MicroRNA-616 promotes the growth and metastasis of non-small cell lung cancer by targeting SOX7

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Abstract. MicroRNAs (miRNAs) are a group of important regulators in human types of cancer including non-small cell lung cancer (NSCLC). miR-616 has been found to be a novel cancer-related miRNA. However, the expression and biological function of miR-616 in NSCLC have not been investigated. In this study, qRT-PCR was performed to evaluate the level of miR-616 in NSCLC tissues. MTT, BrdU and Transwell assay were used to investigate the proliferation and metastasis ability of NSCLC cells. Subcutaneous injection model and tail vein injection model were used to evaluate the effect of miR-616 on the in vivo growth and metastasis of NSCLC cells. It was also found that the expression level of miR-616 was increased in NSCLC tissues and cell lines. Patients with a high level of miR-616 had a significantly shorter overall survival and disease-free survival. Functionally, miR-616 overexpression promoted while miR-616 knockdown inhibited the proliferation, migration and invasion of NSCLC cells. Moreover, miR-616 overexpression enhanced the subcutaneous growth and lung metastasis of NSCLC cells in nude mice. Mechanistically, SOX7 was confirmed to be the downstream target of miR-616 in NSCLC cells. Forced expression of SOX7 prevented the promoting effects of miR-616 overexpression on the proliferation and metastasis of NSCLC cells, while knockdown of SOX7 reversed the inhibitory effects of miR-616 knockdown on the proliferation and metastasis of NSCLC cells. In conclusion, the present study indicates that miR-616 is a promising biomarker and therapeutic target in NSCLC.

Introduction

Non-small cell lung cancer (NSCLC), which accounts for ~80% of all lung cancers, is the leading cause of cancer-related deaths worldwide (1). Although notable improvements have been achieved in the treatment of NSCLC, the prognosis for NSCLC patients remains poor (2). Identifying novel molecules that are critical for the progression of NSCLC may provide new opportunities to find effective treatment options for NSCLC patients (3).

MicroRNAs (miRNAs) are a group of short non-coding RNAs that can post-transcriptionally modulate the expression of targeted genes by inhibiting the translation of targeted mRNAs (4). They have been found to affect many biological functions including cell proliferation, differentiation and metastasis (5-8). Therefore, miRNAs have been demonstrated to be involved in different human diseases including cancers. Numerous studies of NSCLC have confirmed that miRNAs play important roles in the development and progression of NSCLC (9,10). However, the exact molecular mechanisms by which miRNAs affect the progression of NSCLC remain largely unknown.

miR-616 is a novel cancer-related miRNA which has been revealed to play important roles in human types of cancer. It was found to promote the migration and invasion of hepatocellular carcinoma cells by inhibiting phosphatase and tensin homolog (PTEN) (11). In prostate cancer, miR-616 promoted the androgen-independent growth of prostate cancer cells by suppressing tissue factor pathway inhibitor-2 (12). However, the expression status and function of miR-616 in NSCLC remain unknown.

SOX7 is a well-recognized tumor suppressor in many types of human cancer including breast (13), liver (14,15), colorectal (16), gastric (17), as well as lung cancer (18). In lung cancer, its expression was found to be downregulated and associated with the poor prognosis of patients (18). However, the mechanism by which SOX7 expression is regulated in NSCLC remains unknown.

In the present study we found that miR-616 was increased in NSCLC tissues and cell lines. An increased level of miR-616 was correlated with poor prognosis of NSCLC patients. miR-616 promoted the proliferation, migration and invasion of NSCLC cells in vitro. In vivo experiments revealed that miR-616 promoted the subcutaneous growth and lung metastasis of NSCLC cells in nude mice. Notably, SOX7 was identified as the direct downstream target gene of miR-616 in NSCLC. miR-616 exerted its promoting effects on the growth and metastasis of NSCLC cells by inhibiting SOX7.

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Materials and methods

Cell culture. Cell lines including H-358, H-1703, A549 and NL-20 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and the American Type Culture Collection (ATCC; Rockville, MD, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both from Gibco Co., New York, NY, USA). Cell cultures were kept in cell incubators with a humidified atmosphere and 5% CO₂ at 37°C.

Cell transfection. miR-616 mimic and miR-616 inhibitor were obtained from GeneCopoeia (Guangzhou, China). SOX7 expression vector and SOX7-specific siRNA were purchased from Ruibo Biotechnology Co. (Guangzhou, China). The transfection of these vectors into NSCLC cells was performed in 6-well plates with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions.

Clinical NSCLC tissues. Clinical specimens including NSCLC tissues were collected from NSCLC patients who received surgical resection at the Department of Respiratory Diseases, Chinese and Western Combined Hospital of Taizhou, between 2002 and 2011. All these clinical tissues from NSCLC were pathologically confirmed as NSCLC before being used for further experiments in the present study. Informed consent was obtained from every patient involved in the present study. Approvals for the experiments involving the patient samples were obtained from the Institutional Research Ethics Committee of the Chinese and Western Combined Hospital of Taizhou.

Quantitative real-time reverse transcription-PCR (qRT-PCR). The RNA from NSCLC tissues and cells was extracted with TRIzol and a RNAeasy mini kit (Qiagen, Hilden, Germany). Reverse transcriptional reactions and quantitative real-time PCR were performed with the Transcriptional First Strand cDNA Synthesis kit (Applied Biosystems, Foster City, CA, USA) and SYBR-Green PCR Master Mix (Roche Diagnostics Corp., Indianapolis, IN, USA). All primers including those for miR-616, U6 (internal control for miR-616), SOX7 and GAPDH (internal control for SOX7) were purchased from GeneCopoeia.

Western blot analysis. Total protein lysates (30 µg) extracted from NSCLC cells with RIPA buffer were separated in 4-20% SDS-PAGE gels. After being separated on the gels, the protein samples were transferred to polyvinylidene fluoride (PVDF) membranes at 4°C. The membranes were blocked with 5-10% milk/Tris-buffered saline with Tween-20 (TBST), and were incubated with primary antibodies at 4°C overnight. Primary antibodies used in the present study included SOX7 (1:1,000), c-Myc (1:1,000), N-cadherin (1:500), cyclin-D1 (1:1,000), p-Rb (1:500) (all from Cell Signaling Technologies, Danvers, MA, USA) and GAPDH (1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Then, the membranes were incubated with secondary antibodies (1:2,000; Santa Cruz Biotechnology, Inc.). The protein signals on the membranes were detected using ECL reagents (Amersham Biosciences Corp., Piscataway, NJ, USA).

Proliferation assays. To examine the in vitro proliferative ability of NSCLC cells, MTT, BrdU incorporation and colony formation assays were performed. For the MTT assay, 5,000 NSCLC cells transfected with a miR-616 mimic or inhibitor were seeded into 96-well plates. At the 24, 48 and 72 h time-points, these cells stained with MTT (Sigma, St. Louis, MO, USA) for 2 h were subjected to assessment of their absorbance at 490 nm. For the colony formation assay, 1,000 NSCLC cells transfected with different vectors were seeded into the 6-well plates and the cell colonies were stained with crystal violet 3 weeks later. For the BrdU assay, NSCLC cells transfected with different vectors were stained with BrdU following the manufacturer's instructions, and cells positive for BrdU staining were counted under a confocal microscope.

Migration and invasion assays. Transwell inserts were employed to evaluate the migratory and invasive ability of NSCLC cells. NSCLC cells were suspended in serum-free DMEM and they were seeded in the upper chamber. To induce the migration and invasion of NSCLC cells, the lower chambers were filled with 600 µl of DMEM supplemented with 20% fetal bovine serum (FBS). Forty-eight hours after cell seeding, NSCLC cells that had migrated or invaded through the membranes (for the invasion assay, the membranes were covered with 70 µl of Matrigel) were stained with crystal violet for cell counting under a microscope.

Luciferase assay. The wild-type SOX7 3’-UTR sequence containing binding sites for miR-616 or the mutated SOX7 sequence was linked into the pGL3 control vector (Promega, Madison, WI, USA) to construct the wild-type SOX7-3’-UTR vector or mutant SOX7-3’-UTR vector. NSCLC cells in 6-well plates were co-transfected with the wild-type or mutant 3’-UTR of the SOX7 vector along with a miR-616 mimic or miR-616 inhibitor. Forty-eight hours after transfection, the luciferase activity for the wild-type or mutant SOX7 3’-UTR vector was assessed using the single luciferase reporter assay (Promega).

Subcutaneous implantation and tail vein injection assays. The in vivo growth ability of NSCLC cells was examined using a subcutaneous implantation assay. NSCLC cells transfected with a control vector or miR-616 mimic were injected into the subcutaneous flanks of mice. Three weeks later, the subcutaneous tumors were resected and were subjected to volume and weight assessments. To evaluate the in vivo metastatic ability of NSCLC cells, a tail vein injection assay was performed on nude mice. NSCLC cells transfected with the control vector or miR-616 mimic were injected through the tail vein of mice. Six weeks later, the lungs of nude mice were subjected to hematoxylin and eosin (H&E) staining for potential lung metastatic lesions. All animal experiments in the present study were approved by the Animal Care Committee of the Chinese and Western Combined Hospital of Taizhou.

Statistical analysis. All data in the present study are shown as the mean ± standard error (SE). Statistical analysis including Student's t-test, Chi-square, correlation and Kaplan-Meier’s analyses were performed using GraphPad. P<0.05 was considered to indicate a statistically significant result.
**Results**

**miR-616 expression is increased in NSCLC tissues and cells.** To understand the expression status of miR-616 in NSCLC, we extracted RNA from NSCLC and adjacent normal tissues. The results of qRT-PCR revealed that the expression of miR-616 was significantly increased in NSCLC tissues, compared with the adjacent normal tissues (Fig. 1A; P<0.05). Compared with those of small sizes (≤3 cm), tumors of large sizes (>3 cm) had a significantly higher level of miR-616 (Fig. 1B; P<0.05). Furthermore, the levels of miR-616 in patients with metastasis was significantly higher than those without metastasis (Fig. 1C; P<0.05). Lastly, we examined the expression level of miR-616 in NSCLC cell lines. Compared with the NL-20 cells, the expression of miR-616 was significantly higher in NSCLC cell lines including H-358, H-1703 and A549 cells (Fig. 1D; P<0.05).

**Increased miR-616 expression level is associated with unfavorable clinicopathological features and poor prognosis of NSCLC patients.** After confirming the increased expression of miR-616 in NSCLC, we evaluated the prognostic value of miR-616 in NSCLC patients. We divided the NSCLC patients into 2 groups (miR-616 high and miR-616 low expression groups) based on the cutoff value which was defined as the median value of the miR-616 expression level. As shown in Fig. 2A and B, patients with a high expression level of miR-616 had a significantly decreased rate of overall survival (P<0.01; Fig. 2A) and disease-free survival (P<0.01; Fig. 2B). These results indicate that miR-616 is a promising prognostic marker for NSCLC patients.

**miR-616 promotes the proliferation, migration and invasion of NSCLC cells.** Since the expression level of miR-616 was lowest in the H-358 cells and highest in the A549 cells, we overexpressed miR-616 in the H-358 cells while we inhibited miR-616 expression in the A549 cells. Transfection of the miR-616 mimic significantly increased miR-616 expression in H-358 cells (Fig. 3A; P<0.05). Overexpression of miR-616 significantly promoted the cell viability of H-358 cells as determined by MTT assay (Fig. 3B; P<0.05). The proliferative ability of H-358 cells was also significantly increased after miR-616 overexpression, as determined by BrdU incorporation assay (Fig. 3C; P<0.05) and colony formation assay (Fig. 3D; P<0.05). Transwell assays revealed that miR-616 overexpression significantly increased the migration (Fig. 3E; P<0.05) and invasion (Fig. 3F; P<0.05) of H-358 cells. In contrast, the miR-616 inhibitor significantly decreased the expression level of miR-616 in A549 cells (Fig. 4A; P<0.05). Subsequently, inhibition of miR-616 significantly decreased the cell viability (Fig. 4B; P<0.05), BrdU incorporation (Fig. 4C, P<0.05), colony formation (Fig. 4D; P<0.05), migration (Fig. 4E, P<0.05) and invasion (Fig. 4F; P<0.05) of A549 cells.

**miR-616 enhances the in vivo growth and lung metastasis of NSCLC cells in nude mice.** To further confirm the functional influence of miR-616 on NSCLC cells, we performed subcutaneous injection and tail vein injection to assess whether miR-616 could affect the in vivo growth and metastasis of NSCLC cells. Overexpression of miR-616 significantly increased the subcutaneous growth of NSCLC in nude mice, as suggested by the significantly increased volume (Fig. 5B; P<0.05) and weight (Fig. 5C; P<0.05) of the subcutaneous tumors. In addition, tail vein injection experiments revealed that miR-616 overexpression significantly increased the
Figure 2. The prognostic value of miR-616 in NSCLC patients. (A) The overall and (B) disease-free survival rate were compared between patients with a high expression level of miR-616 and those with a low expression level of miR-616. NSCLC, non-small cell lung cancer.

Figure 3. miR-616 overexpression promotes the proliferation, migration and invasion of NSCLC cells. (A) miR-616 mimic significantly increased the expression of miR-616 in H-358 cells. (B) miR-616 overexpression significantly increased the cell viability of H-358 cells. (C) miR-616 overexpression significantly increased the BrdU incorporation of H-358 cells. (D) miR-616 overexpression significantly increased the colony formation of H-358 cells. (E) miR-616 overexpression significantly increased the migration of H-358 cells. (F) miR-616 overexpression significantly increased the invasion of H-358 cells. *P<0.05 by t-test (for the BrdU, colony formation, migration and invasion assays) and ANOVA (for MTT assay). NSCLC, non-small cell lung cancer.
Figure 4. miR-616 inhibition suppresses the proliferation, migration and invasion of NSCLC cells. (A) miR-616 inhibitor significantly decreased the expression of miR-616 in A549 cells. (B) miR-616 inhibitor significantly decreased the cell viability of A549 cells. (C) miR-616 knockdown significantly decreased the BrdU incorporation of A549 cells. (D) miR-616 knockdown significantly increased the colony formation of A549 cells. (E) miR-616 knockdown significantly increased the migration of A549 cells. (F) miR-616 knockdown significantly increased the invasion of A549 cells. n=3 repeats with similar results; \( ^{*} P<0.05 \) by t-test (BrdU, colony formation, migration and invasion assays) and ANOVA (for MTT assay). NSCLC, non-small cell lung cancer.

Figure 5. miR-616 promotes the in vivo growth and lung metastasis of H-358 cells in nude mice. (A) Overexpression of miR-616 promoted the subcutaneous growth of H-358 cells in nude mice. (B) The tumor volume and (C) tumor weight were significantly increased after overexpression of miR-616 in H-358 cells. (D) miR-616 overexpression increased the frequency of lung metastasis of H-358 cells in nude mice. (E) Incidence of lung metastasis and (F) number of lung metastatic nodules were significantly increased after overexpression of miR-616 in H-358 cells. n=5 repeats with similar results; \( ^{*} P<0.05 \) by t-test and Chi-square test.
frequency of lung metastasis (Fig. 5E; \( P<0.05 \)) and the number of metastatic nodules (Fig. 5F; \( P<0.05 \)) in the lungs of nude mice.

**Figure 6.** SOX7 is the downstream target of miR-616 in NSCLC cells. (A) The 3′-UTR of SOX7 contained the binding sequence for miR-616. (B) miR-616 overexpression decreased the luciferase activity of the wild-type SOX7 3′-UTR while miR-616 inhibitor increased the luciferase activity of the wild-type SOX7 3′-UTR. Neither miR-616 overexpression nor miR-616 inhibition affected the luciferase activity of the mutant SOX7 3′-UTR. miR-616 overexpression decreased the (C) mRNA and (D) protein level of SOX7 in H-358 cells. miR-616 inhibition increased the (E) mRNA and (F) protein level of SOX7 in A549 cells. \( n=5 \) repeats with similar results; \( *P<0.05 \) by t-test.

**Figure 7.** miR-616 regulates the expression of the downstream targets of SOX7 in NSCLC cells. (A) miR-616 overexpression increased while miR-616 knockdown inhibited the mRNA level of cyclin-D1, c-Myc, LEF1 and TCF in NSCLC cells. (B) miR-616 overexpression increased while miR-616 knockdown decreased the protein level of c-Myc, cyclin-D1, p-Rb in NSCLC cells. \( n=3 \) repeats with similar results. \( *P<0.05 \) by t-test.

SOX7 is the downstream target of miR-616 in NSCLC cells.

After determining the biological functions of miR-616 in NSCLC cells, we examined what the direct downstream target of
miR-616 was in NSCLC cells. The data from a public database (TargetScan) revealed that the 3'-UTR of SOX7 had a potential binding sequence for miR-616 (Fig. 6A). Luciferase assays revealed that overexpression of miR-616 inhibited the luciferase activity of the wild-type 3'-UTR of SOX7, while it had no effect on that of the mutant SOX7 3'-UTR. On the contrary, miR-616 inhibition increased the luciferase activity of the wild-type 3'-UTR of SOX7 while it had no effect on that of the mutant SOX7 3'-UTR. Furthermore, qRT-PCR and western blot analysis results revealed that miR-616 overexpression significantly decreased the mRNA (Fig. 6C; P<0.05) and protein (Fig. 6D; P<0.05) level of SOX7 in H-358 cells. In contrast, miR-616 knockdown significantly increased the mRNA (Fig. 6E; P<0.05) and protein (Fig. 6F; P<0.05) level of SOX7 in A549 cells.

To further confirm that SOX7 was the downstream target of miR-616 in NSCLC cells, we examined the expression of downstream targets of SOX7 after altering the expression level of miR-616 in NSCLC cells. Previous studies revealed that cyclin-D1, c-Myc, LEF1 and TCF were under the regulation of SOX7. Overexpression of miR-616 significantly increased the mRNA level of cyclin-D1, c-Myc, LEF1 and TCF (P<0.05; Fig. 7A) while miR-616 knockdown significantly decreased the mRNA level of these SOX7 downstream targets (P<0.05; Fig. 7A). Furthermore, miR-616 overexpression significantly increased the protein level of c-Myc, cyclin-D1 and p-Rb (Fig. 7B; P<0.05) while miR-616 inhibition significantly decreased the protein level of c-Myc, cyclin-D1 and p-Rb (Fig. 7B; P<0.05).
Targeting SOX7 is required for the biological functions of miR-616 in NSCLC cells. After confirming that SOX7 was the direct downstream target of miR-616 in NSCLC cells, we investigated whether targeting SOX7 was required for the biological functions of miR-616 in NSCLC cells. Overexpressing SOX7 in H-358 cells (Fig. 8A; P<0.05) transfected with the miR-616 mimic decreased the promoting effects of miR-616 on cell viability (Fig. 8B; P<0.05), BrdU incorporation (Fig. 8C; P<0.05), colony formation (Fig. 8D; P<0.05), migration (Fig. 8E; P<0.05) and invasion (Fig. 8F; P<0.05) of H-358 cells. In contrast, SOX7 knockdown (Fig. 9A; P<0.05) in A549 cells transfected with the miR-616 inhibitor reversed the inhibitory effects of miR-616 knockdown on cell viability (Fig. 9B; P<0.05), BrdU incorporation (Fig. 9C; P<0.05), colony formation (Fig. 9D; P<0.05), migration (Fig. 9E; P<0.05) and invasion (Fig. 9F; P<0.05) of A549 cells.

Discussion
Numerous microRNAs (miRNAs) have been found to be closely associated with cancer development and
progression (19-22). Among these cancer-related miRNAs, miR-616 is a newly identified miRNA closely related with human types of cancer. In the present study, we confirmed for the first time that the expression level of miR-616 was significantly increased in NSCLC tissues and cell lines. An increased expression level of miR-616 was associated with unfavorable clinicopathological features and poor prognosis of NSCLC patients. In vitro functional assays revealed that miR-616 promoted cell viability, proliferation, migration and invasion of NSCLC cells. In vivo experiments also confirmed that miR-616 promoted the growth and metastasis of NSCLC cells in nude mice. These results indicate that miR-616 played an oncogenic role in NSCLC by promoting tumor growth and metastasis.

Previous studies revealed that SOX7 played critical roles in human types of cancer including breast (13), hepatocellular (14), colorectal (16), gastric (17) and lung cancer (18). A study on breast cancer revealed that SOX7 expression was decreased in breast cancer tissues, and inhibition of SOX7 promoted the proliferation and metastasis of breast cancer cells (13). A study of hepatocellular carcinoma revealed that SOX7 was under the regulation of miR-184 and mediated the proliferation of HCC cells by regulating cyclin-D and c-Myc (15). Furthermore, SOX7 was reported to regulate Wnt/β-catenin signaling-related genes including cyclin-1, c-Myc, LEF1 and TCF (15). In the present study, we found that SOX7 contained a binding sequence for miR-616. miR-616 modulated the luciferase activity of the wild-type SOX7 3′-UTR while it had no effect on that of the mutant SOX7 3′-UTR. In addition, miR-616 inhibited the expression of SOX7 in NSCLC cells. Furthermore, miR-616 also regulated the downstream targets of SOX7 including cyclin-D1, c-Myc, LEF1, TCF and p-Rb. All these results support the conclusion that SOX7 was the direct downstream target of miR-616 in NSCLC cells. Furthermore, functional assays revealed that SOX7 overexpression could inhibit the promoting effects of miR-616 on the proliferation and metastasis of NSCLC cells while SOX7 knockdown reversed the inhibitory effects of miR-616 inhibitor on the biological functions of NSCLC cells. These results demonstrated that SOX7 was not only the downstream target of miR-616 in NSCLC cells, but also the functional mediator of miR-616 in NSCLC cells. However, it is worth mentioning in the present study that an miRNA usually has more than one target in cells. In HCC, PTEN was found to be the target of miR-616. In prostate cancer, the downstream target of miR-616 was found to be tissue factor pathway inhibitor-2. Therefore, miR-616 potentially has more than one downstream target in NSCLC, which is worth investigating in the future.

In conclusion, miR-616 expression was increased in NSCLC tissues and cells. miR-616 promoted the progression of NSCLC by contributing to the proliferation and metastasis of NSCLC cells both in vitro and in vivo. Mechanistically, SOX7 was identified to be a downstream target of miR-616 in NSCLC cells. Targeting SOX7 was required for the oncogenic functions of miR-616 in NSCLC.

References