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Combining microRNA-449a/b with a HDAC inhibitor has a synergistic effect on growth arrest in lung cancer

Hyo Sung Jeon[a], Soo Young Lee[b], Eun Jin Lee[a], Seong Cheol Yun[c], Eun Jung Cha[d], Eugene Choi[c], Moon Jun Na[c], Jae Yong Park[e], Jaeku Kang[f,*], Ji Woong Son[b,c,**]

[a] Department of Biochemistry, School of Medicine, Kyungpook National University, Daegu, Republic of Korea
[b] Myunggok Research Institute for Medical Science, Konyang University, Daejeon, Republic of Korea
[c] Department of Internal Medicine, Konyang University Hospital, Daejeon, Republic of Korea
[d] Department of Pathology, Konyang University Hospital, Daejeon, Republic of Korea
[e] Department of Internal Medicine, Kyungpook National University Hospital, Daegu, Republic of Korea
[f] Department of Pharmacology, College of Medicine, Konyang University, Daejeon, Republic of Korea

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ABSTRACT

Histone deacetylases (HDACs) play a crucial role in tumorigenesis. Over-expression of HDACs has been reported in lung cancer. The mechanism of highly expressed HDAC1 in lung cancer has yet not been determined. In the present study, we showed that miR-449a/b regulates HDAC1 by directly binding with the 3′ untranslated region of the HDAC1. The expression of miR-449a/b was down-regulated and the expression of HDAC1 was up-regulated in primary lung cancer. The down expression of miR-449a/b might be one mechanism for over-expression of HDAC1 in lung cancer. miR-449a/b inhibited cell growth and anchorage-independent growth. Furthermore, co-treatment with miR-449a and HDAC inhibitors had a significant growth reduction compared with HDAC inhibitor mono-treatment. These results suggest that miR-449a/b may have a tumor suppressor function and might be a potential therapeutic candidate in patients with primary lung cancer.

1. Introduction

Histone deacetylases (HDACs) are involved in the repression of gene transcription expression by altering chromatin structure [1]. HDACs regulate a variety of biological processes, such as cell cycle progression, apoptosis, differentiation, DNA replication and DNA damage response [2]. Over-expression of HDACs has been reported in several cancer tissues, including stomach, esophagus, colorectal, kidney, breast, and lung [3–8]. Specifically, the expression of HDAC1 is related to advanced non small cell lung cancers (NSCLCs) [9] and an independent predictor of poor prognosis in patients with NSCLC [10].

Because microRNAs (miRNAs) repress gene expression by interacting with messenger RNA (mRNA), by inhibiting mRNA translation or inducing mRNA cleavage, and play important roles in a variety of biological processes, such as cell proliferation, apoptosis, development, and differentiation, we postulated that miRNAs may play a role in modulating HDAC1 expression. We hypothesized that miRNAs play a role in controlling HDAC1 expression, then predicted via computational analysis that miRNAs contribute to HDAC1 regulation. miR-449a/b was predicted to directly bind with the 3′-UTR site of HDAC1 based on the following prediction programs: TargetScan (http://www.targetscan.org/); miRanda (http://www.microrna.org/); and microcosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). In addition, miR-449a induces growth arrest by directly targeting HDAC1 in prostate cancer [11].

In the current study, we examined the differential expression of miR-449 a/b and HDAC1 in lung cancer and normal lung tissues. We hypothesized the miR-449 a/b may have a role in lung cancer cell growth by targeting directly HDAC1. To test this hypothesis, we performed luciferase, cell proliferation and colony formation assays.

2. Materials and methods

2.1. Cell lines

Lung cancer cells (A549, H522, HCC1438, HCC95 and H1299) were maintained in RPMI 1640 medium (GIBCO-BRL, Rockville, MD,

* Corresponding author. Tel.: +82 42 600 6415; fax: +82 42 600 6415.
** Corresponding author at: Department of Internal Medicine, Konyang University Hospital, Gasuwon-dong, Seo-gu, Daejeon, Republic of Korea. Tel.: +82 42 600 8817; fax: +82 42 600 9090.
E-mail addresses: jaeku@konyang.ac.kr (J. Kang), sk1609@hanmail.net (J.W. Son).

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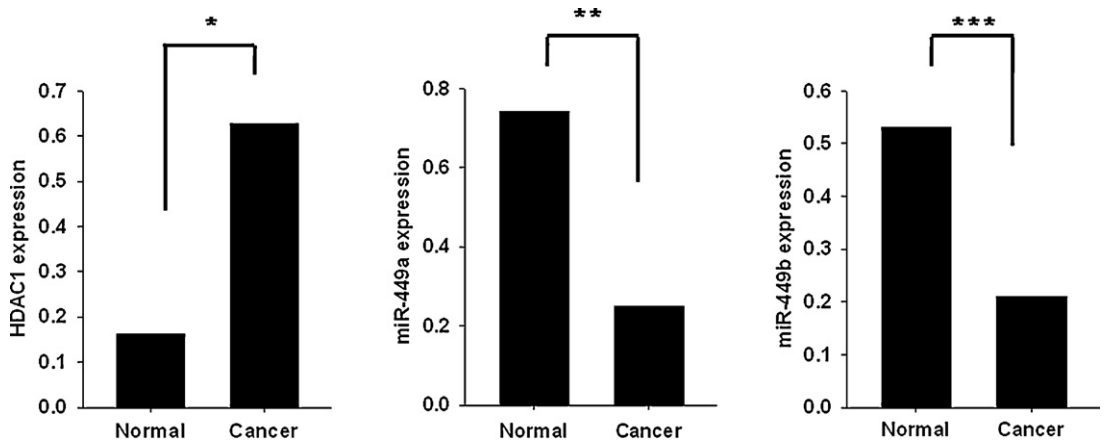

Fig. 1. HDAC1 and miR-449a/b were expressed in opposing directions from lung cancer patients. Real-time RT-PCR analysis of mature miR-449a/b and HDAC1 expression in lung cancer and normal lung tissues prepared from the same patients. $^{*}P = 0.005$, $^{**}P = 0.014$, $^{***}P = 0.031$ versus control.

USA) with 10% FBS and antibiotics (100 units/ml of penicillin and 100 mg/ml of streptomycin).

2.2. Tissues

Tumor and corresponding normal lung tissue specimens were obtained from Korean patients with NSCLC. Twenty-one patients with NSCLC (8 squamous cell carcinomas and 13 adenocarcinomas) who underwent curative resection at the Konyang University Hospital were analyzed (Table 1). None of the patients had received chemotherapy or radiotherapy prior to surgery. Informed consent was obtained from each patient before surgery. This study was approved by the Bioethics Committee of Konyang University Hospital. All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, and were rapidly frozen in liquid nitrogen and stored at −80 °C until analysis. Tissue samples were histologically confirmed by hematoxylin–eosin staining.

2.3. Luciferase assay

We investigated whether or not miR-449a and miR-449b modulate the HDAC1 expression by luciferase assay. The dual luciferase vector psiCHECK2 was purchased from Promega (Madison, WI, USA). The 134 bp fragment was synthesized by PCR using cDNA of 293T cells. The forward primer with an XhoI restriction site (5′-GGGCTCGAG AGG CTC CTA AAG TAA CAT CAG CC-3′) and the reverse primer with a NotI restriction site (5′-GGGCGGCCGC ACT AGC AAC CTC CAC CTG CAG-3′) were used to amplify the HDAC1 3′-UTR region. The PCR products were cloned into the XhoI/NotI 3′-UTR site of the psiCHECK2 plasmid (Promega). The correct sequence of all the clones was verified by DNA sequencing. 293T cells were seeded into a 12-well plate in DMEM medium supplemented with 10% heat-inactivated FBS. The cells were transfected with psiCHECK2-HDAC1 UTR constructs containing 3′-UTR of HDAC1, in the presence or absence of miR-449a and miR-449b precursor (Ambion, Austin, TX, USA) using Effectene (Qiagen, Hilden, Germany). The cells were collected 48 h after transfection, and the cell lysates were prepared according to the Promega's instruction manual. The Renilla luciferase activity was measured using a Lumat LB953 luminometer (EG & G Berthhold, Bad Wildbad, Germany), and the results were normalized by using the activity of luciferase. All experiments were performed in triplicate.

Table 1
Characteristics of the study populations.

Characteristics	
Age	64.19 ± 9.63
Gender	
Male	16
Female	5
Smoking status	
Smoker	14
Never smoker	7
Histology	
Squamous cell carcinoma	8
Adenocarcinoma	13
Pathologic stage	
Stage I/II	11
Stage III/IV	10

2.4. MicroRNA precursor, inhibitor and siRNA transfection

Cells were plated in 6-well plates at a density of 1.5×10^5 cells/well. The next day, cells were transfected with 50 nM Pre-miR miRNA Precursor (Ambion), anti-miR inhibitor (Ambion), Pre-miR miRNA Precursor-Negative control#1 (Ambion) and anti-miR negative control#1 inhibitor (Ambion) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. Also, cells were transfected specific siRNAs for HDAC1 (sc-29343, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a scrambled siRNA (Invitrogen) with Lipofectamine 2000 (Invitrogen).

2.5. Quantitative real-time PCR assay

Total RNA was isolated with TRIzol (GIBCO-BRL, Glasgow, UK) according to the protocols of the manufacturer. The first strand of cDNA was synthesized using the oligo (dT) primer system (SuperScript III First-strand Synthesis System; Invitrogen). Aliquots of the reaction mixture were used for the qPCR amplification with the IQ5 system (Bio-Rad Laboratories, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories). The PCR was run for 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, and elongation at 72 °C for 15 s. Gene expression was quantified by the comparative CT method, with normalizing CT values to the housekeeping gene β-actin. After amplification, melting curve analysis was performed to ensure the specificity of the products.

2.6. TaqMan MicroRNA expression assay

qRT-PCR analysis for miRNAs was performed in duplicate with a TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA,