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Original Article

Effect of miR-18a overexpression on the radiosensitivity of non-small cell lung cancer

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Abstract: The aim of the present study is to investigate whether there is a relationship between miR-18a expression and radiosensitization of non-small-cell lung caner (NSCLC). The relationship between miR-18a expression and clinicopathological characteristics was investigated. To determine whether the miR-18a expression levels were associated with radiotherapeutic efficacy, therapeutic response was evaluated by radiologic Response Evaluation Criteria in Solid Tumors (RECIST). To determine whether miR-18a was required for lung cancer cell radioresistance, A549 cells were treated with different doses of ionizing radiation, following transfection with inhibitor miR-18a or inhibitor NC. We found that the level of miR-18a in NSCLC was strongly correlated with tumor differentiation (P = 0.026), regional lymph node metastasis (P = 0.013) and clinical TNM stage (P = 0.005). According to RECIST, miR-18a expression level was significantly associated with therapeutic response, exhibiting higher expression level in non-responsive patients. Furthermore, the depletion of miR-18a increased A549 cell radiosensitivity. In conclusion, we provide the evidence that down-regulation of miR-18a sensitizes NSCLC to radiation treatment, and it may help to develop a new approach to sensitizing radioresistant lung cancer cells by targeting miR-18a.

Keywords: MicroRNA, miR-18a, NSCLC, lung cancer, radiosensitivity

Introduction

Lung cancer is the leading cause of cancer-related mortality in the word and non-small-cell lung caner (NSCLC) is the most common form of lung cancer [1]. Radiotherapy remains an important form of local and regional cancer therapy [2]. Improving lung cancer radiation sensitivity can increase the local control and radiation effect [3, 4]. Cellular radiosensitivity has been show to correlate with cell apoptosis, cell cycle, and repair of DNA damage. Therefore, identifying markers of radioresistance is an unmet need in the therapeutic management of lung cancer patients [5-7].

MicroRNAs (miRNAs) are a group of small non-coding RNAs which suppress their target expression by binding to the 3' untranslated region (3'UTR). In previous decades, accumulating studies have demonstrated that miRNAs act as key regulators in the development and progression of various types of cancer, including lung cancer [8, 9]. Previously, researchers have found that the expression of miR-18a was increased in lung cancer [10]. We are interested in whether there is a relationship between miR-18a expression and radiosensitization of NSCLC.

Materials and methods

Patients and tissue samples

A total of 120 pairs of fresh NSCLC and matched adjacent normal tissue specimens were collected from patients who underwent surgery at the department of thoracic surgery, Jinling Hospital. The fresh tissue specimens were collected and immediately placed in liquid nitrogen and then stored at -80°C until the isolation of RNA. The pathologic diagnosis was conducted by two pathologists, and any different conclusions were resolved by careful study and discussion. Tumor stage was determined according to the 2009 TNM staging classification system. Clinicopathological features of patients are summarized in **Table 1**. This study was approved

Table 1. Clinicopathological variables and the expression of miR-18a in total NSCLC patients

Clinicopathological variables	Case number	miR-18a expression		*P* value
		High (n = 58)	Low (n = 62)	
Sex distribution				
Female	54	26	28	0.551
Male	66	32	34	
Age (years)				
< 60	72	31	41	0.214
≥ 60	48	27	21	
Smoking history				
Current	25	12	13	0.593
Former	83	41	42	
Never	12	5	7	
Differentiation				
Poor	33	22	11	0.026
Well/Moderate	87	36	51	
TNM stage				
I-II	71	52	19	0.005
IIIa	49	6	43	
Regional lymph node involvement				
Absent	78	39	39	0.013
Present	42	19	23	

by the Research Ethics Committee of Jinling Hospital. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Cell line

The human lung adenocarcinoma cell line A549 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). A549 cell line was cultured in DMEM containing 10% fetal bovine serum (Gibco®, Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator to the log phase of proliferation before harvesting the cells. Normal human bronchial epithelial cells (NHBE) (Clonetics™) were maintained in a culture medium according to the protocol provided by Clonetics™.

MicroRNA isolation and real-time quantitative RT-PCR assay

Total miRNA from cultured cells and tissues was extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The purity and concentration of RNA were determined using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The differentially expressed amount of the miR-18a was validated in triplicate by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Briefly, 2 μg of RNA was added to RT reaction, and then, the cDNA served as the template for amplification of PCR with sequence-specific primers (Sangon Biotech, Shanghai, China) using SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co. Ltd, Dalian, China) on the 7500 Real-Time PCR systems (Applied Biosystems, Carlsbad, CA, USA). The PCR cycling profile was denatured at 95°C for 30 s, followed by 40 cycles of annealing at 95°C for 5 s, and extension at 60°C for 34 s. Small nucleolar RNA U6 was used as an internal standard for normalization. The cycle threshold (C_T) value was calculated. The $2^{-\Delta CT}$ ($\Delta C_T = C_{TmiR18a} - C_{TU6\ RNA}$) method was used to quantify relative amount of miR-18a.

Cell transfection

For the transfection, 1×10^6 cells were treated with 50 nM GMR-miRTM inhibitor miR-18a/inhibitor NC (Shanghai GenePharma) in 1 μl of Lipofectamine™2000 (Invitrogen, USA) according to the manufacturer's instructions.

Radiation exposure

The cells were seeded into 96-well plates and treated with a range of radiation doses (0-10 Gy) using a 250-kV orthovoltage unit the following day (Philips, Amsterdam, The Netherlands).

Colony-forming assay

A colony-forming assay was performed to determine the radiosensitivity of cells. Cells (1×10^5/well) were plated in a 24-well plate and

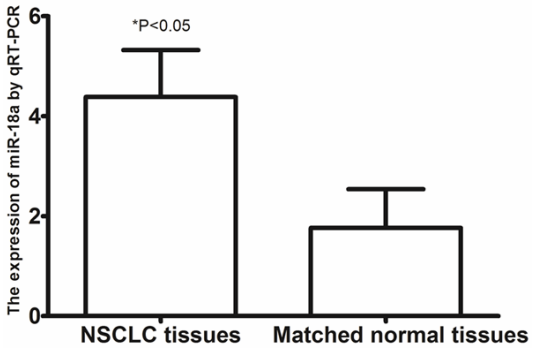

Figure 1. The expression of miR-18a in lung cancer tissues and matched normal tissues.

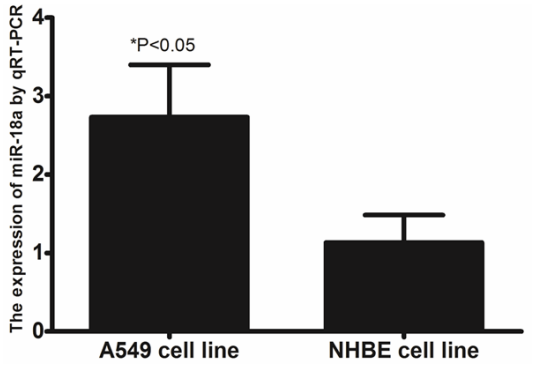

Figure 2. The expression of miR-18a in A549 lung cancer cell line, and NHBE cell line.

transfected with inhibitor miR-18a/inhibitor NC at 20 nmol/l by using Lipofectamine ™2000 (Invitrogen). After 48 h, the cells were collected and seeded (500-1,000/well) in a fresh six-well plates. After 12 days, visible colonies were fixed and stained with crystal violet. A population of > 50 cells was counted as one colony. The number of clones was examined using macroscopic observation.

Statistical analysis

The quantitative values were expressed as mean ± standard deviation (SD), and the hypothesis test for significance between two groups utilized the Student's t test. The chi-square test and Fishers exact tests were used to examine the associations between miR-18a expression and the clinicopathological characters. Statistical significance was set as $P < 0.05$. Statistical analyses were performed using SPSS 18.0 soft-ware (Chicago, Ill., USA) and GraphPad Prism 5 (GraphPad Software Inc., CA, USA).

Results

Expression of miR-18a in lung cancer tissues and A549 cell line

To define the role of miR-18a in human lung cancer tumorigenesis, we compared the expression levels of miR-18a in tissue samples, A549 lung cancer cell, and NHBE cell line. As shown in **Figure 1**, the expression levels of miR-18a were up-regulated in lung cancer tissues as compared to matched normal tissues from the same patients. The expression of miR-18a in the A549 lung cancer cell line was significantly increased as compared to NHBE cell line (shown in **Figure 2**).

Relationships between expression of miR-18a and clinical parameters in lung cancer patients

The relationship between miR-18a expression and clinicopathologic parameters of 120 patients with NSCLC was evaluated. As shown in **Table 1**, the level of miR-18a in NSCLC was strongly correlated with tumor differentiation ($P = 0.026$), regional lymph node metastasis ($P = 0.013$) and clinical TNM stage ($P = 0.005$). However, there were no significant associations between miR-18a expression and other clinical features including sex ($P = 0.551$), age ($P = 0.214$), and smoking status ($P = 0.593$).

MiR-18a expression correlated with radioresistance of lung cancer patients

To determine whether the miR-18a expression levels were associated with radiotherapeutic efficacy, therapeutic response was evaluated by radiologic Response Evaluation Criteria in Solid Tumors (RECIST). According to RECIST, 65% patients responded to radiotherapy with complete response or partial response; 35% patients were not responsive with stable disease or disease progression. The result showed that miR-18a was significantly associated with therapeutic response, exhibiting higher expression level in non-responsive patients ($P = 0.019$, shown in **Table 2**).

MiR-18a regulated sensitivity of A549 lung cancer cells to radiation

To determine whether miR-18a was required for lung cancer cell radioresistance, A549 cells

Table 2. Correlation of miR-18a level and response to radiotherapy

		miR-18a expression		
Treatment response	All case (n = 120)	High (n = 58)	Low (n = 62)	*P* value
Complete response + partial response	78	30	48	0.019
Stable disease + disease progression	42	28	14	

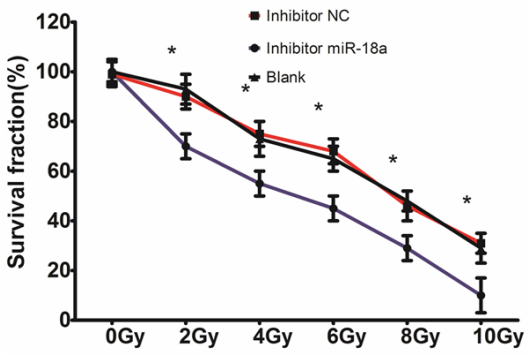

Figure 3. MiR-18a-regulating sensitivity of A549 cells to radiation. The A549 cells transfected with inhibitor miR-18a or inhibitor NC were treated with 0, 2, 4, 6, 8, and 10 Gy irradiation, and survival curves were determined using a colony-forming assay. *P < 0.05.

were treated with different doses of ionizing radiation, from 0 to 10 Gy, following transfection with inhibitor miR-18a or inhibitor NC. Ionizing radiation treatment exhibited a dose-dependent inhibitory effect on the growth of the A549 cells, and the miR-18a depletion enhanced this effect, suggesting that the depletion of miR-18a increased A549 cell radiosensitivity (shown in **Figure 3**).

Discussion

Radiation therapy is one of the most important methods in cancer treatment. However, many types of cancer show radioresistance, thereby affecting the efficacy of radiotherapy in clinical practice. Methods for improving radiosensitivity and reducing radioresistance are accordingly of great interest in cancer radiotherapy [11]. The factors that affect the consequences of radiotherapy include the capability of cancer cells to develop radioresistance, the degree of tumor tissue hypoxia, and the survival ability of the remaining cancer cells postradiotherapy [12]. Thus, overcoming cancer radioresistance is important for achieving better clinical outcomes for patients.

Several previous research studies have shown that miRNAs were closely related to tumor radiosensitivity. This is because miRNAs have the ability to increase and decrease radiosensitivity of tumors [13-15]. Given that miRNAs have the ability to regulate multiple oncogenic processes such as responsiveness to therapy, we must explore the role of miRNAs in radiation resistance.

miR-18a belongs to the miR-17-92 cluster located in the 13q31.1 region, an area partly regulated by the oncogenic transcription factor c-Myc [16]. miR-17-92 cluster consists of seven miRNAs: miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a. Previous studies have confirmed that the miR-17-92 cluster is highly expressed in several types of cancers, such as gastric cancer, breast cancer, melanoma, osteosarcoma, and so on [17-21]. The oncogenic role of the miR-17-92 cluster has also been well-documented, with its overexpression linked to accelerated tumour growth, cell proliferation and progression. Previously, Krysan et al found that prostaglandin E2, abundantly produced by NSCLC and inflammatory cells in the tumor microenvironment, was able to stimulate cell proliferation and promote resistance to pharmacologically induced apoptosis in a c-Myc and miR-17-92-dependent manner [22]. In the present study, we investigated if miR-18a could play an important role in the development of radiosensitization in NSCLC. We found that the expression levels of miR-18a were up-regulated in lung cancer tissues and the A549 lung cancer cell line. Furthermore, the level of miR-18a in NSCLC was strongly correlated with tumor differentiation, regional lymph node metastasis and clinical TNM stage. To determine whether the miR-18a expression levels were associated with radiotherapeutic efficacy, therapeutic response was evaluated by RECIST. According to RECIST, 65% patients responded to radiotherapy with complete response or partial response; 35% patients were not responsive with stable disease or disease progression. The result showed that miR-18a was significantly associated with therapeutic response, exhibiting higher expres-

sion level in non-responsive patients. To determine whether miR-18a was required for lung cancer cell radioresistance, A549 cells were treated with different doses of ionizing radiation, from 0 to 10 Gy, following transfection with inhibitor miR-18a or inhibitor NC. Ionizing radiation treatment exhibited a dose-dependent inhibitory effect on the growth of the A549 cells, and the miR-18a depletion enhanced this effect, suggesting that the depletion of miR-18a increased A549 cell radiosensitivity. Previously, Song et al found an important link between miR-18a-impaired DNA damage response and downregulation of ataxia-telangiectasia mutated (ATM) kinase in breast cancer. Their findings revealed the significance of miR-18a in regulating cell cycle checkpoints, double strand breaks (DSBs) repair and radiosensitivity in breast cancer. To the best of our knowledge, our study was the first to confirm the effect of miR-18a overexpression on the radiosensitivity of NSCLC.

In conclusion, we provide the evidence that down-regulation of miR-18a sensitizes NSCLC to radiation treatment, and it may help to develop a new approach to sensitizing radioresistant lung cancer cells by targeting miR-18a.

Disclosure of conflict of interest

None.

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