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Expression of Parathyroid Hormone-related Protein in Ameloblastomas

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Parathyroid hormone-related protein (PTHrP) was first discovered as a causative protein for hypercalcemia, which is often seen in the malignant tumor. PTHrP binds to the parathyroid hormone 1 receptor (PTH1R) for signal transduction. PTHrP-PTH1R interactions were associated with bone resorption. The present study, therefore, sought to clarify the expression of PTHrP, parathyroid hormone (PTH) and PTH1R in ameloblastoma, using RT-PCR (N = 8), immunohistochemistry (N = 23) and ELISA (N = 11) techniques. PTHrP and B-actin mRNA were detected in the all samples. Expression of PTHrP was also seen in all of the 23 cases in ameloblastoma by immunohistochemistry. There was a significant difference in PTHrP concentration by ELISA between typical unicystic type and solid type including unicystic type 3 (p = 0.0427). Only one exhibited the weak expression of PTH1R mRNA. PTH1R was observed on osteoblasts in bone around the tumor but no expression was observed on ameloblastoma cells in tumor parenchyma by immunohistochemistry. PTH was not detected in ameloblastoma by RT-PCR, immunohistochemistory as well as ELISA. In addition, hypercalcemia and increase of serum PTHrP level was observed in one case of 8 ameloblastomas. It was suggested that PTHrP level may be associated with local bone infiltration and hypercalcemia in ameloblastoma.

Key words: parathyroid hormone-related protein (PTHrP), parathyroid hormone protein (PTH), parathyroid hormone 1 receptor (PTH1R), ameloblastoma

INTRODUCTION

Parathyroid hormone (PTH) acts on the kidney and bone to closely regulate systemic homeostasis of calcium and phosphorousions [1]. PTH-related protein (PTHrP) was first discovered as a causative protein for hypercalcemia, which is often seen during the terminal phase of patients with malignant tumor [2]. These ligands bind to the parathyroid hormone 1 receptor (PTH1R) for signal transduction [3]. In addition, PTH is expressed in the parathyroid and nervous system [1], while PTHrP is wildly expressed in tissues such as the placenta [3, 4], epithelia [5] and mammary tissue [6]. These findings suggest that PTH and PTHrP play different roles [7]. In particular, PTH is found in blood, whereas PTHrP is not found in blood at the normal condition [7]. PTHrP is produced by epithelial cells, and binds to the PTH1R expressed by surrounding mesenchymal cells [8]. In other words, several studies have found that PTHrP acts in a paracrine manner. For example, PTHrP expressed in enamel epithelia binds with PTH1R expressed by osteoblasts around the alveolar bone to facilitate the resorption of alveolar bone [8]. The role of PTHrP during tooth eruption is to facilitate the resorption of alveolar bone [8]. Abdelsayed *et al*. [9] undertook immunohistochemical investigation of PTHrP expression in ameloblastoma cells. However, expression of PTH and PTH1R has not yet been investigated. In addition, association with PTHrP level and histological variation was not also understood. The present study examined the expression of

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Table 1 Expression of PTHrP, PTH and PTH1R in ameloblastomas

Case	Subtype	RT−PCR/immunohistochemistry/intra-tissue concentration			Serum Ca level (mg/dl)
		PTHrP(pmol/wg)	PTH(pg/wg)	PTH1R	
1	unicystic type2	N / + / 0	N / − / *	N / − / N	
2	unicystic type2	N / + / 22.76	N / − / *	N / − / N	
3	unicystic type2	N / + / 37.80	N / − / *	N / − / N	
4	unicystic type2	N / + / 38.48	N / − / *	N / − / N	
5	uncystic type2	+ / + / 0	− / − / *	− / − / N	9.8
6	uncystic type3	+ / + / 262.5	− / − / *	− / − / N	
7	plexform	+ / + / N	− / − / N	− / − / N	
8	plexform	N / + / N	N / − / N	N / − / N	9.3
9	plexform	N / + / N	N / − / N	N / − / N	
10	plexform (peripheral)	N / + / N	N / − / N	N / − / N	
11	plexiform	+ / + / 59.76	− / − / *	− / − / N	9.7
12	follicular	+ / + / 0	− / − / *	− / − / N	9.3
13	follicular	+ / + / 551	− / − / *	+ / − / N	
14	follicular	N / + / N	N / − / N	N / − / N	9.0
15	achantomatous	+ / + / 50	− / − / *	− / − / N	
16	achantomatous	+ / + / 434.3	− / − / *	− / − / N	17.4
17	achantomatous	N / + / N	N / − / N	N / − / N	
18	achantomatous	N / + / N	N / − / N	N / − / N	
19	basal	N / + / N	N / − / N	N / − / N	
20	basal	N / + / N	N / − / N	N / − / N	
21	granular	N / + / N	N / − / N	N / − / N	
22	malignant	N / + / N	N / − / N	N / − / N	8.1
23	malignant	N / + / N	N / − / N	N / − / N	9.4

Abbreviate: N, not done; + , positive; − , negative; * , less than 10 pg/ml.

PTHrP, PTH and PTH1R in ameloblastoma, using RT-PCR, immunohistochemistry and ELISA.

MATERIALS AND METHODS

1. Patients and serum Ca or PTHrP concentration

Surgical materials were retrieved from the pathology files of the Departments of Pathology at Tokai University School of Medicine during the period from 1995 to 2004. The tissue specimens were fixed in 10% buffered-formalin and embedded in paraffin. The tumors were reclassified, according to the WHO criteria [10], were shown in Table 1. Of the 23 tissue specimens, 21 were benign tumors and 2 were malignant tumors Case 6 and 13 were recurrent cases in benign ameloblastoma. Thirteen were male and 10 were female. Their mean age at the time of surgery was 46.5 years (16-76 y). The primary sites were mandible 19, maxilla 3 and gingiva 1. Measurement of serum level of calcium concentration was examined in 8 cases of patients. In addition, case 16 also measured the concentration of serum PTHrP. For analysis of RT-PCR (8 cases) or measurement of intra-tissue concentration (11 cases), fresh frozen tissues of ameloblastoma were used. All materials of this investigation were obtained after the patients were fully

informed about the nature and the aim of the study.

2. RNA Extraction and cDNA Synthesis

Total RNA isolation was performed using ISOGEN reagent (Nippon Gene, Toyama, Japan), in accordance with the manufacturer's instructions. RNA concentrations were determined by absorbance readings at 260 nm with a SmartSpec Plus spectrophotometer (BIO-RAD, Tokyo, Japan). RT was performed as outlined in the instruction manual, using a 1st strand cDNA synthesis kit (Roche Diagnostics Ltd, Lewes, UK).

3. PCR analysis

Oligonucleotide primers of hPTHrP, hPTH, and hPTH1R were described previously [11]. B-actin, were 5'-GGCCATCTCTTGCTC GAAGTC-3' and 5'-ACCTTCAACACCCCAG CCATG-3'. PCR was performed according to the manufacturer's instructions, using Ex Taq (TaKaRa, Tokyo, Japan). The RT product was amplified using Taq DNA polymerase after 10 min of denaturation at 95°C, followed by 28 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, product extension at 72°C for 1 min, and then 72°C for 5 min.

4. Immunohistochemistry

Immunohistochemical staining for PTHrP, PTH or PTH1R antigen was performed using the streptavidin-biotin complex immunoperoxidase method. The slides were then incubated in 3% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity and were pretreated for 10 minutes with 10% normal goat serum (Nichirei, Tokyo, Japan). Anti-PTHrP monoclonal antibody (dilution of 1:100, Yanaihara Lab, Shizuoka, Japan), PTH polyclonal antibody (dilution of 1:100, DAKO, Kyoto, Japan) or PTH1R monoclonal antibody (dilution of 1:100, Santa Cruz Biochemistry, Santa Cruz, USA) was used. In addition, osteopontin polyclonal antibody (cloneLF123, from Dr. L. W. Fisher) was used as a marker of osteoblast. The slides were incubated with primary antibody for 1 hour, followed by exposure to secondary biotinylated antibody (Nichirei) for 10 minutes and exposure to a streptavidin-peroxidase complex reagent (Nichirei) for another 5 minutes. Color was developed with a 0.02% 3,3-diaminobenzidine-tetrahydrochloride solution, containing 0.003% hydrogen peroxide in 0.01 M phosphate buffered saline (PBS), for 10 minutes. In addition, coloration for osteopontin polyclonal antibody used new fuchsin kit (Nichirei). After washing, the immunostained sections were counterstained in hematoxylin or methylgreen, dehydrated, cleared, and mounted. For negative controls, PBS or non-immunized mouse IgG or rabbit IgG was used instead of primary antibodies.

5. Measurement of PTHrP or PTH concentration in tissue

Intratissue concentrations of PTHrP (Mitsubishi Kagaku Yatoron, Tokyo, Japan) or PTH (Kyouwa Medics, Tokyo, Japan) in ameloblastoma were measured by a double antibody sandwich ELISA using a 96-well kit. A standard curve was generated by a plotting optimal density at 450 nm versus log of recombinant human PTHrP or PTH concentration. Results were represented as PTHrP picomol or PTH picograms/wg (wet tissue grams). Mann-Whitney U test was used to analyze the differences between unicystic type and solid type including unicystic 3 type (one case) in PTHrP or PTH concentrations. Significance was considered as $p < 0.05$.

RESULTS

1. Serum calcium or PTHrP

Only one of the 8 patients exhibited hypercalcemia. Level of serum calcium reached 17.4 mg/dl preoperatively in Case 16, but decreased to 8.7 mg/dl after tumor resection. Level of serum PTHrP reached 380 pmol/l preoperatively in Case 16, but decreased to 20 pmol/l after tumor resection. The resected tumor was diagnosed as acanthomatous ameloblastoma. Tumor cells invaded the bone (Fig. 1). Osteoclast and osteoblast are noted around the bone.

2. RT-PCR (Fig. 2)

Amplified products corresponding to PTHrP transcripts were detected in all samples. PTH mRNA was not detected in the ameloblastomas. Only one of case 13 exhibited the weak expression of PTH1R mRNA. B-actin mRNA was detected in all samples.

3. Immunohistochemistry

Expression of PTHrP was seen in all of the 23 cases. Localization of PTHrP

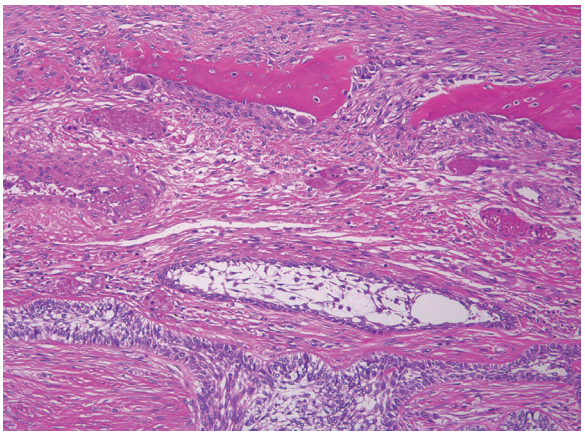

Fig. 1 Phtomicrograph of typical ameloblastoma showing no apparent cellular atypia. Bone invasion is noted.

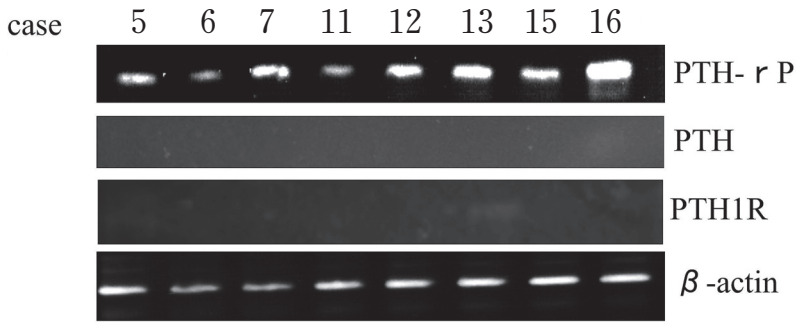

Fig. 2 PTHrP and B-actin mRNA were detected in all ameloblastoma samples by RT-PCR. Only one case, case 13 exhibited weak expression of PTH1R mRNA. PTH mRNA was not detected.

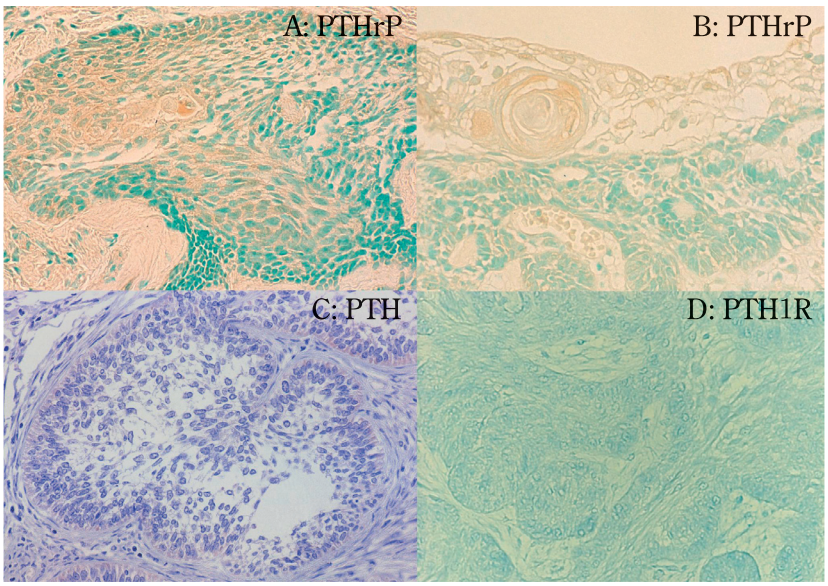

Fig. 3 Expression of PTHrP, PTH, PTH1R in ameloblastoma. (**A**) Plexiform type of ameloblastoma cells with focal squamous metaplasia. (**B**) Expression of PTHrP is observed in tumor cells of cystic type. (**C**) No apparent expression of PTH is noted in the ameloblastoma cells. (**D**) Expression of PTH1R is not detected in the ameloblastoma cells

Table 2 Expression of PTH1R in osteoblast

Case	Subtype	PTH1R
1	unicystic type2	N
2	unicystic type2	N
3	unicystic type2	+
4	unicystic type2	N
5	uncystic type2	+
6	uncystic type3	+
7	plexform	+
8	plexform	+
9	plexform	+
10	plexform(peripheral)	N
11	plexiform	-
12	follicular	+
13	follicular	N
14	follicular	+
15	achantomatous	N
16	achantomatous	+
17	achantomatous	+
18	achantomatous	+
19	basal	-
20	basal	+
21	granular	+
22	malignant	+
23	malignant	+

Abbreviate: N, not include bone in section; + , positive; − , negative.

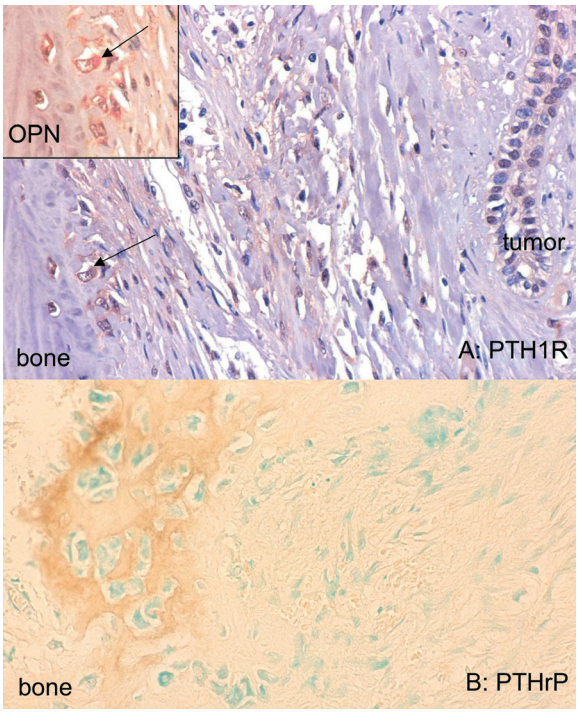

Fig. 4 Expression of PTH1R in ameloblastoma and bone. (**A**) Although expression of PTH1R is absent in tumor parenchyma, osteoblasts (arrow) indicating osteopontin positive reaction (inset) are positive for PTH1R. (**B**) PTHrP positive reaction is observed in bone matrix at invasive front of tumor in solid type ameloblastoma, but no PTHrP expression was noted in osteoblasts.

was mainly observed in ameloblast-like cells and stellate reticulum-like cells in solid type ameloblastoma (Fig. 3A). In particular, tumor cells accompanied by squamous metaplasia exhibited the strongest PTHrP immmunoreaction. In the cystic ameloblastomas, tumor cells of surface in cyst wall were strong positive for PTHrP immunoreactions while weak positive for the PTHrP was observed in basal cells (Fig. 3B). Expression of PTHrP tended to be stronger for solid ameloblastomas than for cystic ameloblastomas. Expression of PTHrP was also detected in malignant ameloblastoma cells, but degree of expression level was low. Expression of PTH (Fig. 3C) and PTH1R (Fig. 3D) was not seen in tumor cells. However, expression of PTH1R was weakly observed in some osteoblasts (Fig. 4A, arrow) indicating osteopontin positive reaction (inset). Expression of PTH1R in osteoblast was seen in 15 of the 17 cases (Table 2). In addition, PTHrP positive reaction was observed in bone matrix at invasive front of tumor, but no PTHrP expression was noted in osteoblasts (Fig. 4B). PTHrP positive reaction of bone matrix was identified in only solid type ameloblastoma (Fig. 4B). No apparent PTH immunoreactivity was noted in mesenchymal cells. No signal was detected for the negative control (data not shown).

4. ELISA

PTHrP was detected in 8 of the 11 cases. The 3 PTHrP(-) cases (cystic ameloblastoma 2 cases and follicular ameloblastomas one case) represented. Mean PTHrP level for the 11 cases was 132.4 pmol/wg (range, 0-551 pmol/wg). Mean PTHrP level was 19.8 ± 19.1 pmol/wg for the 5 cystic ameloblastomas and 226.26 ± 228.5 pmol/wg for the 7 solid ameloblastomas, indicating a significant

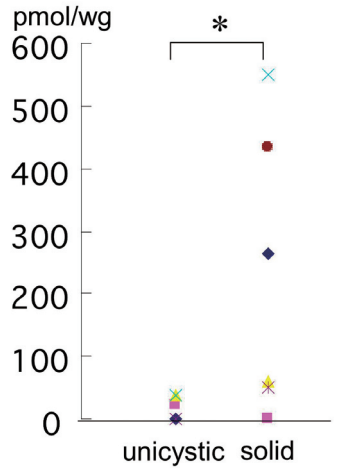

Fig. 5 PTHrP concentration in ameloblastoma tissues. PTHrP concentration in solid type ameloblastoma was higher than that of the cystic type by ELISA ($p = 0.0427$).

difference ($p = 0.0427$)(Fig. 5). PTH was less than 10 pg/ml of cut off level in all cases.

DISCUSSION

Ameloblastoma is the most common odontogenic tumor that resembles the enamel organ [10]. Ameloblastoma is a benign tumor, but grows invasively inside the jaw, and rate of recurrence is relatively high [10]. However, the mechanisms regarding the tumor infiltration into the jaw have not been fully elucidated. PTHrP was identified as a factor inducing hypercalcemia in malignancies such as squamous cell carcinoma of the tongue or lung cancer [12]. PTHrP, a second member of the PTH family composed of 139 amino acids, mimics PTH not only in its genomic structure, but also in its protein configuration [7]. The N-terminal domain of PTHrP, consisting of 13 amino acid residues, is homologous with that of PTH. PTHrP as well as PTH stimulates the PTH1R. Anti-PTHrP antibodies used in this study recognize amino acid 1-36 including N-terminus 1-13, which is essential for PTH1R activation. Recent studies have documented that PTHrP-PTH1R interaction induces the receptor activator of nuclear kappa B (RANK) ligand to osteoblasts, and osteoclasts expressing RANK was enhanced the bone resorption [13-15]. In other words, PTHrP is an important local humoral factor for bone resorption [13].

PTH protein and mRNA could not be expressed in tumor cells, but PTHrP protein and its mRNA were detected in all cases. In expression pattern of PTHrP, the present findings are consistent with previous report by Abdelsayed *et al.* [9] revealed its expression on ameloblastoma by immunohistochemistry. On the other hand, expression of PTH1R was seen in osteoblasts at the invasive front of tumor mass, but not in tumor cells themselves. In PTHrP knockout mice, teeth appear to develop normally but display a failure of tooth eruption [8]. When PTHrP gene was transfected in this mouse, increased levels of PTHrP expression in the enamel epithelium before the formation of the eruption were identified. In addition, PTH1R was expressed in both the mesenchymal tissue of the tooth germ and the alveolar bone [8]. During bone resorption in infiltration of ameloblastoma, it suggests that PTHrP produced by tumor cells may act on osteoblasts in a paracrine manner. Furthermore, protein level of PTHrP which was quantitatively measured using ELISA in tumors was significantly higher in solid ameloblastoma than in cystic ameloblastoma. In general, bone infiltration is rather weak in cystic ameloblastoma than that of solid ameloblastoma [10]. Level of PTHrP was high in Case 16, which exhibited the highest clinical level of bone destruction. In addition, Case 6 and 13 with high level of PTHrP was represented as recurrent cases. These findings suggest that PTHrP level in ameloblastoma may correlate to the tumor infiltration into the bone.

PTHrP secreted from tumor cells plays an important role in the onset of hypercalcemia in malignancy [16]. Only 8 cases of hypercalcemia in ameloblastoma have been reported (malignant, $n = 4$; benign, $n = 4$)[17-21]. However, no reports have documented the relationship between PTHrP expression and hypercalcemia. Case 16 was diagnosed as benign ameloblastoma because of the absence of atypical histological findings and clinical metastasis. However, in Case 16, hypercalcemia and increased blood level of PTHrP were confirmed. Furthermore, ELISA and immunohistochemical staining displayed high levels of PTHrP expression in tumor cells. These abnormalities were normalized after the tumor resection, suggesting that hypercalcemia in Case 16 was caused by PTHrP secreted from the tumor cells. Since

the size in this case was 160 × 100 mm in diameter, tumor size may be an important factor for induction of PTHrP-mediated hypercalcemia.

In summary, since ameloblastoma is develops invasively into the jaw, rate of recurrence is relatively high. Protein level of PTHrP was significantly higher in solid ameloblastoma than in cystic ameloblastoma by ELISA. Hypercalcemia and increased blood level of PTHrP were confirmed in case 16. It was suggested that PTHrP level may be associated with local bone infiltration and hypercalcemia in ameloblastoma. Antibody therapy targeting PTHrP may be possibly effective in the treatment of ameloblastoma.

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