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The differential expression pattern of BMP-4 between the dentigerous cyst and the odontogenic keratocyst

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BACKGROUND: Bone morphogenic protein-4 (BMP-4) is widely expressed in oral cavity and involved in tooth morphogenesis, cellular differentiation and proliferation. The purpose of this study was to compare the difference in expression pattern of BMP-4 in odontogenic keratocysts (OKC) and dentigerous cysts (DC).

METHODS: We evaluated 77 cysts, OKC ($n = 34$) or DC ($n = 43$). The average age of patients with OKC was 29.5 ± 14.4 and that of patients with DC was 36.1 ± 19.4. The male to female ratio was 20:14 for OKC and 27:16 for DC. Ten cases of OKC were recurrences. Expression of BMP-4 was determined by immunohistochemistry and *in situ* hybridization.

RESULTS: The intensity scales were (−) for invisible or trace staining, (+) for visible staining, and (++) for dense, strong staining. OKCs exhibited the following staining patterns: the epithelium in 15/34 specimens and the mesenchymal cells in 17/34 specimens showed (++) stain. In contrast, the staining pattern of DC was (−) for epithelium in 37/43 specimens. The mesenchymal cells showed (−) degree staining in 30/43 specimens. The difference between the groups studied was significant ($P < 0.001$ in epithelium and mesenchymal cells). When recurrent and non-recurrent OKC were compared BMP-4 was expressed more intensely in the recurrent cases ($P = 0.036$ in epithelium). The difference in BMP-4 expression in mesenchymal cells was not significant. *In situ* hybridization demonstrated positive mRNA probes to BMP-4 were localized in epithelium and mesenchymal cells of OKCs and DCs.

CONCLUSIONS: BMP-4 was expressed more intensely in OKC when compared with DC, and was more intensely expressed in recurrent cases.

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Introduction

Both dentigerous cyst (DC) and odontogenic keratocysts (OKC) may contain impacted teeth and they have several other features in common. The important difference between the two cysts is the nature of their lining epithelia, and their respective recurrence rates. The lining epithelium in OKC shows columnar, regimented uniform basal cells and other demonstrates parakeratotic cells with a corrugated appearance on the lumen as surface. In contrast the lining epithelium of DCs is not keratinizing, is of the squamous but can present with several layers of cuboidal or columnar epithelial cells (1). OKCs often tend to recur (1).

The histopathologic difference in the lining epithelium of OKCs and DCs suggests that different factors may be involved in both the development, and the recurrence tendency of these cysts. When large OKCs are treated with decompression and secondary enucleation, the nature of the epithelial lining of the cyst is changed and the lesion is treated successfully (2). The significance of the capsular mesenchymal cells of OKCs, to date, has not been investigated. If the lining of an odontogenic cyst mimics the epithelial elements involved in tooth development, communication between epithelial and mesenchymal tissue could be significant. In the various stages of tooth development, a number of growth factors are involved in this communication (3, 4).

Abnormal epidermal differentiation has been linked to abnormal bone morphogenic protein-4 (BMP-4) expression (5). The key difference between OKC and DC is reflected in lining epithelium. BMP-4 is expressed in buccal mucosa (6), dental pulp (7), and tongue (8). Therefore, postulated the BMP-4 expression pattern between OKC and DC could be different. The purpose of this study was to find differences, if any in BMP-4 expression in the cystic linings of OKCs and DCs. We

also studied the differential expression of BMP-4 between OKC and DC with regard to recurrence, anatomical location of lesion, and patient's age and sex.

Patients and method

Patients

A total of 77 samples were included in this study. The summary of patients was listed in Table 1. We studied the 49 patients diagnosed as OKC ($n = 34$) or DC ($n = 43$). They were hospitalized and treated surgery at Hallym University from January 1, 1999 to April 30, 2000. All cases were treated by enucleation without bone graft. We excluded syndromic cases in this study. The average age of OKC was 29.53 ± 14.37 and that of DC was 36.12 ± 19.41. The male to female ratio was 20:14 in OKC and 27:16 in DC. The location of OKC was 10 cases in the maxilla and 24 cases in the mandible. The location of DC was 12 cases in the maxilla and 31 cases in the mandible. Ten cases were recurrent cases in OKC. There was no recurrence in DC. The mean follow-up was 37.1 ± 6.3 months.

Table 1 Summary of patients

	Odontogenic keratocyst	*Dentigerous cyst*
Number of cases	34	43
Age (years)	29.53 ± 14.37	36.12 ± 19.41
Sex		
Male	20	27
Female	14	16
Location		
Maxilla	10	12
Mandible	24	31
Recurred case	10	0

Immunohistochemical staining

Goat primary polyclonal antibody against BMP-4 was purchased from Santa Cruz Biotechnology (sc 6896) (Santa Cruz, CA, USA) and monoclonal antibody from mouse was purchased from Novocastra Laboratories Inc. (NCL-BMP-4) (Newcastle, UK). The dilution rates were 1:20. Two kinds of antibody were used for this study because polyclonal antibody from goat might show non-specific immunopositive in the epithelium and we wanted to confirm the results via repeated studies. The specificity of the antibodies has previously been demonstrated (9). The positive control was the adenocarcinoma of the prostate and the immunostaining without primary antibody was used as a negative control. Sections of 4 μm thickness were prepared for immunohistochemistry. Immunohistochemical staining was done with Universal LSAB®+ Kits (Dako, Glostrup, Denmark) and the subsequent procedures were done according to the manufacturer's protocol. All slides were undergone immunostain two times those were one for monoclonal antibody and another for polyclonal antibody. This was done to rule out any species-specific false-positive reaction. The adenocarcinoma from prostate was used as a positive control. The sections were counterstained with Mayer hematoxylin.

The reviewing of all slides by two pathologists was blind to the original histologic diagnosis. The minute pattern of expression was different according to the type of the antibody (Fig. 1). However, the intensity and location of staining were not different. The slides were evaluated for intensity of staining. The intensity scales were (−) for invisible or trace staining, (+) for visible stain, and (++) for dense, strongly staining slides.

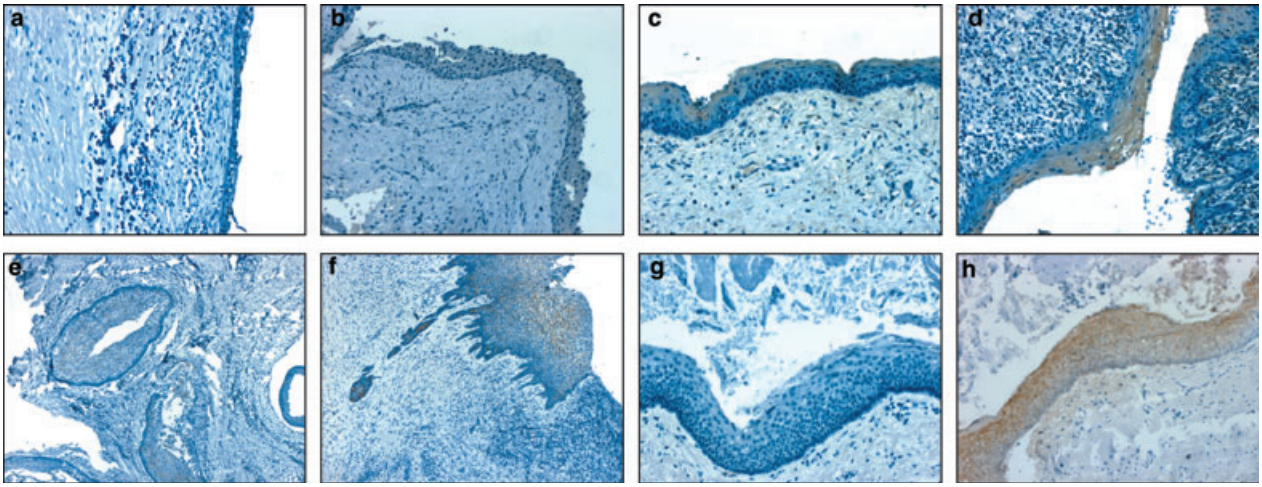

Figure 1 Immunolocalization of bone morphogenic protein-4 (BMP-4) in the dentigerous cyst (DC) and the odontogenic keratocyst (OKC). (a) BMP-4 expression was very weak in DC (original magnification: ×100). (b) BMP-4 expression was visible in the epithelium and the mesenchyme in DC (original magnification: ×100). (c) BMP-4 expression was dense in the epithelium and the mesenchyme in OKC (original magnification: ×100). The strong expression of BMP-4 was particularly well observed in the samples that showed inflammatory reaction (d) (original magnification: ×100). Dense BMP-4 expression was observed in the epithelium of the daughter cysts (e) or in the down-growing epithelium (f) (original magnification: ×40). In the absence of primary antibody, only very weak background was observed (g) (original magnification: ×100). However, the strongly stained epithelium and mesenchyme were observed with primary antibody (h) (original magnification: ×100). All slides were counterstained with Mayer hematoxylin.

In situ hybridization

Total RNA was extracted from homogenized tissue from oral fibroma using TRI Reagent® under the conditions recommended by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH, USA). Poly(A)$^+$ RNA (0.1 μg) was converted to cDNA by reverse transcription using 100 units Molony murine leukemia virus reverse transcriptase (Pharmacia, Uppsala, Sweden). The primers used for amplification of BMP-4 were 5′-GCT AGC CAT TGA GGT GAC TCA C -3′ (forward) and 5′-TCA TCC AGG TAC AGC ATG GAG A-3′ (backward). Sense and anti-sense digoxigenin-labelled single-stranded PCR product (BMP-4 cDNA) was used as a template for probes. RNA probes were synthesized by *in vitro* transcription of BMP-4 cDNAs with DIG RNA Labeling Kit (Roche, Manheim, Germany). *In situ* hybridization was done as described by Matsuki et al. (10) and Denijn et al. (11). Deparaffinization with xylene and hydration with ethanol was done. The specimen was treated in diethylpyrocarbonate (DEPC)–PBS solution for 5 min, and then it was incubated with proteinase K solution (Dako) for 5 min in room temperature. This was again treated with a 4% paraformaldehyde solution for 10 min, and it was placed in 0.2 N HCl solution for 10 min. Then, it was placed in DEPC–PBS solution for 5 min and in 0.1 M triethanolamine (TEA) solution for 5 min. After being placed in 0.1 M TEA solution + 0.25% acetic acid for 5 min, it was treated with DEPC–PBS solution for 10 min. After warming the hybridization solution in water bath, they were mixed with specific digoxigenin (DIG) labelled RNA probe in hybridization solution. Adequate incubation was obtained by incubating on heat plate at 50°C and making a moisture chamber overnight. Incubating on heat plate at 50°C, making a moisture chamber over night incubation was adequate. It was then placed in 2× SSC (saline–sodium citrate)–50% formamide solutions for 30 min in a water bath. After removing the hybrislip (Sigma, St Louis, MO, USA) with a sharp forcep again, it was placed in 2× SSC–50% formamide solutions for 30 min in a 50°C water bath. While treating DIG 1 solution (100 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl) in 37°C water bath for 30 min, we made DIG 2 solution slowly melting 10 gm of block reagent powder in 100 ml of distilled water using a stirrer. The slides were incubated in DIG 2 solution for 2 h. After applying anti-DIG antibody (Roche, 1:100 dilution in DIG 1 solution), it was covered with parafilm (American National Can, Chicago, IL, USA) and then, it was incubated over-night in a refrigerator. After washing in DIG 1 solution for 3 h, it was changed to DIG 1 solution three times. To make the chromogen solution, a fresh tube was prepared with 2 ml of DIG 3 solution (100 mmol/l Tris–HCl, pH 9.5, 100 mmol/l NaCl) and 50 μl of 2 M $MgCl_2$ were added. A 9 μl of nitroblue tetrazolium salt (NBT) solution and 7 μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution were also added. After staining for more than 10 min till a dark blue colour appeared, the reaction was stopped with Tris–EDTA (10 mmmol/l Tris–HCL, 1 mmol/l EDTA, pH 8.0). Counter staining was done with contrast green (methyl green) for 10 min. It was mounted with Dako® Faramount (Dako).

Results

The BMP-4 expression patterns are listed in Table 2. Most DC specimens, the expression of BMP-4 was negligible in both in the epithelium (37/43 cases, 86.0%) and the mesenchymal cells (30/43 cases, 69.8%) with (−) degree staining (Fig. 1a). Some DC specimens, BMP-4 expression was visible in the epithelium (5/43 cases, 11.6%) and the mesenchymal cells (10/43 cases, 23.3%) with (+) degree staining (Fig. 1b). OKC specimens, however, showed the following BMP-4 staining pattern: BMP-4 was strongly expressed from both in the epithelium (15/34 cases, 44.1%) and in the capsular mesenchymal cells (17/34 cases, 50.0%)(Fig. 1c). The strong expression of BMP-4 was particularly well observed in the samples that showed inflammatory reaction (Fig. 1d). When the staining difference between two groups was compared using independent sample *t*-test, the epithelium in OKC showed stronger staining than the epithelium in DC ($P < 0.001$). The mesenchymal cells in OKC also showed stronger staining as compared with those in DC ($P < 0.001$).

Recurrent and non-recurrent cases of OKC were compared (Table 3). Recurrent cases showed that BMP-4 expression was strongly expressed both in the epithelial (7/10 cases, 70.0%) and the capsular mesenchymal cells (7/10 cases, 70.0%) with (++) degree staining. In non-recurrent cases, however, the BMP-4 expression was moderately strong in both epithelium (8/24 cases, 33.3%) and mesenchymal cells (10/24 cases, 41.7%) with (++) degree staining (Table 3). On

Table 2 Immunoreactivity in the dentigerous cyst and the odontogenic keratocyst

	Dentigerous cyst (n = 43) [n (%)]			*Odontogenic keratocyst (n = 34) [n (%)]*		
	0	+	++	*0*	+	++
Epithelium	37 (86.0)	5 (11.6)	1 (2.3)	4 (11.8)	15 (44.1)	15 (44.1)
Capsular mesenchymal cells	30 (69.8)	10 (23.3)	3 (7.0)	1 (2.9)	16 (47.1)	17 (50.0)

$P < 0.001$ in epithelium and capsular mesenchymal cells.

Table 3 Immunoreactivity in recurrent and non-recurrent cases of odontogenic keratocyst

	Recurrent cases (n = 10) [n (%)]			*Non-recurrent cases (n = 24) [n (%)]*		
	0	+	++	*0*	+	++
Epithelium	0 (0.0)	3 (30.0)	7 (70.0)	4 (16.7)	12 (50.0)	8 (33.3)
Capsular mesenchymal cells	0 (0.0)	3 (30.0)	7 (70.0)	1 (4.2)	13 (54.2)	10 (41.7)

$P = 0.036$ in epithelium and $P = 0.127$ in capsular mesenchymal cells.

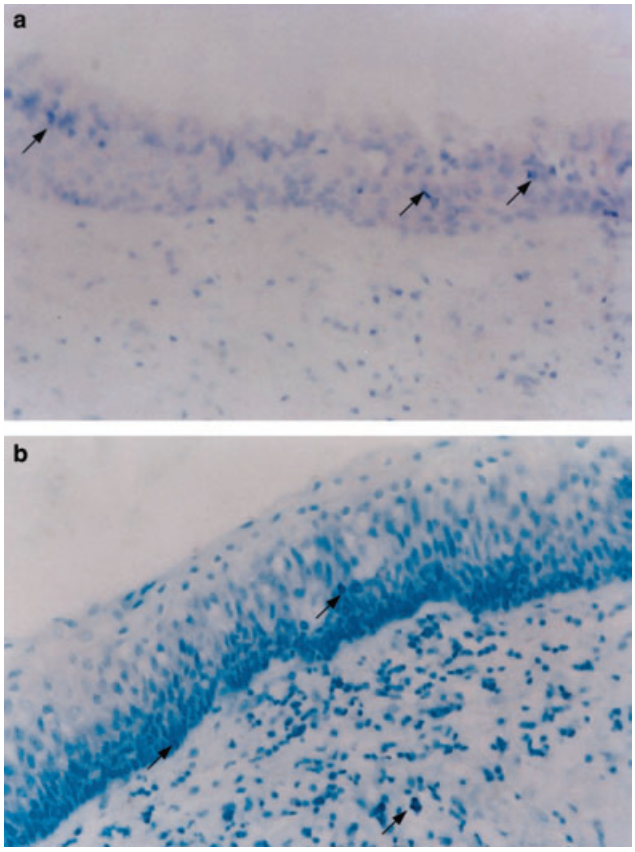

Figure 2 *In situ* hybridization showing the expression of bone morphogenic protein-4 (BMP-4) gene in the lining epithelium and mesenchymal cells (arrows). (a) The sample from dentigerous cyst (DC). (b) The sample from odontogenic keratocyst (OKC). The cells showed a positive to BMP-4 mRNA probe were more frequently observed in the samples from OKC than that from DC (methyl green counterstained; original magnification: ×200).

comparing the differences between groups with independent sample *t*-test, the epithelium in recurrent OKCs showed stronger staining than that of non-recurrent OKCs ($P = 0.036$). The mesenchymal cells in recurrent OKC cases also showed stronger staining, but it was not statistically significant ($P = 0.127$). The recurrent OKC cases showed multiple daughter cysts or the down-growing epithelium. Dense BMP-4 expression was observed in the epithelium of the daughter cysts (Fig. 1d) or in the down-growing epithelium (Fig. 1e). When both groups were compared with respect to sex and anatomic location, there was no statistically significant difference in BMP-4 immunostaining. The BMP-4 expression in OKC and DC was confirmed by *in situ* hybridization. Cells that showed a positive BMP-4 mRNA probe were observed in the samples from OKC and DC (Fig. 2a,b).

Discussion

In this study, we demonstrated that BMP-4 was differentially expressed between OKCs and DCs (Table 2). The strong expression of BMP-4 was observed in OKCs and also appeared to relate to recurrent cases (Table 3). However, BMP-4 expression was appeared unrelated to the anatomical location of the lesion, or patients' age and sex.

OKCs and DCs are believed to be derived from the remnants of odontogenic epithelial cells. The molecular mechanism of human jaw cyst formation as it relates to the expression of morphogenic signalling molecules has yet to be evaluated. Therefore, to know the differential expression of BMP-4 in various odontogenic cysts could help to clarify the molecular mechanism of cyst formation.

BMP-4 is related to transforming growth factor-β (TGF-β). Previous studies on BMP-4 focused on its role in new bone formation (12, 13). Some biomaterials containing BMPs show superior new bone formation ability as components of other materials, and has been used repair of models of bony defects (13). There are recent reports concerning other aspects of BMP-4 is important role in embryogenesis (4, 14, 15). It determines the fate of the undifferentiated mesenchymal cells. The distribution pattern of BMP-4 determines the dorso-ventral pattern of embryo (16). BMP-4 expression also contributes the T-lymphocyte differentiation (17). BMP-4 is very important for head and neck development. BMP-4 and its related molecules participate in the morphogenesis of the face and teeth. The interaction between BMP-4 and FGF-8 determines the morphology of the teeth (3, 15). We also found that the expression of BMP-4 was observed in normal oral epithelium (6). Thus, it can be assumed that BMP-4 has an important role in the development of the odontogenic cyst.

In most OKCs, expression of BMP-4 was found in the cystic epithelium (Table 2). In DC however, BMP-4 expression was only occasionally observed. There are three explanations for why BMP-4 is expressed more intense in the lining of OKC. First, the strong expression of BMP-4 may be because of the compromised upstream signals. As sonic hedgehog (SHH) protein regulates the BMP-4 expression, the strong expression of BMP-4 in OKC may be related to an abnormality of the SHH signalling pathway. The human homologue of the drosophila segment polarity gene patched (PTCH) is a component of SHH signalling pathway (18). It is mutated in the nevoid basal cell carcinoma syndromes (18). SHH signalling pathway has an important role during early murine teeth development (3). The addition of exogenous SHH protein directly to early tooth germs and adjacent to tooth germs results into abnormal epithelial invagination (19). It means that SHH may have a role in epithelial cell proliferation and cyst formation. SHH/PTCH signalling pathway may be abnormal in the nevoid basal cell carcinoma (18). Considering that BMP-4 is related to SHH signalling pathway, abnormal expression of BMP-4 may be because of the abnormality of SHH/PTCH signalling pathway. The daughter cysts in OKC frequently show clonal loss of heterozygosity of common tumor suppressor genes like PTCH (20). Dense BMP-4 expression was observed in the epithelium of the daughter cysts (Fig. 1d).

The second, there may be mutations in the expression regulatory sites in the BMP-4 gene. TGF-β1 gene expression is regulated by autoregulatory loop (21). As

analogy of TGF-β1 gene regulation, BMP-4 expression may be possible to use autoregulatory loop. If there are mutations in expression regulatory area in BMP-4 gene, it will show abnormal expression. Recently, we found mutations in the coding region of BMP-4 in the epithelium of the oral lichen planus (22) and BMP-4 mutations are found in human neural tube defects (23).

The third, the production of BMP-4 by immune cells may induce the production of BMP-4 in cystic lining epithelium. In our recent study, the BMP-4 expression was generally increased in the inflammatory oral epithelium (22). The BMP-4 produced by oscillatory shear stress stimulated an inflammatory response in endothelial cells (24). There have been several reports that inflammation in the underlying connective tissue can change the character of the lining epithelium (2, 25). The strong expression of BMP-4 was particularly well observed in the samples that showed inflammatory reaction (Fig. 1d). Besides, the ectopic application of exogenous BMP-2 or BMP-4 increased cell proliferation in the chick mandibular primordia (26). Thus, they have been shown to function as mitogens. Dense BMP-4 expression was observed in the epithelium of the daughter cysts (Fig. 1e) or in the down-growing epithelium (Fig. 1f). The daughter cysts and the down-growing epithelium are the features of recurrent OKCs (20) and the epithelium in recurrent OKCs showed stronger staining than that of non-recurrent OKCs ($P = 0.036$, Table 3). All these possibilities need to be proven by further study.

In summary, we showed the expression of BMP-4 in OKCs was stronger than that in DCs. In particular, it was strongly stained in recurrent OKCs. Although the exact role of BMP-4 expression in the odontogenic cysts has not been determined at this point, it might be related to the epithelial morphogenesis and invasive growth.

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