Identification and functional characterization of lncRNAs acting as ceRNA involved in the malignant progression of glioblastoma multiforme

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Abstract. Glioblastoma multiforme (GBM) is the most common brain malignancy. Long non-coding RNAs (lncRNAs) are aberrantly expressed in many cancers and involved in pathogenesis, progression and metastasis of tumors. In particular, lncRNAs can function as competing endogenous RNAs (ceRNAs). The functional roles of lncRNA associated-ceRNAs in GBM are not fully understood. Human Exon 1.0 Microarray (Affymetrix) and Human MicroRNA Microarray (Agilent) were used to detect the expression of 955 microRNAs (miRNAs), 33,125 lncRNAs, and 17,453 mRNAs in 8 GBM and 8 normal brain samples. The function of differential mRNA was determined by Gene Ontology (GO) and pathway analysis. The distinctly expressed miRNAs, lncRNAs and mRNAs were subjected to construct miRNA-lncRNA-mRNA interaction network. The expression of miRNAs, lncRNAs and mRNAs in GBM tissues vs. normal brain tissues was examined by quantitative real-time RT-PCR. A total of 41 miRNAs, 398 lncRNAs and 1,995 mRNAs were found to be differentially expressed between the GBM and normal brain groups. GO and pathway analyses had proven that the functions of differentially expressed mRNAs in GBM related closely with many processes important in the cancer pathogenesis. Fifty-five lncRNAs acting as ceRNAs were identified based on the miRNA-lncRNA-mRNA interaction network. The potential roles of the 39 ceRNAs were revealed, which participated in 23 diverse cancer biological pathways, including proliferation, cell apoptosis, adhesion, angiogenesis and metastasis. The identified sets of miRNAs, lncRNAs and mRNAs specific to GBM were verified by qRT-PCR experiment in GBM samples. Our study predicts the biological functions of a multitude of lncRNA associated-ceRNAs in GBM. Moreover, our study provides a road map for the identification and analysis of lncRNA acting as ceRNA in tumors.

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Introduction

Mammalian genomes generate thousands of regulatory RNAs that are either long non-coding RNAs (lncRNAs) or microRNAs (miRNAs) (1,2). lncRNAs are more than 200 nucleotides, and synthesized by RNA polymerase II, spliced and sometimes polyadenylated (3). They are pervasively transcribed, and exhibit spatially and temporally regulated expression patterns (4). Unlike small ncRNAs, lncRNAs can fold into complex secondary and higher order structures to provide greater potential and versatility for both protein and target recognition (5). lncRNAs have been found to play crucial regulatory roles in a diverse range of cellular processes and biological pathways, including genomic imprinting, chromosome inactivation, differentiation and development of many human diseases (6). lncRNAs are emerging as new players in the cancer biology paradigms and their dysfunction are correlated with tumorigenesis and malignancy transformation in various types of cancers (7,8).

miRNAs, the most well characterized ncRNAs, are short endogenous molecules, approximately 22 nucleotides in length, that are processed by the RNase III enzymes Drosha and Dcr. miRNAs post-transcriptionally regulate the gene expression through interaction between the 5' end and the 3' untranslated region (3'UTR) of mRNA. miRNA can guide the RNA-induced silencing complex (RISC) to miRNA response element (MRE) on target transcript, usually resulting in degradation of the transcript or inhibition of its translation (9). Dysregulation of miRNA expression is involved in various

diseases (10). Accumulating evidence highlights the role of miRNA-mediated regulation in cell growth, differentiation, proliferation and apoptosis. Alterations in the miRNA balance in the cell can lead to dysregulation of tumor suppressor genes and/or oncogenes regulated by aberrantly expressed miRNAs, leading to cancer (11,12).

Recent studies have described a complicated interplay among diverse RNA species, including coding and non-coding RNAs. These RNAs inclusive of mRNA, pseudogene, lncRNA or circular RNA, interact and co-regulate with each other by acting as competing endogenous RNAs (ceRNAs). ceRNAs have MRE, and serve as miRNA sponges to control miRNAs available to their target RNAs. ceRNA can sequester miRNAs, thereby protecting their target RNAs from repression (13). Understanding this novel RNA interaction will lead to significant insight into gene regulatory networks in human development and disease. Although lacking 3'UTRs, lncRNAs have been reported to be downregulated by miRNAs and work as ceRNAs. The experimental evidence is already emerging of lncRNAs as competitive platforms for both miRNAs and mRNAs (14,15).

Glioblastoma multiforme (GBM) is the most common and malignant brain tumor with poor prognosis. According to the 2007 World Health Organization classification, gliomas are classified into 4 histopathological grades based on malignancy degree, and GBM is the highest-grade glioma (grade IV) (16). Patients with newly diagnosed GBM exhibit a median survival of approximately 15 months (17). Despite maximal surgical, radiological and chemotherapeutic interventions, these figures have changed little in the past two decades (18). New therapeutic strategies will likely evolve from a better understanding of GBM biology.

Efforts have been made to study the relationship between the lncRNA expression and the GBM pathogenesis (8,19-21), but many more lncRNAs playing crucial roles in GBM remain to be determined. The aberrant miRNA expression has features of GBM (22). Nevertheless, the miRNA-lncRNA-mRNA regulation networks in the GBM, as well as the potential roles of ceRNAs in the biogenesis and development of GBM have not been explored.

In this study, we aimed at profiling the miRNA, lncRNA and mRNA expression signature, and constructing miRNA-lncRNA-mRNA crosstalk by analyzing a cohort of sample-matched exon and miRNA expression microarrays from the Cancer Genome Atlas (TCGA), and predicting the functions of lncRNAs acting as ceRNAs in GBM. The identified sets of lncRNA, miRNA and mRNA specific to GBM were subsequently confirmed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in GBM samples.

Materials and methods

Data-set characteristics. The sample matched whole-transcript and miRNA expression profiling upon GBM were obtained from the TCGA database (https://tcga-data.nci.nih.gov/tcga/). To compare the miRNA, lncRNA and mRNA expression signatures in GBM, we selected 16 data-sets that included 8 GBM and 8 non-tumoral brain samples. Two panels of data-sets were included in our study: Affymetrix Human Exon 1.0 array and Agilent Human MicroRNA array 8x15K.

Data analysis. Two-class differential was used to determine the differentially expressed miRNA, lncRNA and mRNA between the normal and GBM groups. The random variance model (RVM) t-test was applied to filter the differentially expressed genes for it can effectively increase the degrees of freedom in cases of small samples. The false discovery rate (FDR) was calculated to correct the P-value. We selected the differentially expressed miRNAs, lncRNAs and mRNAs according to the P-value and FDR. P-values <0.05 and FDR <0.05 were considered significant.

The differentially expressed probe sets were imported into Cluster and TreeView (Stanford University) to perform hierarchical cluster analysis (HCA) (23).

Gene Ontology (GO) and pathway analysis. A GO analysis was applied to analyze the main functions of the differentially expressed mRNAs (24). Specifically, a two-sided Fisher's exact test and a χ^2 test were used to classify the GO category. We computed P-values of the GO for each differential gene. Enrichment provides a measure for the significant function: As the enrichment increases, the corresponding function is more specific. Within the significant category, the enrichment Re was given as follows: $Re = (n_f/n)/(N_f/N)$, where n_f is the number of flagged genes within the particular category, n is the total number of genes within the same category, N_f is the number of flagged genes in the entire microarray, and N is the total number of genes in the microarray.

Pathway analysis was used to identify the significant pathway of the differential mRNAs according to KEGG, BioCarta and Reactome. We used Fisher's exact test and the χ^2 test to select the significant pathway, and the threshold of significance was defined by P-value and FDR. The enrichment Re was calculated as described above (25).

Construction of lncRNA-mRNA co-expression network. The lncRNA-mRNA networks were built according to the normalized signal intensity of specific mRNA and lncRNA expression in microarray. For each pair of mRNA-lncRNA, mRNA-mRNA or lncRNA-lncRNA, we calculated the Pearson correlation and chose the significant correlation pairs to construct the network (26). In a network analysis, degree is the most important measure of an mRNA or lncRNA centrality within a network. Degree centrality is defined as the link numbers one node has to the other (27). The clustering coefficient represents the density of each gene with the adjacent gene, and the larger the clustering coefficient, the greater importance the gene has in regulating the network.

Patient samples. GBM specimens were derived from patients with GBM who underwent surgical treatment at Beijing Tian Tan Hospital. All histologic diagnoses were made on formalin fixed, paraffin-embedded H&E sections and were reviewed blinded to the original diagnosis according to the 2007 World Health Organization classification. Normal brain tissues were obtained from severe head trauma patients for whom partial resection of normal brain was required during surgery at Beijing Tian Tan Hospital. Samples were collected immediately after surgical resection, snap-frozen and stored in liquid nitrogen. The study was approved by the institutional review board of Beijing Tian Tan Hospital.

Table I. The miRNA, lncRNA and mRNA primers for qRT-PCR.

Primers	Sequences (5'-3')
miR-21	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGATCAACATG
	F: CTCAACTGGTGTCGTGGAGT
	R: ACACTCCAGCTGGGTAGCTTATCAGACTG
miR-27a	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGGAACTT
	F: CTCAACTGGTGTCGTGGAGT
	R: ACACTCCAGCTGGGTTCACAGTGGCTAAG
miR-210	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGATCAGCCGC
	F: CTCAACTGGTGTCGTGGAGT
	R: CACTCCAGCTGGGCTGTGCGTGTGACAG
miR-23a	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAAATCCC
	F: CTCAACTGGTGTCGTGGAGT
	R: CACTCCAGCTGGGATCACATTGCCAGGG
miR-155	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAACCCCTATC
	F: CTCAACTGGTGTCGTGGAGT
	R: ACACTCCAGCTGGGTTAATGCTAATCGTG
miR-139	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAACTGGAGA
	F: CTCAACTGGTGTCGTGGAGT
	R: CACTCCAGCTGGGTCTACAGTGCACGTG
hsa-miR-338	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGACAACAAAAT
	F: CTCAACTGGTGTCGTGGAGT
	R: ACACTCCAGCTGGGTATTGCACTCGTCC
miR-137	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGACTACGCGTA
	F: CTCAACTGGTGTCGTGGAGT
	R: ACACTCCAGCTGGGTTATTGCTTAAGAAT
miR-7	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAACAACAAA
	F: CTCAACTGGTGTCGTGGAGT
	R: CACTCCAGCTGGGTGGAAGACTAGTGAT
miR-124a	RT: TCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCATTCAC
	F: CTCAACTGGTGTCGTGGAGT
	R: CACTCCAGCTGGGTAAGGCACGCGGTGA
miR-15a	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGACACAAACCA
	F: CTCAACTGGTGTCGTGGAGT
	R: CACTCCAGCTGGGTAGCAGCACATAATG
miR-29b	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAAACACTGAT
	F: CTCAACTGGTGTCGTGGAGT
	R: CACTCCAGCTGGGTAGCACCATTTGAAA
miR-29c	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGATAACCGATT
	F: CTCAACTGGTGTCGTGGAGT
	R: CACTCCAGCTGGGTAGCACCATTTGAAA
ENST00000520186	F: GTTGGACCTTACTGAGGCCG
	R: GGAGACACCATGGCTGGAAC
ENST00000559981	F: AGAGTGAAATTTTGTATAAGCACCA
	R: GCCTGGAGACATACTGAGATGG
ENST00000547415	F: TGCCATCTGCAGAGTGAAACT
	R: GGCTTTCCAGTCTAGGGCAG
ENST00000518554	F: TGGCATTTTGTCAGTTTTCCCG
	R: GCAAATGCACACACCACTCC
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA

Table I. Continued.

Primers		Sequences (5'-3')
RNU6	F:	CTCGCTTCGGCAGCACA
	R:	AACGCTTCACGAATTTGCGT
AKT3	F:	TTGCTTTCAGGGCTCTTGAT
	R:	CATAATTTCTTTGCATCATCTGG
PPP3CA	F:	TGTGATATCCTGTGGTCAGA
	R:	CTGACTGTGTTGTGAGTGAA
LAMC1	F:	TGGGCATTCTTCTGTCTGTACAA
	R:	GCCACCCATCCTCATCAATC
TNFRSF1A	F:	TGCCTACCCCAGATTGAGAA
	R:	ATTTCCCACAAACAATGGAGTAG

miRNA, microRNA; lncRNA, long non-coding RNA.

RNA preparation and qRT-PCR. Total RNA from tissue specimens was extracted using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA integrity was analyzed on a 1.2% agarose gel. RNA quantity was determined using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA (1 μg) was reverse transcribed with a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) for cDNA synthesis and genomic DNA removal. For miRNA detection, total RNA was reverse transcribed using miRNA specific primers. qPCRs were performed according to the instructions of the SYBR Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd.) and carried out in the Takara real-time PCR system. Gene-specific primers were designed using online primer designing tools primer-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences are listed in Table I. The lengths of amplifications are between 100 and 250 bp. Standard deviations were calculated from three PCR replicates. The specificity of amplification was assessed by dissociation curve analysis and the relative abundance of genes was determined using the comparative $\Delta\Delta$Ct method.

Results

GBM demonstrates significantly altered miRNA, lncRNA and mRNA expression patterns comparing with that of the normal brain. In terms of the Sanger miRBase database, 866 human and 89 human viral miRNAs were authenticated on the Agilent Human MicroRNA array 8x15K. Based on the NetAffx annotation of the probe sets, the Ensemble, NOCODE3.0, and UCSC annotations of lncRNAs, and the RefSeq, Ensemble and GenBank annotations of mRNAs, we identified 33,125 lncRNAs (corresponding to 44,482 probe sets) and 17,453 mRNAs (corresponding to 22,011 probe sets) represented on the Affymetrix Human Exon 1.0 array (data not shown).

The expression patterns of miRNAs, lncRNAs and mRNAs were detected in 8 GBMs and 8 normal brain samples. We identified 41 miRNAs, 398 lncRNAs and 1,995 mRNAs that had significant differential expression in the GBM group comparing with the normal brain group (fold change ≥2.0 or ≤0.5 and P-value <0.05, data not shown).

The hierarchical clustering analysis showed that with the differential expression of these miRNAs, lncRNAs and mRNAs, samples were non-random partitioned, they were divided into 2 groups, the first group containing 8 normal brain samples and the second group containing 8 GBM samples (Fig. 1). Thus, the miRNA, lncRNA and mRNA expression signatures identified here were likely to be representative.

Construction of miRNA-lncRNA-mRNA interaction network and identification lncRNAs acting as ceRNAs. The miRNA-lncRNA-mRNA network was constructed according to the study flow summarized in Fig. 2.

First, the target mRNAs of the differentially expressed miRNA were analyzed by TargetScan and miRanda method, termed as target 1 mRNAs (6,737 mRNAs, data not shown). The intersection of the target 1 mRNAs and differentially expressed mRNAs in GBM was picked and obtained target 2 mRNAs (1,034 mRNAs, data not shown). Of the target 2 mRNAs, the mRNAs were selected with expression levels negatively correlated with miRNA expression, and were termed the N&T mRNAs (749 mRNAs, data not shown).

Then, GO and pathway analysis were applied to analyze the significant function and pathway of the N&T mRNAs. GO analysis results showed that upregulated and downregulated mRNAs respectively were involved in 156 and 240 items with significant functions (P-value <0.01, data not shown). The pathway analysis revealed that there were 65 and 24 significant pathways corresponding to the up and downregulated mRNAs respectively (P-value <0.01, data not shown).

The third step, the mRNAs that contained both the significant function and pathway were termed G&P mRNAs (248 mRNAs, data not shown). The G&P mRNAs and differently expressed lncRNAs were used to build the lncRNA-mRNA co-expression network, respectively, in the normal and GBM group (data not shown).

The TargetScan method was used to analysis the target lncRNAs of differentially expressed miRNA and obtained the 55 miRNA targeted lncRNAs. These 55 lncRNAs were

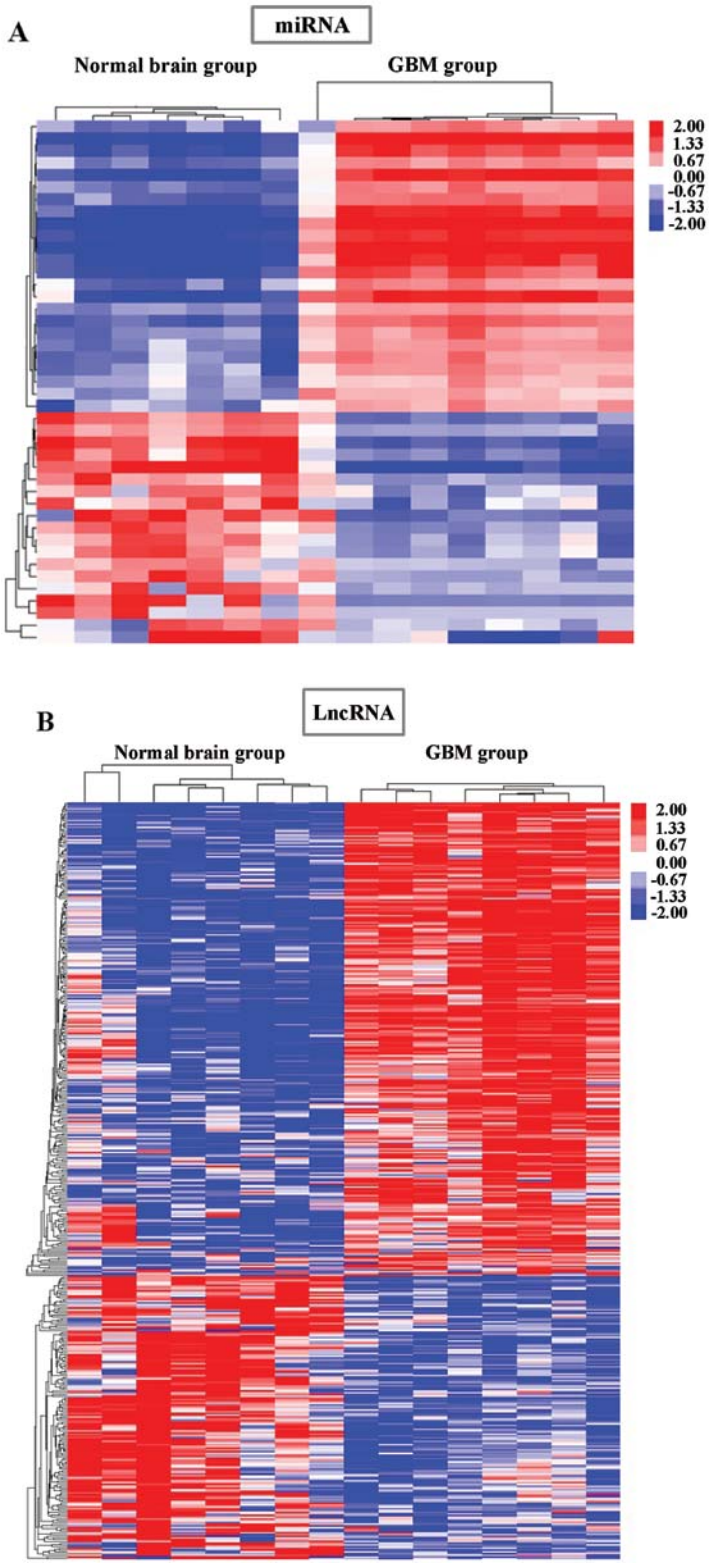

Figure 1. Hierarchical clustering analysis of miRNA, lncRNA and mRNA expression levels change between two groups (normal brain and GBM). (A) Forty-one miRNAs and (B) 398 lncRNAs.

identified ceRNAs. Based on the interaction network of miRNA-mRNA, miRNA-lncRNA and lncRNA-mRNA, we obtained 224 feed-forward loop networks and constructed general miRNA-lncRNA-mRNA feed-forward loop network (data not shown). All of miRNAs, lncRNAs and mRNAs and their relations in this network are listed in Table II.

Biological prediction of lncRNA function as ceRNAs in the GBM. The functions of 55 lncRNAs acting as ceRNAs were predicted through pathway analysis of 67 mRNAs in the miRNA-lncRNA-mRNA interaction network. The results indicated that 30 mRNAs participated in 7 upregulated and 16 downregulated pathways which

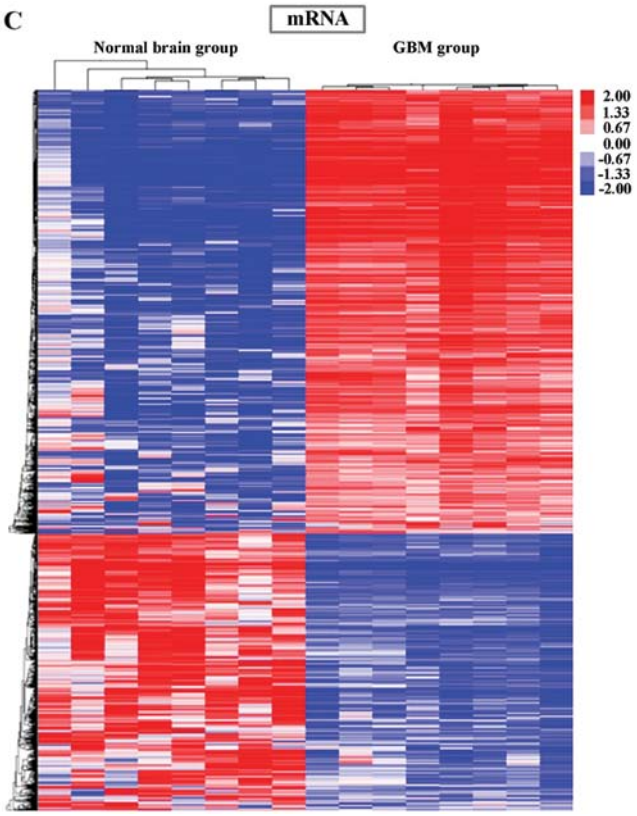

Figure 1. Continued. (C) mRNAs (1,995) are differentially expressed in GBM tissue (≥2-fold or ≤0.5-fold change; P<0.05 and FDR<0.05). Columns represent samples and rows, respectively, represent miRNA, lncRNA or mRNA probe sets. Red, represents high expression; green, represents low expression, indicating expression above and below the median expression value across all of the samples, respectively (log scale, 2; from -1.80 to +1.80). miRNA, microRNA; lncRNA, long non-coding RNA; GBM, glioblastoma multiforme; FDR, false discovery rate.

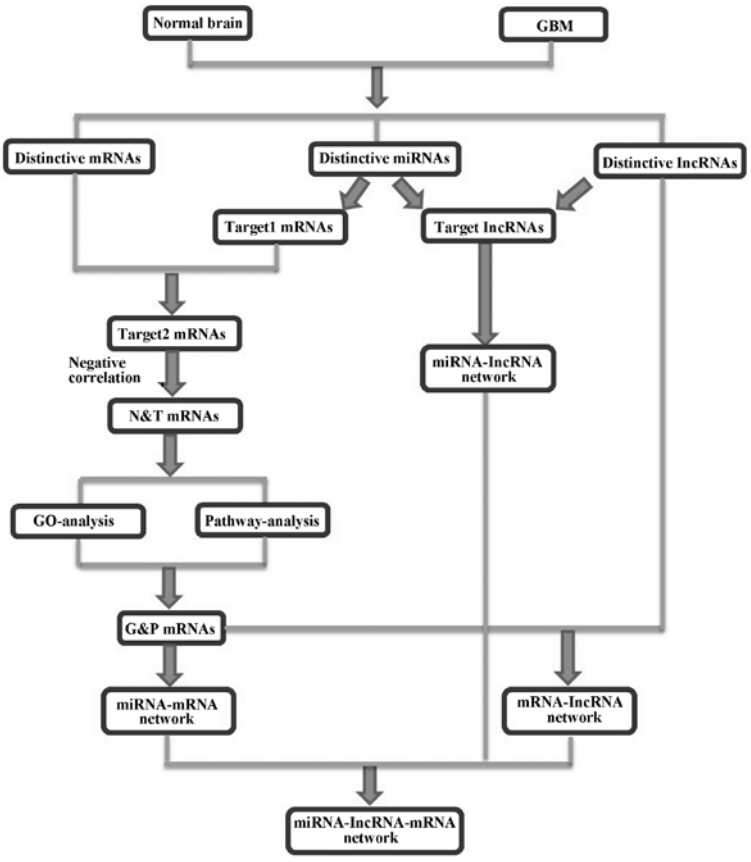

Figure 2. Schematic overview of the study flow for construction of miRNA-lncRNA-mRNA interaction network. miRNA, microRNA; lncRNA, long non-coding RNA.

Table II. The 224 feed-forward loops including miRNAs, lncRNAs and mRNAs.

No.	miRNA	lncRNA	mRNA	No.	miRNA	lncRNA	mRNA
1	miR-15a	n339339	PAK7	53	miR-15b	n338128	PTPRR
2	miR-15a	n339339	CACNA1E	54	miR-15b	ENST00000566630	CACNA1E
3	miR-15a	ENST00000520186	PAK7	55	miR-15b	ENST00000524501	CACNA1E
4	miR-15a	ENST00000533229	PAK7	56	miR-15b	n341995	CACNA1E
5	miR-15a	ENST00000566630	PAK7	57	miR-15b	n406352	CACNA1E
6	miR-15a	n341995	PAK7	58	miR-15b	n410578	SYNJ1
7	miR-15a	n346032	CACNA1E	59	miR-15b	ENST00000524501	NMNAT2
8	miR-15a	n346032	NMNAT2	60	miR-15b	n411142	AKT3
9	miR-15a	ENST00000520186	MAPK9	61	miR-15b	ENST00000492667	AKT3
10	miR-15a	ENST00000520186	AKT3	62	miR-15b	ENST00000434383	SGCD
11	miR-15a	ENST00000559981	VAMP1	63	miR-15b	n411142	SYNJ1
12	miR-15a	ENST00000559981	SYNJ1	64	miR-15b	ENST00000492667	SYNJ1
13	miR-15a	ENST00000596580	VAMP1	65	miR-23a	n339339	SLIT1
14	miR-15a	ENST00000596580	AKT3	66	miR-23a	n339339	RXRG
15	miR-15a	ENST00000596580	SYNJ1	67	miR-23a	n339339	SLC1A1
16	miR-15a	ENST00000596580	SLC9A6	68	miR-23a	ENST00000562191	GABRB2
17	miR-15a	ENST00000569946	VAMP1	69	miR-23a	ENST00000562191	NEFL
18	miR-15a	ENST00000532691	CACNA1E	70	miR-23a	ENST00000562191	MEF2C
19	miR-15a	ENST00000566942	HTR2A	71	miR-23a	n346032	GABRB3
20	miR-15a	n338128	PTPRR	72	miR-23a	n346032	PCLO
21	miR-15a	ENST00000566630	CACNA1E	73	miR-23a	ENST00000522102	SRGAP3
22	miR-15a	ENST00000524501	CACNA1E	74	miR-23a	n385835	GABRA4
23	miR-15a	ENST00000578746	CACNA1E	75	miR-23a	n383510	FUT9
24	miR-15a	n341995	CACNA1E	76	miR-23a	n383510	CADM3
25	miR-15a	n410578	SYNJ1	77	miR-23a	ENST00000559981	ATP6V1C1
26	miR-15a	ENST00000524501	NMNAT2	78	miR-23a	ENST00000559981	SYNJ1
27	miR-15a	ENST00000578746	NMNAT2	79	miR-23a	ENST00000559981	TLN2
28	miR-15a	n411142	AKT3	80	miR-23a	ENST00000555811	GABRB3
29	miR-15a	ENST00000492667	AKT3	81	miR-23a	ENST00000566630	GABRB3
30	miR-15a	ENST00000434383	SGCD	82	miR-23a	ENST00000524501	GABRB3
31	miR-15a	n411142	SYNJ1	83	miR-23a	ENST00000555811	FUT9
32	miR-15a	ENST00000492667	SYNJ1	84	miR-23a	ENST00000555811	PCLO
33	miR-15b	n339339	PAK7	85	miR-23a	n411142	ATP6V1C1
34	miR-15b	n339339	CACNA1E	86	miR-23a	n373066	FUT9
35	miR-15b	ENST00000520186	PAK7	87	miR-23a	ENST00000524501	PCLO
36	miR-15b	ENST00000533229	PAK7	88	miR-23a	ENST00000524501	NRXN3
37	miR-15b	ENST00000566630	PAK7	89	miR-23a	ENST00000434383	RXRG
38	miR-15b	n341995	PAK7	90	miR-23a	ENST00000434383	SGCD
39	miR-15b	n406352	PAK7	91	miR-23a	ENST00000502752	RAB11FIP2
40	miR-15b	n346032	CACNA1E	92	miR-23a	n384012	TLN2
41	miR-15b	n346032	NMNAT2	93	miR-23a	n411142	SYNJ1
42	miR-15b	ENST00000520186	MAPK9	94	miR-30a	n346032	GRM5
43	miR-15b	ENST00000520186	AKT3	95	miR-30a	ENST00000522102	SRGAP3
44	miR-15b	ENST00000559981	VAMP1	96	miR-30a	n373066	CAMK4
45	miR-15b	ENST00000559981	SYNJ1	97	miR-30a	ENST00000520186	PSD3
46	miR-15b	ENST00000596580	VAMP1	98	miR-30a	ENST00000473866	GNAO1
47	miR-15b	ENST00000596580	AKT3	99	miR-30a	ENST00000532691	GRM5
48	miR-15b	ENST00000596580	SYNJ1	100	miR-30a	ENST00000569946	GRM3
49	miR-15b	ENST00000596580	SLC9A6	101	miR-30a	ENST00000569946	CACNA1C
50	miR-15b	ENST00000569946	VAMP1	102	miR-30a	n339481	CACNA1C
51	miR-15b	ENST00000532691	CACNA1E	103	miR-30a	ENST00000532691	B4GALT6
52	miR-15b	ENST00000566942	HTR2A	104	miR-30a	ENST00000569946	NEFL

Table II. Continued.

No.	miRNA	lncRNA	mRNA	No.	miRNA	lncRNA	mRNA
105	miR-30a	ENST00000566630	B4GALT6	157	miR-106b	ENST00000555811	B4GALT6
106	miR-30a	n374560	GDA	158	miR-106b	n345100	RIMS2
107	miR-30a	ENST00000471736	GRIA2	159	miR-106b	n337874	B4GALT6
108	miR-30a	ENST00000569946	NEFM	160	miR-106b	n345100	SCN1A
109	miR-30a	ENST00000530447	B4GALT6	161	miR-106b	n411142	AKT3
110	miR-27a	n339339	IQSEC1	162	miR-106b	ENST00000434383	SGCD
111	miR-27a	n339339	PDE3B	163	miR-25	ENST00000493303	PRKCE
112	miR-27a	ENST00000564076	CACNB2	164	miR-25	ENST00000549205	MAP2K4
113	miR-27a	ENST00000562191	SNAP25	165	miR-25	ENST00000555811	ST6GAL2
114	miR-27a	n346032	GABRB3	166	miR-25	n337874	ST6GAL2
115	miR-27a	n346032	SYT1	167	miR-25	ENST00000566630	ST6GAL2
116	miR-27a	n346032	PCLO	168	miR-25	ENST00000530447	ST6GAL2
117	miR-27a	ENST00000522102	SRGAP3	169	miR-25	ENST00000549205	PRKCE
118	miR-27a	ENST00000566630	GABRB3	170	miR-25	n345100	CACNA1C
119	miR-27a	n406352	GABRB3	171	miR-25	n345100	NEFL
120	miR-27a	n371475	CDS1	172	miR-25	ENST00000555811	RIMS2
121	miR-27a	n338494	CACNB2	173	miR-25	n345100	RIMS2
122	miR-27a	n345100	SYT1	174	miR-25	n345100	SYT1
123	miR-27a	n345100	SNAP25	175	miR-2	n410578	SYNJ1
124	miR-27a	n373066	FUT9	176	miR-25	n406352	PRKCE
125	miR-27a	n339978	SYT1	177	miR-25	n411142	SYNJ1
126	miR-27a	n406352	PDE3B	178	miR-25	ENST00000492667	SYNJ1
127	miR-27a	n406352	ATP6V1A	179	miR-223	ENST00000493303	PRKCE
128	miR-27a	ENST00000492667	ATP2B1	180	miR-223	n371741	ATP2B1
129	miR-34a	n339339	CACNA1E	181	miR-223	n406352	PRKCE
130	miR-34a	n346032	CACNA1E	182	miR-223	ENST00000492667	ATP2B1
131	miR-34a	n346032	GABRA3	183	miR-155	n345100	GABRA1
132	miR-34a	n346032	SYT1	184	miR-155	n346032	DYNC1I1
133	miR-34a	n383510	FUT9	185	miR-155	n341006	RAB11FIP2
134	miR-34a	ENST00000549205	PSD3	186	miR-155	ENST00000596580	ATP6V1C1
135	miR-34a	ENST00000549205	PRKCE	187	miR-155	n345100	CACNA1C
136	miR-34a	ENST00000596580	SYNJ1	188	miR-155	ENST00000535764	ATP6V1C1
137	miR-34a	ENST00000555811	CACNA1E	189	miR-155	n411142	ATP6V1C1
138	miR-34a	ENST00000555811	FUT9	190	miR-155	n345100	SCN1A
139	miR-34a	ENST00000555811	PCLO	191	miR-155	n345100	DYNC1I1
140	miR-34a	n384244	CNTN2	192	miR-155	ENST00000578746	DYNC1I1
141	miR-34a	ENST00000566630	CACNA1E	193	miR-2	ENST00000559981	PPP3CA
142	miR-34a	n406352	CACNA1E	194	miR-21	n411142	PPP3CA
143	miR-34a	n410578	SYNJ1	195	miR-21	ENST00000492667	PPP3CA
144	miR-34a	ENST00000569946	CPLX2	196	miR-92b	ENST00000555811	ST6GAL2
145	miR-34a	n339978	SYT1	197	miR-92b	n337874	ST6GAL2
146	miR-34a	n406352	WASF1	198	miR-92b	ENST00000530447	ST6GAL2
147	miR-34a	n406352	PRKCE	199	miR-92b	ENST00000596580	SYNJ1
148	miR-34a	ENST00000492667	SYNJ1	200	miR-92b	n345100	CACNA1C
149	miR-106b	ENST00000440363	PDE3B	201	miR-92b	n345100	NEFL
150	miR-106b	ENST00000522102	SRGAP3	202	miR-92b	ENST00000555811	RIMS2
151	miR-106b	ENST00000596580	PIP4K2A	203	miR-92b	n345100	RIMS2
152	miR-106b	ENST00000596580	AKT3	204	miR-92b	n345100	SYT1
153	miR-106b	n345100	MAPK9	205	miR-92b	n410578	SYNJ1
154	miR-106b	n337874	PIP4K2A	206	miR-92b	n411142	SYNJ1
155	miR-106b	n337874	GABBR1	207	miR-92b	ENST00000492667	SYNJ1
156	miR-106b	ENST00000555811	RIMS2	208	miR-339	n383510	CADM3

Table II. Continued.

No.	miRNA	lncRNA	mRNA
209	miR-339	ENST00000555811	DAAM2
210	miR-339	ENST00000555811	PCLO
211	miR-339	ENST00000523571	DAAM2
212	miR-339	ENST00000481854	SGCD
213	miR-10b	ENST00000221169	TIAM1
214	miR-10b	ENST00000555811	RIMS2
215	miR-10b	ENST00000524501	RIMS2
216	miR-29b	ENST00000481203	TNFRSF1A
217	miR-29b	ENST00000518554	TNFRSF1A
218	miR-29b	ENST00000547415	LAMC1
219	miR-29b	ENST00000559148	MYBL2
220	miR-377	n338229	LAMC1
221	miR-124	n376998	EDNRB
222	miR-29c	ENST00000547415	LAMC1
223	miR-29c	ENST00000559148	MYBL2
224	miR-29c	ENST00000518554	TNFRSF1A

lncRNAs, long non-coding RNAs; miRNAs, microRNAs.

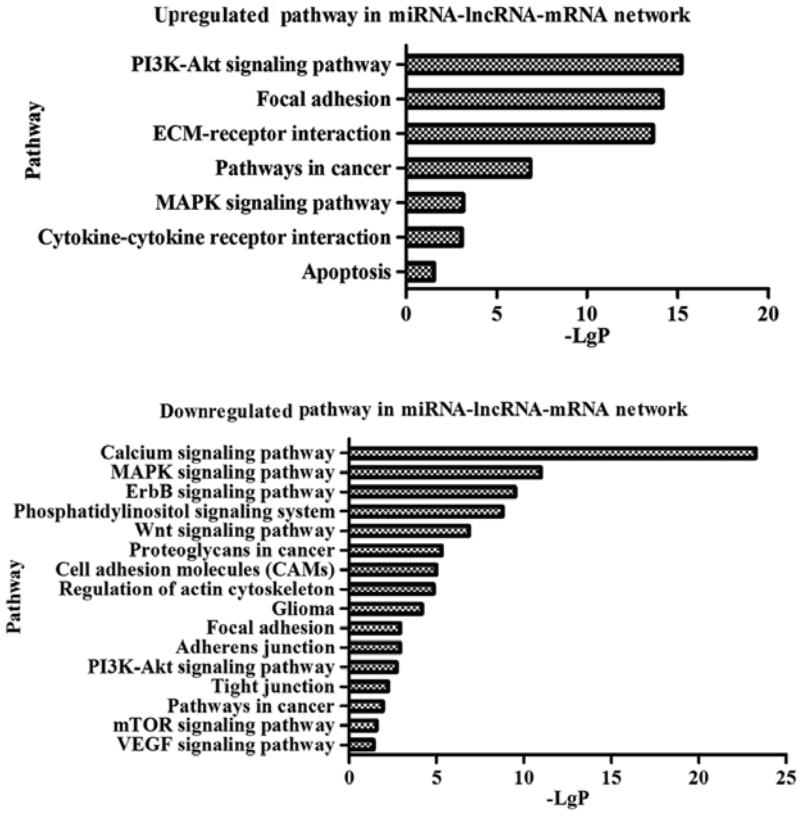

Figure 3. Function prediction of the ceRNAs based on pathway analysis of mRNAs located in miRNA-lncRNA-mRNA interaction network in GBM. The pathway analysis applied for 30 mRNAs showed the 7 upregulated and 16 downregulated tumor associated signaling pathways ($P<0.05$). ceRNAs, competing endogenous RNAs; miRNA, microRNA; lncRNA, long non-coding RNA; GBM, glioblastoma multiforme.

involved in diverse biological processes of cancer, including proliferation, cell apoptosis, adhesion, angiogenesis and metastasis (Fig. 3A and B). As a consequence, we predicted the important roles of the 39 ceRNAs in GBM pathogenesis. The miRNAs, lncRNAs, mRNAs, and their participated pathways are listed in Table III.

Table III. Functional prediction of the lncRNA ceRNAs based on pathway analysis of mRNAs that location together in the miRNA-lncRNA-mRNA feed-forward loop in GBM.

miRNA	lncRNA	mRNA	Pathway
hsa-miR-15a-5p	n339339 ENST00000520186 ENST00000533229 ENST00000566630 n341995	PAK7	ErbB signaling
hsa-miR-15a-5p	n339339 n346032 ENST00000532691 ENST00000566630 ENST00000524501 ENST00000578746 n341995	CACNA1E	Calcium, MAPK signaling
hsa-miR-15a-5p	ENST00000520186	MAPK9	MAPK, ErbB, Wnt signaling, focal adhesion, pathways in cancer
hsa-miR-15a-5p	ENST00000520186 ENST00000596580 n411142 ENST00000492667	AKT3	MAPK, ErbB, PI3K-Akt, VEGF, mTOR signaling, glioma, apoptosis, focal adhesion, pathways in cancer
hsa-miR-15a-5p	ENST00000559981 ENST00000596580 n410578 n411142 ENST00000492667	SYNJ1	Phosphatidylinositol signaling
hsa-miR-15a-5p	ENST00000566942	HTR2A	Calcium signaling
hsa-miR-15a-5p	n338128	PTPRR	MAPK signaling
hsa-miR-15b-5p	n339339 ENST00000520186 ENST00000533229 ENST00000566630 n341995 n406352	PAK7	ErbB signaling
hsa-miR-15b-5p	n339339 n346032 ENST00000532691 ENST00000566630 ENST00000524501 n341995 n406352	CACNA1E	Calcium, MAPK signaling
hsa-miR-15b-5p	ENST00000520186 ENST00000596580 n411142 ENST00000492667	AKT3	MAPK, ErbB, PI3K-Akt, VEGF, mTOR signaling, glioma, apoptosis, focal adhesion, pathways in cancer
hsa-miR-15b-5p	ENST00000559981 ENST00000596580 n410578 n411142 ENST00000492667	SYNJ1	Phosphatidylinositol signaling
hsa-miR-15b-5p	ENST00000520186	MAPK9	MAPK, ErbB, Wnt signaling, focal adhesion, pathways in cancer
hsa-miR-15b-5p	ENST00000566942	HTR2A	Calcium signaling
hsa-miR-15b-5p	n338128	PTPRR	MAPK signaling
hsa-miR-23a-3p	n339339	SLIT1	Phosphatidylinositol signaling

Table III. Continued.

miRNA	lncRNA	mRNA	Pathway
hsa-miR-23a-3p	n339339 ENST00000434383	RXRG	Pathways in cancer
hsa-miR-23a-3p	ENST00000559981 n411142	SYNJ1	Phosphatidylinositol signaling
hsa-miR-23a-3p	ENST00000562191	MEF2C	MAPK signaling
hsa-miR-23a-3p	n383510	CADM3	Cell adhesion molecules
hsa-miR-23a-3p	ENST00000559981	TLN2	Focal adhesion
hsa-miR-23a-3p	ENST00000524501	NRXN3	Cell adhesion molecules
hsa-miR-30a-5p	n346032 ENST00000532691	GRM5	Calcium signaling
hsa-miR-30a-5p	ENST00000569946 n339481	CACNA1C	Calcium signaling, MAPK signaling
hsa-miR-30a-5p	n373066	CAMK4	Calcium signaling
hsa-miR-27a-3p	n339339	PDE3B	Proteoglycans in cancer
hsa-miR-27a-3p	ENST00000564076 n338494	CACNB2	MAPK signaling
hsa-miR-27a-3p	n371475	CDS1	Phosphatidylinositol signaling
hsa-miR-27a-3p	ENST00000492667	ATP2B1	Calcium signaling
hsa-miR-34a-5p	n339339 n346032 ENST00000555811 ENST00000566630 n406352	CACNA1E	Calcium, MAPK signaling
hsa-miR-34a-5p	ENST00000596580 n410578 ENST00000492667	SYNJ1	Phosphatidylinositol signaling
hsa-miR-34a-5p	ENST00000549205 n406352	PRKCE	Tight junction
hsa-miR-34a-5p	n384244	CNTN2	Cell adhesion molecules
hsa-miR-34a-5p	n406352	WASF1	Regulation of actin cytoskeleton, Adherens junction
hsa-miR-106b-5p	ENST00000596580 n337874	PIP4K2A	Phosphatidylinositol signaling, regulation of actin cytoskeleton
hsa-miR-106b-5p	ENST00000596580 n411142	AKT3	MAPK, ErbB, PI3K-Akt, VEGF, mTOR signaling, glioma, apoptosis, focal adhesion, pathways in cancer
hsa-miR-106b-5p	n345100	MAPK9	MAPK, ErbB, Wnt signaling, focal adhesion, pathways in cancer
hsa-miR-25-3p	ENST00000493303 ENST00000549205 n406352	PRKCE	Tight junction
hsa-miR-25-3p	n410578 n411142 ENST00000492667	SYNJ1	Phosphatidylinositol signaling
hsa-miR-25-3p	ENST00000549205	MAP2K4	MAPK, ErbB signaling
hsa-miR-223-3p	ENST00000493303 n406352	PRKCE	Tight junction
hsa-miR-223-3p	n371741 ENST00000492667	ATP2B1	Calcium signaling

Table III. Continued.

miRNA	lncRNA	mRNA	Pathway
hsa-miR-21-5p	ENST00000559981 n411142 ENST00000492667	PPP3CA	Calcium, MAPK, Wnt, VEGF signaling
hsa-miR-92b-3p	ENST00000596580 n410578 n411142 ENST00000492667	SYNJ1	Phosphatidylinositol signaling
hsa-miR-339-5p	ENST00000555811 ENST00000523571	DAAM2	Wnt signaling
hsa-miR-339-5p	n383510	CADM3	Cell adhesion molecules
hsa-miR-10b-5p	ENST00000221169	TIAM1	Regulation of actin cytoskeleton, proteoglycans in cancer
hsa-miR-29b-3p	ENST00000481203 ENST00000518554	TNFRSF1A	MAPK signaling, cytokine-cytokine receptor interaction, apoptosis
hsa-miR-29b-3p	ENST00000547415	LAMC1	PI3K-Akt signaling, focal adhesion, ECM-receptor interaction, pathways in cancer
hsa-miR-377-3p	n338229	LAMC1	PI3K-Akt signaling, focal adhesion, ECM-receptor interaction, pathways in cancer
hsa-miR-29c-3p	ENST00000547415	LAMC1	PI3K-Akt signaling, focal adhesion, ECM-receptor interaction, pathways in cancer
hsa-miR-29c-3p	ENST00000518554	TNFRSF1A	MAPK signaling, cytokine-cytokine receptor interaction, apoptosis

lncRNA, long non-coding RNA; ceRNAs, competing endogenous RNAs; miRNA, microRNA; GBM, glioblastoma multiforme.

Quantitative real-time RT-PCR analysis of the distinctive expression of lncRNAs, miRNAs and mRNAs in GBM samples. To validate the conclusions of microarray analysis, we selected 10 miRNAs with larger fold change from the microarray results and analyzed their expression levels by qRT-PCR in 20 normal brain and 30 GBM samples. Our results confirmed the findings of the miRNA microarray dataset (Fig. 4A and B).

Based on the analysis of 224 miRNA-lncRNA-mRNA feed-forward loops in Table II, we evaluated the expression levels of 4 miRNA, 4 lncRNA and 4 mRNA that, respectively, located in 4 feed-forward loops. The average expression levels of miR-15a and miR-21 were significantly increased, while miR-29b and miR-29c were reduced in GBM compared with normal brain tissues. Analysis showed relatively high expression of miRNA and low expression of lncRNA and mRNA, and low expression of miRNA and high expression of lncRNA and mRNA (Fig. 4C and D). The 4 feed-forward loops detection by qRT-PCR are presented in Fig. 4E.

Discussion

In recent years, the emerging significance of ceRNAs in cancers has drawn attention of researchers. ceRNA activity is determined by factors such as miRNA/ceRNA abundance, ceRNA binding affinity to miRNAs and RNA-binding proteins. The alteration of any of these factors may lead to ceRNA network imbalance and thus contribute to cancer (28). ceRNA study processes generally include: ceRNA prediction, ceRNA validation and ceRNA functional investigation.

Recently, several studies have confirmed the dysregulation of lncRNAs by acting as ceRNAs have profound implications for tumor initiation, maintenance or progression. lncRNAs acting as ceRNAs are involvd in the pathogenesis of several common cancers such as thyroid cancer, gastric cancer and hepatocellular cancer (29-33). The ceRNA activity of lncRNAs has also been shown to have an oncogenic effect: The lncRNA HOTAIR was shown to display ceRNA activity in gastric cancer cells, in which it was found to specifically bind the tumor suppressor miR-331-3p, modulating HER2 derepression (31). The other example of lncRNA-mediated ceRNA regulation involves the tumor suppressor gene BARD1. The lncRNA BARD1 9'L is transcribed by an alternative intronic promoter of the BARD1 gene and share both miR-203 and miR-101 MREs with BARD1 mRNA in their homologous 3'UTRs. BARD1 mRNAs were downregulated by miR-203 and miR-101, and BARD1 9'L counteracted the effect of these miRNAs. These data support a role for BARD1 9'L as

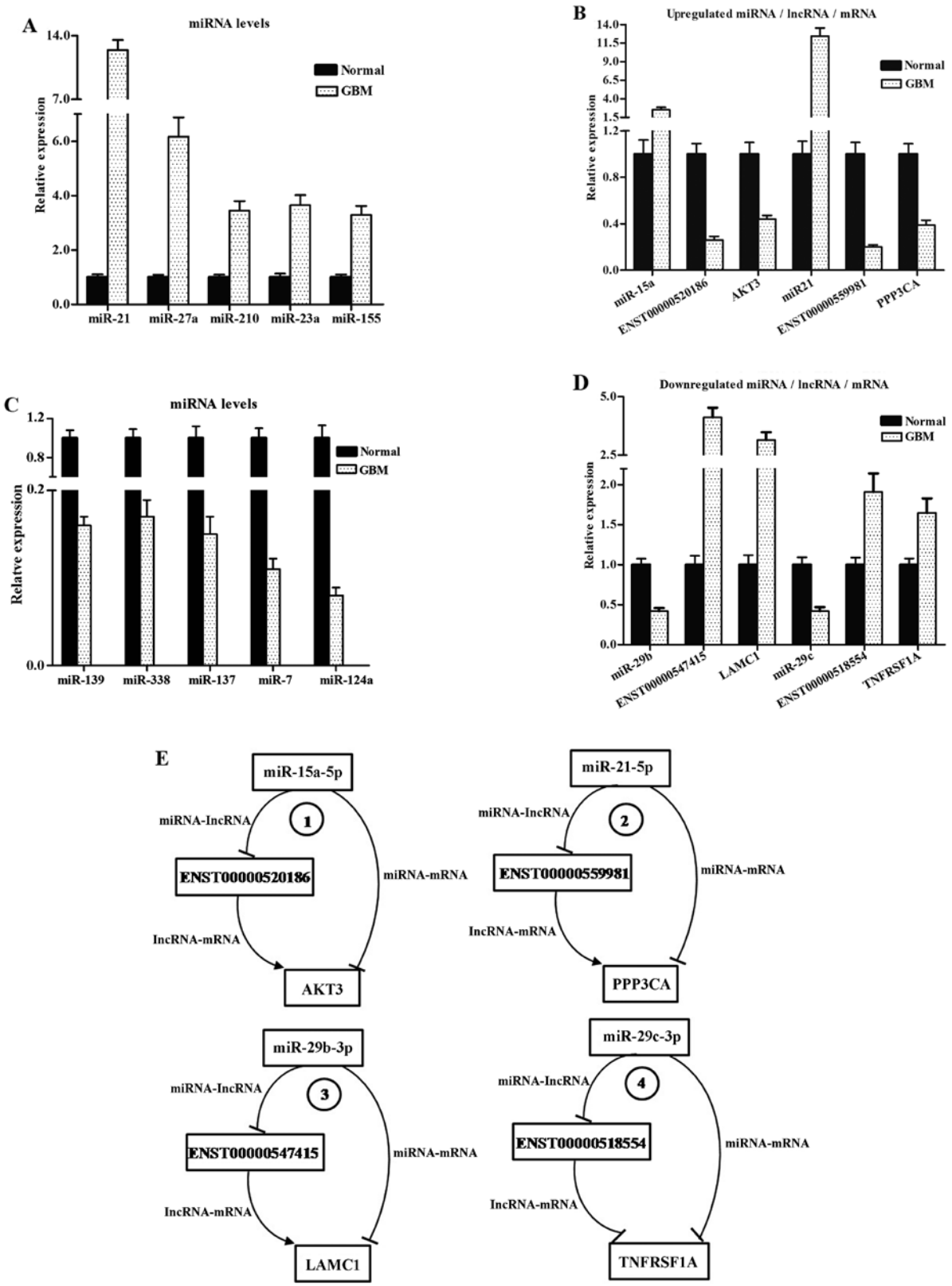

Figure 4. Expression analysis of miRNA, lncRNA and mRNA in normal brain and GBM tissues. (A and B) The expression level of 10 miRNAs, (C and D) 4 miRNAs, 4 lncRNAs and 4 mRNAs that, respectively, located in (E) 4 feed-forward loops were analyzed using qRT-PCR in normal brain (20 samples) and GBM (30 patients) tissues, with the GAPDH (for lncRNA and mRNA) and U6 (for miRNA) gene as an internal control. Error bars represent the standard errors of independent samples. miRNA, microRNA; lncRNA, long non-coding RNA; GBM, glioblastoma multiforme.

a tumor suppressor transcript through its ceRNA activity (33). These findings provide important clues for understanding the key roles of lncRNA-miRNA functional network in cancers. Exploring the interplay of lncRNA function as a ceRNA in cancer provides new insight into cancer pathogenesis and opportunities for therapy exploration.

Understanding the novel miRNA-lncRNA-mRNA cross-talk will lead to significant insight into gene regulatory networks in cancers. In this study, we investigated the miRNA, lncRNA and mRNA expression signatures in GBM, constructed the miRNA-lncRNA-mRNA regulation network, on this basis, identified the lncRNA acting as ceRNAs and predicted the possible biology functions of these ceRNAs.

We re-annotated the Affymetrix Human Exon 1.0 probe sets and identified the lncRNAs and mRNAs on this array. The sample matched miRNA expression profiling of the Agilent Human MicroRNA array 8x15K was analyzed to determine differently expressed miRNAs in GBM. We identified a set of 41 miRNAs, 398 lncRNAs and 1,995 mRNAs with differentiated expression between GBM and normal brain tissues. Such differentiation signified their potential roles in tumorigenesis.

The complexity and diversity of potential ceRNA interactions have been described with the identification of abundant lncRNAs. We discussed the effect of miRNA competition on the regulation of both lncRNAs and mRNAs, as well as the implications of lncRNA function as ceRNA for the development of GBM. To our knowledge, this is the first study to show the roles of lncRNA acting as ceRNAs in GBM. Understanding the key roles of 'miRNA-lncRNA' module will lead to the identification of new therapeutic targets for treating GBM.

Our qRT-PCR expression analysis confirmed there are a series of miRNAs, lncRNAs and mRNAs aberrantly expressed in GBM tissues, which indicated that the differently expressed non-coding and coding RNAs may be one of characters of GBM. The aberrant miR-21, miR-27a, miR-210, miR-23a, miR-155, miR-139, miR-338, miR-137, miR-7, miR-124a, miR-15a, miR-29b and miR-29c expression levels in GBM were detected, our results were in concordance with the previous findings, and these deregulated miRNAs have been reported to be aberrantly expressed in GBM (34-42). In our expression profiling analysis, the lncRNA ENST00000520186, ENST00000559981, ENST00000547415 and ENST00000518554 were separately considered as the ceRNA of miR-15a, miR-21, miR-29b and miR-29c in GBM. So far, these ceRNAs have not been reported implicated in GBM. Four mRNAs may be regulated by these miRNAs and lncRNAs, the PPP3CA have been reported to be aberrantly expressed in other tumors, but have not been studied in GBM; in addition, AKT3, TNFRSF1A and LAMC1 have been studied to different expression in GBM (36,43,44).

Overall, our study identified and analyzed lncRNA function as ceRNA in GBM and showed they may play crucial biological roles during GBM formation and development, and provide important theory and experimental foundations for future study of drug target and treatment for GBM.

Acknowledgements

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