A Histopathological and Immunohistochemical Study on Epithelial Proliferative Activity of Adenomatoid Odontogenic Tumor with Special Reference to Comparison with Ameloblastoma

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Abstract
Adenomatoid odontogenic tumor (AOT), which is histopathologically composed of pseudoductal cells intermingled with polygonal cells, is a rare odontogenic lesion. On the other hand, ameloblastoma (AM) which tends to grow slowly is most common odontogenic tumor of the jaws. The aim of this study was to compare with the proliferative activity in epithelium of AOT and AM. In addition, the role of cellular proliferation in the pseudoductal cells and polygonal cells of AOT was immunohistochemically and histochemically investigated using several proliferative markers as follows: Ki-67, PCNA and AgNORs. These labelling indexes were calculated in them. The mean Ki-67 and PCNA labeling indexes in AOT were statistically lower than those in AM ($p < 0.05$). And about AOT, the mean Ki-67 labeling index in pseudoductal cells was statistically higher than that in polygonal cells ($p < 0.05$). The present study showed the difference proliferative activity between pseudoductal cells and polygonal cells might depend on cell differentiation.

Keywords:
adenomatoid odontogenic tumor, ameloblastoma, Ki-67, PCNA, AgNORs

Introduction
The histopathological characteristics of adenomatoid odontogenic tumor (AOT) are that the neoplastic parenchyma consists of cuboidal to columnar and polygonal cells. The cuboidal to columnar cells are arranged in the periphery of the parenchyma, and form a duct- or rosette-like structure. The polygonal cells present inner side of the parenchyma or around the cuboidal to columnar cell component. The parenchyma is encapsulated by fibrous connective tissue (1–3). AOT was described by Dreblat in 1907 as pseudoadenoma adamantumin (1, 4), and was also called adenoameloblastoma or adenomatoid ameloblastoma as a subtype of ameloblastoma (AM) in the past (4–7). However, since AOT differs from AM in clinical findings, age predilection, site preference, the presence of impacted teeth, and histological morphology, it was described as a separate pathological entity under the 1971 WHO classification (8).

Although AOT is classified as a tumor under the 2004 WHO classification, some opinions claim that AOT is not a true tumor; thus, no conclusion has been reached as yet (3).

A tumor is a mass of tissue resulting from the autonomic over proliferation of cells of the body, and has the fundamental property of undergoing unregulated, unlimited proliferation (9). Therefore, it is important to accurately evaluate the proliferative activity of tumor cells in determining the benign or malignant nature of the tumor and predicting the prognosis, and many researchers have reported on the correlation between the proliferative activity of tumors and clinical features (10, 11). As methods of determining tumor proliferative activity, immunohistochemical methods using anti-human Ki-67 antibody (Ki-67) and anti-human proliferating cell nuclear antigen antibody (PCNA) and histochemical methods using silver-binding nucleolar organizer regions (AgNORs) are known, and many studies have reported that indexes based on these methods are
effective for evaluating proliferative activity (12–15). Several studies in the oral region have the proliferative activity of AM and odontogenic keratocysts (14, 16–22), but few reports have comparatively examined and comprehensively studied the epithelial proliferative activity of AOT in detail.

This study was immunohistochemically and histochemically compared the epithelial proliferative activity of AOT and AM using Ki-67, PCNA, and AgNORs to determine the nature of AOT as a disease.

Materials and Methods
Materials
Eight specimens resected and histopathologically diagnosed as AOT at the Nihon University Dental Hospital at Matsudo were examined. As controls, 10 AM specimens resected at the same hospital were selected such that they were histologically of the follicular or plexiform type and clinically primary, with no confirmed recurrence. The clinical data for 18 cases are summarized in Table 1.

Before collecting specimens, the attending physicians fully explained to the patients all the methods and processes of studies using these specimens and the preparation of this paper, and obtained their consent.

Histopathological examination
The surgically resected tissues were fixed in 10% neutral formalin solution, and embedded in paraffin by routine techniques. Sections were cut at 4 μm, stained with hematoxylin and eosin (HE), and observed under a light microscope. Tumors were diagnosed according to the WHO classification (23).

Immunohistochemical and histochemical stainings for proliferative activity
The above sections were reacted with the primary antibody to Ki-67 (1 : 50, DAKO Japan) and PCNA (1 : 50, DAKO Japan), and stained by the dextran polymer method (Envision+ kit, DAKO Japan) as follows. The sections were deparaffinized in xylene, hydrated in a series of ethanol, immersed in 0.01 M phosphate-buffered saline (PBS, pH 6.4), and subjected to microwave treatment (5 min at 100°C three times; DR-T510, Uchida Seisaku Co., Ltd.). Endogenous peroxidase was blocked by 3% H2O2 for 10 min, and the sections were washed in 0.01 M Tris-buffered saline (TBS, pH 7.4), and reacted with the

| Table 1. Clinicopathological characteristics of patients with AOT |
|-----------------|----------|---|----------------|-----------|
| Diagnosis | Case No. | Sex | Age | Site | |
| AOT | 1 | Female | 17 | Anterior, Maxilla | |
| | 2 | Female | 16 | Anterior, Mandibular | |
| | 3 | Female | 12 | Anterior, Mandibular | |
| | 4 | Female | 60 | Anterior, Mandibular | |
| | 5 | Male | 15 | Premolar area, Maxilla | |
| | 6 | Male | 14 | Premolar area, Maxilla | |
| | 7 | Male | 14 | Premolar area, Maxilla | |
| | 8 | Male | 22 | Posterior, Mandibular | |
| AM | 1 | Male | 47 | Molar area, Mandibular | |
| | 2 | Female | 47 | Anterior area, Mandibular | |
| | 3 | Male | 54 | Anterior area, Maxilla | |
| | 4 | Female | 22 | Molar area, Mandibular | |
| | 5 | Female | 44 | Molar area, Mandibular | |
| | 6 | Female | 38 | Anterior-Molar area, Mandibular | |
| | 7 | Male | 56 | Anterior-Premolar area, Mandibular | |
| | 8 | Female | 8 | Molar area, Mandibular | |
| | 9 | Male | 44 | Anterior-Molar area, Mandibular | |
| | 10 | Female | 35 | Molar area, Mandibular | |
primary antibody at room temperature for 60 min, then with the Envision reagent at room temperature for 60 min. Color was developed with 3,3-diaminobenzidine terahydrochloride (DAB), and the sections were counter stained with Mayer’s hematoxylin, dehydrated, cleared, and mounted. Primary serum instead of the primary antibody was used for a negative control. Squamous cell carcinoma of the tongue served as a positive control.

After deparaffinization, AgNORs staining was performed according to the method of a previous our study (4). Sections were immersed in AgNORs stain for 40 min in the dark, washed in water, left in 0.2% silver nitrate solution for 10 min, washed in water, immersed in a fixed solution (5% sodium thiosulfate), dehydrated, cleared, and mounted. Instead of AgNORs stain, distilled water was used for a negative control, and squamous cell carcinoma of the tongue was used as a positive control.

Cell classification

For microscopic examination, AOT-composing cells were classified into cuboidal to columnar cells (simply referred to as pseudoductal cells below) with a pseudoductal or rosette-like structure and polygonal and/or spindle to columnar cells (simply referred to as polygonal cells below) present around them. AM-composing cells were classified into columnar cells adjacent to the stroma whereas stellate reticulum–like cells in the center of the tumor.

Determination of positive cells, calculation of the Ki–67 labeling index (Ki–67 L.I.), PCNA labeling index (PCNA L.I.) and AgNORs labeling index (AgNORs L.I.), and statistical analysis

Ki–67 or PCNA expression was judged to be positive if cell nuclei were granularly or diffusely stained brown. AgNORs were judged to be positive if nucleoli were ovoid or spindle-shaped, and were stained brown. Under a light microscope, 5 fields of “hot spots” with a high frequency of positive cells at 400-fold magnification were selected, the total cells and positive cells per field were counted, and the percentage of the positive cells to the total cells (positive cell count/total cell count × 100) was calculated as the Ki–67 L.I. or PCNA L.I. Similarly, 5 hot spot fields at 600-fold magnification were counted for positive nucleoli, and the number of the positive nucleoli to the total cells (positive nucleolus count/total cell count) was calculated as the AgNORs L.I. Unpaired Welch’s t-test was used for intergroup comparison. P values less than 5% were considered significant, and those less than 10% were considered to tend to be significant.

Results

Histopathological findings

The AOT parenchyma was encapsulated by a relatively thick fibrous connective tissue, and composed of relatively dense epithelial cells, with scattered pseudoductal or rosette-like structures. Many lumens were near-circular, surrounded by a single layer of pseudoductal cells with an ovoid to spindle-shaped nucleus located opposite the lumen. The lumens were generally empty, but some of them contained eosinophilic amorphous materials. Outside the lumen–forming cells, masses of polygonal and/or spindle cells proliferated, and some areas resembling the stellate reticulum or containing cells with clear cytoplasm were seen. The so-called rosette-like structure with eosinophilic material between cells was also observed. Also, small calcification with a laminated or radiating structure was seen scattered chiefly in masses of polygonal cells of the parenchyma.

The AM parenchyma showed a follicular or plexiform appearance or a mixture of both. Adjacent to the stroma, the parenchyma resembled the enamel organ, and contained an alignment of columnar cells, and stellate reticulum–like cells were present inside of the parenchyma. The columnar cells at the base were relatively high in density, had an oval nucleus, and showed morphology similar to that of ameloblasts. The inner stellate reticulum–like cells were somewhat low in density, had a spindle-shaped nucleus, and showed a squamous epithelium–like structure with cystic formation due to degeneration and disappearance or a tendency toward keratiniza-
tion. The stroma consisted of fibrous connective tissue.

**Immunohistochemical and histochemical findings**

Table 2 shows the observation results.

**Ki-67 staining results**

The mean Ki-67 L.I. of AOT and that of AM were 5.3±1.7% and 7.5±2.5%, respectively, showing a significant difference ($P<0.05$). In AOT, the mean Ki-67 L.I. of pseudoductal cells and that of polygonal cells were 6.9±2.3% and 4.5±1.4%, respectively, showing a significant difference ($P<0.05$) (Fig. 1). In AM, the mean Ki-67 L.I. of columnar cells and that of stellate reticulum-like cells were 9.3±3.0% and 6.6±3.4%, respectively, the former tending to be higher than the latter ($P<0.1$) (Fig. 2).

**PCNA staining results**

The mean PCNA L.I. of AOT and that of AM were 20.7±11.3% and 32.9±9.2%, respectively, showing a significant difference ($P<0.05$). In AOT, the mean PCNA L.I. of pseudoductal cells and that of polygonal cells were 22.1±12.7% and 19.7±10.6%, respectively, showing no tendency toward a significant difference (Fig. 3). In AM, the mean PCNA L.I. of columnar cells and that of stellate reticulum-like cells were 36.3±10.6% and 26.9±10.4%, respectively, the former tending to be higher than the latter ($P<0.1$) (Fig. 4).

<table>
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<tr>
<th>Table 2. The Ki-67 labeling index, PCNA labeling index and AgNORs labeling index in AOT and AM</th>
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<tr>
<td><strong>AOT</strong></td>
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<td>Pseudoductal cells</td>
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a: statistically significant $P<0.01$, b: statistically significant $P<0.05$, c: significant $P<0.1$, d: not significant

Mean Ki-67 labeling index (±standard deviation), mean PCNA labeling index (±standard deviation) and AgNORs labeling index (±standard deviation) are shown for AOT and for AM in each cell type as well as in the whole tumor.
Fig. 1. Adenomatoid odontogenic tumor (AOT) is composed of pseudoductal cells intermixed with polygonal cells (×200).

Fig. 2. Ameloblastoma (AM) consisted of peripheral columnar cells and central stellate reticulum-like cells (×200).

Fig. 3. Ki-67 staining of AOT. A few positive cells are mainly seen in pseudoductal cells (×400).

Fig. 4. Ki-67 staining of AM. Numerous positive nuclei existing from peripheral columnar cells to central stellate reticulum-like cells (×400).

Fig. 5. PCNA staining of AOT. Positive immunoreactivity is observed in nuclei of pseudoductal cells and polygonal cells (×400).

Fig. 6. PCNA staining of AM. Positive nuclei are found in both columnar cells and stellate reticulum-like cells (×400).
AgNORs staining results

The mean AgNORs LI of AOT and that of AM were 1.2 ± 0.3 and 1.5 ± 0.3, respectively, the former tending to be lower than the latter ($P < 0.1$). In AOT, the mean AgNORs LI of pseudoductal cells and that of polygonal cells were 1.3 ± 0.3 and 1.2 ± 0.3, respectively, showing no tendency toward a significant difference. Both pseudoductal and polygonal cells contained small intranuclear, blackish-brown, droplet-like dots of relatively uniform diameter (Fig. 5). In AM, the mean AgNORs LI of columnar cells and that of stellate reticulum-like cells were 1.7 ± 0.3 and 1.3 ± 0.3, respectively, showing a significant difference ($P < 0.01$). Columnar cells contained intranuclear black, small, near-circular or irregularly near-circular dots of various sizes, whereas stellate reticulum-like cells contained intranuclear blackish-brown, near-circular to oval dots of relatively uniform, larger diameters (Fig. 6).

Discussion

Benign tumors show expansive proliferation, whereas malignant tumors characteristically show rapid invasive proliferation and distant metastasis. Morphological methods have been conventionally applied to distinguish benign from malignant tumors, but immunohistochemical methods and various DNA analysis methods including polymerase chain reaction (PCR) have been used in recent studies, many of which have reported the particular usefulness of measurement of cell proliferative activity (24–29). In addition, lesions on the borderline between benign and malignant tumors exist. Many studies comparing malignant, borderline, and benign tumors and normal tissue using proliferative activity as an indicator have reported that the degree of malignancy agrees with the level of cell proliferative activity (13, 15, 24). Furthermore, Lin et al. demonstrated that a higher malignancy grade was associated with a higher cell proliferative activity (30). Odontogenic tumors are no exception, and malignant AM has been reported to have higher proliferative activity than AM (16, 31). However, few studies have examined the proliferative activity of AOT, and it has not been distinctive-

ly characterized as benign (32).

It has been reported that Ki–67 used as an indicator of proliferative activity in this study is expressed in the phases, except the $G_0$ phase, of the cell cycle, that is, the $G_1$, $S$, $G_2$, and $M$ phases, and that its expression is maximal in the $S$ phase, rapidly decreasing from the $M$ phase onward (33–35); therefore, it is useful the detection of cell with proliferative activity. Comparison of Ki–67 LI showed that AOT had a statistically lower proliferative activity than AM. By cell types, the pseudoductal cells of AOT had a statistically higher level of proliferative
activity than polygonal cells.

PCNA is a substance that is closely involved in DNA replication, and is essential for cell proliferation. It has been reported that little or no PCNA occurs in the G₀ phase, but appears in the late G₁ phase chiefly in the nucleolus and in the early S phase in the entire nucleus, disappearing from the late S phase onward (36). However, recent studies have reported that the duration of PCNA metabolism is longer, and it is found in a longer portion of the cell cycle, including part of the G₀ phase (37). It has also been reported that PCNA is expressed not only during the cell proliferation phase but also in the DNA repair activity following DNA injury, such as that due to cell atypia (38, 39). Comparison of PCNA LI. showed that AOT had a statistically lower proliferative activity than AM. PCNA L.I. was higher than Ki–67 L.I., presumably because the above-described relatively long half-life of PCNA and the AOT’s low atypia influenced the measurements.

AgNORs represent the kinetics of RNA polymerase I and other enzymes that are closely involved in the kinetics of cell protein synthesis, and reflect transcription activity (40). Using double-staining for Ki–67/AgNORs or PCNA/AgNORs, Costa et al. showed that the AgNORs counts of Ki–67- and PCNA-positive cells were significantly higher than those of Ki–67- and PCNA-negative cells, and reported that AgNORs correlated with Ki–67 and PCNA, making AgNORs L.I. useful as a measure of proliferative activity (41). Furthermore, a relatively large number of studies have reported that AgNORs are useful in the differentiation of benign and malignant tumors (42–44). In this study, comparison of AgNORs L.I. showed that AOT tended to show a lower proliferative activity than AM. Moreover, Murai et al. reported that the morphology of AgNORs granules in the normal oral mucosa was almost uniform; however, with the increase in cell atypia, the morphology of individual AgNORs granules became non-uniform (45). In this study, AOT cells contained small intranuclear, black, droplet-like dots of relatively uniform diameter, whereas AM cells contained intranuclear black dots of various sizes, suggesting that AOT had lower cellular atypia than AM.

Comparative studies of the two types of cells composing AOT using histopathological, electron microscopic, and immunohistological techniques have indicated that these types of cells have morphological and biological differences (6, 46–48). On the other hand, from the viewpoint of biology, cell proliferative activity is considered to be generally inversely proportional to the degree of differentiation (49). Wistuba et al. have reported that according to this principle, cell proliferation is arrested when cells involved in odontogenesis have undergone functional differentiation (50). In the previous study, we examined AOT cells for amelogenin protein, and found that polygonal cells were positive for it, suggesting that some of the polygonal cells differentiated into cells capable of secreting a hard tissue matrix (48). Analysis of Ki–67 expression in AOT showed that pseudoductal cells had a significantly higher proliferative activity than polygonal cells (p < 0.05), suggesting that the two types of cells differ in the degree of differentiation, and the undifferentiated former cells differentiate into the latter.

In this study, we compared AOT and AM in epithelial proliferative activity by immunohistochemical and histochemical methods using Ki–67, PCNA and/or AgNORs. Relatively many studies using Ki–67, PCNA, or AgNORs have examined the proliferative activity of AM. Since the measurement and evaluation methods differ between these studies and ours, it may not be appropriate to indiscriminately compare all the results, but the previously reported measurements and results were similar to ours. Only de Carmo et al. examined AOT for proliferative activity using AgNORs, and compared the results with histologically or clinically classified AM, but found no significant difference in the AgNORs L.I. between AOT and AM, suggesting that AOT had characteristics of a benign tumor (32). However, in this study, AOT was characterized as a benign tumor with a tendency toward a lower proliferative activity than that of AM.
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