

Expression levels and co-targets of miRNA-126-3p and miRNA-126-5p in lung adenocarcinoma tissues: An exploration with RT-qPCR, microarray and bioinformatic analyses

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Abstract. Lung adenocarcinoma (LUAD) is the most common histological subtype of lung cancer. Previous studies have found that many microRNAs (miRNAs), including miRNA-126-3p, may play a critical role in the development of LUAD. However, no study of LUAD has researched the synergistic effects and co-targets of both miRNA-126-3p and miRNA-126-5p. The present study used real-time quantitative polymerase chain reaction (RT-qPCR) to explore the expression values of miRNA-126-3p and miRNA-126-5p in 101 LUAD and 101 normal lung tissues. Ten relevant microarray datasets were screened to further validate the expression levels of miRNA-126-3p and -5p in LUAD. Twelve prediction tools were employed to obtain potential targets of miRNA-126-3p and miRNA-126-5p. The results showed that both miRNA-126-3p and -5p were expressed significantly lower in LUAD. A significant positive correlation was also present between miRNA-126-3p and -5p expression in LUAD. In addition, lower expression of miRNA-126-3p and -5p was indicative of vascular invasion, lymph node metastasis (LNM), and a later tumor/node/metastasis (TNM) stage of

LUAD. The authors obtained 167 targets of miRNA-126-3p and 212 targets of miRNA-126-5p; 44 targets were co-targets of both. Eight co-target genes (*IGF2BP1*, *TRPM8*, *DUSP4*, *SOX11*, *PLOD2*, *LIN28A*, *LIN28B* and *SLC7A11*) were initially identified as key genes in LUAD. The results of the present study indicated that the co-regulation of miRNA-126-3p and miRNA-126-5p plays a key role in the development of LUAD, which also suggests a fail-proof mode between miRNA-3p and miRNA-126-5p.

Introduction

Lung cancer (LC) is one of the world's most widespread cancers. More than 1.5 million people are diagnosed with LC every year (1,2). Lung adenocarcinoma (LUAD) is the most common histological subtype of LC (3,4). The 5-year survival rate for LUAD patients is less than 15%, as most are diagnosed at advanced stages (5). Therefore, it is urgent to determine the molecular mechanism of LUAD and identify an effective method for early diagnosis and effective treatment.

MicroRNAs (miRNAs) are endogenous, non-coding, small RNAs. They control gene expression by combining with the messenger RNAs (mRNAs) of target genes, causing mRNA degradation or translation suppression (6,7). Numerous studies have demonstrated that the aberrant and disordered expression of miRNAs is involved in many malignant tumors, including LCs (8-12).

Previous research has confirmed that miRNAs can be divided into three different forms, as follows: primary (pri-) miRNA, precursor (pre-)miRNA and mature miRNA. The mature miRNAs designated as miRNA-3p and miRNA-5p are derived from the 3' or 5' arms, respectively, of their pre-miRNAs (13). Theoretically, therefore, all pre-miRNAs can produce two types of mature miRNAs. Previous research assumed that only one mature miRNA took part in regulating target mRNAs; the other was thought to be a byproduct, and it was regarded as functionally irrelevant (14). Increasingly, however, recent investigations have confirmed that both miRNA-3p and miRNA-5p originate from one pre-miRNA

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and can degrade various target mRNAs (15). Studies also have found a correlation and synergistic effects between miRNA-3p and -5p produced by the same pre-miRNA (16). These findings suggest that miRNA-3p and -5p both function during biological processes, implying that they may co-regulate target genes in various diseases.

miRNA-126-3p and miRNA-126-5p (miRNA-126-3p/5p) are both derived from pre-miRNA-126, which is located on human chromosome 9. miRNA-126-3p and -5p have been identified as essential biological factors in the development of certain malignancies (17). Regarding LC, studies with small sample sizes have confirmed that miRNA-126-3p is down-regulated in non-small cell lung cancers (NSCLCs), including LUAD (18). Nevertheless, previous studies concerning LC have mainly focused on miRNA-126-3p; no research has demonstrated a relationship between miRNA-126-5p and LC. It is also worth noting that LUAD research has not investigated or clarified whether a correlation or synergistic effects between miRNA-126-3p and -5p exist. Based on previous research, the authors predicted that both miRNA-126-3p and -5p may be less expressed in LUAD and may co-regulate key target genes of LUAD.

The present study aimed to explore the clinical utility and investigate the correlation and synergistic effects of miRNA-126-3p and miRNA-126-5p in LUAD. A total of 202 tissues (101 LUAD tissues and 101 adjacent normal lung tissues) were collected. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to explore the respective expression values of miRNA-126-3p and -5p in the 202 tissues. A large sample size from the Gene Expression Omnibus (GEO) database was obtained so that a meta-analysis could be performed with the RT-qPCR data from the present study to further identify miRNA-126-3p and -5p expression in LUAD. In addition, 12 prediction software programs were employed to obtain the target genes of miRNA-126-3p and -5p. Then, bioinformatic analysis was conducted to identify the potential molecular mechanisms of miRNA-126-3p and -5p in LUAD.

This is the first study to investigate the co-regulation of miRNA-125-3p and -5p in LUAD. It is hoped that the present study may demonstrate the molecular function and synergistic effects of miRNA-126-3p and miRNA-126-5p. The obtained research findings may reveal the importance of these miRNAs in LUAD diagnosis and treatment.

Materials and methods

Clinical LUAD sample collection. Based on a previous study (19), 202 formalin-fixed, paraffin-embedded (FFPE) tissues were collected at the First Affiliated Hospital of Guangxi Medical University (Nanning, China). Of these 202 FFPE tissues, 101 were LUAD tissues, while the remaining 101 tissues were paired adjacent normal lung tissues. The research protocol for this study has been ratified by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. Consent from all patients was obtained at the time of the sample collection.

RT-qPCR. RT-qPCR is a sensitive technique for quantifying specific RNA targets. RNA was removed from the FFPE

sample tissues using a miRNeasy FFPE kit (Qiagen, Venlo, The Netherlands) as previously described (20,21). A NanoDrop 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to ensure the purity and concentration of the extracted RNA from the FFPE tissues. The specific primers of miRNA-126-3p and miRNA-126-5p were provided by TaqMan MicroRNA Assays (4427975-000468; Applied Biosystems, Life Technologies Europe B.V., Gent, Belgium). The reverse primers were included in TaqMan MicroRNA Reverse Transcription kit (4366596; Applied Biosystems, Life Technologies Europe B.V.) (22). RNU6B was used as the internal control. The forward primer sequences of RNU6B were 5'-CTCGCTTCGGCAGCACA-3' and the reverse primer sequences were 5'-AACGCTTCACGAATTTGCGT-3'. The sequences of miRNA-126-3p were 5'-UCGUACCGUGAGUAAUAAUGC G-3' and the sequences of miRNA-126-5p were 5'-CAUUAU UACUUUUGGUACGCG-3'. The thermocycling conditions were as follows: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The RT-qPCR was analyzed with Applied Biosystems PCR 7900 (Applied Biosystems; Thermo Fisher Scientific, Inc.) to detect miRNA expression values. Relative expression values were calculated using the $2^{-\Delta\Delta C_q}$ method (23).

GEO data extraction. GEO (<https://www.ncbi.nlm.nih.gov/gds/>) is the largest fully disclosed high-throughput molecular abundance database; it is mainly used to store gene expression data. All microarray datasets related to LUAD were filtered out and downloaded from the GEO database. The microarray dataset selection criteria were as follows: i) the samples had to be human tissue; ii) the dataset had to contain LUAD and non-cancerous lung tissue groups; iii) both the LUAD and non-cancerous lung tissue groups had to include at least two samples; and iv) expression values of miRNA-126-3p or -5p had to be available.

Statistical analysis. Statistical Product and Service Solutions (SPSS) version 22.0 (IBM Corp., Armonk, NY, USA) software was applied to analyze the RT-qPCR data. A quantitative variable was computed and presented as the means \pm standard deviation (SD). Student's t-test was applied to evaluate the difference between two continuous variables. A P-value of <0.05 was considered to indicate a statistically significant result. A receiver operating characteristic (ROC) curve based on the RT-qPCR data was applied to estimate the respective distinguishing impact of miRNA-126-3p and -5p on LUAD from non-cancerous tissues. Binary logistic regression and ROC curve analyses were performed for evaluating the combined distinguishing value of miRNA-126-3p and -5p (24,25). Stata statistical software (version 12.0; Stata Corp., College Station, TX, USA) was used to perform a meta-analysis of the GEO data; the results were assessed using the standard mean difference (SMD). The heterogeneity of the GEO data was assessed by I^2 statistics and a Q test. An $I^2 >50\%$ or a P-value <0.05 was deemed to indicate huge heterogeneity. A summary ROC (SROC) analysis based on the selected microarray datasets as well as the results from the present study was performed to determine the potential distinguishing effect of miRNA-126-3p and -5p on LUAD.

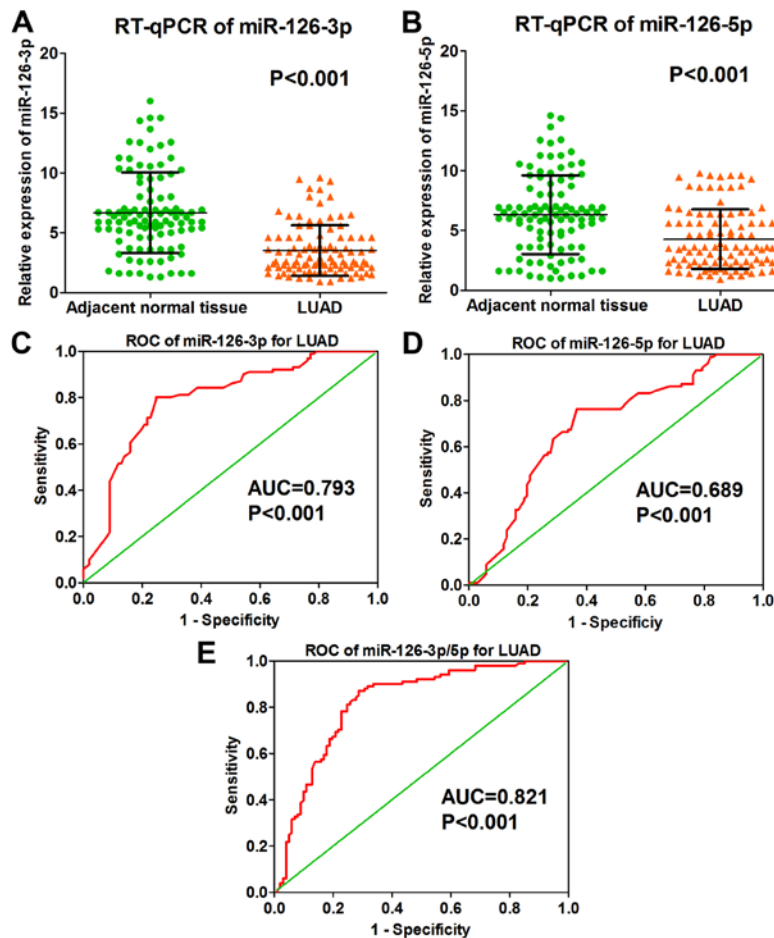


Figure 1. Diagnostic value of miRNA-126-3p and miRNA-126-5p for LUAD according to RT-qPCR. (A) Expression of miRNA-126-3p in 101 LUAD tissues and 101 adjacent normal lung tissues (3.511 ± 2.118 vs. 6.674 ± 3.362 , $P < 0.001$). (B) Expression of miRNA-126-5p in 101 LUAD tissues and 101 adjacent normal lung tissues (4.271 ± 2.501 vs. 6.314 ± 3.289 , $P < 0.001$). (C) ROC curve of miRNA-126-3p for LUAD based on the 101 LUAD tissues and 101 adjacent normal lung tissues (AUC=0.793, $P < 0.001$). (D) ROC curve of miRNA-126-5p for LUAD based on the 101 LUAD tissues and 101 adjacent normal lung tissues (AUC=0.689, $P < 0.001$). (E) Combined ROC curve of miRNA-126-3p and -5p for LUAD based on the 101 LUAD tissues and 101 adjacent normal lung tissues (AUC=0.821, $P < 0.001$). LUAD, lung adenocarcinoma; AUC, area under the receiver operating characteristic (ROC) curve.

Predicting miRNA-126-3p and -5p targets. Twelve target gene prediction software programs (TargetScan, miRWalk, MicroT4, miRDB, miRanda, miRBridge, miRMap, miRNAmap, PITA, PicTar2, RNA22 and RNAhybrid) (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/miRretns-self.html>) were run to obtain the miRNA-126-3p and -5p target genes. Only genes that appeared in more than two of the prediction programs were selected as candidate targets. At the same time, an R function package was used to screen all the overexpressed genes in LUAD from The Cancer Genome Atlas (TCGA) database (<https://cancergenome.nih.gov/>). Fold change (FC) was used to assess the level of gene expression in the TCGA data, and genes were deemed to be overexpressed if the FC > 2. Then, the predicted target genes were overlapped with the overexpressed genes to obtain the miRNA-126-3p and -5p target genes in LUAD.

Bioinformatic analysis. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 (<https://david.ncifcrf.gov/>) was employed for Gene Ontology (GO) analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was used to analyze the function of the above-identified genes in LUAD. A protein-protein

interaction (PPI) network based on the target genes of miRNA-126-3p and -5p was established using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (<http://www.string-db.org/>) and Cytoscape version 3.4.0 (26) was used to determine the correlation between each target.

Results

Expression of miRNA-126-3p and miRNA-126-5p in LUAD tissues according to RT-qPCR

Co-downregulation of the expression of both miRNA-126-3p and miRNA-126-5p in LUAD. The results of the RT-qPCR performed on the 202 collected clinical samples showed that the level of miRNA-126-3p was markedly lower in LUAD tissue than it was in adjacent normal lung tissue (3.511 ± 2.118 vs. 6.674 ± 3.362 , $P < 0.001$; Fig. 1A and Table I); miRNA-126-5p expression was also significantly lower in the LUAD tissues (4.271 ± 2.501 vs. 6.314 ± 3.289 , $P < 0.001$; Fig. 1B and Table I). According to the RT-qPCR data, the ROC curve of miRNA-126-3p in LUAD tissues showed that the area under the curve (AUC) was 0.793 ($P < 0.001$; Fig. 1C); the ROC curve of miRNA-126-5p in LUAD tissues showed an

Table I. Expression of miRNA-126-3p and miRNA-126-5p in LUAD according to RT-qPCR data.

Clinicopathological parameters	N	miRNA-126-3p			miRNA-126-5p		
		Mean ± SD	t	P-value	Mean ± SD	t	P-value
Tissue							
LUAD	101	3.511±2.118	-8.003	<0.001	4.271±2.501	-4.970	<0.001
Adjacent normal lung tissue	101	6.674±3.362			6.314±3.289		
Sex							
Male	56	3.619±2.157	0.572	0.569	4.487±2.359	0.975	0.332
Female	45	3.376±2.083			4.000±2.670		
Age (years)							
<60	41	3.549±1.897	0.149	0.881	4.194±2.081		
≥60	60	3.484±2.271			4.323±2.535		
Smoking history							
+	18	4.306±1.949	1.930	0.060	4.689±2.439	-0.602	0.500
-	26	3.192±1.833			5.141±2.451		
Tumor size (cm)							
≤3	53	3.373±2.250	-0.685	0.495	4.450±2.665	0.755	0.452
>3	48	3.663±1.974			4.073±2.319		
Vascular invasion							
+	31	3.074±1.697	-1.384	0.169	3.045±1.544	-4.195	<0.001
-	70	3.704±2.263			4.814±2.657		
LNM							
+	56	3.161±1.679	-1.798	0.076	3.671±2.090	-3.197	0.002
-	45	3.946±2.514			5.142±2.713		
TNM stage							
I + II	44	4.140±2.583	2.542	0.013	4.923±2.700	2.296	0.024
III + IV	57	3.025±1.527			3.769±2.233		
Pathological grade							
I	17	3.482±2.323	F=1.2	0.305	4.147±2.543	F=1.3	0.271
II	61	3.735±2.087			4.565±2.629		
III	23	2.935±2.024			3.583±2.034		

LUAD, lung adenocarcinoma; N, number of samples; SD, standard deviation; t, t-value of Student's t-test; F, analysis of variance; LNM, lymph node metastasis; +, positive; -, negative.

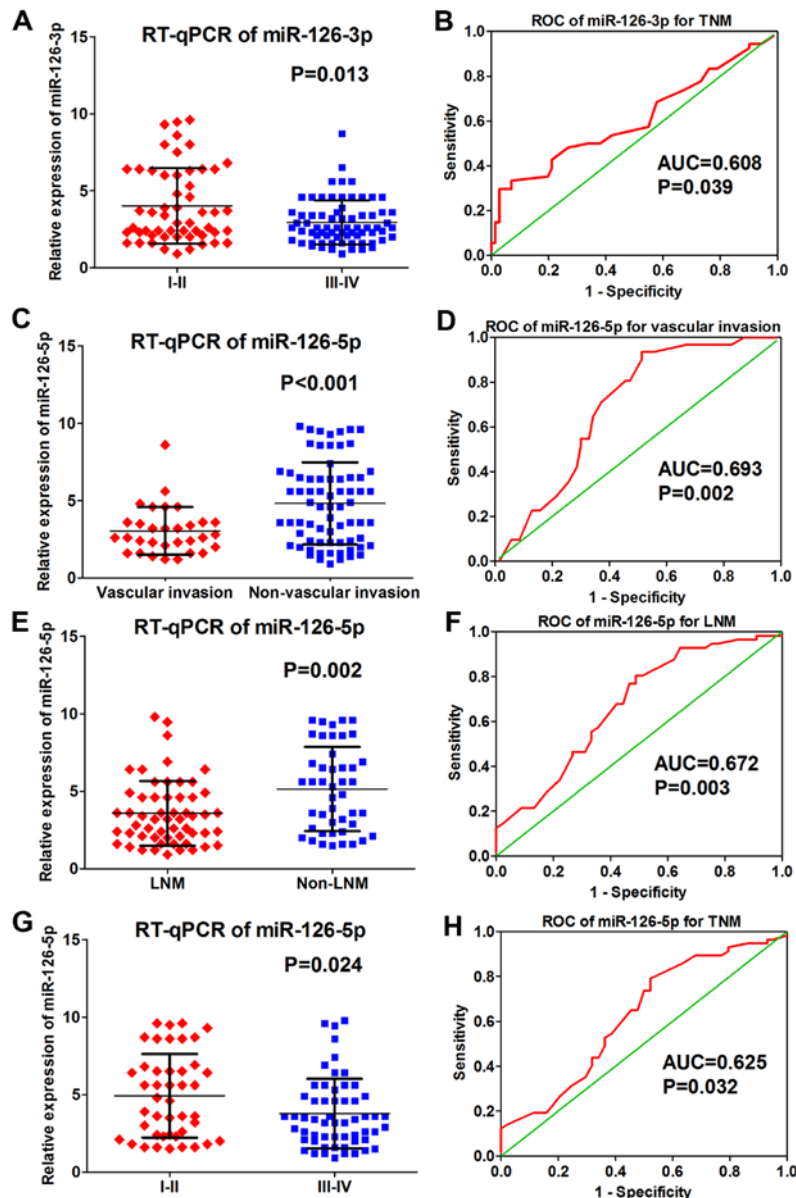


Figure 2. Correlation between miRNA-126-3p and miRNA-126-5p and clinical pathological parameters based on RT-qPCR data. (A) Expression of miRNA-126-3p in early (I-II) and late (III-IV) TNM stages of LUAD (4.140 ± 2.583 vs. 3.025 ± 1.527 , $P < 0.001$). (B) ROC curve of miRNA-126-3p for TNM stages of LUAD (AUC=0.608, $P=0.039$). (C) Expression of miRNA-126-5p in samples with vascular invasion and samples without vascular invasion (3.045 ± 1.544 vs. 4.814 ± 2.657 , $P < 0.001$). (D) ROC curve of miRNA-126-5p for vascular invasion of LUAD (AUC=0.693, $P=0.002$). (E) Expression of miRNA-126-5p in samples with LNM and samples without LNM (3.671 ± 2.090 vs. 5.142 ± 2.713 , $P=0.002$). (F) ROC of miRNA-126-5p for LNM of LUAD (AUC=0.672, $P=0.003$). (G) Expression of miRNA-126-5p in early TNM stage (I-II) and later TNM stage (III-IV) of LUAD (4.923 ± 2.700 vs. 3.769 ± 2.233 , $P=0.024$). (H) ROC of miRNA-126-5p for TNM stage of LUAD (AUC=0.625, $P=0.032$). LUAD, lung adenocarcinoma; AUC, area under the receiver operating characteristic (ROC) curve.

AUC of 0.689 ($P < 0.001$; Fig. 1D). The combined ROC curve of miRNA-126-3p and -5p in LUAD tissues revealed an AUC of 0.821 ($P < 0.001$; Fig. 1E).

Association between miRNA-126-3p and miRNA-126-5p and the clinicopathological parameters of the LUAD samples. The RT-qPCR analysis determined that miRNA-126-3p levels differed significantly according to TNM stage, while miRNA-126-5p levels differed significantly in regards to vascular invasion, lymph node metastasis (LNM) and TNM stage (Table I). Compared with early TNM stages (I-II) of LUAD, miRNA-126-3p was expressed lower in later TNM stages (III-IV) of LUAD (4.140 ± 2.583 vs. 3.025 ± 1.527 ,

$P < 0.001$; Fig. 2A). The ROC of miRNA-126-3p for the TNM stages showed that the AUC was 0.608 ($P=0.039$; Fig. 2B). As for miRNA-126-5p, its level was significantly lower in the samples with vascular invasion (3.045 ± 1.544 vs. 4.814 ± 2.657 , $P < 0.001$; Fig. 2C). The ROC curve of miRNA-126-5p for vascular invasion showed that the AUC was 0.693 ($P=0.002$; Fig. 2D). In addition, miRNA-126-5p expression was lower in samples diagnosed with lymph node metastasis (LNM) (3.671 ± 2.090 vs. 5.142 ± 2.713 , $P=0.002$; Fig. 2E) and the ROC curve relevant to LNM presented an AUC of 0.672 ($P=0.003$; Fig. 2F). miRNA-126-5p expression was also downregulated in later TNM stages (III-IV) of LUAD (4.923 ± 2.700 vs. 3.769 ± 2.233 , $P=0.024$; Fig. 2G), while the relative ROC curve

Table II. The 10 selected microarray datasets.

Authors	Microarray datasets	Year	Country	LUAD tissues	Normal tissues	(Refs.)
Seike <i>et al</i>	GSE14936	2009	USA	19	19	(27)
Ohba <i>et al</i>	GSE19945	2010	Japan	4	8	Not published
Nymark <i>et al</i>	GSE25508	2011	Finland	10	26	(28)
Ma <i>et al</i>	GSE29248	2011	China	3	3	(29)
van Jaarsveld <i>et al</i>	GSE47525	2014	The Netherlands	6	14	(30)
Bjaanaes <i>et al</i>	GSE48414	2014	Norway	154	20	(31)
Arima <i>et al</i>	GSE51853	2014	Japan	76	5	(32)
Robles <i>et al</i>	GSE63805	2015	USA	32	30	(33)
Jin <i>et al</i>	GSE74190	2015	China	36	44	(34)
Yoshimoto <i>et al</i>	GSE77380	2018	Japan	3	12	(35)

LUAD, lung adenocarcinoma.

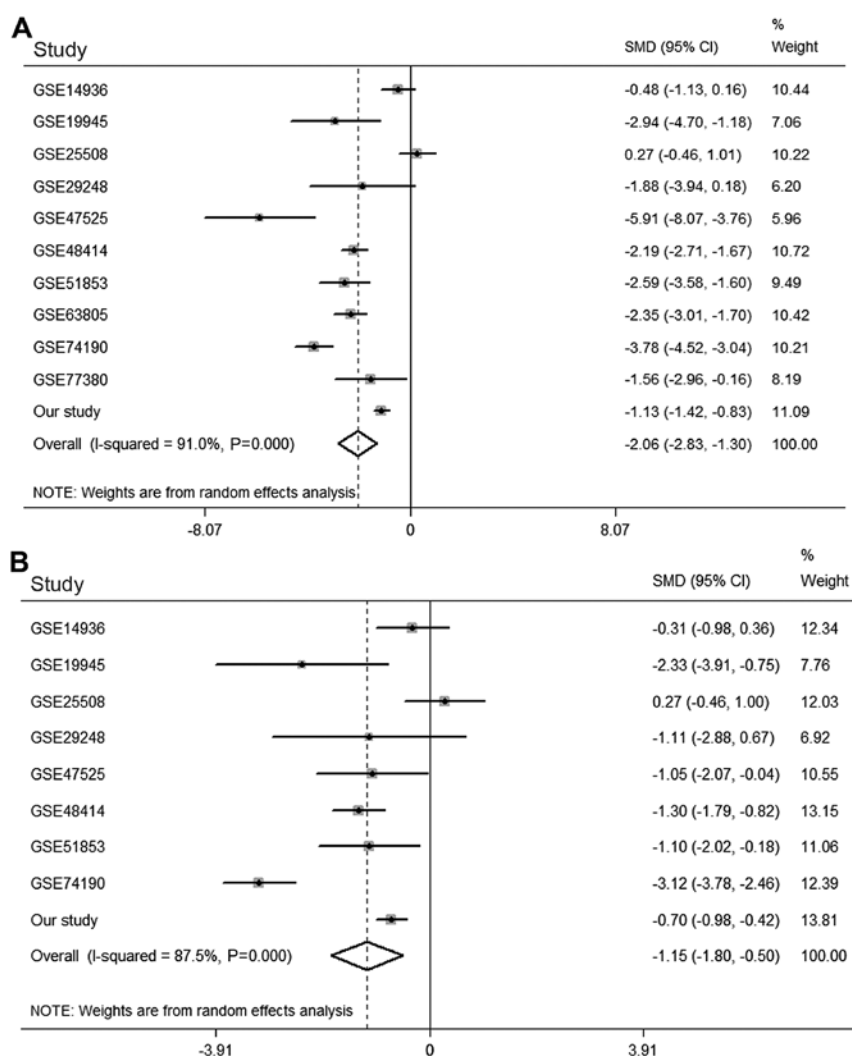


Figure 3. Forest plots of miRNA-126-3p and miRNA-126-5p based on microarray datasets. (A) Forest plot of miRNA-126-3p expression in LUAD according to 10 relevant microarray datasets and the present study. (B) Forest plot of miRNA-126-5p expression in LUAD according to 8 relevant microarray datasets and the present study. LUAD, lung adenocarcinoma; CI, confidence interval; SMD, standard mean difference.

demonstrated that the AUC was 0.625 ($P=0.032$; Fig. 2H). No significant difference was observed between miRNA-126-3p

and -5p and other clinicopathological features (all $P>0.05$; Table I).

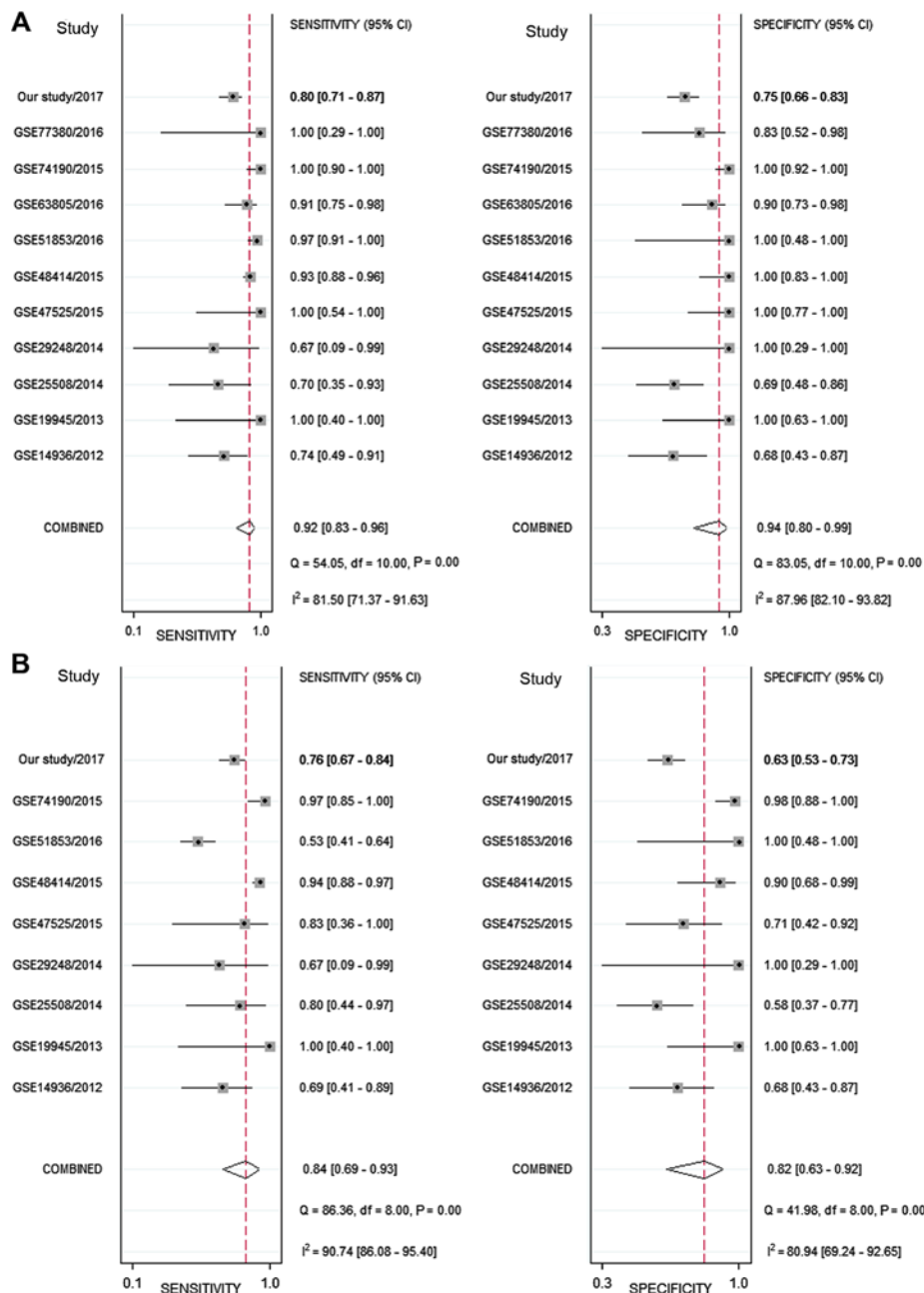


Figure 4. Sensitivity and specificity of the microarray datasets and the present study. Sensitivity and specificity of the 10 relevant microarray datasets and the present study according to the expression of (A) miRNA-126-3p and (B) miR-126-5p. CI, confidence interval.

Meta-analysis of miRNA-126-3p and -5p in LUAD based on the GEO databas. The ten relevant microarray datasets were the following: GSE14936 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14936>), GSE19945 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19945>), GSE25508 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25508>), GSE29248 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29248>), GSE47525 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47525>), GSE48414 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48414>), GSE51853 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51853>), GSE63805 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63805>), GSE74190 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74190>), GSE77380 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77380>),

which contained the expression values of miRNA-126-3p in 388 LUAD and 237 normal lung tissues, were identified and downloaded for meta-analysis with the results from the present study (Table II) (27-35). A random-effects model was used because major heterogeneity existed ($I^2=91.0\%$, $P<0.001$). The meta-analysis demonstrated that miRNA-126-3p expression was significantly lower in LUAD tissues than it was in normal lung tissues [SMD=-2.063, 95% confidence interval [CI]: -2.829 to -1.298, $P<0.001$; Fig. 3A].

Eight microarray datasets (GSE14936, GSE19945, GSE25508, GSE29248, GSE47525, GSE48414, GSE51853 and GSE74190) contained data concerning expression of miRNA-126-5p in 305 LUAD and 139 normal lung tissues. As major heterogeneity also existed in the meta-analysis of miRNA-126-5p ($I^2=87.5\%$, $P<0.001$), a random-effects

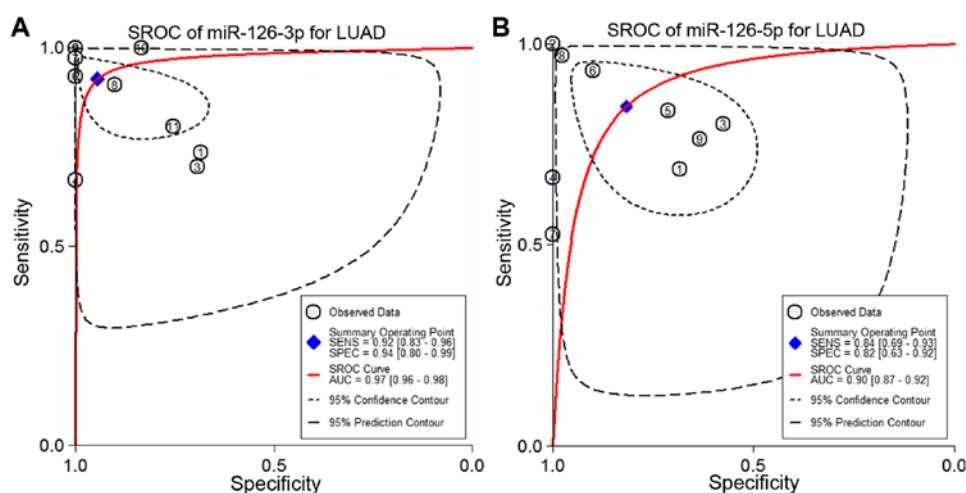


Figure 5. SROC of miRNA-126-3p and miRNA-126-5p for LUAD based on the microarrays and the present study. (A) SROC curve of miRNA-126-3p for LUAD according to the 10 relevant microarray datasets and the present study (AUC=0.97). (B) SROC curve of miRNA-126-5p for LUAD according to 8 relevant microarray datasets and the present study (AUC=0.90). LUAD, lung adenocarcinoma; SROC, summary ROC; AUC, area under the receiver operating characteristic (ROC) curve.

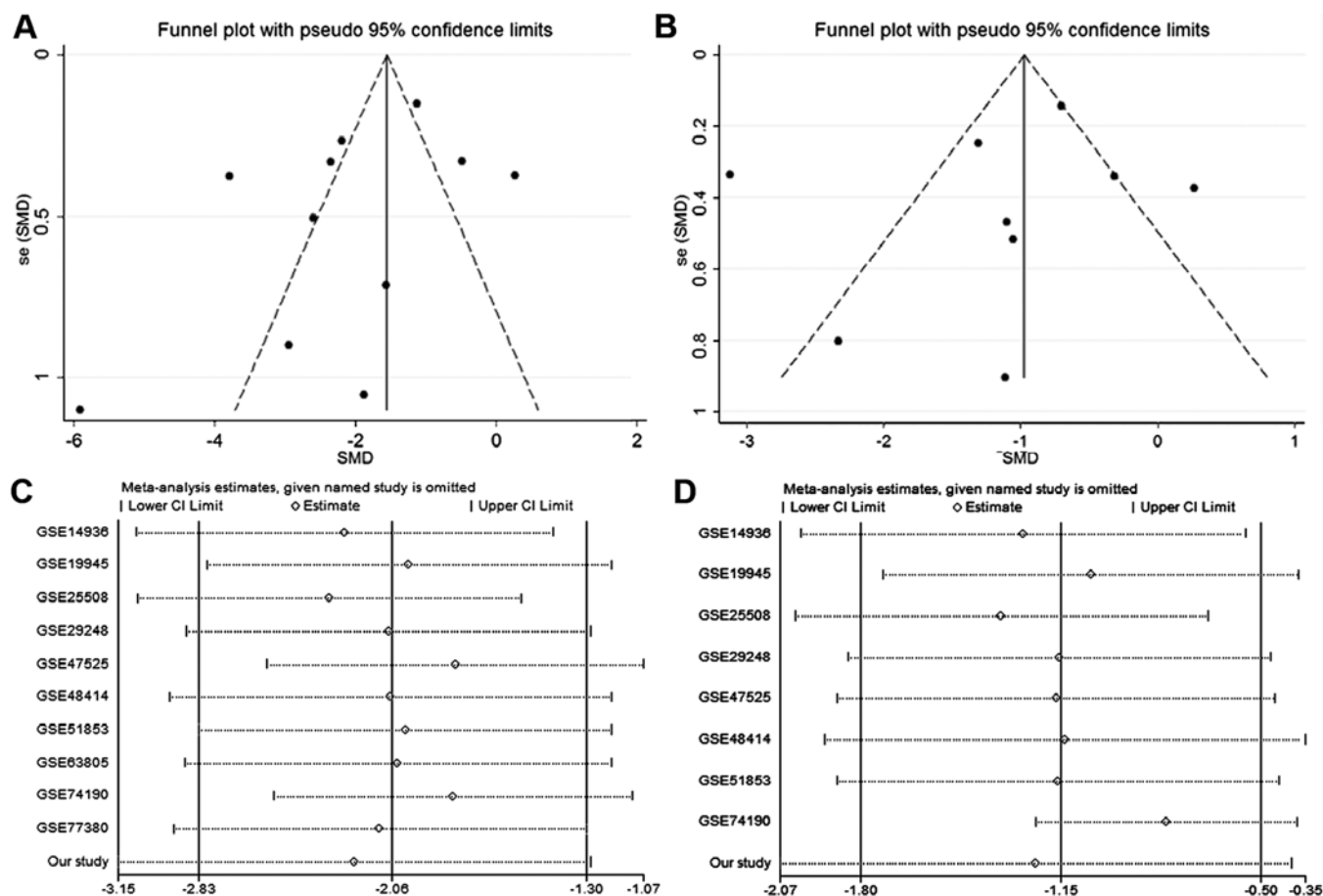


Figure 6. Sensitivity analysis and publication bias of the selected microarray datasets. (A) Funnel plot of the 10 microarray datasets related to miRNA-126-3p and the present study. (B) Funnel plot of 8 microarray datasets related to miRNA-126-5p and the present study. (C) Sensitivity analysis based on the 10 microarray datasets related to miRNA-126-3p and the present study. (D) Sensitivity analysis based on 8 microarray datasets related to miRNA-126-5p and the present study. SMD, standard mean difference.

model was again performed. The results showed that miRNA-126-5p also exhibited significantly low expression in LUAD tissues (SMD=-1.152, 95% CI, -1.804 to -0.499, $P=0.001$; Fig. 3B).

Next, the sensitivity and specificity of each microarray dataset and the present study were extracted (Fig. 4). The SROC curves of miRNA-126-3p and miRNA-126-5p, respectively, for LUAD tissues were performed using the

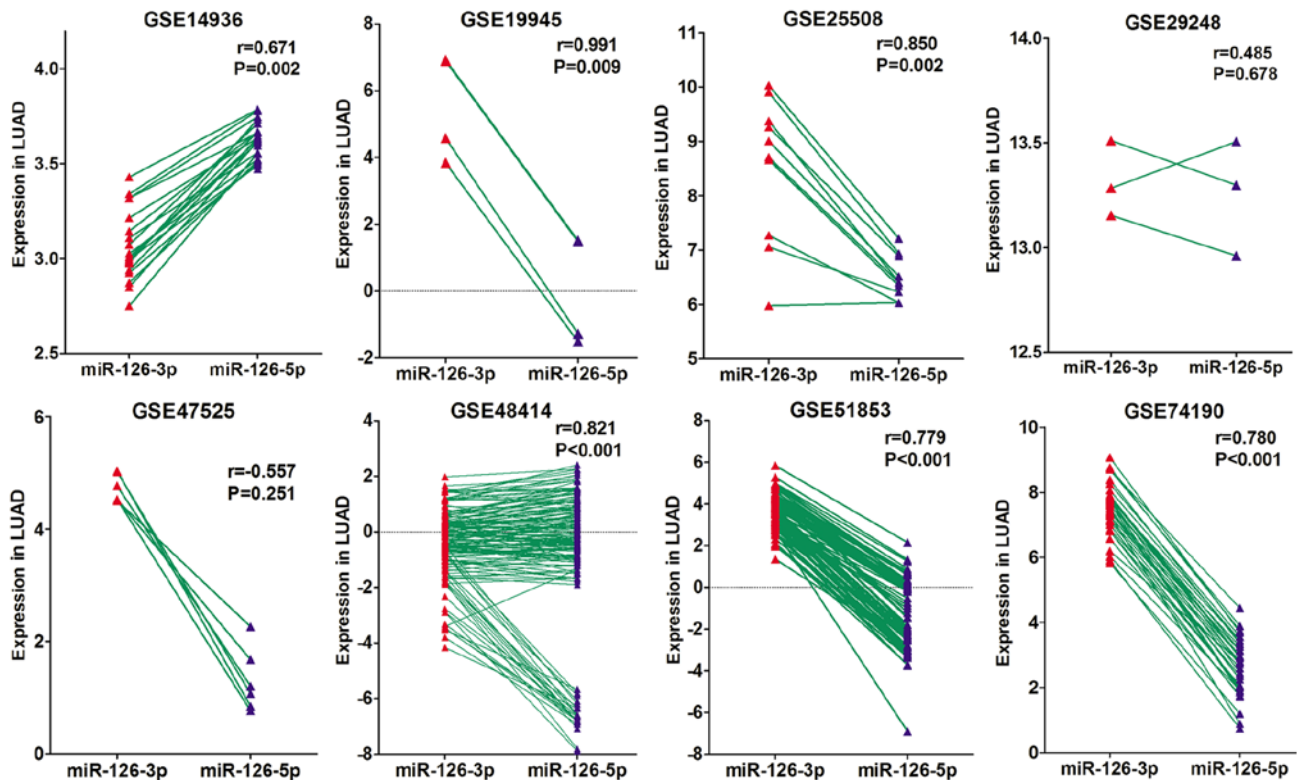


Figure 7. Correlation between miRNA-126-3p and miRNA-126-5p based on GEO data. LUAD, lung adenocarcinoma.

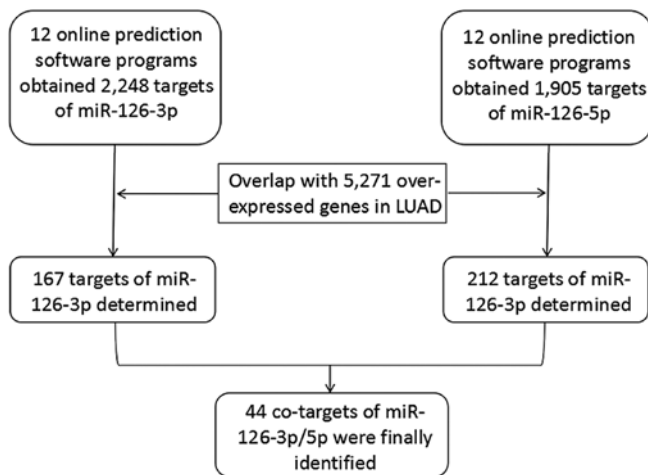


Figure 8. The process of screening the targets. LUAD, lung adenocarcinoma.

GEO data and the present study's RT-qPCR data. The SROC of miRNA-126-3p showed an AUC of 0.97 (Fig. 5A), while the SROC of miRNA-126-5p showed an AUC of 0.90 (Fig. 5B).

Funnel plots were employed to assess the publication bias of the two meta-analyses (Fig. 6A and B). The results of Begg's test ($P=0.533$ and $P=0.533$) and Egger's test ($P=0.592$, $P=0.307$) showed no statistically significant differences for either meta-analysis. Sensitivity analysis for these two meta-analyses revealed that significantly lower expression of miRNA-126-3p and miRNA-126-5p existed regardless of which microarray datasets were removed (Fig. 6C and D).

Correlation analysis between miRNA-126-3p and miRNA-126-5p. A Pearson correlation analysis was performed based on the GEO data to assess the correlation between miRNA-126-3p and miRNA-126-5p expression. Of the eight microarray datasets, six showed significant positive correlations ($r=0.671$ - 0.991 , all $P<0.05$; Fig. 7), one suggested a tendency toward a positive correlation ($r=0.486$; $P=0.678$) and one suggested a trend toward a negative correlation ($r=-0.557$, $P=0.251$).

Bioinformatic analysis based on the target genes of miRNA-126-3p and miRNA-126-5p

Prediction of the target genes. Twelve online prediction tools were employed for acquiring the potential targets of miRNA-126-3p and -5p, respectively. Initially, 2,248 genes and 1,905 genes were separately collected as the target genes of miRNA-126-3p and -5p. In addition, 5,271 upregulated genes in LUAD tissues were also obtained from the TCGA database. After the upregulated genes were overlapped with the 2,248 targets of miRNA-126-3p and the 1,905 targets of miRNA-126-5p, 167 genes and 212 genes, respectively, were identified as the targets of miRNA-126-3p and -5p in LUAD. Combining the 167 targets of miRNA-126-3p and 212 targets of miRNA-126-5p produced 335 total target genes; 44 genes were co-targets of both miRNA-126-3p and miRNA-126-5p (Fig. 8).

GO analysis, KEGG pathways and PPI. Based on these 335 target genes, GO and KEGG pathway analyses were conducted to determine their molecular biological functions in LUAD. The results of the GO analysis indicated that

Table III. GO functional annotation of the 335 target genes.

GO ID	Term	Count	P-value	Gene symbol
Biological process				
GO:0007267	Cell-cell signaling	31	4.43E-07	<i>FGF5, GRIK2, HOXA11, OPRK1, EFNA2, GLRA3, GREM1, IL11, PCSK1, KCNQ5, NPTX1, BARX1, IL17A</i> , and others
GO:0045165	Cell fate commitment	13	8.31E-06	<i>ONECUT1, HOXA11, ONECUT2, NEUROG2, PAX2, VSX2, NR2E1, DLX1, WNT3, NEUROD1, NEUROD4, GAP43, TLX1</i>
GO:0060348	Bone development	11	7.90E-05	<i>PTH1LH, CYP24A1, CASR, CYP27B1, TNFSF11, HOXA11, COL2A1, COL1A1, COL5A2, IGFBP3, MMP13</i>
Molecular function				
GO:0005887	Integral to plasma membrane	43	2.07E-05	<i>MCHR1, CASR, SCN3A, GRIK2, ENPP3, OPRK1, GLRA3, ITGA11, PCDHA1, KCNJ13, KCNQ5, UGT1A8</i> , and others
GO:0034702	Ion channel complex	15	2.35E-05	<i>KCND2, SCN3A, GLRA3, CACNG7, CACNA11, GABRA5, KCTD4, KCNJ13, KCNQ5, KCNQ3, CHRNA5</i> , and others
GO:0031226	Intrinsic to plasma membrane	43	3.52E-05	<i>MCHR1, CASR, SCN3A, GRIK2, ENPP3, OPRK1, GLRA3, ITGA11, PCDHA1, KCNJ13, KCNQ5, UGT1A8, KCNQ3</i> , and others
Cell component				
GO:0015267	Channel activity	23	7.61E-06	<i>KCND2, TRPM8, SCN3A, TRPC5, GRIK2, GLRA3, CACNG7, GABRA5, TRPA1, CACNA11, GRIA4, CNGB3, KCTD4</i> , and others
GO:0022803	Passive transmembrane transporter activity	23	7.90E-06	<i>KCND2, TRPM8, SCN3A, TRPC5, GRIK2, GLRA3, CACNG7, GABRA5, TRPA1, CACNA11, GRIA4, CNGB3, KCTD4</i> , and others
GO:0005216	Ion channel activity	22	9.34E-06	<i>KCND2, TRPM8, SCN3A, TRPC5, GRIK2, GLRA3, CACNG7, GABRA5, TRPA1, CACNA11, GRIA4, CNGB3, KCTD4</i> , and others

GO, Gene Ontology.

Table IV. KEGG pathway analysis based on the 335 target genes.

KEGG ID	Term	Count	P-value	Gene symbol
hsa04080	Neuroactive ligand-receptor interaction	14	2.49E-04	<i>MCHR1, MCHR2, PTH2R, GRIK2, GLRA3, OPRK1, GABRA5, GRIA4, CRHR1, SSTR5, GRM4, HTR2C, TSHR, GABRP</i>
hsa04512	ECM-receptor interaction	8	4.23E-04	<i>ITGA11, COL2A1, COL1A1, COL5A2, THBS2, COL11A1, COL5A1, HMMR</i>
hsa04950	Maturity onset diabetes of the young	4	7.69E-03	<i>ONECUT1, IAPP, NEUROD1, HNF4G</i>
hsa00140	Steroid hormone biosynthesis	4	3.97E-02	<i>AKR1C2, UGT1A10, UGT1A8, CYP21A2, UGT2A3, UGT1A1</i>
hsa00980	Metabolism of xenobiotics by cytochrome P450	4	7.60E-02	<i>AKR1C2, UGT1A10, UGT1A8, ADH4, UGT2A3, UGT1A1</i>

KEGG, Kyoto Encyclopedia of Genes and Genomes.

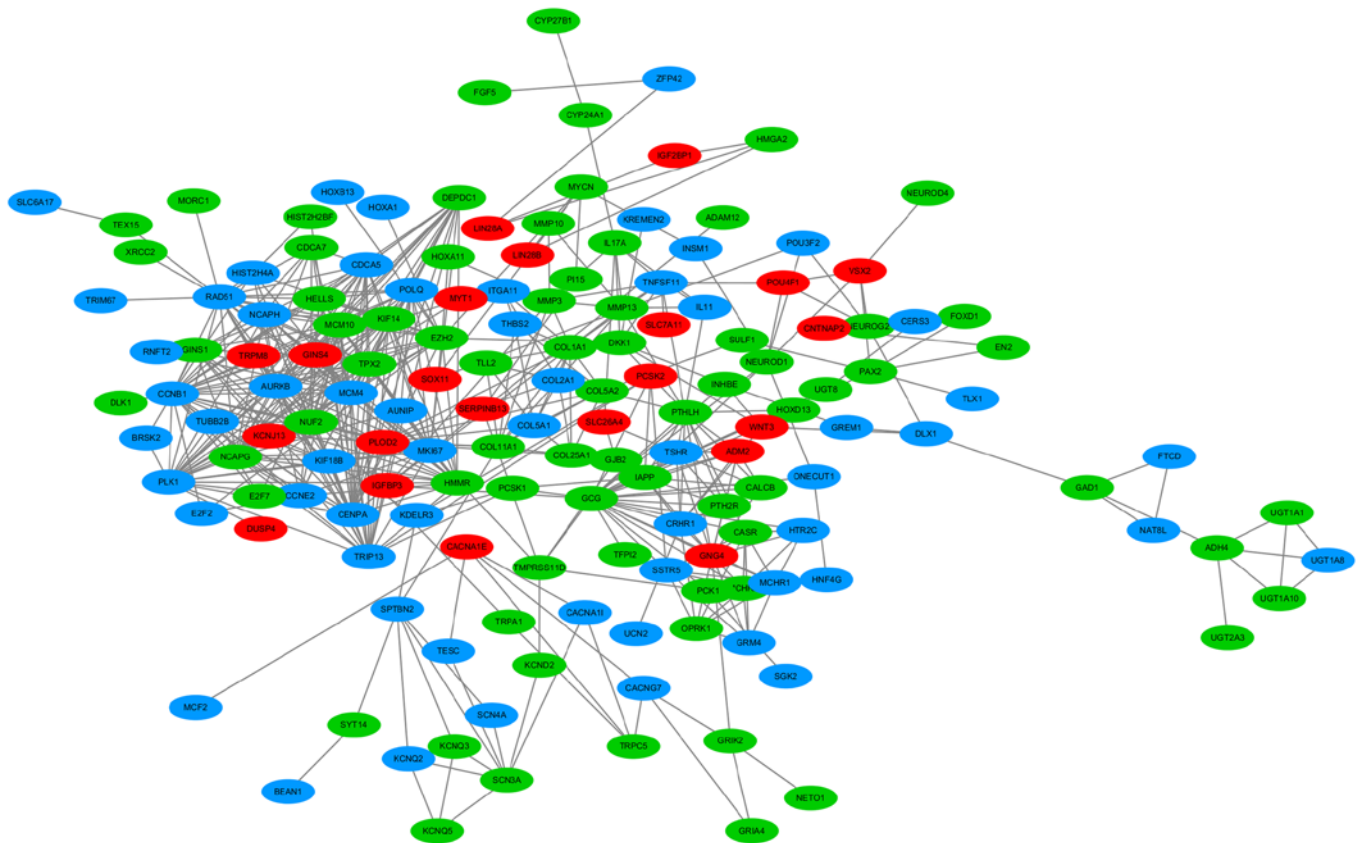


Figure 9. PPI network based on 335 target genes of miRNA-126-3p and miRNA-126-5p. The blue nodes represent the target genes of miRNA-126-3p; the green nodes represent the target genes of miRNA-126-5p; the red nodes represent the co-regulated target genes of both miRNA-126-3p and miRNA-126-5p. The lines between the nodes represent the associations between the different target genes. PPI, protein-protein interaction.

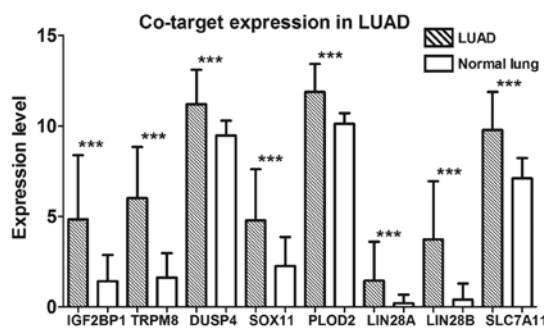


Figure 10. Expression levels of the eight key co-targets of miRNA-126-3p and miRNA-126-5p. The bars in the histogram with oblique lines represent LUAD tissues, and the blank bars represent normal lung tissues. All eight genes were expressed significantly higher in the LUAD tissues (***P<0.001). LUAD, lung adenocarcinoma.

the targets were mainly enriched in cell-cell signaling of biological processes (BPs), which are integral to the plasma membrane of cell components (CCs) and channel activity of molecular functions (MFs) (Table III). The KEGG pathway analysis showed that target genes were significantly enriched in neuroactive ligand-receptor interaction (Table IV). In addition, a PPI network of these 335 target genes was performed (Fig. 9). Eight crucial co-target genes (*IGF2BP1*, *TRPM8*, *DUSP4*, *SOX11*, *PLOD2*, *LIN28A*, *LIN28B* and *SLC7A11*) were selected for further analysis. All were significantly highly expressed in LUAD (all P<0.001; Fig. 10).

Discussion

In recent years, the effect of microRNAs (miRNAs) on the pathogenesis of cancers has been well investigated and clarified. However, even as the investigation of miRNAs has matured and their functions have become clearer, the co-function of miRNA-3p and -5p is rarely mentioned. The present study verified the co-downregulation of the expression of miRNA-126-3p and miRNA-126-5p in lung adenocarcinoma (LUAD) tissues using real-time quantitative polymerase chain reaction (RT-qPCR) and the GEO database. Bioinformatic analysis based on the obtained target genes of miRNA-126-3p and -5p was performed to clarify their co-regulation in LUAD.

The function of miRNA-126-3p has been articulated in previous research. miRNA-126-3p levels have been shown to be downregulated in various malignancies including colorectal, esophageal, oral, liver, breast, thyroid and renal cancers (36-42). In contrast, investigations of miRNA-126-5p in malignant tumors have been rare and limited. Several studies have reported that miRNA-126-5p is expressed at a lower level in colorectal and breast cancers (43,44). As for lung cancer (LC), several studies have reported that miRNA-126-3p expression is downregulated in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), suggesting that it is an LC suppressor (45,46). The targets of miRNA-126-3p have also been described in previous studies. Liu and colleagues pointed out that miRNA-126-3p could inhibit the growth of LC cell lines by

targeting vascular endothelial growth factor (VEGF) (47). Moreover, Song *et al* demonstrated that miRNA-126-3p targeted PIK3R2 and affected the PTEN/PI3K/AKT signaling pathway to suppress the growth of an NSCLC cell line (48). As for the role and effect of miRNA-126-5p in LC, available literature is scarce.

All previous studies concerning miRNA-126 in LC investigated miRNA-126-3p and ignored the function of miRNA-126-5p. To the best of our knowledge, no previous study has explored the co-function of miRNA-126-3p and -5p in LC. The present study first concentrated on the co-function and co-targets of miRNA-126-3p and -5p in LUAD. A total of 202 tissues and 11 microarray datasets were collected to identify the co-expression of miRNA-126-3p and -5p in LUAD. Bioinformatic analysis based on the potential target genes was performed in the hopes of identifying the co-regulation and synergistic effects of miRNA-126-3p and -5p in LUAD.

Both miRNA-126-3p and miRNA-126-5p are derived from precursor miRNA-126 and are associated with angiogenesis and cell proliferation in malignancies. The present study confirmed that both miRNA-126-3p and -5p have lower expression in LUAD tissues, indicating that both may be LUAD tumor suppressors. In addition, expression levels of miRNA-126-3p and -5p were significantly downregulated in late stage LUAD. The miRNA-126-5p expression levels were significantly lower in samples with vascular invasion and LNM, suggesting that miRNA-126-3p and -5p were related to the development, invasion, and metastasis of LUAD. Lower expression of miRNA-126-3p and -5p suggested a worse prognosis of LUAD.

In addition, correlation analysis showed a positive correlation between miRNA-126-3p and -5p expression, meaning that in LUAD, miRNA-126-5p levels decreased as miRNA-126-3p decreased. Thus, miRNA-126-3p and -5p are not expressed independently in LUAD; they are expressed consistently. Analysis of miRNA-126-3p and -5p levels also showed that although these two miRNAs are expressed consistently, the level of miRNA-126-5p was usually lower than that of miRNA-126-3p in the same LUAD tissue sample. This observation conforms to the rule of mature miRNA expression: When miRNA-3p and -5p are developed from the same precursor, one of them is often more highly expressed than the other (14). Previous research improperly assumed that the lower-expressed miRNA was functionally irrelevant, while the higher-expressed one played a leading role in biological processes (13,14). In fact, additional research has demonstrated the important function of both miRNA-3p and -5p (49). The present study confirmed the significantly lower expression of both miRNA-126-3p and -5p in LUAD and demonstrated their association with TNM stage, invasion and metastasis; it also confirmed that both of them perform important functions. Their consistent expression may indicate a synergistic effect in LUAD.

The target predictions further demonstrated the synergistic effects of miRNA-126-3p and -5p. Combining the 167 target genes of miRNA-126-3p with the 212 targets of miRNA-126-5p revealed that these two miRNAs share 44 co-targets. In other words, 26% (44/167) of miRNA-126-3p targets and 20% (44/212) of miRNA-126-5p targets were co-targets of both.

This high target overlap provides credible evidence for their co-regulation in LUAD. The combined 335 targets were mainly enriched in neuroactive ligand-receptor interaction and ECM-receptor interaction of the KEGG pathway. However, when the 167 miRNA-126-3p targets and 212 miRNA-126-5p targets were separately considered to explore the KEGG pathway, the results also showed that the targets were mainly enriched in neuroactive ligand-receptor interaction and ECM-receptor interaction. Thus, miRNA-126-3p and -5p may influence LUAD development together via a similar signaling pathway.

More importantly, many of these co-targets have been identified as key genes in LC development. *IGF2BP1* has been proven to be a target gene of miRNA-491-5p. miRNA-491-5p can downregulate *IGF2BP1* to suppress the growth of NSCLC (50). *TRPM8* was found to contribute to an invasive phenotype in LC (51). Furthermore, *DUSP4* was shown to function as a suppressor and can inhibit the growth of EGFR-mutant LUAD (52). *SOX11* was found to exhibit significantly higher expression in LC and is associated with a prognosis of neuroendocrine tumors of the lung (53). *PLOD2* was proven to be regulated by the PI3K/AKT-FOXA1 axis to promote the metastasis of LC, and its high expression is indicative of a worse prognosis of LUAD (54). miRNA-98 may suppress the expression of *LIN28A* to decrease the invasion and metastasis of LC cells (55), while *LIN28B* expression is high in tumor-initiating cells from NSCLC and functions in the tumorigenesis of lung cancer cells (56). The overexpression of *SLC7A11* was shown to be associated with poor prognosis of LC (57). According to TCGA data, all these co-targets play critical roles and are overexpressed in LUAD. miRNA-126-3p and -5p may directly target these key genes at the same time to co-regulate the development of LUAD. Downregulation of the expression of miRNA-126-3p and -5p reduces their combination with these targets, which decreases the degradation of these target genes and causes them to be highly expressed in LUAD. It will be meaningful to deeply research these potential targets and further validate that they are the downstream targets of miRNA-126-3p and -5p in LUAD. However, the core point of this study was to demonstrate the co-regulation as well as the synergistic effects of miRNA-125-3p and -5p in LUAD, while elucidating the downstream target genes is a secondary viewpoint of our study. Thus, we completed a preliminary identification of these potential targets. Further and intensive investigation is needed to validate them in the future.

It is worth mentioning that the co-function of miRNA-3p and -5p has only recently begun to be recognized and investigated. In fact, many miRNA-3p and -5p pairs have been found to co-regulate the same malignant tumors (49). The present study demonstrated the co-regulation of miRNA-126-3p and -5p in LUAD. The present results and the PPI network provide clear evidence of the existence of the synergistic effects between miRNA-126-3p and -5p in LUAD. The co-regulation of miRNA-126-3p and -5p may be a fail-proof mode of the regulation of miRNAs in LUAD or other malignancies, which can ensure that if either miRNA-126-3p or -5p is disabled by transcriptional inhibition or mutation, the associated biological function may still proceed stably and normally (58). In the regulation of a malignant tumor,

this fail-proof mode may also exist between pairings other than miRNA-3p and -5p or even between miRNAs from two different families.

In conclusion, the present study verified the co-down-regulation of the expression of miRNA-126-3p and -5p in LUAD tissues and discovered the existence of co-target genes between miRNA-126-3p and -5p, many of which have been confirmed to be closely related to the proliferation, invasion, metastasis and poor prognosis of LC. These results indicate that the co-regulation of miRNA-126-3p and -5p plays a significant role in the development of LUAD, which also implies a fail-proof mode between miRNA-3p and -5p. While miRNA-126-5p is by no means equivalent to miRNA-126-3p, the two miRNAs possess vital associations and valuable clinical significance in LUAD and various other malignancies.

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Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding authors on reasonable request.

Authors' contributions

PC designed the study, analyzed the main data and wrote the main part of the manuscript. YYG and FCM carried out the detection of miRNA-126-3p and -5p expression in LUAD, collected the data from GEO database and contributed to the writing of the manuscript. RQH analyzed the data and revised the manuscript. ZYL completed the detection of miRNA-126-3p and -5p expression and analysed the data. GQZ and XL performed the bioinformatics analyses. XHH designed the study and revised the manuscript. LJP and GC designed the study, supervised all experiments and revised the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The research protocol for this study has been ratified by the Ethics Committee of the First Affiliated Hospital of Guangxi

Medical University. All patients content was obtained at the time of the sample collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests, and all authors confirm its accuracy.

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