Ann R Coll Surg Engl 1996; 78: 354–358

Parathyroid hormone-related protein, bone metastases and hypercalcaemia of malignancy

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Key words: Bone metastases; Parathyroid hormone-related protein; Hypercalcaemia

Breast cancer patients frequently develop bone metastases. A newly discovered peptide, Parathyroid Hormone Related Protein (PTHrP), causes humoral hypercalcaemia of malignancy. We have studied whether production of this protein by breast cancers leads to the development of bone metastases or hypercalcaemia.

PTHrP was produced by nearly 60% of early breast cancers and its production by the tumours was associated with malignant mammographic microcalcification, and the development of both bone metastases and hypercalcaemia.

The hypercalcaemia associated with bone metastases has been shown to have a significant humoral component and measurement of plasma PTHrP in patients with hypercalcaemia is diagnostically useful. Potential mechanisms of preventing bone metastasis are discussed.

Bone metastases are a common cause of morbidity in breast cancer. About 70% of women dying from the disease will have bone metastases *(1)*. Nearly 100 years ago, Paget *(2)* proposed that breast cancer cells produced factors (seed) that enabled them to settle in the bone marrow (soil). The nature of the factors responsible for bone metastases have remained elusive.

Hypercalcaemia of malignancy is a common paraneoplastic syndrome which complicates bone metastases from breast cancer in 10–20% of women before death *(1)*. In breast cancer, it is traditionally believed that hypercalcaemia is caused by skeletal metastases secreting local paracrine factors which stimulate osteoclasts and resorb bone in areas of lytic destruction *(3)*. In contrast, in humoral hypercalcaemia of malignancy (HHM), it is well documented that a primary tumour (usually lung) causes distant effects on the bone and kidney. Biochemical changes seen in HHM include increased renal tubular reabsorption of calcium, a decrease in the renal tubular threshold for phosphate reabsorption and increased excretion of nephrogenous cyclic adenosine 5′-phosphate (AMP) *(4)* (Fig. 1).

Recently, a factor produced by cancer cell lines was isolated and shown to be capable of producing increased nephrogenous cyclic AMP production in cell lines. This factor which, when injected into animals, produces similar biochemical effects to those seen on injection of parathyroid hormone was named parathyroid hormone related protein (PTHrP).

Considerable evidence rapidly accumulated that PTHrP was an important humoral mediator of paraneoplastic hypercalcaemia in patients with solid tumours such as squamous carcinoma of the lung *(5)*. However, bone metastases were believed to cause hypercalcaemia by local paracrine rather than humoral effects. Nevertheless, breast cancer bone metastases had been documented to

Based on a Hunterian Lecture delivered to the Surgical Research Society Meeting in Manchester on 6 July 1995

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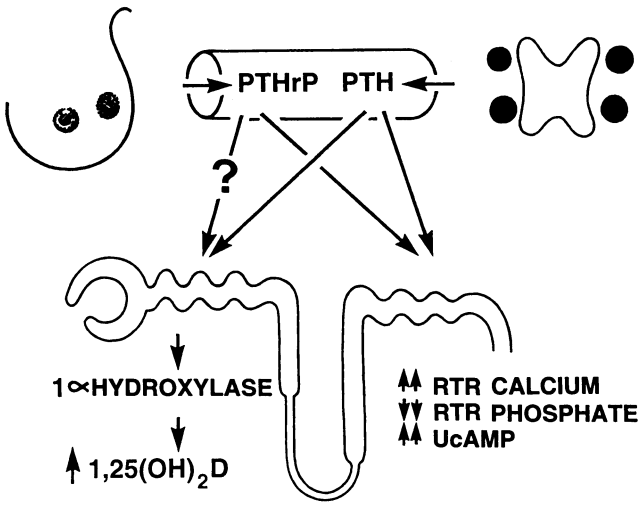

Figure 1. Primary hyperparathyroidism caused by parathyroid hormone secretion from the parathyroid glands (*top left*) increases proximal convoluted tubule conversion of 25 Vitamin D to 1–25 Vitamin D as well as increasing renal tubular reabsorption of calcium and urinary cyclic AMP production. Similar effects on the distal convoluted tubule were noted in hypercalcaemia of malignancy and led to the discovery of PTHrP.

have associated biochemical changes including increased renal tubular reabsorption of calcium and increased urinary cAMP *(6)*. This, together with the fact that bone distant from metastases shows evidence of resorption, implied that humoral factors in breast cancer may be responsible for increased bone breakdown *(7)*.

We therefore undertook a series of studies on breast cancer patients to determine if PTHrP was produced by breast cancers and whether PTHrP had a role in the production, diagnosis and treatment of hypercalcaemia.

Primary tumour production of PTHrP

In a prospective study, breast tumours were snap frozen in liquid nitrogen and subsequently homogenised. PTHrP was extracted by boiling in 1 M acetic acid. Intact-1–86 PTHrP was measured using a two-site immunoradiometric assay. Of 114 early breast cancers, 76% had detectable intact 1–86 PTHrP protein (range 46.5–302 113 pmol/ml; median 190 pmol/ml). In contrast, only nine out of 27 (33%) samples of normal breast expressed PTHrP (range 100–1800 pmol/mg; median 230 pmol/mg). Tumour 1–86 PTHrP levels correlated inversely with age and were significantly higher in premenopausal patients. The proportion of tumours with detectable PTHrP levels was higher in axillary node-positive premenopausal women ($P \leqslant 0.05$).

In a retrospective study, immunohistochemical assessment of PTHrP expression was performed with a 34–67 PTHrP midmolecule polyclonal antibody. PTHrP staining was detected in the epithelial cells of normal and malignant breast using paraffin-fixed sections of breast tissue. PTHrP staining was seen in the epithelium of normal breast lobules and ducts. Of 155 consecutive early breast cancers, 87 (56%) expressed PTHrP compared with 47 (67%) out of 72 primary breast cancers which had developed bone metastases. Of the latter breast cancers, 25 subsequently developed hypercalcaemia and 22 (88%) had PTHrP staining in their primary tumour ($P \leqslant 0.002$) *(8)*.

Mammographic microcalcification is detectable in 40% of early breast cancers. In birds, PTHrP secretion leads to the calcification of eggshells as eggs pass down the oviduct. Comparison between PTHrP staining of breast cancers and radiological assessment of microcalcification in tumours showed a significant association, with 50% of PTHrP-positive breast cancers having microcalcification compared with 21% of PTHrP-negative tumours ($P \leqslant 0.001$; Table I).

Out of the 155 early breast cancer patients who have been followed up for a minimum of 5 years, 28 (18%) have developed bone metastases and six hypercalcaemia. All six women who developed hypercalcaemia had PTHrP-positive primary tumours. Only four, out of 11 prognostic factors, predicted the development of bone metastases, tumour size ($P \leqslant 0.02$), tumour stage ($P \leqslant 0.02$), axillary node status ($P \leqslant 0.05$) and the expression of PTHrP as detected by immunohistochemistry ($P \leqslant 0.005$; Table II) *(9)*. To date, in the prospective study 9/90 (10%) cancers with detectable cytosol 1–86 PTHrP have developed bone metastases compared with 2/42 (5%) with undetected cytosol PTHrP.

Plasma PTHrP levels were measured and were detectable in 19/195 (12%) early breast cancer patients compared with 25/72 (32%) bone metastases patients and 26/31 (84%) women with hypercalcaemia of malignancy owing to breast cancer ($P \leqslant 0.001$).

The median level of PTHrP detectable was significantly

Table I

Breast cancers	*n*	*Mammographic microcalcification*	*Histological microcalcification*
PTHrP Positive	110	52 (50%)	25 (24%)
Negative	75	16 (21%)	8 (11%)

χ^2 test $P \leqslant 0.001$

Table II

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

*Significant
ER, Oestrogen receptor; PR, Progesterone receptor

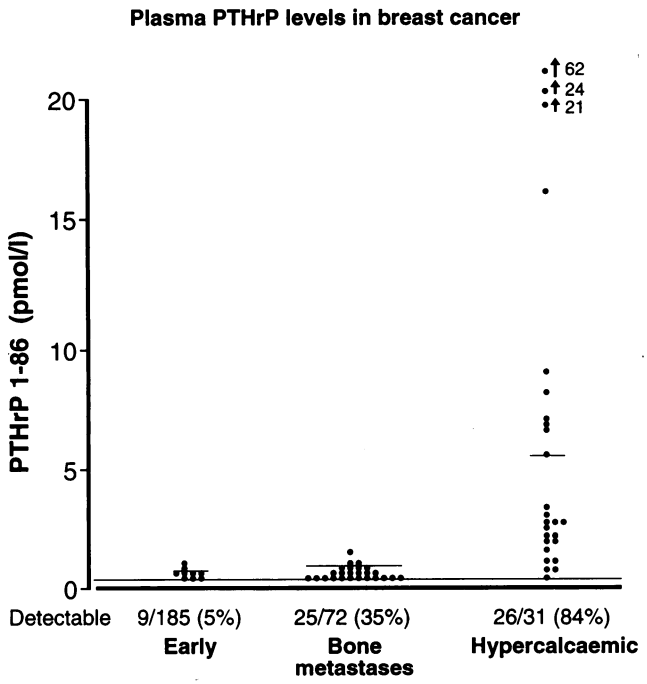

Figure 2. Plasma parathyroid hormone related protein concentrations in women with early breast cancer, bone metastases from breast cancer and women with hypercalcaemia due to breast cancer.

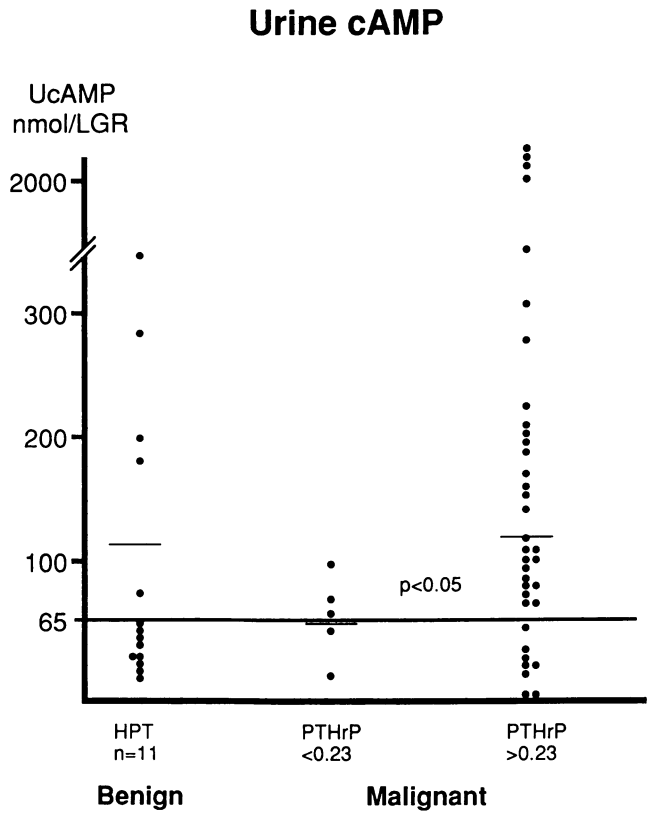

Figure 3. Levels of cyclic adenosine monophosphate in the urine of consecutive hypercalcaemic patients with primary hyperparathyroidism and hypercalcaemia of malignancy were divided into those with detectable PTHrP ($n=37$) or undetectable PTHrP ($n=5$). Levels of UcAMP were significantly higher in patients whose tumours secreted PTHrP.

higher in the hypercalcaemic patients (mean 5.6 pmol/l; range 0.23–66 pmol/l) compared with normocalcaemic patients with or without bone metastases (mean 0.46; range 0.23–1.7) (Fig. 2).

These studies demonstrated that PTHrP was produced by malignant and benign breast epithelial cells, and that tumour cytosol levels may be regulated by oestrogen. Humoral secretion of PTHrP occurs, and raised plasma levels correlate with the development of hypercalcaemia of malignancy. A second series of studies was then initiated to prove that the plasma PTHrP detected correlated with other biological markers of PTHrP action.

Studies on the effects of PTHrP on the kidney and bone

In an initial series of 40 consecutive patients with hypercalcaemia of malignancy seen in Birmingham, detectable PTHrP levels correlated with corrected serum calcium levels ($r=0.34$; $P\leqslant 0.05$). In a second consecutive series of 72 patients with malignancy associated hypercalcaemia, plasma PTHrP levels were elevated in 59 (82%) patients and correlated with serum calcium ($r=0.45$; $P\leqslant 0.001$). Overall, renal tubular reabsorption of calcium was increased in 55 (72%) patients with hypercalcaemia of malignancy and in 91% of patients with a plasma PTHrP $\geqslant 1$ pmol/l, demonstrating the effects of PTHrP on the distal convoluted renal tubule. Urinary cyclic AMP levels were also higher in patients with PTHrP levels $\geqslant 0.23$ pmol/l *(10)* (Fig. 3).

Vitamin D 25 hydroxycholecalciferol levels did not differ between normocalcaemic patients with bone metastases and hypercalcaemic patients. However, proximal convoluted tubular conversion of 25 Vitamin D by 1α hydroxylase to 1–25 dihydroxy-cholecalciferol was inhibited and levels of the 1–25 Vitamin D were significantly lower in patients with hypercalcaemia compared with normocalcaemic patients.

The bone resorption markers, pyrodinolines and deoxypyrodinolines were significantly higher in hypercalcaemic women with breast cancer than normocalcaemic controls, demonstrating that local and humorally mediated osteolysis combined with humorally mediated renal effects are responsible for hypercalcaemia. The detectable plasma PTHrP seen in breast cancer patients clearly has biological effects on the kidney and skeleton.

Diagnosis of hypercalcaemia

A consecutive series of 123 patients presenting to two neighbouring hospitals with undiagnosed hypercalcaemia revealed 72 (59%) had underlying malignancy. Hypercalcaemia was due to benign causes in 42 and parathyroid disease coexisting with malignancy in nine. Bone metastases were present in 32 (72%) of patients with malignancy. Raised plasma PTHrP levels were seen in 59/72 (82%) of patients with hypercalcaemia owing to malignancy, 72% of patients with bone metastases and

91% of patients without bone metastases. Plasma PTHrP levels correlated with serum calcium and patients with PTHrP $\geqslant 1$ pmol/l had increased urinary cAMP and renal tubular reabsorption of calcium ($P \leqslant 0.05$) (Fig. 3). Plasma parathyroid hormone was undetectable in 90% of patients with hypercalcaemia secondary to malignancy. In the remaining 51 patients, 48 had elevated parathyroid hormone levels indicating parathyroid disease, while the remaining three had other benign causes (two chronic renal failure, one sarcoidosis). Plasma PTHrP was undetectable in 41/51 of these patients and modestly increased in the remainder.

The median survival of patients with elevated parathyroid hormone levels was 13 months compared with 3 months for those with hypercalcaemia of malignancy ($P \leqslant 0.02$) indicating the prognostic value of measuring parathyroid hormone in all patients at initial presentation with hypercalcaemia.

A subgroup of 35 symptomatic patients with hypercalcaemia of malignancy (17 without and 18 with bone metastases) were treated with intravenous bisphosphonates to lower serum calcium by inhibiting osteoclast-induced bone resorption. Serum calcium levels fell to a nadir between 3 and 7 days after treatment. Patients who responded to treatment by becoming normocalcaemic had significantly lower basal plasma PTHrP levels (mean 4.06 pmol/l *vs* 8.2 pmol/l ($P \leqslant 0.04$). Urinary cAMP levels fell in all patients. Patients without bone metastases had significantly higher plasma PTHrP levels ($P \leqslant 0.002$), required more doses of bisphosphonates and had a poorer reduction in serum calcium compared with patients with bone metastases, only one of whom required more than one dose.

Thus, measurement of PTH and PTHrP is valuable in the diagnosis and management of patients with hypercalcaemia of malignancy.

Further evidence for the role of PTHrP in bone metastasis

Parallel to our studies, Professor Martin's Research Group in Australia demonstrated that PTHrP (mRNA and protein) was overexpressed in breast cancer bone metastases compared with metastases at other sites (liver, lung, soft tissue) *(11)*. In the USA, Professor Mundy's group transfected the MDA MB231 breast cancer cell line with the PTHrP gene and demonstrated enhanced secretion of PTHrP by the transfected cell line *(12)*. Injection of the transfected cell line into the left ventricle of nude mice, increased the number of osteolytic bone metastases formed and reduced the time taken to form bone metastases compared with the same cell line transfected with antisense to PTHrP. This latter animal model of bone metastasis is associated with multiple bone metastases, yet hypercalcaemia only occurs terminally and plasma PTHrP levels remain virtually undetected until the final stage when hypercalcaemia occurs. This is similar to the clinical situation of multiple osteolytic bone metastases in breast cancer and implies that local production of PTHrP may be important for the development of bone metastasis. Work on other animal models of bone metastasis has suggested that osteoclast activating factors such as interleukin-1α given humorally or locally injected into bone, increase the frequency of bone metastasis after injection of cancer cells into the left ventricle *(13,14)*. The increased bone metastasis seen in this model can be prevented by prior treatment of the animals with bisphosphonates which prevent osteoclast activation. Thus, tumour cells secreting potent osteoclast-activating factors (such as PTHrP or interleukin-1α) are a necessary prerequisite for the development of bone metastases *(14)*.

Clinical studies are already under way to determine if intravenous bisphosphonate therapy given preoperatively can prevent the development of bone metastases after breast cancer surgery. It is to be hoped that within the next 100 years, the critical factors responsible for bone metastasis will have been discovered.

Prostate cancer

Bone metastases occur commonly in prostate cancer, but since they are usually radiologically associated with new bone formation (osteosclerotic) it was assumed osteolysis was not a major feature of such metastasis. Recent work has shown that bone resorption markers (pyrodinolines and deoxypyrodinolines) are elevated in patients with bone metastases from prostate cancer *(15)*. We, and others, have shown that PTHrP is produced by prostate cancer cell lines and acts as an autocrine growth factor, and PTHrP has been identified in prostate primary adenocarcinomas *(16)*, which suggests it may play a role in potentiating bone metastasis in prostate cancer also.

Discussion

We have shown that PTHrP is produced and secreted humorally by breast cancers leading to hypercalcaemia by humoral and osteolytic mechanisms. The biological effects of secretion of PTHrP on the distal convoluted tubules in the kidney have been demonstrated. The low Vitamin 1–25 D levels in hypercalcaemia are likely to be caused by the production of some other humorally secreted factor by the tumour which inhibits renal 1α hydroxylase activity.

Paget's original hypothesis that the cancer seed producing factors which allowed metastases to develop in the soil of distant organs was remarkably perceptive and the mechanisms are beginning to become apparent.

Local secretion of PTHrP in bone increases osteoclast activation and may be responsible for circulating tumour cells lodging in bone and producing bone metastases. Further studies are required to confirm this hypothesis but the demonstration that osteolytic factors secreted by tumours, predispose to bone metastasis, allows us to develop strategies which will inhibit metastatic spread to the skeleton.

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Received 13 September 1995