Upregulation of MiR-1280 Expression in Non-small Cell Lung Cancer Tissues

Li-Min Xu[1], Li-Qin Li[1], Jing Li[1], Hong-Wei Li[2], Qi-Bin Shen[2], Jin-Liang Ping[3], Zhi-Hong Ma[1], Jing Zhong[1], Li-Cheng Dai[1]

[1]Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou, Zhejiang 313000, China
[2]Department of Thoracic Surgery, Huzhou Central Hospital, Huzhou, Zhejiang 313000, China
[3]Department of Pathology, Huzhou Central Hospital, Huzhou, Zhejiang 313000, China

Abstract

Background: Non-small cell lung cancer (NSCLC) is a prolific and high-mortality disease with few effective treatments. Although the detection and surgical techniques for NSCLC continue to advance, the survival rate for the patients with NSCLC remains poor. Enhanced predictive biomarkers such as microRNAs (miRNAs) are needed at the time of diagnosis to better tailor therapies for patients. This study focused on the expression of miR-1280 in NSCLC tissues and distal normal tissues in order to explore the association between miR-1280 expression and NSCLC.

Methods: A total of 72 newly diagnosed primary NSCLC patients were enrolled in this study. Quantitative real-time polymerase chain reaction (PCR) was performed to identify the expression level of miR-1280 in the NSCLC tissues and distal normal tissues of these patients.

Results: The miR-1280 expression was significantly higher in the NSCLC tissues (0.084 ± 0.099) than distal normal tissues (0.014 ± 0.015, $P = 0.009$). In 54 patients (75%), the miR-1280 expression in the NSCLC tissues was upregulated ($2^{-\Delta\Delta ct} > 2$), and no case showed a downregulation of miR-1280 expression.

Conclusions: The expression level of miR-1280 could be regarded as a biomarker for NSCLC.

Key words: Biomarker; miR-1280; Non-small Cell Lung Cancer; Upregulation

INTRODUCTION

Lung cancer is one of the most common malignant tumors and also is the leading cause of cancer mortality worldwide, with non-small cell lung cancer (NSCLC) accounting for approximately 80% of all cases.[1] Approximately 1.6 million cases of lung cancer are newly diagnosed per year throughout the world.[2] According to a report by National Cancer Institute, the new cases and deaths of lung and bronchialus cancer were estimated about 13.5% and 27.2% of all cancer in the US in 2014.[3] Targeted therapeutics such as epidermal growth factor receptor tyrosine kinase inhibitors and anaplastic lymphoma kinase fusion protein inhibitors have significantly improved the therapeutic response of NSCLC and have revolutionized therapy.[4,5] Although the detection and surgical techniques continue to advance, the 5-year survival rate for NSCLC patients remains poor.[2,5] Previously unknown biomarkers such as noncoding RNA gene products may also be associated with NSCLC. Therefore, a better understanding of the novel molecular

mechanisms involved in NSCLC is urgently needed to guide the diagnosis, prognosis, and treatment of this disease.

MicroRNAs (miRNAs) are a class of small noncoding RNA molecules that range in size from 19 to 22 nucleotides (nt) and predominantly regulate gene expression at the posttranscriptional level.[6-8] Evidences have suggested that miRNAs can contribute to the multiple processes of cellular proliferation, differentiation, migration, and apoptosis through regulating specific target mRNA expressions.[8] Overexpression of let-7 miRNA family members has been shown to suppress tumor development in mouse models of lung cancer.[9] In the two most common forms of NSCLC (adenocarcinoma and squamous cell carcinoma), a high expression of miR-155 and a low expression of let-7 correlate with a poor survival.[8,10] Our previous miRNA array showed that miR-1280 is associated with NSCLC. In addition, a recent study has shown that miR-1280 is upregulated in colorectal cancer but downregulated in pancreatic cancer, and further analysis revealed little related information about miR-1280.[11] Moreover, another group has found that miR-1280 is significantly downregulated in bladder cancer cell lines and tumors.[12] Due to the tissue

Address for correspondence: Dr. Li-Cheng Dai,
Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, #198, Hongqi Road, Huzhou, Zhejiang 313000, China
E-Mail: dlc21@126.com

specificity of miR-1280, the aim of this study was to explore the association between miR-1280 expression and NSCLC by focusing on the expression of miR-1280 in NSCLC tissues and distal normal tissues.

METHODS

Patients and tissue specimens

A total of 72 newly diagnosed primary NSCLC patients (48 males and 24 females) were enrolled in this study from September 2010 to December 2013 in Huzhou Center Hospital, Zhejiang University. All the patients were classified according to the 7th International Union Against Cancer tumor, lymph node, and metastasis staging system.[13] None of these patients had received radiotherapy or chemotherapy before surgery. The distribution of ages was from 53 to 76 years old, and the average age was 65.14 ± 6.64 years old. All paired tissue samples (NSCLC and distal normal tissues) were surgically obtained from the newly diagnosed NSCLC patients, instantly frozen in liquid nitrogen, and stored at $-80°C$ until RNA extraction. This study was approved by the Ethics Committee of Huzhou Center Hospital, and all participants provided informed consent.

RNA extraction and reverse transcription

Total RNAs were extracted from tissues by Trizol (Invitrogen, USA), according to the manufacturer's protocol. For reverse transcription (RT) reactions, the All-in-One™ miRNA quantitative RT polymerase chain reaction (qRT-PCR) Detection Kit (No. AOMD-Q050, GeneCopoeia, USA) was applied for cDNA synthesis. The RT mixture contained 2 µg of total RNA (the volume of total RNA was calculated from the concentration detected after extraction), 2.5 U/µl PolyA Polymerase, RTase Mix, 5× Reaction Buffer, and diethylpyrocarbonate-treated H_2O to 25 µl. The prepared reaction mixture was incubated at 37°C for 60 minutes, inactivated at 85°C for 5 minutes, and then stored at 4°C until use.

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR was conducted using the All-in-One™ qPCR Mix (No. AOPR-0200, GeneCopoeia, USA) on the 7500 real-time PCR System (Applied Biosystems, USA). PCR reactions were performed in a total volume of 20 µl including 2× All-in-One qPCR Mix, Universal Adaptor PCR Primer (2 µmol/L), 2 µl of primer, 2 µl of cDNA (diluted 1:5), ROX Reference Dye, and ddH2O. The qPCR conditions included a preincubation stage at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 20 seconds, and primer extension at 72°C for 30 seconds, then a melting curve (90°C for 10 seconds, 60°C for 30 seconds, and 90°C 10 seconds). For normalization, *U48* was used as an internal reference gene according to our preliminary experiments. Six common internal reference genes (*U6*, *U44*, *U47*, *U48*, *U49*, and *U69*) were selected for the preliminary experiments. Only the *U48* gene was stable and had a smaller CT value, while the others showed large fluctuations in different samples. Considering the stability and sensitivity, *U48* was chosen the internal reference gene

rather than *U6*. The primers used for amplification were supplied by All-in-One™ qPCR Primer (GeneCopoeia, USA). Water was used as a negative and quality control, and each sample was measured in triplicate.

Statistical analysis

Statistical analyses were performed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All continuous variable values were expressed as mean ± standard deviation (SD). Comparison between two groups was performed with a two-tailed Student's *t*-tests. The genes with $2^{-\Delta\Delta ct} > 2$ were set as upregulated and $2^{-\Delta\Delta ct} < 0.5$ as downregulated. Therefore, an expression of $0.5 < 2^{-\Delta\Delta ct} < 2$ was regarded as the reference level. A $P < 0.05$ was considered to be statistically significant.

RESULTS

A total of 72 NSCLC patients were enrolled in this study. The clinical characteristics of these patients were presented in Table 1. The expression levels of miR-1280 in the NSCLC tissues and paired distal normal tissues were detected by quantitative real-time PCR, normalized by *U48*, and presented as $2^{-\Delta ct}$.[14] According to statistical analysis, the data were normal distribution. The expression level of miR-1280 was significantly higher in NSCLC tissues (0.084 ± 0.099) than in the distal normal tissues (0.014 ± 0.015, $P = 0.009$) [Figure 1].

To further investigate the correlation of miR-1280 expression with NSCLC, the relative expression level was analyzed by the $2^{-\Delta\Delta ct}$ method.[15] In this study, the values were calculated by the special formula ($\Delta\Delta ct = \Delta ct_{lung\ cancer} - \Delta ct_{distal\ normal\ tissues}$). The miR-1280 expression of 54 (75%) patients was upregulated out of a total of 72 NSCLC patients ($2^{-\Delta\Delta ct} > 2$), and the other 18 cases showed a reference level of expression ($0.5 < 2^{-\Delta\Delta ct} < 2$). As shown in Table 1, the patients were separated into groups according to gender, age, disease type, and differentiation degree

Table 1: The association of miR-1280 expression and clinical characteristics in 72 NSCLC patients (*n*)

Characteristics	Number of patients	Upregulated level* (*n* = 54)	Reference level† (*n* = 18)	*P*‡
Gender				0.774
Male	48	35	13	
Female	24	19	5	
Age				0.682
>60 years old	63	48	15	
≤60 years old	9	6	3	
Disease type				0.525
Adenocarcinoma	56	43	13	
Squamous carcinoma	16	11	5	
Differentiation degree				0.571
Poor	26	18	8	
Moderate	46	36	10	

*Upregulated level: $2^{-\Delta\Delta ct} > 2$; †Reference level: $0.5 < 2^{-\Delta\Delta ct} < 2$. ‡*P* values were obtained by Chi-squared test and represented the expression difference in terms of gender, age, disease type, differentiation degree, respectively. NSCLC: non-small cell lung cancer.
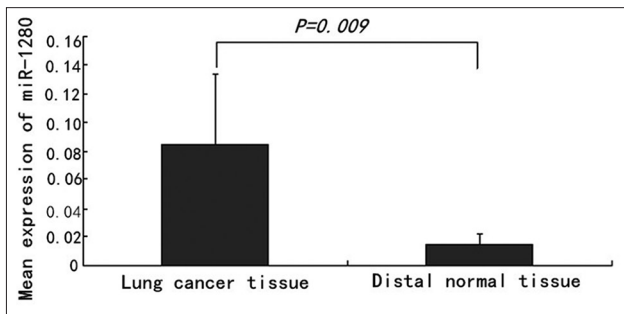
Figure 1: The expression levels of miR-1280 in nonsmall-cell lung cancer tissues and distal normal tissues. The miR-1280 expression was higher in lung cancer tissues than in distal normal tissues ($P = 0.009$).

in order to explore possible influencing factors. The results demonstrated that males and older persons were vulnerable to NSCLC. Meanwhile, adenocarcinoma was more common than squamous carcinoma in our study. In summary, there was no association between miR-1280 expression and gender, age, disease type, or differentiation degree. In addition, the correlation of miR-1280 expression with ten indicators for tumors, such as carcino-embryonic antigen, alpha fetal protein, carbohydrate antigen (CA) 125, CA199, *etc*., was analyzed (data not shown), but no significant correlation was found in this analysis.

DISCUSSION

MiRNAs are nonprotein-coding sequences that can function as oncogenes or tumor suppressor genes.[8,12] RNA molecules are highly conserved across different species and are highly specific for tissues and developmental processes; therefore, they can be biomarkers for lung cancer.[16] MiRNAs play a key role as regulators of gene expression.[8]

In 2005, Lu *et al*.[17] demonstrated the potential for miRNAs as diagnostic tumor markers when they were able to indicate the tumor embryonic origin using miRNA expression profiles. Either upregulation or downregulation of miRNAs has been associated with the clinicopathological parameters of different cancers, including lung cancer,[10] hepatocellular carcinoma,[18] breast carcinoma,[19] and others. Mishra *et al*.[20] have shown that the androgen receptor and miR-21 drive the downregulation of transforming growth factor-β receptor II by acting through a positive feedback loop that inhibits growth responses in prostate cancer. MiR-145 is a tumor suppressor that can be downregulated in breast cancer[19] and colorectal cancer,[21] and its expression is regulated by the p53 pathway.[22] More and more miRNAs are discovered through microarray platforms to the qRT-PCR platforms. Therefore, we detected the miRNAs by microarray and validated the results through qRT-PCR.

More than half of miRNA genes are located in cancer-associated genomic regions; and several miRNAs have low expression levels in lung cancer cell lines, which were first discovered in 2004.[23] In the same year, let-7 miRNA was reported to have a reduced expression in NSCLC patients,[24] and the result was further demonstrated by other independent studies.[25,26] Later, it was shown that the let-7 family had an onco-suppressor activity in NSCLC tumor development in mice xenografts[9] and was

regulated by Ras protein.[25] The tumor suppressor protein p53 directly regulates the expression of miR-125a[27] and miR-34 family members, and the upregulation of the latter miRNA resulted in the inhibition of genes associated with cell cycle control[28] and promotion of apoptosis[29] in lung cancer cells. In addition, Liu *et al*.[30] found that overexpression of serum miR-21 in NSCLC patients was strongly associated with lymph node metastasis and an advanced stage of NSCLC. Meanwhile, the increased miR-21 expression was significantly presented in platinum-based chemotherapy-resistant NSCLC patients and associated with a shorter survival.[31] Other experiments found that miR-21 and miR-205 were frequently upregulated and that miR-126-5p was often downregulated in lung cancer tissues when compared with the corresponding noncancerous lung tissues.[10] Moreover, squamous and nonsquamous NSCLCs can be distinguished according to the expression of miR-205.[8,32]

There is little known about miRNA-1280 as it relates to cancer. One study has shown the miR-1280 expression of 19 colorectal and 17 pancreatic human cancer samples, demonstrating that miR-1280 is upregulated in colorectal cancer and downregulated in pancreatic cancer.[11] Another study first reported that miR-1280 acts as a tumor suppressor and has diagnostic and prognostic potential in bladder cancer as it is significantly downregulated in bladder cancer cell lines and tumor tissues.[12] Fluorescence-activated cell sorting analysis revealed that re-expression of miR-1280 in bladder cancer cells induced G2–M cell cycle arrest and apoptosis. In our study, we reported the association of miR-1280 with NSCLC for the first time. Previous studies have shown that miRNAs are highly tissue specific and can act as tumor suppressor or oncogenes.[33,34] MiR-205 can also play a dual role, depending on the specific tumor context and target genes.[35] In a meta-analysis, the expression of miR-183 family members was most consistently upregulated in colorectal cancer and prostate cancer.[36] Bladder cancer, lung cancer, and hepatocellular carcinoma have been frequently reported to have significant overexpression of miR-96, miR-182, and miR-183, respectively. Breast cancer and gastric cancer had inconsistent regulations, and the members of this family had their own distinct regulation characteristics in different cancers.[36] In terms of thyroid cancer, miR-125b is significantly overexpressed in thyroid follicular carcinoma samples and papillary thyroid cancer but down regulated in anaplastic thyroid carcinomas, relative to normal thyroid tissue.[37,38] Differential tissue distributions of miRNA may suggest that its function may be tissue specific or even stage specific. Therefore, we also analyzed the miR-1280 expression according to paired samples of adenocarcinoma and squamous carcinoma as well as poorly and moderately differentiated cancers. Unfortunately, we found no association. Furthermore, miR-1280 expression was not related with gender, age, or ten indicators for tumors. However, the upregulation of miR-1280 in NSCLC indicated that miR-1280 can be regarded as a biomarker for lung cancer.

In conclusion, we found that the miR-1280 was diagnostic for NSCLC because miR-1280 was upregulated in the

majority of NSCLC tissues compared with distal normal tissues. However, this findings needs to be confirmed in a larger cohort of tissues. Important factors such as smoking history and the 5-year survival rate are necessary to complete this study, but we failed to get the related information. In the future, cultured cancer cell lines should be studied to analyze the pathways and target genes responsible for the cancer development, prevention, and treatment. We hope that some of the miRNA strategies will be further developed and used, alone or in combination with chemotherapy, to improve the specificity of treatment for lung cancer patients.

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