Identifying and Validating a Novel miRNA-mRNA Regulatory Network Associated with Prognosis in Lung Adenocarcinoma

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ABSTRACT

Objective Many studies have revealed the crucial roles of miRNA in multiple human cancers, including lung adenocarcinoma (LUAD). In this study, we sought to explore new miRNA-mRNA pairs that are associated with LUAD prognosis.

Methods A novel miRNA-mRNA regulatory network associated with prognosis in LUAD was identified and validated using the bioinformatic tools including OncomiR database, StarBase, miRnet, GEPIA2, UALCAN.

Results Twenty key miRNAs were compiled after the analysis of the expression and prognostic value in OncomiR and StarBase. Targeted mRNAs of these key miRNAs were predicted in miRnet, and the resulting mRNAs were also analyzed for their prognostic values and expression patterns in GEPIA2 and UALCAN, respectively. Further expression correlation analysis was performed in StarBase. Subsequently, a new miRNA-mRNA network was built, of which each RNA pair showed negative expression correlation, opposite expression pattern, and prognostic value. Protein-protein interaction network was under construction for the mRNAs, and 19 hub genes were determined. Enrichment analysis showed that “Cell Cycle, Mitotic” was the most significantly enriched term. Then, a miRNA-hub gene sub-network was built. We selected and validated the regulatory relationship of some miRNA-hub pairs, including hsa-miR-1976/RFC2, hsa-let-7c-5p/RFC2, hsa-let-7c-5p/ESPL1, hsa-let-7c-5p/CDC25A, and hsa-miR-101-3p/KIF2C. Moreover, over-expression of hsa-miR-1976 and hsa-let-7c-5p resulted in significant cell cycle arrest.

Conclusions Our results determined new prognosis-associated miRNA-mRNA pairs and might shed further light on the mechanism via which miRNA-mRNA network influences prognosis in LUAD.

Key words: lung adenocarcinoma; bioinformatic analysis; miRNA; prognosis; cell cycle

INTRODUCTION

Pulmonary cancer is one of the most common and lethal cancers^[1]. It has two major histological types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Lung adenocarcinoma (LUAD) is the most common subtype of NSCLC and accounts for approximately 50% of NSCLC^[2]. Since LUAD tends to form metastasis at early stages, roughly two-thirds of LUAD patients are diagnosed at advanced stages. In addition, the recurrence rate is high after surgical resection. Thus, the

prognosis of LUAD patients remains poor, with a five-year survival rate below 20%^[2]. Research on the molecular mechanism of LUAD is essential for understanding its pathogenesis, identifying novel biomarkers, and developing potential therapeutic targets.

MicroRNAs (miRNAs) are small non-coding RNA molecules that play critical roles in many cellular processes including cell differentiation, proliferation, and survival. miRNAs can cause mRNA degradation or translational inhibition by binding to complementary target sequences. Dysregulation of miRNAs and their

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target genes is involved in the pathogenesis of multiple human cancers. For example, the *miR-34* family is involved in p53 signal regulation upon stress factors such as arrested cell cycle, programmed cell death, or DNA damage^[3]. The frequent down-regulation of *miR-34* family in lung cancer leads to the up-regulation of target genes^[4]. The up-regulated target oncogenes further lead to cell proliferation and metastasis. In addition, *miR-34a* can inhibit the growth of both NSCLC cells and NSCLC-like stem cells^[5]. Yang *et al.* discovered a novel tumor evasion mechanism in NSCLC patients that PDL1 is regulated by p53 via *miR-34* in PD1/PDL1 signaling^[6]. The dysregulation of miR200 family (*miR-200a/b/c* and *miR-429*) plays a crucial role in epithelial-to-mesenchymal (EMT) transition of lung cancer, thus facilitating cancer cell invasion and spread through regulating EMT-related factors such as ZEB1, ZEB2, E-cadherin, and vimentin^[7,8]. Over-expression of *miR-200* can completely block the transition of lung cancer cells from epithelial state to metastatic state^[9]. Moreover, systemic administration of *miR-200c* in parallel with radiation may enhance radiotherapy sensitivity^[10].

Accordingly, several studies have performed systematic identification of dysregulated miRNAs and construction of their regulatory networks in lung cancer^[11,12]. However, these studies were primarily based on expressions, and no systematic identification of survival-related miRNAs or construction of their regulatory network has been performed. In our current study, focusing on LUAD, we performed a step-by-step analysis through multiple databases in terms of expression pattern, prognostic value, and expression correlation. After a series of analyses, a prognosis-associated miRNA-mRNA network was constructed. A protein-protein interaction (PPI) network was constructed, and the hub genes were determined. We validated some new regulatory relationships between miRNAs and hub genes. Our work might shed further light on the mechanisms of pathogenesis and progression of LUAD.

MATERIALS AND METHODS

Key miRNAs identification

Significant survival-related miRNAs in LUAD were firstly extracted from OncomiR database, an online tool for exploring pan-cancer microRNA dysregulation using data from The Cancer Genome Atlas (by selecting "lung adenocarcinoma", setting $P < 0.05$ and then clicking "retrieve" on the OncomiR webpage; [http://](http://www.oncomir.org/oncomir/search_cancer_surv_miR.html)

www.oncomir.org/oncomir/search_cancer_surv_miR.html)^[13]. The validation of the prognostic values of the obtained miRNAs and then the expression analysis were conducted in Starbase database (by typing the miRNA name into the box and clicking "search", then viewing the results by selecting cancer types) (<http://starbase.sysu.edu.cn/panMirSurvivalExp.php>; <http://starbase.sysu.edu.cn/panMirDiffExp.php>)^[14].

Key mRNAs identification

Prediction of mRNAs for key miRNAs was made in miR-net, an easy-to-use online tool for miRNA-associated studies (<https://www.mirnet.ca/>)^[15]. Prognostic value analysis of obtained mRNAs was conducted in the "Survival" Map webpage of GEPIA database (<http://gepia2.cancer-pku.cn/#survival>). mRNA expression analysis was performed by using the "scan my genes" tool in UALCAN database, which provides easy access to publicly available cancer transcriptome data from The Cancer Genome Atlas (<http://ualcan.path.uab.edu/analysis.html>)^[16]. Expression correlation analysis of miRNA-mRNA pairs was performed in Starbase (<http://starbase.sysu.edu.cn/panMirCoExp.php>). A P value of < 0.05 was considered statistically significant.

Construction of miRNA-mRNA network

After the key miRNAs and mRNAs in LUAD were identified, the miRNA-mRNA network was constructed by Cytoscape software. miRTarBase database was used to search for experimentally validated interactions (<http://mirtarbase.cuhk.edu.cn/php/index.php>)^[17]. The confirmed miRNA-target interactions were indicated in the network by Cytoscape software.

Functional enrichment analysis

FunRich software, a tool used for functional enrichment analysis of genes, was employed to perform pathway-enrichment analysis and GO functional annotation for the identified key mRNAs (<http://www.funrich.org/>)^[18]. Briefly, click "Add dataset", copy and paste the mRNA list into the box to establish a new dataset; click "Gene enrichment"- "Analysis" to select the analysis types and dataset to be analyzed; then, click "OK" to view the results in the window.

Protein-protein interaction network and identification of hub genes

Protein-protein interaction network for dysregulated mRNAs was constructed using the STRING database

that contained predicted functional associations between different proteins (<https://string-db.org/>)^[19]. Cytoscape software was utilized to analyze the degree of connectivity in the PPI network. According to degree, hub genes were identified and their interactive network was established in Cytoscape.

Cell culture and transfection

A549 and H1299 cells were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS)(Gibco) at 37°C in the presence of 5% CO₂. miRNA mimics and scrambled negative control (NC) were provided by Ribobio (Guangzhou, China). Cells were transfected with Lipofectamine 3000 reagents (Invitrogen, USA) according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR) and cell cycle analysis

Total RNA was extracted by TRIzol Reagent (Ambion, life technologies) according to standard protocol. cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (ThermoScientific). Quantitative real-time PCR assays were conducted using QuantiNova™ SYBR®Green PCR Kit (Qiagen) protocol with Bio-

Rad CFX96 PCR system(Bio-Rad). The relative gene expression was derived with the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. Primer sequences for qPCR are presented in **Table 5**.

For the cell cycle assay, the cells were harvested 48 hours after transfection by fixing in 75% ethanol at 4°C. Then, the cells were incubated with 100 µg/mL RNase A and 50 µg/mL propidium iodide (PI) for 30 minutes before analyzing in a Beckman Coulter FC-500MPL flow cytometer.

RESULTS

Identification of key prognosis-associated miRNAs and mRNA in LUAD

We started this study from analysing miRNAs. Firstly, OncomiR, an online database for exploring miRNA dysregulation in cancer, was used to extract significant miRNAs in survival. As showed in **Figure 1A**, there were 109 miRNAs significantly associated with survival in LUAD ($P < 0.05$). The prognostic values of these miRNAs were further validated in Starbase database, which showed only 52 out of the 109 miRNAs were significantly associated with the overall survival in LUAD. Since oncogenes are usually up-regulated in tumors and indicated poor prognosis while tumor-suppressing genes act conversely, the expression patterns of the

Table 5. qPCR primers

Primer name	sequence (5' to 3')	Primer name	sequence (5' to 3')
Q-H-CDT1-F	TGTCAAGGAGCACCACAAGGC	Q-H-BIRC5-F	TCTCAAGGACCACCGCATCT
Q-H-CDT1-R	CGATGTCGGGTACTTCATCCA	Q-H-BIRC5-R	CCAAGTCTGGCTCGTTCTCA
Q-H-CENPI-F	CTCAGATGGGCTCAGTGCTAA	Q-H-KIF2C-F	AGAAATTCGGGCTACTTTGG
Q-H-CENPI-R	TGAAGGAAGTGTGACCGACAA	Q-H-KIF2C-R	TGCTTATTCAGTGGGCGTTT
Q-H-RAD51-F	TGTCAAGGAGCACCACAAGGC	Q-H-KPNA2-F	CGTCGCAGAATAGAGGTCAA
Q-H-RAD51-R	CGATGTCGGGTACTTCATCCA	Q-H-KPNA2-R	GCAGCGGAGAAGTAGCATCA
Q-H-KIF14-F	AAAAGAAAGTCTGGGTGGA	Q-H-CCNB2-F	CGAGGACATTGATAACGAAG
Q-H-KIF14-R	CTGTATCGTTTCAAGGTCAA	Q-H-CCNB2-R	AACTGGCTGAACCTGTAAAA
Q-H-CKS1B-F	TCATGCTGCCCAAGGACATA	Q-H-CDC25A-F	AGGGTCTGGGCAGTGATTAT
Q-H-CKS1B-R	GGACCATCCCTGACTCTGC	Q-H-CDC25A-R	AACAGCTTCTGAGGTAGGGA
Q-H-RFC2-F	CTTCTCAGGATTGGCTTCA	Q-H-FANCI-F	GCTCTGAGGAAGCTGGAACA
Q-H-RFC2-R	GTAGGCTTCGTCAATGTTGG	Q-H-FANCI-R	AGGGCAGTGAGAATGATAGG
Q-H-ESPL1-F	GCTTGTGATGCCATCCTGAGGG	Q-H-CDCA8-F	TGAGTCAGACAGGCAGAACC
Q-H-ESPL1-R	GCCTCTGGGCTTCCTTGTGC	Q-H-CDCA8-R	TTCCTCCAAGGGCGAAGTAG
Q-H-RRM2-F	AACTTGGTGGAGCGATTAG	Q-H-RACGAP1-F	AGCGAAGTGCTCTGGATGTT
Q-H-RRM2-R	ATAGGTAGCCTCTTTGTCCC	Q-H-RACGAP1-R	CATTGCTGCTGGATGGTTGG
Q-H-ACTB-F	ACTCTCCAGCCTTCCTTCC		
Q-H-ACTB-R	TGTTGGCGTACAGGTCTTTG		

52 miRNAs were further analysed. Seventeen out of 52 miRNAs that fitted the criteria were screened out (**Fig. 1A**). To avoid missing any eligible miRNAs, all the miRNAs expressed in LUAD were re-examined. By using the Starbase, we performed additional expression analysis and survival analysis for all miRNAs extracted from OncomiR with a mean expression value $>3[\log_2(\text{RPM}) \text{ normalized}]$ in LUAD, after which another three eligible miRNAs (hsa-miR-526b-5p, hsa-miR-140-3p, and hsa-miR-9-5p) were found (**Fig. 1A**). Hence, a total of 20 miRNAs, made up of 12 down-regulated miRNAs and 8 up-regulated miRNAs, were obtained. Details of expression and prognostic value of the 20 miRNAs are shown in **Table 1**. We defined these 20 miRNAs as key prognosis-associated miRNAs in LUAD and chose them for subsequent analysis.

Subsequently, target mRNAs of the 20 key miRNAs were predicted by miRNet. There were 2679 target mRNAs potentially interacted with the 12 down-regulated miRNAs, while 1724 target mRNAs were expected to interact with eight up-regulated miRNAs (**Fig. 1B**). The prognostic values of these target mRNAs were also assessed in GEPIA2 in its "survival map" page, then significant mRNAs were individually validated. Expression analysis was conducted in the Ualcan database with its "scan my genes" tool that allows simultaneous analysis of multiple genes. After the survival analysis and then the expression analysis, 99 up-regulated

mRNAs targeted by 10 down-regulated miRNAs and 20 down-regulated mRNAs targeted by five up-regulated miRNAs were identified (**Fig. 1B**). According to the functional mechanism of miRNA, the expressions of miRNA and its target mRNA must be negatively correlated. Therefore, the expression correlations of these miRNA-mRNA pairs were analyzed using Starbase. Totally 71 eligible up-regulated mRNAs and 11 eligible down-regulated mRNAs were finally identified (**Fig. 1B**, **Table 2**). The expression details and prognostic values of these mRNAs are presented in **Table 2**. As set out in **Table 3**, these mRNAs formed 97 miRNA/ mRNA pairs with miRNAs ($P < 0.05$). These mRNAs were defined as key prognosis-associated mRNAs in LUAD and used for the next network construction.

Establishment of the prognosis-associated miRNA-mRNA regulatory network in LUAD

Based on the above findings, a key prognosis-associated miRNA-mRNA regulatory network in LUAD was established. As shown in **Figure 2**, this network was divided into sub-network of the down-regulated miRNAs and sub-network of the up-regulated miRNAs. The sub-network of the down-regulated miRNAs was composed of 10 miRNAs and 71 mRNAs, which formed 85 miRNA-mRNA pairs; in contrast, the sub-network of the up-regulated miRNAs contained 5 miRNAs and 11 mRNAs, which formed 12 miRNA-mRNA pairs. Each

Figure 1. Workflow for key prognosis-associated miRNAs and mRNAs identification

(A) The stepwise approach for identifying key prognosis-associated miRNAs. (B) The stepwise approach for identifying key prognosis-associated mRNAs targeted by the key miRNAs.

Table 1. Expression details and prognostic values of key miRNAs in LUAD

miRNA name	miRNA type	Expression details				Prognostic value	
		Cancer Exp	Normal Exp	Fold Change	P Value	Hazard Ration	log-Rank P
hsa-miR-328-3p	Down-regulated / good prognosis	36.93	674.10	0.05	0.0002	0.69	0.014
hsa-miR-1976	Down-regulated / good prognosis	14.73	114.84	0.13	6E-11	0.66	0.0053
hsa-let-7c-5p	Down-regulated / good prognosis	1258.23	5235.5	0.24	2E-18	0.74	0.043
hsa-miR-146b-3p	Down-regulated / good prognosis	227.52	966.56	0.24	0.0002	0.74	0.045
hsa-miR-150-5p	Down-regulated / good prognosis	1108.92	3574.06	0.31	0.0084	0.74	0.046
hsa-miR-140-3p	Down-regulated / good prognosis	908.08	2111.63	0.43	6E-15	0.73	0.035
hsa-miR-30d-5p	Down-regulated / good prognosis	13296.36	23797.41	0.56	2E-05	0.62	0.0017
hsa-miR-101-3p	Down-regulated / good prognosis	11939.12	14362.07	0.83	1E-09	0.65	0.0038
hsa-miR-490-3p	Down-regulated / good prognosis	0.57	2.69	0.21	3E-09	0.72	0.033
hsa-miR-195-3p	Down-regulated / good prognosis	3.42	13.21	0.26	3E-06	0.59	0.00046
hsa-miR-1468-5p	Down-regulated / good prognosis	4.66	9.25	0.5	0.0026	0.64	0.0034
hsa-miR-526b-5p	Down-regulated / good prognosis	7.11	11.19	0.64	2E-07	1.50	0.0093
hsa-miR-582-3p	Up-regulated / poor prognosis	440.1	126.36	3.48	5E-07	1.36	0.039
hsa-miR-21-5p	Up-regulated / poor prognosis	358144.55	38949.90	9.20	6E-101	1.45	0.014
hsa-miR-9-5p	Up-regulated / poor prognosis	3568.81	35.32	101.04	3E-19	1.49	0.0079
hsa-miR-550a-5p	Up-regulated / poor prognosis	4.77	3.24	1.47	0.016	1.50	0.0067
hsa-miR-582-5p	Up-regulated / poor prognosis	20.23	6.00	3.37	5E-05	1.52	0.0051
hsa-miR-873-5p	Up-regulated / poor prognosis	0.47	0.06	8.40	0.037	1.57	0.0031
hsa-miR-1293	Up-regulated / poor prognosis	0.30	0.01	30.34	0.0065	1.88	0.000025
hsa-miR-31-5p	Up-regulated / poor prognosis	17.01	0.42	40.94	1E-08	1.39	0.032

RNA in these pairings showed opposite expression pattern and prognostic values.

To further test the credibility of this network, we examined these pairings in miRTarBase, which contains experimentally validated miRNA-target interactions. It was found that pairings including hsa-miR-30d-5p/KPNB1, hsa-miR-150-5p/PDIA6, hsa-miR-150-5p/BIRC5, hsa-miR-21-5p/DOCK4, and hsa-miR-21-5p/BTG2 had been experimentally validated (**Fig. 2**).

Functional enrichment analysis and construction of miRNA-hub genes network

To explore the functions of the identified key mRNAs in LUAD, we conducted biological pathway analysis in FunRich, which integrates data from multiple sources including KEGG, Reactome, HumanCyc, NCI/Nature, and Cell map. As shown in **Figure 3A**, 11 of 71 up-regulated mRNAs were significantly enriched in the "Cell Cycle, Mitotic" pathway and 5 enriched in the "Validated targets of C-MYC transcriptional activation" pathway (**Table 4**). Both are cancer-related pathways. The 11 down-regulated mRNAs did not enrich any pathway by FunRich analysis.

Enrichment analysis was also performed on three

GO terms (CC: cellular component; MF: molecular function; and BP: biological process). For the 71 up-regulated mRNAs, significantly enriched GO functions included "Centrosome", "Mitochondrion", "Nucleoplasm", and "Nucleus" in the CC category (**Fig. 3B**); no significantly enriched GO terms were identified in the MF and BP categories. The 11 down-regulated mRNAs did not enrich in any GO terms.

PPI network of the 71 up-regulated mRNAs was constructed in STRING database. According to MCC, 19 hub genes were identified and their interactions were re-built using Cytoscape software (**Fig. 3C**). Then, an miRNA-hub gene network was constructed, composed of 10 miRNAs and 19 hub genes (**Fig. 3D**). The 11 up-regulated mRNAs enriched in the "Cell Cycle, Mitotic" term were all hub genes (**Fig. 3D, Table 4**). Few protein-protein interactions existed among the 11 down-regulated mRNAs.

Experimental validation of miRNA-hub gene pairs

Subsequently, we performed experimental validation for some of our identified results. Four miRNAs (hsa-miR-1976, hsa-miR-101-3p, hsa-let-7c-5p, and hsa-miR-140-3p) that targeted most of the hub genes (16

Table 2. Expression details and prognostic values of key mRNAs in LUAD

No.	Gene type	Gene Name	Expression details (Starbase)				Prognostic value (GEPIA2)
			Cancer Exp	Normal Exp	Fold Change	P Value	
1	Onco-mRNA	ESPL1	1.89	0.2	9.36	1.50E-44	0.0023
2	Onco-mRNA	CCNB2	7.76	0.86	9	3.70E-56	0.00079
3	Onco-mRNA	RRM2	11.64	1.12	10.37	1.40E-53	1.5e-05
4	Onco-mRNA	FANCI	4.32	0.99	4.37	2.30E-43	0.043
5	Onco-mRNA	YWHAZ	120.83	61.68	1.96	1.30E-26	0.00023
6	Onco-mRNA	GARS	34.42	15.05	2.29	1.90E-34	0.00018
7	Onco-mRNA	EIF4A3	19.96	11.41	1.75	1.20E-18	0.013
8	Onco-mRNA	RHOV	19.46	1.56	12.48	1.20E-26	0.0023
9	Onco-mRNA	LDHA	141.93	51.1	2.78	1.20E-37	3e-05
10	Onco-mRNA	DIABLO	1.77	1.09	1.63	3.00E-19	0.00058
11	Onco-mRNA	MRPL12	24.41	9.6	2.54	1.20E-14	5e-04
12	Onco-mRNA	POLR2D	9.33	5.24	1.78	7.70E-33	0.0074
13	Onco-mRNA	CALU	66.43	30.23	2.2	4.90E-21	0.016
14	Onco-mRNA	NME4	25.98	9.02	2.88	6.20E-30	0.014
15	Onco-mRNA	PMAIP1	10.42	3.01	3.46	7.50E-13	0.0015
16	Onco-mRNA	RFC2	15.71	8.11	1.94	1.70E-19	0.031
17	Onco-mRNA	IGF2BP3	1.84	0.11	16.91	1.60E-14	0.0017
18	Onco-mRNA	KPNA2	36.71	8.42	4.36	3.10E-38	0.00038
19	Onco-mRNA	KIF2C	7.05	0.54	13.11	1.60E-58	0.016
20	Onco-mRNA	BZW1	20.71	11.81	1.75	5.00E-17	0.012
21	Onco-mRNA	MRPL42	3.48	2.47	1.41	4.10E-07	0.013
22	Onco-mRNA	DDIT4	67.27	28.83	2.33	3.80E-08	0.0017
23	Onco-mRNA	AHCY	46.38	22.08	2.1	1.70E-21	0.0049
24	Onco-mRNA	RACGAP1	8.61	2.77	3.11	1.10E-22	0.00062
25	Onco-mRNA	CDC44	8.52	2.84	3	1.60E-29	0.00081
26	Onco-mRNA	CDC48	8.67	0.95	9.16	2.50E-59	0.0026
27	Onco-mRNA	DNAJC9	4.59	2.3	1.99	2.10E-28	0.00057
28	Onco-mRNA	UCK2	4.75	1.76	2.7	5.50E-09	0.00012
29	Onco-mRNA	SMUG1	7.13	3.22	2.21	7.20E-38	0.004
30	Onco-mRNA	PDIA6	76.66	33.9	2.26	6.90E-26	0.005
31	Onco-mRNA	CACYBP	17.91	9.02	1.98	2.70E-27	0.00041
32	Onco-mRNA	INTS7	6.82	3.39	2.01	7.70E-31	1.3e-05
33	Onco-mRNA	SLC7A11	5.03	0.55	9.14	7.40E-18	0.036
34	Onco-mRNA	MRPS10	26.05	14.3	1.82	1.70E-24	0.01
35	Onco-mRNA	SLC2A1	38.71	3.14	12.33	1.80E-45	2.4e-05
36	Onco-mRNA	BIRC5	11.5	0.82	14.08	3.90E-55	0.00022
37	Onco-mRNA	TTL12	13.47	6.48	2.08	1.50E-15	0.00099
38	Onco-mRNA	TIMM50	9.31	5.16	1.81	1.50E-15	0.03
39	Onco-mRNA	CKS1B	13.34	3.69	3.62	1.90E-32	4.4e-05
40	Onco-mRNA	GPN1	13.77	8.2	1.68	7.20E-19	0.005
41	Onco-mRNA	RAD51	2.43	0.49	4.98	7.10E-40	0.0026
42	Onco-mRNA	TPI1	214.36	90.81	2.36	2.2E-26	0.0031

Continued Table 2. Expression details and prognostic values of key mRNAs in LUAD

No.	Gene type	Gene Name	Expression details (Starbase)				Prognostic value (GEPIA2)	
			Cancer Exp	Normal Exp	Fold Change	P Value	Log rank P	
43	Onco-mRNA	POC1A	4.59	1.41	3.27	1.6E-32	0.00094	
44	Onco-mRNA	DARS2	10.68	4.16	2.57	2E-33	0.00087	
45	Onco-mRNA	CDT1	5.45	0.74	7.33	1.5E-43	0.0019	
46	Onco-mRNA	KIF14	1.56	0.11	14.04	6.6E-57	0.00058	
47	Onco-mRNA	HMGAI	122.27	23	5.32	5.1E-37	0.0014	
48	Onco-mRNA	ALDOA	235.13	94.94	2.48	8.5E-25	0.00021	
49	Onco-mRNA	CENPI	1.4	0.2	7.16	1.1E-39	0.014	
50	Onco-mRNA	NOL10	9.62	5.24	1.84	1.4E-36	0.0054	
51	Onco-mRNA	IMP4	15.07	8.92	1.69	1.9E-26	0.0023	
52	Onco-mRNA	TMCO1	24.58	13.89	1.77	9E-24	0.019	
53	Onco-mRNA	TMEM106B	9.24	4.54	2.03	1.3E-15	0.0051	
54	Onco-mRNA	KIF11	5.96	0.84	7.11	4.5E-52	0.00048	
55	Onco-mRNA	MTHFD2	18.74	5.92	3.17	6.2E-29	0.0038	
56	Onco-mRNA	TWF1	18.66	8.99	2.08	9.5E-23	0.00014	
57	Onco-mRNA	YKT6	35.3	18.72	1.89	5E-22	0.017	
58	Onco-mRNA	GTF2E2	12.55	7.42	1.69	9.8E-20	0.0086	
59	Onco-mRNA	SFXN1	6.8	2.19	3.11	7.2E-67	0.042	
60	Onco-mRNA	GCLC	14.69	2.82	5.21	2.9E-09	0.024	
61	Onco-mRNA	KPNB1	37.6	21.33	1.76	8.6E-24	0.0027	
62	Onco-mRNA	TMED2	108.86	57.26	1.9	5E-25	0.002	
63	Onco-mRNA	CBX3	47.98	20.97	2.29	1.2E-42	0.00063	
64	Onco-mRNA	RPLP0	343.61	203.06	1.69	1.3E-11	0.037	
65	Onco-mRNA	FANCL	7.35	4.66	1.58	4.8E-13	5E-04	
66	Onco-mRNA	SKA1	2.19	0.25	8.85	1.8E-43	0.00043	
67	Onco-mRNA	FKBP4	24.43	10.13	2.41	2E-18	2.4E-05	
68	Onco-mRNA	AGMAT	1.55	0.24	6.39	1.4E-49	0.0096	
69	Onco-mRNA	CDC25A	1.44	0.26	5.52	2.6E-38	0.014	
70	Onco-mRNA	SRM	35.7	15.89	2.25	1.5E-24	0.021	
71	Onco-mRNA	OIP5	2.34	0.42	5.64	1.9E-35	5.7E-05	
72	TS-mRNA	NFIX	13.42	33.09	0.41	5.9E-25	0.007	
73	TS-mRNA	METTL7A	19.34	64.98	0.3	3.7E-41	0.0067	
74	TS-mRNA	BTG2	59.21	131.34	0.45	1.4E-17	0.0014	
75	TS-mRNA	SNX20	2.26	2.99	0.76	0.0000055	0.012	
76	TS-mRNA	PIK3R1	5.03	9.26	0.54	2.8E-20	0.00053	
77	TS-mRNA	LIFR	6.34	17.36	0.37	1.9E-25	0.035	
78	TS-mRNA	ABCB1	0.73	1.92	0.38	5E-18	0.013	
79	TS-mRNA	SNX30	6.97	12.69	0.55	2E-15	0.00091	
80	TS-mRNA	DOCK4	2.87	7.44	0.39	8.1E-36	0.026	
81	TS-mRNA	GPD1L	14.94	23.51	0.64	5.9E-13	0.0036	
82	TS-mRNA	SCD5	4.44	11.89	0.37	4.2E-24	0.035	

Table 3. Expression correlations of miRNA-mRNA pairs

No.	miRNA	gene	Correlation		No.	miRNA	gene	Correlation		No.	miRNA	gene	Correlation	
			R	P value				R	P value				R	P value
1	hsa-let-7c-5p	ESPL1	-0.431	1.25E-24	34	hsa-mir-140-3p	CDC48	-0.268	7.27E-10	67	hsa-mir-30d-5p	MTHFD2	-0.401	3.74E-21
2	hsa-let-7c-5p	CCNB2	-0.384	2.04E-19	35	hsa-mir-140-3p	CDC25A	-0.203	3.79E-06	68	hsa-mir-30d-5p	TWF1	-0.4	4.84E-21
3	hsa-let-7c-5p	RRM2	-0.384	1.81E-19	36	hsa-mir-140-3p	DNAJC9	-0.171	0.000106	69	hsa-mir-30d-5p	YKT6	-0.344	1.21E-15
4	hsa-let-7c-5p	CDC25A	-0.361	3.13E-17	37	hsa-mir-140-3p	UCK2	-0.103	0.0202	70	hsa-mir-30d-5p	GTF2E2	-0.329	2.20E-14
5	hsa-let-7c-5p	HMGAI	-0.348	5.20E-16	38	hsa-mir-150-5p	SMUG1	-0.264	1.23E-09	71	hsa-mir-30d-5p	SFXN1	-0.301	3.59E-12
6	hsa-let-7c-5p	FANCI	-0.332	1.17E-14	39	hsa-mir-150-5p	PDI6	-0.251	8.44E-09	72	hsa-mir-30d-5p	GCLC	-0.245	1.91E-08
7	hsa-let-7c-5p	YWHAZ	-0.296	7.73E-12	40	hsa-mir-150-5p	CACYBP	-0.245	2.00E-08	73	hsa-mir-30d-5p	KPNB1	-0.245	1.95E-08
8	hsa-let-7c-5p	GARS	-0.288	3.19E-11	41	hsa-mir-150-5p	ALDOA	-0.239	4.19E-08	74	hsa-mir-30d-5p	TMED2	-0.214	9.84E-07
9	hsa-let-7c-5p	BZW1	-0.282	7.79E-11	42	hsa-mir-150-5p	INTS7	-0.188	1.93E-05	75	hsa-mir-30d-5p	CBX3	-0.19	1.53E-05
10	hsa-let-7c-5p	EIF4A3	-0.278	1.50E-10	43	hsa-mir-150-5p	SLC7A11	-0.154	0.000489	76	hsa-mir-30d-5p	RPLP0	-0.129	0.00346
11	hsa-let-7c-5p	RHOV	-0.278	1.44E-10	44	hsa-mir-150-5p	MRPS10	-0.153	0.000499	77	hsa-mir-30d-5p	FANCL	-0.119	0.00722
12	hsa-let-7c-5p	LDHA	-0.27	5.22E-10	45	hsa-mir-150-5p	SLC2A1	-0.146	0.000919	78	hsa-mir-328-3p	RAD51	-0.188	1.76E-05
13	hsa-let-7c-5p	DIABLO	-0.254	5.90E-09	46	hsa-mir-150-5p	BIRC5	-0.137	0.00182	79	hsa-mir-328-3p	SKA1	-0.141	0.00133
14	hsa-let-7c-5p	MRPL12	-0.247	1.53E-08	47	hsa-mir-150-5p	TTL12	-0.133	0.00258	80	hsa-mir-328-3p	FKBP4	-0.1	0.0243
15	hsa-let-7c-5p	POLR2D	-0.225	2.52E-07	48	hsa-mir-150-5p	TIMM50	-0.109	0.0139	81	hsa-mir-195-3p	AGMAT	-0.188	1.86E-05
16	hsa-let-7c-5p	CALU	-0.214	9.69E-07	49	hsa-mir-1976	CKS1B	-0.344	1.16E-15	82	hsa-mir-195-3p	CDC25A	-0.302	2.89E-12
17	hsa-let-7c-5p	NME4	-0.181	0.0000372	50	hsa-mir-1976	GPN1	-0.338	4.14E-15	83	hsa-mir-490-3p	SRM	-0.149	0.000748
18	hsa-let-7c-5p	PMAIP1	-0.169	0.000123	51	hsa-mir-1976	RAD51	-0.285	4.67E-11	84	hsa-mir-490-3p	RAD51	-0.151	0.0006
19	hsa-let-7c-5p	INTS7	-0.163	0.000218	52	hsa-mir-1976	TP11	-0.28	1.10E-10	85	hsa-mir-526b-5p	OIP5	0.114	0.00992
20	hsa-let-7c-5p	RFC2	-0.145	0.000963	53	hsa-mir-1976	POC1A	-0.261	2.02E-09	86	hsa-mir-1293	NFIX	-0.235	7.15E-08
21	hsa-let-7c-5p	IGF2BP3	-0.14	0.00147	54	hsa-mir-1976	DARS2	-0.235	7.65E-08	87	hsa-mir-873-5p	METTL7A	-0.19	1.56E-05
22	hsa-mir-101-3p	RRM2	-0.41	3.49E-22	55	hsa-mir-1976	CDT1	-0.215	9.35E-07	88	hsa-mir-873-5p	BTG2	-0.173	8.56E-05
23	hsa-mir-101-3p	KPNA2	-0.388	7.83E-20	56	hsa-mir-1976	KIF14	-0.199	5.61E-06	89	hsa-mir-550a-5p	SNX20	-0.153	0.000531
24	hsa-mir-101-3p	KIF2C	-0.373	2.46E-18	57	hsa-mir-1976	HMGAI	-0.198	6.42E-06	90	hsa-mir-21-5p	BTG2	-0.301	3.61E-12
25	hsa-mir-101-3p	BIRC5	-0.301	3.66E-12	58	hsa-mir-1976	ALDOA	-0.187	2.06E-05	91	hsa-mir-21-5p	PIK3R1	-0.218	6.69E-07
26	hsa-mir-101-3p	BZW1	-0.261	2.12E-09	59	hsa-mir-1976	CENPI	-0.185	2.55E-05	92	hsa-mir-21-5p	LIFR	-0.198	6.29E-06
27	hsa-mir-101-3p	MRPL42	-0.22	5.27E-07	60	hsa-mir-1976	NOL10	-0.18	4.16E-05	93	hsa-mir-21-5p	ABCB1	-0.185	2.44E-05
28	hsa-mir-101-3p	GCLC	-0.216	7.94E-07	61	hsa-mir-1976	IMP4	-0.173	8.01E-05	94	hsa-mir-21-5p	SNX30	-0.172	8.75E-05
29	hsa-mir-101-3p	DDIT4	-0.1	0.0237	62	hsa-mir-1976	RFC2	-0.168	0.00013	95	hsa-mir-21-5p	DOCK4	-0.137	0.00184
30	hsa-mir-140-3p	TP11	-0.403	1.99E-21	63	hsa-mir-1976	TMCO1	-0.16	0.000273	96	hsa-mir-21-5p	GPD1L	-0.133	0.00265
31	hsa-mir-140-3p	AHCY	-0.341	2.01E-15	64	hsa-mir-1976	TMEM106B	-0.108	0.0141	97	hsa-mir-9-5p	SCD5	-0.1	0.023
32	hsa-mir-140-3p	RACGAP1	-0.296	8.31E-12	65	hsa-mir-30d-5p	RRM2	-0.535	2.68E-39					
33	hsa-mir-140-3p	CDCA4	-0.269	5.98E-10	66	hsa-mir-30d-5p	KIF11	-0.416	7.76E-23

Figure 2. The miRNA-mRNA regulatory network associated with prognosis of LUAD. miRNAs are represented by triangles; mRNAs are represented by circles; all up-regulated RNAs are in red; all down-regulated RNAs are in green; experimentally validated interactions (miRTarBase) of RNA pairs are marked by bold blue lines.

Table 4. Enriched pathways of key up-regulated mRNAs

Biological pathway	Genes mapped (from input data set)
Cell Cycle, Mitotic	CCNB2; RRM2; CDC25A; RFC2; KIF2C; BIRC5; CDCA8; CKS1B; CDT1; CENPI; SKA1
Validated targets of C-MYC transcriptional activation	CDC25A; HMGA1; LDHA; PMAIP1; BIRC5; SLC2A1

out of 19) were selected for validation. As showed in **Figure 4A**, the expressions of miRNAs were detected by qPCR, which showed successful transfection. Over-expression of hsa-miR-1976 resulted in significantly lower expressions of CKS1B and RFC2 in A549 cells; similarly, decrease of the mRNA level was observed for RFC2 when hsa-miR-1976 was over-expressed in H1299 cells (**Fig. 4B**). **Figure 4C** shows that the expression of KIF2C was consistently

decreased when the hsa-miR-101-3p mimic was transfected into A549 and H1299 cells. **Figure 4D** demonstrates that, when hsa-let-7c-5p was over-expressed, the expressions of RFC2, ESPL1, and CDC25A were significantly reduced in both A549 and H1299 cells. Over-expression of hsa-miR-140-3p also resulted in significant expression reduction of CDC25A and CDCA8 in A549 cells; however, these results were not observed when hsa-miR-140-3p was over-expressed in H1299 cells (**Fig. 4E**). Because the confirmed target hub genes are involved in cell cycle, we also assessed their influences on cell cycle after miRNAs over-expression. As shown in **Figure 5**, up-regulation of hsa-miR-1976 and hsa-let-7c-5p, but not hsa-miR-101-3p and hsa-miR-140-3p, resulted in remarkable cell cycle arrest in A549 cells compared with the control, with significantly increased proportion of cells in G1 phase and decreased proportion of cells in S phase.

Figure 3. Enrichment analysis and miRNA-hub gene network. (A) Top six enriched pathways of the key up-regulated mRNAs. (B) Top six enriched terms in cellular component of the key up-regulated mRNAs. (C) Hub genes identified from the key up-regulated mRNAs. (D) The miRNA-hub gene network.

DISCUSSION

Lung cancer is one of the most common and lethal malignant tumors worldwide^[1]. LUAD accounts for almost half of all the lung cancer cases^[2]. Recent advances in this field include the development and application of targeted drugs and immunotherapeutics. However, the overall survival of LUAD patients remains poor. Research on the molecular mechanisms underlying LUAD helps identifying potential biomarkers and therapeutic targets. miRNAs are small endogenous non-coding RNAs that are 21-25 bp in length and involved in multiple biological processes^[20]. miRNAs can bind to specific sites in the un-translated regions of coding messenger RNAs (mRNAs) and decrease gene expression at the post-transcriptional or translational levels. These individual miRNAs play crucial roles in human cancers, especially lung cancer^[4-10]. Several

studies also performed systematical identification of miRNAs and construction of their regulatory networks in LUAD based on expression. For example, Wang *et al.* screened out 113 miRNAs, 1633 lncRNAs, and 2507 mRNAs that were differentially expressed in LUAD and built a lncRNA-mRNA-miRNA competing endogenous RNA (ceRNA) network that consisted of 134 lncRNAs, 21 miRNAs, and 34 mRNAs^[11]. A similar work was done in non-small cell lung cancer (NSCLC) that constructed a ceRNA network, including 113 lncRNAs, 12 miRNAs, and 36 mRNAs differentially expressed between NSCLC and non-tumor tissues^[12].

However, to our knowledge, no systematic identification of survival-related miRNAs and construction of their regulatory network in LUAD had been conducted. Hence, in our current study, we identified and constructed a comprehensive miRNA-mRNA network, of which each component was significantly associated

Figure 4. Experimental validation of miRNA-hub gene pairs. Relative miRNA expression (to blank control) after transfected with 1976, 101, let-7c, or 140 in A549 and H1299; relative mRNA expression of predicted targets after over-expression of 1976 (A), 101 (B), let-7c (C), and 140 (D) in A549 and H1299.

NC: natural control mimic; 1976: hsa-miR-1976 mimic; 101: hsa-miR-101-3p mimic; let-7c: hsa-let-7c-5p mimic; 140: hsa-miR-140-3p mimic.

with prognosis in LUAD. After step-by-step analysis of survival, expression pattern, and miRNA-mRNA expression correlation, 20 key miRNAs, and 84 key mRNAs were identified, and a miRNA-mRNA network associated with the prognosis of LUAD was built. The roles of these miRNAs or mRNAs in suppressing and promoting tumor miRNAs or mRNAs have been previously described. For example, miR-582-3p over-expression maintains lung cancer stem cell-like traits through targeting multiple negative regulators of the Wnt/b-catenin pathway, namely, AXIN2, DKK3, and SFRP1^[21]. Luo *et al.* demonstrated that hsa-miR-873-5p directly interacted with TUSC3 in lung cancer^[22]. Hosseini *et al.* suggested that hsa-miR-30d-5p was a possible tumor suppressor in lung cancer initiation and

progression^[23]. These reports supported the crucial roles of our identified miRNAs. Our pathway enrichment analysis showed that the up-regulated mRNAs mostly enriched in "Cell Cycle, Mitotic" pathway. This was similar with previous studies, which showed that "Cell cycle" listed as the top one enriched term for differential expression genes in NSCLC^[12]. Moreover, most of our identified hub genes (11/19) were enriched in the "Cell Cycle, Mitotic" pathway. The functions of most hub genes have been well studied in human cancers. For example, Cdc25A was reported to positively regulate G1-S and G2-M transitions by activating distinct cyclin/cyclin-dependent kinase complexes^[24]. RFC2, a direct target of miR-744, can modulate the cell cycle and promote the proliferation of CRC cells^[25]. KIF2C can

Figure 5. Influences of miRNA over-expression on cell cycle. hsa-miR-1976 and hsa-let-7c-5p over-expression induced the G0/G1 phase arrest of A549 cells.

NC: natural control mimic; 1976: hsa-miR-1976 mimic; 101: hsa-miR-101-3p mimic; let-7c: hsa-let-7c-5p mimic; 140: hsa-miR-140-3p mimic.

modulate the proliferation of NSCLC cells via regulating cell cycle process and control NSCLC cell migration and invasion^[26]. Several miRNA-hub gene pairs including hsa-miR-1976/RFC2, hsa-let-7c-5p/RFC2, hsa-let-7c-5p/ESPL1, hsa-let-7c-5p/CDC25A, and hsa-miR-101-3p/KIF2C were supported by our qPCR validation in both A549 and H1299 cells. As far as we know, the regulatory relationships of all these miRNA/mRNA pairs were new findings. These miRNA/mRNA may play important roles in LUAD initiation and progression. Consistent with the already mentioned roles of RFC2 and CDC25A^[24, 25], transfection with hsa-miR-1976 or hsa-let-7c-5p mimics resulted in remarkable cell cycle arrest in A549 cells. Huang *et al.* suggested that Let-7c-5p might play vital roles in the tumorigenesis of non-small cell lung cancers by targeting multiple genes and affecting distinct pathways^[27]. Chen *et al.* showed that over-expression of miR-1976 inhibited the growth and metastasis of NSCLC cells by directly targeting PLCE1^[28]. Hence, our results added new information about the mechanisms for these two miRNAs, by targeting two crucial cell cycle regulators. However, more well-designed experiments are desired to reveal the

molecular mechanism of the prognosis-associated miRNA-mRNA network in the future.

Competing interests

The authors declare no competing interests.

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Author contributions

TC designed the study and revised the manuscript. WX wrote the manuscript. WX and JY performed the experiments and partial analysis of the data. All authors have read and approved the final manuscript.

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论著

鉴定并验证与肺腺癌预后相关的新的 miRNA-mRNA 调节网络

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摘要

目的 许多研究揭示了 miRNA 在多种人类癌症中的关键作用, 包括肺腺癌 (LUAD)。本研究试图探索与 LUAD 预后相关的新的 miRNA-mRNA 对。

方法 采用生物信息学工具 (如 OncomiR、StarBase、miRnet、GEPIA2 和 UALCAN) 鉴定并验证与肺腺癌预后相关的新的 miRNA-mRNA 调节网络。

结果 首先, 通过在 OncomiR 和 StarBase 中的表达和预后价值分析后得到了 20 个关键 miRNA。在 miRnet 中预测了这些关键 miRNAs 的靶向 mRNAs, 并分别在 GEPIA2 和 UALCAN 中分析了它们的预后价值和表达模式。在 StarBase 中进行进一步的表达相关性分析。随后构建了一个新的 miRNA-mRNA 网络, 其中每个 RNA 对都显示出负的表达相关性、相反的表达模式和预后价值。基于这些 mRNAs 构建了蛋白质相互作用网络, 并从中确定了 19 个枢纽基因。富集分析表明 “Cell Cycle, Mitotic” 是最显著的富集项。然后, 构建了 miRNA-hub 基因网络, 选择并验证了一些 miRNA-hub 基因对的调节关系, 包括 hsa-miR-1976/RFC2、hsa-let-7c-5p/RFC2、hsa-let-7c-5p/ESPL1、hsa-let-7c-5p/CDC25A 和 hsa-miR-101-3p/KIF2C。此外, hsa-miR-1976 和 hsa-let-7c-5p 的过表达还导致显著的细胞周期停滞。

结论 我们的结果确定了新的预后相关的 miRNA-mRNA 对, 进一步阐明了 miRNA-mRNA 网络影响 LUAD 预后的分子调控机制。

关键词: 肺腺癌; 生物信息学分析; miRNA; 预后; 细胞周期

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