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# Epigenetic, metabolic, and transcriptional regulation of immune cell plasticity and functions in cancer and non-cancer diseases

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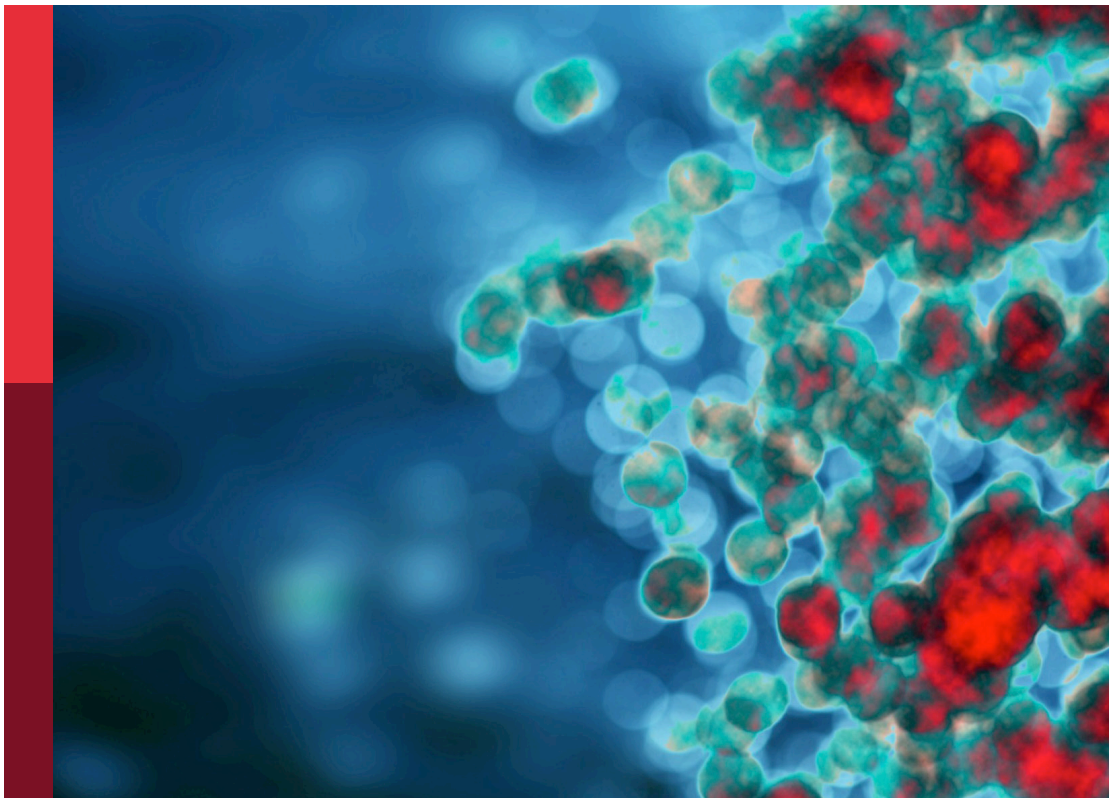

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# Epigenetic, metabolic, and transcriptional regulation of immune cell plasticity and functions in cancer and non-cancer diseases

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# Editorial: Epigenetic, metabolic, and transcriptional regulation of immune cell plasticity and functions in cancer and non-cancer diseases

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Editorial on the Research Topic

Epigenetic, metabolic, and transcriptional regulation of immune cell plasticity and functions in cancer and non-cancer diseases

High plasticity represents an essential characteristic of innate and adaptive immune cells, which allows their multi-directional transition into diverse phenotypes with different (even opposite) functions (1, 2). The significance of this property is further highlighted by an increasing number of recently emerging new phenotypes of immune cells, particularly involving many malignant and non-malignant disorders (3). Mechanistically, the phenotypic transition occurs via reprogramming of gene expression at the transcriptional level primarily driven by complex and interactive mechanisms involving microenvironment, intracellular signaling, transcription factors, epigenetic remodeling, metabolic rewiring, and post-translational modification (PTM) (1, 4–9).

Transcriptional reprogramming for the phenotypic transition of immune cells is primarily governed via various epigenetic mechanisms, including DNA methylation, histone PTMs, non-coding RNA, RNA modification, etc. (1, 10, 11). DNA methylation usually functions to silence the expression of tumor suppressors in most types of cancer (12). In contrast, many oncogenes are often hypomethylated to promote their expression in tumor cells. Interestingly, multiple differential methylation sites (DMSs) have been found in either the gene itself or the promoter of telomerase reverse transcriptase (TERT), a critical enzyme that controls the length of telomere and is thus considered an oncogene (Lin et al.). Moreover, hypermethylation of these DMSs significantly correlates with TERT expression, infiltration of immune cells [e.g., T cells, T helper 2 (Th2) cells, Treg, $CD56^{dim}$ natural killer (NK) cells, activated dendritic cells (DCs), and B cells], and immune checkpoints (e.g., LAG-3) in triple-negative breast cancer (TNBC) (Lin et al.). Of note, hypermethylation of at least some DMSs is associated with poor overall survival of patients with TNBC (Lin et al.). These findings suggest that TERT promoter hypermethylation may

play a role in tumor microenvironment (TME), although the underlying mechanism remains to be explored.

5-methylcytosine (m5C) represents one of the important forms of RNA modification, which occurs on virtually all types of RNA (e.g., mRNA, tRNA, rRNA, lncRNA, and other RNAs) and plays diverse roles in RNA transcription, transportation, and translation (13). Thus, there is no doubt that m5C and its regulatory elements (including "writer", "eraser", and "reader") account for malignant behaviors of both tumor cells and TME involving numerous immune cells (e.g., T cells, B/plasma cells, macrophages, granulocytes, NK cells, DCs, and mast cells) in numerous types of cancer, including solid tumor (e.g., liver, stomach, bladder, prostate, head and neck, breast, pancreas, kidney, and colon and rectum cancer) and hematologic malignancies (e.g., leukemia) (Gu et al.).

Recently, many novel forms of histone and non-histone protein PTMs have been discovered in physiological and/or pathological scenarios (14–17). Among them, lactylation occurs primarily at lysine residues of histones and in some circumstances, also non-histone proteins. Since lactylation is induced by lactate (the end product of glycolysis) (18), it thus represents a key linker between metabolic rewiring (paradigm shift from oxidative phosphorylation to glycolysis or vice versa) and epigenetic remodeling (19, 20). This new PTM may be particularly important in cancer, considering aerobic glycolysis as a metabolic hallmark of cancer known as the Warburg effect (21, 22). Indeed, fast-emerging evidence supports the functional role of lactylation in both tumor cells and TME involving tumor-infiltrating myeloid cells, including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and tumor-associated neutrophils (TANs), especially the communication between them (Su et al.). Thus, more reliable targets will become available for anti-tumor epigenetic therapy and immunotherapy soon.

As another protein PTM, ubiquitination is essential for protein turnover via the ubiquitin-proteasome system (UPS) or autophagy, among many other functions, in both normal and malignant cells (23). Consequently, agents targeting UPS (e.g., proteasome inhibitors such as bortezomib) have been extremely successful in the treatment of plasma cell neoplasms like multiple myeloma that relies on a highly-efficient protein turnover machinery to remove abundant useless but harmful immunoglobulin in tumor cells (24). Notably, many E3 ubiquitin ligases (e.g., WWP1/2, SMURF1/2, ITCH, FBXW7, FBXO3/6/21, HECTD1, and ULF1) deubiquitinases (e.g., USP3/5/7/13/14/15/49) that reciprocally regulate ubiquitination are associated with osteoarthritis (Zheng C. et al.), suggesting that this non-inflammatory degenerative joint arthritis may be another disorder caused by deficient protein turnover. In addition to a number of (de)ubiquitination-targeted agents undergoing development (Zheng C. et al.), it is interesting to see whether the FDA-approved proteasome inhibitors would be effective against this type of (de)ubiquitination-deficient disorders like osteoarthritis.

Among various immunotherapies, immune checkpoint inhibition represents a major therapeutic strategy. The most representative one is anti-PD1/PD-L1 monoclonal antibodies (mAbs), which have been approved for the treatment of many types of solid tumors, including lung cancer (25). However, although anti-PD1/PD-L1 mAbs display a striking efficacy, a considerable number of patients with lung cancer have not been benefited (26). To this end, numerous biomarkers have been discovered to pre-select patients who are most likely to benefit from this immunotherapy, including tumor-related markers (e.g., PD-L1 expression, tumor mutation burden, dMMR/MSI, and many tumor-specific genes), peripheral blood-based markers (e.g., ctDNA, immune cells/T cell receptor, and exosomal PD-L1/cytokines) and gut microbiome (Wang et al.). Exhaustion of T cells represents a major hurdle for immunotherapy (27), which may at least in part explain the unsatisfied efficacy of anti-PD1 mAb in most hematologic malignancies like acute myeloid leukemia (AML). In AML patients, accumulation of a subset of severely exhausted T cells ($CD28^{-}$/$PD\text{-}1^{+}$/$TIGHT^{+}$) correlates with the presence of minimal residue disease, poor therapeutic response, and short disease-free survival (Huang et al.). Thus, a strategy combining agents targeting such exhausted T cells may improve the efficacy of PD-1 blockade in AML and probably other hematologic malignancies as well. Another approach to enhance the efficacy of immunotherapy is to combine it with radiotherapy, based on a potential mechanism of action involving oxidative stress (e.g., ROS) induced by radiation (Zheng Z. et al.). ROS promotes the release of tumor-associated antigens, which promote infiltration and differentiation of immune cells, modulate the expression of immune checkpoints, and remodel TME (Zheng Z. et al.). Interestingly, expression of certain cell cycle-related genes such as CENPE also correlates with the infiltration of immune cells (e.g., DCs, B cells, T cells, $CD4^{+}$ or $CD8^{+}$ memory T cells, macrophages, and mast cells) at least in some types of cancer (e.g., medulloblastoma) (Fang et al.). However, its functional role in TME, other than tumor cells themselves, and immunotherapy remains to be investigated.

In addition, many other immune checkpoints could also serve as potential targets to fill in the gap left behind. One example is CD47/SIRPα (known as a "don't eat me" signal for phagocytosis by macrophages) (28). While this checkpoint has been well investigated, its targeted therapy has however not been as successful as anti-PD1/PD-L1 mAbs thus far, largely due to severe adverse events (e.g., anemia, because red blood cells highly express CD47) (29). In this circumstance, many alternative approaches, rather than mAbs, have been attempted to avoid this dark side (30). Targeting glutaminyl-peptide cyclotransferase-like protein (QPCTL) that catalyzes CD47 pyroglutamylation crucial for the binding between CD47 and SIRPα may be a promising approach for the treatment of glioma, in which QPCTL is highly expressed due to DNA hypomethylation and associated with poor outcomes (Liu et al.).

Last, unlike the rapid advance in immunotherapy for cancer, its application in non-cancer diseases lags way behind (31). Among many obstacles, the most important one may be the lack of defined target immune cells in autoimmune or inflammatory diseases, primarily due to the complexity of immune cells with diverse, plastic phenotypes. To this end, recently developed single-cell-analyzing techniques have been doing a really good job of identifying immune cells with different functions (3). For example, single-cell RNA sequencing (scRNAseq) and high-

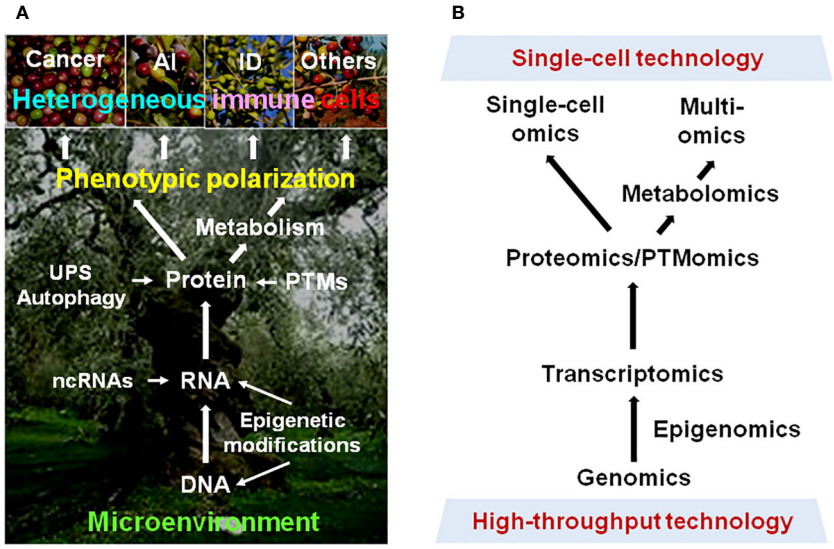


FIGURE 1
Phenotypic plasticity of immune cells in cancer and non-cancer diseases. **(A)** Multiple dimensions involving the mechanisms underlying the polarization of immune cells into heterogeneous phenotypes with diverse (even opposite) functions in different pathological scenarios. **(B)** Modern approaches for resolving the highly complex mechanisms for plasticity of immune cells in a disease-specific manner, thereby leading to immune microenvironment-targeted therapy for cancer and non-cancer diseases. AI, autoimmune disease; ID, inflammatory disease; UPS, ubiquitin-proteasome system; ncRNA, non-coding RNA; PTM, post-translational modification.

dimensional mass cytometry (CyTOF) have defined a hyper-inflammatory signature in peripheral blood mononuclear cells of patients with Prader-Willi syndrome, with a marked increase in $CD16^{+}$ monocytes that likely drive hyper-inflammatory status of this disease and therefore represent a potential target for immunotherapy (Xu et al.).

Together, further basic, translational, and clinical research with the development and utilization of modern technology would provide much deeper insights into the mechanisms underlying immune cell plasticity (Figure 1), leading to the discovery of useful biomarkers or targets for precision medicine against cancer and non-cancer diseases.

## Author contributions

YD: Conceptualization, Funding acquisition, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. DR: Conceptualization, Writing – review & editing. YH: Conceptualization, Writing – review & editing. HY: Conceptualization, Resources, 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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## Publisher's note

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# Loss of CD28 expression associates with severe T-cell exhaustion in acute myeloid leukemia

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**Introduction:** Despite accumulated evidence in T-cell exhaustion in acute myeloid leukemia (AML), the immunotherapeutic targeting exhausted T cells such as programmed cell death protein 1 (PD-1) blockade in AML failed to achieve satisfying efficacy. Characteristics of exhausted T cells in AML remained to be explored.

**Methods:** Phenotypic analysis of T cells in bone marrow (BM) using flow cytometry combining senescent and exhausted markers was performed in de novo AML patients and healthy donors as well as AML patients with complete remission (CR). Functional analysis of T-cell subsets was also performed in de novo AML patients using flow cytometry.

**Results:** T cells experienced a phenotypic shift to terminal differentiation characterized by increased loss of CD28 expression and decrease of naïve T cells. Additionally, lack of CD28 expression could help define a severely exhausted subset from generally exhausted T cells (PD-1$^+$TIGIT$^+$). Moreover, CD28- subsets rather than CD28+ subsets predominantly contributed to the significant accumulation of PD-1$^+$TIGIT$^+$ T cells in AML patients. Further comparison of de novo and CR AML patients showed that T-cell exhaustion status was improved after disease remission, especially in CD28+ subsets. Notably, higher frequency of CD28-TIGIT-CD4$^+$ T cells correlated with the presence of minimal residual disease in AML-CR group. However, the correlation between CD28- exhausted T cells and cytogenetic risk or white blood cell count was not observed, except for that CD28- exhausted CD4$^+$ T cells correlated with lymphocyte counts. Intriguingly, larger amount of CD28-TGITI$^+$CD8$^+$ T cells at diagnosis was associated with poor treatment response and shorter leukemia free survival.

**Discussion:** In summary, lack of CD28 expression defined a severely exhausted status from exhausted T cells. Accumulation of CD28- exhausted T cells was linked to occurrence of AML, and correlated to poor clinical outcome. Our data might facilitate the development of combinatory strategies to improve the efficacy of PD-1 blockade in AML.

KEYWORDS

**CD28, acute myeloid leukemia, exhaustion, T cell, senescence**

# Introduction

Immunotherapies revolute the therapeutic models of cancer by improving anti-tumor immunity, especially T cell function. However, the outcome of clinical trials using immunotherapies targeting T cells such as programmed cell death protein 1 (PD-1) blockade in acute myeloid leukemia (AML) remain frustrating (1). Hence, a more detailed analysis of T cell status, especially T-cell exhaustion in AML is warranted to help us envision more tailored immunotherapeutic strategies for the future of AML treatment.

Due to persistent antigen stimulation in tumors or chronic infections, T cells acquire an exhausted phenotype accompanied by a loss of function (2). T cell exhaustion is characterized by impaired function and high expression of immune checkpoint inhibitory receptors such as PD-1, T-cell immunoglobulin and immunoreceptor tyrosine–based inhibitory motif (ITIM) domain (TIGIT) (3, 4). As we and others reported, exhausted T cells were accumulated in AML patients (4–8). However, PD-1 blockade treatment targeting exhausted T cells in AML showed poor efficacy (1). Severity of T-cell exhaustion correlate to the response to immune check point inhibitors (ICIs) (9). Severe exhaustion of T cells is responsible for the failure of ICIs treatment (9). Coexpression of multiple inhibitory receptors such as PD-1, T cell immunoglobulin and mucin domain 3 (TIM-3), Lymphocyte activation gene-3 (LAG-3) and TIGIT or high expression of PD-1 were used to define severely exhausted T cells in the previous studies (9, 10). Nevertheless, inhibitory receptors are upregulated in activated T cells besides exhausted T cells (11). Moreover, different combinations of inhibitory receptors might incur an inconsistent result. Additionally, due to the consecutive expression of PD-1 on T cells, gating PD-1$^{hi}$ subset of T cells is rather subjective. More markers should be involved to distinguish the exhaustion status in AML.

In tumor settings, T cells could experience a phenotypic shift to terminal differentiation characterized by expressing KLRG-1 and CD57 but loss of CD28 after persistent exposure to neoantigen, which is termed "T-cell senescence" (12). Moreover, proliferative arrest and shortened telomeres are also the characteristic features of senescent T cells. Although senescence and exhaustion mechanically undergo different molecular process, these two states of T cells overlap with regard to the expression of several phenotypic markers (6, 12). As shown in multiple myeloma, T cells with senescent markers like CD57 also express PD-1 (13). As reported, exhaustion of T cells could be largely reversed by ICIs while senescence of T cells is considered as an irreversible process (14). Intriguingly, Kamphorst AO et al. found that CD28 negative exhausted T cells cannot be rescued by PD-1 blockade therapy (15). Recently, George Coukos et al. also demonstrated that tumor-infiltrated T cells activation on anti-PD-1 therapy depends on CD28 costimulation catered by myeloid APCs *in situ* of ovarian cancer (16). These data indicated that CD28 expression might be critical for active T cell response in tumor. Additionally, the IL-7/IL-7Rα axis is involved in the maintenance of naïve and long-living memory cells as well as self-renewal in T cell populations (5, 17, 18). Defect of IL-7 signaling is not only involved in exhaustion but also plays a crucial role in senescence of T cells (17, 19).

In this study, we provide a comprehensive analysis of the expression of senescence-related markers such as CD28, KLRG-1 and CD57 and define severely exhausted T cells using CD28 combining with exhausted markers like PD-1 and TIGIT in bone marrow (BM) samples from AML patients. Since AML blasts as a key role in the compromising immune response are heterogenous and vary along with disease status, the alterations of severely exhausted T cells in different stages of the disease and the correlation to clinical features and prognosis were also evaluated.

# Material and methods

## Patients and clinical evaluation

Bone marrow (BM) of 42 *de novo* AML patients and 32 AML patients in complete remission (CR) as well as from healthy donors (HDs, n = 15) were collected after written informed consent was signed in accordance with the Declaration of Helsinki and approved by the ethics board of the First Affiliated Hospital of Xiamen University. All samples were taken from non-acute promyelocytic leukemia (APL) AML patients. Positivity of minimal residual disease (MRD) was defined as AML cells detected by real-time quantitative polymerase chain reaction (RQ-PCR) or multicolor flow cytometry in BM samples. CR, CR with in complete hematologic recovery (CRi) and partial remission (PR) and no response (NR, induction failure) were defined according to 2017 ELN recommendations (20). Risk stratification by genetics in AML was performed according to 2017 ELN recommendations (20).

## Multiparameter flow cytometry analysis

Surface staining was immediately performed after BM collection. A 100μl BM sample was incubated with directly conjugated monoclonal antibodies for 15 minutes at room temperature; after lysing red blood with 1ml lysing solution (BD Bioscience), the cells were washed and resuspended by phosphate buffered saline (PBS). The monoclonal antibodies used were anti-human CD3-Percp (UCHT1), CD28-FITC (CD28.2), CD4-APC-R700 (RPA-T4), CD8-APC-R700 (RPA-T8), CD45RA-APC-CY7 (HI100), CCR7-BV421 (G043H7), PD-1-PE-Cy7 (EH12.1), CD127-BV510 (A019D5), CD57-PE (HCD57), KLRG-1-APC (SA231A2), TIGIT-BV605 (A15153G) from Biolegend, CA, USA.

## Isolation of bone marrow mononuclear cells and ex-vivo stimulated cytokines production and cytotoxic potentials

BM was diluted 1:1 with PBS before separation of bone marrow mononuclear cells (BMMCs) with lymphocyte separation solution (TBD Science, China) density gradient centrifugation. The isolated

BMMCs were cultured in RPMI-1640 medium (GIBCO) containing 10% fetal bovine serum and stimulated with anti-CD3/CD28 antibody (2 and 5μg/ml) or Phorbol-12-myristate-13-acetate (PMA, 100ng/ml) and ionmycin (1ug/ml) for 5 h. Anti-CD107a-APC (W18263B), CD28-FITC and Golgiplug (BD Pharmingen, San Diego, CA, USA) were added at the start of the incubation. Cells were harvested for surface staining with CD3-Percp, CD8-APCR700, CD45RA-APC-H7, CCR7-V450, PD-1-PE-Cy7, TIGIT-BV605 and intracellularly stained with IFN-γ-BV510(4S.B3).

## Statistical analysis

All flow cytometric data were analyzed using Flowjo 10. software. All data were subjected to normal distribution analysis by the Kolmogorov-Smirnov test using SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). Student's t test was used to analyze data with a normal distribution, while the Mann-Whitney U test or Wilcoxon matched-pairs signed rank test (in paired setting) was used to analyze data without a normal distribution. Fisher's exact test or chi-square test were used to analyze the constituent ratios between *de novo* AML and AML-CR group. P-values <0.05 were considered significant, where *, ** and *** indicated p-values between 0.01 to 0.05, 0.001 to 0.01 and 0.0001 to 0.001, respectively.

# Results

## Patient characteristics

Fifteen HDs (with a median age of 34.5 years, range 25–56 years), forty-two *de novo* AML patients and 32 AML patients in CR (AML-CR) were enrolled in the present study. The characteristics of *de novo* AML patients and AML-CR were summarized in Table 1. Five patients rejected further medical care and three patients died before the evaluation. Two patients dropped out due to transfer to other hospitals. Compared to *de novo* AML group, the percentages of patients at low risk and received idarubicin and cytarabine (IA) regimens tended to be higher in AML-CR group. Fewer patients in *de novo* AML group received low intensity chemotherapy in comparison to AML-CR group. Among 32 *de novo* AML patients,

TABLE 1 Patient characteristics.

| Characteristics | De novo AML (n=42) | AML-CR (n=32) | P value |
|---|---|---|---|
| Gender | | | 1.000 |
| Male (%) | 24 (57.1) | 19 (59.3) | |
| Female (%) | 18 (42.9) | 13 (40.7) | |
| Age ,years, median (range) | 53 (14-83) | 49 (14-78) | 0.869 |
| WBC($\times10^9$/L) at diagnosis | 8.32(0.43-189.48) | 6.32(0.43-179.34) | 0.782 |
| Cytogenetic risk (%) | | | |
| Low | 9 (21.4) | 11(33.3) | |
| Intermediate, | 15(35.7) | 13 (40.6) | |
| High | 13(31.0) | 8 (26.1) | 0. 278 |
| NA | 5 (11.9) | 0 | |
| Induction regimens | | | |
| HMA+Venetoclax based regimen | 6(14.3) | 4(12.5) | |
| Low dose chemotherapy | 5(11.9) | 8(25) | |
| IA based regimen | 24(57.1) | 20(62.5) | 0.937 |
| NA | 7(16.7) | / | |
| Response after induction | | | |
| CR+CRi | 18 (42.9) | / | |
| PR | 1(2.4) | / | |
| NR | 13(30.9) | / | |
| NA | 10(23.8) | / | |
| Chemotherapy Cycles before BM collection | / | 3 (2-5) | |

WBC indicate white blood cell count; NA, not available; HMA, hypomethylation agents; IA, idarubicin + cytarabine; BM, bone marrow; AML, acute myeloid leukemia; AML-CR, patients with acute myeloid leukemia in compete remission; CR, complete remission; Cri, CR with incomplete hematologic recovery; PR, partial remission; NR, no response.

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18 patients achieved CR or CRi while 13 patients showed no response to induction therapy.

## Loss of CD28 expression is linked to terminal differentiation and senescence of T cells in *de novo* AML

T cell differentiation was evaluated in BM samples from *de novo* AML patients (n=42) and HDs (n=15). A significant phenotypic shift to effector memory T cells (TEM) was observed in *de novo* AML patients (Figures 1A, B). Moreover, both $CD8^+$ and $CD4^+$ naïve T cells (Tnaïve) from *de novo* AML patients suffered a significant shrinkage compared with HDs (Figures 1A, B). The frequencies of central memory T cells (TCM) and terminal effector memory expressing $CD45RA^+$ (TEMRA) in $CD4^+$ and $CD8^+$ T cells were comparable in two groups (Figures 1A, B). Besides, more T cells lost CD28 expression in AML group compared with HDs (Figure 1C). A comparison of $CD28^+$ and $CD28^-$ T cells revealed that loss of CD28 was linked to late stage (TEM in $CD4^+$ T cells) or

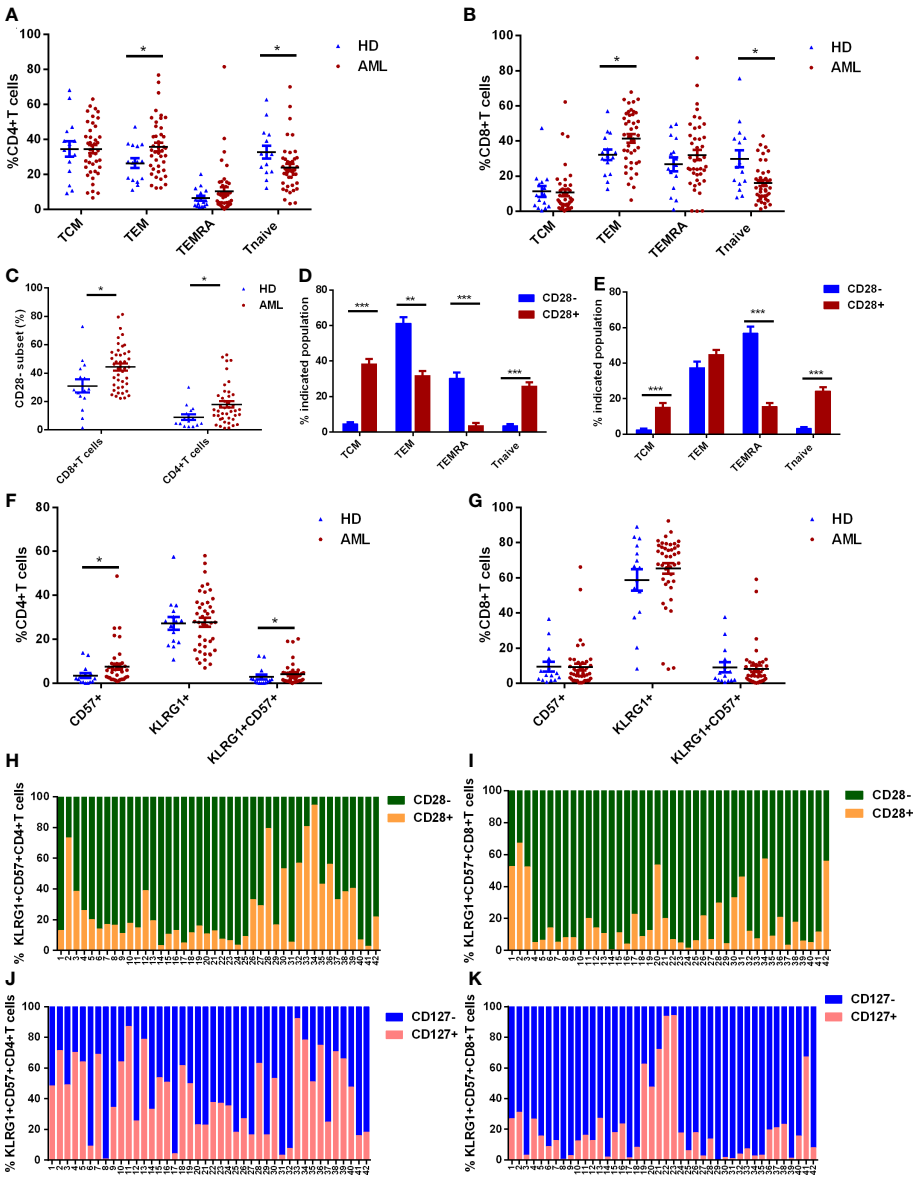


FIGURE 1
T cell differentiation and expression of senescent markers on T cells in *de novo* AML patients (n=42) and healthy donors (HDs, n=15). **(A, B)** The frequencies of naïve T cells (Tnaive, $CCR7^+CD45RA^+$), central memory T cells (TCM, $CCR7^+CD45RA^-$), effector memory T cells (TEM, $CCR7^-CD45RA^-$) and effector memory T cells with expression of CD45RA (TEMRA, $CCR7^-CD45RA^+$) defined by CCR7 and CD45RA in $CD4^+$ **(A)** and $CD8^+$ **(B)** T cells from bone marrow (BM) were compared in *de novo* AML and HDs. Additionally, the percentages of $CD28^-$ subset in $CD4^+$ and $CD8^+$ T cells from BM of *de novo* AML and HDs were evaluated **(C)**. The differentiated status was further analyzed in $CD28^-$ and $CD28^+$ subset of $CD4^+$ **(D)** and $CD8^+$ T cells **(E)**. Senescent markers: CD57 and KLRG-1 expression on $CD4^+$ **(F)** and $CD8^+$ T cells **(G)** in BM were compared between AML patients and HDs. At last, CD28 expression in KLRG-$1^+CD57^+$ subset in $CD4^+$ T cells **(H)** and $CD8^+$ T cells **(I)** and CD127 expression in KLRG-$1^+CD57^+$ subset in $CD4^+$ T cells **(J)** and $CD8^+$ T cells **(K)** were analyzed in *de novo* AML patients. Student's t test or Mann-Whitney U test was used to analyze the data according to normal distribution of data. *, ** and *** indicated p-values between 0.01 to 0.05, 0.001 to 0.01 and 0.0001 to 0.001, respectively.

even terminal differentiated stage (TEMRA in $CD4^+$ and $CD8^+$T cells) of T cells rather than early stage such as Tnaive and TCM (Figures 1D, E). Late or terminal differentiation were generally associated with T-cell senescence. Further analysis showed that significantly increased $CD57^+CD4^+$ T cells and KLRG-$1^+CD57^+CD4^+$ T cells were observed in AML group (Figure 1F). However, no difference was observed in $CD8^+$ T cell compartments (Figure 1G). Lack of CD28 expression was also observed in KLRG-$1^+CD57^+$ subsets in both $CD4^+$ and $CD8^+$ T cells (Figures 1H–K).

## Differentiation status defined by CCR7 and CD45RA could not distinguish a terminal exhausted T-cell subset associated with AML occurrence

The expression pattern of inhibitory receptors (including PD-1 and TIGIT) was also evaluated in AML patients and HDs (Figure 2A). In comparison to HDs, significantly higher percentages of PD-$1^+$ and PD-$1^+$TIGIT$^+$ subsets were observed in both $CD4^+$ and $CD8^+$ T cell compartment of AML patients (Figures 2B, C). Although the percentage of TIGIT$^+$ subset in both $CD4^+$ and $CD8^+$ T cells increased in AML patients, only the subset in $CD4^+$ T cells presented a significant difference (Figures 2B, C). However, when we further analyzed the PD-1 and TIGIT expression pattern in distinctly differentiated subsets like Tnaive, TCM, TEM and TEMRA, the difference varied along with the differentiated status. Higher expression of PD-1 was observed in Tnaive and TEMRA subsets in both $CD4^+$ (Figures 2D, G) and $CD8^+$ T cell compartment (Figures 2H, K). Upregulated TIGIT expression was distributed in relatively early stage in Tnaive and TCM of $CD4^+$ (Figures 2D, E) and $CD8^+$ T cells (Figures 2H, I) as well as in $CD4^+$ TEM (Figure 2F). However, a significant increase of T cells coexpressing of PD-1 and TIGIT was observed in $CD4^+$ and $CD8^+$ Tnaive (Figures 2D, H) and $CD4^+$ TCM (Figure 2E). Neither TEMRA nor TEM showed a significant difference in coexpression of PD-1 and TIGIT in $CD4^+$ (Figures 2F, G) or $CD8^+$ T cells (Figures 2J, K). Taken together, these data suggested it might be hard to further classify exhausted T cells by combination of differentiated markers CCR7, CD45RA and inhibitory receptors PD-1 and TIGIT in BM of *de novo* AML patients.

## CD28 expression could phenotypically classify exhausted T cells in the BM of *de novo* AML patients

$CD28^-$ exhausted T cells were defined as the functionally irreversible subpopulation by ICIs in chronic infection settings. The role of CD28 in classification of exhausted T cells in *de novo* AML patients remained to be assessed. In $CD4^+$ T cell compartment, significant augment of PD-$1^+$ and PD-$1^+$TIGIT$^+$

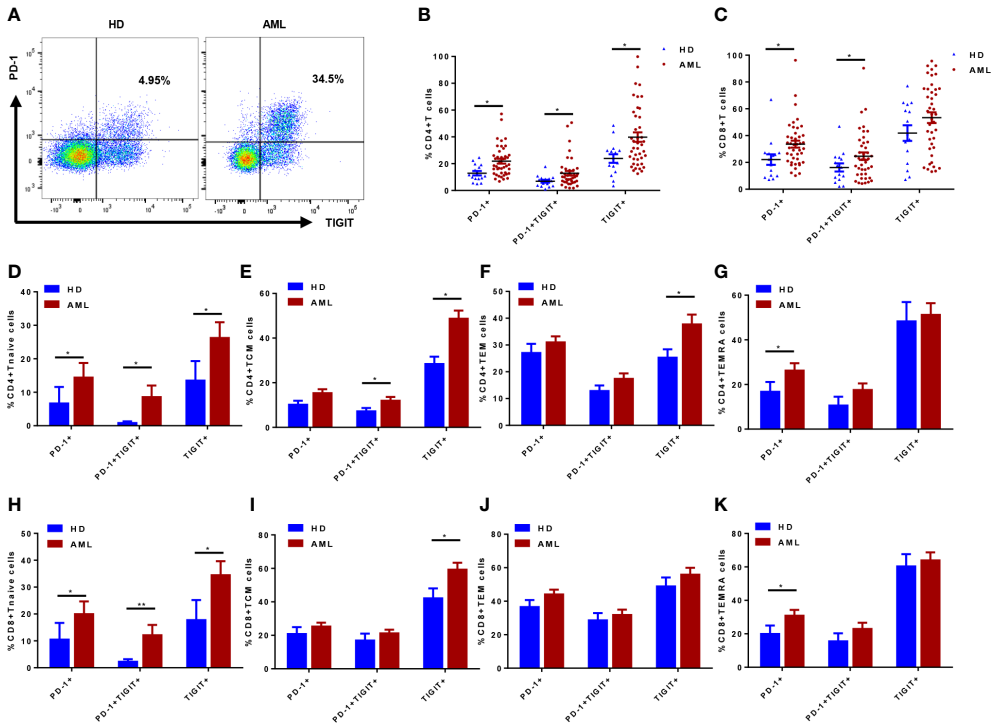


FIGURE 2
Inhibitory receptors PD-1 and TIGIT expression in T cells and their differentiated subsets from bone marrow (BM) of *de novo* AML patients (n=42) and healthy donors (HDs, n=15). PD-1 and TIGIT expression was determined in *de novo* AML patients and HDs **(A)**; The percentages of PD-$1^+$, TIGIT$^+$ and PD-$1^+$TIGIT$^+$ subsets of $CD4^+$ **(B)** and $CD8^+$ **(C)** T cells in BM of *de novo* AML patients and HDs were compared. Moreover, PD-1, TIGIT and coexpression of PD-1 and TIGIT were evaluated in Tnaive, TCM, TEM and TEMRA subsets of $CD4^+$ **(D–G)** and $CD8^+$ **(H–K)** T cells in BM of *de novo* AML patients and HDs. Student's t test or Mann-Whitney U test was used to analyze the data according to normal distribution of data. *, ** indicated p-values between 0.01 to 0.05, 0.001 to 0.01 respectively.

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subsets were observed in $CD28^-$ rather than $CD28^+$ subset of AML patients compared with HDs (Figures 3A, C). However, TIGIT expression was consistently upregulated in $CD28^-$ and $CD28^+CD4^+$ T cells (Figures 3A, C). As for $CD8^+$ T cells, significantly higher percentages of $PD\text{-}1^+$, $TIGIT^+$ and $PD\text{-}1^+TIGIT^+$ in AML patients were merely observed in $CD28^-$ T cells (Figures 3B, D). Additionally, $CD28^-PD\text{-}1^+TIGIT^+$ T cells also displayed a late and terminal differentiated status compared with its $CD28^+$ counterpart (Figures 3E, F). Moreover, other senescent markers like CD57 robustly increased in $CD28^-PD\text{-}1^+TIGIT^+$ T cells compared with $CD28^+PD\text{-}1^+TIGIT^+$ T cells (Figures 3G, H). Similarly, lack of CD127 expression was more frequently observed in $CD28^-PD\text{-}1^+TIGIT^+$ T cells (Figures 3G, H). In summary, CD28 expression could phenotypically classify an exhausted T-cell subset with senescent features that correlated to AML occurrence.

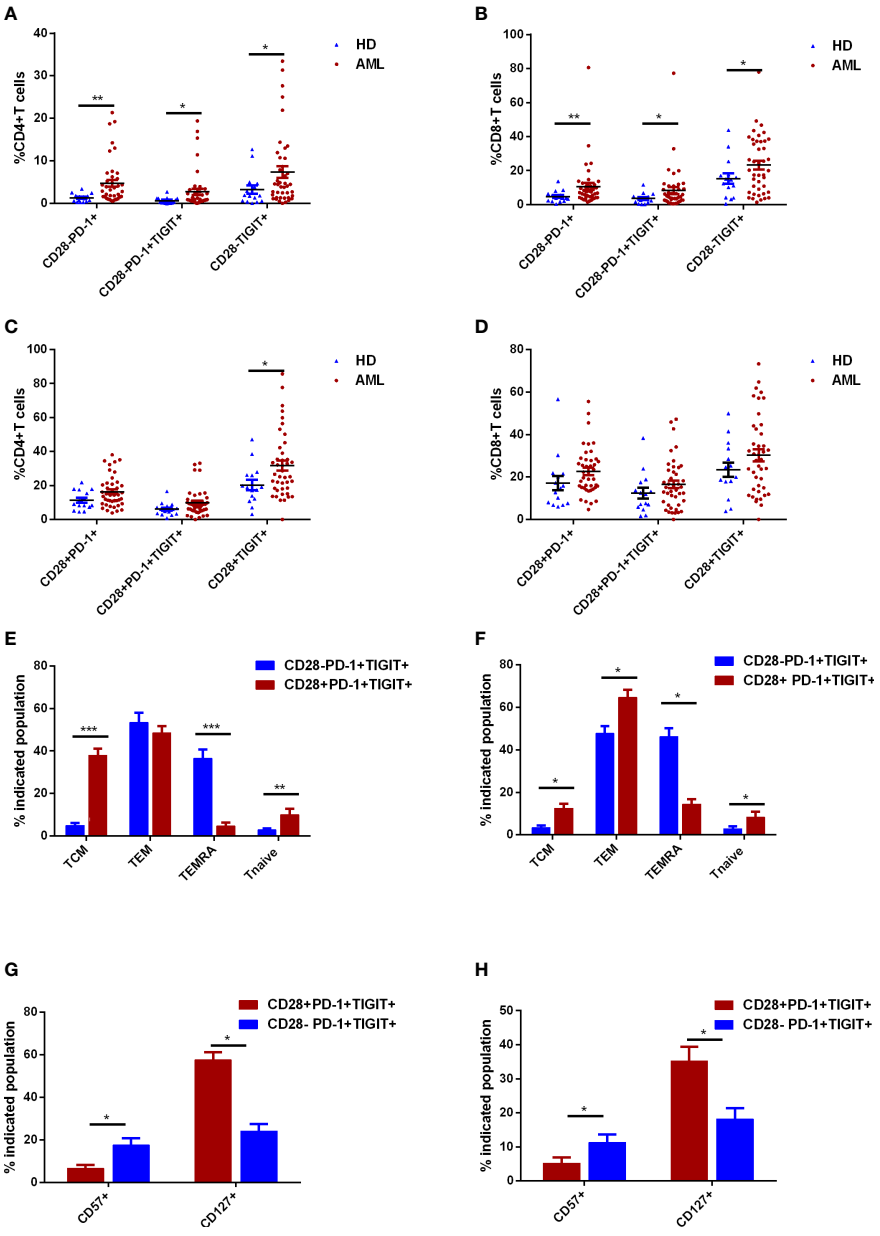


FIGURE 3
Further classification of exhausted T cells in the bone marrow (BM) of *de novo* AML patients using CD28 expression. The frequencies of $CD28^-PD\text{-}1^+$, $CD28^-PD\text{-}1^+TIGIT^+$, and $CD28^-TIGIT^+$ subsets in $CD4^+$ T cells **(A)** and $CD8^+$ T cells **(B)** in BM were analyzed in *de novo* AML patients (n=42) and healthy donors (HDs, n=15). The frequencies of $CD28^+PD\text{-}1^+$, $CD28^+PD\text{-}1^+TIGIT^+$, and $CD28^+TIGIT^+$ subsets in $CD4^+$ T cells **(C)** and $CD8^+$ T cells **(D)** in BM were further evaluated. Thereafter, the differentiated status of $CD28^-PD\text{-}1^+TIGIT^+$ and $CD28^+PD\text{-}1^+TIGIT^+$ subsets of $CD4^+$ T cells **(E)** and $CD8^+$ T cells **(F)** in BM were determined in AML patients. Moreover, CD127 and CD57 expression were also evaluated in $CD28^-PD\text{-}1^+TIGIT^+$ and its counterpart in $CD4^+$ T cells **(G)** and $CD8^+$ T cells **(H)** in BM of *de novo* AML patients. Student's t test or Mann-Whitney U test was used to analyze the data according to normal distribution of data. *, ** and *** indicated p-values between 0.01 to 0.05, 0.001 to 0.01 and 0.0001 to 0.001, respectively.

## Lack of CD28 expression defines a severely functionally exhausted T cells in the BM of *de novo* AML patients

Despite obvious distinction in phenotype in $CD28^-$ and $CD28^+$ T cells, whether lack of CD28 contributes to impairment in function remains to be elucidated. The capacity of degranulation and interferon-gamma (IFN-γ) production were evaluated in $CD28^-$ and $CD28^+$ T cells (Figure 4A). In an ex vivo simulation setting using CD3/CD28 antibody, which is similar to T cell activation *in vivo*, IFN-γ production by $CD4^+$ and $CD8^+$ T cells were significantly compromised by loss of CD28 expression (Figures 4B, C). Similarly, degranulation capacity of $CD28^-$ T cells was remarkably lower than their $CD28^+$ counterparts. To exclude the impact of anti-CD28 antibody stimulation on CD28 expression, ex vivo stimulation with PMA/ionomycin was performed. As a result, loss of CD28 still led to defects in IFN-γ production and degranulation ability in both $CD4^+$ and $CD8^+$ T cells (Figures 4D, E). Further analyses were also performed to determine function of $CD28^-PD\text{-}1^+TIGIT^+$ T cells (Figures 4F, G); Consistent with the total compartment, $CD28^-PD\text{-}1^+TIGIT^+$ subset displayed a more severely dysfunctional status compared with $CD28^+PD\text{-}1^+TIGIT^+$ subset of both CD4+ (Figure 4H) and CD8+T cells (Figure 4I). Additionally, $CD28^-$ $PD\text{-}1^+$ and $CD28^-$ $TIGIT^+$ T cells also showed compromised capacity in degranulation but not IFN-γ production (Supplementary Figures S1A–D). These data demonstrated that loss of CD28 was associated with function impairment and could even define a severely exhausted T-cell subset.

## The improvement of T cell senescence and exhaustion is associated with disease remission after therapy

Although senescent and exhausted T cells were compared between *de novo* AML patients and HDs, whether status of T cells was improved after disease remission remains largely unclear. Senescence and exhaustion, especially the severe exhaustion of T cells was evaluated in the BM of *de novo* AML and AML patients achieving CR. The frequencies of $CD57^+$, $KLRG\text{-}1^+$ and $CD57^+KLRG\text{-}1^+$ subsets in $CD4^+$ T cells were significantly decreased after disease remission (Figure 5A). Similar to HDs, AML-CR group did not show significant alteration in $CD8^+$ T cells expressing either CD57 or KLRG-1 or coexpressing CD57 and KLRG-1(Figure 5B). As for exhausted markers, both $PD\text{-}1^+$ and $PD\text{-}1^+TIGIT^+$ subsets of $CD4^+$ and $CD8^+$ T cells experienced a dramatically decrease after complete remission (Figures 5C, D). Notably, TIGIT expression was not significantly downregulated in $CD4^+$ or $CD8^+$ T cells (Figures 5C, D). Further analysis showed both $CD28^-$ and $CD28^+$ subpopulation of $PD\text{-}1^+$ and $PD\text{-}1^+$ $TIGIT^+$ $CD4^+$ T cells were significantly reduced after disease remission (Figures 5E, G). Moreover, $CD28^-$ $TIGIT^+$ rather than $CD28^+$ $TIGIT^+$ $CD4^+$ T cells were decreased after remission (Figures 5E, G). Additionally, significant decrease of $PD\text{-}1^+TIGIT^+CD8^+$ T cells was observed in $CD28^+$ rather than $CD28^-$ T cells (Figures 5F, H). Both $CD28^-PD\text{-}1^+$ and $CD28^+PD\text{-}1^+$ subsets of $CD8^+$ T cells were significantly diminished after CR (Figures 5F, H). In addition, 12 patients in AML-CR group still have MRD after disease remission. Only $CD28^-TIGIT^+CD4^+$ T cells significantly increased in these patients (Figures 5I, J). These data suggested that $CD4^+$ T cells achieved a more comprehensive recover from senescent and exhausted status than $CD8^+$ T cells after disease remission and could even be associated with MRD status.

## $CD28^-$ Exhausted T cells link to lymphocyte count in peripheral blood and predict poor prognosis in *de novo* AML patients

Due to the heterogeneity of AML, it is critical to evaluate whether the frequencies of $CD28^-$ exhausted T cells correlate with clinical and genetic features. There were no significant differences in the proportion of $CD28^-$ exhausted subsets in $CD4^+$ or $CD8^+$ T cells between higher WBC (≥10×109/L) and lower WBC (<10×109/L) groups (Figures 6A, B). However, higher frequencies of $CD28^-PD\text{-}1^+$, $CD28^-PD\text{-}1^+TIGIT^+$ and $CD28^-$ $TIGIT^+$ subsets of $CD4^+$ T cells but not $CD8^+$ T cells in BM of in *de novo* AML significantly correlated to lower lymphocyte count in peripheral blood (Figures 6C, D). Additionally, no difference of $CD28^-$ exhausted T cells subsets was observed in low, intermediate and high cytogenetic risk group (Figures 6E, F). Intriguingly, only higher frequency of $CD28^-TIGIT^+CD8^+$ T cells was observed in patients with non-CR after induction (Figures 6G, H). Moreover, higher frequency of $CD28^-TIGIT^+CD8^+$ T cells at diagnosis predicted poorer outcome of AML (Figures 6I, J). However, $CD28^-PD\text{-}1^+$, $CD28^-PD\text{-}1^+TIGIT^+CD8^+$T cells did not show significant correlation with the outcome (Supplementary Figures S2A, B); In addition, $CD28^+PD\text{-}1^+$, $CD28^+PD\text{-}1^+TIGIT^+$ and $CD28^+TIGIT^+$ subsets of $CD8^+$ T cells also showed no significance with the outcome (Supplementary Figures S2C–E). These data indicated that $CD28^-TIGIT^+CD8^+$ T cells might be a novel predictor independent of cytogenetic features for clinical response and prognosis in AML patients.

# Discussion

Although accumulation of exhausted T cells in AML was widely reported, extremely poor efficacy of anti-PD-1 therapy in AML warranted deeper phenotypical and functional analysis of T cells status. We defined severely exhausted subsets of T cells that correlated to AML occurrence and predicted the prognosis of AML by combination of senescent marker CD28 and exhausted markers like PD-1 and TIGIT. Our data provided a novel insight into T-cell exhaustion in AML patients.

Consistent with previous studies, more $CD4^+$ and $CD8^+$ T cells showed a shift to terminal differentiation in *de novo* AML. However, the terminal differentiation was characterized by lack of CD28 expression and decrease of Tnaive cells rather than TEM in $CD8^+$ T cells, as reported by F. Brauneck (5). Different sources of T cells

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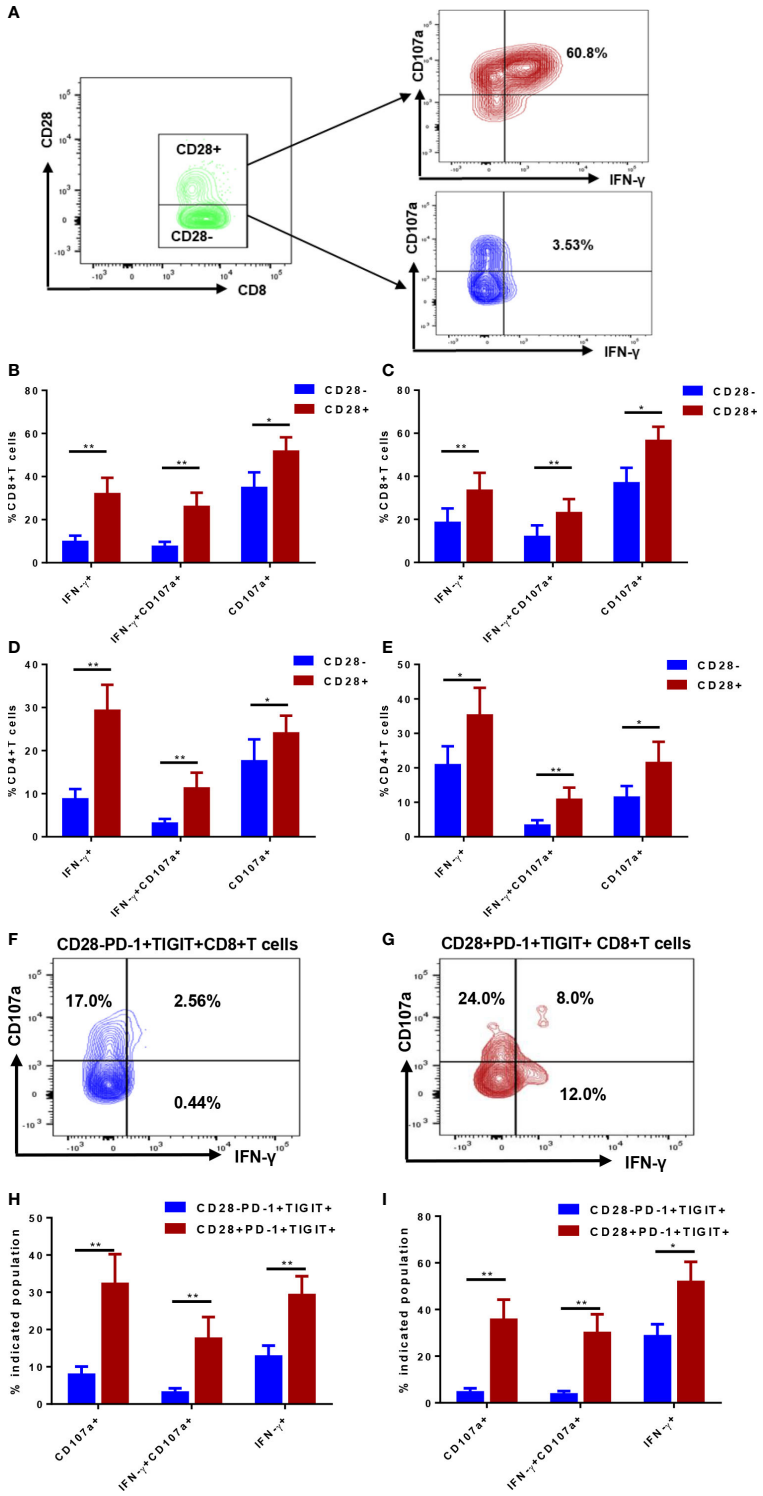


FIGURE 4
Function analysis of CD28$^-$ subsets and the counterparts of T cells in in the bone marrow (BM) of *de novo* AML patients. Degranulation capacity and interferon-gamma (IFN-γ) were determined in CD28$^-$ and CD28$^+$ T-cell subsets **(A)**; CD107a expression and IFN-γ production were simultaneously evaluated in CD28$^-$ and CD28$^+$ subsets of CD4$^+$ T cells **(B)** and CD8$^+$ T cells **(C)** stimulated by anti-CD3/CD28 antibody ex vivo (n=17). Additionally, CD107a expression and IFN-γ production were simultaneously evaluated in CD28$^-$ and CD28$^+$ subsets of CD4$^+$ T cells **(D)** and CD8$^+$ T cells **(E)** stimulated by PMA/ionomycin ex vivo (n=13). Further analyses were performed to evaluate CD28$^-$PD-1$^+$TIGIT$^+$CD8$^+$ **(F)** and its counterpart **(G)** of CD8$^+$ T cells; CD107a expression and IFN-γ production were evaluated in CD28$^-$PD-1$^+$TIGIT$^+$ and its counterpart of CD4$^+$ T cells **(H)** and CD8$^+$ T cells **(I)** stimulated by anti-CD3/CD28 antibody ex vivo (n=17). Paired Student's t test or Wilcoxon matched-pairs signed rank test was used to analyze the data according to normal distribution of data. *, ** indicated p-values between 0.01 to 0.05, 0.001 to 0.01 respectively.

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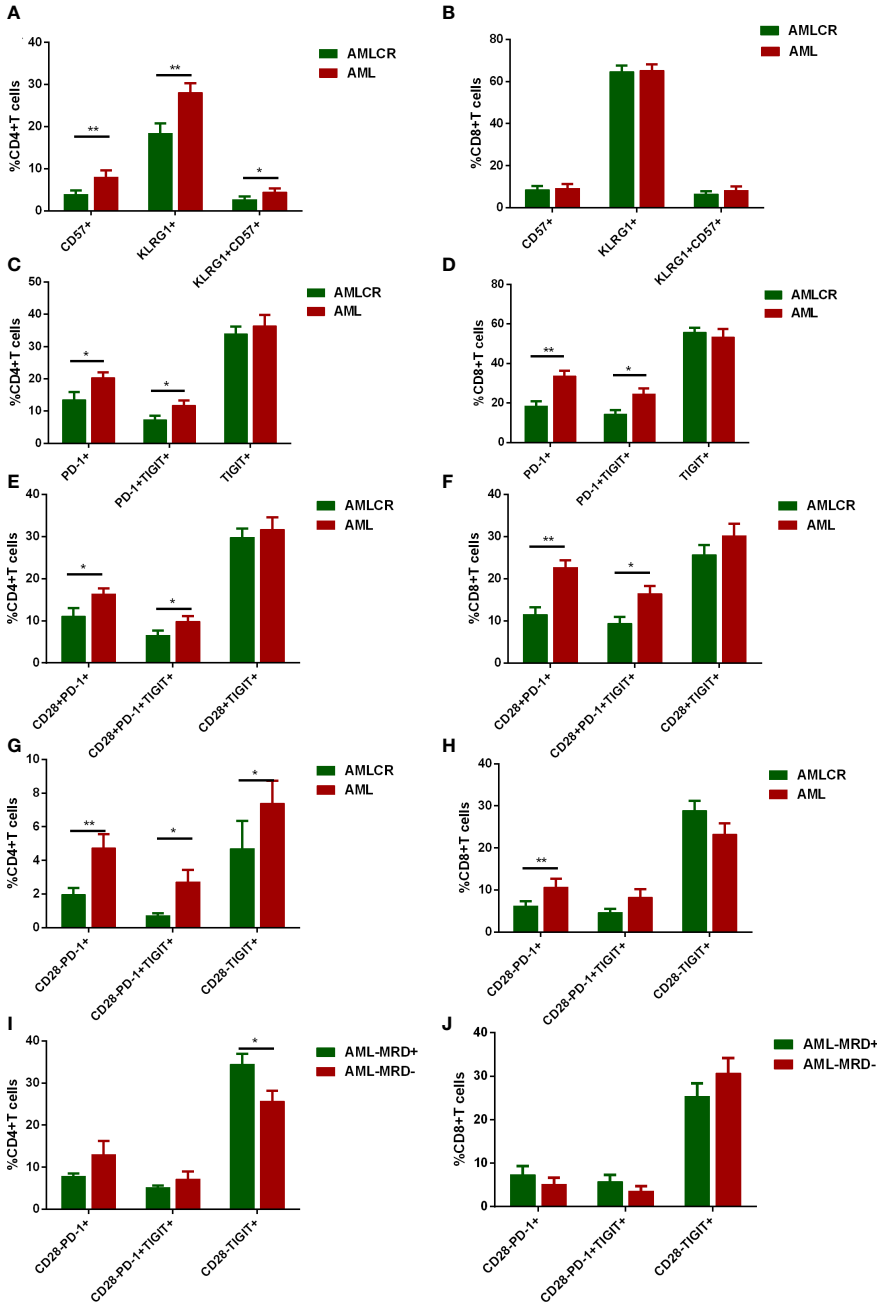


FIGURE 5
Dynamic alteration of senescent T cells and T cell exhaustion in different disease status of AML. CD57 and KLRG-1 expression of $CD4^+$ T cells **(A)** and $CD8^+$ T cells **(B)** were evaluated in *de novo* AML patients (n=42) and AML patients in complete remission (AML-CR, n=32). In addition, the percentages of PD-$1^+$, $TIGIT^+$ and PD-$1^+TIGIT^+$ subsets of $CD4^+$ **(C)** and $CD8^+$ **(D)** T cells in BM of *de novo* AML patients and AML-CR were compared. Further analyses of PD-1 and TIGIT expression on $CD28^+CD4^+$ **(E)** and $CD28^+CD8^+$ **(F)** T cells in BM of *de novo* AML patients and AML-CR were performed. Similarly, PD-1 and TIGIT expression on $CD28^-CD4^+$ **(G)** and $CD28^+$-$CD8^+$ **(H)** T cells in BM of *de novo* AML patients and AML-CR were compared. At last, the expression of PD-1 and TIGIT in $CD28^-$ subsets of $CD4^+$ **(I)** and $CD8^+$ **(J)** T cells were analyzed in AML-CR patients with minimal residual disease (MRD) (n=12) and without MRD (n=20). Student's t test or Mann-Whitney U test was used to analyze the data according to normal distribution of data. *, ** indicated p-values between 0.01 to 0.05, 0.001 to 0.01 respectively.

like BM or PB might contribute to the discrepancy. Tnaive cells are more dependent on the costimulation signaling mediated by CD28 than antigen-experienced T cells such as memory and effector T cells (15). Loss of CD28 expression is not only correlated to terminal differentiation especially in TEMRA stage but also linked to T cell senescence. Accordingly, $CD28^-$ T cells accounted for a predominant part of T cells with senescent phenotype KLRG-$1^+$ $CD57^+$. Recently, Leo Luznik et al. demonstrated that AML blasts could induce KLRG-1 and CD57 expression on $CD8^+$ T cells ex-vivo and was associated with unresponsiveness to PD-1 blockade in AML (21). However, the comparison of KLRG-$1^+CD57^+CD8^+$ T cells in BM of AML patients and HDs was not performed in the

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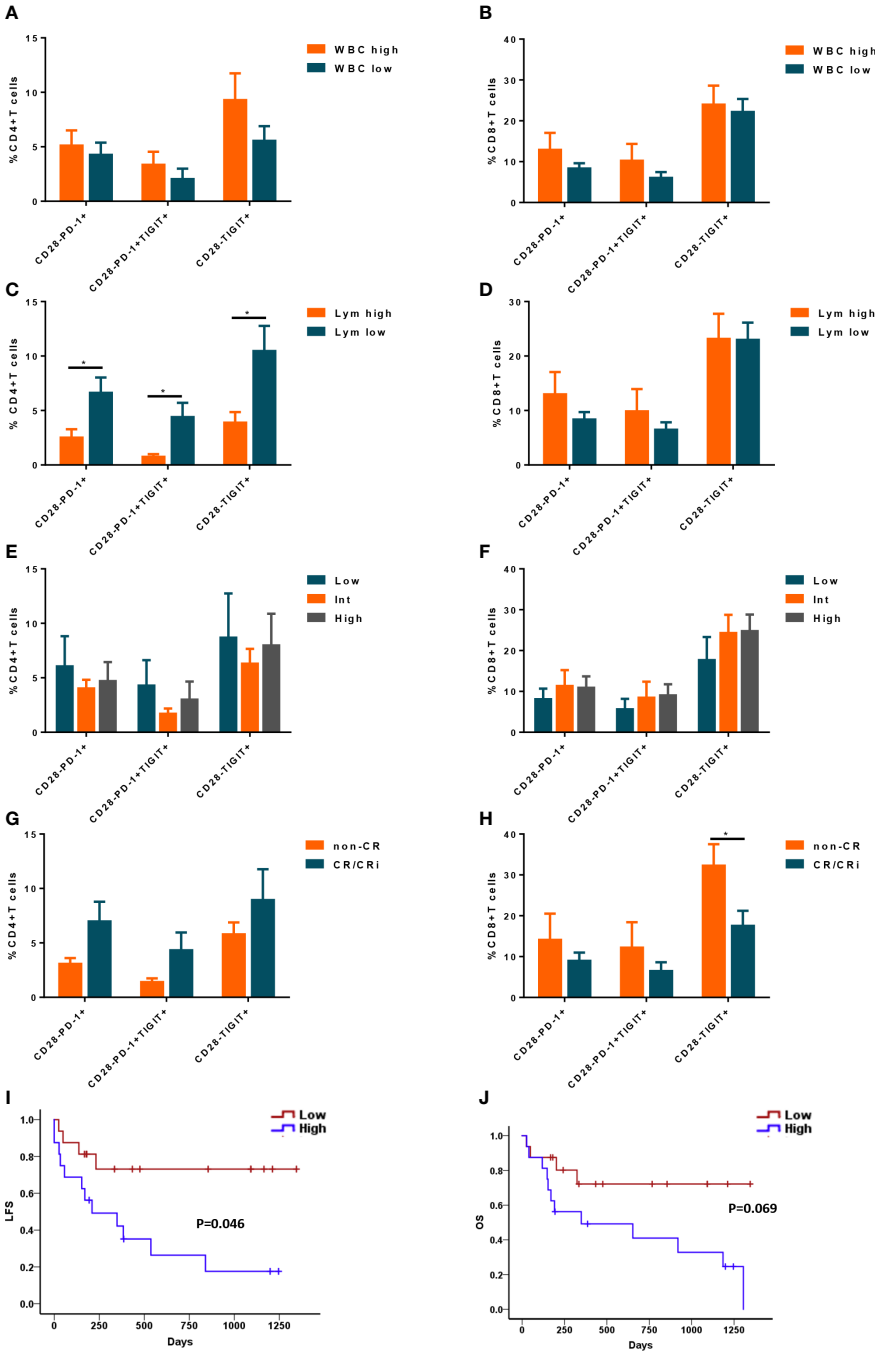


FIGURE 6
The correlation between CD28$^-$ exhausted subsets and clinical features and outcome of AML patients. The percentages of CD28$^-$PD-1$^+$, CD28$^-$PD-1$^+$TIGIT$^+$, and CD28$^-$TIGIT$^+$ subsets in CD4$^+$ T cells **(A)** and CD8$^+$ T cells **(B)** in BM were analyzed in AML patients with low and high level of white blood cell count (WBC) (n=22 and 20 respectively). Likewise, the percentages of CD28$^-$PD-1$^+$, CD28$^-$PD-1$^+$TIGIT$^+$, and CD28$^-$TIGIT$^+$ subsets in CD4$^+$ T cells **(C)** and CD8$^+$ T cells **(D)** in BM were analyzed in AML patients with low(n=22) and high level of lymphocyte count (n= 20 respectively). Moreover, the percentages of CD28$^-$PD-1$^+$, CD28$^-$PD-1$^+$TIGIT$^+$, and CD28$^-$TIGIT$^+$ subsets in CD4$^+$ T cells **(E)** and CD8$^+$ T cells **(F)** in BM were analyzed in AML patients with low (n=9), intermediate(n=20) and high (n=13) cytogenetic risk. Additionally, the correlation between CD28$^-$PD-1$^+$, CD28$^-$PD-1$^+$TIGIT$^+$, and CD28$^-$TIGIT$^+$ subsets in CD4$^+$ T cells **(G)** and CD8$^+$ T cells **(H)** in BM and treatment response were evaluated (n=32). Moreover, the frequency of CD28$^-$TIGIT$^+$CD8$^+$ T cells in BM could also predict the leukemia free survival (LFS, **I**) and overall survival (OS, **J**). Student's t test or Mann-Whitney U test was used to analyze the frequencies of CD28- exhausted subsets in different subgroups according to normal distribution of data. Log-rank test were used to analyze the LFS and OS. * indicated p-values between 0.01 to 0.05.

study. In contrast, our study showed that CD4$^+$ T cells rather than CD8$^+$ T cells displayed higher expression of CD57 and coexpression of CD57 and KLRG-1 in BM of *de novo* AML patients. Senescence of T cells can be triggered by replicative senescence generally incurred by natural age-related process, and premature senescence induced by outside factors such as cell stress and interaction with regulatory T cells (6, 12). Considering that the eventual frequency of senescent T cells results from the multiple

processes in bone marrow niches of AML, it is critical to discriminate the results of ex vivo from *in situ* of BM.

Senescence of T cells referred to an irreversible process while most exhausted T cells could be reinvigorated by ICIs (6, 12, 14). Severity of T cell exhaustion could also contribute to resistance to anti-PD-1 therapy (9). Severely exhausted T cells could be defined by abundance of PD-1 expression and simultaneous expression of other inhibitory receptors (9). However, due to consecutive expression of PD-1 on T cells, expression abundance evaluation is rather subjective. PD-1 and TIGIT were used to define exhausted phenotype of T cell in AML (22), However, there was no significant difference in the frequencies of $CD8^+TIGIT^+$T cells between *de novo* AML group and HDs in our study, which is not consistent with the previous report by others (22). T cells in our study were obtained from BM in AML patients and HDs, while T cells in reported study were derived from PB in HDs and BM in AML patients. Different sample sources might lead to the inconsistence.

CCR7 and CD45RA are generally used to define differentiated status of T cells. However, combination of CCR7 and CD45RA and inhibitory receptors like PD-1 and TIGIT could not well reflect the T-cell subsets correlated to AML occurrence. Kamphorst AO et al. demonstrated that rescue of exhausted T cells by anti-PD-1 therapy was CD28 dependent in mice model of life-long chronic lymphocytic choriomeningitis virus (LCMV) infection (15). However, whether CD28 expression could help to define a severely exhausted subsets remains unclear. We found that lack of CD28 expression in exhausted T cells could not only lead to phenotypical shift to the terminal stage but also result in significant functional impairments compared with $CD28^+$ exhausted T cells. We also observed a senescent phenotype with higher expression of CD57 and lower expression of IL-7 receptor alpha (CD127) in $CD28^-PD\text{-}1^+TIGIT^+$ T cells, which might restrict the capacity of self-renewal capacity and the long-term living. Notably, we found higher expression of PD-1 and TIGIT mainly distributed in $CD28^-$ subsets especially in $CD8^+$ compartment in *de novo* AML group. However, improvement of exhaustion status after CR mainly occurred in $CD28^+PD\text{-}1^+TIGIT^+$ rather than $CD28^-PD\text{-}1^+TIGIT^+$ subset of $CD8^+$ T cells, neither was $TIGIT^+$ T cells significantly changed after CR. Taken together, these data might provide a rational explanation for poor efficacy of PD-1 blockade in AML patients.

WBC and cytogenetic alterations were considered as the main factors impact clinical outcome (23). However, $CD28^-$ exhausted subsets did not correlated to WBC or cytogenetic risk. Additionally, higher frequency of $CD28^-$ exhausted subsets in $CD4^+$ T cells of BM was linked to lower lymphocyte count in PB. Previous studies showed that exhaustion linked to dysfunction and apoptosis of $CD4^+$ T cells in BM (24), however the mechanism of peripheral reduction of lymphocytes needs to be explored in the future. It seems that $CD28^-$ exhausted subsets in $CD4^+$ T cells could be more sensitive to reflect AML load than $CD8^+$ T cells. Both $CD28^-PD\text{-}1^+$ and $CD28^-PD\text{-}1^+TIGIT^+CD4^+$ T cells were significantly decreased after CR while only $CD28^-PD\text{-}1^+CD8^+$ T cells showed the same trend. Moreover, $CD28^-TIGIT^+CD4^+$ T cells could even significantly elevate in AML with positive MRD. However, $CD28^-TIGIT^+CD8^+$ T cells displayed the superior capacity to predict response to induction therapy and prognosis. Higher frequency of $CD28^-TIGIT^+CD8^+$ T cells at diagnosis correlated to non-CR after induction and shorter LFS.

There are some limitations in our study. Despite a larger *de novo* AML cohort than previous studies, a certain proportion of patients dropped out in our study, even if this did not affect the result at diagnosis. However, the small sample size and treatment variations might lead to the biased result in survival analysis.

In summary, lack of CD28 expression could define severely exhausted subsets of T cells with terminally differentiated and senescent features. Higher level of $CD28^-$ exhausted T cells correlated to AML occurrence and poor prognosis. Our findings provide a novel insight into exhausted T cells in AML and the potential combinatory strategies to improve the efficacy of PD-1 blockade.

## 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 Human Ethics Review Committee of the First Affiliated Hospital of Xiamen University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

YTH, HZ, YZ, YH, and LL performed the experiments and data analysis. ZFL, ZJL, JZ, and ZF collected bone marrow samples and clinical data. XY, LL, and BX aided in interpreting the results and worked on the manuscript. XY, LL, and BX conceived and planned the experiments. 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

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# Deciphering the role of QPCTL in glioma progression and cancer immunotherapy

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**Background:** Glioma is the most lethal and most aggressive brain cancer, and currently there is no effective treatment. Cancer immunotherapy is an advanced therapy by manipulating immune cells to attack cancer cells and it has been studied a lot in glioma treatment. Targeting the immune checkpoint CD47 or blocking the CD47-SIRPα axis can effectively eliminate glioma cancer cells but also brings side effects such as anemia. Glutaminyl-peptide cyclotransferase-like protein (QPCTL) catalyzes the pyroglutamylation of CD47 and is crucial for the binding between CD47 and SIRPα. Further study found that loss of intracellular QPCTL limits chemokine function and reshapes myeloid infiltration to augment tumor immunity. However, the role of QPCTL in glioma and the relationship between its expression and clinical outcomes remains unclear. Deciphering the role of QPCTL in glioma will provide a promising therapy for glioma cancer immunotherapy.

**Methods:** QPCTL expression in glioma tissues and normal adjacent tissues was primarily analyzed in The Cancer Genome Atlas (TCGA) database, and further validated in another independent cohort from the Gene Expression Omnibus (GEO) database, Chinese Glioma Genome Atlas (CGGA), and Human Protein Atlas (HPA). The relationships between QPCTL expression and clinicopathologic parameters and overall survival (OS) were assessed using multivariate methods and Kaplan-Meier survival curves. And the proteins network with which QPCTL interacted was built using the online STRING website. Meanwhile, we use Tumor Immune Estimation Resource (TIMER) and Gene Expression Profiling Interactive Analysis (GEPIA) databases to investigate the relationships between QPCTL expression and infiltrated immune cells and their corresponding gene marker sets. We analyzed the Differentially Expressed Genes (DEGs) including GO/KEGG and Gene Set Enrichment Analysis (GSEA) based on QPCTL-high and -low expression tumors.

**Results:** In contrast to normal tissue, QPCTL expression was higher in glioma tumor tissue ($p < 0.05$). Higher QPCTL expression was closely associated with high-grade malignancy and advanced tumor stage. Univariate and multivariate

analysis indicated the overall survival of glioma patients with higher QPCTL expression is shorter than those with lower QPCTL expression ($p < 0.05$). Glioma with QPCTL deficiency presented the paucity of infiltrated immune cells and their matching marker sets. Moreover, QPCTL is essential for glioma cell proliferation and tumor growth and is a positive correlation with glioma cell stemness.

**Conclusion:** High QPCTL expression predicts high grades of gliomas and poor prognosis with impaired infiltration of adaptive immune cells in the tumor microenvironment as well as higher cancer stemness. Moreover, targeting QPCTL will be a promising immunotherapy in glioma cancer treatment.

KEYWORDS

QPCTL, glioma, cancer immunotherapy, immune infiltration, biomarker

# 1 Introduction

Malignant gliomas are the most common primary invasive brain tumors, accounting for approximately 25% of central nervous system tumors (1). WHO divided the gliomas into four grades: grade I and grade II are low-grade gliomas (LGGs) and grade III and grade IV are high-grade gliomas (2). The glioblastoma is the highest grade of glioma, with its mid-survival time within one and a half years after the operation. The common therapies for gliomas include radiotherapy, chemotherapy, and operation. However, therapeutic advances for glioblastoma have been minimal over the past two decades (3). The molecular biomarkers specific to tumor subsets and targeted therapies are promising and important research directions (4). Finding the biomarkers of glioma that can guide the prescription of a particular targeted therapy is necessary and imperative.

The tumor microenvironment (TME) is heterogeneous, mixed with the tumor, stroma, and endothelial cells. It is characterized by the cross-talk between tumor and the innate and adaptive immune cells (5). The immune cells interact with the tumor cells in the development and progression of gliomas (6, 7). The infiltration of immune cells into the TME and the intra-tumor landscape is crucial for cancer immunotherapy and cancer treatment (8–10). A lack of understanding of the cellular complexity and the molecular heterogeneity of immune infiltration in gliomas limit our steps in developing more effective immunotherapies for gliomas (11, 12).

Cancer immunotherapy manipulates immune cells to attack cancer cells by enhancing immune response, including immune checkpoint inhibition and adoptive immunotherapy. Immunotherapy in gliomas is developing fast. For example, the generation of glioma stem cell-specific CAR macrophages primes locoregional immunity for postoperative glioblastoma therapy (13). Targeting immune checkpoint CD47 *via* blocking the CD47-SIRPα pathway has been studied a lot in glioma (14, 15). The metabolic rewiring from glycolysis to fatty acid oxidation (FAO) upregulates the expression of CD47 by the acetylation of NF-kB/RelA, and correspondingly, the FAO promotes the glioma growth with CD47-mediated immune escape (16). To overcome the blood–brain barrier (BBB), a BBB-permeable nanocapsule has been designed to send the anti-CD47 antibody to the brain, and the phagocytosis of macrophages and microglia has been improved (17). The combination therapy RRX-001 (targeting CD47) and temozolomide (a clinical drug for GBM) is in phase I clinical trial (NCT02871843). Besides targeting CD47 in glioma cancer immunotherapy, the GD2-directed chimeric antigen receptor (CAR) T cells are also in phase I clinical trial (NCT04196413) (18). Therefore, cancer immunotherapy targeting glioma is developing fast. However, targeting CD47 may bring side effects like anemia as red blood cells will be removed when CD47 is deficient in their surfaces. The glutaminyl-peptide cyclotransferase-like protein (QPCTL) belongs to a family of enzymes that catalyze the formation of pyroglutamate (pGlu) at the N-terminus of proteins by converting glutamine into pGlu residue (19), and this post-translation modification is named pyroglutamylation. The pyroglutamylation of CD47 on tumor cells is critical for its binding to SIPRα on macrophages (20, 21). Therefore, QPCTL plays a key role in phagocytosis checkpoint immunotherapy (22). A recent study has found that QPCTL regulates macrophage and monocyte abundance and inflammatory signatures in the TME (23). Loss of QPCTL limits chemokine function and reshapes myeloid infiltration to augment tumor immunity (22). Microglia and monocytes are the effector cells of CD47-SIRPα antiphagocytic axis disruption against glioblastoma (24). All of the previous studies indicate that QPCTL has close relations with immune cell infiltration. However, its relations with gliomas require further investigation.

In this study, we have comprehensively explored the diversified role of QPCTL in the progression of gliomas for the first time. We checked the relationship between QPCTL and immune infiltration as well as glioma clinical outcome. Moreover, the DNA methylation status of QPCTL and the association between QPCTL DNA methylation and glioma prognosis were evaluated. The relationship between the QPCTL expression and the glioma stemness was assessed. Our findings shed light on the potential roles of QPCTL in gliomas.

# 2 Materials and methods

## 2.1 Data source

All the mRNA expression data of QPCTL and the matching clinical pathologic information were first checked at The Cancer

Genome Atlas (TCGA) (https://genome-cancer.ucsc.edu/). The mRNA expression level was evaluated by transcripts per kilobase per million mapped reads (TPM). The Chinese Glioma Genome Atlas (CGGA) database (http://www.cgga.org.cn/index.jsp) contains clinical and sequencing data of over 2,000 brain tumor samples from Chinese cohorts and is equipped with a user-friendly web application for data storage and exploration (25). The expression of QPCTL level in CGGA was also explored.

## 2.2 The GEO database and the Human Protein Atlas

The GEO database is a comprehensive gene expression library in the National Center of Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/geo/). The GSE45921 was downloaded from the GEO database and validated for survival analysis. Single-cell sequencing data are also from GEO. The Human Protein Atlas (HPA, https://www.proteinatlas.org/) offers a broad amount of proteomic and transcriptome information of distinct human samples, which consists of cell, tissue, and pathology atlases. Protein immunohistochemistry in normal human tissues and tumor tissues of QPCTL (antibody: HPA040797) was obtained from this online website, and the QPCTL protein level is much higher in the glioma group than in normal groups.

## 2.3 Survival and statistical analysis

To investigate whether QPCTL expression level affects the clinical outcomes of glioma patients, we divided the cancer samples into two groups with high and low expression according to the median mRNA expression value of QPCTL and then we constructed a prognostic classifier using Kaplan–Meier (KM) survival curves to compare the survival disparities (https://kmplot.com/analysis/).

## 2.4 Univariate and multivariate logistic regression analysis

The univariate Cox regression analysis was used for calculating the association between the expression level of QPCTL and the patient's OS. Afterward, a multivariate analysis was used to assess if the QPCTL is an independent prognostic factor for glioma patient survival. QPCTL has statistical significance in Cox regression analysis when the $p$-value is less than 0.05.

## 2.5 Protein–protein interaction comprehensive analysis

Another online tool we used was the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) website (https://string-db.org/). The website hosts a big collection of integrated and consolidated protein–protein interaction data. After importing the QPCTL into the online tool STRING, we obtained the protein–protein interaction (PPI) network information. A confidence score > 0.7 was considered significant. Genemania (http://genemania.org/) is also a function-predicting website for interested genes. We explored the interaction network of QPCTL on this website too.

## 2.6 Immune infiltration analysis

To explore whether the expression of QPCTL is related to the tumor immune microenvironment, we studied the Tumor Immune Estimation Resource (TIMER). TIMER is a public website that covers 32 cancer types and encompasses 10,897 samples from the TCGA database, aiming to assess the abundance of immune inner infiltrates (http://cistrome.org/TIMER/). The correlation of QPCTL expression with the abundance of six types of infiltrating immune cells (CD8+ T cells, CD4+ T cells, B cells, dendritic cells, macrophages, and neutrophils) in glioma patients was evaluated *via* the TIMER database. The relationship between the expression of the QPCTL gene and the tumor purity was also displayed. The tumor immune infiltration analysis with more immune cells (total of 22) was analyzed *via* ssGSEA using the GSVA R package. Original data are from TCGA (https://portal.gdc.cancer.gov/).

## 2.7 Estimation of stromal and immune cells in malignant tumors using expression data

The Estimation of Stromal and Immune Cells in Malignant Tumors using Expression Data (ESTIMATE) is a package that uses gene expression data to predict the content of interstitial cells and immune cells in malignant tumor tissues (26). Based on the enrichment analysis of a single sample gene set (ssGSEA), the algorithm generates three scores: stromal score (recording the presence of stroma in tumor tissue), immune score (representing the infiltration of immune cells in tumor tissue), and estimated score (inferring tumor purity). The Stromal Score, Immune Score, and Estimate Score of QPCTL can be obtained at http://www.sangerbox.com/.

## 2.8 Gene correlation analysis

The Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/index.html) is an online database that consists of 9,736 tumors and 8,587 normal samples from TCGA and GTEx data. It focuses on the analyses of the expression of RNA sequencing. Gene Classes and Isoform Classes exhibit the types of 60,498 genes and 198,619 isoforms. In the GEPIA database, the relation of QPCTL expression with multiple markers for immune cells was investigated. The $x$-axis was presented with the level of QPCTL expression, and the $y$-axis was plotted with other genes of interest. In addition, we used TIMER data to validate the genes that were of significant correlation with QPCTL expression in the GEPIA web.

## 2.9 Identification and enrichment analysis of DEGs

Distinct QPCTL subtype-related DEGs were identified using the "limma" package in R (adj.$p < 0.05$ and $|\log_2 FC| > 2$) (27). The functional and enrichment pathways (GO and KEGG) of DEGs were further explored using the "cluster profiler" package in R (28).

## 2.10 Function and pathway analysis by gene set enrichment analysis

Differentially expressed genes (DEGs) between low- and high-QPCTL expression groups were identified using the DESeq 2 R package. In this study, GSEA was performed using the ggplot2 R package to demonstrate the significant functions and pathways between the two groups. The expression level of QPCTL was used as a phenotype label. An adjusted $p$-value < 0.05, normalized enrichment score (|NES|) > 1.5, and false discovery rate (FDR) < 0.05 were considered a significant difference.

## 2.11 QPCTL gene essential analysis *via* CRISPR screen

To explore whether QPCTL is essential for glioma cells, we first checked BioGRID (https://thebiogrid.org/), which listed the CRISPR screen conducted in the literature. We also checked the cell viability when the QPCTL gene is knocked out from DepMap (https://depmap.org/).

## 2.12 Relation between QPCTL gene and immune checkpoints

From UCSC (https://xenabrowser.net/), we have downloaded a standardized pan cancer dataset: TCGA TARGET GTEx (PANCAN, N = 19,131, G = 60,499). Furthermore, we extracted the expression data of the marker genes of the ENS00000011478 (QPCTL) gene and two types of immune checkpoint pathway genes ($n = 60$) [Inhibitory (24), Stimulatory (29)] from the literature. The immune landscape of cancer in each sample was listed (30). Furthermore, we screened samples from various tumors. We also filtered all normal samples and further performed log2($x$+0.001) transformation on each expression value. Next, we calculated the Pearson correlation between ENSG00000011478 (QPCTL) and the marker genes of five immune pathways. All the above can be realized from http://sangerbox.com/directly.

## 2.13 QPCTL expression in scRNA-seq

Tumor Immune Single-cell Hub 2 (TISCH2) is an scRNA-seq database, which aims to characterize TMEs at single-cell resolution. TISCH2 (http://tisch.comp-genomics.org) has collected 187 sets of high-quality tumor single-cell transcriptome data and corresponding patient information from GEO and ArrayExpress (31). The data cover 50 cancer types, including 6 million cells from more than 1,500 patients. Among them, 40 sets of TISCH2 data are single-cell transcriptome data under different treatment conditions, including immunotherapy, chemotherapy, targeted therapy, and combination therapy.

## 2.14 Relation between the expression of QPCTL and cancer immunotherapy

Tumor Immune Dysfunction and Exclusion (TIDE, website: http://tide.dfci.harvard.edu/) is a computational framework developed to evaluate the potential of tumor immune escape from the gene expression profiles of cancer samples. The highly scored genes in TIDE signatures also present potential regulators of tumor immune escape and resistance to cancer immunotherapies (32, 33).

## 2.15 Gene enrichment analysis

The highly expressed genes in QPCTL-high groups were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis at https://metascape.org.

## 2.16 The combination analysis of GO/KEGG and LogFC

We checked the relations between the expression of QPCTL and the biological process (BP), cellular component (CC), and molecular function (MF) *via* GO analysis; then, we conducted KEGG pathway analysis. On the basis of the GO and KEGG analyses, with the data of logFC, we calculated the $z$ score of each item.

## 2.17 The DNA methylation level of QPCTL in glioma and normal tissue

The DNA methylation level of QPCTL in LGG and GBM was explored from DiseaseMeth (http://bio-bigdata.hubmu.edu.cn/diseasemeth/). This dataset is focused on the methylation of genes in different cancers. The methylation of QPCTL in glioma and normal tissue was checked in DiseaseMeth and verified at https://mexpress.be/. The QPCTL DNA methylation level of Chinese glioma patients was checked at CGGA (http://www.cgga.org.cn/index.jsp).

## 2.18 The correlation between the DNA methylation level of QPCTL and the survival of glioma patients

The correlation between the DNA methylation level of QPCTL and the glioma patients' survival was checked at https://ngdc.cncb.ac.cn/ewas/datahub, and for Chinese glioma patients, it was checked at the CGGA database (http://www.cgga.org.cn/index.jsp).

## 2.19 Relation between QPCTL gene and cancer stemness

We checked the Pearson correlation between the expression of QPCTL and cancer stemness in the pan-cancer atlas by http://sangerbox.com. The stemness was calculated based on the RNA-based stemness scores derived by the stemness group, the DNA methylation-based stemness scores derived by the stemness group, and other stemness probes (219 probes).

# 3 Results

## 3.1 Patient characteristics

We first checked the RNA-sequencing data and the clinical prognostic information of glioma from the TCGA database; 699 glioma samples and 5 normal samples were enrolled in the analysis. A total of 349 patients had a low expression of QPCTL and 350 patients had a higher expression of QPCTL. We summarized the clinical information and their relations with the expression of QPCTL in Table 1.

## 3.2 QPCTL expression is higher in tumor samples than in normal tissues

The mRNA expression of QPCTL in pan-cancer was browsed from the TIMER database (Figure 1A). QPTCL expression was significantly higher in tumor samples than in the normal tissues of LGG (Figure 1B), the combination of both GBM and LGG samples (GBMLGG) (Figure 1C), and glioblastoma multiforme (GBM) (Figure 1D) in TCGA, which was also validated in GEO

TABLE 1 The clinical characteristics of the glioma patients in the test and validation sets.

| Characteristics | Low expression of QPCTL | High expression of QPCTL | *p*-value | Statistic | Method |
|---|---|---|---|---|---|
| *n* | 349 | 350 | | | |
| WHO grade, *n* (%) | | | 8.13459E-38 | 170.8042165 | Chisq test |
| G2 | 166 (26.1%) | 58 (9.1%) | | | |
| G3 | 134 (21%) | 111 (17.4%) | | | |
| G4 | 14 (2.2%) | 154 (24.2%) | | | |
| IDH status, *n* (%) | | | 1.55214E-41 | 182.2648408 | Chisq test |
| WT | 39 (5.7%) | 207 (30%) | | | |
| Mut | 308 (44.7%) | 135 (19.6%) | | | |
| Age, *n* (%) | | | 1.11484E-10 | 41.6089076 | Chisq test |
| ≤60 | 312 (44.6%) | 244 (34.9%) | | | |
| >60 | 37 (5.3%) | 106 (15.2%) | | | |
| Gender, *n* (%) | | | 0.464038535 | 0.536135964 | Chisq test |
| Female | 144 (20.6%) | 154 (22%) | | | |
| Male | 205 (29.3%) | 196 (28%) | | | |
| Histological type, *n* (%) | | | 2.96141E-35 | 163.6679898 | Chisq test |
| Glioblastoma | 14 (2%) | 154 (22%) | | | |
| Astrocytoma | 106 (15.2%) | 90 (12.9%) | | | |
| Oligoastrocytoma | 89 (12.7%) | 46 (6.6%) | | | |
| Oligodendroglioma | 140 (20%) | 60 (8.6%) | | | |
| Primary therapy outcome, *n* (%) | | | 0.024957804 | 9.352113267 | Chisq test |
| PD | 57 (12.3%) | 55 (11.8%) | | | |
| SD | 100 (21.5%) | 48 (10.3%) | | | |
| PR | 43 (9.2%) | 22 (4.7%) | | | |
| CR | 93 (20%) | 47 (10.1%) | | | |
| 1p/19q codeletion, *n* (%) | | | 1.36339E-21 | 91.10366307 | Chisq test |
| Non-codel | 208 (30.1%) | 312 (45.1%) | | | |
| Codel | 141 (20.4%) | 31 (4.5%) | | | |

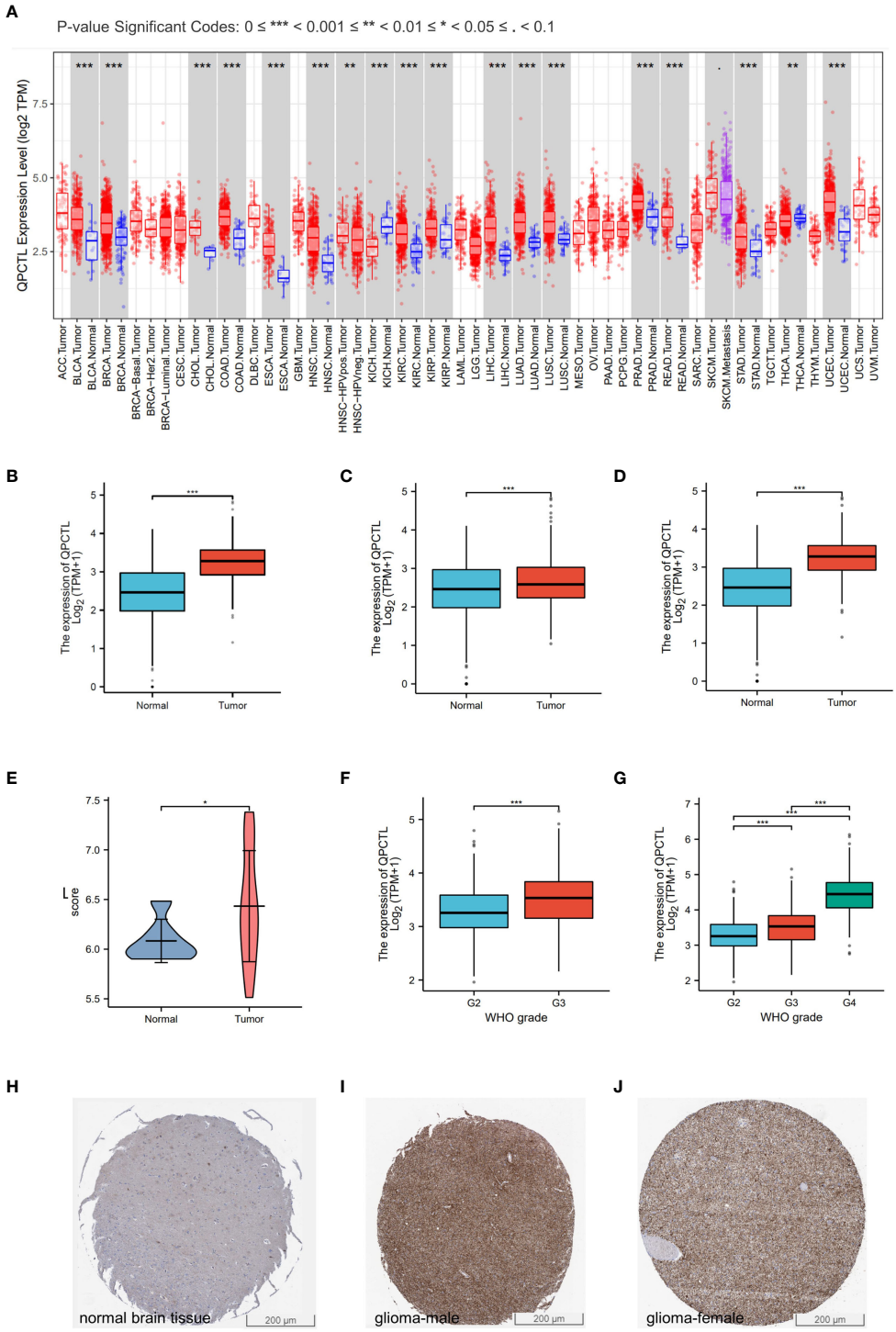


FIGURE 1
Higher QPCTL expression in tumor samples than that in normal tissues. **(A)** QPCTL expression at the mRNA level in pan-cancers, data from the TIMER database. **(B–D)** TCGA data show different QPCTL expression at mRNA levels in normal tissue and LGG **(B)**, GBMLGG **(C)**, and GBM **(D)**. **(E)** Different QPCTL expression in normal and low-grade glioma in the GEO database. **(F, G)** The expression of QPCTL in different stages of glioma in LGG **(F)** and GBMLGG **(G)**. **(H–J)** QPCTL protein expression in normal brain tissue and glioma tissue, data from the Human Protein Atlas. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

database GSE45921 (Figure 1E). As shown in Figure 1F (LGG) and Figure 1G (GBMLGG), QPCTL expression level was observed in the higher tumor stage, as well as in higher grade and glioma classification, indicating that the expression of QPCTL is positively correlated with the glioma pathological stages. Since GBM is a grade IV (stage 4) glioma, the result in Figure 1G of G4 represents GBM data. Subsequently, the protein level of QPCTL was explored in the HPA, and it is shown that the

QPCTL protein is higher in gliomas than in normal tissue (Figures 1E–G). Moreover, there is no significant difference between QPCTL expression and age and gender, but a higher expression of QPCTL indicates a higher grade of glioma. All the above results can also be validated in the CGGA (Supplementary Figure 1).

## 3.3 Higher QPCTL predicts shorter survival in glioma

As per the KM analysis of glioma patients' survival, patients with higher QPCTL expression indicate a shorter overall survival (OS) in the test cohort in LGG (Figure 2A), GBMLGG (Figure 2B),

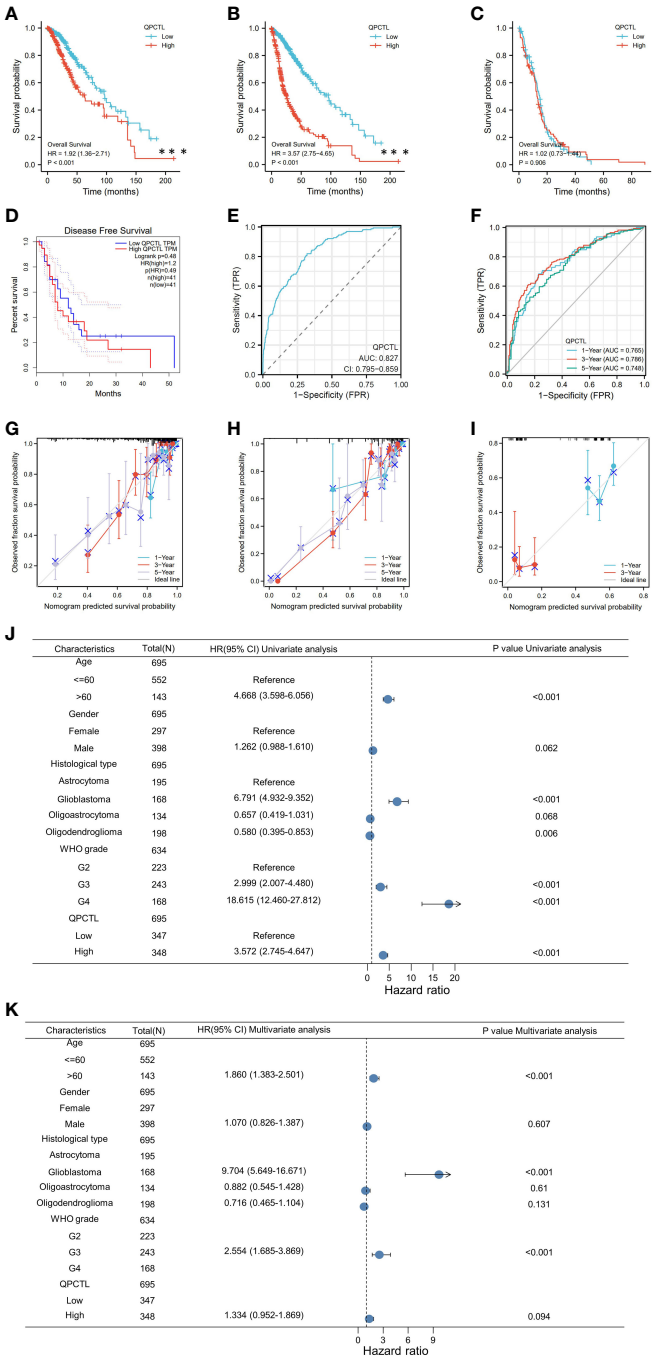


FIGURE 2
Higher QPCTL mRNA expression showing shorter OS in glioma. **(A–C)** The overall survival of QPCTL high and low expression of LGG patients **(A)**, GBMLGG **(B)**, and GBM **(C)** from the TCGA database. **(D)** The disease-free survival of low-grade glioma patients with high and low QPCTL expression. **(E)** The receiver operating characteristic curve (ROC) of QPCTL in GBM in TCGA. **(F)** The time-dependent ROC curve of GBMLGG. **(G–I)** The calibration curve of 1-, 3-, and 5-year survival of LGG patients **(G)**, GBMLGG **(H)**, and GBM **(I)** patients. **(J)** Univariate regression analysis of QPCTL and other clinicopathologic parameters with OS in LGG. **(K)** The multivariate regression analysis of QPCTL and other clinicopathologic parameters with OS in LGG.

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and GBM (Figure 2C). The disease-free survival (DFS) of LGG patients from the GEPIA database also indicated that the higher expression of QPCTL was positively related to a shorter survival (Figure 2D). All the above results were found in CGGA data as well (Supplementary Figure 2).

We then checked whether QPCTL can be a predictor for clinical diagnosis since the evaluation of a risk prediction model is characterized in terms of discrimination and calibration. The receiver operating characteristic (ROC) curve, also named the sensitivity curve, is widely used for evaluating model discrimination and the performance of diagnostic tests. As shown in Figure 2E, the ROC curve indicates that QPCTL can be a prognostic marker of GBMLGG. The time-dependent ROC curve of GBMLGG is shown in Figure 2F. The calibration curve of 1-, 3-, and 5-year survival of LGG (Figure 2G) and GBM (Figure 2H) and 1- and 3-year survival of GBM (Figure 2I) is shown clearly. There are no data on the 5-year survival of GBM since it is a grade IV glioma and the mid-survival is 15 months. The coincidence between the actual incidence and the predicted incidence is the highest in the 5-year survival model. In the univariate Cox model, both higher QPCTL expression and high pathologic grade and stage were negative predictors for OS in glioma patients (Figure 2J). Intriguingly, in multivariate regression analysis, QPCTL expression was an independent factor corrected with OS in LGG patients (Figure 2K).

## 3.4 Differentiated gene analysis of QPCTL-high and -low groups in GBMLGG patients

From the survival analysis and prognosis modal, we found that QPCTL is a more suitable marker for GBMLGG. To explore the possible role of QPCTL in the carcinogenesis of GBMLGG, we divided the patient samples according to QPCTL expression, and the DEG analysis was conducted in QPCTL-high and QPCTL-low groups. The volcano plot in Figure 3A shows the DEGs in both groups, and Figure 3B shows the rank of DEGs. To check how these DEGs are functioning in the biological pathways and cellular functions, we next performed GO and KEGG analysis; as shown in Figure 3C, the high-expressed genes in QPCTL-high groups are enriched in IL-17 signal pathway and cytokine–cytokine receptor interaction. The low-expressed genes in QPCTL-low groups are enriched in nicotine addiction and neuroactive ligand–receptor interaction (Figure 3D). The gene set enrichment analysis (GSEA) shows the high-expressed genes enriched in cytokine–receptor interaction and cell cycle pathway (Figures 3E, F). We verified the analysis at Metascape and found that the enrichment analysis was the highest enriched in the inflammation pathway (Figure 3G). The combinational analysis of GO/KEGG and logFC was also conducted, as shown in Figures 3H, I; the key pathways and involved genes are shown in the figures. Most of them are related to cytokines or other immune function types. We thus checked the relation between QPCTL and the top 8 expressed genes with fold

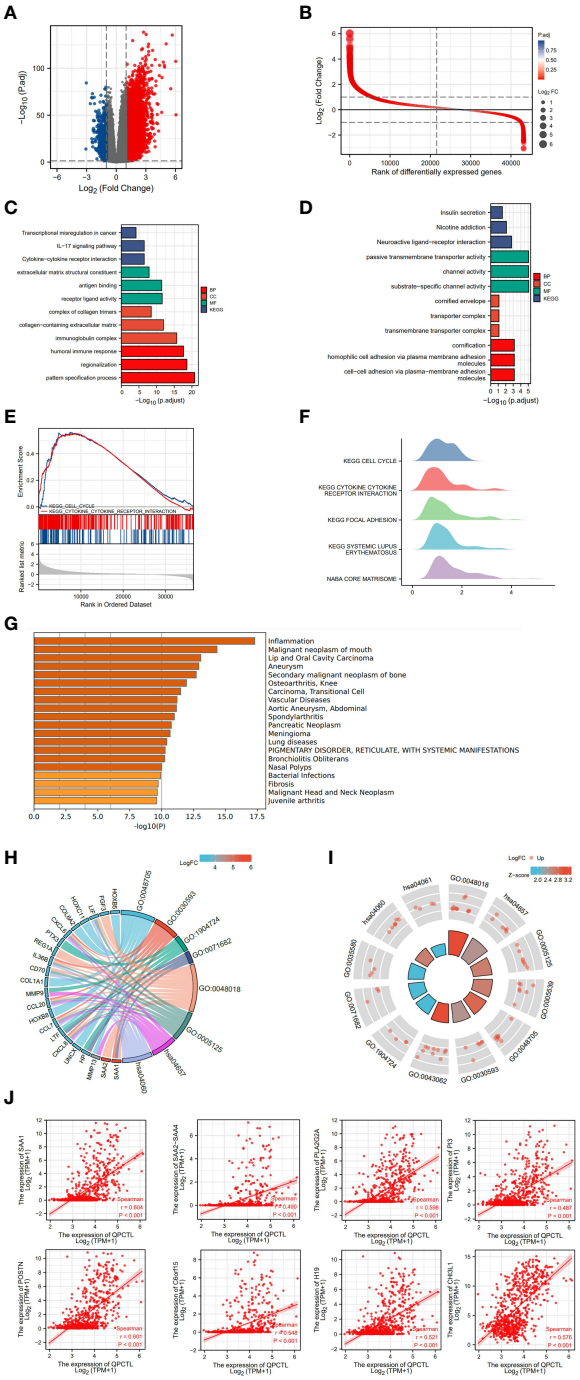



FIGURE 3
Differentiated gene analysis of QPCTL-high and -low groups in LGG patients. **(A)** The volcano plot shows the DEGs in QPCTL-high and -low groups. **(B)** The rank of differentially expressed genes. **(C)** The GO and KEGG analysis of high-expressed genes in QPCTL-high groups. **(D)** The GO and KEGG analysis of low-expressed genes in QPCTL-low groups. **(E)** The GSEA or the DEGs. **(F)** The GSEA in the ridge plot of DEGs. **(G)** The GO analysis of highly expressed genes *via* the website https://metascape.org/. **(H, I)** The combinational analysis of GO and KEGG of high-expressed genes in the QPCTL-high group. **(J)** The Pearson correlations between QPCTL and the top eight highly expressed genes in DEGs.

change more than 4 times and found that the expression of QPCTL was positively correlated with their expression (Figure 3J).

## 3.5 Constructing protein interaction networks

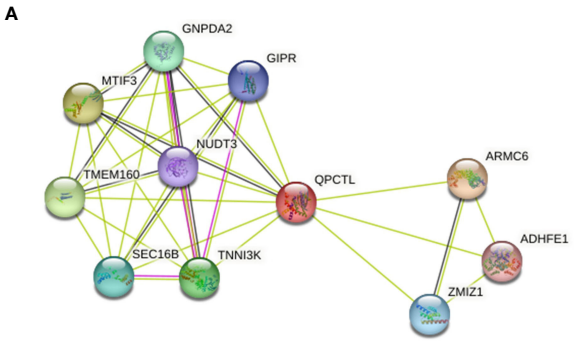
The protein interaction network is necessary for the molecular mechanism of malignancy. Therefore, we used the STRING website to analyze the PPI network of the QPCTL protein to determine their interactions in the progression of glioma. The top 10 proteins and corresponding gene names, annotations, and scores are listed in Figure 4A. We also checked the protein interaction in Geneamia and obtained the network as shown in Figure 4B. The interaction proteins are different from the two other websites, probably due to their different calculation and original database. All the PPI networks explored on the two websites are quite different from our previous analysis; to focus on our study, we used our previous GO and KEGG analysis results to conduct the succeeding analysis; thus, we will focus on the expression of QPCTL and cytokines in the immune cells and the immune cell infiltration in gliomas.

## 3.6 Correlation analysis between QPCTL expression and infiltrating immune cells

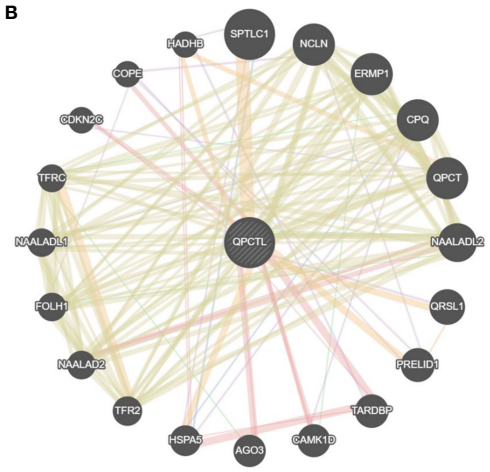
We have a general view of the expression of QPCTL in all different immune cells, as shown in Figure 5A; the single-cell sequencing data from GEO indicate that QPCTL expresses higher in monocyte/macrophages. Single-cell sequencing of GSE131928 (Figure 5B) and GSE135437 (Figure 5C) shows that QPCTL expresses most in mono/macroglia and microglia, respectively.

Tumor-infiltrating lymphocytes are closely associated with the survival of patients with various cancers. Therefore, we analyzed the correlation of QPCTL expression with six kinds of infiltrating immune cells, namely, $CD8^+$ T cells, $CD4^+$ T cells, B cells, dendritic cells, macrophages, and neutrophils, and tumor purity from the TIMER website. The results showed that the expression level of QPCTL has no obvious correlation with the infiltration levels of these six immune cells in GBMLGG (Figure 5D) and in GBM (Figure 5E).

Infiltrating stromal and immune cells form the major fraction of normal cells in tumor tissue and not only perturb the tumor signal in molecular studies but also have an important role in cancer biology (26). They are important factors in the TME. Therefore, we




| Gene symbol | Annotation | Score |
|---|---|---|
| ARMC6 | Armadillo repeat-containing protein 6 | 0.726 |
| MTIF3 | Translation initiation factor IF-3 | 0.698 |
| TMEM160 | Transmembrane protein 160 | 0.681 |
| TNNI3K | Serine/threonine-protein kinase TNNI3K | 0.681 |
| GNPDA2 | Glucosamine-6-phosphate deaminase 2 | 0.679 |
| SEC16B | Protein transport protein Sec16B | 0.654 |
| ZMIZ1 | Zinc finger MIZ domain-containing protein 1 | 0.653 |
| GIPR | Gastric inhibitory polypeptide receptor | 0.653 |
| NUDT3 | Diphosphoinositol polyphosphate phosphohydrolase 1 oligophosphates | 0.652 |
| ADHFE1 | Hydroxyacid-oxoacid transhydrogenase | 0.637 |




| Gene | Description | Rank |
|---|---|---|
| SPTLC1 | serine palmitoyltransferase long chain base subunit 1 | 1 |
| NCLN | nicalin | 2 |
| ERMP1 | endoplasmic reticulum metallopeptidase 1 | 3 |
| CPQ | carboxypeptidase Q | 4 |
| QPCT | glutaminyl-peptide cyclotransferase | 5 |
| NAALADL2 | N-acetylated alpha-linked acidic dipeptidase like 2 | 6 |
| QRSL1 | glutaminyl-tRNA amidotransferase subunit QRSL1 | 7 |
| PRELID1 | PRELI domain containing 1 | 8 |
| TARDBP | TAR DNA binding protein | 9 |
| CAMK1D | calcium/calmodulin dependent protein kinase ID | 10 |

FIGURE 4
QPCTL–interaction proteins. **(A)** Annotation of QPCTL-interacting proteins and their co-expression scores, data from STRING website (https://string-db.org/). **(B)** Annotation of QPCT–interaction proteins and their correlation scores, data from Geneaminia website (http://genemania.org/).

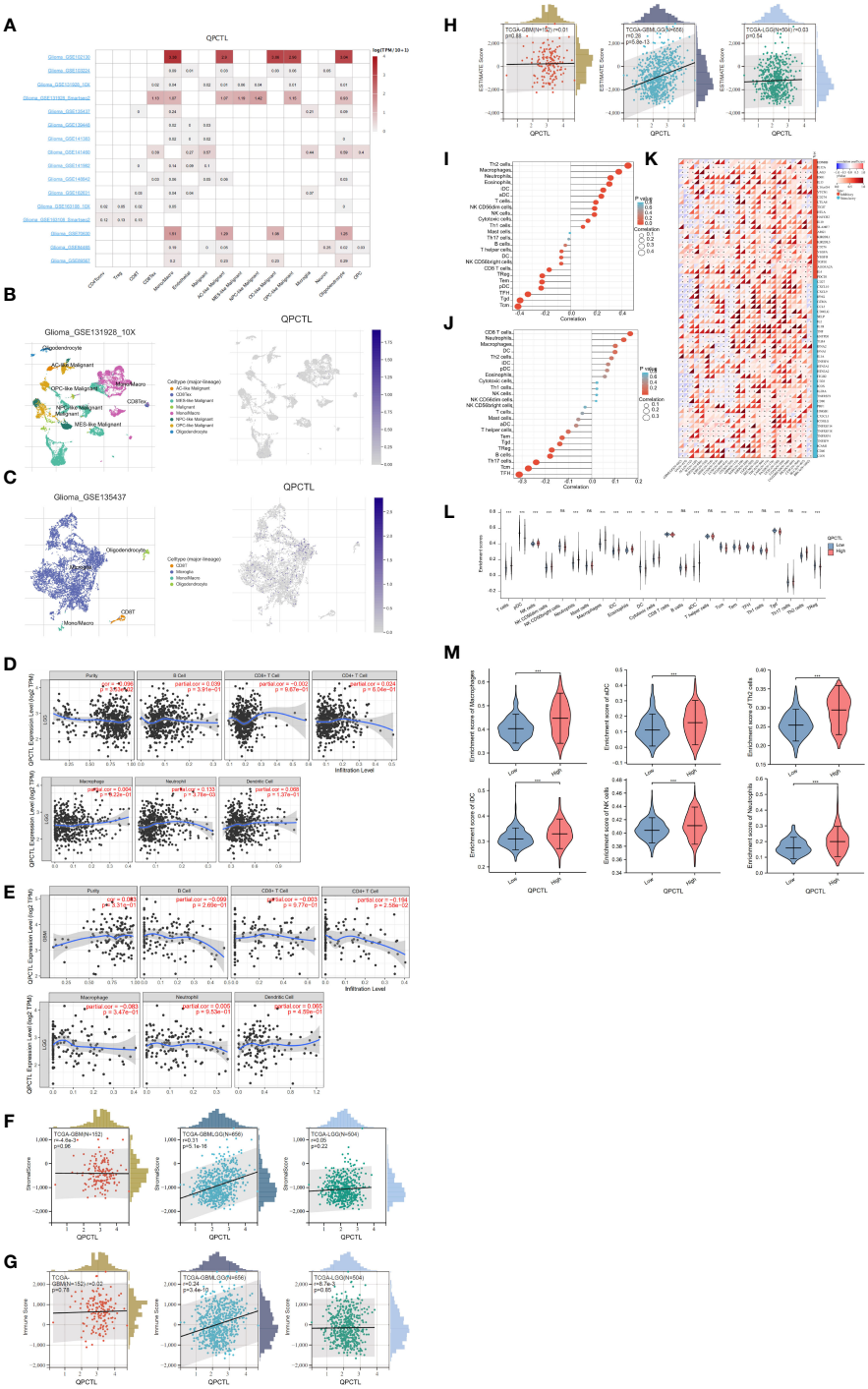


FIGURE 5
Correlation analysis between QPCTL expression and infiltrating immune cells **(A)** The expression of QPCTL in different immune cells, data from GEO data single-cell sequencing. **(B)** QPCTL expression in the glioma single-cell sequencing data (GSE131928). **(C)** QPCTL expression in the glioma single-cell sequencing data (GSE135437). **(D)** Correlation of QPCTL expression with infiltrating immune infiltration in LGG from TIMER database. **(E)** Correlation of QPCTL expression with infiltrating immune infiltration in GBM from the TIMER database. **(F)** Relation between QPCTL expression and stromal score in GBM, GBMLGG, and LGG. **(G)** Correlation of QPCTL expression with infiltrating immune score in GBM, GBMLGG, and LGG from the TCGA database. **(H)** Correlation of QPCTL expression with ESTIMATE score in GBM, GBMLGG, and LGG from the TCGA database. **(I)** The immune cell infiltration in the QPCTL-high and -low groups. **(J)** The immune cells with higher infiltration in QPCTL-high groups. **(K)** The correlation between the expression of QPCTL and the immune checkpoints. **(L)** The enrichment scores of immune cells in QPCTL-high and -low group. **(M)** The enrichment score of macrophage, iDC (immature DC cell), aDC (activated DC cell), Th2 cell, and Neutrophils in the QPCTL-high and -low groups. Data from http://sangerbox.com/, TCGA TARGET GTEx (PANCAN, N = 19,131, G = 60,499). $^{**}p < 0.01$, $^{***}p < 0.001$.

also use ESTIMATE data to check the Stromal Score (Figure 5F), the Immune Score (Figure 5G), and the Estimate Score (Figure 5H) of QPCTL in LGG, GBMLGG, and GBM. The relations between the expression and the above scores indicate that the expression of QPCTL was positively related to the immune infiltration in GBMLGG instead of LGG or GBM. We also explored more immune cell infiltration (a total of 22 immune cells) in Figure 5I for GBMLGG and Figure 5J for GBM *via* ssGSEA. It shows that the expression of QPCTL in GBMLGG was positively correlated with Th2 cells and macrophages, while it was positively correlated with $CD8^+$ T cells and neutrophils in GBM.

QPCTL as an immune checkpoint regulator has been verified as it is a critical enzyme to catalyze the pyroglutamylation of CD47 (20, 34). High expression of checkpoints suppresses the immune response and reduces the efficacy of immunotherapy; we then explore the relationship between the expression of QPCTL and the expression of checkpoint genes in pan-cancer. As shown in Figure 5K, the expression of QPCTL has positive relations with many immune checkpoints. In addition, to explore the relationship between the expression of QPCTL and immune cell infiltration, we checked the infiltration of the immune cells in the QPCTL-high and -low groups (Figure 5L) and found that the innate immune cell infiltration was more than the adaptive immune cell infiltration in the QPCTL-high groups (Figure 5M).

### 3.7 The DNA methylation status of QPCTL and its correlation with glioma patients' survival

The DNA methylation status of the key gene has been widely used as a biomarker in cancer; we then checked the methylation status of QPCTL in gliomas. As shown in Figure 6A (LGG) and Figure 6B (GBM), the DNA methylation of QPCTL promoters in glioma patients is much lower than that in the normal samples. This result is consistent with the mRNA expression of QPCTL in glioma patients since the lower DNA methylation indicates higher gene expression. The DNA methylation level of QPCTL becomes much lower from grade II to grade IV (Supplementary Figure 3), indicating that the DNA methylation of QPCTL predicts the pathological process of glioma. We then checked the correlation between the DNA methylation of QPCTL and the glioma patients' survival and found higher methylation level of QPCTL had a longer survival in GBM (Figure 6C, Supplementary Figure 4), but it varies in different stages of gliomas (Supplementary Figure 4). In summary, the DNA methylation of QPCTL is negatively related to its expression and positively related to glioma patients' survival.

### 3.8 QPCTL is essential for glioma cell proliferation and positively correlated with glioma stemness

To explore the importance of QPCTL in glioma cells, we checked both the DepMap database and the BioGRID database, as shown in Supplementary Table 1 (data from BioGRID) and Supplementary Table 2 (data from DepMap); both CRISPR databases show that QPCTL is the essential gene for glioma cells' proliferation. Among the 74 glioma cells subjected to CRISPR whole-genome screening, 63 cells' proliferation was seriously suppressed (Supplementary Table 1), indicating that QPCTL was essential for glioma cells' proliferation and tumor growth. Mechanistically, knocking out QPCTL attenuated tumor growth *via* disrupting monocyte homeostasis (22). As shown in Figures 7A, B, based on single-cell sequencing data from GEO (GSE 141460, GSE 139448), the expression of QPCTL is higher in the malignant glioma clusters than in other clusters, indicating that the expression of QPCTL has close positive relations with the glioma tumor progression.

Cancer stem cells drive tumor initiation, expansion, and recurrence following chemotherapy (35), and higher cancer stem cells predict poor prognosis. Therefore, we explored the expression of QPCTL and the cancer stemness in pan-cancer including gliomas. As shown in Figure 7C, the expression of QPCTL has a significant positive correlation with GBMLGG, and the correlation coefficient was the highest among all pan-cancers in TCGA, indicating that the QPCTL may also affect the glioma cancer stemness and promote tumor progression.

## 4 Discussion

In this study, we briefly explored the mRNA expression of QPCTL in gliomas and found that it was expressed higher in gliomas than in normal tissue, and the higher expression of QPCTL predicts a poorer prognosis of glioma. QPCTL can serve as an independent prognostic factor for OS. To further explore the function of QPCTL, we divided the gliomas into QPCTL-high and -low groups and then analyzed the DEGs. Interestingly, the GO/KEGG analysis of the QPCTL-high groups was enriched in the immunity-related pathways, especially the cytokine–cytokine receptor pathways. Since QPCTL expression is closely related to cytokine and chemokine function and modulation of QPCTL synergized with anti-PD-L1 expands $CD8^+$ T cells and limits tumor growth, targeting QPTCL constitutes an effective approach for myeloid cell-targeted cancer immunotherapy; it prevents monocyte migration across inflammatory conditions (22). Knocking out QPCTL suppressed tumor growth *via* disrupting monocyte homeostasis, and knocking out QPCTL together with blocking immune checkpoint CD47 has been verified to improve the killing of T-acute lymphoblastic leukemia cells (29, 36). In a recent study, it was found that CD47 deficiency or pyroglutamylation inhibition increases myeloid cell-mediated T-ALL killing; QPCTL deficiency reduced 78% of the binding between CD47 and SIRPα (36). The regulation function of QPCTL of monocyte infiltration under inflammatory conditions had been demonstrated a long time ago (37). In renal cell carcinoma, the high expression of QPCTL leads to sunitinib resistance by promoting angiogenesis (38). In chronic kidney disease, chronic treatment with QPCTL inhibitor PQ529 is a novel and effective

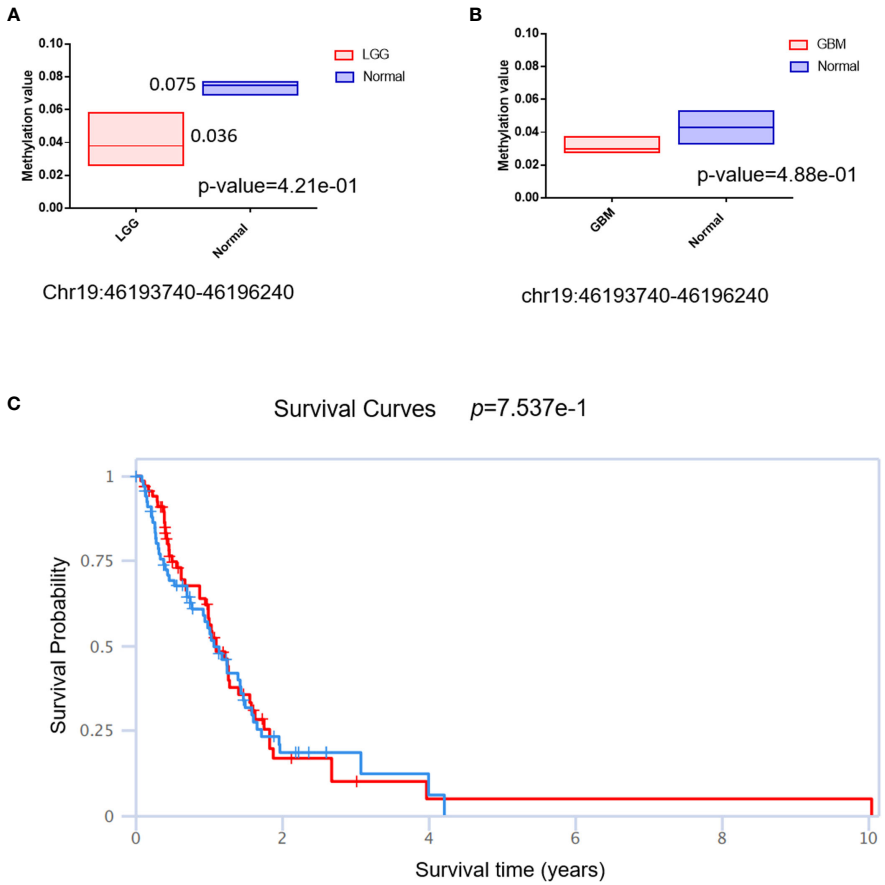



FIGURE 6
The DNA methylation status of QPCTL and its relationship with the glioma patients' survival. **(A)** The DNA methylation level of QPCTL in LGG patients and normal people. **(B)** The DNA methylation level of QPCTL in GBM patients and normal people. **(C)** The correlation between the DNA methylation of QPCTL and the GBM patients' survival.

approach for glomerulonephritis (39). Aside from cancer, targeting QPCTL has been widely studied in Alzheimer's disease as it modified the forms of amyloid-β (Aβ) oligomers to catalyze the generation of pyroglutamate-Aβ (AβpE3), which is more neurotoxic (21, 40). The small-molecule varoglutamstat (formerly PQ912) that inhibits the activity of QPCTL is currently in phase IIb clinical trials (41).

To further analyze the relationship between the expression of QPCTL and the TME, we conducted an immune microenvironment analysis and found that there was a significant difference in the proportion of immune cells between the QPCTL-high and -low expression groups. The infiltration level of different immune cells was significantly correlated with the expression of QPCTL. Our analysis indicates that the adaptive immune cell infiltration in the QPCTL-high groups was worse than that in the QPCTL-low groups. Furthermore, the expression of QPCTL was positively correlated with SAA1, POSTN, PLA2G2A, SAA2, C6orf15, H19, PI3, etc., whose expression also affects cancer progress. It has been found that the higher expression of SAA1 predicts advances and malignancies in various cancers (42–44). POSTN expression is crucial for the angiogenesis of gliomas (45–47).

The DNA methylation status of QPCTL and its relationship with glioma patients' survival indicate that the DNA methylation status can be a predictor for the glioma pathological process. The highly positive correlation between QPCTL expression and glioma stemness also indicates that the expression of QPCTL has close relation with the progression of gliomas.

The advantage of our study is that it provides a novel biomarker of glioma, especially for GBMLGG, and we have explored the working mechanisms from different aspects. We found that the chemokine modulation role of QPCTL in the TME affects the immune responses. Owing to the specific role of QPCTL in the binding between CD47 and SIRPα, targeting QPCTL instead of targeting CD47 in gliomas overcomes the side effects of targeting CD47. It can be easily translated into an easy-to-use clinical assay to identify the efficacy of targeting QPCTL and the potential immunotherapy responders. The small-molecule inhibitors for targeting QPCTL to cure malignancies such as ISM004-1057D are in the pre-clinical stage now. However, there are still limitations in our research. For example, we did not verify the functions of QPCTL in all the gliomas by experiments; we just cited the previous CRISPR result. We will further study this gene and the deep mechanisms of glioma progression in the coming project.

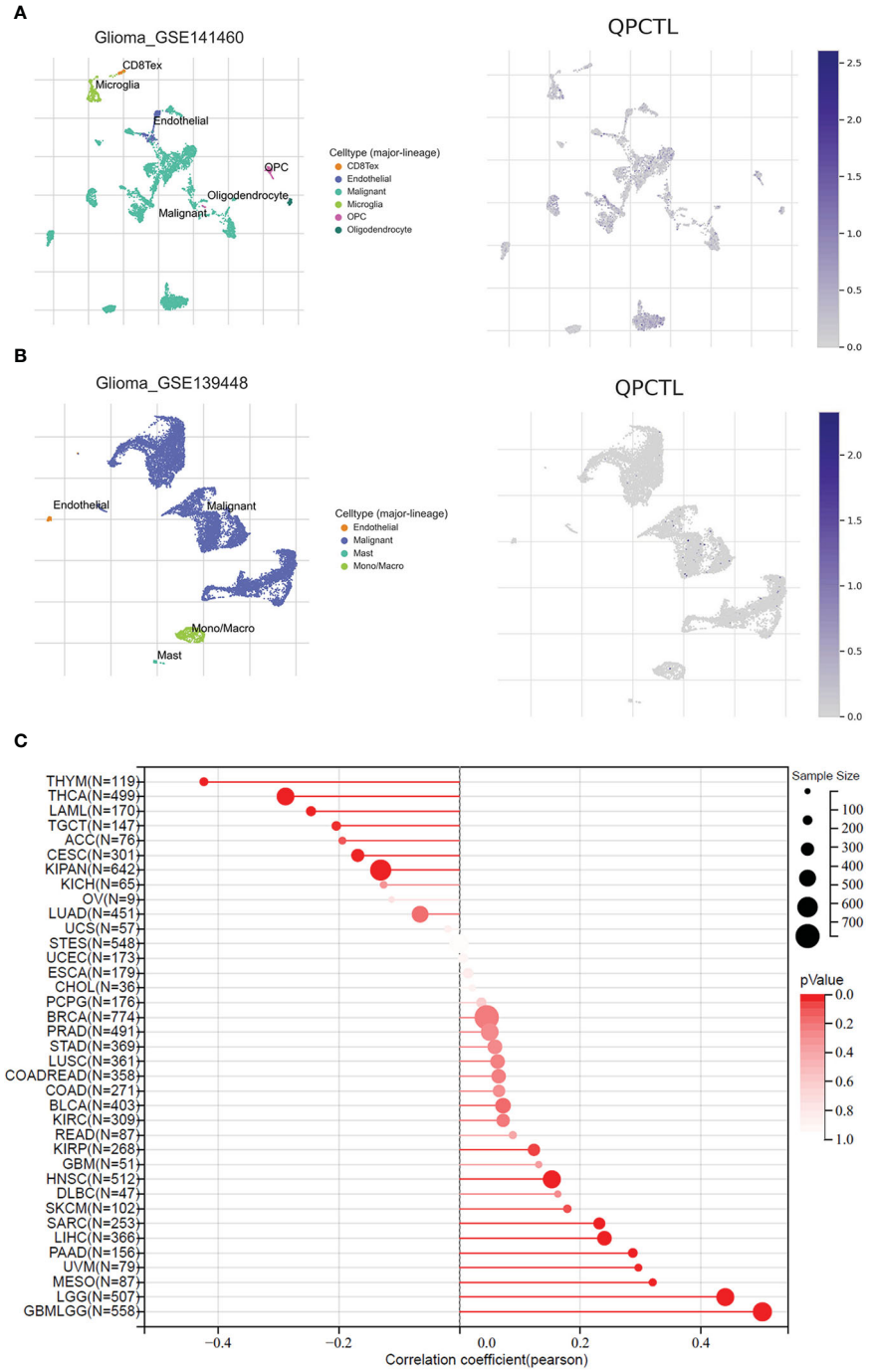



FIGURE 7
QPCTL is essential for glioma cell proliferation and positively correlated with glioma stemness. **(A)** The expression of QPCTL in GSE 141460, data from GEO single-cell sequencing. **(B)** The expression of QPCTL in GSE 139448, data from GEO single-cell sequencing. **(C)** The correlation between the expression of QPCTL and the cancer stemness, data from TCGA.

## 5 Conclusion

In summary, our analysis revealed that the high expression of QPCTL is positively related to unfavorable outcomes in glioma. Further bioinformatic analysis indicates that QPCTL modulates the cytokine and cytokine–receptor pathway and thus affects the adaptive immune cells' infiltration, which enhances the proliferation and progression of the cancer cells. Moreover, the DNA methylation status of QPCTL is closely associated with the patient's survival. QPCTL is the essential gene for glioma cancer cells and its expression has a significantly positive correlation with glioma cancer stemness. Targeting QPCTL overcomes the side effects of targeting CD47; therefore, targeting QPCTL will be a promising strategy in glioma cancer treatment.

## 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.

## Author contributions

KZ, YFS, and YL organized article writing and critically modified the manuscript. SL and YHS modified the manuscript. SY, XC, HY, and L-LW edited the manuscript and sourced literature. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

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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.2023.1166377/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The mRNA expression of QPCTL in the CGGA database **(A)** The expression of QPCTL in different histology of glioma. **(B)** The expression of QPCTL in different grades of glioma. **(C-D)** The expression of QPCTL in IDH-mutant and wild-type gliomas. **(E-F)** the expression of QPCTL in different genders of glioma patients. **(G-H)** the expression of QPCTL in different ages of glioma patients. (LGG, lower-grade glioma; GBM, glioblastoma; A, astrocytoma; O, oligodendroglioma; OA, oligo-astrocytoma; AOA, anaplastic oligo-astrocytoma; AA: anaplastic astrocytoma; rGBM: recurrent glioblastoma; rAA, recurrent anaplastic astrocytoma; rA, recurrent astrocytoma; AO, anaplastic oligodendroglioma; rAO, recurrent anaplastic oligodendroglioma; rO, recurrent oligodendroglioma; rAOA, recurrent anaplastic oligo-astrocytoma.)

SUPPLEMENTARY FIGURE 2
The correlation between QPCTL expression and glioma patients' survival in the CGGA database. **(A)** The correlation between the expression of QPCTL and the survival of primary glioma patients in all WHO grades. **(B)** The correlation between the expression of QPCTL and the survival of recurrent glioma patients in all WHO grades. **(C)** The correlation between the expression of QPCTL and the survival of primary glioma patients in grade II. **(D)** The correlation between the expression of QPCTL and the survival of recurrent glioma patients in grade II. **(E)** The correlation between the expression of QPCTL and the survival of primary glioma patients in grade III. **(F)** The correlation between the expression of QPCTL and the survival of recurrent glioma patients in grade III. **(G)** The correlation between the expression of QPCTL and the survival of primary glioma patients in grade IV. **(H)** The correlation between the expression of QPCTL and the survival of recurrent glioma patients in grade IV.

SUPPLEMENTARY FIGURE 3
The DNA methylation level of QPCTL in the CGGA database. **(A)** The DNA methylation level of QPCTL in different histology of glioma. **(B)** The DNA methylation level of QPCTL in different pathological stages of glioma. **(C)** The DNA methylation level of QPCTL in different genders. **(D)** The different DNA methylation levels of different genders in different pathological stages. **(E)** The DNA methylation level of QPCTL in different ages. **(F)** The different DNA methylation levels of different ages in different pathological stages.

SUPPLEMENTARY TABLE 1
QPCTL CRISPR screen data from BIOGRID.

SUPPLEMENTARY TABLE 2
QPCTL CRISPR screen data from DepMap.

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# CD16$^+$ monocytes are involved in the hyper-inflammatory state of Prader-Willi Syndrome by single-cell transcriptomic analysis

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**Background:** Patients with Prader-Willi syndrome (PWS) have a reduced life expectancy due to inflammation-related disease including cardiovascular disease and diabetes. Abnormal activation of peripheral immune system is postulated as a contributor. However, detailed features of the peripheral immune cells in PWS have not been fully elucidated.

**Methods:** Serum inflammatory cytokines were measured in healthy controls (n=13) and PWS patients (n=10) using a 65- multiplex cytokine assays. Changes of the peripheral immune cells in PWS was assessed by single-cell RNA sequencing (scRNA-seq) and high-dimensional mass cytometry (CyTOF) using peripheral blood mononuclear cells (PBMCs) from PWS patients (n=6) and healthy controls (n=12).

**Results:** PWS patients exhibited hyper-inflammatory signatures in PBMCs and monocytes were the most pronounced. Most inflammatory serum cytokines were increased in PWS, including IL-1β, IL-2R, IL-12p70, and TNF-α. The characteristics of monocytes evaluated by scRNA-seq and CyTOF showed that CD16$^+$ monocytes were significantly increased in PWS patients. Functional pathway analysis revealed that CD16$^+$ monocytes upregulated pathways in PWS were closely associated with TNF/IL-1β- driven inflammation signaling. The CellChat analysis identified CD16$^+$ monocytes transmitted chemokine and cytokine signaling to drive inflammatory process in other cell types. Finally, we explored the PWS deletion region 15q11–q13 might be responsible for elevated levels of inflammation in the peripheral immune system.

**Conclusion:** The study highlights that CD16$^{+}$ monocytes contributor to the hyper-inflammatory state of PWS which provides potential targets for immunotherapy in the future and expands our knowledge of peripheral immune cells in PWS at the single cell level for the first time.

KEYWORDS

Prader-Willi Syndrome, inflammation, CD16+monocytes, single-cell RNA sequencing, mass cytometry

# Introduction

Prader-Willi syndrome (PWS) is a rare, complex, multisystem syndrome with an estimated prevalence of 1 in 10,000–30,000 live births (1), and it was first reported by Prader in 1956. Genetically, PWS is an imprinted disease caused by the lack of active genes located in the paternal chromosome 15q11–q13 region (2). The absence of gene expression in this region mainly occurs through three mechanisms: paternal deletion of the 15q11–q13 region (65–75%), maternal uniparental disomy 15 (20–30%), or imprinting defects (1–3%) (3, 4). The syndrome exhibits a wide clinical presentation spectrum, including hypotonia, developmental delays, cognitive disability, psychiatric phenotypes, sleep disordered breathing and obesity.

PWS patients are at a greater risk for cardiovascular disease and diabetes compared with weight-matched obese controls, which contributes to the most common causes of mortality in PWS (5–9). Furthermore, it was revealed that these comorbidities usually occur at relatively young ages in PWS (10, 11).

These comorbidities were related to chronic inflammation and few studies demonstrated that PWS was associated with increased concentrations of circulating markers of inflammation, such as tumor necrosis factor (TNF) (12), interleukin-6 (IL-6) (13), interleukin-1β (IL-1β) (14), and C-reactive protein (CRP) (13, 15) compared to those with non-syndromic obesity. And these circulating inflammation markers were linked with certain immune cell activation markers (16). It is proposed that the peripheral immune system is activated in PWS, which leads to systemic inflammation manifested by increased cytokine levels and seems to play a critical pathogenic role in the development of these inflammation-related comorbidities. However, detailed characteristics of the peripheral immune cells in PWS have not been fully clarified.

Single-cell RNA sequencing (scRNA-seq) offers an unbiased, comprehensive approach to define cell types and states based on their individual transcriptome and is widely used to reveal immune cell heterogeneity and diversity (17). In this study, we studied the cellular landscape of PWS peripheral immune cells at single-cell resolution *via* scRNA-seq.

# Materials and methods

## Subjects

PWS patients and healthy individuals were included in this study. All study subjects were recruited in Shandong Provincial Hospital affiliated to Shandong First Medical University. Written informed consent was obtained from parent or legal guardian. The diagnosis of PWS had been confirmed by genetic testing and none of the subjects were taking any medications. None of the subjects had a history of cancer, autoimmune disease, diabetes, infections or steroid usage. Serum samples were collected and stored at −80 °C until use.

## Cytokine assay

Serum cytokines were measured for each subject using a 65-multiplex cytokine assays (Cat. No. EPX650-16500-901) on the Luminex 200 system performed at the Laizee Biotech, Shanghai, China. IL-1β levels were measured using high-sensitivity ELISA kits by R&D Systems (Catalog Number HSLB00D) following the instructions exactly.

## PBMCs collection and single-cell RNA-seq

The PBMCs were isolated within 2 hours from fresh EDTA anticoagulated whole blood by density gradient centrifugation using Histopaque-1077 (Sigma, A6929). The single-cell library preparation in our research relied on an available droplet method, the 10x Genomics Chromium Controller. All samples in our study were not pooled. Single-cell RNA-seq libraries were constructed using the Chromium Single Cell 3′ Library & Gel Bead Kit v3·1 (10x Genomics, Pleasanton, CA) according to the manufacturer's instructions. Cells were divided into gel beads-in-emulsions (GEMs) at nanoliter scales. Then, reverse transcription was used to produce the cDNA. Consequently, each cDNA molecule contained a cell barcode and unique molecular identifier (UMI). We constructed and sequenced libraries at a depth of approximately 100,000 reads per cell by using the Novaseq 6000 platform (Illumina, San Diego, CA).

## Single-cell RNA-seq mapping and pre-processing

The raw sequencing data were converted into fastq format using mkfastq (cellranger 10X genomics, v4.0.0). As soon as the reads were de-multiplexed, they were aligned with the human reference

genome (GRCh38; 10x cellranger reference GRCh38 v3.0.0) to obtain the feature-barcode matrices. Then, Cellranger aggr was used to aggregate multiple libraries by default parameters. Seurat R package v4.1.0 was used for subsequent analysis (18). For further analysis, only cells expressing > 800 genes and < 10% mitochondrial genes were included. Doublets and red blood cells were both excluded in downstream analysis. We normalized gene expression for each cell based on the total number of transcripts and log transformation. In order to integrate different datasets, top 2000 highly variable genes for each dataset were recognized *via* using the function FindVariableFeatures with vst method in Seurat (19). Next, samples were integrated with canonical correlation analysis based on the top 20 canonical correlation vectors. The integrated data were scaled and principal component analysis (PCA) was executed. At last, with Seurat's FindClusters function (0.6 resolution), unsupervised clustering was performed and the cells were visualized by uniform manifold approximation and projection (UMAP). For the rest settings, we used default values for the scRNAseq clustering analysis and the UMAP visualization.

## Differentially expressed genes analysis and functional enrichment analysis

Based on the Wilcoxon-test method implemented in the FindAllMarkers function of Seurat package, we analyzed differential expression genes between two groups. Upregulated DEGs were identified according to the following criteria: (1) a logfold change > 0.25, and (2) p-value < 0.05. To find the function of upregulated genes, we used the function clusterProfiler (version 4.0.5) of the R package. We also used Enrichr, the software for gene set enrichment analysis (GSEA) was used for LINCS L1000 dataset to examine potential biological functions for lists of genes (20).

## Cell-cell communication

Cell-cell interactions analysis was conducted using CellChat (version 1.1.2). On the basis of data from scRNA-seq, the CellChat analysis was performed to infer intercellular and intracellular crosstalk between assigned types of cells. For this analysis, we analyzed each group separately and then used the mergeCellChat function to compare differences between two groups. More details about the package can be found in the previous publication (21).

## Weighted gene co-expression network analysis

The WGCNA package (version 1.70) was utilized to generate modules for co-expression. We applied a soft threshold power of six to calculate the adjacency matrix. Then, the adjacency matrix was transformed into a topological overlap matrix (TOM) to construct a gene tree by hierarchical clustering. We merged modules at a cut height of 0.25 and set the minimum module size to ten. To identify modules correlated with clinical traits, Spearman's rank correlation coefficients were measured between the different clinical parameters and module eigengenes.

## Gene set score calculation

The AUCell package (1.14.0) was used to calculate gene set scores. For the parameters default settings were used. Inflammation scores were calculated based on the gene set obtained from the Molecular Signatures Database (MSigDB) (GO:0002864). 15q11–q13 gene scores were calculated based on the gene set which contained all genes in 15q11–q13 region. PWS signature score in each cell type was calculated based on the gene set which consist of upregulated genes in PWS of cell types.

## Hierarchical clustering of different gene expression among disease groups at cell type resolution

We calculated the differential gene expression among obese PWS and obese controls relative to normal weight control. We found distinct transcriptional signatures between obese PWS and obese controls in major cell types and calculated the Pearson correlation coefficient using the above transcriptome characteristics. Hierarchical clustering analysis was performed based on the PCC (22).

## Metal-labeled antibodies

All the antibodies were purchased from BioLegend. A series of antibodies used are listed below: antibodies against CD3 (Cat# 300402, RRID: AB_314056), CD4 (Cat# 300502, RRID: AB_314070), CD8 (Cat# 301002, RRID: AB_314120), CD45 (Cat# 304002, RRID: AB_314390), CD14 (Cat# 301802, RRID: AB_314184), CD16 (Cat# 302002, RRID: AB_314202), CD56 (Cat# 318302, RRID : AB_604092), Tumour necrosis factor α (TNF-α) (Cat# 502902, RRID : AB_315254), IL-1β (Cat# 511605, RRID : AB_2861040). According to Fluidigm's recommendations, antibody conjugations were prepared using the Maxpar Antibody Labeling Kit (Fluidigm, South San Francisco, CA). Metal-labelled antibodies were stored at 4°C at 0.5 mg/mL in PBS-based Antibody Stabilizer (Candor Bioscience).

## CyTOF data acquisition and analysis

PBMCs (~3x106 cells) were spun (300 g, 5 min) and resuspended in calcium magnesium-free phosphate buffered saline (PBS). Mix well and incubated in 1 mL of 5 μM cisplatin (Fluidigm) at room temperature for 5 min. PBMCs incubated with metal-labeled antibodies followed by Ir-intercalator staining. The PBMCs were washed three times by staining buffer. In the next step, EQ beads were mixed 1:10 with MilliQ water solution to adjust cell concentration to $10^6$ cells/ml. The QE beads are used in order to test

if the nebulizer is lined up correctly, by determining the number of events/beads that pass through in a certain amount of time. Before to get normalized data, we calibrated the Helios CyTOF (Fluidigm, South San Francisco,CA). We used Cytobank software to gate the output FCS files to ruled out fragments, dead cells and doublets. Finally, data was clustered and represented in t-SNE maps using the R package Cytofkit (version 1.4.8). Cells were merged by "ceil" in mergeMethod fuction in Cytofkit and the "fixedNum" is 2000. As the mass cytometry data were nonlinear, cytofAsinh was used for data normalization. Data were clustered using the PhenoGraph.

## Statistical analyses

The data were analyzed using SPSS 22.0 or R 4.1.0. The distribution normality was tested using the Shapiro–Wilk test normality test. The Mann - Whitney rank-sum test was used for data with non-normal distribution and the t - test was used for data with normal distribution. Categorical variables were compared by $\chi 2$ or Fisher's exact tests. Multiple linear regression analyses between variables were done using SPSS software (SPSS, Chicago, IL). Regression analyses with the inflammation scores as the outcome predicted by variables BMI, age and PWS scores. Default settings were used for the analysis. We have performed mediation analysis using the bootstrapping method (23) in the 'mediation' package in R (version 4.5.0) to test whether the relationship between PWS score and inflammation scores, is mediated by the level of $CD16^+$ monocyte. Two thousand bootstraps were run to estimate the confidence intervals. The remaining settings were set default.

# Result

## Serum proinflammatory cytokine levels were elevated in PWS

In several studies, it has been reported that patients with PWS have elevated levels of serum inflammatory markers (14, 24), however, the data are limited and conflicting. We applied a multi-omics approach in the study (Figure 1A) and to determine typical proinflammatory cytokines levels in PWS patients, cytokines of 10 PWS patients and 13 controls were measured using a 65- multiplex cytokine assays. There was no significant difference in gender and body mass index (BMI) between the two groups (Supplementary Table 1). Of the 65 indicators, 35 were detectable with 60% of values in the detectable concentration range for the kit in one of the groups. Interestingly, 19 of the 35 indicators (54%) in the PWS group were 1.2-fold higher relative to the control group (Figure 1B; Supplementary Table 2). Most of the increased indicators in PWS were proinflammatory cytokines and chemokines, including TNF-RII, IL-12p70, MIF, MIP-1α and TNF-α (Figure 1B). Due to the limited number of the study participants, only 5 indicators were statistically higher in PWS (Figure 1C; Supplementary Table 2). Previous literature showed that the representative inflammatory cytokine IL-1β was elevated in PWS, but it was not detected by the multi-factor kit. We re-detected IL-1β with Elisa and found in line with previous reports, higher levels of IL-1β were observed in PWS patients compared to the controls (Figure 1D). The difference in serum cytokine levels was still significant when the PWS and control groups had comparable BMIs (21.59 ± 6.17 vs 21.43 ± 10.93) indicating that obesity is not a central driver for the difference in serum. In addition, we found that IL-1β and most of the other indicators had no correlation with BMI in the PWS patients (Figure 1E, Supplementary Figure 1, Supplementary Table 3). Based on preliminary results, we speculated thar the deletion of the genes within the 15q11-q13 region, rather than obesity, may be responsible for the increased levels of inflammatory serum markers.

## Unbiased clustering analysis of PBMCs and cell types identification

Increased pro-inflammatory cytokines in blood are important markers to reflect the state of activation in immune cells. To better understand the PWS peripheral immune cells, we used the scRNA-seq to examine transcriptome of immune cells in the PWS and control groups. Droplet-based scRNA-seq technology was used to profile PBMCs derived from 6 patients with PWS and 12 healthy controls. The PWS and control group were well matched in terms of age, gender, and BMI (Supplementary Table 4). We analyzed a total of 96,067 (control: 74,457; PWS: 21,610) cells in all participants after stringent filtering of the scRNA-seq data (Figure 2A), with an average 6,754 UMIs per cell and 2,037 genes per cell. The total number of cells, median genes per cell, and median UMIs per cell were provided in Supplementary Table 5. After unbiased clustering analysis, data were then visualized by UMAP and the cellular populations in PBMCs were identified using well-known marker genes (Figure 2B; Supplementary Table 6). We identified six major immune cell lineages including $CD8^+$ T cells, $CD4^+$ T cells, gamma delta T cells (gd T), natural killer cells (NK), B cells (BC), and monocytes (Mon) (Figure 2C). In downstream analysis, we focused on the six major cell types. No significant differences were found in the number of cells in the six major immune cell lineages between the two groups and only the number of $CD4^+$ T cells was marginally elevated (Supplementary Figure 2).

## PWS peripheral immune cells were in a hyper-inflammatory state

Consistent with above results, transcription levels of *IL-1β*, *TNF*, and *OSM* were globally elevated in the PWS group (Figure 3A), yet the source of the cytokines in peripheral immune cells remains unclear. We labeled cells with high expressed pro-inflammatory cytokine genes including IL-1β, TNF and OSM, and named these cells as inflammatory cells. The UMAP plot confirmed an expansion of inflammatory cells in PWS (Figure 3B). Globally, we found inflammatory cells increased by approximately 100% in all PBMCs using scRNA-seq analysis (Supplementary Table 7). When examining inter-individual variation in the six major populations, we noted that monocytes were the predominant source of inflammatory cells in PWS (Figure 3C).

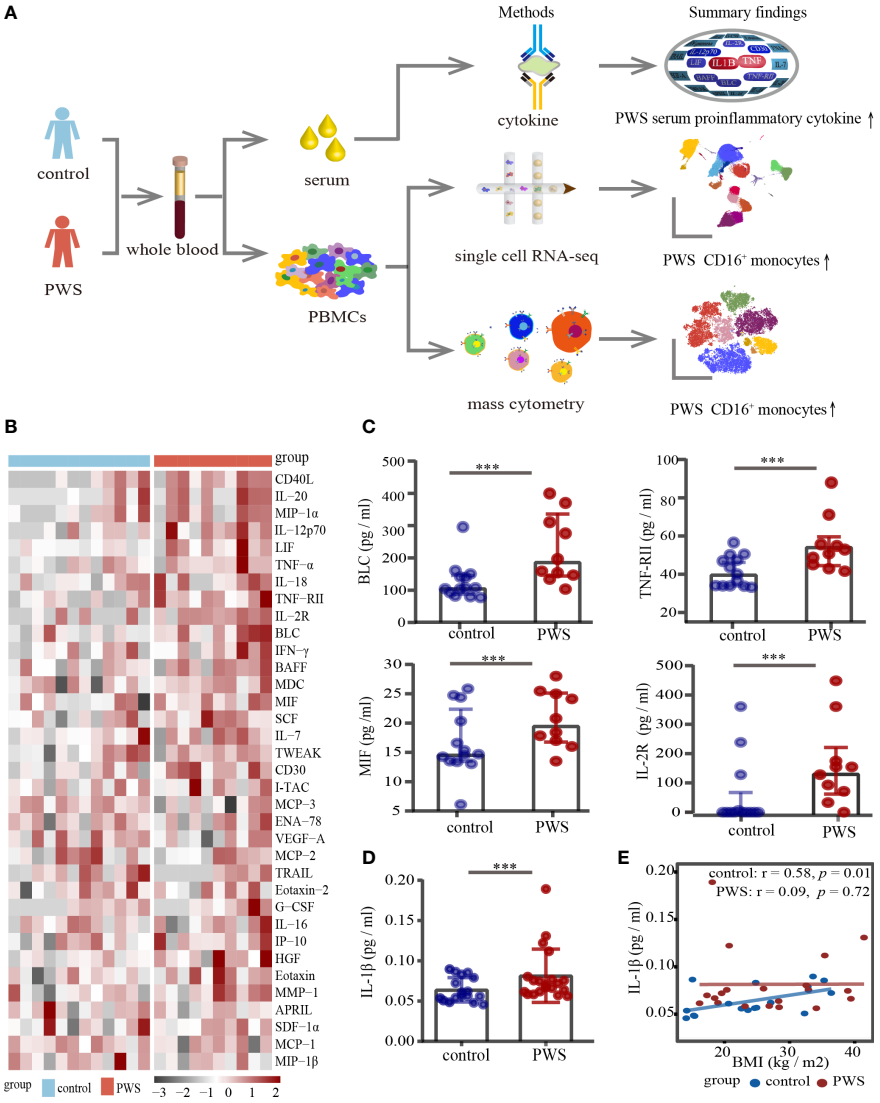


FIGURE 1
Analyses of serum cytokine levels. **(A)** Study overview. **(B)** Heatmap of all cytokines measured in the PWS (n=10) and control groups (n=13). On the x axis, samples are arranged by the study group and on the y axis, cytokines are displayed according to hierarchical clustering. Cytokines are expressed as log (pg/ml), with black to red colors representing lower to higher expression, respectively. **(C)** Dot plots of cytokines in the PWS group (n=10) compared with the control group (n=13). *p*-value is calculated by the Mann-Whitney U test for comparisons. ***$p < 0.05$. **(D)** Dot plots of serum IL-1β in the PWS group (n=20) compared with the control group (n=17). *p*-value is calculated by the Mann-Whitney U test for comparisons. ***$p < 0.05$. **(E)** Spearman correlation between IL-1β serum levels and BMI in the two groups.

An integrated comparative analysis of DEGs in PBMCs from the PWS and control groups was conducted to identify cell-type-specific gene signatures associated with PWS (Supplementary Table 8). A set of 12 genes related to inflammation (e.g., *IER5, JUNB, JUND, NFKBIA, ZFP36*, and *CXCR4*) were found to be upregulated across all major cell types in PWS (Figure 3D). Next, we explored the biological implications of upregulated DEGs using the Gene Ontology (GO) pathway analysis for each major cell type. The generally upregulated genes across major cell types were enriched in inflammatory-related pathways, such as the inflammatory response pathway, NFkB signaling pathway, production of inflammatory cytokines, and stress-related pathways (Figure 3E; Supplementary Table 9). In addition, we have also found PWS-disease-specific inflammatory-related pathways such as aging, cell cycle arrest, and mitotic cell cycle arrest (see Supplementary Table 9). The above result revealed that, in PWS, peripheral blood immune cells may be influenced by common inflammatory mediators regardless of cell type. We also observed cell type-specific enriched pathways in PWS such as regulation of neuron death pathway was enriched in natural killer cells, response to starvation in B cells, positive regulation of cell-cell adhesion in monocytes, and toll-like receptor signaling pathway in $CD8^+$ T cells (Supplementary Table 10).

Differential gene expression analysis revealed that monocytes exhibited the largest number of gene expression changes among the major cell types. Furthermore, the PWS group had significantly higher expression of genes including inflammatory response genes (e.g., *PTGS2, PTGER3* and *ICAM1*), and chemokine (e.g., *CCL2* and *CXCL9*) or cytokine genes (e.g., *IL-1β, OSM* and *TNF*) compared to

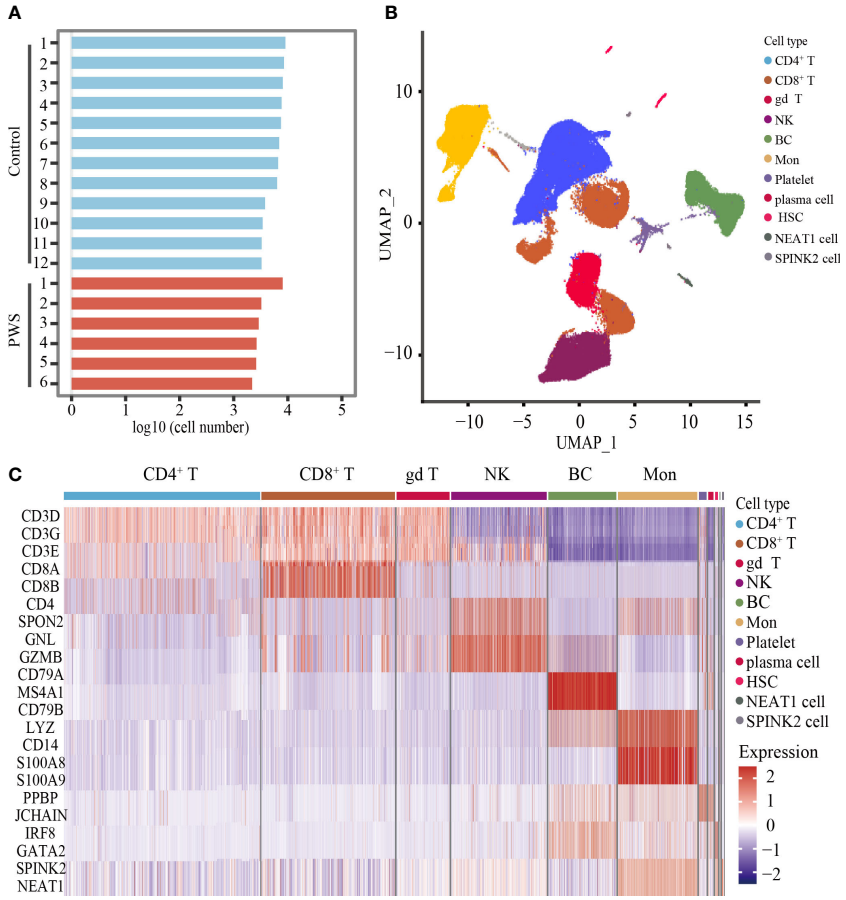


FIGURE 2
Unbiased clustering analysis of PBMCs and cell types identification. **(A)** Bar plot showing the log10 transformed cell number for each participant. **(B)** UMAP plot of all cells derived from scRNA-seq data. **(C)** Heatmaps showing the expression level of cell type-specific genes for each cluster.

the control group. Most of these genes had the highest expression value in monocytes (Figure 3F). Moreover, in the PWS group, a positive correlation was observed between the *IL-1β* transcription levels in monocytes and serum IL-1β levels (Supplementary Figure 3A). A similar result was also observed for the *TNF* transcription levels in monocytes and serum TNF-α levels (Supplementary Figure 3B). These results suggested that monocytes were closely related to the development of inflammation in PWS and that the elevation of serum inflammatory cytokines previously reported in the literature may be largely due to activation of the monocytes.

Traditionally, PWS has been considered as an obesity-related disease, we assessed whether observed transcriptional differences could be attributed to obesity. In contrast to conventional views, we found distinct transcriptional signatures between obese PWS and obese controls in major cell types. To visualize overall transcriptome changes, we performed hierarchical clustering. Surprisingly, all major cell types were clustered together according to the study groups instead of cell types (Figure 3G). In addition, we performed weighted gene co-expression network analysis to identify modules associated with PWS and BMI (Figure 3H). We noted that modules correlated with PWS was not identical to BMI-associated modules which further supporting our hypothesis the observed changes in peripheral immune cells were not mainly due to obesity.

## CD16$^+$ monocytes and their role in promoting PWS inflammation in global single-cell profiling

The findings presented heretofore indicate that, among the six major cell types, the monocytes were most closely related to the development of inflammation in PWS. In order to further uncover PWS-specific transcriptional signatures in monocytes, we performed sub-clustering analysis of monocytes using Seurat and identified ten clusters according to specific markers (Figures 4A, B; Supplementary Figure 4-5). To describe how cell- type composition changed in PWS of monocytes, we separately compared the percentage of each cluster between the PWS and control groups. CD16$^+$ monocytes exhibited the greatest changes and increased by approximately 100% based on the scRNA-seq analysis (Figures 4C, D). CD16$^+$ monocytes, characterized by high expression of CD16 (FCGR3A) and low levels of CD14, are most closely resembled the well-defined nonclassical monocytes (Supplementary Figure 4). To validate the expansion of CD16$^+$ monocytes, we performed CyTOF

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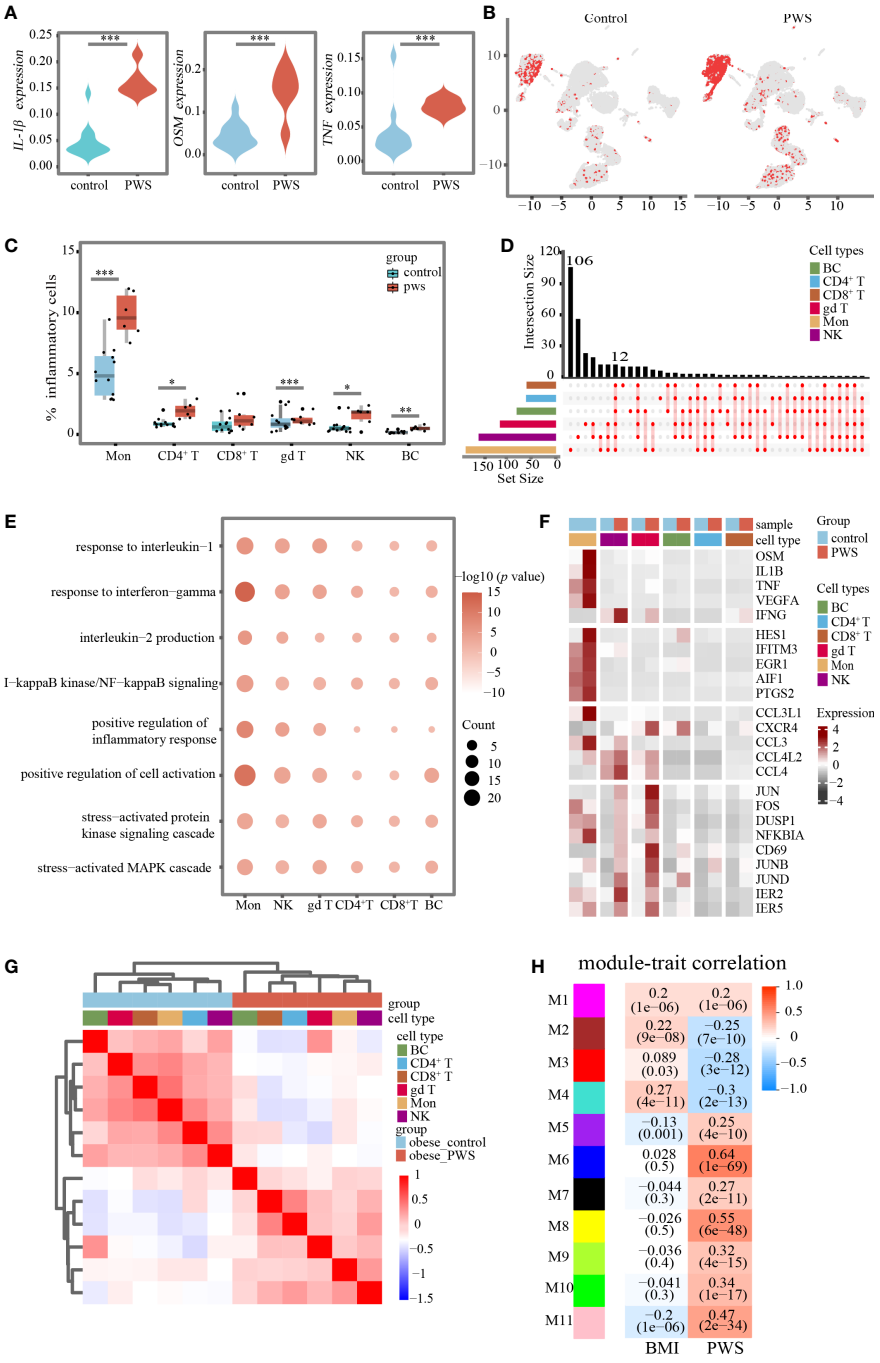


FIGURE 3
Changes in transcriptional landscape of PBMCs in PWS. **(A)** Differential expression levels of inflammatory-related genes between controls and PWS patients in all cells (Mann-Whitney U test was applied. ***$p < 0.05$). **(B)** UMAP plots showing inflammatory cells in controls and PWS patients. **(C)** Percentage of inflammatory cells in major cell types of the two groups(Mann-Whitney U test was applied. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) **(D)** Comprehensive comparative analysis of upregulated DEGs in major cell types between the PWS and control groups. **(E)** Representative GO terms for upregulated genes in PWS patients compared to controls in each major cell type. **(F)** A heatmap showing the scaled expression of inflammatory-related genes in major cell types. **(G)** Hierarchical clustering was based on by Pearson correlation coefficient (PCC). The intensity of the color represents the PCC values. Color bars above the heatmaps indicate the cell type and the study group. **(H)** Heatmap of the correlation between module eigenvalues and clinical traits. Color of the heatmap indicates correlation coefficient. Numerical values in the brackets indicate correlation coefficient and the *p*-value of the correlation coefficient.

analysis of 6 PWS patients and 12 controls using PBMCs. We identified major cell types in PBMC and three types of monocytes including CD14$^+$ monocytes, intermediate monocytes (IM) and CD16$^+$monocytes according to cell markers (Supplementary Figure 6). Remarkably, CD16$^+$ monocytes increased by approximately 80% (Figures 4E, F). The increase in CD16$^+$ monocytes in PWS may be due to the conversion of other types of monocytes to CD16$^+$ monocytes. Previous work has

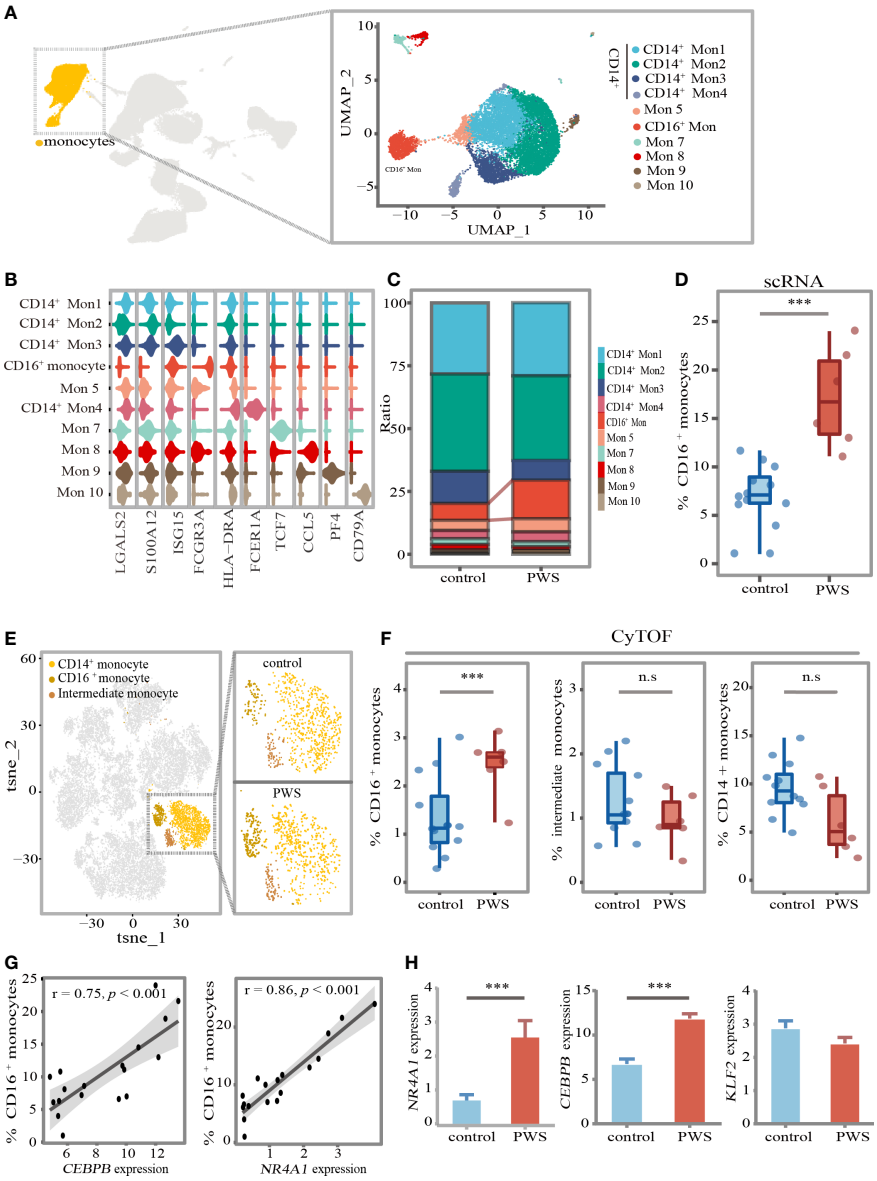


FIGURE 4
CD16$^+$ monocytes increased in PWS patients. **(A)** UMAP visualization of the transcriptional heterogeneity of circulating monocytes. The monocytes are further divided into ten clusters, and their names are annotated on the right. Different colors are used to distinguish each cluster. **(B)** Violin plot showing the signature expression genes of each cell cluster. **(C)** Proportion of cell types in each group. The colors indicate cell types information. **(D)** Percentage of CD16$^+$ monocytes (identified by scRNA-seq) in the monocytes of the control and PWS group. *p*-values were defined by the Mann-Whitney U test. ***$p$<0.05. In the boxplot, each dot represents a sample. Boxes range from the 25th to the 75th percentiles. The upper and lower whiskers extend from the box to the largest and smallest values respectively. **(E)** tSNE representative map of PBMCs clusters derived from PWS patients (n=6) and controls(n=12) by CyTOF and highlighting three monocytes subclusters (dark yellow). **(F)** Percentage of three monocytes subclusters (identified by CyTOF) in PBMCs of the control group and PWS. *p*-values were defined by the Mann-Whitney U test. ***$p$<0.05, n.s., no significance. **(G)** Spearman correlations between the percent of CD16$^+$ monocytes (identified by scRNA-seq) and *NR41A* and *CEBPB* gene expression levels. **(H)** Expression of *NR4A1*, *CEBPB* and *KLFF2* in PWS (n=6) and controls (n=12) of monocytes. *p*-values were defined by the Mann-Whitney U test. ***$p$<0.05.

demonstrated that the transcription factor NR4A1 which regulated by KLF2 or CEBPB is the master regulator of the CD16$^+$ monocytes (25–27). Similarly, we also found that the number of CD16$^+$ monocytes were positively correlated with Nr4a1 and CEBPB (Figure 4G). In PWS patients the expression of the genes *NR4A1* and *CEBPB* were elevated while *KLF2* was not affected, which could partially account for the increase of CD16$^+$ monocytes in PWS (Figure 4H).

We wondered whether CD16$^+$ monocytes not only manifested by increased cellular number but also altered cellular states to elevate levels of inflammation in PWS. Indeed, we identified lots of disease-specific genes expressed genes in CD16$^+$ monocytes (Figure 5A). A large number of upregulated genes with inflammation-related functions were observed in the CD16$^+$ monocytes in PWS, including inflammatory activation-associated genes (e.g., *IRF1*, *HES1*, *NFKBIA*, *ZFP36* and ***ATF3***), and

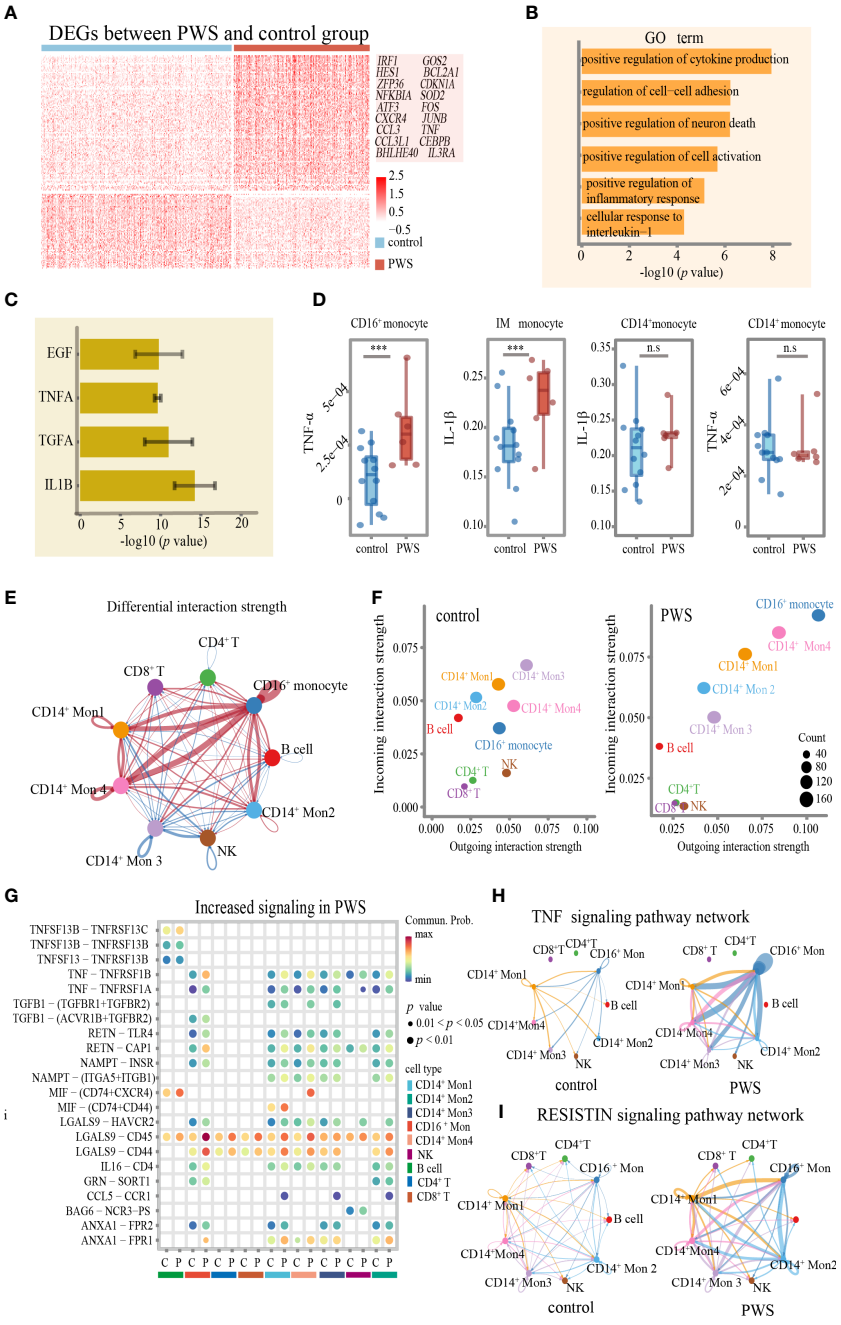


FIGURE 5
Transcriptome of CD16$^+$monocytes in PWS patients. **(A)** Heatmap of differentially expressed genes between PWS patients and healthy controls in the CD16$^+$ monocytes. **(B)** Bar plots of GO terms enriched in CD16$^+$ monocyte from the PWS patients. **(C)** Bar plots showing the average −log10 (*p-value*) values in enrichment analysis using the perturbed genes of different cell lines listed in L1000 LINCS for up-regulated genes in PWS. Error bars indicate standard deviation. **(D)** TNF-α and IL-1β proteomic levels in three monocyte clusters were measured by CyTOF in PWS (n=6) controls (n=12). *p*-values were defined by the Mann-Whitney U test. ***$p$<0.05, n.s., no significance. **(E)** Circle plot of differential interaction strength in PWS compared to the control group. In the circle plot, red (or blue) indicates increased (or decreased) signaling in PWS compared to the control group. Line thickness represents the interaction strength on a continuous scale (thicker = stronger interaction). **(F)** Scatter plot of incoming and outgoing interaction strength of cell types in the control and PWS groups. **(G)** The bubble plot of the communication probability of some significant ligand-receptor pairs from CD16$^+$monocytes to other cell types in PWS. The bubble color gradient and size indicate the communication probability and *p*-values (permutation test), respectively. C, control; P, PWS. **(H, I)** Circle plots of TNF and RESISTIN signaling network in the PWS and control groups. The edge width represents the communication probability. Thicker edge line indicates a stronger signal.

inflammation-related chemokine genes (e.g., *CXCR4*, *CCL3*, and *CCL3L1*). We also found that genes related to aging (e.g., *BHLHE40*, *CDKN1A* and *BCL2A1*), and stress response (e.g., *GOS2* and *SOD2*) were upregulated in PWS (Supplementary Table 11). An extended GO analysis of these genes revealed enrichment in pathways mainly involved in the regulation of the inflammatory response and cytokine production (Figure 5B; Supplementary Table 12). To further understand the biological functions of these genes, we

examined the upregulated DEGs by gene set enrichment analysis using cytokine-responsive gene sets from cytokine-treated cells (LINCS L1000). PWS-upregulated DEGs were enriched by TNF/IL-1β- responsive genes (Figure 5C) and the result was confirmed by CyTOF. We found that the protein expression of TNF-α in $CD16^+$ monocytes was significantly higher in the PWS group than the control group. IL-1β was significantly increased in PWS patients compared to the controls in intermediate monocytes. However, we did not detect increased IL-1β or TNF-α in $CD14^+$ monocyte (Figure 5D).

Interestingly, in $CD16^+$ monocytes pathway analysis showed significant enrichment in the communication between immune cells in PWS (Figure 5B). Given that $CD16^+$ monocytes might regulate the inflammatory process of other cells though cell–cell interactions, we applied CellChat to infer and analyze the intercellular communication networks to identify the alterations of interactions between $CD16^+$ monocytes among other cell types. By comparing the outgoing and incoming signals of cell types in the PWS and control group, we noticed that $CD16^+$ monocytes in the PWS group showed greater changes in transmitted and received signaling compared to those in the control group (Figures 5E, F) which implied that $CD16^+$monocytes may have an increased tendency for interaction with other immune cells in blood vessels. It was notable that the expression of multiple inflammation -related cytokines/receptors were significantly increased in PWS patients such as TNF, RETN, and its receptors, through that $CD16^+$ monocytes may interact with the other monocytes (Figure 5G). Similarly, increased levels of LGALS9 and its receptor were observed in $CD16^+$monocytes of PWS, which suggesting the potential functional interaction of the $CD16^+$monocytes with $CD8^+$ T cells, $CD4^+$ T cells. Chemokines such as MIF, and CCL5 and their respective receptors were also found to be enriched in PWS $CD16^+$monocytes. Correspondingly, we also found that $CD16^+$ monocytes of the PWS group showed more output related to TNF-α signaling and VISFATIN signaling (Figures 5H, I). Overall, these results help illustrate the possible molecular basis for communication between peripheral immune cells of PWS patient leading to a better understanding of the mechanisms about elevated levels of inflammation in PWS.

## The 15q11–q13 region plays a critical role in regulating the peripheral immune cells inflammation

In monocytes, there was no correlation between proinflammatory cytokines and BMI, so we hypothesized that the 15q11–q13 region might be responsible for elevated levels of inflammation. Due to the limited number of healthy controls in this study, we created a new healthy control group which comprised by two groups, one from the control group in this study consisting of 12 healthy individuals and another from our unpublished study consisting of 11 healthy individuals. Additional demographic data are provided in Supplementary Table 13. To assess the impact of the 15q11–q13 region on circulating immune cells, we selected the genes of the 15q11–q13 region and calculated 15q11–q13 gene scores in each cell using AUCell to evaluate gene expression in the 15q11–q13 region in the healthy group. We also calculated the score of the PWS- related transcriptome in the healthy group using the up DEGs in PWS called PWS signature scores (Supplementary Table 14). Surprisingly, there was a negative correlation between the PWS signature scores and 15q11–q13 gene scores in the healthy group (Figure 6A). This result implies that the healthy individuals with lower 15q11–q13 scores tend to display PWS-related transcriptional changes. Furthermore, in the healthy group, negative associations were observed for *IL-1β*, *TNF*, *OSM*, and 15q11–q13 gene scores (Figure 6B) indicating that healthy individuals with low expression of 15q11–q13 gene prone to express more pro-inflammatory cytokines. There was also a negative correlation 15q11–q13 gene scores and percentage of $CD16^+$ monocytes in the healthy group (Figure 6C).

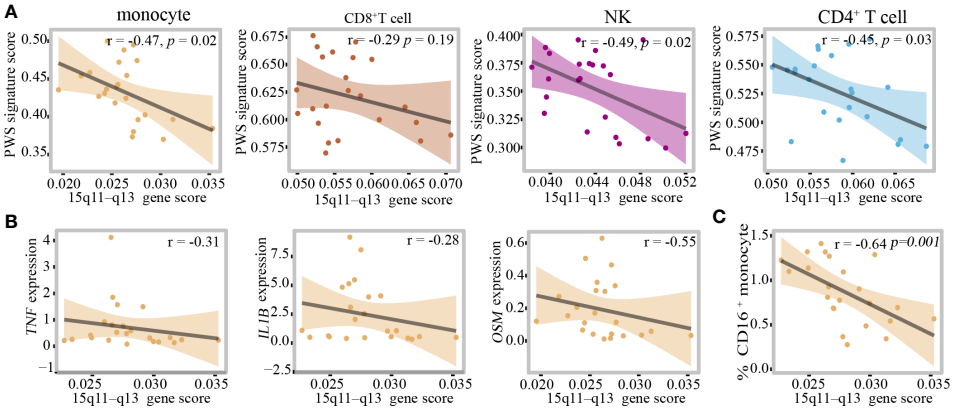


FIGURE 6
The 15q11–q13 region plays a critical role in regulating the peripheral immune cells inflammation. **(A)** Pearson correlation between PWS signature scores and 15q11–q13 gene scores in the healthy group. **(B)** Spearman correlation between 15q11–q13 gene scores and proinflammatory genes expression level in the healthy group. **(C)** Spearman correlation between 15q11–q13 gene scores and the percent of $CD16^+$monocytes in the healthy group.

In order to quantified the individual levels of inflammation, we used inflammation-related gene set which was obtained from Molecular Signatures Database Hallmark to calculate individual inflammation scores. Additionally, 15q11–q13 gene scores were associated with inflammation scores in the healthy individuals after adjusting for the relevant confounders such as BMI and age (Table 1). The regression analysis revealed that 32.6% of the variance in inflammation scores was explained by15q11–q13 gene scores. The results, showed that the 15q11–q13 region affected the overall level of inflammation in healthy person. Next, we made a further analysis to determine if the relationship between 15q11–q13 region gene and inflammation was mediated by $CD16^+$ monocytes by the R process mediation test. Mediation analysis revealed that both the total and indirect effects (ACME $p$ <0.05) of the 15q11–q13 region scores on the inflammation scores were significant; however, the direct effect of the 15q11–q13 region on the inflammation scores was insignificant (ADE $p$ > 0.05) (Table 2). The findings indicated that the lower 15q11–q13 gene expression observed in healthy individuals with a tendency toward higher inflammation score may be explained at least in part by high $CD16^+$ monocytes levels. Collectively, the findings revealed a previously under-appreciated link between 15q11–q13 region peripheral and immune cells inflammation which might provide a theoretical basis for the gene therapy or immunotherapy in PWS patients.

## Discussion

In the present study, we first provide insights into the PBMCs of PWS patients at single-cell resolution. We identified the major cell types in PBMCs and elucidated their contributions to inflammation. We found that monocytes, especially the $CD16^+$ monocytes, had a key role in promoting proinflammatory activities and network in PWS. Moreover, we showed that loss of gene expression from the chromosome 15q11–q13 locus was closely related to inflammation in healthy people.

Multiple studies have shown that PWS confers a relative risk for inflammatory-related diseases including Type 2 diabetes mellitus (T2DM), cardiovascular diseases and mental diseases compared with non-syndromic obesity. It has been reported that T2DM is common in PWS (8) and more than 50% of the PWS patients have developed diabetes before the age of 18 (28, 29). Furthermore, subtle atherosclerosis starts in young patients with PWS has also been demonstrated (10). A number of mental disorders have been widely reported in patients with PWS (30). Importantly, although rare, there were several studies reported that serum levels of circulating inflammatory markers were increased in PWS. Similar to previous reports, pro-inflammatory cytokines such as IL-1β, IL-16, IL-18 and TNF-α which were predominantly derived from activated immune cell were increased in PWS patients according to the study. Notably, the transcriptional levels of IL-1β and the serum concentrations of IL-1β were both significantly increased in PWS. There have been many reports that IL-1β plays a central role in mediating DM, cardiovascular diseases, and progression of mental disorders. In atherosclerotic coronary arteries, IL-1β levels were correlated with disease severity and knocking out IL-1β in atherosclerosis-prone ApoE−/− mice led to attenuation of disease development (31). IL-1β is believed to impair islet function and viability by activation of the inflammasomes in islet inflammatory cells (32). A separate study implied that subclinical inflammation, observed as elevated IL-1β and IL-13 levels, was correlated to several psychopathological symptoms in PWS (14). Thus, we speculated that PWS comorbidities that may be mediated, in part, by immune activation to induce the production of various proinflammatory cytokines. To yield convincing results, clinical trials about the relationship between PWS pro-inflammatory phenotype and clinical presentation need to be done in the future.

We found that there was no significant correlation between most of cytokines and BMIs in PWS patients which suggested that the pro-inflammatory state in PWS patients cannot be attributed totally to overweight or obesity. Nevertheless, in healthy population, negative associations were observed between IL-1β, TNF, OSM, and 15q11–q13 gene scores. In the conventional view, PWS disease is one of the obesity-related diseases. However, according to our results, obesity is unlikely to be the only factors that influence PWS inflammatory status, a more plausible scenario is that PWS hyper-inflammatory state results from the interplay among deletion of genes, obesity and other factors. However, it remains to be determined which of genes in 15q11–q13 region that involved in progression and development of inflammation in PWS.

Increased circulating inflammatory cytokines potentially reflected the alteration of the inflammation profile of peripheral immune cells in PWS. Therefore, we applied scRNA-seq and CyTOF to explore the changes in peripheral immune cells. we found that $CD16^+$ monocytes were significantly increased in PWS. Traditionally, according to the CD14 and CD16 expression patterns, peripheral blood monocytes are divided into three types: classical, intermediate, and nonclassical monocytes. We showed

TABLE 1 Multiple linear regression analysis to assess influence of variables on inflammation scores.

| inflammation score | | | | | |
|---|---|---|---|---|---|
| Model | B | *P* | 95% CI | $R^2$ | *P* |
| PWS score | -0.460 | 0.017 | -2.179 -0.239 | 0.326 | 0.015 |
| BMI (kg/m2) | -0.337 | 0.110 | -0.001 0.000 | | |
| Age(years) | -0.110 | 0.590 | -0.003 0.002 | | |

ß, linear regression coefficient; CI, Confidence interval.

TABLE 2 The mediation analysis of CD16$^+$monocytes in association between 15q11–q13 gene scores and inflammation score.

| | Estimate | 95% CI Lower | 95% CI Upper | *P* |
|---|---|---|---|---|
| ACME | -0.64 | -1.56 | -0.01 | <0.05 |
| ADE | -0.70 | -1.81 | 0.44 | 0.22 |
| Total Effect | -1.34 | -2.37 | -0.24 | <0.05 |

ACM, Average causal mediation effects, namely, indirect effect; ADE, Average direct effects, namely, direct effect.

that CD14$^+$ Mon1-4 appeared most analogous to classical monocytes. Based on numerous studies using scRNA-seq, monocytes that express high levels of CD16 and low levels of CD14 are commonly grouped into a single cluster, which is consistent with our findings (Supplementary Figure 4). This cluster is typically referred to as CD16$^+$ monocytes (33–35). Researchers assumed that CD16$^+$ monocytes most closely resembled the well-defined nonclassical monocytes (35–38). Mon 5 did not form a distinct population but was mainly distributed at the junction between CD14$^+$ monocytes and CD16$^+$ monocytes were more like intermediate monocytes. Few monocytes shared discriminative genes with megakaryocytes, such as PPBP and PF4 and several monocytes expressed the gene signature of NK cells (e.g., high expression levels of FGFBP2, GNLY, GZMA, and IL32), were also identified (Supplementary Figure 5). This illustrates the advantage of the scRNA-seq which could characterize human monocyte clusters in unprecedented detail.

Nr4a1 is necessary for nonclassical monocytes generation and development as Nr4a1-/- mice lack nonclassical monocytes. Graham D et al. identified Klf2 can regulate nonclassical monocyte conversion *via* cell-specific super-enhancer domain E2.The E2 is a single sub-domain (E2) 4 kb upstream of the Nr4a1 transcription start site and was essential for nonclassical monocytes development (26). In Lyz2-cre Klf2$^{flox/flox}$ mice Ly6C$^{hi}$ monocytes were unaffected however Ly6C$^{low}$ nonclassical monocytes were partially reduced. Expression of the monocyte survival factor Nr4a1 is also regulated by C/EBPb which could regulate monocyte differentiation into nonclassical monocyte. In PWS patients the expression of the genes *NR4A1* and *CEBPB* were elevated while *KLF2* was not affected which could partially account for the increase of CD16+ monocytes in PWS.

CD16$^+$ monocytes most closely resembled the well-defined nonclassical monocytes, are usually referred to as anti-inflammatory cells. Nevertheless, it was recently shown that CD16$^+$ monocytes are also capable of exerting proinflammatory responses depending on the disease context. Ratnadeep Mukherjee demonstrated that CD16$^+$ monocytes were the primary producers of TNF-α and IL-1β upon *ex-vivo* activation of whole blood with lipopolysaccharides (39). The observed phenomena are explained by high basal levels of phosphorylated NF-κB in CD16$^+$ monocytes which is a transcription factor for pro-inflammatory cytokines (40). Moreover, CD16$^+$ monocytes enter the peripheral tissues and differentiate into inflammatory macrophages (M1), and are involved in regulating inflammation in peripheral tissue (41). Clinical data showed that CD16$^+$ monocytes were increased in various inflammatory conditions, such as coronary artery disease (42), liver fibrosis (43), NAFLD (44), aging (40), and systemic sclerosis (45). CD16$^+$ monocytes are thought to contribute to the chronic inflammation associated with these diseases, and they may also be involved in the destruction of healthy tissues. Thus, we hypothesized that CD16$^+$ monocytes may have proinflammatory and anti-inflammatory functions, with a greater inclination towards anti-inflammatory functions under normal conditions. However, in certain disease contexts, the balance of pro-inflammatory and anti-inflammatory functions in CD16$^+$ monocytes may become disrupted, leading to an increased expression of pro-inflammatory properties. We also found that PWS CD16$^+$ monocytes upregulated DEGs were enriched by TNF/IL-1β- responsive genes. We further clarified their role in promoting PWS inflammation at global single-cell profiling. In addition, we made a further analysis to determine the relationship between 15q11–q13 region gene and inflammation was mediated by CD16+ monocytes by the R process mediation test (Table 2). Given lots of changes displayed by CD16$^+$monocytes in PWS, and the previously reported etiological link between CD16$^+$monocytes activity and inflammation-related diseases, we put forward a plausible view that the changes in CD16$^+$ monocytes state participated in hyper-inflammatory phenotype and comorbidities of PWS.

Despite some important findings made in this study, article had several limitations. First, as a result of the limited number of PWS patients enrolled in our study, the differences we identified between PWS patients and controls need to be future validated by larger clinical trials. Second, since it was a single time point study, drawing causal conclusions was not possible. The study did not provide evidence as to whether CD16$^+$ monocytes are associated with the severity of inflammatory diseases in PWS.

In conclusion, based on an unbiased scRNA-seq approach, we established an atlas of PWS circulating immune cells and offered insights into the function of CD16$^+$ monocytes which partially contributed to a hyper-inflammatory state in PWS. Further study is needed to elucidate the mechanism underlying the CD16$^+$monocytes and its broader implications, and thus help identify novel therapeutic target for PWS patients.

## Data availability statement

The sequencing data has been deposited in GSA-Human (https://ngdc.cncb.ac.cn/) under the accession number HRA003585.

## Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Shandong Provincial Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

JZ, YFS, LG conceived the study. YX, HG, XF and ZY performed most of the bioinformatics analysis and wrote the manuscript. GL, YW, XH, CX and YS collected the samples. 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.2023.1153730/full#supplementary-material

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# Vital roles of $m^5C$ RNA modification in cancer and immune cell biology

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RNA modification plays an important role in epigenetics at the posttranscriptional level, and 5-methylcytosine ($m^5C$) has attracted increasing attention in recent years due to the improvement in RNA $m^5C$ site detection methods. By influencing transcription, transportation and translation, $m^5C$ modification of mRNA, tRNA, rRNA, lncRNA and other RNAs has been proven to affect gene expression and metabolism and is associated with a wide range of diseases, including malignant cancers. RNA $m^5C$ modifications also substantially impact the tumor microenvironment (TME) by targeting different groups of immune cells, including B cells, T cells, macrophages, granulocytes, NK cells, dendritic cells and mast cells. Alterations in immune cell expression, infiltration and activation are highly linked to tumor malignancy and patient prognosis. This review provides a novel and holistic examination of $m^5C$-mediated cancer development by examining the exact mechanisms underlying the oncogenicity of $m^5C$ RNA modification and summarizing the biological effects of $m^5C$ RNA modification on tumor cells as well as immune cells. Understanding methylation-related tumorigenesis can provide useful insights for the diagnosis as well as the treatment of cancer.

KEYWORDS

RNA modification, $m^5C$, cancer, immune cells, cancer immunity

## Introduction

Modifications of biological macromolecules, such as DNA, RNA and proteins, are essential for life. RNA modification at the posttranscriptional level, which does not alter the genome, plays a large role in epigenetics. The first RNA modification site, pseudouridine (Ψ), was discovered in the 1950s (1), and a total of 334 types of RNA modifications have been identified since then (2). Commonly recognized RNA modification sites include $N^6$-methyladenosine ($m^6A$), 5-methylcytosine ($m^5C$), 7-methylguanosine ($m^7G$), $N^1$-methyladenosine ($m^1A$), $N^4$-acetylcytidine ($ac^4C$), $N^6$-acetyladenosine ($ac^6A$),

pseudouridine (Ψ), uridylation, and phosphorylation (3–5). The deposition, removal and recognition of RNA modification sites are realized through three groups of responsible proteins. "Writers" and "erasers" refer to proteins capable of catalyzing the deposition and removal of a specific RNA modification site, respectively, while "readers", sometimes also called "binders", mainly recognize and bind to these modification sites (6, 7). Previous studies have demonstrated that RNA modification occurs not only in messenger RNA (mRNA) but also in noncoding RNAs, such as transfer RNA (tRNA), ribosomal RNA (rRNA), long noncoding RNA (lncRNA), microRNA (miRNA) and small nuclear RNA (snRNA) (8–11). For example, $m^6A$ and $m^5C$ modifications of mRNA are crucial in embryo development and stem cell fate determination (12), $m^7G$ modification of tRNA influences pathogenic infectivity of thermophilic bacteria (13), and $m^6A$ modification of lncRNA is likely to participate in the process of cell senescence (14).

Among the types of RNA modifications that have been discovered, $m^6A$ modification is the most widely and comprehensively investigated because of its abundance in eukaryotic cells (15, 16). $m^5C$ modification, comparatively, is less understood than $m^6A$ modification, as it is only moderately abundant (2, 17). A study in 2015 discovered that $m^5C$ consists of approximately 1%, 0.01% and 1% of cytosine residues in the samples extracted from mouse brain, E. coli and HEK293T (human embryonic kidney 293 T) cells (18). However, $m^5C$ has attracted increasing attention from researchers in recent years as detection methods for $m^5C$ have progressed (17, 19), showing the presence of $m^5C$ in mRNA, tRNA, rRNA and viral RNA infecting mammalian cells (20, 21). RNA sequencing methods are commonly used in the detection of $m^5C$ RNA modification, including RNA bisulphite sequencing, immunoprecipitation-based RNA sequencing and third generation sequencing (21–23). Immunoprecipitation-based RNA sequencing can be further divided into several categories, such as methylated RNA immunoprecipitation sequencing (MeRIP-Seq), 5-azacytidine-mediated RNA immunoprecipitation sequencing (5-azaIP-Seq) and methylation-individual nucleotide resolution crosslinking immunoprecipitation sequencing (miCLIP-Seq) (21, 23). Other detection methods include mass spectrometry, total base composition analysis, nearest neighbor analysis, etc. (24).

Up to now, an increasing amount of evidence has unveiled the importance of $m^5C$ in the modulation of gene expression, metabolism and diseases (25–27). Specifically, in the field of oncology, posttranscriptional RNA modification has been discovered to play important roles in the development and pathological process of various types of cancers since more than half a century ago (10, 28, 29), which introduced a promising new area of mechanistic exploration and therapeutic innovation. Alterations in $m^5C$ modifications of both coding RNAs and noncoding RNAs are also highly linked to cell proliferation (30, 31), metabolism (32) and tumor metastasis (33, 34) and appear in various kinds of cancer types, such as hepatocellular carcinoma (35), breast cancer (36) and bladder cancer (32). Moreover, $m^5C$ has vital impacts on different kinds of immune cells, including B cells, T cells, NK cells, granulocytes and macrophages (37, 38). Obviously, the $m^5C$-associated biological changes in immune cells are not to be neglected in the process of cancer development, but there is no comprehensive summary regarding the relationship between $m^5C$-associated tumorigenesis and alterations in immune cells.

In this review, we provide a novel and holistic review of $m^5C$-mediated cancer development by examining the exact mechanisms underlying the oncogenicity of $m^5C$ RNA modification and summarize the biological effects of $m^5C$ RNA modification on tumor cells as well as immune cells.

## The mechanism and basic biological functions of $m^5C$ RNA modification

There are three main groups of molecular effectors in the process of $m^5C$ RNA modification, namely, "writers", "erasers" and "readers" (Table 1). "Writers" refer to proteins that facilitate the formation of methylation sites, such as DNMT2 (DNA methyltransferase homolog 2) and the NSUN (NOL1/NOP2/SUN domain) family proteins. "Readers" are related recognition proteins that bind and identify methylation sites, such as ALYREF (Aly/REF export factor) and YBX1(Y-box binding protein 1). Although they do not directly take part in catalysis, the abnormality of "readers" is often associated with metabolic disorders and diseases. "Erasers", in contrast, facilitate the deletion of methylation sites, such as TET (ten-eleven translocation) family genes and ALKBH1 (AlkB homolog 1), creating a dynamic balance between the two antagonizing biological processes (Figures 1–3).

### Writers

To date, the $m^5C$ methylation of RNA, including mRNA, rRNA and tRNA, is believed to be mainly mediated by two groups of RNA methyltransferases, DNMT2 and the NSUN protein family.

DNMT2, also known as TRDMT1 (tRNA methyltransferase 1), generally influences tRNA methylation (25). The $m^5C$ site is located on cytosine 38 in the anticodon loop of $tRNA^{Asp\text{-}GUC}$, $tRNA^{Gly\text{-}GCC}$, $tRNA^{Val\text{-}AAC}$ (39, 40), and receives a methyl group from the cofactor S-adenosyl-methionine (SAM) (115). Studies showed that simultaneous knockout of DNMT2 and NSUN2 led to deficient tRNA methylation, protein synthesis and cellular differentiation, causing the death of experimental mice, although deficiency of either DNMT2 or NSUN2 alone did not show detectable effects. The results suggested that DNMT2 plays a role in tRNA methylation and cell survival (41). In addition, the role of DNMT2 in mRNA methylation and expression modulation was also reported. DNMT2 deficiency is associated with alterations in mRNA expression and methylation profiles and the inhibition of cell proliferation and migration (42).

The NSUN family also utilizes SAM as a methyl donor (116). The NSUN family consists of seven members, NSUN1-7, and each target different types of RNAs. The RNA targeting specificity of the NSUN family was reviewed in 2019 by Katherine E. Bohnsack et al., with

TABLE 1 Different types of $m^5C$ writers, readers and erasers and their biological functions.

| Types | Proteins | Target RNAs and $m^5C$ sites | Cellular functions | Mechanisms | References |
|---|---|---|---|---|---|
| Writers | DNMT2 | $tRNA^{Asp\text{-}GUC}$, $tRNA^{Gly\text{-}GCC}$, $tRNA^{Val\text{-}AAC}$ (C38 in the anticodon loop) | enhances protein synthesis and cellular differentiation | / | (39–41) |
| | | mRNA | cell proliferation and migration | / | (42) |
| | NSUN1 | 28S rRNA (C4447) | ribosome biogenesis | / | (43) |
| | | / | | regulates pre-rRNA processing by binding to the 5'-ETS region of pre-rRNA transcript, forming a noncatalytic complex together with box C/D snoRNAs | |
| | | / | cell proliferation | / | (44) |
| | | 26S rRNA (C2982) | healthspan modulation | / | (45) |
| | | / | promotes HIV-1 viral latency | competes with HIV-1 Tat protein to interact with HIV-1 TAR RNA | (46) |
| | NSUN2 | tRNA | preserves synaptic signaling at prefrontal cortex pyramidal neurons and suppresses contextual fear memory | / | (47–49) |
| | | mRNA | cell proliferation and migration | $m^5C$-methylates GRB2 and CD44 | (50) |
| | | mRNA | gastric cancer (GC) development | $m^5C$-methylates PIK3R1, PCYT1A and FOXC2 mRNAs; represses p57Kip2 by destabilizing its mRNA in a $m^5C$-dependent manner | (30, 34, 51) |
| | | mRNA | esophageal squamous cell carcinoma (ESCC) development | $m^5C$-methylates GRB2 *via* LIN28B-dependent way, thus activating PI3K/AKT and ERK/MAPK signaling pathway; promotes TIGAR | (52, 53) |
| | | lncRNA | hepatocellular carcinoma (HCC) development | $m^5C$-methylates H19 lncRNA, leading to MYC stimulation; modulates Ras signaling pathway and cell cycle | (35, 54) |
| | | mRNA | hypopharyngeal squamous cell carcinoma (HPSCC) development | $m^5C$-methylates TEAD1 mRNA, thus upregulating its expression | (55) |
| | | mRNA | prostate cancer development | $m^5C$-methylates and stabilizes androgen receptor (AR) mRNA | (56) |
| | | mRNA | cervical cancer development | $m^5C$-methylates KRT13 mRNA, enhancing its binding with $m^5C$ reader YBX1 | (57) |
| | | / | nasopharyngeal carcinoma (NPC) development | negatively regulates immune cell infiltration in tumor microenvironment (TME) | (58) |
| | | / | uveal melanoma development | / | (59) |
| | | mRNA (C466) | enhances IL-17A secretion of T cells | $m^5C$-methylates IL-17A mRNA in T cells | (60) |
| | | mRNA | enhances p21 expression under conditions of oxidative stress-induced cellular senescence | $m^5C$-methylates p21 mRNA at the 3'-UTR | (61) |
| | | / | promotes ALYREF's nuclear-cytoplasmic shuttling, RNA-binding affinity and associated mRNA export | / | (62) |
| | NSUN3 | / | possibly promotes low-grade glioma development | / | (63) |
| | | / | promotes the development of head and neck squamous cell carcinoma (HNSCC) | promotes tumor progression by regulating immune cell infiltration | (64) |
| | | $tRNA^{Met}$ (C34 at the anticodon loop) | | produces methylated $tRNA^{Met}$ needed for initiation and elongation of mitochondrial mRNA translation | (27, 65) |

*(Continued)*

TABLE 1 Continued

| Types | Proteins | Target RNAs and $m^5C$ sites | Cellular functions | Mechanisms | References |
|---|---|---|---|---|---|
| | | | facilitates mitochondrial mRNA translation, thus promoting metastasis | | |
| | | / | facilitates CD8+ T cells infiltration | / | (66) |
| | | / | facilitates M2 macrophages infiltration | / | (64) |
| | | / | preserves mitochondrial functions | / | (67) |
| | NSUN4 | 12S rRNA (C911) | facilitates mitoribosomal assembly | $m^5C$-methylates 12S rRNA and interacts with MTERF4 | (68) |
| | | tRNA (C34) | facilitates adaptation to higher temperatures | ensures translation efficiency of UUG-rich transcripts and fertility | (69) |
| | | mRNA | chondrogenic differentiation | $m^5C$-methylates the 3'-UTR of Sox9 mRNA | (70) |
| | | / | HCC development | / | (71, 72) |
| | | / | neutrophil infiltration | / | (66) |
| | NSUN5 | rRNA (C3782 in human and C3438 in mice) | protein synthesis and cell proliferation | / | (73, 74) |
| | | / | HCC development | strengthens ribosome functions and global protein translation | (75) |
| | | / | colorectal cancer (CRC) development | / | (76) |
| | NSUN6 | $tRNA^{Cys}$, $tRNA^{Thr}$ (C72) | / | / | (77, 78) |
| | | / | suppresses triple-negative breast cancer (TNBC) | potential regulation of infiltration of CD4+ T cells | (36) |
| | | mRNA | suppresses pancreatic cancer | promotes tumor-suppressive CDK10 | (31) |
| | | mRNA | suppresses testis, thyroid and ovary cancers | higher expression and translation levels of $m^5C$-methylated mRNAs | (79) |
| | | / | CRC development | / | (80, 81) |
| | | mRNA | promotes cell cycle dysfunction | / | (82) |
| | | / | infiltration of B cells and CD8+ T cells | / | (80) |
| | | mRNA | formation of antibody-secreting plasma cells | / | (83) |
| | NSUN7 | eRNA | enhances transcriptional coactivator function of PGC-1α | $m^5C$-methylates eRNA associated with PGC-1α | (84) |
| Readers | ALYREF | / | HCC development | promotes eIF4A3 expression; disrupts cell cycle and mitosis regulation | (72, 85, 86) |
| | | mRNA | glioblastoma development | stabilizes MYC mRNA; activates the Wnt/β-catenin signaling pathway | (87, 88) |
| | | / | glioma development | / | (63) |
| | | / | neuroblastoma development | forms a nuclear coactivator complex with MYCN to stimulate USP3 transcription | (89) |
| | | mRNA (3'-UTR) | lung adenocarcinoma development | binds with 3'-UTR of YAP mRNA, increasing its stability and thus enhancing exosome secretion, tumor malignancy and drug resistance | (90, 91) |
| | | / | HNSCC development | enhances mitochondrial activity and intracellular energy metabolism, ensuring continuous energy supplies | (92, 93) |

*(Continued)*

TABLE 1 Continued

| Types | Proteins | Target RNAs and $m^5C$ sites | Cellular functions | Mechanisms | References |
|---|---|---|---|---|---|
| | | mRNA | bladder cancer development | binds and stabilizes PKM2 mRNA, enhancing PKM2-mediated glycolysis | (32) |
| | | lncRNA | breast cancer development | binds with the NEAT1 lncRNA promoter region, enhancing its transcription | (94, 95) |
| | | / | suppresses colon adenocarcinoma development | / | (81) |
| | | mRNA | inhibits adipogenesis | recognizes and exports YBX2 and CDKN1A mRNAs into the cytoplasm, leading to increased YBX2 and CDKNIA protein expression levels which inhibit adipogenesis | (96, 97) |
| | | mRNA | promotes myogenesis | recognizes and exports SMO mRNA into the cytoplasm, leading to increased SMO protein expression levels which promote myogenesis | (97) |
| | | / | promotes retrovirus replication | / | (98) |
| | | / | possibly promotes abdominal aortic aneurysm (AAA) and infiltration of CD45+ leukocytes and CD3+ T cells | / | (99) |
| | YBX1 | mRNA | GC development | recognizes and binds with NSUN2-mediated $m^5C$ sites on FOXC2 mRNA to stabilize it | (51) |
| | | mRNA | bladder cancer development | stabilizes oncogenic HDGF mRNA by targeting the $m^5C$-modified site on its 3'-UTR and recruiting ELAVL1 | (100) |
| | | / | glioblastoma development | / | (101) |
| | | / | CRC development | / | (76, 102) |
| | | lncRNA | cholangiocarcinoma development | recognizes and stabilizes $m^5C$-modified NKILA | (103) |
| | | mRNA | suppresses the development of ccRCC | YBX1/ELAVL1 complex binds and stabilizes PEBR1 mRNA, which negatively modulates ccRCC | (104) |
| | | mRNA | prostate cancer development | recognizes and binds with NSUN2-mediated $m^5C$ sites on AR mRNA to stabilize it | (56) |
| | | / | epithelial ovarian cancer development | modulates the expression of a variety of downstream targets, including CD44, thus enhancing chemoresistance | (105) |
| | | mRNA | cervical cancer development | recognizes and binds with NSUN2-mediated $m^5C$ sites on KRT13 mRNA to stabilize it | (57) |
| | | / | embryonic brain development | / | (101) |
| | | mRNA | facilitates the maternal-to-zygotic transition | recognizes and stabilizes $m^5C$-modified mRNAs by recruiting Pabpc1a, preventing maternal mRNA decay | (106) |
| Erasers | TET1 | mRNA | ensures proper completion of DNA repair and survival of cells after DNA damage | mediates mRNA $m^5C$-demethylation, thus promoting mRNA-dependent recombination | (107) |
| | TET2 | / | convert $m^5C$ into $hm^5C$ | decreases $m^5C$ | (108, 109) |
| | | / | possibly promotes low-grade glioma | / | (63) |
| | | / | possibly suppresses the development of ccRCC | / | (110, 111) |
| | | / | possibly inhibits ovarian cancer | / | (112) |
| | | / | possibly inhibits prostate adenocarcinoma | potentially promotes immune cell infiltration | (113) |
| | TET3 | / | possibly promotes prostate cancer | / | (38) |

*(Continued)*

TABLE 1 Continued

| Types | Proteins | Target RNAs and $m^5C$ sites | Cellular functions | Mechanisms | References |
|---|---|---|---|---|---|
| | ALKBH1 | tRNA, mRNA | converts $m^5C$ into $hm^5C$ | decreases $m^5C$ levels | (109) |
| | | $tRNA^{Leu\text{-}CAA}$ (C34) | converts $m^5C$ into $hm^5C$ or $f^5C$ | promotes the decoding of Leu codons under stress | (109, 114) |
| | | $tRNA^{Met}$ (C34) | converts $m^5C$ into $f^5C$ | promotes the translation of AUA, a non-universal codon in mammalian mitochondria which is significant for mitochondrial functions | (114) |

ETS, external transcribed sequence; snoRNA, small nucleolar RNA; Tat, transactivator; TAR RNA, transactivation response RNA; GRB2, growth factor receptor-bound protein 2; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PCYT1A, phosphate cytidylyltransferase 1 choline-alpha; FOXC2, Forkhead box protein C2; $p57^{Kip2}$, the cyclin-dependent kinase (CDK) inhibitor; LIN28B, protein lin-28 homolog B; PI3K/AKT, phosphatidylinositol 3-kinase/protein kinase B; ERK/MAPK, extracellular-signal-regulated kinases/mitogen-activated protein kinases; TEAD1, first member of TEA/ATTS domain transcription factor family; AR, androgen receptor; KRT13, keratin 13; YBX1, Y-box binding protein 1; TME, tumor microenvironment; TIGAR, TP53-induced glycolysis and apoptosis regulator; MTERF4, mitochondrial transcription termination factor 4; SOX9, SRY-box transcription factor 9; CDK10, cyclin-dependent kinases 10; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; eIF4A3, eukaryotic translation initiation factor 4A3; USP3, ubiquitin specific peptidase 3; YAP, Yes-associated protein; PKM2, pyruvate kinase M2; NEAT1, nuclear enriched abundant transcript 1; YBX2, Y-box-binding protein 2; CDKN1A, cyclin-dependent kinase inhibitor 1A; SMO, smoothened; HDGF, heparin binding growth factor; ELAVL1, ELAV-like RNA binding protein 1; NKILA, NF-kappa B interacting lncRNA; PEBR1, phosphatidylethanolamine binding protein 1; Pabpc1a, poly A binding protein cytoplasmic 1a; $hm^5C$, 5-hydroxymethylcytosine; $f^5C$, 5-formylcytidine.

NSUN1, 4, and 5 responsible for rRNA methylation, NSUN2, 3, and 6 responsible for tRNA methylation (NSUN2 also promotes mRNA methylation), and NUSN7 responsible for enhancer RNA (eRNA) methylation (25). However, within the last three years, studies have provided new insights in this regard. Among the seven RNA methyltransferases, NSUN2 was the first discovered and most widely studied. The role of NSUN2 in promoting tRNA methylation is wellknown, and NSUN2 deficiency directly causes a decrease in tRNA $m^5C$ levels (47). Notably, NSUN2 is predominantly distributed in the nucleus and catalyzes methylation of cytoplasmic tRNA; it is also capable of introducing $m^5C$ to mitochondrial tRNA (48, 49). However, NSUN2 silencing did not significantly affect mitochondrial tRNA stability (49), which suggested that NSUN2 may not be necessary for tRNA methylation within the mitochondria. NSUN2 is also responsible for biological processes, including cell proliferation (50) and carcinogenesis. Enhanced levels

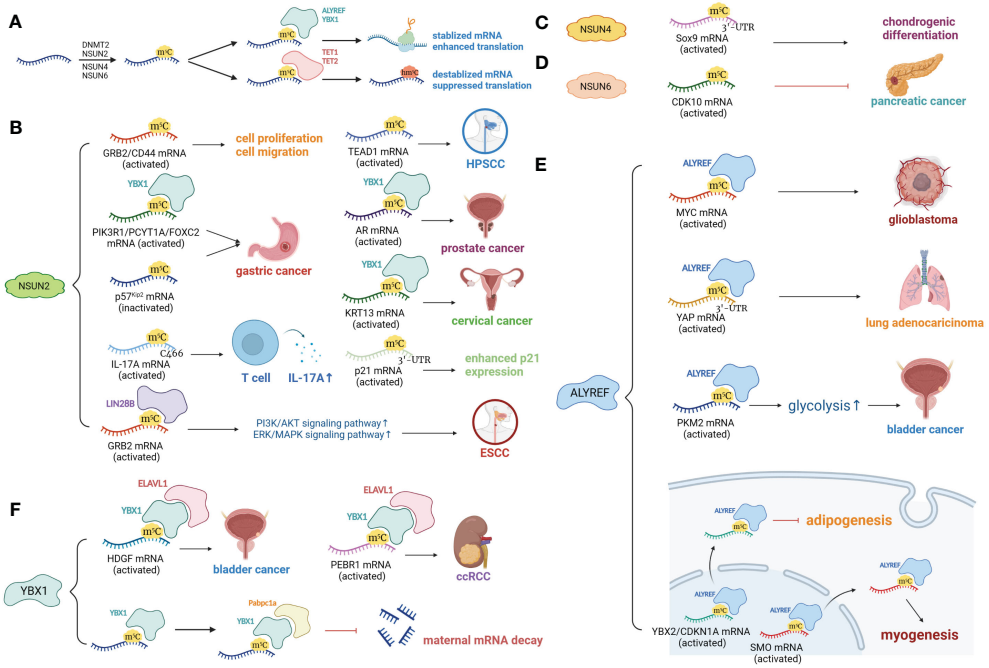


FIGURE 1
$m^5C$ modifications of mRNA. **(A)** mRNA is methylated at $m^5C$ sites by DNMT2, NSUN2, NSUN4 and NSUN6. ALYREF and YBX1 bind and stabilize $m^5C$-modulated mRNAs. TET1, TET2 and ALKBH1 remove $m^5C$ sites by turning them into $hm^5C$. **(B)** Target mRNAs of NSUN2 include GRB2, CD44, PIK3R1, PCYTIA, FOXC2, p57Kip2, TEAD1, AR, KRT13, IL-17A, and p21. Most target mRNAs are stabilized due to $m^5C$ modification, with the exception of p57Kip2, and are associated with enhanced cell proliferation and migration. **(C)** NSUN4-mediated $m^5C$ at the 3'-UTR of Sox9 mRNA promotes chondrogenic differentiation. **(D)** NSUN6-mediated $m^5C$ modification suppresses pancreatic cancer by promoting tumor-suppressive CDK10. **(E)** ALYREF binds with MYC, YAP, PKM2 and NEAT1 lncRNA, promoting tumor development and drug resistance. ALYREF facilitates nuclear export of YBX2, CDKN1A and SMO mRNAs, inhibiting adipogenesis and enhancing myogenesis. **(F)** YBX1 binds to $m^5C$ sites on the FOXC2, HDGF, PEBRQ, AR and KRT13 mRNAs, leading to enhanced mRNA translation and promoting tumorigenesis. YBX1 recognizes and stabilizes $m^5C$-modified mRNAs by recruiting Pabpc1a, facilitating maternal-to-zygotic transition.

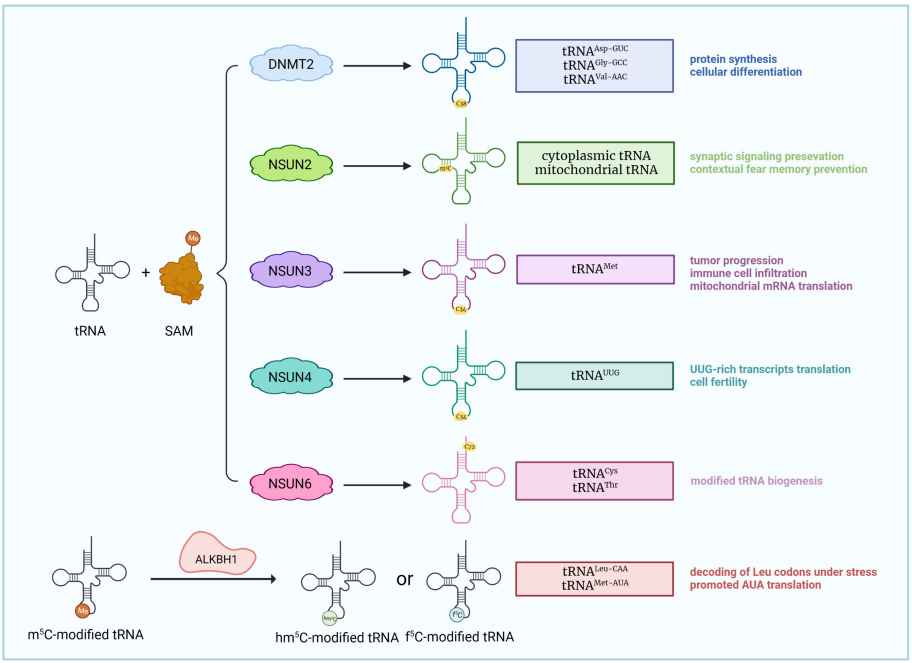


FIGURE 2
$m^5C$ modifications of tRNA. SAM induces tRNA $m^5C$ modification. DNMT2, NSUN2-4, and NSUN6 catalyze $m^5C$ modification of various tRNAs at different sites, causing distinct biological effects. ALKBH1 removes m5C sites from $tRNA^{Leu-CAA}$ and converts them into $f^5C$ (5-formylcytidine) sites, promoting the decoding of Leu codons under stress.

of NSUN2 and NSUN2-mediated $m^5C$ are observed in patients with gastric cancer (GC) (30, 34, 51), esophageal squamous cell carcinoma (ESCC) (52, 53), hepatocellular carcinoma (HCC) (35, 54), hypopharyngeal squamous cell carcinoma (HPSCC) (55), prostate cancer (56), cervical cancer (57), nasopharyngeal carcinoma (58) and uveal melanoma (59). NSUN2 also affects immune cells, as hyperexpression of NSUN2 in T cells promotes IL-17A secretion by methylating IL-17A mRNA at cytosine C466 both *in vitro* and *in vivo*, which stimulates its translation (60). In addition, under conditions of oxidative stress-induced cellular senescence, NSUN2-mediated $m^5C$, together with METTL3/METTL14-mediated $m^6A$, synergistically upregulates the expression of p21 (61). Note that NSUN2 is also involved in the functioning of $m^5C$ readers, including ALYREF, whose nuclear-cytoplasm transportation is partly modulated by NSUN2 (62). Other members of the NSUN family are also active in catalyzing $m^5C$ RNA modifications. NSUN1, or NOP2 (nucleolar protein 2), catalyzes rRNA $m^5C$ modifications, thus affecting biological processes including ribosome biogenesis (43), cell proliferation (44), healthspan modulation (45) and HIV-1 viral latency (46). NSUN3, a putative tRNA methyltransferase, plays a role in tumor progression (27, 63, 64), immune cell infiltration (64, 66) and multisystem mitochondrial diseases (67). Mechanistically, $m^5C$ modification of tRNA occurs at C34 in the anticodon loop (65, 117). Current studies have revealed that NSUN3 expression is upregulated in patients with low-grade glioma (63) and head and neck squamous cell carcinoma (HNSCC) (64), and NSUN3-mediated $m^5C$ modification of tRNA enhances metastasis by stimulating the translation of mitochondrial mRNA (27). NSUN3-associated immune cell infiltration mainly includes CD8+ T cells (66) and M2 macrophages (64). Deficiency of NSUN3 also leads to severe dysfunction within the mitochondria, such as combined oxidative phosphorylation deficiency, which may lead to early-onset encephalomyopathy and seizures (67). NSUN4 facilitates mitoribosomal assembly by methylating C911 in 12S rRNA and interacting with MTERF4 (mitochondrial transcription termination factor 4) (68). In addition to rRNA methylating activity, NSUN4 also acts as a tRNA (69) and mRNA (70) methyltransferase, and NSUN4-mediated $m^5C$ modification in the 3'-UTR (3'-untranslated region) of SOX9 (SRY-box transcription factor 9) mRNA is necessary for adaptation to higher temperatures (69) and chondrogenic differentiation regulated by SOX9 (70). NSUN4 also promotes HCC generation (71, 72) and neutrophil infiltration (66). NSUN5 participates in rRNA methylation, introducing $m^5C3782$ into human and $m^5C3438$ into mouse 28S rRNA (73, 74). Overexpression of NSUN5 is associated with tumorigenesis in HCC (75) and colorectal cancer (CRC) patients (76), while NSUN5 deficiency causes a reduction in total protein synthesis, thus impairing cell proliferation (73). In patients with tetralogy of Fallot (TOF) (118) and William's-Beuren syndrome (WBS) (119), NSUN5 is drastically downregulated. Previous studies regarded NSUN6 as at RNA methyltransferase, which identifies C72 at the 3′ end of the tRNA acceptor stem and targets $tRNA^{Cys}$ and $tRNA^{Thr}$ (77, 78), but recent investigations have discovered that NSUN6 exhibits mRNA methylating bioactivity (79, 82, 120, 121). mRNA methylated by NSUN6, which primarily targets the 3'-UTR at the consensus sequence motif CTCCA, increased in transcript and protein levels (79). The role of NSUN6 in cancer development remains unclear, but studies have shown that NSUN6 acts as a protective factor against triple-negative breast cancer (TNBC) (36), pancreatic cancer (31), testis cancer (79), thyroid cancer (79) and

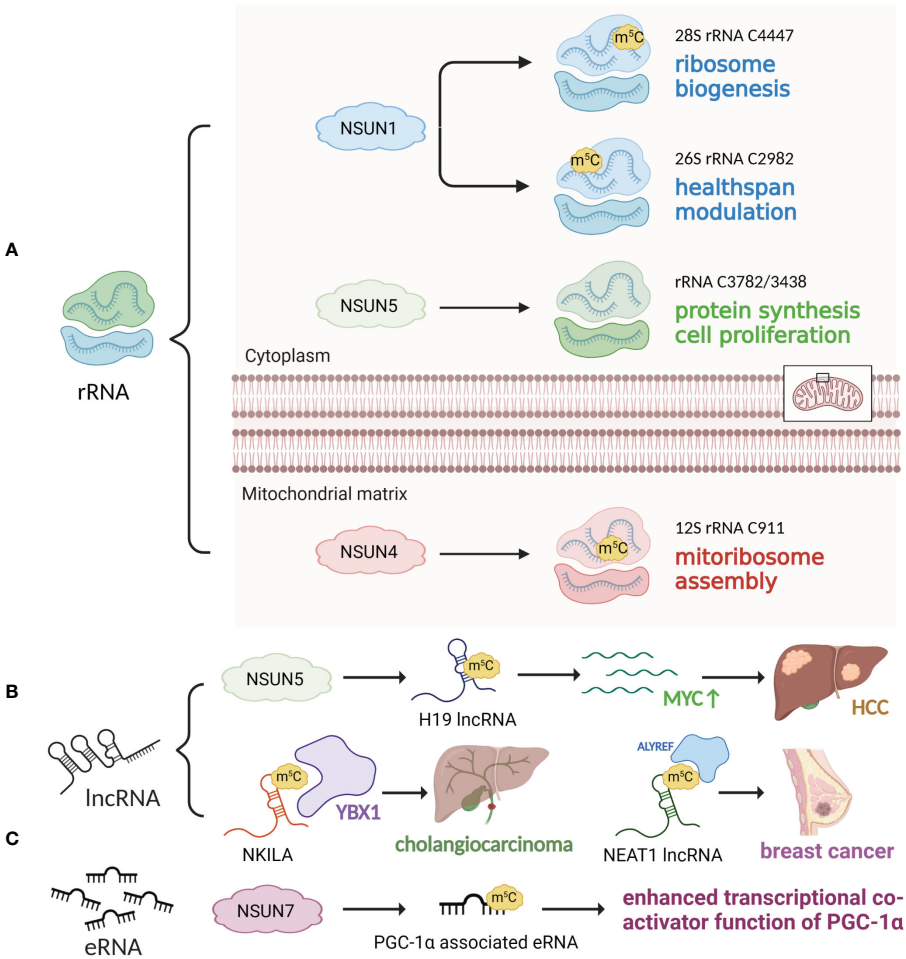


FIGURE 3
$m^5C$ modifications of rRNA, lncRNA and eRNA. **(A)** rRNA $m^5C$ modification is mediated by NSUN1, NSUN4 and NSUN5, facilitating ribosome biogenesis, healthspan modulation, mitoribosomal assembly, protein synthesis and cell proliferation. **(B)** NSUN2 catalyzes $m^5C$ modification of H19 lncRNA, which stimulates MYC expression and HCC development. YBX1 recognizes and stabilizes $m^5C$-modified NKILA, promoting cholangiocarcinoma development. **(C)** NSUN7 $m^5C$-methylates eRNA associated with PGC-1α, promoting its transcriptional coactivator function.

ovary cancer (79) but is a risk factor for CRC (80, 81). One possible explanation of the controversial role of NSUN6 in different types of cancers is that NSUN6 expression level in different immune cells within the TME differs based on the tumor context. For instance, NSUN6 is mainly expressed in Tregs in TNBC (36), but in exhausted CD8+ T cells, proliferating T cells and myofibroblasts in CRC (80). In addition, NSUN6 is also related to the promotion of the cell cycle (82), infiltration of B cells, CD4+ T cells and CD8+ T cells (36, 80), and formation of antibody-secreting plasma cells (83). NSUN7 methylates eRNA, a noncoding RNA associated with transcription modulation, and enhances the expression of mRNAs coding for Pfkl, Sirt5, Idh3b and Hmox2 in a peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α)-dependent manner. These effects are likely to facilitate adaptive metabolic alterations under starvation (84).

## Readers

Readers, or binding proteins of $m^5C$ sites, include ALYREF and YBX1. ALYREF is reported to be an important oncogenic factor and is associated with poor prognosis in patients with various types of cancer, including HCC (72, 85, 86), glioblastoma (87, 88), glioma (63), neuroblastoma (89), lung adenocarcinoma (90, 91), HNSCC (92, 93), bladder cancer (32) and breast cancer (94, 95). For example, elevated levels of ALYREF in HCC patients were found to be responsible for upregulated eIF4A3 expression (86) and abnormal cell cycle and mitosis (72). In lung adenocarcinoma patients, ALYREF, together with NSUN2, promotes $m^5C$ modification of YAP (Yes-Associated Protein) mRNA in the 3'-UTR, thus increasing the stability of YAP mRNA and causing enhanced exosome secretion, tumor malignancy and drug resistance (91). In contrast, ALYREF is considered a protective factor against colon adenocarcinoma (81), but the mechanism remains to be elucidated. In addition, ALYREF also participates in the regulation of adipogenesis (96), myogenesis (96, 97) and retrovirus replication (98) and may have biological activity in the context of abdominal aortic aneurysm (AAA) (99).

YBX1 is another $m^5C$ reader that has multiple functions in cancer development and embryo development. Oncogenic effects of YBX1 are found in GC (51), bladder cancer (100), glioblastoma

(101), CRC (76, 102), cholangiocarcinoma (103), clear cell renal cell carcinoma (ccRCC) (104), prostate cancer (56), epithelial ovarian cancer (105) and cervical cancer (57), mainly owing to YBX1-RNA interactions that have stabilizing effects on target RNAs. For example, in GC patients, YBX1 binds with FOXC2 (Forkhead box protein C2) mRNA, which is $m^5C$-modulated by NSUN2, to enhance its tumor-promoting ability (51). In bladder cancer patients, YBX1 stabilizes oncogenic HDGF (heparin binding growth factor) mRNA by targeting the $m^5C$-modified site on its 3′-UTR (100). Interestingly, in addition to its oncogenic effects, YBX1 is also important in normal cell proliferation and embryo development. For instance, animal experiments revealed that YBX1 is essential for embryonic brain development in mice (101), and YBX1 deficiency causes early gastrulation defects in zebrafish embryos (106).

## Erasers

Currently discovered $m^5C$ demethyltransferases are the TET family and ALKBH1. The TET family is a group of Fe(II) and alpha-ketoglutarate-dependent $m^5C$ dioxygenases that convert 5-methylcytosine ($m^5C$) to 5-hydroxymethylcytosine ($hm^5C$) (122). These enzymes were originally discovered in the translocation breakpoint of t(10;11) in patients with infant acute myeloid leukemia (AML), hence the name (123). Overall, the scarcity of research focusing on $m^5C$ erasers limits the comprehensive understanding of these proteins. TET1-mediated mRNA $m^5C$ demethylation is essential for completion of DNA repair and survival of cells in the context of DNA damage (107). TET2 mainly facilitates the conversion of $m^5C$ into $hm^5C$, thus leading to the elimination of $m^5C$ modification in RNA (108, 109), but the effects are not as strong as ALKBH1 (109). TET2 expression was measured in patients with various types of cancers, and it was upregulated in low-grade glioma patients (63) but downregulated in ccRCC (110, 111), ovarian cancer (112) and prostate adenocarcinoma (113) patients. Note that the potential tumor-suppressive effect of TET2 in prostate adenocarcinoma is likely to be linked with enhanced immune cell infiltration (113). TET3-mediated $m^5C$ elimination has not been clarified to date. However, some researchers have reported that upregulated TET3 expression in prostate cancer patients might be associated with poor prognosis (38).

ALKBH1 has been identified as a demethyltransferase for both RNA and DNA (124), but most studies focused on ALKBH1-demethylated DNA modifications, with fewer researchers concentrating on the RNA part. So far, studies have revealed that ALKBH1 takes part in the transformation of $m^5C$ RNA modifications to either $hm^5C$ or $f^5C$ (5-formylcytidine) RNA modifications (109, 114). More specifically, in cytoplasmic tRNA, ALKBH1 targets the wobble position (position 34) of $tRNA^{Leu\text{-}CAA}$ and converts $m^5C$ RNA modifications to $hm^5C$ or $f^5C$, promoting the decoding of Leu codons under stress (109, 114). At the same position (position 34) of mitochondrial $tRNA^{Met}$, only the alteration from $m^5C$ to $f^5C$ was found, which was proved indispensable for the translation of AUA, a non-universal codon in mammalian mitochondria, indicating that ALKBH1-mediated $m^5C$ RNA modification removal is significant for mammalian mitochondrial functions (114). Interestingly, an *in vitro* experiment showed that ALKBH1 first hydroxylates $m^5C$ to form $hm^5C$, and then oxidizes $hm^5C$ to form $f^5C$, meaning ALKBH1-mediated biogenesis of $hm^5C$ and $f^5C$ is actually two relevant and coherent processes (114).

# $m^5C$ RNA modification in cancer cells

As discussed above, RNA $m^5C$ modification has been discovered to be an important biological process in many types of diseases, including cancer. Dysfunction or alterations in the expression levels of $m^5C$ writers, readers and erasers influence tumor development, malignancy and metastasis by changing both mRNA and noncoding RNAs at the expressional and transcriptional levels (Figure 4). Here, we present a detailed overview of $m^5C$-mediated alterations within tumor cells that have been clarified to date (Table 2). Understanding the molecular mechanisms of $m^5C$-mediated tumorigenesis is vastly important for increasing the therapeutic efficiency of antitumor treatments.

## Hepatocellular carcinoma (HCC)

Previous studies have demonstrated a clear relationship between high $m^5C$ levels and HCC development, migration and malignancy. Recent studies have mainly focused on lncRNAs. For example, during HCC, the expression levels of $m^5C$-associated genes, including NSUN2 (35, 54), NSUN4 (71), NSUN5 (75) and ALYREF (86), increase. NSUN2-mediated $m^5C$ modulation of H19 lncRNA increases its stability, leading to enhanced recruitment of the G3BP1 (Ras-GTPase-activating protein-binding protein 1) oncoprotein, a potential enhancer of MYC accumulation (35). NSUN2 also modulates the Ras signaling pathway as well as the cell cycle, thus allowing for tumor escape from chemotherapy (54). A bioinformatics analysis discovered that NSUN5 overexpression was positively associated with enhanced ribosome functions and protein translation within HCC cells (75). ALYREF dysfunction is responsible for aberrant cell cycle regulation and mitosis of HCC cells (72) and promotes HCC possibly *via* stimulation of eIF4A3 expression (86). Thus, suppressors for ALYREF and eIF4A3, such as miR-4666a-5p and miR-6124, are promising therapeutic agents (86). Moreover, $m^5C$ modulation of circRNA is also important in HCC development (125). In addition, alterations in the tumor microenvironment (TME) and immune cell infiltration also contribute to $m^5C$-mediated HCC development (135, 136). Currently, researchers are investigating new methods for prognosis prediction in HCC patients and constructed speculating models based on $m^5C$-related modulators, such as the NSUN family, TET1, and YBX1 (72, 137, 138). These findings may have profound clinical implications.

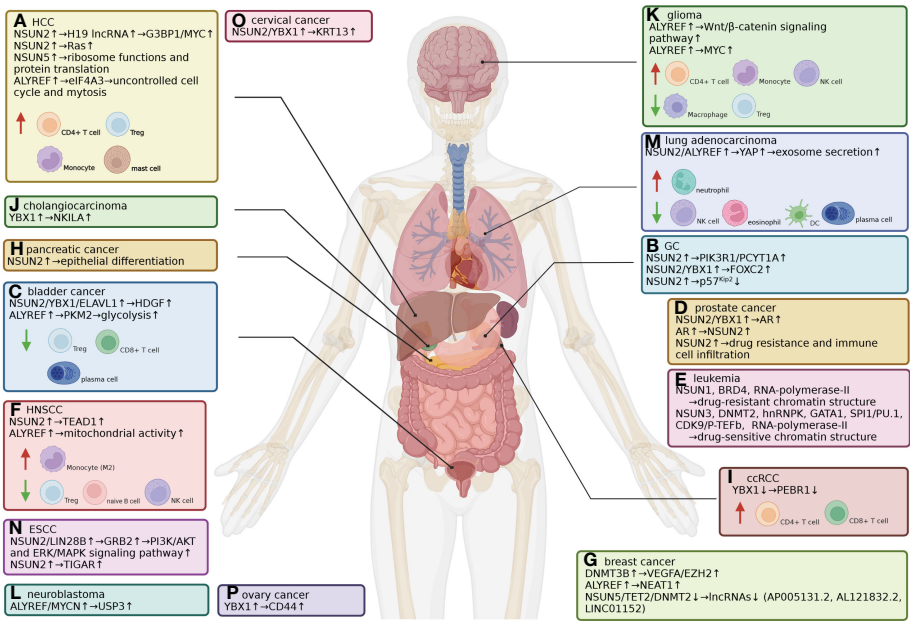


FIGURE 4
Expression of m$^5$C-related genes and immune cell infiltration in different cancer types. **(A)** HCC. NSUN2-mediated m$^5$C modulation of H19 lncRNA increases its stability, leading to enhanced recruitment of G3BP1 and MYC. NSUN2 also promotes HCC progression by modulating the Ras signaling pathway, the cell cycle and drug resistance. NSUN5 facilitates ribosomal functions and protein translation. ALYREF upregulates eIF4A3 expression, which leads to uncontrolled mitosis. The abundance of CD4+ T cells (including Tregs), M0, M1 and M2 macrophages and resting mast cells is higher in HCC tissues from patients with poor prognoses. **(B)** GC. NSUN2 methylates PIK3R1 and PCYT1A mRNA, stabilizing them and activating downstream cancerous signaling pathways. NSUN2 methylates FOXC2 mRNA, enhancing its interaction with the m$^5$C reader YBX1. NSUN2 destabilizes tumor-suppressive p57Kip2 mRNA by m$^5$C-methylation in its 3′-UTR. **(C)** Bladder cancer. ALYREF binds to the 3′-UTR of PKM2 mRNA, stabilizing it and enhancing PKM2-mediated glycolysis. NSUN2 mediates m$^5$C modification in the 3′-UTR of oncogenic HDGF mRNA; YBX1 recruits ELAVL1 to form a m$^5$C-binding complex to stabilize HDGF mRNA. Lower Treg, CD8+ T-cell and plasma cell infiltration rates indicate poor prognosis. **(D)** Prostate cancer. NSUN2 catalyzes and YBX1 recognizes m$^5$C modification sites on androgen receptor (AR) mRNA, and AR positively regulates NSUN2 transcription in return. NSUN2 expression also leads to drug resistance and immune cell infiltration. **(E)** Leukemia. NSUN1 forms an active drug-resistant chromatin structure with BRD4 and RNA polymerase-II, while SUN3 and DNMT2 form a drug-sensitive structure with hnRNPK, GATA1, SPI1/PU.1, and CDK9/P-TEFb to recruit RNA polymerase-II. **(F)** HNSCC. NSUN2 promotes HNSCC by suppressing immune infiltration and methylates and stabilizes TEAD1 mRNA. ALYREF increases mitochondrial activity to ensure tumor cells are supplied with energy. Lower Treg, naïve B-cell and NK cell infiltration indicates poor prognosis, while higher M2 macrophage infiltration indicates poor prognosis. **(G)** Breast cancer. ALYREF promotes breast cancer by enhancing the transcription of NEAT1 lncRNA. DNMT3B targets VEGFA and EZH2 as tumor promoters. NSUN5, TET2 and DNMT2 exert inhibitory effects on breast cancer by modifying three lncRNAs. **(H)** Pancreatic cancer. NSUN2 promotes pancreatic cancer and epithelial differentiation. **(I)** ccRCC. YBX1 negatively modulates ccRCC by binding and stabilizing PEBR1 mRNA. The abundance of CD4+ T cells and CD8+ T cells was higher in ccRCC tissues. **(J)** Cholangiocarcinoma. YBX1 promotes tumor development by stabilizing m$^5$C-methylated NKILA. **(K)** Glioma. ALYREF activates the Wnt/β-catenin signaling pathway and stabilizes MYC mRNA, promoting the development of glioblastoma, a malignant type of glioma. The infiltration of CD4+ T cells, monocytes and NK cells decreases in glioma tissues, while macrophage and Treg infiltration increases. **(L)** Neuroblastoma. ALYREF forms a nuclear coactivator complex with MYCN to stimulate USP3 transcription, which promotes tumorigenesis. **(M)** Lung adenocarcinoma. SUN2 and ALYREF increase YAP mRNA stability, thus enhancing exosome secretion, tumor malignancy and drug resistance. Lower plasma cell, eosinophil, NK cell and DC infiltration rates and higher neutrophil infiltration rates indicate poor prognosis. **(N)** ESCC. NSUN2 methylates GRB2 in a LIN28B-dependent manner, thus activating the PI3K/AKT and ERK/MAPK signaling pathways. NSUN2 also promotes TIGAR to enhance tumor growth. **(O)** Cervical cancer. NSUN2 and YBX1 promote cervical cancer by increasing the expression levels of KRT13 mRNA. **(P)** Ovarian cancer. YBX1 modulates CD44 expression to enhance chemoresistance.

## Gastric cancer (GC)

The role of m$^5$C RNA modification in GC is generally oncogenic, with high levels of m$^5$C indicating poor prognosis and a low overall survival (OS) rate (126). Modifications of both mRNA (30, 34, 51) and lncRNA (126, 139) have been observed during GC progression, and risk models based on m$^5$C levels were developed for prognosis prediction (139). NSUN2 is the main oncogenic m$^5$C-methyltransferase in GC, targeting the mRNAs of PIK3R1 (phosphoinositide-3-kinase regulatory subunit 1) (34), PCYT1A (phosphate cytidylyltransferase 1 choline-alpha) (34), FOXC2 (Forkhead box protein C2) (51) and p57$^{Kip2}$ (a type of cyclin-dependent kinase (CDK) inhibitor) (30). After m$^5$C modulation and binding to m$^5$C readers, such as YBX1, the transcriptional activity of PIK3R1, PCYT1A and FOXC2 mRNA was increased, while tumor-suppressive p57$^{Kip2}$ mRNA was destabilized as a result of m$^5$C modulation in the 3′-UTR. Consequently, the elevated NSUN2 levels in GC patients lead to enhanced proliferation, migration, and invasion of cancerous cells. NSUN2 activators, such as small ubiquitin-like modifier (SUMO)-2/3, which directly interact with NSUN2 to stabilize and mediate its nuclear transport, promote the development of GC (34). The oncogenic interaction between NSUN2 and FOXC2 mRNA can be facilitated by lncRNA FOXC2-AS1 (FOXC2 antisense RNA 1) (51). The oncogenic role of

TABLE 2 Expression of $m^5C$-related genes and tumor-promoting/suppressing mechanisms in different types of tumors.

| Cancer Types | Related Enzymes | Expression | Target RNAs | Effects | Mechanisms | References |
|---|---|---|---|---|---|---|
| HCC | NSUN2 | upregulated | H19 lncRNA | tumor-promoting | $m^5C$-methylates H19 lncRNA, leading to MYC stimulation | (35) |
| | | | / | tumor-promoting | modulates Ras signaling pathway and cell cycles, causing drug resistance | (54) |
| | NSUN4 | upregulated | / | tumor-promoting | / | (71) |
| | NSUN5 | upregulated | / | tumor-promoting | facilitates ribosome functions and protein translation | (75) |
| | ALYREF | upregulated | / | tumor-promoting | causes uncontrolled cell cycle and mitosis | (72) |
| | | | / | tumor-promoting | stimulates eIF4A3 expression | (86) |
| | / | / | circRNA | tumor-promoting | / | (125) |
| GC | NSUN2 | upregulated | PIK3R1 and PCYT1A mRNA | tumor-promoting | $m^5C$-methylates PIK3R1 and PCYT1A mRNA, stabilizing them and activating downstream cancerous signaling pathways | (34) |
| | | | FOXC2 mRNA | tumor-promoting | $m^5C$-methylates FOXC2 mRNA, enhancing its interaction with $m^5C$ reader YBX1 | (51) |
| | | | $p57^{Kip2}$ mRNA | tumor-promoting | destabilizes tumor-suppressive p57Kip2 mRNA by $m^5C$-methylation in its 3'-UTR | (30) |
| | / | / | / | tumor-promoting | immune suppression | (126) |
| bladder cancer | NSUN2, YBX1 | upregulated | HDGF mRNA | tumor-promoting | NSUN2 mediates $m^5C$ modification in 3'-UTR of oncogenic HDGF mRNA; YBX1 recruits ELAVL1 and together forms a $m^5C$-binding complex to stabilize HDGF mRNA | (100) |
| | ALYREF | upregulated | PKM2 mRNA | tumor-promoting | stabilizes PKM2 mRNA by binding to its 3'-UTR, enhancing PKM2-mediated glycolysis | (32) |
| prostate cancer | NSUN2, YBX1 | upregulated | AR mRNA | tumor-promoting | NSUN2 reciprocally increasesAR translation *via* $m^5C$-modulating AR mRNA in a YBX1-dependent manner | (56) |
| | NSUN2 | upregulated | / | tumor-promoting | affects drug resistance and immune cell infiltration | (37) |
| | TET3 | upregulated | / | tumor-promoting | / | (38) |
| leukemia | NSUN1 | / | / | tumor-promoting | forms an active chromatin structure with BRD4 and RNA-polymerase-II, which responds poorly to 5-AZA treatment | (127) |
| | NSUN3, DNMT2 | / | / | tumor-suppressive | bind directly with hnRNPK which interacts with GATA1, SPI1/PU.1 and CDK9/P-TEFb to recruit RNA-polymerase-II at precursor RNA, forming chromatin structures that are sensitive to 5-AZA treatment | (127) |
| HNSCC | NSUN3 | upregulated | / | tumor-promoting | promotes tumor progression by regulating immune infiltration | (64) |
| | NSUN2 | upregulated | / | tumor-promoting | negatively regulates immune cell infiltration in TME, promoting nasopharyngeal carcinoma (NPC) | (58) |
| | | upregulated | TEAD1 mRNA | tumor-promoting | $m^5C$-methylates oncogenic TEAD1 mRNA and upregulates its expression level, promotes hypopharyngeal squamous cell carcinoma (HPSCC) | (55) |
| | ALYREF | upregulated | / | tumor-promoting | enhances mitochondrial activity and intracellular energy metabolism, which ensures continuous energy supplies for timorous tissues | (92) |

*(Continued)*

TABLE 2 Continued

| Cancer Types | Related Enzymes | Expression | Target RNAs | Effects | Mechanisms | References |
|---|---|---|---|---|---|---|
| breast cancer | NSUN2 | upregulated | / | tumor-promoting | / | (36) |
| | ALYREF | upregulated | NEAT1 lncRNA | tumor-promoting | binds with oncogenic NEAT1 lncRNA promoter region, enhancing its transcription | (94) |
| | DNMT3B | upregulated | / | tumor-promoting | targets VEGFA and EZH2 | (95) |
| | NSUN6 | downregulated | / | tumor-suppressive | / | (36) |
| | NSUN5, TET2, DNMT2 | downregulated | lncRNA | tumor-suppressive | modifies three lncRNAs | (128) |
| pancreatic cancer | NSUN2 | upregulated | mRNA | tumor-promoting | regulates pancreatic tumorigenesis and epithelial differentiation through mRNA methylation | (129) |
| | / | / | / | tumor-promoting | $m^5C$ modification causes immune evasion and enhances PD-L1 expression | (130) |
| | NSUN6, DNMT3A | downregulated | / | tumor-suppressive | / | (31, 131) |
| ccRCC | DNMT3B, NSUN1, NSUN2, NSUN5 | upregulated | / | tumor-promoting | / | (110, 111, 132) |
| | NSUN6, TET2 | downregulated | / | tumor-suppressive | / | (111) |
| | YBX1 | downregulated | PEBR1 mRNA | tumor-suppressive | YBX1/EVAVL1 complex binds and stabilizes PEBR1 mRNA, which negatively modulates ccRCC | (104) |
| CRC | NSUN5, NSUN6, ALYREF, YBX1 | upregulated | / | tumor-promoting | / | (76, 81) |
| | / | / | / | tumor-promoting | inhibits tumor infiltration of immune cells | (133) |
| cholangiocarcinoma | YBX1 | upregulated | NKILA | tumor-promoting | recognizes and stabilizes $m^5C$-methylated NKILA | (103) |
| glioma | NSUN1-5, NSUN7, DNMT1, DNMT3B, YBX-1 | upregulated | / | tumor-promoting | / | (63, 101, 134) |
| | NSUN6 | downregulated | / | tumor-suppressive | / | (63, 134) |
| glioblastoma | ALYREF | upregulated | / | tumor-promoting | activates Wnt/β-catenin signaling pathway and reciprocally stabilizes MYC mRNA | (87, 88) |
| neuroblastoma | ALYREF | upregulated | / | tumor-promoting | forms a nuclear coactivator complex with MYCN to stimulate USP3 transcription | (89) |
| lung adenocarcinoma | NSUN2, ALYREF | upregulated | YAP mRNA | tumor-promoting | increase YAP mRNA stability, thus enhancing exosome secretion, tumor malignancy and drug resistance | (91) |
| ESCC | NSUN2 | upregulated | GRB2 mRNA | tumor-promoting | $m^5C$-methylates GRB2 *via* LIN28B-dependent way, thus activating PI3K/AKT and ERK/MAPK signaling pathway; promotes TIGAR | (52, 53) |
| cervical cancer | NSUN2, YBX1 | upregulated | KRT13 mRNA | tumor-promoting | promote KRT13 mRNA methylation and translational activation | (57) |

*(Continued)*

TABLE 2 Continued

| Cancer Types | Related Enzymes | Expression | Target RNAs | Effects | Mechanisms | References |
|---|---|---|---|---|---|---|
| ovarian cancer | YBX1 | upregulated | / | tumor-promoting | YBX1 modulates the expression of a variety of downstream targets, including CD44, thus enhancing chemoresistance | (105) |
| | NSUN6 | downregulated | / | tumor-suppressive | / | (79) |
| testis cancer | NSUN6 | downregulated | / | tumor-suppressive | / | (79) |
| thyroid cancer | NSUN6 | downregulated | / | tumor-suppressive | / | (79) |
| uveal melanoma | NSUN2 | upregulated | / | tumor-promoting | / | (59) |

VEGFA, vascular endothelial growth factor A; EZH2, enhancer of zeste homolog 2; BRD4, bromodomain-containing protein 4; 5-AZA, 5-azacitidine; hnRNPK (heterogeneous nuclear ribonucleoprotein K; GATA1, GATA binding protein 1; SPI1/PU.1, recombinant spleen focus forming virus proviral integration 1/purine rich box-1; CDK9/P-TEFb, cyclin-dependent kinase 9/positive transcription elongation factor b.

$m^5C$ modulation may also be linked to immune suppression, as patients with lower levels of $m^5C$ modulation were found to have higher levels of immune activation and longer progression-free survival (PFS) and OS (126).

## Bladder cancer

$m^5C$-mediated cell proliferation is considered one of causes of bladder cell malignancy. Overexpressed ALYREF in bladder cancer cells interacts with the 3'-UTR of PKM2 (pyruvate kinase M2) mRNA, causing its stabilization and enhanced PKM2-associated glycolysis (32). ALYREF stimulators, such as hypoxia-inducible factor-1alpha (HIF-1α), significantly increase the expression levels of ALYREF and PKM2 and are correlated with poor prognosis (32). Another $m^5C$ reader, YBX1,maintains the stability of its target mRNA, oncogenic HDGF mRNA methylated by NSUN2, by forming a 3'-UTR-binding complex with ELAVL1 (ELAV like RNA binding protein 1) (100). Moreover, high expression of immune cells, including regulatory T cells (Tregs), CD8+ T cells, plasma cells and activated dendritic cells, is related to a good prognosis, while high expression of resting CD4+ memory T cells, M0 macrophages, M1 macrophages, M2 macrophages and neutrophils show the opposite trend (140).

## Prostate cancer

Several prognostic models based on $m^5C$ modulators (38) or $m^5C$-related lncRNAs (141) have been developed for prostate cancer patients. Specifically, experimental results showed that increased NSUN2 (56), YBX1 (56) and TET3 (38) levels correlate with a poor prognosis. Posttranscriptional $m^5C$ modification of androgen receptor (AR) mRNA by NSUN2 is recognized by YBX1, increasing AR mRNA stability and translation (56). Interestingly, AR positively regulates NSUN2 at the transcriptional level (56), forming a reciprocal activation loop. High NSUN2 expression is also associated with low chemotherapeutic sensitivity and immune cell infiltration (37). In addition to NSUN2, immune cell infiltration characteristics are associated with many other $m^5C$ regulators, such as NSUN6 and TET1-3 (38, 113).

## Leukemia

RNA $m^5C$ modifications affect tumor malignancy and drug resistance not only in solid tumors but also in nonparenchymal tumors, including leukemia. In 2018, David G. Courtney et al. determined that NSUN1 is partly responsible for the formation of the 5-AZA (5-azacitidine)-insensitive chromatin structure during leukemia, which causes drug resistance (127). Mechanistically, NSUN1 forms an active chromatin structure with BRD4 (bromodomain-containing protein 4) and RNA-polymerase-II, which responds poorly to 5-AZA but well to the BRD4 inhibitor JQ1 or miRNA targeting NSUN1. In contrast, another two $m^5C$ regulators, NSUN3 and DNMT2, bind directly with heterogeneous nuclear ribonucleoprotein K (hnRNPK), a conserved RNA-binding protein that interacts with the lineage-determining transcription factors GATA binding protein 1 (GATA1), recombinant spleen focus forming virus proviral integration 1/purine rich box-1 (SPI1/PU.1) and cyclin-dependent kinase 9 (CDK9)/positive transcription elongation factor b (P-TEFb) to recruit RNA-polymerase-II to RNA precursors, forming chromatin structures that are sensitive to 5-AZA (127).

## Head and neck squamous cell carcinoma (HNSCC)

HNSCC refers to a group of epithelium-derived cancers that occur in the mucosal surfaces of the head and neck, including the oral and nasal cavity, oropharynx, nasopharynx, larynx and

hypopharynx. To date, statistics have revealed that almost all $m^5C$ regulators show elevated expression levels during HNSCC, with the exception of NSUN7 and TET2 (93, 142, 143), suggesting that they play different roles in HNSCC tumorigenesis. Mechanistically, NSUN3 promotes tumor progression by regulating immune infiltration (64), and ALYREF enhances mitochondrial activity and intracellular energy metabolism, which ensures continuous energy supplies for timorous tissues (92). In nasopharyngeal carcinoma (NPC) specifically, NSUN2 negatively regulates immune cell infiltration in the TME (58). In addition, NSUN2 promotes hypopharyngeal squamous cell carcinoma (HPSCC) by $m^5C$-methylating oncogenic TEAD1 (TEA domain transcription factor 1) mRNA, which upregulates its expression level (55).

## Breast cancer

$m^5C$ RNA modification has dual effects on breast cancer development. Recent studies suggest NSUN2 (36), ALYREF (94, 95) and DNMT3B (95) as risk factors, while NSUN5 (128), NSUN6 (36), TET2 (128), and DNMT2 (128)are protective factors. Mechanistically, ALYREF, which was found amplified both at the mRNA and protein levels, binds with the oncogenic NEAT1 lncRNA promoter region, enhancing its transcription (94). Additionally, enrichment analysis revealed that vascular endothelial growth factor A (VEGFA) and enhancer of zeste homolog 2 (EZH2) were potential targets of DNMT3B (95). In addition, NSUN5, TET2, and DNMT2 modified three lncRNAs, namely, AP005131.2, AL121832.2, and LINC01152, to be protective factors against breast cancer (128).

## Pancreatic cancer

As in many other types of cancers, NSUN2 regulates pancreatic tumorigenesis and epithelial differentiation through mRNA methylation (129). In contrast, NSUN6 (31) and DNMT3A (131) have inhibitory effects on pancreatic cancer and suppress the proliferation of cancerous cells, but the mechanisms remain to be elucidated. $m^5C$ modification profoundly influences the tumor immune microenvironment (130, 144), interfering with the infiltration of CD8+ T cells and upregulating PD-L1 expression (130). Risk models based on $m^5C$-related lncRNAs have also been constructed to provide prognostic information (145).

## Clear cell renal cell carcinoma (ccRCC)

$m^5C$ modification has dual effects on ccRCC development. YBX1 negatively modulates ccRCC by binding and stabilizing PEBR1 mRNA, a tumor suppressor gene (104). Other $m^5C$-related genes, such as DNMT3B, NSUN1, NSUN2 and NSUN5, are highly expressed in ccRCC patients and correlate with worse prognosis (110, 111, 132), while NSUN6 and TET2 mainly function as protective factors (111). Notably, the role of NSUN4 in ccRCC remains controversial, as studies have obtained opposing results (110, 111).

## Other cancer types

In CRC patients, increased levels of $m^5C$-related regulators, such as NSUN5, NSUN6, ALYREF and YBX1, were found (76, 81). The $m^5C$ levels of peripheral blood immune cells showed higher CRC diagnostic value than that of common blood tumor biomarkers (76), which is correlated with the discovery that $m^5C$ modification inhibits tumor infiltration of immune cells (133). In cholangiocarcinoma patients, the $m^5C$-modified functional lncRNA NKILA (NF-kappa B interacting lncRNA), which is recognized and stabilized by YBX1, is associated with advanced TNM stage and poor prognosis (103). In glioma patients, $m^5C$-associated genes, including NSUN1-5, NSUN7, DNMT1, DNMT3B and YBX-1, are upregulated, with the exception of NSUN6 (63, 101, 134). In patients with glioblastoma, the most aggressive diffuse glioma, upregulated ALYREF plays an oncogenic role by activating the Wnt/β-catenin signaling pathway and stabilizing MYC mRNA (87, 88). Interestingly, MYC also exerts positive impacts on ALYREF, forming a positive feedback loop (87). In neuroblastoma patients, the $m^5C$ reader ALYREF forms a nuclear coactivator complex with MYCN to stimulate USP3 transcription, which promotes the tumorigenesis of neuroblastoma (89). In lung adenocarcinoma patients, NSUN2 and ALYREF were found to be oncogenic through interacting with YAP mRNA. The $m^5C$ modification in the 328-331 3'-UTR of YAP mRNA increases its stability, enhances exosome secretion, and stimulates the transcription of seven downstream exosome-promoting genes. Together, $m^5C$-mediated YAP stimulation leads to increased tumor malignancy and drug resistance (91). In addition, risk models based on $m^5C$ regulators or $m^5C$-related lncRNAs were also developed for prognosis prediction (11, 90, 146). In patients with ESCC, NSUN2 promotes ESCC progression and chemoresistance by promoting TIGAR (TP53 induced glycolysis regulatory phosphatase) (53) and GRB2 (growth factor receptor bound protein 2) (52). The positive influence on GRB2 is achieved by NSUN2-mediated LIN28B-dependent $m^5C$ modification of GRB2 mRNA, which indirectly activates the PI3K/AKT and ERK/MAPK signaling pathways (52). In cervical cancer patients, NSUN2 and YBX1, which catalyze and recognize methylation sites, respectively, induce KRT13 mRNA methylation and translational activation (57). In ovarian cancer patients, YBX1 modulates the expression of a variety of downstream targets, including CD44, thus enhancing chemoresistance (105). In contrast, RNA $m^5C$ modification mediated by the methyltransferase NSUN6 suppresses testis, thyroid and ovary cancers (79). Finally,

NSUN2-mediated RNA $m^5C$ modification modulates uveal melanoma cell proliferation and migration, although the exact mechanisms remain unknown (59).

# $m^5C$ RNA modification in immune cells

Current studies have revealed that most immune cells, including T cells from different subgroups (e.g., CD4+ T cells, CD8+ T cells, Tregs), B cells and plasma cells, NK cells, NKT cells, macrophages, granulocytes and mast cells, manifest alterations in cell expression, infiltration and recruitment rate, which is concluded by several prognostic models constructed based on $m^5C$-related lncRNAs (147), $m^5C$-regulated genes (38, 148) or $m^5C$-related differentially expressed genes (DEGs) (149), especially in different types of cancers, and leads to a varied immune microenvironment (Figure 4), although most scattered studies did not provide systematized and convincing results (Table 3).

## T cells

Subgroups of T lymphocytes (mainly CD4+ and CD8+ T cells) are distinguished in T cells by surface markers using flow cytometry (154). Upon leaving the thymus, naïve CD4+ T cells further differentiate into T cell subsets according to different stimulating signals, such as T helper (Th) cells (e.g., Th1, Th2, Th9, Th17, Th22), T follicular helper (Tfh) cells and regulatory T cells (Tregs) (155).

Recent studies have mainly focused on the $m^5C$ RNA modification of T cells in the context of cancer. For instance, the

TABLE 3 Biological functions of $m^5C$-related genes in immune cells.

| Immune cells and subgroups | | Disease type | Associated $m^5C$-related genes | Target genes | Biological functions | References |
|---|---|---|---|---|---|---|
| T cells | CD4+T cells | prostate cancer | NSUN2 | / | / | (37) |
| | | CRC | DNMT3A | / | / | (80) |
| | | / | TET1, TET2 | / | TET1 and TET2 convert $m^5C$ into its oxidative derivatives, regulating CTCF-dependent pre-mRNA splicing, which affects gene expression | (150) |
| | | SLE | NSUN2 | mRNA | NSUN2 levels decrease along with mRNA $m^5C$ levels of CD4+ T cells | (151) |
| | | HIV-1 infection | NSUN1, NSUN2 | / | NSUN1 suppresses viral replication; NSUN2 facilitates the methylation and replication of HIV-1 transcripts | (46, 152) |
| | | / | NSUN2 | IL-17A mRNA | NSUN2 enhances IL-17A secretion of T cells by methylating IL-17A mRNA at C466, stimulating its translation | (60) |
| | CD8+ T cells | / | NSUN3, NSUN6, TET1, TET3 | / | / | (38, 66, 80) |
| B cells | memory B cells | prostate cancer | NSUN2 | / | / | (37) |
| | naïve B cells | prostate cancer | NSUN6, TET1, TET3 | / | / | (38) |
| | B cells | CRC | NSUN6, DNMT3A | / | / | (80) |
| | | / | NSUN6 | / | NSUN6 is dispensable for germinal center B-cell formation but necessary for the formation of antibody-secreting plasma cells | (83) |
| macrophages | | prostate cancer | NSUN6, TET1, TET3 | / | / | (38) |
| | | HNSCC | NSUN3 | / | NSUN3 promotes infiltration of M2 macrophages but suppresses infiltration of M1 macrophages | (64) |
| | | AAA | ALYREF | lncRNAs | ALYREF-interacting lncRNAs are involved in immune system regulation and macrophage infiltration | (99) |
| neutrophils | | lung squamous cell carcinoma | NSUN4 | / | / | (66) |
| | | / | TET2, TET3 | socs3b mRNA | TET2 and TET3 influences neutrophil granulation, phagocytosis and cytokine signaling by demethylating and destabilizing socs3b mRNA | (153) |
| NK cells | | prostate cancer | NSUN2 | / | / | (37) |
| DC | | CRC | DNMT3A | / | / | (80) |

CTCF, CCCTC-binding factor; socs3b, suppressors of cytokine signaling 3b.

abundance of CD4+ T cells is higher in patients with soft tissue sarcoma (STS) (149), ccRCC (110) and HCC (148) but the opposite is found in glioma patients (156). Tregs appear to be slightly different from common CD4+ T cells, as they correlate with poor prognosis in STS (149) and HCC (138) patients and positive outcomes in HNSCC (147) and bladder cancer (140) patients. The role of CD8+ T cells in cancer is also controversial since they are considered protective factors in bladder cancer (140) and lung adenocarcinoma (157) patients but risk factors in ccRCC (110) patients.

For CD4+ T cells, associated $m^5C$-related regulators include NSUN1 (46), NSUN2 (37, 60, 151, 152), DNMT3A (80), TET1 (150) and TET2 (150). Downregulated NSUN2 expression, along with decreased mRNA $m^5C$ levels of CD4+ T cells, was observed in systemic lupus erythematosus (SLE) patients, while the number of $m^5C$-containing RNAs increased. In addition, $m^5C$ sites were mainly distributed in mRNA translation initiation sites, and hypermethylated $m^5C$ and/or upregulated genes in SLE were enriched immune-related and inflammatory pathways, including immune system signaling pathway, cytokine signaling pathway, and interferon signaling pathway (151). In HIV-1-infected CD4+ T cells, NSUN2, as the primary HIV-1 $m^5C$ methyltransferase, facilitates HIV-1 transcript methylation as well as viral replication (152). Note that NSUN2 inactivation did not reduce HIV-1 mRNA expression levels but did downregulate protein expression, suggesting the role of $m^5C$ in HIV-1 translation. Additionally, $m^5C$ loss dysregulates the alternative splicing of viral RNAs (152). In contrast, NSUN1 deficiency caused latently infected HIV-1 proviruses to reactivate, revealing the viral suppressive effects of NSUN1 (46). Moreover, NSUN2 enhances IL-17A secretion by T cells by methylating IL-17A mRNA at C466, stimulating its translation (60). Finally, the $m^5C$ erasers TET1 and TET2 regulate pre-mRNA splicing in a CCCTC-binding factor (CTCF)-dependent manner.

Moreover, $m^5C$-related regulators affecting CD8+ T cells include NSUN3, NSUN6, TET1 and TET3 (38, 66, 80), but no further studies were found.

## B cells and plasma cells

B cells are derived from hematopoietic stem cells (HSCs) in the bone marrow. Naïve B cells, once properly activated, mature into plasma cells, the antibody-secreting form of B cells, following an intrinsic developmental process (158). Recent studies on B cells mainly concentrated on alterations in $m^5C$ RNA modifications during tumor pathology. In HNSCC patients, a higher number of naïve B cells is negatively correlated with the risk score for poor prognosis (147). Activated plasma cells exert similar effects in bladder cancer (140) and lung adenocarcinoma (157) patients. Also, a prognostic model for pancreatic cancer based on $m^6A$/$m^5C$/$m^1A$-associated lncRNAs showed that the low-risk group has a significantly higher concentration of naïve B cells and plasma cells within the TME (159), suggesting the protective role of B cells and plasma cells. Genes associated with $m^5C$ RNA modifications in B cells include NSUN2, NSUN6, DNMT3A, TET1 and TET3 (37, 38, 80). Specifically, although NSUN6 is dispensable for germinal center (GC) B-cell formation, it plays vital roles in the formation of antibody-secreting plasma cells (83).

## Macrophages

Monocytes and macrophages stem from hematological precursors in the bone marrow and are important in the innate immune system due to their phagocytic and antigen-presenting activity (160). Monocytes accumulate in peripheral blood, while macrophages are tissue-resident mature monocytes (161). Classically activated macrophages, or M1 macrophages, are proinflammatory, while alternatively activated macrophages, or M2 macrophages, are anti-inflammatory (162). Similar to T cells and B cells, most studies on $m^5C$ RNA modifications in macrophages are in the context of cancer development, especially macrophage infiltration in the TME. The risk scores based on $m^5C$-related genes of patients with four types of cancer (HCC, HNSCC, glioma and pancreatic cancer) were positively correlated with infiltration of resting macrophages (M0), M1 or M2 macrophages (138, 147, 148, 156, 159). Analysis of the TME in prostate cancer patients showed differentially expressed NSUN6, TET1 and TET3 in M1 and M2 macrophages (38). NSUN3 promotes infiltration of M2 macrophages but suppresses M1 macrophage infiltration in HNSCC patients (64). Moreover, in AAA patients, ALYREF-interacting lncRNAs are involved in immune system processes and macrophage infiltration (99).

## Granulocytes

Granulocytes refer to a group of leukocytes with specific cytoplasmic granules distinguished by Romanowsky staining into three main subsets, namely, neutrophils, eosinophils and basophils (163). Currently, the scarcity of research focusing on $m^5C$ RNA modification of eosinophils and basophils makes it difficult to conclude $m^5C$-related alterations in these two types of granulocytes. Only in lung adenocarcinoma patients is eosinophil infiltration correlated with a favorable prognostic pattern (157). Neutrophil abundance is generally associated with poor prognosis (138, 147), and a study on lung squamous cell carcinoma patients discovered that NSUN4 exerts a regulatory effect on neutrophil $m^5C$ RNA modification (66). Studies have also reported that TET2 and TET3 influence neutrophil granulation, phagocytosis and cytokine signaling by demethylating and destabilizing socs3b (suppressors of cytokine signaling 3b) mRNA, a member of the suppression of cytokine signaling gene family (153). TET2/3-defective embryos showed aberrant granule formation, defective phagocytosis and dysregulation of cytokine signaling in neutrophils due to accumulation of socs3b mRNA, which binds the Jak receptor to prevent Stat phosphorylation and downstream signaling *via* the Jak/Stat pathway (153).

## Others

Natural killer (NK) cells constitute a first line of innate immunity against tumors due to their capabilities of killing aberrant cells (164). Regarding $m^5C$ RNA modifications in NK cells, both resting and activated NK cells are correlated with positive outcomes in HNSCC (147), glioma (156) and lung adenocarcinoma (157) patients, with NSUN2 being the most closely associated $m^5C$ gene (37).

Dendritic cells (DCs) are generally regarded as the most potent antigen-presenting cells, thus modulating both immunity and tolerance (165). In lung adenocarcinoma patients, more DC infiltration was discovered in the low-risk group, suggesting the protective role of $m^5C$ in DCs (157). In contrast, a prognostic model for pancreatic cancer based on $m^6A/m^5C/m^1A$-associated lncRNAs showed that the high-risk group has a significantly higher concentration of activated DCs within the TME (159), showing the tumor-promoting effects of activated DCs. The conflicting results might result from the differences in cancer types, DC subtypes, activation extent and patient characteristics. Consequently, rigorous future studies are needed in this regard. In CRC patients, the $m^5C$ writer DNMT3A was found to be involved in regulating DCs (80).

Mast cells are tissue-resident cells that function in inflammatory responses and tissue homeostasis (166). These cells are usually recognized clinically for their roles in IgE-mediated degranulation and allergic inflammation (167). A risk model based on $m^6A/m^1A/m^5C$-regulated genes in HCC patients indicated the links between poor prognosis and high infiltration of resting mast cells (148).

In summary, the roles of $m^5C$ RNA modification of immune cells in the context of tumorigenesis largely remain to be further clarified. A better understanding of the mechanisms by which alterations in cell expression, infiltration and activation are regulated by methylation can be extremely helpful for the development of novel methods for tumor diagnosis as well as treatment.

## Summary and perspectives

In this review, we provide a detailed review concerning the roles of $m^5C$ RNA modifications in cancer by discussing $m^5C$ RNA-related genes and alterations in gene expression and immune cell infiltration. The modifications involve mainly mRNAs but also other noncoding RNAs such as tRNA, rRNA, and lncRNA, and are regulated by RNA $m^5C$ writers, readers and erasers, leading to changes in RNA processes, including transcription, transportation, translation and metabolism. Risk models made for prognosis prediction are based on $m^5C$ regulators as well as genes with $m^5C$ modification, which indicates the relationship between prognosis and alterations in immune cell infiltration in the TME.

However, numerous questions regarding oncogenic $m^5C$ RNA modifications remain to be elucidated. For example, the mechanisms by which $m^5C$ writers, readers and erasers function have only been investigated in a limited manner. What are the target genes of RNA $m^5C$ modifications? How are these genes linked to cell signal transduction and tumor malignancy? Additionally, we now know that RNA $m^5C$ modifications are related to immune cell infiltration within the TME, but scarce and controversial study results offer no comprehensive and fully convincing conclusions. What alterations do RNA $m^5C$ modifications cause indifferent groups of immune cells? How are these alterations linked to the progression of cancer? Is immune cell infiltration protective or destructive for patients with malignant tumors? These questions might provide deeper insights into the diagnosis and treatment of different cancer types.

Nevertheless, studies on RNA $m^5C$ modifications in cancer patients have massively progressed within the last five years, providing new analytical results from clinical samples. It should be pointed out that most of the studies based on clinical samples were only limited at the laboratory level, and no $m^5C$-related clinical trials against cancer has been performed so far. However, as detection methods for RNA $m^5C$ sites continue to improve, cancerous RNA $m^5C$ modifications will most likely remain a popular scientific topic in the years to come and, hopefully, instill new hope for millions of patients fighting cancer.

## Author contributions

XG and XM have equal contributions to this study. XG and HZ designed the whole study. XG and XM drafted the manuscript. CC, JG, JW, and SW made the relevant edits to the manuscript. XG and XM 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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# Elucidating the role of ubiquitination and deubiquitination in osteoarthritis progression

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Osteoarthritis is non-inflammatory degenerative joint arthritis, which exacerbates disability in elder persons. The molecular mechanisms of osteoarthritis are elusive. Ubiquitination, one type of post-translational modifications, has been demonstrated to accelerate or ameliorate the development and progression of osteoarthritis *via* targeting specific proteins for ubiquitination and determining protein stability and localization. Ubiquitination process can be reversed by a class of deubiquitinases *via* deubiquitination. In this review, we summarize the current knowledge regarding the multifaceted role of E3 ubiquitin ligases in the pathogenesis of osteoarthritis. We also describe the molecular insight of deubiquitinases into osteoarthritis processes. Moreover, we highlight the multiple compounds that target E3 ubiquitin ligases or deubiquitinases to influence osteoarthritis progression. We discuss the challenge and future perspectives *via* modulation of E3 ubiquitin ligases and deubiquitinases expression for enhancement of the therapeutic efficacy in osteoarthritis patients. We conclude that modulating ubiquitination and deubiquitination could alleviate the osteoarthritis pathogenesis to achieve the better treatment outcomes in osteoarthritis patients.

KEYWORDS

**ubiquitination, deubiquitination, osteoarthritis, E3 ligase, DUBs**

**Abbreviations:** DDAH1, dimethylarginine dimethylaminohydrolase-1; ADMA, metabolite asymmetric dimethylarginine; ECM, extracellular matrix; ERS, endoplasmic reticulum stress; FOXO3, forkhead box O3; H2O2, hydrogen peroxide; HIF-1α, hypoxia-inducible factor-1α; IL-1β, interleukin-1β; ITCH, itchy E3 ubiquitin protein ligase; LPS, lipopolysaccharide; MMP-1, matrix metalloproteinases; NEDL1, neural precursor cell expressed, developmentally down-regulated protein 1; NEDD4, neural precursor cell expressed developmentally down-regulated 4; NF-κB, nuclear factor κB NLRP3, Nod-like receptor protein 3; NOX4, NADPH oxidase 4; OA, osteoarthritis; ROS, reactive oxygen species; SMURF1, SMAD ubiquitination regulatory factor 1; TRAF6, tumor necrosis factor-receptor-associated factor 6; TNF-α, tumor necrosis factor alpha; USP7, ubiquitin specific protease 7; VEGF, vascular endothelial growth factor; WWP1, WW domain-containing protein 1.

## Introduction

Osteoarthritis (OA) is one common type of non-inflammatory degenerative arthritis, which is a joint disease and influences older persons for walk, leading to disability in elder persons (1, 2). Pain is the primary symptom in osteoarthritis patients. The features of osteoarthritis are the destructions of cartilage extracellular matrix (ECM), loss of mobility, joint dysfunction and synovial inflammation (3, 4). Aging and injure are two common reasons for the development of osteoarthritis. The exact mechanisms of osteoarthritis are not fully understood, but it is thought to involve a complex interplay of genetic, environmental, and biomechanical factors (5–9). The treatments often include pain control and reduction of inflammation (10). It is of great importance to discover the mechanisms of osteoarthritis and develop the novel therapeutic strategies for osteoarthritis (11, 12).

In general, protein is synthesized from the genetic code in DNA and translated into a polypeptide chain. Protein could be modified *via* the chemical changes, which is named as post-translational modification (PTM) (13, 14). PTMs are found to modulate protein functions *via* an effect on various protein behavior, such as activity, localization, stability and interactions with other proteins (15). Many common PTM types include methylation (addition of a methyl group), acetylation (addition of an acetyl group), ubiquitination (attachment of a small ubiquitin protein), sumoylation (attachment of the small protein SUMO), glycosylation (addition of sugar molecules) and phosphorylation (addition of a phosphate group), which affect localization, stability, interaction, folding, and gene expression (16–19). PTMs are critical in governing some biological processes, such as cell signaling pathways, cell metabolism, differentiation, metastasis, cell cycle and proliferation (20).

Ubiquitination is one type of PTMs, which involves the covalent attachment of ubiquitin to a target substrate. Ubiquitination affects substrate protein functions *via* regulation of localization and stability (21). The ubiquitination process is performed by E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase. E1 enzymes activate ubiquitin and transfer it to E2 enzyme. Then, E2 enzymes interact with E3 enzymes, leading to the transfer of ubiquitin from the E2 enzymes to the specific target proteins (22, 23). The ubiquitinated proteins can be recognized and degraded by the proteasome machinery (24, 25). Hence, the E3 ligases determine the specificity of the ubiquitination reaction due to recognizing and interacting with specific substrates (Figure 1). Several types of E3 ligases are RING (really interesting new gene) ligases, HECT (homologous to E6-AP carboxyl terminus) ligases, RBR (RING-between-RING) ligases, U-box ligases and PHD-finger ligases (26, 27). Ubiquitination can be reversed by deubiquitination, which is a process of cleaving ubiquitin from target proteins (28) (Figure 1). Deubiquitination is carried out by a class of deubiquitinases (DUBs), including ovarian tumor proteases (OTUs), ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), and Josephin domain-containing proteins (29). Among these DUBs, USPs are the largest family to cleave ubiquitin from substrates. Dysregulation of USPs have been implicated in various diseases, such as neurodegeneration, inflammation and cancer (30–32). It is clear that the balance between ubiquitination and deubiquitination is pivotal for maintaining proper protein levels and their functions (33).

In recent years, ubiquitination and deubiquitination have been found to play a potential role in the various pathologies, including osteoarthritis (34). In this review, we will describe the role of ubiquitination in the development and progression of osteoarthritis. Moreover, we discuss the function of deubiquitination in regulating osteoarthritis development. Furthermore, we describe the compounds that target ubiquitination and deubiquitination to influence the osteoarthritis progression. Lastly, we provide the challenge and future perspectives for targeting ubiquitination and deubiquitination to treat with osteoarthritis patients.

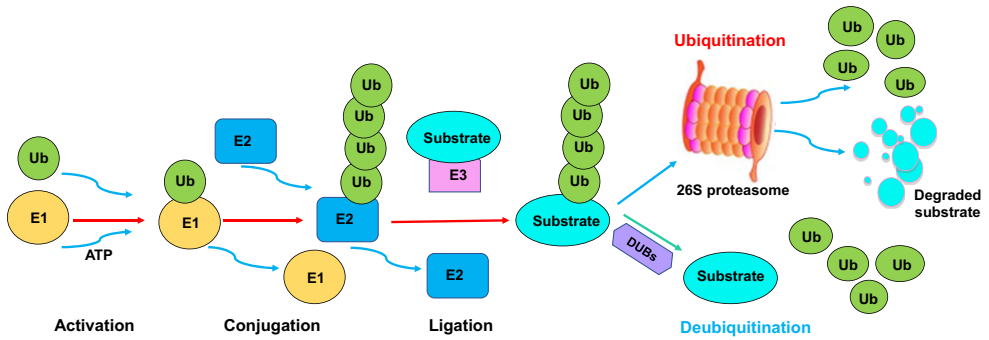


FIGURE 1
The ubiquitination and deubiquitination processes. The ubiquitination process is performed by E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase. E1 enzymes activate ubiquitin and transfer it to E2 enzyme. Then, E2 enzymes interact with E3 enzymes, leading to the transfer of ubiquitin from the E2 enzymes to the specific target proteins. The ubiquitinated proteins can be recognized and degraded by the proteasome. Ubiquitination can be reversed by deubiquitination, which is a process of cleaving ubiquitin from target proteins. Deubiquitination is carried out by a class of deubiquitinases (DUBs).

# The role of ubiquitination in osteoarthritis

## NEDD4 E3 ubiquitin ligases in osteoarthritis

NEDD4 E3 ubiquitin ligases are a family of enzymes that share a C2 domain at N-terminal and HECT domain at C-terminal, which regulate protein degradation, membrane trafficking and signaling transduction (35). This family includes several members, such as NEDD4, NEDD4-2, WWP1, WWP2, ITCH, Smurf1, Smurf2, NEDL1 and NEDL2. NEDD4 family has been involved in regulation of various diseases, including cancer, inflammation, and osteoarthritis (36–38). In the following paragraphs, we will discuss the mechanisms of NEDD4 E3 ubiquitin ligases in osteoarthritis initiation and progression (Table 1).

## WWP1

WWP1 (WW domain-containing protein 1), an E3 ubiquitin ligase, plays an essential role in regulating protein degradation, cellular signal transduction, and gene expression. WWP1 targets proteins and make them for degradation by the proteasome (52). Recent studies have implied that WWP1 may involve in the development and progression of osteoarthritis. One bioinformatics analysis using cartilage samples showed that several genes are potential biomarker of osteoarthritis, such as WWP1, MDM2, OAS2, MSH2, UBE2E3, BCL2, RB1, TYMS and EGFR (39). In addition, miR-5692, miR-548e-5p and miR-3613-3p were associated with the abovementioned genes. It is required to determine whether WWP1 could be a useful biomarker of osteoarthritis (39).

## WWP2

WWP2 (WW domain-containing protein 2) belongs to the NEDD4 family of E3 ubiquitin ligases. WWP2 contains several domains, such as a C2 domain, a HECT domain, and multiple WW domains. These domains are responsible for transferring the ubiquitin to target proteins (53). WWP2 controls the protein stability and biological functions *via* providing ubiquitin molecules to targets, resulting in proteasomal degradation or modulation of protein activity or changing protein subcellular localization (54). Due to that WWP2 is involved in a wide range of biological functions, dysregulation of WWP2 leads to various disease states, such as neurodegeneration, cancer, inflammation and viral infections (55, 56). WWP2 was involved in chondrogenesis and osteoarthritis by regulating cartilage-specific transcription factors (57). WWP2 overexpression led to an inhibition of COL2A1, STC2, ACAN, GDF10 and GJA1, and an upregulation of EPAS1 expression using 3D chondrocyte pellet culture system. In addition, miR-140 overexpression caused an increased WWP2 and WDR1 in chondrocytes (58).

Yang et al. reported that miR-140 co-expressed with WWP2-C isoform, which can be accelerated by Sox9. Silencing of miR-140 reduced chondrogenic proliferation *via* targeting Sp1 (59). NFAT3 (nuclear factor of activated T-cells protein 3) and TGF-β/SMAD3 (mothers against decapentaplegic homolog 3) controlled miR-140 expression in osteoarthritis (60). Depletion of NFAT5 inhibited the expression of miR-140 and WWP2. NFAT3 and SMAD3 can bind to miR-140, but depletion of NFAT3 reduced miR-140 expression without altering WWP2 (60). Mice without WWP2 and mice with inactivation type of WWP2 E3 ligase (WWP2-C838A) had spontaneous and induced osteoarthritis. WWP2 regulated the expression of Runx2 *via* poly-ubiquitination and degradation and reduced Adamts5, contributing to cartilage homeostasis. WWP2

TABLE 1 E3 ubiquitin ligases regulate osteoarthritis development and progression.

| Name | Targets | Functions | Ref |
|---|---|---|---|
| WWP1 | N/A | Involves in osteoarthritis development | (39) |
| WWP2 | Runx2 | Modulates osteoarthritis | (40) |
| Smurf1 | N/A | Regulates osteoarthritis | (41) |
| Smurf2 | SIRT1 | Regulates apoptosis, proliferation, inflammation in osteoarthritis chondrocytes | (42) |
| ITCH | JAG1 | Regulates chondrocyte damage and cartilage damage | (43) |
| FBXW7 | MKK7 | Regulates chondrocyte degeneration and osteoarthritis | (44) |
| FBXO3 | IL-18, IL-1β, pyroptosis-related proteins | Regulates knee osteoarthritis | (45) |
| FBXO6 | MMP14 | Controls post-injury osteoarthritis development | (46) |
| FBXO21 | ERK | Regulates osteoarthritis-associated cartilage degeneration | (47) |
| HECTD1 | Rubicon | Controls stress-induced chondrocyte death and osteoarthritis | (48) |
| Cbl-b | TrkA | Regulates osteoarthritis pain | (49) |
| UFL1 | NO, PGE2, iNOS, COX-2, MMP-3, MMP-13, ADAMTS-4, ADAMTS-5 | Involves in osteoarthritis development | (50) |
| HRD1 | OS9 | Regulates osteoarthritis development | (51) |

mRNA injection attenuated the severity of osteoarthritis in mouse joints (40). One methylation quantitative trait loci (mQTLs) analysis showed that WWP2 was linked to osteoarthritis genetic risk (61). In a word, WWP2 is involved in modulation of osteoarthritis.

## SMURF1 and SMURF2

Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2 belong to the family of NEDD4 ubiquitin ligase. Both Smurf1 and Smurf2 are involved in regulation of protein levels in the ubiquitin-dependent manner, which control the cell cycle progression, cell growth and differentiation (62). Although Smurf1 and Smurf2 share many functional similarities, they have distinct roles in governing various cellular processes to maintain cellular homeostasis. Dysregulation of Smurf1 and Smurf2 has been linked to cancer, inflammation and developmental disorders (63–65). One group used chondrogenic progenitor cells (CPCs) and meniscus progenitor cells (MPCs) and found that both Smurf1 and Smurf2 existed in articular cartilage and meniscus. An increased expression of Smurf1 alleviated the levels of TGFBR1, RUNX2 and SOX9, while upregulation of Smurf2 inhibited the levels of RUNX2 and GFFBR1, but not SOX9 levels. Depletion of Smurf2 increased the levels of SOX9 and RUNX2 in CPCs, but not in MPCs. Inhibition of Smurf1 did not affect the levels of RUNX2, SOX9 and TGFBR1 (41). Smurf1 protein was upregulated more in mice with Smurf2 depletion than wildtype mice upon TFG-β3 stimulation (66). Smurf2 has been observed to regulate osteoarthritis *via* targeting β-catenin in cartilage (67). Smurf2 was strongly expressed in human osteoarthritis compared with nonarthritic cartilage. Mice with Smurf2 overexpression displayed less articular cartilage area, osteophytes, clefting, subchondral sclerosis, and fibrillation. These mice exhibited the high expression of MMP-13 and type X collagen in articular cartilage. Moreover, Smurf2-transgenic mice had a decreased TGF-β pathway and an increased degradation of pSmad3 (68). Mice with Smurf2 deficiency exhibited higher expression of SOX, Col2 and Acan compared with wildtype mice (66). Chondrocytes with Smad3 deficiency accelerated differentiation and activated BMP signaling pathway, but failed to increase the expression of Smurf2, Sno, TGFRII in chondrocytes (69).

Increased expression of Smurf2 in cells of the chondrogenic lineage blocked differentiation of chondrocytes and enhanced maturation as well as promoted osteoblast differentiation. This phenotype could be due to upregulation of beta-catenin in the chondrocytes after Smurf overexpression, indicating that Smurf2 might be responsible for the development of osteoarthritis (70). One study showed that Smurf2 mediated degeneration of articular cartilage *via* promoting degradation of GSK-3beta and induction of beta-catenin in chondrocytes (71). LncRNA TM1-3P targeted miR-144-3p and ONECUT2 (one cut homeobox 2), governed cell apoptosis, inflammation and proliferation in osteoarthritis (72). LncRNA-CRNDE depletion elevated the ubiquitination of SIRT1 induced by SMURF2, resulting in reduction of SIRT1 stability. Overexpression of lncRNA-CRNDE stimulated the interaction of collagen 2 promoter and SOX9 (42). Circ_0116061 regulated the expression of miR-200b-3p and SMURF2, leading to modulation of cell apoptosis, proliferation, and inflammation in osteoarthritis chondrocytes (73). Lead, an environmental toxin, impaired TGF-β signaling pathway *via* upregulation of Smurf2 and downregulation of pSmad2 and pSmad3, leading to osteoarthritis in articular chondrocytes (74).

## ITCH

ITCH (also known as AIP4), a member of NEDD4 family of E3 ubiquitin ligases. It is pivotal to regulate signaling pathways and immune system *via* degradation of different proteins (75–77). One group reported that mice with ITCH deficiency in macrophages exhibited more severe joint damage compared with wildtype mice after post-traumatic osteoarthritis surgery. Mice with ITCH deficiency displayed promotion of the inflammatory macrophage infiltration in the synovium (78). ITCH protein levels were decreased during post-traumatic osteoarthritis. Macrophages with ITCH depletion were stimulated with IL-1β and exhibited pro-inflammatory phenotype. Hence, ITCH attenuated the progression of post-traumatic osteoarthritis *via* suppression of macrophage polarization (78). Another group identified that ITCH alleviated LPS-mediated chondrocyte injury through regulating JAG1 ubiquitination in osteoarthritis (43). ITCH expression was decreased in osteoarthritis samples compared with normal cartilaginous samples, whereas JAG1 expression was elevated in osteoarthritis specimens. Upregulation of ITCH or depletion of JAG1 induced proliferation, attenuated inflammation and ECM degradation and blocked apoptosis in LPS-mediated chondrocytes (43). Specifically, ITCH interacted with JAG1 *via* the WW-PPXY motif and caused JAG1 degradation *via* K48-linked ubiquitination, leading to inactivation of Notch1 pathway. Upregulation of JAG1 restrained the ITCH-mediated protection of chondrocyte damage that was induced by LPS. In conclusion, LPS-mediated chondrocyte injury and osteoarthritis-mediated articular cartilage damage were mitigated by ITCH in part *via* inhibition of Notch1 activation by enhancement of JAG1 ubiquitination and degradation (43).

## F-box proteins in osteoarthritis

The F-box protein is one of the subunits of the Skp1-Cullin1-F-box (SCF) complex, which acts as the substrate recognition component (79). The F-box protein contained a conserved F-box motif, which binds to a scaffold protein Skp1 to provide the link for the F-box protein to the rest of the SCF complex. Cullin1 interacts with Skp1 and F-box protein to form a core structure of the SCF complex. It is important to note that the variable region of F-box proteins determines its specificity for various substrates, leading to the ubiquitination of specific target proteins and degradation by the proteasome (80). F-box proteins regulate cell growth, apoptosis, differentiation, autophagy, cell cycle and metastasis. Abnormal

expression of F-box proteins could participate in the development of cancer, inflammation and neurodegenerative disorders (81–83). For example, FBXO45 targeted GGNBP2 for ubiquitination and degradation and enhanced progression of esophageal squamous cell carcinoma (84). In the next paragraphs, we will mention the function of numerous F-box proteins in governing osteoarthritis initiation and progression (Table 1).

## FBXW7

FBXW7 (F-box and WD repeat domain-containing 7) has been well studied in regulation of various cellular processes. Numerous targets of FBXW7 have been identified, including c-Myc, c-Jun, Notch, cyclin E and Mcl-1 (85). Trough targeting these proteins, FBXW7 controls cell proliferation, survival, cell cycle, differentiation and apoptosis. Mutations in FBXW7 were reported in various human malignancies. In tumorigenesis, FBXW7 has been revealed to be a tumor suppressive protein (86, 87). In recent years, FBXW7 was characterized to take part in osteoarthritis development. Mechanical overloading led to acceleration of senescence in chondrocyte and in mouse articular cartilage, which accompanied with downregulation of FBXW7 in chondrocytes and mouse cartilage (44). In line with this finding, FBXW7 was decreased in cartilage tissues in ageing mice, osteoarthritis mice, osteoarthritis patients. Depletion of FBXW7 in chondrocytes enhanced cartilage catabolism and caused chondrocyte senescence *via* promotion of p16, p21 and Colx and reduction of Col2a1 and ACAN, contributing to the exacerbation of osteoarthritis (44). On the contrary, overexpression of FBXW7 by intra-articular injection of adenovirus restrained osteoarthritis in mice. Further experiments indicated that mechanical overloading reduced the transcription of FBXW7 and alleviated FBXW7-induced degradation of MKK7, leading to activation of JNK signaling pathway (44).

Circular RNA VMA21 (circVMA21) expression was impeded by IL-1β in chondrocytes and C28/I2 cells. Ectopic expression of circVMA21 elevated the expression of Bcl-2 and reduced Bax and C-caspase-3 in C28/I2 cells after IL-1β stimulation, resulting in viability promotion and apoptosis attenuation (88). In addition, upregulation of circVMA21 reduced the MMP1 and MMP13 expressions, elevated COL2A1 expression and attenuated the production of NO, PGE2, TNF-α and IL-1β. Mechanistically, circVMA21 sponged miR-495-3p and consequently upregulated the expression of FBXW7, a target of miR-495-3p in chondrocytes (88). Zhu et al. reported that FBXW7 participated in IL-1β-involved chondrocytes degeneration *via* modulation of HIF-1α and VEGF pathways (89). In particular, lower expression of FBXW7 and higher expression of VEGF and HIF-1α were observed in osteoarthritis cartilage and IL-1β-mediated degenerated chondrocytes. Inhibition of HIF-1α led to reduction of VEGF levels, leading to upregulation of aggrecan, SOX9 and collagen II, and downregulation of collagen I and Runx-2 expression. Therefore, FBXW7 impeded HIF-1α/VEGF pathway and displayed a protective function in IL-1β-mediated chondrocyte degeneration *via* altering the expression of collagen I, collagen II, SOX9, aggrecan and Runx-2 (89).

## FBXO3

FBXO3 has been found to stimulate cytokine secretion *via* controlling the degradation of Fbxl2 and subsequent upregulation of TNFR-associated factor (TRAF) proteins (90). FBXO3 was also reported to potentiated vascular inflammation and atherosclerosis (91). One study used rat fibroblast-like synoviocytes (FLSs) to construct knee osteoarthritis cell model. This cell model exhibited elevation of IL-18, IL-1β, apoptosis rate, and increased expression of pyroptosis-associated proteins. The abovementioned indicators were downregulated by up-modulation of miR-219a-5p, while downregulation of miR-219a-5p exhibited the opposed results (45). FBXO3 expression was inhibited by miR-219a-5p, and depletion of FBXO3 repressed the expression of IL-18, IL-1β and pyroptosis-related proteins. FBXO3 counteracted the effects of miR-215a-5p-inhibited IL-18, IL-1β and pyroptosis-related proteins. Additionally, miR-219a-5p alleviated knee joint injury and elevated step size of rat with knee osteoarthritis partly *via* inhibition of FBXO3 and inactivation of NLRP3 (45).

## FBXO6

Wang et al. explored the effects of FBXO6 on the osteoarthritis pathogenesis. The human osteoarthritis samples and several mouse osteoarthritis models were used to detect the expression of FBXO6. This study showed that FBXO6 was downregulated in the cartilage from aged mouse samples, spontaneous osteoarthritis samples, ACLT (anterior cruciate ligament transaction)-induced osteoarthritis samples (46). Osteoarthritis process was accelerated in mice with cartilage conditional knockout or global knockout of FBXO6. FBXO6 reduced the MMP14 protein levels *via* ubiquitination and degradation, contributing to inactivation of MMP13 proteolytic ability. Notably, TGF-β-SMAD2/3 axis can upregulate the expression of FBXO6. Overall, upregulation of FBXO6 attenuated post-injury osteoarthritis development, suggesting that induction of FBXO6 in cartilage could be a useful strategy for treating osteoarthritis (46).

## FBXO21

Lin et al. evaluated the effects of FBXO21 in osteoarthritis degeneration and its underlying molecular mechanism. They reported that osteoarthritis patient cartilages had higher expression of FBXO21. Ageing rats and monosodium iodoacetate-driven osteoarthritis rats exhibited an increased expression of FBXO21 in cartilages (47). FBXO21 expression was elevated in chondrocytes after treatment with lipopolysaccharide, IL-1β and TNF-α. Additionally, downregulation of FBXO21 alleviated osteoarthritis-associated cartilage degeneration, which accompanied with promotion of autophagy and anabolism, reduction of apoptosis as well as catabolism. On the contrary, overexpression of FBXO21 accelerated cartilage degeneration. Furthermore, FBXO21 performed their effects on cartilage degeneration *via* suppression of autophagy by phosphorylating

ERK in chondrocytes (47). Strikingly, JUNB directly targeted the promoter of FBXO21 and upregulated the expression of FBXO21, thus contributing to cartilage degeneration in chondrocytes and SW1353 cells. In a word, JUNB/FBXO21/ERK pathway governs cartilage degeneration *via* blockade of autophagy in osteoarthritis, indicating that downregulation of FBXO21 might be a novel approach for osteoarthritis treatment (47). Jia et al. reported that circRNA-MSR inhibited miR-761 expression and consequently upregulated the expression of FBXO21. FBXO21 and circRNA-MSR expression levels were increased in osteoarthritis, while miR-761 expression levels were decreased in osteoarthritis (92). Depletion of circRNA-MSR facilitated the autophagy of LPS-stimulated cells. Upregulation of FBXO21 abolished the induced autophagy by miR-761 overexpression in chondrocytes. Hence, FBXO21 plays a potential role in osteoarthritis development (92).

## Other E3 ligases

The E3 ubiquitin ligase HECTD1 (HECT domain E3 ubiquitin protein ligase 1) has been proposed to control autophagy and osteoarthritis pathogenesis *via* degradation of Rubicon (48). The expression of HECTD1 was remarkably reduced in patients with osteoarthritis compared with healthy cartilage tissues. Surgery- and aging-triggered osteoarthritis pathogenesis was largely enhanced in cartilage with conditional depletion of HECTD1. Consistently, upregulation of HECTD1 alleviated pathogenesis of osteoarthritis in mouse joints. HECTD1 can interact with Rubicon at K534 and cause ubiquitination and proteasomal degradation of Rubicon, contributing to attenuation of stress-mediated chondrocyte death and osteoarthritis progression (48). Casitas B-lineage lymphoma b (Cbl-b) E3 ubiquitin ligase has been demonstrated to regulate cancer immunotherapy (93). One group identified that Cbl-b targeted Tropomyosin-related kinase A (TrkA) for ubiquitination and degradation (94). It has been known that TrkA is a one of nerve growth factors, which participates in the osteoarthritis pain (95). Knee osteoarthritis that was induced by DMM (destabilization of the medial meniscus) exhibited the dissociation of TrkA and Cbl-b E3 ligase in dorsal root ganglion (DRG) neurons, leading to impaired Cbl-b-mediated degradation of TrkA, thereby contributing to TrkA-dependent pain sensitization (49). In line with this result, overexpression of Cbl-b reduced the levels of TrkA protein and attenuated heat hyperalgesia and mechanical allodynia in DRG neurons of mice with osteoarthritis (49).

Ubiquitin-fold modifier 1-specific ligase 1 (UFL1) E3 ligase had a lower expression level in articular tissues of osteoarthritis. IL-1β treatment alleviated the expression of UFL1 in chondrocytes. Increased expression of UFL1 promoted viability of IL-1β-mediated chondrocytes, which was accompanied with inhibition of NO and PGE2 production and suppression of iNOS and COX-2 expression levels (50). IL-6 and TNF-α levels were elevated after IL-1β induction, which was abrogated by UFL1. Moreover, UFL1 reduced the production of ADAMTS-4, ADAMTS-5, MMP-3 and MMP-13 and inactivated the NF-κB signaling pathway in IL-1β-mediated chondrocytes (50). 3-Hydroxy-3-methylglutaryl reductase degradation (HRD1) E3 ubiquitin ligase exhibits a key role in endoplasmic reticulum (ER)-associated degradation (ERAD) *via* regulating unfolded protein response (UPR) to maintain cellular proteostasis after stress stimulation (96). HRD1 deficient cells had the upregulation of OS9 expression, which was required for degradation of ERAD substrates. HRD1 levels were inversely associated with OS9 expression in clinical synovium tissues of osteoarthritis and rheumatoid arthritis patients (51).

# The role of deubiquitination in osteoarthritis

USPs act as deubiquitinating enzymes, which function to remove Ub from Ub conjugates and control the ubiquitination and degradation of proteins. It has been known that USP play a vital role in bone and bone-related diseases (34). For example, one study identified that a total of 463 ubiquitinated peptides were associated with injured articular cartilage tissues after mechanical injury. These ubiquitinated proteins participated in endoplasmic reticulum (ER)-related protein degradation, such as USP5, YOD1 deubiquitinase, BRCA1/BRCA2-containing complex subunit 3 (BRCC3) E3 ligase, Ataxin 3 (ATXN3) deubiquitinating enzyme. The ER stress regulators were also altered in injured articular cartilage, including ubiquilin 1, RAD23 and VCP/p97 (97). ER stress markers were also elevated after cartilage injury. This work suggested that activation of some DUBs and ER stress regulators involved in regulation of cartilage tissue injury and osteoarthritis (97). Herein, we describe the molecular insight of USPs into osteoarthritis processes (Table 2).

## USP3

Ubiquitin-specific protease 3 (USP3) has been reported to inhibit type I interferon signaling *via* deubiquitination of RIG-I-like receptors and antiviral immunity (106). USP3 was found to be downregulated in osteoarthrosis. USP3 upregulation attenuated IL-1β-mediated apoptosis of chondrocytes and inactivation of NF-κB pathway (107). TRAF6 is an adaptor protein for NF-κB pathway and involves in immune response and inflammation. IL-1β elevated the ubiquitination of TRAF6, which can be abrogated by overexpression of USP3 in chondrocytes (107). Increased USP3 expression recued IL-1β-induced cell senescence in rat primary chondrocytes (98). USP3 maintained the protein levels of SIRT3 *via* inhibition of SIRT3 ubiquitination. Consistently, suppression of SIRT3 attenuated the function of USP3 upregulation in cell senescence in rat chondrocytes (98). Similarly, upregulation of SIRT3 abolished USP3-depletion-mediated ROS production and cell senescence. Mechanistically, upregulation of SIRT3 reduced chondrocyte senescence in part *via* FOXO3 deacetylation (98).

TABLE 2 USP regulate osteoarthritis progression.

| Name | Targets | Functions | Ref |
|---|---|---|---|
| USP3 | SIRT3 | Reduces chondrocyte senescence | (98) |
| USP5 | TRAF6 | Promotes proinflammatory cytokine production | (99) |
| USP7 | NOX4, SOX9 | Controls osteoarthritis progression | (100, 101) |
| USP13 | PTEN/AKT | Inhibits osteoclastogenesis, synovial hyperplasia, cartilage damage, inflammation | (102) |
| USP14 | IκBα | Influences chondrocyte dedifferentiation | (103) |
| USP15 | ERK2 | Suppresses osteoarthritis progression | (104) |
| USP49 | Axin | Regulates chondrocyte apoptosis | (105) |

## USP5

Evidence has suggested that USP5 is critical involved in inflammation development and processes. For example, USP5 regulated neuropathic and inflammatory pain *via* promotion of CaV3.2 channel activity (108). Impairment of USP5 and Cav3.2 interaction attenuated inflammatory and neuropathic pain (109). A cell-permeant peptide targeting the cUBP domain of USP5 abolished neuropathic pain and inflammatory (110). IL-1β was an essential mediator to regulate the interaction between Cav3.2 and USP5 in the pain process (111). Impairing the binding between USP5 and Cav3.2 protected mechanical hypersensitivity in female mice with peripheral inflammation (112). USP5 was reported to be related with proinflammatory function in RA-FLS (rheumatoid arthritis-fibroblast-like synoviocytes (99). It has been known that RA is a common chronic autoimmune inflammatory disease. USP5 expression was increased in RA-FLS, while the expression of USP5 was decreased in OA-FLS. USP5 expression was increased after IL-1β stimulation in a time-dependent way (99). Overexpression of USP5 aggravated activation of NF-κB pathway and promoted proinflammatory cytokine production. In addition, depletion of USP5 reduced the cytokines release and inactivated NF-κB activation. USP5 can bind with TRAF6 and stabilize TRAF6 *via* deubiquitination. USP5 overexpression controls inflammatory processes *via* maintenance of TRAF6 stability in RA-FLS (99).

## USP7

One study revealed that hydrogen peroxide ($H_2O_2$) induced the expression of USP7 and increased ROS levels and reduced proliferation in rat chondrocytes. Depletion of USP7 abolished $H_2O_2$-mediated pyroptosis and ROS induction and inactivated NLRP3 inflammasome activation in rat chondrocytes (100). Consistently, upregulation of USP7 enhanced pyroptosis, IL-1β and IL-18 levels, MMP-1, MMP13, and NLRP3 inflammasome activation *via* upregulation of ROS levels in rat chondrocytes. Moreover, USP7 interacted with NOX4 and promoted its ubiquitination in rat chondrocytes (100). In addition, USP7 and NOX4 were highly expressed in osteoarthritis patients. Suppression of NOX4 abrogated the function of USP7-mediated pyroptosis, ROS induction and NLRP3 activation in rat chondrocytes. P22077, one USP7 inhibitor, repressed osteoarthritis process in mice with monosodium iodoacetate (MIA) injection. Hence, USP7 regulated NOX4/ROS/NLPR3 pathway to contribute to osteoarthritis progression (100). Ubiquitination of LKB1 increased activation of AMPK pathway and repressed NLRP3 inflammasome response and blocked chondrocyre pyroptosis in osteoarthritis (113).

USP7 expression was decreased in the knee joint cartilage of mice with osteoarthritis. Silencing of USP7 by siRNAs or its inhibitors recued cell proliferation and accelerated apoptosis in chondrocyte. Depletion of USP7 reduced inflammatory response during inflammation process (114). Inhibition of USP7 by its inhibitors promoted cartilage destruction in osteoarthritis mice *via* activation of the BiP-eIF2α-ATF4-CHOP pathway in ERS and promotion of NF-κB/p65 pathway. Several compounds, including QNZ and 4-PBA, and CHOP siRNAs decreased USP7 expression and contributed to inhibition of chondrocyte proliferation and induction of apoptosis and reduction of inflammatory response after TNF-α stimulation (114). One group found that ADMA promoted SOX9 destabilization, which was mediated by DDAH1 in osteoarthritis. DDAH1 expression was decreased, while DDAH1 was increased in osteoarthritis patients (101). Because DDAH1 was an ADMA hydrolase, mice with global or chondrocyte-conditional knockdown of DDAH1 displayed a rapid development of osteoarthritis. ADMA promoted osteoarthritis progression through induction of degeneration and senescence and disruption of ECM deposition in chondrocytes. ADMA interacted with SOX9 and USP7, protecting the SOX9 deubiquitination by USP7 and resulting in promotion of SOX9 degradation. Hence, upregulation of DDAH1 to inhibit ADMA levels and regulate USP7-mediated SOX9 deubiquitination could be a useful strategy for the treatment of osteoarthritis (101).

## USP13

USP13 has been known to participate in the pathogenesis of infection, cancers, inflammation and neurodegenerative diseases (115). For example, USP13 inhibited lung inflammation *via* stabilization of the anti-inflammatory receptor IL-1R8/single immunoglobin interleukin-1-related receptor (Sigirr) (116). Inhibition of miR-19a-3p reduced sepsis-mediated lung injury through upregulation of USP13 expression (117). USP13

impaired the symptoms of lipopolysaccharides-mediated sepsis *via* deubiquitination of IRAK4 (118). USP13 inhibited sepsis-induced inflammation and cardiomyocyte oxidative stress *via* induction of nuclear factor erythroid 2-related factor 2 (Nrf2) (119). One work showed that USP13 regulated PTEN to ameliorate osteoarthritis *via* reducing oxidative stress, regulating apoptosis and inflammation (102). Specifically, USP13 expression was increased in synovial specimens from RA patients. Interestingly, USP13 expression was decreased in human-FLSs after stimulation by LPS, TNF-α and IL-1β. Upregulation of USP13 repressed inflammatory response in H-FLSs upon TNF-α or IL-1β challenge due to the improvement of PTEN and reduction of AKT phosphorylation as well as inactivation of NF-κB pathway (102). USP13 performed the protective functions due to the upregulation of Nrf-2 and downregulation of caspase-3. Mechanistically, USP13 bound with PTEN and regulated AKT activation. Moreover, upregulation of USP13 suppressed the expression of osteoclast-related genes and repressed osteoclastogenesis. In collagen-mediated arthritis (CIA) mice, USP13 attenuated synovial hyperplasia, cartilage damage and inflammation and bone loss (102).

## USP14

USP14 has been known to regulate protein degradation and associate with carcinogenesis, neurodegenerative diseases and viral infections (120, 121). Li et al. reported that USP14 exacerbated activation of NF-κB signaling pathway and accelerated IL-1β-induced chondrocyte dedifferentiation *via* modulation of IκBα degradation (103). USP14 was highly elevated in osteoarthritis articular cartilage and IL-1β-induced chondrocytes. ACHP, an inhibitor of IKK-β, inactivated NF-κB pathway and abrogated USP14 upregulation. In turn, USP14 enhanced IκBα deubiquitination and promoted NF-κB activation. Suppression of NF-κB abolished USP14-mediated dedifferentiation effect of IL-1β on chondrocytes (103). Hence, USP14 might be a useful target for osteoarthritis intervention.

## USP15

USP15 has been uncovered to participate in several cellular processes and tumorigenesis as well as other noncancer diseases (122). USP15 plays the multifaceted roles in various diseases *via* regulating diverse signaling pathways by deubiquitination of target proteins (123). In addition, USP15 has been implied to involve in immune and inflammatory processes by regulation of several pathways, including TLR signaling, NF-κB, RIG-1 pathway, TGF-β, and p53 pathways (124). USP15 was found to stimulate TGF-β/SMAD2 signaling. Moreover, USP15 was required for ERK2 to affect TGF-β/SMAD2 signaling and to control the cartilage phenotype. Mechanistically, USP15 interacted with ERK2 and form a complex to govern the ubiquitination of ERK2. USP15 elevated the levels of phosphor-ERK1/2, but not ERK2 stability, to activate the TGF-β/SMAD2 signaling pathway. Overall, USP15 suppressed osteoarthritis progression *via* targeting ERK and TGF-β/SMAD2 signaling (104).

## USP49

Ubiquitin-specific protease 49 (USP49) has been reported to govern the tumorigenesis. For instance, Fbxo45 promoted cell proliferation and invasion *via* induction of USP49 for ubiquitination and degradation (125). USP49 targets Yes-associated protein 1 (YAP1) for maintaining the stability of YAP1 in gastric cancer, leading to induction of cell proliferation, metastasis, chemoresistance (126). USP49 controlled the oncogenic ability of glucocorticoid receptor beta in glioblastoma cells (127). USP49 was reported to regulate rat chondrocyte apoptosis *via* governing Axin deubiquitination (105). In osteoarthritis patients, the expression of USP49 was lower compared with normal healthy persons. IL-1β stimulation on primary rat chondrocytes caused the downregulation of USP49 expression. Consistently, overexpression of USP49 reduced chondrocyte apoptotic death that was induced by IL-1β stimulation *via* enhancement of Axin deubiquitination, leading to upregulation of Axin protein levels. The increased Axin protein led to β-catenin degradation and suppression of Wnt/β-catenin pathway (105).

# Targeting ubiquitination to regulate osteoarthritis

Numerous compounds have been discovered to target ubiquitination and regulate osteoarthritis (Table 3). Digoxin has been approved by FDA to treat several medical conditions, such as heart failure, atrial fibrillation, supraventricular tachycardia, and cardiomyopathy (133, 134). One study showed the effects of digoxin on the inflammatory microenvironment and chondrogenesis in osteoarthritis (128). Digoxin was found to alleviate osteoarthritis synovitis *via* suppression of the M1-like polarization of synovial macrophages in patients with osteoarthritis. The similar results were found in collagenase-mediated osteoarthritis mice (128). By analysis of exosomes produced by macrophages and M1-like macrophages after digoxin treatments, this study found that digoxin governed inflammatory microenvironment of osteoarthritis and enhanced chondrogenesis *via* inhibition of the synovial macrophage M1-like polarization and exosomal miR-146b-5p, USP3 and SOX5 pathways in osteoarthritis (128).

It has been known that [6]-gingerol protected against osteoarthritis *via* regulation of a number of factors, such as Nrf2 and GSTA4-4 (135, 136). One group showed that [6]-gingerol, an anti-osteoarthritis compound, increased the protein levels of USP49 and inhibited the activation of the Wnt/β-catenin signaling pathway in primary rat chondrocytes (105). Resveratrol was reported to inhibit IL-1β-mediated apoptosis *via* regulation of caspase-3 activation and PARP cleavage, and reduce inflammatory signaling *via* inhibition of the degradation of IκBα in articular chondrocytes, suggesting that resveratrol might be a natural compound for treating osteoarthritis (129). ALLN (N-Ac-Leu-norleucinal), the proteasome inhibitor, was also found to repress the degradation of IκBα in chondrocytes and decreased

TABLE 3 Compounds target ubiquitination in osteoarthritis.

| Name | Targets | Functions | Ref |
|---|---|---|---|
| Digoxin | USP3, SOX5, miR-146b-5p | Regulates inflammatory microenvironment of osteoarthritis, enhances chondrogenesis, inhibits synovial macrophage M1-like polarization. | (128) |
| 6-gingerol | USP49, Wnt/β-catenin | Protects against osteoarthritis | (105) |
| Resveratrol | IL-1β, caspase-3, PARP, IκBα | Inhibits apoptosis, reduces inflammatory signaling in chondrocytes | (129) |
| N-Ac-Leu-norleucinal | IκBα | Decreases chondrocyte apoptosis | (129) |
| 5-azacytidine | TGF-β, BMP, Smurf2, Smads | Promotes chondrocyte maturation | (130) |
| Spermidine | RIP1, CYLD, TNF-α, NF-κB | Performs anti-inflammatory effects on osteoarthritis | (131) |
| Alpinetin | TNF-α, IκB, MMP13, ADAMTS-5, COL2A1, ERK | Attenuates cartilage matric degradation | (132) |

chondrocyte apoptosis (129). 5-azacytidine promoted maturation *via* modulation of TGF-β (transforming growth factor-beta) and BMP (bone morphogenetic protein) pathways in articular chondrocytes (130). 5-azacytidine increased the expression of Smurf2 and decreased the protein levels of Smad2 and Smad3, but elevated the expression of Smad1 and Smad5 as well as inactivation of TGF-β in articular chondrocytes (130).

Spermidine, a natural inducer of autophagy, performs antiaging, anti-inflammation and antitumor activities (137). Spermidine exhibits anti-inflammatory effects on osteoarthritis *via* attenuation of synovitis, cartilage degeneration and osteophyte formation (131). Spermidine facilitated RIP1 deubiquitination by CYLD to block TNF-α-mediated NF-κB signaling pathway in osteoarthritis (131). Alpinetin, a natural flavonoid compound, displayed antitumor, anti-inflammation and antibacterial ability (138). Alpinetin attenuated the TNF-α-mediated upregulation of MMP-13 and ADAMTS-5 and downregulation of COL2A1 expression. Alpinetin also inhibited p65 nuclear translocation and inactivated IκB phosphorylation and regulated ERK phosphorylation. In DMM-induced rats, alpinetin abolished cartilage matrix degradation (132).

## Conclusions and discussions

In conclusion, ubiquitination and deubiquitination play a potential role in osteoarthritis progression *via* regulating protein degradation (Figure 2). Although E3 ubiquitin ligases and DUBs have demonstrated to modulate osteoarthritis processes, several issues should be mentioned. In some studies, the researchers reported the role of ubiquitination in osteoarthritis pathogenesis. However, these studies did not point out the E3 ubiquitin ligases or deubiquitinases. For example, RUNX2 stability was regulated by lncRNAs LINC02035 and LOC100130207, leading to hypertrophic changes in human chondrocytes (139). Depletion of these two lncRNAs facilitated ubiquitin-involved proteasomal degradation of RUNX2 and attenuated hypertrophic differentiation of chondrocytes. Overexpression of LINC02035 and LOC100130207 promoted the stability of RUNX2 protein and induced hypertrophic changes. Therefore, LINC02035 and LOC100130207 might be potential targets for blocking osteoarthritis development *via* delaying chondrocyte hypertrophy (139).

Besides lncRNAs, circRNAs are also involved in osteoarthritis progression (140, 141). Circ_DHRS3 sponged miR-183-5p and

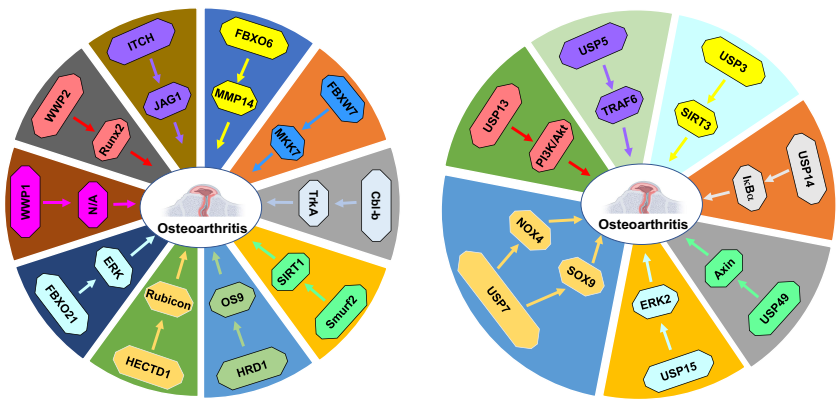


FIGURE 2
E3 ubiquitin ligases and DUBs in osteoarthritis progression. E3 ubiquitin ligases and DUBs regulate protein ubiquitination and degradation to participate in osteoarthritis development and progression.

elevated the expression of GREM1, resulting in influencing IL-1β-induced chondrocyte proliferation, apoptosis and ECM degradation (142). Circ-9119 intercepted the miR-26a/PTEN axis and prevented chondrocyte apoptosis after IL-1β treatment (143). Circ_0134111 impaired the interaction between miR-224-5p and CCL1 and enhanced osteoarthritis progression (144). CircSERPINE2 protected IL-1β–induced apoptosis and ECM degradation *via* modulation of miR-495/TGFBR2 pathway in chondrocytes (145). CircFNDC3B regulated miR-525-5p and HO-1 pathways and subsequently governed osteoarthritis and oxidative stress (146).

In addition, m6A ($N^6$-methyladenosine) modification has been known to regulate osteoarthritis (147–149). METTL3, an enzyme promoted the m6A formation on the mRNA, modulated inflammatory response and apoptosis and increased osteoarthritis development (150). METTL3 affected ECM degradation and governed the inflammatory response, leading to osteoarthritis progression (151). METTL3 reduced the chondrocyte autophagy and apoptosis *via* regulation of Bcl-2 stability by YTHDF1-involved m6A modification (152). METTL3 induced ATG7 m6A modification and enhanced cellular senescence and osteoarthritis progression *via* targeting autophagy and GATA4 pathways (153). METTL3 stimulated LINC00680 expression and facilitated osteoarthritis *via* SIRT1 m6A modification (154). AC008 was increased after m6A modification, leading to accelerating osteoarthritis progression *via* modulation of miR-328-3p and AQP1/ANKH pathways (155). One study implicated that m6A ($N^6$-methyladenosine)-modified circRNA RERE regulated osteoarthritis *via* control of β-catenin ubiquitination and degradation (156).

One group found that knockdown of FBXO32 did not alter cartilage destruction in mouse osteoarthritis, although FBXO32 was elevated in osteoarthritis cartilage, suggesting that FBXO32 was not necessary for cartilage destruction in mouse osteoarthritis (157). Besides osteoarthritis, E3 ubiquitin ligases have been revealed to regulate rheumatoid arthritis pathogenesis. For example, E3 ubiquitin ligase TRIM32 (tripartite motif protein 32) overexpression accelerated the production of pro-inflammatory cytokines in FLS of patients with rheumatoid arthritis (158). The expression of TRIM32 was higher in FLS of rheumatoid arthritis patients than that in FLS of osteoarthritis patients. TRIM32 activated NF-κB pathway and bound to TRAF2 to induce the K63-linked polyubiquitination of TRAF2 in rheumatoid arthritis FLS (158). CUL1 affected rheumatoid arthritis *via* alteration of lymphocyte signal transduction (159). E3 ubiquitin ligase STUB1/CHIP regulated the ubiquitination of aryl hydrocarbon receptor and led to the imbalance between Treg and Th17 in rheumatoid arthritis (160).

Like ubiquitination, other PTMs, such as acetylation, methylation, phosphorylation and SUMOylation, have also participated in osteoarthritis progression. Aging increased superoxide dismutase 2 acetylation, which was dependent on Sirtuin 3 in cartilage, leading to osteoarthritis progression (161). GDF11 (growth differentiation factor 11) suppressed the abnormal adipogenesis of chondrocytes and reduced TMJ condylar cartilage degeneration *via* modulation of PPARγ SUMOylation in cartilage of osteoarthritis (162). Desumoylation of aggrecan and collagen II by SENP2 triggered osteoarthritis after IL-1β treatment (163). SIRT1 maintained chondrocyte ECM upon activation of SOX9 *via* deacetylation of FOXO4 (164). Acetylation attenuated SOX9 nuclear accumulation and transactivation of ACAN gene in chondrocytes (161). Protein arginine methyltransferase PRMT1 regulated AKT/FOXO1 pathway and enhanced ECM degradation and apoptosis of chondrocytes in joint osteoarthritis (165). The activity of PRMT1 was elevated and controlled osteoarthritis *via* induction of DHX9 arginine methylation and activation of NF-κB signaling pathway (166). Suppression of PRMT5 reduced MAPK and NF-κB signaling pathway and blocked cartilage degradation in osteoarthritis (167).

There are cross-talks between several PTMs. For instance, SIRT1 restoration regulated PTEN-involved EGFR ubiquitination and accelerated chondrocyte autophagy in osteoarthritis (168). SIRT1 depletion diminished the acetylation of PTEN and then elevated the expression of PTEN. Inactivation of PTEN alleviated EGFR ubiquitination and aggravated EGFR expression *via* destabilization of the EGFR-Cbl complex, contributing to suppression of extracellular matrix degradation in cartilage and promotion of autophagy in chondrocyte (168). In addition, it is necessary to describe that ubiquitin conjugating enzymes involved in osteoarthritis pathogenesis (169, 170). For example, miR-101a-3p increased apoptosis of chondrocytes *via* targeting FZD4 and ubiquitin-conjugating enzyme 2D1 (UBE2D1), leading to involvement of pathogenesis of temporomandibular joint osteoarthritis (169). Ubiquitin conjugating enzyme E2 M (UBE2M) induced apoptosis of chondrocyte *via* upregulation of Wnt/β-catenin signaling in osteoarthritis (170). Without a doubt, it is pivotal to further explore the mechanisms of ubiquitination and deubiquitination in involvement of osteoarthritis development.

## Author contributions

CZ searched the literature and drafted the original manuscript. JC, YW, XW, and YL made the figures and tables and edited the manuscript. LS and WL corrected and edited the manuscript. PW conceived and supervised this study and edited 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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# Research progress of biomarkers in the prediction of anti-PD-1/PD-L1 immunotherapeutic efficiency in lung cancer

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Currently, anti-PD-1/PD-L1 immunotherapy using immune checkpoint inhibitors is widely used in the treatment of multiple cancer types including lung cancer, which is a leading cause of cancer death in the world. However, only a limited proportion of lung cancer patients will benefit from anti-PD-1/PD-L1 therapy. Therefore, it is of importance to predict the response to immunotherapy for the precision treatment of patients. Although the expression of PD-L1 and tumor mutation burden (TMB) are commonly used to predict the clinical response of anti-PD-1/PD-L1 therapy, other factors such as tumor-specific genes, dMMR/MSI, and gut microbiome are also promising predictors for immunotherapy in lung cancer. Furthermore, invasive peripheral blood biomarkers including blood DNA-related biomarkers (e.g., ctDNA and bTMB), blood cell-related biomarkers (e.g., immune cells and TCR), and other blood-related biomarkers (e.g., soluble PD-L1 and cytokines) were utilized to predict the immunotherapeutic response. In this review, the current achievements of anti-PD-1/PD-L1 therapy and the potential biomarkers for the prediction of anti-PD-1/PD-L1 immunotherapy in lung cancer treatment were summarized and discussed.

KEYWORDS

**lung cancer, biomarker, anti-PD-1/PD-L1 immunotherapy, immune checkpoint, dMMR/MSI, CtDNA, bTMB, cytokines among these immune checkpoints**

## 1 Introduction

Lung cancer is the leading cause of cancer death in the world, which is traditionally divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (1, 2). NSCLC accounts for approximately 85% of lung cancer, which mainly includes lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LSCC), and lung large cell carcinoma (LLCC) (2). Surgery, radiation therapy, chemotherapy, targeted therapy,

immunotherapy, and combined therapy are current treatment strategies of lung cancer (3). Importantly, the efficiency of these treatment strategies varies from the type and stage of lung cancer. Surgery is the main treatment choice for early-stage (stage I and II) patients, which may provide a longer survival time (4). Primary radiation therapy (such as stereotactic body radiotherapy) is an alternative therapeutic strategy for patients unsuitable for surgery and patients who are medically inoperable (4). However, more than 70% of patients diagnosed with lung cancer are at stage III or IV (5). The standard strategy is adjuvant chemotherapy postoperatively for stage IIIA patients who can benefit from surgery (6). Moreover, the concurrent chemoradiotherapy (CCRT) followed by programmed death 1 ligand (PD-L1) inhibitor treatment is the standard strategy for unresectable stage III lung cancer patients (7).

It was well known that the targeted therapy was the first-line treatment for lung cancer with specific targetable oncogenic drivers, while the platinum-based combination chemotherapy was the standard treatment for lung cancer without specific targetable oncogenic drivers (6). However, in recent years, the immunotherapy was introduced to the treatment of stage IV lung cancer patients and improved the therapeutic effect and survival time of patients (8–10). The immune checkpoint blockade (ICB) therapy including anti-programmed death 1 (PD-1) therapy, anti-programmed death 1 ligand (PD-L1), and anti-cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4) therapy has been proven to be beneficial in some lung cancer patients in clinical trials (8–12). The immune checkpoints are important regulators of the immune system that maintain self-tolerance, protect tissue from damage, and prevent autoimmune responses by modulating the duration and intensity of the immune response in normal states (13). However, the immune checkpoints also affect the antitumor immunity because of their role as mediators in tumor immune evasion. PD-1 on T lymphocytes and its principal ligand PD-L1 on tumor cells are two well-known immune checkpoints that deliver inhibitory signals of T-cell proliferation, cytokine production, and cytotoxicity when they are bound (14). Therefore, blockage of PD-1/PD-L1 by their inhibitors could boost the killing effect of immune system on tumor cells (Figure 1). It was confirmed that the anti-PD-1/PD-L1 immunotherapy has shown significant antitumor efficiency and good safety in the treatment of lung cancer (12). Nevertheless, some side effects occurred in the process of lung cancer anti-PD-1/PD-L1 therapy, and some patients do not respond well to their treatment (12). It was reported that the expression of PD-L1, tumor mutation burden (TMB), tumor-specific genes, dMMR/MSI, gut microbiome, and the invasive peripheral blood biomarkers including blood DNA-related biomarkers, blood cell-related biomarkers, and other blood-related biomarkers were potential biomarkers to predict clinical response of anti-PD-1/PD-L1 therapy in cancers (15). Therefore, it is of importance to explore some biomarkers to predict the response to immunotherapy, which is helpful for the precision medicine of lung cancer patients (Figure 2).

Here, we summarized and discussed the current achievements of anti-PD-1/PD-L1 therapy and the potential biomarkers for

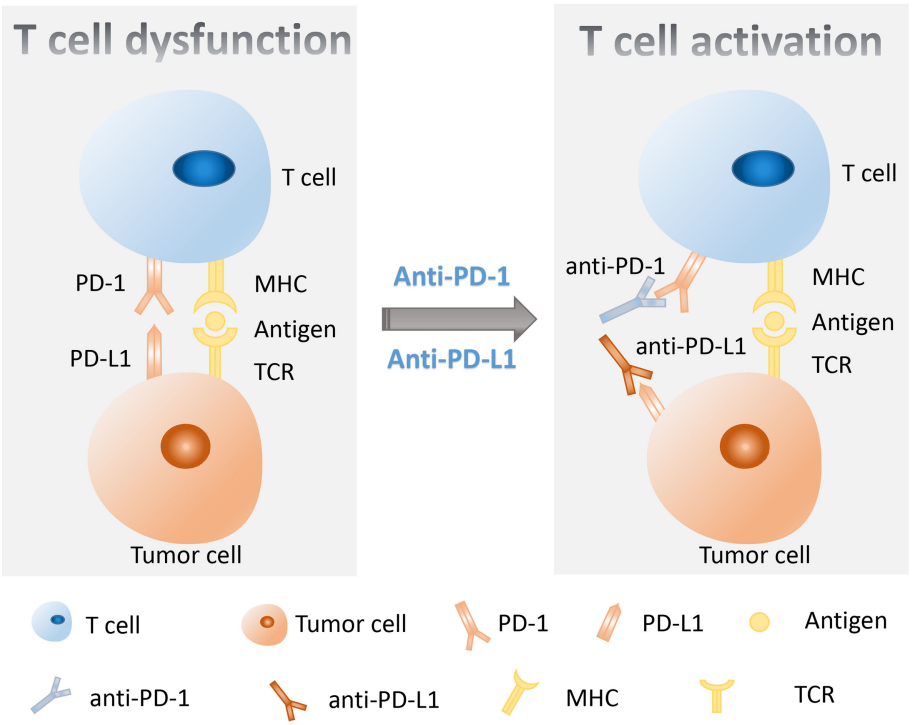


FIGURE 1
A schematic diagram of the molecular mechanism using anti-PD-1/PD-L1 therapy to restore T-cell functions. PD-1 on T lymphocytes and PD-L1 on tumor cells are two important immune checkpoints that, when combined, transmit inhibitory signals for T-cell activation. The inhibitors of PD-1 or PD-L1 could block the PD-1/PD-L1 axis and rescue the T-cell functions. PD-1, programmed death 1; PD-L1, programmed death 1 ligand.

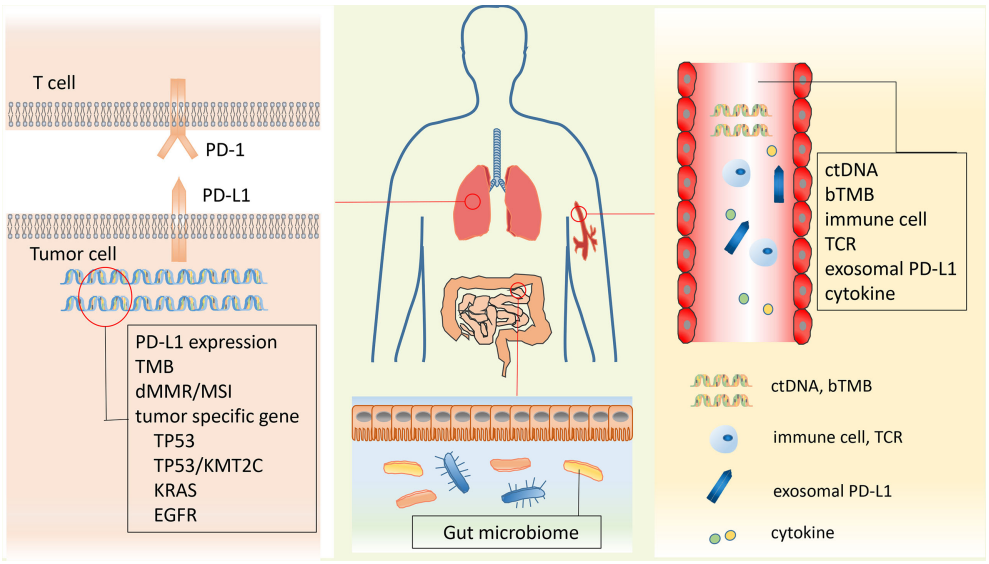


FIGURE 2
Potential predictive biomarkers of response for anti-PD-1/PD-L1 therapy. Biomarkers could be found from two traditional biopsy samples and the peripheral blood. Biomarkers isolated from traditional biopsy mainly include PD-L1 expression, TMB, dMMR/MSI, and tumor-specific genes (TP53, TP53/KMT2C, KRAS, and EGFR). Peripheral blood biomarkers include ctDNA, bTMB, immune cells, TCR, soluble PD-L1, and cytokines. Gut microbiome is also a promising predictor for immunotherapy in lung cancer. PD-L1, programmed death 1 ligand; TMB, tumor mutation burden; dMMR, MMR deficiency; ctDNA, circulating tumor DNA; bTMB, blood tumor mutation burden; TCR, T-cell receptor.

the prediction of anti-PD-1/PD-L1 immunotherapy in lung cancer treatment. Abbreviations and their full names were shown in Table 1.

## 2 Mechanisms of anti-PD-1/PD-L1 immunotherapy

Cancer immunotherapy, which functions by leveraging the cytotoxic potential of human immune system to kill cancer cells, has become a powerful strategy for cancer treatment. A large number of antibodies and small molecules targeting immune checkpoints are currently undergoing pre-clinical and clinical studies (16). The immune checkpoint proteins under study mainly include PD-1, PD-L1, CTLA-4, lymphocyte-activation gene 3 (LAG3), and T-cell immunoglobulin and mucin domain 3 (TIM3) (17). Notably, PD-1 and PD-L1 are the focus of all these immune checkpoints, which are also well studied (16). PD-1 is a type I transmembrane protein that is mainly expressed on activated T cells, B cells, and natural killer (NK) cells (18). PD-L1 is a member of the B7 protein family and is mainly expressed on the tumor cells, tumor-infiltrating cells, and antigen-presenting cells (APCs) (19).

The activation of T cells relies on at least two signals. The first signal is the T-cell receptor (TCR) recognition of the antigen presented by the major histocompatibility complex (MHC) antigen in the form of peptides. The second signal is the co-stimulatory signal provided by antigen-presenting cells (APCs), which is generated by the interaction between co-stimulatory ligands on APCs and corresponding receptors on the surface of T cells (20). Co-stimulatory signals are necessary for the induction of productive immune responses as it is essential for the optimal proliferation, differentiation, and survival of T cells. Under physiological conditions, the PD-1/PD-L1 axis is crucial in the development of immune tolerance, which can prevent excessive immune cell activity-induced tissue destruction and autoimmunity by regulating the quantity and activity of antigen-specific T cells (21). However, in tumor environments, the interaction between PD-1 and PD-L1 could inhibit T-cell activation and cause T-cell apoptosis, reduced cytokine production, T-cell lysis, and induction of tolerance to antigen, thus enabling the tumor to evade immune surveillance (22). PD-1 is mainly expressed on activated T cells and can serve as a brake for T-cell activation when combined with its ligands PD-L1. After binding with PD-L1, PD-1 is phosphorylated at its immune receptor tyrosine-based inhibitory motif (ITIM) and immune receptor tyrosine-based switch motif (ITSM), leading to the recruitment of tyrosine phosphatase SHP2 (Src homologous phosphatase 2) and subsequent dephosphorylation of TCR and CD28, thereby inhibiting T cell-related signaling (23–26). In addition to inhibiting some early activation pathways of T cells, a recent study has shown that PD-1 can directly prevent antigen recognition by disrupting the cooperative TCR–pMHC–CD8 trimolecular interaction (27). Under the intervention of PD-1/PD-L1 immune checkpoint inhibitors (ICIs), the membrane motif of PD-1 cannot be phosphorylated, resulting in the inability of cells to recruit SHP-2. Then, the dephosphorylation of TCR and CD28 is blocked, leading to effective transmission of activation signals to downstream proteins and signaling pathways, ultimately stimulating T-cell proliferation and differentiation. Ultimately, the immune function of T cells is effectively exerted.

TABLE 1 Summarization of abbreviations and their full name.

| Abbreviation | Full name |
|---|---|
| SCLC | Small cell lung cancer |
| NSCLC | Non-small cell lung cancer |
| LUAD | Lung adenocarcinoma |
| LSCC | Lung squamous cell carcinoma |
| LLCC | Lung large cell carcinoma |
| CCRT | Concurrent chemoradiotherapy |
| PD-1 | Programmed death 1 |
| PD-L1 | Programmed death 1 ligand |
| CTLA-4 | Cytotoxic-T-lymphocyte-associated protein 4 |
| anti-PD-1 | Anti-programmed death 1 |
| anti-PD-L1 | Anti-programmed death 1 ligand |
| anti-CTLA-4 | Anti-cytotoxic-T-lymphocyte-associated protein 4 |
| FDA | Food and Drug Administration |
| ORR | Overall response rate |
| OS | Overall survival |
| PFS | Progression-free survival |
| EGFR | Epidermal growth factor receptor |
| ALK | Anaplastic lymphoma kinase |
| TMB | Tumor mutation burden |
| mut/Mb | Mutations/megabase |
| IV | Intravenously |
| TMB-H | TMB-high |
| ES-SCLC | Extensive-stage SCLC |
| ICIs | Immune checkpoint inhibitors |
| WES | Whole exome sequencing |
| MMR | Mismatch repair |
| MSI | Microsatellite instability |
| dMMR | MMR deficiency |
| VAF | Variant allele fraction |
| bTMB | Blood TMB |
| ctDNA | Circulating tumor DNA |
| TILs | Tumor infiltrating lymphocytes |
| QIF | Quantitative immunofluorescence |
| SCFAs | Short chain fatty acids |
| TME | Tumor microenvironment |
| CAR-T | Chimeric antigen receptor T |
| NSqNSCLC | nonsquamous non-small-cell lung cancer |
| nab | nano-particle albumin-bound |
| ECOG | Eastern Cooperative Oncology Group |
| TPS | Tumor Proportion Score |

## 3 Mechanisms of resistance for anti-PD-1/PD-L1 immunotherapy

In recent years, anti-PD-1/PD-L1 immunotherapy has achieved surprising effects in the treatment of various malignant tumors (28, 29). However, many patients have developed resistance to PD-1/PD-L1 inhibitors, which severely limits the wide application of this therapy and has become a serious clinical problem in this field. Therefore, it is necessary to deeply reveal the molecular mechanism of PD-1/PD-L1 inhibitor resistance and therefore improve the response rate of cancer patients to anti-PD-1/PD-L1 immunotherapy.

The resistance to anti-PD-1/PD-L1 therapy can be classified into primary resistance and acquired resistance based on clinical outcomes (30). In primary resistance, patients failed to exhibit clinical response when treated with PD-1/PD-L1 inhibitors (30). In contrast, acquired resistance means that patients respond to anti-PD-1/PD-L1 therapy at the beginning of treatment, but then the therapeutic effect of the therapy is significantly weakened or unresponsive (30). The mechanism of primary resistance mainly includes lack of immunogenic antigens (31), restriction of T-cell infiltration (32), lack of interferon responsiveness (33), abnormal gut microbiome composition (34), epidermal growth factor receptor (EGFR) mutations, or anaplastic lymphoma kinase (ALK) rearrangements (35). The mechanism of acquired resistance may be associated with the weakened recognition of tumor antigens by immune cells, the loss of neoantigen, the change of the tumor microenvironment (TME), and aberrant cellular signaling transduction (18, 19).

Tumor cells can avoid processing and presenting tumor antigens by silencing or altering the expression of antigen-presenting machinery, beta-2-microglobulin (B2M), or major histocompatibility complex (MHC) molecules (36). B2M plays an important role in supporting MHC class I molecules to present tumor-specific peptide antigens to T cells. The loss of functional B2M may be a mechanism of tumor resistance to T cell-mediated immune responses (37).

Neoantigen loss is also a mechanism of acquired resistance to immune checkpoint therapy (38). The analyses on matched pretreatment and resistant tumors showed that resistant clones lost 7 to 18 putative mutation-associated neoantigens (38). This result proved that the loss of neoantigen could augment acquired resistance as an escape mechanism after PD-1/PD-L1 blockade therapy (38).

In immunosuppressive TME, tumor cells can interact with the stromal cells and immune cells to prevent immune surveillance and immune system killing (39). In TME, in addition to tumor cells, other components that might be associated with primary or acquired resistance include exhausted T cells, T regulatory cells, myeloid-derived suppressor cells (MDSCs), macrophages, immunosuppressive cytokines, and gut microbiome (40). Moreover, the alterations of metabolic landscape of the TME could also suppress the infiltration of immune cells and other antitumor immune functions by producing immunosuppressive metabolites (41). For primary resistance, a study has shown that TGFβ shaped the TME to attenuate tumor response by restricting T-cell infiltration in anti-PD-L1 therapy (32).

Abnormal cellular signaling transduction is also an important factor leading to immunotherapy resistance. For example, the IFN-γ pathway has been proved to be associated with resistance to checkpoint blockade therapy (42). Interferon-γ produced by tumor-specific T cells that have recognized their cognate antigen on tumor cells or APCs could induce effective antitumor immune response. Lack of IFN responsiveness resulted in resistance to anti-PD-1 therapy (33). However, the duration of tumor interferon signaling allowed immuno-editing of tumors and resulted in adaptive resistance to immune checkpoint blockade therapy (42).

The abnormal gut microbiome composition may be responsible for primary resistance to immune checkpoint blockade therapy. A study has shown that antibiotics reduced the clinical benefit of immune checkpoint blockade therapy for patients with advanced cancer (34). The antitumor effects of PD-1 blockade therapy on germ-free or antibiotic-treated mice can be enhanced by performing fecal microbiota transplantation (FMT) from cancer patients who responded to ICIs, but not from the nonresponding patients (34). Metagenomics of patient stool samples at diagnosis showed that the clinical response of patients to ICIs was related to the relative abundance of Akkermansia muciniphila (34).

EGFR mutations and ALK rearrangements are associated with primary resistance to PD-1/PD-L1 blockade therapy in NSCLC. A retrospective analysis evaluated the efficacy of anti-PD-1/PD-L1 therapy on EGFR-mutant, ALK-positive, and EGFR wild-type/ALK-negative patients who received anti-PD-1/PD-L1 therapy (35). The results revealed that the NSCLC patients who harbored EGFR mutations or ALK rearrangements are associated with low objective response rates to anti-PD-1/PD-L1 therapy, which may be due to low rates of co-localized PD-L1 expression and CD8(+) tumor-infiltrating lymphocytes (TILs) (35).

# 4 Anti-PD-1 immunotherapies for lung cancer

## 4.1 Nivolumab

Nivolumab, a fully human antibody targeting PD-1, has been approved for the treatment of several cancers including lung cancer by the Food and Drug Administration (FDA) (43). In 2015, nivolumab was approved as the second-line treatment strategy for advanced squamous NSCLC patients with progression during or after platinum-based chemotherapy based on the CheckMate 017 study (44). The median overall survival (OS) of patients treated with nivolumab was 9.2 months, which is longer than that of the patients treated with docetaxel (6.0 months), proving the positive effect of nivolumab for patients with advanced squamous NSCLC. In 2020, the combination therapy of nivolumab and ipilimumab (an CTLA-4 inhibitor) was approved for the treatment of patients with metastatic NSCLC without epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) genomic aberrations (45, 46). The CheckMate-9LA study is a randomized and open phase III study that involved stage IV or recurrent NSCLC patients (45, 46). This study compared the clinical benefit of combination therapy (nivolumab plus ipilimumab with two cycles of chemotherapy) and chemotherapy alone. Compared with 10.7 months median OS of chemotherapy, the combination of nivolumab plus ipilimumab with two cycles of chemotherapy improved OS to 11.4 months. The approval of the CheckMate-9LA regimen benefited from the safety and efficacy data of CheckMate 568 and CheckMate 227 (47, 48). Moreover, CheckMate 568 is an open phase II study and aimed to evaluate the efficacy and safety of nivolumab combined with ipilimumab in the treatment of advanced/metastatic NSCLC, and to investigate the correlation between PD-L1 expression and TMB on the treatment efficacy (47). The results proved the safety and effectiveness of the combination and also indicated that 10 or more mutations/megabase (mut/Mb) of TMB were associated with better response and longer progression-free survival (PFS) regardless of PD-L1 expression (47). In line with this finding, the CheckMate-227 study was performed as an open phase III study. The results indicated that the combination of nivolumab and ipilimumab exhibited a longer OS time than chemotherapy in NSCLC patients without causing new safety concerns (48).

In 2022, FDA approved the combination of nivolumab and chemotherapy as adjuvant therapy for resectable NSCLC based on CheckMate 816 clinical trials (49). In the study, stage IB to IIIA resectable NSCLC patients were treated with nivolumab and platinum-based chemotherapy or platinum-based chemotherapy alone before resection. The median event-free survival of the combination of nivolumab and platinum-based chemotherapy was 31.6 months, compared with the 20.8 months of chemotherapy alone. Importantly, compared with the 2.2% pathological complete response for chemotherapy alone, the pathological complete response was 24.0% for the patients who received the combined therapy. Moreover, the combined therapy did not increase adverse events or hinder the surgery feasibility. Therefore, the combination of nivolumab and platinum dramatically improved the clinical benefit of treatment for patients with resectable NSCLC compared with chemotherapy alone.

Furthermore, nivolumab was also approved for the treatment of SCLC as a salvage regimen (43). In the CheckMate-032 study, the efficiency and safety of nivolumab as a monotherapy or in combination with ipilimumab (an CTLA-4 inhibitor) in the treatment of multiple types of tumors were evaluated (43). Those SCLC patients who failed in previous platinum-based chemotherapy were treated with nivolumab or a combination of nivolumab and ipilimumab. The overall response rate (ORR) for the single treatment of nivolumab and the combination of nivolumab and ipilimumab was 10% and 23%, respectively. However, the median OS was 4.4 months and 7.7 months, respectively. These results indicated that both the monotherapy of nivolumab and the combination therapy of nivolumab and ipilimumab showed significant efficacy in the treatment of SCLC.

## 4.2 Pembrolizumab

Pembrolizumab, the humanized monoclonal antibody targeting and blocking the PD-1, was approved as the second-line treatment for advanced NSCLC in 2015 based on the KEYNOTE-001 study, which aimed to assess the efficacy and safety of pembrolizumab in the

treatment of NSCLC (50–52). The inclusion criteria of this study are the locally advanced or metastatic non-small cell lung cancer patients with or without treatment previously. The study presented 22.3 months and 10.5 months median OS for the naive patients and previously treated patients, respectively. Therefore, pembrolizumab provided acceptable antitumor activity and tolerable safety for the treatment of patients with advanced NSCLC, paving the way for the FDA approval (52, 53). Importantly, in 2016, pembrolizumab was approved as the first-line treatment of NSCLC patients with high PD-L1 expression based on the KEYNOTE-024 trial, which aimed to test its therapeutic effect on metastatic treatment-naive NSCLC (51, 54, 55). In this clinical trial, 154 previously treatment-naive patients with advanced NSCLC received pembrolizumab treatment and at least 50% of tumors cells of these patients expressed PD-L1 without EGFR or ALK genomic aberrations. The other 151 patients received platinum-based chemotherapy. Conclusively, the PFS of the pembrolizumab group and chemotherapy group was 10.3 months and 6.0 months, respectively. Furthermore, the pembrolizumab group exhibited a better OS and improved response rate. Moreover, the serious adverse events were 56.6% and 26.6% in the chemotherapy group and in the pembrolizumab group, respectively (55).

In 2017, FDA approved the combination of pembrolizumab, pemetrexed, and carboplatin for the treatment of patients with previously untreated metastatic NSCLC based on the KEYNOTE-021 study (56). The results of this study indicated that the combination group (pembrolizumab plus chemotherapy) achieved better ORR and PFS than the chemotherapy group. Moreover, in 2018, based on the results of the KEYNOTE-189 study, the combination of pembrolizumab, pemetrexed, and platinum was approved as the first-line treatment for the patients with metastatic non-squamous non-small-cell lung cancer (NSqNSCLC) and the patients lack EGFR or ALK genomic tumor aberrations (57). The results from the KEYNOTE-189 study showed that the combination of pembrolizumab, pemetrexed, and platinum dramatically increased the ORR and PFS. In 2018, based on the clinical results of the KEYNOTE-407 study, FDA also approved the combination of pembrolizumab, carboplatin, and paclitaxel or nab-paclitaxel as first-line treatment for metastatic squamous NSCLC (58). The results showed that the median OS of the pembrolizumab combination group was 15.9 months, which is longer than that of the placebo combination group (11.3 months). Moreover, the median PFS was 6.4 months in the pembrolizumab combination group and 4.8 months in the placebo combination group. Therefore, these results indicated that the application of pembrolizumab in chemotherapy with carboplatin and paclitaxel or nab-paclitaxel significantly improved clinical benefit. In 2019, based on the study of KEYNOTE-024, FDA approved pembrolizumab as first-line treatment for patients with stage III NSCLC (59). The results showed that compared with platinum-based chemotherapy, pembrolizumab treatment significantly prolonged the OS of patients, suggesting that pembrolizumab treatment was a reasonable treatment option for NSCLC patients with low PD-L1 TPS and without EGFR mutation or ALK translocation.

Interestingly, in 2019, pembrolizumab was approved for the treatment of patients with metastatic SCLC based on clinical results of the KEYNOTE-028 trial and the KEYNOTE-158 trial (60). The KEYNOTE-028 trial was a phase Ib trial that aimed to study the tolerability and efficiency of pembrolizumab on 20 tumor types including SCLC (61). In this study, they found a 33.3% ORR, 1.9 months of median PFS, and 9.7 months of median OS. In the phase II KEYNOTE-158 study, SCLC patients were treated by 200 mg of pembrolizumab every 3 weeks, and the ORR was 18.7% and median PFS was 2 months (61). Moreover, Hyun et al. performed a pooled analysis of KEYNOTE-028 and KEYNOTE-158 trials and found that pembrolizumab exhibited a durable antitumor activity in patients with recurrent or metastatic SCLC who had received two or more previous lines of therapy with good tolerance (62). Furthermore, pembrolizumab was approved for the treatment of patients with unresectable or metastatic TMB-high (TMB-H) solid tumors (≥10 mut/Mb) and yielded a 29% ORR of the TMB-H SCLC patients (63).

### 4.3 Cemiplimab

Cemiplimab, a human PD-1 monoclonal antibody that binds to PD-1 and blocks its interaction with PD-L1 and PD-L2, has been approved as monotherapy for advanced NSCLC with PD-L1 expression in more than 50% tumor cells (64, 65). The phase III study EMPOWER-Lung 1 provided clinical data for the treatment of cemiplimab in advanced NSCLC with PD-L1 expression in at least 50% tumor cells (66). In this study, patients were treated with cemiplimab or platinum-doublet chemotherapy and the transition from chemotherapy to cimilizumab was allowed when disease progressed. The median PFS of the cemiplimab group and chemotherapy group was 8.2 months and 5.7 months, respectively. Compared with chemotherapy, cemiplimab monotherapy significantly improved the OS and PFS of NSCLC patients (at least 50%).

## 5 Anti-PD-L1 immunotherapies for lung cancer

### 5.1 Atezolizumab

Atezolizumab, a fully humanized monoclonal antibody targeting PD-L1, is the first FDA-approved PD-L1 inhibitor for the second-line therapy of NSCLC patients (51). The clinical study NCT01375842 was performed to evaluate the tolerability, safety, and pharmacokinetics of atezolizumab in the treatment of several cancers (67). In this study, NSCLC patients treated with atezolizumab showed 28% of 3-year survival rates, which proved that atezolizumab was well tolerated and had long-term clinical benefits in the treatment of NSCLC patients.

In 2018, FDA approved atezolizumab in combination with bevacizumab, paclitaxel, and carboplatin as the first-line treatment of metastatic non-squamous NSCLC with wild-type EGFR and ALK (68). Results of the IMPOWER150, a randomized, open-label, phase III study, indicated that patients treated with the combination of the four-drug regimen had improved survival rate compared with patients treated with the combination of bevacizumab, carboplatin, and paclitaxel (68).

Furthermore, atezolizumab was approved in combination with paclitaxel and carboplatin to treat metastatic non-squamous NSCLC without EGFR or ALK genomic aberrations based on the IMpower-130, which was a multicenter, randomized, open-label, phase III trial and aimed to assess the clinical efficacy and safety of atezolizumab in combination with chemotherapy versus chemotherapy alone to treat non-squamous NSCLC (69). The median OS of patients treated by atezolizumab in combination with chemotherapy was 18.6 months, and it was 13.9 months for the patients treated by chemotherapy alone. Moreover, the median PFS of the combination group was 7.0 months, and it was 5.5 months for the chemotherapy treatment group. Together, the significant improvement of median OS and median PFS in atezolizumab plus chemotherapy group led to the FDA approval of atezolizumab.

Importantly, the atezolizumab is also the first ICI that was approved to treat extensive-stage SCLC (ES-SCLC) in combination with carboplatin and etoposide (70). In the multinational IMpower133 trial, 403 previous untreated patients with ES-SCLC were divided into two groups, one is the atezolizumab group in which the patients received the combination treatment of carboplatin, etoposide, and atezolizumab, and the other is the placebo group in which the patients received the combination treatment of carboplatin, etoposide, and placebo (71, 72). At a median follow-up of 13.9 months, the median OS of the atezolizumab group was 12.3 months, and it was 10.3 months for the placebo group. Accordingly, the median PFS of the atezolizumab group and placebo group was 5.2 months and 4.3 months, respectively. Therefore, the combination of atezolizumab and chemotherapy resulted in improved clinical benefit compared with chemotherapy alone as the first-line treatment of ES-SCLC.

## 5.2 Durvalumab

Durvalumab is a human IgG1 monoclonal antibody that can block the PD-L1 to restore T-cell activity (73). In 2018, durvalumab was applied in the treatment of patients with unresectable stage III NSCLC without progression after platinum-based concurrent chemoradiotherapy (cCRT) (74, 75). The data of the PACIFIC study indicated that durvalumab treatment significantly improved PFS of patients; therefore, durvalumab could be applied as a maintenance therapeutic strategy after chemoradiotherapy for patients with advanced unresectable stage III lung cancer (74, 75). Furthermore, in 2020, based on the CASPIAN study, durvalumab was approved in combination with chemotherapy as the first-line therapy to treat patients suffering from extensive stage small cell lung cancer (76). In the study, ES-SCLC patients who had not received first-line chemotherapy were treated by combination therapy or chemotherapy alone. The median OS of patients treated by durvalumab and platinum-based chemotherapy was 13.0 months, while it was 10.3 months for the patients who received chemotherapy alone. Furthermore, they observed 34% and 25% of 18-month survival rates for these two groups, respectively, which proved the advantage of the combination of durvalumab and platinum-based chemotherapy in the treatment of SCLC.

# 6 Potential predictive biomarkers for anti-PD-1/PD-L1 immunotherapy of lung cancer

Although anti-PD-1/PD-L1 therapy has demonstrated clinical benefits in the treatment of lung cancer, only a limited proportion of patients would benefit from the immunotherapy. For example, only 20%–25% of patients with NSCLC showed a sustainable response to ICIs (77). Therefore, it is urgent and important to identify effective predictive biomarkers for patients suffering from cancer before they are given immunotherapy.

## 6.1 PD-L1 expression

It was reported that the level of PD-L1 expression might be a biomarker for the prediction of the patient response to anti-PD-1/PD-L1 immunotherapy in clinical trials (53, 78, 79). Some results suggested that the high expression of PD-L1 is associated with increased response rates and clinical benefits of anti-PD-1/PD-L1 therapy (80, 81). In the Keynote-001 study, pembrolizumab treatment resulted in a longer median OS for the advanced NSCLC patients with a PD-L1 proportion score of ≥50% than those with a proportion score of 1–49% (53). The Keynote-052 study indicated that the subgroup with PD-L1 expression above 10% showed a higher objective response rate than the subgroup with PD-L1 expression below 1% (39% vs. 11%) in urothelial cancer patients treated with pembrolizumab (82). However, not all patients with high PD-L1 expression will respond well to anti-PD-1/PD-L1 immunotherapy while some patients with negative PD-L1 expression can also benefit from this therapy (83, 84), which indicated that there remain challenges in defining the predictive function of PD-L1 expression. The possible reasons might be as follows: (1) the methods and antibodies used for IHC to detect the PD-L1 level vary from different clinical studies; (2) the scoring system determining the quantification of PD-L1 expression of tumor cells, tumor-infiltrating immune cells, or both, is not consistent and it is difficult to confirm the best cutoff value; and (3) the expression of PD-L1 suffers from heterogeneity in space and time. Studies also showed that the expression of PD-L1 differs in primary and metastatic tumor sites and the PD-L1 expression may be affected by previous chemotherapy (85, 86). The accuracy of histological specimen may be affected by the small size of biopsy tissue (87). Despite these disadvantages, PD-L1 expression remains a promising predictive biomarker for anti-PD-1/PD-L1 immunotherapy.

## 6.2 TMB

TMB, the total number of mutations (including synonymous and non-synonymous) in tumor cells, could be predictive biomarkers for immunotherapy. Advanced sequencing technologies can better characterize the mutational landscape to

identify patients who are more likely to gain clinical benefits from immunotherapy. Rizvi et al. performed whole exome sequencing (WES) on NSCLC samples from the patients treated with pembrolizumab and found that in the process of pembrolizumab treatment, the patients having tumors with a high asynchronous mutation burden exhibited improved objective response and a lasting clinical benefit (88). Yarchoan et al. also observed a significant correlation between the tumor mutational burden and the objective response rate in anti-PD-1/PD-L1 therapy against multiple cancer types (89). Importantly, the patients with high TMB and high PD-L1 expression (>50%) had the longest PFS and OS compared with patients with only a single factor, suggesting that the integration of TMB with PD-L1 expression might be more precise to identify the patients who are more likely to respond to anti-PD-1/PD-L1 therapy (90). However, the assessment of TMB needs large sequencing panels that require large amounts of tumor tissue (91). The limited amount of DNA obtained from a conventional tumor biopsy or a fine needle biopsy may make TMB evaluation challenging or even impossible (91). Moreover, the standardization of TMB assessment has not been determined. Although the terms "low TMB" or "high TMB" are commonly used in study, the threshold to define them has not been clearly established. Therefore, the limitations that prevent TMB from becoming a promising predictive marker for immunotherapy should be overcome before it can be used in a clinical setting.

## 6.3 dMMR/MSI

DNA mismatch repair (MMR) is a system that aims to identify and repair mutations that occurred during DNA replication and recombination. Dysfunction of the DNA mismatch repair system will lead to the accumulation of mutations. Moreover, the microsatellite instability (MSI) is a genetically hyper-mutational state that is a phenotypic result of MMR deficiency (dMMR). Studies have shown that MSI-H/dMMR can predict the response to ICIs in patients with colon cancer and endometrial cancer (92, 93). Recently, the role of the MMR system in the response to ICIs in NSCLC was assessed and the alteration of MMR system-related genes in NSCLC seems to be related to the enhanced response to nivolumab immunotherapy (94).

## 6.4 Tumor-specific genes

It was reported that the tumor-specific driver gene mutations are associated with the efficiency of immunotherapy (95). Wang et al. fully analyzed the clinical, genomic, and transcriptomic data of lung adenocarcinoma patients in a public database and evaluated the impact of TP53 variant allele fraction (VAF) on tumor immune microenvironment and clinical outcomes of LUAD patients treated with ICIs (96). They found that compared with patients from the high TP53 VAF group and the wild-type group, low TP53 VAF group patients demonstrated more immune cell infiltration and superior response to anti-PD-1/PD-L1 therapy, proving that low TP53 VAF could be a predictive marker for better clinical outcomes of anti-PD-1/PD-L1 therapy (96). Notably, the KMT2C/TP53 co-mutations might be a promising predictive marker of clinical benefit for immunotherapy due to the high correlation of KMT2C/TP53 co-mutations with naive CD8+ T cells, Th1, Th2, and gd T cells (97). In line with this notion, it was found that KRAS mutation was associated with an inflammatory TME and tumor immunogenicity, leading to higher response of patients to anti-PD-1/PD-L1 immunotherapy in NSCLC patients (98). Interestingly, EGFR mutation is reported to be associated with decreased PD-L1 expression, low TMB, and decreased CD8+ T-cell infiltration (99). In a clinical trial, pembrolizumab showed no effect on the treatment of advanced NSCLC patients with EGFR-mutant and positive PD-L1 expression (≥1%) (100). Comprehensive analysis of EGFR mutation as a predictive biomarker of immunotherapy should be widely carried out in NSCLC (101).

## 6.5 Peripheral blood-related biomarkers

### 6.5.1 ctDNA and bTMB

Blood DNA-related biomarkers mainly include circulating tumor DNA (ctDNA) and blood TMB (bTMB). The amount of circulating tumor DNA in plasma is related to the tumor burden and clinical outcome (102). It was reported that pretreatment ctDNA is associated with the durable clinical benefit of NSCLC patients treated with ICIs (103). Using ctDNA to detect TMB in peripheral blood has also been developed to estimate response to immunotherapy. Gandara et al. found that TMB in peripheral blood (bTMB) can identify patients who had the clinical improvement in progression free survival after atezolizumab treatment, proving that high bTMB may be a marker for immunotherapy efficiency in NSCLC (104).

### 6.5.2 Immune cells and TCR

Blood cell-related biomarkers mainly include immune cells and T-cell receptor (TCR) immunophenotyping. Notably, the type and number of immune cells and TCR immunophenotyping can reflect the treatment effect of anti-PD-1/PD-L1 immunotherapy (105, 106). Immune cells in peripheral blood can reflect the functions and subtypes of tumor-infiltrating lymphocytes (TILs). The paired whole exome DNA sequencing and multiplexed quantitative immunofluorescence (QIF) were performed in pretreatment samples from NSCLC patients treated with anti-PD-1 immunotherapy to figure out the role of intratumoral T cells and their relationship with the tumor genomic landscape (107). The results elucidated that the level of CD3+ TILs was related to a favorable response of patients to immunotherapy (107). Furthermore, Han et al. performed sequencing of complementarity-determining region 3 of TCRβ chains isolated from PD-1+ CD8+ T cells to evaluate its value as a biomarker to predict the response to anti-PD-1/PD-L1 therapy in NSCLC patients (108). Those results showed that the TCR diversity and clonality of PD-1+ CD8+ T cells in peripheral blood may be promising predictors of response to anti-PD-1/PD-L1 therapy and survival outcomes in NSCLC patients (108).

### 6.5.3 Exosomal PD-L1 and cytokines

Exosomal PD-L1 and cytokines are other important blood cell-related biomarkers. PD-L1 was present on exosomes isolated from the plasma of NSCLC patients, and the PD-L1-positive exosomes can impair immune functions by inhibiting cytokine secretion and inducing apoptosis of CD8+ T cells in lung cancer patients (109). In support of this notion, Wang et al. analyzed the blood samples of 149 NSCLC patients and found that the exosomal PD-L1 was correlated with the clinical response of patients to ICI treatment, implying that exosomal PD-L1 could be used for the evaluation of immunotherapeutic efficacy in lung cancer (110). Furthermore, a prospective study of 26 NSCLC patients who received pembrolizumab or nivolumab treatment was conducted (111). The values of IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-12 were measured by flow cytometry at the time of diagnosis and at 3 months after initiation of anti-PD-1 therapy (111). Their results showed that high levels of cytokines including IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, and IL-8 are associated with improved response to immunotherapy and prolonged OS of NSCLC patients (111). Moreover, the other study reported that the elevated baseline serum IL-8 levels are associated with poor outcome in NSCLC patients who received ICIs (112). The above results together showed that the cytokine levels may be potential predictive biomarkers in selecting NSCLC patients who can benefit from anti-PD-1/PD-1 immunotherapy.

## 6.6 Gut microbiome

Accumulating lines of evidence indicated that gut microbiota could regulate the host response to chemotherapeutic drugs and is related to the pharmacological effects of chemotherapies and immunotherapies such as anti-PD-L1 and anti-CLTA-4 therapies (113, 114). Takada et al. performed a retrospective study for 294 patients with advanced or recurrent NSCLC who received nivolumab or pembrolizumab monotherapy at three medical centers in Japan (115). Their univariate analyses indicated that the utilization of probiotics was associated with better disease control and overall response of NSCLC patients with anti-PD-1 therapy (115). These results implied that the gut microbiota might be a novel predictor of clinical response to anti-PD-1/PD-L1 immunotherapy in NSCLC. In accordance, a metabolomics analysis was conducted to detect volatile and non-volatile metabolites of the gut microbiota in 11 NSCLC patients who received nivolumab, and the results showed that 2-Pentanone (ketone) and tridecane (alkane) were associated with early progression, while short-chain fatty acids (SCFAs) (i.e., propionate, butyrate), lysine, and nicotinic acid were associated with long-term clinical benefit, suggesting that gut microbiota metabolic pathways may play an important role in clinical response to immunotherapy (116). However, further in-depth studies are warranted to validate the clinical significance of the gut microbiota as a biomarker of immunotherapeutic efficacy.

# 7 Discussion

Recently, the anti-PD-1/PD-L1 immunotherapy has shown great clinical efficacy in many cancers including lung cancer. Anti-PD-1 drugs, including nivolumab, pembrolizumab, and cemiplimab, and anti-PD-L1 drugs, including atezolizumab and durvalumab, provided significant antitumor activity for lung cancer and improved the survival time of lung cancer patients (Figure 3). Although immune checkpoint blockade has achieved clinical

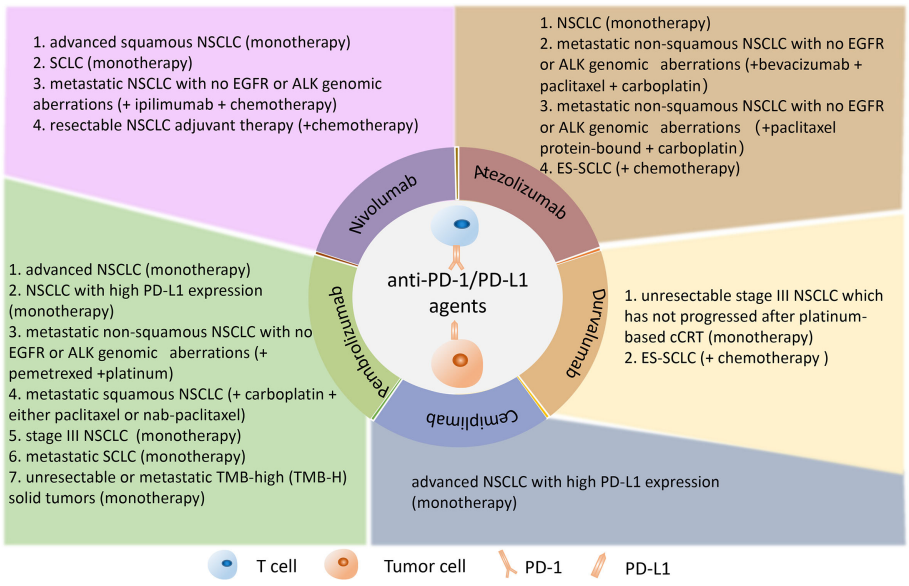


FIGURE 3
Anti-PD-1/PD-L1 inhibitors are approved by FDA for the treatment of lung cancer. The PD-1 inhibitors such as nivolumab, pembrolizumab, and cemiplimab and the PD-L1 inhibitors such as atezolizumab and durvalumab were illustrated in the panel. PD-1, programmed death 1; PD-L1, programmed death 1 ligand.

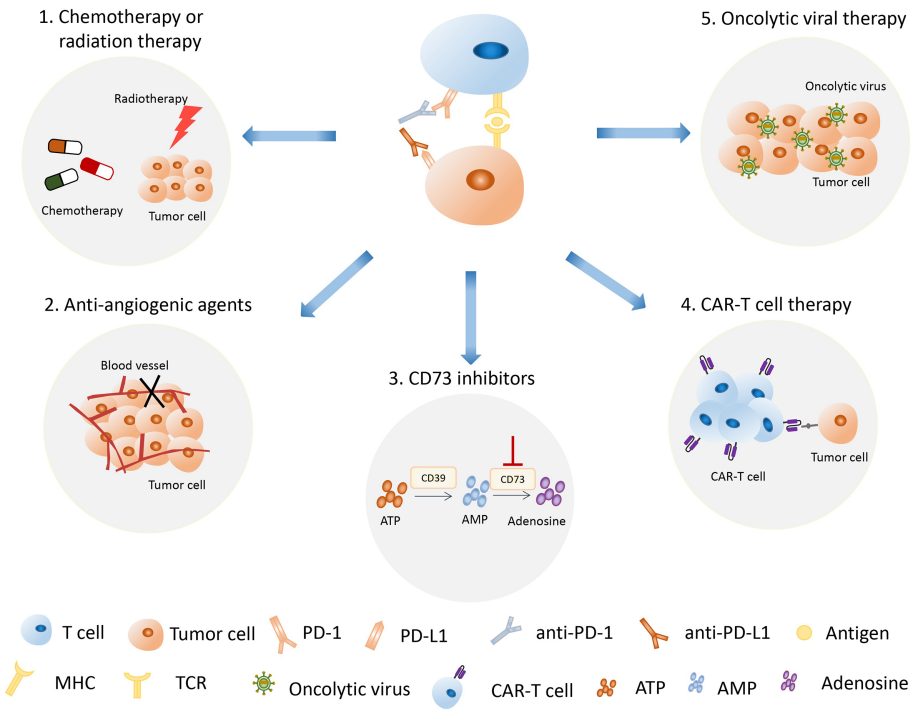


FIGURE 4
The combination of anti-PD-1/PD-L1 therapy with other therapies has become an important treatment strategy. The strategies used in combination with anti-PD-1/PD-L1 therapy include radiotherapy, chemotherapy, oncolytic viral therapy, anti-angiogenic therapy, CAR-T therapy, and CD73 inhibition therapy. PD-1, programmed death 1; PD-L1, programmed death 1 ligand; CAR-T therapy, chimeric antigen receptor T therapy.

success in cancer treatment and several drugs have been approved by FDA to treat cancers, a great proportion of patients could not exhibit sustained clinical response to the anti-PD-1/PD-L1 immunotherapy (117). Current research on anti-PD-1/PD-L1 has shown that some patients who have high PD-L1 expression cannot achieve ideal therapeutic benefit, while some patients who have lower or even negative PD-L1 expression can benefit from anti-PD-1/PD-L1 therapy (18). It is important to explore suitable biomarkers for prediction of immune efficacy and screen suitable patients for anti-PD-1/PD-L1immunotherapy. Here, we summarized and comprehensively discussed the current achievements of anti-PD-1/PD-L1 therapy and the potential biomarkers for the prediction of anti-PD-1/PD-L1 immunotherapy in lung cancer.

A previous study showed that a large proportion of patients cannot demonstrate sustained clinical response to anti-PD-1/PD-L1 immunotherapy (117). To conquer the problem of low response in patients treated with single immunotherapy, the combination therapy with anti-PD-1/PD-L1 therapy has been developed and used in clinical trials, and they exhibited synergized clinical efficacy and decreased the immune toxicity (Figure 4). Notably, radiotherapy (RT) can not only kill tumor cells by damaging the tumor DNA, but also stimulate the immune system by releasing tumor antigens (118). Moreover, preclinical studies indicated that radiotherapy could upregulate PD-L1 expression on tumor cells, which synergistically improved antitumor effect when combined with anti-PD-L1 therapy (119, 120). The combination therapy of RT and anti-PD-1/PD-L1 therapy proved to be a safe strategy by the clinical trial of 187 NSCLC patients (121). In support of this notion, studies have shown that chemotherapy may enhance the efficiency of anti-PD-1/PD-L1 therapy by inducing a tumor-specific adaptive immune response (122). The combined treatment of atezozumab, carboplatin, and etoposide, and the combined treatment of duvarumab and chemotherapy have been successively approved by FDA as the first-line treatment of ES-SCLC (70, 76). Recently, the combination of nivolumab and chemotherapy has been approved as adjuvant therapy for resectable NSCLC (49). Notably, oncolytic viral therapy can not only kill tumor cells, but also increase cytokines such as interferon gamma and interleukins, which are present in the TME, resulting in an improved innate immunologic response to tumor cells (123). The combination of oncolytic viral therapy and anti-PD-1/PD-L1 therapy might have the potential to enhance antitumor efficacy and reduce adverse events (123–125). Furthermore, angiogenic factors play important roles in inducing an immunosuppressive state and anti-angiogenic agents have been proven to enhance tumor immunity response. A clinical study has shown that the combined therapy of anti-angiogenic agents anlotinib and PD-1 inhibitor camrelizumab had promising efficacy and manageable toxicity in the treatment of NSCLC (126). Interestingly, the combination of different immunotherapy could achieve better therapeutic effect and outcomes. Chimeric antigen receptor T (CAR-T) cell therapy belongs to the immunotherapy that can specifically identify and kill tumor cells through the genetically modified T cells *in vitro* (127). More notable, the combination of CAR-T therapy and anti-PD-1/PD-L1 therapy has been proven to enhance antitumor efficacy and improve safety on cancer treatment (128, 129). Sanaz

Taromi et al. reported that the combined therapy of CAR-T cells, anti-PD-1-antibody, and CD73 inhibitor can specifically eliminate chemo-resistant tumor stem cells and overcome SCLC-mediated T-cell inhibition in a humanized orthotopic SCLC mouse model (129).

Anti-PD-1/PD-L1 immunotherapy has been widely used in the treatment of lung cancer patients. However, a great proportion of patients could not exhibit sustained clinical response to the anti-PD-1/PD-L1 immunotherapy. It is of significance to screen out patients suitable for immunotherapy and to improve immunotherapy efficacy. Accumulating lines of evidence demonstrated that predictive biomarkers play an important role in predicting response and safety of anti-PD-1/PD-L1 therapy and in the identification of lung cancer patients who will benefit from anti-PD-1/PD-L1 immunotherapy. Current commonly used markers include PD-L1 expression level of tumor tissue, TMB, dMMR/MSI, and specific driver gene mutations. However, the predictive precision was affected by the intrinsic limitation of the markers when they are used alone to predict the response, and then the combination of multiple biomarkers is usually used in the prediction process (130–132). The surgical approach and a minimally invasive intervention to collect tumor tissues are commonly used traditional biopsy methods, while liquid biopsy can easily be retrieved from plasma or serum (102). Liquid biopsy has been developed to identify circulating cancer biomarkers including blood DNA-related biomarkers, blood cell-related biomarkers, and others such as PD-L1 and cytokines (133). Furthermore, novel techniques, such as high-dimensional single-cell analysis, have also been developed to predict response to anti-PD-1/PD-L1 immunotherapy (134).

Although anti-PD-1/PD-L1 therapy has achieved clinical success in the treatment of various cancer types, the immune-related adverse events (irAEs) should not be ignored (135). Among patients who received anti-PD-1/PD-L1 therapy, the incidence of any grade irAEs and severe grade irAEs was 26.82% (95% CI, 21.73–32.61; $I^2$, 92.80) and 6.10% (95% CI, 4.85–7.64; $I^2$, 52.00), respectively (136). The irAEs caused by immune checkpoint blockade thereby mainly involve abnormality on skin, gastrointestinal tract, endocrine glands, lung, liver, and other tissues. During nivolumab treatment, the most common irAE involves skin and gastrointestinal tract, with an incidence of approximately 13% (136). During pembrolizumab treatment, the most common irAE was hypothyroidism with an incidence of approximately 8% (136). Immune-related cholangitis was observed in patients receiving nivolumab and avelumab, which has raised concerns about liver damage induced by ICI drugs (137).

There remain challenges in the treatment of cancer using anti-PD-1/PD-L1 therapy, including screening out patients who may benefit from immunotherapy, improving therapeutic effect, and reducing the side/adverse effects. Therefore, more prospective studies are still warranted to validate the detailed function of biomarkers in clinical trials by increasing the tumor types and number of enrolled cancer patients. In the future, more efforts should be exerted to the screening of more predictive biomarkers that will help make an accurate therapeutic strategy and identify suitable patients who should be given the precision medicine to reduce their suffering from cancer. On the basis of anti-PD-1/PD-L1 therapy, the combined therapy also needs to be further optimized to improve the treatment of lung cancer with reduced adverse events.

## Author contributions

LW and ZY wrote the manuscript and drew the pictures with partial help from FG, YC, and JW. XZ and XD 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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# Prognostic value and immune landscapes of TERT promoter methylation in triple negative breast cancer

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**Background:** Treatment options for patients with triple-negative breast cancer (TNBC) remain limited to mainstay therapies owing to a lack of efficacious therapeutic targets. Accordingly, there is an urgent need to discover and identify novel molecular targets for the treatment and diagnosis of this disease. In this study, we analyzed the correlation of telomerase reverse transcriptase (TERT) methylation status with TERT expression, prognosis, and immune infiltration in TNBC and identified the role of TERT methylation in the regulation TNBC prognosis and immunotherapy.

**Methods:** Data relating to the transcriptome, clinicopathological characteristics and methylation of TNBC patients were obtained from The Cancer Genome Atlas (TCGA) database. TERT expression levels and differential methylation sites (DMSs) were detected. The correlations between TERT expression and DMSs were calculated. Kaplan–Meier curves was plotted to analyze the relationship between the survival of TNBC patients and the DMSs. The correlations of DMSs and TERT expression with several immunological characteristics of immune microenvironment (immune cell infiltration, immunomodulators, immune-related biological pathways, and immune checkpoints) were assessed. The results were validated using 40 TNBC patients from Sun Yat-sen University Cancer Center (SYSUCC).

**Results:** Six DMSs were identified. Among them, four sites (cg11625005, cg07380026, cg17166338, and cg26006951) were within the TERT promoter, in which two sites (cg07380026 and cg26006951) were significantly related to the prognosis of patients with TNBC. Further validation using 40 TNBC samples from SYSUCC showed that the high methylation of the cg26006951 CpG site was associated with poor survival prognosis ($P$=0.0022). TERT expression was significantly correlated with pathological N stage and clinical stage, and cg07380026 were significantly associated with pathological T and N stages in the TCGA cohort. Moreover, the methylation site cg26006951, cg07380026 and TERT expression were significantly correlated with immune cell infiltration, common immunomodulators, and the level of the immune checkpoint receptor lymphocyte activation gene 3 (LAG-3) in TNBC patients.

**Conclusion:** TERT promotertypermethylation plays an important role in TERT expression regulation and tumor microenvironment in TNBC. It is associated with overall survival and LAG-3 expression. TERT promoter hypermethylation may be a potential molecular biomarker for predicting response to the TERT inhibitors and immune checkpoint inhibitors in TNBC.

KEYWORDS

**triple negative breast cancer (TNBC), telomerase reverse transcriptase (TERT), methylation, prognostic value, immune infiltration**

## Introduction

Breast cancer is the most common malignant tumor in women according to the latest epidemiological data from 2022 (1). Breast cancer is classified into different subtypes based on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) expression (2). Chemotherapy, radiotherapy, endocrine therapy, and targeted therapy are all appropriate postoperative treatment options for each subtype (3, 4). Triple-negative breast cancer (TNBC) constitutes a breast cancer subtype with obvious heterogeneity regarding histomorphology, genetic background, treatment response, and prognosis (5, 6). Chemotherapy remains the first-choice treatment for TNBC owing to a lack of other, more effective treatment methods as well as the aggressive biological behavior of this cancer (7), which is associated with a high risk of early recurrence, particularly visceral metastasis (8, 9). However, over recent years, immunotherapy has brought hope for patients with TNBC (10). Current research hotspots involving the immune microenvironment in TNBC include PD-L1 expression, evaluation of tumor-infiltrating lymphocytes (TILs) (11), and distribution of immune cell subsets, among others (USCAP, 2022) (12). The biggest advantage of immunotherapy lies in that the body's own immune system is used to attack the tumor cells (13). Additionally, the tumor cells are specifically recognized by the immune system (adaptive/acquired immunity), thus potentially avoiding the targeting of non-cancerous cells. Moreover, immunological memory results in that once tumor-specific memory lymphocytes are generated, they will exist for life, and will play a role in tumor eradication after recurrence or metastasis. Finally, immunotherapy has relatively weak cytotoxicity. Because TNBC is associated with a high mutation rate, TIL ratio, and PD-L1 expression, patients with this type of breast cancer represent a population that may potentially benefit from immunotherapy (14).

The telomerase reverse transcriptase (TERT) gene, which encodes a catalytic subunit of the telomerase enzyme, plays an important role in tumorigenesis by maintaining telomere homeostasis and the proliferative capacity of cells (15, 16). TERT is located on the short arm of human chromosome 5 (5p15.33) and is 42 kb long, including 15 introns, 16 exons, and a 330-bp promoter region (17). Most human somatic cells do not possess telomerase activity as TERT transcription is suppressed during embryonic development (18, 19). However, TERT expression and telomerase activity are reactivated during tumorigenesis and tumor progression and are considered to be biomarkers of tumor cells (20, 21). Telomerase acts to maintain the length and stability of telomeres, as well as the ability of cells to proliferate indefinitely, as observed in cancer cells (22, 23). Recent studies have shown that, in addition to maintaining telomere length, TERT is also involved in multiple signaling pathway, such as those associated with the MAPK and PI3K/Akt/mTOR signaling pathway, the WNT/β-catenin signaling pathway, the NF-κB signaling pathway, and epithelial-mesenchymal transition-related pathways (24–27).

TERT has been reported to function as an oncogene in various types of cancer (28–31). Many factors contribute to the abnormal elevation of TERT expression levels, including transcriptional activators, TERT copy number variation, TERT promoter mutation, and TERT hypermethylation, among others (32). TERT is mutated in many solid tumors, including central nervous system malignancies, thyroid cancer, and melanoma (33–36). The two most common mutated sites are 1,295,228 C>T and 1,295,250 C>T (C228T and C250T, respectively) (37, 38). Mutations mentioned above, which result in changes in transcription factor binding sequences, enhance TERT transcriptional activity, which is one of the main mechanisms underlying the upregulation of TERT expression. But TERT promoter hypermethylation is more prevalent (>70%) in cancer types without TERT mutation (e.g., lung, breast, prostate, and colon cancers) (18, 39), implying that epigenetic mechanisms are the drivers of upregulation of TERT expression in these cancer types (40, 41). However, the exact locations of the epigenetic modifications and their effect on TERT promoter activation remain unclear. A methylation map based on next-generation sequencing showed that DNA methylation was not uniform along the TERT promoter region (18).

To further clarify the specific situation of TERT promoter methylation and its clinical significance, in this study, we explored the association of the CpG sites in the TERT promoter and TERT expression with clinicopathological characteristics, immunological features, and survival outcome in patients with TNBC.

# Methods

## Data retrieval

### The Cancer Genome Atlas (TCGA) cohort

Data relating to the transcriptome, clinicopathological characteristics, and methylationome for the BRCA cohort were downloaded from TCGA database (https://portal.gdc.cancer.gov/). TNBC-related data were extracted from the whole BRCA cohort.

The Infinium HumanMethylation450 BeadChip methylation data were processed using the R package "ChAMP". A gene expression matrix was constructed based on Fragments per Kilobase Million values. CpG sites in the human TERT (hTERT) gene were identified based on the methylation data of TNBC patients. Analysis of all CpG sites in hTERT between TNBC and normal adjacent tissues was conducted using the R package "edgeR" (version 3.34.1) (42, 43). In addition, the "pheatmap" (version 1.0.12) and "ggplot2" (version 3.3.5) packages were utilized to plot a heatmap and boxplot, respectively, for data visualization. The R package "DESeq2" (44) was employed to identify differences in TERT gene expression between TNBC samples and normal samples from TCGA database. The acquired data were visualized using a boxplot created using the "ggplot2" package (version 3.3.5). Correlations between the TERT gene and DMSs were calculated using the R package "ggstatsplot" (version 0.9.1). To analyze the relationship of DMSs with the survival of TNBC patients, Kaplan-Meier curves were constructed using the R package "survminer" (version 0.4.9).

### Sun Yat-sen University Cancer Center (SYSUCC) cohort

For validation, a total of 40 patients were collected from SYSUCC, including 40 tumor tissue samples and 27 adjacent normal tissue samples (total 67 samples) from TNBC patients who underwent mastectomy or lumpectomy from December 2010 to November 2012.

## Methylation analysis

The methylation status of the cg26006951 and cg07380026 CpG sites was validated in the SYSUCC cohort using pyrosequencing. Briefly, DNA was extracted from clinical specimens using a TIAN amp Genomic DNA Kit (Cat. No: 4992254; ID: DP304-03, TIANGEN) and then subjected to bisulfite conversion following the manufacturer's instructions. Purified bisulfite-converted DNA was amplified by PCR using primers covering the cg26006951 and cg07380026 CpG sites. The PCR products were subjected to pyrosequencing using the PyroMark Q96 pyrosequencing and quantification platform following the manufacturer's instructions (Shanghai Biotechnology Corporation, Shanghai, China). The sequences of the primers used for amplification of the cg26006951 and cg07380026 target regions were forward: 5′-AGAAAGGGTGGGAAATGG-3′; reverse: 5′-ACCAAA TATTAACCTCATCTACCA-3′ and forward: 5′-GAGAAAGGG TGGGAAATGGA-3′; reverse: 5′-ATAACCAAATATTAACCT CATCTACCA-3′, respectively.

## Antibodies, reagents, and immunohistochemistry

Slides were deparaffinized, rehydrated, immersed in an antigen-retrieval solution (pH 6), and boiled three times, 10 min each time, at a medium baking temperature in a microwave. After blocking with 3% BSA, the sections were incubated first with primary antibodies against TERT (dilution: 1:100; ID: ab32020, Company: Abcam; https://www.abcam.com/) and LAG-3 (dilution: 1:100; Cat No. 29548-1-AP, Company: Proteintech; https://www.ptglab.com/) at 4°C overnight in a humidified container and then with HRP-labeled anti-rabbit IgG secondary antibody. The specimens were counterstained with hematoxylin. The slides were scanned using a 3DHISTECH scanner (3DHISTECH Ltd, Budapest, Hungary). The immunostaining was evaluated independently by two pathologists blinded to the clinicopathologic information. The expression of TERT and LAG-3 (also known as CD223) was represented by the H-score. Antibody staining intensity was categorized as follows: no staining = 0, weak = 1, moderate = 2, and strong = 3. A five-scale system was used to categorize the percentage of stained cells, as follows: 0, no positive cells; 1, <25% positive cells; 2, 25%-50% positive cells; 3, 50%-75% positive cells; and 4, >75% positive cells. The H-score (range: 0-12) for each tissue was calculated by multiplying the intensity index with the percentage scale. The median value of the H-score for TERT staining served as the cutoff. Tumors with H-scores for TERT staining lower than or equal to the median were designated as "low expression", whereas those with scores higher than the median were designated as "high expression".

## Analysis of immune cell infiltration

The single-sample gene set enrichment analysis (ssGSEA) algorithm based on 24 types of immune cells was used to calculate immune cell infiltration in the tumor microenvironment in TNBC samples and normal samples from TCGA database. In addition, differences in immune cell infiltration between the TNBC and normal groups were analyzed using the rank-sum test ($p<0.05$) and visualized using the R package "ggplot2" (version 3.3.5). Subsequently, the package "ggstatsplot" (version 0.9.1) was employed to further analyze the relationships of the TERT gene and DMSs with 24 types of immune cells. Immune-related biological pathways in TNBC patients were further quantified using the R package "gsva" (version 1.40.1) (45).

## Analysis of correlations with immunomodulators

Spearman's algorithm was used to analyze the correlations among TERT gene expression, TERT DMSs, and 74 immune modulators. The data were visualized in a heatmap.

## Follow-up

Follow-up was performed by telephone or through a regular outpatient surveillance system to record the condition of patients or the cause and date of death if the patient had already died. Follow-up duration was measured from the date of diagnosis to the date of the last visit or the date of death.

## Statistical analysis

Chi-square tests (categorical variables) or Mann–Whitney U tests (continuous variables) were used for comparisons between two groups. An optimal cut-off point for continuous variables was determined using maximally selected rank statistics. The significance of the Kaplan–Meier survival curve was assessed based on the log-rank test. Statistical analysis was conducted using GraphPad Prism (Version 8) and R (Version 4.0.3) software. Correlation coefficients were calculated using Spearman's rank correlation. All p-values were from a two-tailed t-test and a p-value <0.05 was considered significant.

## Ethics statement

The clinical specimens used in this study were obtained from the Tumor Biobank of SYSUCC. The study protocol for the SYSUCC cohort was approved by the Institutional Research Ethics Committee of SYSUCC (B2022-332-01).

# Results

## TERT promoter was hypermethylated in tumor tissues compared with normal adjacent tissues in TNBC

A total of 91 TNBC tissues in TCGA (84 tumor tissues and 7 normal tissues) with both methylation and expression data were selected for the combined methylation and transcriptome analysis. According to the annotation of the Infinium HumanMethylation450 BeadChip, 100 probes are located in TERT gene, 12 of which missing values. Accordingly, 88 valid TERT probes were used for detection (Supplementary Table 1). Using a β-value >0.4 and a p-value <0.05 as a cutoff, six CpG sites (Figures 1A, B) were found to be significantly differentially methylated, namely, cg11625005, cg07380026, cg17166338, cg26006951, cg19977628, and cg00576086. Among them, cg11625005, cg07380026, cg17166338, and cg26006951 were hypermethylated and cg19977628 and cg00576086 were hypomethylated. Moreover, these four sites (cg11625005, cg07380026, cg17166338, and cg26006951) were located in TERT TSS1500 or 5'UTR.

## TERT promoter methylation was positively correlated with TERT expression in patients with TNBC

To investigate the role of TERT in TNBC, we analyzed TERT expression in both tumor tissues and normal tissues. The results showed that TERT mRNA expression was significantly higher in TNBC tissues than in normal tissues (Figure 2A). The methylation of gene promoter plays an important role in the regulation of expression. To investigate the effect of TERT promoter methylation on the regulation of TERT expression, we analyzed the correlation between the degree of methylation of the four CpG sites and TERT expression. We found that TERT expression was significantly and positively correlated with hypermethylation of cg11625005 ($p < 0.0001$, $r = 0.29$), cg17166338 ($p = 0.01$, $r = 0.25$), and cg26006951 ($p = 0.03$, $r = 0.23$) (Figure 2B).

## TERT promoter methylation predict overall survival in patients with TNBC

To explore the prognostic value of TERT promoter methylation status in TNBC patients, we conducted Kaplan-Meier survival analyses relating to the four differentially methylated CpG sites with overall survival in TNBC patients from TCGA cohort. The results indicated that hypermethylation of the two sites (cg07380026 [$p = 0.003$, HR = 1.48, 95% CI: 1.25-1.93] and cg26006951 [$p < 0.001$, HR = 1.47, 95% CI: 1.27-1.80]) were associated with poor overall survival (Figures 3A–D).

To confirm the above results, we examined the methylation status of the cg26006951 and cg07380026 CpG sites in 40 TNBC samples from SYSUCC by pyrosequencing. The results showed that both cg26006951 and cg07380026 CpG sites were significantly hypermethylated in tumor tissues compared with that in normal adjacent tissues (Figures 4A, B) and hypermethylation of cg26006951 was associated with poor overall survival (cg07380026 [HR = 1.30, 95% CI: 0.54–1.61, log-rank p = 0.14] and cg26006951 [HR = 1.62, 95% CI: 1.15–1.97,log-rank p = 0.0022]) (Figures 4C, D).

Based on the above analysis, we showed that there is abnormal hypermethylation of the TERT promoter, and the hypermethylation of the CpG site cg26006951 is closely related to the poor prognosis in TNBC. Therefore, we speculate that the CpG site cg26006951 is a potential predictive biomarker for TNBC prognosis. Next, we focused on cg26006951 for further analysis. We divided the above 40 patients of SYSUCC into a cg26006951 hypermethylation group and a cg26006951 hypomethylation group according to the median value and analyzed the relationship between the clinicopathological characteristics and the methylation level of cg26006951 in TNBC patients. The results showed that there was no statistical correlation between the degree of methylation of cg26006951 and the patient's age, T stage, N stage, clinical stage, and Ki-67 (Table 1).

According to the results of the TCGA database, we showed that cg07380026 and cg26006951 were potential poor prognostic factors for TNBC patients, and externally validated with 40 cases of TNBC tissues from the SYSUCC. Although the cg07380026 CpG site did not show statistical significance, the trend of the results was consistent with the TCGA database. Considering the existence of unavoidable interference factors such as small sample size, single-center samples and experimental errors, we believe that it is not possible to simply generalize from a statistical point of view and

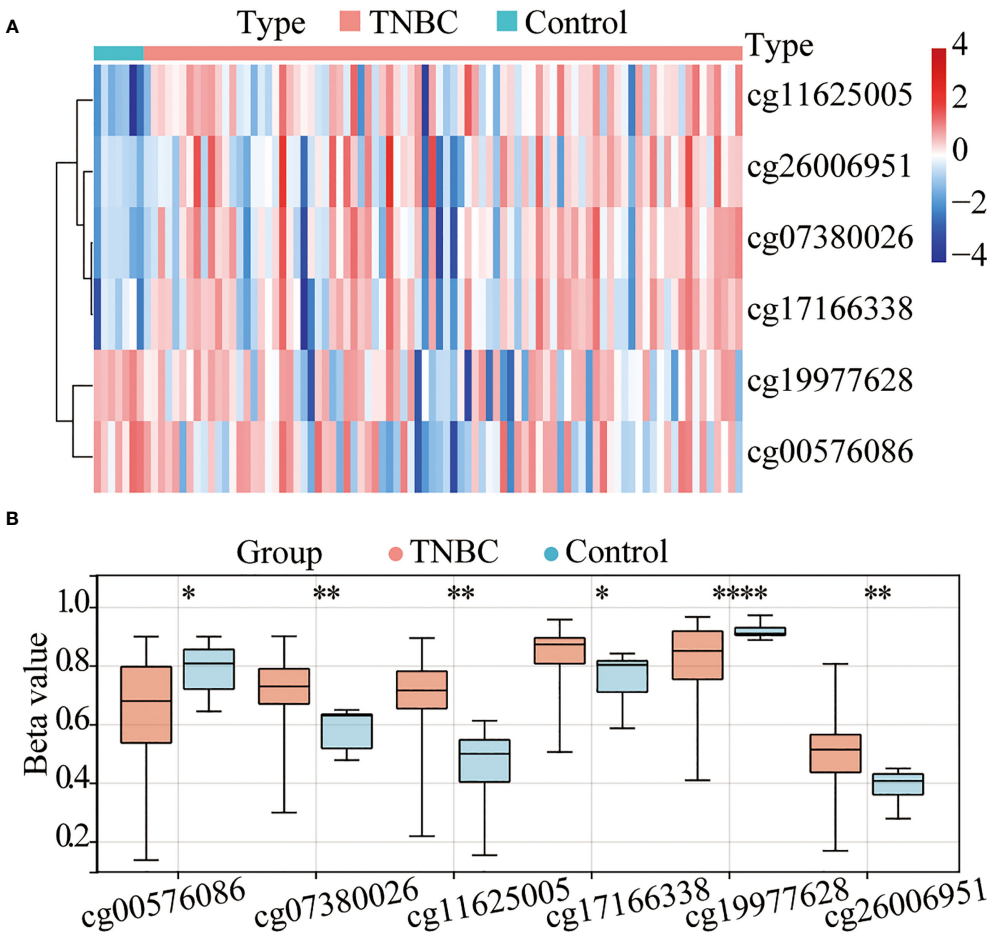


FIGURE 1
The TERT promoter is hypermethylated in triple-negative breast cancer (TNBC). **(A)** Heatmap of the differentially methylated CpG sites in TERT between tumor and adjacent normal tissues using data from The Cancer Genome Atlas (TCGA) cohort. **(B)** The boxplot of relative methylation levels of six differentially methylated CpG sites (cg00576086, cg07380026, cg11625005, cg17166338, cg19977628, and cg26006951) between tumor and adjacent normal tissues in the TCGA cohort. *P < 0.05; ** P < 0.01; **** P < 0.0001.

ignore its true clinical significance. Therefore, in the following further analysis of the TCGA database, we included TERT expression, cg07380026 and cg26006951 methylation levels into analysis.

## TERT gene expression and DMSs were closely related to the clinicopathological features of TNBC patients

To further explore the association between TERT expression, cg07380026, cg26006951, and clinicopathological features of TNBC patients, we combined the information of age, T, N, M stage and clinical stage of TNBC patients of TCGA database (Supplementary Table 2) for correlation analysis. The results showed that TERT expression was significantly correlated with pathological N stages, specifically: N0 and N3 (p<0.0001), N0 and N2 (p=0.01), N1 and N2 (p=0.05) and N1 and N3 (p=0.03) and in clinical stages: stage II and stage III (p=0.03) (Figures 5A, B). Meanwhile cg07380026 had significant differences between N1 and N3 (p=0.04) and between T2 and T3 (p=0.02) (Figures 5C, D). The CpG site cg26006951 still shows no correlation with clinicopathological features in the TCGA cohort. This result is consistent with that of SYSUCC cohort.

## TERT gene expression and DMSs were correlated with immune infiltration in TNBC patients

ssGSEA of the samples from TNBC patients and normal samples identified significant differences in the infiltration levels of 16 immune cell types, including activated dendritic cells (aDCs), B cells, $CD8^+$ T cells, and DCs, indicating that the immune microenvironment played a crucial role in TNBC (Figure 6A). We further analyzed the correlation among TERT expression, DMSs, and 24 types of immune cells, and found that high TERT expression and hypermethylated CpG sites within the TERT promoter (cg07380026 and cg26006951) were associated with more extensive immune infiltration of aDCs, B cells, cytotoxic cells, $CD56^{dim}$ NK cells, T cells, T helper 2 (Th2) cells, and regulatory T cells (Tregs). We also showed that low TERT expression and hypomethylation of CpG sites within the TERT

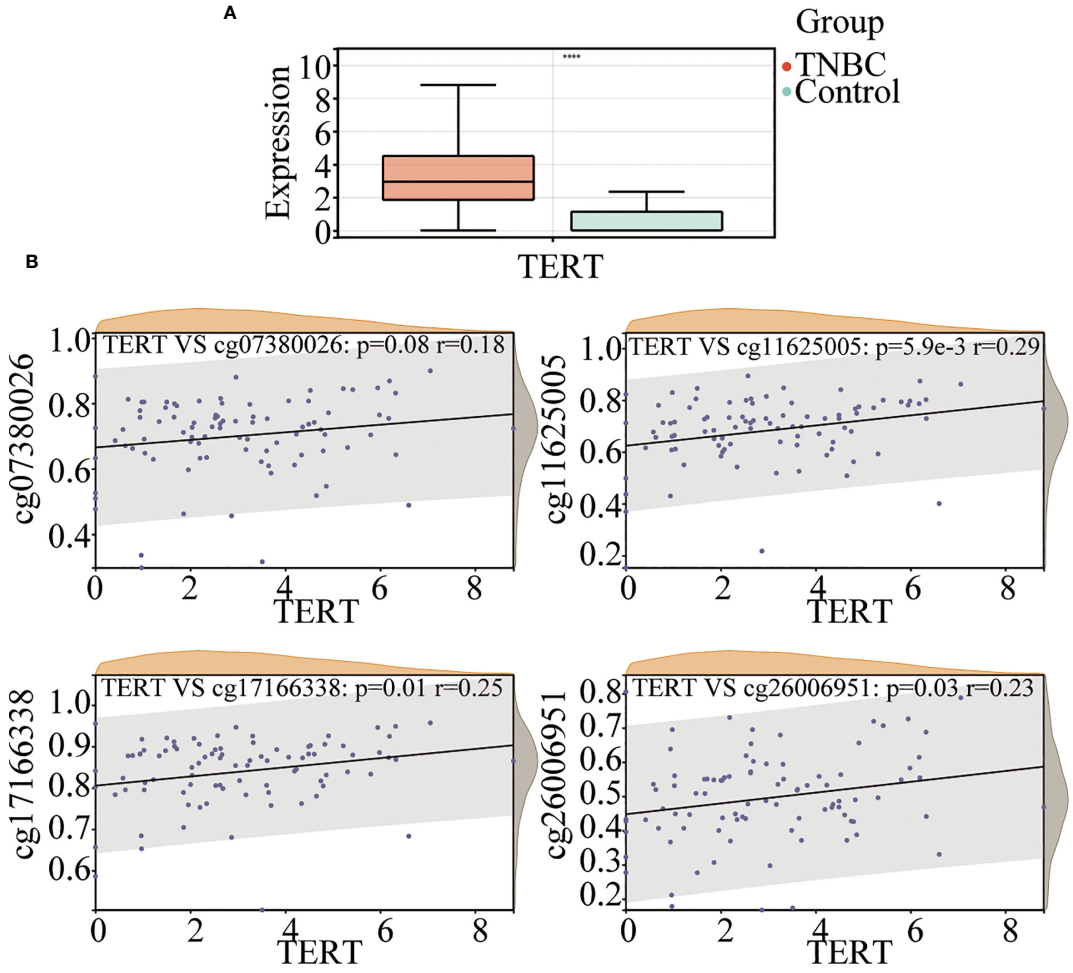


FIGURE 2
TERT promoter methylation is correlated with TERT expression in TNBC. **(A)** Comparison of TERT mRNA level between tumor and normal adjacent tissues in the TCGA cohort. **(B)** The correlation of cg07380026, cg11625005, cg17166338, and cg26006951 methylation status with TERT expression in tumor tissues in the TCGA cohort. **** P < 0.0001.

promoter (cg07380026 and cg26006951) were associated with increased immune infiltration of eosinophils, iDCs, mast cells, neutrophils, CD56$^{bright}$ NK cells, NK cells, T helper cells, central memory T cells, effector memory T cells, Th1 cells, and Th17 cells. Among the 24 immune cells examined, the increases in iDC and mast cell infiltration levels were statistically significant (Figure 6B). Moreover, we divided the TNBC patients into high-risk and low-risk groups according to the median value of TERT expression and the beta-value of TERT DMSs (cg07380026 and cg26006951) and then quantified 17 immune-related biological pathways in TNBC patients from TCGA using the R package "gsva" (version 1.40.1). The results showed that high TERT expression was correlated with lower epithelial mesenchymal transition 2 (EMT 2), pan-fibroblast TGFβ response signature (Pan-F-TBRS), angiogenesis, and type II interferon (IFN) response scores. Additionally, high TERT expression was associated with higher CD8$^+$ T effector scores and higher co-stimulation T-cell scores, indicative of abundant immune cell infiltration in the tumor microenvironment (Figure 6C).

Furthermore, we also analyzed the correlation of the cg07380026 and cg26006951 methylation status with 17 immune-related pathways. We found that cg07380026 hypomethylation was associated with lower CD8+ T effector, antigen processing machinery, APC co-stimulation and MHC-I HLA scores and with higher EMT1, EMT2, Pan-F-TBRS and angiogenesis scores (Figures 6D, E). Moreover, cg26006951 hypomethylation was associated with higher angiogenesis and type II IFN response scores. These results suggest that TERT, cg07380026, and cg26006951 exerted similar effects on angiogenesis, implying that patients with elevated TERT expression and cg07380026 and cg26006951 hypermethylation were likely to be insensitive to anti-angiogenesis therapy.

## TERT gene expression and DMSs were correlated with key immunomodulators in TNBC patients

Spearman's algorithm was adopted to analyze the correlations among TERT gene expression, TERT DMSs, and 74 immunomodulators. Significant differences were found for 27

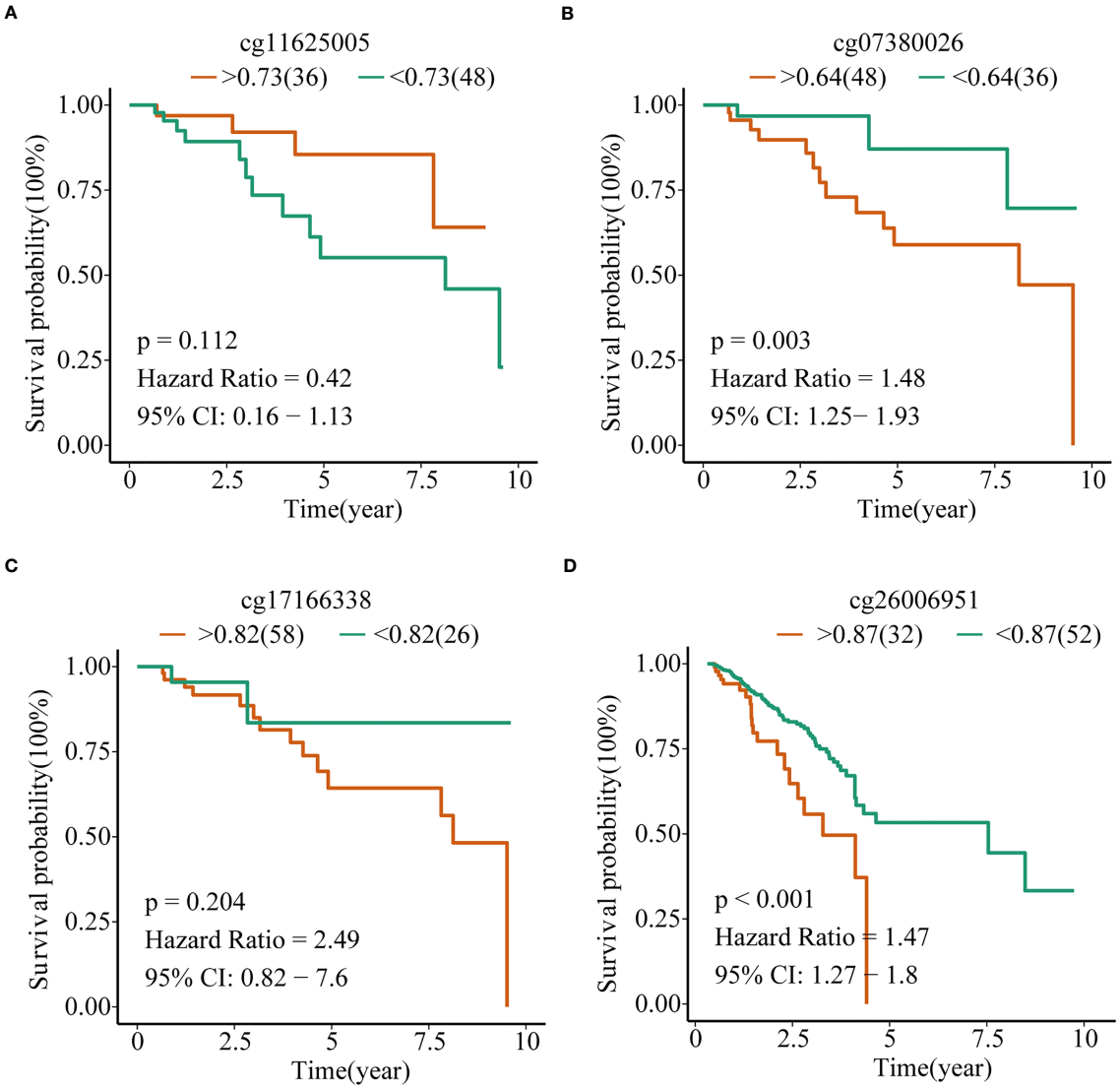


FIGURE 3
TERT promoter methylation predict overall survival in TNBC in the TCGA cohort. Kaplan–Meier analysis of overall survival relating to the methylation level of **(A)** cg11625005, **(B)** cg07380026, **(C)** cg17166338, and **(D)** cg26006951 in patients in the TCGA cohort, respectively.

immunomodulators (Figure 7A; Supplementary Table 3). We also analyzed the correlations among TERT expression, cg07380026, cg26006951 and routine immune checkpoint molecules (LAG-3, PD-1, PD-L1, CTLA4, TIGIT, and PD-L2), and showed that LAG-3 was significantly positively associated with TERT expression and cg07380026 and cg26006951 hypermethylation (Figures 7B–D).

To verify the association between LAG-3 and TERT promoter methylation or TERT expression, we examined the expression of LAG-3 and TERT and the methylation status of the TERT promoter in 40 TNBC tissues in the SYSUCC cohort. We found that cg26006951 hypermethylation was positively correlated with TERT and LAG-3 expression (Figures 8A-C). These results further validated that TERT promoter methylation was associated with overall survival and immunomodulator-LAG-3 in TNBC patients.

## Discussion

Epigenetics refers to a gene expression regulatory mechanism that affects transcription and translation without altering the DNA sequence (46, 47). Although genetic factors also underlie the etiology of many tumors, epigenetic abnormalities lead to more in-depth and extensive perturbations of cell signaling pathways and are thus more conducive to tumor occurrence and development (48, 49). The mechanisms involved in epigenetic regulation include DNA methylation, histone modifications, chromatin remodeling, and RNA interference (50). DNA methylation refers to the covalent modification of cytosine (51). In cancer, abnormal DNA methylation usually comprises hypermethylation of the promoters of suppressor genes and hypomethylation of those of oncogenes (52). The dysregulated methylation of CpG islands in the promoter

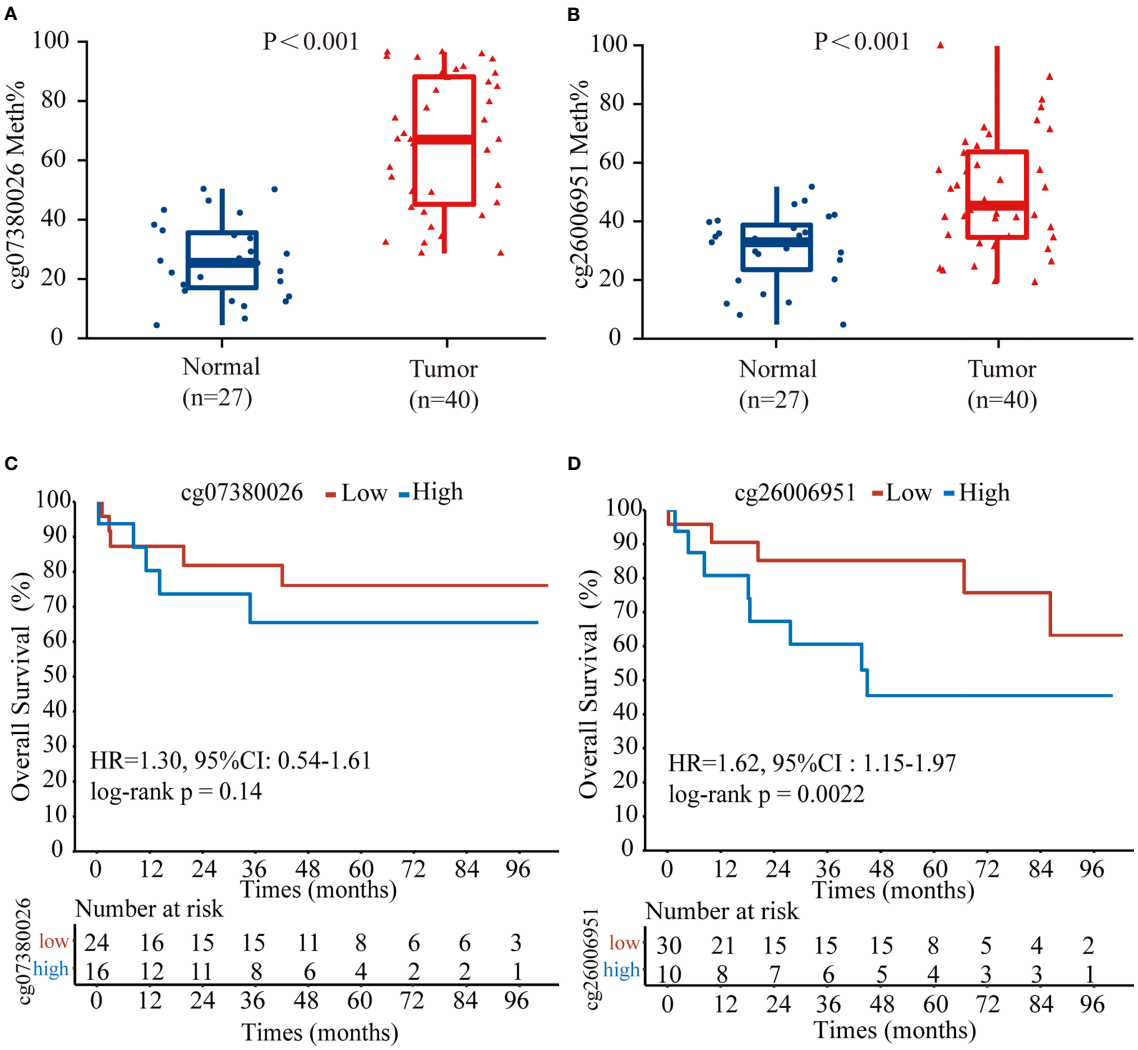


FIGURE 4
The CpG site cg26006951 predict overall survival in TNBC from Sun Yat-Sen University Cancer Center (SYSUCC) cohort. Comparison of the relative **(A)** cg07380026 and **(B)** cg26006951 methylation levels between tumor and normal adjacent tissues in the SYSUCC cohort. **(C, D)**. Kaplan–Meier survival analysis associated with cg07380026 and cg26006951 methylation level in the SYSUCC cohort, respectively.

TABLE 1 The relationship between cg26006951 and clinicopathologic characteristics in the SYSUCC cohort.

| Characteristic | Total | cg26006951 | | *P* |
|---|---|---|---|---|
| | n=40 | Low | High | |
| **Age (years) median (IQR)** | 47 (39-56) | 41 (36-46) | 43 (37-48) | 0.190 |
| **Tumor stage** | | | | 0.216 |
| T1 | 16 (40.0) | 8 (50.0) | 8 (50.0) | |
| T2 | 22 (55.0) | 12 (54.5) | 10 (45.5) | |
| T3 | 1 (2.5) | 1 (100.0) | 0 (0.0) | |
| T4 | 1 (2.5) | 0 (0.0) | 1 (100.0) | |
| **Node stage** | | | | 0.083 |
| N0 | 22 (55.0) | 11 (50.0) | 11 (50.0) | |
| N1 | 11 (27.5) | 4 (36.4) | 7 (63.6) | |

*(Continued)*

TABLE 1 Continued

| Characteristic | Total | cg26006951 | | *P* |
|---|---|---|---|---|
| | n=40 | Low | High | |
| N2 | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| N3 | 7 (17.5) | 6 (85.7) | 1 (14.3) | |
| **Clinical stage** | | | | 0.078 |
| I | 8 (20.0) | 4 (50.0) | 4 (50.0) | |
| II | 19 (47.5) | 13 (68.4) | 6 (31.6) | |
| III | 13 (32.5) | 8 (61.5) | 5 (38.5) | |
| **Ki-67** | | | | 0.154 |
| >14% | 31 (77.5) | 21 (67.7) | 10 (32.3) | |
| ≤14% | 9 (22.5) | 6 (66.7) | 3 (33.3) | |

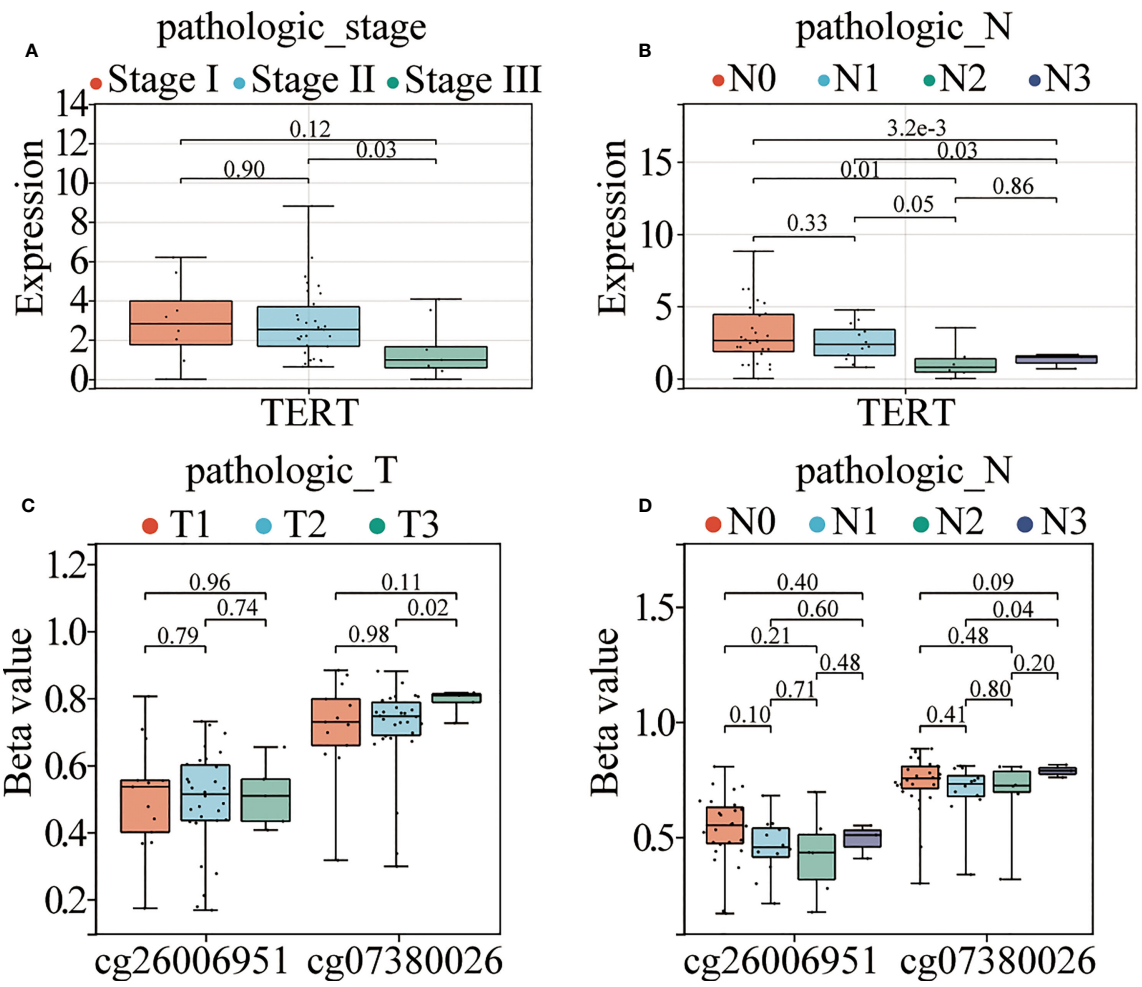


FIGURE 5
TERT promoter hypermethylation and elevated expression correlate with aggressive clinical phenotypes in TNBC. **(A, B)**. TERT expression in different TNM stages and pathological N stages among patients from the TCGA cohort. **(C, D)**. The relative methylation levels of cg26006951 and cg07380026 in different pathological N and T stages in patients in the TCGA cohort.

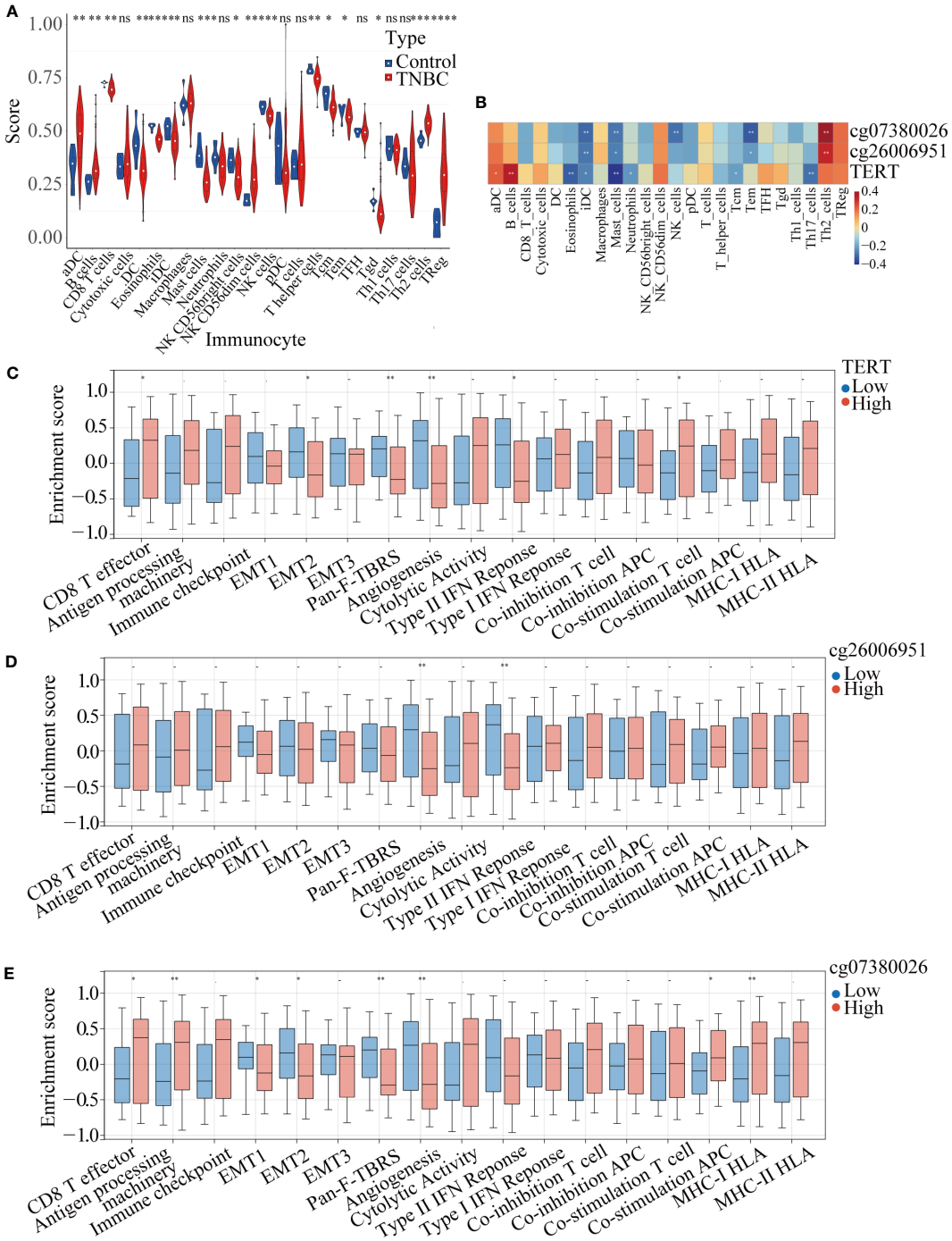


FIGURE 6
TERT expression and promoter methylation were correlated with immune cell infiltration in TNBC. **(A)**. Immune infiltration scores for tumor and normal tissues in TNBC. **(B)**. Correlation heatmap of TERT expression and two differentially methylated CpG sites (cg26006951 and cg07380026) with 24 types of immune cells in the TCGA cohort. **(C–E)**. Relative enrichment scores for 17 immune-related pathways in the high-TERT-expression and low-TERT-expression groups based on the median scores for TERT expression or cg26006951 and cg07380026 methylation level. * P < 0.05; ** P < 0.01; ns, not statistically.

regions of suppressor genes can result in their silencing and inactivation. This affects the normal expression of the affected genes, leading to uncontrolled cell proliferation and the promotion of tumor occurrence and development (52).

Telomere maintenance, which enables tumor cells to replicate indefinitely, is an important feature of most tumors (53–55). Most cells do not display telomere maintenance mechanisms (56). However, in stem cells, germ cells, and activated memory lymphocytes, among other cell types, the telomerase complex actively maintains telomere length (22, 57). Previous studies have shown that telomerase is tightly regulated in normal cells (22, 58, 59). TERT is the key rate-limiting catalytic subunit of telomerase

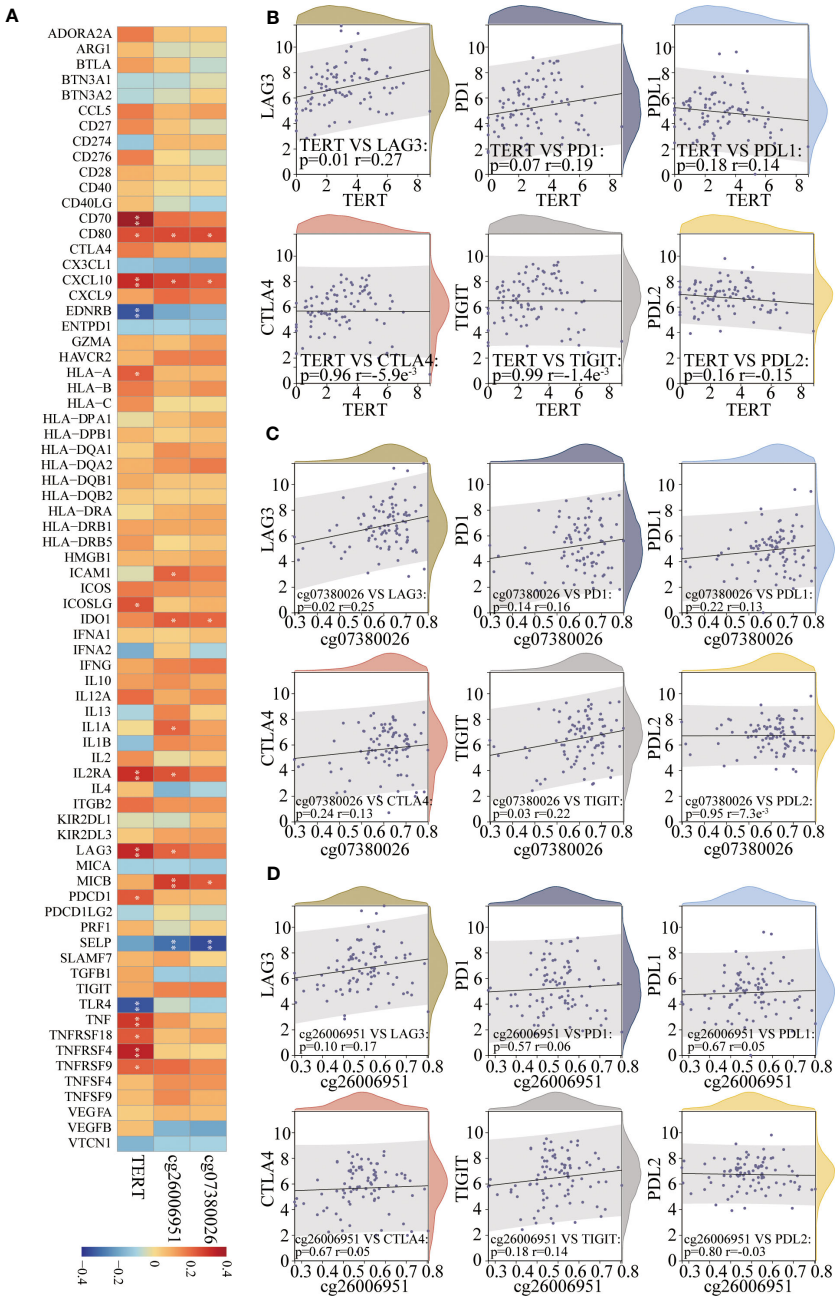


FIGURE 7
TERT expression and promoter CpGs were associated with the expression of key immunomodulators in TNBC. **(A)**. Correlation heatmap of TERT expression and two differentially methylated CpG sites (cg26006951 and cg07380026) with 74 key immunomodulators in the TCGA cohort. **(B–D)**. The correlation of TERT expression, cg26006951, and cg07380026 methylation level with the immune checkpoint molecules LAG-3, PD-1, PD-L1, CTLA4, TIGIT, or PD-L2, respectively. * P < 0.05; ** P < 0.01.

and most tumor cells maintain telomere length by abnormally upregulating TERT expression (22, 60).

In this study, we showed that TERT expression was elevated in patients with TNBC and four differential CpG sites in the TERT promoter were hypermethylated. Further correlation analysis revealed that three of them were significantly positively correlated with TERT expression. These findings suggest that aberrant TERT promoter hypermethylation might be responsible for the elevated TERT expression in TNBC. Similarly, different studies in other tumor types have drawn similar conclusions with ours. It has shown that the TERT promoter is hypermethylated in both tumor tissues and cancer cells and is positively correlated with TERT mRNA expression and telomerase activity (61–64). In view of the widespread abnormal hypermethylation of the TERT promoter in different tumor types, many scholars have also explored its clinical prognostic value. Pedro Castelo-Branco et al. from the University of Toronto in Canada investigated whether TERT promoter methylation can be used as a biomarker of malignancy and a marker of prognosis in pediatric brain tumor patients (65). They found that TERT promoter hypermethylation was positively correlated with TERT expression,

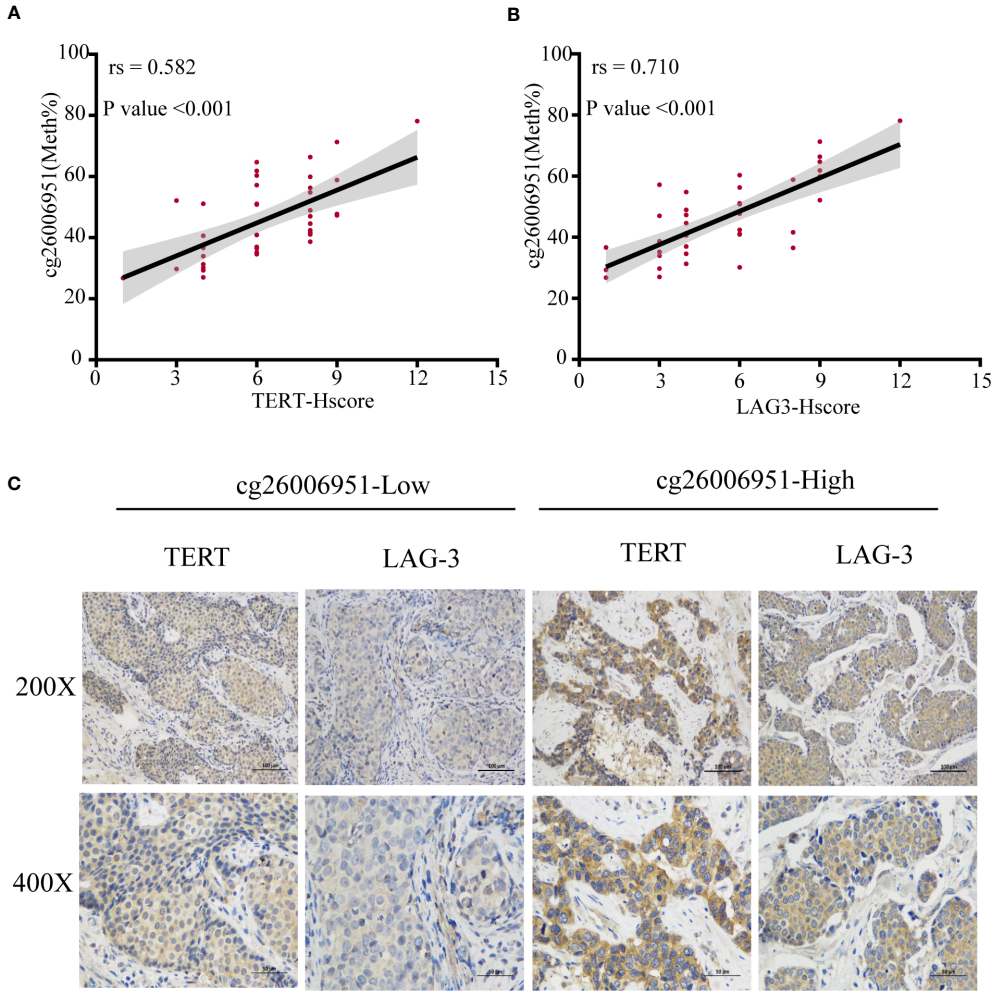


FIGURE 8
The methylation of cg26006951 is positively correlated with the expression of TERT and LAG-3. **(A, B)**. The correlation of cg26006951 methylation with LAG-3 and TERT expression in the SYSUCC cohort. **(C)** Representative immunohistochemistry images of LAG-3 and TERT expression in the cg26006951 hyper- and hypomethylation groups in the SYSUCC cohort.

and TERT promoter hypermethylation was associated with disease progression and poor prognosis in children with brain tumors. They also found that regarding the identified CpG site cg11625005, in 79 normal brain tissues and low-grade tumor tissues, 78 samples (99%) showed no hypermethylation, while among the 201 malignant tumor samples, 145 samples (72%) were hypermethylated, and the results were statistically significant. Meritxell Oliva et al. (66) generated array based DNAm profiles describing methylation and transcriptome correlations for 987 human samples (9 tissue types from 424 subjects) and found that hypermethylation of cg07380026 might affect TERT expression in breast and ovarian cancer. Subsequently, a series of studies have shown that hypermethylation of TERT promoter is associated with poor prognosis in tumors such as melanoma (67), gastric cancer (68), bladder cancer (69), prostate cancer (70) and thyroid cancer (71). Although studies mentioned above have shown that TERT promoter hypermethylation is positively correlated with TERT expression, some studies have obtained contradictory results. One study has concluded that the causal relation between TERT promoter methylation and gene expression remains to be established (72). What's more, in another liver cancer study, Iliopoulos et al. showed that there was a significant negative correlation between TERT expression and TERT promoter methylation (73). The reason for the opposite conclusion may be that TERT promoter methylation detection regions selected in different studies are different because each region contains different CpG methylation sites, and the methods of detecting methylation are also different in different studies. In view of the above reasons, there may be some differences in the results of different studies, and even some conclusions are controversial.

In the present study, we also sought to identify immune biomarkers related to high TERT expression and TERT promoter hypermethylation. We found that LAG-3, an immune checkpoint receptor protein, was positively correlated with high TERT expression and TERT promoter hypermethylation. The primary function of LAG-3 involves the negative regulation of T-cell function, the maintenance of immune system homeostasis, and the promotion of tumor immune escape (74). LAG-3 shows great potential as a target in tumor immunotherapy (75). Additionally, LAG-3 may be a better

immunotherapeutic target than PD-1 or CTLA-4 given that, although antibodies against the PD-1 and CTLA-4 immune checkpoints can activate effector T cells, they cannot inhibit Treg activity (76). Studies have shown that a LAG-3 antibody can activate T effector cells while inhibiting Treg activity (76). Like PD-1 and CTLA-4, LAG-3 is not expressed on naive T cells but can be induced on $CD4^+$ and $CD8^+$ T cells upon antigenic stimulation (77). LAG-3 is also expressed in subsets of $CD4^+$ T cells with suppressive function. $Foxp3^+$ Tregs constitutively express LAG-3 (78). Taken together, our study demonstrated that TERT promoter hypermethylation was not only associated with TERT expression and overall survival in TNBC but also significantly positively correlated with expression of immune checkpoint molecules–LAG-3. It is intriguing to continue to investigate whether TERT is capable of regulating the expression of LAG-3 directly. If so, the combination of TERT inhibitors and LAG-3 inhibitors in solid tumors is a promising strategery.

Although we reported this important finding for the first time, this study had some limitations. Because patients with TNBC account for approximately only 15% of all breast cancer patients, the sample size in this study (40 TNBC patients) was relatively small and the validation cohort was derived from only one single cancer center (SYSUCC). We searched for other databases that could serve as external validation cohorts, such as CPTAC. However, we could only find five samples from TNBC patients. Considering that the sample size was too small, we excluded these five samples from the analysis. Furthermore, we only used immunohistochemical methods to indirectly demonstrate that high TERT expression was positively correlated with high LAG-3 expression at the protein level. Additionally, we did not elucidate the mechanism underlying the identified correlation between the high TERT and LAG-3 expression levels. These limitations provide a direction and reference for our future research.

## 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 clinical specimens used in this study were obtained from the Tumor Biobank of SYSUCC. The study protocol for the SYSUCC cohort was approved by the Institutional Research Ethics Committee of SYSUCC (B2022-332-01).

## Author contributions

FL, JH, LG, WD and HL conceived of the study and participated in its design and coordination. FL, JH, TJ, WZ, WX, JG and MC performed the experiments and statistical analysis. FL, WD and MC drafted and revised the manuscript. All authors read and approved the final 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.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1218987/full#supplementary-material

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# Primary microcephaly gene CENPE is a novel biomarker and potential therapeutic target for non-WNT/non-SHH medulloblastoma

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**Background:** Non-WNT/non-SHH medulloblastoma (MB) is one of the subtypes with the highest genetic heterogeneity in MB, and its current treatment strategies have unsatisfactory results and significant side effects. As a member of the centromere protein (CENP) family, centromeric protein E (CENPE) is a microtubule plus-end-directed kinetochore protein. Heterozygous mutations in CENPE can leads to primary microcephaly syndrome. It has been reported that CENPE is upregulated in MB, but its role in MB development is still unknown.

**Methods:** We downloaded the relevant RNA seq data and matched clinical information from the GEO database. Bioinformatics analysis includes differential gene expression analysis, Kaplan-Meier survival analysis, nomogram analysis, ROC curve analysis, immune cell infiltration analysis, and gene function enrichment analysis. Moreover, the effects of CENPE expression on cell proliferation, cell cycle, and p53 signaling pathway of non-WNT/non-SHH MB were validated using CENPE specific siRNA *in vitro* experiments.

**Results:** Compared with normal tissues, CENPE was highly expressed in MB tissues and served as an independent prognostic factor for survival in non-WNT/non-SHH MB patients. The nomogram analysis and ROC curve further confirmed these findings. At the same time, immune cell infiltration analysis showed that CENPE may participate in the immune response and tumor microenvironment (TME) of non-WNT/non-SHH MB. In addition, gene enrichment analysis showed that CENPE was closely related to the cell cycle and p53 pathway in non-WNT/non-SHH MB. *In vitro* experimental validation showed that knockdown of CENPE inhibited cell proliferation by activating the p53 signaling pathway and blocking the cell cycle.

**Conclusion:** The expression of CENPE in non-WNT/non-SHH MB was positively correlated with poor prognosis. CENPE may affect tumor progression by regulating cell cycle, p53 pathway, and immune infiltration. Hence, CENPE is highly likely a novel biomarker and potential therapeutic target for non-WNT/non-SHH MB.

KEYWORDS

CENPE, non-WNT/non-SHH medulloblastoma, microcephaly, bioinformatics analysis, cell cycle, p53

# Introduction

Medulloblastoma (MB) is the most common cerebellar malignant tumor in children, which accounts for a large proportion of both cancer-related incidence rate and mortality in this age group (1). MB is divided into at least four subgroups: Wingless-activated (WNT), Sonic Hedgehog-activated (SHH), Group 3 and Group 4, which are based on different molecular expression profiles, clinical manifestations, and prognostic characteristics (2). In 2021, the Groups 3 and Group 4 of MB were summarized as non-WNT/non-SHH subtype in the WHO classification of central nervous system tumors (3). The characteristic of this subtype is high heterogeneity, with varying biological characteristics, genetic basis, and clinical course. This subtype accounts for approximately 60% of all cases and remains the most genetically heterogeneous and least understood subset of MB cases (4). In terms of treatment, there is still a lack of effective treatment targets for non-WNT/non-SHH MB. The current treatment methods mainly include surgical resection combined with postoperative cranial spinal cord irradiation (CSI) and chemotherapy. Although the survival period of some children has been extended, their mortality rate is still high, and some children cannot tolerate the side effects of radiation and chemotherapy, resulting in poor long-term quality of life and frequent neurological and endocrine sequelae (5). Therefore, it is necessary to search for new biomarkers and safer and more effective therapeutic targets for non-WNT/non-SHH MB.

As a member of the centromere protein (CENP) family, centromeric protein E (CENPE) is a microtubule plus-end-directed kinetochore protein. It plays an important role in spindle assembly checkpoint (SAC), chromosome congression and spindle microtubule capture at kinetochores during mitosis (6, 7). Pan-cancer analysis indicates that CENPE is associated with various types of tumorigenesis. Its high expression is associated with poor prognosis of non-small cell lung cancer, colorectal cancer and breast cancer (8–10). In addition, CENPE is involved in the malignant proliferation and migration of lung adenocarcinoma and ovarian cancer (11, 12).

In research on the nervous system, it has been found that that as a key gene in primary microcephaly (MCPH) syndrome, CENPE mutations can lead to a decrease in brain capacity (13). However, inhibiting CENPE expression in MB cells can cause p53 dependent cell apoptosis (14). Although the origin of MB is unknown, it is clear that MB cells share many common molecular features with radial glial cells and cerebellar granulosa progenitor cells. Based on the above foundation, mutated genes in primary MCPH syndrome have been proposed as potential therapeutic targets for MB (15).

In this study, we evaluated the expression changes of CENPE in MB and its potential value in diagnosis and prognosis, and explored the relationship between CENPE and immune infiltration. In addition, enrichment analysis showed that CENPE is associated with cell cycle, p53 signaling pathway in non-WNT/non-SHH MB, and this result was validated in cell experiments. In summary, our results indicate that CENPE can serve as a novel biomarker and potential therapeutic target for non-WNT/non-SHH MB.

# Materials and methods

## The expression levels of CENPE

GSE124814 and GSE85217 datasets were retrieved from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The platform for GSE124814 included the transcriptome sequencing data of 1350 MB samples and 291 normal cerebellum samples. The platform for GSE85217 included 763 MB samples of transcriptome sequencing data and matched clinical information. Box plot and scatter plot were used to compare the expression levels of CENPE in MB samples and normal cerebellum tissues.

## Survival analysis

The median expression of CENPE was selected to divided the patients into CENEP$^{high}$ expression group and CENPE$^{low}$ expression group. The R software package survminer was used to analyze the

correlation between CENPE expression and the overall survival of patients with MB. The relationship between CENPE expression and prognosis of MB patients was analyzed by Spearman and logistic regression analysis, and visualized by R package ggplot2.

## Predictive nomogram construction

We used the R package rms to integrate data on age, gender, tumor subtypes, and survival time. Using Cox proportional hazard regression analysis established a nomogram to evaluate the prognostic significance of these features in 599 samples. We evaluated the diagnostic and predictive capabilities of CENPE by drawing Receiver operating characteristic (ROC) curves. The area under the curve (AUC) was calculated, AUC>0.7 indicates satisfactory predictive ability.

## Immune infiltration analysis

We selected the TIMER method in the R package IOBR to evaluate the infiltration level of immune cells in non-WNT/non-SHH MB sample in dataset GSE85217. Spearman correlation analysis was used to analyze the correlation between CENPE expression and the infiltration of immune cells, which was visualized by R package ggplot2 (4.1.3).

## Functional enrichment analysis

We analyzed the correlation between CENPE and all genes in the GSE85217 dataset through the R package tidyverse, and after ranking according to correlation, the 100 genes with the strongest positive correlation and the 100 genes with the strongest negative correlation with CENPE were selected. These 200 genes were subjected to Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis using the R package clusterProfiler. Gene set enrichment analysis (GSEA) was performed using the GSEA software (version 3.0) derived from GSEA (http://software.broadinstitute.org/gsea/index.jsp) to investigate the differences in biological functions and KEGG pathways between the $CENEP^{high}$ and $CENPE^{low}$ non-WNT/non-SHH MB groups (cutoff value was 50%). To evaluate relevant pathways and molecular mechanisms, based on gene expression profiling and phenotype grouping, with a minimum gene set of 5 and a maximum gene set of 5000, with 1000 resampling, $P < 0.05$ and FDR < 0.25 were considered statistically significant.

## Protein–protein interaction network analysis

The protein-protein interaction (PPI) network of CENPE co-expressed genes in non-WNT/non-SHH MB was analyzed using the search tool (STRING database, https://string-db.org/, version 11.0b) for the retrieval of interacting genes. The Betweenness score of each node analyzed by Cytoscape 3.7.2, and the top 50 with the highest score were screened to draw the skeleton network and correlation heat map.

## Cell culture and transfection

The non-WNT/non-SHH MB cell lines D283 and D341 were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA) at 37°C (16). The cells were grown to ~50% confluency in six-well plates before transfection. We transfected the small interfering RNA (siRNA) sequences with Lipofectamine 3000 (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. The siRNA sequences of CENPE in D283 and D341 cells were used as follows: siRNA-CENPE (5′-GGCUGUAAUAUAAAUCGAA-3′), and siRNA negative control (NC) (5′-TTCTCCGAACGTCACGT-3′).

## Western blot

D283 and D341 cells were lysed with protease inhibitor, and RIPA cleavage buffer (Thermo Fisher Scientific, Shanghai, China). The protein lysates were resolved *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and imprinted on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) for analysis. Anti-CENEP (1:1,000 dilution, DF7745; Affinity Biosciences, OH, USA), anti-P53 (1:500 dilution, AF0879; Affinity Biosciences, OH, USA), anti-P21 (1:500 dilution, AF6290; Affinity Biosciences, OH, USA), anti-CDK1 (1:500 dilution, DF6024; Affinity Biosciences, OH, USA) and anti-β-Actin (1:5,000 dilution, AF7018; Affinity Biosciences, OH, USA) were incubated overnight. Goat Anti-Rabbit IgG (H+L) HRP-conjugated secondary antibodies (1:5,000 dilution; S0001, Affinity Biosciences, OH, USA) were added at room temperature for 2 h. The membrane was detected with BeyoECL Plus developer (Beyotime, Shanghai, China).

## Cell cycle analysis

D283 and D341 cells were cultured in six-well plates with $2 \times 10^5$ cells for 24 h and were transfected with CENPE siRNA for 48 h. The cells were collected, and then fixed with 70% ethanol at 4°C overnight. The cells were stained with RNase A-containing PI buffer (C1052; Beyotime, Shanghai, China) at 37°C for 30 min. The cell cycle distribution was detected by flow cytometry (Beckman Coulter Quanta SC System).

## Cell counting kit-8 assay

D283 and D341 cells transfected with CENPE siRNA were cultured in a 96-well plate at $1 \times 10^3$/well and allowed to adhere overnight. 10 μL CCK-8 solution was added to each well and incubated for 1.5 h. A microplate reader (Thermo Fisher Scientific, Shanghai, China) was used to detected the absorbance at 450 nm every day.

## Immunofluorescence staining

D283 and D341 cells transfected with CENPE siRNA were fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with 0.2% TritonX-100 for 15 min. After blocking in 10% goat serum for 30 min, the cells were incubated with anti-ki67 (1:200 dilution, AF0198; Affinity Biosciences, OH, USA) antibody at 4°C overnight. Then the cells were incubated with AlexaFluor 488 (goat anti-rabbit IgG, Abcam, Cambridge, UK, 1:1,000 dilution) at room temperature for 40 min. At last, the cells were stained with Dapi (1 μg/ml) for 10 min in dark place at room temperature and viewed with an inverted IX71 microscope system (Olympus, Tokyo, Japan). The mean intensity was measured by ImageJ software.

## Statistical analysis

R (4.1.3) software and GraphPad Prism (8.0.0) software were used for statistical analyses, the main R packages used in the study were as follows: "survminer", "tidyverse", "IOBR", "clusterProfiler", "rms" and "ggplot2". All in experiments were repeated three times. A t-test was used in comparing the means of two groups, $p < 0.05$ means statistical significance.

# Results

## CENPE was highly expressed and served as an independent prognostic factor for overall survival in MB

Our results showed that the expression of CENPE was significantly higher in MB than in normal cerebellum tissue (Figure 1A). Moreover, the expression of CENPE was also elevated in different subtypes of MB (Figure 1B). Kaplan-Meier survival analysis showed that the expression of CENPE in MB was not statistically significant with the survival prognosis of MB patients (Figure 1C). However, the expression of CENPE in the non-WNT/non-SHH subtype and SHH subtype was negatively correlated with the survival prognosis of patients. The Hazard Ratio (HR) value of CENPE in survival of non-WNT/non-SHH MB patients was higher than that in SHH subtype (Figures 1D–F).

## Diagnostic and predictive ability of CENPE in MB

To analyze the diagnostic value of CENPE expression in MB, we performed ROC curve and nomogram analysis on the CENPE

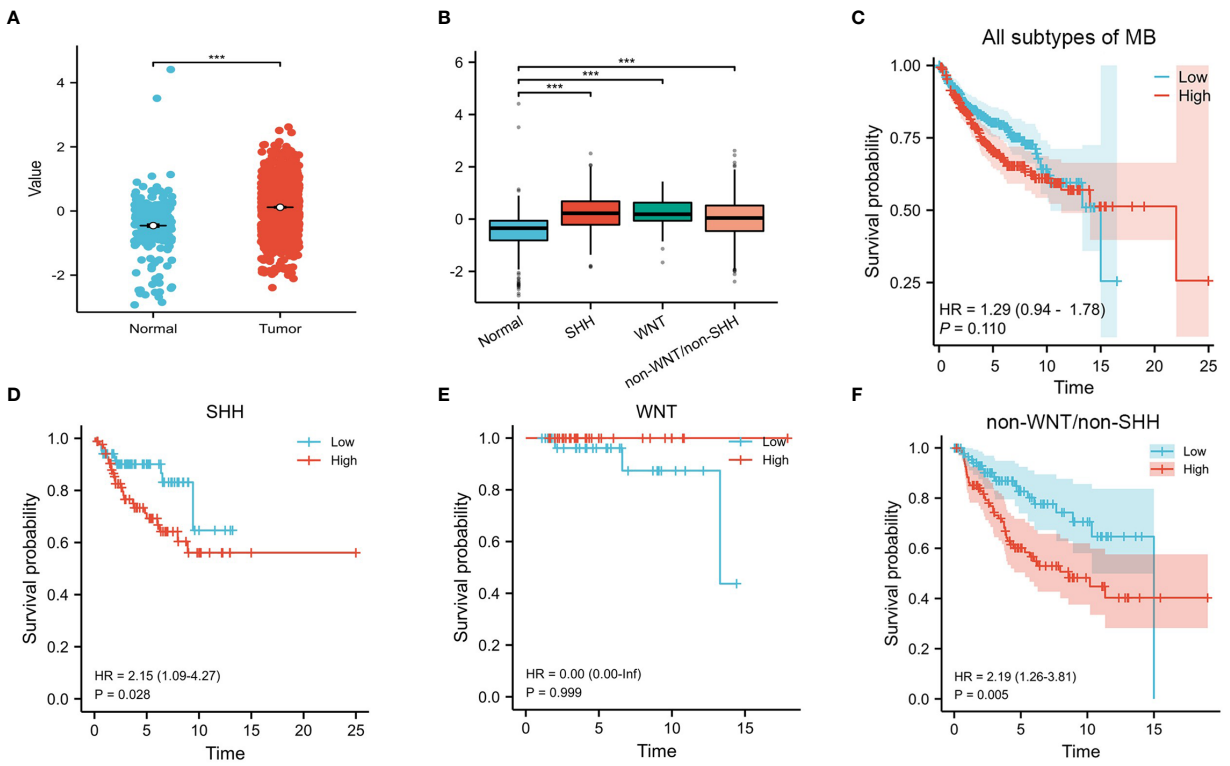


FIGURE 1
CENPE's overall expression and survival analyses in MB. **(A, B)** The expression of CENPE in different subtypes of MB and normal cerebellum tissue. **(C–F)** Kaplan–Meier survival curve for impacts of CENPE on overall survival of all subtypes MB patients. ***$p < 0.001$.

expression data of GSE85217 database to evaluate the diagnostic value of the gene. The area under the ROC curve (AUC) was 0.726 (Figure 2A), indicating the good predictive ability. We combined the expression level of CENPE with clinical information to construct a nomogram to predict 1-, 3-, and 5-year survival in patients with MB. The nomogram showed that the subtypes of MB and the expression of CENPE were good prognostic indicators (Figure 2B). We further constructed a nomogram among the non-WNT/non-SHH subtypes with the worst prognosis in MB to predict 1-, 3-, and 5-year survival of patients. The results indicated that prognostic prediction of the expression level of CENPE in non-WNT/non-SHH MB was better than other traditional clinical features (Figure 2C). Moreover, the results of Figures 1C–F indicated that among all subtypes of MB patients, high expression of CENPE was most strongly correlated with poor prognosis in non-WNT/non-SHH MB. Therefore, we chose to further investigate the role of CENPE in the occurrence and development of non-WNT/non-SHH MB.

## The correlation between CENPE and immune cell infiltration in non-WNT/non-SHH MB

To explore the correlation between the expression level of CENPE and tumor immune response, we first used CIBRSORT to evaluate the differences in immune cell infiltration in non-WNT/non-SHH MB with high and low expression of CENPE. The results showed that CD4+ central memory T cells (Tcm), Class-switched memory B cells, Eosinophils, NKT, regulatory T cells (Tregs) were highly infiltrated in the low CENPE expression group. In contrast, the expression of Activated dendritic cells (aDC), B cells, CD4+ memory T cells (Tem), CD8+ Tcm, DC, conventional DC, immature DC, Macrophages, Macrophages M1, Mast cells, NK cells, pro B-cells, Tgd cells, Th1 cells, Th2 cells were highly infiltrated in the high CENPE expression group (Figures 3A, B). We further analyzed the correlation between the expression level of CENPE and immune infiltration in non-WNT/non-SHH MB. The results showed that CENPE expression correlated positively with the infiltration of Tergs (r=0.118, p=0.010), NKT(r=0.106, p=0.022), but negatively with Tgd cells (r=-0.149, p=0.001), pro B cells (r=-0.110, p=0.0017), Th2 cells (r=-0.186, p<0.001), CD4+ Tem (r=-0.131, p=0.004) (Figures 3C–H).

## Biological pathways correlated with CENPE in non-WNT/non-SHH MB

We then used the R package tidyverse to further explore the biological function of CENPE. 5840 genes were positively correlated with the expression of CENPE, and 5272 genes were negatively correlated with the expression of CENPE (Figure 4A). Heat map showed the top 50 genes positively and negatively correlated with CENPE expression (Figures 4B, C). The top 200 co-expressed genes of CENPE were annotated by GO and KEGG functional enrichment analysis. The KEGG analysis showed that co-expression of CENPE was mainly involved Cell cycle, Human T-cell leukemia virus 1 infection, Cellular senescence, p53 signaling pathway, Fanconi anemia pathway (Figure 4D). GO functional annotations showed that CENPE co-expressed genes were mainly involved in the organelle fission (GO-BP) (Figure 4E), chromosomal region (GO-CC) (Figure 4F), tubulin binding (GO-MF) (Figure 4G). PPI network and correlated analysis were used to identify the interactions between the top 50 proteins related to CENPE. We found that most proteins in the network have strong positive or negative correlations with each other. (Figures 5A, B).

## Difference of biological pathways between high and low CENPE groups in non-WNT/non-SHH MB

To further characterize the function of CENPE, we identified 8404 differentially expressed genes (DEGs) between the CENPE$^{high}$

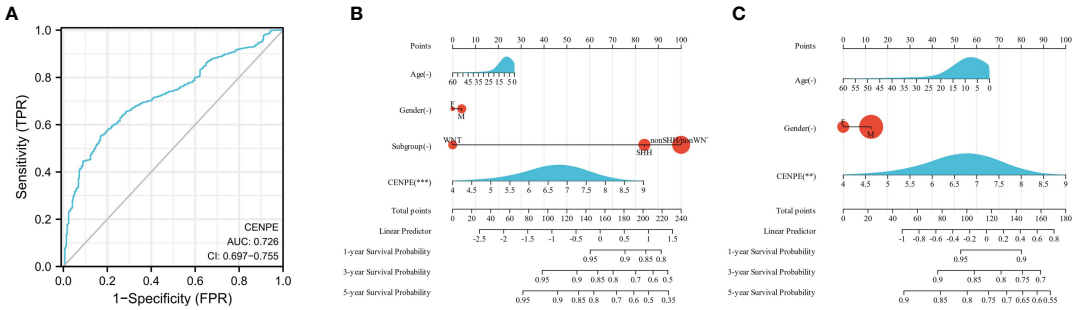


FIGURE 2
Predictive ability of CENPE for MB. **(A)** ROC curve analysis of CENPE expression in MB and normal cerebellum tissues. **(B)** Nomograms to predict 1 -, 3 -, and 5-year survival for patients with all subtypes MB. **(C)** Nomograms to predict 1 -, 3 -, and 5-year survival of patients with non-SHH/non-WNT MB. $^{**}p < 0.01$ and $^{***}p < 0.001$.

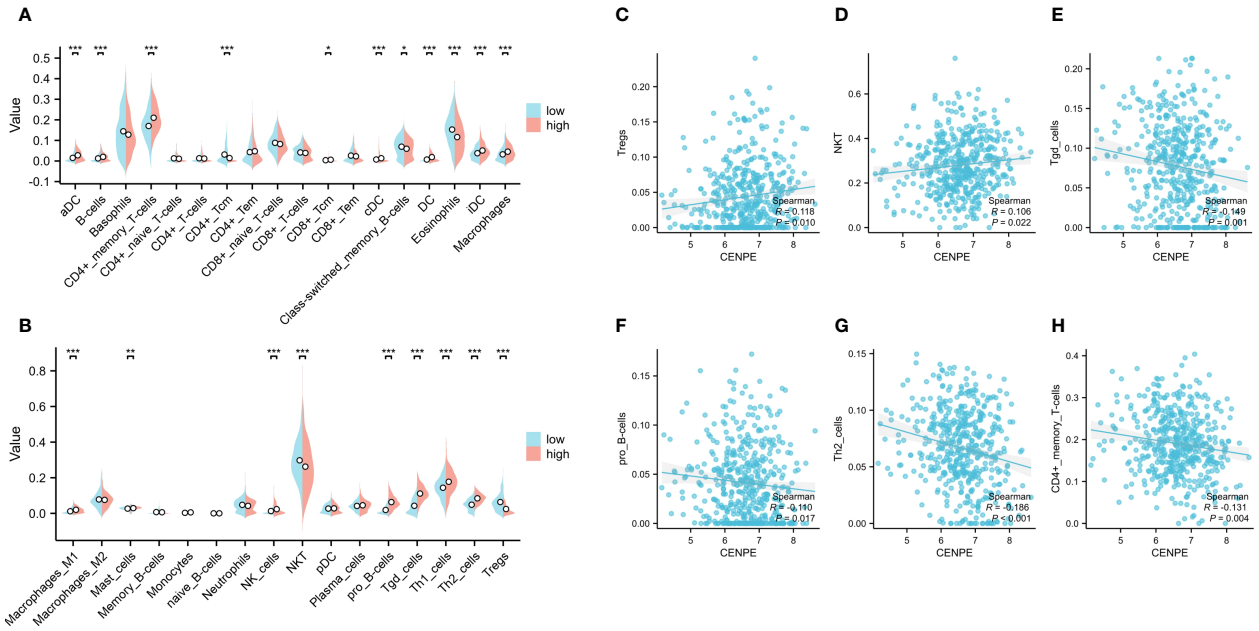


FIGURE 3
Correlation analysis between CENPE expression and immune infiltration in non-WNT/non-SHH MB. **(A, B)** Differential distribution of immune cells in patients with high and low CENPE expression. Correlation between CENPE expression and immune infiltration in non-WNT/non-SHH MB: **(C)** Tregs, **(D)** NKT, **(E)** Tgd cells, **(F)** pro B cells, **(G)** TH2 cells, **(H)** CD4+ Tem. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

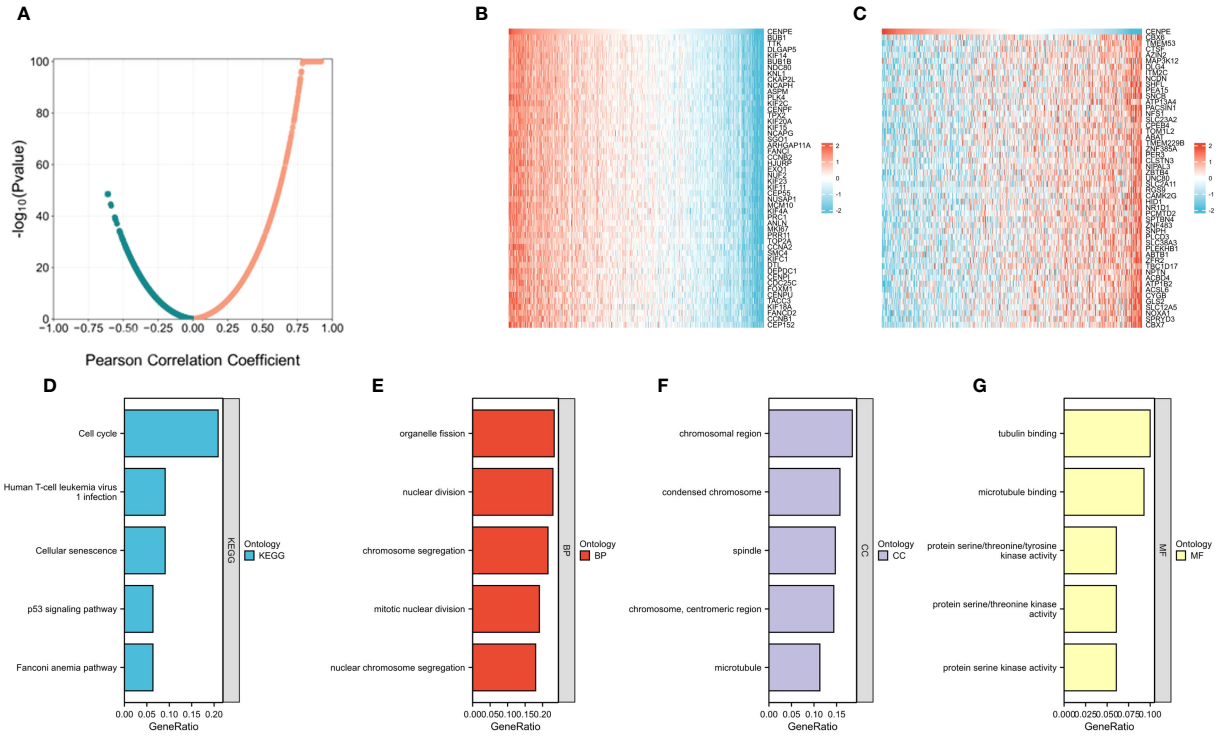


FIGURE 4
Functional Enrichment Analysis of CENPE co-expressed genes in non-WNT/non-SHH MB. **(A)** Volcano map showed CENPE co-expression genes in non-WNT/non-SHH MB. **(B, C)** Heat maps showed top 50 genes positively and negatively correlated to CENPE. **(D)** Enrichment analysis of KEGG terms for CENPE co-expression genes. **(E–G)** Enrichment analysis GO terms for CENPE co-expression genes.

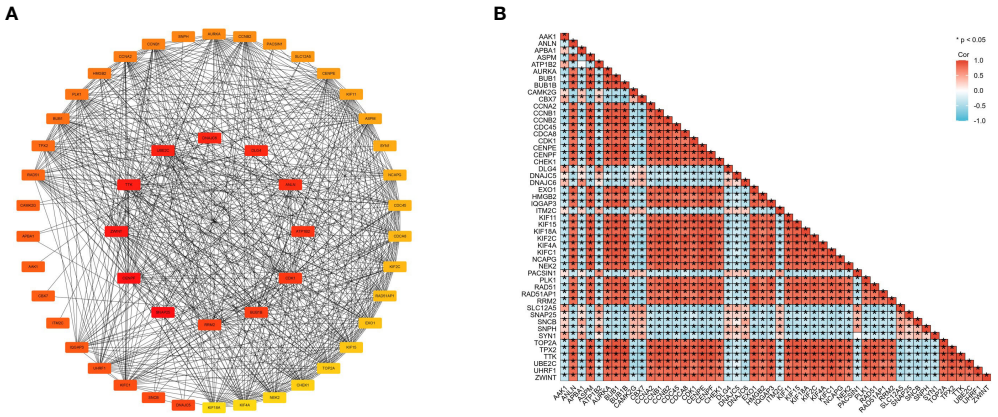


FIGURE 5
Cross-gene interactions were retrieved from the STRING database. The top 50 CENPE-related genes interaction network **(A)** and correlation matrix **(B)**.

and CENPE$^{low}$ non-WNT/non-SHH MB samples, including 4517 upregulated and 3887 downregulated genes (Figure 6A). The heat map showed the top 50 upregulated/downregulated genes (Figure 6B). Then the differentially expressed genes were used for KEGG analysis by GSEA. We ranked signal pathways based on standardized enrichment scores (Figure 7A). The eight KEGG-annotated pathways positively associated with high CENPE expression were in Cell cycle, DNA replication, Ribosome, Fanconi anemia pathway, Homologous recombination, Oocyte meiosis, Cellular senescence, p53 signaling pathway (Figures 7B–I). The above research results indicated that in non-WNT/non-SHH MB, the genes co expressed with CENPE and the DEGs between high and low CENPE groups both included cell cycle and P53 signaling pathway in their gene function enrichment results.

To further verify the relationship between CENPE and cell cycle, we analyzed the correlation between CENPE and cell cycle regulatory genes in non-WNT/non-SHH MB. The cycle regulatory genes BUB1B, ESPL1, PTTG1, PCNA, PKMYT1, CDC45, PLK1, MCM2, MCM4, MCM6, E2F1, CCNA2, CCNB1, CCNB2, CCNE1, CDC6, CDC20, CDC25A, CDC25C and CDK1 were positively correlated with CENPE (Figure 8).

## Knockdown of CENPE induced cell cycle arrest and p53 pathway activation in non-WNT/non-SHH MB

To explore the regulatory effect of CENPE on the cell cycle and p53 pathway, CENPE siRNA was used to interfere with CENPE expression. The protein expression levels of CENPE, p53, p21, and CDK1 were detected by Western blotting. The results showed that after transfection with CENPE, the expression of CENPE significantly decreased and the expression of p53 increased (Figures 9A–C). The cell cycle analysis revealed that knocking down CENPE caused accumulation of cells in G2/M phase in both non-WNT/non-SHH MB cell lines (Figures 9F–H). The expression of key factors p21 and CDK1 downstream of p53 responsible for cell cycle regulation (G2/M) had also changed.

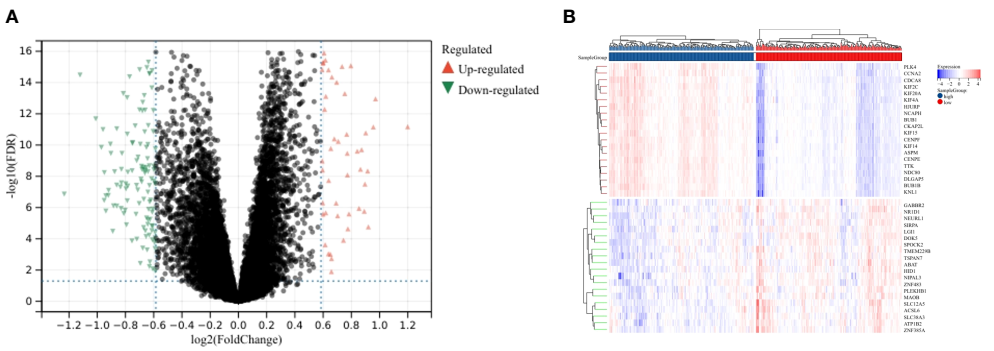


FIGURE 6
DEGs between CENPE$^{high}$ and CENPE$^{low}$ groups. **(A)** Volcano plot showing the DEGs according to the median expression of CENPE. **(B)** Heat map showing the top 20 upregulated and downregulated genes between CENPE$^{high}$ and CENPE$^{low}$ groups.

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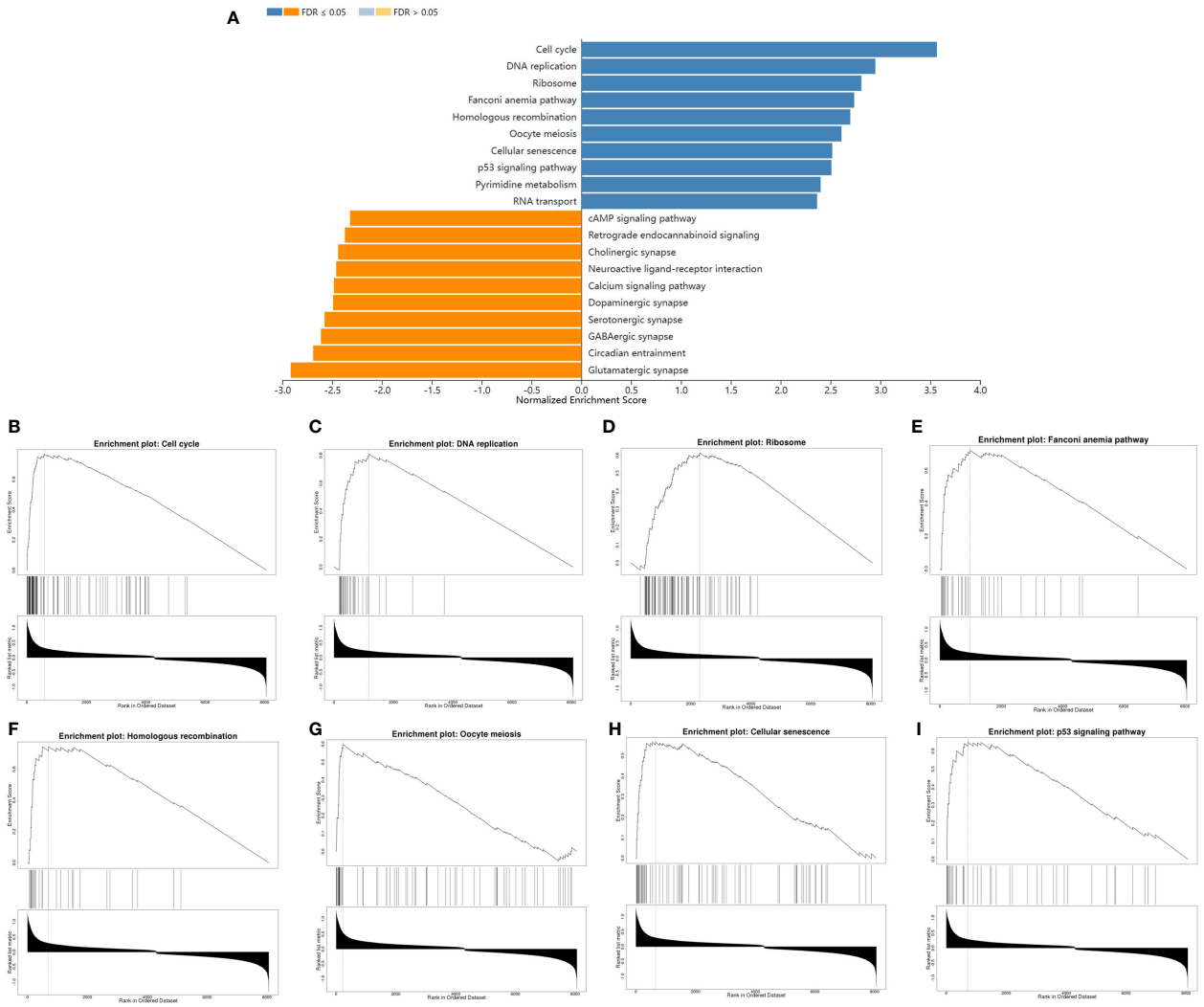


FIGURE 7
The differences in biological pathways between the CENPE$^{high}$ and CENPE$^{low}$ groups. **(A)** Bar graph showing the NES values of the top 10 KEGG pathways positively and negatively correlated with CENPE. **(B–I)** Enrichment plots showing the top 8 positively correlated KEGG pathways.

Compared with the control group, knocking down CENPE resulted in an increase in p21 expression (Figures 9A, D) and a decrease in CDK1 expression (Figures 9A, E). These data indicated that CENPE may regulate cell cycle through the p53 pathway.

## Knockdown of CENPE Inhibits the proliferation of non-WNT/non-SHH MB

CCK-8 assays were performed to investigate the effect of CENPE on cell proliferation. The results showed that silencing CENPE significantly inhibited the proliferation of both cell lines at 24 h, 48 h, and 72 h after seeding (Figures 10A–C). In addition, immunofluorescence evaluation found that the average fluorescence intensity of cell proliferation protein Ki67 in the CENPE knockdown group was lower than that in the control group (Figures 10D, E). Based on the analysis of cell cycle, the inhibitory effect of knocking down CENPE on the proliferation of non-WNT/non-SHH MB cell lines may be partially mediated by cell cycle arrest.

# Discussion

Each subtype of MB has different molecular characteristics and targeted treatment strategies. A common strategy for developing new anticancer therapies is to directly target molecules that drive mutation changes. For example, various SHH pathway inhibitors, including Vimodji and Eremodeji, have been developed as a treatment for SHH subtype MB (17). Unfortunately, only some patients are sensitive to these drugs, and resistance may also occur in these patients (18, 19). In response to the high heterogeneity of

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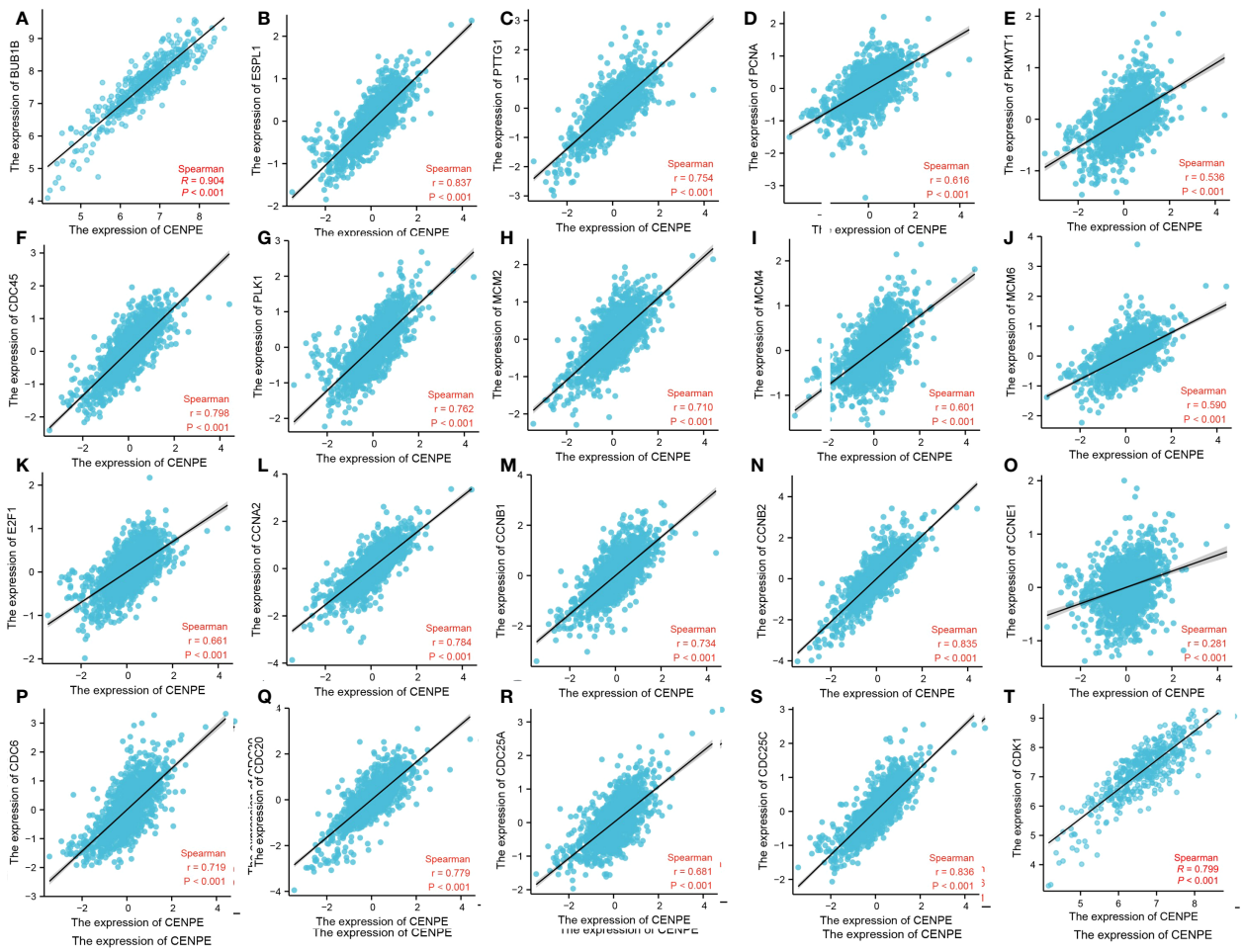


FIGURE 8
CENPE is closely related to cell cycle regulatory genes in non-WNT/non-SHH MB. **(A)** BUB1B, **(B)** ESPL1, **(C)** PTTG1, **(D)** PCNA, **(E)** PKMYT1, **(F)** CDC45, **(G)** PLK1, **(H)** MCM2, **(I)** MCM4, **(J)** MCM6, **(K)** E2F1, **(L)** CCNA2, **(M)** CCNB1, **(N)** CCNB2, **(O)** CCNE1, **(P)** CDC6, **(Q)** CDC20, **(R)** CDC25A, **(S)** CDC25C, **(T)** CDK1.

non-WNT/non-SHH MB, another targeting strategy is represented by the class non-oncogene proteins that are essential for tumor growth and progression, despite not having mutations (20, 21). These protein molecules mediate biological changes during the process of cell carcinogenesis. In this study, we first reported that CENPE was overexpressed in MB, and CENPE had high sensitivity and specificity in distinguishing MB tissue from normal cerebellar tissue. In addition, our analysis suggests that CENPE was an independent risk factor for the prognosis of non-WNT/non-SHH MB patients. Patients with higher expression of CENPE had a shorter OS. These results indicate that CENPE is an important factor in the development of non-WNT/non-SHH MB.

As a microtubule plus-end-directed kinetochore protein, CENPE interacts with mitotic centromere-associated kinesin during mitosis to regulate chromosome-microtubule end-on attachment (6, 22). Microtubule is an important component of cytoskeleton, which is essential for cell division, vesicle transport and other cellular functions. CENPE heterozygous mutations can cause MCPH13 syndrome, characterized by reduced head circumference, sloping forehead and large ears (13, 23). In cancer treatments, various microtubule targeting agents (MTA), including paclitaxel and vincristine, have been developed for the treatment of cancer. However, due to the non-specific targeting of microtubules in cancer and normal cells by these drugs, the use of MTA in tumor treatment often leads to various adverse reactions such as neuropathy, bone marrow suppression, nausea, and febrile neutropenia-like septic death (21, 24, 25). Targeting protein molecules that act on microtubule dynamics but are only essential for cancer cells may be a specific target for MB therapy (26). They are necessary for the proliferation of neural progenitor cells and brain tumor cells. Inhibiting its function can mimic the effect of MTA without affecting the post mitotic cells of the central nervous system (27). This study analyzed the molecular mechanisms of tumor progression and poor prognosis mediated by CENPE in non-WNT/non-SHH MB. We conducted GO and KEGG functional enrichment analysis of CENPE co-expressed genes and found that

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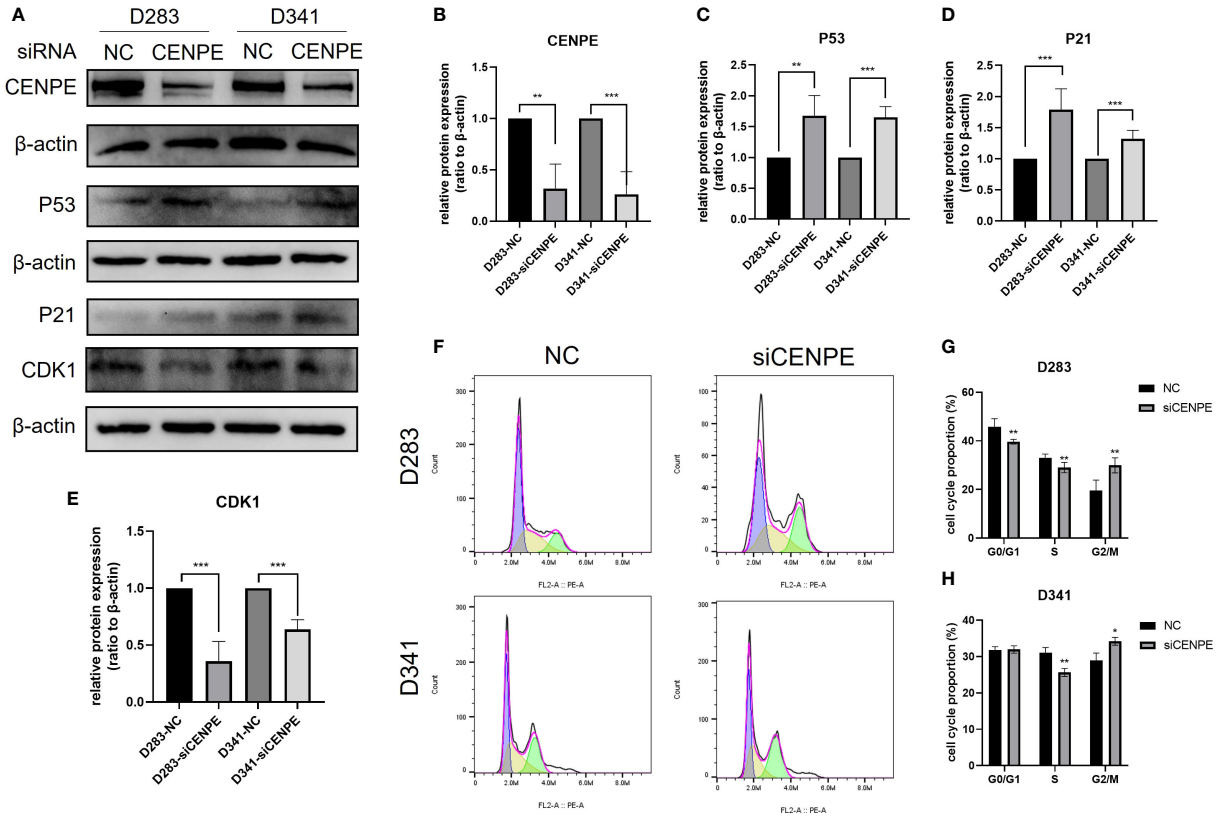


FIGURE 9
CENPE regulates the cell cycle and p53 pathway of non-WNT/non-SHH MB. **(A–E)** Western blot showed that the protein level of CENPE, P53, P21 and CDK1 by the knockdown of CENPE (mean ± SD, n = 3). **(F–H)** Flow cytometry was used to detect the cell cycle (mean ± SD, n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

the cell cycle gene set enrichment was most significant. This conclusion was also validated by differential genes GSEA analysis. Further analysis showed that CENPE was positively correlated with the expression of cell cycle regulator gene such as CCNB1 and CDK1. CDK1 plays an important role in the G2/M phase transition of cells (28). Our cell experiments showed that knockdown of CENPE can inhibit the expression of CDK1 and induce G2/M phase arrest in non-WNT/non-SHH MB cells.

In addition, pathway enrichment analysis also indicated a close correlation between CENPE and the p53 signaling pathway. As an important effector molecule downstream of the p53 pathway, p21 protein participates in the regulation of cell cycle (29). P21 inhibits cell G2/M phase transition by binding to the CCNB1/CDK1 complex to block the activation of cell division cycle factor 25 (CDC25) and cyclin dependent kinase activated kinases (CAK) (30). Our research results indicated that downregulation of CENPE can activate the p53 pathway and increase the expression levels of p53 and p21. The reason for P53 activation may be related to oblique cell division after loss of CENPE expression (31, 32). Therefore, we suggest that CENPE inhibition may exert a regulatory effect on cell cycle through the p53 pathway. The results of CCK-8 and Ki67 immunofluorescence staining indicated that CENPE affected the malignant proliferation of non-WNT/non-SHH MB cells by regulating the p53 pathway and cell cycle.

Tumor microenvironment (TME) plays an important role in the occurrence, development and metastasis of tumors (33). The regulatory effect of immune infiltrating cells in the tumor microenvironment on tumor cells can be a double-edged sword, which can inhibit tumor progress, and can also create an immunosuppressive microenvironment (34, 35). This study explored the correlation between CENPE expression and immune infiltration levels in non-WNT/non-SHH MB. The results showed that CENPE was positively correlated with the infiltration levels of aDC, B cells, CD4+ Tem, CD8+ Tcm, DC, conventional DC, immature DC, Macrophages, Macrophages M1, Mast cells, NK cells, pro B-cells, Tgd cells, Th1 cells and Th2 cells; while negatively correlated with the infiltration levels of CD4+ Tcm, Class-switched memory B cells, Eosinophils, NKT and Tregs. These findings suggest that CENPE may play an important role in the regulation of tumor immune microenvironment and serve as a potential immunotherapy related biomarker in non-WNT/non-SHH MB.

To summarize, our study found that the expression of CENPE was significantly upregulated and closely related to poor prognosis in patients with non-WNT/non-SHH MB. CENPE has reasonable accuracy in predicting the diagnosis and prognosis of non-WNT/non-SHH MB. CENPE may affect the progression of tumor by regulating cell cycle, p53 pathway, and immune infiltration. It can serve as a novel biomarker and potential therapeutic target for

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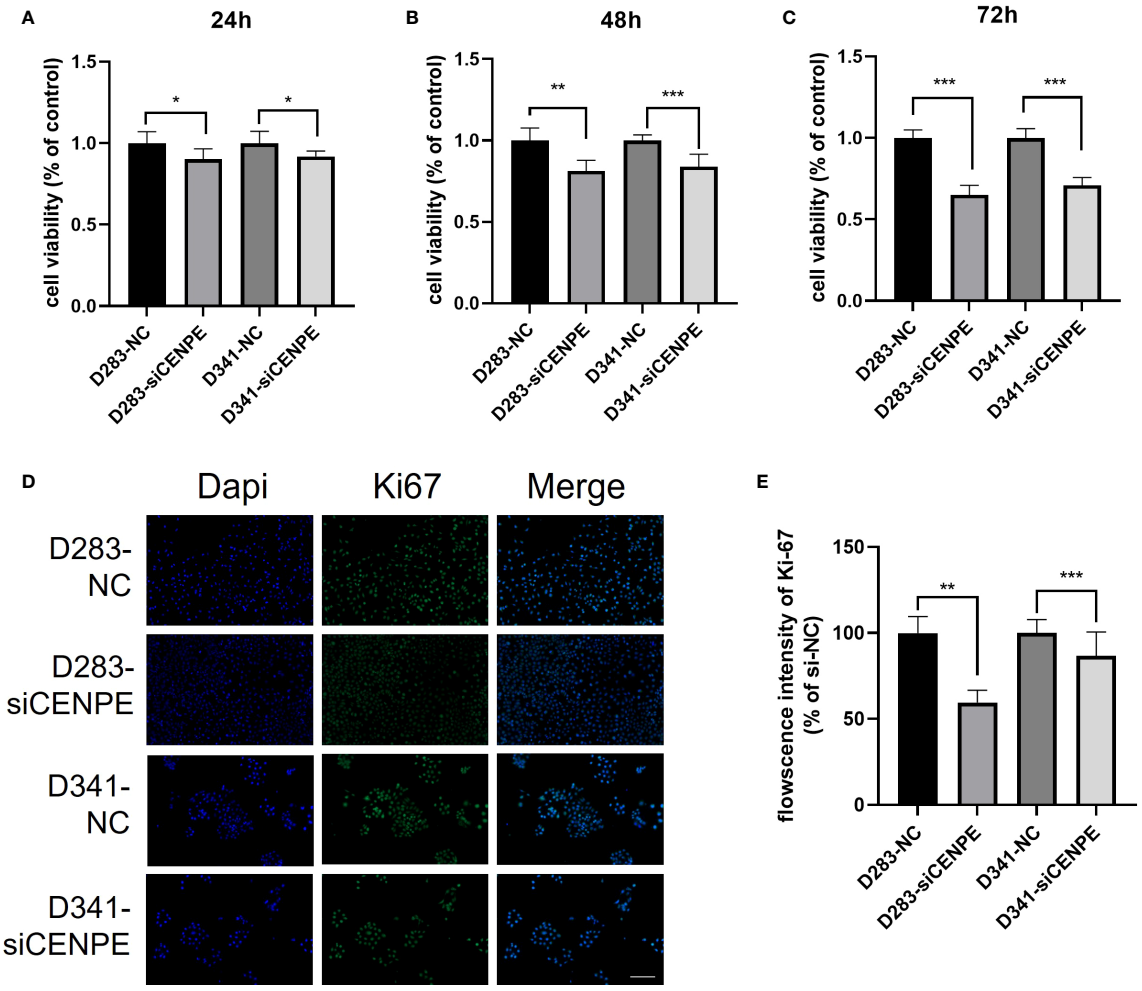


FIGURE 10
CENPE regulates the proliferation of non-WNT/non-SHH cells. **(A–C)** CCK-8 was used to evaluate the proliferation ability of D283 and D341 cells (mean ± SD, n = 3). **(D, E)** Immunofluorescence staining showed the Ki67 protein expression in D283 and D341 cells (scale bar, 100 μm), (mean ± SD, n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

patients with non-WNT/non-SHH MB. However, this study still has some limitations. We lack direct evidence that CENPE affects prognosis through its involvement in immune infiltration, and the mechanism by which CENPE regulates immune infiltration needs to be elucidated through animal experiments.

## Data availability statement

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

## Author contributions

HF, JL, and HS conceived the study. HF, YZ, CL, JL, and HS designed the study. HF, YZ, CL, ZS, and WW performed experiments. HF, YZ, and CL analyzed the data. HF and YZ drafted the manuscript. HF, CL, and JL 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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# Mechanisms and applications of radiation-induced oxidative stress in regulating cancer immunotherapy

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Radiotherapy (RT) is an effective treatment option for cancer patients, which induces the production of reactive oxygen species (ROS) and causes oxidative stress (OS), leading to the death of tumor cells. OS not only causes apoptosis, autophagy and ferroptosis, but also affects tumor immune response. The combination of RT and immunotherapy has revolutionized the management of various cancers. In this process, OS caused by ROS plays a critical role. Specifically, RT-induced ROS can promote the release of tumor-associated antigens (TAAs), regulate the infiltration and differentiation of immune cells, manipulate the expression of immune checkpoints, and change the tumor immune microenvironment (TME). In this review, we briefly summarize several ways in which IR induces tumor cell death and discuss the interrelationship between RT-induced OS and antitumor immunity, with a focus on the interaction of ferroptosis with immunogenic death. We also summarize the potential mechanisms by which ROS regulates immune checkpoint expression, immune cells activity, and differentiation. In addition, we conclude the therapeutic opportunity improving radiotherapy in combination with immunotherapy by regulating OS, which may be beneficial for clinical treatment.

KEYWORDS

**radiotherapy, reactive oxygen species, oxidative stress, tumor immune microenvironment, immunotherapy**

## 1 Introduction

Immunotherapy, a treatment that uses the immune system to eliminate cancer cells, has been touted as a potential cure for cancer in recent years (1). Immunotherapy eliminates cancer cells in the body by enhancing the recognition of tumor-associated antigens (TAAs) and specific killing effect of immune system (2). However, due to the

heterogeneity of the tumor microenvironment (TME) and the immunosuppressive response of tumor cells, most patients do not benefit from the immunotherapy regimen.

Radiotherapy (RT) is a critical nonsurgical treatment for cancer. RT usually induces cell death by increasing the level of reactive oxygen species (ROS) in tumor cells (3). RT causes the production of a variety of ROS in tumor cells, which is one of the main ways of radiation-induced DNA damage and cell death (4). RT can directly induce DNA base damage. RT can also trigger ionization of water molecules, resulting in the production of large amounts of free radicals and ROS that damage DNA, lipids and proteins, leading to metabolic and functional changes and ultimately apoptosis (5). In addition, RT can favorably modulate immunological response, leading to increased tumor antigen presentation, priming of tumor-specific cytotoxic T cells, as well as enhanced T-cell homing, engraftment, and function in tumors (6).

Radiotherapy can enhance the effect of immunotherapy through a variety of mechanisms (Figure 1). Firstly, irradiation (IR) can increase the recognition of immune cells. IR damages DNA and proteins directly or indirectly through free radical production, which leads to an increase in neoantigens released by tumor cells for immune recognition. Tumor cells also release damage-associated molecular patterns (DAMPs) after IR, including high-mobility group box 1 (HMGB1), heat shock proteins (HSPs), and calreticulin (CRT), which mediate phagocytosis of antigen-presenting cells (APCs) and initiate tumor-specific T cell activation. In addition, T cell activation is mediated by the recognition and binding of major histocompatibility complex (MHC) molecules to which T cell receptor (TCR) bind peptides. IR can increase the expression of MHC-I in tumor cells, making it easier for cytotoxic T cells to recognize. Secondly, IR activates innate immune response and immune checkpoint upregulation. activation of the stimulator of interferon genes (STING) is an important part of innate immune response. The damaged DNA fragments after IR are released into the cytoplasm and recognized by cyclic GMP-AMP synthase (cGAS) to synthesize cyclic GMP-AMP (cGAMP), which induces the production of type I interferon (IFN) through the stimulation of STING-TBK1-IRF3 signal axis. Type I IFN regulates dendritic cells (DCs) function and helper T cell differentiation, mediating innate immune response. However, STING may also induce IR resistance and immunosuppression. On the one hand, STING can induce up-regulation of programmed cell death-ligand 1 (PD-L1) to promote immune escape. On the other hand, STING can promote IR and immune resistance of tumors through myeloid-derived suppressor cells (MDSCs) mobilization and indoleamine 2,3-dioxygenase (IDO) activation. IR-induced DNA damage can also directly up-regulate the expression of PD-L1 on tumor cells through ATM/ATR/Chk1 kinase, or indirectly regulate the expression of PD-L1 by increasing the secretion of IFN-γ. Thirdly, radiation-regulated

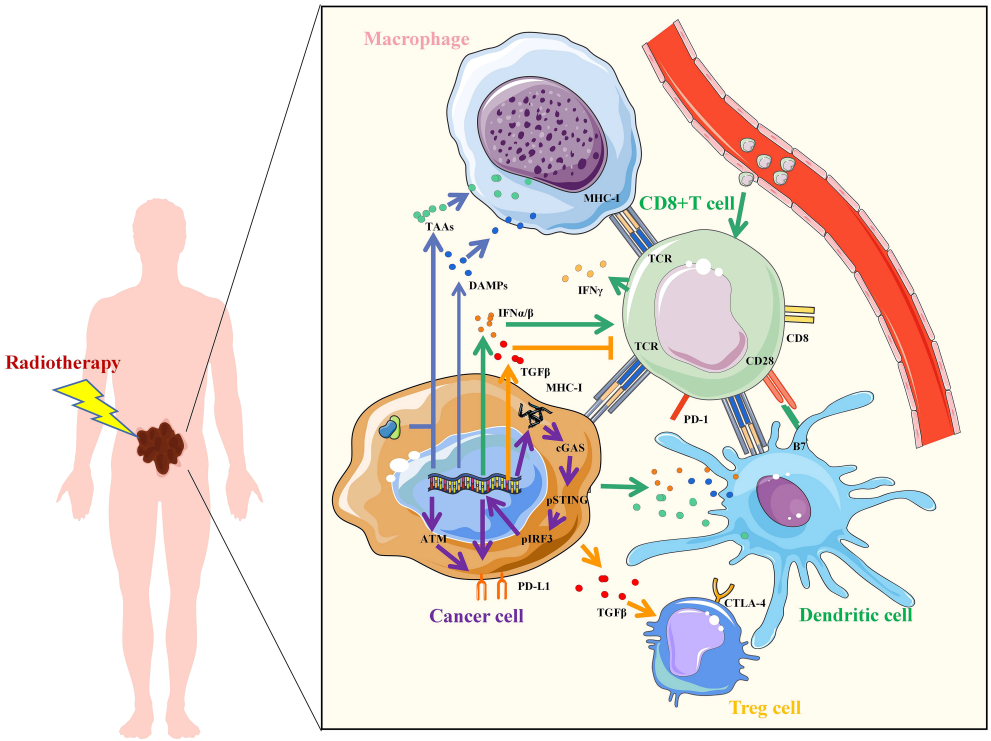


FIGURE 1
Mechanisms of radiotherapy to enhance immunotherapy. Irradiation increased the upregulation of tumor antigens, TAAs, tumor-associated antigens; DAMPs, damage-associated molecular patterns; IFN, interferon; TGF, transforming growth factor; MHC, major histocompatibility complex; TCR, T cell receptor; CD, Cluster of Differentiation; PD-L1, programmed cell death-ligand 1; PD-1, Programmed death 1; B7, CD80 and CD86; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; IRF3, interferon regulatory factor 3; ATM, ataxia telangiectasia mutated protein.

immune microenvironments. IR can increase $CD8^+$T cell infiltration and IFN-γ, promote the normalization of tumor vasculature and induce the polarization of M2-like macrophages towards M1 phenotype. However, IR can also promote the secretion of transforming growth factor β (TGF-β), inhibit $CD8^+$T cells and increase the proportion of regulatory T cells (Tregs), leading to immunosuppression in tumors. To enhance the antitumor effect, the combination of RT and immunotherapy has become a new strategy for cancer care.

Recent evidence suggests that ROS-induced oxidative stress (OS) can regulate multiple tumor immune responses. ROS plays a mediator role of pivotal functions such as phagocytosis, antigen presentation and recognition, cytolysis and phenotypical differentiation in immune cells (7). Moderate level of ROS contribute to activation and differentiation of T cells, whereas high level of ROS impair T cell survival and function (8). Tumor-associated macrophage (TAM), MDSCs and Tregs are the main immunosuppressive cells in TME (9). ROS is an important regulator of their immunosuppressive function. Increased ROS level in TME may promote the differentiation of TAM into M2 subtypes and regulate PD-L1 expression (10). Tregs are less susceptible to cell death induced by OS compared to other $CD4^+$T cells and ROS may promote the expression of forkhead box protein 3 (FOXP3) in Tregs and maintain the immunosuppressive function (11, 12). ROS is essential for MDSCs in their undifferentiated state. The release of ROS molecules mediates the suppression of T cells and the activation of Tregs (9, 13). Production of several immunosuppressive cytokines is also regulated by ROS, such as IL-6, IL-10 and TGF-β, which can block the function of immune cells (14, 15). In addition, increased ROS can mediate the expression of immune checkpoints such as PD-L1 to promote tumor immune escape (16). Given the regulatory role of ROS in antitumor immunity, it is necessary to summarize the role of IR-induced OS in cancer immunotherapy.

In this review, we summarize several mechanisms by which RT induces ROS production and OS in tumors. The molecular mechanism underlying the interaction between OS and anti-tumor immunity is further discussed, with emphasis on the regulatory role of ROS on TME. On this basis, we attempted to find a treatment method for regulating OS during RT to enhance the efficacy of radiotherapy combined with immunotherapy.

# 2 IR-induced OS promotes tumor cell death

Increased ROS is a critical way to kill tumor cells exposed to RT. After exposure, water molecules are broken down to produce large amounts of ROS, including the superoxide anion, Hydroxyl agent and hydrogen peroxide, respectively (17). RT-induced ROS promotes the death of tumor cells mainly by damaging macromolecular substances such as DNA, lipids, and proteins in cells. When RT-induced ROS level exceed their own antioxidant level, tumor cells death will occur in a variety of ways, including apoptosis, autophagy, and ferroptosis and others. In addition, injured and dead tumor cells activate the anti-tumor immune mechanism and induce immunogenic death of tumor cells (Figure 2).

## 2.1 Apoptosis and autophagy

DNA is one of the main targets of ROS, which can induce DNA damage and induce DNA damage response (18). When the level of ROS-induced DNA damage exceeds the cell's ability to repair it, the cell initiates the apoptosis program. P53 is an important molecule that regulates apoptosis. Increased ROS can lead to p53 activation and its downstream upregulation of p53 upregulated modulator of apoptosis, the pro-oxidant genes (19). Furthermore, ROS-induced p53 has been demonstrated to downregulate antioxidant proteins and the anti-oxidant transcriptional factor, including superoxide dismutase 2 and nuclear factor–erythroid 2 related factor 2 (Nrf2) (20, 21). Caspases are a family of proteases in cells, activated by death receptor-dependent pathway and mitochondrial-dependent pathway. ROS can cause the activation of caspase 8 by regulating the expression of the death receptors and its ligand such as Fas and Fas L. Another way of apoptosis is mediated by mitochondria. ROS can not only damage mitochondrial DNA, but also damage mitochondrial electron transport chain, causing mitochondrial dysfunction. Mitochondrial dysfunction results in the release of proapoptotic protein cytochrome c and activates caspases to induce apoptosis. B-cell lymphoma-2 (Bcl-2) is a key anti-apoptotic protein molecule in apoptosis cells. ROS can down-regulate the expression of Bcl-2 and promote apoptosis through oxidative modification.

Autophagy is another important form of RT-induced tumor cell death. ROS accumulation induced by IR can regulate autophagy through a variety of pathways. Autophagy protein (ATG)4 is the core of autophagy regulation, and ROS can directly oxidize ATG4, leading to the accumulation of autophagosomes. ROS can also activate adenosine 5'-monophosphate-activated protein kinase signaling and induce autophagy initiation through ATG1 complex. Similarly, radiation-induced ROS can also activate mitogen-activated protein kinase (MAPK) signaling pathway, which mediates the phosphorylation of mechanistic target of rapamycin (mTOR) and the transport of ATG9, thus promoting the initiation of autophagy. Mitophagy is mainly mediated by BCL2/adenovirus E1B 19kDa interacting protein 3/Nip3-like protein X, and Parkin/PINK1 (PTEN induced putative kinase 1) (22). Radiation induced the accumulation of mitochondrial ROS, which resulted in mitochondrial damage that was in turn recognized by Parkin (23).

## 2.2 Immunogenic cell death

Immunogenic cell death (ICD), known as immunogenic apoptotic cell death or immunogenic apoptosis, has been defined as a "form of regulated cell death that is sufficient to activate an adaptive immune response in immunocompetent hosts" (24). Cellular redistribution and extracellular release of DAMPs are the main features and mechanisms of ICD, including CRT, HSPs,

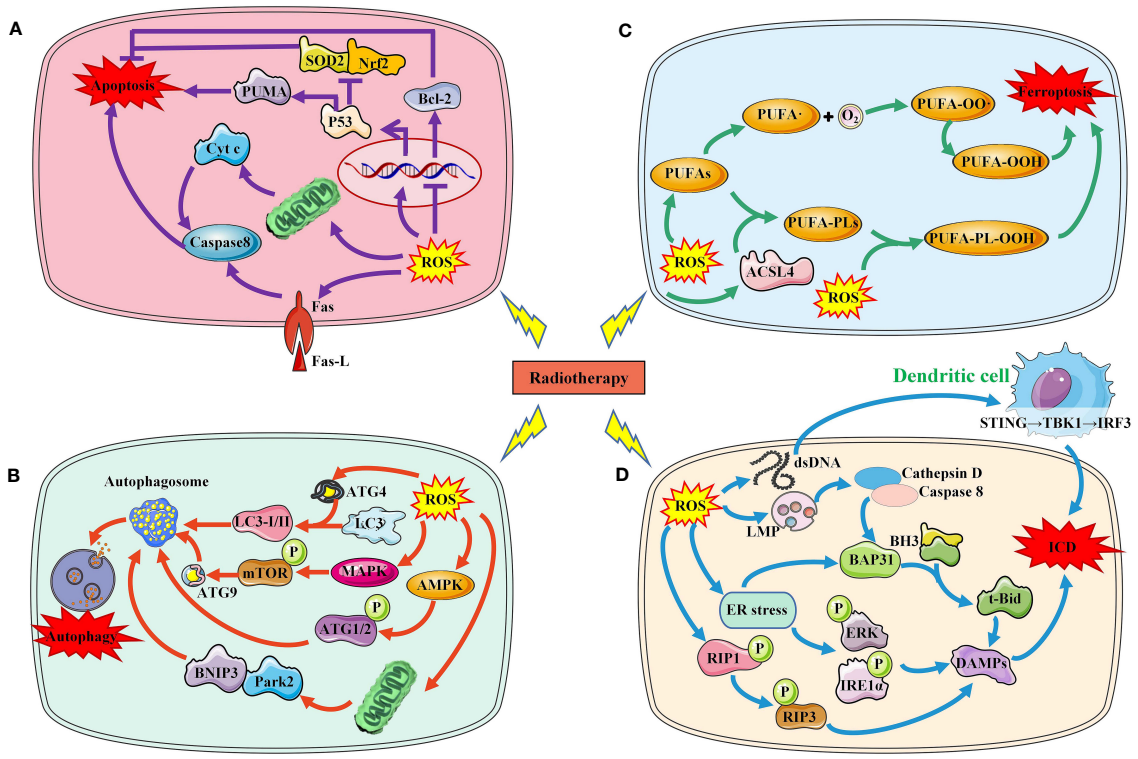


FIGURE 2
Several major ways in which RT-induced ROS induce tumor cell death. **(A)**. RT-induced ROS induces tumor cell apoptosis through P53 pathway, mitochondrial pathway and death receptor ligand pathway. **(B)** RT-induced ROS induces autophagy of tumor cells by activating AMPK, MAPK and LC3. **(C)** RT-induced ROS increased lipid peroxidation induced tumor cell ferroptosis. **(D)** RT-induced ROS increased ICD by increasing DAMPs production and immune cell activation.SOD2, superoxide dismutase 2; Nrf2, nuclear factor-erythroid 2 related factor 2; PUMA, p53 upregulated modulator of apoptosis; Bcl-2, B-cell lymphoma-2; Cty c, Cytochrome C; ROS, reactive oxygen species; ACSL4, acyl-CoA synthetase long-chain family member 4; PUFAs, polyunsaturated fatty acids; LMP, Lysosome membrane permeability; BH3,; BAP31, BCR associated protein Bap 31; ER, endoplasmic reticulum; RIP, receptor interacting protein; ERK, extracellular regulated protein kinases; IRE1, Inositol-requiring enzyme 1; DAMPs, damage-associated molecular patterns; ICD, immunogenic cell death; ATG, autophagy protein; MAPK, mitogen-activated protein kinase; AMPK, adenosine 5`-monophosphate-activated protein kinase; mTOR, mechanistic target of rapamycin; BNIP3, BCL2/adenovirus E1B 19kDa interacting protein 3; Park2, Recombinant Parkinson Disease Protein 2.

HMGB1, adenosine 5-triphosphate (ATP), spliceosome-associated protein 130, defensins and S100 proteins.

IR-induced OS can induce immunogenic death of tumor cells through multiple mechanisms (25). (1) Endoplasmic reticulum (ER) stress, caused by overproduction of ROS, has been shown to be responsible for ICD (26–30). CRT is overexpressed and released during ER stress. IR-mediated ER stress produces DAMPs molecules via PERK and IRE1-α phosphorylation, especially CRT, acting as an 'eat me' signal to stimulate the antigen presenting function of dendritic cells, acting as an 'eat me' signal to stimulate the antigen presenting function of DCs (31). Currently, various therapies, including photodynamic therapy, hyperthermia, and nanomaterials have been explored as ways to enhance ER stress to promote ICD (26, 32, 33). (2) Mitochondria is another important pathway of ROS production induced by IR. Increased ROS level can be directly detected by receptor-interacting protein 1 and leading to autophosphorylation on serine residue 161 (34). And then recruits RIP3 and induces the formation of a functional necrosome with pore formation and the release of DAMPs (35, 36). After IR, mitochondrial DNA is oxidized in tumor cells, which will be phagocytosed by DCs and activate the STING-TBK1-IRF3-IFN-β pathway in DCs, which subsequently cross-presented irradiated tumor cell-derived antigens to $CD8^+$T cells and elicited antitumor immunity (37). (3) Lysosome membrane permeability (LMP): After the radiation-induced ROS were perceived by the cells, LMP changed, which would trigger further downstream approaches to induce ICD (38). After the lysosomal membrane is destroyed, cathepsin D and the prozymogenic form of caspase 8 are released into the cytoplasm (39). Caspase 8 induces ER-associated BAP31 cleavage through downstream caspase activation and cleaves the BH3-only protein Bid into its truncated form, t-Bid, which promotes CRT expression on the cell surface by inducing mitochondrial outer membrane permeation and anterograde ER-Golgi traffic (39–41).

## 2.3 Ferroptosis

Ferroptosis is another way of radiation-induced tumor cell death, which is caused by lipid peroxidation caused by iron metabolism disorder and ROS accumulation. Lipid peroxidation is an important marker of ferroptosis. IR-induced ROS can remove

electrons from polyunsaturated fatty acids (PUFAs) to form fatty acid free radicals (PUFA•), which rapidly react with molecular oxygen to form lipid peroxyls (PUFA-OO•) and form lipid hydroperoxides (PUFA-OOH) through Fenton reaction, which lead to lipid peroxidation of membrane phospholipids, eventually resulting in ferroptosis (42). In addition, IR-induced acyl-CoA synthetase long-chain family member 4 (ACSL4) expression increased PUFA-PL biosynthesis, which together with ROS drove PUFA-PL peroxidation (PUFA-PL-OOH) and ferroptosis (43, 44).

Ferroptosis is an important way of tumor cell death induced by RT, and lipid peroxidation induced by OS is the basis of ferroptosis. RT not only induced ROS production, but also induced the expression of ACSL4, a lipid metabolizing enzyme required for ferroptosis, leading to elevated lipid peroxidation and ferroptosis (44). IR-induced ROS attack PUFAs to produce lipid peroxides (L-OOH), resulting in the death of iron. The peroxidation process is mediated by lipoxyphenase and phosphatidyl ethanolamine binding protein 1. L-OOH can be reduced to the corresponding alcohol by glutathione peroxidase or by panthenol by Fenton reaction L-OO (45–47). Recombinant solute carrier family 7, member 11 (SLC7A11 or xCT) can promote glutathione (GSH) synthesis and reduce L-OOH production to reduce the occurrence of ferroptosis (48). Radiation activates the ataxic-telangiectasia mutant (ATM), which inhibits SLC7A11 expression and promotes ferroptosis (49). It has been suggested that tumor ferroptosis is a new intersection between RT and adaptive immune response. IFN-γ released by immunotherapy-activated $CD8^+$T cells down-regulates the expression of SLC3A2 and SLC7A11, inhibits cystine uptake in cancer cells, and enhances ferroptosis specific lipid peroxidation in tumor cells (50). Nanoparticle loaded miR-21-3p increases ROS production by directly targeting thioredoxin reductase 1, thereby enhancing lipid peroxidation to promote IFN-γ-mediated ferroptosis and acting synergistically with anti-PD-1 antibodies (51). Radiation-induced ATM activation is also an important source of IFN-γ, which synergies with IFN-γ derived from $CD8^+$T cells to inhibit SLC7A11 and increase ferroptosis in tumor cells (52). In an oxygen-deficient environment, neutrophils are able to transfer granules containing myeloperoxidase into tumor cells, thereby inducing the accumulation of lipid peroxides and iron in tumor cells and triggering ferroptosis (53). On the other hand, immune cells in the TME also undergo ferroptosis. CD36 mediates the uptake of fatty acids by tumor-infiltrating $CD8^+$T cells, induces lipid peroxidation and ferroptosis, and leads to reduced cytotoxic cytokine production and reduced anti-tumor ability (54). Since Tregs are more resistant to ROS-induced ferroptosis, therapeutic strategies that can specifically induce lipid peroxidation and ferroptosis accumulation in Tregs need to be developed (55). Ferroptosis is more likely to occur in M2 TAM than M1 (56). Hsieh et al. (57) found that zero-valent iron (ZVI), a nanoparticle capable of inducing ferroptosis in tumor cells, was able to convert M2 type TAM into M1 type, lifting the inhibition of anti-tumor immunity. However, in pancreatic cancer, OS was able to induce secretion of KRASG12D-containing exosomes, which were taken up by macrophages to mediate autophagy-dependent ferroptosis and switch TAM to the M2 phenotype (58). In addition, in TME, the expression of proteins associated with ferroptosis and lipid peroxidation in natural killer cells (NKs) was increased, suggesting that the dysfunction of NKs may be related to ferroptosis (59). Similarly, ferroptosis in DCs decreases antigen presentation and antitumor ability, and prevents $CD8^+$T cells from producing IFN-γ (60). RT is often used in combination with ICIs, but lacks the expected survival benefit in some tumors. In this subset of patients, combining ferroptosis inducers with immunotherapy and RT may be a good strategy for enhancing tumor ferroptosis and sensitizing such tumors to immunotherapy and RT. Drugs have also been developed to promote ferroptosis, inducing ferroptosis while also enhancing the immune response caused by immune checkpoint inhibitors (61). However, Due to the contradictory effects of ferroptosis on tumor cells and immune cells, the clinical application value of ferroptosis in immune cells needs further exploration (Figure 3).

# 3 OS regulates TME

## 3.1 OS regulates immune checkpoint expression

IR is considered as a precipitating factor of immune checkpoint expression (62, 63), and ROS plays a key role in this process (64). In macrophages, treatment with ROS inducers resulted in increased expression of PD-L1 (65). Mechanistically, ROS accumulation activated nuclear factor kappa-B (NF-κB) signaling to promote PD-L1 transcription and immunosuppressive chemokine release. In pancreatic cancer, ROS can also induce the expression of fibroblast growth factor receptor 1, which can directly induce the expression of PD-L1 or by activating protein kinase B (AKT) signaling (66). ROS induced by IR is one of the main causes of DNA double-strand breaks (DSBs). DSBs activate ATM kinase, which is able to convert signals to ataxia telangiectasia and Rad3-related (ATR), thereby upregulating checkpoint kinase 1 (Chk1) activity to promote PD-L1 expression, mediated through STAT1/3-IRF1 pathway (67). The fragment DNA produced by DSBs can also be sensed by intracellular cGAS, thereby activating STING-TBK1-IRF3 signal to increase the secretion of type I IFN, which is an important way to induce the expression of PD-L1 (68, 69). Similarly, during OS in mitochondria, oxidized mitochondria DNA is released into the cytosol and then it induces IFN signaling via cGAS-STING-TBK1, which upregulates PD-L1 and IDO-1 expression to inhibit T-cell activation (16). In addition to PD-L1, ATR-Chk1 activation also contributes to integrin-associated protein (CD47) expression, which is mediated by STAT3 (70). CD47 is another immunoglobulin highly expressed on the surface of tumor cells, which can inhibit macrophage-mediated phagocytosis by binding to its major ligand signal regulatory protein α, thereby negatively regulating anti-tumor immunity (71). However, it has also been reported that ROS can also down-regulate the expression of CD47, which is mediated by hypoxia-inducible factor 1α (HIF-1α) (72, 73).

## 3.2 OS regulates immune cells

IR-induced ROS also regulates the function and activation of immune cells (74). In TME, several major immune cells, such as T cells, Tregs, NKs, DCs, TAMs and MDSCs, are regulated by OS

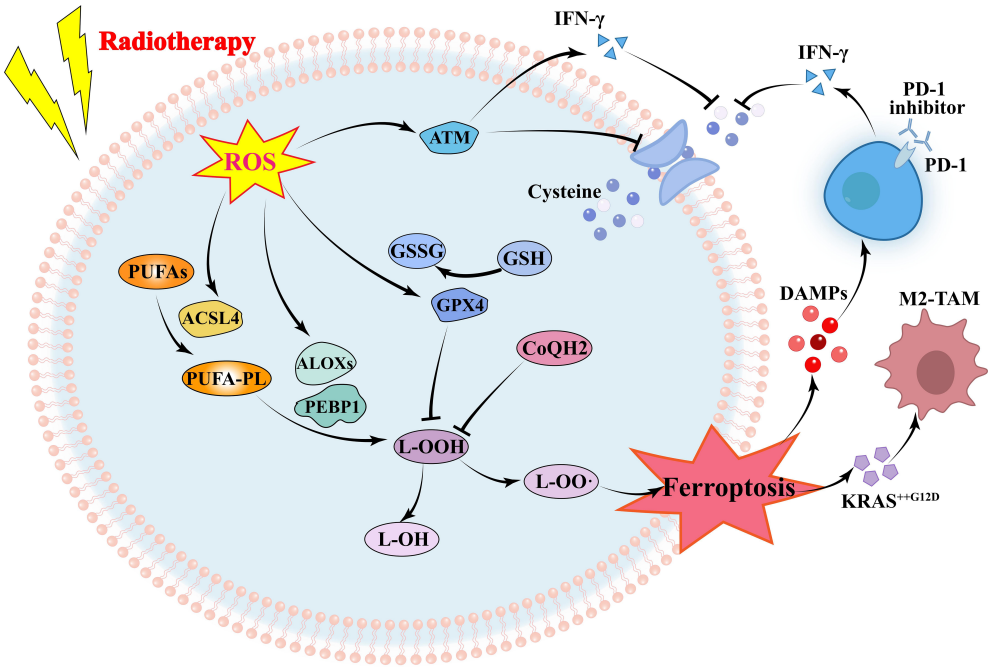


FIGURE 3
Mechanism of radiotherapy and immunotherapy in regulating ferroptosis of tumor cells. RT-induced ROS increases ferroptosis by increasing lipid peroxidation and decreasing glutathione. Immunotherapy and RT-activated T cells secreting IFN-γ inhibit glutathione synthesis by reducing glutamine transport, to promoting ferroptosis. ROS, reactive oxygen species; ATM, ataxia telangiectasia mutated protein; IFN, interferon; GSH, glutathione; GSSG, Glutathione Oxidized; GPX4, glutathione peroxidase 4; PUFAs, polyunsaturated fatty acids; ACSL4, acyl-CoA synthetase long-chain family member 4; ALOXs, Lipoxygenases; PEBP1, Phosphatidylethanolamine-binding Protein 1; DAMPs, damage-associated molecular patterns; TAM, tumor-associated macrophage.

(Figure 4). Among T cells, $CD8^+$T cells are the main specific immune killer cells that perform tumor killing. Of course, T cells also include many helper T cells. NKs are the main innate anti-tumor immune cells, which can directly kill tumor cells. DCs is the primary antigen presenting cell responsible for delivering TAAs to other immune killer cells. TAMs are divided into M1 type and M2 type, in which M1 type is an inflammatory phenotype and contributes to anti-tumor, while M2 type has the effect of promoting tumor immune escape. MDSCs and Tregs are the two main cells that inhibit anti-tumor immunity, and their increase often indicates immune escape of tumor cells.

### 3.2.1 T cells

ROS concentration is a necessary precursor for the activation and function of $CD8^+$T cells, especially during TCR signal transduction after tumor antigen stimulation (75). It is well known that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is an important inhibitory molecule in the PI3K/AKT/mTOC pathway and has immunosuppressive function. SUMO-specific protease 7 (SENP7), a reversible posttranslational modification protease, can sense OS to induce PTEN degradation, thereby maintaining metabolic fitness and effector functions of $CD8^+$T cells (76). $CD8^+$Tc9 (cytotoxic T lymphocyte subset 9) cells exert greater persistence and antitumor efficacy than Tc1 cells. Tc9 cell-derived IL-9 activated STAT3, upregulated fatty acid oxidation and mitochondrial activity, and rendered Tc9 cells with reduced lipid peroxidation and resistance to ROS-induced ferroptosis in the TME (77). The increase of ROS significantly down-regulates the expression of miR-155-5p in tumor cells. Targeting miR-155-5p may be an effective method to improve TME by down-regulating PD-L1 and increasing infiltration of $CD8^+$T cells (78). Appropriate level of ROS is a key signaling molecule involved in directing T cell activation and differentiation. However, with too high level, it inhibits proliferation, damages DNA, and induces apoptosis (79). It has been suggested that elevated ROS is a driver of T cell depletion. ROS elevation drives elevated nuclear factor of activated T cells localization and sustained signaling, inducing depleted T cell phenotypes with elevated PD-1, T cell immunoglobulin domain and mucin domain-3, and T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif domains (80). In addition, ROS induced decreased IFN-γ secretion in T cells and increased IL-10 production through the STAT3-STAT5 axis (81). ROS can also cause changes in Th1/Th2 polarity, which is conducive to the production of more Th2 cells, which is conducive to the tumor (82). NF-κB is believed to be the key factor in inhibiting T cell function and inducing T cell death induced by OS (79). Chronic or persistent low-dose OS down-regulates NF-κB in naive and mature activated/memory T cells, and the synergistic effect of Ros-mediated NF-κB suppression and elevated TNF-α level leads to T cell death (83).

Tregs are recruited into the TME to mediate immunosuppression and are susceptible to OS in the TME (84). Cellular OS state induces

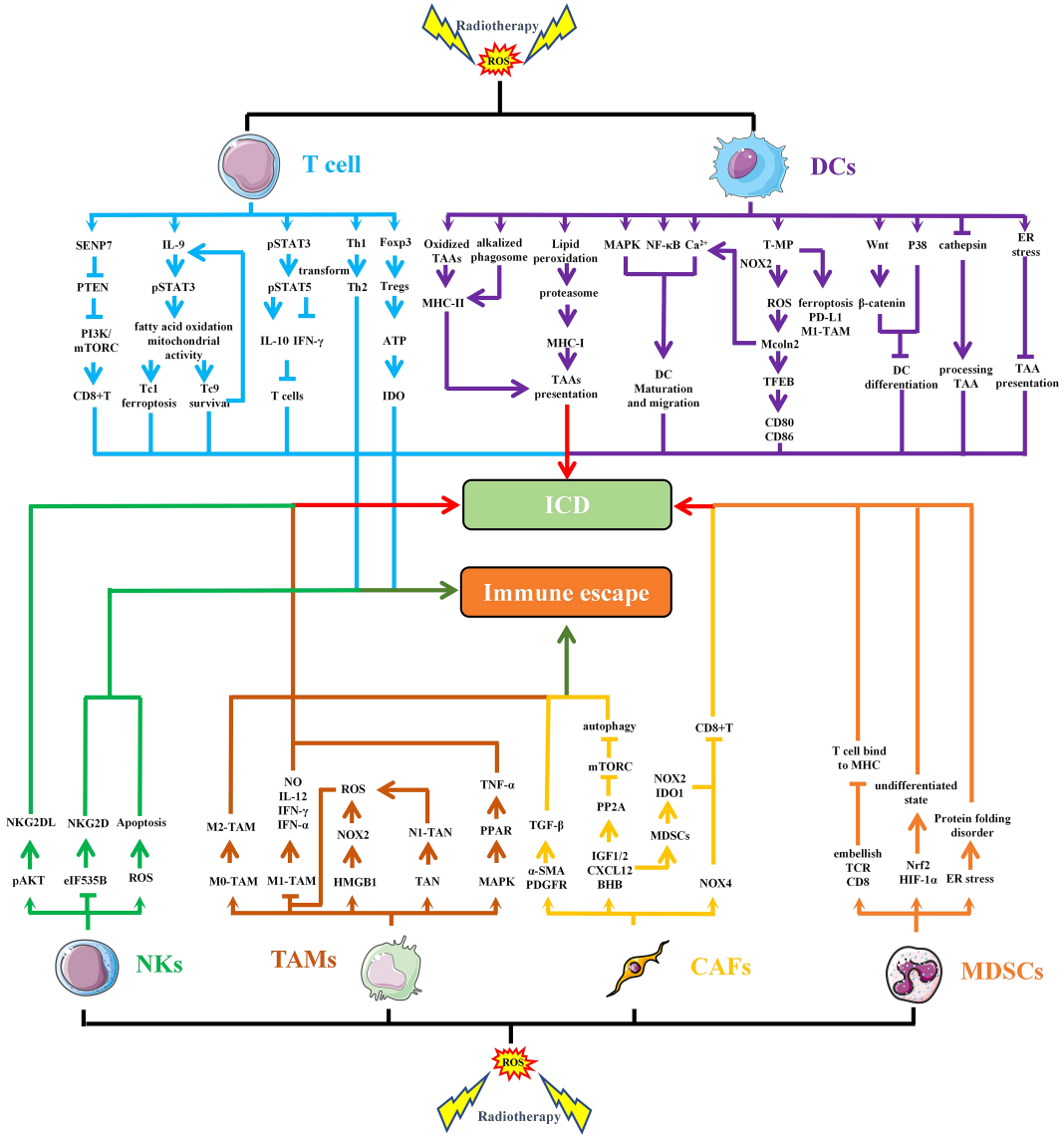


FIGURE 4
RT-induced ROS regulates the function and activation of immune cells. IR-induced ROS regulates the function of different cells in TME through multiple pathways, leading to immunogenic death or immune escape of tumor cells. SENP7, SUMO-specific protease 7; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PI3K, Phosphoinositide-3 kinase; IL, interleukin; STAT, signal transducer and activator of transcription; IFN, interferon; CD, cluster of differentiation; Foxp3, forkhead box protein 3; ATP, adenosine 5-triphosphate; IDO, indoleamine 2,3-dioxygenase; MHC, major histocompatibility complex; TAAs, tumor-associated antigens; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-B; T-MP, tumor cell-derived microparticles; NOXs, NADPH oxidases; EB, transcription factor EB; ICD, immunogenic cell death; NO, nitrous oxide; TANs, Tumor-associated neutrophils; TNF, tumor necrosis factor; PPAR, peroxisome proliferator activated receptor; TGF, transforming growth factor; α-SMA, α-smooth muscle actin; PDGFR, platelet—derived growth factor receptor; PP2A, protein phospholipase; IGF, insulin like growth factor; CXCL, chemokine (C-X-C motif) ligand; BHB, β-hydroxybutyrate.

and maintains the epigenetic modification, transcription, translation, and post-translational stability of FoxP3, the master regulator of Tregs (85). In TME with OS, Tregs are able to compete with DCs and effector T cells for cysteine, the feedstock of GSH, resulting in inhibition of DCs function and downregulation of T cell activation and proliferation (86). In addition, the weak Nrf2-related antioxidant system in Tregs transforms them into apoptotic Tregs in oxidized TME, which can self-supply and release ATP, and convert it to adenosine to mediate, sustain, and amplify powerful suppression in the TME (84)

### 3.2.2 DCs

DCs are fundamental for the initiation and maintenance of immune responses against cancer cells and have been shown to generate ROS during antigen presentation (87, 88). Immature DCs mainly have the ability to migrate, while mature DCs can mediate antigen presentation. During the differentiation of monocytes into DCs, expression of the DNA repair proteins XRCC1, ligase IIIα, poly (ADP-ribose) polymerase-1, and catalytic subunit of DNA-dependent protein kinase become up-regulated, making DCs repair-competent and ROS-resistant (89). Oxidized antigens have

higher immunogenicity, which is reflected in enhanced MHC-II antigen processing and presentation by DCs (90). ROS can promote maturation of DCs and promote antigen presentation through phagosomal alkalinization (87). In DCs, ROS production and lipid peroxidation promote the escape of antigens from endosomes into the cytoplasm so that the proteasome can be degraded into peptides and processed into MHC I (91). In addition, transient intracellular ROS rapidly induced cytosolic mobilization of $Ca^{2+}$, differential activation of mitogen-activated protein kinases, and nuclear translocation of NF-κB, which activate immature DCs to mature and potently enhance migration (92). DNA fragments generated by IR can promote the production of cGAMP, which can enter the TME and be ingested by DCs, activating STING signal (37, 93). STING-dependent cytosolic DNA sensing in DCs initiates antitumor immune responses, OS as a metabolic regulator promotes STING-mediated DCs antitumor immune responses and highlights SENP3 as an overflow valve for STING signaling induction in the metabolically abnormal TME (94). Tumor cell-derived microparticles (T-MP) increase lysosomal pH via NADPH oxidase 2 (NOX2, previously known as $gp91^{phox}$)-catalyzed ROS production, promoting the formation of MHC class I-tumor antigen peptide complexes (95). It has been reported that radiation and chemotherapy can produce microparticles from tumor cells. Irradiation-induced T-MP can be taken up by tumor cells and TAMs, resulting in increased ferroptosis, PD-L1 expression, and M1 phenotype TAMs (96). Moreover, T-MP increased ROS activates the lysosomal $Ca^{2+}$ channel Mcoln2, leading to $Ca^{2+}$ release and activation of the transcription factor EB, a lysosomal master regulator that directly binds to CD80 and CD86 promoters and promotes gene expression, which facilitates antigen presentation (95). However, ROS has also been reported to have harmful effects on DCs. High glucose medium impaired the differentiation of monocytes to DCs by inducing ROS to activate Wnt/β-catenin and p38 MAPK (97). Overproduction of ROS also inhibits the hydrolytic function of cathepsin in DC, thus inhibiting antigen processing (98). Meanwhile, chronic activation of endoplasmic reticulum stress responses induced by excess ROS inhibits the ability of DC to present antigens to T cells (99, 100). Mitochondrial metabolism in plasmacytoid DCs (pDCs) which acquire the cross-presentation ability after stimulation by toll-like receptor (TLR) ligands is important to the induction of adaptive immune responses. The reduction of mitochondrial ROS production dramatically decreases the cross-presentation capacity of pDCs, leading to a strong reduction of their capacity to trigger $CD8^+$T cells responses (101).

### 3.2.3 NKs

NKs are lymphocytes capable of eliminating tumor cells without prior sensitization to a specific antigen (102). However, in TME, ROS is quite detrimental to NK survival and function. ROS induced in NK promotes NK cell apoptosis, while activation of antioxidant pathway increases NK resistance to OS (103, 104). Inhibition of NOx2-derived ROS in combination with cell-activating cytokines or checkpoint inhibitors can enhance NK and T cell function (105). NKs-expressed activating receptor Natural Killer Group 2D (NKG2D) acts as a "master switch" in controlling the arousal state of NK cells. NKG2D-mediated cytotoxicity is related to the level of NKG2D ligands (NKG2DLs) expressed by tumor cells. It has been reported that ROS-induced AKT phosphorylation induces the expression of NKG2DLs, which would sensitize tumor cells to NKs (106). In breast cancer, ROS reduces translation-initiation rate-limiting factor (eIF535B) phosphorylation at Ser2 and down-regulates NKG2D expression to inhibit NK cell activity (107).

### 3.2.4 TAMs and tumor-associated neutrophils

In the tumor microenvironment, TAMs mainly include M1 and M2 phenotypes, among which M1 type has highly pro-inflammatory and anti-tumor functions, while M2 type contributes to suppress immune response. ROS plays a critical role in the differentiation of TAMs (108). M1 macrophages are more sensitive to OS, so ROS can polarize M0 macrophages into a tolerant M2 phenotype (109). It has also been reported that ROS is a critical trigger of cyclooxygenase-2–mediated macrophage differentiation from monocytes (108). Inhibition of ROS could transform TAMs into M1 phenotype, promote the secretion of inducible nitric oxide synthase, IL-12, IFN-γ and TNF-α (110). HMGB1 is a key molecule in irradiation induced ICD. A study has confirmed that in hepatocellular carcinoma, HMGB1 binds to TLR2 on the surface of TAMs to produce ROS via NOX2 (111). The increase of ROS promotes an increase in autophagy, which polarizes TAMs toward the M2 phenotype. In melanoma, ROS is also capable of ectopic peroxisome proliferator activated receptor-γ from the nucleus into the cytoplasm via mitogen-activated protein kinase kinase 1, thereby inducing an increase in TNF-α, which not only plays an antitumor role, but also converts TAMs into a phenotype that promotes tumor invasion (112).

Tumor-associated neutrophils (TANs) play an important role in TME. Irradiation can activate NOXs in neutrophils and produce ROS, which can increase phagocytosis (113). TANs include both antitumor (N1) and proto-tumor (N2) phenotypes, in which N1 TAN can be induced by RT. RT-induced N1 TAN produced more ROS, leading to tumor cell apoptosis and subsequent activation of tumor-specific cytotoxic T lymphocytes (CTLs) (114).

### 3.2.5 MDSCs

There is a close relationship between the immunosuppressive function of MDSCs and ROS. ROS molecules are essential for maintenance of MDSCs in their undifferentiated state. Scavenging ROS induces differentiation of immature myeloid cells into macrophages, while differentiate into macrophages and DCs in the absence of NOX activity (115, 116). In polymorphonuclear MDSC, ROS-induced ER stress is the main factor driving MDSC function. ROS accumulation can induce oxidative modification of ER coelomins and thus affect protein folding (117, 118). Clearing ROS significantly attenuates MDSC immunosuppression by inhibiting the unfolded protein response. In addition, the release of ROS by MDSCs is one of the main mechanisms used to inhibit human T cells. ROS produced and secreted by MDSCs can modify TCR and CD8 molecules in T cells, resulting in $CD8^+$T cells losing the ability to bind MHC (119). Furthermore, beyond their role in MDSC-mediated immune-suppression, ROS molecules are

intrinsically involved in activation of transcription factors such as Nrf2 and HIF-1α, which can induce transcriptional and metabolic reprogramming of MDSCs and influence their differentiation and maintenance (9).

### 3.2.6 Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) are key stromal cells in TME and promote tumor growth by releasing various growth factors. Myofibroblasts (an activated form of fibroblast) express smooth muscle actin (alpha-SMA) and platelet-derived growth factor receptors after IR, activating TGF-β signaling and angiogenesis via mitochondrial ROS to promote tumor growth (120). In addition, Insulin-like growth factor-1/2(IGF1/2), chemokine (C-X-C motif) ligand 12(CXCL12), and beta-hydroxybutyrate produced by CAFs can induce autophagy of cancer cells after RT, and promote recovery of irradiated cancer cells and tumor regeneration after RT. These CAFs-derived molecules, IGF1/2, CXCL12 and beta-hydroxybutyrate, increased ROS level after radiation, thereby enhancing protein phosphatases 2A activity, inhibiting mTOR activation, and increasing autophagy in cancer cells (121). CAFs are thought to promote immune escape through a variety of mechanisms and are regulated by ROS induced by NOX4 (122). NOX4 inhibition is capable of promoting $CD8^+$T cell infiltration within tumors and restoring the immunotherapeutic response of CAFs-rich tumors. CAFs secrete CXCL2, facilitating the migration of monocytes to the local TME in lung squamous cell carcinoma and promoting monocyte-to-MDSCs differentiation. CAFs-induced MDSCs further inhibit $CD8^+$T cells proliferation by upregulating the expression of NOX2 and IDO 1 to generate excessive ROS (123).

## 4 Regulating OS to enhance the efficacy of immunotherapy

Given the effect of OS on TME, regulation of OS is considered an effective protocol for enhancing immunotherapy efficacy (Table 1).

### 4.1 Induced ROS production

The histopathological feature of glioblastoma is the significant invasion of microglia and peripheral macrophages (the primary innate immune cells of the central nervous system) resident in the tumor. Researchers have designed a compound Au-OMV using gold nanoparticles and bacterial outer membrane vesicles to increase intracellular ROS production (124). ROS induced OS promotes the expression of macrophage/monocyte chemoattractant protein-1 (MCP-1) and MCP-2. The combination of Au-OMV with RT increases chemotaxis of macrophages and radiation sensitivity in glioblastoma. TLR7 agonist imiquimod binding RT-induced ROS accelerates autophagy in melanoma through MAPK and NF-κB signaling pathways and increases CD4 and $CD8^+$T cell ratios through IFN-γ and TNF-α production (125). Alternol is a naturally occurring compound that promotes the production of DAMPs and pro-inflammatory cytokines through elevated ROS, thereby promoting $CD8^+$T cell initiation and T cell infiltration in tumors (126). To improve the therapeutic efficacy of RT-radiodynamic therapy, an oxygen-rich X-ray nanoprocessor Hb@Hf-Ce1 nanoparticles were designed in which an encapsulated oxygen carrier (hemoglobin, Hb) was used to regulate oxygen balance in anoxic TME. In contrast, radioluminescence stimulated by Hf under X-ray IR can activate the photosensitizer Ce6 to produce ROS, thus inducing a comprehensive anti-tumor immune response (127). G-CSF can stimulate the release of neutrophils and monocytes and enhance the phagocytosis of macrophages. It could amplify the tumor-specific immune response induced by RT, which is mediated by ROS production from N1 TAN stimulated by G-CSF (114). Anti-angiogenic drugs have the effect of normalizing blood vessels, which will improve tumor hypoxia and increase ROS production after radiotherapy (128). Immunotherapy also has this effect, so anti-angiogenic drugs (bevacizumab) can also increase the therapeutic efficacy of immune checkpoint inhibitors (129). Anlotinib is another antiangiogenic drug, and in colorectal cancer, Anlotinib benefits the anti-PD-L1 immunotherapy by activating ROS/JNK/AP-1 pathway to upregulate PD-L1 expression (130).

### 4.2 Photodynamic therapy

Photodynamic therapy (PDT) uses photosensitizers (PSs) to absorb certain wavelengths of ultraviolet light and produce free radicals or ions, which in turn produce ROS to suppress tumors (131). Importantly, PDT may well initiate ICD. First, PDT promotes the transfer of CRT from the endoplasmic reticulum to the surface of cell membranes and enhances the expression of HSP, providing "eat me" signals (132). In addition, PDT-induced ROS can promote the secretion of various cytokines (IFN-γ, IFN-α, etc.) by immune cells, and promote the infiltration of APCs and $CD8^+$T cells (133). Many preclinical studies have found beneficial antitumor effects of PDT in combination with immunotherapy. In one study, Zhang et al. developed phenyporphyrin-based nMOFs, and PSs (TBP) combined with αPD-1 to restore the activity of suppressed CTLs in immunosuppressive TME (134). In another study, Lin et al. constructed a cationic nMOF (W-TBP) that is highly loaded with the anion CpG, which enhances the release of TAAs and effectively enhances the maturation of DCs (135). In addition, a self-assembled nano-coordination polymer (NCP) nanoparticle loaded with oxaliplatin induced an increase in $CD8^+$T cell infiltration in both primary and remote tumors blocking PD-L1 and production of large amounts of ROS (136). Another temophen-supported nanodrug delivery system (VES-CSO/TPGS-RGD) has been shown in colorectal cancer to promote PD-L1 expression via HIF-1α and synergistically increase DCs maturation and ICD with PD-L1 blocking (137). Conventional PDT is limited in clinical application by the depth of light penetration, while X-rays can break shallow penetration limits as an energy source (138). In turn, ROS produced by PDT can increase DNA damage and lipid peroxidation damage, thus

TABLE 1 Potential approachs to modulating oxidative stress to enhance radiotherapy in combination with immunotherapy.

| Classification Criteria | Treatment | Tumors | Characteristics |
|---|---|---|---|
| Induced ROS production | Au-OMV+RT | Glioblastoma | Increasing intracellular ROS, promoting MCP-1 and MCP-2, to increase chemotaxis of macrophages. |
| | Imiquimod+RT | Melanoma | Accelerating autophagy through MAPK and NF-κB signaling pathways, increasing CD4 and CD8+T cell ratios through IFN-γ and TNF-α production. |
| | Hb@Hf-Ce1 nanoparticles +radiotherapy-radiodynamic therapy | Melanoma Colorectal cancer Mammary carcinoma | activating the photosensitizer Ce6 to produce ROS, inducing a comprehensive anti-tumor immune response. |
| | G-CSF+RT | Prostate carcinoma Mammary carcinoma Thymoma | stimulating ROS release of N1 TAN, enhancing the phagocytosis of macrophages. |
| | Anlotinib+ anti-PD-L1 | Colorectal Cancer | Anlotinib could activate ROS/JNK/ AP-1 signaling pathway to increase the expression levels of PD-L1, IFN-α/β/γ, and CXCL2. |
| Photodynamic therapy | TBP-nMOF+αPD-1+PDT | Breast cancer | The efficient ROS production at low oxygen concentration, restoring the activity of suppressed CTLs in TME. |
| | nMOFs(W-TBP)+PDT | Breast cancer | Delivering immunostimulatory CpG oligodeoxynucleotides to DCs, enhancing the release of TAAs and the maturation of DCs. |
| | NCP@pyrolipid+PDT | Colorectal cancer | producing large amounts of ROS, blocking PD-L1, increasing $CD8^+$T cell infiltration. |
| | VES-CSO/TPGS-RGD+PDT | Colorectal cancer | blocking PD-L1, increasing DCs maturation and ICD |
| Nanomedicine | Cu-NCPs+RT | Colorectal cancer | Eliminatiing GSH and producing ROS, augmenting radioimmunotherapy and T-cell infiltration |
| | M-FDH+RT | Breast cancer | Producing a 10.98-fold tumor oxygenation, causing efficient production of ROS, resulting in over 90% elimination of CAFs, enhancing $CD8^+$T cells. |
| | MGTe+RT | Breast cancer | Inducing ROS production, up-regulating the ratio of M1 macrophages and levels of multiple cytokines. |
| | HfMOF-PEG-FA+RT | Colorectal cancer | increasing radiation dose deposition and production of ROS, promoting IFN regulatory factor stimulation, enhancing immune activation. |
| | RAN-PC+RT | Breast cancer | Enhancing $CD8^+$T cells and IFN-γ expressing subtype, notably reduced immunosuppressive cells. |
| | LipC6 | liver tumors | Inhibiting ROS production in TAM, promoting the polarization of the M1 phenotype, reducing the inhibition of $CD8^+$T cells. |
| Ameliorate hypoxia to induce OS | Hyperbaric oxygen therapy | Multiple malignancies | blocking HIF1-α, reducing adenosine production, enhancing $CD8^+$T cells, reducing Treg cell infiltration, decreasing expression of PD-L. |
| | PLGA-R837@Cat+RT+αCTLA-4 | Breast cancer Colorectal cancer | Relieving the tumor hypoxia, inducing strong immune memory, increasing TAAs. |

enhancing RT sensitivity (139). This suggests that the development of PDT is a potential sensitization method for radiotherapy combined with immunotherapy.

## 4.3 Nanomedicine

A variety of nanomaterials have been developed to amplify OS-induced ICDs during RT. Cu-based nanoscale coordination polymers (Cu-NCPS) with mixed-valence ($Cu/Cu^{2+}$) have been shown to trigger GSH elimination and ROS production, to augmented radiotherapy combined with immunotherapy and T-cell infiltration (140). Hypoxic TME limits ROS production during RT. A microvesicle-inspired oxygen-delivering polyfluorocarbon nanosystem loading DiIC18(5) and halofuginone (M-FDH) produced a 10.98-fold enhancement of tumor oxygenation and caused efficient production of ROS upon radiation, which resulted in over 90% elimination of CAFs,

enhancement of $CD8^+$T cells, and elimination of suppressive immune cells (141). A hybrid nanoplatform (designated as MGTe) composed of tumor- and bacteria-derived immunomodulators and nano-radiosensitizer was acted as a radiosensitizer to enhance the efficacy of RT by ROS generation and ICD (142). MGTe-induced ROS would up-regulate the ratio of M1 macrophages and level of multiple cytokines. The hafnium foundation organic framework (HfMOF-PEG-FA) modified with folate increases radiation dose deposition and subsequent production of ROS, promotes IFN regulatory factor (IRF) stimulation, and in combination with the TLR7 agonist imiquimod enhances immune activation, characterized by an increase in $CD8^+$ and proliferative T cells (143). A ROS-responsive albumin nanocomplex of anti-PD-L1 and cabazitaxel (RAN-PC) was designed to promote their intratumor delivery with controllable release capacity and potentiate radiation-mediated antitumor immunity for cancer therapy (144). RAN-PC+radiotherapy treatment produced a 3.61- and 5.10-fold enhancement in $CD8^+$T cells and IFN-γ expressing subtype, respectively, and notably reduced versatile immunosuppressive cells. A nanoliposome loaded C6-ceremide (LipC6) was developed to inhibit ROS production in TAM to promote the polarization of the M1 phenotype and reduce the inhibition of $CD8^+$T cells (110).

### 4.4 Ameliorate hypoxia to induce oxidative stress to enhance radiotherapy combined with immunotherapy

Hypoxia is a hallmark of solid tumors that reduces their susceptibility to radiation therapy and is a driver of immunotherapy resistance (145). Given the enormous benefits of targeting hypoxia for RT and immunotherapy, a number of regiments have been developed to ameliorate hypoxia to increase the efficacy of radiotherapy combined with immunotherapy, including increasing oxygen supply to tumors, inhibiting mitochondrial oxidative phosphorylation (OXPHOS), inhibiting HIF activity (146). Hyperbaric oxygen therapy can be used to counteract the anoxic effect in the TME to achieve the large number of ROS produced after IR, which can improve the efficacy of RT (147). In addition, hyperoxia blocks the hypoxia-HIF1α-adenosine pathway to reduce adenosine production, thereby enhancing $CD8^+$T cells and reducing Treg cell infiltration. Meanwhile, oxygen-depleted MDSC inhibits antigen-specific and nonspecific T cell activity via HIF-1α, which can be inhibited by hyperoxygen, leading to decreased expression of PD-L1 (147). Core-shell nanoparticle based poly (lactate-glycolic) acid (PLGA) was prepared by encasing water-soluble catalase (Cat) to decompose H2O2 into O2, and imiquimote (R837), a toll-like receptor-7 agonist, was loaded into the shell. The formed PLGA-R837@Cat nanoparticles modulate the immunosuppressive TME by relieving tumor hypoxia. TAAs produced after RT induce intense ICD with the help of PLGA-R837@Cat. Combined with CTLA-4 checkpoint blocking, tumor metastasis is effectively inhibited through a powerful distant effect (148).

## 5 Conclusion

Radiotherapy can induce DNA strand breaks, damage of large molecules such as lipids and proteins, and other reactions. ROS is considered to be an important cytotoxic and signaling molecule in these reactions, inducing oxidative stress response of tumor cells. With the combination of radiotherapy and immunotherapy, the effect of oxidative stress on immune response has gained attention. In fact, apoptosis, autophagy and ferroptosis induced by oxidative stress are related to immunogenic death of tumor cells. A variety of immune cells and immune checkpoints in the immune microenvironment are also regulated by ROS. Appropriate ROS is a necessary condition for the activation of some immune cells, but excessive ROS may be an important reason why many immune cells have difficulty in performing anti-tumor functions and become resistant to immunotherapy. Therefore, a variety of therapeutic approaches are being explored to regulate ROS production during radiotherapy to enhance the efficacy of combination radiotherapy. In the future, while focusing on the changes of oxidative stress in tumor cells, we still need to pay attention to the influence of ROS in the TME on immunotherapy.

## Author contributions

Conceptualization: XJ. Methodology: ZZ and JS. Software: XB. Validation: JX. Formal analysis: HW. Investigation: CB. Resources, XJ. Data curation: ZZ. Writing—original draft preparation: ZZ, JS, and CB. Writing—review and editing: XB, QZ, and XJ. Visualization: XJ. Supervision: XJ. Project administration: XJ. Funding acquisition: XJ. 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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# Glossary

| | |
|---|---|
| RT | radiotherapy |
| ROS | reactive oxygen species |
| OS | oxidative stress |
| TAAs | tumor-associated antigens |
| TME | tumor immune microenvironment |
| IR | irradiation |
| DAMPs | damage-associated molecular patterns |
| HMGB1 | high-mobility group box 1 |
| HSPs | heat shock proteins |
| CRT | calreticulin |
| APCs | antigen-presenting cells |
| MHC | major histocompatibility complex |
| TCR | T cell receptor |
| STING | stimulator of interferon genes |
| cGAS | cyclic GMP-AMP synthase |
| cGAMP | cyclic GMP-AMP |
| DCs | dendritic cells |
| PD-L1 | programmed cell death-ligand 1 |
| MDSCs | myeloid-derived suppressor cells |
| IDO | indoleamine 2, 3-dioxygenase |
| TGF-β | transforming growth factor β |
| Tregs | regulatory T cells |
| TAM | tumor-associated macrophage |
| NKs | natural killer cells |
| FOXP3 | forkhead box protein 3Nrf2, nuclear factor–erythroid 2 related factor 2 |
| Bcl-2 | B-cell lymphoma-2 |
| ATG | autophagy protein MAPK, mitogen-activated protein kinase |
| mTOR | mechanistic target of rapamycin |
| ICD | immunogenic cell death |
| ATP | adenosine 5-triphosphate |
| ER | endoplasmic reticulum |
| LMP | lysosome membrane permeability |
| PUFAs | polyunsaturated fatty acids |
| PUFA• | fatty acid free radicals |
| ACSL4 | acyl-CoA synthetase long-chain family member 4 |
| L-OOH | lipid peroxides |
| SLC7A11 or xCT | solute carrier family 7, member 11 |

*(Continued)*

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| | |
|---|---|
| GSH | glutathione |
| ATM | ataxic-telangiectasia mutant |
| ZVI | zero-valent iron |
| NF-κB | nuclear factor kappa-B |
| AKT | activating protein kinase B |
| DSBs | DNA double-strand breaks |
| ATR | ataxia telangiectasia and Rad3-related |
| Chk1 | checkpoint kinase 1 |
| PTEN | phosphatase and tensin homolog deleted on chromosome 10 |
| SENP7 | SUMO-specific protease 7 |
| NOX2 | NADPH oxidase 2 |
| pDCs | plasmacytoid DCs |
| NKG2D | Natural Killer Group 2D |
| TLR | toll-like receptor |
| CTLs | tumor-specific cytotoxic T lymphocytes |
| CAFs | cancer-associated fibroblasts |
| IGF1/2 | insulin-like growth factor-1/2 |
| CXCL12 | chemokine (C-X-C motif) ligand |
| MCP-1 | macrophage/monocyte chemoattractant protein-1 |
| PDT | photodynamic therapy |
| PSs | photosensitizers |
| Cu-NCPS | Cu-based nanoscale coordination polymers |
| LipC6 | nanoliposome loaded C6-ceremide |
| OXPHOS | oxidative phosphorylation |
| PLGA | poly (lactate-glycolic) acid |
| Cat | catalase |

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# Functions and mechanisms of lactylation in carcinogenesis and immunosuppression

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As critical executors regulating many cellular operations, proteins determine whether living activities can be performed in an orderly and efficient manner. Precursor proteins are inert and must be modified posttranslationally to enable a wide range of protein types and functions. Protein posttranslational modifications (PTMs) are well recognized as being directly associated with carcinogenesis and immune modulation and have emerged as important targets for cancer detection and treatment. Lactylation (Kla), a novel PTM associated with cellular metabolism found in a wide range of cells, interacts with both histone and nonhistone proteins. Unlike other epigenetic changes, Kla has been linked to poor tumor prognosis in all current studies. Histone Kla can affect gene expression in tumors and immunological cells, thereby promoting malignancy and immunosuppression. Nonhistone proteins can also regulate tumor progression and treatment resistance through Kla. In this review, we aimed to summarize the role of Kla in the onset and progression of cancers, metabolic reprogramming, immunosuppression, and intestinal flora regulation to identify new molecular targets for cancer therapy and provide a new direction for combined targeted therapy and immunotherapy.

KEYWORDS

**immunosuppression, lactylation (Kla), metabolic reprogramming, tumor microenvironment (TME), Warburg effect**

## 1 Introduction

Metabolic reprogramming is a vital characteristic of tumor cells and plays an important role in tumor therapy, with the Warburg effect being the most widely studied. Even when sufficient oxygen is available to support mitochondrial oxidative phosphorylation, tumor cells tend to convert glucose into lactate (1). Lactate was formerly considered to be a

metabolic waste; however, recent research has shown that it can promote the formation and development of cancers through activating the $G_i$ protein-coupled receptor 81 (GPR81) signaling pathway (2), influencing cell metabolism (3), and modulating the tumor microenvironment (TME) (4).

Protein posttranslational modifications (PTMs) refer to the process of changing the biochemical properties of proteins through adding chemical groups to one or more amino acid residues so that precursor proteins can have specific functions. Common modifications include acetylation, ubiquitination, methylation, phosphorylation, and glycosylation (5). PTMs are closely related to carcinogenesis, and their pathological significance involves all cancer characteristics, including maintenance of proliferation signals, resistance to cell death, induction of angiogenesis, and activation of invasion (6). PTMs also play vital roles in TME regulation. Swamy et al. (7) found that activated effector T cells contain more O-GlcNAcylation-modified proteins than naïve T cells, suggesting that O-GlcNAcylation plays an important role in T cell activation. Forkhead/winged helix transcriptional factor P3 (FoxP3) is a transcriptional regulator that plays an important role in regulatory T cell (Treg) growth. Acetylation can enhance its ability to bind to chromatin, thereby increasing the number and activity of Treg cells and inhibiting the antitumor effects of the immune system (8). Therefore, targeting PTMs to key proteins or pathways is an emerging strategy for improving early cancer detection and treatment.

Lactylation (Kla) is a novel PTM. Zhao et al. (9) conducted the first study on histone Kla in 2019, demonstrating that Kla refers to the addition of a lactyl (La) group to a lysine residue in the histone tail. Later studies established that Kla is prevalent in immune-related cells (10), non-small cell lung cancer (NSCLC) (11), and ocular melanoma (12), and that it is strongly associated with the development of malignancies (13). This systematic review aimed to examine the important role of Kla in tumor cell metabolism, microenvironment, and immunosuppression, explore the possibility of targeting Kla sites and catalytic enzymes for cancer therapy, and provide a new direction for combined targeted cancer therapy and immunotherapy.

## 2 Lactate regulation of tumor metabolism and microenvironment

Glucose primarily generates energy *via* two metabolic pathways: glycolysis and oxidative phosphorylation (Figure 1). Both pathways begin with the conversion of glucose to pyruvate, followed by the production of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). When oxygen is available, the pyruvate produced during glycolysis is carried into the mitochondria and transformed into acetyl-CoA, which subsequently enters the tricarboxylic acid (TCA) cycle and generates considerable energy (14, 15). However, in the absence of oxygen, pyruvate is converted to lactate by lactate dehydrogenase (LDH) (16). Under aerobic conditions, the glycolytic process in normal mammalian cells is blocked, which is known as the Pasteur effect (17). However, the glycolytic metabolism of tumor cells is significantly active even in the presence of an ample oxygen supply, which is known as the Warburg effect (1).

The causes of the Warburg effect remain contentious, and several theories provide preliminary explanations. One possible explanation is that, under oxygen-sufficient conditions, the efficiency of ATP production is not a limiting factor for cell multiplication. When the capability of glucose metabolism to produce ATP is impaired in normally proliferating cells, they undergo cell cycle arrest and reactivate catabolism while activating signaling pathways, such as AMP-activated protein kinase (AMPK) and activating protein kinase B (AKT), to maintain energy homeostasis (18, 19). Furthermore, cancer cells constantly divide and require additional metabolic intermediates to form macromolecules for their daughter cells. However, mitochondrial oxidative phosphorylation converts all glucose into $CO_2$ to maximize ATP generation, in conflict with the requirements of growing cells. During glycolysis, glucose can be transformed into additional macromolecular precursors, such as acetyl-CoA for fatty acid synthesis, glycolytic intermediates for nonessential amino acid synthesis, and ribose for nucleotide synthesis, to enhance cell proliferation (9). In addition, it has been shown that adverse metabolic conditions in TME can lead to the activation of transcription factors such as Krüppel-like factor 4 (KLF4) and nuclear factor-kappa B (NF-κB), leading to the selection of the Warburg phenotype through transcriptional reprogramming (20).

Previously, the lactate produced through glycolysis was considered to be merely metabolic waste. However, in recent years, an increasing number of studies have shown that lactate plays a role in tumor growth. Lactate can be employed as a GPR81 ligand to activate the GPR81 signaling pathway, which can then influence the expression of metabolism-related genes and promote tumor growth (2). In addition, Vegran et al. (21) found that lactate can enter endothelial cells through monocarboxylate transporter 1 (MCT1), causing degradation and phosphorylation of IκBα, and then stimulating the autocrine NF-κB/interleukin (IL)-8 pathway to cause cell migration and blood vessel formation. Lactate is also closely related to TME (22). Proton-coupled lactate transport in cancer or stromal cells creates an acidic environment with a pH ranging from 6–6.6, leading to tumor development and treatment resistance (23). Unlike exogenous pathogens, cancer cells are derived from normal cells and express almost all the proteins that normal cells express, so they are more difficult to distinguish by the immune system. In lymphocytes, B cells can act as antigen-presenting cells and secrete cytokines to exert anti-tumor effects (24); Cytotoxic T lymphocytes (CTLs) can specifically recognize tumor-associated antigens through major histocompatibility complex (MHC) I on their surface, bind to tumor cells and produce perforin and other cytotoxins to kill cancer cells (25); Natural killer (NK) cells have no MHC or human leukocyte antigen restriction and can release perforin, granzyme and cytokines to destroy tumor cells (26). Among myeloid cells, the frequency of dendritic cells in tumors was associated with a good prognosis, while tumor-associated macrophages (TAM), especially M2 macrophages, were associated with a poor prognosis (27). It has been reported that lactate generated from tumor cells can activate the production of vascular endothelial growth factor (VEGF) and

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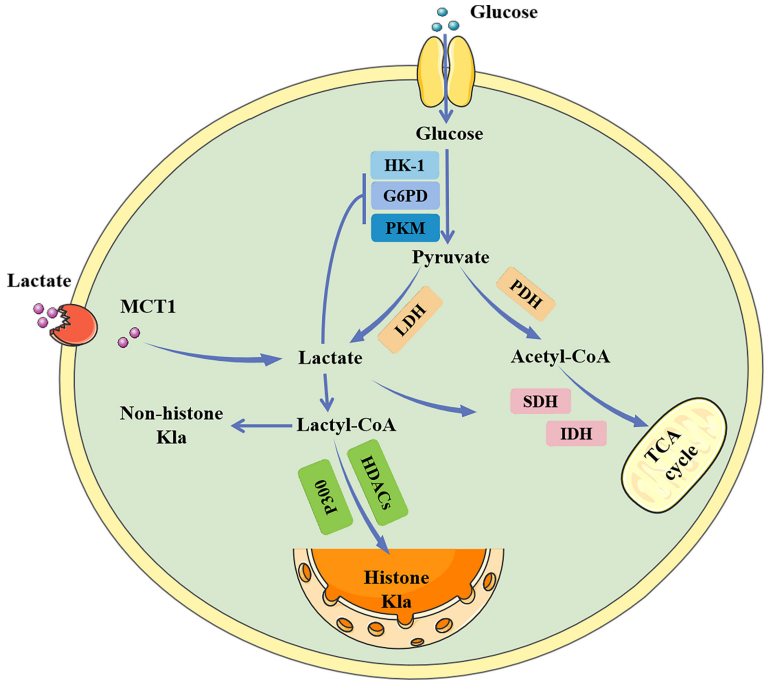


FIGURE 1
The metabolic process of lactate. Lactate can be created intracellularly by glycolysis catalyzed by LDH, or it can be taken up from the outside via MCT1. Intracellular lactate inhibits glycolytic enzymes while promoting the tricarboxylic acid cycle, generating a negative feedback loop. Lactate can also be converted to lactoyl-CoA, which is involved in the lactylation of histone and non-histone proteins. HK-1, hemokinin-1; G6PD, glucose 6-phosphate dehydrogenase; PKM, glycolytic enzyme pyruvate kinase M; MCT1, monocarboxylate transporters 1; LDH, lactate dehydrogenase; PDH, Pyruvate dehydrogenase; SDH, succinate dehydrogenase; IDH, isocitrate dehydrogenase; TCA, Tricarboxylic acid; HDAC, histone deacetylase; Kla, lactylation.

arginase 1 (Arg1), promoting TAM polarization to M2 and assisting TAM in promoting tumor growth (28). Besides, the lactate concentration in the TME can limit the lactate efflux of CTLs, lowering the production of cytokines, perforin, and granzyme B, and inhibiting CTL cytotoxicity (29). NK cells are potent innate immune effectors that serve as the first line of defense against cancer. Lactate induces NK cell apoptosis (30). Natural killer T (NKT) cells are another type of immune cells with anti-tumor activity. Lactate can inhibit the production of interferon-γ (IFNγ) and IL-4 by NKT cells, inhibit their survival and proliferation, and promote tumor development (31).

However, the specific mechanisms underlying lactate uptake and utilization by tumor cells have not yet been fully elucidated. The accumulation of lactate in the TME can be reduced through increasing its uptake and utilization by tumor cells, thus reducing its influence on tumor cells and immune cells, or through finding effective targets to cause tumors to undergo aerobic metabolism instead of the Warburg effect, thereby inhibiting tumor invasion. These issues require urgent attention in the field of lactate metabolism in tumors.

## 3 Lactylation: a new posttranslational modification

Kla is a PTM protein first reported in 2019 (32). Subsequent studies have further confirmed that Kla has an important function in relation to lactate and is involved in important activities such as tumor proliferation (33), nervous system regulation (34), and metabolic regulation (35).

Lysine is a basic amino acid and its sixth amino group is highly active. After modification, this amino acid changes from basic to acidic, leading to changes in protein polarity and function (36). Lysine acylation is a widespread and highly conserved PTM that affects various physiological and pathological processes through epigenetic regulation. Using mass spectrometry analysis, Zhao et al. (32) found a mass shift of 72.021 Da on lysine residues in three proteolytic peptides, which increased in a dose-dependent manner with an increase in lactate. Subsequently, they showed the existence of Kla through tracking isotopic sodium l-lactate ($^{13}C_3$). Inhibition of pyruvate dehydrogenase kinase (PDK) and LDH production by dichloroacetic acid (DCA) and oxalate was found to cause Kla inhibition (37, 38), whereas inhibition of mitochondrial oxidative phosphorylation by rotenone caused an increase in Kla. This implies that lactate levels are inextricably linked to local lactate concentrations. Furthermore, stressors such as hypoxia and bacterial infection can promote a change in cellular energy metabolism into a glycolysis-dependent pattern, resulting in increased lactate production and the activation of histone Kla (39).

Kla has also been shown to cause changes in the expression of genes related to inflammatory responses (40), macrophage polarization (32), and other processes. Several studies have shown that Kla plays an important role in disease occurrence and development (33, 34). However, research into “writers” and

"erasers," which are necessary for the occurrence and removal of Kla, remains in its infancy and has yet to be expanded.

Lactate, a chiral molecule, typically exists as three optical isomers: D-lactate, L-lactate, and racemic DL-lactate. L-Lactate is the most common type of lactate. It increases dramatically in pathological conditions such as tumors, sepsis, and autoimmune disorders (41). According to previous research, L-lactate can be activated through acyl-CoA synthetase and transported to lysine residues by histone acetyltransferases such as p300 (32). D-Lactate is primarily synthesized in cells through the metabolism of-carbonyl aldehydes with high glycation activity, such as methylglyoxal (MGO) (42). D-Lactate concentrations can increase dramatically when intestinal function is impaired (43). Galligan et al. (44) found that methylglyoxal, a byproduct of glycolysis, can combine with glutathione to form lactoylglutathione (LGSH), which is catalyzed by glyoxalase 1 (GLO1). Simultaneously, GLO2 hydrolyzes LGSH, cycling glutathione, and produces D-lactate. LGSH can be employed as a donor to supply lactate molecules to enhance the Kla of target proteins; this reaction does not require enzyme catalysis. In addition, Zhao et al. (45) found that common deacetylases HDAC1-3 and SIRT1-3 have the ability to eliminate Kla, among which HDAC3 is the most effective "eraser" for L- and D-lactylation (46) (Figure 2).

The study of Kla and its associated enzymes offers new perspectives for targeted cancer treatment and has promising practical applications. As an increasing number of histone Kla sites have been identified, the role of Kla in nonhistone proteins is becoming increasingly understood and recognized. Among glycolytic enzymes, Kla has been found to be widespread. For instance, the Kla of aldolase A (ALDOA) is present in numerous human tumor cell lines and can control glycolysis through controlling the activity of metabolic enzymes (47). Through thoroughly analyzing the tumor and nearby liver, Young et al. (48) identified 9,275 Kla sites, of which 9,256 were found on proteins other than histones, indicating that Kla is a broad alteration that extends beyond histone and transcriptional regulation. They also showed that the p53 pathway is controlled by adenylate kinase 2 (AK2) Kla in hepatocellular carcinoma (HCC), which contributes to a poor prognosis.

# 4 Lactylation promoting the occurrence and development of tumors

## 4.1 Lactylation affecting the metabolism of tumor cells

The metabolic reprogramming of tumor cells, which promotes rapid cell growth and proliferation through altering the metabolism,

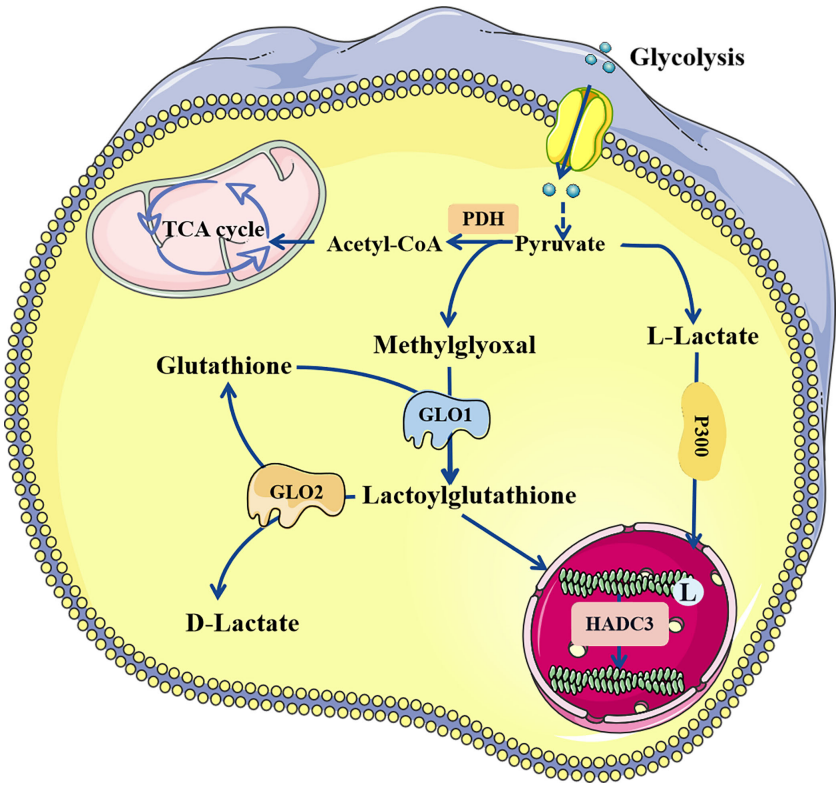


FIGURE 2
The regulatory process of lactylation. There are two known modification pathways for lactylation. L-lactate can form lactoyl-CoA, which mediates lactylation under the catalysis of P300. In addition, methylglyoxal can form LGSH under the catalysis of GLO1, and LGSH can also mediate the lactylation of proteins, which does not require enzyme catalysis. LGSH can be decomposed into glutathione and D-lactate under the action of GLO2. Both L-and D-lactate-mediated lactylation were abolished by HDAC3. LGSH, Lactoylglutathione; GLO, glyxoalase.

is considered to represent a new cancer characteristic (49). The most prevalent and basic type of research on tumor metabolism is aberrant glucose metabolism (50). Tumor cells have a distinctive glucose metabolism pattern compared with normal cells; even under oxygen-sufficient conditions, they prefer glycolysis to the tricarboxylic acid (TCA) cycle to obtain energy. Recent studies have also found that many other metabolic pathways, including fatty acid metabolism (51), cholesterol metabolism (52), and glutamine metabolism (53), undergo reprogramming changes in tumor cells.

Hypoxia-inducible factor alpha (HIF-1α) is a key gene that regulates the metabolic mode of tumor cells (54). Lactate concentration can inhibit the activity of proline hydroxylase (PHD) and weaken the ubiquitination and degradation of HIF-1α by PHD, which indicates that lactate is closely related to tumor metabolism (55). Previous studies have demonstrated that lactate can be used as a source of three-carbon compounds in mammals. Circulating lactate also allows uncoupling of glycolysis and TCA, allowing glucose use to be regulated according to more advanced bodily demands (56). Besides, lactate and pyruvate together can be used as a circulating REDOX buffer to balance the NADH/NAD ratio in cells and tissues (57). Chiarugi et al. (3) demonstrated that lactate can activate the key enzymes ATP citrate lyase and acetyl-CoA carboxylase in fatty acid synthesis, as well as the cholesterol pathway, by redirecting citrate in prostate cancer cells. Meanwhile, the current study showed that the accumulation of circulating lactate is negatively correlated with fat oxidation (58). In recent years, research concerning Kla has gain considerable momentum, and its role in tumor metabolism has gradually been revealed.

Wan et al. (47) found that Kla regulates numerous glycolysis-related enzymes. For example, Kla at the K147 site of fructose-bisphosphate ALDOA is found in both humans and animals. After Kla treatment, ALDOA activity was reduced, and its catalytic function was suppressed, establishing a negative feedback loop. Kla may also influence the function of other enzymes involved in glycolysis. Kla of the human recombinant enolase (ENO1) disrupts its interaction with substrates. Given the prevalence of metabolic dysfunction in the TME and high lactate levels resulting from the Warburg effect, it is plausible that Kla serves as a crucial link between lactate, tumor metabolism, and patient prognosis.

In a study concerning hepatocellular cancer, Kla was found to have primarily affected enzymes involved in metabolic pathways such as the TCA cycle, glucose, amino acid, fatty acid, and nucleotide metabolism. Kla at the K28 site then inhibited the function of AK2 and promoted the growth and spread of liver cancer cells (48). Studies concerning NSCLC have shown that increased histone Kla levels may cause downregulation of hemokinin-1 (HK-1) and pyruvate kinase M (PKM) in glycolysis, and upregulation of succinate dehydrogenase (SDHA) and isocitrate dehydrogenase 3 gamma (IDH3G) in the TCA cycle. As a result, glucose absorption and glycolysis are inhibited, mitochondrial homeostasis is maintained, and tumor development occurs (11). Furthermore, Kla has been shown to control the activity of enzymes involved in lipid metabolism in a nonalcoholic fatty liver disease model. The presence of Kla at the K673 site of fatty acid synthase inhibits its function and decreases lipid accumulation in the liver (59).

Tumor metabolic reprogramming can successfully increase tumor cell proliferation, growth, migration, and invasion, among other important biological functions (49). Therefore, targeting tumor metabolism through exploiting metabolic variations between tumor and normal cells is a promising anticancer technique. As a new PTM, Kla plays an important role in the regulation of tumor metabolism, especially glucose metabolism. However, there have been few investigations of other basic metabolic pathways such as lipid and amino acid metabolism. Further exploration of the regulatory mechanism of Kla in tumor metabolism and the search for more Kla sites could provide more reliable targets for tumor therapy.

## 4.2 Regulation of TME

Changes in TME are also important factors affecting carcinogenesis. Lactate is one of the most prominent metabolites in TME. Lactate produced by tumor cells via the Warburg effect can enter the extracellular microenvironment and promote angiogenesis through upregulating the expression of angiogenesis-related proteins. At the same time, it can regulate the metabolism of immune cells and inhibit the proliferation of $CD8^+$ T cells, NK cells, and dendritic cells, thereby mediating immune escape (60, 61). The degree of histone lysine Kla has been found to be higher in TAM than in other tissues, implying that Kla plays an important regulatory role in TME, which may provide a new direction for tumor immunotherapy, antiangiogenesis therapy, and targeted therapy.

Lactate accumulation can stimulate angiogenesis by activating VEGF, transforming growth factor (TGF), IL-1, HIF-1α, and other substances (62, 63). According to previous studies, Kla plays a crucial role in this process. The high expression of hyaluronic acid (HA)-binding protein KIAA1199 has been reported to be positively correlated with tumor stage, overexpression of HIF-1α and upregulation of angiogenesis markers (64, 65). Lactate enters prostate cancer cells via MCT1 and promotes the Kla of HIF-1α, keeping it stable under normoxic circumstances and boosting KIAA1199 transcription. This discovery could lead to a promising target for antiangiogenic tumor therapy (66).

Immune cells play a significant anticancer role in TME. They can detect extracellular lactate levels and send intracellular signals to alter their function in TME (29). As a result, the impact of Kla on the efficacy of immunotherapy has attracted interest (Figure 3). Tumor-infiltrating myeloid cells (TIMs) include TAMs, myeloid-derived suppressor cells (MDSCs), and tumor-associated neutrophils (TANs). They are closely related to tumor immune escape and their functions are regulated through a variety of epigenetic processes. The increased expression of methyltransferase-like 3 (METTL3) in TIMs has been linked to poor prognosis in patients with colon cancer (67). Lactate stimulates METTL3 expression in TIMs through the H3K18 Kla. METTL3 mediates the m6A modification of Janus kinase (JAK1) mRNA, enhancing its protein translation efficiency and mediating an increase in the phosphorylation level of its downstream protein signal transducer and activator of transcription 3 (STAT3).

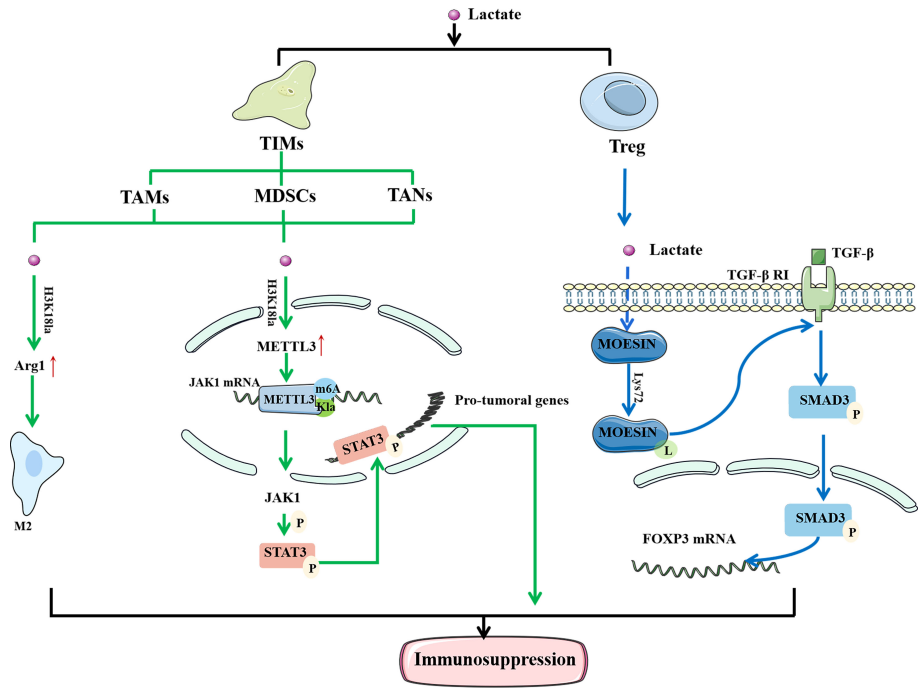


FIGURE 3
Lactylation regulates the function of immune cells to mediate immunosuppression. In TAM, histone lactylation will lead to the up-regulation of M2 phenotypically related genes, thereby mediating the M2 polarization. In TIMs, histone lactylation promote the expression of METTL3, and METTL3 mediates the m6A modification of JAK1 mRNA. At the same time, there are also lactylation sites in METTL3 that allow it to bind to the target RNA. Moesin can reduce the expression of TGF-β receptor and inhibit the production of Treg cells to restore anti-tumor immunity. Lactate can inhibit the function of moesin by promoting Lys72 site lactylation to mediate the generation of Treg cells and promote the immune escape of tumor cells. In conclusion, lactylation is closely related to the immunosuppression of tumors. LPS, Lipopolysaccharide; IFN γ, Interferon-gamma; IL-4, interleukin-4; IL-13, interleukin-13; TAM, tumor-associated macrophages; TIM, tumor -infiltrating myeloid cells; Arg1, arginase 1; METTL3, methyltransferase-like 3; JAK1, Janus Kinase 1; M6A, N6-methyladenosine; TGF-β, transforming growth factor beta.

Phosphorylated STAT3 can act as a transcription factor to regulate the production and secretion of cytokines, such as IL-6 and IL-10, resulting in immunosuppression. In addition, the presence of two Kla sites in the zinc-finger domain of METTL3, which are required for it to capture target RNA, has been confirmed. These findings highlight the importance of Kla in promoting the immunosuppressive capacity of TIMs (68).

Macrophages are one of the most important innate immune cells in the human body and are classified into M1 and M2 phenotypes. Lipopolysaccharide (LPS) and IFNγ activate M1 macrophages, while IL-4 or IL-13 stimulate M2 macrophages (69). TAMs are common in the TME and associated with the formation and progression of malignancies (70). Seventy percent of TAMs exhibit the M2 phenotype, which suppresses the immune system and promotes tumor development and metastasis (71). Studies have shown that M1 macrophages undergo metabolic reprogramming during aerobic glycolysis to produce lactate during polarization. In contrast, polarization of M2 macrophages enhances aerobic phosphorylation and fatty acid metabolism (72). Zhao et al. (32) found that activation of M1 macrophages leads to the activation of glycolysis, accompanied by the production of a large amount of lactate and upregulation of H3K18la. Histone Kla can cause an increase in M2 phenotype-related proteins, indicating that it promotes the transformation of TAMs from the proinflammatory, anticancer M1 phenotype to the antiinflammatory, procancer M2 phenotype. In a study concerning prostate cancer, Patnaik et al. (73) discovered that reducing phosphatidylinositol-3 kinase (PI3K) resulted in a decrease in the synthesis of lactate in tumor cells, thereby inhibiting the histone Kla of TAM and enhancing immune efficacy. In conclusion, histone Kla is closely related to M2 polarization of TAMs, and increased levels of Kla tend to cause immunosuppression and reduce the efficacy of immunotherapy.

TGF-β is a multifunctional cytokine belonging to the transforming growth factor superfamily. It plays a crucial role in stem cell differentiation and T cell regulation (74). When activated, T cells effectively attack and kill tumor cells. However, when the immune response is complete, TGF-β delivers a signal to naïve T cells to become Treg cells to regulate and destroy activated proinflammatory T cells, ensuring that they do not create too many immune factors and cause damage to their own cells and tissues (75). In tumor tissues, Treg cells suppress immune responses and protect cancer cells from killer T cells. Moesin reduces the expression of TGF-β receptors and inhibits the production of Treg cells to restore antitumor immunity (76). Studies have found that lactate can inhibit the function of moesin by promoting Kla at the Lys72 site to mediate the generation of Treg cells and promote the immune escape of tumor cells. Therefore, it is feasible to improve the efficacy of immunotherapy by inhibiting moesin Kla (10). In summary, the discovery of Kla provides a new direction for tumor immunotherapy.

## 4.3 Lactylation affecting the growth and distribution of intestinal flora

Kla provides a new perspective on the regulatory function of lactate, which plays an important role in eukaryotic cell metabolism and gene transcription (32). Lactate is a key carbon source for bacteria and is linked to stress tolerance, cell wall remodeling, and virulence (77, 78). However, if Kla occurs in prokaryotes, the biological functions it may be involved in need further investigation.

YiaC and CobB are common lysine acetyltransferases and deacetylases found in prokaryotes, respectively (79). Dong et al. found that YiaC catalyzes Kla in *Escherichia coli*, whereas CobB scavenges PTM. CobB enhances glycolysis and bacterial growth through eliminating the Kla of pyruvate kinase I (PykF) (80), which provides a strong basis for controlling the growth and distribution of certain bacteria through regulating specific Kla sites.

Intestinal flora refers to a group of bacteria that live in the intestinal tract of humans and animals. They play important roles in regulating digestion, immunity, and nutrient absorption. In recent years, an increasing number of investigations have shown that certain bacteria can cause malignancies in gastrointestinal tissues (Figure 4). For example, *Helicobacter pylori* (HP) is closely related to atypical hyperplasia, intestinal metaplasia, and gastric cancer progression (81). Moreover, *Fusobacterium nucleatum* is closely related to the transformation of colorectal adenomas into adenocarcinomas (82). The carcinogenic mechanism of the intestinal flora is not yet fully understood. Several studies have shown that some members of *Enterobacteriaceae* produce colibactin, which results in DNA damage and promotes malignancies (83). In addition, they can affect cytokine synthesis and secretion, thereby activating various carcinogenic pathways (84). Furthermore, several Toll-like receptors (TLR), such as TLR4 and TLR5, have been linked to the interaction between malignancies and microorganisms. Kong et al. (85) found that *F. nucleatum* promotes colorectal cancer by activating TLR4/Keap1/NRF2 signaling. Moreover, studies have shown that lactate regulates the proliferation of intestinal flora and has a carcinogenic effect (86).

Panagiotou et al. (87) found that the excessive growth of intestinal *Candida* in patients with lung cancer was related to an increase in lactic-producing bacteria and a decrease in short-chain fatty acid production. *Candida* species use lactate as a nutrient source to compete with other fungi in the intestine. In addition, gut microbes can participate in disease pathogenesis via their interaction with the host genome through epigenetic mechanisms, such as long non-coding RNA. Liu et al. (88) quantitatively analyzed transcriptomic changes in a human colon cell line after infection with the common intestinal pathogen *Salmonella typhimurium* SL1344. They found that LINC00152 expression was significantly increased and associated with intestinal microbiome-derived LPS. LPS induces histone Kla at the LINC00152 promoter and decreases its binding efficiency to the transcription factor yin-yang 1 (YY1), leading to increased LINC00152 expression, thereby promoting the migration and invasion of colon cancer cells.

Bacteria promote carcinogenesis by causing DNA damage and activating oncogenic pathways. Additionally, bacteria can affect tumors through controlling the body's immune system and metabolism (89). It has been shown that the level of Kla affects the growth and dissemination of bacteria. However, whether specific Kla sites exist in different bacteria and whether tumor progression can be controlled by modulating the Kla level in the intestinal flora needs to be further explored.

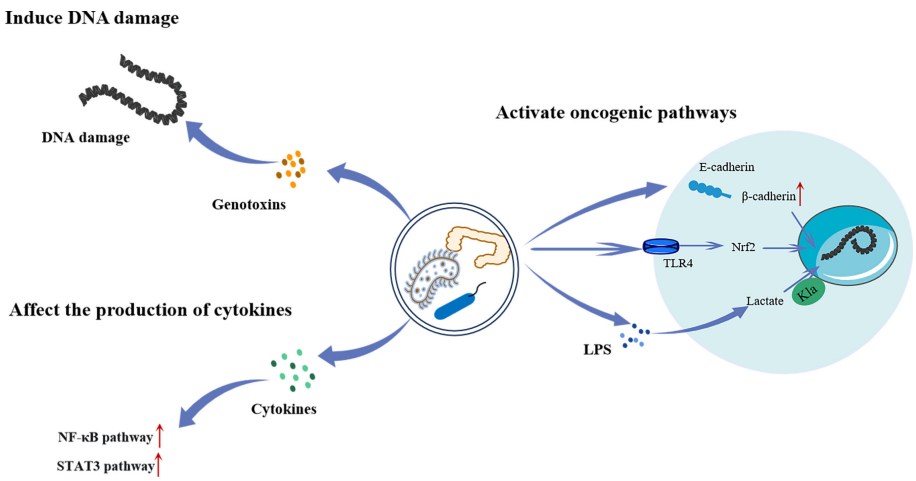


FIGURE 4
The mechanism of intestinal flora regulating the occurrence and development of tumors. Intestinal bacteria can directly cause intracellular DNA damage by producing genotoxins, or activate the NF-κB and STAT3 pathway by promoting cytokines secretion to promote tumorigenesis. Intestinal bacteria can also activate a range of oncogenic pathways. Increased release and translocation of β-catenin into the nucleus through degradation of the E-cadherin/β-catenin complex leads to aberrant activation of WNT signaling associated with various cancers. Besides, they can lead to the invasion and metastasis of cancer by activating TLR4/Keap1/NRF2 signaling. Furthermore, LPS produced by bacteria has been shown to promote the Kla of histones, which in turn promotes tumor progression. LPS, Lipopolysaccharide; TLR4, Toll-like receptor 4; Nrf2, Nuclear factor E2-related factor 2; NF-κB, Noncanonical nuclear factor-kappaB; STAT3, ignal transducer and activator of transcription 3.

# 5 Lactylation providing a new direction for targeted therapy and immunotherapy

Kla is involved in a variety of processes such as tumor metabolism, angiogenesis, and immunosuppression, and is closely related to the poor prognosis of tumors (32, 47). Therefore, targeting specific sites that inhibit the occurrence of Kla may be an effective cancer treatment strategy (Table 1).

## 5.1 The role of lactylation in tumor targeted therapy

In tumor cells, glucose undergoes glycolysis to produce pyruvate, which then reacts with LDH to form lactate. Lactate can inhibit glycolysis and promote the TCA cycle by regulating key enzymes and producing a negative feedback loop (95). Studies have shown that Kla levels are positively correlated with intracellular lactate content (32). Therefore, key enzymes involved in lactate metabolism may be potential targets for tumor treatment. LDH is a key enzyme in the conversion of pyruvate to lactate during glycolysis. It maintains glycolysis and ATP production by regenerating NAD to form NADH (96). Baumann et al. (97) showed that the carcinogenicity of LDH-deficient tumor cells was significantly reduced and, after LDH knockdown, the metabolic phenotype of the cells was disturbed and their proliferation ability was significantly decreased. Furthermore, the enzyme ULK1, as an upstream regulator, could mediate the phosphorylation of LDH and enhance its enzymatic activity to promote lactate production, thereby promoting tumor progression (98). In addition to being produced within cells, lactate can be secreted between cells through MCTs to participate in the regulation of cellular physiological and pathological processes (99). Several studies have shown that targeting MCT1 or MCT4 can effectively inhibit the growth of multiple types of tumors, such as breast (100), liver (101), and bladder cancers (102).

p300 and SIRT3 are present as "writer" and "eraser" for Kla. Studies have shown that p300 is involved in tumor proliferation, migration, and invasion (103). SIRT3 can eliminate lysine Kla, and cyclin E2 (CCNE2) acts as a substrate. SIRT3 induces apoptosis in HCC cells through regulating the Kla level of CCNE2 and prevents HCC growth *in vivo* (91). Therefore, further exploration of the mechanism of action of Kla and identification of Kla-related catalytic enzymes may provide more targets for tumor treatment.

In addition to the enzymes related to lactate metabolism and Kla, the identification of an increasing number of Kla and their

TABLE 1 The regulation of lactylation in cancer cells.

| Cancers | Lactylated protein(s)/site(s) | Function | References |
|---|---|---|---|
| Prostate cancer | HIF-1α | Promote angiogenesis | (66) |
| | H3K18 | Neuroendocrine differentiation | (90) |
| T-cell acute lymphoblastic leukemia | ALDOA | Metabolic Reprogramming | (47) |
| Colorectal cancer | ALDOA | Metabolic Reprogramming | (47) |
| Liver cancer | AK2 | Regulate p53 pathway and lead to poor prognosis | (48) |
| | CCNE2 | Promote tumor proliferation | (91) |
| | H3K9la, H3K56la | Promote tumorigenesis | (92) |
| Non-small cell lung cancer | H3 | Inhibit glycolysis | (11) |
| | H3K18 | Neuroendocrine differentiation | (90) |
| Ocular melanoma | H3K18 | Upregulate the oncogene YTHDF2 and promote tumorigenesis | (12) |
| Anaplastic thyroid cancer | H4K12 | Cause cell cycle dysregulation and promote tumor proliferation | (93) |
| Clear Cell Renal Cell Carcinoma | H3K18 | Activate the transcription of PDGFRβ and promote tumor proliferation | (94) |
| Breast cancer | H3K9,18,23,27,56,122<br>H4K5,8,12,31,77,91 | N/A | (9) |
| Cervical cancer | H3K9,18,23,27,56,122<br>H4K5,8,12,31,77,91<br>H2AK11,13,115<br>H2BK5,11,15,16,20,23,43,85,<br>108,116,120 | N/A | (9) |

HIF-1α, Hypoxia-inducible factor-1alpha; ALDOA, aldolase A; AK2, adenylate kinase 2; CCNE2, cyclin E2; YTHDF2, Human YTH domain family 2; PDGFRβ, platelet-derived growth factor receptor β.
N/A means "not applicable". It means that the function of these Kla sites has not been reported.

regulatory sites opens up new directions for tumor therapy. The BRAFV600E oncogene has been shown to increase the level of the intracellular protein Kla through enhancing glycolytic flux. It can promote the proliferation of anaplastic thyroid cancer through inducing H4K12 Kla, mediating driver gene transcription, and cell cycle dysregulation (93). The ninth member of the proprotein convertase family, subtilisin-kexin type 9 (PCSK9), maintains lipoprotein homeostasis. Alterations in PCSK9 expression have been linked to carcinogenesis and progression (104). The levels of lactate, protein Kla, and macrophage migration inhibitory factor (MIF) in colon cancer cells were found to have significantly increased after PCSK9 overexpression, which inhibited the polarization of M1 macrophages and promoted the occurrence and development of colon cancer, providing a potential therapeutic method for the clinical control of colon cancer (33). Pan et al. (92) identified a triterpene antitumor drug that inhibited the carcinogenesis of liver cancer stem cells through interfering with H3K9la and H3K56la Kla. Inactive von Hippel-Lindau (VHL) protein is associated with metabolic reprogramming and is important in the development of clear cell renal cell carcinoma (ccRCC) (105). VHL inactivation promotes the progression of ccRCC through triggering histone Kla to activate the transcription of platelet-derived growth factor receptor β (PDGFRβ). Meanwhile, PDGFRβ signaling in turn stimulates histone Kla, thereby forming a positive feedback loop for carcinogenesis in ccRCC. The combined reduction in histone Kla and PDGFRβ signaling was shown to substantially increase the therapeutic efficacy. This suggests that addressing the positive feedback loop between histone Kla and PDGFRβ signaling could be a viable treatment for patients with ccRCC (94). Additionally, histone Kla could stimulate the production of YT521-B homology domain family member 2 (YTHDF2), which leads to the degradation of m6A-modified period1 and TP53 and accelerates the development of ocular melanoma (12).

It has also been reported that Kla has a role in the neuroendocrine transition of adenocarcinoma (90). Neuroendocrine differentiation in adenocarcinoma is an important cause of resistance to tumor treatment (106). Most neuroendocrine cancer cells are glycolytic in nature and contain fragmented mitochondria with low membrane potential (107). Numb plays a crucial role in mitochondrial quality control by attaching to Parkin and facilitating Parkin-mediated mitophagy (90). In prostate and lung adenocarcinomas, loss of the Numb/Parkin pathway results in metabolic reprogramming marked by a significant increase in lactate generation, which in turn causes an increase in histone Kla and the activation of the transcription of genes linked to neuroendocrine function. Collectively, Numb/Parkin is a promising therapeutic target for modulating cancer cell plasticity by regulating histone Kla.

At present, some drugs targeting lactate metabolism have entered clinical trials. Lonidamine (LND), a dechlorinated derivative of indolazole-3-carboxylic acid, can destroy tumor cells by decreasing lactate production and pyruvate uptake in mitochondria and interrupting the mitochondrial transmission chain. LND does not have good anticancer activity when used alone, but it has been widely researched in combination with standard chemotherapy for the treatment of solid tumors (108). Madrid et al. (109) revealed that the combination of LND and cisplatin was more effective than cisplatin alone in reducing tumor growth in MX-1 breast cancer and A2780 ovarian cancer. Dichloroacetate (DCA) is an oral small-molecule medication that reduces tumor growth by inhibiting pyruvate dehydrogenase kinase and encouraging glucose oxidation rather than glycolysis (110). DCA has shown promising efficacy in both recurrent glioblastoma and locally advanced head and neck squamous cell carcinoma (111, 112). AZD3965, a dual MCT1 and MCT2 inhibitor, has previously been shown to be effective in the treatment of breast cancer (113). A recently completed phase 1 clinical trial validated the safety and efficacy of AZD3965 in patients with advanced solid malignancies and lymphoma (114). As a result, we anticipate that investigating Kla will provide more possibilities for tumor treatment.

## 5.2 Lactylation affecting immunotherapy

Kla is crucial in immunotherapy. The therapeutic efficacy of programmed cell death protein 1 (PD-1) blocking therapy is determined by the competition for the reactivation of PD-1-expressing CD8+ T cells and Treg cells in TME (115). Nishikawa et al. (4) showed that Treg cells express more PD-1 than effector T cells in highly glycolytic malignancies. Under low-glucose conditions, Treg cells rapidly absorb lactate through MCT1, stimulating the translocation of the nuclear factor of activated T cell 1 (NFAT1) into the nucleus and consequently increasing PD-1 expression, whereas effector T cells suppress PD-1 expression. Treatment failure has been reported to occur because PD-1 inhibition stimulated PD-1-expressing Treg cells. Furthermore, studies have shown that lactate can also promote the expression of PD-L1 on macrophages and neutrophils to mediate immune resistance (116, 117). Preclinical studies have indicated that combining the MCT1 inhibitor AZD3965 with anti-PD-1 therapy lowers lactate release into the TME and boosts anti-tumor immunity (118). Lu et al. (10) found that Kla levels in the Treg cells of patients with HCC who responded to anti-PD-1 therapy were lower. Moreover, the combination of anti-PD-1 drugs and lactate dehydrogenase inhibitors has a stronger antitumor effect than anti-PD-1 drugs alone. This indicates that combining Kla inhibition with immunotherapy may be effective in treating tumors.

Kla has been found to be abundant on histone and nonhistone proteins in tumor cells, and it is linked to a poor prognosis. Therefore, exploration of Kla and its regulatory sites could help identify additional safe and effective therapeutic targets for cancer treatment and provide new directions for combination therapies.

# 6 Conclusions and future perspectives

Kla plays an important role in tumor metabolic reprogramming, angiogenesis, immune escape, and regulation of intestinal flora. It has also been linked to the occurrence and progression of malignancies. However, Kla-targeting techniques and clinical translation remain in their infancy. Numerous issues have yet to be addressed, such as, the need to investigate the

synergistic or antagonistic effects of lactate and other epigenetic modifications to better understand the mechanism of tumor metabolic reprogramming, the need to investigate more nonhistone Kla sites to identify potential "writers" and "erasers," and the need to investigate the effect of Kla on immune cells for improving the safety and effectiveness of immunotherapy. Targeted suppression of Kla coupled with standard chemotherapy, radiotherapy, and immunotherapy can be expected to provide additional cancer treatment options in the future.

## Author contributions

Conceptualization: YX and XJ. Methodology: JS. Software: ZZ. Validation: YX, and XJ. Formal analysis: SC. Investigation: CB. Resources: XJ. Data curation: ZZ. Writing-original draft preparation: JS and HY. Writing-review and editing: JS and YX. Visualization: XJ. Supervision: XJ. Project administration: XJ. Funding acquisition: XJ. 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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# Glossary

| | |
|---|---|
| PTMs | post-translational modifications |
| Kla | Lactylation |
| GPR81 | Gi-protein-coupled receptor 81 |
| TME | tumor microenvironment |
| HDAC | histone deacetylase |
| CBP | CREB-binding protein |
| HDAC | histone deacetylase |
| SIRT | sirtuins |
| La | lactyl;' |
| NSCLC | non-small cell lung cancer |
| ATP | adenosine triphosphate |
| NADH | nicotinamide adenine dinucleotide |
| TCA | tricarboxylic acid |
| LDH | lactate dehydrogenase |
| AMPK | AMP-activated protein kinase |
| AKT | activating protein kinase B |
| KLF4 | kruppel-like factor 4 |
| NF-κB | nuclear factor-kappa B |
| MCT1 | moncarboxylate transporter 1 |
| TAM | tumor-associated macrophages |
| VEGF | vascular endothelial-derived growth factor |
| Arg1 | arginase 1 |
| CTLs | cytotoxic T lymphocytes |
| NK | Natural killer |
| NKT | Natural killer T |
| IFNγ | interferon-γ |
| IL | interleukin |
| PDK | pyruvate dehydrogenase kinase |
| DCA | dichloroacetic acid |
| MGO | methylglyoxal |
| LGSH | lactoylglutathione |
| GLO1 | glyoxalase 1 |
| ALDOA | aldolase A |
| AK2 | adenylate kinase 2 |
| HCC | hepatocellular carcinoma |
| HIF-1α | hypoxia-inducible factor alpha |
| PHD | proline hydroxylase |
| ENO1 | enolase |
| HK-1 | hemokinin-1 |
| PKM | pyruvate kinase M |
| SDHA | succinate dehydrogenase |
| IDH3G | isocitrate dehydrogenase 3 gamma |
| TGF | transforming growth factor |
| LPS | lipopolysaccharide |
| PI3K | phosphatidylinositol-3 kinase |
| TIMs | tumor-infiltrating myeloid cells |
| METTL3 | methyltransferase-like 3 |
| JAK1 | janus kinase |
| PykF | pyruvate kinase I |
| HP | helicobacter pylori |
| TLR | toll-like receptors |
| YY1 | yin-yang 1 |
| SDH | succinate dehydrogenase |
| IDH | lactate dehydrogenase |
| CCNE2 | cyclin E2 |
| PCSK9 | proprotein convertase subtilisin-kexin type 9 |
| MIF | macrophage migration inhibitory factor |
| VHL | von Hippel-Lindau |
| ccRCC | clear cell renal cell carcinoma |
| PDGFRβ | platelet-derived growth factor receptor β |
| YTHDF2 | YT521-B homology domain family member 2 |
| PD-1 | programmed cell death protein 1 |
| NFAT1 | nuclear factor of activated T cells 1 |
| MDSCs | myeloid-derived suppressor cells |
| STAT3 | signal transducer and activator of transcription 3 |
| FoxP3 | Forkhead/winged helix transcriptional factor P3 |
| MHC | major histocompatibility complex |
| LND | lonidamine |

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