Expression and clinical significance of microRNA-21, PTEN and p27 in cancer tissues of patients with non-small cell lung cancer

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Abstract. Expression and clinical significance of microRNA-21, PTEN and p27 in cancer tissue of patients with non-small cell lung cancer (NSCLC) were investigated. In this study, cancer tissue and adjacent tissue specimens from 230 patients with NSCLC were collected from thoracic surgery department in Hubei Cancer Hospital from March 2010 to February 2016. The expression of miRNA-21, PTEN and p27 in cancer tissue and adjacent tissue of patients with NSCLC was detected by RT-PCR. Combined with clinical information, the correlation among miRNA-21, PTEN, p27 and clinical features of NSCLC was analyzed. The expression of miRNA-21, PTEN, p27 in NSCLC was significantly lower than that in adjacent tissue by RT-PCR (P<0.05). There was no significant difference in age, sex and course of disease (P>0.050), but there were differences in smoking, lymph node metastasis, TNM stage and differentiation degree classification (P<0.050). By comparing the 3-year survival rate in the group with high and low expression of miRNA-21, PTEN and p27, it was found that the 36-month survival rate of patients with high expression of miRNA-21 was 85.19% (P<0.05), and of patients with low expression of miRNA-21 it was 95.90% (P<0.05). The 36-month survival rate of patients with high expression of PTEN was 85.59% (P<0.05), of patients with low expression of PTEN it was 94.96% (P<0.05) and in patients with high expression of p27 it was 84.91% (P<0.05). The 36-month survival rate of patients with low expression of p27 was 94.35% (P<0.05). The survival rates of miRNA-21, PTEN and p27 low expression groups were significantly higher than those of high expression groups (P<0.05). In conclusion, the expression of miRNA-21, PTEN and p271 in cancer tissue of NSCLC patients was low. The three indexes have good diagnostic efficacy based on ROC curve analysis, and are expected to be excellent indexes for early clinical diagnosis and prognosis of NSCLC.

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Introduction

Lung cancer is the leading cause of cancer-related death in the world, the most common of which is non-small cell lung cancer (NSCLC) (1). At present, smoking is considered to be the most important high risk factor for lung cancer (2). Multi-chain aromatic hydrocarbons and nitrosamines can cause DNA damage in bronchial epithelial cells through a variety of mechanisms, which can lead to cell transformation and eventually carcinogenesis. According to the annual statistics of GLOBOCAN2018, lung cancer is the most commonly diagnosed cancer in both sexes, with a total of 11.6% of the cases. Lung cancer accounts for 18.4% of the cancer deaths, which is also the main cause of cancer death in men (3). At present, the early diagnosis of NSCLC is mainly based on computerized tomography scanning, magnetic resonance imaging, positron emission tomography, sputum cytology examination or histology of bronchoscopy. These techniques are usually not practical, and are expensive, for most early NSCLC detection (4). According to Hu *et al* (5) and Chen *et al* (6), serum miRNA, as a potential biomarker for the diagnosis and prediction of prognosis of lung cancer, and may be useful for the detection of NSCLC and provides high sensitivity and specificity (7).

miRNA (microRNA) is a small class of non-coding RNA, which can be used as endogenous RNA interference to regulate the expression of target genes and is involved in the regulation of various physiological and pathological functions (8). Bioinformatics data show that a single miRNA can be bound to hundreds of target mRNAs to play an important role in various biological processes (9). There is increasing evidence of the association between miRNA and tumorigenesis (10,11). Phospholipase and tensin homologue (PTEN) are lipid phosphatase, which is one of the tumor inhibitor genes that are often mutated or deleted during cancer progression. Some studies have shown that inactivated PTEN can convert cancer genes into anti-oncogene in the process of disease progression (12). P27 is essentially an unstructured multifunctional protein, which can affect various biological processes from cell cycle regulation to cell migration and transcriptional

regulation, and was initially found to be the key regulator of cell proliferation (13).

Therefore, the expression of miRNA-21, PTEN and p27 in NSCLC patients was studied, in order to provide the basis for the diagnosis and treatment of NSCLC.

Patients and methods

General materials. Cancer tissue and adjacent tissue specimens from 230 patients with NSCLC were collected from the Thoracic Surgery Department of the Hubei Cancer Hospital (Wuhan, China) from March 2010 to February 2016. The samples were cryopreserved in liquid nitrogen within 5 min of resection. The cancer tissue was treated as the study group. There were 92 males and 23 females, aged 45-68 years, with a mean age of 51.05±10.47. Adjacent tissue was included in the control group. The number and mean age of the study group were consistent with those of the study group. The collection of clinical specimens was approved by the Medical Ethics Committee of the Hubei Cancer Hospital. All the patients signed informed consent forms.

Inclusion and exclusion criteria. Inclusion criteria. Patients diagnosed and treated in Hubei Cancer Hospital; cancer tissue and adjacent tissue were obtained by resection of lung cancer in thoracic surgery of the hospital; patients aged 35-70 years and with an education of primary school and above; patients cooperating with the research; patients with no other serious organ diseases; patients or lineal consanguinity signed the informed consent forms.

Exclusion criteria: Patients who died during treatment; patients with injury in important organs; patients complicated with other tumors, cardiovascular and cerebrovascular diseases; patients with physical disability; pregnant patients; patients with other autoimmune diseases or chronic diseases; patients transferred to other hospitals; patients with surgical contraindications, mental diseases and language dysfunction; patients with diseases that affect the results of this study.

Main reagent. TRIzol reagent and miRNA reverse transcriptase kit were purchased from Invitrogen Company of the United States. SYBR Green Master Mix was purchased from American Applied Biological Systems Co., Ltd. ABI StepOne Plus fluorescence quantitative PCR instrument was purchased from American Applied Biological Systems Co., Ltd. NanoDrop 2000 spectrophotometer, KH19A desktop high speed and high performance centrifuge were purchased from KAIDA Co., Ltd. Cryogenic refrigerator (-80˚C) was purchased from Thermo Fisher Scientific, Inc. The sequence of miRNA-21 primers was designed and synthesized by Shanghai Bioengineering Co., Ltd. (Table I).

qRT-PC detection. Total RNA was extracted in strict accordance with the instructions using TRIzol reagent in PCa cancer tissue and PCa adjacent tissue. The concentration and purity of extracted RNA were detected by ultraviolet spectrophotometer. The OD value of total RNA solution: A240/A300 was in the range of 1.8-2 1. If the standard was not met, it would be extracted again. The integrity of RNA was detected by 1% denatured agarose gel electrophoresis. Configuration of reaction system and reverse transcription synthesis of cDNA for total RNA were carried out by miRNA reverse transcription kit (-20˚C storage for use). ABI StepOne Plus fluorescence quantitative PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for determination. The reaction system was arranged according to the specification, and the 12.33 μl of the reaction system was filled with DEPC water to 20 μl. Reaction conditions: 95˚C for 5 min; 95˚C for 45 sec; 60˚C for 60 sec; 72˚C for 45 sec; 45 sec for a total of 45 cycles. U6 was used as the internal parameter of the reaction. The experiment was repeated 3 times. $2^{-\Delta\Delta ct}$ method was used to analyze the results.

Observation indicators and evaluation criteria. miRNA-21, PTEN, p27 expression levels were compared between the two groups; diagnostic value of miRNA-21, PTEN, p27 in NSCLC; correlation among miRNA-21, PTEN, p27 and clinical pathology; the prognosis of high and low expression levels of miRNA-21, PTEN and p27 in the two groups was compared for 3-year survival; the prognostic value of miRNA-21, PTEN and p27 in the prognosis of death.

Follow-up. The patients were followed-up in March, June, September and December every year for 3 years, and the survival of the patients was recorded by telephone and outpatient medical records.

Statistical analysis. Data were processed and analyzed using SPSS 24.0 software system (Beijing Strong-Vinda Information Technology Co., Ltd.). All graphics were plotted by GraphPad 8 (Shenzhen Tianruiqi Software Technology Co., Ltd.) and the results were checked twice. The counting data were tested by χ^2. t-test was used for detection of measurement data, which was expressed as mean ± standard deviation (mean ± SD). ROC curve was used for diagnostic value analysis to evaluate diagnostic effectiveness and to calculate sensitivity and specificity. The survival rate was calculated using the Kaplan-Meier method, and the survival rate was compared using the log-rank test. A value of $P<0.05$ was considered as indicating a statistically significant difference.

Results

Comparison of miRNA-21, PTEN and p27 expression levels between the two groups. The expression levels of miRNA-21, PTEN and p27 of cancer tissue in the study group were 1.35±0.46, 1.48±0.16 and 2.41±0.34, respectively. The expression levels of miRNA-21, PTEN and p27 of adjacent tissue in the control group were 2.87±1.03, 3.10±0.09 and 4.29±1.01, respectively. The expression was low in cancer tissue of patients with NSCLC, and there were significant differences between the two groups ($P<0.050$) (Table II).

Diagnostic effectiveness of miRNA-21, PTEN and p27 in NSCLC. ROC curve analysis showed that when the cut-off value was 1.558, the sensitivity and specificity of miRNA-21 in the diagnosis of NSCLC were 72% and 74%, respectively, the AUC was 0.756. When the cut-off value was 1.408, the sensitivity, and specificity of PTEN in the diagnosis of NSCLC were 82% and 56%, respectively, the AUC was 0.687. When the

Table I. Primer sequences.

Gene	Upstream sequence	Downstream sequence
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
miRNA-21	5'-AACGCTTCACGAATTTGCGT-3'	5'-TGGTGTCGTGGAGTCG-3'

Table II. Comparison of miRNA-21, PTEN and p27 expression levels between the two groups.

Group	Case	miRNA-21	PTEN	p27
Study group	115	1.35±0.46	1.48±0.16	2.41±0.34
Control group	115	2.87±1.03	3.10±0.09	4.29±0.19
t-value		14.450	94.630	51.760
P-value		0.001	0.001	0.001

Table III. Diagnostic effectiveness of miRNA-21, PTEN and p27 in NSCLC.

Index	miRNA-21	PTEN	p27
AUC	0.756	0.687	0.732
Standard error	0.489	0.054	0.050
95% CI	0.660-0.851	0.581-0.793	0.635-0.830
P-value	0.001	0.001	0.001
Cut-off	1.558	1.408	2.296
Sensitivity	72%	82%	82%
Specificity	74%	56%	62%

cut-off value was 2.206, the sensitivity and specificity of p27 in the diagnosis of NSCLC were 82% and 62%, respectively, the AUC was 0.732 (Table III and Fig. 1).

Correlation of miRNA-21, PTEN and p27 in clinicopathological features of NSCLC. There was no significant difference in age, sex and course of disease in the study group (P>0.050), while there was a difference in smoking, lymph node metastasis, TNM stage and differentiation (P<0.050) (Tables IV-VI).

Survival rate at 3 years in patients with high and low expressions of miRNA-21, PTEN and p27. According to the median value of miRNA-21, PTEN and p27 expression levels, there were 108 cases in miRNA-21 low expression group (≥0.82) and 122 cases in group with high expression of miRNA-21 (<0.82). There were 111 cases in low expression group of PTEN (≥2.52) and 119 cases in group with high expression of PTEN (<2.52). There were 106 cases in p27 low expression group (≥3.18) and 124 cases in p27 high expression group (<3.18). Patients were randomly visited for 36 months, and the termination time and the termination event were March 2018 and the death of patients. The success rate of follow-up in 36 months was 95.22% (219/230). A total of 16 patients died in the miRNA-21 low expression group, and the 36-month survival rate was

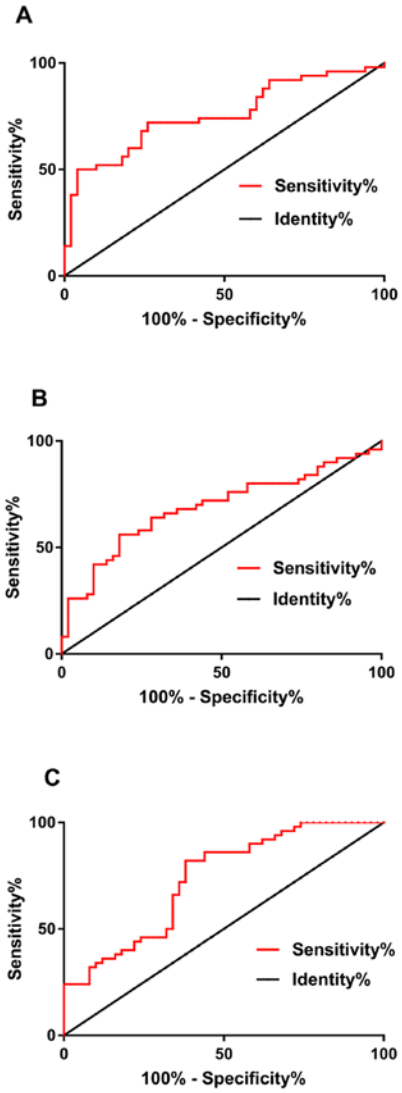

Figure 1. Diagnostic effectiveness of miRNA-21, PTEN and p27 for NSCLC. (A) When the cut-off value was 1.558, the sensitivity and specificity of miRNA-21 in the diagnosis of NSCLC were 72 and 74%, respectively, the AUC was 0.756. (B) When the cut-off value was 1.408, the sensitivity, and specificity of PTEN in the diagnosis of NSCLC were 82 and 56%, respectively, the AUC was 0.687. (C) When the cut-off value was 2.206, the sensitivity and specificity of p27 in the diagnosis of NSCLC were 82 and 62%, respectively, the AUC was 0.732. NSCLC, non-small cell lung cancer.

85.19%. A total of 5 patients died in miRNA-21 high expression group, and the 36-month survival rate was 95.90%. A total of 16 patients died in the PTEN low expression group, and the 36-month survival rate was 85.59%. A total of 6 patients died in TEN high expression group, and the 36-month survival rate was 94.96%. A total of 16 patients died in the p27 low expression group, and the 36-month survival rate was 84.91%. A total

Table IV. Correlation between miRNA-21 and clinicopathological features in NSCLC.

Clinicopathological feature	n (115)	miRNA-21	F	P-value
Age, years			0.480	0.632
>50	62	1.36±0.45		
≤50	53	1.32±0.44		
Sex			0.206	0.837
Male	92	1.33±0.42		
Female	23	1.31±0.40		
Course of disease, weeks			1.032	0.304
>5	60	1.41±0.23		
≤5	55	1.37±0.18		
Smoking			2.582	0.011
Yes	104	1.72±0.69		
No	11	1.18±0.12		
Lymph node metastasis			3.487	0.007
Yes	69	1.48±0.75		
No	46	1.09±0.13		
TNM stage			3.410	0.009
I-II	41	1.06±0.17		
III-IV	74	1.45±0.72		
Differentiation degree			5.073	0.001
Poorly differentiated	77	1.08±0.20		
Moderately and highly differentiated	38	1.51±0.69		

Table V. Correlation between PTEN and clinicopathological features in NSCLC.

Clinicopathological feature	n (115)	PTEN	F	P-value
Age, years			1.129	0.899
>50	62	1.33±0.42		
≤50	53	1.34±0.41		
Sex			0.480	0.631
Male	92	1.37±0.45		
Female	23	1.32±0.43		
Course of disease, weeks			1.068	0.288
>5	60	1.39±0.21		
≤5	55	1.35±0.19		
Smoking			3.468	0.007
Yes	104	1.76±0.58		
No	11	1.15±0.11		
Lymph node metastasis			3.666	0.004
Yes	69	1.58±0.71		
No	46	1.19±0.15		
TNM stage			6.066	0.001
I-II	41	1.05±0.21		
III-IV	74	1.62±0.58		
Differentiation degree			7.420	0.001
Poorly differentiated	77	1.12±0.21		
Moderately and highly differentiated	38	1.67±0.58		

Table VI. Correlation between p27 and clinicopathological features in NSCLC.

Clinicopathological feature	n (115)	p27	F	P-value
Age, years			0.446	0.656
>50	62	2.42±0.31		
≤50	53	2.39±0.41		
Sex			0.518	0.605
Male	92	2.43±0.42		
Female	23	2.38±0.39		
Course of disease, weeks			0.755	0.451
>5	60	2.45±0.33		
≤5	55	2.40±0.38		
Smoking			3.950	0.001
Yes	104	2.92±0.57		
No	11	2.23±0.29		
Lymph node metastasis			7.238	0.004
Yes	69	2.89±0.63		
No	46	2.19±0.22		
TNM stage			5.284	0.001
I-II	41	2.28±0.31		
III-IV	74	2.75±0.52		
Differentiation degree			6.730	0.001
Poorly differentiated	77	2.31±0.26		
Moderately and highly differentiated	38	2.78±0.49		

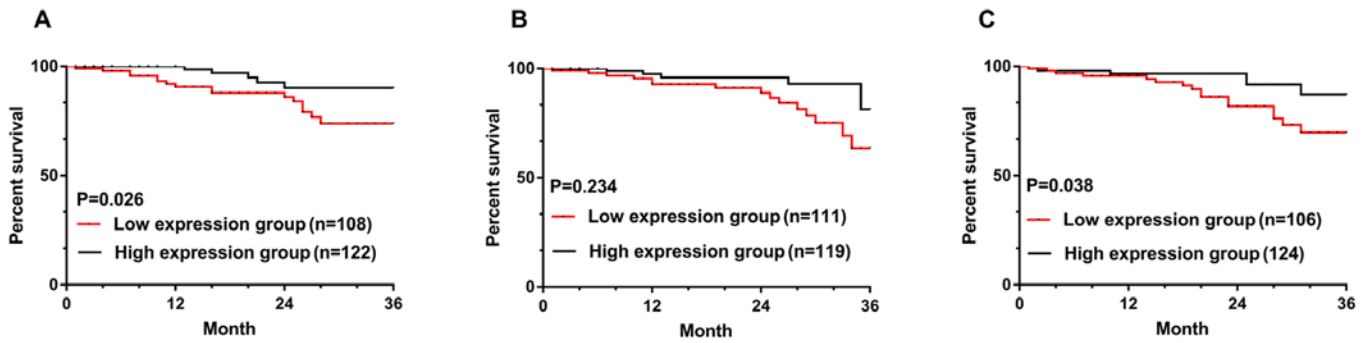

Figure 2. Three-year survival rate of patients with high and low expression of miRNA-21, PTEN and p27. (A) The 36-month survival rate of patients with low expression of miRNA-21 was 85.19%, the 36-month survival rate of patients with high expression of miRNA-21 was 95.90%. (B) The 36-month survival rate of patients with low expression of PTEN was 85.59%. The 36-month survival rate of patients with high expression of PTEN was 94.96%. (C) The 36-month survival rate of patients with low expression of p27 was 84.91% (P<0.05). The 36-month survival rate of patients with high expression of p27 was 94.35%. The survival rate of patients with high expression of miRNA-21, PTEN and p27 was higher than that of patients with low expression.

of 7 patients died in the p27 high expression group, and the 36-month survival rate was 94.35% (Fig. 2).

Prognostic value of miRNA-21, PTEN and p27 in the prognosis of survival. When the cut-off value was 1.905, the sensitivity and specificity of miRNA-21 were 85.24 and 60%, respectively, and the AUC was 0.786. When the cut-off value was 1.724, the sensitivity and specificity of PTEN were 95.24 and 55%, respectively, and the AUC was 0.776. When the cut-off value was 2.739, the sensitivity and specificity of p27 were 81.5 and 55%, respectively, the AUC was 0.682 (Table VII and Fig. 3).

Discussion

NSCLC accounts for 80-85% of all lung cancer and is still one of the leading causes of cancer-related death in the world (14). The World Health Organization (WHO) estimates that lung cancer is the cause of 1.37 million deaths worldwide each year and that 71% of these deaths are caused by smoking, indicating that ~400,000 people die of lung cancer each year (15). Because the etiology and pathogenesis of PHC have not been determined yet, some studies (16-18) have shown that the diagnosis of NSCLC by genetic index is more reliable and the

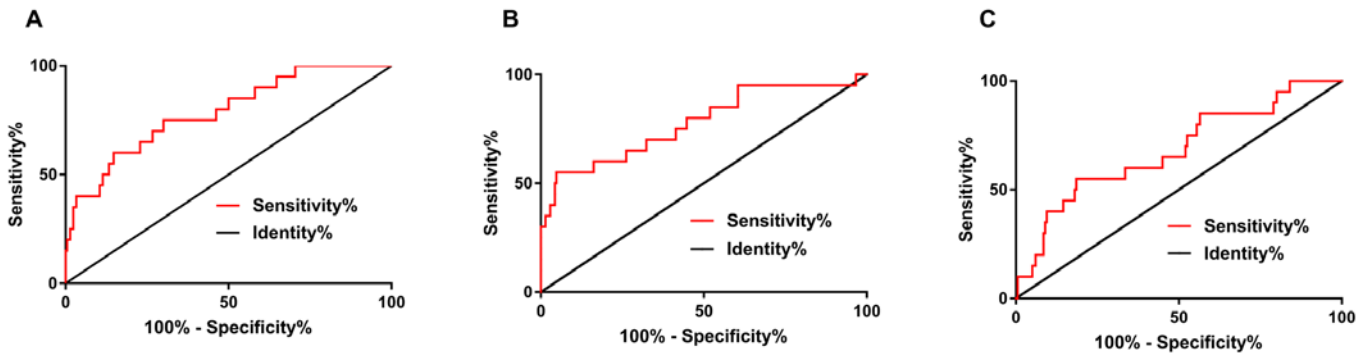

Figure 3. Prognostic value of miRNA-21, PTEN and p27 in the prognosis of death. (A) When the cut-off value was 1.905, the sensitivity and specificity of miRNA-21 were 85.24 and 60%, respectively, the AUC was 0.786. (B) When the cut-off value was 1.724, the sensitivity and specificity of miRNA-21 were 95.24 and 55%, respectively, the AUC was 0.776. (C) When the cut-off value was 2.739, the sensitivity and specificity of p27 were 81.5 and 55.%, respectively, the AUC was 0.682.

Table VII. The prognostic value of miRNA-21, PTEN and p27 for survival.

Index	miRNA-21	PTEN	p27
AUC	0.786	0.776	0.682
Standard error	0.054	0.063	0.065
95% CI	0.680-0.891	0.653-0.899	0.554-0.809
P-value	0.001	0.001	0.007
Cut-off	1.905	1.724	2.739
Sensitivity	85.24%	95.24%	81.5%
Specificity	60.00%	55.00%	55.00%

detection method is convenient. Therefore, it is very important to find an index that can accurately reflect the occurrence, progression and change of NSCLC and a convenient means of detection. Therefore, the expression of miRNA-21, PTEN and p27 in NSCLC patients were explored, which is of great significance for the early screening of NSCLC.

miRNA is a class of endogenous non-coding short-stranded RNA, which can degrade the target gene and inhibit the translation of the target gene, thus completing the silencing after gene transcription (19). It has been reported (20) that nearly 30% of the coding proteins in human body are affected and regulated by miRNAs. miRNA is differentially expressed in tumors, which can inhibit or promote the occurrence and progression of tumors by regulating the target genes.

In this study, the expression of miRNA-21, PTEN and p27 in cancer tissue of 230 patients with NSCLC was detected by qRT-PCR, and it was found that the expression levels of miRNA-21, PTEN and p27 in cancer tissue were significantly lower than those in adjacent tissue. In the study results of Zhu (21), Marsit *et al* (22) and Catzavelos *et al* (23), the expression was also decreased, which can further confirm the results of the present study. According to the ROC curve of miRNA-21, PTEN and p27, the AUC was, respectively, 0.756, 0.687 and 0.732, with high sensitivity. This showed that miRNA-21, PTEN and p27 had very good diagnostic value in NSCLC. Through the analysis of the correlation between the low expression of miRNA-21, PTEN, p27 and clinical medical records, it was found that smoking, lymphoid metastasis, TNM analysis and differentiation of tissue types were correlated with the expression of miRNA-21, PTEN and p27. In the study of Liu *et al* (24), miR-21 significantly inhibited the growth, migration and invasion of NSCLC cells. miRNA-21 was associated with TNM stage and lymph node metastasis, and may be an independent prognostic factor in NSCLC patients. miR-21 significantly inhibited the growth migration and invasion of NSCLC cells. In the study of Zhang *et al* (25), it was shown that the cells transfected with miR-21 inhibitor showed significantly decreased cell growth and invasion. This is basically consistent with the results of this study. A previous study shows that PTEN is a tumor inhibitor gene, and its protein product is inversely proportional to the phosphorylated Akt in endometria and breast cancer cell lines (26). In the study of Marsit *et al* (22), PTEN was often lost in lung tumors, but PTEN loss may also be a favorable prognostic marker. This suggests that miRNA-21, PTEN and p27 can be used as potential biomarkers for the diagnosis of NSCLC. The human epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases (RTKs) and plays an important role in the pathogenesis and progression of different cancers, thus it has become a major subject for scholars (27,28). In the study by Wang *et al* (29), EGFR mutation-induced drug resistance has become a major threat to the treatment of NSCLC; the resistance mechanism involves the modification of intracellular signaling pathways. According to Xu *et al* (30), molecular genetic analysis showed that KRAS mutations were frequent in NSCLC. However, there is no previous study on the relationship between EGFR mutation, KRAS mutation and expression of miRNA-21, PTEN and p27.

The clinical value of miRNA-21, PTEN and p27 was preliminarily proven through the above experiments, but for example, we did not conduct cell-based experiments. Therefore, further research is still required.

In conclusion, the expression of miRNA-21, PTEN and p27 in cancer tissue of NSCLC patients were low. ROC curve analysis shows that the three indexes have good diagnostic efficacy and are expected to be excellent indexes for early clinical diagnosis and prognosis 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

LY analyzed and interpreted the general data of the patients. JY performed PCR and was responsible for the analysis of the observation indicators. LY wrote the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Hubei Cancer Hospital (Wuhan, China). Patients who participated in this research, signed an informed consent and had complete clinical data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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