Chromatin, Gene, and RNA Regulation

The Novel miR-7515 Decreases the Proliferation and Migration of Human Lung Cancer Cells by Targeting c-Met

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Abstract

MicroRNAs (miRNA) are small noncoding RNAs that regulate gene expression in human diseases, including lung cancer. miRNAs have oncogenic and nononcogenic functions in lung cancer. In this study, we report the identification of a novel miRNA, miR-7515, from lung cancer cells. The novel miR-7515 was characterized using various predictive programs and experimental methods. miR-7515 was able to forming a stem-loop structure and its sequence was conserved in mammals. The expression level of miR-7515 in lung cancer cells and tissues was profiled using TaqMan miRNA assays. miR-7515 was downregulated in lung cancer compared with normal human lung cells and tissues. The target of miR-7515 was determined using a dual luciferase reporter assay. Expression of the target gene was determined by quantitative RT-PCR and Western blot analysis after transfection with miR-7515. miR-7515 directly suppressed human mesenchymal–epithelial transition factor (c-Met) by binding to the 3′ untranslated region (UTR). Overexpression of miR-7515 significantly decreased cell-cycle–related proteins downstream of c-Met through c-Met inhibition. Cell proliferation and migration were examined using the XTT proliferation assay and the Transwell migration assay. miR-7515 led to decreased cell proliferation, migration and invasion in a lung cancer cell line. These results suggest that miR-7515 plays an important role in the proliferation and migration of lung cancer cells through c-Met regulation. Mol Cancer Res; 11(1); 43–53. ©2012 AACR.

Introduction

The first microRNA (miRNA) was discovered in Caenorhabditis elegans. The first miRNA in mammals was found in Drosophila melanogaster, and it was named lin-4. MicroRNAs (miRNA) and RNA interference (RNAi) are crucial for maintaining cellular homeostasis and playing important roles in many biologic processes in eukaryotic organisms, including cell identity and developmental timing (12). Recently, various cancer-related miRNAs have been reported that may have potential roles as therapeutic targets for cancer treatment (13). Approximately, 80% of all lung cancer patients have non–small-cell lung cancer (NSCLC) type (14, 15). Despite improvements in clinical treatment strategies, NSCLC remains an aggressive malignant tumor with a high mortality rate (16). Several factors contribute to lung cancer malignancy. One of these factors, human mesenchymal–epithelial transition factor (c-Met), was upregulated in various
cancers, including lung cancers (17). c-Met, which is the receptor tyrosine kinase (RTK) for hepatocyte growth factor (HGF), regulates the growth and invasive ability of lung cancer cells (18).

A recent study showed that the recognition of target gene 3' UTRs by a miRNA is achieved by base pairing the nucleotides in the miRNA seed sequence (nucleotides 2–8; ref. 19). It was also recently discovered that miRNAs can bind to 5' UTRs miRNA seed region allowing the recognition of multiple sites in target mRNAs (20). miRNAs have been found to play important roles in silencing target genes (21). Several reports have shown that miRNAs are involved in apoptosis, differentiation, inflammation, metastasis, and migration in cancer cell lines (22). For example, miR-212 increases apoptosis in NSCLC by targeting antiapoptotic proteins (23). miR-34b, miR-34c, and miR-199 negatively regulate c-Met expression, which promotes the migration of melanoma-derived primary cells (24). miR-203 regulates several genes in metastatic prostate cancer that are attractive candidates for inhibition (25). Although many studies examining the association of miRNAs with cancer have been performed, more research on this topic is needed.

In this study, we identified and characterized the novel miR-7515 from lung cancer cells. We analyzed the expression pattern of miR-7515 in lung cancer tissues and searched for target genes. We report the identification of a novel miRNA and suggest that miR-7515 may play critical roles in human lung cancers.

Materials and Methods

Cell culture

The human cell lines were obtained from the American Type Culture Collection (ATCC) and Korean cell line bank (KCLB). The WI-38 diploid normal lung cell line was maintained in Eagle’s minimal essential medium (EMEM; Lonza). The WI-38 VA-13 simian virus 40 (SV40)-transformed lung cells, NCI-H596 lung adenosquamous carcinoma cells, A549 lung carcinoma cells, and HCC-1588 lung squamous cell carcinoma cell lines were maintained in RPMI-1640 (Lonza). All cell lines were supplemented with 10% FBS obtained from Hyclone (Lonza), 100 U/mL penicillin, and 100 µg/mL streptomycin (Welgene) and incubated at 37°C in a humidified atmosphere incubator containing 5% CO2.

RNA isolation

Briefly, total RNA was extracted using the TRIZOL reagent (Molecular Research Center, Inc.) according to the manufacturer’s protocol. Following a wash with 80% ethanol, the RNA was resuspended in 0.1% DEPC-treated water (Sigma) and stored at −80°C.

Small RNA cloning and sequencing

All of the RNAs smaller than 200 nt were extracted from lung cancer cells using the mirVana RNA isolation kit (Ambion). The small RNAs were cloned into vectors using a DynaExpressmiRNA Cloning Kit (BioDynamics Laboratory Inc.). Small RNAs were isolated by RNA precipitation and then purified on a 15% denaturing PAGE. The 18 to 30 nt RNAs were dephosphorylated using alkaline phosphatase. Next, the small RNAs were ligated to 3' linkers. The 3' linker-ligated products were blocked at the 3' end, which prevented recircularization with the 5' linker. These products were separated on a 15% denaturing PAGE gel, and the products between 36 and 46 nt were extracted and purified. Ligation of the two linkers was performed by PAGE followed by staining. The 50 to 60 nt miRNAs were cut out of the gel. The resulting products were reverse-transcribed to create cDNAs. The PCR products were directly ligated into the T vector using the TA cloning kit (Promega), cloned, and sequenced (Macrogen).

Bioinformatic analysis

Putative small RNA sequences were evaluated for miRNA-like characteristics using a web-based program. The location of the putative small RNA sequences in the human genome was determined by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) analysis. The potential for each putative small RNA to form a stem-loop was examined using the RNAfold webserver.

TaQMan miRNA assays

The expression of miRNA was quantified by real-time PCR analysis using TaQMan miRNA Assays (Applied Biosystems; ref. 26). The goal of the TaQMan miRNA Assays is to identify differences between two group samples, often normal human lung tissue and cancerous lung tissue (27). The TaQMan miRNA assays were performed using miRNA-specific primers. The primers used were as follows: miR-7515 5'-AGAAGGGAGATGGTGAC-3'.

For miRNA expression analysis, 10 ng of total RNA was used along with the miR-specific primers supplied with the TaqMan miRNA Assays. Customized RT primers were synthesized complementary to the sequences of the mature miRNAs. Complementary DNA templates were standardized to RNU48 and subjected to 40 PCR cycles according to the manufacturer’s instructions. Data were generated using the CFX Manager software program (Bio-Rad). The relative gene expression was calculated via the 2−DDCt method.

Lung cancer patient tissues

Tissue samples were obtained from the Korea Lung Tissue Bank, which is supported by the Korea Science and Engineering Foundation in the Ministry of Science & Technology. The tissue samples used were described in our previous study (28).

miRNA transfections

Transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. Mock miRNAs [negative control (NC), miR-7515, si-c-Met and Anti-7515] were purchased from Genolution Pharmaceuticals, Inc. NC values were used as a universal control: UCACACCUCGUAGAAAGUAGA. Anti-7515, an miR-7515 inhibitor, and si-c-Met, an siRNA targeting c-Met, were chemically synthesized.
Dual luciferase reporter assay

The pGL3-reporter plasmids were constructed by inserting the 3' UTRs of the predicted target genes into the pGL3 vector. To generate the c-Met 3' UTR mutant construct (c-Met-3'UTR-mut), seed regions were mutated using the Muta-direct Site-directed Mutagenesis kit (iNtRON). A549 cells (5 x 10^4) were plated in 24-well plates and subsequently cotransfected with 100 nmol miR-7515 or the NC (a pGL3-reporter for the predicted target genes and a pRL-TK vector) for 48 h. The pRL-TK Renilla luciferase vector was cotransfected as an internal control. Firefly and Renilla luciferase activities were consecutively measured using a Dual-Luciferase Reporter Assay System kit (Promega), and luminescence was measured on a VICTOR3 analyzer (PerkinElmer).

Quantitative RT-PCR analysis

Real time PCR was performed using a QuantiTect SYBR green PCR kit (QIAGEN) and the Real-Time PCR Detection System (Bio-Rad). The primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'-TGCACCACCAACTGCTTAGC-3' and 5'-GGCATGGACTGTGGTCCAGAGACTTGGCTG-3'; c-Met 5'-GTCTGTGCTGAG3' and 5'-ATCCACTTCACTGCGAGCT-3' (29).

Western blotting

Proteins extracted from the cells were immunoblotted with different antibodies according to the manufacturer's protocol. The proteins were extracted from cells lysed with Pro-prep Lysis Buffer (iNtRON). The proteins were separated on an SDS-PAGE gel and subjected to immunoblot analysis using c-Met, ERK1/2, phosphorylated-ERK1/2 (p-ERK1/2), Akt, phosphorylated-Akt (p-Akt), Rb, phosphorylated-Rb (p-Rb), cyclin E, CDK2 (Santa Cruz), and GAPDH primary antibodies (AB Frontier) and the appropriate secondary antibodies (Santa Cruz). GAPDH was used as an internal control.

XTT proliferation assay

Cell proliferation was investigated using a commercially available proliferation kit (XTT, Roche). Briefly, A549 cells were subcultured in 96-well culture plates at a density
of $1 \times 10^4$ cells per well. The XTT test solution [50 μL, sodium 3-(1-phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate mixed at a ratio of 50:1] was prepared by mixing 5 mL of the XTT-labeling reagent with 100 μL of the electron coupling reagent. This mixture was added to each well, and cell viability was determined according to the manufacturer’s protocol. After 4 h of incubation in a 37°C incubator supplemented with 5% CO₂, the absorbance was measured on an ELISA reader at a wavelength of 490 nm.

**Wound healing assay**

A549 cells were subcultured in six-well culture plates at a density of $3 \times 10^5$ cells per well. The cell layer was scratched with a plastic pipette tip and reincubated. The cells were subsequently observed under the microscope.

**Transwell migration assay**

Cell migration was assessed using the Corning Transwell system. Twenty-four hours after transfection, $2 \times 10^4$ A549 cells in 0.2 mL serum-free medium were plated in the top chamber with a 0.1% gelatin-coated membrane (24-well; pore size, 8 mm; Corning). Next, 0.5 mL of medium containing 10% serum was added to the lower chamber. The cells were incubated for 48 h. The membrane was fixed in 95% ethanol and stained with 0.2% crystal violet (Sigma) for 30 min at 37°C and later washed with PBS. The values for cell migration were expressed as the average number of cells per microscopic field over three fields per one filter. The experiments were performed in triplicate.

**Statistical tests**

All data were expressed as mean ± SD. Student’s t test was used to compare values of test and control. $P \leq 0.05$ was considered to be statistically significant.

**Results**

Cloning and identification of miR-7515 from lung cancer cells

In this study, purified small RNAs were obtained from lung cancer cells. After sequence analysis, small RNA clones were identified by NCBI BLAST analysis. From the candidate small RNAs, we identified miR-7515, which was predicted by the RNAfold program to form a secondary structure (Fig. 1A). The mature miRNA sequence of miR-7515 was as follows: 5′-AGAAGGGAAGAUGGUGAC-3′. The sequence for miR-7515 was located on chromosome 2p25.2 at sites 6790550-6790567 (Fig. 1B). miR-7515 was transcribed from an intergenic region, and its location was confirmed by NCBI BLAST analysis. miR-7515 was 100% conserved in other mammals, including orangutans and monkeys (Fig. 1C). We knocked down Dicer and observed that miR-7515 was downregulated in si-Dicer–transfected cells compared with NC-transfected cells (Supplementary Fig. 1).

Expression of miR-7515 in various cell types and lung tissue samples

We performed an expression analysis of miR-7515 in various human cell lines using poly A-tailed RT-PCR (30). As shown in Supplementary Fig. 2, miR-7515 was expressed in various human cell types. The novel miRNA was cloned...
from a lung cancer cell line, and its expression level was quantified by TaqMan miRNA Assay. As shown in Fig. 2A, the expression of miR-7515 was approximately two times lower in WI-38 VA-13, HCC1588, A549, and NCI-H596 cells compared with WI-38 cells.

Next, we analyzed the expression of miR-7515 in tissues from 10 lung cancer patients using the TaqMan miRNA Assays. The expression level of miR-7515 in human lung cancer tissue samples was compared with the expression in normal human lung tissue samples. The expression of miR-7515 was decreased in lung cancer tissues compared with normal lung tissues (Fig. 2B).

Regulation of c-Met by miR-7515
To characterize the function of miR-7515, we identified target genes predicted to be upregulated in lung cancer (http://www.ebi.ac.uk/gxa/experiment/E-MTAB-37). From this list, we selected predicted miR-7515 target genes based on sequence identity with the miR-7515 seed region. Four of these genes (IGF1R, BAD, c-Met, and VCAM1) were identified as potential miR-7515 targets using the dual luciferase reporter assay. For all 4 of these genes, the luciferase activity of the c-Met 3' UTR construct decreased following transfection with miR-7515. The expression of the other genes (IGF1R, BAD, and VCAM1) was not obviously altered (Fig. 3A). The luciferase activity in A549 cells cotransfected with miR-7515 and the vector containing Figure 3. c-Met is a potential target of miR-7515. A, the predicted miR-7515 target genes with potential binding sites in the 3' UTRs. Luciferase activity in A549 cells cotransfected with predicted target genes in the pGL3 vector and miRNAs mimics (Mock, NC, or miR-7515). The sequences of the miR-7515 mimics are displayed. B, the mutations of the c-Met binding site are shown below the seed region. The data were normalized by the ratio of Firefly to Renilla luciferase control activity. These results were obtained from at least three experiments. (bars, SD; *, P < 0.05).
miR-7515 were conserved at the c-Met 3' UTR, conserved across different species. To confirm these results, we mutated the c-Met 3' UTR predicted binding site (positions 503–509 nt) to generate c-Met-3'UTR-mut in which the binding site was converted to GGGGGGG (Fig. 3B). Mutation of the c-Met 3' UTR binding site completely abolished the ability of miR-7515 to regulate luciferase activity. This result suggests that c-Met was an authentic target of miR-7515.

Downregulation of c-Met by miR-7515

The effect of miR-7515 on c-Met was determined using quantitative RT-PCR (Fig. 4A) and Western blot analysis (Fig. 4B). The level of c-Met expression was determined after the cells were treated with NC, the miR-7515 mimic, anti-7515 inhibitors, or si-c-Met. Dose-dependent expression of miR-7515 was detected in transfected cells compared with NC cells (Supplementary Fig. 4). A specific siRNA-targeting c-Met was included as a control. Transcription and translation of c-Met significantly decreased in miR-7515–transfected cells compared with NC cells. The anti-7515 inhibitor did not affect the c-Met levels through miR-7515 inhibition. Taken together, these results suggest that miR-7515 directly regulated c-Met in A549 cells.

Downregulation of downstream of c-Met and cell-cycle–related protein by miR-7515

We determined the expression of downstream signaling pathways after downregulation of c-Met by miR-7515. Downregulation of c-Met by miR-7515 led to decreased p-Akt and p-ERK1/2 in A549 cells. Translation of total Akt and ERK1/2 was not affected in miR-7515 compared with the NC (Fig. 5A). Transfection with anti-7515 inhibitors did not affect p-Akt and p-ERK.

Next, we assessed the effect of p-Akt and p-ERK1/2 on cell-cycle–related proteins, such as p-Rb, CDK2, and cyclin E, are downregulated by miR-7515 (Fig. 5B). The expression of the Rb protein was not affected by miR-7515. Taken together, these results suggest that miR-7515 led to decreased cell-cycle–related protein in A549 cells through c-Met targeting.
Overexpression of miR-7515 inhibits A549 cellular proliferation, migration, and invasion

To understand better the functionality of miR-7515 in A549 transformation, we tested whether miR-7515 could exert an effect on cell growth and motility in A549 cells. As shown in Fig. 6A, the overexpression of miR-7515 inhibited cell proliferation, as assessed by the XTT assay. miR-7515 inhibited cell proliferation in a time-dependent manner: cells transfected with miR-7515 showed 60% lower proliferation than cells

Figure 5. The inhibition of c-Met by miR-7515 regulated downstream cell-cycle-related proteins. A, the expression of p-Akt, p-ERK, which is downstream of the target gene, was determined in miR-7515-transfected cells by Western blotting analysis. B, cell-cycle–related protein levels were determined in miR-7515–transfected cells compared with NC. These results were obtained from at least three experiments. The results were quantified using NIH ImageJ (bars, SD; *, P < 0.05).
transfected with the NC. Knockdown of c-Met expression significantly inhibited cell proliferation in the A549 cell line. As shown in Fig. 6B and C, miR-7515 or si-c-Met repressed cell motility and migration, as determined by the wound healing assay and the Transwell migration assay. These data suggest that miR-7515 negatively regulates the motility and migration of A549 cells and that this effect may be at least partly attributed to the targeting of c-Met.

**Discussion**

In this study, we discovered the novel miRNA miR-7515 in lung cancer cells profiled its expression and characterized its function. The approximately 70 nt miR-7515...
miR-7515 Inhibits Proliferation in Lung Cancer

Because mature miRNAs are processed from precursor miRNAs by Dicer (31), we knocked down Dicer and observed that miR-7515 was downregulated in si-Dicer–transfected cells compared with NC-transfected cells. miR-7515 was therefore dependent on the Dicer-regulated pathway (Supplementary Fig. 1). miR-7515 is located on chromosome 2 and is transcribed from an intergenic region (Fig. 1B). The mature miRNA is conserved in mammals (Fig. 1C).

Recently, several studies have reported that miRNAs are downregulated in cancer cells and tissues. For example, miR-1 is significantly downregulated in lung cancer cells compared with normal cells, and the presence of miR-1 inhibits cell proliferation (32). miR-15a and miR-16 are specifically downregulated in NSCLC cells, and miR-15a/miR-16 induce G1 arrest via the downregulation of cyclin D1, D2, and E1 (33). Furthermore, the expression of miR-16 in tumor samples is thought to be a prognostic marker for NSCLC (34). miR-145 is downregulated in lung cancer and may be a marker for advanced NSCLC (35). In this study, we determined the expression profile of miR-7515 in various cell types, including lung-derived cells (Supplementary Fig. 2). The expression of miR-7515 was approximately two times lower in WI-38 VA-13, HCC1588, A549, and NCI-H596 cells than in WI-38 cells (Fig. 2). WI-38 VA-13 cells are derived from WI-38, which is a normal human fibroblast cell line transformed by SV40 infection. WI-38 VA-13 cells have many properties of tumor cells, including the loss of growth control (36). miR-7515 was downregulated in lung tumorigenesis cells, such as WI-38 VA-13, HCC1588, A549, and NCI-H596. Expression of miR-7515 was downregulated in lung cancer tissues. However, lung tissue samples not find any correlation about clinical characteristics (Supplementary Table 1).

Although the functions of miRNAs are largely unknown, miRNAs regulate target genes in various biologic processes (37, 38). We searched for potential miR-7515 target genes that are upregulated in lung cancers and related to cell proliferation and migration. Because miR-7515 was downregulated in lung cancer cells and tissues (Fig. 2B), we hypothesized that its target genes would be upregulated in lung cancers. As upregulated genes in lung cancers play roles in carcinogenesis, including the promotion of cell proliferation or migration (35), we selected for potential target genes those involved in proliferation and migration. We used the European Molecular Biology Laboratory (EMBL) databases to identify genes related to lung cancer proliferation and migration. In many other previous studies, target gene searches were performed using the TargetScan and PicTar programs (38, 39), which can be used for previously registered miRNAs. The putative target genes for the novel miR-7515 were selected by searching for perfect base pairing between the 7 nt on the 5′ end miR-7515 and the 3′ UTRs of the genes (40, 41). Using a luciferase reporter assay, we next sought to determine which of predicted target genes (IGF1R, BAD, c-Met, and VCAM1) was regulated by miR-7515 (Fig. 3A). The expression of one of the target genes, c-Met, was directly regulated by miR-7515.

Inhibition of c-Met downstream signaling might be effective in the treatment of lung cancer (42). miR-1 regulates cell proliferation and motility by downregulating c-Met (43). The downregulation of c-Met results in the inhibition of cancer cell proliferation, migration, and also increases apoptosis (44). In this study, we show that miR-7515 downregulates the transcription and translation of c-Met (Fig. 4). In addition to regulating c-Met, miR-7515 also reduced the expression of two c-Met downstream proteins, p-Akt and p-ERK1/2. miR-7515 also downregulated cell-cycle–related proteins, such as p-Rb, CDK2, and cyclin E (Fig. 5). The cyclin E-CDK2 complex controls the G1/S transition in proliferating cells by phosphorylating p-Rb (45). miR-7515 did not directly downregulate c-Met downstream and cell-cycle–related proteins by binding to their 3′ UTR regions. Instead, base pairing between the miR-7515 seed region and the c-Met 3′ UTR inhibited c-Met expression, which led to the downregulation of these downstream cell-cycle–related molecules. In addition, miR-7515 inhibited proliferation and migration via downregulation of c-Met (Fig. 6), and could therefore inhibit lung cancer progression through c-Met suppression. Anti-7515 inhibitors did not affect in results because of low expression of miR-7515 in A549.

In conclusion, in this study, we identified and characterized the novel miR-7515 in lung cancer cells. miR-7515 was downregulated in lung cancer tissues. In addition, miR-7515 directly regulated c-Met expression and altered the signaling of downstream cell-cycle–related proteins. Thus, miR-7515 inhibited proliferation and migration via the downregulation of c-Met. These results suggest that modulating miR-7515 expression may serve as a novel strategy for lung cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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