Risk assessment model constructed by differentially expressed IncRNAs for the prognosis of glioma

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Abstract. A risk assessment model was constructed using differentially expressed long non-coding (lnc)RNAs for the prognosis of glioma. Transcriptome sequencing of the lncRNAs and mRNAs from glioma samples were obtained from the TCGA database. The samples were divided into bad and good prognosis groups based on survival time, then differently expressed lncRNAs between these two groups were screened using DEseq and edgeR packages. Multivariate Cox regression analysis was performed to establish a risk assessment system according to the weighted regression coefficient of lncRNA expression. Survival analysis and receiver operating characteristic curve were conducted for the risk assessment model. Furthermore, the co-expression network of the screened lncRNAs was constructed, followed by the functional enrichment analysis for associated genes. A total of 117 lncRNAs were screened using edgeR and DEseq packages. Among all differently expressed lncRNAs, five lncRNAs (RP3-503A6, LINC00940, RP11-453M23, AC009411 and CDRT7) were identified to establish the risk assessment model. The risk assessment model demonstrated a good prognostic function with high area under the curve values in the training, validation and entire sets. The risk score was certified as an independent prognostic factor for gliomas. Multiple genes were screened to be co-expressed with these five lncRNAs. Functional enrichment analysis demonstrated that they were involved in cytoskeleton, adhesion and Janus kinase/signal transducer and activator of transcription signaling pathway-associated processes. The present study established a risk assessment model integrating five significantly different expressed lncRNAs, which may help to assess the prognosis of patients with glioma with increased accuracy.

Introduction

Glioma is one of the most common primary tumors in brain and it accounts for the majority of central nervous system oncology cases in adults globally in 2016 (1). In general, glioma infiltrates into the brain and affects the cerebral hemispheres of patients (1). Though aggressive therapies including surgery, chemotherapy, and radiotherapy have been developed and applied widely, glioma remains associated with high recurrence rates and there are no efficient treatment options (2). The prognosis of glioma patients remains poor (3). Therefore, the development of novel treatments, the identification of new prognostic biomarkers and a clearer understanding of the molecular mechanisms underlying disease progression are essential and urgently required.

Long non-coding RNAs (lncRNAs) account for 80% of non-coding RNAs and are defined as the endogenous cellular RNAs that are >200 nucleotides in length (4,5). With the development of biological computing research tools including lncRNAdb, ChiPBase, LNCipeida and lncRNAtor, the number of lncRNAs being identified is rapidly increasing. In total, >8,000 lncRNA genes have been identified within 4 years and the number of human lncRNAs is estimated to be between 10,000 and 20,000 (6,7). A previous study revealed that lncRNAs are associated with multiple critical cellular functions, including transcriptional, posttranscriptional and epigenetic functions (8). LncRNAs have attracted considerable attention in cancer, since they may be involved in oncogenic and tumor suppressive pathways (6). In the last decade, accumulating evidence demonstrated that the expression levels of lncRNAs were correlated with the development and progression of several types of cancer, through affecting the biological processes including growth, proliferation, metastasis and invasion (9,10). In addition, the functional roles that they may serve in tumor prognosis have been also investigated (11).

The important roles of lncRNAs in gliomas have been revealed in multiple studies. It was reported that lncRNAs may regulate cellular proliferation, apoptosis and migration in glioma (3,12,13). In addition, aberrant expression of lncRNAs has been linked with differential treatment responses in patients with glioma and lncRNAs may be promising as diagnostic and prognostic biomarkers in glioma (14). Ma et al (15) reported that lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) may regulate the progression of glioma, thus serving as a potential prognostic biomarker. Using univariate Cox
regression analysis, the overexpression of lncRNA AB073614 was identified as a poor prognostic biomarker in glioma (16). Despite these findings, our understanding of the prognostic role of lncRNAs in glioma remains unclear.

In the present study, the lncRNA sequencing (lncRNA-seq) data and mRNA-seq expression profiles of glioma samples were downloaded from the TCGA database and the differentially expressed IncRNAs were screened. Furthermore, multivariate Cox regression analysis was used to establish a risk assessment system based on the weighted regression coefficient of IncRNA expression. Subsequently, survival analysis for the risk assessment model was conducted in training, validation and entire sets. Finally, the co-expression networks of IncRNAs in the risk assessment model were constructed and the function enrichment was performed for the genes associated with these IncRNAs.

Materials and methods

Data and data processing. The lncRNA-seq data and mRNA-seq expression profiles of glioma samples were downloaded from the TCGA database (gdc-portal.nci.nih.gov). Glioblastoma samples were used. A total of 173 glioma samples were obtained. Following matching the barcodes of lncRNA-seq data and mRNA-seq expression profiles, 154 glioma samples remained. Finally, 140 samples were selected for analysis following removal of the samples without survival information and overall survival time <6 months. The IncRNAs were annotated using human genome annotation GTF format in the GENCODE database (www.gencodegenes.org) (17). These 140 samples were randomly divided into two groups as the ratio of 1:1, namely training and validation sets.

Screening differently expressed IncRNAs. The 70 glioma tissue samples in the training group were divided into bad prognosis (patients succumbed within 6 months) and good prognosis (patients with a survival time >12 months) groups. Subsequently, the differentially expressed IncRNAs between these two groups were screened using DEseq 1.16.0 (www.bioconductor.org/packages/release/bioc/html/DESeq.html) (18) and edgeR 3.8.5 (www.bioconductor.org/packages/release/bioc/html/edgeR.html) (19) package in R 3.1.0 (www.r-project.org) with the threshold of false discovery rate (FDR) <0.05 and |log_2 fold change| >0.263. Then, the differentially expressed IncRNAs were screened for prognostic factors (threshold, P<0.05) using the log-rank test and univariate Cox regression analysis in R 3.1.0.

Establishment of a risk assessment model. Based on the previously obtained differentially expressed lncRNAs, multivariate Cox regression analysis was performed to establish a risk assessment system according to the regression coefficient weighted lncRNA expression. The risk score for each patient was based on a linear combination of mRNA expression values after weighting regression coefficients. The risk assessment for each patient was scored according to the following equation: Risk score = $\beta_{\text{lncRNA1}} \cdot \text{expr}_{\text{lncRNA1}} + \beta_{\text{lncRNA2}} \cdot \text{expr}_{\text{lncRNA2}} + \ldots + \beta_{\text{lncRNA}} \cdot \text{expr}_{\text{lncRNA}}$, where $\text{expr}$ means the expression level of lncRNA. The $\beta$ value obtained in the training set was also used to assess the risk of patients in the validation set.

Survival analysis for risk assessment model. The risk score of samples in validation set was calculated according to the risk assessment system. Subsequently, the samples were divided into high and low-risk types based on the median risk score. Finally, Cox regression was performed for correlation analysis in combination with corresponding clinical data of samples. Kaplan-Meier survival analysis was performed to compare the overall survival (OS) rates of patients with high and low-risk scores. Receiver Operating Characteristic (ROC) curve was used to evaluate the classification efficiency of the obtained risk assessment model. Furthermore, the distribution of signature IncRNAs in training validation and entire sets were analyzed and displayed. In addition, the Kaplan-Meier survival analysis and ROC curve were also performed for each of these IncRNAs in the risk assessment model.

Correlation between risk assessment model and clinical features. The risk scores of samples in the validation set were calculated according to the risk assessment system. Subsequently, the samples were divided into high and low-risk types based on the median risk score. The correlations between prognosis and the clinical features, including risk score, age, sex, chemotherapy and pharmaceutical-therapy, were evaluated using univariate and multivariate Cox regression analyses.

Subsequently, hierarchical analysis was performed for the screened clinical features, which were significantly correlated with prognosis risk. The association between the risk groups and survival prognosis was analyzed under the same clinical condition.

Construction of co-expression network. The mRNA expression data matching the IncRNAs was identified based on the sample number. The co-expression network of IncRNA and mRNA was constructed using MEM software (20,21) with the screening threshold of P<0.05. Then, the interaction between the mRNAs in the co-expression network was identified using the STRING (string-db.org) database (22). The interaction pairs with interaction score above 0.4 were selected. Cytoscope 3.4 (www.cytoscape.org) was used to visualize the networks (23).

Functional enrichment analysis. To identify the signaling pathways and biological processes that may be associated with the prognosis of gliomas, Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed for the mRNAs, which were co-expressed with the IncRNAs in risk assessment model, by Database for Annotation, Visualization and Integrated Discovery (david.ncifcrf.gov) (24) with P<0.05.

Statistical analysis. In the present study, the data were presented as the mean ± standard deviation. An unpaired t-test was used to compare the difference between two groups using R 3.1.0. P<0.05 was considered to indicate a statistically significant difference.

Results

Differentially expressed lncRNAs. The clinical information for the training, validation and complete sets is provided in Table I. For the training set, a total of 171 differentially
expressed lncRNAs were screened using edgeR (Fig. 1A), whereas 167 significantly differentially expressed lncRNAs were screened using DEseq between the bad and good prognosis groups (Fig. 1B). The 117 overlapping lncRNAs were screened for further analysis (Fig. 1C).

Risk assessment model. In the training set, a total of 117 differentially expressed lncRNAs were analyzed via univariate Cox regression analysis and 35 lncRNAs were identified to be significantly correlated (P<0.05) with prognosis. Subsequently, multivariate Cox regression analysis was conducted for these 35 lncRNAs and 5 of them were screened to build the risk assessment model (Table II). The risk score was calculated using these five lncRNAs as follows: Risk score = (-1.05854) \times \text{exp} \text{RP3-503A6} - 503A6 + (-1.03947) \times \text{exp} \text{LINC00940} + (1.017787) \times \text{exp} \text{RP11-453M23} + (0.83919) \times \text{exp} \text{AC009411} + (1.095126) \times \text{exp} \text{CDRT7}.

Survival analysis for five lncRNAs in training, validation and entire sets. For the training set, patients in the low-risk group were associated with longer survival time compared with the patients in the high-risk group (15.29±11.29 vs. 9.41±7.13 months; P=0.0117). Kaplan-Meier analysis confirmed the significant difference of survival time between low-risk group and high-risk group (P<0.05; Fig. 2A). Consistent with the results of the training set, the patients in the low-risk group exhibited significantly longer survival time compared with the patients in high-risk group in the validation and the entire sets (validation set: 16.13±12.84 vs. 10.19±8.69 months, P=0.0273, Fig. 2B; entire set: 15.71±12.01 vs. 9.81±7.89 months, P=0.0207, Fig. 2C). Additionally, the area under the curve (AUC) was 0.910, 0.84 and 0.896 for the ROC of these 5 lncRNAs in the training set (Fig. 2D), validation set (Fig. 2E) and entire set (Fig. 2F). The Youden index was 0.600, 0.621, and 0.62 in the training, validation and entire sets, respectively.

There were significant differences in the expression levels of these five lncRNAs in training, validation and entire sets (P<0.05; Fig. 3). The expression levels of RP3-503A6 and LINC00940 were significantly increased in high-risk groups, whereas the expression levels of RP3-503A6 and LINC00940 were significantly decreased compared with the low-risk group (P<0.05). Consistently, the results of Kaplan-Meier survival analysis demonstrated that increased expression of RP3-503A6 and LINC00940 and decreased expression of RP11-453M23, AC009411 and CDRT7 indicated improved prognoses (Fig. 4). The AUC values for ROC curves of RP3-503A6, LINC00940, RP11-453M23, AC009411 and CDRT7 were 0.691, 0.679, 0.615, 0.652 and 0.716, respectively.

Prognostic factors for glioma. The results obtained from the univariate and multivariate Cox regression analysis are provided in Tables III-V. In the training, validation and entire sets, the risk score was significantly associated with the prognosis of patients and it was an independent prognostic factor. The impact of high and low risk on

Table I. Clinical information for training, validation and complete set.

| Clinical characteristic | Training set (n=70) | Testing set (n=70) | Entire set (n=140) |
|-------------------------|---------------------|-------------------|-------------------|
| Age (years, mean ± SD)  | 59.33±11.86         | 60.91±14.43       | 60.12±13.19       |
| Sex (male/female)       | 45/25               | 45/25             | 90/50             |
| Chemotherapy (yes/no/unknown) | 20/43/7         | 24/36/10          | 44/79/17          |
| Drug therapy (yes/no/unknown) | 8/55/7           | 11/48/11          | 19/103/18         |
| Pharmaceutical-therapy (yes/no/unknown) | 24/42/4        | 30/31/9           | 54/73/13          |
| Radiation-therapy (yes/no/unknown) | 5/61/4          | 12/49/9           | 17/110/13         |
| Targeted molecular-therapy (yes/no/unknown) | 9/53/8          | 8/53/9            | 17/106/17         |
| Mortality (deceased/alive) | 51/19             | 51/19             | 102/38            |
| Overall survival time, months | 12.35±9.83      | 13.16±11.29       | 12.75±10.55       |

SD, standard deviation.

Table II. Information on the five lncRNAs screened to build the risk assessment model.

| lncRNA       | Coefficient | HR   | 95% CI      | P-value |
|--------------|-------------|------|-------------|---------|
| RP3-503A6    | -1.0585     | 2.8821 | 0.1719-7.003| 0.0031  |
| LINC00940    | -1.0395     | 2.8277 | 0.1721-7.265| 0.0047  |
| RP11-453M23  | 1.0178      | 0.3614 | 1.3416-5.7070| 0.0059 |
| AC009411     | 0.8392      | 0.4321 | 1.2657-4.2325| 0.0064  |
| CDRT7        | 1.0951      | 0.3345 | 1.3582-6.5803| 0.0065 |

lnc, long non-coding; HR, hazard ratio.
prognosis under the same clinical features was also analyzed using hierarchical analysis. It was demonstrated that the low-risk group had a significantly improved prognosis compared with the high-risk group in the subgroups of age >60 years (P=0.00862), no chemotherapy (P=0.0113) and no pharmaceutical therapy (P=0.0142; Fig. 5). No
Table III. Univariate and multivariate Cox regression analysis for malignant glioma in training set (n=70).

| Variable               | Univariate analysis | Multivariate analysis |
|------------------------|---------------------|-----------------------|
|                        | HR      | 95% CI     | P-value | HR      | 95% CI     | P-value |
| Risk score             |         |            |         |         |            |         |
| High/low               | 1.283   | 1.116-1.474| 0.0002  | 1.318   | 1.108-1.568| 0.0019  |
| Age, years ≤60/>60     | 1.444   | 0.823-2.534| 0.1978  | 2.123   | 1.088-4.142| 0.0273  |
| Sex                    |         |            |         |         |            |         |
| Male/female            | 0.8142  | 0.461-1.438| 0.4779  | 0.942   | 0.487-1.823| 0.8590  |
| Chemotherapy           |         |            |         |         |            |         |
| Yes/no                 | 0.3947  | 0.199-0.781| 0.0062  | 1.355   | 0.587-1.901| 0.1280  |
| Pharmaceutical therapy |         |            |         |         |            |         |
| Yes/no                 | 0.223   | 0.0994-0.499| 0.0003 | 0.381   | 0.261-0.446| 0.0092  |

HR, hazard ratio; CI, confidence interval.

Table IV. Univariate and multivariate Cox regression analysis for malignant glioma in validation set (n=70).

| Variable               | Univariate analysis | Multivariate analysis |
|------------------------|---------------------|-----------------------|
|                        | HR      | 95% CI     | P-value | HR      | 95% CI     | P-value |
| Risk score             |         |            |         |         |            |         |
| High/low               | 1.186   | 0.738-1.964| 0.0395  | 1.147   | 0.742-1.027| 0.0435  |
| Age, years ≤60/>60     | 1.772   | 0.983-3.195| 0.0571  | 1.264   | 0.647-2.470| 0.4930  |
| Sex                    |         |            |         |         |            |         |
| Male/female            | 0.862   | 0.486-1.528| 0.6110  | 0.939   | 0.486-1.815| 0.8520  |
| Chemotherapy           |         |            |         |         |            |         |
| Yes/no                 | 0.532   | 0.279-1.012| 0.0506  | 0.881   | 0.3312-2.339| 0.7990 |
| Pharmaceutical therapy |         |            |         |         |            |         |
| Yes/no                 | 0.405   | 0.216-0.759| 0.0048  | 0.462   | 0.179-1.187| 0.1090  |

HR, hazard ratio; CI, confidence interval.

Table V. Univariate and multivariate Cox regression analysis for malignant glioma in entire set (n=140).

| Variable               | Univariate analysis | Multivariate analysis |
|------------------------|---------------------|-----------------------|
|                        | HR      | 95% CI     | P-value | HR      | 95% CI     | P-value |
| Risk score             |         |            |         |         |            |         |
| High/low               | 1.037   | 0.943-1.141| 0.0482  | 1.447   | 0.918-2.281| 0.0197  |
| Age, years ≤60/>60     | 1.57    | 1.052-2.344| 0.0272  | 1.447   | 0.918-2.281| 0.1120  |
| Sex                    |         |            |         |         |            |         |
| Male/female            | 0.815   | 0.547-1.215| 0.3160  | 0.841   | 0.539-1.312| 0.4460  |
| Chemotherapy           |         |            |         |         |            |         |
| Yes/no                 | 0.485   | 0.308-0.762| 0.0017  | 1.414   | 0.641-3.119| 0.3910  |
| Pharmaceutical therapy |         |            |         |         |            |         |
| Yes/no                 | 0.339   | 0.214-0.539| <0.0001 | 0.253   | 0.114-0.563| 0.0008  |

HR, hazard ratio; CI, confidence interval.
significant differences of survival ratio were observed between low-risk and high-risk groups in the subgroups of age <60 years (P=0.625), chemo-therapy (P=0.613) and pharmaceutical therapy (P=0.508; Fig. 5).
Functional enrichment for co-expressed mRNAs of signature lncRNAs. The co-expressed network for each lncRNA is demonstrated in Fig. 6. GO functional biology processes and KEGG pathways enriched by genes in the networks are shown in Fig. 7. The genes involved in membrane organization, vesicle-mediated transport, intracellular signaling...
cascade, cytoskeleton organization and cell motion biological process (BP) terms. For cell component (CC) term, cell leading edge, cytoskeleton, cell projection, vesicle and cytoplasmic vesicle were the significantly enriched ones. For the molecule function (MF) term, GTPase activity, cytoskeletal protein binding and actin binding were important. It was also noted that the genes associated with these five lncRNAs were mainly enriched in cell adhesion (hsa04510: Focal adhesion) and Janus kinase (Jak)-signal transducer and activator of transcription (STAT) signaling pathway (hsa04630: Jak-STAT signaling pathway).

Discussion

A total of 117 lncRNAs were identified between bad and good prognosis groups in the training set using edgeR and DEseq packages. According to univariate and multivariate Cox regression analyses, five lncRNAs were screened to establish a risk assessment model. The risk assessment model demonstrated good prognostic function with increased AUC in training, validation and entire sets. There were significant differences in the expression levels of these five lncRNAs in the training, validation and entire set. Furthermore, risk score calculated using these five lncRNAs was certified as an independent prognostic factor for glioma. According to the results of hierarchical analysis, the prognostic function of the risk assessment model may be useful for patients >60 years who did not receive chemotherapy and pharmaceutical therapy.

Multiple genes were screened to be co-expressed with these five lncRNAs and these were mainly involved in cell adhesion, Jak-STAT signaling pathway, intracellular signaling cascade, cell motion, cytoskeleton, GTPase activity, cytoskeletal protein binding and actin binding.

Metastasis accounts for the vast majority of cancer-associated mortalities and is a result of cancer cells moving from the primary site (site of the origin of cancer) to a distant site or other organ (25). The spatial and temporal reorganization of the cytoskeleton serves a critical role in the movements and alterations of cell shape throughout the complex processes of metastasis (26). The importance of cytoskeleton-associated proteins and pathways involved in glioma has also been reported in numerous previous studies (27,28). In addition, increasing evidence revealed that lncRNAs are associated with the occurrence and development of glioma by regulating the structure of reorganization of cytoskeleton-associated proteins and signaling pathways (29,30). Additionally, the association between metastasis or cytoskeleton and the prognosis of glioma has also been reported (31,32). According to our analysis, the biological process of cytoskeleton organization and actin cytoskeleton organization, the cell component of cytoskeleton, and the molecular function of cytoskeletal protein binding were enriched by the genes associated with the five lncRNAs of the constructed risk assessment model. Therefore, it may be reasonable to infer that the cytoskeleton-associated process may be one of the underlying mechanisms for the prognostic risk assessment model in glioma.

Consistently, the focal adhesion pathway, which is associated with cell invasion and metastasis, was screened as a significant pathway enriched by the genes associated with the five lncRNAs based on the KEGG analysis. Focal adhesions are known as the large macromolecular assemblies through which regulatory signals and mechanical forces are transmitted between interacting cells and the extracellular matrix (ECM) (33). Focal adhesion functions as the mechanical linkage to the ECM and as a biochemical signaling hub to concentrate and direct numerous signaling proteins at sites of integrin binding and clustering (34). Yue et al (35) demonstrated that a cytoskeleton protein of microtubules ending-binding 2 (EB2) may regulate the dynamics of focal adhesion through MAP4K4. Importantly, adhesion to ECM has been demonstrated to contribute to the resistance to chemotherapy and radiotherapy in tumor cells (36,37). Therefore, focal adhesion and ECM may have a prognostic role for tumors (38).

The Jak-STAT signaling pathway is associated with multiple biological processes, including cell survival, growth, differentiation and development (39). In the central nervous system, the Jak-STAT signaling pathway is mainly associated with gene regulation during development, inflammation, hormone release and tumorigenesis (39). A previous study reported that inhibiting the STAT3 signaling pathway resulted in depressed proliferation and increased apoptosis in various cancer types including glioma (40). It was speculated that the regulation of the Jak-STAT signaling pathway may be an important factor for the prognostic function of the risk assessment model in glioma.

Multiple genes, including metastasis suppressor protein 1 (MTSS1), drebrin 1 (DBN1), transforming growth factor βl induced transcript 1 (TGFB1I1), and cyclin D1 (CCND1) are associated with cytoskeleton and adhesion or Jak-STAT signaling pathway-associated processes. MTSS1 is reported to be associated with the progression and metastasis of tumor in multiple organ sites, possibly via an interaction with the actin cytoskeleton (41). DBN1 serves a role in cell migration, the extension of neuronal processes and plasticity of dendrites and is overexpressed during cancer metastasis (42). TGFB1I1 functions as a molecular adapter for coordinating various protein-protein interactions associated with focal adhesion (43). Multidimensional analysis of gene expression reveals that TGFB1I1-induced EMT contributes to the malignant progression of astrocytomas (44). The activation of CCND1 affects the prognosis of glioma by promoting cell proliferation, migration and invasion (45). Therefore, it was inferred that the lncRNAs in the risk assessment model may serve critical functions for the prognosis of glioma by regulating the expression or function of these genes. The prognosis of glioma patients may be predicted by detecting the expressions of the five lncRNAs.

To conclude, lncRNAs serve important roles in the development and progression of tumors and may affect tumor prognosis. The present study provided a risk assessment model consisting of five differentially expressed lncRNAs, which may help to assess the prognosis of glioma with increased accuracy, as suggested by the survival analysis and increased AUC value. The risk assessment model indicates the cytoskeleton, adhesion and Jak-STAT signaling pathway may be the most important mechanism in glioma prognosis. However, as all expression data were downloaded from only one database, there may be possible bias in the present study and further studies are required to verify the conclusions.
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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

CH performed data analyses and wrote the manuscript. YZ and CL collected the dataset, contributed to data analyses and manuscript revision. YK conceived and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

In the original creation of the datasets, the local institutional review boards of all participating centers approved the trials and informed consent to participate was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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