POLR2J is a potential biomarker for abnormal tumor progression, vorinostat sensitization, immune infiltration, and prognosis of glioblastoma multiforme

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Abstract

Glioma is one of the most primary malignant brain tumors, and glioblastoma multiform (GBM) is the most common and highly aggressive glioma. Most GBM are high malignant, poor prognosis, resistant to conventional therapy, and prone to recurrence. Therefore, it is crucial to explore novel therapeutics strategies for the treatment and prognosis of GBM. In this study, we elucidated that the maximal overexpression of DNA-directed RNA polymerase II subunit J-1 (POLR2J) was observed in GBM compared with normal tissues among all cancer types, and high expression of POLR2J or its co-expressed genes predicted poor outcome of GBM patients. DNA replication were significantly enriched in the GBM clinical samples with high POLR2J expression, and POLR2J suppression inhibited the proliferation and triggered cell cycle G1/G0 phase arrest of GBM cells. HDAC inhibitors, such as vorinostat, are identified as effective agents against GBM. We showed that POLR2J silence activated UPR and significantly enhanced anti-GBM activity of vorinostat via suppressing cell proliferation and inducing apoptosis. In addition, POLR2J promoted epithelial-mesenchymal transition (EMT) and the metastatic potentials of GBM cells. Furthermore, POLR2J expression was negatively relevant to the number of B cells, neutrophil, myeloid dendritic cells, CD4 + T cells and etc. Meanwhile, the expression of POLR2J was negatively correlative to the expression of immunotherapy-related genes. Our study confirmed a novel oncogene POLR2J in GBM progression as well as provided a promising strategy for the chemotherapy and immunotherapy of GBM treatment.

Introduction

Glioma is one of the most prevalent types of primary malignant brain tumors in adults, accounting for approximately 81% of malignant brain tumors [1]. Glioma can be divided into I-IV malignancy grades and various histological subtypes, including astrocytoma, glioblastoma multiform (GBM), oligodendroglioma and mixed tumors [2]. GBM is a grade IV glioma and the most common and highly aggressive malignant, with 5-year overall survival rate less than 10% [3]. The standard treatment for GBM patients is surgical resection followed by radiotherapy with concurrent and adjuvant temozolomide chemotherapy, but the prognosis remains poor [4]. Since most GBM are genetically unstable, highly infiltrative, angiogenic, resistant to conventional therapy, and prone to recurrence [5], it is crucial to explore novel targets for the treatment and prognosis of GBM.

In eukaryotes, RNA polymerase II is a multiprotein complex that transcribes DNA into precursors of mRNA and most small nuclear RNA and microRNA [6]. RNA polymerase II is composed of nine subunits, including RPB1, RPB2, RPB3, RPB4, RPB5, RPB6, RPB7, RPB8 and RPB11 [7]. DNA-directed RNA polymerase II subunit J-1 (POLR2J, also named RPB11) is a subunit of RNA polymerase II [8]. Besides, RNA polymerase II assembles with general transcription factors into a pre-initiation complex that opens promoter DNA to initiate transcription [9]. For example, transcription factor ATF4 was identified as a critical component of RNA polymerase II containing POLR2J, and ATF4 activated transcription by directly interacting with RNA polymerase II in the region of the heterodimer of α-like subunits (RPB3-RPB11) [10]. Meanwhile, RPB3 is a crucial subunit of RNA polymerase II that can interact with ATF4 and enhance the
activation of ATF4 by promoter recognition [11]. Interestingly, POLR2J is considered as a promising prognostic biomarker for patients with testicular germ cell tumor [12]. Meanwhile, POLR2J is also overexpressed in rectal tumor organoids compared with than normal rectum organoids [13]. However, the functions and mechanism of POLR2J in cancer progression remains to be fully understood.

In this context, we explored the fundamental functions and mechanisms of POLR2J in GBM. Firstly, the maximal upregulation of POLR2J was observed in GBM compared with normal tissues among all cancer types. Meanwhile, POLR2J facilitated cell proliferation, metastasis and EMT in GBM. In addition, POLR2J suppression enhanced the anti-GBM activity of vorinostat. Furthermore, the expression of POLR2J is correlated with immune infiltration of GBM. Collectively, POLR2J might be a prospective prognostic biomarker and therapeutic target for GBM.

**Materials And Methods**

**Reagents and antibodies**

Minimum Essential Medium (MEM) and High-glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (Logan, UT, USA) for cell culture. Trypsin-EDTA Solution (0.25%) was purchased from Gibco (Grand Island, NY, USA). BCA Protein Assay Kit and RIPA Lysis Buffer were obtained from Beyotime Biotechnology (Shanghai, China). Pre-stained protein marker was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Jet PRIME Transfection Reagent was provided by Polyplus Transfection SA (Strasbourg, France). Vorinostat (purity > 99%) was purchased from Aladdin (Shanghai, China). N-acetyl-L-cysteine (NAC) was provided by Beyotime Institute of Biotechnology (Shanghai, China). Anti-POLR2J (16403-1-AP, 1:1000 dilution), anti-E-Cadherin (20874-1-AP, 1:1000 dilution), anti-N-Cadherin (22018-1-AP, 1:1000 dilution), anti-EGFR (66455-1-Ig, 1:1000 dilution) and anti-Cyclin B1 (28603-1-AP, 1:1000 dilution) antibodies were obtained from Proteintech (Wuhan, China). The anti-p-EIF2α (Ser51) (3398, 1:1000 dilution), anti-Cyclin A2 (4656, 1:1000 dilution) and anti-Cleaved PARP (5625, 1:1000 dilution) antibodies were provided by Cell Signaling Technology (Danvers, MA, USA). The anti-STAT3(sc-482, 1:500 dilution), anti-p-AKT1/2/3 (Ser473) (sc-7985-R, 1:500 dilution) and anti-c-Myc (sc-40, 1:500 dilution) antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-p-STAT3(Tyr705) (ab76315, 1:1000 dilution) antibody was obtained from Abcam (Cambridge, MA, USA). The anti-GAPDH (db106, 1:10000 dilution) antibody was obtained from Diagbio. The anti-FLAG-tag (ABT2010, 1:10000 dilution) antibody was obtained from Abbkine, Inc. (San Diego, CA, USA).

**Cell Culture**

Glioma cell lines (T98G, U251, and A172) were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). T98G was culture with MEM containing non-essential amino acids with 10% fetal bovine serum (FBS), U251 and A172 cells were maintained in DMEM with 10% FBS. All the cells were kept in a 37°C condition with 5% CO₂.
Quantitative reverse transcription-PCR (qRT-PCR)

The total RNA from cultured cell lines was extracted with Trizol reagent (Takara, Tokyo, Japan), and the concentration was measured with Nano-300 Nucleic Acid Analyzer (Allsheng, Hangzhou, China). Subsequently the total RNA was converted to cDNA by the HiScript II 1st Strand cDNA Synthesis Kit according to the manufacturers’ recommendations (Vazyme Biotech, Nanjing, China). The qRT-PCR analysis was then conducted with HiScript II Q RT SuperMix (Vazyme Biotech, Nanjing, China). The 2−\(\Delta\Delta C_t\) method was used to calculate the relative levels of target genes among groups. A list of all primer sequences is provided in supplementary Table 1.

Plasmid And Sirna Transfection

Cells were seeded into 6-well plates with a density of 1.0 × 10^5 cells per well. At 30–50% confluence, siRNA was transfected using jetPRIME transfection reagent (Polyplus Transfection SA, USA). siRNAs were obtained from GenePharma (Shanghai, China) and the sense sequences were shown as follows: siPOLR2J-1, 5'-AGGACACCAAAGGUACCCAAUTT-3', siPOLR2J-2, 5'-AGAAGAAGAUCACUAUAAATT-3', and negative control siRNA, 5'-UUCUCCGAA CGUGUCACGUTT-3'. The POLR2J plasmid was obtained from GenScript Biotech (Piscataway, NJ, USA).

Sulforhodamine B (SRB) assay

After 24 h siRNA transfection, U251 and A172 cells were cultured into 96-well plates at a density of 3 × 10^3 cells/well. At 30–50% confluence, cells were treated with 1–6 µM vorinostat at 37°C for 72 h. The following steps are performed as described previously [14]. The OD value was detected at 540 nm with a microplate reader (Bioteck, Winooski, VT, USA).

Colony Formation Assay

U251 and T98G cells (2 × 10^3/well) were seeded into 6-well plates for overnight and were then transfected with siRNA, the culture medium containing the siRNA was replaced every 2–3 days for 10–14 days at 37°C. Following the supernatant is discarded, and the 6-well plates were carefully rinsed with PBS at three times, and then fixed with 4% paraformaldehyde for 30 min and 1% crystal violet stained for 20 min at room temperature, then washed with PBS and photographed.

Cell Cycle Analysis

U251 cells were seeded into 6-well plates at a density of 12 × 10^4 cells/well. At 30% confluence, cells were transfected with siPOLR2J for 48 h at 37°C, and then cells were collected and fixed with cold 75% ethanol at -20°C for overnight and washed with 500 µl PBS at three times. Subsequently, 1ml U251 cell
suspensions were hatched with 5 µl Propidium iodide (PI) solution for 5 min at room temperature and cell cycle analysis was then performed with a FACSCalibur flow cytometer (USA).

**Cell Apoptosis Assay**

U251 cells (2 × 10^4/well) were cultured in 6-well plates. At 30% confluence, cells were transfected with siPOLR2J for 24 h and treated with 2.5 mM NAC for 6 h, afterwards treated with 4 µM vorinostat at 37°C for 48 h. The cells were harvested, cleaned with cold PBS, and resuspended in binding buffer solution mixed with 5 µl annexin V and 5 µl PI and cultivated in the dark for 15 min according to the kit's (BD Biosciences, USA) instructions and the fluorescence property was analyzed with a FACSCalibur flow cytometer (USA). The Flowjo software was applied to measure the figures.

**Wound Healing Assay**

The transfected cells were plated in 24-well plates for overnight. Artificial wounds were created using 10 µl pipette tip, and then rinsed with 500 µl PBS for 2–3 times to remove cell debris. Cell culture medium without FBS were added and then wound distances were recorded by microscope as 0 h distance. After 24 h, wound distance was recorded, and the percentage of wound healing was measured as (the wound distance of 0 h − 24 h) / 0 h wound distance× 100%.

**Transwell Assay**

After 12 h of serum starvation, the transfected cells were collected and resuspended in the serum-free medium with the density of 3×10^5/ml. The Transwell insert membranes were coated with or without a Matrigel for invasion or migration assay, respectively. The upper chamber (8 µm pore size) was seed with 200 µl of cell suspension and a 600 µl medium with 20% FBS was added to the lower chamber. After 24 h, cells on the bottom surface of Transwell insert membranes were fixed with methanol for 15 min and stained with 1% crystal violet for 30min at room temperature. Images were photographed and measured by ImageJ software.

**Western Blotting**

Western Blotting

Cellular protein lysate buffering solution was applied for protein extraction. Treated cells were collected and dissolved with cold RIPA lysate and the protein samples were separated by using 8–15% SDS-PAGE and translocated onto a PVDF (Schleicher, USA) membrane, followed by blocked with 5% skimmed milk for 1 h at room temperature. The bands were incubated overnight with specific antibodies at 4°C. Afterwards, underwent 1 h incubation at room temperature with the corresponding secondary antibody. The bands were visualized using ECL detection system (Millipore, Germany).
Co-immunoprecipitation (Co-ip) Assay

U251 cells transfected with POLR2J overexpressed plasmid for 48 h were lysed with IP buffer and centrifugation at 120,000rpm for 30 min at 4°C. Afterwards, the cell lysates were divided equally hybridized with 20 µl mouse IgG magnetic beads and anti-Flag Immunomagnetic beads, respectively, for 16 h at 4°C with gentle shaking. Subsequently, standing on the magnetic stand for 5 min, add 500 µl of PBST (NaCl 136.89 mM, KCl 2.67 mM, Na₂HPO₄ 8.1 mM, KH₂PO₄ 1.76 mM, 0.5% Tween 20) to the above precipitate, and redispersed the beads by gently blowing, then flip the sample up and down for 5 min and removed the supernatant after magnetic separation and repeated the above steps 3 times. Finally, resuspend with SDS loading buffer and performed SDS-PAGE detection.

Statistical analysis

All data are presented as the mean ± SD. Statistical significance was determined by unpaired two-tailed Student’s t test or one-way ANOVA followed by Tukey’s post hoc test using GraphPad Prism 7 software. P-values < 0.05 were considered statistically significant.

Results

POLR2J was overexpressed and predicted poor prognosis in GBM patients

Firstly, we compared the mRNA expression of POLR2J between tumor and normal tissue across 24 cancer types using UALCAN, compared with normal tissues, the maximal upregulation of POLR2J was observed in GBM, and GBM exhibited the highest level of POLR2J among all cancer types (Fig. 1A and 1B) [15, 16]. Meanwhile, the protein level of POLR2J was overexpressed in GBM compared with normal tissues (Fig. 1C) [17], and high expression of POLR2J predicted poor outcome of GBM patients (p = 0.008, Fig. 1D) [18]. In addition, DNA methylation of POLR2J was associated with longer survival time of GBM patients (p = 0.047, Fig. 1E) [19]. Furthermore, the alteration of POLR2J predicted poor overall survival and disease-free survival of GBM patients (p < 0.05, Fig. 1F and 1G) [20, 21]. Meanwhile, POLR2J expression exhibited higher level in GBM from high-risk patients compared with that from low-risk patients (p = 4.57e-13, Fig. 1H and 1I). These data suggested that POLR2J might participate in development of glioma and the overexpression of POLR2J was correlated with poor prognosis of glioma patients.

POLR2J co-expressed genes in GBM predicted poor prognosis

To further demonstrated the prognostic value of POLR2J in GBM patients, we identified the co-expressed genes of POLR2J in GBM using LinkedOmics [22]. The Pearson coefficient test was used to identify the 8187 positive co-expressed genes of POLR2J with red label and 11473 negative co-expressed genes of POLR2J with green label in GBM (Fig. 2A). The top 50 crucial genes which were positively (left panel) and
negatively (right panel) associated with POLR2J were showed in the heat map (Fig. 2B). 39 co-expression genes that had an important positive correlation with POLR2J were used to establish a network of connections between protein-protein visualized by Cytoscape (Fig. 2C) [23]. The potential prognostic value of 39 co-expression genes correlation with POLR2J were identified using ExSurv [18]. As shown in Fig. 2D, 27 of 29 co-expression genes correlation with POLR2J predicted poor outcomes of GBM patients. These data indicated that the expression of POLR2J and its co-expressed genes were relevant to worse prognosis of GBM patients.

**POLR2J suppression inhibits the proliferation of GBM cells**

The GSEA analysis was performed to annotate the biological function of POLR2J in GBM tumorigenesis using LinkedOmics [22]. The enrichment plots of GSEA revealed that the POLR2J signature was positively correlated with citrate cycle, DNA replication, DNA mismatch repair, generation of precursor metabolites and energy, and metabolic pathways (Fig. 3A). These data elucidated that POLR2J might be participated in the proliferation of GBM cells. Meanwhile, we generated a Venn diagram through over-representation analysis (ORA) using LinkedOmics, which demonstrated that cell cycle was significantly enriched in the GBM clinical samples with high POLR2J expression among KEGG pathway, Panther pathway, Reactome pathway, and Wikipathway (Fig. 3B). GSEA enrichment demonstrated that POLR2J was positively correlated with DNA replication in GBM (Fig. 3C) [22]. Thus, we were interested in investigating the role of POLR2J in proliferation of GBM cells. Indeed, the inhibition of POLR2J was seriously restrained the proliferation of GBM cells (Fig. 3D and 3E). Furthermore, POLR2J knockdown suppressed the clone formation of GBM cells (Fig. 3F and 3G). In addition, GSEA enrichment demonstrated that POLR2J was positively correlated with cell cycle in GBM patients’ samples, and POLR2J silence significantly triggered cell cycle G1/G0 phase arrest in GBM cells (Fig. 3H). Meanwhile, POLR2J knock down suppressed multiple signaling pathway involved in cell proliferation, including EGFR and AKT pathways (Fig. 3I).

**POLR2J suppression enhanced the anti-GBM activity of vorinostat**

During cancer development and metastasis, tumor cells accommodate to oxidative stress by improving NADPH production [24]. GSEA enrichment demonstrated that POLR2J was positively correlated with NADH dehydrogenase complex assembly, oxidoreductase complex, NADH dehydrogenase complex, oxidoreductase activity, and unfolded protein in GBM (Fig. 3A and 4A). Thus, we hypothesized that POLR2J might participate in cancer progression by regulating oxidoreductase activity and unfolded protein response (UPR). The tight interconnection between UPR and oxidative stress is detected and associated with tumor development [25]. Our data showed that POLR2J silence significantly activated UPR by enhancing the phosphorylation of eIF2α (Fig. 4B). Meanwhile, NAC reversed the activated UPR induced by POLR2J silence in GBM (Fig. 4C). The strategies to adapt oxidative stress may be used as an effective approach to overcome chemotherapy resistance in cancer treatment [26]. Our results demonstrated that POLR2J might participate in drug metabolism, and POLR2J silence dramatically strengthened the cytotoxicity of vorinostat in GBM cells (Fig. 4D and 4E). In addition, POLR2J knockdown
increased the vorinostat induced apoptosis in GBM cells, which could be rescued by treated with NAC (Fig. 4F). Furthermore, POLR2J silence enhanced the cleavage of PARP induced by vorinostat (Fig. 4G). Meanwhile, NAC reversed the apoptotic effect induced by POLR2J knockdown plus vorinostat (Fig. 4H). These data suggested that POLR2J silence activated UPR and strengthened the anti-GBM effective of vorinostat by regulating UPR.

**POLR2J promotes the migrative and invasive abilities of GBM cells**

Epithelial-mesenchymal transition (EMT) is considered to be the critical procedure in initiating tumor invasion and metastasis, and EMT alters cell shape, adhesion, and movement [27]. The GSEA demonstrated that high POLR2J expression was negatively correlated with regulation of cell morphogenesis, cell-cell adhesion, cell junction organization, cell-cell junction, ECM-receptor interaction (Fig. 5A and Supplementary Fig. 1A) [22]. Thus, we assumed that POLR2J might take prominent part in the EMT and metastatic potential of GBM cells. Indeed, transwell assay indicated that POLR2J suppression could remarkably restrict the migrative and invasive abilities of GBM cells, including U251 and A172 cells (Fig. 5B – 5E). Likewise, wound healing assay also revealed that POLR2J inhibition could significantly inhibit the migration ability of GBM cells (Fig. 5F and 5G). In addition, POLR2J knockdown suppressed the progress of EMT in GBM cells by increasing the E-cadherin expression and reducing the N-cadherin expression (Fig. 5H). In contrast, transwell assay demonstrated that POLR2J overexpression significantly promoted the migrative and invasive abilities of GBM cells (Fig. 6A – 6D). Simultaneously, POLR2J overexpression significantly promoted the migrative ability of GBM cells in wound healing assay (Fig. 6E and 6F). In addition, POLR2J reinforced the EMT progress of GBM cells by facilitating the N-cadherin expression and lessening the E-cadherin expression (Fig. 6G). These data suggested that POLR2J accelerated the migrative and invasive abilities of GBM cells.

**POLR2J interacts with STAT3**

POLR2J co-expressed with Signal transducer and activator of transcription 3 (STAT3) in diffuse glioma and brain low grade glioma patients' samples (Fig. 7A; diffuse glioma: Spearman = 0.24, p = 0.0307, Pearson = 0.57, p = 5.03e-8; brain low grade glioma: Spearman = 0.20, p = 6.58e-4, Pearson = 0.12, p = 0.0408) [21, 28–30]. As previously described, RNA polymerase II interacts with general transcription factors into a pre-initiation complex that opens promoter DNA to trigger transcription. We hypothesized that POLR2J may interact with STAT3 to promote GBM malignant behaviors. Interestingly, the result showed that POLR2J could bind to STAT3 which was confirmed by immunoprecipitation in U251 cells (Fig. 7B). Multiple studies revealed that STAT3 takes a significant part in the proliferation and metastasis of tumor[31]. To further elucidated that POLR2J regulated metastasis via the STAT3 pathway, we performed experiments to elucidate whether overexpression of POLR2J could rescue STAT3 silence-mediated migration and invasion. Firstly, the wound healing assay showed that POLR2J overexpression could significantly promote the migration of GBM cells, while downregulation of STAT3 rescued the increased migration caused by POLR2J overexpression (Fig. 7C and D). Meanwhile, the transwell assay
confirmed that upregulating POLR2J expression rescues the change in STAT3 downregulation-mediated migration and invasion (Fig. 7E-G). Furthermore, our results showed that the protein level of p-STAT3 Tyr705 was down-regulated when POLR2J was knocked down (Fig. 7H). In addition, we suppressed the expression of STAT3 while POLR2J was overexpressed. The results revealed that the elevated protein levels of N-Cadherin, E-Cadherin, Cyclin A2, STAT3 and p-STAT3 Tyr705 due to POLR2J overexpression were rescued by STAT3 inhibition (Fig. 7I). These results demonstrated that POLR2J regulated the metastasis and EMT process by promoting the STAT3 signaling pathway in glioma cells.

The Correlation Between Polr2j Expression And Tumor-infiltrating Immune Cells (Tics) In Gbm

The heat map was drawn to demonstrate the correlation between the POLR2J gene and TICs in GBM patients (Fig. 8A) [32]. As shown in Fig. 8B, POLR2J expression was negatively related to B cells, neutrophil, myeloid dendritic cells, CD4+ T cells and cancer associated fibroblast (p < 0.001). Furthermore, the enrichment plots of GSEA demonstrated that the POLR2J signature was negatively relevant to B cell activation (Fig. 8C). In addition, the correlation between POLR2J expression and gene levels of common immune checkpoints were investigated. Our data suggested that the expression of POLR2J had obviously negative correlation with the expression of immunotherapy-related genes, including CD86, CTLA4, HAVCR2, LAG3, and TNFSF18 (Fig. 8D).

Discussion

GBM is the most frequent primary malignant brain tumor with high morbidity and mortality, because of highly invasion and infiltration of GBM, complete resection is hard to realize, it will relapse even after a complete resection [33]. Therefore, it is urgent to explore promising biomarkers for the diagnosis and therapy of GBM patients. The current study first demonstrated that the maximum upregulation of POLR2J was observed in GBM compared with normal tissues among all cancer types, and high level of POLR2J predicted poor outcome of GBM patients. Meanwhile, POLR2J co-expressed genes were also associated with poor prognosis of GBM patients. In addition, POLR2J suppression dramatically suppressed cell proliferation, and POLR2J silence significantly induced cell cycle G1/G0 phase arrest in GBM cells. In addition, POLR2J reinforced the EMT progress, the migrative and invasive abilities of GBM cells. In this context, our data, for the first time, revealed that POLR2J functions as an oncogene in the tumorigenesis of GBM, and it was a promising prognostic and therapeutics biomarker for GBM.

Temozolomide (TMZ) is widely used to treat GBM, but at least 50% of TMZ treated patients do not respond to TMZ [34]. Moreover, the overall 5-year survival after TMZ treatment is only 9.8%, thus it is urgent to seek alternative options for GBM treatment [35]. The HDAC inhibitors, such as vorinostat, have been well tolerated as a monotherapy in patients with recurrent GBM and exhibit modest single-agent activity [36]. HDAC inhibitors induce nuclear translocation of the transcription factors and activate cytoprotective autophagy, which contributes to therapeutic resistance. Furthermore, combining HDAC
inhibitors with autophagy modulating drugs enhance the anti-cancer activity in high-risk neuroblastoma cells [37]. It had been reported that POLR2J could interact with transcription factor and activate transcription [10]. Thus, we hypothesized POLR2J silence might enhance the anti-cancer activity of vorinostat by regulating transcription factors. In this study, we demonstrated, for the first time, that POLR2J might participate in drug metabolism, and POLR2J silence significantly enhanced anti-GBM activity of vorinostat by suppressing cell proliferation and inducing apoptosis. ER stress/UPR is involved in the pathophysiology of GBM, overexpression of UPR marker ATF4 is correlated with poor overall survival and plays vital role in GBM progression [38]. GSEA enrichment demonstrated that POLR2J was positively correlated with oxidoreductase activity and UPR in GBM, POLR2J silence activated UPR by enhancing the phosphorylation of eIF2α, and ROS inhibitor NAC reversed the activated UPR induced by POLR2J silence in GBM. These data indicated that POLR2J was correlated with oxidoreductase activity and UPR in GBM, and POLR2J knockdown enhanced ROS-UPR in GBM cells. HDAC inhibitors induce apoptosis in cancer cells by ROS accumulation [39]. Thus, POLR2J silence might enhance the anti-cancer activity of vorinostat by increasing ROS accumulation, and POLR2J silence might be a novel chemosensitization strategy for vorinostat treatment against GBM.

The potential mechanisms that POLR2J may promote the progression of glioma remains unclear. Therefore, we further investigated and integrated online databases and conducted a series of experiments, the results showed that POLR2J was positively related to STAT3 and could interact with STAT3. STAT3 is demonstrated to take a crucial part in regulating cell proliferation, apoptosis, oncogenesis and metastasis in various kinds of tumors including glioma [40–43]. By knocking down POLR2J expression, we found that POLR2J suppression can significantly reduce the protein level of p-STAT3, indicating that POLR2J can regulate cell proliferation and invasion through STAT3 pathways. Interestingly, our results also manifested that suppression of STAT3 rescued the POLR2J overexpression-mediated metastasis and EMT process, suggesting that POLR2J promoted the metastasis of glioma cells by activating STAT3 signaling. Despite these discoveries, the mechanisms of how POLR2J interacted with STAT3 remain unknown and how POLR2J specifically influenced STAT3 phosphorylation need further investigation.

Although immunotherapy achieved some notable successes in the treatment of multiple types of cancer such as skin, lung or breast cancer [44]. Innovative immune-targeting strategies, such as cancer vaccines, oncolytic viruses, checkpoint blockade inhibitors, adoptive cell transfer, and CAR-T cells have been investigated for GBM patients [45]. However, therefore no FDA-approved immunotherapy against GBM currently exists. There are multiple challenges needed to be solved in the progression of immunotherapy for GBM, including the immunosuppressive tumor microenvironment, tumor heterogeneity, tumor genomic characteristics, and efficiency of drug penetration through the BBB [45]. GBM cells can express multiple immune checkpoints, such as programmed cell death protein 1 ligand (PD-L1), which can restrain T cell proliferation and activation, and GBM intra-tumoral T cells are scarce [46]. B-cells, acting as antigen-presenting cells, directly present antigens to T-cells via both MHC class I and II and have the ability to stimulate T-cell proliferation [47]. Thus, novel strategies to enhance immune B cells infiltration of GBM are urgently needed for GBM patients’ immunotherapy. In this context, POLR2J had negative
correlation with B cells, CD4+ T cells, neutrophil, and etc. Meanwhile, GSEA enrichment manifested that the POLR2J was negatively relevant to B cell activation. Furthermore, POLR2J had negative correlation with the expression of immunotherapy-related genes. In addition, it had been reported that POLR2J co-expressed gene PTCD1 was also negatively associated with immune cells' infiltrations, immune functions, and checkpoints in cancer samples [48]. Thus, these data suggested that the suppression of POLR2J might be a novel strategy to enhance immune B cells infiltration and the efficacy of immunotherapy for GBM patients.

To sum up, our current study demonstrated that POLR2J was overexpressed in GBM compared with normal tissues, and high level of POLR2J predicted poor outcome of GBM patients. Meanwhile, POLR2J functioned as an oncogene in the tumorigenesis of GBM, and POLR2J silence might be a novel chemosensitization strategy for vorinostat treatment against GBM. Furthermore, POLR2J promoted the development and progression of GBM by regulating STAT3 signaling pathway. In addition, POLR2J had negative correlation with immune cell infiltration in GBM. Thus, POLR2J might be a novel prognostic and therapeutics biomarker for GBM treatment.

**Declarations**

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**Conflict of Interest Statement**

The authors have declared that no competing interest exists.

**Author Contribution Statement**

Conception and design: LHZ and CZ. Acquisition of data: ZDL, YHL, SYS, FYS, WYL. Analysis and interpretation of data: ZDL. Writing, review, and/or revision of the manuscript: LHZ and CZ. Study supervision: LHZ and CZ.

**Ethics Statement**

None

**Data Availability Statement**

All data generated or analyzed during this study are included in this published article.
References


Figures
Figure 1

**POLR2J was overexpressed and predicted poor prognosis in GBM patients.** (A-C) The data were obtained from UALCAN (http://ualcan.path.uab.edu). Gene: POLR2J; TCGA dataset: Glioblastoma multiforme; Pan-cancer view (A) and Expression (B); CPTAC dataset: Glioblastoma multiforme (C). (D) The data was collected from ExSurv (https://exsurv.soic.iupui.edu). Gene: POLR2J; Cancer type: GBM; Divided by: Median. (E) The data was collected from MethSurv (https://biit.cs.ut.ee/methsurv/). TCGA cancer
datasets: Glioblastoma multiforme (GBM) TCGA March 2017; Gene: POLR2J; Relation to island: Island; Genomic Region: TSS200; CpG site: cg17970286; Split by: Best. (F-G) The data was collected from cBioPortal (http://www.cbioportal.org). Studies: CNS/Brain-Pediatric Brain Cancer (CPTAC/CHOP, Cell 2020); Gene: POLR2J; Survival: OS (F) and DFS (G). (H-I) The data was collected from SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp).

Figure 2

A POLR2J Association Result

B Pearson Correlation Coefficient (Pearson test)

D
The co-expressed genes of POLR2J in GBM predicted poor prognosis. (A) The data were obtained from LinkedOmics (http://www.linkedomics.org/admin.php). Search dataset: TCGA_GBM; Target dataset: TCGA_GBM; Attribute: POLR2J; Patients: 153; Statistical Method: Pearson Correlation test. All expressed genes relevant with POLR2J in glioma. (B) Top 50 significant genes positively and negatively correlated with POLR2J in glioma. (C) Cytoscape and MCODE were established to estimate the most indispensable module in the protein-protein interaction networks. Protein-protein connection network and MCODE analysis of POLR2J co-expressed genes. (D) The data was collected from ExSurv (https://exsurv.soic.iupui.edu).
Figure 3

POLR2J suppression inhibits the proliferation of GBM cells. (A) The data were obtained from LinkedOmics (http://www.linkedomics.org/admin.php). Search dataset: TCGA_GBM; Target dataset: TCGA_GBM; Attribute: POLR2J; Patients: 153; Statistical Method: Pearson Correlation test; Select Tool: Gene Set Enrichment Analysis (GSEA); Enrichment Analysis: GO analysis (Biological process) or GO analysis (Cellular Component) or GO analysis (Molecular Function) or KEGG pathway or WikiPathway;
Select Rank Criteria (from LinFinder table): FDR; Minimum Number of Genes: 3; Simulations: 500.

Visualization of positively functional profiles for POLR2J based on GSEA. (B) The data were obtained from LinkedOmics (http://www.linkedomics.org/admin.php). Search dataset: TCGA_GBM; Target dataset: TCGA_GBM; Attribute: POLR2J; Patients: 153; Statistical Method: Pearson Correlation test; Select Tool: Overrepresentation Enrichment Analysis (ORA); Select Rank Criteria (from LinFinder table): FDR; Select Sign (or direction): Positive correlated. (C) GSEA was utilized to investigate the enrichment of DNA replication in GBM process according to the expression of POLR2J. (D) GBM cells were transfected with siPOLR2J or negative siRNA for 48 h, and then RT-PCR was used to detect the knock-down efficiency of siPOLR2J in GBM cells. (E) GBM cells were incubated with siPOLR2J or negative siRNA for 24 h, and then transferred to 96-wells plate, the cell proliferation was determined after 1, 2, 3, 4, and 5 days. (F-G) GBM cells were transfected with siPOLR2J or negative siRNA, and the number of cell clone was counted. (H) GBM cells were transfected with siPOLR2J or negative siRNA for 24 h, and the cell cycle distribution was tested by PI staining followed by flow cytometer. (I) GBM cells were transfected with siPOLR2J or negative siRNA for 48 h, the expression of proteins was detected by western blot.
Figure 4

POLR2J suppression enhanced the anti-GBM activity of vorinostat. (A) GSEA was utilized to investigate the enrichment of oxidative stress in GBM according to the expression of POLR2J using LinkedOmics online tool. (A) GBM cells were transfected with siPOLR2J or negative siRNA for 48 h, the expression of proteins was detected by western blot. (B) GBM cells were transfected with siPOLR2J or negative siRNA for 6 h, and then cells were incubated with NAC for 48 h, finally the expression of proteins was detected by western blot.
by western blot. (C) GSEA was utilized to investigate the enrichment of drug metabolism in GBM according to the expression of POLR2J using LinkedOmics online tool. (D) GBM cells were cultured with siPOLR2J or negative siRNA for 24 h, and then incubated with vorinostat at the indicated concentrations for 72 h, SRB assay was performed to detect the proliferation of GBM cells. (E-F) GBM cells were incubated with siPOLR2J or negative siRNA for 24 h, and then treated with vorinostat at the indicated concentrations for 48 h, Annexin V-FITC/PI staining assay was performed to detect the apoptosis of GBM cells. (G) GBM cells were cultured with siPOLR2J or negative siRNA for 24 h, and then treated with vorinostat at the indicated concentrations for 48 h, the expression of protein was detected by western blot. (H) GBM cells were transfected with siPOLR2J or negative siRNA for 24 h, pretreated with NAC for 6 h, and then incubated with vorinostat for 48 h, finally the expression of protein was detected by western blot.
POLR2J silencing inhibited the migration and invasion of glioma cells. (A) The data were obtained from LinkedOmics. Visualization of negatively functional profiles for POLR2J based on GSEA. (B-C) GBM cells were transfected with siPOLR2J or negative siRNA for 24 h, and then transferred to the upper chamber of transwell, the migrated cells were stained with crystal violet and counted. (D-E) GBM cells were transfected with siPOLR2J or negative siRNA for 24 h, and then transferred to the upper chamber of transwell, the invaded cells were counted.
transwell with a Matrigel coating on the insert membrane, the invaded cells were stained with crystal violet and counted. (F-G) GBM cells were incubated with siPOLR2J or negative siRNA for 24 h and wound healing assay was performed. (H) GBM cells were transfected with siPOLR2J or negative siRNA for 48 h, and then western blot was performed.

Figure 6
POLR2J promoted the migration and invasion of glioma cells. (A-B) GBM cells were transfected with POLR2J plasmid or empty vector for 24 h, and then transferred to the upper chamber of transwell, the migrated cells were stained with crystal violet and counted. (C-D) GBM cells were incubated with POLR2J plasmid or empty vector for 24 h, and then transferred to the upper chamber with a Matrigel coating on the insert membrane, the invaded cells were stained with crystal violet and counted. (E-F) GBM cells were transfected with POLR2J plasmid or empty vector for 24 h, and then wound healing assay was performed. (G) GBM cells were transfected with POLR2J plasmid or empty vector for 48 h, and then western blot was performed.
Figure 7

(A) The data were collected from cBioPortal (http://www.cbioportal.org). Studies for visualization & analysis: Diffuse Glioma (GLASS Consortium, Nature 2019), n=444, left panel or Brain low grade glioma (TCGA, Firehose Legacy), n=283, right panel; Gene: POLR2J and STAT3. (B) U251 cells were incubated with POLR2J plasmid for 48 h, an immunoprecipitation assay was carried out to assess the interaction between POLR2J and STAT3. (C-D) GBM cells were transfected with POLR2J plasmid or empty vector for
24 h and transfected with siSTAT3 or negative siRNA for 24 h, and then wound healing assay was performed. Images were acquired under a microscope. (E-G) GBM cells were transfected with POLR2J plasmid or empty vector for 24 h and transfected with siSTAT3 or negative siRNA for 24 h, and then transferred to the upper chamber of transwell with or without a Matrigel coating on the insert membrane, the migrated and invaded cells were stained with crystal violet and counted. (H) GBM cells were incubated with siPOLR2J or negative siRNA for 48 h, and then western blot was performed. (I) GBM cells were cultured with POLR2J plasmid or empty vector for 24 h and transfected with siSTAT3 or negative siRNA for 24 h, and then western blot was conducted.
The correlation between POLR2J expression and tumor-infiltrating immune cells (TICs) in GBM. (A-B) The data were collected from TIMER 2.0 (The http://timer.cistrome.org/). (C) GSEA was utilized to investigate the enrichment of B cell activation in GBM according to the expression of POLR2J using LinkedOmics. (D) The spearman’s coefficient of immune checkpoints and POLR2J expression were analyzed using TIMER 2.0.
Supplementary Files

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- FigureS1.jpg
- SupplementaryTable1primer.xls