

Correlation of microRNA-335 expression level with clinical significance and prognosis in non-small cell lung cancer

Wen Huo, MD^a, Man Zhang, MD^a, Chunhua Li, MD^a, Xinying Wang, MD^a, Xiuli Zhang, MD^b, Xiaona Yang, MD^c, Haitao Fei, MD^{a,*}

Abstract

Although treatments have improved significantly in recent years, the prognosis of patients with non-small cell lung cancer (NSCLC) remains poor. miR-335 has been demonstrated to play the antitumor role in several cancer types. Its expression was reduced in NSCLC tissues relative to noncancerous adjacent tissues. Furthermore, downregulation of miR-335 in A459 lung cancer cells promoted cell proliferation. In the present study, we aimed to investigate the clinical significance and prognostic value of miR-335 in NSCLC.

The lung cancer tissues and adjacent nontumor lung tissues were obtained from 131 patients who underwent the primary surgical resection at Lianyungang First People's Hospital. Student *t* test was used to distinguish differences between groups. χ^2 test was involved for analysis of clinicopathological data. The overall survival was analyzed by the Kaplan-Meier method and the log rank test. Multiple Cox proportional hazards regression analysis was carried out to identify the independent factors that had a significant impact on patient survival.

miR-335 was significantly lower in NSCLC samples compared to non-cancerous samples ($P < .001$). The expression level of miR-335 was significantly correlated with tumor histology ($P = .028$), lymph node metastasis ($P = .002$), differentiation degree ($P < .001$), and pathological TNM stage ($P < .001$). The log-rank test indicated that patients with decreased miR-335 expression experienced poor overall survival in NSCLC ($P = .029$).

The results of the present study indicated that miR-335 was down-expressed in NSCLC, and is associated with tumor progression and poor prognosis, suggesting that the expression of miR-335 might be an independent prognostic factor of overall survival in patients with NSCLC.

Abbreviations: 3'-UTR = 3'-untranslated region, ccRCC = clear cell renal cell carcinoma, HCC = hepatocellular carcinoma, miRNAs = microRNAs, NSCLC = non-small cell lung cancer, PCR = polymerase chain reaction.

Keywords: expression, lung cancer, microRNA, miR-335, NSCLC, prognosis

1. Introduction

Lung cancer is a leading cause of cancer-related morbidity and mortality worldwide.^[1] Non-small cell lung cancer (NSCLC) accounts for 80% of lung cancer cases, including adenocarcino-

ma, squamous cell carcinoma, and large cell carcinoma.^[2] Although treatments have improved significantly in recent years, the prognosis of patients with NSCLC remains poor.^[3] Therefore, further elucidation of the molecular mechanisms underlying tumorigenesis, invasion, and metastasis is required to develop an efficient strategy for the therapy of NSCLC.

MicroRNAs (miRNAs) are part of a class of 21 ~ 25 nucleotides of noncoding RNAs that are known to post-transcriptionally regulate gene expression by binding to their 3'-untranslated region (3'UTR).^[4] Previous studies have reported that miRNAs are involved in cell proliferation, growth, apoptosis, and differentiation.^[5] MiRNAs act as oncogenes and tumor suppressors by regulating the expression of key molecular cellular proliferation, growth, apoptosis, and mobility.^[6] Emerging evidence indicates that miRNAs may play a critical role in cancer and could serve as biomarkers, depending on the tumor type.^[7]

miR-335 has been demonstrated to play the antitumor role in several cancer types, including colorectal cancer, gastric cancer, bladder cancer, thyroid cancer, osteosarcoma, clear cell renal cell carcinoma (ccRCC), hepatocellular carcinoma (HCC), and breast cancer.^[8–15] Previous study found that miR-335 expression was reduced in NSCLC tissues relative to noncancerous adjacent tissues. Furthermore, downregulation of miR-335 in A459 lung cancer cells promoted cell proliferation through

Editor: Sayed S. Daoud.

The authors report no conflicts of interest.

WH and MZ contributed equally to this study.

^aDepartment of Respiratory and Critical Care Medicine, ^bDepartment of Anesthesiology, ^cPain Department, The First People's Hospital of Lianyungang, The First Affiliated Hospital of Kangda College of Nanjing Medical University, Jiangsu, P. R. China.

*Correspondence: Haitao Fei, No. 182, Tongguan North Road, Haizhou District, Lianyungang, Jiangsu 222002, P.R. China (e-mail: feihaitaomed@sina.com).

Copyright © 2020 the Author(s). Published by Wolters Kluwer Health, Inc. This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial License 4.0 (CCBY-NC), where it is permissible to download, share, remix, transform, and buildup the work provided it is properly cited. The work cannot be used commercially without permission from the journal.

How to cite this article: Huo W, Zhang M, Li C, Wang X, Zhang X, Yang X, Fei H. Correlation of microRNA-335 expression level with clinical significance and prognosis in NSCLC. *Medicine* 2020;99:34(e21369).

Received: 19 May 2019 / Received in final form: 9 December 2019 / Accepted: 18 June 2020

<http://dx.doi.org/10.1097/MD.0000000000021369>

upregulation of Tra2 β , mediated via activation of the AKT/mTOR signaling pathway, suggesting that miR-335 might have potential as a novel therapeutic target for NSCLC.^[16] In the present study, we aimed to investigate the clinical significance and prognostic value of miR-335 in NSCLC.

2. Materials and methods

2.1. Clinical tissue samples

This research was approved by the department of Ethics Committee of Lianyungang First People's Hospital. And all patients had written informed consent. The lung cancer tissues and adjacent nontumor lung tissues were obtained from 131 patients who underwent the primary surgical resection at Lianyungang First People's Hospital. None of the patients with NSCLC received treatment (chemotherapy, radiotherapy, or target therapy) before surgery. Tissues were immediately frozen in liquid nitrogen after resection and stored at -80°C . The pathological diagnosis for these tissues was conducted by 2 independent pathologists. The clinical and pathological data of all patients with lung cancer are presented in Table 1.

2.2. Quantitative real-time PCR analysis

We used Trizol (Invitrogen) reagent to isolate total RNA from tissues according to the manufacturer's protocol. First-strand cDNA was synthesized by reverse transcription of 500 ng of total RNA according to the manufacturers' protocol. Quantitative polymerase chain reaction (PCR) was synthesized according to the manufacturer's protocol at 95°C for 30 seconds, 95°C for 5 seconds, 60°C for 34 seconds, 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds, for 40 cycles. U6 was used for

normalization of qRT-PCR data. All the experiments were performed 3 times independently. Data were analyzed using the comparative Ct method ($2^{-\Delta\Delta\text{Ct}}$).

2.3. Statistical analysis

Student *t* test was used to distinguish differences between groups. χ^2 test was involved for analysis of clinicopathological data. The overall survival was analyzed by the Kaplan-Meier method and the log rank test. Multiple Cox proportional hazards regression analysis was carried out to identify the independent factors that had a significant impact on patient survival. In this study, the data were analyzed using SPSS 18.0 (SPSS Inc., Chicago, IL) and GraphPad Prism (version 5.01; GraphPad Software, Inc, La Jolla, CA) statistical software. $P < .05$ was regarded as significant.

3. Results

3.1. The tissue expression level of miR-335 in NSCLC

We assessed miR-335 expression level in 131 NSCLC samples and paired noncancerous lung samples. The expression of miR-335 was significantly lower in NSCLC samples compared to noncancerous samples ($P < .001$, shown in Fig. 1). We then divided 131 NSCLC patients into 2 groups according to miR-335 expression level. The cutoff point was the median expression level of miR-335 in NSCLC samples (low miR-335 expression group, $n = 66$; high miR-335 expression group, $n = 65$).

3.2. Correlation of miR-335 expression with clinicopathological characteristics of NSCLC

The associations of miR-335 expression with various clinicopathological parameters of NSCLC are summarized in Table 1. The median expression level of miR-335 was used to classify the patients with NSCLC into two groups. The expression level of miR-335 was significantly correlated with tumor histology ($P = .028$), lymph node metastasis ($P = 0.002$), differentiation degree ($P < .001$), and pathological TNM stage ($P < .001$).

Table 1
Correlation between miR-335 expression and clinical features in patients with NSCLC.

Variables	Cases (n)	miR-335 expression		P
		Low (n=66)	High (n=65)	
Sex				
Male	62	35	27	.222
Female	69	31	38	
Age, y				
<60	60	27	33	.295
≥ 60	71	39	32	
Smoking history				
Yes	58	32	26	.381
No	73	34	39	
Tumor size, cm				
<5	77	37	40	.596
≥ 5	54	29	25	
Histology				
Adenocarcinoma	98	55	43	.028
Squamous cell carcinoma	33	11	22	
Lymph node metastasis				
Present	52	35	17	.002
Absent	79	31	48	
Differentiation degree				
High-moderate	81	25	56	<.001
Low	50	41	9	
Pathological TNM stage				
I/II	89	33	56	<.001
III	42	33	9	

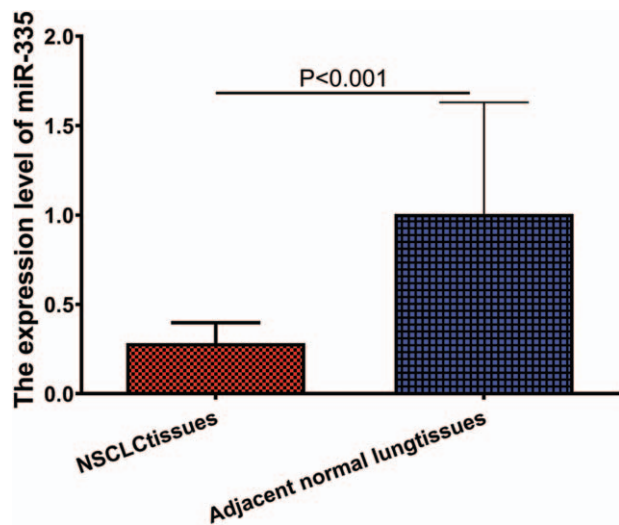


Figure 1. The expression of miR-335 was significantly lower in NSCLC samples compared to noncancerous samples ($P < 0.001$). NSCLC = non-small cell lung cancer.

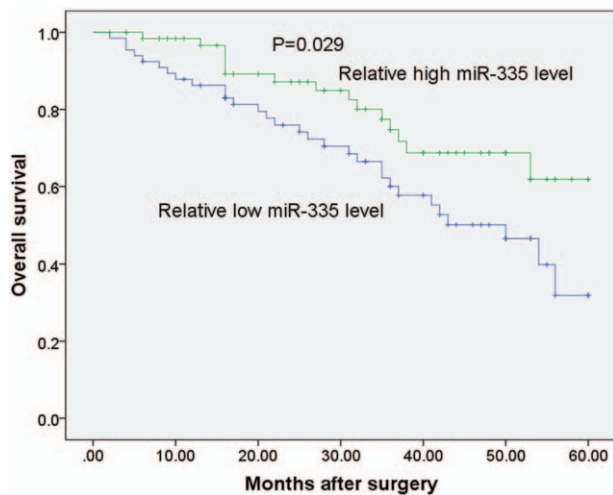


Figure 2. Kaplan-Meier survival plots and log-rank analyses indicated that patients with low miR-335 expression level experienced poor overall survival in non-small cell lung cancer ($P = .029$).

However, no significant correlation was observed between miR-335 expression and other clinicopathologic characteristics of NSCLC, including age, sex, smoking history, and tumor size (all $P > 0.05$).

3.3. Decreased miR-335 expression predicts the favorable prognosis of NSCLC

Using Kaplan-Meier survival plots and log-rank analyses, we evaluated the association of miR-335 expression with overall survival of NSCLC patients. The log-rank test indicated that patients with decreased miR-335 expression experienced poor overall survival ($P = .029$, shown in Fig. 2). To determine the possibility of miR-335 as an independent risk factor for poor prognosis, both clinicopathological factors and the level of miR-335 expression were evaluated by multivariate Cox regression analysis. Results showed that the tumor histology ($P = .038$), lymph node metastasis ($P = .011$), differentiation degree ($P = 0.009$), pathological TNM stage ($P = .007$), and miR-335 expression level ($P = .019$) were independent factors in predicting the overall survival of NSCLC patients (shown in Table 2).

Table 2			
Multivariate analysis of overall survival in patients with NSCLC.			
Variable	Hazard ratio	95% CI	P
Gender	0.728	0.283–1.829	.812
Age	1.372	0.784–2.937	.381
Smoking history	1.738	0.927–3.657	.185
Tumor size	2.017	0.827–4.516	.091
Histology	2.839	1.829–7.283	.038
Lymph node metastasis	2.819	1.738–10.923	.011
Differentiation degree	3.271	2.037–11.937	.009
Pathological TNM stage	3.293	2.263–14.294	.007
miR-335 expression level	2.937	1.355–8.537	.019

CI = confidence interval, NSCLC = non-small cell lung cancer.

4. Discussion

Previous study found that miR-335 expression was reduced in NSCLC tissues relative to noncancerous adjacent tissues. Furthermore, downregulation of miR-335 in A459 lung cancer cells promoted cell proliferation through upregulation of Tra2 β , mediated via activation of the AKT/mTOR signaling pathway, suggesting that miR-335 might have potential as a novel therapeutic target for NSCLC.^[16] However, the clinical significance and prognostic value of miR-335 in NSCLC have not been investigated. In the present study, we aimed to investigate the clinical significance and prognostic value of miR-335 in NSCLC. We assessed miR-335 expression level in 131 NSCLC samples and paired noncancerous lung samples. The expression of miR-335 was significantly lower in NSCLC samples compared to noncancerous samples. We then divided 131 NSCLC patients into 2 groups according to miR-335 expression level. The associations of miR-335 expression with various clinicopathological parameters of NSCLC are investigated. The expression level of miR-335 was significantly correlated with tumor histology, lymph node metastasis, differentiation degree, and pathological TNM stage. Using Kaplan-Meier survival plots and log-rank analyses, we evaluated the association of miR-335 expression with overall survival of NSCLC patients. The log-rank test indicated that patients with decreased miR-335 expression experienced poor overall survival. To determine the possibility of miR-335 as an independent risk factor for poor prognosis, both clinicopathological factors and the level of miR-335 expression were evaluated by multivariate Cox regression analysis. Results showed that the tumor histology, lymph node metastasis, differentiation degree, pathological TNM stage, and miR-335 expression level were independent factors in predicting the overall survival of NSCLC patients.

The prognostic value of miR-335 has also been investigated in other cancer types. For example, Wang et al found that cervical cancer patients with reduced tissue miR-335 level had shorter survival time, compared with those with high levels of miR-335 expression ($P = .011$, log-rank = 6.458). Through Cox regression, they found that miR-335 expression was associated with the survival of cervical cancer (RR = 0.251, 95% CI 0.095–0.663, $P = .005$), suggesting that lower tissue miR-335 expression resulted in poorer survival in patients with cervical cancer.^[17] Sandoval-Bórquez et al found that reduced miR-335 expression level was significantly associated with depth of invasion, lymph node metastasis, and poor prognosis in gastric cancer.^[11] Not only the expression of miR-335 in cancer tissues has prognostic value, but also the expression of miR-335 in serum has prognostic value. For example, Cui et al found that HCC patients had significantly lower miR-335 levels than hepatitis patients and healthy controls. Lower serum miR-335 levels were closely associated with more progressive clinical features, including a higher mean serum AFP level, more vascular invasion, cirrhosis, and larger tumor size. Response rates to transarterial chemoembolization were higher in patients with high miR-335 compared to those with low miR-335 level. Patients with lower serum miR-335 levels had significantly poorer prognosis than patients with higher serum miR-335 levels.^[18]

The researchers also investigated the mechanism and regulatory network of miR-335 in other cancers. For example, Dong et al found that miR-335 was downregulated in breast cancer tissues, and miR-335 overexpression suppressed migration and invasion in MCF-7 and MDA-MB-231 breast cancer cells.

Furthermore, EphA4 was a direct target gene of miR-335 and miR-335 suppressed breast cancer cell proliferation and motility in part by directly inhibiting EphA4 expression.^[12] Wang et al found that miR-335 inhibited colorectal cancer cells growth, migration, invasion, and epithelial-mesenchymal transition process. And miR-335 exhibited tumor suppressive effects possibly by inhibition of Twsit1 and thus inactivating NF- κ B and Wnt/ β -catenin pathways.^[10] Luo et al found that the expression level of miR-335 was downregulated in thyroid cancer. miR-335 was able to inhibit invasion and metastasis of thyroid cancer cells by targeting intercellular adhesion molecule 1.^[9] Liu et al found that miR-335 suppressed cell proliferation and migration by upregulating CT10 regulator of kinase-like protein (CRKL) in bladder cancer.^[8] Guo X et al found that the expression of miR-335 in osteosarcoma stem cells was lower than their differentiated counterparts. Furthermore, miR-335 negatively regulated osteosarcoma stem cell-like properties by targeting POU5F1, and miR-335 could target CSCs to synergize with traditional chemotherapeutic agents to overcome osteosarcoma.^[13] Wang K et al showed that the expression level of miR-335 was significantly downregulated in ccRCC tissues versus corresponding non-tumor tissues and the low expression of miR-335 was significantly associated with lymph node metastasis, larger tumor size, and poor T stage. Furthermore, miR-335 inhibited the proliferation and invasion of ccRCC cells through direct suppression of BCL-W.^[14] There are some limitation of the present study. First, we have not investigated the expression level of miR-335 in the serum of NSCLC patients, and its prognostic value. Second, we have not investigated the specific mechanism of miR-335 in NSCLC. In particular, the differences of its mechanism in different types of NSCLC.

In conclusion, the results of the present study indicated that miR-335 was down-expressed in NSCLC, and is associated with tumor progression and poor prognosis, suggesting that the expression of miR-335 might be an independent prognostic factor of overall survival in patients with NSCLC.

Author contributions

Data curation: Wen Huo, Man Zhang.

Formal analysis: Wen Huo.

Investigation: Haitao Fei, Wen Huo, Man Zhang, Chunhua Li, Xinying Wang, Xiuli Zhang, Xiaona Yang.

Methodology: Wen Huo, Man Zhang, Chunhua Li, Xinying Wang, Xiaona Yang.

Project administration: Man Zhang.

Software: Man Zhang, Chunhua Li.

Writing – original draft: Wen Huo, Man Zhang, Chunhua Li, Xinying Wang, Xiuli Zhang.

Writing – review & editing: Haitao Fei, Wen Huo.

References

- [1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA: a cancer journal for clinicians* 2019;691:7–34.
- [2] Torre LA, Siegel RL, Jemal A. Lung Cancer Statistics. *Advances in experimental medicine and biology* 2016;893:1–9.
- [3] Genestreti G, Di Battista M, Cavallo G, Bartolotti M, Brandes AA. Maintenance therapy in non-small cell lung cancer. *Expert review of anticancer therapy* 2015;157:839–46.
- [4] Kim YK, Kim VN. Processing of intronic microRNAs. *The EMBO journal* 2007;263:775–83.
- [5] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;1162:281–97.
- [6] Iorio MV, Croce CM. microRNA involvement in human cancer. *Carcinogenesis* 2012;336:1126–33.
- [7] Castro D, Moreira M, Gouveia AM, Pozza DH, De Mello RA. MicroRNAs in lung cancer. *Oncotarget* 2017;846:81679–85.
- [8] Liu XK, Chen D, Li X. MiR-335 suppresses cell proliferation and migration by upregulating CRKL in bladder cancer. *European review for medical and pharmacological sciences* 2019;236:2399–408.
- [9] Luo L, Xia L, Zha B, Zuo C, Deng D, Chen M, et al. miR-335-5p targeting ICAM-1 inhibits invasion and metastasis of thyroid cancer cells. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2018;106:983–90.
- [10] Wang J, Wang X, Liu F, Fu Y. microRNA-335 inhibits colorectal cancer HCT116 cells growth and epithelial-mesenchymal transition (EMT) process by targeting Twist1. *Die Pharmazie* 2017;728:475–81.
- [11] Sandoval-Borquez A, Polakovicova I, Carrasco-Veliz N, Lobos-Gonzalez L, Riquelme I, Carrasco-Avino G, et al. MicroRNA-335-5p is a potential suppressor of metastasis and invasion in gastric cancer. *Clinical epigenetics* 2017;9:114.
- [12] Dong Y, Liu Y, Jiang A, Li R, Yin M, Wang Y. MicroRNA-335 suppresses the proliferation, migration, and invasion of breast cancer cells by targeting EphA4. *Molecular and cellular biochemistry* 2018;439(1–2):95–104.
- [13] Guo X, Yu L, Zhang Z, Dai G, Gao T, Guo W. miR-335 negatively regulates osteosarcoma stem cell-like properties by targeting POU5F1. *Cancer cell international* 2017;17:29.
- [14] Wang K, Chen X, Zhan Y, Jiang W, Liu X, Wang X, et al. miR-335 inhibits the proliferation and invasion of clear cell renal cell carcinoma cells through direct suppression of BCL-W. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine* 2015;369:6875–82.
- [15] Liu H, Li W, Chen C, Pei Y, Long X. MiR-335 acts as a potential tumor suppressor miRNA via downregulating ROCK1 expression in hepatocellular carcinoma. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine* 2015;368:6313–9.
- [16] Liu J, Bian T, Feng J, Qian L, Zhang J, Jiang D, et al. miR-335 inhibited cell proliferation of lung cancer cells by target Tra2beta. *Cancer science* 2018;1092:289–96.
- [17] Wang C, Jiang T. MicroRNA-335 represents an independent prognostic marker in cervical cancer. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine* 2015;368:5825–30.
- [18] Cui L, Hu Y, Bai B, Zhang S. Serum miR-335 Level is Associated with the Treatment Response to Trans-Arterial Chemoembolization and Prognosis in Patients with Hepatocellular Carcinoma. *Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology* 2015;371:276–83.