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Original research

Inactivation of dermatopontin via histone deacetylation in human oral cancer

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ABSTRACT

Objective: Dermatopontin (DPT), a regulator of tumoral invasiveness and metastasis, is down-regulated in oral squamous cell carcinoma (OSCC); however, the DPT regulatory mechanism is poorly understood. Epigenetic alterations of chromatin such as histone acetylation do not alter the DNA sequence but modify the chromatin architecture and its accessibility, resulting in repressed gene transcription.
Methods: To investigate whether epigenetic or genetic mechanisms control *DPT* expression in OSCC, we assessed the effects of a histone deacetylase (HDAC) inhibitor and mutation on *DPT* expression. Human normal oral keratinocytes and DPT-overexpressed OSCC cells were used for chromatin immunoprecipitation to assess the effects of HDAC inhibitors on *DPT* expression.
Results: DPT mRNA was significantly ($p < 0.05$) up-regulated in OSCC-derived cells after treatment with HDAC inhibitors, trichostatin A and sodium butyrate, whereas there was no mutation in the entire coding region of the *DPT* gene. We found significant ($p < 0.05$) enrichment of histone H3 lysine 9 in the DPT promoter region in OSCC-derived cells.
Conclusions: These results suggested that *DPT* down-regulation in OSCC is associated closely with histone deacetylation and that HDAC inhibitors can reactivate the epigenetically silenced *DPT* gene.

1. Introduction

Tumoral invasion and metastasis are major causes of deaths from cancer, including oral squamous cell carcinoma (OSCC) [1]. Molecular changes in a number of oncogenes and tumor suppressor genes associated with progression of OSCC can be important clues to preventing tumoral invasion and metastasis [2,3].

Dermatopontin (DPT), a noncollagenous extracellular matrix (ECM) protein, initially is co-purified as a 22-kDa protein from bovine dermal extracts during the course of decorin purification [1]. To date, the functional characterization of DPT has not been clarified fully in cancer research. Recently, DPT was reported to be a multifunctional adhesion molecule in epidermal cells [4]. Our previous study also found that DPT plays an important role in regulating tumoral invasiveness and metastasis in OSCCs and might be not only a novel diagnostic marker of regional lymph node metastasis but also an emerging potential target for therapeutic intervention in the treatment of OSCCs [5].

Epigenetic mechanisms have emerged recently as major determinants of gene expression and were implicated in the regulation of complex differentiation and developmental processes [6]. Among them, histone modification, particularly histone acetylation, is a key factor in epigenetic regulation of cancer-related genes [7]. Histone deacetylation is related to a repressed chromatin state and tightly controlled by two enzymes, histone acetyltransferase and histone deacetylase (HDAC) [8]. Numerous studies have reported that HDAC inhibitors are therapeutic for hematologic malignancies and solid tumors [9].

In the current study, we showed that treatment with the HDAC inhibitors restored *DPT* mRNA expression in the OSCC-derived cells, suggesting that histone acetylation is associated with the epigenetic regulation of *DPT* in OSCCs.

☆ AsianAOMS: 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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2. Methods

2.1. Mutational analysis

To screen the sequence variations of the *DPT* gene fragments encompassing each of the four coding exons of DPT, the corresponding splice junctions were amplified (Table 1). Direct DNA sequencing was performed as described previously [6]. Sequence homogeneity was confirmed by comparison with all available sequences in the GenBank database using BLAST [Basic Local Alignment Search Tool] (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2. Transfection of DPT plasmid

The RIKEN BRC provided OSCC-derived cell lines, Sa3 and Ca9-22, through the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology (Ibaraki, Japan). The identity of these cell lines was confirmed by short tandem repeat profiling. All cells were grown in Dulbecco's Modified Eagle Medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) and 50 units/ml penicillin and streptomycin (Sigma). OSCC-derived cells were transfected with DPT plasmid designed to overexpress DPT cDNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously reported [5]. After transfection, the cells that stably overexpressed DPT were isolated by neomycin (Invitrogen, Carlsbad, CA). Two to 3 weeks after transfection, viable colonies were transferred to new dishes.

2.3. Evaluation of DPT mRNA expression

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was generated from 5 μg of total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK) and oligo(dT) primer (Hokkaido System Science, Hokkaido, Japan) according to the manufacturer's instructions. Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to evaluate *DPT* mRNA expression using a Light Cycler II 480 (Roche Diagnostics, Penzberg, Germany). The primer sequences for *DPT* were forward 5′-CGAGGAGCAACAACCACTTT-3′ and reverse 5′-CGGCACATTATGAACTTCCA-3′. The transcript amounts for the target genes were estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (forward 5′-GCTCTCTGCTCCTCCTGTTC-3′ and reverse 5′-ACGACCAAATCCGTTGACTC-3′). qRT-PCR was repeated three times using cDNA prepared from three independent experiments.

2.4. Western blot analysis

The cellular pellets from cultured cells were incubated on ice in a lysis buffer with a proteinase inhibitor cocktail (Roche Diagnostics). The protein extracts were electrophoresed on 4–12% Bis-Tris gel and transferred to nitrocellulose membranes (Invitrogen). The membrane was incubated with antibodies against DPT (Proteintech Group, Chicago, IL, USA) for 4 h at room temperature and β-actin for 1 h at room temperature. The Western blot data were visualized by exposing the membrane to a cooled CCD camera system, Light-Capture II (ATTO, Tokyo, Japan). Signal intensities were quantitated using the CS Analyzer version 3.0 software (ATTO).

2.5. Treatment with HDAC inhibitors and chromatin immunoprecipitation

OSCC-derived cells were treated with trichostatin A (TSA) (Wako, Osaka, Japan) or sodium butyrate (NaB) (Wako). Briefly, the cells were plated at 50% confluence and treated with TSA (0.5 or 1.0 mM) or NaB (2.5 or 5.0 mM). After incubation for 24 h, the total RNA was isolated for qRT-PCR.

The chromatin immunoprecipitation (ChIP) assay for histone acetylation was performed using an EpiScope ChIP Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. One percent formaldehyde was used to cross-link the proteins of OSCC-derived cells to the DNA for 5 min at room temperature. The cells were lysed in 1 ml cytoplasmic lysis buffer (Takara Bio) sheared with a Biomic 7040 Ultrasonic Processor (Seiko, Tokyo, Japan) to obtain 200–1000-basepair DNA fragments. For cross-linked ChIP, 200 μl of magnosphere anti-mouse IgG (Takara Bio) was washed with RIPA buffer-1 (Takara Bio), incubated with 4 μg of anti-acetyl histone H3 lysine 9 (H3K9ac), mouse monoclonal antibody (MAB Institute, Hokkaido, Japan), or 4 μg of control antibody (normal goat IgG, Santa Cruz). Each sample was analyzed by qRT-PCR, and the results were expressed as percentages of the input DNA. The primer sequences designed for DPT promoter regions were forward: 5′-CTCAGCCGGACTGTAGAAGG-3′, and reverse: 5′-AGGTGAGCCTGGTCTCTGAA-3′.

2.6. Statistical analysis

The statistical significance of the DPT expression levels was evaluated using the Fisher's exact test or Mann-Whitney *U* test. $P < 0.05$ was considered significant. The data are expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Mutational analysis

We screened DNA samples obtained from human normal oral keratinocytes (HNOKs) and Sa3 and Ca9-22 cell lines. Direct sequencing did not detect mutations in the entire coding region of the *DPT* gene.

3.2. Effects of HDAC inhibitors on DPT expression

OSCC-derived cells (Sa3 and Ca9-22) were treated with an HDAC inhibitor, TSA or NaB, to assess the effects of histone acetylation on DPT expression. In the Sa3 and Ca9-22 cell lines, *DPT* was significantly ($p < 0.05$) up-regulated after treatment with TSA or NaB for 24 h compared with untreated cells (Fig. 1).

3.3. Establishment of DPT-overexpressed cells

OSCC-derived cells (Sa3 and Ca9-22) transfected with a human DPT stable expression vector were cloned. To assess *DPT* mRNA and protein expressions in DPT-overexpressed cells, we performed qRT-PCR and Western blot analyses. The *DPT* mRNA expression in DPT-overexpressed cells was significantly ($p < 0.05$) (Fig. 2A) higher than that in mock-transfected cells. The DPT protein levels also increased significantly ($p < 0.05$) in the DPT-overexpressed cells (Fig. 2B).

3.4. Decreased H3K9ac in OSCC-derived cells

To examine the relative contribution of histone deacetylation to DPT silencing, we measured the acetylation status of H3K9 on the effect of regained DPT expression in OSCC-derived cells. We conducted ChIP assays in primary cultures of HNOKs and OSCC-derived cells (Sa3 and Ca9-22). Overexpressed cells showed a higher level of H3K9ac. The cells treated with TSA or NaB increased acetylation levels similar to DPT-overexpressed cells (Fig. 3).

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