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Community series in post-translational modifications of proteins in cancer immunity and immunotherapy, volume II

Edited by
Xiangpeng Dai, Zichuan Liu and Naoe Taira Nihira

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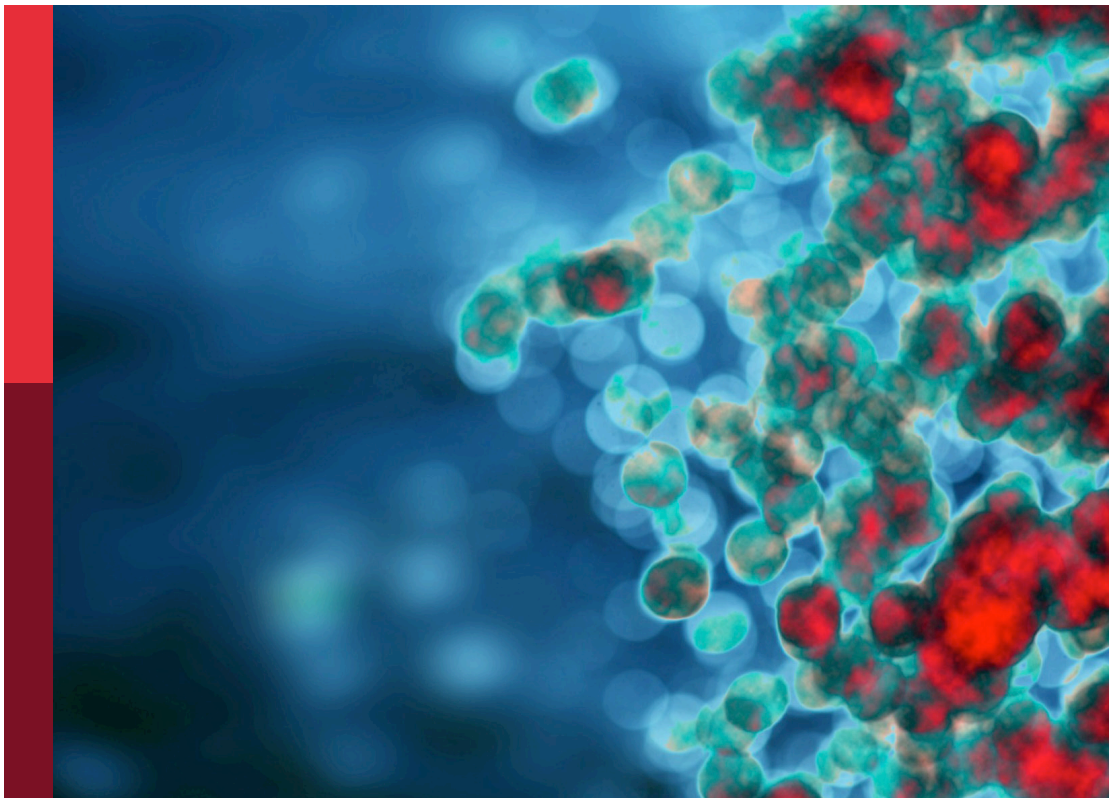

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Community series in post-translational modifications of proteins in cancer immunity and immunotherapy, volume II

Topic editors

Xiangpeng Dai — Jilin University, China

Zichuan Liu — Tianjin University, China

Naoe Taira Nihira — St. Marianna University Graduate School of Medicine, Japan

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EDITED AND REVIEWED BY
Paola Cappello,
University of Turin, Italy

*CORRESPONDENCE
Zichuan Liu
zichuan.liu@tju.edu.cn
Xiangpeng Dai
daixiangpeng@jlu.edu.cn

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Editorial: Community series in post-translational modifications of proteins in cancer immunity and immunotherapy, volume II

Naoe Taira Nihira[1], Tomohiko Ohta[1], Zichuan Liu[2*] and Xiangpeng Dai[3,4*]

[1]Department of Translational Oncology, St. Marianna University Graduate School of Medicine, Kawasaki, Japan, [2]School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, China, [3]Key Laboratory of Organ Regeneration and Transplantation of Ministry of Education, First Hospital of Jilin University, Changchun, China, [4]National-Local Joint Engineering Laboratory of Animal Models for Human Disease, First Hospital of Jilin University, Changchun, China

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Editorial on the Research Topic
Community series in post-translational modifications of proteins in cancer immunity and immunotherapy, volume II

The emergence of immune checkpoint inhibitors (ICIs) allows cancer immunotherapy to move to the frontiers of cancer therapy. ICIs targeting CTLA-4, PD-1, and PD-L1 are applied to patients with advanced malignancies and prolong their survival. Among the ICIs, therapeutic antibodies against the PD-1/PD-L1 improve the clinical outcome, supporting the evidence that PD-1/PD-L1 axis strongly contributes to anti-tumor immunity. Nevertheless, it is also reported that some cancer patients exhibit limited response rates and resistance to ICIs, therefore restricting the clinical application of ICIs. To overcome this issue, elucidating molecular mechanisms underlying cancer immunity and optimizing the combination therapy of ICIs with other therapeutic strategies using molecular target drugs would be required. Especially, the enzymes that catalyze post-translational modifications (PTMs) would be promising molecular targets for immunotherapy or combined therapy. During the past few years, immune checkpoint molecules, including TIM-3, LAG-3, and TIGIT, have been newly identified. However, most papers listed in this Research Topic focused on PTMs of PD-L1. Therefore, we summarized the current pieces of evidences on PTMs of PD-L1 in this editorial.

PTMs in regulating the PD-L1 expression

The PD-L1 stability was regulated by many E3 ligases and deubiquitinases, since PD-L1 expression is tightly regulated in a context-dependent manner. To date, the E3 ubiquitin ligases β-TRCP, SPOP, STUB1, HRD1, DCUN1D, NEDD4, RNF144A, c-Cbl, and ARIH1 were reported to regulate PD-L1 expression. Contrary, it is reported that PD-L1 can be deubiquitinated by CSN5, USP22, USP7, USP9X, and OTUB1 (Feng et al.).

Cell cycle

PD-L1 expression fluctuates during the cell cycle; it increases in the M and early G1 phases and rapidly decreases in the late G1 and S phases. SPOP promotes the rapid reduction by the poly-ubiquitination of PD-L1. Since Cyclin D/CDK4 enhances the PD-L1 ubiquitination, CDK4/6 inhibitors, Palbociclib and Ribociclib, increase PD-L1 expression and enhance the efficacy of anti-PD-1 immunotherapy *in vivo* (1).

Inflammatory stimulation

Upon TNFα stimulation, NF-κB up-regulates expression of deubiquitinase CSN5, resulting in increased PD-L1 expression without affecting the transcription level of PD-L1 (2). Curcumin, a CSN5 inhibitor, can be used with anti-CTLA4 antibody together to decrease PD-L1 level and inhibit PD-L1 to enhance the efficacy of immunotherapy (2). Therefore, manipulation of PD-L1 stability could be promising strategy for increased efficacy of combination treatment with immunotherapy.

Cancer metabolisms

Dysregulation of cellular metabolism is one of the hallmarks of cancer progression, which was defined by Hanahan and Weinberg in 2011. Two independent studies demonstrated that energy metabolism regulates PD-L1 status at both transcriptional and post-translational levels. Dai et al. demonstrated that energy deprivation activates AMPK kinase, which subsequently phosphorylates PD-L1 at Ser283, resulting in PD-L1 degradation (3). CMTM4 and CMTM6 are critical regulators for lysosomal degradation of PD-L1. Mechanistically, phospho-mimic PD-L1 S283D mutation or treatment with AMPK-specific agonist A-769662 markedly attenuated the interaction between PD-L1 and CMTM4, and the dissociation triggers lysosomal degradation. Simultaneously, AMPK induces the expression of IFN- and antigen presentation-related genes through phosphorylation of EZH2 (3) (Figure 1). Furthermore, the mouse tumor model showed that ketogenic diet- or A-769662-induced PD-L1 degradation enhanced the CTLA-4 immune checkpoint blockade efficacy (3).

On the other hand, Lin et al. showed that high glucose condition increases expression of PD-L1 depending on NF-κB activation. Glycolytic enzyme hexokinase 2 (HK2) activation induced by high concentration of glucose phosphorylates IκBα at Thr291 and triggers its degradation, and then NF-κB translocates into the nucleus to upregulate PD-L1 transcription (Figure 1). In cancer cells, glucose uptake and the production of lactate are dramatically increased due to the expression of glucose transporters (GLUTs) and activation of glucose-responsible kinases. This characteristic might increase *PD-L1* expression and trigger immune surveillance in tumors.

PTMs regulating the cellular distribution of PD-L1

Not only the expression control, the subcellular localization of PD-L1 is also tightly regulated by PTMs. After translation in the endoplasmic reticulum, translocation of newly synthesized PD-L1 to the plasma membrane from Golgi is regulated by K63-linked ubiquitination by mind bomb homolog 2 (MIB2) (4). Yu et al. screened the E3 ubiquitin ligases using shRNA library and found that knockingdown of *MIB2* reduces PD-L1 expression on the plasma membrane without affecting the total amount of PD-L1

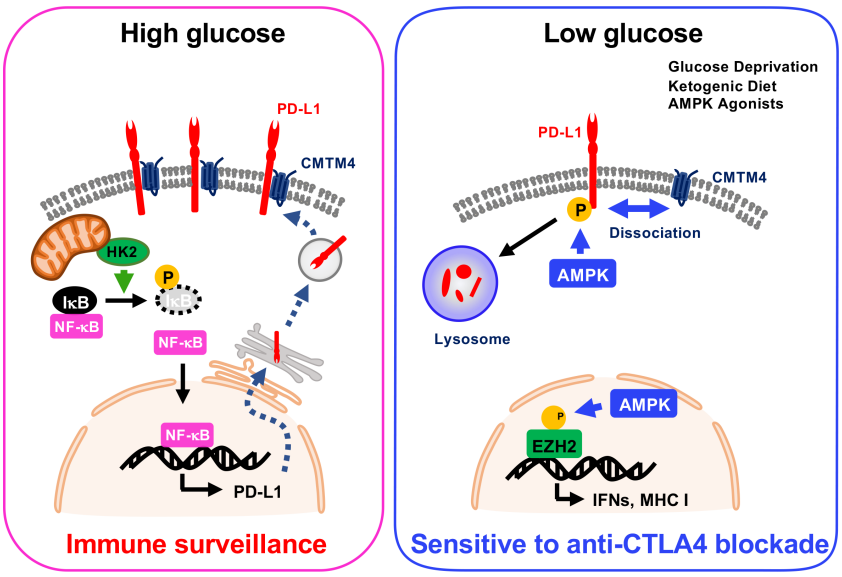

FIGURE 1
PTMs in the regulation of PD-L1 expression in cancer metabolism.

(4). K63-linked poly-ubiquitination of PD-L1 at Lys136 in trans-Golgi network triggers RAB-8-mediated exocytosis, and then expresses on the plasma membrane (4).

On the other hand, several research groups reported that small portions of PD-L1 function in the cytoplasm and nucleus. Notably, Lys263 residue within PD-L1 is constitutively acetylated by acetyltransferase p300 (5). HDAC2-mediated PD-L1 deacetylation enables it to bind with regulators of sub-cellular localization, including Adaptin β2, Clathrin heavy chain, Vimentin, and Importin α1. RNA sequence analysis reveals that the expression of immune response-, NF-κB signaling-, type I IFN-, and type II IFN-related genes are induced by nuclear PD-L1 (5). It was reported that inhibition of nuclear translocation of PD-L1 by HDAC2 inhibitor increases the efficacy of anti-PD-1immunotherapy in mouse tumor models (5). To date, numerous HDAC inhibitors have developed and exhibited improved outcomes for cancer patients (Lian et al.). Among HDAC inhibitors, a class I HDAC inhibitor, Romidepsin, in combination with PD-1 blockade increases the efficacy of immunotherapy in lung adenocarcinoma. In addition, Entinostat is also implied in the combination treatment with ICIs (Lian et al.).

Novel PTM regulating the tumor immunity

Aerobic glycolysis is also an important feature of cancer metabolism, which is well-known as the Warburg effect. Production of aerobic glycolysis, lactate, promotes the proliferation of tumor-related immune cells to build an immunosuppressive tumor microenvironment (TME). It is newly reported that protein lactylation is involved in the regulation of gene expression, which is considered the other function of lactate. Histone lactylation on Lys residues functions as an epigenetic hallmark during macrophage polarization (6). Thus, protein lactylation might be an essential biological event that links cancer metabolism to tumor immunity. Yang et al. compared the differentially expressed genes in tumor versus normal tissue and identified 11 lactylation-related genes. Among the 11 candidate genes, *HIBCH* expression is negatively correlated with tumor grade and TME score. Furthermore, the expression of *CTLA-4* and *PD-1* is also negatively linked with *HIBCH* expression, suggesting that HIBCH would be a potential biomarker to speculate ICI response.

Conclusion

This Research Topic provides an overview of PTMs of PD-L1 and the combination therapy with ICIs. Further research about PTMs of PD-L1 must expand the choice of drugs that can be combined with ICIs.

Author contributions

NN: Writing – original draft, Writing – review & editing. TO: Writing – original draft, Writing – review & editing. ZL: Writing – original draft, Writing – review & editing. XD: Writing – original draft, Writing – review & editing.

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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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EDITED BY
Xiangpeng Dai,
Jilin University, China

REVIEWED BY
Xueqiong Zhu,
Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, China
Lixia Wang,
Soochow University, China

*CORRESPONDENCE
Xiaosong Chen
chenxiaosong0156@hotmail.com
Kunwei Shen
kwshen@medmail.com.cn

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Inhibition of histone deacetylases attenuates tumor progression and improves immunotherapy in breast cancer

Bi Lian, Xiaosong Chen* and Kunwei Shen*

Department of General Surgery, Comprehensive Breast Health Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Breast cancer is one of the common malignancies with poor prognosis worldwide. The treatment of breast cancer patients includes surgery, radiation, hormone therapy, chemotherapy, targeted drug therapy and immunotherapy. In recent years, immunotherapy has potentiated the survival of certain breast cancer patients; however, primary resistance or acquired resistance attenuate the therapeutic outcomes. Histone acetyltransferases induce histone acetylation on lysine residues, which can be reversed by histone deacetylases (HDACs). Dysregulation of HDACs *via* mutation and abnormal expression contributes to tumorigenesis and tumor progression. Numerous HDAC inhibitors have been developed and exhibited the potent anti-tumor activity in a variety of cancers, including breast cancer. HDAC inhibitors ameliorated immunotherapeutic efficacy in cancer patients. In this review, we discuss the anti-tumor activity of HDAC inhibitors in breast cancer, including dacinostat, belinostat, abexinostat, mocetinotat, panobinostat, romidepsin, entinostat, vorinostat, pracinostat, tubastatin A, trichostatin A, and tucidinostat. Moreover, we uncover the mechanisms of HDAC inhibitors in improving immunotherapy in breast cancer. Furthermore, we highlight that HDAC inhibitors might be potent agents to potentiate immunotherapy in breast cancer.

KEYWORDS

HDAC, inhibitors, breast cancer, immunotherapy, targets

Introduction

Breast cancer is one of the common tumors worldwide. Approximately 2.3 million new breast cancer cases were estimated in 2020 in the 185 countries (1). It has been estimated that there are 297,790 new cases of breast cancer and 59,910 deaths due to this deadly disease in the United States (2). Approximately 11%-20% of breast cancer patients are triple negative breast cancer (TNBC) due to lack of expression of HER2, ER and PR (3). TNBC patients often have aggressive behavior, metastasis and poor prognosis (4). For the treatment of local breast cancer, there are surgery and radiation, while the systemic

therapies of breast cancer include chemotherapy, hormone therapy, targeted drug therapy and immunotherapy (5, 6). Histone acetyltransferases can lead to histone acetylation on lysine residues, which can be reversed by histone deacetylases (HDACs) (7, 8). It has been known that HDACs function on remodeling of chromatin and modulation of gene expression by specific epigenetic regulation (9). There are 18 HDACs that have been characterized to regulate various biological processes, which are classified into four groups (I-IV). Class I includes HDAC1, HDAC2, HDAC3 and HDAC8, which are related to RPD3 gene. Class II includes HDAC4, HDAC5, HDAC6, HDAC-7, HDAC9 and HDAC10. Class III includes sirtulin 1-7 and class IV includes HDAC11 (10–12). Dysregulation of HDACs *via* mutation and abnormal expression contributes to oncogenesis and tumor progression (10–12). Therefore, modulation of HDACs could be a potent strategy for cancer treatment.

Role of HDAC in immunotherapy

Immunotherapy has emerged for fighting cancer *via* using the patient's own immune system (13). Immunotherapy includes monoclonal antibodies, chimeric antigen receptor (CAR) T-cell therapy, CAR NK cell therapy, tumor infiltrating lymphocyte (TIL) therapy, endogenous T cell (ETC) therapy, immune checkpoint inhibitors (ICIs), cancer vaccines, cytokines and immunomodulators (14–17). It has been known that ICIs block immune checkpoints, which allow immune cells to respond to tumor. Inhibitory immune checkpoint molecules include programmed cell death ligand (PD-1), programmed death ligand (PD-L1), PD-L2, B7-H3 (CD276), B7-H4 (VTCN1), LAG3, TIM-3, and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (18, 19). Although immunotherapy has improved the survival of certain cancer patients, primary resistance and acquired resistance in immunotherapy attenuate the cancer treatment outcomes (20, 21). Hence, it is pivotal to uncover the mechanism of immunotherapy resistance and to develop the compounds that improve immunotherapy.

Several HDAC inhibitors have been developed and exhibited the potent anti-tumor activity in a various cancer types, including inhibition of tumor growth, metastasis and drug resistance (22–24). For instance, abexinostat, givinostat and mocetinostat decreased the expression of Slug and increased the expression of E-cadherin in mammary tumor cells (25). Breast epithelial cells with E-cadherin depletion were sensitive to several HDAC inhibitors, including entinostat, vorinostat, pracinostat, and mocetinostat, due to inhibition of proliferation and upregulation of cell apoptosis (26). Here, we discuss the function of HDAC inhibitors in tumorigenesis, especially in improving immunotherapy in breast cancer.

Vorinostat

Vorinostat, also known as SAHA (suberoylanilide hydroxamic acid), is an oral inhibitor of class I and II of HDACs, which was the first time to approve for clinical application in patients with cutaneous T-cell lymphoma in 2006 (27–29). Vorinostat has been determined by preclinical experiments and clinical trials to decide its therapeutic efficacy in combination with other antitumor drugs in breast cancer (30). Vorinostat plus CDK inhibitor flavopiridol treatments exhibited synergistic lethality in breast cancer cells *via* suppression of ERK1/2 and AKT pathways and regulation of apoptosis pathways (31). Using breast cancer brain metastatic cells and intracranial xenograft model, radio-sensitivity was increased by vorinostat (32). Vorinostat accelerated radio-sensitivity of breast tumor cells, leading to suppression of lung metastasis *via* inhibition of MMP-9, DNA repair proteins and modulation of autophagy and endoplasmic reticulum stress (33).

TRAIL-resistant breast cancer cells became more sensitive after vorinostat treatment in BALB/c nude mice because vorinostat inhibited the expression of NF-κB, cyclin D1, Bcl-2, Bcl-xL, VEGF, MMP-2, MMP-9, HIF-1α, IL-6, IL-8, increased the expression of DR4, DR5, p21, PUMA, TIMP-1, TIMP-2, Bax, Bak, Bim and Noxa (34). It has been reported that vorinostat overcame apoptosis-inducing ligand Apo2L/TRAIL resistance *via* regulation of Bax, DR5, caspase-3, caspase-8, caspase-9 and PARP cleavage in human MDA-MB-231 breast cancer cells (35). Vorinostat increased the sensitivity of olaparib, one PARP inhibitor, in TNBC cells *via* induction of DNA damage, apoptosis and autophagy (36). Vorinostat restrained brain metastasis and stimulated DNA double-strand breaks and induced the downregulation of Rad52 in a TNBC model (37). Vorinostat promoted taxol-mediated cell death and triggered inhibition of cell growth and induced cell cycle arrest at G2/M phase in breast cancer (38). Vorinostat in combination with Aurora kinase inhibitor (MK-0457) displayed synergistical inhibition of proliferation of breast cancer cells (39). Vorinostat activated the expression of estrogen receptor α (ERα) and sensitized a ligand of the aryl hydrocarbon receptor, aminoflavone, -mediated growth inhibition in mesenchymal-like TNBC cells, such as MDA-MB-231 and Hs578T cells (40). Co-treatment with vorinostat and simvastatin exhibited synergistic functions on cell proliferation and apoptosis *via* inhibition of Rab7 prenylation in TNBC cells (41). It has been found that tamoxifen sensitivity was enhanced by vorinostat treatment in TNBC cells (42).

Vorinostat in combination with chemotherapeutic agent decitabine increased sensitivity of Fas ligand (FasL)-induced apoptosis and CTL immunotherapy *via* promotion of CD8+ T cells in colon cancer cells (43). Vorinostat increased sensitivity of anti-GD2 monoclonal antibody (mAb) treatment and reduced tumor growth through elevation of macrophage effector cells with high expression of Fc-receptors and reduction of MDSC number in neuroblastoma (44, 45). In pancreatic cancer, vorinostat and sorafenib co-treatment enlarged the efficacy of anti-PD-1 antibody *via* promotion of CD8+ cells, M1 macrophages and NK cells in mice (46). A combination therapy by vorinostat and anti-PD-L1 to abrogate the immune escape has been reported *via* induction of cell apoptosis and G1 phase arrest in melanoma (47). In head and neck and salivary cancer patients with vorinostat plus pembrolizumab treatments, NLR, neutrophils, lymphocytes and T helper cells were associated with poor overall survival (48). The MDA-MB-231 breast carcinoma cells and LNCaP

prostate cancer cells displayed sensitivity to vorinostat therapy *via* enhancement of the immune evasion, leading to promotion of T-cell-induced lysis. HDAC1 was further identified to play a pivotal role in tumor immune escape in breast cancer cells (49). Data from ER-positive breast cancer patients after vorinostat, tamoxifen and pembrolizumab treatments revealed that exhausted T cell signature was linked to immunotherapy response (50). Hence, combination of HDAC inhibitors and immunotherapy could obtain synergistic effects in cancer therapy in breast cancer.

Entinostat

Entinostat, a class I HDACs inhibitor, has been uncovered to attenuate cell proliferation and stimulated cell apoptosis in breast cancer (51, 52). Moreover, entinostat was critically involved in reversal of tumor immune escape in breast cancer (51). One study revealed that entinostat promoted lapatinib efficacy *via* inhibition of AKT phosphorylation, activation of FOXO3 transcription, leading to elevation of Bim1 expression in breast cancer cells with HER2 overexpression (53). Entinostat can attenuate the resistance of trastuzumab/lapatinib-resistant breast cancer cells with HER2 overexpression to the trastuzumab/lapatinib treatment (53). Entinostat plus MEK inhibitor pimasertib retarded cell growth in TNBC cells and inflammatory breast cancer (IBC) cells, and reduced tumor growth in mice *via* regulation of NOXA-participated MCL1 degradation (54).

One study used microarray analysis and revealed that doxorubicin and entinostat regulated numerous gene expressions related to differentiation, inflammation and proliferation. Entinostat sensitized doxorubicin-mediated cell cycle arrest at G2 phase (55). Doxorubicin and entinostat inhibited the expression of E2F and Myc genes, elevated interferon genes and increased the numbers of tumor-infiltrating lymphocytes. Moreover, entinostat and doxorubicin enhanced the expression of tumor testis antigens, such as IL13RA2, and elevated the expression of ICOSL and GITRL in MDA-MB-231 cells, which were immune checkpoint agonists (55). PD-L1 expression was increased by entinostat and reduced by doxorubicin treatment. Entinostat, all-trans retinoic acid, and doxorubicin together stimulated cell death and differentiation, leading to regression of tumor growth in mice by a xenograft model of TNBC (55). A combination of entinostat, all-trans retinoic acid, and doxorubicin caused tumor regression *via* targeting tumor-initiating cells in TNBC and modulating the ESE-1 and ELF-3 (56).

Entinostat, a cancer vaccine, and an IL15 agonist N-803 displayed a synergistic effect on tumor growth *via* upregulation of infiltration of CD8+ T cells, promotion of tumor inflammation-related gene expressions, enhancement of T cell responses to antigens, reduction of VISTA expression in 4T1 TNBC murine carcinoma model and MC38-CEA colon mouse model (57). Combined treatments with vaccine, entinostat, ICIs, and chemotherapy had exhibited a potential efficacy in advanced breast cancer (58). The breast cancer cells and prostate tumor cells exhibited sensitivity to entinostat by T-cell-involved lysis (49). Entinostat altered the tumor-related antigens, including PSA, brachyury, CEA and MUC1, and elevated the expression of several proteins that governed tumor immune recognition and antigen processing (49). Entinostat combined with immunotherapy could be a potential strategy for breast cancer therapy.

Romidepsin

Romidepsin (FK228), a class I HDAC inhibitor, has been reported to inhibit the tumor growth in different types of cancers (59, 60). For example, in colon cancer cells, romidepsin attenuated cellular immune functions *via* upregulation of PD-L1 expression by enhancing the acetylation of histones H3 and H4 and modulation of BRD4 (61). Romidepsin accelerated the number of FOXP3+ regulatory T cells, reduced the number of IFN-γ+ CD8+ T cells, and alleviated Th1/Th2 ratio in TME in subcutaneous model and colitis-related cancer mice. Moreover, Romidepsin-mediated tumor suppression was abrogated by anti-PD-1 antibody treatment in colon cancer cells (61). One case report showed that romidepsin might be safe and effective for treatment of anaplastic large cell lymphoma (ALCL), which did not impair cellular immunity to HTLV-1 (62).

Romidepsin increased paclitaxel sensitivity and blocked tumor metastasis in inflammatory breast cancer (63). Specifically, romidepsin impaired tumor emboli and lymphatic vascular structure, and suppressed the expression of VEGF and HIF-1α in inflammatory breast cancer. Moreover, romidepsin induced the expression of acetylated Histone 3 proteins, triggered cell apoptosis and upregulated p21 expression level (63). Recently, romidepsin treatment upregulated the expression of chemokines, stimulated T-cell infiltration, and promoted T-cell-induced tumor regression. A combination of romidepsin and PD-1 blockade elevated T-cell infiltration and increased the efficacy of anti-PD-1 immunotherapy in lung adenocarcinoma (64). One group reported that a triple combination (gemcitabine, romidepsin, cisplatin) accelerated cell death in MDA-MB-231 and MDA-MB-468 cells (65). Moreover, a triple combination treatment using gemcitabine, romidepsin and cisplatin inhibited cell survival and invasion *via* targeting EMT in an ROS-dependent way, leading to suppression of tumor development, recurrence, and metastasis in TNBC (66).

Panobinostat

It has been known that panobinostat (LBH589), a pan-HDAC inhibitor, performs a tumor suppressive function in various cancer types (67, 68). The function of panobinostat has bene verified in breast carcinogenesis and progression. Panobinostat enhanced the acetylation of GRP78 (glucose-regulated protein 78) and increased endoplasmic reticulum stress *via* upregulation of p-eIF2α, CHOP and ATF4, and elevation of BIK, BIM, Bax and BAK expression, acceleration of the caspase-7 activity and UPR in breast cancer cells (69). Panobinostat inhibited proliferation of breast cancer cells *via* modulation of aromatase gene expression, and synergized the anti-

tumor function of letrozole in hormone-dependent breast cancer (70). In addition, panobinostat exposure elevated histone acetylation, induced G2/M cell cycle arrest and alleviated cell proliferation in TNBC cells. Panobinostat increased the expression of E-cadherin and changed the cell morphology in MDA-MB-231 cells (71). Another study showed that panobinostat inhibited the expression of ZEB family (ZEB1 and ZEB2) and led to suppression of tumor metastasis in TNBC (72).

The proliferation of breast cancer cells with aromatase inhibitor resistance was mitigated by panobinostat in part *via* inactivation of NF-κB1 pathway (73). The invasive and migratory ability of breast cancer cells was also repressed by panobinostat *via* induction of E-cadherin and alteration of Slug, MTA3 and Snail (74). Using a claudin-low TNBC PDX model, one group revealed that panobinostat inhibited the mesenchymal phenotype, such as inhibition of collagen expression (75). Panobinostat accelerated the expression of APCL and blocked Wnt/β-catenin pathway *via* promotion of β-catenin degradation in breast cancer, resulting in inactivation of β-catenin targets, including c-Myc, CD44, Cyclin D1 and c-Jun, which contributed to inhibition of tumor growth and metastasis (76). Panobinostat plus rapamycin led to increased efficacy against TNBC on inhibition of proliferation, invasion, migration and induction of apoptosis, which could be due to overproduction of ROS ad activation of endoplasmic reticulum stress in breast cancer (77). Panobinostat inhibits tumor growth by induction of autophagy and accelerated secretory autophagy *via* targeting Vps34/Rab5C pathway in breast cancer (78). Panobinostat has shown the treatment benefits in oncolytic herpes simplex virus in combination with anti-PD-1/PD-L1 therapy in glioma and squamous cell carcinoma (79). The efficacy of panobinostat was spatially correlated with multiple gene expressions, including galectin-3, cleaved caspase-3, PD-L1, neuropilin-1 and calrecticulin in breast cancer, suggesting that panobinostat (80). Without a doubt, the function of panobinostat in altering immunotherapy warrant to further exploration in breast cancer.

Mocetinotat

Mocetinostat, a class I/IV HDAC inhibitor, has been identified to suppress the tumorigenesis and tumor development in a various types of human cancers (81). Mocetinostat increased PD-L1 expression and elevated the expression of antigen presentation genes in NSCLC (82). Mocetinostat interacted with the promoters of a class I HDAC and increased active histone marks, and enhanced IFN-γ activity in governing class II transactivator. In mice, mocetinostat reduced the number of Tregs and MDSCs, but elevated the number of CD8+ population in tumors. Mocetinostat and PD-L1 antibody displayed a synergistic function in mouse lung tumor models (82). Mocetinostat plus the BET inhibitor JQ1 reduced viability of breast cancer cells *via* modulation of cell cycle-associated gene expressions. Mocetinostat and JQ1 cotreatment upregulated the expression of USP17 family members in breast cancer cells, resulting in inactivation of Ras/MAPK pathway and attenuation of cell viability (83).

Fyn-related kinase (FRK) has been known to be repressed in cancer cells due to its promoter CpG methylation (84). Cell migration and invasion were reduced by FRK overexpression *via* inactivation of MAPK, AKT and JAK/STAT pathways and blockade of EMT in breast cancer cells, including inhibition of slug, vimentin, fibronectin, and upregulation of E-cadherin (85). Mocetinostat and entinostat can induce re-expression of FRK at mRNA and protein levels in basal B breast cancer cells, contributing to tumor regression (86). Similarly, mocetinostat exhibited anti-cancer functions in basal-like breast cancer cells with HDAC2 overexpression (87). Moreover, mocetinostat plus azacytidine increased chemotherapeutic sensitivity in mammary mesenchymal tumors *via* targeting EMT process (25). One group used TCGA database and found that mocetinostat and vorinostat exhibited the functional similarity with the FDA-approved drugs for the treatment of HER2-postive breast cancer (88). Mocetinostat combined with capecitabine showed a synergistic effect on suppression of proliferation and induction of apoptosis in 4T1 breast cancer cells *via* targeting Bax, Bcl-2, PI3K/AKT, c-Myc, PTEN, p53, caspase-7, -9, and cleaved PARP (89). It is required to further dissect the function of mocetinostat in improving immunotherapy in breast cancer.

Abexinostat

Abexinostat (PCI-24781, CRA-024781) is a Pan-HDACs mainly targeting HDAC1. It has been reported that abexinostat increased tumor radio-sensitivity in NSCLC (90). PCI-24781 was developed to decrease cell proliferation, differentiation and metastasis *via* influencing calcium influx by activation of RGS2 in breast cancer (91). Abexinostat triggered the differentiation of cancer stem cells in breast cancer with low level of lncRNA Xist expression (92). Moreover, low expression of lncRNA Xist could indicate abexinostat response in breast tumor PDXs and linked to an inhibition of cancer stem cells in breast cancer (92). Interestingly, administration of abexinostat did not change the expression of ESR1, ERα, and ESR1-associated genes in xenograft models (93). This study indicated that it is doubtable to use a combination of abexinostat and hormone therapy for the management of breast cancer patients. Due to unclear role of abexinostat in immune response, it is pivotal to define the function of abexinostat in regulation of immunotherapy of breast cancer patients.

Belinostat

Belinostat (Beleodaq, PXD101) is a HDACi with antineoplastic function in part *via* targeting HDAC6. One study showed that TNBC cells and HER2-enriched breast cancer cells were remarkably sensitive to belinostat and panobinostat treatment. Moreover, belinostat and panobinostat increased doxorubicin sensitivity in TNBC cells (94). Belinostat and SAHA sensitized TNBC cells to the PARP inhibitor olaparib treatment, showing the synergistic inhibition of proliferation of TNBC cells and induction of cell apoptosis (95). Belinostat plus Hsp90 inhibitor 17-AAG displayed a

synergistic effect on suppression of invasion and cell growth in TNBC cells *via* inhibiting the expression of TEAD family proteins and elevating YY1AP1 phosphorylation and MLC1 (modulator of VRAC current 1) (96). Chemotherapeutic drugs led to cancer stem cell (ALDH+/CD44+) abundance in breast cancer, which was abrogated by belinostat exposure (97). One group has demonstrated that belinostat stimulated the expression of CXCL1 in TBNC cells, suggesting that CXCL1 clone evolution could be an indicator for TNBC prognosis (98).

Dacinostat

Dacinostat (LAQ-824) has been observed to tackle cancer chemoresistance in multiple myeloma ad acute myeloid leukemia (99). One study demonstrated that dacinostat and givinostat can restore the activity of cytotoxic T lymphocytes in in pancreatic cancer cells (100). NVP-LAQ824 attenuated tumor growth and angiogenesis and enhanced VEGFR inhibitor PTK787/ZK222584-mediated inhibition of angiogenesis *via* upregulation of p21 and downregulation of angioprotein-2, Tie-2, VEGF, HIF-1α, and survivin (101). Using an orthotopic breast tumor model, NVP-LAQ824 plus PTK787/ZK222584 induced a greater suppression of tumor growth (101). LAQ824 can regulate the expression of miRNAs in SKBR-3 breast cancer cells (102). It has been known that noncoding RNAs, including microRNAs, lncRNAs and circRNAs, are critical in carcinogenesis in a variety of human cancers (103–105). LAQ824 increased 22 miRNA expressions and decreased 5 miRNA expressions in breast cancer cells (102). LAQ824 in combination with 5-Aza-2'-deoxycytidine, known as decitabine, displayed a greater antineoplastic effect on breast cancer cells (106). LAQ824 reduced the expression of ERα, PRβ, c-Myc, cyclin D1 and HDAC6 in breast cancer cells, leading to suppression of cellular proliferation (107). LAQ-824 sensitized drug sensitivity, including taxotere, epothilone B, trastuzumab and gemcitabine, *via* downregulation of HER-2 expression in breast cancer cells (108). LAQ824 was found to work as a sensitizer to immunotherapy with adoptive T-cell transfer in melanoma (109). Further exploration is pivotal to determine the LAQ824-enhanced immunotherapy in cancer patients *via* improving the anticancer function of tumor antigen-specific lymphocytes.

Other HDACs

Pracinostat (SB939) attenuated tumor growth and metastasis *via* blocking the IL6/STAT3 pathway in breast cancer (110). YF479, a HDACi, exhibited antitumor functions in breast cancer, including suppression of growth, metastasis and recurrence (111). NK-HDAC-1 was designed and synthesized for fighting breast cancer, which induced apoptosis and cell cycle arrest *via* upregulation of p21 and inhibition of Cyclin D1 (112). Givinostat (ITF2357) increased cell death and reduced cell proliferation in urothelial carcinoma cells and acute lymphocytic leukemia (113, 114). Givinostat enhanced CTL sensitivity in pancreatic cancer cells (100). In addition, givinostat reduced cancer stemness and reversed transformed phenotype in glioblastoma (115, 116). The function of givinostat is breast tumorigenesis is unclear, which should be explored in the future. Tubastatin A and alisertib reduced the number of pulmonary metastases *via* suppression of HDAC6 and AURKA in breast tumor xenograft models (117). Tubastatin A in combination with palladium nanoparticles triggered cell apoptosis in breast cancer cells (118). MPT0G211, a HDAC6 inhibitor, exhibited an inhibition of tumor metastasis *via* attenuation of HDAC6 activity in breast cancer cells (119).

Trichostatin A (TSA) inhibited the expression of DNMT1 (DNA methyltransferase 1) *via* reduction of DNMT1 mRNA stability in Jurkat T leukemia cells (120). TSA decreased the transcript and protein levels of aromatase CYP19 and phospholipase C gamma-1 (PLC-γ1) in MCF-7 breast cancer cells (121, 122). SK-7041, a HDACi *via* a hybrid of TSA and MS-275, induced cell apoptosis and G2/M arrest in breast cancer cells (123). MAGE-C1 (melanoma-associated antigen-C1) and MAGE-C2 expressions were linked to advanced tumor grade and poor survival in breast cancer patients. TSA treatment increased 5-aza-CdR-induced MAGE-C2 transcription in breast cancer cells, indicating that MAGE-C2 could be a target for cancer immunotherapy (124). Tucidinostat, an inhibitor of HDAC1, HDAC2, HDAC3 and HDAC10, has shown a remarkable anticancer activity and a synergistic ability with immunotherapy (125). Tucidinostat combined with selinexor, an exportin 1 inhibitor, showed a greater antitumor effect on TP53 wild-type breast cancer (126). Breast cancer patients with HR+/HER2- received CDK4/6 inhibitor treatment and then obtained tucidinostat-based therapy, which displayed better clinical outcomes (127). DNMT inhibitor 5-zazcytidine and HDACi butyrate ameliorated the tumorigenicity of CSCs and retarded breast tumor growth (128). We believe more HDAC inhibitors will be developed for potentiating immunotherapy in the future.

Conclusion and perspectives

In conclusion, HDAC inhibitors improve immunotherapy *via* targeting HDACs and their downstream targets in breast cancer (Figure 1). Although HDAC inhibitors might be useful to enhance tumor immunotherapy, several concerns should be mentioned. So far, only five HDAC inhibitors have been approved by FDA for cancer therapy, including vorinostat, belinostat, panobinostat, pracinostat and romidepsin (129). These HDAC inhibitors exhibited clinical advantage in hematological malignancies. It is required to measure the efficacy of HDAC inhibitors in solid tumors (130). Sirtuins inhibitors, such as nicotinamide, sirtinol and splitomicin, have shown their activities in regulation of metabolism, DNA repair, proliferation, drug resistance and immunotherapy (131). Due to limited space, we do not discuss the role of sirtuins inhibitors in modulation of breast cancer immunotherapy. Among dozens of HDAC inhibitors, which one is the best choice for enhancement of immunotherapy in breast cancer? The development of inhibitors based on the differential expression of HDAC isoforms is pivotal to rationally develop selective and effective inhibitors for personalized-medicine

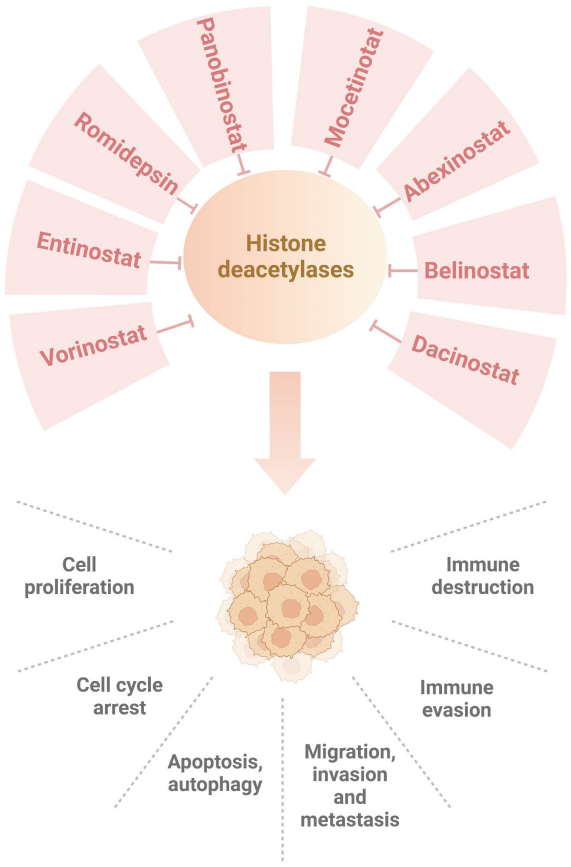

FIGURE 1
Numerous HDAC inhibitors suppress histone deacetylases in breast cancer. Dacinostat, belinostat, abexinostat, mocetinotat, panobinostat, romidepsin, entinostat and vorinostat, inhibit histone deacetylases and regulate breast tumorigenesis, progression and immunotherapy.

treatment (132, 133). Notably, HDAC inhibitors also have adverse side effects and cause drug resistance, which should be overcome. The resistant reasons of HDAC inhibitors are still incomplete. This might be due to cancer cell types, tumor-specific mutations, tumor microenvironmental conditions, upregulation of efflux pumps (P-glycoprotein), overexpression of HDAC enzymes. Lastly, triple combination of HDACi, immunotherapy and other inhibitors could be a promising approach for the treatment of breast cancer.

Author contributions

BL wrote the manuscript and made the figure. XC and KS edited and revised the manuscript.. All authors contributed to the article and approved the submitted version.

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.

Publisher's note

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Glossary

ATF	activating transcription factor
BET	bromodomain and extra C-terminal
Bim1	a BH3 domain-containing pro-apoptotic protein
CDK	cyclin-dependent kinase
CHOP	CAAT/enhancer binding protein homologous protein
eIF2	eukaryotic translation initiation factor
ER	estrogen receptor
ERK1/2	extracellular signal-regulated kinase 1/2
HER2	human epidermal growth factor receptor 2
HIF-1	hypoxia-inducible factor-1
HLA	human leukocyte antigen
HTLV-1	human T-lymphotropic virus type 1
IFN-g	interferon gamma
LAG3	lymphocyte activation gene-3
MAPK	mitogen-activated protein kinase
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NF-kB	nuclear factor-kappa B
NLR	neutrophil-to-lymphocyte ratio
PARP	poly ADP-ribose polymerase
PDX	patient-derived xenograft
PI3K	phosphatidyl inositol 3 kinase
PTEN	phosphatase and tensin homolog
PR	progesterone receptor
ROS	reactive oxygen species
STAT	signal transducers protein kinase
TIM-3	T-cell immunoglobulin domain and Mucin domain 3
TIMP-1	tissue inhibitor of metalloproteinase-1
TNBC	triple-negative breast cancer
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Treg	T-regulatory cell
UPR	unfolded protein response
VEGF	vascular endothelial growth factor.

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Xiangpeng Dai,
Jilin University, China

REVIEWED BY
Zhijun Zhou,
University of Oklahoma Health Sciences Center, United States
Jianping Guo,
Sun Yat-sen University, China

*CORRESPONDENCE
Jun Gong
gjun@hust.edu.cn

†These authors have contributed equally to this work

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N6-methyladenosine reader YTHDF family in biological processes: Structures, roles, and mechanisms

Lin Chen[1†], Yang Gao[1†], Simiao Xu[2†], Jinxiong Yuan[1], Min Wang[1], Tianyu Li[3] and Jun Gong[1*]

[1]Department of Biliary-Pancreatic Surgery, Affiliated Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2]Division of Endocrinology, Affiliated Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Branch of National Clinical Research Center for Metabolic Disease, Wuhan, China, [3]Trauma Center/Department of Emergency and Traumatic Surgery, Affiliated Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

As the most abundant and conserved internal modification in eukaryote RNAs, N6-methyladenosine (m^6A) is involved in a wide range of physiological and pathological processes. The YT521-B homology (YTH) domain-containing family proteins (YTHDFs), including YTHDF1, YTHDF2, and YTHDF3, are a class of cytoplasmic m^6A-binding proteins defined by the vertebrate YTH domain, and exert extensive functions in regulating RNA destiny. Distinct expression patterns of the YTHDF family in specific cell types or developmental stages result in prominent differences in multiple biological processes, such as embryonic development, stem cell fate, fat metabolism, neuromodulation, cardiovascular effect, infection, immunity, and tumorigenesis. The YTHDF family mediates tumor proliferation, metastasis, metabolism, drug resistance, and immunity, and possesses the potential of predictive and therapeutic biomarkers. Here, we mainly summary the structures, roles, and mechanisms of the YTHDF family in physiological and pathological processes, especially in multiple cancers, as well as their current limitations and future considerations. This will provide novel angles for deciphering m^6A regulation in a biological system.

KEYWORDS

M6A, YTHDF, biological process, cancer, clinical applications

1 Introduction

In recent years, more than 170 different chemical RNA modifications have been identified, drawing more attention to the epitranscriptome (1). Among them, N6-methyladenosine (m^6A), which adds a methyl group to the sixth nitrogen atom of adenine, is the most abundant internal transcriptome modification in eukaryotes (2, 3). By identifying the consensus motif "RRACH" (R = A/G; H = A/C/U), m^6A usually occurs in the

3' untranslated region (3'UTR) and coding sequence (CDS), especially in the vicinity of stop codons (4, 5). Accordingly, m^6A modification regulates the metabolism of multiple types of RNAs and are ultimately participating in various pathophysiological processes.

The m^6A methylation is dynamic and reversible, regulated by a series of m^6A-modifying enzymes which can be classified into "writers", methyltransferases that install m^6A modifications, and "erasers", demethylases that remove m^6A from mRNA, as well as "readers" that recognize and bind to m^6A-modified mRNA to mediate their ultimate fate. Methyltransferase complex (MTC) is the main "writers", including methyltransferase like 3/14 (METTL3/14), Wilms' tumor 1-associating protein (WTAP) (6, 7). They catalyze the formation of m^6A methylation synergistically. Conversely, the fat mass and obesity-associated protein (FTO) and AlkB homolog 3/5 (ALKBH3/5) that belong to the "erasers" act as key proteins in m^6A demethylation (8, 9). Moreover, "readers" are important m^6A binding proteins such as YTHDFs, YTH domain-containing 1/2 (YTHDC1/2), heterogeneous nuclear ribonucleoproteins (HNRNP) family, insulin-like growth factor 2 mRNA-binding proteins (IGF2BP1/2/3), and eukaryotic initiation factor 3 (eIF3) (5, 10–16). They influence RNA splicing, export, translation, and decay, and then regulate diverse downstream signaling pathways.

The YTHDF family is the most studied "readers" of m^6A, which includes YTHDF1, YTHDF2, and YTHDF3. They regulate the translation and stability of target mRNAs to alter the expression of downstream molecules, thus affecting diverse biological processes (10, 17). In this review, we summarize the structures and functions of the YTHDF family, especially the m^6A-binding specificity. Moreover, we focus on its underline mechanisms in multiple physiological and pathological processes, especially in tumors, hoping to provide possible application value.

2 M^6A methylation regulators

In "writers", MTC is the main component that catalyzes the formation of m^6A. Among them, METTL3 installs methyl groups in S-adenosylmethionine to RNA target sites, while METTL14 selects RNA adenine bases and stabilizes the catalytic process (6, 18, 19). WTAP, RBM15/15B, VIRMA, and ZC3H13 are also components of the MTC, directing complexes to nuclear speckles as well as RNA sites (7, 20–22). In addition to MTC, METTL16, ZCCHC4, and METTL5 also can catalyze m^6A modification of specific RNAs (23–25). In contrast, FTO and ALKBH3/5 act as key "erasers" proteins in m^6A demethylation (8, 9, 26). FTO and ALKBH5 target mRNA and are associated with obesity and spermatogenesis, respectively (9, 27). Whereas ALKBH3 removes m^6A on tRNA (26).

Moreover, "readers" are required in m^6A-regulated diverse downstream signaling pathways. For example, YTHDC1 promotes mRNA splicing in the nucleus as well as nuclear export (11, 12). Furthermore, YTHDC1 accelerates the function of XIST to silence the transcription of genes on the X chromosome (20). Interestingly, YTHDC2 promotes mRNA translation with a concomitant decrease in mRNA abundance and has ATPase and 3' to 5' RNA helicase activities (13, 28). In addition, the HNRNP family regulates the alternative splicing of mRNA through an "m^6A-switch" mechanism (29–33). IGF2BPs stabilize target mRNAs in different ways under normal and stress conditions (15). And eIF3 binds m^6A on the 5'UTR of mRNA and promotes mRNA translation in a cap-independent manner (16).

The YTHDF family was identified by selecting proteins containing the YTH domain and subsequently obtained in pull-down experiments using methylated RNA bait (5, 34, 35). Now, the features of the YTHDF family have been gradually unraveled. The YTH domains of YTHDFs have a hydrophobic pocket, which is critical to the recognition of m^6A in the cytoplasm (36). But the role of each protein is different, for example, YTHDF1 promotes RNA translation, YTHDF2 facilitates RNA decay, and YTHDF3 exhibits a dual function depending on its binding partner (37). Thus, the YTHDF family is closely associated with many cancers and other biological processes (Figure 1).

3 The structures and functions of the YTHDF family

The YTHDF family is composed of a C-terminal YTH domain and an N-terminal domain rich in P/Q/N (Pro/Gln/Asn). The YTH domain is the basis of recognizing m^6A RNA specifically and its targeted position and consensus sequence are similar to the distribution pattern of m^6A sites on mRNA (20, 38). YTH domain can also directly bind to N1-methyladenosine (m^1A), but with a lower affinity than m^6A (39). The prion-like low-complexity sequence regions (LCRs) of the N-terminal domain are associated with the liquid-liquid phase separation (LLPS) (40). The mRNA-YTHDF complexes are located in different membrane-less compartments in the cytoplasm, such as processing bodies (P-bodies), stress granules (SGs), or neuronal granules, which are the result of LLPS and can be enhanced by multivalent m^6A modifications (41). Proteomic studies revealed that YTHDFs can be phosphorylated and myristoylated to regulate their expression and clustering (42). Additionally, the EGFR/SRC/ERK pathway stabilizes YTHDF2 protein by phosphorylating YTHDF2 at serine39 and threonine381 in glioblastoma cells (43). YTHDF2 can also be SUMOylated at site K571, thereby enhancing its binding affinity with m^6A-modified mRNAs and accelerating cancer advancement (44). Therefore, targeting post-translational modifications represent a novel opportunity for YTHDFs to regulate their functions.

The crystal structures of the three YTH domains and their complexes with an m^6A mononucleotide (or m^6A oligoribonucleotides) have been revealed (45, 46). The YTH domains share a mixed α-helix-β-sheet fold, where the α-helices surround a barrel-shaped center arranged by the β-sheets. The surface of the YTH domain has a positively-charged groove in which m^6A is tightly locked. Specifically, m^6A is located in a hydrophobic pocket formed by three highly conserved aromatic residues called an aromatic cage. In the YTHDF-m^6A complex, the m^6A adenine moiety is sandwiched between the rings of two aromatic residues, paralleling them (Trp411 and Trp470 in YTHDF1, Trp432, and Trp491 in YTHDF2, Trp438, and Trp497 in YTHDF3). And the methyl group of m^6A points to the ring of one aromatic residue (Trp465 in YTHDF1, Trp486 in

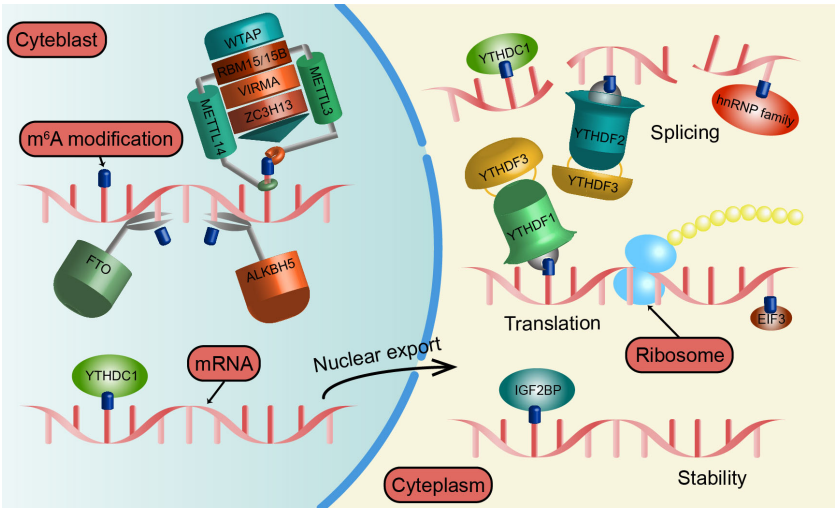

FIGURE 1
The regulation mechanism of m6A modification. METTL3, METTL14, WTAP, RBM15, VIRMA, and ZC3H13 all belong to the "writers" and catalyze the formation of m6A modification by constituting MTC. The "erasers" includes FTO and ALKBH5, which act as key proteins in m6A demethylation. YTHDF1/2/3, YTHDC1, IGF2BP, hnRNP family, and EIF3 as "readers" that bind to m6A and affect RNA splicing, output, translation, and decay.

YTHDF2, Trp492 in YTHDF3) (36, 47, 48). As well as aromatic residues, some amino acids (aa) of the YTH domain also interact with m^{6}A. For example, the backbone NH of Tyr397 in YTHDF1 and Tyr418 in YTHDF2 form hydrogen bonds with the N3 of m^{6}A. The carbonyl oxygen of Cys412 in YTHDF1, Cys433 in YTHDF2, and Cys439 in YTHDF3 bind to the N6 of m^{6}A by hydrogen bonding. To sum up, the Π-Π interactions between the m^{6}A adenine moiety and the aromatic cage, the cation-Π interactions between the methyl group and the aromatic cage, and a series of hydrogen bonds lay a foundation for m^{6}A recognition (36) (Figure 2).

Evidence confirms that the YTHDF family plays an integral role in the translation and degradation of m^{6}A-modified mRNAs. YTHDF2 is the most explored YTHDFs and is generally expressed at much higher levels than YTHDF1 and YTHDF3 in most cells (42). YTHDF2 binds to m^{6}A-modified mRNAs and recruits the CCR4-NOT deadenylase complex through its N-terminal 101-200 aa to initiate deadenylation, which is a prior condition of P-body localization and decay of targeted mRNAs (10, 49, 50). Additionally, m^{6}A-modified mRNAs can also bind to YTHDF2 in an HRSP12-dependent manner, and subsequently cleaved by RNase P/MRP (endoribonucleases) (51, 52). In particular, HRSP12 bridges the N-terminal 100 aa of YTHDF2 and RNase P/MRP, contributing to the rapid degradation of mRNAs. And m^{6}A-containing circular RNAs (circRNAs) are also degraded by this pathway. Interestingly, under heat shock stress, nuclear-translocated YTHDF2 protects m^{6}A motifs in the 5' untranslated region (5'UTR) of stress-induced transcripts and activates cap-independent translation initiation (53). The N-terminal of YTHDF1 (100-200 aa) is in charge of the translation of mRNAs with m^{6}A modifications (54). YTHDF1 not only transports more mRNAs to translation machinery and promotes ribosome occupancy, but also enhances the translation-initiation rate by correlating eIF4G-mediated loop structure through interaction with eIF3 in a cap-dependent manner (17). YTHDF1 can also trigger translational elongation through interaction with elongation factors in some cancer cells (55–57). Apart from the above results, Li et al. found that YTHDF1 interacts with Argonaute 2 (AGO2) to stimulate the production of P-bodies for mRNA degradation (58). In addition, YTHDF3 augments m^{6}A-mRNA translation by cooperating with YTHDF1 and interacting with the 40s/60s ribosome subunits (59). Besides that, YTHDF3 recruits eIF4G2 to m^{6}A sites, driving translation initiation of circRNAs (60). YTHDF3 also promotes m^{6}A-modified mRNA decay by working together with YTHDF2 (37). A recent study found that the effect of YTHDF3 in regulating targeted mRNA deadenylation during somatic cell reprogramming relies on the recruitment of the PAN2-PAN3 deadenylase complex (61).

Interestingly, the YTHDF family forms a classic functional model: upon entry into the cytoplasm, m^{6}A-modified mRNAs are first bound by the YTHDF3 or YTHDF3-YTHDF1 complex and then recognized by YTHDF2, thereby regulating the different fates of the targeted mRNA (62). Nevertheless, it has recently been discovered that YTHDFs have redundant functions to a large extent (63). Those three YTHDFs share highly homologous structures (about 85% of aa sequence similarity) (64), similar RNA-binding properties (20), and a similar set of binding proteins, jointly regulating mRNA destiny in an m^{6}A-dependent manner (65). Indeed, the distinct functions of YTHDFs depend on their expression levels, spatial locations, and post-translational modifications. Also, YTHDFs are affected by additional RNA-binding proteins that interact with YTHDFs, such as fragile X mental retardation protein (FMRP) (66, 67), and Proline-rich coiled-coil 2 A (Prrc2a) (68). Collectively, the role of YTHDFs in regulating gene expression is complex and requires further investigation.

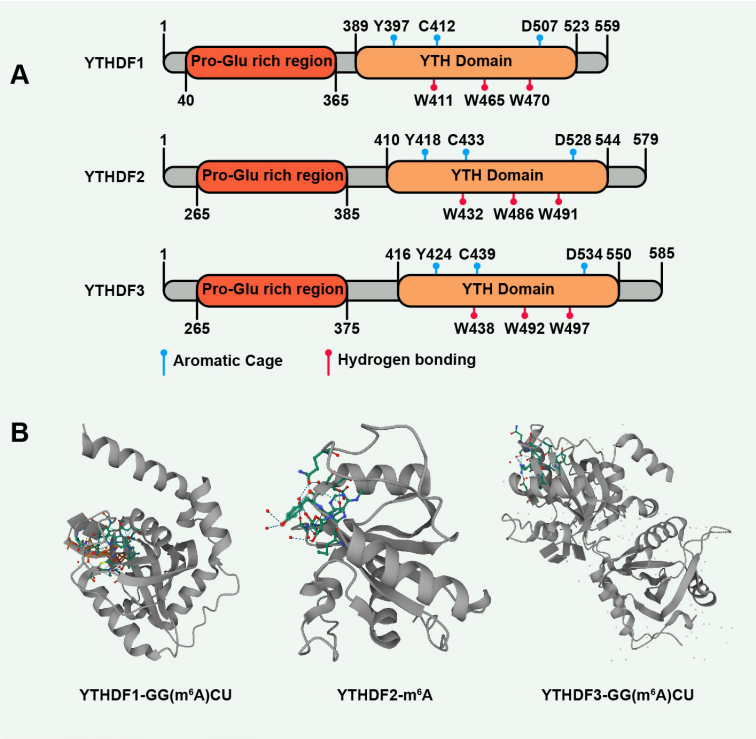

FIGURE 2
The structures of the YTHDF family, especially the YTH domain. **(A)** The YTH domain of YTHDFs: YTHDF1 (UniProt ID: Q9BYJ9), YTHDF2 (UniProt ID: Q9Y5A9), YTHDF3 (UniProt ID: Q7Z739). **(B)** Structures of YTHDFs in complex with m6A. YTHDF1 (PDB ID:4RCJ), YTHDF2 (PDB ID:4RDN), YTHDF3 (PDB ID:6ZOT). The secondary structures of proteins are shown in gray, and RNA molecules are shown in color.

4 The roles of the YTHDF family in physiological and pathological processes

4.1 Embryonic development

Among the three YTHDFs, YTHDF2 is expressed and plays a pivotal role throughout mammalian gametogenesis. YTHDF2-knockout female mice are infertile while male mice are hypo fertile (65, 69). Specifically, YTHDF2 is intrinsically required for oocyte competence to support early zygotic development rather than MII oocytes formation and fertilization process (69). YTHDF2 regulates appropriate maternal transcript dosage during oocyte maturation by selectively mediating transcript destabilization. Additionally, YTHDF2 clears m^6A-dependent matrix metallopeptidase transcripts to promote the adhesion and proliferation of spermatogonia during spermatogenesis (70). Knockout of YTHDF2 results in morphologically deformed and functionally impaired sperm, even severe loss (65, 71).

Intriguingly, unlike the previous view that maternal mRNAs clearance and maternal-to-zygotic transition (MZT) are dependent on YTHDF2, Kontur et al. found that individual YTHDFs deletion does not prevent embryonic development, whereas double mutations of YTHDF2/YTHDF3 disrupts oogenesis and triple YTHDF depletion causes lethality in zebrafish (72, 73). Despite evidence for the redundant functions of YTHDFs in early mouse embryonic development, depletion of YTHDF2 causes lethality at late embryonic development stages with embryos exhibiting severe neurological deficits (65, 74). Zheng et al. found that YTHDF3 reduction is an adaptive mechanism under a hypoxic environment in early embryonic development (75). Specifically, YTHDF3 binds to the m^1A site of insulin-like growth factor 1 receptor (IGF1R) mRNA and degrades IGF1R mRNA, hindering migration and invasion of trophoblast.

4.2 Stem cell fate

Somatic cells are reprogrammed into induced pluripotent stem cells (iPSCs), which have unlimited proliferation and pluripotent differentiation potential similar to human embryonic stem cells (ESCs) (76). YTHDF2 and YTHDF3 play an essential role in this reprogramming process by clearing somatic mRNAs, especially Tead2, through distinct m^6A-dependent deadenylation mechanisms (61). While YTHDF1 is capable of increasing the expression of the transcription factor Btg2 and promoting the reprogramming of induced neuronal cells (77). In terms of iPSCs functions, the YTHDF1/YTHDF2 orchestration is involved in METTL3-m^6A-mediated maintenance of pluripotent state in porcine iPSCs by elevating JAK2 level, reducing SOSC3 expression, and provoking STAT3/KLF4/SOX2 signal axis (78). YTHDF1 upregulation depends on MATR3 and maintains a MATR3-mediated pluripotent state in human iPSCs by maintaining the expression of OCT4 and LIN28A

transcripts (79). Importantly, YTHDF2 is overexpressed and disrupts the expression of a group of m^{6}A-modified mRNAs associated with neurodevelopment, thereby blocking neural differentiation and promoting pluripotency in human iPSCs (80). Similarly, YTHDF3 reduces gene expression associated with the formation of three germ layers, and the absence of YTHDF3 impairs pluripotency in ESCs (81).

Several studies have revealed that the specification and characteristics of hematopoietic stem cells (HSCs) are significantly regulated by YTHDF2. The m^{6}A-YTHDF2-mediated decay of Notch1 mRNA is critical for the generation of the earliest hematopoietic stem/progenitor cells (HSPCs) during the endothelial-to-hematopoietic transition (EHT) in both zebrafish and mice embryos (82, 83). Li et al. first reported that YTHDF2 specifically mediates the ex vivo expansion of human HSCs due to the regulation of the stability of multiple mRNAs essential for HSC self-renewal (84). Therefore, inhibition of YTHDF2 makes it possible to obtain a sufficient number of HSCs from human umbilical cord blood (hUCB), which facilitates the application of hUCB HSCs transplantation. Furthermore, YTHDF2 deletion also promotes the expansion and regeneration of HSCs by eliminating the decay of both WNT-targeted and survival-related genes under stress conditions (85). Interestingly, although YTHDF2 is dispensable for steady-state multilineage hematopoiesis, long-term deficiency of YTHDF2 dramatically impairs HSCs activity and blocks reconstitution of multilineage hematopoiesis (86). Given that hematopoietic-specific YTHDF2 deficiency-induced long-term HSCs impairment is consistent with the adverse consequences of inflammation in HSCs, the inflammation-induced increase in YTHDF2 may be a protective mechanism for the long-term integrity of HSCs. YTHDF3 is also involved in the regulation of HSCs. YTHDF3 binds m^{6}A on the 5'UTR of CCND1 mRNA and cooperates with PABPC1 and EIF4G2 to promote the expression of CCND1, a positive regulator of HSCs reconstitution capacity (87). While YTHDF3 facilitates the translation of FOXM1 and ASXL1 transcripts and is critical for maintaining HSC properties under stress conditions (88).

YTHDF1 is indispensable for maintaining intestinal stem cells (ISCs) during regeneration after intestinal damage by driving a positive feedback loop of the YTHDF1/TCF4/WNT signaling axis (89). Similarly, YTHDF1 sustains the stemness of ISCs through a targeted translation of transcriptional-enhanced associate domain 1 (TEAD1) (90). In addition, YTHDF1 is also involved in the m^{6}A-mediated self-renewal of mouse female germline stem cells (mFGSCs) (91).

4.3 Fat metabolism

YTHDFs play key roles in adipogenesis, particularly YTHDF2. YTHDF2 binds and degrades JAK1 mRNA to block the JAK1/STAT5/C/EBPβ pathway, thereby inhibiting the adipogenic differentiation of bone marrow stem cells (92). Similarly, YTHDF2-mediated silencing of the JAK2/STAT3/C/EBPβ pathway impedes adipogenesis (93). Indeed, YTHDF2 also impairs adipogenesis by degrading multiple target transcripts through methylation-dependent modifications. Cell cycle factors, including CCNA2, CDK2, and CCND1 promote cell cycle progression and mitotic clonal expression in adipocytes (94, 95). Epigallocatechin gallate (EGCG) and metformin reduce CCNA2 and CDK2 levels by increasing m^{6}A modification in an FTO-YTHDF2-dependent manner (96, 97). Conversely, Zinc finger protein (Zfp217) binds and sequesters YTHDF2 to reduce m^{6}A levels, thus reversing CCND1 mRNA degradation (98). YTHDF2 also reduces the content of FAM134B, fatty acids synthesis-related proteins such as FASN, and autophagy-related proteins, including ATG5 and ATG7, which inhibit adipogenesis (99–101). Furthermore, the liver Bmal1 regulates the circadian clock of lipid metabolism by controlling the abundance of m^{6}A modifications on transcripts (102). Mechanistically, Bmal1 knockdown inhibits PPARα expression in an m^{6}A-YTHDF2-dependent manner, which increases lipid accumulation. Moreover, AMPK upregulates CD36 levels through YTHDF2-dependent Parkin reduction, which enhances intestinal long-chain fatty acid uptake and induces obesity in high-fat diet mice (103).

Intriguingly, YTHDF1 inhibits ovine adipogenesis and promotes porcine adipogenesis by promoting the expression of PNPLA2 and MTCH2, respectively (62, 104). Chen et al. found that YTHDF1 restrains PPARγ expression in mice by promoting the translation of m^{6}A-modified TRAF4 transcripts, while curcumin exerts an anti-obesity role by reducing the effect of ALKBH5 demethylation on TRAF4 m^{6}A modification (105). In addition, YTHDF1 together with METTL3 amplifies the function of Rubicon that inhibits autophagy by stabilizing Rubicon mRNA, and further blocks the clearance of lipid droplets (LDs) in mouse nonalcoholic fatty liver disease (NAFLD) (106).

4.4 Neuromodulation

YTHDF1 mainly regulates axonal function as well as learning and memory, and YTHDF2 is mainly involved in neural development and differentiation. Functional axon regeneration under peripheral nervous system injury is supported by m^{6}A-YTHDF1-derived increases in global protein translation (107). And YTHDF1 is a key player in enhancing Robo3.1 mRNA translation and guidance of pre-crossing commissural axons in the spinal cord, whereas YTHDF1 is inhibited by floor plate-induced signals in post-crossing axons guidance (108). Furthermore, dual depletion of YTHDF1/YTHDF3 affects spine morphology and excitatory synaptic transmission in hippocampal neurons (109). Further study revealed that YTHDF1 accelerates basal transmission and long-term potentiation of synapses by advancing neuronal stimulation-induced protein translation, thereby promoting learning and memory, especially long-term memory (110). In a Drosophila short-term memory experiment, memory-storing neurons require YTHDF to maintain normal memory function during aging (111). Furthermore, YTHDF1-mediated Dvl1 mRNA translation has a synergistic effect with YTHDF2-mediated Wnt5a mRNA degradation in inhibiting axon growth of cerebellar neurons (112).

During neural development, YTHDF2 is overexpressed and positively regulates early brain development by promoting the proliferation and differentiation of neural stem/progenitor cells (NSPCs) (74). Knockout of YTHDF2 significantly reduces cerebral cortical thickness and induces differentiated neurons to produce abnormal stress-sensitive neurites. Interestingly, YTHDF2-silenced NSPCs cannot differentiate into glial cells. Wu et al. showed that YTHDF2 competes with Prrc2a for binding to Olig2 mRNA, resulting

in impaired oligodendrocyte specification and myelination (68). Moreover, YTHDF2 is detrimental to the extension and maintenance of retinal ganglion cell (RGC) dendritic arborization (113).

YTHDFs are also involved in a variety of brain disorders. For example, downregulated miR-421-3p in microglia after cerebral artery occlusion/reperfusion (MCAO/R) relieves the repression of YTHDF1, thereby promoting p65 mRNA translation, leading to aggravated inflammation and brain injury (114). Impairments of fine motor and cognitive function in young mice exposed to multiple sevoflurane are attributable to a specific decrease in YTHDF1 expression (115). Overexpression of YTHDF1 ameliorates diabetes-induced cognitive impairment (116). Additionally, elevated YTHDF2 under persistent light impedes cognitive behavior in mice by perturbing the stability of TrkappaB mRNA (117). And a recent case report found that most individuals with YTHDF3 haploinsufficiency show intellectual disability and/or developmental delay of variable degrees (118).

4.5 Cardiovascular effect

YTHDF1 promotes cardiomyocyte (CM) differentiation, whereas YTHDF3 does the opposite (81). YTHDF1, which is positively regulated by ALKBH5, also promotes CM proliferation in injury-induced cardiac regeneration by enhancing YAP mRNA translation (119). Xu et al. indicated that YTHDF2 degrades Myh7 mRNA to mitigate cardiac hypertrophy during heart failure development (120). Conversely, lncRNA MIAT-induced YTHDF2 high expression stimulates cardiac hypertrophy by downregulating CPT-1a levels in the PPARα pathway (121). Moreover, YTHDF1 and YTHDF2 promote ocular pathological angiogenesis *via* the METTL3-m6A-LRP6 axis and the FTO-m6A-FAK axis, respectively (122, 123). YTHDF1/YTHDF2 cooperation stimulates the atherogenic inflammatory cascade in the vascular endothelium by upregulating NLRP1 and downregulating KLF4 (124). Furthermore, loss of either YTHDF1 or YTHDF2 alleviates the proliferation of pulmonary arterial smooth muscle cells and pulmonary hypertension under hypoxia. Mechanistically, YTHDF1 promotes the translation of MAGED1 mRNA while YTHDF2 activates the PI3K/AKT signaling pathway by degrading PTEN mRNA (125, 126). And YTHDF3 knockout protects lung epithelial cells from inflammatory injury by inhibiting inflammatory cytokine secretion after hypoxia/reoxygenation (127).

4.6 Viral infection

YTHDFs play anti-viral roles in the life cycle of Epstein-Barr virus (EBV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Zika virus (ZIKV), and enterovirus 71 (EV71) (128–133). For example, the knockdown of each DF in EBV-infected cells promotes EBV lytic replication and reactivation. Mechanistically, YTHDF1 attracts ZAP, DDX17, and DCP2 forming RNA degradation complexes to accelerate the decapping of m^6A-modified RNAs and degrade EBV cleavage gene transcripts (128). Furthermore, activation of caspases cleaves D166 and D367 sites on YTHDF2 upon EBV reactivation reduces YTHDF2 expression, thereby increasing caspase-8 protein levels and enhancing EBV replication (129). Alternatively, YTHDFs inhibit HCV infection by reducing viral particle production rather than blocking viral RNA replication (131). During the chronic HCV infection state, YTHDFs relocate to lipid droplets, bind to the m^6A site in the HCV E1 region, and antagonize viral packaging caused by the binding of the viral core protein to the non-m^6A site in the E1 region. In contrast, YTHDF2 promotes simian virus 40 (SV40) and influenza A virus (IAV) replication (134, 135). Moreover, YTHDF1 and YTHDF3 induce severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, YTHDF1 inhibits chikungunya virus (CHIKV) infection, and YTHDF2 functions opposite to that of YTHDF1 in both SARS-CoV-2 and CHIKV (136–138).

Notably, the regulation of YTHDFs in the transcription and replication of human immunodeficiency virus type 1 (HIV-1) and Kaposi's sarcoma-associated herpesvirus (KSHV) remains controversial. Evidence suggests that YTHDFs hinder HIV-1 replication in target cells contradicting previous views that YTHDFs increase viral transcript and protein levels (139–141). Specifically, after HIV-1 infection into cells, YTHDFs impede HIV-1 reverse transcriptase by degrading incoming HIV-1 genomic RNA (gRNA) in an m^6A-dependent manner, thereby limiting viral replication (139). Nevertheless, YTHDFs facilitate HIV-1 structural protein Gag synthesis and virus release, while forming a complex with HIV-1 Gag protein and viral and cellular RNAs in virus-producing cells (140). To ensure optimal HIV-1 infectivity, HIV-1 protease cleaves YTHDF3, which enters the virion in a nucleocapsid-dependent fashion (142). Additionally, Hesser et al. showed that YTHDF2 exerts pro- and anti-KSHV effects in iSLK and B cell lines, respectively (143). Instead, Tan et al. observed that YTHDF2 inhibits KSHV gene expression and virion production in iSLK cells (144). Together, the paradoxical phenomenon of YTHDFs in viral regulation may be explained by differences in cell types, viral life cycle stages, and experimental approaches.

4.7 Immunity

The type I interferon (IFN) signaling pathway relies on the expression of IFN-stimulated genes (ISGs) to mediate a powerful innate antiviral immune response. YTHDF1-mediated upregulation of IFITM1, a subset of ISGs, initiates antiviral responses (145). Another study showed that YTHDF1 prevents viral double-stranded RNA (dsRNA)-driven IFN responses (146). YTHDF1 induces the IFN-mediated expression of ADAR1, which disrupts the secondary structure of dsRNA in an adenosine-to-inosine (A-to-I) RNA editing manner. Furthermore, YTHDF2 deletion enables increased levels of IFN-βand inflammatory factors, including interleukin-6 (IL-6) by stabilizing host antiviral transcripts (147, 148). YTHDF2 also binds and sequesters m^6A-modified viral RNA, which protects viral RNA from RIG-I recognition, thereby inhibiting RIG-I activation and the downstream IFN signaling pathway (149, 150). In contrast, YTHDF2 is an essential cofactor for the IFN-α-induced degradation of m^6A-methylated HBV RNA by ISG20 (151). Additionally, enterovirus 2A proteases cleave YTHDFs and limit antiviral responses during early viral infection (152). Among them, the cleavage of YTHDF3 dampens the IFN-I-stimulated JAK/STAT signaling pathway. Interestingly, only YTHDF3 attenuated ISGs expression in the absence of viral infection (153). Mechanistically, YTHDF3 rapidly translates forkhead box

protein O3 (FOXO3) mRNA through cooperation with PABP1 and eIF4G2 in an m^{6}A-independent way, thereby suppressing ISGs expression.

Inflammatory responses are also an important part of immunity. YTHDF1 counteracts the excessive and persistent development of inflammation in the septic response by promoting the expression of SOCS1, a negative regulator of macrophage-mediated inflammation (154). However, YTHDF1 knockout suppressed inflammatory lung or intestinal damage (155, 156). Macrophage-specific YTHDF1 knockdown may be a protective therapy against brain injury in severe sepsis rats with ECMO by enhancing adaptive immune function and alleviating inflammatory damage (157). YTHDF2 also negatively regulates inflammation. YTHDF2 inhibits the MAPK and NF-κB signaling pathways by downregulating the expression of MAP2K4, MAP4K4, STAT1, and PPAR-γ, and subsequently prevents macrophage polarization and proinflammatory cytokine secretion (158–160). And YTHDF2-dependent decay of KDM6B mRNA restricts H3K27me3 demethylation, which impedes transcription of proinflammatory cytokine genes (161).

Strikingly, the expression of YTHDFs has a strong relationship with the immune regulation of various tumors. The expression of YTHDF1 is not only the highest in normal immune cells but also dramatically correlated with tumor immune-infiltrated cells in cancer, especially CD8$^+$ T cells, macrophages, and dendritic cells (DCs) (162). Han et al. revealed that YTHDF1 is an important target for anti-tumor immunotherapy (163). YTHDF1 depletion accelerates tumor antigen presentation and cross-priming of CD8$^+$ T cells by retarding lysosomal cathepsin translation in DCs in an m^{6}A-dependent manner. And the loss of YTHDF1 recruits DCs and activates IFN-γ receptor 1 and JAK/STAT1 signaling pathways, thereby promoting antitumor immunity in GC (164). Li et al. demonstrated that YTHDF1 hinders CD8$^+$ T cell infiltration and increases immune checkpoint expression, such as PD-L1 and V-domain Ig suppressor of T cell activation (VISTA), in CRC (165). To this end, YTHDF1 consumption can be synergistic with anti-PD-1/PD-L1 immunotherapy for effective anti-tumor therapy. Similarly, YTHDF2-deficient tumors increased the sensitivity to anti-PD-1/PD-L1 immunotherapy by stabilizing PD-L1 mRNA in ICC (166). However, YTHDF2 participates in anti-tumor and anti-viral infection by regulating the maturation, proliferation, and effector functions of NK cells (167) (Figure 3).

5 The role of the YTHDF family in cancers

5.1 Digestive system cancers

5.1.1 Liver cancer

Studies have reported that YTHDF1 is an oncogene that is highly expressed and positively correlates with the pathology stage in hepatocellular carcinoma (HCC) (168, 169). YTHDF1 is also an independent factor for an unfavorable HCC prognosis. Lin et al. suggested that Snail induces epithelial-mesenchymal transition (EMT) to enhance the metastasis of HCC cells. Mechanistically, m^{6}A-modified CDS facilitates translational elongation of the Snail mRNA in a YTHDF1/eEF2-dependent manner (55). In addition, the YTHDF1-mediated aggressive phenotypes are also associated with the activation of the AKT/GSK-3β/β-catenin pathway (170). Chi et al. perceived that the effect of YTHDF1 in enhancing HCC proliferation can be antagonized by hsa-miR-139-5p (171). YTHDF1 also promotes HCC cell growth by upregulating the PI3K/AKT/mTOR signaling pathway (172). Hu et al. showed that METTL3-m^{6}A-YTHDF1-mediated RBM14 overexpression promotes Kupffer cell polarization and HCC progression (173). Furthermore, YTHDF1 is involved in the regulation of HCC under hypoxic stress. For example, hypoxia-inducible factor-1α (HIF-1α)-mediated upregulation of YTHDF1 promotes autophagy-associated genes ATG2A and ATG14 translation, thus aggravating HCC malignancy behavior (174). FOXO3 is a negative regulator of hypoxia-induced autophagy and mediates the sorafenib sensitivity in HCC (175). Importantly, YTHDF1 binds to METTL3-methylated m^{6}A modification in the FOXO3 mRNA 3'UTR and increases its mRNA stability rather than translation. Moreover, under the sublethal heat stress from insufficient radiofrequency ablation (IRFA), YTHDF1 binds to the m^{6}A site on the 5'UTR of EGFR mRNA and triggers EGFR translation, eventually resulting in HCC recurrence after IRFA (176).

Notably, YTHDF3 is also reported as a potential oncogene in HCC. YTHDF3 enhances HCC metastasis by maintaining ZEB1 mRNA stability in an m^{6}A-dependent mechanism (177). YTHDF3/integrin subunit alpha 6 (ITGA6) is positively regulated by the lysine-specific demethylase 5B (KDM5B)/microRNA-448 axis and thereby enhances the self-renewal of HCC cells (178).

Intriguingly, YTHDF2 has a paradoxical effect on HCC in different studies. Zhong et al. professed that hypoxia-induced YTHDF2 downregulation reverses the repression of YTHDF2 on the ERK/MAPK signaling pathway, subsequently removing the inhibitory effect of YTHDF2 on the proliferation and growth of HCC cells (179). Mechanistically, YTHDF2 suppresses the activation of the ERK/MAPK signaling pathway by selectively recognizing the m^{6}A site at the 3'UTR and triggering EGFR mRNA degradation. Hou et al. confirmed that YTHDF2 is significantly downregulated in HCC cells and YTHDF2 deficiency elicits inflammation, vascular abnormalization, and metastatic progression (180). Specifically, YTHDF2 destabilizes the mRNA of m^{6}A-modified interleukin 11 (IL11) and serpin family E member 2 (SERPINE2) to exert an inhibitory effect. Conversely, YTHDF2 is also considered a tumor-promoting factor in HCC (181, 182). Yang et al. discovered that microRNA-145 targets the 3'UTR of YTHDF2 mRNA to attenuate its expression and thereby inhibits the proliferation of HCC cells (183). And YTHDF2 participates in METTL3-m^{6}A-mediated HCC malignancy by shortening the half-life of the suppressor of cytokine signaling 2 (SOCS2) mRNA (184). Additionally, YTHDF2 increases the m^{6}A levels in the 5'UTR of OCT4 mRNA in tandem with promoting OCT4 expression, eventually accelerating the HCC cancer stem cell (CSC) phenotype and metastasis (185). And PA2G4 depends on YTHDF2 to stabilize FYN mRNA and promote EMT-induced HCC metastasis (186). The discrepancy in the effect of YTHDF2 on HCC may be due to different cellular microenvironments or tumor heterogeneity (187).

In addition, YTHDF1 and YTHDF2 facilitate the advancement of intrahepatic cholangiocarcinoma (ICC) through increasing EGFR mRNA translation and IFIT2 mRNA decay, respectively (188, 189). Meanwhile, YTHDF2 silencing restrains ICC resistance to the exposure of cisplatin by reversing the degradation of cyclin-dependent kinase inhibitor 1B (CDKN1B) mRNA (190).

5.1.2 Gastric cancer

YTHDF1 mutations occur in approximately 7% of gastric cancer (GC) patients, and high expression of YTHDF1 is correlated with high-risk progression and poor prognosis in patients (191–193). YTHDF1 deficiency is capable to attenuate GC progression, including proliferation and metastasis *in vitro* and *in vivo*. Mechanistically,

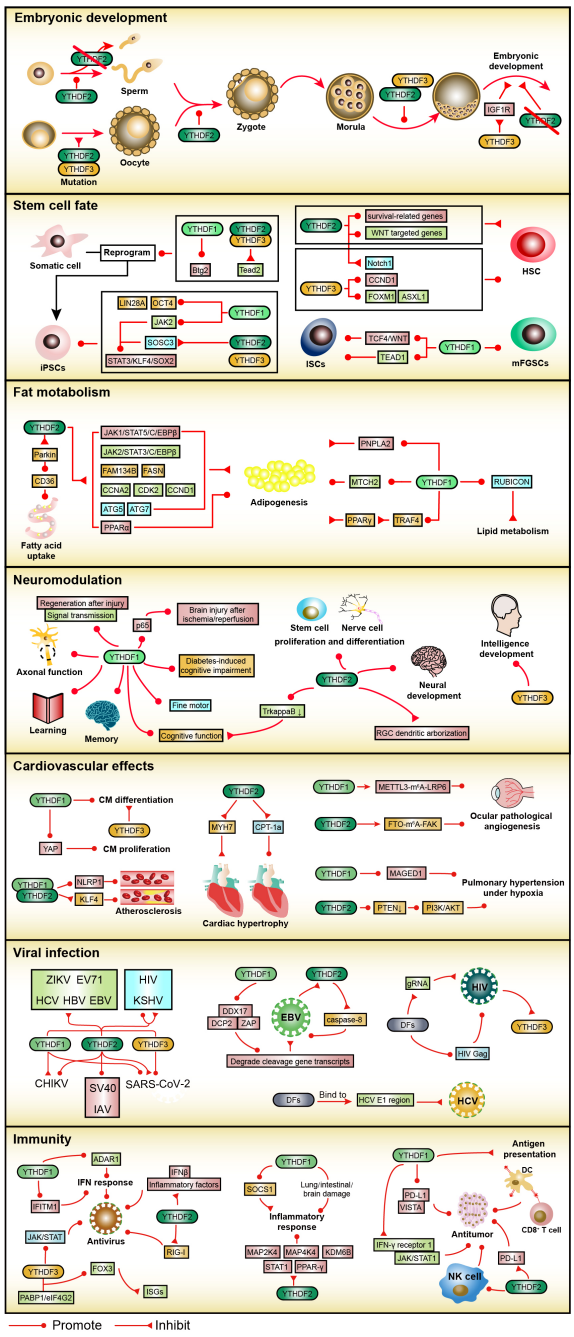

FIGURE 3
The roles of the YTHDF family in embryonic development, stem cell fate, fat metabolism, neuromodulation, cardiovascular effect, viral infection, and immunity. In embryonic development, YTHDF2 is essential for sperm, oocyte, zygote, and embryo formation. In stem cell fate, the YTHDF family promotes somatic cell reprogramming and the properties of iPSCs. In addition, YTHDF2 and YTHDF3 participate in the fate of HSC, and YTHDF1 in the fate of ISCs as well as mFGSCs. In fat metabolism, YTHDF1 and YTHDF2 regulate adipogenesis and fatty acid metabolism. In neuromodulation, YTHDF1 affects axonal function as well as learning and memory, YTHDF2 regulates neural development and differentiation, and YTHDF3 participates in intellectual development. In cardiovascular effect, YTHDF1 and YTHDF2 are closely related to the fate of CM, vascular endothelial cells, and pulmonary artery smooth muscle cells. In a viral infection, the YTHDF family is involved in the life cycle of several viruses, especially EBV, HCV, and HIV. In immunity, the YTHDF family plays an important role in antiviral immunity, inflammatory immunity, and anti-tumor immunity.

YTHDF1 relies on m^{6}A modification to promote the translation of frizzled7 (FZD7) and USP14, which transmit WNT/β-catenin signaling and AKT/ERK signaling, respectively (192, 193). In addition, METTL3 promotes the malignancy behavior of GC through YTHDF1/eIF3a-dependent post-transcriptional translation of SPHK2 (194).

Zhang et al. showed that the knockdown of YTHDF2 inhibits GC cell proliferation and accelerates apoptosis *in vitro* (195). And lncRNA LINC00470 relies on YTHDF2 to degrade m^{6}A-containing PTEN mRNA and thus promote GC advancement (196). Additionally, the HIF-1α-induced increase of lncRNA-CBSLR suppresses ferroptosis and chem-sensitive under hypoxic stress through the YTHDF2-CBS-ACSL4 axis (197). Specifically, CBSLR contributes to CBS mRNA destabilization by binding to the m^{6}A site on the CDS of CBS mRNA by recruiting YTHDF2. However, Shen et al. found that YTHDF2 plays a suppressive role in GC by destabilizing FOXC2 mRNA (198).

5.1.3 Pancreas cancer

Among the YTHDF family, YTHDF2 is the most studied protein in pancreatic cancer. YTHDF2 is elevated in pancreatic cancer and orchestrates the migration/proliferation dichotomy (199). Specifically, YTHDF2 prevents EMT, migration, and invasion by downregulating YAP signaling and enhances proliferation by activating AKT/GSK3B/CCND1 pathway. However, YTHDF2 downregulates the levels of PERP and PER1 mRNA to promote cell proliferation and migration in an m^{6}A-dependent manner (200, 201). METTL3-m^{6}A-YTHDF2-mediated decay of nucleobindin 1 (NUCB1) mRNA counteracts the effects of NUCB1 in halting pancreatic cancer growth and augmenting the antitumor with gemcitabine (GEM) (202). Conversely, another study showed that the rs142933486 G>T polymorphism in PIK3CB improves PIK3CB mRNA and protein levels by derailing m^{6}A-YTHDF2-dependent degradation mechanisms, which is significantly associated with the poor prognosis of PTEN-deficient pancreatic cancer patients (203). And compared with PIK3CB[T], YTHDF2 mainly binds to PIK3CB[G]. Similarly, FTO reverses YTHDF2-regulated degradation of platelet-derived growth factor C (PDGFC) mRNA and promotes cell proliferation by reactivating the AKT signaling pathway (204). Notably, YTHDF1 is associated with the immune microenvironment and prognosis of pancreatic cancer (205–207). A recent study found that a novel antineoplastic drug, Olean-28,13β-lactam (B28), inhibits glutamine metabolism by reducing the expression of YTHDF1, which induces pancreatic cancer cell death (208). In addition, YTHDF3-mediated downregulation of lncRNA DICER1-AS1 reverses the repression of glycolysis by miR-5586-5p in pancreatic cancer (209).

5.1.4 Colorectal cancer

In colorectal cancer (CRC), YTHDF1 may be a molecular target for diagnosis and treatment (210). Mechanistically, elevated YTHDF1 in CRC is mainly attributed to an increase in DNA copy number (211). The oncogene c-MYC, WNT signaling, and APC mutation can also upregulate YTHDF1 expression at the translational level (89, 212). Further studies found that YTHDF1 promotes tumorigenicity and CSC-like activity by amplifying the WNT/β-catenin pathway with little effect on normal intestinal development (211). And deletion of YTHDF1 in ISCs shrinks tumor size and prolongs the lifespan of CRC-formed mice substantially. YTHDF1 can promote CRC progression and metastasis by translating m^{6}A-modified Rho/Rac guanine nucleotide exchange factor 2 (ARHGEF2) mRNA and activating RhoA signaling (213). Furthermore, circular RNA protein tyrosine kinase 2 (circPTK2) restores the miR-136-5p-mediated repression of YTHDF1 by competitively binding to miR-136-5p, resulting in the CRC advancement and chemoresistance (214). Chen et al. suggested that YTHDF1-mediated glutamine metabolism reduces the sensitivity of CRC cells to cisplatin (215). Specifically, YTHDF1 targets the m^{6}A of glutaminase 1 (GLS1) mRNA 3'UTR to promote its translation. And METTL3 deletion inhibits LDHA mRNA translation by reducing the binding of YTHDF1 to LDHA mRNA CDS, thereby hindering glycolysis and promoting 5-fluorouracil sensitivity in CRC cells (216). Interestingly, the rs8100241 G>A mutation in ANKLE1 increases ANKLE1 levels in an m^{6}A-YTHDF1-dependent fashion, thereby inhibiting proliferation and maintaining the genomic stability of CRC (217).

In addition, YTHDF2 often collaborates with "writers" and participates in CRC progression. For example, METTL3 downregulates YPEL5 in an m^{6}A-YTHDF2-dependent manner and boosts CRC progression (218). METTL14 exerts an inhibitory effect in CRC by promoting the degradation of SYR-related high-mobility-group box 4 (SOX4) mRNA and long noncoding RNA XIST, which is dependent on YTHDF2 (219, 220). Han et al. deciphered that glutaminolysis inhibition increases ATF4 expression through FTO-mediated demethylation and YTHDF2-regulated decay, which further inactivates mTOR and promotes pro-survival autophagy of CRC cells (221). Moreover, in CRC, silencing of microRNA-6125 destabilizes GSK3β mRNA by upregulating the expression of YTHDF2, ultimately increasing WNT/β-catenin/Cyclin D1 pathway-related proteins and promoting CRC growth (222). Intriguingly, Zhou et al. found that HIF-1α-induced upregulation of lncRNA STEAP3-AS1 activates the WNT/β-catenin signaling pathway through overexpression of STEAP3, leading to CRC progression in a hypoxic environment (223). Specifically, after combining YTHDF2, STEAP3-AS1 prohibits STEAP3 mRNA from binding with YTHDF2, thus antagonizing STEAP3 mRNA decay.

Moreover, Ni et al. revealed that the long noncoding RNA GAS5-YAP-YTHDF3 axis forms a feedback loop in CRC (224). In detail, the downregulation of GAS5 enhances CRC proliferation and invasion by inhibiting phosphorylation and ubiquitin-mediated decay of YAP, which positively regulates YTHDF3. And YTHDF3 promotes the degradation of GAS5 mRNA by recognizing the m^{6}A in GAS5 mRNA. Furthermore, YTHDF3 recruits eIF2AK2 and eIF3A on the 5'UTR of target mRNAs and promotes translation in oxaliplatin-resistant CRC (225).

5.2 Respiratory system cancers

The expression of YTHDF1 and YTHDF2 is markedly upregulated in tumor tissues of lung cancer series and possesses tumor-promoting activities (226). Shi et al. demonstrated that YTHDF1 is amplified and increases the translation of key regulators of the G0/G1 cell cycle

transition, including CDK2, CDK4, and cyclin D1 mRNAs, intensifying non-small cell lung cancer (NSCLC) progression under normoxia conditions (227). In addition, microRNA-376c, delivered by endothelial cells through extracellular vesicles, inhibits the YTHDF1 and WNT/β-catenin pathway in NSCLC cells, resulting in the malignant progression of NSCLC cells (228). Nevertheless, under cisplatin-induced oxidative stress, YTHDF1 deficiency activates the antioxidant Nrf2-AKR1C1 axis by inhibiting the Keap1 mRNA transition, which resulted in cisplatin resistance and poor prognosis. Furthermore, the YTHDF1-m^{6}A-enolase1 (ENO1) translation axis is a crucial pathway for stimulating glycolysis and tumorigenesis (229). In KRAS and TP53 co-mutated lung adenocarcinomas, YTHDF1 recognizes m^{6}A modification and contributes to tumor proliferation and poor prognosis through the upregulation of cyclin B1 (230).

In addition, YTHDF2 promotes translation but not clearance of 6-phosphogluconate dehydrogenase (6PGD) mRNA in an m^{6}A-dependent manner by interacting with eIF3a/b, which enhances the pentose phosphate pathway (PPP) flux for tumor growth (231). The transcriptional repressor ZBTB4 and the tumor suppressor DAPK2 are negatively regulated by YTHDF2 and significantly associates with smoking-induced lung cancer (232, 233). However, ALKBH5 attenuates YTHDF2-mediated downregulation of oncogenic drivers such as SOX2, SMAD7, and MYC, contributing to the progression of aggressive lung cancer with KRAS mutation/LKB1 loss (234). Furthermore, YTHDF2 produces a positive effect on lung adenocarcinoma progression through the mRNA decay of AXIN1, a negative regulator of the WNT/β-catenin pathway (235). YTHDF2 produces the same effect in a VIRMA-m^{6}A-dependent fashion in lung adenocarcinoma and NSCLC by reducing BTG2 mRNA and DAPK3 mRNA stability, respectively (236, 237). Nevertheless, YTHDF2 induces sensitivity of lung adenocarcinoma to gefitinib *via* cleavage of circASK1 (238). Interestingly, YTHDF2 promotes proliferation and downregulates the FAM83D-TGFβ1-SMAD2/3 pathway to inhibit migration and invasion in lung adenocarcinoma cells (239). In lung squamous cell carcinoma, up-regulation of YTHDF2 under hypoxic conditions activates the mTOR/AKT signaling pathway and induces EMT to play a tumor-promoting role (240).

Interestingly, YTHDF1 and YTHDF2 regulate YAP expression by competitively binding to YTHDF3-m^{6}A-YAP mRNA, thereby aggravating and attenuating the malignancy behavior of NSCLC, respectively (241). YTHDF1/3 recruits eIF3a/b to promote YAP mRNA translation, while YTHDF2/3 recruits AGO2 to promote YAP mRNA decay. And YTHDF3 indirectly increased YAP levels to empower NSCLC progression and drug resistance by enhancing MALAT1 mRNA stability (242).

5.3 Urogenital system cancers

5.3.1 Bladder cancer

YTHDF family plays a tumor-promoting role in bladder cancer. Specifically, METTL3 and YTHDF1 are closely related to malignant transformation and tumorigenesis in the presence of chemical carcinogens, with the m^{6}A-methylated 3'UTR promoting oncogene CDCP1 translation (243). Moreover, YTHDF1/3 promotes aggressive phenotypes by translating ITGA6 mRNA, while YTHDF2 facilitates migration by degrading the mRNAs of the tumor suppressors SETD7 and KLF4 (244, 245).

5.3.2 Prostate cancer

YTHDF2 acts as a facilitator and is negatively regulated by miR-493-3p in prostate cancer (PCa) (246). Du et al. considered that KDM5A abrogates the inhibition of miR-495 on YTHDF2, and then upregulated YTHDF2 intensifies PCa progression by inducing m^{6}A-MOB3B mRNA decay (247). In addition, YTHDF2 clears METTL3-mediated m^{6}A-dependent mRNA of LHPP, NKX3-1, and USP4 (248, 249). The decrease of LHPP and NKX3-1 causes PCa proliferation and migration by inducing AKT phosphorylation. And downregulated USP4 promotes ARHGDIA expression by reducing ELAVL1 protein, thus accelerating invasion and metastasis of PCa. METTL14-mediated m^{6}A modification of Thrombospondin 1 (THBS1) mRNA promotes PCa proliferation in a YTHDF2-dependent manner of transcriptome degradation (250).

5.3.3 Breast cancer

In breast cancer, high expression of YTHDF1 and YTHDF3 is associated with gene copy number amplification and induces a poor prognosis (251, 252). YTHDF1 targets FOXM1 mRNA and positively regulates breast cancer progression (253). Additionally, hypoxia-mediated downregulation of miR-16-5p restored YTHDF1 expression, thereby promoting tumor glycolysis by enhancing PKM2 mRNA translation (254). Sun et al. demonstrated that YTHDF1 stabilizes E2F8 mRNA, which accelerates DNA damage repair and chemoresistance to adriamycin, cisplatin, and the PARP inhibitor olaparib in breast cancer cells (255). YTHDF1/eEF1-mediated translational elongation of KRT7 mRNA and YTHDF3-induced mRNAs translation of ST6GALNAC5, GJA1, and EGFR is involved in breast cancer lung and brain metastasis, respectively (57, 256). And YTHDF3 can be antagonized by miR-106b-5p (257). Moreover, YTHDF3 stabilizes ZEB1 mRNA to promote the invasion and migration of triple-negative breast cancer (TNBC) cells (258). Furthermore, YTHDF2 is upregulated in TNBC cells and prevents cell apoptosis (259, 260). YTHDF2 also targets the m^{6}A site 5'UTR region of ATF3 mRNA to mitigate the resistance of breast cancer cells to tamoxifen (261).

5.3.4 Ovarian cancer

YTHDF1 and YTHDF2 are considered oncogenes in ovarian cancer. YTHDF1 is recruited to the m^{6}A site of EIF3C mRNA and stimulates EIF3C as well as overall protein translation (262). YTHDF1 also confers cisplatin-resistant ovarian cancer cells with CSC-like traits by promoting m^{6}A-TRIM29 mRNA translation (263). Furthermore, FBW7 abrogates the mRNA degradation of YTHDF2 on pro-apoptotic gene BMF by inducing YTHDF2 decay, disrupting ovarian cancer progression (264). Moreover, YTHDF2 can be directly targeted and inhibited by miR-145 in ovarian cancer cells (265).

5.3.5 Cervical cancer

In cervical cancer (CC) cells, YTHDF1 accelerates m^{6}A-augmented glycolysis and cancer progression by promoting translational elongation of pyruvate dehydrogenase kinase 4 (PDK4) mRNA and

stabilization of hexokinase 2 (HK2) mRNA (56, 266). Specifically, the YTHDF1/eEF-2 complex binds the m^{6}A site of PDK4 mRNA at the 5'UTR and YTHDF1 recognizes the m^{6}A-modified 3'UTR of HK2 mRNA. Furthermore, YTHDF1 plays a tumor-promoting role by facilitating mitosis-associated RANBP2 mRNA translation in an m^{6}A-mediated approach, while YTHDF2 exerts the same role by degrading the tumor suppressor GAS5 mRNA (267, 268). YTHDF2 deficiency suppresses the proliferation of CC cells, promotes apoptosis, and arrests the cells at the S phase (269). YTHDF2 can also facilitate EMT and cisplatin resistance in CC cells by stabilizing AXIN1 mRNA (270).

5.3.6 Endometrial cancer

YTHDF1 and YTHDF2 modulate the negative regulator PHLPP2 and positive regulator mTORC2 of AKT respectively, which is unfavorable to the tumorigenicity of the AKT pathway in endometrial cancer (EC) (271). In addition, YTHDF2-mediated transcript degradation of IRS1 is accompanied by inhibition of the AKT/MMP9 signaling pathway, thereby impairing the activity of endometrial cells (272). And YTHDF2 deficiency activates the WNT signaling pathway by reducing the decay of HOXB13 mRNA, and thus promotes EC invasion and metastasis (273). Conversely, YTHDF2 degrades lncRNA FENDRR to enhance the expression of SOX4, which ultimately promotes EC cell proliferation and hinders apoptosis (274).

5.4 Cancers in other systems

5.4.1 Glioblastoma

YTHDF1 and YTHDF2 were found to be highly overexpressed in glioblastoma (GBM) tissues compared to normal tissues (275). YTHDF1 is required for maintaining GBM CSC properties and promoting proliferation, migration, and chemoresistance (276). And Musashi-1(MSI1) is a GBM hyper-oncogenic regulator and positively regulates YTHDF1 expression. YTHDF1 also assists METTL3 in increasing levels of ADAR1 and thereby stimulates GBM cell growth (277). In addition, YTHDF2 is positively regulated by the EGFR/SRC/ERK pathway and facilitates the malignancy progression of GBM by degrading downstream transcripts, including LXRα, HIVEP2, UBXN1, and ASS1 mRNAs in an m^{6}A-dependent manner (43, 278, 279). Among them, LXRα and ASS1 are related to cholesterol homeostasis and arginine metabolism, respectively. Strikingly, YTHDF2 recognizes m^{6}A methylation to maintain MYC mRNA stability, thereby promoting the expression of the downstream effector IGFBP3, leading to GBM CSC growth (280). And this process occurs specifically in GBM CSCs but not in normal neural stem cells (NSCs). Chen et al. verified that YTHDF2 promotes temozolomide desensitization in GBM cells (281). Mechanistically, YTHDF2 activates PI3K/AKT and NF-κB signaling pathways by targeting the 3'UTR and downregulating the mRNAs stability of EPHB3 and TNFAIP3.

5.4.2 Melanoma

YTHDF1 is amplified in melanoma, and the combination of YTHDF1 and HNRNPA2B1 significantly increases the diagnostic validity (282). However, YTHDF1 inhibits ocular melanoma progression by facilitating HINT2 mRNA translation (283). YTHDF2 knockdown promotes tumor growth and reduces the sensitivity of anti-PD-1 therapy by enhancing the mRNAs stability of the intrinsic genes PD-1 (PDCD1), CXCR4, and SOX10 in an m^{6}A-dependent fashion (284). Yu et al. discovered that histone lactylation promotes YTHDF2 expression in ocular melanoma, and YTHDF2 stimulates tumorigenesis by degrading m^{6}A-modified PER1 and TP53 mRNAs (285). Similarly, YTHDF3 also promotes ocular melanoma progression by promoting CTNNB1 mRNA translation in an m^{6}A-dependent manner (286).

5.4.3 Merkel cell carcinoma

The occurrence of Merkel cell carcinoma (MCC) is mostly attributed to the attack of the small T antigen of Merkel cell polyomavirus (MCPyV) (287). Meanwhile, overexpression of YTHDF1 improves the proliferative and clonogenic capacity of MCC cells by recruiting eIF3a/b to promote the translation initiation of small T antigen mRNA. Mechanistically, overexpression of YTHDF1 is caused by increased gene copy number.

5.4.4 Acute myeloid leukemia

Nguyen et al. first reported that YTHDF2 is identified as a novel acute myeloid leukemia1 (AML1) T translocation partner gene (288). Notably, YTHDF2 is highly expressed in different AML subtypes (289). And inhibition of YTHDF2 specifically impairs AML initiation and progression while expanding hematopoietic stem cells (HSCs) and maintaining normal hematopoietic function. In detail, YTHDF2 promotes the development and propagation of AML CSCs by degrading multiple m^{6}A-modified mRNAs such as TNF receptor superfamily member 1b (TNFRSF1b) that are associated with the functional integrity of AML CSCs. Moreover, the AML1/ETO-HIF1α loop transactivates the YTHDF2 promoter to promote t (8, 21) AML cell proliferation (290). However, YTHDF2 may interfere with the glycolytic process of AML cells by destabilizing transcripts of phosphofructokinase platelet (PFKP) and lactate dehydrogenase B (LDHB) (291). Interestingly, the three YTHDFs can jointly degrade the associated transcripts and inhibit the differentiation of AML cells (63) (Figures 4–6) (Tables 1–3).

6 Limitations and perspectives

Although it has been revealed that the YTHDF family is involved in a variety of biological processes as the "readers" of m^{6}A modification, there are still many mysteries about the YTHDF family that need to be discovered and solved in terms of structure, function, and treatment.

The discussion of the structure and function of YTHDFs is partially doubtful due to the limitations of technology and conditions. The reason why YTHDFs select the same or different target mRNAs and m^{6}A sites on mRNAs, and why YTHDFs pair with different cooperating m^{6}A regulators, has not been reached. In addition, YTHDFs can be localized in different cellular compartments and may re-enter the nucleus or transport out of the cell membrane, thus expanding the regulation of YTHDFs. The post-

transcriptional modifications of YTHDFs and interactions of YTHDFs with other proteins also add to the structure and function complexity of YTHDFs. Therefore, the development of emerging technologies, the control of various conditions, and the change of different stimulus states are necessary to further investigations in the YTHDF family.

At present, many experiments have successfully constructed the YTHDF1/2/3 genetic KO mouse model using different techniques. First, the whole-body YTHDF1/2/3 KO mice are generated directly based on CRISPR/Cas9 by deleting a certain exon or inducing the premature appearance of a stop codon (87, 88, 110, 213). Second, the Cre/LoxP technique is used to generate cell-specific conditional YTHDF1/2/3 KO mice (74, 84, 108, 165, 167, 174, 227, 289). This represents an improvement in experimental research moving from *in vitro* to *in vivo*. However, the specific mutation of functional RNA binding sites of YTHDFs in mice needs to be further realized. In addition, one of the important purposes of experimental research is clinical transformation, so it is of great need to explore the application value of targeting YTHDFs in the clinic, especially in tumors. Many clinical-related studies have analyzed the expression profile of the m^{6}A regulator in tumors and its association with the immune microenvironment, grading, staging, therapeutic effect, and prognosis. For example, the analysis of 162 HCC samples from the Zhou et al. and 177 HCC samples from the Nakagawa et al. showed that YTHDF1 was related to poor prognosis of HCC and YTHDF2 was related to HCC recurrence, respectively (169, 182). YTHDF1 was associated with a poor prognosis of GC in a study of 379 patients with GC (164). Interestingly, high expression of YTHDF1 and YTHDF2 was associated with a better prognosis in 603 cases of resected NSCLC, which might be due to increased tumor-infiltrating lymphocytes (TILs) and decreased co-inhibitor molecule PD-L1 (226).

In addition, an assessment of single nucleotide polymorphisms (SNPs) in the YTHDF1 gene in 313 cases of hepatoblastoma showed that rs6090311 A>G was correlated with a reduced risk of hepatoblastoma (292). A similar SNPs assessment found that the YTHDF2 rs3738067 variant significantly increased glioma risk in 171 pediatric patients (293). Moreover, increasing evidence confirms the efficacy of bioinformatics analysis based on TCGA and other databases for the YTHDFs-associated model. To sum up, the expression of YTHDFs is significantly correlated with the grades and stages of various tumors and may be used as indicators to judge the occurrence and development of tumors. YTHDFs may act as independent prognostic factors for many tumors and affect survival-related indicators such as overall survival (OS), disease-free survival (DFS), and progression-free survival (PFS). At the therapeutic level, targeting YTHDFs can not only directly modulate the malignancy behavior of tumors, but also affect the sensitivity of chemotherapy and immunotherapy. Besides, YTHDFs also have the possibility of effective clinical application in non-cancer, including hematopoietic, anti-obesity, anti-viral, and anti-inflammatory.

However, studies of YTHDFs are still in the preclinical stage and many issues need attention. First, the clinical application of YTHDFs in different diseases, alone or in combination with other targets, requires further investigation. Second, the effectiveness of YTHDFs in diagnosing and predicting prognosis may vary across disease types, grades, and stages. Most importantly, the specific molecules targeting YTHDFs have not yet been developed. So how can YTHDFs be used in clinical treatment? The expression of YTHDFs can be regulated by other strategies. Targeting upstream or metabolic mechanisms of YTHDFs is an alternative approach to indirectly regulate the levels of YTHDFs (Figure 7). YTHDF2 has the capability of inhibiting the

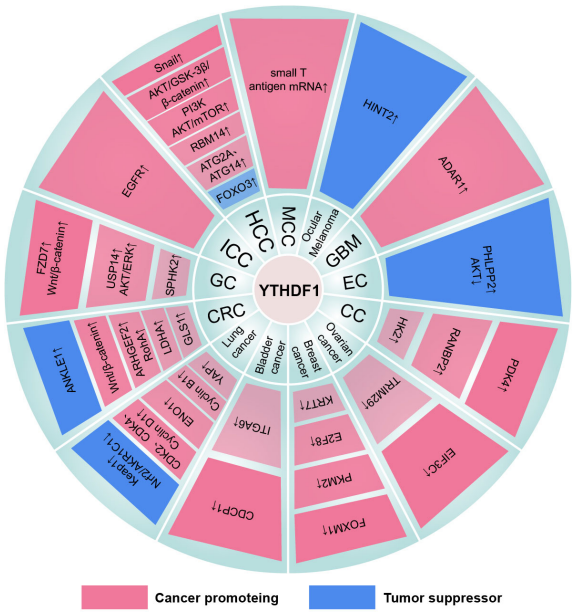

FIGURE 4
The mechanism of the YTHDF1 family in cancers. "↓" is the decrease of target mRNAs. "↑" is the increase of target mRNAs.

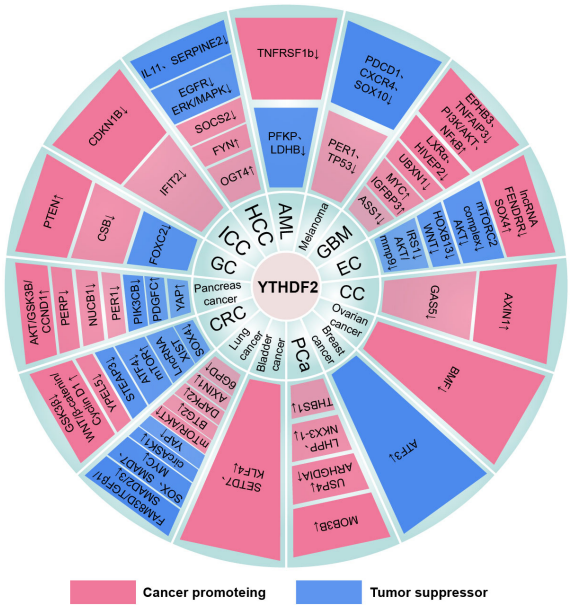

FIGURE 5
The mechanism of the YTHDF2 family in cancers. "↓" is the decrease of target mRNAs. "↑" is the increase of target mRNAs.

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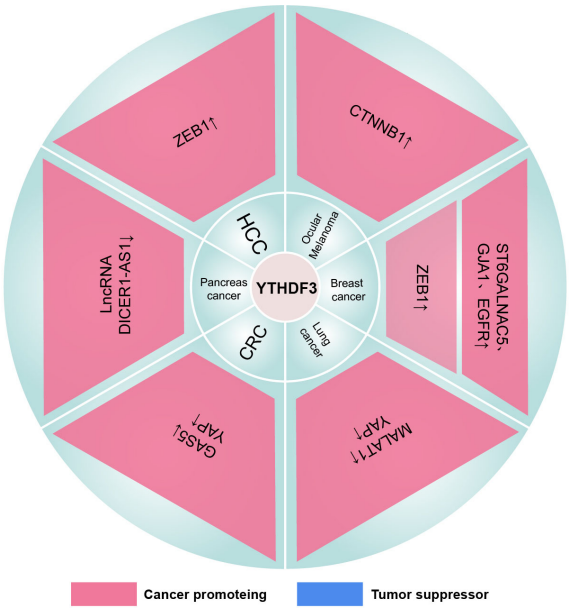

FIGURE 6
The mechanism of the YTHDF3 family in cancers. "↓" is the decrease of target mRNAs. "↑" is the increase of target mRNAs.

progression of HCC, and this effect can be antagonized by HIF-2α (180). Therefore, the HIF-2α antagonist (PT2385) can indirectly restore the effect of YTHDF2. And CDK1 inhibitors promote YTHDF2 proteolysis in AML (294). Furthermore, the delivery of target genes using viral vectors is also a feasible approach to target YTHDFs. YTHDF1 overexpression therapy can be achieved by injecting adeno-associated virus (AAV)-YTHDF1 into the hippocampus of diabetic cognitively impaired mice (116). In conclusion, clarifying the limitations of YTHDFs is conducive to better clinical transformation.

7 Conclusions

With multi-omics advancement, the roles of m^{6}A modification have been gradually and seriously excavated. By binding to m^{6}A, the YTHDF family plays an important role in the regulation of various physiological and pathological processes, including embryonic development, stem cell fate, fat metabolism, neuromodulation, cardiovascular effect, viral infection, immunity, and especially in tumors. In particular, YTHDFs regulate multiple tumor phenotypes such as proliferation, metastasis, metabolism, drug resistance, and immunity. Additionally, YTHDFs can be used as biomarkers for the diagnosis, treatment, and predictors of

TABLE 1 The role of the YTHDF1 in cancers.

Cancers	Roles	Cooperative m6A regulators	Target mRNAs	"Reading" position	The mechanism of target mRNAs	Functional classification	References
Hepatocellular carcinoma	Oncogene	METTL3	Snail	CDS	Promoting translation	EMT and metastasis	(55)
		METTL3	RBM14	–	Promoting expression	Growth and metastasis; Kupffer cells polarization	(173)
		–	ATG2A and ATG14	CDS	Promoting translation	Hypoxia-induced autophagy, growth, and metastasis	(174)
		METTL3	EGFR	5'UTR	Promoting translation	Viability and metastasis	(175)
	Tumor suppressor	METTL3	FOXO3	3'UTR	Increasing stability	Sorafenib sensitivity	(176)
Intrahepatic cholangiocarcinoma	Oncogene	–	EGFR	3'UTR	Promoting translation	Proliferation, migration, and invasion	(188)
Gastric cancer	Oncogene	–	FZD7	3'UTR	Promoting translation	Proliferation and metastasis	(192)
		–	USP14	CDS	Promoting translation	Proliferation and metastasis	(193)
		METTL3	SPHK2	–	Promoting translation	Proliferation, migration, and invasion	(194)
Colorectal cancer	Oncogene	–	ARHGEF2	3'UTR	Promoting translation	Growth and metastasis	(213)
		–	GLS1	3'UTR	Promoting translation	Cisplatin resistance	(215)
		METTL3	LDHA	CDS	Promoting translation	Glycolysis and 5-fluorouracil resistance	(216)
	Tumor suppressor	METTL3/14 and WTAP	ANKLE1	–	Promoting translation	Proliferation	(217)

(Continued)

TABLE 1 Continued

Cancers	Roles	Cooperative m6A regulators	Target mRNAs	"Reading" position	The mechanism of target mRNAs	Functional classification	References
Lung cancer	Oncogene	–	CDK2, CDK4, and cyclin D1	–	Promoting translation	Proliferation	(227)
		METTL3 and ALKBH5	ENO1	CDS	Promoting translation	Glycolysis and growth	(229)
		–	cyclin B1	3'UTR	Promoting translation	Proliferation	(230)
		METTL3 and ALKBH5	YAP	–	Promoting translation	Growth and metastasis	(241, 242)
	Tumor suppressor	–	Keap1	–	Promoting translation	Cisplatin sensitivity	(227)
Bladder cancer	Oncogene	METTL3 and ALKBH5	CDCP1	3'UTR	Promoting translation	Growth	(243)
		METTL3 and ALKBH5	ITGA6	3'UTR	Promoting translation	Adhesion, migration, and invasion	(244)
Breast cancer	Oncogene	–	FOXM1	CDS	Promoting translation	Proliferation and metastasis	(253)
		–	PKM2	CDS	Promoting translation	Glycolysis, growth, and metastasis	(254)
		METTL14	E2F8	–	Increasing stability	Growth, DNA damage repair, and chemoresistance	(255)
		FTO	KRT7	CDS	Promoting translation	Lung Metastasis	(57)
Ovarian cancer	Oncogene	–	EIF3C	–	Promoting translation	Proliferation and metastasis	(262)
		–	TRIM29	3'UTR	Promoting translation	The CSC-like phenotype	(263)
Cervical cancer	Oncogene	METTL3	PDK4	5'UTR	Promoting translation	Glycolysis, proliferation, and doxorubicin resistance	(56)
		METTL3	HK2	3'UTR	Increasing stability	Warburg effect and Proliferation	(266)
		–	RANBP2	–	Promoting translation	Growth, migration, invasion, and apoptosis	(267)
Endometrial cancer	Tumor suppressor	METTL3/14	PHLPP2	–	Promoting translation	Proliferation	(271)
Glioblastoma	Oncogene	METTL3	ADAR1	–	Promoting translation	Proliferation	(277)
Ocular melanoma	Tumor suppressor	METTL3 and ALKBH5	HINT2	3'UTR	Promoting translation	Growth and migration	(283)
Merkel cell carcinoma	Oncogene	–	small T antigen	–	Promoting translation	Proliferation and Cloning	(287)

The meaning of the symbol "-" is that the specific content has not yet been revealed in the corresponding research.

TABLE 2 The role of the YTHDF2 in cancers.

Cancers	Roles	Cooperative m6A regulators	Target mRNAs	"Reading" position	The mechanism of target mRNAs	Functional classification	References
Hepatocellular carcinoma	Oncogene	METTL3	SOCS2	–	Promoting degradation	Proliferation, migration, and colony formation	(184)
		–	OCT4	5'UTR	Promoting translation	CSC phenotype and cancer metastasis	(185)
		–	FYN	–	Increasing stability	EMT and metastasis	(186)
	Tumor suppressor	–	EGFR	3'UTR	Promoting degradation	Proliferation and growth	(179)
		–	IL11 and SERPINE2	3'UTR	Promoting degradation	Inflammation, vascular reconstruction, and metastatic progression	(180)
Intrahepatic cholangiocarcinoma	Oncogene	METTL3	IFIT2	–	Promoting degradation	Proliferation, apoptosis, cell cycle process, invasion, and migration	(189)
		METTL3	CDKN1B	–	Promoting degradation	Proliferation, apoptosis, cell cycle process, and cisplatin resistance	(190)
Gastric cancer	Oncogene	METTL3	PTEN	–	Promoting degradation	Proliferation, migration, and invasion	(196)
		METTL3	CBS	CDS	Decreasing stability	Ferroptosis and chemoresistance	(197)
	Tumor suppressor	–	FOXC2	–	Decreasing stability	Proliferation, migration, and invasion	(198)
Pancreas cancer	Oncogene	METTL14	PERP	3'UTR	Decreasing stability	Growth and metastasis	(200)
		ALKBH5	PER1	3'UTR	Promoting degradation	Proliferation and metastasis	(201)
		METTL3	NUCB1	5'UTR	Promoting degradation	Growth and GEM resistance	(202)
	Tumor suppressor	METTL3/14 and WTAP	PIK3CB	–	Decreasing stability	Proliferation and migration	(203)
		FTO	PDGFC	3'UTR	Decreasing stability	Proliferation	(204)
Colorectal cancer	Oncogene	METTL3	YPEL5	CDS	Promoting degradation	Growth and metastasis	(218)
		–	GSK3β	3'UTR	Promoting degradation	Proliferation	(222)
	Tumor suppressor	METTL14	SOX4	–	Promoting degradation	migration, invasion, and metastasis	(219)
		METTL14	XIST	–	Promoting degradation	Proliferation and metastasis	(220)
		FTO	ATF4	–	Decreasing stability	Autophagy	(221)
		METTL14	STEAP3	–	Promoting degradation	Proliferation and metastasis	(223)
Lung cancer	Oncogene	–	6PGD	3'UTR	Promoting translation	Growth	(231)
		METTL3	DAPK2	–	Decreasing stability	Proliferation and migration	(233)
		–	AXIN1	–	Promoting degradation	Proliferation and metastasis	(235)
		VIRMA	BTG2	3'UTR	Decreasing stability	Proliferation and metastasis	(236)

(Continued)

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TABLE 2 Continued

Cancers	Roles	Cooperative m6A regulators	Target mRNAs	"Reading" position	The mechanism of target mRNAs	Functional classification	References
		VIRMA	DAPK3	3'UTR	Promoting degradation	Proliferation, migration, and invasion	(237)
	Tumor suppressor	ALKBH5	SOX2, SMAD7, and MYC	–	Decreasing stability	Proliferation and migration	(234)
		METTL3	circASK1	–	Promoting degradation	Gefitinib sensitivity	(238)
		–	FAM83D	–	Promoting degradation	Migration and invasion	(239)
		METTL3 and ALKBH5	YAP	–	Promoting degradation	Growth and metastasis	(241)
Bladder cancer	Oncogene	METTL3	SETD7 and KLF4	–	Promoting degradation	Migration	(245)
Prostate cancer	Oncogene	–	MOB3B	–	Promoting degradation	Proliferation, migration, invasion, and apoptosis	(247)
		METTL3	LHPP and NKX3-1	–	Promoting degradation	Proliferation and migration	(248)
		METTL3	USP4	CDS	Promoting degradation	Invasion and metastasis	(249)
		METTL14	THBS1	–	Promoting degradation	Proliferation	(250)
Breast cancer	Tumor suppressor	–	ATF3	5'UTR	Decreasing stability	Tamoxifen sensitivity	(261)
Ovarian cancer	Oncogene	–	BMF	3'UTR	Promoting degradation	Proliferation	(264)
Cervical cancer	Oncogene	ALKBH5	GAS5	–	Promoting degradation	Growth and metastasis	(268)
		–	AXIN1	–	Increasing stability	EMT and cisplatin resistance	(270)
Endometrial cancer	Oncogene	FTO	FENDRR	–	Promoting degradation	Proliferation and apoptosis	(274)
	Tumor suppressor	METTL3/14	mTORC2	–	Promoting degradation	Proliferation	(271)
		METTL14 and ALKBH5	IRS1	CDS	Promoting degradation	Proliferation and invasion	(272)
		FTO	HOXB13	3'UTR	Promoting degradation	Invasion and metastasis	(273)
Glioblastoma	Oncogene	–	LXRα and HIVEP2	–	Promoting degradation	Proliferation, invasion, and cholesterol dysregulation	(43)
		METTL3	UBXN1	–	Promoting degradation	Proliferation and migration	(278)
		METTL14	ASS1	–	Promoting degradation	Proliferation, migration, and invasion	(279)
		METTL3	MYC	–	Increasing stability	CSC growth	(280)
		–	EPHB3 and TNFAIP3	3'UTR	Decreasing stability	Temozolomide resistance	(281)
Melanoma	Tumor suppressor	FTO	PDCD1, CXCR4, and SOX10	5'UTR and 3'UTR	Promoting degradation	Growth and anti-PD-1 blockade immunotherapy sensitivity	(284)

(Continued)

TABLE 2 Continued

Cancers	Roles	Cooperative m6A regulators	Target mRNAs	"Reading" position	The mechanism of target mRNAs	Functional classification	References
Ocular melanoma	Oncogene	–	PER1 and TP53	3'UTR	Promoting degradation	Proliferation and migration	(285)
Acute myeloid leukemia	Oncogene	–	TNFRSF1b	–	Promoting degradation	The development and propagation of AML CSCs	(289)
		–	TNFRSF1b	3'UTR	Decreasing m^6A levels	Proliferation	(290)
	Tumor suppressor	FTO	PFKP and LDHB	–	Promoting degradation	Glycolysis	(291)

The meaning of the symbol "-" is that the specific content has not yet been revealed in the corresponding research.

TABLE 3 The role of the YTHDF3 in cancers.

Cancers	Roles	Cooperative m6A regulators	Target mRNAs	"Reading" position	The mechanism of target mRNAs	Functional classification	References
Hepatocellular carcinoma	Oncogene	–	ZEB1	–	Increasing stability	Metastasis	(177)
Pancreas cancer	Oncogene	–	DICER1-AS1	–	Decreasing stability	Glycolysis, proliferation, and metastasis	(209)
Colorectal cancer	Oncogene	–	GAS5	–	Promoting degradation	Proliferation and invasion	(224)
Lung cancer	Oncogene	METTL3	MALAT1	–	Increasing stability	Cisplatin resistance, growth, and metastasis	(242)
Breast cancer	Oncogene	–	ST6GALNAC5, GJA1, and EGFR	–	Promoting translation	Brain metastasis	(256)
		–	ZEB1	–	Increasing stability	Migration, invasion, and EMT	(258)
Ocular melanoma	Oncogene	–	CTNNB1	–	Promoting translation	Proliferation and migration	(286)

The meaning of the symbol "-" is that the specific content has not yet been revealed in the corresponding research.

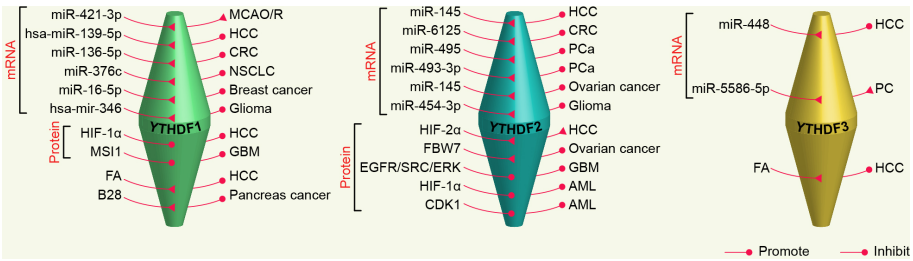

FIGURE 7
The upstream regulations of the YTHDF family.

prognosis evaluation. On-going explorations of YTHDFs in modeling disease progression are still warranted for a better and deeper understanding of epigenetic modifications.

Author contributions

LC collected the related papers and drafted the manuscript. YG made the figures and revised the manuscript. SX edited and revised the manuscript. JG designed the framework and revised the manuscript. JY, MW, and TL revised the manuscript. 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.

Publisher's note

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EDITED BY
Zichuan Liu,
Tianjin University, China

REVIEWED BY
Xin Mu,
Tianjin University, China
Zhi-Wei Wang,
Wenzhou Medical University, China
Naoe Taira Nihira,
St. Marianna University School of Medicine, Japan

*CORRESPONDENCE
Xueju Wang
xueju@jlu.edu.cn

†These authors share first authorship

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The role of E3 ubiquitin ligases and deubiquitinases in bladder cancer development and immunotherapy

Xuemei Wang†, Ying Zhang†, Yao Wu, Hongjing Cheng and Xueju Wang*

Department of Pathology, China-Japan Union Hospital, Jilin University, Changchun, Jilin, China

Bladder cancer is one of the common malignant urothelial tumors. Post-translational modification (PTMs), including ubiquitination, acetylation, methylation, and phosphorylation, have been revealed to participate in bladder cancer initiation and progression. Ubiquitination is the common PTM, which is conducted by E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-protein ligase. E3 ubiquitin ligases play a key role in bladder oncogenesis and progression and drug resistance in bladder cancer. Therefore, in this review, we summarize current knowledge regarding the functions of E3 ubiquitin ligases in bladder cancer development. Moreover, we provide the evidence of E3 ubiquitin ligases in regulation of immunotherapy in bladder cancer. Furthermore, we mention the multiple compounds that target E3 ubiquitin ligases to improve the therapy efficacy of bladder cancer. We hope our review can stimulate researchers and clinicians to investigate whether and how targeting E3 ubiquitin ligases acts a novel strategy for bladder cancer therapy.

KEYWORDS

E3 ligases, bladder cancer, immunotherapy, resistance, ubiquitination

Abbreviations: ANKRD1, ankyrin repeat domain 1; BET, bromodomain and extraterminal domain; CSN5, COP9 signalosome subunit 5; CTGF, connective tissue growth factor; CYR61, cysteine-rich angiogenic inducer 61; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; GRIM19, gene associated with retinoid-interferon-induced mortality-19; LAPTM5, lysosomal-associated multispanning membrane protein 5; mTORC1, mammalian target of rapamycin complex 1; MIBC, muscle invasive bladder cancer; MRE11, meiotic recombination 11 homolog; NSCLC, non-small cell lung cancer; NSR-SCCUB, non-schistosomiasis related-squamous cell carcinoma of urinary bladder; NEDD4, neuronally expressed developmentally downregulated 4; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand-1; PI3K, phosphoinositide 3-kinase; POI, protein of interest; PROTACs, proteolysis targeting chimeras; PTMs, post-translational modifications; qRT-PCR, quantitative real-time polymerase chain reaction; SIRT1, silent information regulator sirtulin 1; SOX2, Sex-determining region Y-box 2; SREBP1a, sterol regulatory element-binding protein 1a; TCGA, the Cancer Genome Atlas; TCR, T cell receptor; TILs, tumor-infiltration T cells; USP7, ubiquitin-specific processing protease 7.

Introduction

Bladder cancer is one of the common malignant tumors worldwide (1). It was estimated that there are 82,290 new cases and 16,710 deaths in 2023 in the United States. In men, bladder cancer was the eight-leading cause of cancer-associated death in the United States (2). In the world, there were approximately 573,278 new cases with bladder cancer and 212,536 deaths due to this disease (3, 4). Tobacco smoking could be a reason for bladder cancer incidence. In addition, some risk factors, such as chemicals and aromatic amines, arsenic contamination and aluminum, could increase the bladder cancer development. The treatments of bladder cancer often include endoscopic resection, chemotherapy, radiation, intravesical immunotherapy and combination therapy (5–7). The gold standard therapy for MIBC was chemotherapy and radical cystectomy. Bladder-sparing trimodal therapy is also available for MIBC patients. Chemoimmunotherapy is the key strategy for bladder cancer with metastatic feature (8). The treatment of immunotherapy and immune checkpoint inhibitors has not shown the good efficacy in bladder cancer patients (9). However, bladder cancer exhibits immune evasion and poor outcomes, suggesting that novel therapies need to be developed for treating bladder cancer (10).

Several genes have been known to regulate development and aggressiveness in bladder cancer, including Wnt, STAT3, PI3K, AKT, mTOR and PTEN (11–15). For instance, monocarboxylate transporter isoform 1 (MCT1) has been found to govern aggressive and metabolic phenotypes in bladder cancer because higher expression of MCT1 was associated with lymph node, poor survival and distant metastasis (16, 17). Silencing of MCT1 blocked proliferation, invasion, migration and altered the expression of EMT-associated proteins (16). MCT1, MCT4 and CD147 displayed a prognostic implication and a potential role in bladder cancer metabolism (18). MCT1 and CD147 also participated in cisplatin resistance and tumor aggressiveness in bladder cancer (19). In addition, some proteins could be post-translationally modified, including phosphorylation, ubiquitination, acetylation, glycosylation, methylation and SUMOylation (20). Post-translational modification (PTM) has been known to govern tumorigenesis and progression in various cancer types, including bladder cancer (21, 22).

PTMs include ubiquitination, acetylation, phosphorylation, methylation, hydroxylation, lipidation, palmitoylation, and glycosylation (23–27). Autophagy-lysosome pathway and the ubiquitin-proteasomal system (UPS) are common PTMs to control protein stability (28, 29). Ubiquitination is an ATP-mediated process: an E1 ubiquitin-activating enzyme activates ubiquitin, E2 ubiquitin-conjugating enzyme links ubiquitin via a transesterification reaction, E3 ubiquitin-protein ligase makes the binding between E2 enzyme and substrate proteins, leading to ubiquitin transfer from E2 to the specific substrate (30, 31). E3 ubiquitin ligases are critically involved in oncogenesis and progression as well as drug resistance in bladder cancer (32, 33). Targeting E3 ubiquitin ligases has demonstrated to be a novel approach for bladder cancer therapy (34). In this review, we summarize current knowledge regarding the roles of E3 ubiquitin ligases in bladder oncogenesis. Furthermore, we discuss the insights of E3 ubiquitin ligases in regulation of immunotherapy in bladder cancer. Moreover, we highlight the efforts on targeting E3 ubiquitin ligases to improve the efficacy of bladder cancer treatments. We hope our review can encourage researchers to explore how can improve the benefit of bladder cancer therapy via targeting E3 ubiquitin ligases.

Deubiquitinases in bladder cancer

USP2a

USP2a has been reported to regulate oncogenesis and progression in a variety of human cancers (35–37). USP2a mRNA expression was reduced in bladder cancer tissues compared with age-matched bladder tissues, and USP2a mRNA expression was decreased in higher stage of MIBC (38). Kim et al. found that USP2a increased tumor progression in part via regulation of cyclin A1 in bladder cancer (39). Specifically, overexpression of USP2a increased cell invasion, migration, chemotherapeutic drug resistance and proliferation. Downregulation of USP2a showed the opposite effects in bladder cancer. USP2a overexpression increased the Erk/MAPK phosphorylation after HB-EGF stimulation in T24 cells. Overexpression of USP2a in T24 cells caused more resistance to cisplatin-induced apoptosis due to inhibition of the cleaved form of PARP (c-PARP). USP2a interacted with cyclin A1 and blocked the ubiquitination of cyclin A1, contributing to cyclin A1 accumulation, which led to promotion of cell proliferation in bladder cancer (39). Frizzled 8-associated APF (antiproliferative factor) maintained the stability of p53 via modulation of USP2a and murine double minute 2 (MDM2) (40). APF decreased USP2a expression and caused MDM2 ubiquitination, leading to inhibition of the interaction between p53 and MDM2, thereby impairing p53 ubiquitination (40). Overexpression of USP2a increased cell growth through upregulation of cyclin D1 at the mRNA and protein levels, while depletion of USP2a reduced cell proliferation in part via increased cellular p53 levels in T24 cells (40).

USP21

USP21 has been gradually uncovered the essential role in carcinogenesis (41, 42). One integrative assay of 1q23.3 copy number gain in urothelial cancer patients with metastasis after platinum-based chemotherapy demonstrated that USP21, F11R, PPOX, DEDD, PFDN2 genes were closed linked to poor outcomes (43). Similarly, USP14 and USP21 were found to be associated with chemoresistance in bladder urothelial carcinoma with metastasis (44). Another study showed that USP21 expression was elevated in bladder cancer. High expression of USP21 was closely correlated with tumor metastasis and tumor size. Intriguingly, poorer survival rate was found in bladder cancer patients with higher levels of USP21 (45). In bladder cancer cells,

increased expression of USP21 promoted cell proliferation, stimulated cell migration and invasion, enhanced tumor metastasis (45). Notably, overexpression of USP21 led to the development of EMT. Mechanistically, USP21 deubiquitinated EZH2 and stabilized its protein levels. USP21 could be a potential target for bladder cancer therapy (45). PD-L1 is observed in membrane of immune cells ad tumor cells. PD-L1 can bind to PD-1, leading to protection of tumor cells from an immune attack. The inhibitors of PD-1/PD-L1 can impair this binding and enhance the immune response against tumor cells (46, 47). USP21 has been identified to act as a deubiquitinase of PD-L1. Increased USP21 elevated PD-L1 abundance, whereas depletion of USP21 promoted PD-L1 degradation. Hence, targeting USP21 could be helpful to improve tumor immunotherapy (48).

USP22

USP22 has been known to involve in tumor cell proliferation, invasion, stemness, cell cycle arrest, metastasis, immune response and drug resistance in human cancer (49). In bladder cancer, silencing USP22 by siRNAs induced cell cycle arrest and attenuated cell proliferation (50). USP22 siRNA transfection increased the expression of p53 and p21, decreased cyclin E expression in bladder cancer cells. Silencing of USP22 promoted the degradation of MDM2 in bladder cancer cells. USP22 siRNA transfection induced cell cycle at G0/G1 phase via upregulation of p53, p21 and downregulation of cyclin E in bladder cancer cells (50). Depletion of USP22 expression retarded the tumor growth of implanted bladder cancer cells in mice (50). Another study also revealed that USP22 depletion reduced cell cycle progression and retarded tumor growth in animal models of bladder cancer, liver cancer, lung cancer, breast cancer and ovarian cancer (51). USP22 has been reported to regulate immune evasion and drug sensitivity in cancer (52). USP22 has been identified to work as a new regulator of PD-L1. USP22 interacted with PD-L1 and maintained PD-L1 stability via deubiquitination in A549, H1299 and H1792 NSCLC cells (53). USP22 also interacted with CSN5 and kept its stability via deubiquitination. Either CSN5 or USP22 enhanced the binding of PD-L1 with the other one. The K6, K11, K27, K29, K33 and K63-linked ubiquitin chains were removed by USP22 in PD-L1 and CSN5 in HEK293FT cells. Hence, USP22 governed the PD-L1 protein levels via CSN5/PD-L1 pathway in HEK293FT cells (53). Silencing of USP22 enhanced T cell cytotoxicity and blocked lung tumorigenesis. This study showed a critical role of USP22 in regulation of immune evasion via maintenance of PD-L1 protein levels (53). It is required to define the role of USP22 in bladder tumorigenesis.

E3 ubiquitin ligases in bladder cancer

FBXW7

FBXW7 belongs to F-box protein family and shows a tumor suppressive function in cancer development (54). F-box proteins target numerous substrates and regulate proliferation, metastasis, EMT, cancer stem cells, and drug resistance (55–57). FBXW7 exhibited single nucleotide variants or insertion or deletion in non-schistosomiasis related-squamous cell carcinoma of urinary bladder (NSR-SCCUB) patients (58). NSR-SCCUB is not common type in urothelial carcinoma, which could have genomic alterations (58). FBXW7 targeted an epigenetic regulator ZMYND8 for ubiquitination and degradation in bladder cancer (59). ZMYND8 increased cell viability and colony formation, migrative ability in bladder cancer. FBXW7 interacted with and degraded ZMYND8 in a polyubiquitin-dependent manner. By a gene set enrichment analysis, ZMYND8 was observed to be positively correlated to tumor stemness markers, including FOXM1, SOX2 and NANOG (59). One group revealed that overexpression of p65 increased cell migration via FBXW7-induced ubiquitination and degradation of RhoGDIα protein in bladder cancer (60). RhoGDIα protein was found to be a p65 downstream target and mediated p65-induced cell migration in bladder cancer. Mechanistically, p65 enhanced FBXW7 stability via attenuating the mRNA transcription of PTEN (60). Hence, p65 inhibited PTEN mRNA transcription and subsequently promoted FBXW7 stability, leading to degradation of RhoGDIα in bladder cancer cells (60). Liu et al. found that upregulation of FBXW7 reduced the invasion and growth of bladder cancer cells, caused cell cycle arrest at G0/G1 phase. Increased FBXW7 activated GSK-3β phosphorylation and inhibited the expression of SREBP1a in bladder cancer cells (61). SREBP1 is a transcription factor, including two isoforms, SREBP-1a and SREBP-1c, which regulates the expression of lipogenesis genes. Studies have shown that SREBP1 regulates the expression of stearoyl-CoA desaturase, fatty acid synthase, and acetyl-CoA carboxylase (62). FBXW7 can bind with SREBP1a by a co-immunoprecipitation assay. *In vivo* study further validated the role of FBXW7 in regulation of SREBP1a (61). The role of FBXW7 in bladder cancer indicated that targeting FBXW7 is a novel approach for bladder cancer therapy.

MDM2

MDM2 (mouse double minute 2 homologue) is involved in tumorigenesis mainly targeting p53 protein in different cancer types, including bladder cancer (63, 64). In 1994, upregulation of MDM2 and p53 expression was observed in bladder cancer patients (65). Moreover, p53 and MDM2 were found to be key factors in the progression of bladder cancer (66). There was an association between TP53 (codon 72, arginine> proline), MDM2 (SNP309, T>G) polymorphisms and patient's survival in bladder cancer after chemoradiotherapy (CRT) (67). Patients with MDM2 T/G + G/G genotypes exhibited a good survival rate after CRT. TP53 and MDM2 with more than two of variant alleles exhibited an improved survival (67). For example, MDM2 SNP309 G-variant was revealed to be correlated with tumor cell invasive growth and the risk of bladder cancer (68, 69). Mao et al. found that OCT3/4 increased tumor immune escape via upregulation of TET1 and NRF2 expression, leading to enhancement of MDM2 expression, which contributed to acceleration of tumor immune evasion in

bladder cancer (70). Small-molecule MDM2 inhibitors have been detected in clinical trials for improving the efficacy of cancer treatment (71, 72). MDM2 inhibitor APG-115 was reported to enhance the efficacy of PD-1 blockade via increasing anticancer immunity in the tumor microenvironment (73). One MDM2 inhibitor, AMG-232, sensitized tumor cells to T-cell-induced killing in tumors with high expression of MDM2 (74). The MDM2 ligand Nutlin-3 modulated the expression of PD-L1 and CD276 (75). Nutlin-3 induced the expression of PD-L1, while MDM2 did not bind PD-L1 (75). Suppression of MDM2 by HDM201 inhibitor facilitated anticancer responses via interaction with the stromal and immune microenvironment in tumor cells with p53 wild-type (76). MDM2 gene amplification could be a useful biomarker for prediction of a better response for targeted therapies in PD-L1 positive or negative urothelial bladder cancer (77).

TRIM38

TRIM38 functions as a SUMO ligase or an E3 ubiquitin ligase and targets several cellular signaling components (78). Glucose transporter type 1 (GLUT1) was upregulated in bladder cancer and correlated with poor survival rate and poor prognosis in patients with bladder cancer (79, 80). Moreover, GLUT1 was identified as an independent biomarker for prognosis in bladder cancer patients after radical cystectomy treatment (81). GLUT1 was also taken part in cisplatin resistance in bladder cancer, which can be regulated by miR-218 (82). According to TCGA bladder cancer database, TRIM38 expression was low in bladder cancer patients. Lower expression of TRIM38 was linked to shorter survival rate and worse prognosis in patients with bladder cancer (83). TRIM38 was further found to regulate proliferation, stemness and invasion of bladder cancer cells. Strikingly, TRIM38 had an interaction with GLUT1 and enhanced the ubiquitination and degradation of GLUT1 in bladder cancer cells. Accordingly, BAY-876, an inhibitor of GLUT1, inhibited proliferation and tumor growth in bladder cancer cells and mouse models (83).

Other deubiquitinases and E3 ubiquitin ligases

Accumulating evidence has shown that many E3 ubiquitin ligases are involved in bladder tumorigenesis. For instance, the E3 ubiquitin ligase cIAP2 (cellular inhibitor of apoptosis protein 2) was elevated after inhibition of histone deacetylase (HDAC) in bladder cancer. MRE11, which regulates DNA repair pathways and double-strand breaks, was also inhibited by HDAC inhibitors (84). The cIAP2 was found to bind with MRE11 and governed radio-sensitization after HDAC inhibitor treatment. cIAP2 modulated the ubiquitination of MRE11 and caused the downregulation of MRE11 in bladder cancer cells (84). Therefore, cIAP2 might be a promising target for improving chemoradiation strategy in bladder cancer. Suppression of GRIM19 expression impaired ubiquitination-mediated degradation of Bcl-xL in bladder cancer cells, conferring to promotion of cisplatin chemoresistance (85). Overexpression of GRIM19 potentiated cisplatin sensitivity and reduced the invasion and proliferation of bladder cancer cells, which was due to attenuation of Bcl-xL polyubiquitination and degradation (85).

Yes-associated protein (YAP) is one of key effectors in the Hippo tumor suppressor pathway, which regulates organ size and tissue growth and tumorigenesis (86, 87). Luo et al. reported that MINDY1, a DUB enzyme, interacted with YAP and acted as a deubiquitylase of YAP to stabilize YAP protein levels in bladder cancer (88). Consistently, silencing of MINDY1 reduced proliferation of bladder cancer cells. Overexpression of YAP abrogated the MINDY1 depletion-induced inhibition of cell proliferation in bladder cancer cells (88). Connective tissue growth factor (CTGF) controls differentiation, adhesion and proliferation, and involves in Hippo pathway, NF-κB and p53 pathways, leading to regulation of cancer, inflammation and fibrosis (89). Cysteine-rich protein 61 (CYR61) was reported to involve in the development of melanoma (90), glioma (91) and esophageal squamous cell carcinoma (92). Exosomal miR-217 mimic promoted migration and proliferation in 5637 and T24 cells via upregulation of YAP and its targets, such as CTGF, CYR61 and ANKRD1 (93). Downregulation of MINDY1 disrupted the YAP stabilization and inhibited the expression of YAP downstream genes, such as CTGF, CYR61 and ANKRD1 in bladder cancer (88). MINDY1 could be a possible biomarker and therapeutic target for bladder cancer (88). RNF126 (ring finger protein 126), acting as a E3 ubiquitin ligase, has been reported to be overexpressed in numerous cancer types and correlated with tumorigenesis (94). RNF126 expression was elevated in bladder cancer tissues via a TCGA database analysis. Depletion of RNF126 remarkably impaired proliferation and metastasis of bladder cancer cells via modulation of the EGFR/PI3K/AKT pathway. RNF126 silencing reduced EGFR expression and AKT phosphorylation, slightly inhibited PI3K expression, and remarkably increased the PTEN protein levels in UMUC3 and T24 cells. The mRNA levels of AKT and EGFR were reduced after RNF126 downregulation, but PTEN mRNA levels did not change in RNF126-silencing cells. Notably, PTEN was identified as a new substrate of RNF126 (95). RNF126 bound to PTEN and led to polyubiquitination and degradation of PTEN. Inhibition of RNF126 oncoprotein could be a novel approach for bladder cancer therapy (95). It has been known that c-Cbl is an E3 ubiquitin ligase that targets its substrates for degradation (96). C-Cbl was reported to target the EGFR for ubiquitination and degradation (97). Another study revealed that USP8 can regulate SOX2 ubiquitination and degradation in bladder cancer (98).

Deubiquitinases and E3 ubiquitin ligases regulate immunotherapy

The E3 ubiquitin ligases have been approved as important factors to govern the tumor microenvironment and affect immunotherapy in human cancers (99). Evidence has dissected that the E3 ubiquitin ligases control PD-1/PD-L1 protein levels and

enhance tumor immunotherapy (100). For example, FBXO38, FBXW7 and C-Cbl target PD-1, whereas SPOP and FBXO22 target PD-L1. In addition, USP7, USP8 and USP22 target PD-L1 to maintain the PD-L1 protein levels (100, 101).

RNF144A regulates PD-L1

RNF144A is an E3 ubiquitin ligase for the degradation of DNA-PKcs (DNA-dependent protein kinase catalytic subunit), leading to promotion of apoptosis during DNA damage (102, 103). RNF144A governed PARP inhibitor sensitivity via targeting PARP1 in ubiquitin-dependent manner in breast cancer cells (104). In addition, RNF144A expression was decreased due to promoter hypermethylation in breast cancer cells (105). Moreover, RNF144A targeted the stability of HSPA2 via ubiquitin-dependent regulation in breast cancer (106). Furthermore, RNF144A degraded YY1 and inhibited the expression of GMFG as well as suppressed oncogenesis in breast cancer (107). RNF144A maintained the activation of EGFR signaling pathway to enhance EGF-involved cell proliferation (108). RNF144A controlled the stability of LIN28B *via* the uniquitin-proteasome manner and inhibited stem cell properties in ovarian cancer cells (109).

In bladder cancer cells, depletion of RNF144A elevated the stabilization of PD-L1 protein and enhanced carcinogen-mediated bladder oncogenesis (110). Mice with RNF144A deficiency were more prone to initiation of bladder cancer after carcinogen exposure. RNF144A knockout mice displayed the higher expression of PD-L1. RNF144A can bind with PD-L1 and enhanced ubiquitination and disruption of PD-L1 in the intracellular vesicles and plasma membrane (110). RNF144A depletion in mice caused a decrease of tumor infiltration CD8+ T-cells in the carcinogen-induced bladder cancer. Moreover, RNF144A depletion stimulated cellular differentiation, showing that a luminal subtype marker GATA3 was increased in RNF144A knockout tumors (110). This phenotype could be due to that RNF144A maintained EGFR expression. Hence, depletion of RNF144A increased the expression of PD-L1, DNA-PKcs and BMI1, resulting in the carcinogen-mediated the development of bladder cancer (110).

NEDD4 regulates PD-L1

An E3 ubiquitin ligase NEDD4 (also known as NEDD4-1) belongs to NEDD4 family, which has shown a critical function in carcinogenesis and progression (111, 112). NEDD4 performs its biological functions via targeting numerous substrates for ubiquitination and degradation (113, 114). NEDD4 has been revealed to regulate many functions, including growth, cell cycle, proliferation, differentiation, invasion, motility, apoptosis, necrosis, autophagy and metastasis (115). NEDD4 has been identified to take part in bladder cancer initiation and development. Inhibition of LAPTM5 blocked cell viability and growth and caused cell cycle arrest at G0/G1 phase via inhibition of p38 and ERK1/2 activation in bladder cancer (116). Depletion of NEDD4 suppressed the transportation of LAPTM5 from Golgi to lysosome, which could affect bladder tumorigenesis (116). Suppression of NEDD4 displayed antitumor activity in bladder cancer cells (117). Mao et al. found that NEDD4 can bind to KLF8 (Kruppel-like factor 8) and target the miR-132 and NRF2 (nuclear factor E2-related factor 2) axis in bladder cancer, contributing to acceleration of tumor growth, recurrence and lung metastasis (118). NEDD4 depletion reduced K63-linked polyubiquitination of KLF8 and inhibited the stability and transcriptional ability of KLF8 (118). NEDD4 promoted the interaction between KLF-8 and miR-132 promoter region, resulting in suppression of miR-132. Moreover, miR-132 inhibited the expression of NRF2 in bladder cancer cells, leading to repression of cell migration and viability (118).

Fibroblast growth factor receptor 3 (FGFR3) has been known to play a key role in bladder cancer development. FGFR3 rearrangements and missense mutations were reported in bladder cancer (119). One study showed that suppression of FGFR3 increased PD-L1 protein levels in FGFR3-expressing bladder cancer due to influencing its ubiquitination, leading to suppression of the anticancer activity of CD8+ T cells. FGFR3 expression was negatively associated with PD-L1 expression levels in bladder cancer tissues. FGFR3 activation can promote NEDD4 phosphorylation. NEDD4 catalyzed K48-linked polyubiquitination of PD-L1 *via* their interactions. CD8+ T-cell infiltration and anticancer ability were largely impaired because of upregulation of PD-L1 in bladder tumor cells in mice with NEDD4 knockout bladder cancer. Targeting FGFR3 and PD-L1 increased CD8+ T-cell-induced anticancer efficacy and exhibited effective tumor suppression in bladder cancer. This work provided a molecular clue among NEDD4, PD-L1 and FGFR3, suggesting that targeted therapy in combination with immune therapy could be much better for the treatment of bladder cancer. Therefore, NEDD4 targets PD-L1 for ubiquitination and destruction in FGFR3-overexpressing bladder cancer, indicating that NEDD4 is associated with immune surveillance *via* regulation of PD-L1 in bladder cancer (120). One group showed that a natural compound lycorine downregulated the expression of NEDD4 in bladder cancer, leading to suppression of cell growth and invasiveness (121). Hence, natural compounds targeting NEDD4 could be useful to improve immunotherapy in bladder cancer.

USP7 regulates PD-L1 expression

USP7 (ubiquitin-specific protease 7), also named as HAUSP (herpesvirus-associated protease), has been discovered to be associated with oncogenesis in some cancer types, including bladder cancer (122–125). USP7 has been revealed to control the anti-tumor immune responses. Inhibition of USP7 by its inhibitors impedes the activity of Treg cells, enhances polarization of tumor-related macrophages in tumor cells (126). It has been reported that USP7 modulated the expression levels of CCDC6 in bladder cancer. One USP7 inhibitor, P5091, regulated CCDC6 degradation and enhanced cell sensitivity to PARP inhibitors. Combined therapy with DNA damage inducer RRx-001 and P5091 promoted the tumor cell sensitivity to PARP inhibitors (127).

DNA methylation is regulated by DNMTs (DNA methyltransferases). SB216763, an inhibitor of GSK3 (glycogen synthase kinase-3), increased cell proliferation and upregulated the expression of pGSK3β, β-catenin and DNMT1 (128). The expression of USP7, DNMT1, UHRF1 and β-catenin was inhibited after re-expression of WIF-1 and treatment with DNMT1 inhibitor DAC (128). One study revealed that PD-L1 expression was positively associated with USP7 levels in gastric cancer patients. USP7 directly bound to PD-L1 and stabilize it (129). Abrogation of USP7 impaired the interaction between PD-1 and PD-L1, leading to sensitization of cancer cells to T cell killing in cancer cells and in mice. In addition, inhibition of USP7 by its inhibitor reduced cell proliferation due to p53 stabilization in gastric cancer cells (129). Hence, USP7 suppression by its inhibitors not only blocked gastric tumor cell proliferation but also inhibit the expression of PD-L1 to improve anti-cancer immune response in gastric cancer (129). It is required to explore whether USP7 inhibitors could enhance the immune response of bladder cancer. USP7 inhibitors have been developed to perform anticancer ability in various cancer types (130). It is necessary to determine whether these USP7 inhibitors can improve immunotherapy in bladder cancer.

Other E3 ubiquitin ligases regulate immunotherapy

One group used TCGA and GEO database to analyze ubiquitination-related molecular subtypes for bladder cancer (131). This group found a total of four ubiquitination-related molecular subtypes of bladder cancer. These four subgroups had various tumor microenvironment, prognosis, clinical characteristics and PD-L1 expression level. In addition, six ubiquitination-related genes (URGs), including HLA-A, UBE2D1, UBE2T, USP5, TMEM128 and UBE2N, could be useful for prognostic markers (131).

Compounds regulate E3 ligases in bladder cancer

In recent years, some compounds have been uncovered to regulate the expression of E3 ubiquitin ligases in human malignancies, including bladder cancer (132–134). β-lactam cephalosporin antibiotic cefepime has been uncovered to deplete PD-L1 and promote tumor DNA damage and increase sensitivity of DNA-damaging compounds in multiple tumor cell lines, such as bladder cancer, melanoma, GBM (glioblastoma multiforme) and ovarian cancer (135). Cefepime inhibited tumor PD-L1 via regulation of its ubiquitination, enhanced efficacy of DNA-damaging compounds in mice, stimulated immunogenic tumor STING pathway. Ceftazidime exhibited the similar performance as cefepime in regulation of PD-L1 and DNA-damaging agent therapeutic efficacy. Taken together, cefepime and ceftazidime could improve immunotherapy and DNA-damaging agent efficacy in bladder cancer (135). Hispolon from Phellinus linteus is a natural polyphenol and conducted a function as a cancer killer via targeting several signaling pathways (136). Hisplon inhibited tumor cell growth via upregulation of p21 in bladder cancer cells (137). Hispolon promoted the ubiquitination and degradation of MDM2 in bladder cancer cells. ERK1/2 was activated and recruited to MDM2 and led to MDM2 ubiquitination. Inhibition of ERK1/2 by U0126 blocked hispolon-mediated caspase-7 cleavage. Hence, hispolon downregulated MDM2 via degradation in bladder cancer (137).

Allyl isothiocyanate was often obtained from cruciferous vegetables and caused mitotic arrest via upregulation of ubiquitination and degradation of alpha and beta-tubulin in bladder cancer cells (138). PR-619 was an inhibitor of deubiquitylating enzymes and overcame cisplatin resistance via the inhibition of c-Myc in bladder urothelial carcinoma cells (44). Stevioside was identified by high-throughput screening as a useful compound to increase cell apoptosis via activation of GSK-3β and induction of FBXW7, contributing to downregulation of MCL-1 in bladder cancer (139). Similarly, OSU-T315, an inhibitor of integrin-linked kinase, was observed to inhibit Mcl-1 expression levels via targeting the GSK-3β/FBXW7 axis in bladder cancer cells (140). Green tea polyphenol EGCG plays a tumor suppressive role in bladder cancer via inactivation of NF-kappa B. Moreover, EGCG promoted the anticancer activity of doxorubicine via modulation of NF-κB/MDM2/p53 pathway in bladder cancer (141). Proguanil, which is often used as an anti-malarial drug, inhibited the cell growth by promotion of EGFR degradation and induction of autophagy in bladder cancer (97). Proguanil enhanced the interaction between EGFR and Caveolin-1, leading to endocytosis and recruiting c-Cbl to elevate EGFR degradation via the lysosomal pathway (97). 4-hydroxynonenal (HNE), a pro-oxidant compound, conducted tumor suppressive function via altering several signaling pathways. HNE upregulated YAP phosphorylation and ubiquitination, caused promotion of YAP proteasomal degradation in bladder cancer cells (142). One compound ChlA-F blocked cell invasion via inhibition of SOX2 protein by USP8-mediated SOX2 degradation in bladder cancer (98). Therefore, compounds can regulate E3 ubiquitin ligases to enhance the ubiquitination and degradation of specific targets, which lead to antitumor activity in bladder cancer (Table 1).

Noncoding RNAs target E3 ligases

Multiple studies have shown that noncoding RNAs govern carcinogenesis in bladder cancer (143–146). Noncoding RNAs are transcribed from DNA, but not translated into proteins, including microRNAs (miRNAs), lncRNAs (log noncoding RNAs), siRNAs (small interfering RNAs), snRNAs (small nuclear RNAs) and piRNAs (147–149). Noncoding RNAs target E3 ubiquitin ligases to control bladder cancer initiation and progression. For example, miR-143 inhibited the expression of MDM2 and performed a tumor suppressive function via inhibition of cell growth and migration in bladder cancer (150). LncRNA SNHG1 sponged miR-9-3p expression and upregulated the expression of MDM2 in bladder

TABLE 1 Compounds regulate E3 ligases in bladder cancer.

Item	Target	Function	Ref.
Cefepime	PD-L1 ubiquitination, activation of STING.	Enhances efficacy of DNA-damaging compounds	(135)
Ceftazidime	PD-L1 ubiquitination	Increases immunotherapy and DNA-damaging agent efficacy	(135)
Hispolon	MDM2 ubiquitination and degradation, p21 upregulation.	Inhibits tumor cell growth	(137)
Allyl isothiocyanate	Alpha and beta-tubulin ubiquitination and degradation	Causes mitotic arrest	(138)
PR-619	Inhibits c-Myc expression	Overcomes cisplatin resistance	(44)
Steviosode	Activates GSK-3β/FBXW7, inhibits Mcl-1.	Increases cell apoptosis	(139)
OSU-T315	Inhibits Mcl-1, targets GSK-3β/FBXW7	Reduces cell growth and increases apoptosis	(140)
EGCG	Targets NF-κB/MDM2/p53	Increase antitumor activity of doxorubicine	(141)
Proguanil	Promotes EGFR degradation	Induces autophagy	(97)
HNE	Upregulates YAP phosphorylation and ubiquitination and degradation	Performs tumor suppressive function	(142)
ChlA-F	Inhibits SOX2 *via* USP8-mediated degradation	Blocks cell invasion	(98)

cancer cells. MDM2 targeted PPARγ for ubiquitination and degradation, leading to facilitating the development of bladder cancer (151). LncRNA LNPPS displayed a tumor suppressive function via modulation of MDM2/p53 degradation in bladder cancer (152). LncRNA SNHG18 was downregulated in tumor specimens of bladder cancer patients. The bladder cancer patients with high expression of SNHG18 had a better survival rate. Upregulation of SNHG18 reduced proliferation of bladder cancer cells and decreased tumor sizes in mice (153). SNHG18 impaired the expression of c-Myc via targeting its ubiquitination and degradation, resulting in p21 upregulation in bladder cancer (153).

LncRNA PVT1 promoted the expression of MDM2 and accelerated the p53 ubiquitination and degradation, leading to promoting cell invasion and cell resistance to doxorubicin (154). AURKB (Aurora kinase B) was increased after MDM2 upregulation induced by lncRNA PVT1 in bladder cancer cells. AURKB further promoted the p53 ubiquitination that was induced by MDM2 (154). LncRNA LOC572558 overexpression was downregulated in tumor tissues of bladder cancer patients. In T24 and 5637 bladder tumor cells, upregulation of LOC572558 suppressed cell growth and invasion, induced apoptosis and caused cell cycle arrest, which was correlated with p53 phosphorylation, MDM2, AKT dephosphorylation (155). Chen et al. reported that a circRNA circNUDT21 altered the miR-16-1-3p/MDM2/p53 axis and accelerated tumor progression in bladder cancer (156). Hence, noncoding RNAs are pivotal to regulate E3 ubiquitin ligases in bladder tumorigenesis (Figure 1).

Conclusions and future perspectives

In conclusion, E3 ubiquitin ligases are critical in bladder cancer initiation and development via targeting specific substrates. E3 ubiquitin ligases alter tumor immunotherapy and drug resistance in bladder cancer (Figure 2). Targeting E3 ubiquitin ligases could be an effective strategy for bladder cancer therapy. It is necessary to mention several points regarding the roles of E3 ubiquitin ligases in bladder cancer. First, besides ubiquitination, there are many other

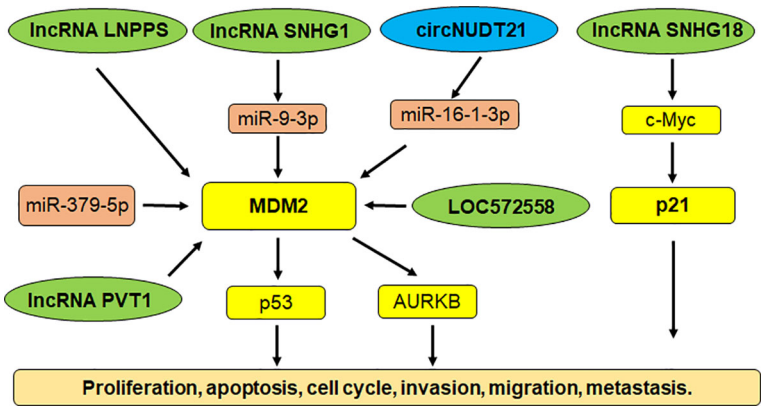

FIGURE 1
Noncoding RNAs regulate E3 ubiquitin ligases in bladder cancer.

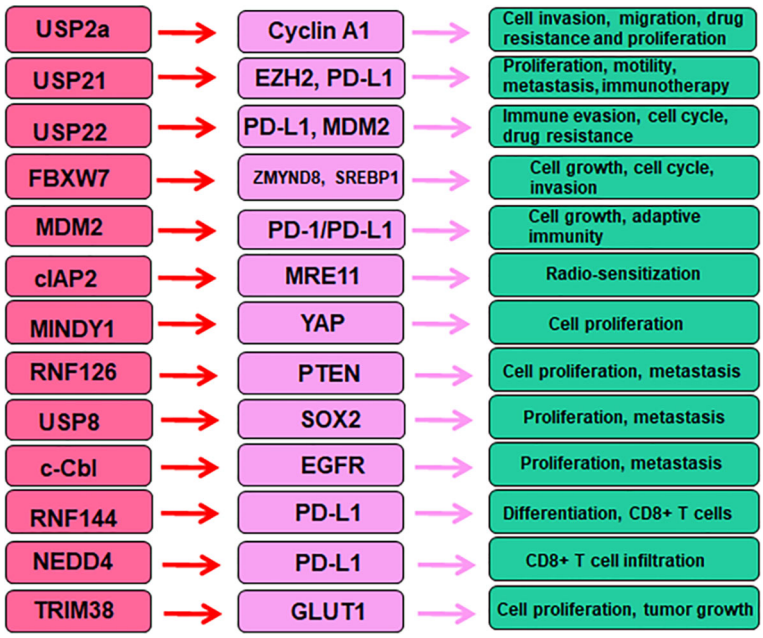

FIGURE 2
E3 ubiquitin ligases regulate several proteins in bladder cancer.

types of PTMs to involve in bladder tumorigenesis. For example, activation of autophagy altered acetylation profile relevant for mechanotransduction in bladder tumor cells (157). PD-L1 methylation was found to be an independent biomarker for patient survival in bladder cancer (158). Histone demethylase JMJD1A promoted glycolysis via coactivation of HIF1α and led to promotion of urinary bladder cancer progression (159). SIRT1 (silent information regulator sirtulin 1), a NAD+ dependent deacetylase, elevated the expression of GLUT1 and stimulated tumor progression in bladder cancer via modulation of glucose uptake (160).

Second, in addition to E3 ubiquitin ligases, E2 enzyme has also been involved in bladder carcinogenesis. Ubiquitin-conjugating enzyme E2S (UBE2S) is a type of E2 enzyme in the ubiquitin system, which has displayed several activities in carcinogenesis (161). UBE2S has been suggested to promote the ovarian cancer development via targeting PI3K/AKT/mTOR pathway and modulating cell apoptosis and cell cycle (162). UBE2S reduced cell chemosensitivity via regulation of PTEN-AKT pathway in hepatocellular carcinoma (163). UBE2S expression was increased in urinary bladder cancer cells. Knockdown of UBE2S led to reduction of proliferation and induction of cell apoptosis, while upregulation of UBE2S resulted in an inverse phenotype in bladder cancer cells (164). Moreover, UBE2S performed the oncogenic functions via modulation of the mTORC1 pathway in bladder cancer cells. UBE2S targeted tuberous sclerosis 1 (TSC1) for ubiquitous degradation (164). Collectively, UBE2S promoted bladder cancer progression via degradation of TSC1 and activation of mTOR signaling pathway.

Third, noncoding RNAs have been identified as potential biomarkers for bladder cancer prognosis (165, 166). Besides lncRNAs, miRNAs and circRNAs, one study showed that PIWI-interacting RNAs (piRNAs) and snRNAs are important in bladder carcinogenesis (167, 168). In this work, it has been shown that 106 piRNAs were increased and 91 piRNAs were decreased in bladder tumor specimens. Upregulation of piRABC reduced proliferation, colony formation, but enhanced cell apoptosis in bladder cancer cells. Moreover, piRABC increased the expression of TNFSF4 protein in bladder cancer cells (167). Fourth, several F-box proteins have been described to target PD-1/PD-L1 in cancers; however, whether other F-box proteins can regulate immunotherapy is unclear. For example, FBXO45 has shown an essential role in tumorigenesis and malignant progression (169–171). FBXO22 targeted PD-L1 for degradation and sensitized tumor cells to DNA damage (172). FBXO1, FBXO20, FBXO22, FBXO28, FBXO32 and FBXO45 have been found to be associated with immune infiltration in pancreatic cancer (173). Hence, it is required to explore whether these F-box proteins are involved in immunotherapy in bladder cancer.

Fifth, it has been validated that PROTACs are novel tools for the enhancement of immunotherapy in human cancers (174). PROTACs have been designed to degrade a protein of interest (POI), resulting in a reduction of the expression of the POI (175, 176). One study has shown that one BET (bromodomain and extraterminal domain) inhibitor mivebresib synergized with a Bcl-xL PROTAC degrader PZ703b increased cell apoptosis through the mitochondrial pathway in bladder cancer (177). Another study showed that BRD4 PROTAC degrader QCA570 increased the degradation of BRD4 protein, leading to induction of cell apoptosis and cell cycle arrest, which caused antiproliferation ability in bladder cancer (178). All in a word, E3 ubiquitin ligases are essential for the initiation and progression of bladder cancer. Regulation of E3 ubiquitin ligases might be a potential therapeutic strategy for bladder cancer treatment.

Data availability statement

The data in this study are available from the corresponding author on reasonable request.

Author contributions

XMW and YZ wrote the manuscript. YW and HC made the tables and figures. XJW edited and revised the manuscript. 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.

Publisher's note

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EDITED BY
Zichuan Liu,
Tianjin University, China

REVIEWED BY
Yuling Han,
Cornell University, United States
Jean-Marc Barret,
GamaMabs Pharma, France

*CORRESPONDENCE
Yan Kuang
kuangyan_2004@163.com

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The overexpression of actin related protein 2/3 complex subunit 1B(ARPC1B) promotes the ovarian cancer progression *via* activation of the Wnt/β-catenin signaling pathway

Junning Huang, Haiqin Zhou, Caichun Tan, Shien Mo, Tingji Liu and Yan Kuang*

Department of Gynecology, First Affiliated Hospital of Guangxi Medical University, Nanning, China

Introduction: Ovarian cancer is one of the most fatal malignancies of the female reproductive system. The purpose of this study is to explore the mechanism of Actin Related Protein 2/3 Complex Subunit 1B(ARPC1B) in the progression of ovarian cancer.

Methods: The expressions and prognostic value of ARPC1B in ovarian cancer were identified using the GEPIA database and the Kaplan-Meier Plotter database. The expression of ARPC1B was manipulated to evaluate its impact on the malignant phenotypes of ovarian cancer. The cell proliferation ability was analyzed through CCK-8 assay and clone formation assay. The cell migration and invasion capacity was evaluated through wound healing assay and trans well assay. Mice xenografts were conducted to measure the effects of ARPC1B on tumor development *in vivo*.

Results: Our data suggested that ARPC1B was overexpressed in ovarian cancer, which was correlated with a poorer survival compared to low mRNA expression of ARPC1B in ovarian cancer patients. The overexpression of ARPC1B promoted cell proliferation, migration, and invasion of ovarian cancer cells. Conversely, the knockdown of ARPC1B resulted in the opposite effect. Additionally, ARPC1B expression could activate Wnt/β-catenin signaling pathway. The administration of the β-catenin inhibitor XAV-939 abolished the promotion of cell proliferation, migration, and invasion activities induced by ARPC1B overexpression *in vitro*.

Conclusions: ARPC1B was overexpressed in ovarian cancer and was correlated with poor prognosis. ARPC1B promoted ovarian cancer progression through activation of Wnt/β-catenin Signaling Pathway.

KEYWORDS

ovarian cancer, actin related protein 2/3 complex subunit 1B, β-catenin, XAV-939, prognosis, oncogene

1 Introduction

Over the past several decades, significant progress has been made in improving cancer survival rates for most types of cancer (1). However, ovarian cancer remains a significant challenge. As the fifth most common cause of cancer-related death among women and the leading cause of mortality among gynecologic malignancies (1), ovarian cancer presents a critical public health concern. Unfortunately, the absence of specific symptoms and diagnostic biomarkers often leads to late diagnosis, with more than 70% of patients being diagnosed at clinical stage III or IV according to the Federation International of Gynecology and Obstetrics (FIGO) classification system (2). This results in a high mortality rate, with more than 75% of women with advanced ovarian cancer succumbing to the disease. In contrast, when ovarian cancer is diagnosed at an early stage, with the tumor confined to one or two sides of the ovaries, the cure rate can reach 90% (3). The current main therapeutic strategies for ovarian cancer include chemotherapy, surgery, and targeted therapy (4). Despite abundant research on the pathogenesis and therapy for ovarian cancer, there is still a lack of authoritative treatment. Therefore, the identification of effective predictive biomarkers for early diagnosis and personalized treatment is an urgent need in the field of ovarian cancer research.

Actin Related Protein 2/3 Complex Subunit 1B (ARPC1B), also known as ARC41, P41-ARC, P40-ARC, PLTEID, and IMD71, encodes one of seven subunits of the human Arp2/3 protein complex (5). This complex has been implicated in a variety of crucial biological functions, including regulation of cell differentiation, migration, adhesion, and cargo transport (6, 7). Recent studies have demonstrated that ARPC1B promotes cancer cell invasion and metastasis in several types of cancer, including glioblastoma and prostate cancer (8, 9). Additionally, ARPC1B has been linked to radiotherapy resistance, as ARPC1B-deficient patients exhibit increased sensitivity to ionizing radiation and the drug bleomycin (10).Furthermore, the overexpression of ARPC1B has been shown to promote radiotherapy resistance and maintain mesenchymal phenotype in glioma stem cells (11). Despite its known role in other types of cancer, the role of ARPC1B in ovarian cancer has not yet been reported in the literature.

β-catenin, also known as CTNNB1, is the key downstream component of the canonical Wnt/β-catenin signaling pathway (12). This pathway plays a crucial role in tumorigenesis and is activated in many ovarian epithelial carcinomas (13). Upon activation of the Wnt/β-catenin pathway, β-catenin is released from the cell membrane and redistributes to the nuclei and cytoplasm of tumor cells (14). Wnt/β-catenin signaling pathway is associated with tumor proliferation, metastasis, epithelial-to-mesenchymal transition (EMT), recurrence, chemoresistance, and anti-tumor immune regulation (15, 16). As such, the Wnt/β-catenin signaling pathway represent important targets for the development of new therapeutic strategies for ovarian cancer.

In this work, we conducted a bioinformatics analysis and found that the expression of ARPC1B was significantly elevated in ovarian cancer patients. Survival analysis revealed that high expression of ARPC1B was associated with poor overall survival and progression-free survival in these patients. We then explored the effects of modulating ARPC1B expression on ovarian cancer cells and found that it significantly influenced cell proliferation, metastasis, and invasion *in vitro*, as well as the growth of ovarian cancer tumors *in vivo*. Further investigation revealed that these effects were linked to the regulation of the Wnt/β-catenin signaling pathway.

2 Materials and methods

2.1 Databases and data analysis

The relationship between ARPC1B expression level and overall survival in ovarian cancer was assessed using Gene Expression Profiling Interactive Analysis (GEPIA) and Kaplan-Meier Plotter (KM plotter) databases that include the Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA), Genotype-Tissue Expression (GTEx), and The Cancer Genome Atlas (TCGA).

2.2 Clinical specimens

The study was approved by the Institutional Research Ethics Committee of Guangxi Medical University. In the study, ovarian specimens were collected from patients who were hospitalized in the First Affiliated Hospital of Guangxi Medical University from January 2021 to November 2022. The specimens were taken from both normal and cancer tissues and stored at -80°C for further experiments. All patients signed informed consent forms and none of them received any treatment before surgery. The normal ovarian specimens were taken from patients who underwent adnexectomy for uterine myoma or adenomyosis. The patients were between 18-70 years old with an average age of 46 years and all diagnoses were determined by pathological examination of the ovarian tissues.

2.3 Cell culture, cell transfection, and reagents

The ovarian epithelial cell line IOSE80 and ovarian cancer cell lines A2780, CAOV3, and SKOV3 were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China). The cells were propagated in RPMI-1640 medium (Procell, Wuhan, China) containing 10% fetal bovine serum (Procell) and incubated at 37°C under a humidified atmosphere containing 5% CO_2. Plasmid vectors expressing small hairpin RNA (shRNA) targeting ARPC1B were named shRNA1 or shRNA2. The complementary cDNAs of ARPC1B were synthesized and the plasmid overexpressed vectors pLV3-CMV-3×FLAG-CopGFP-Puro (Miaolingbio, Wuhan, China) of ARPC1B were constructed as ov-ARPC1B. The shRNA and scramble control sequences were listed in Table 1. The plasmid vectors were transfected into A2780 and SKOV3 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. To determine the effect of ARPC1B on the Wnt/β-catenin signaling

TABLE 1 The sequences of shRNAs and scramble control.

Group	Sequence
Scramble control	5'-CAACAAGATGAAGAGCACCAAT-3'
shRNA1	5'-GTGTGATCTCCATCTGTTATT-3'
shRNA2	5'-CCAAGGTGCACGAGCTCAAGG-3'

pathway, we used XAV-939 (MedChemExpress, USA), an inhibitor of β-catenin, at a concentration of 5.0 μM.

2.4 RNA extraction, rt-PCR, and RT-qPCR

The extraction of RNA was performed from ovarian cancer tissue or cell lines using a Total RNA Extraction Kit (Axygen, USA) according to the manufacturer's protocol. The genomic DNA present in the RNA samples was eliminated, and cDNA was synthesized using an RNA Reverse Transcription Kit (Servicebio, Wuhan, China). Subsequently, RT-qPCR was carried out using 2x Universal Blue SYBR Green qPCR Master Mix (Servicebio) on a CFX Touch Real-Time PCR Machine (Bio-rad, USA). The reaction mixture was subjected to denaturation at 95°C for 30 seconds, followed by 40 cycles of 15 seconds at 95°C, 10 seconds at 55°C, and 30 seconds at 72°C. The expression levels of ARPC1B was quantified using the $2^{-\Delta\Delta Ct}$ method. The primers for ARPC1B and β-actin were synthesized by GeneSys (Nanning, China), and their sequences are listed in Table 2.

2.5 Protein extraction and western blot

Total protein was extracted from ovarian cancer tissue or cell lines as per the manufacturer's instruction using RIPA (Solarbio, Beijing, China). The protein concentration was quantified using BCA Protein Assay Kit (Beyotime, Shanghai, China), and 40 μg of protein was subjected to electrophoresis on a 10% SDS-PAGE gel. The proteins were then transferred onto a 0.22 μm PVDF membrane (Merck, USA). The protein bands were incubated with primary antibodies at 4°C overnight, after being blocked with Non-Protein Blocking Solution (Servicebio). Subsequently, the membranes were incubated with dylight-800 labeled secondary antibodies (Invitrogen; 1:10000) at 37°C for one hour. The protein probes were visualized on Odyssey CLx (LI-COR, USA). The primary antibodies used were ARPC1B (Proteintech, Wuhan, China; 1:3000), β-tubulin (Proteintech; 1:2000), β-catenin (Servicebio; 1:1000), cyclin D1 (Huabio, Hangzhou, China; 1:2000), c-myc (ABmart, Shanghai, China; 1:500).

2.6 Cell counting Kit-8 assay (CCK-8 assay)

In accordance with the manufacturer's protocol, the Cell Counting Kit-8 (CCK-8) assay was employed to determine the effect of ARPC1B on cell proliferation. Infected cells were plated into 96-well plates and were cultured for 0 hours, 24 hours, 48 hours, 72 hours, and 96 hours. Then, a 10 μL aliquot of the CCK-8 kit (Servicebio) was added to each well and incubated at 37°C with 5% CO_2 for 2 hours. The optical density (OD) value was measured at 450 nm using a modular multimode microplate reader machine, the Synergy H1 (BioTek, USA).

2.7 Colony formation assay

After being infected for 24 h, 500 cells of SKOV3 or 1000 cells of A2780 were seeded in each well of a six-well plate to assess the impact of ARPC1B on cell clonogenesis. The cells were allowed to grow for 10-14 days, forming colonies which were then fixed with methanol and stained with 0.5% crystal violet. The number of colonies was subsequently counted.

2.8 Wound healing assay

The cellular migration ability was evaluated using the wound healing assay. After being infected for 24 hours, infected cells were plated into 6-well plates. After 24 hours of cell culture, a wound was achieved in each well by 1 mL pipette tips. The cells were rinsed with a serum-free medium. Photographic documentation of the distance between cells was taken at 0 hours, 24 hours, and 48 hours.

2.9 Transwell assay

After being infected for 24 h, infected cells were harvested and placed in the upper chambers of transwell inserts (Corning, USA), with a non-serum medium. In the lower chambers, 900 mL of medium containing 10% FBS was added, and the system was maintained for 48 hours. The cells were then fixed with methanol

TABLE 2 The primers for qRT–PCR analysis.

Gene	Primer	Sequence
ARPC1B	Forward	5'- GACAAGAAGATGGCCGTCGC -3'
	Reverse	5'- TGCGAGCTCTGCTTAGGAAC -3'
β-actin	Forward	5'- CTCAGGATTTAAAAACTGGAACG -3'
	Reverse	5'- GACAAAAAAGGGGGAAGGG -3'

and stained with 0.5% crystal violet (Servicebio). The number of migrated cells in the lower chambers was quantified to reveal the cell invasion ability.

2.10 Tumor xenograft model

Six-week-old BALB/c nude mice (Guangxi Medical University, Nanning, China) were handled and managed in accordance with the agreement approved by Guangxi Medical University. Exponentially growing infected cells (either SKOV3 or A2780 cells) were subcutaneously inoculated into the armpit region of the mice. One week after the injection, the tumor diameter was measured every 4 days and used to calculate the tumor volume. The mice were sacrificed on the 27th day after inoculation and the tumors were then utilized for Western Blot analysis.

2.11 Statistical analysis

The statistical analyses in this study were executed utilizing the R software version 4.2.2. The data were represented as the mean and standard deviation, derived from a minimum of three independent experiments. To assess the differences between two groups, the Student's t-test was employed, whereas, for comparisons among three or more groups, an ANOVA was conducted. A p-value of less than 0.05 was considered as statistically significant.

3 Results

3.1 The expression of ARPC1B and its co-relation of the overall survival of ovarian cancer

The relationship between ARPC1B expression level and overall survival in ovarian cancer was assessed using Gene Expression Profiling Interactive Analysis (GEPIA) and Kaplan-Meier Plotter (KM plotter) databases that include the Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA), and The Cancer Genome Atlas (TCGA). Our results showed that ARPC1B was significantly overexpressed in ovarian cancer tissues compared to normal tissues (Figures 1A). The survival analysis indicated that a high expression level of ARPC1B was associated with poorer overall

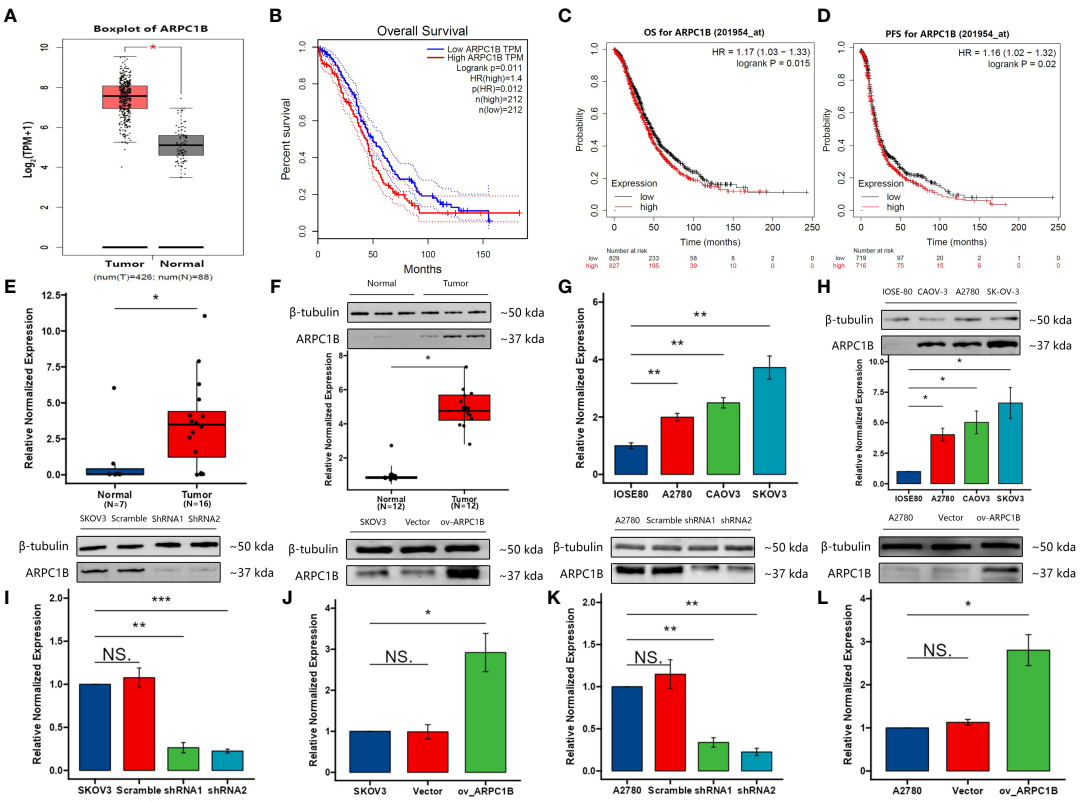

FIGURE 1
The expression of ARPC1B and its co-relation of the overall survival of ovarian cancer. **(A)** Boxplot of ARPC1B in ovarian cancer. TPM, transcripts per million. **(B)** Overall survival time between patients with high and low ARPC1B expression by GEPIA with median cut-off. Dotted lines indicated the 95% confidence interval. HR, hazard ratio. **(C, D)** Overall survival time and progression-free survival time between patients with high and low ARPC1B expression by KM Plotter with median cut-off. **(E, F)** Relative mRNA and protein expression of ARPC1B in normal ovarian tissues and ovarian cancer tissues. **(G, H)** Relative mRNA and protein expression of ARPC1B in ovarian epithelial cell line IOSE80 and ovarian cancer cell lines A2780, CAOV3, and SKOV3. **(I, J)** Relative protein expression of ARPC1B in infected SKOV3 cells. **(K, L)** Relative protein expression of ARPC1B in infected A2780 cells. Measurement data were expressed as mean ± SD of three independent experiments. NS, Not statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.

survival and progression-free survival in ovarian cancer patients (Figures 1B–D), suggesting the prognostic significance of ARPC1B. We determined the mRNA and protein expression of ARPC1B in ovarian tissue and ovarian cells, including ovarian epithelial cell line IOSE80 and ovarian cancer cell lines A2780, CAOV3, and SKOV3. The results demonstrated that ARPC1B was significantly overexpressed in ovarian cancer tissues compared to normal ovarian tissues, and significantly overexpressed in ovarian cancer cell lines compared to ovarian epithelial cell line (Figures 1E–H). ARPC1B expression was low in A2780 cells and high in SKOV3 cells (Figures 1G, H). We then artificially regulated the expression of ARPC1B in A2780 and SKOV3 cells and confirmed through Western Blot analysis (Figures 1I–L).

3.2 Effects of ARPC1B on the progression of ovarian cancer cells

Using the clone formation assay and CCK-8 assay, we evaluated the proliferation ability of ovarian cancer cells. The results of the clone formation assay showed that knocking down ARPC1B significantly decreased the proliferation of A2780 and SKOV3 cells, but the effect was weaker in A2780 cells (Figures 2A, B). Overexpression of ARPC1B significantly enhanced the proliferation of A2780 and SKOV3 cells, but the effect was weaker in SKOV3 cells (Figures 2C, D). The results of the CCK-8 assay were consistent with those of the clone formation assay. Knocking down ARPC1B significantly reduced the proliferation of A2780 and SKOV3 cells, but the effect was weaker in A2780 cells (Figures 2E, F). Overexpression of ARPC1B significantly increased the proliferation of A2780 and SKOV3 cells, but the effect was weaker in SKOV3 cells (Figures 2G, H). We also assessed the migration ability of ovarian cancer cells using the Transwell assay and wound healing assay. The results of the Transwell assay showed that knocking down ARPC1B significantly reduced the migration ability of A2780 and SKOV3 cells (Figures 3A, B), while overexpression significantly increased the migration ability of A2780 and SKOV3 cells, but the effect was weaker in SKOV3 cells (Figures 3C, D). The results of the wound healing assay were consistent with those of the Transwell assay. Knocking down ARPC1B significantly reduced the migration ability of A2780 and SKOV3 cells, but the effect was weaker in A2780 cells (Figures 3E, F). Overexpression of ARPC1B slightly increased the migration ability of A2780 and SKOV3 cells (Figures 3G, H). Overall, our results suggest that knocking down ARPC1B reduces the proliferation and migration ability of A2780 and SKOV3 cells, and this effect is generally more significant in SKOV3 cells than in A2780 cells. On the other hand, overexpression of ARPC1B enhances the proliferation and migration ability of A2780 and SKOV3 cells, and this effect is generally more significant in A2780 cells than in SKOV3 cells.

3.3 Effects of ARPC1B on the Wnt/β-catenin signaling pathway in ovarian cancer cells

To further explore the mechanism of the effects caused by ARPC1B, the expression of key proteins (β-catenin, c-myc, and

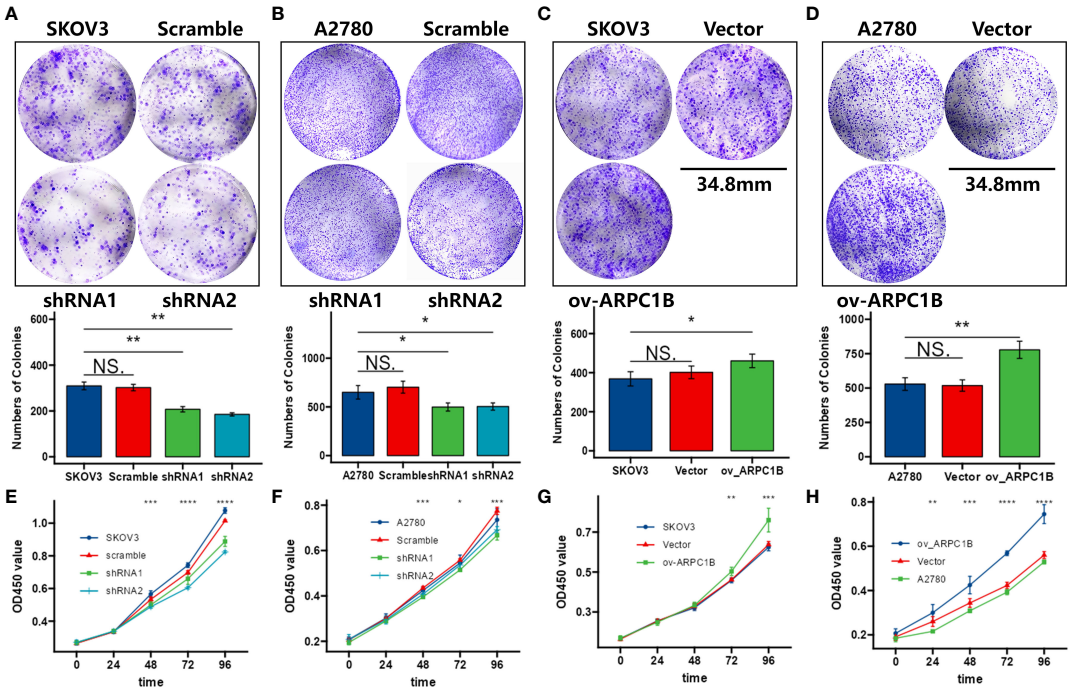

FIGURE 2
Effects of ARPC1B on the proliferation of ovarian cancer cells. **(A–D)** Cellular clone forming ability was evaluated by colony formation assay. **(E–H)** Cell proliferation ability was assessed by CCK-8 assay. Measurement data were expressed as mean ± SD of three independent experiments. NS, Not statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.

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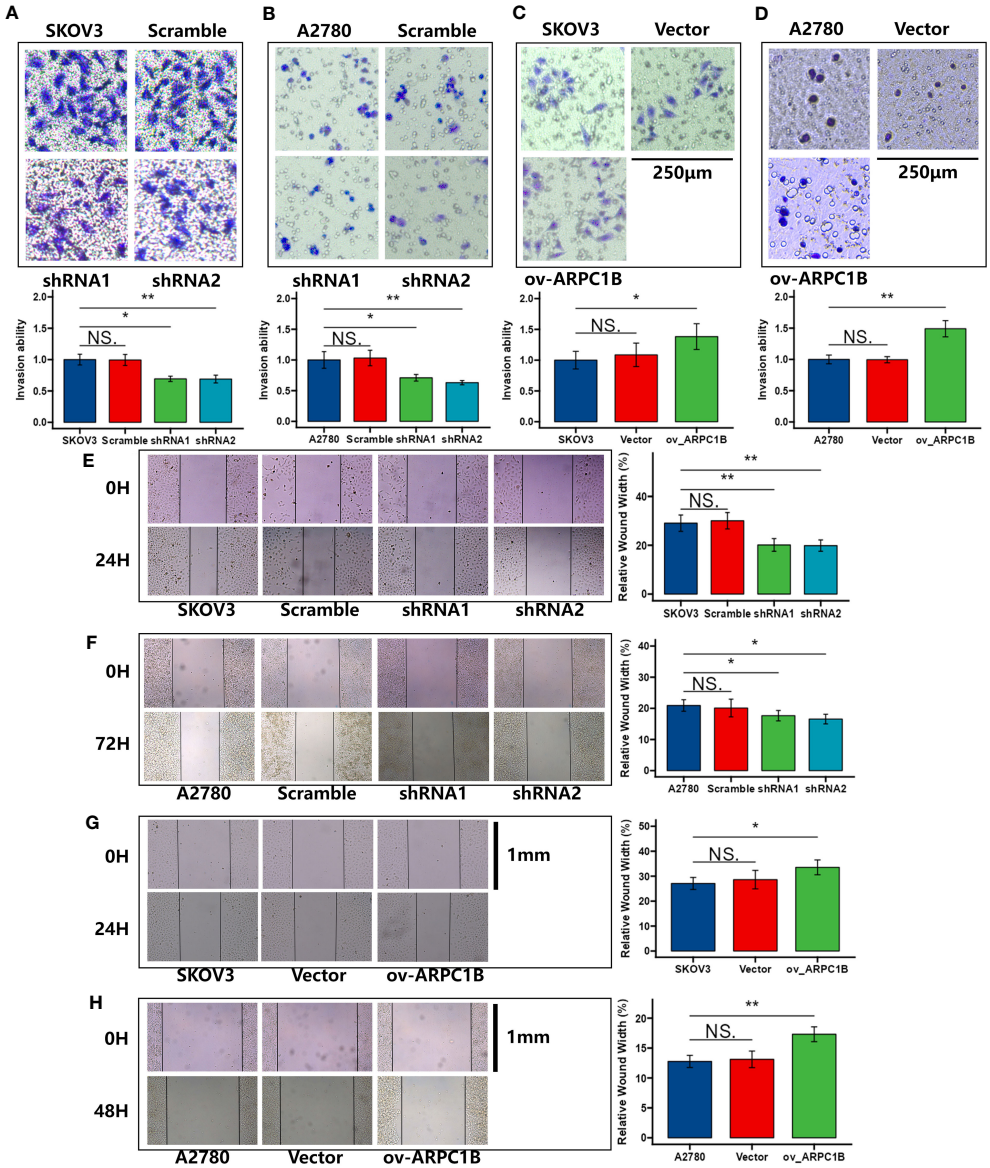

FIGURE 3
Effects of ARPC1B on the migration of ovarian cancer cells. **(A–D)** Cell invasion ability was detected by transwell assay. **(E–H)** Cell migration ability was assessed by wound healing assay. Measurement data were expressed as mean ± SD of three independent experiments. NS, Not statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.

cyclin D1) in the Wnt/β-Catenin signaling pathway was evaluated. Western Blot demonstrated that the knockdown of ARPC1B attenuated the expression of β-catenin in SKOV3 cells, leading to the low expression of c-myc and cyclin D1 (Figure 4A). The overexpression of ARPC1B promoted the expression of β-catenin in A2780 cells, causing the up-regulation of c-myc and cyclin D1, and the promotion was abolished by the administration of β-Catenin inhibitor XAV-939 (Figure 4B). The results of the colony formation assay, transwell assay, wound healing assay, and CCK-8 showed that ARPC1B overexpression-induced promotion of cellular proliferation and migration was abolished by the administration of β-Catenin inhibitor XAV-939 (Figures 4C–F). These data confirmed that the administration of β-Catenin inhibitor XAV-939 could reverse the malignant process caused by the up-regulated ARPC1B in ovarian cancer. Our results suggested the possibility that the overexpression of ARPC1B promoted the expression of cell proliferation or metastasis-related proteins by the activation of the Wnt/β-Catenin signaling pathway in ovarian cancer.

3.4 Effects of ARPC1B on the growth of ovarian cancer tumor *in vivo*

To confirm the effects of ARPC1B on the growth of ovarian cancer tumor *in vivo*, xenograft tumor models were built with

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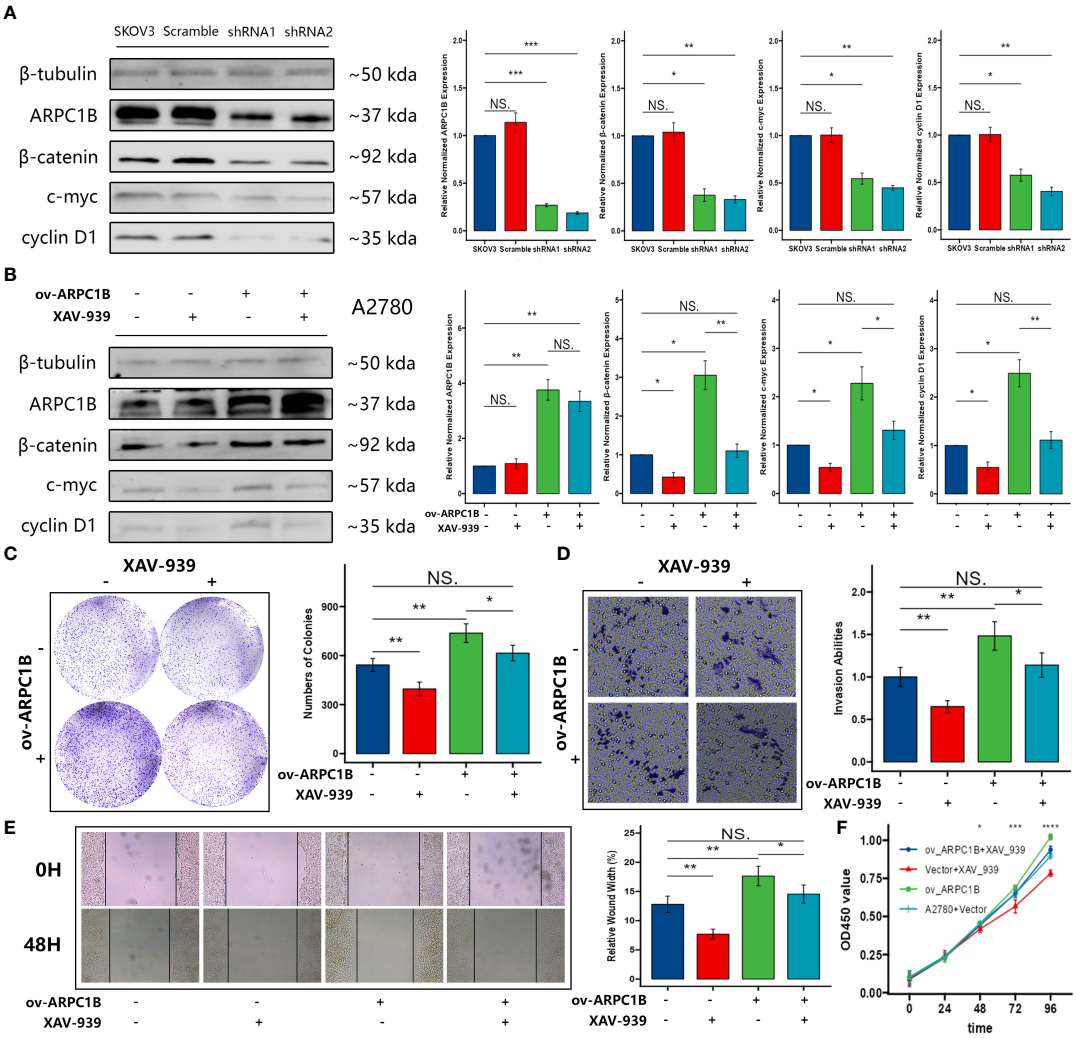

FIGURE 4
Effects of ARPC1B on the Wnt/β-Catenin signaling pathway in ovarian cancer cells. **(A)** The protein expression of β-catenin, c-myc, cyclin D1 in SKOV3 cells following ARPC1B knockdown. **(B)** The protein expression of β-catenin, c-myc, cyclin D1 in A2780 cells following ARPC1B overexpression. **(C)** Cellular clone forming ability was evaluated by colony formation assay. **(D)** Cell invasion ability was detected by transwell assay. **(E)** Cell migration ability was measured by wound healing assay. **(F)** Cell proliferation ability was assessed by CCK-8 assay. Measurement data were expressed as mean ± SD of three independent experiments. NS, Not statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.

SKOV3 cells infected with shRNA1 and shRNA2 or A2780 cells infected with ov-ARPC1B. Compared to scramble control, ARPC1B interference markedly ameliorated the average tumor volume. However, compared to vector, the overexpression of ARPC1B significantly increased the average tumor volume (Figures 5A–D). Then, we verified the expression of ARPC1B and further proved the association between ARPC1B and Wnt/β-Catenin signaling pathway using Western Blot. The protein expression of β-Catenin was weakened in tumors derived from the infected SKOV3 cells model, causing the attenuated expression of c-myc and cyclin D1 (Figures 5E). The protein expression of β-Catenin was elevated in tumors derived from the A2780 cells model, leading to the up-regulation of c-myc and cyclin D1 (Figures 5F). The results suggested that the overexpression of ARPC1B promoted the growth of ovarian cancer tumors *in vivo via* the activation of the Wnt/β-Catenin signaling pathway.

4 Discussion

Despite a decrease in incidence rates in recent years due to increased use of oral contraceptives (17), ovarian cancer remains the leading cause of death from gynecologic cancer, with a five-year survival rate of only 48% (18). The cure rate of early stage ovarian cancer can reach 90% (3), however, screening for ovarian cancer remains challenging due to its vague and nonspecific symptoms. The positive predictive value of routine screening methods, including ultrasound and serum CA-125, is less than 50% with a false-positive rate of up to 44% (19). Treatment of ovarian cancer typically involves a combination of chemotherapy and surgery, including surgical staging of affected tissue, tumor debulking surgery, subsequent chemotherapy, and target therapy such as PARP inhibitors (20, 21). However, late diagnosis and drug resistance present significant challenges in the treatment of

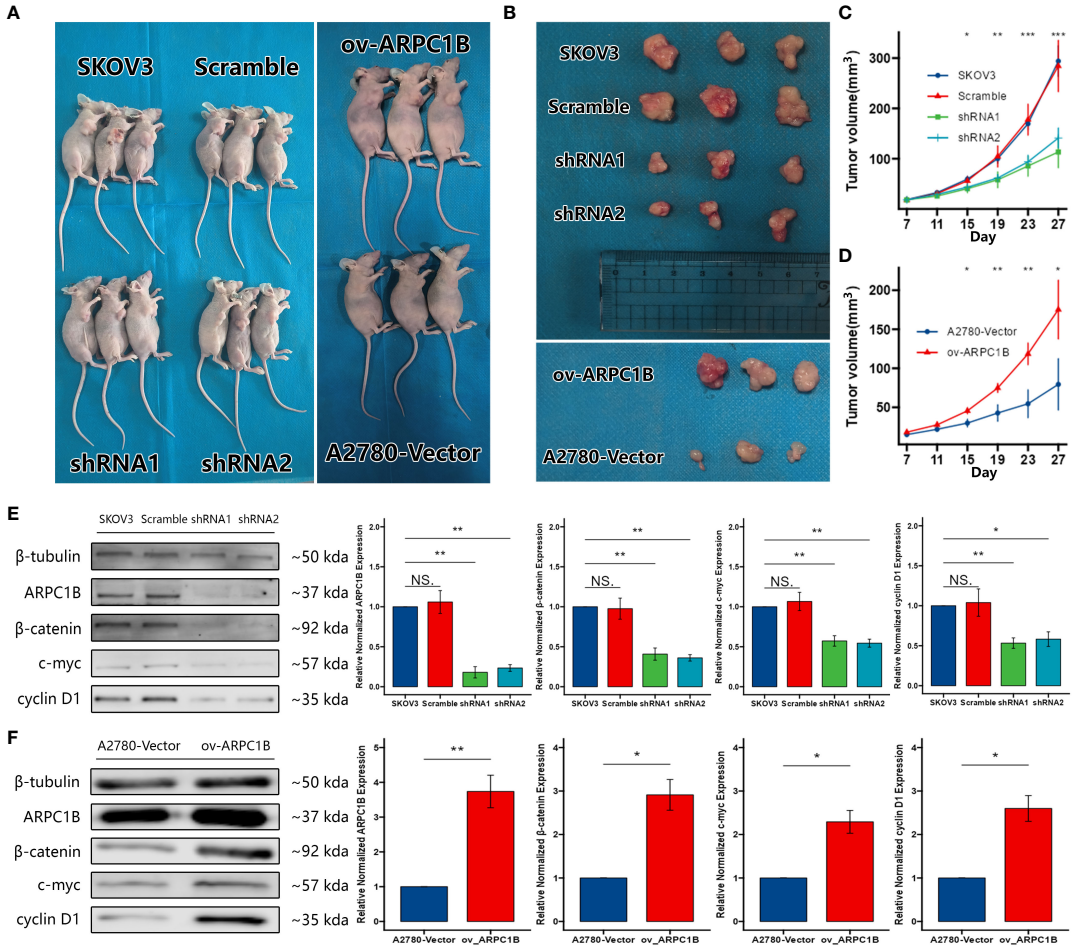

FIGURE 5
Effects of ARPC1B on the growth of ovarian cancer tumor *in vivo*. Xenograft tumor models were built with infected SKOV3 cells or A2780 cells. **(A–D)** The volume of xenograft tumors. The tumors were extracted on day 27 **(E, F)** Relative protein expression of β-catenin, c-myc, cyclin D1. Measurement data were expressed as mean ± SD of three independent experiments. NS, Not statistically significant. *p < 0.05, **p < 0.01, ***p < 0.001.

ovarian cancer. To address these challenges, immediate research priorities should focus on developing novel diagnosis marker and therapy for ovarian cancer (22). It is well established that certain genes are associated with an increased risk of ovarian cancer (17, 23). Mutations in the BRCA1, BRCA2, and MMR genes can increase the risk of ovarian cancer from 1.6% to 40%, 18%, and 10%, respectively (24). To identify novel oncogenes in ovarian cancer, we conducted a data mining analysis of public cancer genomics databases.

In this study, we conducted a bioinformatics analysis and found that the expression of ARPC1B was overexpressed in ovarian cancer. Further analysis revealed that patients with high expression of ARPC1B had a poorer overall survival and progression-free survival compared to those with low expression. To investigate the effect of ARPC1B on ovarian cancer progression, we modulated its expression in ovarian cancer cell lines SKOV3 and A2780. The results showed that overexpression of ARPC1B enhanced cell proliferation and migration *in vitro* through activation of the Wnt/β-Catenin signaling pathway. Conversely, knockdown of ARPC1B resulted in the opposite effect. Furthermore, administration of β-Catenin inhibitor XAV-939 was observed to abolish the proliferation and migration induced by ARPC1B overexpression. Our findings *in vivo* confirmed that ARPC1B overexpression facilitated the growth of ovarian cancer xenograft tumors, while ARPC1B interference suppressed tumor growth. This is the first report to suggest that ARPC1B is involved in ovarian cancer progression and may act as an oncogene in ovarian cancer.

The oncogene role of ARPC1B has been observed in other cancers as well. The overexpression of ARPC1B in glioma cells has been shown to maintain the malignant phenotype, including migration, invasion, and epithelial-to-mesenchymal transition (EMT) status (8, 11). On the other hand, knockdown of ARPC1B in prostate cancer cells has been observed to reduce the proliferation, migration, and invasion and cause cell cycle arrest at the G2/M phase *via* the downregulation of AURKA (9). Further research is needed to explore the effects of ARPC1B on the cell cycle in ovarian cancer and its underlying mechanisms. In the field of immunology, the overexpression of ARPC1B has been shown to promote macrophage recruitment through the activation of the NF-κB and STAT3 pathways (8). Neutrophils were defective in actin microfilament reorganization due to a mutation in ARPC1B or

inhibition of its upstream regulator, and Rac2 lose their ability to upregulate complement receptor immunoglobulin expression (25). ARPC1B is also associated with radiotherapy resistance, and patients with ARPC1B deficiency have increased sensitivity to ionizing radiation and bleomycin (11). ARPC1B has been found to promote radiotherapy resistance and maintenance of the mesenchymal phenotype in glioma stem cells (12). Additionally, ARPC1B plays a crucial role in metabolism and the upregulation of ARPC1B in the hypothalamic arcuate nucleus has been linked to the improvement of high-fat diet induced hypothalamic inflammation and leptin resistance (26). The exploitation of cancer metabolism is providing new insight into cancer biology and can potentially lead to the development of more effective targeted treatments for patients (27).

ARPC1B encodes one of seven subunits of the human Arp2/3 protein complex (5), which is the only molecular machine that generates branched actin networks (7), and plays a crucial role in the regulation of various biological functions, including cell differentiation, migration, adhesion, as well as cargo transport (6, 7). Immunohistochemical analysis has revealed that Arp2/3 subunits are overexpressed in a number of cancers, including bladder (28), breast (29), colorectal (30), gastric (31), gliomas (32), and lung cancers (33). In this study, we have discovered that ARPC1B promotes the progression of ovarian cancer by activating the Wnt/β-Catenin signaling pathway. However, it is still unclear whether ARPC1B indirectly activates the pathway through the Arp2/3 protein complex or whether it directly activates a known molecule within the pathway. Further research is needed to clarify this mechanism.

Interestingly, our results suggest that knocking down ARPC1B leads to a more significant decrease in the proliferation and migration abilities of ovarian cancer cells in SKOV3 cells than in A2780 cells. Conversely, overexpression of ARPC1B leads to a more significant increase in the proliferation and migration abilities of ovarian cancer cells in A2780 cells than in SKOV3 cells. We hypothesize that this difference may be due to the characteristics of the cells, as A2780 cells are derived from primary tissues of ovarian adenocarcinoma, while SKOV3 cells are derived from ascites fluids of patients with ovarian serous carcinoma (34, 35). A wound healing assay involving 10 types of ovarian cancer cell lines showed that at 30 hours, the healing rate of SKOV3 cells could reach over 80%, while the healing rate of A2780 cells was less than 20% (36). This suggests that active cell lines like SKOV3 may be more suitable as models for observing anticancer effects, while less active cell lines like A2780 may be more suitable as models for observing cancer-promoting effects. Alternatively, adjusting the observation time points flexibly may be necessary to ensure that the most significant differences between different treatment groups can be observed. However, this speculation is based on limited experimental evidence and cannot completely rule out the possibility of accidental circumstances. We also concern the involvement of other proteins in a compensation process, which could mask the effects of ARPC1B. Additionally, the effects observed *in vivo* appear to be clearer than those observed *in vitro*, suggesting that cellular interactions between tumor cells and the tumor microenvironment (TME) may play a role *in vivo* that is not observable *in vitro*. We will continue to pay attention to these issues in future research.

In summary, this study advances our knowledge of the molecular pathogenesis of ovarian cancer. Further research is needed to fully understand the mechanism by which ARPC1B activates the Wnt/β-catenin signaling pathway in ovarian cancer cells and to assess the potential therapeutic value of targeting ARPC1B. Future studies should focus on examining a greater number of tissue samples using immunohistochemistry to further validate the diagnostic value of ARPC1B and exploring the impact of ARPC1B on the tumor microenvironment, metabolism, drug resistance, and radiotherapy resistance in ovarian cancer.

5 Conclusion

The expression levels of ARPC1B were found to be elevated in ovarian cancer tissues. The overexpression of ARPC1B has been shown to contribute to the malignant phenotype in ovarian cancer *via* activation of the Wnt/β-Catenin signaling pathway. These findings suggest that ARPC1B may be a novel target in the arsenal to treat ovarian cancer.

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 Institutional Research Ethics Committee of Guangxi Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Research Ethics Committee of Guangxi Medical University.

Author contributions

JH conceived and designed the experiments. JH, HZ, CT, and SM performed the experiments. JH, HZ, CT, SM, TL, and YK collected the clinical specimens. JH performed the statistical analysis. JH wrote the manuscript. YK made revision of the manuscript. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1182677/full#supplementary-material

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EDITED BY
Xiangpeng Dai,
Jilin University, China

REVIEWED BY
Xiaoming Dai,
Beth Israel Deaconess Medical Center and Harvard Medical School, United States
Linyong Du,
Wenzhou Medical University, China
Xiaowei Qi,
Army Medical University, China

*CORRESPONDENCE
Leina Ma
leinama@gmail.com
Qiang Wang
wangqiang401@139.com
Zhimin Lu
zhiminlu@zju.edu.cn

†These authors have contributed equally to this work

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Glycolytic enzyme HK2 promotes PD-L1 expression and breast cancer cell immune evasion

Jichun Lin[1,2,3†], Wenshuo Fang[1,2,3†], Zhuo Xiang[4], Qingqing Wang[4], Huapeng Cheng[4], Shimin Chen[1,2,3], Jing Fang[1,2,3], Jia Liu[5], Qiang Wang[4*], Zhimin Lu[6,7*] and Leina Ma[1,2*]

[1]Department of Oncology, the Affiliated Hospital of Qingdao University, Qingdao, China, [2]Qingdao Cancer Institute, Qingdao, China, [3]School of Basic Medicine, Qingdao University, Qingdao, China, [4]Oncology Department, Shandong Second Provincial General Hospital, Jinan, China, [5]Department of Pharmacology, School of Pharmacy, Qingdao University, Qingdao, China, [6]Zhejiang Provincial Key Laboratory of Pancreatic Disease, The First Affiliated Hospital and Institute of Translational Medicine, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China, [7]Cancer Center, Zhejiang University, Hangzhou, Zhejiang, China

Immune therapies targeting the PD-1/PD-L1 pathway have been employed in the treatment of breast cancer, which requires aerobic glycolysis to sustain breast cancer cells growth. However, whether PD-L1 expression is regulated by glycolysis in breast cancer cells remains to be further elucidated. Here, we demonstrate that glycolytic enzyme hexokinase 2 (HK2) plays a crucial role in upregulating PD-L1 expression. Under high glucose conditions, HK2 acts as a protein kinase and phosphorylates IκBα at T291 in breast cancer cells, leading to the rapid degradation of IκBα and activation of NF-κB, which enters the nucleus and promotes PD-L1 expression. Immunohistochemistry staining of human breast cancer specimens and bioinformatics analyses reveals a positive correlation between HK2 and PD-L1 expression levels, which are inversely correlated with immune cell infiltration and survival time of breast cancer patients. These findings uncover the intrinsic and instrumental connection between aerobic glycolysis and PD-L1 expression-mediated tumor cell immune evasion and underscore the potential to target the protein kinase activity of HK2 for breast cancer treatment.

KEYWORDS

HK2, PD-L1, IκBα, NF-κB, immunotherapy, metabolism, breast cancer

1 Introduction

Breast cancer is commonly diagnosed cancer and is a leading cause of cancer-related deaths in females worldwide (1). Accumulated evidence has indicated that the immune system response is critical for the therapeutic efficacy and survival of breast cancer patients. In addition, breast cancer cells exhibit immune evasion capabilities (2, 3). Tumor cell membrane protein programmed cell death ligand1 (PD-L1, also known as B7-H1) binds to the receptor protein programmed cell death 1 (PD-1) on the surface of T lymphocyte cells, resulting in the blockage of T cell proliferation, cytokine production, and the inhibition of the immune response (4–6). PD-L1 expression is often upregulated in breast cancer cells and plays a role in immune evasion (7, 8). A study on breast cancer patients showed that the abnormal expression of PD-L1 was closely related to the reduction of overall survival rate and poor prognosis (9). PD-1/PD-L1 immune checkpoint inhibitors have been used in various cancer treatments, including clinical trials in breast carcinoma. However, a portion of patients did not respond to the immunotherapy (2, 10). Therefore, further research on the regulation of PD-L1 expression in breast cancer cells will shed light on the mechanism underlying breast cancer cell immune evasion and help increase immune checkpoint therapy's clinical effectiveness.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a nuclear transcription factor highly expressed in breast cancer tissues (11, 12). In unstimulated cells, NF-κB composed of Rel A (p65)/p50 dimers is bound by IκBα protein and sequestrated in the cytoplasm. In response to cytokine stimulation, IκBα undergoes rapid ubiquitylation-mediated proteasome degradation that releases the bound, cytoplasmic NF-κB dimers (13). Then, NF-κB enters the nucleus and promotes PD-L1 transcription (14, 15). NF-κB can be regulated by hexokinase (HK) in glioblastoma cells (16). HK is a rate-limiting enzyme in aerobic glycolysis, which converts glucose to the metabolic intermediate glucose-6-phosphate (G-6-P) (17). Four isotypes of the HK family are founded in mammals: HK1, HK2, HK3, and HK4 (18, 19). HK2 binds to mitochondrial outer membrane voltage-dependent anion channel 1 (VDAC1) protein (20, 21), which enables HK2 to utilize ATP produced by mitochondria for glycolysis. High glycolysis-produced large amount of G-6-P disassociates HK2 from the mitochondria by a feedback-regulated mechanism (22). The expression of HK2, which can be induced by erbB2/Neu (23), was significantly increased in breast cancer specimens compared to normal tissue (24). HK2 deletion inhibited breast cancer metastasis (25). HK2 not only has the function of a glycolytic enzyme but also has non-metabolic functions (16, 26, 27). A recent study demonstrated that HK2 in glioblastoma cells acts as a protein kinase and phosphorylates IκBα, resulting in IκBα degradation and NF-κB activation for PD-L1 transcription (16). However, the relationship between HK2 and immunoregulation in breast cancer remains unclear.

In this study, we demonstrated that aerobic glycolysis induces PD-L1 expression in an HK2-dependent manner. HK2 phosphorylates IκBα at T291, resulting in IκBα rapid degradation and NF-κB activation, resulting in enhanced PD-L1 transcription and breast cancer cell immune evasion.

2 Materials and methods

2.1 Materials

Rabbit antibodies that recognize human HK2 (Cat#ab209847; RRID: AB2904621) and p65 (Cat#ab32536; RRID: AB776751) were obtained from Abcam (Shanghai, China). Rabbit antibodies against PD-L1 (Cat#ab13684; RRID: AB2687655) and α-tubulin (Cat#ab2125; RRID: AB2619646) and mouse antibody against IκBα (Cat#ab4814; RRID: AB390781) were purchased from Cell Signaling Technology. Rabbit antibodies against Flag (Cat#20543-1-AP; RRID: AB11232216) and histone H3 (Cat#ab17168; RRID: AB2716755) were purchased from Proteintech (Wuhan, China). Rabbit polyclonal anti-IκBα pT291 from Signalway Biotechnology (Pearland. TX). Goat anti-rabbit IgG (H+L) secondary antibody (Cat#A-11008; RRID: AB-143165) was obtained from Invitrogen. G-6-P (Cat#D9434) was purchased from Sigma (Shanghai, China). Glucose (Cat#A501991) was obtained from Sangon Biotech (Shanghai, China). CHX (HY-12320) was purchased from MedChemExpress (Shanghai, China). Lipofectamine 2000 (L3000015) transfection reagents and Blasticidin (Cat#R21001) were obtained from Thermo Fisher Scientific (Waltham, MA).

2.2 Cell culture and cell transfection

Human breast cancer MCF-7 (RRID: CVCL 0031), BT-549 (RRID: CVCL 1092), SK-BR-3 (RRID: CVCL 0033), and human embryonic kidney 293T (RRID: CVCL LF52) cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) or McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37° C with 5% CO_2. The transfection using Lipofectamine2000 reagent (Invitrogen) was performed as previously described (28). For G-6-P treatment, 1M G-6-P was mixed with 5 μl Lipofectamine2000 in OPTI-MEM for 30 minutes at room temperature and supplemented into the culture medium in a 6-well plate.

2.3 Subcellular fractionation

Nuclear and cytosolic fractions were prepared as previously described (29). Briefly, Flag-HK2 or vector was transfected into MCF-7 cells with Lipofectamine2000 reagent (Invitrogen). 48 h later, cells were collected and suspended in 300 μl Buffer A (10 mM HEPES, 10 mM KCL, 0.1 mM EDTA,0.1 mM EGTA, 0.15% NP-40, protease inhibitors), shaken by hand, and placed on ice for 10 min, 13000 rpm at 4°C for 30 seconds, and the supernatant is the cytoplasm. Then, the precipitate was suspended with 700 μl Buffer A, left for 3min, 13000 rpm for 30 seconds at 4°C to clean the nuclear components. Repeat the above steps 2 times to wash the remaining pulp components from the core. Discard the supernatant and add 70 μl CST lysis, 25% ultrasonic for 6 times, centrifuged at 13000 rpm for 20 min at 4°C. The supernatant is the nuclear component.

2.4 Quantitative PCR

Quantitative PCR analyses were performed as described previously (30). Total RNA was extracted from cells using TRIzol reagent and reverse transcribed with Maxima Reverse Transcriptase according to the manufacturer's instructions. Quantitative PCR analysis was carried out using a 7500 Real-Time PCR system (Applied Biosystems) with an SYBR Premix ExTaq kit (Bimake). The relative expression was determined using the ΔΔCT method of normalization. The following primers were used for quantitative PCR, Human CD274 forward: 5'-CTGCACTTTTAGGAGATTAGATC-3'; Human CD274 reverse: 5'-CTACACCAAGGCATAATAAGATG-3'; Human β-actin forward: 5'-TGGCACCCAGCACAATGAA-3'; Human β-actin reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'.

2.5 Western blot analysis

Total proteins were extracted with CST lysis buffer containing protease and phosphatase inhibitors. The protein concentration was determined using a Bradford reagent kit (Thermo Fisher Scientific), and proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% milk for 1 hour and then incubated with primary antibody at 4°C overnight. Membranes were washed with Tris-buffered saline containing Tween-20, incubated with secondary antibodies, and developed with an enhanced chemiluminescence kit.

2.6 Flow cytometry analysis

Flow cytometry analysis was performed as described previously (31). Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then were washed with PBS. An anti-PD-L1 antibody was added to the cells for 1 hour at room temperature. The cells were washed with PBS three times. A fluorescence antibody was added to the cells for 30 minutes at room temperature. After incubation, the cells were washed with PBS and detected by a Beckman cytometer.

2.7 Immunoprecipitation analysis

Immunoprecipitation analysis using antibodies as described previously (32). Briefly, cells were collected and lysed in CST lysis buffer (20 mM Tris-HCl [pH7.5], 150 mM NaCl, 1 mM $Na_2EDTA.2H_2O$, 1 mM EGTA, 1% TritonX-100 and 2.5 mM $Na_4P_2O_7$) containing protease inhibitor cocktail (Bimake) and phosphatase inhibitor cocktails (Bimake). For coimmunoprecipitation, the cell lysate supernatant was mixed with indicated antibodies overnight at 4°C and incubated with 30 μl protein A/G agarose beads for 3 hours at 4°C on a rocking platform and then washed the beads 3 times with NETN buffer (20 mM Tris-HCl [pH8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) and boiled with 50 μl of 2×SDS loading buffer for 10 min. Finally, the obtained proteins were subject to Western blotting.

2.8 Lentiviral generation and infection

Lentiviral constructs expression shControl and shHK2 were co-transfected into HEK293T cells with package plasmids with PEI (Invitrogen) as described previously (33). Lentivirus was collected 72 hours after transfection and was filtered by a 0.45 μm filter membrane. The filter lentivirus was infected with MCF-7 using 10 μg/ml polybrene. Screening stable expression cells by Blasticidin.

2.9 Patients and tissue samples

We retrospectively collected 220 human breast carcinoma specimens from Shandong Second Provincial General Hospital (Jinan, China), and obtained clinical data by reviewing the patients' medical histories.

2.10 Ethics statement

The studies involving human breast cancer specimens and the database were approved by the institutional research ethics committee of the Oncology Department, Shandong Second Provincial General Hospital. All patients involved in the study were conducted strictly with the national ethical policy. Informed consent was obtained from all the patients whose tissue samples were allowed to be used for scientific research, and patient privacy was protected.

2.11 Immunohistochemical analysis

IHC staining was performed using the VECTASTAIN ABC kit (Vector Laboratories) according to the manufacturer's instructions. Human breast cancer tissues were stained with antibodies HK2 (dilution 1:500), PD-L1 (dilution 1:400), IκBα pT291 (dilution 1:50) or nonspecific IgG (as a negative control). We quantitatively scored the sections based on the percentage of positive cells and the intensity of staining of the sections (34). The staining intensity is scored as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong. The IHC scores were assessed by independent pathologists. We then multiply the intensity and percentage of positive cells to obtain a total score.

2.12 TIMER database analysis

TIMER (http://timer.cistrome.org/) is an estimating immune cell infiltration database and provides comprehensive analysis and

visualization functions of tumor infiltrating immune cells which uses data from TCGA (35–37). In the study, we examined the correlation between HK2 mRNA levels and CD274 mRNA levels. Then, we examined tumor-infiltrating $CD4^+$ T cells through TIMER algorithm and tumor-infiltrating $CD8^+$ T cells through CIBERSORT algorithm in TIMER2.0 database. Spearman's rho value was used to evaluate the degree of their correlation. HK2 expression and breast cancer patient survival analysis was tested using the Kaplan-Meier Plotter (https://kmplot.com/analysis/) (38, 39), which searched for breast cancer cohorts in NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) and in the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/).

3 Results

3.1 High glucose enhances PD-L1 expression in an HK2-dependent manner

To determine whether changes of glucose level modulate PD-L1 expression in breast cancer cells, we treated MCF-7 and BT-549 cells with different concentrations of glucose. We found that a high glucose concentration increased PD-L1 expression (Figure 1A). In addition, flow cytometry analyses revealed that high glucose concentration enhanced PD-L1 expression on the surface of MCF-7 cells (Figure 1B). This increase was decreased by treatment with both protein synthesis inhibitor cycloheximide

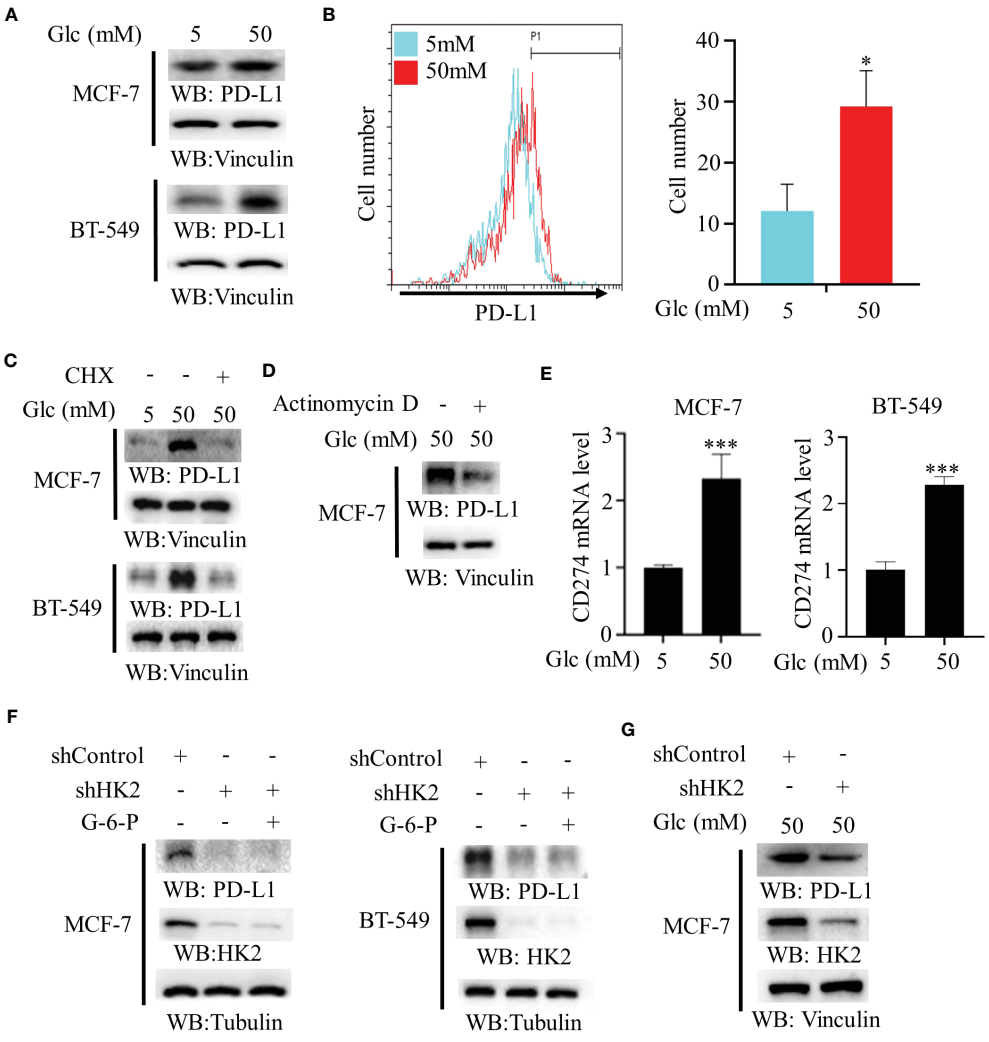

FIGURE 1
High glucose enhances PD-L1 expression in an HK2-dependent manner. **(A)**, MCF-7 and BT-549 cells were treated with the indicated glucose concentrations for 24 h. Immunoblotting analyses were performed with the indicated antibodies. **(B)**, MCF-7 cells were treated with low (5 mM) or high glucose (50 mM) for 24 h. Flow cytometry analyses were performed. *p < 0.05. **(C)**, MCF-7 and BT-549 cells were treated with the indicated glucose concentrations for 24 h in the presence or absence of cycloheximide (CHX) (100 μg/ml). Immunoblotting analyses were performed with the indicated antibodies. **(D)**, MCF-7 cells were cultured with high glucose (50 mM) for 24 h with or without pretreatment with actinomycin D (1 μg/ml). **(E)**, Real-time PCR analyses of *CD274* mRNA in MCF-7 cells and BT-549 cells cultured with the indicated glucose concentrations for 24 h. Data are the means ± SD of 3 independent experiments. ***p < 0.001. **(F)**, MCF-7 and BT-549 cells stably expressing a control shRNA or HK2 shRNA were treated with or without G-6-P for 12 h. Immunoblotting was performed with the indicated antibodies. **(G)**, MCF-7 cells stably expressing a control shRNA or HK2 shRNA were cultured in medium containing high glucose (50 mM). Immunoblotting analyses were performed with the indicated antibodies. *p<0.05.

(CHX) (Figure 1C) and transcription inhibitor actinomycin D (Figure 1D), suggesting extracellular glucose levels regulate PD-L1 at both transcriptional and posttranslational levels. Consistent with this finding, quantitative PCR analyses showed that high glucose treatment increased mRNA expression of the *CD274* gene (encoding PD-L1) in MCF-7 and BT-549 cells (Figure 1E). Notably, depletion of HK2 by expression of its shRNA in MCF-7 and BT-549 cells reduced PD-L1 expression, and this reduction was not rescued by supplementation with HK2 product G-6-P (Figure 1F), suggesting that glycolytic reactions downstream of HK2 are not involved in the regulation of PD-L1 expression. Consistently, HK2 depletion decreased PD-L1 expression in MCF-7 cells under high glucose conditions (Figure 1G). These results indicated that high glucose upregulates PD-L1 expression in an HK2-dependent manner.

3.2 HK2-mediated IκBα phosphorylation reduces IκBα expression

HK2 phosphorylates IκBα T291 and promotes IκBα degradation in glioblastoma cells (16). To define the mechanism underlying HK2-upregulated PD-L1 expression in breast cancer cells, we performed co-immunoprecipitation analyses and showed that endogenous HK2 interacted with endogenous IκBα in MCF-7 and BT-549 cells (Figure 2A). In addition, high glucose-induced

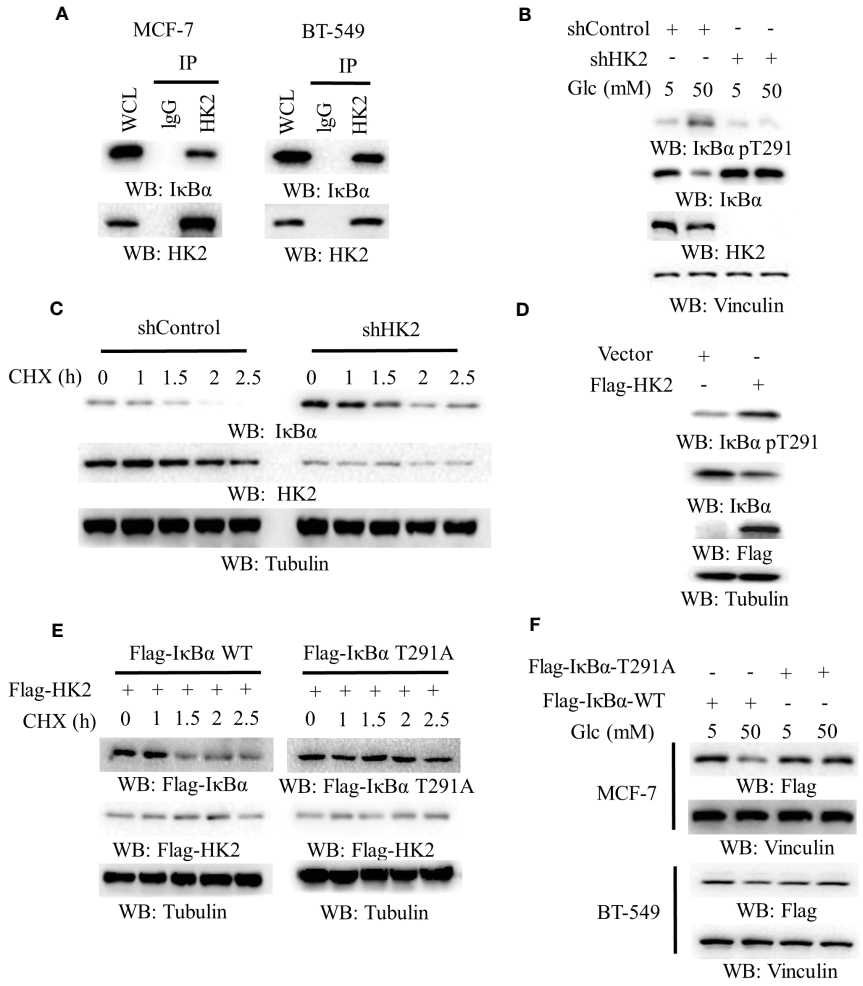

FIGURE 2
HK2-mediated IκBα phosphorylation reduces IκBα expression. **(A)**, MCF-7 and BT-549 cells were analyzed by immunoprecipitation and immunoblotting analyses with the indicated antibodies. **(B)**, MCF-7 cells stably expressing a control shRNA or HK2 shRNA were cultured in medium containing the indicated concentrations of glucose for 24 h. Immunoblotting analyses were performed with the indicated antibodies. **(C)**, MCF-7 cells with or without HK2 shRNA were treated with cycloheximide (CHX) (100 μg/ml) and harvested at the indicated periods of time. Immunoblotting analyses were performed with the indicated antibodies. **(D)**, A control vector or a vector expression Flag-HK2 was transfected into MCF-7 cells. Immunoblotting analyses were performed with the indicated antibodies. **(E)**, MCF-7 cells expressing Flag-HK2, WT Flag-IκBα or Flag-IκBα T291A were treated with CHX (100 μg/ml) for the indicated periods of time. Immunoblotting analyses were performed with the indicated antibodies. **(F)**, WT Flag-IκBα or Flag-IκBα T291A was expressed in MCF-7 and BT-549 cells. The cells were cultured with the indicated concentrations of glucose for 24 h.

IκBα T291 phosphorylation and decreased IκBα expression. Notably, this change was abrogated by HK2 depletion (Figure 2B), which prolonged the half-life of IκBα (Figure 2C). Consistently, Flag-HK2 overexpression considerably enhanced IκBα T291 phosphorylation and reduced IκBα expression (Figure 2D) and decreased the half-life of wild-type (WT) IκBα compared to that of IκBα T291A (Figure 2E). In contrast to WT Flag-IκBα, Flag-IκBα T291A displayed resistance to degradation in MCF-7 and BT-549 cells upon high glucose treatment (Figure 2F). These results indicated that HK2 phosphorylates IκBα T291 phosphorylation and decreases IκBα expression under high glucose conditions.

3.3 Overexpression of HK2 induces nuclear translocation of p65 and CD274 transcription

To determine whether aerobic glycolysis regulates the NF-κB in breast cancer cells, we overexpressed Flag-HK2 in MCF-7 cells. We

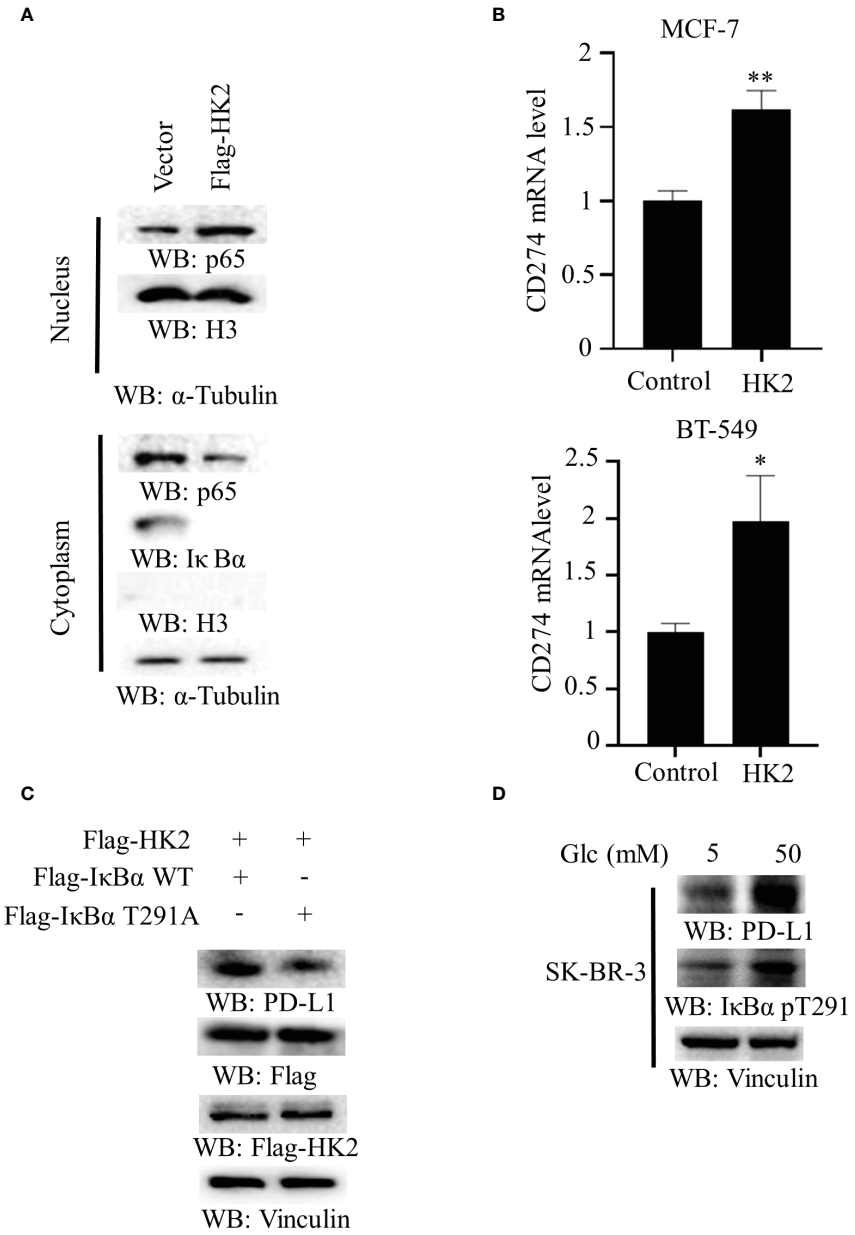

FIGURE 3
Overexpression of HK2 induces nuclear translocation of p65 and *CD274* transcription. **(A)**, Cytoplasmic and nuclear fractions of MCF-7 cells with or without expressing Flag-HK2 were analyzed by immunoblotting analyses with the indicated antibodies. **(B)**, MCF-7 and BT-549 cells were transfected with a control vector or Flag-HK2 for 48 hours. A real-time PCR analysis was performed. Data are the means ± SD of 3 independent experiments. **p < 0.01, *p < 0.05. **(C)**, Flag-HK2, WT Flag-IκBα or Flag-IκBα T291A was expressed in MCF-7 cells. Immunoblotting analyses were performed with the indicated antibodies. **(D)**, SK-BR-3 cells were cultured in medium containing low (5 mM) or high glucose (50 mM) for 24 h. Immunoblotting analyses were performed with the indicated antibodies.

found that Flag-HK2 expression promoted the nuclear translocation of p65 with a corresponding decrease of IκBα expression in the cytosol (Figure 3A). In addition, HK2 overexpression considerably elevated the mRNA level of CD274 in both MCF-7 and BT-549 cells (Figure 3B) and increased expression of WT Flag-IκBα to a higher level than that of IκBα T291A (Figure 3C). Notably, high glucose conditions also enhanced IκBα T291 phosphorylation and PD-L1 expression in HER2-positive SK-BR-3 breast cancer cells (Figure 3D), suggesting that HK2-regulated PD-L1 expression is independent of HER2 expression. These results suggested that HK2-mediated IκBα T291 phosphorylation promotes nuclear translocation of p65 and PD-L1 expression.

3.4 HK2 expression is positively correlated with CD274 expression and negatively associated with CD8$^+$ T cell infiltration and survival time of breast cancer patients

To determine whether HK2 expression is correlated with PD-L1 expression in human breast cancer specimens, we analyzed 1100 breast cancer cases in The Cancer Genome Atlas (TCGA) database. We revealed that HK2 mRNA levels were positively associated with CD274 mRNA levels (correlation: 0.169, p=1.63e-08) (Figure 4A). Analyses of the associations between HK2 expression and immune cells infiltration using the TIMER2.0 database (40), which showed that HK2 mRNA levels in breast cancer specimens were inversely correlated with the

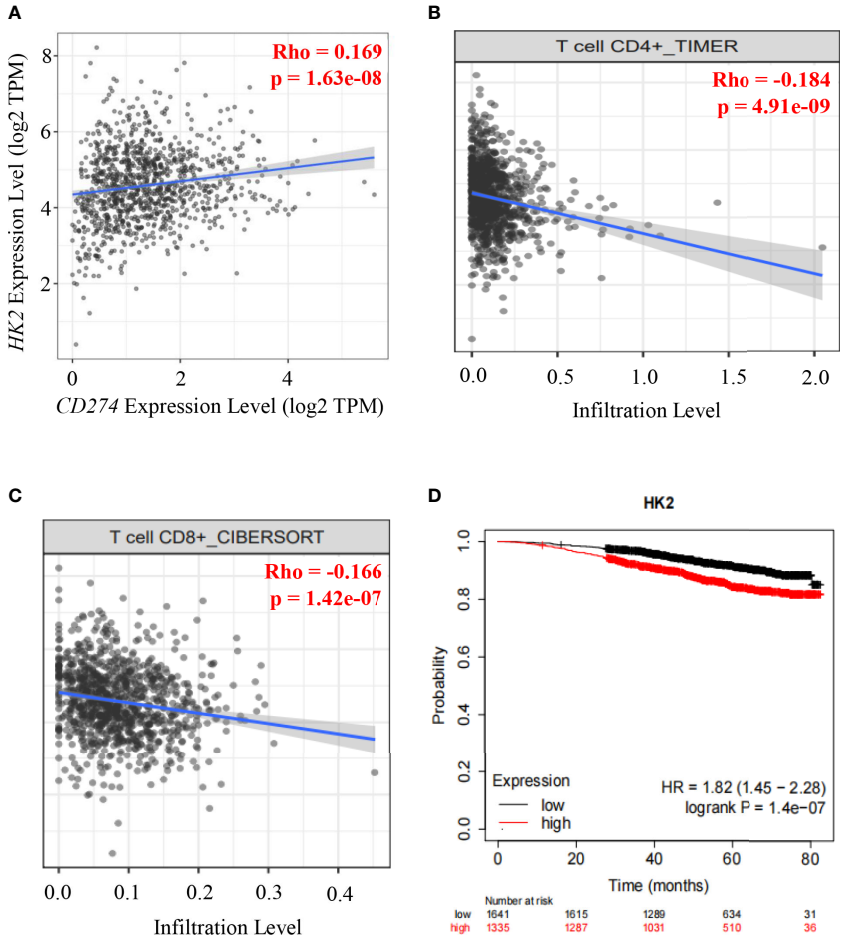

FIGURE 4
HK2 expression is positively correlated with CD274 expression and negatively associated with CD8$^+$ T cell infiltration and survival time of breast cancer patients.**(A)**, Correlative expression of *CD274* mRNA with HK2 mRNA expression in the TCGA cohort of BRCA samples (n = 1100) was analyzed. Spearman's rho value is presented for correlations. **(B)**, The correlation between HK2 mRNA expression levels and the infiltrating levels of CD4$^+$ T cells was analyzed by TIMER algorithm in the TIMER2.0 database in breast cancer patients specimens (n=1100). Spearman's rho value is presented for correlations. **(C)**, The correlation between HK2 mRNA expression levels and the infiltrating levels of CD8$^+$ T cells was analyzed through CIBERSORT algorithm in TIMER2.0 database in breast cancer patients specimens (n=1100). Spearman's rho value is presented for correlations. **(D)**, The association between HK2 mRNA expression levels and breast cancer patient survival was analyzed using the Kaplan Meier plotter database.

infiltration of $CD4^+$ T cells (correlation: 0.184, p=4.91e-08) (Figure 4B) and $CD8^+$ T cells (correlation: 0.166, p=1.42e-07) (Figure 4C) through TIMER algorithm and CIBERSORT algorithm analyses, respectively. In addition, analyses of the association between HK2 expression and breast cancer patient survival using the Kaplan Meier plotter database (https://kmplot.com) revealed that HK2 expression levels were inversely correlated with the survival time of breast cancer patients (Figure 4D). These results indicated that HK2 expression is positively correlated with CD274 expression and negatively associated with $CD8^+$ T cell infiltration and survival time of breast cancer patients.

3.5 HK2 expression is positively correlated with IκBα T291 phosphorylation and PD-L1 expression in human breast cancer specimens

To further determine the clinical significance of HK2-mediated IκBα T291 phosphorylation, thereby promoting the expression of PD-L1 in breast cancer patients, we performed immunohistochemistry (IHC) analyses of 220 breast cancer specimens with a specificity-validated anti-IκBα T291antibody and antibodies against HK2 and PD-L1 (16). We analyzed the correlation between HK2 expression and clinicopathological characteristics. We found a positive correlation of HK2 expression levels with larger tumor sizes, progesterone receptor (PR)-negative expression, and higher Ki67 levels (Table 1). In addition, IHC staining showed that HK2 expression levels were positively correlated with levels of IκBα T291 phosphorylation and PD-L1 expression (Figure 5A). Statistical analysis showed that these correlations were significant (Figure 5B). These results support the role of HK2-mediated IκBα T291 phosphorylation in upregulated PD-L1 expression in breast cancer specimens.

4 Discussion

Metabolic reprogramming and immune evasion are characteristic of many cancers (41). PD-L1 is overexpressed in various tumors, including breast cancer, leading to immune evasion (42). PD-L1 can be regulated by different mechanisms. A recent study showed that energy deprivation activates AMPK kinase, which phosphorylates and promotes PD-L1 degradation (7, 43). Our study showed that high glucose regulates the transcription of PD-L1 in a NF-κB-dependent manner. In breast cancer cells, HK2

TABLE 1 The Correlation between HK2 Expression and Clinicopathological Characteristics in Breast Cancer Patients (n=220 cases).

Characteristic	Number (%)	HK2 expression		p value
Total	220	Positive (103, 46.82%)	Negative (117, 53.18%)	
Age, years				
<50	64 (29.09%)	24 (10.91%)	40 (18.18%)	0.076
≥50	156 (70.91%)	79 (35.91%)	77 (35.00%)	
Tumor size, cm				
≤2	62 (28.18%)	20 (9.09%)	42 (19.09%)	0.021
2~5	123 (55.91%)	63 (28.64%)	60 (27.27%)	
≥5	35 (15.91%)	20 (9.09%)	15 (6.82%)	
Histological grades				
I	23(10.45%)	7 (3.18%)	16 (7.27%)	0.151
II	127 (57.73%)	70 (31.82%)	67 (30.45%)	
III	70 (31.82%)	26(11.82%)	34 (15.45%)	
Lymph node status				
0	138 (62.73%)	60 (27.27%)	78 (35.45%)	0.436
1-3	42 (19.09%)	22 (10.00%)	20 (9.09%)	
≥4	40 (18.18%)	21 (9.55%)	19 (8.64%)	
ER				
Positive	156 (70.91%)	68 (30.91%)	88 (40.00%)	0.134
Negative	64 (29.09%)	35 (15.91%)	29 (13.18%)	
PR				

(Continued)

TABLE 1 Continued

Characteristic	Number (%)	HK2 expression		p value
Total	220	Positive (103, 46.82%)	Negative (117, 53.18%)	
Positive	144 (65.45%)	60 (27.27%)	84 (38.18%)	0.035
Negative	76 (34.55%)	43 (19.55%)	33 (15.00%)	
HER2				
Positive	46 (20.91%)	26 (11.82%)	20 (9.09%)	0.138
Negative	174 (79.09%)	77 (35.00%)	97(44.09%)	
Ki67				
≥30%	110 (50.00%)	62 (28.18%)	48 (21.82%)	0.006
<30%	110 (50.00%)	42 (18.64%)	69 (31.36%)	

ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2. Two-sides Chi-Square tests.

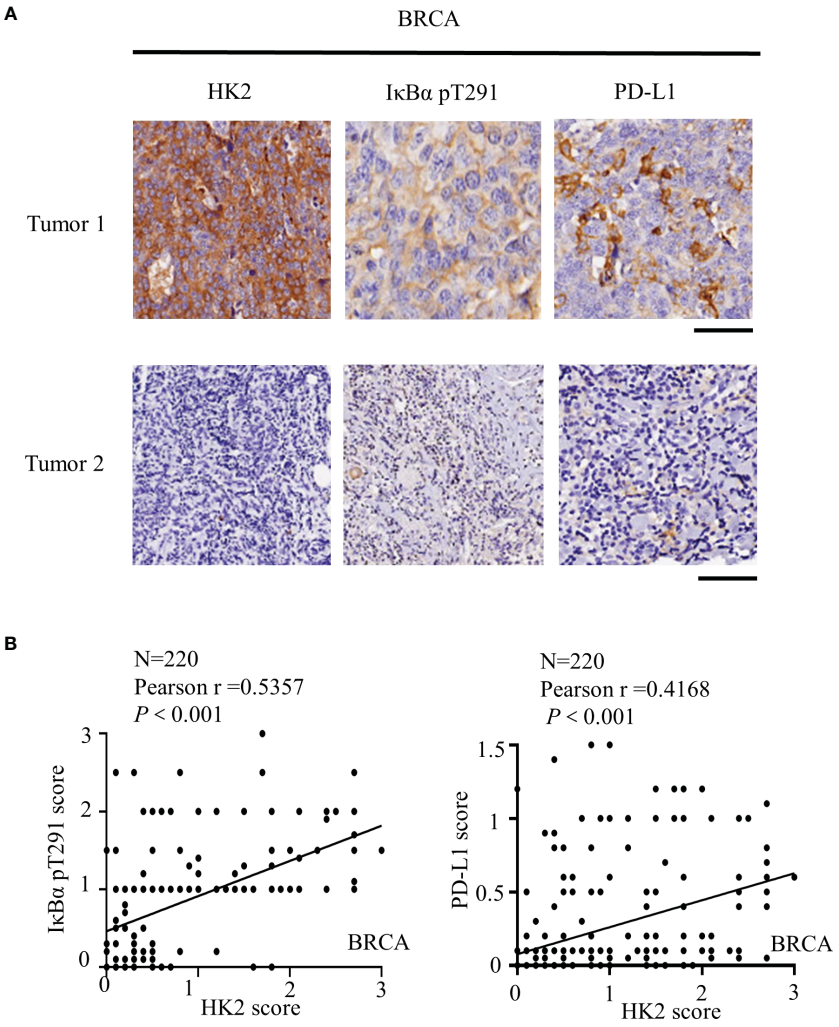

FIGURE 5
HK2 expression is positively correlated with IκBα T291 phosphorylation and PD-L1 expression in human breast cancer specimens. **(A)**, IHC analyses 220 human breast cancer specimens with HK2, PD-L1 and IκBα T291 antibodies. Two representative tumor IHC staining images were shown. Scale bars, 100μM. **(B)**, IHC staining was scored, and the correlations between the expression levels of HK2, PD-L1, and IκBα T291 phosphorylation were analyzed by Pearson correlation test. Note that some of the dots on the graphs are overlapped.

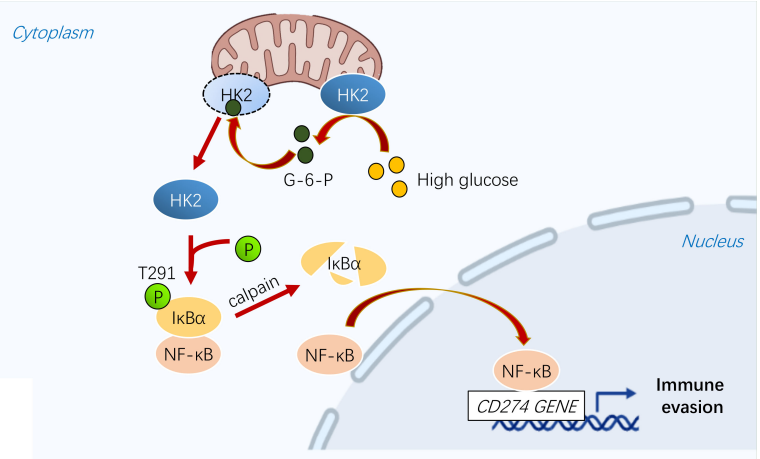

FIGURE 6
A schematic diagram shows that glycolytic enzyme HK2 acts as a protein kinase and phosphorylates IκBα to promote PD-L1 expression.

is highly expressed and is associated with the occurrence and progression of breast cancer (3, 44, 45). We demonstrated that HK2 plays a key role in regulating PD-L1 in breast cancer cells in response to high glucose (Figure 6).

Cell metabolism and gene expression are two fundamental biological processes that can be mutually regulated (27). Recent research demonstrated that metabolic enzymes could possess protein kinase activity to phosphorylate protein substrates (46). For instance, phosphoenolpyruvate carboxykinase1 (PCK1) (29), phosphoglycerate kinase 1 (PGK1) (47–49), ketohexokinase (KHK)-A (50, 51), pyruvate kinase M2 isoform (PKM2) (52–54), choline kinase α (CHKα) (55, 56) phosphorylate a variety of protein substrates thereby regulating instrumental cellular activities, such as gene expression. Intriguingly, it was shown that fructose-1,6-bisphosphatase 1 (FBP1) functions as a protein phosphatase to dephosphorylate histone H3, highlighting the critical control of protein phosphorylation and dephosphorylation by metabolic enzymes (57, 58). We showed here that HK2, acting as a protein kinase, phosphorylates IκBα at T291 in breast cancer cells, leading to IκBα degradation and subsequent activation of NF-κB for upregulation of PD-L1 transcription. Bioinformatic analysis showed that HK2 expression is associated with upregulated CD274 mRNA expression, reduced infiltration of $CD4^+$ and $CD8^+$ T cells in breast cancer specimens, and decreased survival time of breast cancer patients. In addition, the clinical significance of HK2-upregulated PD-L1 expression is evidenced by the positive correlation of HK2 with IκBα T291 phosphorylation and PD-L1 expression in human breast cancer samples. Our findings highlight the interplay between metabolic enzymes and tumor immunity, suggesting that HK2 serves as an effective molecular biomarker for PD-L1 antibody therapy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by The institutional research ethics committee of the Oncology Department, Shandong Second Provincial General Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

LM, ZL, QiaW, and JLiu conceived and designed the study; JLin, WF, ZX, QiaW, HC and SC performed the experiments; JLin, WF, QiaW performed the statistical analysis and wrote the original draft. ZL and LM contributed to the final draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

ZL owns shares in Signalway Biotechnology Pearland, TX, which supplied rabbit antibodies that recognize IkBa pT291. ZL's

interest in this company had no bearing on its being chosen to supply these reagents.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1189953/full#supplementary-material

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EDITED BY
Xiangpeng Dai,
Jilin University, China

REVIEWED BY
Jiateng Zhong,
Xinxiang Medical University, China
Jingjing Wang,
Central South University, China

*CORRESPONDENCE
Yingchao Zhao
yingchaozhao@icloud.com
Kai Xu
kxutongji@hotmail.com

†These authors share first authorship

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Emerging role of deubiquitination modifications of programmed death-ligand 1 in cancer immunotherapy

Yao Jiang[1,2†], Kai Hong[3†], Yingchao Zhao[1,2*] and Kai Xu[4*]

[1]Cancer Center, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2]Institute of Radiation Oncology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [3]Department of Medical Ultrasound, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [4]Department of Otolaryngology-Head and Neck Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Immune evasion is essential for carcinogenesis and cancer progression. Programmed death-ligand 1 (PD-L1), a critical immune checkpoint molecule, interacts with programmed death receptor-1 (PD-1) on immune cells to suppress anti-tumor immune responses. In the past decade, antibodies targeting PD-1/PD-L1 have tremendously altered cancer treatment paradigms. Post-translational modifications have been reported as key regulators of PD-L1 expression. Among these modifications, ubiquitination and deubiquitination are reversible processes that dynamically control protein degradation and stabilization. Deubiquitinating enzymes (DUBs) are responsible for deubiquitination and have emerged as crucial players in tumor growth, progression, and immune evasion. Recently, studies have highlighted the participation of DUBs in deubiquitinating PD-L1 and modulating its expression. Here, we review the recent developments in deubiquitination modifications of PD-L1 and focus on the underlying mechanisms and effects on anti-tumor immunity.

KEYWORDS

deubiquitinating enzymes, deubiquitination, cancer immunotherapy, post-translational modification, programmed death-ligand-1 (PD-L1)

1 Introduction

Immune evasion is essential for carcinogenesis and cancer progression. Cancer cells have developed multiple mechanisms to evade immune surveillance, including reducing immunogenicity, limiting antigen recognition, inducing T cell exhaustion, and expressing inhibitory immune checkpoint proteins (1). Among these checkpoint molecules, programmed death-ligand 1 (PD-L1) is one of the most critical players. PD-L1 interacts with programmed

death receptor-1 (PD-1) on immune cells, including T cells, dendritic cells, macrophages, and natural killer (NK) cells to restrain anti-tumor immunity (2). Elevated PD-L1 expression has been observed in multiple cancers including cervical cancer, non-small cell lung cancer (NSCLC), and hepatocellular cancer (3). Currently, PD-1/PD-L1-targeting treatments have significantly affected cancer treatment approaches, and PD-L1 expression has emerged as an indicator for the selection of patients who are more likely to benefit from PD-1/PD-L1 inhibitors (4, 5). Therefore, exploring PD-L1 regulatory mechanisms is of great importance.

The expression of PD-L1 is modulated at various levels, including epigenetic, transcriptional, post-transcriptional, and post-translational mechanisms (3, 6–8). Deubiquitination and ubiquitination are among the most important post-translational modifications, which dynamically control protein degradation and stability, thereby influencing cellular processes. Deubiquitination, mediated by deubiquitinating enzymes (DUBs), involves the covalent cleavage of conjugated monoubiquitin or polyubiquitin chains from various substrates (9, 10). To date, approximately 100 DUBs have been discovered. Based on their structural homology, DUBs can be classified into seven categories as follows: ubiquitin-specific proteases (USPs), otubain proteases (OTUs), and JAB1/MPN/Mov34 metalloenzymes (JAMMs), ubiquitin C-terminal hydrolases, Machado-Joseph disease proteases, MIU-containing novel DUB family proteases, and Zn-finger and UFSP domain proteins. With the exception of JAMMs, all of these DUBs belong to the cysteine protease family.

DUBs have been extensively studied in a variety of cellular activities including cell proliferation, apoptosis, cell cycle control, and adaptive immune response (11). Several DUBs have been demonstrated to deubiquitinate PD-L1 and regulate its expression in cancer (7) (Figure 1). Here, we provide a comprehensive review of the recent advancements in deubiquitination modifications of PD-L1, focusing on their impact and the underlying mechanisms related to anti-tumor immunity (Table 1).

2 Emerging progress on the regulation of PD-L1 by DUBs in cancer

2.1 CSN5

The constitutive photomorphogenesis 9 signalosome 5 (CSN5) contains a conserved JAMM motif, which belongs to the JAMM subfamily (32). On one hand, CSN5 could directly interact with a variety of molecules, including c-Jun and p53, thereby influencing tumor proliferation (12). On the other hand, as a deubiquitinase, CSN5 deubiquitinates PD-L1, promoting tumor progression and immune escape (12). One study demonstrated that the activation of nuclear factor κB (NF-κB), further transactivates CSN5, leading to PD-L1 deubiquitination and stabilization (12). Moreover, PD-L1 and CSN5 expression levels are positively correlated in breast cancer (12). The application of a CSN5 inhibitor, curcumin, results in PD-L1 destabilization, increases the cytotoxic activity of T cells, and synergizes with anti-cytotoxic T-lymphocyte associated protein 4 antibodies (12).

Multiple mechanisms regulate CSN5-mediated PD-L1 deubiquitination. Golgi membrane protein 1 upregulates the expression of PD-L1 in hepatocellular cancer cells through the CSN5-mediated deubiquitination of PD-L1, leading to the suppression of $CD8^+$ T cells (14). Ma et al. reported that protein disulfide isomerase family A member 6 interacts with CSN5 and promotes the deubiquitination of PD-L1 in pancreatic cancer cells (15). In colorectal cancer, macrophages-derived C-C motif chemokine ligand 5 (CCL5) promotes the activation of NF-κB p65 activation, which binds to the *CSN5* promoter, increases CSN5 expression, and upregulates PD-L1 protein level (16). In triple negative breast cancer, long non-coding RNA GATA binding protein 3 antisense RNA 1 stabilized PD-L1 *via* the miR-676-3p/CSN5 axis (13). Interestingly, berberine, an established anti-inflammatory drug, interacts with CSN5 and inhibits CSN5/PD-L1 interaction, resulting in PD-L1 ubiquitination (17).

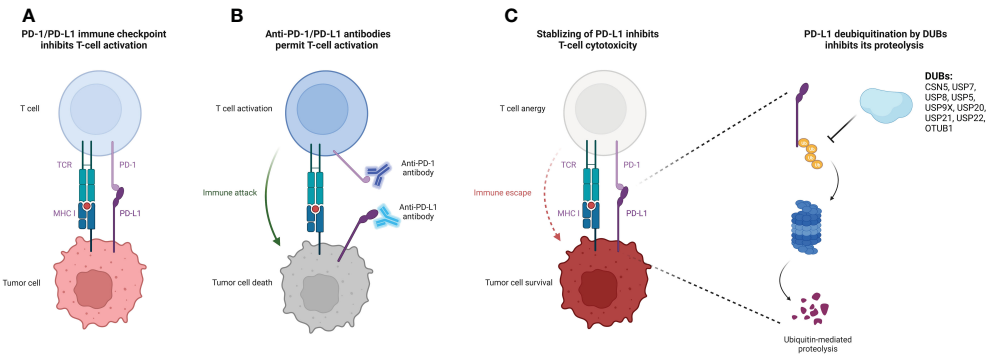

FIGURE 1
Deubiquitination of PD-L1 protein by DUBs causes increased PD-L1 stability and suppressed T-cell cytotoxicity. **(A)** Binding of PD-L1 on the tumor cell surface to their receptor PD-1 on the T cell surface releases the immune suppression signal, thereby inhibiting T cell activation and cytotoxicity. **(B)** The administration of specific antibodies to PD-L1/PD-1 reverses the T cell activation suppression signal, facilitating for the immune attack form of cytotoxic T cells to target tumor cells. **(C)** DUBs of PD-L1 stabilize PD-L1 and protect it from ubiquitin-mediated proteolysis, promoting tumor cell immune escape from T cell attack. The figure was created with Biorender.com. PD-L1, programmed death-ligand-1; DUB, deubiquitinating enzyme; MHC-I, major histocompatibility complex class I; PD-1, programmed death receptor-1; TCR, T cell receptor.

TABLE 1 Major deubiquitinating enzymes (DUBs) of PD-L1 and their biological effects in cancer.

DUBs	Categories	Mechanism	Types of cancer	Related molecules	Effects	References
CSN5	JAMM	Removes K48-linked ubiquitin on PD-L1	Breast cancer	NF-κB p65, GATA-AS1	Inhibits anti-tumor function of T cells	(12, 13)
			Hepatocellular cancer	GOLM1	Inhibits $CD8^+$ T cell cytotoxicity	(14)
			Pancreatic cancer	PDIA6	Inhibits NK cell function	(15)
			Colorectal cancer	CCL5, NF-κB p65	Inhibits $CD8^+$ T cell cytotoxicity	(16)
			NSCLC	BBR	Inhibits intratumor T cell infiltration	17)
USP7	USP	Stabilizes PD-L1 through deubiquitination	Glioma	N/A	Inhibits $CD8^+$ T cell cytotoxicity	(18)
			Gastric cancer	N/A	Inhibits T cell mediated cytotoxicity and tumor cell proliferation	(19)
USP8	USP	Removes K63-linked ubiquitin on PD-L1	Multiple cancer types	TRAF6, NF-κB	Inhibits MHC-I-dependent antigen presentation	(20)
		Stabilizes PD-L1 through deubiquitination	Pancreatic cancer	N/A	Inhibits $CD8^+$ T cell cytotoxicity	(21)
			NSCLC	LncRNA SNHG12, HuR	Inhibits $CD8^+$ T cell cytotoxicity	(22)
USP5	USP	Stabilizes PD-L1 through deubiquitination	NSCLC	N/A	Inhibits $CD8^+$ T cell cytotoxicity	(23)
USP9X	USP	Stabilizes PD-L1 through deubiquitination	Oral cancer	N/A	Inhibits T cell cytotoxicity	(24)
USP20	USP	Stabilizes PD-L1 through deubiquitination	Breast cancer	TINCR	N/A	(25)
USP21	USP	Removes K48-linked ubiquitin on PD-L1	Lung cancer	N/A	N/A	(26)
			Colorectal cancer	STAT3, Foxp3	Promotes Treg cell function	(27)
USP22	USP	Stabilizes PD-L1 through deubiquitination	NSCLC	CSN5	Inhibits T cell cytotoxicity	(28)
			Liver cancer	N/A	Inhibits intratumor T cell infiltration	(29)
OTUB1	OTU	Removes K48-linked ubiquitin on PD-L1	Breast cancer	N/A	Inhibits T cell cytotoxicity	(30)
			NSCLC	PKP3	Inhibits $CD8^+$ T cell infiltration	(31)

PD-L1, programmed death-ligand-1; DUBs, deubiquitinating enzymes; USP, ubiquitin-specific proteases; OTU, otubain proteases; JAMM, JAB1/MPN/Mov34 metalloenzymes; NF-κB, nuclear factor κB; GATA-AS1, GATA binding protein 3 antisense RNA 1; CSN5, The constitutive photomorphogenesis 9 (COP9) signalosome 5; GOLM1, Golgi membrane protein 1; PDIA6, protein disulfide isomerase family A member 6; CCL5, C-C motif chemokine ligand 5; NSCLC, non-small cell lung cancer; BBR, berberine; N/A, not applicable; TRAF6, TNF receptor associated factor 6; LncRNA SNHG12, lncRNA small nucleolar RNA host gene 12; HuR, human antigen R; TINCR, tissue differentiation inducing non-protein coding RNA; STAT3, signal transducer and activator of transcription 3; Foxp3, forkhead box P3; PKP3, plakophilin 3; MHC-I, major histocompatibility complex class I; NK, natural killer.

2.2 USP7

USP7 is a deubiquitinase that contains a USP domain (33), and is aberrantly expressed in several human cancers. USP7 mediates cell cycle control, tumor growth, chemoresistance, and tumor immunity by regulating multiple cellular signaling pathways, including the p53 and Wnt pathways (33, 34).

Regulatory T cells (Tregs) suppress the activity of effector T cells and promote immune escape (35). Moreover, Tip60, a histone acetyltransferase, promotes the acetylation and dimerization of the key transcription factor, forkhead box P3 (Foxp3), and regulates the activity of Tregs (35). A previous study discovered that USP7 directly deubiquitinates Foxp3 and stabilizes it. Further, USP7 depletion disrupts the immunosuppressive functions of Tregs *in vivo* (36). Another investigation revealed that USP7 is a deubiquitinase of both Tip60 and Foxp3, which enhances Tregs functions by increasing protein abundance (37).

Emerging studies have demonstrated that USP7 expression correlates with PD-L1 levels in cancer (18, 38). One recent investigation observed the overexpression of USP7 and PD-L1 proteins in glioma (18). USP7 mediates the deubiquitination of PD-L1, leading to increased PD-L1 expression. Abrogated USP7 expression promotes $CD8^+$ T cell proliferation, elevates tumor necrosis factor (TNF) alpha and interferon gamma (IFN-γ) levels, and inhibits glioma cell immune evasion, which can be reversed by PD-L1 overexpression (18). Similarly, USP7 expression is

upregulated and positively associated with PD-L1 in gastric cancer. Silencing USP7 decreases PD-L1 expression on cell surfaces, and augments the T cell-mediated killing of cancer cells (19). However, the regulatory relationship between USP7 and PD-L1 appears to be context-dependent. A negative association between the USP7 and PD-L1 expression in lung adenocarcinoma was revealed using The Cancer Genome Atlas data (38). In addition, the targeted inhibition of USP7 significantly increases PD-L1 protein levels in both cancer cells and the tumor microenvironment. Furthermore, abrogated USP7 expression inhibits the M2 macrophages transformation and their function, and promotes IFN-γ^+CD8$^+$ T cells infiltration, augmenting anti-tumor immunity. Additionally, a USP7 inhibitor, P5091, has shown a synergistic anti-tumor effect with a PD-1 inhibitor *in vivo* (38, 39).

2.3 USP8

Increasing evidence suggests that USP8 expression is upregulated, which stabilizes multiple oncogenes, in various cancers (22, 40). Additionally, USP8 is involved in T cell development and homeostasis. It is also essential for thymocyte maturation, proliferation, and the suppressive function of Treg cells on γδ T cells. Mechanistically, USP8 interacts with Gads and 14-3-3β, forming a complex with the T cell receptor (TCR)−CD28 cluster upon stimulation. Subsequently, USP8 is degraded *via* a caspase-dependent pathway, leading to the downregulation of interleukin-7 receptor subunit alpha (IL-7Rα) levels through the Forkhead box protein O1−IL-7Rα axis (41). Another study demonstrated that USP8 deubiquitinates and increases the expression of the type II transforming growth factor-β receptor (TβRII) in tumor-derived extracellular vesicles (TEVs). The inhibition of USP8 reduces the abundance of TβRII$^+$ circulating TEVs and prevents CD8+ T cell exhaustion (42).

In a screening study performed by Xiong et al., it was revealed that DUBs-IN-2, a USP8 inhibitor, significantly increases PD-L1 protein levels in multiple cancer cell lines (20). Furthermore, a negative association between USP8 and PD-L1 was confirmed in lung squamous cancer tissues. Mechanistically, USP8 specifically removes K63-linked ubiquitination, but promotes the K48-linked ubiquitination of PD-L1, which finally promotes PD-L1 degradation. Furthermore, by deubiquitinating the K63-linked modification of TNF receptor associated factor 6 (TRAF6), USP8 up-regulates the expression of most genes in the major histocompatibility complex class I pathways, which limits the NF-κB signaling pathway and inhibits the immune response and antigen presentation. A USP8 inhibitor synergizes with anti-PD-1/PD-L1 treatments, dramatically inhibits tumor growth, and improves survival rates in mouse colon cancer models (20). Conversely, a recent study showed that the expression levels of USP8 and PD-L1 are positively correlated in pancreatic cancer. USP8 deficiency decreases PD-L1 protein abundance by promoting PD-L1 ubiquitination-mediated degradation. Moreover, a combined strategy comprising a USP8 inhibitor and PD-L1 inhibitor decreases tumor growth and enhances CD8$^+$ T cell mediated killing of cancer cells (21).

2.4 USP5

USP5 belongs to the USP subfamily and can specifically recognize unconjugated polyubiquitin and cleave ubiquitin linkages. USP5 participates in multiple cellular procedures, including inflammatory responses (43, 44). The NLR family pyrin domain-containing 3 (NLRP3) inflammasome is critical for defense against microbial pathogens, and its dysregulation is implicated in various inflammatory diseases. Notably, USP5 is involved in regulating NLRP3 inflammasome activity, unrelated to its DUBs function. Mechanistically, USP5 acts as a pivotal scaffold protein recruiting a specific E3 ligase to NLRP3, promoting its ubiquitination and autophagic degradation. In addition, in alum-induced peritonitis mouse models, the overexpression of USP5 reduces interleukin 1 beta (IL-β) levels and polymorphonuclear infiltration (45). Furthermore, recent studies have demonstrated that USP5 directly deubiquitinates and stabilizes PD-L1. In NSCLC tissues, elevated USP5 expression correlates with PD-L1 expression, indicating of unfavorable clinical outcomes. Moreover, the inhibition of USP5 suppresses tumor growth *in vivo* by downregulating PD-L1 expression (23).

2.5 USP9X

USP 9, X-linked (USP9X) is a positive regulator of the TCR signaling pathway. Silencing of USP9X *in vivo* suppresses T-cell growth, cytokine production, and the differentiation of T helper (Th) cells, without affecting T-cell survival and the development of specific T-cell populations in the thymus. Moreover, *USP9X* knockdown in human and mouse T-cell lines attenuates the TCR signaling-mediated activation of NF-κB through the deubiquitination of Bcl10 (46). *USP9X* knockout also results in a proliferation defect in both CD4$^+$and CD8$^+$ T cells, impairs the development of T cells in the thymus, and downregulates proximal TCR signaling. *In vivo* studies demonstrated that the T cell-specific knockout of *Usp9x* elevates PD-1-expressing T cell populations, leading to the incidence of specific autoimmune disease (47). In B lymphocytes, USP9X is necessary for the kinase activity of protein kinase C beta after B cell antigen receptor-dependent activation (48). In a model of sepsis with liver injury, USP9X promotes CD8$^+$ T cells dysfunction in the liver through the inhibition of autophagy, which can be reversed by the conditional depletion of mechanistic target of rapamycin (49). Moreover, USP9X directly binds PD-L1, and USP9X reduces PD-L1 ubiquitination and increases its protein abundance. Additionally, a positive association was found between USP9X and PD-L1 expression in oral cancer (24).

2.6 USP20

USP20 has been linked to antiviral response, metabolic disease, neuroinflammation, and tumor progression (50–52). Tax is a viral oncoprotein which persistently activates NF-κB signaling and causes adult T cell leukemia. Through deubiquitination of TRAF6

and Tax, USP20 suppresses activation of NF-κB signaling and inhibits proliferation of leukemia cells (53).

A recent study demonstrated that USP20 interacts with PD-L1 and deubiquitinates it, which can be regulated by a long non-coding RNA, tissue differentiation inducing non-protein coding RNA (TINCR). Mechanistically, LncRNA TINCR acts as a competing endogenous RNA, which promotes stability of USP20 mRNA and upregulates the expression of PD-L1 in breast cancer (25).

2.7 USP21

Numerous studies have implicated USP21 in regulating cancer cell stemness, tumor growth, and metastasis (26, 54). In regard to immune regulation, Yang et al. have demonstrated a direct interaction between USP21 and PD-L1, whereby USP21 removes polyubiquitin chains from PD-L1, leading to its stabilization. Notably, the expression of USP21 is upregulated in lung cancer tissues, showing a positive association with PD-L1 protein levels (26). Additionally, USP21 has a role in regulating Treg cell functions (27). Li et al. demonstrated that USP21 suppresses the transformation of Th1-like Treg cells by deubiquitinating and stabilizing Foxp3. Mouse models of *Usp21* depletion in Tregs exhibit spontaneous T cell activation and the expanded transformation of Tregs toward the Th1-like phenotype (55). Furthermore, emerging evidence suggests that USP21 suppresses antiviral responses in various immune cell types, including mouse embryonic fibroblasts and bone marrow-derived dendritic cells. This is achieved through the binding and deubiquitination of retinoic acid-inducible gene-I, which restricts type-I interferon production and antiviral immune defense (56). Notably, *Usp21*-knockout mice display enhanced resistance to vesicular stomatitis virus infection with increased production of interferons (56).

2.8 USP22

USP22, of which expression levels are elevated in various cancer types, is correlated with disease progression and an unfavorable prognosis (57, 58). This DUB plays a critical role in regulating PD-L1 stability. On one hand, USP22 directly deubiquitinates PD-L1. On the other hand, USP22 interacts with CSN5 and deubiquitinates it, thereby facilitating the interaction between PD-L1 and CSN5 (28). Moreover, USP22 and PD-L1 protein levels are positively correlated in NSCLC samples. The inhibition of USP22 enhances the cytotoxicity of T cells and reduces tumor growth (28). Another study revealed that USP22 interacts with the C terminus of PD-L1 and deubiquitinates it. In mouse models of hepatocellular carcinoma, knockout of *Usp22* increases the infiltration of tumor-infiltrating lymphocytes, augments anti-tumor immunity, and synergizes with anti-PD-L1 treatments and chemotherapy (29). Furthermore, USP22 plays a part in regulating the tumor microenvironment. The knockout of *USP22* in pancreatic ductal adenocarcinoma cells results in reduced myeloid cells infiltration and increased tumor infiltration of NK cells and T cells, leading to a synergistic response with combined immunotherapy (59). USP22 is also involved in regulating invariant NK T (iNKT) cells. USP22 suppression inhibits the development of iNKT cells, and attenuates iNKT1 and iNKT17 cell differentiation, while favoring iNKT2 polarization (60).

2.9 OTUB1

OTUB1, a member of the OTU superfamily, exhibits a preference for deubiquitinating K-48 and K-63 ubiquitin chains. Its involvement in cancer development and progression has also been observed (61). Extensive research has highlighted the role of OTUB1 in modulating immune cell responses. Depletion of *OTUB1* activates NK cells and $CD8^+$ T cells, leading to increased tumor infiltration of NK cells, DCs and T cells. Additionally, *OTUB1* depletion enhances the cytokine production and the proliferation of $CD4^+$ T cells (62, 63).

Moreover, OTUB1 has been reported to specifically interact with of PD-L1, wherein it removes the K48-linked ubiquitin chain from PD-L1 and stabilizes it. Functionally, *OTUB1* depletion decreases PD-L1 expression, and increases the cytotoxicity of human peripheral blood mononuclear cells against tumor cells. The expression of OTUB1 is positively correlated with PD-L1 expression in breast cancer samples. Furthermore, OTUB1 depletion increases $CD8^+$ T cell infiltration, elevates serum IFN-γ, and augments anti-tumor immune responses in mouse models (30). Liu et al. reported that circIGF2BP3 acts competitively to upregulate plakophilin 3 (PKP3) expression, which further stabilizes OTUB1 mRNA. CircIGF2BP3/PKP3 suppression synergized with anti-PD-1 treatment in mouse models of lung cancer (31).

3 Discussion

Over the past few decades, the application of anti-PD-1/PD-L1 treatments has significantly improved the clinical prognoses of patients with cancer. Nevertheless, the clinical response to single-agent anti-PD-1/PD-L1 antibody therapy is limited to only a subset of patients (64, 65). Combinatorial treatments comprising anti-PD-1/PD-L1 antibodies with antiangiogenic drugs, chemotherapy, and targeted therapy have resulted in more promising clinical outcomes (66). As described above, several DUBs are participated in deubiquitination modifications of PD-L1, and regulated its expression. Thus, developing small-molecule inhibitors targeting these DUBs and the combination therapy represent an attractive therapeutic strategy.

However, despite the importance of these reported DUBs, there are still questions remain to be elucidated. One such question is determining which specific DUB plays the predominant role in regulating PD-L1 expression within a particular type of cancer. Second, although some studies have demonstrated synergistic efficacy of DUBs inhibitors with anti-PD-1/PD-L1 treatments, further exploration through clinical trials is needed to validate these findings and assess their potential for clinical application. In recent years, multiple selective inhibitors of DUBs, including inhibitors of USP7, USP8, and USP9X, have been developed (11). Accordingly, further investigation of these inhibitors in clinical trials is required.

Author contributions

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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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Xiangpeng Dai,
Jilin University, China

REVIEWED BY
Xiaoling Zhang,
Jilin University, China
Bo Xu,
Xuzhou Medical University, China

*CORRESPONDENCE
Xiao-Hong Li
xhli18@tju.edu.cn
Chong Chen
calphen_cc_gz@163.com

†These authors have contributed equally to this work

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Post-translational modifications and immune responses in liver cancer

You-Wei Wang†, Jia-Chen Zuo†, Chong Chen* and Xiao-Hong Li*

Academy of Medical Engineering and Translational Medicine, Medical College of Tianjin University, Tianjin, China

Post-translational modification (PTM) refers to the covalent attachment of functional groups to protein substrates, resulting in structural and functional changes. PTMs not only regulate the development and progression of liver cancer, but also play a crucial role in the immune response against cancer. Cancer immunity encompasses the combined efforts of innate and adaptive immune surveillance against tumor antigens, tumor cells, and tumorigenic microenvironments. Increasing evidence suggests that immunotherapies, which harness the immune system's potential to combat cancer, can effectively improve cancer patient prognosis and prolong the survival. This review presents a comprehensive summary of the current understanding of key PTMs such as phosphorylation, ubiquitination, SUMOylation, and glycosylation in the context of immune cancer surveillance against liver cancer. Additionally, it highlights potential targets associated with these modifications to enhance the response to immunotherapies in the treatment of liver cancer.

KEYWORDS

hepatocellular carcinoma, post-translational modifications, immune surveillance, phosphorylation, ubiquitination, SUMOylation, glycosylation

Introduction

Liver cancer is one of the most common malignancies worldwide and directly causes nearly one million deaths each year (1). According to global cancer statistics in 2020, liver cancer is the sixth most diagnosed cancer and the third most common cause of cancer death (2). In 2020, about 900,000 people worldwide were diagnosed with liver cancer and about 800,000 died of liver cancer. It is estimated that the number of liver cancer diagnoses could reach 1.3 million by the year of 2040 (3). Primary liver cancer mainly includes four types: hepatoblastoma (HB), hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), and combined hepatocellular carcinoma and cholangiocarcinoma (cHCC-CCA) (4). HCC is the main type of primary liver cancer, accounting for approximately 75% of the

total number of liver cancer cases worldwide. CCA is the second most common primary liver cancer, of which intrahepatic cholangiocarcinoma (ICC) is a highly heterogeneous primary epithelial liver cancer (5). While novel therapeutic approaches have demonstrated notable clinical efficacy or promising prospects in cancer treatment (6), the current primary approach for liver cancer therapy is still surgical intervention.

Protein translational modifications (PTMs) are covalent attachment of functional groups to protein substrates and can alter the activity, stability, protein interaction, and intracellular localization of target proteins (7). These modifications involve addition of chemical groups (methylation, acetylation, phosphorylation, etc.), addition of polypeptide chains (ubiquitination, SUMOylation, etc.), amino acid modification (racemization, citrullination, etc.), and addition of complex molecules (palmitoylation, oxidation, glycosylation, etc.) (8, 9). PTMs, whether direct or indirect, have a significant impact on the immunogenicity of cancer cells, thereby affecting their recognition and susceptibility to immune system. Furthermore, these modifications also play a crucial role in shaping the response of various immune cells, influencing their interactions with liver tumor cells within the microenvironment. PTMs exert a significant influence on the initiation, progression, immune evasion, and immunotherapy of cancers. By investigating PTMs, we can gain valuable insights into the mechanisms governing cancer-immune cell interactions and potentially develop novel strategies to enhance anti-cancer immune responses.

Acetylation and methylation have received extensive attention in previous reviews (10, 11). In this review, our primary focus will be the profound influence of phosphorylation, ubiquitination, glycosylation, and SUMOylation on liver cancers, with a particular emphasis on their immunological significance.

Phosphorylation

Phosphorylation, a highly conserved type of PTM (12), primarily targets serine, threonine, or tyrosine residues, and involves a reversible reaction mediated by protein kinases and protein phosphatase (13). This essential modification plays a pivotal role in numerous biological processes, including protein interactions, stability, signal transduction, transcriptional regulation, and intracellular localization (14).

T cells play a central role in the immune system and tumor immune response. Some immunotherapies that target T cells, such as CAR (Chimeric Antibody Receptor)-T cell therapy and checkpoint inhibitors (15, 16), have shown promising results in cancer immunotherapy. T-cell development, differentiation, and activation are intricately regulated by phosphorylation events which target various transcription factors. These phosphorylation events play a critical role in dictating T cell fates and functions. The phosphorylation of specific transcription factors, such as signal transducer and activator of transcription 1 (STAT1) in Th1 cells, STAT6 in Th2 cells, and STAT3 in Th17 cells, contributes to their differentiation and functional specialization (17–21). In patients with HCC, Th1 cytokines of serum level are often suppressed, while Th2 cytokines are frequently elevated (22). Interleukin-6 (IL-6), one of the Th2 cytokines, has been observed to exhibit a negative correlation with overall survival rate and can independently serve as a predictive factor for survival. Conversely, increase of Th1 cytokine responses have been linked to favorable immunological effects on the prognosis of HCC (23). An increase in Th1-related cytokines and a decrease in Th2-related cytokines was observed in a study on primary HCC after radiofrequency ablation (RFA) treatment (22). Th17 cells, a specific subset of T-helper cells, play a pivotal role in immune responses through the production of IL-17 (24, 25). IL-17 acts on HCC cells and triggers the activation of AKT (protein kinase B) through phosphorylation. This activation leads to the production of IL-6 by HCC cells (26). In patients with HCC, there is an elevated presence of Th17 cells compared to healthy individuals, and as the severity of HCC malignancy worsens, the levels of Th17 cells further escalate (27).

Macrophages are the main effector cells in chronic inflammation, a known driver of carcinogenesis (28). Serine/threonine-protein kinase 4 (STK4) was considered as a pivotal tumor suppressor gene in HCC. Notably, significant downregulation of STK4 expression observed in macrophages isolated from HCC patients. This decrease in STK4 expression shows a strong inverse correlation with the levels of IL-1 receptor-associated kinase 1 (IRAK1). Through its interaction with IRAK1 and subsequent phosphorylating it, STK4 exerts inhibitory effects on the secretion of proinflammatory cytokines, including IL-6, IL-1β, and tumor necrosis factor-α (TNF-α), particularly following the activation of Toll-like receptor 4/9 (TLR4/9). This implies that the regulatory mechanism mediated by STK4 attenuates the chronic inflammatory response and significantly reduces the probability of HCC development (29).

Macrophages can be categorized into two subpopulation based on their distinct functions: M1 macrophages, which promote inflammatory responses, and M2 macrophages, which support tissue repair and cell proliferation (30). In liver cancers, macrophages tend to exhibit excessive M2-like polarization, thereby suppressing immune responses against cancer cells. Recent findings highlight the importance of protein phosphorylation in the cancer microenvironment for macrophage polarization (31). Sirtuin 1 (SIRT1) has been shown to enhance the infiltration of M1-like macrophages and inhibit HCC metastasis. This effect is mediated by SIRT1's ability to enhance nuclear factor kappa-B (NF-κB) activation and promote the phosphorylation of p65, IκB, and IκB kinase (IKK) (22). Zinc finger protein 64 (ZFP64), a gene upregulated in HCC patients with unfavorable prognosis in anti-PD1 treatment, undergoes direct phosphorylation at S226 by protein kinase Cα (PKCα), leading to its nuclear translocation and the transcriptional activation of macrophage colony-stimulating factor (CSF1). CSF1 derived from HCC cells further promotes macrophage polarization towards M2 phenotype.

NK (natural killer) cells earned their name due to their remarkable ability to "naturally" eliminate cancer cells without

the need for prior sensitization, and without being restricted by the major histocompatibility complex (MHC) (32). Upon entering the tumor microenvironment (TME) or encountering cancer cells, NK cells can eliminate cancer cells through self-destruction mechanisms (perforin/granzyme mediated) or +antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism (33). In contrast to the NK cells found in peripheral blood, the liver harbors two distinct types of NK cells: one shares similarities with circulating NK cells (cNK cells), while the other primarily resides within liver tissue (trNK cells) (34). Despite various pathways easily active NK cell cytotoxicity, the killing capacity of NK cells can also be easily inhibited, especially within the TME of HCC. The PI3K/AKT/mTOR (phosphoinositide 3-kinase, protein kinase B, and mammalian target of rapamycin) signaling pathway plays a crucial role in the development of HCC and the immune response of NK cells against HCC. Aberrant activation of the PI3K/AKT/mTOR pathway confers HCC cells with enhanced metabolic capacity, promoting their proliferation and metastasis (35). The development and cytotoxic capability of NK cells also heavily rely on the activation of the PI3K/AKT/mTOR signaling pathway (36). PI3K consists of a catalytic subunit, p100, and a regulatory adapter subunit, p85. The p85 subunit is responsible for linking p100 to activated receptor tyrosine kinases (RTKs), thereby activating PI3K and initiating the PI3K/AKT/mTOR signaling pathway (37). Tim-3 is one of the checkpoint molecules expressed on the surface of NK cells. Its expression levels are significantly elevated in HCC. Bind with phosphatidylserine induces phosphorylation of Tim-3, which further interferes with PI3K/AKT/mTOR pathway in NK cells. By competitively binding to p85, phosphorylated Tim-3 reduces the opportunity for PI3K p110 to bind with p85 and leads to decreased activity of the downstream AKT/mTOR pathway, thereby suppressing the activity of liver NK cells, including cNK and trNK (38).

Ubiquitination and SUMOylation

Ubiquitination is a posttranslational modification wherein ubiquitin molecules are covalently attached to target proteins (39). This process relies on the coordinated action of three key adaptor proteins: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3) (40). The canonical ubiquitination pathway involves the attachment of ubiquitin lysine amino acids (Ub) to glycine residues located at the C-terminus of target proteins, while the atypical pathways involve the conjugation of ubiquitin to cysteine, serine, and threonine residues on target proteins (41). Ubiquitination can facilitate various downstream responses, including degradation, alterations in activity, changes in subcellular localization, or modulation of protein-protein interactions (42–44). Modulating ubiquitin levels has been shown to have a profound impact on T cell activation and can effectively enhance antitumor responses, as indicated by reference (45). Here, we will shift our focus towards the impact of ubiquitination on other immune cells.

IL-2, IL-15, and IL-21 are members of the common gamma chain receptor family cytokines. While they share numerous similarities, these cytokines also show distinct functions within NK cells. IL-15 is primarily involved in promoting NK cell maturation, whereas IL-2 enhances NK cell cytotoxicity (32). IL-21 facilitates NK cell proliferation without causing telomere shortening (46). However, the mechanisms underlying the discriminatory capacity of NK cells among these closely related cytokines, despite their shared receptors, have not been fully elucidated. IL-15 serves as a critical regulator in the development and maturation of NK cells (47), and it has demonstrated the ability to restore NK cell dysfunction that is impaired by HCC (48). Ubiquitination and deubiquitination processes also play vital roles during IL-15-mediated NK cell maturation. Similar to IL-2, IL-15 binds to its receptor trigger not only phosphorylation, but also ubiquitination of AKT. Otub1, a deubiquitinases enzyme, is involved in inhibiting the ubiquitination of AKT. This negative regulation exerted by Otub1 serves as a checkpoint mechanism, influencing the function of NK cells (49). IL-2 and IL-15 share two identical chains in their receptors, and their downstream effects in NK cells are highly similar. However, Otub1 has minimal impact on the activation of AKT by IL-2. Investigating the differential ubiquitination patterns of downstream molecules may provide new insights and potential avenues for fully understanding the function and signal transduction mechanism of these common gamma chain cytokines.

Although the application of CAR-T cell therapy in liver cancer is still in its early stages, it holds tremendous promise for future advancements. A major hurdle in the effectiveness of CAR-T cell therapy lies in the rapid ubiquitination and subsequent degradation of CAR upon interaction with tumor antigens. This phenomenon presents a significant challenge in maintaining the sustained efficacy of CAR-T cell therapy. Fortunately, recent studies have shown that by introducing specific mutations that target the amino acid residues involved in CAR ubiquitination, the long-term killing capacity of CAR-T cells can be significantly improved (50). Ubiquitination is also linked to other protein or gene regulatory mechanisms. For instance, in a study focusing on Treg cells in HCC, it was observed that the expression level of long noncoding RNA lnc-EGFR (Epidermal Growth Factor Receptor) was elevated, showing a positive correlation with tumor size and EGFR/forkhead box protein 3 (Foxp3) expression levels. By directly binding to EGFR protein, lnc-EGFR preventing its ubiquitination and subsequently stabilizing EGFR, thereby enhancing Treg function and promoting the progression of HCC (51).

SUMO (or SUMOylation), which stands for Small Ubiquitin-like Modifier, is a protein modification process that commonly targets lysine residues, involving the attachment of small regulatory peptides of approximately 11 KDa. Like ubiquitin, this post-translational modification regulates various biological processes such as cell division, DNA replication/repair, signal transduction, and cell metabolism (52). HCC-derived exosomes play a significant role in remodeling the TME and promoting HCC progression (53). One key factor involved in this process is the pyruvate kinase M2

isoform (PKM2) found within these exosomes (54, 55). HCC-derived exosomal PKM2 not only induces metabolic reprogramming in monocytes but also triggers the phosphorylation of nuclear STAT3. This phosphorylation leads to the up-regulation of differentiation-associated transcription factors, promoting M2-like macrophage differentiation. The SUMOylation of PKM2 is responsible for its plasma membrane targeting and subsequent excretion through interaction with arrestin-domain-containing protein 1 (ARRDC1). Additionally, the cytokines and chemokines secreted by macrophages further reinforce the association between PKM2 and ARRDC1 in HCC. This reinforcement occurs through a CCL1-CCR8 axis-dependent mechanism, ultimately promoting the excretion of PKM2 from HCC cells. Consequently, a feed-forward regulatory loop is formed, contributing to tumorigenesis (55).

Glycosylation

Glycosylation is a form of co-translational and post-translational modification that involves the attachment of glycans to proteins. It is primarily categorized into two types: N-chain glycosylation, where the glycan is linked to asparagine residues, and O-chain glycosylation, where the glycan is attached to oxygen atoms on the hydroxyl groups of serine or threonine amino acid residues within protein (56). Many tumor-associated antigens related to HCC are highly glycosylated proteins, and their glycosylation profiles undergo significant changes in HCC patients (56). Aberrant glycosylation not only promotes the proliferation and metastasis of HCC but also plays an important role in immune recognition and immune escape.

Abnormally expressed alpha-fetoprotein (AFP) in HCC has an inhibitory effect on tumor immune surveillance. It has long been observed that AFP in HCC undergoes different glycosylation compared with normal AFP (57). Tumor-derived AFP exhibits stronger immunosuppressive effects, characterized by lower dendritic cell maturation and decreased T cell activation (58). Recent studies using single-cell metabolic profiling and single-cell energetic metabolism by profiling translation inhibition techniques have found that HCC-derived AFP binds significantly more polyunsaturated fatty acids than normal AFP. Phagocytosis of HCC-derived AFP reduced fatty acid uptake by dendritic cells, increased glucose uptake and metabolism, decreased expression of co-stimulatory molecules, and increased expression of immune checkpoint molecules such as PD-L1. These mechanisms help the tumor evade T cell mediated immune surveillance (59).

IL-12 is a cytokine of significant importance in promoting T cell differentiation and IFN-γ production. IL-12 not only activates $CD8^+$ T cells and NK cells in HCC tumors (60) but also enhances the cytotoxicity of Glypican-3-targeting CAR-T cells (61). IL-12 (p70) is composed of two subunits, p30 and p40. The free p40 subunit can act as a negative regulator by blocking the binding of IL-12 to its receptor, thereby inhibiting the biological activity of IL-12 (62). The IL-12 cytokine and its family members are glycoproteins (63). Post-translational glycosylation is a critical step in regulating IL-12 secretion (64). Through molecular biology techniques, mutations in the N-glycosylation site (N220) of the p40 subunit, a component of the Th1 cytokine IL-12, have been shown to reduce the secretion of free p40. However, these mutations have minimal impact on IL-12 secretion. As a result, they significantly enhance long-term $CD8^+$ T cell responses and provide protection against tumor attacks. These mutations can be utilized as adjuvants to generate long-term memory T cells (65).

Keratinocyte-associated protein 2 (KRTCAP2) is a critical protein involved in N-glycosylation processes, which play a fundamental role in the modification of proteins with complex sugar molecules in various cellular contexts. In HCC, there is a notable upregulation of KRTCAP2 expression, highlighting its potential significance in HCC pathogenesis and progression. Interestingly, high levels of KRTCAP2 are associated with a decreased infiltration of $CD8^+$ T cells and $CD68^+$ macrophages, both in the tumor region and the surrounding stroma. Furthermore, the expression level of KRTCAP2 shows a negative correlation with the expression of PD-L1 in HCC (66). The interaction between PD-1 and PD-L1 serves as a critical immune checkpoint and has gained significant recognition as a prominent target for cancer immunotherapy. Elucidating the precise role of KRTCAP2 in the modulation of the TME holds considerable scientific significance and translational potential for overcoming immunosuppression in HCC.

Summary and discussion

Liver cancer is a common malignant tumor, which poses a great threat to human health and life. Protein posttranslational modification and immune response play an important role in the development of liver cancer, the immune surveillance against liver cancer, and the treatment of patients with liver cancer. Figure 1 summarized a mechanism by which PTM contributed in cytokine mediated cancer immune surveillance. Numerous studies have shown promising therapeutic potential in targeting PTM for liver cancer treatment. STT3A is a endoplasmic reticulum-associated N-glycosyltransferase, which glycosylates PD-L1 and maintain its stability (67). One notable finding is that spermine, a natural polyamine compound, can activate β-catenin, a protein involved in cell adhesion and signaling pathways. Activation of β-catenin leads to the transcriptional expression of PD-L1 and N-glycosyltransferase STT3A (68). Targeting STT3A might be a potential strategy for improving the response to checkpoint inhibitors in HCC patients.

In the treatment of HCC, certain drugs have been observed to induce alterations in glycosylation. Sorafenib, for instance, has been identified as capable of modifying the glycosylation patterns of multiple proteins in HCC. Further research is needed to determine whether these changes can be targeted to enhance the efficacy of this HCC therapeutic drugs (69). Additionally, researchers are exploring novel approaches that focus on the

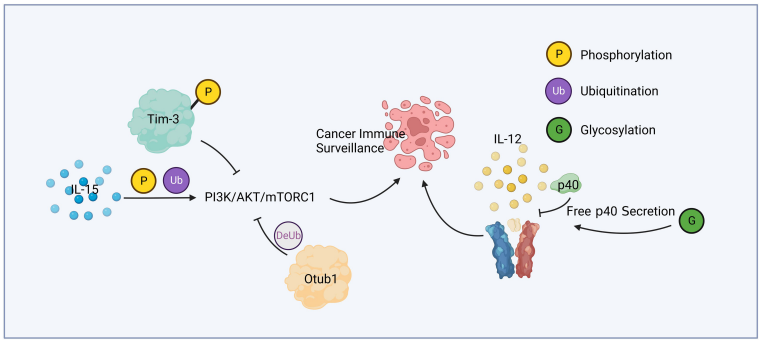

FIGURE 1
Cytokine relevant post-translational modification and immune surveillance. IL-15 active PI3K/AKT/mTORC1 pathway through phosphorylation and ubiquitination. Phosphorylated Tim-3 competitively inhibits this pathway, while Otub1 downregulates it by deubiquitination. Glycosylation of p40 increases the secretion of free p40, leading to the attenuation of IL-12 signaling.

TABLE 1 Examples of PTM targeting immunotherapy studies for HCC.

Drug	Immune cells	PTM	Treatment rationale	References
TLR3 agonist with sorafenib	DCs	Phosphorylation	Decreasing phosphorylation of AKT, MEK1/2, ERK1/2 and played an anti-HCC role.	(71)
MY1340	DCs	Phosphorylation	Inhibiting tumor growth *in vivo* by blocking the VEGF-NRP-1 axis through phosphorylation of p65 NF-κB and ERK1/2.	(72)
Caffeic acid (C9H8O4)	Macrophages, T cells	Ubiquitination	Inducing ubiquitination-mediated mortalin degradation to inhibit angiogenesis and reverse sorafenib resistance.	(73)
DMC	$CD8^+$T cells	Ubiquitination	Promoting the ubiquitin degradation of PD-L1 in HBx-induced HCC and showing an anti-hepatoma function.	(74)
Targeting MUC1 Glycosylation	CAR-T cells	Glycosylation	Targeting MUC1 aberrant O-glycosylation can control HCC growth.	(75)

MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor; NRP, neuropilin; NF-κB, nuclear factor kappa-B; DMC, 2,5-dimethylcelecoxib; HBx, hepatitis B virus X; MUC1, Mucin1.

aberrant glycosylation sites of tumor-associated antigens in HCC. These strategies involve the utilization of antibodies or antigen specific T cells with the aim of converting specific tumor-associated antigens into tumor-specific antigens. Although these studies are still in their early stages, promising preclinical prospects have already emerged (70). Some studies aiming to establish PTM based immunotherapy strategies against HCC were listed in Table 1.

In this review, we summarized the current knowledge of post-translational modification of protein in liver cancer cells, tumor infiltrated immune cells, and the microenvironment of liver cancer. Unraveling the intricate network of post-translational modifications in liver cancer holds great promise for advancing our understanding of this disease and undoubtedly contributes to the development of more effective and personalized treatments.

Author contributions

Y-WW and J-CZ contributed equally to this study. 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.

Publisher's note

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EDITED BY
Xiangpeng Dai,
Jilin University, China

REVIEWED BY
Jianli Tao,
Boston Children's Hospital and Harvard Medical School, United States
Lu Yang,
Sichuan University, China
Jia Hu,
Huazhong University of Science and Technology, China

*CORRESPONDENCE
Yasheng Zhu
zys0562@foxmail.com
Chuanliang Xu
chuanliang_xu@126.com

†These authors have contributed equally to this work and share first authorship

‡These authors share last authorship

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E3 ubiquitin ligases and deubiquitinases in bladder cancer tumorigenesis and implications for immunotherapies

Maoyu Wang[1†], Zhensheng Zhang[1†], Zhizhou Li[1†], Yasheng Zhu[1,2*‡] and Chuanliang Xu[1*‡]

[1]Department of Urology, Shanghai Changhai Hospital, Naval Medical University, Shanghai, China, [2]Department of Urology, Shanghai Changzheng Hospital, Naval Medical University, Shanghai, China

With the rapidly increasing incidence of bladder cancer in China and worldwide, great efforts have been made to understand the detailed mechanism of bladder cancer tumorigenesis. Recently, the introduction of immune checkpoint inhibitor-based immunotherapy has changed the treatment strategy for bladder cancer, especially for advanced bladder cancer, and has improved the survival of patients. The ubiquitin–proteasome system, which affects many biological processes, plays an important role in bladder cancer. Several E3 ubiquitin ligases and deubiquitinases target immune checkpoints, either directly or indirectly. In this review, we summarize the recent progress in E3 ubiquitin ligases and deubiquitinases in bladder cancer tumorigenesis and further highlight the implications for bladder cancer immunotherapies.

KEYWORDS

bladder cancer, E3 ubiquitin ligase, deubiquitinases, immunotherapy, tumorigenesis

1 Introduction

Bladder cancer (BCa) is one of the most common types of cancer, with 550,000 new cases and 200,000 deaths annually (1). While the 5-year survival rate of all bladder cancer patients is 77.1%, the rate drops dramatically to 36.3% for regional disease and 4.6% for metastatic disease (2). Therefore, adjunctive therapy is needed to improve the prognosis of invasive and metastatic diseases. Cisplatin and gemcitabine combination chemotherapy has been applied for advanced bladder cancer (3); however, no major improvements in survival rate have been achieved until recently. The 5-year survival rate for patients with metastasis is 15% (3).

Immunotherapy, especially immune checkpoint inhibitors, is widely used for the treatment of different cancers (4, 5). BCa has been reported to be relatively sensitive to immunotherapy (6, 7). In May 2016, atezolizumab was the first PD-L1 inhibitor approved

by the Food and Drug Administration (FDA) for bladder cancer (8). Since then, another four immune checkpoint inhibitors targeting PD-1 or PD-L1 for locally advanced and metastatic bladder cancer, including Nivolumab, Pembrolizumab, Avelumab, and Durvalumab have been approved by FDA for bladder cancer (8–10). However, owing to a lack of response, only a small group of patients with BCa can benefit from these agents (11). Taking PD-L1 as example, many studies have verified that PD-L1 expression is correlated with anti-PD-1/PD-L1 treatment, where high PD-L1 expression is equal to a good response to anti-PD-1/PD-L1 treatment (12). Thus, exploring the mechanism and identifying other reagents that can improve the efficacy of immune checkpoint blockade (ICB) is urgently needed (13). A series of mechanisms of PD-L1 regulation by post-translational modifications have been revealed in different cancers among recent research, including bladder cancer (14–16).

Ubiquitination and deubiquitinating modifications are highly conserved posttranslational modifications (PTMs) in mammals that play important roles in many biological processes and diseases, including cancers. The ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3 contribute to the step-by-step process of ubiquitination. Ubiquitination involves the transfer of the C-terminal glycine of ubiquitin to the -NH2 group of the substrate lysine residue. Monoubiquitination, multiubiquitination, and polyubiquitination, which lead to proteolysis and signal transduction, are the three main types of ubiquitination (17). On the other hand, deubiquitinases (DUBs) can reverse ubiquitination by removing ubiquitin chains, thereby preserving the expression of the substrate protein while preventing ubiquitination. Most elements of biological activity depend on the interplay between ubiquitination and deubiquitination (13).

Numerous studies have demonstrated that the ubiquitin proteasome system (UPS) is related to the occurrence and progression of bladder cancer and that E3 ubiquitin ligases may be promising therapeutic targets (18–21). Meanwhile, the interaction between ubiquitination modification and immune-related molecules is emerging as a crucial regulatory mechanism and has recently draws great research interest (16, 22–25).

In this review, we summarize recent findings on protein ubiquitination and deubiquitinating enzymes in bladder cancer tumorigenesis and progression, as well as recent advances in the regulation of cancer immunotherapy effects.

2 Roles and mechanisms of E3 ubiquitin ligases in bladder cancer

2.1 The category of E3 ubiquitin ligases

Over 600 types of E3 ubiquitin ligases involved in the degradation of proteins have been discovered in humans (26). E3 ligases are classified into three subtypes: the interesting new gene (RING)-type, the homologous to E6AP carboxyl terminus (HECT)-type, and the RING-between-RING (RBR)-type (27). RING E3 ligases contain multiple subtypes, including monomers (c-CBL, E4B), homodimers (cIAP, CHIP), heterodimers (MDM2-MDMX), cullin-RING ligases (CRLs), and other RING E3s (28). CRLs are comprised of multiple subunits, which consist of four components: a cullin (CUL1,2,3,4A,4B,5,7,9), an adaptor protein, a substrate-recognizing receptor, and one RING protein (29–32). Moreover, SCF is the largest complex, consisting of SKP1, Cullin1, RBX1, and F-box proteins (29, 33). HECT structures are divided into three subfamilies: NEDD4 subfamily, HERC subfamily, and other HECT E3 ligases (34). RBRs are grouped into the Ariadne family and other RBRs (35). In particular, E3 ubiquitin ligases determine substrate specificity in the ubiquitination process.

2.2 Roles of E3 ubiquitin ligases in bladder cancer

In addition to maintaining the balance of intracellular proteins, E3 ligases are involved in multiple non-degradable functions including intracellular transport, autophagy, DNA damage repair, and metabolism (36). Thus, E3 ubiquitin ligases are critical for cellular processes. Therefore, their dysregulation may have a potential effect on the pathogenesis of cancer. Disorders of E3 ligases result in aberrant activation or inactivation of signaling pathways and the accumulation of misfolded or dysfunctional proteins (37), which promotes the occurrence and progression of cancer.

Numerous E3 ligases have been reported to be involved in bladder cancer tumorigenesis. They are involved in the regulation of key molecules including PD-L1, PTEN, and p53 (Table 1). In this section, we provide a detailed description of each E3 ligase in bladder cancer.

2.2.1 RNF126

RNF126 is a RING domain E3 ligase. A group of RNF126 substrates has been identified, including frataxin (62–64), epidermal growth factor receptor (64), pyruvate dehydrogenase kinases (65) and insulin-like growth factor II receptor (66). RNF126 is highly expressed in various cancers and strongly associated with tumorigenesis, including bladder cancer (38, 67–69). In BCa, RNF126 directly binds to PTEN via its C-terminal containing the RING domain and promotes the polyubiquitination and degradation of PTEN through the proteasome pathway (38). *In vivo* and *in vitro* studies have demonstrated that PTEN acts as an anti-oncogene, and PTEN silencing is closely related to the poor prognosis of patients with BCa (70). RNF126 silencing stabilizes PTEN, which antagonizes PI3K/AKT signaling pathway (38, 39), and promotes cell proliferation and metastasis when activated.

Moreover, previous studies revealed that RNF126 promotes the repair of DNA double-strand breaks via NHEJ and HR through different mechanisms (71, 72). The Ku70-Ku80 heterodimer recognizes DNA double-strand breaks (DSBs) and recruits proteins responsible for DNA repair by non-homologous end joining (NHEJ). While prolonged retention of Ku70/80 at DSBs prevents the completion of DNA repair, RNF126 ubiquitylates

TABLE 1 E3 ligases in bladder cancer tumorigenesis.

E3	Function	Substrate	Pathway	Reference
RNF126	Promoting/oncogene	PTEN	PI3K/AKT	(38, 39)
RNF144A	Promoting/oncogene	PD-L1		(40, 41)
NEDD4	Promoting/oncogene	PD-L1		(16, 42)
		KLF8	microRNA-132/NRF2	(43, 44)
		PTEN		(42)
RBX1	Promoting/oncogene	p-IκBα	NF-κB	(45)
		DEPTOR	mTOR	(46)
		SUFU	RBX1-SUFU-GLI2	(47)
cIAP2	DNA damage response	MRE11		(48, 49)
FBW7	Tumor suppressor	ZMYND8		(50)
		RhoGDIα	p65/PTEN/FBW7/RhoGDIα	(51)
TRAF4	Promoting/oncogene		BMP/SMAD	(21)
TRIM21	Promoting/oncogene	ZHX3		(52)
TRIM65	Promoting/oncogene	ANXA2		(53)
TRIM25	Promoting/oncogene	RBPJ	Notch1	(54)
TRIM26	Promoting/oncogene		AKT/GSK3β/β-catenin	(55)
CUL4B	Promoting/oncogene	H2AK119	CUL4B/miR-372/373/PIK3CA/AKT	(56)
TRIM38	Promoting/oncogene	GLUT1		(57)
RFWD3,HUWE1 MDM2,DTL	Promoting/oncogene			(58–61)

Ku80 at DSBs and promotes Ku70/80 dissociation from DSBs. In contrast, RNF126 can ubiquitinate and quench RNF168 function in the DNA damage response (71). Cisplatin has been widely used as first-line treatment for patients with advanced BCa (73). Furthermore, cisplatin induces cell apoptosis by accumulating DNA double-strand breaks. RNF126 depletion markedly increases the effect of cisplatin in inducing apoptosis in BCa cells (38). It has also been reported that RNF126 can directly bind and regulate PTEN stability through polyubiquitination, making RNF126 an attractive target for augmenting cisplatin-based chemotherapy and regulating bladder cancer tumorigenesis.

2.2.2 RNF144A

RNF144A belongs to the RBR E3 ubiquitin ligase family. Epigenetic depletion of RNF144A has been detected in numerous human cancers, including glioblastoma (74), breast cancer (75), and bladder cancer (40), indicating that RNF144A may act as a tumor suppressor. Previous studies have found that RNF144A is upregulated by various DNA-damaging agents (76) and further promotes cancer cell apoptosis of cancer cells by ubiquitinating and degrading DNA-PKcs and BMI1 (74, 77).

In a recent study, the basal-squamous subtype of bladder cancer has been found to express relatively low levels of RNF144A and high levels of immune checkpoint protein programmed cell death ligand-1(PD-L1) (41). The carboxyl-terminal region (aa 250–292) of RNF144A is responsible for its interaction with PD-L1, and RNF144A mainly targets glycosylated PD-L1 for degradation (40), further indicating a complex mechanism between protein ubiquitination and glycosylation.

2.2.3 NEDD4

NEDD4 is a HECT family E3 ubiquitin ligase (78). Mounting evidence has demonstrated that NEDD4 participates in the tumorigenesis of human cancers, such as cervical cancer (79), hepatocellular carcinoma (80), and breast cancer (81). NEDD4 is highly expressed in bladder cancer and promotes tumor cell migration and invasion (42, 43). KLF8 acts as a transcription factor in the Sp/KLF family and stimulates and promotes migration of bladder cancer cells. Moreover, miR-132 is downregulated by KLF8, which is overexpressed in bladder cancer. NEDD4 is conformed to interact with KLF8 (44). In bladder cancer, NEDD4 depletion significantly downregulated endogenous KLF8 ubiquitination, which affected the K63-linked polyubiquitination of KLF8, while K48-linked polyubiquitination remained unchanged. NEDD4 intensifies the stability and transcriptional activity of KLF8 through ubiquitination and affects the miR-132/NRF2 axis, thereby promoting tumor progression (44).

The ubiquitin ligase activity of NEDD4 can be promoted by FGFR1 and EGFR activation via tyrosine phosphorylation of NEDD4 (82). Previous studies have demonstrated that there is relatively decreased expression of PD-L1 in bladder cancer with

FGFR3 mutations or high expression (41, 83, 84). Jing et al. (16) have indicated that the activation of FGFR3 promoted NEDD4 binding and phosphorylation and it had been reported that NEDD4 can be phosphorylated to greatly improve its ubiquitination capacity. NEDD4 depletion using CRISPR/Cas9-sgRNA remarkably upregulated PD-L1 expression in bladder cancer cells. NEDD4 targets and catalyzes the K48-linked polyubiquitination of PD-L1. These results reveal that NEDD4 is a critical regulator of PD-L1 expression in bladder cancer upon FGFR3 activation. This study provides powerful evidence for the combination of anti-PD-1 antibody therapy and erdafitinib, a tyrosine kinase inhibitor of FGFR1–4 (16).

As mentioned earlier, PTEN acts as an oncogene in bladder cancer. NEDD4 regulates PTEN levels in several types of human cancers (85). In bladder cancer, PTEN levels were increased by NEDD4 silencing (42). NEDD4 downregulation inhibits cell proliferation and apoptosis. However, the precise mechanism by which NEDD4 regulates PTEN expression has not been fully elucidated.

2.2.4 RBX1

The cullin/RING ubiquitin ligase (CRL)family is the largest UPS E3 family (86). RBX1 forms the catalytic core of CRL complexes with different Cullin subunits (87). RBX1 is widely reported to be associated with poor clinical prognosis and is highly expressed in many cancers, including bladder cancer. In particular, RBX1 expression is significantly higher in muscle-invasive BCa and positively correlated with epithelial–mesenchymal transition (EMT) via inhibition of mTOR kinase activity by accumulation of the cullin-RING ligase (CRL) substrate mTOR-inhibitory protein DEPTOR (46).

Moreover, RBX1 has been confirmed to be positively correlated with activation of the NF-κB signaling pathway and nuclear p65 expression (45). p65 plays a key role in the canonical NF-κB pathway and is inactive in the cytoplasm upon binding to IκBα. Upon receiving the relevant signals, IκBα is phosphorylated, which is then ubiquitinated and degraded. Finally, p65 enters the nucleus and activates gene transcription (88). Therefore, IκBα-p65 is a key regulatory factor in the NF-κB signaling pathway. Activation of the NF-κB signaling pathway promotes tumor progression (89). By enhancing p-IκBα ubiquitination and degradation, RBX1 activates NF-κB signaling, which promotes p65 nuclear translocation and causes the transcription of several metastasis-related target genes including matrix metalloproteinase 9 (MMP9), vascular cell adhesion molecule 1 (VCAM1), and urokinase-type plasminogen activator receptor (uPAR) (45). Recently, Wang et al. demonstrated that RBX1 can activate the hedgehog pathway through the ubiquitinate suppressor of fused homolog (SUFU) for degradation, and dysregulation of the RBX1–SUFU–GLI2 axis play a pivotal role in bladder cancer progression (47).

2.2.5 cIAP2

IAP family members have been indicated to act as a key role in the regulation of NF-κB signaling and participate in intrinsic and extrinsic cell death pathways (90). cIAP2 is a RING-type E3 ligase in the IAP family and has been demonstrated to play a pivotal role in DNA repair (91, 92). Although the expression of cIAP1 examined by immunohistochemical testing is highly correlated to bladder cancer TNM stage, tumor grade, disease recurrence, and tumor-related death (93) and cIAP2 precise function and substrate specificity is unclear, previous studies have a common sense that there is redundancy between cIAP1 and cIAP2 in the regulation of cell death (94, 95). Recently, cIAP2 was reported to be involved in regulating radiosensitization in bladder cancer (48).

Histone deacetylase (HDAC) inhibitors exhibit low toxicity in normal cells, and panobinostat, an HDAC inhibitor, is a promising radiosensitizer (96). Panobinostat downregulates MRE11 (49), which is a key player in DNA repair, leading to a decreased ability to repair DNA, thereby enhancing radio sensitization. In T24 cells, transfecting cIAP2 into cells in increasing quantities, a growing decrease in MRE11 levels was observed. cIAP2 downregulates MRE11 via proteasomal pathways and increases the ubiquitination of MRE11. Furthermore, T24 cells became more radiosensitive after panobinostat treatment when cIAP2 was silenced.

2.2.6 FBW7

F-box and WD repeat domain-containing 7(FBW7) is a member of the RING E3 ligase family, which is a subunit of the SKP1, cullin1, and F-box protein ubiquitin ligase complex (29). Low expression and mutation of FBW7 has been frequently detected in various human tumors such as breast cancer (97), colon cancer (98), and gastric cancer (99). Therefore, FBW7 is generally considered a tumor suppressor. According to the analysis of public datasets TCGA-BLCA and GSE13507, it has been verified that the mRNA expression levels of FBW7 are significantly downregulated in bladder tumors compared with normal samples (50). Kaplan–Meier analysis suggested that patients with BCa with high FBW7 expression levels exhibited longer survival times. Collectively, these results indicate that FBW7 may serve as a tumor suppressor in bladder cancer. ZMYND8 was acted as a common oncogene in numerous tumors, including bladder cancer (50). Bioinformatics predictive analysis from the UbiBrowser platform (http://ubibrowser.ncpsb.org/) and ubiquitination assays demonstrated that in T24 cells, ZMYND8 was a substrate target of FBW7. FBW7 is a tumor suppressor that is and downregulated in BCa. Low expression of FBW7 can increase the protein levels of ZMYND8 and promote BCa progression (50). This result was further confirmed in clinical samples.

Moreover, FBW7 was verified to be an NF-κBp65 downstream effector. Through promoting RHO guanosine diphosphate dissociation inhibitor (RhoGDIα) protein degradation, FBW7 significantly inhibited BCa migration (51). Mechanistically, p65 inhibited PTEN mRNA transcription, whereas PTEN accelerated FBW7 protein degradation. This revealed the function of the p65/PTEN/FBW7/RhoGDIα axis in mediating bladder cancer migration and expands the theoretical support for the regulation of the NF-κBp65 and PTEN pathways in BCa treatment.

2.2.7 MDM2

MDM2 is reported to mainly target p53 protein in various types of cancer, including bladder cancer (100). The SNP309

polymorphisms of MDM2 is associated with an improved survival rate of bladder cancer (101). MDM2 is upregulated by the OCT3/4/TET1/NRF2 axis, which contributes to increased immune escape in bladder cancer (102). Amounts of inhibitors, such as MDM2 exerted an influence on immunity in the tumor microenvironment, such as APG-115 and AMG-232. APG-115 can enhance the efficacy of PD-L1 blockade (103) and AMG-232 (104) can increase the ability to kill T cells. Furthermore, gene amplification of MDM2 can act as a predictive marker for PD-L1 targeted therapy response (105).

2.3 Other E3 ubiquitin ligases

Several other E3 ubiquitin ligases are also involved in bladder tumorigenesis. RFWD3 is highly expressed in bladder cancer tissue and correlates with a higher N stage and poorer prognosis (58). A bladder cancer genome-wide CRISPR/Cas9 KO screen showed that HUWE1 was correlated with cisplatin sensitivity in bladder cancer; however, the underlying mechanism has not been elucidated (59). MDM2 binds to PPARγ to ubiquitinate and downregulate its PPARγ expression (60). Denticleless E3 ubiquitin protein ligase homolog (DTL) is overexpressed in BCa, and increased DTL expression correlates with malignant biological behavior and promotes BCa progression through the AKT/mTOR pathway (61). A pan-cancer study also showed that DLT could be a potential immunotherapy biomarker (106).

TRAF4 can bind to and target another E3 ligase, SMURF1, for proteasomal degradation (21). As SMURF1 is a negative regulator of the BMP/SMAD signaling pathway, TRAF4 can promote BMP/SMAD signaling and inhibit bladder cancer progression (21). TRIM21 acts as a ubiquitin E3 ligase to degrade ZHX3, which is involved in bladder cancer progression and metastasis (52). The expression level of TRIM65 is frequently upregulated and ANXA2 is ubiquitinated and degraded by TRIM65. Bladder cancer patients with low ANXA2 expression and high TRIM65 expression showed the poorest outcome (53). RITA1 recruits TRIM25 to ubiquitinate RBPJ to accelerate its degradation via the proteasome, which leads to transcriptional inhibition of Notch1 downstream targets (54). TRIM26 plays an oncogenic role in bladder cancer by regulating cell proliferation, migration, and invasion via the AKT/GSK3β/β-catenin pathway (55). CUL4B is a scaffold protein in the CUL4B–RING ubiquitin ligase (CRL4B) complexes. CUL4B levels are overexpressed and positively associated with the malignancy of BCa, and CUL4B epigenetically represses the transcription of miR-372/373 by catalyzing the monoubiquitination of H2AK119 in the gene cluster encoding miR-372/373, which further leads to the upregulation of PIK3CA and activation of AKT (56).

Reprogramming cell metabolism is a hallmark of cancer (107, 108). Aerobic glycolysis has been extensively studied in several cancers, including bladder cancer (107). It is characterized by increased glucose uptake and lactate production under normal oxygen conditions. Elevated glycolytic flux in cancer cells is mediated by glycolysis-associated signature genes, including GLUT1 (109). GLUT1 driven glycolytic reprogramming is considered necessary for tumor cell growth (110).Wang et al. identified GLUT1 as the downstream substrate of TRIM38 and TRIM38 can constrain bladder tumor progression through ubiquitination and degradation of GLUT1 (57). TRIM38 has been verified to be a predictive biomarker related to prognosis, with low expression in BCa (57).

3 Deubiquitinases in bladder cancer

3.1 Overview of deubiquitinases

Deubiquitinases (DUBs) are proteases that remove ubiquitin from substrates or cleave ubiquitin chains to regulate ubiquitination (111). It is important to regulate the processes of deubiquitination and ubiquitination (112). DUBs consist of cysteine proteases and metalloproteinases that specifically cleave ubiquitin molecules on protein substrates (113). Approximately 100 different DUBs can be broadly classified into seven distinct superfamilies (114). Six of these families are cysyrine-based DUBs, including Ub C-terminal hydrolases (UCHs), Ub-specific proteases (USPs), Machado-Josephin domain proteases (MJDs), ovarian tumor proteases (OTUs), motifs interacting with the Ub-containing novel DUB family (MINDY), zinc-finger-containing Ub peptidase (ZUP1), and Jab1/Mov34/MPN+ protease (JAMM) family members, which are zinc-binding metalloproteases (115).

Numerous studies have demonstrated that the effect of protein deubiquitination is associated with the occurrence and development of cancers, such as prostate cancer, lung cancer, stomach cancer, and bladder cancer (116–120). A summary of the deubiquitinases involved in BCa is presented in Table 2.

3.2 Roles of deubiquitinases in bladder cancer

3.2.1 OTUD5

There are 16 types of cysteine protease OTU family members, including OTUB, OTUD, A20-like, and OTULIN subfamily (113). The OTUD family is one of the subfamilies including OTUD1, OTUD2/YOD1, OTUD3, OTUD4, OTUD5/DUBA, OTUD6A, OTUD6B, and ALG13 (113, 136). OTUD5 has been the focus of numerous studies and plays pivotal roles in various cellular processes. The first report of function of OTUD5 is to negatively regulate IFN-1 expression by cleaving the polyubiquitin chains on TRAF3 (137). Furthermore, OTUD5 regulates DNA damage repair, transcription, and innate immunity (138, 139).

In bladder cancer, OTUD5 has been shown that is highly expressed in tumor tissues compared with normal urothelial cells (121). OTUD5 knockdown inhibited the cell proliferation, and OTUD5 positively regulated the mTOR signaling pathway to promote cell proliferation. Specifically, OTUD5 stabilizes RNF186 by deubiquitination, leading to sestrin2 degradation, which acts as a feedback inhibitor of the mTOR signaling pathway (140, 141). Everolimus treatment, an mTOR inhibitor, with simultaneous OTUD5 knockdown seems to be an ideal strategy for bladder cancer treatment (121).

TABLE 2 Deubiquitinases in bladder cancer tumorigenesis.

DUBs	Function	Substrate	Pathway	Reference
OTUD5	Promoting/oncogene	RNF186	mTOR	(121)
OTUB1	Promoting/oncogene	ATF6α		(122)
		SLC7A11		(123)
MINDY1	Promoting/oncogene	YAP		(119)
UCHL5	Promoting/oncogene	c-Myc	AKT/mTOR	(124)
	Cisplatin resistance		β-catenin, c-Myc	(125)
USP24	Promoting/oncogene	GSDMB	GSDMB/STAT3	(126)
USP13	Tumor suppressor	PTEN		(127)
USP7	Tumor suppressor	CCDC6		(128, 129)
USP8	Promoting/oncogene	AUF1	USP8/AUF1/RhoGD1β	(130)
USP38	Tumor suppressor	METTL14		(131)
USP22 USP18,USP28	Promoting/oncogene			(132–135)

3.2.2 OTUB1

The deubiquitinase OTUB1 is significantly more highly expressed in bladder cancer tumor tissues than in normal tissues (122). Kaplan–Meier survival analysis confirmed that bladder cancer patients with low OTUB1 expression had significantly superior overall survival compared to those with high OTUB1 expression. It has been found that OTUB1 can stabilize activating transcription factor 6α (ATF6α) in response to endoplasmic reticulum stress and promote bladder cancer progression (122). Numerous studies have indicated that ferroptosis is an important and independent mechanism of tumor suppression (142). Solute carrier family 7, membrane 11 (SLC7A11), a 12-pass transmembrane protein, acts as a potential biomarker for protecting cancer cells from oxidative stress and ferroptosis (143). Liu et al. discovered a distinct mechanism by which OTUB1 mediates ferroptosis in bladder cancer via the stabilization of SLC7A11 (123).

3.2.3 MINDY1

MINDY1 (also known as FAM63A) has been reported that contains MIU motifs with high selectivity for binding and cleaving K48-linked polyUb (144). The Hippo signaling pathway has emerged as a critical pathway in the regulation of bladder cancer tumorigenesis, and TAZ and YAP are important effectors of this pathway (145–147). MINDY1 removes the K48-linked ubiquitin chain from YAP, thus inhibiting proteasome-mediated YAP degradation, which will in turn promote the expression of YAP downstream genes, CTGF, ANKRD1, and CYR61 (119).

3.2.4 UCHL5

UCHL5 is abnormally upregulated in human cancer tissues and cell lines, such as pancreatic adenocarcinoma, gastric cancer, endometrial cancer, and bladder cancer (124, 148–150). Upregulation of the TGF signaling pathway is the main mechanism by which UCHL5 modulates malignant tumor progression (151–153). UCHL5 is overexpressed in patients with bladder cancer patients, and high expression is associated with poor prognosis and tumor progression. Mechanistically, UCHL5 activates the AKT/mTOR signaling pathway and increases c-Myc expression, which promotes tumor occurrence and progression (124). Meanwhile, it has been reported that the UCHL5 inhibitor b-AP15 suppresses bladder cancer stemness by inhibiting the β-catenin and c-Myc signaling pathways and overcomes cisplatin resistance (125). b-AP15 has been demonstrated to have synergistic effects in combination with cisplatin, gefitinib, gemcitabine, and vinorelbine in lung cancer cells (154). In bladder cancer cell lines and mouse xenograft models, b-AP15 combined with cisplatin showed superior therapeutic effects compared to cisplatin monotherapy (125). These studies indicate that UCHL5 may act as a potential therapeutic target, and that b-AP15 may be a new choice for patients with cisplatin resistance.

3.2.5 USP24

Ubiquitin-specific peptidase 24 (USP24), consisting of 2,620 amino acids, serves as a deubiquitinase (155). However, the biological function of USP24 in cancer is poorly understood. It has been reported that USP24 binds to GSDMB to deubiquitinate and stabilize GSDMB. GSDMB promotes cancer cell growth by activating STAT3, which increases the expression of HK2, LDNA, ENO2, and IGFBP3 to enhance glycolysis in bladder cancer cells (126). EOAI3402143, a USP24 inhibitor, can block this process, which provides a therapeutic strategy for inhibiting the GSDMB/STAT3 axis (126).

3.2.6 USP13

USP13 belongs to the Ub-specific protease subfamily of deubiquitinase family. USP13 has been indicated in suppressing tumor occurrence by deubiquitinating anti-oncogenes, including p53

(156), PTEN (157), and MITF (158), and subsequently stabilizing these proteins. As mentioned above, PTEN acts as a key tumor suppressor in bladder cancer via inhibition of the PI3K/AKT/mTOR signaling pathway. Otherwise, NF-κB activation has been reported to be essential for inhibition of PTEN expression (159, 160). PTEN is deubiquitinated by USP13 in bladder cancer, and its stabilized expression suppresses tumor progression (127). There is also a potential regulatory loop in which NF-κB induces miR-130b/301b overexpression, decreasing USP13 expression and subsequently leading to the downregulation of PTEN overexpression (127).

3.2.7 USP2a/7/8/18/22/28/38

Several studies have demonstrated that other USPs serve as oncogenes in BCa tumorigenesis (128, 130, 132–134, 161). Jeong et al. detect the mRNA expression of USP2a in bladder cancer tissues and normal tissues. The results indicate that the expression of USP2a in bladder cancer is downregulated and that high stage muscle invasive bladder cancer (MIBC) has lower USP2a expression. USP2a can be specifically used as a potential marker to stratify the more invasive phenotype of MIBC (132).

USP7 has been reported to modulate CCDC6 levels in bladder cancer and lung neuroendocrine cancers (129). CCDC6 acts as a tumor suppressor, its deficiency determines the sensitivity of PARP-inhibitors (162, 163). In a recent study, P5091, an inhibitor of USP7, promoted CCDC6 degradation and sensitized bladder cancer cells to the cytotoxic effect of the PARP-inhibitor olaparib (128).

The non-canonical NF-κB subunit p52 upregulates USP8 expression at the transcriptional level, and USP8 modulates AUF1 protein degradation. USP8 plays a significant role in the p52/miR-145/Sp1/USP8/AUF1/RhoGD1β axis, which can act as a positive regulator of bladder cancer invasion (130).

USP22 is a positive regulator of tumor growth. Silencing USP22 by interfering with RNA inhibits proliferation and induces cell cycle arrest in BCa cells (133). USP18 and USP28 have been reported to serve as prognostic markers for bladder cancer (134, 135). A study also revealed a feedback loop of USP38 and METTL14 in bladder cancer to suppress BCa progression. METTL14 stabilizes USP38 mRNA expression through YTHDF2-dependant m6A modification and USP38 enhances the stability of METTL14 by deubiquitination of METTL14 (131).

4 Role of E3 ligases and DUBs in immunotherapy of bladder cancer

The concept of immunotherapies for bladder cancer can be divided into cytokine-based treatment, genetically engineered immune cells (adoptive cell therapy), oncolytic viruses, bispecific antibodies, intravesical therapy with Bacillus Calmette–Guerin (BCG) vaccine, immune checkpoint inhibitors (ICIs), and antibody–drug conjugates (ADCs) (10, 164, 165).

BCG immunotherapy remains the gold standard treatment for patients with non-muscle-invasive bladder cancer (NMIBC) at a high risk of progression or recurrence (166). Although it has been used in clinical practice since 1976, the mechanism of the BCG vaccine in BCa is not completely understood. Upon attachment to the urothelium and internalization, it is thought to induce innate and adaptive immune responses. However, whether a combination of reagents targeting E3 ligases or DUBs can augment the response to BCG or conquer certain patients' unresponsiveness to BCG warrants further exploration (167).

The adoption of ICIs in bladder cancer has dramatically changed its treatment landscape (168). ICIs are now approved for the treatment of BCa at all stages, depending on the specific tumor characteristics (10). Immune checkpoint inhibitors can enhance T-cell responses and provide promising clinical outcomes in bladder cancer. However, this treatment strategy has only a 13%–24% response rate among patients with bladder cancer. A deeper exploration of the mechanisms that regulate PD-1/PD-L1 expression and stability may help increase clinical effectiveness. During the last decade, intensive evidence has demonstrated that PD-1/PD-L1 protein expression is regulated by the ubiquitin-mediated proteasome degradation pathway (169–172).

RNF144A and NEDD4 have been reported to participate in the regulation of PD-L1 expression (Figure 1). The basal-squamous subtype of bladder cancer expresses relatively low levels of RNF144A and high levels of immune checkpoint protein programmed cell death ligand-1 (PD-L1) (41). The carboxyl-terminal region (aa 250–292) of RNF144A is responsible for its interaction with PD-L1 and RNF114A mainly targets glycosylated PD-L1 for degradation (40). PD-L1, primarily in the insoluble fraction, interacts with RNF144A, which contains the plasma membrane and intracellular vesicles (40). RNF114A knockout stabilizes PD-L1 and leads to a reduction in tumor-infiltrating CD8+ T-cell populations in BBN-induced bladder tumors (40). Thus, RNF144A E3 ligase may be a promising therapeutic target for immunotherapy or combined therapy.

FGFR3 is an eligible target for the treatment of bladder cancer. p-FGFR3 and NEDD4 co-localized at the cell surface of bladder cancer cells. It has been demonstrated that NEDD4 can be phosphorylated to greatly improve its ubiquitination capacity by FGFR3 (16). NEDD4 depletion using CRISPR/Cas9-sgRNA remarkably upregulated PD-L1 expression in bladder cancer cells. NEDD4 targets and catalyzes the K48-linked polyubiquitination of

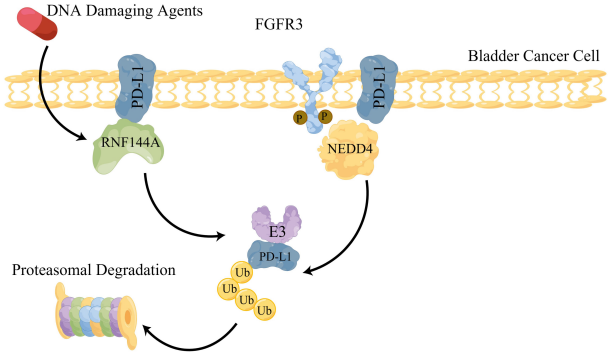

FIGURE 1
Graphic model of interaction between PD-L1 and E3 ligases in bladder cancer.

PD-L1. These results revealed that NEDD4 is a critical regulator of PD-L1 expression in bladder cancer with FGFR3 activation (16). Thus, NEDD4 E3 ligase may be a promising therapeutic target in the bladder with immunotherapy or combined therapy.

USP7 has been shown to regulate anti-tumor immune responses. The activity of Treg cells is impeded by its inhibitor and the polarization of tumor-related macrophages is enhanced (173). One study reported that USP7 expression is positively related to PD-L1 expression and USP7 directly binds to PD-L1 which stabilized it in gastric cancer (117).However, the function of USP7 inhibitors in enhancing the immune response in bladder cancer remains unclear. Therefore, it is essential to investigate the role of USP7 in bladder cancer.

Although some other DUBs, including USP22 (174) and USP9X (175), have been shown to regulate PD-1/PD-L1 expression, no research has been conducted on bladder cancer. Because ubiquitination or deubiquitination of certain molecules can be cellular context-dependent, E3 ligases and DUBs targeting PD-1/PD-L1 in other tumors should be further verified in bladder cancer. Several E3 ligases and DUBs, especially DUBs, can be directly targeted by small molecular drugs; thus the combination of specific inhibitors and ICIs might be attractive and promising for enhancing ICI treatment effects (176). Notably, deubiquitinating enzymes are potential biomarkers for treatment selection and prognosis prediction (177).

In addition to PD-1 or PD-L1 based immunotherapy, antibody–drug conjugates (ADCs) have recently shown great progress. An ADC targeting nectin-4 (Enfortumab Vedotin) has shown significantly prolonged survival in patients with locally advanced or metastatic urothelial carcinoma who previously received platinum-containing chemotherapy and progressed after treatment with a PD-1 or PD-L1 inhibitor (178). For patients who are not eligible for cisplatin-containing chemotherapy, Enfortumab Vedotin Plus Pembrolizumab may be a safe and effective surrogate for previously untreated advanced bladder cancer patients (179, 180). Nectin-4 is a transmembrane protein overexpressed in bladder cancer and several other malignancies, making it an appropriate target antigen for ADCs. However, little is known about its role in tumor development, progression, and immunomodulatory functions. It might also be interesting to investigate the regulation of stabilization and degradation (180).

Casitas B lymphoma-b (Cbl-b) is an E3 ligase that can modulate PD-L1 ubiquitination and degradation after inhibition of PI3K/Akt, Jak/Stat, and MAPK-Erk signaling (181). Cbl-b can also target the ubiquitination of PI3K NEDD4, PLCγ, and the zeta-subunit of TCR. Interestingly, Cbl-b also serves as a downstream regulator of both CD28 and CTLA-4 signaling pathways. Thus, both innate and adaptive immune cells are regulated by E3 ubiquitin ligase, promoting an immunosuppressive tumor microenvironment. This implicated a complex regulatory loop between CTLA-4, E3 ligase Cbl-b, and PD-L1. Novel Cbl-b inhibitors offer antigen-specific immune stimulation and are promising therapeutic tools in the field of immune-oncology (182).

5 Summary and perspectives

In summary, patients with advanced bladder cancer have poor survival rates, and immunotherapy may be a promising method for these patients. The use of single-agent immunotherapy or combined immunotherapy may be a further direction for treating advanced bladder cancer. A better understanding of bladder cancer progression and its regulation of immune-related molecules will help us to develop better therapeutic drugs and select appropriate patients. However, the overall efficacy is unsatisfactory, and a large number of patients cannot benefit from these agents due to a lack of response. PTMs have been indicted to play a significant role in the regulation of protein stabilization of the PD-1/PD-L1 axis. The ubiquitinase–protease system plays a pivotal role in bladder cancer, including in tumor progression, cisplatin resistance, tumor suppression, and predictive biomarkers. Notably, numerous E3 ligases and DUBs act as oncogenes, including RBX1, cIAP2, CUL4B, OTUD5, MINDY1, and USP24. FBW7, USP13, USP2a, USP8, and USP7 serve as tumor suppressors. Furthermore, emerging evidence has demonstrated that RNF114A and NEDD4 can modulate PD-L1 ubiquitination, which in turn leads to the subsequent modulation of immunosuppression and anticancer effects.

This review highlights the significant role of the UPS in bladder cancer carcinogenesis and in the regulation of certain immune therapy-related molecules, including PD-1/PD-L1. These findings indicate that E3 ligases and DUBs may act as potential targets for bladder cancer therapy or a promising therapeutic approach to promote immunotherapy effectiveness by regulating ubiquitination and deubiquitination.

Author contributions

MW, ZZ, and ZL wrote the manuscript and YZ and CX edited it. All authors contributed to the article and approved the submitted version.

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Figure 1 is drawn using Figdraw. Export ID is YTIYU24832 (https://www.figdraw.com).

Conflict of interest

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

Publisher's note

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EDITED BY
Zichuan Liu,
Tianjin University, China

REVIEWED BY
Jun Yue,
Sun Yat-sen University, China
Weiqi Wang,
Nantong University, China

*CORRESPONDENCE
Tao Zhang
taozhang@jlu.edu.cn

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Regulation of post-translational modification of PD-L1 and advances in tumor immunotherapy

Chong Feng[1], Lening Zhang[1], Xin Chang[2], Dongliang Qin[1] and Tao Zhang[3]*

[1]Thoracic Surgery, China-Japan Union Hospital of Jilin University, Changchun, Jilin, China, [2]Ophthalmology, China-Japan Union Hospital of Jilin University, Changchun, Jilin, China, [3]Gastrointestinal and Colorectal Surgery, China-Japan Union Hospital of Jilin University, Changchun, Jilin, China

The immune checkpoint molecules programmed cell death receptor 1 (PD-1) and programmed death ligand 1 (PD-L1) are one of the most promising targets for tumor immunotherapy. PD-L1 is overexpressed on the surface of tumor cells and inhibits T cell activation upon binding to PD-1 on the surface of T cells, resulting in tumor immune escape. The therapeutic strategy of targeting PD-1/PD-L1 involves blocking this binding and restoring the tumor-killing effect of immune cells. However, in clinical settings, a relatively low proportion of cancer patients have responded well to PD-1/PD-L1 blockade, and clinical outcomes have reached a bottleneck and no substantial progress has been made. In recent years, PD-L1 post-translation modifications (PTMs) have gradually become a hot topic in the field of PD-L1 research, which will provide new insights to improve the efficacy of current anti-PD-1/PD-L1 therapies. Here, we summarized and discussed multiple PTMs of PD-L1, including glycosylation, ubiquitination, phosphorylation, acetylation and palmitoylation, with a major emphasis on mechanism-based therapeutic strategies (including relevant enzymes and targets that are already in clinical use and that may become drugs in the future). We also summarized the latest research progress of PTMs of PD-L1/PD-1 in regulating immunotherapy. The review provided novel strategies and directions for tumor immunotherapy research based on the PTMs of PD-L1/PD-1.

KEYWORDS

post-translational modification, tumor immunotherapy, programmed death ligand 1, glycosylation, ubiquitination, phosphorylation, acetylation, S-palmitoylation

1 Introduction

In the past decade, tumor immunotherapy has emerged as a therapeutic tool and been characterized as the most important scientific breakthrough of the year by *SCIENCE* magazine in 2013 due to its high specificity toward tumor cells and low adverse effects on patients. Immunotherapy mainly stimulates the human immune system to produce tumor-specific immune response in an active or passive way to enhance the immunity of the body against tumor and control/kill tumor cells. At present, five main types of tumor immunotherapy are known: 1) molecular targeted therapy; 2) immune checkpoint inhibitors (PD-1/L1 and CTLA-4 inhibitors); 3) adoptive cellular immunotherapy (CAR-T cellular immunotherapy, TCR-T cellular immunotherapy, etc.); 4) cytokine therapy; 5) tumor vaccines. T cells are activated by recognition of T cell receptor (TCR) peptide-major histocompatibility complexes (MHC) in antigen-presenting cells (APCs) or other target cells and participate in the immune response (1, 2). This process is regulated by a combination of co-stimulatory and co-inhibitory factors involved in the immune checkpoint system. Under normal physiological conditions, the balance between co-stimulatory and co-inhibitory molecules (3) and the balance of immune checkpoint molecules, maintains the optimum immune effect of T cells (4). However, tumor cell growth can disrupt this balance, causing an abnormal upregulation of co-suppressor molecules and their related ligands, such as PD-1 and PD-L1 (5). Pardoll and his co-workers (6) showed that blocking co-inhibitory molecules from binding to ligands (blocking the PD-1/PD-L1 signaling pathway) can reverse the tumor immune microenvironment and enhance and maintain the endogenous anti-tumor effect, resulting in durable tumor control (7). Therefore, immune checkpoint blocker anti-PD-1 and anti-PD-L1 antibodies have now become one of the most promising directions in antitumor therapy (5, 7–9).

Proteins are important performers in the regulation of cellular functions in the organism and affect almost all aspects of normal cell biology and pathogenesis. PTMs are required for proteins to perform their biological functions, and they alter protein stability and activity, are one of the most important modifications in the regulation of protein biological functions. Recent studies on PD-L1/PD-1 have demonstrated that PD-L1 protein levels harbor dynamic changes in the development of the tumor, and corresponding expression changes also occur after immunotherapy, and these dynamic changes are partially regulated by posttranslational modifications (PTMs) (10). Given that PTMs machineries are often therapeutic targets for pharmacological inhibition of cancer, targeting PD-L1 PTMs may be a novel strategy for enhancing antitumor immune responses. Therefore, in this review, we summarized and discussed currently defined multiple PTMs of PD-L1 and the latest research progress of PD-L1/PD-1PTMs in regulating cancer immunotherapy. The review provided references for development of novel strategies and directions for tumor immunotherapy regarding with the PTMs of PD-L1/PD-1.

2 PD-L1 and immune escape

PD-L1 (also known as CD274 or B7-H1) serves as the primary ligand for PD-1. The frequency of PD-L1 presence is typically low in the steady state, but it can be expressed in malignant cells, lymphocytes, APCs, hematopoietic cells and epithelial cells in response to certain inflammatory or tumor cell stimuli. In malignant cells, the PD-1/PD-L1 signaling pathway is aberrantly activated, and PD-1 and PD-L1 bind with each other to regulate the proliferation and activity of T cells. This reduces their immune response to surrounding tissues, helping tumor cells achieve immune escape (11, 12). In addition, PD-L1 can protect tumor cells from the cytotoxic effects mediated by interferon and cytotoxic lymphocytes (CTL), even in the absence of PD-1 of T cells (13). Thus, the role of PD-L1 in tumor immunity is remarkably crucial than that of PD-1 because of its characteristics.

The examination of molecular mechanisms of tumor immune escape is one of the core challenges in immuno-oncology research, in which PD-1/PD-L1-mediated immune escape mechanisms are particularly important. PD-1 exerts its effects mainly because of three structural domains in the extracellular, intracytoplasmic and transmembrane parts, which also contain the immunoreceptor tyrosine-based switch motif (ITSM) and the immunoreceptor tyrosine-based inhibitory motif (ITIM) (14). PD-L1 does not have a typical signaling motif because its tail consists of a shorter cytoplasmic group. The interaction of the extracellular structural domain of PD-1 with PD-L1 results in a change in PD-1 conformation and tyrosine phosphorylation in the PD-1 cytoplasmic structural domain, which leads to an increase in the linkage of SHP-2 tyrosine phosphatase to ITSM (15). The increase in SHP-2 leads to a decrease in phosphorylation of TCR molecules. PD-1/PD-L1 inhibitors can block the combination of both, thereby restoring the immune cell-mediated killing of tumor cells (16, 17).

3 PTMs of PD-L1 and immunotherapy

Post-translational modification is an important and reversible process for protein regulation. Currently reported PTMs of PD-1/PD-L1 include glycosylation, ubiquitination, phosphorylation, acetylation, palmitoylation. These modifications not only regulate the expression level and stability of PD-L1, but also play an important role in regulating PD-1/PD-L1-related signaling pathways and improving the anti-tumor performance of T cells (18, 19). Therefore, PTMs of PD-1/PD-L1 may emerge as a novel strategy to enhance the efficacy of target PD-1/PD-L1-related drugs.

3.1 Glycosylation of PD-L1

Glycosylation modifications are fundamental to the stable expression and normal physiological function of membrane proteins and affect protein activity (20). PD-L1 is inserted into

the endoplasmic reticulum to begin the process of glycosylation and is processed and transported through the secretory pathway. The process is completed within the Golgi apparatus. Glycosylated PD-L1 is transferred to the cell membrane to participate in immune regulation of the cell. Once glycosylation is dysregulated, aberrant or non-glycosylated PD-L1 can be recognized by endoplasmic reticulum-associated protein degradation (ERAD) and E3 ligase. This is followed by polyubiquitination, translocation from the endoplasmic reticulum to the cytoplasm and degradation by the proteasome (21). Depending on the glycosidic bond site, glycosylation modifications of proteins can be classified into two types: N-glycosylation and O-glycosylation (22). N-glycosylation is the process that the N-glycan chain is covalently attached to the dissociative NH2 group of the aspartic acid of the protein. O-glycosylation is the process that the O-glycan chain is covalently linked to the dissociative OH group of the serine or threonine of the protein. Glycosylation of proteins usually leads to the observation of heterogeneous patterns on western blot, such as PD-L1 (B45 kDa) (23). After removal of the entire n-glycan structure with recombinant glycosidase (peptide-n -glycosidase F; PNGase F) followed by western blot analysis of cell lysates, it was found that the size of PD-L1 was reduced from 45 kDa to 33 kDa, but O-glycosidase failed to produce a similar effect (24). This indicates that the higher molecular weight PD-L1 is indeed attributed to glycosylated form, which is mainly N-glycosylation. N-glycosylation plays a key role in determining protein structure and function, especially the glycosylation of membrane receptor proteins is important for protein interactions (e.g., between ligands and receptors) and has been shown to affect protein activity (25). N-glycosylation was divided into three subtypes, complex, mixed and mannose-rich, according to the composition of their glycan chains, and the glycosylation type of PD-L1 was mainly complex N-glycosylation. The mass spectrometry analysis showed that the asparagine residues of PD-L1 extracellular structural domains N35, N192, N200, and N219 were highly glycosylated (24). Glycosylation is involved in the stability of PD-L1 structure and PD-1/PD-L1-mediated tumor immunosuppressive function and affects the accuracy of PD-L1 detection.

Three main effects of glycosylation on PD-L1 are known (24): firstly, glycosylation of PD-L1 at the N192, N200 and N219 sites impedes the recognition of PD-L1 binding by E3 ubiquitin ligases, which protects PD-L1 from degradation and enhances its protein stability (24). Secondly, N-glycosylation modification of PD-L1 is fundamental for its binding to PD-1 and its immunosuppressive function. Upon N-glycosylation, it enhances protein stability by blocking phosphorylation and subsequent ubiquitination degradation of the adjacent region of the T180/S184 site (24, 25), which in turn binds to PD-1 and inhibits CLT activity (21, 26). Third, Lee et al. (23), also found that the affinity of glycosylated PD-L1 to PD-L1 antibodies was significantly reduced, which may be due to the fact that deglycosylase PD-L1 may eliminate the gap that exists in the space that would be detected by the antibody. Thus, the use of anti-PD-L1 immunohistochemistry can significantly improve the sensitivity of PD-L1 recognition. Furthermore, the deglycosylation of PD-L1 also has potential acting as a diagnostic biomarker that can well predict the response to PD-L1 immunotherapy and more accurately assess PD-L1 protein levels.

PD- L1 can also express in exosomes as a soluble protein (27). Exosomal PD- L1 exists in a highly N-glycosylated form and plays an important role in the regulation of immune escape. Experiments by Zhu et al, showed that exosomal PD- L1 glycosylation is the key structural basis for PD - L1/PD-1 interaction and inhibition of $CD8^+$ T cell proliferation (27). Glycosylated PD- L1 in exosomes may be a promising new target for immunotherapy.

Due to the aforementioned role of glycosylation on PD-L1, Pu et al, proposed the concept of "non-glycosylated PD-L1" for tumor cellular immunotherapy (28). Glycosyltransferase 1 domain-containing 1 (GLT1D1), the staurosporine temperature sensitivity 3 (STT3), G-fructose amidotransferase1 (GFAT1), Glyco-PD-L1-processing enzymes, as well as several molecules and proteins, can be blocked. Non-glycosylated PD-L1 can effectively reduce the interaction between PD-L1 and PD-1, weaken the regulatory effect of tumor cells on T cells, and inhibit the immune escape of tumor cells.

3.1.1 Glycosyltransferase 1 domain 1

GLT1D1 is an enzyme that translocates polysaccharides to target proteins. Current analysis of clinical specimens has demonstrated high expression of GLT1D1 in B-cell non-Hodgkin's lymphoma and early relapsed diffuse large B-cell lymphoma (29). High expression of GLT1D1 increases PD- L1 glycosylation and promotes tumor immune escape and tumor growth. Hence, it is negatively correlated with patient prognosis. Downregulation of GLT1D1 significantly decreases PD- L1 glycosylation, which leads to a significantly higher proportion of non-glycosylated PD-L1, enhances cytotoxic activity of cytotoxic T cells against lymphoma cells, and influences PD- L1/PD-1 interaction, implying that GLT1D1 can be a novel target for immunosuppressive therapy in non-Hodgkin's lymphoma (29).

3.1.2 STT3

The ER-associated N-glycosyltransferase STT3 is essential for N-glycosylation and PD- L1 stability. Epithelial-mesenchymal transition (EMT) enriches PD-L1 in CSCs by the EMT/β-catenin/STT3/PD-L1 signaling axis, in which EMT transcriptionally induces N-glycosyltransferase STT3 through β-catenin, and subsequent STT3-dependent PD-L1 N-glycosylation stabilizes and upregulates PD-L1 (30). In hepatocellular carcinoma (HCC), IL- 6 activates JAK1/PD-L1 phosphorylation at the Y112 site, followed by stimulation of STT3A recruitment and subsequent glycosylation to increase PD-L1 expression. Inhibition of the IL- 6/JAK1 pathway resulted in loss of STT3A and subsequent decrease in PD- L1 stability (26). Etoposide (ETO) (30), a DNA topoisomerase inhibitor, inhibits STT3 expression induced by EMT, thereby suppressing STT3-mediated PD-L1 glycosylation modification. More non-glycosylated PD-L1 reduces the stability of PD-L1 proteins in tumor stem cells and promotes the clearance of tumor-infiltrating T lymphocytes (TILs) from tumor stem cells. In addition, ETO improves the therapeutic effect of TIM-3 (hepatitis A virus cellular receptor 2) monoclonal antibody in a tumor-bearing mouse model, further enhancing the CD8+ T cell-mediated anti-tumor immune response. Secondly, in colorectal cancer,

KYA1797K inhibits the β-catenin/STT3 signaling pathway and downregulates the expression of STT3, thereby inhibiting PD- L1 glycosylation, reducing its stability and suppressing immune escape (31, 32). In addition, a recent study in nasopharyngeal carcinoma found that transforming growth factor β (TGF-β) promotes upregulation of the expression of glycosyltransferase STT3A via c-Jun, which promotes PD-L1 glycosylation and enhances its stability (33). Therefore, the efficacy of immune checkpoint blockade may be enhanced by interfering with TGF-β or combining it with PD-1/PD-L1 blockade strategies.

3.1.3 G-fructose amidotransferase1

GFAT1 is the rate-limiting enzyme of the hexosamine biosynthetic pathway, generating the glycosylation precursor uridine diphosphate-N-acetyl-glucosamine (UDP-GlcNAc), which is fundamental for the glycosylation of many proteins (34), also one of the substances necessary for the stability of PD-L1 protein. Inhibition of GFAT1 significantly reduces PD-L1 glycosylation and protein stability which leaving more PD- L1 in a non-glycosylated state. And it promotes T cell activity and NK cell antitumor activity, significantly enhancing tumor immunity. This mechanism has been demonstrated in lung cancer (35).

3.1.4 Glyco-PD-L1-processing enzymes

Glyco-PD-L1-processing enzymes are also involved in the regulation of N-linked polysaccharide-mediated PD- L1 modification in tumor cells. Biochemical tests revealed that resveratrol (RSV) (36) can directly inhibit N-linked glycan chain modifying enzymes, retaining unglycosylated PD- L1 in the endoplasmic reticulum, reducing the stability of PD- L1, preventing the migration of fully glycosylated PD- L1 to the cell membrane, and affecting the interaction between extra-membrane PD- L1 and PD- 1, limiting immune escape to a certain extent and significantly enhancing the activity of T lymphocytes. The enhancement of T-cell immune function by RSV mediated by targeting PD-1/PD- L1 immune checkpoints is an important mechanism for its oncogenic function.

3.1.5 Glucose analogues 2-deoxyglucose

2-DG inhibits N-glycosylation modifications. 2-DG, as a glucose analogue with glycolysis inhibitor, significantly inhibits the glycosylation modification of PD-L1 (37). Inhibition of protein n-linked glycosylation using 2-DG enhances anti-tumor T-cell immunity in TNBC *in vivo* (38, 39). Furthermore, co-culture experiments with human peripheral blood mononuclear cells (PBMCs) showed that the combination of 2-DG and Olaparib significantly attenuated the immunosuppressive effect of Olaparib monotherapy on PBMCs and enhanced PBMC-mediated killing of tumor cells (40). 2-DG can play a role in immunotherapy treatment of tumors not only directly but also in combination with other drugs through non-glycosylation, achieving a 1 + 1>2 effect.

3.1.6 β-1,3-N-acetylglucosaminyl transferase

As a type II transmembrane protein in the Golgi apparatus, β-1,3-N-acetylglucosaminyl transferase B3GNT3 (25), is responsible for catalyzing the synthesis reaction of poly-N-acetylgalactose sugar chains and is involved in the glycosylation of various proteins. Li et al, confirmed in cytological experiments that B3GNT3 promotes the glycosylation of PD-L1 in response to EGF stimulation and enhances the binding of PD-L1 to PD-1, thereby inhibiting the killing effect of T cells on tumor cells (25). This effect can be blocked by N-glycosylation inhibitors.

3.1.7 PD-L1-associated chaperone Sigma1

Sigma1 facilitates PD-L1 glycosylation in the endoplasmic reticulum, preventing autophagic degradation of PD-L1, thereby stabilizing PD-L1 in tumor cells. Maher et al, confirmed in their molecular cell experiments that (41) (1-(4-iodophenyl)- 3 -(2-adamantyl) guanidine) (IPAG), an inhibitor of the endoplasmic reticulum molecular chaperone Sigma1, can inhibits the glycosylation modification of PD-L1 and reduces the binding ability of PD-L1 to PD-1, which promotes the killing of tumor cells by $CD8^+$ T cells.

3.1.8 Other genes involved in the regulation of glycosylation

In addition, there are other genes involved in the regulation of glycosylation. fK506-binding protein 51 spliceosomes (FKBP51s) act as chaperone molecules for PD-L1 and stabilize PD-L1 in tumor cells by assisting its folding in the endoplasmic reticulum, thereby promoting PD-L1 glycosylation (42). Induction of GSK3β inactivation by EGF in tumor cells leads to increased glycosylation PD-L1. Glycogen synthase kinase 3β (GSK3β) induces phosphorylation-dependent proteasomal degradation of PD-L1 via β-TrCP, but this interaction is antagonized by PD-L1 glycosylation at N192, N200 and N219. Hence, inactivated GSK3β helps increase PD-L1 stability and aids tumor immune escape (24). The Chinese herbal medicine *Shikonin* may inhibit PD-L1 glycosylation through NF-κB and signal transducer and activator of transcription 3 (STAT3), which in turn promotes its degradation (43–45). These enzymes, molecules or their inhibitors can reduce the glycosylation of PD-L1 and therefore may become new targets for immunotherapy (46). The most widely used glycosylation inhibitor tunicamycin (42), however, cannot be used clinically because its own specific structure can affect the glycosylation of many glycoproteins, and the matter that toxin production associated with endoplasmic reticulum stress cannot be resolved.

3.2 Ubiquitination of PD-L1

Ubiquitination is the binding of ubiquitin as a monomer or multimer to specific amino acids of proteins with the participation of ubiquitin-activating enzymes. Damaged or unwanted proteins in cells need to be modified by ubiquitination before they can be recognized and degraded by the proteasome (47). The ubiquitin-proteasome pathway is responsible for most of the intracellular protein degradation and involves three enzymes: ubiquitin activating enzymes (E1s), ubiquitin binding enzymes (E2s) and ubiquitin ligases (E3s) The conjugative cascade of the ubiquitin pathway consists of

three enzymatic reaction steps (48, 49): first, the ubiquitin C -terminal glycine residue is activated by E1 in an ATP-dependent manner; second, the activated ubiquitin is delivered to the E2 enzyme cysteine residues; third, the E3 enzyme catalyzes the covalent attachment of ubiquitin to the lysine residues of the substrate protein. Ubiquitination and deubiquitination together regulate the half-life of PD-L1. Degradation of PD-L1 by ubiquitination greatly reduces its half-life, decreases its stability, and affects the regulation of binding to PD-1, preventing immune escape of tumor cells. The process of ubiquitination can be reversed by deubiquitination (50, 51).

The different ubiquitin chain modifications of ubiquitination have different functions (52–55), among which the polyubiquitin chains linked by K48 and K11 can be recognized by the 26S proteasome, which in turn degrades the substrate protein. The K63-linked polyubiquitin chain can also be sorted into endosomes by internalization and finally degraded by lysosomes. However, in recent years, strong evidence has shown that PD-L1 protein expression is usually regulated by the ubiquitin (UB)-mediated proteasomal degradation pathway. Therefore, here, we mainly discuss the degradation via the proteasome pathway.

3.2.1 β-TrCP

β-TrCP (also known as BTRC) E3 ubiquitin ligase, often functions in combination with SCF (SKP1-CUL1-F-box protein) to form the SCFβ-TrCP E3 ubiquitin ligase complex. β-TrCP mediates the ubiquitination of proteins involved in cell cycle progression, signal transduction, and transcription in a phosphorylation-dependent manner (56). Phosphorylation of PD-L1 by GSK3β (see Phosphorylation below for details) leads to the association of PD-L1 with the E3 ligase β-TrCP, resulting in the degradation of PD-L1 in the cytoplasm (24). β-TrCP binds to the DSG motif on PD-L1 (where D is aspartate, S is serine, and G is glycine) to catalyze its ubiquitination at the K48 site and subsequent degradation of PD-L1 via the 26S proteasome. Glycosylation of N192, N200 and N219 creates a spatial barrier that disrupts the interaction between GSK3β and PD-L1 (24), leading to stabilization of PD-L1 protein. Inhibition of β-TrCP or certain specific molecules that inactivate GSK3β can in turn block PD-L1 ubiquitination, promote its stability, and ultimately induce cancer immunosuppression. In addition, recent *in vivo* experiments in mice revealed that eukaryotic elongation factor 2 kinase (eEF2K) (57) promotes immunosuppression of melanoma via PD-L1 stabilization mediated by GSK3β inactivation. Screening of the FDA-approved antitumor drug library identified Cytarabine as a potentially clinically applicable eEF2K inhibitor (58). The association revealed that cytarabine could be used synergistically with the downward regulation of the oncogene expression SPP1 by other BET inhibitors (for example I-BET282E) (59) for the treatment of melanoma. This finding was also confirmed in a clinical trial of 38 patients with melanoma treated with anti-PD-1 therapy, providing a potential combination treatment strategy to improve the efficacy of immunotherapy.

3.2.2 SPOP

Speckled POZ protein (SPOP) is a typical CRL3 adaptor protein. SPOP interacts with Cullin scaffold protein 3 in CRL3, and 3 (cullin 3)-SPOP interacts with the 283-290 region of PD-L1 to promote polyubiquitination and degradation of PD-L1 in a cell cycle-dependent manner (49). This process can be catalyzed by the cell cycle protein D-CDK4 (60). Then conversely, SPOP inactivating mutations reduce the ubiquitinated degradation of PD-L1, significantly upregulate PD-L1 expression and reduce the number of tumor-infiltrating lymphocytes at tumor sites. *In vivo* studies have shown that when the MATH structural domain of SPOP polypeptide chain was mutated, the protein content of PD-L1 was significantly increased in the tumor tissues of tumor-bearing mice, improving the therapeutic effect of PD-L1 monoclonal antibody. It is also noteworthy that inhibition of CDK4/6 blocked cell cycle protein cyclin D-CDK4-mediated phosphorylation of SPOP, which significantly increased the expression of PD-L1 protein. Paboxicillin and Ribociclib (61), inhibitors of CDK4/6 combined with PD-L1 antibody enhance the ability of $CD8^+$ T cells to secrete IFN-γ (interferon-γ), promote T cell-mediated anti-tumor immune response and significantly increase survival (61).

3.2.3 STUB1

STUB1 ubiquitin ligase is considered a tumor suppressor because it promotes the ubiquitination and degradation of some oncogenic proteins. It also negatively regulates the suppressive activity of regulatory T cells (Treg) by promoting the degradation of the transcription factor Foxp3, which is often reduced or absent in cancer cells (62, 63). Similarly, STUB1 ubiquitin ligase catalyzes the ubiquitination of lysine sites in the cytoplasmic region of PD-L1, promoting PD-L1 degradation in a proteasome-dependent manner and is involved in regulating PD-L1 stability. The knockdown of CMTM6 induces polyubiquitination of PD-L1 in circulating endosomes, which leads to degradation via the lysosomal pathway (53, 64). In addition, Mezzadra et al. (53), and Burr et al. (64), showed that the binding of CMTM6 to PD-L1 mediates the internalization of a larger fraction of PD-L1 on the cell membrane and recirculation to the plasma membrane and circulating endosomes. Furthermore, CMTM6 also specifically regulates PD-L1 in these two cell compartments. This results in reduced ubiquitination of PD-L1 during the cell cycle and lysosomal degradation of PD-L1 during the cell cycle, thereby prolonging its half-life, inducing and stabilizing PD-L1 expression at the cell membrane, and enhancing the ability of PD-L1-expressing tumor cells to suppress T cells and cancer cells for evasion of immune surveillance. Notably, STUB1 downregulation leads to a significant upregulation of PD-L1 expression in CMTM6-insufficient cells compared with that in CMTM6-proficient cells, which suggests that STUB1 initiates the ubiquitination of PD-L1, either indirectly or through direct regulation of the lysine in the PD-L1 cytoplasmic domain.

3.2.4 HMG-coenzyme A reductase degradation protein 1

In the endoplasmic reticulum, the E3 ubiquitin ligase HRD1 is involved in the immune regulation of antigen-presentation function of dendritic cells and sensitization of T and B lymphocytes. Deletion of the HRD1 gene results in a corresponding decrease in the number of T cells, and the clonal expansion and differentiation of

T cells is inhibited. Therefore, HRD1 is considered to be a positive phase regulator of T cell activity. HRD1 induces polyubiquitination of aberrantly glycosylated PD-L1, leading to degradation of PD-L1 via the ERAD pathway (65). Reduction of HRD1 significantly blocked metformin-stimulated ubiquitination of endogenous PD-L1 and revealed that HRD1 acts as a positive regulator of T-cell activity in immune regulation (21).

3.2.5 Defective cullin neddylation 1 domain-containing 1

Defective cullin neddylation 1 domain-containing 1/squamous cell carcinoma-related oncogene (DCUN1D1/SCCRO) is a ring-finger domain ubiquitin E3 enzyme that is involved in the growth and metastasis of malignancies such as colorectal (66), glioma (67) and prostate cancers (68). A recent study revealed the oncogenic mechanism of DCUN1D1 in non-small cell lung cancer (69). Upregulation of the expression of DCUN1D1 significantly increased the levels of PD - L1 protein in non-small cell lung cancer cells. The regulatory mechanism may be related to FAK pathway; however, the exact mechanism of action is not fully understood.

3.2.6 Other enzymes and factors involved in the regulation of ubiquitination

Additionally, there are several enzymes and factors involved in the regulation of ubiquitination of PD-L1. Metformin-stimulated amp-activated protein kinase (AMPK) phosphorylates PD-L1 at the S195 site, blocking its ER-to-Golgi translocation and leading to the degradation of PD-L1 by the ERAD system (21). E3 ligase Neural precursor cell express developmental downregulated protein 4 (NEDD4) can be inhibited by activated fibroblast growth factor receptor 3 (FGFR3) in tumor cells, contributing to polyubiquitination and degradation of PD-L1 (70). The membrane-bound ubiquitin ligase RNF144A interacts with PD-L1 in the cytosolic membrane and intracellular vesicles to promote polyubiquitination and proteasomal degradation of PD-L1 (71). Epidermal growth factor (EGF) (72) induces PD-L1 monoubiquitination and increases protein expression. In addition, epidermal growth factor receptor (EGFR) inhibitors Osimertinib significantly reduce PD-L1 ubiquitination (73, 74). The Cbl family members, c-Cbl and Cbl-b, are RING finger E3 enzymes that catalyze the transfer of ubiquitin from specific E2 enzymes to target substrates. They can inhibit PD-L1 expression by inactivating STAT, AKT, and ERK signaling pathways and are also promising therapeutic manipulation targets for anti-PD-1/PD-L1 tumor immunotherapy. CDK5 can mediate activation by phosphorylation of FBXO22, which acts as E3 to promote degradation of PD-L1 in lung cancer cells (75). A recent study identified ARIH1 as an E3 ubiquitin ligase responsible for targeting PD - L1 degradation associated with GSK3α phosphorylation (see below for details of the mechanism involved) and promoting antitumor immunity, suggesting that ARIH1 may be a potential drug target for enhancing antitumor immunity and immunotherapy (76).

A growing body of data shows that, blocking the interaction between PD-1 and PD-L1 by anti-PD-1/PD-L1 monoclonal antibody has shown great anti-tumor efficacy in various kinds of solid tumors (77). However, many immune related adverse reactions with fatal consequences of monoclonal antibodies have been reported in these years. Based on this situation, small molecule immunotherapy has emerged. PROTACs are ternary chemical complexes that usually consist of three functional parts, an E3 ligase-recruiting chemical ligand, a POI-binding chemical ligand and a linker (78, 79). It uses ubiquitination mechanism to degrade the target protein via both proteasomal and lysosomal pathways to achieve the inhibition of the target protein, and enable targeted degradation of proteins hard to target by conventional methods. We have mentioned above that PD-L1 protein is subject to ubiquitin-mediated proteasomal degradation, therefore, it is feasible to design novel PD-L1 small molecule degraders based on PROTAC technology. Typically, von Hippel–Lindau disease tumor suppressor (VHL) and Cereblon (CRBN) are the most commonly used endogenous E3 ligases in the PROTAC field. Cheng et al. (80), synthesized a novel resorcinol diphenyl ether-based PROTAC molecule P22 for the first time, targeting the involvement of PD-1/PD-L1 pathway. Not only did it inhibit PD-1/PD-L1 interaction, but also moderately reduced PD-L1 protein levels in a lysosome-dependent manner, enhancing the anti-tumor effect of PD-L1 antibody. Liu et al, designed a group of PROTACs consisting of multiple PD-L1 extracellular segment ligands (BMS-37) -junction -CRBN ligands in three parts. The most active PROTAC molecule, BMS-37-C3, has been confirmed to significantly enhance the killing ability of T cells while reducing the protein level of PD-L1 in various molecular tests (81, 82). Besides the traditional inhibitor based PROTAC of PD-L1, Cotton et al, developed first antibody-based PROTACs (AbTACs) inducing the degradation of PD-L1, which can target both PD-L1 and the E3 ligase RNF-43 to induce the lysosomal degradation of PD-L1 (83).

3.3 Deubiquitination of PD-L1

As mentioned above, ubiquitination is an essential protein post-translational modification process that degrades proteins via the proteasomal pathway, thereby affecting various physiological metabolisms within the cell. Deubiquitination is the reverse process of ubiquitination modification, and this process requires the involvement of deubiquitinase (DUB). DUB reverse regulates the ubiquitination process by removing individual ubiquitin molecules or polyubiquitin chains from the tagged target protein by hydrolyzing the peptide bond at the carboxyl terminus of ubiquitin, ester bond or isopeptide bond. The reversible regulation of protein ubiquitination modification and deubiquitination puts the protein expression level in dynamic equilibrium, maintains the stability of its expression level and function, and affects the function of proteins in cellular life activities.

3.3.1 COP9 signalosome 5

The constitutive COP9 signalosome 5 acts as a large multiprotein complex, similar to the 19S lid of the 26S proteasome and plays an integral role in the regulation of cullin-

RING ubiquitin E3 ligases (CRLs). CSN5 is the fifth member of the CSN family and is involved in a subgroup of biological processes that including transcription factor specificity, denuclearization modification of NEDD8 and nuclear to cytoplasmic translocation of primary molecules. CSN5 is associated with cancer survival and is considered a biomarker of poor prognosis in some tumors. Consistently, CSN5 acts as a DUB with deubiquitinating activity and is a negative regulator of PD-L1 ubiquitination (52, 84). Macrophages secrete the pro-inflammatory cytokine TNF-α to activate NF-κB and induce tumor cells to express CSN5 (85), which subsequently inhibits ubiquitination and degradation of PD-L1, thereby enhancing PD-L1/PD-1 interactions and evading immune surveillance by T cells. However, the CSN5 inhibitor curcumin reversed this situation and improved the therapeutic efficacy of CTLA4 blockade therapy (52). CC chemokine receptor 5 (CCR5) and its ligand ligands (e. g. CCL5) are involved in the suppressive effect of tumor-associated macrophages (TAMs), one of the most potent immune cell types in the cancer tumor microenvironment, on $CD8^+$ T cell immunity. They demonstrate oncogenic and immunosuppressive effects. The activity is further enhanced by the production of NF-kB p65/signal transducer and activator of transcription 3 (STAT3) complexes linked to the CSN5 promoter by macrophage-derived CCL5 (85). The NF-KB inhibitor Shikonin (46) decreased PD-L1 glycosylation and increased PD-L1 degradation, whereas activated STAT3 and overexpressed CSN5 reversed these trends. Thus, stabilization of PD-L1 by inhibiting NF-kB/CSN5 is a potential strategy to treat cancer-associated inflammation. Overall, CSN5 plays an important role in PD-L1 regulation and may be a promising therapeutic target in tumor immunotherapy.

3.3.2 Ubiquitin-specific proteases

A variety of USPs are involved in the regulation of PD-L1 deubiquitination mediated by different mechanisms. USP22 is observed in a variety of malignancies and is especially highly expressed in HCC. It interacts with the C-terminus of PD-L1, deubiquitinates PD-L1 (86), and inhibits its degradation via the USP22-CSN5-PD-L1 axis (87), which is closely associated with the prognosis of HCC (88). Moreover, in NSCIC (86), USP22 deletion can promote the therapeutic effect of PD-L1-targeted tumor immunotherapy. Recent experiments in mouse models of lung cancer have shown that (89) targeting USP7 with USP7 inhibitor P5091 upregulates the expression of PD-L1 protein in Lewis tumor cells and blocks PD-1, leading to an effective anti-tumor response in lung cancer. However, the underlying mechanism of its inhibition of PD-L1 upregulation is still unclear. Another ubiquitin-specific protease, USP9X (also known as FAM), which reduces PD-1 expression on T cells is involved in immune regulation of tumors. As a member of DUBs, ubiquitin-specific peptidase 9, Xlinked (USP9X) has a role in the control of tumor cell proliferation, adhesion, and apoptosis, among other things (90). And it has been found to be inappropriately expressed in non-small cell lung cancer, melanoma as well as breast cancer (91–93). Next, USP9X was found to be expressed at high levels in EGR-positive prostate cancer and the USP9X inhibitor WP1130 was found to induce ERG degradation and thus inhibit tumor growth (94). In addition, USP9X is highly expressed in oral squamous cells carcinoma (OSCC) cells. The high expression of USP9X in OSCC cells increases the stability of PD-L1 in OSCC cells by deubiquitinating the tumor and promoting immune escape (95). Therefore, targeting PD-1/PD-L1 by inhibiting the activity of USP9X may be a promising anti-cancer therapeutic strategy. In addition, USP21 (96), a novel deubiquitinating enzyme of PD-L1, was recently identified. Its overexpression significantly upregulated PD-L1 expression.

3.3.3 OTU domain, ubiquitin aldehyde binding 1

The deubiquitinating enzyme OTUD1 is involved in the deubiquitination of apoptosis inducing factor (AIF) and plays a role in regulating apoptosis. It interacts with the K48-linked polyubiquitin chain in the intracellular region of PD-L1 and impedes the degradation of PD-L1 via the ERAD pathway by mediating its deubiquitinase activity (97). In non-small cell lung cancer experiments, the overexpression of the cyclic RNA insulin-like growth factor 2 mRNA-binding protein 3 (circIGF2BP3) upregulates the expression of OTUD1 by stabilizing mRNA (98). This, in turn, antagonizes the ubiquitinated degradation of PD-L1 and suppresses $CD8^+$ T cell function, leading to immune escape. In addition, in mice with breast cancer (97), the number of infiltrating $CD8^+$ T cells and the serum levels of IFN-γ were significantly increased after PD-L1 destabilization induced by OTUD1 deletion, promoting tumor immunotherapy.

3.4 Phosphorylation of PD-L1

Phosphorylation modification is the process of transferring ATP phosphate groups to amino acid (tyrosine, serine, threonine) residues of substrate proteins catalyzed by protein kinases. The phosphorylation sites of PD-L1 are mainly concentrated in the extracellular structural domain and are often found together with the sites of glycosylation and ubiquitination (24). However, different kinases induce phosphorylation of different sites of PD-L1 with completely different effects. Among the five widely studied protein kinases that mediate PD-L1 phosphorylation, GSK3β/α and AMPK-mediated phosphorylation of PD-L1 leads to degradation by ubiquitination in the cytoplasm, whereas JAK1 and NIMA-associated kinase 2 (NEK2)-mediated phosphorylation of PD-L1 stabilizes PD-L1 by promoting PD-L1 glycosylation and inhibiting PD-L1 ubiquitination.

3.4.1 Glycogen synthase kinase 3β/α

GSK3β induces phosphorylation of non-glycosylated PD-L1 at the T180 and S184 sites of its extracellular structural domain by binding to the post-translational motif of PD-L1 (S/TXXXS/T, where S is serine, T is threonine and X is any amino acid) (24). The phosphorylated PD-L1 is further ubiquitinated by K48 via binding to the E3 ligase β-TrCP. This induces degradation of PD-L1 via polyubiquitination in the cytoplasm. For PD-L1 that has undergone glycosylation, the glycosylation of N192, N200 and

N219 blocks its spatial site, inhibiting the effect of GSK3β on PD-L1 and preventing PD-L1 phosphorylation and subsequent degradation. Here, β-TrCP mediates the GSK3β-dependent phosphorylation of PD-L1 for PD-L1 degradation. In contrast, GSK3β-independent PD-L1 needs to be induced by mTORC1/p70S6K to phosphorylate it, which, in turn, leads to β-TrCP-mediated PD-L1 degradation (99). PARP1 inhibitor Olaparib (100, 101), tyrosine kinase inhibitors (TKIs) (100), and resveratrol (36) inhibit GSK3β activity, which further affects the interaction between PD-L1 and β-TrCP. In addition, epidermal growth factor (EGF) acts as an upstream signal that regulates the expression and function of GSK3β protein. Gefitinib, Erlotinib, Osimertinib and ES-072 are inhibitors of the EGF receptor EGFR (24, 76, 102, 103), induce phosphorylation of non-glycosylated PD-L1, rendering it susceptible to degradation. Secondly, the novel apropionate derivative SA-49 (104) can enhance the killing of co-cultured tumor cells by NK cells and T cells via the PKCα-GSK3β cascade, which ultimately promotes PD-L1 lysosomal degradation.

In addition, GSK3α also plays a role similar to that of GSK3β (24). GSK3α phosphorylates the S279 and S283 sites of PD-L1, and the phosphorylated PD-L1 is then degraded by the proteasome mediated by the E3 ubiquitin ligase ARIH1 mentioned above (76). Therefore, GSK3α/GSK3β may be a potential target for regulating PD-L1 phosphorylation/ubiquitination.

3.4.2 Metformin activates the AMP-activated protein kinase

AMPK is a key molecule that stimulates glucose utilization by phosphorylating targets involved in glucose transporter transport. It is an enzyme necessary for the body to maintain glucose homeostasis. AMPK phosphorylation is also involved in the regulation of PD-L1, and although it phosphorylates different sites of PD-L1 to promote PD-L1 degradation, the mechanisms are not the same. First, phosphorylation of PD-L1 at the S195 site (21) leads to abnormal PD-L1 glycosylation. The aberrantly glycosylated PD-L1 cannot be transferred from the endoplasmic reticulum to the Golgi apparatus, resulting in a large amount of aberrantly glycosylated PD-L1 being degraded via the ERAD pathway of endoplasmic reticulum-associated degradation. The study confirmed that D-mannose (105) can activate AMPK, which is involved in regulating the whole process mentioned above. Combined treatment with D-mannose and PD-1 blockade therapy in mice greatly inhibited the growth of TNBC and prolonged the survival of tumor-bearing mice. Metformin (106), an AMPK agonist, induced PD-L1 degradation via the ERAD pathway (105) by activating its serine protein kinase activity. It is a process that inhibits the immune escape of tumor cells by reducing the stability and membrane localization of PD-L1. Second, because abnormal energy status leads to cancer development, and energy deprivation activates AMPK, the activated AMPK mediates PD-L1 phosphorylation at the S283 site (107), which disrupts its interaction with chemokine superfamily member 4 (CMTM4) and leads to PD-L1 degradation. In addition, Cha et al. (108), demonstrated that metformin significantly improved the therapeutic effect of immune checkpoint CTLA-4 (cytotoxic T-lymphocyte associated protein 4) monoclonal antibody in mice. The combination of these two drugs promoted the secretion of granzyme B (GZMB) by $CD8^+$ T cells and enhanced the killing effect of TILs cells in breast cancer cells. These findings indicate a novel hypothesis for cancer immunotherapy.

3.4.3 Tyrosine kinase JAK1

In 2019, Chan et al, demonstrated that the tyrosine kinase JAK1 induces phosphorylation of PD-L1 at the Y112 site upon interleukin-6 (IL-6) stimulation (26). JAK1 binds to PD-L1 in the endoplasmic reticulum and promotes phosphorylation at the PD-L1 Y112 site, thereby recruiting oligosaccharyltransferase (OST). OST acts on the same position of the n-glycosyltransferase STT3A to catalyze PD-L1 glycosylation and upregulate PD-L1 expression, thereby promoting PD-L1 stability and inducing tumor immune escape. This has been demonstrated by the results of *in vitro* $CD8^+$ T cell killing experiments (26), where the protein stability of PD-L1 in cancer cells was reduced when a point mutation of Y112 was noted in PD-L1, rendering PD-L1 unable to bind to PD-1 on the membrane surface of CD8+ T cells. This, enhanced the killing effect of $CD8^+$ T cells on tumor cells. Phosphorylation of PD-L1 and its stability is closely related to its stability and may also serve as a potential target for enhanced immunotherapy. There are already drugs based on JAK1 such as to Tofacitinib, Ruxolitinib and Fedratinib (76, 109, 110). And the combination of IL-6 inhibitor with anti-T cell immunoglobulin mucin-3 (anti-Tim-3) enhances the efficacy of T cell-mediated killing of tumor cells (26, 76).

3.4.4 NIMA related kinase 2

In a study of cytological experiments in pancreatic cancer, Zhang et al (111), confirmed that dephosphorylation of PD-L1 by NEK2 is one of the main reasons for the poor immunotherapeutic effect observed with pancreatic cancer. NEK-binding motifs (F/LXXS/T) could be identified in the glycosylation-rich region of PD-L1 (112). NEK2 interacts with PD-L1, phosphorylates T194/T210 residues and prevents ubiquitin-proteasome pathway-mediated degradation of PD-L1 in the ER lumen. This is followed by further promotion of glycosylation at the N192, N200 and N219 sites, preventing PD-L1 degradation and promoting PD-L1 stability (111). Hence, we can speculate that the inhibition of NEK2 and PD-L1 is a promising anti-cancer strategy. And a small molecule drug NCL 00017509, based on NEK2, is currently in preclinical studies (111).

In addition, phosphorylation of heat shock transcription factor 1 (HSF1) (113), a major regulator of the proteotoxic stress response, at the Thr120 site induces its binding to the PD-L1 promoter and upregulates PD-L1 expression. It is expected to be a new immune target.

3.5 Acetylation of PD-L1

Acetylation is the process of adding acetyl groups to protein residues, which is mediated by acetyltransferases of acetyl coenzymes A (114, 115) [including histone acetyltransferases

(HATs), lysine acetyltransferases (KATs), and Nα-acetyltransferases (NATs) (116)]. And this process can be reversed by deacetylases of acetylated proteins [including histone deacetylases (HDACs) and Sirtuins (SIRTs) (117)]. PD-L1 acetylation can promote tumor immune escape along with glycosylation, but they show completely different mechanisms. As mentioned above, PD-L1 glycosylation inhibits its ubiquitination-mediated degradation, prolongs the half-life of PD-L1, maintains PD-L1 stability, and promotes its binding to PD-1 to help tumor immune escape. Differently, PD-L1 acetylation is able to translocate PD-L1, which is mainly expressed on the cell membrane to function, into the nucleus (118). The accumulation of PD-L1 in the nucleus helps tumor cells evade immune surveillance during metastasis and promotes further tumor development and metastasis.

3.5.1 Histone acetyltransferase P300

PD-L1 is acetylated by p300 at the Lys263 site in the cytoplasmic structural domain, promoting the translocation of PD-L1 to the nucleus via cytocytosis and nucleoplasmic translocation pathways (118). The increased levels of PD-L1 in the nucleus lead to its bindings to DNA and is involved in the regulation of IFN, nuclear factor κB (NF-κB), major histocompatibility complex I (MHCI), and other immune response genes, thus promoting tumor immune escape. HDAC2 inhibitors Trichostatin A in combination with anti-PD-1 antibodies enhance tumor growth inhibition and improve survival in MC38 homozygous use models (119, 120). Therefore, inhibition of HDAC2 in combination with PD-1/PD-L1 blockade is a new strategy for tumor immunotherapy. In addition, binding of hepatitis B virus X-interacting protein (HBXIP) to P300 in breast cancer enhances acetylation of the PD-L1 K270 site, leading to stabilization and accumulation of PD-L1 in cancer cells, thereby enhancing tumor immune escape (121). A study by Gao et al. (118), found that HDAC2 genetic depletion or pharmacologic inhibition of HDAC2 reduced the nuclear portion of PD-L1, thereby enhancing the antitumor efficacy of PD-1 blockers.

3.5.2 Huntingtin interacting protein 1-related

After deacetylation of PD-L1 on the cell membrane by unacetylated PD-L1 or by HDAC2, HIP1R binds specifically to the C-terminus of PD-L1, allowing the β-subunit (AP2B1) of the lattice protein-dependent endocytic junction protein complex (AP2) to recognize HIP1R via a double leucine motif D/E-x-xx-x-L-L/I and bind to PD-L1 to form a complex. The formation of this complex is mediated by lattice proteins to achieve endocytosis, accumulation in the nucleus, and enhancement of the activation of multiple immune response pathways (122). PD-L1 acetylation can mediate its translocation and degradation, but its role in these processes requires further investigation. High expression of PD-L1 is widely used as a marker for patient selection, and we also speculate PD-L1 nuclear expression or PD- L1 acetylation status could be a useful biomarker for future cancer immunotherapy.

3.6 S-palmitoylation of PD-L1

Protein palmitoylation, also known as S-palmitoylation, is a reversible form of PTM of protein lipidation in which palmitoyl groups are attached to the sulfhydryl groups of cysteine residues of proteins by thioester bonds. This process is usually catalyzed by a family of DHHC protein acyltransferases (DHHC-PATs) containing the Asp-His-His-Cys active center (123, 124). S-palmitoylation mainly affects protein membrane anchoring, transport, and degradation.

3.6.1 Aspartate-histidine-histidine-cysteine rich sequence

Yang et al. (125), first reported that palmitoyl transferases can interact with PD-L1 in breast cancer and catalyze its palmitoylation at the Cys272 site. Yao et al (126, 127), recently reported a similar finding in a mouse colon cancer model. C272 is the main site of palmitoylation, and after palmitoylation of PD-L1 at the C272 site in the cytoplasm, PD-L1 inhibits the ubiquitination of PD-L1 and prevents its movement to the multivesicular body (MVB). This prevents PD-L1 from being degraded by the lysosomal degradation pathway, thus inducing the development of tumor immune escape. The mutated C272 site inhibits PD-L1 palmitoylation, reduces PD-L1 levels on the membrane surface and its binding to PD-1, and continuously activates T cell-mediated cytotoxicity. Bromohexadecanoic acid (2-bromopalmitate, 2-BP) (126), as an inhibitor of DHHC-PATs enzyme activity, reduces the protein stability of PD-L1 by inhibiting its palmitoylation modification and is the only validated effective PD-L1 palmitoylation targeting drug. However, the inhibitory effect of 2-BP on DHHC-PATs is not specific (127). In the subsequent studies, the development of targeted drugs that specifically inhibit DHHC3/9 could be considered to achieve specific modulation of PD-L1 palmitoylation, and targeting PD-L1 palmitoylation could increase the sensitivity of tumor cells toward T-cell-mediated killing and retard tumor growth. Next, the investigators designed a PD-PALM peptide based on the amino acid sequence profile near the C272 site. It inhibits the palmitoylation of endogenous PD-L1 by competitively binding DHHC3 enzyme, reduced the level of PDL1 expression in tumor cells, and enhanced T cell-mediated anti-tumor immune responses. Clinical studies (114) also found PD-L1 palmitoylation in cisplatin-resistant bladder cancer cells, and inhibition of fatty acid synthase (FASN) inhibited PD-L1 palmitoylation and its expression. The PTMs of PD-L1 and immunotherapy summarized above are detailed in Table 1.

4 Interactions and correlations of PTMs of PD-L1

In the regulation of different post-translational modifications of PD-L1, they do not work singularly, but rather are closely related and participate in the regulation of PD-L1 in collaboration.

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TABLE 1 Various PTMs of PD-L1.

	Related enzymes	Modification site	Biological effects	Cancer type	Molecules	References
N-glycosylation	GLT1D1	N35 /N192 /N200 /N219	Enhanced stability of PD-L1	Non-Hodgkin's		(29)
	STT3	N35 /N192 /N200 /N219	Enhanced stability of PD-L1	Liver cell carcinoma	IL- 6	(30)
				Colon cancer		(31, 32)
				Nasopharyngeal Carcinoma	TGF-β	(33)
	GFAT1	N35 /N192 /N200 /N219	Enhanced stability of PD-L1	Lung cancer		(34)
	Glyco-PD-L1-processing enzymes		Reduced stability of PD- L1; Blocking PD-L1 from binding to PD- 1 binding	Breast cancer		(36)
	2-DG		Blocking PD-L1 from binding to PD- 1 binding	Triple negative breast cancer		(37–40)
	B3GNT3	N192 /N200	Promoting PD-L1 binding to PD-1		EGF	(25)
	Sigma1		Enhanced stability of PD-L1	Prostate Cancer/Triple negative breast cancer		(41)
	FKBP51s		Enhanced stability of PD-L1	Glioma	GSK3β,b-TrCP	(42)
	STAT3		Suppresses glycosylation of PD-L1, Activates the NF-kB/STAT3 and NF-kB	Pancreatic cancer		(43–46)
Ubiquitination	β-TrCP	K48	Catalytic degradation of PD-L1	Breast cancer	GSK3β, mTORC1/ p70S6K	(56, 57)
	SPOP		Decreases PD-L1 level	Prostate cancer	Cullin 3, D-CDK4/6	(60, 61)
	STUB1		Downregulates level of PD-L1	Melanoma	CMTM6	(53, 62–64)
	HRD1		Downregulates level of PD-L1, Positively regulates T-cell immunity	Breast cancer	ERAD, Metformin	(65)
	DCU N1D1		Increases PD-L1 level	Colorectal cancer, Glioma, Prostate cancer and Lung cancer	FAK Pathway	(68, 69)
	NEDD4	K48	Promotes PD-L1 degradation	Bladder caner	FGFR3	(70)
	RNF 144A		Promotes PD-L1 degradation	Bladder Tumor	EGFR	(71)
	c-Cbl Cbl-b		Inhibition of PD-L1 expression	Melanoma, Gastric cancer, NSCLC	STAT5a, AKT, and ERK signaling pathways	(73, 74)
	ARIH1		Degradation of PD - L1	Breast cancer	GSK3α	(76)
Deubiquitination	CSN5		suppresses degradation of PD-L1	Triple negative breast cancer, NSCLC	TNF-α, NF-κB signaling pathway	(52, 84)
	USP22		Enhanced stability of PD-L1	Liver Cancer, NSCLC, PDA	CSN5	(86–88)

(Continued)

TABLE 1 Continued

	Related enzymes	Modification site	Biological effects	Cancer type	Molecules	References
	USP7		Enhanced stability of PD-L1	Lewis lung carcinoma	FOXP3	(89)
	USP9X		Enhanced stability of PD-L1	OSCC, prostate cancer	EGR	(94, 95, 128)
	OTUD1	K48	Blocking PD-L1 degradation	Triple negative breast cancer	ERAD, circIGF2BP3	(97, 98)
Phosphorylation	GSK3β	T180/S184;	Promotes β-TrCP-mediated PD-L1 degradation	Gastrointestinal tumors, Breast cancer, Lung cancer, Renal cell carcinoma, etc.	β-TrCP, EGF, EGFR	(24, 99, 100, 104)
	GSK3α	S279/S283	Promotes ubiquitinated degradation of PD-L1	Colon cancer, Cervical cancer, Pancreatic cancer, Lung cancer, Prostate cancer	ARIH1	(24, 76)
	JAK1	Y112				(26, 76)
	AMPK	S195	Aberrant glycosylation of PD-L1 upon degradation by ubiquitination	Breast cancer	ERAD, D-mannose	(21, 105, 106)
		S283	Induction of PD-L1 degradation	Breast cancer	metformin, CMTM4	(107)
	NEK2	T194/T210	Glycosylation of PD-L1 to promote its stability	Liver Cancer	IL-6, OST, STT3A	(111, 112)
Acetylation	P300	K263/K270	Promotes PD-L1 internal transfer	Liver cell carcinoma	NF-κB, MHCI, HDAC2	(118, 119, 121)
	HIP1R		Promotes PD-L1 internal transfer	AP2B1		
S-palmitoylation	DHHC	C272	Colon cancer, Breast cancer, Bladder cancer	Protects PD-L1 from degradation by lysosomes		(126, 127)

Glycosylation of PD-L1 is involved in the regulation of its ubiquitination and phosphorylation. GSK3β-induced phosphorylation of non-glycosylated PD-L1 activates β-TrCP-mediated degradation of PD-L1 (99). Glycosylation antagonizes the binding of GSK3β to PD-L1 and inhibits PD-L1 phosphorylation (24). Thus, glycosylation of PD-L1 can directly inhibit its phosphorylation and indirectly inhibit its ubiquitination and degradation. PD-L1 glycosylation does not affect its acetylation and nuclear translocation. AMPK phosphorylates PD-L1 at the Ser195 site leads to aberrant glycosylation of PD-L1 and impaired translocation from the endoplasmic reticulum to the Golgi apparatus, resulting in the accumulation of PD-L1 in the endoplasmic reticulum and promoting degradation via. ubiquitination (21). With IL-6 stimulation, JAK1 binds to PD-L1 in the endoplasmic reticulum and promotes phosphorylation at the PD-L1 Y112 site, lead to the n-glycosyltransferase STT3 to catalyze PD-L1 glycosylation and upregulate PD-L1 expression, thereby promoting PD-L1 stability (26). NEK2 (111) induced phosphorylation of PD-L1 inhibits PD-L1 ubiquitination, thereby increasing PD-L1 stability. S-palmitoylation of PD-L1 at the C272 site in the cytoplasm, PD-L1 inhibits the ubiquitination of PD-L1 and prevents its movement to the multivesicular body (MVB) (116, 117). Peracetylation of forkhead box P3(Foxp3), has been reported to inhibit its ubiquitination and degradation (129). And Lysine acetylation of non-histone proteins can compete with ubiquitination and thus affect the stability or subcellular localization of the protein (115). However, there is no clear support for whether PD-L1 acetylation will prevent its ubiquitination in cancer cells and further studies are needed. (See Figure 1 for details of the relationship between several PTMs of PD-L1).

Glycosylation-targeted drugs prevent immune escape of tumor cells by inhibiting glycosylation and increasing the number and proportion of non-glycosylated PD-L1. Ubiquitination-targeted drugs mainly promote the process of PD-L1 ubiquitination to exert their anti-tumor effects. Phosphorylation-targeted drugs exert their anti-tumor effects mainly by regulating related kinases to achieve the effect of inhibiting glycosylation or promoting ubiquitination degradation. Acetylation-targeted drugs inhibit PD-L1 acetylation and prevent its translocation into the nucleus, preventing immune cells from evading immune surveillance to exert their antitumor effects. Palmitoylation-targeted drugs promote palmitoylation, prevent PD-L1 glycosylation, and reduce PD-L1 stability (See Table 2 for details of specific drugs and mechanisms of action).

5 Discussion

PD-L1/PD-1 has garnered significant interest in recent years as a signaling pathway that inhibits immune cell activation and

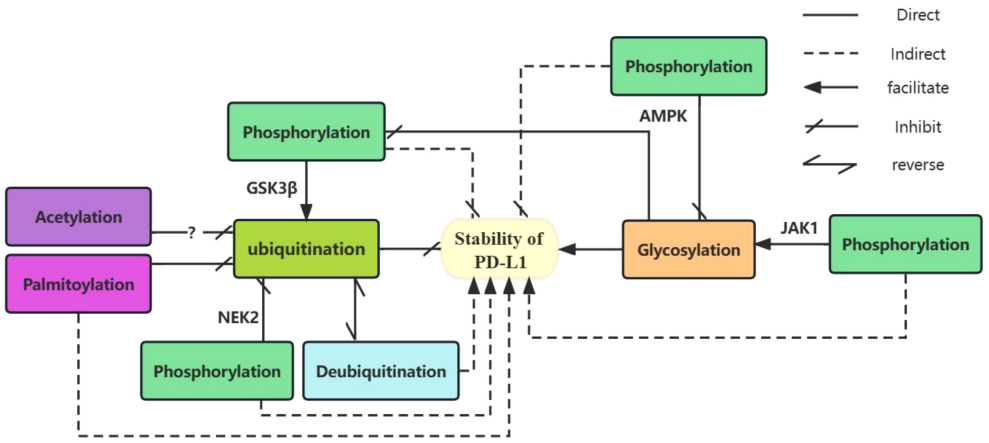

FIGURE 1
The relationship between several PTMs of PD-L1. Glycosylation and ubiquitination of PD-L1 play a direct role in promoting and inhibiting its stability. Deubiquitination indirectly promotes the stability of PD-L1 by antagonizing its ubiquitination. Different enzyme-mediated phosphorylation of PD-L1 plays different roles. GSK3β-mediated phosphorylation which can be inhibited by glycosylation promotes ubiquitination of unglycosylated PD-L1 and indirectly inhibits its stability. AMPK-mediated phosphorylation inhibits glycosylation of PD-L1 and indirectly reduces its stability. In contrast, JAK1-mediated phosphorylation facilitates PD-L1 glycosylation, indirectly contributing to its stability. Both NEK2-mediated phosphorylation and palmitoylation indirectly promote PD-L1 stability by inhibiting ubiquitination of PD-L1. Acetylation of PD-L1 may inhibits its ubiquitination.

promotes immune escape of tumor cells. Given that PTMs are often therapeutic targets for drug-mediated inhibition of cancer, it is crucial to better understand PD-L1 PTMs in malignant tumors. This review summarizes the types of post-translational modifications of PD-L1 that have been identified, the mechanisms underlying regulation of PD-L1 by different post-translational modifications, and the PTMs that have been identified and used to target PD-L1, providing a solid theoretical basis for the improvement of immunotherapeutic effects and combination of drugs. We aim to provide new ideas and directions for tumor immunotherapy research. (See Figure 2 for the six PTMs of PD-L1. See Figure 3 for the pathways of PTMs related to PD-L1 in tumor cells and the main related molecules).

PTMs of PD-L1 have made some achievements. Firstly, an increasing number of mechanisms have been translated into clinical drugs (see Table 2 for details).

Secondly, PD-L1/PD-1 related immunotherapy has more side effects and only some cancer patients respond well to PD-1/PD-L1 blockade, thus a tumor vaccine with PD-L1/PD-1 immunotherapy strategy is proposed to overcome these limitations. Recent studies identified Local tumor photothermal treatment with the near-infrared light at the second window (NIR-II) (131) is a boosting strategy in triggering the *in-situ* tumor vaccination (ISTV) for cancer therapy. It is responsible to reverse the immunosuppressive microenvironment of tumors, increasing the antigen presentation efficacy and promoting the immunological responses of T-cells to attack the remaining tumor cells (131). However, most of the previously developed ISTV adjuvants may indiscriminately damage tumor cells and immune cells, limiting the overall effect of the immune response. Fan et al, designed a "cocktail" nano adjuvant that significantly enhanced the immune response to NIR-II light-induced DC mutation and T-cell differentiation, and had a stronger inhibitory effect on tumor growth (132). Moreover, Hu et al, explored a synergistic strategy to combine *in situ* vaccination and gene-mediated anti-PD therapy. It was generated by unmethylated cytosine-phosphate-guanine (CpG) and pshPD-L1 gene co-delivery. PEI worked as the delivery carrier to co-deliver the CpG and pshPD-L1 genes, the formed PDC (PEI/DNA/CpG) nanoparticles were further shielded by aldehyde modified polyethylene glycol (OHC-PEG-CHO) via pH responsive Schiff base reaction for OHC-PEG-CHO-PEI/DNA/CpG nanoparticles (P(PDC) NPs) preparation (133). In mouse experiments, the synergistic effect of this step was rapid and effective (133).

However, some challenges still remain to be addressed. Firstly, the effects of various PD-L1 PTMs on tumor immune escape mentioned in this paper have been largely clarified, but due to the diversity and complexity of post-translational modification forms and mechanisms, the regulatory mechanisms of PD-L1 PTMs still need to be explored further. For example, the effects of various PTMs on the subcellular localization and physiological functions of PD-L1, and the existence of novel PD-L1 PTMs processes need to be further investigated.

Secondly, most of the corresponding intervention strategies or combination drug regimens for PTM are still in the experimental stage in cellular and animal models. Therefore, future studies will continue to investigate in depth at the mechanistic level on the one hand, and focus on the clinical translation and combination of existing intervention strategies for PD-L1-based PTM on the other hand.

Thirdly, different forms of PTMs mentioned in the text also have their particular breakthrough points.

Glycosylation and Phosphorylation: Glycosylation of PD-L1 inhibits 26S proteasome-mediated protein degradation, which, in turn, maintains PD-L1 stability, and GSK3β is the central node that regulates PD-L1 stability (24). Furthermore, glycosylated PD-L1 inhibits GSK3β phosphorylation and β-TrCP ubiquitination-mediated degradation (99), but the mechanism of the translocation of ER-bound PD-L1 into the cytoplasm and degradation by the 26S proteasome is unclear. Future work may focus on how phosphorylation and glycosylation of PD-L1 regulate the ERAD

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TABLE 2 PTMs of PD-L1 currently exist for clinical treatment.

	Drug name	Combination therapy	Target	Action mechanism	References
N-Glycosylation	Etoposide	Etoposide + TIM-3 mAb	STT3	Inhibiting EMT-induced STT3 expression and reducing PD-L1 stability	(30)
	2-DG	2-DG + Olaparib	2-DG	Inhibition of PD-L1 Glycosylation Modification	(40)
	IPAG	Unknow	Sigma1	Inhibition of PD-L1 Glycosylation Modification	(41)
	Shikonin	Unknow	STAT3	Inhibition of PD-L1 Glycosylation Modification	(43–46)
Ubiquitination	Resveratrol	Unknow	β-TrCP	Blocking PD-L1 from binding to PD- 1 binding	(36)
	Cytarabine	Unknow	GSK3β	Down-regulation of the expression of oncogenic gene SPP1	(58, 59)
	Palbociclib	Palbociclib + PD-1 mAb	CDK4/6	Promotes T cell-mediated anti-tumor immune response	(60)
	Curcumin	Curcumin + CTLA-4 mAb	CSN5	Promotes ubiquitination and degradation of PD-L1	(52)
	PROTACs PD- LYSO	Unknow		PD- L1 degradation	(81–83, 130)
Phosphorylation	Metformin	Metformin + CTLA-4 mAb	AMPK	Induced PD-L1 degradation via ERAD pathway	(21, 37, 108)
	SA-49	Unknow	PKCα-GSK3β	Promotes PD-L1 lysosomal degradation	(104)
	Erlotinib, Osimertinib and ES-072	Erlotinib, Osimertinib and ES-072 + PD-1 mAb	EGFR	Induces phosphorylation of non-glycosylated PD-L1	(24, 76, 102, 103)
	Tofacitinib, Ruxolitinib and Fedratinib	Unkonw	JAK1	Downregulation of PD- L1 via suppression of STAT1/3- mediated transcription	(76, 109, 110)
	IL-6 mAb	IL-6 mAb + TIM-3	IL-6	Inhibits downstream JAK1 activation and promotes PD-L1 degradation	(26, 76)
Acetylation	Trichostatin A	Trichostatin A+ PD-1 mAb	HDAC2	Inhibition of PD-L1 translocation to the nucleus	(119, 120)
S-Palmitoylation	2-BP	Unknow	DHHC family	Decreased palmitoylation and thus increased degradation of PD- L1	(126, 127)

pathway. In addition, EGFR-activated AKT is associated with cytomembrane PD-L1 expression and survival in patients with lung cancer (134–137). EGFR-activated AKT inhibits GSK3β activity via Ser9 phosphorylation, suppresses EGF signaling in basal-like breast cancer (BLBC) cells, reduces PD-L1 stability, and decreases cancer cell immune escape, thereby demonstrating a therapeutic benefit. Similar regulation was observed by Akbay et al. (138), in PD-L1 mouse lung tumor cells. However, no study has yet indicated whether AKT can directly regulate PD-L1 expression. More in-depth analysis is needed to demonstrate the role of AKT in EGFR-mediated PD-L1 protein stabilization. The effect of the catalysis of ATP uptake mediated by the endoplasmic reticulum on PD-L1 phosphorylation in the endoplasmic reticulum is not understood. Furthermore, the mechanism of how AMPK is localized in the lumen of the endoplasmic reticulum is also unclear. Ubiquitination: Novel small molecule immunotherapeutic agents, PROTACs, generated against PD-L1 ubiquitination have

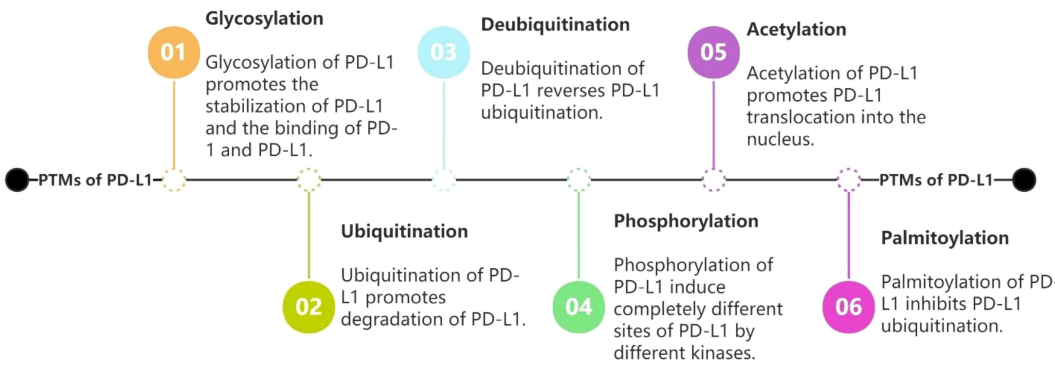

FIGURE 2
Regulations of PD-L1 by glycosylation, ubiquitination, deubiquitination, phosphorylation, acetylation and palmitoylation.

10.3389/fimmu.2023.1230135

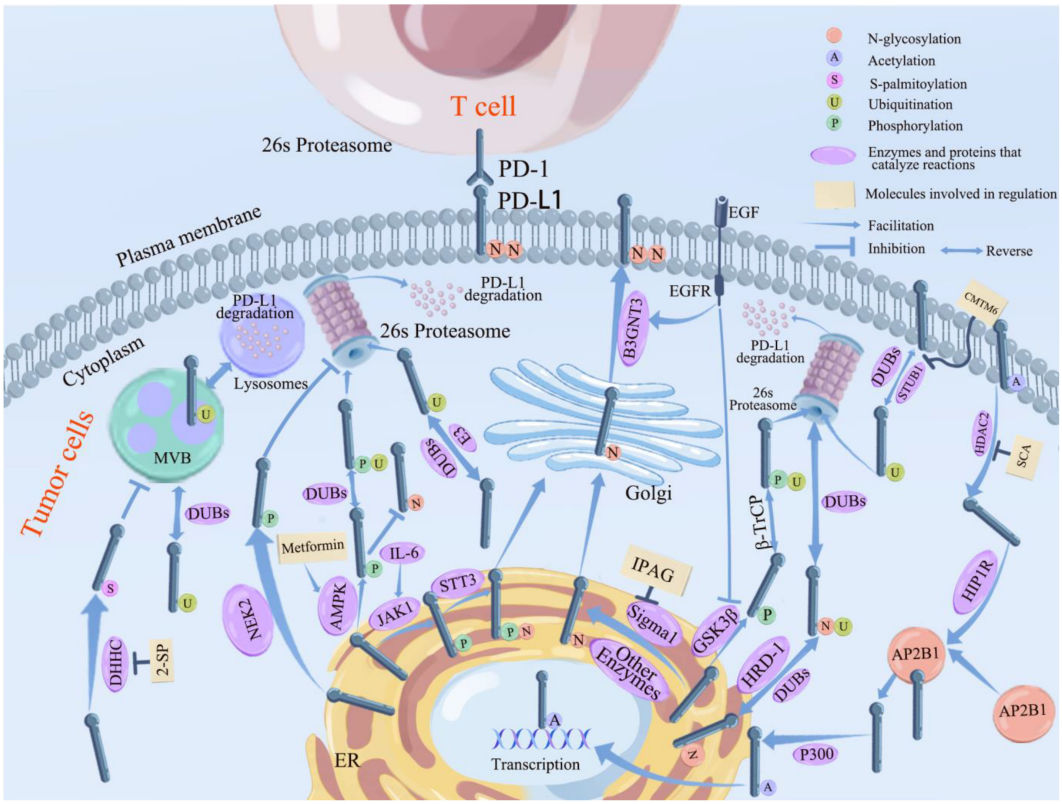

FIGURE 3
Pathways related to PTMs of PD-L1 in tumor cells and the main related molecules. PD-L1 glycosylation begins in the ER and ends in the Golgi, where it reaches the cell surface and binds to PD-L1 on the surface of T cells, inhibiting lymphocyte function and causing immune escape of tumor cells. STT3, Sigma 1 (which can be inhibited by IPGA), and other enzymes that promote glycosylation (see previous section for details) all promote PD-L1 glycosylation in the endoplasmic reticulum. B3GNT3 (which can be inhibited by EGFR) further promotes the completion of PD-L1 glycosylation in Golgi. Ubiquitination of PD-L1 takes place in the lysosome and 26s proteasome. β-TrCP promotes ubiquitination of PD-L1 after phosphorylation by GSK3β; along with STBU1 (which can be boosted by CMTM6) and other E3 enzymes (see previous section for details on particular enzymes), HRD1 encourages the ubiquitination of abnormally glycosylated PD-L1 so that it can be degraded via the 26s proteasome pathway. Ubiquitination of PD-L1 can be reversed by DUBs including CSN5 and USPs, etc, which inhibitthe degradation of PD-L1. PD-L1 phosphorylated by JAK1 undergoes glycosylation mediates by STT3; PD-L1 phosphorylated by NEK2 inhibits its ubiquitination; PD-L1 phosphorylation mediated by AMPK (which can be inhibited by metformin) inhibits its glycosylation and promotes the degradation of aberrantly glycosylated PD-L1 by ubiquitination; and phosphorylation of PD-L1 mediated by GSK3β (which can be inhibited by EGFR) all promotes it ubiquitination and can be degraded by the 26s proteasome. HDAC2 deacetylates PD-L1 (inhibited by SCA) into the cytoplasm and forms a complex with AP2B1 mediated by HIP1R, which is later reacetylated by P300 and translocated into the nucleus through the action of P300. DHHC promotes PD-L1 palmitoylation and inhibitis its transport to MVB, preventing PD-L1 degradation via the lysosomal pathway.

been generated. However, whether these PROTACs can show better clinical outcomes than primary antibodies need to be explored in depth (83). Acetylation: Nuclear PD-L1 expression is higher in metastatic tumors than in primary tumors (118), but the mechanism by which nuclear PD-L1 is increasing the aggressiveness of tumors is currently unclear. Furthermore, whether PD-L1 acetylation is associated with drug resistance is unclear and needs to be explored.

Fourthly, PD-1 modification is also critical in the anti-cancer immune response. However, the mechanisms underlying the regulation of PD-1 PTMs remain largely unknown.

Fifthly, Although PD-L1 has emerged as an important target for drug development, and several approved drugs and related clinical trials targeting PD-L1 are available demonstrating the potential of PD-L1 as a drug target, the response rate is still below 40% in most cancer types. Post-translational modulation of PD-1/PD-L1 and the proposed combination therapeutic strategies to improve the PD-1/PD-L1 blockade efficacy by providing new avenues.

Finally, Most FDA-approved therapeutic antibodies are typically produced by E. coli or other host microorganisms that do not exhibit PTMs. This renders the detection of PD-L1 suboptimal, and therefore, new technologies are needed to improve the efficacy of antibody therapy.

Author contributions

CF write the article. LZ revised the article. XC and DQ retrieved literature. TZ revised and reviewed the article. All authors contributed to the article and approved the submitted version.

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.

Publisher's note

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EDITED BY
Naoe Taira Nihira,
St. Marianna University School of Medicine, Japan

REVIEWED BY
Wentao Zhang,
Tongji University, China
Liangyou Gu,
People's Liberation Army General Hospital, China

*CORRESPONDENCE
Jing Xiong
alex7023@126.com
Bin Fu
urofbin@163.com
Songhui Xu
songhuixu007@gmail.com

†These authors share first authorship

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Prognostic and tumor microenvironmental feature of clear cell renal cell carcinoma revealed by m6A and lactylation modification-related genes

Lin Yang†, Xiaoyu Wang†, Jiahao Liu†, Xiaoqiang Liu†, Sheng Li, Fuchun Zheng, Qianxi Dong, Songhui Xu*, Jing Xiong* and Bin Fu*

Department of Urology, First Affiliated Hospital of Nanchang University, Nanchang, China

Background: Both lactylation and m6A modification have important implications for the development of clear cell renal cell carcinoma (ccRCC), and we aimed to use crosstalk genes of both to reveal the prognostic and immunological features of ccRCC.

Methods: Our first step was to look for lactylation-related genes that differed between normal and tumor tissues, and then by correlation analysis, we found the genes associated with M6A. Following that, ccRCC subtypes will be identified and risk models will be constructed to compare the prognosis and tumor microenvironment among different subgroups. A nomogram was constructed to predict the prognosis of ccRCC, and *in vitro*, experiments were conducted to validate the expression and function of key genes.

Results: We screened 100 crosstalk genes and identified 2 ccRCC subtypes. A total of 11 prognostic genes were screened for building a risk model. we observed higher immune scores, elevated tumor mutational burden, and microsatellite instability scores in the high-risk group. Therefore, individuals classified as high-risk would derive greater benefits from immunotherapy. The nomogram's ability to predict overall survival with a 1-year AUC of 0.863 demonstrates its significant practical utility. In addition, HIBCH was identified as a potential therapeutic target and its expression and function were verified by *in vitro* experiments.

Conclusion: In addition to developing a precise prognostic nomogram for patients with ccRCC, our study also discovered the potential of HIBCH as a biomarker for the disease.

KEYWORDS

lactylation, M6A, clear cell renal cell carcinoma, immunotherapy, tumor microenvironment

Background

Renal cell carcinoma (RCC), particularly clear cell cancers which account for approximately 80% of cases, are highly aggressive and heterogeneous tumors (1). Due to the lack of specific symptoms in early RCC, nearly 30% of patients present with distant metastases at the time of initial diagnosis, which is one of the major reasons for the poor prognosis of RCC patients (2, 3). An excellent outcome is usually achieved by surgically resecting the primary lesion of a low-risk limited clear cell renal cell carcinoma (ccRCC), but a significant proportion of patients still recur within a short period (approximately 40%). Patients with high-risk metastatic or limited ccRCC must undergo systemic therapy to improve their prognosis (4, 5). In recent years, combination therapies based on anti-angiogenic agents and immune checkpoint inhibitors have been shown to improve the survival of ccRCC patients (6). Nevertheless, the current problem is that effective long-term treatment responses can only be observed in a small number of patients (7, 8). In the era of precision medicine, it is crucial to thoroughly understand the tumor microenvironment (TME) and identify biomarkers associated with therapeutic response to effectively manage ccRCC patients in the long term.

Aerobic glycolysis is an important feature of tumor cell energy metabolism known as the "Warburg effect", which leads to a large accumulation of lactic acid in the TME (9). Recent findings suggest that lactate in TME can regulate immune cell metabolism through mitochondrial metabolic pathways, thereby affecting immune surveillance and escape-related behaviors (10, 11). A study by Zhao et al. proposed a novel epigenetic modification that translates the cellular metabolic state into a stable gene expression pattern through histone lactylation modification (12). This provides a new direction for understanding the mechanisms by which lactate regulates cellular metabolism and immune function. Currently, it has been demonstrated that lactylation plays a key role in the progression of ccRCC. Yang et al. found that Inactive von Hippel-Lindau-triggered (VHL) histone lactylation can drive the progression of ccRCC (13). More interestingly, Yu and Xiong et al. found that histone lactylation drives N6-adenylation methylation modifications (m6A) to promote tumor progression and immunosuppression (14, 15). Moreover, ccRCC progression and immune landscapes are strongly influenced by m6A modification (16). However, the impact of crosstalk between histone lactylation and m6A modification on the ccRCC TME is unclear.

Therefore, we utilized the interaction between histone lactylation and m6A modification-related genes to forecast patient survival and assess the response to immunotherapy in ccRCC.

Abbreviations: RCC, Renal cell carcinoma; ccRCC, Clear cell renal cell carcinoma; TME, Tumor microenvironment; VHL, Von Hippel-Lindau-triggered; m6A, N6-adenylation methylation modifications; TCGA, The Cancer Genome Atlas; FC, Fold change; DEGs, Differentially expressed genes; DECGs, Differentially expressed crosstalk genes; TMB, Tumor mutational load; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCS, Principal component analysis; OS, Overall survival; HLA, Human leukocyte antigen; LASSO, Least absolute shrinkage and selector operation analysis; K-M, Kaplan-Meier; GSEA, Gene set enrichment analysis, IPS, Immunophenotype score; ROC, Receiver Operating Characteristics; AUC, Area Under Curve; DCA, Decision curve analysis; MTGs, Mitochondria genes; FBS, Fetal bovine serum; qPCR, Quantitative PCR.

Methods

Datasets

A training set of TCGA-KIRC data was downloaded from the Cancer Genome Atlas (TCGA) database, which contained gene expression data from 541 tumor tissues and 72 normal tissue samples, as well as corresponding clinical information. The E-MTAB-1980 validation set, which contains clinical and gene expression data from 101 patients with ccRCC, was generated from ArrayExpress. Our next step was to remove genes with raw counts below 10 in more than 25% of the samples. The TPM data were transformed into log2 (TPM+1). There are 1223 genes associated with lactylation modification according to Zhang et al. (12).

Correlation and difference analysis

The 1223 lactylation modification-related genes were subjected to Pearson correlation analysis with 23 m6A genes to obtain crosstalk genes with screening criteria of correlation>0.5 and padj<0.01. To identify differentially expressed genes (DEGs) between cancer and paracancerous tissues, we used the "DESeq2" package [padj <0.05, |log2fold change (FC)|>1]. Subsequently, the crosstalk genes were merged with DEGs and the intersection was taken to finally obtain the differentially expressed crosstalk genes (DECGs). The correlation results between DECGs were visualized using the "circlize" package.

Each sample's tumor mutation load (TMB) was calculated using the "Maftools" package. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of DECGs were performed using the "clusterProfiler" package.

Identification of ccRCC subtypes

We identified ccRCC clinical subtypes using the consensus clustering R package "ConsensusClusterPlus" (17). The ability of DECGs to discriminate between subtypes was assessed using principal component analysis (PCA). After that, we compared the differences between subtypes in terms of clinical variables (age, gender, grade, and stage) as well as overall survival (OS).

The immune landscape of different subtypes

Every sample was analyzed for the TME score using the "estimate" package. In each TCGA-KIRC sample, immune cell

infiltration was assessed using the online analysis tool TIMER2.0 (CIBERSORT algorithm). Based on our knowledge of the close relationship between immune-inhibitory, immune-stimulatory, and human leukocyte antigen (HLA) genes and TME, a comparison of expression levels between subtypes was made (18, 19).

Constructing risk model

Univariate COX regression analysis was first performed on the DECGs to screen for genes associated with OS according to $p<0.05$, and they were used to perform a least absolute shrinkage and selection operation (LASSO) analysis to screen for genes most associated with prognosis for constructing the risk model and to derive a risk coefficient for each gene. The expression of each modeled gene was multiplied by the risk coefficient to calculate the risk score for each patient. Each group of patients was categorized according to the median risk score. To test whether the model was able to discriminate between patients at different risks, PCA and Kaplan-Meier (K-M) survival analyses were performed. In addition, the relationship between clinical variables and risk scores for different clinical characteristics in the high- and low-risk groups was assessed.

Immune landscape and enrichment analysis

Based on the above results, we evaluated the differences between the two risk groups in terms of TME scores, immune cell infiltration, and immune-related gene expression. Then, we calculated immune-related function scores using the " GSEABase" and "GSVA" packages based on the " immune. gmt" file. The DEGs were determined using the "limma" package, followed by GO, KEGG, and gene set enrichment analysis (GSEA) using "ClusterProfiler". The "enrichplot" and "GseaVis" packages were used to visualize the enrichment analysis results.

As a result of the analysis above, we obtained the sample TMB and then downloaded the MSI score file *via* the "cBioPortalData" package. We defined samples as MSI when the score exceeded 0.3, and MSS if the score was below it. Moreover, the "easier" package calculates an immunotherapy response score based on TME characteristics, where higher scores indicate greater immunotherapy sensitivity (20). We downloaded each patient's immunophenotype score (IPS) from the Cancer Immunome Atlas (TCIA, https://tcia.at/home) and divided it into <=8 and >8 groups according to IPS. The relationship between these metrics and risk scores was finally evaluated to reflect the predictive value of risk scores on immunotherapy response.

Constructing a nomogram

Through multivariate and univariate COX regressions, several independent predictors of OS were identified. A nomogram was then created with the help of the "survival" and "rms" packages. Calibration plots, Receiver Operating Characteristics (ROC), and Area Under Curve (AUC) were used to assess the predictive capability of the nomogram. Decision curve analysis (DCA) was used to determine the clinical value of the nomogram.

Comprehensive analysis of key genes

Both the metabolism of lactate and the lactylation modification process are closely related to the function of mitochondria. We obtained 1136 mitochondria genes (MTGs) from the MitoCarta 3.0 database (https://www.broadinstitute.org/) and then took intersections with risk model genes to obtain key genes. Following the pan-cancer analysis, key genes were assessed for differential expression and prognostic value across multiple cancer types. An assessment of the association between clinical features and TME of ccRCC was conducted using the TISIDB database (http://cis.hku.hk/TISIDB/). The ChEA3 database (https://maayanlab.cloud/chea3/#top) was used to obtain potential TF regulating key genes, and we selected the "Mean Rank" panel and took the top 10 genes for subsequent analysis and further screened the TF regulating key genes by differential analysis and K-M survival analysis. Patients with ccRCC were categorized into two groups according to the expression of key genes. After performing a differential analysis using the "limma" package, downstream pathways were identified using GO, KEGG, and GSEA analyses.

Cell culture, transfection, and infection

Cell lines used in this study were obtained from Procell Life Science&Technology Co., Ltd (Wuhan, China). An incubator containing 37°C and 5% CO2 was used to grow HK-2 and ACHN cells. Medium: MEM + 10% fetal bovine serum (FBS) + 1% antibiotics (HK-2 and ACHN), 1640 + 10% FBS + 1% antibiotics (786-O).

The overexpression plasmids and control plasmids of HIBCH were synthesized by Obio Technology (Shanghai) Corp., Ltd. We transfected the plasmid into 293T cells using calcium phosphate transfection to collect the viral fluid, which was then used to infect ACHN and 786-O cells, resulting in elevated levels of HIBCH expression in the cells. The RNA extraction was performed 48 hours after cell infection, and PCR was carried out to determine overexpression efficiency. Meanwhile, further cell phenotyping experiments were carried out.

Quantitative PCR

For RNA extraction, we used TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) (Eight pairs of ccRCC cancer and paracancerous tissue specimens were obtained from the Human Genetic Resources Center, The First Affiliated Hospital of Nanchang University.), and then reverse transcribed by Takara PrimeScript RT kit (Takara Bio, Inc., Otsu, Japan). The qPCR was performed on a Roche LightCycler96 real-time fluorescent

quantitative PCR system using an SYBR premix Ex Taq kit (Takara Bio, Inc., Otsu, Japan). The relative expression of genes was calculated based on the 2^-ΔΔCt method. Primer sequences: HIBCH-F: 5'-GGAGTTGGTCTCTCAGTCCATG-3', HIBCH-R: 5'-CCAAGTTTTCCTTGGAGTCGTGG-3'.

Cell migration

Cell migration was measured using 24-well transwell chambers, each upper chamber was inoculated with approximately 30,000 cells, and 200ul of FBS-free medium was added; the lower chamber was filled with 600ul of medium containing 20% FBS and counted after 36 hours. Cells were cultured to 80% density in 6-well plates, scratched, and then switched to an FBS-free medium and photographed in the same field of view at 0 h and 24 h, respectively.

Statistical analysis

Statistical analyses were conducted using R (version 4.2.2) or GraphPad Prism (version 9.0), and $p<0.05$ was considered statistically significant. Analysis of variance was used to compare categorical variables, and t-tests were used to compare continuous variables. Correlations between continuous variables were examined by Spearman or Pearson correlation analysis. Non-parametric samples comparing two independent samples were compared using Wilcoxon, while multiple independent samples were compared using Kruskal-Wallis.

Results

Screening and analysis of DECGs

By correlation analysis, we obtained 604 crosstalk genes, including 105 DEGs (Figure 1A). Then we removed 5 genes that were not detected in the validation cohort and finally obtained 100 DECGs. As shown in the volcano plot, there were 27 low and 73 high-expressed genes in the tumor tissue (Figure 1B). Subsequently, correlation network plots demonstrated a close association between DECGs (Figure 1C). the results of GO and KEGG analysis suggested that DECGs may be involved in biological processes such as protein modification and energy metabolism, and may play an important role in the HIF-1 signaling pathway (Figure 1D). Interestingly, the VHL/HIF pathway is linked to lactate production in ccRCC (13), which certainly suggests to us that these DECGs deserve to be studied in depth. According to Figure 1E, DECGs are mutated in 45.83% of samples, with MTOR showing the highest mutation frequency (18%).

Clinical features of the two ccRCC subtypes

Two subtypes of ccRCC were identified (Figure 2A), and the PCA confirmed this (Figure 2B). A comparison of the clinical characteristics of the two subtypes was conducted following that. In the TCGA cohort, age and gender did not differ between the two groups, whereas the distribution of grading and stage showed significant differences, with the C2 group having a higher nuclear grade and a more advanced clinical stage (Figure 2C). Although the E-MTAB-1980 cohort also showed the same trend, the C2 group in the cohort had more male patients (Figure 2D). The C2 group suffered a worse prognosis in both cohorts according to the K-M survival analysis (Figure 2E). In addition, the Sankey diagram more visually demonstrates the close association between subtypes and clinical features (Figure 2F).

Two subtypes of the immune landscape

The results showed that all scores, except for tumor purity, showed higher levels in the C2 group (Figure 3A). Figure 3B shows that the two subtypes infiltrated differently with immune cells, for example, the C1 group had more abundant monocytes and macrophages; the C2 group was infiltrated with more T cell follicular helper (TFH), T cell regulatory (Tregs), and NK cell activated. In addition, it is clear from the heat map that most of the immunoinhibitory, immunostimulatory, and HLA genes were expressed at higher levels in the C1 group (Figures 3C–E). In conclusion, all of the above results indicate that the ccRCC subtypes identified by DECGs have distinct TME and clinical characteristics. Therefore, an in-depth analysis of the predictive value of DECGs for the prognosis and immunotherapy of ccRCC is warranted.

Prognostic risk characteristics of ccRCC patients

For the risk model, LASSO regression analysis identified 11 prognostic genes in the training set (Figure 4A). All were associated with a better prognosis, except TTLL3 and CHFR (Figure 4B). Risk score per patient = SORL1*(-0.056) + HIBCH*(-0.122) + KDR*(-0.047) + VASH1*(-0.016) + VWA7*(-0.036) + TMEM25*(-0.202) + PLCL1*(-0.116) + PRUNE2*(-0.055) + TTLL3*0.069+CHFR*0.501+ABCG1*(-0.054). According to PCA, these 11 risk genes (RGs) could be assigned to different risk groups of ccRCC patients (Figure 4C). K-M survival curves show that the high-risk group has shorter long-term survival times (Figure 4D). The validation cohort also demonstrated similar results. Figures 4E, F demonstrate the distribution of survival status and RGs with a risk score, which is almost consistent with the trend in both cohorts.

Subsequently, we analyzed the risk characteristics of the different clinical variables. Among patients of different ages (<=65 vs >65), gender (Male vs Female), grades (G1/2 vs G3/4), and stages (Stage I/II vs Stage III/IV), the cumulative risk was increasing year by year in the high-risk group and was consistently higher than in the low-risk group (Figures 5A–D). We also found large differences in risk scores between variables within each variable. Although risk scores did not differ significantly between the two age groups, male,

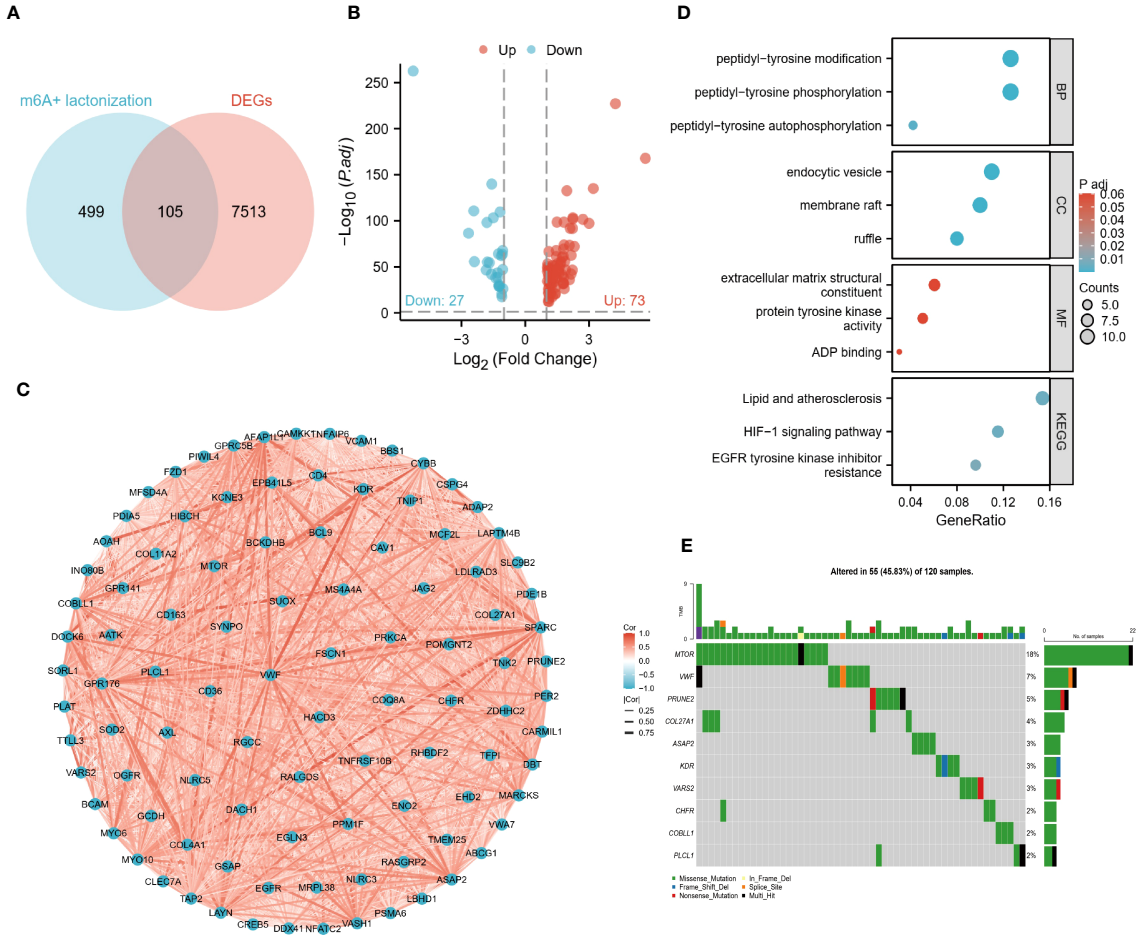

FIGURE 1
Identification and preliminary analysis of DECGs. **(A)** Venn diagram of crosstalk genes and DEGs intersected. **(B)** Volcano plots show the differential distribution of DECGs. **(C)** Correlation network diagram of DECGs. **(D)** DECGs are enriched for biological functions and BP, Biological Process; MF, Molecular function; CC, Cellular component). **(E)** Mutational landscape of DECGs.

high-core graded (G3/4), and late-stage (Stage III/IV) patients tended to have higher risk scores (Figures 5E–H). In the Sankey diagram depicting the correlation between C1/C2 subtypes and risk groupings, we can observe that the C2 group with a worse prognosis is almost exclusively distributed in the high-risk group, whereas the C1 group, with a slightly better prognosis, is in a homogeneous distribution in the two risk groups (SF1 I).

Immunological characterization and enrichment analysis of the two risk groups

In the TME score, both the immune score and the estimated score were higher in the high-risk group (Figure 6A). And the higher abundance of CD8 T cells and TFH clustered in the high-risk group (Figure 6B), which usually exerts anti-tumor immune effects. Additionally, only a few HLA genes showed differential expression, but most of the immunoinhibitory and immunostimulatory were different from them. High-risk patients expressed more CD96, CTLA4, IL10RB, LAG3, LGALS9, PDCD1, and TIGIT levels among immunoinhibitory molecules. The same is true for immunostimulatory, most of which are highly expressed in high-wind samples, such as CD70, IL6, and TNFRSF18 (Figure 6C). A similar expression pattern was observed in cohort E-MTAB-1980 (Figure 6D).

The immune-related function scores were generally higher in the high-risk group than in the other group (Figures 7A, B). Furthermore, the results of GO, KEGG, and GSEA also showed a strong association of risk grouping with immune-related functions. Biological pathways and functions related to immunity were enriched in DEGs between high- and low-risk groups (Figure 7B). Moreover, primary immunodeficiency and cytokine-cytokine receptor interaction pathways were enriched in the high-risk group (Figure 7C). The series of results suggest a close association between risk groups and the immune microenvironment, especially in high-risk groups.

Prediction of immunotherapy response

Patients with ccRCC were analyzed based on TMB, MSI, IPS, and easier scores to predict their response to immunotherapy.

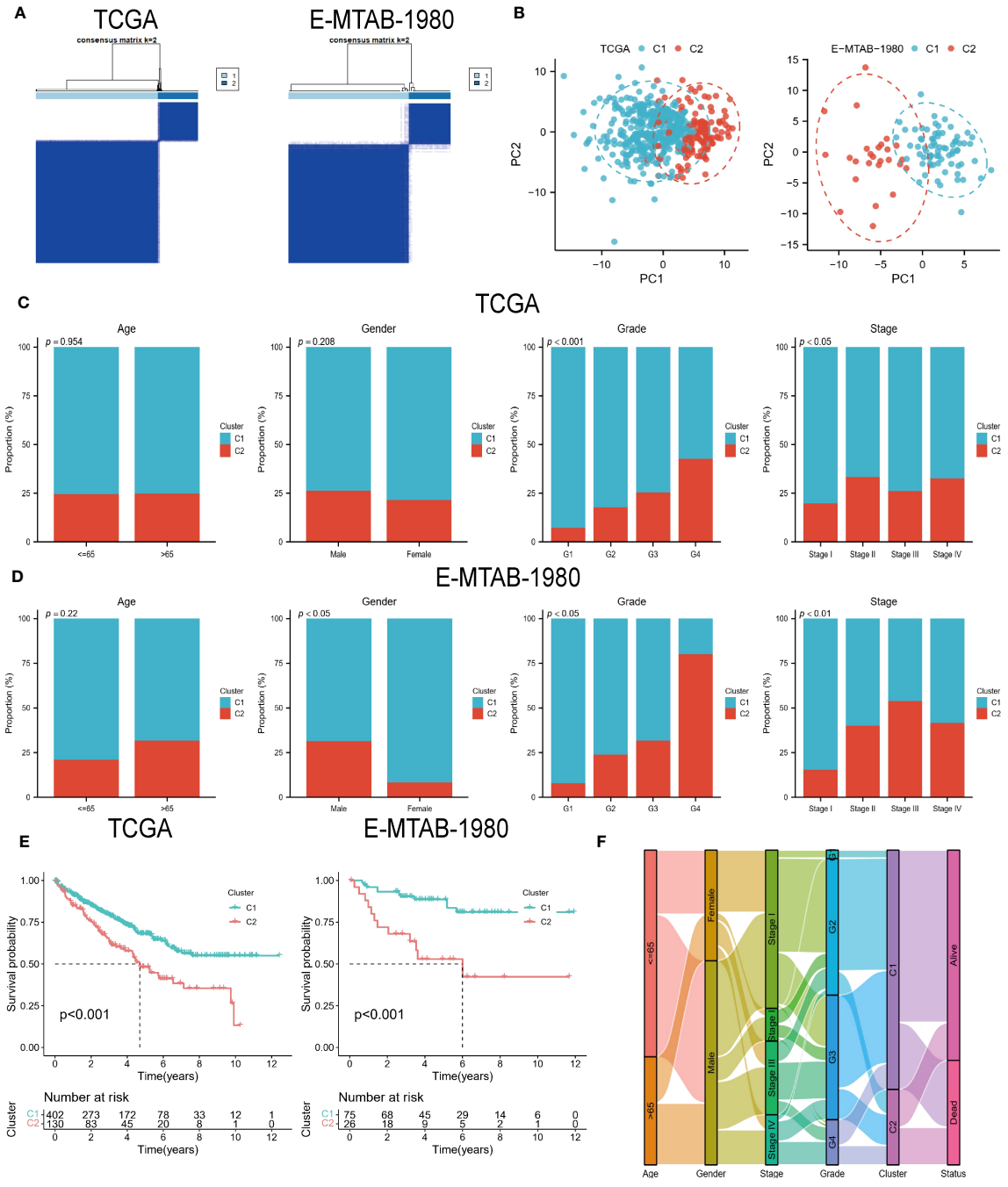

FIGURE 2
DECGs-based ccRCC subtypes. **(A)** Consensus clustering identified 2 ccRCC subtypes. **(B)** Principal component analysis based on DECGs. **(C, D)** Distribution of the 2 subtypes across clinical variables in the TCGA and E-MTAB-1980 cohorts. **(E)** K-M analysis to compare survival differences between the 2 subtypes. **(F)** Sankey diagrams show the interchangeable relationships between clinical variables, subtypes, and survival status.

High-risk samples showed significantly higher levels of TMB and easier scores, and the samples in the MSI group had higher risk scores (Figures 8A–C). Similarly, higher scores in the IPS scores about immune checkpoints were associated with higher risk scores (Figure 8D). According to the above results, TME differs greatly between the two risk groups, and the immunotherapy response may be more durable and effective in patients at high risk. Interestingly, we got the same results in a bladder cancer immunotherapy cohort. We found that patients in the immune-responsive group had higher risk scores and more high-risk patients (Figure 8E).

Nomogram accurately predicts survival of ccRCC patients

Using the training set, we performed univariate and multivariate COX regression analysis to identify independent

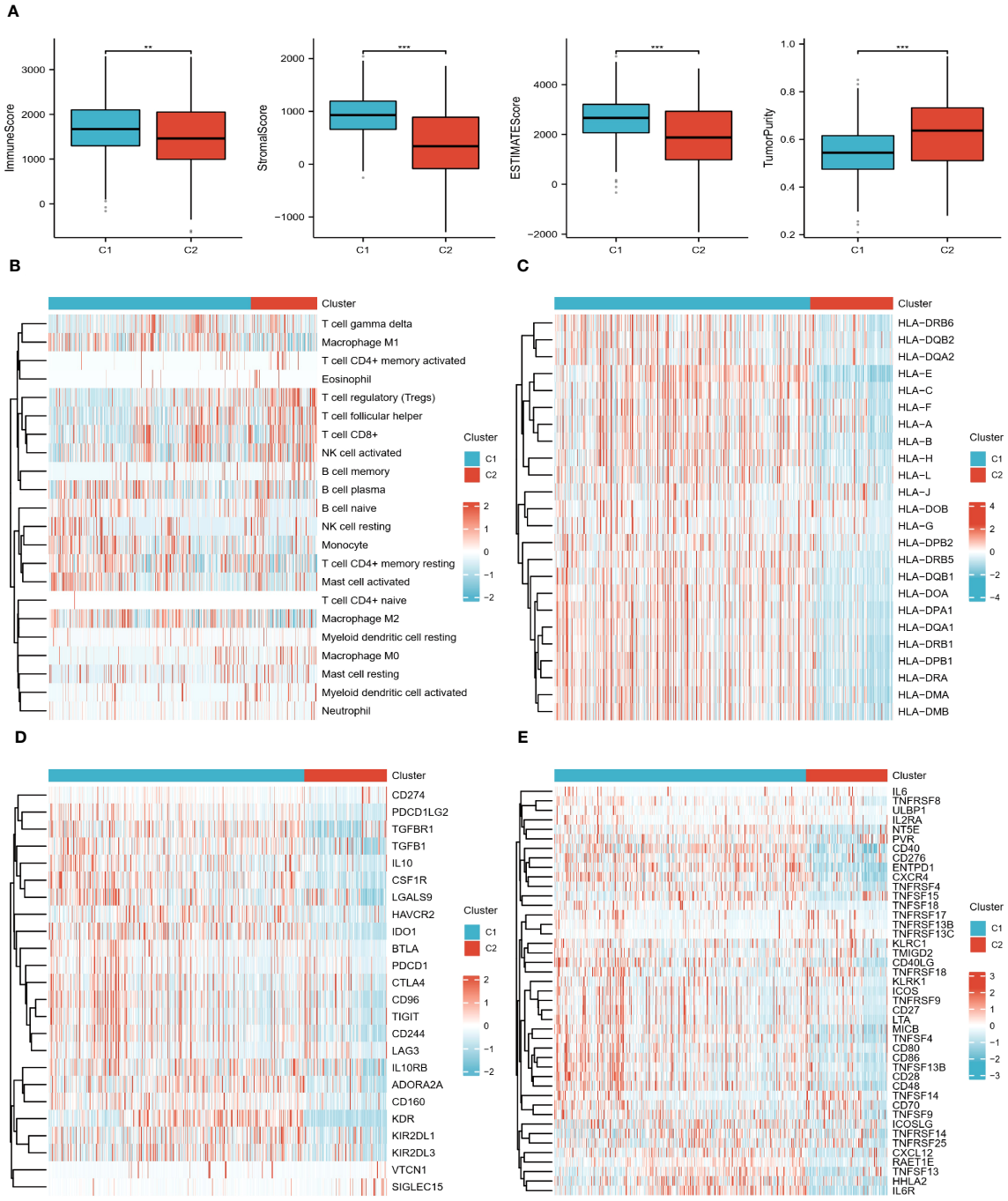

FIGURE 3
Immune landscape of the 2 subtypes. **(A)** Differences in TME scores between C1 and C2 groups. **(B)** Heat map of the distribution of 22 immune cells in the two subtypes. **(C–E)** Heat map of the difference in expression levels of HLA genes, immunoinhibitory, and immunostimulatory in the two subtypes. "**" <0.01, and "***" <0.001.

predictors of OS, including age, stage, and risk score (Figures 9A, B). Based on the sum of the corresponding scores for each factor, a nomogram was constructed to predict patient survival at 1, 3, and 5 years (Figure 9C). Both the training and validation data sets showed the predicted probabilities to be almost in line with the actual probabilities (Figure 9D). Furthermore, the results of the ROC analysis also showed strong predictive performance of the model with 1-year AUC=0.863 for the TCGA cohort and 1-year AUC=0.900 for the validation cohort (Figure 9E). The DCA demonstrated that the nomogram was superior to the TNM staging for clinical purposes (Figure 9F).

The role of the key gene HIBCH in ccRCC

From risk model genes, we identified HIBCH, a gene closely related to the mitochondrial function that may play a role in the development of ccRCC (Figure 10A). To begin with, HIBCH is

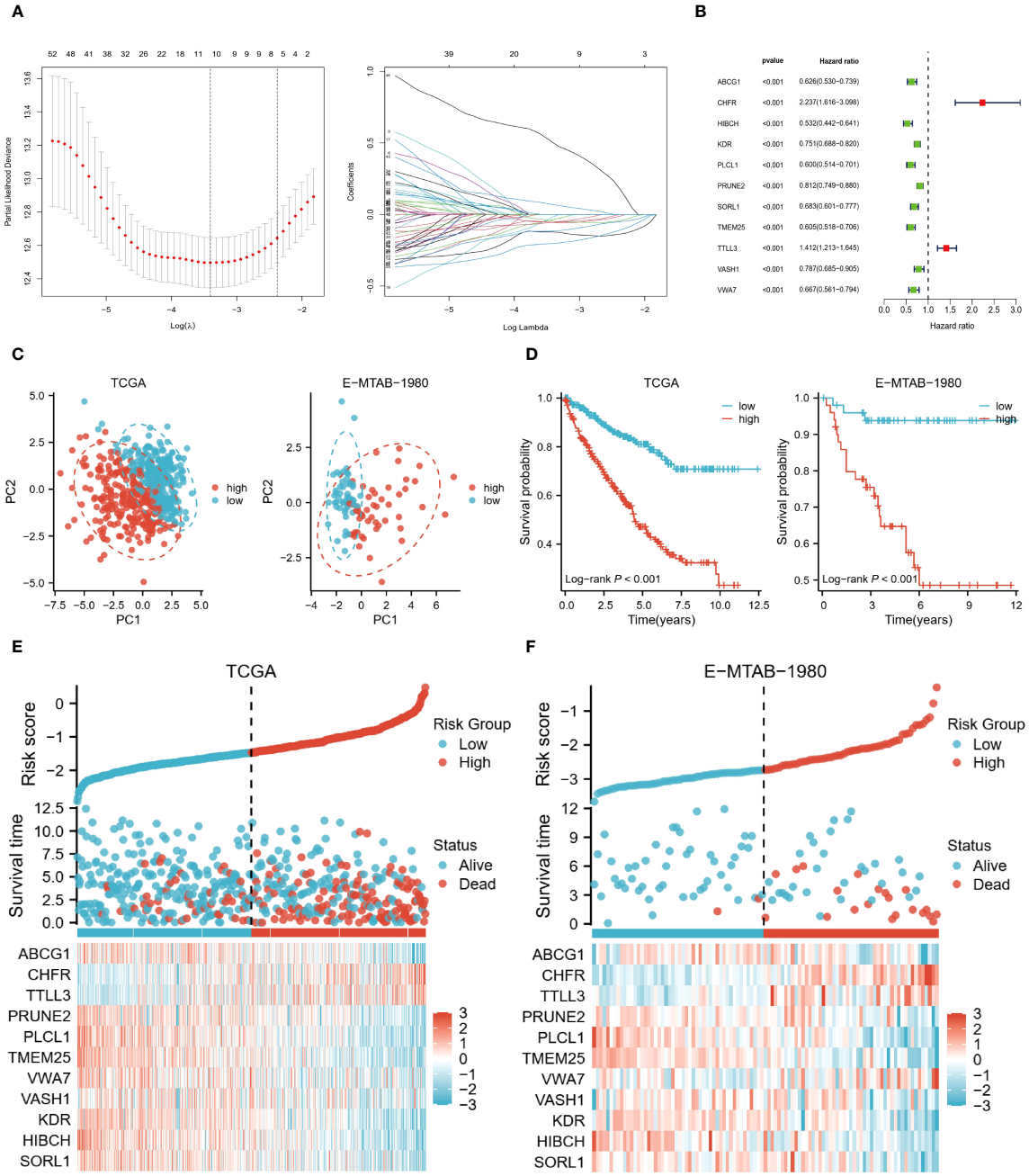

FIGURE 4
Risk model based on 11 genes. **(A)** LASSO regression analysis was performed to identify the genes used to construct the risk model. **(B)** Hazard ratio of the 11 model genes. **(C)** Principal component analysis based on model genes. **(D)** K-M analysis comparing OS of high and low-risk groups. **(E, F)** Trends in the distribution of survival status and model gene expression levels with changes in risk scores.

expressed at lower levels in tumor tissues, and high levels are associated with better clinical outcomes (Figures 10B, C). High levels of HIBCH expression are associated with lower tumor grades and stages, reflecting its relationship to clinical variables (Figure 10D). The immune microenvironment and HIBCH also appear closely linked, which classify ccRCC into 6 immune subtypes and may be useful to classify different types of ccRCC according to their immune response (Figure 10E). In addition to having a negative correlation with immune cell infiltration, HIBCH expression was also found to be negatively correlated with TME scores (Figures 10F, G). More interestingly, the immune checkpoints CTLA4 and PDCD1 showed a negative correlation with the expression level of HIBCH as well (Figure 10H). According to the pan-cancer analysis, HIBCH displayed similar effects in numerous cancers (SF2). Based on these findings, there may be a mechanism through which HIBCH interacts with ccRCC's immune microenvironment, which may influence tumor development and treatment response.

To further explore the regulatory mechanisms of HIBCH in ccRCC, we identified several potential TFs that regulate HIBCH

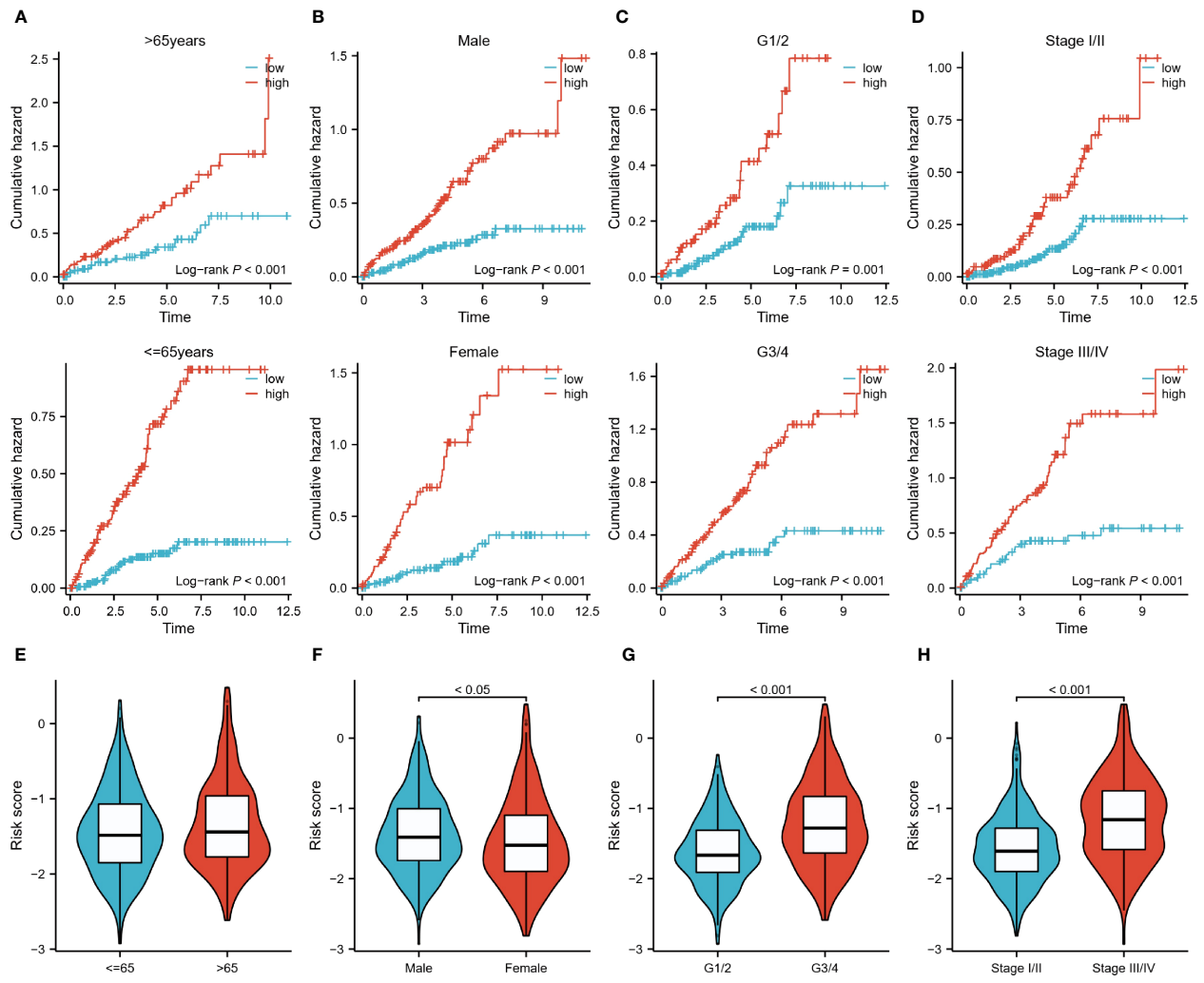

FIGURE 5
Risk characteristics for each subgroup of clinical variables. **(A–D)**: Cumulative hazard over time for <=65 and >65 years of age, male and female, G1/2 and G3/4, and Stage I/II and III/IV patients in high and low-risk groups. **(E–H)**: Differences in risk scores between patients <=65 years and >65 years, male and female patients, G1/2 and G3/4 patients, StageI/II and StageIII/IV.

expression, as well as potential pathways that inhibit ccRCC development. The co-expression heat map demonstrated the relationship between HIBCH expression and the top 10 TFs, most of which had high correlation coefficient values (Figure 11A). Difference analyses revealed significant differences between tumors and normal tissues in the expression levels of SPI1, GATA1, NR1H3, FLI1, SP2, MYBL2, and TFAP2C (Figure 11B). Subsequent K-M survival analysis of these DEGs revealed that FLI1, SP2, MYBL2, and TFAP2C were associated with OS (Figure 11C). Moreover, SP2 was lowly expressed in tumor tissues and associated with a good prognosis, while MYBL2 was highly expressed in tumor tissues and associated with a poorer prognosis. It is more likely that these two genes play a role in the regulation of HIBCH, but more experiments are needed to verify this conjecture. Following enrichment analysis, DEGs between high and low HIBCH expression groups had enriched biological functions associated with immunity (Figure 11D). More importantly, the GSEA results suggested that HIBCH is closely associated with FCGR-related pathways (Figure 11E). This gene family encodes the receptor for the Fc portion of immunoglobulin G, which is involved in a range of immune processes. This further suggests a complex mechanism of interaction between HIBCH and the immune microenvironment and has the potential to be a relevant biomarker for immunotherapeutic response.

Experimental verification results

Our *in vitro* studies revealed that HIBCH was higher expressed in HK2 than in ACHN and 786-O (Figure 12A). Moreover, by extracting RNA from kidney cancer and paraneoplastic tissues for qPCR, the same results were obtained, and the cancer tissues expressed lower levels of HIBCH (Figure 12B). Subsequently, we further explored the effect of abnormal expression of HIBCH on the migration ability of kidney cancer cells to elucidate its role in the metastasis of kidney cancer. Figure 12C showed that we successfully

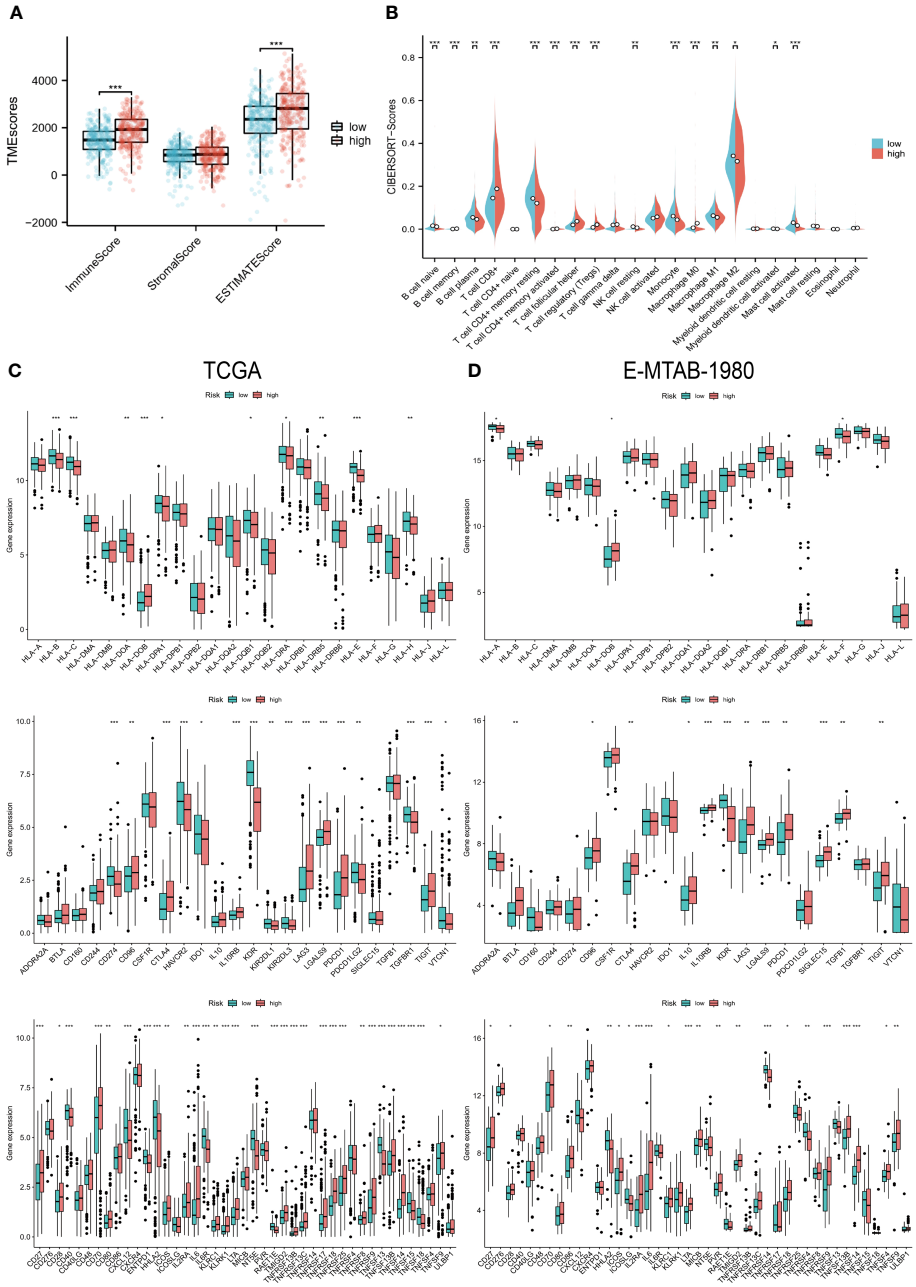

FIGURE 6
Immunological characteristics of the two risk groups. **(A)** Differences in TME scores between the high and low-risk groups. **(B)** Levels of infiltration of 22 immune cell types in the two risk groups. **(C, D)** HLA gene, immunoinhibitory, and immunostimulatory expression levels in the TCGA and E-MTAB-1980 cohorts in the high- and low-risk groups. "*" <0.5, "**" <0.01, and "***" <0.001.

overexpressed HIBCH in ACHN and 786-O. As compared to the overexpression group, the number of cells in the control group was significantly higher (Figure 12D); in the scratch assay, the control cell migration rate was also higher (Figures 12E, F), and these results were statistically significant.

Discussion

There are two primary mechanisms responsible for tumor occurrence and development: inactivation of tumor suppressors and activation of tumor promoters. In this process, epigenetic modifications play a key role in regulating the expression of genes (21). Current studies have shown that epigenetic aberrations are common in RCC, especially histone modifications, and are closely associated with their prognosis and treatment (22, 23). According to Zhang et al., histone lactylation modification (12) is a new epigenetic modification modality that provides new insights into the pathogenesis of RCC. Cancer is characterized by two crucial features: immune escape and metabolic reprogramming. Linking these aspects together is lactate, a metabolite that facilitates immunosuppression through lactylation modification. Recent

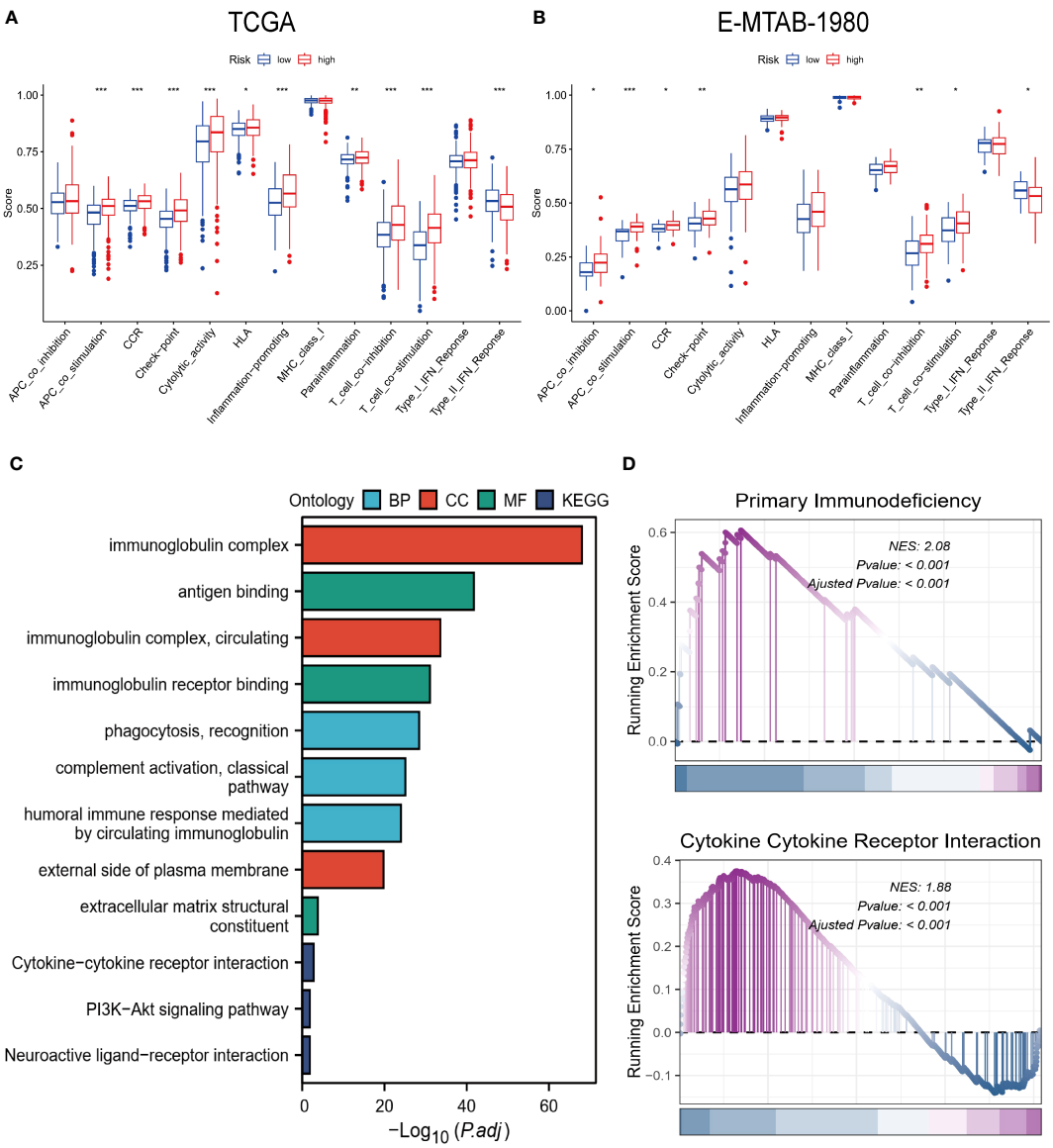

FIGURE 7
Biofunctional and pathway analysis. **(A, B)** Comparison of immune-related functions between high and low-risk groups. **(C)** Results of GO and KEGG enrichment analysis. **(D)** Visualization results of GSEA. "*" <0.5, "**" <0.01, and "***" <0.001.

research reveals that high levels of lactate in the tumor microenvironment (TME) hinder T cell-mediated immune responses, effectively facilitating tumor immune evasion. Additionally, histone lactylation in macrophages drives a transition toward an immunosuppressive M2 macrophage phenotype (10, 13, 24). This evidence suggests that tumor metabolism and lactylation modification can modulate each other and influence the function of immune cells in TME (25, 26). As a result, it is imperative to investigate in depth the effect of histone lactylation modifications on TME in ccRCC to predict patient survival and immunotherapeutic response.

In this study, we identified DEGs with histone lactylation modifications interfering with m6A, which were used to reveal the prognosis and TME characteristics of ccRCC. At first, the DECG obtained by screening classified ccRCC patients into two subtypes with different clinical and immunological characteristics, and then we constructed a risk model based on 11 prognostic genes. Patients at higher risk have shorter survival but had higher levels of TMB, MSI, and anti-tumor immune cell infiltration, and the easier score suggested that this group of patients was more sensitive to immune checkpoint inhibitors. The screening of patients suitable for immunotherapy is an urgent clinical problem, and our results are certainly instructive for the design of future prospective studies. The nomogram is a practical prediction tool that has important reference value in both the clinical decision-making of ccRCC and the long-term management of the disease.

As seen in our findings, patients in different risk groups have very different TME characteristics, with high-risk patients having higher immune scores. However, we also observed that the C2 subtype, which has a worse prognosis, has a lower immunization

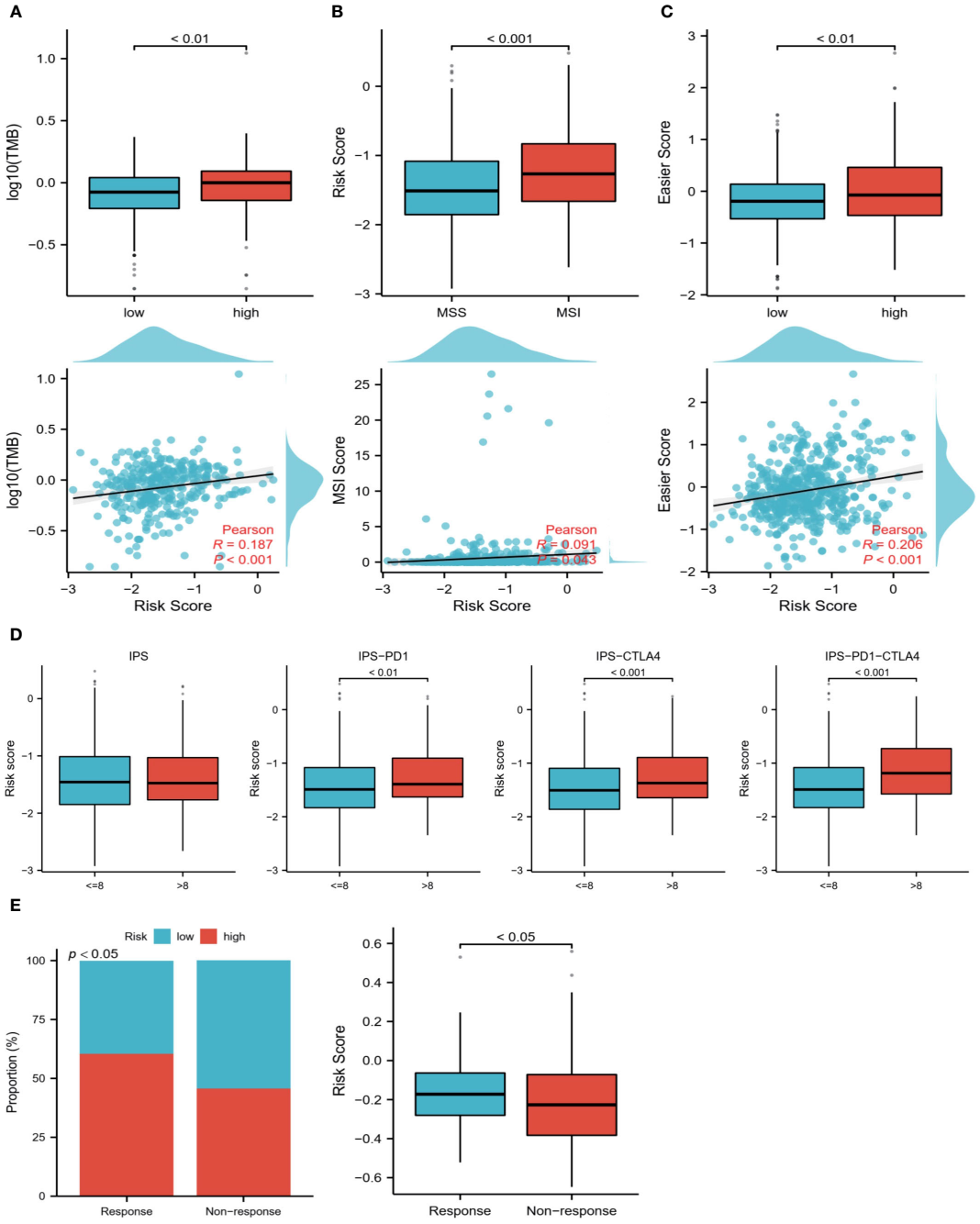

FIGURE 8
Prediction of the immune response. **(A)** TMB levels in high and low-risk groups, Pearson correlation analysis of TMB and risk scores. **(B)** Differences in risk scores between MSI and MSS groups, Pearson correlation analysis of risk scores with MSI scores. **(C)** Differences in easier scores in high and low-risk groups, and Pearson correlation analysis with risk scores. **(D)** The relationship between IPS grouping and risk scores. **(E)** Relationship between immunotherapy response and risk scores in a bladder cancer immunotherapy cohort.

score than the C1 group, which may seem paradoxical. A Sankey diagram of the correlation between the C1/C2 subtypes and the risk grouping may explain this phenomenon, and that the C1 group, which has a slightly better prognosis, is not all distributed in the low-risk group, and that it contains a significant portion of high-risk patients. Therefore, there is some heterogeneity within the C1 group, and a more detailed delineation is needed in the future.

It is well known that the TME at which the tumor cells are located is one of the key reasons for this difference (27, 28). Various cells, stroma, and non-cellular components together constitute the TME, and not only do these components have complex interactions with each other, but they are also influenced by other factors such as metabolic and epigenetic modifications (19, 29, 30). Both lactylation modifications and m6A modifications can influence the TME, and

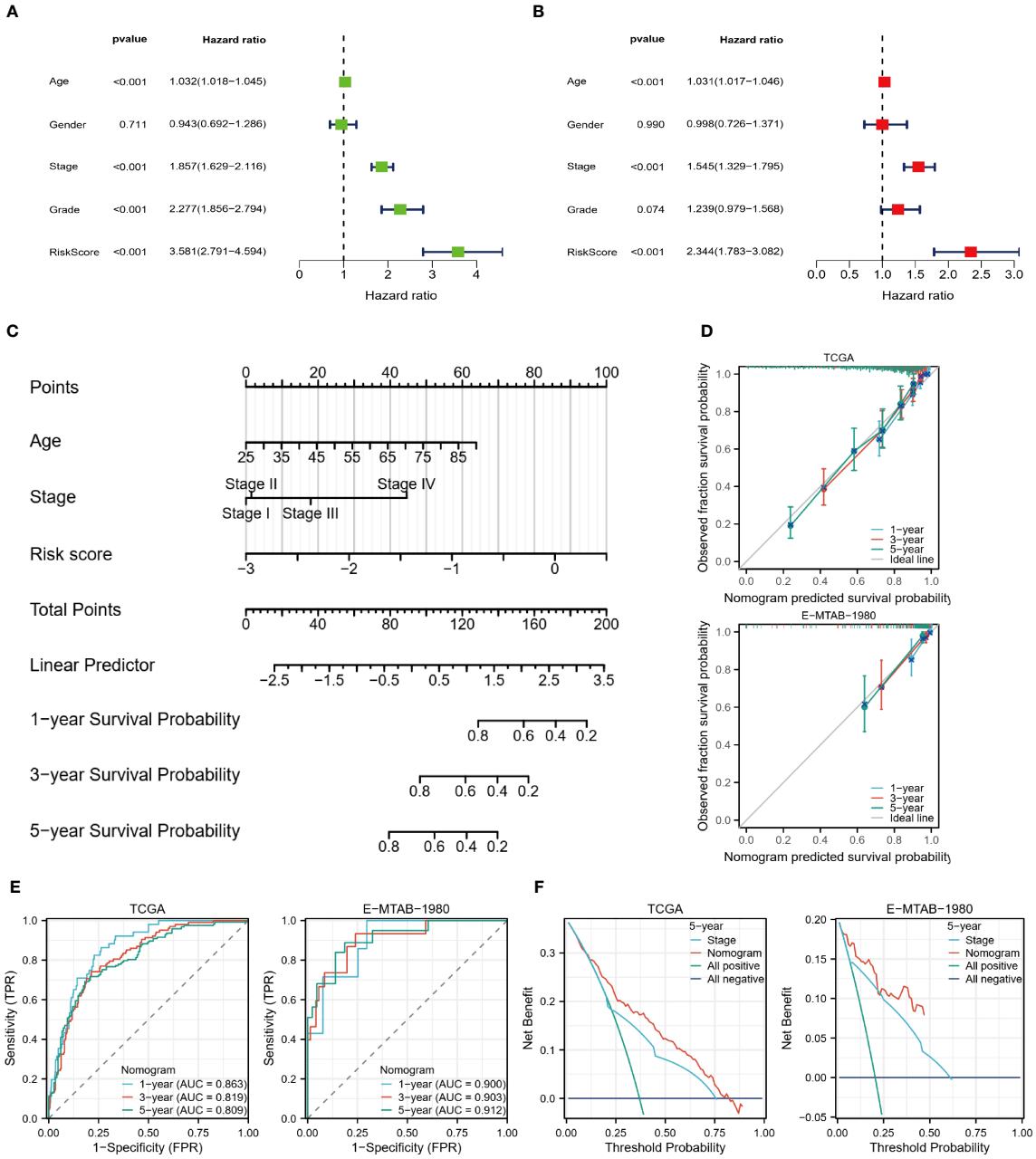

FIGURE 9
Nomogram predicts survival probability. **(A, B)** Univariate and multivariate COX regression analysis. **(C)** Nomogram constructed based on TCGA cohort. **(D)** Nomogram calibration plots for 1, 3, and 5 years. **(E)** 1, 3, and 5-year ROC curves and ACU to assess the predictive performance of the model. **(F)** 5-year DCA curves to assess the clinical application value of the model.

they can not only affect the chemotaxis and activation of immune cells, but also regulate the molecules on the surface of immune cells, and thus the function of immune cells and the intensity of immune responses (31–33). The study by Jia Xiong et al. confirmed the effect of the interaction between these two epimodification modalities on the immune microenvironment (15), in other words, they may have synergistic effects in the immune microenvironment of tumors, jointly affecting tumor growth and the effectiveness of immunotherapy. For the first time, we have combined the analysis of these two epigenetic modifications for exploring the heterogeneity of ccRCC in terms of TME. More importantly, our model is not only able to accurately predict the long-term survival of patients but also has implications for immunotherapy.

Furthermore, we have identified a crucial gene in the model known as HIBCH (3-Hydroxyisobutyryl-CoA Hydrolase), which is an enzyme that plays a vital role in the metabolism of fatty acids (34). It also means that HIBCH is not only closely related to histone lactylation modification (12), but may also influence the process of

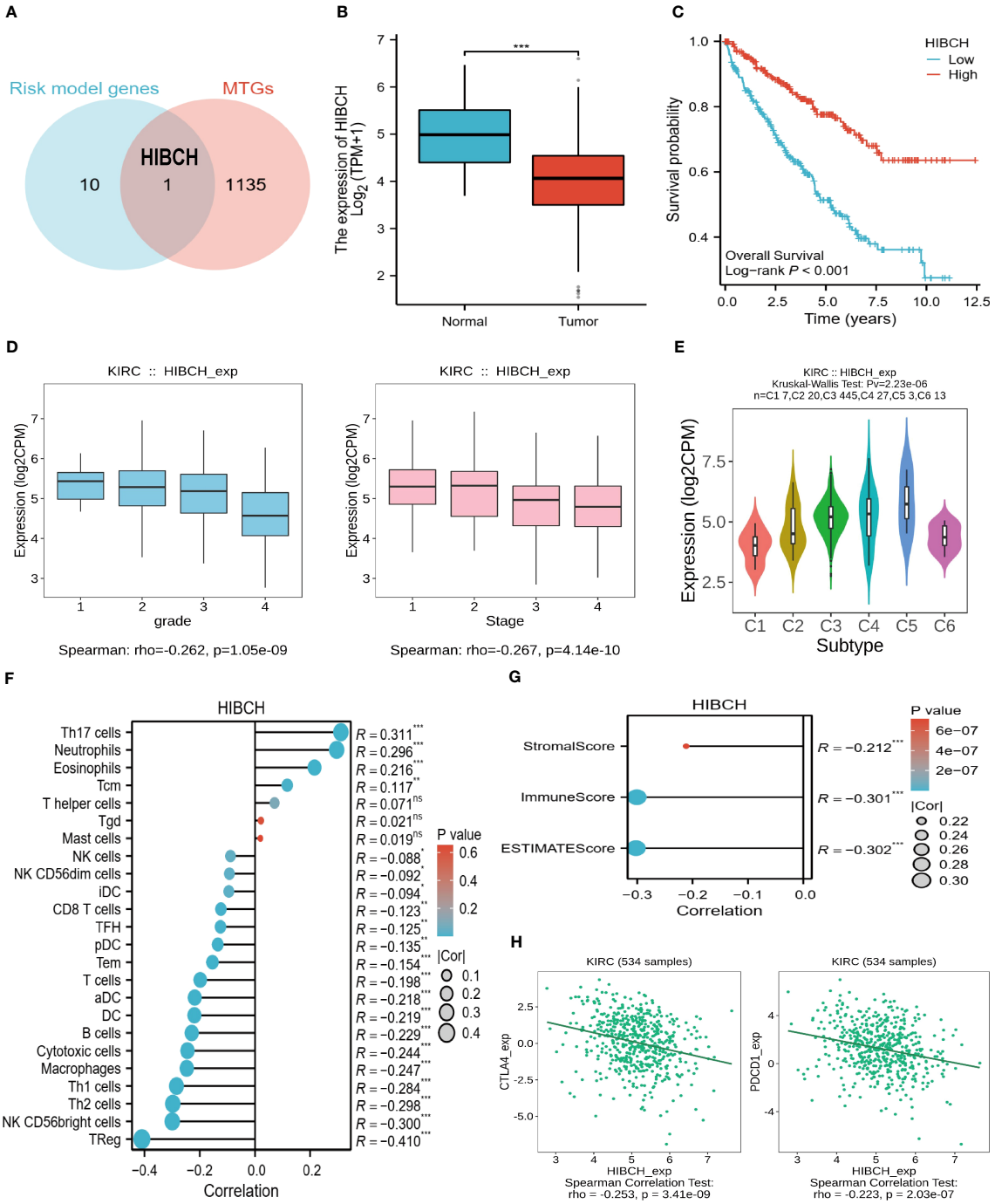

FIGURE 10
The significance of HIBCH in ccRCC. **(A)** The Venn diagram identified the key gene HIBCH. **(B)** HIBCH expression levels in tumor and normal tissues. **(C)** K-M survival curves between high and low expression groups of HIBCH. **(D)** HIBCH expression levels in samples of different grades and stages. **(E)** HIBCH can classify ccRCC into 6 immune subtypes. **(F, G)** Lollipop plot of the correlation between HIBCH and immune cell infiltration level, TME score. **(H)** Correlation of HIBCH with the expression of CTLA4, PDCD1. "***" <0.001.

mitochondrial energy metabolism. HIBCH's role in cancer is currently unknown in the current state of research. Shan et al. delved into the implications of HIBCH in the progression and treatment of colorectal cancer. Colorectal cancers express higher levels of HIBCH, and its function depends on its localization in mitochondria, and blocking the function of HIBCH not only can inhibit the growth of cancer cells but also can improve the efficacy of targeted therapy (35). In our study, HIBCH was suggested to be reduced in expression in ccRCC and associated with a good prognosis, and the results of *in vitro* experiments also showed

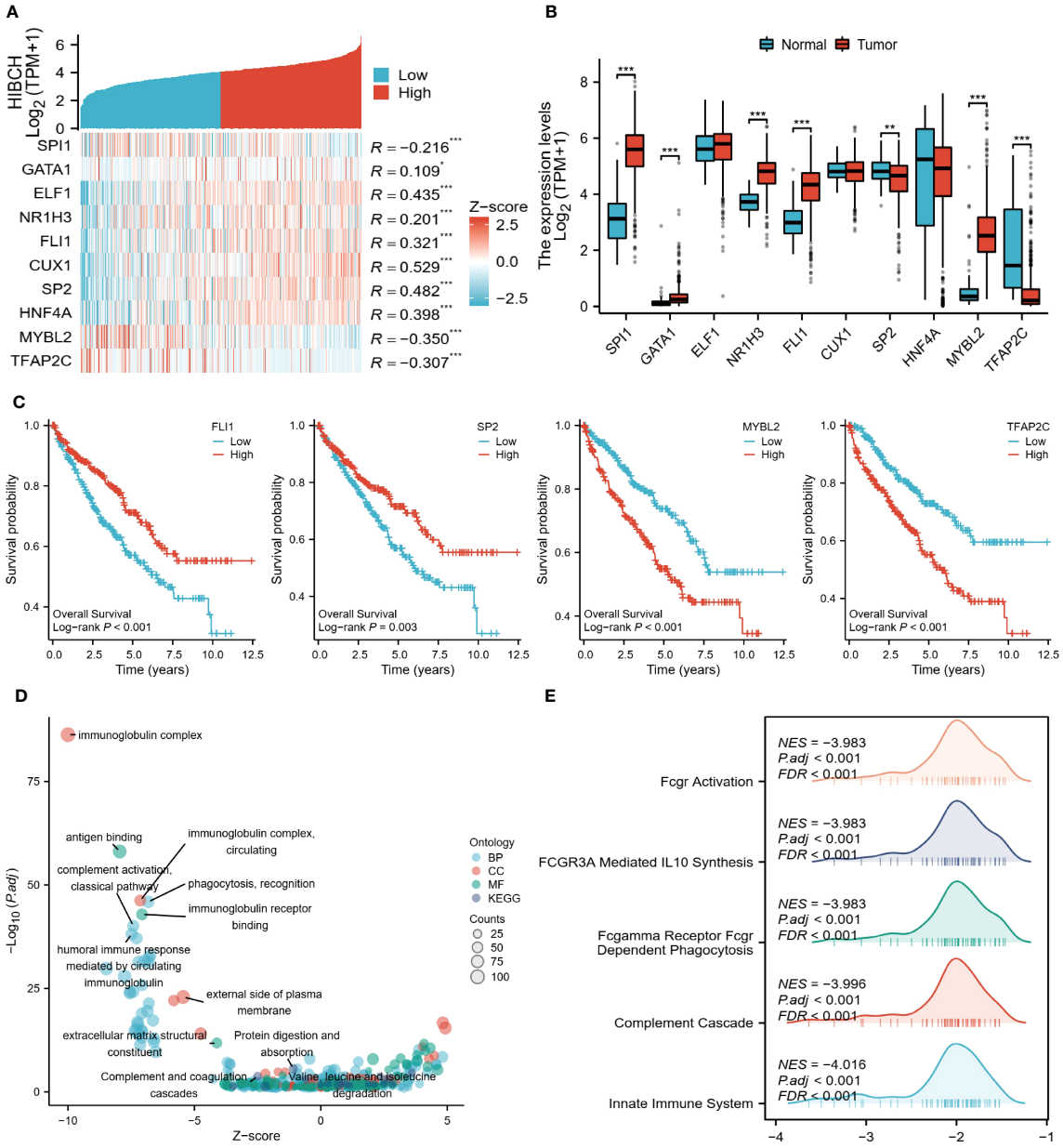

FIGURE 11
Regulatory mechanisms of HIBCH. **(A)** Heat map of HIBCH co-expression with the top 10 potential TFs. **(B)** Expression levels of potential TFs in tumor and normal tissues. **(C)** K-M survival curves of the screened TFs. **(D)** Visualized scatter plots of GO and KEGG analysis. **(E)** Visualized-mountain range plot of the top 5 of |NES| in GSEA analysis results. "*" <0.5, "**" <0.01, and "***" <0.001.

that HIBCH inhibits the migration ability of kidney cancer cells. To our knowledge, mitochondrial energy metabolism is not only closely related to the process of lactate metabolism (36) but also plays an important role in the progression of ccRCC (37). Therefore, we believe that HIBCH is important for finding new biomarkers in the field of ccRCC, however, more rigorous *in vitro* and *in vivo* experiments are still needed in the future to clarify the specific mechanism of HIBCH action in ccRCC.

In conclusion, our study provides a novel perspective on the prognostic significance and characteristics of the tumor microenvironment (TME) in clear cell renal cell carcinoma (ccRCC). We have developed a reliable nomogram and identified a potentially valuable biomarker. However, it is important to acknowledge certain limitations in our study. The first limitation of our study is that we rely mainly on retrospective data collected from public databases. It also means that we lacked much valuable clinical information to perform a comprehensive analysis. A second limitation is that we did not fully elucidate the specific mechanisms driving the key gene functions. While our study has limitations, it

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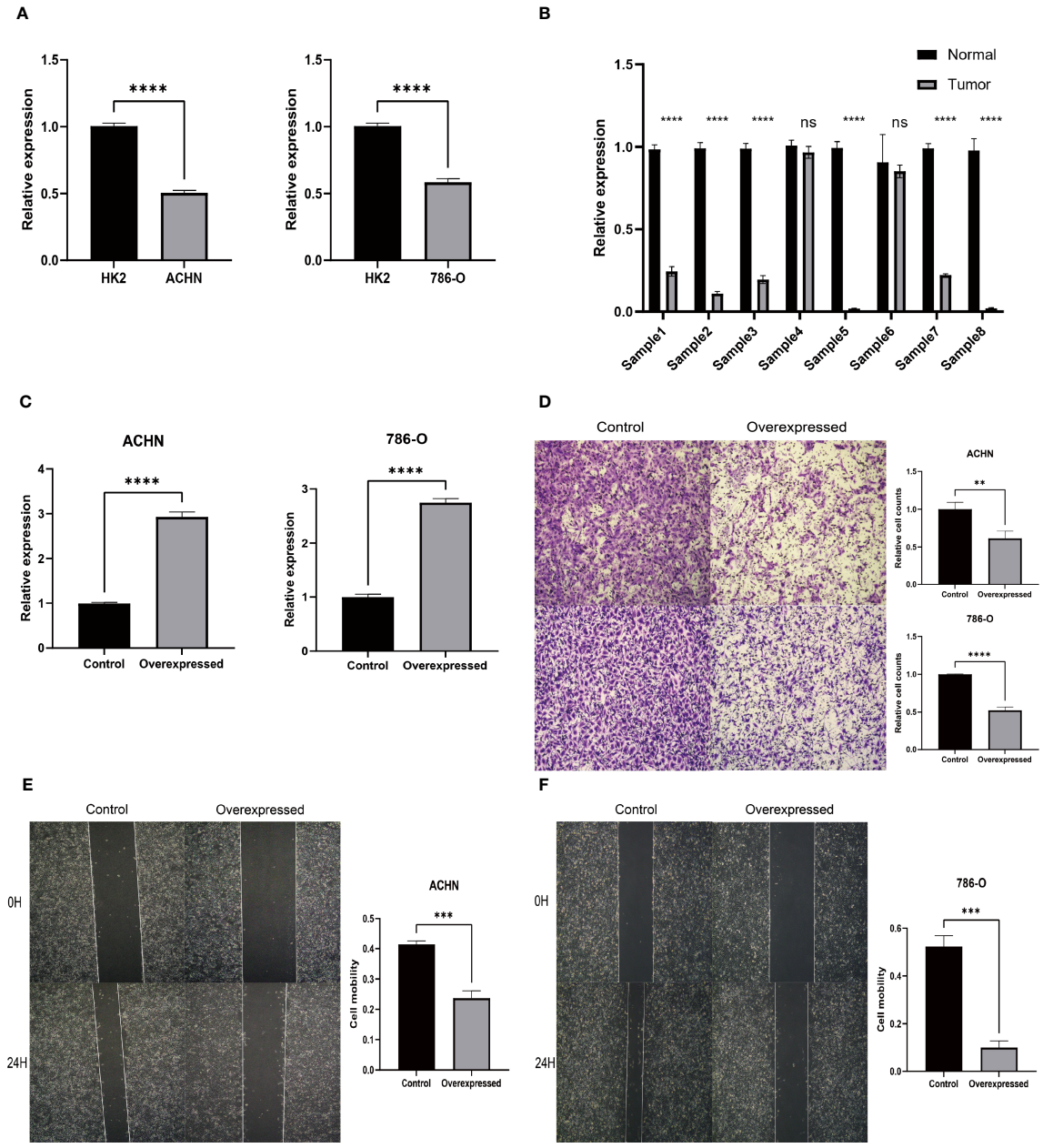

FIGURE 12
Experimental verification results. **(A)** Expression levels of HIBCH in HK-2, ACHN, and 786-O cell lines. **(B)** Differential expression of HIBCH in kidney cancer and paraneoplastic tissues. **(C)** Overexpression efficiency of HIBCH. **(D)** Transwell assay results in ACHN and 786-O cell lines. **(E, F)** Results of the scratch assay in ACHN and 786-O cell lines. "**" <0.01, "***" <0.001, and "****" <0.0001.

contributes to our understanding of ccRCC and provides a basis for future research.

Conclusion

From an epigenetic standpoint, our research has uncovered distinct traits within the TME of ccRCC. Moreover, we have successfully established robust prognostic models that accurately predict patient outcomes and offer valuable insights for the effective utilization of immunotherapy. Furthermore, our data analysis and *in vitro* experiments have pinpointed a promising therapeutic target for ccRCC treatment, namely HIBCH. These findings hold great potential for advancing the field of ccRCC research and potentially improving patient outcomes.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Medical Research Ethics Committee of the First Affiliated Hospital of Nanchang University. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent for participation was not required from the participants or the participants' legal guardians/ next of kin in accordance with the national legislation and institutional requirements.

Author contributions

LY, JX, and JL: data curation, formal analysis, writing - original draft, writing - review & editing. XW: data curation, formal analysis, writing - review & editing. SL, XL, FZ, and QD: data curation, formal analysis. BF and SX: conceptualized research, writing - review & editing. 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1225023/full#supplementary-material

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EDITED BY
Xiangpeng Dai,
Jilin University, China

REVIEWED BY
Kai Xu,
Huazhong University of Science and Technology, China
Wei Su,
The First Affiliated Hospital of Xinxiang Medical University, China
Jia Liu,
Qingdao University, China

*CORRESPONDENCE
Hu Hei
tigerblack@126.com

†These authors share first authorship

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Advances in post-translational modifications of proteins and cancer immunotherapy

Yanqing Li†, Runfang Zhang† and Hu Hei*

Department of Thyroid and Neck, Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, China

Protein post-translational modification (PTM) is a regulatory mechanism for protein activity modulation, localization, expression, and interactions with other cellular molecules. It involves the addition or removal of specific chemical groups on the amino acid residues of proteins. Its common forms include phosphorylation, ubiquitylation, methylation, and acetylation. Emerging research has highlighted lactylation, succinylation, and glycosylation. PTMs are involved in vital biological processes. The occurrence and development of diseases depends on protein abundance and is regulated by various PTMs. In addition, advancements in tumor immunotherapy have revealed that protein PTM is also involved in the proliferation, activation, and metabolic reprogramming of immune cells in tumor microenvironment. These PTMs play an important role in tumor immunotherapy. In this review, we comprehensively summarize the role of several types of PTMs in tumor immunotherapy. This review could provide new insights and future research directions for tumor immunotherapy.

KEYWORDS

post-translational modification, tumor immunotherapy, phosphorylation, ubiquitylation, succinylation

1 Introduction

Tumor immunotherapy is a novel and effective treatment that overcomes tumor immune escape by activating or reversing immune cells with failed functions, thereby inhibiting or killing tumor cells (1). According to molecular mechanisms, it includes immune checkpoint inhibitors (ICIs), acceptance and commitment therapy (ACT), and monoclonal antibody therapy (2). ICIs can block the inhibitory effect of tumor cells on immune cells. In the 1990s, immunologists James P. Alison and Tasuku Honjo discovered ICIs, which marked the new era of tumor immunotherapy (3). In 2011, ipilimumab, a cytotoxic T lymphocyte antigen-4 antibody, was first used to treat melanoma (4). ACT suppresses tumors mainly by injecting specific immune cells targeting cancer cells into patients after being expanded and cultured *in vitro* (5). Anti-CD19 chimeric antigen receptor T-cell therapy (CAR-T) for B-cell lymphoma has been approved for clinical use (6). Monoclonal antibody therapy can inhibit tumors mainly by recruiting T cells to the tumor site and directly targeting tumor cells (7). Monoclonal antibodies are widely used in the field of tumor immunotherapy. Currently, the Food and Drug Administration has approved more

than 100 monoclonal antibody products to enter the market (8). Immunotherapy can treat various solid and hematological tumors. New immunotherapy targets and corresponding immunotherapeutic drugs have been continuously discovered. Thus, treatment strategies for tumors have gradually shifted from inhibiting malignant proliferation and invasion of tumor cells to exploring the complex relationship between the tumor and the microenvironment around the tumor (9).

Post-translational modification (PTM) is a covalent modification of the side chains of amino acids in translated proteins. Under physiologic and pathologic conditions, it can expand the functional diversity of proteins by regulating protein folding, activity, stability, localization, signal transduction, and binding (10). Its main forms include ubiquitin, phosphorylation, methylation, acetylation, glycosylation, and succinylation (11). It is closely related to immune cell activation, signal regulation, immune response, and tumor metabolic reprogramming (12–14). It can affect the efficacy of immunotherapy directly or indirectly by regulating immune checkpoints or remodeling tumor immune microenvironment (15–17). In this review, we summarize potential mechanisms of several types of PTMs affecting cancer development and immunotherapy (Figure 1).

2 Phosphorylation and tumor immunity

Phosphorylation is a classical and reversible PTM in which phosphate groups are covalently modified to amino acid residues after catalysis of protein kinases. It is the most common and essential PTM in eukaryotes. Approximately 30% of the proteins in mammals can be phosphorylated (18). Protein phosphorylation plays an important role in cell division, signal transduction, gene expression regulation, and protein interaction (19). Therefore, mutation of a protein phosphate site can lead to the occurrence and progression of cancer by inducing tumor cell proliferation, invasion, and metastasis and inhibiting apoptosis (18, 20). The activation or inhibition of mitogen-activated protein kinases, such as phosphoinositide 3-kinase (PI3K) and Akt kinase, and other signaling pathways is related to the phosphorylation and dephosphorylation of related proteins or enzymes in tumors, followed by regulation of the proliferation, differentiation, apoptosis, and migration of tumor cells (18).

The progression and inhibition of breast cancer are significantly related to the phosphorylation of upstream and downstream regulatory factors of nitric oxide (NO) (21). The growth and proliferation of breast cancer cells are partly induced by NO synthase, which maintains the phosphorylation of Akt and mitogen-activated protein kinase 1/2 (extracellular signal-regulated protein kinases 1 and 2 [ERK1/2]) (22, 23). However, a high NO concentration can induce apoptosis of breast cancer cells through dephosphorylation of Akt and ERK (24). Nuclear transcription factor kappa B-interacting long noncoding RNA (lncRNA) can inhibit breast cancer metastasis by blocking inhibitor of nuclear factor kappa B (NF-?B) phosphorylation (25). Chao et al.'s study revealed that fructose-1,6-bisphosphatase 1 and 6-phosphofructose-2-kinase/fructose-2,6-bisphosphatase 3 can promote breast cancer cell genesis, glycolysis, and paclitaxel resistance through phosphorylation by proviral insertion in murine lymphomas 2 (26, 27). The PI3K–Akt–mammalian target of rapamycin kinase pathway is abnormally activated in non-small cell lung cancer, and overexpression of phosphorylated Akt leads to tumor cell proliferation (28). Phosphorylation of S308 and S30 of cyclase-associated protein 1 can stimulate the proliferation, migration, and metastasis of lung cancer cells (29).

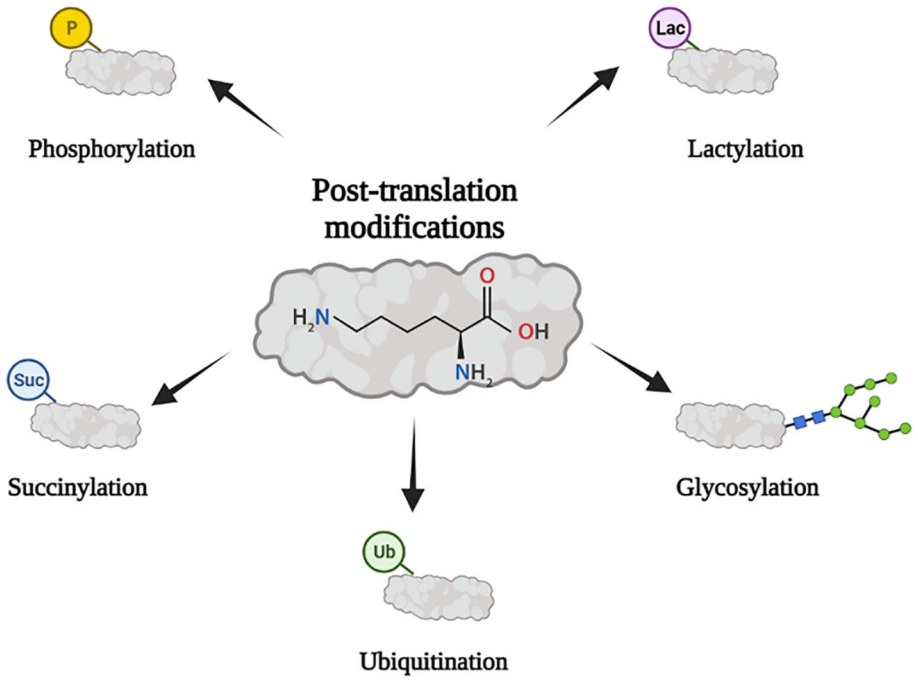

FIGURE 1
PTM is closely related to tumor immunity. Its main forms include ubiquitination, phosphorylation, glycosylation, succinylation, and lactylation.

The search for immune checkpoints and ICIs is a research direction in the field of tumor immunotherapy (30). Considering the significant effect of phosphorylation on tumor characteristics, inhibitors targeting phosphokinases or phosphorylated molecules can be used as targets for tumor therapy (31). Phosphorylated transforming growth factor beta (TGF-β)-induced factor homeobox 2 (TGIF2) can induce epithelial–mesenchymal transition (EMT) and metastasis of lung adenocarcinoma, and p-TGIF2 is a potential therapeutic target for lung adenocarcinoma metastasis (32). The expression level of the CD274 molecule programmed cell death ligand-1 (PD-L1) in tumors is regulated in many aspects of translation and post-translation. Guo proved that hexokinase 2 can be used as a protein kinase to phosphorylate the Thr291 site of I?Bα, thereby promoting combined protease u-calpain and I?Bα and degrading I?Bα, in turn promoting the entry of the NF-?B transcription subunit into the nucleus and the PD-L1 expression, ultimately leading to the immune escape of the tumor (33). Combined hexokinase 2 inhibitor and PD-1 antibody in glioma can significantly improve the therapeutic effect of the PD-1 antibody. Li showed that epidermal growth factor receptor (EGFR) overexpression in tumors inhibited the phosphorylation of PD-L1 through glycogen synthase kinase-3β/α (GSK3β/α), which hindered the ubiquitination and improved the stability of PD-L1 (34). In contrast, the EGFR inhibitor osimertinib can interfere with the aforementioned process, induce ubiquitin, degrade PD-L1, and enhance the antitumor immune function of T cells (35, 36). Chen et al. found that interleukin (IL)-6 can phosphorylate PD-L1 by activating Janus kinase 1 (JAK1), in turn catalyzing PD-L1 glycosylation, enhancing its stability, and promoting tumor immune escape (37). In an animal model, the anti-IL-6 antibody combined with anti-T-cell immunoglobulin 3 induced the synergistic T-cell killing effect (37). Drugs targeting phosphokinases can be used as the focus of tumor immunotherapy. Thus, the potential application value of PTM in tumor immunotherapy has been sufficiently proven (Figure 2).

Some immune drugs related to phosphorylation have been developed and applied. MYCi975, a small-molecule compound, can increase the degradation of MYC by enhancing the phosphorylation of MYC on threonine-58, mediated by proteasome. In addition, it can upregulate PD-L1 and make tumors sensitive to PD1 immunotherapy (38). Simvastatin, a potential therapeutic drug for immunotherapy in colorectal cancer, inhibits the phosphorylation of YAP, mediated by the lncRNA SNHG29, and promotes antitumor immunity by inhibiting the PD-L1 expression (39). Ursodeoxycholic acid, a clinically approved compound, can enhance antitumor immunity by phosphorylating TGF-β at T282 along the Takeda G-protein-coupled receptor 5–cyclic adenosine monophosphate–protein kinase A axis and inhibiting the differentiation and activation of T_{reg} cells in mice (40). Elesclomol was identified in the differential cytotoxicity screening of the internal tool compound library. It promoted YAP phosphorylation and inhibited its nuclear accumulation through the reactive oxygen species/large tumor suppressor kinase 1 kinase signaling pathway (41). In addition, the PD-L1 expression and signal transducer and activator of transcription 3 phosphorylation increased after the nintedanib therapy for lung cancer. Nintedanib combined with αPD-L1 can enhance the therapeutic response of ICIs, further activating the tumor immune microenvironment and showing remarkable antitumor effects (42) (Table 1).

Targeted phosphorylation performs therapeutic and predictive functions in tumor immunity. The immunohistochemistry of ERK1/2 phosphorylation can predict the overall survival rate of patients with independent recurrent glioblastoma after blocking PD-1 (43). Therefore, the development of related kits would help adjust the treatment plan of patients with PD-1 blockade.

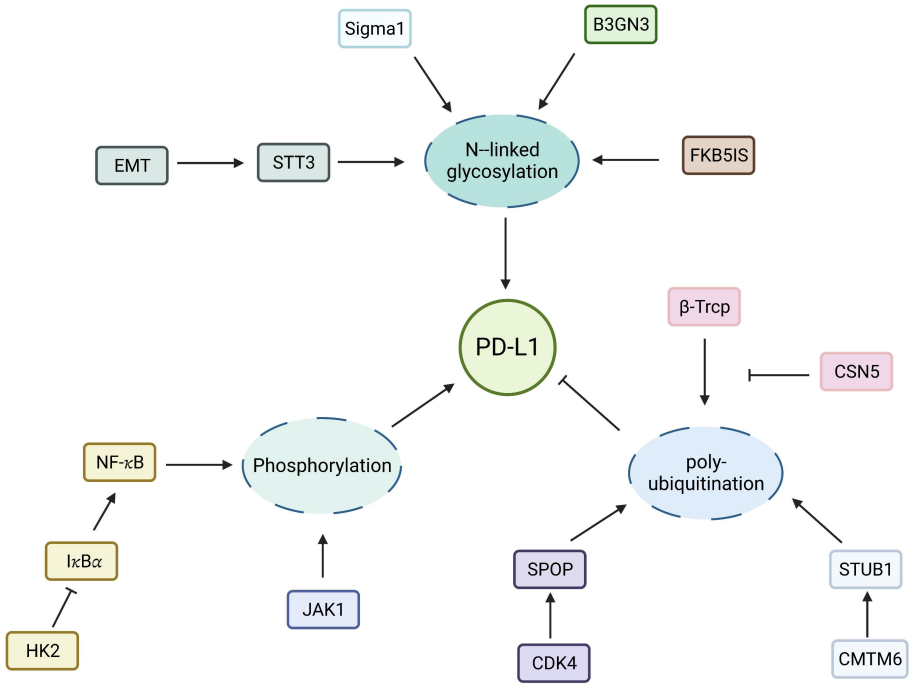

FIGURE 2
Regulation of PD-L1 by PTMs. Molecular regulation of PD-L1 N- linked glycosylation, phosphorylation and ubiquitination.

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TABLE 1 PTM-related immunotherapy drugs.

PTM-related immunotherapy drugs		
Phosphorylation	drug	Molecular target
	osimertinib	EGFR
	MYCi975	MYC
	Simvastatin	YAP
	nintedanib	STAT3
	Metformin	PD-L1
	2DG	HK2
Ubiquitination	M435-1279	UBE2T
	ES-072	EGFR
	HUWE1	TMUB1
	Albendazole	UBQLN4
	gefitinib	EGF
	61	OTUB1/USP8
	BC-1471	STAMBP
	HOIPIN-8	LUBAC
glycosylation	NGI-1	B7-H4
	gPD-L1	PD-L1
	Stattic	PD-L2
	all-trans retinoic	MGAT3
	polyphenol resveratrol	PD-L1
	tunicamycin	PTX3
	rituximab	Fc
	SAR566658	huDS6
	gefitinib	PD-L1
	swainsonine	MAN2A1

3 Ubiquitylation and tumor immunity

In ubiquitination, ubiquitin molecules are covalently attached to specific residues of substrate proteins. Ubiquitination, a dynamic and reversible process, plays important roles in protein localization, metabolism, function, regulation, and degradation. Signal transduction proteins are regulated by PTM, and their ubiquitin level is second only to phosphorylation (44). Some ubiquitin-modified proteins change their function and location (45), while most are degraded by the ubiquitin–proteasome system (UPS) or lysosome degradation pathways, thus regulating various life activities, such as cell cycle, proliferation, apoptosis, differentiation, gene expression, transcriptional regulation, signal transduction, damage repair, inflammation, and immunity (46, 47). Currently, three ubiquitin enzymes are involved in ubiquitination modification: ubiquitin-activating enzyme (E1), ubiquitin-binding enzyme (E2), and ubiquitin ligase (E3). Ubiquitination is terminated by deubiquitinating proteins (DUBs). Among them, the E3 ubiquitin ligase can recognize the type of substrate protein; therefore, the specificity of the ubiquitin ligase is mainly realized by the ubiquitin ligase. The E3 ubiquitin ligase is believed to play an important role in tumor immunity (48, 49).

Casitas B-lineage lymphoma proto-oncogene b (CBL-b), as a E3 ubiquitin ligase, is an immune tolerance factor directly related to T-cell activation (50). Naramura's study revealed that c-cbl-knockout T cells were more responsive to CD3 stimulation and promoted T-cell receptor beta variable 20/OR9-2 clearance on the cell surface, thereby inhibiting T-cell activation (51). Mutations in the ubiquitin-mediated protein degradation system can be involved in at least 10% of tumorigenesis and development (47, 52). The ubiquitin protein ligase E3 component n-recognin 5 (UBR5), an E3 ubiquitin ligase, is essential for the embryonic development of mammals (53). Elevated UBR5 expression is closely related to the survival and poor prognosis of patients with ovarian cancer (54). The E3 ubiquitin ligase regulates the activity and function of immune cells and plays an important role in regulating tumor cells and microenvironment (55).

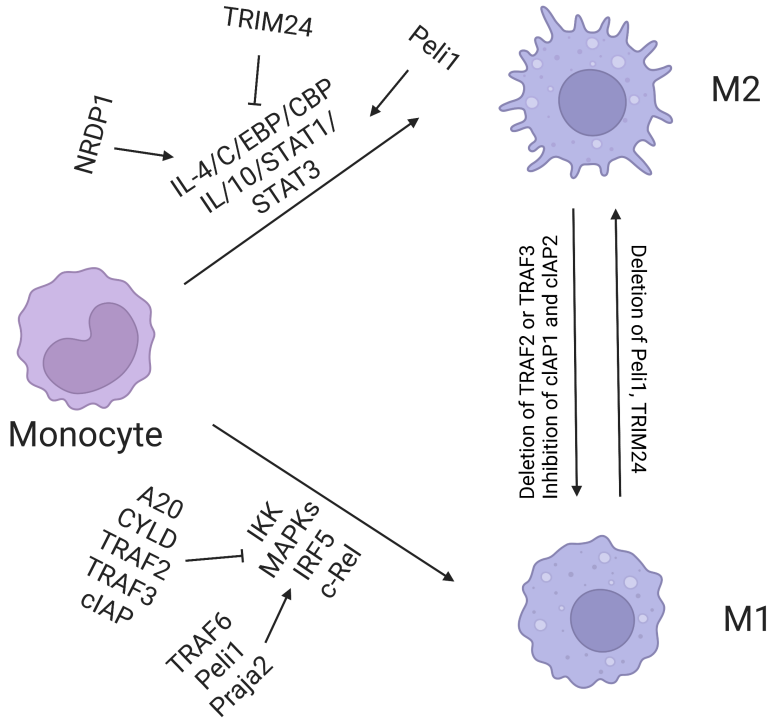

FIGURE 3
Regulation of macrophage polarization by ubiquitination. E3 ligases TRAF6, Peli1, Praja2, TRAF2, TRAF3, and cIAP promote M1 polarization, among which A20 and CYLD inhibit this process. Nrdp1 promotes the expression of M2 gene induced by IL-4 by mediating ubiquitination of K63 and activation of transcription factor C/EBP. TRIM24 inhibits M2 polarization by ubiquitination of acetyltransferase CBP.

UPS is the intracellular system responsible for protein degradation. Abnormal activation of the system accelerates the degradation of intracellular proteins. UPS can affect the survival of tumor cells by promoting the degradation of tumor-suppressor proteins, such as the tumor protein p53, or blocking the degradation of carcinogenic proteins. Song's study proved that tumor-derived UBR5 plays a dual role in promoting tumorigenesis and affecting immune microenvironment. UBR5 can regulate tumor spheroid formation of ovarian cancer through the p53–β–catenin pathway and then enhance immunosuppression by recruiting tumor-associated macrophages (TAMs) (54). Considering the aforementioned mechanism, targeted UBR5 significantly inhibits tumor growth, eliminates the ability of ovarian cancer to resist conventional chemotherapy and immunotherapy, and significantly improves the effect of the standard treatment of ovarian cancer. In lung cancer, ubiquitin ligase interleukin 17 receptor B (CRL4) and WD repeat domain 4 promotes the progression of lung cancer through ubiquitin degradation of the promyelocytic leukemia protein. Therefore, targeted regulation of the E3 ubiquitin ligase or its substrate protein can provide new opportunities for tumor immunotherapy (56). Yu et al. showed that the ubiquitin-binding enzyme E2T (UBE2T) can promote the entry of β-catenin into the nucleus through ubiquitin degradation of the receptor for activated C kinase 1, thus promoting the occurrence and development of gastric cancer (57). The team further targeted the upstream ubiquitin-binding enzyme UBE2T to develop a small molecular inhibitor M435-1279 with low cytotoxicity that can inhibit the progression of gastric cancer *in vivo* and *in vitro* (Figure 3).

Owing to its role in tumor immunity, ubiquitination would be helpful in clinical immunotherapy to find new E3s and DUBs for antitumor immunomodulation and clarify their functional mechanisms. Finding and developing specific inhibitors targeting the E3 ligase and DUBs are important for clinical applications of ubiquitination. In addition to developing inhibitors, adoptive cell therapy has a clinical application potential, such as knocking-out specific E3 and DUB to improve the therapeutic effect. This method is particularly attractive for adoptive T-cell and natural killer cell therapy based on the chimeric antigen receptor.

4 Succinylation and tumor immunity

Succinylation modification is a type of PTM that mainly occurs in lysine residues. Compared with methylation and acetylation modification, succinylation modification has a greater effect on the structure and function of proteins. Enzymes in cell metabolism, particularly mitochondrial metabolism, are widely regulated by succinylation modification. Currently, the regulatory enzyme system of succinylation (including transferase and de-modifier enzyme) and biological function have become hot research topics.

Lu et al. first discovered histone-succinylated transferase-lysine acetyltransferase 2A, which can use succinyl coenzyme A as a substrate to catalyze the succinylation of the histone H3K79 site, thus promoting the transcription of oncogenes, tumorigenesis, and cancer development (58). In addition, glutaminase was overexpressed in pancreatic ductal carcinoma. Compared with normal cells, the growth and survival of

pancreatic ductal cancer cells depended more on glutamine metabolism. In addition, glutaminase was overexpressed in pancreatic ductal carcinoma. Compared with normal cells, the growth and survival of pancreatic ductal cancer cells depended more on glutamine metabolism. Succinylation modification occurred on the glutaminase protein. Succinylation at the K311 site of the glutaminase protein was directly mediated by succinyl coenzyme A, which promoted the conversion of glutaminase from the monomer to the tetramer form. As a result, catabolism of glutamine was enhanced (59).

Tumor immunotherapy is closely related to protein succinylation modification. Tumor immune metabolism in immune cell proliferation, differentiation, response, and outcome is a research frontier worldwide. Advancements in this research would aid our understanding of immune cell biology in theory and exhibit an application prospect in maintaining immune homeostasis and tumor immunotherapy. When activated by lipopolysaccharide, macrophages consume abundant glucose, enhance glycolysis, express M1 molecular markers, and produce abundant inflammatory factor IL-1β. The mechanism involves the accumulation of succinate, the intermediate product of glucose metabolism. Succinate promotes hypoxia inducible factor-1 (HIF-1α) to enhance the transcription of IL-1β, in which the activation of pyruvate kinase M2 (PKM2), a key enzyme in glycolysis, plays an important role. Yang et al. further discovered that PKM2 is desuccinylated by sirtuin-5 (60). In SIRT5-deficient cells, the succinylation level of PKM2 increased; PKM2 transformed from the tetramer to the dimer form; and pyruvate kinase activity decreased. Dimerized PKM2 enters the nucleus and cooperates with HIF-1α to bind to the promoter region of IL-1β, which significantly enhances the transcription of IL-1β and glycolysis of macrophages. These results suggest that SIRT5 regulates macrophage metabolism and plays an important role in the malignant transformation of colitis and even colitis cancer. Metabolic changes in tumor microenvironment significantly regulate tumor immune sensitivity, but the underlying mechanism remains unclear. Cheng et al. found that tumors deficient in fumarate hydratase (FH) showed functional $CD8^+$ T-cell activation, expansion, and inhibition and enhanced malignant proliferation (61). Regarding the mechanism, FH depletion in tumor cells can accumulate fumarate in tumor interstitial fluid. Elevated fumarate levels can directly succinate ZAP70 at C96 and C102 sites and eliminate the activity of infiltrating $CD8^+$ T cells, thus inhibiting the activation of $CD8^+$ T cells and antitumor immune response *in vitro* and *in vivo*. In addition, removing fumarate by increasing the FH expression can significantly enhance the antitumor effect of anti-CD19 CAR T cells. Thus, these findings prove the role of fumarate in controlling TCR signal transduction and suggest that the accumulation of fumarate in tumor microenvironment is a metabolic disorder of the antitumor function of $CD8^+$ T cells. Potentially, fumarate depletion may be an important strategy for tumor immunotherapy.

Immune-targeted drugs for succinylation have not been confirmed in the clinical treatment of cancer. They remain in the research stage *in vivo* and *in vitro*. Nevertheless, in a previous study, 90Y-labeled succinylated streptavidin significantly inhibited the growth of breast cancer in the pre-targeted radiotherapy group ($p < 0.05$) (62). In ovarian cancer cells, inhibition of dihydrothionyl succinyltransferase, a subunit of α-KGDC in the tricarboxylic acid cycle, reduced oxidative phosphorylation and the expression and function of immunosuppressant markers in myeloid cells (63). Lactb is a positive regulator of the NF-κB signal in dendritic cells, and succinylation of the lysine 288 residue is inhibited by Suclg2. Therefore, the development of succinylated immune-targeting drugs may be a research direction for immunotherapy.

5 Lactylation and tumor immunity

Lactic acid is a metabolite of cellular glycolysis. However, it has been considered a simple cellular energy substance and metabolite. Its regulatory role in biological function has been unknown. Lactic acid-mediated protein PTM lactylation plays a regulatory role in immune cells and cancer metabolism.

Regarding the tumor metabolism, Zhao et al. found that lactic acid accumulated during metabolism can be used as a precursor to induce lactylation of histone lysine and participate in the homeostasis regulation of M1 macrophages infected by bacteria (64). In addition to a study on the epigenetic regulation of histone lactic acid modification, Gao et al. drew a panoramic map of lactic acid modification in hepatocellular carcinoma for the first time. Lactylation occurs in histones and plays a global regulatory role in hepatocellular carcinoma by affecting widely distributed non-histone proteins. E1A-binding protein p300. Histone deacetylase causes the activation and deactivation of non-histone lactylation (65). Lu et al. found that the metabolite lactic acid affects the tumor microenvironment and promotes tumorigenesis by regulating the lactylation of M protein Lys72 in T_{reg} cells and enhancing TGF-β signal transduction, which provides a new theoretical basis for cancer immunotherapy by targeting T_{reg} cells (66). Zhao et al. found that activated macrophages play an important role in ulcerative colitis. Lactic acid can enhance histone H3K18 lactylation in macrophages, inhibit macrophage coking, and restore intestinal immune function (67). In addition, Zhang et al. found that lactic acid-mediated lactylation of PKM2 at the K62 site can enhance the pyruvate kinase activity of PKM2 to inhibit the Warburg effect and ultimately promote the transition of macrophages from the proinflammatory to the repair phenotype (68). The underexpression of sirtuin 3 in hepatocellular carcinoma promotes the lactylation of cyclin E2, which in turn promotes tumor progression, and sirtuin 3 is a potential therapeutic target for hepatocellular carcinoma (69).

The lactate score model can be used to predict tumor immune escape (70). A lactic acid-related model study on gastric cancer revealed numerous infiltrated immune cells (macrophages to the highest degree), characterized by an increased lactic acid score. ICIs showed a decreased response rate in gastric cancer with a high lactate score. Tumors with a high lactate fraction have high tumor immune dysfunction, implying higher risks of immune escape and dysfunction. These findings indicate that the lactate score can be used to predict malignant progression and immune evasion of gastric cancer. However, the application of lactic acid drugs in clinical tumor immunity remains under development.

6 Glycosylation and tumor immunity

Glycosylation is an important protein PTM in which O-linked N-acetylglucosamine (O-GlcNAcylation) refers to addition of monosaccharide modification to serine and/or threonine residues of

protein in cells, which is the most common glycosylation form in eukaryotes (71). This modification is a highly dynamic modification method, which would change with the nutritional status in cells and extracellular stimuli. It widely occurs in intracellular proteins and regulates important biological processes, such as gene transcription, signal transduction, protein synthesis, and metabolic reprogramming.

As early as 1991, Crowley et al. discovered the effect of non-enzymatic glycosylation on the function of mesangial cells (72). Khidekel discovered a new strategy to monitor the glycosylation kinetics of O-GlcNAc using protein omics based on quantitative mass spectrometry (73). Fogel et al. found that site-specific N-glycosylation affects the structure and function of binding synaptic cell adhesion molecule interaction (74). In addition, some studies have summarized the current knowledge of immunoglobulin glycosylation and paid special attention to the research and vaccination for infectious diseases, considered to be a field with many interesting opportunities (75). Hu et al. analyzed 83 high-grade serous ovarian cancer and 23 non-tumor tissues prospectively with comprehensive protein omics and glycochemistry. Tyagi et al. comprehensively summarized the discovery of RNA glycosylation, conceptually understood its previous potential discovery and its biological consequences, and explained the dynamic impact of this modification on its molecular versatility, determining the immunological fate of cancer and the potential impact of glycosylation on cell interaction, signal transduction, immunomodulation, cancer escape, and proliferation (76). Shi et al. found that glucose metabolism in TAM was modified by enhancing O-GlcNAcylation, promoting tumor metastasis and chemotherapy resistance. They revealed that M2-like TAM is the immune cell subgroup with the strongest glucose uptake ability in tumor microenvironment and discovered the new function of O-linked N-acetylglucosamine transferase located in lysosomes (77). They clarified the significance of competitive uptake and utilization of glucose by TAM, particularly M2-like TAM, in shaping cell-specific tumor-promoting function, which provided a potential target for tumor treatment.

The star molecule of glycosylation in tumor immunotherapy is PD-L1. Glycosylation can stabilize PD-L1, which prevents PD-L1 from degradation of 26S proteasome mediated by GSK3β, thus enhancing its interaction with PD-1 on $CD8^+$ T cells. In addition, the catalytic subunit of oligo-glycosyltransferase STT3 transfers the core glycan structure to PD-L1, which leads to EMT. Combined PD-L1 and PD-1 is also influenced by the glycosylation of PD-L1. In a study on EGF/EGFR signal transduction, PD-L1/PD-1 interaction requires β 1,3-N- acetylglucosamine transferase 3 (B3GNT3) to mediate the glycosylation of poly-N-acetyllactosamine on PD-L1 N192 and N200. The 4T1 cells lacking B3GNT3 expression grew in severe combined immunodeficiency mice, but not in immunocompetent BALB/c mice (78). Therefore, glycosylation targeting PD-L1 is a breakthrough in tumor immunotherapy.

7 Conclusions

PTMs are chemical changes that occur after protein synthesis that play a vital role in regulating protein function, stability, localization, and interactions. In addition to the types of PTMs summarized above, other PTMs play a vital role in tumor immunity. Currently, immune-related PTM drugs mostly include phosphorylation inhibitors. In addition, some PTM models can predict tumor immune evasion, such as the lactic acid score model. Immune-targeted drugs for succinylation have not been confirmed in the clinical treatment of cancer and remain in the research stage *in vivo* and *in vitro*. In addition to developing inhibitors, adoptive cell therapy carries clinical application potential, such as knocking-out specific PTM-related proteins to improve the therapeutic effect. This method is particularly attractive for adoptive T-cell and natural killer cell therapy based on the chimeric antigen receptor. This may be the direction of PTM immunotherapy in the future. In general, protein PTM is a regulator of tumor immunity. Its disorders affect various immune processes, including T-cell activation, immune checkpoint regulation, cytokine production, and immune cell interaction in the tumor microenvironment. Understanding its role in tumor immunity may provide insights for the development of new immunotherapies and targeted therapies for cancer.

Author contributions

Conceptualization: YL and HH. Formal analysis: YL. Investigation: HH. Resources: YL. Data curation: HH. Writing—original draft preparation: YL. Writing—review and editing: HH. Visualization: HH. Supervision: RZ. Project administration: RZ. Funding acquisition: YL. All authors have read and agreed to the published version of the manuscript.

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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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