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Original Article

Up-regulation of MicroRNA-93 predicates advanced clinicopathological features and serves as an unfavorable risk factor for survival of patients with non-small cell lung cancer

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Abstract: MiRNA-93-5p (miR-93) has been reported to be differentially regulated in a variety of neoplastic diseases. However, there are few studies of clinical relevance of miR-93 on non-small cell lung cancer (NSCLC), particularly its relationship with prognosis in patients with NSCLC. In this study, we investigated the expression levels of Micro-93 (miR-93) in non-small cell lung cancer tissues and adjacent non-tumor tissues and the relationship with clinical significance. Expression levels of miR-93 in 104 pairs of NSCLC and adjacent non-tumor tissues were measured by quantitative real-time PCR (qRT-PCR). All the patients were divided into 2 groups according to the different expression levels of miR-93. Then, clinic-pathological features and expression levels of miR-93 were compared. In order to explore its prognostic value, overall survival (OS) and progression-free survival (PFS) were assessed using the Kaplan-Meier method, and multivariate analysis was estimated using the Cox-proportional hazards regression model. It was observed that miR-93 expression level was significant higher in NSCLC tissues compared with adjacent non-tumor tissues (P<0.001), and miR-93 was correlated with lymph nodes metastasis and clinical stage, while was not correlated with age, gender, tumor size, smoking, etc. Univariate Kaplan-Meier analysis revealed a significant correlation between high miR-93 expression level and poor progression-free survival (P=0.020) and overall survival (P=0.008). Furthermore, a multivariate Cox regression analysis revealed high miR-93 expression (HR=5.93, 95% CI=1.25-11.38) and clinical stage (HR=4.90, 95% CI=0.29-15.73) were predictor of poor prognosis of the patients with NSCLC. We conclude that up-regulation of MiR-93 is correlated with worse clinic-pathological features, indicating it serves as an independent marker for poor prognosis in patients with Non-small cell lung cancer.

Keywords: Non-small cell lung cancer (NSCLC), MicroRNA-93, clinical prognosis, survival analysis

Introduction

Lung carcinoma is the leading diagnosed neoplastic disease and cause of cancer-related deaths, accounting for 1.59 million deaths worldwide in 2012 and an estimated 486555 deaths in China in 2010 [1, 2]. Almost 85% of all lung cancer cases is non-small cell lung cancer (NSCLC). Although considerable improvements have been achieved in diagnosis, treatment and our understanding of molecular alterations in tumorigenesis of NSCLC, a high rate of local recurrences and distant metastasis aggravates the clinical prognosis of NSCLC, which has remained poor with a 5-year overall survival rate of approximately 11% [3]. Therefore, accurate biomarkers are urgent needed to identify early lung carcinoma with good sensitivity and specificity, and to support the identification of novel drug targets for more effective and less toxic treatment. Recently, numerous studies have proven that non-coding small RNAs may be involved in pathogenesis and malignant progression of NSCLC [4-7]. The measurement of miRNA in tissues and biological fluids including blood, plasma, sputum has been utilized to grade various pathological conditions in lung carcinoma. Furthermore, specific microRNA

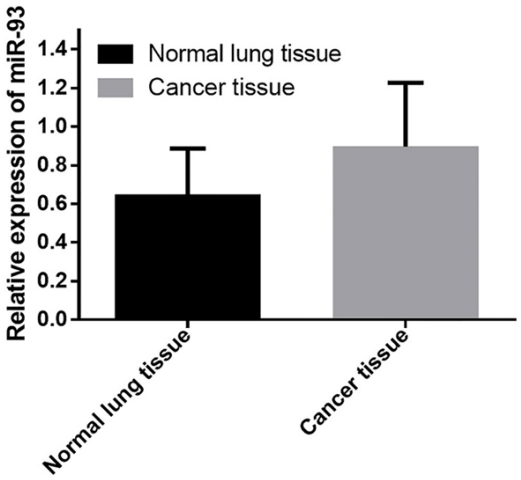

Figure 1. MiR-93 expression levels were detected by quantitative real time polymerase chain reaction (qRT-PCR) in 104 pairs of NSCLC tissues and adjacent non-cancer lung tissues normalized to U6 snRNA. The expression of miR-93 was significantly up-regulated in NSCLC tissues compared as paired normal lung tissue (mean ± SD: 0.89±0.331 vs. 0.65±0.237, P<0.001).

(miR) signatures from biological specimens have great potential to become valuable non-invasive diagnostic and prognostic tools. Thus, investigation of aberrant miRNA levels could contribute to the discovery of novel miRNA biomarkers for NSCLC.

In the past, up to 2000 miRNAs have been reported accounting for 1-3% of human genes. MiRNAs are a class of short (19-24 nucleotides) non-coding RNAs that can predict to regulate the expression of at least half of the human transcriptome involving in diverse biological activities, including differentiation, proliferation, metabolism and apoptosis [8-10]. Many of these miRNAs have been reported potentially to be biomarkers for diagnosis, prognosis and personalized therapy in cancers, in which aberrant expression patterns of miRNAs have been indicated to serve as oncogene or tumor suppressor.

MiRNA-93-5p (miR-93) belongs to miR-106b-25 cluster located in intron 13 of the host gene MCM7 at chromosome 7q22. MiR-93 has been confirmed to be differentially regulated in a variety of carcinomas including esophageal cancer [11], colon cancer [12], breast cancer [13], head and neck squamous cell carcinoma (HNSCC) [14]. Previous studies demonstrated that miR-93 can stimulate tumor cells proliferation, migration and invasion through the oncogenic c-Met/PI3K/Akt pathway and inhibit apoptosis by directly target PTEN and CDKN1A expression in human hepatocellular carcinoma [15]. At present, a growing number of miR-93 target genes have been identified, for instance TP53INP1, PTEN, FUS-1, NEDD4L and DAB2, indicating miR-93 may considerably contribute to tumorigenesis and aggressiveness [16-18]. However, there are few studies of clinical relevance of miR-93 in NSCLC, particularly its correlation with prognosis in patients with NSCLC.

In the current study, we investigated the expression levels in NSCLC tissues and addressed the questions whether the altered levels of miR-93 are correlated to clinic-pathological features and the prognosis of patients with NSCLC.

Materials and methods

Patients

Specimens of lung tissue were collected from 104 patients with primary NSCLC who underwent surgeries in the Qingpu Branch of Zhongshan Hospital affiliated Fudan University, China, from January 2007 and December 2012. No patients recruited into current study received any treatment prior to surgery. Samples of tumor tissue and paired adjacent non-tumor tissue (5 cm away from the tumor) were obtained during the surgery after obtaining informed consent from all the patients. All the specimens were preserved in liquid nitrogen within 5 min of excision and stored at -80°C until RNA extraction. Histological diagnosis of the tumor and paired non-tumor tissues, and tumors grade were confirmed by two pathologists according to the World Health Organization classification of lung tumors. Clinical information was retrieved from medical history and follow-up records of patients. This study was performed in accordance with the ethical standards of the Declaration of Helsinki and approved by the ethics committee of Zhongshan Hospital of Fudan University.

RNA extraction and quantitative RT-PCR

Total RNA from the tissues of 104 paired samples was isolated with the miRNeasy Mini kit (Qiagen, Germany) following the manufacturer's instructions. Quantity and quality of isolated

Table 1. Correlation analysis for miR-93 expression level and clinic-pathological features of NSCLC

		N	Low miR-93	High miR-93	χ^2	*P* value
Age (years)	<59.1	40	22 (21.2%)	18 (17.3%)	0.650	0.420
	≥59.1	64	30 (46.8%)	34 (32.7%)		
Gender	Male	74	34 (32.7%)	40 (38.5%)	1.686	0.194
	Female	30	18 (17.3%)	12 (11.5%)		
Tumor size	0-3 cm	36	24 (23.1%)	15 (14.4%)	3.323	0.068
	>3 cm	68	28 (26.9%)	37 (35.6%)		
Lymph node status	N0	38	27 (25.9%)	13 (12.5%)	7.963	0.005
	N+	66	25 (24.1%)	39 (37.5%)		
Clinical stage	Stage I II	49	33 (31.7%)	16 (15.4%)	11.153	0.001
	Stage III IV	55	19 (18.3%)	36 (34.6%)		
Histologic type	Adenocarcinoma	32	12 (11.5%)	20 (19.2%)	2.889	0.089
	Squamous carcinoma	72	40 (38.5%)	32 (30.8%)		
Smoking	Non-smoker	29	12(11.5%)	17 (16.3%)	1.195	0.274
	Former or current smoker	75	40 (38.5%)	35 (33.7%)		

RNA was determined by UV spectrophotometry (A260/A280 ratio of 1.8-2.0). Total RNA samples were reversely transcribed using a TaqMan microRNA assay miRNA-specific stem-loop primers and TaqMan reverse Transcription Kit (Applied Biosystems, USA). Quantitative RT-PCR was performed with TaqMan Universal PCR Master Mix and ABI7900 Sequence Detection System (Life Technologies) following the manufacturer's instructions. PCR conditions were as follows: at 95°C for 10 min, following by 40 cycles (95°C for 15 s, 60°C for 1 min). U6 small nuclear RNA was used as an internal control. The PCR primer for miR-93 and U6 were designed as follows: sense; 5'-GGCAGCAAAGTTCTGAGACAC-3', and antisense; 5'-GTGCAGGGTCCGAGGTATTC-3'. U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. Levels of miR-93 were analyzed quantitatively relative to U6 snRNA by the $2^{-\Delta CT}$ method, where ΔCT= (CTmiR-93-CTU6). All samples were run in triplicates.

Statistical analysis

Statistical analysis was performed using SPSS software (version 19). Differences of miR-93 levels between the tumor tissues and adjacent normal tissues were compared with Student's *t* test. Correlation significance between the levels of miR-93 and clinic-pathological parameters was analyzed with Chi square test. Overall survival (OS) was calculated as the time from the date of primary carcinoma diagnosis to the date of NSCLC related death within the follow-up interval (events). Progression-free survival (PFS) was calculated from the date of primary tumor diagnosis to the date of the first local recurrence, lymph node or distant metastasis, second primary tumor, or date of NSCLC related death within the follow-up period (events). The method of Kaplan-Meier was utilized to estimate survival distributions. Differences between groups were determined by log-rank test. A multivariate Cox proportional hazard model was applied to assess the association between miR-93 levels and overall and progression-free survival of NSCLC patients, together with the covariates age (continuous variable), clinical stage (IV vs. I vs), gender (female vs. male), tumor size, TNM stage, histological type, alcohol and tobacco consumption (never vs. former and current consumer). The validity of the proportional hazards assumption was tested with the Supreme Test for proportional hazards assumption and was met for all covariates. In all statistical tests, the results were regarded as statistically significant at $P \leq 0.05$.

Results

Up-regulation of miR-93 in NSCLC compared to adjacent normal tissue

To investigate the role of miR-93 in NSCLC, miR-93 expression levels were detected by qRT-PCR in 104 pairs of NSCLC tissues and

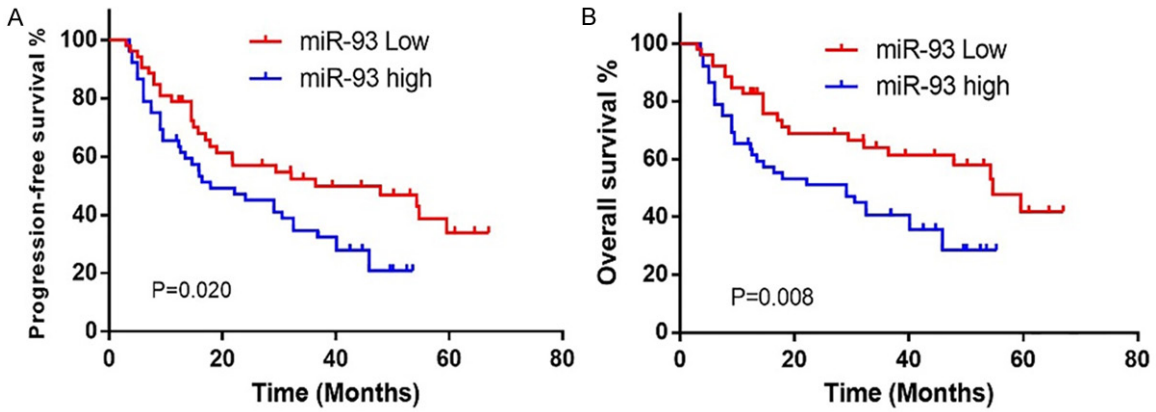

Figure 2. Correlation between expression levels of miR-93 and progression-free and overall survival. Kaplan Meier analysis was performed for progression-free survival (A) and overall survival (B), and the survival difference between groups was compared using the log-rank test. The patients with high expression of miR-93 had shorter PFS (χ^2=5.387, P=0.020), OS (χ^2=7.096, P=0.008) and lower 5-year OS rate (28.5% vs. 41.8%) as compared to the miR-93 low expression group.

Table 2. Multivariate analysis of overall survival for NSCLC patients of the combined cohort

Risk factors	HR (95% CI)	*P*-value
Age		
<59.1 vs. ≥59.1	1.90 (0.64-5.74)	0.26
Gender		
Male vs. Female	1.87 (0.71-5.12)	0.16
Tumor size		
>3 cm vs. 0-3 cm	2.21 (0.84-8.31)	0.12
Lymph node status		
N+ vs. N0	3.81 (0.63-9.45)	0.09
Clinical stage		
Stage III IV vs. Stage I II	4.90 (0.29-15.73)	0.03
Histologic type		
Adenocarcinoma vs. squamous carcinoma	1.71 (0.60-4.74)	0.31
Smoking		
Non-smoker vs. Former or current smoker	2.19 (0.49-6.54)	0.17
MiR-93 levels		
High vs. low	5.93 (1.25-11.38)	0.02

adjacent non-cancer lung tissues normalized to U6 snRNA. As shown in **Figure 1**, the expression levels of miR-93 in cancerous tissues exhibited significantly higher compared with the paired normal lung tissues (mean ± SD: 0.89±0.331 vs. 0.65±0.237, P<0.001).

Association between miR-93 mRNA levels and clinic-pathological features

To gain further insight into the clinical relevance of miR-93, we divided the 104 NSCLC patients into two subgroups, a low level group (n=52) and a high level group (n=52), according to the miR-93 median expression level (0.86) in all NSCLC specimens. Subsequently, we compared the expression pattern with clinical and histopathological features including age, gender, TNM status, clinical grade and histological type (**Table 1**). The differences in the distribution of miR-93 levels between different groups of NSCLC patients were evaluated using the Chi square test. Our study cohort revealed a high level of miR-93 expression was correlated with advanced clinical stage and positive lymph node metastasis (*P*<0.05). However, there were no significant association of miR-93 expression with other clinical features such as age, gender, tumor size, histologic type, and tobacco consumption (*P*>0.05).

Correlation of miR-93 expression with overall and progression-free survival

To address the question, whether miR-93 expression serves as prognostic biomarker for NSCLC, we performed Kaplan Meier analysis for progression-free survival (PFS) and overall survival (OS). We found a significant correlation between the high level of miR-93 and poor PFS

(χ^2=5.387, P=0.020), while patients with low level of miR-93 were characterized by improved PFS. Moreover, univariate analysis revealed that patients with high expression of miR-93 had shorter OS (χ^2=7.096, P=0.008) and lower 5-year OS rate (28.5% vs. 41.8%) as compared to patients with the low expression of miR-93 (**Figure 2**). Hence, our data demonstrated aberrant miR-93 expression in the pathogenesis of NSCLC and NSCLC patients with high expression were associated with an unfavorable clinical outcome.

High expression level of miR-93 serves as an independent prognostic marker for OS in NSCLC patients

Next, we performed multivariate Cox regression analysis to evaluate prognostic significance of miR-93 expression level, and putative prognostic factors, including age, gender, clinical stage, tumor size, TNM status, tobacco consumption. A multivariate Cox regression analysis revealed high miR-93 expression (HR=5.93, 95% CI=1.25-11.38) and clinical stage (HR= 4.90, 95% CI=0.29-15.73) were predictor of poor prognosis of the patients with NSCLC (**Table 2**). In summary, our study clearly indicates that high expression of miR-93 could serve as an independent risk factor for poor clinical outcome in NSCLC.

Discussion

Novel prognostic and predictive biomarkers for NSCLC are urgently needed for early detection and treatment to individual patients. Deregulated miRNAs play a significant character in a range of human malignancies and many studies demonstrated miR-93 as a prognostic biomarker. Several studies have proved that miR-93 expression is up-regulated in various carcinomas, including esophageal cancer, breast cancer, hepatocellular carcinoma, cervical cancer, head and neck squamous cell carcinoma (HNSCC), etc. Consistent with most reports, we found expression levels of miR-93 in cancerous tissues exhibited significantly higher compared to the paired normal lung tissues. Conversely, few studies reported that the level of miR-93 was down-regulated in glioblastoma and colon cancer [19, 20]. The controversial outcome could indicate various histological types of tumor exhibited diverse expression levels of miR-93.

Next, we assessed the association between miR-93 levels and clinic-pathological features of 104 patients with NSCLC and found that high expression of miR-93 was significantly correlated with advanced clinical stage and positive lymph node metastasis. Previous reports have shown miR-93 regulates tumor metastasis via regulating various metastasis genes and signal pathways. In breast carcinoma, miR-93 promoted the epithelial mesenchymal transition (EMT) by targeting the inhibitory Smad7 protein and activating the TGF-beta signaling [21]. Moreover, another study indicated the high expression level of miR-93 was associated with tumor progression and metastasis in HNSCC, suggesting miR-93 might be a critical regulator in tumorigenesis and progression and a promising biomarker for the prediction of distant metastasis and clinical prognosis in HNSCC [14]. In lung carcinoma cells, overexpression of miR-93 promoted TGF-beta-mediated EMT via down-regulating the expression of neural precursor cell expressed developmentally downregulated gene 4-like (NEDD4L), indicating the carcinogenesis character of miR-93 in lung tumorigenesis and metastasis [22]. Taken together, these findings suggest that miR-93 is involved in tumor invasion and metastasis. However, the studies from Fang and colleagues [16] confirmed that endogenous miRNA-93 has an important role in promotion of tumor growth. It sums up that cells with miR-93 overexpression not only promoted angiogenesis but also developed a correlation with blood vessels assisting cancer cells to survive and to grow perniciously. In our present study there was no significant association of miR-93 expression with tumor size, this controversial result may be explained by the limited number of patients. To our knowledge, we have proved the expression of miR-93 was closely correlated with tumor metastasis and worse clinical stage by clinical data.

Besides, the relationship between miR-93 and prognosis in NSCLC patients has been investigated in this study. Based on the Kaplan-Meier survival analysis and log-rank test, our data revealed that patients with high expression of miR-93 had shorter PFS, OS and lower 5-year OS rate as compared to patients with the low expression of miR-93. The multivariate Cox regression analysis revealed high miR-93 expression and advanced clinical stage were predictor

of poor prognosis of the patients with NSCLC. Intriguingly, another important study shown miR-93-directed disabled homolog 2 (DAB2) gene down-regulation fulfills an important role in lung tumorigenesis and poor survival of lung carcinoma patient. The dysregulation of miR-93/DAB2 pathway might be a potential marker for treating lung carcinoma patients. In summary, our data clearly indicates that high expression of miR-93 could serve as an independent risk factor for poor clinical outcome in NSCLC, which is consistent with other studies in gastric cancer, ovarian cancer and HNSCC [14, 23, 24].

In conclusion, our clinical investigations demonstrate elevated miR-93 expression is correlated to lymph node metastasis, advanced tumor stage and poor prognosis of NSCLC patients. Furthermore, our comprehensive analyses show miR-93 is an independent prognostic factor in NSCLC patients, indicating miR-93 might be a promising biomarker for predicting the outcome for these patients. Given the increasing evidences of the clinical value of miRNAs in cancer therapeutics and diagnostics, molecular regulatory mechanisms related to miR-93 will be further explored in the near future.

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Disclosure of conflict of interest

None.

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