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Original Article

MicroRNA-148b is down-regulated in non-small cell lung cancer and associated with poor survival

Hui Ge, Bin Li, Wen-Xia Hu, Rui-Juan Li, Hao Jin, Meng-Meng Gao, Cui-Min Ding

Department of Respiratory Medicine, The Fourth Hospital of Hebei Medical University, Shijiazhuang 050011, Hebei, China

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Abstract: Background: The aim of this study was to clarify the clinicopathological significance of miRNA-148b (miR-148b) expression in NSCLC, and to explore the correlation between miR-148b level and the prognosis of patients with NSCLC. Methods: 151 patients diagnosed with NSCLC between May 2007 and April 2012 were included in the present study. Real-time RT-PCR method was used to assess the expression levels of miR-148b. The differences between two groups were assessed using Student's t -test, and the Kaplan-Meier method was used to estimate overall survival. Results: The expression of miR-148b was decreased in tumor tissues compared to corresponding adjacent normal lung tissues (0.37 ± 0.12 vs. 1.00 ± 0.53, $P < 0.05$). Low miR-148b expression was significantly associated with TNM stage ($P = 0.014$), lymph node metastasis ($P = 0.031$), and distant metastasis ($P = 0.008$). Kaplan-Meier survival analysis showed that patients with low expression of miR-148b had significantly worse overall survival rates compared with those who had cancers with high miR-148b expression (log-rank test $P = 0.039$). Furthermore, multivariate Cox proportional hazards model analysis showed that miR-148b expression was independently associated with overall survival of patients with NSCLC (HR = 2.357, 95% CI: 1.612-9.212, $P = 0.011$). Conclusion: our data indicate that decreased expression of miR-148b in NSCLC tissues has prognostic value.

Keywords: MicroRNAs, miR-148b, NSCLC, survival

Introduction

Lung cancer still remains the most common cancer and the most common cause of cancer-related death worldwide [1]. Non-small cell lung cancer (NSCLC), which accounts for about 85% of lung cancer cases, is further divided to histological subtypes of adenocarcinoma, squamous cell carcinoma and large cell carcinoma. The 5-year survival rate of NSCLC remains low as 15-16%, despite improvement of treatment in the past decades. Furthermore, few histology-dependent prognostic biomarkers are available for routine use in clinical practice, especially in resectable patients.

MicroRNAs (miRNAs), a group of non-coding RNAs, can repress the expression of multiple target genes through endogenous RNA interference machinery [2, 3]. Increasing evidence suggests that miRNAs confer delicate biological processes and provides robustness via regulation of target networks [4]. MiRNAs are dysregulated in a variety of human cancers, and specific signatures of aberrantly expressed miRNAs harbor diagnostic, prognostic and therapeutic implications. MiRNA-148b (miR-148b) is located at chromosome 12q13 and recent studies have found it is downregulated in oral, pancreatic, colon and gastric cancer tissues [5-10]. Furthermore, an increasing number of reports indicate that miR-148b acts as a tumor suppressor by targeting specific oncogenes. Previously, Liu et al reported that miR-148b functioned as a tumor suppressor in NSCLC by targeting carcinoembryonic antigen (CEA) [11]. However, to our knowledge, the clinical significance of miR-148b in NSCLC has not been addressed yet. In the present study, we investigated the association of miR-148b with clinicopathological characteristics and overall survival of the patients with NSCLC.

Materials and methods

Patients and specimens

A total of 151 patients diagnosed with NSCLC who underwent surgical resection at the Fourth

Table 1. Relationship between miR-148b expression and clinicopathological characteristics of patients with NSCLC

Variables	Cases (n)	miR-148b expression		*P* value
		Low	High	
Age (years)				
< 60	59	28	31	0.38
≥ 60	92	49	43	
Gender				
Male	84	45	39	0.82
Female	67	32	35	
TNM stage				
I + II	91	34	57	0.014
III + IV	60	43	17	
Lymph node metastasis				
Yes	49	38	11	0.031
No	102	39	63	
Distant metastasis				
Yes	18	17	1	0.008
No	133	60	73	
Histological type				
Adenocarcinoma	66	32	34	0.78
Squamous cell carcinoma	70	36	34	
Others	15	9	6	
Type of Surgery				
Lobectomy/bilobectomy	69	35	34	0.61
Pneumonectomy	61	32	29	
Atypical resection	21	10	11	
Smoking History				
Current smoker	45	29	16	0.21
Former smoker	101	47	54	
Never smoker	5	1	4	
Family history of lung cancer				
Yes	32	19	13	0.09
No	119	58	61	

Hospital of Hebei Medical University between May 2007 and April 2012 were included in the present study. None of these patients had received radiotherapy or chemotherapy prior to surgery. The histomorphology of all the tissue specimens was confirmed by the Department of Pathology, Fourth Hospital of Hebei Medical University. Patients' clinical information, such as age, sex and differentiation status, was collected and stored in a database. The follow-up information of all the participants was updated every 3 months by a telephone call and questionnaire letters. The death of the participants was ascertained by a report from the family and verified by the review of public records. The present study was approved by the Research Ethics Committee of the Fourth Hospital of Hebei Medical University. Written informed consent was obtained from all patients. Clinicopathological characteristics of all patients with NSCLC are shown in **Table 1**.

Real-time PCR

Total RNA and enrichment of small RNA from fresh samples was isolated using the mirVana miRNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and then stored at -70°C until use. Total RNA from fresh cultured cells was carried out with TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. Real-time RT-PCR method was used to assess the expression levels of miR-148b with Express SYBR® GreenER qPCRs supermix Universal kit (Invitrogen) on a Rotor-gene 6000 system (Qiagen, Valencia, CA, USA). U6 RNA was used as an endogenous reference for normalizing the expression levels of miR-148b. Initially, we calculated a $^{\Delta}$ctt (target-reference), which is equal to the difference between threshold cycles for miR-148b (target) and those for U6 RNA (reference). The fold-change between cancer tissues and normal ovary tissue control for miR-148b was calculated with the $2^{\Delta\Delta ct}$ method, in which $^{\Delta\Delta}$ct = $^{\Delta}$Ct (target-reference in tumor samples)- $^{\Delta}$Ct (target-reference in normal samples). The relative expression levels of miRNAs in cancer compared to their non-tumorous controls were calculated using the method of $2^{-\Delta\Delta ct}$.

Statistical analysis

Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS) software 18.0 (SPSS Inc., Chicago, IL, USA). The differences between two groups were assessed using Student's t-test, and the Kaplan-

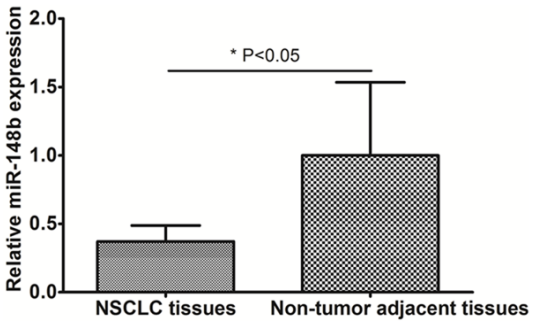

Figure 1. miR-148b was downregulated in tumor tissues compared to corresponding adjacent normal lung tissues (0.37 ± 0.12 vs. 1.00 ± 0.53, $P < 0.05$).

Meier method was used to estimate overall survival. Differences in survival between two groups were analyzed using the log-rank test. Multivariate Cox proportional hazard regression analysis with stepwise selection was used to evaluate independent prognostic factors associated with patient survival. Two-tailed tests were used, and P values < 0.05 were considered to indicate statistical significance.

Results

miR-148b was down-regulated in human NSCLC tissues

To compare the miR-148b expression levels in the NSCLC tissues, we performed real-time RT-PCR analysis on pairs of tissue containing normal and tumor tissue samples from the same donor. As shown in **Figure 1**, miR-148b was downregulated in tumor tissues compared to corresponding adjacent normal lung tissues (0.37 ± 0.12 vs. 1.00 ± 0.53, P < 0.05). According to the median level of miR-148b, 77 cases of NSCLC were classified as low miR-148b expression group, while 74 cases were classified as the high expression group.

Association of miR-148b expression levels with the clinicopathological characteristics

We further investigated the association of miR-148b expression with clinicopathological characteristics of 151 NSCLC patients to explore the potential role of miR-148b in tumor progression. Results showed that aberrant miR-148b expression was significantly associated with TNM stage ($P = 0.014$), lymph node metastasis ($P = 0.031$), and distant metastasis ($P = 0.008$). These results showed that decreased expression of miR-148b in NSCLC was associated with significantly aggressive pathologic features, indicating that miR-148b might inhibit NSCLC progression. However, miR-148b expression was not found to be associated with the gender ($P = 0.82$), age ($P = 0.38$), histological type ($P = 0.78$), type of surgery ($P = 0.61$), smoking history ($P = 0.21$), and family history of lung cancer ($P = 0.09$).

Survival analysis

Kaplan-Meier survival analysis and log-rank test demonstrated a significant difference in the outcomes of patients who were divided into two groups based on their miR-148b expression level. Specifically, patients with low expression of miR-148b had significantly worse overall survival rates compared with those who had cancers with high miR-148b expression (log-rank test $P = 0.039$, shown in **Figure 2**). We further evaluated whether miR-148b could serve as an independent prognostic marker for patients with NSCLC by multivariate Cox proportional hazards model analysis. In Cox proportional hazards model adjusted for sex, age, TNM stage, lymph node metastasis, distant metastasis, histological type, type of surgery, smoking history, and family history of lung cancer, miR-148b expression was found to be independently associated with overall survival of patients with NSCLC. The HR of the low miR-148b expression group was 2.357 (95% CI: 1.612-9.212, $P = 0.011$, shown in **Table 2**), indicating that patients with low miR-148b expression had poor overall survival. These results proved that miR-148b could be an independent prognostic factor of overall survival for patients with NSCLC.

Discussion

Although recent advances have been made in clinical diagnosis and therapeutic treatment, the overall 5-year mortality rate of lung cancer has remained unfavorable since the 1970s [12]. One of the major obstacles to improving patient survival is the lack of understanding the molecular mechanisms of the early events of lung cancer. Therefore, there is a need to identify new, noninvasive prognostic biomarkers for NSCLC in order to improve postoperative treatment strategies [13]. According to the recent studies, miRNAs may offer a new regulatory model of gene expression, and miRNA expression signatures correlate well with specific clini-

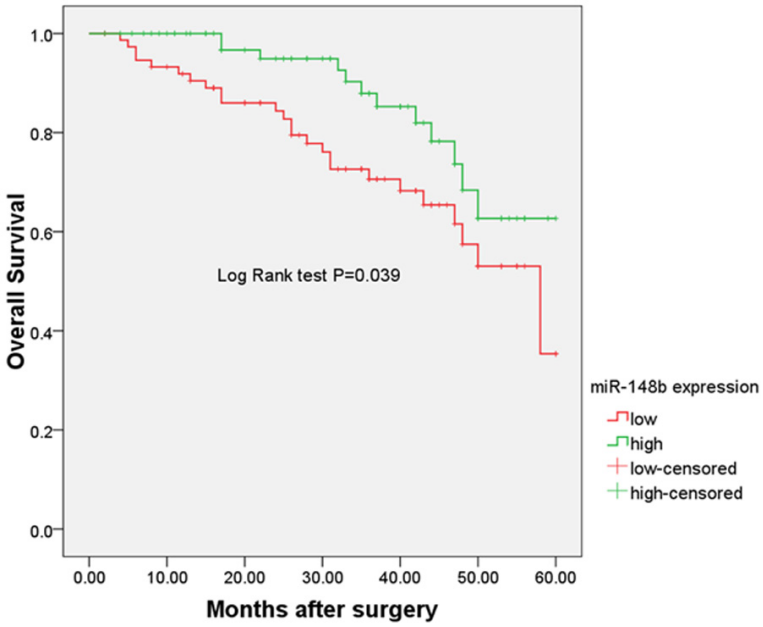

Figure 2. Patients with low expression of miR-148b had significantly worse overall survival rates compared with those who had cancers with high miR-148b expression (P = 0.039).

Table 2. Multivariate analyses for overall survival by Cox regression model

Variable	Hazard ratio	95% CI	*P*-value
Age	1.271	0.671-2.191	0.554
Gender	0.622	0.351-3.178	0.781
TNM stage	2.001	1.527-8.621	0.021
Lymph node metastasis	1.876	0.952-6.656	0.083
Distant metastasis	3.598	1.654-10.542	0.009
Histological type	0.543	0.265-3.654	0.851
Type of Surgery	0.682	0.412-2.971	0.723
Smoking History	1.752	0.627-1.281	0.261
Family history of lung cancer	1.827	0.923-5.227	0.062
miR-148b expression level	2.357	1.612-9.212	0.011

cal characteristics of cancer, so that they can be used to classify normal and cancerous tissues, as well as for prognosis [14-18].

The down-regulation of miR-148b is a frequent event in various cancers, suggesting that miR-148b may play an important role in tumorigenesis and tumor progression. Indeed, several key oncogenic functions have been attributed to miR-148b in the context of tumorigenesis. Zhao et al reported that overexpression of miR-148b induced the apoptosis and cell-cycle arrest of pancreatic cancer cells by targeting AMPKa1 [5]. Furthermore, miR-148b was found to play an important role in regulating the metastasis of hepatocellular carcinoma cells through targeting CCK2R [6]. In breast cancer, ectopical expression of miR-148b inhibits the invasion, survival to anoikis, extravasation, and lung metastasis formation of breast cancer cells [19]. In non-Hodgkin's Lymphoma (NHL), miR-148b was reported to increase the radiosensitivity of NHL cells [20]. Liu et al analyzed the expression level of miR-148b in NSCLC clinical samples and cells and found that miR-148b was notably down-regulated in NSCLC. They then investigated the function of miR-148b in NSCLC. Their data suggested that miR-148b functioned as a tumor suppressor in NSCLC by targeting carcinoembryonic antigen (CEA). However, to our knowledge, the clinical significance of miR-148b in NSCLC has not been addressed yet. In the present study, we investigated the associations of miR-148b with clinicopathological characteristics and overall survival of the patients with NSCLC. We found that miR-148b was downregulated in NSCLC tissues compared to corresponding adjacent normal lung tissues. We then investigated the associations of miR-148b expression with clinicopathological characteristics of 151 NSCLC patients to explore the potential role of miR-148b in tumor progression. Results showed that aberrant miR-148b expression was significantly associated with TNM stage, lymph node metastasis, and distant metastasis. These results showed that decreased expression of miR-148b in NSCLC was associated with significantly aggressive pathologic features, indicating that miR-148b might inhibit NSCLC progression. Furthermore, Kaplan-Meier survival analysis showed that patients with low expression of miR-148b had significantly worse overall survival rates compared with those who had cancers with high miR-148b expression. Multi-

variate Cox proportional hazards model analysis showed that miR-148b expression was independently associated with overall survival of patients with NSCLC, indicating that miR-148b could be an independent prognostic factor of overall survival for patients with NSCLC.

The prognostic value of miR-148b has also been investigated in several types of cancers. For example, Zhang et al showed that decreased miR-148b expression was associated with poor overall survival of patients with hepatocellular carcinoma, indicating the potential role of miR-148b as a prognostic marker in clinical practice [21]. Zhao et al found that the deregulated miR-148b was correlated with increased tumor size, late tumor-node-metastasis stage, lymphatic invasion, distant metastasis, and worse survival in pancreatic cancer, implicating the potential effects of miR-148b on prognosis of pancreatic cancer [5]. To the best of our knowledge, this is the first study showing a significant correlation between miR-148b expression level and the prognosis of NSCLC.

In conclusion, our analysis showed that the level of miR-148b was downregulated in NSCLC patients, and associated with tumor stage. Furthermore, a lower miR-148b level correlated with a less favorable long term outcome. Based on these results, miR-148b may serve as a prognostic marker for NSCLC.

Disclosure of conflict of interest

None.

Address correspondence to: Cui-Min Ding, Department of Respiratory Medicine, The Fourth Hospital of Hebei Medical University, 169 Tianshan Road, Hebei 050011, Shijiazhuang, P. R. China. Tel: 086-0311-66696405; Fax: 086-0311-66696405; E-mail: hebei_dingcuimin@126.com

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