Basic Transcription Factor 3 Like 4 Enhances Malignant Phenotypes through Modulating Tumor Cell Function and Immune Microenvironment in Glioma

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Glioma is a prevalent intracranial brain malignancy associated with poor prognosis and a mortality rate of approximately 80% after the first year of diagnosis, especially for patients with glioblastoma (GBM).1 Despite advancements in glioma molecular classification that have proposed potential therapeutic goals for targeting potential oncogenic pathways, the overall prognosis has remained relatively unchanged.2 The glioma microenvironment is characterized by its diverse cellular components that can evade both immune surveillance and targeted therapies.3 Moreover, the microenvironment undergoes significant changes in response to treatment, driven by various forms of molecular dysregulation, resulting in extensive intertumoral and intratumoral heterogeneity.3,4 Identifying clinical biomarkers for predicting patient prognosis is thus paramount to improving treatment outcomes.

Recent investigations into the tumor microenvironment have provided insights into the limited response of glioma progression to immunotherapy. However, the specific involvement of basic transcription factor 3 like 4 (BTF3L4) in glioma progression and its correlation with immune cell infiltration remain areas of uncertainty that require further exploration. In the current study, BTF3L4 expression was delineated by using gene expression profiling/interactive analysis and multiplex-immunohistologic staining of tissue microarrays. The prognostic value of BTF3L4 was then assessed by using Cox regression models and Kaplan-Meier methods, and in vitro experiments were conducted to investigate how BTF3L4 protein affects the proliferation, migration, and invasion capabilities of glioma cells. Furthermore, the CIBERSORT and ESTIMATE methods were used to quantify immune cells that correlate to BTF3L4 expression, and multiplex-immunohistologic staining was applied to investigate its correlation with infiltrated immune cells in glioma tissues. These findings revealed higher BTF3L4 expression in glioma tissues compared with non-tumor brain tissues, which correlated with clinical characteristics and worse patient prognosis. Furthermore, the down-regulation of BTF3L4 protein in the glioma cell line had a detrimental effect on cell migration, invasion, and proliferation. In addition, the association between BTF3L4 and key immune molecules in glioma, particularly with the infiltration of C066B+ neutrophils and programmed death-ligand 1 expression, was identified. These results highlight the prognostic significance of BTF3L4 and propose BTF3L4 as a potential target for glioma immune therapy. (Am J Pathol 2024, 11: 1–13; https://doi.org/10.1016/j.ajpath.2024.01.011)

Basic transcription factor 3 like 4 (BTF3L4), initially verified in the context of chondrogenic differentiation, has been identified as assisting in the application of mesenchymal stem cells in cell-based therapy for cartilage regeneration.6 BTF3L4 has also been implicated as a potentially lethal gene candidate, influencing protein binding and contributing to the growth and metastasis of clear cell renal carcinoma, thyroid tumors, and colorectal cancer, as well as involvement in the inhibition of gastric cancer cell growth.6,7 Notably, it has been shown that BTF3L4 can also

B.L. and T.L. contributed equally to this work.

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maintain neuronal morphology and influence the structural characteristics of brain malignancies. However, the precise cellular mechanisms underlying the associations between BTF3L4 expression and glioma progression remain largely unclear.

To date, glioma treatment research has been focused on immunotherapy to enhance antitumor immune response; at best, the outcomes have been inconsistent. Immuno-therapy faced unique obstacles and showed very modest success in treating gliomas, which exhibited limited efficacy and met distinctive challenges. Early clinical trials have suggested that increased infiltration of immune cells could lead to better treatment outcomes, especially in glioma. Neoadjuvant administration of programmed cell death 1 blockade enhances the local and systemic antitumor immune response and may represent a more efficacious approach to glioma treatment. Moreover, immune cell–based analyses have identified valuable therapeutic targets for predicting patient prognosis; similar discoveries for glioma are limited, which might be due to distinct immunosuppressive mechanisms specific to glioma, highlighting the need to focus on immune cells for prognosis prediction. Consequently, it is crucial to identify immune cell–related prognosis indicators in glioma, which might further lead to more significant advancements in this field.

Exploring prognostic biomarkers within the dynamic glioma tumor microenvironment (TME) is crucial for understanding immune responses, predicting patient outcomes, and assessing immunotherapy efficacy. In this context, the relationship between BTF3L4 expression, patient prognosis, and the presence of tumor-infiltrating immune cells was investigated. In addition, to gain direct insights into the specific role of BTF3L4 in glioma development, in vitro experiments were conducted to observe how the down-regulation of BTF3L4 affected the phenotype of glioma cells.

Materials and Methods

Pan Cancer and Glioma Data Collection and Processing

Public data available from The Cancer Genome Atlas (TCGA) were retrieved for this study. RNA-sequencing profiles with corresponding clinical data from the TCGA pan cancer and the TCGA GBM and lower grade glioma (GBM&LGG) samples were collected and standardized by using the University of California Santa Cruz database (https://xenabrowser.net, last accessed January 1, 2024). We then assessed the differential expression of the BTF3L4 gene by extracting the expression data of the BTF3L4 gene (ENSG00000134717) (NIH, National Library of Medicine, https://www.ncbi.nlm.nih.gov/gene/91408, last accessed January 19, 2024) from each sample that had complete transcriptome data and patient overall survival information. All data included in this study were available to the public.

Analysis of the Relative Abundance of Tumor-Infiltrating Immune Cells

To evaluate the potential association between BTF3L4 expression and tumor-infiltrating immune cells, a gene expression–based deconvolution algorithm known as CIBERSORT (CIBERSORTx, http://cibersort.stanford.edu, last accessed January 19, 2024) was used. The aim was to investigate the association between differential BTF3L4 expression and immune responses involving tumor-infiltrating immune cells in glioma.

Clinical Materials

Tissue specimens were collected from 193 patients diagnosed with primary glioma and 29 patients with non-tumor brain diseases at Nantong University Affiliated Hospital between March 2013 and November 2017. Clinical and laboratory information was retrieved from retrospective data, and the glioma pathologic type and World Health Organization grade were determined in accordance with the 2021 WHO Classification of Tumors of the Central Nervous System. The IDH1R132H mutation was assessed by using immunohistochemistry methods and examined by a pathologist. This study included patients with a confirmed pathologic diagnosis of primary gliomas who had undergone surgical resections, comprising 113 men and 80 women, with a median age of 53.79 ± 13.03 years (range, 21 to 86 years) and a median 5-year survival time of 26.94 ± 26.78 months. Surgically resected glioma and adjacent non-tumor brain tissue samples were placed on ice within 30 minutes of lesion excision. Western blot analysis was conducted on six tumor samples and six adjacent non-tumor brain tissue samples.

Ethical approval for the use of human samples in this study was obtained from the Human Research Ethics Committee of the Affiliated Hospital of Nantong University (grant 2018-K020).

Multiplex-Immunohistochemistry Staining, Identification, and Expression Analysis of the Infiltrated Immune Cells

Formalin-fixed paraffin-embedded primary glioma tissue blocks were used to construct tissue microarrays, which consisted of blocks of 2 mm diameter, manually created by using the Tissue Microarray System Quick Ray (UNITMA, Seoul, Republic of Korea). Multiplex-immunohistochemistry (mIHC) staining was performed by using the Opal 7-Color Manual IHC Kit (NEL810001KT; Akoya Biosciences, Marlborough, MA). Tissue microarray sections were first deparaffinized and rehydrated. Microwave treatment was performed with the appropriate AR69 antigen retrieval buffer (AR600250ML/AR900250ML; Akoya Biosciences). The slides were then blocked with tissue-blocking buffer (ARD1A01EA; Akoya Biosciences) to inhibit endogenous
peroxidase activity before primary antibody incubation, followed by incubation with Opal polymer horseradish peroxidase (anti-Ms + Rb) secondary antibody (ARH1001EA; Akoya Biosciences). Fluorophores (Opal 520, Opal 540, Opal 570, Opal 620, Opal 650, and Opal 690; Akoya Biosciences) were used for fluorescent visualization. Multiplex staining was accomplished by serially repeating staining cycles, with microwave treatment procedures in between each step to remove the antibody complex. Fluoromount (F6057; Sigma, NY) with DAPI was used to visualize the nuclei for the final step.

The primary antibodies used in this experiments included: anti-BTF3L4 antibody (1:500, Orb511711; Biobyrtd, Cambridge, UK), anti-CD3 antibody (1:500, 85061; CST, MIA), anti-CD4 antibody (1:5000, ab133616; Abcam, Cambridge, UK), anti-CD8 antibody (1:500, 85365; CST), anti-CD20 antibody (1:1000, ab78237; Abcam), anti-CD25 antibody (1:100, MA5-12680; Invitrogen, Carlsbad, CA), anti-CD68 antibody (1:500, 76437; CST), anti-CD10 antibody (1:200, 3576; CST), anti-CD83 (1:1000, MAB1774; R&D Systems, Minneapolis, MN), anti-CD66b antibody (1:1000, ARG66287; Airgibo, Shanghai, China), anti—programmed death-ligand 1 (PD-L1) (1:1000, 13684T; CST), and anti—CTLA 4 (1:500, NB100-64894; Novus Biologicals, Littleton, CO).

The slides with fluorescence staining were digitally scanned by using the Vectra 3.0 Automated Quantitative Pathology Imaging System (Akoya Biosciences). Subsequently, the fluorescence intensity was quantified by using InForm Tissue Analysis software version 26.1 (Akoya Biosciences). Specific algorithms were designed for BTF3L4 and the immune cell markers to automatically compute scores based on fluorescence intensity. The expression levels of BTF3L4 and immune cells were assessed as percentage values (the number of positively stained cells divided by the number of nuclei, multiplied by 100%) for each tissue category.

Cell Culture and Transfection

The four glioma cell lines (U251, U87mg, SHG44, and T98G) used in this study were obtained from the Cell Bank/ Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). U87mg, SHG44, and T98G cell lines were cultured in vitro with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (#04-001-IAC; Biological Industries, Beit-Haemek, Israel) and 100 U/mL penicillin/streptomycin (C022; Beyotime, Shanghai, China). U251 cells were cultured in minimal essential medium with 10% fetal bovine serum, nonessential amino acids (C0332; Beyotime), and sodium pyruvate (C0331; Beyotime). All glioma cell lines underwent monthly mycoplasma testing and were cultured at 37°C with 5% carbon dioxide. Two distinct siRNA sequences targeting BTF3L4 (siRNA-1/2) and a control (siRNA—negative control) were designed by using the Invitrogen online software BLOCK-iT RNAi Designer (Thermo Fisher Scientific, https://rnaidesigner.thermofisher.com/mairexpress) and synthesized by the Oligobio Company (Beijing, China). The siRNA sequences targeting BTF3L4 were si-BTF3L4-1 (5'-CCTGATGTACAGTTGAGATT-3') and si-BTF3L4-2 (5'-CAAATCGAAATGCTAGCATGT-3'), respectively. Glioma cells were transfected using the Lipofectamine 3000 transfection reagent kit (L3000001; Invitrogen) following the manufacturer’s instructions. Lastly, the cells were collected for Western blot analysis, cell invasion and migration assays, and cell cycle experiments.

Transwell Assay

Transwell assays were performed to analyze the migration and invasion abilities of glioma cells. Briefly, U251 glioma cells (5 × 10^4) were introduced into chambers coated with Matrigel (for cell invasion analysis) or left uncoated (for cell migration analysis). The upper chamber was filled with serum-free medium, while the lower chamber received complete Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. After 24 to 48 hours of incubation, the migrating or invading cells were fixed by using 4% paraformaldehyde and stained with 0.1% crystalline violet (C0121; Beyotime). Quantitative analysis was performed by using a light microscope.

Cell Cycle Analysis and Flow Cytometry Analysis

The distribution of cells across the cell cycle phases, including the G₀/G₁, S, and G₂/M phases, was assessed by using a staining kit (C1052; Beyotime). After achieving confluence, the cells were adjusted to a concentration of 1 × 10^5 cells/mL, then seeded into a 6-well culture plate with 3 mL of culture medium per well and placed in a 37°C, 5% carbon dioxide incubator for 24 hours. Next, the cells transfected with siRNA and siRNA—negative control were cultured for 48 hours; 1 × 10^5 cells were then harvested, washed once with phosphate-buffered saline, resuspended to obtain a single-cell suspension, and fixed and stained according to the manufacturer’s instructions. The stained samples were subjected to flow cytometry analysis (BD-FACSVerse; Becton Dickinson, San Jose, CA) directly, and the data were analyzed by using CellQuest software version 5.1 (BD Biosciences, San Jose, CA).

Western Blot Analysis

Total protein was extracted from both glioma tissue and cell samples by using radioimmunoprecipitation assay buffer (P0013B; Beyotime), with the addition of protease and phosphatase inhibitors (P1260; Solarbio, Beijing, China). The lysates obtained from tissue and cells were then centrifuged at 12,000 × g for 15 minutes at 4°C, and the resulting supernatant was transferred to a clean tube. The total protein concentration in each sample was quantitated...
by using a BCA Protein Assay Kit (P0010; Beyotime). Subsequently, the total protein in each sample was diluted 1:1 with loading buffer (two times, P0015B; Beyotime) and boiled for 5 minutes at 95°C. Equal amounts of protein were loaded onto 10% SDS-PAGE gels (AR0138; Boster, Wuhan, China) and transferred onto polyvinylidene fluoride membranes with a pore size of 0.22 μm (#GSWP04700 M; MilliporeSigma, Merck KGaA, Darmstadt, Germany). To minimize nonspecific binding, the membranes were blocked by using 5% bovine serum albumin in tris-buffered saline + Tween 20 for 2 hours at room temperature. Next, the membranes were incubated overnight with primary antibodies, including anti-BTF3L4 antibody (1:1000, Orb511711; Biorybt) and anti-tubulin antibody (1:5000, M2005L; Abmart, Shanghai, China). Afterward, the membranes were probed with a secondary antibody (goat anti-rabbit IgG–horseradish peroxidase conjugate, 1:5000, SA00001-2; Proteintech, Wuhan, China) for 2 hours at room temperature. The protein band signals were visualized using an ECL chemiluminescence reagent (BL520A; Bio-sharp, Beijing, China) and captured using a Chemiluminescent Imaging System (Tanon 4600 SF, NJ, China). Finally, the protein bands were quantified by using Gel-Pro Analyzer version 4.0.0.0.01 (Media Cybernetics LP, China).

Statistical Analysis

The optimal cutoff point for BTF3L4 expression (cutoff value = 50; range, 0 to 100) was determined by using X-tile statistics software version 3.6.1 (Rimm Lab, Yale School of Medicine, New Haven, CT). Survival analysis was performed by using the Kaplan-Meier method with the log-rank test in SPSS version 22.0 (IBM SPSS Statistics, IBM Corporation, Armonk, NY). The experiments involving cell proliferation, Western blot analysis, and cell cycle analysis were independently replicated thrice. Comparisons between two different groups were assessed by using unpaired or paired two-tailed t-tests, and the analysis was conducted by using GraphPad Prism software version 9.0.0 (GraphPad Software, La Jolla, CA). All quantitative data are expressed as means ± SD or mean ± 95% CI. P values <0.05 were considered statistically significant.

Results

Identification of BTF3L4 mRNA Expression and its Prognostic Significance

Expression of BTF3L4 mRNA in glioma and non-tumor brain tissues was first investigated by using the TCGA and GTEx (Genotype-Tissue Expression) databases, which showed a significant up-regulation of BTF3L4 transcription in the TCGA-GBM data set (tumor, 6.00 ± 0.49; non-tumor, 4.18 ± 1.32; P = 2.1e-83) and the TCGA-GBM&LGG data set (tumor, 5.70 ± 0.54; non-tumor, 4.18 ± 1.32; P = 1.3e-7) (Figure 1A). In glioma tissue and non-tumor brain tissue samples, BTF3L4 mRNA expression was significantly higher in the glioma tissues (Figure 1B). Furthermore, high BTF3L4 expression was correlated with poor patient prognosis in both the TCGA-GBM&LGG [hazard ratio (HR), 1.56 to 2.6; P = 1.3e-75] and TCGA-LGG (HR, 1.00 to 2.12; P = 0.05) data sets (Supplemental Figure S1). Kaplan-Meier survival analysis revealed that elevated BTF3L4 mRNA expression in tumor tissues was associated with reduced overall survival (Figure 1C). Taken together, these findings indicate that BTF3L4 mRNA expression could be a prognostic indicator for glioma patients.

Associations Between BTF3L4 Protein Expression and Clinical Characteristics of Patients with Glioma

We then explored the relationship between BTF3L4 protein expression and clinical features in patients with glioma. Given that mRNA abundance may not always correlate with protein levels due to translational processes, BTF3L4 protein expression was assessed to provide more reliable clinical insights. In GBM, translational modifications of mRNA are often more extensive than changes at the transcriptional level, underscoring the significance of protein-level analysis. Therefore, tissue microarray–mIHC staining was used to evaluate BTF3L4 protein expression in glioma, which revealed the predominant localization of the BTF3L4 protein in the cytoplasm region of the glioma cells (Figure 2, A and B). The proportion of BTF3L4 protein was significantly higher in glioma tissues compared with non-tumor brain tissues (P < 0.05) (Figure 2C). Consistently, elevated BTF3L4 protein expression was observed in glioma tissues compared with paired non-tumor brain tissues from surgically resected samples (paired, n = 6) (Figure 2, D and E), consistent with previous findings at the mRNA level. We subsequently assessed the relationship between BTF3L4 protein expression and clinical characteristics in the glioma patient cohort. The patients were categorized into a low or no BTF3L4 protein expression group and a high BTF3L4 expression group based on an optimized cutoff value, and we found that those with high BTF3L4 protein expression were associated with glioma World Health Organization grade (χ² = 12.876, P = 0.004) and histologic type (χ² = 9.860, P = 0.043) (Table 1).

Prognostic Potential of BTF3L4 Protein Expression in Glioma Patients

Univariate and multivariate Cox regression analysis was also performed to examine the relationship between BTF3L4 expression and patient overall survival. Among the clinical parameters analyzed, BTF3L4 protein expression (HR, 1.801; P = 0.006), age (HR, 2.093; P < 0.001), sex (HR, 1.601; P = 0.005), glioma histopathologic type (HR, 0.855; P = 0.033), and glioma World Health
Organization grade (HR, 1.920; \( P < 0.001 \)) were associated with patient survival in the univariate analysis. In the multivariate analysis, BTF3L4 protein expression (HR, 2.339; \( P < 0.001 \)), age (HR, 1.668; \( P = 0.002 \)), and World Health Organization grade (HR, 1.934; \( P < 0.001 \)) were identified as independent significant predictors of patient survival (Table 2). In line with these findings, the Kaplan-Meier survival curve illustrated that high BTF3L4 protein expression in patients with glioma was associated with shorter overall survival (Figure 2F), supporting the potential of BTF3L4 as a promising prognostic indicator for glioma patient survival.

High BTF3L4 Expression Contributed to Malignant Phenotypes of Glioma Cells

Although BTF3L4 has been recognized as an important transcriptional factor, its role in the oncogenesis of solid
Basic Transcription Factor 3 Like 4 (BTF3L4) differential expression and its prognostic value in glioma patients. A and B: The representative multiplex-immunohistochemistry images of BTF3L4 protein expression in clinical patient tissue microarrays (A1 and B1 indicated the magnified images of the box regions). C: BTF3L4 protein expression in glioma (n = 193) and non-tumor brain tissue (n = 29) of clinical patients. D and E: BTF3L4 protein expression in six pairs of fresh surgical excision of glioma tissues (T) and adjacent non-tumor brain tissues (N). F: High BTF3L4 protein expression was correlated with a poor prognosis in the clinical glioma patient cohort (N = 193). *P < 0.05, **P < 0.01. Scale bar = 20 μm (A1 and B1).

BTF3L4 Correlated to Infiltrated Immune Cells in the Glioma TME

Considering that the TME plays a pivotal role in determining the response to immune therapy in glioma, the correlation scores of BTF3L4 expression and immune cell infiltration were evaluated based on its gene expression profile (Figure 4A and Supplemental Figure S2). In addition, BTF3L4 showed positive associations with gamma delta T cells, T helper cells, and T helper 2 cells and was negatively associated with CD56bright natural killer cells, regulatory T cells, dendritic cells (DCs), plasmacytoid DCs, and CD56dim natural killer cells (P < 0.05) (Figure 4B).

Interestingly, BTF3L4 displayed the strongest associations with stromal, immune, and estimated scores but was negatively associated with infiltrated immune cells in the TCGA-GBM, TCGA-GBM&LGG, and TCGA-LGG data sets (P < 0.01) (Figure 4C–E).

To further verify the correlation between BTF3L4 protein expression and immune cell abundance in the glioma immune microenvironment, the quantified tissue microarray--mHIC data were then analyzed. The results showed that CD8+ T
Table 1  Relationship Between BTF3L4 Protein Expression and Clinical Characteristics in Glioma Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>BTF3L4 expression</th>
<th></th>
<th></th>
<th>Pearson χ²</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>Low or no expression (%)</td>
<td>High expression (%)</td>
<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>193</td>
<td>104 (53.89)</td>
<td>89 (46.11)</td>
<td>0.106</td>
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<td>Sex</td>
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<td>113</td>
<td>64 (56.64)</td>
<td>49 (43.36)</td>
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<td>40 (50.00)</td>
<td>40 (78.31)</td>
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<tr>
<td>&lt;60</td>
<td>121</td>
<td>63 (52.07)</td>
<td>58 (47.93)</td>
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<td>≥60</td>
<td>72</td>
<td>41 (56.94)</td>
<td>31 (43.06)</td>
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<td>Histopathologic type</td>
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<tr>
<td>a</td>
<td>120</td>
<td>74 (61.67)</td>
<td>46 (38.33)</td>
<td>9.860</td>
<td>0.043*</td>
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<tr>
<td>b</td>
<td>26</td>
<td>19 (73.08)</td>
<td>7 (26.92)</td>
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<tr>
<td>c</td>
<td>9</td>
<td>5 (55.56)</td>
<td>4 (44.44)</td>
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<tr>
<td>d</td>
<td>13</td>
<td>7 (53.85)</td>
<td>6 (46.15)</td>
<td></td>
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<tr>
<td>e</td>
<td>25</td>
<td>13 (52.00)</td>
<td>12 (48.00)</td>
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<tr>
<td>Molecular type</td>
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<td></td>
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<tr>
<td>IDH1R132H/mut</td>
<td>176</td>
<td>97 (55.11)</td>
<td>79 (44.89)</td>
<td>0.049</td>
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<td>IDH1R132H/WT</td>
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<td>6 (35.29)</td>
<td>11 (64.71)</td>
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<td>WHO grade</td>
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<td>1 and 2</td>
<td>40</td>
<td>19 (47.50)</td>
<td>21 (52.50)</td>
<td>12.876</td>
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<td>3</td>
<td>43</td>
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<td>18 (41.86)</td>
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<td>110</td>
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<td>173</td>
<td>91 (52.60)</td>
<td>82 (47.40)</td>
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<td>Radiotherapy</td>
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<td>Yes</td>
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<td>25 (62.50)</td>
<td>15 (37.50)</td>
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<td>0.635</td>
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<td>153</td>
<td>79 (51.63)</td>
<td>74 (48.37)</td>
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Table 2  Univariate and Multivariable Analyses of Prognostic Factors for 5-Year Survival in Glioma Patients

<table>
<thead>
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<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>HR (95% CI)</td>
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<tr>
<td>BTF3L4 expression: high vs low or none</td>
<td>1.801 (1.217–2.665)</td>
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<tr>
<td>Age: ≤60 y vs &gt;60 y</td>
<td>2.093 (1.510–2.902)</td>
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<tr>
<td>Sex: male vs female</td>
<td>1.601 (1.149–2.232)</td>
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<td>Histologic type: a vs b vs c vs d vs e</td>
<td>0.855 (0.581–1.891)</td>
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<tr>
<td>Molecular type: IDH1mut vs IDH1WT</td>
<td>1.048 (0.799–0.985)</td>
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<tr>
<td>WHO grade: 1 and 2 vs 3 vs 4</td>
<td>1.920 (1.543–2.390)</td>
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<tr>
<td>Chemotherapy: yes vs no</td>
<td>0.680 (0.399–1.160)</td>
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<tr>
<td>Radiotherapy: yes vs no</td>
<td>0.737 (0.499–1.089)</td>
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</tbody>
</table>

*P < 0.05.

a, Astrocytoma, IDH1R132H mutant; b, oligodendroglioma, IDH1R132H mutant; BTF3L4, Basic Transcription Factor 3 Like 4; c, glioblastoma, IDH1R132H wild type; d, pilocytic astrocytoma; e, pleomorphic xanthoastrocytoma; HR, hazard ratio; WHO, World Health Organization.
Association of BTF3L4 Protein Expression with the Immune Checkpoints

Moreover, the efficacy of immune checkpoint blockade therapies, such as programmed cell death 1/PD-L1 inhibitors, has been linked to tumor mutational burden. Clinical studies have successfully used tumor mutational burden as a marker to predict treatment response.22 Thus, the paired expression of BTF3L4 in glioma and non-tumor brain tissues was analyzed in relation to the gene mutation landscape and genomic heterogeneity using TCGA data sets; it was identified as positively correlated to BTF3L4 and tumor mutational burden, a known indicator of a favorable response to anti—programmed cell death 1/PD-L1 treatment (Figure 6A). Furthermore, an assessment of the relationships between BTF3L4 protein expression and key immune checkpoint molecules within the glioma patient cohort (Figure 6, B and C) showed that BTF3L4 protein expression was negatively correlated with PD-L1 expression in lymphocytes (P = 0.05) (Figure 6C), implying a potential role of BTF3L4 in attenuating the immune response in glioma cells. Collectively, these findings suggest a negative association between BTF3L4 and PD-L1 expression in patients with gliomas.

**Figure 3** High expressed Basic Transcription Factor 3 Like 4 (BTF3L4) protein was associated with a malignant glioma phenotype. A and B: BTF3L4 protein expression in four glioma cell lines (U251, U87mg, SHG44, and T98G). C and D: The interference efficiency of siRNA1/2 in the U251 glioma cell line. E–G: Knock-down of BTF3L4 had an effect on the invasion and migration ability of U251 cells. H and I: Fluorescence-activated cell sorting analysis showing cell cycle progression after BTF3L4 knock down on U251 glioma cells. **P < 0.01, ***P < 0.005, ****P < 0.0001. Scale bar = 100 μm. n.s., no significance; si, small interfering.
The distinct microenvironment and inherent cellular characteristics of brain tumors have made them resistant to conventional and cutting-edge treatments.23 Although various strategies for treating gliomas have been developed, only a few have received clinical approval, highlighting the need for more research to discover promising biomarkers.

Immunotherapy has played a crucial role in cancer treatment, with impressive results in many types of cancer.24,25 Hence, more reliable immune-based biomarkers capable of predicting patient prognosis are needed to improve glioma treatment and patient outcomes.

The central nervous system is now recognized as having specialized immune properties, allowing the infiltration of immune cells.26 However, glioma patients often experience substantial immunologic dysfunctions as tumor cells create an immunosuppressive microenvironment to escape immune surveillance.27 Therefore, identifying prognostic-predictive genes and proteins related to infiltrated immune cells has been a significant focus of current research. Consequently, the primary focus of current research is on identifying genes and proteins that can predict prognosis and relate to infiltrating immune cells. Kaplan-Meier and Cox regression analyses revealed that BTF3L4 was an independent prognostic biomarker, which predicted an unfavorable prognosis for glioma patients. Meanwhile, in vitro experiments further showed that knockdown of BTF3L4 expression significantly increased the proliferation, migration, and invasion capacities of glioma cell lines, suggesting that BTF3L4 may play a vital role in glioma oncogenesis by promoting cell proliferation, migration, and invasion.

The heterogeneous components of the TME play a pivotal role in interacting with malignant cells throughout various stages of tumor progression, including metastasis, treatment resistance, and the induction of angiogenesis.28 Mechanistically, the TME influences cancer cells via complicated and
dynamic pathways to regulate cancer-related signaling involving ligand–receptor interactions (i.e., PD-L1 binding to programmed cell death 1). Quantitative miHc analysis revealed an increased proportion of patients in the neo-adjuvant group that exhibited focal up-regulation of PD-L1. However, insufficient endogenous immune cell was activated to hamper tumor cell proliferation. In this study, we observed down-regulation of the immune-checkpoint ligand PD-L1 in glioma tissues, together with high BTF3L4 expression and impaired patient overall survival. In addition, BTF3L4 was closely related to immune cell infiltration, especially with increased infiltration of natural killer CD56bright/dim cells, gamma delta T cells, regulatory T cells, T helper cells, DCs, plasmacytoid DCs, and T helper 2 cells, indicating a "nonsupportive" glioma TME. Among them, BTF3L4 protein expression was related to extensive CD66B+ neutrophil infiltration in the glioma TME. The TME plays a critical role in shaping the characteristics of CD66B+ neutrophils by influencing the permeability of blood vessels to immune and cancer cells. Specifically, the crosstalk between tumor-associated neutrophils and tumor cells actively contributes to tumor progression and significantly affects patient survival. Recent research has linked neutrophil extracellular traps, produced by tumor-infiltrating neutrophils, to poor patient prognosis in hepatocellular carcinoma. In addition, neutrophil extracellular traps have been implicated in mediating the crosstalk between glioma progression and the TME. Thus, targeting CD66B+ neutrophils to inhibit formation of neutrophil extracellular traps could be a promising approach to hinder glioma progression.

Nonetheless, there are limitations to this research. We did not fully elucidate the dynamic interactions between BTF3L4 and the receptors/ligands on the neutrophil surface within the TME. Future research should aim to uncover the...
molecular mechanisms and regulatory pathways involved in glioma development and progression, particularly in the context of BTF3L4 interactions.

In summary, these findings highlight the potential of dysregulated BTF3L4 expression as an independent prognostic factor for glioma patients. Moreover, this study found that the BTF3L4 protein plays a direct role in driving the malignant progression of glioma. As a result, BTF3L4 holds promise as both a prognostic marker for glioma and a target for immune therapy for improving the treatment of glioma patients in the future.

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Author Contributions

Author contributions were as follows: B.L. and T.L., conceptualization and writing—original draft; J.S., investigation and validation; P.S. and X.Z., data curation and visualization; L.Y., methodology and resources; and Z.W. and J.H., supervision and funding acquisition and writing—review and editing. Informed consent was obtained from all co-authors for publication of this manuscript.

Disclosure Statement

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Supplemental Data

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