

# Correlative analysis of miR-34b and p53 with pathological characteristics of NSCLC

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**Abstract.** The expression of miR-34b and p53 in non-small cell lung cancer (NSCLC) was investigated to explore its relationship with clinical pathology of NSCLC. Reverse transcription-quantitative PCR (RT-qPCR) method was used to quantitatively analyze miR-34b and p53 in cancer tissue and adjacent paraneoplastic (PTLC) tissue in 54 cases of NSCLC. The relationship between gene expression and clinical pathological data was analyzed. The expression of miR-34b in tumor tissues of NSCLC patients was significantly downregulated in comparison with PTLC. The expression level of miR-34b was negatively correlated with lymph node metastasis. It was positively correlated with the degree of differentiation and negatively correlated with the pathological stage ( $P<0.05$ ). There was no significant association in the expression of miR-34b with age, sex, histological type, and gross classification (all  $P>0.05$ ). The expression of p53 in the tumor tissue of NSCLC patients was significantly reduced in comparison with PTLC, and its expression was negatively correlated with the pathological stage, lymph node metastasis, and was positively correlated with the degree of differentiation. The expression of p53 in adenocarcinoma was generally higher than that of squamous cell carcinoma and large cell carcinoma. The expression of p53 in central type cancer was significantly higher than that in peripheral type ( $P<0.05$ ). The expression of miR-34b and p53 was positively correlated in NSCLC tissues ( $r=0.797$ ,  $P<0.001$ ). The high expression of miR-34b and p53 is closely related to the clinical stage and pathological grade of NSCLC. miR-34b and p53 may serve as important tumor markers for NSCLC.

## Introduction

Lung cancer is one of the most common malignancies in the world. Its morbidity and mortality have increased year by year. More than 80% of patients have non-small cell lung cancer (NSCLC) (1). Most diagnoses are made at an advanced stage. More than 30% of NSCLC patients diagnosed as stage I have relapse after treatment, the prognosis of lung cancer is extremely poor. The five-year survival rate does not exceed 10% (2). The progress of science and technology, and lung cancer detection and diagnostic technology have made a great progress, but the early detection rate of lung cancer is still low (3). Molecular gene detection and targeted therapies are new treatments/detections for a specific gene or expression product, with excellent specificity and sensitivity.

miRNAs are small-molecule single-stranded RNAs produced by the cleavage of Pri-miRNAs and Pre-miRNAs in the body, consisting of about 19-25 bp nucleotides; many genes have a non-translated sequence downstream of the coding region called 3'-UTR, miRNAs can be post-transcriptionally regulated by base complementary binding to the target gene mRNA (4). The miR-34 family contains three miRNAs: miR-34a, miR-34b and miR-34c. miR-34b and miR-34c are derived from the same gene, and miR-34a is transcribed from the transcript of another gene (5). Based on mouse experiments, the miR-34 family has a certain tissue specificity. The expression of miR-34a in brain tissue is much higher than that in other tissues, and miR-34b/c is mainly found in lung tissue (6). At present, the molecular pathogenesis of NSCLC has not been studied clearly (7).

p53 is an important anti-oncogene, and wild-type causes apoptosis of cancer cells, thus preventing carcinogenesis. It also has the function of helping gene repair defects, and mutations increase the chance of carcinogenesis (8). Targeted regulatory networks with p53 as the radiation center can inhibit the formation of tumors, and the miR-34 family is one of its downstream target genes that are directly regulated (4). Therefore, the quantification of miR-34b and p53 in 54 NSCLC tissues and normal lung tissues was analyzed by reverse transcription-quantitative PCR (RT-qPCR) method and their correlation with clinicopathological data was analyzed in order to provide new insights into the gene targets or tumor markers of NSCLC.

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Table I. Reaction system.

Reverse transcription	Amount	PCR	Amount
5X Reverse transcription primers	3 $\mu$ l	SYBR-Green	10 $\mu$ l
100 mmol/l dNTP	0.15 $\mu$ l	Upstream primers	0.4 $\mu$ l
Reverse transcriptase	1 $\mu$ l	Downstream primers	0.4 $\mu$ l
Buffer	1.5 $\mu$ l	cDNA	2 $\mu$ l
RNA inhibitor	0.19 $\mu$ l		
Total RNA	1 g		
Add RNase free H <sub>2</sub> O to	15 $\mu$ l	Add RNase free H <sub>2</sub> O to	20 $\mu$ l

## Patients and methods

**Research subjects.** The specimens and clinical data of 54 patients with pathologically diagnosed NSCLC surgically resected in Xiangyang Center Hospital, Hubei University of Arts and Science (Xiangyang, China) from February 2017 to February 2018 were collected. NSCLC cancer tissue and adjacent paraneoplastic (PTLC) tissue specimens were obtained from each patient. The patients' age was  $59.24 \pm 16.84$  years, including 26 females and 28 males. Lymph node dissection was performed in all patients.

This study was approved by the Medical Ethics Committee of Xiangyang Center Hospital, Hubei University of Arts and Science. Signed informed consents were obtained from the patients or guardians.

**Inclusion and exclusion criteria.** Inclusion criteria were: All the patients underwent pathological examination and diagnosis; in line with IASLC 2009 International Association for Lung Cancer Research IASLC 2009 version of the NSCLC diagnosis and staging criteria (9); in line with the WHO NSCLC classification criteria (10); age >18 years; patients are from the first visit to the Xiangyang Center Hospital Affiliated to Hubei University of Arts and Science for initial treatment of NSCLC; patients who signed an informed consent.

Exclusion criteria were: Patients who received preoperative radiotherapy and chemotherapy and targeted gene therapy; patients with brain or other organ metastases found before surgery; patients with incomplete or missing clinical data; incomplete or missing follow-up data.

**Material and reagents.** TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); All-in-One First-Strand cDNA Synthesis kit (Guangzhou Qiyun Biotechnology Co., Ltd., Guangzhou, China); PowerUp SYBR® Green Master Mix (Thermo Fisher Scientific China Co., Ltd., Beijing, China); Fluorescent Quantitation PCR instrument ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.); Primer sequences (Shanghai Sangon Biological Co., Ltd., Shanghai, China).

**RT-qPCR detection of miR-34b and p53.** TRIzol extraction was used to extract total RNA from NSCLC lung cancer tissues and adjacent PTLC tissues. A UV spectrophotometer (Bio Rad, Hercules, CA, USA) was used to identify the purity and concentration of the RNA and the purity reached the

standard when A260/A280 was between 1.8 and 2.0. cDNA was synthesized strictly in accordance with the instructions of the reverse transcription kit. After preparing the reaction system according to Table I, p53 was reverse transcribed at a temperature of 50°C for 30 min and at a temperature of 94°C for 2 min. miR-34b was reverse transcribed at 95°C for 3 min, 60°C for 39 sec, and 72°C for 9 min.

The synthesized cDNA was amplified according to the PCR kit and amplified on a fluorescence quantitative PCR machine. The predenaturation temperature was set to 95°C for 1 min. In 40 cycles, the denaturation was set to 95°C for 15 sec, annealing was 60°C for 20 sec, and the elongation was 70°C for 15 sec. Using U6 as the internal reference, the upstream primer sequence was 5'-CGCTTCGGCAGCACATATAC-3', the downstream primer sequence was 5'-TTCACGAATTTCGTGTCAT-3', the upstream primer sequence of p53 was 5'-TGAAGCCCTCCGAGTGTC-3', and the downstream primer sequence was 5'-TCGGGTGGCTCATAAGGT-3'. The upstream primer sequence of miR-34b was 5'-AGGCAGTGTCTATTAGCTGATTGT-3', and the downstream primer sequence was 5'-ACAATCAGCTAATGACACTGCCT-3'. The relative expression levels of miR-34b and p53 were expressed by the  $2^{-\Delta\Delta C_q}$  method (11).

**Statistical analysis.** Analysis and processing was performed in SPSS19.0 (Asia Analytics Formerly SPSS China) software system. Spearman's correlation analysis was used to analyze the correlation between the expression levels of p53 and miR-34b. The relative expression levels obtained by RT-qPCR were expressed as mean  $\pm$  standard deviation (mean  $\pm$  SD), and t-test was used for the comparison between the two groups. Analysis of variance was used to compare multiple groups and the post hoc test was Dunnett's test. Partial correlation analysis was used for the correlation between the expression of miR-34b and p53.  $P < 0.01$  was considered to indicate a statistically significant difference.

## Results

**Expression of miR-34b in NSCLC and PTLC.** The relative expression of miR-34b in NSCLC was  $(5.70 \pm 3.77)$ , which was significantly lower than that of the adjacent PTLC  $(8.89 \pm 3.93)$ . Compared with PTLC, 46 cases of miR-34bC in 54 patients were downregulated in NSCLC, with a rate of 85.19%. The mean downregulation of NSCLC was  $57.24 \pm 6.81\%$  compared with PTLC, as shown in Fig. 1.

Table II. Relationship between miR-34b expression and clinical characteristics in NSCLC.

Clinical characteristics	n	mir-34b expression level	t/F-value	P-value
Age			0.84	0.21
>65 years	30	5.35±2.52		
≤65 years	24	5.19±3.21		
Sex			0.62	0.54
Male	35	5.73±2.21		
Female	19	5.24±2.71		
Pathological stage			6.87	<0.001
I+II	26	8.76±3.19		
III+VI	28	3.14±2.86		
Histological type			0.03	0.99
Adenocarcinoma	24	5.92±3.17		
Squamous cell carcinoma	20	5.24±4.14		
Large cell carcinoma	10	5.01±3.52		
Differentiation			4.33	<0.001
Poor differentiation	28	3.15±3.25		
High-medium differentiation	26	7.92±3.15		
Lymph node metastasis			3.56	<0.001
Yes	26	3.46±2.72		
No	28	6.32±3.97		
Gross classification			0.93	0.08
Central type	28	5.13±4.25		
Peripheral type	26	5.22±3.76		

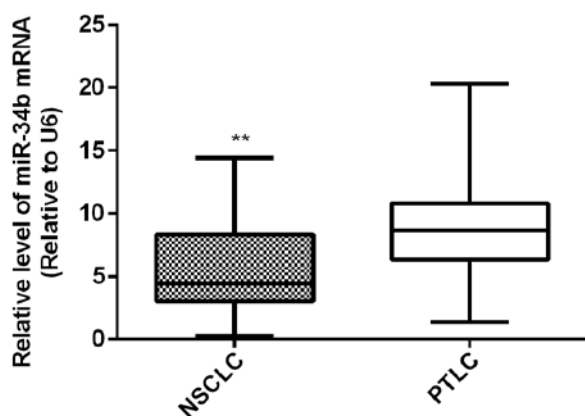


Figure 1. The expression of miR-34b in NSCLC and PTLC. The relative expression of miR-34b in NSCLC was (5.70±3.77), which was significantly lower than that of the adjacent PTLC (8.89±3.93). \*\*P<0.01 compared with the adjacent PTLC.

**Relationship between miR-34b expression and clinical characteristics in NSCLC.** t-test analysis was performed on the results of miR-34b expression in relation with age, sex, pathological stage, differentiation, lymph node metastasis, and gross classification. Analysis of variance was performed on the results of miR-34b expression in relation with histology type. It was revealed that the expression of miR-34b was significantly associated with pathological stage, differentiation, and lymph node metastasis (P<0.01), but there was no association with age, sex, histological type and gross classification (P>0.05) (Table II).

The Spearman's correlation analysis between miR-34b and pathological stage, differentiation and lymph node metastasis revealed that the expression of miR-34b was negatively correlated with lymph node metastasis (r=-0.64, P<0.01) and positively correlated with the degree of differentiation (r=0.27, P<0.01). There was a negative correlation with pathological stage (r=-0.32, P<0.01) (Fig. 2).

**Expression of p53 in NSCLC and PTLC.** The relative expression of p53 in NSCLC was (1.82±0.90) and in PTLC was (4.07±2.59). The relative expression of p53 in NSCLC was significantly lower than that of PTLC. Compared with PTLC, there were 48 cases out of 54 showing upregulated p53, and the rate of increase was 88.89%. Compared with PTLC, the average increase of NSCLC was 64.82±15.72%, as shown in Fig. 3.

**Relationship between p53 expression and clinical characteristics in NSCLC.** t-test was used to analyze the expression of p53 in relation with age, sex, pathological stage, differentiation, lymph node metastasis and gross classification. The histological type was analyzed by analysis of variance. The expression of p53 was associated with pathological stage, histological type, degree of differentiation, lymph node metastasis, gross classification (P<0.05), and not associated with age and sex (P>0.05). The expression of p53 in adenocarcinoma was generally higher than that of squamous cell and large cell carcinoma, and its expression was higher in squamous cell carcinoma than in large cell carcinoma (P<0.01).

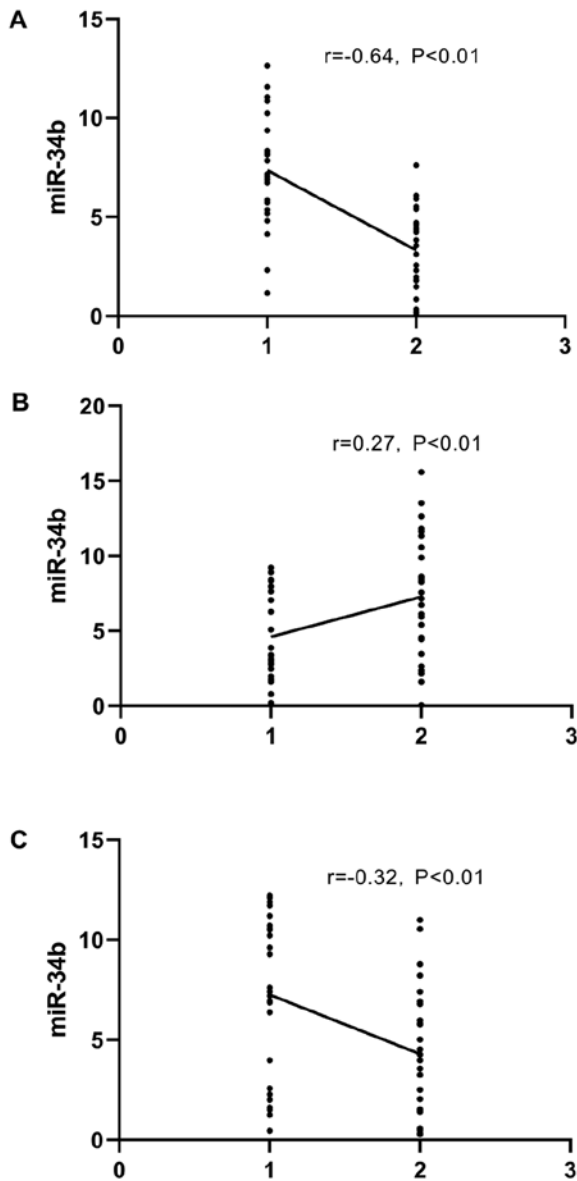


Figure 2. Correlation analysis of miR-34b with lymph node metastasis, differentiation and pathological stage. (A) The expression of miR-34b was negatively correlated with lymph node metastasis ( $r=-0.64, P<0.01$ ), 1=no metastasis, 2=metastasis. (B) miR-34b expression was positively correlated with the degree of differentiation ( $r=0.27, P<0.01$ ), 1=low differentiation, 2=medium-high differentiation. (C) miR-34b expression was negatively correlated with pathological stage ( $r=-0.32, P<0.01$ ), 1=I+II, 2=III+IV.

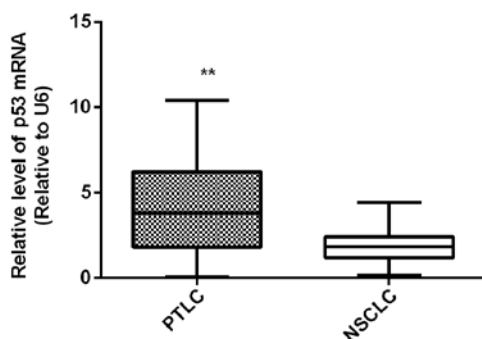


Figure 3. The expression of p53 in NSCLC and PTLC. The relative expression of p53 in NSCLC was  $(1.82\pm0.90)$  and in PTLC was  $(4.07\pm2.59)$ . The relative expression of p53 in NSCLC was significantly lower than that of PTLC. \*\* $P<0.05$  compared with the adjacent PTLC.

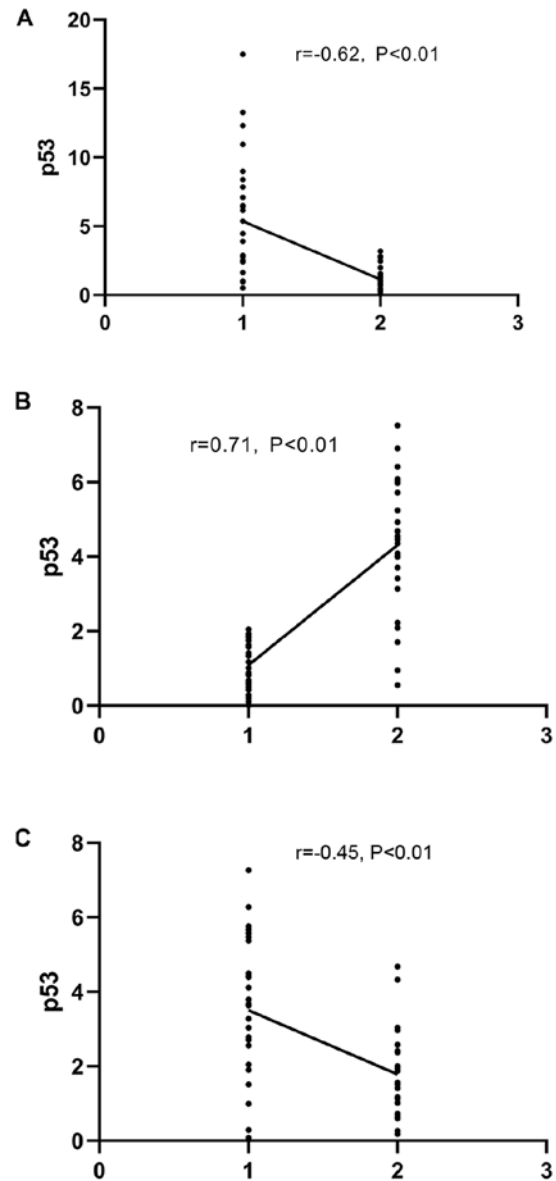


Figure 4. Correlation analysis of p53 with lymph node metastasis, differentiation and pathological stage. (A) The expression of p53 was negatively correlated with pathological stage ( $r=-0.62, P<0.01$ ), 1=II+IV, 2=I+II. (B) p53 expression was positively correlated with the degree of differentiation ( $r=0.71, P<0.01$ ), 1=medium-high differentiation, 2=low differentiation. (C) p53 was negatively correlated with lymph node metastasis ( $r=-0.45, P<0.01$ ), 1=metastasis, 2=no metastasis.

Compared with the peripheral type, the expression of p53 in central type was significantly upregulated ( $P<0.01$ ) (Table III).

The Spearman's correlation analysis between p53 and pathological stage, differentiation degree and lymph node metastasis revealed that the expression of p53 was negatively correlated with the pathological stage ( $r=-0.62, P<0.01$ ), was positively correlated with the degree of differentiation ( $r=0.71, P<0.01$ ), and negatively correlated with lymph node metastasis ( $r=-0.45, P<0.01$ ) (Fig. 4).

**Correlation analysis of miR-34b and p53 in NSCLC tissues.** Partial correlation analysis showed that the expression of miR-34b and p53 was positively correlated in NSCLC tissues ( $r=0.797, P<0.001$ ; Fig. 5).

Table III. Relationship between p53 expression and clinical characteristics in NSCLC.

Clinical characteristics	n	p53 expression level	t/F-value	P-value
Age			0.74	0.46
>65 years	30	1.86±0.81		
≤65 years	24	1.77±1.03		
Sex			0.44	0.66
Male	35	1.83±0.82		
Female	19	1.85±0.98		
Pathological stage			9.75	<0.001
I+II	26	1.14±1.02		
III+VI	28	3.86±5.93		
Histological type			30.76	<0.001
Adenocarcinoma	22	3.04±1.19		
Squamous cell carcinoma	18	2.12±0.87		
Large cell carcinoma	9	1.76±0.57		
Differentiation			7.30	<0.001
Poor differentiation	28	4.32±2.12		
High-medium differentiation	26	1.14±0.76		
Lymph node metastasis			5.10	<0.001
Yes	26	3.46±1.87		
No	28	1.45±0.97		
Gross classification			8.41	<0.001
Central type	28	3.87±1.25		
Peripheral type	26	1.22±0.57		

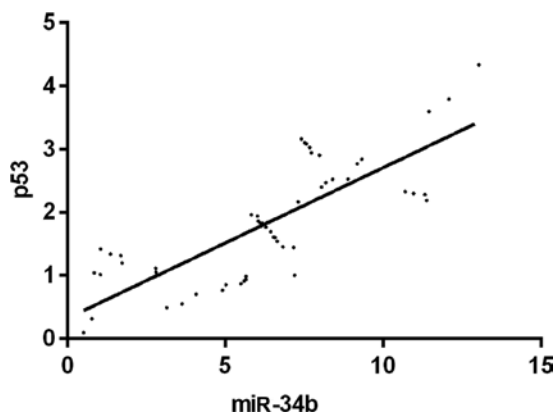


Figure 5. Correlation of miR-34b and p53 in NSCLC organization. Partial correlation analysis showed that the expression levels of miR-34b and p53 were positively correlated in NSCLC tissues ( $r=0.797$ ,  $P<0.001$ ).

## Discussion

With the decline of air quality and changes in people's living habits, lung cancer is consistently high, with early metastasis and rapid proliferation (12), and 80% of lung cancers are NSCLC (13). p53 is one of the most important tumor suppressor genes involved in many signaling pathways such as TRIM29-p53-Wnt, and its mutation can lead to the production of many malignant tumors including lung cancer (14). miRNAs are short-chain non-coding RNAs naturally occurring in the human body and play an important role in tumors. Studies

have shown a general decline in the miR-34b levels detected in a variety of cancers (15,16), suggesting that miR-34b may play a role in endogenous tumor suppression (17).

The expression of miR-34b in NSCLC was significantly downregulated compared with PTLC, and the expression of miR-34b was significantly correlated with lymph node metastasis, pathological stage, and differentiation. The expression level of miR-34b was negatively correlated with lymph node metastasis, positively correlated with the degree of differentiation, and negatively correlated with the pathological stage. There was no significant association between the miR-34b expression and patients' sex, age, histological type, and gross classification. Hu *et al* (18) also found that miR-34b is downregulated in NSCLC and has a good sensitivity and specificity for diagnosing NSCLC. Halvorsen *et al* (19) found that the expression of miR-34b in serum of NSCLC patients was high compared with that of normal individuals. According to the present investigation and other literature, it was found that miR-34b was downregulated in cancer tissues (20). The specific reasons are worth further exploring. Balça-Silva *et al* (20) found that the miR-34b expression was downregulated in cancer tissues, and the expression level was significantly negatively correlated with lymph node metastasis, which is consistent with our results. However, this study considers miR-34b is not correlated with a pathological type and degree of cell differentiation. This may be due to the individual differences and diversity as well as the size of the sample, so it may need a larger number of samples for further

study. The relative expression of p53 in NSCLC was significantly lower than that of PTLC. The expression of p53 was negatively correlated with the pathological stage and lymph node metastasis, and positively correlated with the degree of differentiation. In routine pathological work, the pathological histology of NSCLC is generally judged by the results of HE staining. This morphological method is not easy to judge when poorly differentiated, and the accuracy rate is not high, because the structure is not clear or typical due to poor differentiation. It is difficult to identify squamous cell carcinoma or adenocarcinoma. In particular, when the size of the specimen is small and the area is not large enough, the difficulty in identification is increased, such as specimens obtained through fine needle or bronchoscopic puncture (21). In this study, the expression of p53 in adenocarcinoma was generally higher than that in squamous cell carcinoma and large cell carcinoma. The expression of p53 in squamous cell carcinoma was higher than that in large cell carcinoma. Compared with the peripheral type, the expression of p53 was significantly upregulated in the central type. Therefore, the expression level of p53 can be used to assist the histological typing of NSCLC and improve the accuracy. Cortez *et al* (22) studied the expression regulation mechanism of PD1/PDL1 in NSCLC and found that the control axis of immune escape in NSCLC was: p53/miR-34/PDL1: p53 can regulate the expression of miR-34a, and the activated miR-34a can regulate PD-L1, therefore inhibiting immune escape.

However, no immunohistochemistry was performed in this study. RT-qPCR results should be compared with the results of immunohistochemistry. The two results should be mutually validated, making the results more credible. Balça-Silva *et al* (20) found that miR-34b is not only a p53 effector, but the overexpression of miR-34b can also increase the radiosensitivity to low-dose irradiation. The downregulation of miR-34b in NSCLC makes this sensitivity also be reduced, so theoretically NSCLC requires a higher dose of radiation therapy, in the latter test, the correlation of radiation dose and the miR-34b expression can be analyzed to verify this conjecture. Wang *et al* (23) found that methylation can limit the expression of miR-34b. The methylation status of miR-34b is closely related to the prognosis and recurrence of NSCLC. Therefore, in a follow-up study, based on the test of the miR-34b expression level, it should also be analyzed whether the methylation level is related to clinical stage and pathological grade.

In summary, the expression of miR-34b and p53 is closely related to the clinical stage and pathological grade of NSCLC. miR-34b and p53 may serve as tumor markers of NSCLC.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

SY and HZ collected and analyzed the general information of patients. SY, HZ and QC were responsible for PCR. SY and QC were involved in the drafting of the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The study was approved by the Ethics Committee of Xiangyang Center Hospital, Hubei University of Arts and Science (Xiangyang, China). Signed informed consents were obtained from the patients or guardians.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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