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Effects of silencing Id-1 in cell culture of human adenoid cystic carcinoma

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SUMMARY

Adenoid cystic carcinoma (ACC) is a slow growing but highly invasive cancer with a high recurrence rate. Id (inhibitor of DNA binding) proteins are dominant regulators of basic helix–loop–helix transcription factors that control malignant cell behavior in many different tissues. This study aimed to identify the potential role of inhibiting DNA binding-1 (Id-1) in human salivary adenoid cystic carcinoma (SACC) progression. First, we compared the Id-1 protein expression in a human salivary adenoid cystic carcinoma cell line (ACCM) against three other cell lines and found that Id-1 protein expression in ACCM to be significantly higher. Then we measured Id-1 mRNA and protein expression in ACCM before and after RNA interference (RNAi), which showed successful inhibition of Id-1. Further studies then demonstrated that the proliferation and invasiveness of ACCM cells were dramatically down-regulated, and increased numbers of apoptotic cells were detected after Id-1 silencing. Consequently, our data suggest that Id-1 is a potential target in the treatment of human salivary adenoid cystic carcinoma.

Introduction

Adenoid cystic carcinoma (ACC), the most common malignancy of the submandibular and minor salivary glands, represents about 20% of all malignant salivary gland tumors[1]. ACC is generally slow-growing but highly malignant neoplasm with a remarkable capacity for invasion and metastasis[2]. Most ACC patients are in their fifth and sixth decades of life, and females are slightly more affected than males[3]. The long-term overall survival of these patients is poor, owing to the occurrence of late recurrences or metastases, even for low-grade tumors[4]. Even with optimal surgical resection and radiotherapy, the majority of patients eventually recur, often with disseminated disease[5].

Id (Inhibitor of DNA binding) proteins belong to the helix–loop–helix (HLH) family. There are four known Id proteins (Id-1, Id-2, Id-3, and Id-4), which share a homologous HLH domain, but differ in their DNA binding regions. They function as global regulators of gene expression during cell growth and differentiation by binding to and antagonizing the activities of several classes of transcriptional regulator, such as bHLH proteins[6–8]. They function depending on their binding partners, they have been found to either inhibit tissue-specific transcription and differentiation or stimulate cell proliferation[9].

Id protein family is also implicated in the regulation of cellular differentiation, cell cycle progression, senescence, and apoptosis[6]. There also exists evidence to suggest that Id proteins may be key regulators of oncogenic transformation and tumor progression in a subset of tumors[10].

Currently, however, among the four Id proteins, Id-1 has been much more extensively researched and better demonstrated to correlate with tumor behavior, though little is known of its role in ACC. In this study, we aimed to elucidate the significance of Id-1 in the progression, invasion and apoptosis of this common malignant salivary gland tumor.

Materials and methods

Cell culture

ACCM cells were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). SAS cells, 8113 cells and ACC2 cells were obtained from Qilu Hospital of Shandong University. They were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Gibco), at 37 °C in 5% CO_2, without penicillin or streptomycin.

RNA interference

ACCM cells were divided into three groups for transfection: lipofectamine only, scrambled siRNA, or Id-1 siRNA. ACCM cells

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Table 1
SiRNA sequences.

Gene	SiRNA sequences
Id-1 (NM_002165)	5′-UGAGUAACAGCCGUUCAUGUCGUAG-3′ 5′-CUACGACAUGAACGGCUGUUACUCA-3′
Scrambled	Unknown because of commercial reason

were transfected with lipofectamine 2000 and opti-MEM (invitrogen). 1×10^5 cells/well were planted in 6-well plates and maintained in culture media with 10% fetal bovine serum for about 24 h. The cells reached approximately 50% confluence, then the media were changed to opti-MEM without serum. 100 pmol Id-1stealth siRNA (nm_002165_stealth_297 siRNA, invitrogen) plus 5 μl lipofectamine 2000 was added to each well. The sequences of Id-1 siRNA and scrambled siRNA (invitrogen) are summarized in Table 1.

RNA isolation and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

According to the manufacturer's instructions, total RNA was isolated by Trizol (Invitrogen, Carlsbad, CA). The reverse transcriptase reaction mixture was used for RT-PCR reaction with a DNA thermal cycler (Eppendorf, Hamburg, Germany). The primers used in the PCR reactions are summarized in Table 2. Amplified PCR products were electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining. The intensities of bands were measured by computerized image analysis (Image J).

Western blotting analysis

Briefly, the cells were washed with phosphate-buffered saline (PBS), lyzed with denaturing SDS–PAGE sample buffer using standard methods. 20 mg proteins were denatured and fractionated by electrophoresis on 15% (w/v) SDS–PAGE and transferred to a PVDF membrane (BD PharMingen).Then the blots were blocked and incubated with the primary antibody (SC-488,rabbit against mouse, Santa Cruz Biotechnology)with 1 μg/ml concentration in 5% nonfat dry milk in TBST (PBS containing 0.05% Tween 20) overnight at 4 °C. After washing, the blots were incubated with the secondary antibody (antirabbit IgG, Sigma, St. Louis, MO) conjugated to horseradish peroxidase at 1:1000 in TBST for 1 h at room temperature. Proteins were visualized with Chemiluminescent Substrate (Pierce) according to the manufacturer's instruction. The intensities of acquired bands were measured by computerized image analysis (IPP 5.0) and normalized to β-Actin as the internal control.

MTT assay

The cells were seeded in flat-bottom 96-well plates at 1×10^4 cells/well. At 24 h, 48 h, 72 h after siRNA transfection, 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) were added to each well. After cells were incubated at 37 °C for another 4 h, the medium was removed, 150 μl dimethyl sulfoxide (DMSO, Sigma–Aldrich Co.) was added, and the cells were agitated for 10 min with protection from light. Absorbance was determined by spectrophotometry (Infinite M200, Grödig, Austria) using a wavelength of 570 nm with 630 nm as a reference.

Boyden chamber invasion assays

Invasion assays were performed in a BioCoat Matrigel invasion chamber (Becton Dickinson, Bedford, MA) with an 8-μm porous membrane bottom for 24-well plates. Membranes were coated with 15 μl Matrigel. Cells (1×10^4) were added to the upper chamber in 100 μl of DMEM. The lower chamber was filled with 600 μl of DMEM and conditioned medium as a chemotactic factor. After a 20-h incubation, non-invading cells were removed from the top of the wells with a cotton swab, and cells that transferred to the inverse surface of the membrane were stained with hematoxylin for 10 min. The cells were counted under a microscope at 200 × magnification.

Annexin V-FITC assay

To assess the degree of apoptosis following treatment with siRNA, the extent of Annexin V-FITC/PI staining was determined by flow cytometry using the Annexin V/PI staining kit from Bender MedSystems (Vienna, Austria). Samples were read on an Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA) and were analyzed by WinMDI 2.8 software.

Statistical analysis

Values are presented as mean ± standard deviation (SD). Results were analyzed with the Student *t* test and a one-way repeated-measures analysis of variance (ANOVA) using SPSS software package (SPSS, USA). In this study, $P < 0.05$ was considered statistically significant.

Results

To compare levels of Id-1 expression of among our four cell lines, we utilized western blot and demonstrated that Id-1 expression is significantly increased in ACCM cells in comparison to the other three cell types (Fig. 1A,B). To determine whether Id-1 transcripts were knocked down through siRNA transfection, RT-PCR and western blotting were performed. The levels of Id-1 mRNA (Fig. 1C,D) and Id-1 protein (Fig. 1E,F) in the group transfected with Id-1 siRNA were significantly decreased in comparison to the two non-transfected groups, demonstrating that the Id-1 siRNA was effective in silencing the Id-1 gene.

To determine whether Id-1 has an effect on ACCM proliferation, we transfected Id-1 siRNA into ACCM cells by lipofectamine 2000 and analyzed its effect using MTT assay. The number of viable cells was determined at 24 h, 48 h, and 72 h. Transfection with Id-1 siRNA significantly decreased the proliferation in ACCM cells at 24 h, 48 h, and 72 h. Transfection with Id-1 siRNA significantly decreased the proliferation in ACCM cells at 24 h, 48 h, and 72 h

Table 2
Prhner sequences and size of PCR products.

Target mRNA	Primers	PCR Protocol	Expected size of PCR products (bp)
Id-1	5′-CATTCTGTTTCAGCCAGTCG-3′ 5′-AGCTCCTTGAGGCGTGAGTA-3′	95 °C for 5 m, 94 °C for 40 s, 56 °C for 40 s, 72 °C for 40 s, and for 36 cycles	245
β-Actin	5′-TCACCCACACTGTGCCCATCTACGA-3′ 5′-CAGCGGAACCGCTCATTGCCAATGG-3′		300