

Upregulated expression of miR-421 is associated with poor prognosis in non-small-cell lung cancer

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Background: Non-small-cell lung cancer (NSCLC) represents the most frequent subtype of lung cancer. MicroRNAs (miRNAs) have attracted a lot of attention with regard to their clinical significance and crucial biological functions in various human cancers. This study aimed to investigate the prognostic significance of microRNA-421 (miR-421) and its correlation with tumor progression in NSCLC.

Materials and methods: Expression levels of miR-421 in both serum and tissue samples were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The prognostic value of miR-421 was evaluated using Kaplan–Meier survival analysis and Cox regression assay. To explore the functional role of miR-421 during NSCLC progression, cell experiments were carried out.

Results: Expression of serum and tissue miR-421 was upregulated in the NSCLC patients compared with the normal controls (all $P < 0.001$), and the expression showed a significant correlation between the serum samples and tissues ($R = 0.475$, $P < 0.001$). The increased miR-421 expression was associated with positive lymph-node metastasis and advanced TNM stage (all $P < 0.05$). Moreover, patients with high miR-421 expression had poor overall survival compared with those with low expression (log-rank $P = 0.007$). The overexpression of miR-421 proved to be an independent prognostic factor for NSCLC (HR=1.991, 95% CI=1.046–3.791, $P = 0.036$). According to the cell experiments, the proliferation, migration and invasion of NSCLC cells were suppressed by knockdown of miR-421.

Conclusion: Overexpression of miR-421 serves as a prognostic biomarker and may be involved in the promotion of tumor progression in NSCLC.

Keywords: miR-421, prognosis, progression, non-small-cell lung cancer

Introduction

Lung cancer is one of the most common cancers worldwide and represents a leading cause of deaths due to malignancies.^{1,2} Previous research reported that the occurrence of lung cancer is influenced by diverse factors, including genetic damage to DNA and some epigenetic changes, mainly caused by smoking and air pollution.^{3,4} Two major subtypes are included in lung cancer: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC represents the most frequent type, which accounts for ~85% of all lung cancer cases.⁵ Thus, great efforts have been made to advance NSCLC treatment, and various therapeutic strategies, such as surgery, chemotherapy, radiotherapy and other anti-tumor therapy, are applied in clinical practice.^{6,7} However, the curative effect is limited in advanced stage NSCLC cases, with the 5-year survival rate being only <15%.⁸ Therefore, improving the prognosis appears to be crucial to meet the clinical requirements for patients diagnosed with NSCLC.

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Emerging studies have focused on the investigation of prognostic markers to develop cancer prognosis.⁹ Among these available biomarkers, microRNAs (miRNAs) have attracted tremendous attention for their clinical application in the diagnosis and prognosis of human cancers.¹⁰ miRNAs refer to a class of short and single-stranded endogenous RNAs (18–24 nucleotides) with the regulatory function of gene expression.¹¹ It is generally considered that miRNAs can bind the 3'-untranslated region (3'-UTR) of the target messenger RNAs (mRNAs), leading to the inhibition of translation or degradation of mRNAs.¹² A wide of oncogenes and tumor suppressors have been found to be regulated by miRNAs according to in-silico analysis, indicating their pivotal roles in the malignancies.^{13,14} Using in-silico analysis, the target genes and related signaling pathways involved in the effects of miRNAs can be identified.^{15,16} Thus, the molecular mechanisms underlying the role of miRNAs acting in human cancers could be further uncovered. The dysregulation of miRNAs is involved in the progression of tumors, such as proliferation, differentiation, metastasis, invasion and apoptosis of tumor cells.^{17,18} Therefore, identification of the miRNAs involved in the progression of NSCLC may help us to better understand this complex malignancy.

MicroRNA-421 (miR-421) is an extensively studied member of the miRNAs. It has been found to be upregulated in some human cancers as an oncogene.¹⁹ Increased expression of miR-421 has also been reported in lung adenocarcinoma.²⁰ However, the detailed role of miR-421 in NSCLC remains elusive and needs to be further investigated.

In this study, we aimed to examine the expression patterns of miR-421 in NSCLC serum and tissue samples, as well as its prognostic significance for cancer patients. In addition, the effects of miR-421 on cell proliferation, migration and invasion were analyzed in NSCLC cells.

Materials and methods

Patients and sample collection

This study included a total of 132 NSCLC patients who underwent surgery in Shouguang People's Hospital from May 2004 to April 2010. None of the patients had ever received any comprehensive therapy (chemotherapy, radiotherapy or other anti-tumor therapy) prior to the surgery. In addition, 68 healthy volunteers were recruited to act as healthy controls. Serum samples were collected from the cancer patients and healthy controls before surgery. Cancerous tissues and matched adjacent non-cancerous tissues

were collected from the patients during surgery and snap frozen in liquid nitrogen. After surgical resection, each patient was followed up for 5 years (range 3–60 months) to collect their information for survival analysis. The clinical characteristics of the NSCLC patients were recorded and are listed in Table 1. All these patients were anonymous and their personal information was protected. Written informed consent was received from the participants and the protocols of this study were approved by the Ethics Committee of Shouguang People's Hospital.

Cell cultures and transfection

Human NSCLC cell line HCC827 was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and kept at 37°C in a humidified atmosphere with 5% CO₂. The miR-421 inhibitor and its negative control (NC) were synthesized by RiboBio (Guangzhou, China). They were transfected into the HCC827 cell line with Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Table 1 Association of miR-421 with clinicopathological features of NSCLC patients

Features	All cases	miR-421 expression		P
		Low	High	
Total	132	61	71	
Age (years)				0.816
≤60	57	27	30	
>60	75	34	41	
Gender				0.857
Female	53	25	28	
Male	79	36	43	
Smoking history				0.112
No	51	28	23	
Yes	81	33	48	
Tumor size (cm)				0.145
≤3	56	30	26	
>3	76	31	45	
Lymph-node metastasis				0.008
Negative	53	32	21	
Positive	79	29	50	
Differentiation				0.339
Good/moderate	83	41	42	
Poor	49	20	29	
TNM stage				0.004
I–II	58	35	23	
III–IV	74	26	48	

Note: $P < 0.05$ are shown in bold.

Abbreviations: miR-421, microRNA-421; NSCLC, non-small-cell lung cancer.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated from tissue samples using Trizol reagent (Invitrogen) following the manufacturer's instructions. Single-stranded cDNA was synthesized from the RNA with the TaqmanTM miRNA reverse transcription kit. The expression of miR-421 was estimated by qRT-PCR, which was conducted using SYBR1 Premix Ex TaqTM (Takara, Shiga, Japan). The primers of miR-421 used in these reactions were as follows: 5'-CTCACTCACATCAACAGACATTAATT-3' (forward) and 5'-TATGGTTGTTCTGCTCTCTGTGTC-3' (reverse). *U6* was used to normalize the relative expression of miR-421 with the primers: 5'-GGAACGCTTCACGAATTTG-3' (forward) and 5'-ATTGGAACGATACAGAGAAGATT-3' (reverse). All these reactions were run on the IQ5 Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The final relative miR-421 expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to *U6*.

MTT assay

To investigate the effect of miR-421 expression on NSCLC cell proliferation, the MTT assay was conducted. Cells transfected with miR-421 inhibitor or NC were added into 96-well plates (2×10^4 cells/well) and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 3 days. The number of cells was calculated using the MTT assay every 24 hours. In brief, MTT (0.5 mg/mL; Sigma-Aldrich Co., St Louis, MO, USA) was added into each well and incubated for 1 hour. DMSO (Sigma-Aldrich) was then added into the wells following low-speed oscillation for 10 minutes. The viable cell number was measured by evaluating the absorbance at 490 nm with an enzyme-linked immune monitor (Thermo Fisher Scientific, Waltham, MA, USA).

Transwell assay

The effects of miR-421 expression on cell migration and invasion were examined using a transwell assay. The wells with Matrigel[®] were used for invasion analysis, and those without Matrigel for migration analysis. The upper chambers were filled with serum-free medium and the lower chambers filled with medium supplemented with 10% FBS. The transfected cells were seeded in the upper chambers with cell density of 2×10^4 cells/well, then incubated at 37°C for 24 hours. After the incubation, the cell number in the lower chamber was counted under an inverted microscope.

Statistical analysis

Differences between the two groups were assessed by the Student's *t*-test. Relationships between miR-421 expression and the clinicopathological features were analyzed via the chi-squared test. Pearson correlation analysis was performed to examine the degree of dependency between variables. Survival analysis was carried out based on the expression of miR-421 in NSCLC patients using the Kaplan–Meier method. Differences distributed between the survival curves were analyzed with the log-rank *t*-test. Cox regression analysis for miR-421 and clinical parameters was conducted to identify those factors that might have a significant influence on the overall survival. Differences were considered statistically significant when $P < 0.05$.

Results

Baseline characteristics of patients and expression levels of miR-421

In total, 132 pathologically diagnosed NSCLC patients were recruited in the study, comprising 79 males and 53 females with an average age of 58.34 ± 15.42 (mean $\pm$ SD) years. Of these patients, 58 were diagnosed at TNM I–II stage and 74 cases were TNM III–IV stage. The other clinical characteristics of the patients are summarized in Table 1.

According to the qRT-PCR, the serum expression of miR-421 was higher in NSCLC patients than in the healthy controls ($P < 0.001$) (Figure 1A). Similar results were obtained in tissue specimens, where the overexpression of miR-421 was detected more in the NSCLC tissues compared with the normal controls ($P < 0.001$) (Figure 1B). In addition, the serum miR-421 expression was shown to be positively correlated with the tissue miR-421 expression levels ($R = 0.475$, $P < 0.001$) (Figure 1C).

Relationship between miR-421 and clinicopathological features of NSCLC patients

To analyze the role of miR-421 in the progression of NSCLC, its association with clinicopathological data of NSCLC patients was assessed. The clinical characteristics included age, gender, smoking history, tumor size, lymph-node metastasis, differentiation and TNM stage. The results of the chi-squared test shown in Table 1 revealed that the increased expression of miR-421 was significantly correlated with positive lymph-node metastasis ($P = 0.008$) and advanced TNM stage ($P = 0.004$), which was because the tumors with positive lymph-node metastasis had high miR-421 levels,

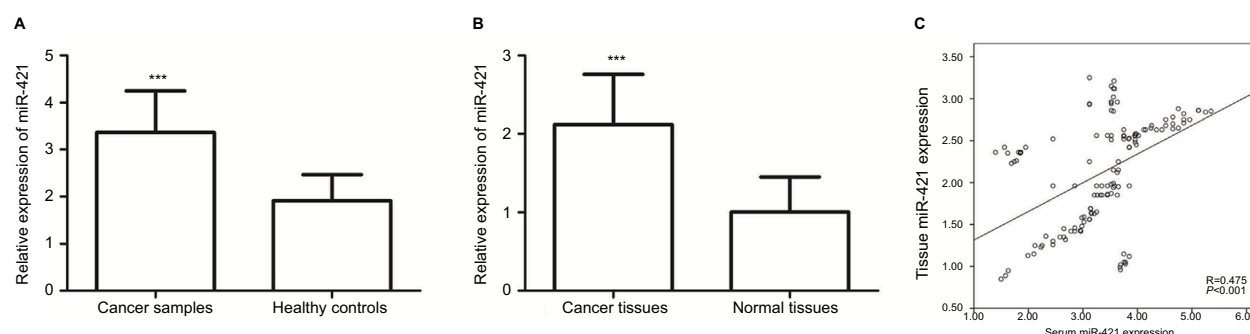


Figure 1 Expression levels of miR-421 evaluated by qRT-PCR in serum and tissue samples collected from NSCLC patients.

Notes: (A) Upregulated serum expression of miR-421 was identified in NSCLC patients compared with healthy individuals ($***P<0.001$). (B) Expression of miR-421 was higher in cancerous tissues than in normal controls ($***P<0.001$). (C) Serum expression of miR-421 was positively correlated with miR-421 expression in tissues ($R=0.475$, $P<0.001$).

Abbreviations: miR-421, microRNA-421; qRT-PCR, quantitative real-time polymerase chain reaction; NSCLC, non-small-cell lung cancer.

and advanced TNM stage tumors usually showed upregulated miR-421 expression. Conversely, no relationship was found between miR-421 expression and age, gender, tumor size, smoking history or differentiation (all $P>0.05$).

Prognostic value of miR-421 in patients with NSCLC

To uncover the relationship of miR-421 expression with overall survival of NSCLC patients, Kaplan–Meier survival analysis was carried out. The survival curves showed that overall survival was poor in patients with high miR-421 expression levels compared to those with low expression of miR-421 (log-rank $P=0.007$) (Figure 2). Moreover, multivariate Cox regression was conducted to analyze the potential factors independently correlated with overall survival. The results shown in Table 2 indicated that miR-421 was correlated with overall survival of NSCLC and acted as an independent prognostic factor for NSCLC patients (HR=1.991, 95% CI=1.046–3.791, $P=0.036$).

Effects of miR-421 on cell proliferation, migration and invasion in NSCLC cells

To further explore the functional role of miR-421 in NSCLC, miR-421 inhibitor was transfected in HCC827 cells to decrease the expression of miR-421. qRT-PCR was used to verify the expression of miR-421 and significantly downregulated miR-421 expression was found in the miR-421 inhibitor cells compared with the miR-421 NC cells ($P<0.01$) (Figure 3), indicating that the transfected cells were constructed successfully. The MTT assay results revealed that cell proliferation was suppressed by knockdown of miR-421 ($P<0.05$) (Figure 4A). From the transwell analysis, cell migration and invasion were also inhibited in the cells transfected with miR-421 inhibitor (all $P<0.001$) (Figure 4B, C).

Discussion

NSCLC is the most frequent subtype of lung cancer, and represents a serious worldwide health burden for humans.²¹ Many NSCLC patients are diagnosed with advanced disease at the initial diagnosis, contributing to the increased rate of mortality.²² Despite great progress in therapeutic strategies, the prognosis of NSCLC remains dismal. Thus, reliable prognostic biomarkers are necessary for the improvement of NSCLC treatment. Altered expression of miRNAs has been observed in various malignancies, and is significantly correlated with tumor diagnosis and prognosis.^{23,24} miRNAs have been reported to be involved in cancer progression by regulating the expression of oncogenes and tumor suppressors.²⁵ They can also serve as oncogenes or tumor suppressors in different kinds of malignancies.²⁶ For example, increased expression of miR-182 was detected in colorectal cancer tissue and serum samples, and served as a candidate biomarker for early diagnosis of colorectal cancer.²⁷ In gastric cancer, aberrant expression of miR-185 was observed in cancerous tissues compared with normal controls, and could act as a prognostic biomarker and regulate cell proliferation and migration.²⁸ Some miRNAs with aberrant expression patterns have also been identified in NSCLC, and are involved in tumor development and progression. For example, increased expression of miR-150 was detected in NSCLC samples in the study by Gu et al, which demonstrated that miR-150 could promote cell proliferation and suppress apoptosis in tumor cells.²⁹ As another example, miR-136 has been reported to be upregulated and to act as a therapeutic target in NSCLC.³⁰ All these findings indicate the crucial roles of miRNAs in the management of NSCLC. To better understand the progression of NSCLC and improve its prognosis, we investigated the expression profiles, functional role and clinical significance

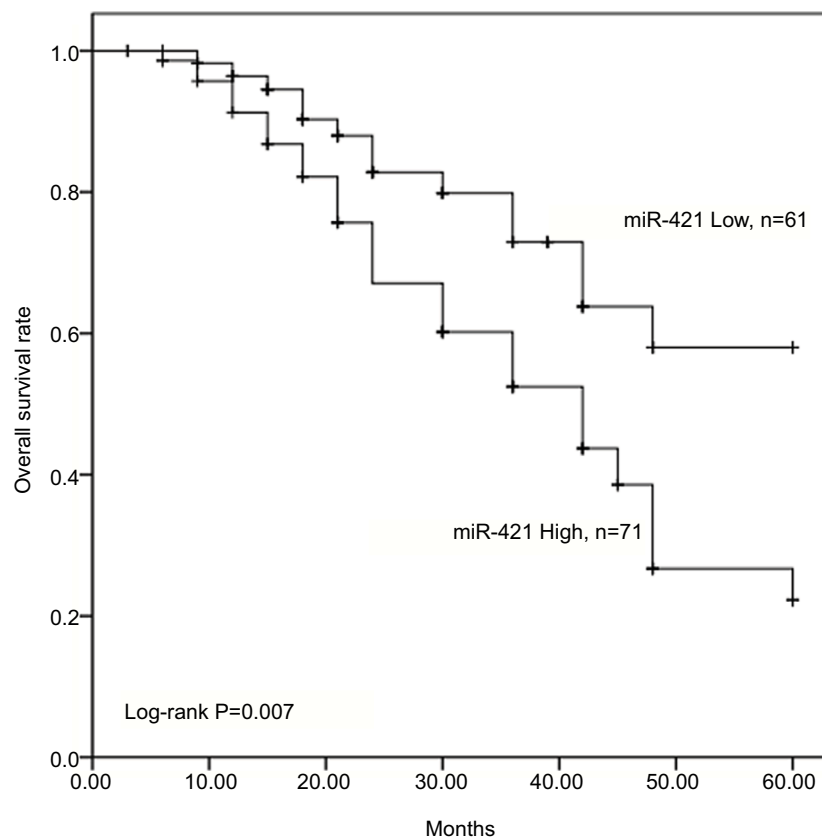


Figure 2 Kaplan-Meier survival analysis for the NSCLC patients based on different miR-421 expression.

Note: Upregulated miR-421 expression was found to be correlated with poor overall survival of patients with NSCLC (log-rank $P=0.007$).

Abbreviations: miR-421, microRNA-421; NSCLC, non-small-cell lung cancer.

Table 2 Multivariate Cox regression analysis for miR-421 expression in patients with NSCLC

Characteristics	Multivariate analysis		
	HR	95% CI	P
miR-421 (Low vs High)	1.991	1.046–3.791	0.036
Age (≤60 years vs >60 years)	1.364	0.746–2.496	0.313
Gender (Female vs Male)	1.092	0.618–1.930	0.762
Smoking history (No vs Yes)	1.026	0.563–1.869	0.934
Tumor size (≤60 cm vs >60 cm)	1.038	0.562–1.920	0.904
Lymph-node metastasis (Negative vs Positive)	1.020	0.557–1.868	0.949
Differentiation (Good/moderate vs Poor)	1.243	0.686–2.252	0.474
TNM stage (I–II vs III–IV)	1.631	0.918–2.898	0.095

Abbreviations: miR-421, microRNA-421; NSCLC, non-small-cell lung cancer.

of miR-421 in NSCLC, which have been rarely reported in this disease.

According to qRT-PCR analysis, we found significantly upregulated expression of miR-421 in NSCLC serum and

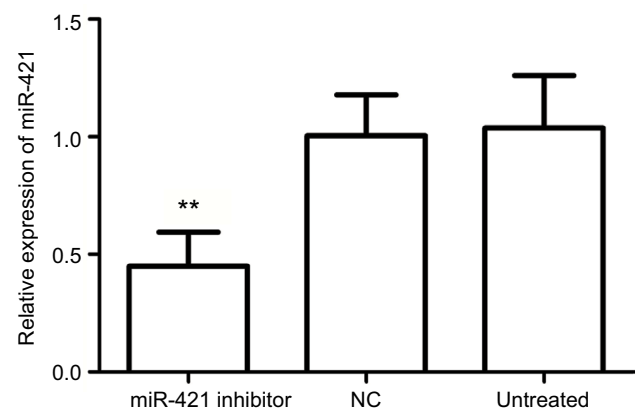


Figure 3 Expression of miR-421 in NSCLC cells regulated by miR-421 inhibitor.

Note: The expression of miR-421 was significantly downregulated in NSCLC cells transfected with miR-421 inhibitor compared with the untreated and NC cells (** $P<0.01$).

Abbreviations: miR-421, microRNA-421; NC, negative control; NSCLC, non-small-cell lung cancer.

tissue specimens compared to normal controls, and the expression in serum and tissues exhibited an obviously positive correlation. Increased miR-421 expression has also been reported in other malignancies, such as breast cancer and gastric cancer.^{31,32} Moreover, the increased expression

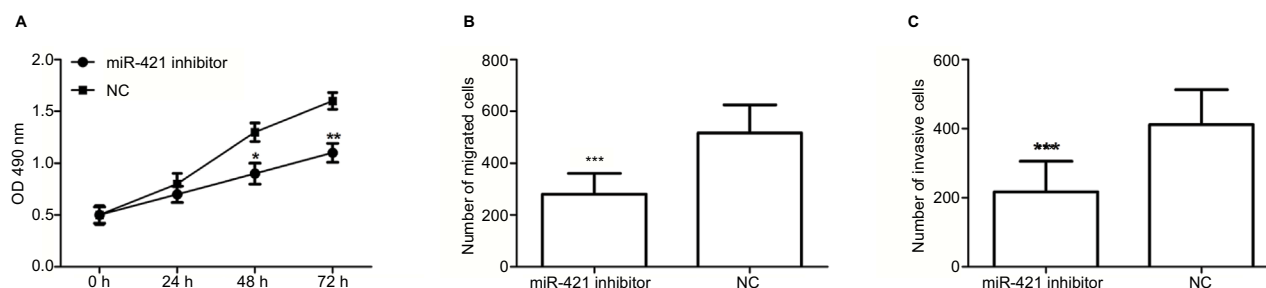


Figure 4 Effects of miR-421 expression on cell proliferation, migration and invasion of NSCLC cells in vitro.

Notes: (A) The MTT assay result indicated that the knockdown of miR-421 could suppress cell proliferation (* $P < 0.05$, ** $P < 0.01$). (B) NSCLC cell migration was significantly inhibited by miR-421 reduction (*** $P < 0.001$). (C) The invasion ability was markedly suppressed in the cells transfected with miR-421 inhibitor (*** $P < 0.001$).

Abbreviations: miR-421, microRNA-421; NC, negative control; NSCLC, non-small-cell lung cancer.

of miR-421 was first demonstrated to be associated with positive lymph-node metastasis and advanced TNM stage in NSCLC patients. These results indicate that miR-421 is a candidate oncogene and may be involved in tumor development in NSCLC. Similar results for miR-421 have also been reported in other malignancies. Yang et al found that the overexpression of miR-421 in gastric cancer tissues was associated with lymph-node metastasis and clinical stage.³² In neuroblastoma tissues, elevated miR-421 was observed and shown to be involved in tumor development by targeting menin.³³

In some human cancers, the clinical significance of miR-421 for diagnosis and prognosis has been assessed. Liu et al showed that increased expression of miR-421 was a predictor for poor prognosis of gastric cancer.³⁴ The expression of miR-421 was found to be upregulated in both serum and tissue samples collected from osteosarcoma patients, which may serve as a useful diagnostic and prognostic biomarker.³⁵ Considering the alteration of miR-421 expression in NSCLC, we further explored its prognostic significance. From the Kaplan–Meier survival curves, patients with high expression levels of miR-421 had poorer overall survival than those with low levels of miR-421. Following the multivariate Cox regression assay, the upregulated expression of miR-421 was shown to be an independent prognostic factor in patients with NSCLC.

To understand the functional role of miR-421 in tumor progression in NSCLC, its effects on cell proliferation, migration and invasion were investigated in tumor cells using miR-421 inhibitor. The results of the analysis revealed that cell proliferation, migration and invasion were all suppressed by knockdown of miR-421, indicating that miR-421 could promote tumor progression in NSCLC. However, the molecular mechanisms underlying the role of miR-421 in NSCLC remain unclear. Yang et al³² demonstrated that miR-421 may

enhance the proliferation, invasion and metastasis of gastric cancer cells by targeting Claudin 11 (*CLDN11*). In the study by Wu et al,³⁶ overexpression of miR-421 promoted cell growth and suppressed apoptosis in gastric cancer cells, and its anti-tumor effects may be exerted by targeting caspase-3. In human nasopharyngeal carcinoma, upregulated expression of miR-421 was involved in the promotion of cell proliferation and resistance of cell apoptosis through downregulation of forkhead box protein O4 (*FOXO4*).³⁷ The upregulated expression of miR-421 was also detected in biliary tract cancer, which acted as an oncogene via downregulation of farnesoid receptor (*FXR*).³⁸ However, the precise mechanisms of miR-421 acting in NSCLC remain elusive and need to be explored in further studies.

Conclusion

To our knowledge, this is the first research on the clinical performance and functional role of miR-421 in NSCLC. All the data in the present study revealed that upregulated expression of miR-421 serves as a candidate prognostic biomarker for NSCLC patients, and is associated with lymph-node metastasis and TNM stage in NSCLC. In addition, the increased miR-421 expression can promote NSCLC cell proliferation, migration and invasion, and may therefore be a potential therapeutic target for NSCLC treatment. However, the molecular mechanisms underlying the role of miR-421 in NSCLC remain unclear and warrant further investigation.

Disclosure

The authors report no conflicts of interest in this work.

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