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MiR-10b-5p Impairs TET2-Mediated Inhibition of PD-L1 Transcription Thus Promoting Immune Evasion and Tumor Progression in Glioblastoma

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Glioblastoma (GBM) is a highly aggressive primary brain tumor that shows intratumoral heterogeneity at the cellular and molecular level. Activation of programmed death receptor 1 (PD-1) interaction with its ligand PD-L1 is a well-known mechanism requisite for immune evasion deployed by malignant tumors including GBM. Herein, we set out to dissect the mechanism explaining the regulation of PD-L1 gene expression in GBM. The clinical samples consisted of 37 GBM tissues and 18 normal brain tissues. GBM cell model was treated by microRNA (miRNA) inhibitor, DNA constructs, and siRNAs. Assays of CCK-8 and Transwell insert were employed to assess the survival, migratory and invasive ability of GBM cell model. The immunosuppressive factor production, T cell apoptosis, and T cell cytotoxicity to GBM cells were evaluated in the co-culture system. GBM exhibited more miR-10b-5p abundance than normal at both tissue and cellular level. Suppression of miR-10b-5p weakened the ability of GBM cell model to survive, migrate, and invade, decreased the release of immunosuppressive factors, reduced T cell apoptosis, and strengthened the T cell cytotoxicity to GBM cell model. MiR-10b-5p conferred a negative control of Ten-eleven translocation 2 (TET2) that was downregulated in GBM. The functions of miR-10b-5p on GBM cell aggressiveness and immune evasion were mediated by TET2. TET2 recruited histone deacetylases HDAC1 and HDAC2 into the PD-L1 promoter region thus inhibiting its transcription. The study demonstrated the importance of miR-10b-5p-mediated repression of TET2 in PD-L1-driven immune evasion and their potential for immunotherapeutic targeting in GBM.

Keywords: glioblastoma; immune evasion; miR-10b-5p; PD-L1; TET2
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Introduction

Gliomas constitute a vast group of malignant tumors primarily occurring in the adult central nervous system (CNS) and encompass multiple subtypes with unique characteristics and varying descriptive epidemiology, contributing to substantial morbidity and mortality on a global scale (Miller et al. 2021). Glioblastoma (GBM) is the most commonly diagnosed subtype of gliomas and categorized as a grade 4 astrocytoma by the World Health Organization (WHO), accounting for about 57% of all gliomas and 48% of all primary malignant CNS tumors (Tan et al. 2020; Thakur et al. 2022). The incidence of GBM increases with advancing age, with a peak incidence occurring at 75 to 84 years of age and a slight predominance in men (Grochans et al. 2022). Despite much improvement in multimodal therapies for GBM incorporating surgical excision, radiotherapy, and adjuvant chemotherapy with temozolomide, the long-term survival of at least 2 years from diagnosis is rare (Gately et al. 2018; Verdugo et al. 2022). Due to extensive heterogeneity at cellular and molecular levels, GBM exhibits diversified phenotypes with multifaceted molecular signatures in each tumor mass, with a main hurdle in identification of a specific therapeutic target (Cruz et al. 2022). In addition to standard treatment paradigm, ongoing studies investigate the effectiveness of several immunotherapeutic interventions, such as immune checkpoint inhibitors and/or oncolytic virotherapy, which have provided new opportuni-

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ties to improve GBM outcomes (Fekrirad et al. 2022; Rong et al. 2022). However, there are limited phase III clinical trials convincing the GBM immunotherapy due to profound GBM-mediated immunosuppression or the presence of chemical and physical barriers preventing successful delivery of most anti-tumor drugs into the target in appreciable amounts (Lechpammer et al. 2022).

T cell development can ensure an exquisite balance between immune tolerance and immune surveillance while keeping immunological memory (Kumar et al. 2018). Programmed death protein-1 (PD-1) is expressed on the surface of cancer-specific cytotoxic lymphocytes and binds to its ligand, programmed cell death ligand 1 (PD-L1) harbored by tumor cells, leading to T cell apoptosis and functional exhaustion followed by tumor cell immune invasion (Kumagai et al. 2020). Blockade of the PD-1/PD-L1 axis as one of immune checkpoint inhibitor therapies has achieved unprecedented success in clinical applications (Diskin et al. 2020). A histopathological analysis based on cancerous brain tissue samples collected from 235 patients with gliomas showed 31.5% of tumor-infiltrating lymphocytes (TILs) expressing PD-1 protein and 6.1% of tumors expressing PD-L1 protein. Higher PD-1 and PD-L1 expressions were more likely observed in higher grade tumors (Garber et al. 2016). GBM cells can express PD-L1 protein when exposed to interferon-gamma (IFN-γ) in the tumor microenvironment and activate PD-1/PD-L1 checkpoint, which explain the occurrence of immunosuppressive escape during an anti-tumor immune response (Jung et al. 2022). Therefore, dissecting the mechanisms behind PD-L1 expression on cancer cells is a critical step to develop immunotherapies blocking PD-1/PD-L1 checkpoint activation and prevent immune evasion of cancer cells. A previous study found that Ten-eleven translocation 2 (TET2) deficiency may confront with anti-PD-L1 therapy in murine melanoma and colon tumor cells (Xu et al. 2019). High miR-10b-5p expression were correlated with poor patient outcome in GBM, and transgenic expression of miR-10b-5p enhances the stem cell phenotype of GBM cells by targeting TET2 expression from previous evidence (Lopez-Bertoni et al. 2022). Therefore, we proposed a hypothesis that miR-10b-5p could mediate the repression of TET2 to promote PD-L1 transcription, thus helping immune evasion deployed by GBM cells. In this work, we attempt to reveal the mechanisms behind PD-L1 gene activation in GBM, in a bid to enhance the responsiveness to anti-PD-L1 therapy in GBM.

Materials and Methods

Ethics approval and consent to participate

The study protocols were approval by the Ethics Committee of the General Hospital of Central Theater Command. All participants provided written informed consent to participate in the study.

Clinical sample collection

The study included cancerous brain tissues originated from 37 patients undergoing surgical resection for GBM in the General Hospital of Central Theater Command, from January 2020 to July 2022. GBM was first diagnosed by enhanced magnetic resonance imaging (MRI) prior to surgery and then pathologically confirmed after surgical resection. No patient had been treated by radiotherapy or chemotherapy prior to surgery. In addition, normal brain tissues in the non-functional area were collected from 18 patients undergoing intracranial decompression during the same period and same hospital.

Cell culture

Human GBM cell lines (U87-MG, U251, A127) and normal human astrocytes (NHA), all provided by ATCC (Manassas, VA, USA), were maintained in the Dulbecco's Modified Eagle Medium (DMEM) in a 5% CO_2 incubator (CB60, Binder, Berlin, Germany) at 37°C. The medium was added with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, San Jose, CA, USA), penicillin (50 μIU/mL) and streptomycin (50 μg/mL). Interferon-γ (IFN-γ, 100 ng/mL, Thermo Fisher Scientific) was added to induce PD-L1 expression.

miRNA, siRNA, DNA constructs, and transient transfection

The human full length TET2 was inserted into the pcDNA3.0 vector (Invitrogen, Carlsbad, CA, USA) and delivered into U251 cells for construction of TET2 overexpression cell model. The miR-10b-5p inhibitors and TET2 siRNA oligos (GenePharma, Shanghai, China) were used to construct miR-10b-5p inhibition cell model and TET2 knockdown cell model. For transient transfection, cells were transfected for 48 h using Lipofectamine 3000 (Invitrogen) in line with the instructions attached by the manufacturer.

Quantitative real-time PCR (qRT-PCR)

The Trizol reagents (Invitrogen) were used for total RNA isolation, and the M-MLV reverse transcriptase (Promega, Madison, WI, USA) was used for cDNA synthesis. Each transcript was quantified by qPCR using specific qPCR primers (Table 1) and shown with the $2^{-\Delta\Delta Ct}$ comparative method, using the SYBR Master Mixture (Takara, Tokyo, Japan) and the LightCycler 480 II System (Roche Diagnostics, Indianapolis, IN, USA). All data are normalized to either U6 or GADPH expressions.

Evaluation of cell viability

U251 cells were seeded into 96-well plates and harvested overnight. Viable U251 cells were examined at 0, 1, 2, 3, 4, and 5 by using the CCK-8 kits (Beyotime Biotechnology, Shanghai, China). The CCK-8 reaction solution (10 μl) was added to test and the optical density after 4 h.

Table 1. The qPCR primers.

Transcript	Forward primer (5' to 3')	Reverse primer (5' to 3')
miR-10b-5p	TACCCTGTAGATCCGAATTTGTG	ATTCCCCTAGATACGAATTTGTGA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
TET2	CACTGCATGTTTGGACTTCTG	TGCTCATCCTCAGGTTTTCC
TGF-β	AGCGACTCGCCAGAGTGGTTA	GCAGTGTGTTATCCCTGCTGTCA
VEGF	CTTCTGAGTTGCCCAGGAGA	GGATGGAGGAAGGTCAACCA
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA

Evaluation of cell migration and invasion ability

A Transwell insert (Corning Incorporated, Corning, NY, USA) equipped with polycarbonate filters with 8-μm pores between the apical chamber and the basolateral chamber was utilized to analyze the migratory ability of U251 cells. The apical chamber contained U251 cells suspended in 100 μL serum-free medium and the basolateral one contained 600 μL normal medium with 30% FBS. After 24 h, the vectorial transport of U251 cells were microscopically observed (Olympus, Tokyo, Japan). Migratory cells per field were recorded. The invasive capacity of U251 cells was evaluated using the same Transwell insert with same procedures except 1 mg/mL Matrigel coated on the polycarbonate filters.

Western blotting

Protein lysates were extracted using modified radioimmunoprecipitation (RIPA) buffer. The samples with identical amounts of protein were plied to gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, and then the separated protein bands were blotted onto the polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Billerica, MA, USA). The membrane was immunoblotted by using the primary antibody against TET2 (#36449, Cell Signaling Technology, Beverly, MA, USA), CD274/PD-L1 (#13684, Cell Signaling Technology), and GAPDH (#5174, Cell Signaling Technology). The GAPDH band was used for normalization.

Luciferase activity assays

The pMIR-Report luciferase reporter cassette spanning the human TET2 3′UTR containing binding sites of miR-10b-5p either being wild-type (pMIR-TET2-wt) or mutated (pMIR-TET2-mut) was provided by RiboBio (Guangzhou, China). The pMIR-TET2-wt or pMIR-TET2-mut was introduced into HEK293T cells (Procell Life Science & Technology Co., Ltd., Wuhan, China). The luciferase activity in the presence of miR-10b-5p inhibitor or inhibitor normal control (NC) was detected using the Dual-Luciferase assay kit (Promega) and analyzed using a GLomax20/20 Luminometer (Promega).

Co-culture experiments

At the beginning, Jurkat T cells were activated with 500 ng/ml ionomycin and then co-cultured with U251 cells undergoing different treatment protocols for 12 h at a ratio of 1:4 (U251 cells:T cells) for 24 h. The supernatants from the co-culture system were measured for concentrations of immunosuppressive factors, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE2), using the ELISA kits (R&D Systems, Minneapolis, MN, USA). The T cells were obtained from the co-culture system for their apoptosis with the aid of flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using Annexin V-FITC and propidium iodide (PI) stains. A plus of early-stage apoptosis (Annexin V+/PI− cells) and late-stage apoptosis (Annexin V+/PI+ cells) were used to estimate T cell apoptosis. The supernatant in the co-culture system of U251 cells and Jurkat T cells was collected to detect the activity of produced lactic dehydrogenase (LDH) by applying the LDH Cytotoxicity Assay Kit (ab197004, Abcam, Cambridge, UK). The LDH activity released reflected the cytotoxicity of T cells to U251 cells.

Chromatin immunoprecipitation (ChIP) assays

The ChIP assays were conducted by using the Simple ChIP Enzymatic Chromatin IP (Cell Signaling Technology). The DNA-protein cross-links were formed in U251 cells using 10% formaldehyde and then sonicated to gain chromatin fragments. The ChIP products were generated using anti-TET2 (#36449, Cell Signaling Technology), anti-H3K27ac (#4353, Cell Signaling Technology), anti-HDAC1 (#34589, Cell Signaling Technology), anti-HDAC2 (#57156, Cell Signaling Technology) or normal IgG (#5946, Cell Signaling Technology) and then obtained using protein Agarose/Sepharose beads. The qRT-PCR detection of the ChIP products and the corresponding inputs was performed to measure the enrichment levels of TET2, H3K27ac, HDAC1, and HDAC2 at promoter of PD-L1 using the primers (forward: 5'-CCCCAACCTGAATGAGCCAT-3' and reverse: 5'-GTGAGGGACGAAAAGGCAGA-3').

Statistical analysis

The outcomes were described using mean ± standard deviation once the data were verified for the normal distribution. The GraphPad prism 8.0 (GraphPad Software, San Diego, CA, USA) was employed to conduct statistical comparisons including unpaired t-test, one-way analysis of variance (ANOVA), repeated measures ANOVA, and figure creation. The level of statistical significance for all tests

was $P < 0.05$.

Results

Depletion of miR-10b-5p prevented GBM cell aggressiveness

We analyzed the raw data available in the GSE65626 and found 34 differentially expressed miRNAs consisting of 13 upregulated ones and 21 downregulated ones between GBM tissues and the matched adjacent normal brain tissues ($\log_2|FC| > 1$ and adjusted $P < 0.05$, Fig. 1A). It was found that miR-10b-5p was upregulated with the largest $\log_2FC$ ($\log_2FC = 4.56$) between GBM tissues and the matched adjacent normal brain tissues. Therefore, we chose to further analyze the role of miR-10b-5p in the context of GBM. More miR-10b-5p abundance was observed in 37 GBM tissues than 18 normal brain tissues, revealed by results of qRT-PCR ($P < 0.001$, Fig. 1B). It was shown that the expression of miR-10b-5p was correlated with the overall survival of patients with GBM ($P < 0.001$, Fig. 1C). Concordantly, GBM cell model exhibited more miR-10b-5p abundance than normal cell model ($P < 0.001$, Fig. 1D), and U251 cells showed a largest miR-10b-5p abundance. MiR-10b-5p inhibition cell model was prepared by delivery of miR-10b-5p inhibitor into U251 cells, and decreased miR-10b-5p expression was confirmed by qRT-PCR ($P < 0.001$, Fig. 1E). MiR-10b-5p inhibition cell model was demonstrated with reduced cell viability, compared with control cell mode, by CCK-8 detection of OD values at day 1 to day 5 ($P < 0.001$, Fig. 1F). MiR-10b-5p inhibition cell model also showed weaker ability to migrate and invade compared with control cell model ($P < 0.001$, Fig. 1G). It was suggested that high expression of miR-10b-5p made contribution to GBM progression.

MiR-10b-5p functions in GBM immune evasion

GBM cells are believed to secrete immunosuppressive factors thus preventing the killing effects of immune cells on tumor cells. Our next study focus was shifted to the investigation of miR-10b-5p functions in GBM immune evasion. As previous evidence shown, TGF-β, VEGF, and PGE2 have been regarded as tumor and host dependent immunosuppressive cytokines responsible for immune evasion (Nicolini and Carpi 2009). Accordingly, we first carried out the qRT-PCR and ELISA detections to quantify the production of several immunosuppressive factors released from U251 cells, including TGF-β, VEGF, and PGE2. The lower mRNA levels of TGF-β and VEGF ($P < 0.001$, Fig. 2A) as well as lower supernatant levels of TGF-β, VEGF, and PGE2 ($P < 0.001$, Fig. 2B) were detected in miR-10b-5p inhibition cell model when comparable to control cell model. Subsequently, we endeavored to examine T cell apoptosis in the co-culture system with U251 cells by flow cytometric analysis, with results determining fewer apoptotic T cells in the co-culture system with miR-10b-5p inhibition cell model than that with control cell model ($P <$

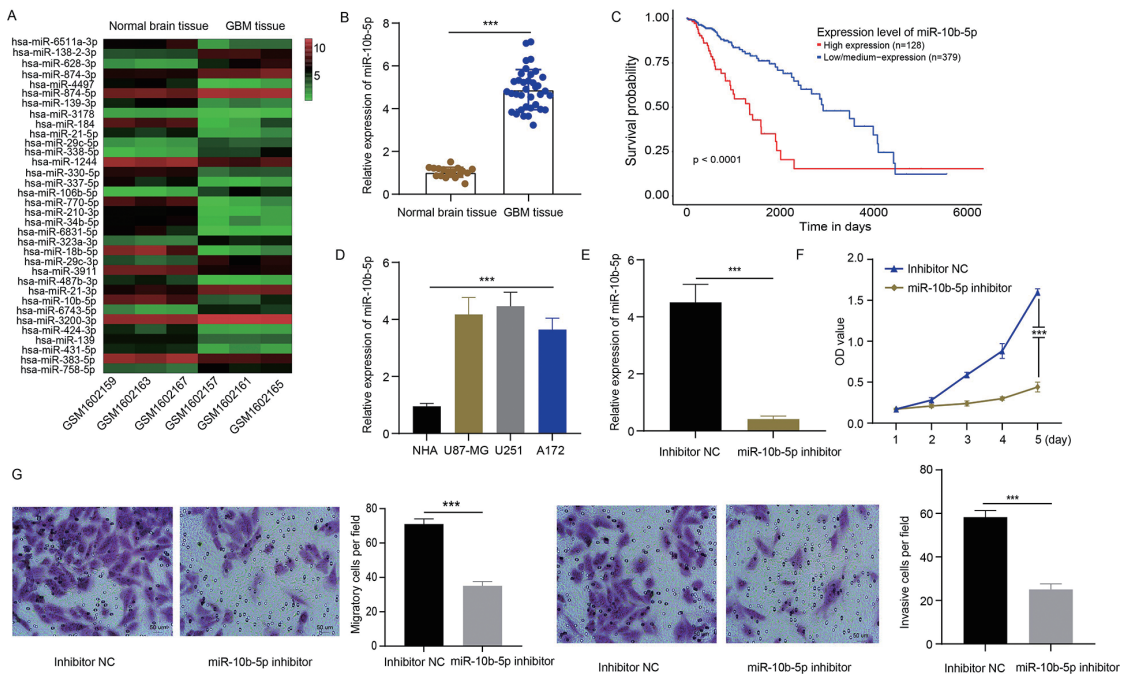

Fig. 1. Depletion of miR-10b-5p prevented GBM cell aggressiveness.
(A) A total of 34 differentially expressed miRNAs between GBM tissues and the matched adjacent normal brain tissues by analyzing the miRNA profiles in the GSE65626. (B) qRT-PCR detection of miR-10b-5p in normal brain tissues (n = 18) and GBM tissues (n = 37). (C) The survival analysis of miR-10b-5p in clinical samples of the Cancer Genome Atlas (TCGA). (D) qRT-PCR detection of miR-10b-5p in GBM cell models and normal cell model. (E) Verification of miR-10b-5p inhibition cell model. (F) Evaluation of viable U251 cells after miR-10b-5p inhibition. (G) Evaluation of migratory and invasive U251 cells per view (200 ×) after miR-10b-5p inhibition. ***$P < 0.001$ by unpaired t test for panel B, D, F, by one way ANOVA for panel C, and by repeated measures ANOVA for panel E.

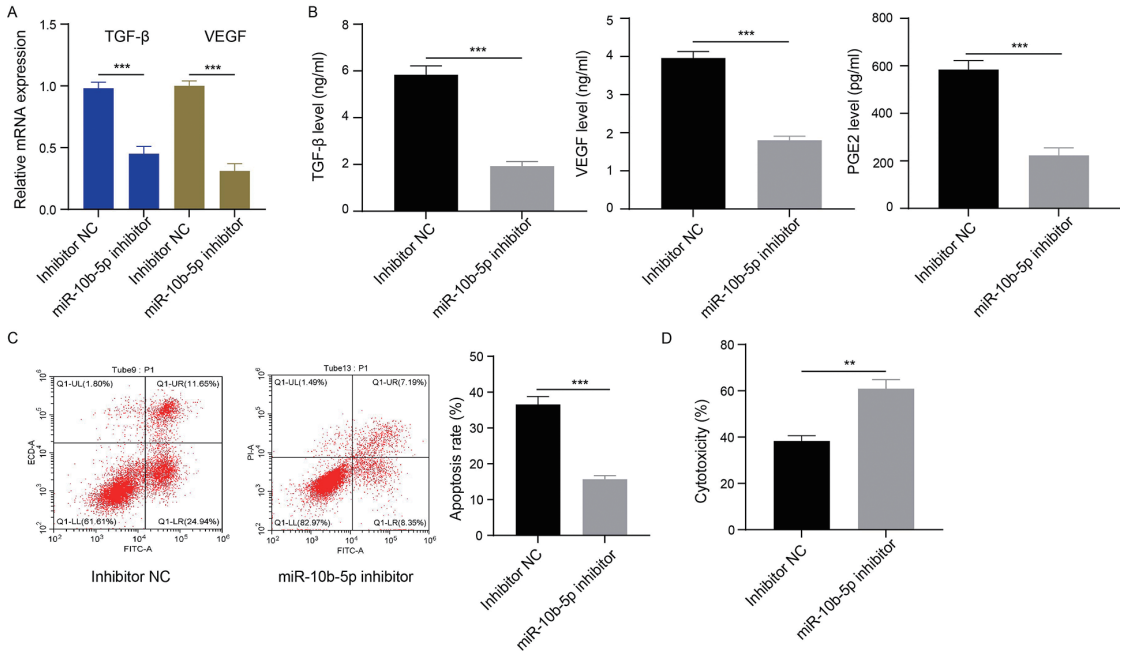

Fig. 2. MiR-10b-5p functions in GBM immune evasion.
(A) qRT-PCR detection of TGF-β and VEGF mRNA levels in the co-culture system with T cells and miR-10b-5p inhibition cell model. (B) ELISA method of supernatant levels of TGF-β, VEGF, and PGE2 in the co-culture system with T cells and miR-10b-5p inhibition cell model. (C) T cell apoptosis in the co-culture system by flow cytometric analysis. (D) Evaluation of T cell cytotoxicity to U251 cells by LDH activity assays. **P < 0.01 and ***P < 0.001 by unpaired t test for all panels.

0.001, Fig. 2C). Additionally, we detected the activity of released LDH in the co-culture system to evaluate the T cell cytotoxicity to U251 cells. Results revealed a stronger cytotoxicity from T cells to U251 cells in the co-culture system with miR-10b-5p inhibition cell model than that with control cell model (P = 0.01, Fig. 2D). It was suggested that depletion of miR-10b-5p could prevent the immune evasion in GBM.

MiR-10b-5p control of TET2 expression in GBM

TET2 directly targeted by miR-10b-5p is supported by i) miRNA-mRNA prediction among the TargetScan, miRDB, miRNA.org and DIANA databases all showing TET2 as the target gene of miR-10b-5p (Fig. 3A), and ii) luciferase activity assays with the observations that miR-10b-5p inhibitor transfection induced luciferase activity more than 2-fold in HEK293T transfected with pMIR-TET2-wt but exerted no effect in HEK293T transfected with pMIR-TET2-mut (P < 0.001, Fig. 3A). According to the principle of miRNA control of mRNA, upregulated miR-10b-5p concomitant with downregulated TET2 in the context of GBM is believed. As validated by qRT-PCR detection and western blotting of TET2 expressions in clinical brain samples, GBM tissues exhibited declined TET2 mRNA and protein expressions compared to the normal brain tissues (P < 0.001, Fig. 3B). The expression of TET2 was found to be correlated with the overall survival of patients with GBM (P < 0.0001, Fig. 3C). GBM cell model compared to the normal cell model (P < 0.001, Fig. 3D). MiR-10p-5p and TET2 expression levels were found to be inversely correlated in GBM tissues (r = −0.683, Fig. 3E). TET2 overexpression cell model was prepared by delivery of expression vector harboring the TET2 cDNA into U251 cells. When the TET2 siRNA sequence was introduced into U251 cells in the presence of miR-10b-5p inhibitor, no difference being statistically considered existed compared with the control vector. All these were confirmed by qRT-PCR (P < 0.001, Fig. 3F).

MiR-10b-5p affected GBM cell aggressiveness and immune evasion by modulating TET2

TET2 overexpression cell model was demonstrated with reduced cell viability, compared with control cell mode, by CCK-8 detection of OD values at day 1 to day 5 (P < 0.001, Fig. 4A), as well as weaker ability to migrate and invade compared with control cell model by Transwell chamber assays (P < 0.001, Fig. 4B). Next, we found lower mRNA levels of TGF-β and VEGF (P < 0.001, Fig. 4C) concurrent with lower supernatant levels of TGF-β, VEGF, and PGE2 (P < 0.001, Fig. 4D) in TET2 overexpression cell model than control cell model. These levels did not differ in cell model when co-transfection of miR-10b-5p inhibitor and TET2 siRNA sequence compared to control cell model. As for analysis of T cell apoptosis in the co-culture system, we found fewer apoptotic T cells in the co-culture system with TET2 overexpression cell model than that with control cell model (P < 0.001, Fig. 4E). No difference concerning T cell apoptosis was statistically found in the co-culture

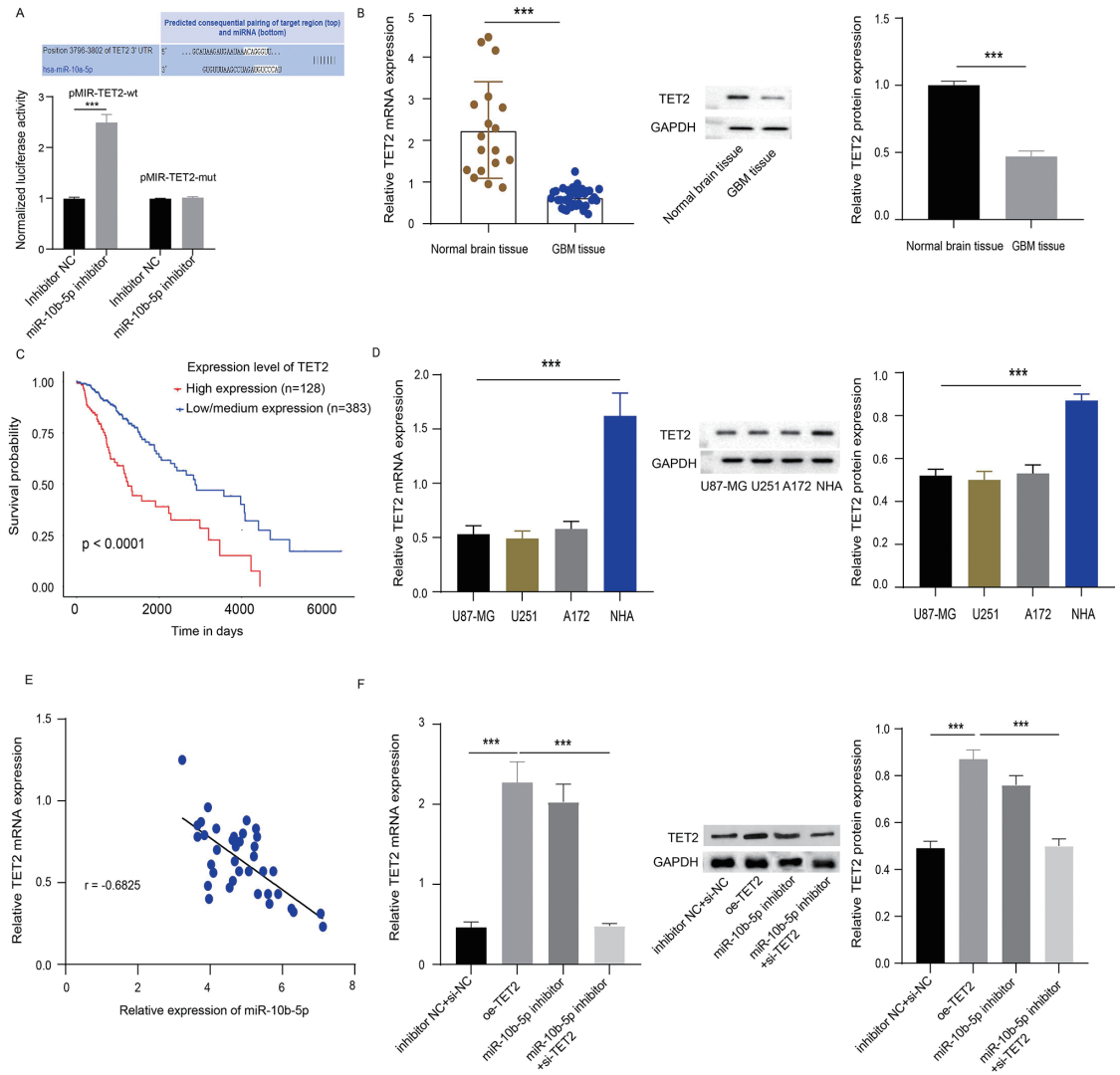

Fig. 3. MiR-10b-5p control of TET2 expression in GBM.
(A) The effects of miR-10b-5p inhibitor transfection on the luciferase activity in HEK293T transfected with either pMIR-TET2-wt or pMIR-TET2-mut. (B) qRT-PCR detection of TET2 mRNA level as well as western blotting of TET2 protein in normal brain tissues (n = 18) and GBM tissues (n = 37). (C) The survival analysis of TET2 in clinical samples of TCGA. (D) qRT-PCR detection of TET2 mRNA level as well as western blotting of TET2 protein in GBM cell models and normal cell model. (E) Pearson correlation analysis of miR-10b-5p and TET2 levels in the GBM tissues. (F) qRT-PCR detection of TET2 mRNA level as well as western blotting of TET2 protein in U251 cells after TET2 overexpression or miR-10b-5p inhibition plus TET2 knockdown. ***P < 0.001 by unpaired t test for panel A, B, and by one way ANOVA for panel C and E.

system between miR-10b-5p inhibitor plus TET2 siRNA sequence and control vector. In light of T cell cytotoxicity to U251 cells, an enhanced killing effect of T cells on U251 cells was demonstrated in the co-culture system with TET2 overexpression cell model than that with control cell model ($P < 0.001$, Fig. 4F). Not surprisingly, neither weaker nor stronger cytotoxicity from T cells to U251 cells was examined in the co-culture system between miR-10b-5p inhibitor plus TET2 siRNA sequence and control vector. These data suggested that miR-10b-5p-mediated inhibition of TET2 elicits GBM cell aggressiveness and immune evasion.

The involvement of the miR-10b-5p/TET2/PD-L1 axis in regulating GBM cell aggressiveness and immune evasion

PD-L1 is requisite for tumor immune evasion in some human cancers. Although TET2 can actively causes demethylation of 5-methylcytosine (5mC), previous evidence showed that TET2 was associated with epigenetic alternations of PD-L1 dependent on its histone deacetylase activity, especially on the H3K27ac, rather than 5mC dioxygenase activity (Shen et al. 2021). Given that, we decided to investigate whether TET2 affected the expression of PD-L1 through its histone deacetylase activity in GBM. At the beginning, we demonstrated that the PD-L1 expression was declined in TET2 overexpression cell model and miR-

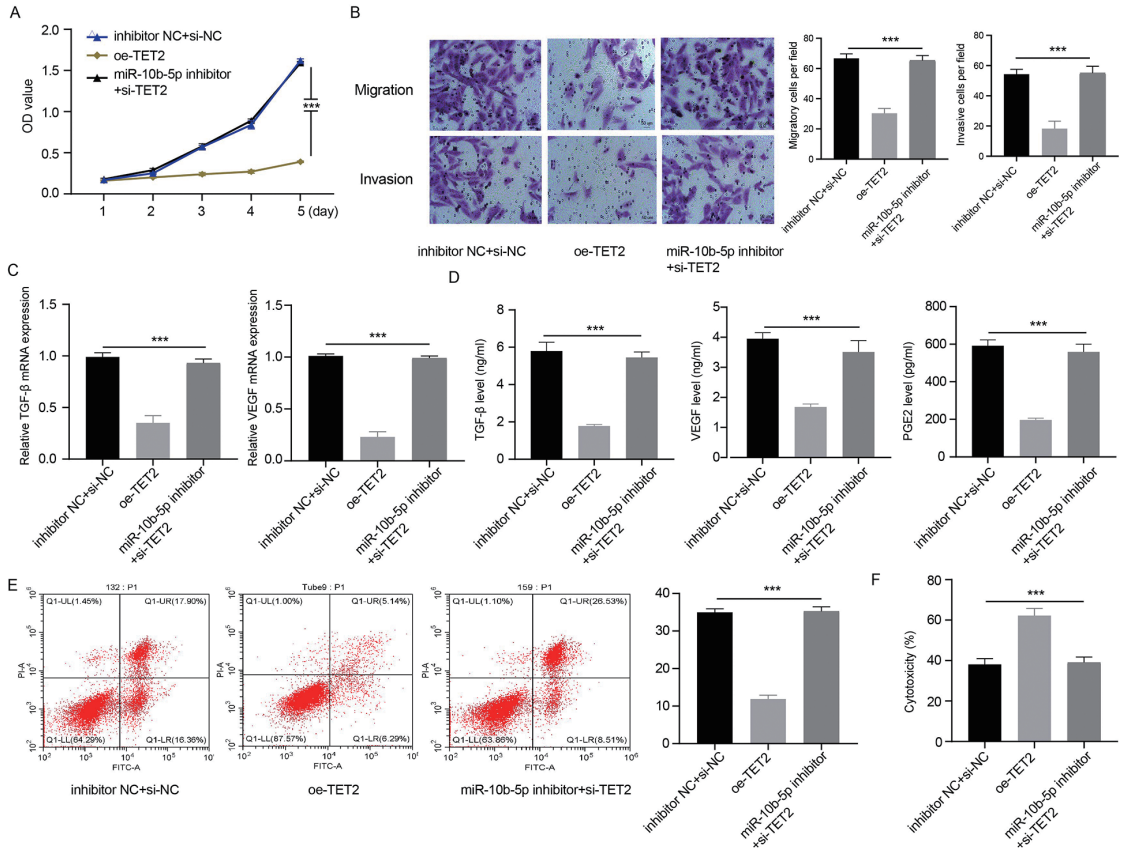

Fig. 4. MiR-10b-5p affected GBM cell aggressiveness and immune evasion by modulating TET2.
(A) Evaluation of viable U251 cells after TET2 overexpression or miR-10b-5p inhibition plus TET2 knockdown. (B) Evaluation of migratory and invasive U251 cells per view after TET2 overexpression or miR-10b-5p inhibition plus TET2 knockdown. (C) qRT-PCR detection of TGF-β and VEGF mRNA levels in the co-culture system with T cells and U251 cells (TET2 overexpression or miR-10b-5p inhibition plus TET2 knockdown). (D) ELISA method of supernatant levels of TGF-β, VEGF, and PGE2 in the co-culture system with T cells and U251 cells (TET2 overexpression or miR-10b-5p inhibition plus TET2 knockdown). (E) T cell apoptosis in the co-culture system by flow cytometric analysis. (F) Evaluation of T cell cytotoxicity to U251 cells by LDH activity assays. ***P < 0.001 by repeated measures ANOVA for panel A and by one way ANOVA for panel B-F.

10b-5p inhibition cell model than control cell model and cell model with combined transfection, as evidenced by western blotting results (P < 0.001, Fig. 5A). The ChIP assays were performed for further validation. Our ChIP-qPCR using anti-TET2 antibody demonstrated the TET2 occupancy at the promoter of PD-L1 (P < 0.001, Fig. 5B) in control cell model, with less TET2 occupancy determined in TET2 overexpression cell model and miR-10b-5p inhibition cell model. The ChIP assays detected reduced H3K27ac enrichments at the PD-L1 promoter in TET2 overexpression cell model and miR-10b-5p inhibition cell model than control cell model and cell model with combined transfection (P < 0.001, Fig. 5C). Subsequent ChIP-qPCR analysis of histone deacetylases HDAC1 and HDAC2 demonstrated TET2 overexpression and miR-10b-5p inhibition led to higher binding of HDAC1 and HDAC2 to the PD-L1 promoter in U251 cells (P < 0.001, Fig. 5D). These data revealed that TET2 recruited histone deacetylases into the PD-L1 promoter region thus inhibiting its transcription.

Discussion

Tumor-immune system interplay in GBM is complex, which hinders the development of immunotherapies (Cao et al. 2022). One of important findings in our study is that loss of TET2 function enhanced PD-L1 expression stimulated by IFN-γ, enabling GBM cells to flight with anti-tumor immunity together with lowering the effectiveness of anti-PD-L1 therapy. Our results demonstrated miR-10b-5p-mediated repression of TET2 in PD-L1-driven immune evasion and their potential for immunotherapeutic targeting in GBM. TET2 was associated with epigenetic alternations of PD-L1 dependent on its histone deacetylase activity rather than 5mC dioxygenase activity. TET2 inhibited the PD-L1 transcription by recruitment of histone deacetylases HDAC1 and HDAC2 into its promoter region, providing a mechanistic explanation of TET2-mediated anti-tumor immunity.

Under pathological conditions, frequent mutations in TET genes have been reported in hematopoietic malignancy

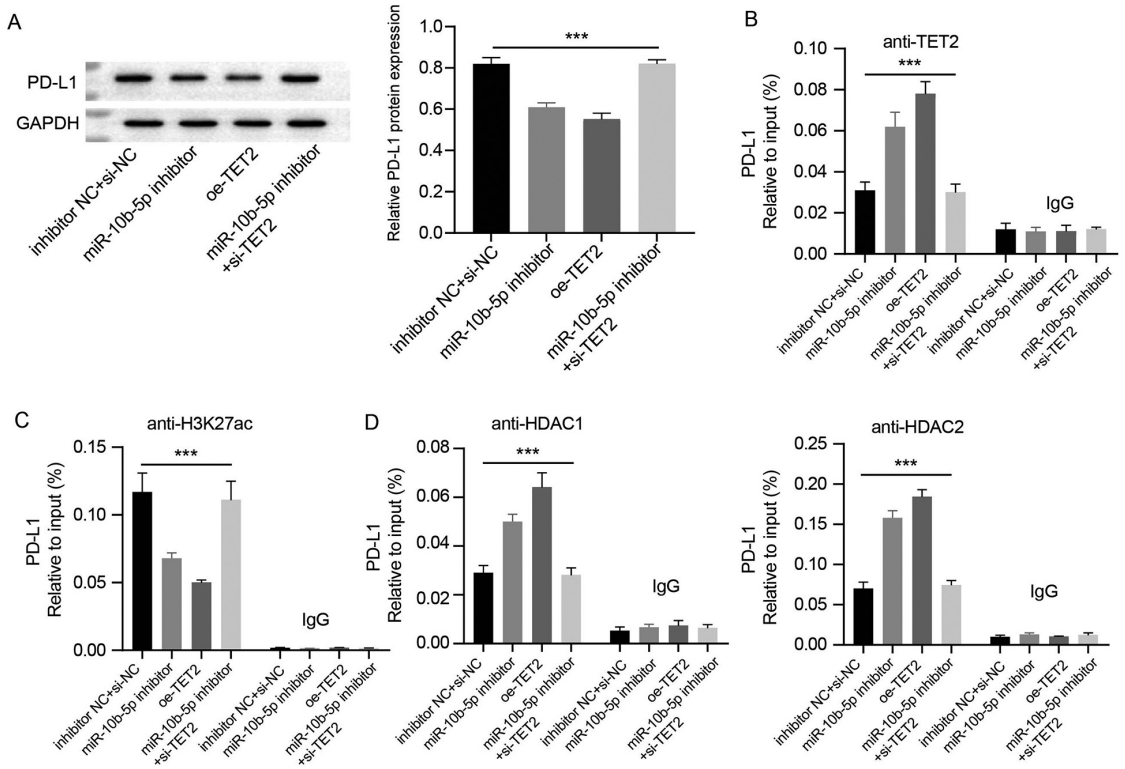

Fig. 5. The involvement of the miR-10b-5p/TET2/PD-L1 axis in regulating GBM cell aggressiveness and immune evasion. (A) Western blotting of PD-L1 protein in different cell models. (B) ChIP-qPCR of the TET2 occupancy at the promoter of PD-L1 in different cell models. (C) ChIP-qPCR of the H3K27ac occupancy at the promoter of PD-L1 in different cell models. (D) ChIP-qPCR analysis of the binding of HDAC1 and HDAC2 to the PD-L1 promoter in different cell models. ***$P < 0.001$ by one way ANOVA for all panels.

of both myeloid and lymphoid lineages (Pethusamy et al. 2022). Lose-of-function mutation in the TET2 gene is deemed as one of frequently occurring genetic variations resulting in an abnormal self-renewal of hematopoietic stem cells during the development of hematopoietic malignancy (Cobo et al. 2022). In approximately 20% of acute myeloid leukemia patients showing exclusive manner with TET2 mutations, TET2 enzyme is believed to be catalyzed and inactivated by d-2-hydroxyglutarate which is an oncometabolite as a result of isocitrate dehydrogenases 1 and 2 mutations (Figueroa et al. 2010). Loss or inactivation of TET2 is emerging as an oncogenic event and thus enhance the invasiveness of cancer stem cells and aggressive phenotypes in GBM (Kraus et al. 2015). TET2-mediated DNA hydroxymethylation was demonstrated as a key mechanism elucidating the pathogenesis of gliomas (Bragiel-Pieczonka et al. 2022). Chen et al. (2017) showed the glioma grade advanced as the TET2 expression decreased partially due to Zinc finger E-box-binding homeobox 1 in non-stem-like cell GBM models. When concerning TET2 involvement in lymphoid and myeloid cell development and its functional roles, we discussed how TET2 activities are modulated by microRNAs. As previously reported by Ren and Xu (2019), TET2 was identified as a downstream gene of miR-19a-5p and inhibited GBM cell proliferation and metastasis. We found, during the development of GBM, TET2 directly targeted by miR-10b-5p, and high expression of miR-10b-5p made a contribution to GBM progression and enabled GBM evading from anti-tumor immunity, in agreement with a previous study (Lopez-Bertoni et al. 2022). MiR-10b-5p is downregulated (or not expressed) in normal brain tissues while being upregulated in cancerous brain tissues of GBM patients. Importantly, overexpression of miR-10b-5p may be closely linked with the aggressiveness as well as immune evasion occurring in GBM. Concurring with the notion that therapeutic potential of miR-10b-5p in GBM, El Fatimy et al. (2017) reported the miR-10b edited by the CRISPR-Cas9 system exerted inhibitory effects on GBM. As they demonstrated, loss of miR-10b-5p was demonstrated to repress the survival of GBM cell cultures along with the tumor growth of mouse GBM xenografts. In line with this information, we believed, with the aid of the antagomiRNA approach, miR-10b-5p can serve as a pharmaceutical target for GBM treatment (Ananta et al. 2016; Teplyuk et al. 2016).

TET2 mutations is also found to be associated with lineage specification of conventional and unconventional T cells, such as invariant natural killer T cells, myeloid cell, and B-cell, indicating previse biological role of TET2 in immune response (Aijo et al. 2022). TET2 deficiency in regulatory T cells resulted in a lethal hyperproliferation, promoted Foxp3 destabilization, and caused conversion into

IL-17-producing cells in the spleen and mesenteric lymph nodes (Nakatsukasa et al. 2019). IL-17-expressing Tregs were found in the brain tissues obtained from patients with high-grade glioma, which could suppress the anti-tumor activity of CD8(+) T cells by producing IL-17 and TGF-β expression (Liang et al. 2014). Apart from their mediation on the DNA demethylation activity, increasing evidence shows that TET proteins recruit HDACs into the promoter region of the target genes causing transcriptional repression in immune cells (Zhang et al. 2015; Xue et al. 2016; Tanaka et al. 2020). Expectedly, our findings showed that recruitments of HDAC1/2 by TET2 leading to the deacetylation of H3K27ac at PD-L1 promoter explained its inhibition on PD-L1 gene expression in GBM. Concerning the poor outcomes yielded by anti-PD-1/L1 immunotherapy in GBM, we believe that targeting TET2 with anti-PD-L1 immunotherapy combined with the inhibitors of HDAC and may produce promising results for improving GBM patients with limited response to anti-PD-1/L1 immunotherapy.

In conclusion, the data obtained from our work support the concept that PD-L1 gene transcription may results from changes of TET2-HDAC1/2 complex due to more miR-10b-5p abundance in the context of GBM. Of note, the molecular mechanism through which TET2 inhibits PD-L1 gene transcription in GBM should be validated by MeDIP-qPCR and hMeDIP-qPCR using anti-5mC antibody and anti-5hmC antibody, and the enrichments of other well-studied histone modifications, such as H3K4me3 and H3K27me3 at the PD-L1 promoter region. More efforts are warranted centering to the regulatory mechanism and functional role of TET2/PD-L1 axis in the anti-tumor immunity using more cell lines or experimentally constructed mouse GBM xenografts. Further investigations based on more follow-up data from our institution will be required to validate the prognostic role of miR-10b-5p and TET2 in GBM.

Availability of Data and Materials

The dataset (accession number: GSE65626) supporting the conclusions of this article is available in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE65626). Other data analyzed during this study are included in this published article.

Author Contributions

D.W. conceived the study and wrote the first draft of manuscript. C.D.Y., W.K., and Y.D. contributed to data collection. G.Z.Q. and X.G.Z. were involved in data analysis and visualization. Y.G.J. completed manuscript revision. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

References

Aijo, T., Theofilatos, D., Cheng, M., Smith, M.D., Xiong, Y., Baldwin, A.S. & Tsagaratou, A. (2022) TET proteins regulate T cell and iNKT cell lineage specification in a TET2 catalytic dependent manner. *Front. Immunol.*, **13**, 940995.

Ananta, J.S., Paulmurugan, R. & Massoud, T.F. (2016) Tailored nanoparticle codelivery of antimiR-21 and antimiR-10b augments glioblastoma cell kill by Temozolomide: toward a "Personalized" anti-microRNA therapy. *Mol. Pharm.*, **13**, 3164-3175.

Bragiel-Pieczonka, A., Lipka, G., Stapinska-Syniec, A., Czyzewski, M., Zybura-Broda, K., Sobstyl, M., Rylski, M. & Grabiec, M. (2022) The profiles of tet-mediated DNA hydroxymethylation in human gliomas. *Front. Oncol.*, **12**, 621460.

Cao, T.Q., Wainwright, D.A., Lee-Chang, C., Miska, J., Sonabend, A.M., Heimberger, A.B. & Lukas, R.V. (2022) Next steps for immunotherapy in glioblastoma. *Cancers* (*Basel*), **14**, 4023.

Chen, B., Lei, Y., Wang, H., Dang, Y., Fang, P., Wang, J., Yang, J. & Liu, L. (2017) Repression of the expression of TET2 by ZEB1 contributes to invasion and growth in glioma cells. *Mol. Med. Rep.*, **15**, 2625-2632.

Cobo, I., Tanaka, T.N., Chandra Mangalhara, K., Lana, A., Yeang, C., Han, C., Schlachetzki, J., Challcombe, J., Fixsen, B.R., Sakai, M., Li, R.Z., Fields, H., Mokry, M., Tsai, R.G., Bejar, R., et al. (2022) DNA methyltransferase 3 alpha and TET methylcytosine dioxygenase 2 restrain mitochondrial DNA-mediated interferon signaling in macrophages. *Immunity*, **55**, 1386-1401. e10.

Cruz, J.V.R., Batista, C., Afonso, B.H., Alexandre-Moreira, M.S., Dubois, L.G., Pontes, B., Moura Neto, V. & Mendes, F.A. (2022) Obstacles to glioblastoma treatment two decades after temozolomide. *Cancers* (*Basel*), **14**, 3203.

Diskin, B., Adam, S., Cassini, M.F., Sanchez, G., Liria, M., Aykut, B., Buttar, C., Li, E., Sundberg, B., Salas, R.D., Chen, R., Wang, J., Kim, M., Farooq, M.S., Nguy, S., et al. (2020) PD-L1 engagement on T cells promotes self-tolerance and suppression of neighboring macrophages and effector T cells in cancer. *Nat. Immunol.*, **21**, 442-454.

El Fatimy, R., Subramanian, S., Uhlmann, E.J. & Krichevsky, A.M. (2017) Genome editing reveals glioblastoma addiction to microRNA-10b. *Mol. Ther.*, **25**, 368-378.

Fekrirad, Z., Barzegar Behrooz, A., Ghaemi, S., Khosrojerdi, A., Zarepour, A., Zarrabi, A., Arefian, E. & Ghavami, S. (2022) Immunology meets bioengineering: improving the effectiveness of glioblastoma immunotherapy. *Cancers* (*Basel*), **14**, 3698.

Figueroa, M.E., Abdel-Wahab, O., Lu, C., Ward, P.S., Patel, J., Shih, A., Li, Y., Bhagwat, N., Vasanthakumar, A., Fernandez, H.F., Tallman, M.S., Sun, Z., Wolniak, K., Peeters, J.K., Liu, W., et al. (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*, **18**, 553-567.

Garber, S.T., Hashimoto, Y., Weathers, S.P., Xiu, J., Gatalica, Z., Verhaak, R.G., Zhou, S., Fuller, G.N., Khasraw, M., de Groot, J., Reddy, S.K., Spetzler, D. & Heimberger, A.B. (2016) Immune checkpoint blockade as a potential therapeutic target: surveying CNS malignancies. *Neuro Oncol.*, **18**, 1357-1366.

Gately, L., McLachlan, S.A., Philip, J., Ruben, J. & Dowling, A. (2018) Long-term survivors of glioblastoma: a closer look. *J. Neurooncol.*, **136**, 155-162.

Grochans, S., Cybulska, A.M., Siminska, D., Korbecki, J., Kojder, K., Chlubek, D. & Baranowska-Bosiacka, I. (2022) Epidemiology of glioblastoma multiforme-literature review. *Cancers* (*Basel*), **14,** 3698.

Jung, M.Y., Aibaidula, A., Brown, D.A., Himes, B.T., Cumba Garcia, L.M. & Parney, I.F. (2022) Superinduction of immunosuppressive glioblastoma extracellular vesicles by IFN-γ through PD-L1 and IDO1. *Neurooncol. Adv.*, **4**, vdac017.

Kraus, T.F., Greiner, A., Steinmaurer, M., Dietinger, V., Guibourt, V. & Kretzschmar, H.A. (2015) Genetic characterization of

ten-eleven-translocation methylcytosine dioxygenase alterations in human glioma. *J. Cancer*, **6**, 832-842.

Kumagai, S., Togashi, Y., Kamada, T., Sugiyama, E., Nishinakamura, H., Takeuchi, Y., Vitaly, K., Itahashi, K., Maeda, Y., Matsui, S., Shibahara, T., Yamashita, Y., Irie, T., Tsuge, A., Fukuoka, S., et al. (2020) The PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. *Nat. Immunol.*, **21**, 1346-1358.

Kumar, B.V., Connors, T.J. & Farber, D.L. (2018) Human T cell development, localization, and function throughout life. *Immunity*, **48**, 202-213.

Lechpammer, M., Rao, R., Shah, S., Mirheydari, M., Bhattacharya, D., Koehler, A., Toukam, D.K., Haworth, K.J., Pomeranz Krummel, D. & Sengupta, S. (2022) Advances in immunotherapy for the treatment of adult glioblastoma: overcoming chemical and physical barriers. *Cancers* (*Basel*), **14**, 1627.

Liang, H., Yi, L., Wang, X., Zhou, C. & Xu, L. (2014) Interleukin-17 facilitates the immune suppressor capacity of high-grade glioma-derived CD4 (+) CD25 (+) Foxp3 (+) T cells via releasing transforming growth factor beta. *Scand. J. Immunol.*, **80**, 144-150.

Lopez-Bertoni, H., Johnson, A., Rui, Y., Lal, B., Sall, S., Malloy, M., Coulter, J.B., Lugo-Fagundo, M., Shudir, S., Khela, H., Caputo, C., Green, J.J. & Laterra, J. (2022) Sox2 induces glioblastoma cell stemness and tumor propagation by repressing TET2 and deregulating 5hmC and 5mC DNA modifications. *Signal Transduct. Target. Ther.*, **7**, 37.

Miller, K.D., Ostrom, Q.T., Kruchko, C., Patil, N., Tihan, T., Cioffi, G., Fuchs, H.E., Waite, K.A., Jemal, A., Siegel, R.L. & Barnholtz-Sloan, J.S. (2021) Brain and other central nervous system tumor statistics, 2021. *CA Cancer J. Clin.*, **71**, 381-406.

Nakatsukasa, H., Oda, M., Yin, J., Chikuma, S., Ito, M., Koga-Iizuka, M., Someya, K., Kitagawa, Y., Ohkura, N., Sakaguchi, S., Koya, I., Sanosaka, T., Kohyama, J., Tsukada, Y.I., Yamanaka, S., et al. (2019) Loss of TET proteins in regulatory T cells promotes abnormal proliferation, Foxp3 destabilization and IL-17 expression. *Int. Immunol.*, **31**, 335-347.

Nicolini, A. & Carpi, A. (2009) Immune manipulation of advanced breast cancer: an interpretative model of the relationship between immune system and tumor cell biology. *Med. Res. Rev.*, **29**, 436-471.

Pethusamy, K., Seethy, A., Dhar, R., Karmakar, A., Chaudhary, S., Bakhshi, S., Palanichamy, J.K., Chopra, A., Chauhan, S.S. & Karmakar, S. (2022) Loss of TET2 with reduced genomic 5-hmC is associated with adverse-risk AML. *Leuk. Lymphoma*, **63**, 3426-3432.

Ren, S. & Xu, Y. (2019) AC016405.3, a novel long noncoding RNA, acts as a tumor suppressor through modulation of TET2 by microRNA-19a-5p sponging in glioblastoma. *Cancer Sci.*, **110**, 1621-1632.

Rong, L., Li, N. & Zhang, Z. (2022) Emerging therapies for glioblastoma: current state and future directions. *J. Exp. Clin. Cancer Res.*, **41**, 142.

Shen, Y., Liu, L., Wang, M., Xu, B., Lyu, R., Shi, Y.G. & Tan, L. (2021) TET2 inhibits PD-L1 gene expression in breast cancer cells through histone deacetylation. *Cancers* (*Basel*), **13**, 2207.

Tan, A.C., Ashley, D.M., Lopez, G.Y., Malinzak, M., Friedman, H.S. & Khasraw, M. (2020) Management of glioblastoma: state of the art and future directions. *CA Cancer J. Clin.*, **70**, 299-312.

Tanaka, S., Ise, W., Inoue, T., Ito, A., Ono, C., Shima, Y., Sakakibara, S., Nakayama, M., Fujii, K., Miura, I., Sharif, J., Koseki, H., Koni, P.A., Raman, I., Li, Q.Z., et al. (2020) Tet2 and Tet3 in B cells are required to repress CD86 and prevent autoimmunity. *Nat. Immunol.*, **21**, 950-961.

Teplyuk, N.M., Uhlmann, E.J., Gabriely, G., Volfovsky, N., Wang, Y., Teng, J., Karmali, P., Marcusson, E., Peter, M., Mohan, A., Kraytsberg, Y., Cialic, R., Chiocca, E.A., Godlewski, J., Tannous, B., et al. (2016) Therapeutic potential of targeting microRNA-10b in established intracranial glioblastoma: first steps toward the clinic. *EMBO Mol. Med.*, **8**, 268-287.

Thakur, A., Faujdar, C., Sharma, R., Sharma, S., Malik, B., Nepali, K. & Liou, J.P. (2022) Glioblastoma: current status, emerging targets, and recent advances. *J. Med. Chem.*, **65**, 8596-8685.

Verdugo, E., Puerto, I. & Medina, M.A. (2022) An update on the molecular biology of glioblastoma, with clinical implications and progress in its treatment. *Cancer Commun*. (*Lond*), **42**, 1083-1111.

Xu, Y.P., Lv, L., Liu, Y., Smith, M.D., Li, W.C., Tan, X.M., Cheng, M., Li, Z., Bovino, M., Aube, J. & Xiong, Y. (2019) Tumor suppressor TET2 promotes cancer immunity and immunotherapy efficacy. *J. Clin. Invest.*, **129**, 4316-4331.

Xue, S., Liu, C., Sun, X., Li, W., Zhang, C., Zhou, X., Lu, Y., Xiao, J., Li, C., Xu, X., Sun, B., Xu, G. & Wang, H. (2016) TET3 inhibits type I IFN production independent of DNA demethylation. *Cell Rep.*, **16**, 1096-1105.

Zhang, Q., Zhao, K., Shen, Q., Han, Y., Gu, Y., Li, X., Zhao, D., Liu, Y., Wang, C., Zhang, X., Su, X., Liu, J., Ge, W., Levine, R.L., Li, N., et al. (2015) Tet2 is required to resolve inflammation by recruiting Hdac2 to specifically repress IL-6. *Nature*, **525**, 389-393.