A Pathomorphological Study of Fractal Analysis in Parenchymal–stromal Border on Keratocystic Odontogenic Tumor— with Special Reference to Proliferative Activity and Vascular Distribution—

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Abstract
Keratocystic odontogenic tumor shows a relatively high rate of recurrence with daughter cysts and epithelial islands. Determining evidence of the daughter cysts or epithelial islands is therefore important for deciding treatment and assessing the prognosis. However, the evidence is often difficult to confirm from preoperative imaging and biopsy. Using histopathological and immunohistochemical methods on surgical specimens, the author conducted fractal analysis of tumor parenchymal–stromal border shape, provided feedback on biopsy materials, and investigated the utility of the methodology. The materials used in the present study were histopathologically divided into a group with neither daughter cysts nor epithelial islands (Group 1) and a group with either daughter cysts or epithelial islands or both (Group 2). In the epithelial basal lamina, rete ridges presenting an inverted conical shape were directly observed in Group 1 and “budding” with an alveoli–like spherical shape was seen in Group 2. The results of fractal analysis on the rounded form of the basement membrane showed a complex shape with a significantly higher fractal dimension in Group 2 than in Group 1. Immunohistochemically, the MIB-1 labeling index in the basal lamina, indicative of cell proliferative activity, the microvessel density (MVD) measured 200 µm immediately below the basal lamina, indicative of vascular distribution for tumor growth, and the microvessel area (MVA) were all significantly higher in Group 2 than in Group 1. These findings suggested that fractal analysis of the basement membrane curve shape and a comparative analysis of MVD and MVA were useful as factors indicating the presence of daughter cysts and epithelial islands, which are causes of recurrence, suggesting a close association with the neoplastic characteristics.

Keywords :
kERATOCYSTIC odontogenic tumor, pathomorphology, fractal analysis, daughter cyst, epithelial island

Introduction
Keratocystic odontogenic tumor (KCOT) is thought to originate from dental lamina, enamel organs, or residual epithelium. Histopathologically, KCOT is a cystic tumor in which the intramural surface is lined with parakeratotic stratified squamous epithelium. Because the tumor has the characteristics of locally invasive growth and a tendency for recurrence, KCOT is classified as a neoplastic lesion (1), and the presence of daughter cysts and epithelial islands may contribute to recurrence (2–4). Following the histopathological diagnosis of KCOT from biopsy specimens, therefore, assessment of daughter cysts and epithelial islands, particularly during biopsy, is thought to be important in deciding the treatment course and determining prognosis.

The formation of daughter cysts or epithelial islands is important to identify on computed tomography, magnetic resonance imaging, or other imaging examinations conducted preoperatively, and the presence or absence of these structures is then confirmed by histopathological examination. However,
biopsy specimens allow observation of only one part of a lesion, making it difficult to determine whether daughter cysts or epithelial islands are present in the entire tumor. Therefore, information on whether daughter cysts or epithelial islands are present in the tumor could be obtained from KCOT biopsy specimens using morphological methods would be helpful in deciding the treatment course and determining prognosis.

The shape of the KCOT epithelial basal lamina ranges from flat and smooth to uneven, irregular, and complex. The lining epithelium often exhibits a budding–like proliferation of the basilar layer (5). The findings of “budding” show teardrop–shaped cell clusters similar to dental germ formation. The shape of the basal lamina is reflected in the parenchymal–stromal border. The parenchymal–stromal border shape can be observed even in biopsy specimens limited to one region of the tumor.

Fractal analysis is a method for expressing such geometric shapes with objective numbers (6). Mandelbrot (7) has suggested the use of the fractal dimension (FD), which takes non–integers, as a quantitative analysis method for complex shapes. The value of FD lies between 1 and 2, with increasing values of FD correlating with increasing irregularity of the outline (6). The parenchymal–stromal border on section specimens is a curve. In such an analysis, the value approaches “1” as the shape becomes simpler and more linear, and moves closer to “2” as the shape becomes more complex. The use of fractal analysis in pathology can be found in the study of malignant epithelial tumors (8–15). However, only a few studies on the fractal analysis of KCOT have been reported.

In immunohistochemical investigations of KCOT, the MIB–1 labeling index (MIB–1 LI) is known to be useful as a prognostic marker (16). However, few investigations on the vascular distribution of the stroma include the parenchymal cells of this tumor (17) and no comparative investigations of the relationship between fractal analysis of the parenchymal–stromal border and cell proliferative activity or vascular distribution have been performed.

In the present study, therefore, to implement a more appropriate and beneficial assessment of the treatment course and prognosis for KCOT, a pathomorphological study of fractal analysis of the parenchymal–stromal border on KCOT was conducted, with special reference to proliferative activity and vascular distribution.

**Materials and Methods**

**Materials**

Specimens were surgically removed at the Nihon University Hospital at Matsudo and cases from the past 5 years in which a definitive diagnosis of KCOT was obtained histopathologically were used in the present study (42 cases). The study was approved by the ethics committee of Nihon University School of Dentistry at Matsudo (ethics committee recognition number: EC 09–018). In selecting the research specimens, the tumors showing secondary inflammatory changes were excluded.

The specimens were divided into a group with no daughter cysts or epithelial islands (Group 1, 11 cases), and a group with daughter cysts and/or epithelial islands (Group 2, 31 cases).

Specimens were immediately fixed in 10% neutral formalin solution for 24–48 h at room temperature, and then paraffin blocks were prepared according to conventional methods. Paraffin blocks were sliced at a thickness of 4 μm. These sections were then deparaffinized with xylene according to conventional methods and dehydrated in a descending alcohol solution series. The sections were subjected to hematoxylin–eosin (HE) double staining, periodic acid–Schiff (PAS) staining, and immunohistochemical staining.

**Immunohistochemical staining**

Immunohistochemical staining was performed as follows using monoclonal mouse anti–human Ki–67 antigen (MIB–1) (Clone MIB–1, IgG1, dilution 1: 50; Dako Cytomation, Glostrup, Denmark), polyclonal rabbit anti–cytokeratin, wide spectrum screening (cytokeratin) (dilution 1: 500; Dako Cytomation, Glostrup, Denmark), and monoclonal mouse anti–human CD34 Class II (CD34) (clone QBEnd10, IgG1,
dilution 1 : 50; Dako Cytomation, Glostrup, Denmark) as primary antibodies. Goat serum (10%) was used for blocking. For MIB-1, 10 mmol/l Tris-buffered solution containing 1 mmol/l EDTA at pH 9.0 was used and processing was performed for 10 min at 111 °C in a pressure container at 1.54 atm. Cytokeratin was treated with proteinase K (Dako Cytomation, Glostrup, Denmark) for 5 min at room temperature and CD34 antigen was activated by microwave treatment for 15 min using 10 mmol/l citrate buffer solution at pH 6.0. ChemMate Envision (Dako Cytomation, Glostrup, Denmark) was used for secondary antibodies. DAB+ Liquid (Dako Cytomation, Glostrup, Denmark) was used as the chromogenic substrate. Counterstaining was performed with Mayer’s hematoxylin. Mouse IgG1-negative control (Dako Cytomation, Glostrup, Denmark) or normal rabbit serum were used as negative controls and tissue containing healthy oral mucosa was used as a positive control.

Fractal analysis
Sites with numerous undulations in the parenchymal-stromal border on HE-stained specimens were photographed under original magnification ×200 (Fig. 1). Next, the shape of the parenchymal-stromal border on the image was manually traced and binary images were prepared. FD was obtained from these binary images using the fractal box counting method (box size = 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024) of ImageJ (http://rsb.info.nih.gov/ij/) (18). The parenchymal-stromal border was confirmed on the slide specimens stained with PAS and immunohistochemically stained for cytokeratin (Figs. 2 and 3).

Cell proliferative activity of the basal cell layer
The areas of HE-stained specimens showing remarkable positive findings were selected and photographed under an original magnification ×400 (Fig. 4).

MIB-1 LI (%) = (number of MIB-1-positive basal cells in a single field/number of total basal cells in that field) × 100

Three fields were selected for each case at random and mean values were used.

Calculation of microvessel density (MVD) and microvessel area (MVA)
MVD and MVA were calculated using CD34 immunohistochemically stained specimens. MVD was taken to be the number of CD34-positive capillaries in the stroma (200 μm × 200 μm) bordering the tumor parenchyma. MVA was taken to be the percentage of the CD34-positive capillary luminal space in the stroma (200 μm × 200 μm) bordering the tumor parenchyma. First, a search was performed at weak magnification for sites of high CD34 expression, and the stroma adjacent to the parenchyma was photographed under an original magnification ×200 to include the tumor parenchyma (Fig. 5a). A stromal area of 200 μm × 200 μm adjacent to the tumor parenchyma was cut from each photograph (Fig. 5b). These images were then divided into CD34-positive vascular lumen regions and other regions, and were binarized by manual tracing (19) (Fig. 5c). MVD and MVA were then calculated from the binary images. MVA was calculated from the following formula:

(number of black pixels/total number of pixels) × 100

Three fields were selected for each case at random, and mean values were used.

Statistical analysis
Welch’s t-test was performed for significant differences in the mean values for each group. Spearman’s rank correlation was performed to show correlations between items (statistical software: R version 2.14.0, R Development Core Team).

Results
Histopathological findings
Histopathologically, the tumor parenchyma indicated cystic proliferation of parakeratotic stratified squamous epithelium with acanthosis and basilar hyperplasia. Fibrous connective tissue was present under the epithelium with dilatation and proliferation of capillaries. In Group 1, the rete
Fig. 1. Histopathological feature and fractal dimension (FD) of the basal lamina in keratocystic odontogenic tumor. Histopathological features showed inverted cone shape (a) and spherical shape (b) of the basement membrane, and selected areas for FD analysis of inverted conical shape (c) and spherical shape (d). (HE, original magnification × 200) Each binarized image was shown in the areas of inverted conical shape (e) and spherical shape (f).
Fig. 2. Feature of PAS staining
PAS-positive basement membrane was indicated in the parenchymal-stromal border. (original magnification ×400)

Fig. 3. Immunohistochemical feature for cytokeratin
Positive immunoreactivity was observed in both the inverted conical area (a) and budding area (b) (original magnification ×400)
ridges were relatively flat, and some partly shown an inverted conical shape, but in Group 2, the elongation of rete ridges and acanthosis were remarkable. Accumulation of basal cells and spherical budding of small alveoli were also observed. The basal lamina ranged from flat to greatly undulating. Group 2 showed a tendency for marked dilatation and proliferation of capillaries in the fibrous connective tissue compared with Group 1, in which dilatation and proliferation of capillaries and fibrous connective tissue with mucinous degeneration was present. The basement membrane was present between the basal lamina and fibrous connective tissue, but in some parts, it was unremarkable.

**Immunohistochemical findings**

Immunohistochemically, MIB-1 positive findings were found in the area from the basal lamina to the prickle layer of the epithelium. CD34-positive findings were observed in stromal capillary endothelial cells of various sizes in all cases. In budding areas that showed a teardrop-shaped structure, numerous
Fig. 5. Immunohistochemical feature for CD34 and vascular distribution
Immunohistochemical features showed that CD34-positive blood vessels were observed in the connective tissue below the epithelium (a), and selected image of 200 μm × 200 μm area for analysis of vascular distribution (b). (original magnification ×200) Image used for calculating microvessel density and microvessel area (c).
MIB-1-positive cells were found from the basal lamina through the prickle layer (Fig. 4). In addition, cytokeratin-positive findings were seen in tumor cells, and the tumor parenchyma and stroma could be clearly differentiated. Mean MIB-1 LI±2 SD was 12.0±16.3 in all cases, 7.0±11.9 in Group 1, and 13.8±16.3 in Group 2 (Fig. 6, Table 1). A significant difference was seen between Groups 1 and 2 (p<0.05).

Mean MVD±2 SD was 8.7±6.9 in all cases, 5.5±4.7 in Group 1, and 9.8±6.2 in Group 2 (Fig. 7, Table 1). A significant difference was observed between Groups 1 and 2 (p<0.001). Mean MVA±2 SD was 3.0±3.4 in all cases, 1.2±1.4 in Group 1, and 3.6±3.0 in Group 2 (Fig. 8, Table 1). A significant difference was confirmed between Groups 1 and 2 (p<0.001).

**Fractal analysis**

Mean FD±2 SD was 1.044±0.054 in all cases, 1.026±0.010 in Group 1, and 1.053±0.052 in Group 2 (Fig. 9, Table 1). A significant difference was seen between Groups 1 and 2 (p<0.001).
Discussion

KOOT is generally treated by surgical enucleation of the tumor with curettage of surrounding healthy tissues or marginal mandibullectomy (resectioning of the jaw bones) (2, 20). However, there remains a possibility of recurrence caused by incomplete surgery of the primary lesion or remaining daughter cysts or epithelial islands (2-4). When considering lesion recurrence, therefore, it is important to assess the presence of the daughter cysts, epithelial islands, and budding of the basal lamina.

The presence or absence of epithelial islands or daughter cysts can be expressed as 0 or 1, while the presence or absence of budding is difficult to express as 0 or 1. Moreover, budding is a finding in which continuity with the tumor parenchyma is seen, but both daughter cysts and epithelial islands are observed in fibrous connective tissues separate from the tumor parenchyma. In the present study, therefore, daughter cysts and epithelial islands were regarded as risk factors of the same level for recurrence of KOOT. Consequently, cases were classified into two groups in the present study: one with neither epithelial islands nor daughter cysts (Group 1) and one with epithelial islands and/or daughter cysts (Group 2).

The basement membrane is present on the border between the tumor parenchyma and stroma in epithelial tumors. KOOT has a weak and discontinuous linear staining for laminin and collagen IV (21, 22). The FD of the tumor parenchymal–stromal border was obtained from HE and PAS, which stained the basement membrane, and immunohistochemical staining for cytokeratin, a marker of epithelial cells. The FD of the parenchymal–stromal border shape was compared between Groups 1 and 2, and was found to be significantly higher in Group 2.

The shape of the basal lamina is such that findings of budding are seen in parts of sites that show complex forms with numerous undulations. In budding, epithelial basal cells accumulate and alveoli-like shapes form, resembling the morphology of dental germ development. If the shape of the parenchymal–stromal border is separated into types, as shown in

Statistical Analysis

Based on the correlation coefficients between items, significant correlations (p<0.01) were recognized for FD–MIB-1 LI (r=0.40, n=42), FD–MVD (r=0.46, n=42), FD–MVA (r=0.50, n=42), MIB-1 LI–MVD (r=0.42, n=42), and MIB-1 LI–MVA (r=0.41, n=42).
In contrast, the morphogenesis of tissue consists mainly of cell proliferation and vascular distribution. The cell proliferative activity (MIB-1 LI) of the basal lamina was significantly higher in Group 2 than in Group 1. In addition, a significant correlation (p<0.05) was confirmed between the FD value and MIB-1, suggesting that if the cell proliferative activity in the basal lamina is high, then the shape of the parenchymal-stromal border will become complex. Conversely, in the vicinity of the epithelial basal lamina, when the part in which the cell population shows alveolar shape was compared with the part in which rete ridge extensions form inverted cones, hyperchromation was conspicuous, and cell proliferative activity (MIB-1 LI) was high. These findings suggested that in Group 2, budding, indicative of high proliferative activity, was associated with not only more complex parenchymal-stromal border shapes, but also the formation of daughter cysts or epithelial islands.

The distribution and density of blood vessels are essential to histogenesis and the proliferation of tumor cells. These factors are evaluated by MVD and MVA (23). If nutrient consumption in the tumor parenchyma exceeds nutrients provided by blood circulation and the tumor cells are in a hypoxic state, the tumor cells produce vascular endothelial cell growth factor and ensure the provision of necessary nutrients and oxygen by promoting angiogenesis (24). The diffusion distance of nutrients and oxygen is reported to be 70–200 μm (25, 26). In the 200 μm range

Fig. 10. Fractal dimension (FD) between Group 1 and Group 2
FD was significantly higher in Group 1 (n=11) than in Group 2 (n=31).

Fig. 11. Pathomorphological diagram of basement membrane
(left: smooth; center: normal epithelial elongation; right: budding).
immediately under the epithelium, which was the measurement range in the present study, both MVD and MVA were significantly higher in Group 2 than in Group 1. Thus, it appears that samples in Group 2 had higher cell proliferative activity (MIB-1 LI), because of the high consumption of nutrients and oxygen. In addition, significant correlations (p < 0.01) were confirmed between MIB-1 LI and MVD, and between MIB-1 LI and MVA. These results agree with those in the literature (27) and are thought to be evidence for tumor cell growth by neovascularization induced by tumor cells. In addition, the significant correlations seen between FD and MVA, between FD and MVD (p < 0.01) showed a relationship among morphogenesis, cell proliferation, and angiogenesis and suggested that Group 2 had greater neoplastic characteristics. These neoplastic characteristics are also conjectured to be associated with budding and the formation of daughter cells and epithelial islands.

Based on the results of a comparative analysis of the above pathological findings, fractal analysis, cell proliferative activity (MIB-1 LI), and vascular distribution factors MVD and MVA, a relationship is possible between budding findings and epithelial island and daughter cyst formation as prognostic factors that have not been observed in previous investigations into KCOT, and that may be extremely useful in deciding treatment strategies and determining prognosis.

It is necessary to compare biopsy and surgical specimens from the same patients and further investigate cases with recurrence.

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References
