Expression of Cytokeratin 14 and 19 in Process of Oral Carcinogenesis

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Abstract

Cytokeratins (CK) are abundant in keratinized cells, particularly CK14 and CK19, which are expressed in stratified squamous epithelial cells. In this study, expression of CK14 and 19 was examined in human epithelial and dysplastic tissues. Surgical specimens from patients with clinically diagnosed oral leukoplakia or early cancer were stained with hematoxylin and eosin and classified into normal, low grade dysplasia (LGD), high grade dysplasia (HGD), or squamous cell carcinoma (SCC). The sections were examined by immunostaining and reverse transcription-polymerase chain reaction (RT-PCR) for CK14 and CK19. Expression and the results of RT-PCR for CK14 showed a decrease in the order of LGD, HGD, and SCC, whereas those of CK19 showed an increase in that order. These results suggest that decreased expression of CK14 and increased expression of CK19 serve as indicators of potential for malignant transformation.

Key words: Cytokeratin 14 — Cytokeratin 19 — Oral carcinogenesis — Dysplasia — Squamous cell carcinoma

Introduction

Many genes and proteins have been specified as inducing oral carcinogenesis¹,⁷. A number of these, including p53 and β-catenin, were analyzed by our group to establish criteria for potential for malignant transformation¹⁵,¹⁷. In another study, our department conducted a comprehensive microarray analysis of precancerous and early cancerous tissues of the human tongue to investigate their potential for malignant transformation. The results clearly showed expression of cytokeratin 14 (CK14) and cytokeratin 19 (CK19)²⁰. In the present study, our focus was on these specific cytokeratins.

This paper was a dissertation submitted in March 2007 by Kyoko Yoshida to the Graduate School of Tokyo Dental College for the degree of Doctor of Philosophy.
The expression pattern of CKs in healthy human tissues, cultured cell lines, and cancer cells was first characterized by Moll et al. in 1982. Since then, monoclonal anti-keratin antibodies have been utilized to detect epithelial tumor markers. However, unlike in tumors derived from glandular epithelium, the complex patterns of CK polypeptides observed in tumors arising in squamous epithelium differ from those in their tissues of origin. Cytokeratins are a group of water-insoluble proteins with a molecular weight in the range of 40–70 kD that form 8 to 11-nm intermediate filaments in a wide variety of epithelial cells. These proteins are classified into two subfamilies: type I acidic (CK9–20); and type II, neutral or basic (CK1–8). Cytokeratin 19 is characterized by its expression and localization, and has been utilized as an indicator gene in peripheral blood cancer cells in the diagnosis of breast, lung, stomach, and colorectal cancer. In addition, an attempt was made to evaluate squamous epithelial carcinomas based on localization of CK. The main CKs in human squamous cell carcinomas were reported to be CK6 and CK16. Therefore, the purpose of the present study was to examine differences in expression of CK14 and CK19 histopathologically and molecular-biologically to investigate their relationship with potential for malignant transformation.

### Materials and Methods

1. **Human tissue samples**

   Ethical clearance for the study protocol (number 102) was obtained from the Ethics Committee of Tokyo Dental College, along with written informed consent from the tissue donors. Epithelial dysplasia or early cancer samples and their corresponding healthy tissues were subsequently obtained by excisional biopsy from 15 patients (Table 1).

2. **Preparation of frozen human tissue samples**

   The obtained tissue samples were cut using the Bread Loaf Step Sectioning method at a slice width of approximately 2 mm. The samples were then placed in a cryo-dish and embedded in Optimal Cutting Temperature compound.

   Cryofixation was performed using isopentane and preservation in liquid nitrogen at −80°C. Sections approximately 5 μm in thickness were cut from frozen samples in a cryostat at −20°C.

3. **Histological examination** (Table 2)

   Thin frozen sections were stained with hematoxylin and eosin (H-E) using the standard

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>M</td>
<td>Gingiva</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>F</td>
<td>Gingiva</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>M</td>
<td>Buccal mucosa</td>
</tr>
<tr>
<td>4</td>
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<td>M</td>
<td>Tongue</td>
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<tr>
<td>5</td>
<td>60</td>
<td>M</td>
<td>Tongue</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>F</td>
<td>Gingiva</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>F</td>
<td>Tongue</td>
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<tr>
<td>8</td>
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<td>M</td>
<td>Tongue</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>M</td>
<td>Gingiva</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>M</td>
<td>Gingiva</td>
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<tr>
<td>11</td>
<td>85</td>
<td>F</td>
<td>Tongue</td>
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<tr>
<td>12</td>
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<td>34</td>
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<td>Tongue</td>
</tr>
<tr>
<td>15</td>
<td>57</td>
<td>M</td>
<td>Buccal mucosa</td>
</tr>
</tbody>
</table>

M: Male, F: Female

### Table 1 Clinical data on 15 patients

<table>
<thead>
<tr>
<th>H-E stain</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (NO)</td>
<td>15</td>
</tr>
<tr>
<td>Low grade dysplasia (LGD)</td>
<td>9</td>
</tr>
<tr>
<td>High grade dysplasia (HGD)</td>
<td>8</td>
</tr>
<tr>
<td>Squamous cell carcinoma (SCC)</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
</tr>
</tbody>
</table>

### Table 2 Distribution of number of experimental lesions by H-E stain
technique. Based on the results of H-E staining, 15 histological specimens were classified as normal (NO), low grade dysplasia (LGD), high grade dysplasia (HGD), or squamous cell carcinoma (SCC) based on the WHO Epithelial Dysplasia Criteria\(^\text{16}\). In its 1997 list of 13 histological criteria for epithelial dysplasia classification, the WHO defined LDG as mild or moderate dysplasia; and HGD as severe dysplasia or carcinoma \textit{in situ}\(^\text{16}\). Tissues from the surgical margins of each specimen were used as samples of healthy tissue. Samples which did not reveal typical findings of LGD, HGD or SCC were excluded. A total of 44 samples were thus obtained.

4. Histological examination by H-E staining

Of the 15 specimens, 9, 8, and 12 were classified as LGD, HGD, and SCC, respectively. In LGD, the basal cell layer was intact, and nuclear dysplasia was mild (Fig. 1-a). In HGD, the epidermal rete pegs were elongated (Fig. 2-a). In SCC, marked nuclear dysplasia was observed (Fig. 3-a).

5. Immunohistochemical methods

Immunohistochemistry for each antigen was performed on approximately 5-μm paraffin sections of each tissue. Slides were stained for CK14 (Ventana Medical Systems, Inc., Arizona, U.S.A.) and CK19 (Ventana Medical Systems, Inc.) using the Ventana Discovery
(Ventana Medical Systems, Inc.) automated staining system with the DAB Map kit (Ventana Medical Systems, Inc.). All slides were counterstained with hematoxylin, dehydrated, cleared, and mounted. More than 500 epithelial cells were counted in each lesion, and characterized as NO, LGD, HGD, or SCC. The counts were performed in one microscopic field in each section at 200× magnification. The percentage of positive cells was calculated in each sample together with the mean for all 44 samples. These values were used as labeling indices (LIs) and compared statistically using the Student’s t-test. A p-value of less than 0.01 was considered significant.

### 6. Microdissection

A tissue microdissection method was employed to obtain carcinoma samples without excessive contamination from healthy tissue\(^ {35}\). Single 10-μm paraffin-embedded tissue sections were cut with a microtome and placed on glass slides. Each type of lesion (NO, LGD, HGD, or SCC) was microdissected as carefully as possible.

### 7. Reverse transcription-polymerase chain reaction (RT-PCR) (Table 3)

The RT-PCR of CK14 mRNA, CK19 mRNA, and β-actin mRNA templates yielded fragment sizes of 111, 148, and 154 bp, respectively. The primer sequences were as follows: 5′-CCGAGCACCCTTCTCATGAGCTG for CK14 mRNA; 5′-CTGAGTGACATGCGAAGCCAATA and 5′-CAGTAACCTCGGACCTGCTCATC for CK19 mRNA; and 5′-CAGTGTTGCGGTCTACAGGT and 5′-TCATCACCATTGGAATGAG for β-actin mRNA. Ten μl reverse transcription buffer containing 1 μg RNA, 0.2 μg oligo-dT primers, 0.5 mM dNTP, 5 U of RNasin, and 100 U of MMLV-RT (Takara Bio Inc., Shiga, Japan) was incubated at 42°C for 15 min and the reaction terminated by heating at 95°C for 2 min. Two μl of this cDNA solution was removed for use in PCR amplification, adding samples to 20 μl solution containing 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, down-stream and up-stream primers, 2.0 units RNase inhibitor, and 2.5 units TaKaRa Ex Taq (Takara Bio Inc.). First, the samples were incubated at 30°C for 10 min, followed by 42°C for 40 min. Amplification was then performed in a Smart Cycler (Takara Bio Inc.) for 40 cycles (10 sec, 95°C; 5 sec, 50°C; 20 sec, 60°C). After amplification, the entire PCR reaction mixture (20 μl) was analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

### 8. Statistical analysis

The levels of cytokeratin 14 and 19, their LIs, and the results for lesion classification (NO, LGD, HGD, or SCC) were compared statistically using the Student’s t-test. A p-value of less than 0.01 was considered significant.

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**Table 3** Primers for reverse transcription-polymerase chain reaction amplification of cytokeratin 14 mRNA, cytokeratin 19 mRNA, and β-actin mRNA

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequences 5′–3′</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 14</td>
<td>F CCGAGCACCCTTCTCATGAGCTG</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Cytokeratin 19</td>
<td>F CTTGAGTGACATGCGAAGCCAATA</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>R CAGTAACCTCGGACCTGCTCATC</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F CACTGTGGTGGCGGTACAGGT</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>R TCATCACCATTGGAATGAG</td>
<td></td>
</tr>
</tbody>
</table>

F: forward primer, R: reverse primer
Results

1. Immunohistochemical examination

Cells positive for CK14 were frequently observed in the basal and prickle cell layers in LGD (Fig. 1-b), partly in the basal and prickle cell layers in HGD (Fig. 2-b), and partly in the prickle layer in SCC (Fig. 3-b). The CK14 LIs in LGD, HGD, and SCC were 70.3 ± 34.7%, 19.6 ± 13.1%, and 6.4 ± 7.2%, respectively; thus, the CK14 LI in LGD was the highest, and significantly differed from that in SCC (p < 0.01, Fig. 4).

Cells positive for CK19 were virtually absent in LGD (Fig. 1-c), and were observed only partly in the basal cell layer in HGD (Fig. 2-c) and partly in the basal and prickle cell layers in SCC (Fig. 3-c). The CK19 LIs in LGD, HGD, and SCC were 0.8 ± 1.3%, 27.6 ± 34.4%, and 48.0 ± 34.62%, respectively; thus, the CK19 LI in SCC was the highest, and significantly differed from that in LGD (p < 0.01, Fig. 5).

2. Analysis of expression level of CK14 and CK19 mRNA by RT-PCR (Fig. 6)

Electrophoresis revealed that level of expression of CK14 mRNA fell in the order of LGD > HGD > SCC, while that of CK19 mRNA level rose in the order of NO < LGD < HGD < SCC.

Discussion

Leukoplakia, a lesion covered by a thick, keratinized layer of oral mucosal epithelium, has been reported to undergo malignant transformation in 5–10% of cases39. The oral mucosal cells of leukoplakia, a precancerous lesion, contain abundant CKs, which form the intermediate filaments of the cytoskeleton. To date, 21 different CKs have been reported.
in human epithelia. A combination of a type I CK with an acid isoelectric point and a type II CK with a neutral-to-basic isoelectric point results in the formation of stable filaments of keratin\(^1\). Studies in knockout mice reported that mutations in these CK pairs caused a variety of genetic diseases and pathological states\(^2\). In the basal layer of the oral mucosal epithelium, keratin filaments are composed of type I CK14 and type II CK5\(^8\). In this study, CK14 was observed in the basal cells in NO. However, in LGD, CK14 was noted in all epithelial layers, and yielded the highest result in RT-PCR. As basal cell dysplasia progressed in severity from HGD to SCC, expression of CK14 fell, however, and RT-PCR gave similar results. This suggests that, while CK14 may be involved in change from healthy epidermis to precancerous lesion, its expression then decreases as malignancy progresses. Opinion on CK19, however, is divided. One study, for example, reported that poorly differentiated oral cancer was associated with higher expression of CK19\(^10\), while another study using frozen sections reported that well-differentiated oral cancers were also positive for CK19\(^12\). Many studies have analyzed CK19 expression levels according to differences in degree of differentiation, and some have investigated localization of CK19. In this study, expression levels of CK19 in different grades of dysplasia in precancerous lesions were compared. Expression levels of CK19, as determined by immunostaining and RT-PCR rose in the order of LGD<HGD<SCC. Immunostaining revealed that CK19 was expressed in HGD and SCC in the following order of increasing intensity: LGD<HGD<SCC. These findings indicate that CK19 is expressed at higher levels in epithelial dysplasias and cancer cells than in normal cells. It has been reported that CK19 is expressed, not in normal tissue or dysplasias, but in carcinomas in situ\(^20\); that is, changes in CK19 expression occur in lesions undergoing transition to cancer. In addition, one study reported that CK19 was expressed in early invasive oral cancers, but not in advanced cancers\(^20\). We speculate that changes in expression of CK19 in basal cells are involved in malignant transformation and cancer proliferation. It has been suggested that, clinically, the detection of blood or urinary CK19 in breast cancer\(^20\), lung cancer\(^21\), stomach cancer\(^24\), or colon cancer\(^11\) is useful in the early diagnosis of cancer metastasis. The present results also suggest CK14 and 19 as a potential marker of malignant change in the oral cavity.

Therefore, we believe that reduced expression of CK14 in normal tissue and elevated expression of CK19 in association with increasing epithelial dysplasia serves as an indicator of potential for malignant transformation.

References


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