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Parathyroid Hormone Related Protein and Skeletal Morbidity in Breast Cancer

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The presence of parathyroid hormone related protein (PTHRP) in human breast cancers has been assessed by immunohistochemistry using a polyclonal antiserum specific for the mid-region sequence 37–67 in an immunoperoxidase technique. The primary tumours from 155 normocalcaemic, consecutive women with early breast cancer who had been followed up for a minimum of 5 years were assessed. Dewaxed paraffin sections of formalin fixed tissue was used throughout. Positive PTHRP staining was detected in 56% of the cancers and was unrelated to standard prognostic factors, recurrence or survival. However, PTHRP positivity was related to the development of bone metastases ($P \leq 0.03$) and hypercalcaemic episodes. PTHRP is implicated as the humoral factor responsible for hypercalcaemia associated with breast cancer and tumour positivity may be a useful predictor of which women will develop bone metastases.
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INTRODUCTION

BONE METASTASES affect 70% of women with advanced breast cancer and cause considerable skeletal morbidity [1, 2]. Hypercalcaemic episodes occur in 10% of patients with bone metastases and are associated with a poorer survival [1–3]. Bisphosphonate therapy for bone metastases from breast cancer reduces skeletal morbidity when used in conjunction with hormonal or chemotherapy regimens [4]. Potentially bisphosphonates might be used in the early prevention of the morbidity associated with bone metastases but no good marker exists to predict when women are likely to develop skeletal metastases [4].

Parathyroid hormone related peptide (PTHRP), the putative cause of humoral hypercalcaemia of malignancy, stimulates bone resorption in animal models [5] and it has been suggested that the production of PTHRP by breast cancer cells may facilitate the development of skeletal metastases [6, 7]. The aim of this study was to determine the frequency of expression of PTHRP in human breast cancers by immunohistochemistry and its relationship to the subsequent skeletal morbidity experienced.

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PATIENTS AND METHODS

Patients

A consecutive series of 155 normocalcaemic women with early invasive breast cancer who were presented to Selly Oak Hospital from August 1984 to December 1985 were studied. None of the patients had clinical evidence of bone metastases at presentation and all were normocalcaemic. The upper limit of the reference range for corrected calcium is 2.65 mmol/l. The following prognostic factors were recorded on a computerised database:- age, tumour size (mm) and TNM stage, histological grade (modified Bloom and Richardson [8] method) and type, pathological lymph node status, oestrogen and progesterone receptor status, and menstrual status. Oestrogen and progesterone receptor status was measured using a dextran-coated charcoal method and Scatchard analysis [9]. Tumours containing hormone receptor levels $\geq$ 5 fmol/mg protein were considered steroid receptor positive. Menstrual status was classified by designating the patients as premenopausal (less than 12 months since last menstrual period) or postmenopausal (more than 12 months since last menstrual period, hysterectomised—ovariectomised or hysterectomised and 50 or more years of age). Patients were treated by either mastectomy with axillary clearance or node sampling (n = 97) or breast conserving operations (wide local excision and radiotherapy, n = 58). All patients were seen every

4 months in the first year after surgery and thereafter at 6 month intervals. Patients who presented with symptoms of recurrent disease, either at a clinic visit or during the interval between visits, were investigated by conventional radiography (including bone scintigraphy) and ultrasound of the liver; and in the case of locoregional recurrence by surgical biopsy. Bone metastases were diagnosed by the combination of a positive bone scan and conventional radiography. All patients were followed up for a minimum of 5 years or until death.

Immunohistochemistry

All tissue samples were fixed in 4% formaldehyde in saline and routinely processed to paraffin. The primary antiserum was raised in a rabbit to the mid-region 37–67 sequence of PTHRP; this antiserum has previously been shown to cross-react with both native and synthetic sub-fragments of PTHRP, but not PTH [10].

After dewaxing of the section, non-specific binding sites were blocked with non-immune swine serum diluted 5-fold for 10 min. Excess serum was removed and the primary antiserum diluted 100-fold was applied for 1 h at room temperature. This was followed by application of biotinylated-swine anti-rabbit immunoglobulin antiserum and streptavidin–biotin peroxidase complex (both Dako-patts Corp., Copenhagen), followed by further washing in Tris-HCl buffer (pH 7.6). Peroxidase was localised using diaminobenzidine–hydrogen peroxide, and nuclei counterstained with Mayer's haematoxylin.

The controls for the immunohistochemistry included omission of the primary antibody; and preincubation of the primary antibody with PTHRP37-67 (0.5 mg/ml) for 16 h at 4°C. A negative control in which the primary antibody was replaced by non-immune serum and a positive control consisting of squamous carcinoma of lung were included in each batch of slides. Staining of tumours was assessed by two independent observers. If interpretation differed, further slides were stained until agreement was achieved. Only cytoplasmic staining was considered positive.

Statistical analysis

The association between PTHRP expression in tumours and the various prognostic factors was assessed by χ^2 test, and the difference between the median tumour size in PTHRP positive and negative tumours was compared using the Mann–Whitney test (Table 1). Overall survival, relapse free survival and bone metastases free survival curves were constructed by the Kaplan–Meier method. Differences between the two survival curves for tumour positivity and negativity were tested statistically by the log rank test. This analysis was carried out using SAS Version 6 software supplied under license by the SAS Institute Inc, Cary, North Carolina, USA.

RESULTS

Positive staining for PTHRP was detected in 87 (56%) out of 155 early breast cancers. The proportion of cells staining in individual tumours varied with some additional variation in intensity, while surrounding stromal tissue was consistently negative [11]. Positive staining was abolished by omission of the primary antiserum and immunoabsorption of the antiserum by PTHRP 37–67. No association between any of the standard prognostic factors and PTHRP staining was observed (Table 1).

PTHRP staining was not related to either relapse free survival [31 out of 87 (35%) positive staining tumours have recurred versus 16 out of 68 (24%) negative staining tumours] ($P = 0.091$) or survival (24 out of 87 (28%) positive have died versus 19 out of 68 (28%) negative) ($P = 0.74$) (Table 2). In the PTHRP negative group, 6 women died with uncontrolled locoregional recurrence of their primary tumour. Despite this increased local recurrence rate, overall survival was not influenced by surgical treatment ($P = 0.51$).

28 women developed first recurrence in the skeleton and 6 had an episode of hypercalcaemia with a corrected serum calcium greater than 2.70 mmol/l. All 6 women who had a hypercalcaemic episode had tumours expressing PTHRP as did 21 out

Table 1. Relationship of tumour positivity for PTH-related protein and prognostic factors in breast cancer patients

Prognostic factor	Number	PTHRP Staining Positive $n = 87$	PTHRP Staining Negative $n = 68$	P
Age	155			
≤ 50 years		32	33	0.14
≥ 51 years		55	35	
Size: Median (range)	110	30 (5–90)	30 (9–75)	0.92
Histological type:	155			
Infiltrating ductal		73	53	0.43
Other types		14	15	
Histological grade:	155			
I		15	15	0.27
II		47	41	
III		25	12	
Node status: (Pathological)	98			
All nodes negative		20	21	0.71
Any node positive		30	27	
Menstrual status:	155			
Premenopausal		23	20	0.80
Postmenopausal		64	48	
ER status:	100			
Negative		21	15	0.62
Positive		34	30	
PR status:	86			
Negative		18	13	0.99
Positive		32	23	

All statistical comparisons were by χ^2 test, except for tumour size which was compared by Mann–Whitney. None of the differences are statistically significant.

Table 2. Relationship of prognostic factors and PTHRP staining to survival in patients with breast cancer

Prognostic factor	Bone metastases free survival (P)	Overall survival (P)
Age	0.076	0.65
Tumour size (mm)	0.011*	0.0003*
Tumour stage	0.012*	0.0009*
Histological grade	0.31	0.046*
Histological type	0.81	0.41
Node status	0.048*	0.043*
Menstrual status	0.66	0.53
ER status	0.067	0.0045*
PR status	0.25	0.962
PTHRP stain	0.029*	0.74

The association of survival time with co-variates was tested by a univariate χ^2 for the log-rank test. * Significant.

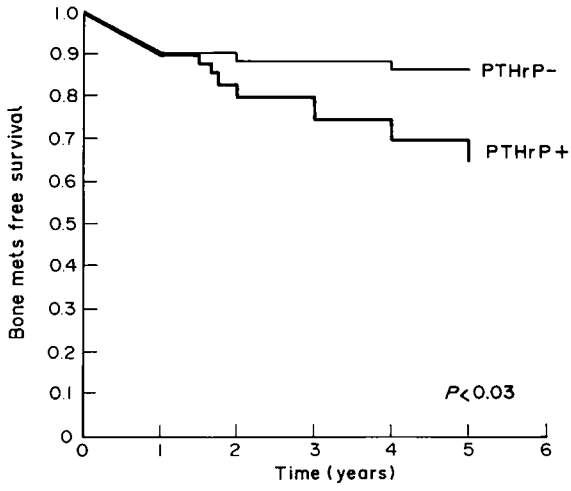

Fig. 1. Relationship of PTHRP immunostaining of the primary tumour and the development of bone metastases.

of 28 women who developed bone metastases. The time to the development of bone metastases in the patients with PTHRP positive or negative tumours are compared in Fig. 1. The differences in these curves are significant ($P < 0.03$). Tumour stage, size, histological grade, lymph node status and oestrogen receptor status were prognostic for overall survival but only tumour size, stage, lymph node status and PTHRP staining were prognostic for the development of skeletal metastases (Table 2).

DISCUSSION

PTHRP was first isolated in 1987 from a breast cancer associated with humoral hypercalcemia of malignancy [12]. This study suggests that PTHRP is commonly expressed in breast cancers and confirms the findings of a recent study which reported a similar frequency of PTHRP positivity using an antiserum of NH_2-terminal specificity [6]. Unlike the previous study, we are unable to show an association between positive PTHRP staining and steroid hormone receptor status.

The skeleton is the site of metastases in 20% of early and 75% of advanced breast cancer patients [1, 2]. Single prognostic factors are unreliable in indentifying which patient will develop bone metastases. In a large series of patients, tumour size and the extent of axillary lymph node involvement were said to predict tumours which recur in the skeleton [1] and we have confirmed the importance of these factors. Although oestrogen receptor (ER) rich tumours are claimed to preferentially metastasise to bone [1, 2], the data from the present study did not reach statistical significance ($P < 0.067$). This study suggests that tumours which produce the potent bone resorbing factor PTHRP [5] may preferentially select bone as the site of recurrence. PTHRP expression occurred in 75% of tumours developing bone secondaries and was closely related to recurrence in bone. Whether production of PTHRP by tumour cells confers an advantage in the seeding or survival of tumour cells in bone or directly enhances the ability of cells to invade bone, remains to be established. If PTHRP expression in the primary tumour can be shown prospectively to identify women at risk of developing skeletal metastases, it would allow their selection for specific treatment (e.g. bisphosphonates) aimed at early prevention of the morbidity associated with bone metastases [4].

Up to one third of patients with bone metastases and two thirds of hypercalcaemic breast cancer patients may present with increased renal tubular calcium absorption and increased nephrogenous cyclic AMP, [13, 14], suggesting that the tumours secrete a factor with PTH-like actions on calcium homeostasis. In this study PTHRP was localised in 56% of primary tumours and all six primary tumours from women who subsequently developed hypercalcaemia. Furthermore PTHRP has been localised in 90% of bone metastases from breast cancer patients [7]. These findings suggest that tumour derived PTHRP may be the humoral factor responsible for hypercalcaemia associated with breast cancer. It is of note that the first validated immunoassays for plasma PTHRP have found elevated levels in a high proportion of selected patients with hypercalcaemia and breast cancer [11, 15, 16].

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