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Original Article

Increased levels of tissue microRNA-221 in human non-small cell lung cancer and its clinical significance

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Abstract: Objective: MicroRNAs (miRNAs) play a potential role as diagnostic and prognostic biomarkers of cancers. The present study was to investigate the expression level of miR-221 in non small cell lung cancer (NSCLC), and to determine whether it can be used as a noninvasive prognostic biomarker for NSCLC. Methods: 120 pairs of fresh NSCLC and matched adjacent normal tissue specimens were collected from patients who underwent surgery at the department of thoracic surgery from 2007 to 2013. miR-221 expression in NSCLC tissues and paired normal adjacent tissues was measured by reverse transcription PCR (RT-PCR). The survival curves of the patients were determined using the Kaplan-Meier method and Cox regression, and the log-rank test was used for statistical evaluations. Results: The expression level of miR-221 was significantly upregulated in NSCLC tissues (mean relative expression level: 5.27, SD = 1.28) when compared with matched adjacent normal tissues (mean relative expression level: 1.93, SD = 0.83, $P < 0.0001$). We found significant correlations between miR-221 expression levels and clinical stage ($P = 0.003$), distant metastasis ($P = 0.006$), and tumor differentiation ($P = 0.012$). Patients with higher levels of miR-221 expression had poorer survival rates than those with lower levels of miR-221 expression ($P < 0.001$). Multivariate Cox regression analysis showed that miR-221 expression level ($P = 0.008$) was an independent factors in predicting the overall survival of NSCLC patients. Conclusion: Our results revealed that the increased expression level of miR-221 was associated with poor prognosis, suggesting that it can be used as a noninvasive prognostic biomarker for NSCLC.

Keywords: NSCLC, survival, prognosis, miR-221

Introduction

Lung cancer is one of the most common malignancies and one of the leading causes of cancer related deaths in the world. Almost 85% of lung cancer cases belong to non small cell lung cancer(NSCLC) [1]. Optimization of treatment with better surgery, cytotoxic agents and radiation therapy has not altered the prognosis much. We are now in an era where personalized medicine and targeted therapies may give new hope for this patient group. Identification of novel molecular markers which can improve diagnosis and prognostic stratification and serve as possible therapeutic targets will be of great importance in the near future.

MicroRNAs (miRNAs) are a recently discovered class of small (approximately 18-24 nucleotides in length), noncoding regulatory RNAs that negatively regulate gene expression at the posttranscriptional and/or translational level. miRNAs can trigger cleavage of target mRNAs or inhibit protein translation through sequence-specific interactions with the 3'-untranslated regions (3'-UTRs) of the target mRNAs [2, 3]. Although the full extent of the biological functionalities of miRNAs has yet to be identified, they have been suggested to act as intrinsic regulators of many cellular processes including cell invasion, differentiation, proliferation, and apoptosis [4, 5]. Furthermore, aberrant expression of miRNAs has been linked to the development and progression of cancer and has been shown to have prognostic significance in several tumor types [6-11].

miR-221 has been reported to be overexpressed in human tumor tissues, such as breast cancer, colorectal cancer and glioblas-

Table 1. Correlation between the clinicopathologic characteristics and expression of miR-221 in NSCLC

Characteristics	Number of patients	miR-221 expression		*P* value
		High	Low	
Gender				
Female	53	27	26	0.542
Male	67	33	34	
Age (years)				
≥ 60	47	26	21	0.765
< 60	73	34	39	
Smoking				
No	31	14	17	0.334
Yes	89	46	43	
Clinical stage				
I-II	67	26	41	0.003
III-IV	53	34	19	
T classification				
T1-T2	63	14	49	0.073
T3-T4	57	46	11	
N classification				
N0-N1	86	33	53	0.061
N2-N3	34	27	7	
Distant metastasis				
No	111	52	59	0.006
Yes	9	8	1	
Histological subtypes				
Adenocarcinoma	69	36	33	0.343
Squamous cell carcinoma	51	24	27	
Differentiation status				
Well	34	10	24	0.012
Moderate	47	25	22	
Poor	39	25	14	

toma [5, 6, 12-14]. However, the expression level of miR-221 and its clinical significance in NSCLC have not been reported now. The present study was to investigate the expression level of miR-221 in NSCLC, and to determine whether it can be used as a noninvasive prognostic biomarker for NSCLC.

Materials and methods

Patients and clinical samples

This study was approved by the Research Ethics Committee of Yantaishan Hospital. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards. The selection criteria for patients with NSCLC were as follows: (1) pathologically confirmed patients with NSCLC; (2) the patients had no previous history of other cancers; (3) the patients haven't received preoperative treatment such as radiation or chemotherapy. All patients were diagnosed and treated at the department of thoracic surgery, Yantaishan Hospital from 2007 to 2013. All subjects underwent clinical examination; plain chest radiograph; CT scan of the chest, upper abdomen, and brain; fiberoptic bronchoscopy; and bone scan. The pathologic diagnosis was conducted by two pathologists, and any different conclusions were resolved by careful study and discussion. Tumor stage was determined according to the 2009 TNM staging classification system. The duration of follow-up was calculated from the date of surgery to death or last follow-up, and patients were excluded if they had incomplete medical records or inadequate follow-up. Overall survival (OS) time was calculated from the date of the initial surgery to death. For qRT-PCR, 120 pairs of fresh NSCLC and matched adjacent normal tissue specimens were collected from patients who underwent surgery at the department of thoracic surgery, Yantaishan Hospital. The fresh tissue specimens were collected and immediately placed in liquid nitrogen and then stored at-80°C until the isolation of RNA. Clinicopathological features of patients are summarized in **Table 1**.

RNA isolation and qRT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of all RNA samples were detected by NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Houston, TX, USA). NCode™ SYBR® Green miRNA qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA) was used to synthesize specific cDNA of miR-221 and U6B (as an internal control), and perform qRT-PCR, which was analyzed with the DNA Engine Opticon 2 Real-Time Cycler (MJ Research Inc., Waltham, MA, USA) according to the manufacturer's instructions. Each sample was examined in triplicate and analyzed by the comparative threshold cycle (Ct) method. The expression levels of miR-221 were normalized to U6B.

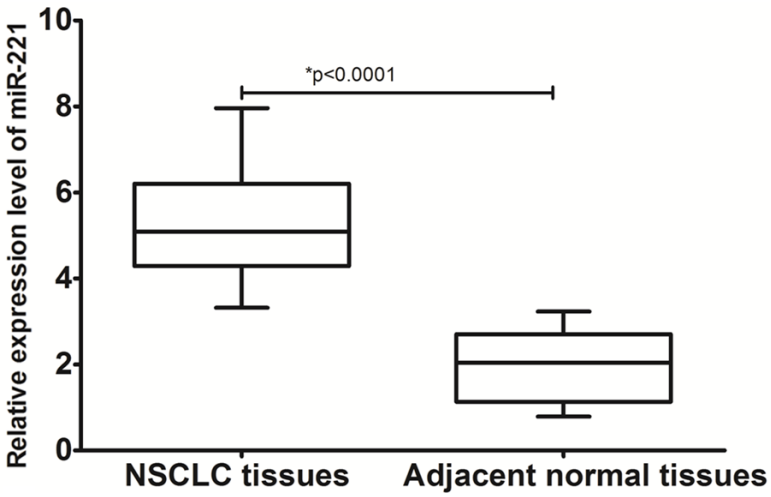

Figure 1. miR-221 expression in 120 pairs of NSCLC and matched adjacent normal tissue specimens by qRT-PCR. All data were normalized to U6B.

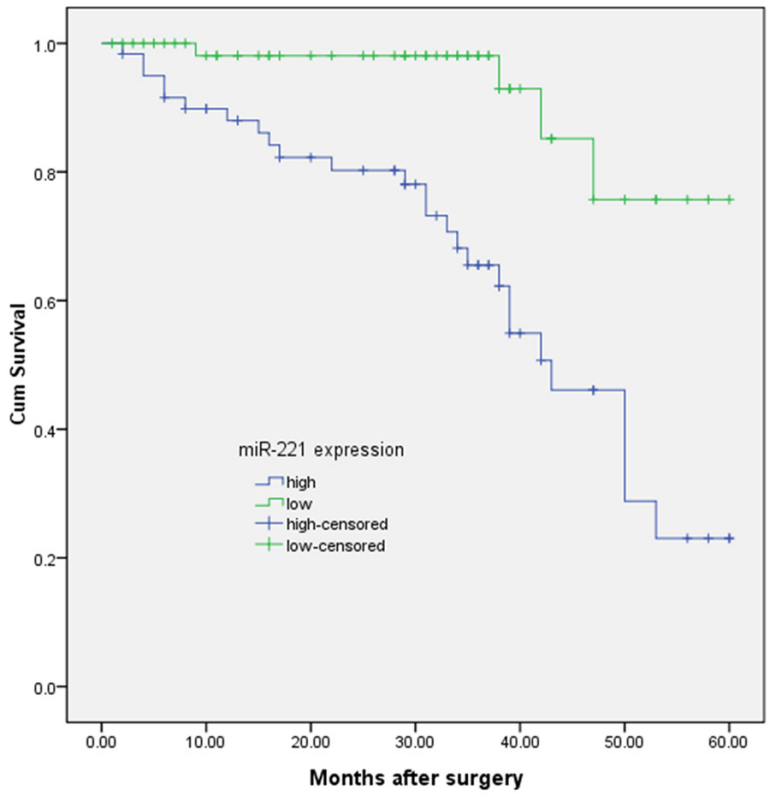

Figure 2. The expression of miR-221 in relation to overall survival in the patients with NSCLC. Patients with higher levels of miR-221 expression had poorer survival rates than those with lower levels of miR-221 expression ($P < 0.001$).

Statistical analysis

Statistical analysis was conducted using the SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). The chi-square test was used to assess miR-221 expression with respect to clinicopathological parameters. The survival curves of the patients were determined using the Kaplan-Meier method and Cox regression, and the log-rank test was used for statistical evaluations. Univariate Cox regression was performed on each clinical covariate to examine its influence on patient survival. Final multivariate models were based on step-wise addition. A Wald statistic of $P < 0.05$ was used as the criterion for inclusion in final multivariate models. Data were expressed as the mean and standard deviation and analyzed using one-way analysis of variance. $P < 0.05$ was considered to indicate a significant difference.

Results

Expression level of miR-221 in NSCLC samples

The expression level of miR-221 was significantly upregulated in NSCLC tissues (mean relative expression level: 5.27, SD = 1.28) when compared with matched adjacent normal tissues (mean relative expression level: 1.93, SD = 0.83, $P < 0.0001$, shown in **Figure 1**).

Correlation between miR-221 expression level and clinicopathological variables

The relative miR-221 expression level was classified as high or low in relation to the median value. The relationships between miR-221 expression levels and clinicopathological characteristics in individuals with NSCLC are summarized in **Table 1**. We found significant correlations between miR-221 expression levels and clinical stage ($P = 0.003$),

Table 2. Summary of multivariate Cox regression analyses of overall survival duration

	Multivariate analysis		
Parameter	*P*	HR	95% CI
Gender	0.672	0.862	0.278-1.954
Age	0.189	1.283	0.781-3.019
Smoking	0.276	1.223	0.653-2.764
Clinical stage	0.009	5.016	2.964-8.324
T classification	0.073	3.765	0.842-6.543
N classification	0.053	5.764	0.954-7.865
Distant metastasis	0.011	4.875	3.556-10.875
Pathology classification	0.852	0.842	0.567-2.342
Differentiated degree	0.012	4.554	2.677-9.654
miR-221 expression	0.008	5.711	3.545-10.882

distant metastasis ($P = 0.006$), and tumor differentiation ($P = 0.012$). However, we did not find a significant association of miR-221 expression levels with gender ($P = 0.542$), age ($P = 0.765$), smoking status ($P = 0.334$), T classification ($P = 0.073$), N classification ($P = 0.061$), and pathology classification ($P = 0.343$).

miR-221 expression correlates with prognosis of NSCLC patients

To investigate the prognostic value of miR-221 expression for NSCLC, we assessed the association between the expression levels of miR-221 and patient survival using Kaplan-Meier analysis with the log-rank test. In 120 NSCLC cases with prognosis information, we observed that the level of miR-221 expression was significantly correlated with the overall survival of NSCLC patients (shown in **Figure 2**). Patients with higher levels of miR-221 expression had poorer survival rates than those with lower levels of miR-221 expression ($P < 0.001$). To determine the possibility of miR-221 as an independent risk factor for poor prognosis, both clinicopathological factors and the level of miR-221 expression were evaluated by multivariate Cox regression analysis. Results showed that clinical stage ($P = 0.009$), distant metastasis ($P = 0.011$), differentiated degree ($P = 0.012$), and miR-221 expression level ($P = 0.008$) were independent factors in predicting the OS of NSCLC patients (shown in **Table 2**).

Discussion

Approximately two thirds of NSCLC cases were diagnosis at locally advanced (27.6%) or metastatic (38.1%) disease as the typically asymptomatic at early stages [15]. It is well known that pathologic TNM category, age, sex, and cell type are all important prognostic factors for the patients with NSCLC [16]. The advances in molecular biology have enabled researchers to focus on molecular or biological markers in NSCLC.

A complete understanding of the molecular mechanisms underlying tumor initiation and progression is essential for novel therapeutic approaches aimed at improving the outcome of patients with cancer. Further more, there is still a need to improve early detection screening methods and to identify new prognostic biomarkers. Ideal biomarkers should be easy to measure and have a strong association with clinical outcome. miRNAs could match these proposed criteria, so they could be used as biomarkers for diagnosis and prognosis of cancer. miR-221 is considered as a microoncogene. miR-221 plays an important role in epithelial-to-mesenchymal transition (EMT). It has been identified as a basal-like subtype-specific miRNA that downregulates the expression of epithelial-specific genes and enhances the expression of mesenchymal-specific genes. Furthermore, miR-221 increases cell migration and invasion [17-19]. The basal-like transcription factor, FOSL1, can directly stimulate the transcription of miR-221 [19]. The abundance of miR-221 reduces with the suppression of mitogen-activated or extracellular signal-regulated protein kinase (MEK) [19]. The miR-221-mediated reduction in E-cadherin is dependent on the targeting of the 3'-UTR of trichorhinophalangeal syndrome type 1 (TRPS1). TRPS1 inhibits EMT by directly repressing the expression of Zinc finger E-box-binding homeobox 2 (ZEB2) [20]. Thus, miR-221 could contribute to the aggressive clinical behavior of various types of cancers.

miR-221 has been reported to be overexpressed in human tumor tissues, such as breast cancer, colorectal cancer and glioblastoma [5, 6, 12-14]. Furthermore, high level of miR-221 expression is correlated with metastasis, tumor capsular infiltration, tumor stage, and poor prognosis. However, the expression level of miR-221 and its clinical significance in NSCLC have not been reported now. In the present study, we found that the expression level of miR-221 was significantly upregulated in NSCLC

tissues when compared with matched adjacent normal tissues. Significant correlations were observed between miR-221 expression levels and clinical stage, distant metastasis, and tumour differentiation, suggesting that miR-221 might be involved in the carcinogenesis and metastasis of NSCLC. More importantly, patients with higher levels of miR-221 expression had poorer survival rates than those with lower levels of miR-221 expression. Multivariate Cox regression analysis showed that miR-221 expression level was an independent factors in predicting the overall survival of NSCLC patients (HR: 5.711; 95% CI: 3.545-10.8228, $P = 0.008$). Empirically, HR of more than 1.5 is considered to be a strong prognostic factor. So miR-221 might have a considerable potential in prognosis for patients with NSCLC.

In summary, our study has shown that the higher expression of miR-221 is associated with poor survival in patients with NSCLC, suggesting that it can be used as a noninvasive prognostic biomarker for NSCLC. More clinical studies should be carried out before the application of miR-221 in prognosis of patients with NSCLC.

Disclosure of conflict of interest

None.

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