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Original contribution

Aberrant expression of the tight junction molecules claudin-1 and zonula occludens-1 mediates cell growth and invasion in oral squamous cell carcinoma☆

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Summary We reported that altered cell contact mediated by E-cadherin is an initial event in the pathogenesis of oral epithelial malignancies. To assess other effects of cell adhesion, we examined the expression levels of tight junction (TJ) molecules in oral carcinoma in situ (CIS) and squamous cell carcinoma (SCC). To identify changes in the expression of TJ molecules, we conducted an analysis of the immunohistochemical profiles of claudin-1 (CLDN-1) and zonula occludens-1 (ZO-1) in surgical specimens acquired from patients with oral SCC containing foci of epithelial dysplasia or from patients with CIS. We used immunofluorescence, Western blotting, reverse-transcription polymerase chain reaction, and RNA interference to evaluate the functions of CLDN-1 and ZO-1 in cultured oral SCC cells. TJ molecules were not detected in normal oral epithelial tissues but were expressed in SCC/CIS cells. ZO-1 was localized within the nucleus of proliferating cells. When CLDN-1 expression was inhibited by transfecting cells with specific small interference RNAs, SCC cells dissociated, and their ability to proliferate and invade Matrigel was inhibited. In contrast, although RNA interference–mediated inhibition of ZO-1 expression did not affect cell morphology, it inhibited cell proliferation and invasiveness. Our findings indicated that the detection of TJ molecules in the oral epithelia may serve as a marker for the malignant phenotype of cells in which CLDN-1 regulates proliferation and invasion.

1. Introduction

Dysregulation of cell adhesion, including cell-cell and cell–extracellular matrix contacts, is a characteristic feature of

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multistep carcinogenesis and is associated with tumor progression [1]. Loss of cell-cell contact promotes the dissociation of cancer cells from their sites of origin, leading to invasion of adjacent tissues and metastasis [2]. Moreover, altered cell adhesion modulates gene activity via cell-cell junction-to-nuclear pathways such as β-catenin signaling [3]. We reported that loss of E-cadherin expression and nuclear translocation of β-catenin is associated with cell proliferation in oral epithelial dysplasia and carcinoma in situ (CIS) [4]. Recently, tight junction (TJ) proteins have attracted attention as important modulators of cancer cell functions [5,6].

TJ, which serves as a cell adhesion device for glandular epithelial cells or vascular endothelial cells that form luminal spaces, maintains cell polarity (fence function) and regulates fluid flow (barrier function) [7]. Further, TJs are present in stratified squamous epithelia such as the epidermis [8]. TJs contribute to the activities of signaling pathways that mediate cell proliferation and differentiation under pathophysiological conditions such as mammary gland development and breast cancer [6,9].

The primary constituents of TJs are members of the claudin (CLDN) family, which comprises at least 27 members [6]. For example, the function of CLDN-1 is the subject of intensive investigations. In epidermal keratinocytes, CLDN-1 deficiency causes abnormal keratinization, eventually leading to functional failures [10]. However, whether CLDN-1 functions in the oral epithelium that harbors keratinocytes is not completely understood [11]. Other components of TJs include zonula occludens (ZO) proteins that belong to the membrane-associated guanylate kinase family [12]. ZO-1 binds directly to the C-terminal domain of CLDNs [12] and serves as a linker between CLDNs, the cytoskeleton, and cell signaling pathways [13]. ZO-1 is a member of the family of proteins that localize to adhesion sites and the nucleus in many cell types such as the epithelial cells of kidney or small intestine origin [14]. However, the expression and functions of ZO-1 in the oral epithelium have not been investigated.

Therefore, in the present study, we determined the expression profiles of the representative TJ molecules CLDN-1 and ZO-1 to determine the roles of TJ molecules in the progression of oral squamous cell carcinoma (SCC).

2. Materials and methods

2.1. Tissue samples

Surgical specimens resected from 72 patients with oral CIS/SCC were routinely fixed in 10% formalin and embedded in paraffin. After review by 2 pathologists, we selected specimens that simultaneously contained areas of normal epithelia, foci of epithelial dysplasia, CIS, and/or SCC. The number of patients and total number of foci by the anatomical sites are shown in Supplementary Tables 1 and 2. Serial sections (4-μm thick) were prepared for hematoxylin and eosin staining and immunohistochemistry. The Ethical Board of the Niigata University Graduate School of Medical and Dental Sciences (Oral Life Science) reviewed and approved the experimental protocol for analyzing surgical materials.

2.2. Antibodies

The antibodies used were as follows: mouse monoclonal antibody against human CLDN-1 (clone XX7, IgG2a, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), mouse monoclonal antibody against human ZO-1 (clone ZO1-1A12, IgG1, Thermo Fisher Scientific, Inc, Waltham, MA), mouse monoclonal antibody against β-actin (clone mAbcam 8226, IgG1, Abcam plc, Cambridge, United Kingdom), mouse monoclonal antibody against keratin (K) 17 (clone E3, IgG2b, Dako, Glostrup, Denmark), and mouse monoclonal antibody against Ki-67 (clone MIB-1, IgG1, Dako).

2.3. Immunohistochemistry

Immunohistochemical analysis of paraffin sections was performed using the Chem-Mate Envision System (Dako). To analyze CLDN-1 and ZO-1 expression, sections were autoclaved in 10 mM Tris buffer (pH 9.0) containing 1 mM EDTA at 121°C for 10 minutes. Primary antibodies were used at the following dilutions: 1:100, K17, Ki-67, and CLDN-1; and 1:50, ZO-1. In the controls, the primary antibodies were replaced with the appropriate preimmune mouse IgG subclasses (Dako).

2.4. Analysis of the expression of TJ molecules

We used representative sections of each case with confirmed foci to determine CLDN-1 and ZO-1 expression levels according to the stained area in the cell border, cytoplasm, or both, and separately in the nuclei. Foci occupying ≥10% of stained area were considered positive.

2.5. Cells

ZK-1, ZK-2, and MK-1 cells of oral SCC origin were cultured as monolayers in Dulbecco's minimal essential medium (Gibco, Thermo Fisher Scientific Inc) containing 10% fetal calf serum (Gibco), 50 IU/mL penicillin, and 50 μg/mL streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air [15]. When the cells reached 50% or 80% confluence, they were subjected to analysis using reverse-transcription polymerase chain reaction (RT-PCR), Western blotting, cell fractionation, RNA interference (RNAi), immunofluorescence, and the functional assays described in Sections 2.11 to 2.14.

2.6. RNA isolation and RT-PCR

ZK-1, ZK-2, MK-1, and transfected cells cultured in 60-mm dishes were lysed with ISOGEN (Nippon Gene, Tokyo, Japan)