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Clinical impact of serum miR-661 in diagnosis and prognosis of non-small cell lung cancer

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Abstract. – OBJECTIVE: The aim of this study was to evaluate whether serum miR-661 could serve as a biomarker for the diagnosis and prognosis of non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: The expression of serum miR-661 was detected in 150 cases of NSCLC and 114 cases of normal healthy controls by Real-time PCR. Receiver-operating characteristic (ROC) curve analysis was applied to analyze diagnostic value of serum miR-661. The relationship between serum miR-661 expression and clinicopathological characteristics was investigated. Overall survival was analyzed using Kaplan-Meier method. Moreover, Cox proportional-hazards regression analyses were performed to determine the prognostic value of serum miR-661 in NSCLC patients.

RESULTS: We found that the expression of serum miR-661 was significantly upregulated in NSCLC compared with healthy controls ($p < 0.01$). The expression level of serum miR-661 was positively correlated with histological grade ($p = 0.011$), lymph node metastasis ($p = 0.003$), distant metastasis ($p = 0.021$) and clinical stage ($p = 0.005$). Then, ROC curve analysis showed that serum miR-661 has considerable diagnostic accuracy, with an area under the ROC curve (AUC) of 0.726 ($p = 0.001$). Moreover, NSCLC patients with serum miR-661 higher expression have shown significantly poorer overall survival than those with lower serum miR-661 expression ($p = 0.004$). Furthermore, multivariate analyses showed that serum miR-661 expression levels were an independent prognostic factor for survival in NSCLC patients.

CONCLUSIONS: Overall, these findings indicate that serum miR-661 may be a potential biomarker for the diagnosis and prognosis of NSCLC.

Key Words:
Serum miR-661, NSCLC, Prognosis, Diagnosis.

Introduction

Lung cancer remains a leading cause of cancer-related mortality and morbidity worldwide, and non-small cell lung cancer (NSCLC) accounts for approximately 80% of lung cancer diagnoses[1,2]. Although advancements of diagnosis and treatment have improved the survival of patients with NSCLC, the 5-year overall survival of NSCLC patients is reported to be only 30-60%[3,4]. High rates of recurrence and metastasis are the major reasons responsible for the poor outcome[5]. Until now, several independent prognostic factors for survival have been identified: age, sex, and disease stage[6]. However, these factors alone are not sufficient to predict prognosis of NSCLC patients. Thus, more efforts should be made to identify NSCLC biomarkers, which might improve the outcome of NSCLC patients.

MicroRNAs (miRNAs) are a type of short non-coding RNAs with conserved sequences, which target the 3'untranslated regions (3'-UTRs) of certain genes to regulate their expression[7]. There is a large body of evidence that microRNAs play important roles in regulation of various biological processes such as proliferation, survival and development[8]. Recent findings showed that aberrant expression of many miRNAs was linked to human diseases, especially cancer[9-11]. More and more studies indicated that abnormal expression of miRNAs was found in the blood[12]. Importantly, miRNAs were easier to detect in blood comparison with cancerous tissues. Several serum miRNAs were reported to serve as useful biomarkers in tumor detection[13,14]. However, most serum miRNAs in NSCLC have not been identified

MiR-661 is encoded by its own transcript at chromosome 8q24.3. It has been demonstrated that miR-661 acted as either an oncogene or tumor suppressor in various human tumors including NSCLC[15,16]. However, the role of serum miRNA-661 in the progression of NSCLC remains unknown. The aim of the present work was

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to explore the expression and clinical significance of serum miR-661 in NSCLC patients.

Patients and Methods

Patients and specimens

One hundred and fifty serum samples from NSCLC patients were collected from Jining No. 1 People's Hospital (Jining, China). The diagnosis of all samples was histopathologically confirmed by two pathologists. None of the patients had received neoadjuvant chemo- or radio-therapy before surgery. The clinicopathological findings were determined according to the classification of malignant tumors by the World Health Organization. Overall survival (OS) was defined as the time interval between the date of surgery and the date of death. Informed consent was obtained from all participants, and the study was approved by the Ethics Committee of the Jining No. 1 People's Hospital.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from serum samples using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany); both miRNA and mRNA were reverse transcribed to cDNA. Amplification and detection of serum miR-661 were performed using a miRNA-specific TaqMan miRNA assay kit (Applied Biosystems, Foster City, CA, USA) under ABI PRISM 7900 Sequence Detection System applied (Applied Biosystems, Foster City, CA, USA). U6 snRNA was used as a loading control. The primer sequences were presented as follows: miR-661 5'-ACACTCCAGCTGGGTGCCTGGGTCTCTGGCCT-3' (forward) and 5'- CTCAACTGGTGTCGTGGA' (reverse), U6 5'- CTCGCTTCGGGCAGCACA-3' and 5'- AACGCTTCACGAATTTGCGT-3'. Gene expression was normalized to the level of U6 within each sample using the relative $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All statistical analyses were performed using the SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). Each experiment was repeated at least three times. The x^2-test was used to investigate the significance of serum miR-661 expression as correlated with clinicopathologic features in NSCLC. The receiver operating characteristic (ROC) curve was performed to calcu-

Table I. Correlation between miR-661 expression and clinicopathological variables of NSCLC cases.

	MiR-661 expression			
Variable	**Number**	**Low**	**High**	**χ^2 test *p*-value**
Age				0.835
< 60	65	31	34	
≥ 60	85	42	43	
Sex				0.567
Male	91	46	45	
Female	59	27	32	
Tumor size				0.301
< 5 cm	105	54	51	
≥ 5 cm	45	19	26	
Histological grade				0.011
I	100	56	44	
II-III	50	17	33	
Histology type				0.644
Adenocarcinoma	50	23	27	
Squamous	100	50	50	
Lymph node metastasis				0.003
Yes	46	14	32	
No	104	59	45	
Distant metastasis				0.021
Yes	44	15	29	
No	106	58	48	
Clinical stage				0.005
I-II	96	55	41	
III-IV	54	18	36	

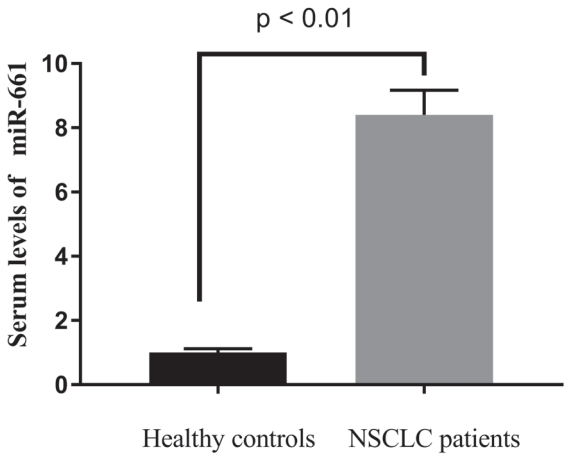

Figure 1. Serum miR-661 expression in patients with NSCLC and in healthy controls was analyzed by Real-time qRT-PCR.

late the sensitivity, specificity and area under curve (AUC) for serum miR-661 levels. Differences in NSCLC patient survival were assessed using the Kaplan-Meier method and analyzed using the log-rank test in a univariate analysis. A Cox proportional hazards model was used for multivariate analysis. All the reported *p*-values were two-sided, and $p < 0.05$ was considered significant.

Results

Serum miR-661 Expression is Upregulated in NSCLC

Previous study has indicated that miR-661 was significantly up-regulated in NSCLC tissues. However, its expression in the sera from NSCLC patients has not been previously investigated until now. We firstly detected serum miR-661 expression in NSCLC patients and healthy controls. As shown in Figure 1, we found that expression of serum miR-661 was significantly upregulated in NSCLC compared with healthy controls ($p < 0.01$).

Diagnostic Potential of Serum miR-661 Levels in NSCLC

In order to explore diagnostic value of serum miR-661 expression in discriminating NSCLC from healthy controls, ROC curves and the area under the ROC curves (AUC) were performed on data from all subjects, including 150 NSCLC patients and 114 healthy controls. As shown in Figure 2, we observed that the serum miR-661 level was a potential biomarker for screening NSCLC patients from healthy controls with the area under the ROC curve (AUC) of 0.726. When the cut-off value was set to 1.69, the sensitivity and specificity of discriminating serum miR-661 in NSCLC patients were 60.7% and 84.6%, respectively.

Correlation of Serum miR-661 Expression with Clinicopathological Characteristics of NSCLC Patients

To identify the clinical relevance of serum miR-661 expression in NSCLC, we divided 150 NSCLC patients into a high expression group (n =77) and a low expression group (n =73) based on the mean expression of serum miR-661. The relationships between serum miR-661 expression levels and clinicopathological features were shown in Table I. We found that the expression level of serum miR-661 was positively correlated with histological grade ($p = 0.011$), lymph node metastasis ($p = 0.003$), distant metastasis ($p = 0.021$) and clinical stage ($p = 0.005$). However, serum miR-661 expression levels were reversely correlated with patients' sex, age, tumor size, and histology type (all, $p > 0.05$).

Serum miR-661 Overexpression is Poor Prognostic factor for NSCLC Patients

In NSCLC patients with prognosis information, we found that NSCLC patients with high serum miR-661 expression level had distinctly shorter overall survival than patients with low serum miR-661 expression level ($p = 0$ .004, Figure 3).

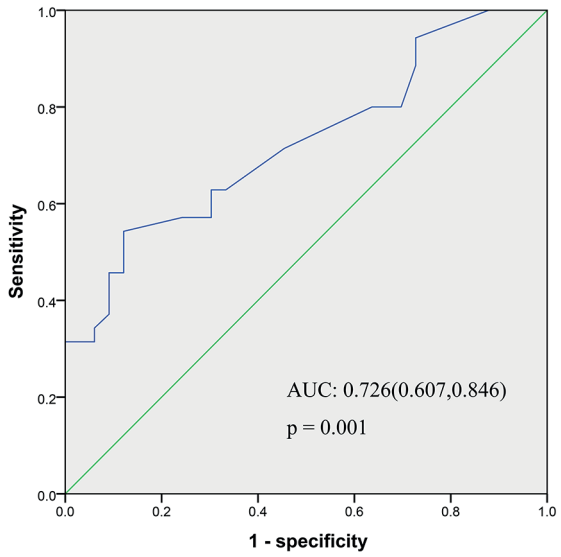

Figure 2. The ROC curve analysis for discriminative ability between NSCLC cases and normal healthy controls (AUC = 0.726, sensitivity: 0.607, specificity: 0.846).

Table II. Univariate and multivariate analysis of overall survival in 150 patients with NSCLC.

	Univariate analysis		Multivariate analysis	
Variable	**HR**	***p*-value**	**HR**	***p*-value**
Age	1.478	0.381	-	-
Sex	1.231	0.619	-	-
Tumor size	1.834	0.131	-	-
Histology type	1.311	0.217	-	-
Histological grade	3.452	0.011	3.131	0.017
Lymph node metastasis	4.349	0.003	3.988	0.005
Distant metastasis	2.891	0.029	2.362	0.033
Clinical stage	3.883	0.007	3.341	0.009
Serum miR-661 expression	3.732	0.002	3.553	0.001

Then, univariate and multivariate analyses were used to explore whether the serum miR-661 was independent prognostic parameter. As shown in Table II, our results showed that serum miR-661 was an independent prognostic factor for overall survival in patients with NSCLC.

Discussion

NSCLC is becoming one of most mortal threats to human health and life. Although radiotherapy and chemotherapy are effective for the treatment of NSCLC patients, NSCLC is usually resistant to radiotherapy and chemotherapy[17,18]. It has been reported that early NSCLC has a good prognosis compared to advanced NSCLC[19]. Current diagnostic system is hard to be used in the mass screening. Thus, it is important to develop novel methods to improve the findings of early NSCLC. On the other hand, in clinical practice, accurate prediction in prognosis of NSCLC patients is helpful for the decision of treatment strategy[20]. To date, although many markers were reported to be associated the prognosis of NSCLC patients, most of these markers had not been proven to be sufficiently effective[21]. Thus, identification of novel effective molecular markers is important for the improvement of diagnostic and prognostic techniques.

Recently, serum miRNAs attracted scientist' attention. Growing studies focused on the effect of serum miRNAs to act as early detection and/or prognostic biomarkers for various human cancers. For instance, Yue et al[22] reported that serum microRNA-205 have diagnostic value in the differentiation of glioma patients from healthy controls. Furthermore, they also found that serum miR-205 expression was independently associated with overall survival in glioma patients. Cai et al[23] found that abnormal expression of serum miR-195 could be used to screen osteosarcoma and predict poor prognosis. Another study by Li et al[24] showed that serum miR-499 was an independent prognostic factor for NSCLC. By ROC assay, they proved serum miR-499 as promising biomarker for early detection of NSCLC. These findings suggested that serum miRNAs have potential in the diagnosis and assessment of prognosis of NSCLC.

The role and function of miR-661 have been reported in progression of several tumors. For instance, Li et al[25] found that over-expression of miR-661 suppressed glioma cell proliferation, mi-

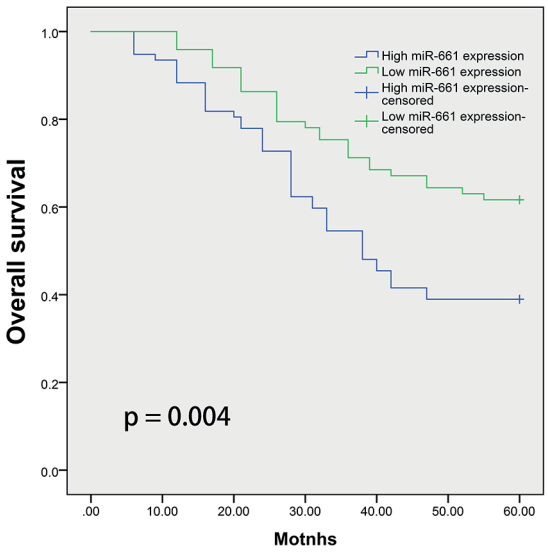

Figure 3. Kaplan-Meier curves stratified according to expression levels of serum miR-661.

gration and invasion by targeting hTERT. Fan et al[26] also reported that miR-661 served as a tumor suppressor in osteosarcoma by modulating the expression of cytochrome c1. However, Zhu et al[27] revealed that miR-661 upregulation promoted cell proliferation of ovarian cancer by repressing INPP5J expression. These results indicated that the function of miR-661 was different according to the types of tumors. Liu et al[16] reported that miR-661 was upregulated in NSCLC tissues, and its overexpression could promote tumor invasion and metastasis by directly inhibiting RB1. Furthermore, they firstly provided evidence that high miR-661 expression was associated with poor prognosis of NSCLC patients. However, to our best knowledge, the expression and the clinical significance of serum miR-661 levels in NSCLC have not been described.

In the present study, we firstly found that serum miR-661 was up-regulated in NSCLC patients relative to healthy controls. Importantly, high expression of serum miR-661 was found to associate with low histological grade, positive lymph node metastasis, distant metastasis and advanced clinical stage. Furthermore, the results of ROC assay indicated that serum miR-661 can be used to effectively discriminate between patients with NSCLC and healthy controls. Subsequently, we analyzed prognostic significance of serum miR-661 in NSCLC patients. Kaplan-Meier analysis with the log-rank test indicated that patients with serum higher expression have shown significantly poorer overall survival than those with lower serum miR-661 expression. Moreover, in the multivariate Cox proportional hazards analysis, high serum miR-661 expression was independently associated with poor survival. Taken together, our findings indicated that serum miR-661 have potential in predicting the diagnosis and prognosis of NSCLC patients.

Conclusions

We showed a strong relation between the expression of serum miR-661 in NSCLC and healthy controls, indicating that serum miR-661 could be a useful biomarker in the diagnosis and prognosis of NSCLC. Further study with a larger case population is needed to validate these results.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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