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Original research

The expression and roles of Id1 and Id2 in the aggressive phenotype of human oral squamous cell carcinoma cells☆

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ABSTRACT

Objective: Id (inhibitor of DNA binding or differentiation) proteins are dominant-negative regulators of basic helix-loop-helix transcription factors that control malignant cell behavior in many different tissues. The study aim was to investigate the effects of Id on human oral squamous cell carcinoma (OSCC) cells.
Materials and methods: The expression spectrum of Id1 and Id2 was examined in 6 different OSCC cell lines. Next, antisense vector infection or siRNA-mediated gene silencing was used to knockdown Id1 and Id2 expression in the SAS and HSC-2 cell lines (only Id1) to determine the effects on proliferation and invasion in vitro.
Results: The poorly differentiated SAS cell line and the differentiated HSC-2 cell line abundantly expressed Id1, whereas the other 4 cell lines showed little or undetectable Id1 expression. Id2 was generally expressed at a lower level than Id1 in the SAS cell line but showed little to no expression in the other OSCC cell lines. The knockdown of Id1 significantly repressed cell proliferation and invasion and decreased telomerase activities in the SAS and HSC-2 cell lines, whereas the knockdown of Id2 in the SAS cell lines showed no effect on proliferation and invasion.
Conclusion: We conclude that Id1 plays a more important role than Id2 in the proliferation and invasion of human OSCC cells.

1. Introduction

Many genetic alterations are involved in the proliferation, migration, and metastasis of cancer cells. Thus, clarifying the functions of these genes is essential to understanding the mechanisms of cancer progression and to develop diagnostic and treatment methods for cancer. Recently, strong expression of the Id (inhibitor of DNA binding or differentiation) genes has been identified in cell lines derived from a wide variety of tumors, and a recent report implicates Id proteins in the cancers of many organs [1]. Basic helix-loop-helix (bHLH) transcription factors are key regulators of lineage- and tissue-specific gene expression in a number of mammalian and nonmammalian organisms. bHLH proteins act as obligate dimers, dimerizing through the HLH domains and binding DNA through composite basic domains to regulate the transcription of target genes containing E-boxes (CANNTG) in their promoters. Id proteins are dominant negative regulators of bHLH function and act by dimerizing with the bHLH proteins [2]. Because Id proteins lack basic domains, the Id-bHLH heterodimers fail to bind DNA, thereby inhibiting bHLH function. Constitutive expression of Id proteins has been shown to inhibit the differentiation of various cell types [3,4]. To date, 4 members of the Id gene family have been described (Id1, Id2, Id3, and Id4) [5,6], with each showing different types of expression patterns and functions.

Id proteins, especially Id1, are up-regulated in various human primary tumors, and Id1 expression has been primarily associated with the more aggressive and invasive phenotypes, as well as less differentiated ones. Further, Id1 has been regarded as a marker for the diagnosis and progression of some kinds of cancers, and plays the most crucial role in head and cervical cancer among the 4 subtypes [7–9]. However, little is known about its role in oral squamous cell carcinoma (OSCC), which is one of the common human cancers. The treatment of OSCC has improved over the years, but the survival rate of patients with OSCC remains poor [10]. Therefore,

Abbreviations: Id, inhibitor of DNA binding or differentiation; OSCC, oral squamous cell carcinoma; bHLH, basic helix-loop-helix; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TRAP, telomeric repeat amplification protocol.

☆ Asian AOMS: Asian Association of Oral and Maxillofacial Surgeons; ASOMP: Asian Society of Oral and Maxillofacial Pathology; JSOP: Japanese Society of Oral Pathology; JSOMS: Japanese Society of Oral and Maxillofacial Surgeons; JSOM: Japanese Society of Oral Medicine; JAMI: Japanese Academy of Maxillofacial Implants.

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a new treatment modality for OSCC needs to be introduced. The purpose of this study was to investigate the effects of Id on human OSCC cells.

2. Materials and methods

2.1. Cell culture

Human cell lines derived from squamous cell carcinoma lesions in the head and neck (SAS [11], HSC-2 [12], HSC-3 [12], OSC2 [13], OSC5 [13], and Ca9-22 [14]) were obtained from the American Type Culture Collection (ATCC, Manassas, VA); OSC2 and OSC5 cell lines were a generous gift from Prof. Osaki (Kochi University, Kochi, Japan). SAS cells are poorly differentiated and originated from the gingiva [11]. Meanwhile, HSC-2 cells are well differentiated and were isolated from the oral floor [12], whereas HSC-3 cells are poorly differentiated and possess metastatic potential [12]. OSC2 and OSC5 were isolated from a metastatic cervical lymph node [13]. Ca9-22 cells are well differentiated and represent a low-grade malignancy [14].

The cell lines were cultured in phenol red containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/ml penicillin, and 100 mg/ml streptomycin at 37 °C in the presence of 5% CO_2.

2.2. Western blotting analysis

Cells were lysed in 2× Laemmli buffer [15] and stored at −70 °C. Samples (20–30 μg) were separated by SDS-PAGE and transferred to a PVDF membrane (Hybond-P; Amersham Biosciences, Piscataway, NJ) according to standard methods. The membrane was blocked for 1 h at room temperature with TBST (20 mM Tris base, 137 mM NaCl, 3.8 mM HCl, and 0.1% Tween 20) containing 5% nonfat milk, and blots were probed with anti-Id1, anti-Id2 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-actin antibodies (Chemicon, Temecula, CA) for 1 h. The membrane was washed, incubated with secondary antibody (Santa Cruz Biotechnology), washed again, and developed for enhanced chemiluminescence by using the Amersham ECL-plus kit, according to the supplier's instructions.

2.3. RNA extraction, northern blot analysis, and microarray analysis

The cells were cultured in 10% serum prior to being harvested for RNA extraction. Total cellular RNA was isolated and purified as described by Chomczynski and Sacchi [16]. Fifteen micrograms were electrophoretically separated in formaldehyde-agarose gels and transferred to a nylon membrane (Hybond N; Amersham Biosciences, Piscataway, NJ). The membrane was hybridized with ^{32}P-labeled human Id-1 [17] and Id-2 [18] cDNA probes, washed, and then exposed to XAR-5 film (Kodak Rochester, NY) for autoradiography, as described previously [19]. Ribosomal 28S and 18S RNA were used to control for RNA integrity and quantitation. A panel of microarray data sets (Affymetrix, Santa Clara, CA) was used for the microarray analysis. Samples from the Ca9-22 cell line were used as a control.

2.4. siRNA experiments

siRNA sequences targeting human Id1 and Id2 (GenBank accession number for Id1, NM_002165; for Id2, NM_002166) were designed and synthesized in vitro by BBI (B-Bridge International, Inc., Cupertino, CA). Ten siRNAs with different nucleotide sequences designed to target either Id1 or Id2 were transfected into SAS cells, which show the most abundant expression of these genes, using the Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY). The siRNA sequences are shown in Table 1. The efficiency of the RNAi suppression was determined by western blot analysis. For the experiments, we used the siRNAs (E4 for Id1 and F9 for Id2) revealed to be the most efficient by the western blotting analysis. For each transfection, 10 nmol of Id1 or Id2 siRNA was used, and 1×10^5 cells were added to each well in 2 6-well plates. RNA interference was started 24 h after cell plating, according to the supplier's instructions. In one plate, 3 wells were used for RNA interference for a period of 48 h, while the other 3 wells were nontranfected and were used as a control. In the other plate, the same procedures were followed, except that the observation period was 72 h.

Table 1
The design of siRNAs targeting Id1 and Id2.

Target sequences for Id1		Target sequences for Id2	
E1	AGUCGCCAGUGGCAGCACC	F1	GCCUUCAGUCCCGUGAGGU
E2	UGGUGCGCUGUCUGUCUGA	F2	GUGAGGUCCGUUAGGAAAA
E3	CUACGACAUGAACGGCUGU	F3	ACAGCCUGGGCAUCUCCCG
E4	CUAGAACGGCUGUUACUCA	F4	AACAUGAACGACUGCUACU
E5	UGAGCAAGGUGGAGAUUCU	F5	CCAGCAUCCCCCAGAACAA
E6	GUCAUCGACUACAUCAGGG	F6	AGAACAAGAAGGUGAGCAA
E7	CAUCAGGGACCUUCAGUUG	F7	AAAUCCUGCAGCACGUCAU
E8	AACUCGGAAUCCGAAGUUG	F8	AGAACCAGGCGUCCAGGAC
E9	CCCUCAACGGCGAGAUCAG	F9	UUCCCUUCUGAGUUAAUGU
E10	ACGAUCGCAUCUUGUGUCG	F10	AAUGACAGCAAAGCACUGU

2.5. pBabe-Id retroviral vector and virus production

Full-length human *Id1* and *Id2* cDNAs were excised from CMV-*Id1* and cloned into pBabe-puro. Clones in which the *Id1* and *Id2* cDNAs were inserted in the antisense orientation (pBabe-*Id1* and pBabe-*Id2*) were selected for use. pBabe-*Id1* and pBabe-*Id2* were transfected into the TSA54 packaging cell line (Cell Genesis, Foster City, CA) by using calcium phosphate [20]. Viral titers were determined by reverse-transcriptase activity. Briefly, thawed aliquots of harvested media were incubated with poly(A) (20 ng/ml), oligo dT (10 ng/ml), and [^{3}H]TTP (0.1 mCi/ml) in a reaction buffer (50 mM Tris–HCl, 75 mM KCl, 0.5 mM EDTA, and 5 mM $MgCl_2$) for 30 min at 37 °C. The reaction mixture was spotted on Whatman DE81 paper, which was washed with 2× SSC, and the viral cells were counted in a scintillation counter. One unit of MMLV reverse transcriptase (Life Technologies, Inc. Grand Island, NY) was subjected to the same reaction, and the amount of [^{3}H]TTP thus incorporated was defined as 1 RT unit. The retroviral titer (RT units/ml) was determined by comparing the amount of [^{3}H]TTP incorporated by the virus-containing medium with that incorporated by MMLV reverse transcriptase.

2.6. Retroviral infection

In 100-mm dishes, we mixed approximately 8 RT units of either pBabe-puro or pBabe-*Id1* and pBabe-*Id2* retrovirus with 5 ml of a medium containing 4 mg/ml polybrene and added this to the SAS and HSC-2 cells (only pBabe-*Id1*), which had the most abundant expression of Id1. Cells expressing the retroviral genes were selected in 0.6 mg/ml puromycin, which killed all of the mock-infected cells within 3 days, whereas 80% of the pBabe-puro and 30% of the pBabe-*Id1*- and pBabe-*Id2*-infected cells survived. These puromycin-resistant cells are referred to as SAS-Ctl, SAS-pBabe-Id1AS, SAS-pBabe-Id2AS, HSC-2-Ctl and HSC-2-pBabe-Id1AS. To establish single-cell clones, the population was plated at 1–2 cells/well in 24-well tissue culture plates. Clones that grew in the wells were expanded for further experimentation.

2.7. Cell proliferation assay

The cell proliferation ability was determined by counting the cell number. Cells were digested with trypsin–EDTA at 37 °C for 5 min. Next, 8 ml of conditioned medium containing 10% FBS was added to