Co-expression of BMP-2 and -7 in the Tumoral Epithelium of CEOT with Selective BMP-7 Expression in Amyloid Materials

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Abstract: The calcifying epithelial odontogenic tumor (CEOT) is a benign but locally-invasive odontogenic neoplasm believed to take origin from the stratum intermedium of the developing tooth germ. Bone morphogenetic proteins (BMPs) are multifunctional signaling molecules that regulate diverse cellular processes including epithelial-mesenchymal interactions during odontogenesis. Aberrant BMP activity has been enumerated in the ameloblastoma but information about its distribution in the CEOT remains limited. The aim here was to investigate BMP expression in CEOT and to speculate on its significance. Immunolabelling for BMP-2 and BMP-7 was performed on archival tissues of six CEOT cases and the level of expression was quantified as negative (0), mild (+), moderate (2+) and strong (3+). Results disclosed that CEOT epithelium demonstrated co-expression of BMP-2 and -7 suggesting upregulation of these proteins at sites of tumor differentiation. Distribution patterns were distinct with some overlap. Their localizations were largely membranous and/or cytoplasmic. Amyloid-like materials strongly expressed BMP-7 but were nonreactive for BMP-2, implicating that these signaling proteins play differential roles in the formation of these extracellular products. Mineralized substances including Liesegang rings were mostly negative for both BMPs suggesting that calcification process is associated with repression of these molecules. Stromal endothelium and fibroblasts were stained variably positive. BMP was heterogeneously detected in the CEOT epithelium at the tumor advancing front suggesting their upregulation at active sites and downregulation in quiescent areas. Present findings suggest that BMP-2 and BMP-7 most likely play differential roles in the cellular differentiation and progression of CEOT. BMP-7 accumulation within amyloid-like protein is a novel finding.

Key words: Bone morphogenetic proteins (BMPs), BMP-2; BMP-7, Calcifying epithelial odontogenic tumor (CEOT), Amyloid, Liesegang rings

Introduction

The calcifying epithelial odontogenic tumor (CEOT) is a benign but locally-invasive odontogenic neoplasm that accounts for approximately 0.4-3.8% of all odontogenic neoplasms. It is unique for its tumoral growth pattern and the presence of amyloid materials that may become calcified. The CEOT is believed to originate from remnants of the dental lamina or stratum intermedium of the developing tooth germ. Clinically, CEOT has been reported in all age groups, peaking at age 40 years and shows an even sex distribution. The mandible is more frequently involved than the maxilla, and the molar region is the site of predilection. Most CEOTs occur centrally in the jaws and appear as unilocular or multilocular radiolucencies with occasional radiopaque deposits or association with an unerupted tooth. Peripheral variants are rare. Microscopically, the tumor is characterized by presence of sheets, strands, or discrete islands of polyhedral epithelial cells supported by a fibrous connective tissue stroma. These tumor cells have hyperchromatic and pleomorphic nuclei. Their cytoplasm is deeply eosinophilic with well-delineated borders and prominent intercellular bridges. A clear cell variant has also been reported. A distinguishing feature
of CEOT is the presence of amorphous and sometimes hyaline extracellular eosinophilic material that stains positive for amyloid. Calcifications are usually present. Noncalcifying CEOT is rare.

Bone morphogenetic proteins (BMPs) were first identified by Marshall Urist in 1965 as proteins with an ability to induce bone or cartilage formation when implanted subcutaneously or intramuscularly in animals. These polypeptides are secreted ligands that formed the largest subgroup within the transforming growth factor-β (TGFβ) superfamily. There are 30 types of BMP and these are present in all tissues and organs. BMP-2 induces osteogenic differentiation whereas BMP-7 stimulates chondrogenic phenotype of adipose tissue-derived mesenchymal stem cells. Recent evidence suggests that BMPs are multifunctional proteins involved in numerous cellular processes including proliferation, differentiation, chemotaxis and apoptosis of normal tissues during embryonic development and postnatally. BMP signaling is mediated via specific type I and type II serine–threonine kinase receptors (BMPR). Its ligand/receptor binding to type II receptor leads to oligomerization of the receptor complex, resulting in phosphorylation of the type I receptor and recruitment of downstream signaling protein namely Smad1, Smad5, and Smad8.

It is known that BMPs especially BMP-2, -4 and -7 are involved in modulating epithelial–mesenchymal interactions during tooth development. However reports of aberrant BMP activity in neoplastic odontogenic tissues remain conflicting. One study found positive BMP expression in benign cementoblastomas, dentinomas, odontogenic fibromas but was unable to demonstrate these proteins in ameloblastoma, adenomatoid odontogenic tumor and calcifying epithelial odontogenic tumor. In contrast, another study observed that BMP-2, -4 and -7 were identified in all ameloblastoma subsets with BMP-7 overexpression in the acanthomatous variant. These findings were further supported by a recent study that further demonstrated presence of these BMPs as well as BMP-3 in the tumoral and nontumoral component of ameloblastoma with upregulation of BMP-2,-3, -4 and -7 in the desmoplastic variant.

Odontogenic tumors constitute a clinically significant group of jawbone neoplasms in the Asian region and are therefore a subject of considerable research. Among the most well-studied are ameloblastomas, adenomatoid odontogenic tumor, keratocystic odontogenic tumor and calcifying cystic odontogenic tumor. These works have enumerated the clinicopathologic characteristics and clarified the various signaling molecules implicated in the development of the various tumor entities. Limited work has been published on Notch signaling proteins in the squamous odontogenic tumor. Our current ongoing work focuses on the immunohistochemical characterization of key signaling protein molecules namely Notch in the CEOT. Because of an earlier report that negates the presence of BMP activity in the CEOT, this study was undertaken to examine independently whether indeed these proteins are absent in this neoplastic entity.

Materials and methods

Tissue samples

The test sample consisted of six CEOT cases that fulfilled the diagnostic criteria of the WHO classification of tumors. All cases were intrabony lesions that demonstrated amyloid production and calcifications. This is a nonclinical study which was exempted from institutional ethics board approval (Research Grant No. FP028/2010A).

From the archival formalin-fixed, paraffin-embedded tissue blocks of these cases, new 5-μm-thick sections were prepared for staining with hematoxylin-eosin, Congo red and for immunohistochemistry with mouse monoclonal anti-BMP-2 (Abcam code: ab6285) and rabbit polyclonal anti-BMP-7 (Abcam code: ab56023).

Immunohistochemistry

Immunostaining for BMP-2 and -7 was performed using the Envision technique as previously described. Briefly, pretreatment of deparaffinized sections for antigen retrieval was done by microwaving (99°C) in 10 mM of citrate buffer (pH 6, 20 min). For blocking endogenous peroxidase, the sections were immersed in 0.3% methanol containing 3% hydrogen peroxide for 20 min and rinsing in 0.05 M Tris-buffered saline (TBS) (5 min, two times) before immersing in blocking solution (Dako Corporation, Carpintera, CA, USA) for 20 min at room temperature. Subsequently the sections were incubated with the primary antibody against BMP-2 (1:200) and -7 (1:200) for 1 h at room temperature. Immunoreactions were performed using the Envision Kit (Dako Corporation, Carpintera, CA, USA). The antigenic sites were visualized using diaminobenzidine (DAB) substrate chromogen (Dako Corporation, Carpintera, CA, USA) and counterstained with Mayer’s hematoxylin. Appropriate positive controls were applied. For negative control, sections were treated as above but without the primary antibody. All the control sections were negative.

Immunohistochemical analysis

BMP distribution pattern and levels of staining intensity in all six CEOT cases were evaluated using descriptive and semiquantitative methods. In the latter, the immunostained sections were systematically sampled and the level of expression for BMP-2 and -7 was quantified according to the staining intensity and percentage of immunopositive tumoral components present (CEOT neoplastic epithelial cells, amyloid-like substances and calcifications including Liesegang rings): (-) negative when none of these tumoral components are positively stained; (+) mild when
staining is present in focal areas (<25%); (+) moderate when staining is evident in significant areas (25-50%); and (+++) strong when staining is present in extensive areas (>50%). Stromal immunoreactivity was also assessed in a similar manner.

Results

**Microscopic findings**

Histological examination revealed lesional tissues composed of sheets, strands and nests of polyhedral epithelial cells arranged in a pavementing pattern (Fig. 1A-C). These epithelial cells have abundant eosinophilic cytoplasm, centrally placed nuclei and prominent intercellular bridges. Nuclear pleomorphism was occasionally observed. No mitoses were found. Interspersed extracellularly among these epithelial sheets are amyloid-like substances occurring as eosinophilic amorphous globules (Fig. 1B) or as diffuse stromal deposits. These amyloid globules were Congo-red positive (Fig. 1D) and gave an apple-green birefringence under polarized light (Fig. 1E). Scattered foci of basophilic acellular calcified materials, some exhibiting concentric laminations (Leisegang rings) were present in all six cases (1C).

![Image 1A](image1A.png) ![Image 1B](image1B.png)

Figure 1. Microscopic features of the calcifying epithelial odontogenic tumor: A, low-power sheets of polyhedral epithelial cells with scattered amorphous eosinophilic globules and calcifications (H&E); B, pavementing pattern of tumoral epithelium with interspersed amyloid globules (H&E); C, large irregular calcified mass with Liesegang ring formation juxtaposed with tumor epithelium (H&E); D, amyloid globules stained positive for Congo red (Congo red); and E, apple green birefringence of amyloid under polarized light (Congo red).

![Image 1C](image1C.png) ![Image 1D](image1D.png)

Staining intensity scores for A, BMP-2 and B, -7 in the tumoral and nontumoral components of CEOT.

![Image 1E](image1E.png)
Figure 3. BMP-2: A, positive expression in CEOT epithelial cells; boxed areas are detailed in B and C: B, localization is predominantly membranous and/or cytoplasmic; C, nonreactivity in amyloid globules; D, positive expression in tumoral epithelium and nonreactivity in bone at the tumor advancing front (arrowheads).

Figure 4. BMP-7: A, positive expression in CEOT epithelial cells and amyloid globules; note peripheral fibrous condensation resembling a capsule (arrowheads); B, localization is predominantly membranous and/or cytoplasmic, and in the osteocytes of bone at advancing tumor front (arrowheads); C, overexpression in amyloid globules; D, positive expression in the Liesegang rings.
The tumor showed partial fibrous condensation resembling a capsule in some peripheral areas of the tumor while in others, have infiltrative features. On the basis of these histological findings, a diagnosis of CEOT was made.

**Immunohistochemical findings**

### BMP-2 expression

BMP-2 protein distribution was detected in all six CEOT cases (Fig. 2A, 3A-D). Positive staining was observed heterogeneously in the sheets, strands and islands of polyhedral epithelial cells (Fig. 3A). Expression levels ranged from mildly focal to moderately strong and extensive (Fig. 3A). Immunopositivity was mainly localized in the cytoplasm and/or cell membrane (Fig. 3B). Amyloid-like protein products were consistently negative for BMP-2 (Fig. 3C). Calcified materials and Liesegang rings were nonreactive for BMP-2 (Fig. 5A). Stromal vascular structures and fibroblasts stained weakly for this protein. Bone and its osteoblasts at the tumor advancing front were nonreactive for BMP-2 (Fig. 3D).

### BMP-7 expression

All six CEOT cases demonstrated overexpression for BMP-7 in both the tumoral epithelium as well as the amyloid protein globules (Fig. 2B, Fig. 4A-D). Protein localization was membranous and/or cytoplasmic. Variable immunoreactivity for this molecule was observed in areas of calcifications (Fig. 4D, Fig. 5B) and in Liesegang rings (Fig. 5B). In the stromal endothelium and fibroblasts, BMP-7 was weak to barely detectable. At the bone infiltrating front, BMP-7 strongly stained CEOT tumoral epithelium but was mildly detected in the osteoblasts of the juxtaposed bone (Fig. 4B). No osteoclastic activity was encountered.

**Discussion**

Mammalian BMP2-7 are members of the decapentaplegic-Vg-related (DVR) gene family belonging to the TGF-β superfamily. The aim of this study was to clarify the distribution of two members, BMP-2 and -7 in six cases of CEOT. Our immunohistochemical results revealed that these two proteins were co-expressed in the tumoral epithelium but differ with respect to their distribution in amyloid globules. These findings contrasted with those of Gao et al.12 who found that BMP was absent in their two cases of CEOT. Accordingly, BMP was not detected in CEOT because the tumor cells might be too immature to synthesize detectable BMP12. In view of these discrepancies in findings between published12 and present study, examination of BMP activity in larger series of CEOT is warranted.

BMP-2 and -7 putatively play an important role in modulating epithelial-mesenchymal interaction necessary for induction of tooth formation. BMP-2 and -7 are present in the dental epithelium during the dental lamina, bud and cap stages28. However at bell stage, their expression patterns would shift from dental epithelium to the dental mesenchyme where BMP-2 is localized in differentiated odontoblasts and BMP-7 to the dental
mesenchyme\textsuperscript{28–31}. Current data revealed that BMP localization in the CEOT epithelium showed some similarities and differences with the expression patterns of these molecules in the developing dental epithelium during tooth morphogenesis\textsuperscript{9–11}. Positive expression for BMP-2 and -7 in CEOT epithelium was comparable with the co-distribution of these proteins in the presumptive dental epithelium during the initiation stages (bud and cap) of tooth development\textsuperscript{6,11,28–31}. From these observations, it might be possible that the CEOT epithelium arises from dental epithelium belonging to the earlier stage of cellular differentiation before lineage commitment.

The CEOT is a benign but locally-aggressive neoplasm with a tendency to invade bone. In this study, we also examined the CEOT-bone interface for BMP activity. We found that CEOT epithelium at the tumoral advancing front showed positive expression for both BMP-2 and -7, and that their expression levels were heterogeneous. This observation seems to suggest that those peripheral tumor areas showing BMP overexpression most probably represented active advancing tumor fronts whereas the underexpressed areas corresponded with quiescent sites. Along the bone interface, only BMP-7 was detected in the osteoblasts and osteocytes whereas BMP-2 was absent. It is known that BMP-2 induces osteoblastic differentiation whereas BMP-7 is implicated in chondrogenic differentiation in adipose tissues. Although the reason for the lack of BMP-2 expression in the bone adjacent to CEOT remains unknown, we speculated that BMP signaling within the tumoral environment might be different from physiological states. Recently, BMP expression was demonstrated in osteoclasts implicating their role in bone remodelling processes\textsuperscript{32}). However in this study we did not encounter any osteoclastic activity at the tumor advancing front in all six cases of CEOT evaluated and therefore is unable to verify this.

Amyloid is a fine nonbranching fibrillar protein with a cross â-pleated sheet structure. It is identifiable in tissues as pink amorphous substances that stained positively with Congo red to produce an apple-green birefringence under polarized light. Amyloid deposits may be local or systemic. In the latter, the primary form commonly complicated plasma cell dyscrasia whereas secondary amyloidosis is a frequent outcome of chronic inflammatory processes\textsuperscript{33}). CEOT is unique in that it synthesizes amyloid globules which are not native to the developing tooth primordium. In this study we demonstrated positive BMP-7 expression in these amyloid globules. BMP-2 was not detected. The significance of this finding remains unknown. In other systemic diseases such as Alzheimer disease, BMP-6 accumulations surrounding diffuse and mature amyloid-β plaques purportedly have deleterious effects on neurogenesis\textsuperscript{34}).

Calcifications and Leisegang rings in the CEOT were mostly nonreactive for both BMPs. The reason for this is not yet known but it may be related to the downregulation of these signaling molecules during the process of calcification. Gao et al\textsuperscript{32} suggested that the mechanism of calcification is possibly different and independent of BMP signaling pathway.

This study also revealed that the expression patterns of BMPs in the CEOT are distinctive from other odontogenic epithelial neoplasm such as the ameloblastoma\textsuperscript{41}. We speculated that these differences might be related to different functional activities of the BMP signaling molecules during the oncogenesis and cytodifferentiation of these tumor entities.

In summary, a study to investigate the distribution of BMP-2 and -7 in six cases of CEOT was carried out. Results revealed positive BMP expression and distribution patterns suggesting that these protein molecules most likely participate in the process of cellular proliferation and differentiation in this neoplasm. The strong BMP-7 expression in CEOT-associated amyloid-like substances is a novel finding.

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levels in the brains of Alzheimer's disease patients and APP transgenic mice are accompanied by impaired neurogenesis.