ITGA3 Is a Reliable Biomarker and Putative Therapeutic Target for Glioma

Qing Zhang
Department of Neurosurgery, The First Hospital of China Medical University, Shenyang, Liaoning, China
Chen Zhu
Department of Neurosurgery, The First Hospital of China Medical University, Shenyang, Liaoning, China
Gefei Guan
Department of Neurosurgery, The First Hospital of China Medical University, Shenyang, Liaoning, China
Shuai Shen
Department of Neurosurgery, The First Hospital of China Medical University, Shenyang, Liaoning, China
Yunhe Han
Department of Neurosurgery, The First Hospital of China Medical University, Shenyang, Liaoning, China
Lianhe Yang
Department of Pathology, the College of Basic Medical Sciences and First Affiliated Hospital of China Medical University, Shenyang, Liaoning, China
Wen Cheng
Department of Neurosurgery, The First Hospital of China Medical University, Shenyang, Liaoning, China
Anhua Wu (ahwu@cmu.edu.cn)
China Medical University https://orcid.org/0000-0002-7218-8765

Research

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Abstract

Background: Glioma is the most prevalent and malignant primary central nervous system tumor in adults. As a member of the integrin alpha chain family of proteins, integrin subunit alpha 3 (ITGA3) has been found to play a critical role in the occurrence and progression of several cancers, including lung, ovarian, and pancreatic cancers. However, the role of ITGA3 in glioma remains unclear.

Methods: The Cancer Genome Atlas (TCGA), Chinese Glioma Genome Atlas (CGGA), REMBRANDT, GSE16011, GSE59612, and GSE4290 datasets were used to analyze relevant characteristics of ITGA3 in glioma. R language and GraphPad Prism 7.00 were employed as major tools for statistical analysis and graph manipulation.

Results: We identified that ITGA3 expression was upregulated in glioma and related to unfavorable outcomes of glioma patients. Expression of ITGA3 also tended to be enriched in aggressive subtypes of glioma. We demonstrated that expression of ITGA3 was negatively correlated with glioma purity. In gliomas with high ITGA3 expression, the anti-glioma immune response was inhibited. ITGA3 also regulated angiogenesis within the glioma microenvironment and promoted the epithelial–mesenchymal transition (EMT) and autophagy of glioma cells. GSEA analysis revealed that ITGA3 could activate ERK1/2 and PI3K/AKT/mTOR pathways.

Conclusion: Our data suggested that ITGA3 promoted the malignant progression of glioma by regulating the immunity of glioma as well as the EMT and autophagy of glioma cells, which could act as a therapeutic target for glioma.

Background

Gliomas, including low grade glioma (LGG) and glioblastoma (GBM), are the most frequent, invasive, and malignant primary tumors of the brain, with a grave prognosis and limited treatment methods available(1, 2). Although great efforts have been made in surgery, chemotherapy, radiotherapy, and combination therapy to improve therapeutic efficacy, the outcome of glioma remains dismal; the median survival time for GBM is less than 15 months(3). Therefore, to improve treatment effects, it is imperative to discover novel therapeutic targets and develop more effective strategies for glioma treatment.

Integrins are multifunctional heterodimeric transmembrane receptor molecules that generally consist of non-covalently combined α and β subunits. These molecules play a vital role in signal transduction and homeostasis by mediating the interaction between cells and of these cells with the extracellular matrix(4). Growing evidence indicates that integrins and integrin-related biological process play critical roles in regulating tumor stem-like properties, cancer invasion, and treatment resistance(5).

As a member of the family of integrin subunits, ITGA3 has been correlated with unfavorable prognoses in cancers such as non-small cell lung cancer(6), ovarian cancer(7), and pancreatic adenocarcinoma(8). In addition, ITGA3 has been found to regulate cancer cell migration and invasion in bladder cancer(9),

colorectal cancer(10) and head and neck cancer(11). In nasopharyngeal carcinoma, ITGA3 was shown to simultaneously regulate the migration of tumor cells and angiogenesis within the tumor microenvironment(12). In ovarian cancer, the monoclonal antibody OV-Ab 30 − 7, which specifically neutralizes ITGA3, restrained tumor progression and lengthened the survival of tumor-bearing mice, indicating that ITGA3 might be a potential therapeutic target for ovarian cancer(13). Although accumulating studies have begun to focus on the role of ITGA3 in tumors, its specific role and underlying mechanisms are still unclear. In particular, very little is known about the role of ITGA3 in gliomas.

In this study, we identified ITGA3 as a prognostic factor in glioma and revealed that its expression is enriched in aggressive subtypes of glioma. We also found that ITGA3 was closely related to the immune microenvironment of glioma and could regulate EMT and autophagy of glioma cells.

Methods

Data source

The Cancer Genome Atlas (TCGA) glioma RNA sequencing data, as well as clinical and pathological information were obtained from TCGA database (https://portal.gdc.cancer.gov/). The Chinese Glioma Genome Atlas (CGGA), including CGGA (mRNAseq_325) (when not specified, CGGA refers to CGGA mRNAseq_325 data) and CGGA (mRNA-array), and REMBRANDT gene expression data and corresponding clinicopathological information were acquired from CGGA (http://www.cgga.org.cn). GSE16011, GSE59612, and GSE4290 datasets were acquired from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Background correction and normalization of the raw GEO data was conducted using the robust multi-array analysis (RMA) method via the *affy* package in R. The Kamoun database and TCGA (Aglient-4502A) GBM data were obtained from GlioVis(14).

Screening integrins related to the prognosis of glioma

Univariate Cox regression analysis was used to select integrins correlated with the overall survival (OS) of glioma or GBM patients and was implemented using the *survival* package in R. Kaplan-Meyer survival analysis was employed to analyze the OS rate between different groups of glioma patients. Survival curves were plotted using the *survival* package.

Determination of tumor purity and immune cells enrichment level of glioma

The *ESTIMATE* R package was employed to calculate the stromal and immune scores of glioma samples based on the gene expression data(15). The immune cell enrichment scores were calculated via *GSVA* package in R(16). All heat maps were plotted using the *pheatmap* package in R. Genes related to the immune response were acquired from AmiGO 2 (http://amigo.geneontology.org/amigo).

Functional enrichment analysis

Gene set enrichment analysis (GSEA) was implemented using GSEA v4.1.0 to explore the hallmark gene sets, and Gene Ontology (GO) biological processes enriched in ITGA3 high- and low-expression gliomas. The differentially expressed genes in ITGA3 high- and low-expression gliomas were determined using the *limma* R package. GO analysis was implemented using the *clusterProfiler* package.

Patients and samples

Forty-five primary glioma samples were gathered from the First Hospital of China Medical University (CMU) from September, 2016 to September, 2018 (15 cases including grades II, III, and IV). Six non-tumor brain tissue specimens collected from partial lobectomy in epilepsy patients were used as a negative control. The qPCR and western blotting test were performed for all specimens. Immunohistochemistry staining was conducted for randomly selected samples (five cases of grades II, III, and IV, and two cases of non-tumor brain tissue). The Medical Ethics Committee of the First Hospital of CMU authorized the study, and each patient provided a written informed consent. Detailed information of the 45 glioma patients is shown in Supplementary Table 1.

Immunohistochemistry

Following fixation (10% neutral formalin) and embedding in paraffin, all samples were sliced as 4-μm-thick sections. Following deparaffinization in xylene, the sections were rehydrated in gradient ethanol. Citrate buffer (pH 6.0) was used for antigen retrieval. Hydrogen peroxide (3%) was employed to eliminate endogenous peroxidase activity (10 min) and the normal goat serum was employed to block nonspecific antigen (15 min). Anti-ITGA3 rabbit polyclonal antibody (1:100, no. ab131055, Abcam) was added at 4°C overnight. After washing with phosphate-buffered saline (PBS), sections were incubated with secondary antibody (SP-9001, ZSGB-BIO) for 15 min at 27°C. Then, 3,3'-diaminobenzidine (DAB; MaiXin, Fuzhou, China) was applied for developing. Expression of ITGA3 was assessed by three investigators using the German Immunohistochemical Score(17).

Cell cultures and reagents

U87, U251, LN229, T98, U373 and normal human astrocytes cell line (NHA) were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human brain microvascular endothelial cells (HBMECs) were purchased from FENGHUISHENGWU (Changsha, Hu Nan, China). Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin was used to culture the cells. Knockdown small-interfering RNA (siRNA) targeting ITGA3 and the negative control RNA (si-NC) were purchased from RIBOBIO (Guangzhou, China). The siRNA transfection was implemented using Lipofectamine 3000 (Life Technologies, USA) according to the manufacturer's

instructions. The RFP-GFP-LC3 plasmid was used to monitor the autophagic flux, which was indicated by the formation of RFP-GFP-positive puncta. G418 (Gentihold, Beijing, China) was used to screen cells stably transfected with RFP-GFP-LC3.

Migration and invasion assays

The wound healing assay was used to evaluate the migration ability of glioma cells. A 200-μl pipette tip was used to inflict a wound when seeded cells in the 6-well plates reached a density of approximately 90% confluence. After washing with PBS to remove debris, the cells were cultivated in serum-free DMEM. The wounds were recorded and photographed at 0 h and 24 h. ImageJ software (Bethesda, MD, USA) was used to measure the wound area.

The Transwell invasion assay was adopted to assess the invasion potential of glioma cells. Transwell chambers (Costar; Corning Inc., Corning, NY, USA) plated with Matrigel (BD Biosciences, San Jose, CA, USA) were applied to perform the cell invasion assays. Following placement on 24-well plates, 100 μL of serum-free DMEM suspended with 10^5 cells was added to the upper chamber; the lower chamber was injected with 800 μL of DMEM containing 20% fetal bovine serum. After culturing at 37 °C in 5% CO_2 for 20 h, the cells on the lower wall of the chamber were fixed and stained. In each chamber, the cells were counted in randomly selected five high power fields. Each experimental group was performed in triplicate.

Tube formation assay

In vitro angiogenesis evaluation was performed by Matrigel assay(18). Briefly, after coating with 100 μL of Matrigel, 100 μL cell suspension containing 10^5 HBMECs was added to each well of a 96-well culture plate and the cells were cultured for 16 h. Branched tubules were imaged and counted using a fluorescence microscope (DP71; Olympus, Tokyo, Japan).

Real-time quantitative PCR (qPCR)

TRIzol reagent (Invitrogen) was applied to isolate total RNA from cells or tissues. RNA concentration was detected using a NanoDrop 2000. Then, total RNA was reverse transcribed to cDNA. Real-time qPCR was performed according to the instructions of the SYBR Green PCR master mix kit (Takara, Japan). Each sample was repeated three times. Primer sequences for GAPDH, and ITGA3 were as follows:

GAPDH forward: 5'-GGAGCGAGATCCCTCCAAAAT-3';

GAPDH reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3';

ITGA3 forward: 5'-TGTGGCTTGGAGTGACTGTG-3';

ITGA3 reverse: 5'-TCATTGCCTCGCACGTAGC-3'.

Western blotting

Western blotting was conducted as described previously(19). We extracted total protein from cells or tumor tissues using RIPA buffer and PMSF (Beyotime, Beijing, China) in a ratio of 10:1. The concentration of total protein was determined using the bicinchoninic acid (BCA) (Beyotime) method. Equal amounts (30 μg) of protein were separated by 10% gel electrophoresis and transferred onto polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). After blocking with 5% skim milk, the membranes were incubated with primary antibodies against ITGA3(1:1000, no. ab131055, Abcam), E-Cadherin, N-Cadherin, β-Catenin, Vimentin, Snail, Claudin-1 (1:1000; no. 9782T, Cell Signaling Technology), VEGFA (1:1000, no.ab1316, Abcam), TGFB1 (1:1000, no.3709S, Cell Signaling Technology), LC3 (1:1000, no.14600-1-AP, Proteintech), ERK1/2 (1:1000, no.4695S, Cell Signaling Technology), and p-ERK1/2 (1:1000, no.9101S, Cell Signaling Technology) at 4°C for 15 h. The membranes were washed with Tris-buffered saline with Tween-20 (TBST) and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Proteintech; 1:5000) at 25 °C for 2 h. Immunoreactive proteins were photographed by Tanon 5200. GAPDH (1:1000, no.10494-1-AP, Proteintech) was used as a loading control.

Data analysis

All statistical analyses were conducted using R (version 4.0.2) or GraphPad Prism version 7.00 (GraphPad Software Inc., USA). Quantitative data are displayed as the mean ± standard deviation (SD). The Wilcoxon test was used to compare the statistical differences between two groups, while the differences between multiple groups were compared by Kruskal–Wallis H test. P-values < 0.05 were considered statistically significant. The Venn diagram was drawn using jvenn(20). *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001, ns: P ≥ 0.05.

Results

Expression characteristics and prognostic significance of ITGA3 in glioma are more prominent than other integrins

Integrins produce an important effect in cancer biology, and some subunits are considered potential targets for tumor therapy(21).To screen for integrin subunits that impact the occurrence and progression of glioma, we first used public databases to compare the expression of all integrins in normal brain tissues and GBM samples. the expression levels of most integrins in GBM were significantly higher than that in normal brain tissue in the GSE4290, REMBRANDT, and GSE59612 datasets (Figure 1A–C). In these three datasets, the number of non-tumor brain tissues was greater than 15, which strongly illustrated the expression characteristics of integrins in GBM and normal brain tissues. Eleven significantly differently

expressed overlapped integrin subunits in the three datasets were selected for further analysis (Figure 1D). In the TCGA cohort, we conducted univariate Cox regression analysis using the 11 integrin subunits and found that the expression level of ITGA3 was most significantly correlated with the OS of all glioma patients as well as GBM patients (Figure 1E–F). Therefore, we further investigated the role of ITGA3 in glioma.

ITGA3 predicts poor prognosis in glioma

Based on the results of the above univariate Cox regression analysis, we believed that the expression level of ITGA3 was correlated with the prognosis of glioma patients. To confirm this, we used the Kaplan–Meier method to perform survival analysis based on ITGA3 expression in the TCGA, CGGA, and GSE16011 databases. The results of the survival analysis were consistent with those of the univariate Cox analysis. In all glioma patients, the higher the expression level of ITGA3, the worse the prognosis of the patient (Figure 2A–C). In GBM and LGG, ITGA3 had the same prognostic significance (Figure 2D–I), indicating that ITGA3 could act as a prognostic indicator for glioma patients. In addition, glioma patients receiving radiotherapy or chemotherapy in the high ITGA3 expression group had a worse outcome than patients in the low expression group in the TCGA and CGGA datasets (Supplementary Figure 1A–D). These results further conformed that ITGA3 could act as a biomarker in predicting the sensitivity of glioma patients to radiotherapy and chemotherapy.

ITGA3 expression is enriched in aggressive subtypes of glioma

We have found that the expression level of ITGA3 in GBM was higher compared with non-tumor brain tissue. We further evaluated the expression of ITGA3 in different grades of gliomas in TCGA, CGGA, CGGA (mRNA-array), and GSE16011 databases. We found that the higher the grade of glioma, the higher the expression level of ITGA3 (Figure 3A–D). Integrated genomic analysis grouped GBM into proneural (PN), neural (NE), classical (CL), and mesenchymal (ME) transcriptomic subtypes(22, 23). Different transcriptomic subtypes have different prognoses for glioma, among which the NE subtype has a relatively favorable prognosis and the ME subtype has a relatively poor outcome. Because the expression level of ITGA3 was significantly related to the prognosis of glioma, we further detected the expression characteristics of ITGA3 in different transcriptomic subtypes. As shown in Figure 3E–H, the expression level of ITGA3 in the ME subtype was significantly higher than that in the other three subtypes in TCGA, CGGA, GSE16011, and REMBRANDT datasets. In addition to transcriptomic subtypes, IDH mutation and chromosome 1p19q codeletion status also played a critical role in the occurrence and progression of glioma(24, 25). In TCGA, CGGA, GSE16011, and Kamoun datasets, we discovered that the expression level of ITGA3 was not only higher in IDH wild-type gliomas compared with IDH mutant gliomas (Figure 3I–L), but also higher in 1p19q non-codeletion gliomas than that in codeleted gliomas (Figure 3M–Q). These results suggested that the expression level of ITGA3 was correlated with the malignant subtype of

glioma. These results also explained the correlation between the expression level of ITGA3 and the prognosis of glioma. However, in the CGGA cohort, there was no significant difference in the expression of ITGA3 and MGMT promoter methylation status (data not shown).

To explore whether ITGA3 could be a diagnostic marker for glioma, we used the GETxPortal (https://www.gtexportal.org/home/documentationPage) to compare the expression of ITGA3 in different normal tissues of the human body. We found that the expression level of ITGA3 in the central nervous system was lower than that in most other tissues (Supplementary Figure 2A). We also compared the expression of ITGA3 in different tumor cell lines using the cancer cell line encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle). The expression level of ITGA3 in glioma cell lines was noticeably higher than that of most other tumor cell lines. These results indicated that ITGA3 was closely linked with the occurrence and progression of glioma, and ITGA3 might serve as a diagnostic marker for glioma. To further confirm the expression characteristics of ITGA3 in glioma, we tested the mRNA and protein expression level of ITGA3 in glioma samples and glioma cell lines. As shown in Figure 4A–B and Supplementary Figure 2C–G, the mRNA and protein expression levels of ITGA3 in glioma samples were evidently higher than those in normal brain tissue, and as the grade of glioma increased, ITGA3 expression was upregulated. In addition, the mRNA and protein expression levels of ITGA3 in different glioma cell lines (T98, LN229, U373, U251, and U87) were also significantly higher than that in NHA cells (Figure 4C–D). Immunohistochemical detection of ITGA3 protein expression levels in different grade glioma specimens and normal brain tissue samples was consistent with qPCR and western blotting results (Figure 4E). These results indicated that ITGA3 was preferentially expressed in aggressive subtypes of glioma, and thus may be useful as a diagnostic marker for glioma.

Relationship between ITGA3 and tumor purity of glioma

Our previous study found that the purity of glioma was closely related to the clinical and pathological characteristics of glioma, and could accurately predict prognosis(26). An important factor affecting the purity of glioma is the content of immune cells in the glioma microenvironment. ITGA3 is a transmembrane protein that can transduce and regulate signals inside and outside the cell. Therefore, we speculated that ITGA3 might be linked to the purity of glioma. The ESTIMATE score analysis suggested that the expression of ITGA3 was significantly positively correlated with ESTIMATE, immune, and stromal scores, but negatively correlated with tumor purity of gliomas in TCGA, CGGA, and GSE16011 datasets (Figure 5A–H, Supplementary Figure 3A–D). Correlation analysis between the expression of ITGA3 and the degree of enrichment of immune cells found that ITGA3 was positively correlated with the degree of enrichment of most immune cells in TCGA, CGGA, and GSE16011 cohorts (Figure 5I–J, Supplementary Figure 3E). Among the immune cells whose enrichment degree was positively correlated with the expression of ITGA3 and the correlation coefficient was greater than 0.4 in the three cohorts, there were six overlapped immune cell types in TCGA, CGGA, and GSE16011 datasets (Figure 5K). Figure 5L–M and Supplementary Figure 3F show the relationship between the enrichment of these six types of immune cells and the expression level of ITGA3 in TCGA, CGGA, and GSE16011 datasets. It had been confirmed

that high immune cell infiltration resulted in a more unfavorable prognosis in glioma(27). These results indicated that ITGA3 reduced the purity of glioma by promoting the enrichment of immune cells in the glioma microenvironment, thereby affecting the prognosis of glioma.

ITGA3 is closely related to immune checkpoints in glioma

Among the six overlapped types of immune cells (Figure 5K–M and Supplementary Figure 2F), all are anti-tumor immunocytes except plasmacytoid dendritic cells, which have a tumor-promoting function. But the outcome of glioma patients with high ITGA3 expression was worse. This might be because ITGA3 could promote anti-tumor immunosuppression in glioma. Considering the important role of immune checkpoints in promoting tumor immunosuppression(28-30), we hypothesized that ITGA3 could promote anti-glioma immunosuppression by regulating the expression of immune checkpoints. To verify this hypothesis, we assessed the correlation between ITGA3 and immune checkpoints. Expression of ITGA3 was significantly positively correlated with the expression level of most immune checkpoints in TCGA, CGGA, and GSE16011 cohorts (Figure 6A–B, Supplementary Figure 4A). There were six immune checkpoints (CD276, NRP1, CD274, CD44, TNFRSF14, and CD40) with correlation coefficients greater than 0.4 that overlapped in TCGA, CGGA, and GSE16011 datasets (Figure 6C). The correlation between these immune checkpoints and ITGA3 is also shown in Figure 6D–O, and Supplementary Figure 4B–G. The correlation between ITGA3 and immune checkpoints indicated that ITGA3 could inhibit anti-glioma immunity by promoting the expression of immune checkpoints.

ITGA3-related immune signatures in glioma

Because ITGA3 could affect tumor purity and regulated the expression of immune checkpoints, it might also influence other aspects of glioma immunity. To determine the ITGA3 related immune signature in glioma, gene sets associated with the immune response (http://amigo.geneontology.org/amigo/landing) were filtered out. The optimal correlated genes to ITGA3 (Pearson's $|r| > 0.4$) were determined, including 352, 401, and 403 genes in TCGA, CGGA, and GSE16011 datasets, respectively (Figure 7A–B, Supplementary Figure 5A). GO analysis was employed to identify the biofunctions of the overlapped genes positively related to ITGA3 in TCGA, CGGA, and GSE6011 (Supplementary Figure 5B). The results indicated that these genes were significantly enriched in GO biological processes related to neutrophil regulation, T cell, and leucocyte function in immune response (Figure 7C). GSEA analysis also found that innate immune response, inflammatory response, and adaptive immune response were highly enriched in ITGA3 high-expressing gliomas in TCGA, CGGA, and GSE16011 databases (Figure 8A–C). These results suggested that ITGA3 played a vital role in immune response in glioma.

ITGA3 regulates the EMT of glioma cells

To further explore the role of ITGA3 in glioma, we performed GSEA analysis based on hallmark gene sets. GSEA analysis revealed that the EMT was highly enriched in ITGA3 high-expressing gliomas in TCGA, CGGA, and GSE16011 cohorts (Figure 9A–C). To further confirm the effect of ITGA3 on the EMT of glioma cells, we assessed the migration and invasion potential of glioma cells after knocking down ITGA3. Wound healing and Transwell assays revealed that the migration and invasion ability of glioma cells significantly decreased after silencing ITGA3 (Figure 9D–F). In addition, the expression of ITGA3 was significantly positively correlated with the expression levels of the EMT markers (Vimentin, N-Cadherin, Claudin-1, SNAI1) in TCGA, CGGA, and GSE16011 datasets (Supplementary Figure 6A–L). In U87 and U251 cells, the expression levels of EMT markers (N-Cadherin, β-Catenin, Vimentin, SNAIL, Claudin-1) were significantly downregulated after silencing of ITGA3, while the expression of E-Cadherin was upregulated (Figure 9G). These results confirmed that ITGA3 could regulate the EMT of glioma cells.

ITGA3 promotes angiogenesis in glioma

In addition to the EMT, GSEA analysis also found that angiogenesis was highly enriched in ITGA3 high-expressing gliomas (Figure 10A–C). The tube formation capacity of HBMECs induced with glioma cell-conditioned medium was also significantly suppressed following knockdown of ITGA3 in glioma cells (Figure 10D). Glioma cells can produce many pro-angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin and pleiotropic factors, pleiotrophin and transforming growth factor-β (TGF-β) (31, 32). To further explore the relationship between ITGA3 and angiogenesis within the glioma microenvironment, we evaluated the relationship between ITGA3 and the pro-angiogenic factors (VEGFA, TGFB1, PDGFA, and ANGPT2) in TCGA, CGGA, and GSE16011 datasets. Expression of ITGA3 in gliomas was significantly positively correlated with the expression levels of these pro-angiogenic factors (Supplementary Figure 7A–L). In addition, after silencing ITGA3 in glioma cells, the expression levels of VEGFA and TGFB1 were also significantly reduced (Figure 10E). The above results indicated that ITGA3 regulated angiogenesis in the glioma microenvironment by regulating the expression of pro-angiogenic factors in glioma cells.

Silencing ITGA3 inhibits autophagy in glioma cells

Many studies have regarded ITGA3 as an autophagy-related gene(33). However, whether ITGA3 can regulate autophagy in glioma cells is still unclear. We used glioma cells stably transfected with RFP-GFP-LC3 to explore the effect of ITGA3 on autophagy. After knockdown of ITGA3 in glioma cells stably transfected with RFP-GFP-LC3, the numbers of yellow puncta were significantly reduced (Figure 11A–B). In addition, in U87 and U251 cells, silencing ITGA3 significantly inhibited the expression of LC3-II (Figure 11C). These results indicated that ITGA3 could promote autophagy in glioma cells.

Given that ITGA3 played an important role in glioma, we further explored the signaling pathways through which it produces these effects. GSEA analysis found that ERK1/2 and PI3K-AKT-mTOR signaling pathways were significantly enriched in ITGA3 high-expressing gliomas in TCGA, CGGA, and GSE16011

datasets (Figure 11D–F, Supplementary Figure 8A–F). In vitro experiments also found that silencing ITGA3 inhibited the phosphorylation of ERK1/2 (Figure 11G–H).

Discussion

Although great efforts have been made to improve glioma treatment, the prognosis of glioma patients is still poor(1, 2). New effective treatment methods and reliable treatment targets are urgently needed for the treatment of glioma. In addition, new molecular markers for improving the diagnostic and prognosis prediction efficiency of glioma are also necessary.

Our study found that the expression level of ITGA3 was significantly upregulated in glioma compared with that of normal brain tissue, and tended to be enriched in aggressive subtypes of glioma. In addition, the expression of ITGA3 was also closely correlated with the prognosis of glioma patients. This indicated that ITGA3 could not only act as a diagnostic marker for glioma, but also serve as a prognostic predictor of glioma.

The tumor microenvironment, especially immune cell components, plays a crucial role in tumor progression. Our previous study confirmed that glioma purity was an independent prognostic factor for glioma(26). In this study, we revealed that ITGA3 was strongly correlated with glioma purity. Additionally, the expression level of ITGA3 in gliomas was also significantly positively correlated with the enrichment level of most immune cells. Furthermore, ITGA3 could also regulate the anti-glioma immune response through immune checkpoints and other immune-related genes. These findings suggested that expression levels of ITGA3 affected the characteristics of the glioma microenvironment and influenced the anti-glioma immune response state. In addition to immune regulation, we also found that ITGA3 could regulate angiogenesis in the glioma microenvironment. Anti-angiogenesis therapies have become an important treatment method for many cancers including glioma(32, 34–37). Furthermore, angiogenesis also has an important impact on the immune composition in the glioma microenvironment(38). Thus, ITGA3 could also affect the immune microenvironment of glioma through angiogenesis, and inhibiting ITGA3 not only prevented angiogenesis, but also regulated anti-glioma immunity.

In addition to the microenvironmental factors that affect the progression of glioma, the factors of glioma cells themselves should not be ignored. We found that ITGA3 promoted the EMT of glioma cells to improve their migration and invasion capabilities. The high invasiveness of glioma cells is an important factor that makes gliomas easy to relapse and difficult to treat. Although autophagy plays dual roles, which include anti-cancer effects as well as helping with therapy resistance in cancer chemoradiotherapy(39–41), we believe that the promotion effect of ITGA3 on autophagy of glioma cells might reduce the chemotherapy sensitivity of glioma cells. This is because that the prognosis of glioma patients with high ITGA3 expressing receiving radiotherapy or chemotherapy was significantly worse than that of the ITGA3 low expressing patients.

ITGA3 played the above roles in glioma, but its specific mechanism was still unclear. The ERK1/2 pathway participates in many biological processes, such as cell proliferation, differentiation, cell

migration, survival(apoptosis and senescence)(42, 43), the EMT(44), autophagy(45), angiogenesis(45, 46), and inflammatory and immune responses(47). The PI3K/AKT/mTOR pathway play a critical role in basic intracellular functions in many neoplasms(48, 49). A common function of the PI3K/AKT/mTOR pathway is regulation of autophagy(50). The PI3K/AKT/mTOR and ERK1/2 pathways are also critical for exploring potential cancer therapeutic targets. The relationship between ITGA3 and these pathways shows the important role ITGA3 plays in glioma. It also reflects the possibility of ITGA3 as a target for glioma treatment.

Conclusions

In summary, our study explored the prognostic value, expression characteristics, and biological processes of ITGA3 in glioma. Our results revealed that ITG3 not only regulated the immune characteristics and angiogenesis within the glioma microenvironment, but also regulated the EMT and autophagy of glioma cells. These findings indicated that ITGA3 might be a potential therapeutic target for glioma.

Abbreviations

TCGA: The Cancer Genome Atlas;

CGGA: Chinese Glioma Genome Atlas;

EMT: Epithelial–mesenchymal transition;

LGG: Low grade glioma;

GBM: Glioblastoma;

GEO: Gene expression omnibus;

OS: Overall survival;

GSEA: Gene set enrichment analysis;

GO: Gene ontology;

CMU: China Medical University;

PBS: Phosphate-buffered saline;

siRNA: Small-interfering RNA;

DMEM: Dulbecco's Modified Eagle's medium;

PN: Proneural;

NE: Neural;

CL: Classical;

ME: Mesenchymal;

WT: Wild type;

CCLE: Cancer cell line encyclopedia;

NHA: Normal human astrocytes cell line;

HBMEC: Human brain microvascular endothelial cell;

Declarations

Ethics approval and consent to participate

The Medical Ethics Committee of the First Hospital of CMU authorized the study, and each patient provided a written informed consent.

Consent for publication

Not applicable

Data Availability Statement

The cohorts used in this study can be acquired through: http:// www.cgga.org.cn/ (CGGA, CGGA mRNA-array, REMBRANDT), https://portal.gdc.cancer.gov/ (TCGA), https://www.ncbi.nlm.nih.gov/geo/ (GSE16011, GSE4290, GSE59612), and http://gliovis.bioinfo.cnio.es/ (TCGA-GBM Aglient-4502A, Kamoun data).

Conflict of Interest

The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

The project was designed by Qing Zhang and Anhua Wu, and implemented by Qing Zhang. Qing Zhang wrote the initial draft and Anhua Wu revised and edited it. All authors reviewed the final version of the manuscript and consented its publication.

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Figures

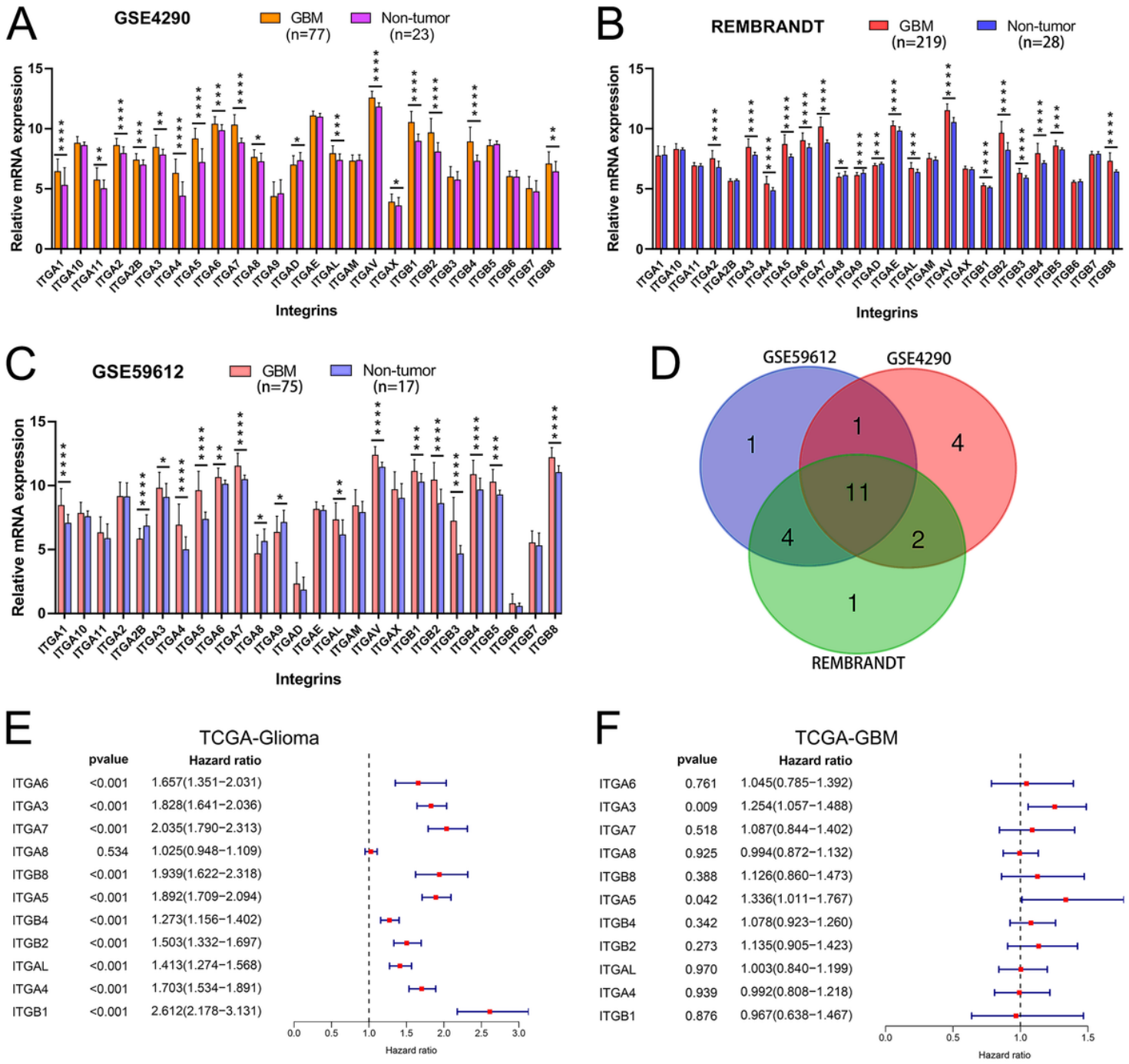

Figure 1

The expression pattern and prognostic effect of integrins in GBM and non-tumor brain tissues. (A–C) The expression characteristics of integrins in GBM and non-tumor brain tissues in GSE4290 (A), REMBRANDT (B), and GSE59612 (C). (D) The intersection of significantly differently expressed integrins in GBM and non-tumor brain tissues in the three datasets. (E–F) In the TCGA (E) and TCGA-GBM (F) databases, univariate Cox regression analysis was applied to identify the prognostic value of overlapped integrins.

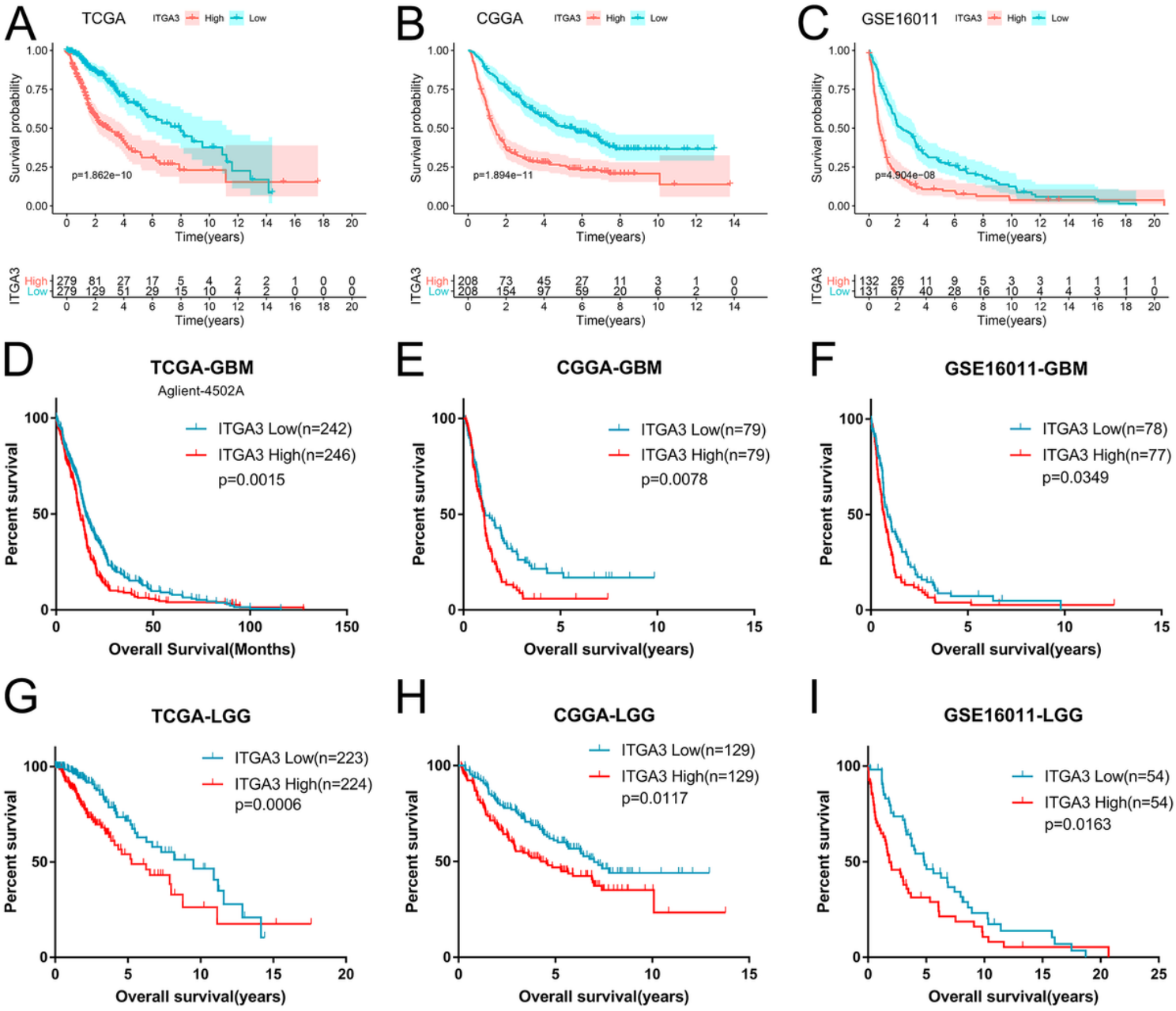

Figure 2

The prognostic value of ITGA3 in glioma. (A–C) In TCGA (A), CGGA (B), and GSE16011 (C) datasets, the higher the expression level of ITGA3, the worse the prognosis of glioma patients. (D–F) In TCGA(Aglient-4502A) (D), CGGA (E), and GSE16011 (F) datasets, the higher the expression level of ITGA3, the worse the prognosis of GBM patients. (G–I) In TCGA (G), CGGA (H), and GSE16011 (I) datasets, the higher the expression level of ITGA3, the worse the prognosis of LGG patients.

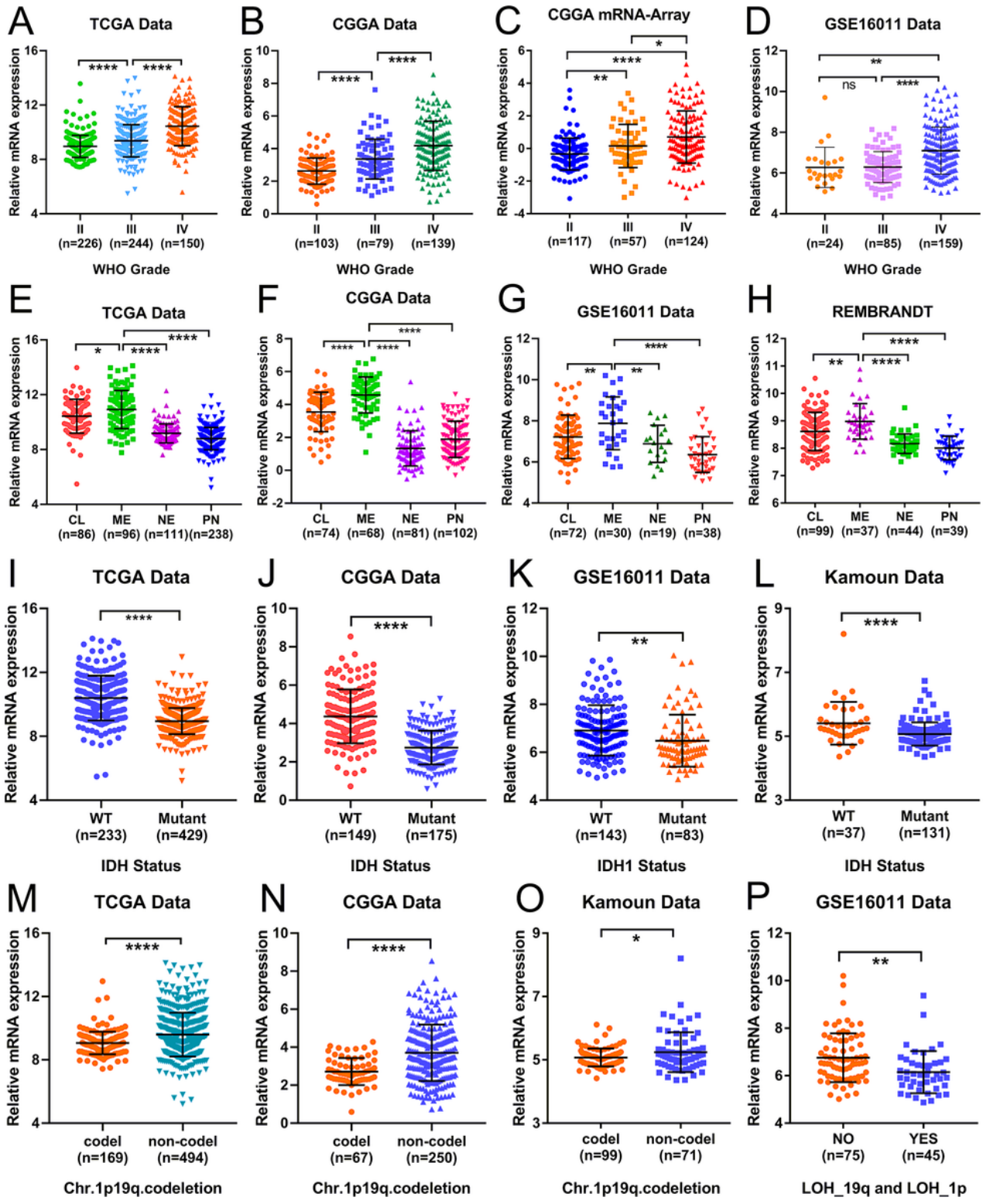

Figure 3

ITGA3 was enriched in aggressive subtypes of glioma. (A–D) The expression level of ITGA3 was significantly upregulated along with increased WHO grade of glioma in TCGA (A), CGGA (B), CGGA (mRNA-array) (C), and GSE16011 (D) datasets. (E–H) The expression level of ITGA3 in ME subtypes was significantly higher than that in other transcriptomic subtypes in TCGA (E), CGGA (F), GSE16011 (G), and REMBRANDT (H) datasets. (I–L) ITGA3 was enriched in IDH wild-type glioma in TCGA (I), CGGA (J),

GSE16011 (K), and Kamoun (L) cohorts. (M–P) The expression level of ITGA3 was significantly upregulated in chromosome 1p19q non-codeleted glioma in TCGA (M), CGGA (N), Kamoun (O), and GSE16011 (P) datasets. In GSE16011, LOH stands for loss of heterozygosity.

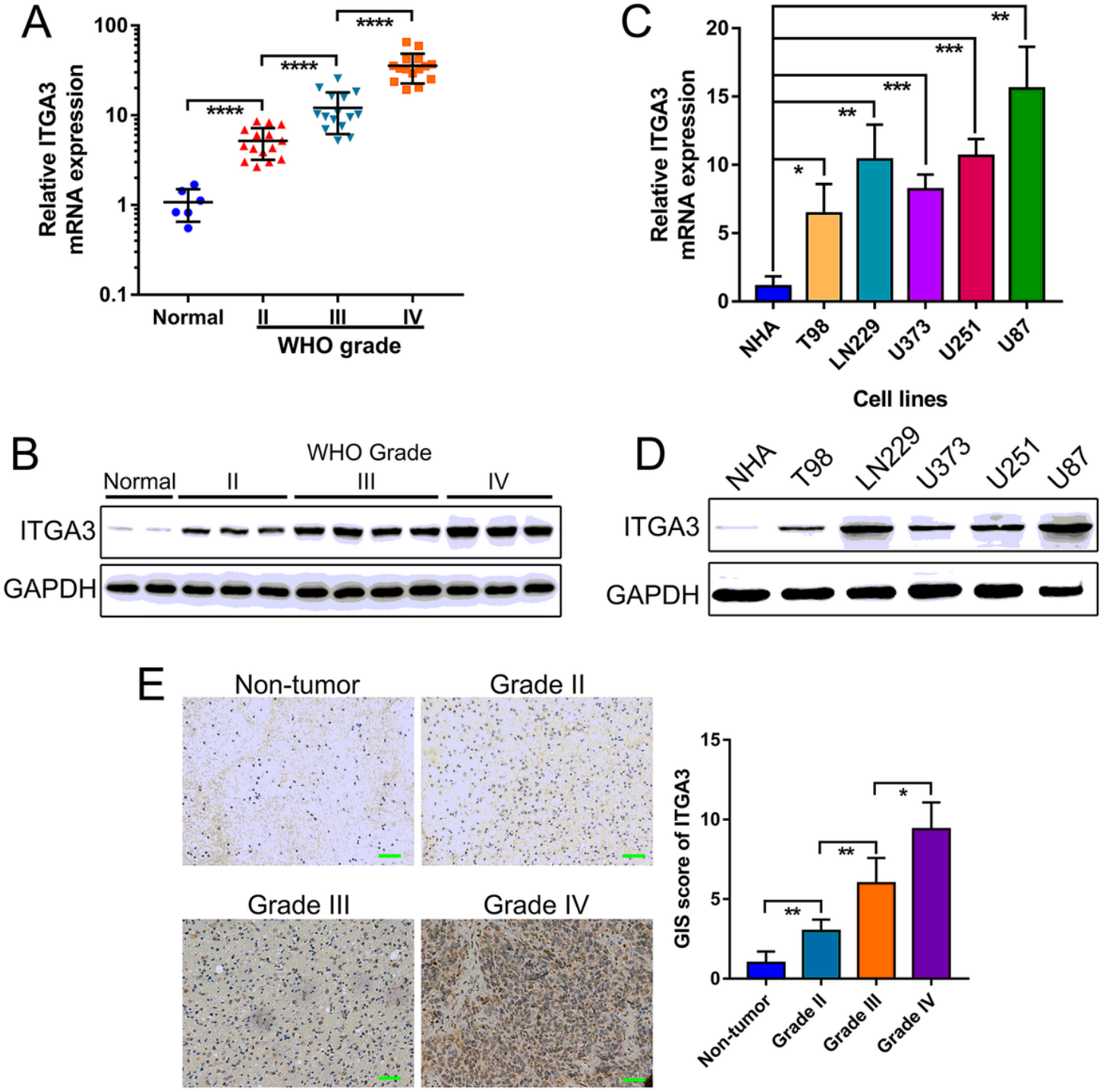

Figure 4

ITGA3 expression in glioma samples and glioma cell lines. (A–B) ITGA3 mRNA (A) and protein (B) expression in glioma samples was higher than that in non-tumor brain tissue. (C–D) ITGA3 mRNA (C) and protein (D) expression in glioma cell lines was higher than that in NHA.

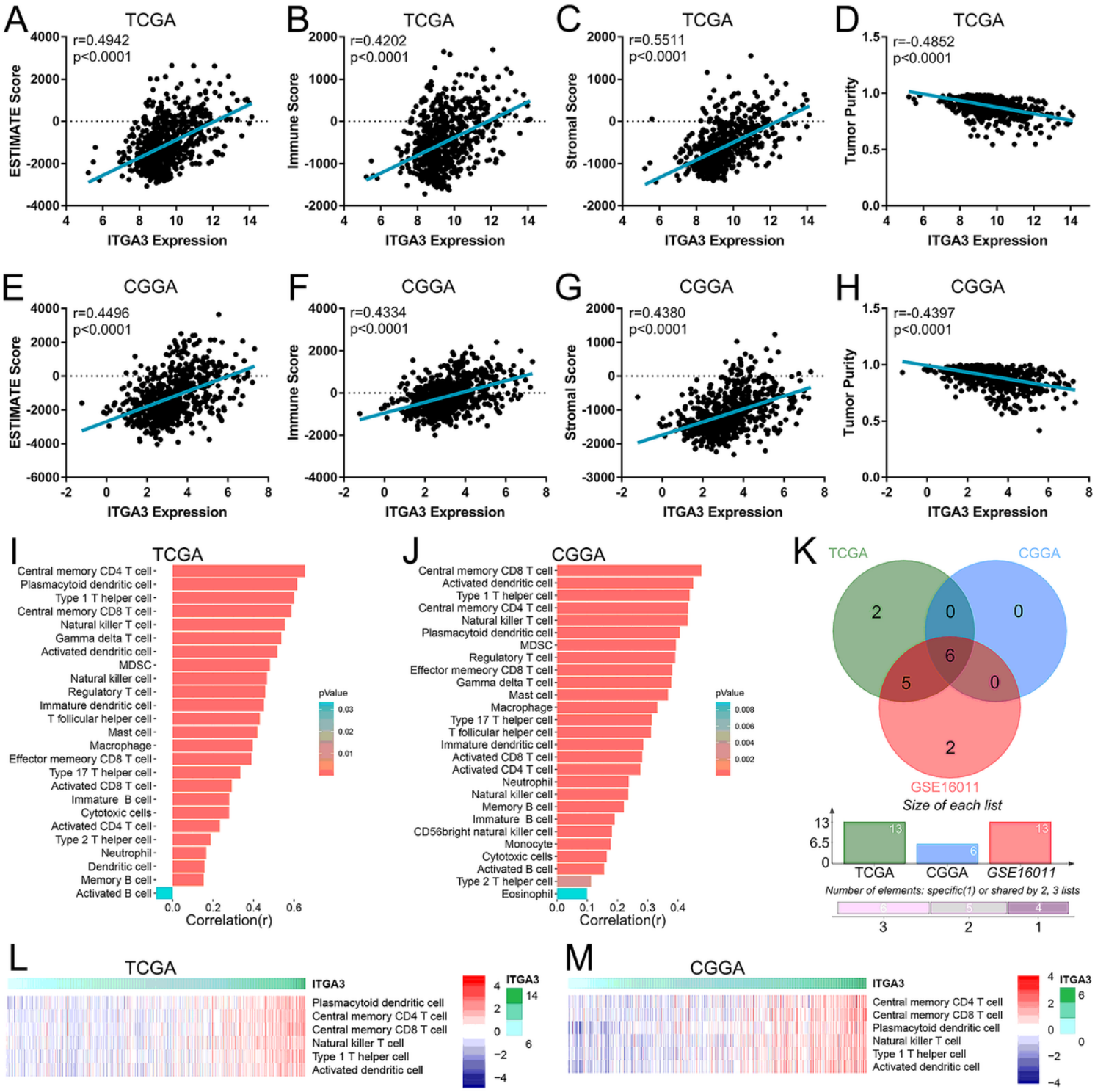

Figure 5

ITGA3 affected the purity of glioma. (A–H) The expression of ITGA3 was significantly positively correlated with ESTIMATE, immune, and stromal scores, but negatively correlated with glioma purity in TCGA (A–D) and CGGA (E–H) datasets. (I–J) The expression level of ITGA3 in gliomas was significantly positively correlated with the enrichment of most immune cells within the glioma microenvironment in TCGA (I) and CGGA (J) datasets. (K) The intersection of immune cells with correlation coefficient ≥ 0.4 or ≤ -0.4 significantly correlated with ITGA3 expression level in TCGA, CGGA, and GSE16011 datasets.

(L–M) Enrichment characteristics of overlapped immune cells in glioma in TCGA (L) and CGGA (M) datasets.

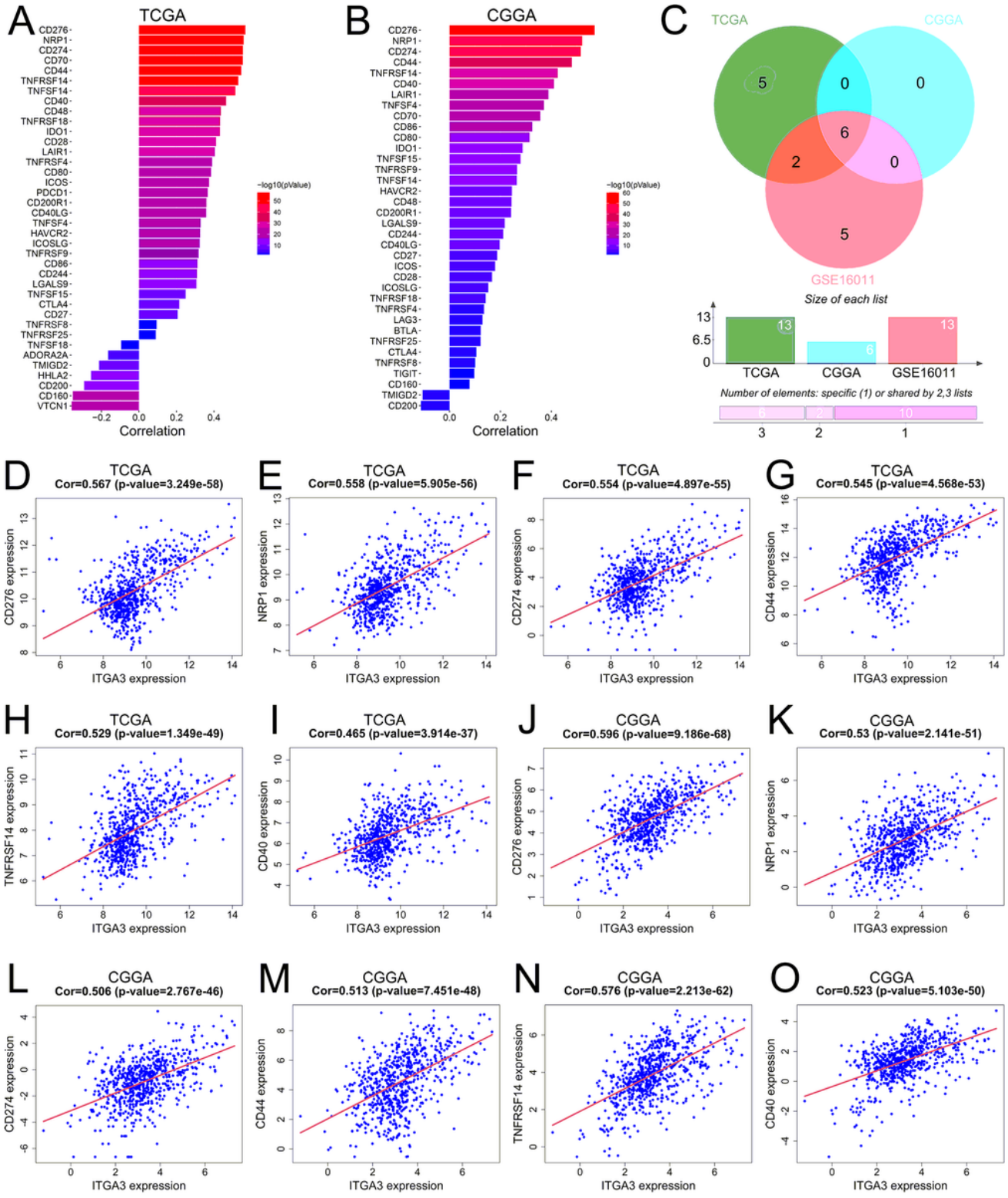

Figure 6

The relationship between ITGA3 and immune checkpoints in glioma. (A–B) The expression of ITGA3 was significantly positively correlated with most immune checkpoints in TCGA (A) and CGGA (B) datasets. (C) The intersection of immune checkpoints with correlation coefficient ≥ 0.4 or ≤ -0.4 significantly

correlated with ITGA3 expression level in TCGA, CGGA, and GSE16011 datasets. (D–O) The correlation between ITGA3 and six overlapped immune checkpoints (CD276, NRP1, CD274, CD44, TNFRSF14, and CD40) in TCGA (D–I) and CGGA (J–O) datasets.

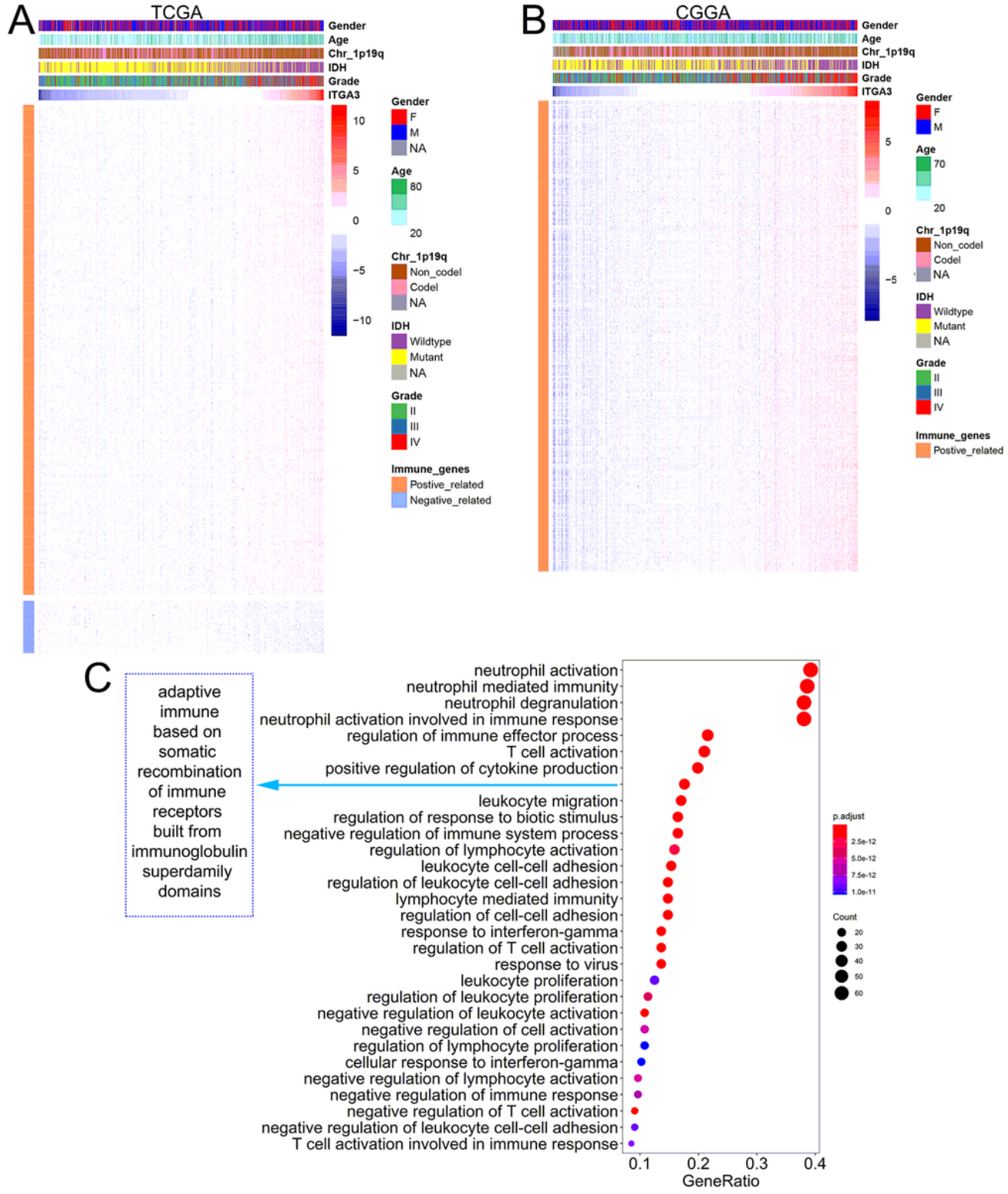

Figure 7

ITGA3 related immune genes and GO processes in glioma. (A–B) ITGA3 showed a strong positive correlation with most immune genes. (C) ITGA3 was closely linked with the immune cell response in

glioma.

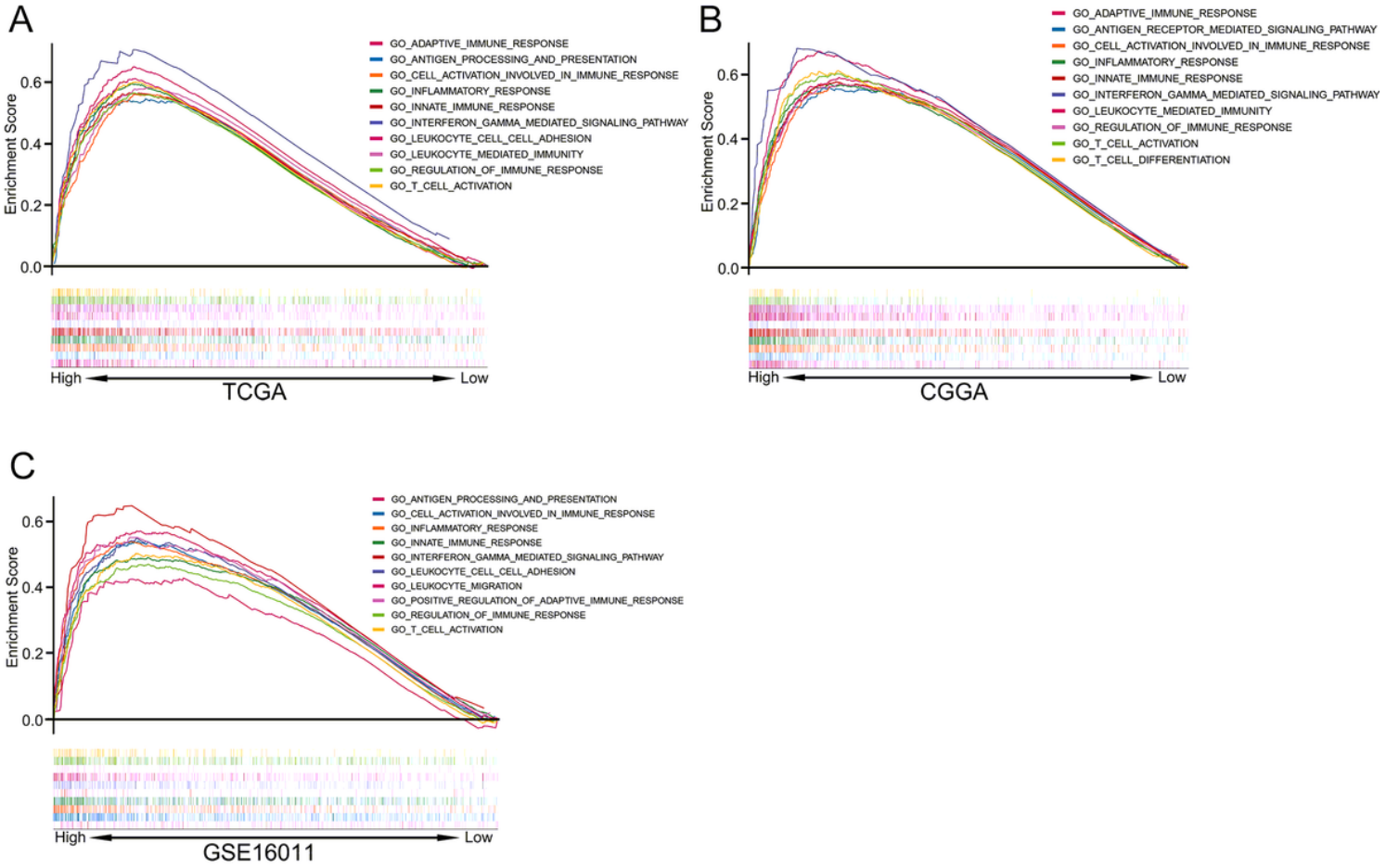

Figure 8

ITGA3 was tightly correlated with immune response in glioma. (A–C) There was a high enrichment of inflammatory and immune responses in ITGA3 high expression gliomas in TCGA (A), CGGA (B), and GSE16011 (C) datasets.

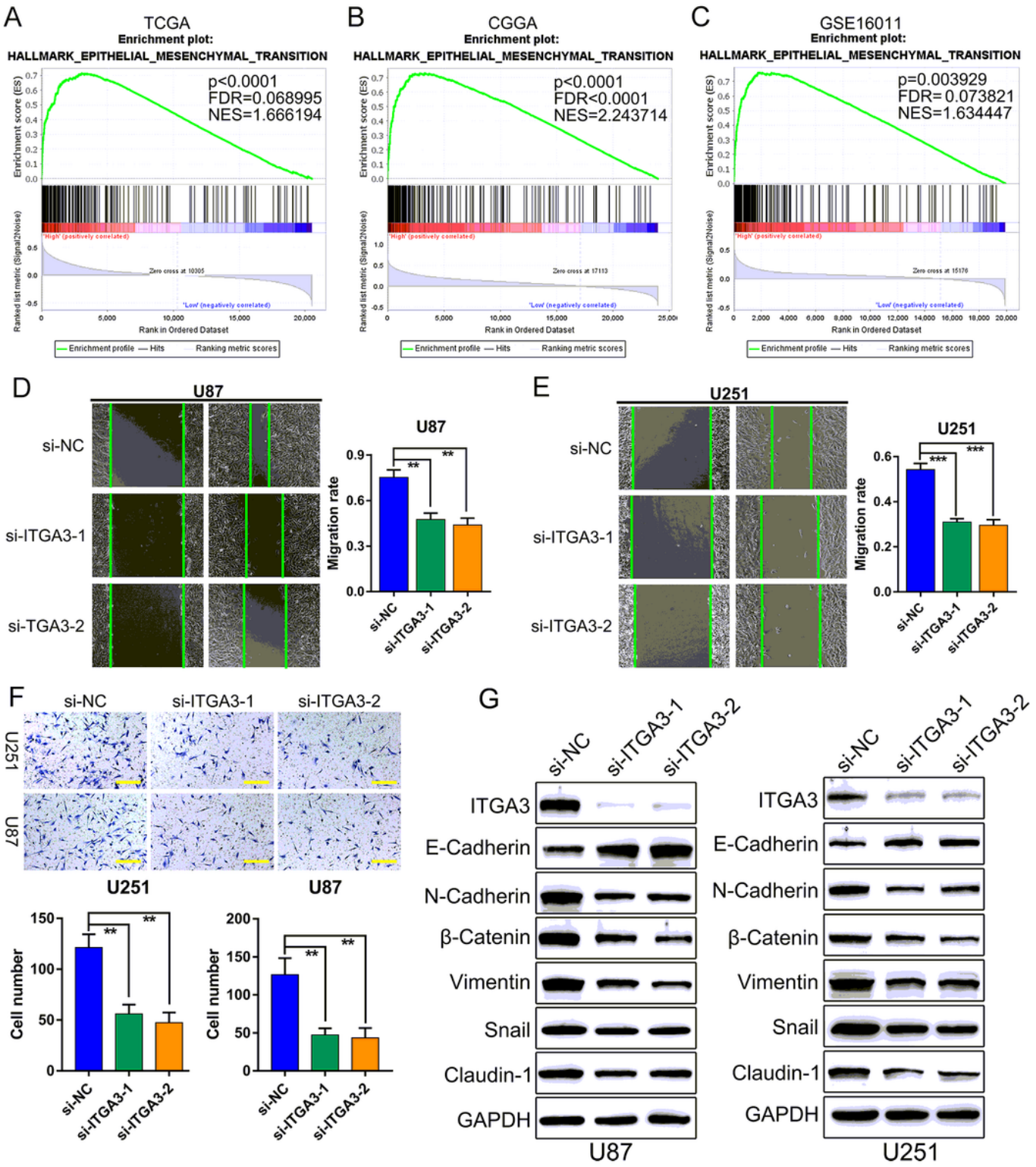

Figure 9

ITGA3 promoted the EMT of glioma cells. (A–C) the EMT was significantly enriched in ITGA3 high-expressing gliomas in TCGA (A), CGGA (B), and GSE16011 (C). (D–E) Silencing ITGA3 inhibited the migration ability of U87 (D) and U251 (E) cells. (F) Knockdown of ITGA3 inhibited the invasion ability of U87 and U251 cells. Scar bar: 50 μm. (G) The expression of EMT-related markers was regulated by ITGA3.

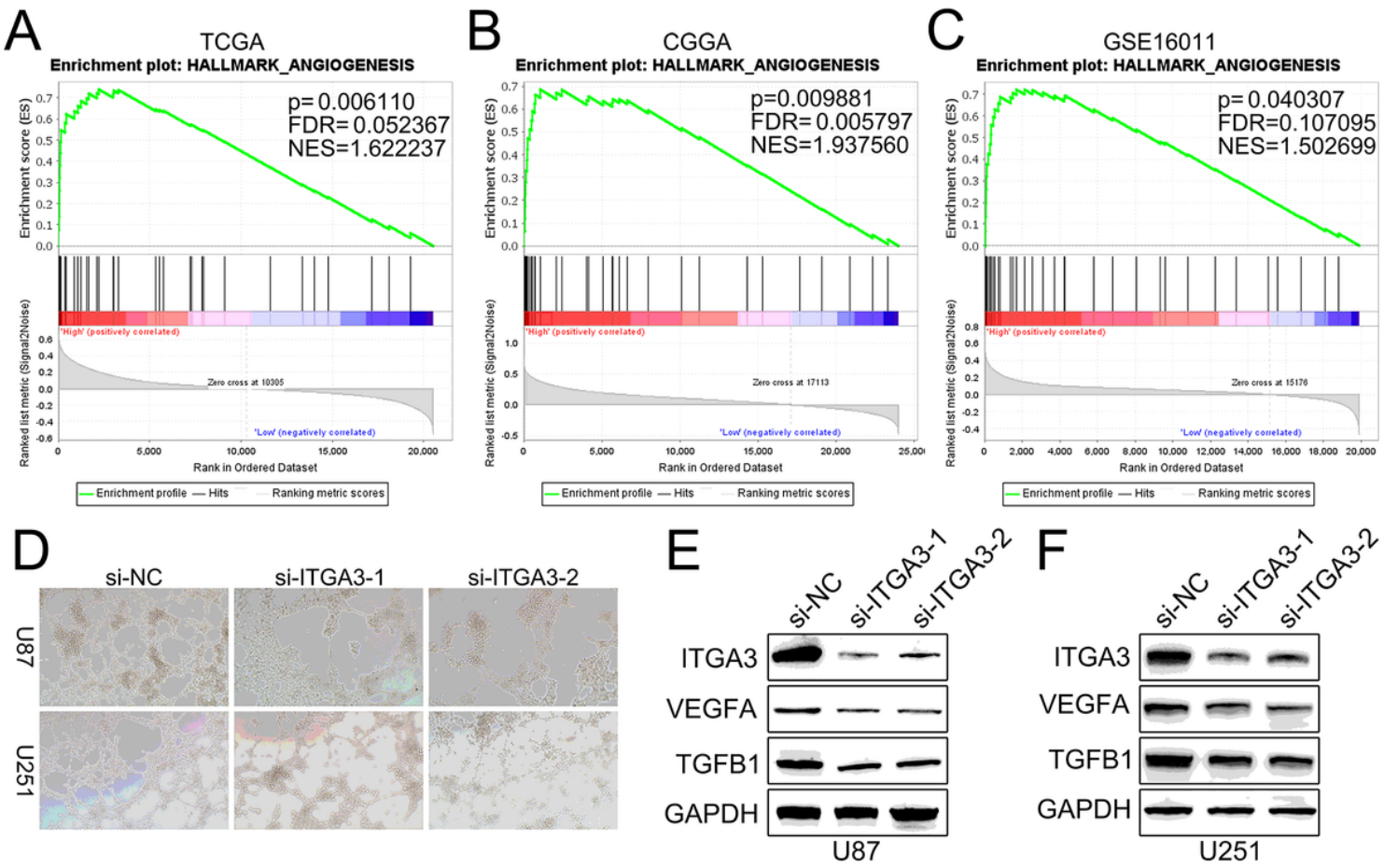

Figure 10

ITGA3 induced angiogenesis within the glioma microenvironment. (A–C) GSEA analysis found that angiogenesis was enriched in ITGA3 high-expressing gliomas in TCGA (A), CGGA (B), and GSE16011 (C) datasets. (D) ITGA3 knockdown inhibited tube formation by HBMECs incubated with conditioned medium from U87 and U251 cells. (E–F) Silencing ITGA3 suppressed the expression of VEGFA and TGFB1 in U87 and U251 cells.

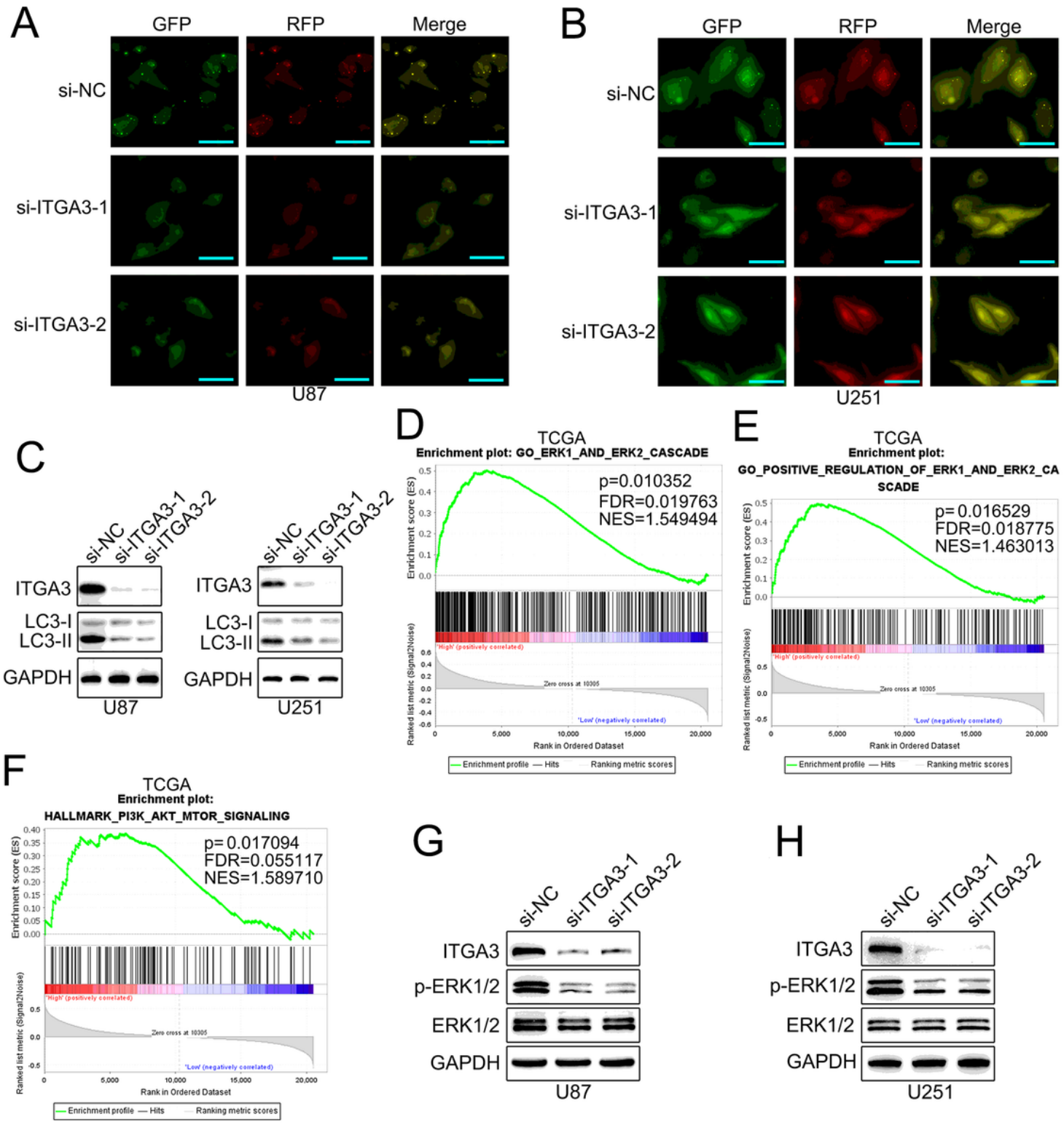

Figure 11

ITGA3 promoted autophagy in glioma cells. (A–B) Knockdown of ITGA3 inhibited the formation of autophagosomes in U87 (A) and U251 (B) cells. Scar bar: 20 μm. (C) ITGA3 regulated the expression of LC3-II. (D–F) ERK1/2 and PI3K/AKT/mTOR pathways were enriched in ITGA3 high-expressing gliomas in TCGA cohort. (G–H) Knockdown ITGA3 inhibited phosphorylation of ERK1/2 in U87 (G) and U251 (H) cells.

Supplementary Files

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- Additionalfile2.pdf