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Original article

Down-regulation of microRNA-181b is a potential prognostic marker of non-small cell lung cancer

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ABSTRACT

The aim of this study was to investigate the clinical significance of microRNA-181b (miR-181b) expression in non-small cell lung cancer (NSCLC).

MiR-181b expression in 126 pairs of surgically removed NSCLC tissues and their corresponding normal lung tissues was measured by real-time quantitative RT-PCR assay. Additionally, the correlation of miR-181b expression with clinicopathological factors or prognosis of patients was analyzed.

At first, miR-181b expression was significantly down-regulated in NSCLC tissues as compared with their normal counterparts ($P < 0.001$). Then, the low miR-181b expression was found to be closely correlated with larger tumor size ($P = 0.02$), higher p-TNM stage ($P = 0.008$) and positive lymph node metastasis ($P = 0.03$) of NSCLC patients. After that, survival analysis found that the overall survival ($P = 0.001$) and disease-free survival ($P = 0.008$) of NSCLC patients with low miR-181b expression were both significantly poorer compared to those patients with high miR-181b expression. Finally, both univariate and multivariate analyses demonstrated that low miR-181b expression may be a poor prognostic marker of NSCLC patients.

This is the first study to indicate that down-regulation of miR-181b may be correlated with aggressive disease progression and poor prognosis of NSCLC patients, suggesting that miR-181b might be involved in lung carcinogenesis and become a potential prognostic marker for NSCLC.

Introduction

Lung cancer represents the most widespread cancer worldwide. It is the leading cause of cancer-related death in the developed world, accounting for 26–29% of estimated cancer deaths [1]. With the advanced development of the society, lung cancer has become a global health problem with a poor clinical outcome. More than 1 million deaths annually attributed to lung cancer and less than 10% of people with this disease live longer than 5 years after diagnosis [2]. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancers, and the majority (60–80%) of patients are diagnosed at an advanced stage, for which the prognosis of NSCLC patients remains very poor with a 5-year survival of 15%, although there has been some progress in the treatment of NSCLC in recent years [3,4]. Therefore, it is of great significance to investigate the molecular mechanisms involved in lung carcinogenesis, and to identify diagnostic and prognostic markers for early detection and targeted treatment of lung cancer.

MicroRNAs (miRNAs) are a group of small, noncoding, endogenous single-stranded RNAs that regulate the expression of ~60% human genes. Mammalian miRNAs are generally encoded in introns in pre-messenger RNA (mRNA) or the 3′ untranslated region of mRNA [5]. They reduce gene expression by binding to complementary regions of mRNA and either blocking translation or degrading mRNA through the RNA-induced silencing complex. Recent studies have demonstrated that miRNAs are evolutionary conserved and tissue-specific, and are involved in tuning of many important pathways, including developmental and oncogenic pathways [6–8]. Thus, the alteration of miRNA regulation may play a critical role in development and differentiation processes of tissues and organs, and their aberrant expression may be implicated in carcinogenesis and disease progression [9–12]. MicroRNA-181b (miR-181b) belongs to the miR-181 family, which consists of 4 members, miR-181a, miR-181b, miR-181c, and miR-181d, which are thought to have regulatory roles at a post-transcriptional level, through complementarity to target mRNAs [13]. This miRNA family has been predicted or experimentally confirmed in a wide number of vertebrate species, such as rat, zebrafish, and in pufferfish [14]. The biological functions of the miR-181 family were firstly identified when miR-181a was recognized as a contributor to hematopoietic lineage commitment and differentiation [15]. Later

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studies identified miR-181b as a regulator of the B cell primary antibody repertoire based upon its ability to restrict the activity of activation-induced cytidine deaminase [16]. Several studies have detected the abnormal expression of miR-181b in multiple tumors and leukemia/lymphoma. It functions as a tumor suppressor or a tumor promoter in various human malignancies. For example, Visone et al. [17] found that miRNA-181b was down-regulated in aggressive B-cell chronic lymphocytic leukemia with 11q deletion as compared with the indolent form of chronic lymphocytic leukemia; Shi et al. [18] reported the downregulation of miR-181b in both human gliomas and glioma cell lines, confirming the data of Ciafrè et al. [19], and they also showed that transfection of miR-181b triggered growth inhibition, apoptosis, and inhibited invasion of gliomas [18]. In contrast, Jiang et al. [20] reported that the expression of miR-181b was significantly overexpressed in gastric tumors compared to normal gastric tissues. Kaplan–Meier survival analysis revealed that the low level of miR-181b might be closely associated with better patient's overall survival. Interestingly, the previous study of Zhu et al. [21] found that miR-181b was downregulated in multidrug-resistant human lung cancer cell line. However, its involvement in clinical NSCLC has not been fully elucidated. The aim of this study was to investigate the clinical significance of miR-181b expression in NSCLC.

Materials and methods

Patients and tissue samples

This study was approved by the Research Ethics Committee of People's Hospital of Tangshan City, PR China. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

A total of 126 patients analyzed in this study underwent resection of the primary NSCLCs at People's Hospital of Tangshan City between 1999 and 2003. One hundred and twenty-six pairs of NSCLC tumors and their matched histologically normal lung parenchyma adjacent to the tumors (5 cm distant from the discrete tumor margin) were immediately cut and snap-frozen in liquid nitrogen before stored at −80 °C until RNA extraction. All patients did not receive chemotherapy or radiotherapy prior to surgery. Disease histology was determined in accordance to the criteria of the World Health Organization. Pathologic staging was performed in accordance to the current International Union against Cancer tumor-lymph node-metastasis classification. The clinicopathologic features of all patients are summarized in Table 1.

Clinical follow-up was available for all patients (median, 18 months; range, 1–87 months). Follow-up was conducted every 3 months by telephone after the primary surgical treatment. The final date of follow-up was July 23, 2012. At the last follow-up, 36 patients were still alive, whereas 90 had died. Overall survival time was calculated from the date of the initial surgical operation to death. Disease-free survival was calculated from the date of the initial surgical operation to the date of second cancer, tumor recurrence, distant metastases or death from any cause. Patients who died of diseases but without direct relation to their NSCLCs or of unexpected events were excluded from this study.

Real-time quantitative RT-PCR for miRNA

Real-time quantitative RT-PCR for miRNA was performed to detect the expression levels of miR-181b in clinical NSCLC and nontumorous lung tissues. Total RNA was extracted from tissue samples of 126 primary NSCLC and matched nontumorous lung tissues using TRIzol (Invitrogen) according to the manufacturer's protocol. The concentrations of samples were measured spectrophotometrically, and the quality was checked on agarose gel. Then, the miR-181b and RNU6B-specific cDNA were synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA assays protocol (Applied Biosystems, Foster City, CA, USA). The miRNA-specific primer sequences were 5′-ACGCAAATTCGTGAAGCGTT-3′ for RNU6B and 5′-TTGTAAGTAACGACAGCCACCC-3′ for miR-181b. RNU6B small nuclear RNA was used as an internal control in the quantitative RT-PCR to normalize RNA input. Reverse transcriptase reactions contained 10 ng of total RNAs, 50 nmol/l stem-loop RT primer, 1× RT buffer, 0.25 mmol/l each of deoxynucleotide triphosphate (dNTP), 3.33 U/μl MultiScribe reverse transcriptase, and 0.25 U/μl RNase Inhibitor. The 10-μl reaction volumes were incubated in Bio-Rad i-Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a 96-well plate for 30 min at 15 °C, 30 min at 40 °C, 5 min at 85 °C, and then held at 4 °C. Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system. The 10-μl PCR included 0.6 μl of RT products, 1X TaqMan Universal PCR master mix, and 1 μl of primers and probe mix of the TaqMan MicroRNA Assays. Relative quantification of target miRNA expression was evaluated using the comparative cycle threshold (CT) method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to RNU6B. Each sample was examined in triplicate. Mean normalized gene expression ± standard deviation (SD) was calculated from independent experiments.

Table 1
Correlation between miR-181b expression and different clinicopathological features of clinical NSCLC patients.

Clinicopathological features	No. of cases	miR-181b expression		*P*
		High (*n*, %)	Low (*n*, %)	
Age				
<56	66	33 (50.0)	33 (50.0)	NS
≥56	60	23 (38.3)	37 (61.7)	
Gender				
Male	82	37 (45.1)	45 (54.9)	NS
Female	44	19 (43.2)	25 (56.8)	
Tumor size (cm)				
≤3.0	55	33 (60.0)	22 (40.0)	0.02
≥3.0	71	23 (32.4)	48 (67.6)	
Histological type				
Squamous cell carcinoma	52	23 (44.2)	29 (55.8)	NS
Adenocarcinoma	67	31 (46.3)	36 (53.7)	
Other	7	2 (28.6)	5 (71.4)	
Differentiation grade				
High	18	9 (50.0)	9 (50.0)	NS
Moderate	50	22 (44.0)	28 (56.0)	
Low	58	25 (43.3)	33 (56.7)	
p-TNM stage				
I–II	35	28 (80.0)	7 (20.0)	0.008
III–IV	91	28 (30.8)	63 (69.2)	
Lymph node metastasis				
Negative	17	10 (58.8)	7 (41.2)	0.03
Positive	109	46 (42.2)	63 (57.8)	

Statistical analysis

All computations were carried out using the software of SPSS version12.0 for Windows (SPSS Inc, IL, USA). Data were expressed as mean ± SD. For comparison of means between paired clinical NSCLC and nontumorous lung tissue groups, a paired *t*-test was used. The analysis of variance (ANOVA) was used to determine the statistical differences among groups. The Kaplan–Meier method was used to estimate survival rates, and the log-rank test was used to assess survival differences between groups. The Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to survival. Differences were considered statistically significant when p was less than 0.05.

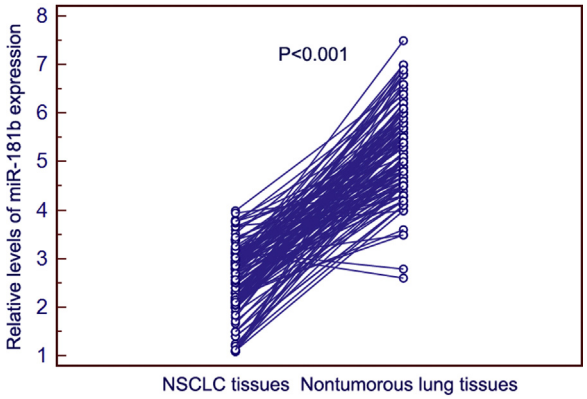

Fig. 1. MiR-181b expression in 126 pairs of clinical NSCLC and nontumorous lung tissues were respectively detected by real-time quantitative RT-PCR assay. After normalization to RNU6B expression levels, the expression level of miR-181b in NSCLC tissues (mean ± SD: 2.5 ± 0.7) was significantly lower than that in non-tumor tissues (mean ± SD: 5.3 ± 0.9, $P < 0.001$).

Results

Patient characteristics

Among the 126 patients with NSCLC, 82 were male and 44 were female. The median age of the NSCLC subjects was 55.8 years (range: 26–82 years). The subtypes included 52 squamous-cell carcinomas and 67 adenocarcinomas. Of 126 patients with NSCLC, 35 (27.8%) patients were in p-TNM stage I–II, and 91 (72.2%) patients were in p-TNM stage II–IV. According to the differentiation grade, 18 (14.3%), 50 (39.7%), and 58 (46.0%) cases had high, moderate, and low differentiation grade, respectively. In addition, there were 109 (86.5%) patients with positive lymph node metastasis. Details are shown in Table 1.

MiR-181b expression in clinical NSCLC and nontumorous lung tissues

MiR-181b expression in 126 pairs of primary NSCLC and nontumorous lung tissues was detected by real-time quantitative RT-PCR. As shown in Fig. 1, after normalization to RNU6B expression levels, the relative levels of miR-181b expression (mean ± SD: 2.5 ± 0.7) in NSCLC tissues were significantly lower than in corresponding nontumorous lung tissues (mean ± SD: 5.3 ± 0.9, $P < 0.001$). NSCLC tissue samples expressing miR-181b at levels less than the median expression level (2.6) were assigned to the low expression group (mean expression value 2.0, $n = 70$), and those samples with expression above the median value were assigned to the high expression group (mean expression value 3.2, $n = 56$).

Correlation of miR-181b expression with clinicopathological parameters of clinical NSCLC patients

Subsequently, the correlation of miR-181b expression with clinicopathological features of NSCLC patients is shown in Table 1. By statistical analyses, we showed that low miR-181b expression was closely correlated with larger tumor size ($P = 0.02$), higher p-TNM stage ($P = 0.008$), and positive lymph node metastasis ($P = 0.03$) of NSCLC patients. However, the expression of miR-181b was not correlated with other factors of patients, including age, gender, histological type, and differentiation grade (all $P > 0.05$).

Correlation of miR-181b expression with prognosis in clinical NSCLC patients

The correlation of miR-181b expression with prognosis in clinical NSCLC patients was further investigated by Kaplan–Meier analysis and log-rank test. As shown in Fig. 2, the 5-year overall survival ($P = 0.001$, Fig. 2A) and the 5-year disease-free survival ($P = 0.008$, Fig. 2B) of NSCLC patients with low miR-181b expression were both significantly poorer compared to those patients with high miR-181b expression. Univariate analysis showed that p-TNM stage, lymph node metastasis and low miR-181b expression were significantly correlated with poor overall survival ($P = 0.006$, 0.01 and 0.001, respectively; Table 2) and disease-free survival ($P = 0.01$, 0.03 and 0.008, respectively; Table 2) of NSCLC patients. Multivariate analysis using the Cox proportional hazard model indicated that p-TNM stage and low miR-181b expression were independent prognostic factors for both overall survival ($P = 0.01$ and 0.008, respectively; Table 3) and disease-free survival of NSCLC patients ($P = 0.03$ and 0.02, respectively; Table 3), while the status of lymph node metastasis was only an independent prognostic factor for overall survival ($P = 0.03$; Table 3) of NSCLC patients.

Discussion

As one of the most common malignancies in the world, the prognosis of NSCLC patients remains very poor although recent

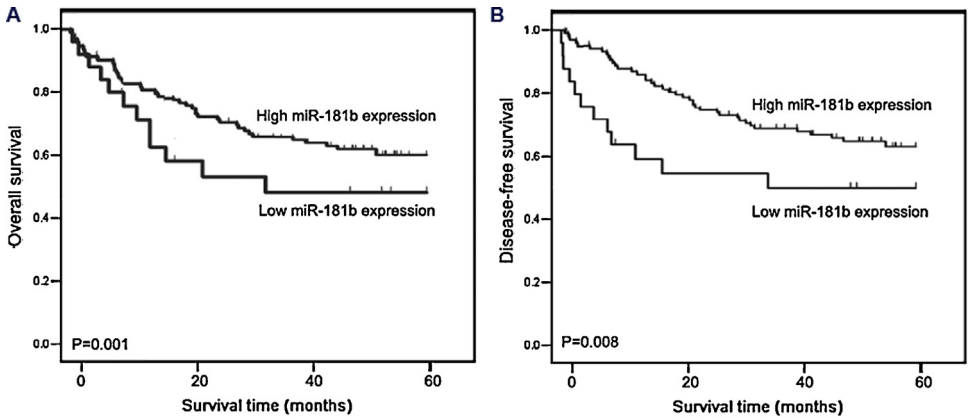

Fig. 2. Kaplan–Meier curves for survival time in patients with NSCLCs divided according to miR-181b expression. Overall survival and disease-free survival of patients with high vs. low miR-181b expression levels are shown. (A) The 5-year overall survival rate of NSCLC patients with low miR-181b was significantly poorer compared to those patients with high miR-181b ($P = 0.001$). (B) The 5-year disease-free survival rate of NSCLC patients with low miR-181b was significantly poorer compared to those patients with high miR-181b ($P = 0.008$).

Table 2
Univariate analysis of prognostic parameters in patients with NSCLCs by Cox regression analysis.

Variables	Overall survival		Disease-free survival	
	P value	Relative risk (RR)	*P* value	Relative risk (RR)
Age at diagnosis (years)				
<56 vs. ≥56	0.33	0.82	0.71	0.58
Gender				
Male vs. female	0.62	0.61	0.89	0.46
Histological type				
Squamous cell carcinoma vs. adenocarcinoma and other	0.13	1.26	0.15	1.21
Differentiation grade				
High vs. moderate and low	0.27	0.99	0.31	0.87
p-TNM stage				
I–II vs. III–IV	0.006	4.25	0.01	3.25
Tumor size				
<5 cm vs. ≥5 cm	0.08	1.68	0.10	1.32
Lymph node metastasis				
Negative vs. positive	0.01	3.79	0.03	2.53
MiR-181b expression				
High vs. low	0.001	5.01	0.008	3.89

advances have been made in improving diagnosis and treatment strategies. Accumulating studies have indicated that miRNAs may play important roles in carcinogenesis and have become potential biomarkers for early diagnosis and prognosis in various human cancers. The key finding of the present study is that the down-regulation of miR-181b may be associated with the progression of NSCLC. Results of real-time RT-PCR show that miR-181b is down-regulated in primary NSCLC specimens. The expression level of miR-181b significantly correlates with the tumor size, p-TNM, and the status of lymph node metastasis of NSCLC patients. Statistical analyses reveal that patients with low expression of miR-181b have a poorer prognosis. Taken together, the aforementioned data suggest for the first time that miR-181b is a significant predictor of advanced progression and poor prognosis for NSCLC patients.

MiR-181b expression in cancer is controversial, because recent studies have indicated that it is up-regulated or down-regulated in different cancer tissues as compared with normal tissues. MicroRNA expression profiles in different cancers have found that miR-181b expression is down-regulated in astrocytoma [22] and acute myeloid leukemia [23], but is up-regulated in gastric cancer [20], retinoblastoma [24], pancreatic ductal adenocarcinoma [25], osteosarcoma [26], as well as head and neck cancers [27]. In the present study, we observed that miR-181b was markedly down-regulated in clinical NSCLC tissues as compared with nontumorous lung tissues, which is consistent with the previous report of Zhu et al. [21] in human lung cancer cell line A549/cisplatin. These findings indicate that reduced miR-181b expression in NSCLC might be important for tumor progression of this disease. To examine this possibility, we analyzed the correlation of miR-181b expression with clinicopathological features of clinical NSCLC patients. Our results demonstrated that the low miR-181b expression was more frequent in NSCLC tissues with larger tumor size, higher pathological TNM stage, and positive lymph node metastasis, suggesting the correlation of altered miR-181b expression with the aggressiveness of human NSCLC. More interestingly, several previous studies have demonstrated the prognostic value of miR-181b in various human cancers [20,22]. In the current study, we also identified miR-181b down-regulation as an independent predictor for short overall survival and disease-free survival of NSCLC patients.

However, precise molecular mechanisms for the altered expression of miR-181b in NSCLC remain unclear thus far. Recent studies have demonstrated that miR-181b may be involved in tumorigenesis and tumor progression by regulating various targets, such as tissue inhibitor of metalloproteinase 3 (TIMP3) [28], Insulin-like growth factor I receptor (IGF-1R) [18], and cAMP responsive element binding protein 1 (CREB1) [29]. Interestingly, one explanation for its involvement in NSCLC could be that it was caused by the aberrant expression of Neuropilin 1 (NRP-1), a mediator of lung branching and angiogenesis in embryonic development and angiogenesis in cancer [30]. Previously, our data showed overexpression of NRP-1 in NSCLC tissues as compared with normal lung tissues, and demonstrated that NRP-1 might be closely correlated with invasion and metastasis of NSCLC [31]. Cui et al. [32] confirmed NRP-1 as a target of miR-181b. Considering these findings, we supposed that downregulation of miR-181b might play critical roles in NSCLC by posttranscriptionally regulating NRP-1 expression.

In conclusion, this is the first study to indicate that the down-regulation of miR-181b may be correlated with aggressive disease progression and poor prognosis of NSCLC patients, suggesting that miR-181b might be involved in lung carcinogenesis and become a potential prognostic marker for NSCLC. Further studies of the molecular mechanisms by which miR-205 contributes to the initiation and progression of NSCLC are warranted.

Table 3
Multivariate analysis of prognostic parameters in patients with NSCLCs by Cox regression analysis.

Variables	Overall survival		Disease-free survival	
	P value	Relative risk (RR)	P value	Relative risk (RR)
p-TNM stage				
I–II vs. III–IV	0.01	3.41	0.03	2.26
Lymph node metastasis				
Negative vs. positive	0.03	2.17	0.06	1.62
MiR-181b expression				
High vs. low	0.008	4.09	0.02	2.55

Conflict of interest

None.

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