miR-210-3p regulates the proliferation and apoptosis of non-small cell lung cancer cells by targeting SIN3A

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Abstract. Previous studies have indicated that microRNA (miR)-210-3p is upregulated in NSCLC, however, the specific mechanism underlying the role of miR-210-3p in NSCLC pathogenesis requires further investigation. The aim of the present study was to explore the roles of miR-210-3p in NSCLC and the associated mechanisms. A total of 30 NSCLC tissues and paired adjacent normal tissues were collected for study. Reverse transcription-quantitative polymerase chain reaction was performed to compare the expression of miR-210-3p in the 30 paired cancerous and adjacent normal tissues. Additionally, the expression of miR-210-3p in different NSCLC lines and normal human lung epithelial cell line BEAS-2B were also compared. Furthermore, A549 and H1299 NSCLC cells were cultured and transfected with miR-210-3p inhibitors, and MTT and propidium iodide/annexin V assays were performed to investigate the effects of miR-210-3p inhibition on the proliferation and apoptosis of the cells. RT-qPCR and western blot analyses were also performed to determine the effects of miR-210-3p on the expression levels of SIN3A, B-cell lymphoma 2 (Bcl-2) and Caspase-3. Finally, a reverse experiment was conducted by transfecting A549 cells with miR-210-3p inhibitor and SIN3A small interfering (si)RNA, and a dual-luciferase reporter assay was performed to confirm that SIN3A is a direct target of miR-210-3p. The results of the dual-luciferase reporter assay demonstrated that SIN3A is a direct target of miR-210-3p. Collectively, these findings indicate that can regulate the proliferation and apoptosis of NSCLC cells by targeting SIN3A. These results suggest that miR-210-3p has the potential to become a novel therapeutic target for the treatment of NSCLC.

Introduction

Lung cancer is one of the most common malignancies and the leading cause of cancer-related mortality every year worldwide (1). Multiple risk factors, including environmental deterioration, smoking, exposure to radon and the overexpression or mutation of certain key proteins (for example, epidermal growth factor receptor) may contribute to the occurrence and development of the disease (2-4). Due to the heterogeneity of the disease, the lack of early diagnostic markers and the high recurrence rate (~50% of cases), the therapeutic efficacy of current anti-lung cancer therapies remains unsatisfactory (5), the therapeutic efficacy of current anti-lung cancer therapies, including surgical resection, radiotherapy and chemotherapy remain unsatisfactory, and the prognosis for patients with lung cancer is poor (5-year survival rate, <16%) (6,7). Although lung cancer is divided into various subtypes, non-small cell lung cancer (NSCLC) is the most common, accounting for >85% of cases (8) NSCLC can be classified into three subtypes, including adenocarcinoma, squamous cell carcinoma and large cell carcinoma. In recent years, efforts have been made to explore the pathogenesis of NSCLC (9-12); however, the mechanism underlying the carcinogenesis and development of NSCLC remains poorly understood. Elucidating the molecular mechanisms involved in NSCLC may help researchers to identify effective therapeutic targets, and also provide novel biomarkers for the risk assessment and early diagnosis of NSCLC (13).

In recent years, the roles of non-coding RNA in various diseases have become a hot topic among scientists and
physicians. MicroRNA (miR) are a group of small, non-coding RNA that may negatively regulate the expression of their target genes (14). Abnormal behaviors of miR in various types of cancer have been observed, and the roles of miR as tumor suppressors or oncomiR have been reported previously (15-17). miR-210 has been demonstrated to serve important roles in a variety of cancer types. For example, it was revealed that the deletion of miR-210-3p may promote the carcinogenesis of renal cell carcinoma (18), while miR-210 is increased in osteosarcoma and may contribute to the malignant progression of the disease (19). Furthermore, in breast cancer, miR-210 may interact with F-box only protein 31 and regulate the proliferation and migration of human breast cancer cells (20).

In NSCLC, the upregulation of miR-210-3p has been demonstrated in several previous studies (21-23); however, the role of miR-210-3p in the pathogenesis NSCLC requires further investigation. The aim of the present study was to investigate the expression and biological functions of miR-210-3p in NSCLC, and to elucidate the underlying molecular mechanisms in NSCLC. The expression of miR-210-3p in NSCLC tumor tissues and cell lines was examined, and the associations between the expression of miR-210-3p and the clinical features of patients with NSCLC were investigated. Furthermore, the effect of miR-210-3p on cell proliferation and apoptosis was explored.

Materials and methods

Patients and clinical information. The present study enrolled 30 patients (Average age 62.1±9.8 years, Age range, 35-79 years; 18 males and 12 females) who had been diagnosed with NSCLC between August 2010 and December 2015 at Handan First Hospital (Handan, China). For each patient, paired cancer tissues and adjacent normal tissues were collected during surgery and immediately stored in liquid nitrogen until use. Patients who received chemotherapy or radiotherapy were excluded from the study. The clinical information of the patients is summarized in Table I. The Research Ethics Committee of Handan First Hospital approved the present study, and each patient signed an informed consent form.

Cell culture. The human NSCLC cell lines A549, H358, H1650 and H1299, and normal human lung epithelial cell line BEAS-2B, were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin / streptomycin solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in an incubator at 37°C with 5% CO₂.

Cell transfection. The miR-210-3p inhibitors (5'-UCAGCCC GCUGUCACACGCAAG-3'; 50 nM), miR-210-3p inhibitor negative control (NC; 5'-CAGUCUUUGUGUAGUAA CA-3'; 50 nM) and SIN3A small interfering (si)RNA (20 nM; forward, 5'-CUAGCUCUAGGAACCUTT-3' and reverse 5'-UAGGUCUUGAGACGUAGTT-3') were synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). The sequences of the SIN3A siRNA were: Cell transfection was performed using Lipofectamine® RNAi Max (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were divided into five groups: Control group (untreated cells), NC group (miR-210-3p inhibitor NC-transfected group), inhibitor group (miR-210-3p inhibitor-transfected group), inhibitor + SIN3A siRNA group (miR-210-3p inhibitor + SIN3A siRNA-transfected group), NC + SIN3A siRNA group (miR-210-3p inhibitor NC + SIN3A siRNA-transfected group). The effects of the miR-210-3p inhibitor on A549 or H1299 cells were examined using various assays at 48 h after transfection.

Cell proliferation analysis. The effects of the miR-210-3p inhibitor on the proliferation of A549 and H1299 cells was determined by using an MTT assay (the purple formazan was dissolved in DMSO) at 12, 24 and 48 h, as well as a Cell Proliferation kit I (MTT; Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. Viability was determined by the optical density values at 490 nm.

Cell apoptosis analysis. After transfection for 48 h, A549 and H1299 cells were double-stained with annexin V-fluorescein isothiocyanate (FITC) at 4°C for 15 min and propidium iodide (PI) and at 4°C for 5 min using a PI/Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA). The rates of apoptosis of the cells in different groups were examined with a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The data was analyzed using FlowJo (version 7.6.5; Tree Star, Inc., Ashland, OR, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells and the tissue samples using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and RT-qPCR was performed. The expression of miR-210-3p was examined using a Hairpin-it™ MicroRNA Quantitation kit (Shanghai GenePharma Co., Ltd.), with U6 (RNU6B; Shanghai GenePharma Co., Ltd.) used for normalization. The thermocycling profiles were as follows: 95°C for 3 min; followed by 40 cycles of 95°C for 15 sec and 62°C for 30 sec. The data were analyzed using the 2⁻ΔΔCq method (24). The mRNA expression levels of SIN3A were examined by performing reverse transcription with a PrimeScript™ RT Master Mix (Takara Biotechnology Co., Ltd., Dalian, China) at 37°C for 15 min. PCR was performed using a SYBR® Fast qPCR Mix (Takara Biotechnology Co., Ltd.), with GAPDH for normalization. RT-qPCR was conducted on an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The primer sequences were as follows: miR-210-3p forward, 5'-GTGCCAGGTCGCCAGGT-3' and reverse, 5'-TATCTG TGGGGTTCGCAGCAGGCT-3'; U6 forward, 5'-CTCGCT TGCAGCAGCACA-3' and reverse, 5'-AAGCGTCTACACG AGTTTGCCTG-3'; SIN3A forward, 5'-TTAAACATTCAGGAC TCAGACAC-3' and reverse, 5'-AGGATTTCAGTACCTC CA-3'; GAPDH forward, 5'-CATTCTCGTGGATGACAA CGA-3' and reverse, 5'-GTCTACAGGCGAAGTGGAG-3'. The thermocycling profiles were as follows: 95°C for 30 sec; followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The data were analyzed using the 2⁻ΔΔCq method.
Western blot analysis. A549 cells were harvested at 48 h after transfection and lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China). The concentration of the total protein was determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Subsequently, 8% SDS-PAGE gel was used to separate the proteins, which were then transferred to polyvinylidene difluoride membranes and blocked with 5% non-fat milk at room temperature for 1 h. Subsequently, the membranes were incubated overnight at 4˚C with the following primary antibodies (1:1,000) obtained from Wuhan Boster Biological Technology, Ltd. (Wuhan, China): Anti-SIN3A (cat. no. BM5270), anti-B-cell lymphoma 2 (Bcl-2; cat. no. A00040-1), anti-Caspase-3 (cat. no. BM3957) and anti-GAPDH (cat. no. A00227). The following day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. A0208, 1:1,000; Beyotime Institute of Biotechnology) at room temperature for 1 h. Subsequently, the membranes were incubated overnight at 4˚C with the following primary antibodies (1:1,000) obtained from Wuhan Boster Biological Technology, Ltd. (Wuhan, China): Anti-SIN3A (cat. no. BM5270), anti-B-cell lymphoma 2 (Bcl-2; cat. no. A00040-1), anti-Caspase-3 (cat. no. BM3957) and anti-GAPDH (cat. no. A00227). The following day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. A0208, 1:1,000; Beyotime Institute of Biotechnology) at room temperature for 45 min and treated with BeyoECL Plus enhanced chemiluminescent reagent (Beyotime Institute of Biotechnology). The signals were detected and imaged using a ChemiDoc™ XRS+ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed by using ImageJ software (version 1.47; NIH, Bethesda, MD, USA).

Statistical analysis. All statistical analysis was performed using SPSS v. 22.0 (IBM Corp., Armonk, NY, USA). Data were presented as the mean ± standard deviation of three repeated experiments. The differences between the expression levels of miR-210-3p and SINA3 in the paired tumor tissues and adjacent tissues were analyzed using a paired Student's t-test, while the differences among multiple groups for in vitro studies were analyzed using one-way analysis of variance followed by a Turkey's post hoc test. Pearson's correlation coefficient was used for correlation analysis. Cox regression model was applied for univariate analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of the expression of miR-210-3p in tissue samples and cell lines. Initially, paired lung cancer tissues and adjacent normal tissues were collected from 30 patients with NSCLC, and the levels of miR-210-3p in the different tissues were examined using RT-qPCR (Fig. 1). The expression of miR-210-3p was significantly increased in the cancer tissues compared with the level in the adjacent tissues (P<0.01; Fig. 1A). The
expression level of miR-210-3p was positively correlated with tumor size (P=0.005), histological grade (P=0.024) and metastasis (P=0.01) (Table 1). Furthermore, the expression levels of miR-210-3p in four NSCLC cell lines (A549, H358, H1650, H1299) and a normal human lung epithelial cell line (BEAS-2B) were also examined. As demonstrated in Fig. 1B, the expression of miR‑210‑3p was significantly increased in NSCLC cell lines compared with the level in the BEAS-2B cell line (P<0.05). A549 and H1299 exhibited the highest expression of miR-210-3p and as such were utilized for further analysis.

Effect of transient miR-210-3p knockdown on the proliferation and apoptosis of A549 and H1299 cells in vitro. To further explore the roles of miR-210-3p in the pathogenesis of NSCLC, A549 and H1299 cells were cultured and transfected with miR-210-3p inhibitors or inhibitor NCs, and the effects of miR-210-3p on the proliferation and apoptosis of A549 cells were examined using an MTT assay and flow cytometry, respectively. The transient downregulation of miR-210-3p in the inhibitor group significantly suppressed the cell growth at 24 and 48 h after transfection, as compared with the control group (P<0.01; Fig. 2). Furthermore, the results of the flow cytometry analysis indicated that transfection of A549 and H1299 cells with miR-210-3p inhibitors induced a marked increase in the rate of cell apoptosis in vitro, as compared with the level in the control group (P<0.01; Fig. 3).

SIN3A is a direct target of miR-210-3p in NSCLC. Using bioinformatics tools (TargetScan (25)), SIN3A was predicted as a target of miR-210-3p. Therefore, the association between miR-210-3p and SIN3A in the pathogenesis of NSCLC was explored. The expression level of SIN3A was demonstrated to be significantly decreased in lung cancer tissues compared with the level in the paired adjacent tissues (P<0.01; Fig. 4A), and the expression level of miR-210-3p was negatively correlated with that of SIN3A (r=-0.273; P=0.009; Fig. 4B).

Knockdown of miR-210-3p induced a significant decrease in the expression level of miR-210-3p and a significant increase in the expression of SIN3A in A549 and H1299 cells at the mRNA and protein levels, when compared with the control group (P<0.05; Figs. 5 and 6). Furthermore, compared with the control group, knockdown of miR-210-3p significantly decreased the expression level of the anti-apoptotic factor Bcl-2 and increased the expression of the pro-apoptotic factor Caspase-3 (P<0.05; Fig. 6B and C). On the other hand, co-transfection with miR-210-3p inhibitor and SIN3A siRNA partially blocked the miR-210-3p inhibitor-induced pro-apoptotic effects (Fig. 7). Co-transfection with miR-210-3p NC and SIN3A siRNA further reduced the expression of SIN3A (P<0.05), when compared with the NC group, increased the proliferation and inhibited the apoptosis of A549 cells (but with no significant difference compared with control; P>0.05).
Finally, a dual-luciferase reporter assay was used to investigate whether miR-210-3p directly targets SIN3A. It was revealed that transfection of the cells with miR-210-3p mimics significantly suppressed the luciferase activity of the SIN3A-3'UTR reporter compared with the activity in the NC (P<0.01); whereas miR-210-3p mimics had no significant effect on cells transfected with the SIN3A-MUT reporter (Fig. 8). These results suggest that miR-210-3p directly targets the 3'UTR of SIN3A.

Discussion

The roles of miR-210 in various cancer types have been discussed in numerous previous studies. miR-210 has been demonstrated to be upregulated in the majority of cancer types, including pancreatic cancer (26,27), colorectal cancer (28,29), breast cancer (30-32) and renal cell carcinoma (33-35), suggesting that it may act as an oncomiR. The roles of miR-210 in lung cancer have also been discussed previously. Zhang et al (36) reported that miR-210 was upregulated in the plasma of patients with early-stage NSCLC, suggesting that miR-210 has the potential to be a biomarker for the early diagnosis of NSCLC that may be detected by non-invasive techniques. Eilertsen et al (21) demonstrated that the expression of miR-210 in stromal cells and cancer cells may serve as a prognostic marker in NSCLC, and Li et al (37) suggested that the serum levels of miR-210 may serve as a diagnostic and prognostic marker for NSCLC. However, the majority of these studies have focused on the diagnostic and prognostic value of miR-210, and investigations into the effects of miR-210 on lung cancer cell behavior, including cell growth, apoptosis and migration, as well as the specific mechanisms underlying the role of miR-210 in the pathogenesis of NSCLC, have been limited. In the present study, increased miR-210-3p expression in cancer tissues and different NSCLC cell lines was observed, which was consistent with previous findings. Furthermore, knockdown of miR-210-3p in the A549 and H1299 lung cancer cell lines led to the significant suppression of cell proliferation and increase of cell apoptosis. These results suggest that...
miR-210-3p is upregulated in NSCLC and that it may regulate the proliferation and apoptosis of lung cancer cells.

Using bioinformatics tools, SIN3A was predicted as a target gene of miR-210-3p; however, to the best of our knowledge, the association between miR-210-3p and SIN3A in NSCLC has not yet been discussed. SIN3A is a transcriptional regulator that contains a number of protein-interaction domains. Previous studies have indicated that SIN3A is involved in the processes of cell proliferation, apoptosis, differentiation and migration, as well as the regulation of the cell cycle and embryonic development, via interacting with certain proteins, including Myc, Myc-associated factor X, Max dimerization protein and methyl-CpG binding protein 2. Previous studies have identified SIN3A as a tumor suppressor in NSCLC. Suzuki et al. observed decreased expression of SIN3A in NSCLC, and Das et al. demonstrated that downregulation of SIN3A could increase the invasive behavior of A549 cells.

In the present study, a series of experiments were performed to explore the association between miR-210-3p and SIN3A in NSCLC. First, the expression levels of SIN3A in NSCLC tissues and adjacent normal tissues were compared, and it was revealed that SIN3A was downregulated in NSCLC, which was consistent with the results described by Suzuki et al. Previous studies have identified SIN3A as a tumor suppressor in NSCLC, and Das et al. demonstrated that downregulation of SIN3A could increase the invasive behavior of A549 cells.

In the present study, a series of experiments were performed to explore the association between miR-210-3p and SIN3A in NSCLC. First, the expression levels of SIN3A in NSCLC tissues and adjacent normal tissues were compared, and it was revealed that SIN3A was downregulated in NSCLC, which was consistent with the results described by Suzuki et al. A subsequent correlation analysis indicated that the expression of SIN3A was significantly negatively correlated with the expression of miR-210-3p, suggesting that the upregulation of miR-210-3p may lead to inhibited expression of SIN3A in lung cancer tissues. The transfection of A549 and H1299 cells with an miR-210-3p inhibitor induced a significant increase in the expression of SIN3A, and also led to a significant decrease in...
the expression of the downstream anti-apoptotic protein, Bcl-2, and an increase in the expression of the pro-apoptotic protein, Caspase-3. Furthermore, co-transfection with miR-210-3p inhibitor and SIN3A siRNA partially blocked miR-210-3p inhibitor-induced pro-apoptotic effects. Finally, a dual-luciferase reporter assay indicated that SIN3A is a direct target of miR-210-3p. As discussed, miR-210-3p may regulate the proliferation and apoptosis of A549 cells, and SIN3A is a key regulator of cell proliferation and apoptosis; thus, miR-210-3p may promote the proliferation and inhibit the apoptosis of lung cancer cells, at least partially, through targeting SIN3A.

The present study has limitations. First, due to ethical issues, only 30 clinical samples were included; therefore, the results should be verified with a larger sample size. Second, the present study includes only a clinical study and in vitro cell studies, and in vivo animal studies should also be performed to confirm the roles of miR-210-3p and SIN3A in NSCLC in the future.

In conclusion, the results of the present study indicate that miR-210-3p is upregulated in NSCLC and may regulate the proliferation and apoptosis of lung cancer cells by targeting SIN3A. These results may provide a novel therapeutic target for the treatment of NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors' contributions

XL and HD analyzed the patient data. LS performed histological examination of samples. JuZ and LZ performed some of the cell experiments. JR performed most of the cell experiments and he was a major contributor in writing the manuscript. JIiZ designed the study and wrote part of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Research Ethics Committee of Handan First Hospital approved the present study. Each patient signed an informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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