



Immune-related lncRNAs, LINC01268 and CTB-31O20.2, as favorable prognostic markers for glioma inhibition

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Background: Glioblastoma (GBM) is the most common and fatal tumor in the central nervous system. Recent studies have found that long non-coding RNAs (lncRNAs) serve as competitive endogenous RNAs (ceRNAs) and play an important role in GBM by regulating immune responses. The aim of the present study was to identify lncRNAs with immune relevance and functions in GBM.

Methods: We analyzed GBM datasets from The Cancer Genome Atlas (TCGA) database to obtain 356 significantly differentially expressed lncRNAs (DE-lncRNAs), 4,951 DE-mRNAs, and 34 DE-miRNAs in GBM, respectively. For mRNAs, 369 DE-mRNAs were identified as immune-related genes in the ImmPort database. For DE-lncRNAs, univariate analysis identified 39 DE-lncRNAs with prognostic significance, and 9 DE-lncRNAs were included in the ImmLnc database. Combined analysis was then conducted by integrating 9 immune-related DE-lncRNAs, 369 immune-related DE-mRNAs, and 34 DE-miRNAs. A ceRNA network composed of 2 upregulated lncRNAs (LINC01268 and CTB-31O20.2), 3 downregulated miRNAs, and 5 upregulated mRNAs was generated.

Results: Kaplan-Meier survival analysis and univariate and multivariate Cox regression analyses showed that LINC01268 and CTB-31O20.2 serve as independent favorable prognostic markers in GBM. LINC01268 and CTB-31O20.2 overexpression was conducted in GBM cell U251. Cell Counting Kit-8 (CCK8), Transwell assay, and scratch healing assay indicated that LINC01268 and CTB-31O20.2 inhibit GBM cell line, U251, proliferation, invasion, and migration.

Conclusions: LINC01268 and CTB-31O20.2 are independent prognostic immune-related markers, and reduce cancer cell proliferation and metastasis in GBM.

Keywords: Glioblastoma; long non-coding RNAs (lncRNAs); immune-related genes; competitive endogenous RNAs (ceRNAs); prognosis

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Introduction

Glioblastoma (GBM) is the most common and fatal tumor in the central nervous system and has a poor prognosis. It accounts for 82% of primary malignant brain tumors and is classified as a grade IV tumor by the World Health Organization (1). Evidence suggested that the risk factors for

GBM are cumulative genetic alterations and environment, such as aging, family history, ionizing radiation, and viruses (2,3). However, the causes of GBM are still unclear. Although some novel therapeutic methods, such as molecular targeted therapy, gene therapy, and antiangiogenic agent treatment, have been developed (4,5), the treatment of GBM remains

a challenge. With the advent of advanced sequencing technologies, an abundance of gene expression data, distinct molecular and immune-related genetic alterations have been revealed (6). Producing immunosuppressive factors and modulating cell surface receptors and immune cell subsets impairs GBM in both the local central nervous system and systemic immune system functions (7). GBM alters major immunogenic signaling pathways and cellular immunity inside and outside the brain (8). A deeper understanding of the molecular biology of GBM can promote the development of various biomarkers and new therapeutic strategies.

The competitive endogenous RNA (ceRNA) hypothesis was first proposed by Salmena *et al.*, and revealed a new mechanism for RNA interaction (9). ceRNA competitively combines to microRNA through microRNA response elements to regulate gene expression. Long non-coding RNA (lncRNA) is a ceRNA, and is an RNA molecule >200 bases in length (10). With the advancement of high-throughput sequencing technology and the progress of abundant research on lncRNA, the potential function of lncRNA has been revealed in many human diseases, such as cancer, diabetes and Alzheimer's disease (11-16). RNA-Seq data has helped identify a variety of biomarkers currently used for cancer prognosis and treatment. For instance, Liang *et al.* identified a prognostic signature, made up of the following 6 lncRNAs, that could improve the prognosis prediction of GBM: C20orf166-AS1, LINC00645, LBOX2-AS1, LINC00565, LINC00641, and PRRT3-AS1 (17). P73-AS1 was found to promote temozolomide resistance in GBM stem cells, and high TP73-AS1 as a biomarker had poor prognosis in GBM (18). TTC28-AS1, AC090425.1, LINC00092, and HCG18 are closely related to glioma (19). lncRNA with prognostic value in GBM warrants further attention.

Many published studies on lncRNA as key regulator in complex immune response are emerging, highlighting that lncRNA plays an important role in the immune system (20,21). A recent analysis of transcriptome identified many differentially expressed lncRNAs (DE-lncRNAs), which were involved in regulating the immune response. SNHG14/miR-5590-3p/ZEB1 was found to promote large B-cell lymphoma progression and immune evasion by regulating programmed cell death 1 (PD-1)/CD274 molecule (PD-L1) checkpoints (22). Moreover, lncRNA, Sros1, accelerated IFN- γ -mediated activation of immune responses by stabilizing STAT1 (23). With increasingly updated research, immune-related lncRNAs have been revealed in many cancer types. For example, OSTN-AS1 is an immune-related molecule and has the potential function

of immunotherapy in triple-negative breast cancer (24). The effect of lncRNA in immune regulation is complicated, and until now, neither a ceRNA network to describe the immune-related predictions nor immune-related lncRNAs with a prognostic role have been reported. Therefore, in the present study, we generate a ceRNA network and focus on the prognostic role of immune-related lncRNA in GBM.

We identified DE-lncRNAs, DE-mRNAs, and DE-miRNAs based on a cohort study of GBM patients from The Cancer Genome Atlas (TCGA) database. Combining the ImmLnc and ImmPort databases, we focused on immune-related lncRNAs and constructed an immune-associated ceRNA network. We then identified the prognostic value of immune-related lncRNAs in the ceRNA network. We identified 2 immune-related lncRNAs for the prognosis of GBM. Finally, the function of LINC01268 and CTB-31O20.2 in GBM cell line, U251, was investigated. We present the following article in accordance with the MDAR reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-546/rc>).

Methods

Data collection and preprocessing

Transcriptome data (including lncRNA, mRNA, and miRNA) and the clinical data of GBM patients were downloaded from the Genomic Data Commons data portal provided by TCGA database. A total of 155 GBM and 5 control samples were obtained. A total of 155 GBM patients were divided into the high expression group and low expression group according to the 9 lncRNAs with prognostic value. The DE-lncRNAs, DE-mRNAs, and DE-miRNAs between GBM and normal samples were analyzed by edgeR package of R. Volcano plots and heatmaps were drawn using R script. Fold change (FC) >2 and False discovery rate (FDR) <0.05 were used as cut-off values. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Identification of immune-related DE-lncRNAs

The ImmLnc database was developed by Li *et al.* (25). It charts the landscape of immune-related lncRNA regulation in 33 cancer types and can be used to identify potential carcinogenic biomarkers (26). A total of 934 immune-related lncRNAs of GBM from the ImmLnc database were downloaded (available online: <https://cdn.amegroups.cn/static/public/10.21037/tcr-22-546-1.xlsx>), and were used to

determine the potential function of immune-related DE-lncRNAs in GBM.

Identification of immune-related DE-mRNAs

The ImmPort database contains a large number of immune-related genes and includes 17 immune-related pathways according to different molecular functions (27). These include antimicrobials, antigen processing, presentation, BCR signaling, chemokine receptors, natural killer cell cytotoxicity, TNF family members, TGF- β family members, and the TCR signaling pathway. A total of 1,424 immune-related genes were downloaded from the ImmPort database, and were identified as immune-related DE-mRNAs in GBM.

Construction of a ceRNA network

The LncBase and miRDB databases were used to predict corresponding target lncRNAs and mRNAs of miRNAs, respectively (28,29). Only immune-related lncRNAs and mRNAs that co-target miRNAs were eligible for the construction of the ceRNA networks. Finally, immune-related lncRNAs, miRNAs, immune-related mRNAs, immune-related pathways, and immune cells were selected to construct the immune-related ceRNA network using Cytoscape visualization software (30).

Cell culture

The human GBM cell line, U251, was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences, Shanghai, China. U251 cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA). All cells were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C.

LNC01268 and CTB-31O20.2 overexpression

The full length of lncRNAs LNC01268 and CTB-31O20.2 was reverse transcribed into cDNA, polymerase chain reaction amplified, and cloned into the pCDNA3.1 vector (Invitrogen, Shanghai, China). The empty pCDNA3.1 vector was used as the control. The cell lines were transfected with a Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. To detect the efficiency of transfection, the expression of LNC01268 and CTB-

31O20.2 was verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Cell proliferation assays

Cell proliferation was measured using Cell Counting Kit-8 (CCK8) assay. After 48 h of transfection, cells were seeded onto 96-well plates with a density of 5×10^3 per well and cultured in an incubator for 0, 24, 48 and 72 h at 37 °C. Then, 10 μ L of CCK8 (Beyotime Biotechnology, Shanghai, China) was added, and the cells were cultured in an incubator for 2 h. OD450 was measured using a spectrophotometer (DS-11 FX; DeNovix, Wilmington, NC, USA).

Scratch wound assay

Cell migration ability was evaluated using a scratch wound assay. U251 cells were seeded and cultured onto 6-well plates for 24 h. A pipette tip was then used to create wounds in then culture plate. The wound healing process was observed at 0 and 48 h.

Transwell assay

Transwell assay was used to investigate the invasion ability of U251 cells. U251 cells were plated on the upper side of a Transwell chamber (Costar, Cambridge, MA, USA), and 20% fetal bovine serum was added into the lower compartment. After the cells were cultured and washed twice with phosphate-buffered saline, the chamber was fixed with methanol for 30 min and stained with 0.1% crystal violet. Cells were counted using a microscope.

Statistical analysis

Kaplan-Meier survival analysis, nomogram analysis, and univariate and multivariate Cox regression analyses were performed by SPSS software version 22 (IBM, Armonk, NY, USA) and R software version 3.2.3. All statistical analyses were performed using GraphPad Prism version 8. All cell experiments were replicated 3 times, and $P < 0.05$ was considered statistically significant.

Results

Identification of DE-mRNAs, DE-miRNA, and DE-lncRNAs in GBM

To determine the mRNA expression changes, we used the

gene expression profile of 5 normal samples and 155 GBM samples from TCGA database to identify DE-lncRNAs. As shown in *Figure 1A*, clustering of 4,951 DE-mRNAs of high or low expression was observed. *Figure 1B* shows the distinct cluster distribution of these DE-mRNAs in normal and GBM samples. To construct an immune-related ceRNA network, 34 DE-miRNAs in normal and GBM groups were also identified (FC >2, FDR <0.05) (*Figure 1C*). The results of the heatmap demonstrated that these DE-miRNAs showed distinct expression pattern between groups (*Figure 1D*). Furthermore, 356 DE-lncRNAs were selected (FC >2, FDR <0.05) (*Figure 1E,1F*) for further analysis.

Identification of immune-related pathways in GBM

To explore immune-related pathways in GBM, we obtained 369 immune DE-mRNAs by overlapping the 4,951 DE-mRNAs and immune-related mRNAs of the ImmPort database. As shown in *Figure 2A*, 239 upregulated DE-mRNAs and 130 downregulated DE-mRNAs were identified. *Figure 2B* shows the clustering of these genes in all samples. Afterwards, we built a regulatory network between genes and immune-related pathways, and 16 immune-related to immune pathways were enriched. These were cytokines, chemokines, chemokine receptors, cytokine receptors, and interleukins receptors, as shown in *Figure 2C*.

Identification of immune-related DE-lncRNAs with prognostic significance in GBM

We then found that 39 DE-lncRNAs can be used as independent prognosis factors by univariate analysis, and their general performance was showed in *Figure 3A*. The cluster heatmap of these 39 DE-lncRNAs in the normal group and the tumor group is shown in *Figure 3B*. Considering that the expression of lncRNA is related to immune cell infiltration in tumors, we took the intersection between prognostic DE-lncRNAs and lncRNAs included in ImmLnc database GBM dataset (*Figure 3C*), and constructed a network of lncRNA-immune cell, as shown in *Figure 3D*. In the immune cell-related lncRNA network, a total of 7 DE-lncRNAs were upregulated and 2 DE-lncRNAs were downregulated.

Construction of immune-related ceRNA networks

To construct an immune-related ceRNA network, the LncBase and miRDB databases were used to predict

corresponding target lncRNAs and mRNAs of DE-miRNAs, respectively. Only immune-related lncRNAs and mRNAs that co-target miRNAs were eligible for the construction of ceRNA networks. The LncBase and miRDB databases were searched to determine the relationship of lncRNA-miRNA and miRNA-mRNA, respectively. We integrated all the information of the LncBase database, miRDB database, ImmLnc database, and ImmPort database, and constructed an immune-related ceRNA network, as shown in *Figure 4A*. This network contained 5 immune-related pathways, which were TNF family members, cytokines, antimicrobials, the TCR signaling pathway, and cytokine receptors. It included 5 DE-mRNAs (TNFSF14, SLC11A1, NCK1, NOX4, and NR5A2). Furthermore, LINC01268 and CTB-31O20.2 were obtained from the network, as well as has-miR-23b-5p and has-miR-139-3p.

Identifying LINC01268 and CTB-31O20.2 as immune-related prognostic markers for GBM

To further determine whether CTB-31O20.2 and LINC01268 could act as independent predictors of GBM, a Cox regression analysis and multivariate analysis were conducted. The univariate Cox regression analysis demonstrated that the expressions of LINC01268 and CTB-31O20.2 were associated with overall survival (OS), as shown in *Figure 4B*. The multivariate analysis showed that sex, CTB-31O20.2, and LINC01268 were significantly associated with OS, as shown in *Figure 4C*. Subsequently, a nomogram was constructed to project the 1-, 3-, and 5-year OS of GBM patients, as shown in *Figure 4D*.

LINC01268 and CTB-31O20 inhibit cell proliferation, migration, and invasion in GBM

To study the function of LINC01268 and CTB-31O20.2 in GBM, LINC01268 and CTB-31O20.2 were overexpressed in U251. qRT-PCR results showed that the expressions of LINC01268 and CTB-31O20.2 were significantly elevated (*Figure 5A*). CCK8 assay was conducted, and LINC01268 and CTB-31O20.2 significantly reduced U251 cell proliferation (*Figure 5B*). The effect of LINC01268 and CTB-31O20.2 on the migration and invasion abilities of GBM cells was determined using Transwell assay and scratch wound assay, respectively. Transwell invasion assay results indicated that the overexpression of LINC01268 and CTB-31O20.2 was reduced in GBM cell invasion (*Figure 5C,5D*). Similarly, scratch wound assay results

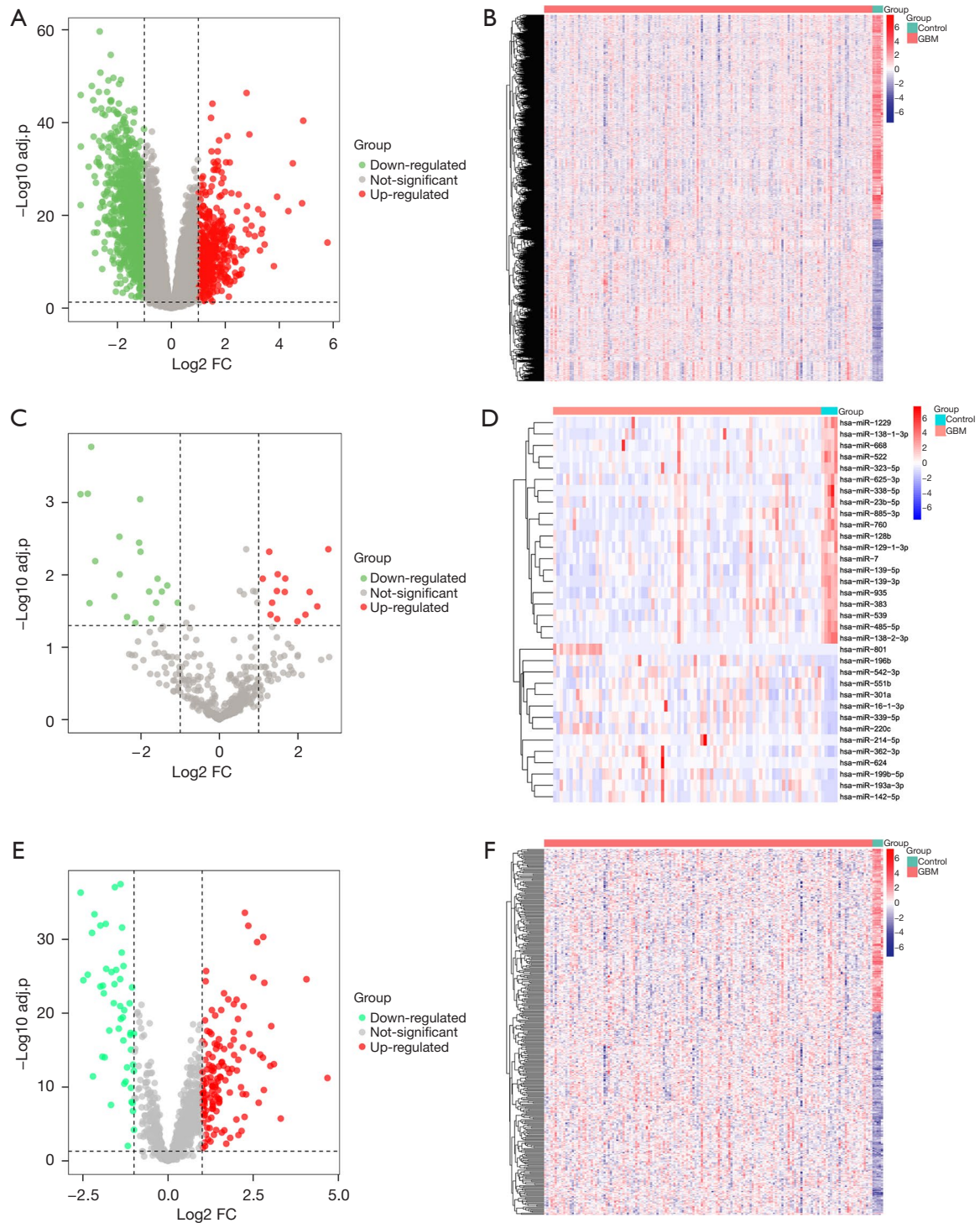


Figure 1 Identification of DE-mRNAs, DE-miRNA, DE-lncRNAs in GBM. (A,B) Volcano plot and heatmap of DE-mRNAs between GBM and control groups. (C,D) Volcano plot and heatmap of DE-miRNAs between the GBM and normal groups. (E,F) Volcano plot and heatmap of DE-lncRNAs between the GBM and normal groups. FC, fold change; DE-mRNA, differentially expressed mRNA; DE-lncRNA, differentially expressed lncRNA; GBM, glioblastoma.

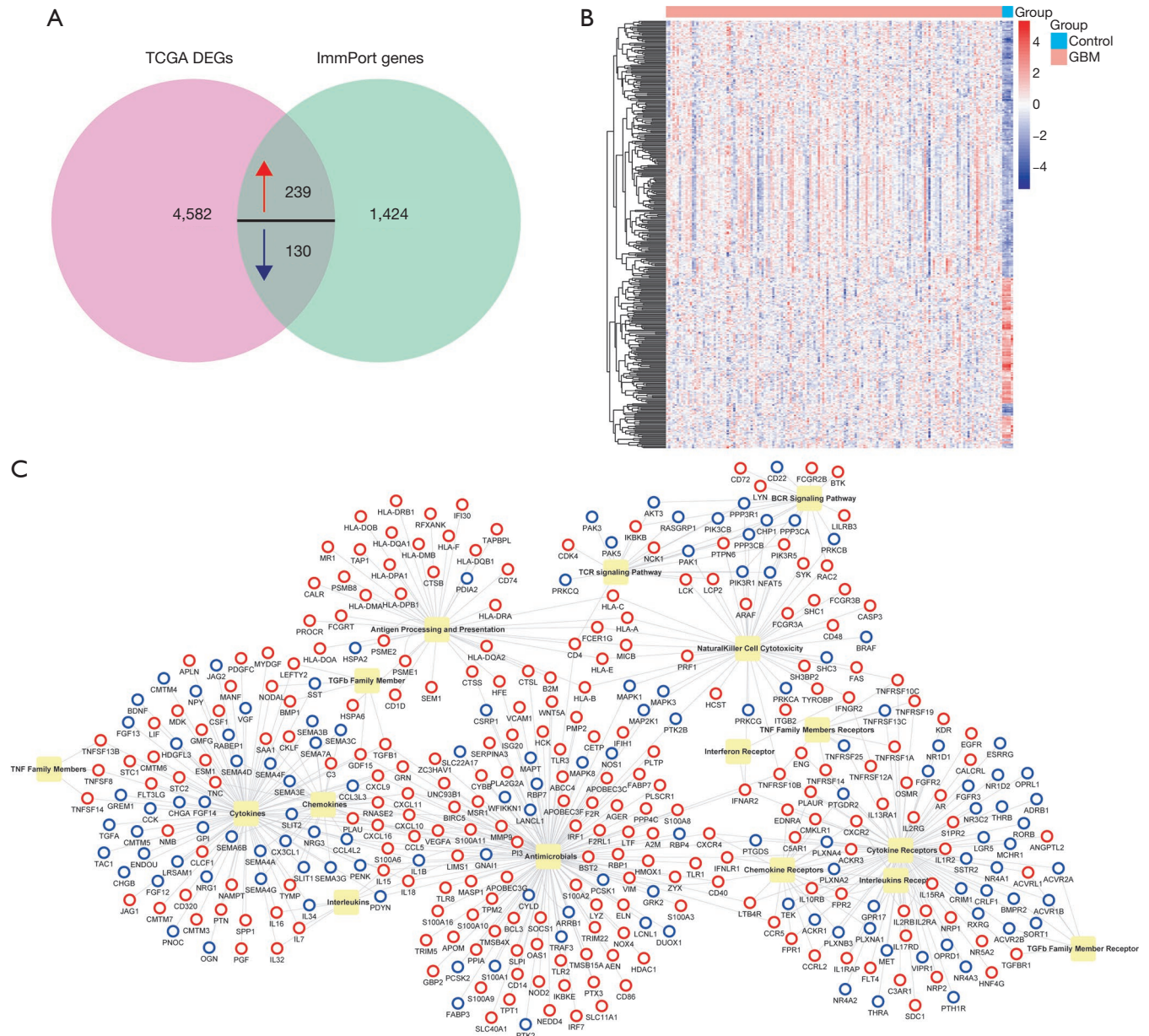


Figure 2 Identification of immune-related DE-mRNAs in GBM. (A) Venn analysis of DE-mRNAs in TCGA database and immune-related genes in the ImmPort database. (B) Heatmap of common immune-related DE-mRNAs. (C) Network diagram of 369 immune-related DE-mRNAs and immune pathways. Red circle represents upregulated mRNAs, blue represents downregulated mRNAs, and yellow squares represent immune-related pathway. TCGA, The Cancer Genome Atlas; DEG, differentially expressed genes; GBM, glioblastoma; DE-mRNA, differentially expressed mRNA.

showed that LNC01268 and CTB-31O20.2 inhibited migration capability in GBM U251 cells (Figure 5E,5F). These results indicate that LNC01268 and CTB-31O20.2 play a tumor-suppressing role in cell proliferation, migration, and invasion in GBM.

Discussion

Primary GBM is known for its aggressiveness and resistance to treatment, and because of its high recurrence, patients often die from the disease. Current treatment strategies

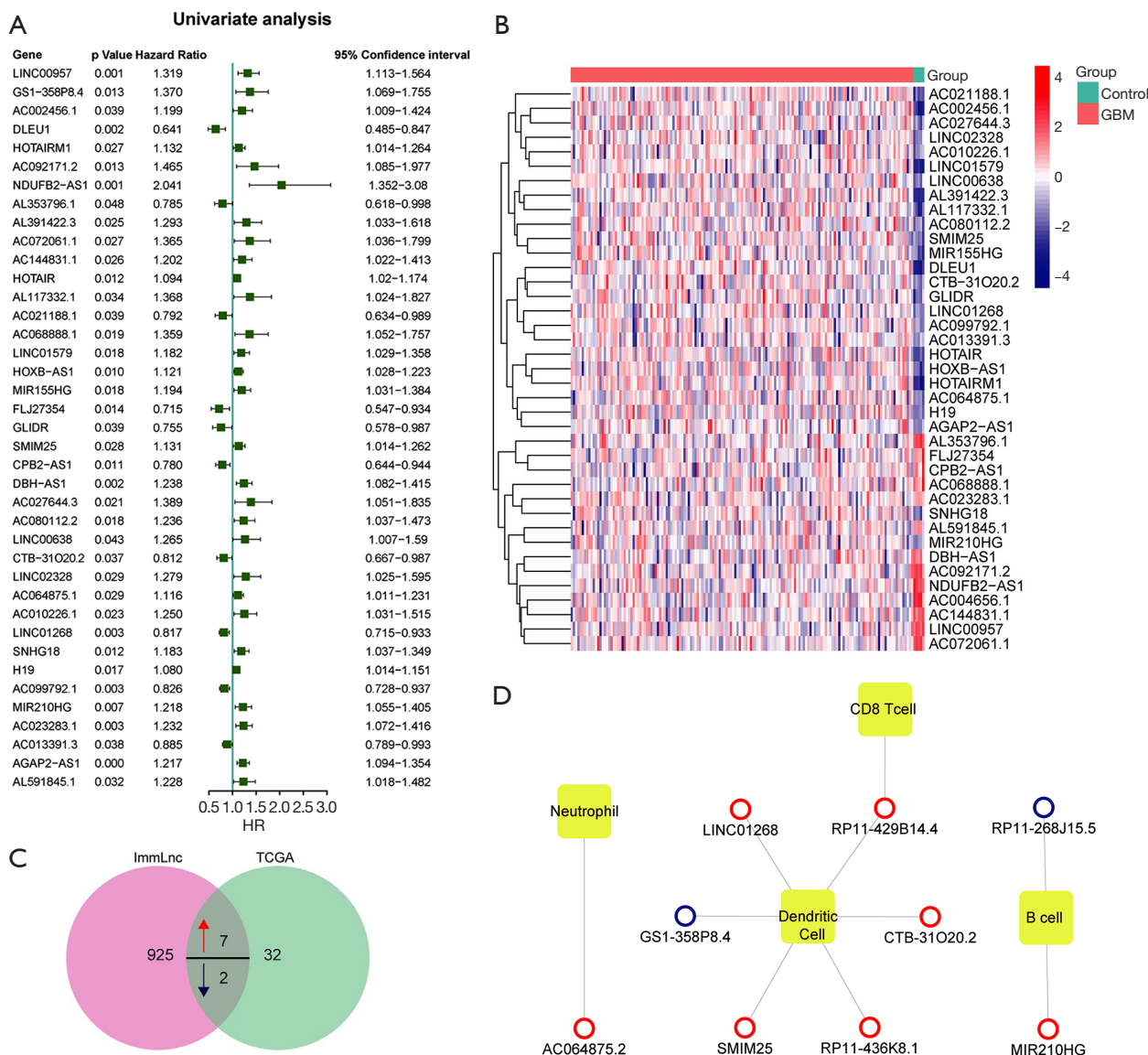


Figure 3 Identification of immune-related DE-lncRNAs with prognostic significance in GBM. (A) Univariate Cox regression analysis of risk score of 39 DE-lncRNAs. (B) Cluster analysis of 39 DE-lncRNAs with prognostic effect. (C) Venn analysis of 39 DE-lncRNAs in TCGA database and 925 immune-related lncRNAs in the ImmLnc database. (D) Network of immune-related lncRNAs. Red circle represents upregulated lncRNAs, blue represents downregulated lncRNAs, and yellow squares represent immune cells. DE-lncRNA, differentially expressed lncRNA; GBM, glioblastoma; TCGA, The Cancer Genome Atlas.

include surgery, with postoperative chemotherapy or radiotherapy. Also, many novel therapies have emerged with the development of medical technology, such as molecular targeted agents, immunotherapy, and nanotechnology (3,31). GBM alters major immunogenic signaling pathways and cellular immunity inside and outside the brain (8). Tumor-related immune responses and immunotherapy

have been used in the treatment of malignant cancer (32). With the advent of targeted therapy and immunotherapy, the discovery of new biomarkers is urgent. In addition, the advancement of high-throughput sequencing technology has made it possible for revealing the role of non-coding RNA, especially the study of specifically expressed genes as biomarkers for treatment, diagnostic and prognostic in

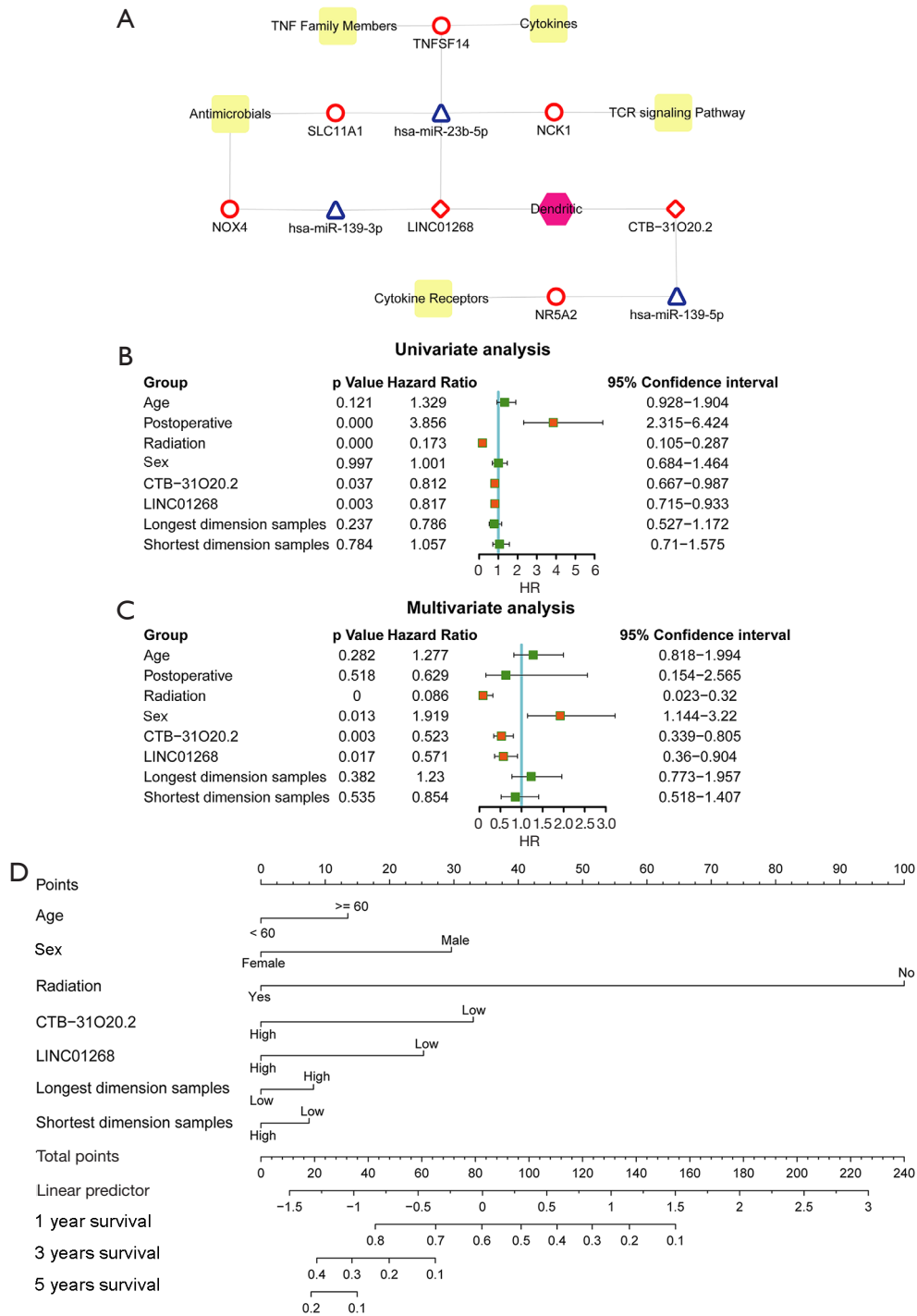


Figure 4 Identifying LINC01268 and CTB-31O20.2 as prognostic markers for GBM. (A) Construction of immune-related ceRNA network. Red circles represent upregulated mRNAs, red squares represent upregulated lncRNAs, blue triangles represent downregulated miRNAs, yellow squares represent immune-related pathways, and purple hexagons represent immune cells. (B,C) Univariate and multivariate Cox regression analyses of risk score and clinicopathological characteristics in GBM. (D) Nomogram integrating the signature risk score with the clinicopathological characteristics. GBM, glioblastoma; TCGA, The Cancer Genome Atlas; ceRNA, competitive endogenous RNA; mRNAs, messenger RNAs; lncRNAs, long non-coding RNAs.

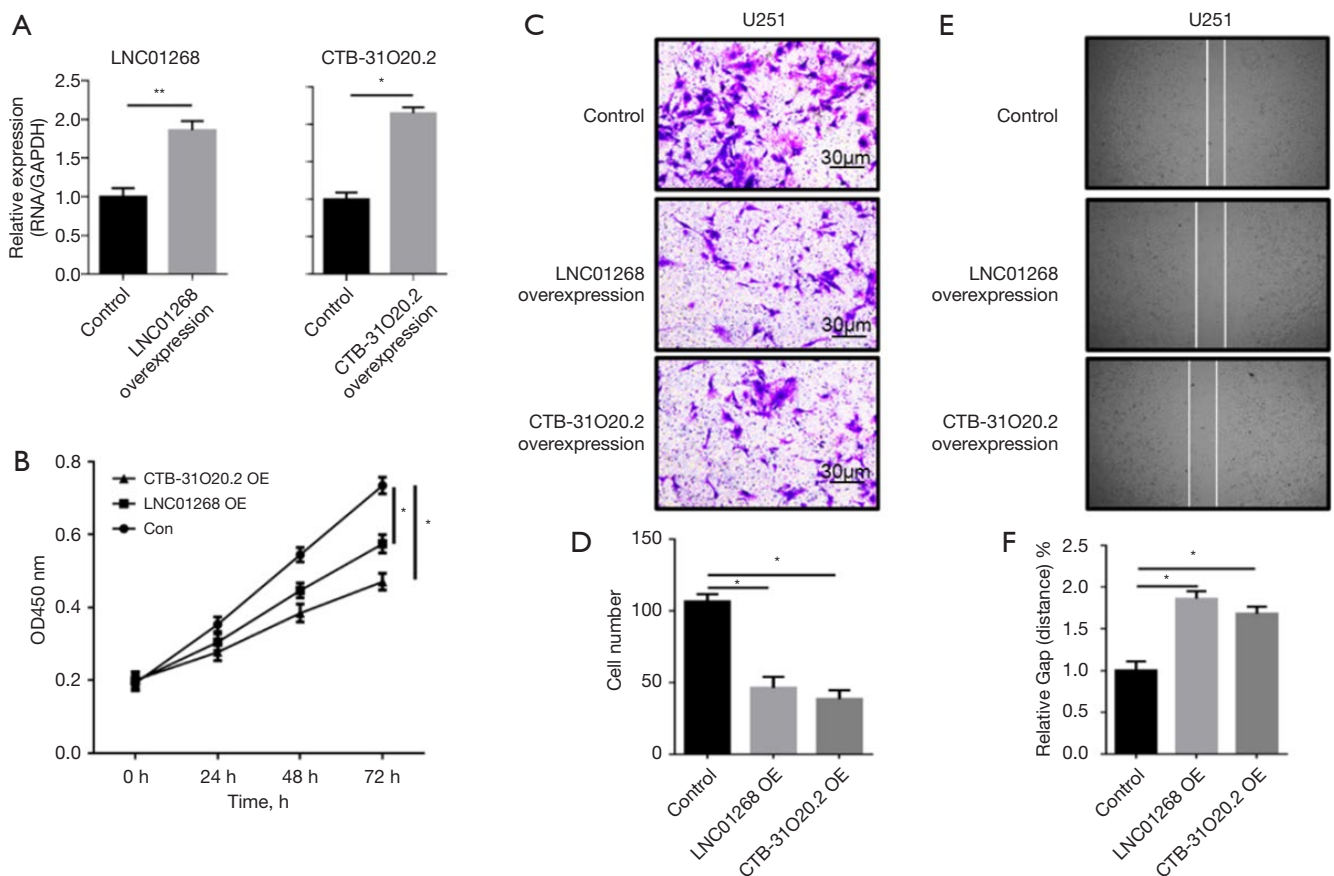


Figure 5 LNC01268 and CTB-31O20.2 overexpression inhibits glioblastoma cell proliferation, migration, and invasion. (A). LNC01268 and CTB-31O20.2 were overexpressed in U251 cells. (B) Cell Counting Kit-8 assay showed that LNC01268 and CTB-31O20.2 reduced U251 proliferation. (C,D). Transwell assay showed that LNC01268 and CTB-31O20.2 inhibited U251 invasion. Cells were stained with 0.1% crystal violet for 30 min. (E,F) Scratch wound assay showed that LNC01268 and CTB-31O20.2 inhibited U251 migration. U251 cells were seeded and cultured onto 6-well plates for 24 h. A pipette tip was then used to create wounds in then culture plate. The wound healing process was observed at 0 and 48 h under the microscope. All experiments were replicated 3 times. *, $P < 0.05$; **, $P < 0.01$.

tumor (33-35). However, there are still few biomarkers used for the diagnosis and prognosis in GBM, and this warrants further study.

Many studies have reported that lncRNAs not only regulate gene expression in some tumors but also can be used as biomarkers for diagnosis and prognosis. For example, Sun *et al.* found that LOXL1-AS1 up-regulates the expression of USF1 as a ceRNA via sponging miR-708-5p (36). LncRNA RPPH1 is significantly up-regulated and was associated with poor prognosis in colorectal cancer (37). LncRNA HOTTIP could mediate HOXA9 to enhance the Wnt/ β -catenin pathway by combining with WDR5 in pancreatic cancer stem cells, and the HOTTIP/WDR5/HOXA9/Wnt axis is expected to be potential therapeutic

target for pancreatic cancer (38). These results have given us a deeper understanding of lncRNAs, but are still not sufficient. In our study, we used the sequencing data of GBM and normal groups in TCGA database to obtain DE-lncRNAs, DE-mRNAs, and DE-miRNAs through gene differential expression analysis. These differentially expressed genes serve as the basis for subsequent research.

With increasing development in research on immune infiltration in the tumor microenvironment, immune-related genes have become the focus of attention. Guo *et al.* found 7 immune-related lncRNAs with prognostic significance in melanoma (39).

Wang *et al.* found 9 immune-related lncRNAs to have prognostic value for anaplastic gliomas (40). Zhou *et al.*

found that a 6-lncRNA signature could be an independent prognostic factor in GBM, with significantly different survival in high-risk and low-risk groups (41). Therefore, it is necessary to explore the characteristics of immune-related molecules and evaluate the function of immune genes in GBM, which does not only reveal the immune mechanism but also new therapeutic targets for GBM. In the present study, immune-related mRNAs were obtained from the ImmPort database and overlapped with DE-mRNAs; 369 immune-related genes were selected. Furthermore, we took the intersection of 925 in the ImmLnc database and 32 prognostic-value DE-lncRNAs and obtained 9 immune-related lncRNAs related to the prognosis. Similarly, we identified 34 DE-miRNAs; all the above data were used in the following analysis. LncRNA AC064875.2 shows potential prognostic biomarker and may regulate neutrophil infiltration in glioma (42). Similarly, MIR210HG was reported up-regulation upon hypoxia exposure in glioma cells, and was considered a resistance in GBM therapy (43,44). Transcriptome sequencing and qRT-PCR confirmed that lncRNA SMIM25 had high expression in cerebral cavernous malformations (45). LINC01268 could influence emotional regulation and was found to be involved in gene regulation of potential immune response (46). Study reported that high expressions of PDK3 (GS1-358P8.4) were poor prognostic factors of acute myeloid leukemia (47). Here, the role of RP11-429B14.4, CTB-31O20.2, RP11-436K8.1, and RP11-268J15.5 were not reported, and will be validated in the future.

The mechanism of lncRNAs as ceRNAs in GBM have been the focus of a number of studies (48,49). Therefore, we constructed an immune ceRNA network by predicting the relationship between lncRNA-miRNA and miRNA-mRNA. In this ceRNA network, 2 lncRNAs were enriched. LINC01268 could regulate the expression of SLC11A1, NCK1, TNFSF14, and NOX4 by adsorbing hsa-miR-23b-5p and hsa-miR-139-3p, thereby affecting immune-related responses. CTB-31O20.2 can regulate NR5A2 by adsorbing hsa-miR-139-5p to affect immune-related responses. To the best of our knowledge, LINC01268 and CTB-31O20.2 are novel lncRNAs that have never been reported in GBM. The univariate, multivariate, and nomogram analyses indicated that these 2 lncRNAs are prognostic markers for GBM. These results revealed the prognostic role of LINC01268 and CTB-31O20.2 in GBM via the immune ceRNA network, although challenges exist on the clinical applications of LINC01268 and CTB-31O20.2, they are helpful to deepen understanding of the underlying

mechanism in immune responses and GBM.

In conclusion, our study screened out 2 innovative molecules, LINC01268 and CTB-31O20.2, which play a crucial role in GBM and have the potential to predict the prognosis of GBM. LINC01268 and CTB-31O20.2 reduce cancer cell proliferation and metastasis in GBM. Our results provide new insight into the discovery of biomarkers for the prognosis of GBM. However, *in vivo* experiments need to be further verified.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-546/rc>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-546/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. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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