

ORIGINAL ARTICLE

Microarray expression profile of microRNAs in lung adenocarcinoma patients with the Cancer Genome Atlas and its clinical validation

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Summary

Purpose: To mine differentially expressed microRNAs (miRs) in lung adenocarcinoma (LUAD) via The Cancer Genome Atlas (TCGA).

Methods: The transcriptome and pathological data of LUAD patients were downloaded from TCGA. The differentially expressed genes were screened using "edgeR" package in R and analyzed by univariate and multivariate Cox regressions. The expression and clinic value of serum miR-548v in 50 patients with LUAD (study group) and 50 healthy individuals (control group) was detected by the quantitative real time polymerase chain reaction (qRT-PCR), and the diagnostic value of miR-548v in LUAD was analyzed by the receiver operating characteristic (ROC) curve.

Results: A total of 233 differentially expressed genes were found, of which 115 were highly expressed and 118 were lowly expressed. Multivariate Cox regression showed that

hsa-mir-142 and hsa-mir-548v were independent prognostic factors for patients with LUAD. The study group showed significantly higher serum miR-548v expression than the control group ($p < 0.001$). miR-548v was related to TNM staging, lymph node metastasis, and tumor size of patients ($p < 0.05$). The area under the curve (AUC) of miR-548v in diagnosing LUAD was 0.960, and that in determining TNM staging, lymph node metastasis and tumor size was 0.829, 0.851 and 0.881 respectively.

Conclusion: Differential expression of miR-548v predicts poor prognosis of patients with LUAD and is closely related to TNM staging, lymph node metastasis and tumor size. Therefore, it is expected to be a potential diagnostic and prognostic marker for LUAD.

Key words: The Cancer Genome Atlas, lung adenocarcinoma, miR expression profile

Introduction

The incidence of cancer is high and increasing year by year [1]. There were 18.1 million new cancer patients and 9.6 million deaths worldwide in 2018, of which lung cancer (LC) accounted for the highest proportion [2]. More than 80% of LC are non-small cell lung cancer (NSCLC), which can be

divided into lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) according to the pathological type, and LUAD is more common [3,4]. Recently, the incidence rate of LUAD has increased significantly faster than LUSC's, and it is prone to distant metastasis, with a very low response rate

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[5,6]. In addition, the early LUAD may not affect the daily life of patients, so most patients are diagnosed with advanced disease after admission, missing the optimal time for surgical treatment [7]. Although radiotherapy and chemotherapy are used to improve the patient's condition, the long-term survival is still low [8]. The main reason for the above is the lack of highly specific diagnostic indicators with high specificity, so seeking a new one is of great significance [9,10].

MicroRNA (miR), a non-small coding RNA distributed in eukaryotes, regulates the expression of target genes by binding to the 3' untranslated region (3' UTR) and 5' UTR of downstream target genes, thus participating in numerous intercellular signal regulation [11]. A study showed that miR plays a regulatory role in cell proliferation, invasion and migration [12-14]. With the continuous improvement of the second-generation sequencing technology, an ever increasing tumor research programs have been carried out, the most famous of which is The Cancer Genome Atlas (TCGA) of the United States. Mapping human cancer genomes by second-generation high-throughput gene sequencing and chip technology is more favorable for researchers to study the occurrence and molecular mechanism of tumors, so as to find new potential therapeutic targets [15,16]. In this study, differentially expressed miRs in LUAD were screened out through TCGA, which were verified by clinical practice, in order to provide new biological markers for the early diagnosis and treatment of LUAD.

Methods

Data download from TCGA

The LUAD miR file data downloaded from <https://portal.gdc.cancer.gov/> was collected by TCGA core sample repository, then sequenced and analyzed by standardized processing scheme (<http://can-cergenome.nih.gov/cancergenomics/tissuesamples>). A total of 528 cases, including 483 LUAD samples and 45 matched samples, were obtained through IlluminaHiSeq2000 sequencing platform. Perl script was used to merge and convert the files into miRNAmatrix files.

Clinical sample collection

Fifty LUAD patients treated in Nanjing Gaochun People's Hospital from January 2017 to March 2018 comprised the study group, with an average age of 59.4 ± 9.2 years. In addition, 50 normal individuals who underwent physical examination in this hospital comprised the control group, with an average age of 58.7 ± 7.7 years. This study was approved by the Medical Ethics Committee of Nanjing Gaochun People's Hospital.

Inclusion criteria: Patients meeting TNM staging [17] issued by American Association of Cancer (AJCC) in 2009; patients pathologically diagnosed with LUAD;

patients with complete clinical data and signing an informed consent form.

Exclusion criteria: Patients receiving radiotherapy and chemotherapy before this study; patients complicated with other tumors; patients with life expectancy less than 3 months; patients not cooperating with the follow-up; patients with immunodeficiency. There was no statistical difference in sex and age between the two groups ($p > 0.05$), indicating a comparability.

Data preprocessing

Data of patients whose survival time was shorter than 30 days were deleted. Due to the presence of < 1 and > 0 in a single sample gene expression, \log_2 conversion was performed. Since the conversion results might become negative, the sample was subjected to $\log_2(X+1)$ conversion to make the data more similar to gene expression.

Detection of miR expression in patients' serum

Samples of 5-mL peripheral blood were extracted from patients, kept still for 30 min, then centrifuged at

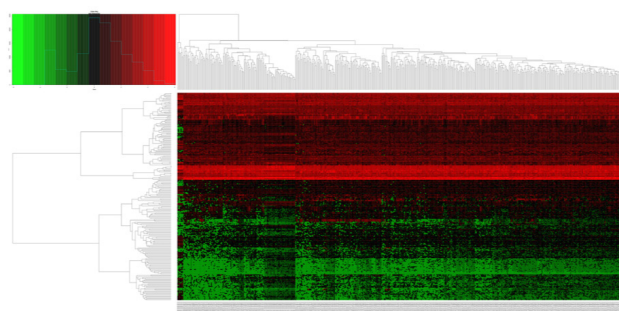


Figure 1. Thermogram of differentially expressed miRs in LUAD. Red represents highly expressed miRs and green represents lowly expressed miRs.

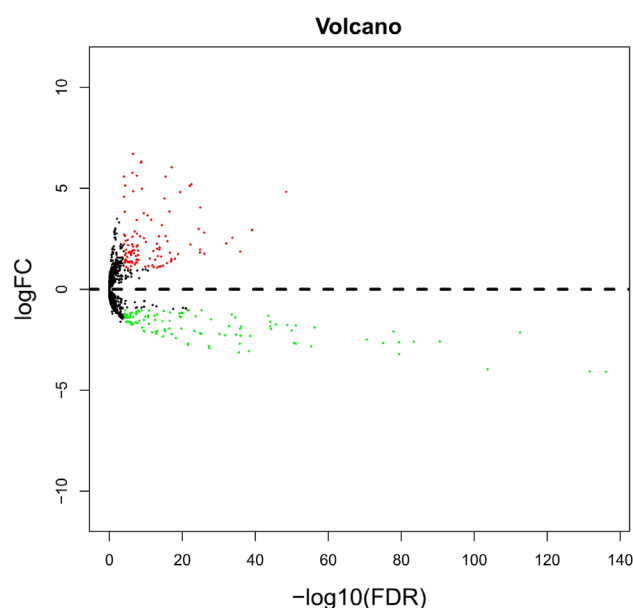


Figure 2. Volcano plot of differentially expressed miRs in LUAD. Red represents highly expressed miRs and green represents lowly expressed miRs.

3000 rpm for 10 min to collect the serum. The samples were measured by quantitative real time polymerase chain reaction (qRT-QPCR). Total RNA was extracted from serum using an EasyPure miRNA Kit (TransGen Biotech, Beijing, China), and the purity, concentration and integrity were detected by an UV spectrophotometer and agarose gel electrophoresis. Reverse transcription of total RNA was carried out using the reverse transcription kit provided in TransScript Green miRNA Two-Step qRT-PCR SuperMix (TransGen Biotech, Beijing, China). The reverse transcribed cDNA was collected and amplified using TransScript Green miRNA Two-Step qRT-PCR SuperMix and 7500PCR (ABI) instrument. PCR amplification system: 1 µL of cDNA, 0.4 µL of each upstream and downstream primer, 10 µL of 2×TransTaq® Tip Green qPCR SuperMix, 0.4 µL of Passive Reference Dye (50X), and finally made up to 20 µL with ddH₂O. PCR reaction conditions: pre-denaturation at 94°C for 30 s, denaturation at 94°C for 5 s, annealing at 60°C for 30 s, for a total of 40 cycles. Each sample was tested in 3 repeated wells, and the experiment was carried out 3 times. U6 was used as an internal reference and 2^{-ΔΔct} was used to analyze the data [18].

Statistics

The edgeR package in R was applied to analyze the difference of gene expression in TCGA, and the data met

the requirements of p<0.0001 and fold change =1. Univariate and multivariate Cox regression analysis were utilized to perform tumor time files. SPSS 20.00 software was used to statistically analyze the collected data, and Graph Pad 7 to plot figures. Kolmogorov-Smirnov (K-S) test was performed to analyze the data distribution, and t-test was used for normal distributed data, while the comparison between groups was conducted by independent sample t-test. The counting data expressed as percentage (%) were tested by chi-square (x²) test. A ROC curve was employed to assess the diagnostic value of miR-548v in LUAD. Area under the curve (AUC) greater than 0.5 indicated a high value. P<0.05 was considered to be statistically significant.

Results

Differentially expressed miRs in TCGA

In this study, 233 differentially expressed genes were found through screening. There were 115 highly expressed genes, and top 10 were hsa-mir-210, hsa-mir-96, hsa-mir-708, hsa-mir-21, hsa-mir-183, hsa-mir-182, hsa-mir-7-1, hsa-mir-301a, hsa-mir-153-2, and hsa-miR-141. There were 118 lowly expressed genes, and top 10 were

Table 1. Top 10 highly expressed miRs

miR mean	logFC	logCPM	p value	FDR
hsa-mir-210	4.835	10.132	4.09E-51	3.40E-49
hsa-mir-96	2.955	4.874	1.27E-41	7.45E-40
hsa-mir-708	2.930	6.870	1.58E-41	8.94E-40
hsa-mir-21	1.876	18.433	2.90E-38	1.39E-36
hsa-mir-183	2.563	13.557	4.86E-36	1.97E-34
hsa-mir-182	2.283	14.550	2.46E-34	9.06E-33
hsa-mir-7-1	1.767	4.562	2.97E-28	9.60E-27
hsa-mir-301a	2.812	4.203	3.34E-28	1.06E-26
hsa-mir-153-2	4.061	4.320	3.98E-27	1.19E-25
hsa-mir-141	1.834	10.658	5.29E-27	1.51E-25

logFC: log fold change, logCPM: log counts per million, FDR: false discovery rate

Table 2. Top 10 lowly expressed miRs

miR mean	logFC	logCPM	p value	FDR
hsa-mir-486-1	-4.071	7.488	5.18E-140	8.19E-137
hsa-mir-486-2	-4.060	7.476	2.96E-135	2.34E-132
hsa-let-7d	-2.132	9.499	5.29E-116	2.79E-113
hsa-mir-4732	-3.952	1.623	5.56E-107	2.20E-104
hsa-mir-197	-2.578	8.667	8.95E-94	2.83E-91
hsa-mir-139	-2.587	6.254	1.44E-86	3.81E-84
hsa-mir-6892	-2.610	2.455	1.87E-82	4.11E-80
hsa-mir-144	-3.200	7.760	2.08E-82	4.11E-80
hsa-mir-195	-2.080	5.710	8.08E-81	1.42E-78
hsa-mir-328	-2.654	5.722	5.63E-78	8.91E-76

logFC: log fold change, logCPM: log counts per million, FDR: false discovery rate

hsa-mir-486-1, hsa-mir-486-2, hsa-let-7d, hsa-mir-4732, hsa-mir-197, hsa-mir-139, hsa-mir-6892, hsa-mir-144, hsa-mir-195, and hsa-mir-328 (Figures 1 and 2 and Tables 1 and 2).

Cox analysis of survival

Univariate Cox regression was carried out on tumor.time files, and 22 miRs with difference were

found. The top 15 with the most significant difference were hsa-mir-101-1, hsa-mir-101-2, hsa-mir-200a, hsa-mir-148a, hsa-mir-450a-2, hsa-mir-31, hsa-mir-429, hsa-mir-450a-1, hsa-mir-29b-2, hsa-mir-584, whereas multivariate Cox regression showed that there were 2 miRs with difference, hsa-mir-142 and hsa-mir-548v, which were independent prognostic factors for LUAD patients (Tables 3 and 4).

Table 3. Univariate Cox regression analysis

Factor	β value	Z value	p value	HR	95CI%
hsa-mir-101-1	0.032	-3.638	0.000	0.032	0.005-0.203
hsa-mir-101-2	0.032	-3.635	0.000	0.032	0.005-0.204
hsa-mir-200a	0.292	-2.931	0.003	0.292	0.128-0.665
hsa-mir-148a	0.095	-2.886	0.004	0.095	0.019-0.469
hsa-mir-450a-2	1.597	2.867	0.004	1.597	1.160-2.199
hsa-mir-31	1.307	2.814	0.005	1.307	1.085-1.574
hsa-mir-429	0.465	-2.689	0.007	0.465	0.266-0.812
hsa-mir-450a-1	1.606	2.659	0.008	1.606	1.133-2.278
hsa-mir-29b-2	0.310	-2.427	0.015	0.310	0.120-0.798
hsa-mir-584	1.823	2.370	0.018	1.823	1.110-2.996

HR: hazard ratio

Table 4. Multivariate Cox regression analysis

Factor	β value	Z value	p value	HR	95CI%
hsa-mir-548v	0.724	-3.638	0.045	0.724	0.527-0.993
hsa-mir-142	0.115	-2.917	0.004	0.115	0.027-0.492

HR: hazard ratio

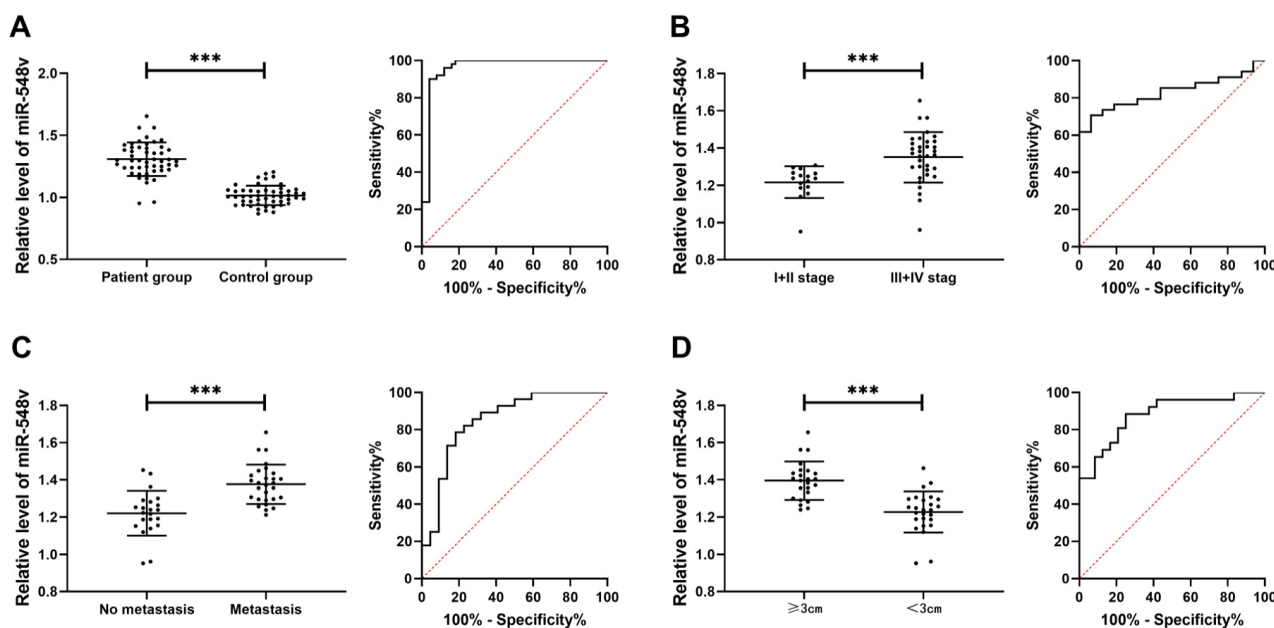


Figure 3. Expression and diagnostic value of miR-548v. **A:** expression and diagnostic value of miR-548v in patients in the study and control groups. **B:** expression and diagnostic value of miR-548v in diagnosing clinical staging. **C:** expression and diagnostic value of miR-548v in diagnosing lymph node metastasis. **D:** expression and diagnostic value of miR-548v in diagnosing tumor size. *** p<0.001.

Table 5. Analysis on miR-548v expression and pathological data

Factor	miR-548v expression	95CI%	t value	p value
Sex		-0.066-0.104	0.453	0.653
Male (n=35)	1.314±0.133			
Female (n=15)	1.294±0.146			
Age (years)		-0.116±0.037	1.040	0.304
<60 (n=24)	1.329±0.121			
≥60 (n=26)	1.289±0.148			
TNM staging		0.060-0.209	3.644	<0.001
I+II (n=16)	1.263±0.085			
III+IV (n=34)	1.351±0.135			
Lymph node metastasis		0.091-0.221	4.862	<0.001
Yes (n=28)	1.376±0.106			
No (n=22)	1.221±0.121			
Tumor size		-0.230--0.107	5.535	<0.001
≥3cm (n=24)	1.395±0.103			
<3cm (n=26)	1.227±0.111			
Smoking history		-0.133-0.062	0.736	0.465
Yes (n=40)	1.336±0.157			
No (n=10)	1.301±0.132			

Table 6. ROC curve

Indicators	AUC	95CI%	Specificity (%)	Sensitivity (%)	Youden's index (%)	Cut-off value
LUAD diagnosis	0.960	0.916-1.000	96.00	90.00	86.00	<1.115
Diagnosis of TNM staging	0.829	0.717-0.941	93.75	70.59	64.34	>1.296
Diagnosis of lymph node metastasis	0.851	0.738-0.963	81.81	78.57	60.39	>1.293
Diagnosis of tumor size	0.881	0.788-0.975	75.00	88.46	63.46	<1.344

Expression and clinical value of miR-548v

As the relationship between miR-142 and lung cancer has been reported in many studies, miR-548v was chosen for further research. This study found that the expression of miR-548v in the study group (1.308±0.136) was significantly higher than that in the control group (1.016±0.079) ($p<0.001$). And miR-548v was related to TNM staging, lymph node metastasis and tumor size of the patient ($p<0.05$). ROC curve showed that the AUC of miR-548v was 0.960 and the optimal specificity (96.00%) and sensitivity (90.00%) were obtained when cut-off was <1.115, as shown in Figure 3 and Tables 5 and 6.

Discussion

There were 4.222 million new cancer patients and 2.842 million deaths in China in 2015 [19]. The death and onset of lung cancer are at the top of the list, with LUAD patients accounting for more than 50%. The main factors leading to high morbidity

and mortality are as follows: 1. The improvement of people's living standard, irregular daily routine and diet [20]; 2. The difficulty of early diagnosis of LC makes it easy for patients to miss the best timing for treatment [21]; 3. As LC is resistant to chemotherapy and radiotherapy, there is no other treatment but surgery [22]. The above problems are mainly caused by the lack of clinically specific biological indicators. Therefore, seeking a biological indicator with high specificity and sensitivity is of great significance.

As a single-stranded non-coding small molecule RNA with a length of about 22 nucleotides, miR regulates the expression of target genes and inhibits/promotes the function of target genes by targeting the 3' non-coding region of downstream RNA molecules for complementary pairing [23,24]. A study [25] has reported that miR has significant effects in the diagnosis and prognosis evaluation of LUAD. Therefore, in this study, we screened differentially expressed miRs in LUAD via TCGA. A total of 233 differentially expressed genes were

screened out, of which 115 were highly expressed and 118 were lowly expressed. The clinical data of patients were collected to carry out univariate and multivariate Cox regression analysis, and it was found that hsa-mir-142 and hsa-mir-548v were independent prognostic factors for LUAD patients. Since there are many studies on miR-142 in LUAD, miR-548v was chosen in this study for clinical verification. miR-548v, a member of miR-548 family, is located in the short arm (region 22) of chromosome 8, which has been currently hardly studied. We found that the expression of miR-548 in the paracancer group was significantly higher than that in the control group through database. Moreover, clinical experiments also showed that miR-548v was highly expressed in LUAD, indicating that miR-548 was expected to be a potential observation indicator for LUAD. Therefore, we further plotted the ROC curve of miR-548v in patients with LUAD. The result showed that the AUC was 0.960, suggesting that miR-548v had extremely high diagnostic value in LUAD and can become a potential diagnostic marker for LUAD. However, the diagnostic value of miR-548v in lung cancer is unclear. At present, there are more and more indicators for the diagnosis of LUAD, but there are few indicators for distinguishing patients with early or late LUAD. This study also demonstrated that miR-548v varied in patients with different TNM staging, lymph node metastasis, and tumor size, and the expression in serum of patients with III+IV stage, lymph node metastasis and tumor ≥ 3 cm was higher than that of the corresponding group, which indicated that miR-548v was valuable in diagnosing TNM staging, lymph node metastasis, and tumor size. ROC curve showed that the AUC of miR-548v in evaluating TNM stage, lymph node

metastasis, and tumor size was 0.829, 0.851, and 0.881, respectively, suggesting that miR-548v was a potential diagnostic marker for TNM staging, lymph node metastasis and tumor size.

In this study, miR-548v was identified as a potential diagnostic marker for LUAD patients and had the function of distinguishing LUAD stagings, lymph node metastasis and tumor size by TCGA screening. However, there are still limitations. Firstly, the relationship between miR-548v and survival of the patients was not observed. A previous study by Lin et al [28] reported that the total survival of patients in miR-548v high expression group was significantly higher than that in low expression group, but the specific mechanism was not clear. And the survival curve was not drawn due to the short sample collection time. Secondly, the relevant mechanisms of miR-548v remained unclear. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment were not carried out and a protein-protein interaction (PPI) network was not constructed. Therefore, we hope to conduct long-term follow-up and find the target genes and co-expression network through bioinformatics analysis, as well as carry out basic research to prove and supplement our findings.

To sum up, the differential expression of miR-548v predicts the poor prognosis of LUAD patients, and is closely related to TNM staging, lymph node metastasis, and tumor size. Therefore, it is expected to be a potential diagnostic and prognostic marker for LUAD.

Conflict of interests

The authors declare no conflict of interests.

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