An Immunohistochemical Study of Oral Carcinoma Cuniculatum

Yan Sun,¹ Kayo Kuyama,² Arne Burkhardt,³ and Hirotugu Yamamoto⁴

¹Nihon University Graduate School of Dentistry at Matsudo, Oral Pathology, Matsudo, Chiba 271-8587, Japan
²Department of Oral Pathology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan
³Department of Pathology, Kreiskliniken Reutlingen, Reutlingen, Germany

Correspondence to:
Kayo Kuyama
E-mail: kuyama.kayo@nihon-u.ac.jp

Abstract
An immunohistochemical study of oral carcinoma cuniculatum (CC) was performed to clarify the factors that contribute to the unique architectural features in CC. Sixteen lingual specimens, consisting of six CC, six conventional squamous cell carcinoma (SCC), and four verrucous carcinoma (VC), were studied. From the immunohistochemical findings, cytokeratin 10 and cytokeratin 13 showed greater positive expression in the prickle layer of CC than conventional SCC and VC. Gene-related p53, Ki-67, and p63 revealed low immunopositive rates in CC and high positive rates in the basal layer of conventional SCC and VC. These results suggested that the tumor cells of CC were well-differentiated with low proliferative activity. Regarding cell–cell adhesion molecules, laminin 5γ2 was revealed strongly in the basal layer of CC, with thinner and discontinuous distribution in conventional SCC. Although E-cadherin was negative or weakly positive in conventional SCC and VC, it was remarkable in the prickle and basal layers of CC compared with the other two lesions. It was supposed that cell–cell adhesion molecules participate in the construction of the unique architecture of CC. From these results and a review of the literature, independent branching crypts such as the rabbit caves of CC could be formed by the restriction of invasiveness and migration by the comparatively firm cell–cell linkage and low proliferative activity of tumor cells.

Keywords:
carcinoma cuniculatum, immunohistochemical study, cytokeratins, differentiation, proliferation, cell–cell adhesion

Introduction
Carcinoma cuniculatum (CC) is a rare tumor of the oral cavity. In 2005, oral CC was first described in the World Health Organization (WHO) guidelines as a new variant characterized by cuniculatum architecture, which was similar in appearance to rabbit burrows, and formed by keratin-filled branching crypts and keratin cores (1), although some authors described CC as the same lesion as verrucous carcinoma (2). The special growth pattern of burrows and unique architecture make it different from other variants of oral squamous cell carcinoma (SCC). Case reports (3, 4) and some series studies (5, 6) of oral CC have described its clinical and histopathological attributes. From immunohistochemical studies of oral CC, the expression of p53 is negative (7) and Ki–67 shows high proliferative activity (8). Keratins are the typical intermediate filament proteins of epithelia, showing an outstanding degree of molecular diversity (9). Cell cycle-regulated proteins often exhibit aberrant patterns of expression in tumor lesions. Cell–cell and extracellular proteins are found on the surface of all cells and play an important role in tumor cell proliferation and transforming activity (10). Some adhesion molecules are crucial in the development of recurrence, invasion, and distant metastasis, which have important consequences for tumor growth (10, 11). It is hypothesized that cell differentiation, proliferative activity, and cell–cell adherence of tumor cells contribute to the unique construction of CC. However, comparative studies among CC, conventional SCC, and VC are
The purpose of this research was to clarify the factors such as proliferation, differentiation, and cell–cell adhesion that contribute to the unique architecture of CC. A comparison study among CC, conventional SCC, and VC with special reference to immunohistochemical staining of cyto-keratins, cell cycle-related genes, and cell-adhesive proteins was performed.

**Materials and Methods**

**Histopathological specimens**

Sixteen lingual specimens were taken from oral pathology files at the Dental Hospital of the Nihon University School of Dentistry at Matsudo. They consisted of six CC, six conventional SCC, and four VC. One set of sections was stained with hematoxylin and eosin (H.E.) and reviewed by oral pathologists to confirm the diagnosis and observe the histological characteristics and the other set was used for an immunohistochemical study. The protocol was approved by the Committee on Studies Involving Human Beings of Nihon University School of Dentistry at Matsudo E05-002. Informed consent was obtained from all patients before retrieving the pathological specimens.

**Immunohistochemical staining**

Paraffin-embedded tissue sections (4 μm thick) were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed with citrate buffer (pH 6.0) or 1 mM Tris–EDTA (pH 9.0) depending on the antibodies, followed by incubation with 3% hydrogen peroxide to quench endogenous peroxidase. The sections were then incubated in primary antibody incubation for 1 h at room temperature. Information on the antibodies is shown in Table 1. Sections were then incubated in the secondary antibody by means of the polymer method (ChemMate Envision, K5027; Dako, Glostrup, Denmark) for 1 h, developed in diaminobenzidine (Dako), and counterstained in Mayer’s hematoxylin. Positive controls for p53, Ki-67, and laminin 5γ2 were specimens of SCC of the endocervix, that for integrin α6 was specimen of inflammatory granulation tissue, and those for E-cadherin, cytokeratin 10 (CK10), and cytokeratin 13 (CK13) were specimens of healthy mucosa. For negative control studies, the primary antibodies were replaced with mouse and rabbit universal negative controls (Dako).

**Immunohistochemical evaluation**

Two observers independently evaluated the immunostaining results. The concordance ratio was >90%. Differences of opinion were resolved by reaching a consensus with the assistance of a third evaluator. The intensity of tissue staining was graded semiquantitatively on a 4-point scale: negative (−), weak positive (+), moderate positive (±), and strong positive (+ +).

The immunohistochemical expression modes of p53, Ki-67, and p63 were evaluated by counting immunopositive cells in five unit fields (1 mm²). Using ×200 magnification, the numbers of positive–stained nuclei for p53, Ki-67, and p63 were counted in 1,000 cells. The positivity rates of p53, Ki-67, and p63 were calculated in basal layer cells and/or all tumor cells.

**Results**

**Histopathological findings**

The histopathological and immunohistochemical findings are shown in Table 2. The histopathological

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**Table 1. The antibodies detailed of immunohistochemical study**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>CK10</td>
<td>Dako</td>
<td>DE-K10</td>
<td>×100</td>
</tr>
<tr>
<td>CK10/13*</td>
<td>Dako</td>
<td>DE-K13</td>
<td>×50</td>
</tr>
<tr>
<td>p53</td>
<td>Dako</td>
<td>DO-7</td>
<td>×50</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Dako</td>
<td>TEC-3</td>
<td>×50</td>
</tr>
<tr>
<td>p63</td>
<td>GeneTex</td>
<td>4A4</td>
<td>×200</td>
</tr>
<tr>
<td>Laminin 5γ2</td>
<td>Dako</td>
<td>4G1</td>
<td>×50</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Dako</td>
<td>NCH-38</td>
<td>×50</td>
</tr>
<tr>
<td>Integrin α6</td>
<td>abcam</td>
<td>MP4F10</td>
<td>×50</td>
</tr>
</tbody>
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*Antibody cytokeratin CK10/13 reacts with the 53kDa protein corresponding to cytokeratin 13 on formalin fixed paraffin-embedded tissue sections.
characters of CC, conventional SCC, and VC appear in Fig. 1a–3a. In CC, the unique architecture of rabbit burrows from which the Latin term “cuniculatum” was derived was seen and was composed of branching crypts. They were filled with keratin and surrounded by well-differentiated squamous epithelium (Fig. 1a). In conventional SCC, tumor cells proliferated and destroyed the basement membrane and formed compact masses that invaded the subjacent connective tissue. Well-differentiated cancer cells had transformed into keratinized squamous cells and formed characterized keratinous pearls (Fig. 2a). Verrucous carcinoma was characterized by a verrucous growth pattern with marked keratosis arranged in a “church-spire” configuration. The verrucous growth was usually limited to the lamina propria, consistent with club-shaped papillae and blunt stromal invaginations (Fig. 3a).

**Immunohistochemical findings**

In the three lesions, CK10 showed positive expression in the prickle layer, although the intensity was different among lesions. The immunopositive stain was stronger and more defined in CC than in VC and SCC (Figs. 1b–3b). Concerning CK13, CC had more positive expression in the prickle layer. Conventional SCC showed scattered positive expression and VC showed moderately positive expression (Figs. 1c–3c). The p53-positive cells of CC accounted for 33.7% of all tumor cells and 27.6% of cells in the basal layer; conventional SCC had the highest immunopositive rate of 63.2% in all tumor cells and 68.4% in the basal layer; the immunopositive rate of VC was between those of CC and conventional SCC, with 43.9% of all cells and 64.5% of cells in the basal layer (Figs. 1d–3d). The proliferation marker Ki-67 had a 20.2% immunopositive rate in CC with 19.4% positive in the basal layer; it was highest in conventional SCC at 58.6%, while VC was 42.0%. However, SCC and VC had almost the same positive rate in the basal layer (Figs. 1e–3e). The immunopositive rates of p63 in the basal layer were 58.5% in CC, 83.0% in conventional SCC, and 88.2% in VC (Figs. 1f–3f). Laminin 5γ2 stained strongly in the basal layer in CC, but showed a thinner, weaker, and discontinuous appearance in conventional SCC, and a negative or sparsely positive appearance in VC (Figs. 1g–3g). E-cadherin showed strongly in the prickle layer of CC and negatively or weakly positive in conventional SCC and VC (Figs. 1h–3h). For integrin α6, positive findings were observed in the prickle and basal layers of CC. In conventional SCC, findings were negative in the basal layer and weakly positive in the prickle layer. In VC, findings were weakly positive in the basal layer and moderately positive in the prickle layer (Figs. 1i–3i).

**Discussion**

Carcinoma cuniculatum, a rare subtype of oral SCC, is characterized by the unique architecture of rabbit burrows consisting of branching crypts and keratin cores. Because the histological architecture
resembled that of rabbit burrows, the entity was named carcinoma cuniculatum (“cuniculatum” means rabbit burrows in Latin). This distinctive morphology characterized by burrowing channels became the point of discrimination between CC and other SCC variants. This comparative study using immunohistochemical staining was performed for the purpose of clarifying the factors that contribute to the unique architecture of CC.

Keratins are the intermediate filament proteins that constitute epithelial cells and they have a differential expression in epithelial tissues (12). CK10 was found to be expressed in the terminally differentiating epithelial cells (13) and loss of CK10 led to increased keratinocyte turnover (9). In oral SCC, CK10 was sparser in poorly differentiated tumors but could still be detected in nearly 50% of cases (9). CK10 appeared more strongly positive in the prickle layer of CC than in that of conventional SCC and VC in the present study. The positive cells with terminal differentiation meant CC was a low malignant tumor. CK13 is also particularly important as a component of mucosal stratified squamous epithelium. CK13 is usually localized through the healthy epithelial layers except the basal layer (14). In CC, CK13 had the same immunohistochemical expression strength as CK10 and presented a stronger positive expression in CC than in the other lesions. The absence of CK13 in conventional SCC and VC suggested that the cancer cells lost some capacity for keratin differentiation. Moreover, a previous study demonstrated that the loss of CK13 expression in lingual SCC was a possible sign of local recurrence, which indicated stronger aggression of the tumor (15). The immunopositive results of CK10 and CK13 in CC constituted the keratinized stratified squamous epithelium located in the circumference of the branching crypts, which are one of the characteristic organization constructions, and showed clearly that a tumor was well differentiated.

Cell cycle-regulated proteins often exhibit aberrant patterns of expression in tumor lesions. As a well-known tumor suppressor gene, p53 is a dominant transforming oncogene and the up-regulation of p53 might result in defective apoptosis and subsequent tumor progression in the oral cavity and tongue (16, 17). In our three lesions, immunopositive rates of p53 were 80.2% in conventional SCC, 76.1% in VC, and 20.3% in CC. The positive rates in the basal layer cells of conventional SCC and VC were much higher than that of CC. Presumably, high immunopositive basal layer cells that had lost regulation of apoptosis might gain high proliferation and migration abilities.

Ki-67 is a classic proliferation-associated human nuclear antigen and is expressed in all continuously cycling cells of G1, S, G2, and M phases, but not in resting cells in G0 (18). Overexpression of Ki-67 indicates active proliferation of tumor cells. In the three lesions of this study, the positive expression was stronger in conventional SCC and VC, as compared with CC. The higher Ki-67 positive rates of conventional SCC and VC showed the conspicuous aggressiveness of these two lesions and suggested the low
proliferation biological behavior of CC.

The p53 family member p63 plays an essential role in the developing epithelium, governing the establishment and maintenance of multilayered epithelium (19). Romano et al. (20) mentioned that p63 expression often increased with increasing tumor grade. A statistical relevance was found between the grading of the neoplasm and the p63 expression (21). Of our three lesions, p63 expression decreased in CC, while it was diffuse and highly positive in the basal layer of conventional SCC and VC. The high positive rate of p63 has been associated with a poor degree of differentiation (20) and a poor prognosis (21). Conversely, a previous report (19) mentioned that p63 down-regulation caused cell cycle arrest in keratinocytes with p53-dependence and that subsequent p63 up-regulation reversed cell cycle arrest.

The adhesive interactions of cell-cell and cell-extracellular matrix are an essential function controlling differentiation, adhesion, invasion, and formation of epithelial barriers (10). Evidence to date (11) suggests that cell adhesion molecules might be associated with invasion and metastasis in various human malignancies. Evaluation of the cell-cell adhesion molecules in oral CC has not yet been studied.

Laminin 5, consisting of α3, β3, and γ2 chains, is an important cell adhesion molecule. Overexpression of laminin 5γ2 has been observed at the invasive front of the tumors, which might be associated with the depth of invasion (22). The intensely immunopositive basal layer cells of CC indicated the invasion front and suggested deep invasion, which may reflect the growth pattern of deep burrows. Moderate discontinuous cytoplasmic positivity had also been shown in conventional SCC with a diffuse pattern of expression, which might be a result of fragmentation generated by basement membrane degradation during tumor proliferation (23) and suggests a comprehensive invasion into surrounding tissues.

Cadherins are a large family of cell-cell adhesion molecules and E-cadherin is the major mediator of cell-cell adhesion in epithelial cells, required for the formation of intermediate and adherent junctions (24). Loss of E-cadherin expression has been noted with poorly differentiated morphology in a large number of malignancies (26). Down-regulation of E-cadherin enhances the proliferation of head and neck cancer (25). In our series, E-cadherin showed stronger and thicker positive expression in the basal layer of CC, in contrast to the negative or sparse immunopositive findings in conventional SCC and VC. The low immunopositive expression of E-cadherin in conventional SCC and VC might indicate reduced cell-cell adhesiveness and was associated with a loss of contact inhibition of proliferation, thereby allowing tumor cells to escape from growth control. Conversely, with a high immunopositive expression of E-cadherin, CC possessed relatively firm cell-cell contact, making rapid uncontrolled growth difficult. E-cadherin accumulation in tumor cells is hypothesized to be one factor of the independent branching crypt construction that is characteristic of CC.

Integrins are important extracellular matrix (ECM) receptor proteins located on cell surfaces. There are 18α and 8β subunits that can assemble into 24 different receptors with different binding properties and different tissue distributions (27). Of these, integrin α6β4 is highly selective for laminin 5 receptors (28) and the immunopositive integrin α6 subunit demonstrates the distribution of the hemidesmosome integrin α6β4 (29). Loss of the α6 and β4 subunits is more common in poorly differentiated tumours (30). In line with this, immunolocalization of integrin α6 was shown to be negative or sparsely positive in the prickle layer of conventional SCC, while it was more extended in the prickle and basal layers of CC. The continuous, stronger expression in the ECM of CC suggests a stable physical linkage of cells to each other and to the ECM, which restricts invasiveness and migration. There is evidence that the lack of restricted basal polarization of α6 could be an early but non-specific marker of oral malignancy (31).

Our findings suggest that CC is a wild tumor variant and in concordance with the description of the biological character in the World Health Organi-
zation guidelines: lacking obvious cytological features of malignancy. Consequently, it was supposed that cell-cell adhesion molecules participated in the construction of CC. Furthermore, the expression of cell-cell adhesion molecules reflected the growth pattern of deep burrows and stable physical linkage of cells limited the epithelial barriers, consequently forming the typical branching crypts.

References
23. Tamaki Y, Kuyama K, Sun Y: A histopathological
and immunohistochemical study of basal membrane alterations, with special reference to advance of dysplastic grading. IJOMS, 10: 12–19, 2011.