A Histopathological and Immunohistochemical Study of Cell–Cell Interactions: With Special Reference to Advance of Dysplastic Grading

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Abstract
A histopathological and immunohistochemical study was conducted to elucidate the changes in cell-cell interactions and important related factors in oral precursor lesions and oral squamous cell carcinoma (OSCC). Gingivae from 36 cases (5 healthy epithelium, 7 hyperkeratosis, 7 mild dysplasia, 7 moderate dysplasia, 5 severe dysplasia, and 5 OSCC) were examined. From immunohistochemical analysis, the positive distribution of E-cadherin was observed as pericellular meshes that strongly appeared in the upper spinous layer in moderate dysplasia, decreased in severe dysplasia, and disappeared in OSCC. Integrin α6 gradually expanded to all spinosum layers with the advance of dysplastic grading. Weakly irregular distribution was observed in all tumor cells of OSCC. Epidermal growth factor receptor (EGFR) was shown in the spinosum layer of mild dysplasia, expanded with the advance of dysplastic grading, and was seen in all tumor cells in OSCC. Anti-lysyl oxidase (LOX) gradually increased with the advance of dysplastic grading. Perlecan showed similar results to LOX except that was decreased in OSCC. Briefly, the loss of E-cadherin in the lower half of the spinosum layer, integrin α6 in the spinosum layer with lost polarity, and the appearance of EGFR in the basal layer were observed in moderate dysplasia. Furthermore, LOX and perlecan distributions were frequently found in high-grade dysplastic lesions. The present study of oral precursor lesions found that instability or decreased cell adhesion related to the environment was observed in moderate dysplasia and higher grades of dysplasia. Consequently, it was supposed that moderate dysplasia was the stage at which lesions became tumorous.

Keywords:
Leukoplakia, dysplasia, cell–cell interaction

Introduction
Leukoplakias, defined as altered epithelium with an increased likelihood for progression to oral squamous cell carcinoma (OSCC), are precursor lesions with a wide range of microscopic appearances from simple hyperkeratosis to severe dysplasia (1). Clinico-pathologically, the malignant transformation rate of leukoplakia to OSCC is very broad, 3–28% (2). The clinical diagnostic entity “leukoplakia” is divided into four categories: hyperkeratosis and mild, moderate, and severe dysplasias. It is difficult to predict histologically which of the precursor lesions will progress to OSCC, although it is widely accepted that the malignant transformation rate increases with the advance of dysplastic grading (3).

For the purpose of analyzing the lesions that acquired a malignant character, the interrelationship among stromal reaction (4), basal membrane alteration (5), and oral precursor lesions with special reference to the advance of dysplastic grading was studied and inflammation, neovascularization, up-regulation of extracellular matrix-related proteins, and instability of basal membrane components were examined from moderate dysplasia. Consequently, it
was supposed that moderate dysplasia was the stage at which the malignant transformation occurred from the phenomenon of stromal reaction and basal membrane instability.

Concerning the structure of epithelium, cell adhesion molecules constitute stratified squamous epithelium and adhesion is essential to structural integrity. E-cadherin and integrin play regulatory roles in the mediation of both cell-cell and cell-matrix adhesions and their absence is associated with abnormal stratification. Unusual distribution of E-cadherin and integrin in malignant tumors has been reported for various internal organs (6, 7), but there are few reports that observed their distribution in oral precursor lesions of OSCC with dysplastic grading (8, 9). Very few reports have analyzed the cell environment in connection with the instability of cell adhesion in detail and assessed malignant transformation.

The objective of this study was to clarify the interrelationship between oral precursor lesions and cell-cell interactions with special reference to advancement of dysplastic grading using histopathology and immunohistochemistry.

Materials and Methods
Histopathological specimens

Thirty-six specimens of gingivae were selected from the pathology files of the Department of Oral Pathology, Nihon University School of Dentistry at Matsudo. They consisted of 5 cases of healthy epithelium, 7 of hyperkeratosis, 19 of precursor lesions (7 mild dysplasia, 7 moderate dysplasia, 5 severe dysplasia), and 5 of OSCC, with the entire lesion contained in the gingiva. One set of sections was stained with hematoxylin and eosin (H.E.) by the usual method and reviewed by oral pathologists to confirm the diagnosis and observe the histological characteristics and the other set was studied by immunohistochemistry. The degrees of epithelial dysplasia were determined according to the World Health Organization (WHO) guidelines (1). In addition, the cases secondarily accompanied by inflammation were excluded. The protocol was approved by the Committee on Studies Involving Human Beings of Nihon University School of Dentistry at Matsudo (EC 05-002). Informed consent was obtained from all patients before retrieving the pathological specimens.

Immunohistochemical assessment

Immunohistochemical studies were conducted using 10% neutral formalin solution-fixed, paraffin-embedded tissue from all cases. Sections (4 μm thick) were deparaffinized in xylene and hydrated in graded ethanol solution. The EnVision+ Polymer System (Dako Glostrup, Denmark), which also carried secondary antibody molecules, was used for antigen detection. Primary antibodies used were directed against the following antigens: E-cadherin (NCH-38, 1: 50; Dako Glostrup, Denmark); integrin α6 (MP 4F10, 1: 100; Abcam, England); epidermal growth factor receptor (EGFR, H11, 1: 200; Dako Glostrup, Denmark); and anti-lysyl oxidase (LOX, 1: 100; Imgenex, USA). The VECTASTAIN Elite ABC kit (Vector Laboratories, USA) was used for heparin sulfate proteoglycan (perlecan, A7L6, 1: 100; Millipore, USA). Antigen retrieval was performed in a pressure pot with citrate buffer solution (pH 6.0 for LOX, E-cadherin, and perlecan and pH 9.0 for EGFR). The sections were developed in a solution of 3, 3’-dianibobenzidine tetrahydrochloride (DAB). Finally, all sections were counterstained with Mayer's hematoxylin. Positive controls for EGFR and LOX were specimens of SCC of the endocervix, that for integrin α6 was specimens of inflammatory granulation tissue, that for perlecan was specimens of mammalian tissue, and that for E-cadherin was specimens of healthy skin. For evaluation of the immunohistochemical staining technique, as a negative control, mouse and rabbit universal negative controls (Dako Glostrup, Denmark) were used during the staining procedure instead of primary antibodies.

Results
Histopathological findings

The immunohistochemical findings are summarized in Table 1. Representative images of oral mucosal lesions and healthy control tissues are
Table 1. Result of immunohistochemical findings

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>E-cadherin</th>
<th>Integrin α6</th>
<th>EGFR</th>
<th>LOX</th>
<th>Perlecan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spinosum</td>
<td>Spinosum</td>
<td>Spinosum</td>
<td>Spinosum</td>
<td>Spinosum</td>
</tr>
<tr>
<td>Healthy epithelium</td>
<td>Basal 5</td>
<td>+ + ±</td>
<td>± ± ±</td>
<td>- - -</td>
<td>± ± ±</td>
</tr>
<tr>
<td>Hyperkeratosis</td>
<td>Basal 7</td>
<td>+ + ±</td>
<td>± ± ±</td>
<td>- - -</td>
<td>± ± ±</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>Basal 7</td>
<td>- - -</td>
<td>± ± ±</td>
<td>- - -</td>
<td>± ± ±</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>Basal 7</td>
<td>- - -</td>
<td>+ + +</td>
<td>± ± ±</td>
<td>- - -</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>Basal 5</td>
<td>- - -</td>
<td>± ± ±</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>SCC, early invasive</td>
<td>Basal 5</td>
<td>- - -</td>
<td>± ± ±</td>
<td>± ± ±</td>
<td>± ± ±</td>
</tr>
</tbody>
</table>

++: strong positive, +: moderate positive, ±: weak positive, -: negative

No. *: Number of cases

shown in Figs. 1–6. In hyperkeratosis, thicken keratinized layer with acanthosis and without cellular atypia was seen (Fig. 2a); in mild dysplasia, general architectural disturbance limited to the lower third of the epithelium accompanied by minimum cellular atypia was seen (Fig. 3a); in moderate dysplasia, general architectural disturbance extending into the middle third of the epithelium was seen (Fig. 4a); in severe dysplasia, greater than two-thirds of the epithelium showing architectural disturbance associated with cellular atypia was seen (Fig. 5a). Additionally, the characteristic two-phase appearance was frequently seen in moderate and severe dysplasias (Figs. 4a, 5a). In early invasive OSCC, cancer cells with squamous cell characteristics were proliferating and invading into the superficial subepithelial zone (Fig. 6a).

**Immunohistochemical staining**

Immunohistochemical findings of E-cadherin showed that positive distribution was observed as pericellular meshes in the basal and spinosum layers of healthy epithelium and hyperkeratosis and in the spinosum layer of mild dysplasia (Figs. 1b–3b). Positive findings of E-cadherin strongly appeared in the upper spinosum layer of moderate dysplasia (Fig. 4b), decreased in severe dysplasia (Fig. 5b), and disappeared in OSCC (Fig. 6b).

Integrin α6 gradually appeared from healthy tissue to mild dysplasia of the basal and lower spinosum layers and it was expanded to all spinosum layers in moderate and severe dysplasias (Figs. 1c–5c). Its distribution was irregular and its weakly positive distribution was observed in all the tumor cells of OSCC (Fig. 6c).

EGFR was negative in healthy epithelium and slightly positive in hyperkeratosis (Fig. 1d). EGFR-positive findings were shown in the spinosum layer of mild dysplasia (Fig. 2d) and increased with the advance of dysplastic grading, until they were observed in all tumor cells of OSCC (Figs. 3d–6d).

Positive findings for LOX gradually increased with the advance of dysplastic grading (Figs. 1e–5e). The LOX-positive finding in OSCC was strong, but irregular (Fig. 6e). Perlecan has a similar positive reaction to LOX (Figs. 1f–5f) except for its decrease in OSCC (Fig. 6f). More intense reaction was observed in the spinosum layer of mild dysplasia, moderate dysplasia, and severe dysplasia in particular (Figs. 3f–5f).

**Discussion**

With regard to malignant transformation, an imbalance between proliferation and apoptosis in tissues may contribute to the advance of dysplastic grading and initial carcinogenesis. Although many papers about these relevancies to oral leukoplakia have been reported (3, 10, 11), there are few reports that observed the cell environment in connection with the instability of cell adhesion in oral precursor lesions of OSCC with dysplastic grading.

Cell adhesion molecules, which are essential for
Figs. 1–6. Histopathological and immunohistochemical findings in healthy epithelium (1), precursor lesions in 4 dysplastic grading: hyperkeratosis (2), mild dysplasia (3), moderate dysplasia (4), severe dysplasia (5), and OSCC (6).

a: Histopathological appearances of epithelial lesion (H.E. stain, ×10).
b: E-cadherin, positive distribution was decreased in severe dysplasia, and disappeared in OSCC. (×20)
c: Integrin α6 was expanded to all spinosum layers in moderate and severe dysplasias, and weakly irregular distribution was observed in all the tumor cells of OSCC. (×20)
d: Positive finding of EGFR was expanded with dysplastic grading, and it was observed by all the tumor cells in OSCC. (×10)
e: Positive findings for LOX was gradually increased from healthy, hyperkeratosis, mild to severe dysplasias (×10).
f: More intensive reaction for perlecan was increased with dysplastic grading, but decreased in OSCC (×20).

structural integrity, constitute stratified squamous epithelium. The cadherins are a family of calcium-dependent cell adhesion molecules and E-cadherin, a 120-kD transmembrane molecule, is involved in cell-cell adhesion between epithelial cells of both healthy basal and differentiating keratinocytes (6). The role of E-cadherin is to maintain epithelial cellular cohesion; it plays a role as an invasion suppressor molecule. Infiltrative and invasive activities were associated with a low level of E-cadherin in in vitro and immunohistochemical studies (8). In this study, positive pericellular findings strongly appeared in the spinosum layers in moderate dysplasia, decreased in severe dysplasia, and disappeared in OSCC. The function of invasion suppressor molecules was lost from the basal and lower spinosum layers of moderate dysplasia. Furthermore, it was suggested that the loss of E-cadherin from the basal and lower spinosum layers was involved in the formation of the two-phase appearance (12) in moderate dysplasia. The cause of E-cadherin loss could be suppression of its translation by hypermethylation (12). On the basis of the present results, the loss of E-cadherin in the lower half of the all of the layer in moderate dysplasia was considered to be partially attributable to methylation of the E-cadherin promoter region (12).

Integrins are a family of transmembrane receptors that mediate both cell-cell and cell-matrix adhesions, including epithelial keratinocytes. They are heterodimers composed of α and β transmembrane glycoprotein subunits and 22 different integrins are found in nature (13). Integrin α6 complexes are receptors for laminin, which plays important roles in regulating malignant transformation in tumors (13). Local loss of integrin α6 in the basal layer coincides with the loss of basement membrane components; the quantities of laminin and type IV collagen change during tumor progression in oral precursor lesions (5, 9, 14). Consequently, integrin α6 was considered to be an important marker of malignant potentiality (15). Integrin α6, which is concentrated in the basal cells adjacent to the basal membranes in healthy epithelium (16), was even found in the spinosum layer with lost polarity in the process of tumor progression in this study. Concerning integrin polarity, its loss in SCC was discussed (9) and the same tendency was observed in an in vitro study (17). Localization of integrin in epithelial cells is recognized to be related to the order of keratinocyte layers (17). In short, adhesion in epithelial cells is regulated by integrin polarization.

E-cadherin regulation is based on a kinase of the erbB growth factor receptor family containing EGFR (18). Increased expression of EGFR and down-regulation of E-cadherin are associated with epithelial malignancies including OSCC (19). EGFR, a surface receptor with intrinsic tyrosine kinase activity, is one of several known pivotal intermediates in many epithelial malignancies (20). Positive EGFR expression was expanded from the spinosum to basal layers in moderate dysplasia and was irregularly observed in all of the epithelial cells in severe dysplasia and OSCC. EGFR activation regulates the rate of proliferation and differentiation of dysplastic cells (20). It was suggested that loss of the order of epithelial architecture could begin from moderate dysplasia. This result was consistent with another description (21) and supported the theory that EGFR up-regulation might be associated with down-regulation of E-cadherin as early events in OSCC invasion (22).
The lysyl oxidase gene family comprises five members acting as extracellular modulating enzymes and the first identified and the better-studied isoform of this family is LOX (23). Arachidonic acid metabolism, whose main pathway requires LOX, is associated with carcinogenesis (24). LOX was generally absent in healthy epithelial cells and was increased in SCC in the role of facilitating anti-apoptosis and cell proliferation in the early stage of malignant transformation (25). Conversely, LOX was identified to be over-expressed in human hypoxic cells (26). As for the LOX immunoreactivity of OSCC, there are two theories of up-regulation (27) and down-regulation (28). Our result was concordant with the one showing the highest LOX expression level was observed in severe dysplasia. Pre-invasive steps occur in an avascular environment because the epithelium is separated from the blood vessels by a basement membrane. Hypoxia induced by cell proliferation is the initial step, followed by modulated tumor progression, in the previous reports (29, 30). Namely, hypoxia enhanced the tumor progression environment, such as angiogenesis and epithelial mesenchymal transition by E-cadherin down-regulation (31). E-cadherin decreased gradually, although up-regulation of LOX with dysplastic upgrading occurred as a result. We hypothesized that oxygen supply by neovascularization (4) might be acquired when cancer cells begin invading. Up-regulation of LOX with the increase in dysplastic grading was identified, although E-cadherin decreased in this study.

The perlecan protein is a large multidomain proteoglycan that binds to and cross-links many extracellular matrices and cell-surface molecules of basal membranes and connective tissues (32). In the oral epithelial layer, perlecan is present between the pericellular spaces of dysplastic cells in precursor lesions (33). It was thought that perlecan retention was increased in the pericellular spaces with the decrease of E-cadherin in the dysplastic lesions. Deposition of perlecan in the basal and spinosum layers in moderate and severe dysplasias was thought to be caused by decreased cell adhesion. Therefore, tumor progression provided epithelial cells with a hypoxic environment and resulted in down-regulation of E-cadherin.

The present study of oral precursor lesions found that instability or decreased cell adhesion related to the environment was observed in moderate dysplasia and higher grades of dysplasia. Consequently, it was supposed that moderate dysplasia was the stage at which the malignant transformation occurred, just as in studies of stromal reaction (4) and basement membrane alteration (5).

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351