Original Article

Comprehensive analysis to identify the relationship between CALD1 and immune infiltration in glioma

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Background: An accumulating number of studies show that CALD1 is associated with a variety of tumor microenvironments (TME) and is closely related to patients' survival. However, to the best of our knowledge, few studies examined the role of CALD1 in the immune microenvironment of glioma. The aim of this study is to investigate the potential correlation between CALD1 and the pathogenesis and progression of glioma, aiming to identify a novel therapeutic target.

Methods: We assessed the role of CALD1 in pan-cancer and investigated the correlation between CALD1 and TME of glioma by bioinformatic analysis and experimental verification.

Results: We found that CALD1 expression in glioma was associated with a variety of infiltrating immune cells. CALD1 can promote the development of glioma by affecting M2 macrophage infiltration. Also, we found that CALD1 was closely associated with tumor mutation burden, microsatellite instability, copy number variation, methylation, and stem cell index. Our clinical correlation study demonstrated that CALD1 was associated with overall survival, progression-free interval, and disease-specific survival in a variety of tumors. We verified the significantly high expression of CALD1 in glioma using quantitative real-time polymerase chain reaction (PCR) and Western blotting. Meanwhile, we also conducted relevant cell experiments to prove that CALD1 can affect the proliferation and migration ability of glioma cells in vitro.

Conclusions: Our results confirmed that CALD1 may be a prognostic marker for glioma and a potential target for immunotherapy in the future.

Keywords: CALD1; glioma; immune infiltration; biomarker

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Introduction

Glioma is the most commonly occurring type of malignant brain tumor, and it causes the highest tumor-related morbidity and mortality in children and adults (1). Despite great advances in therapeutic approaches in recent decades, the 5-year survival rate in patients with glioma, especially glioblastoma (GBM), is only 4.7% (2). A large part of the treatment failure is due to intracranial molecular complexity and the specific intracranial microenvironment (3,4). Therefore, an in-depth investigation of the intracranial molecular mechanism will greatly contribute to the personalized treatment and survival prognosis of glioma patients.

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CALD1 is a gene that encodes a calmodulin with two major isoforms. The high molecular weight calmodulin is restricted to visceral and vascular smooth muscle cells, and the low molecular weight isoform is found in non-smooth muscle cells (5). CALD1 binds to calmodulin and actin and regulates smooth muscle contraction. It also regulates cell morphology, cell motility, and cytoplasmic division and may play a part in tumor metastasis and proliferation (6). Several studies showed that CALD1 overexpression is associated with metastasis and poor prognosis in several tumor types, such as stomach, colorectal and oral cancer (7-9). The tumor microenvironment (TME) is a complex multi-factor, multi-cellular microenvironment that gradually forms during tumor growth (10) and supports the proliferation and metastasis of various tumor cells (11). Tumor stromal cells are an important component of the TME, and cancer-associated fibroblasts (CAFs) have an important impact on tumor cell growth and metastasis (12). Du *et al.* demonstrated that CALD1 was an important gene associated with CAFs and played an important role in regulating the bladder cancer (BLCA) stromal microenvironment and immune microenvironment (13). Cheng *et al.* also verified that stromal cell aggregation in the glioma microenvironment was significantly associated with CALD1 overexpression (14). However, to our knowledge, the immune-specific functions of CALD1 expression in gliomas have not been evaluated.

Therefore, we investigated the correlation of CALD1 with immune infiltration and immune-related markers in the glioma TME and immune checkpoints using a bioinformatics approach. We also investigated the association of CALD1 with glioma clinical features, tumor mutation burden (TMB), microsatellite instability (MSI), copy number variation (CNV), methylation, and stemness index. We further confirmed the differential expression of CALD1 in gliomas by experiments and demonstrated its important role *in vitro* and *in vivo*. In conclusion, CALD1 is a promising new therapeutic target for glioma and a potential predictive target for immune infiltration and poor outcome. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-216/rc).

Highlight box

Key findings

- Our results confirmed that CALD1 may be a prognostic marker for glioma and a potential target for immunotherapy in the future.

What is known and what is new?

- CALD1 overexpression is associated with metastasis and poor prognosis in several tumor types, such as stomach, colorectal and oral cancer.
- The most important finding of our study is that we confirmed that CALD1 expression positively correlated with the degree of infiltration of multiple immune cells in glioblastoma and low-grade glioma. And CALD1 may playe an immunosuppressive role by promoting CCL2 expression and tumor-associated macrophages polarization.

What is the implication, and what should change now?

- The presence of CALD1 may contribute to the establishment of immunosuppressive microenvironment in glioma. Inhibition of CALD's activity holds promise for enhancing the immune response against glioma in the future.

Methods

Data collection and processing

We first downloaded pan-cancer sequencing data, TMB, MSI, and prognostic information, such as overall survival (OS), progression-free interval (PFI), and disease-specific survival (DSS), of tumor patients from UCSC Xena (https://tcga.xenahubs.net). TMB represents the total number of mutations present in tumor samples, and it predicts the effectiveness of immunotherapy (15). MSI is another marker of genomic instability that results from functional defects in DNA mismatch repair in tumor tissue. We downloaded the stemness index information of pan-cancer from the study of Malta *et al.* (16), which primarily includes the mRNA expression-based stemness index (mRNAsi) and the DNA methylation-based stemness index (mDNAsi).

Differential expression of CALD1

We analyzed the differential expression of CALD1 using R software on tumor information from The Cancer Genome Atlas Program (TCGA) database and normal tissue information from the Genotype Tissue Expression (GTEx) database. The differential expression of CALD1 was confirmed in Tumor Immune Estimate Resource (TIMER, https://cistrome.shinyapps.io/timer/), which is a web portal for comprehensive analysis of tumor-infiltrating immune cells (17-19).

CNV and methylation analysis

Gene Set Cancer Analysis (GSCA, https://bioinfo.life.hust.edu.cn/GSCA/#/) is an online platform for genomic, pharmacogenomic, and immunogenomic gene set cancer analysis. We investigated the correlation of CALD1 expression with CNV and methylation using GSCA.

Survival analysis and relationship with clinical stage

To investigate whether CALD1 predicts prognosis, we analyzed the correlations between CALD1 and patient OS, PFI, and DSS using the "SURVIVAL" package and plotted Kaplan-Meier (K-M) survival curves and receiver operating characteristic (ROC) validation curves. We also investigated the correlation between CALD1 and cancer grading.

Gene set enrichment analysis (GSEA)

To further evaluate the molecular mechanisms involved in CALD1 in the progression of glioma, we performed CALD1 expression correlation analysis on glioma data in TCGA using R software. The differentially expressed genes with a correlation threshold Log_2[fold change (FC)] >1 and P<0.05 were selected for subsequent analyses.

Immune infiltration analysis

We analyzed the correlation between CALD1 and immune cell infiltration in gliomas using the "GSVA" package of R software. We used the "Correlation" module of the TIMER database to analyze the correlation between different markers of immune infiltrating cells and the expression of CALD1. Immune checkpoint-related genes may be strongly associated with the clinical outcomes of immunotherapy (20). Therefore, we further evaluated the correlation of CALD1 with immune checkpoint-related genes. To further assess immune microenvironment differences, we calculated the ImmuneScore, StromalScore, and ESTIMATEscore separately for glioma patients using the "ESTIMATE" package.

Cell culture

Patient-derived GBM stem-like cells GBM#BG7 and GBM#BG5 were isolated and functionally characterized from GBM surgical specimens (21,22). The glioma cell lines U118MG, A172, U251MG, and LN229 were obtained from the Type Culture Collection of the Chinese Academy of Sciences. All media were supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Invitrogen, USA). All cells were incubated in complete medium in a constant temperature incubator at 37 ℃ in an atmosphere of 5% CO_2 and saturated humidity. Normal human astrocytes (NHAs) were obtained from Lonza, and the medium was supplemented with rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine, and 5% FBS.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from cultured cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The PrimeScript™ RT reagent kit (Takara, Dalian, China) was used for the reverse transcriptase reaction. RT-PCR analyses were performed using SYBR Premix Ex Taq II (Takara) and a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). The mRNA expression level of CALD1 was normalized to GAPDH. The following primers were used for CALD1: forward primer: 5'-TGGAGGTGAATGCCCAGAAC-3', reverse primer: 5'-GAAGGCGTTTTTGGCGTCTTT-3'. Fold differences were calculated for each group using normalized cycle threshold (CT) values.

Western blotting analysis

The tissue samples were washed with cold phosphate-buffered saline (PBS) and homogenized with lysis buffer, and the supernatant was collected via centrifugation at 12,000 r/min for 10 min at 4 ℃. A bicinchoninic acid assay (BCA) kit (KeyGEN Biotech, Nanjing, China) was used for protein concentration measurement. The proteins were diluted by adding 10% sodium dodecyl sulfate (SDS) to the sample buffer and denatured by heating at 100 ℃ for 5 min. The proteins were separated and transferred to polyvinylidene difluoride membranes (PVDF membranes, Millipore, Burlington, MA, USA), blocked, and incubated with primary antibody overnight at 4 ℃. The antibodies used in the present study were rabbit anti-CALD1 (1:1,000) and anti-GAPDH.

Tissue samples

All specimens used in this study were from the neurosurgical

pathology of The Second Hospital of Shandong University. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The Second Hospital of Shandong University (KYLL2024456) and informed consent was obtained from all individual participants and/or their legal guardian(s). According to the World Health Organization (WHO) grading standard, pathological classification can be divided into WHO grade II, WHO grade III and WHO grade IV.

Immunohistochemical (IHC) staining

For the formalin-fixed paraffin-embedded (FFPE) brain tissue, deparaffinization, rehydration and antigen retrieval were performed. The slides were incubated with CALD1 antibody for 30 min in a humid chamber at 37 ℃. The slides were then incubated with goat anti-rabbit polyclonal antibody for 30 min in a humid chamber at 37 ℃. After complete rinsing in PBS, the slides were developed in 0.05% freshly prepared diaminobenzidine solution (DAB) for 10 min and then restained with hematoxylin. Finally, slides were dehydrated in rising concentrations of ethanol, air dried, and mounted.

Immunofluorescence

NHA/LN229 cell lines were mounted on slides for immunofluorescence analysis. NHA and LN229 cells were fixed, blocked and treated with CALD1 antibody (diluted 1:200) at 4 ℃ overnight. They were then treated with secondary antibody (Goat Anti-Rabbit IgG, IF488 Conjugate). DAPI is used to stain the nucleus. Phalloidin stains the cytoskeleton.

Transwell assay

To assess the migration capacity of cells, transwell experiments were performed. The transfected cells were placed in the upper chamber for 24 h. The cells migrated to the lower chamber were fixed and stained with methanol and crystal violet respectively. All data were presented as the mean ± standard deviation (SD) of three independent experiments.

EdU (5-ethynyl-2-deoxyuridine) experiment

Proliferation was assessed using the EdU incorporation assay (Yefluor 488 EdU Imaging Kits, YEASEN Biotech Co.) following the manufacturer's instruction. In simple terms, 24 hours after transfection, cells were cultured for 48 hours. Then they were fixed and stained. Fluorescence microscopy was used for observation. All data were presented as the means ± SD of three independent experiments.

Clone formation assay

There were 1,500 cells planted in six-well plates and cultured for 14 days. Then the cells were fixed and stained with methanol and crystal violet respectively. Take pictures with a professional camera. All data were presented as the means ± SD of three independent experiments.

Statistics analysis

The Wilcoxon test was used for comparisons between two groups, and the Kruskal-Wallis test was used for comparisons between more than two groups. All correlation analyses in this study, including CALD1 with TMB, MSI, stemness index, CNV, methylation, and immune cell infiltrating correlations, were performed using the Spearman correlation test. Patient survival analyses were performed using univariate Cox regression analysis. A P value less than 0.05 was considered significantly different. All analyses were performed using R software (version 4.1.2).

Results

Expression levels of CALD1 in pan-cancer

The expression levels of CALD1 in pan-cancer are shown in *Figure 1A,1B*. The results showed that CALD1 was differentially expressed in most tumors. We further mapped the expression of CALD1 in TCGA and GTEx databases (*Figure 1C,1D*). The TIMER database was used to validate the differential expression of CALD1 in pan-cancer. These results suggested CALD1 expression varies significantly among tumors and was highly expressed in most tumors which indicated CALD1 may be an oncogene.

Analysis of CNV of CALD1 expression

We investigated the correlation between the mRNA expression of CALD1 and its CNV (*Figure 1E-1G*). The results showed that the mRNA expression of CALD1 positively correlated with its CNV in low-

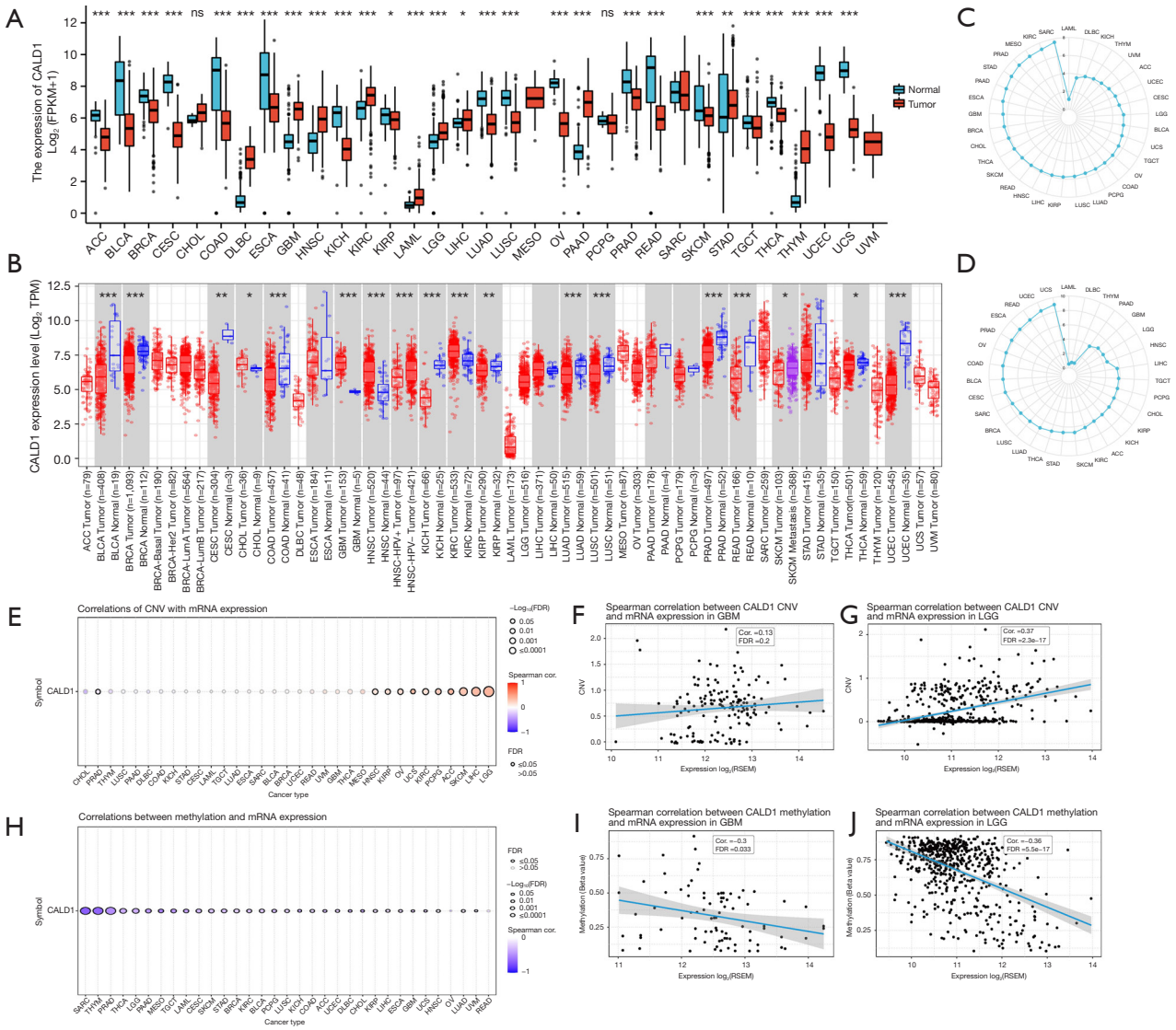

Figure 1 CALD1 expression in pan-cancer. (A) Differential expression of CALD1 in pan-cancer with data downloaded from UCSC Xena by R software. (B) Identification of differential expression of CALD1 in pan-cancer by TIMER. (C) The expression of CALD1 in TCGA database. (D) The expression of CALD1 in the GTEx database. (E) The correlation between the mRNA expression of CALD1 and its CNV in pan-cancer. The correlation between the mRNA expression of CALD1 and its CNV in GBM (F) and LGG (G). (H) The methylation levels of CALD1 in pan-cancer. The methylation levels of CALD1 in GBM (I) and LGG (J). ns, P≥0.05; *, P<0.05; **, P<0.01; ***, P<0.001. TPM, transcript per million; CNV, copy number variation; FDR, false discovery rate.

grade glioma (LGG), liver hepatocellular cancer (LIHC), skin cutaneous melanoma (SKCM), adrenocortical cancer (ACC), pheochromocytoma and paraganglioma (PCPG), kidney clear cell carcinoma (KIRC), uterine carcinosarcoma (UCS), ovarian cancer (OV), kidney papillary cell carcinoma (KIRP), and head and neck cancer (HNSC), and it negatively correlated in prostate cancer (PRAD). The results of the correlation study showed that the mRNA expression of CALD1 in LGG positively correlated with CNV (r=0.37, P<0.001), but its expression in GBM was not significantly correlated with CNV (r=0.13, P=0.20).

Analysis of CALD1 methylation

We also investigated the methylation levels of CALD1 in different tumors (*Figure 1H-1J*). The results showed a significant correlation between CALD1 methylation levels and mRNA expression in a variety of tumors, except OV and rectal cancer (READ). The correlation results showed that the mRNA expression of CALD1 significantly negatively correlated with methylation in LGG (r=–0.36, P<0.001) and GBM (r=–0.3, P=0.03).

Identification of the association between CALD1 and TMB and MSI

We observed that CALD1 significantly correlated with TMB in 14 tumors (Figure S1), with significant positive correlations in six tumors, such as glioma (GBM + LGG) (R=0.450, P<0.001), LGG (R=0.282, P<0.001), acute myeloid leukemia (LAML) (R=0.275, P=0.002), kidney cancer (R=0.134, P<0.001), thymoma (THYM) (R=0.477, P<0.001), and OV (R=0.123, P=0.03). It significantly negatively correlated with TMB in eight tumors, including lung adenocarcinoma (LUAD) (R=–0.107, P=0.02), stomach and esophageal carcinoma (STES) (R=–0.223, P<0.001), stomach cancer (STAD) (R=–0.260, P<0.001), PRAD (R=–0.2705, P<0.001), HNSC (R=–0.173, P<0.001), LIHC (R=–0.106, P=0.045), thyroid carcinoma (THCA) (R=–0.107, P=0.02), and uveal melanoma (UVM) (R=–0.237, P=0.04). CALD1 significantly correlated with MSI in twelve tumors, with significant positive correlations in three tumors, including mesothelioma (MESO) (R=0.252, P=0.02), testicular germ cell tumors (TGCT) (R=0.214, P=0.009), and ACC (R=0.243, P=0.03), and significant negative correlations in nine tumors. These results indicated that CALD1 significantly correlated with TMB and MSI in a variety of tumors and may be a marker for predicting the effect of immunotherapy. We also studied the relationship between the CALD1 and stemness index. The results suggested that CALD1 expression may have a significant correlation with tumor progression and recurrence.

Correlation analysis of CALD1 and tumor staging

We investigated the correlation between the CALD1 expression level and tumor stage (*Figure 2*). The results showed that CALD1 expression levels positively correlated with tumor stage in BLCA, esophageal carcinoma (ESCA), READ, STAD, and glioma. We observed that higher CALD1 expression levels were related to a higher WHO grade in glioma patients, and there were statistically significant differences between all grades. We used the data from TCGA to analyze the correlation between CALD1 and the clinical characteristics of glioma patients. The results demonstrated that CALD1 expression correlated significantly with patient age (≥60 years), male sex, isocitrate dehydrogenase (IDH) wild type, 1p/19q non-codeletion, and pathological type (*Figure 2*). The higher the malignancy of glioma, the higher the CALD1 expression, which indicated that CALD1 may be used as a molecular marker to predict the malignancy of glioma.

Correlation analysis of CALD1 and prognosis of pan-cancer

We performed univariate Cox regression analysis of patient OS, PFI, and DSS using tumor information from the TCGA database, and forest plots were drawn using R software (*Figure 3*). The results indicated that CALD1 expression in BLCA, LGG, KIRC, STAD, and MESO significantly correlated with patients' OS. CALD1 expression significantly correlated with patient PFI in LGG, BLCA, and PRAD. CALD1 expression significantly correlated with patient DSS in BLCA, LGG, and KIRC.

We further plotted K-M curves of CALD1 with OS, PFI, and DSS in GBM, LGG, and glioma patients and plotted ROC curves to verify their predictive ability. The results showed that CALD1 expression significantly correlated with the OS, PFI, and DSS of glioma patients, and the differences were statistically significant. The areas under the curves (AUCs) for OS-related ROC curves were 0.811, 0.830, and 0.848 for 1, 2, and 3 years, respectively, and for PFI-related ROC curves were 0.783, 0.763, and 0.748 for 1, 2, and 3 years, respectively. The results of GBM and LGG were shown in Figure S2.

Correlation and CALD1-related gene enrichment analysis

To further investigate the molecular pathways involved in CALD1 during glioma development and progression, we selected genes that positively correlated with CALD1 expression (log_2FC >1) for GSEA. A total of 68 related pathways were enriched. The first ten pathways are shown in *Figure 4A*. We then performed an in-depth analysis of the immune pathways in which these 68 genes may be involved

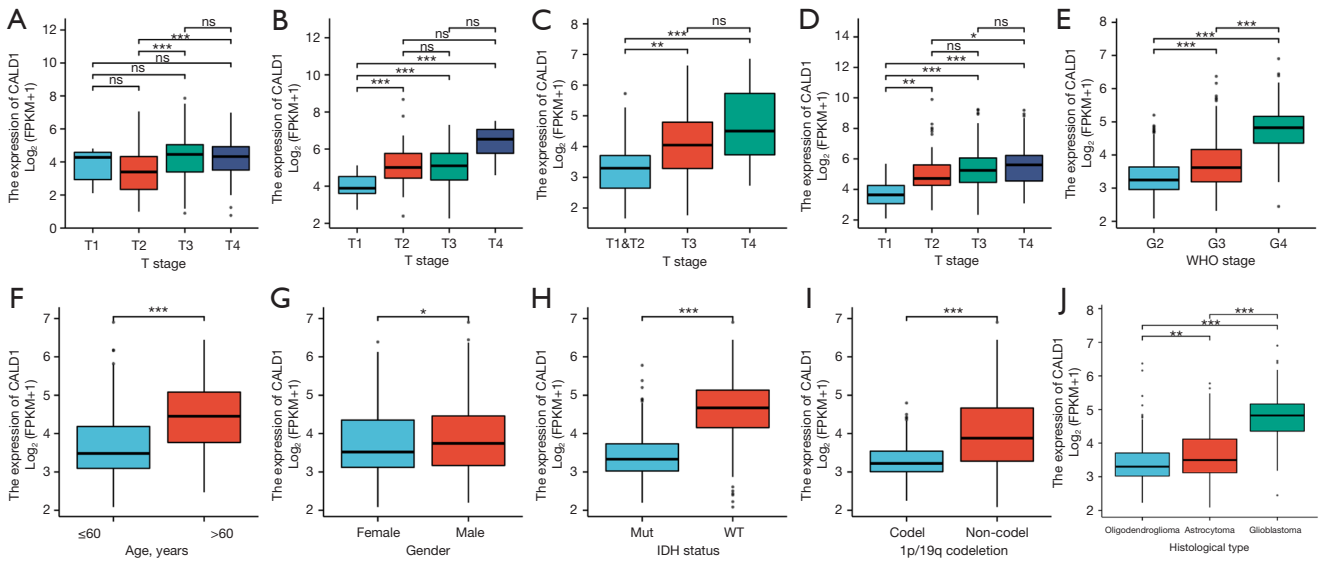

Figure 2 Correlation analysis of CALD1 and clinical factors. The expression levels of CALD1 positively correlated with tumor stage in BLCA (A), ESCA (B), READ (C), STAD (D), and glioma (E). The expression levels of CALD1 correlated significantly with patient old age (≥60 years) (F), male sex (G), IDH wild type (H), 1p/19q non-codeletion (I), and pathological type (J) in glioma. ns, P≥0.05; *, P<0.05; **, P<0.01; ***, P<0.001. FPKM, fragments per kilobase of transcript per million mapped reads; WHO, World Health Organization; IDH, isocitrate dehydrogenase.

(*Figure 4B*). The results showed that these genes clustered mainly in B cell receptor signaling pathway and regulation of B cell proliferation pathway. This suggests that CALD1 is extensively involved in glioma immune infiltration pathways, especially adaptive immune pathways.

Relationship between CALD1 expression and immune cell infiltration

The GSEA results suggested that CALD1 was involved in the development of glioma via immune pathways. Therefore, we analyzed the correlation between CALD1 expression and infiltrating immune cells using the "GSVA" R package. The results showed that all infiltrating immune cells in LGG, except dendritic cells (DCs), CD8 T cells, and mast cells, significantly correlated with CALD1. All cells in GBM, except cytotoxic cells, T cells, CD8 T cells, B cells, and NK CD56bright cells, correlated with CALD1 expression (*Figure 5*). This result indicated that CALD1 played an important role in immune escape function in the glioma microenvironment. We analyzed the correlation between CALD1 and immune cell infiltration and survival of glioma patients using TIMER. The results showed that M2 macrophage infiltration was associated with poor prognosis in GBM and LGG (*Figure 5*). To further investigate the correlation of CALD1 expression with individual immune infiltrating cells, we also analyzed the correlation of CALD1 with marker genes of immune cells. We further investigated the correlation of CALD1 with immune checkpoint genes. The results showed that CALD1 expression in LGG positively correlated with all immune checkpoint genes, and the differences were significant. However, CALD1 only positively correlated with the expression of TIGIT, PDCD1LG2, and CD274 in GBM. In our study of the relationship between CALD1 and TME, we analyzed glioma scRNA-seq data obtained from the Gene Expression Omnibus (GEO) database. The results showed that CALD1 was highly expressed in glioma cells but not in immune cells (*Figure 5G-5I*). Analysis of the Tumor Immunotherapy Gene Expression Resource (TIGER) database of GBM patients treated with anti-programmed cell death-1 (PD-1) immunotherapy revealed that CALD1 hindered the effectiveness of treatment and resulted in poor patient survival (*Figure 5J*). We investigated the effect of CALD1 expression on the microenvironment of glioma by calculating the Immunocore, StromalScore, and ESTIMATEscore of tumors using the R software "ESTIMATE" package (Figure S3). The results showed

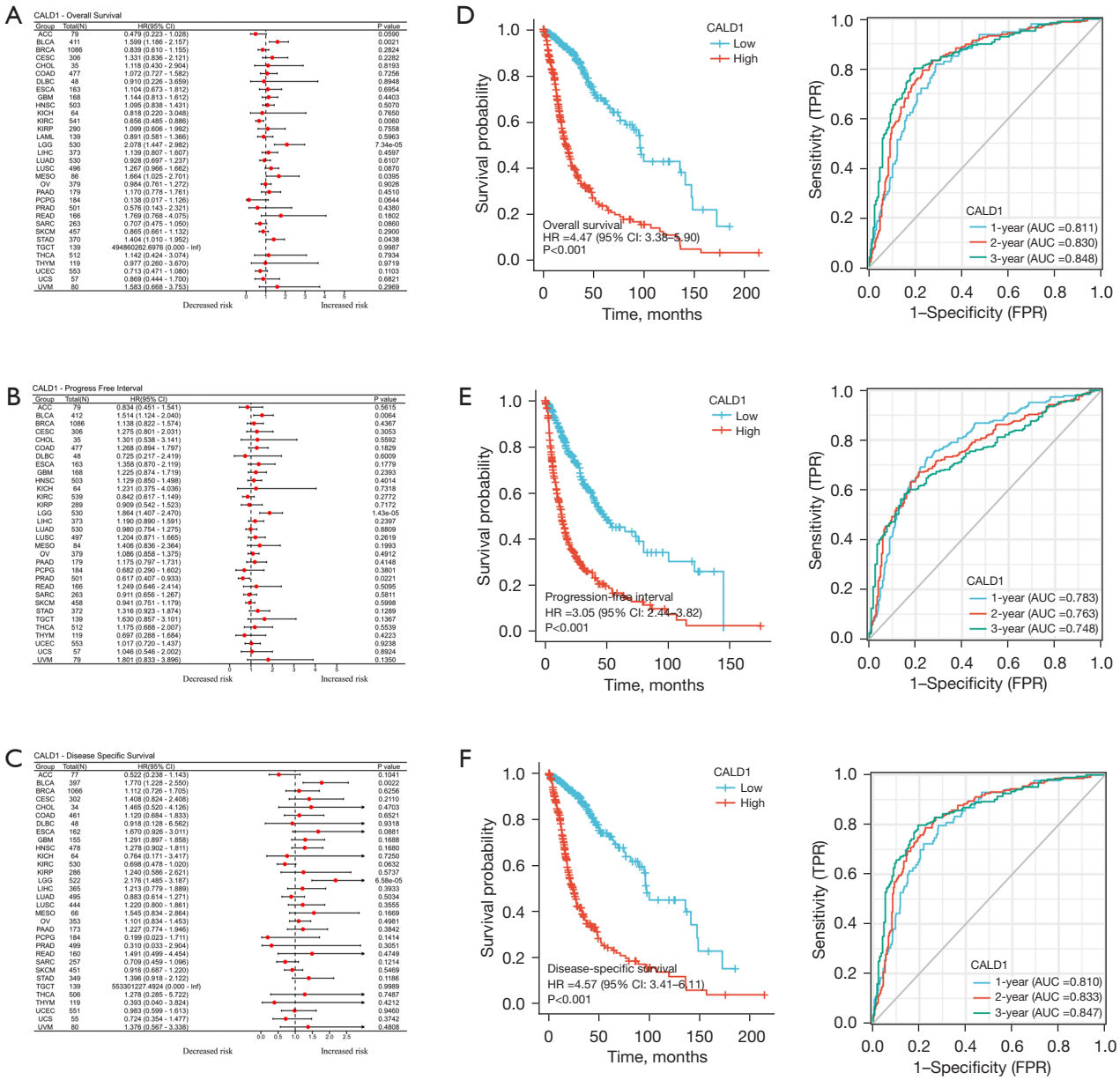

Figure 3 Prognostic value of CALD1 in glioma. The relationship between the expression level of CALD1 and OS (A), PFI (B), and DSS (C) in pan-cancer. The Kaplan-Meier survival curve and ROC curve of OS (D), PFI (E), and DSS (F) in glioma. CI, confidence interval; OS, overall survival; PFI, progression-free interval; DSS, disease-specific survival; TPR, true positive rate; FPR, false positive rate; HR, hazard ratio; ROC, receiver operating characteristic; AUC, area under the ROC curve.

that CALD1 positively correlated with Immunocore, StromalScore, and ESTIMATEscore in LGG and glioma patients. However, CALD1 expression only positively correlated with StromalScore but not with Immunocore or ESTIMATEscore in GBM.

Identification of CALD1 expression in GBM cell lines and pathological specimens

We used RT-PCR and Western blotting to verify the differential expression of CALD1 in different cell lines of GBM and NHA. RT-PCR showed that CALD1 was highly

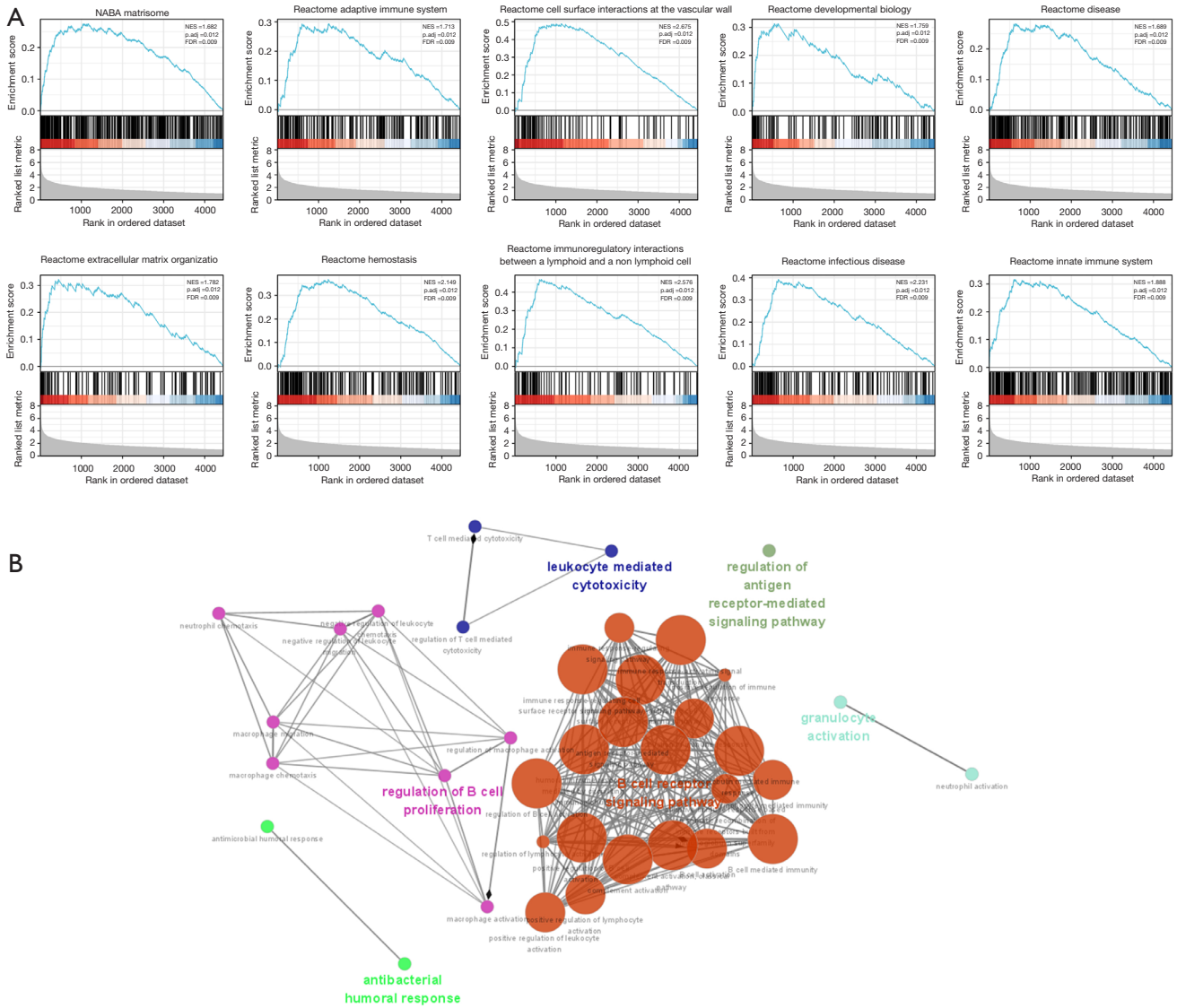

Figure 4 Correlation and CALD1-related gene enrichment analysis. (A) The first ten pathways of CALD1-related gene enrichment. (B) The immune-related pathways involved CALD1-related genes according to the pathway analysis tool, ClueGO. They clustered mainly in B cell receptor signaling pathway and regulation of B cell proliferation pathway. NES, normalized enrichment score; FDR, false discovery rate.

expressed in the LN229 cell line. Western blot results showed that CALD1 was highly expressed in U118MG, A172, U251MG, LN229, GBM#BG7 and GBM#BG5 cells. Previous studies have found that CALD1 expression may be associated with tumor metastasis (8,23,24). Our immunofluorescence results showed that CALD1 was mainly expressed in the cytoskeleton in NHA as well as in LN229 cell line, which was co-localized with F-actin. CALD1 expression was more pronounced at the cell synapse, again verifying that its expression may affect tumor cell motility. We further performed IHC studies using pathological specimens obtained from the clinic and showed a positive correlation between CALD1 expression and patient grade in glioma (*Figure 6*).

Knockdown of CALD1 inhibits glioma cells proliferation

In order to verify whether knockout of CALD1 can affect cell proliferation, we conducted EdU proliferation experiment and Colony formation assay (*Figure 7*). The

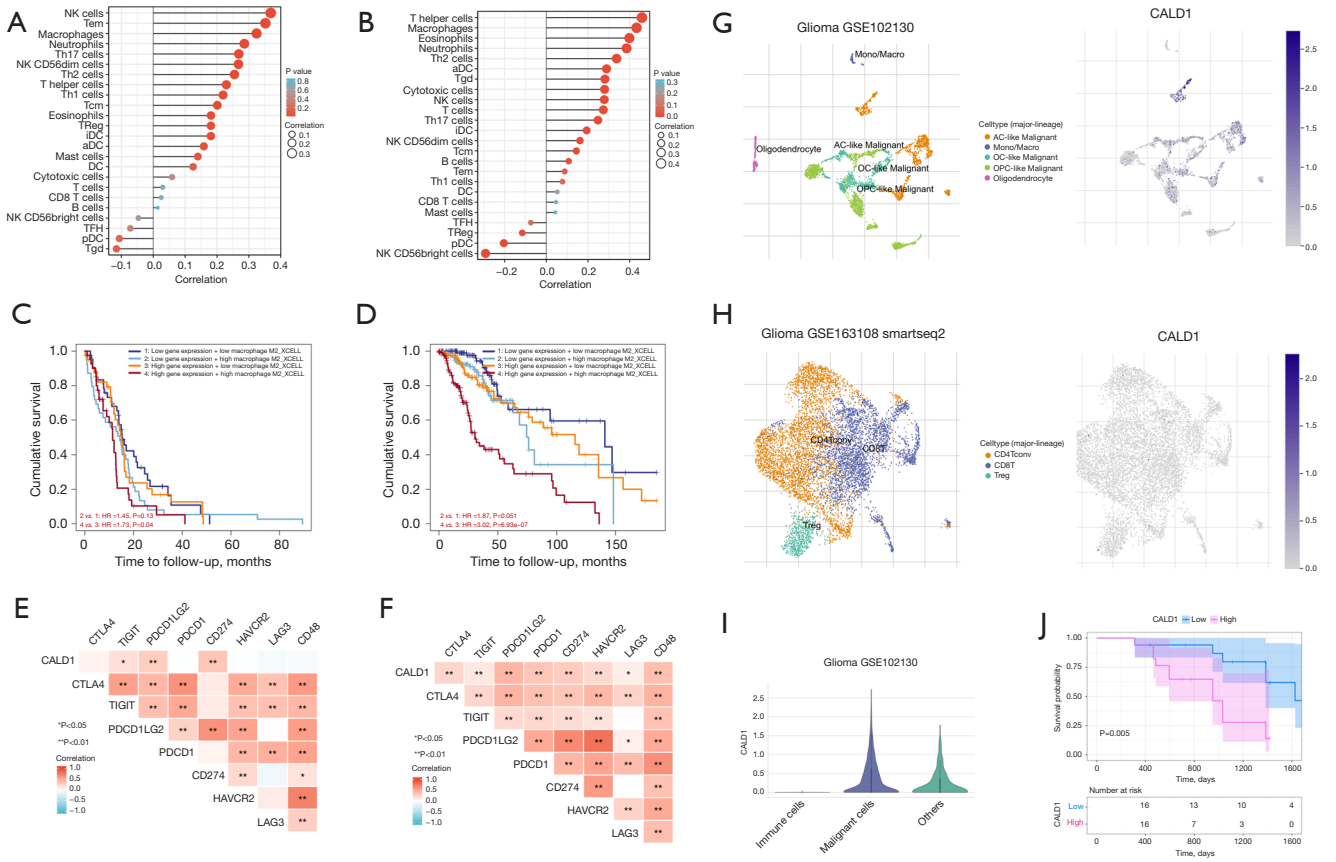

Figure 5 Correlation between CALD1 and infiltrating immune cells. The correlation between CALD1 expression and infiltrating immune cells in GBM (A) and LGG (B). The correlation between CALD1 and immune cell infiltration and patient survival in GBM (C) and LGG (D) using TIMER. The association between CALD1 expression and immune checkpoint genes in GBM (E) and LGG (F). UMAP demonstrates the major immune cell populations in the TME of glioma (GSE102130) (G) and (GSE163108) (H). (I) UMAP demonstrates the distribution of CALD1 expression in various cells. (J) Effect of CALD1 on the survival of GBM patients undergoing anti-PD-1 immunotherapy. NK, natural killer; HR, hazard ratio; PD-1, programmed cell death-1.

results of EdU experiment showed that the number of cells in the proliferative stage (green fluorescence) was significantly reduced in the CALD1 knockout group compared with the control group, and the difference was statistically significant. Similarly, there were significantly fewer colonies in the experimental group than in the control group. All these results suggest that CALD1 plays an important role in the proliferation of GBM cell lines.

Knockdown of CALD1 inhibits glioma cells migration

In order to further study the effect of CALD1 knockout on the migration ability of glioma, we performed Transwell experiment. We found that after siRNA knockdown, the percentage of cells migrated through the transwell plate decreased significantly. This result indicate the importance role of CALD1 in migration of glioma.

Discussion

Glioma is a rare and aggressive malignancy with a poor prognosis (25-27). With the development of bioinformatics, we aim to find new therapeutic targets for glioma treatment. Previous studies found that CALD1 positively correlated with the malignancy of several tumors and correlated with patient prognosis. Chang *et al.* showed that oral cancer patients with lymph node metastases had increased CALD1 expression compared to patients without metastases (8).

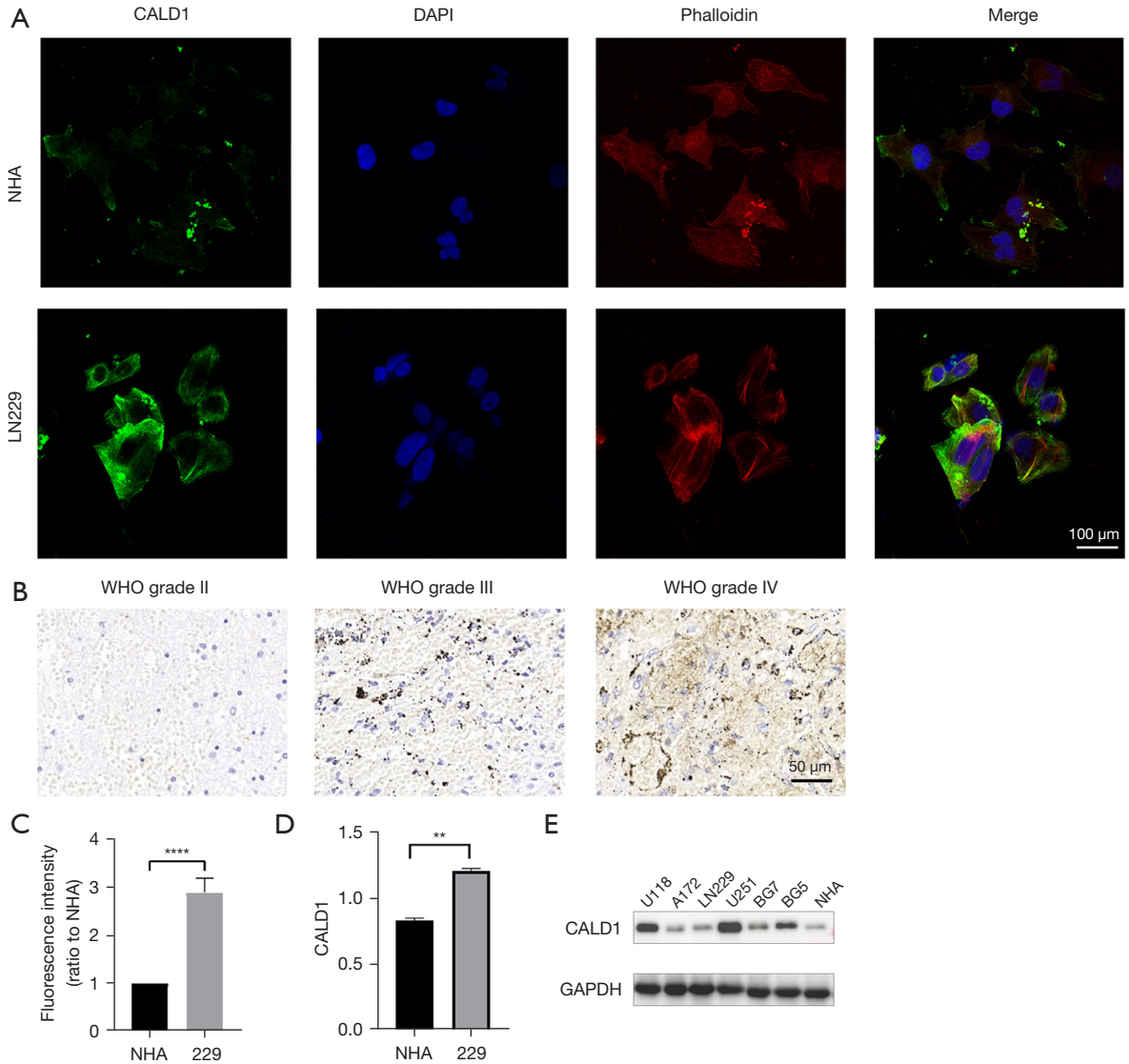

Figure 6 The expression of CALD1 in glioma. (A) Immunofluorescent staining of CALD1 in NHA and LN229 cell lines. (B) Immunohistochemistry of CALD1 in pathological specimens of WHO grade II, grade III and grade IV. (C) Comparing CALD1 expression difference in NHA and LN229 cell lines. (D) The mRNA expression levels of CALD1 in the human astrocyte cell line NHA and the glioma cell line LN229 using RT-PCR. (E) The transcription and protein expression of CALD1 in the human astrocyte cell line NHA and the glioma cell lines, U118MG, A172, LN229, U251MG, GBM#BG7, and GBM#BG5 by western blotting (The grouping of gels cropped from different parts of the same gel, Figure S4). **, P<0.01; ****, P<0.0001. NHA, Normal human astrocytes; WHO, World Health Organization; DAPI, 4',6-diamidino-2-phenylindole.

Du *et al.* found that CALD1 promoted prostate cancer progression by remodeling the TME (13). Zheng *et al.* demonstrated a positive correlation between CALD1 and the degree of M2 macrophage infiltration in patients with grade III/IV colorectal cancer. CALD1 promoted the proliferation, invasion, and metastasis of colorectal cancer cells (28). Cheng *et al.* showed that stromal cell aggregation in the glioma microenvironment was significantly associated with CALD1 overexpression (14). However, to the best of our knowledge, there is a lack of studies correlating CALD1 with the immune microenvironment of glioma. Therefore, the present study determined the correlation between

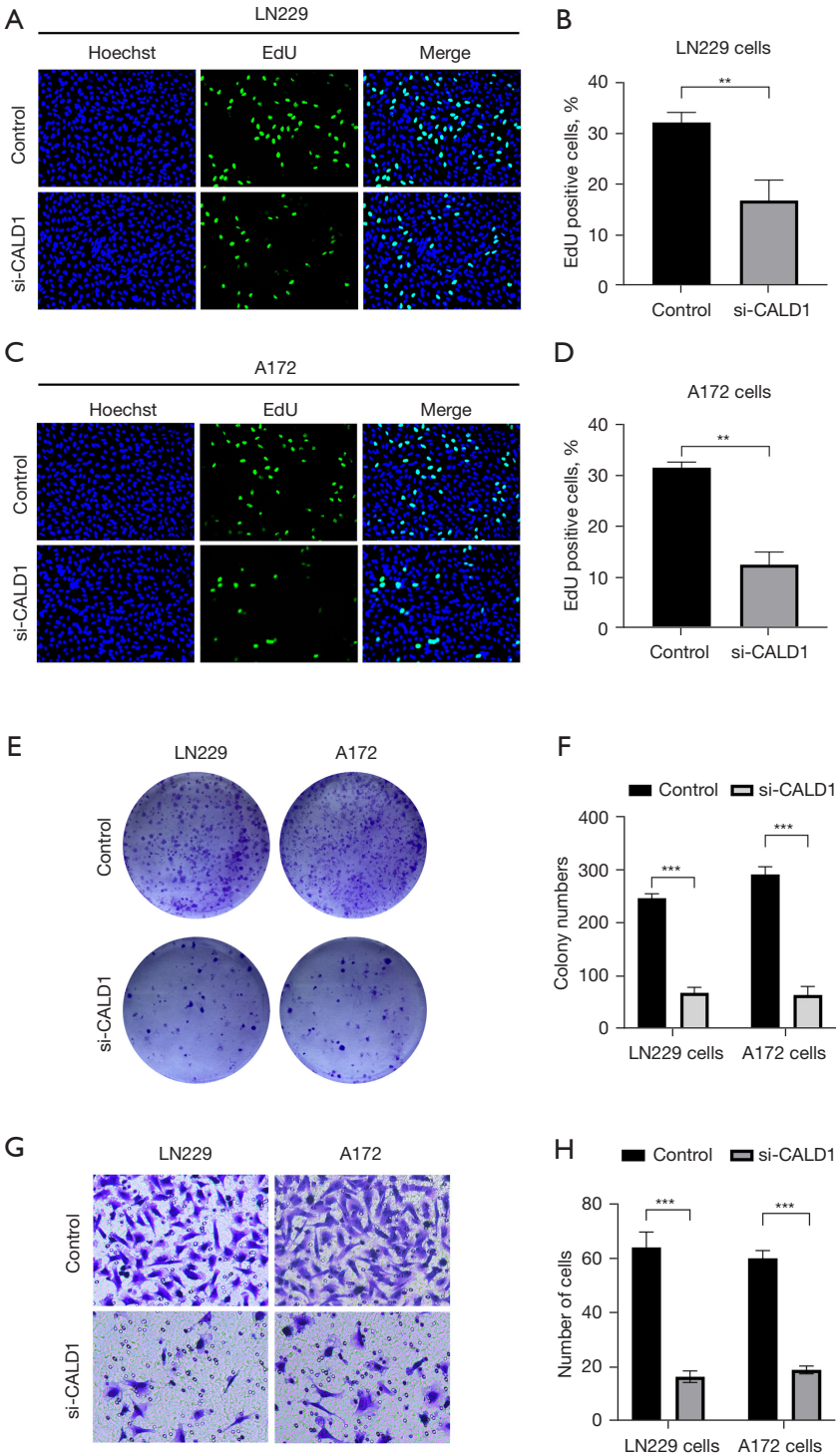

Figure 7 Knockdown of CALD1 Inhibits glioma cell proliferation. Fluorescence images of EdU assays performed on LN229 cells (A) and A172 cells (C) transfected with si-CALD1. Nuclei were stained with DAPI (blue). Graphic representation of the ratios of EdU positive cells in LN229 cells (B) and A172 cells (D) transfected with si-CALD1. (E,F) Colony formation assay. Cells with CALD1 knockdown exhibited a significant decrease in the numbers of colonies, compared with the control group. Cells were fixed and stained with crystal violet, and colonies were counted. (G,H) Transwell assay. The migration ability of glioma cells decreased after knockdown of CALD1. Cells were fixed and stained with crystal violet, and cells were counted (100×) (**, P<0.01; ***, P<0.001).

CALD1 and the glioma immune microenvironment and further investigated whether it may be a target for predicting glioma prognosis.

We first investigated the differential expression of CALD1 in 33 cancers and demonstrated that CALD1 was differentially expressed in 28 cancers but not cholangiocarcinoma (CHOL), MESO, PCPG, sarcoma (SARC), and UVM. This result indicates that CALD1 is closely related to the occurrence and progression of tumors. We further verified its significantly high expression in GBM using RT-PCR and Western blotting. We investigated the correlation of CNV and methylation with CALD1 expression in pan-cancer. We also investigated the correlation between CALD1 expression and TMB, MSI, and the stemness index, and the results showed that CALD1 correlated significantly with TMB, MSI, and the stemness index in various tumors. These results suggest that CALD1 is a potential molecular marker to predict the efficacy and prognosis of immunotherapy in tumor patients. To investigate whether CALD1 effectively predicts patient prognosis, we further investigated the correlation between CALD1 and OS, PFI and DSS of pan-cancer. CALD1 positively correlated with clinical stage in BLCA, ESCA, READ, STAD, and glioma. These results suggest that CALD1 effectively predicts the prognosis of various tumors and is involved in the process of tumor progression. The results of clinical studies showed that CALD1 expression significantly correlated with old age, male sex, IDH wild-type, 1p/19q non-codeletion, and pathological type of glioma patients. The results of single-cell studies suggest that CALD1 is involved in the metastatic and apoptotic processes of GBM. To investigate the molecular mechanisms of CALD1 in glioma development and progression, we performed GSEA of genes co-expressed with CALD1 in gliomas, and the results showed that the chosen related genes were enriched in multiple immune-related pathways.

Because of the blood-brain barrier, the brain is considered an immune-privileged organ (29,30). However, there is existing evidence showing that patients with glioma also benefit from immunotherapy (31,32). The lack of adequate understanding of the immune microenvironment of glioma has led to unsatisfactory treatment outcomes (33-36). The most important finding of this study is that we confirmed that CALD1 expression positively correlated with the degree of infiltration of multiple immune cells in GBM and LGG, and CALD1 significantly correlated with multiple molecular markers of infiltrating immune cells. Some studies demonstrated that tumor-associated macrophages (TAMs) in the TME promoted tumor proliferation and metastasis (11,37). The TAM molecular marker CCL2 plays an important role in immunosuppression by activating and recruiting TAM cells (38,39). Our study found that CALD1 significantly positively correlated with CCL2 in GBM ($r=0.318$, $P<0.001$) and LGG ($r=0.302$, $P<0.001$). We also found that CALD1 expression positively correlated with M2 macrophage aggregation. High CALD1 expression and M2 macrophage aggregation correlated with the poor prognosis of patients. Therefore, we hypothesized that CALD1 played an immunosuppressive role by promoting CCL2 expression and TAM polarization. Of course, this hypothesis must be further confirmed in future experiments.

Pembrolizumab and nivolumab achieved promising therapeutic results in melanoma (40) and non-small cell lung cancer (41). These drugs have been approved by the United States Food and Drug Administration (USFDA) as PD-1 antibodies. PD-1/programmed cell death-ligand 1 (PD-L1)-targeting therapy is approved for many other cancers, including BLCA (42), colorectal cancer (43), clear cell renal cell carcinoma (44) and head and neck carcinoma (45). However, the results of a phase III clinical trial for recurrent GBM showed that nivolumab was not effective in extending patient survival compared with the bevacizumab-treated control set (46). The prognostic relevance of PD-1/PD-L1 in glioma remains controversial. Therefore, it is necessary for us to find new biomarkers. Our study found that the expression of CALD1 positively correlated with TIGIT, PDCD1LG2, and CD274 in GBM. However, CALD1 positively correlated with all immune checkpoint genes in LGG patients, and the difference was significant. Therefore, CALD1 may be a predictive target for the efficacy of immune blockade therapy in glioma patients, especially in LGG patients.

Conclusions

In conclusion, our results demonstrate that CALD1 is significantly highly expressed in GBM and LGG and correlates with patient prognosis. The expression of CALD1 positively correlated with glioma malignancy and may play an important role in glioma progression via immune cell infiltration. Therefore, CALD1 may be a molecular target for glioma treatment and prognosis.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-216/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). The study was approved by the Ethics Committee of The Second Hospital of Shandong University (KYLL2024456) and informed consent was obtained from all individual participants and/or their legal guardian(s).

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