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The role of miR-506 in transformed 16HBE cells induced by anti-benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide

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ABSTRACT

Growing evidence indicates that the alteration of microRNA (miRNA) expression in tumors that is induced by chemical carcinogens plays an important role in tumor development and progression. However, the mechanism underlying miRNA involvement in lung carcinogenesis induced by anti-benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide (anti-BPDE) remains unclear. In our study, we used the malignant transformation of human bronchial epithelial cells (16HBE-T) induced by anti-BPDE to explore the mechanisms of human lung carcinogenesis. We found that expression of miR-506 was reduced in 16HBE-T transformed malignant human bronchial epithelial cells compared with 16HBE normal human bronchial epithelial cells. Restoration of miR-506 in 16HBE-T cells led to a decrease in cell proliferation, G0/G1 phase cell cycle arrest, as well as significantly suppressed anchorage-dependent growth *in vitro* and tumor growth inhibition in a nude mouse xenograft model. In addition, we provided novel evidence regarding the role miR-506 potentially plays in negatively regulating the protein and mRNA expression level of N-Ras in cancer cells. Together, these findings revealed that miR-506 acts as an anti-oncogenic miRNA (anti-oncomir) in malignantly transformed cells. The identification of tumor suppressive miRNAs could provide new insight into the molecular mechanisms of chemical carcinogenesis.

1. Introduction

Lung cancer is by far the leading cause of cancer death worldwide, and tobacco smoking is the primary risk factor. Cigarette smoking accounts for 80% of lung cancer cases in men and 50% in women worldwide (Thun et al., 2010). Benzo[a]pyrene (B[a]P) is one of the numerous compounds found in tobacco smoke (Sharma et al., 2008) and is also a ubiquitous environmental pollutant; it is a representative polycyclic aromatic hydrocarbon (PAH), metabolically activated by three enzymatic pathways (Jiang et al., 2007). Anti-benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide (anti-BPDE), the most important metabolite of B[a]P, exerts its genotoxic effects by binding covalently to DNA (Rojas et al., 2004) therefore enabling the activation or inactivation of cancer-related genes (Zhao et al., 2010). However, the molecular mechanisms involved in chemical carcinogenesis are complex and poorly understood. The discovery of microRNAs (miRNA), a class of non-coding endogenous RNAs 20–23 nucleotides in length that function as negative regulators of gene expression, has provided new opportunities in the study of the molecular mechanisms of cancer (Guarnieri and DiLeone, 2008). MiRNAs are able to negatively regulate expression of protein-coding genes through mRNA degradation or translational inhibition by binding with the 3′-untranslated regions (3′-UTR) of their mRNA targets (Engels and Hutvagner, 2006; Lim et al., 2005). Molecular alterations that target inactivation of tumor suppressor genes and activation of proto-oncogenes play a key role in the development of multistage carcinogenesis (Spandidos, 2007). Many previous studies have demonstrated that miRNA can influence the development and progression of tumors through the targeting of certain oncogenes or tumor suppressor genes.

We established 16HBE-T, a malignantly transformed cell line of the 16HBE human bronchial epithelial cell line induced by anti-BPDE, in our laboratory. We found that N-Ras expression at both the mRNA and protein levels in 16HBE-T cells were the highest among three Ras members (H-Ras, K-ras, N-Ras) and that this elevated expression plays an important role in chemically induced oncogenesis (Zhou et al., 2008). In our previous study, there were 55 significantly differentially expressed miRNAs in 16HBE-T cells identified by microarray (Shen et al., 2009). In this study, we explored the role of miRNAs in regulation of N-Ras in malignant transformation induced by anti-BPDE.

To further elucidate the potential role of miRNAs in the regulation of N-Ras, we initially utilized bioinformatic forecasts to

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rapidly filter miRNAs that target N-Ras in mammalian cells, and subsequently we employed Western blot, quantitative real-time polymerase chain reaction (qRT-PCR), assessment of cell viability, cell cycle, and apoptosis, as well as the soft agar assay and tumorigenicity to identify the function of the miRNA. We found that miR-506 functions as an anti-oncogene, suppresses cell proliferation, and decreases the degree of malignancy. However, the tumor suppressor role of miR-506 is weakened in 16HBE-T because it is down-regulated after exposure to anti-BPDE, a molecule that promotes the tumor progression. This is a novel report concerning the role of miR-506 in carcinogenesis. The results of this study have increased the available knowledge regarding the anti-oncomir properties of miRNA in anti-BPDE–induced transformed human bronchial epithelial cells.

2. Materials and methods

2.1. Cell culture

16HBE-T cells and the control untransformed cell line 16HBE-N cells, treated with dimethyl sulfoxide, were established in our laboratory and cultured in minimum essential medium (Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and incubated at 37 °C in a humidified chamber with 5% CO_2. Cells were passaged two to three times per week with 0.02% (w/v) ethylenediamine tetraacetic acid (EDTA) and 0.25% (w/v) trypsin.

2.2. Cell transfection

The 16HBE-T cells were seeded in plates the day before transfection to ensure approximately 30% confluence on the day of transfection. The over-expression study was performed with miR-506 specific miRNA mimic (miR-506-mimic), FAM-conjugated miRNA mimic negative control (miR-nc) (50 nM final concentration), Lipofectamine 2000 negative control (mock), and no treatment to monitor the effect of miR-506. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The medium was replaced 6 h after transfection. The transfection efficiency, examined by FAM-conjugated negative control and fluorescence microscopy (Nikon Corporation, Japan), reached 80% in the cells. At 1 or 2 days after transfection, the cells were subjected to further assays and RNA/protein extraction. The RNA oligoribonucleotides were purchased from Genepharma (Shanghai, China). All pyrimidine nucleotides in the miR-506-mimic or miR-nc were substituted with 2′-*O*-methyl analogs to improve RNA stability.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. To detect the expression of mature miR-506, first strand cDNA was generated by TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) using 50 ng total RNA. We performed qRT-PCR for mature miR-506 with the TaqMan MicroRNA assay and the TaqMan Universal PCR Master Mix (Applied Biosystems) using the Applied Biosystems 7500 real-time PCR system. The U6 snRNA was used as an internal control. The miRNA qRT-PCR conditions were as follows: polymerase activation 10 min at 95 °C, 45 cycles at 95 °C for 15 s, and 60 °C for 1 min. In order to detect the expression level of the Ras gene, cDNA was generated using the PrimeScript® RT reagent Kit (TaKaRa, Dalian, China). Next, qRT-PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa) according to the manufacturer's instructions. Reactions were performed on a Roter-Gene 6000 (Corbett Research, Sydney, Australia) with the following conditions: polymerase activation 30 s at 95 °C, 45 cycles at 95 °C for 5 s, and 60 °C for 1 min; β-actin served as an internal control. PCR products were identified by melting curve analysis. The data were calculated using the $2^{-\Delta\Delta C_t}$ method normalized to the individual internal control level. All primers were synthesized by Invitrogen. The primer sets used in Ras mRNA qRT-PCR are as follows:

β-actin-F: 5′-GGATGCAGAAGGAGATCACTG-3′,
β-actin-R: 5′-CGATCCACACGGAGTACTTG-3′;
N-Ras-F: 5′-TTTGCCAACAAGGACAGTTGA-3′,
N-Ras-R: 5′-TGGCGTATTTCTCTTACCAGTGT-3′;
K-Ras-F: 5′-GGACTGGGGAGGGCTTTCT-3′,
K-Ras-R: 5′-GCCTGTTTTGTGTCTACTGTTCT-3′;
H-Ras-F: 5′-GGAAGCAGGTGGTCATGAT-3′,
H-Ras-R: 5′-ATGGCAAACACACACAGGAA-3′.

2.4. Western blot

Total cellular protein extraction and Western blot procedures were conducted as previously described (Zhou et al., 2008). Briefly, one membrane containing both N-Ras protein and β-actin was divided into two pieces according to the molecular mass of prestained protein standards (Beyotime, Shanghai, China). The N-Ras and β-actin proteins were incubated with primary antibody: rabbit anti-human β-actin (Bioworld Technology, St. Louis Park, MN, USA) at a concentration of 1:1500 and rabbit anti-human N-Ras (Origene, Rockville, MD, USA) at a concentration of 1:500, respectively. Signals were detected using secondary antibodies labeled with IRDye 800 (Rockland Immunochemicals, Lincoln, NE, USA) and signal intensity was determined with an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

2.5. Cell viability

For the cell viability assay, 16HBE-T cells were plated in 96-well plates (3×10^3 cells per well) and cultured for 24 h and then transfected with miRNA mimics. After 24 h and 48 h incubation, the absorbance at 450 nm was determined for each well using a Synergy 2 microplate reader (BioTek, Winooski, VT). Each test was performed five times. Cell viability was evaluated with a cell counting kit CCK-8 (Dojindo, Tokyo, Japan). The cell viability (% of control) was expressed as the percentage of $(OD_{test} - OD_{blank})/(OD_{control} - OD_{blank})$, where OD_{test} was the optical density of the transfected cells, $OD_{control}$ was the optical density of 16HBE-T cells, and OD_{blank} was the optical density of the wells without 16HBE-T cells.

2.6. Cell cycle analysis

Upon 24 h of serum-starvation, and 48 h after transfection, cells were harvested and fixed in 70% ice-cold ethanol at 4 °C overnight, washed twice with 0.1% (v/v) Triton X-100 in phosphate buffer solution (PBS), treated with RNAse A (200 mg/l) at 37 °C for 30 min, stained with 20 mg/l propidium iodide (PI), and finally immediately analyzed by flow cytometry with FACScan (Becton Dickinson, Franklin, NJ). Data were gated using the FlowJo FACS analysis software (Tree Star Inc., Ashland, OR).

2.7. Apoptosis assay

Apoptosis was evaluated with the Annexin V-FITC/PI apoptosis kit (KeyGen Biotech, Nanjing, China). For the Annexin V/FITC binding assay, cells were harvested using 0.25% trypsin without EDTA 48 h post-transfection, washed twice with ice-cold PBS, and then resuspended in 500 μl binding buffer. Then cells were stained with 5 μl Annexin V-FITC and 5 μl PI for 15 min in the dark at room temperature, and then analyzed by flow cytometry (Becton Dickinson). Results were calculated as the percentage of apoptotic cells out of the total number of cells counted.

2.8. Soft agar assay

Soft agar plates were prepared in six-well plates with a bottom layer of 0.6% low-melting-point agarose (Sigma) in minimum essential medium containing 10% fetal bovine serum. The cells transfected with miRNA mimics for 24 h and negative control cells were trypsinized, and 1000 cells were suspended in 2 ml of 0.35% (w/v) low-melting-point agarose with 10% fetal bovine serum supplemented minimum essential medium, and then seeded onto the bottom agar layer. Three wells were plated for each group of parental cells. Colonies with at least 50 cells were counted at 2 weeks post-transfection.

2.9. Tumorigenicity study

Four week old Balb/c nude mice were provided by Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). All experimental procedures involving animals were performed in accordance with the University Guide for the Care and Use of Laboratory Animals and were performed in accordance with the institutional ethical guidelines for experiments with animals. At 24 h post-transfection, transfected and untransfected 16HBE-T cells were harvested by trypsinization, washed twice, and suspended in PBS. Four mice per group received 4×10^6 cells in 150 μl of PBS injected subcutaneously into the fold inguen of each mouse. The mice remained in a pathogen-free environment and were monitored every 5 days for tumor formation. The mice were sacrificed 20 days post-injection and the tumor xenografts were excised and weighed. The growth curves were plotted as the mean tumor volume ± SD of the four mice in each group.

2.10. Statistical analysis

All statistical analyses were performed with SPSS 11.5 software. Values are expressed as mean ± SD and all experiments were performed a minimum of three times. Differences between groups were analyzed using the double-sided Student's *t*-test for comparison of two groups, and multiple comparisons were