MicroRNA-15b promotes proliferation and invasion of non-small cell lung carcinoma cells by directly targeting TIMP2

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Abstract. MicroRNA-15b (miR-15b) plays an important role in tumor development and progression. miR-15b functions differently in various types of malignant tumors. However, the expression pattern and role of miR-15b in non-small cell lung cancer (NSCLC) have not been elucidated. In the present study, we investigated the expression of miR-15b in the occurrence and development of lung cancer and the underlying mechanism. Lung cancer cell lines A549 and LTEP-a-2 were transfected with miR-15b inhibitor or mimic, respectively. Real-time PCR revealed that the expression level of miR-15b was significantly higher in human NSCLC tissues and NSCLC cells, than that of normal tissues and cells, respectively (P<0.05). Moreover, the effect of miR-15b on A549 and LTEP-a-2 cell viability, cell cycle, migration and invasion was further evaluated. Experiments indicated that miR-15b knockdown inhibited the viability, cell cycle, migration and invasion in A549 cells, while upregulation of miR-15b exhibited the opposite effect. Tissue inhibitor of metalloproteinases 2 (TIMP2) protein and mRNA levels were downregulated after miR-15b overexpression in A549 and LTEP-a-2 cells, respectively. The dual-luciferase reporter gene assay implied that TIMP2 is a direct target gene of miR-15b. Our results indicate that high expression of miR-15b is associated with NSCLC and suggest that miR-15b expression may be a novel biomarker for predicting clinical outcomes in NSCLC patients. The inhibition of miR-15b may even provide helpful therapeutic strategies for the treatment of NSCLC.

Introduction

Lung cancer is one of the most malignant cancers worldwide. The mortality rate of lung cancer in China is higher than other solid tumors (1). According to the characteristics of pathology, lung cancer is mainly classified into 2 types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the most common form of lung cancer, accounting for ~80-85% of all cases (2). NSCLC has a high mortality, and the 5-year survival rate is less than 15% despite improvement in cancer treatment (3). The majority of patients will have developed an aggressive form of NSCLC and even metastases to other organs by the time of diagnosis, which is often at a late stage, seriously troubling doctors. Thus, it is urgent to explore novel biomarkers for early detection, thereby improving treatment outcome.

MicroRNAs (miRNAs) are single-stranded, endogenous non-coding, small (~22 nucleotides in length) RNAs which play crucial roles in the regulation of genes by directly binding to the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs) (4,5). miRNAs are implicated in a broad range of important cellular processes (6-8). More and more evidence demonstrates that miRNAs act as tumor suppressors or novel oncogenes according to the roles of their target genes, and aberrant miRNA expression is common in various types of human cancer including NSCLC (9-11).

miR-15b belongs to the miR-15/16 cluster which includes miR-15a, miR-195 and miR-16-1/2 (12,13). Previous studies have found that miR-15b is upregulated in several types of cancer, suggesting its pivotal role in tumorigenesis and progression (14,15). On the contrary, in other tumors miR-15b acts as a tumor suppressor (16-19). These findings suggest that the functions of miR-15b may be different in various cells. To date, many miRNAs have been identified in NSCLC, including miR-30c, miR-4500, miR-193a, miR-4782-3p and miR-138 (20,21). The role of miR-15b in the progression of NSCLC and its underlying mechanism, however, remain unclear.

In the present study, we demonstrated that miR-15b was significantly upregulated in NSCLC tissues and cell lines. In vitro experiments revealed that downregulation of miR-15b inhibited NSCLC cell growth, migration and invasion, while upregulation of miR-15b promoted NSCLC cell growth, migration and invasion. Moreover, downregulation of miR-15b suppressed tumor growth in vivo. We also identified TIMP2 as a direct and functional target of miR-15b. Our research firstly suggests that miR-15b may be a new target for the diagnosis and treatment of NSCLC in the future.
Materials and methods

Tissue samples. Forty-two human NSCLC and 42 normal tissues far from the cancerous area (located >5 cm from the edge of tumor tissues) were collected from patients who underwent primary surgical treatment in The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) between May 2013 and August 2015. All patients had not been subjected to preoperative radiotherapy or chemotherapy in the 6 months prior to the surgery. The mean age of the included patients was 62.6±14.7 years. Among them, 19 were male and 23 were female. Tissue samples were obtained at the time of surgery, histologically confirmed to be tumorous or non-tumorous tissues, and were rapidly frozen in liquid nitrogen and stored at -80°C until analysis. Information on tumor classification, stage and lymph node status was extracted from the medical records of patients and pathology studies. The smoking history information was collected from each patient using a structured questionnaire. The present study was approved by the Medical Ethics Committee of Wenzhou Medical University, and informed written consent was obtained from patients before enrollment.

Cell lines and culture. NSCLC cancer cell lines A549, LTEP-a-2, H358 and SPCA1, and normal cell line MRC-5 were obtained from The American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin at 37°C in a humidified incubator with 5% CO₂.

Cell transfection. Cells were seeded into 6-well plates at a density of 3x10⁵ cells/well. miR-15b mimics (5 µl) and miR-15b inhibitor were mixed with 200 µl of FBS-free medium for 15 min at room temperature. Then, Lipofectamine 2000 was mixed and incubated with miR-15b mimics or miR-15b inhibitor for 15 min at room temperature. Then, Lipo2000 was mixed and then transferred to the upper chambers (2x10⁴ cells/well) with a 10-µl tip in each well. After being scratched, the cells were continuously cultured in a medium with 3% FBS for 72 h. Subsequently, microscopic images of the cultures were captured. The experiments were performed 3 times, independently.

Wound healing assay. Cell migration was assessed using a wound healing assay in 6-well plates. A fine line was scraped with a 10-µl tip in each well after cultured cells became fully confluent. After being scratched, the cells were continuously cultured in a medium with 3% FBS for 72 h. Subsequently, microscopic images of the cultures were captured. The experiments were performed 3 times, independently.

Cell invasion assay. The Transwell chambers with 8-µm pores were obtained from Corning (Corning, NY, USA). The Transwell membrane (filter) was pre-coated with 30 µl of Matrigel (1:3 mixed with PBS; BD Biosciences, Heidelberg, Germany) and incubated for 48 h. The transfected cells were harvested and resuspended in 100 µl of serum-free medium, and then transferred to the upper chambers (2x10⁵ cells/well). Medium (600 µl) supplemented with 10% FBS was added to the lower chamber. After incubation for 24 h, the Transwell membrane was fixed with methanol, stained with crystal violet, and then the cells were counted under a light microscope.

Flow cytometric assay. Cultured cells were seeded onto the 6-well plates at a density of 1x10⁵ cells/well, and incubated overnight. After transfection for 48 h, the cells were harvested and washed with PBS. The cells were then stained with 5 µl of propidium iodide (PI) for 5 min before they were subjected to FACS analysis. The DNA contents of the stained cells were analyzed using the ModFit LT software (Verity Software House Inc., Topsham, ME, USA).

Western blotting. After transfection, the cells were harvested and protein was extracted. Protein concentrations were determined using a BCA protein assay kit (Beyotime, Beijing, China). Proteins (30 µg) were separated by 10% SDS denatured polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membranes with a pore size of 0.45 µm (Millipore, Billerica, MA, USA). After blocking in 5% skim milk in Tris-based saline with Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated with rabbit anti-human antibodies at the recommended dilution (1:1,000) overnight at 4°C. After being washed in TBST, the membranes were further incubated with horseradish...
peroxidase-conjugated secondary antibodies (1:2,000) for 1 h. The following antibodies purchased from Cell Signaling Technology (CST; Boston, MA, USA) were used for western blotting: E-cadherin (CST, 3195), N-cadherin (CST, 13116), TIMP2 (CST, D18B7), β-actin (CST, 13E5), mouse anti-rabbit IgG (CST, 5127). Enhanced chemiluminescence (ECL) solution was added onto the membranes and the protein expression was quantified using The Laboratory Work Image Acquisition and Analysis Software (UVP, Upland, CA, USA). β-actin was used as a loading control.

Dual-luciferase reporter assay. Cells were cultured in 24-well plates and transfected with wild-type (WT) or mutated (Mut) 3’-UTR of TIMP2, along with hsa-miR-15b mimic (miR-15b) or negative control (miR-NC). After post-transfection for 48 h, Dual-Luciferase Activity Assays (Promega) were performed following the manufacturer’s instructions.

Animal experiments. Six female nude (BALB/c-nu) mice (4-5 weeks old and weighing 10-14 g) were purchased from Sun Yat-sen University Laboratory Animal Center (Guangzhou, China). Animal experiments were approved by the Animal Ethics Committee of Wenzhou Medical University. All procedures involving animals were in accordance with the Institutional Animal Welfare Guidelines of Wenzhou Medical University. The mice were bred and maintained under specific pathogen-free conditions with 5 in a cage, provided with sterilized food and water, and housed in a barrier facility with a 12 h light/dark cycle. The mice were randomly divided into 2 groups (n=3). A549 cells were transfected with miR-15b inhibitor and inhibitor negative control. The cells (2x10^6) at an exponential stage were harvested, and were then mixed and injected into the left flank sides of the mice. Treatment started when tumors reached a 0.5-cm mean diameter. All animals were humanely euthanized by ethyl ether inhalation after 3 weeks. Freshly frozen tumors were used for western blotting.

Statistical analysis. All data are expressed as the mean ± SD. Differences between 2 groups were assessed using Fisher’s exact test or Student’s t-tests, while differences among multiple groups were analyzed using one-way ANOVA followed by Bonferroni’s multiple comparison test. P<0.05 was considered statistically significant. Statistical analysis was performed with the SPSS statistical software program (version 13.0; SPSS, Inc., Chicago, IL, USA).

Results

miR-15b is upregulated in NSCLC patient tissues and NSCLC cell lines. The expression of miR-15b was detected by RT-qPCR in tumorous tissue and adjacent normal tissue of 42 patients. Our data revealed that the expression of miR-15b in tumor tissues was significantly higher than that in the normal tissues (Fig. 1A). Consistently, all tested NSCLC cell lines had significantly upregulated miR-15b levels compared with the normal cell line MRC-5 (Fig. 1B). These data demonstrated the important role of miR-15b in the development of NSCLC.

Correlation between miR-15b expression level and clinicopathological factors. The relationship between miR-15b expression and clinicopathological factors was investigated. As shown in Table I, high expression of miR-15b was significantly associated with TNM stage (P=0.0482) and lymph node status (P=0.0293). However, no significant differences were detected between miR-15b expression and gender, age, smoking history or histology.

miR-15b promotes growth and the cell cycle of NSCLC cells. To evaluate the effects of miR-15b in NSCLC cell lines, a series of experiments were performed for its detection. We first determined the effect of miR-15b on cell growth. We found that downregulated expression of miR-15b significantly inhibited the viability of A549 cells compared to their corresponding controls (Fig. 2A), while upregulated expression of miR-15b significantly promoted the viability of LTEP-a-2 cells compared to their corresponding controls (Fig. 2B). Furthermore, colony formation assay revealed that cells transfected with miR-15b inhibitor had lower colony formation than cells transfected with the control (Fig. 2C), and the opposite effect was observed in the cells transfected with miR-15b mimic (Fig. 2D). In addition, using flow cytometry to assess cell cycle status, we found that cells transfected with miR-15b inhibitor had an increased number of cells in the G1 phase, but a decreased number of cells in the G2 phase (Fig. 2E and F) in A549 cells. In LTEP-a-2 cells, the cells transfected with miR-15b mimic had a decreased number of cells in the G1 phase, but an increased number of cells in the G2 phase (Fig. 2G and H).

miR-15b promotes migration and invasion of NSCLC cells in vitro. To further assess the effects of miR-15b on malignant
tumor progression and metastasis, we performed wound healing and Transwell assays. The wound healing assay revealed that knockdown of miR-15b inhibited cell migration in A549 cells (Fig. 3A). As shown in Fig. 3B-E, knockdown or upregulation of miR-15b significantly suppressed or promoted the cell invasion in A549 or LTEP-a-2 cells, respectively. In addition, western blotting revealed that miR-15b inhibitor enhanced the expression of E-cadherin, while it downregulated the expression of N-cadherin in A549 cells (Fig. 3F and G). miR-15b mimic downregulated the expression of E-cadherin, while it enhanced the expression of N-cadherin in LTEP-a-2 cells (Fig. 3H and I).

**TIMP2 is a direct target of miR-15b.** To elucidate the underlying mechanisms by which miR-15b exerts its function, we used
TargetScan bioinformatics algorithm. TIMP2 was found to be a potential target (Fig. 4A). To confirm this relationship, a dual-luciferase reporter assay was performed. We found that miR-15b suppressed the luciferase activity of the wild-type TIMP2 3'-UTR (WT), but not the Mut 3'-UTR of TIMP2 in A549 and LTEP-a-2 cells (Fig. 4B). qRT-PCR analysis demonstrated that TIMP2 mRNA expression was inhibited after transfection of miR-15b mimic in A549 and LTEP-a-2 cells (Fig. 4C). Similar results were also achieved with western blotting. miR-15b mimic decreased the protein expression level of TIMP2 in A549 and LTEP-a-2 cells (Fig. 4D). Collectively, these data demonstrated that miR-15b directly targets TIMP2 in NSCLC cells.

miR-15b promotes tumorigenicity in vivo. To further demonstrate the role of miR-15b, we injected A549 cells infected with miR-15b inhibitor and negative control plasmids into the female nude mice. As revealed by the results obtained, the tumors derived from the miR-15b inhibitor-transfected A549 cells grew much more slowly than the NC group, and the tumor weight was also significantly decreased when compared to the NC group (Fig. 5A-C). In addition, in vivo western blotting concerning the expression of E-cadherin and N-cadherin was also performed. We found that miR-15b inhibitor enhanced the expression of E-cadherin and downregulated the expression of N-cadherin in the xenograft tumors (Fig. 5D and E).

Knockdown of TIMP2 promotes viability and invasion in A549 cells. Since miR-15b promotes proliferation and invasion in NSCLC cells and TIMP2 is a direct target of miR-15b, we transfected A549 cells with si-TIMP2 to determine whether
miR-15b PROMOTES PROLIFERATION AND INVASION BY TARGETING TIMP2

Figure 4. miR-15b negatively regulates TIMP2 by binding to the TIMP2 3'-UTR. (A) The wild-type and the mutated sequences of the TIMP2 mRNA 3'-UTR. (B) The luciferase activity of the wild-type TIMP2 3'-UTR (WT) and mutant TIMP2 3'-UTR (Mut) co-transfected with miR-15 mimics or a negative control (miR-NC) was assessed. (C) The mRNA level of TIMP2 in A549 and LTEP-a-2 cells transfected with miR-15b mimic or the corresponding control (miR-NC) was determined by qRT-PCR. (D) Western blotting was used to detect the protein level of TIMP2 in cells transfected with miR-15b mimic or the corresponding control; β-actin was used as an internal control. Error bars represent the SD, n=3. *P<0.05 compared with the control. TIMP2, tissue inhibitor of metalloproteases 2.

Figure 5. Suppression of tumor growth by miR-15b downregulation in the xenograft mouse model. Six female BALB/c-nu mice were randomly separated into 2 groups of 3 mice each. A549 cells were transfected with miR-15b inhibitor or the corresponding control and then injected into left subaxillary flanks of the mice as described in the text. (A) Tumor growth was assessed every 2 days after the injection. (B and C) Images and tumor weights of xenograft tumors. Tumors were harvested on day 18 and weighed. (D) Western blotting was used to detect the protein level of E-cadherin and N-cadherin in vivo; β-actin was used as an internal control. (E) Quantification of the results obtained from western blotting. Error bars represent the SD, n=3. *P<0.05 compared with the control.
downregulation of TIMP2 had a phenocopy of miR-15b overexpression. The expression of TIMP2 was confirmed by qRT-PCR (Fig. 6A). Results from an MTT assay revealed that knockdown of TIMP2 significantly promoted A549 cell viability (Fig. 6B). Moreover, a Transwell assay revealed that knockdown of TIMP2 significantly promoted invasion in A549 cells (Fig. 6C and D). These results indicated that the effect of miR-15b on NSCLC cells is involved in its suppression of TIMP2.

Discussion

Although the molecular mechanisms of various miRNAs have been elucidated in the process of carcinogenesis, specific patterns of miRNA expression in NSCLC development still remain poorly understood. Hence, it is of great value to explore the function of miRNAs specifically involved in NSCLC carcinogenesis and to screen new targets for its diagnosis and therapy. Aberrant expression of miR-15b has been found in several types of cancer. miR-15b overexpression has been observed to inhibit cellular proliferation, invasion and tumor metastasis in various cancer cell lines (16-18), suggesting a tumor-suppressive role for miR-15b. On the contrary, various studies have shown that miR-15b is statistically overexpressed in tumor tissues (14,22), suggesting that miR-15b functions as an onco-miRNA. This variation indicated that dysregulation of miR-15b was different in diverse organic tissues. However, few studies exist concerning the potential function of this miR-15b in human NSCLC progression. In the present study, we investigated the potential role of miR-15b in the malignant progression of NSCLC.

In the present study, we assessed the levels of miR-15b in 42 pairs of human NSCLC and normal tissues as well as different cell lines by real-time PCR. We found that miR-15b was frequently upregulated in malignant tissue specimens and malignant cells. Prior to the present study, however, the role of miR-15b and its target genes was unclear in NSCLC. Thus, we supposed that miR-15b may be a novel tumor oncogene miRNA and its dysregulation may be connected with advanced progression of human NSCLC. Therefore, the functions and molecular mechanisms of miR-15b in human NSCLC were the focal point of our research.

Previous studies have indicated that abnormal miRNA expression is involved in various biological processes (23). In the present study, we demonstrated that increased miR-15b expression promoted cell viability, cell migration and invasion. In addition, we investigated the effect of miR-15b on progression of the cell cycle. Knockdown of miR-15b induced G0/G1 phase arrest in A549 cells, while miR-15b overexpression prompted cell cycle progression in LTEP-a-2 cells, suggesting that the promoting role of miR-15b on cell proliferation may be associated with the regulation of the cell cycle. The downregulation of the expression of E-cadherin and the upregulation of the expression of N-cadherin after miR-15b overexpression further indicated that miR-15b promoted cancer invasion ability in NSCLC. To investigate the in vivo potential of miR-15b in NSCLC, we established a down-regulated miR-15b NSCLC cancer xenograft mouse model. Growth curve results revealed that knockdown of miR-15b significantly suppressed the growth of NSCLC cancer xenografts in nude mice.

To further determine how miR-15b functions as an oncogene, we screened potential targets using bioinformatics analysis, TargetScan 6.2. Luciferase activity assay suggested direct targeting of TIMP2 by miR-15b. We also found that both mRNA and protein levels of TIMP2 were decreased in
A549 cells transfected with miR-15b. These results demonstrated that TIMP2 was a target of miR-15b in NSCLC cells.

TIMP2 is a member of the tissue inhibitor of metalloproteinase (TIMP) family, which inhibits the activity of matrix metalloproteinases (MMPs) by binding with a 1:1 stoichiometry to the active site (24). It was reported that TIMP2 suppressed tumor cell proliferation and metastasis in numerous types of cancer including gastric, pancreatic and breast cancer (25-27). In recent years, TIMP2 was revealed to be regulated by several miRNAs in cancers. For example, Zhu et al (28) demonstrated that miR-106a regulates gastric cancer cell proliferation, migration and invasion by targeting TIMP2. Dai et al (29) observed that miR-200b suppresses the expression of TIMP2 at both the messenger RNA and protein levels in human endometrial cancer cell line HEC-1A. However, Yang et al (30) demonstrated that miR-221/222 regulates cell proliferation, cell cycle, apoptosis, invasion, metastasis, and angiogenesis in glioma cell lines by targeting TIMP2. Here, we revealed that TIMP2 is a direct target of miR-15b in NSCLC, and demonstrated that knockdown of TIMP2 promotes viability and invasion in A549 cells. These results shed new light on the regulation of TIMP2.

In conclusion, we firstly demonstrated that the expression of miR-15b was upregulated in NSCLC tissues and cells. miR-15b promoted cell growth, migration and invasion by targeting TIMP2 in human NSCLC cells. These results suggest that miR-15b may therefore play an important role in the initiation and development of NSCLC through the regulation of TIMP2. All of these results indicate that miR-15b may be a biomarker and may represent a new molecular target for NSCLC treatment.

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References