Original Article

Significance of the Parathyroid Hormone-related Protein Expression in Breast Carcinoma

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Background: Parathyroid hormone-related protein (PTHrP) is produced by various neoplasms and is known to be a causative factor of hypercalcemia of malignancy. It has also been suggested to act as a cytokine for tumor progression. The purpose of this study was to clarify the significance of PTHrP expression in breast carcinoma.

Methods: PTHrP expression was examined in 177 surgically resected breast carcinoma specimens by immunohistochemistry using a monoclonal antibody against the for PTHrP, The relationship of PTHrP expression with clinicopathological factors was analyzed and the clinical courses of the patients are reported.

Results: Positive PTHrP staining was detected in 113 (64%) of the breast tumors. Among the positive cases, 36 (32%) of the tumors clearly showed strong expression. When the PTHrP expression was divided into three categories, a significant positive relationship was found between PTHrP expression and histological grade of tumor. PTHrP expression was also significantly related to bone metastasis but the staining degree of PTHrP was not. The patients with positive PTHrP tended to have poor outcome in proportion to the staining degree. Univariate analysis demonstrated a significantly shorter overall survival for patients expressing PTHrP, and in multivariate analysis showed that PTHrP status and nodal status were associated with a significantly shorter overall survival.

Conclusion: Our results suggest that PTHrP expression is not only correlated with bone metastasis but is also related to the progression of breast carcinoma, and that overexpression of PTHrP may be a potential prognostic factor for human breast carcinoma.

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Key words: Breast carcinoma, PTHrP, Survival, Bone metastasis

Parathyroid hormone-related protein (PTHrP) was originally discovered as a main causative factor of humoral hypercalcemia of malignancy[1]. This protein mimics the action of parathyroid hormone (PTH) in classic PTH target tissues such as bone and kidney. PTHrP is 70% homologous to the first amino acids of the N-terminal portion of PTH, and likewise binds to PTH receptors[2]. In addtion, PTHrP seems to regulate diverse biological activities in a wide variety of normal adult and fetal tissues, and serves as a growth factor during fetal development[3,4]. PTHrP is also produced by a variety of tumors without accompanying hypercalcemia. Expression of PTHrP has been shown in 60% of primary breast cancer cases[5]. Increased levels of PTHrP expression have been demonstrated in skeletal metastasis of human breast cancer[6-8]. Furthermore, PTHrP expression has been correlated with tumor proliferation and progression in gastric[9], colorectal[10], prostate[11], and thyroid tumors[12]. To clarify the clinical significance of PTHrP expression in breast carcinoma, we examined PTHrP expression in surgically resected tissue, its relationship with clinicopathological factors, and the clinical courses of the patients.

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Abbreviations:
PTHrP, Parathyroid hormone-related protein; ER, Estrogen receptor; PgR, progesterone receptor; EGF, Epidermal growth factor; TGB-β, Transforming growth factor-β

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Materials and methods

A total of 177 surgically resected breast carcinoma specimens were obtained from patients operated upon at Kanagawa Cancer Center be-

tween 1990 and 1996. The tumors were histologically graded by two of the authors (AY, YK) according to the criteria proposed by Bloom and Richardson, as modified by Elston and Ellis[13]. The estrogen receptor (ER) and progesterone receptor (PgR) were measured by standard dextran-coated charcoal assay. The medical records of each patient were reviewed. The age of the patients ranged from 27 to 87 years old (mean 53.4 years). There were 109 stage I patients, 29 stage II patients, 23 stage III patients and 6 stage IV patients. The mean follow-up period for the patients was 6.1 years, during which time 45 of 171 patients (excluding stage IV) showed disease recurrence and 30 of 177 patients died from their disease. For analysis of the relationship between distant metastasis and PTHrP expression, the patients were divided into three groups. Twenty-nine of 177 patients showed metastasis to bone with or without other metastases (Group A). The diagnosis of bone metastasis was made by scinitigraphy and plain X-ray, with 6 detected at the time of operation and 23 developing during follow-up. Twenty-two patients showed disease recurrence other than bone (Group B). The remaining 126 patients had no disease recurrence during follow-up (Group C).

Immunostaining for PTHrP was performed on paraffin-embedded tumor tissues, which were fixed in neutral formalin for exacting 24 hours, using the standard streptavidine biotin peroxidase method (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA, USA). Deparaffinized sections were preincubated with normal goat serum and were incubated overnight at 4℃ with an optimal dilution (5μg/mL) of a primary antibody against PTHrP (monoclonal; Oncogene Science, Inc., Uniondale, NY, USA14)). The reaction product was prepared by incubating the section with 3,3'-diaminobenzidine tetrahydrochloride as the chromogen, and the slides then were counterstained with hematoxylin. Negative controls were prepared by replacing the primary antibody with non-immunized mouse serum. The criteria used for assessing the immunostaining of the breast tumors were as follows. The intensity of staining of the tumor was graded on a three-point scale: 0 =no positive staining of tumor cells; 1=weak positive staining; 2=strong positive staining of tumor cells. The percentage of stained cells in each section was divided into three grades: 0=no positive tumor cells; 1=1-20% of the tumor cells were positive; 2=>20% were positive. The degree of staining was taken to be the sum of the staining intensity and percentage of cells stained: negative (−)=0-1; weakly positive (+)=2-3; and strongly positive (++)=4. Almost all strongly positive (++) had a widely stained area.

The chi-square test was performed to evaluate the relationship between PTHrP expression and clinicopathological features and bone metastasis. Survival curves were plotted using the Kaplan-Meier method, and their statistical significance was calculated by use of the log-rank test. The multivariate analysis concerning bone metastsis was evaluated by logistic regression analysis, and overall survival was evaluated by Cox regression analysis. All analyses were performed using the Statistical Package for Social Science (SPSS) statistical software program (SPSS Inc., Chicago, IL, USA).

Results

None of the cases in this study exhibited humoral hypercalcemia. Expression of PTHrP was observed throughout the cytoplasm of the tumor cells. Sixty-four (34%) of 177 cases were negative (−) for PTHrP, 76 (43%) were weakly positive (+), and 37 (21%) were strongly positive (++) for PTHrP expression in malignant cells (Fig 1). There was almost no immunostaining in the surrounding normal breast epithelial cells. The relationship between PTHrP expression and other clinicopathological features is shown in Table 1. No relationship was demonstrated between PTHrP expression and patient age, tumor size (major axis), nodal status, presence or absence of distant metastasis and estrogen receptor status. However, a significant positive relationship was found between PTHrP expression and histological grade ($p=0.002$).

Relationship between bone metastasis and PTHrP expression

Table 2 shows the relationship between distant metastasis and PTHrP expression. The incidence of weakly positive (+) tumors tended to be high in group A. When PTHrP expression was divided into two categoies, negative (−) and positive (+, ++), the positive of PTHrP expression in group A was siginificantly higher than that in group C ($p<0.05$). However, this relationship was not seen between groups B and C. The combined effect of

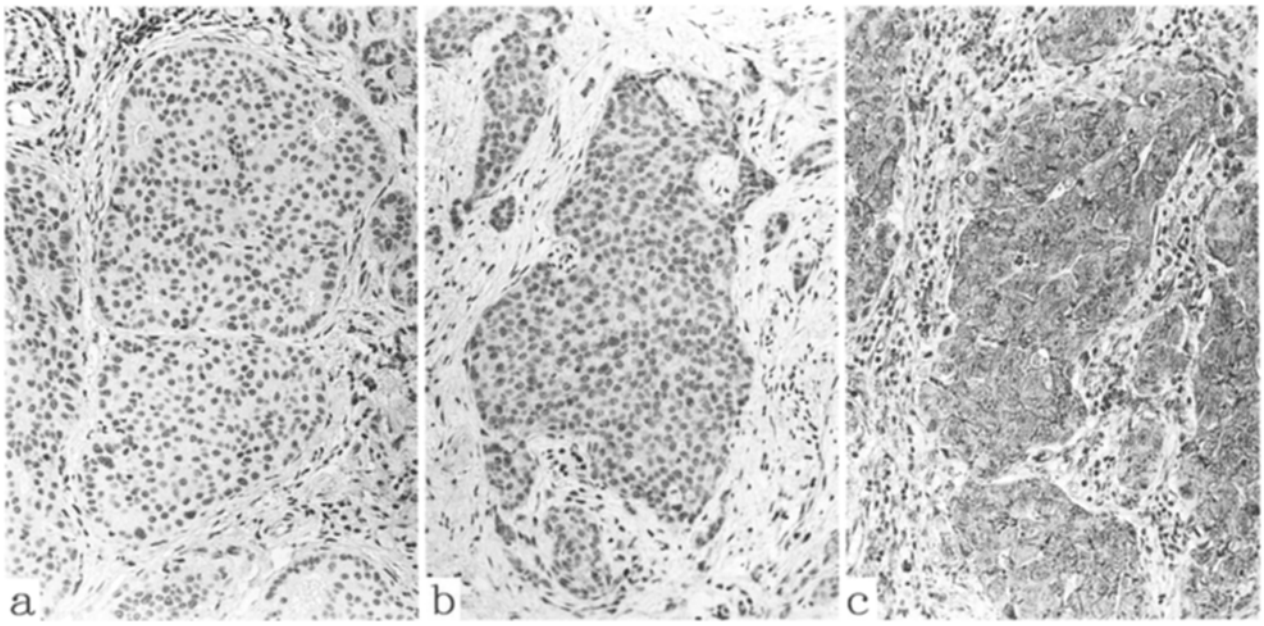

Fig 1. Representative immunostaining for PTHrP in breast carcinoma. a: negative (−), b: weakly positive (+), c: strongly positive (++).

Table 1. Relationship Between PTHrP Expression and Clinicopathological Factors in Breast Carcinomas

Factor		N	PTHrP expression Number of patients − (%)	+ (%)	++ (%)	χ^2-test
Age (y/o)	≦50	80	26 (32)	35 (44)	19 (44)	n.s.
	>50	97	38 (39)	41 (42)	18 (19)	
Tumor size (cm)	≦2.5	97	40 (41)	40 (41)	17 (18)	n.s.
	>2.5	80	24 (30)	36 (45)	20 (25)	
Nodal status	(−)	100	39 (39)	41 (41)	20 (20)	n.s.
	(+)	77	25 (33)	35 (45)	17 (22)	
Distance metastsais	M0	171	63 (37)	72 (42)	36 (21)	n.s.
	M1	6	1 (17)	4 (66)	1 (17)	
Histological grade	I	60	33 (55)	21 (35)	6 (10)	p=0.002
	II	65	17 (26)	33 (51)	15 (23)	
	III	52	14 (27)	22 (42)	16 (31)	
Estrogen receptor	(−)	88	27 (31)	41 (46)	20 (23)	n.s.
	(+)	89	37 (42)	35 (39)	17 (19)	

Table 2. Relationship Between Distant Metastasis and PTHrP Expression

Metastatic site	PTHrP expression Number of patients − (%)	+ (%)	++ (%)
Bone (Group A)	5 (17.2)	19 (65.5)	5 (17.2)
Visceral and / or soft tissue (Group B)	7 (31.8)	8 (36.4)	7 (31.8)
No distant metastasis (Group C)	52 (41.3)	49 (38.9)	25 (19.8)

PHTrP status, patient age, tumor size, nodal status, histologic grade and ER status on bone metastasis evaluated by logistic regression analysis is shown in Table 3. In the multivariate analysis, PTHrP status was the second most important factor concerning bone metastasis,

Table 3. Multivariate Analysis of Bone Metastasis in 177 Patients with Breast Carcinomas (Logistic Regression Analysis)

		Coefficient	*P*	Odds ratio
Age	(<50y/o, ≧50y/o)	−0.404	0.354	0.668
Tumor size	(<2.5cm, ≧2.5cm)	0.062	0.891	1.063
Nodal status	(+,−)	1.461	0.002	4.310
Histological grade	(I, II, III)	−0.172	0.558	0.842
Estrogen receptor	(+,−)	−0.026	0.953	0.974
PTHrP	(−,+)	1.141	0.039	3.131
Constant		−2.33	0.026	-

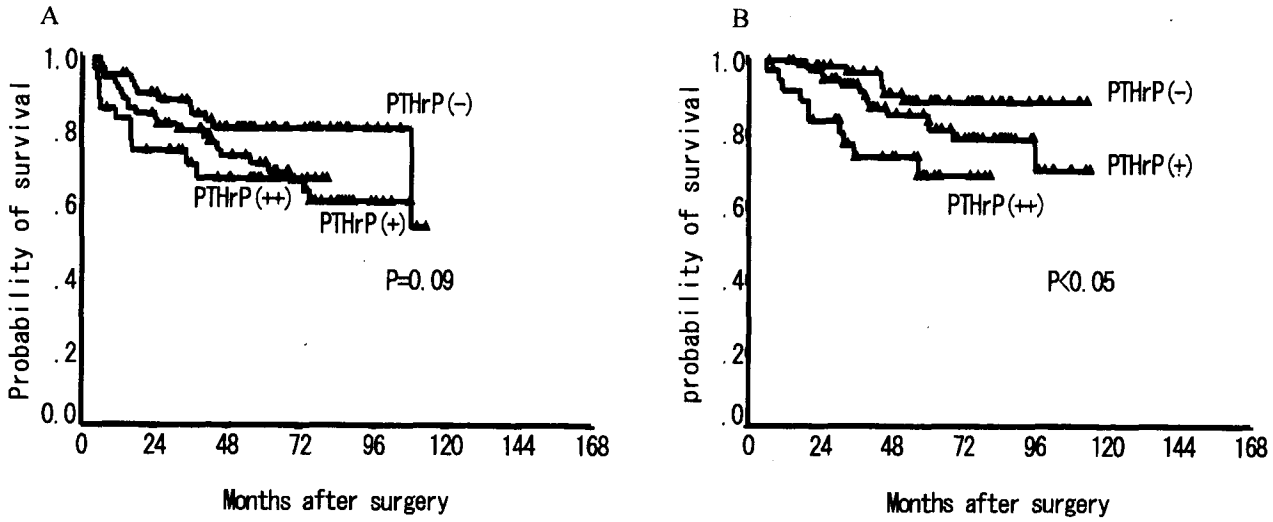

Fig 2. Kaplan-Meier life table analysis for disease-free survival curves (A) and overall survival curves (B) in 171 patients with breast carcinoma.

Table 4. Multivariate Analysis of Overall Survival in 171 Patients Intially Staged as M0 (Cox Regression Analysis)

		Overall survival RR (95%CI)	*P*
Age (y/o)	(<50, ≧50)	0.824 (0.372-1.827)	0.633
Tumor size (cm)	(<2.5, ≧2.5)	1.061 (0.467-2.407)	0.888
Nodal status	(−,+)	4.191 (1.661-10.578)	0.002
Histological grade	(I, II, III)	1.424 (0.816-2.487)	0.214
Estrogen receptor	(+,−)	0.593 (0.260-1.354)	0.215
PTHrP	(−,+,++)	1.979 (1.090-3.590)	0.025

following nodal status. However, when the PTHrP expression was divided into three categories; (−, +, ++), this relationship disappeared.

The relationship between PTHrP expression and clinical course

In the group of patients initially staged M0 (n=171), univariate analysis of disease-free survival and overall survival was performed according to PTHrP status (Fig 2). The patients with positive PTHrP expression tended to have poor outcome in proportion to their staining degree. There was a significant difference in overall survival curves. The joint effect of PHTrP status, patient age, tumor size, nodal status, histological grade, and ER status on overall survival, as evaluated by Cox regression analysis, is shown in Table 4. Multivariate analysis by Cox regression showed that, PTHrP status and nodal status were associated with significantly shorter overall survival.

Discussion

Positive PTHrP staining was detected in 64% of the breast tumors in this study. About 20% of tumors clearly showed strong expression, and when PTHrP expression was divided into three categories, it was seen to be directly related to histological grade. There have been many studies concerning PTHrP expression in breast carcinoma by immunohistochemistry[5-7,15-19]. However, no studies have shown an association with histological grade.

PTHrP expression was significantly related to bone metastasis when divided into two categories, positive and negative. However, staining degree was not related to bone metastasis. Several immunohistochemical studies using antibody against the amino-terminal portion of PTHrP exhibited a close relationship between PTHrP expression and bone metastasis[6,7,15]. In those studies, it was suggested that because of the PTH-like properties of PTHrP produced by tumor cells, tumors may cause increased osteoclastic bone resorption and facilitate bone metastasis formation. The discrepancy between our results and these studies might be attributable to a difference in the sensitivities of the primary antibodies used. The primary antibodies in each of the other immunohistochemical studies was generated against a different region of the PTHrP molecule. Mature PTHrP contains several amino acid residues that allow it to be cleaved into smaller fragments[20]. Distinct biological properties have been attributed to the different PTHrP peptides. The amino-terminal portion has homology with PTH and mediates the growth-regulating and hypercalcemic effects of the molecule. The physiological role of the other portion of PTHrP has yet to be fully elucidated.

Primary antibodies generated against different regions of the same target molecule might vary in their sensitivity as immunohistochemical reagents. It has been indicated that the immunoreactivivity of PTHrP in gastric cancer depends on antibodies for the amino- or carboxy-terminal residue of PTHrP[21]. The antibody used in our study was monoclonal and specific for an epitope in the mid-region of PTHrP. To our knowledge, there have been no previous studies of breast carcinoma using this antibody. Interestingly, studies performed on various other tumors such as thyroid[12], colorectal[10] and gastric tumors[9], using the same antibody as in our study showed that PTHrP expression was correlated with poor tumor differentiation and progression. It is suggested that mid-region and amino-terminal PTHrP fragments are packaged and secreted separately by the same carcinoma cells[20]. Together with these data, our results indicate that the mid-region fragment of PTHrP might be well preserved in tumor cells with histological dedifferentiation.

In our study, the patients with positive PTHrP staining tended to have poor outcome proportional to the staining degree. Univariate analysis demonstrated that significantly shorter overall survival and survival after recurrence was proportional PTHrP expression. In multivariate analysis, PTHrP expression and positive nodal status were associated with significantly shorter overall survival.

In the regulation of PTHrP transcription and translation, many cytokines and oncogenes are involved. Human breast carcinoma expresses a variety of growth factors that evidently regulate the growth of cancer cells. EGF[22] and TGF-β[23] are associated with tumor progression in breast carcinoma, and these growth factors up-regulate the PTHrP gene in some cell lines. EGF or TGF-β and PTHrP may also play a cooperative role in the development and/or progression of breast carcinoma[24,25]. In vitro experimental data have shown that when the 8 701 BC primary breast cancer cell line was subdivided according to the ability to express PTHrP mRNA, PTHrP-positive clones displayed more aggressive growth behavior than PTHrP-negative clones[26]. PTHrP has been shown to have growth factor-like activity[27] and to stimulate plasminogen activator in various cell lines[28,29]. It has been suggested that PTHrP might influence local control of invasive breast carcinoma. Furthermore, PTHrP is known to act as an autocrine growth factor in the breast carcinoma cell line MCF-7, known to express both the protein and the receptor for PTHrP[30]. Moreover, it has been reported that the PTHrP receptor is present in vivo in a majortiy of primary breast carcinomas[18,19]. These findings suggest that PTHrP is an autocrine growth factor for human breast carcinoma.

Our results suggest that PTHrP expression is not only correlated with bone metastasis but that it is also related to progression of breast carcinoma, and that overexpression of PTHrP may be a potential prognostic factor for human breast carcinoma.

References

1) Suva LJ, Winslow GA, Wettenhall REH, *et al*: A Parathyroid hormone-related protein implicated in malignant hypercalcaemia; Cloning and expression. *Science* 237:893-896, 1987.
2) Schipani E, Karga H, Karaplis AC, *et al*: Identical complementary deoxyribonucleic acids encode a human renal bone parathyroid hormone (PTH)/PTH-related peptide receptor. *Endocrinol* 132:2157-2165, 1993.
3) Roskams T, Desmet V: Parathyroid hormone-related peptides: a new class of mutifunctional proteins. *Am J pathol* 150:779-785, 1997.
4) Philbrrick WM Wysolmerski JJ, Galbraith S, *et al*: Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiol Rev* 76:127-173, 1996.
5) Southby J, Kissin MW, Danksa JA, *et al*: Immunohistochemical localization of paratyhyroid hormone-related protein in human breast cancer. *Cancer Res* 50:7710-7716, 1990.
6) Powell GJ, Southby J, Danks JA, *et al*: Localization of parathyroid hormone-related protein in breast cancer metastases; Increased incidence in bone compared with other site. *Cancer Res* 51:3059-3061, 1991.
7) Kohno N, Kitazawa S, Fukase M, *et al* : The expression of parathyroid hormone-related protein in human breast cancer with skeletal metastases. *Jpn J Surg* 24:215-220, 1994.
8) Bouizar Z, Spyratos F, Deytieux S *et al*: Polymerase chain reaction analysis of parathyroid hormone-related protein gene expression in breast cancer patients and occurrence of bone metastases. *Cancer Res* 53:5076-5071, 1993.
9) Alipov GK, Ito M, Nakashima M, *et al*: Expression of parathyroid hormone-related peptide(PTHrP) in gastric tumors. *J Pathol* 182:174-179, 1997.
10) Nishihara M, Ito M, Tomioka T, *et al*: Clinicopathological implication of parathyroid hormone-related protein in human colorectal tumors. *J Pathol* 187:217-222, 1999.
11) Iwamura M, Sant'Agnee PA, Wu G, *et al*: Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. *Cancer Res* 53:1724-1726, 1993.
12) Nakashima M, Ohtsuru A, Luo WT, *et al*: Expression of parathyriod hormone-related peptide in human thyroid tumors. *J Pathol* 175:227-236, 1995.
13) Elston CW and Ellis IO: Pathological prognostic factors in breast cancer. I . The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19:403-410, 1991.
14) Kramer S, Reynolds F, Castillo M, *et al*: Immunological identification and distribution of parathyroid hormone-like protein polypeptides in normal and malignant tissues. *Endocrinol* 128:1927-1937, 1991.
15) Kohno N, Kitazawa S, Sakoda Y, *et al*: Parathyroid hormone-related protein in beast carcer tissues: relationship btween primary and metastatic sites. *Breast Cancer* 1:43-49, 1994.
16) Bundred NJ, Walker, WA, Ratcliffe J. *et al*: Parathyroid hormone-related protein and skeletal morbidity in breast cancer. *Eur J Cancer* 28:690-692, 1992.
17) Liapis H, Crouch EC, Grosso LE, *et al*: Expression of parathyroidlike protein in nomal, proliferative, and neoplastic human breast tissues. *Am J Pathol* 143:1169-1178, 1993.
18) Downey SE, Hoyland J, Freemont AJ, *et al*: Expression of the receptor for parathyroid hormone-related protein in normal and malignant brest tissue. *J Pathol* 183:212-217, 1997.
19) Iezzoni JC, Bruns ME, Frierson HF, *et al*: Coexpression of parathyroid hormone-related protein and its receptor in breast carcinoma: a potential autocrine effector system. *Mod Pathol* 11:265-270, 1998.
20) Broadus AE, Stewart AF: Parathyroid hormone-related protein structure, processing, and physiological action. In: Bilezkan JP ed, The parathyroids, Raven, New york, pp259-294, 1994.
21) Abdeen O, Pandol SJ, Burton DW, *et al*: Parathyroid hormone-related peptide expression in human gastric adenocarcinoma not associated with hypercalcemia. *Am J Gastroenterol* 90:1864-1867, 1995.
22) Osborne CK, Hamilton B, Titus G, *at al*: Epidermal growth factor stimulation of human breast cancer cells in culture. *Cancer Res* 40:2361-2366, 1980.
23) Dalal BI, Keown PA, Greenberg AH: Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. *Am J Pathol* 143:381-389, 1993 .
24) Rodan SB, Wesolowski G, Ianacone J, *et al*: Production of parathyroid hormone-like peptide in a human osteosarcoma cell line: stimulation by phorbol esters and epidermal growth factor. *J Endocrinol* 122:219-227, 1989.
25) Kiriyama T, Gillespie MT, Glatz JA, *et al*: Transforming growth factor β stimulation of parathyroid hormone related-peptide (PTHrP); A paracrine regulator? *Mol Cell Endocrinol* 92:55-62, 1993.
26) Luparello C, Ginty AF, Callagher JA, *et al*: Transforming growth factor-β 1, β 2, and β 3, urokinase and parathyroid hormone- related peptide expression in 8701-BC breast cancer cells and clones. *Differentiation* 55:73-80, 1993.
27) Insogna KL, Stewart AF, Morris CA *et al*: Native and a synthetic analogue of the malignancy associated parathyroid hormone-like protein have in vitro transforming growth factor-like activities. *J Clin Invest* 83:1057-1060, 1989.
28) Grills BL, Gallagher JA, Allen EH, *et al*: Identification of plasminogen activator in osteoclasts. *J Bone Miner Res* 5:499-505, 1990 .
29) Luparello C, Burtis WJ, Raue F, *et al*: Parathyroid hormone-related peptide and 8701-BC breast cancer cell growth and invasion in vitro: evidence for growth -inhibiting and invasion-promoting effects. *Mol cell Endocrinol* 111:225-232, 1995.
30) Birch MA, Carron JA, Scott M, *et al*: Parathyroid hormone (PTH) / PTH-related protein (PTHrP) receptor expression and mitogenic response in human breast cancer cell lines. *Br J Cancer* 72:90-95, 1995.