MicroRNA-625 serves as a tumor suppressor in non-small cell lung cancer through targeting SOX4

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Received June 28, 2017; Accepted January 4, 2018; Epub April 15, 2018; Published April 30, 2018

Abstract: MicroRNAs (miRNAs) have been implicated in tumor development and progression in many types of human cancers. The purpose of this study was to determine the expression pattern and biological function of miR-625 in human non-small cell lung cancer (NSCLC). 104 NSCLC and their corresponding non-cancerous tissue samples were collected. Cell proliferation, migration and invasion and the cell cycle distribution were detected by CCK-8 assay, transwell assay, wound healing assay and Annexin V analysis. The target genes of miR-625 were further explored. A tumor xenograft animal model was used to detect the role of miR-625 on NSCLC cell growth in vivo. We found that miR-625 was markedly down-regulated in NSCLC and low expression level of miR-625 was correlated with unfavorable prognosis of NSCLC patients. Over-expression of miR-625 led to decreased NSCLC cell proliferation, migration and invasion. Furthermore, increased miR-625 expression inhibited NSCLC tumor growth in the xenograft animal model. MiR-625 over-expression reduced the mRNA and protein levels of SOX4. Luciferase assay confirmed that miR-625 could directly bind to the 3'UTR of SOX4. These results suggest that deregulation of miR-625 is involved in the etiology of NSCLC partially through direct targeting SOX4, which suggests that miR-625 may provide novel diagnostic and therapeutic options for NSCLC in the future.

Keywords: Non-small cell lung cancer (NSCLC), miR-625, SOX4, prognosis, cell cycle

Introduction

Lung cancer is the leading major cause of cancer-related death around the world [1]. Non-small cell lung cancer (NSCLC), including squamous cell carcinoma, adenocarcinoma, adenosquamous cell carcinoma, and large cell carcinoma, accounts for approximately 80% of all lung cancer cases [2]. Despite recent advances in clinical and experimental oncology, the prognosis of NSCLC remains unfavorable, with a 5-year overall survival rate of approximately 10% [3]. Therefore, there exists an urgent need to understand the molecular mechanisms underlying NSCLC progression, which contribute to exploration of novel diagnostic and therapeutic targets for this fatal disease.

Numerous genetic and epigenetic alterations are associated with NSCLC [4]. MicroRNAs (miRNAs or miRs), a class of endogenous expressed, small (18-25 nucleotides in length), well-conserved non-coding RNAs, regulate posttranscriptional gene expression by binding to the 3'-untranslated region (UTR) of their target mRNAs, leading to target mRNA degradation and/or translation inhibition [5]. Nearly 50% of human miRNAs are located at fragile sites and genomic regions associated to cancers [6]. miRNAs are consistently dysregulated across various types of human cancers, suggesting that miRNAs may act as oncogenes or tumor suppressors [7]. Dysregulation of miR-625, which is located on chromosome 14q23.3, was recently observed in many types of human tumors. However, the functional role and mechanism of action of miR-625 in NSCLC have not been clearly elucidated.

Therefore, in the present study, we investigated the relationship between miR-625 expression and NSCLC. We focused on the functional roles and further molecular mechanism of miR-625 in NSCLC cells. Our results will help clarify the
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Table 1. Correlation between miR-625 expression and clinicopathological characteristics of NSCLC patients

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<td>21</td>
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</table>

Role of miR-625 in NSCLC progression and its potential as a therapeutic target.

Materials and methods

Patients and tissue samples

One hundred and four pairs of NSCLC tissues and their matched non-tumorous adjacent tissues (more than 5 cm away from the tumor) were obtained from patients undergoing surgery for NSCLC at West China Hospital (Sichuan, China). None of these patients received any preoperative chemotherapy or radiotherapy. All these tissue samples were snap-frozen immediately after resection, and stored in liquid nitrogen until further analysis. This study was approved by the Ethics Committee of West China Hospital and complied with the Declaration of Helsinki. All participants or their relatives have given written informed consent. Clinicopathological characteristics of these patients were summarized in Table 1.

Cell culture and transfection

Four NSCLC adenocarcinoma cell lines (A549, H1975, H1299 and SPCA1), two NSCLC squamous carcinomas cell lines (H1703 and SK-MES-1), and a normal human bronchial epithelial cell line (16HBE), purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) under an humidified air atmosphere of 5% CO2 at 37°C.

The miR-625 mimics (miR-625), miR-625 inhibitor and their corresponding negative control (miR-NC and anti-miR-NC) were purchased from GenePharma (Shanghai, China). The full-length cDNA sequence of SOX4, amplified by RT-PCR, was inserted into the pcDNA3.1 (GeneScript, Piscataway, NJ, USA) vectors (pcDNA-SOX4). Transfection was performed using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer’s instructions. At 48 h post transfection, cells were harvested for further analysis.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from cultured cells or tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed into cDNA using Reverse Transcription Kit (Thermo Fisher Scientific). For mRNA detection, a Q-PCR Detection Kit (Thermo Fisher Scientific) was used to perform qRT-PCR on the ABI Prism 7500 (Applied Bio systems, Foster City, CA, USA). For miRNA detection, an MiRNA Q-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA) was used. The relative expression of individual genes was determined by 2^ΔΔCT methods [8]. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) and U6 were used as endogenous controls. The specific primer pairs used are listed in the Table 2. All reactions were performed in triplicate.
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Protein extraction and western blotting

Tissue samples or cultured cells were lysed with RIPA peptide lysis buffer (Beyotime, Shanghai, China) containing 1% protease inhibitors (Pierce, Rockford, IL, USA). The total protein concentration was determined using a BCA Protein Assay Kit (Vigorous Biotechnology, Beijing, China). Equal amounts of total cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated with the diluted primary antibiotics. Then the membranes were incubated with horseradish peroxidase-conjugated second antibodies. Positive protein signals were detected with an enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Waukesha, USA). GAPDH was used as an internal control.

Cell proliferation assay

Cell proliferation was determined using Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Kumamoto, Japan). Briefly, cells were plated in 96-well plates at a density of 2 × 10³ cells/well. Cell viability was documented at 24, 48, 72 and 96 h after transfection. 10 μl of CCK8 solution was added to each well, and the absorbance was measured at 450 nm using a microplate reader.

Flow cytometry analysis of cell cycle

After transfection, the cells were typsinsized, washed twice with PBS, and then fixed with 75% ethanol on ice for 2 h. The fixed cells were resuspended in 600 μl PBS containing 0.1% RNase (Sigma) for RNA digestion at 37°C for 1 h. Finally, the cells were stained with PI for 15 min in the dark room before being measured by flow cytometry. A FACS Calibur instrument (BD Biosciences) was used for this assay.

Migration, invasion and wound healing assays

5 × 10³ cells suspended in serum-free medium were placed into the upper chamber of an insert pre-coated with or without Matrigel (Chemicon, CA, USA). Medium with 10% FBS was added to the lower chamber as chemoattractant. After 48 hours, the cells remaining on the upper surface of the membrane were removed, whereas the cells that had traversed the membrane were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet, imaged and counted under a microscope.

To perform wound healing assay, the cells were seeded in six-well plates and cultured to 90% confluence. An artificial wound was created in the cell monolayer using a sterile plastic micropipette tip, then the cells were washed in PBS and cultured for another 48 hours. The wound closure rate was observed and calculated.

Dual luciferase report assay

The wild-type (WT) and mutant (MUT) 3'-UTR of SOX4 were designed and cloned into the psiCHECK2 vector (Promega, Madison, WI, USA), referred to WT-SOX4 and MUT-SOX4. For the luciferase assay, A549 cells were plated and cultured in 12-well plates. Cells were co-transfected with miR-625 mimics or miR-NC and WT/Mut SOX4-3'-UTR reporter plasmid. Luciferase assays were performed 48 h post-transfection.

Table 2. Primer sequences used in the present study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
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<tr>
<td>miR-625-RT</td>
<td>5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGAGGG-3′</td>
</tr>
<tr>
<td>U6-RT</td>
<td>5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA-3′</td>
</tr>
<tr>
<td>miR-625 Forward primer</td>
<td>5′-GACTATAGAACTTTCC-3′</td>
</tr>
<tr>
<td>miR-625 Reverse primer</td>
<td>5′-GTGCCAGGTCGAGGT-3′</td>
</tr>
<tr>
<td>U6 Forward primer</td>
<td>5′-CTCGCTTCGCCAGCACATATAC-3′</td>
</tr>
<tr>
<td>U6 Reverse primer</td>
<td>5′-ACTGCTTCAGAATTGTGCCTGC-3′</td>
</tr>
<tr>
<td>SOX4 Forward primer</td>
<td>5′-GTAGCCGAGATGTCTCGGG-3′</td>
</tr>
<tr>
<td>SOX4 Reverse primer</td>
<td>5′-CAGGTTGGAGATGCTGGACTC-3′</td>
</tr>
<tr>
<td>GAPDH Forward primer</td>
<td>5′-CCACATCGCTCAGACACCAT-3′</td>
</tr>
<tr>
<td>GAPDH Reverse primer</td>
<td>5′-ACCAGGCCGCCCCAATA-3′</td>
</tr>
</tbody>
</table>
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Using a dual luciferase reporter gene assay kit (BioVision, Milpitas, CA, USA). Renilla luciferase activity was normalized to firefly luciferase activity.

In vivo tumorigenicity

Ten male BALB/c athymic nude mice (5- to 6-week old, 18-20 g), purchased from Shanghai Laboratory Animals Center (Shanghai, China), were maintained under specific pathogen-free (SPF) conditions under a 12 h light/dark cycle. To establish a NSCLC xenograft model, $2 \times 10^6$ A549 cells were suspended in PBS and inoculated subcutaneously into the flanks of nude mice (five in each group). MiR-625 mimics or miR-NC were injected directly into the implanted tumor every 4 days. When palpable tumors arose, xenograft volume (V), detected every three days through measuring the length (L) and width (W) with calipers, was calculated as $V = \frac{0.5 \times L \times W^2}{2}$ (width). 4-week after seeding the tumor cells, the mice were killed, and the tumors were removed. All animal procedures were approved by the Animal Care and Use Committee of West China Hospital, and every effort was made to minimize animal suffering [9].

Results

MiR-625 is down-regulated in NSCLC tissues and cell lines

We first performed qRT-PCR in order to verify the expression pattern of miR-625 in NSCLC, and found that miR-625 levels in 104 NSCLC tissues were markedly lower than that of in their normal counterparts (Figure 1A). In addition, as shown in Figure 1B, miR-625 expression was significantly lower in all six NSCLC cell lines than that in 16HBE cells. A549 and H1703 cells were used in subsequent studies.

Next, we examined the potential clinical significance of miR-625 in NSCLC. 104 NSCLC patients were divided into two groups according to their miR-625 expression levels: the high expression group (n=42, fold change $\geq$ mean ratio) and the low expression (n=62, fold change $\leq$ mean ratio). We evaluated the correlation of miR-625 expression with patients’ clinicopathological parameters. As listed in Table 1, low miR-625 expression was positively correlated

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) and SPSS 20.0 software (IBM, Chicago, IL, USA). All data were presented as the mean $\pm$ S.D. (standard deviation) of at least three independent experiments. Differences between individual groups were analyzed by Student’s two-tailed t-test. Chi-square test was used to compare the levels of miR-625 expression and the clinicopathological parameters of NSCLC patients. The overall survival (OS) and disease-free survival (DFS) of NSCLC patients were analyzed by the Kaplan-Meier method with the log-rank test. The data were considered significant if the P value was <0.05.
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Kaplan-Meier survival analysis was conducted to investigate the correlation between miR-625 expression and NSCLC patient prognosis. The results showed that NSCLC patients with high miR-625 expression had longer median OS (P=0.030; Figure 1C) and DFS (P=0.029; Figure 1D) than patients with low miR-625 expression. Thus, reduced expression of miR-625 might be an important factor in NSCLC progression and development.

Over-expression of miR-625 inhibits NSCLC cell proliferation and induces cell cycle arrest

To further determine the anticancer role of miR-625 in NSCLC, we transfected the NSCLC cell lines A549 with miR-625 mimics, H1703 with miR-625 inhibitor, and examined the effects on cellular proliferation. As expected, qRT-PCR analyses confirmed that miR-625 levels were increased remarkably in A549 cells transfected with miR-625 mimics, whereas miR-625 was substantially down-regulated in H1703 cells via

Figure 2. Overexpression of miR-625 inhibits NSCLC cell proliferation and induces cell cycle arrest. A. Transfection efficacy was confirmed by qRT-PCR analysis. B. CCK-8 assay showed that overexpression or inhibition of miR-625 significantly arrested or accelerated NSCLC cell proliferation. Error bars represent the S.D. from at least three independent experiments. *P<0.05. C. Overexpression or inhibition of miR-625 induced or inhibited cell cycle arrest in A549 or H1730 cells.
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Figure 3. Overexpression of miR-625 inhibits NSCLC cell migration and invasion. A. Transwell assay showed that overexpression or inhibition of miR-625 significantly suppressed or enhanced NSCLC cell migration and invasion. B. Wound healing assay showed that overexpression or inhibition of miR-625 significantly suppressed or enhanced NSCLC cell mobility. Error bars represent the S.D. from at least three independent experiments. *P<0.05.

transfection of miR-625 inhibitor (Figure 2A). CCK-8 assay revealed that, compared to miR-NC-transfected cells, over-expression of miR-625 significantly decreased the growth rate of A549 cells, and miR-625 knockdown promoted H1703 cell proliferation (Figure 2B). A reduction in cell proliferation is often accompanied by cell cycle arrest [10]. The flow cytometry assay was thus employed to measure cell cycle distribution. We observed a significant increase in the percentage of cells in the G1/G0 phase and a decrease in the percentage of cells in the S phase in miR-625-overexpressing cells (Figure 2C). In contrast, miR-625 knockdown promoted cell cycle progression in H1703 cells.

Overexpression of miR-625 inhibits NSCLC cell migration and invasion

Invasion and metastasis of cancer cells is a critical aspect of cancer progression. Therefore, we hypothesized a relationship between miR-625 and NSCLC cell migration and invasion. In transwell assays, the migratory and invasive capacities of A549 cells were significantly reduced after transfection with the miR-625 mimics (Figure 3A). Similarly, over-expression of miR-625 suppressed the mobility of A549 cells, indicated by wound healing assay (Figure 3B). In contrast, miR-625 knockdown enhanced the migratory and invasive capacities of H1703 cells.

SOX4 is a target gene of miR-625

A miRNA usually performs its function by reducing the expression of target genes. Thus we performed a bioinformatic search (TargetScan, PicTar, and miRanda) for putative targets of miR-625 and found a complementary miR-625 sequence in the 3'-UTR of the SOX4 mRNA (Figure 4A). Using qRT-PCR and western blot, we verified that SOX4 was significantly decreased in both mRNA and protein levels after the over-expression of miR-625 in A549 cells (Figure 4B, 4C). As shown in Figure 4D, compared with the control group, luciferase activity was significantly suppressed in WT-SOX4-transfected A549 cells.

SOX4 reintroduction attenuates the inhibitory effects of miR-625 on NSCLC cell migration and invasion

Next we aimed to determine whether the down-regulation of SOX4 was involved in miR-625-
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mediated suppression of cell migration and invasion. Western blot analysis proved that SOX4 expression was significantly increased in the pcDNA-SOX4-transfected cells (Data not shown). As shown in Figure 5A, introduction of SOX4 evidently restored the effects of miR-625 on the migratory and invasive capacities of A549 cells. Similar effects were also observed in wound healing assay (Figure 5B).

Over-expression of miR-625 represses tumor growth in vivo

To confirm the tumor suppressor role of miR-625 in vivo, we established a BALB/c nude mouse xenograft model using A549 cells. Consistent with in vitro results, over-expression of miR-625 significantly inhibited tumor growth in vivo (Figure 6A). The weight of subcutaneous tumors derived from miR-625-overexpressing cells was significantly reduced compared to that of controls (Figure 6B). qRT-PCR and western blot analyses of tumor tissues confirmed elevated miR-625 with reduced
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Discussion

In recent years, an increasing number of oncogenic and tumor-suppressive miRNAs have been discovered in NSCLC [11], such as miR-15b [12] and miR-29a [13]. Therefore, finding novel miRNAs involved in the development of NSCLC represents an opportunity to improve patient outcome. This report might be the first direct investigation of the relationship between loss of miR-625 and tumorigenic potential of NSCLC cells. Taken together, the observations of this study indicate that miR-625 may serve as an oncogene and may play an important role in NSCLC development and progression.

Recently, several studies have demonstrated that miR-625 expression is frequently reduced and that this molecule functions as a tumor suppressor in multiple tumor types, such as hepatocellular carcinoma [14], breast cancer [15] and malignant melanoma [16]. Li et al. reported that the down-regulation of miR-625 predicts unfavorable prognosis of patients with esophageal squamous cell carcinoma [17]. In the present study, we found that miR-625 was significantly low expressed in NSCLC samples and correlated with a poor prognosis of NSCLC patients. Functional assays demonstrated that overexpression of miR-625 inhibited the NSCLC cell proliferation, migration and invasion, while miR-625 knockdown reversed it. The A549 xenograft model showed that miR-625 also suppressed tumor growth in vivo. These in vitro findings along with in vivo results provide promising evidence that supports miR-625 as a tumor suppressor in NSCLC.

Although miR-625 has been suggested to act as a tumor suppressor in various malignancies, the underlying mechanism by which miR-625-mediated gene expression participates in tumorigenesis remains to be clarified. To date, several targets of miR-625 have been identified, such as ILK, Sox2, IGF2BP1 and HMGA1. To explore the molecular mechanisms by which miR-625 affects NSCLC development, we predicted that SOX4 might be a direct target of miR-625 in NSCLC with the help of bioinformatics prediction. SOX4 is a member of the SOX (SRY-related HMG-box) family of transcription factors that is involved in embryonic development and cell-fate determination during organogenesis [18]. Aberrant SOX4 expression has been linked to development, progression in multiple tumor types, including NSCLC [19, 20]. It is demonstrated that increased expression of SOX4 is a biomarker for malignant status and poor prognosis in NSCLC patients [21]. SOX4

SOX4 protein levels in miR-625-overexpressing tumors (Figure 6C, 6D).

Figure 6. Overexpression of miR-625 represses tumor growth in vivo. A. Tumor volume in nude mice. B. Tumor weight in nude mice. C. The expression level of miR-625 in xenograft tissues. D. The expression level of SOX4 protein in xenograft tissues. Error bars represent the S.D. from at least three independent experiments. *P<0.05.
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can strengthen cisplatin-resistance of NSCLC cells [22]. In the present study, we found that SOX4 was significantly decreased in miR-625-overexpressed cells at both mRNA and protein levels, and overexpression of SOX4 effectively abrogated miR-625-induced cell migration and invasion inhibition.

Taken together, the present study demonstrated that the expression of miR-625 was reduced in NSCLC tissue samples and cell lines. Moreover, we observed that miR-625 overexpression inhibits NSCLC cell proliferation, migration and invasion in vitro, as well as suppress tumor growth in vivo. The newly identified miR-625/SOX4 signaling provides a novel molecular mechanism for the development of NSCLC, and may function as potential therapeutic targets for NSCLC in the future.

Disclosure of conflict of interest

None.

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