Expression of Cytokeratin 13, 14, 17, and 19 in 4-nitroquinoline-1-oxide-induced Oral Carcinogenesis in Rat

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Abstract

The management of epithelial dysplastic spread around an oral squamous cell carcinoma is very important, particularly intraoperatively. Both cytokeratin (CK) 14 and CK19 are believed to be involved in the development of precancerous lesions, and their expression profiles are quite specific in these and early cancer lesions. Here, expression of CK13, 14, 17, and 19 was investigated in a rat model of 4-nitroquinoline-1-oxide-induced tongue cancer during a series of carcinogenetic processes to determine their value in assessing the features of epithelial dysplastic spread around a cancer. Based on tissue conditions, the results showed that expression levels of CK13 and 14 decreased in the order of no change, dysplasia, and cancer, whereas those of CK17 and 19 increased in the same order. Expression of CK13 showed a significant difference among no change, dysplasia, and cancer. This indicates that comparing the immunohistochemical staining profiles of CKs, especially CK13, could help in assessing the characteristics of epithelial dysplastic spread around a cancer.

Key words: Cytokeratin — Epithelial dysplasia — Early cancer — Rat — Tongue
Introduction

Managing epithelial dysplastic spread around an oral squamous cell carcinoma is very important, particularly intraoperatively, as such lesions can become malignant or acquire the characteristics of cancer, thus conferring a risk of tumor recurrence\(^1\). Epithelial dysplasia is currently graded histopathologically as mild, moderate, or severe based on morphological features according to diagnostic standards laid down by the WHO\(^6\). Some recent reports have indicated that the expression of several proteins might be useful in assessing epithelial dysplasia\(^13,14\). Histopathologically, squamous cell carcinoma accounts for more than 90% of oral cancers. Even after cancerization, epithelial cells always contain cytokeratin (CK), a type of intermediate cellular skeletal filament that maintains cell morphology. Large quantities of CKs have been found in keratinized cells in histopathological and molecular investigations into change in expression of CK14 and CK19 in human lesions. Moreover, their involvement in early cancer and precancerous lesions has also been investigated (Yoshida et al., unpublished observations). The basal cell layer of normal oral mucosal epithelia expresses CK14\(^7\) and normal epithelial cells express CK19. Cytokeratin 13 is expressed in normal tissue, but levels are lower in cancer cells, and particularly in poorly differentiated squamous cell carcinoma\(^3,5,7,9\). The non-keratinization marker CK13 is found in simple epithelia and some stratified epithelia\(^1\). Cytokeratin 17, which is found in simple epithelia and some stratified epithelia\(^1\), may indicate malignant transformation in oral epithelial squamous cells\(^21\). Here, the expression profiles of CK13, 14, 17, and 19 were investigated during a series of carcinogenic processes using a rat model of 4-nitroquinoline-1-oxide(4NQO)-induced tongue cancer to determine whether these expression profiles could be used to assess the characteristics of epithelial dysplastic spread around a cancer. The present study focused on leukoplakia-type change, as Takaki\(^15\) found that this was the most common in macroscopically classified lesions in rats with 4NQO-induced tongue cancer and that epithelial dysplasia and early invasive carcinoma were the most frequent among leukoplakia-type lesions.

Materials and Methods

1. Animals and treatment

Male Sprague-Dawley rats were housed and provided with care in accordance with the Guidelines for Experimental Animals at the Animal Facility of Tokyo Dental College and fed the MF Koito diet (Oriental Yeast, Tokyo, Japan). Tap water was replaced with water containing 50 ppm carcinogenic 4NQO\(^3\) when the body weight of the rats reached 200 g each. The study was carried out in accordance with the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College.

2. Tissue preparation

Tongue samples were obtained from animals given 4NQO for 4–5 months when macroscopic changes in the midline of the tongue dorsum, including white lesions and a rough tongue surface, were observed. The animals were sacrificed by intraperitoneal injection of pentobarbital (25 mg/kg). Dissected tongues were cut into sagittal sections; one part of the tissue was fixed in 10% formalin for 24 hr and embedded in paraffin. Serial sections 5 \(\mu\)m in thickness were prepared for various types of staining. Another part was embedded in OCT compound, cryofixed in isopentane, and stored in liquid nitrogen at \(-80^\circ\)C. Sections 10 \(\mu\)m in thickness were prepared for RNA extraction.

3. Experimental lesions

One serial section was stained with hematoxylin-eosin (HE). A total of 60 sections were obtained. From each section, 12 samples each of normal epithelium with no carcinogenic change (no change), epithelial dysplasia (dysplasia), and invasive carcinoma (cancer) were extracted from the tongue sample of each rat,
for a total of 36 parts (Fig. 1). Early cancerous lesions and dysplasia were observed histologically through an optical microscope. Grade of epithelium was determined by counting the applicable items among the 13 items provided in the WHO Epithelial Dysplasia Criteria. The sum (WHO score) was used as a parameter of histological findings in investigating differences between histological results and gene expression in each lesion. Epithelium that appeared morphologically normal was classified as ‘no change’. Epithelium located around a cancer, with atypia, pleomorphism, nuclear division of nuclei in the basal layers, and hyperplastic spinous layers but with an apparently normal epithelial structure was classified as ‘dysplasia’.

4. Immunohistochemical study

Localization of CKs was evaluated using immunohistochemical staining with streptavidin-biotin for CK13, CK14, CK17, and CK19 as follows. Serial sections (5 μm) were deparaffinized in xylene, dehydrated in a graded ethanol series, and rinsed in distilled water. Endogenous peroxidase was then inactivated by incubation with 0.3% hydrogen peroxide in methanol for 15 min. Antigen retrieval slides were heated 3 times in 10 mM citric acid buffer in a microwave oven for 5 min. The diluent and wash buffer used throughout the immunostaining procedure was PBS. After blocking endogenous peroxidase and nonspecific reactivity, the sections were incubated with anti-CK13 rabbit polyclonal antibody (dilution ×500; ab58744, Abcam, London, UK), anti-CK14 mouse monoclonal antibody (dilution ×100; clone LL002; Novocastra, Newcastle upon Tyne, UK), anti-CK17 mouse monoclonal antibody (dilution ×200; clone E3, Novocastra, Newcastle upon Tyne, UK), and anti-CK19 mouse monoclonal antibody (dilution ×500; clone b170; Novocastra, Newcastle upon Tyne, UK) at room temperature for 1 hr. The sections were incubated with biotinylated secondary antibody and then peroxidase-labeled streptavidin (LSAB2 kit, DAKO, Tokyo, Japan). Immunolabeled proteins were visualized using 3,3’-diaminobenzidine. The sections were then counterstained with Mayer’s hematoxylin and mounted. Negative controls were processed in the same manner.

5. Evaluation of immunostaining

Over 500 epithelial cells were counted in the central part of each lesion and labeled as no change, dysplasia, or cancer. Counts were performed in one microscopic field in each section at 200× magnification. The following areas were identified: no change, upper layer normal epithelium including the basal cell layer; dysplasia, upper area of epithelial dysplasia located on the basal and parabasal cell layer; cancer, cancerous lesion including interstitial cells. Color revision was performed on the images, which were taken at 200× magnification. Counts were performed in all fields of vision. The ratio (%) of positive cells was calculated and the mean values obtained
from each lesion used as labeling indices (LI).

6. Microdissection

Immunohistochemical staining was performed on 12 of the sections prepared for RNA extraction; 8 samples each with no change, dysplasia, and cancer were extracted from the same section of each case. A total of 24 carcinoma samples were obtained by microdissection to avoid excessive contamination from normal tissues. Individual 10-μm-thick frozen tissue sections were cut using a microtome and placed on glass slides. The central part of individual areas showing no change, dysplasia, or cancer was microdissected.

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

Fragments were amplified from the mRNAs for CK13, 14, 17, 19, and GAPDH using 111-, 148-, 134-, 159-, and 307-bp RT-PCR templates, respectively; Table 1 shows the primer sequences. A total of 10 μl reverse transcription buffer containing 1 μg RNA, 0.2 μg oligo-dT primers, 0.5 mM dNTP, 5U RNAsin, and 100 U MMLV-RT (Takara Shuzo Co., Kyoto, Japan) was incubated at 42°C for 15 min; the reaction was terminated by heating at 95°C for 2 min. From this cDNA solution, 2 μl was removed to be subsequently used for the RT-PCR reaction. The TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA, US), which contains 0.2 μl forward and reverse primers and 0.2 μl TaqMan probe was used at 20 μl/tube. The RT-PCR assay was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) with the following profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles each 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.

8. Statistical analysis

Levels of CK13, 14, 17, and 19, together with their LI and RT-PCR findings in no change, dysplasia, and cancer samples were statistically compared using Tukey’s HSD test. A p-value of below 0.05 was considered significant.

Results

1. Immunohistochemical analysis

Figure 2 shows staining for CK13. In no change, positive cells were detected in the basal cell and prickle cell layers (Fig. 2a); in dysplasia, positive cells were seen in the basal cell and prickle cell layers (Fig. 2b); in cancer, posi-
tive cells were hardly detected in keratinocytes around the cancer pearl (Fig. 2c). The LI for no change, dysplasia, and cancer was 63.2 ± 12.8%, 49.8 ± 17.7%, and 19.8 ± 8.7%, respectively. The level of CK13 expression was the highest for no change, which significantly differed from that for cancer (p < 0.01). The level also significantly differed between dysplasia and cancer (p < 0.01; Fig. 3).

Figure 4 shows staining for CK14. In no change, positive cells were detected in the basal cell layers (Fig. 4a); in dysplasia, positive cells were seen in the basal cell layers (Fig. 4b); in cancer, positive cells were hardly detected in keratinocytes around the cancer pearl (Fig. 4c). The LI for no change, dysplasia, and cancer was 33.2 ± 9.9%, 29.1 ± 10.8%, and 20.5 ± 9.2%, respectively. The level of CK14 expression was the highest for no change, and significantly differed from that for cancer (p < 0.01; Fig. 5).

Figure 6 shows staining for CK17. In no change, positive cells were detected in the basal cell and prickle cell layers (Fig. 6a); in dysplasia, positive cells were seen in the basal cell and prickle cell layers, and the rate of expression was higher than for no change (Fig. 6b); in cancer, positive cells were detected in keratinocytes around the cancer pearl (Fig. 6c). The LI for no change, dysplasia, and cancer was 13.4 ± 7.9%, 19.2 ± 10.8%, and 36.7 ± 19.9%, respectively. The level of CK17 expression was the highest for cancer, which significantly differed from that for no change (p < 0.01). The level also significantly differed between dysplasia and cancer (p < 0.01; Fig. 7).

Figure 8 shows CK19 staining. In no change, positive cells were detected in the basal cell layers (Fig. 8a); in dysplasia, positive cells were seen in the basal cell and prickle cell layers (Fig. 8b); in cancer, positive cells were seen in the basal cell and prickle cell layers, and the rate of expression was higher than for no change (Fig. 8b); in
cancer, positive cells were detected in keratinocytes around the cancer pearl (Fig. 8c). The LI for no change, dysplasia, and cancer was 50.0 ± 19.2%, 53.2 ± 18.5%, and 62.7 ± 19.8%, respectively. The level of CK19 expression was the highest for cancer, which significantly differed from that for no change (p < 0.01; Fig. 9).

2. Expression of mRNA assessed by RT-PCR

The level of CK13 mRNA for no change, dysplasia, and cancer was 92.5 ± 13.1%, 73.6 ± 21.0%, and 44.7 ± 15.0%, respectively. The level of CK13 expression was the highest for no change. The level differed significantly between each condition (p < 0.05; Fig. 10).

The level of CK14 mRNA for no change, dysplasia, and cancer was 69.6 ± 26.1%, 59.1 ± 14.0%, and 58.0 ± 16.0%, respectively. The level did not differ significantly between each condition (Fig. 11).

The level of CK17 mRNA for no change, dysplasia, and cancer was 50.6 ± 19.4%, 64.2 ± 10.3%, and 94.4 ± 10.4%, respectively. The level of CK17 expression was the highest for cancer, which significantly differed from that for no change (p < 0.01). The level differed significantly between dysplasia and cancer (p < 0.01; Fig. 12).

The level of CK19 mRNA for no change, dysplasia, and cancer was 75.6 ± 17.6%, 87.3 ± 11.4%, and 106.4 ± 9.9%, respectively. The level of CK19 expression was the highest for cancer, which significantly differed from that for no change (p < 0.01). The level also significantly differed between dysplasia and cancer (p < 0.01; Fig. 13).

Discussion

The handling of epithelial dysplastic spread around a cancer is very important, and morphological features are presently assessed by histopathological means. However, recent
studies have indicated that the expression of several proteins, including p53 and Ki-67, is also useful in assessing epithelial dysplasia.

Currently, the most effective way of assessing the malignant potential and cancerous properties of epithelial dysplasia around a cancer is to thoroughly assess the cellular morphological features of tissue samples by breadloaf step sectioning and then to measure the expression of various proteins on serial sections.

Cytokeratins have been used as tumor markers for epithelial tumors since Moll et al. identified their expression profiles in normal human tissues, cultured cell lines, and cancer cells. Furthermore, the characteristics of CK in squamous cell carcinoma differ from those in normal tissues, unlike in glandular epithelial tumors.

Low CK14 and high CK19 expression in early cancer and precancerous lesions could serve as indicators of malignant potential. Hence, it is clear that change in the expression of cytokeratin 13 and 17 is related to carcinogenesis. Combined assessment of CK13 and CK17 may make the early clinical diagnosis of the malignant potential of epithelial dysplasia possible. Furthermore, poorer differentiation is associated with lower CK13 expression and CK17 is expressed during the malignant transformation of oral squamous epithelial cells. In the present study, poorer differentiation was associated with lower expression of CK13 and CK14 and higher expression of CK17 and CK19.

The non-keratinization marker CK13 is found in simple epithelium and in some stratified epithelia. Expression of CK13 weakens as the level of differentiation decreases from normal epithelium to epithelial dysplasia and squamous cell carcinoma. The basal cell layer of normal oral mucosal epithelia expresses CK14 and normal epithelial cells express CK19. Less differentiated oral cancers are associated with higher CK19 expression.
Cytokeratin 19 is expressed in the basal cell layer of the oral mucosa during early invasive cancer, but not in advanced cancer\(^{20}\), normal tissue, or epithelial dysplasia. However, CK19 is expressed in carcinoma \textit{in situ}\(^{16}\), suggesting that change in CK19 expression is involved in carcinogenesis. Cytokeratin 17 exists in simple epithelia and some stratified epithelia\(^{1}\). Domachowske et al. suggested that the transcriptional activity of the CK17 gene depends on nuclear factor \(\kappa B\)^{2}, the anti-apoptotic properties of which are activated by apoptotic stimulation due to tumor necrosis factors in various cultured head and neck cancer cell lines\(^{10}\). Cytokeratin 17 is associated with cellular growth and protein synthesis by activating the cytoplasmic mTOR signal series due to 14-3-3 proteins\(^{4}\). Only CK17 is proven to be involved with carcinogenesis, and the involvement of other CKs awaits clarification.

The present study found that the immunostaining LI for CK13 and 14 was the highest for no change, decreasing in the order of dysplasia and cancer; whereas that for CK17 and 19

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Fig. 8 Immunohistochemical staining of CK19 under each condition
(a) No change (original magnification \(\times 200\)), (b) Dysplasia (original magnification \(\times 200\)), (c) Cancer (original magnification \(\times 200\)).

Fig. 9 Average CK19 labeling index under each condition
Horizontal center line in each box indicates median and vertical line staining ratio (%). Upper edge of each box shows third and lower edge first quartile deviation. **\(p<0.01\)

Fig. 10 RT-PCR relative values of CK13 under each condition
Horizontal center line in each box indicates median and vertical line relative mRNA expression ratio of CK13 and GAPDH. Upper edge of each box shows third and lower edge first quartile deviation. *\(p<0.05\) **\(p<0.01\)
was the highest for cancer, decreasing in the order of dysplasia followed by no change. Similar results were obtained by RT-PCR. The immunostaining levels of each CK for dysplasia were between those for no change and cancer. Because the expression of CK13 significantly differed among no change, dysplasia, and cancer, we speculated that malignant transformation involved loss of CK13 expression. Also, significantly more CK17 and 19 was expressed in cancer than in no change or dysplasia, suggesting that CK17 and 19 are involved in the progression from epithelial dysplasia to cancer. Expression of CK14 did not significantly differ between each condition.

The immunohistochemical staining profiles of CK13 and CK14 showed a similar tendency, but CK14 was expressed only in the basal cell layer, whereas CK13 was expressed in the basal cell and prickle cell layers. This suggests that CK13 has stronger diagnostic potential than CK14, even allowing for error.

In addition, neither CK13 nor CK14, which represent a higher molecular form of keratin, were expressed when the degree of differentiation was low, whereas CK17 and 19 were, albeit at a low molecular weight. This suggests that type of CK expression could indicate degree of differentiation.

The present results indicate that evaluating the immunohistochemical staining profile of CK13 aids the morphological assessment of epithelial dysplasia. In addition, they also indicate that CK13 offers higher diagnostic value than CK14, 17, or 19.

The present study focused on CK, but further multifaceted assessments can be achieved by combining the present findings with the results of previous studies\textsuperscript{13,14}. More human studies can be based on these data, and microarrays should be developed to investigate the
expression of various proteins to identify those that would be useful in evaluating the characteristics of epithelial dysplasia. Resection stumps should be assessed using serial sections prepared by microdissection and a diagnostic cytological test should be established to screen for malignant potential.

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