

LINC01268 and CTB-31020.2 as Prognostic and Functional Protective Immune Related lncRNAs in Glioblastoma

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Research Article

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Abstract

Glioblastoma (GBM) is the most common and deadly tumor in the central nervous system. Recent studies illuminated that long noncoding RNAs (lncRNAs) serve as competitive endogenous RNAs (ceRNAs) and play an important role in GBM by regulating immune responses. Here, GBM datasets from TCGA database were analyzed to obtain 356 significantly differentially expressed lncRNAs (DE-lncRNAs), 4951 DE-mRNAs, and 34 DE-miRNAs in GBM, respectively. For mRNAs, 369 DE-mRNAs were identified as immune-related genes in Immport database. For DE-lncRNAs, univariate analysis identified 39 DE-lncRNAs with prognostic significance, and 9 DE-lncRNAs are included in ImmLnc database. Then, combined analysis was conducted, by integrating 9 immune related DE-lncRNAs, 369 immune related DE-mRNAs and 34 DE-miRNAs, and generated a ceRNA network composed of 2 upregulated lncRNAs (LINC01268 and CTB-31020.2), 3 downregulated miRNAs and 5 upregulated mRNAs. Then we focused on LINC01268 and CTB-31020.2, and Kaplan-Meier survival, univariate and multivariate Cox regression analysis showed that LINC01268 and CTB-31020.2 serve as independent protective prognostic markers in GBM. Finally, LINC01268 and CTB-31020.2 overexpression was conducted in GBM cell U251. CCK8, transwell and scratch healing assay indicated that LINC01268 and CTB-31020.2 inhibits GBM cell line U251 proliferation, invasion and migration. To sum up, LINC01268 and CTB-31020.2 are independent prognostic immune related markers, and reduces cancer cell proliferation and metastasis in GBM.

1. Introduction

As one of the most feared type disease with poor prognosis, GBM accounts for 82% of primary malignant brain tumor and classified as grade IV tumors by the World Health Organization (WHO) [1]. Ample evidence suggested that the risk factors of GBM arising are cumulative genetic alterations and environment such as aging problem, family history, ionizing radiation, and virus [2, 3]. However, the occurring of most GBMs are accidental and the causes are still unclear. Though some novel therapeutic methods such as molecular targeted therapy, gene therapy, and antiangiogenic agent treatment have appeared [4, 5], the treatment of GBM remains a serious challenge. With the advent of advanced sequencing technologies, an abundant of gene expression data, distinct molecular, and immune-related genetic alterations have been revealed [6]. Through producing immunosuppressive factors and modulation of cell surface receptors and immune cell subsets, GBM impairs both local central nervous system (CNS) and systemic immune system functions [7]. Besides, GBM alters major immunogenic signaling pathways and altering 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.

Competitive endogenous RNA (ceRNA) hypothesis was first proposed by Salmena and revealed a new mechanism for RNA interaction [9]. CeRNA competitively combined to microRNA through microRNA response elements (MREs) to regulate gene expression. Long non-coding RNA (lncRNA) was one of ceRNA, which was an RNA molecule greater than 200 bases in length [10]. With the advancement of high-throughput sequencing technology and the progress of abundant researches on lncRNA, the potential

function of lncRNA has been revealed in many human disease [11–16]. Remarkably, a mass of RNA-Seq data helped identify a variety of biomarkers currently used for prognosis and treatment in cancer. For instance, Liang et al. identified prognostic six-lncRNA signature may improve prognosis prediction of GBM, including C20orf166-AS1, LINC00645, LBX2-AS1, LINC00565, LINC00641, and PRRT3-AS1 [17]. P73-AS1 demonstrated that it could promote temozolomide resistance in glioblastoma stem cells, and revealed that high TP73-AS1 as a biomarker had a poor prognosis in glioblastoma [18]. LncRNA with prognostic value in GBM deserves our attention

Many works on lncRNA as key regulator in complex immune response were emerging, which illustrated that lncRNA plays an important role in immune system [19, 20]. Recently, the analysis of transcriptome identified many differentially expressed lncRNAs (DE-lncRNAs), which were involved in regulating the immune response. Such as SNHG14/miR-5590-3p/ZEB1 was pointed out that the positive feedback promoted large B cell lymphoma progression and immune evasion by regulating PD-1/PD-L1 checkpoints [21]. Moreover, lncRNA Sros1 accelerated IFN- γ -mediated activation of immune responses by stabilizing Stat1 [22]. With increasingly updated research, immune-related lncRNAs have been revealed in many type cancers. For example, OSTN-AS1 was an immune-related molecule and had a potential function of immunotherapy in triple-negative breast cancer [23]. The effect of lncRNA in immune regulation was complicated, and until now plenty of immune-related lncRNAs with prognostic role have not reported in GBM. Therefore, we focus on the prognostic role of immune-related lncRNA in GBM.

Here, we identified DE-lncRNAs, DE-mRNAs, and DE-miRNAs based on cohort study of GBM patients from TCGA database. Combining ImmLnc and ImmPort databases, we focus on the immune-related lncRNA and constructed an immune-associated ceRNA network. Then we identified the prognostic value of immune-related lncRNA in the ceRNA network. Our research identified 2 immune-related lncRNAs for prognosis of GBM. Finally, the function of LINC01268 and CTB-31O20.2 in GBM cell line U251 was investigated.

2. Materials And Methods

2.1 Data collection and preprocessing

The transcriptome data (include lncRNA, mRNA, and miRNA) and clinical data of GBM patients were downloaded from the Genomic Data Commons (GDC) data portal provided by TCGA database. A total of 155 GBM and 5 control samples were obtained. Then, 155 GBM patients were divided into high expression group and low expression group according to the 9 lncRNAs with prognostic value. The differentially expressed lncRNAs, mRNAs, and miRNAs between GBM and normal samples were analyzed by edgeR package of R, drawing volcano plot and heatmap by R script. The fold change > 2 and FDR < 0.05 were used for cutoff value.

2.2 Identification of immune-related DE-lncRNAs

ImmLnc database is a database developed by Yongsheng Li et al., which chart the landscape of immune-related lncRNA regulation in 33 cancer types and can be used to identify potential carcinogenic biomarkers [24]. A total of 934 immune-related lncRNAs of GBM from ImmLnc database were downloaded as shown in **Table 1**, which used to recognize the potential function of immune-related DE-lncRNAs in GBM.

2.3 Identification of immune-related DE-mRNAs

ImmPort database contains a large number of immune-related genes and includes 17 immune-related pathways according to different molecular function [25]. Such as antimicrobials, antigen processing, presentation, BCR signaling, chemokine receptors, natural killer cell cytotoxicity, TNF family members, TGF β family members, TCR signaling pathway. 1,424 immune-related genes were downloaded from the ImmPort database, which was identified immune-related DE-mRNAs in GBM.

2.4 Construction a ceRNA network

Database of LncBase [26] and miRDB [27] were used to predict corresponding target lncRNAs and mRNAs of miRNAs, respectively. Only immune-related lncRNAs and mRNAs that co-target miRNAs were eligible for the construction of 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.

2.5 Cell Culture

The human glioblastoma cell line U-251 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 (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). All cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

2.6 LncRNA LNC01268 and CTB-31O20.2 overexpression

Full length of LNC01268 and CTB-31O20.2 was reverse transcribed into cDNA, PCR-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, the United States) in accordance with the kit instructions. To detect the efficiency of transfection, the expression of LNC01268 and CTB-31O20.2 was verified by qRT-PCR.

2.7 Cell Proliferation Assays

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

2.8 Scratch Wound Assay

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

2.9 Transwell Assay

Transwell assay were used to investigate the invasion ability of U251 cells. U251 cells were plated in the upper side of a Transwell chamber (Costar, USA), and 20% FBS medium was added into the lower compartment. After cultured and washed twice with PBS, the chamber was fixed with methanol for 30 min, stained with 0.1% crystal violet. The cells were counted using a microscope.

2.10 Statistical analysis

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

3. Results

3.1 Identification of DE-mRNAs, DE-miRNA, DE-IncRNAs in GBM.

The impact of DE coding genes is often the most intuitive and basic change of molecular, because many coding genes have an important function in human diseases. In order to intuitively clarify the expression changes of mRNA, we used gene expression profile of 5 normal samples and 155 GBM samples from TCGA database for identifying the DE-IncRNAs. As shown in Fig. 1A, clustering of 4951 DE-mRNAs of high or low expression level were exhibited and the Fig. 1B showed distinct cluster distribution of these DE-mRNAs in normal and GBM samples. To construct immune-related ceRNA network, 34 DE-miRNAs in normal and GBM groups were also identified ($FC > 2$, $FDR < 0.05$, Fig. 1C). The results of heatmap demonstrated that these DE-miRNAs were hierarchical clustering in each group (Fig. 1D). Furthermore, 356 DE-IncRNAs were selected ($FC > 2$, $FDR < 0.05$, Fig. 1E-F).

3.2 Identification of immune-related pathways in GBM.

To explore the immune-related pathways in GBM, we obtained 369 immune DE-mRNAs by overlapping the 4951 DE-mRNAs and immune-related mRNAs of ImmPort database. As shown in Fig. 2A, 239 upregulated DE-mRNAs and 130 downregulated DE-mRNAs were identified. Figure 2B showed 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 in all. They were cytokines, chemokines, chemokine receptors, cytokine receptors, and interleukins receptors, etc. as shown in Fig. 2C.

3.3 Identification of immune-related DE-IncRNAs with prognostic significance in GBM

Next, we found that 39 DE-IncRNAs can be used as independent factors which related to prognosis by univariate analysis, and the general description was showed in Fig. 3A. Then, the cluster heatmap of these 39 DE-IncRNAs in the normal group and the tumor group was shown in Fig. 3B. Considering that the expression of IncRNA was related to immune cell infiltration in tumors, we took the intersection of common genes by used the IncRNA and 39 DE-IncRNAs related to glioblastoma in the ImmLnc database Fig. 3C, and constructed a network of IncRNA-immune cell as shown in Fig. 3D. In the immune cell-related IncRNA network, a total of 7 DE-IncRNAs was up-regulation, 2 DE-IncRNAs was down-regulation.

3.4 Construction of immune-related ceRNA networks

To construct immune-related ceRNA network, database of LncBase and miRDB were used to predict corresponding target IncRNAs and mRNAs of DE-miRNAs, respectively. Only immune-related IncRNAs and mRNAs that co-target miRNAs were eligible for the construction of ceRNA networks. Searching the LncBase database and miRDB database to predict the targeting relationship of IncRNA-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 Fig. 4A. This network contained 5 immune-related pathways, which were TNF family members, cytokines, antimicrobials, TCR signaling pathway, and cytokine receptors. And included 5 DE-mRNAs that they were TNFSF14, SLC11A1, NCK1, NOX4, and NR5A2. Furthermore, LINC01268 and CTB-31020.2 were obtained from network, has-miR-23b-5p and has-miR-139-3p were also acquired.

3.5 Identifying LNC01268 and CTB-31020.2 as immune-related prognostic markers for GBM

To further determine whether the CTB-31020.2 and LINC01268 could be acted an independent predictor of GBM patients, we carried out Cox regression analysis and multivariate. the univariate Cox regression analysis demonstrated LINC01268 and CTB-31020.2 expression level were associated with the overall survival (OS) in the Fig. 4B. The multivariate analysis showed Gender, CTB-31020.2, and LINC01268 were significantly associated with the OS in the Fig. 4C. Subsequently, a nomogram was constructed to project the 1-year, 3-year, and 5-year OS of GBM patients as shown in Fig. 4D.

3.6 LNC01268 and CTB-31020 inhibit cell proliferation, migration and invasion in glioblastoma

To study the function of LNC01268 and CTB-31020.2 in glioblastoma, LNC01268 and CTB-31020.2 was overexpressed in U251. qRT-PCR result showed that LNC01268 and CTB-31020.2 was significantly elevated (Fig. 5A). Then CCK8 assay was conducted, and LNC01268 and CTB-31020.2 significantly reduced U251 cells proliferation (Fig. 5B). The effect of LNC01268 and CTB-31020.2 on the migratory and invasive abilities of glioblastoma cells were determined using transwell and scratch wound assay,

respectively. Transwell invasion assay results indicated that LNC01268 and CTB-31020.2 overexpression reduced in glioblastoma cells invasion (Fig. 5C-5D). Similarly, scratch wound assay result showed that LNC01268 and CTB-31020.2 inhibited the migrating capability in glioblastoma U251 cells (Fig. 5E-5F). These results indicate that LNC01268 and CTB-31020.2 plays an tumor suppressing role in cell proliferation, migration and invasion in glioblastoma.

4. Discussion

Over the past decade, primary GBM is known for its aggressiveness and resistance to treatment, and because of its high recurrence, patients often die from the disease. Many novel therapies have emerged with the development of medical technology, such as surgery, chemotherapy, radiotherapy, molecular targeted agents, immunotherapy and nanotechnology [3, 28]. As we known, GBM alters major immunogenic signaling pathways and altering cellular immunity inside and outside the brain [8]. Tumor-related immune responses and immunotherapy have been used in the treatment of malignant cancer [29]. With the advent of targeted therapy and immunotherapy, the discovery of new biomarkers is urgent. In addition, the advancement of high-throughput sequencing technology made it possible for more and more function of non-coding RNA to be explored. Especially the study of some specifically expressed genes as a biomarker for treatment, diagnostic and prognostic in all kinds of tumors [30–32]. However, there are still few biomarkers used for diagnosis and prognosis in GBM, and further needed to explore.

Many researches 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, Qi Sun etc. found that LOXL1-AS1 upregulate the expression of USF1 as a ceRNA via sponging miR-708-5p [33]. LncRNA RPPH1 was significantly upregulated and was associated with poor prognosis in colorectal cancer [34]. LncRNA HOTTIP could mediate HOXA9 to enhance the Wnt/β-catenin pathway by combining with WDR5 in pancreatic cancer stem cells, HOTTIP/WDR5/HOXA9/Wnt axis was expected to be potential therapeutic targets for pancreatic cancer [35]. These results have given us a newer understanding of lncRNA, but they are not sufficient. In our study, we used the sequencing data of GBM and normal groups in the TCGA database to obtain DE-lncRNAs, DE-mRNAs, and DE-miRNAs through gene differential expression analysis. These DEGs serve as the basis for subsequent research.

Recently, with the increasing development of research on immune infiltration in the tumor microenvironment, immune-related genes have immediately become the focus of attention. Just as, 63 immune-related genes were founded associated with overall survival of melanoma and showed a powerful predictive ability in study of Rongzhi Huang etc. [36]. Wen Wang etc. revealed 9 immune-related lncRNAs have prognostic value for anaplastic gliomas [37]. Meng Zhou etc. verified that six-lncRNA signature could be an independent prognostic factor in GBM multiforme, with significantly different survival in high-risk and low-risk groups [38]. Therefore, it is necessary to explore the characteristics of immune-related molecules and evaluate the function of immune genes in GBM, which not only revealing the immune mechanism but also finding new therapeutic targets in GBM. In this study, immune-related mRNAs were obtained from ImmPort database and overlap 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. An article was illustrated AC064875.2 as potential prognostic biomarker may regulate neutrophil infiltration in glioma [39]. MIR210HG was reported up-regulation upon hypoxia exposure in glioma cells, and was considered a resistance in GBM therapy [40, 41]. Transcriptome sequencing and qRT-PCR confirmed that lncRNA SMIM25 was high expression in cerebral cavernous malformations on the before study [42]. LINC01268 could influence the emotional regulation, and was involved in gene regulation of potential immune response [43]. The alias of GS1-358P8.4 is PDK3, the study reported that high expressions of PDK3 were poor prognostic factors of acute myeloid leukemia [44]. Besides, RP11-429B14.4, CTB-31O20.2, RP11-436K8.1, and RP11-268J15.5 have not been reported, and further needs to explore in the future.

The mechanism of lncRNAs as ceRNAs in GBM have been attracted many researchers [45, 46]. Therefore, we constructed an immune ceRNA network by predicting the targeting relationship between lncRNA-miRNA and miRNA-mRNA. In this ceRNA network, two 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 related immune responses. CTB-31O20.2 can regulate NR5A2 by adsorbing hsa-miR-139-5p to affect related immune responses. Otherwise, LINC01268 and CTB-31O20.2 could directly affect dendritic cell in the GBM. CTB-31O20.2 is a novel lncRNA that haven't been reported. LINC01268 and CTB-31O20.2 are novel lncRNA that have never been reported in GBM. Besides, the univariate, multivariate, and nomogram analysis indicated that these two lncRNAs are prognostic markers for GBM. The results above revealed the prognostic role of LINC01268 and CTB-31O20.2 in GBM via immune ceRNA network, which are helpful to have a better understanding of the underlying mechanism in immune responses and GBM.

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

Declarations

Ethics approval and consent to participate:

All participants are consent to this study.

Consent for publication:

All authors have read and agreed to publish the manuscript.

Availability of data and materials:

All the data will be provided by reasonable request from the corresponding author.

Competing interests:

The authors declare that they have no conflicts of interest.

Funding:

None.

Authors' contributions:

Dong-Hui Liu conceived the idea, conducted cell experiment, and write the manuscript. Xiu-Yang, Han-Meng, and Gui-Yun Zhang collected and performed analysis the data. Shang-Hang Shen provided idea and modify of the article. All authors read and approved the final manuscript.

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Authors' information (optional):

None.

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Supplemental

Table S1 is not available with this version.

Figures

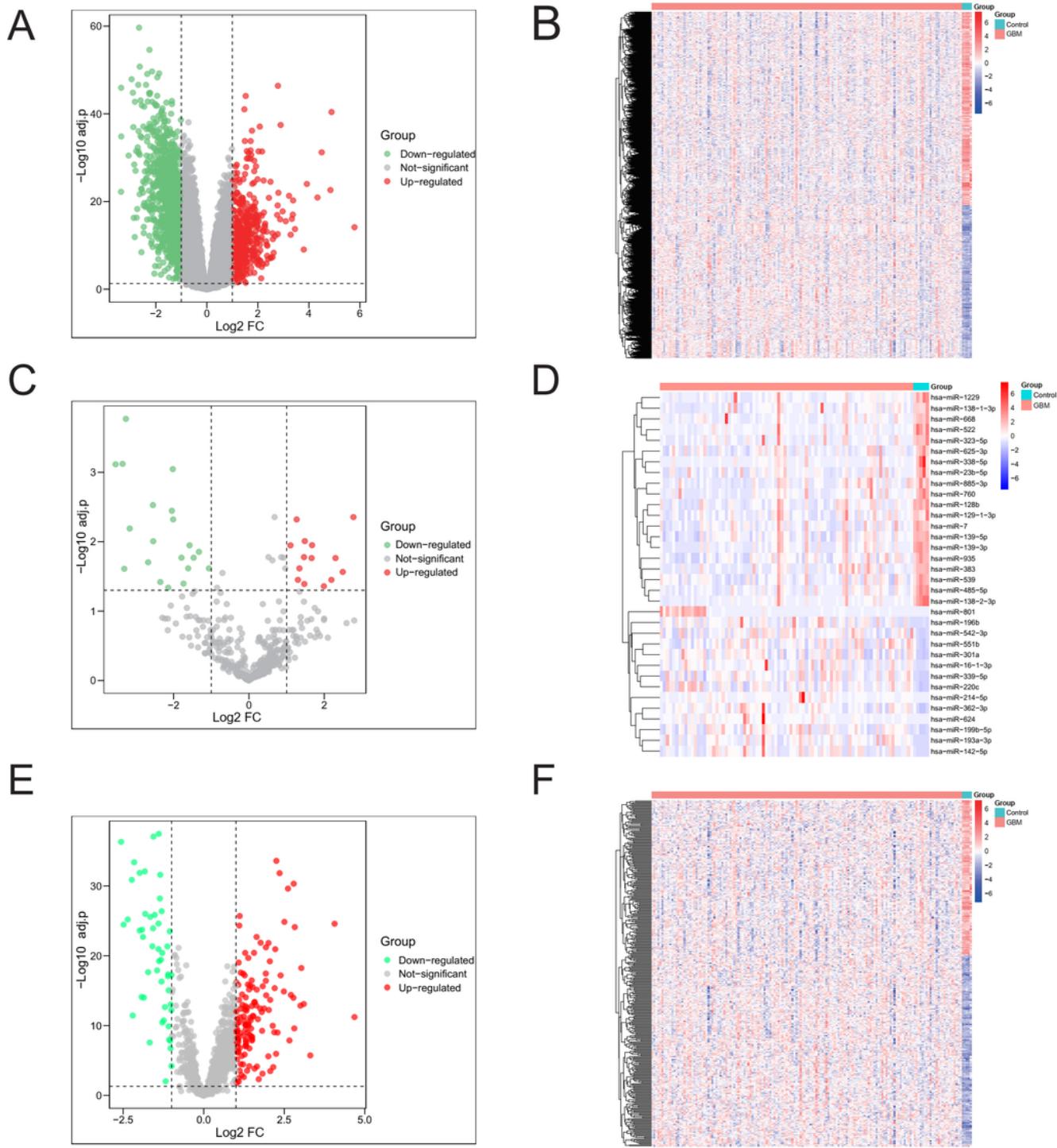
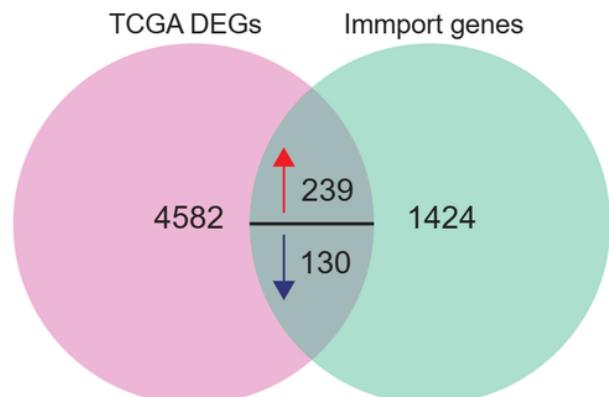


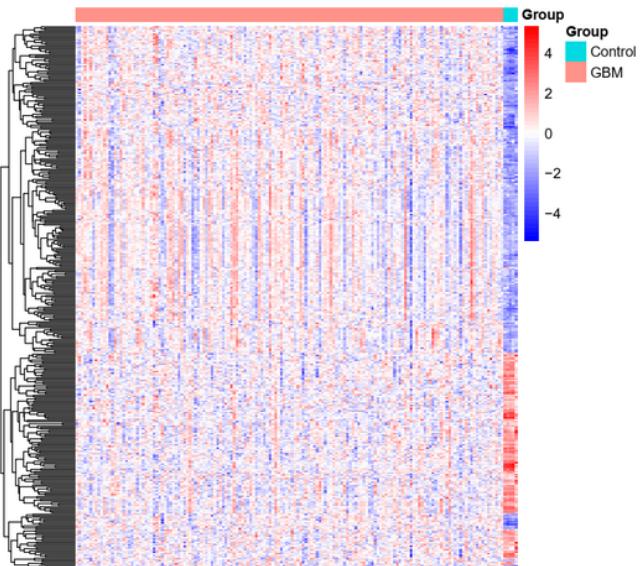
Figure 1

Identification of DE-mRNAs, DE-miRNA, DE-lncRNAs in GBM. (A-B) Volcano plot and heatmap of DE-mRNAs between glioblastoma and normal group. (C-D) Volcano plot and heatmap of DE-miRNAs between glioblastoma and normal group. (E-F) Volcano plot and heatmap of DE-lncRNAs between glioblastoma and normal group.

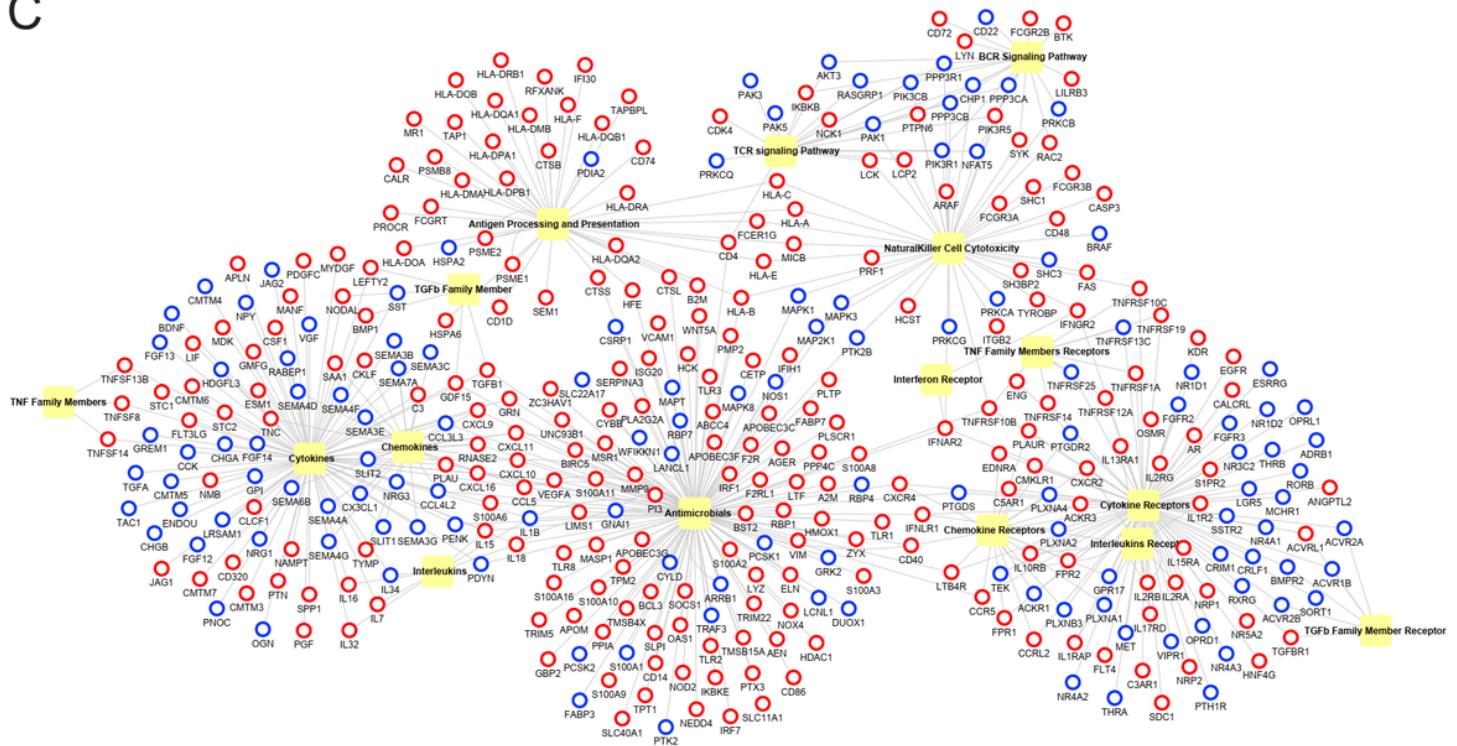
A



B



C

**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) The heatmap of common immune-related DE-mRNAs. (C) Network diagram of 369 immune-related DE-mRNAs and immune pathways. Red circle represents upregulated mRNAs, and blue represents downregulated, yellow squares represent immune-related pathway.

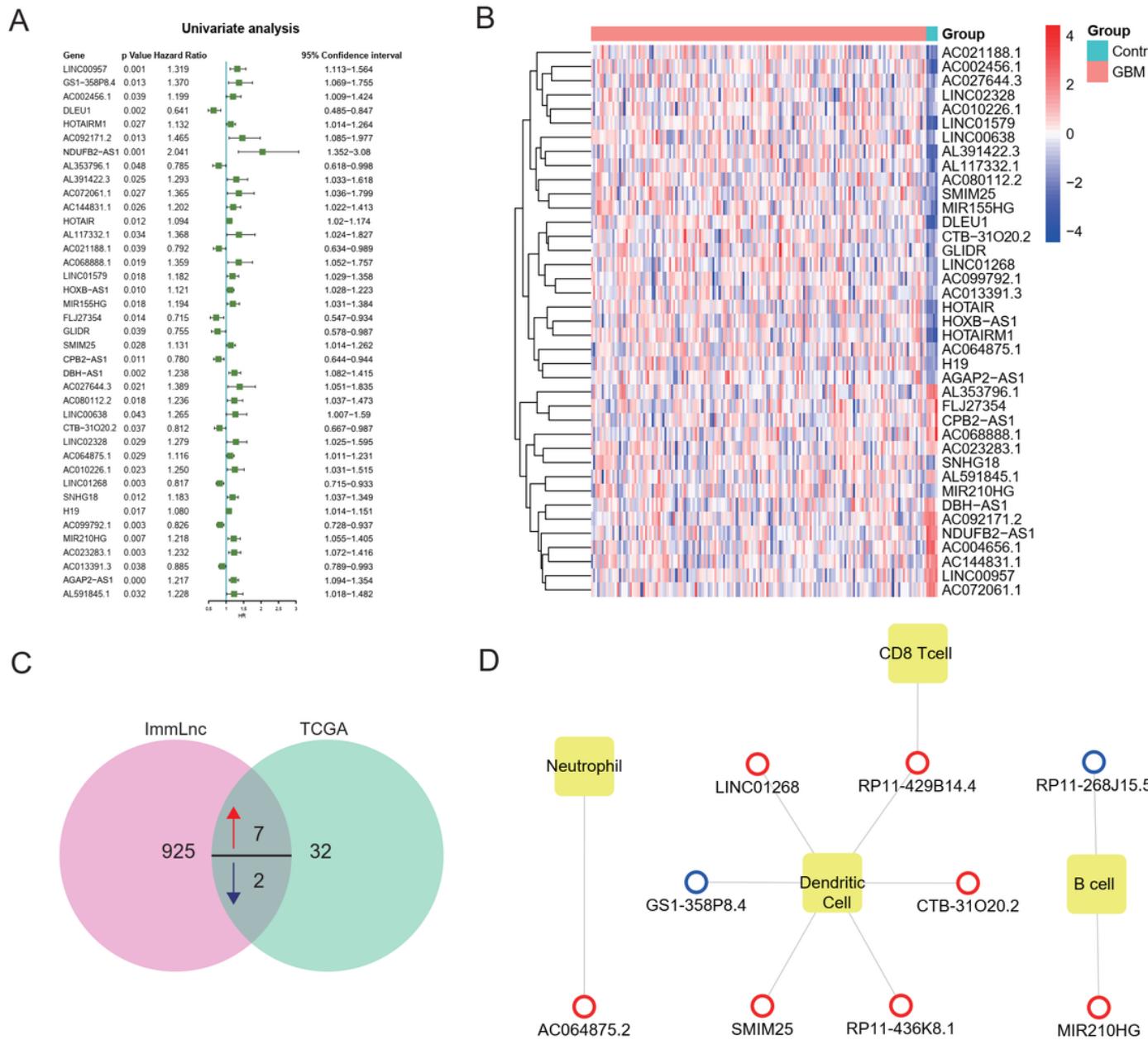
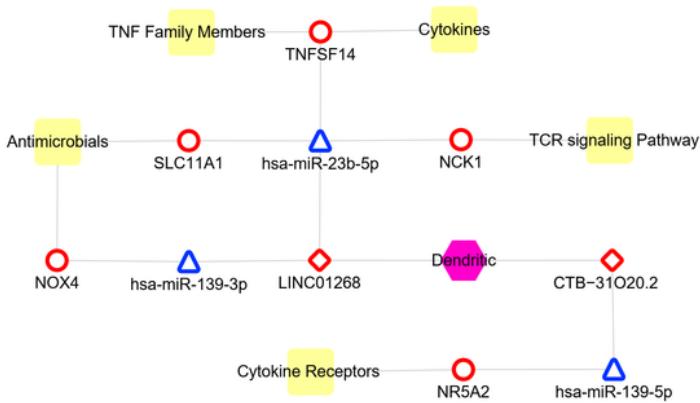


Figure 3

Identification of immune-related DE-lncRNAs with prognostic significance in GBM (A) Univariate Cox regression analyses 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 ImmLnc database. (D) The network of immune-related lncRNAs. The red circle represents up-regulated lncRNAs, and the blue represents down-regulated. The yellow squares represent immune cells.

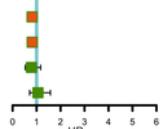
A



B

Univariate analysis

Group	p Value	Hazard Ratio	95% Confidence interval
Age	0.121	1.329	0.928–1.904
Postoperative	0.000	3.856	2.315–6.424
Radiation	0.000	0.173	0.105–0.287
Gender	0.997	1.001	0.684–1.464
CTB-31O20.2	0.037	0.812	0.667–0.987
LINC01268	0.003	0.817	0.715–0.933
Longest dimension samples	0.237	0.786	0.527–1.172
Shortest dimension samples	0.784	1.057	0.71–1.575



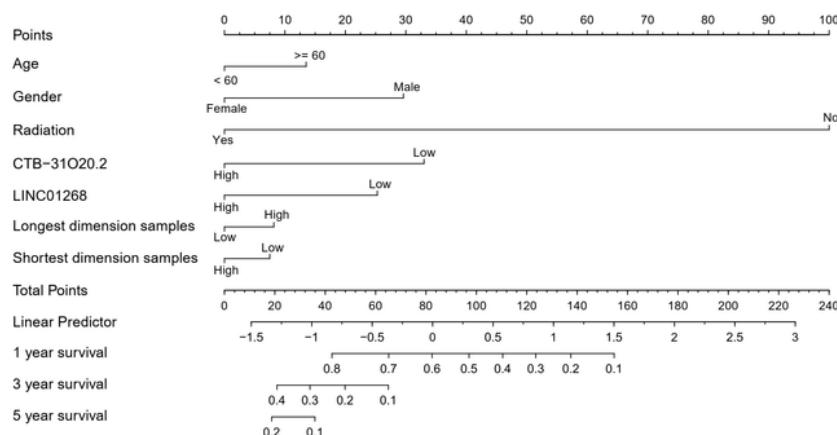
C

Multivariate analysis

Group	p Value	Hazard Ratio	95% Confidence interval
Age	0.282	1.277	0.818–1.994
Postoperative	0.518	0.629	0.154–2.565
Radiation	0	0.086	0.023–0.32
Gender	0.013	1.919	1.144–3.22
CTB-31O20.2	0.003	0.523	0.339–0.805
LINC01268	0.017	0.571	0.36–0.904
Longest dimension samples	0.382	1.23	0.773–1.957
Shortest dimension samples	0.535	0.854	0.518–1.407



D

**Figure 4**

Identifying INC01268 and CTB-31O20.2 as prognostic markers for glioblastoma (A) Construction of immune-related CeRNA network. Red circle represents upregulated mRNAs, red square represents upregulated lncRNAs, blue triangle represents downregulated miRNAs, yellow square represents immune-related pathways, and purple hexagon represent immune cell. (B-C) Univariate and multivariate Cox

regression analyses of risk score and clinicopathological characteristics in glioblastoma. (D) A nomogram integrating the signature risk score with the clinicopathological characteristics.

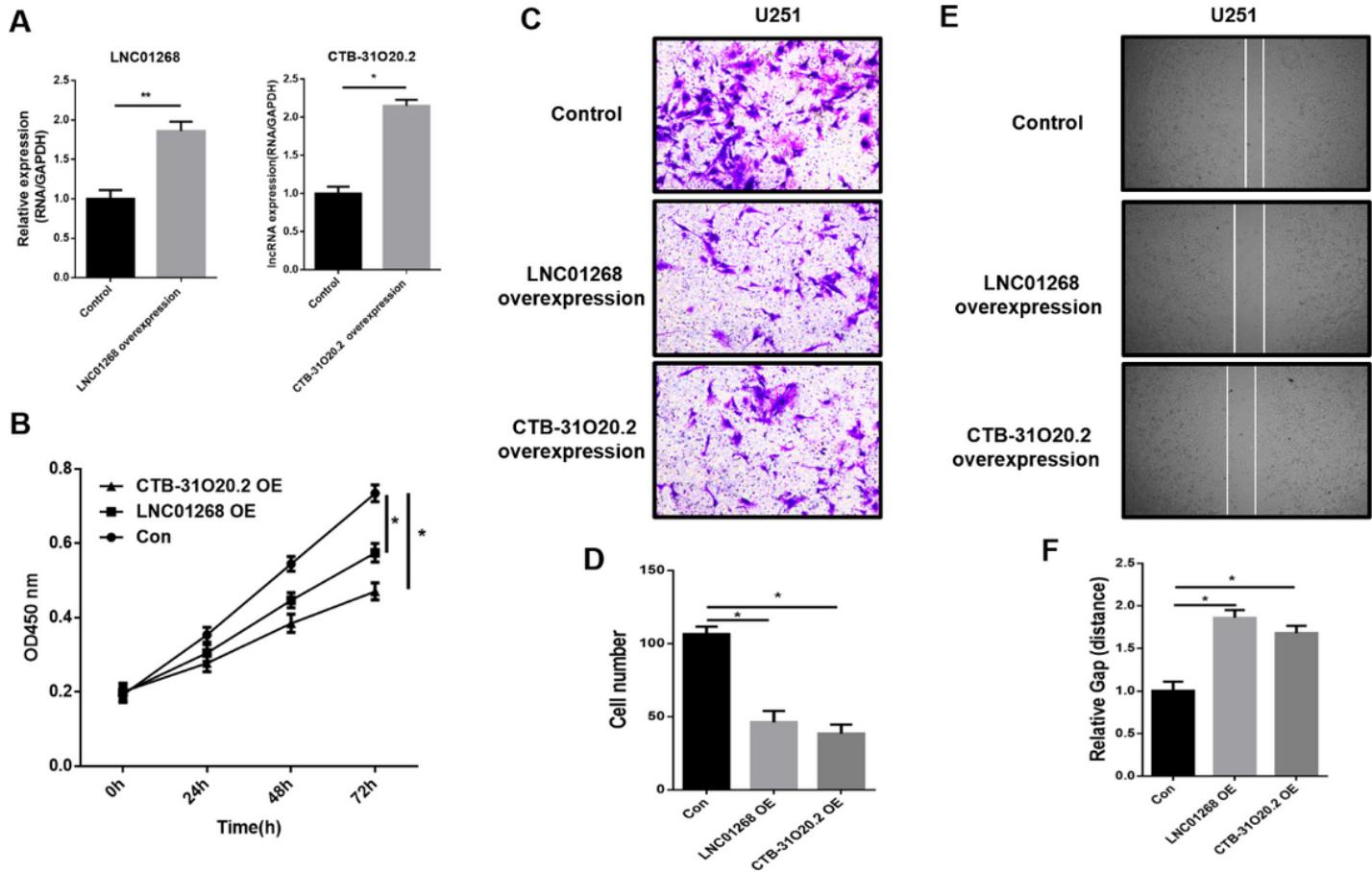


Figure 5

LNC01268 and CTB-31O20.2 overexpression inhibits glioblastoma cell proliferation, migration and invasion. (A). LNC01268 and CTB-31O20.2 was overexpressed in U251 cells; (B). CCK8 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; (E-F). Scratch wound assay showed that LNC01268 and CTB-31O20.2 inhibited U251 migration; All experiments were replicated three times; *, P<0.05; **, P<0.01;