ECM components that form a physical barrier against immune cell infiltration (Mhaidly and Mechta-Grigoriou, 2021). LncRNAs can also act as tumor promoter genes and suppressor genes to regulate immune cell and immune response in TME (Xue et al., 2021). For example, lncRNA NKILA promotes tumor immune escape in the lung cancer microenvironment by regulating T cell sensitivity to activation-induced cell death (AICD) and increasing CTL infiltration (Huang et al., 2018). Exosomes contain membrane surface related antigens, immune stimulation and inhibitory factors, and biological active substances, which can participate in the immune response (Xu et al., 2020).

There is evidence that the interaction between CAFs and lncRNAs can also mediate immune responses. Domvri et al. found that PCAT-1 in the lung tumor microenvironment promotes a pre-metastatic niche formation by immunosuppressive miR-182/miR-217 signaling and p27/CDK6 regulation. PCAT-1-activated CAFs also enhanced the transformation of tumor-associated macrophages (TAMs) into a tumor-supporting M2 phenotype. This leads to an increase in infiltrating macrophages in the microenvironment, which then

progresses to an immunosuppressive phenotype (Hegab et al., 2019; Domvri et al., 2020). Teng et al. (2019) showed lncRNA profiling in NSCLC. The lncRNAs differentially expressed between the paired CAFs and adjacent normal fibroblasts of the three patients were detected by gene microarray. Compared with NFs, we found 322 up-regulated and 444 down-regulated lncRNAs in CAFs. Bioinformatics analysis methods such as Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to analyze these differentially expressed lncRNAs, and it was found that these dysregulated lncRNAs were associated with immune function. These results suggest that CAF-specific lncRNAs mediate immune responses during the progression of lung cancer. However, there are relatively few studies on CAFs directly regulating lncRNAs to induce immunosuppression. Most of them are due to the interaction between various cytokines secreted by CAFs (such as TGF, IL-6, IL-8, etc.) and lncRNAs to exert immunosuppression (Ghafouri-FardAbak et al., 2021). For example, lncRNA LINC00301 significantly accumulated Tregs and inhibited $CD8^+$ T cell infiltration in the nude mouse tumor microenvironment of NSCLC cell lines by targeting TGF-β (Sun et al., 2020). The mechanism of CAF regulating lncRNAs to further play immune escape needs to be further explored. This provides a promising target for further study of immune regulation in lung cancer.

Therapeutic Resistance

Accumulating studies have found that tumor drug resistance is not only related to tumor cells, but also closely related to TME. TME-mediated resistance can be induced by chemokine and cytokine secreted by tumor cells and stroma. Mink et al. (2010) found that 24% of CAFs in tumor matrix were generated by EMT-derived tumor cells via green fluorescent protein-tagging experiment, and expressed epithelial membrane protein-1, a gefitinib resistance biomarker, to promote EGFR-TKI resistance. LncRNAs also play significant roles in the regulation of drug resistance. CAFs can directly or indirectly lead to abnormal expression of lncRNAs in tumor cells. This abnormality may alter the drug sensitivity of tumor cells by affecting downstream ceRNA. Zhang et al. (2017) found Midkine derived from CAFs increases the expression of lncRNA ANRIL in lung cancer cells, ovarian cancer cells, and oral squamous cell carcinoma cells through paracrine action, thereby promoting the upregulation of ABC family proteins MRP1 and ABCC2, and ultimately leading to the resistance of tumor cells to cisplatin. In contrast, silencing lncRNA ANRIL can overcome MK-induced cisplatin resistance via activating the caspase-3-dependent apoptotic pathway. Similarly, CAFs upregulate the expression of lncRNA HOTAIR to inactivate caspase-3/BCL-2 signaling pathway and promote cisplatin resistance (Sun and Chen, 2021).

In addition, it has been shown that CAFs affect the therapeutic resistance behavior of tumor cells through the release of exosomes containing lncRNA. CAFs can activate the β-catenin pathway by transferring exosome lncRNA H19, promoting chemotherapy resistance in colorectal cancer (Ren et al., 2018). Exosomes with high expression of H19 can also promoted gefitinib resistance of NSCLC cells (Lei et al., 2018). Based on the above studies, it is possible to explore whether H19-exosomes in NSCLC also derive from CAF. LncRNA KCNQ1OT1 is also packaged into exosomes and secreted by CAFs, thus inducing cancer cell proliferation, promoting the G1/S transformation of cancer cells, and increasing radiotherapy resistance (Mao, 2019). The development of potential drugs that can target lncRNA and CAFs may be a solution to improve treatment resistance.

THE DIAGNOSTIC, PROGNOSTIC AND THERAPEUTIC VALUE OF LONG NON-CODING RNAS IN TUMOR MICROENVIRONMENT

As functional RNA molecules, lncRNAs and lncRNAs-enriched exosomes control crucial pathways in microenvironment and are expressed in specific ways at specific stages of progression. They have gradually become new biomarkers and therapeutic targets for cancer. We provide our perspective on the clinical use of lncRNAs as therapeutic targets or prognostic/predictive biomarkers, contributing to the development of precision therapy.

Application of Long Non-Coding RNAs as Biomarkers

Previous studies have found that abnormal expression of lncRNAs can regulate the interaction between tumor cells and stromal cells, which can be used to predict tumor behavior and patient prognosis. The ability to capture tumor interstitial correlation signals may be of great significance. The lipid bilayer of exosomes provides a protective membrane that encapsulates and protects biologically active molecules. Therefore, exosomes exist stably in various body fluids such as blood, saliva, bronchoalveolar lavage fluid, and sputum. Due to their small size, exosomes are easier to penetrate into a variety of body fluids, mediating signal and substance exchange between tumor cells and CAFs. Exosomes can be used as promising diagnostic and prognostic value due to these unique characteristics (Chung et al., 2020). Precise liquid biopsies can be performed by non-invasive methods to obtain exosomes abundant in body fluids. It is a promising method to improve early diagnosis and cancer prognosis. Abnormal expression of exosomes from tumor cells and mesenchymal cells are also associated with different stages of cancer (Barile and Vassalli, 2017). Dynamic monitoring of differential expression of bioactive molecules (such as lncRNAs, miRNAs) in exosomes can reflect tumor development more accurately and timely. However, the reliability and stability of extraction technology are the premise of exosome research. However, due to their nanoscale volume, efficient purification of exosomes from body fluids is full of challenges. In addition, the high cost of the test may limit its widespread use and large prospective clinical trials must be conducted to provide evidence of its clinical utility. At present, these are still great challenges to its clinical application as biomarker.

Potential of Targeted Long Non-Coding RNAs Therapy

The roles of CAFs in cancer development makes them potential therapeutic targets. Nevertheless, due to the lack of specific CAFs markers, it is difficult to accurately target CAFs without damaging normal tissues. In addition, CAFs have both protumorigenic and antitumorigenic effects. Ongoing attempts to treat CAFs still face a number of challenges. A few of receptor antagonists targeting CAFs-derived paracrine factors are being developed to prevent tumor-stromal interactions. Siltuximab, as an anti-IL-6 monoclonal antibody, was found to have strong tumor suppressive effects in lung cancer xenograft models treated with CAFs (Nikanjam et al., 2019).

According to recent studies, targeting lncRNAs in CAFs is also a promising direction of exploration. Although miRNAs in the tumor microenvironment have been extensively studied. The regulation of mRNAs is realized by targeting miRNAs, and then the transcription and post-transcription regulation of functional genes are realized. Because one miRNA can regulate multiple mRNAs, and one mRNA is regulated by different miRNAs. Compared to targeting miRNAs, lncRNAi is equivalent to simultaneously targeting multiple different miRNAs molecules, thus improving the efficiency of tumor growth inhibition. Several approaches can target lncRNAs, such as antisense oligonucleotides (ASOs), short hairpin RNAs (shRNAs), short interfering RNAs (siRNAs), small molecule antagonists, and nucleic acid aptamers (Chen et al., 2019a). The degradation of lncRNAs in CAFs by ASO interferes with their phenotypes and tumor-friendly functions. In a mouse xenograft model, MALAT1 ASO is a potential treatment targeting cancer-associated lncRNAs, which can effectively inhibit lung cancer spreading (Gutschner et al., 2013). As a large hydrophilic compound, delivery of ASOs to target organs *in vivo* is one of the biggest problems encountered in drug development (Levin, 2019). The efficacy of oligonucleotide drugs in locally administered diseases is significantly better than that in systemic diseases. Nusinersen, the most successful antisense oligonucleotide drug, is administered by intrathecal injection for spinal muscular atrophy (Chiriboga, 2017). As the stable structure gradually became the marker of lncRNA, the specific identification of lncRNA domain by small molecule drugs was used to regulate the phenotype. And compared with ASOs, small molecule drugs have higher tunability properties. With molecular docking and high-throughput screening, a small-molecule compound AC1Q3QWB was identified to interfere with the expression of lncRNA HOTAIR and increase the expression of tumor suppressor (Li et al., 2019). It is currently difficult that targeting lncRNAs in a scalable and replicable way with small, drug-like molecules. In addition, screening methods should be improved to identify drug-like lead molecules with appropriate pharmacological properties and identify RNA motifs with sufficient informative content (Warner et al., 2018).

Exosomes are effective carriers for communication of genetic material and other information between cells. Inhibiting the production and secretion of exosomes can be a potential therapeutic method. Several compounds have been identified as potential inhibitors of exosome production. The most common exosome inhibitor is a non-competitive inhibitor of membrane neutral sphingomyelinase (nSMase) inhibitor GW4869. According to the bilayer lipid structure of exosomes, exogenous SMase can induce exosomes formation. Accordingly, the use of GW4869 can inhibit lipid metabolism and reduce exosome production, thus interfering with tumor progression (Catalano and O'Driscoll, 2020). Some studies have found that GW4869 blocks lncRNA ASLNCS5088-enriched exosomes in M2 macrophages, and then inhibits the regulation of M2 macrophages on fibroblast activation (Chen et al., 2019b). Ras-related protein RAB27A belonging to the RAB GTPase superfamily is involved in protein transport and small GTPase mediated signal transduction as a membrane binding protein. It has been found to play an important role in the production and secretion of exosomes. Reducing the expression of RAB27A can effectively reduce the secretion of exosomes (Zhang Y. et al., 2020). Sulfisoxazole (SFX), an FDA-approved oral antibiotic, has recently been found to act as an inhibitor of exosomes by interfering with endothelin receptor A (ETA) in breast cancer (Im et al., 2019). Interestingly, the mechanisms by which these compounds block exosome secretion vary from each other. Further studies are needed to better understand the role and targets of exosome in intercellular communication. In addition, most exosome inhibitors cannot selectively identify exosomes that are involved in pathology, rather than those that perform necessary physiological roles currently. The study of targeted inhibition of exosome secretion still needs substantial efforts.

CONCLUSION AND PERSPECTIVES

Cancer-associated fibroblasts (CAFs) are key components in TME that support tumor growth, generate a physical barrier against drug and immune invasion, and help regulate malignant progression. Activation and function of CAFs are mainly controled by a large number of factors, among which lncRNAs act in indispensable roles. Through lncRNAs, CAFs regulate tumor cell proliferation, metabolism, immune escape and treatment resistance. Tumor cells and CAF-derived lncRNA-loaded exosomes are also extensively involved in this interaction. Therefore, it is widely accepted that therapies designed to block the interaction between tumor cells and CAFs can enhance the effectiveness of anti-cancer therapies. Although there are only a few reports of lncRNAs in CAFs. To sum up, the results provide new insights into the communication between tumor stroma and cancer cells, and demonstrate the therapeutic potential of targeting lncRNA mediated cross-talk between cancer and stromal cells in cancer therapy. At present, there are still many obstacles and challenges to carry out targeted work on CAFs. The design of novel therapies targeting the tumor stroma depends on a better understanding of TME's interactions with cancer cells.

AUTHOR CONTRIBUTIONS

YC and JW contributed to the conception of the study; WT collected the data analyses and wrote the manuscript; YC supervised the study; All authors read and approved the final version of the manuscript.

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REVIEW
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Review of LINC00707: A Novel LncRNA and Promising Biomarker for Human Diseases

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Long noncoding RNAs (lncRNAs) are a major type of noncoding RNA greater than 200 nucleotides in length involved in important regulatory processes. Abnormal expression of certain lncRNAs contributes to the pathogenesis of multiple diseases, including cancers. The lncRNA LINC00707 is located on chromosome 10p14 and is abnormally expressed in numerous disease types, and particularly in several types of cancer. High LINC00707 levels mediate a series of biological functions, including cell proliferation, apoptosis, metastasis, invasion, cell cycle arrest, inflammation, and even osteogenic differentiation. In this review, we discuss the main functions and underlying mechanisms of LINC00707 in different diseases and describe promising applications of LINC00707 in clinical settings.

Keywords: LINC00707, clinical characteristic, function, mechanism, lncRNA

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1 INTRODUCTION

In recent years, a shift toward a growing interest in long non-coding RNAs (lncRNAs) has been evident (Ali and Grote, 2020). Applications of newly developed genomic high-throughput sequencing toolkits are constantly revealing unprecedented structures and emerging functions of lncRNAs (Qian et al., 2019).

LncRNAs are 200 nucleotides or longer. They belong to the noncoding RNA family (Kopp and Mendell, 2018; Bridges et al., 2021) and are incapable of coding proteins. Most lncRNAs can regulate the biological processes of diverse diseases by affecting target gene expression (Bhan et al., 2017; Tan et al., 2021). The common mechanisms mainly act through competitive endogenous RNA (ceRNA) to sponge microRNAs (miRNAs) or in direct combination with proteins (Ferrè et al., 2016). Further study of lncRNA-mediated processes may contribute to the diagnosis and therapy of relevant diseases (Tan et al., 2021).

Long intergenic non-protein coding RNA 707 (LINC00707), genomic location chromosome 10p14, and has been found to dysregulate many diseases (e.g., pneumonia, spinal cord injury (SCI), osteogenic differentiation, and multiple types of cancers). LINC00707 is implicated in the regulation of biological functions, including cell proliferation, cell apoptosis, cell migration and invasion, cell cycle control, inflammation, vasculogenic mimicry (VM) development, and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (HBMSCs). LINC00707 is closely associated with clinical manifestations of disease, like tumor size, stage, grade, lymphatic and distant metastasis, shorter overall survival times, and antitumor drug sensitivity. LINC00707 is considered a promising biomarker for the diagnosis, treatment, and prognosis of specific diseases.

TABLE 1 | LINC00707 expression and clinical characteristics in diverse diseases.

Disease type	Expression	Clinical characteristics	Refs
Lung adenocarcinoma	upregulated	TNM stage, tumor size, lymphatic metastasis, poor prognosis, and cisplatin (DDP) resistance	34,486,476 29,482,190 31,788,103
Osteosarcoma	upregulated	—	34316513
Breast cancer	upregulated	—	32,432,749 32,273,767
Cervical Cancer	upregulated	—	34,258,298
Ovarian cancer	upregulated	overall survival	34,062,972
Bladder cancer	upregulated	tumor stage, grade, and shorter overall survival	34,192,702
M	upregulated	—	29,436,619
Gastric cancer	upregulated	tumor stage, tumor size, lymph node metastasis and poor prognosis	30,502,359
Glioma	upregulated	Karnofsky performance status (KPS) score, and WHO staging	33,542,193 33,107,401
Colorectal cancer	upregulated	tumor size, stage, lymphatic metastasis, distant metastasis, and poor survival	32,010,320 31,213,848 31,115,001
Hepatocellular carcinoma	upregulated	—	30,488,589 30,317,590
Clear cell renal cell carcinoma	upregulated	—	32,633,350
Pneumonia	upregulated	—	33,604,647
Spinal cord injury	upregulated	—	31,272,297
Osteogenic differentiation	upregulated	—	31,957,814 30,795,799
Osteogenic differentiation	downregulated	—	32,705,245

TABLE 2 | Functions and mechanisms of LINC00707 in diverse diseases.

Disease type	Cell lines	Role	Functions	Related mechanisms	Refs
Lung adenocarcinoma	SPCA1, A549, DDP-resistant A549	tumor promoter	cell proliferation, cell cycle, apoptosis, migration, and invasion	Cdc42, and miR-145	34,486,476 29,482,190 31,788,103
Osteosarcoma	Saos-2, MG-63, U-2 OS, HOS, and SW-1353	tumor promoter	cell proliferation, migration, and invasion	miR-338-3p, and AHSA1	34,316,513
Breast cancer	MCF-10AT, MDA-MB-231, and MDA-MB-468	tumor promoter	cell proliferation, cell cycle, apoptosis, invasion, and migration	miR-30c, CTHRC1, AKBA, miR-206, Estrogen Receptor-α	32,432,749 32,273,767
Cervical cancer	HCC94, CaSki, MS751, HT-3, and C-33A	tumor promoter	cell proliferation, migration, and invasion	miR-382-5p, and VEGFA	34,258,298
Ovarian cancer	SKOV3	tumor promoter	—	—	34,062,972
Bladder cancer	UMUC3, and T24T	tumor promoter	cell proliferation, colony formation, apoptosis, and metastasis	miR-145, and CDCA3	34,192,702
Melanoma	—	tumor promoter	—	—	29,436,619
gastric cancer	BGC-823, and SGC-7901	tumor promoter	cell proliferation, and metastasis	HuR, and VAV3/F11R	30,502,359
Glioma	U87, and U251	tumor promoter	cells proliferation, migration, invasion, and VM formation	miR-613, HNRNPD, ZHX2, miR-651-3p, SP2, MMP2, MMP9, and VE-cadherin	33,542,193 33,107,401
Colorectal cancer	LoVo, HCT116, HT29, SW620, and SW480	tumor promoter	cell proliferation, cell cycle, invasion, and migration	miR-206, NOTCH3, TM4SF1, miR-485-5p, and FMNL2	32,010,320 31,213,848 31,115,001
Hepatocellular carcinoma	HepG2, Huh7, Hep3B, and SNU449	—	cell proliferation, cell cycle, colony formation, apoptosis, invasion, and migration	miR-206, CDK14, and ERK/JNK/AKT pathway	30,488,589 30,317,590
Clear cell renal cell carcinoma	786-O, and 769-P	tumor promoter	cell proliferation, migration, and invasion	EMT pathway	32,633,350
Pneumonia	MRC-5	—	cell viability, apoptosis, and inflammation	miR-223-5p	33,604,647
Spinal cord injury	PC-12	—	cell inflammation, and apoptosis	miR-30a-5p, and Neurod 1	31,272,297
Osteogenic differentiation	HBMSCs	the regulator of osteogenic differentiation	osteogenic differentiation	miR-103a-3p, DKK1, miR-145, LRP5, Wnt/β-catenin, miR-370-3p, and WNT2B	32,705,245 31,957,814 30,795,799

The aim of this review is to present an overview of the clinical features, biological functions, relevant mechanisms, and future clinical applications of LINC00707.

2 THE EXPRESSION AND ROLE OF LINC00707 IN DISEASE

LINC00707 is upregulated in disease such as lung cancer (Ma et al., 2018; Zhang et al., 2019; Shao et al., 2021), pneumonia (Zou et al., 2021), melanoma (Yang et al., 2018), colorectal cancer (CRC) (Shao et al., 2019; Zhu H. et al., 2019; Wang et al., 2020), glioma (Liu and Hu, 2020; Yu et al., 2021), clear cell renal cell carcinoma (Pang et al., 2020), breast cancer (BC) (Jiang et al., 2020; Yuan et al., 2020), osteosarcoma (Zhang et al., 2021), cervical cancer (Guo et al., 2021), bladder cancer (Gao and Ji, 2021), ovarian cancer (Guo et al., 2021), gastric cancer (Xie et al., 2019), hepatocellular carcinoma (Tu et al., 2019; Wang et al., 2019), and SCI (Zhu S. et al., 2019). LINC00707 dysregulation contributes to the process of osteogenic differentiation (Jia et al., 2019; Cai et al., 2020; Liu J. et al., 2020) of HBMSCs. Changes in LINC00707 expression levels are correlated with specific clinicopathological features (**Table 1**). LINC00707 has an extensive role in the activation of various biological functions via different mechanisms (**Table 2**).

In subsequent sections, we focus on the expression level, clinicopathological features and biological functions of LINC00707 in a variety of diseases.

2.1 Cancer

2.1.1 Lung Cancer

Lung cancer is a molecularly heterogeneous disease. The most common type is non-small cell lung cancer (NSCLC), which is diagnosed based on microscopic examination of morphological cell characteristics (Molina et al., 2008; Sankar et al., 2020). Most lung cancers in the United States are NSCLCs (prevalence, approximately 85%). The three major NSCLC subtypes are lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and lung large cell carcinoma (LULC) (Herbst et al., 2018; Zhao et al., 2020; Shao et al., 2021). Despite ever-improving clinical targeted therapies, the long-term survival rate among patients with LUAD is less than 20% (Molina et al., 2008). Therefore, studies to find new biomarkers to provide novel therapeutic targets and improve LUAD prognosis are needed.

Tumor hypoxia in LUAD contributes to a poor clinical outcome (Terry et al., 2020; Vito et al., 2020) by affecting the tumor immune microenvironment. LINC00707 is markedly upregulated in 64 LUAD tissues collected from First Affiliated Hospital of Nanjing Medical University and SPCA1 (Ma et al., 2018), A549, H1299, and H1975 cell lines and has been identified as a hypoxia-related lncRNA (Shao et al., 2021). High levels of LINC00707 are significantly associated with LUAD tumor size, TNM stage, distant metastasis, overall survival, and a poor prognosis (Ma et al., 2018; Shao et al., 2021). *In vitro* and *in vivo* experiments revealed that LINC00707 is involved in modulation of LUAD progression via regulation of diverse biological functions, including cell proliferation, apoptosis, and migration (Ma et al., 2018) of SPCA1 and A549 cells (**Figure 1**).

2.1.2 Osteosarcoma

Osteosarcoma is the most common type of primary malignant bone tumor, with a peak in incidence in children, and in adolescents 15–19 years of age (Ritter and Bielack, 2010; Kansara et al., 2014). Osteosarcomas are heterogeneous tumors that lack specific targets (Corre et al., 2020) for therapy. Current combined therapeutic management approaches fail to improve overall survival of patients (Kansara et al., 2014; Corre et al., 2020; Chen et al., 2021). Genomic technologies are being applied to examine new aspects of the molecular pathogenesis of osteosarcoma to provide comprehensive insights into therapeutic strategies (Kansara et al., 2014). LINC00707 is highly expressed in osteosarcoma cell line types such as Saos-2, MG-63, U-2 OS, HOS, and SW 1353 (Zhang et al., 2021). LINC00707 can also function as an oncogene in osteosarcoma progression by regulating cell proliferation, migration, and invasion of MG-63 and Saos-2 cells (Zhang et al., 2021).

Moreover, osteogenic differentiation is the key process of development in osteosarcomas (Zhang et al., 2014; Liu et al., 2017), which has been widely explored for osteosarcomas studies. Understanding of molecular mechanisms that regulate osteogenic differentiation may hold promise for the novel treatment of human osteosarcoma (Chen et al., 2014; Modi et al., 2019; Lanzillotti et al., 2021). Recently, LINC00707 has also been found to exert an important pro-osteogenic differentiation contribution in human bone marrow mesenchymal stem cells (HBMSCs) (Jia et al., 2019; Cai et al., 2020; Liu J. et al., 2020). Most studies have found that LINC00707 expression is markedly elevated during osteogenic differentiation. The key mechanisms via which LINC00707 positively regulates osteogenic differentiation are by functioning as miR-145 (Cai et al., 2020) sponges to affect the role of LRP5 and thereby initiating the Wnt/β-catenin pathway and by sponging miR-370-3p to upregulate WNT2B (Jia et al., 2019) levels. However, one study found the opposite; LINC00707 levels were significantly decreased, and overexpression of LINC00707 repressed osteoblastic induction of HBMSCs via combination with miR-103a-3p to upregulate DKK1 expression (Jia et al., 2019).

2.1.3 Breast Cancer

BC (DeSantis et al., 2011; Yin et al., 2017; Liang et al., 2020) is one of the most common female malignancies worldwide and is associated with notable incidence and mortality. Breakthroughs in the use of genomic technologies for identification and diagnosis have provided unprecedented insights into the understanding of pathogenic mechanisms of BC (Libson and Lippman, 2014; Peairs et al., 2017; Liang et al., 2020). These findings may contribute to the development of more effective systemic therapies.

LINC00707 is significantly upregulated in BC tissues, cell lines (MDA-MB-468, SK-BR-3, MDA-MB-415, Hs 362.T, and MDA-MB-231 cells), and precancerous cells (MCF-10AT cells) (Jiang et al., 2020; Yuan et al., 2020). LINC00707 has roles in carcinogenesis in cell proliferation and apoptosis, and in the

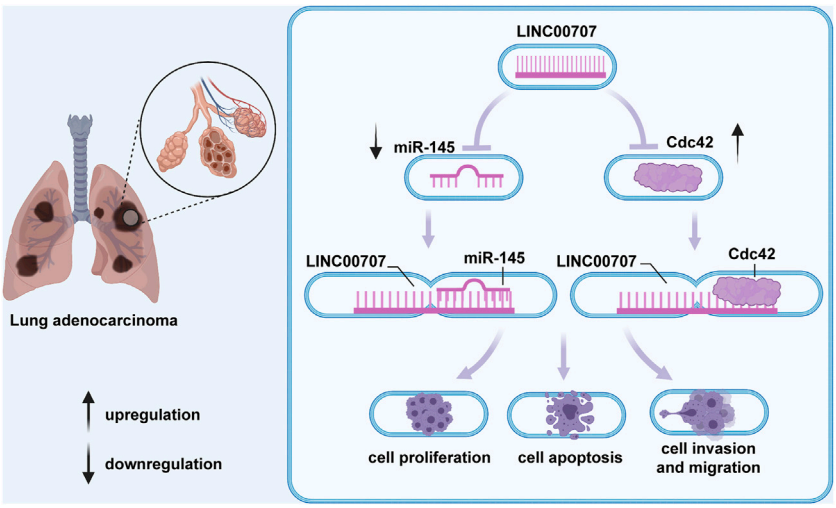

FIGURE 1 | In lung adenocarcinoma, LINC00707 participates in mediation of the processes of cell proliferation, apoptosis, and migration by upregulating Cdc42 expression or combining with miR-338-3p and further increasing AHSA1 level.

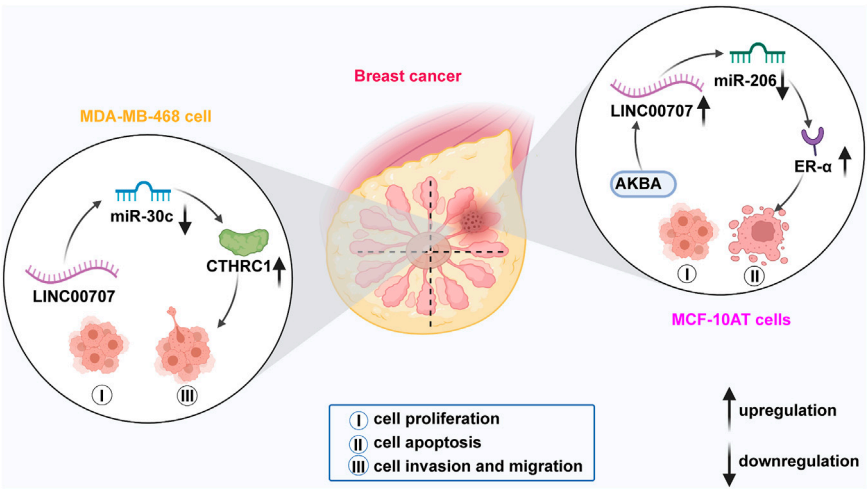

FIGURE 2 | In breast cancer, LINC00707 sponges miR-30c to modulate expression of CTHRC1 and thus enhance the proliferative, invasive, and migratory abilities of MDA-MB-468 cells. LINC00707 also interacts with miR-206 to upregulate ER-α expression, which promotes the processes of proliferation and apoptosis of MCF-10AT cells.

invasion and migration of BC MDA-MB-231, MDA-MB-468, and MCF-10AT cells (**Figure 2**).

2.1.4 Cervical Cancer

Cervical cancer (Moore, 2006; Li et al., 2019; Gao et al., 2020) is one of the most common gynecological cancers in women worldwide. Infection with human papilloma virus (Burd, 2003; Olusola et al., 2019) is the leading pathogenic risk factor for development of cervical cancer. Newly developed molecular technologies have been used to reveal a variety of new molecular characteristics for clinical therapies (The Cancer Genome Atlas Research Network, 2017). LINC00707 is markedly overexpressed in 20 cervical cancer tissues and cell lines (e.g., HCC94, CaSki, MS751, HT-3, and C-33A) (Guo et al., 2021). Xenograft mouse models have revealed that LINC00707 overexpression is strongly linked to increased tumor volumes and weights. LINC00707 exerts its oncogenic functions to prompt cervical cancer development by modulating biological processes such as cell proliferation, migration, and invasion in C-33A and HT-3 cells.

2.1.5 Bladder Cancer

Bladder cancer (Dobruch et al., 2016; Aghaalikhani et al., 2019; Cao et al., 2021) is the most frequent urinary system malignancy. LINC00707 expression is markedly upregulated in bladder cancer (Gao and Ji, 2021) tissues from 103 patients at the Weifang

People's Hospital and UMUC3 and T24T cells. Overexpression of LINC00707 is associated with tumor stage, grade, poor overall survival, and disease-free survival. Biological functional studies have found that LINC00707 is involved in the induction of cell proliferation, colony formation, and metastasis of UMUC3 and T24T cells.

2.1.6 Gastric Cancer

Gastric cancer (Jemal et al., 2011; Yasunaga et al., 2013) is the most common malignancy of the human digestive system. Findings of mechanism studies highlight that LINC00707 has emerged as a crucial promotor in gastric cancer initiation and development. LINC00707 is markedly expressed in 60 gastric cancer patients' tissues (Xie et al., 2019) and cell lines. Upregulation of LINC00707 is clinically linked to increased TNM stage, tumor size, lymphatic metastasis, and an unfavorable prognosis in patients with gastric cancer. LINC00707 also has pivotal oncogenic functions in the processes of cell proliferation and metastasis in BGC-823 and SGC-7901 cells.

2.1.7 Glioma

LINC00707 is overexpressed in glioma tissues (Liu and Hu, 2020; Yu et al., 2021) and cells. This high expression is associated with poor glioma clinicopathological parameters, including Karnofsky performance status score and WHO grade (Liu and Hu, 2020). LINC00707 enables modulation of diverse biological behaviors in gliomas with respect to cell proliferation, invasion, and migration, and together with generation of VM of U87 and U251 cells. (Liu and Hu, 2020; Yu et al., 2021).

2.1.8 Colorectal Cancer

As an oncogene, LINC00707 participates in the modulation of CRC (Shao et al., 2019; Zhu H. et al., 2019; Wang et al., 2020). High levels of LINC00707 expression occur in both CRC tissues and cell lines and are strongly associated with large tumor size (Zhu H. et al., 2019; Wang et al., 2020), advanced tumor stage, lymphatic and distant metastases, and poor survival. LINC00707 exerts cancer-promoting activity, as it participates in cell proliferation, invasion, and migration of LoVo, HT29, SW480, SW620, and HCT116 cells.

2.1.9 Hepatocellular Carcinoma

Noncoding RNAs are implicated in carcinogenesis, including of HCC (Bruix and Sherman, 2011; Galuppo et al., 2014). LINC00707 is highly expressed in HCC cell lines (e.g., HepG2, Huh7, Hep3B, and SNU449) and tissues. It can regulate the cell cycle, enhance cell proliferation, migration, and invasion capacity, and repress cell apoptosis of HepG2, Huh7, Hep3B, and SNU449 cells.

2.1.10 Ovarian Cancer

A study of biomarkers for ovarian cancer in patients exposed to bisphenol A (Zahra et al., 2021) found that LINC00707 levels are significantly increased in ovarian cancer tumor tissues compared with adjoining healthy tissues. Using the SKOV3 cell exposed to bisphenol A, LINC00707 has been found to associate with poor overall survival.

2.1.11 Melanoma

Expression of LINC00707 is distinctly higher during early-stage melanoma (Yang et al., 2018). More importantly, LINC00707, which is contained within the six-lncRNA signature, may have potential roles in melanoma risk-stratification and survival prognosis via signaling pathway types such as the mitogen-activated protein kinase (MAPK) pathway, immune and inflammation-related pathways, the neurotrophin signaling pathway, and focal adhesion pathways.

2.1.12 Clear Cell Renal Cell Carcinoma

LINC00707 is overexpressed in 526 clear cell renal cell carcinoma tissues (Pang et al., 2020) and cell lines. It promotes proliferative, migratory, and invasive biological processes of 786-O and 769-P cells via the epithelium-to-mesenchymal transition pathway.

2.2 Other Diseases

2.2.1 Pneumonia

Pneumonia (Liu et al., 2018; Schicke et al., 2020) is the most frequent and fatal infectious disease of the lower respiratory tract worldwide. It typically manifests as a series of lung inflammatory responses. Precise identification of the underlying associated pathogens to implement effective anti-infective therapy remains a challenge (Meyer Sauteur, 2020). Sensitive diagnostic tools to guide pneumonia treatment (Naydenova et al., 2016) are required. LINC00707 (Zou et al., 2021) is highly expressed in damage caused by lipopolysaccharide (LPS) in MRC-5 cells. LINC00707 participates in biological functions mediated by LPS, such as cell viability, apoptosis, and inflammatory status in MRC-5 cells.

2.2.2 Spinal Cord Injury

LINC00707 is a vital regulator involved in the progression of SCI (Zhu S. et al., 2019). Its expression is significantly increased in SCI LPS-treated PC-12 cells, where it consequently exerts pro-apoptotic and pro-inflammatory effects on LPS-treated cells by binding to miR-30a-5p and inhibiting Neurod 1 expression. These results may reveal a new strategy for clinical treatment of SCI.

3 MECHANISMS OF LINC00707 IN DISEASE

LINC00707 participates in the regulation of a diverse range of biologic functions, including tumor cell proliferation, apoptosis, invasion, metastasis, cell cycle regulation, and osteogenesis. The following sections mainly focus on the molecular mechanisms of LINC00707 in regulating biological functions of diseases.

3.1 Cell Proliferation

Dysregulation of the cell cycle leads to unlimited cell proliferation and further tumor formation (Tu et al., 2020). LINC00707 has a pro-oncogenic role and promotes cell proliferation in cancers such as LUAD (Ma et al., 2018), osteosarcoma, BC (Yuan et al.,

2020), CC (Guo et al., 2021), bladder cancer (Gao and Ji, 2021), glioma (Liu and Hu, 2020), HCC (Tu et al., 2019; Wang et al., 2019), and clear cell renal cell carcinoma (Pang et al., 2020).

In LUAD SPCA1 and A549 cells, LINC00707 fosters cell proliferation via direct enhancement of expression of target gene cell division cycle 42 (Cdc42) (Ma et al., 2018). Experiments on osteosarcoma MG-63 and Saos-2 cells revealed that LINC00707 exploits positive regulatory effects on cell proliferation by acting as a competing endogenous RNA (ceRNA) of miR-338-3p and further heightening AHSA1 expression (Zhang et al., 2021). In BC (Yuan et al., 2020), LINC00707 sponges miR-30c to affect CTHRC1 expression, thus enhancing the proliferative ability of BC MDA-MB-231 and MDA-MB-468 cells (Yuan et al., 2020). LINC00707 can also interact with miR-206 to regulate expression of target protein ER-α, which affects proliferation of precancerous breast MCF-10AT cells (Jiang et al., 2020). Likewise, in cervical cancer HT-3 (Guo et al., 2021) and C-33A cells, LINC00707 functions as an miRNA sponge to restrain levels of miR-382-5p and elevate expression of VEGFA to result in more intense cell proliferation. In bladder cancer (Gao and Ji, 2021), LINC00707 is involved in Wnt/β-catenin signaling to contribute to the proliferation of UMUC3 and T24T cells, through sponging miR-145 to modulate CDCA3 expression. In GC (Xie et al., 2019), LINC00707 can regulate the stability of its downstream target VAV3/F11R mRNAs to promote proliferation of GC BGC-823 and SGC-7901 cells by interacting with the mRNA stabilizing protein HuR. In glioma (Yu et al., 2021), LINC00707 directly binds miR-613/miR-651-3p, and thus promotes proliferation of U87 and U251 cells. In CRC, LINC00707 accelerates cell proliferation, and even the cell cycle from the G1 to S phase, via sponging miR-206 to increase expression of target proteins FMNL2 (Shao et al., 2019) in SW620, and HCT 116 cells, NOTCH3 (Zhu H. et al., 2019) and TM4SF1 in LoVo and HCT116 cells, or via binding with miR-485-5p (Wang et al., 2020) in HT29 and HCT116 cells. In HCC (Tu et al., 2019; Wang et al., 2019), LINC00707 knockdown inhibits HCC cell proliferation, cell cycle progression, and colony formation via depression of the ERK/JNK/AKT signaling pathway in Hep3B and SNU449 cells or via regulation of miR-206 and CDK14 *in vivo* and in HepG2 and Huh7 cells.

3.2 Cell Apoptosis

Cell apoptosis (Liu et al., 2009; Yamanouchi et al., 2010; Seyrek et al., 2019) is the common process of programmed cell death. Abnormal regulation of apoptosis has an important role in cancer pathogenesis. In LUAD (Ma et al., 2018), LINC00707 suppresses cell apoptosis by activating Cdc42 expression. LINC00707 directly inhibits miR-145 expression to reduce NSCLC A549 cell apoptosis (Zhang et al., 2019). Large studies of patients with pneumonia found that LINC00707 binds to miR-223-5p, and subsequently regulates p38 MAPK and nuclear factor-κB (NF-κB) signaling pathways to aggravate LPS-mediated MRC-5 cell apoptosis (Zou et al., 2021). In BC, LINC00707 is hypothesized to be involved in inhibition of MCF-10AT cell apoptosis (Jiang et al., 2020) and to drive the cell cycle from the G1 to S and the G2 phase via blocking miR-206 expression and upregulating ESR1 expression. LINC00707 serves as a ceRNA to combine with miR-145 and thus affect CDCA3 expression and decrease the apoptotic cell number of bladder cancer (Gao and Ji, 2021) UMUC3 and T24T cells. In HCC (Tu et al., 2019; Wang et al., 2019), the antiapoptotic effects of LINC00707 are via mediation of the activity of the ERK/JNK/AKT pathway in Hep3B and SNU449 cells or via interaction with miR-206 to regulate CDK14 expression in HepG2 and Huh7 cell (**Figure 3**).

3.3 Cell Migration and Invasion

Invasion and migration are the major causes behind the high mortality rate of diverse types of cancer (Liu L. et al., 2020) and high rates are characteristic of advanced malignancies. In LUAD, LINC00707 overexpression can induce cell migration and invasion via triggering Cdc42 expression (Ma et al., 2018). In osteosarcoma, LINC00707 binds to miR-338-3p and increases AHSA1 expression to promote cell migration and invasion (Zhang et al., 2021). In BC MDA-MB-231 cells and MDA-MB-468 cells, LINC00707 acts as a miRNA sponge to interact with miR-30c and actively stimulate CTHRC1 expression; it thus promotes BC cell invasion and migration (Yuan et al., 2020). CC mechanism studies revealed that LINC00707 can combine with miR-331-3p to regulate expression of target VEGFA, and thus contribute to cervical cancer (Guo et al., 2021) HT-3 and C-33A cell migration and invasion.

In vitro bladder cancer assays (Gao and Ji, 2021) revealed LINC00707 knockdown leads to damaged premetastatic abilities of UMUC3 and T24T cells via targeting miR-145 and further inhibition of CDCA3 expression. In GC (Xie et al., 2019), LINC00707 combines with HuR, thereby reinforcing the stability of the mRNA targets VAV3 and F11R and promoting BGC-823 and SGC-7901 tumor cell metastasis. In glioma (Liu and Hu, 2020; Yu et al., 2021), LINC00707 enhances migration and invasion capacities of glioma U87 and U251 cells via direct binding of LINC00707 to miR-613 or miR-651-3p. In CRC, LINC00707 modulates protein expression of FMNL2 (Shao et al., 2019), NOTCH3 (Zhu H. et al., 2019), and TM4SF1 by sponging miR-206 and thus making LoVo, SW620, and HCT 116 cells more aggressive. During HCC migration and invasion (Tu et al., 2019; Wang et al., 2019), LINC00707 induces cell migration and invasion of HepG2, Huh7, Hep3B, and SNU449 cells by downregulating miR-206 to increase CDK14 levels or via deactivation of the ERK/JNK/AKT pathway.

3.4 *In Vivo* Studies

A LUAD xenograft tumor model that found smaller tumor sizes, lower tumor weights, and slower tumor growth after LINC00707 knockdown (Ma et al., 2018) further supported the tumor-promoting properties of LINC00707. In a GC nude mouse model (Xie et al., 2019), the phenomenon of larger tumor volumes and weight, a faster tumor growth and epithelium-to-mesenchymal transition process, and fewer metastatic nodules further supported the hypothesis that LINC00707 can promote GC cell tumorigenesis and metastasis. A xenograft experiment using glioma nude mice (Yu et al., 2021) found that LINC00707 knockdown attenuates the process of tumor growth, results in a

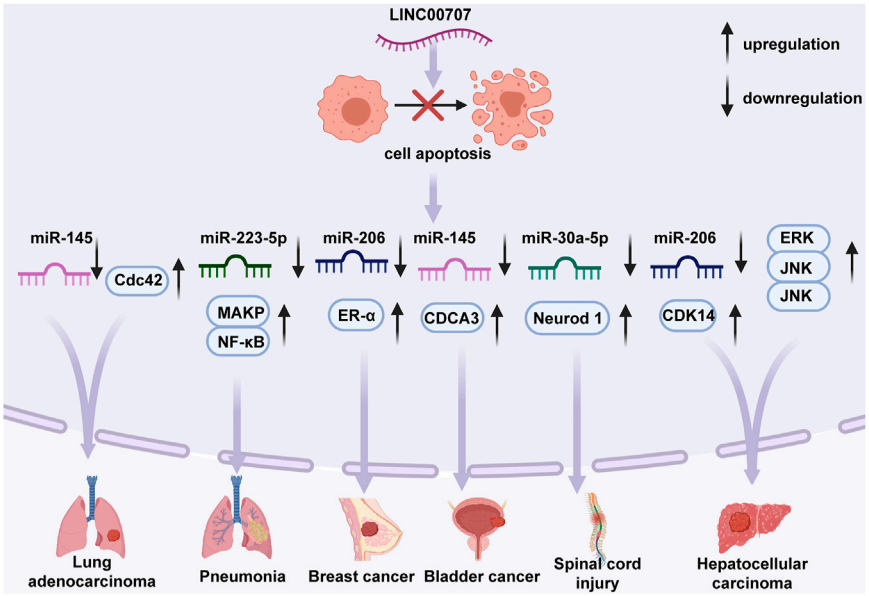

FIGURE 3 | Regulative functions of LINC00707 in the apoptotic mechanisms of certain cell types. In lung adenocarcinoma, LINC00707 suppresses cell apoptosis by activating expression of Cdc42 or inhibiting expression of miR-145. In pneumonia, LINC00707 binds to miR-223-5p and regulates p38 mitogen-activated protein kinase (MAPK), and nuclear factor-κB (NF-κB) signaling pathways. In breast cancer, LINC00707 suppresses miR-206 expression and upregulates ESR1 expression. In bladder cancer, LINC00707 combines with miR-145 and affects expression of CDCA3. In hepatocellular carcinoma, LINC00707 mediates activity of the ERK/JNK/AKT pathway or interacts with miR-206 to regulate expression of CDK14. In spinal cord injury, LINC00707 binds to miR-30a-5p and inhibits expression of Neurod 1.

better survival rate and less VM formation, and revealed the value of the protumor for the occurrence and development of glioma. A tumor xenograft experiment that was performed in mice with cervical cancer (Guo et al., 2021) revealed reduced tumor volumes and tumor weights when LINC00707 was lacking. These results supported the hypothesis that LINC00707 has a role in cervical cancer growth. A CRC *in vivo* xenograft nude mice model (Shao et al., 2019) revealed that tumors grow more slowly under conditions of LINC00707 knockdown and further support the cancer-promoting ability of LINC00707. A BALB/c mouse animal study of HCC (Tu et al., 2019) also found that downregulation of LINC00707 hinders HCC carcinogenesis.

4 LINC00707 IN CLINICAL SETTINGS

4.1 LINC00707 as a Diagnosis and Prognosis Biomarker

Study findings support the hypothesis that abnormal LINC00707 expression in cancer tissues is a useful marker for the clinical diagnosis and prognosis of cancers including lung adenocarcinoma (Ma et al., 2018), BC (Jiang et al., 2020; Yuan et al., 2020), bladder cancer (Gao and Ji, 2021), melanoma (Yang et al., 2018), GC (Xie et al., 2019), and CRC (Wang et al., 2020). The differential expression of LINC00707 in normal and pathological tissues and cell lines has made it the hopeful and powerful tool of choice for disease diagnosis. However, the expression of LINC00707 in some types of disease and specific physiological processes remains unclear, such as the process of osteogenic differentiation. Considering the limited research recently, further studies are necessary to confirm the exact expression in diseases. In addition, there is little evidence concerning the stability and distribution of LINC00707 in human plasma, serum, urine and other body fluids at present, hindering the LINC00707 application in diagnosis. Blood and urine are easily available body fluids which are capable of reflecting the systemic metabolic activity. Further studies for the expression, sensitivity and stability of LINC00707 in non-invasive body fluids are required to make LINC00707 an ideal tool for disease diagnosis.

A study of LUAD in hypoxic conditions found that a constructed seven-lncRNA prognostic model that contained LINC00707 had reliable predictive ability, based on area under the receiver operating characteristic curve results (Shao et al., 2021). LINC00707 overexpression to reduces disease-free and overall survival (Ma et al., 2018). In hypoxic conditions, LINC00707 also mirrors the tumor immunological homeostatic response; its use improves the prognosis (Ma et al., 2018; Shao et al., 2021) of patients with LUAD.

4.2 LINC00707 as a Disease Treatment Target

LINC00707 is widely engaged in the processes of cell proliferation, apoptosis, and metastasis for a range of diseases, including LUAD (Ma et al., 2018; Zou et al., 2021), osteosarcoma (Jiang et al., 2020; Zhang et al., 2021), BC (Yuan et al., 2020), glioma (Liu and Hu, 2020; Yu et al., 2021), CRC (Shao et al., 2019; Zhu H. et al., 2019), and pneumonia (Zou et al., 2021). The differential expression of LINC00707 in a broad range of diseases

and physiological processes can also provide a new insight into drug candidate for the disease treatment. We can employ the pro-oncogenic role of LINC00707 in some disease to achieve a therapeutic effect by suppressing its expression. And the application of the anti-oncogenic role in other disease can also attain the therapeutic impact via enhancing its level. On the other hand, LINC00707 knock-down or agonist/antagonist addition based on its molecular mechanisms in disease may act as essential druggable targets for the development of new therapies. For example, LINC00707 knockdown enables relief of LPS-induced cell injury and inflammation, which is a potential novel therapeutic approach for patients with pneumonia (Zou et al., 2021). A study of BC revealed that acetyl-11-keto-β-boswellic acid (Jiang et al., 2020) may have therapeutic effects on precancerous breast lesions via the LINC00707/miR-206 axis, and therefore mediate the ER-α protein of ESR1 expression to inhibit the estrogen signaling pathway. Remarkably, one study found that LINC00707 involvement is necessary for the regulation of VM formation via the HNRNPD/ZHX2/LINC00707/miR-651-3p/SP2 axis. These results suggest a novel approach for anti-VM therapy for glioma (Yu et al., 2021). It is also found to intensify the DDP IC50 value and consequent involvement in the progression of cisplatin (DDP) resistance in NSCLC A549 cells (Zhang et al., 2019). However, current information regarding LINC00707 for treatment application is limited, the efficacy, stability and safety of LINC00707-targeted drugs need further pre-clinical and clinical studies.

Taken together, these findings all suggest that LINC00707 is a promising treatment target for a variety of diseases. Further studies for the expression, sensitivity, and stability of LINC00707 in non-invasive body fluids for disease diagnosis in addition to the efficacy, and safety of LINC00707-targeted drugs for disease treatment was required.

5 CONCLUSION

LINC00707 is now widely accepted to have critical functions in the regulation of diverse pathological and biological processes, together with tumor development and progression through specific pathways. Recently, an increasing number of studies has revealed that LINC00707 is a dysregulated lncRNA in multiple types of diseases. Results indicate it has considerable involvement in a variety of complex clinicopathological characteristics, such as advanced tumor TNM stage, larger tumor size, lymphatic metastasis, distant metastasis, and a shorter overall survival time.

LINC00707 widely participates in various cellular processes, including cellular proliferation, apoptosis, and migration in most types of tumors, and in the process of osteogenic differentiation and the inflammatory conditions induced by LPS. Results of studies on mechanistic pathways suggest that LINC00707 mainly exerts its biological function by binding with relevant miRNA and consequently regulating expression of downstream targets, including miR-338-3p/AHSA, miR-382-5p/VEGFA, miR-145/CDCA3, miR-30c/CTHRC1, miR-103a-3p/DKK1, and miR-370-3p/WNT2B. Taken together, results of studies of LINC00707 could provide us with breakthroughs for the diagnosis, prognosis, and treatment of various diseases, including cancers. Given that LINC00707 research is currently in its nascent stages and studies have not fully revealed its functions, in-depth studies are needed to further elucidate the regulatory mechanisms of LINC00707 in numerous diseases.

AUTHOR CONTRIBUTIONS

QY and ZH wrote the main manuscript text. QY completed the tables and relevant figures. DC conducted the manuscript revision. All authors reviewed and approved the final manuscript.

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Exploration of Potential Roles of m5C-Related Regulators in Colon Adenocarcinoma Prognosis

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Objectives: The purpose of this study was to investigate the role of 13 m^5C-related regulators in colon adenocarcinoma (COAD) and determine their prognostic value.

Methods: Gene expression and clinicopathological data were obtained from The Cancer Genome Atlas (TCGA) datasets. The expression of m^5C-related regulators was analyzed with clinicopathological characteristics and alterations within m^5C-related regulators. Subsequently, different subtypes of patients with COAD were identified. Then, the prognostic value of m^5C-related regulators in COAD was confirmed *via* univariate Cox regression and least absolute shrinkage and selection operator (LASSO) Cox regression analyses. The prognostic value of risk scores was evaluated using the Kaplan-Meier method, receiver operating characteristic (ROC) curve. The correlation between the two m^5C-related regulators, risk score, and clinicopathological characteristics were explored. Additionally, Gene Set Enrichment Analysis (GSEA), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Gene Ontology (GO) analysis were performed for biological functional analysis. Finally, the expression level of two m^5C-related regulators in clinical samples and cell lines was detected by quantitative reverse transcription-polymerase chain reaction and through the Human Protein Atlas database.

Results: m^5C-related regulators were found to be differentially expressed in COAD with different clinicopathological features. We observed a high alteration frequency in these genes, which were significantly correlated with their mRNA expression levels. Two clusters with different prognostic features were identified. Based on two independent prognostic m^5C-related regulators (NSUN6 and ALYREF), a risk signature with good predictive significance was constructed. Univariate and multivariate Cox regression analyses suggested that the risk score was an independent prognostic factor. Furthermore, this risk signature could serve as a prognostic indicator for overall survival in subgroups of patients with different clinical characteristics. Biological processes and pathways associated with cancer, immune response, and RNA processing were identified.

Conclusion: We revealed the genetic signatures and prognostic values of m^5C-related regulators in COAD. Together, this has improved our understanding of m^5C RNA

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modification and provided novel insights to identify predictive biomarkers and develop molecular targeted therapy for COAD.

Keywords: colon adenocarcinoma, epigenetics, RNA modification, 5-methylcytosine, gene expression profile, prognostic signature, TCGA

INTRODUCTION

Changes in gene expression are closely associated with the development of disease, and epigenetic processes are heritable changes in gene expression that do not alter the nucleotide sequence (Wu and Morris, 2001). Traditional epigenetic modifications, including chromatin remodeling, DNA methylation, and histone modifications, are involved in various biological processes related to the occurrence and progression of tumors, including gastrointestinal cancers (Dawson and Kouzarides, 2012; Darwiche, 2020; Grady et al., 2021). With considerable progress in zymology and high-throughput sequencing technology, epitranscriptomics has attracted significant attention recently (Angelova et al., 2018; Porcellini et al., 2018; Minervini et al., 2020; Song et al., 2020; Zhao et al., 2020; Schaefer, 2021). Research investigating the physiological and pathological functions of RNA modifications have identified multiple dynamic modifications of RNA, including N6-methyladenosine, 2-O-dimethyladenosine, 5-methylcytosine (m^5C), 7-methylguanosine, N1-methyladenosine, and pseudouridylation (Roundtree et al., 2017; Shi et al., 2020). Increasing evidence suggests that RNA modifications play critical roles in tumorigenesis and the progression of different cancers (Barbieri and Kouzarides, 2020; Begik et al., 2020). m^5C RNA modification is found in a variety of RNAs, including messenger RNAs, transfer RNAs, ribosomal RNAs. This modification introduces a methyl group in the fifth carbon atom of cytosine (Yu-Sheng Chen et al., 2021). Based on published data, m^5C RNA modification plays a critical role in the translation, transport, and stability of mRNAs, and is also closely associated with the biogenesis and function of other RNA species (Xue et al., 2020; Hussain, 2021). As a dynamic and reversible process, m^5C RNA modification is primarily regulated by "writers" (adenosine methyltransferases) and "erasers" (demethylases), and achieves different functions by interacting with "readers" (m^5C-binding proteins). The "writers" include the NOL1/NOP2/Sun domain RNA methyltransferase family NSUN1-NSUN7 and DNMT2. m^5C "erasers" include enzymes in the TET family (TET1, TET2, TET3) and ALKBH1. The "readers", such as ALYREF and YBX1, recognize and bind to methylated RNAs to realize different functions (Nombela et al., 2021; Xie et al., 2020).

Globally, colorectal cancer (CRC) is the third most common cancer and the second most deadly neoplasm (Bray et al., 2018). Colon adenocarcinoma (COAD) is the most common pathological type of CRC, and despite considerable progress in diagnosis and therapeutic strategies for COAD, the prognosis of patients with COAD remains poor due to advanced stage and postsurgical recurrence (White et al., 2017; Miller et al., 2019). Therefore, the identification of novel biomarkers for early detection and effective therapeutic targets for treating patients with COAD is critical and urgent.

In this study, we analyzed a TCGA dataset for m^5C-related regulators involved in COAD, the correlation between the expression levels of 13 m^5C-related regulators and clinicopathological features, as well as potential independent prognostic m^5C-related regulators and a risk signature to predict the prognosis of patients with COAD.

MATERIAL AND METHODS

Acquisition of Datasets

The RNA-seq transcriptome data (fragments per kilobase million, FPKM) from 437 samples (Mortazavi et al., 2008), copy number variant (CNV) data from 825 samples, single nucleotide variant (SNV) data from 399 samples, and clinical information from 385 patients with COAD in TCGA database (http://cancergenome.nih.gov/) were downloaded for our study. Patients with complete clinicopathological and survival information were included for further assessment (**Table 1**).

Selection of m^5C-Related Regulators

Based on published data, 14 m^5C-related regulators, including NOP2 (NSUN1), NSUN2, NSUN3, NSUN4, NSUN5, NSUN6, NSUN7, DNMT2, TET1, TET2, TET3, ALKBH1, ALYREF, and YBX1 were used in our study. DNMT2 was not found to be expressed in COAD from TCGA datasets. Therefore, the remaining 13 m^5C-related regulators were used for further analysis.

Tumor Classification and Principal Component Analysis

To explore the function of m^5C-related regulators in COAD, a consistent clustering algorithm was used to determine the clustering of samples and estimate the stability of the clustering. Using the "Consensus ClusterPlus" R package (Wilkerson and Hayes, 2010), two different subgroups (cluster Ⅰ and cluster Ⅱ) were identified based on the following classification parameters: 1) slow growth rate of the cumulative distribution function value; 2) high correlation in the subgroup; and 3) no small clusters in the clustering data. Furthermore, principal component analysis (PCA) was used to assess gene expression patterns in different subgroups using the "Limma" R package (Ritchie et al., 2015).

Analysis of Clinicopathological Features and Prognosis

The correlation between m^5C-related regulators and clinicopathological features was analyzed. Then, to filter the

TABLE 1 | Clinicopathological features of patients included in this study.

	Total patients (337)		High-risk group (163)		Low-risk group (168)		*p*-value
	Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)	
Fustat							0.009
Alive	279	82.8	125	76.7	148	88.1	
Dead	58	17.2	38	23.3	20	11.9	
Age							0.178
≤65	135	40.1	72	44.2	61	36.3	
>65	202	59.9	91	55.8	107	63.7	
gender							0.714
female	156	46.3	78	47.9	76	45.2	
male	181	53.7	85	52.1	92	54.8	
Stage							
I	59	17.5	28	17.2	30	17.9	
II	137	40.7	61	37.4	73	43.5	
III	87	25.8	42	25.8	44	26.2	
IV	54	16.0	32	19.6	21	12.5	
Stage T							0.016
T1	7	2.1	5	3.1	2	1.2	
T2	59	17.5	26	16.0	32	19.0	
T3	235	69.7	106	65.0	124	73.8	
T4	36	10.7	26	16.0	10	6.0	
Stage M							0.105
M0	283	84.0	131	80.4	147	87.5	
M1	54	16.0	32	19.6	21	12.5	
Stage N							0.202
N0	203	60.2	92	56.4	107	63.7	
N1	76	22.6	37	22.7	38	22.6	
N2	58	17.2	34	20.9	23	13.7	

m^5C-related regulators that were highly correlated with overall survival (OS), univariate Cox regression analysis was performed. Next, the Lasso Cox regression algorithm was used to identify m^5C-related regulators with powerful prognostic significance. According to the best penalty parameter λ, the selected regulators' coefficients were calculated. The risk score (RS) was estimated using the following formula:

$$RS = \sum_{i=1}^{n} Coef\,(i) X\,(i)$$

Where Coef(i) is the coefficient and X(i) represents the expression levels of the selected m^5C-related regulators. Using the obtained median risk score as the demarcation value, patients with COAD were classified in two groups: high-risk group and low-risk group. The OS and clinicopathological features were compared between these subgroups. Kaplan-Meier analysis and the receiver operating characteristic (ROC) curves were used to validate the predictive efficiency (Hanley and McNeil, 1982). Additionally, the prognostic value of the RS was verified using univariate and multivariate Cox regression analyses. The hazard ratio (HR) with 95% confidence intervals and log-rank *p*-value were calculated using the "glmnet" and "survival" R packages (Simon et al., 2011).

Biological Function Analysis

To explore the biological functions associated with m^5C RNA modification, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, Gene Ontology (GO) analysis and Gene Set Enrichment Analysis (GSEA) were performed. The genes that were differentially expressed between the high-risk group and the low-risk group were functionally annotated using GO analysis and KEGG pathway analysis. Next, GSEA was conducted to determine the signaling pathways related to different clusters. Later, to explore the latent biological function of the m^5C-related genes in COAD, GSEA for the m^5C-related regulatory genes with powerful prognostic value was performed. The flow chart of bioinformatic analysis was shown in **Figure 1**.

Cell Culture

The COAD cell lines LS174T and normal colon mucosal epithelial cell line NCM460 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). All cells were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (Life Technologies) at 37°C in a humidified atmosphere with 5% CO_2.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, China) according to manufacturer's instruction. Reverse transcription was carried out according to the manufacturer's instructions using the PrimeScript RT Reagent Kit (Takara, China). The SYBR PrimeScript RT-PCR Kit (Takara)

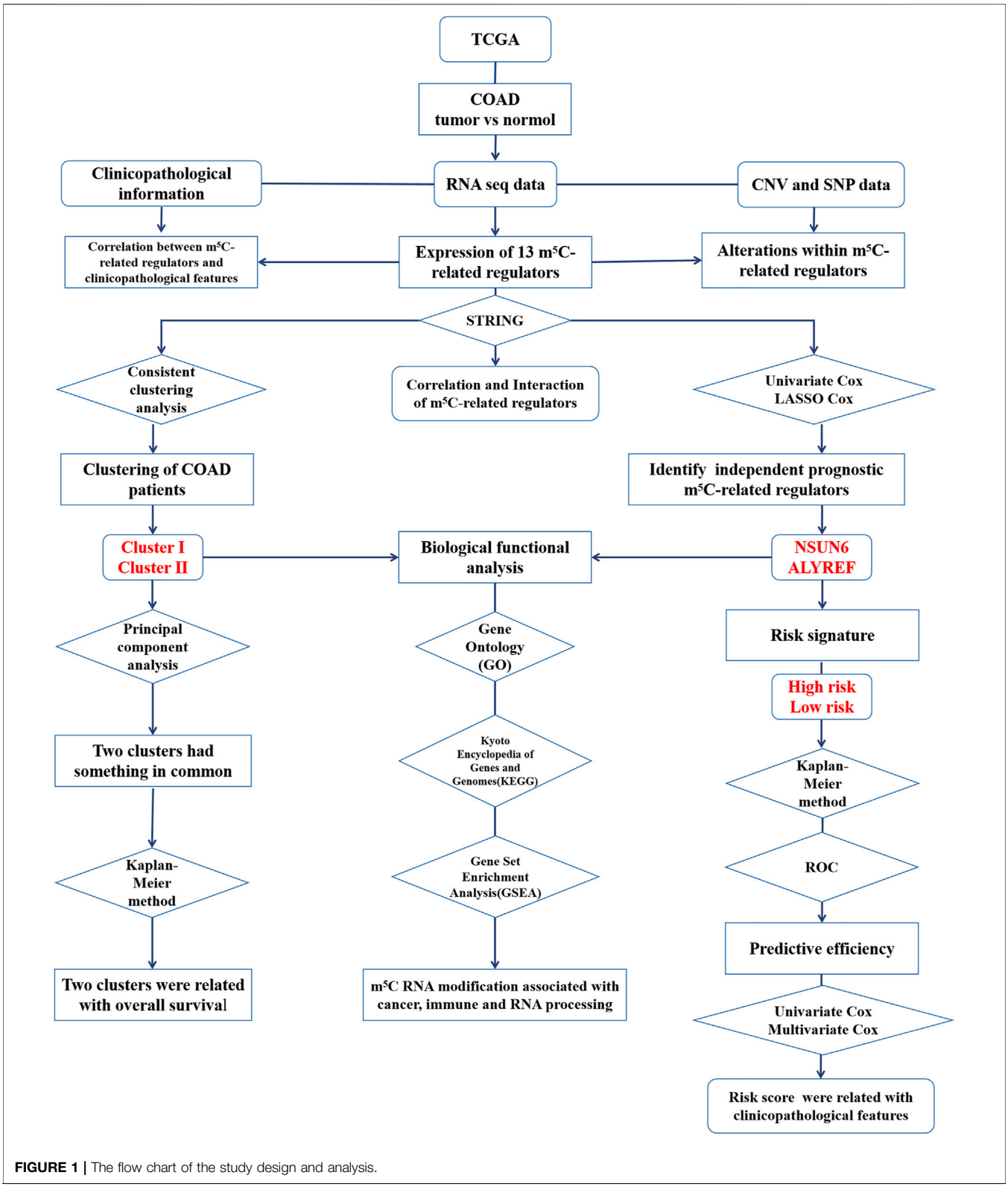

FIGURE 1 | The flow chart of the study design and analysis.

was applied for the analysis of quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Related mRNAs expression levels were calculated using the 2-ΔΔCT method and the related GAPDH mRNA expression was used as an endogenous control. Primers sequences used in our study were as follows: GAPDH forward 5′-GGACCTGACCTGCCGTCT

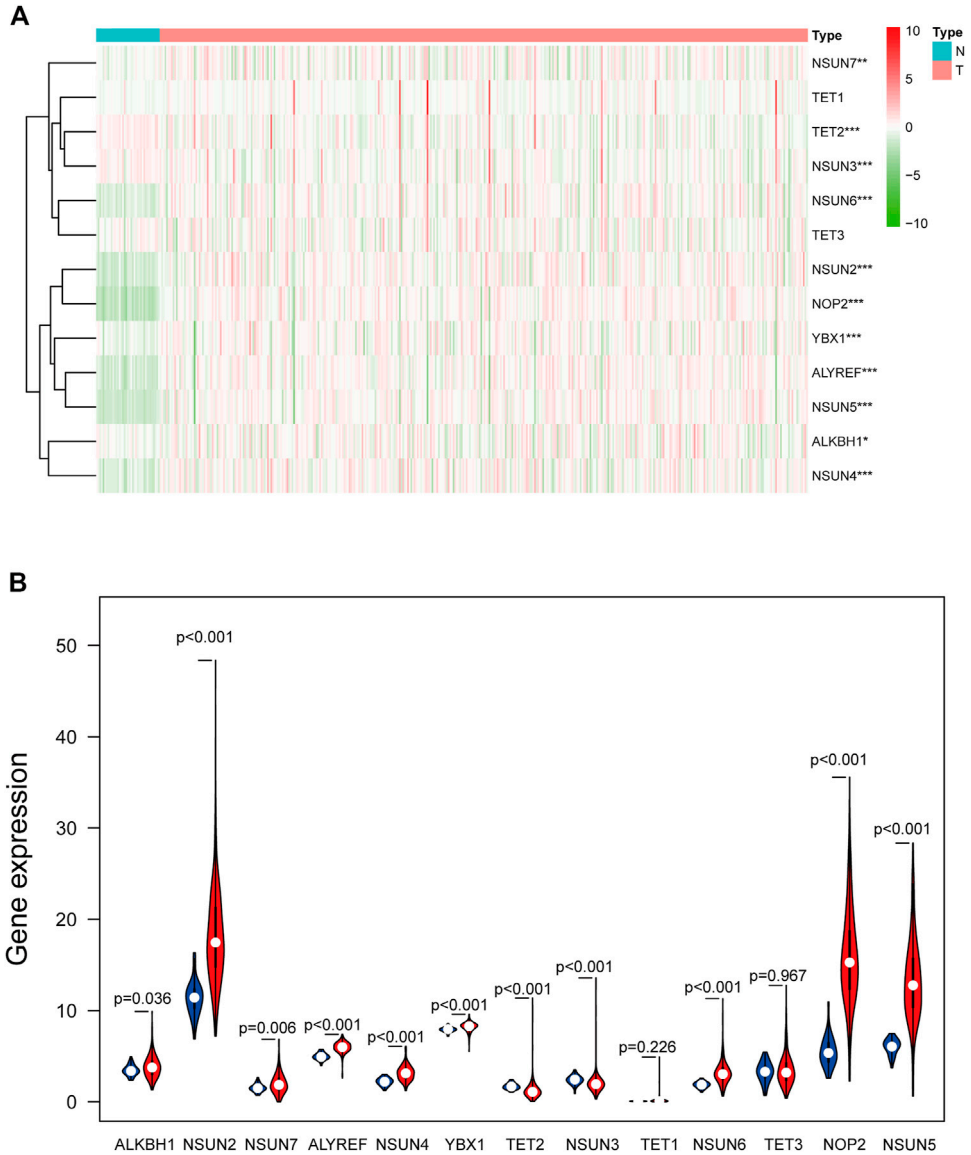

FIGURE 2 | The expression of 13 m^{5}C-related regulators in TCGA database between the tumor group and the normal group. **(A)** Heatmap of the expression of 13 m^{5}C-related regulators. The depth of red represents the level of high expression, and the depth of green represents the level of low expression *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(B)** The violin diagram showed the median expression of 13 m^{5}C-related regulators in COAD, and the position of white spots on the way represented the median value of the expression.

AG-3′, and reverse 5′-GTAGCCCAGGATGCCCTTGA-3′; NSUN6 forward 5′-TTTGCCATCTGCCTTAGT-3′, and reverse 5′-GTGTGTTGTTTTCCCTCC-3′; ALYREF forward 5′-GCAGGCCAAAACAACTTCCC-3′, and reverse 5′-AGT TCCTGAATATCGGCGTCT-3′.

Validation of the Protein Expression Levels of the m^{5}C-Related Regulators *via* the Human Protein Atlas

To verify the protein expression levels of NSUN6 and ALYREF in COAD and normal tissues, immunohistochemistry (IHC) data were downloaded from the Human Protein Atlas (HPA, http://www.proteinatlas.org). The HPA online database provides IHC expression data for nearly 20 different cancers (Asplund et al., 2012) and enables the validation of the differential protein expression levels between tumor and normal tissues.

Statistical Analysis

The expression data of m^{5}C-related regulators in tumor tissues and adjacent mucosa of COAD obtained from TCGA were compared using one-way analysis of variance (ANOVA); the clinical characteristics and m^{5}C-related regulators of different groups were compared using the chi-square test; the Kaplan-

Meier method was used to perform a bilateral logarithmic rank test in overall survival analysis; p-values < 0.05 were regarded as statistically significant. All statistical analyses were implemented using Rv4.0.3 (https://www.r-project.org/).

RESULTS

RNA-Seq Transcriptome Data of m^{5}C-Related Regulators in COAD

Based on RNA-seq transcriptome data of COAD from TCGA database, the expression of 13 m^{5}C-related regulators between tumor tissues and adjacent mucosa was compared (**Figure 2**). With the exceptions of TET1 and TET3, the expression levels of the other 11 factors were significantly different in the tumor tissues and the adjacent mucosal tissues. Compared with the adjacent mucosa, the expression of NSUN3 ($p < 0.001$) and TET2 ($p < 0.001$) in the tumor group was significantly downregulated. The expression of ALKBH1 ($p = 0.036$), ALYREF ($p < 0.001$), NOP2 ($p < 0.001$), NSUN2 ($p < 0.001$), NSUN4 ($p < 0.001$), NSUN5 ($p < 0.001$), NSUN6 ($p < 0.001$), NSUN7 ($p = 0.006$), and YBX1($p < 0.001$) were significantly upregulated in tumor tissues compared with the adjacent mucosa.

Correlation and Interaction of m^{5}C-Related Regulators in COAD

The correlations between the m^{5}C-related regulators were analyzed using the "corrplot" package in R and their interrelationships were retrieved from the STRING database (https://string-db.org/). The expression levels of the seven "writers" were correlated with each other, except for NSUN2 and NSUN7, NSUN5 and NSUN7, NSUN2 and NSUN3, and NSUN5 and NSUN6. There were also close and complicated relationships between each regulator in the protein-protein interaction (PPI) network. We also found that the expression of TET family genes (TET1, TET2, TET3) were highly related to each other and had little correlation with ALKBH1. However, the TET family was associated with ALKBH1 in the PPI network and had interrelationships with the "writer" genes *via* ALKBH1. In addition, there was evidence supporting the interaction between the "reader" genes ALYREF and YBX1 in the PPI network. The expression of these genes was also positively associated with each other (**Figure 3**).

CNVs and SNPs of m^{5}C-Related Regulators in COAD

Regarding CNVs, we found that 10 of the 13 m^{5}C-related regulators were significantly different between the tumor tissue and the adjacent mucosa from 825 samples with CNV data. Furthermore, it was found that CNVs affect the expression of m^{5}C-related regulators. The highest frequency of CNVs occurred in the "writer" gene NSUN5 (24.47%), followed by the "eraser" gene ALKBH1 (19.53%). The "eraser" gene TET3 had the lowest CNV frequency (2.35%) (**Table 2**). The "writer" genes NOP2, NSUN2, NSUN5, and NSUN7, the "eraser" genes TET2 and ALKBH, and the "reader" gene ALYREF displayed a significant difference in expression due to CNVs (**Figure 4**).

Regarding SNPs, we found that all of the m^{5}C-related regulators had missense mutations, and missense mutations were the highest frequency mutation in 399 COAD cases with available sequencing data. Among them, the m^{5}C "eraser" gene TET2 had the highest frequency of mutation events (96/399), followed by TET3 and TET1 (both 39/399). In addition, the "writer" genes NSUN2 and NSUN7, the "eraser" gene TET2, and the "reader" gene ALYREF displayed significant differences in expression levels due to SNPs. Next, we evaluated the effect of SNPs on patient prognosis, but no difference was observed due to the relatively few numbers of mutations (**Figure 5**).

Consensus Clustering of Patients With COAD

Based on the expression levels of 13 m^{5}C-related regulators, consistent clustering analysis of patients with COAD was performed, and they were clustered into two subgroups because there was minimal interference between the two subgroups (**Figures 6A–D**).

PCA showed that the RNA expression levels in patients with COAD in clusters I and II were specific (**Figure 6E**). Nevertheless, there were many overlapping areas between each cluster on the whole, indicating that the clusters had something in common. The cluster II had a longer survival time than cluster I when analyzed using the Kaplan-Meier method, but they had no significant different (**Figure 6F**).

Prognostic Value of m^{5}C-Related Regulators in COAD Prognosis

To evaluate the prognostic value of these 13 m^{5}C-related regulators in COAD, univariate Cox regression analysis was used to identify m^{5}C-related regulators that were highly correlated with the OS in patients with COAD, and two regulators with prognostic significance ($p < 0.05$) were found: NSUN6 and ALYREF. Specifically, ALYREF was considered a protective factor with HR < 1 in patients with COAD, and NSUN6 was considered as a risk factor with HR > 1 (**Figure 7A**). To further evaluate the prognostic significance of these two m^{5}C-related regulators, LASSO Cox regression analysis was performed and it was revealed that NSUN6 (Coef = 0.300256795278519) and ALYREF (Coef = 0.00796895949684636) could serve as powerful prognostic factors in COAD (**Figures 7B–C**).

Based on NSUN6 and ALYREF, a risk signature was constructed and the risk score was calculated. Using the median risk score as the demarcation value, patients with COAD ($n = 525$) were classified into two groups, namely the high-risk and low-risk groups. To test the prognostic role of the two gene risk signatures, survival and ROC curve analyses were conducted. Based on the Kaplan-Meier (KM) survival analysis, the low-risk group had significantly longer survival time than the high-risk group (**Figure 7D**). In particular, compared with the 46.4% 5-year survival rate in the high-risk group, that of the low-

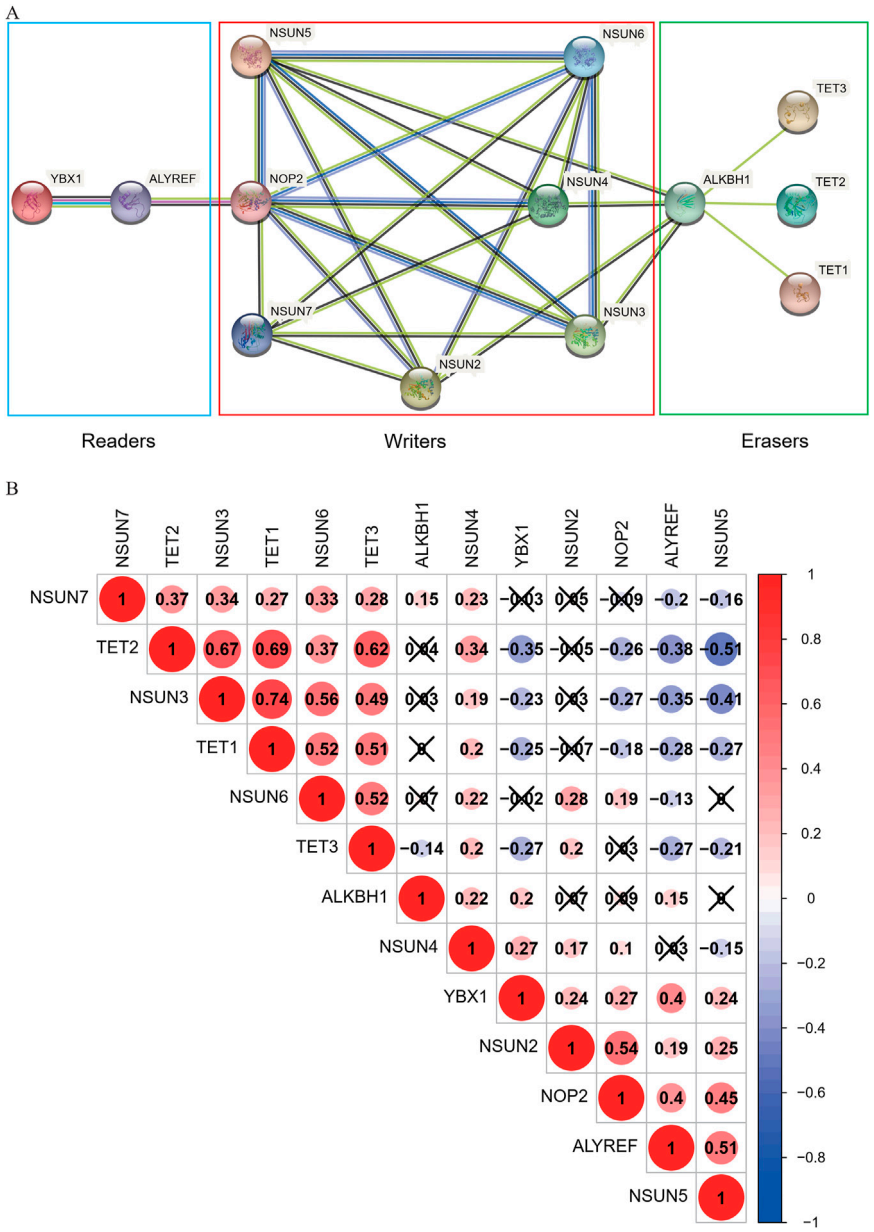

FIGURE 3 | Correlation and interaction of m^{5}C-related regulators in COAD. **(A)**The PPI network of the 13 m^{5}C-related regulators was constructed using STRING. **(B)** Spearman correlation analysis of the 13 m^{5}C-related regulators.

risk group was 78.7%. The area under the curve (AUC) value in the time-dependent ROC curve was 0.754, suggesting good prediction performance of the survival model (**Figure 7E**).

Correlation Between the Two m^{5}C-Related Regulators, Risk Score, and Clinicopathological Characteristics in COAD

We further analyzed the relationship between the two m^{5}C-related regulators, risk score, and different clinical variables. KM survival analysis showed a close association of the two m^{5}C-related regulators (NSUN6 and ALYREF) with the OS of patients with COAD (**Figures 8A,B**). In terms of TMN stage, the expression of ALYREF was differentially expressed between T3 stage and T4 stage and between M0 stage and M1 stage (**Figure 8C**). However, the expression of NSUN6 was not significantly different across groups in the TMN stage (**Figure 8D**). The expression of the two m5C-related regulators and the distribution of clinicopathological characteristics in the high-risk and low-risk groups are displayed as a heatmap (**Figure 8E**). Evident differences

TABLE 2 | Copy number variants (CNV) of m5C related regulators in colon adenocarcinoma.

Function	Genes	Diploid	Deletion	Amplification	CNV sum	Deletion	Amplification	Percentage
Writers	NOP2	379	6	40	46	13.04%	86.96%	10.82%
	NSUN2	385	7	33	40	17.50%	82.50%	9.41%
	NSUN3	406	5	14	19	26.32%	73.68%	4.47%
	NSUN4	406	17	2	19	89.47%	10.53%	4.47%
	NSUN5	321	1	103	104	0.96%	99.04%	24.47%
	NSUN6	405	9	11	20	45.00%	55.00%	4.71%
	NSUN7	391	32	2	34	94.12%	5.88%	8.00%
Erasers	TET1	405	13	7	20	65.00%	35.00%	4.71%
	TET2	396	26	3	29	89.66%	10.34%	6.82%
	TET3	415	2	8	10	20.00%	80.00%	2.35%
	ALKBH1	342	78	5	83	93.98%	6.02%	19.53%
Readers	ALYREF	377	13	35	48	27.08%	72.92%	11.29%
	YBX1	401	19	5	24	79.17%	20.83%	5.65%

between the two groups according to stage T ($p < 0.05$) and fustat ($p < 0.01$) were observed.

To evaluate whether the risk score could serve as a prognostic indicator for OS in subgroups of patients with different clinical characteristics, we stratified subgroups by age (age ≤ 65 and age > 65), gender (female and male), clinical stage (stage I-II and stage III-IV), stage T (T1-2 and T3-4), stage M (M0 and M1) and stage N (N0 and N1-2). As the result shown in **Figures 9A–D**, the OS of the low-risk patients based on age ($p < 0.001$ in age ≤ 65), sex ($p < 0.001$ in male), and stage T ($p < 0.005$ in stage T1-2 and T3-4) was significantly higher than those of the high-risk patients.

To further examine whether the risk score was an independent prognostic factor, univariate and multivariate Cox regression analyses were conducted. This revealed that the risk score was significantly associated with OS in univariate analysis, in addition to age at diagnosis, pathological stage, and TNM stage ($p < 0.05$). However, only the age at diagnosis and risk score were correlated with OS ($p < 0.05$) in the multivariate Cox regression analysis (**Figures 9E,F**).

Biological Functional Analysis

As we clustered the patients with COAD into cluster I and cluster II, genes that were significantly upregulated (fold change >1 and $p < 0.05$) or downregulated (fold change <1 and $p < 0.05$) between the high-risk group and low-risk group were identified using the "edgeR" package in R. GO and KEGG pathway analysis were used for biological functional analysis.

Concerning GO analysis, the differentially expressed genes were associated with immune-related biological processes, such as "antigen binding" and "immunoglobulin receptor binding," and pre-mRNA-related biological processes, such as "pre-mRNA 5′-splice site binding" and "pre-mRNA binding." (**Figure 10A**). KEGG pathway analysis results were correlated with immune-related pathways, including "complement and coagulation cascades" and "NOD-like receptor signaling pathway," and RNA-related pathways, including "RNA transport" and "spliceosome." Moreover, cancer-related pathways were enriched, such as "transcriptional misregulation in cancer" and "MAPK signaling pathway" (**Figure 10B**).

Next, we used GSEA to predict the functional difference between clusters I and II. The results showed that cluster I had a worse OS and lower 5-year survival rate associated with malignancy-associated pathways, including the ATP-binding cassette transporter (NES = 1.79, normalized $p = 0.006$) and phosphatidylinositol signaling system (NES = 1.63, normalized $p = 0.03$) (**Figures 10C,D**).

Furthermore, as NSUN6 and ALYREF were shown to be important regulators of m^5C in our study, GSEA was performed to investigate the potential biological processes associated with NSUN6 and ALYREF in COAD pathogenesis. GSEA suggested that increased expression of NSUN6 and ALYREF is involved in various biological functions in RNA processing, such as spliceosome, RNA polymerase, and RNA degradation. Upregulation of these genes was associated with malignancy-associated pathways, such as the cell cycle (**Figures 10E,F**).

Validation of the Expression Levels of the m^5C-Related Regulators in Cell Lines and Clinical Samples

For validating the expression levels of the two m^5C-related prognostic regulators from prognostic signature, we detected the expression levels in the COAD cell lines LS174T and normal colon mucosal epithelial cell line NCM460 by qRT-PCR. Our results showed that NSUN6 and ALYREF were significantly upregulated in LS174T compared with NCM460 (**Figures 11A,B**). IHC data from the HPA online database also demonstrated that the protein levels of NSUN6 and ALYREF were more highly expressed in cancer tissues than in normal tissues (**Figure 11C**).

DISCUSSION

RNA modifications have been increasingly demonstrated in tumorigenesis and tumor progression, suggesting that RNA epigenetic regulators may play an important role in COAD. Previous studies have shown that m^6A RNA modification not only plays a critical role in the tumorigenesis and progression of CRC, but also has powerful significance in the diagnosis and prognosis of CRC patients (Li et al., 2021). Additionally, a

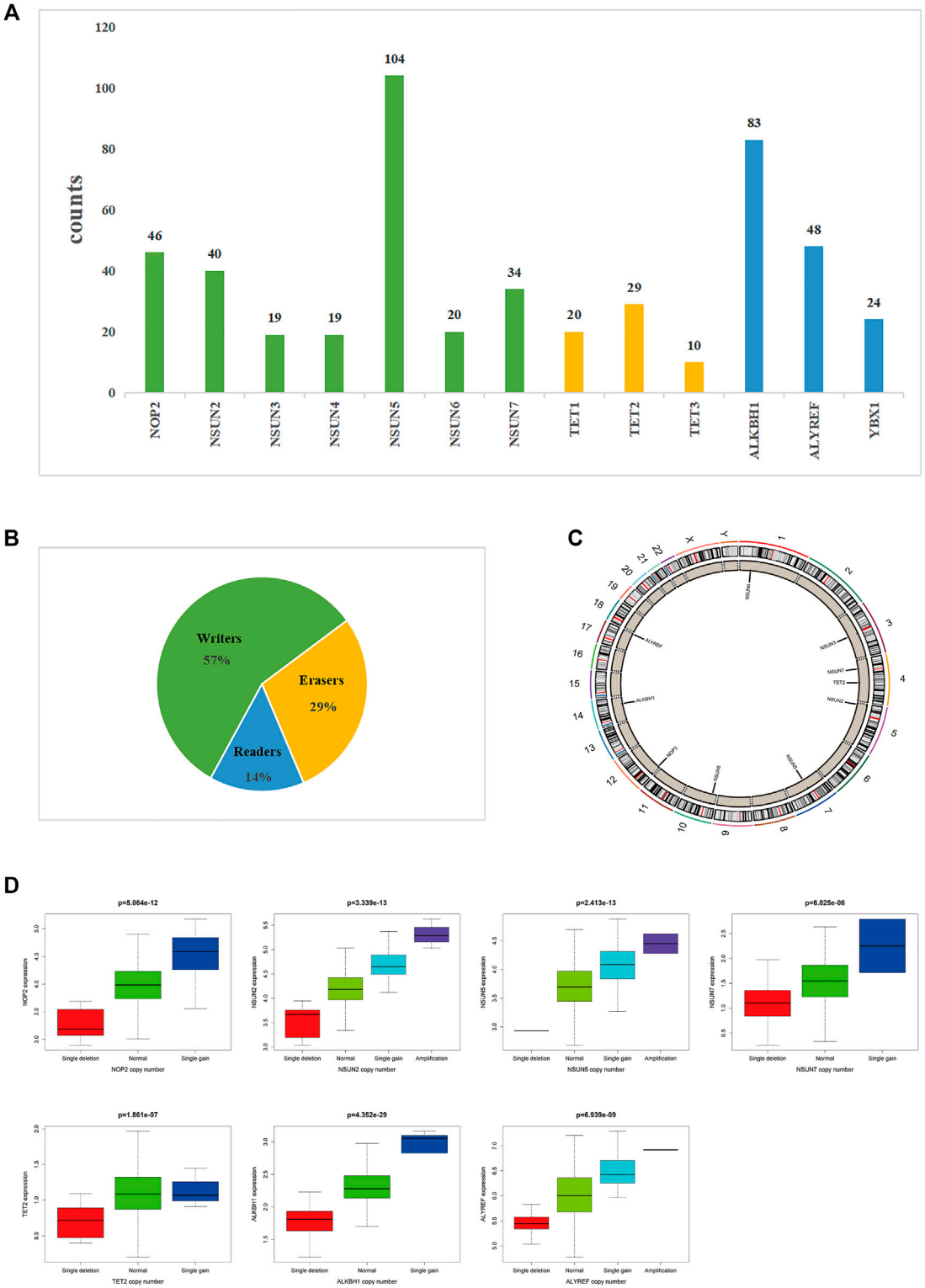

FIGURE 4 | The landscape of CNV of m^{5}C-related regulators in COAD.**(A,B)** Frequency of CNV of 13 m^{5}C-related regulators in COAD. **(B)** Percentage of CNV of 13 m^{5}C-related regulators in COAD. **(C)** Location of CNV alteration of 13 m^{5}C-related regulators on chromosomes. **(D)** NOP2, NSUN2, NSUN5, NSUN7, TET2, ALKBH, and ALYREF displayed a significant difference in expression due to CNVs.

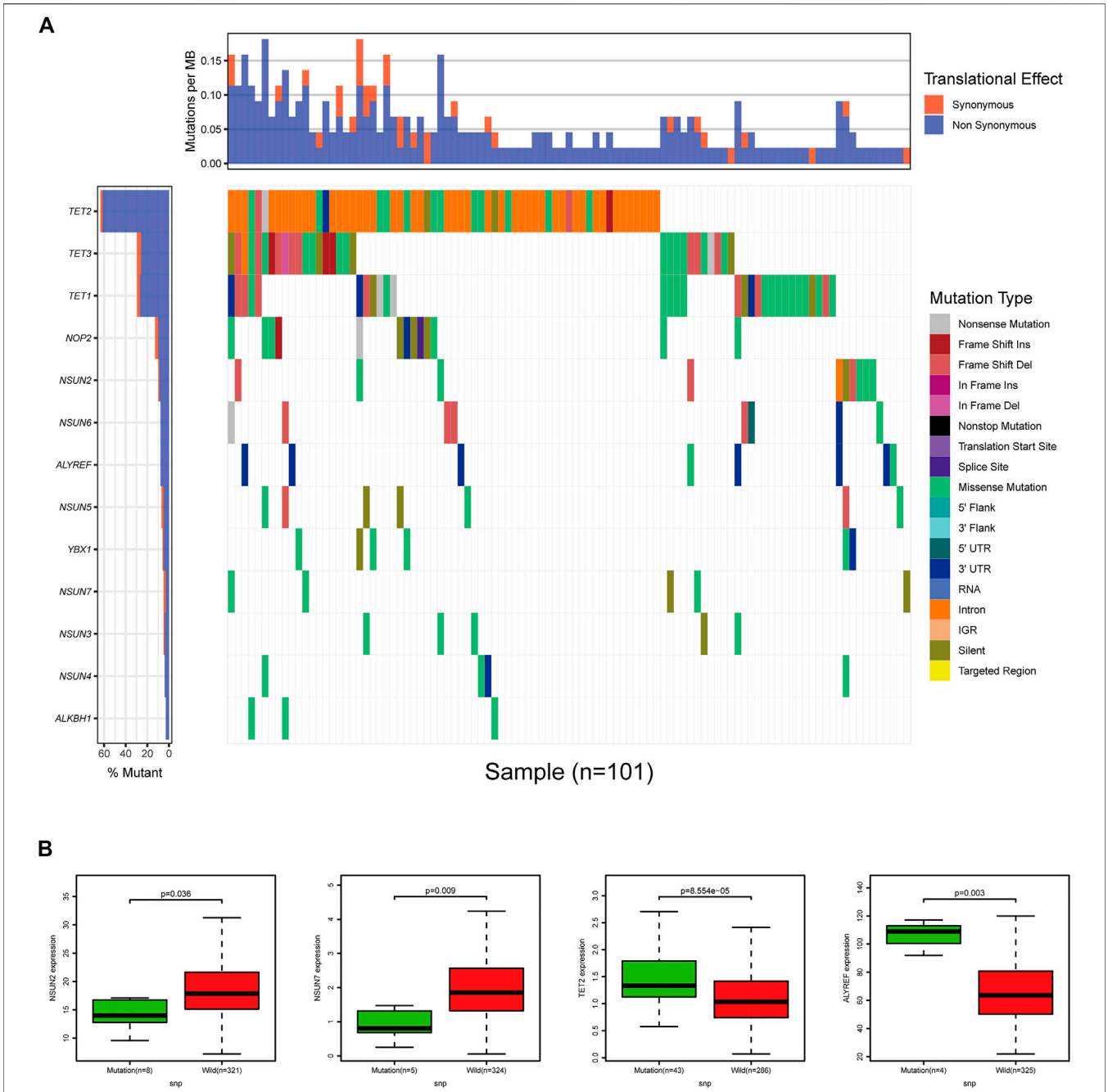

FIGURE 5 | The landscape of SNP of m^{5}C-related regulators in COAD. **(A)** Waterfall plot of SNP of 13 m^{5}C-related regulators in COAD. **(B)** NSUN2, NSUN7, TET2, and ALYREF displayed significant differences in expression levels due to SNPs.

growing body of evidence shows that m^{5}C-related regulators could be latent predictive biomarkers in a variety of cancer (Huang et al., 2021a; Huang et al., 2021b; Pan et al., 2021). However, the literature on CRC and m^{5}C has largely focused on DNA methylation (Zhu et al., 2018). Little is known about the relationship between m^{5}C-related RNA modifications and CRC, which calls our attention to investigate the aberrant expression of m^{5}C-related regulators in COAD and explore whether m^{5}C-related regulators could serve as ideal biomarkers for COAD prognosis and participate in COAD initiation and progression.

In our study, we showed that the expressions of m^{5}C-related regulators were significantly altered between tumor tissues and adjacent mucosa and had a strong correlation with the tumor progression and prognosis. This indicated that m^{5}C-related regulators play a crucial role in COAD. First, the "writer" genes NSUN1-NSUN7, the "eraser" genes TET2 and ALKBH1, and the "reader" genes ALYREF and YBX1 were significantly

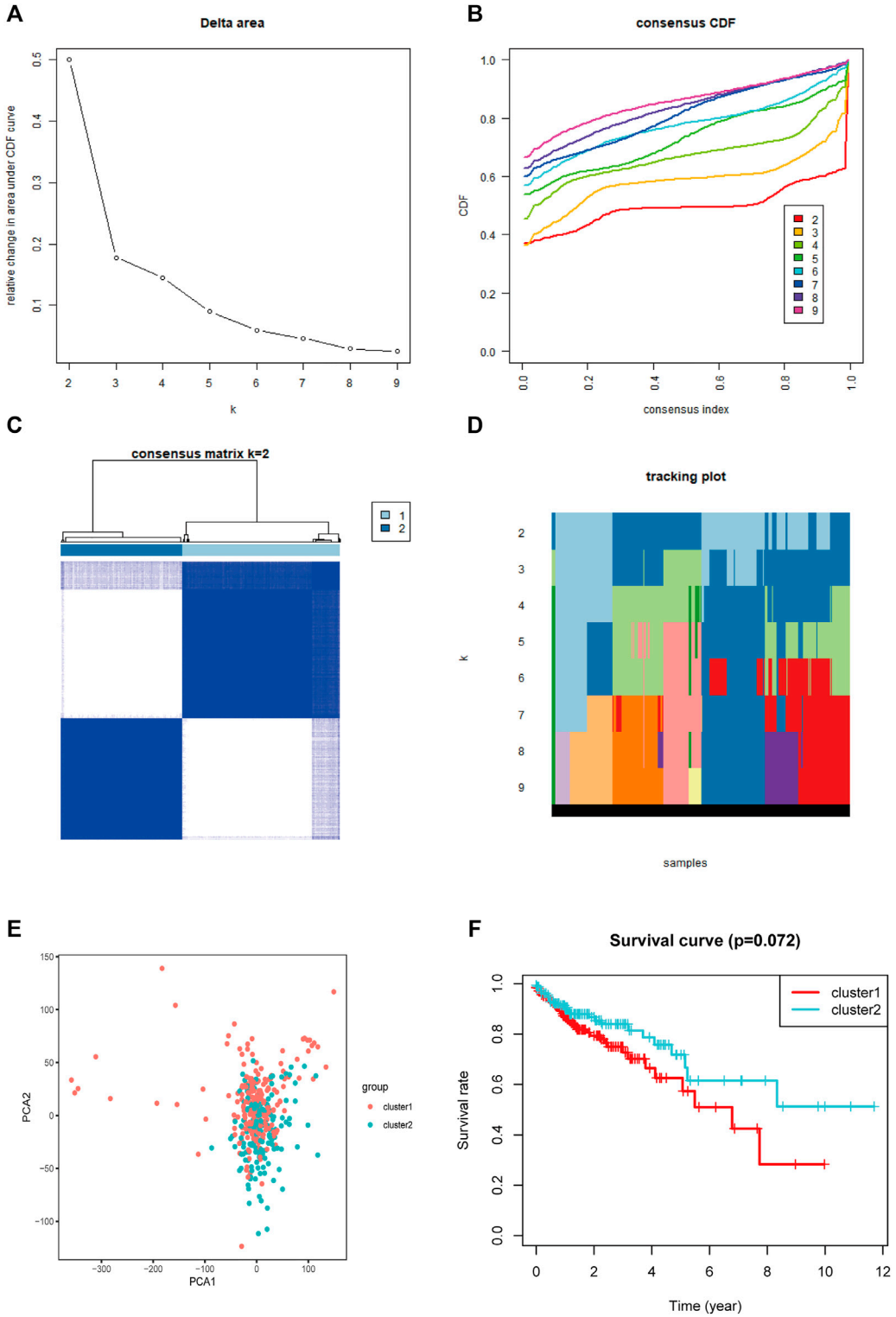

FIGURE 6 | Consistent cluster analysis and principal component analysis of COAD. **(A)** The consistency clustering cumulative distribution function (CDF) when k is between 2 and 10. **(B)** The relative change of the area under the CDF curve from 2 to 10 of k. **(C)** At k = 2, the correlation between groups. **(D)** The distribution of the sample when k is between 2 and 10. **(E)** Principal component analysis of 2 clusters of total RNA expression profile after consistency analysis. **(F)** Comparison of Kaplan-Meier overall survival curves for COAD patients in cluster I and II.

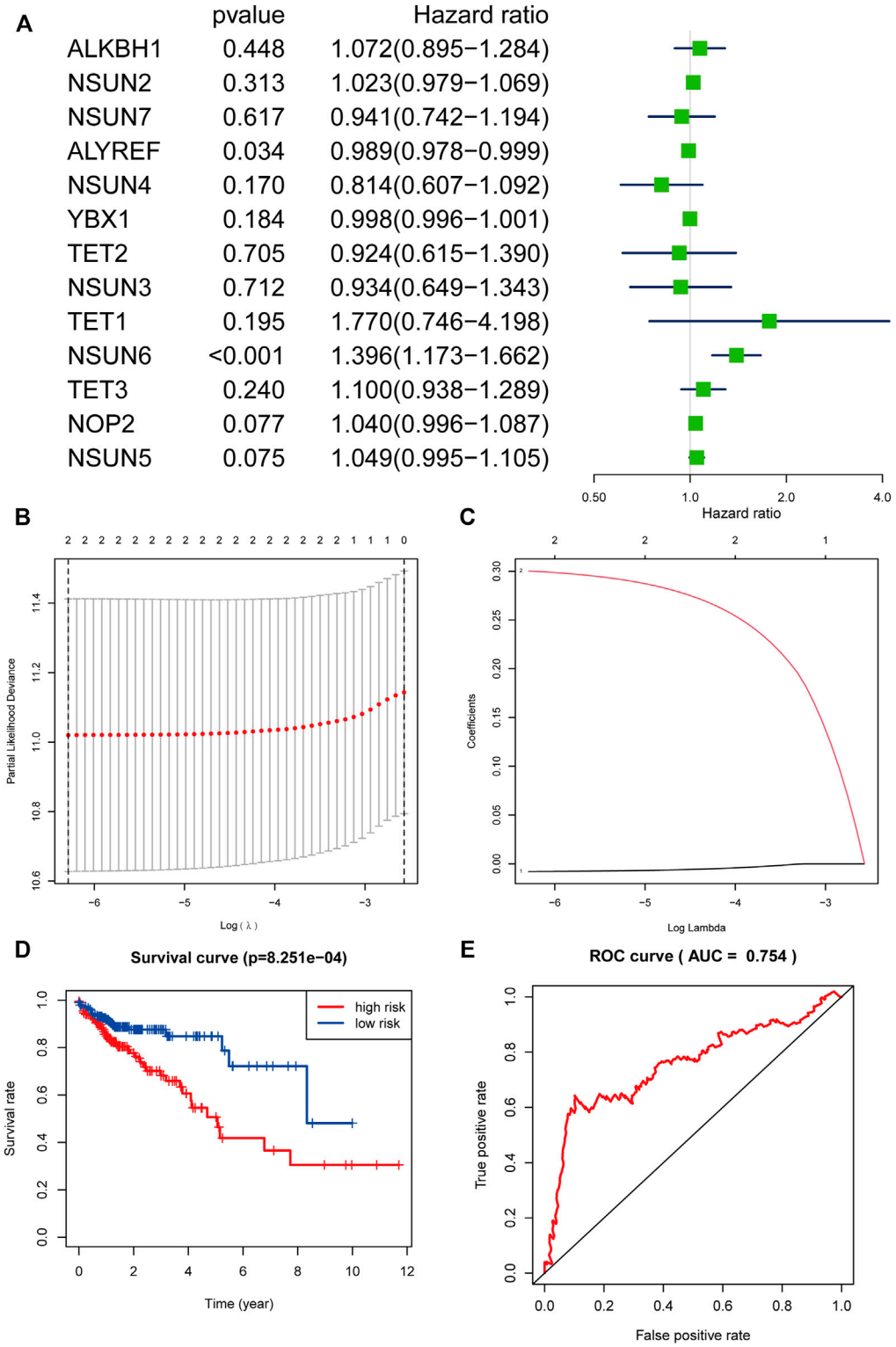

FIGURE 7 | The process of constructing the signature based on NSUN6 and ALYREF and evaluating its prognostic value. **(A)** The Hazard ratio (HR), 95% confidence interval (CI) of 13 m^5C-related regulators estimated by univariate Cox regression. **(B)** The point with the smallest cross verification error corresponds to the number of factors included in the Lasso regression model. **(C)** The lines of different colors represent the trajectory of the correlation coefficient of different factors in the model with the increase of Log Lamda. **(D)** Kaplan-Meier overall survival curves for patients in high-risk group- and low-risk group divided according to the risk score. **(E)** ROC analysis and AUC value of the ROC curve suggested the sensitivity and specificity for risk signature.

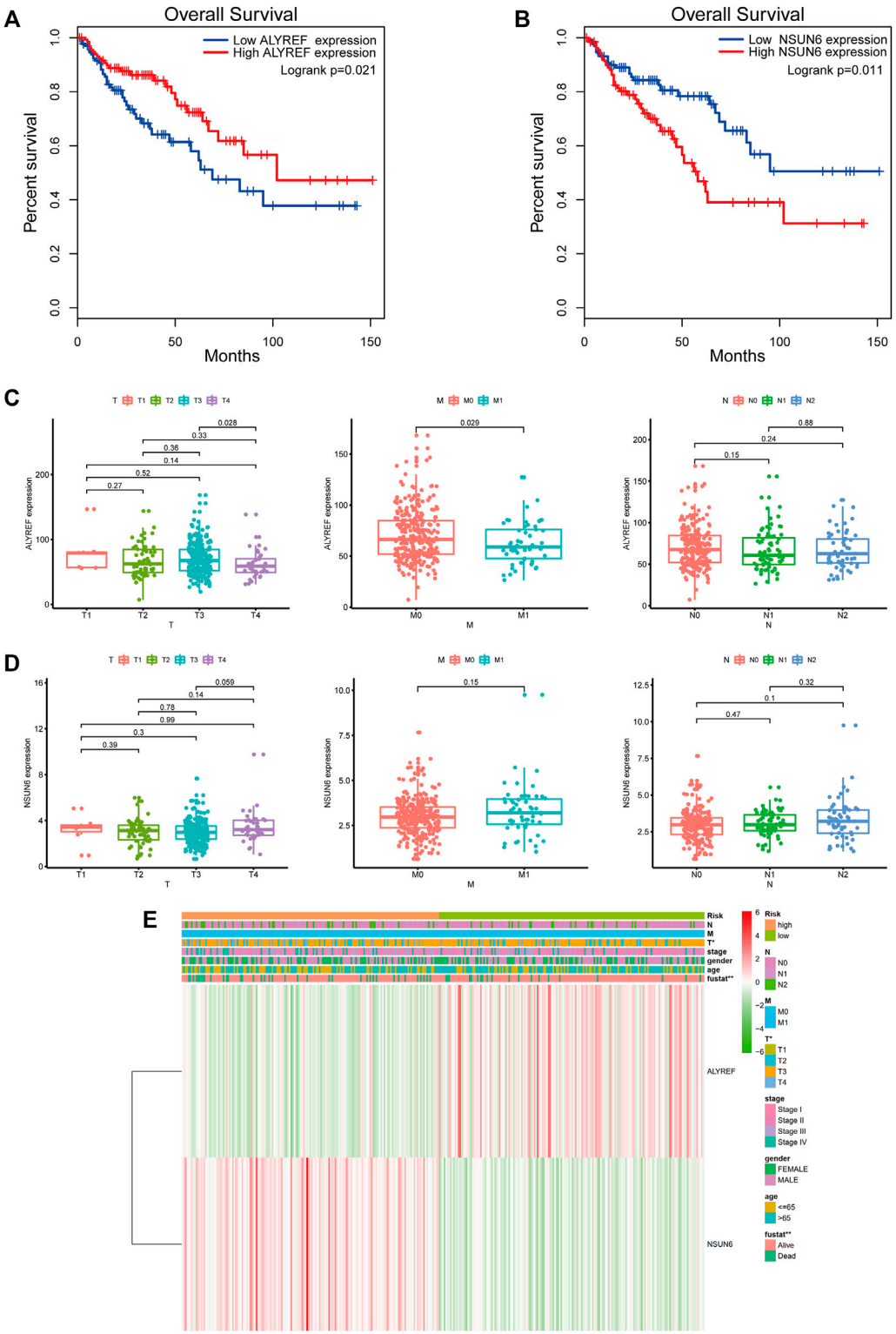

FIGURE 8 | Survival analysis and clinicopathological characteristics of the two m^{5}C-related regulators. **(A)** Kaplan-Meier survival curve of ALYREF in high- and low-expression groups. **(B)** Kaplan-Meier survival curve of NSUN6 in high- and low-expression groups. **(C)** Analysis of the relationship between the expression of ALYREF and TMN stage. **(D)** Analysis of the relationship between the expression of NSUN6 and TMN stage. **(E)** The heatmap shows the expression of NSUN6 and ALYRE in high-risk and low-risk. The distribution of clinicopathological characteristics was compared between the high-risk and low-risk groups. *$p < 0.05$, **$p < 0.01$.

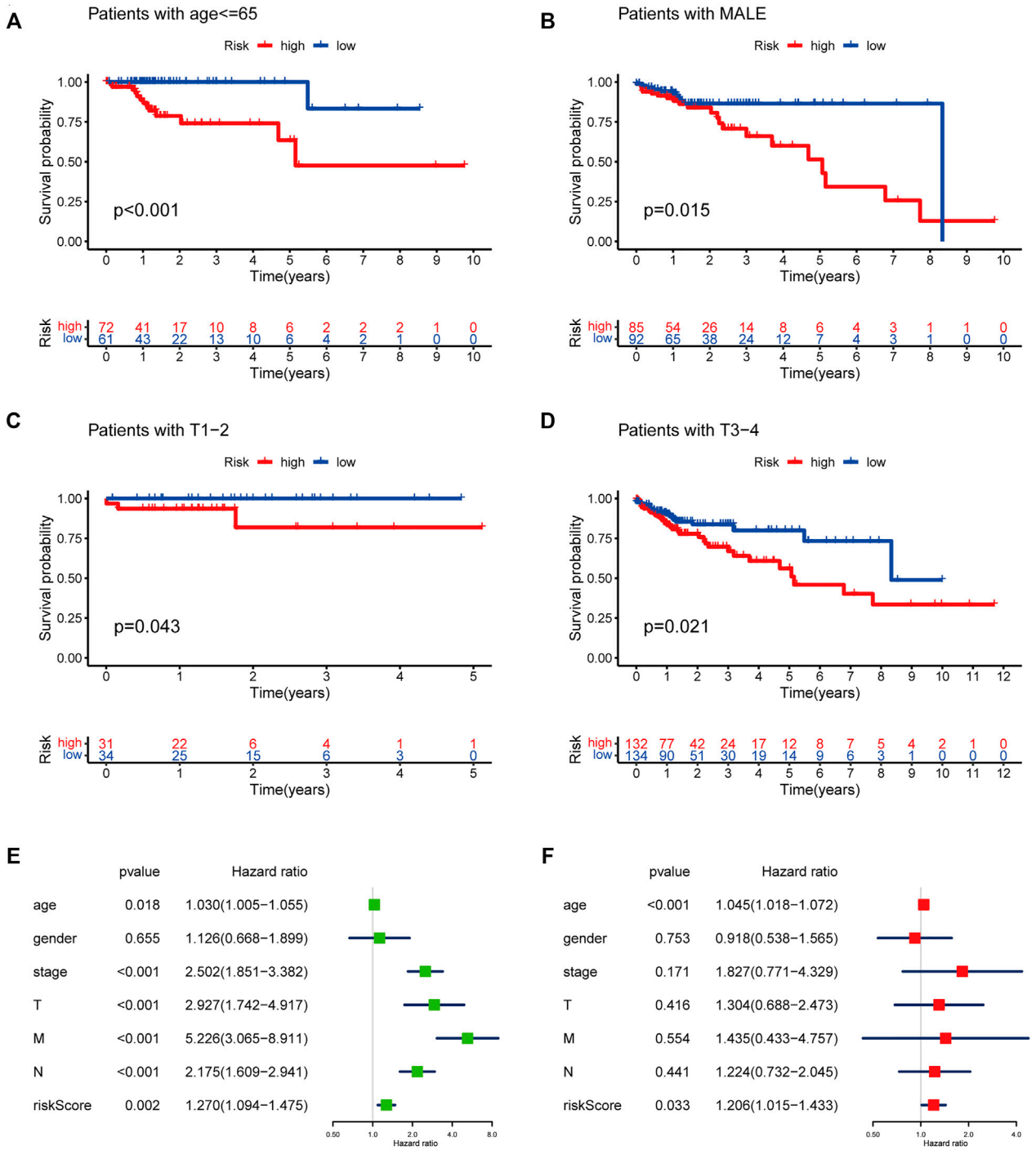

FIGURE 9 | Subgroup analysis with risk score in different clinicopathological features and Prognostic risk model verification. **(A)** age ≤ 65. **(B)** male. **(C)** T1-T2. **(D)** T3-T4. **(E)** Univariate Cox regression analysis of risk score combined with clinicopathological factors. **(F)** Multivariate Cox regression analysis of risk score combined with clinicopathological factors.

upregulated or downregulated in tumor tissues, suggesting these genes may be critical in m^{5}C-related occurrence and progression of COAD. To investigate the relationship between CNVs or SNPs of m^{5}C-related regulators and their mRNA expression levels, COAD samples with CNV or SNP data from TCGA were analyzed. Regarding CNVs, the copy number of seven m^{5}C-related regulators increased or was lost, and their mRNA expression was upregulated or downregulated accordingly and was significantly correlated. SNPs in TET2 and ALYREF were highly correlated with their high mRNA expression, while SNPs

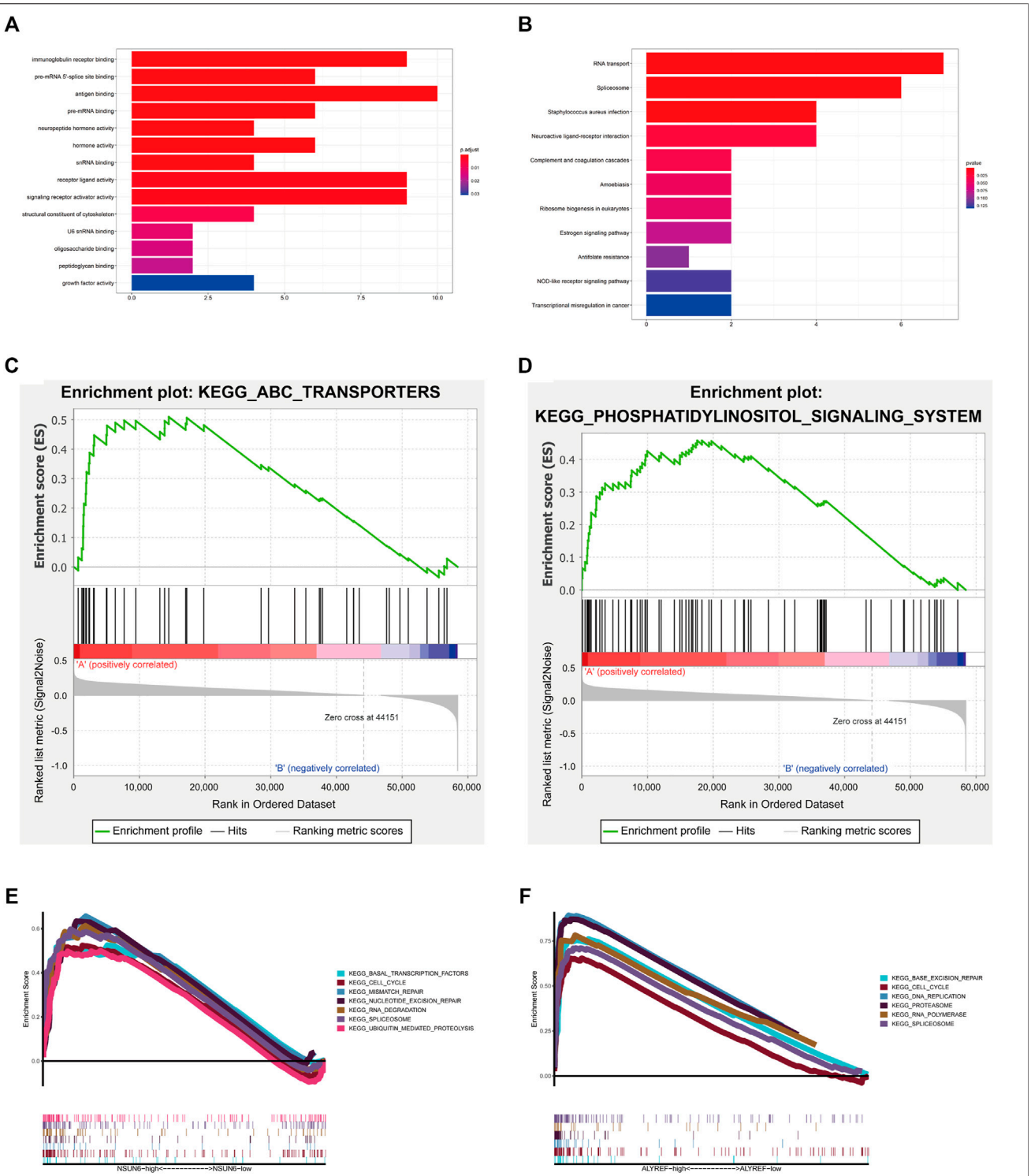

FIGURE 10 | Biological functional analysis. **(A,B)** GO analysis and KEEG pathways analysis of the genes significantly upregulated or downregulated between cluster I and cluster II. **(C,D)** Cluster I had a worse overall survival and lower 5-year survival rate associated with malignancy-associated pathways, including the ATP-binding cassette transporter and phosphatidylinositol signaling system. **(E)** GSEA results for NSUN6 in COAD. **(F)** GSEA results for ALYREF in COAD.

of NSUN2 and NSUN7 were significantly correlated with their low mRNA expression levels. Additionally, m^5C-associated mutations in COAD could be studied in RMVar and RMdisease database, which were recently constructed and focused on genetic variants in RNA modifications (Kunqi Chenet al., 2021; Xin Luo et al., 2021).

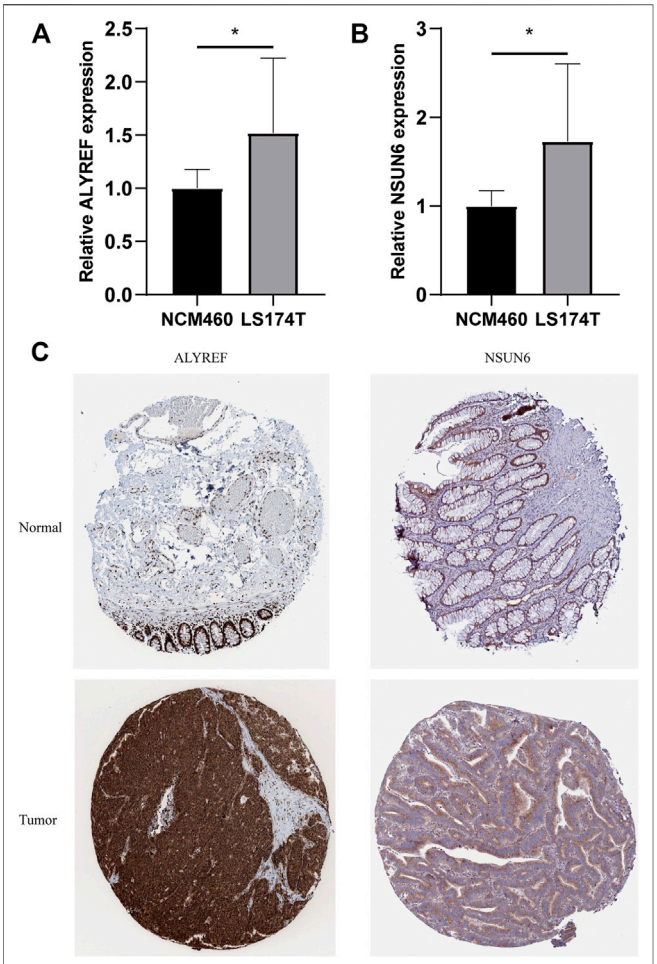

FIGURE 11 | Validation of the Expression Levels of the m^{5}C-Related Regulators in Cell Lines and Clinical Samples. **(A,B)** Expression of ALYREF and NSUN6 in COAD cell lines LS174T and normal colon mucosal epithelial cell line NCM460. *$p < 0.01$. **(C)** IHC analysis of the protein level expression of ALYREF and NSUN6 in COAD and normal tissues in HPA online database.

Thereafter, based on the expression of the m^{5}C-related regulators, patients with COAD were clustered into two subgroups (cluster Ⅰ and cluster Ⅱ), an the cluster II had a longer survival time than cluster I. To further study the effect of m^{5}C-related regulators on the prognosis and clinicopathological characteristics of COAD, we constructed a prognostic risk signature using two identified m^{5}C-related regulators (NSUN6 and ALYREF) and were able to assign patients with COAD into high- and low-risk groups. The correlation between the groups and clinicopathological characteristics was assessed, which revealed that the high-risk group was linked with stage T and fustat. Based on the risk value, the established ROC curve showed a satisfactory prediction performance. Moreover, the risk score can be used as an independent prognostic factor for COAD, suggesting that NSUN6 and ALYREF may be vital m^{5}C-related regulators and significant prognostic factors for patients with COAD. Furthermore, this m5C-related regulators prognostic model could serve as a prognostic indicator for OS in subgroups of patients with different clinical characteristics, especially age ≤65, male, and stage T. The results presented above indicated that NSUN6 and ALYREF can be used as potential biomarkers, and a reliable risk model is critical for providing the necessary evidence for clinical adoption. Apart from our results, there was another study similarly demonstrated that a risk score developed from the three-m^{5}C signature represented an independent prognostic factor for patients with COAD (Geng et al., 2021).

Recently, many studies have indicated that m^{5}C RNA modification is involved in all types of human cancer. NSUN2 is the most studied m^{5}C methyltransferase and participates in various cancers, such as bladder cancer, gallbladder carcinoma, and hepatocellular carcinoma (Chen et al., 2019; Gao et al., 2019; Sun et al., 2020). It was reported that NSUN2 is highly expressed in colon cancers (Okamoto et al., 2012), which was corroborated in our results. NSUN2 mainly exerts an oncogenic role by maintaining the stability of oncogenic RNA (Chellamuthu and Gray, 2020), but whether NSUN2 plays the same role in COAD requires further research. With respect to the two m^{5}C-related regulators (NSUN6 and ALYREF) identified in our results, there have been some studies on cancer and related mechanisms. The role of NSUN6 in regulating cell proliferation and pancreatic cancer tumor growth was recently confirmed, and NSUN6 performs well in evaluating tumor recurrence and survival among pancreatic cancer patients (Yang et al., 2021). Next, ALYREF was found to be upregulated in hepatocellular carcinoma and oral squamous cell carcinoma, and it may have an effect on tumorigenesis *via* cell cycle regulation and mitosis (Saito et al., 2013; He et al., 2020).

To provide a comprehensive analysis, GO, KEEG pathway, and GSEA analyses of m^{5}C-related regulators were also conducted. Several biological processes and pathways associated with the occurrence and progression of COAD were enriched, including "MAPK signaling pathway" and "cell cycle" (Koveitypour et al., 2019; Malki et al., 2020). Moreover, previous studies have reported that m^{5}C-related RNA modifications are closely associated with mRNA translation, transport, and stability. Here, we found that the m^{5}C-related regulators were associated with "pre-mRNA 5′-splice site binding" and "spliceosome," suggesting they play important roles in RNA processing. In addition, it should be noted that a number of biological processes and pathways associated with immune response were identified. While extensive literature reports have demonstrated that N6-methyladenosine plays important role in immune evasion and immune response (Xiaoting Lou et al., 2021; Shulman and Stern-Ginossar, 2020) and bioinformatic analysis have shown that the m^{5}C-related regulators were related to tumor immune microenvironment and affected the abundance of tumor-infiltrating immune cells in COAD (Geng et al., 2021), there have been few experimental reports about the relationship between m^{5}C-related RNA modifications and immune response, suggesting that further research is required.

However, there are some limitations associated with our research. Firstly, the m^{5}C-related regulators we selected included some DNA demethylase, such as TET1, TET2, TET3, and ALKBH1. The specific role of these genes in m^{5}C RNA modification and DNA methylation

of COAD and the crosstalk between m^5C RNA modification and DNA methylation in COAD need to be further explored. Secondly, our research mainly focused on bioinformatic analysis, more experimental studies exploring the function of m^5C on the different types of RNA and sites in COAD are in urgent need in future work. The m^5C-Atlas database, a comprehensive database for decoding and annotating the m^5C epitranscriptome, may be useful in the research (Ma et al., 2022). Thirdly, overall survival between cluster I and cluster II had no significant difference, more m^5C-related regulators and more cohort need to be included in future analysis.

CONCLUSION

In this study, we first found that there was a significant correlation between the expression of m^5C-related regulators and clinicopathological features and OS of patients with COAD. This revealed that a prognostic signature obtained using m^5C-related regulators (NSUN6 and ALYREF) had significant value in COAD and could effectively predict the survival of patients with COAD. Additionally, biological processes and pathways associated with m^5C-related RNA modifications were identified, which may facilitate the malignant development of COAD, thus improving our understanding of the role of m^5C-related RNA modifications in the occurrence and progression of COAD. This work also provides important evidence towards the development of predictive biomarkers and molecular targeted therapy for COAD Bray et al., 2018.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study, which can be found in the Cancer Genome Atlas (TCGA) database and the Human Protein Atlas (HPA) online database.

ETHICS STATEMENT

This study met the publication guidelines stated by TCGA (https://cancergenome.nih.Gov/publications/publicationguidelines) HPA (https://www.proteinatlas.org/about/publications). All data used in the study were obtained from TCGA and HPA, and ethics approval and informed consent were not required.

AUTHOR CONTRIBUTIONS

YH and YW conceived and designed the study. CH, XJ, and YY organized the database and performed statistical analyses. YH wrote the first draft of the manuscript. CH, XJ, and YY prepared the figures and tables and were involved in manuscript writing. KZ, PL, FL, and YW revised and proofread the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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A Pan-Cancer *In Silico* Analysis of the COVID-19 Internalization Protease: Transmembrane Proteaseserine-2

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The new coronavirus (2019-nCoV) is an emerging pathogen that can cause severe respiratory infections in humans. It is worth noting that many of the affected COVID-19 patients have malignant tumors. In addition, cancer has been identified as a personal risk factor for COVID-19. Transmembrane proteaseserine-2 (TMPRSS2) is a crucial host protease that mediates S protein activation and initially promotes virus entry into host cells. Moreover, it is abnormally expressed in a variety of tumors. However, the systematic analysis of TMPRSS2 aberrations in human cancer remains to be elucidated. Here, we analyzed the genetic changes, RNA expression, and DNA methylation of TMPRSS2 in more than 30 tumors. It has been reported that TMPRSS2 is overexpressed in tumors such as prostate adenocarcinoma (PRAD), and in contrast, the expression of TMPRSS2 is decreased in tumors such as head and neck cancer (HNSC). In addition, TMPRSS2 low DNA methylation was also found in most of these TMPRSS2 high-expressing tumors in this study. Clinical studies have found that there is a significant correlation between the expression of TMPRSS2 and the prognosis of some tumor patients. The expression of TMPRSS2 is also related to the infiltration of cancer-related fibroblasts, and the potential pathways and functional mechanisms were analyzed through KEGG/GO enrichment. In the end, our study planned the genetic and epigenetic variation of TMPRSS2 in human malignant tumors for the first time and provided a relatively comprehensive understanding of the carcinogenic effects of TMPRSS2.

Keywords: COVID-19, tmprss2, expression, methylation, correlation

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INTRODUCTION

The pandemic respiratory disease sweeping through 2020 and 2021 is a new type of coronavirus pneumonia (coronavirus disease 2019, COVID-19) caused by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) (Surkova et al., 2020). Current studies have proven that the invasion of SARS-CoV-2 into host cells mainly depends on the activation of viral spike protein (S protein) by certain proteases (Ward et al., 2020). Transmembrane proteaseserine-2 (TMPRSS2) is a key host protease that mediates S protein activation and

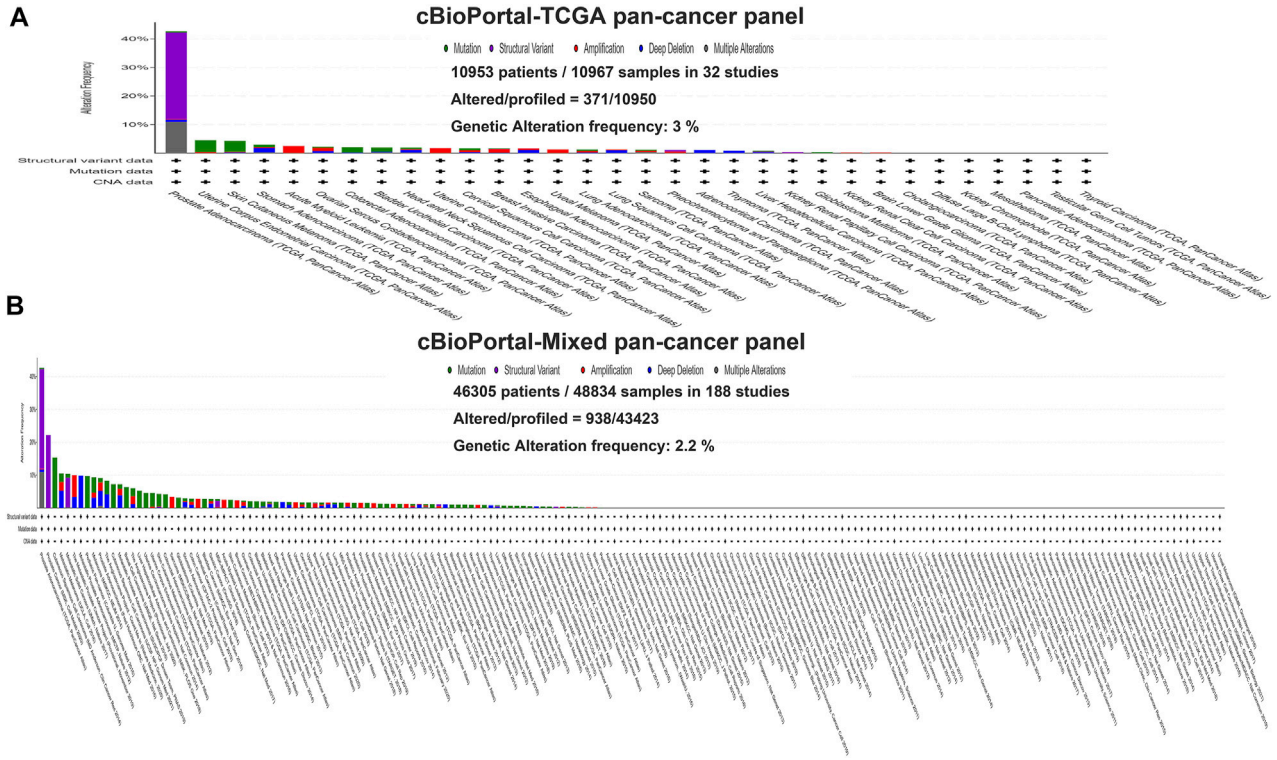

FIGURE 1 | Genetic variation of TMPRSS2 in tumors. **(A)** The TCGA pan-cancer panel was obtained from the cBioPortal dataset, which included 10,953 patients/10967 samples in 32 studies. **(B)** The mixed pan-cancer panel was obtained from the cBioPortal dataset, which also included 46,305 patients/48,834 samples in 188 studies.

initially promotes virus entry into host cells (Hoffmann et al., 2020). Moreover, it is abnormally expressed in a variety of tumors. Hence, cancer has been identified as an individual risk factor for COVID-19 (Stopsack et al., 2020). In 1997, Antonarakis et al. identified the TMPRSS2 gene for the first time and found that the gene encodes a multimeric protein with a serine protease domain (Paoloni-Giacobino et al., 1997). Since then, due to the high expression of the TMPRSS2 gene in the prostate, research on TMPRSS2 has mainly focused on the related diseases of prostate cancer (Hong et al., 2020). Due to the impact of the new coronavirus epidemic, coupled with the expression of TMPRSS2 in the epithelial cells of the respiratory system, enthusiasm for research on TMPRSS2 has further increased (Kimura et al., 2020). However, the systematic analysis of TMPRSS2 aberrations has not been characterized in human cancers. Then, we planned a pan-cancer analysis of TMPRSS2 in malignant tumors.

Our study is the first to explore the TCGA database to perform pan-cancer analysis on TMPRSS2. We also included a set of factors, such as gene expression, survival status, DNA methylation, genetic changes, protein phosphorylation, immune infiltration, and related cell pathways, to study the potential molecular mechanisms of TMPRSS2 in the pathogenesis or clinical prognosis of different cancers.

MATERIALS AND METHODS

Analysis of Genetic Changes

This study obtained the pan-cancer data of "TMPRSS2" from the cBioPortal database (https://www.cbioportal.org/) (Gao et al., 2013). In the "Cancer Type Summary" module, we observed the change frequency, mutation type, and CNA (copy number change) results of all TCGA tumors. The mutation site information can be displayed in the protein structure diagram or 3D (three-dimensional) structure through the "Mutations" module. We also took advantage of the "comparison" module to obtain data on overall survival and disease-free survival in TCGA cancer cases with or without TMPRSS2 gene alterations.

Survival Prognosis Analysis

The GEPIA2 database (http://gepia.cancer-pku.cn/index.html), also known as the gene expression interactive analysis database, was researched and developed by the team of Professor Zemin Zhang from Peking University (Tang et al., 2017). Gene expression analysis was based on tumor and normal samples from the TCGA database. We utilized the "survival map" module of GEPIA2 to obtain the differential expression of TMPRSS2 in tumors and corresponding normal tissues, survival differences, and

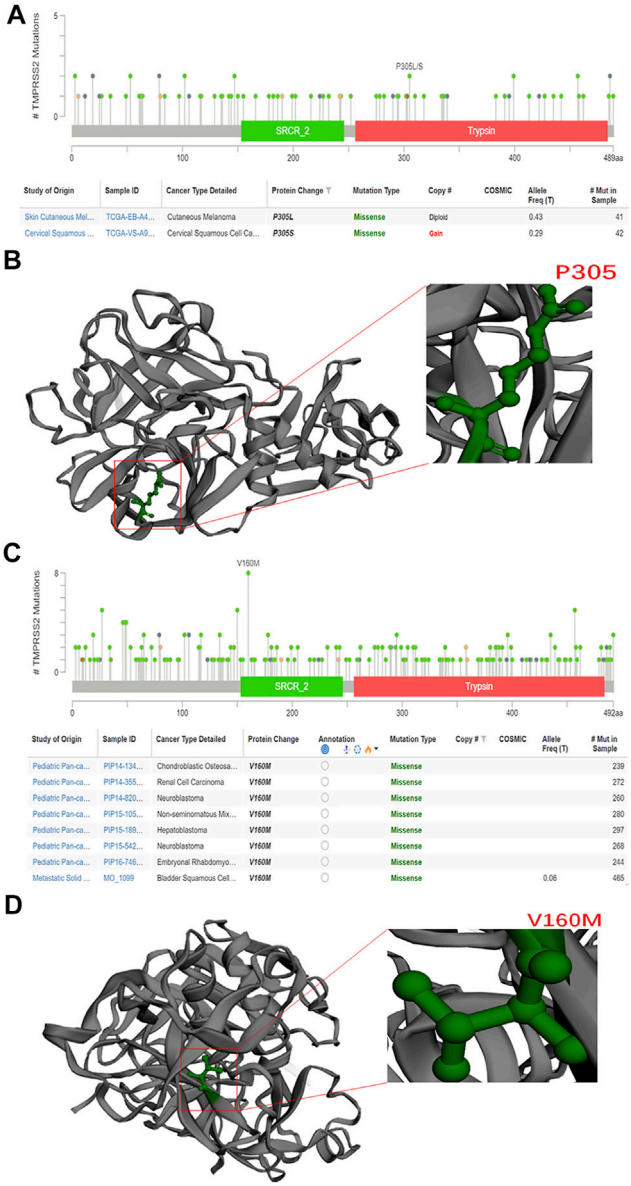

FIGURE 2 | Methylation level of TMPRSS2 in pan-cancer. **(A)** TMPRSS2 promoter DNA methylation probe. **(B)** Three tumors with high expression of TMPRSS2 showed reduced levels of TMPRSS2 DNA methylation, including colon adenocarcinoma (COAD), prostate adenocarcinoma (PRAD), and rectal adenocarcinoma (READ), $^{**}p < 0.01$. **(C)** Tumors with downregulated TMPRSS2 expression, including breast invasive carcinoma (BRCA), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), lung squamous cell carcinoma (LUSC), sarcoma (SARC), skin cutaneous melanoma (SKCM), and thyroid carcinoma (THCA), exhibit increased levels of DNA methylation. $^{**}p < 0.01$.

other related genes. OS (overall survival) and DFS (disease-free survival) saliency map data in all TCGA tumors.

First, the GEPIA2 database further verified the TMPRSS2 expression results and obtained the available experimentally determined TMPRSS2 binding protein. We operated the "Similar Gene Detection" module of GEPIA2 to obtain the top 100 targeted genes related to TMPRSS2 based on all TCGA tumor and normal tissue datasets. We also applied the "Correlation Analysis" module of GEPIA2 to perform paired gene Pearson correlation analysis on TMPRSS2 and selected genes. The *p* value and correlation coefficient (R) are exhibited in the plots. In addition, we used the "Gene_Corr" module of TIMER2 to provide heatmap data for selected genes, including the partial correlation (cor) and *p* value in the purity-adjusted Spearman rank correlation test. In addition, we combined the two sets of data for KEGG pathway analysis and GO enrichment analysis.

Methylation Level Analysis

The UALCAN portal (http://ualcan.path.uab.edu/analysis-prot.html) (Chandrashekar et al., 2017) is an effective tool for online analysis and mining cancer data and is mainly used for the Cancer Genome Atlas (TCGA) project. This website can be regarded as a platform for computer verification of target genes and identification of tumor subgroup-specific candidate biomarkers. The methylation level between the primary tumor and normal tissues was entered by "TMPRSS2." The available datasets for six tumors were selected, namely, breast cancer, ovarian cancer, colon cancer, clear cell renal cell carcinoma, uterine corpus endometrial carcinoma, and lung adenocarcinoma.

Gene Expression Analysis

TMPRSS2 was searched in the "Gene_DE" module by running the GRPIA2 database to observe the difference in TMPRSS2 expression between the tumor and adjacent normal tissues. Different tumors or specific tumor subtypes of the TCGA project were observed. Through the "Pathological Staging Diagram" module, a violin diagram of the expression of all TCGA tumors in different pathological stages (stage I, stage II, stage III, and stage IV) was obtained.

Immune Infiltration Analysis

TIMER2.0 is a database (http://timer.cistrome.org/) that can comprehensively analyze tumor and immune interactions (Li et al., 2017). The database covers 32 types of tumors and provides online analysis, including gene expression, clinical results, somatic mutations, and somatic copy number changes. The relationship between TMPRSS2 expression and immune infiltration in all TCGA tumors was explored through the "immune gene" module. TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, XCELL, MCPCOUNTER, and EPIC algorithms were applied to the estimation of immune infiltration. The *p* value and the partial correlation (cor) value were obtained through the Spearman rank correlation test with purity adjustment.

Analysis of the TMPRSS2 Interaction Protein Network and the Related Biological Processes

The STRING database (https://www.string-db.org/) (von Mering et al., 2003) is a well-known database for predicting

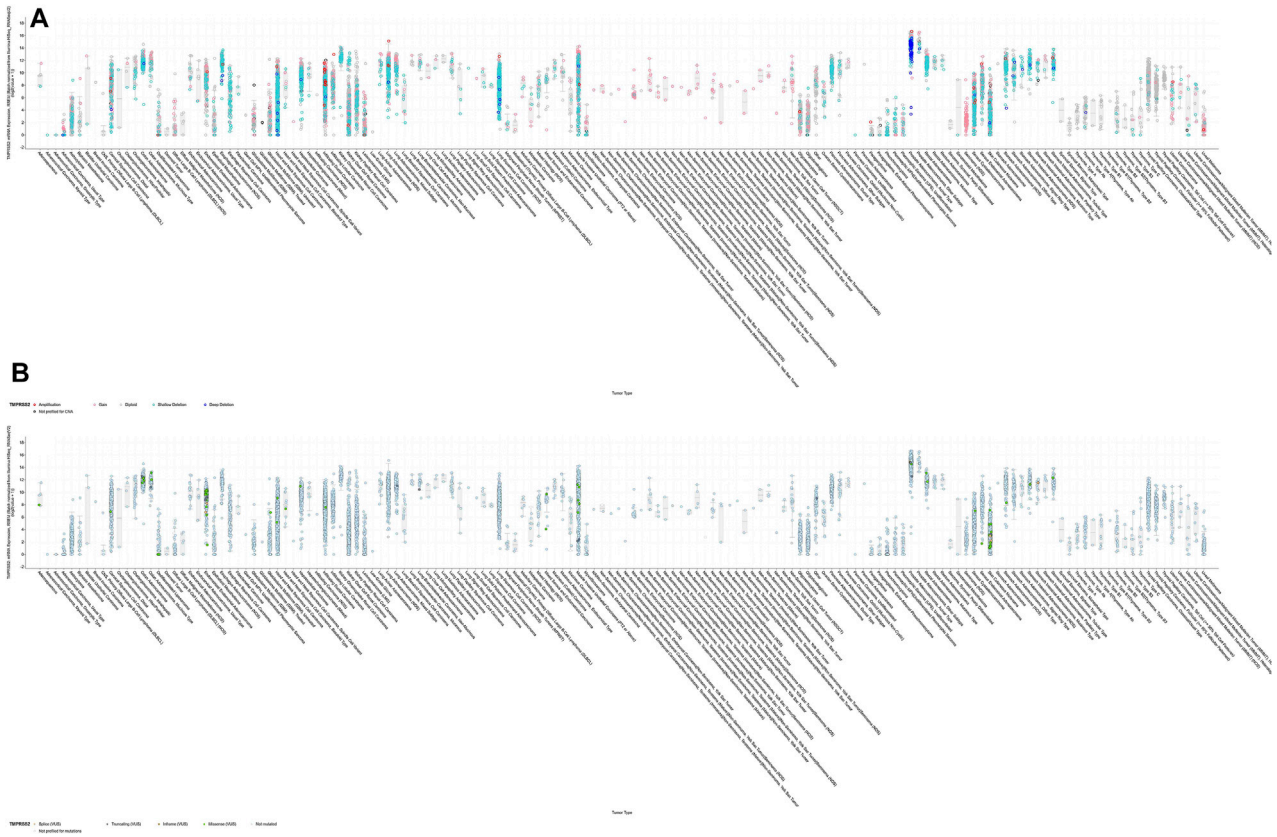

FIGURE 3 | RNA expression of TMPRSS2. **(A)** Exploration of the differential expression of TMPRSS2 through the GEPIA2 database (upper panel; red, tumor samples; green, normal samples) and the UALCAN database (lower panel; blue, tumor samples; red, normal samples). **(B)** TMPRSS2 is overexpressed in colon adenocarcinoma (COAD), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), kidney chromophobe (KICH), prostate adenocarcinoma (PRAD), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS), and rectum adenocarcinoma (READ). **(C)** TMPRSS2 is downregulated in breast invasive carcinoma (BRCA), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), lung squamous cell carcinoma (LUSC), sarcoma (SARC), skin cutaneous melanoma (SKCM), testicular germ cell tumors (TGCTs), and thyroid carcinoma (THCA).

protein–protein interactions. The database mainly predicts protein interactions through computational prediction, information transfer between different species, and aggregation of other database information. The STRING database was used to analyze the protein network interacting with TMPRSS2 and the biological processes involved in these interacting proteins.

The Interactive Venn diagram viewer (Bardou et al., 2014) and Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) online analysis tool (Jiao et al., 2012) were utilized for gene ontology (GO) analysis and Kyoto Encyclopedia of Gene and Genome (KEGG) pathway enrichment. Among them, GO functional enrichment analysis included cell composition, biological process, and molecular function enrichment analysis of genes, thereby obtaining a significantly enriched signaling pathway ($p < 0.05$). The smaller the p value, the higher the significance.

RESULTS

The Expression Level and Genetic Variation of TMPRSS2 in Each Tumor

In this study, we exploited the cBioPortal database to study the mutations of TMPRSS2 in different cancers, including the mutation site, type, amino acid changes, and the corresponding three-dimensional structure of the protein. To study the relationship between TMPRSS2 copy number changes in different cancers and gene expression, we looked for genes that were coexpressed with TMPRSS2 and presented them in the form of a scatter plot sequentially. The survival curve of TMPRSS2 was searched to lay the foundation for the analysis of survival prognosis. The results were obtained from the cBioPortal dataset, including 10,953 patients/10967 samples in more than 30 studies (**Figure 1**). According to the TCGA and mixed pan-cancer databases, the main mutation sites of TMPRSS2 are P305 L/S and V160 M (**Figure 2**), and the mutation of

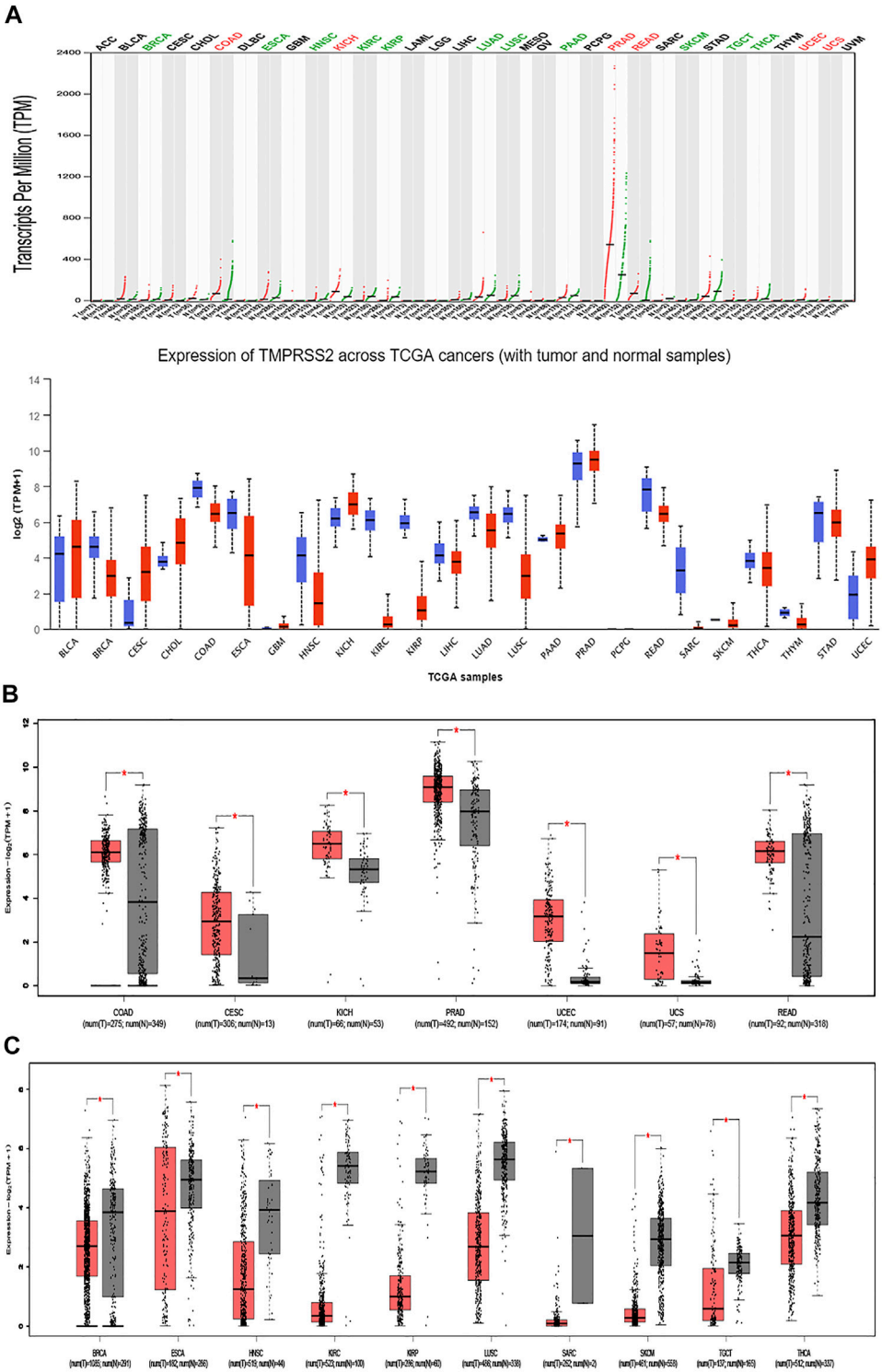

FIGURE 4 | Overall survival (OS) data for malignant tumors overexpressing TMPRSS2. **(A)** Survival picture of tumors overexpressing TMPRSS2; **(B)** OS in tumors overexpressing TMPRSS2.

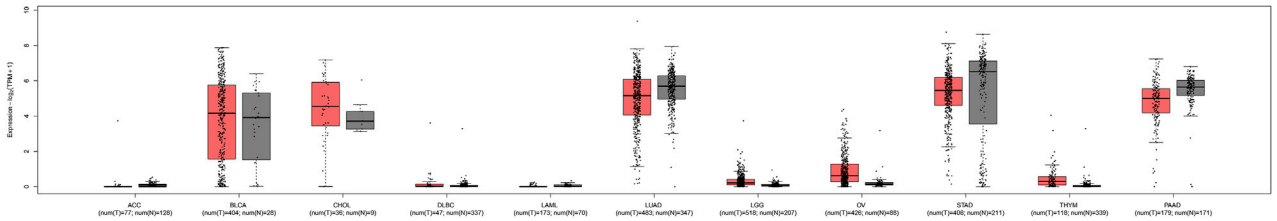

FIGURE 5 | Mutations of TMPRSS2 in different tumors. **(A)** The mutation site of TMPRSS2 is displayed in the plot based on TCGA database from cBioPortal. **(B)** 3D structure of TMPRSS2 and the mutation site in TCGA. **(C)** The mutation site of TMPRSS2 is displayed in the plot based on the mixed pan-cancer cohort from cBioPortal. **(D)** 3D structure of TMPRSS2 and the mutation site in the mixed pan-cancer cohort.

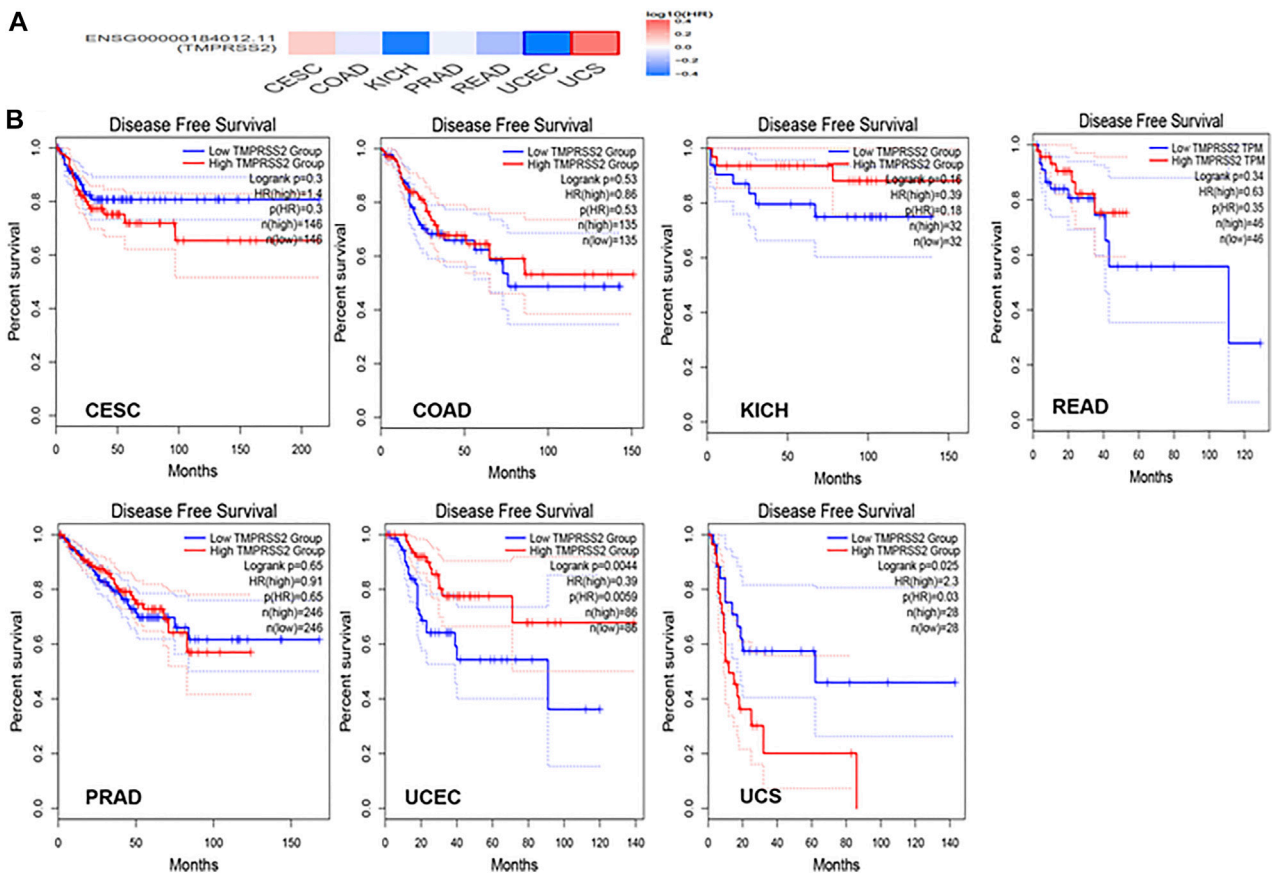

FIGURE 6 | OS data of malignant tumors with low expression of TMPRSS2. **(A)** Survival picture of tumors with low expression of TMPRSS2; **(B)** OS in tumors with low expression of TMPRSS2.

TMPRSS2 in different tumors was also analyzed and is shown in **Figure 3**.

Differential Expression and Survival Analysis of TMPRSS2

GEPIA2 has been explored to dynamically analyze the differential expression of TMPRSS2 in normal and tumor tissues, including RNA sequencing expression data of more than 9,000 tumor samples and more than 8,000 normal samples from TCGA and GTEx. The data have proven that TMPRSS2 is overexpressed in colon adenocarcinoma (COAD), cervical squamous cell carcinoma and endometrial carcinoma (CESC), kidney chromophobe (KICH), prostate adenocarcinoma (PRAD), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma

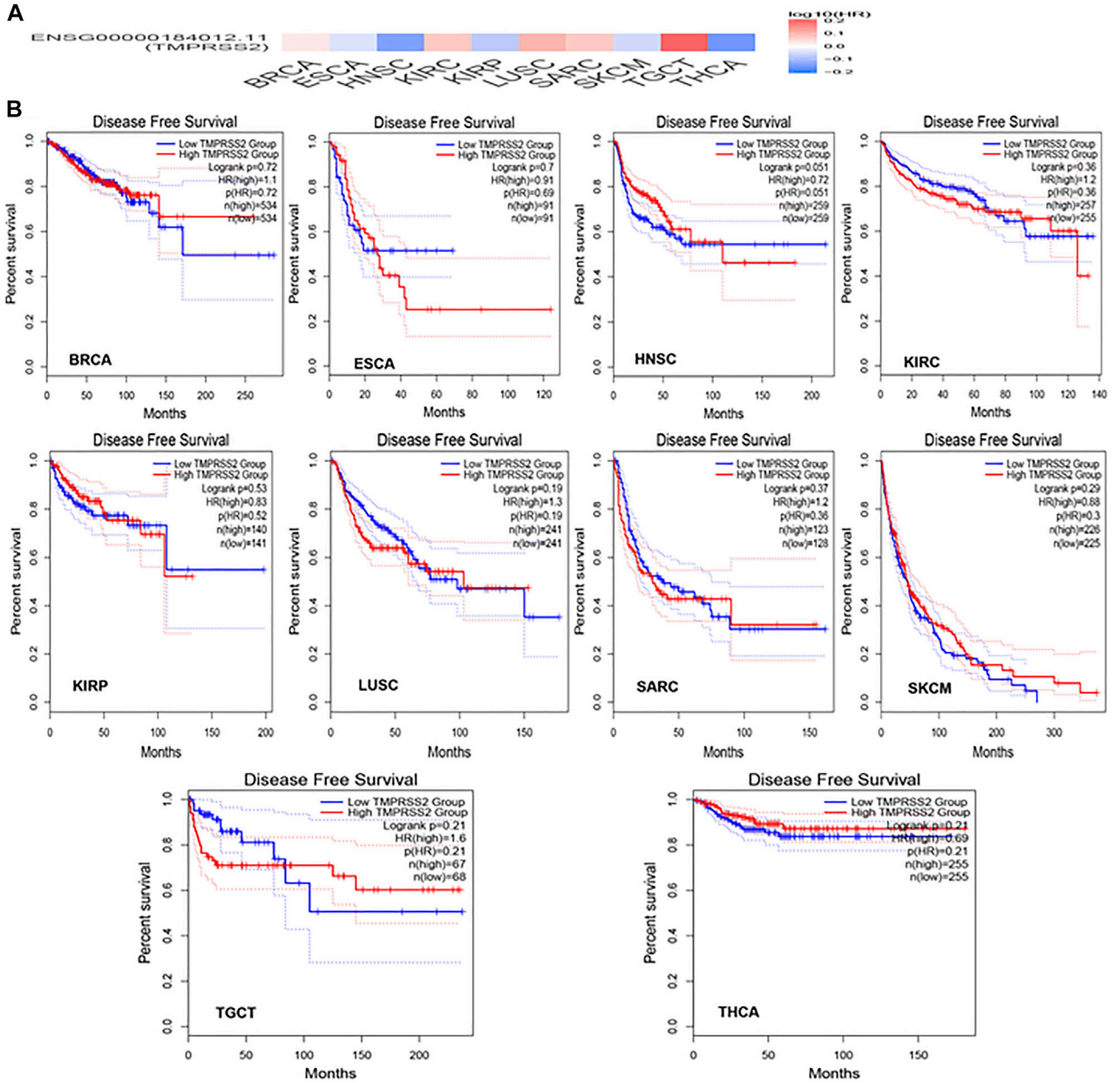

FIGURE 7 | Correlation between genetic disorders and TMPRSS2 expression. **(A)** In most cases, there was no statistical correlation between DNA copy variation and RNA TMPRSS2 expression. **(B)** The mutation has nothing to do with RNA expression.

(UCS), and rectal adenocarcinoma (READ). However, the expression of TMPRSS2 in breast invasive cancer (BRCA), esophageal cancer (ESCA), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), rectal adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), testicular germ cell tumor (TGCT), and thyroid cancer (THCA), including lung squamous cell carcinoma (LUSC), was downregulated (**Figure 4**). The mining data still revealed that there was no difference in the expression of TMPRSS2 in some tumors and normal tissues, including lung adenocarcinoma (LUAD) (**Figure 5**). The disease-free survival of UCES and UCS with high expression of TMPRSS2 was significantly different from that of the low-expression group (**Figure 6** and **Figure 7**). The overall survival analysis found that BRCA with high expression of TMPRSS2 was associated with poor prognosis (**Figure 8** and **Figure 9**).

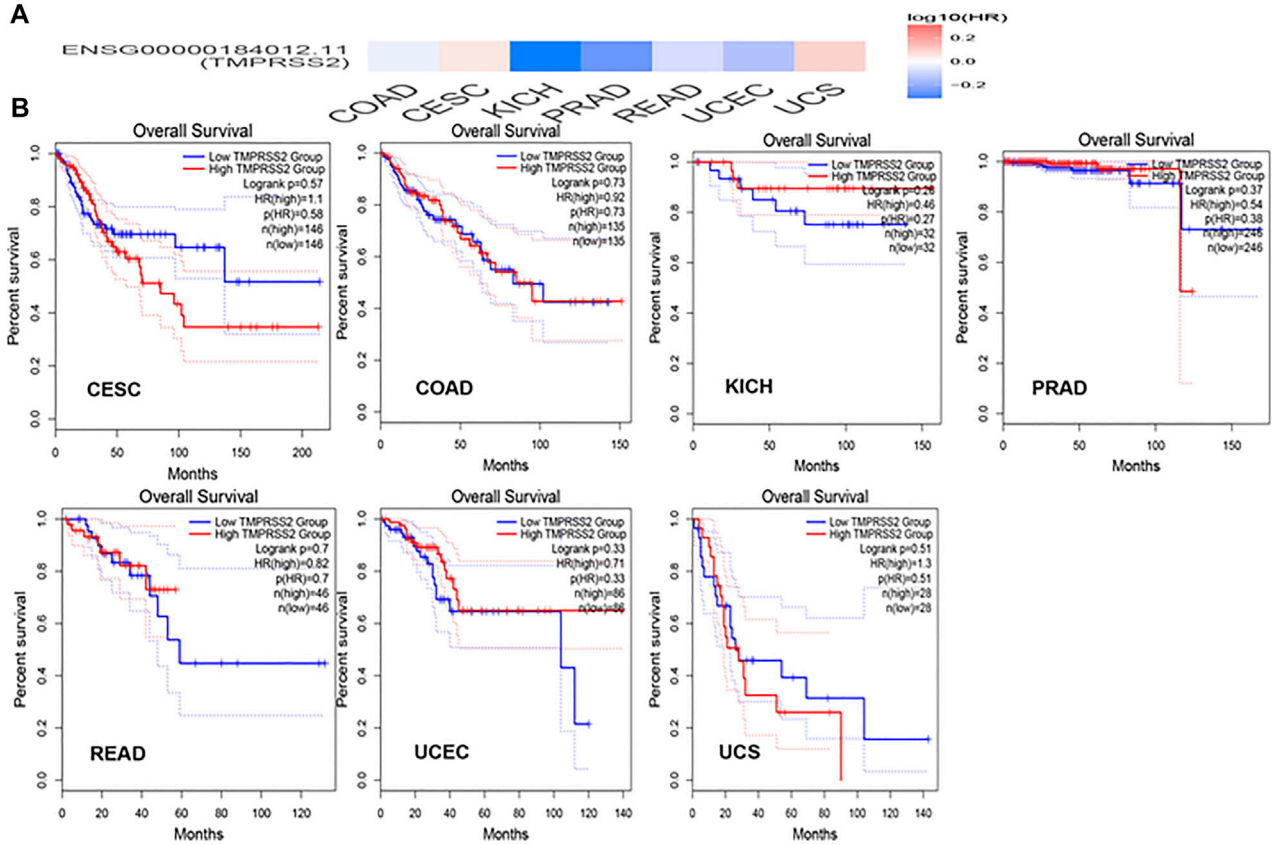

FIGURE 8 | Expression level of TMPRSS2 in different pathological stages. Based on TCGA data, the expression level of TMPRSS2 was analyzed according to the main pathological stages (stage I, stage II, stage III, and stage IV) of KICH, ESCA, KIRC, TGCT, THCA, and KIRP.

Methylation Level of TMPRSS2 in Pan-Cancer

The potential relationship between TMPRSS2 DNA methylation and the pathogenesis of different tumors in the TCGA project was detected using the MEXPRESS method. In COAD, PRAD, and READ with high expression of TMPRSS2, the methylation level was lower than that of normal tissues, and the difference was statistically significant. In contrast, tumors with downregulated TMPRSS2 expression, such as BRCA, ESCA, HNSC, KIRC, KIRP, LUSC, SARC, SKCM, and THCA, displayed increased levels of DNA methylation, and the difference was statistically significant (**Figure 10**).

The Relationship Between TMPRSS2 and Tumor Pathological Staging

The relationship between TMPRSS2 gene expression and KICH, ESCA, KIRC, TGCT, THCA, and KIRP pathological stages was analyzed and is presented in **Figure 11**, Pr (>F) ≤ 0.05. These results further indicated that the expression level of TMPRSS2 can function as a staging indicator for judging patients with KICH, ESCA, KIRC, TGCT, THCA, and KIRP.

Correlation Analysis Between TMPRSS2 Expression and Tumor-Associated Fibroblast Immune Infiltration

The TIMER database was explored to evaluate the relationship between TMPRSS2 expression and tumor-associated fibroblast immune infiltration in different tumor tissues. The TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, XCELL, MCPCOUNTER, and EPIC algorithms were adopted to detect the potential relationship between different immune cell infiltration levels and TMPRSS2 gene expression in different cancer types. The findings illustrated that the expression of TMPRSS2 was closely related to the immune infiltration of tumor-associated fibroblasts in COPD, ESCA, HNSC, STAD, LIHC, and TGCT (**Figure 12**).

Enrichment Analysis of TMPRSS2 Related Genes

To further investigate the molecular mechanism of the TMPRSS2 gene in tumorigenesis, the TMPRSS2 binding protein and TMPRSS2 expression–related genes for a series of pathway enrichment analyses were screened. Based on the

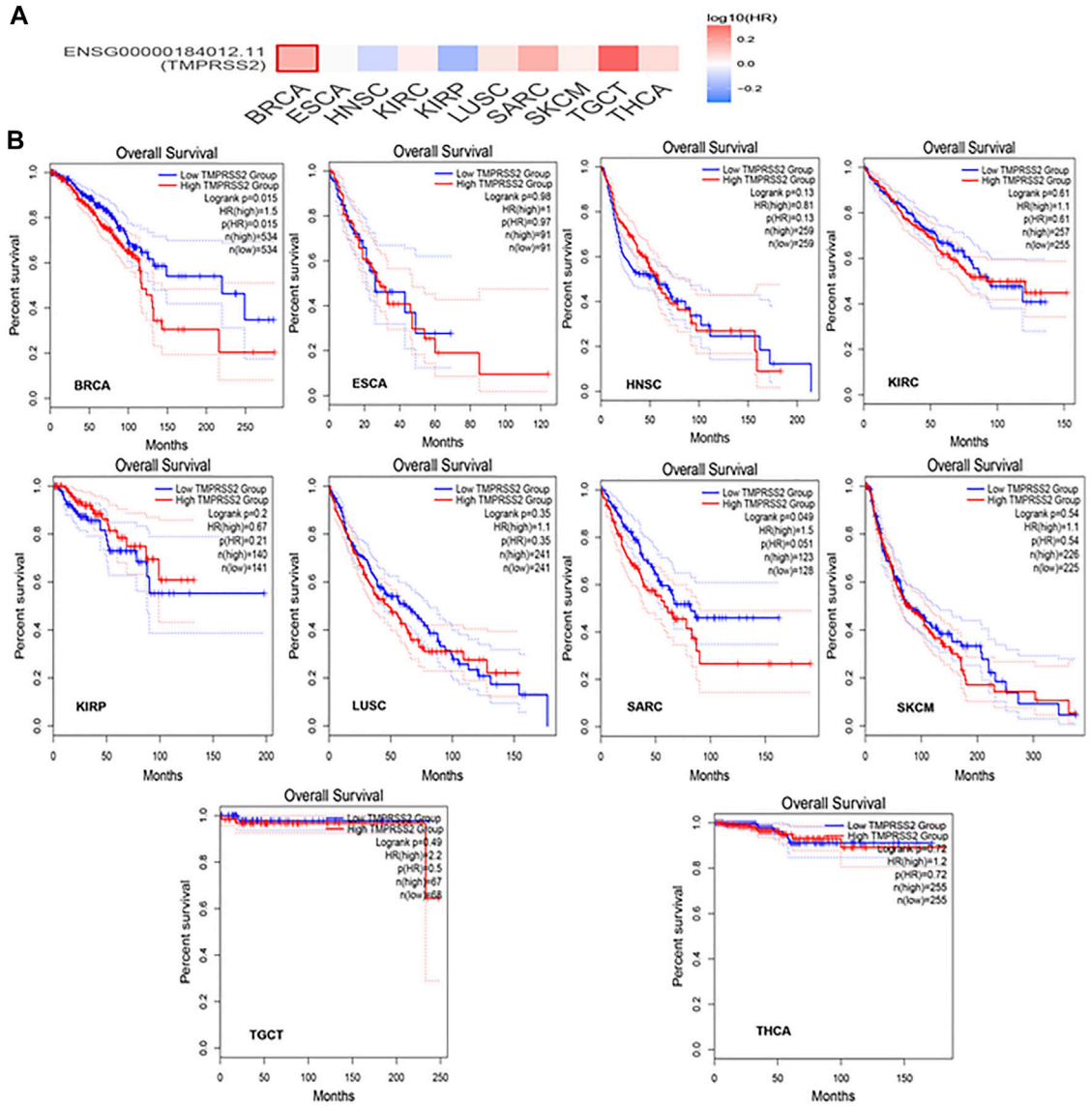

FIGURE 9 | TMPRSS2 expression unchanged in some of the tumors. There was no difference in the expression of TMPRSS2 in some tumors and normal tissues, including adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), cholangiocarcinoma (CHOL), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), acute myeloid leukemia (LAML), brain lower grade glioma (LGG), ovarian serous cystadenocarcinoma (OV), stomach adenocarcinoma (STAD), thymoma (THYM), pancreatic adenocarcinoma (PAAD), and even lung adenocarcinoma (LUAD).

STRING tool, the obtained binding proteins were supported by experimental evidence, and **Figure 13A** reveals the interaction network. The GEPIA2 tool was applied to obtain the top 100 genes related to TMPRSS2 expression. The corresponding heatmap data also indicated the correlation between TMPRSS2 and the top 10 genes in most detailed cancer types (**Figure 13C**). In addition, KEGG/GO enrichment analysis data further showed that most of these genes were related to endosome membrane, presynapse, Golgi vesicle transport, Ras protein signal transduction, axonogenesis, regulation of neuron projection development, prostate cancer, Rab protein signal transduction, and other related pathways or cell biology (**Figures 13D,E**).

DISCUSSION

Previous research evidence has shown that TMPRSS2 plays an important role in monitoring the infection process of severe acute respiratory syndrome (Khoury et al., 2020; Suarez-Farinas et al.) virus and Middle East respiratory syndrome (Lavin et al., 2017) coronavirus (Khan and Khan, 2021). Similarly, in a research report on SARS-CoV-2, TMPRSS2

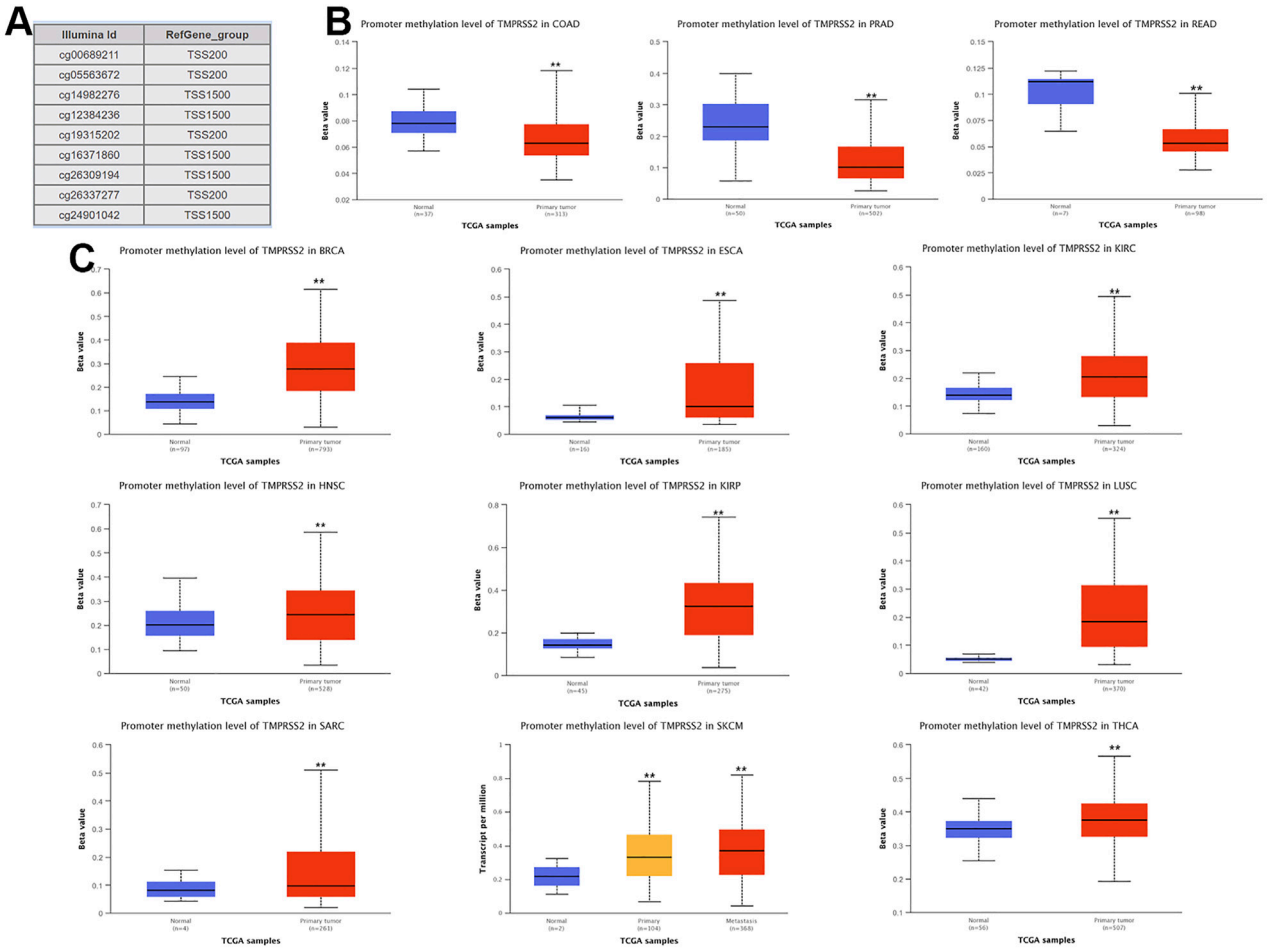

FIGURE 10 | Disease-free survival (DFS) data in malignant tumors with TMPRSS2 overexpression. **(A)** Survival picture of tumors overexpressing TMPRSS2; **(B)** DFS in TMPRSS2-overexpressing tumors.

and angiotensin converting enzyme 2 (ACE2) were coexpressed in human air and alveolar and blood air (Lukassen et al., 2020; Suarez-Farinas et al., 2021). ACE2 has been identified as a functional receptor for pathogenic SARS-CoV-2, which is related to the transport of the virus from the cell membrane to the cytoplasm (Barnes et al., 2020; Liu K. et al., 2021). TMPRSS2 promotes the binding of SARS-CoV-2 to ACE2 in the host cell by activating the S protein and assists the virus in entering the host cell (Kusmartseva et al., 2020). TMPRSS2 may also be the key to SARS-CoV-2 replication, and its expression greatly promotes the replication of the virus and the formation of syncytial virus–infected cells (Buchrieser et al., 2021; Kruger et al., 2021). The expression of TMPRSS2 greatly promoted the replication of the virus and the formation of syncytia (Iwata-Yoshikawa et al., 2019). Another study showed that in the presence of TMPRSS2, the number of SARS coronaviruses entering cells increased by 2.6 times, and the targeted elimination of TMPRSS2 could significantly reduce the number of SARS coronaviruses entering cells (Kawase et al., 2012). In addition, cancer has been identified as a personal factor of COVID-19, so the significance of TMPRSS2 expression in pan-cancers is more likely to be connected to the susceptibility of SARS-CoV-2 to tumor patients (Kuderer et al., 2020; Moris et al., 2020). Some researchers have suggested that although patients with underlying diseases are more susceptible to SARS-CoV-2 infection, patients suffering from head and neck cancer or lung cancer have reduced expression of TMPRSS2 in the body (Sacconi et al., 2020), which makes these patients less susceptible to SARS-CoV-2. Our research has genetically engineered the genetic and epigenetic variation of TMPRSS2 in humans for the first time.

TMRPSS2 expression was overexpressed in seven different types and downregulated in 10 different types (**Figure 4**). Since COVID-19 is mainly spread through the airway, we are particularly concerned about respiratory tumors. TMRPSS2 was significantly decreased in LUSC but remained unchanged in LUAD (**Figure 5**). Nine other types of tumors, including BRCA, ESCA, HNSC, KIRC, KIRP, SARC, SKCM, TGCT, and

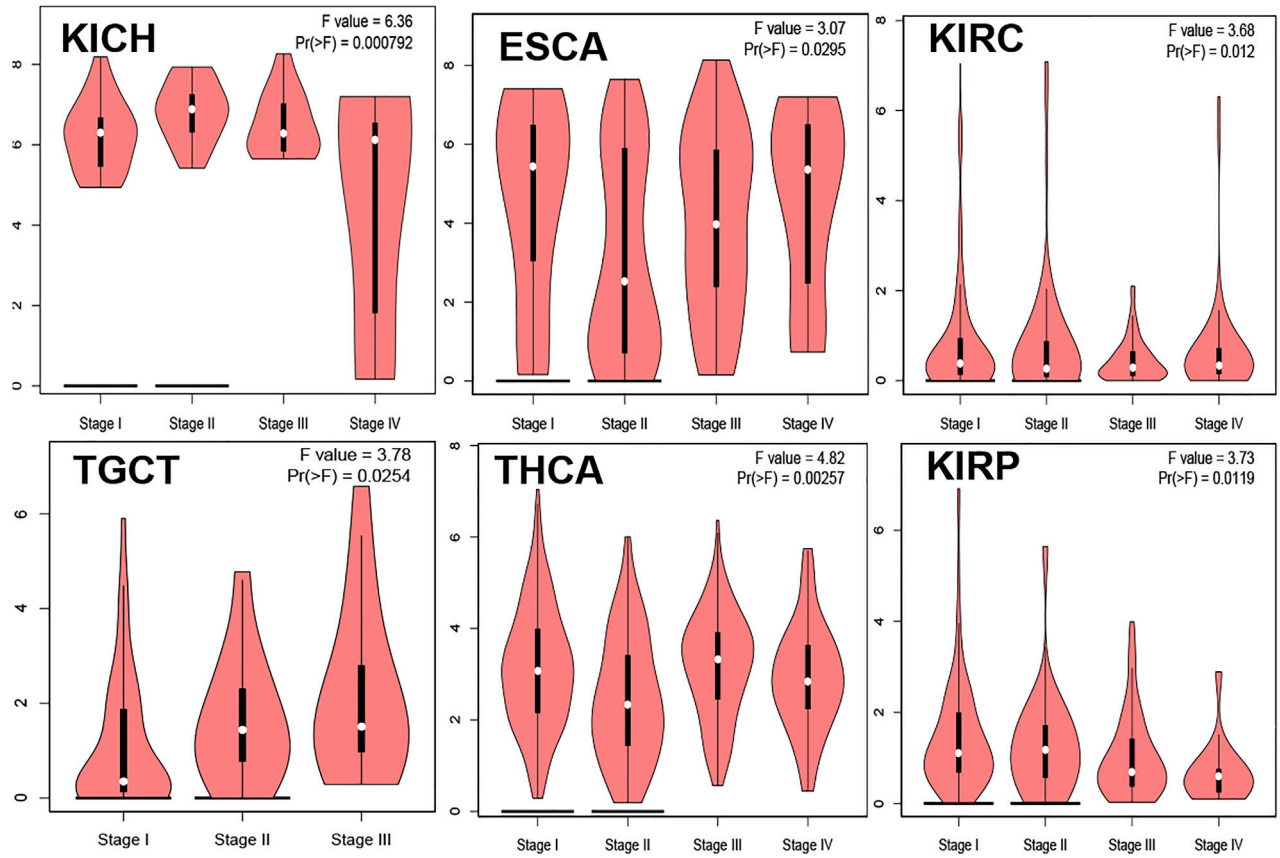

FIGURE 11 | DFS data in malignant tumors with low TMPRSS2 expression. **(A)** Survival picture of tumors with low TMPRSS2 expression; **(B)** DFS in tumors with low TMPRSS2 expression.

THCA, exhibited downregulation of TMPRSS2. The correlation between genetic diseases and TMRPSS2 expression was also confirmed in this research. In addition, there was no correlation between DNA/RNA mutations and TMRPSS2 expression (**Figure 3**). Our results implied that there are some differences in the frequency of TMPRSS2 variants in patients with different tumors. The existence of genetic variation does not always affect gene expression. Therefore, the upregulation of TMPRSS2 expression may not be caused by genetic mutations.

Nine probes in the TMRPSS2 promoter were used to detect the DNA methylation level of TMRPSS2 (**Figure 10A**). The findings verified that three tumors with high expression of TMPRSS2 manifested reduced levels of TMPRSS2 DNA methylation, including COAD, PRAD, and READ (**Figure 10B**). In other respects, among the nine tumors downregulated by TMPRSS2, BRCA, ESCA, HNSC, KIRP, SARC, SKCM, THCA, and even LUSC showed increased DNA methylation levels (**Figure 10C**). Since there is no DNA methylation dataset available for KICH normal controls, global DNA methylation levels of KICH cannot be compared. Thus, the DNA methylation levels of KICH at different tumor stages were compared, and the outcomes further found the enhancement of DNA methylation levels. In addition, the level of DNA methylation in other tumors with differential expression of TMPRSS2 remained unchanged, indicating that DNA methylation may not be the only cause of abnormal TMPRSS2 expression, such as histone modification and glycosylation. The high level of methylation in the promoter region can also silence the gene transcription process. Therefore, in some tumors, such as LUSC, the high level of methylation in the TMPRSS2 promoter may lead to a decrease in its transcriptional expression level.

The connection of clinical information with TMPRSS2 expression was monitored by the GEPIA2 database. The disease-free survival of UCES and UCS with high expression of TMPRSS2 was significantly different from that of the low-expression group (**Figures 6**, **7**). The overall survival analysis found that BRCA with high expression of TMPRSS2 was related to poor prognosis. However, low expression of TMPRSS2 was irrelevant to the prognosis of tumor patients (**Figures 8**, **9**). These data suggested that

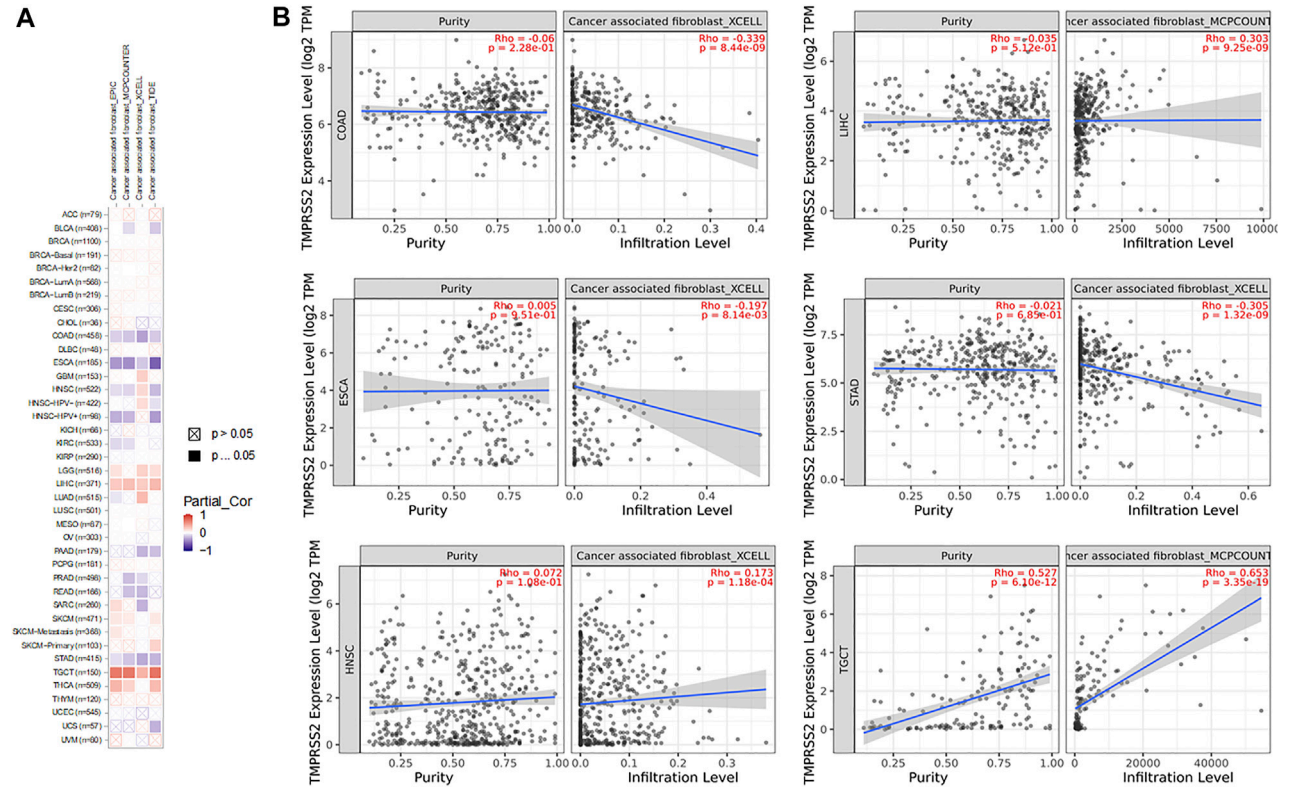

FIGURE 12 | Correlation analysis of TMPRSS2 expression and immune infiltration of cancer-related fibroblasts. **(A)** Different algorithms were used to explore the potential correlation between the expression level of the TMPRSS2 gene and the infiltration level of cancer-related fibroblasts in all types of cancers in TCGA. **(B)** Enumerated XCELL infiltration of COPD, ESCA, HNSC, and STAD and MCPCOUNTER infiltration of LIHC and TGCT.

TMPRSS2 may be a double-edged sword in tumor patient prognosis. Therefore, other clinical features should also be fully considered. Second, more in-depth molecular experimental evidence is needed to determine whether the high expression of TMPRSS2 plays an important role in the occurrence of the aforementioned tumors or if it is just the result of normal tissues resisting tumor alterations.

As reported, TMPRSS2 has been shown to be an important regulator of tumorigenesis (Bao et al., 2020). TMPRSS2-ERG gene fusion occurs in approximately 50% of prostate cancer (PCa) cases, and the fusion product is a key driver of prostate cancer. Cell signaling to ablate the TMPRSS2-ERG oncoprotein may be beneficial in the treatment of PCa (Hong et al., 2020). Studies have shown that regardless of the size of the primary tumor, deletion of TMPRSS2 in tumor-bearing mice can significantly reduce metastasis (Kang et al., 2021). This is consistent with the emerging view that metastasis and primary tumor growth are controlled by different factors and suggests that TMPRSS2 may be essential for tumor metastasis behavior. By using a large number of single-cell RNA sequencing datasets, scientists systematically studied the expression of ACE2 and TMPRSS2 in human tumors and normal colorectal tissues and found that these two receptors are highly expressed in colorectal epithelial cells. They further found that patients with colorectal cancer and COVID-19 were more prone to lymphopenia with higher respiratory rates and high-sensitivity C-reactive protein levels than patients with COVID-19 alone (Liu C. et al., 2021). The expression of TMPRSS2 is downregulated in patients with head and neck cancer, which implies more resistance to SARS-CoV-2 infection (Sacconi et al., 2020).

In this research, the potential correlation between tumor-associated fibroblast immune infiltration in all TCGA tumors and TMPRSS2 was explored using the Timer 2.0 database. Information on TMPRSS2 binding components and TMPRSS2 expression–related genes in more than 30 tumors was obtained simultaneously by a series of enrichment analyses. The findings demonstrated that most of these genes were related to endosome membrane, presynapse, Golgi vesicle transport, Ras protein signal transduction, axonogenesis, regulation of neuron projection development, prostate cancer, Rab protein signal transduction, and other related pathways or cell biological functions.

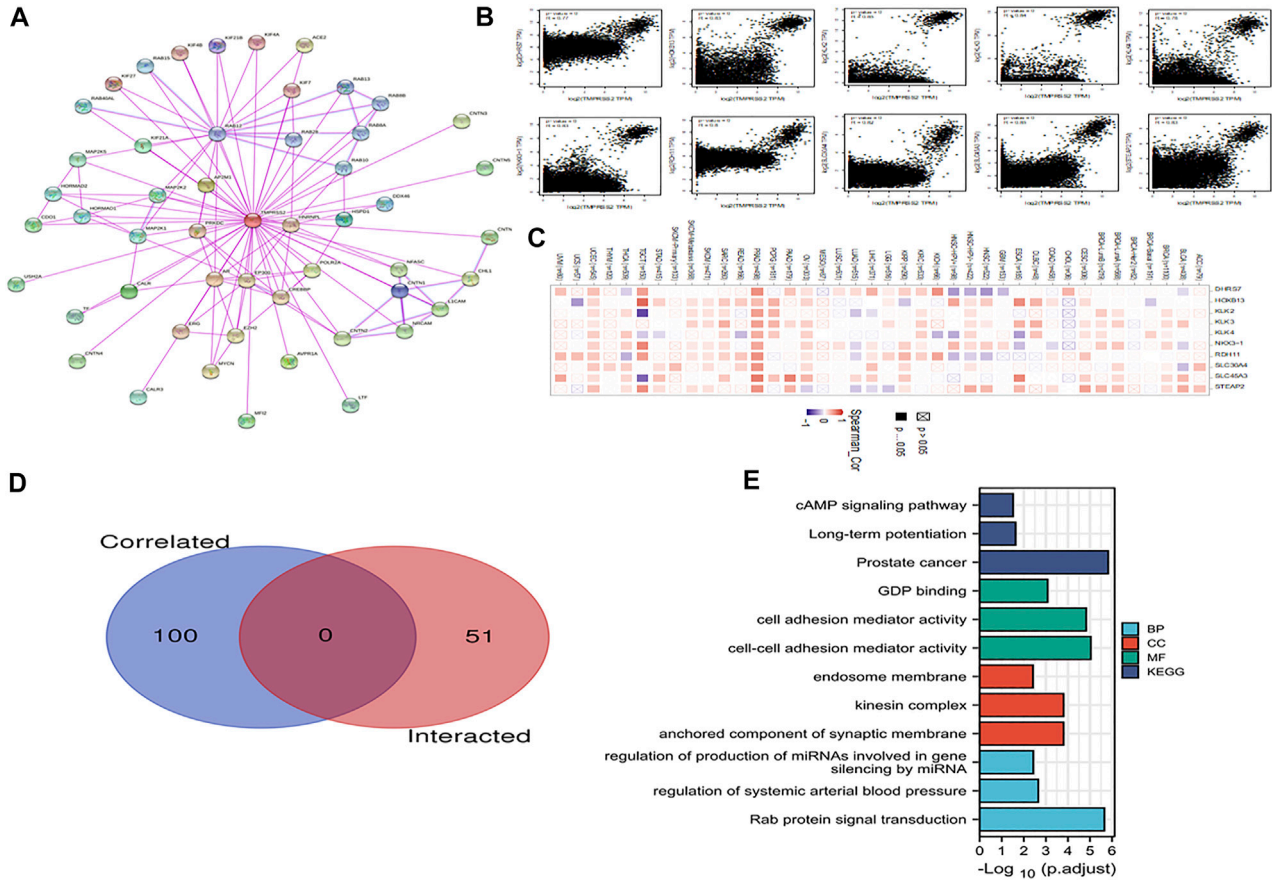

FIGURE 13 | Enrichment analysis of TMPRSS2-related genes. **(A)** The STRING tool was utilized to obtain available TMPRSS2 binding proteins. **(B)** Using the GEPIA2 method, we also obtained the top 100 TMPRSS2-related genes in the TCGA project, including DHRS7, HOXB13, KLK2, KLK3, KLK4, NKX3-1, RDH11, SLC30A4, SLC45A3, and STEAP2. **(C)** The plot demonstrates the correlation data between TMPRSS2 and the related top 10 genes in various cancers. **(D)** Cross-analysis of TMPRSS2 binding genes and related genes was performed. **(E)** GO/KEGG pathway analysis based on TMPRSS2 binding genes and interacting genes.

In conclusion, the genetic changes, RNA expression, and DNA methylation of TMPRSS2 were analyzed in more than 30 tumors. These findings suggest the need for priority precautions for COAD, CESC, KICH, PRAD, UCEC, UCS, and READ during the COVID-19 pandemic. In addition, low DNA methylation of TMPRSS2 was also found in most of these tumors with high TMPRSS2 expression. In the end, our study planned the genetic and epigenetic variation of TMPRSS2 in human malignant tumors for the first time. However, the specific molecular mechanism of TMPRSS2 in the formation and development of tumors *in vivo* has not been clarified. Since our research is a bioinformatics test, it is necessary to conduct further functional and clinical verification.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

HP, TY, and YM designed research studies; JS and GZ performed data analysis and used the tool. YM wrote the manuscript. All authors helped with reviewing the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.805880/full#supplementary-material

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Epigenetic Alterations of DNA Methylation and miRNA Contribution to Lung Adenocarcinoma

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This study focused on the epigenetic alterations of DNA methylation and miRNAs for lung adenocarcinoma (LUAD) diagnosis and treatment using bioinformatics analyses. DNA methylation data and mRNA and miRNA expression microarray data were obtained from The Cancer Genome Atlas (TCGA) database. The differentially methylated genes (DMGs), differentially expressed genes (DEGs), and differentially expressed miRNAs were analyzed by using the limma package. The DAVID database performed GO and KEGG pathway enrichment analyses. Using STRING and Cytoscape, we constructed the protein–protein interaction (PPI) network and achieved visualization. The online analysis tool CMap was used to identify potential small-molecule drugs for LUAD. In LUAD, 607 high miRNA-targeting downregulated genes and 925 low miRNA-targeting upregulated genes, as well as 284 hypermethylated low-expression genes and 315 hypomethylated high-expression genes, were obtained. They were mainly enriched in terms of pathways in cancer, neuroactive ligand–receptor interaction, cAMP signaling pathway, and cytosolic DNA-sensing pathway. In addition, 40 upregulated and 84 downregulated genes were regulated by both aberrant alternations of DNA methylation and miRNAs. Five small-molecule drugs were identified as a potential treatment for LUAD, and five hub genes (*SLC2A1*, *PAX6*, *LEP*, *KLF4*, and *FGF10*) were found in PPI, and two of them (*SLC2A1* and *KLF4*) may be related to the prognosis of LUAD. In summary, our study identified a series of differentially expressed genes associated with epigenetic alterations of DNA methylation and miRNA in LUAD. Five small-molecule drugs and five hub genes may be promising drugs and targets for LUAD treatment.

Keywords: DNA methylation, miRNA, epigenetics, lung adenocarcinoma, mRNA

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1 INTRODUCTION

Lung cancer is a fatal malignancy featuring the highest incidence (11.6%) and mortality (18.4%) globally. Lung adenocarcinoma (LUAD), increasing yearly, is the most common histological type of lung cancer (Bray et al., 2018). LUAD often arises peripherally and has adenoid tissue differentiation and mucin production in histology (Chen et al., 2014). It has been documented that the pathogenesis of LUAD involves inflammation, oxidative stress, mitochondrial dysfunction, changes in lipid metabolism, and epigenetic changes (Lindskog et al., 2014; Tan et al., 2016; Zhang C. et al., 2021). Unlike other subtypes, LUAD has a high rate of gene mutations. Although targeted therapy has improved the survival rate and quality of life of nearly 60% of LUAD patients with corresponding

driver gene mutations in recent years, drug resistance is still inevitable. The long-term survival rate of LUAD is still not satisfactory (Kulasingam and Diamandis, 2008; Hirsch et al., 2017). A large amount of literature studies show that environmental factors and genetic and epigenetic factors will affect the occurrence and development of lung adenocarcinoma (Fabrizio et al., 2020; Gong et al., 2020; Wang et al., 2020; Huang et al., 2021; Shi et al., 2021). Although detailed knowledge about the processes of initiation and progression of LUAD is still unknown and remains a major stumbling block on the road to LUAD treatment, robust and accurate development of biomarkers will greatly facilitate early diagnosis and treatment of biological characteristics of LUAD. Therefore, there is an urgent need to identify new therapeutic targets and some chemicals of LUAD.

Carcinogenesis is a complex process involving genetic and epigenetic changes. Abnormal genetic and epigenetic changes are the hallmarks of cancer. Epigenetic modifications can modify the gene expression without altering the DNA sequence. In cancer, deviant epigenetic regulation includes miRNA gene silencing, DNA methylation, mRNA and non-coding RNA methylation, histone methylation, and histone acetylation (Maruyama et al., 2011). The aforementioned processes are closely related and affect protein synthesis. Interference with each operation may lead to dysfunction.

miRNAs are small non-coding RNA sequences about 19–23 nucleotides in length, which are highly conserved in regulating post-translational modifications (Bartel, 2004). miRNAs can exhibit carcinogenic effects or suppressor tumors by regulating target genes. These two miRNAs are termed oncomiR and tumor suppressor (TS) miRNA, respectively

miRNAs can show carcinogenic effects or suppressor tumors by regulating target genes. These two miRNAs are termed oncomiR and tumor suppressor (TS) miRNA, respectively (Zhang et al., 2014). They have emerged as promising biomarkers for diagnostic, therapeutic, and prognostic applications due to their association with LUAD (Gu et al., 2017; Wang et al., 2019; Yuan et al., 2019). For instance, miR-196b-5p displays high expressions, whereas its target gene RSPO2 (R-Spodin 2) is expressed low in the cancer tissues and normal in para-cancer tissues, promoting proliferation and migration and invasion of LUAD (Xu and Xu, 2020).

DNA methylation is a genetic modification that does not change the DNA sequence (Santos et al., 2005). DNA methylation is associated with the subtypes and prognosis of multiple tumors, including LUAD (Fleischer et al., 2017; Long et al., 2019; Ding et al., 2020; Xu et al., 2020). Shen et al. (2019) discovered that the hypermethylation of HOXA9 and hypomethylation of TULP2, CCND1, and KRTAP8-1 could be used as biomarkers for the early detection of LUAD in the undetermined lung nodules.

As yet, although a large number of studies have demonstrated the abnormal DNA methylations or the global methylation level and miRNA level in LUAD, the comprehensive regulatory network and pathways analyses of DNA methylation levels and miRNA epigenetic alterations have not yet been conducted.

This study systematically analyzed the data on DNA methylation microarrays, miRNA expression microarrays, and mRNA expression profiling microarrays from TCGA database to identify the core genes and pathways that lead to the occurrence and development of LUAD *via* epigenetic regulation.

2 MATERIALS AND METHODS

2.1. Microarray Data

In this study, the data on DNA methylation microarrays (including 437 LUAD and 29 adjacent normal tissue samples), miRNA expression microarrays (including 483 LUAD and 45 adjacent normal tissue samples), and mRNA expression profiling microarrays (including 497 LUAD and 54 adjacent normal tissue samples) were obtained from TCGA database (https://portal.gdc.cancer.gov/).

2.2. Data Process

The Perl script (Perl version 5.18.4) was used to process expression data to obtain mRNA and miRNA matrix. R (version 4.0.2) and Bioconductor packages were used to preprocess the raw gene expression profiles, including background correction, normalization, and logarithmic conversion. Differentially methylated probes (DMPs), differentially expressed miRNAs (DEMs), and differentially expressed genes (DEGs) were performed by using the limma package in R. DMPs were screened with P. adjust <0.05 and |logFC | >0.2 as the cut-off criteria. DEMs were screened with P. adjust <0.05 and |logFC |>2 as the cut-off criteria, and DEGs were screened with P. adjust <0.05 and |logFC |>1 as the threshold. Draw Venn Diagram online software (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to find overlapping genes from DMPs, DEMs, and DEGs. Aberrant methylated and expressed genes were overlapped to obtain hypermethylated low-expression genes and hypomethylated high-expression genes. Subsequently, high miRNA-targeting downregulated genes and low miRNA-targeting upregulated genes were obtained *via* overlapping potential targets of DEMs and DEGs. The Kaplan–Meier plotter database (https://kmplot.com/analysis/index.php?p=service&cancer=lung) was used for the survival analysis of hub genes.

2.3. Prediction of Potential Targets of miRNAs and Construction of the miRNA–mRNA Network

The targets of DEGs were predicted by microT-CDS online software of DIANA TOOLS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) and the miRWalk database (http://mirwalk.umm.uni-heidelberg.de/). In addition, the Cytoscape tool (v3.7.2) was used to construct the entire miRNA–mRNA regulatory network.

2.4. Functional and Pathway Enrichment Analysis

Gene ontology (GO) analyses, including the biological process (BP), cellular component (CC), and molecular function (MF), were conducted for the upregulated genes, downregulated genes, hypermethylation-low-expression genes, and hypomethylation-high-expression genes selected by DAVID (https://david.ncifcrf.

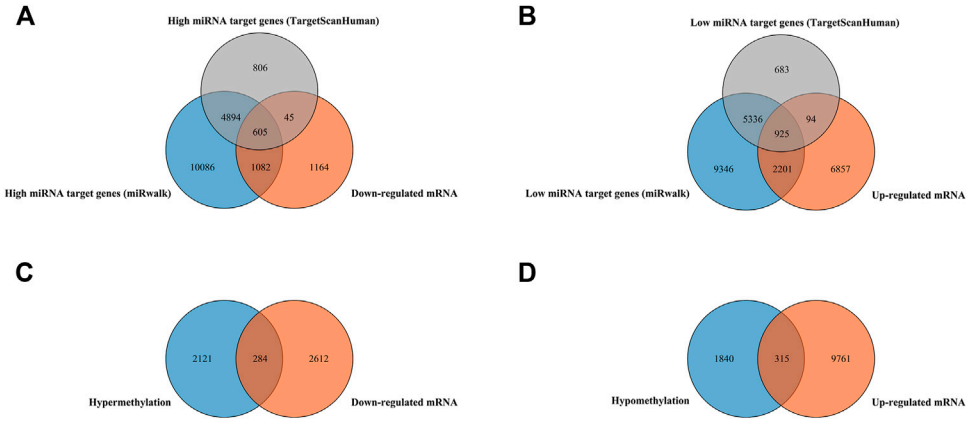


FIGURE 1 | Identification of target genes of differentially expressed miRNAs and mRNA, as well as aberrantly methylated differentially expressed genes between cancer and adjacent samples from LUAD patients. **(A,B)** Target genes of differentially expressed miRNAs and mRNAs; miRNA target genes were predicted by microT-CDS online software and the miRWalk database, respectively. **(C,D)** Aberrantly methylated differentially expressed genes.

gov/). Subsequently, we performed the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses for the high miRNA-targeting downregulated genes, low miRNA-targeting upregulated genes, hypermethylation-low-expression genes, and hypomethylation-high-expression genes. All analyses were performed with $p < 0.05$ as the screening condition.

2.5. Protein–Protein Interaction Network Construction and Module Analysis

We used the Search Tool of the Retrieval of Interacting Genes (STRING) online tool to perform PPI networks of hypermethylation-low-expression genes and hypomethylation-high-expression genes, respectively. cytoHubba in Cytoscape software was used to obtain hub genes within the PPI (top 10 nodes ranked by degree). The functional and pathway enrichment analysis of the genes in each module was performed by DAVID with $p < 0.05$ as the threshold.

2.6. Real-Time Quantitative PCR

PC9 and BEAS-2B cell lines were purchased from Zhejiang Meisen Cell Technology Co., Ltd. (MeisenCTCC). The PC9 cell line was cultured using 1640 + 10% FBS+1% anti-anti. The BEAS-2B cell line was cultured using BEGM, with 1% anti-anti added to the culture. Real-time Quantitative PCR was performed using Bio-Rad CFX96. After cell culture, the cells were washed three times with iced PBS. The RNA isolater Total RNA Extraction Reagent (Vazyme) was used to isolate the total RNA from cells. Then, 1 μg of total RNA and HiScript III-RT SuperMix for qPCR (Vazyme) were used for reverse transcription, according to the manufacturer's instructions. Amplification reactions were set up in 20 μL volume containing ChamQ Universal SYBR qPCR Master Mix (Vazyme) and amplification primers according to the manufacturer's instructions. The primer sequences used for real-time PCR are listed as follows. An amount of 5ng of cDNA was used in each amplification reaction.

The primer sequences for PCR amplification were as follows: SLC2A1, forward: 5′-TCTGGCATCAACGCTGTCTTC-3′ and reverse: 5′-CGATACCGGAGCCAATGGT-3′; PAX6, forward: 5′-TGGGCAGGTATTACGAGACTG-3′ and reverse: 5′-ACT CCCGCTTATACTGGGCTA-3′; LEP forward: 5′-TGCCTT CCAGAAACGTGATCC-3′ and reverse: 5′-CTCTGTGGA GTAGCCTGAAGC-3′; KLF4 forward: 5′-CGGACATCAACG ACGTGAG-3′ and reverse: 5′-GACGCCTTCAGCACGAACT-3′; FGF forward: 5′-CAGTAGAAATCGGAGTTGTTGCC-3′ and reverse: 5′-TGAGCCATAGAGTTTCCCCTTC-3′; and β actin forward: 5′-CATGTACGTTGCTATCCAGGC-3′ and reverse: 5′-CTCCTTAATGTCACGCACGAT-3′.

2.7. Drug Exploration in CMap

The Connectivity Map (CMap) database (https://www.broadinstitute.org/) contains gene expression profiles of human cells treated with small bioactive molecules. Researchers can use CMap to identify connections among small molecules that share a physiological process, chemicals, and actions and then predict the potential drugs (Lamb et al., 2006). We used the CMap database to identify potential small-molecule drugs that reverse or induce DEGs' modified expression in LUAD cell lines (mean range from −0.5 to 0.5 and $p < 0.01$).

2.8. Statistical Analyses

All the results were analyzed and processed by GraphPad Prism 8 software. The unpaired *t*-test was used for statistical analysis, and the data were expressed as mean ± standard deviation. $p < 0.05$ was considered statistically significant; * meant $p < 0.05$, ** meant $p < 0.01$, *** meant $p < 0.001$, and **** meant $p < 0.0001$.

3 RESULTS

3.1. Identification of Abnormal Methylated Differentially Expressed Genes in LUAD

The characteristics of mRNA and miRNA transcriptome profiling and DNA methylation profiling based on the TCGA database are shown in **Supplementary Table S1**. In mRNA expression profiling microarrays of TCGA, a total of 12972

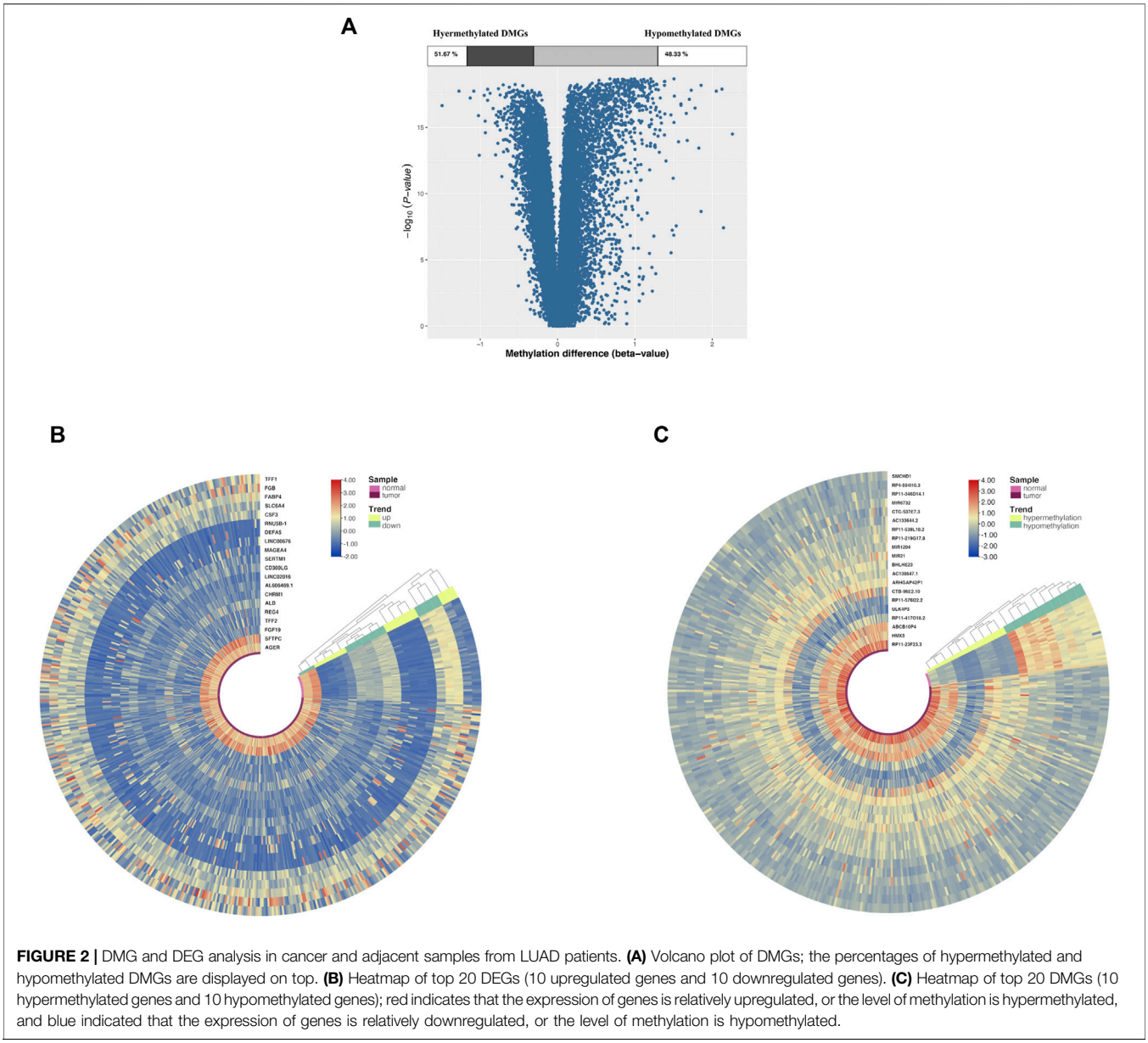


FIGURE 2 | DMG and DEG analysis in cancer and adjacent samples from LUAD patients. **(A)** Volcano plot of DMGs; the percentages of hypermethylated and hypomethylated DMGs are displayed on top. **(B)** Heatmap of top 20 DEGs (10 upregulated genes and 10 downregulated genes). **(C)** Heatmap of top 20 DMGs (10 hypermethylated genes and 10 hypomethylated genes); red indicates that the expression of genes is relatively upregulated, or the level of methylation is hypermethylated, and blue indicated that the expression of genes is relatively downregulated, or the level of methylation is hypomethylated.

DEGs were screened in cancer tissue samples from LUAD, including 10076 upregulated genes and 2896 downregulated genes. Simultaneously, 13 high-expressed miRNAs and 18 low-expressed miRNAs were identified in miRNA expression microarrays of TCGA database. **Supplementary Table S2** records the characteristics of the top five differentially expressed miRNAs and their potential target DEGs. As to DNA methylation microarrays, 2405 hypermethylated genes and 2155 hypomethylated genes were found.

Finally, 607 high miRNA-targeting downregulated genes and 925 low miRNA-targeting upregulated genes were screened *via* overlapping target genes of DEMs and DEGs (**Figures 1A,B**). In addition, 284 hypermethylation-low-expression genes and 315 hypomethylation-high-expression genes by overlapping abnormal methylation and regulated genes were identified (**Figures 1C,D**).

Of all DMGs, 51.76% were hypermethylated, and 48.33% were hypomethylated (**Figure 2A**). Moreover, **Figures 2B,C** suggested that the DEGs and DMGs (top 20 upregulated and top 20 downregulated genes, as well as top 20 hypermethylation and top 20 hypomethylation genes) can be differentiated between LUAD and normal samples.

3.2. DEGs Associated With Altered Targeting miRNAs

3.2.1. Low-Expression miRNAs and Upregulated Genes

For low-expression miRNAs and upregulated genes, 168 GO terms were screened with the thresholds of $p < 0.05$, which were mainly associated with the regulation of transcription and cell adhesion (**Figure 3A**). The most enriched KEGG pathways were

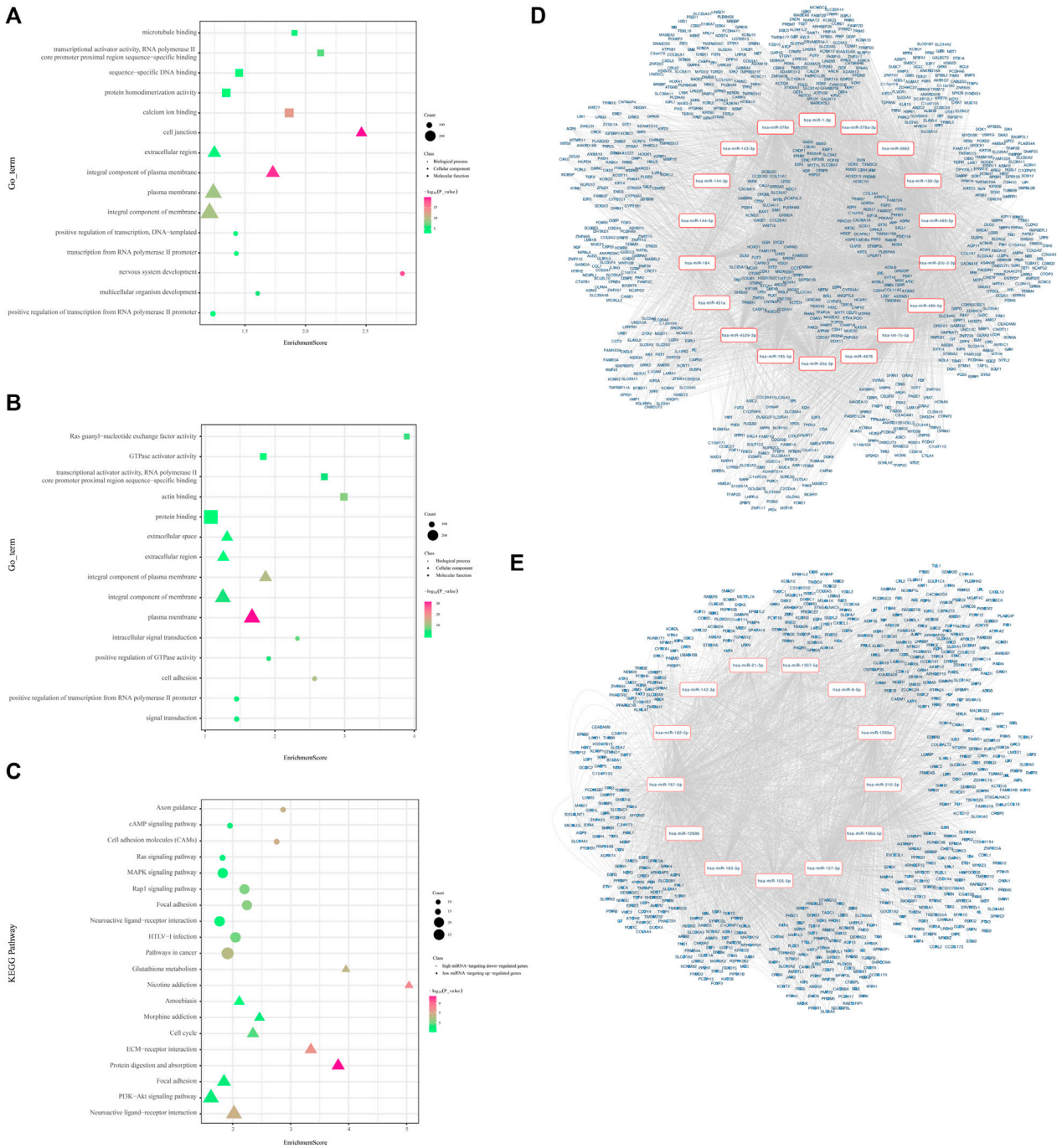


FIGURE 3 | Visualized regulatory network and enrichment bubble graph for miRNA-targeting DEGs. **(A)** Enrichment bubble graph for upregulated genes. **(B)** Enrichment bubble graph for downregulated genes. **(C)** KEGG enrichment bubble chart of low-expression miRNAs and upregulated genes and high-expression miRNAs and downregulated genes. **(D)** Regulatory network graph of 18 low-expression miRNAs. **(E)** Regulatory network graph of 13 high-expression miRNAs.

the neuroactive ligand–receptor interaction, PI3K-Akt signaling pathway, focal adhesion, protein digestion, and absorption, and ECM–receptor interaction. The KEGG enrichment chart of low-expression miRNAs and upregulated genes is shown in **Figures 3C,D**. It also shows the miRNA-mRNA network of the 925 upregulated genes.

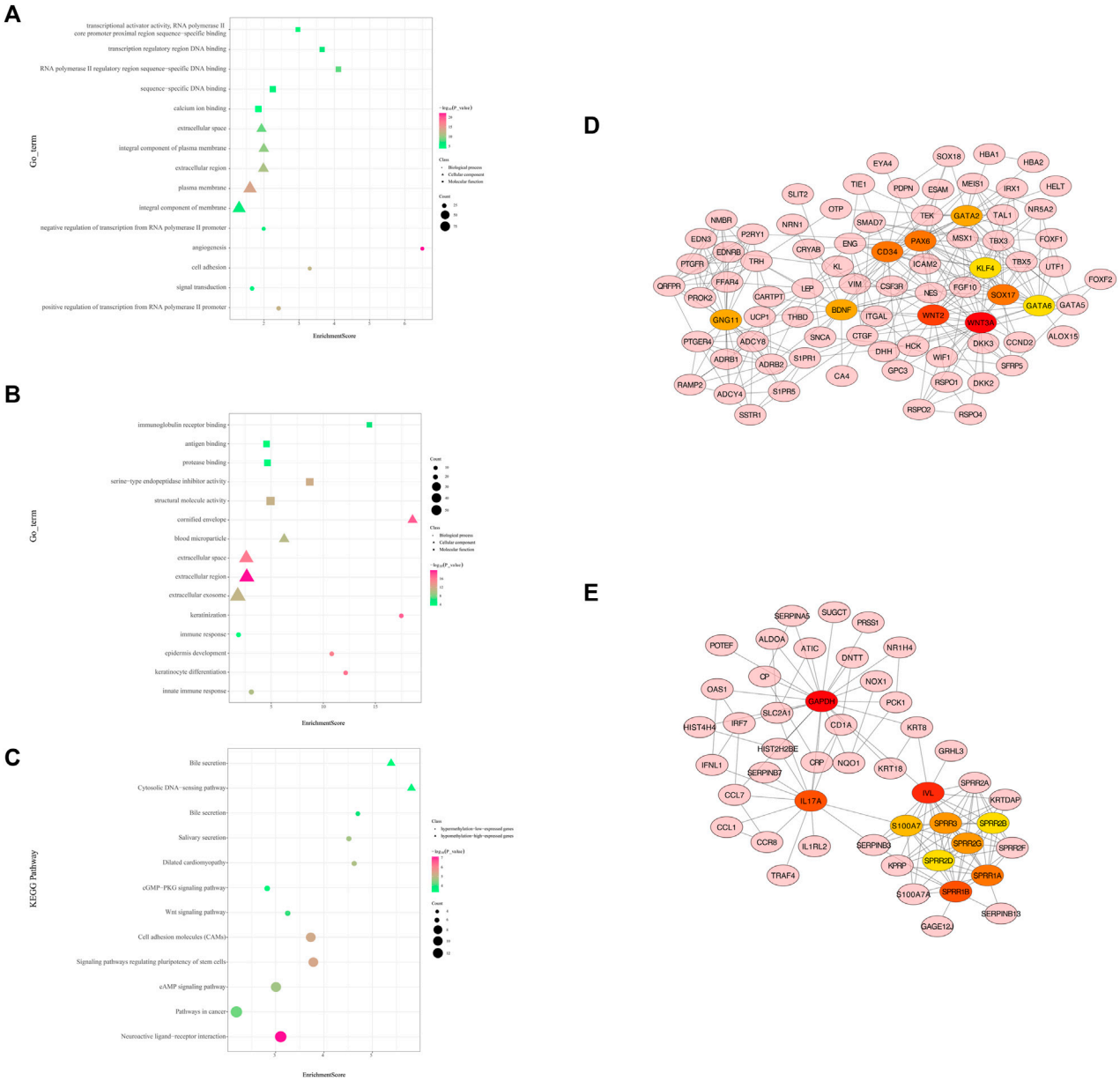


FIGURE 4 | Protein-protein interaction (PPI) network and enrichment bar graph for methylation-related DEGs. **(A)** Enrichment bubble graph for hypermethylated downregulated genes. **(B)** Enrichment bubble graph for hypomethylated upregulated genes. **(C)** KEGG enrichment bubble chart of hypermethylated downregulated genes and hypomethylated upregulated genes. **(D)** PPI network of hypermethylation and low-expression genes between cancer and adjacent samples from LUAD patients (only displays the 10 hub genes and the expanded subnetwork identified by the cytoHubba app in Cytoscape). **(E)** PPI network of hypomethylation and high-expression genes between cancer and adjacent samples from LUAD patients (only displays the 10 hub genes and the expanded subnetwork identified by the cytoHubba app in Cytoscape).

3.2.2. High-Expression miRNAs and Downregulated Genes

A total of 607 high-expression miRNAs and downregulated genes were enriched in 169 GO terms with the thresholds of $p < 0.05$, which were mainly associated with the regulation of transcription, signal transduction, and cell adhesion (**Supplementary Table S3**, **Figure 3B**). The most enriched KEGG pathways were pathways in cancer, HTLV-I infection, neuroactive ligand–receptor interaction, focal adhesion, and the Rap1 signaling pathway. The KEGG enrichment chart of high-expression miRNAs and downregulated genes is shown in **Figure 3C**. Meanwhile, we constructed the miRNA-mRNA network to reveal further significant miRNA/mRNAs regulated in LUAD progression (**Figure 3E**).

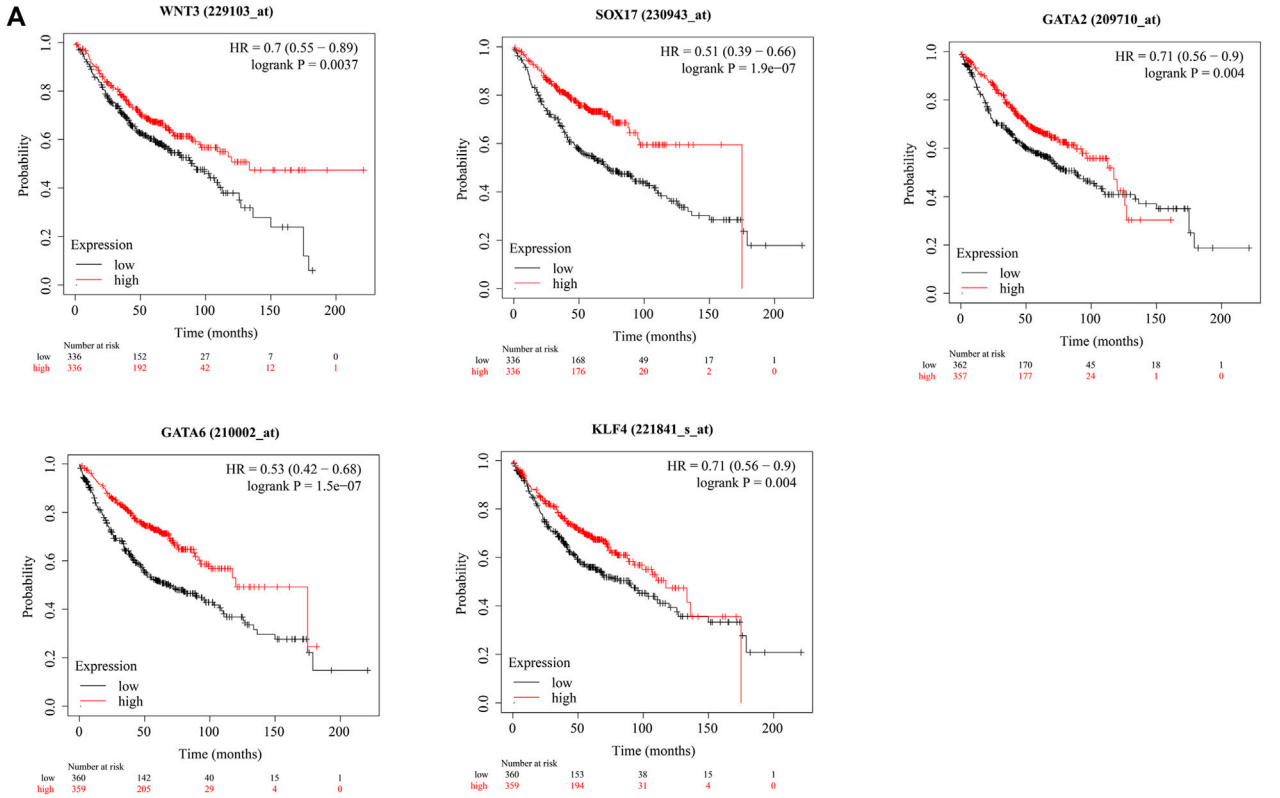


FIGURE 5 | (Continued).

3.3. DEGs Associated With Altered DNA Methylation

3.3.1. Hypermethylation and Low-Expression Genes

Functional enrichment analysis of hypermethylation and low-expression genes suggested that 141 GO terms were recognized with the thresholds of $p < 0.05$, such as the regulation of transcription, signal transduction, and cell adhesion (**Figure 4A**). The most enriched KEGG pathways were the neuroactive ligand–receptor interaction, pathways in cancer, cAMP signaling pathway, signaling pathways regulating pluripotency of stem cells, and cell adhesion molecules (CAMs) (**Supplementary Table S4**, **Figure 4C**). In total, 191 nodes and 486 edges are shown in the PPI network (**Supplementary Figure S1A**).

WNT3A, *WNT2*, *SOX17*, *CD34*, *PAX6*, *GATA2*, *GNG11*, *BDNF*, *GATA6*, and *KLF4* were identified as hub genes by the degree rank with the cytoHubba app in Cytoscape (**Figure 4D**, **Supplementary Table S5**). In these 10 hub genes, *WNAT3A* is calculated with the highest degree (degree = 24). The Kaplan–Meier survival analysis showed that low-expression *WNT3*, *SOX17*, *GATA2*, *GATA6*, and *KLF4* were correlated significantly with poor OS (**Figure 5A**).

3.3.2. Hypomethylation and High-Expression Genes

As for hypomethylation and high-expression genes, 39 GO terms were identified with the thresholds of $p < 0.05$ (**Figure 4B**). KEGG pathway analysis recognized enriched cytosolic DNA-sensing pathway and bile secretion (**Supplementary Table S4**, **Figure 4C**). In total, 133 nodes and 238 edges were shown in the PPI network (**Supplementary Figure S1B**).

GAPDH, *IVL*, *IL17A*, *SPRR1B*, *SPRR1A*, *SPRR3*, *SPRR2G*, *S100A7*, and *SPRR2A* were identified as hub genes by degree rank with the cytoHubba app (**Figure 4E**, **Supplementary Table S5**). GAPDH is calculated with the highest degree (degree = 23). The Kaplan–Meier survival analysis showed that high-expression *GAPDH*, *SPRR1B*, *SPRR1A*, *SPRR3*, and *S100A7* were all significant with poor OS (**Figure 5B**).

3.4. DEGs Associated With Both Abnormal miRNA and DNA Methylation

We found that several DEGs were regulated by both abnormal miRNA and DNA methylation. It suggested that these DEGs might be of vital importance in the occurrence and development of LUAD. A total of 84 genes such as *FAT4*, *KLF4*, and *EPB41L3* were downregulated under the regulation of both increased miRNA and hypermethylation (**Figure 6A**). Coincidentally, 40 genes such as *SUGCT*, *RNF43*, and *UGT2B15* were upregulated under the regulation of both decreased miRNA and hypomethylation (**Figure 6B**). The modulatory miRNA and binding sites, as well as the DNA methylation site, cg ID, and its relation to CpG island, are summarized in **Supplementary**

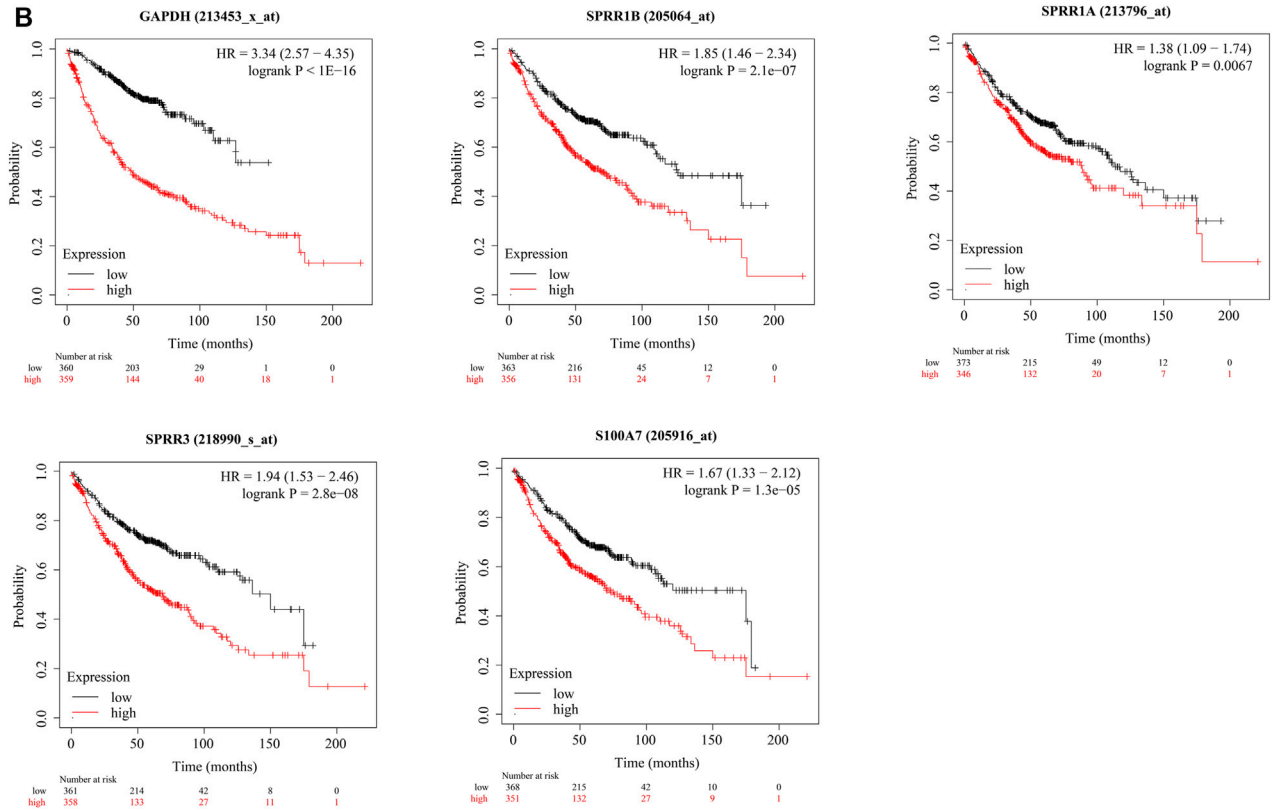


FIGURE 5 | (Continued). Kaplan–Meier plots of 10 hub genes. **(A)** Kaplan-Meier analysis of patients with low (black curve) and high (red curve) expressions of *WNT3*, *SOX17*, *GATA2*, *GATA6*, and *KLF4*. **(B)** Kaplan-Meier analysis of patients with low (black curve) and high (red curve) expressions of *GAPDH*, *SPRR1B*, *SPRR1A*, *SPRR3*, and *S100A7*. p values, log-rank test.

Table S6. In total, 86 genes, including 29 hypomethylation miRNA-targeting upregulated genes and 57 hypermethylation miRNA-targeting downregulated genes (11 hypomethylated miRNA-targeting upregulated genes and 26 hypermethylated miRNA-targeting downregulated genes without corresponding Affymetrix Probe Set ID on GPL96 cannot be used for CMap), were submitted to the CMap online tool to predict potential drugs in the therapy for LUAD depending on the expression alteration. By ranking the *p*-value in the ascending order and filtering the mean range from −0.5 to 0.5, five small-molecule chemicals were identified as latent treatment options for LUAD (**Table 1**). Furthermore, a PPI network for all the abnormal expressed genes, including 40 upregulated genes and 84 downregulated (63 nodes and 61 edges) genes were constructed (**Figure 6C**). Five hub genes were identified for further analysis, including *SLC2A1* with up-regulated expression levels under both low miRNA and hypomethylation regulation, as well as four genes with downregulated expression levels under both high miRNA and hypermethylation regulation of *PAX6*, *LEP*, *KLF4*, and *FGF10*. The Kaplan–Meier survival analysis showed that high-expression *SLC2A1* and low-expression *KLF4* were all significant with poor OS (**Figure 6D**). To verify the difference in the expression of the five hub genes in TCGA database, we used qRT-PCR to evaluate the expression of the five hub genes at the transcription level and found that the expression levels of *SLC2A1* mRNA ($p < 0.0001$, **Figure 7**), *LEP* mRNA ($p < 0.05$, **Figure 7**), and *FGF4* mRNA (($p < 0.05$, **Figure 7**) in the PC9 cell line were significantly higher than those in the BEAS-2B cell line. The expression levels of *KLF4* mRNA ($p < 0.0001$, **Figure 7**) and *PAX6* mRNA ($p < 0.001$, **Figure 7**) in the PC9 cell line were significantly lower than those in the BEAS-2B cell line. A CpG island prediction has been proceeded, and the results are shown in **Figure 8A**. The JASPER database predicted the sequence of four possible transcription factors of *SLC2A1* and *KLF4*, as shown in **Figures 8B,C**. However, further clinical trials are required to verify these findings.

4 DISCUSSION

CpG island-specific methylation in the promoter region of genes is associated with gene silencing (Morgan et al., 2018), which changes the expression of downstream hub genes and promotes abnormal cell proliferation (Kulis and Esteller, 2010). DNA methylation and miRNA expression can make a real difference in LUAD by up- or downregulating gene expressions (Herbst et al., 2018; He et al., 2021). Aberrant DNA methylation and

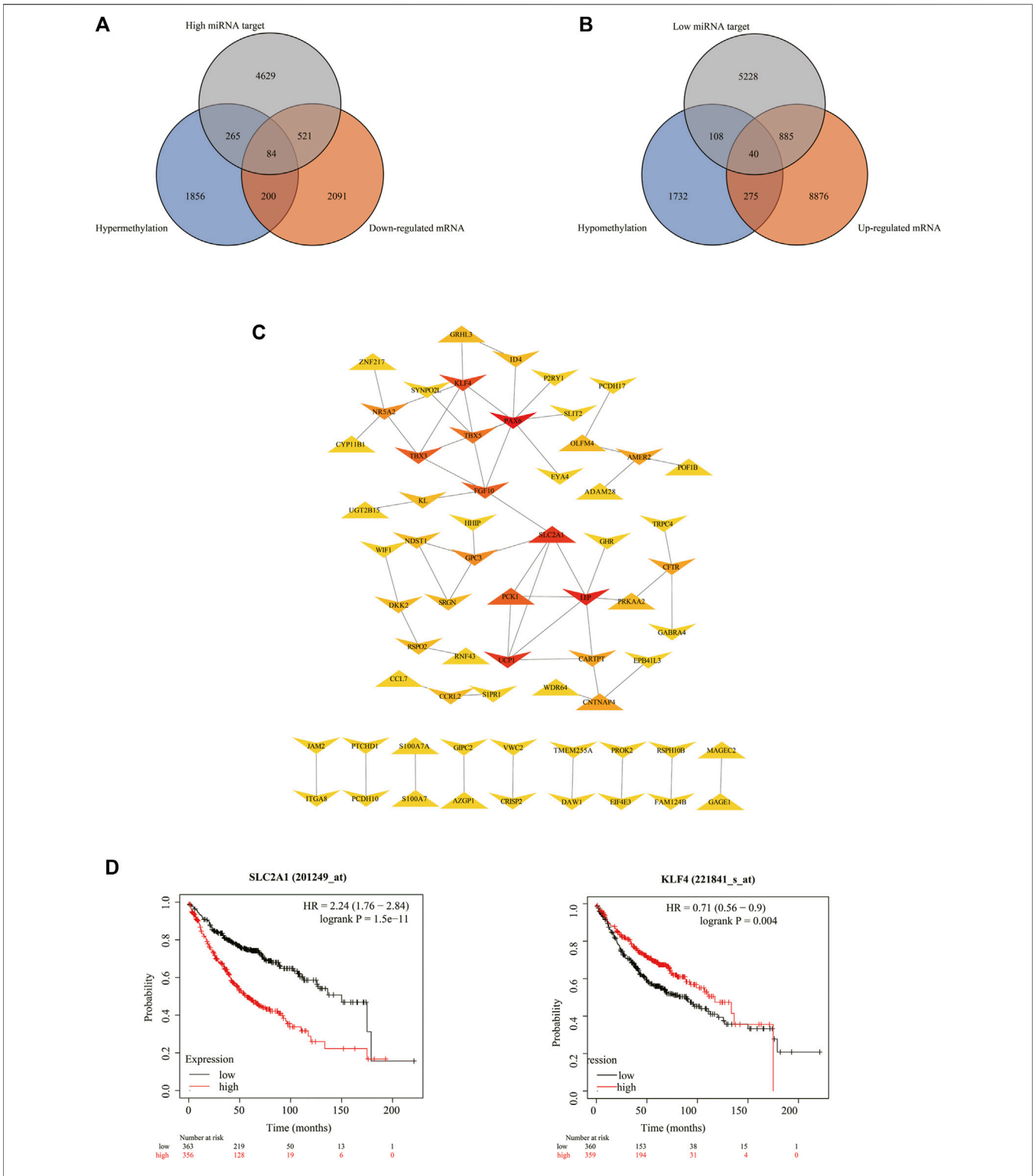
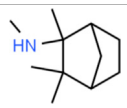
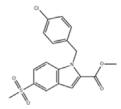
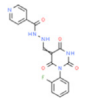
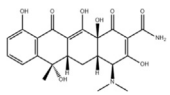
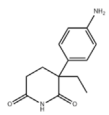


FIGURE 6 | Details for all the overlapped genes. **(A,B)** Venn graph for all the overlapped genes including 84 downregulated genes and 40 upregulated genes, respectively. **(C)** PPI network of all the overlapped genes including 84 downregulated genes and 40 upregulated genes. **(D)** Kaplan–Meier analysis of patients with low (black curve) and high (red curve) expressions of *SLC2A1* and *KLF4*.

TABLE1 | Five chemicals were predicted as putative therapeutic agents for LUAD.

CMap name	Chemical formula	Mean	n	Enrichment	*P*
Mecamylamine		−0.817	3	−0.988	0
LM-1685		−0.613	3	−0.909	0.00132
5182598		−0.722	2	−0.963	0.00308
Tetracycline		0.578	5	0.733	0.0031
Aminoglutethimide		0.556	3	0.853	0.00605

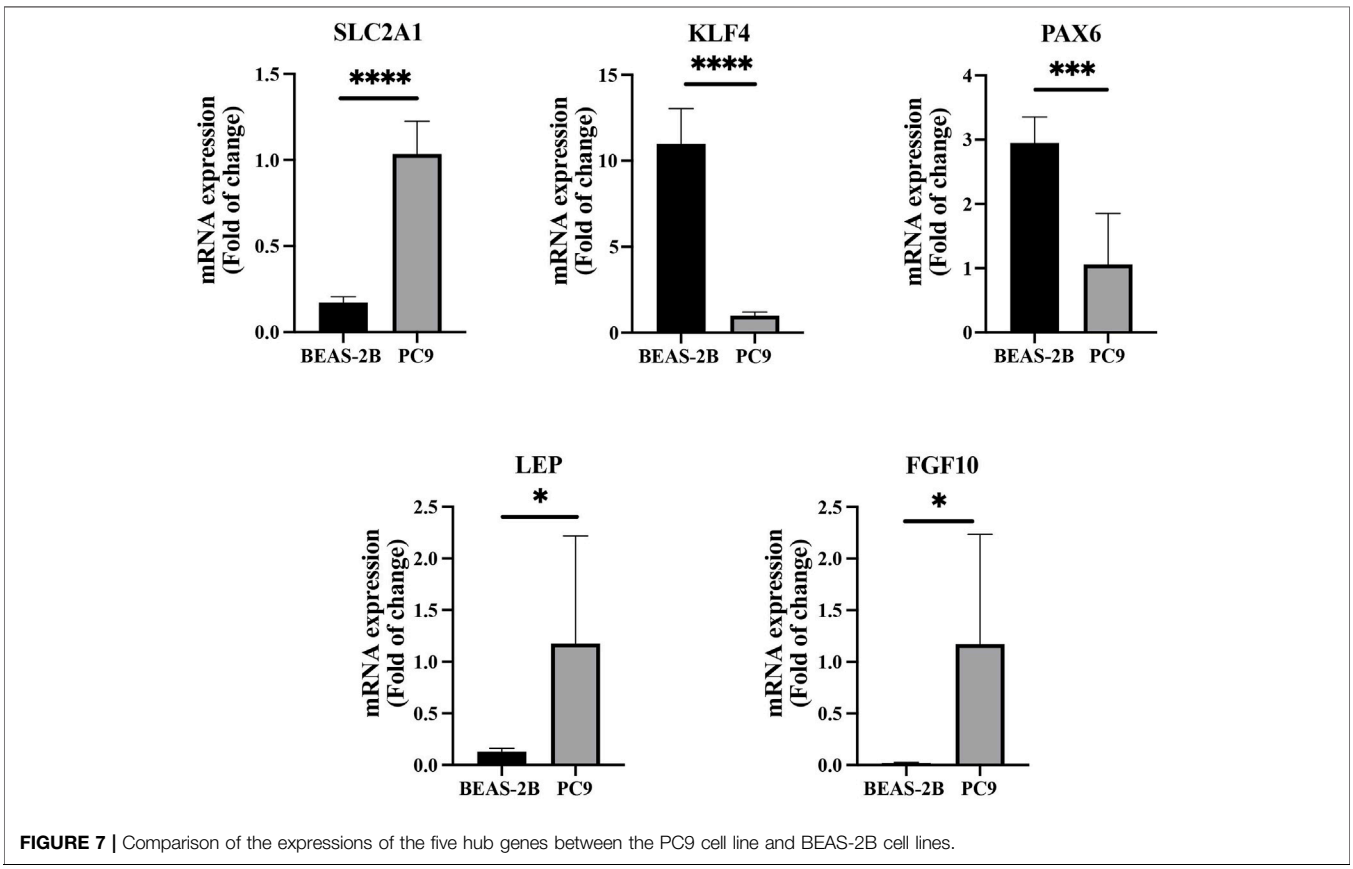


FIGURE 7 | Comparison of the expressions of the five hub genes between the PC9 cell line and BEAS-2B cell lines.

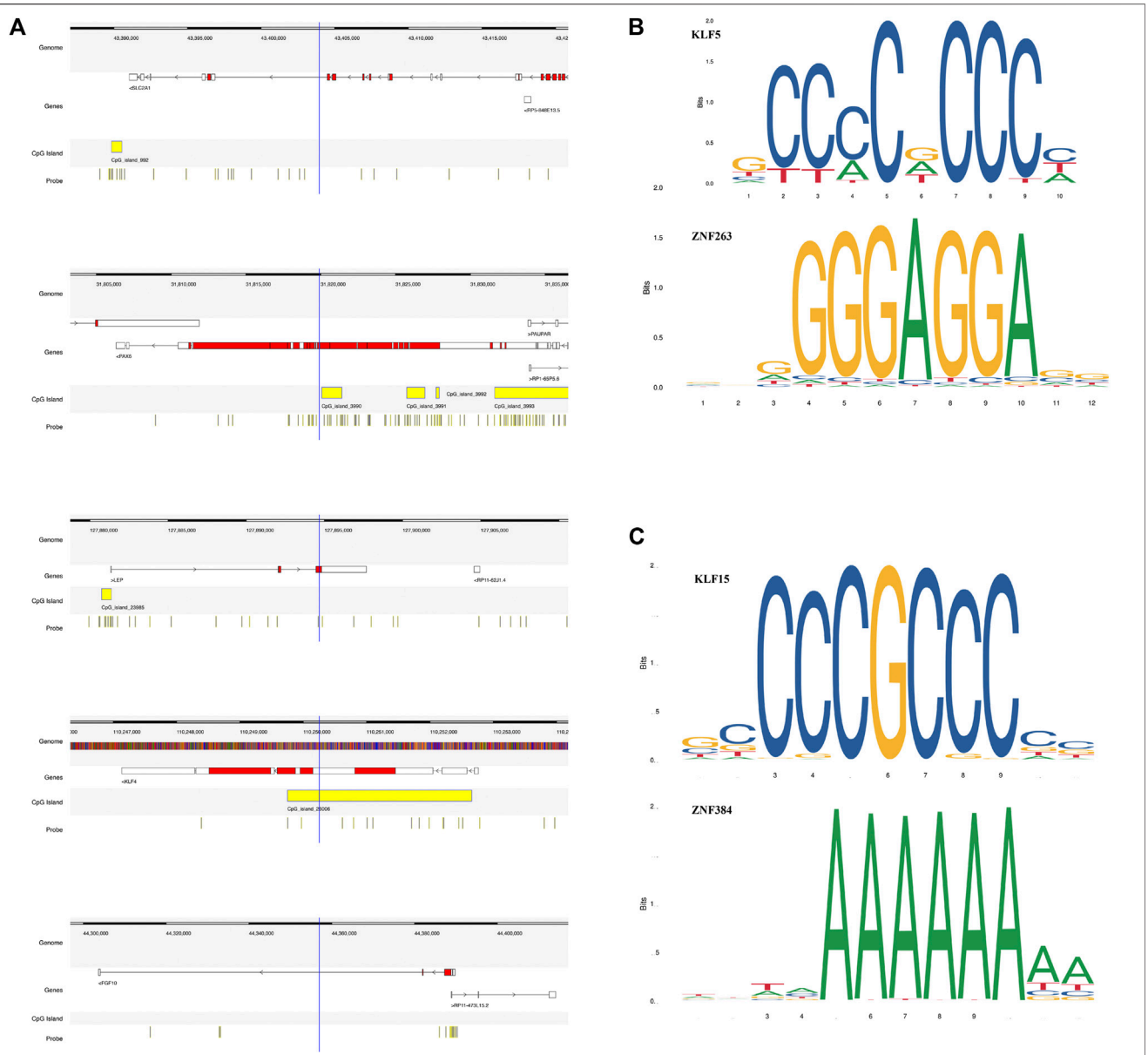


FIGURE 8 | Details of the five screened genes. **(A)** Epigenetic regulatory patterns of both miRNA and DNA methylation-regulating gene expression, as well as CpG island prediction analysis of five screened genes. **(B)** The JASPER database predicted the sequence of two possible transcription factors of *SLC2A1*. **(C)** The JASPER database predicted the sequence of two possible transcription factors of *KLF4*.

miRNA expression can be regarded as impactful biomarkers to distinguish LUAD from normal samples (Ren et al., 2019; Sherafatian and Arjmand, 2019), which would be helpful in diagnosis, assessment of treatment, and prediction of prognosis (Ye et al., 2021). In this present study, data on DNA methylation microarrays, miRNA expression microarrays, and mRNA expression profiling microarrays (the aforementioned data are all obtained from TCGA database) were methodically analyzed, which compare the differential profiling between cancer and adjacent samples from LUAD patients. Hub genes and core pathways have been enriched to screen pivotal events in epigenetic alteration regulated by DNA methylation and miRNA.

A total of 607 high miRNA-targeted downregulated genes were identified through overlapping targets of DEMs and DEGs. The GO analysis showed that these 607 genes are primarily enriched in the cellular component in LUAD, reminding us of the potential regulation of membrane-related metabolism in LUAD. Furthermore, for molecular function, these genes were significantly enriched in protein binding, which indicated an interaction of any protein or protein complex in LUAD. As for KEGG pathway analysis, the target genes were most

enriched in pathways in cancer, which suggested that these genes may participate in the tumorigenesis in LUAD. Previous research has indicated that hsa-miR-1269a had the most target genes among the 13 high-expression miRNAs, including *NEGR1*, *ITGA8*, *CLDN18*, *JAM2*, and *JAM3*, associated with cell adhesion. Cell adhesion molecules are a type of membrane surface glycoprotein molecules involved in regulating inflammatory response and promoting the metastasis of LUAD (Liu et al., 2021). Loss of cell adhesion is one of the characteristics of epithelial-to-mesenchymal transition (EMT), and the low expression of adhesion molecules is associated with distant metastasis in LUAD (Kim et al., 2013).

A total of 925 low miRNA targeted upregulated genes by overlapping targets of DEMs and DEGs were finally exhibited. The GO term analysis indicated that the upregulated genes were primarily enriched in integral components of membrane, positive regulation of transcription from RNA polymerase II promoter, and calcium ion binding, which indicated a regulatory role in RNA translation and transcription. The previous study has shown that the activation of Ca2+ in cells may be related to tumorigenicity and metastasis in LUAD (Li et al., 2018). KEGG analysis revealed pathways including the neuroactive ligand–receptor interaction, PI3K-Akt signaling pathway, focal adhesion, and protein digestion and absorption. GABA receptors are regulated by neuroactive steroids and are considered to control cell proliferation (Watanabe et al., 2006). A PI3K-Akt signaling pathway is a key signal medium that activates EMT-induced transcription factors (Karimi Roshan et al., 2019). According to the research, hsa-let-7c-5p upregulated 236 genes, including *COL1A1*, *COL24A1*, *LAMA1*, *ITGA2*, and other genes, and mainly enriched in focal adhesion is associated with EMT (Wu et al., 2021).

Until now, 284 hypermethylation and low expression genes were obtained *via* overlapping strategies of DMGs and DEGs. KEGG pathway analysis showed that hypermethylation-induced disorder of Neuroactive ligand-receptor interaction and Pathways in cancer might cause LUAD. The PPI network of hypermethylation and low-expression genes shows their functional connections; not only the top 10 hub genes among them but also five genes related to prognosis, such as *WNT3*, *SOX17*, *GATA2*, *GATA6*, and *KLF4*, were also selected. Interestingly, eight out of the top 10 hub genes were enriched in the biological process of positive regulation of transcription from the RNA polymerase II promoter.

As for 315 low-methylation and high-expression genes, overlapping hypomethylation and upregulation in LUAD, GO, and KEGG pathway analysis showed enrichment in the innate immune response and cytosolic DNA-sensing pathway. A series of studies indicated that the cytosolic DNA-sensing pathway was associated with antitumor immunity (Amouzegar et al., 2021; Verrier and Langevin, 2021). Therefore, hypomethylation-induced aberrance of high-expression genes may affect the antitumor immunity and promote the progression of LUAD. *GAPDH*, *SPRR1B*, *SPRR1A*, *SPRR3*, and *S100A7* associated with the prognosis of LUAD are obtained through the PPI network. GAPDH is indeed the internal reference used in PCR and Western blot analysis, but GAPDH has also been shown to be dysregulated in the lung, kidney, breast, stomach, glioma, liver, colorectal, melanoma, prostate, pancreatic, and bladder cancers, and GAPDH is generally upregulated in many types of cancer. GAPDH could be utilized as a reference gene for normalizing lung cell lines, while it was de-regulated in non–small cell lung cancer specimens (Schmidt et al., 2005; Nguewa et al., 2008). The de-regulation of GAPDH in tumor tissues or cells demonstrated that the utilization of GAPDH as a reference gene/protein should be chosen very carefully (Guo et al., 2013). In normal tissues, small proline-rich proteins (SPRRs) are involved in the structural integrity of the cornified cell envelope (Patel et al., 2003), and the upregulation of SPRRs is also common under various pathophysiological conditions. Compelling evidence shows that SPRR downregulates p53 and promotes EMT (Demetris et al., 2008; Mizuguchi et al., 2012). A large number of studies have confirmed that SPRR is related to the progression of a variety of tumors (Carregaro et al., 2013). SPRR1 B activates the MAPK signaling pathway involved in LUAD proliferation and metastasis (Zhang Z. et al., 2021), but the influence of other genes of the SPRR family on the progression of LUAD is poorly understood, and further experimental elucidation is needed.

Interestingly, the abnormal expression of DEGs may be regulated by combining the epigenetic alterations of DNA methylation and miRNA. Forty genes such as *SUGCT*, *RNF43*, and *UGT2B15* were upregulated due to the regulation of both decreased DNA methylation and miRNAs, while under the modulation of both increased DNA methylation and miRNAs, 84 genes including *FAT4*, *KLF4*, and *EPB41L3* were downregulated. GO analysis for 40 low miRNA-targeting high-expression hypomethylation genes identified enrichment in cell adhesion, glucose homeostasis, and cellular response to interleukin-1. The three upregulated genes (*CCL7*, *ADAMTS12*, and *PCK1*) are involved in the cellular response to tumor necrosis factor. Moreover, the aforementioned genes are also involved in the glucagon signaling pathway, insulin resistance, bile secretion, and adipocytokine signaling pathway. For 84 high miRNA-targeting and low-expression hypermethylation genes, GO analysis screened the most significantly enriched CC, BP, and MF are integral components of membrane, positive regulation of transcription from the RNA polymerase II promoter, and sequence-specific DNA binding, respectively. Two significant results, neuroactive ligand–receptor interaction and signaling pathways regulating pluripotency of stem cells, were retrieved from the KEGG pathway analysis. Three of the five hub genes (*PAX6*, *LEP*, and *KLF4*) are involved in the aforementioned two pathways; the low expression of KLF4 is associated with poor prognosis. In addition, qRT-PCR was performed to verify the differential expressions of the five hub genes in LUAD. The mRNA expression level of SLC2A1 in the PC9 cell line was observed to be significantly higher than that in the BEAS-2B cell line, and the expression level of mRNA of KLF4 in the PC9 cell line was significantly lower than that in the BEAS-2B cell line. The results of qRT-PCR are consistent with those of bioinformatics analysis, meaning that SLC2A1 may be an oncogene in LUAD, while KLF4 may be a tumor suppressor gene.

Although chemotherapy, targeted therapy, and immunotherapy have brought hope to LUAD patients, drug

resistance is still inevitable. It is urgent to find new therapeutic targets, explore new drugs, or reuse existing drugs; online databases can help us predict drugs. By far, the effectiveness of the CMap database has been confirmed by a large number of studies due to its practical value in drug prediction (Aramadhaka et al., 2013; Wang et al., 2016). From the CMap database, five compounds, including mecamylamine, LM-1685, 5182598, tetracycline, and aminoglutethimide, may have significant therapeutic effects on LUAD. Mecamylamine is a nicotinic acetylcholine receptor (nAChR) antagonist; research by Zhu et al. (2003) showed that mecamylamine could reverse the increase in VEGF and circulating endothelial progenitor cells (EPC) caused by secondhand smoke, thereby inhibiting tumor growth and angiogenesis. LM-1685 is a kind of selective COX-2 inhibitor, which induces cancer cell apoptosis and cell cycle arrest and inhibits tumor angiogenesis (Wu et al., 2004; Grosch et al., 2006; Liggett et al., 2014). It was reported that the selective COX-2 inhibitor might enhance the effect of conventional antitumor treatments by intensifying the sensitivity of lung cancer cells to NK cell-mediated cytotoxicity (Kim et al., 2020). It is observed that 5182598 has been reported to be an effective anti-tumor drug from the group of benzylisoquinoline alkaloids (Cordell et al., 2001).

Our research still has some shortcomings. The drug prediction results from the CMap database require a large number of rigorous clinical trials to corroborate their availability in the treatment of LUAD. In addition, the effects of both abnormal DNA methylation and miRNA expression on gene expression also need to be verified by corresponding experiments.

This study indicated that a cavalcade of abnormal methylated differentially expressed genes is related to the epigenetic changes of DNA methylation and miRNAs in LUAD. In total, 607 high miRNA-targeting downregulated genes and 925 low miRNA-targeting upregulated genes were identified by overlapping targets of DEMs and DEGs, which were enriched in the pathways in cancer and the PI3K-Akt signaling pathway, respectively. Furthermore, 284 hypermethylated downregulated genes and 315 hypomethylated upregulated genes obtained by overlapping DMGs and DEGs were associated with the neuroactive ligand–receptor interaction and cytosolic DNA-sensing pathway. Interestingly, 40 genes were upregulated under the co-regulation of hypomethylation and decreased miRNA, while 84 were downregulated under the co-regulation of hypermethylation and increased miRNA. Five small-molecule drugs were identified as potential therapeutic agents for LUAD. Finally, from these genes, *SLC2A1*, *PAX6*, *LEP*, *KLF4*, and *FGF10* were identified as hub genes, especially *SLC2A1* and *KLF4*, which were related to the prognosis of LUAD, and might be used as biomarkers for the precise diagnosis and treatment of LUAD.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: TCGA.

AUTHOR CONTRIBUTIONS

WC was responsible for the statistical analysis and wrote the manuscript. MJ and HG contributed to the review and revision of the manuscript. JW and ZX were accountable for the design. WH and HG performed the experiments. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.817552/full#supplementary-material

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