Integrated analysis of long non-coding RNA-associated ceRNA network reveals potential lncRNA biomarkers in human lung adenocarcinoma

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Abstract. Accumulating evidence has highlighted the important roles of long non-coding RNAs (lncRNAs) acting as competing endogenous RNAs (ceRNAs) in tumor biology. However, the roles of cancer specific lncRNAs in lncRNA-related ceRNA network of lung adenocarcinoma (LUAD) are still unclear. In the present study, the 465 RNA sequencing profiles in LUAD patients were obtained from The Cancer Genome Atlas (TCGA) database, which provides large sample RNA sequencing data free of charge, and 41 cancer specific lncRNAs, 25 miRNAs and 1053 mRNAs (fold change >2, P<0.05) were identified. Then, the lncRNA-miRNA-mRNA ceRNA network of LUAD was constructed with 29 key lncRNAs, 24 miRNAs and 72 mRNAs. Subsequently, we selected these 29 key lncRNAs to analyze their correlation with clinical features, and 21 of them were aberrantly expressed with tumor pathological stage, TNM staging system, lymph node metastasis and patient outcome assessment, respectively. Furthermore, there were 5 lncRNAs (BCRP3, LINC00472, CHIAP2, BMS1P20 and UNQ6494) positively correlated with overall survival (OS, log-rank P<0.05). Finally, 7 cancer specific lncRNAs were randomly selected to verify the expression in 53 newly diagnosed LUAD patients using qRT-PCR. The expression results between TCGA and qRT-PCR were 100% in agreement. The correlation between AFAP1-AS1 and LINC00472 and clinical features were also confirmed. Thus, our results showed the lncRNA expression profiles and we constructed an lncRNA-miRNA-mRNA ceRNA network in LUAD. The present study provides novel insight for better understanding of lncRNA-related ceRNA network in LUAD and facilitates the identification of potential biomarkers for diagnosis and prognosis.

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Introduction

Lung cancer is the most commonly diagnosed and fatal cancer, which was estimated as 1.8 million new cases and almost 1.6 million deaths occurring worldwide in 2012 (1,2). Lung adenocarcinoma (LUAD) is major pathologic subtype of lung cancer in non-smoking males, and in females (smokers or non-smokers) (3,4). Based on the GLOBOCAN series, the incidence of LUAD has significantly increased over the past few decades in Africa, America and Asia (5). LUAD mainly occurs in the peripheral region of the lungs, with ability to metastasize to the lymph nodes and other organs. Previous studies have shown that several genetic abnormalities were associated with the initiation and development of LUAD (6-10), but the pathogenesis contributing to biological properties of LUAD remain inconclusive.

Non-coding RNAs (ncRNAs) are a class of RNA molecules with no protein-coding function, which are widely expressed in organisms. ncRNAs are divided into several subtypes, such as microRNAs (miRNAs), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), ribosomal RNA (rRNA), transfer ribonucleic acidtRNA (tRNA) and long non-coding RNA (lncRNA).

Recent studies indicate that ncRNAs play important biological roles in various biological processes, such as regulate gene expression, cell proliferation, differentiation and apoptosis (11-15). lncRNAs, ranging from 200 nucleotides to 100 kb in length, have become an area of increased research (12,16). lncRNAs may regulate gene expression at the transcriptional, post-transcriptional and epigenetic levels (17-19). Because of the stronger tissue specificity, lncRNAs can be more effective as early diagnosis and screening of cancer biomarkers (20). Increasing number of studies on lncRNAs has been performed in various types of cancer, such as lung, esophageal, gastric, hepatocellular and colorectal cancer (21-25.) The aberrantly expressed lncRNAs, which

regulate initiation, development, invasion and metastasis of tumors have been detected in LUAD (10,26).

The ceRNA (competing endogenous RNA) hypothesis was proposed as a novel regulatory mechanism between non-coding RNA and coding RNA (27). lncRNA can interact with the miRNA by miRNA-binding sites (MREs), thereby regulating gene expression (28). The complex crosstalk of ceRNA network has been detected in many different diseases (19). Zhang *et al* (29) analyzed the lncRNA profiles in 372 hepatocellular cell carcinoma (HCC) patients from The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/) and NCBI GEO Omnibus (GSE65485), and constructed an lncRNA-miRNA-mRNA network in HCC. Based on the ceRNA hypothesis, Li *et al* (30) selected 361 RNA sequencing profiles of gastric cancer (GC) patients from TCGA, and further analysis of the associations between these RNA data and related patient clinical information. Then, the lncRNA-miRNA-mRNA ceRNA network of GC was constructed.

A recent study showed the differential expression of lncRNA profile of LUAD using high-throughput microarray analysis from 33 LUAD patients (31). However, studies with large scale samples and microarray detection is still rare, and the relationship between cancer specific lncRNAs and clinical features are unclear. TCGA database is a public platform, in which sequencing data of lncRNA, miRNA and mRNA for LUAD can be downloaded. To improve the reliability and accuracy of the studies, we detected lncRNAs in LUAD using data sets from TCGA database.

In the present study, the RNA sequencing data of 521 LUAD samples and 49 adjacent non-tumor lung tissues samples were collected from TCGA database. This is the first study to investigate the cancer specific lncRNA from large scale sequencing database (TCGA) and ceRNA network constructed in LUAD. Quantificational real-time polymerase chain reaction (qRT-PCR) was used to verify the bioinformatic analysis results in LUAD tumor tissues and adjacent non-tumor lung tissues from 53 newly diagnosed LUAD patients. The present study can help elaborate the function of lncRNAs through lncRNA-miRNA-mRNA ceRNA network in LUAD.

Materials and methods

Patients and samples. This study was approved by the ethics committee of the Zhongda Hospital Southeast University. A total of 521 LUAD cases were obtained from the TCGA database. The exclusion criteria were set as follows: i) histologic diagnosis is not LUAD; ii) suffering of other malignancy except LUAD; iii) patients samples without complete data for analysis; and iv) overall survival (OS) >5 years. Overall, a total of 465 LUAD patients were included in this study. lncRNA expression profiles for normal lung tissue samples were obtained from adjacent non-tumor lung tissues (n=49). In addition, there are 170 LUAD patients with lymph node metastasis and 295 LUAD patients with non-lymph node metastasis. According to the Union for International Cancer Control (UICC), well and moderately-differentiated LUAD (stage I-II) were 359 patients, poorly-differentiated LUAD (stage III-IV) were 106 patients. Both RNA expression data and clinical data, including staging information and outcome of LUAD patients downloaded from the TCGA Data Portal, are open-access and publicly available. The present study meets the publication guidelines provided by TCGA (http://cancergenome.nih.gov/publications/publicationguidelines).

In addition, 53 frozen tissue specimens (tumor tissues and their paired adjacent non-cancerous tissues) from LUAD patients collected between 2006 and 2015 were obtained from Nanjing Chest Hospital Medical School of Southeast University. Tissues were snap-frozen in RNAlater (Ambion, Austin, TX, USA) and stored in liquid nitrogen immediately after surgical resection until total RNA extraction and analysis. Samples were received with a pathology report and a quality assessment report verifying collection of tumor and/or adjacent non-tumor lung tissues. Informed consent forms were obtained from all patients included in the present study.

RNA sequence data procession and computational analysis. The LUAD RNA expression data (level 3) of the corresponding patients were downloaded from the TCGA Data Portal (March 2016). The RNA sequencing raw reads (lncRNA and mRNA) were post-processed and normalized by TCGA RNASeqV2 system. Level 3, normalized miRNA expression data (the calculated expression for all reads aligning to a particular miRNA per sample) were downloaded from the TCGA Data Portal performed using Illumina HiSeq and Illumina GA microRNA sequencing platforms (Illumina, Inc., Hayward, CA, USA) and quantile normalized before performing analysis. No further normalizations were applied to the lncRNA, miRNA and mRNA expression profile data in level 3, because these data were already normalized by TCGA. To detect the differential expression of lncRNA, miRNA and mRNA, samples were divided into LUAD patient tumor tissues vs. adjacent non-tumor lung tissues, lymph node metastasis of LUAD patients vs. non-lymph node metastasis of LUAD patients, well and moderately-differentiated LUAD (stages I-II) vs. poorly-differentiated LUAD (stages III-IV), respectively. For further analysis, intersection of lncRNA, miRNA and mRNA was selected. The flow chart for bioinformatics analysis is depicted in Fig. 1.

Functional enrichment analysis. To understand the underlying biological processes and pathways of aberrantly expressed intersection genes, we use the DAVID (Database for Annotation, Visualization, and Integrated Discovery) Bioinformatics resources (http://david.abcc.ncifcrf.gov/) (32), to conduct functional enrichment analysis, and we were only interested in GO (Gene Ontology) biological processes and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways at the significant level ($P<0.05$ and an enrichment score of >1.5).

Construction of lncRNA-miRNA-mRNA ceRNA network. The lncRNA-miRNA-mRNA ceRNA network was constructed based on the relationship among lncRNA, miRNA and mRNA. It is established that the post-transcriptional regulation of mRNA transcripts could be bound by single-stranded miRNAs. In the present study, the lncRNA-miRNA-mRNA ceRNA network, which was based on the theory that lncRNAs can have a direct interaction by invoking the miRNA sponge to regulate the activity of mRNAs (33). To construct ceRNA network, the human miRNA-mRNA and miRNA-lncRNA

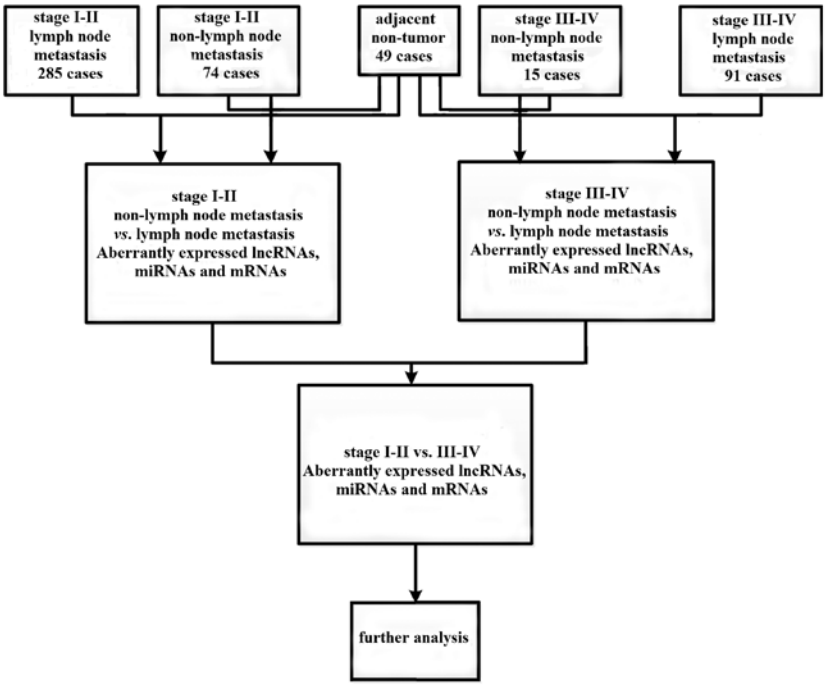

Figure 1. Flow chart of bioinformatics analysis.

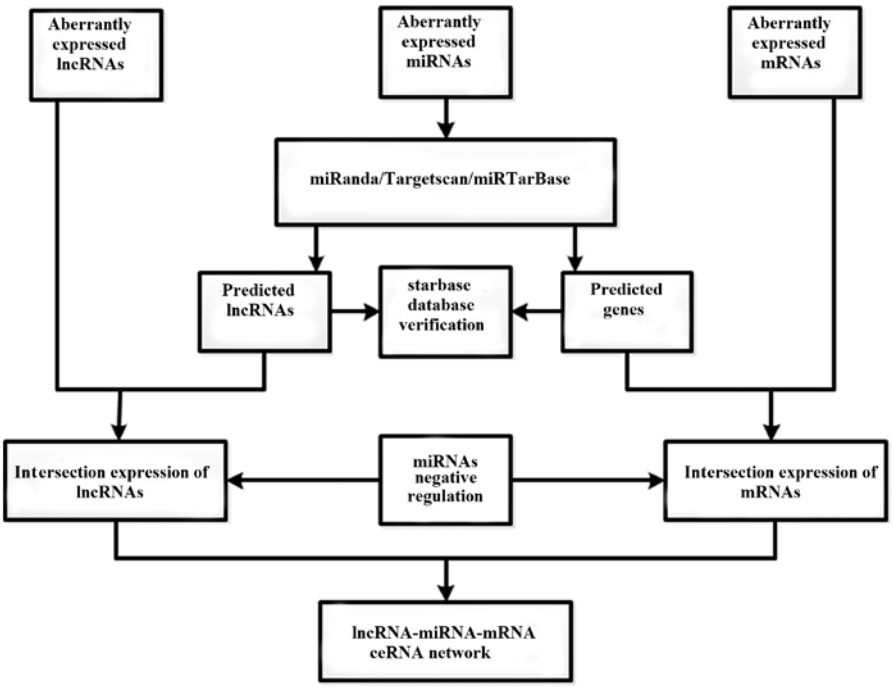

Figure 2. The flow chart of ceRNA network analysis.

interactions could be downloaded from the starBase V2.0 database (http://starbase.sysu.edu.cn/) (34), then aberrantly expressed intersection lncRNA, miRNA and mRNA with fold change >2.0 and/or fold change <0.5 and P<0.05 were retained. The miRanda tools (http://www.microrna.org/microrna/home.do) were used to predict the lncRNA-miRNA interactions. Targetscan (http://www.targetscan.org/) and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) (35) were performed to predict the mRNAs targeted by miRNAs. The results could be verified in starBase database. Furthermore, in the present study the predicted miRNAs and aberrantly expressed data of TCGA were combined to select the intersection lncRNAs and mRNAs. To construct ceRNA network, the miRNAs negatively regulated intersection expression of lncRNAs and mRNAs were selected, based on the theory of ceRNA. Cytoscape v3.0 (30) was performed to construct and visualize the lncRNA-miRNA-mRNA ceRNA network. The flow chart for ceRNA network construction is described in Fig. 2.

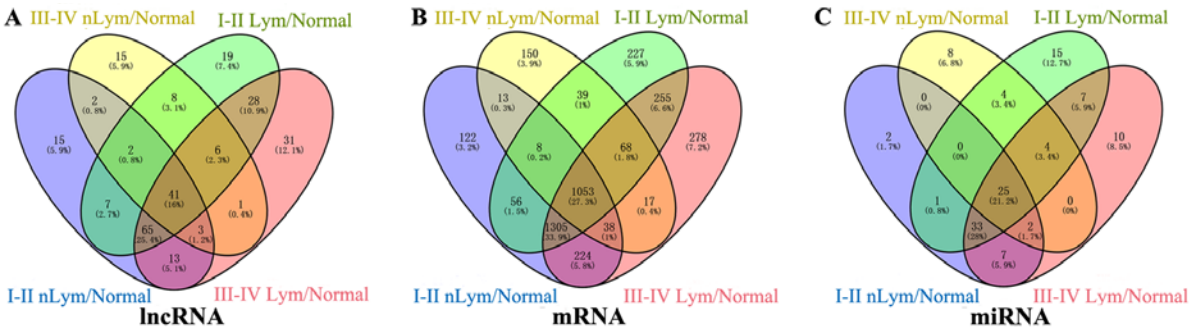

Figure 3. Venn diagram analysis of aberrantly expressed (A) lncRNAs, (B) mRNAs and (C) miRNAs between I-II Lym/Normal, I-II nLym/Normal, III-IV Lym/Normal and III-IV nLym/Normal. Lym, lymph node metastasis; nLym, non-lymph node metastasis; normal represent adjacent non-tumor lung tissues.

The correlations between LUAD specific lncRNAs and clinical features. According to the bioinformatics analysis and the ceRNA network, specific lncRNAs were selected. We further analyzed the clinical features including gender, tumor pathological stage, TNM staging system, lymph node metastasis and patient outcome assessment. Subsequently, to identify the specific lncRNAs with prognostic characteristics, the univariate Cox proportional hazards regression model was used to analyze the association between the specific lncRNAs and the LUAD patient survival time.

Total RNA extraction and qRT-PCR verification. We randomly selected 7 of the specific lncRNAs in lncRNA-miRNA-mRNA ceRNA network, and validated the reliability and validity of the results in 53 newly diagnosed LUAD patients using qRT-PCR.

The total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol, and RNA purity was detected by NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription reactions using A3500 reverse transcription system kit (Promega, Madison, WI, USA) was conducted in two steps according to the manufacturer's protocol. QRT-PCR was carried out to detect the expression levels of candidate lncRNAs with the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). QRT-PCR was then performed using A6001 GoTaq qPCR Master Mix (Promega) according to the manufacturer's protocol. All primers were purchased from Generay Biotech Co., Ltd. (Shanghai, China). The Ct-value for each sample was calculated with the ΔΔCt method (10), and fold change results were presented as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{RNAs} - Ct_{GAPDH})_{\text{tumor}} - (Ct_{RNAs} - Ct_{GAPDH})_{\text{adjacent non-tumor tissues}}$.

Statistical analysis. All data are presented as mean ± SD, and statistically compared performing the paired t-test. The significance level was set as 0.001 as default to control the false discovery rate (FDR). Values of P<0.05 were considered statistically significant. The statistical analyses were performed by the SPSS 21.0.

Results

LUAD specific lncRNAs in LUAD patients. We identified a total of 1806 lncRNAs from TCGA database. Analysis of lncRNA expression profiles in LUAD patient tissues (n=465) compared with adjacent non-tumor lung tissues (n=49) identified 256 aberrantly expressed lncRNAs (absolute fold change >2, P<0.01). Of these, 103 lncRNAs were downregulated and 153 lncRNAs were upregulated. Based on these data, further analysis was performed between tumor stage (lymph node metastasis) LUAD patient tissues and adjacent non-tumor lung tissues. A total of 148 aberrantly expressed lncRNAs were selected between stages I-II (non-lymph node metastasis) LUAD patient tissues and adjacent non-tumor lung tissues, 176 aberrantly expressed lncRNAs were selected between stages III-IV (non-lymph node metastasis) LUAD patient tissues and adjacent non-tumor lung tissues, 78 aberrantly expressed lncRNAs were selected between stages I-II (lymph node metastasis) LUAD patient tissues and adjacent non-tumor lung tissues, 188 aberrantly expressed lncRNAs were selected between stages III-IV (lymph node metastasis) LUAD patient tissues and adjacent non-tumor lung tissues. To further enhance the data reliability, we selected 41 aberrantly expressed lncRNAs (17 downregulated and 24 upregulated) in the intersection of the above 4 groups to build the lncRNA-miRNA-mRNA ceRNA network (Fig. 3A and Table I).

Functional enrichment analysis. In order to understand the functions of aberrantly expressed genes in this study, the intersection mRNAs were analyzed by DAVID bioinformatics resources. In total, we identified 18633 mRNAs from TCGA database. A total of 3853 aberrantly expressed mRNAs were found between LUAD patient tissues and adjacent non-tumor lung tissues (absolute fold change >2, P<0.01). Based on these data, aberrantly expressed genes were further analyzed; 2819 and 1386 aberrantly expressed mRNAs were respectively identified between stages I-II (non-lymph/lymph node metastasis) LUAD patient tissues and adjacent non-tumor lung tissues, 3011 and 3238 aberrantly expressed mRNAs were, respectively, identified between stages III-IV (non-lymph/lymph node metastasis) LUAD patient tissues and adjacent non-tumor lung tissues. Subsequently, 1053 mRNAs were selected from the intersection of the above 4 groups, and divided into downregulated and upregulated genes for further functional analysis (Fig. 3B).

It revealed enrichment of 668 GO categories and 87 KEGG categories (P-value of <0.05 and an enrichment score of >1.5; Figs. 4 and 5). It was found that the top GO biological process of downregulated and upregulated genes was G-protein coupled receptor signaling pathway (GO:0007186) and mitotic cell cycle (GO:0000278) (Fig. 4), respectively. The

Table I. Aberrantly expressed intersection lncRNAs between I-II Lym/Normal, I-II nLym/Normal, III-IV Lym/Normal and III-IV nLym/Normal[a,b,c].

lncRNAs	Gene ID	Chromosome	Regulation	Fold-change	-Log(p)	-Log(FDR[d])
WWC2-AS2	152641	4	Downregulation	-3.306±0.220	6.757	5.243
LINC00472	79940	6	Downregulation	-7.018±0.423	7.000	6.490
AGPAT4-IT1	79992	6	Downregulation	-3.200±0.086	3.916	2.406
CES1P1	51716	16	Downregulation	-7.407±2.079	6.240	4.583
FAM95B1	100133036	9	Downregulation	-2.581±0.390	3.334	1.950
LINC00930	100144604	15	Downregulation	-4.124±1.099	5.140	3.480
RAMP2-AS1	100190938	17	Downregulation	-4.819±0.520	6.903	5.513
BRE-AS1	100302650	2	Downregulation	-3.922±1.397	4.080	2.534
CHIAP2	149620	1	Downregulation	-43.011±6.456	6.903	5.513
LINC01105	150622	2	Downregulation	-5.797±1.526	3.721	2.254
LINC00961	158376	9	Downregulation	-6.061±0.683	7.000	5.774
LINC00908	284276	18	Downregulation	-3.810±0.695	4.337	2.748
BCRP3	644165	22	Downregulation	-2.759±0.328	4.190	2.625
MGC27382	149047	1	Downregulation	-11.799±0.396	7.000	6.490
RPL13AP17	399670	7	Downregulation	-47.619±10.808	7.000	6.490
SIGLEC16	400709	19	Downregulation	-3.478±0.259	3.413	2.023
TPTEP1	387590	22	Downregulation	-3.200±1.353	4.274	2.525
C1orf220	400798	1	Upregulation	4.635±0.649	5.078	3.409
LINC00319	284836	21	Upregulation	6.750±1.475	4.638	3.027
LINC00471	151477	2	Upregulation	2.423±0.135	3.423	2.033
ZNF252P-AS1	286103	8	Upregulation	3.685±0.681	3.623	2.190
DDX12P	440081	12	Upregulation	4.395±1.037	4.232	2.683
DGCR5	26220	22	Upregulation	9.073±2.637	5.049	3.384
FER1L4	80307	20	Upregulation	21.753±9.600	5.199	3.515
ANKRD36BP2	645784	2	Upregulation	5.205±1.082	3.520	2.109
LOC100132111	100132111	1	Upregulation	3.048±0.504	3.361	1.984
LOC100133985	100133985	2	Upregulation	2.888±0.318	3.471	2.069
LINC00896	150197	22	Upregulation	5.338±0.378	7.000	6.245
LOC285629	285629	5	Upregulation	9.730±5.681	7.000	6.245
LRRC37A6P	387646	10	Upregulation	2.860±0.538	3.322	1.954
LOC399815	399815	10	Upregulation	8.038±1.458	7.000	6.245
AFAP1-AS1	84740	4	Upregulation	46.210±7.498	7.000	6.245
BMS1P20	96610	22	Upregulation	4.468±0.767	3.550	2.132
PVT1	5820	8	Upregulation	5.335±1.037	5.949	4.199
RAET1K	646024	6	Upregulation	3.463±0.161	7.000	5.568
SNHG4	724102	5	Upregulation	3.625±0.117	5.505	3.791
UCA1	652995	19	Upregulation	5.445±0.844	6.456	4.695
TCAM1P	146771	17	Upregulation	4.053±0.409	4.205	2.661
CECR7	100130418	22	Upregulation	4.055±0.821	4.428	2.850
ALOX12P2	245	17	Upregulation	3.623±0.261	3.787	2.321
UNQ6494	100129066	9	Upregulation	2.330±0.881	3.549	1.940

[a]41 LUAD specific lncRNAs for ceRNA network construction with absolute fold change >2.0, P<0.05. [b]I, II, III and IV, TNM stage I, II, III and IV. [c]Lym, lymph node metastasis; nLM, non-lymph node metastasis; normal represents adjacent non-tumor lung tissues; [d]FDR, the false discovery rate of the lncRNAs, using Benjamini and Hochberg (1995) method.

top 15 KEGG pathways of downregulated and upregulated genes are also described in Fig. 5. Of these above pathways, PI3K-Akt signaling pathway, p53 signaling pathway, MAPK signaling pathway and Wnt signaling pathway are involved in development, invasion and metastases of cancer. In addition, there were other cancer-related pathways including pathways in cancer, small cell lung, pancreatic and bladder cancer and glioma.

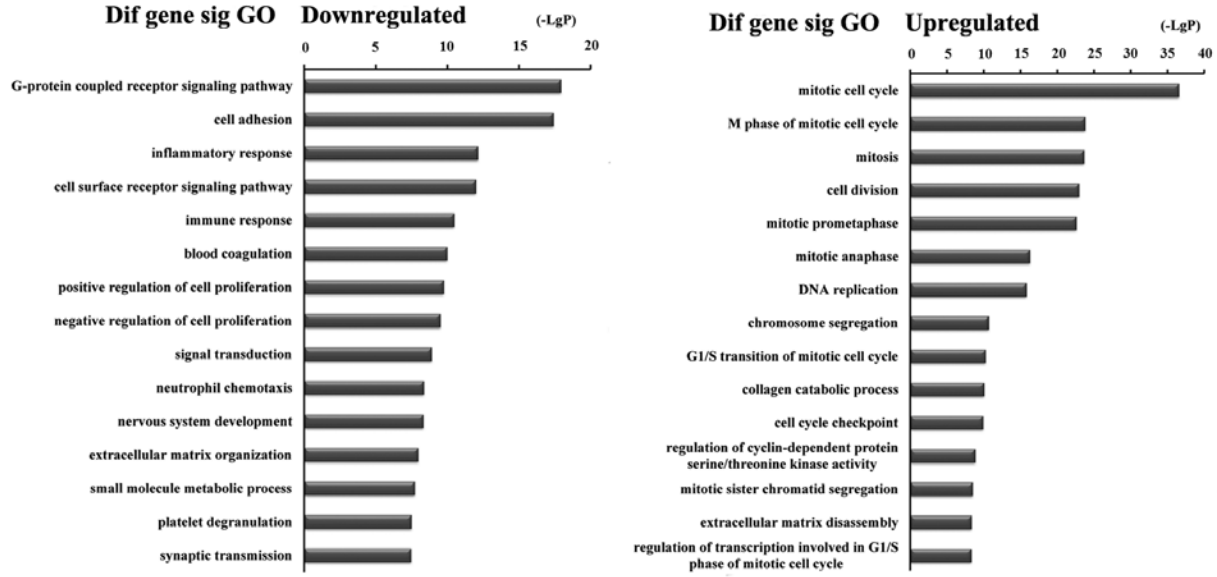

Figure 4. Top 15 enrichment of GO terms for aberrantly expressed intersection mRNAs (the bar plot shows the enrichment scores of the significant top 15 enrichment GO terms).

Table II. miRNAs that may target LUAD specific lncRNAs.

lncRNAs	miRNAs
LINC00961	hsa-miR-127-5p, hsa-miR-96-5p
SIGLEC16	hsa-miR-127-5p
ALOX12P2	hsa-miR-133a-3p, hsa-miR-30a-3p , hsa-miR-486-5p
BCRP3	hsa-miR-135b-5p
LINC01105	hsa-miR-135b-5p, hsa-miR-143-5p, hsa-miR-182-5p, hsa-miR-708-5p, hsa-miR-96-5p
FER1L4	hsa-miR-139-3p, hsa-miR-30c-2-3p
LINC00319	hsa-miR-139-3p
LINC00896	hsa-miR-139-3p, hsa-miR-30c-2-3p
DDX12P	hsa-miR-139-5p,
TCAM1P	hsa-miR-139-5p, hsa-miR-221-5p, hsa-miR-30c-2-3p
TPTEP1	hsa-miR-142-3p, hsa-miR-708-5p
FAM95B1	hsa-miR-143-5p, hsa-miR-9-5p
LINC00472	hsa-miR-143-5p, hsa-miR-96-5p
LINC00930	hsa-miR-143-5p,
RAMP2-AS1	hsa-miR-143-5p
CHIAP2	hsa-miR-182-5p, hsa-miR-708-3p,
LOC399815	hsa-miR-30a-3p, hsa-miR-3614-5p, hsa-miR-378a-3p, hsa-miR-378c
LRRC37A6P	hsa-miR-30a-3p, hsa-miR-338-5p, hsa-miR-378a-3p, hsa-miR-378c
BMS1P20	hsa-miR-30c-2-3p, hsa-miR-378a-3p, hsa-miR-378c
LOC285629	hsa-miR-30c-2-3p,
MGC27382	hsa-miR-33a-5p
AFAP1-AS1	hsa-miR-3614-5p, hsa-miR-378a-3p, hsa-miR-378c, hsa-miR-451a
SNHG4	hsa-miR-3614-5p
UCA1	hsa-miR-3614-5p
CECR7	hsa-miR-378a-3p, hsa-miR-378c
RAET1K	hsa-miR-378a-3p, hsa-miR-378c
PVT1	hsa-miR-378c,
UNQ6494	hsa-miR-378c
DGCR5	hsa-miR-486-5p

Table III. miRNAs targeting LUAD-specific mRNAs.

miRNAs	mRNAs
hsa-miR-127-5p	ATOH8, PKNOX2, SYNPO2L
hsa-miR-135b-5p	ADARB2, ARC, NTNG1, PCYT1B, PGR, PRIMA1, SCN2B
hsa-miR-139-3p	SOX11
hsa-miR-139-5p	MEX3A, NRK, TRIM9
hsa-miR-143-5p	FAT3, TPPP
hsa-miR-182-5p	AATK, ANGPTL1, BDNF, CACNB4, DKK2, EPAS1, FAM107A, FAT3, FIGF, KCNMB2, LDB3, NRN1, ST6GALNAC3, ZFP36
hsa-miR-210-3p	HIF3A
hsa-miR-221-5p	CACNA1E, NAT8L, RAD51
hsa-miR-30a-3p	KCNE4
hsa-miR-30c-2-3p	INHBE, MCHR1, MNX1, NAT8L, PACSIN1
hsa-miR-338-5p	ONECUT2
hsa-miR-33a-5p	AFF3, SLC24A4
hsa-miR-3607-3p	CDH13, NECAB1, TNXB
hsa-miR-3614-5p	ELAVL2, EPN3, FAM155B, GAP43, THBS2, UBE2QL1
hsa-miR-378a-3p	SLC2A1, SULF1
hsa-miR-378c	SLC2A1, SULF1
hsa-miR-708-3p	AFF2, AFF3, MDGA1, CNTFR, GPM6A, MASP1, MDGA1
hsa-miR-9-5p	CNTFR, GABRB2, NTNG1, SCN2B, SHC3, TMTC1, TSPAN18
hsa-miR-96-5p	AHNAK, CACNB4, COL13A1, FREM1, HBEGF, LDB3, LRRC4, NR4A3, RIMS4, SCNN1G, SLC1A1, SOX5, TLL1

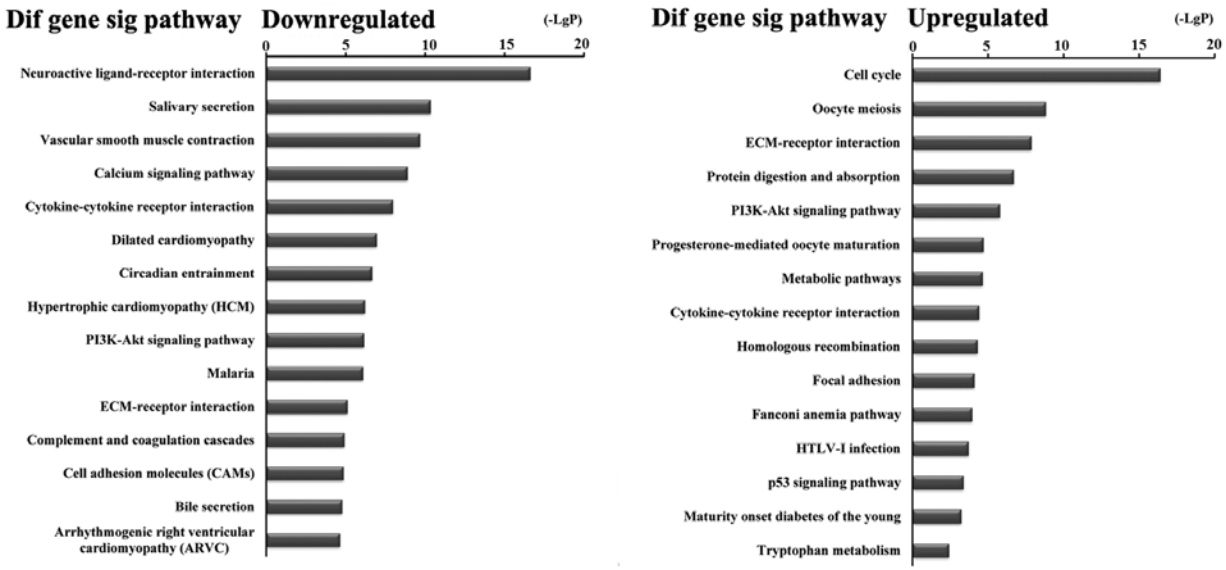

Figure 5. Top 15 enrichment of KEGG pathways for aberrantly expressed intersection mRNAs (the bar plot shows the enrichment scores of the significant top 15 enrichment KEGG pathways).

Prediction of miRNA targets and ceRNA network construction. In order to establish lncRNA-miRNA-mRNA ceRNA network, lncRNAs and mRNAs targeted by miRNAs were identified from the above data. In the present study, in total we identified 1030 miRNAs from TCGA database. A total of 118 aberrantly expressed miRNAs were found between LUAD patient tissues and adjacent non-tumor lung tissues (absolute fold change >2, P<0.05). Based on comparison of the above 4 groups, 25 miRNAs were selected from the intersection aberrantly expressed miRNAs (Fig. 3C). Subsequently, we focused on the targeted relationship between these 25 miRNAs and the above 41 specific intersection lncRNAs. In the lncRNA-miRNA-mRNA ceRNA network, miRNAs interact with lncRNAs through MREs, starBase v2.0 was performed to detect the potential MREs. The results showed that 22 specific miRNAs interacted with 29 specific lncRNAs (Table II).

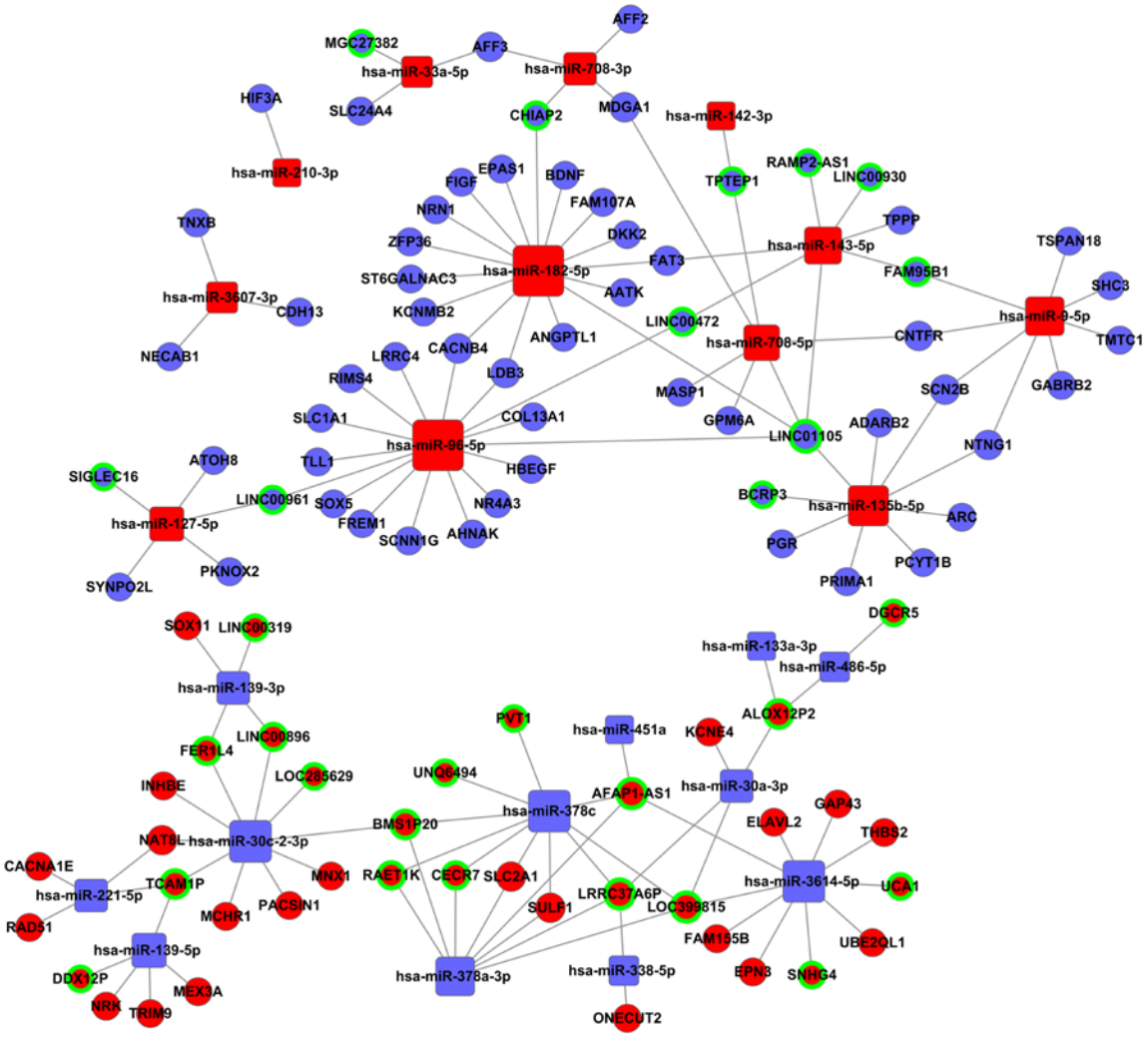

Figure 6. The lncRNA-miRNA-mRNA ceRNA network. Blue diamonds, downregulated miRNAs; blue balls, downregulated mRNAs; blue balls surrounded by green rings, downregulated lncRNAs. Red diamonds, upregulated miRNAs; red balls, upregulated mRNAs; red balls surrounded by green rings, upregulated lncRNAs.

Table IV. The correlations between LUAD specific lncRNAs from ceRNA network and clinical features.

Comparisons	Downregulated	Upregulated
Gender (female vs. male)	BCRP3	ALOX12P2, TCAM1P, UCA1, LOC399815, SNHG4
Race (White vs. Asian)		LINC00896, SNHG4
Tumor pathological stage (I-II vs. III-IV)	BCRP3, LINC01105	AFAP1-AS1, SNHG4, CECR7, RAET1K, PVT1
TNM staging system (T1 + T2 vs. T3 + T4)	LINC01105, TPTEP1, LINC00472, RAMP2-AS1	DDX12P
Lymph node metastasis (no vs. yes)	BCRP3, LINC00472, LINC00930	
Patient outcome assessment (dead vs. alive)	LINC00961, TPTEP1, LINC00472, LINC00930, MGC27382	FER1L4, LINC00896, BMS1P20

In the next step, mRNA targeted by miRNAs was performed. Based on the miRNAs described in Table II, TargetScan and miRTarBase were employed to predict miRNA-targeted mRNA. We identified 19 specific miRNAs related to the 72 intersection mRNAs (Table III). Some of their targets are cancer-associated genes, such as BDNF, CACNB4, DKK2, EPAS1, FIGF, RAD51, THBS2 and SLC2A1.

Based on the above data (Tables II and III), the lncRNA-miRNA-mRNA ceRNA network was constructed. The ceRNA network was plotted with Cytoscape 3.0. Fig. 6, shows

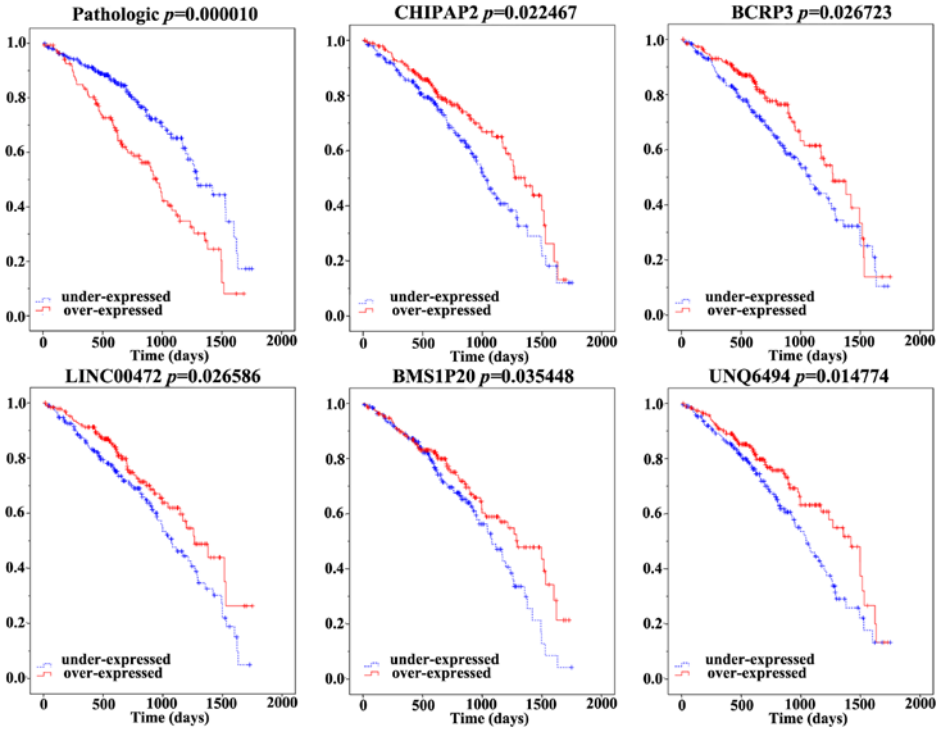

Figure 7. Kaplan-Meier survival curves for 5 lncRNAs associated with overall survival. Horizontal axis, overall survival time, days; vertical axis, survival function.

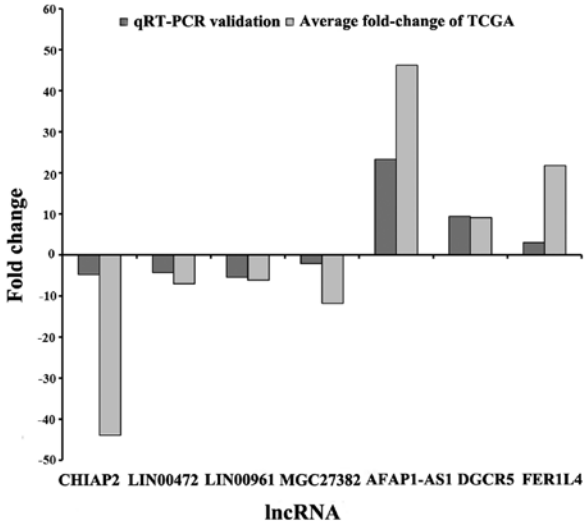

Figure 8. qRT-PCR validation of 7 differentially expressed key lncRNAs. Comparison of fold change ($2^{-\Delta\Delta Ct}$) of lncRNAs between TCGA and qRT-PCR results.

29 lncRNAs, 24 miRNAs and 72 mRNAs that are involved in the proposed ceRNA network.

The correlations between LUAD specific lncRNAs and clinical features. The 29 specific lncRNAs from the ceRNA network were further analyzed according to clinical features including gender, race, tumor pathological stage, TNM staging system, lymph node metastasis, and patient outcome assessment at diagnosis in TCGA database. There were 21 LUAD-specific lncRNAs, the expression levels of which were significantly aberrantly expressed in clinical feature comparisons (P<0.05; Table IV). Six lncRNAs (BCRP3, ALOX12P2, TCAM1P, UCA1, LOC399815 and SNHG4) were aberrantly expressed in gender, 2 lncRNAs (LINC00896 and SNHG4) were aberrantly expressed in race, 7 lncRNAs (BCRP3, LINC01105, AFAP1-AS1, SNHG4, CECR7, RAET1K and PVT1) were aberrantly expressed in tumor pathological stage, 5 lncRNAs (LINC01105, TPTEP1, LINC00472, RAMP2-AS1 and DDX12P) were aberrantly expressed in TNM staging system, 3 lncRNAs (BCRP3, LINC00472 and LINC00930) were aberrantly expressed in lymph node metastasis, and 8 lncRNAs (LINC00961, TPTEP1, LINC00472, LINC00930, MGC27382, FER1L4, LINC00896 and BMS1P20) were aberrantly expressed in patient outcome assessment.

In order to identify the 29 specific lncRNAs with prognostic characteristics, these lncRNAs in the TCGA database were profiled with the univariate Cox proportional hazards regression model and 5 specific lncRNAs were found significantly associated with OS (log-rank P<0.05). While the remaining 5 (BCRP3, LINC00472, CHIAP2, BMS1P20 and UNQ6494) were positively correlated with OS (Fig. 7).

qRT-PCR verification. Finally, we randomly selected 7 specific lncRNAs (AFAP1-AS1, CHIAP2, DGCR5, FER1L4, LINC00472, LINC00961 and MGC27382) to validate the reliability and validity of the above analysis results. We applied the paired t-test to assess the differences between the LUAD tumor tissues and the adjacent non-tumor lung tissues. The results showed that CHIAP2, LINC00472, LINC00961 and MGC27382 were downregulated in LUAD tumor tissues when compared with adjacent non-tumor lung tissues, while AFAP1-AS1, CHIAP2 and FER1L4 were

Table V. Relative expression of 7 lncRNAs in the LUAD tumor tissues and the adjacent non-tumor lung tissues.

lncRNAs	Tissues	ΔCт (mean ± SD)	ΔΔCт (mean ± SD)	$2^{-\Delta\Delta Ct}$	P-value[a]
CHIAP2	C	11.492±3.274	2.267±3.834	0.206	0.000[b]
	N	9.225±2.448			
LINC00472	C	10.463±2.038	0.275±3.143	0.826	0.002[b]
	N	10.188±3.091			
LINC00961	C	11.361±2.027	2.109±2.917	0.232	0.000[b]
	N	9.253±2.372			
MGC27382	C	8.419±2.559	2.443±3.059	0.184	0.000[b]
	N	5.977±2.397			
AFAP1-AS1	C	9.143±3.779	-4.539±4.813	23.244	0.000[b]
	N	13.682±4.450			
DGCR5	C	13.504±2.427	-3.227±4.183	9.365	0.000[b]
	N	16.669±3.591			
FER1L4	C	7.887±1.983	-1.585±2.840	2.999	0.000[b]
	N	9.472±1.994			

[a]P-value, ΔΔCт of LUAD tumor tissues compared with adjacent non-tumor lung tissues. [b]P<0.01.

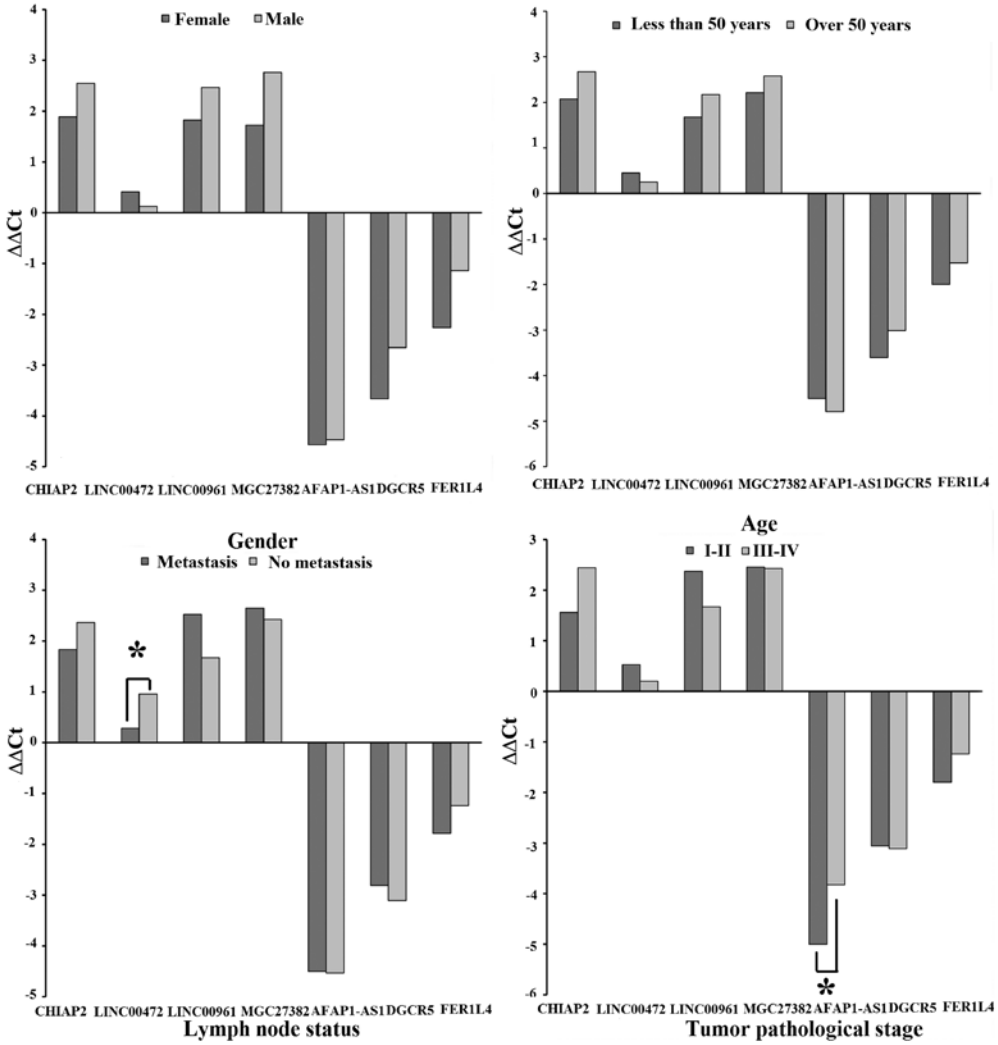

Figure 9. Association between the ΔΔCт of AFAP1-AS1, CHIAP2, DGCR5, FER1L4, LINC00472, LINC00961 and MGC27382 and clinicopathological characteristics in 53 LUAD (*P<0.05).

upregulated in LUAD tumor tissues (Fig. 8 and Table V). The results from the qRT-PCR validation in 53 newly diagnosed LUAD patients were consistent with the above bioinformatics results (Table I). Then, we analyzed the 7 lncRNAs with clinical features. The result showed that AFAP1-AS1 was significantly related with tumor pathological stage, and LINC00472 was significantly related with lymph node metastasis (Fig. 9). The clinical relevance of the 2 lncRNAs was substantially identical with the results of bioinformatics analysis. The above results revealed that our bioinformatics analysis was credible.

Discussion

Lung cancer is current the neoplasia with the highest global incidence and mortality (36,37). LUAD, a cancer that originates in lung glandular cells, is the most frequent type of lung cancer (38). It has been reported that LUAD is steadily rising in both men and women replacing lung squamous cell carcinoma as the most common pathological type of lung cancer in Korea (39). While major improvements in diagnosis, surgical skills, and medical treatments of lung cancer have been applied over the past few years, the average 5-year survival rate remains ~10% (40), mainly because diagnosis cannot find all early stage of the disease. Although several investigative methods are available to support diagnosis and staging of LUAD, such as biopsy sampling, mediastinoscopy, and bronchoscopy, these methods are time-consuming, which may lead to delays in early treatment (41). Therefore, to improve this situation, the identification of cancer-related genes and the exact regulatory mechanism of LUAD initiation and development have received increasing attention.

Aberrant expression of lncRNAs has been widely observed in various diseases, and studies have shown that the dysregulated lncRNAs emerge as key roles in vital biological functions of cancers (42,43). However, only a few studies have described lncRNA profiles in LUAD by microarray analysis (31). Song *et al* (44) have constructed lncRNA-mRNA network by dysregulated lncRNAs and mRNAs based on ceRNA hypothesis. To date, only a few studies represent the interaction between lncRNA and mRNA, or lncRNA and miRNA in LUAD, the results of which showed that lncRNAs can function as a key part of ceRNA network, but such ceRNA network is still poorly described (45-48). The ceRNA hypothesis has been proposed as a novel regulatory mechanism functioning through miRNA competition (27,49). With further research of ceRNA crosstalk, studies showed that ceRNA genes were mediated by miRNAs, which interacted with lncRNAs, in the complex ceRNA network (50).

In the present study, we identified lncRNAs, miRNAs and mRNAs from TCGA database, which were related with TNM stage and lymph node metastasis in LUAD. Then, we constructed the lncRNA-miRNA-mRNA ceRNA network with TNM stage and lymph node metastasis-related LUAD-specific lncRNAs, miRNAs and mRNAs, which provides integrated biological views of the ceRNA network. The constructed LUAD-related lncRNA-associated ceRNA network provides important hints for detecting the key RNAs of ceRNA-mediated gene regulatory network in the initiation and development of LUAD. Next, we selected the cancer specific key lncRNAs from ceRNA network and investigated their distributions in different LUAD clinical features and their associations with overall survival on the basis of RNA sequencing profile of 465 LUAD tissues and 49 adjacent non-tumor lung tissues. We randomly selected 7 specific lncRNAs (AFAP1-AS1, CHIAP2, DGCR5, FER1L4, LINC00472, LINC00961 and MGC27382) and analyzed their expression levels in the 53 newly diagnosed tumor tissues of LUAD patients and adjacent non-tumor lung tissues using qRT-PCR.

Based on the RNA sequence data from TCGA database, we divided LUAD patients into 4 groups with TNM stage and lymph node metastasis as condition, and they were compared with adjacent non-tumor lung tissues, respectively. Then, we selected the intersection RNAs. In the next step, we investigated bioinformatics of aberrantly expressed mRNAs, and the front enriched functional annotation of GO and KEGG pathway might be the potential function of lncRNA. The results of GO biological process terms showed that the aberrantly expressed genes mainly concentrate on cellular functions, metabolism and immune functions. Based on KEGG pathway analysis, a few cancer-related pathways were detected, including p53 signaling pathway, MAPK signaling pathway and Wnt signaling pathway. Wu *et al* (51) merged two datasets to find novel target genes and pathways to explain the pathogenicity of LUAD, and they found that PPM1D and GADD45B may regulate LUAD progression through p53 signaling pathway. Wang *et al* (52) established a co-culture system of A549 cells and bone marrow-derived cells (HS-5) to investigate the molecular mechanism of BMP9 in LUAD and the bone metastatic microenvironment. Then, he found BMP9 can inhibit the growth and migration of LUAD A549 cells through the MAPK/ERK and NF-κB pathways. It was also reported that Wnt signaling pathways were related with LUAD (53-55).

Growing body of evidence has presented that lncRNAs function as a crucial component of ceRNA network by modulating other RNA transcripts (19,30,33,56-58). For example, HOTAIR may act as an endogenous sink by binding miR-331-3p, thereby abolishing the miRNA-induced inhibitory activity on the HER2 3'-UTR and increasing an additional level of post-transcriptional regulation (59). Hence, the potential connection lncRNA, miRNA and mRNA may exist in initiation and development of LUAD. In the present study, we constructed the lncRNA-miRNA-mRNA ceRNA network to reveal a novel ceRNA regulatory network in LUAD. We found some cancer special lncRNAs, such as FER1L4 (60), MGC27382 (61), UCA1 (25), AFAP1-AS1 (62), were also reported in cancers acting as potential diagnosis and prognostic biomarkers. In addition, we found THBS2 (63), RAD51 (64), SLC2A1 (65), BDNF (66), in the ceRNA network were also related with initiation and development of cancers. In the present study, we analyzed the LUAD-specific lncRNA indirectly related mRNA signal pathways involved in ceRNA network. The results of pathway analysis showed that there were 9 pathways related with cancer. Therefore, our results suggested that the key lncRNAs may play an important role in initiation and development of LUAD and the cancer genes related pathways.

With respect to the associations between 29 cancer specific lncRNAs from ceRNA network and clinical features, including

gender, tumor pathological stage, TNM staging system, lymph node metastasis and patient outcome assessment, we found that 21 lncRNAs were related to clinical features. Eight of these 21 lncRNAs were reported to be associated with cancer. For example, UCA1 was reported to be an indicator of early gastric cancer (67). However, there is no report on the association between LUAD and the above lncRNAs features. Then we analyzed the associations between 29 cancer-specific lncRNAs and the patient survival, and we found that 5 lncRNAs were related to LUAD OS.

Finally, 7 cancer specific lncRNAs were randomly selected to verify the expressions of specific lncRNAs and credibility of bioinformatics analysis using qRT-PCR. The expression data from TCGA and verification result of 53 newly diagnosed LUAD patients were 100% in agreement. Then, we performed the correlation analysis between the 7 cancer specific lncRNAs and clinical features. The results showed that AFAP1-AS1 and LINC00472 were significantly correlated with tumor pathological stage and lymph node metastasis, respectively. These above results revealed that our bioinformatics analysis is credible.

Based on these above LUAD OS results, only 1 of these 5 LUAD-OS related lncRNAs (LINC00472) was aberrantly expressed in clinical feature. Therefore, lncRNAs that aberrantly expressed in clinical feature comparisons may not be necessarily related with OS (29). Among these 5 lncRNAs only LINC00472 has been reported in the survival of carcinoma and the other lncRNAs (BCRP3, CHIAP2, BMS1P20 and UNQ6494) have not been reported (68). Furthermore, a recent study also found that high LINC00472 expression could significantly reduce risk of relapse and death in breast cancer patients, and LINC00472 could also suppress breast cancer cell proliferation and migration (69). It implies that LINC00472 may play an important role in LUAD.

It has been reported that upregulated AFAP1-AS1 was associated with the poor prognosis of non-small cell lung cancer (NSCLC) patients (70). Furthermore, AFAP1-AS1 was also reported to be associated with prognosis, cell proliferation and invasion of other cancers (62,71,72). Combined with the present study (Tables I, IV, V and Figs. 6-8), showing that the expression of AFAP1-AS1 and LINC00472 were dysregulated in LUAD from TCGA database and qRT-PCR verification, and they were related with tumor pathological stage, and lymph node metastasis and OS, respectively, it reveals that AFAP1-AS1 and LINC00472 may be potential biomarkers in LUAD.

In the present study, we identified the LUAD-specific lncRNAs from hundreds of candidate lncRNAs detected from large scale samples in TCGA database, and revealed aberrant expression profiles of cancer specific lncRNAs under different clinical features. The aberrantly expressed key lncRNAs, which were identified in LUAD, may shed light on sensitive biomarkers in LUAD. Importantly, we have constructed the lncRNA-miRNA-mRNA ceRNA network to clarify the unknown ceRNA regulatory network in LUAD. In addition, qRT-PCR verification was used for the reliability and validity of expression of key lncRNAs and bioinformatics analysis. Our findings provide novel insight into better understanding of lncRNA-related ceRNA network in LUAD and potential biomarkers for diagnosis and prognosis.

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