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Clinicopathological correlation between expression of PTHrP receptor and various prognostic factors in breast cancer without axillary lymph node metastasis

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Purpose ; Expression of PTHrP is immunohistologically found in mammary tissues of all the normal cell, hyperplasia and malignant cell. In patients of breast cancer, positive ratio of PTHrP is reportedly 60-69%. Higher positive ratios of PTHrP are found in breast cancer showing bone metastasis than those without bone metastasis. And, breast cancer patients with PTHrP positive show poor prognosis suggesting a relationship to histological grade. Regarding PTHrP receptor, there is few report on its physiological role and relationship to other prognostic factors. The aim of this study was to determine the correlation between PTHrP receptor and various prognostic parameters of breast cancer patients with no axillary node metastasis.

Material and methods ; We employed 26 breast cancer patients, who showed negative axillary node metastasis and did not receive postoperative therapy. We examined relationship between PTHrP receptor and various prognostic factors such as hormone receptors, MIB-1 index, proliferation of cell nuclear antigen (PCNA), DNA ploidy, S-phase fraction, age, tumor diameter, c-erbB-2, and Cathepsin D.

Results ; No relationship between expression of PTHrP receptor and the cancer recurrence. PTHrP receptor correlated MIB-1 index alone ($p<0.044$). Conclusions ; This findings suggest PTHrP receptor is related to the tumor proliferation of breast cancer.

Key words: Parathyroid hormone-related protein (PTHrP), PTHrP receptor, Breast cancer

INTRODUCTION

PTHrP was discovered in 1987 from cell lines of pulmonary squamous cell carcinoma, breast cancer and renal cell carcinoma accompanied by hypercalcemia in all diseases above. PTHrP is known as a causative substance to develop humoral hypercalcemia of malignancy (HHM) and is found in plasma of over 90% patients showing HHM [1-3]. Physiological role of PTHrP is not clearly identified, but there are several reports to suggest the physiological roles. For example, PTHrP contributes to calcium metabolism in the bone and kidney [4], and PTHrP shows such local contributions as to inhibit final differentiation and proliferation of keratinocyte, suggesting that PTHrP presents influences on proliferation and differentiation of cells [5]. PTHrP reportedly is an autocrine growth factor in renal cell line of carcinoma. A breast cancer cell line called MCF-7 that develops mRNA of both PTHrP and PTHrP receptor, and PTHrP increases cAMP to proliferate the cell, indicating that PTHrP participates in proliferation of breast cancer cell [6, 7]. From a clinical aspect, there are reports indicated that 60-69% of breast cancer immunohistochemically showed PTHrP positive [8, 9], and 92% of breast cancer with metastasis to the bone was PTHrP positive, but only 17% of breast cancer showing metastasis to sites other than the bone was the PTHrP positive [10]. Other report indicated that breast cancer with PTHrP positive would have a higher

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incidence of bone metastasis and showed relationship to histological grade of breast cancer resulting in poor prognosis [11, 12]. Thus, these findings suggest relationship between PTHrP and bone metastasis of breast cancer. On the other hands, another report [9] pointed out no relationship between PTHrP and various prognostic factors of breast cancer patients. These findings indicate no clear physiological characteristics of PTHrP in breast cancer. Regarding to PTHrP receptor, there is few report on its physiological role and relationship to other prognostic factors of the breast cancer patients.

MATERIALS AND METHODS

In the present study, we employed 26 breast cancer patients (Table 1), who showed negative axillary lymph node metastasis and did not receive postoperative therapy but were capably confirmed of their survival, death or recurrence of breast cancer. The 26 patients underwent surgery from June, 1980 to September, 1987 in the Department of Surgery Tokai University, School of Medicine. We also reviewed cases reported by Khanna *et al.* [13]. In the present study, we examined relationship between PTHrP receptor and various prognostic factors such as hormone receptors, MIB-1 index, proliferation of cell nuclear antigen (PCNA), DNA aneuploidy, S-phase fraction, patient age, tumor diameter, c-erbB-2, presence or absence of hormone receptors and Cathepsin D. Breast cancer tissue was fixed in formalin and was embedded in paraffin to be cut for immunohistological staining.

Estrogen and progesterone receptors

Levels of estrogen and progesterone receptors were examined in all patients by using dextran coated charcoal method.

Immunostaining

The immunohistochemical staining was done on paraffin sections by MIB-1 recognizing Ki-67 (antibody: MIB-1, Immunotech, Marseille, France) [14], PCNA (antibody: anti PCNA, Dako, Glostrup, Denmark) [15], c-erbB-2 (antibody: polyclonal, Dako, Glostrup, Denmark) [16] and Cathepsin D (antibody: NCL-CDp, Novocastra, Newcastle, UK) [17]. After deparaffinization of the sections by using xylene/rehydration and ethanol, the endogenous peroxidase activity was bloked by using methanol/H_2O_2. Then, for different antibodies, indirect or avidin biotin peroxidaze complex (ABC) methods was employed. The diaminobendine was used as a chromogen, and counterstaining was conducted by using methyl green. For MIB-1, we performed antigen retrieval method by using microwave.

For nuclear antigens (MIB-1, PCNA), 1000 tumor cells were countered to obtain an index of positive cells to total number of the cells.

The c-erbB-2 expressions were graded according to the percentage of positive tumor cells with the staining on the cell membrane among the total tumor cells: −, no staining; +, less than 25%; ++, 25-50%; +++, 50-75% ; and ++++, more than 75%.

Formalin-fixed and paraffin-embedded sections were submitted for immunohistochemical study. The antibody

Table 1 Clinical features of the patients

	Recurrence (n=9)	Non-recurrence (n=17)	
Age	49.3±12.3	45.0±8.7	N.S.
Tumor size (cm)	3.0±8.7	2.2±1.3	N.S.
Menopausal status			
pre-menopausal	6	12	
post-menopausal	3	3	
Hysterectomy	0	2	N.S.

N.S ; not significant

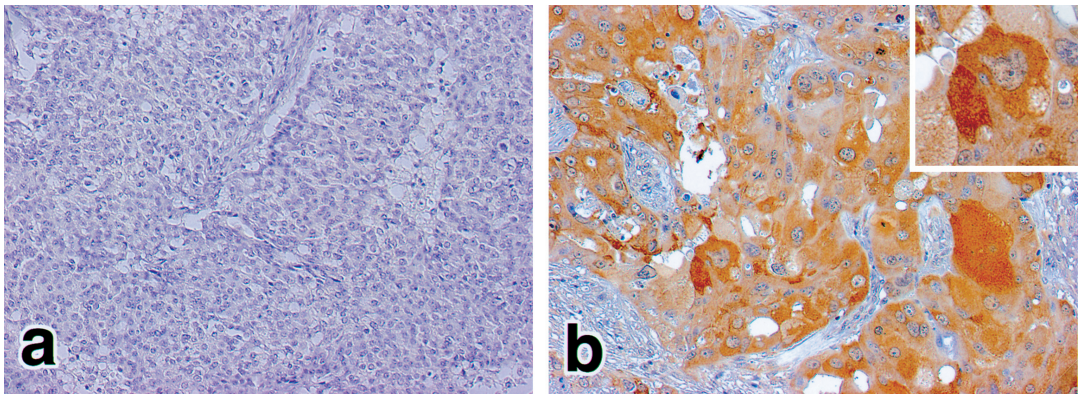

Fig. 1 **a**: Negative immunostaining for PTHrP receptor
b: Strongly positive immunostaining for PTHrP receptor
This slide was interpreted as having +++ immunostaining

used was anti-PTH receptor antibody (clone; OK-1, Berkely antibody con. USA). The PTHrP receptor expressions were graded according to the percentage of positive tumor cells with the cytoplasmic staining among the total tumor cells: −, no staining; +, less than 25% staining; ++, 25-59%; and +++, 50-75% (Fig. 1).

Flow DNA ploidy and S-phase fraction

Flow cytometric analysis was carried out on the cell preparation from paraffin sections. The method by Hedley *et al.* [18] was used with some modifications by using the FAC-scan (Becton Dickinson. USA). Normal human lymphocytes served as an internal control for normal (diploid) DNA content. The cell cycle analysis was done by the grating method and the S-phase qualification was obtained by using a RFIT model.

Statistical analysis

Student's paired t-test was used to identify presence or absence of either PTHrP receptor and the cancer recurrence, and Student's paired t-test, Fisher's exact test, χ^2 test were employed to confirm factors such as MIB-1, PCNA, aneuploidy, S-phase, tumor diameter, patient age, c-erbB-2, Cahepsin D, hormone receptor and PTHrP receptor.

RESULTS

The 26 patients underwent surgery from June, 1980 to September, 1987 in the Department of Surgery Tokai University, School of Medicine. We reviewed all cases on December, 2004. The 9 patients had recurrence, and 17 had no recurrence. All of the recurrent patients have died because of breast cancer, and other 17 patients are all alive. Table 1 shows patients backgrounds in detail. No significant difference was found in presence or absence of the cancer recurrence, patient age, tumor diameter and menopausal situation. Expression of PTHrP receptor included was estimated as negative in 12 patients (46.2%), 1+ in 4 (15.4%), 2+ in 2 (7.7%), and 3+ in 8 (30.8%), and no significant differences were found in expression level of PTHrP receptor and presence or absence of the cancer recurrence (Table 1). In the group of patients with recurrence, expression of PTHrP receptor was negative in 3 patients (33.3%); 1+, 2 (22.2%); 2+, 1 (11.1%); 3+, 3 (33.3%), and in the group of non-recurrent patients, negative, 9 patients (52.9%); 1+, 2 (11.7%), 2+, 1 (5.8%); 3+, 5 (29.6%). Neither significant difference was seen in expression level of PTHrP receptor (Table 2). Non-recurrence group had the tendency of negative PTHrP receptor. Results of our investigation on relationship between prognostic factors of breast cancer, factors indicating the cancer malignancy and PTHrP receptor expression showed causal relationship only between MIB-1 and PTHrP receptor expression (Table 3, $p=0.044$). And other prognostic markers, tumor diameter ($p=0.59$, Student's paired t-test), patient age ($p=0.76$, Student's paired t-test), overexpression of c-erbB-2 ($p=0.33$, Fisher's exact test), estrogen receptor situation ($p=0.42$, Fisher's

Table 2 Correlation between PTHrP receptor and recurrence status

	Recurrence (n=9)	Non-recurrence (n=17)	
Expression level of PTHrP receptor			
-	3 (33.3%)	9 (52.9%)	
+	2 (22.2%)	2 (11.7%)	
++	1 (11.1%)	1 (5.8%)	
+++	3 (33.3%)	5 (29.6%)	N.S.

N.S ; not significant

Table 3 Correlation between PTHrP receptor expression and various prognostic parameters

	PTHrP receptor(-) (n=12)	PTHrP receptor(+) (n=14)	
MIB-1 index	8.35±4.39	15.02±10.02	p=0.044
PCNA index	8.21±7.34	13.12±9.92	N.S.
DNA ploidy			
Diploidy	6	16	
Aneuploidy	3	3	N.S.
S-phase fraction	6.59±4.6	23.14±31.02	N.S.
Tumor size (cm)	2.36±1.22	2.66±1.54	N.S.
Age	47.2±10.6	45.9±9.9	N.S.

N.S ; not significant — mean±S.D.

	PTHrP receptor(-) (n=12)	PTHrP receptor(+) (n=14)	
c-*erb*B-2			
1+/2+	11	10	
3+	1	4	N.S.
Estrogen receptor			
-	7	4	
+	5	10	N.S.
Progesteron receptor			
-	7	8	
+	5	6	N.S.
Cathepsin D			
-/1+	7	5	
2+/3+	5	5	N.S.

N.S ; not significant

exact test), progesterone receptor situation ($p>0.99$, Fisher's exact test), Cathepsin D ($p=0.99$, χ^2 test), S-phase fraction ($p=0.08$, Student's paired t-test), DNA ploidy ($p=0.59$, Fisher's exact test), and PCNA ($p=0.17$, Student's paired t-test) had no significant correlation with expression of PTHrP receptor.

DISCUSSION

In the present study, we investigated relationship between PTHrP receptor expression and parameters previously regarded as prognostic factors of breast cancer patients or indexes of tumor malignancy, such as tumor diameter, patient age, c-erbB-2, hormone receptor situation, S-phase fraction, MIB-1 index, Cathepsin D, PCNA and DNA ploidy. The present study showed that only MIB-1 index had relationship with expression of PTHrP receptor ($p=0.044$). MIB-1 is a monoclonal antibody against proliferating antigen, Ki-67, in human nuclei, which appears in all the cell cycle including G1, S, G2 and M. On the other hand, Ki-67 does not appear at the resting cell cycle (G0). These findings suggest that PTHrP receptor may have a role for tumor cell proliferation in breast cancer.

Parathyroid hormone (PTH) and PTHrP share in the same receptor, and have a function to transmit their signals into the cytoplasm. PTH and PTHrP have similar 8 units of amino acid sequence in the N-terminal, however in other sites the homology of these two proteins was low. Cloning for cDNA of PTH/PTHrP receptor was carried out in 1991 by Juppner *et al.* using the opossum kidney (OK) cells [19]. The clone has 585 units of amino acid to make G protein related-type receptor. The PTH/PTHrP receptor shows a high homology with receptors such as calcitonin, secretin, glucagons, glucagons-like peptide-1 (GLP-1), growth hormone releasing hormone (GHRH), vasoactive intestinal peptide (VIP), VIP-2, pituitary adenylyl cyclase-activating peptide (PACAP), gastric inhibitory peptide (GIP) and corticotropin releasing factor (CRF). PTH/PTHrP receptor has formed a new receptor family with these receptors above [14]. The role of PTH/PTHrP receptor in breast cancer has not been fully understood. Hoey RP reported that PTHrP increased cAMP and DNA synthesis in a cell line of MCF-7 transfected and overexpressing PTHrP receptor stably, and the receptor antagonist reduced DNA synthesis in MCF-7 transfected by PTHrP gene [20]. It is speculated that DNA synthesis is increased by an autocrine mechanisms via PTHrP receptor. However, it has not been well demonstrated in clinical studies the relation between cell proliferating activity and PTHrP receptor that was shown in the present study.

Our present study showed no significance between expression and expression level of PTHrP receptor and recurrence situation of breast cancer. Linforth R, *et al.* reported that PTHrP receptor expression was more in bone metastases than early primary cancer. The PTHrP receptor was well correlated with increasing patient age, but not with tumor size, histological grade, hormone receptor status and lymph node status [21]. Individually PTHrP and PTHrP receptor both correlated with a reduced disease-free survival and receptor alone with reduced overall survival. PTHrP receptor predicted the worse clinical outcome [21]. The correlation between PTHrP receptor expression and bone metastasis is an issue to be further examined by larger number of the patients.

To conclude, we have shown that PTHrP receptor is expressed in breast cancer, and correlate with MIB-1 index. These data suggest PTHrP receptor had the relationship to tumor proliferation.

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