Cytokeratin 13, Cytokeratin 17, Ki-67 and p53 Expression in Upper Layers of Epithelial Dysplasia Surrounding Tongue Squamous Cell Carcinoma

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Abstract

Early detection of oral squamous cell carcinoma (OSCC) improves its prognosis and aids in selecting the appropriate treatment, which may also have a positive effect on quality of life. Early detection, therefore, is an important issue in the treatment of this disease. The purpose of this study was to investigate expression of cytokeratin 13 (CK13), CK17, Ki-67 and p53 as potential markers of tongue SCC. Five areas in 12 specimens were examined: the upper and lower layers of normal epithelium; those of dysplastic epithelial tissue surrounding the cancerous lesion; and the lesion itself. Strong expression of each of the following mRNAs and proteins was observed; CK13 in upper layers of normal epithelium; Ki-67 and p53 in lower layers of normal epithelium; CK13 and CK17 in upper layer of epithelial dysplasia; and CK17, Ki-67, and p53 in lower layer of epithelial dysplasia and cancerous lesions. These results indicate that the characteristic pattern of expression of CK13 and CK17 differs between normal and dysplastic oral epithelium. Oral epithelial dysplasia adjacent to OSCC has high malignant potential, and is similar to early-stage OSCC. This suggests that evaluation of these markers could be a useful secondary procedure for improving detection of early-stage OSCC.

Key words: Oral squamous cell carcinoma—Oral epithelial dysplasia—Cytokeratin 17—Cytokeratin 13—Laser capture microdissection

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Introduction

The oral cavity plays an important role in eating, swallowing, breathing, speaking, and facial aesthetics. Delays in detecting oral cancer can increase the risk of complications, which may or may not affect its prognosis. Early detection of oral cancer is therefore essential in ensuring a high quality of life for the patient. Visual detection of early-stage oral cancer in a clinical setting, however, is often difficult. Initial detection by screening can later be confirmed and treatment commenced more rapidly. In order to facilitate its detection our group has attempted to clarify the characteristics of early-stage oral squamous cell carcinoma (OSCC) in number of studies, focusing particularly on dysplastic epithelial tissue surrounding such lesions as it has the potential for malignant transformation. A number of markers are considered useful in diagnosing early-stage OSCC, and our group has focused on their expression in the upper layer of dysplastic epithelial tissue. These surface areas are those the most likely to be subjected to vital staining and inspection with optical instruments, and cells from this layer may be gathered by exfoliative cytology. The purpose of this study was to investigate expression of cytokeratin 13 (CK13), CK17, Ki-67, and p53 mRNA and protein in the upper and lower layers of oral epithelium surrounding tongue SCC as potential markers of this disease.

Materials and Methods

1. Patients

A total of 12 specimens of tongue SCC were resected from 12 different patients (7 men and 5 women; mean age, 61.5 years; range 39 to 75 years) treated at the Ichikawa General Hospital over a 5-year period from 2008 to 2012 (Table 1). All specimens were histopathological-grade well-differentiated SCC and included a region of epithelial dysplasia surrounding a cancerous lesion. Written informed consent was obtained from all study participants and this study was approved by the Ethics Committee of Tokyo Dental College (approval no.259).

2. Sampling criteria

Frozen sections were used for immuno-histochemical examination of expression of mRNA. Samples were collected from surgical specimens. Dissected specimens were embedded in optimal cutting temperature compound (Sakura, CA, USA) and preserved by isopentane cooled in liquid nitrogen for storage at −80°C. A series of 5-μm frozen sections was cut and stained with hematoxylin and eosin (H-E) using standard techniques. Each H-E stained section was assessed by an experienced oral pathologist in accordance with WHO classifications, in which the following areas (sampling criteria) are identified (Fig. 1): UN, upper layer of normal epithelium, excluding the parabasal cell layer surrounding the cancer; LN, lower layer of normal epithelium, including the parabasal cell layer surrounding the cancer; UD, upper area of epithelial dysplasia, excluding second layer located on basal and parabasal cells; LD, lower area of epithelial dysplasia located

<table>
<thead>
<tr>
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<td>W</td>
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<tr>
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<td>39</td>
<td>W</td>
<td>T4aN0M0</td>
</tr>
</tbody>
</table>

M: men, W: women

Table 1 Clinical and pathological characteristics of 12 tongue cancer patients
on the basal and parabasal cell layer; and C, cancerous lesion (including interstitial cells).

Normal epithelium, epithelial dysplasia, and cancerous lesion were classified as follows: normal epithelium was epithelium that appeared morphologically normal; epithelial dysplasia was epithelium that had cytologic atypia extending into the middle third of the epithelium (moderate dysplasia); cancerous lesion was the OSCC. All five areas were present in each section^{12,17}.

3. Evaluation of mRNA expression

For analysis of mRNA expression, serial 8-μm frozen sections were cut and placed on membrane-film slides (Meiwa Shoji, Japan). Each section was fixed with 100% methanol for 3 min and stained with cresyl violet acetate (LCM Staining Kit; Ambion) at room temperature. Five samples were collected per section by laser capture microdissection using the PALM™ LPC-Micro Beam System (Carl Zeiss, Germany) based on the sampling criteria. Purification of total RNA was performed using the RNasy Micro Kit (QIAGEN) in accordance with the manufacturer’s instructions. For synthesis of cDNA, 20-μl reaction mixtures containing 16 μl total RNA and 4 μl High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) were used. Synthesis of cDNA was carried out using the Gene Amp PCR System 9700 (Applied Biosystems) with the following cycle parameters: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, and at 4°C. The resulting cDNA was stored at −20°C until use.

Real-time reverse transcription-PCR (real-time PCR) of the following 4 genes was performed: CK13 (Hs00999762), CK17 (Hs00356958), Ki-67 (Hs01032443), p53 (Hs99999147) and GAPDH (endogenous control, Hs99999905). For real-time PCR, 20-μl reaction mixtures containing 2 μl sample cDNA, 10 μl TaqMan
Gene Expression Master Mix (Applied Biosystems), 1 μl TaqMan Gene Expression Assay (Applied Biosystems), and 7 μl RNA-free water were used. The real-time PCR program was run using the 7500 Fast Real-time PCR System (Applied Biosystems) as follows: 1 cycle at 95°C for 10 min, 50 cycles at 95°C for 15 sec, and 60°C for 1 min. Levels of mRNA expression were calculated as a ratio against GAPDH expression levels.

4. Evaluation of protein expression by immunohistochemistry

Immunohistochemical staining was performed in an incubation chambers (Cosmo Bio Co., Japan) at room temperature. A serial of 6-μl frozen sections was cut and placed on clean silane-coated glass slides. Sections were fixed with 10% paraformaldehyde for 30 min and then stained using the two-step polymer method (EnVision™ + HRP (DAB); DAKO, Japan). The washing solution used was phosphate-buffered saline (PBS, pH 7.4). Four mouse monoclonal antibodies, against CK13 (×100 dilution; KS-1A3, Novocastra), CK17 (×50 dilution; E3, DAKO), Ki-67 (×100 dilution; Ki-67, DAKO), and p53 (×100 dilution; DO-7, DAKO), were used to determine immunohistochemical expression. Counterstaining was performed with Mayer’s hematoxylin. The analysis of the results was performed with Image J 1.47d cell-counter software (National Institutes of Health, Bethesda). The Labeling Index is given as positive staining ratios, which were calculated from the percentage of cells positive for CK13 and CK17, or nuclei positive for Ki-67 and p53.

5. Statistical analysis

The Steel-Dwass test was used for the statistical analysis. The R 2.15.0 software package (R Foundation, GNU project) was used to determine p-values and perform a multiple comparison analysis. A p-value of <0.05 was considered to be statistically significant. Measurements of central tendency are expressed as medians ± quartile deviation (Med ± Q.D.).

Results

1. mRNA expression

Figure 2 shows mRNA expression of CK13, CK17, Ki-67, and p53. Characteristic mRNA expression patterns were observed for each of the four markers. Expression of CK13 was higher in UN and UD than in C, LD, or LN; that of CK17 was higher in UD and C than in LN, UN, or LD; and that of Ki-67 and p53 was higher in C, LD, and LN than in UN or UD.

2. Immunohistochemical expression

Representative results for immunohistochemical staining are shown in Fig. 3. Immunohistochemical data on expression of CK13, CK17, Ki-67, and p53 are shown in Fig. 4. The staining ratio of CK13 was higher in UN and UD than in LN, LD, or C; that of CK17 was higher in UD and C than in LN, UN, or LD; and that of Ki-67 and p53 was higher in C, LD, and LN than in UN or UD. The mRNA expression and immunohistochemical staining data were similar. Table 2 shows the characteristic expression patterns for each protein marker in each of the five areas.

Discussion

Epithelial dysplasia adjacent to OSCC is recognized as having the potential for malignant transformation and shows similarities with early-stage OSCC. Therefore the present study focused on the upper layer of dysplastic epithelial tissue surrounding tongue SCC. Laser capture microdissection allows selectively harvesting of cells. Genes are better preserved when cells are harvested from frozen sections, making this technique particularly suited to gene assay of a target area. Here, laser capture microdissection was used to evaluate the characteristic expression in each region selected with the basal and para-basal cell layer acting as border.

Cytokeratin is an intermediate filament in the cytoskeleton. In normal oral epithelium, these filaments are involved in cell-cell adhesion, and it has been proposed that changes
in these filaments are related to malignant transformation and progression. Bongers et al. reported that some cytokeratins are suitable markers for screening or monitoring of premalignant lesions, as well as for following malignant lesions. Cytokeratin 13 is a 54-kDa type I cytokeratin expressed throughout normal oral epithelium, except in the basal cell layer. In contrast, CK17 is 46-kDa type I cytokeratin expressed in OSCC. Noguchi et al. noted that the expression of these cytokeratins was associated with carcinogenesis in oral epithelial dysplasia, and that it was a useful indicator in the diagnosis of malignant potential and detection of early-stage OSCC. The present study focused on patterns of expression of CK13 and CK17 in the upper layers of epithelial dysplasia surrounding...

Fig. 2 mRNA expression levels of CK13, CK17, Ki-67, and p53
Horizontal center line in each box indicates median, and vertical line relative mRNA expression level. Upper edge of each box shows third and lower edge first quartile deviation.
The results showed that expression of CK13 and CK17 differed between the types of tissue examined. Of particular note was the finding that CK13 and CK17 were both expressed in the upper layer of epithelial dysplasia.

Proliferation marker Ki-67 is mainly expressed in basal and parabasal cell layers in epithelium, which is composed of actively proliferating cells. The level of Ki-67 expression depends on the degree of oral epithelial dysplasia. Malignance marker p53 is a protein encoded by the TP53 tumor-suppressor gene, and is associated with apoptosis, the cell cycle, and genetic homeostasis. Immunohistochemical expression of p53 is also mainly observed in the basal and parabasal cell layers, and its levels are increase depending on the severity of oral epithelial dysplasia, similarly to Ki-67 levels.

The present results are similar to those of a number of earlier studies showing that expression of Ki-67 and p53 in the lower layers of oral epithelial dysplasia is higher than in the upper layers. On the other hand, expression of p53 was higher in the lower layers of normal epithelium, as well as in the lower layers of adjacent dysplastic epithelium.
Cytokeratin 13 and 17 Expression in Dysplasia

This may reflect the fact that malignant transformation at the gene level has already occurred in epithelial tissues adjacent to OSCC\(^{21}\).

In conclusion, oral epithelial dysplasia adjacent to OSCC is characterized by high expression of CK13 and CK17 in the upper layer and high expression of Ki-67 and p53 in the lower layer. In a clinical setting, however, it is necessary to be able to evaluate

Table 2  Characteristic expression patterns for each protein marker in each of the five areas

<table>
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<tbody>
<tr>
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<td>p53</td>
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(*: positive expression, ○: negative expression)
surface cells showing mild atypia, and oral exfoliative cytology is often used as a means of screening for OSCC. Detecting early-stage OSCC based on cellular atypia in surface tissue, however, is difficult. Therefore, evaluation of a combination of such protein markers, and particularly CK13 and CK17, may offer a useful secondary tool in improving the accuracy of diagnoses based on oral exfoliative cytology.

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Conflict of Interest

The authors have no conflicts of interest related to the experiments in this study.

References


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