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The Expression Levels of Mir-33b-5p and FGFRL1 in Non-Small Cell Lung Cancer Tissues and Their Relationship with Clinical Pathological Features and Prognosis

Chen Wang[1,a,*], Huang Li[2,b,*]

[1]Yueyang Municipal People's Hospital, Yueyang, China
[2]Yueyang Hospital of Traditional Chinese Medicine, Yueyang, China
[a]1987504541@qq.com, [b]llhhlay1007@vip.163.com
**Both individuals are considered first authors*

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Abstract: This study collected non-small cell lung cancer (NSCLC) tissues and corresponding adjacent non-tumor tissues resected intraoperatively from 92 NSCLC patients admitted between June 2019 and June 2021, aiming to investigate the expression levels of microRNA (miRNA)-33b-5p and fibroblast growth factor receptor-like protein 1 (FGFRL1) in NSCLC tissues, as well as their relationships with clinicopathological features and prognosis. The conclusion we drew is that the expression of miR-33b-5p is significantly decreased and the expression of FGFRL1 is significantly increased in non-small cell lung cancer (NSCLC) tissues. Both are correlated with the clinicopathological features and prognosis of patients, and the combined detection of miR-33b-5p and FGFRL1 can be used to evaluate the prognosis of NSCLC patients.

1. Introduction

Non-small cell lung cancer (NSCLC) is a malignant tumor originating from bronchial mucosa, glands, and alveolar epithelium, mainly including adenocarcinoma and squamous cell carcinoma [1]. The pathogenesis of NSCLC is complex, and its etiology has not been fully clarified so far. Some scholars believe that it may be caused by the long-term interaction of factors such as individual factors, genetics, and environment [2]. The early symptoms of NSCLC are not typical and the onset is insidious. Symptoms caused by the primary lesion include cough, fever, and hemoptysis, and chest pain and other symptoms occur after intrathoracic spread. Most patients are diagnosed at an advanced stage and miss the best opportunity for treatment [3-4]. Although medical technology has been continuously improved, the 5-year survival rate of patients is still relatively low. Therefore, it is particularly important to find indicators related to NSCLC in clinical practice to improve the prognosis of patients. MicroRNA (miRNA) is a type of non-coding RNA that can pair with target mRNA to inhibit target gene translation, thereby regulating gene expression. miRNAs are abnormally expressed in malignant tumors[5]. Studies have found that miR-33b-5p is down-regulated in gastric cancer cells, and up-

regulating its expression can inhibit the proliferation and epithelial-mesenchymal transition of gastric cancer cells and increase the sensitivity of gastric cancer cells to chemotherapeutic drugs [6]. Fibroblast growth factor receptor-like protein 1 (FGFRL1) is a target marker for a variety of malignant tumors, which can regulate processes such as cell proliferation and differentiation, and promote the proliferation and migration of various malignant tumor cells [7-8]. The ENCORI website predicts that there is a targeted relationship between miR-33b-5p and FGFRL1, and it is speculated that miR-33b-5p may be involved in the progression of NSCLC by targeting FGFRL1. However, there are few relevant research reports at present. Therefore, this study aims to investigate the expression levels of miR-33b-5p and FGFRL1 in NSCLC tissues and their relationships with clinicopathological features and prognosis.

2. Materials and Methods

2.1 General Information

A total of 92 NSCLC patients admitted from June 2019 to June 2021 were selected. Fresh NSCLC tissues and corresponding adjacent non-cancerous tissues (more than 5 cm away from the tumor edge) resected during surgery were collected. Among them, there were 60 males and 26 females, aged 41-81 years, with an average age of (60.75 ±8.31) years.

Inclusion criteria: (1) Conforming to the diagnostic criteria of NSCLC [9]; (2) Confirmed by pathology and imaging examinations; (3) All underwent surgical resection (radical resection of lung cancer); (4) Complete clinical data; (5) Patients signed the informed consent form.

Exclusion criteria: (1) Received radiotherapy or chemotherapy before surgery; (2) Complicated with other malignant tumors; (3) Suffered from failure of important organs; (4) Had immune or hematological system diseases; (5) Had infectious diseases; (6) Had abnormal mental status and difficulty in normal communication.

2.2 Methods

2.2.1 Detection of miR-33b-5p and FGFRL1 mRNA Expression Levels by qRT-PCR

A part of the resected fresh NSCLC tissues was quickly frozen in liquid nitrogen and stored in a -80℃ refrigerator. During extraction, the tissues were ground and crushed. Total RNA was extracted from NSCLC tissues and adjacent non-cancerous tissues using Trizol reagent. The concentration and purity of total RNA were evaluated using an ultra-micro spectrophotometer. After reverse transcription into cDNA, the cDNA was stored. Using cDNA as a template, qRT-PCR was performed to detect the mRNA levels of miR-33b-5p and FGFRL1, with U6 and GADPH as internal references. The primer sequences are shown in Table 1. The qRT-PCR reaction system was 20 μL in total: 10 μL of 2×miRNA qPCR master mix, 0.5 μL of forward primer, 0.5 μL of reverse primer, 2 μL of DNA template, 1 μL of ROX reference dye (L), and high-purity water was added to make up to 20 μL. The reaction conditions were as follows: 95℃ for 15 minutes, followed by 40 cycles of 94℃ for 15 seconds, 55℃ for 30 seconds, and 72℃ for 30 seconds. Finally, the mRNA expressions of miR-33b-5p and FGFRL1 were calculated using the 2-ΔΔCt method.

Table 1 Primer Sequences for qRT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
miR-33b-5p	AACCAGCGCATGGACAGTTA	GACTTGACCACCGAACCCAT
U6	ACCAGCGCATGGACAGT	ACTTGACCACCGAACCC
FGFRL1	AGGACGGCTCCTCTAACCAT	AGCGGCTCCACAAGTAAGAC
GADPH	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

2.2.2 Detection of FGFRL1 Expression in NSCLC and Adjacent Non-Cancerous Tissues by Immunohistochemistry

NSCLC tissues and adjacent non-cancerous tissues were made into paraffin sections, followed by antigen retrieval and removal of peroxidase. After serum blocking, FGFRL1 antibody (1:100, ab95940) was added and incubated overnight at 4℃. Then, secondary antibody (1:1000, ab6721) was added and incubated at room temperature for 1 hour. Finally, staining was completed by DAB color development and counterstaining. Two physicians observed the staining of the sections under a high-power microscope (using a double-blind method). The scoring standard is shown in Table 2. The results were evaluated based on the score of the percentage of positive cells and the score of the staining intensity of positive cells. Negative expression was defined as < 3 points, and positive expression was defined as ≥ 3 points [10].

Table 2 Scoring Standard

Score	Score of the Percentage of Positive Cells	Score of the Staining Intensity of Cells
0	≤5%	No staining
1	6%~25%	Yellow
2	26%~50%	Brownish yellow
3	51%~75%	Brown
4	>75%	

2.3 Follow-Up

Patients were followed up systematically after surgery (by phone call or hospital admission examination). The follow-up period started from the end of the surgery, lasting for 36 months, with the latest cut-off time of June 2024. The post-operative survival status of patients was recorded, and patients were divided into the poor prognosis group (deceased) and the good prognosis group according to the prognosis.

2.4 Statistical Analysis

Statistical analysis was performed using SPSS 25.0 software. Continuous data (conforming to normal distribution) were analyzed by t-test and expressed as ($x \pm s$); counting data were expressed as n (%), and analyzed by χ^2 test. Kaplan-Meier survival curve analysis was used to analyze the relationships of miR-33b-5p and FGFRL1 with the prognosis of NSCLC patients. Cox regression analysis was used to identify the influencing factors of prognosis in NSCLC patients. Receiver operating characteristic (ROC) curve was plotted to analyze the predictive value of miR-33b-5p and FGFRL1 for poor prognosis in NSCLC patients. A P value < 0.05 was considered statistically significant.

3. Results

3.1 Comparison of miR-33b-5p and FGFRL1 mRNA Expression Levels Between the Two Tissue Groups

The expression level of miR-33b-5p in NSCLC tissues was significantly lower than that in adjacent non-cancerous tissues (P < 0.05), and the mRNA expression level of FGFRL1 in NSCLC tissues was significantly higher than that in adjacent non-cancerous tissues (P < 0.05). (See Figure 1.)

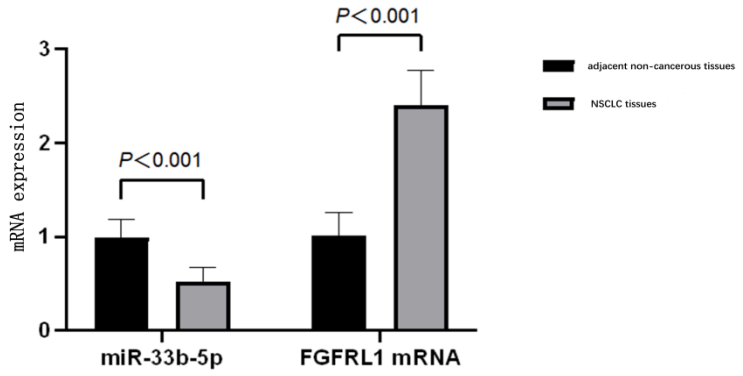

Figure 1 Comparison of miR-33b-5p and FGFRL1 mRNA Expression Levels between the Two Tissue Groups

3.2 Expression of FGFRL1 Protein in the Two Tissue Groups

The positive expression rate of FGFRL1 in NSCLC tissues was significantly higher than that in adjacent non-cancerous tissues ($P < 0.05$). (See Table 3 and Figure 1.)

Table 3 Expression of FGFRL1 Protein in the Two Tissue Groups

Group	Number of Cases	FGFRL1 Positive [n (%)]	FGFRL1 Negative [n (%)]	χ^2
Adjacent non-cancerous tissues	92	24 (26.09)	68 (73.91)	15.281
NSCLC tissues	92	50 (54.35)	42 (45.65)	15.281
P	-	< 0.001	< 0.001	-

3.3 Targeted Relationship between miR-33b-5p and FGFRL1

Correlation analysis showed a negative correlation between miR-33b-5p and FGFRL1 ($r = 0.575$, $P < 0.05$). The ENCORI prediction website showed that there was a targeted relationship between miR-33b-5p and FGFRL1. (See Figure 2.)

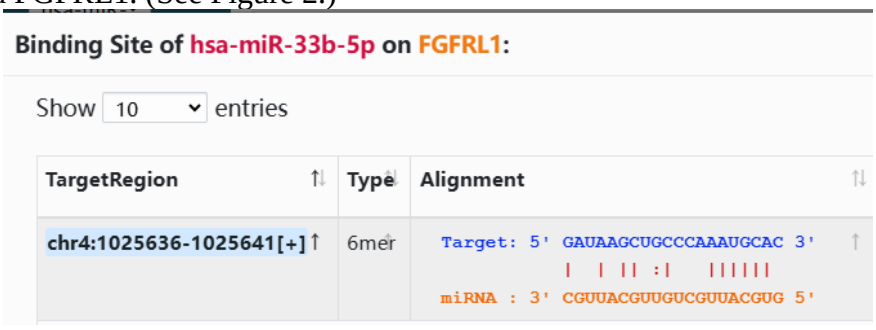

Figure 2 Targeted Relationship Between miR-33b-5p and FGFRL1

3.4 Relationships of miR-33b-5p and FGFRL1 Expressions in NSCLC Tissues with Clinicopathological Features

Taking the average value of miR-33b-5p expression level in NSCLC tissues as the boundary, ≥ 0.46 was defined as high expression, and < 0.46 was defined as low expression. FGFRL1 protein expression was divided into positive and negative. Among NSCLC patients, those with high miR-

33b-5p expression and negative FGFRL1 expression had lower proportions of TNM stage II, poor differentiation, and lymph node metastasis compared with patients with low miR-33b-5p expression and positive FGFRL1 expression ($P < 0.05$), and there were no significant differences in other data ($P > 0.05$). (See Table 4.)

Table 4 Relationships of miR-33b-5p and FGFRL1 Expressions in NSCLC Tissues with Clinicopathological Features

Pathological Features	Number of Cases (92)	miR-33b-5p		χ^2	P	FGFRL1		χ^2	P
		High Expression (n=44)	Low Expression (n=48)			Positive (n=50)	Negative (n=42)		
Age (years)				0.013	0.910			0.043	0.836
≥60	58	28(48.28)	30(51.72)			32(55.17)	26(44.83)		
<60	34	16(47.06)	18(52.94)			18(52.94)	16(47.06)		
Gender				0.076	0.782			0.127	0.722
Male	64	30(46.88)	34(53.13)			34(53.13)	30(46.88)		
Female	28	14(50.00)	14(50.00)			16(57.14)	12(42.86)		
Pathological Type				0.744	0.689			1.104	0.576
Adenocarcinoma	48	25(52.08)	26(47.92)			28(58.33)	20(41.67)		
Squamous Cell Carcinoma	32	14(43.75)	18(56.25)			15(41.67)	17(53.13)		
Adenosquamous Carcinoma	12	5(41.67)	7(58.33)			7(58.33)	5(41.67)		
TNM Stage				12.759	<0.001			10.638	0.001
Stage I	58	36(62.07)	22(37.93)			24(41.38)	34(58.62)		
Stage II	34	8(23.53)	26(76.47)			26(76.47)	8(23.53)		
Tumor Diameter				0.005	0.941			0.077	0.782
<3.0 cm	54	26(48.15)	28(51.85)			30(55.56)	24(44.44)		
≥3.0 cm	38	18(47.37)	20(52.63)			20(52.63)	18(47.37)		
Differentiation Degree				15.538	<0.001			13.086	<0.001
Moderate to High Differentiation	56	36(64.29)	20(35.71)			22(39.29)	34(60.71)		
Poor Differentiation	36	8(22.22)	28(77.78)			28(77.78)	8(22.22)		
Lymph Node Metastasis				13.700	<0.001			14.405	<0.001
No	55	35(79.55)	20(41.67)			21(38.18)	34(61.82)		
Yes	37	9(20.45)	28(58.33)			29(78.38)	8(21.62)		
Smoking History				0.134	0.714			0.012	0.912
Yes	52	24(46.15)	28(53.85)			28(53.85)	24(46.15)		
No	40	20(50.00)	20(50.00)			22(55.00)	18(45.00)		

3.5 Relationships of miR-33b-5p and FGFRL1 Expressions in NSCLC Tissues with Prognosis

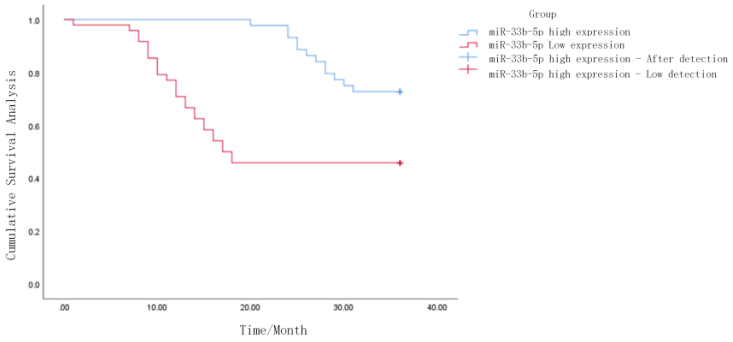

Figure 3 Relationship Between miR-33b-5p Expression and Prognosis of Patients

The 3-year survival rate of patients with high miR-33b-5p expression was (32/44, 72.73%), and that of patients with low miR-33b-5p expression was (22/48, 45.83%). The survival rate of patients with high expression was higher than that of patients with low expression (Log-rank $\chi^2 = 11.215$, $P <$

0.05); the 3-year survival rate of patients with positive FGFRL1 expression was (22/50, 44.00%), and that of patients with negative FGFRL1 expression was (32/42, 76.19%). The survival rate of patients with positive expression was lower than that of patients with negative expression (Log-rank χ^2 = 14.239, P < 0.05). (See Figure 3 and Figure 4.)

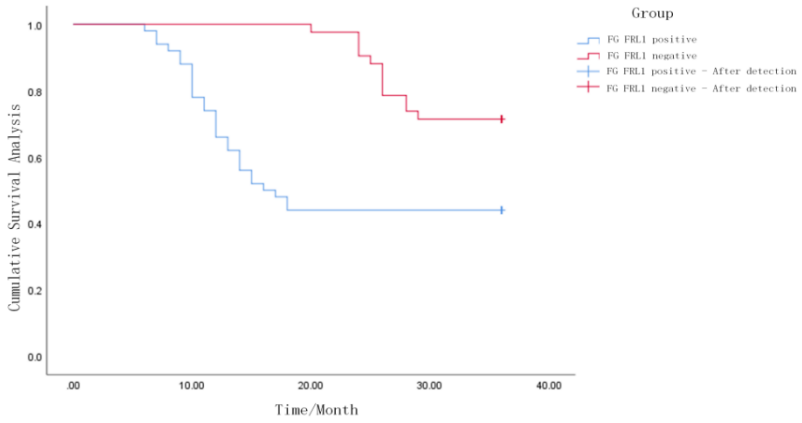

Figure 4 Relationship Between FGFRL1 Expression and Prognosis of Patients

3.6 Comparison of miR-33b-5p and FGFRL1 mRNA Expression Levels Between the Two Prognosis Groups

Compared with the good prognosis group, the expression level of miR-33b-5p was significantly decreased and the mRNA expression level of FGFRL1 was significantly increased in the poor prognosis group (P < 0.05). (See Table 5.)

Table 5 Comparison of miR-33b-5p and FGFRL1 mRNA Expression Levels Between the Two Prognosis Groups

Group	Number of Cases	miR-33b-5p	FGFRL1 mRNA
Good prognosis group	54	0.75±0.20	1.37±0.26
Poor prognosis group	38	0.20±0.06	3.86±0.52
t	-	16.416	30.265
P	-	<0.001	<0.001

3.7 Analysis of Influencing Factors of Prognosis in NSCLC Patients

Taking whether NSCLC patients died within 3 years as the dependent variable (yes = 1, no = 0), and miR-33b-5p, FGFRL1, and clinicopathological features (TNM stage, degree of differentiation, lymph node metastasis) as independent variables, the assignment is shown in Table 6. Multivariate Cox regression analysis showed that FGFRL1, TNM stage, degree of differentiation, and lymph node metastasis were risk factors affecting the prognosis of NSCLC patients (P < 0.05), while miR-33b-5p was a protective factor (P < 0.05). (See Table 7.)

Table 6 Assignment of Variables

Variable	Assignment Method
miR-33b-5p	Continuous variable
FGFRL1	Continuous variable
TNM stage	Stage I = 0, Stage II = 1
Degree of differentiation	Moderate to high differentiation = 0, Poor differentiation = 1
Lymph node metastasis	No = 0, Yes = 1

Table 7 Analysis of Influencing Factors of Prognosis in NSCLC Patients

Factor	Univariate Analysis			Multivariate Analysis		
	OR	95%CI	P	OR	95%CI	P
miR-33b-5p	0.513	0.293-0.899	0.020	0.432	0.247-0.757	<0.001
FGFRL1	4.012	3.153-5.106	<0.001	4.021	2.638~6.128	<0.001
TNM stage	2.147	1.151~4.004	0.016	3.278	2.576-4.172	<0.001
Degree of differentiateon	1.771	1.201-2.611	0.004	5.124	2.210-11.879	<0.001
Lymph node metastasis	3.454	1.353-8.815	0.010	4.012	3.153-5.106	<0.001

3.8 Predictive Value of miR-33b-5p and FGFRL1 for Poor Prognosis in NSCLC Patients

According to the ROC curve, the area under the curve (AUC) for predicting poor prognosis in NSCLC patients with miR-33b-5p, FGFRL1, and their combination is 0.856, 0.815, and 0.894, respectively. The combination is superior to each marker's individual prediction ($Z = 2.678$, $Z = 2.785$, both $P < 0.05$), as shown in Figure 5 and Table 8.

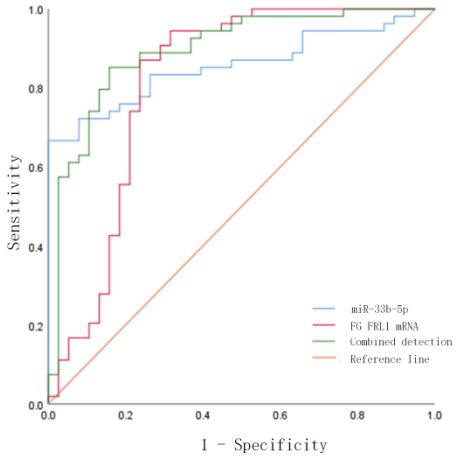

Figure 5 Predictive Value of miR-33b-5p and FGFRL1 for Poor Prognosis in NSCLC Patients

Table 8 Predictive Value of miR-33b-5p and FGFRL1 for Poor Prognosis in NSCLC Patients

Item	AUC	95%CI	Sensitivity (%)	Specificity (%)	Cut-Off Value
miR-33b-5p	0.856	0.779~0.933	77.62	83.46	0.312
FGFRL1	0.815	0.712~0.918	779.68	78.76	3.521
Combined prediction	0.894	0.825~0.963	94.67	76.75	-

4. Discussion

The main clinical manifestations of NSCLC include recurrent cough, hemoptysis, etc. Its occurrence may be related to smoking, genes, etc. [11]. The clinical symptoms of early NSCLC are not typical, and most patients are diagnosed at an advanced stage, missing the best treatment opportunity, with a poor prognosis, which seriously threatens the life safety of patients [11-12]. Therefore, finding markers related to the pathological features and prognosis of NSCLC in clinical practice can provide a reference for improving the prognosis of patients.

miRNA is mainly located in eukaryotes and is related to cell growth, apoptosis, etc. It can affect the progression of the body through a variety of pathways. Abnormal expression of miRNA can cause organ lesions and affect the proliferation and migration of malignant tumor cells [13-15]. As a

member of the miR-33 family, miR-33b-5p can regulate cholesterol homeostasis, carbohydrate metabolism, and the progression of malignant tumors. It is significantly down-regulated in osteosarcoma tissues and can promote the survival, migration, and invasion of osteosarcoma cells by regulating the SIRT6 signaling axis [16]. miR-33b-5p is significantly down-regulated in lung adenocarcinoma cells, and its up-regulation can significantly inhibit the apoptosis of lung adenocarcinoma cells [17]. The results of this study found that the expression level of miR-33b-5p in NSCLC tissues was significantly decreased, which was consistent with previous studies. In addition, among NSCLC patients, those with high miR-33b-5p expression had lower proportions of TNM stage II, poor differentiation, and lymph node metastasis compared with patients with low miR-33b-5p expression, indicating that miR-33b-5p may be involved in the progression of NSCLC.

FGFRL1 is the fifth member of the FGFR family, which can participate in inhibiting angiogenesis, inhibiting DNA synthesis and cell proliferation, and regulating the cell cycle to promote tumor cell proliferation [17-18]. The expression of FGFRL1 is increased in esophageal cancer tissues, and inhibiting its expression can inhibit the proliferation and invasion of esophageal cancer cells [19]. In addition, targeted regulation of FGFRL1 pathway activity can regulate the expression of migration-related proteins and the migration of drug-resistant gastric cancer cells [20]. Another study found that the expression of FGFRL1 is increased in NSCLC samples, and its expression level is also related to clinical stage, survival time, etc. Patients with high FGFRL1 expression have high tumor malignancy, strong drug resistance during chemotherapy, and poor prognosis [8]. Up-regulating FGFRL1 can promote the proliferation of NSCLC cells [21]. The results of this study found that the mRNA expression level and positive rate of FGFRL1 in NSCLC tissues were significantly increased, and among NSCLC patients, those with negative FGFRL1 expression had lower proportions of TNM stage II, poor differentiation, and lymph node metastasis compared with patients with positive FGFRL1 expression, indicating that FGFRL1 may also be involved in the occurrence and development of NSCLC.

This study further found that patients with high expression of miR-33b-5p and positive FGFRL1 had a higher survival rate than those with low expression of miR-33b-5p and negative FGFRL1. Moreover, in the poor prognosis group, miR-33b-5p levels were decreased, and FGFRL1 mRNA levels were increased, indicating that both are associated with the prognosis of NSCLC patients. FGFRL1, miR-33b-5p, TNM stage, degree of differentiation, and lymph node metastasis are factors influencing the prognosis of NSCLC patients, suggesting that monitoring changes in their levels in clinical practice can assess disease progression. The combined prediction of poor prognosis in NSCLC patients using miR-33b-5p and FGFRL1 has a higher AUC than each marker alone, indicating that their combination improves the predictive value for poor prognosis and provides a reference for clinicians in diagnosis and treatment strategy formulation. This study also found that miR-33b-5p and FGFRL1 are negatively correlated, with a targeted relationship between miR-33b-5p and FGFRL1, suggesting that miR-33b-5p may target FGFRL1 to participate in the progression of NSCLC.

In conclusion, miR-33b-5p expression is significantly decreased, while FGFRL1 expression is significantly increased in NSCLC tissues. Both are related to the clinicopathological features and prognosis of patients. The combination of the two can be used to evaluate the prognosis of patients. The limitations of this study are the small sample size and the failure to explore the mechanism of miR-33b-5p and FGFRL1 in NSCLC. In the future, the sample size will be expanded for further analysis, in order to provide a direction for improving the prognosis of NSCLC.

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