RESEARCH COMMUNICATION

MicroRNA-663 Targets TGFB1 and Regulates Lung Cancer Proliferation

Liu Zhi-Yong[1&], Zhang Guang-Ling[2&], Wang Mei-Mei[2], Xiong Ya-Nan[2], Cui He-Qin[3*]

Abstract

MicroRNAs (miRNAs) play critical roles in many different cellular processes, including metabolism, apoptosis, differentiation, and development. In this study, miR-663 was shown to be highly expressed in patients with lung cancer. Furthermore, miR-663 contributed to lung cancer cell proliferation of by regulating TGFB1, P53, Bax, and Fas directly or indirectly. Our results demonstrated that miR-663 plays an important role in the biology of lung cancer and may be useful in developing therapies targeting genes.

Keywords: miR-663 - lung cancer - oncogene - A549 - TGFB1

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Introduction

Lung cancer is a disease that is characterized by frequent mutations, amplifications, and epigenetic changes in various cancer-related genes, and is the major cause of death from neoplastic disease worldwide. The main types of lung cancer are small cell lung carcinoma and non-small cell lung carcinoma (Tiozzo et al., 2009; Young et al., 2009). The most common cause of lung cancer is long-term environmental exposure and genetic predisposition. Early studies suggest potential involvement of altered regulation of microRNAs (miRNAs) in the pathogenesis of a limited range of human cancers (Hiyoshi et al., 2009; Aguda et al., 2008; Mathonnet et al., 2007).

MicroRNAs (miRNAs) are a class of endogenous small, single-stranded, non-coding RNAs. MicroRNAs negatively regulate the expression of target genes through interacting with complementary sites in the 3' untranslated region (3'UTR) of their target genes (Bartel et al., 2004). MiRNAs are considered to play crucial roles in many cellular processes, such as proliferation, development, differentiation, and apoptosis (Agrawal et al., 2009; Matsubara et al., 2007; Venugopal et al., 2010). Studies have shown that miRNAs may function as a novel class of oncogenes or tumor suppressor genes. These oncogene miRNAs usually promote tumor development by negatively inhibiting tumor suppressor genes. The amplification of an oncogenic miRNA could eliminate the expression of a miRNA-target tumor suppressor gene and lead to cancer progression (Osada et al., 2007).

In the present study, we looked for alterations of miR-663 in ten patients with lung cancer. We here have documented the first evidence of frequent and marked overexpression of miR-663 in lung cancers. We also have shown an inhibitory effect of miR-663 on the expression of TGFB1, which we preselected based on the potential significance of its putative TargetScan algorithm–predicted target genes. TGFB1 is a potent growth inhibitor implicated in the development of several cancers (Elliott et al., 2005). In addition, our findings suggest that miR-663 regulates lung cancer cell proliferation and apoptosis through targeting TGFB1, and also represents a promising therapeutic approach in lung cancer.

Materials and Methods

Cell culture

The A549 cells were maintained in RPMI 1640 (Gibco, Grand Island) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100 μg/ml streptomycin at 37ºC in humidified air containing 5% carbon dioxide.

RNA extraction and real-time analysis

Briefly, total RNA was extracted from 10 paired samples of lung cancer and normal lung tissues by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. These tissues were provided by Tian Jin hospital. The mature species of miR-663 was then measured by quantitative real-time PCR specific for miR-663 [23]. Accordingly, 2 μg total RNA was reverse transcribed to cDNA using the following stem loop RT primers, 5'- GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGAT ACGACGCGG TCC -3'. The PCR primers were miR-663-forward, 5'- TGCGGAGGCGGGGCGCCGC GGG -3', and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'. The PCR cycles were as follows: initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s , 56 °C for 30 s, and 72 °C for 30 s. Concentrations of the miR-663 were normalized by the average of the control samples.

Transfection

A549 cells were plated at a density of 5×105 cells per well in 6-well plates. Transfection was performed using Lipofectamine 2000 at a final concentration of 1 μg/ml (Invitrogen, USA). The final concentrations of DNA transfected into A549 cells were oligonucleotides (200 nM), and plasmids (1 μg/ ml).

*[1]Hebei United University Affiliated Hospital, [2]Basic Medical College, [3]Life Science College, Hebei United University, Tang Shan, China [&]Equal contributions *For correspondence: cuiheqin@yahoo.cn*

Growth curve

A549 cells were plated at a density of 5×105 per well in 6-well plates, and were transfected as described above after 24 h of incubation. After transfection, the cells were counted once per day for 10 days. Each treatment was carried out in triplicate.

MTT assay

Cells were seeded into 96-well plates at a density of 20% per well in a fixed volume of 100 μL and incubation under 5% CO2 at 37 °C overnight. Then cells were tranfected. After 72 h incubation, 20 μL of MTT solution (5mg/mL)were added to each well. Incubation with MTT was terminated. Then, the 96-well plate was kept at 5 °C for 1-2 h. Finally, the absorption was measured at 570 nm.

Soft agar assay for colony formation

For colony formation assay, 5 x103 cells in 2× RPMI 1640 mixed equally with 1% agar were distributed on a total of ten 6-cm-diameter dishes, and were incubated at 37°C. A colony was counted only if it contained more than 50 cells, and the number of colonies was counted the 9th day after seeding. Each treatment was carried out in triplicate.

DNA construction and fluorescent reporter assays

TGFB1 expression plasmid with 3'UTR or without 3'UTR was cloned. These two DNA fragments from cDNA library both cloned into pcDNA3.1 vector by EcoRI/XbaI site. Primers of full length TGFB1 without 3'UTR: forward primer, 5'-CCGGAATTCGCCACCATGCCGCCCTCCGGGCTG-3' ; reverse primer, 5'-CTAGTCTAGA TCAGCTGCACTTGCAGGAG-3'. Primers of full length TGFB1 with 3'UTR: forward primer, 5'-CCGGAATTCGCCACCATGCCGCCCTCCGGGCTG-3' ; reverse primer, 5'- CTAGTCTAGA CCGCAGTCCTCTCTCCATC-3'. The 3' UTR of TGFB1 (144 bp) containing the TGFB1-miR-663 response element was cloned into the PmeI/XbaI site of pmirGLO Luciferase vector (Promega). Mutant 3'UTR of TGFB1 lacks 1-54 gene fragment were synthesized and cloned into the PmeI/XbaI site of pmirGLO Luciferase vector (Promega). We transfected A549 cells with 0.2 μg of the pmirGLO Vector reporter vector with wild TGFB1 3'UTR or mutation TGFB1 3'UTR and 0.1 μg of the pmirGLO control vector. Twenty-four hours after transfection, we transfected cells with 200 ng of miR-663 LNA; and the control LNA was transfected in parallel. We collected and assayed luciferase expression level 48 h after transfection. Each treatment was carried out in triplicate.

Western blot

Cells were resuspended in protein extraction buffer, as described previously[18]. Proteins were separated on 10% SDS-polyacrylamide gel, and electrophoretically transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were incubated with antibodies overnight at 4°C. TGFB1 antibodies and β-tublin were purchased from Abcam (Cambridge, MA, USA). The antibodies were used at 1:100 and 1:2,000 dilutions, as indicated. After incubating the membranes with secondary antibodies conjugated to peroxidase, the signal was revealed with Immobilon Western (Millipore, USA).

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis utilized two-tailed Student's t test and statistical significance was set as $P < 0.05$.

Results

MiR-663 was highly expressed in lung cancer patients

To validate the expression pattern of miR-663 of lung cancer, we first performed microRNA specific qRT-PCR on 10 pairs of lung cancer tissues and their adjacent normal tissues. Moreover, the lung cancer A549 cells were also analyzed. As shown in Figure 1, miR-663 was markly up-regulated in lung cancer tissues and A549 cells.

Reduction of miR-663 inhibits the proliferation ablility of A549 cells

Because miR-663 is expressed at a high level in lung cancer cells, the miR-663 LNA (locked nucleic acid) was transfected into A549 cells. We then used cell growth curve and colony formation assay to validate the effects of miR-663 on the proliferation of A549 cells. As shown in Figure 2, these results clearly demonstrate that decreased level of miR-663 can significantly inhibit the proliferation of lung cancer cells.

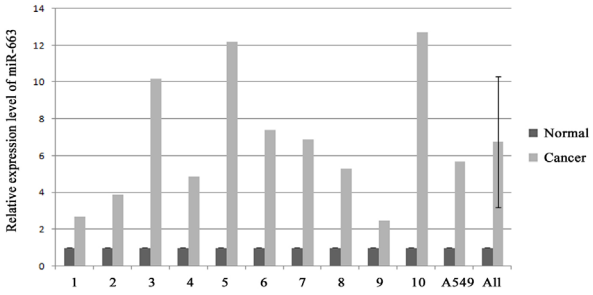

Figure 1. Expression of the Mature Species of the MiR-663. After extracting the total RNA from 10 specimens of lung cancer and from 10 specimens of normal lung, miR-663 level was determined by quantitative RT-PCR. U6snRNA was used as endogenous normalizer. The histogram shows the relative levels of miR-663 expression.

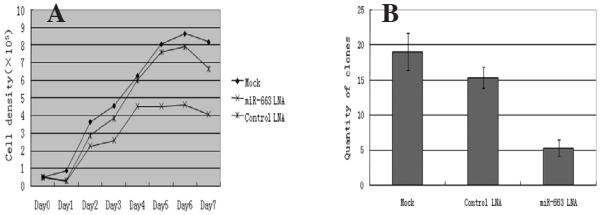

Figure 2. Reduction of MiR-663 Inhibits the Proliferation of A549 Cells. A. A549 cells were plated at a density of 5×105 per well in 6-well plates, and were transfected with miR-663 LNA or control LNA. Starting at 24 h after transfection, the cells were counted once per day for 7 days. Each data point represents the average of three independent experiments. B. A549 cells were plated at a density of 5×103 per well in 6-well plates, and treated as in A, after 10 days, the number of visible colonies was counted using a dissecting microscope. Each data point represents the average of three independent experiments.

TGFB1 is a direct target of miR-663

Recent studies indicate that miRNAs repress gene expression through targeting the 3'UTR region. Thus we used the miRNA target prediction program Targetscan to find the candidate target genes of miR-663. As shown in figure 3A, TGFB1 was select for the candidate target gene of miR-663 because the 3'UTR of TGFB1 mRNA carries several binding sites matching with the "seed region" of miR-663. Then we investigate the protein expression level of TGFB1 in A549 with altered expression levels of miR-663 by Western blotting. We transfected miR-663 LNA into A549 cells and scrambled oligonucleotides were used as negative control. As shown in figure 3B and 3C, TGFB1 protein level were up-regulated in A549 cells at 48 h after transfection with miR-663 LNA. To validate the direct interaction of TGFB1 and miR-663, we cloned the 3'UTR of TGFB1 (predicted to interact with miR-663) into a luciferase reporter vector, and co-transfected with miR-663 LNA or scrambled oligonucleotides into the A549 cells. We found that suppression of the expression of miR-663 in A549 cells could pecifically enhance the

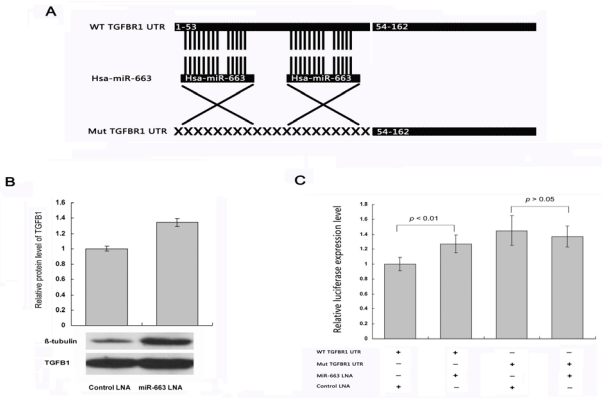

Figure 3. Identification of TGFB1 as a Direct Target Gene of MiR-663. A. The 3'UTR of TGFB1 mRNA carries a binding site for miR-663 and the mutation sites of miR-663 seed region. B. A549 cells were transfected with luciferase reporter plasmid with wild type TGFB1 3'UTR (TGFB1-3'UTR) along with either miR-663 LNA or control LNA. The luciferase intensity was detected and normalized by the amount of total protein. C. A549 cells were transfected with luciferase reporter plasmid with WT TGFB1 3'UTR or Mut TGFB1 3'UTR along with either miR-663 LNA or control.LNA. The luciferase intensity was detected and normalized by the amount of total protein. The fluorescence value of TGFB1-3'UTR group was set at 1, and the relative luciferase intensity is shown. Each data point represents the average of three independent experiments.

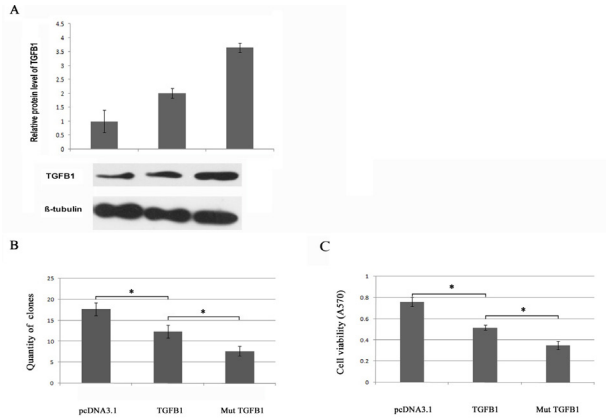

Figure 4. Highly Expressed TGFB1 Inhibits A549 Cell Proliferation. A. A549 cells were plated at a density of 5×10^3 per well in 6-well plates, and were transfected with pcDNA3/TGFB1 or pcDNA3 as control. After 10 days, the number of visible colonies was counted using a dissecting microscope. B and C. A549 cells were plated at a density of 1×10^4 per well in 96-well plates, and were trasfected as in A. 48 h after transfection, cell viability was analyzed by MTT assay. Each data point represents the average of three independent experiments.

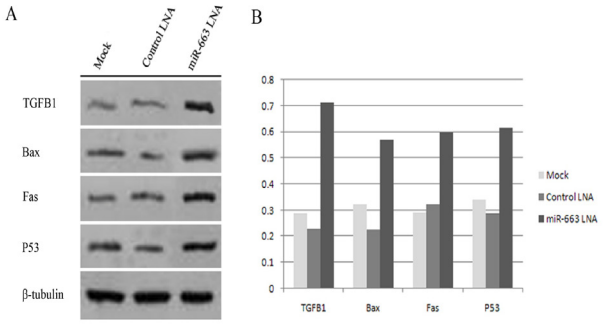

Figure 5. A549 cells were plated at a density of 5×105 per well in 6-well plates, and were transfected with miR-663 LNA or control LNA as control. 48 h after transfection, cells were lysated, and the protein level of TGFB1, Bax, Fas, and P53 were detected. β-tubulin was used as the loading control.

expression level of a reporter gene that carries a TGFB1 3'UTR. Moreover, no marked difference were observed in cells transfected with mutant TGFB1-3UTR no matter the real level of miR-663 ($p > 0.05$). These results demonstrated that miR-663 can specifically interacted with TGFB1 mRNA and repress TGFB1 expression at post-transcriptional level.

TGFB1 inhibits A549 cell proliferation

In order to verify that miR-663 regulates A549 cell proliferation through targeting TGFB1, we further elucidate the real role of TGFB1 in vitro. We constructed the plasmid expressing TGFB1 (pcDNA3/TGFB1, without 3'UTR) to increase the protein level of TGFB1 in A549 cells and investigated the possible effects of TGFB1 on cell proliferation ability. As shown in Figure 4A, the protein level of TGFB1 was increased when pcDNA3/TGFB1 was transfected into A549 cells. Furthermore, the viability and capacity of cell proliferation were both significantly reduced through MTT and a soft agar assay (Figure 4B and 4C).

MiR-663 regulates P53, Fas, and Bax expression through targeting TGFB1

Since miR-663 and TGFB1 has been shown to be related with cell growth and proliferation and TGFB1 is a direct target of miR-663, we further investigate the possible mechanism in lung cancer proliferation about miR-663 and TGFB1. It has been reported that TGFB1 is a multifunctional cytokine and involved in mulitiple cell progesses including cell apoptosis and proliferation. Therefore, we test the expression level of three apoptosis-related genes, P53, Fas, and Bax, in the situations of alternated levels of miR-663 and TGFB1. As shown in figure 5, obviously increased level of these three genes were observed in TGFB1 over-expressed group. These result indicated that miR-663 may exert its function through target TGFB1 and other three genes, P53, Fas, and Bax, may be the downstream genes of TGFB1 in regulating lung cancinogenesis.

Discussion

Work in recent years has identified over 1000 kinds of microRNAs in humans. MicroRNAs play critical roles in many different cellular processes, including metabolism, apoptosis, differentiation, and development (Agrawal et al., 2009; Matsubara et al., 2007; Venugopal et al., 2010).

Our data presented herein demonstrate that miR-663 is highly expressed in lung cancer, and plays a critical role in lung cancer proliferation. To find the direct target genes of miR-663, we performed bioinformatics analysis. According to the microRNA target prediction database Targetscan, we identified TGFB1 as a candidate target gene of miR-663. We further validated TGFB1 as a direct

target gene of miR-663 through a luciferase reporter assay.

TGFB1 is a multifunctional cytokine that involved in the control of several biological processes, including cell proliferation, differentiation, migration, and apoptosis (Datto et al., 1995; Berndt et al., 2007; Mao et al., 2006; Aluwihare et al., 2009). It is one of the most commonly altered cellular signaling pathways in human cancers (Wrana et al., 1994). In mammalian cells, TGFB1 is the most abundant isoform of TGF-ß family (which includes TGFB1, TGFB2, and TGFB3). TGFB1 signals the target cell by binding to a heterodimeric complex of two transmembrane receptors, transforming growth factor beta receptor types I and II (TGFRBI and TGFBRII). Once bound to TGFB1, TGFBR2 recruits and phosphorylates the type I TGF-ß receptor (TGFBR1). Activated TGFBR1 then phosphorylates two downstream transcription factors, SMAD2 and SMAD3, allowing them to bind to SMAD4 (Attisano et al., 2002; Shi et al., 2003; Nakao et al., 1997). Early studies also have suggested that TGFB1 interacts with oncogenes and tumor suppressed genes (Katakura et al., 1999; Inman et al., 2000; Ewan et al., 2002; Depoortere et al., 2000). Some of the downstream targets of TGF-β signaling are important cell-cycle checkpoint genes, including CDKN1A (p21), CDKN1B (p27) and CDKN2B (p15), and their activation leads to growth arrest (Massaguéet al., 2000). Therefore, TGF-β serves as a tumor suppressor in the normal intestinal epithelium by inhibiting cell proliferation and inducing apoptosis. Studies indicate that TGFB1 inhibits the proliferation of normal intestinal epithelial cells, but promotes the growth of malignant colorectal cancer cells (Derynck al., 2001). But in MCF-7 cells, TGFB1 can suppress leptin-stimulated cell proliferation (Perera al., 2008). In addition, TGFB1 Suppresses Nonmetastatic Colon Cancer at an Early Stage of Tumorigenesis (Engle al., 1999). In our study, TGFB1 was found to inhibit cell proliferation and down-regulated in miR-663 over-expressed A549 cells. After inhibit miR-663 express, TGFB1 and some tumor suppressor genes, P53, Bax, and Fas, were all upregulated. All of the three genes are play important role in anti cancer mechanism. The result indicate that miR-663 can regulate the expression level P53, Bax, and Fas directly or mediated by TGFB1.

In conclusion, we have presented herein the novel finding that miR-663 contributes to A549 cell proliferation through regulation of the expression of TGFB1, P53, Bax, and Fas directly or indirectly. Owing to its dual complex role in cancer, TGFB1 still remains a challenge as a potential therapeutic target. Thus, understanding of the precise mechanisms of miR-663 and TGFB1 will improve patient stratification and provide new therapeutic targets for improving the efficacy of cancer therapy.

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