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Original

Expression of activation-induced cytidine deaminase in oral epithelial dysplasia and oral squamous cell carcinoma

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Abstract: Oral epithelial dysplasia is thought to be a precursor state of carcinogenesis and may harbor gene alterations. Recently, it was reported that gene editing enzyme, activation-induced cytidine deaminase (AID), is expressed in precursor and cancer epithelial cells during carcinogenesis associated with chronic inflammation/infection and that this enzyme induces mutation of tumor-suppressor genes. Thus, AID may have a role in carcinogenesis via oral epithelial dysplasia. In this study, we classified oral mucosal epithelium exhibiting epithelial dysplasia as squamous intraepithelial neoplasia (SIN) grades 1-3, according to the 2005 World Health Organization classification, and used immunohistochemical techniques to examine AID expression in oral mucosal epithelium exhibiting SIN and oral cancer tissues. AID was observed in prickle cells in oral mucosal epithelium with epithelial dysplasia and in oral cancer cells. Additionally, to investigate the mechanism of AID expression and its role in cancer progression, we incubated the oral cancer cell line HSC-2 with inflammatory cytokines. In the HSC-2 cell line, AID expression was enhanced by TNF-α via NF-κB activation and promoted expression of N-cadherin by regulating Snail expression. These findings suggest that AID has a role in the development of oral epithelial dysplasia and promotes progression of oral cancer.
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Keywords: activation-induced cytidine deaminase (AID); oral squamous cell carcinoma (OSCC); dysplasia-carcinoma sequence; epithelial-mesenchymal transition (EMT); cancer progression.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most frequently occurring cancers. It is an invasive epithelial neoplasm of the oral cavity and exhibits various degrees of squamous differentiation and a propensity for early and extensive lymph node metastasis (1).

It has been proposed that OSCC carcinogenesis is triggered by chronic inflammation or epithelial dysplasia (1,2), and that cancer development and progression occur due to accumulation of gene alterations (3,4). Kusama et al. suggested that *p53* mutation is involved in the early stages of the dysplasia-carcinoma sequence in oral squamous epithelium (5); however, the mechanism of cancer development and progression from chronic inflammation and/or precursor lesions is not known.

During cancer invasion, an epithelial-mesenchymal transition (EMT) occurs, i.e., epithelial cells assume the features of mesenchymal cells. Cadherin is a representative molecular marker of this phenomenon (6). E-cadherin is the major cadherin expressed in epithelial cells of normal tissues, and reduced E-cadherin expression and increased expression of the mesenchyme-associated molecule N-cadherin have been associated with invasiveness in a variety of epithelial neoplasms (6).

Recently, gene editing enzyme—activation-induced

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Table1 Details of tissue samples and results of immunohistochemical staining

	Oral epithelial mucosa			OSCC		Total
	SIN 1	SIN 2	SIN 3	Well	Moderate	
Mean age	56.7	63.4	69.9	71.9	67.7	66.2
Sex (male:female)	11:10	20:15	13:10	12:30	6:05	62:70
Tongue	5	15	16	15	1	52
Gingiva	12	12	4	17	6	51
Buccal mucosa	3	5	2	4	4	18
Others	1	3	1	6	0	11
Total	21	35	23	42	11	132

OSCC, oral squamous cell carcinoma; SIN, squamous intraepithelial neoplasia

cytidine deaminase (AID)—was originally reported (7). AID is expressed only in activated B-cells under normal conditions (7) and preferentially deaminates cytosine in WRC motifs (W: A or T; R: A or G) to generate uracil, thereby changing a DNA C:G pair to a U:G mismatch (8). This mismatch induces somatic hypermutation and class-switch recombination in immunoglobulin genes, thereby producing high-affinity antibodies against specific antigens (7-9). It has also been reported that AID is expressed in cancer cells based on inflammatory conditions (3,10-15), and that it induces *p53* mutation in gastric epithelial cells (16). These findings suggest that AID is associated with development of precancerous lesions and progression of malignant tumors.

We previously studied the relation of AID expression with epidermal growth factor, sodium butyrate (NaB), and inflammatory cytokines such as interleukin-1β, interferon-γ, tumor necrosis factor (TNF)-α in the OSCC-derived cell lines Ca9-22, HSC-2, HSC-3, and HSC-4 and found that epidermal growth factor and TNF-α promote expression of AID mRNA in HSC-2 cells (17). This suggests that AID contributes to OSCC progression. In the present study we investigated the association of AID expression with development of oral epithelial dysplasia and EMT during progression of human OSCC.

Materials and Methods

Tissue preparation

Specimens were collected from the archives of the Department of Diagnostic and Therapeutic Sciences, Division of Pathology, Meikai University during the period 2006-2011. The samples comprised 79 cases of epithelial dysplasia (including carcinoma in situ) and 53 cases of OSCC (Table 1). Data on patient age, sex, and lesion site were obtained from information that had been recorded on surgery-request forms. The paraffin-embedded tissue blocks were sliced into sections (thickness, 5 μm) for subsequent histologic examination. The tissue sections were stained with hematoxylin-eosin for histologic diagnosis according to the World Health Organization (WHO) histologic classification. In this study, cases of epithelial dysplasia were classified as squamous intraepithelial neoplasia (SIN) 1 (mild dysplasia), SIN 2 (moderate dysplasia), and SIN 3 (severe dysplasia and carcinoma in situ), and cases of OSCC were classified according to the degree of differentiation (well, moderately, poorly).

Table 2 List of antibodies used in present study

Name	Source	Dilution	Company
AID	rabbit	1/100	AbD Serotec
E-cadherin	rabbit	1/200	Santa Cruz
N-cadherin	mouse	1/100	Santa Cruz
SNAIL	goat	1/100	Santa Cruz
NF-κB	rabbit	1/100	Santa Cruz
GAPDH	rabbit	1/400	Santa Cruz

AID, activation-induced cytidine deaminase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

The study protocol was reviewed and approved by the Research Ethics Committee of Meikai University Graduate School of Dentistry (A0832).

Immunohistochemistry

The serial sections were deparaffinized and immersed in methanol containing 0.3% (v/v) hydrogen peroxide for 15 min at room temperature, to block endogenous peroxidase activity. After washing with running water and phosphate-buffered saline (PBS, pH 7.4), the sections were heated in a microwave oven while immersed in 0.01 M citrate buffer (pH 6.0) for 15 min at low power (for antigen retrieval). Appropriately diluted polyclonal antibodies (Table 2) were applied to each section overnight at room temperature. The sections were then incubated with peroxidase-labeled dextran polymer (Simple Stain MAX-PO; Nichirei Bio, Tokyo, Japan) for 60min, and the reaction products were visualized by immersing the

Table 3 Numbers[a] of tissue samples with positive reactions for anti-AID antibody

	Oral epithelial mucosa			OSCC		Total
	SIN 1	SIN 2	SIN 3	Well	Moderate	
Tongue	4/5	6/15	0/16	3/15	0/1	13/52
Gingiva	6/12	2/12	0/4	8/17	5/6	21/51
Buccal mucosa	0/3	0/5	0/2	2/4	1/4	3/18
Others	1/1	0/3	0/1	2/6	0/0	3/11
Total	11/21	8/35	0/23	15/42	6/11	40/132

OSCC, oral squamous cell carcinoma; SIN, squamous intraepithelial neoplasia
[a] number of positive specimens/total number of specimens

sections in a freshly prepared 2-mM diaminobenzidine (DAB) solution containing 0.03% hydrogen peroxide. Nuclei were lightly stained with Mayer's hematoxylin. A case was defined as positive when at least one cell showed a positive reaction.

Cells

The human tongue squamous cell carcinoma-derived cell line HSC-2 was purchased from RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. The cells were cultured routinely in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 U/mL penicillin-streptomycin (GIBCO Invitrogen, Carlsbad, CA, USA), 10 U/mL Fungizone (GIBCO Invitrogen), and 10% fetal bovine serum (GIBCO Invitrogen) in a humidified atmosphere of 5% CO_2 at 37˚C.

Real-time reverse-transcription polymerase chain reaction (real-time RT-PCR)

The cells were incubated overnight with 100 ng/mL TNF-α with or without siRNA. After cell culture, total RNAs were extracted in accordance with the instructions supplied with the PAREx kit (Takara Bio, Shiga, Japan) and adjusted to 0.1 μg/mL. As in our previous study, real-time RT-PCR was performed using a Thermal Cycler Dice Real Time System (Takara Bio) with specific primers for AID (forward: 5'-tcttgatgaaccggaggaag; reverse: 5'-agccgttcttattgcgaaga) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5'-gcaccgtcaaggctgagaac; reverse: 5'-tggtgaagacgccagtgga).

RT-PCR

RT-PCR was performed in accordance with the instructions supplied with the OneStep RT-PCR Kit (Qiagen, Hamburg, Germany). Each PCR mixture (final reaction volume, 25 μL) contained 12.4 μL of sterile water, 5 μL of 5x QIAGEN OneStep RT-PCR Buffer, 1.0 μL of dNTP Mix (containing 10 mM dNTP), 1 μL of QIAGEN OneStep RT-PCR Enzyme Mix, 0.3 μL of forward primer (100 pmol/μL), 0.3 μL of reverse primer (100 pmol/μL), and 5 μL (0.1 μg/mL) of total RNA. PCR conditions were 50˚C for 30 min and 95˚C for 15 min, followed by 35 cycles of 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 1 min. After all cycles, final extension was carried out at 72˚C for 10 min. Amplification of PCR products was identified by 3% agarose gel electrophoresis.

Western blotting

Cellular proteins were extracted using lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1% NP-40). To estimate NF-κB activation, the cells were incubated for 24 h with 10 μg/mL NF-κB SN50, a cell-permeable inhibitor peptide (Calbiochem, San Diego, CA, USA). Then, proteins from cytoplasmic and nuclear fractions were isolated by using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Aliquots were subjected to 10% sodium dodecyl sulfate-polyacrylamide-gel electrophoresis and electroblotted onto pure nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Specific proteins on the membranes were detected by incubation with a specific primary antibody (Table 3) overnight at 4˚C, followed by a species-specific secondary antibody conjugated with peroxidase and DAB/H_2O_2 solution.

Results

Clinicopathologic findings

The sex ratio (male: female) of the patients was 62:70, age range was 31-94 years, and mean age was 66.2 years. The most common lesion site was the tongue (52 cases), followed by the gingiva (51 cases), buccal mucosa (18 cases), and others (11 cases: hard palate 8, mouth floor 2, lip 1). The lesions investigated in this study included 21 SIN 1 cases, 35 SIN 2 cases, 23 SIN 3 cases, and 53 OSCC cases. Among the OSCC cases, 42 were well differentiated and the remaining 11 were moderately differentiated SCCs. No cases of poorly differentiated

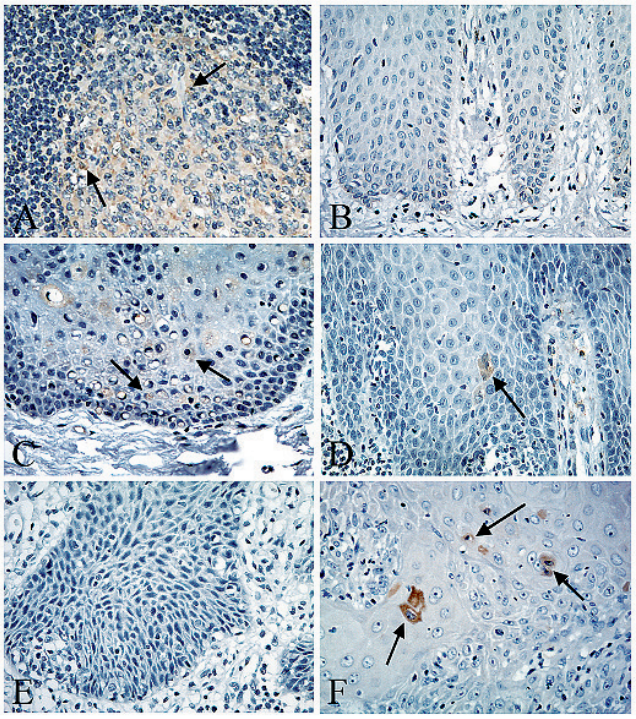

Fig. 1 Immunohistochemical staining of AID.
Positive reactivity for AID is observed in the germinal center (arrows in A), prickle cells in SIN 1 (arrows in C) and SIN 2 (arrow in D) specimens, and a cancer cell (arrows in F). There is no reactivity in morphologically normal epithelium (B) or SIN 3 tissue (E). Original magnification: ×200 (A-F).

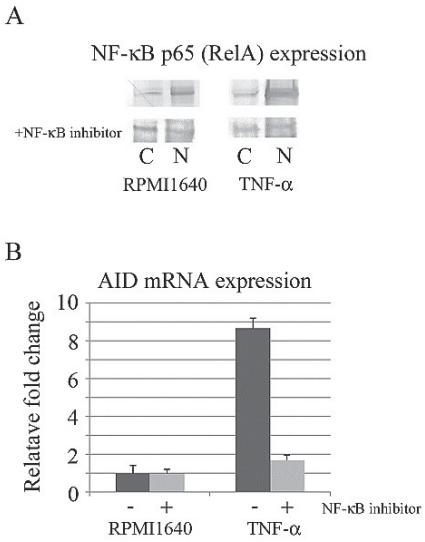

Fig. 2 NF-κB activation by inflammatory cytokines.
After incubation of HSC-2 cells with TNF-α, proteins were isolated from cytoplasmic and nuclear fractions, as described in Materials and Methods. Western blotting showed that TNF-α induced translocation of most NF-κB p65 (Rel A) into the nucleus (A) and that NF-κB inhibitor (NF-κB SN50) blocked the translocation of NF-κB p65 (Rel A) (A). Real-time RT-PCR showed that NF-κB inhibitor suppressed expression of AID mRNA stimulated by TNF-α (B). Each column and bar represents the mean ± SD of duplicate cultures.
C: protein isolated from cytoplasmic fraction; N: protein isolated from nuclear fraction.

SCC were included in this study (Table 1).

Immunohistochemical findings

Five normal cervical lymph nodes obtained by neck dissection were stained with anti-AID antibody as a positive control. Positive reactivity for AID was observed in activated germinal center B cells (Fig. 1A). In addition, AID positivity was observed in many prickle cells in 11 SIN 1 cases (Fig. 1C). In tissues from SIN 2 cases, AID positivity was observed in prickle cells in eight cases (Fig. 1D); however, fewer than five positive cells were seen in all these cases. In contrast, AID positivity was not observed in normal oral mucosal epithelium (Fig. 1B) or in SIN 3 tissues (Fig. 1E). The incidence of AID positivity was almost the same in tongue and gingiva (Table 1B).

In OSCC, AID positivity was observed in 21 cases (19 cases of well differentiated OSCC and two cases of moderately differentiated OSCC; Fig. 1F, Table 3) and was more evident in gingiva than in tongue (Table 3).

AID involvement in epithelial-mesenchymal transition

In HSC-2 cells, NF-κB translocation into the nucleus was enhanced by TNF-α (Fig. 2A), and enhancement of AID mRNA expression by TNF-α was inhibited by the NF-κB SN50, cell-permeable inhibitor peptide (Fig. 2B).

To examine the role of AID in cancer progression, HSC-2 cells were incubated with TNF-α and/or AID siRNA (siAID). Western blotting revealed that expressions of N-cadherin and Snail, a regulator of N-cadherin expression, were increased by TNF-α and that this enhancement was suppressed by siAID. There was no significant effect on E-cadherin expression (Fig. 3A). RT-PCR using specific primers for AID revealed that siAID suppressed expression of AID mRNA (Fig. 3B). In addition, we incubated HSC-2 cells with siRNA for Snail (siSnail) to confirm the relation between Snail and AID. Although expression of N-cadherin was inhibited by siSnail (Fig. 4A), AID mRNA expression was not suppressed (Fig. 4B). We confirmed that control siRNA (Santa Cruz) had no effect on the expressions of these molecules (data not shown).

Discussion

AID is gene editing enzyme that can alter gene sequences. It is expressed only in activated B-cells in normal lymph

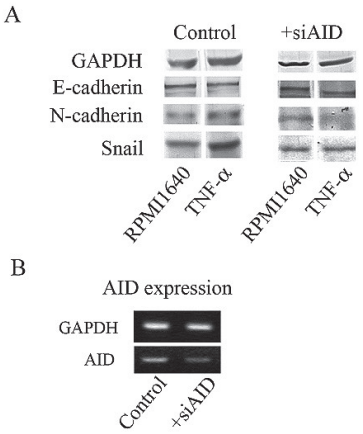

Fig. 3 Effect of AID siRNA on cadherin and Snail expression.
After incubation of HSC-2 cells with TNF-α and/or AID siRNA (siAID), cadherin expression was examined by Western blotting. TNF-α and siRNA had no effect on E-cadherin expression (A). Expression of N-cadherin and Snail was enhanced by TNF-α, and this enhancement was inhibited by siAID (A). RT-PCR revealed that expression of AID mRNA was suppressed by siAID (B).

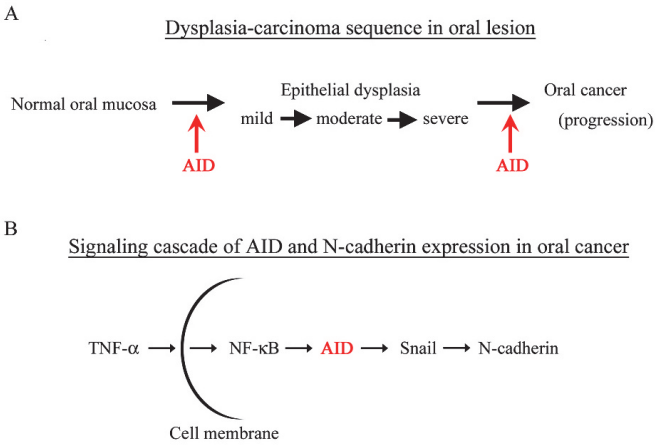

Fig. 5 Association of AID with dysplasia-carcinoma sequence and N-cadherin expression.
By inducing gene mutation, AID may have a role in initiating the dysplasia-carcinoma sequence and/or oral cancer progression (A). TNF-α induced by cancerous inflammation enhanced N-cadherin expression by inducing AID expression in OSCC (B).

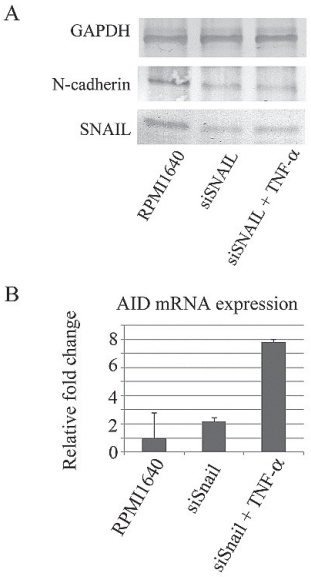

Fig. 4 Effect of siRNA for Snail on N-cadherin, Snail, and AID expression.
After incubation of HSC-2 cells with TNF-α and/or Snail siRNA (siSnail), expressions of N-cadherin and Snail were examined by Western blotting, and expression of AID mRNA was examined by real-time RT-PCR. siSnail inhibited expression of N-cadherin stimulated by TNF-α (A) but had no significant effect on AID expression (B). Each column and bar represents the mean ± SD of triplicate cultures.

nodes (7) and is related to somatic hypermutation and class-switch recombination (7-9). AID transgenic mice have an increased risk of developing malignant lymphoma, hepatocellular carcinoma, gastric cancer, and lung cancer (18,19). In addition, AID induced alteration of *p53* in a human gastric cancer-derived cell line (16). These previous reports suggest that carcinogenesis occurs through accumulation of gene mutations caused by AID.

Oral epithelial dysplasia is thought to be a precursor state, referred to as altered epithelium, that increases the likelihood of progression to SCC and may harbor gene alterations. We classified oral mucosal epithelium exhibiting epithelial dysplasia as SIN grades 1-3, according to the 2005 WHO classification (1), and used immunohistochemical techniques to examine AID expression in SIN and oral cancer tissues. We noted AID positivity in prickle and basal cells in SIN 1 and 2 specimens. AID was re-expressed in OSCC tissue, although no expression was evident in SIN 3 tissue. These observations suggest that AID may trigger development of oral epithelial dysplasia as part of a dysplasia-carcinoma sequence (Fig. 5A), that only monoclonal proliferation of gene-mutated cells occurs in SIN 3 tissue (where gene alteration may not have occurred), and that AID contributes to cancer progression by inducing N-cadherin expression as part of EMT (Fig. 5A, B).

Examples of carcinogenesis resulting from chronic inflammation and/or infection include gastric cancer due to chronic atrophic gastritis caused by *Helicobacter pylori* infection, hepatocellular carcinoma developing from hepatic cirrhosis/chronic hepatitis due to hepatitis B or C virus infection, and colon cancer developing from ulcerative colitis (20-23). In addition, AID is expressed in epithelial cells of inflammatory lesions (10-15). However, in the present SIN 1 and 2 tissues, immunohistochemical analysis revealed that AID tended to be expressed in oral

epithelial dysplasia with less inflammation. Thus, several processes may be involved in the development of oral epithelial dysplasia.

In this study, gingiva exhibited the highest degree of AID positivity, and NaB slightly increased AID expression in HSC-2 cells in culture. NaB/butyric acid (BA) is a short-chain fatty acid produced by *Porphyromonas gingivalis* and was reported to function as a histone deacetylase inhibitor and epithelial cell migration-inducing factor (24,25). These findings suggest that chronic periodontitis induces AID expression, leading to exacerbation of oral lesions, including OSCC. Not all oral lesions exhibit AID expression; thus, those that do may have a higher potential for malignancy than those that lack such expression.

In 1995, Hay proposed that EMT is a mechanism underlying cancer progression. In EMT, epithelial cells assume mesenchymal features and invade the mesenchyme (6). Representative markers of EMT include decreased E-cadherin expression and promotion of N-cadherin expression, a change known as the "cadherin switch" (26). It has been suggested that several factors, such as β-catenin, NF-κB, Snail, and TGF-α, induce EMT (27-32) and that EMT is related to embryonic development and/or cancer progression (6). However, little is known of the molecular mechanisms involved.

We found that AID promotes Snail and N-cadherin expression in an HSC-2 cell line, while E-cadherin expression was not affected. This suggests that AID is upstream of Snail and N-cadherin and has a role in EMT by regulating N-cadherin expression (Fig. 5B). However, E-cadherin expression was not affected. Thus, there may be multiple pathways regulating EMT, and degradation of E-cadherin expression may be independent of AID expression/activation.

AID might induce carcinogenesis and cancer progression by transforming cytosine to uracil in WRC motifs (W = A or T; R = A or G) (8), as many WRC sequences are believed to be present in the genome. This gene editing enzyme may be involved in altering numerous genes, through processes such as alternative splicing. Further study will need to clarify the role of AID and other gene editing enzymes, such as apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) (33,34) and adenosine deaminase acting on RNA (ADAR) (35-37), in the development of tissue disorders and cancer progression in the oral cavity.

In conclusion, our findings suggest that AID induces pathogenesis and development of oral epithelial dysplasia and promotes progression of oral carcinoma via regulation of N-cadherin expression.

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