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ORIGINAL ARTICLE

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Immunohistochemical localization of parathyroid hormone-related protein (PTHrP) and serum PTHrP in normocalcemic patients with oral squamous cell carcinoma

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Abstract Cancer cells produce parathyroid hormone-related protein (PTHrP) in the early phase of malignancy development, before hypercalcemia occurs. The relationship between PTHrP and the clinicopathologic features of oral squamous cell carcinoma is poorly understood. We studied 60 patients (43 men, 17 women; mean age, 64.8 ± 11.2 years) with primary oral squamous cell carcinoma, from whom pretreatment biopsy specimens were obtained. We examined the relationship among immunohistochemical PTHrP expression, serum PTHrP levels, clinical characteristics of the tumor, and histopathologic aspects of the tumor. The mean calcium concentration for the 60 patients was 9.1 ± 0.4 mg/dl. No patients had laboratory evidence of hypercalcemia before treatment. Six patients had serum levels of C-terminal (C)-PTHrP higher than the normal level of 55.3 pmol/l. There were no significant differences in serum C-PTHrP levels according to TNM stages. Abundant positive immunoreactivity for anti-PTHrP (1-34) antibody was recognized diffusely in the whole cytoplasm of many tumor cells. Anti-PTHrP (38-64) antibody staining tended to localize as small granules in the cytoplasm, especially close to the nuclear periphery. There was no correlation between the serum C-PTHrP concentration and the intensity of either immunostain. The intensity of PTHrP was proportionally related to the degree of differentiation or extent of keratinization ($P < 0.05$) and the histologic malignancy grade of the tumor ($P < 0.05$), when using antibody against PTHrP (1-34), but not when using antibody against PTHrP (38-64). Serum C-PTHrP levels did not correlate with the intensity of cellular PTHrP expression and characteristics of the tumor at the initial patient visit. The fragment that includes PTHrP (1-34) may be involved in the differentiation of oral squamous cell carcinoma. The differences between immunoreactivities may have been due to differing tissue malignancies and the use of different antibodies. The results suggest the need for caution when interpreting immunoreactivities of PTHrP in malignancies.

Key words PTHrP · Oral cancer · Squamous cell carcinoma · Immunohistochemistry · Radioimmunoassay

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Introduction

Parathyroid hormone-related peptide (PTHrP) was determined to be a causative factor for malignancy-associated hypercalcemia (MAH) in 1987.[1] Subsequently, PTHrP was isolated and its amino-acid sequence was determined from breast, lung, and kidney cancer cells.[2,3] A large proportion of MAH is due to humoral hypercalcemia with malignancy (HHM), in which PTHrP is the primary factor released from cancer cells that results in hypercalcemia. MAH frequently arises in malignant solid tumors such as squamous cell carcinoma of the head and neck, lung, and uterus; and adenocarcinoma of the breast, stomach, and kidney. Multiple myeloma, malignant lymphoma, and adult T-cell lymphoma/leukemia (ATL) are also associated with a high incidence of hypercalcemia.[4–6] Hypercalcemia has been reported in 4.2% to 90% of these malignancies.[5,7,8] In squamous cell carcinoma of the head and neck, including oral cancer, the incidence varies from 0.7% to 55%.[5,9–16]

Measurement of serum levels of PTHrP can be useful for predicting hypercalcemia as part of a paramalignant syndrome affecting multiple organ systems, including the neurologic, gastrointestinal, renal, cardiovascular, and musculoskeletal systems.[17,18] Little is known about the relationship between HHM and serum PTHrP levels in oral squamous cell carcinoma. Rikimaru et al.[14] reported a correlation between serum C-terminal (C)-PTHrP levels and serum calcium levels in head and neck cancers. We noted high serum calcium levels and increased PTHrP levels among patients who had late-stage oral squamous cell

carcinoma.[19] HHM is usually associated with malignancies in the late or developed stage. In the present study, we examined the serum calcium and PTHrP levels in patients with oral squamous cell carcinoma at the time of their initial visit for treatment of malignancies.

PTHrP binds to a common receptor, PTH/PTHrP receptor, on the osteoblast and affects bone resorption similarly to parathyroid hormone (PTH). Therefore, PTHrP may be related to bone metastasis or bone involvement in cancers. In breast cancer, PTHrP is highly expressed in the tissue of bone metastatic lesions, in contrast to little expression in nonskeletal metastatic lesions.[20,21] Similar results were reported in a study of PTHrP mRNA, suggesting that PTHrP influences tumor characterization.[22] Furthermore, it has been recognized that the *PTHrP* gene is expressed not only in cancers but also in most normal tissues in maturity and fetal life.[23] The physiologic functions of the protein include regulation of smooth muscle tone;[24] regulation of transepithelial calcium transport in renal, placental,[25] oviduct, and mammary gland cells;[26] and regulation of tissue and organ development, differentiation, and proliferation in endochondral bone, teeth, and mammary glands.[23,27] PTHrP is also expressed in the normal skin and mucosa.[28–31] In keratinocyte cell-line studies, Kaiser et al.[32–34] found that PTHrP acted as an effective inhibitor of cell growth and enhanced differentiation. These physiologic functions suggest that PTHrP may be involved in the physiologic characteristics of malignancies beyond skeletal involvement. It is assumed that cancer cells begin to produce PTHrP in early phases of malignant development before serum calcium concentrations rise. Therefore, we examined the relationship among PTHrP immunostaining in tumor cells, clinical tumor characteristics, degree of cellular differentiation, grade of malignancy, and serum PTHrP levels in patients with oral squamous cell carcinoma at their initial visit.

Subjects, materials, and methods

The study included 60 patients (43 men, 17 women; mean age, 64.8 ± 11.2 years) with primary oral squamous cell carcinoma who underwent pretreatment biopsy at the Second Department of Oral and Maxillofacial Surgery, The Nippon Dental University School of Dentistry at Niigata, University Hospital, from May 1986 to April 1996. The sites of the primary lesion were the tongue ($n = 17$), lower gingiva ($n = 15$), maxillary gingiva ($n = 9$), buccal mucosa ($n = 8$), floor of the mouth ($n = 7$), maxillary sinus ($n = 3$), and lip ($n = 1$). Clinical stage was defined according to the International Union Against Cancer (UICC) TNM classification (1997),[35] as follows: stage I ($n = 9$), stage II ($n = 11$), stage III ($n = 17$), and stage IV ($n = 23$). Paraffin blocks from the tissue archives of the Department of Oral Pathology were processed for histopathologic studies.

Radioimmunoassay

Serum specimens collected from patients before treatment were preserved at –80°C and used for C-PTHrP measurement. All specimens were defrosted fewer than two times. C-terminal PTHrP levels in the serum were determined by commercially available radioimmunoassay kits (Daiichi RI Laboratories, Tokyo, Japan). There were no cross-reactions with PTH (1-84), PTH (39-84), PTHrP (1-34), and PTHrP (107-138) for this measurement.

The procedure for measurement was as follows. The patient serum (200 µl) or standard C-PTHrP (PTHrP109-141, 200 µl, ^{125}I-labeled C-PTHrP, 100 µl), and antiserum C-PTHrP (100 µl) were incubated at 25 ± 5°C for 20 to 24 h. Boud/Free (B/F) separation was accomplished in tubes with 60 min of incubation. After centrifuging the tubes at 2000 g at 4°C for 30 min, the supernate was aspirated. The radioactivity of the pellet was measured by using an automatic well counter (Auto Well Gamma System; Aloka JDC-761, Tokyo, Japan). An assay standard curve was attained for standard C-PTHrP measurements. Patients who had abnormal serum creatinine concentrations were excluded from the study, because inadequate kidney function interferes with accurate measurement of C-PTHrP. The normal C-PTHrP concentration was determined as a range of 13.8 to 55.3 pmol/l.[36] Serum calcium was measured by the OCPC method. We compensated for calcium levels below 4.0 mg/dl via the following equation:

Compensated calcium concentration = measured serum calcium value (mg/dl) + (4 – serum albumin valuve [mg/dl]).[37,38]

Immunohistochemical study

Immunohistochemistry was performed for PTHrP using a labeled streptavidin-biotin (LSAB) method. Polyclonal rabbit antiserum against synthetic human peptide PTHrP (1-34) (kindly supplied by Yuka Medias, Tokyo, Japan) and monoclonal murine antibody specific for human PTHrP (38-64; Oncogene Science, Uniondale, NY, USA) (Ab-1) were used as the primary antibodies. Both antibodies were diluted 1:1000 for use. Formalin-fixed, paraffin-embedded 4-µM sections of the biopsy specimens from the 60 patients were examined. The sections were deparaffinized and rehydrated through alcohols to distilled water. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. Following a 30-min application of normal swine serum (10%; Dako, Copenhagen, Denmark), for the polyclonal antibody, and normal rabbit serum (10%, Dako), for the monoclonal antibody, to reduce background staining, the primary antibodies were applied overnight at 4°C. After being left for 30 min at room temperature, the slides were washed for 5 min each time in three changes of 0.01-M phosphate-buffered saline (PBS; pH 7.2) and incubated with 1:800 diluted biotinylated antirabbit immunoglobulin swine serum for the polyclonal antibody (Dako), and 1:400 diluted biotinylated antimouse immunoglobulin rabbit

Table 1. Malignancy grading system of oral squamous cell carcinoma

Histologic grading of malignancy of tumor cell population

Morphologic parameter	Score 1	Score 2	Score 3	Score 4
Degree of keratinization	Highly keratinized (>50% of thecells)	Moderately keratinized (20% to 50% of the cells)	Minimal keratinization (5% to 20% of the cells)	No keratinization (0% to 5% of the cells)
Nuclear polymorphism	Little nuclear polymorphism (>75% mature cells)	Moderately abundant nuclear polymorphism (50% to 75% mature cells)	Abundant nuclear polymorphism (25% to 50% mature cells)	Extreme nuclear polymorphism (0% to 25% mature cells)
Number of mitoses/HPF	0 to 1	2 to 3	4 to 5	>5

Histologic grading of malignancy of tumor-host relationship

Morphologic Parameter	Score 1	Score 2	Score 3	Score 4
Pattern of invasion	Pushing, well – delineated infiltrating borders	Infiltrating, solid cords, bands, and/or strands	Small groups or cords of infiltrating cells ($n > 15$)	Marked and widespread cellular dissociation in small groups of cells ($n < 15$) and/orin single cells
Stage of invasion (depth)	Carcinoma in situ and/or questionable invasion	Distinct invasion, but involving lamina propria only	Invasion below lamina propria adjacent to muscles, salivary gland tissues, and periosteum	Extensive and deep invasion replacing most of the stromal tissue and infiltrating jawbone
Lymphoplasmacytic infiltration	Marked	Moderate	Slight	None

HPF, high power field (×400)
From Anneroth et al.[39]

serum for the monoclonal antibody (Dako) for 30 min. Following rinsing with PBS, the sections were incubated with 1:800 diluted peroxidase-labeled streptavidin-biotin complex reagent for 30 min, dipped into PBS containing 0.03% diaminobenzidine and 0.005% H_2O_2 for 5 min, washed, and counterstained with hematoxylin. Sections employing the polyclonal antibody were subjected to antigen retrieval by heating in a microwave oven in 20% $ZnSO_4$ for 10 min after deparaffinization. The specificity of the PTHrP 1-34 polyclonal antibody was established by preabsorbing the antibody with PTHrP-NH_2 1-86 and observing reduced staining of tumors when used in the immunohistochemistry protocol.

Immunohistochemical staining was scored by assessing the staining intensity. A staining intensity that was stronger than the background staining in more than 10% of the field under 200-fold magnification was evaluated as positive. The positive staining was rated as slight (+), moderate (++), and intense (+++) based on the intensity of staining. Background staining was considered as the staining in normal connective tissue around the tumor.

Degree of keratinization, nuclear polymorphism, number of mitoses, pattern of invasion, stage of invasion, and lymphoplasmacytic infiltration were evaluated, by using the histologic grading system of Anneroth et al.,[39] in specimens stained with hematoxylin and eosin (H&E; Table 1). The differentiation status of histopathologic sections stained with H&E was classified as: well-differentiated (grade 1), moderately differentiated (grade 2), and poorly differentiated (grade 3).[40]

Statistical analysis

Comparisons were performed using the χ^2 test, Mann-Whitney *U*-test, Spearman rank correlation, and Student's *t*-test for unpaired data. A *P* value of less than 0.05 was considered statistically significant.

Results

Analysis of serum C-PTHrP concentration

The mean calcium concentration for the 60 patients was 9.1 ± 0.4 mg/dl. No patients had serum calcium levels greater than 10.2 mg/dl. The average C-PTHrP concentration was 41.1 ± 16.4 pmol/l. Six patients had C-PTHrP levels greater than 55.3 pmol/l, the maximum value of the normal range of serum C-PTHrP. There was no significant difference in serum calcium concentrations between the 54 patients whose C-PTHrP levels were in the normal range (9.07 ± 0.14 mg/dl) and the 6 patients whose concentrations were higher than the normal range (9.03 ± 0.22 mg/dl), according to Student's *t*-test. The PTHrP levels of these two groups of patients were 36.8 ± 9.3 pmol/l and 79.1 ± 20.1 pmol/l, respectively. There was no correlation between the concentration of C-PTHrP and the serum calcium (R = 0.03; *P* = 0.80). The mean concentration of serum C-PTHrP was 32.7 ± 10.9 pmol/l in the 11 patients who had T1 disease, 41.0 ± 11.5 pmol/l in the 22 patients who had T2 disease, 54.1 ± 22.1 pmol/l in the 10 patients who had

T3 disease, and 38.9 ± 16.9 pmol/l in the 17 patients who "had T4 disease. There was no significant difference in serum C-PTHrP levels according to the T classifications (χ^2 test). There was also no significant difference in serum C-PTHrP levels according to the stage (χ^2 test), with a mean of 34.1 ± 11.6 pmol/l in stage I (9 patients), 38.5 ± 9.2 pmol/l in stage II (11 patients), 46.9 ± 21.7 pmol/l in stage III (17 patients), and 40.8 ± 14.7 pmol/l in stage IV (23 patients).

The histopathologic grading of cell differentiation was evaluated in H&E stained specimens. The mean serum C-PTHrP was 44.5 ± 23.2 pmol/l in patients with poorly differentiated lesions, 43.4 ± 16.6 pmol/l in patients with moderately differentiated lesions, and 36.6 ± 10.3 pmol/l in patients with well-differentiated lesions, reflecting no significant differences in the C-PTHrP concentration based on primary tumor differentiation (χ^2 test). The degree of keratinization in the Anneroth classification[39] is characterized as highly, moderately, minimal, and not keratinized. Respective mean values of C-PTHrP according to these degrees of keratinization were 37.4 ± 10.0 pmol/l, 46.4 ± 18.6 pmol/l, 31.3 ± 5.0 pmol/l, and 47.4 ± 25.0 pmol/l. No significant difference in C-PTHrP concentration was seen according to keratinization status (χ^2 test), and there was no significant difference in serum C-PTHrP values based on the histologic grade of malignancy (χ^2 test).

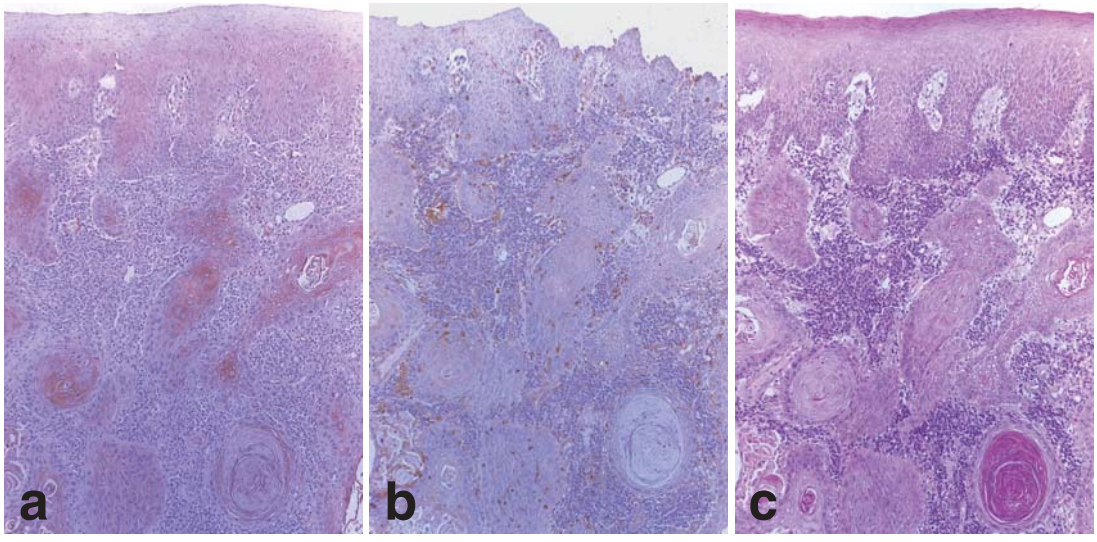

Fig. 1. **a** Immunostaining with anti-parathyroid hormone-related protein (PTHrP) (1-34) antibody was localized diffusely in the keratinized region of oral squamous cell carcinoma. **b** Anti-PTHrP (38-64) antibody immunostaining is dispersed in some tumor cells. **c** H&E stain. **a** ×100; **b** ×100; **c** ×100

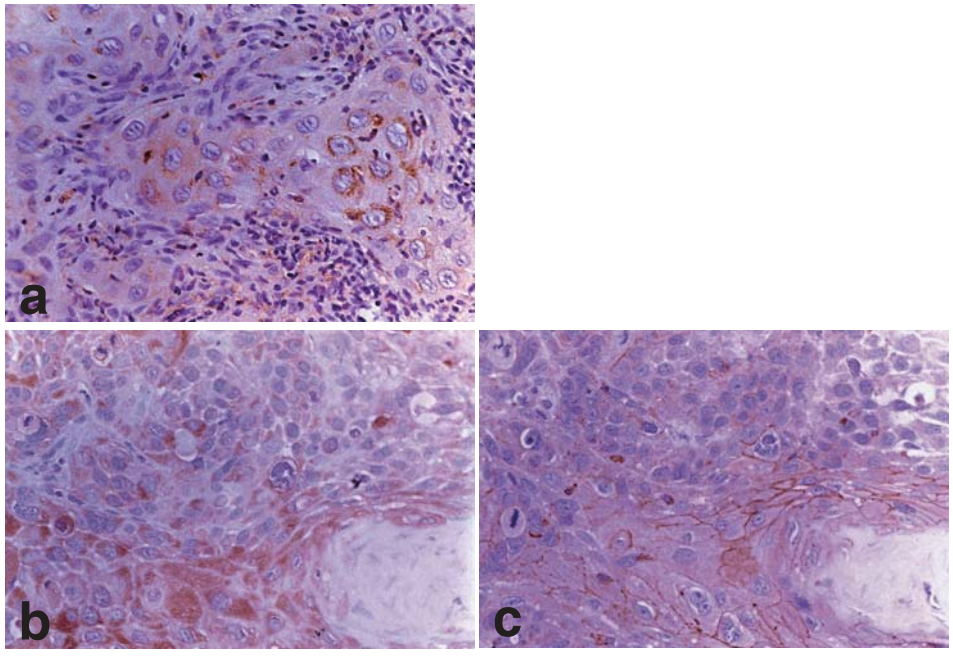

Fig. 2. **a** Staining with anti-PTHrP (38-64) antibody, showing small granules in the nuclear periphery. **b** Well-differentiated or keratinized oral squamous cell carcinoma showed stronger diffuse immunostaining in the cytoplasm with anti-PTHrP (1-34) antibody. **c** However, immunoreactivity with anti-PTHrP (38-64) antibody was localized in the circumferential area of the cellular membrane in the same field as that shown in **b**. **a** ×400; **b** ×400; **c** ×400

Table 2. Relationship between anti-PTHrP (1-34) antibody staining and tumor classification

	PTHrP (1-34)		PTHrP (1-34)	
	No-Slight (n = 24)		Moderate-Intense (n = 46)	
	No.	(%)	No.	(%)
T-Classification (TNM)[a]				
T1	4	(17)	7	(19)
T2	8	(33)	14	(39)
T3	5	(21)	5	(14)
T4	7	(29)	10	(28)
Stage category (TNM)[a]				
Stage I	4	(17)	5	(14)
Stage II	4	(17)	7	(19)
Stage III	8	(33)	9	(25)
Stage IV	8	(33)	15	(42)
Histopathologic grading (WHO)*				
Well-differentiated (grade 1)	5	(21)	17	(47)
Moderately differentiated (grade 2)	11	(46)	17	(47)
Poorly differentiated (grade 3)	8	(33)	2	(6)

* $P < 0.01$; Mann-Whitney U-test
[a] Not significant, χ^2 test

PTHrP localization

Of the 60 primary oral squamous cell carcinomas, 56 showed positive PTHrP immunoreactivity, at a variety of staining intensities, when using anti-PTHrP (1-34) antibody. The immunostain was diffusely dispersed in the cytoplasm of oral cancer cells. The staining was prominent in the more keratinized cells compared with less- or not-keratinized cells, although there was very little localization in completely keratinized or denucleotized areas (Fig. 1). Positive immunoreactivity for anti-PTHrP (38-64) antibody was also documented in 58 of 60 tumors. However, staining by anti-PTHrP (38-64) antibody appeared as small granules in the cytoplasm of the cancer cells, especially in the periphery of the nucleus (Fig. 2a). In some tumors, immunostaining for the anti-PTHrP (38-64) antibody was localized in the circumferential area of the cell membrane (Fig. 2c). Staining by both anti-PTHrP (1-34) antibody and anti-PTHrP (38-64) antibody was present in almost identical areas in 35 of 60 tumors (58.3%), with the remainder of cases showing different patterns or localizations of the stains.

PTHrP localization and tumor characteristics

The relationships between PTHrP distribution and tumor characteristics (TNM classification and histopathologic grading) are shown in Tables 2 and 3. There were no significant differences between the intensities of PTHrP staining with anti-PTHrP (1-34) antibody based on the T-classification or stage category of the TNM classification (χ^2 test; Table 2). There were significant PTHrP staining differences based on the WHO histopathologic differentiation grading ($P < 0.01$; Mann-Whitney U-test). No significant difference in anti-PTHrP (38-64) antibody staining was evident according to the T-classification or the stage category of the TNM classification, or the WHO histopathologic grading (Table 3; χ^2 test, Mann-Whitney U-test).

PTHrP localization and serum C-PTHrP level

Neither of the PTHrP immunostains correlated with serum C-PTHrP levels (χ^2 test). With the anti-PTHrP (1-34) antibody staining, the mean C-PTHrP level in the 24 cases with slight staining was 41.3 ± 21.8 pmol/l, the mean level in the 22 cases with moderate staining was 40.8 ± 10.4 pmol/l, and the mean level in the 14 cases with intense staining was 41.0 ± 13.1 pmol/l. Corresponding mean C-PTHrP levels were 43.3 ± 18.9 pmol/l with slight anti-PTHrP (38-64) antibody staining (n = 33 cases), 41.0 ± 13.1 pmol/l with moderate staining (n = 18 cases), and 33.2 ± 8.0 pmol/l with intense staining (n = 9 cases).

PTHrP localization and keratinization scores of the Anneroth[39] classification system

The respective mean keratinization scores of the Anneroth[39] classification were 2.3 ± 1.1, 1.7 ± 0.9, and 1.4 ± 0.6 for slight, moderate, and intense staining with anti-PTHrP (1-34) antibody. The keratinization scores for moderately and intensely immunostained tumors were lower than the score for slightly immunostained tumors ($P < 0.01$; $P < 0.05$, Spearman rank correlation, Student's t-test; Fig. 3a). Highly keratinized tumors demonstrated increasing immunostaining with anti-PTHrP (1-34) antibody. There were no significant differences between keratinization scores according to intensity of immunostaining with the anti-PTHrP (38-64) antibody. The mean keratinization scores were 2.1 ± 1.1 (intense stain), 1.6 ± 1.0 (moderate stain), and 1.7 ± 0.9 (slight stain).

Table 3. Relationship between anti-PTHrP (38-64) antibody staining and tumor classification

	PTHrP (38-64)		PTHrP (38-64)	
	No-Slight (n = 24)		Moderate-Intense (n = 46)	
	No.	(%)	No.	(%)
T-Classification (TNM)[a]				
T1	3	(9)	8	(30)
T2	12	(36)	10	(37)
T3	7	(21)	3	(11)
T4	11	(33)	6	(22)
Stage category (TNM)[a]				
Stage I	2	(6)	7	(26)
Stage II	8	(24)	3	(11)
Stage III	9	(27)	8	(30)
Stage IV	14	(42)	9	(33)
Histopathologic grading (WHO)[b]				
Well-differentiated	9	(27)	13	(48)
Moderately differentiated	17	(52)	11	(41)
Poorly differentiated	7	(21)	3	(11)

[a] Not significant, χ^2 test
[b] Not significant, Mann-Whitney U-test

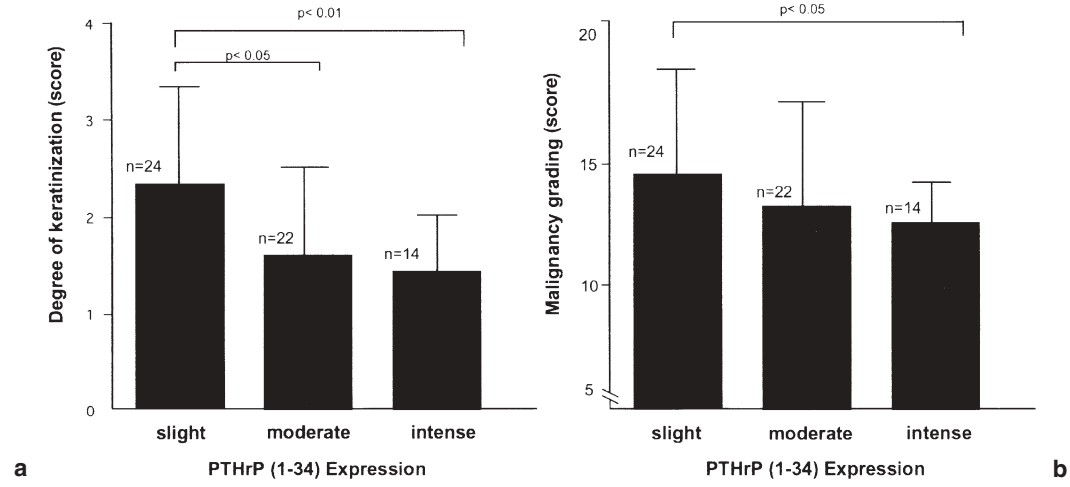

Fig. 3. a PTHrP Expression and keratinization scores according to the Anneroth[39] classification system, The keratinization scores of moderately and intensely immunostained tumors were lower than the score of slightly immunostained tumors ($P < 0.01$; $P < 0.05$; Spearman rank correlation, Student's t-test). Highly keratinized tumors demonstrated increasing immunostaining with anti-PTHrP (1-34) antibody. There were no significant differences between keratinization scores according to the intensity of immunostaining with anti-PTHrP (38-64) antibody. **b** PTHrP expression and the malignancy grading system. In the anti-PTHrP (1-34) antibody staining study, the score for malignancy grade of the Anneroth classification[39] was higher for slightly stained tumors than for intensely stained tumors ($P < 0.05$; Spearman rank correlation), which indicates that tumors which were not expressing PTHrP or expressing only small amounts had higher malignancy grades than tumors expressing more PTHrP. The anti-PTHrP (38-64) antibody staining did not show any significant correlation with the malignancy scores

PTHrP localization and the malignancy grading system

In the anti-PTHrP (1-34) antibody staining study, the malignancy score of the Anneroth classification was higher[39] for slightly stained tumors than for intensely stained tumors ($P < 0.05$; Spearman rank correlation; Fig. 3b), which indicates that the tumors which were not expressing PTHrP, or expressing only small amounts, had higher malignancy grades than tumors which were expressing more PTHrP. The malignancy scores for intense, moderate, and slightly positive tumors were 12.6 ± 1.8 (n = 14), 13.1 ± 4.0 (n = 22), and 14.7 ± 3.5 (n = 24), respectively. The anti-PTHrP (38-64) antibody staining did not show any significant correlation with malignancy scores. The malignancy scores were 12.7 ± 3.3 (n = 9) for intense stain, 13.1 ± 3.5 (n = 18) for moderate stain, and 14.2 ± 3.6 (n = 33) for slight stain.

Discussion

No patients in this study, had hypercalcemia (serum calcium >10.2 mg/dl), although 23 patients had stage IV malignancies. Goodwin and Chardler[9] reported that hypercalcemia

was rarely found at the time of diagnosis in patients who had squamous cell carcinoma of the head and neck; they found only 2 instances among 307 patients at the time of diagnosis. Hypercalcemia subsequently developed in 10 of 139 patients (7.2%) who did not remain disease-free 2 years after surgery or at the time of death. High percentages of oral or head and neck cancers are associated with hypercalcemia at the time of cancer-related death: 50% to 55% for tongue and larynx cancer and 35% to 45% for gingiva and esophagus cancer.[5] We previously reported hypercalcemia in 7 of 10 patients (70%) with oral squamous cell carcinoma who were in the late stage of the disease 3 months before death.[19] The results of the present study document that hypercalcemia becomes apparent in the late phases of oral squamous cell carcinoma, as reported in other malignancies.

In this study, 10% of patients (6/60) had high serum PTHrP values, although no patients had serum calcium concentrations greater than 10.2 mg/dl. Rikimaru et al.[14] reported high serum PTHrP levels in 4 patients with hypercalcemia among 37 patients with oral squamous cell carcinoma, including maxillary sinus carcinoma, and they documented a statistically significant correlation between serum C-PTHrP and calcium levels. In our previous study of 75 patients with oral squamous cell carcinoma, including end-stage, follow-up, and primary tumor patients, there was a positive correlation between C-PTHrP and serum calcium levels, with high C-PTHrP concentrations in patients with end-stage disease, all of whom also had hypercalcemia.[19] In contrast, there was no relationship between C-PTHrP and serum calcium levels in the current study. Furthermore, serum calcium concentrations were not significantly different between the patients who had normal and those who had high PTHrP levels. Calcium metabolic hormones, such as parathyroid hormone (PTH), 1,25 $(OH)_2$ vitamin D_3, and calcitonin, may compensate for marginal increments over the normal limit of serum calcium in these patients. One patient in the previous study had high serum C-PTHrP concentrations in advance of the development of hypercalcemia.[19] Possibly, hypercalcemia develops after a sufficient increase in serum PTHrP concentrations disturbs the control of serum calcium levels. Therefore, it is important to monitor serum PTHrP levels in patients with oral cancer who are in follow-up or have late-stage disease to predict the possible development of hypercalcemia. Furthermore, such measurements could differentiate hypercalcemia caused by PTHrP from that caused by other factors.

Many forms of PTHrP, such as N-terminal, mid-region, and C-terminal regions, are present in the circulation of patients who have HHM.[41–45] The fragments result from posttranslational processing, postsecretion processing, and degradation. Full-length PTHrP is probably a precursor protein that is processed into smaller peptides that have different biologic functions. The plasma half-life of the N-terminal fragments is 6 to 8 min.[46,47] The plasma half-life of C-terminal fragments may be longer than that of N-terminal fragments. Circulating PTHrP has been recognized by using radioimmunoassays (RIAs) that are immunologically targeted to PTHrP (1-34), (1-36), (1-40), (63-78), (109-138), and (109-141), as well as by two-site PTHrP immunoradiometric assays (IRMAs). Two-site IRMAs span much broader sequences than RIAs, including amino-terminal and mid-region sequences directed against PTHrP (1-72), (1-74), (1-84), (1-86), (1-108), and (1-141).[6,41–45,48] N-Terminal-detecting assays can recognize the portion, including amino acids (1-13), that is biologically active for exerting hypercalcemia by binding to PTH/PTHrP receptors on osteoblasts and epithelial cells of renal acinar canaliculi. IRMAs detect much longer amino acids than RIAs. Furthermore, the detection limit of the IRMAs is sensitive, to as low as 0.1 to 1.0 pmol/l. Thus, IRMAs are appropriate for detecting biologically active PTHrP. We used the C-terminal PTHrP RIA employing an antibody raised against amino acids 109–141 because it was capable of measuring the antibody in frozen-stored serum without protease inhibitor preparation. Other N-terminal peptide assays cannot measure antibody in serum that has been stored frozen. The minimum detection limit of the assay we used was 2 pmol/l. Because C-terminal fragments increase with insufficient renal function, even among patients who have normal intact PTHrP blood levels, we excluded samples that showed high serum creatinine levels from this study.

Can serum PTHrP be a marker to characterize oral squamous cell carcinoma? In our study, the mean C-PTHrP concentration in patients who had T3 or T4 disease was higher than that in patients who had T1 or T2 disease, although the difference was not statistically significant. There also was no statistically significant correlation between serum C-PTHrP levels and TNM stages. Blood PTHrP concentrations are frequently elevated in patients with breast cancer who have bone metastasis.[49] However, in a previous study using bone scintigraphy, we could not demonstrate any difference in C-PTHrP concentrations between patients with carcinoma of the gingiva who had normal and those who had increased mandibular bone uptake.[50]

When comparing serum C-PTHrP concentrations and the histologic characteristics of primary tumors, we used the malignancy grading system for oral squamous cell carcinoma proposed by Anneroth et al.,[39] which is a histopathologic evaluation system based on the degree of keratinization, nuclear pleomorphism, pattern of invasion, host response, and mitotic activity. The system has been shown to have good prognostic value when applied to the least differentiated tumor at the deep invasive front of oral carcinomas.[39,51,52] The serum C-PTHrP concentration did not correlate with the grade of malignancy or degree of keratinization. Thus, serum C-PTHrP was not an appropriate marker for demonstrating tumor characteristics at the time of the patient's first visit.

Many solid tumor cells, such as lung, oral, pharyngeal, esophageal, renal, breast, skin, prostate, cervical, and ovarian, occasionally express aberrant levels of PTHrP. A high proportion of squamous cell carcinomas (oral, pharyngeal, esophageal, lung, cervical, and skin) secrete PTHrP. No study has focused on correlations between PTHrP and the histopathologic characteristics of oral squamous cell carcinoma. We examined the relationship by using two

antibodies raised against different recognition sites, PTHrP (1-34) and PTHrP (38-64). Almost all oral squamous cell carcinomas expressed PTHrP with both antibody studies. Danks et al.[28] reported similar results, with 34/34 (100%) squamous cell carcinomas, including those of the floor of the mouth, lip, and esophagus, localizing PTHrP in a study using rabbit polyclonal antiserum against PTHrP (1-16). We observed abundant PTHrP (1-34)-positive immunoreactivity dispersed throughout the cytoplasm of many tumor cells. In contrast to the diffuse expression of PTHrP (1-34), anti-PTHrP (38-64) antibody staining was localized as small granules throughout the cytoplasm, especially close to the nuclear membrane, and, likely, inside Golgi bodies. In some specimens, the intercellular distribution appeared like latticework.

The *PTHrP* gene transcribes three isoforms of peptides – (1-139), (1-141), and (1-173) – by a process of alternative splicing.[53–56] Because recognition sites for both of the antibodies we used are included in all three isoforms, it seems that alternative splicing did not cause a difference in staining. Isoforms of PTHrP are processed to smaller fragments by proteolysis, with modification by glycosylation. The fragments exert specific individual biologic functions.[23,57,58] Specific splicing, posttranslational processing, and modification patterns may depend on the tissues or pathologic conditions, although this still is not clear.[55,59,60]

Heterogeneous immunoreactivity similar to our findings was found when antibodies to PTHrP regions (1-36) and (37-74) were used for an immunofluorescence study in human renal carcinoma cells and rat insulinoma cells stably transfected with the cDNA for PTHrP (1-141).[61] In that study, Soifer et al.[61] observed that cells stained with the anti-PTHrP (1-36) antibody disclosed a perinuclear staining pattern, which is typical for peptides located within the Golgi apparatus. Little or no peripheral staining was seen. In contrast, cells stained with antibody against the amino acid (37-74) region revealed perinuclear staining plus prominent fluorescence at the cell periphery, a location typical for proteins contained in secretory granules. Soifer et al.[61] suggested that keratinocytes secreted the amino-terminal species in a Golgi pattern via consecutive pathways, with the mid-region fragments being secreted in a secretory granule pattern. Our immunohistochemical results indicate that oral keratinocytes may secrete these different PTHrP fragments similarly.

It has been known that PTHrP acts in a paracrine or autocrine manner, but recently an intracrine function, without binding to the PTH/PTHrP receptors, has been reported.[62] Immunohistochemical studies show that PTHrP is transported to the intranuclear or intranucleolan region in osteoblast-like cells and COS-7 cells transfected with the *PTHrP* gene.[63] Similarly, PTHrP is targeted to the nucleolus in keratinocytes.[64] Therefore, the differences in immunohistochemical staining in our study may have been due to the different production, pathway degradation, or exocytosis of peptides after translation.

In our study, there was no correlation between serum C-PTHrP concentration and the intensity of immunostaining of either the antibody against PTHrP (1-34) or that against PTHrP (38-64). These results are in accordance with the outcome of a study of normocalcemic patients with primary breast cancer.[65] Similar results were reported regarding the relationship between circulating PTHrP and peptides or the mRNA expression of PTHrP in lung squamous cell carcinoma and adenocarcinoma.[66] Neither N-terminal detecting assays, two-site IRMAs, nor immunofluorometric assay could reveal a correlation.[65,66] It appears that circulating PTHrP does not consistently correspond to the status of the cellular expression of mRNA and peptides, especially in early phases of malignancies. This may be due to differences in the secretion extent, posttranslational processing, and degradation processes of PTHrP.

Is there any relationship between PTHrP and tumor characteristics? We found no statistically significant relationship among immunostaining, using both PTHrP antibodies, and the clinical tumor grade, tumor size, and nodal status. Similar results were reported in breast cancer[65,67] and renal cell carcinoma.[68] However, we found that the histopathologic score for keratinization was high in cases of increased PTHrP localization, suggesting that well-differentiated cancers possess more PTHrP. This correlation was shown in the anti-PTHrP (1-34) antibody immunostaining, but not in the anti-PTHrP (38-64) antibody study. Normal skin keratinocytes express PTHrP, and immunohistochemical studies using antibody raised against PTHrP, including NH_2-terminal, have shown that PTHrP stain localizes in the keratinized portion of normal human skin keratinocytes.[28–31,69,70] Kitazawa et al.[30] have suggested that PTHrP plays an important physical role in the normal keratinization of skin. An antisense RNA study also indicates that PTHrP acts to enhance differentiation in an established human keratinocyte cell line.[34] Results of studies of PTHrP-knockout mice that have been rescued by the transgenic replacement of the peptides and keratin-14 -PTHrP transgenic mice suggest that PTHrP regulates the rate of keratinocyte differentiation in the skin of adult mice.[71] In human cervical keratinocytes, PTHrP (1-34, 1-141) acts as an autocrine negative growth regulator. However, PTHrP (67-86) had no influence on cell growth in either normal or immortalized keratinocytes.[72] In an immunohistochemical clinical study of squamous cell carcinoma in the lung, well-differentiated and moderately differentiated tumors showed intense immunoreactivity to recombinant PTHrP (1-34), but two of five poorly differentiated tumors did not show the immunostain.[30] Similar results were also reported in an immunohistochemical study using a rabbit polyclonal antiserum against PTHrP (1-16).[28] These reports support the possibility that PTHrP affects the differentiation and inhibition of proliferation of oral squamous cell carcinoma.

In contrast to our findings, other studies have indicated that increased PTHrP immunoreactivity correlates with more aggressive tumors, and adverse outcomes related to this increased immunoreactivity were reported in other regional malignancies. The intensity of immunostaining, using an antibody raised against amino-acid fragment PTHrP (109-141), appeared to correlate directly with increasing histopathologic tumor grade in prostate carci-

noma.[73] Extensive staining was observed in poorly differentiated prostate carcinomas. Similar results were reported in prostate cancer, gastric tumors, and colorectal cancer, using antibodies raised against PTHrP (53-64), PTHrP (38-64), and PTHrP (38-64), respectively.[74–76] Squamous cell cervical carcinomas were immunohistochemically positive for PTHrP, but almost all adenocarcinomas were negative for staining by an antibody raised against PTHrP (1-34).[77] PTHrP induces both increased and decreased cellular differentiation in miscellaneous malignancies. The differences in function appear to depend on the histopathologic features of the malignancies. Antibodies recognizing different portions of PTHrP may also produce different immunostains. We observed differences in immunostaining between antibodies against PTHrP (1-34) and PTHrP (38-64).

In conclusion, we found serum C-PTHrP levels to be elevated in a subset of normocalcemic patients with oral squamous cell carcinoma at the time of the first visit. The serum C-PTHrP level was not correlated with the cellular expression of PTHrP or the characteristics of tumors. Immunohistochemically, the cellular expression of PTHrP was related to the extent of differentiation or keratinization and the histologic malignancy grade of the tumor when using antibody against PTHrP (1-34), but not when using antibody against PTHrP (38-64). The results of immunostaining in this study were not in accordance with some other studies, in which extensive staining of PTHrP was observed in poorly differentiated tumors. This difference may be related to the choice of antibodies. It is also possible that different tissues or malignancies have different posttranslational processing of peptides, pathways of exocytosis, and degradation. Immunostaining for PTHrP in patients with malignancy should be interpreted cautiously in the context of the immunohistochemical study examined.

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