MicroRNA-661 promotes non-small cell lung cancer progression by directly targeting RUNX3

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Received February 12, 2017; Accepted June 8, 2017

DOI: 10.3892/mmr.2017.6827

Abstract. Lung cancer is the primary cause of cancer-associated mortality in men and women worldwide. Increasing evidence indicates that abnormal microRNA (miRNA) expression contributes to the carcinogenesis and progression of multiple human cancers, including non-small cell lung cancer (NSCLC). Therefore, miRNAs exhibit the potential to act as biomarkers for the diagnosis, treatment and prognosis of human malignancies. miRNA-661 (miR-661) has previously been demonstrated to be important in the development of various human cancer types. However, the expression levels, functions and underlying mechanisms of miR-661 in NSCLC remain to be elucidated. The present study demonstrated that miR-661 was upregulated in NSCLC tissues and cell lines. In addition, miR-661 expression levels were significantly correlated with differentiation and tumor stage lymph node metastasis of NSCLC patients. Functional experiments demonstrated that miR-661 downregulation inhibited NSCLC cell proliferation and invasion in vitro. Furthermore, runt-related transcription factor 3 (RUNX3) was expressed at a low level in NSCLC tissues and was negatively correlated with the miR-661 expression level. Further experiments revealed that RUNX3 knockdown significantly rescued the effects of miR-661 undertargeting on NSCLC cell proliferation and invasion. In conclusion, the present findings indicated a role for miR-661 as an oncogene in NSCLC via direct targeting of RUNX3, thus suggesting that miR-661 may be used to develop novel therapies for NSCLC patients.

Key words: non-small cell lung cancer, microRNA-661, runt-related transcription factor 3, progression, proliferation, invasion

Introduction

Lung cancer is the most common malignancy in humans and is the primary cause of cancer-related mortality in both men and women globally (1,2). Previous studies demonstrated that accumulation of permanent genetic alterations in combination with dynamic epigenetic alterations results in lung cancer formation and progression (3,4). Lung cancer can be divided into two major groups based on pathological features: Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (5). NSCLC is clinically characterised by rapid progression, strong invasiveness and high mortality rate (6). NSCLC accounts for ~85% of all lung cancer cases and includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (7). Although considerable progress in surgery, diagnostic method, radiotherapy and new chemotherapy regimens has been made, the prognosis of NSCLC patients remains poor (8). Recurrence and metastasis, even after radiation therapy and/or chemotherapy, are the major causes of death among NSCLC patients (5). Therefore, further understanding of the underlying molecular mechanisms during NSCLC initiation and progression is necessary. Moreover, the development of novel efficient therapeutic strategies for patients with this malignancy is urgently needed.

MicroRNAs (miRNAs) are a large group of endogenous, single-strand, non-coding and short RNAs that are between approximately 19 and 25 nucleotides in length (9). MiRNAs serve as key regulators of gene expression through direct binding to the 3′-untranslated region (UTR) of their target genes in a sequence-specific manner, leading to either translation inhibition or messenger RNA (mRNA) degradation (10). In recent years, miRNAs have emerged as powerful regulators of various physiological and pathological processes, such as growth, apoptosis, differentiation, angiogenesis, inflammation and tumourigenesis (11). Increasing evidence has shown that more than half of miRNA genes are located at fragile sites (12-14). Several studies reported that numerous miRNAs are aberrantly expressed in various human cancers and play significant roles in tumourigenesis and tumour development, including NSCLC (15-17). Obviously, miRNAs can function as either tumour suppressors or oncogenes in different human cancers depending on the characteristics of their target genes (18). Therefore, miRNAs are potential biomarkers for diagnosis, treatment and prognosis of human malignancies.
owing to their tissue- and disease-specific expression and regulatory functions (19).

MicroRNA-661 (miR-661) has been reported to be abnormally expressed in several tumors (20-22). However, the miR-661 expression and functions in NSCLC are yet to be investigated. The present study aims to elucidate the miR-661 expression, clinical significance and biological roles in NSCLC and to investigate its underlying molecular mechanisms.

Materials and methods

Tissue samples. Forty-seven pairs of NSCLC tissues and adjacent non-cancer tissues were obtained from patients who had undergone surgical resection at The First Affiliated Hospital of Jinzhou Medical University (Liaoning, China) between February 2013 and June 2015. All of these patients had not been treated with any pre-operative cancer treatment, such as radiotherapy or chemotherapy. All tissues were rapidly snap-frozen in liquid nitrogen and stored at -80°C for subsequent experiments. The protocol of the present study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University. Written informed consent was also provided by all patients who enrolled in the present study.

Cell lines, culture and transfection. Non-tumorigenic bronchial epithelium BEAS-2B cells and four NSCLC cell lines (A549, H460, SK-MES-1 and SPC-A1) were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). BEAS-2B cells were cultured in LHC-9 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). NSCLC cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.). NSCLC cell lines were isolated from tissues or cells with TRIzol reagent (Invitrogen; Carlsbad, CA, USA). RNA was reverse-transcribed to cDNA using a TaqMan RT Reagent kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an Applied Biosystems 7500 Real-time PCR System (Thermo Fisher Scientific, Inc.). For quantification of RUNX3 mRNA, cDNA was synthesized using PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China), followed by qPCR with SYBR Premix Ex Taq mastermix (Takara Biotechnology Co., Ltd.). RUN6B and GAPDH were used as internal control for miR-661 and RUNX3 mRNA, respectively. The primers used in the present study were as follows: miR-661 forward, 5'-GTG CCT GGG TCT CTT GCG-3', and reverse, 5'-CGT CAT GAT GTG CTG GCC TCC-3'; and reverse, 5'-ACG CTT CAC GTA ATTT GCG TGC T-3'. RUNX3 forward, 5'-GAC AGC CCA CAC TTCT CTT-3', and reverse, 5'-CAC AGT CAC CAC CGT ACC AT-3'; GAPDH forward, 5'-GGGT GCT GAA CAC GTA AGT-3', and reverse, 5'-GAC CGT TGT CAC CTG AGT-3'. The 2-△△Ct method was used to calculate the relative expression of miRNA and mRNA (23).

Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was measured with CCK8 assay (Dojindo Laboratories, Kumamoto, Japan). Cells were collected and seeded into 96-well plates at a density of 3000 cells per well the day before transfection. Cells were then transfected with miR-661 inhibitor, NC inhibitor, si-RUNX3 or si-NC. Subsequent to being incubated at 37°C in a 5% CO2 incubator for 0-72 h, 10 μl CCK-8 reagent was added into each well and incubated at 37°C for additional 4 h. The optical density (OD) at 450 nm for each well was determined using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were repeated in triplicate.

Cell invasion assay. Cell invasion assays were performed using Transwell chambers (pore size, 8 mm; Millipore Corp., Billerica, MA, USA) pre-coated with Matrigel (BD Biosciences, Bedford, MA, USA). Transfected cells were harvested at 48 h post-transfection, re-suspended in FBS-free DMEM and then seeded into the upper Transwell chambers. In the lower chamber, DMEM containing 10% FBS was added. After 48 h of incubation at 37°C with 5% CO2, the non-invasive cells were removed carefully with cotton swabs. The cells that had invaded through the chamber membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Subsequent to washing with PBS, cells in five randomly selected visual fields were photographed and counted under an inverted microscope (Olympus Corporation, Tokyo, Japan).

miRNA target prediction. Human miRNA target prediction algorithms: PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/) were used to predicate the potential targets of miR-661.

Luciferase reporter assays. For reporter assays, the human RUNX3 wild type or mutated 3'-UTR sequence containing the miR-661 binding site was inserted into psiCHECK-2 vector to develop psiCHECK2-RUNX3-3'UTR-Wt and psiCHECK2-RUNX3-3'UTR-Mut. Cells were seeded into 24-well plates at 5x104 cells per well and transfected with miR-661 inhibitor or NC inhibitor, together with psiCHECK2-RUNX3-3'UTR-Wt or
psiCHECK2-RUNX3-3’UTR-Mut. Following incubation at 37°C for 48 h, the Firefly and Renilla luciferase activities were determined using Dual-luciferase reporter system according to the manufacturer’s instructions (Promega, Madison, WI, USA). Luciferase activities were normalized to Renilla activities.

Western blot analysis. Tissues or cells were solubilized in cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and protein concentrations were measured by the standard BCA method (BCA™ Protein Assay kit, USA). Equal amounts of protein were separated by 10% SDS-PAGE electronically and transferred onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBST) buffer and incubated overnight at 4°C with the following primary antibodies: mouse anti-human monoclonal RUNX3 antibody (sc-376591; 1:1,000 dilution; Santa Cruz Biotechnology, CA, USA) and mouse anti-human monoclonal GAPDH antibody (sc-47724; 1:1,000 dilution; Santa Cruz Biotechnology). Subsequent to washing three times with TBST, the membranes were incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (sc-2005; 1:5,000 dilution; Santa Cruz Biotechnology) at room temperature for 2 h. The immunoblot was detected by an enhanced chemiluminescence solution (Pierce Biotechnology, Inc., Rockford, IL, USA). GAPDH was used as an internal control for RUNX3. Densitometric analysis was conducted using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. All data were presented as mean ± standard errors. Statistical significance between groups were evaluated by Student’s t-test or one-way analysis of variance (ANOVA). SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. P<0.05 was considered statistically significant.

Results

MiR-661 is upregulated in NSCLC tissues and cell lines. To investigate whether or not miR-661 levels were altered in NSCLC, we measured miR-661 expression in 47 pairs of NSCLC tissues and adjacent non-cancer tissues using RT-qPCR. *P<0.05 compared with non-cancer tissues. (B) Relative expression of miR-661 was detected in four NSCLC cell lines (A549, H460, SK-MES-1 and SPC-A1) and non-tumourigenic bronchial epithelium BEAS-2B cells. *P<0.05 compared with BEAS-2B.

Table I. Association between microRNA-661 expression and clinicopathologic factors of non-small cell lung cancer.

<table>
<thead>
<tr>
<th>Clinicopathological factors</th>
<th>microRNA-661 expression</th>
<th>Cases</th>
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*P<0.05.
expression level was strongly correlated with differentiation \((P=0.011)\), tumor stage \((P=0.013)\) and lymph node metastasis \((P=0.029)\), but not with gender, age, smoker and tumour size \((all P>0.05)\).

RT-qPCR was further carried out to quantify miR-661 expression levels in non-tumorigenic bronchial epithelium BEAS-2B cells and four NSCLC cell lines (A549, H460, SK-MES-1 and SPC-A1). Consistent with the results observed in clinical tissues, NSCLC cell line exhibited significantly higher miR-661 expression levels compared with BEAS-2B \((P<0.05)\). These results suggested that miR-661 might play key roles in NSCLC formation and progression.

**Downregulation of miR-661 suppresses NSCLC proliferation and invasion.** To investigate the potential role of miR-661 in NSCLC, miR-661 inhibitor was transfected into H460 and SK-MES-1 cells, which expressed relatively higher miR-661 expression. RT-qPCR analysis confirmed that miR-661 was markedly downregulated in H460 and SK-MES-1 cells after transfection with miR-661 inhibitor \((P<0.05)\). The effect of miR-661 underexpression on NSCLC cell proliferation was evaluated using CCK-8 assay. As shown in Fig. 2B and C, miR-661 downregulation inhibited H460 and SK-MES-1 cell proliferation compared with NC inhibitor. We also assessed the ability of miR-661 to regulate NSCLC cell invasion using cell invasion assay. The results indicated that miR-661-underepressed H460 and SK-MES-1 cells showed significantly less invasiveness than cells transfected with NC inhibitor \((P<0.05)\). These findings suggested that miR-661 may play an oncogenic role in NSCLC.

**RUNX3 is a direct target of miR-661 in NSCLC.** We sought to identify the direct target genes of miR-661 to explore the mechanism underlying its tumour-promoting activity in NSCLC. A considerable number of potential targets were predicted using bioinformatic analysis, and RUNX3 was selected for further confirmation \((P<0.05)\) because it was downregulated in NSCLC tissues and contributed to NSCLC initiation and progression \((P<0.05)\). To determine whether RUNX3 is a direct target of miR-661, luciferase reporter assay was performed in H460 and SK-MES-1 cells co-transfected with psiCHECK2-RUNX3 3’UTR Wt or psiCHECK2-RUNX3 3’UTR Mut and miR-661 or NC inhibitor. As shown in Fig. 3B, co-transfection with miR-661 inhibitor increased the luciferase activities of wild-type RUNX3 3’UTR plasmid compared with NC inhibitor \((P<0.05)\). However, the luciferase activities of mutant RUNX3 3’UTR constructs was not affected by miR-661 inhibitor. Further RT-qPCR and Western blot demonstrated that RUNX3 expression at both mRNA \((P<0.05)\) and protein \((P<0.05)\) levels in H460 and SK-MES-1 cells was significantly increased after transfection with miR-661 inhibitor. These results revealed that RUNX3 is the direct target of miR-661 in NSCLC.

**RUNX3 is downregulated and negatively correlated with miR-661 in NSCLC tissues.** To further determine the relationship between miR-661 and RUNX3, RT-qPCR and Western blot were performed to measure RUNX3 expression at mRNA and protein levels in NSCLC tissues and adjacent non-cancer tissues. We found that RUNX3 expression level was decreased
at both mRNA (Fig. 4A, P<0.05) and protein (Fig. 4B, P<0.05) levels in NSCLC tissues compared with that in adjacent non-cancer tissues. Additionally, we assessed the association between RUNX3 mRNA and miR-661 expression levels in NSCLC tissues. The results of Spearman’s correlation analysis indicated a statistically inverse association between RUNX3 mRNA and miR-661 expression levels in NSCLC tissues (Fig. 4C; r=-0.6960, P<0.0001).

**Discussion**

Increasing evidence indicated that abnormal miRNA expression contributes to the carcinogenesis and progression of multiple human cancers (26-28). Numerous studies have shed light on tumour-targeting therapies using miRNAs as novel diagnostic and therapeutic tools (29-31). Therefore, elucidating the expression level, clinical significance, biological roles and underlying mechanisms of specific miRNA in NSCLC will provide novel therapeutic targets for the diagnosis and therapy of patients with this disease. In the present study, we found that the miR-661 expression was upregulated in NSCLC tissues and that knocking down RUNX3 rescued the effects induced by miR-661 inhibitor on NSCLC cells. Rescue experiments were carried out to explore whether or not RUNX3 is responsible for the functional effect of miR-661 in NSCLC cells. Si-RUNX3 or si-NC was introduced into H460 and SK-MES-1 cells, and Western blot confirmed that RUNX3 was downregulated in si-RUNX3-transfected H460 and SK-MES-1 cells (Fig. 5A, P<0.05). Subsequently, CCK-8 and cell invasion assays were performed, which identified that RUNX3 knockdown significantly rescued the effects of miR-661 inhibitor on H460 and SK-MES-1 cell proliferation (Fig. 5B and C, P<0.05) and invasion (Fig. 5D, P<0.05). These findings suggested that miR-661 exerts its biological roles in NSCLC through negative regulation of RUNX3.
miR-661 IN NSCLC

and cell lines. The miR-661 expression level was significantly correlated with differentiation, tumor stage and lymph node metastasis of NSCLC patients. Functional assays indicated that miR-661 underexpression attenuated NSCLC cell proliferation and invasion in vitro. With regard to the mechanism, our results demonstrated that RUNX3 functions as a direct downstream target of miR-661 in NSCLC. These results demonstrated that miR-661 may play significant roles in NSCLC.

Previously, miR-661 was revealed to be aberrantly expressed in a number of human malignancies and play a crucial role in tumourigenesis and cancer progression. For example, Li et al. (20) reported that miR-661 was lowly expressed in glioma tissues. Restoration of miR-661 expression inhibited glioma cell proliferation, migration and invasion; induced cell cycle arrest and apoptosis in vitro; and decreased cell growth in vivo. Zhu et al. (21) found that miR-661 was upregulated in both ovarian cancer tissues and cell lines. Upregulation of miR-661 promoted cell proliferation, colony formation and anchorage-independent growth in vitro. Reddy et al. (22) demonstrated that ectopic miR-661 expression repressed cell migration, invasion and anchorage-independent growth and tumourigenicity of breast cancer. These findings indicated that miR-661 may be investigated as an effective therapeutic target for these types of cancer.

The identification of the direct target gene of miR-661 is important in understanding its roles in NSCLC and is also essential in the investigation of novel therapeutic target for NSCLC patients. Several targets of miR-661 have been reported, including hTERT (20) in glioma, INPP5J (21) in ovarian cancer and MTA1 (22) in breast cancer. In the present study, RUNX3 was identified as a direct target gene of miR-661 in NSCLC. Initially, the bioinformatic analysis revealed that RUNX3 may be a potential target for miR-661. Subsequently, this predication was further confirmed by the luciferase report assay. The results demonstrated that the 3’-UTR of RUNX3 could be directly targeted by miR-661 in NSCLC. Moreover, the regulatory effect of miR-661 on endogenous RUNX3 expression in NSCLC cells was determined using RT-qPCR and western blot, and demonstrated that downregulation of miR-661 significantly increased RUNX3 expression at the mRNA and protein levels in NSCLC cells. Besides, RUNX3 was downregulated and negative correlated with miR-661 expression level in NSCLC tissues. Finally, RUNX3 knockdown rescued the effects observed as a result of miR-661 underexpression in NSCLC cells.

Runt-related gene family includes three members, namely, RUNX1, RUNX2, and RUNX3, and all play key roles in the normal developmental process and tumourigenesis (32). RUNX3, which is located on chromosome 1p36, has been reported to be downregulated in many human cancer types, such as bladder cancer (33), oesophageal squamous cell

Figure 5. RUNX3 knockdown reverses the effects of miR-661 inhibitor on H460 and SK-MES-1 cells proliferation and invasion. (A) Western blot analysis indicated that RUNX3 protein in H460 and SK-MES-1 cells was downregulated following transfection with si-RUNX3. *P<0.05 compared with si-NC. (B-D) CCK-8 and cell invasion assays detected the proliferation and invasion of H460 and SK-MES-1 cells transfected with NC inhibitor or miR-661 inhibitor or miR-661 inhibitor co-transfected with si-RUNX3. *P<0.05 compared with NC inhibitor and miR-661 inhibitor+si-RUNX3.
carcinoma (34), breast cancer (35), gastric cancer (36) and glioma (32). In addition, RUNX3 is regarded as a tumour suppressor gene that inhibits cell proliferation, migration and invasion and induces cell cycle arrest and apoptosis (37-39). Previous studies also found that RUNX3 was reduced in NSCLC tissues, which was associated with promoter hypermethylation (24). Additionally, RUNX3 methylation was obviously correlated with NSCLC clinical stage, lymph node metastasis and differentiation (40). Functional experiments revealed that RUNX3 is involved in crucial regulation of cell proliferation (41), epithelial-mesenchymal transition (42) and tumourigenesis (25) in NSCLC. These findings indicated that RUNX3 may contribute to NSCLC progression. The present study identified RUNX3 as a direct gene target of miR-661, suggesting that the miR-661/RUNX3 axis may be a promising therapeutic target for the treatment of NSCLC patients.

In conclusion, we found that miR-661 expression level was increased in NSCLC tissues and cell lines. In addition, miR-661 expression level was significantly correlated with differentiation, tumor stage and lymph node metastasis of NSCLC patients. Furthermore, miR-661 acted as an oncogene in NSCLC, at least in part through RUNX3 targeting. These observations suggest that miR-661 may serve as a novel therapeutic target for the treatment of NSCLC patients.

References


