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Epigenetic silencing of microRNA-373 to epithelial-mesenchymal transition in non-small cell lung cancer through IRAK2 and LAMP1 axes

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ABSTRACT

The role of microRNAs (miRNAs) in carcinogenesis as tumor suppressors or oncogenes has been widely reported. Epigenetic change is one of the mechanisms of transcriptional silencing of miRNAs in cancer. To identify lung cancer-related miRNAs that are mediated by histone modification, we conducted microarray analysis in the Calu-6 non-small cell lung cancer (NSCLC) cell line after treatment with suberoylanilide hydroxamic acid (SAHA), a histone deacetylase (HDAC) inhibitor. The expression level of miR-373 was enhanced by SAHA treatment in this cell line by microarray and the following quantitative RT-PCR analyses. Treatment with another HDAC inhibitor, Trichostatin A, restored the levels of miR-373 expression in A549 and Calu-6 cells, while demethylation drug treatment did not. Importantly, miR-373 was found to be down-regulated in NSCLC tissues and cell lines. Transfection of miR-373 into A549 and Calu-6 cells attenuated cell proliferation, migration, and invasion and reduced the expression of mesenchymal markers. Additional microarray analysis of miR-373-transfected cells and computational predictions identified *IRAK2* and *LAMP1* as targets of miR-373. Knockdown of these two genes showed similar biological effects to those of miR-373 overexpression. In clinical samples, overexpression of IRAK2 correlated with decreased disease-free survival of patients with non-adenocarcinoma. In conclusion, we found that miR-373 is silenced by histone modification in lung cancer cells and identified its function as a tumor suppressor and negative regulator of the mesenchymal phenotype through downstream *IRAK2* and *LAMP1* target genes.
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Introduction

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 22 nucleotides that control the expression level of specific proteins by attenuating the translation of target messenger RNAs (mRNAs). Their functions cover a wide range of biological processes including cell proliferation, migration, and invasion of cancer cells [1–4]. In addition, the expression levels of various miRNAs have been reported to change during the carcinogenic process [5–8]. MicroRNAs are therefore thought to act as tumor suppressors or oncogenes that modulate oncogenic pathways [9–13].

Several causes of aberrant expression of miRNA in cancers have been postulated, such as mutations in the pri-miRNA sequence, gene copy number alterations, and abnormal transcription [14–16]. Epigenetic modification of DNAs, such as DNA promoter methylation and histone modification, contributes to chromatin remodeling and the general regulation of gene expression in mammalian development and human diseases [17–19]. Recent studies have demonstrated evidence of epigenetic aberration of miRNA expression in human cancers [20–23]. Furthermore, in cancer cell lines in which DNA hypermethylation represses miRNA expression, treatment with demethylating drugs and histone deacetylase (HDAC) inhibitors has been found to restore miRNA expression [24–28].

Lung cancer has a high incidence and mortality rate, making it the most deadly malignant disease in the world [29]. Accumulation of multiple genetic aberrations, as well as epigenetic changes, which affect key cellular genes such as oncogenes and tumor suppressor genes, plays a key role in lung carcinogenesis [30–32]. MicroRNAs have been reported as targets of epigenetic events during carcinogenesis. In lung cancer, several miRNAs, such as miR-9-3, miR-34b, miR-124-1, miR-124-2, miR-124-3, mir-126, miR-137, miR-152, miR-193a, miR-503, and miR-886-3p, are silenced by DNA

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hypermethylation [33–41]. Furthermore, histone modification may be another possible epigenetic mechanism underlying aberrant miRNA expression. However, little evidence exists to suggest that miRNAs are affected by this mechanism in lung cancer.

In the present study, we identify that miR-373 is silenced by histone modification and participates as a tumor suppressor in lung cancer cells. After overexpression of miR-373 in A549 and Calu-6 lung cancer cells, we searched its downstream target genes by microarray and the following computational database analyses. We observed that the miR-373 plays as negative regulator of the mesenchymal phenotype through downstream target genes, interleukin-1 receptor-associated kinase-like 2 (*IRAK2*) and *lysosomal-associated membrane protein 1 (LAMP1)*.

Materials and methods

Cell lines and tissue specimens

Eight non-small cell lung cancer (NSCLC) cell lines (A549, Calu-6, H460, EKVX, H23, H322M, H332M, and H358) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Tissue specimens surgically resected from primary lung cancer patients were randomly collected in the Asan Medical Center. Informed consent from all patients and ethical approval by the Institutional Review Board of Asan Medical Center were obtained prior to any experiments.

Deacetylation and demethylation assays

A549 and Calu-6 cells were seeded at 3×10^5 cells/well in 60 cm^2 dishes and cultured for 24 hours. Calu-6 cells were then exposed to 3 μM or 5 μM suberoylanilide hydroxamic acids (SAHA) for 72 hours. A549 and Calu-6 cells were treated with 300 nM Trichostatin A (TSA) as HDAC inhibitors, and 3 μM 5-aza-2′-deoxycytidine (AZA) as a DNA demethylating drug for 72 hours. These three reagents were purchased from Sigma Aldrich (St. Louis, MO).

RNA extraction and qRT-PCR

Total RNA was isolated from cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). For single-stranded complementary DNA synthesis, 1 μg of total RNA was reverse-transcribed by MultiScribe™ Reverse Transcriptase (Applied Biosystems, Carlsbad, CA). The primer sets and amplification conditions for PCR are listed in Appendix: Supplementary Table S1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA and 18s ribosomal RNA expressions were used as endogenous controls. For the quantification of miRNA, 20 ng of total RNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) with specific primers for U6 small nuclear RNA (RNU6B) and miR-373 (Applied Biosystems). PCR amplifications were subsequently performed in triplicate according to the TaqMan MicroRNA Assay protocol (Applied Biosystems). The expression levels of miR-373 were normalized against those of the endogenous control RNU6B by the $2\text{-}\Delta\Delta C_t$ methods.

Western blotting

Cells were lysed using the Cell Lysis Buffer (#9803; Cell Signaling Technology, Danvers, MA) with a protease inhibitor cocktail kit (P3100-005; GenDEPOT, Barker, TX) and a phosphatase inhibitor (sc-45065; Santa Cruz Biotechnology, Santa Cruz, CA). Aliquots containing 30 μg of cell lysates were denatured in 5 × sample buffer, electrophoretically resolved on SDS-polyacrylamide gels, and then transferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK) in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH 8.3)] at 300 mA at 4 °C for 90 minutes. The membranes were blocked with 7% skim milk in PBS containing 0.1% Tween 20 for 60 minutes at room temperature and then incubated with the antibodies listed in Appendix: Supplementary Table S2. The secondary antibody used was an HRP-conjugated goat anti-rabbit IgG (1:2000; Cell Signaling Technology). The blots were developed using the ECL western blotting analysis system (GE Healthcare, Buckinghamshire, UK).

Synthetic miRNA/siRNA transfection

Two NSCLC cell lines, A549 and Calu-6, were transfected with Precursor Molecule mimicking miR-373 (Applied Biosystems), scrambled sequence miRNA (Scrambled Negative Control; Applied Biosystems), siRNAs targeting *IRAK2* (IRAK-2 sense 5′ CAGCAACGUCAAGAGCUCUAAUU 3′ antisense 5′ UUAGAGCUCUUGACGUUGCUGUU 3′) and *LAMP1* (LAMP1 sense 5′ AGAAAUGCAACACGUUAUU 3′ and antisense 5′ UAACGUGUUGCAUUUCUUU 3′) or a scrambled sequence siRNA (sense 5′ CCUCGUGCCGUUCCAUCAGGUAGUU 3′ and antisense 5′ CUACCUGAUGGAACGGCACGAGGUU 3′) to a final concentration of 5–100 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested for assaying at 24–72 hours after transfection.

Cell proliferation assay

The effect of miR-373 on cell proliferation was evaluated by MTT assay. Briefly, 10^3 A549 and Calu-6 cells were treated with precursor miR-373 in 96-well plates. After incubation for 1, 3, and 5 days at 37 °C, 20 μL of 10 mg/mL MTT (Sigma Aldrich) was added to each well. Four hours after incubation with MTT, the supernatant was discarded and the precipitate of formazan was dissolved in 200 μL of dimethylsulfoxide. The solution was measured on a microplate reader at 540 nm.

Wound healing assay

Transfected cells were seeded at 3×10^5 cells/well in 6-well plates at a density, which was expected to reach 70–80% confluence as a monolayer after 24 hours of growth. A scratch was made through the center of each well using a 200-μL pipette tip, and dislodged cells were removed by three washes with complete culture media. The plates were then incubated with RPMI 1640 with 1% FBS for 48 hours, and the scratch was microscopically observed.

Cell migration and invasion assays

We tested the motile and invasive capacity of A549 and Calu-6 cells transfected with mimic RNA oligos using BD Biocoat Tumor Invasion System (BD Biosciences, San Diego, CA) according to the manufacturer's protocol with minor modifications. Briefly, A549 and Calu-6 cells were transfected with mimic miR-373, siIRAK2, siLAMP1, or negative control for 24 hours. These transfected cell lines were then seeded into the upper chamber of the transwells with serum-free medium (3×10^4 cells). The bottom wells in the system were filled with medium containing 1% FBS. After 72 hours of incubation, the cells in the upper chamber were removed, and the cells infiltrating through the chamber membrane were microscopically counted.

Microarray analysis and miRNA target prediction

Total RNA was extracted from A549 and Calu-6 cells at 24 hours after transient transfection. Complementary DNA microarray analysis was conducted by DNA Chip Research (Macrogen, Korea) with whole Human Genome oligo DNA arrays (Agilent Technologies, Santa Clara, CA). The predicted targets of miR-373 and their binding sites were analyzed by miRBase, TargetScan and RegRna.

Tissue microarray generation and immunohistochemical staining

Tissue microarrays were constructed from paraffin-embedded blocks of 392 NSCLC cases as previously described [42]. Histological typing and grading were performed according to the World Health Organization guidelines.

Sections at a thickness of 4 μm were generated from tissue microarray blocks and mounted on silane-coated slide-glasses (MUTO Pure Chemicals Co. Ltd, Tokyo, Japan). The slides were deparaffinized through a series of xylene rinses and rehydrated stepwise by soaking in graded alcohol-distilled water solutions. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature. For antigen retrieval, sections were placed in a 10 mM sodium citrate buffer (pH 6.0) at 95 °C. Non-specific binding was blocked by incubating in 5% normal goat serum for 30 minutes at room temperature. A primary rabbit anti-IRAK2 polyclonal antibody (1:100; Cell Signaling Technology) was applied for 1 hour at room temperature. The intensity of staining was categorized as 0 (negative), 1 (weak), or 2 (strong). The score was dichotomized into negative (no and low expression, score 0 and 1) and positive (high expression, score 2).

Quantitative methylation analysis (EpiTyper)

Genomic DNA was subjected to sodium bisulfite treatment using the EZ methylation kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. Quantitative DNA methylation was determined by MassArray EpiTyper technology (Sequenom, San Diego, CA) as previously described [43,44]. These data were corrected using DNA methylation standards (0, 20, 40, 60, 80, and 100% methylated genomic DNA). The primers used are as follows: forward 5′-GTAGCAGGATGGCCCTAGAC-3′; and reverse 5′-CGCCCTCTGAACCTTCTCTT-3′. The locations of EpiTyper amplicons are illustrated in Appendix: Supplementary Fig. S1.

Statistical analysis

Associations between categorical variables were analyzed by Pearson's chi-square and Fisher's exact tests. Survival curves were calculated by the Kaplan–Meier method, and statistical significance was evaluated using the log-rank test and the Cox proportional hazards regression model. *P*-values less than 0.05 were