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Advances in Brief

Polymerase Chain Reaction Analysis of Parathyroid Hormone-related Protein Gene Expression in Breast Cancer Patients and Occurrence of Bone Metastases[1]

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Abstract

Parathyroid hormone-related protein (PTHrP) is associated with the syndrome of humoral hypercalcemia of malignancy. A high incidence of positive staining for PTHrP is observed in breast cancer and positivity is more frequent in patients who develop bone metastases. We assessed the presence of PTHrP mRNA by using the polymerase chain reaction in 38 normocalcemic breast cancer patients with long-term follow-up (minimum, 5 years) selected for the presence or absence of later bone metastasis development. In all the patients except one, the PTHrP gene was expressed in the breast tumor. The level of amplified PTHrP complementary DNA was inversely related to age ($P < 0.02$) and positively related to the proportion of invaded nodes ($P < 0.02$) but was not related to the other usual prognostic factors. The level of PTHrP mRNA was not different between the group of patients without recurrence or metastases ($n = 11$) and the group of patients who later developed metastases in soft tissues ($n = 10$). By contrast, patients who subsequently developed bone metastases ($n = 17$) showed higher PTHrP gene expression than patients in the other two groups ($P < 0.001$). This study suggests that strong PTHrP gene expression in breast tumors is associated with the onset of bone metastases.

Introduction

PTHrP[3] is the cause of humoral hypercalcemia of malignancy (1–3). The PTHrP gene contains 6 exons and its primary RNA transcript gives rise, through alternative splicing, to three mRNAs. These transcripts encode three related but COOH-terminally distinct proteins (1–139, 1–173, 1–141) with a common 139-amino acid sequence (2, 3). The first 13 NH_2-terminal amino acids are 70% homologous with the corresponding region of PTH, and this sequence homology accounts for similar biological activity. In a variety of biological assays, recombinant PTHrP(1–34) induces hypercalcemia and bone resorption, stimulates phosphate transport, and increases cyclic AMP production in kidney and bone cells (4).

Human and bovine milk contain large amounts of PTHrP (5, 6). Lactating rat breast tissue expresses PTHrP mRNA (7), and cultured mammary epithelial cells produce PTHrP (8). In 60% of normocalcemic women with breast cancer, immunoperoxidase staining of PTHrP is positive in tumoral tissue (9, 10). PTHrP positivity was found slightly more frequently in patients who later developed a bone metastasis (10). PTHrP might be involved in osteolytic metastasis. Indeed, most hypercalcemic patients with breast cancers metastasizing to bone had elevated plasma levels of PTHrP (11–13). PTHrP mRNA was detected by *in situ* hybridization more frequently in bone metastases than in nonbone metastases (14). Given the known propensity for breast cancers to metastasize to bone and the ability of PTHrP to increase bone resorption, it has been suggested that PTHrP production might be related to the ability of breast cancer to establish and grow in bone. This hypothesis is based on immunocytochemical studies using various polyclonal antibodies, which may not be optimally sensitive for PTHrP. Furthermore, this morphological method does not permit quantification. Studies using classical molecular biology methods are hampered by the frequent degradation of RNA in these tumors.

To overcome these difficulties, we used the reverse transcriptase PCR technique to identify and quantify PTHrP cDNA in samples from a retrospective group of breast cancer patients with long-term follow-up, selected for the presence or absence of bone metastases.

Patients and Methods

Patients. The 38 patients in this study (mean age, 58 years; range, 38–79 years) were treated at the Centre René Huguenin, St. Cloud, France, between 1981 and 1989. The follow-up was at least 5 years. Patients were selected from a regularly updated computerized data base and the corresponding tumor specimens were taken from a liquid nitrogen tumor bank containing 1600 malignant breast lesions, according to the following criteria: primary, M_0, operable, unilateral breast cancer; estrogen and progesterone receptors assayed on the primary tumor; no other primary cancer; full follow-up at the Centre René Huguenin; complete information on the variables; and sufficient tumor remaining for the assay of PTHrP gene expression. Three groups of patients were selected for the presence or absence of bone metastases: patients without recurrence or metastases ($n = 11$); patients who later developed metastases in soft tissues ($n = 10$); and patients who subsequently developed bone metastases ($n = 17$). Tumor specimens had been obtained during surgery. Immediately after removal, they were chilled on crushed ice and selected by the pathologist. One portion was kept for histoprognostic grading and the other was immediately frozen and stored in liquid nitrogen. Macroscopic tumor size was defined as the largest tumor diameter measured by a pathologist at the time of excision. The histoprognostic grade of the tumor was determined according to the methods of Bloom and Richardson (15) and Scarff and Torloni (16). Estrogen and progesterone receptors were assayed at the time of surgery by means of the dextran-coated charcoal method (17) with a detection limit of 10 fmol/mg protein. Quality control was assured by frequent testing with both internal controls and European Organisation for Research and Treatment of Cancer standards. Table 1 shows the relevant data for these patients.

Reverse Transcriptase Reaction. Total RNA was isolated by a guanidinium thiocyanate-phenol-chloroform method (18). The first cDNA strand was synthesized from 6 μg of denatured total RNA at 37°C for 60 min in a 20-μl reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.3) at 23°C, 2.5 mM $MgCl_2$, 100 μg/ml of nuclease-free bovine serum albumin, 20 units of RNase inhibitor (Promega; Coger, Paris, France), 1 mM concentrations of each deoxynucleotide triphosphate, 200 units of Moloney murine leukemia virus reverse transcriptase (BRL; Gibco France, Cergy, France), and 50 pmoles of a 3′ oligo$(dT)_{15}$ primer (Promega; Coger, Paris, France). The reaction was then stopped, and RNA and cDNA strands were separated at 95°C for 7 min.

PCR. Eighty μl of PCR buffer containing 50 pmol of each specific upstream and downsteam primer were added to the reverse transcriptase reaction mix. The primers used for amplification were 5′-GCGACGATTCT-TCCTTCACC-3′ and 5′-AGAGTCTAACCAGGCAGAGC-3′, corresponding to the sense and antisense strands of exon 4, respectively. Two units of AmpliTaq DNA polymerase (Promega) were added. Control of mRNA amplifica-

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[3] The abbreviations used are: PTHrP, parathyroid hormone-related protein; PTH, parathyroid hormone; PCR, polymerase chain reaction; cDNA, complementary DNA.

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Table 1 *Clinical and biological characteristics and PTHrP-amplified cDNA values (arbitrary units; mean ± SEM) in 38 patients with breast cancer*

	Patients		
	No.	%	PTHrP
Age			
≤50 yr	13	34	1.39 ± 0.23
>51 yr	25	66	1.02 ± 0.14
Menopausal status			
Pre-	14	37	1.39 ± 0.14
Post-	24	63	1.03 ± 0.16
Clinical tumor size (cm)			
≤2	4	11	1.65 ± 0.45
>2	32	84	1.03 ± 0.35
nd	2	5	1.89 ± 0.52
UICC[a] stage			
I	4	11	1.9 ± 0.9
II	21	11	0.90 ± 0.11
III	13	34	1.34 ± 0.25
Pathological nodal status			
ND	12	31	0.87 ± 0.19
1–3	11	29	1.03 ± 0.21
>3	14	37	1.54 ± 0.23
nd	1	3	0.36
Histological type			
IDC	34	89	1.18 ± 0.14
ILC	2	5	1.15 ± 0.43
AC	1	3	1.11
MC	1	3	1.01
Estrogen receptor status			
<10 fmol/mg of protein	15	39	1.00 ± 0.18
≤10 fmol/mg of protein	23	61	1.27 ± 0.17
Progesterone receptor status			
<10 fmol/mg of protein	23	61	0.99 ± 0.15
≤10 fmol/mg of protein	15	39	1.42 ± 0.21
Tumor calcification			
Absent	19	50	1.14 ± 0.21
Present	11	29	1.13 ± 0.17
nd	8	21	0.32 ± 0.1
Histopathological grade of Scraff, Bloom, and Richardson			
I	0	00	
II	19	50	1.42 ± 0.18
III	17	45	0.920 ± 0.22
nd	2	5	0.781 ± 0.43

[a] UICC, Union Internationale Contre le Cancer; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; AC, adenocarcinoma; MC, medullary carcinoma.

tion was performed in the absence of Moloney murine leukemia virus reverse transcriptase and after digestion of RNA by RNase A; four total RNA samples were treated with RNase A for 1 h at 37°C before PCR. Amplification was performed on a programmable thermal cycler (Techne; PHC-3) for 25 cycles. The reaction started with a denaturation step at 95°C for 30 s. Annealing was performed at 55°C for 30 s and elongation at 72°C for 30 s (19).

Amplified products were analyzed by agarose gel (2%) electrophoresis in TEB (Tris EDTA/Borate, electrophoresis buffer pH 8) buffer, and DNA fragments were visualized by ethidium bromide staining and photographed under UV light using positive/negative instant film (Polaroid 665; Kodak). DNA molecular weight markers with sizes of 2176, 1766, 1230, 1033, 653, 394, 334, 298, 220, and 154 base pairs were used. The negative films were analyzed by using a densitometric scanner (Transidyne General Corporation), and results were expressed as arbitrary units. The densitometric value is the mean of three scans.

PCR experiments and densitometric scanning were performed twice for 12 tumors and the average values of PTHrP cDNA obtained in these two independent experiments were strongly correlated ($r = 0.92$; $P < 0.005$).

Statistical Methods. Data were analyzed using parametric (analysis of variance and regression analysis) and nonparametric tests (Kruskal Wallis test; Mann- Whitney and Spearman rank correlation). Differences or correlations were considered significant at $P < 0.05$.

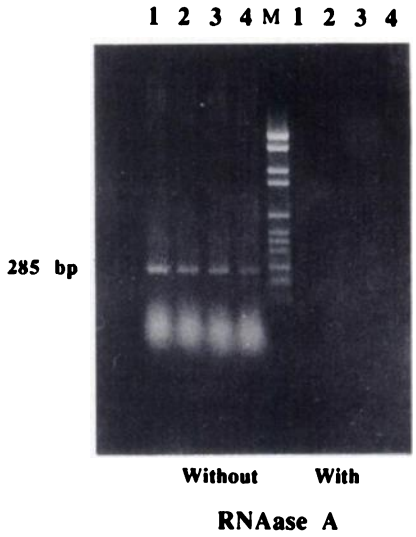

Fig. 1. Treatment with RNase A. Total RNA samples (6 μg) from four patients were treated or not treated with RNase A (3 mg/ml) for 1 h at 37°C. Then PCR reaction was performed. Conditions and procedures are described in the text. *M*, DNA molecular weight markers with a size of 2176, 1766, 1230, 1033, 653, 394, 334, 298, 220, 220, and 154 base pairs. The 285 base pairs disappear in the samples treated with RNase A.

Results and Discussion

Ethidium bromide staining of products amplified using PTHrP-specific primers in exon 4 revealed a single band of the expected size (285 base pairs) in positive cases. The band was not observed after RNase A digestion of RNA, proving the specificity of the reaction (Fig. 1). PTHrP gene expression increased linearly with the amount of mRNA (2–8 μg) (Fig. 2). PTHrP expression was detected in all cases except one (liver metastasis). This nearly uniform expression of the PTHrP gene in breast cancer has not been reported previously. Using *in situ* hybridization on paraffin sections, a less sensitive method than PCR, PTHrP mRNA was observed in only 10 of 17 primary tumors (14). It is possible that PTHrP mRNA is not translated in every case; PTHrP was detected by means of immunocytochemistry in only 60% tumors (9, 10), but this method might underestimate the presence of the protein.

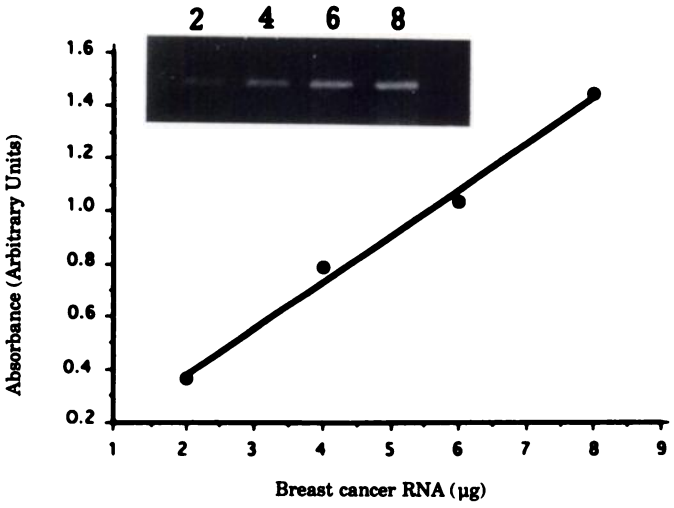

Fig. 2. Quantification of PTHrP-amplified cDNA using increasing RNA concentration. Total RNA was extracted from the tumors, reverse transcribed, and amplified as described in "Patients and Methods." PCR experiments was performed with serial dilutions of PTHrP RNAs corresponding to 2, 4, 6, and 8 μg, and products were separated on 2% agarose gel containing ethidium bromide in TEB buffer as described in "Patients and Methods." The regression curve and coefficient were calculated ($r = 0.995$; $P < 0.05$).

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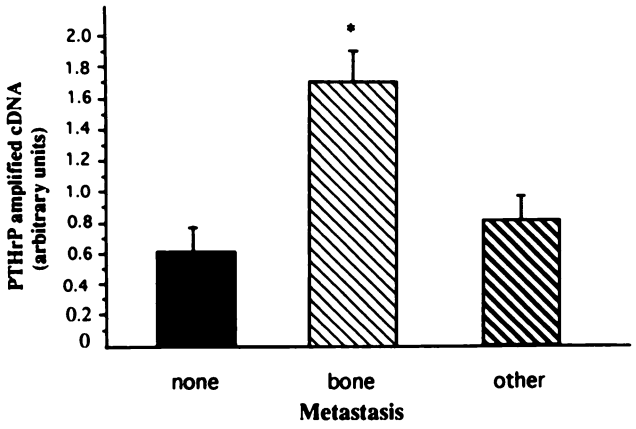

Fig. 3. Amplified cDNA of PTHrP in breast cancer in three groups of patients. PTHrP cDNA were quantified as described previously. PTHrP cDNA is higher in patients who develop bone metastasis than in patients without metastasis or with extraskeletal metastasis ($P < 0.001$). *Bars*, SEM.

No relationship was observed between PTHrP expression and the usual prognostic factors, with the exception of histological node involvement (rs = 0.394; $P < 0.02$). These data are in line with previous studies based on immunocytochemistry (10). However, the correlation observed between the amount of PTHrP cDNA and the percentage of lymph nodes invaded suggests that expression of the PTHrP gene is enhanced in the more aggressive forms of the disease. The quantity of PTHrP cDNA amplified was inversely correlated to the age of the patients ($r = -0.379$; $P < 0.02$), probably because transcription of the PTHrP gene is decreased or the stability of the PTHrP messenger is diminished in older patients.

Patients were divided into three groups according to the follow-up data. Eleven patients had no recurrences or metastases, 17 patients developed bone metastasis, and 10 patients developed soft-tissue metastases; no statistical difference in the usual clinical and biological factors was found between these small groups. By contrast, the level of PTHrP gene expression was dramatically increased in patients who developed bone metastases relative to the other two groups ($P < 0.001$) (Fig. 3).

These data confirm and extend previous immunocytochemical results suggesting that the presence of PTHrP in the primary tumor could predict the subsequent occurrence of metastases (10). Indeed, the difference between patients with bone metastasis and other patients was marked in this study.

In conclusion, the level of PTHrP gene expression in the primary tumor might be a valuable predictive factor in breast cancer, as patients with high levels are at risk of developing bone metastases. Multiparametric retrospective (20) and prospective studies of a larger number of patients are now required.

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