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Original Article

Long Non-Coding RNA *CRNDE, LINC00957,* and *AC072061.1* as a Promising Diagnostic and Prognostic Biomarker in Glioblastoma Multiforme

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Abstract

Background: Glioblastoma multiforme (GBM) is one of the most invasive types of brain cancer. LncRNAs can be considered a new prognostic and diagnostic biomarker in GBM. This study comprehensively explored the interaction of lncRNAs with mRNAs in the TCGA database and proposed a novel promising biomarker with favorable diagnostic and prognostic values.

Methods: The public data of RNA-seq and related clinical data were downloaded from the TCGA database. Differential expression analysis was conducted in R. GO and KEGG signaling pathways were used for enrichment. The STRING database was used for PPI analysis. CE-network was constructed by STAR database. Kaplan-Meier survival analysis and ROC curve analysis to indicate the biomarkers' diagnostic and prognostic values.

Results: Differentially expressed data illustrated that 4428 mRNAs were differentially expressed in GBM. The GO and KEGG pathway analysis showed that the differentially expressed mRNAs were enriched in critical biological processes. The PPI showed that *WEE1, BARD1,* and *CDK6* were the important PPI hubs. The ceRNA network data demonstrated critical lncRNAs. The data revealed that the lncRNA *CRNDE*, *LINC00957*, *AC072061.1*, *AC068888.1,* and *DBH-AS1* are potential diagnostic prognostic biomarkers in the GBM patients.

Conclusion: Altogether, we demonstrated lncRNA, and mRNA interaction and mentioned regulatory networks, considered a therapeutic option in GBM. In addition, we proposed potential diagnostic and prognostic biomarkers for the patients.

Keywords: Glioblastoma multiforme; Tumorigenesis; Long non-coding RNAs

Introduction

Glioblastoma multiforme (GBM) is one of the most invasive and dreadful types of brain cancer in adults. The incidence of GBM is 2–3 per 100.000 in Europe and North America yearly (1). GBM is also considered as a very heterogeneous cancer in the clinicopathological and molecular investigation. A dismal number of the patients survive more than 3 years with a poor prognosis (2). The average survival rate of the patient is not more than 14.6 months. Despite radical surgery, radiotherapy, and chemotherapy, treatment and diagnosis of GBM have remained so challengeable and controversial (3, 4). Majority of the patients in the late stages of GBM demonstrate TMZ resistance which causes frustrating survival rate and dismal prognosis (5). A large number of studies have shown that early diagnosis in the lower stages of GBM could improve treatment and prognosis. However, the exact underlying molecular mechanism and etiology of GBM are not entirely illustrated yet (6).

In the recent decade, numerous investigations focused on the establishment of multigene-expression signatures for GBM diagnosis, patients' classification, and also the prognosis of the disease (7, 8). Recently, long non-coding RNAs (lncRNAs) have great potential characteristics to be a valuable biomarker for the investigation of multigene-expression signatures (9-13). LncRNAs, with more than 200 nucleotides, play main roles in many human diseases, such as different cancers. lncRNAs fine tune the expression of downstream genes by sponging miRNAs in the cancer cells (14, 15). LncRNAs contributed in numerous biological functions, such as cell proliferation, differentiation and apoptosis, inflammation, autophagy, and immunity (16).

In this study, we comprehensively considered lncRNAs, and mRNAs expressions from a public database, "Cancer Genome Atlas (TCGA)" and we constructed a ceRNA network in GBM. Furthermore, we demonstrated novel potential diagnostic and prognostic biomarkers for GBM patients.

Materials and Methods

Sample and data collection

The clinical data of the patients were retrieved from the TCGA database. Totally, 599 GBM were enrolled in this study. Three hundred and ten participants had age > 59 yr and 289 patients had age ≤ 59 and 366 and 230 patients were male and female, respectively (Table 1).

Table 1: Clinicopathological characteristics of GBM patients

Characteristics	*N*	*(%)*
Age (yr) (mean ± SD)	57.83	14.40
Age > 59	310	51.75
Age ≤ 59	289	48.25
Sex		
Male	366	61.10
Female	230	38.40
Not Available	3	0.50
Ethnicity		
Hispanic or Latino	13	2.17
Not Hispanic or Latino	490	81.80
Not Available	96	16.03
Race		
Asian	13	2.17
Black or African American	51	8.51
White	507	84.64
Not Available	28	4.67
Vital status		
Alive	102	17.03
Dead	492	82.14
Not Available	5	0.83

RNA-seq data analysis

The molecular data (RNA-Seq Level 3) of GBM were downloaded from the TCGA database. The raw count of the reads of RNA-Seq data was normalized by Voom and TMM normalization methods. The "limma" package was used to indicate the differentially expressed genes. The concluded data were filtered based on the |log2 fold change (FC)|>1 for DEmRNA, and DElncRNA. *P*-value<0.05 and false discovery rate (FDR) <0.05 were considered as significant thresholds.

In Silico functional enrichment analysis and protein-protein interaction (PPI) network

Gene ontology (GO) in three domains including biological processes, cellular components, and molecular functions, and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathways were used for functional enrichment analysis.

CeRNA network construction

LncRNA-miRNA-mRNA ceRNA network was constructed by the"GDCRNATools" package in R software based on the starbase database [14]. The nodes and edges were virtualized by Cytoscape 3.7.2.

Statistical Analysis

All the differentially expressed data were analyzed by using R software (3.5.2). Kaplan-Meier survival analysis (log-rank test) was utilized to indicate the patient's survival time. ROC curve analysis and univariate Cox regression analysis were conducted by SPSS v21. LncRNADisease v2.0 (http://www.rnanut.net/lncrnadisease/) and GEPIA datasets (http://gepia.cancer-pku.cn/) have been utilized for linking related cancer to the lncRNAs. *P*-value<0.05 was considered a significant threshold.

Results

Differentially Expressed Genes

Differentially expressed data illustrated that 4428 mRNA including 2582 upregulated and 1846 down-regulated were differentially expressed in GBM. Furthermore, 299 lncRNAs including 143 upregulated and 156 down-regulated were indicated as differentially expressed lncRNA in the patients. The data are shown in Figs. 1, 2 and Tables 2, 3.

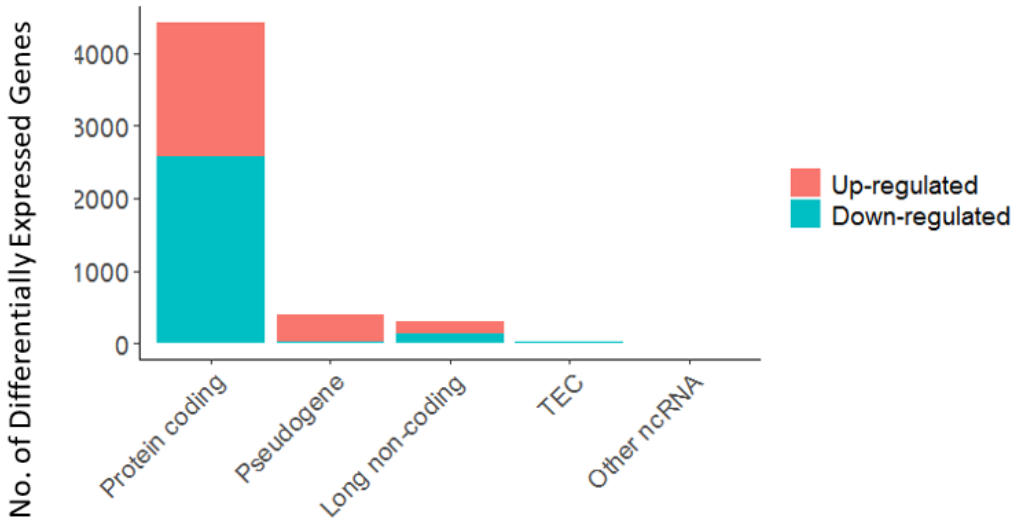

Fig. 1: Bar graph of differentially expressed genes in the GBM samples. TEC: To be Experimentally Confirmed; TR: T cell receptor; IG: Immunoglobulin

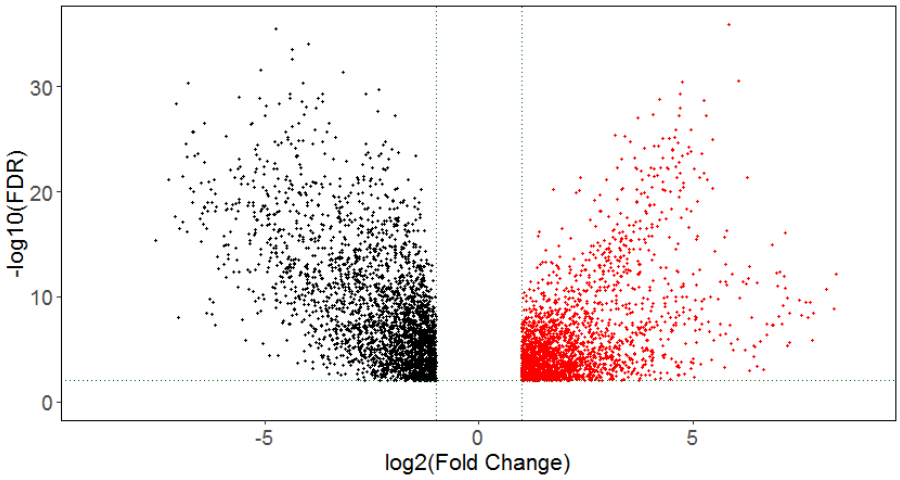

Fig. 2: Volcano plot of differentially expressed genes. Up- and down-regulated genes are demonstrated in red and black, respectively

Table 2: Top 5 up-regulated mRNAs, and lncRNAs

Protein_coding			
symbol	logFC	PValue	FDR
SHOX2	8.37	0.00	0.00
HOXD10	8.31	0.00	0.00
HOXA5	8.14	0.00	0.00
NKX2-5	7.82	0.00	0.00
HOXC10	7.80	0.00	0.00
Long_non_coding			
symbol	logFC	PValue	FDR
AP000924.1	7.17	0.00	0.00
FOXD3-AS1	7.03	0.00	0.00
HOXA-AS2	7.02	0.00	0.00
HOXD-AS2	6.96	0.00	0.00
HOTAIR	6.22	0.00	0.00

Table 3: Top 5 down-regulated mRNAs, and lncRNAs

Protein_coding			
Symbol	logFC	PValue	FDR
GRIN1	-7.55	0.00	0.00
GABRA5	-7.25	0.00	0.00
SV2B	-7.10	0.00	0.00
PRKCG	-7.06	0.00	0.00
SLC17A7	-7.01	0.00	0.00
Long_non_coding			
symbol	logFC	PValue	FDR
RFPL1S	-5.21	0.00	0.00
AC023301.1	-4.92	0.00	0.00
PART1	-4.47	0.00	0.00
LINC00599	-4.32	0.00	0.00
AC107398.3	-3.83	0.00	0.00

GO enrichment and KEGG pathway analysis

Thereby GO enrichment analysis, we indicated several prominent roles of the DEmRNAs, Biological process of GO illustrated that the DEmRNAs are majorly assigned to modulation of chemical synaptic transmission, regulation of trans-synaptic signaling, and axonogenesis, regulation of neuron. Moreover, the cellular component of GO depicted that the genes were significantly classified in neuron-to-neuron synapse, postsynaptic specialization, and synaptic membrane. Moreover, the GO molecular function part showed that the DEmRNAs were dominantly enriched in tubulin binding, microtubule binding, and calmodulin binding (Fig. 3). Furthermore, KEGG pathway analysis showed that the DEmRNAs are remarkably attributed to Circadian entrainment, Glutamatergic synapse, and and MAPK signaling pathway (Fig. 4).

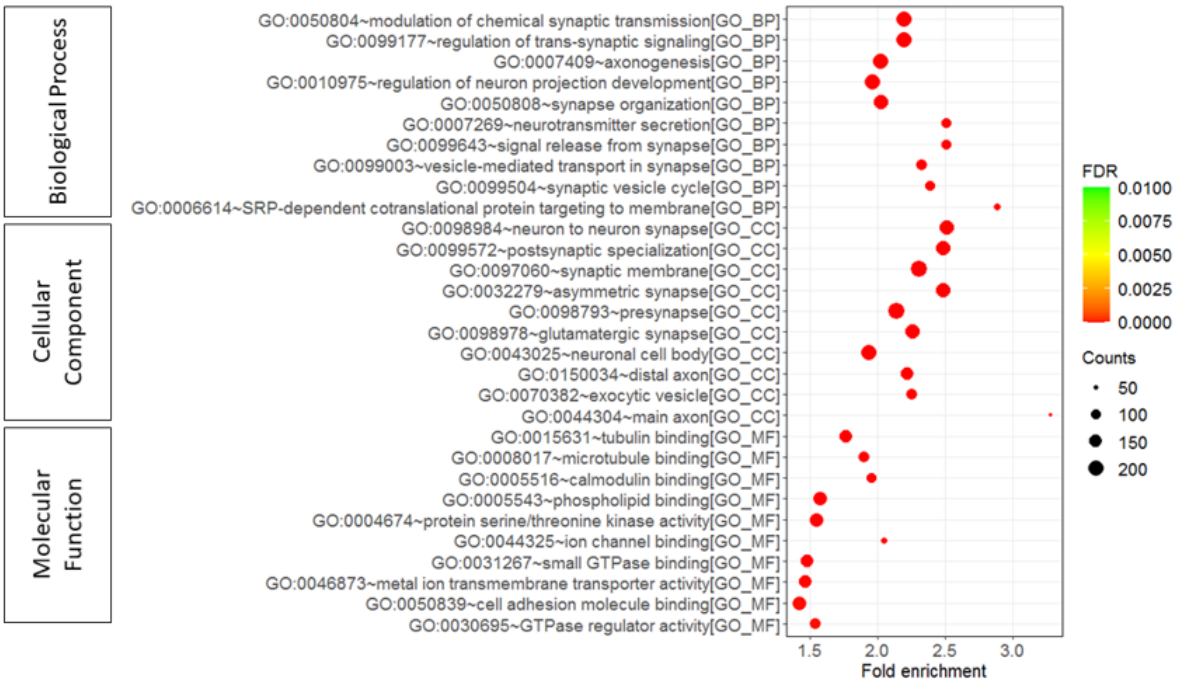

Fig. 3: GO enrichment analysis (Biological Process, Cellular Component, Molecular Function) of the differentially expressed mRNAs in GBM patients (Top 10 GO enrichments are presented)

PPI network construction

For a better understanding of the protein-protein interactions, we constructed a PPI network of the DEmRNAs via the STRING database. The data showed that WEE1, BARD1, CDK6 etc. were the important PPI hubs (Fig. 5). Furthermore, the data showed that several crucial cancerous proteins which linked to the hub such as KCNC1, KCNC2, etc. Among the dominant hubs several cancer key proteins are presented such as PLK, CXCR4, GNG and SSTR.

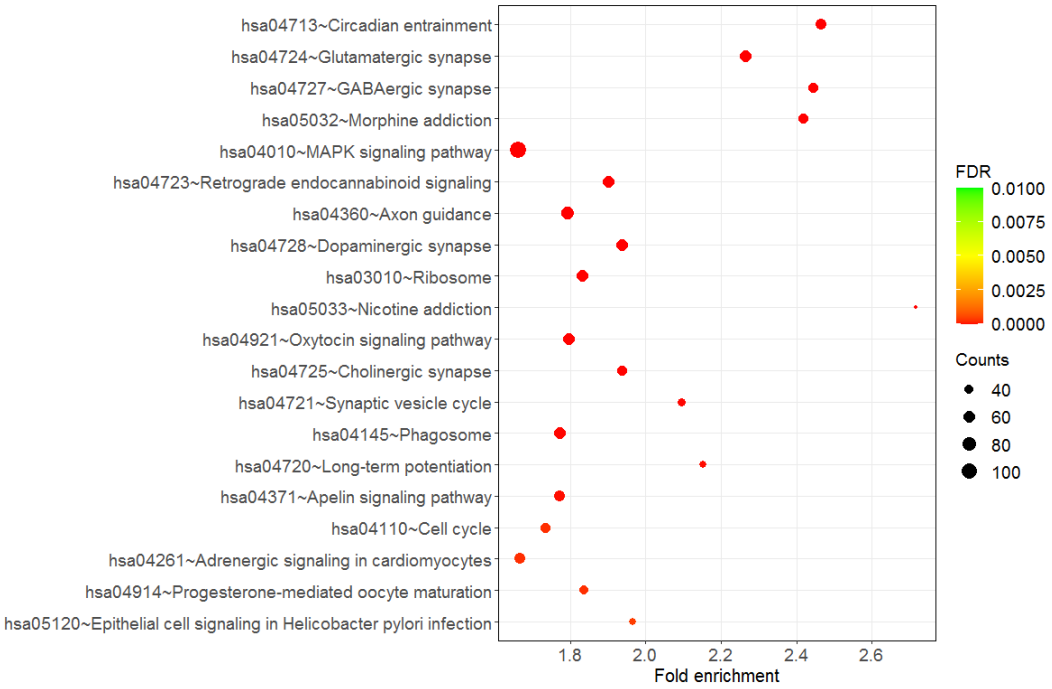

Fig. 4: KEGG signaling pathway analysis of differentially expressed mRNAs in GBM. (Top 20 KEGG terms are presented)

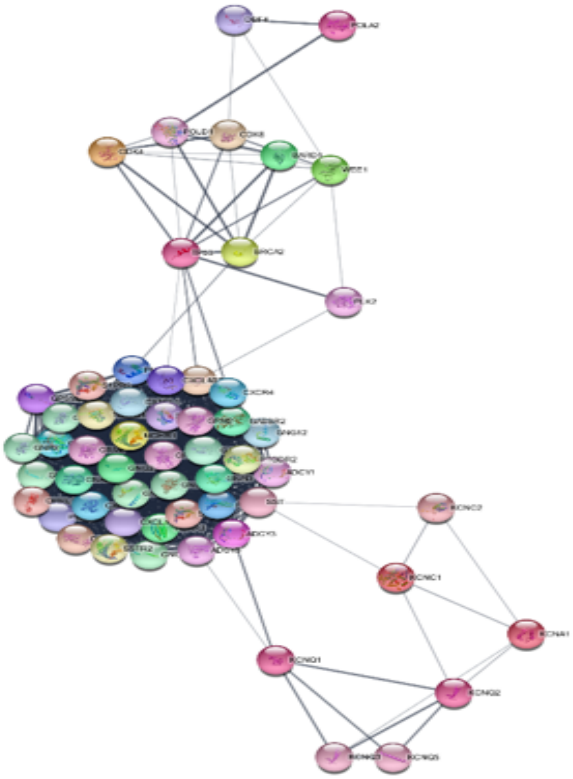

Fig. 5: PPI network of the DE mRNAs in GBM (score > 0.4) with Node:57, eadge:823, MCADE score: 29.393

LncRNA-miRNA-mRNA ceRNA network construction

Based on the competing endogenous RNA (ceRNA) hypothesis, which explains that lncRNAs regulate mRNA expression by competing with shared miRNAs in cells, a ceRNA network was built based on the differentially expressed genes data. The ceRNA network data demonstrated critical lncRNAs including *GAS5, OIP5-AS1, AC093157.1, ASB16-AS1, AC004656.1, LRPC75A-AS1, AC108488.1, HCG11*, and *NORAD* which have an important role in the development of GBM (Fig. 6). Furthermore, the data showed that miRNAs modulatory function between the lncRNAs and mRNAs. Through this ceRNA network, it has been shown miR-30, miR-137, miR-320, miR-15, miR-590, miR-144, miR-155, miR-27, miR-185, miR-330, and miR-326 have modulatory effects between critical lncRNAs and its target mRNAs.

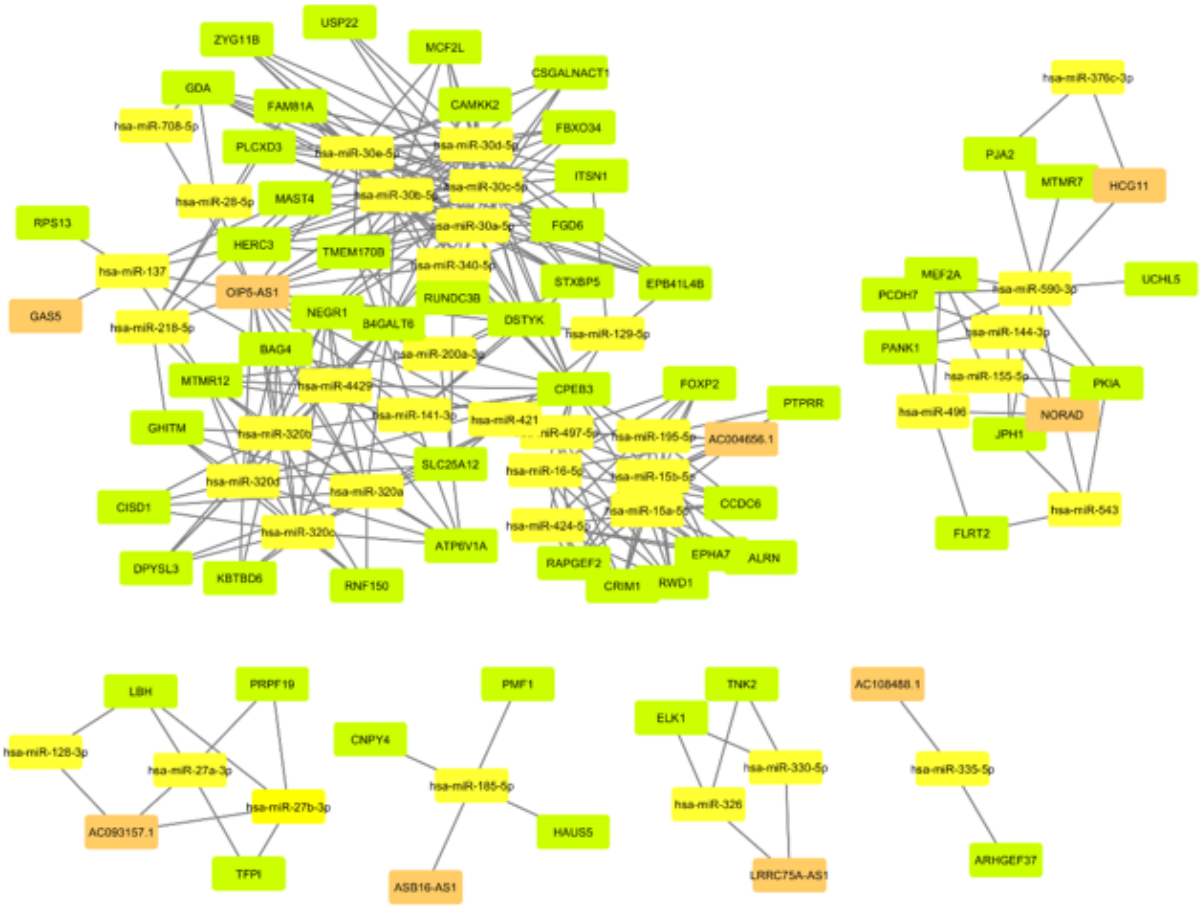

Fig. 6: LncRNA-miRNA-mRNA ceRNA network construction of GBM. (Orange: LncRNA, Yellow: miRNA, and Green: mRNA)

Kaplan-Meier survival analysis of differentially expressed genes

In order to explore the association of differential expression of the genes and the GBM patient's prognosis, a Kaplan-Meier survival analysis was conducted over the differentially expressed genes. The data indicated that 300 mRNAs and 19 lncRNAs were associated with the overall survival rate in the patients. The top 5 hits of each group are presented in Table 4.

Table 4: Top 5 mRNAs, and lncRNAs that were associated with overall survival

mRNA				
symbol	HR	lower95	upper95	*P*-value
PTPRN	2.015783	1.399687	2.903063	4.75E-05
UBXN10	1.915275	1.322754	2.773213	0.000162
RPP25	1.889961	1.306174	2.734667	0.000256
LBH	1.821627	1.254755	2.644602	0.000359
NELL1	1.816388	1.266157	2.605731	0.000691
LncRNA				
	HR	lower95	upper95	*P*-value
CRNDE	1.619327	1.12814	2.324375	0.00595
LINC00957	1.612148	1.124505	2.311259	0.00717
AC072061.1	1.595938	1.115527	2.283243	0.00859
AC068888.1	1.548292	1.082151	2.215224	0.0136
DBH-AS1	1.527936	1.065361	2.191361	0.0166

Diagnostic analysis of differentially expressed lncRNAs

For demonstrating the diagnostic value of each DElncRNAs, AUC curve analysis was accomplished in the GBM samples. All 299 DElncRNAs indicated remarkable diagnostic values in the patients. The top 5 hits of the lncRNAs are presented in Table 5.

Table 5: Top 10 lncRNAs that had remarkable diagnostic value

LncRNA	***AUC***	**P-*value***	***CI 95%***		***Expression***
WAC-AS1	1	0	0	0	Low
SNAI3-AS1	1	0	0	0	Low
AL158212.3	1	0	0	0	Low
AP001486.2	1	0	0	0	Low
AC005070.3	1	0	0	0	Low

Novel Diagnostic and prognostic lncRNA biomarkers

Hereby merging the prognostic, and the diagnostic value data, we found that 19 lncRNAs had high ranks in prognostic and diagnostic areas, considered GBM biomarkers. The top 5 data are presented in Table 6 and Fig. 7.

Table 6: The lncRNAs as diagnostic and prognostic biomarkers in GBM

Symbol	***HR***	***Lower95***	***Upper95***	**P-*value***	***AUC***	**P-*value***	***logFC***	**P-*value***
CRNDE	1.62	1.13	2.32	0.01	0.99	0.00	5.21	0.00
LINC00957	1.61	1.12	2.31	0.01	0.99	0.00	-2.42	0.00
AC072061.1	1.60	1.12	2.28	0.01	0.96	0.00	-1.39	0.00
AC068888.1	1.55	1.08	2.22	0.01	0.90	0.00	-1.27	0.00
DBH-AS1	1.53	1.07	2.19	0.02	0.85	0.01	-1.68	0.00

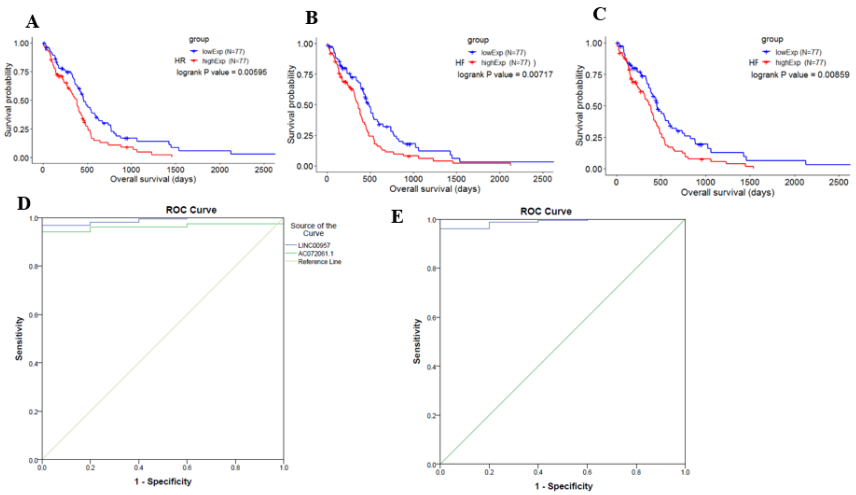

Fig. 7: Kaplan-Meier and ROC curve analysis. A. Kaplan-Meier curve of *CRNDE*. *B.* Kaplan- Meier curve *of LINC00957*. C. Kaplan-Meier curve of *AC072061.1*. D. ROC curve of the lncRNA *LINC00957* and *AC072061.1*. E. ROC curve of the lncRNA *CRNDE*.

The lncRNAs are related to different types of cancer

In the previous section of our study, the lncRNA *CRNDE, LINC00957,* and *AC072061.1* have shown promising diagnostic and prognostic values in the GBM patients. In order to illustrate the lncRNA's contribution to different sorts of cancer, LncRNADisease v2.0 and GEPIA datasets have been considered comprehensively. There are several studies that demonstrates the lncRNA *CRNDE, LINC00957,* and *AC072061.1* contributions to different cancers (Table 7).

Table 7: The lncRNAs and related cancers

LncRNA	***LncRNADisease v2.0***	***GEPIA***
CRNDE	colorectal cancer	Adenoid Cystic Carcinoma
	stomach cancer	cervical squamous cell carcinoma
	cervical cancer	Colon adenocarcinoma
	breast cancer	Diffuse Large B-Cell Lymphoma
	Glioma	Glioblastoma
	gallbladder cancer	Kidney renal clear cell carcinoma
	malignant glioma	Low-grade gliomas
	renal cell carcinoma	Liver Hepatocellular Carcinoma
	ovarian cancer	Pancreatic adenocarcinoma
	pancreatic cancer	Rectum Adenocarcinoma
		Tenosynovial Giant Cell Tumor
		Thymus Cancer
		Uterine carcinosarcoma
LINC00957	-	cervical squamous cell carcinoma endocervical adenocarcinoma
		Glioblastoma
		Kidney Chromophobe
		Low-grade gliomas
		Uterine carcinosarcoma
AC072061.1	Lymphoma	-
	breast cancer	
	cervical cancer	
	colorectal cancer	
	hepatocellular carcinoma	
	malignant glioma	
	melanoma	
	neuroblastoma	
	non-small cell lung carcinoma	
	ovarian cancer	

Discussion

There is a large amount of evidence that shows lncRNA plays an important role in cell biological function. (17). A large body of studies has shown that disrupting the regulation of lncRNAs leads to cell growth, proliferation, and finally carcinogenesis (18). We showed that lncRNA expression in GBM samples has significantly changed compared to normal counterpart brain tissue. Moreover, the results showed the relationship between lncRNA, mRNA, and miRNA expression, which plays an important role in GBM pathogenicity and prognosis (19, 20). Furthermore, GO enrichment and KEGG signaling pathway analysis showed the differentially expressed genes majorly enriched in canonical pathways, particularly in tumorigenesis such as the MAPK signaling pathway. The MAPK signaling pathway is involved in various types of tumor development and progression (21, 22). Downregulation of *MALAT1* leads to cancer cell proliferation and progression, while overexpression of *MALAT1* reduces cell proliferation and invasion. The tumor suppressor effect of *MALAT1* on glioma is mediated by mitogen-activated protein kinase (MAPK) and metalloproteinase 2 (MMP2) in the cells (23).

The ceRNA network data demonstrated critical lncRNAs including *GAS5, OIP5-AS1*, *AC093157.1*, *ASB16-AS1, AC004656.1*, *LRPC75A-AS1, AC108488.1*, *HCG11*, and *NORAD* which have an important role in the development of GBM.

Decreased expression of lncRNA *HCG11* has been identified in GBM. Overexpression of lncRNA *HCG11* effectively inhibits cell proliferation, cell cycle, and apoptosis. lncRNA *HCG11* plays a tumor suppressor role in GBM. Analysis of the KEGG pathway showed a potential link between lncRNA *HCG11* and the Wnt signaling pathway (24). LncRNA *ASB16-AS1* drives cell proliferation, chemoresistance and stemness of the cancer cells by activating the NF-κB pathway. lncRNA *ASB16-AS1* has been shown to be upregulated in NSCLC tissues and cells which contributes to promoting cell proliferation and inhibits apoptosis of NSCLC cells through the Wnt/β-catenin signaling pathway (25).

LncRNA *GAS5* has been demonstrated as a tumor-suppressor gene in cells. Downregulation of *GAS5* could increase cell cycle promotion in the cells (17). *GAS5* is also downregulated in gastric cancer and acts as a poor prognostic factor for the survival rate of gastric cancer patients. More investigation has to be conducted to understand the exact mechanism of GAS5 used for the diagnosis and treatment of different cancers (17).

LncRNA NORAD has been reported to have an important role in the development and growth of tumor cells by regulation of proliferation and migration in the cells (26). NORAD act as a ceRNA sponges for miR-608, and besides, upregulates the expression of FOXO6. NORAD could act as a diagnostic and prognostic biomarker for CRC. Further studies on the role of NORAD in tumorigenesis might be useful for therapeutic approaches to different types of cancers (26).

Wide number of studies demonstrated prominent miRNA roles in cancer (27). In the ceRNA network, we showed crucial miRNAs in cancer which play important modulatory function particularly in GBM development. MiR-30 induces cell apoptosis, and ameliorates cell proliferation and invasion in glioblastoma cells (28). MiR-137 has been demonstrated to be down-regulated in GBM patients and suppresses of Cox-2 (29). MiR-137 inhibits cell proliferation, angiogenesis and induces differentiation of tumor stem cells of glioblastoma multiforme (30). MiR-320 acts as a tumor-suppressor miRNA in GBM cells. miR-320 inhibits cell growth and proliferation by down regulating PBX3 and E2F1 in glioma cells (31, 32). MiR-590 has been depicted that inhibited cell invasion, migration, and epithelial–mesenchymal transition (EMT) through Down-regulating ZEB1 and ZEB2 in glioblastoma multiforme cells (33). A large body of studies demonstrated anti-tumor activity of miR-144 in GBM. miR-144 inhibits cell proliferation and metastasis by targets c-Met in GBM (34). miR-155 has demonstrated oncogenic function in GMB

cells. miR-155 enhances cell progression and growth by augmenting Wnt/β-catenin pathway and suppressing GABA receptors respectively in GBM (35, 36). Numerous investigations demonstrated that miR-27 has a prominent tumorigenesis role in GBM. MiR-27 overexpression in glioma clinical samples. Furthermore miR-27 expression is associated with STAT3, c-myc and cyclin D1 expression in glioma cells (37). MiR-185 has been detected to have dominant effects on tumorigenesis of glioblastoma growth and progression. Leucine-rich repeat C4 (LRRC4) has been shown to reduce cell growth and metastasis of glioma cells by modulating miR-185- Dependent Pathway (38). Furthermore, miR155HG/miR-185/ANXA2 loop contributes to cell proliferation and progression in GBM cells (39). A large number of investigations showed that miR-330 inhibits cell progression and invasion of GBM cells through down regulation of MSI1 and ITGA5 expression of glioma (40, 41). Recently, miR-326 is associated with desired prognosis and good clinical outcome in GBM patients (42). MiR-326 is a tumor suppressor miRNA which inhibits SMO oncogene and PI3 kinase pathway in glioma cells (43, 44).

In the last part of our results, we demonstrated the lncRNA-related cancers according to the datasets. This part emphasizes on the lncRNA contributions to different cancers. Even though numerous reports explained lncRNA roles in GBM, in this work, we thoroughly presented lncRNA, miRNA, and mRNA networks and proposed a novel lncRNA-based biomarker for the patients.

Conclusion

Altogether, in our study, we demonstrated lncRNA, miRNA, and mRNA interaction and mentioned regulatory networks, which can be considered as a therapeutic approach in GBM. In addition, the results demonstrated that the lncRNA *CRNDE, LINC00957,* and *AC072061.1* have potential diagnostic and prognostic characteristics for the patients.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Conflict of interest

The authors declare that there is no conflict of interests.

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