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Identification of differential expression lncRNAs in gastric cancer using transcriptome sequencing and bioinformatics analyses

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Abstract. The current study aimed to identify novel long non-coding RNAs (lncRNAs) associated with gastric cancer (GC). Transcriptome sequencing of the lncRNAs and mRNAs from GC tissues and normal adjacent tissues was performed. The data were analyzed using bioinformatics analysis, specifically analysis of differentially expressed lncRNAs and mRNA, target gene prediction and functional enrichment analysis. A total of 1,181 differentially expressed mRNA and 390 differentially expressed lncRNAs were identified. The targets of upregulated lncRNAs were significantly enriched in functions associated with collagen fibril organization, whereas the downregulated lncRNA were significantly associated with ion transmembrane transport and regulation of membrane potential. A total of 7 lncRNAs were verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following RT-qPCR validation, AC016735.2, AP001626.1, RP11-400N13.3 and RP11-243M5.2 were considered to be consistent with the prediction of the bioinformatics analysis. Transcriptome sequencing and RT-qPCR experiments identified 4 lncRNAs, including AC016735.2, AP001626.1, RP11-400N13.3 and RP11-243M5.2 to have an important role in the carcinogenesis of GC.

Introduction

Gastric cancer (GC), developing from the lining of the stomach, is one of the most common cancers worldwide, particularly in East Asia and China (1). It is the third leading cause of death from cancer and accounts for 9% of mortality worldwide (2). If untreated, tumor cells often metastasize to other parts of the body, particularly the lungs, liver, bone, and lymph nodes; therefore, the prognosis of GC is generally unfavorable (3). The 5-year survival rate for GC is reported to be <10% (4). In China, the majority of patients with GC are diagnosed at a late stage and the prognosis is unfavorable (1). Therefore, understanding of the molecular mechanisms and identification of the key biomarkers associated with GC progression is essential for the diagnosis and therapy of GC.

The conventional view of gene regulation in biology is primarily concentrated on the protein-coding genes. However, the human genome project suggested that ~1.2% of the mammalian genome encodes proteins (5,6) and most of the genome is transcribed into long non-coding RNAs (lncRNAs) (7,8). LncRNAs are RNA molecules >200 nucleotides in length. Dysregulated lncRNAs have been demonstrated to have an important role in tumorigenesis and cancer metastasis (9-11). The association between aberrant expression of lncRNAs and GC has been previously investigated. For example lncRNA-HMlincRNA717 was determined to have a crucial role during GC occurrence and progression (12). Song *et al* (13) performed lncRNA microarray analysis and identified 135 differentially expressed lncRNAs between GC and normal tissues (13). However, numerous lncRNAs have been identified they are not sufficient for the treatment of GC.

In the present study, the lncRNA sequencing for GC tissues was performed using a transcriptome sequencing technique. The differentially expressed lncRNAs between GC and normal adjacent tissues were identified. The bioinformatics analysis included prediction of target genes and function enrichment analysis. Finally, the lncRNAs predicted by the present study were verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The current study aimed to investigate the additional lncRNAs associated with GC, which may be used as potential markers for the diagnosis and treatment of GC.

Materials and methods

Tissue samples. Between October 2015 and January 2016, a total of 3 male patients with GC (aged 65-76 years old) were included in the current study, whose diagnoses were pathologically confirmed. The cancer tissues and the normal adjacent tissues were obtained from clinically ongoing surgical specimens, were snap frozen with liquid nitrogen and subsequently stored at -80˚C until RNA extraction.

All patients have provided written informed consent prior to participating in the present study. The procedures in the current study were approved by the Protection of Human

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Ethics Committee of Shanghai Shuguang Hospital Affiliated with Shanghai University of TCM (Shanghai, China).

Transcriptome sequencing. Total RNAs from gastric cancer tissues (3 samples) and normal adjacent tissues (3 samples) were extracted using the RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China). Evaluation of the quality and integrity of the total RNA was performed using 1% agarose gel electrophoresis (visualized using ethidium bromide), and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Following this, a cDNA library was established using the NEBNext Ultra RNA Library Prep kit (New England Biolabs, Inc., Ipswich, MA, USA) prior to Illumina sequencing. The transcriptome sequencing of mRNA and lncRNA was performed on an Illumina gene analyzer (Illumina, Inc., San Diego, USA).

The data have been deposited at National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession number: SRP092509.

Quality control of the sequencing data was performed to identify the clean reads using the FASTX-toolkit (version 0.0.13) (14). The obtained clean reads were aligned to the human reference genome hg19 using TopHat software (version 2.10) (15). Then based on the mRNA and lncRNA annotation information provided by gencode version 24 (mapped to GRCh37) (16) database, the Fragments Per Kilobase of transcript per Million mapped reads values of mRNA and lncRNA and the reads number of lncRNA were identified using StringTie tool (version 1.2.3) (17).

Bioinformatics analysis of sequencing data. The differentially expressed lncRNAs and genes (DEGs) between the cancer group and the control group were identified using the limma package (18) in R (version 3.2.5) with the following criteria: $|\log_2$ fold change (FC)$|>1$ and $P<0.05$.

The downstream target genes of the differentially expressed lncRNAs were predicted based on the co-expression associations between lncRNAs and mRNAs. The threshold values were correlation coefficient >0.8 and P<0.05. Additionally, the network of lncRNAs and their target genes was constructed using Cytoscape (version 3.0) (19).

Subsequently, DEGs and target genes of lncRNAs were used to perform Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses with the clusterprofiler package (20) in R.

RT-qPCR verification of the expression of lncRNAs. Total RNA was extracted from tissues (3 GC tissues and 3 normal adjacent tissues) using RNAiso Plus (9109; Takara Biotechnology Co., Ltd.). The concentration and purity of the isolated RNA was determined using TECAN infinite M100 PRO Biotek microplate reader (Tecan Group, Ltd., Mannedorf, Switzerland) and reverse transcription (37˚C for 15 min and 85˚C for 5 sec) was performed according to the PrimeScript RT Master mix RR036A (Takara Biotechnology Co., Ltd.). qPCR was performed using SYBRGreen kit (cat no. 4367659; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reaction procedures were as follows: 50˚C for 3 min, 95˚C for 3 min, 95˚C for 10 sec, and 60˚C for 30 sec, for 40 cycles, the melting process was 60 to 95˚C (increments of 0.5˚C for 10 sec). According to the results of bioinformatics analysis, the expressions of 7 lncRNAs, including AC016735.2, RP11-243M5.2, RP11-400N13.2, RP11-400N13.3, AP001626.1, LINC01139 and RP11-54H7.4, were detected with the primers presented in Table I. The expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Statistical analysis. Data are presented as the mean ± standard error of mean. The statistical analysis was performed by Graphpad Prism (version 5.01) using the Student's t-test (Graphpad Software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

High-throughput sequencing. From the 6 samples, a total of 3,4290 mRNAs and 10,148 lncRNAs were identified, which were expressed in at least one sample. With the criteria of $|\log_2 FC|>1$ and P<0.05, a total of 1,181 DEGs were identified, 902 were upregulated and 279 were downregulated. Additionally, 390 differentially expressed lncRNAs, including 163 upregulated and 227 upregulated lncRNAs were identified (Fig. 1). The top 10 differentially expressed lncRNA with higher FCs, including RP11-171I2.1, RP11-171I2.1 and AC016735.2 are presented in Table II.

Bioinformatics analysis of lncRNA sequencing data. Based on the threshold values of correlation coefficient >0.8 and P<0.05, 157 differentially expressed lncRNAs were selected for target prediction and a total of 231 target genes were identified. Among the 157 differentially expressed lncRNAs, 13 lncRNAs (12 upregulated and 1 downregulated) predicted an additional 10 target genes (Table III), including RP11-400N13.2, RP11-400N13.3, AP001626.1 and RP11-54H7.4. The regulatory network constructed with 13 lncRNAs and their target genes is presented in Fig. 2.

Due to the high number of lncRNAs identified, the functions of individual lncRNAs were not analyzed. The present study focused on the functions of the 13 aforementioned lncRNAs. A total of 50 and 12 target genes were predicted for the upregulated and downregulated lncRNAs, respectively. Functional enrichment analysis of the 62 target genes determined that the upregulated lncRNAs were significantly enriched in functions associated with collagen fibril organization, whereas the downregulated lncRNA was significantly associated with ion transmembrane transport and regulation of membrane potential (Table IV).

RT-qPCR verification of the expression of lncRNAs. According to the findings of the transcriptome sequencing and bioinformatics analyses, AC016735.2, RP11-243M5.2, RP11-400N13.2, RP11-400N13.3, AP001626.1, LINC01139 and RP11-54H7.4 were upregulated, and only RP11-243M5.2 was downregulated.

The RT-qPCR validation confirmed that AC016735.2, AP001626.1 and RP11-400N13.3 were upregulated, whereas RP11-243M5.2, RP11-400N13.2, LINC01139 and RP11-54H7.4 were downregulated in GC tissues compared with normal adjacent tissues. It is of note that although upregulation and downregulation were detected, no significant difference was

Table I. Primer sequences of the long noncoding RNAs for reverse transcription- quantitative polymerase chain reaction.

Gene	Forward (5'-3')	Reverse (5'-3')
AC016735.2	CTGCTTCTCACTGCCTCG	TTTCCCAAATGGTCCTCC
RP11-243M5.2	TTGCGTGAAAGCGTATGG	GAAAGCAGCCTTGAGAACAGAG
RP11-400N13.2	CCCCTGTCCTCCTGCTCTT	CGGGCAGTGTCAGTCTTCA
RP11-400N13.3	GCAGATGGCAAAGGATAAAGC	GGTGATATACGATGCAACGGTG
AP001626.1	AGCTGCACCAAGGAGAATC	CAAAGCCAAGGTCCACTGTT
LINC01139	ACCAGTCACCCAACCAGAGC	AAGCGTAAGAATGAAGACCAGTG
RP11-54H7.4	TCCACTCTAGGTTCCCACG	CCTGACATTCCTGCCTTCTT
GAPDH	TGACAACTTTGGTATCGTGGAAGG	AGGCAGGGATGATGTTCTGGAGAG

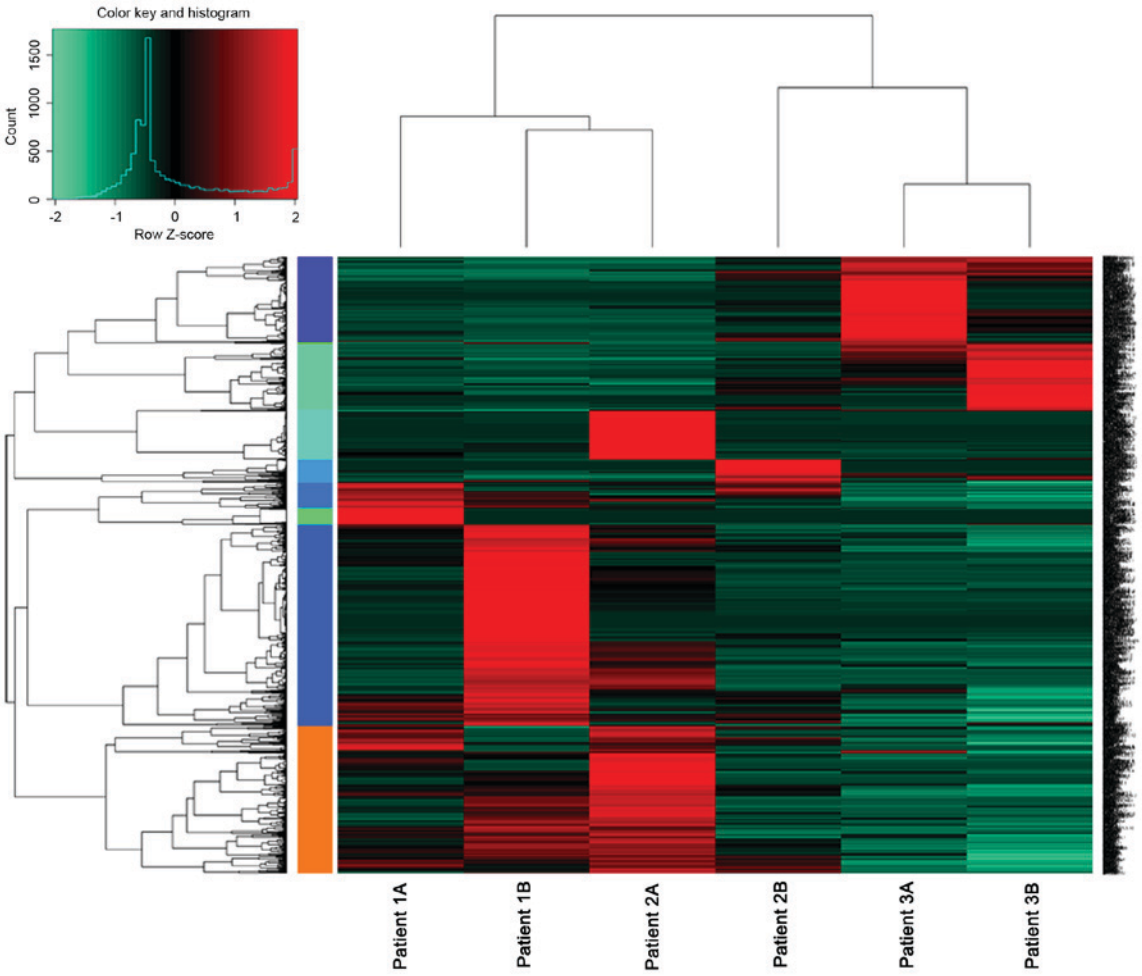

Figure 1. Heatmap of differentially expressed lncRNAs. Red indicates high expression and green indicates low expression. Patient 1A and Patient 1B are samples derived from the same patient; Patient 2A and Patient 2B are samples derived from the same patient; and Patient 3A and Patient 3B are derived from the same patient. A, tumor tissue; B, normal tissue.

identified (Fig. 3). The findings of AC016735.2, AP001626.1, RP11-400N13.3 and RP11-243M5.2 were considered to be consistent with the predicted lncRNAs in the bioinformatics analysis.

Discussion

Previous studies have identified lncRNAs to be important in the governing of fundamental biological processes, where aberrant expression may be associated with various human cancers (22,23). The present study identified 390 differentially expressed lncRNAs between GC and normal adjacent tissues via transcriptome sequencing and bioinformatics analysis. The upregulated lncRNAs were significantly enriched in functions associated with collagen fibril organization, whereas the downregulated lncRNA was significantly associated with ion transmembrane transport and regulation of membrane potential. Following RT-qPCR validation, AC016735.2, AP001626.1,

Table II. Top 10 differentially expressed upregulated and downregulated lncRNAs.

A, Downregulated

ID	logFC	P-value
RP11-171I2.1	-4.549491715	0.006324281
RP5-994D16.9	-4.73821376	0.002778706
RP11-139H15.6	-4.802835645	0.002652066
RP11-637N19.1	-4.807460785	0.005211747
RP11-243M5.2	-4.87029533	0.011518821
RP11-382A20.5	-4.909287572	0.002375869
AC003090.1	-5.054470484	0.002583845
FGF14-IT1	-5.152474325	0.004193094
AC053503.12	-5.200695133	0.045876461
RP11-16P20.4	-5.309441725	0.00370217

B, Upregulated

ID	logFC	P-value
RP3-416H24.1	6.312295751	0.002010312
RP5-1185I7.1	5.627125267	0.002013113
LINC01087	5.562031916	0.00323185
RP11-1007G5.2	5.506776995	0.000837008
LINC00483	5.440975498	0.01629253
LINC00618	5.425270941	0.000784189
RP11-120K24.4	5.413330508	0.002061786
AC016735.2	5.383341519	0.030647411
AC110769.3	5.338202236	0.001217988
LA16c-325D7.1	5.296535904	0.002461903

FC, fold-change

RP11-400N13.3 and RP11-243M5.2 were considered to be consistent with the results of the bioinformatics prediction, suggesting that they may have a role in the tumorigenesis of GC.

RP11-400N13.3, AP001626.1 and AC016735.2 were all upregulated lncRNAs, and were predicted to regulate >10 target genes, including collagen type I a 1 (*COL1A1*), *COL1A2*, and arachidonate 15-lipoxygenase (*ALOX15*). It is of note that the three target genes were also DEGs. *COL1A1* and *COL1A2* encode type I collagen, which is the most abundant collagen of the human body that forms collagen fibers. *COL1A1* and *COL1A2* were identified to be significantly enriched in GO function associated with collagen fibril organization. Type I collagen has an important role in fibrosis and cancer progression (24). A previous study determined that collagen is a major contributor to diffusive hindrance in human tumors (25). Additionally, type I collagen is a prevalent component of the stromal extracellular matrix (26). The stromal extracellular matrix is a barrier to a progressing cancer cell, changes of which contribute to metastasis in cancer (27). Therefore, it is possible that the three lncRNAs may be involved in GC metastasis by regulating *COL1A1* and *COL1A2*.

Table III. Long noncoding RNAs with more than 10 target genes.

ID	No of target genes	log_2FC	P-value
AC005609.18	16	5.258702066	0.002564097
AC016735.2	23	5.383341519	0.030647411
AP001626.1	30	3.480674967	0.039101426
CTD-2034I4.2	19	3.225981981	0.039614051
LINC01139	27	3.784674378	0.034659687
RP11-210L7.3	15	3.183358792	0.03741905
RP11-243M5.2	12	-4.87029533	0.011518821
RP11-400N13.2	32	4.411473414	0.015832445
RP11-400N13.3	32	4.01570403	0.031992561
RP11-537P22.2	19	3.519296946	0.027224444
RP11-54H7.4	25	3.227024693	0.043767147
RP11-782C8.3	11	3.386174545	0.029429789
RP4-781K5.7	18	3.607427164	0.025935631

ALOX15 encoding protein is part of the lipoxygenases family. Human lipoxygenases are widely distributed in human organs, tissues and cells (28), catalyzing peroxidation of unsaturated fatty acid producing various types of eicosanoids (29). It has been previously reported that many cancers are driven by lipoxygenases and their metabolites (30,31). A previous study determined that ALOX15 is an important factor in the regulation of colorectal epithelial cell terminal differentiation and apoptosis (32). In the current study, *ALOX15* was significantly enriched in engulfment of apoptotic cell (GO:0043652), which may confirm its role in apoptosis. Therefore, RP11-400N13.3, AP001626.1 and AC016735.2 may also regulate *ALOX15* and have a role in the progression of GC.

RP11-243M5.2 was a downregulated lncRNA and had 12 target genes, including ATPase Na+/K+ transporting subunit a 4 (*ATP1A4*) and sodium voltage-gated channel a subunit 7 (*SCN7A*), which were significantly involved in functions associated with ion homeostasis and ion transmembrane transport. It has been previously established that cells require a balance of ions across their cell membrane in order to ensure cell survival. The homeostatic intracellular ionic environment is necessary for the correct functioning of gene expression, hormone release and cellular proteins (33,34). It is of note that Bortner and Cidlowski (35) have reported that intracellular ion homeostasis has an important role in the regulation of the cell death and changes may alter the apoptotic rate of cells. Evading apoptosis by generating genetic mutations is a key mechanism of carcinogenesis (36). Therefore, the present study suggested that RP11-243M5.2 may have a role in the carcinogenesis of GC by regulating *ATP1A4* and *SCN7A* to participate in functions associated to ion homeostasis and ion transmembrane transport.

AC016735.2, AP001626.1, RP11-400N13.3 and RP11-243M5.2 were verified by RT-qPCR; however, the results were not statistically significant. This may be due to the heterogeneity between the samples used in the transcriptome sequencing and RT-qPCR.

Table IV. Functional enrichment analysis of the target genes of the 13 lncRNAs with more than 10 target genes.

A, Upregulated

Ontology	ID	Function	Count	FDR
BP	GO:0043588	Skin development	5	0.011708982
BP	GO:0030199	Collagen fibril organization	3	0.011708982
BP	GO:0071230	Cellular response to amino acid stimulus	3	0.012776741
BP	GO:0043589	Skin morphogenesis	2	0.012776741
BP	GO:0070208	Protein heterotrimerization	2	0.016397947
CC	GO:0005583	Fibrillar collagen trimer	3	0.000116529
CC	GO:0098643	Banded collagen fibril	3	0.000116529
CC	GO:0098644	Complex of collagen trimers	3	0.000426968
CC	GO:0005581	Collagen trimer	3	0.016659231
CC	GO:0044420	Extracellular matrix component	3	0.037435206
MF	GO:0048407	Platelet-derived growth factor binding	3	0.000119244
MF	GO:0005201	Extracellular matrix structural constituent	3	0.016290955
F	GO:0005198	Structural molecule activity	6	0.036696011
MF	GO:0019838	Growth factor binding	3	0.040871579
KEGG	hsa04974	Protein digestion and absorption	3	0.002473586
KEGG	hsa05146	Amoebiasis	3	0.002473586
KEGG	hsa04933	AGE-RAGE signaling pathway in diabetic complications	3	0.002473586
KEGG	hsa04611	Platelet activation	3	0.003235067
KEGG	hsa04512	ECM-receptor interaction	2	0.026270733

B, Downregulated

Ontology	ID	Function	Count	FDR
BP	GO:0055078	Sodium ion homeostasis	2	0.164234693
BP	GO:0042391	Regulation of membrane potential	3	0.164234693
BP	GO:0034220	Ion transmembrane transport	4	0.164234693
BP	GO:0007416	Synapse assembly	2	0.164234693
BP	GO:0035725	Sodium ion transmembrane transport	2	0.164234693
CC	GO:1902495	Transmembrane transporter complex	3	0.02856848
CC	GO:1990351	Transporter complex	3	0.02856848
CC	GO:0098794	postsynapse	3	0.02856848
CC	GO:0030424	Axon	3	0.02856848
CC	GO:0030426	Growth cone	2	0.041273443
MF	GO:0015075	Ion transmembrane transporter activity	4	0.011272955
MF	GO:0022891	Substrate-specific transmembrane transporter activity	4	0.011272955
MF	GO:0022857	Transmembrane transporter activity	4	0.011272955
MF	GO:0005216	Ion channel activity	3	0.011272955
MF	GO:0046873	Metal ion transmembrane transporter activity	3	0.011272955

FDR, false discovery rate; Count, number of enriched genes; GO, gene ontology; BP, biological process; MF, molecular function; CC, cellular component; KEGG, Kyoto Encyclopedia of Genes and Genomes.

In conclusion, by transcriptome sequencing and RT-qPCR experiments the present study identified 4 lncRNAs, including AC016735.2, AP001626.1, RP11-400N13.3 and RP11-243M5.2 to have an important role in the pathogenesis of GC. They may be used as potential diagnosis or treatment biomarkers of GC in the future.

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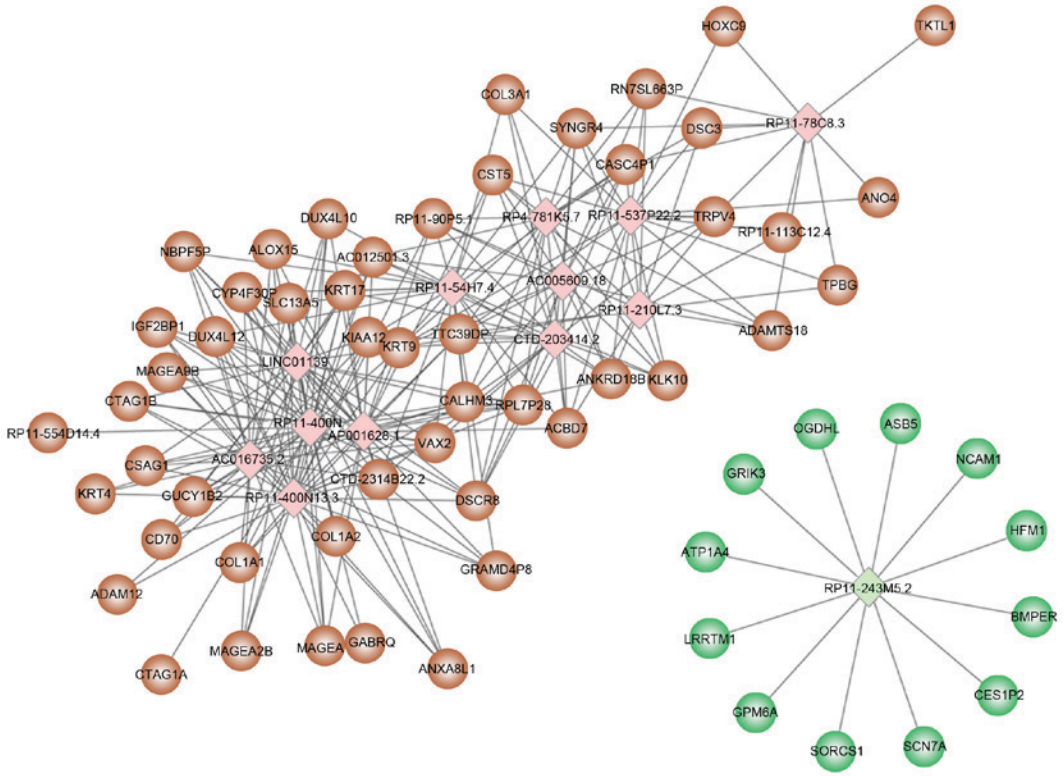

Figure 2. Regulatory network constructed with 13 lncRNAs and their target genes. Brown circles indicate upregulated target genes and green circles represent downregulated target genes. Pink rhombus, upregulated lncRNA; green rhombus, downregulated lncRNA. lncRNA, long noncoding RNA.

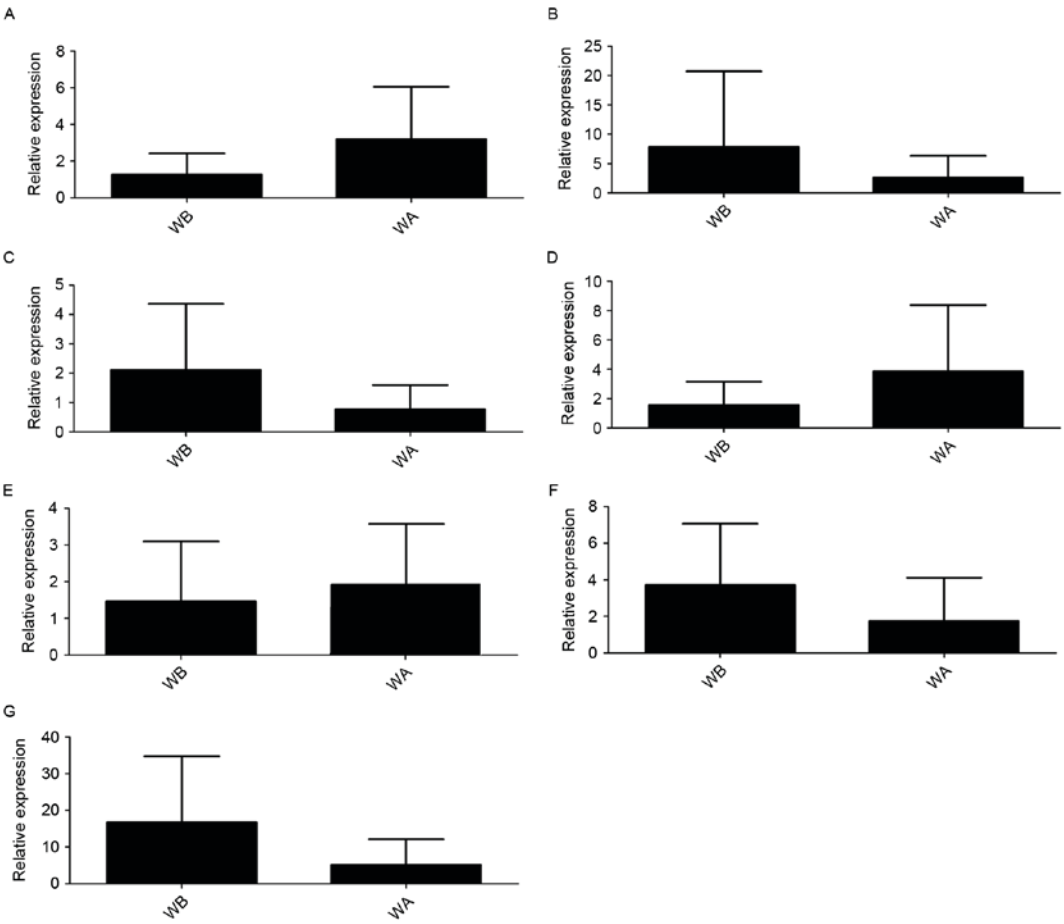

Figure 3. LncRNA levels verified by reverse transcription-quantitative polymerase chain reaction. (A) AC016735.2, (B) RP11-243M5.2, (C) RP11-400N13.2, (D) RP11-400N13.3, (E) AP001626.1, (F) LINC01139 and (G) RP11-54H7.4. WA, cancer tissue; WB, normal adjacent tissues.

Availability of data and materials

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

Authors' contributions

YW drafted the manuscript and acquired and analysed the data. JZ interpreted the data and revised the manuscript.

Ethics approval and consent to participate

The procedures in the current study were approved by the Protection of Human Ethics Committee of Shanghai Shuguang Hospital Affiliated with Shanghai University of TCM (Shanghai, China).

Consent for publication

All patients have provided written informed consent prior to participating in the present study.

Competing interests

The authors declare that they have no competing interests.

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