

Original Article

Clinical significance of miR-199a-5p expression in non-small cell lung cancer patients

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Abstract: Background: Low miR-199a-5p expression was associated with cancer prognosis. However, its clinical significance in non-small cell lung cancer (NSCLC) is still unclear. This study was aimed to evaluate the prognostic significance of miR-199a-5p in NSCLCs and verified the effects of miR-199a-5p on NSCLC cells. Methods: Radically resected NSCLC tissues and adjacent non-tumor tissues (NTs) were collected from 61 NSCLC patients who underwent lung resection between January 2012 and December 2014. We used quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays to detect miR-199a-5p expression. The association of miR-199a-5p expression with clinicopathological factors was statistically analyzed. Effects of miR-199a-5p overexpression on proliferation, apoptosis and invasion on NSCLC cells *in vitro* were detected. Results: Compared with the adjacent non-tumor group, miR-199a-5p expression was significantly downregulated in NSCLCs ($P < 0.001$). Low miR-199a-5p expression was significantly correlated with the *lymph node metastasis, high II+III stage, lymphatic permeation, pleural involvement and vascular invasion*. MiR-199a-5p overexpression decreased invasion, proliferation and induced apoptosis in NSCLC cells *in vitro*. Conclusions: The expression level of miR-199a-5p was significantly downregulated in NSCLC tissues compared with the adjacent non-tumor tissues ($P < 0.001$). Downregulation of miR-199a-5p was correlated with adverse clinical features. MiR-199a-5p could serve as a promising biomarker and therapeutic targets in NSCLC.

Keywords: Non-small cell lung cancer, miR-199a-5p, marker

Introduction

Lung cancer (LC) is a highly invasive malignancy that has a strong tendency to distant metastasis at early stages. LC is comprised into two major clinicopathological categories: small-cell (SCLC) and non-small-cell lung carcinoma (NSCLC). The latter consists of more than 85% of lung cancers. Despite advances in early diagnosis and standard treatment, the prognosis of patients with NSCLC remains poor, with a 5-year survival less than 15% [1]. Early stage NSCLC patients who undergo complete surgical tumor resection still develop distant metastases in 50% to 70% of cases, resulting in an overall 5-year survival rate of only 40% [2]. Therefore, molecular studies aiming at early detection and targeted treatment of lung cancers draw intensive research interest.

Despite the devastating problem of NSCLC and the estimated 51% increased numbers of cases of this disease since 1985 [3], a panel of reliable serum biomarkers has not yet been identified. Existing lung cancer protein biomarkers include tumor-liberated proteins such as CEA, NSE, TPA, chromogranin, CA125, CA19-9, and Cyfra 21-1. While these are the best options currently available in the clinic, they each have limitations as detailed by Tarro et al [4].

MicroRNAs (miRNAs) are a series of small non-coding cellular RNAs regulating gene expression at the posttranscriptional level [5]. Many genes that involves in the basic biological functions, such as cellular differentiation, proliferation, and apoptosis are targets of miRNAs. It has found that profiles of miRNA expression differ between normal tissues and tumor tissues and vary among tumor types [6].

MicroRNA-199a-5p expression in non-small cell lung cancer

Table 1. Relation of serum miR-199a-5b expression and characteristics of patients with NSCLC

Age	Total N	miR-199a-5b expression		p-value
		High (N)	Low (N)	
65 years	28	8	20	0.264
>65 years	33	11	22	
<i>Gender</i>				0.437
Male	39	12	27	
Female	22	7	15	
<i>Tumour size (cm)</i>				0.218
3	34	10	24	
>3	27	9	18	
<i>Lymph node metastasis</i>				0.0026
Positive	21	5	16	
Negative	40	14	26	
<i>Disease stage (p-stage)</i>				0.0017
I	38	6	22	
II+III	23	13	10	
<i>Lymphatic permeation</i>				0.002
Positive	26	5	21	
Negative	35	14	21	
<i>Vascular invasion</i>				0.013
Positive	37	6	31	
Negative	24	13	11	
<i>Pleural involvement</i>				0.03
Positive	20	4	16	
Negative	41	15	15	
<i>Tumor differentiation</i>				0.174
Well/Moderately	44	13	31	
Poorly	17	6	11	
<i>Smoking habit</i>				0.179
Yes	43	12	31	
No	18	7	11	
<i>Histological type</i>				0.683
Adenocarcinoma	51	14	37	
Squamous cell carcinoma	9	5	4	

The expression levels of miRNAs are tumor specific. This property of the cancer cell is being utilized in cancer diagnosis for early and accurate cancer diagnosis [6, 7]. Depending on their downstream signaling effect on genes and gene products, miRNAs may be up or down-regulated in cancers [8]. The ones which are up-regulated in cancers are proposed to have an oncogenic potential. Alternatively, the miRNAs which are down-regulated in malignancies are proposed to have a tumor suppressor effect.

Recent studies have revealed that miRNA-199a-5p (miR-199a-5p) functions as an anti-tumor effect in many cancer cells, such as follicular thyroid carcinoma [9] and gastric cancer [10]. In hepatocellular carcinoma, a significant down-regulation of miR-199a-5p was observed in 65.2% of HCC tissues and in four of five cell lines, and miR-199a-5p overexpression inhibited invasion of HCC cells [11].

In the present study, we aimed to examine the miRNA-199a-5p expression in samples of NSCLC and to explore their clinical significance in disease development and progression. We then verified the function of miRNA-199a-5p in NSCLC cells *in vitro*.

Materials and methods

Patients

After informed consent and approval by appropriate Institutional Review Board/Independent Ethical Committee of People's hospital of Laiwu, China, NSCLC tissues and adjacent non-tumor tissues (NTs) used for qRT-PCR were collected from 61 NSCLC patients who underwent lung resection between January 2012 and December 2014 at the affiliated hospital of Qingdao University. Tissues were snap frozen in liquid nitrogen immediately after resection and then stored at -80°C until use. No preoperative chemotherapy and/or radiotherapy case was included. Clinical

data were obtained from each patient's medical records and summarized in **Table 1**. Clinical stage was assessed according to the seventh edition of the Lung Cancer Staging International Division, which was published by the Union for International Cancer Control (UICC) and the International Association for the Study of Lung Cancer (IASLC) in 2009. To use these samples for research purposes, prior informed consent from the patients and approval from the Institute Research Ethics Committee of people's hospital of Laiwu were obtained.

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Cell lines and culture

NSCLC cell lines H292, H358, HCC827, H1650, and H1975 were obtained from the American Type Culture Collection (Shanghai, China), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in 5% CO₂/95% air at 37°C. *In vitro* assays were performed at 60-70% cell density.

MiR-199a-5p construct and transfection

The oligonucleotides encoding miR-199a-5p were subcloned into linearized pcDNA3.1 according to the miR-199a-5p sequence documented in miRNA registry database, and verified by DNA sequencing (GenePharma Co., Ltd, Shanghai, China). A negative control vector was established by inserting a mature miRNA, but does not target any known vertebrate gene (Invitrogen). The miR-199a-5p vector and control vector were transfected into H358 and HCC82 cells using siPORT™ NeoFX™ Transfection Agent (10 µl in 200 µl of OPTI-MEM® I medium without serum) for 72 h, cells were harvested and analyzed.

qRT-PCR

The total RNA, including microRNA, was extracted from cells and samples of these patients. Real-time RT-PCR method was used to assess the expression levels of miR-9 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-199a-5b. Initially, we calculated a ΔCt (target-reference), which is equal to the difference between threshold cycles for miR-199a-5b (target) and those for U6 RNA (reference). The fold-change between cancer tissues and normal breast tissue control for miR-199a-5b was calculated with the $2^{\Delta\Delta\text{Ct}}$ method, in which $\Delta\Delta\text{Ct} = \Delta\text{Ct}$ (target-reference in tumor samples) - ΔCt (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 $2^{-\Delta\Delta\text{Ct}}$. The quantitative real-time PCR primers for miRNA were designed according to Chen et al [12]. The reaction parameters were: 58°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 55°C for 1 minute. PCR cycle threshold

(Ct) values were recorded for each target gene and for normalization controls and were averaged across three independent runs. Primers for miR-199a-5b were custom-ordered from Shanghai, China. In addition, each measurement was performed in triplicate.

Wound healing assay

Cells in different groups were seeded onto six-well plates and a scratch was made across the cell monolayer when they reached 100% confluence. Cells were gently washed with PBS and new media containing hydroxyurea 30 m (Sigma, Shanghai, China) were added to block cell division. Cells were incubated for 24 h and photographed using an inverted tissue culture microscope at $\times 100$ magnification.

Invasion assay

The upper chamber of each transwell was coated with Matrigel (BD Biosciences, MA, USA) 1:6 diluted with DMEM at 37°C for 3 h. Cells (2×10^4) were seeded in upper chambers in DMEM and incubated in 24-well-plates with 10% FBS supplemented DMEM. After 48 h of incubation, cells remaining on the upper surface of the membrane were removed with a cotton swab. Cells that invaded through the Matrigel-precoated membrane filter were fixed. The cells were counted at $\times 100$ magnification in 10 random fields of view under a microscope. Three independent experiments were performed in each case.

Flow cytometer for apoptosis assay

Using Annexin V-FITC apoptosis detection kit (BD Biosciences, Guangzhou, China), Annexin V-staining followed by a FACScan flow cytometer was used to detect cell apoptosis according to the manufacturer's instructions. The Cell-Quest software was used to analyze the data (Becton-Dickinson).

Cell viability assay

The cell viability in different groups was quantitatively assessed by MTT assay. The cells were incubated in 500 mg/ml MTT solution for 4 h. After solubilization of formazan crystals in DMSO, the optical density of each well was determined by a spectrophotometric reader at 570 nm (Senago, Shanghai, China).

MicroRNA-199a-5p expression in non-small cell lung cancer

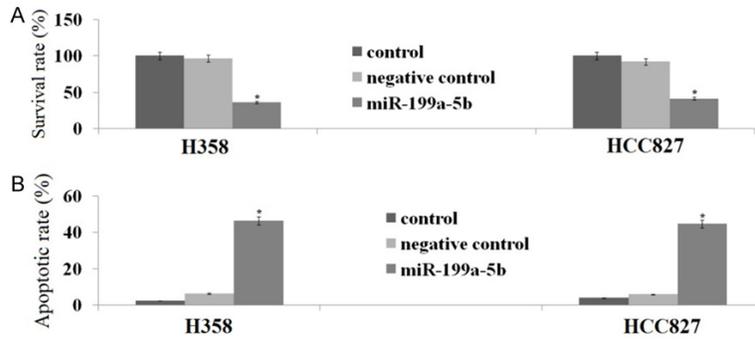


Figure 1. miR-199a-5p inhibits cell viability and induces apoptosis of H358 and HCC827 cells. H358 and HCC827 cells were transfected with miR-199a-5p for 72 h. A. miR-199a-5p inhibits cell viability by MTT assay; B. miR-199a-5p induces cell apoptosis by Flow cytometric assay; Data represented is the means \pm SD of three independent experiments. * $P < 0.01$, compared with the control group.

Statistics

Differences in miR-199a-5b expression and clinicopathologic variables were analyzed using the χ^2 test. Age was dichotomized at the median value. Overall survival (OS) was defined as the time between surgery and death from any cause. Survival curves were calculated using the method of Kaplan-Meier and compared using the log-rank test. Factors shown to be of prognostic significance in the univariate models were evaluated using a multivariate Cox regression model. Mann Whitney test was used to compare the miR-199a-5b expression in NSCLC tissues and adjacent non-tumor tissues; Student's *t*-test (two-tailed) was performed to analyze the data *in vitro* and *in vivo*. A *p* value less than 0.05 was considered statistically significant. All statistical manipulations were performed using the SPSS.17 software program (SPSS Inc., Chicago, IL, USA).

Results

Decreased miR-199a-5b expression in tissues of NSCLC

The expression levels of miR-199a-5b in 61 NSCLC tissues and adjacent non-tumor tissues (NTs) were examined using qRT-PCR. Using U6 RNA as normalization control, the relative levels of median miR-199a-5b expression was 0.03 ± 0.006 (miR-199a-5b/U6) in NSCLC tissues, which was significantly lower than that of in the adjacent non-tumor tissues 0.14 ± 0.02 (miR-199a-5b/U6) ($P < 0.001$, Mann Whitney test).

Correlation between miR-199a-5b and the clinicopathologic features of patients with NSCLC

The 61 patients with NSCLC were divided into high and low miR-199a-5b expression groups (19 and 42 patients, respectively) using the median miR-199a-5b value as the cutoff point. As shown in

Table 1, MiR-199a-5b expression was significantly decreased in the groups of lymph node metastasis, II+III stage, Lymphatic permeation, Vascular invasion and Pleural involvement. No relation was found between miR-199a-5b expression and other characteristics of patients with NSCLC (**Table 1**). These results suggest that decreased MiR-199a-5b expression may be correlated with NSCLC progression.

MiR-199a-5p induces apoptosis and inhibits viability of NSCLC cells *in vitro*

The expression levels of miR-199a-5b in NSCLC cell lines H292, H358, HCC827, H1650, and H1975 were examined using qRT-PCR. Using U6 RNA as normalization control, the relative levels of miR-199a-5b expression was 0.027 ± 0.005 , 0.015 ± 0.04 , 0.012 ± 0.001 , 0.048 ± 0.001 and 0.035 ± 0.003 in H292, H358, HCC827, H1650 and H1975 cells, respectively. Lower miR-199a-5b mRNA was found in H358 and HCC827 cells. So we selected the two cell lines for further investigation.

H358 and HCC827 cells were transfected with miR-199a-5b for 72 h. The cell viability and cell apoptosis was detected by MTT and flow cytometry analysis. Our results showed that transfection of miR-199a-5b for 72 h resulted in more than 60% cell growth inhibition in both of the cells, respectively ($P < 0.01$, **Figure 1A**). In addition, transfection of miR-199a-5b for 72 h resulted in more than 40% cell apoptosis in both of the cells, respectively ($P < 0.01$, **Figure 1B**).

MiR-199a-5p inhibits invasion and migration of NSCLC cells *in vitro*

To determine the effect of miR-199a-5p on the migration and invasion of NSCLC cells, H358

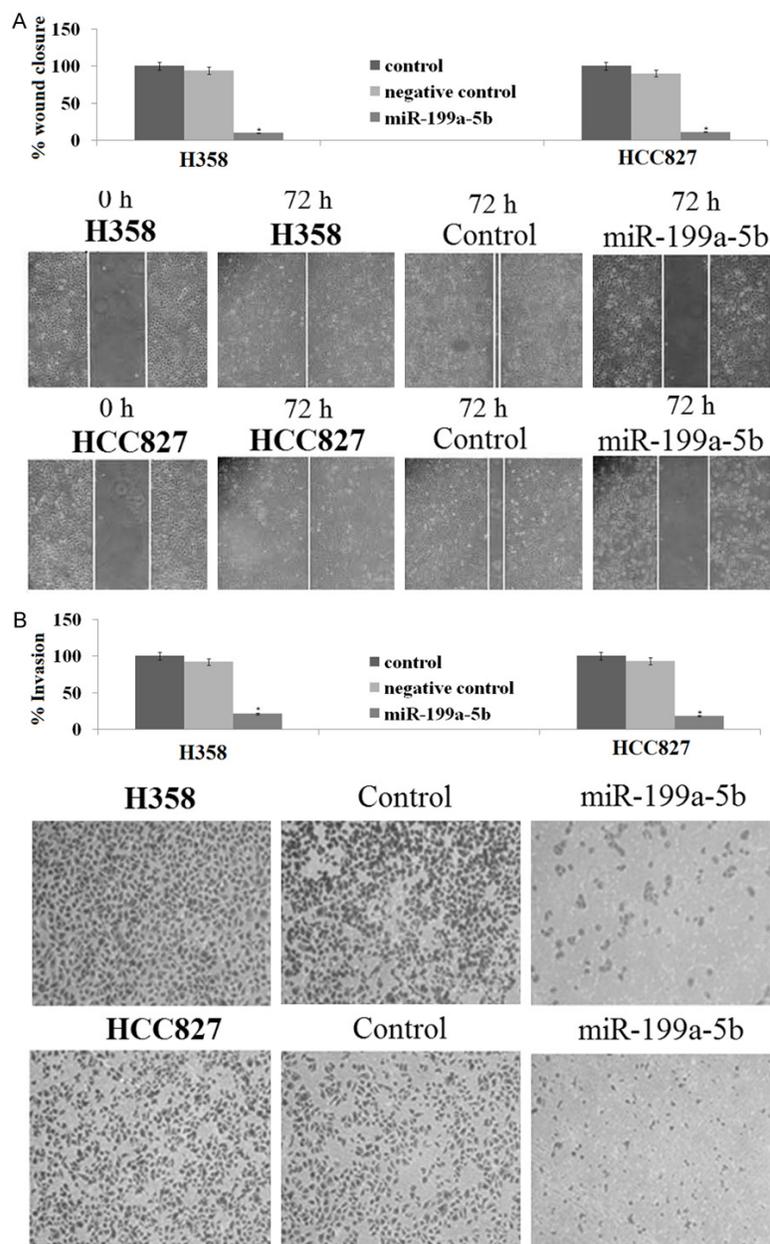


Figure 2. miR-199a-5p inhibits cell invasion and migration of H358 and HCC827 cells. H358 and HCC827 cells were transfected with miR-199a-5p for 72 h. A. Wound-healing assay; B. Matrigel invasion assay; Data represented is the means \pm SD of three independent experiments. * $P < 0.01$, compared with the control group.

and HCC827 cells were transfected with miR-199a-5b for 72 h, matrigel invasion and wound-healing assays were performed. As shown in **Figure 2A**, MiR-199a-5p significantly inhibited migration and invasion in both of the cells, respectively ($P < 0.05$). Transfection with MiR-199a-5p for 72 h showed about 80-90% wound closure in H358 and HCC827 cells ($P < 0.01$, **Figure 2B**).

Discussion

Numerous studies have found that deregulation of miRNAs acts as a factor in the initiation and progression of cancer, though the mechanisms are still largely unknown [13, 14]. Among all of these miRNAs, down-regulation of miR-199a-5b was closely relevant to the metastasis, invasion, proliferation, apoptosis and prognosis of a certain type of cancers [11, 15-18]. MiR-199a-5p is found to be expressed in a broad array of tissues, such as the liver, vascular and visceral smooth muscle, brain, ovarian and testicular tissue, endothelial cells and cardiomyocytes [19].

However, miR-199a-5b was downregulated in a broad array of cancer tissues, including gastric cancer [10], hepatocellular carcinoma [11, 20], osteosarcoma [21], and colorectal cancer [22], and higher miR-199a-5p expression level was associated with increased likelihood of lymph node metastasis and bad prognosis [10, 22].

In the present study, we have examined the role of miR-199a-5p as a prognostic factor in non-small cell lung cancer patients. In our series of patients, miR-199a-5p expression was significantly downregulated in patients with tumor samples compared to

the adjacent non-tumor tissues. Moreover, low miR-199a-5p expression was significantly correlated with the lymph node metastasis, high tumor stage, lymphatic permeation, pleural involvement and vascular invasion. Our results suggest that patients with NSCLC who have lower miR-199a-5p expression are especially likely to have bad outcomes after lung resection. Therefore, we suggested that miR-199a-

5p could serve as a promising biomarker and therapeutic targets in NSCLC.

To verify the potential role of miR-199a-5p on NSCC cells, we selected NSCC cell lines (H358 and HCC827) with lower levels of miR-199a-5p expression. Using H358 and HCC827 cells, we found increase of miR-199a-5p expression by pre-miR-199a-5p transfection in both cells significantly inhibited viability and induced apoptosis. In addition, the invasive and migrate ability of the H358 and HCC827 cells was significantly decreased with the miR-199a-5p expression. These results suggest that restoration of miR-199a-5p expression has an important anti-tumor and anti-metastasis effect on NSCLC cells.

Conclusion

These findings suggest that low miR-199a-5p expression may be involved in more aggressive behavior of NSCLC. Our investigation provides evidence on miR-199a-5p involvement in cell proliferation, apoptosis, invasion and suggests a critical role for miR-199a-5p in the tumor progress. Taken together, these data support the development of miR-199a-5p mimics as new potential therapeutic agents in the treatment of NSCLC.

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Disclosure of conflict of interest

None.

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