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Serum Levels of miR-19b and miR-146a as Prognostic Biomarkers for Non-Small Cell Lung Cancer

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MicroRNA (miRNA) is a type of small non-coding RNA molecule that has important roles in cancer initiation, promotion and progression by negatively regulating gene expression. In this study, we explored the role of miRNAs in the prognosis of patients with non-small cell lung cancer (NSCLC). The miRNA expression profiles were determined in 5 pairs of NSCLC and paracancerous tissues (3 adenocarcinomas and 2 squamous cell carcinomas). Aberrantly expressed miRNAs were validated by quantitative real-time PCR (qRT-PCR) in 61 pairs of NSCLC and paracancerous tissues. Differentially expressed miRNAs were further analyzed in sera from 94 healthy subjects and 94 advanced NSCLC patients receiving platinum-based chemotherapy. Three miRNAs (miR-19b, miR-146a, and miR-223) were significantly dysregulated in NSCLC tissues ($P < 0.05$). High miR-19b and low miR-146a expression in NSCLC tissues were associated with higher TNM stage, lymph node metastasis and poorer survival ($P < 0.05$). The serum levels of miR-19b in NSCLC patients were significantly higher ($P < 0.001$), whereas serum levels of miR-146a were significantly lower ($P < 0.001$), compared with those in controls. Serum levels of miR-19b and miR-146a were associated with overall survival of NSCLC patients ($P < 0.05$). Patients with low serum level of miR-19b and high serum level of miR-146a achieved a higher overall response rate and longer survival time ($P < 0.05$). These data suggest that miR-19b and miR-146a are potential biomarkers for the prediction of survival and response to chemotherapy in NSCLC.

Keywords: chemotherapy; lung cancer; microRNA; miR-19b; miR-146a
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Introduction

Lung cancer is one of the leading causes of cancer deaths worldwide (Jemal et al. 2010). Although the survival rate for lung cancer has increased because of improvements in diagnosis and treatment of lung cancer, the prognosis remains poor. To date, the molecular network of lung carcinogenesis on protein and gene levels has been partly clarified (Boland and Burtness 2013). However, individualized lung cancer therapy based on genetics has progressed in the last 10 years with no significant improvement in the five-year mortality rate.

MicroRNAs (miRNAs) are abundant small non-coding RNA that can negatively regulate mRNA degradation and then inhibit protein translation by binding to the 3′ untranslated region of mRNA to yield an RNA-induced silencing complex. Approximately 50% of miRNA genes are located in tumor-related genes or fragile sites (Calin et al. 2004). The miRNA expression profiles vary from one type of cancer to another (Volinia et al. 2006). Some upregulated miRNAs can function as oncogenes by inhibiting tumor suppressor genes or other genes that modulate cell differentiation and apoptosis, to stimulate cell proliferation, vasculogenesis, and tumorigenesis (Sumazin et al. 2011). By contrast, some downregulated miRNAs may act as cancer inhibitors by negatively modulating oncogenes or genes that inhibit cell differentiation and apoptosis (Iorio and Croce 2009). Many studies have shown that miRNAs participate in tumorigenesis and progression of various types of cancer, including lung (Chen et al. 2013a; Kesanakurti et al. 2013), breast (Li et al. 2012a; Wang et al. 2013a), gastric (Zhou et al. 2013) and colorectal cancers (Dassow and Aigner 2013). Since miRNAs have important roles in tumorigenesis and cancer progression, miRNAs may also be a potential target

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for cancer treatment (van Kouwenhove et al. 2011; Dassow and Aigner 2013; Shen et al. 2013).

Although miRNAs in cancer tissues can be developed as novel biomarkers for progression, invasive therapies or punctures are usually necessary to obtain cancer tissues (Patnaik et al. 2010). These procedures may be difficult for most cancer patients, particularly those who are in advanced cancer stages. This may hinder wide applications of tissue miRNAs in diagnosis and prognosis prediction. Therefore, peripheral blood is preferred because it is easily obtained during physical examination. The unique expression profile of circulating miRNA may function as the fingerprint of different diseases because miRNAs are highly stable in serum and plasma (Chen et al. 2008). Circulating miRNA has been considered as a potential non-invasive biomarker in tumor diagnosis and prognosis (Sanfiorenzo et al. 2013; Chen et al. 2013b; Lin et al. 2013; Greenberg et al. 2013; Kim et al. 2013; Rani et al. 2013).

To determine novel miRNA biomarkers used to predict the prognosis in non-small cell lung cancer (NSCLC), we initially performed miRNA expression profiling using TaqMan low density array. Differentially expressed miRNAs were further validated in 61 pairs of lung cancer and paracancerous tissues and serum samples from 94 advanced NSCLC patients using quantitative real-time PCR (qRT-PCR). We also evaluated the relationship between miRNA expression and clinical outcomes of two independent NSCLC patient populations.

Materials and Methods

Patients

A total of 66 pairs of NSCLC and paracancerous tissues were collected between 2009 and 2012 from Zhongshan Hospital. Tissue samples were preserved in liquid nitrogen at −80°C prior to RNA isolation. In addition, serum samples from 94 advanced NSCLC patients, and age- and sex-matched 94 healthy control subjects with no history of cancer were also recruited. Ninety-four advanced NSCLC patients were treated with at least 2 circles of platinum-based chemotherapy. All cases were pathologically confirmed as NSCLC, and samples were obtained before anticancer treatment, including surgery, chemotherapy and radiotherapy. This study was approved by the Ethics Committees of Zhongshan Hospital. All participants were informed about the purpose, procedures, and potential risks and benefits of the study, and their written informed were obtained.

We initially determined the miRNA expression profiles in 5 pairs of NSCLC and paracancerous tissues (3 adenocarcinomas and 2 squamous cell carcinomas), and then evaluated the expression levels of miR-19b, miR-223, and miR-146a in 61 pairs of NSCLC and paracancerous tissues (cohort 1). The relationships between miRNA expression and clinical features and survival were evaluated. Further cohort of 94 advanced patients receiving platinum-based chemotherapy (cohort 2) was used as an independent validation of association and prognostic analysis.

The miRNA expression profiles

Tissue RNA and serum RNA were extracted using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) and a miRNeasy mini kit (Qiangen, Germany), respectively, according to the manufacturers' instructions. RNA was eluted in an adsorption column and dissolved in 100 μl of RNase-free water.

The miRNA profiling was performed using 5 pairs of lung cancer and paracancerous tissues. Reverse transcription was performed using TaqMan miRNA reverse translation kit and Megaplex RT primers. The reaction system comprised 20 ng of total RNA. The reverse transcription parameters were set as follows: 40 cycles of 16°C for 2 min; 42°C for 1 min; and 50°C for 1 s. To improve the test sensitivity of the TaqMan low density array, a TaqMan PreAmp Mastermix was used for cDNA pre-amplification. The reaction system comprised 2.5 μl of cDNA, 12.5 μl of 2 × TaqMan PreAmp Mastermix, and 2.5 μl of 10 × Megaplex PreAmp Primers. The pre-amplification parameters were set as follows: 95°C for 10 min; 55°C for 2 min; 75°C for 2 min; 12 cycles of 95°C for 15 s; and 60°C for 4 min. The pre-amplified product was diluted with 75 μl of nuclease-free water. Expression profiling was performed using a TaqMan low density array (Applied Biosystems, CA, USA) that can analyze 365 miRNAs according to the manufacturer's instructions. U6, RNU44, and RNU48 were used as internal references. *Caenorhabditis elegans* miR-39 with low homology was used as a negative control. Amplification was performed using an Applied Biosystems 7900 HT thermal cycler (Applied Biosystems, CA, USA) according to the manufacturer's recommended cycling conditions. Cycle threshold (Ct) was obtained using SDS version 2.4 software (Applied Biosystems, CA, USA).

qRT-PCR

Reverse transcription of the total RNA was performed using a TaqMan miRNA reverse translation kit and miRNA specific stem loop primers (Applied Biosystems, CA, USA). The reaction system (7.5 μl) comprised 0.75 μl of 10 × reverse translational buffer, 0.095 μl of RNase inhibitor, 0.075 μl of 100 mM dNTPs, 0.5 μl of MultiScribe reverse transcriptase, 1.5 μl of primers, and 2.5 μl of total RNA templates. The reaction conditions were set as follows: 16°C for 30 min; 42°C for 30 min; and 85°C for 5 min.

The reaction system (20 μl) for qPCR comprised 3 μl of cDNA, 10 μl of TaqMan Universal PCR Master Mix, 1 μl of TaqMan miRNA assay mix, and 6 μl of water. Amplification was performed in a 7500 real-time PCR system (Applied Biosystems, CA, USA) under the following reaction conditions: 95°C for 2 min; 95°C for 15 s; and 40 cycles of 60°C for 30 s. RNU6 was used as an internal control for tissue samples, whereas the expression levels of serum miRNAs were normalized to the cel-miR-39 (Mitchell et al. 2008). Relative quantification of miRNA expression was calculated with $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Differences in miRNA levels between NSCLC and paired paracancerous tissues were compared using a paired Student's *t*-test. Wilcoxon tests were conducted to determine the intergroup differences in serum miRNA expression. Patients were divided into two groups according to median miRNA expression levels. Bilateral χ^2 test was used to determine the correlations of serum miR-19b, miR-146a, and miR-223 with the clinical factors of lung cancer patients. Kaplan-Meier method was used to analyze the survival curve. Cox regression was conducted to analyze the survival risk. $P < 0.05$ indicated significant difference. These statistical analyses were performed using SPSS (SPSS, IL, USA). Figures were constructed using GraphPad Prism 5.0 (Graphpad software Inc., CA, USA).

Results

The miRNA expression profile

Five pairs of NSCLC and paracancerous tissues were subjected to TaqMan low-density array to determine the miRNA expression profiles. Microarray expression analysis showed that 20 miRNAs were significantly upregulated, whereas 18 miRNAs were downregulated ($P < 0.05$) (Table 1). Only miR-19b, miR-223 and miR-146a remained significant differences in gene expression after Bonferroni cor-

Table 1. Differentially expressed miRNAs in NSCLC tissues.

Regulation	miRNA	Fold change	*P* value
Up	miR-221	2.42	8.28×10^{-4}
Up	miR-376a	7.98	2.90×10^{-4}
Up	miR-191	2.55	2.34×10^{-3}
Up	Let-7a	2.14	4.34×10^{-3}
Up	miR-27b	3.87	1.51×10^{-4}
Up	miR-21	3.25	6.87×10^{-3}
Up	miR-17	8.87	3.89×10^{-3}
Up	miR-133b	4.69	2.58×10^{-2}
Up	miR-155	5.75	1.54×10^{-4}
Up	miR-248	5.24	3.01×10^{-4}
Up	miR-373	2.54	1.41×10^{-3}
Up	miR-338-3p	5.77	2.03×10^{-2}
Up	miR-19b	2.86	8.23×10^{-6}
Up	miR-106a	4.23	2.50×10^{-4}
Up	miR-106b	4.33	2.38×10^{-4}
Up	miR-27a	2.39	5.49×10^{-4}
Up	miR-223	2.08	5.66×10^{-5}
Up	miR-200c	3.32	2.85×10^{-3}
Up	miR-498	2.04	5.61×10^{-3}
Up	miR-557	7.36	1.92×10^{-4}
Down	miR-1295p	0.21	1.44×10^{-2}
Down	miR-602	0.07	8.50×10^{-3}
Down	miR-122	0.25	7.64×10^{-3}
Down	miR-140-3p	0.03	6.42×10^{-4}
Down	miR-30a	0.31	8.94×10^{-3}
Down	miR-145	0.12	1.06×10^{-3}
Down	miR-143	0.39	4.04×10^{-3}
Down	miR-133a	0.47	1.02×10^{-2}
Down	miR-301a	0.35	7.73×10^{-3}
Down	miR-328	0.23	8.31×10^{-4}
Down	miR-195	0.05	3.83×10^{-4}
Down	miR-199a-5p	0.4	5.97×10^{-3}
Down	miR-130b	0.41	6.52×10^{-3}
Down	miR-130a	0.01	1.14×10^{-2}
Down	miR-936	0.48	6.05×10^{-3}
Down	miR-494	0.37	2.79×10^{-3}
Down	miR-193b	0.37	3.06×10^{-3}
Down	miR-146a	0.41	1.93×10^{-6}

Bonferroni corrections: $P < 0.05/365 = 0.000137$

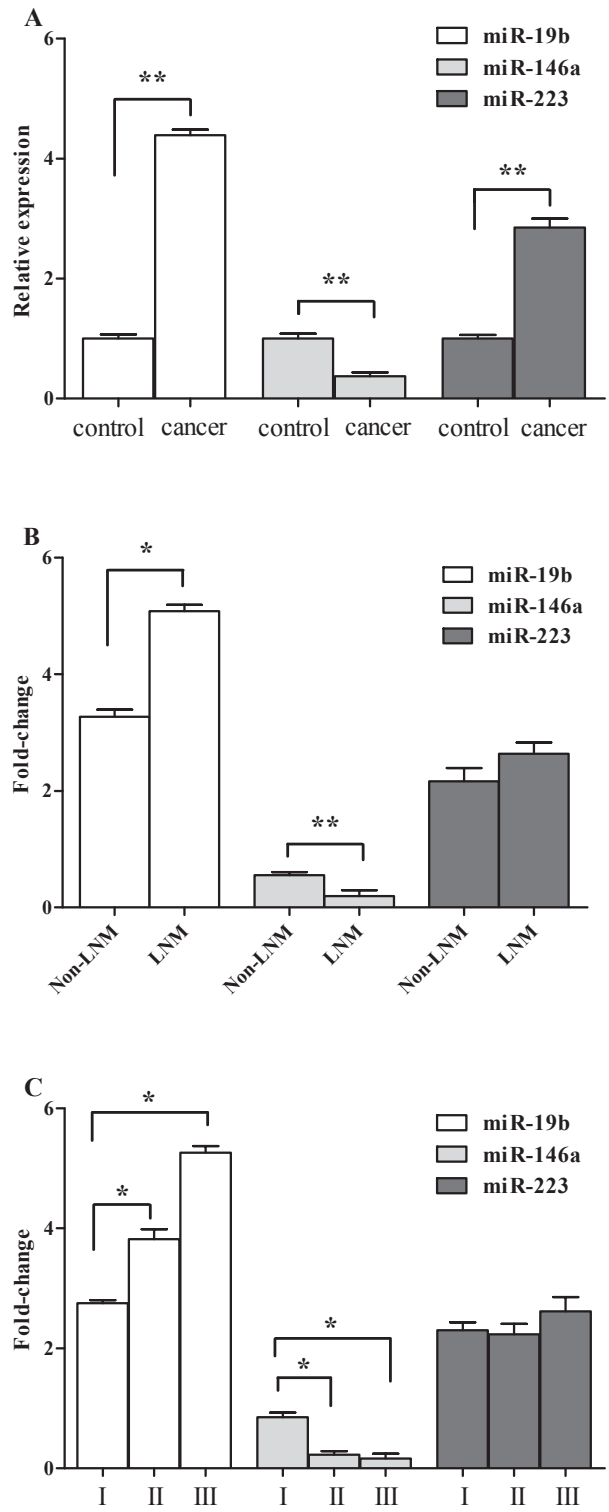

Fig. 1. The expression levels of miR19b, miR-146a and miR-223 in 61 NSCLC patients.
A, the expression levels of miR19b, miR223 and miR146a in 61 pairs of NSCLC and adjacent non-tumor tissues. B, the expression levels of miR-19b, miR-146a and miR-223 subdivided by lymph node status. C, the expression levels of miR-19b, miR-146a and miR-223 subdivided by TNM stage. $*P < 0.05$; $**P < 0.001$.

rections ($P < 0.05/365 = 0.000137$). Importantly, no difference was found between adenocarcinoma and squamous cell carcinoma. Thus, miR-19b, miR-146a and miR-223 were selected for further analysis.

The tissue levels of miR-19b, miR-223 and miR-146a in 61 NSCLC patients

The expression levels of miR-19b, miR-223, and miR-146a in 61 pairs of lung cancer and paracancerous tissues were analyzed by qRT-PCR. The expression levels of miR-19b and miR-223 were higher in NSCLC tissues than those in paracancerous tissues ($P < 0.001$), but miR-146a showed lower expression in NSCLC tissues ($P < 0.001$) (Fig. 1A). In addition, miR-19b and miR-146a were differentially expressed in NSCLC, depending on lymph node metastasis (LNM) ($P < 0.05$) (Fig. 1B). The expression level of miR-19b was significantly higher with increased TNM stages ($P < 0.05$), whereas the expression levels of miR-146a were significantly higher in patients with TNM stage I than those in patients with TNM stage II or III ($P = 0.001$) (Fig. 1C).

The association of miR-19b and miR-146a with clinical outcome of 61 NSCLC patients

High miR-19b and low miR-146a expression in NSCLC tissues were significantly associated with LNM and higher TNM stage ($P < 0.05$) (Table 2). Furthermore, miR-19b expression was also significantly associated with sex ($P = 0.018$). No association between miR-223 expression and clinicopathological features was observed.

The median survival time of all 61 patients was 29.0 months [95% confidence interval (CI) 23.0-35.0]. In the univariate survival analyses, both high miR-19b expression [hazard ratio (HR) = 3.591, 95% CI: 1.564-8.246, $P = 0.003$] and low miR-146a expression (HR = 3.852, 95% CI: 1.647-9.010, $P = 0.002$) correlated with decreased overall survival (OS) (Table 3, Fig. 2). Upon multivariate analysis, high miR-19b expression (HR = 3.466, 95% CI: 1.389-8.650, $P = 0.008$), low miR-146a expression (HR = 2.657, 95% CI: 1.100-6.417, $P = 0.030$) and TNM stage (HR = 3.169; 95% CI: 1.150-8.733, $P = 0.026$) were independent factors affecting OS (Table 3). No significant correlation between miR-223 expression and survival was observed. We further evaluated the cumulative effects of miR-19b and miR-146a on survival in NSCLC patients. Patients with high miR-19b and low miR-146a expression had worse survival than those with low miR-19b and high miR-146a expression (HR = 6.210, 95% CI: 1.921-12.078, $P = 0.002$) after adjusting for TNM stage.

Table 2. Relationship between miR-19b, miR-146a and miR-223 and clinicopathological characteristics in 61 NSCLC patients.

Clinicopathological features	miR-19b			miR-146a			miR-223		
	Low ($n = 31$)	High ($n = 30$)	*P* value	Low ($n = 31$)	High ($n = 30$)	*P* value	Low ($n = 31$)	High ($n = 30$)	*P* value
Age (%)			0.309			0.202			1.00
< 60	19 (51)	14 (23)		14 (23)	19 (31)		17 (28)	16 (26)	
≥ 60	12 (20)	16 (26)		17 (28)	11 (18)		14 (23)	14 (23)	
Gender (%)			0.018			0.434			0.067
Male	24 (39)	14 (23)		21 (34)	11 (18)		23 (37)	15 (25)	
Female	7 (12)	16 (26)		10 (16)	13 (21)		8 (13)	15 (25)	
Smoking status (%)			0.916			0.171			0.459
Former	7 (12)	9 (15)		8 (13)	8 (13)		9 (15)	7 (12)	
Current	12 (20)	12 (20)		16 (26)	8 (13)		13 (21)	11 (18)	
Never	7 (12)	7 (12)		5 (8)	9 (15)		5 (8)	9 (15)	
TNM (%)			0.015			0.001			0.100
I	12 (20)	3 (5)		2 (3)	13 (21)		11 (18)	4 (7)	
II	10 (16)	9 (15)		9 (15)	10 (16)		7 (12)	12 (20)	
III	9 (15)	18 (30)		20 (33)	7 (12)		13 (21)	14 (23)	
LNM (%)			0.005			0.002			0.444
No	20 (33)	8 (13)		8 (13)	20 (33)		16 (26)	12 (20)	
Yes	11 (18)	22 (36)		23 (38)	10 (16)		15 (25)	18 (30)	
Histology (%)			0.174			0.559			0.125
Adenocarcinoma	9 (15)	12 (20)		13 (21)	8 (13)		13 (21)	8 (13)	
Squamous cell carcinoma	6 (10)	10 (16)		8 (13)	8 (13)		10 (16)	6 (10)	
Adenosquamous	11 (18)	7 (12)		8 (13)	10 (16)		5 (8)	13 (2)	
Other	5 (8)	1 (2)		2 (3)	4 (7)		3 (5)	3 (5)	

Table 3. Univariate and multivariate Cox regression analysis of overall survival in 61 NSCLC patients.

Features	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Sex (male vs. female)	0.869 (0.406-1.859)	0.717		
Age (< 60 vs. ≥ 60)	1.489 (0.714-3.102)	0.258		
smoking status (smokers vs. nonsmokers)	0.882 (0.347-2.242)	0.793		
TNM (III vs. I, II)	2.525 (1.452-4.391)	0.001	3.169 (1.150-8.733)	0.026
Histology	1.134 (0.541-2.375)	0.740		
LNM (yes vs. no)	3.324 (1.416-7.805)	0.006	0.466 (0.102-2.123)	0.324
miR-19b (high vs. low)	3.591 (1.564-8.246)	0.003	3.466 (1.389-8.650)	0.008
miR-146a (low vs. high)	3.852 (1.647-9.010)	0.002	2.657 (1.100-6.417)	0.030
miR-223 (high vs. low)	1.973 (0.893-4.359)	0.093		
miR-19b+miR-146a				
low miR-19b and high miR-146a expression	1		1	
high miR-19b or low miR-146a expression	3.078 (1.112-8.521)	0.030	2.023 (0.614-6.666)	0.247
high miR-19b and low miR-146a expression	9.660 (3.313-19.169)	< 0.001	6.210 (1.921-11.078)	0.002

The serum levels of miR-19b, miR-223 and miR-146a in 94 advanced NSCLC patients

We further validated results in serum samples from 94 healthy controls and 94 advanced NSCLC patients receiving platinum-based chemotherapy. Two miRNAs, miR-19b and miR-146a, were differentially expressed between advanced NSCLC patients and controls ($P < 0.05$) (Fig. 3A). The serum levels of miR-19b in advanced NSCLC patients were significantly higher than those in controls ($P < 0.001$), but miR-146a showed lower levels in advanced NSCLC patients ($P < 0.001$). Furthermore, the serum levels of miR-19b were significantly higher in patients with TNM stage IV than those in patients with TNM stage III ($P = 0.019$) (Fig. 3B). We found no significant expression difference of serum miR-223 between cases and controls.

The association of serum miR-19b, miR-223 and miR-146a with clinical outcome of 94 advanced NSCLC patients

The relationships between serum miRNAs and clinical features of 94 advanced NSCLC patients receiving platinum-based chemotherapy were analyzed. Table 4 listed the relationship between serum miR-19b, miR-146a, and miR-223 and the clinicopathological features. The expression level of serum miR-19b was closely related to TNM ($P = 0.007$). No other difference was observed between serum miR-146a and miR-223 and the clinicopathological features. Although single miRNA showed no association with overall response rate ($P > 0.05$), the combination of serum miR-19b and miR-146a was significantly related to overall response rate ($P = 0.024$). The odds ratio for resistance to chemotherapy in patients with high miR-19b and low miR-146a expression relative to those with low miR-19b and high miR-146a expression was 4.571 (95% CI: 1.452-14.389, $P = 0.009$).

The overall survival rates at 1 and 3 years were 53.1% and 11.1%, respectively. Univariate analysis revealed that patients with high expression of serum miR-19b had shorter survival time than those with low expression of serum miR-19b (HR = 2.243, 95% CI: 1.328-3.790, $P = 0.003$) (Table 5, Fig. 4). Furthermore, the low serum level of miR-146a was significantly associated with worse survival in advanced NSCLC patients (HR = 1.911, 95% CI: 1.156-3.159, $P = 0.012$). Upon multivariate analysis, the serum level of miR-19b was shown to be the only independent factor negatively affecting survival (HR = 1.800; 95% CI: 1.008-3.216, $P = 0.047$). Combination of serum miR-19b and miR-146a was analyzed in 94 patients. Using the low-risk group (low miR-19b and high miR-146a expression) as a reference, patients with high miR-19b and/or low miR-146a expression had 3.422- (95% CI: 1.630-7.182, $P = 0.001$) and 3.175-fold (95% CI: 1.521-6.628, $P = 0.002$) increased risk of death, respectively.

Discussion

Although the complex relationship between NSCLC cells and their microenvironment have been elucidated, techniques and tools that can be used to identify patients with different therapeutic responses have not been provided yet. The success of NSCLC treatment is impeded by high relapse rates and lack of proper prognostic biomarkers (Jemal et al. 2010). In recent decades, mechanisms and biomarkers of postoperative relapse have been investigated at a molecular level of cancer tissues due to the limitations of histopathology. High-throughput miRNA profiling has revealed that miRNAs are either upregulated or downregulated in almost all types of cancer (Lu et al. 2005; Calin and Croce 2006). Different types of cancers exhibit unique patterns of miRNA expression. In contrast to mRNAs and proteins, miRNAs are not involved in complex post-transcriptional and post-transcriptional modification. Considering the limitations of condition and time for tissue sampling (Freidin et al. 2012), circulating miRNAs are optimal prog-

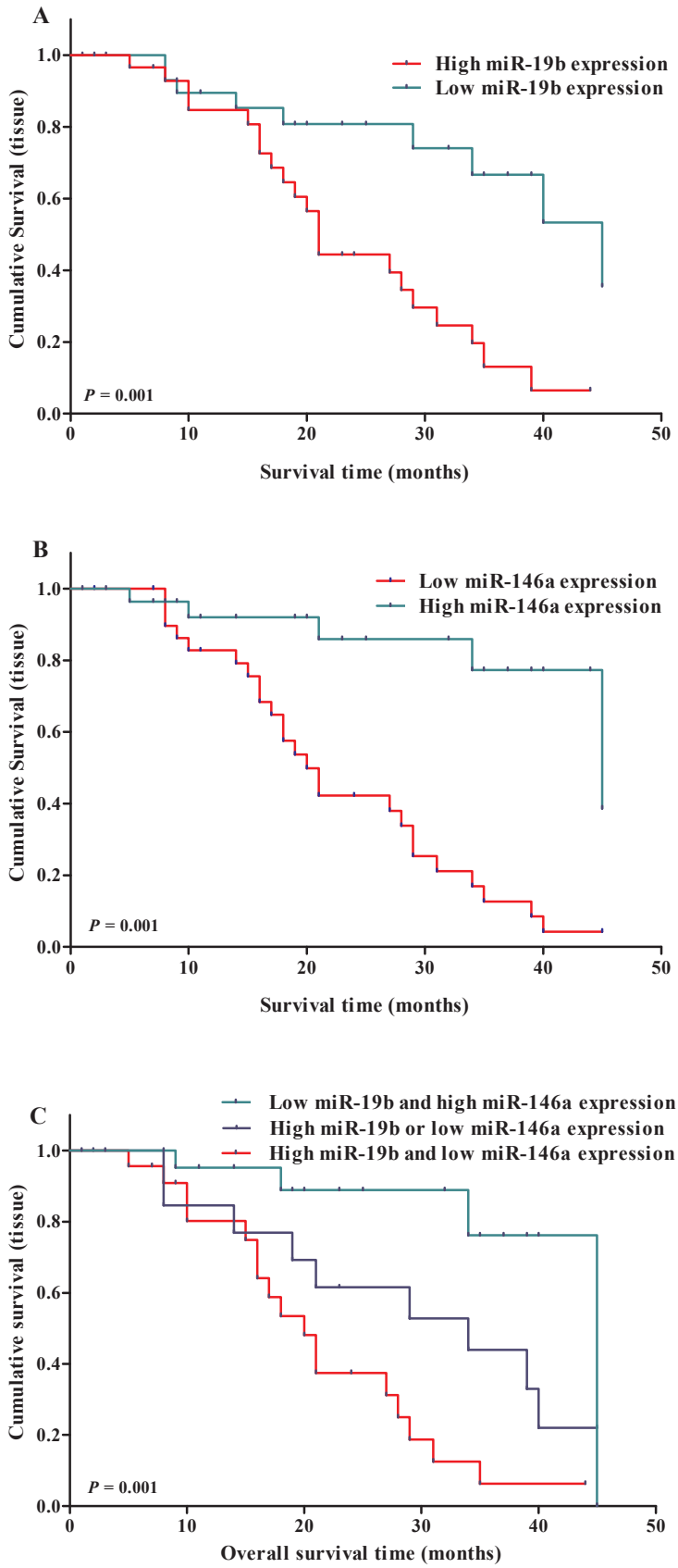

Fig. 2. Kaplan-Meier survival curves of NSCLC patients according to the expression levels of miR-19b and miR-146a in primary tumors from 61 NSCLC patients.
A, miR-19b. B, miR-146a. C, combination of miR-19b and miR-146a.

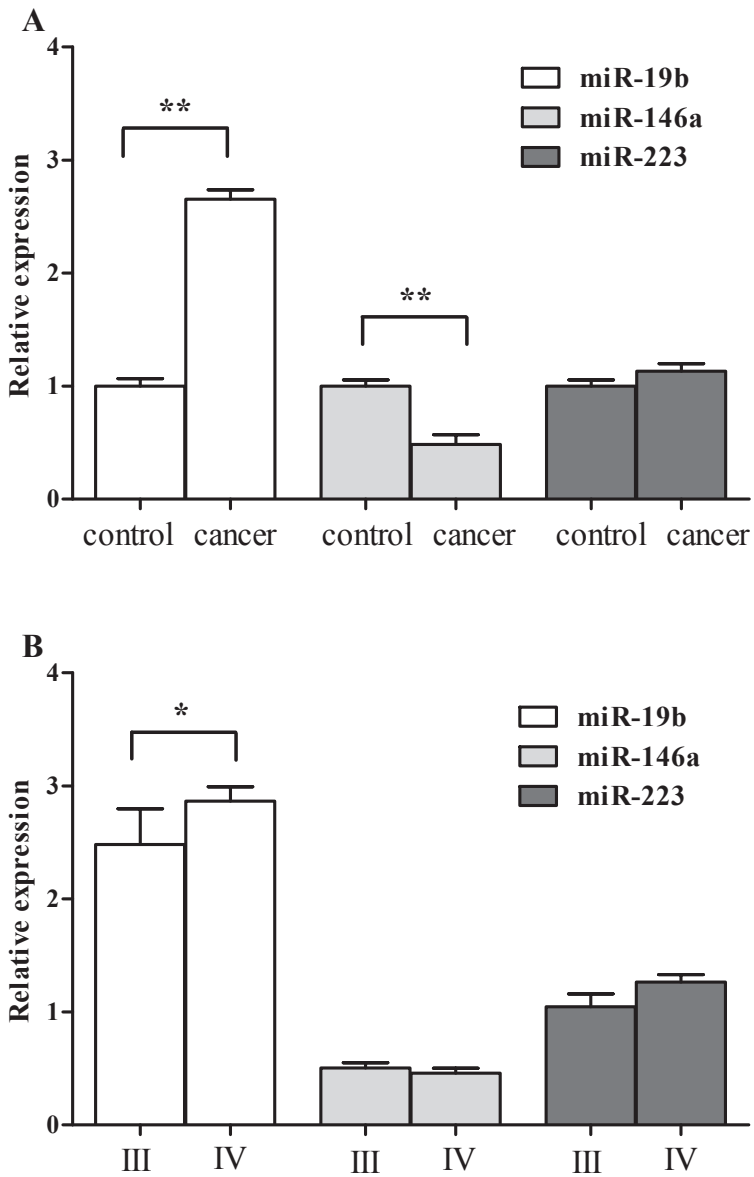

Fig. 3. The serum levels of miR19b, miR-146a and miR-223 in 94 advanced NSCLC patients.
A, the serum levels of miR19b, miR146a and miR223 in NSCLC patients and controls. B, the expression levels of miR19b, miR146a and miR223 subdivided by TNM stage. *$P < 0.05$; **$P < 0.001$.

nostic biomarkers for cancer (Sanfiorenzo et al. 2013; Chen et al. 2013b; Lin et al. 2013; Greenberg et al. 2013; Kim et al. 2013). However, further studies should be conducted before miRNA can be extensively used in clinical applications. One of the main reasons is the heterogeneity of lung cancer. Lung cancer caused by different factors has distinct expression profiles. Effective serum miRNA related with tumor progression has not been identified yet because of the limitation of sample collection. In addition, circulating miRNAs may be produced from the immunocytes in the tumor microenvironment or inflammatory responses in other organs mediated by parahormones (Cortez et al. 2011) if not by cancer cells (Michael et al. 2003). Thus, further investigations are needed to determine whether or not circulating miRNAs are produced by cancer cells in the lesion.

In this study, both miR-19b and miR-146a were aberrantly expressed in tissues and sera from NSCLC patients. In addition, serum levels of miR-19b and miR-146a presented the same trend as those in tissues, which was consistent with that in a previous study (Boeri et al. 2011). This result indicated the comparable effectiveness of using circulating miRNA as a prognostic indicator. The miR-19b belongs to the miR-17-92 gene cluster family with upregulated expression in various tumors (Chen et al. 2013b). The target genes of miR-19b are involved in cell apoptosis, DNA recombination and repair (Engelmann and Spang 2012; Kurokawa et al. 2012). The miR-19b, as a regulator of tumor suppressor genes, participates in the occurrence of

Table 4. Relationship between serum levels of miR-19b, miR-146a and miR-223 and clinicopathological characteristics in 94 advanced NSCLC patients receiving platinum-based chemotherapy.

Clinicopathological features	miR-19b			miR-146a			miR-223		
	Low (n = 47)	High (n = 47)	P value	Low (n = 47)	High (n = 47)	P value	Low (n = 47)	High (n = 47)	P value
Age (%)			0.836			0.534			1.000
< 60	25 (27)	27 (29)		28 (30)	24 (26)		26 (28)	26 (28)	
≥ 60	22 (23)	20 (21)		19 (20)	23 (24)		21 (22)	21 (22)	
Sex (%)			0.394			0.200			0.670
Male	27 (29)	32 (34)		33 (35)	26 (28)		31 (33)	28 (30)	
Female	20 (21)	15 (16)		14 (15)	21 (22)		16 (17)	19 (20)	
Smoking status (%)			0.697			0.149			0.286
Former	12 (13)	14 (15)		9 (10)	17 (18)		16 (17)	10 (11)	
Current	20 (21)	15 (16)		19 (20)	16 (17)		17 (18)	18 (19)	
Never	12 (13)	11 (12)		14 (15)	9 (10)		9 (10)	14 (15)	
TNM (%)			0.007			0.061			0.300
III	33 (35)	19 (20)		21 (22)	31 (33)		23 (25)	29 (31)	
IV	14 (15)	28 (30)		26 (28)	16 (17)		24 (25)	18 (19)	
Histology (%)			0.987			0.778			0.590
Adenocarcinoma	17 (18)	17 (18)		18 (19)	16 (17)		8 (9)	16 (17)	
Squamous cell carcinoma	13 (14)	13 (14)		12(13)	14 (15)		11 (12)	15 (16)	
Adenosquamous	14 (15)	13 (14)		15 (16)	12 (13)		15 (16)	12 (13)	

Table 5. Univariate and multivariate Cox regression analysis of serum levels of miR-19b, serum miR-146a and miR-223 in 94 advanced NSCLC patients receiving platinum-based chemotherapy.

Features	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Sex (male vs. female)	0.602 (0.349-1.040)	0.069		
Age (< 60 vs ≥ 60)	0.989 (0.606-1.614)	0.965		
Smoking status (smokers vs nonsmokers)	1.246 (0.941-1.650)	0.125		
TNM (III vs. IV)	1.684 (1.023-2.771)	0.040	1.406 (0.845-2.340)	0.189
Histology	1.323 (0.790-2.215)	0.287		
miR-19b (high vs. low)	2.243 (1.328-3.790)	0.003	1.800 (1.008-3.216)	0.047
miR-146a (low vs. high)	1.911 (1.156-3.159)	0.012	1.412 (0.812-2.455)	0.221
miR-223 (high vs. low)	0.950 (0.580-1.558)	0.840		
miR-19b+miR-146a				
low miR-19b and high miR-146a expression	1		1	
high miR-19b or low miR-146a expression	3.466 (1.682-7.143)	0.001	3.175 (1.521-6.628)	0.002
high miR-19b and low miR-146a expression	3.597 (1.724-7.509)	0.001	3.422 (1.630-7.182)	0.001

lymphoma (Mavrakis et al. 2011) and is involved in invasion and metastasis of cervical cancer (Xu et al. 2012). As a tumor suppressor gene, the miR-146a is downregulated in various types of cancer cells (Chen et al. 2013a; Zhou et al. 2013). In mice, knockout of the miR-146a gene results in the dysregulation of nuclear factor-κB (NF-κB) and then drives the development of myeloid malignancies (Zhao et al. 2011). Overexpression of miR-146a can also inhibit NF-κB activity, thereby inhibiting proliferation and invasion of cancer cells as well as inducing apoptosis (Paik et al. 2011; Li et al. 2012a; Chen et al. 2013a). However, miR-146a is upregulated in diffuse large B-cell lymphoma (Zhong et al. 2012) and some invasive human breast cancer cell lines (Wang et al. 2013b). Li et al. (2012b) reported that miR-146a was highly expressed in brain metastasis in colorectal cancer. Therefore, miR-146a may function as tumor suppressor and oncogene, depending on the context. Taken together, these results showed that miR-19b and miR-146a play important roles in tumorigenesis and cancer progression.

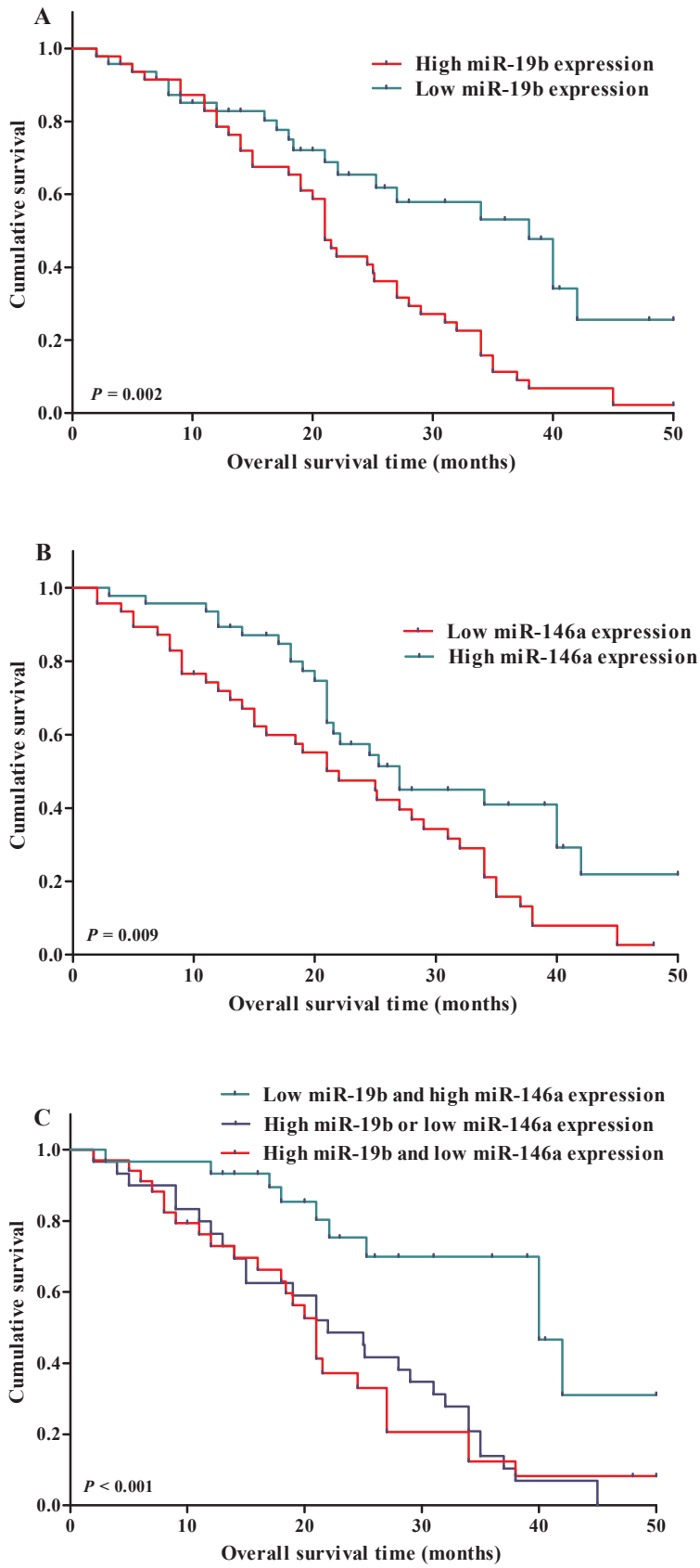

Fig. 4. Kaplan-Meier survival curves of NSCLC patients according to the serum levels of miR-19b and miR-146a. A, miR-19b. B, miR-146a. C, combination of miR-19b and miR-146a.

Metastasis is an important factor in the prognosis of NSCLC patients and the main reason for therapeutic failure. In this study, miR-19b expression was related to LNM as well as TNM. Both the tissue and serum levels of miR-19b were increased as TNM stage increased, indicating the important function of miR-19b in the occurrence, progression, and metastasis of NSCLC. Several studies reported that the upregulation of miR-146a inhibits cancer metastasis (Kogo et al. 2011; Hwang et al. 2012; Chen et al. 2013a). Rong et al. (2014) found that low expression of miR-146a was associated with TNM and LNM in patients with hepatocellular carcinoma. In the present study, we found that the lower tissue level of miR-146a was associated with LNM. The tissue levels of miR-146a in patients with TNM stages II and III were significantly lower than those with TNM stage I. However, we found no significant expression difference of the serum level of miR-146a between TNM stage III and IV. Like other tumor suppressor genes, miR-146a is dramatically downregulated in the early stage of NSCLC.

Previous studies demonstrated that miR-19b overexpression was related to 5-FU resistance in colon cancer cells (Kurokawa et al. 2012) and was an adverse prognostic marker of recurrence and overall survival in patients with colorectal cancer (Kahlert et al. 2011). Aberrant expression of miR-146a was correlated with complete remission rate, higher overall response rate and longer progression-free survival time in patients with diffuse large B-cell lymphoma (Zhong et al. 2012). In this study, the tissue levels of miR-19b and miR-146a significantly influenced the OS in NSCLC patients with TNM stages I, II and III. We also found that the mortality rate was increased significantly in advanced NSCLC patients receiving platinum-contained chemotherapy as a result of the abnormal expression of serum miR-19b and miR146a. In addition to single miRNA analysis, combination of miR-19b and miR-146a analysis showed that patients with high miR-19b and low miR-146a expression had the highest mortality risk. Patients with low serum level of miR-19b and high serum level of miR-146a achieved a higher overall response rate and longer survival time. This indicates that advanced NSCLC patients with low serum level of miR-19b and high serum level of miR-146a may gain the greatest benefit in terms of prolonging survival when receiving platinum-based chemotherapy.

In conclusion, the present study showed that both tissue and serum levels of miR-19b and miR-146a were associated with clinical outcomes of NSCLC patients. Serum levels of miR-19b and miR-146a may serve as predictive and prognostic biomarkers for NSCLC patients receiving platinum-based chemotherapy. Our findings provide novel ideas to investigate the pathogenesis and prognostic prediction of NSCLC. Further studies are needed to investigate the function of miR-19b and miR-146a in NSCLC pathogenesis.

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Conflict of Interest

The authors declare no conflict of interest.

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