Bromodomain-containing protein 4 activates cardiotrophin-like cytokine factor 1, an unfavorable prognostic biomarker, and promotes glioblastoma *in vitro*

Shang-Hang Shen[1]^, Jian-Feng Guo[1], Ju Huang[2], Qian Zhang[3], Yi Cui[3]

[1]Department of Neurosurgery, The First Affiliated Hospital of Xiamen University, Medical College of Xiamen University, Xiamen, China; [2]College of Life and Environment Sciences, Shanghai Normal University, Shanghai, China; [3]School of Biotechnology, East China University of Science and Technology, Shanghai, China

Contributions: (I) Conception and design: SH Shen; (II) Administrative support: JF Guo; (III) Provision of study materials or patients: J Huang; (IV) Collection and assembly of data: Q Zhang; (V) Data analysis and interpretation: Y Cui; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Qian Zhang; Yi Cui. School of Biotechnology, East China University of Science and Technology, Shanghai 200237, China. Email: zhangqian_988@163.com; cuiyi0422@hotmail.com.

Background: Glioblastoma (GBM) is one of the most common and malignant brain tumors. Cardiotrophin-like cytokine factor 1 (CLCF1) is a member of the IL-6 superfamily. However, the clinical significance, potential role, and molecular mechanism of CLCF1 in GBM remain obscure. Here, the expression and prognostic significance of CLCF1 was investigated in GBM.

Methods: The Cancer Genome Atlas (TCGA) GBM and Chinese Glioma Genome Atlas (CGGA) datasets were downloaded and analyzed by using Gene Expression Profiling Interactive Analysis (GEPIA). Next, 3 shRNAs targeting CLCF1 were designed, and silencing efficiency was examined with real-time polymerase chain reaction (PCR). Cell Counting Kit 8 (CCK-8), flow cytometry, transwell, and wound healing assays were used to study the function of CLCF1 in glioma cells.

Results: We found increased expression of CLCF1 as an unfavorable prognostic marker in GBM. Functionally, down-regulation of CLCF1 significantly reduced cell proliferation, induced cell apoptosis and cell cycle G2 phase arrest, and weakened the migration and invasion of GBM cells. Downstream pathway analysis was conducted, and potential targets in cytokine receptors, extracellular matrix (ECM) receptors, apoptosis, and the cell cycle were uncovered. Finally, transcriptional regulators were analyzed, and bromodomain-containing protein 4 (BRD4) was found to activate CLCF1 in GBM.

Conclusions: CLCF1, transcriptionally activated by BRD4, promotes glioma and serves as an unfavorable marker in GBM.

Keywords: Cardiotrophin-like cytokine factor 1 (CLCF1); glioblastoma (GBM); prognosis; cytokine receptors; bromodomain-containing protein 4 (BRD4)

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^ ORCID: 0000-0002-9207-6461.

Introduction

Glioblastoma (glioblastoma multiforme, GBM) is one of the most common and malignant grade IV glial tumors (1,2). Mutations (MUT) in isocitrate dehydrogenase (IDH) and O6-methylguanine-methyltransferase (MGMT) promoter methylation are 2 prognostic biomarkers of GBM (3). Typical molecular changes include MUT in receptor tyrosine kinase (RTK), rat sarcoma (RAS), phosphoinositide 3-kinase (PI3K), p53, and retinoblastoma protein (RB) signaling genes (4,5). Although surgery combined with radiation therapy and chemotherapy has been widely used for the treatment of GBM, the prognosis is still poor. Thus, it is necessary to identify all potential therapeutic targets for GBM and clarify their underlying mechanisms.

Increasing evidence indicates that immune cells and cytokines (6) in the tumor microenvironment are involved in GBM progression (7), prognosis (8), and therapy response (9). Cardiotrophin-like cytokine factor 1 (CLCF1) is a member of the IL-6 superfamily (10) and forms a heterodimer complex with cytokine receptor-like factor 1 (CRLF1). As a potent neurotrophic factor, CLCF1 competes with ciliary neurotrophic factor (CNTF) and binds ciliary neurotrophic factor receptor (CNTFR) (11). CLCF1 was also reported to bind and activate the ILST/gp130 receptor and Janus kinase (JAK)-STAT signaling cascade (12). Previous studies have shown that CLCF1 is involved in osteogenesis (13), osteoporosis (14), hematopoiesis (15), atherosclerosis (16,17), fibrosis (18), nerve regeneration (19), cold-induced sweating syndrome (20), inflammatory diseases (12), and lung cancer (11,16,21). CLCF1 was significant prognostic genes in both IDH-wild type (WT)/PTEN-MUT GBM and LGG patients, and was bond by the small molecule compound (+)-JQ1 as potential therapy for PTEN-mut glioma (22). However, further studies are needed to uncover the detailed function and regulation of CLCF1 in GBM.

Recently, the bioinformatics research and microarray technology has enabled us to understand the occurrence, development and metastasis of glioma at the molecular level. Bioinformatics research revealed that therapeutic molecular targets and the theoretical basis of tumor with systematic, accurate and effective way, which makes it possible to study the genetic changes and molecular mechanisms of glioma and provides a new idea for studying the molecular pathogenesis of various diseases. Bioinformatics research including gene ontology (GO), Kyoto encyclopedia of genes and genomes (KEGG), protein to protein interaction analysis (GSEA), etc. These methods help us identify the core driving genes of the disease and the abnormal regulatory pathways in glioblastoma. Gene Expression Profiling Interactive Analysis (GEPIA) database including the genes expression and the survival prognosis of glioblastoma patients.

Here, we first studied the expression changes and prognostic significance of CLCF1 in GBM based on the GEPIA database. Then, the function of CLCF1 in glioma cells was investigated with gene loss-of-function strategies. Next, the downstream target of CLCF1 in GBM was analyzed. Finally, we analyzed the transcriptional activators for high expression of CLCF1 in GBM tumor samples. This study deepens our understanding of CLCF1 in GBM and provides a potential target for GBM therapy. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1164/rc).

Methods

Differential expression and prognostic significance of CLCF1 in GBM datasets

The expression and prognostic significance of CLCF1 was investigated with The Cancer Genome Atlas (TCGA) GBM and Chinese Glioma Genome Atlas (CGGA) datasets using Gene Expression Profiling Interactive Analysis (GEPIA) (23). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell culture and transfection

The human GBM cell lines U87 and U251 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences, Shanghai, China. U87 and U251 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Three shRNAs targeting CLCF1 were designed, and silencing efficiency was examined with real-time polymerase chain reaction (PCR).

The sequencing of shRNAs for CLCF1 were as follows: shRNA1 (5'-3'): GGCTGGGACCTATCTGAACTA; shRNA2 (5'-3'): GCTGGGACCTATCTGAACTAC; shRNA3 (5'-3'): GCGAAGCCTCAATGACAAACT. The primer sequences of CLCF1 and GAPDH (housekeeping gene) were as follows: CLCF1 F(5'-3'): TTTCAACGAGCCAGACTTCAAC, R(5'-3'): GAGGCCACGCAAGTAACACA; GAPDH F(5'-3'): GGAGCGAGATCCCTCCAAAAT, R(5'-3'): GGCTGTTGTCATACTTCTCATGG. Gene expression

quantification was performed with the $2^{-\Delta\Delta CT}$ method in this study.

Cell proliferation assays

Cell proliferation was measured using the Cell Counting Kit 8 (CCK-8) assay. After 48 h of transfection, cells were seeded in 96-well plates at a density of 5×10^3 per well and cultured in an incubator for 0, 24, 48, and 72 h at 37 ℃. Then, 10 μL of CCK-8 solution (Beyotime Biotechnology, Shanghai, China) was added, and the cells were cultured in an incubator for 2 h. The OD450 was measured using a spectrophotometer (DS-11 FX; DeNovix, Wilmington, USA).

Cell cycle assay by flow cytometry

The effect of CLCF1 on cell cycle arrest in GBM cells was assessed using a flow cytometer. Cells were harvested, washed twice using phosphate-buffered saline (PBS), and fixed with 75% ethanol at 4 ℃ for at least 4 h. Then, the cells were collected and stained with PI solution/RNase A at 4 ℃ in the dark for 30 min, followed by flow cytometric analysis.

Apoptosis assay by flow cytometry

The effect of CLCF1 on apoptosis in GBM cells was assessed using a flow cytometer. U251 cells were transfected and cultured for 24 h. Then, the cells were digested, washed, and resuspended in PBS. Finally, the cells were stained using Annexin V-FITC at 4 ℃ in the dark for 20 min and analyzed by flow cytometry.

Wound healing assay

Take cells from each group and add them into the well plate. There are 5 horizontal lines and 5×10^5 cells. After overnight incubation, 200 μL gun head is perpendicular to the horizontal line scratch on the back, and wash away the scratched cells. After 48 hours of treatment, take samples and take photos.

Transwell assay

U251 cells were routinely digested, centrifuged and collected, and cultured with serum-free DMEM. The cells were resuspended in the medium and made into cells with a density of 2.5×10^4 cells/mL suspension. 200 mL cell suspension was inoculated into the upper chamber of transwell chamber, 800 mL DMEM medium containing 10% fetal bovine was added to the lower chamber. Incubate in 37 ℃ incubator for 48 h. Take out the transwell chamber, gently wipe the non-invasion cells in the upper chamber with a cotton swab, wash with PBS for 3 times, and dry them at room temperature for about 10 min. The cells were stained with 100 mL of crystal violet. Let it stand at room temperature for 20 min, and then wash it with PBS for 3 times. The invasion cells were observed and counted under the microscope. The experiment was repeated three times.

Gene set enrichment analysis (GSEA) of CLCF1-related pathways

GBM samples were separated into 2 groups (high and low) based on the median CLCF1 expression. GSEA was applied to identify the differences in the MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp) C2 collection between the high group and low group.

Dual luciferase assay

HEK-293T cells were plated on a 12-well plate and cultured at 37 ℃ in a humidified incubator with 5% CO_2. One day after culture, the cells in each well were co-transfected with pcDNA3.1. The cells were also co-transfected with pcDNA3.1-CLCF1-WT or pcDNA3.1-CLCF1-MUT using Renilla luciferase expression vectors. The bromodomain-containing protein 4 (BRD4) inhibitor JQ1 was added to the 293T cells. After 48 hours of culture, luciferase activity was detected according to the steps of double luciferase reporter gene detection kit.

Statistical analysis

The statistical analysis was conducted with GraphPad Prism 8.0. A *t*-test or one-way ANOVA was used to compare the difference between 2 or more groups. All cell experiments were replicated 3 times, and a P value <0.05 was considered statistically significant.

Results

CLCF1 is an unfavorable prognostic marker in GBM

To explore the clinical significance of CLCF1 expression in GBM, we first analyzed the differential expression of CLCF1 in GBM cancer and non-cancerous samples with

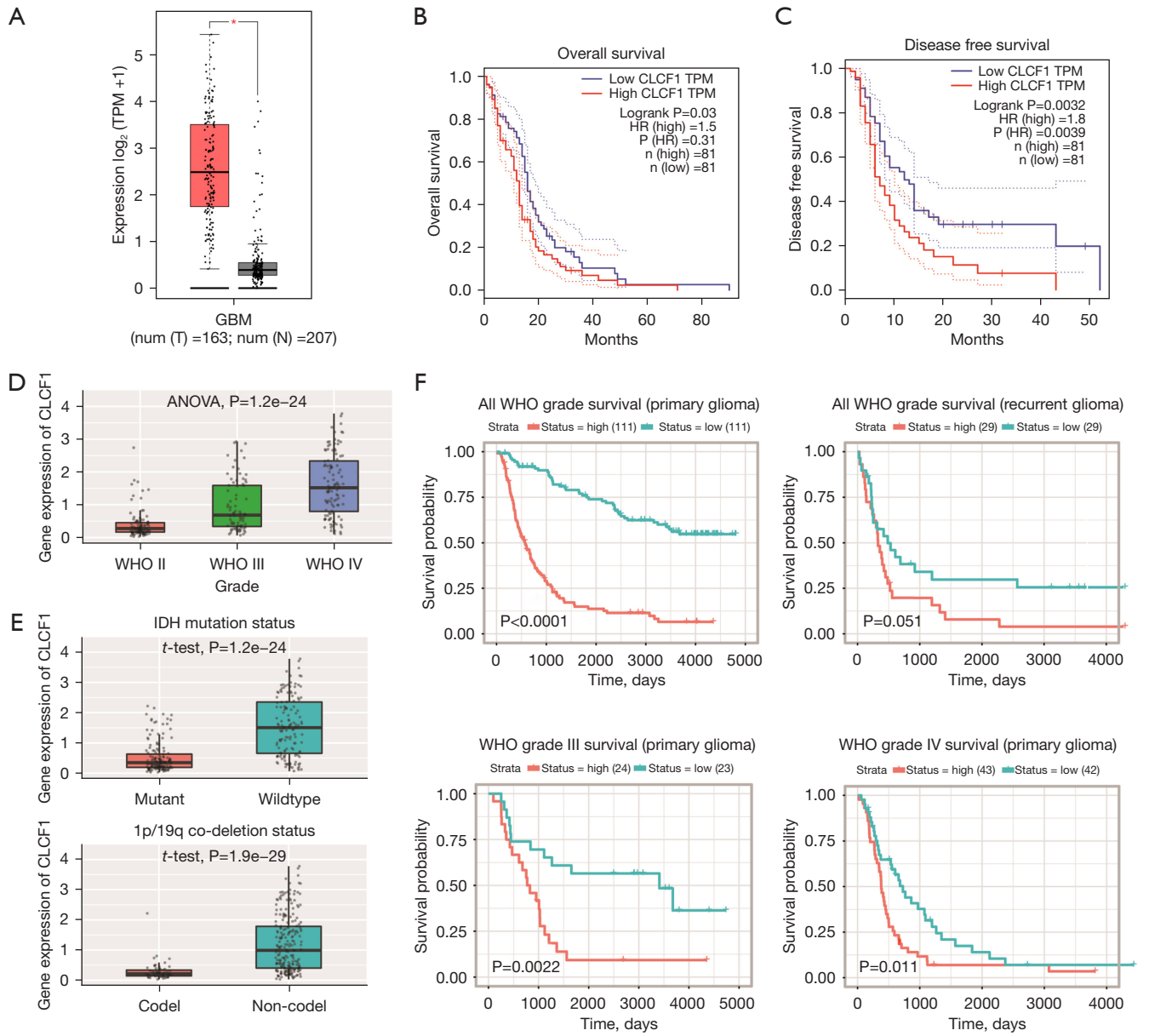

Figure 1 Clinical significance of CLCF1 expression in glioblastoma. (A) CLCF1 showed increased expression in TCGA GBM cancer samples; *, P<0.01; (B,C) Kaplan-Meier plot showing the overall survival rate (B) and disease-free survival rate (C) in the CLCF1 high and low GBM groups; the blue solid line represents low CLCF1 survival curve, and red solid line represents high CLCF1 survival curve; the blue dotted line represents the confidence interval of low CLCF1, red dotted line represents the confidence interval of high CLCF1; (D) expression level of CLCF1 in WHO grade II, III, and IV glioma samples; (E) expression level of CLCF1 in IDH mutant/wildtype and 1p/19q co-deletion and non-co-deletion glioma samples; (F) Kaplan-Meier survival plot showing the prognostic performance of CLCF1 in primary, recurrent, grade III, and IV glioma patients. CLCF1, cardiotrophin-like cytokine factor 1; TPM, transcripts per million; HR, hazard ratio; IDH, isocitrate dehydrogenase; TCGA, The Cancer Genome Atlas; GBM, glioblastoma.

the TCGA GBM dataset. As shown in *Figure 1A*, CLCF1 had significantly higher expression in the cancer group, indicating an oncogenic role of CLCF1 in GBM. Then, we analyzed the prognostic significance of CLCF1 in GBM patients. As shown in *Figure 1B,1C*, GBM patients with higher CLCF1 expression had a significantly lower overall

survival (OS) rate [hazard ratio (HR) =1.5, P value =0.031] and disease-free survival rate (HR =1.8, P value =0.0039), implying that CLCF1 may serve as a risk factor for GBM patients. CGGA datasets were utilized to validate the prognostic significance of CLCF1 in GBM. As shown in *Figure 1D*, increased expression of CLCF1 was observed in higher WHO grade GBM samples. Additionally, CLCF1 was found to be highly expressed in IDH1 wild-type and non1p/19q co-deletion samples (*Figure 1E*). Finally, CLCF1 was found to be significantly related to prognosis in primary glioma (P<0.0001), recurrent glioma (P=0.051), grade III (P=0.0022), and IV glioma patients (P=0.011) (*Figure 1F*).

CLCF1 silencing suppresses cell proliferation, migration, and invasion and enhances apoptosis in GBM in vitro

To investigate the biological functions of CLCF1 in GBM U87 and U251 cells, including cell proliferation, migration, invasion, cell cycle, and apoptosis, shRNAs were constructed. Expression of CLCF1 in the CLCF1 knockdown groups was down-regulated compared to that in the control groups (*Figure 2A*). Cell proliferation in the CLCF1 knockdown groups was found to be significantly lower than that in negative control (NC) in both cell lines (*Figure 2B*,*2C*). As CLCF1 silencing exhibited a more pronounced effect in U251 cells, the subsequent assays were conducted in U251 cells. As shown in *Figure 2D*, CLCF1 knockdown significantly reduced the proportion of cells in G0/G1 phase but slightly induced G2 cell cycle arrest. A higher percentage of apoptotic cells was found in the CLCF1 knockdown groups than in the control groups (*Figure 2D*). These results suggest that CLCF1 might enhance the cell growth of GBM. The effect of CLCF1 on the migratory and invasive abilities of GBM cells was determined using transwell and wound healing assays, respectively. The transwell invasion assay results indicated that knockdown of CLCF1 reduced cell migration in GBM cells (*Figure 2E*). Migration in GBM cells was slower in the CLCF1 silencing group than in the control groups (*Figure 2F*). These results indicate that CLCF1 plays an oncogenic role by promoting cell migration and invasion *in vitro*.

Related downstream mechanisms of CLCF1 in GBM

To uncover the molecular mechanism of CLCF1 in GBM, we divided GBM cancer samples into CLCF1 high/low groups and performed GSEA. The top 10 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the CLCF1 high and low groups are shown in *Figure 3A*. For the CLCF1 high group, immune- and cell proliferation-related pathways, such as cytokine-cytokine receptor interaction (*Figure 3B*), extracellular matrix (ECM) receptor interaction (*Figure 3C*), and apoptosis (*Figure 3D*), were significantly enriched. For the CLCF1 low groups, we noticed that the gene set in the cell cycle (*Figure 3E*) was one of the most significantly enriched terms. To further analyze potential targets of CLCF1, a protein-protein interaction (PPI) network of CLCF1 was constructed using the String database (*Figure 3F*). According to *Figure 3F*, CLCF1 was predicted to be related to leukemia inhibitory factor receptor (LIFR), cardiotrophin-1 (CTF1), JAK1, JAK2, signal transducer and activator of transcription 3 (STAT3), CNTF, CNTFR, CRLF1, IL-6 signal transduction (IL6ST), and tyrosine kinase 2 (TYK2). In fact, CLCF1 is a member of the IL-6 cytokine family, and its potential role in inflammatory diseases and cancers has been reported (12). All of these results suggest that CLCF1 may play a complex role in GBM by repressing cell proliferation and regulating the tumor microenvironment through cytokine receptors and ECM receptors.

BRD4 transcriptionally activates CLCF1 in GBM

As CLCF1 showed significantly higher expression in GBM, we explored the factors that impact CLCF1 expression. First, we analyzed histone activation marks, such as H3K4me3 and H3K27ac peaks, in glioma cells and samples from the ChIP-Atlas database (24). As shown in *Figure 4A*, strong binding peak signals were not only found for H3K4me3 and H3K27ac, but also transcription factors such as BRD2, BRD4, MYC, MYC-associated protein X (MAX), and Mediator Subunit 1 (MED1) were found to bind to the CLCF1 promoter. Then, we validated the expression of 5 transcription factors with CLCF1 in the CGGA glioma datasets. As shown in *Figure 4B*, there was a strong positive correlation coefficient between BRD2, BRD4, MYC, and MAX and CLCF1 in both primary and recurrent glioma samples, suggesting that CLCF1 may be regulated by these transcription factors. As BRD4 showed the highest correlation coefficient with CLCF1 (0.359 for primary glioma and 0.28 for recurrent glioma), we chose BRD4 for subsequent validation. A dual luciferase assay was then conducted to test whether mutation of the BRD4 binding cis-element in the CLCF1 promoter impacts CLCF1 expression. Binding sequencing was mutated, as

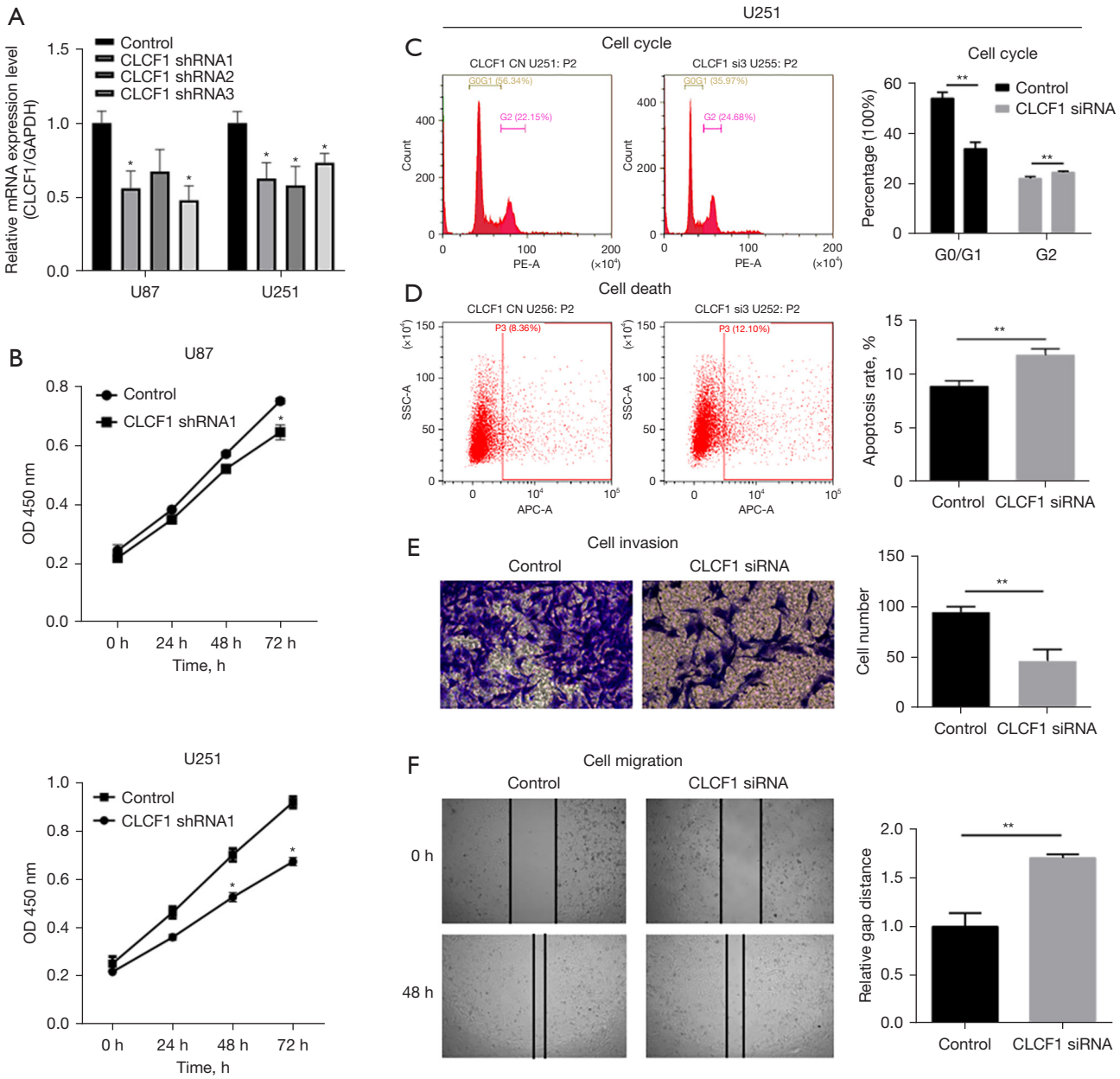

Figure 2 The effect of CLCF1 silencing in glioblastoma cells. (A) CLCF1 mRNA expression after shRNA transfection as measured by qRT-PCR; (B) CCK-8 assay of the viability of U251 and U87 cells after knockdown of CLCF1; (C) effect of CLCF1 silencing on the cell cycle distribution of U251 cells by used flow cytometry assay; (D) CLCF1 down-regulation induced apoptosis in U251 cells by used flow cytometry assay; (E) CLCF1 silencing in U251 cells reduced cell invasion were observed under electron microscope by stained with 0.1% crystal violet (magnification, 100×); (F) wound healing assay indicated that reduction of migration capability after CLCF1 knockdown in U251 cells were observed under electron microscope (magnification, 40×). *, P<0.05; **, P<0.01. CLCF1, cardiotrophin-like cytokine factor 1; OD, optical density; qRT-PCR, quantitative real-time polymerase chain reaction; CCK-8, Cell Counting Kit 8.

shown in *Figure 4C*, and the BRD4 inhibitor JQ1 was used to antagonize BRD4 function. The BRD4 inhibitor JQ1 significantly reduced luciferase activity, while no significant difference was found in the mutant group (*Figure 4D*), implying that elevated CLCF1 was transcriptionally activated by BRD4 in glioma.

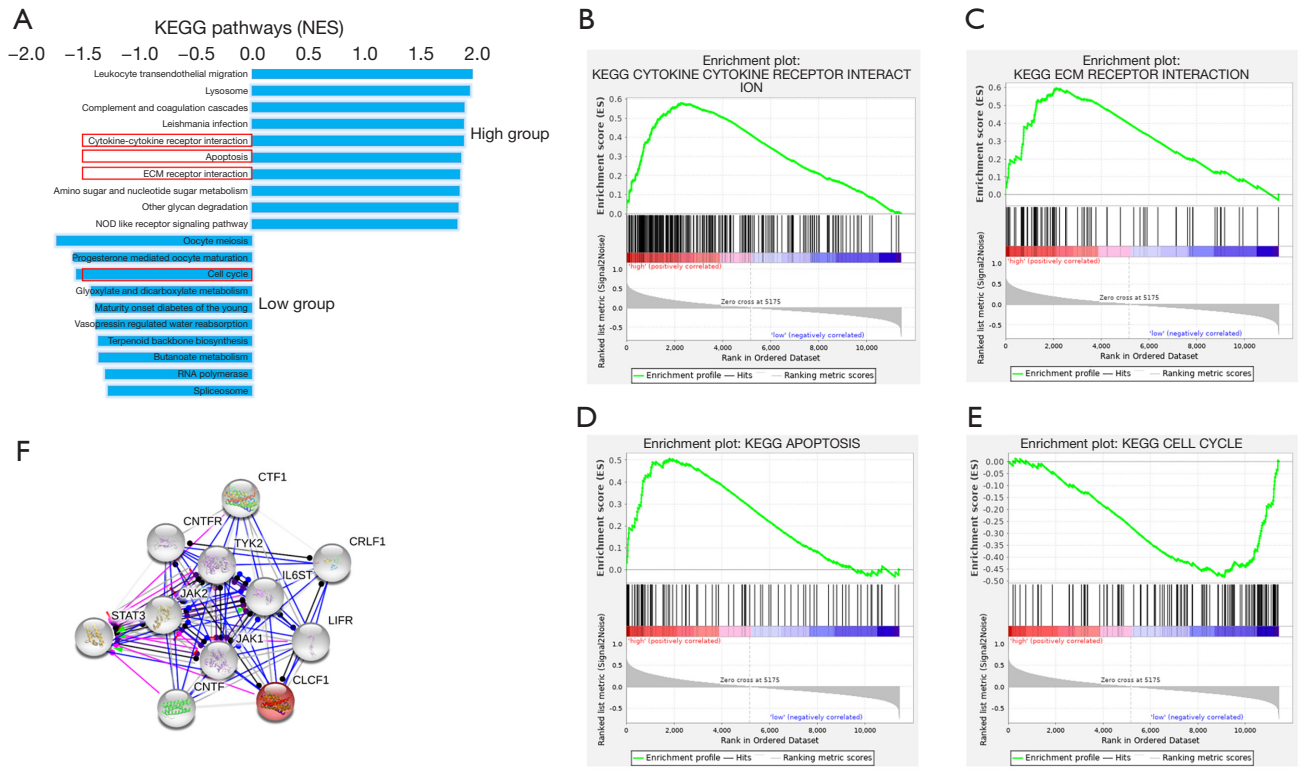

Figure 3 GSEA and protein-protein interaction analysis revealed pathways and targets of CLCF1. (A) Top 10 most enriched pathways in the CLCF1 high and low groups, revealed by GSEA; enrichment plots of cytokine-cytokine receptor interactions (B) ECM receptor interactions (C), apoptosis (D), and the cell cycle (E) are shown; (F) protein-protein interaction network of CLCF1. KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, non-epileptic seizures; ECM, extracellular matrix; NOD, non diabetic; CTF1, cardiotrophin-1; CNTFR, ciliary neurotrophic factor receptor; TYK2, tyrosine kinase 2; CRLF1, cytokine receptor-like factor 1; JAK, Janus kinases; IL6ST, IL6 signal transduction; STAT3, signal transducer and activator of transcription 3; LIFR, leukemia inhibitory factor receptor; CNTF, ciliary neurotrophic factor; CLCF1, cardiotrophin-like cytokine factor 1; GSEA, gene set enrichment analysis.

Discussion

Accumulating evidence indicates that cytokines are widely involved in GBM progression and therapy response (6). Previous studies found that the high expression of lncRNA MIR155 host gene (MIR155HG) was a prognostic biomarker and associated with poorer overall survival in glioblastoma multiforme (25). Shugoshin 2 (SGO2) is a biomarker that is predictive of WHO pathological grading and patient survival in patients with gliomas (26). Nicotinamide phosphoribosyltransferase (NAMT), and C1q/TNF-related protein 1 (CTRP1) were reported to be potential prognostic and therapeutic biomarker for glioblastoma (27,28). In this study, we focused on the cytokine CLCF1. We uncovered its clinical significance and function in glioma cells and finally identified potential downstream targets and BRD4 as a transcription activator for the elevated expression of CLCF1 in GBM. By binding to CNTFR, CLCF1 mediates interactions with the co-receptors glycoprotein 130 (gp130) and LIFR. In lung cancer, CLCF1-CNTFR signaling has been observed in cancer-associated fibroblasts (CAFs) and acts to promote cancer cell growth (21). Additionally, a decoy receptor/antagonist (16) engineered to target CLCF1/CNTFR signaling proved effective in inhibiting tumor growth, and the mechanism was related to guanosine triphosphate loading (11). Therefore, this study may provide a new therapeutic target and prognostic marker in GBM.

Given that CLCF1 showed significantly higher expression in GBM cancer samples, we then focused on the CLCF1 downstream pathways and targets to analyze the role of CLCF1 in GBM. As expected, the GSEA pathway analysis identified potential targets in 4 cellular processes: cytokines and receptors, ECM and receptors,

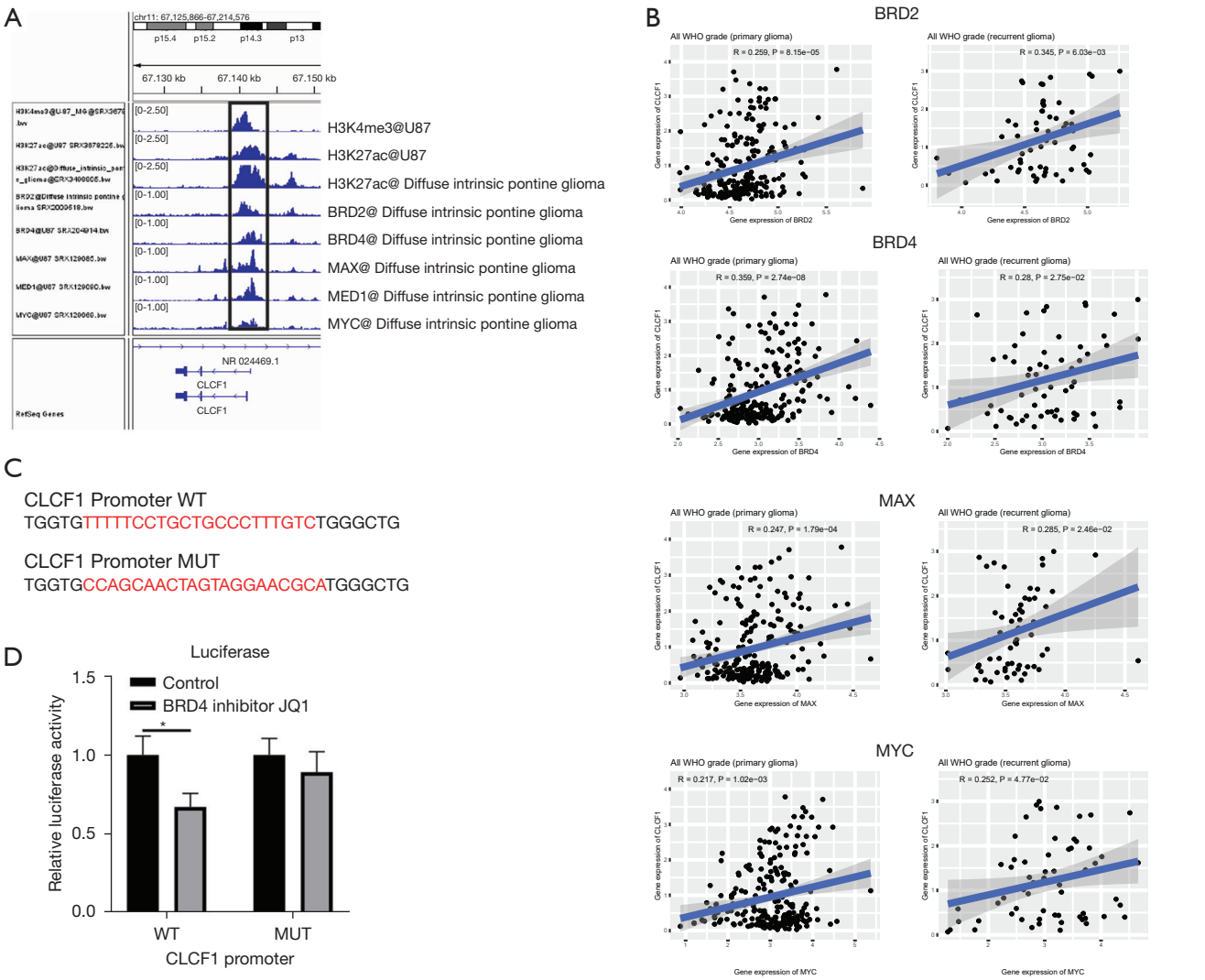

Figure 4 Transcriptional analysis suggests BRD4 as a potential transcription activator of CLCF1 in glioblastoma. (A) ChIP-Seq showing transcription activation marks of H3K4me3, H3K27ac, and transcription factor binding peaks in the CLCF1 promoter region of glioma cells and samples; (B) expression correlation between BRD2, BRD4, MAX, MYC, and MED1 and CLCF1 in CGGA glioma samples; (C) the binding of BRD4 with the CLCF1 promoter cis-element was mutated, and a subsequent dual luciferase assay was conducted; (D) a dual luciferase assay was conducted to study the regulation of CLCF1 by BRD4 in glioma. *, P<0.05. BRD, bromodomain-containing protein; MAX, MYC-associated protein X; MED1, Mediator Subunit 1; MYC, Myc gene; CLCF1, cardiotrophin-like cytokine factor 1; WT, wild type; MUT, mutations.

apoptosis, and the cell cycle (*Figures 3,4*). Interestingly, CLCF1 showed positive and negative correlations with pro-apoptosis markers (FAS, BAX, and CASP7) and cell cycle (CCNA2, CCNB1, and CDKN1C) markers, suggesting that CLCF1 may inhibit cancer cell growth in GBM. Furthermore, CLCF1 was positively correlated with cytokines (LIF, CXCL3, and CSF1), cytokine receptors (IL4R, TGFBR2, and CCR1), ECM proteins (COL1A1, COL1A2, and LAMA2), and ECM receptors (ITGA5, ITGA3, and ITGB5), most of which have been reported as oncogenes in GBM (29-31). As cytokines, ECM proteins and their receptors are well-recognized participants in cell-cell communications of the microenvironment (6,32).Their significant correlations with CLCF1 imply that CLCF1 may also play a tumor-promoting role by regulating the GBM microenvironment. In summary, CLCF1 may play a

complex role in GBM by repressing cell proliferation and regulating the tumor microenvironment through cytokines and ECM proteins and their respective receptors.

Transcription factors (33) are known regulators of gene expression and are markers in GBM. In this study, we found a strong binding peak and a significant positive correlation between 4 transcription factors and CLCF1, suggesting their transcriptional regulation of CLCF1 expression, and subsequent analysis identified BRD4 as a transcription factor for CLCF1.

BRD4, a member of the bromodomain and extraterminal (BET) protein family, plays an important role in controlling oncogene expression and genome stability (34). In glioma, BRD4 has been reported to promote glioma cell stemness (35-37) and progression (38-41). Recently, studies have indicated that BRD4 is a prospective therapeutic target for the treatment of GBM for its ability to penetrate the blood-brain barrier and target glioma tumor tissues with little side effects (42,43). Therefore, understanding the mechanism of CLCF1 as the target of BRD4 would provide another perspective for the treatment of glioma with BRD4 inhibitors.

Here, this study reveals the expression and prognosis of CLCF1 in glioblastoma based on public information database and bioinformation analysis technology, in order to further clarify the mechanism in the occurrence and development of glioblastoma from a new perspective, seek new effective treatment targets and prolong the survival of patients. Collectively, by integrated bioinformatics analysis, this study revealed CLCF1 as a hazardous prognostic marker in GBM *in vitro*. CLCF1 might play a complex role in GBM by regulating GBM cancer cells and the immune microenvironment through cytokines, the ECM, and receptors. Finally, BRD4 may be involved in transcriptional activation of CLCF1 in GBM.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1164/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1164/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1164/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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