An immune-related long non-coding RNA signature predicts prognosis in glioblastoma patients

Type
Research paper

Keywords
prognosis, glioblastoma, immune, long non-coding RNA

Abstract
Introduction
This study aimed to explore the prognostic value of immune-related long non-coding RNAs (lncRNAs) in glioblastoma (GBM).

Material and methods
Expression and clinical data were acquired, including GSE111260 dataset: 67 GBM and 3 normal brain samples; GSE103227 dataset: 5 GBM and 5 normal brain samples; and TCGA data: 187 GBM samples. Immune-related genes were retrieved from ImmPort database. Immune-related differentially expressed genes (DEGs) and IncRNAs were screened. Prognostic IncRNAs were then screened to establish prognostic risk score model. Survival analysis and differential expression analysis were performed in high- vs. low-risk groups, followed by protein-protein interaction network and IncRNA-mRNA co-expression network.

Results
A total of 251 immune-related DEGs were screened. After correlation analysis, 387 immune-related IncRNAs that co-expressed with 140 immune-related DEGs were screened. Univariate analysis identified 18 IncRNAs that were significantly associated with prognosis. The prognostic risk score could be able to stratify GBM patients into high- and low-risk groups, and patients with high risk scores displayed worse outcomes than those with low risk scores in both training set and validation set. A total of 272 genes had abnormal expression between high- and low-risk groups. Of which, 22 genes were immune-related, such as SNAP25, SNAP91, SNCB, and RAB3A. These genes were mainly enriched in synaptic vesicle cycle/exocytosis and insulin secretion. The co-expression network contained 22 genes and 11 IncRNAs, and IncRNA LINC01574 co-expressed with the great number of mRNAs.

Conclusions
We identified 18 immune-related prognostic IncRNAs, and the established IncRNAs-based prognostic risk model could stratify GBM patients into different risks.
An immune-related long non-coding RNA signature predicts prognosis in glioblastoma patients

**Running title:** Prognostic IncRNAs in GBM

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Highlights:

1. We identified 18 immune-related lncRNAs that were associated with survival of GBM patients.
2. The 18 lncRNA signature-based prognostic model could stratify patients into high- and low-risk groups.
3. Patients with high risk scores had worse outcomes than those with low risk scores.
4. DEGs in high- vs. low-risk groups were involved in synaptic vesicle-related pathways.
Abstract

Background: This study aimed to explore the prognostic value of immune-related long non-coding RNAs (lncRNAs) in glioblastoma (GBM).

Methods: Expression and clinical data were acquired, including GSE111260 dataset: 67 GBM and 3 normal brain samples; GSE103227 dataset: 5 GBM and 5 normal brain samples; and TCGA data: 187 GBM samples. Immune-related genes were retrieved from ImmPort database. Immune-related differentially expressed genes (DEGs) and lncRNAs were screened. Prognostic lncRNAs were then screened to establish prognostic risk score model. Survival analysis and differential expression analysis were performed in high- vs. low-risk groups, followed by protein-protein interaction network and lncRNA-mRNA co-expression network.

Results: A total of 251 immune-related DEGs were screened. After correlation analysis, 387 immune-related lncRNAs that co-expressed with 140 immune-related DEGs were screened. Univariate analysis identified 18 lncRNAs that were significantly associated with prognosis. The prognostic risk score could be able to stratify GBM patients into high- and low-risk groups, and patients with high risk scores displayed worse outcomes than those with low risk scores in both training set and validation set. A total of 272 genes had abnormal expression between high- and low-risk groups. Of which, 22 genes were immune-related, such as SNAP25, SNAP91, SNCB, and RAB3A. These genes were mainly enriched in synaptic vesicle cycle/exocytosis and insulin secretion. The co-expression network contained 22 genes and 11 lncRNAs, and lncRNA LINC01574 co-expressed with the great number of
mRNAs.

**Conclusion:** We identified 18 immune-related prognostic lncRNAs, and the established lncRNAs-based prognostic risk model could stratify GBM patients into different risks.

**Keywords:** glioblastoma, long non-coding RNA, immune, prognosis
Background

Glioma is the most common malignant tumor of the central nervous system (CNS). As the most malignant pathological type of glioma, glioblastoma (GBM) has strong aggressiveness and high recurrence rate, accounting for approximately 52% of gliomas [1]. At present, the standard treatment for GBM is still surgical resection supplemented by radiotherapy and chemotherapy [2]. However, it is difficult to completely excise the tumor due to the invasive growth of GBM tumor cells and the restriction of tumor site. Moreover, chemoradiotherapy can not specifically identify tumor cells and may result in drug resistance. Therefore, the prognosis of GBM is still poor with a overall median survival of 15 months and 5-year survival rate less than 10% [3]. The CNS has long been known to be a immunological privileged region [4]. However, in recent years, a large number of studies have shown that the immune system in the brain is in a high level of activation and can interact with brain tumors [5]. Therefore, it is very important to investigate the immune landscape for the treatment of GBM.

Immune cells represent a main population of non-neoplastic cells in tumor microenvironment of GBM, and different GBM subtypes show differences in their microenvironment [6]. Identification of molecular markers has been demonstrated to effectively distinguish tumor subtypes, degree of malignancy, and response to chemoradiotherapy [7]. For example, Zhou et al. indicated that high expression of ribonucleotide reductase subunit M2 (RRM2) was associated with poor survival of patients with GBM, suggesting that RRM2 could be a prognostic biomarker in GBM.
Han et al suggested that the interaction of miR-431-5p-erythrocyte membrane protein band 4.1 (EPB41L1) is involved in GBM progression, implying the potential of miR-431-5p and EPB41L1 in predicting tumor progression [9]. Long non-coding RNAs (lncRNAs) act as major regulators of gene expression, and dysregulation of lncRNAs is involved in a variety of biological processes in many cancers [10,11]. Aberrant expression of lncRNAs participates in brain glioma development and may be critical regulators of tumorigenesis [12]. Ma et al. demonstrated that lncRNA-HULC was highly expressed in glioma tissues and cells and might serve as a therapeutic target for human glioma [13]. Mazor et al. indicated that high expression of lncRNA TP73 Antisense RNA 1 (TP73-AS1) predicted aggressiveness and poor prognosis of GBM and might contribute to temozolomide resistance in GBM cancer stem cells [14]. Lu et al. showed that over-expression of lncRNA Small Nucleolar RNA Host Gene 12 (SNHG12) promoted the cells to acquire temozolomide resistance, which could predict poor survival for GBM patients [15]. Despite these, the immune-related lncRNAs that have prognostic value remain largely unknown.

In this study, we identified immune-related lncRNAs associated with the prognosis of GBM, constructed an immune-related lncRNA signature-based prognostic risk score model, and explored the underlying differences on molecular expression, biological function and regulatory mechanism between high- and low-risk groups. Overall, we hypothesized that the identified immune-related lncRNAs played pivotal roles in GBM progression, and the identified prognostic signature of 18 immune-related lncRNAs could be a novel prognostic biomarker and therapeutic target for GBM,
contributing to improve the prognosis prediction and treatment of this disease.

Methods

Dataset acquisition and preprocessing

GSE111260 and GSE103227 datasets were downloaded from GEO database [16]. GSE111260 contained 70 human samples, including 67 glioma samples and 3 normal brain samples. The data were generated on GPL5175 [HuEx-1_0-st] Affymetrix Human Exon 1.0 ST Array platform. GSE103227 contained 5 GBM samples and 5 normal brain samples, and the platform was GPL16956 Agilent-045997 Arraystar human lncRNA microarray V3. The data were standardized preprocessed utilizing the robust multi-array average (RMA) method [17] in Limma package [18] in R3.4.3. The probes were annotated on the basis of the annotation files of the platform. The probes matching no gene symbol was removed, and mean value was selected as the final expression value of the gene when multiple probes matching to one gene symbol. In addition, the FPKM RNA-seq data and clinical phenotype data of 167 GBM samples were obtained from The Cancer Genome Atlas (TCGA) database. The Ensemble gene was converted into gene symbol utilizing the annotation file (hg38, gencode.V22.) of GENCODE database [19]. Mean value was selected as the final expression value of the gene when multiple Ensemble genes matching to one gene symbol. Then mRNAs and lncRNAs were obtained based on the gene symbol types, of which mRNAs and lncRNAs without expression in more than 80% of samples were removed. These three datasets (GSE111260, GSE103227 and TCGA-GBM datasets) were integrated
analyzed in this study, and the analysis procedure is shown in Figure S1.

Screening of differentially expressed genes (DEGs) and immune-related DEGs

Differential expression analysis in GBM vs normal brain samples was carried out utilizing Limma package in R 3.4.3, and genes with \(|\log\text{FC}|>1\) and P value <0.05 were considered as DEGs. The common up-regulated and down-regulated DEGs from the two databases (GSE111260 and GSE103227) were screened and used in the following analysis. The immune-related genes in The Immunology Database and Analysis Portal (ImmPort) database were downloaded from InnateDB [20] (https://www.innatedb.ca/index.jsp). By Venn analysis, the overlapped genes between immune-related genes and DEGs were screened as immune-related DEGs.

Screening of immune-related lncRNAs

The expression data of immune-related DEGs in TCGA-GBM dataset were retrieved. Correlation analysis between immune-related DEGs with all annotated lncRNAs was then conducted utilizing cor.test in R 3.4.3. Immune-related lncRNAs were screened by \(|\text{Pearson correlation coefficient}| > 0.6\) and P value < 0.001.

Construction of lncRNA-based prognostic risk model

Among the 167 GBM samples, seven samples were removed due to their survival time less than one month, and the remaining samples were randomly divided into training set (n = 80) and validation set (n = 80) with a ratio of 5:5. Univariate Cox regression analysis was performed to screen the immune-related lncRNA that was significantly correlated with prognosis. Then, the lncRNAs with log-rank P < 0.05 in univariate analysis were further included in multivariate Cox regression analysis.
Then the prognostic risk model was constructed according to the formula: Risk score = \( \sum \text{Coefficients} \times \text{Expression values} \). Of which, Coeff represents the correlation coefficient in multivariate Cox regression analysis, and Exp represents the expression value of the lncRNAs in TCGA dataset. Patients were divided into high- and low-risk groups based on the risk score calculated by the prognostic risk model, and Kaplan–Meier (KM) survival curves and log-rank test were utilized to conduct survival analysis to confirm the prognostic value of the risk model in training set, validation set and total set (training set+ validation set).

**DEGs in high risk and low risk group**

Based on the risk score calculated by the prognostic risk model, samples in TCGA set were divided into high- and low-risk groups. DEGs in high- vs. low-risk were screened utilizing Limma package with the cut-off value of |\( \log_{2} FC \)|>0.585 and \( P < 0.05 \). The overlapped genes between DEGs and immune-related genes from ImmPort database were screened as key immune-DEGs, and functional enrichment analysis was performed for these overlapped genes. The clusterProfiler tool [21] was utilized to conduct functional enrichment analysis, including Gene Ontology (GO) annotation terms and KEGG pathways. The significant enriched results were screened by threshold of count \( \geq 2 \) and \( P < 0.05 \). Additionally, the protein-protein interactions (PPIs) among those genes were predicted utilizing STRING database [22] with PPI score of 0.4. The PPI network was visualized by Cytoscape [23].

**Construction of lncRNA-mRNA co-expression network**

Correlation analysis between key immune-DEGs and lncRNA was conducted, and
IncRNA-mRNA pairs with |Pearson correlation coefficient| > 0.3 and P value < 0.05 were screened to construct IncRNA-mRNA co-expression network. To further investigate the function of these IncRNAs, clusterProfiler was utilized to explore the involved KEGG pathways of mRNAs in IncRNA-mRNA network. The significant enriched pathways were screened with P < 0.05.

Results

Screening of DEGs and immune-related DEGs

A total of 5569 DEGs between GBM and normal brain samples were screened from GSE103227 dataset, including 2886 up- and 2683 down-regulated genes (Figure 1A). Moreover, 1429 DEGs (including 558 up- and 871 down-regulated) were screened from GSE111260 dataset (Figure 1B). Venn analysis showed that there were 819 overlapped DEGs in the two datasets, including 257 up- and 562 down-regulated overlapped DEGs (Figure 1C).

In order to screen the immune-related genes from the DEGs, 4677 immune-related genes were retrieved from ImmPort database. A total of 215 overlapped genes between immune-related genes and DEGs were identified as immune-related DEGs (Figure 1D).

Identification of immune-related IncRNAs by correlation analysis

A total of 5934 IncRNAs and 16863 mRNAs were annotated from TCGA-GBM dataset. By correlation analysis of 4677 immune-related genes and 5934 IncRNAs, 1902 IncRNA-mRNAs co-expression pairs were obtained, involving 387 IncRNAs
and 140 immune-related DEGs. These 387 lncRNAs were considered as immune-related lncRNAs.

Construction of IncRNA-based prognostic risk model

The results of univariate Cox regression analysis showed that 18 immune-related lncRNAs were significantly associated with prognosis (Table 1), which were further included in multivariate Cox regression analysis to calculate the β coefficient (Coef in formula) (Table 2).

Prognostic risk score models were established in training set, validation set and total set (training set + validation set). Then, the risk score was calculated for each sample, and patients were divided into high- and low-risk groups based on the medial risk score. KM survival curves showed that patients with high risk scores had a shorter survival than those with low risk scores in training set (P < 0.0001), validation set (P = 0.024) and total set (P < 0.0001) (Figure 2). These data suggested that the risk score model could stratify GBM patients with different prognosis.

Screening of DEGs in high- vs. low-risk groups

All the TCGA-GBM samples were divided into high- and low-risk groups based on median risk score. A total of 272 DEGs in high- vs. low risk groups were obtained, including 58 up- and 214 down-regulated genes (Figure 3A). Among the 272 DEGs, there were 22 immune-related DEGs after merging with the 4677 immune-related genes. To explore the interactions among proteins encoded by these 22 immune-related DEGs, PPI network was constructed (Figure 3B). The PPI network contained 18 nodes and 53 interactions. SNAP25 (synaptosome associated protein 25,
degree = 12), SYT1 (synaptotagmin 1, degree = 10), SNCB (synuclein beta, degree = 10), SNAP91 (Ras-related protein rab-3A, degree = 9) and RAB3A (degree=8) were considered as hub nodes in this PPI network with higher degree.

**Functional enrichment analysis of immune-related DEGs in high- vs. low-risk groups**

We further investigated the involved GO annotation terms and KEGG pathways of the 22 immune-related DEGs. A total of 174 GO-biological processes were enriched, such as synaptic vesicle cycle, synaptic vesicle exocytosis, neurotransmitter secretion and transport (Figure 4A). A total of 53 GO-cellular component annotations were enriched, such as glutamatergic synapse, synaptic vesicle and distal axon, etc. In terms of GO-molecular function, SNARE binding, syntaxin−1 binding and others such as protein C−terminus binding were enriched (Figure 4C). Additionally, two KEGG pathways, including synaptic vesicle cycle and insulin secretion were significantly enriched (Figure 4D).

**Construction of lncRNA-mRNA co-expression network**

Correlation analysis of the expression levels between 18 IncRNAs for prognosis risk model construction and the 22 immune-related DEGs was performed. As results, 55 IncRNA-mRNA co-expression pairs were obtained, involving 22 mRNAs and 11 IncRNAs (Figure 5). Among 11 IncRNAs, Long Intergenic Non-Protein Coding RNA 1574 (LINC01574) was co-expressed with the greatest number of immune-related DEGs, such as SNAP25, SYT1, SNCB, and SNAP91, etc. Furthermore, these IncRNAs were involved in different GO biological processes and KEGG pathways. For
examples, LINC01574 was associated with GO functions associated with synaptic vesicle cycle, vesicle-mediated transport in synapse, glutamate secretion, etc. RP11-1398P2.1 was implicated in GO functions associated with positive regulation of cyclin-dependent protein kinase activity, cerebral cortex/telencephalon/forebrain cell migration. LINC01574 was involved in key pathways, such as synaptic vesicle cycle and insulin secretion, and RP11-455F5.5 was enriched in insulin secretion and neuroactive ligand-receptor interaction pathways (Figure 6).

Discussion

GBM is a deadliest brain malignancy with poor outcomes due to the limited effect of existing treatments, including surgical and chemoradiotherapy [24]. Identification of key molecular markers affecting the prognosis of GBM is helpful to optimize the treatment and improve the clinical outcome. In the present study, 18 immune-related lncRNAs were identified to have significant impact on the prognosis of patients. The prognostic risk score model integrating these 18 immune-related lncRNAs was constructed, which could stratify GBM patients into groups with different risks and predict the prognosis of patients. Furthermore, lncRNA LINC01574 was co-expressed with more immune-related DEGs between high- and low-risk groups, such as SNAP25, SNAP91, SNCB and RAB3A. Besides, the lncRNAs in the co-expression network were enriched in different biological functions and pathways.

In recent years, immunotherapy has successfully altered the landscape for the treatment of many types of cancers, including GBM [25]. Nevertheless, the initial
immunotherapy trials show dismal results in GBM because of the complexities within intratumoral molecular heterogeneity [26]. Screening of key immune-related molecular markers is essential to design personalized immunotherapy for GBM. Increasing studies have suggested that immune-related lncRNAs could indicate prognosis of glioma patients [27,28]. For example, Wang et al. showed that their identified a 9-immune-related lncRNA signature could predictive role for anaplastic gliomas patients who had different OS and progression-free survival, and this signature was independent from other clinical factors [29]. Zhou et al. found that a 6-lncRNA signature could stratify GBM patients into high/low-risk groups with obvious diverse survival, and this signature was implicated in tumorigenesis by taking part in immune-related pathways [30]. Wang et al. established 5-immune-related lncRNA signature could stratify glioma patients into diverse tumor immune subtypes, and their expression showed positive correlations with immune indicators, such as programmed cell death-1 [31]. The identified prognostic signature of 18 immune-related lncRNAs in this study could stratify GBM patients into groups with different risks and predict the prognosis of patients. Given the low 5-year survival rate (less than 10%) [3], we believe that the constructed prognostic risk score model based on 18-immune-related lncRNA signature could guide doctors to judge the disease severity and evaluate the prognosis, and contribute to the development of more reasonable and effective personalized treatment plans, so as to maximize the benefits of GBM patients.

Furthermore, lncRNA LINC01574 was co-expressed with more immune-related
DEGs that were involved in synaptic vesicle, such as SNAP25, SNAP91, SNCB, and RAB3A, etc. The communication among glioma tumor cells and the non-glioma brain cells has been proved to be implicated in the development and progression of brain tumor. Extracellular vesicles-mediated crosstalk resulted in an elevated synaptic activity in neurons, and inhibiting the release of extracellular vesicles could inhibit glioma growth [32]. Gao et al. had demonstrated that the SNAP91 expression showed direct positive correlation with tumor grade and was associated with survival, suggesting that SNAP91 might be a biomarker in GBM [33]. RAB proteins are small GTP-binding proteins that function in intracellular vesicle trafficking. Kim et al. indicated that the expression of RAB3A was increased in glioma and GBM, and overexpression of RAB3A could facilitate proliferation and tumorigenicity of cells [34]. Although LINC01574 has not been investigated in GBM, the results of this study prompted us to speculate that LINC01574 might be implicated in the progression of GBM by regulating the genes involved in synaptic vesicle.

Other lncNRAs in the co-expression network were found to be enriched in different functions and pathways, RP11–1398P2.1 co-expressed with CDK5R1 was implicated in positive regulation of cyclin–dependent protein kinase activity and cell migration. RP11 has been found to regulate glioma progression [35], tumorigenesis and epithelial-mesenchymal transition [36], and glioma growth [37]. Zhang et al. demonstrated that as a downstream gene of RE1 silencing transcription factor, CDK5R1 was implicated in the proliferation and migration of GBM cells [38]. RP11–455F5.5 co-expressed with MAG (myelin-associated glycoprotein), which
functions significant effect in inhibiting adhesion and migration of U87MG glioma cells by regulating Nogo-66 receptor [39]. These studies suggested that these lncRNAs had important roles in GBM. However, the expression and functions of these lncRNAs in GBM have not been investigated by experiments, such as real-time quantitative PCR, western blot, and knockdown assays. This is a limitation of this study. Besides, the prognostic value of the key lncRNAs has not been confirmed by clinical data.

Conclusion

In conclusion, we identified 18 immune-related prognostic lncRNAs, and established a prognostic risk score model. The risk score model showed the ability to distinguish GBM patients into different risk groups, and patients with high risk scores displayed worse survival. DEGs in high- vs. low-risk groups were involved in the progression of GBM by regulating synaptic vesicle. This study would provide novel prognostic biomarkers and potential therapeutic targets for the treatment of GBM.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials:
Not applicable. This study was only the primary research, and further study has been in progress.

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

Xiangdong Lu and Peng Qiu carried out the Conception and design of the research, Siquan Zhu and Yunfeng Ma participated in the Acquisition of data. Shouqing Zhang and Siquan Zhu carried out the Analysis and interpretation of data. Feng Si and Fei Chen participated in the design of the study and performed the statistical analysis. Xiangdong Lu and Peng Qiu conceived of the study, and participated in its design and coordination and helped to draft the manuscript and revision of manuscript for important intellectual content. All authors read and approved the final manuscript.

Acknowledgement

Not applicable.

References


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Figure legends

**Figure 1.** Screening of differentially expressed genes (DEGs) between glioblastoma (GBM) and normal brain samples and immune-related DEG.

Volcano plots of DEGs in GSE103227 dataset (A) and GSE111260 dataset (B); Venn plot shows the overlapped DEGs in GSE103227 and GSE111260 datasets (C); Venn plot shows the overlapped genes between immune-related genes from ImmPort database and overlapped DEGs in the two datasets (D).

**Figure 2.** Kaplan–Meier (KM) survival curves.

KM survival curves show the survival status of patients with high and low risk scores in training set (A), validation set (B) and total set (C).

**Figure 3.** DEGs in high- vs. low-risk groups

Volcano plots of DEGs between high-risk and low-risk groups in TCGA-GBM dataset (A); Protein-protein interaction (PPI) network of immune-related DEGs between high-risk and low-risk groups (B). Green nodes represent down-regulated immune-related DEGs.

**Figure 4.** Functional enrichment analysis of immune-related DEGs in high- vs. low-risk groups.

Histogram shows the top 20 enriched Gene Ontology (GO) biological processes (A), cellular component (B), molecular function (C), and KEGG pathways (D).

**Figure 5.** The lncRNA-mRNA co-expression network.

Green circles and red triangles represent down-regulated and up-regulated immune-related DEGs, respectively; orange rhombus represent lncRNAs.
**Figure 6. Functional enrichment analysis for lncRNAs in co-expression network.**

Bubble diagrams shows the significantly enriched GO biological processes (A) and KEGG pathways (B) for lncRNAs. Bubble size represents gene count; bubble color from blue to red represent significant from low to high.

**Figure S1. The workflow of this study.**
Table 1. The 18 immune-related prognosis lncRNAs screened by univariate Cox regression analysis.

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Table 2. The β coefficient calculated by multivariate Cox regression analysis.

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</tbody>
</table>
A Cutoff for log2FC is 0.565
The number of up-regulated genes is 58.
The number of down-regulated genes is 214.

B

Gene interactions and networks, including genes like CDK5R1, STXB1B, RAB3A, SYT1, SNAP25, SNCB, CCK, SH3GL2, PACSIN1, MAG, CALD1, MOG, with connections highlighting regulatory relationships.