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The expression of amelogenin in oral cystic lesions

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Subcutaneous injection of enamel matrix derivative into rat back tissue results in the formation of eosinophilic round bodies (ERBs). When a large amount of ERBs are formed, they induce cartilage. Our previous studies have shown that ERB is a part of amelogenin exon-5. We therefore artificially created synthesised peptide (SP) with the same sequence as ERB. Subcutaneous injection of SP into rat back tissue induces bone and endochondral bone formation. Since amelogenin plays many important roles, we hypothesised that ERB, which is a part of amelogenin, may also play an important role. For this reason we produced a mouse monoclonal antibody (A-ERB) using SP, to investigate the function of ERB. In the present study, immunohistochemical staining with A-ERB was performed on oral cystic lesions in the expectation that A-ERB would react with odontogenic epithelium. We selected as test material seven human oral cystic lesions, the radicular cyst, dentigerous cyst, dermoid cyst, epidermoid cyst, orthokeratinised odontogenic cyst, odontogenic keratocyst, and unicystic ameloblastoma. They were sectioned and immunohistochemically stained using A-ERB. Although ERBs were expressed in the lining epithelium of dermoid cysts, they were not expressed in the epidermoid cysts. Although epidermis with cutaneous appendages expressed ERB, epidermis without cutaneous appendages did not. Dermoid and epidermoid cysts are now classified as the same lesion. However, the character of the lining epithelium of the two is different. Perhaps the two lesions should not be in the same category. (J Osaka Dent Univ 2023; 57: 137-146)

Key words: Immunohistochemical staining; Synthetic oligopeptide; Monoclonal antibody; Odontogenic epithelium

INTRODUCTION

Subcutaneous injection of enamel matrix derivative into rat back tissue induces the formation of eosinophilic round bodies (ERBs). When a large number of ERBs are formed, cartilage is induced.[1] Our previous studies have revealed that ERBs are a part of amelogenin exon-5.[1] We therefore artificially created synthesised peptide (SP) with the same sequence as ERBs. Subcutaneous injection of SP into the rat back tissue induces bone and endochondral ossification.[2] Since amelogenin plays an important role in life phenomena,[3] SP is also thought to also be involved in important life phenomena. In particular, SP promotes hard tissue formation of human periodontal ligament fibroblasts,[4] human periodontal ligament stem cells,[5] bone marrow cells,[6] and human dental pulp stem cells.[7] SP promotes the proliferation of gingival epithelial cells *in vitro* during the first three days, but inhibits them after that.[8] In rat back tissue, SP induced bone and endochondral ossification.[2] To investigate the function of ERB, a mouse monoclonal antibody to SP (A-ERB) has been produced.[9] In the hope that A-ERB would react with odontogenic epithelium, in this study we performed immunohistochemical staining of human oral cystic lesions with A-ERB.

MATERIALS AND METHODS

Tissue samples

We studied seven human oral cystic lesions: radicular cysts, dentigerous cysts, dermoid cysts, epidermoid cysts, orthokeratinized odontogenic cysts, odontogenic keratocysts, and unicystic ameloblastomas. Three cases were chosen for each disease. Patients who presented to Osaka Dental University Hospital and had a histopathological diagnosis were selected for this study. Their clinical data are shown in Table 1. Cystic lesions in or around the mandible were selected for this study, because the lining epithelium of cystic lesions in the maxilla tends to show ciliated epithelium metaplasia. This study was approved by the Ethics Committee of Osaka Dental University (Approval No.111204-0).

Sectioning and immunohistochemical staining

Formalin-fixed, paraffin-embedded sections from each cystic lesion were cut at 3-5 μm thickness. Immunohistochemical staining with anti-eosinophilic round bodies antibody (A-ERB), anti-cytokeratin 14 (CK14) antibody, anti-cytokeratin 19 (CK19 and N-CK19) antibodies, anti-beta-catenin (β-cate) antibody, and anti-ki-67 (Ki-67) antibody, as well as haematoxylin-eosin (HE) staining were performed on thin section specimens. There are differences in the expression of CK19 on healthy oral mucosa among researchers.[10] As the staining properties of anti-CK19 antibodies differ greatly depending on the epitope recognized, we used two types of anti-CK19 antibodies. The antibodies and their uses are shown in Table 2. The immunohistological staining method using A-ERB has already been described.[9] Briefly, thin sectioned specimens were pre-treated with proteinase K (Agilent Technologies, Santaclara, CA, USA) for five min at room temperature. A-ERB was diluted 1:5,000 in antibody diluent (Agilent Technologies) and reacted at 4°C overnight. As a secondary antibody, Envision+ system (Agilent Technologies) was used and reacted at room temperature for 60 min. All antigen-antibody mixtures were visualized by the 3,3'-diaminobenzidine reaction. The reacted slides were counter-stained with hematoxylin. Tissues of rat mandible were used as a control. They were cut at 5 μm and

Table 1 Clinical findings

Patient	Age (yrs)	Sex	Site	Diagnosis
1	55	M	Molar region of mandible	Radicular cyst
2	81	M	〃	〃
3	40	M	〃	〃
4	39	M	〃	Dentigerous cyst
5	46	M	〃	〃
6	20	F	〃	〃
7	51	M	Neck	Dermoid cyst
8	18	F	Sublingual region	〃
9	32	M	Mouth floor	〃
10	47	M	Tongue	Epidermoid cyst
11	5	F	Mouth floor	〃
12	86	M	Buccal region	〃
13	24	M	Molar region of mandible	Orthokeratinized odontogenic cyst
14	34	M	〃	〃
15	31	M	〃	〃
16	32	F	〃	Odontogenic keratocyst
17	41	F	Ramus of mandible	〃
18	30	M	Molar region of mandible	〃
19	57	M	〃	Unicystic ameloblastoma
20	20	F	〃	〃
21	37	M	〃	〃

Table 2-1 Primary antibodies and usage

Abbreviation	First antibody	Dilution	Pretreatment
A-ERB antibody	Anti-eosinophilic round body antibody that we made	1:5,000	Proteinase K
CK14	Anti-cytokeratin 14 (Roche Diagnostics)	1:1	Heat in CCl buffer
CK19	Anti-human cytokeratin 19 (Agilent Technologies)	1:200	Heat in CCl buffer
N-CK19	Anti-cytokeratin 19 (Leica Biosystems)	1:150	0.1% Trypsin in Tris buffer
β-cate	Anti-beta-catenin (Leica Biosystems)	1:50	Heat in Envision FLEX high pH
Ki-67	Anti-human Ki-67 antigen (Agilent Technologies)	1:1	Heat in Envision FLEX low pH

Table 2-2 Second antibodies

Abbreviation	Second antibody
A-ERB antibody	Envision+ system (Agilent Technologies)
CK14	HRP multimer (Roche diagnostics)
CK19	HRP multimer (Roche diagnostics)
N-CK19	Envision+ system (Agilent technologies)
β-cate	Envision-FLEX (Agilent technologies)
Ki-67	Envision-FLEX (Agilent technologies)

stained immunohistochemically using each antibody and with HE. The protocol for this animal experiment was approved by the Osaka Dental University Institutional Animal Care and Use Committee (Approval No.12-03005).

Number of Ki-67 positive cases and calculation of the Ki-67 index

Specimens stained with Ki-67 were observed using a light microscope, and the number of positive cells were counted. To calculate the Ki-67 index, an area of equal size was set for three cases for each lesion, the number of positive cells in each epithelial tissue sample was counted, and the mean and median values were calculated.

RESULTS

Immunohistochemical staining

A-ERB

Ameloblasts, basal cells of the gingival epithelium, and epithelial rests of Malassez were A-ERB positive in the rat tissues used as controls (Fig. 1). The basal cells in the lining epithelium of all of the dermoid cysts (Fig. 2 C), one case each of the dentigerous cysts and orthokeratinised odontogenic cysts, and two cases of the unicystic ameloblastomas were ERB positive. However, no ERB-positive areas were found in the radicular cysts, epidermoid cysts (Fig. 2 D) or odontogenic keratocysts. ERB expression was detected in parts of the lining epithelium of the dentigerous cysts, in the basal cell layer of the lining epithelium of orthokeratinised odontogenic cysts, and in parts of the odontogenic epithelium of unicystic ameloblastomas (Fig. 3).

CK14

Anti-CK14 antibodies were positive in the lining epithelium of all of the cystic diseases (Table 3). Among the radicular cysts, one case showed positivity in the basal to spinous cell layer (Fig. 4 B) and two cases in all layers of the lining epithelium. In the dentigerous cysts, one case was positive in the basal and parabasal cell layers, and two cases in all layers of the lining epithelium. In the dermoid cysts, one case was positive from the basal cell layer to the granular layer, one from the basal cell layer to the spinous cell layer, and the remaining one in the basal cell layer and parabasal cell layer. In the epidermoid cysts, one case was positive in the basal and parabasal cell layers, and the other two in all layers of the lining epithelium. In the orthokeratinised odontogenic cysts, all three cases were positive from the basal cell layer to the granular layer. In the odontogenic keratocysts, one case was positive from the basal cell layer to the middle layer of cells, and the remaining two cases were positive in all layers of the lining epithelium. In the unicystic ameloblastomas, all three cases were positive in all parenchyma.

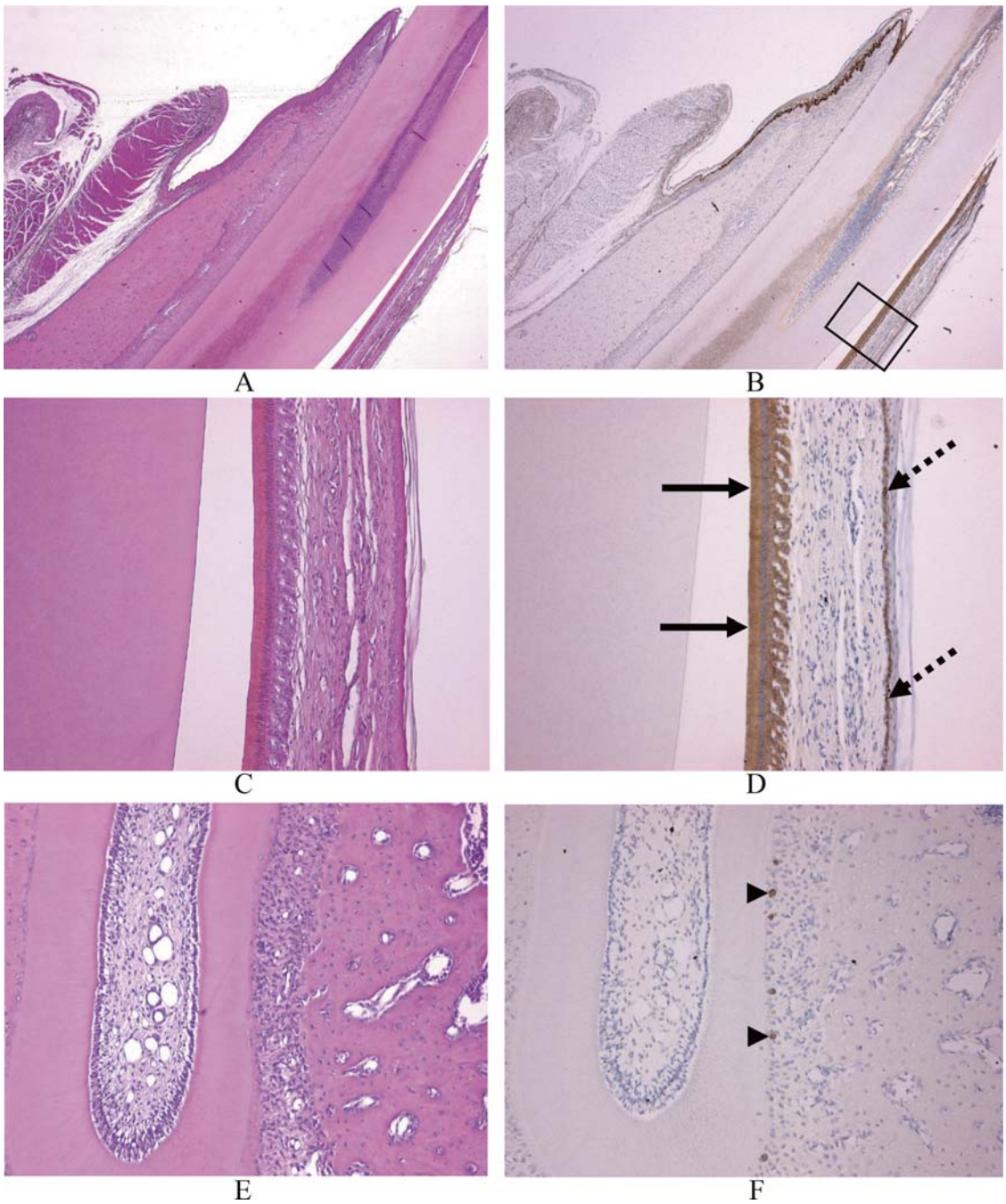

Fig. 1 Immunohistochemical staining using anti-ERB antibody and HE staining where (A-D) are rat incisor and the periodontal tissue, (E and F) are molor and periodontal tissue. (C and D) are higher magnifications of (A and B). Arrows show ameloblasts, broken line arrows are the basal cell layer of stratified flattened epithelium, and arrowheads show epithelial rests of Malassez.
A and B: Original mag × 2, C and D: Original mag × 10, E and F: Original Mag × 2.

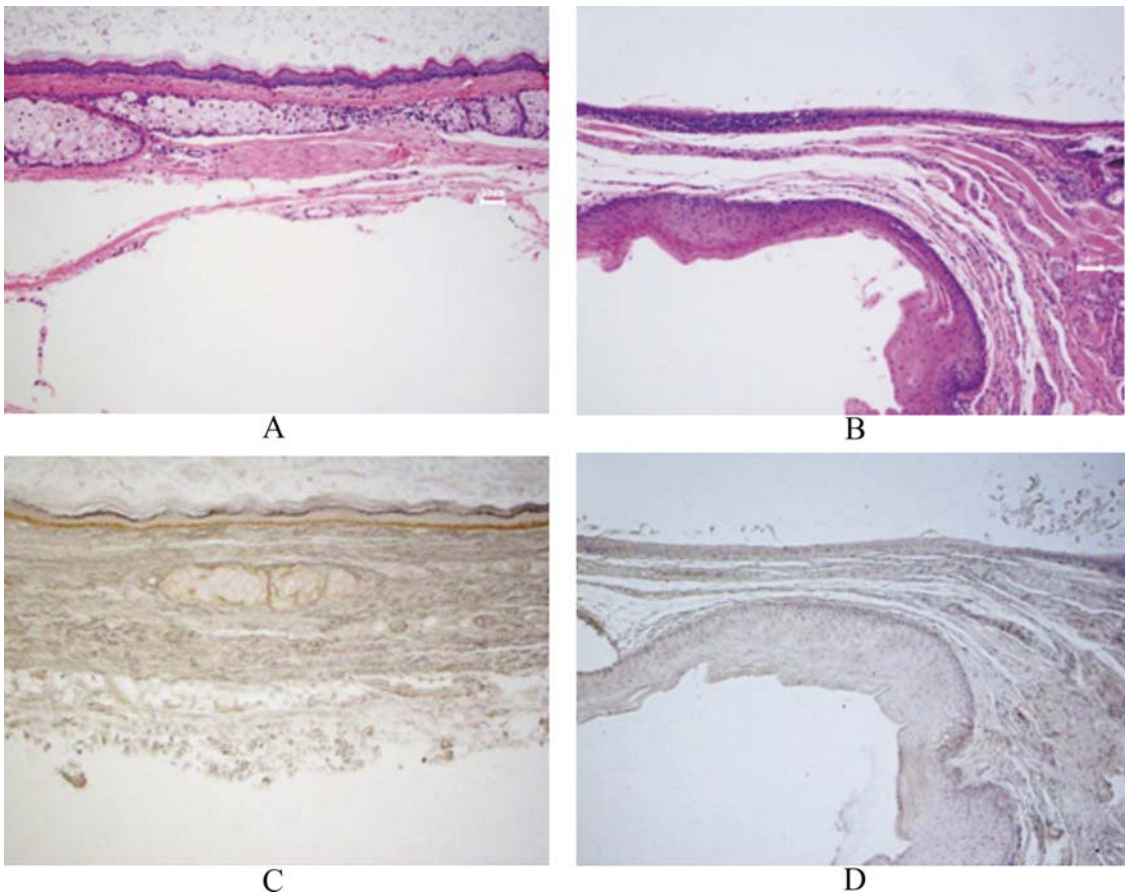

Fig. 2 Immunohistochemical staining of dermoid cysts and epidermoid cysts using anti-ERB antibody and HE staining where (A and C) are dermoid cysts, and (B and D) are epidermoid cysts. (A and B) were stained with HE, while (C and D) were immunohistochemically stained using anti-ERB antibody.
ERB: Eosinophilic round bodies, (A-D): Original mag × 10, Bars: 50 μm.

Table 3 Results of positive cases in the lining epithelium

Diagnosis	A-ERB	CK14	CK19	N-CK19	β-cate	Ki-67 (%)
Radicular cyst	0/3	3/3	1/3	2/3	3/3	11.2
Dentigerous cyst	*1/3	3/3	0/3	3/3	3/3	11.0
Dermoid cyst	3/3	3/3	0/3	*1/3	3/3	14.0
Epidermoid cyst	0/3	3/3	1/3	1/3	3/3	17.9
Orthokeratinized odontogenic cyst	1/3	3/3	0/3	1/3	*3/3	31.2
Odontogenic keratocyst	0/3	3/3	1/3	2/3	3/3	13.8
Unicystic ameloblastoma	*2/3	3/3	0/3	2/3	3/3	5.8

The Ki-67 value shows the average counted on each slide of three cases (*Positive reaction detected in a small area of the lining epithelium).

CK19

Anti-CK19 antibodies were negative in most cystic diseases (Figs. 4 C and 5 B), but reacted in one case each of the radicular cysts, epidermoid cysts, and odontogenic keratocysts. In particular, it was positive in the basal cell layer to the spinous cell layer of the radicular cysts and the basal cell layer of the epidermoid cysts and odontogenic keratocysts. Each specimen was stained with another CK 19 (N-CK19) antibody because the immunohisto-

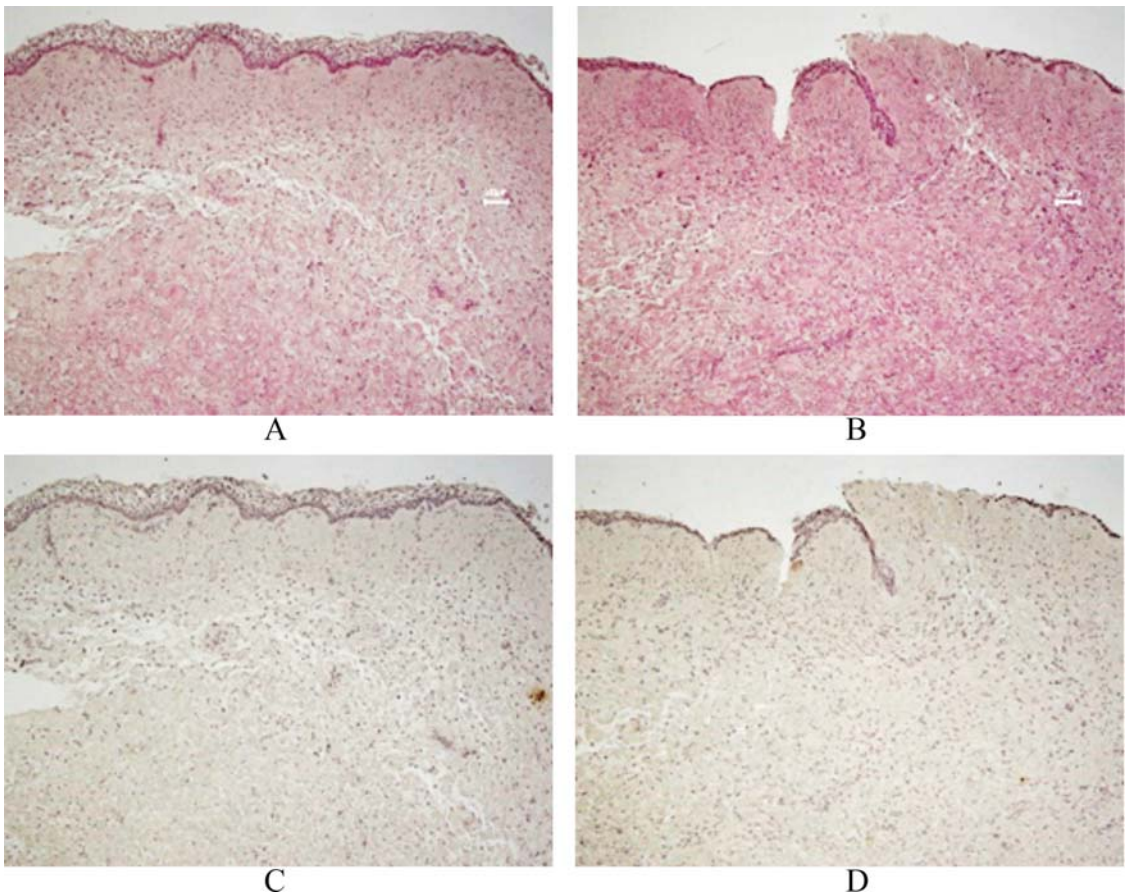

Fig. 3 Immunohistochemical staining of unicystic ameloblastoma using anti-ERB antibody and HE staining where (A and C) are the epithelium lining area, and (B and D) are the budding regions. (A and B) were stained with HE, while (C and D) were immunohistochemically stained using anti-ERB antibody.
(A-D): Original mag×10, Bars: 50 μm.

chemical stainability of anti-CK19 antibody is unstable. Anti-N-CK19 antibodies were positive in more cases than anti-CK19 antibodies (Table 3). Anti-CK 19 antibodies were positive in at least one case each of the cystic diseases (Figs. 4 D and 5 C).

β-cate

Anti-*β*-cate antibodies were positive in the lining epithelium of all cystic lesions (Table 3). All or parts of the lining epithelium had positive areas, and in the radicular cysts, dentigerous cysts and orthokeratinised odontogenic cysts, *β*-cate was expressed at the cell membrane (Fig. 6 C). Dermoid cysts, epidermoid cysts, unicystic ameloblastoma and some odontogenic keratocysts showed *β*-cate expression in the cytoplasm (Fig. 6 D).

Calculation of the Ki-67 positive ratio

The results for the Ki-67 positive ratio are shown in Table 3 and Fig. 7.

The mean and median values of the orthokeratinised odontogenic cysts (Fig. 6 E) showed a higher Ki-67 index than other cysts (Fig. 6 F).

DISCUSSION

The seven types of cystic disease used in this study can be divided into two main categories. One is a disease associated with the odontogenic epithelium (radicular cysts, dentigerous cysts, orthokeratinised odontogenic cysts, odontogenic keratocysts, and unicystic ameloblastomas), and the other is a disease not-associated with the odontogenic epithelium (dermoid cysts and epidermoid

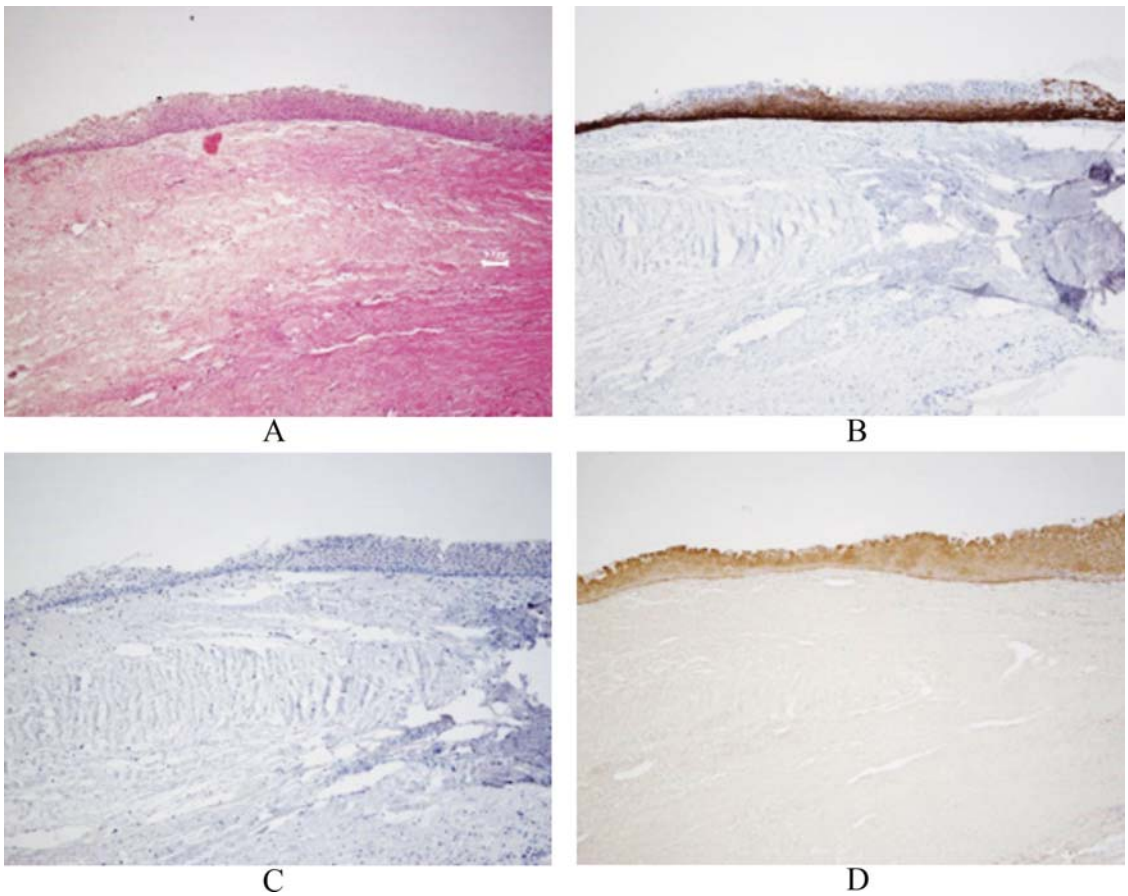

Fig. 4 Immunohistochemical staining of a radicular cyst stained with (A) HE, (B) anti-CK14, (C) CK19 and (D) N-CK19. (A-D): Original mag×10, Bar: 50 μm.

cysts). When A-ERB reacts with the odontogenic epithelia, it is useful for histopathological diagnosis. We had hoped that ERB would be expressed in the epithelium of these odontogenic diseases, especially in radicular cysts, dentigerous cysts, orthokeratinized cysts, odontogenic keratocysts, and unicystic ameloblastomas. However, ERB expression was detected in very few of them (Table 3). The expression of ERB, which is a part of amelogenin, may not be related to it being an odontogenic epithelium. Furthermore ERB was expressed in the lining epithelium of dermoid cysts, but not in epidermoid cysts. In other words, ERB was observed in the lining epithelium of cystic walls with skin appendages and not in the lining epithelium of cystic walls without skin appendages.

The homology of amelogenin sequences is very high within species,[11] and in Xenopus tropicans, amelogenin is expressed in the skin.[12] In the present study, ERB expression was identified in the odontogenic epithelia; ameloblasts, epithelial rests of Malassez, and mucosal (gingival) epithelium of rats (Figs. 1 B and 1 D). Amelogenin seems to express in the dermis, because of an evolutionary process in vertebrates that shows reinforcement of the dermis.[13] Hence, amelogenin may be detected in the skin. Our results demonstrated that the character of the lining epithelium of dermoid and epidermoid cysts is different. Although dermoid and epidermoid cysts are now classified as the same lesion,[14] these two lesions may need to be separated as different diseases.

β-cate is expressed at the cell membrane in innocuous diseases, and in the cytoplasm and nucleus in more aggressive ameloblastomas.[15] We obtained similar results. β-cate was observed at the

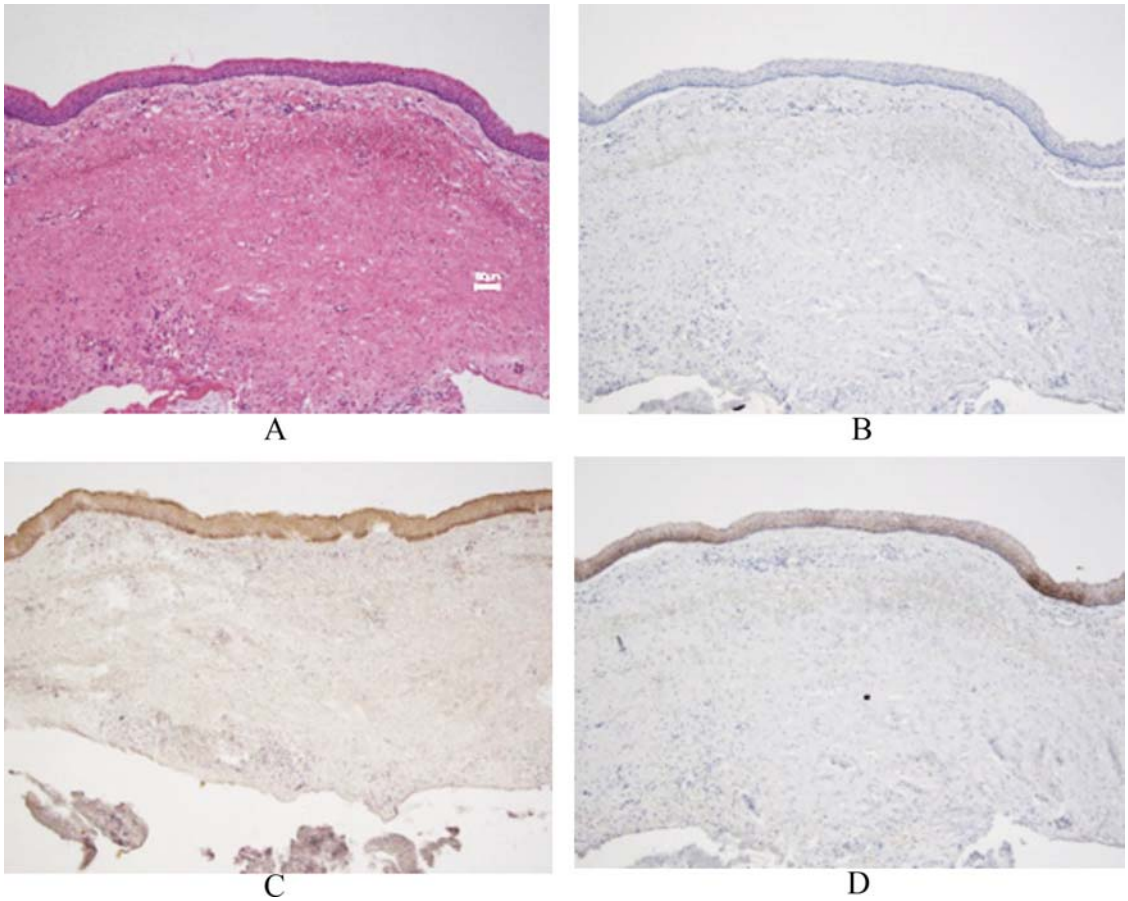

Fig. 5 Immunohistochemical staining of a dentigerous cyst stained with (A) HE, (B) anti-CK19, (C) N-CK19, and (D) β-cate. (A-D): Original mag×10, Bar: 50 μm.

cell membrane of the lining epithelium in radicular cysts, dentigerous cysts and orthokeratinised odontogenic cysts. β-cate was observed in the cytoplasm of epithelial tissue in some odontogenic keratocysts and unicystic ameloblastomas. When β-cate is expressed in the cytoplasm, the lesions seem to have a tendency to recur. All cases, including lesions with the non-odontogenic epithelium, were immunohistochemically CK14-positive. In the present study, staining properties of β-cate were similar to those of CK14. Both CK14 and β-cate are markers of odontogenic epithelium.[10, 15]

CK19 positive cells are said to be of odontogenic epithelium in origin.[16] The number of CK19-positive cases was lower than that of the N-CK19-positive cases. This was thought to be due to the difference between the epitope of CK19 and that of N-CK19. CK14 and CK19 are markers of the odontogenic epithelium.[17] CK14-positive areas were more common than those of CK19.[10] CK19 is more frequently expressed in dysplastic epithelium and squamous cell carcinomas than in healthy epithelium. Decreased CK14 expression and increased CK19 expression indicate malignant transformation.[18] Scattered expression of CK19 in potentially malignant oral disease is a false positive, according to Rathore *et al.*[19] In the present study, we used two anti-CK19 antibodies and did not see such a staining pattern. Although the Ki-67 index of orthokeratinised odontogenic cysts was higher than that of the other six lesions, the reason for this was unclear. The Ki-67 index of odontogenic keratocysts is said to range from 0.54% to 23.40%.[20] The Ki-67 index of the odontogenic keratocysts in our study was 13.8% on average in the three cases, which is consistent with the literature. It is said that although

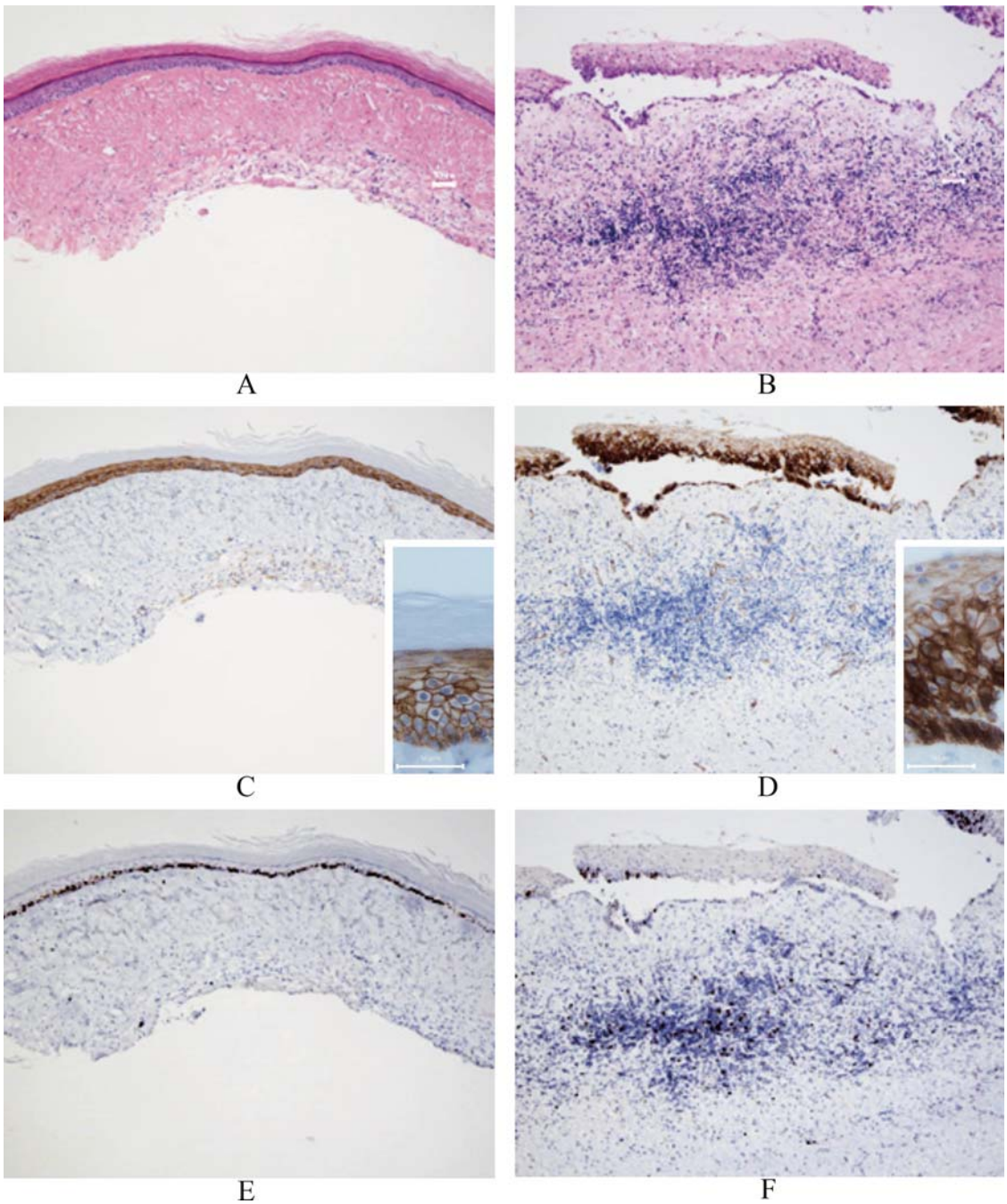

Fig. 6 Immunohistochemical staining of an orthokeratinized odontogenic cyst and an odontogenic keratocyst stained using anti-β-cate antibody, anti- Ki-67 antibody and HE. (A, C and E) are orthokeratinized odontogenic cysts, and (B, D and F) are odontogenic keratocysts. (A and B) were stained with HE and (C and D) were immunohistochemically stained using anti- β-cate antibody. (E and F) were immunohistochemically stained using anti- Ki-67 antibody.
(A-F): Original mag×10, Insets (C and D): Original mag×40, Bars: 50 μm.

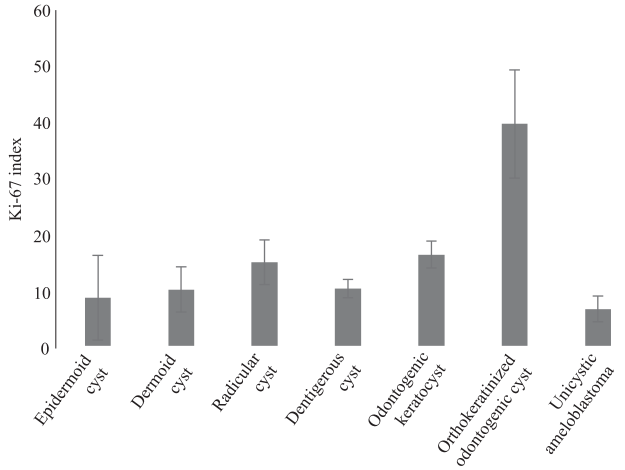

Fig. 7 Median value of Ki-67 index.

the biological behaviour of odontogenic keratocysts cannot be clarified by assessing the Ki-67 index alone,[20] the character of orthokeratinized odontogenic cysts is similar to that of odontogenic keratocysts.

The authors declare no conflicts of interest associated with this study.

REFERENCES

1. Kim N-H, Tominaga K, Tanaka A. Analysis of eosinophilic round bodies formed after injection of enamel matrix derivative into the backs of rats. *J Periodontol* 2005; **76**: 1934-1941.
2. Hida T, Tominaga K, Tanaka A. Tissue reaction to synthetic oligopepide derived from enamel matrix derivative in rats. *Oral Sci Int* 2010; **7**: 26-33.
3. Tominaga K, Takeuchi T, Honda S, Okamura T, Wato M, Tanaka A. Effect of newly-developed bone-inducing peptide derived from enamel matrix derivative. *Journal of Japanese Society of Periodontology* 2017; **59**: 39-47.
4. Kawanaka A, Tominaga K, Tanaka A. Effect of peptide derived from Emdogain® on human periodontal ligament fibroblasts. *J Osaka Dent Univ* 2009; **43**: 111-117.
5. Takeuchi T, Masuno K, Kato H, Taguchi Y, Umeda M, Okusa N, Tanaka A, Tominaga K. A human amelogenin-derived oligopeptide enhances osteogenic differentiation of human periodontal ligament stem cells. *J Hard Tissue Biology* 2019; **28**: 251-258.
6. Yasui N, Taguchi Y, Tanaka A, Ueda M, Umeda M. Biological effects of Emdogain-derived oligopeptides on rat bone marrow cells *in vitro. J Oral Tissue Engin* 2011; **9**: 126-135.
7. Nakano Y, Kato H, Taguchi Y, Imai K, Nakata T, Tominaga K, Umeda M. Effects of amelogenin peptide derived from enamel matrix derivative on human dental pulp stem cells. *J Osaka Dent Univ* 2022; **56**: in printing.
8. Nando M, Tominaga K, Takahashi T, Tanaka A, Umeda M. Effects of novel synthetic peptide during early wound healing of periodontal artificial defects in rats.*J Oral Tissue Engin* 2014; **12**: 69-79.
9. Ikeda C, Tominaga K, Nishikawa T, Okamura T, Masuno K, Wato M, Cui J, Hou Y N, Yin J, Okusa N, Kaji K, Tanaka A. Synthetic calcium particles as a new scaffold have porosity and extended-release properties.*J Osaka Dent Univ* 2021; **55**: 53-58.
10. Mehrpouya M, Pourhashem Z, Yardehnavi N, Oladnabi M. Evaluation of cytokeratin 19 as a prognostic tumoral and metastatic marker with focous on improved detection methods. *J Cell Physiol* 2019; **234**: 21425-21435.
11. Asano Y, Uekita T, Kasai K, Endo H, Yamada T, Saburi S, Yamanouchi K, Tojo H, Natori M, Tachi C. Studies on the recovery of genomic DNA and functional genes from mammalian pelt specimens. *Anim Sci J* 1999; **70**: J 651-J 362.
12. Okada M, Tazawa I, Nakajima K, Yaoita Y. Expression of the amelogenin gene in the skin of xenopus tropicalis. *Zool Sci* 2013; **30**: 154-159.
13. Kawasaki K, Suzuki T, Weiss KM. Genetic basis for the evolution of vertebrate mineralized tissue. *Proc Natl Acad Sci USA* 2004; **101**: 11356-11361.
14. Chiosea S, Gnepp DR, Wenig BM. Dermoid and teratoid cysts. In: El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ eds. WHO classification of head and neck tumours 4th edition. Lyon: IARC, 2017: 147-158.
15. Prakash S, Swaminathan U, Nagamalini BR, Krishnamurthy AB. Beta-catenin in disease. *J Oral Maxillofac Pathol* 2016; **20**: 289-299.
16. Hayashi I, Takeda Y, Toida M, Abe K, Yamashita T. A case of botryoid odontogenic cyst of the anterior maxilla. *Jpn J Oral Maxillofac Surg* 2021; **67**: 537-541.
17. Kishino M, Ishibashi M, Koizumi H, Sato S, Murakami S, Ogawa Y, Kogo M, Toyosawa S. Central odontogenic fibroma of the maxilla: a case report with immunohistochemical study. *Oral Med Pathol* 2009; **14**: 29-32.
18. Yoshida K, Sato K, Tonogi M, Tanaka Y, Yamane G, Katakura A. Expression of cytokeratin 14 and 19 in process of oral carcinogenesis. *Bull Tokyo Dent Coll* 2015; **56**: 105-111.
19. Rathore AS, Katyal S, Jain A, Shetty DC. Biochemical analysis of cytokeratin fragment 21-1 concentration and expression of cytokeratin 19 in oral potentially malignant disorders. *J Cancer Res Ther* 2020; **16**: 452-457.
20. Portes J, Cunha KSG, Silva LE, Silva AKF, Conde DC, Junior AS. Computerized evaluation of the immunoexpression of Ki-67 protein in odontogenic keratocyst and dentigerous cyst. *Head Neck Pathol* 2020; **14**: 598-605.