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Level of Expression of Parathyroid Hormone Related Protein and its Receptor in Human Breast Cancer and its Correlation with the Clinical Outcome

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Abstract: In this study we examined the level of the expression of PTHrP and its receptor (PTHrP-R) in human breast cancer and correlated their level of expression with the clinical outcome. Primary Breast cancer samples (n = 124) and adjacent non-cancerous breast tissues obtained from patients in cohort (n = 30) were processed for RNA extraction and cDNA synthesis. Levels of expression were assessed quantitatively by Q-PCR and were analyzed against tumour's stage, grade, nodal status, local relapse, distant metastasis and survival after a median follow-up of 120 months period. Tissue distribution of PTHrP and its receptor (PTHrP-R) protein in human breast cancer tissues were assessed using immunohistochemical analysis. Breast tumour tissues had significantly higher levels of PTHrP transcript compared with normal tissues (breast cancer 64.2 ± 32 copies vs normal tissue 19.4 ± 8.5 copies; $p = 0.05$). The levels of PTHrP transcript were high in node positive tumours (117 ± 74 copies) compared to node negative tumours (24.8 ± 8.7), although the difference was not statistically significant ($p = 0.23$). Increased levels of PTHrP mRNA expression appears to be positively correlated with disease progression as levels were high in samples of patients who had distant metastasis (66.5 ± 64 ; $p = 0.56$) and/or die of breast related causes (306 ± 23.5 ; $p = 0.26$) when compared to those who were disease free (25.9 ± 8). PTHrP expression was also increased with increasing grade and TNM status. No difference of PTHrP-R levels was found between normal and tumour tissues ($p = 0.73$), node negative and positive tumours ($p = 0.15$). Ductal carcinomas had a higher level of PTHrP transcripts (75 ± 39 copies), compared with less malignant lobular (17 ± 12). However, ductal carcinomas had a lower level PTHrP-R (1.7 ± 0.4) than lobular carcinomas (7.6 ± 7.0). PTHrP and PTHrP-R staining was cytoplasmic as well as showed some degree of staining in the nuclei. Tumour cells displayed stronger staining compared with normal mammary epithelial cells. We conclude that here, PTHrP expression is increased in human breast cancer, particularly in patients with aggressive tumours and with mortality. Over-expression of PTHrP in breast cancer cells result in more aggressive phenotype. Together, it suggests that PTHrP plays a role beyond the initial metastasis repressor and is involved in disease progression in this cancer type.

Key words: Breast cancer, parathyroid hormone related protein, clinical outcome

INTRODUCTION

Parathyroid Hormone-related Protein (PTHrP) is a polypeptide with PTH activity and is produced by many different normal and malignant cells (Massfelder *et al.*, 2003). It regulates cell growth, differentiation and death (Martin *et al.*, 1997). It has 140 amino acid residues and is encoded

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by a gene on human chromosome 12. PTHrP was originally identified as the tumour factor responsible for the common paraneoplastic syndrome known as humoral hypercalcaemia of malignancy (Dunbar and Wysolmerski, 1999). It was purified and cloned 10 years ago as a factor responsible for the hypercalcaemia associated with malignancy (Guise *et al.*, 1997; Philbrick *et al.*, 1996). Hypercalcaemia is usually defined as a serum calcium concentration greater than 12 mg dL^{-1} , corrected for the serum albumin concentration (Solimando *et al.*, 2001). This syndrome is generally considered to be due to over production of PTHrP by tumour cells and its subsequent entry into the general circulation (Falzon and Du, 2000). Breast cancer accounts for 25% of the cases of hypercalcaemia in cancer (Martin and Moseley, 2000).

PTHrP and PTHrP receptor (PTHrP-R) are present in both normal breast epithelium and breast tumour tissues, suggesting that PTHrP is a potential autocrine growth factor in primary breast carcinoma (Cataisson *et al.*, 2000; Cross *et al.*, 2002; Downey *et al.*, 1997). It is also secreted by normal breast cells during lactation to increase bone resorption and liberate skeletal calcium stores for the purpose of milk production (DeMauro and Wysolmerski, 2005). PTHrP has many important functions in normal physiology related to growth and development, reproductive function and smooth muscle relaxation (Guise and Mundy, 1996). Recent evidence also has shown that, directly after translation, PTHrP is able to enter the nucleus and/or nucleolus and influence cell cycle progression and apoptosis (Falzon and Du, 2000).

Breast cancer is the commonest form of malignancies in females in the U.K and metastasis of breast cancer is common. About 7% of patients with breast cancer present with widespread metastases at the initial presentation (Mansel *et al.*, 2000). The sites most frequently visited by metastasis in breast cancer are bone, lungs, liver, chest wall and central nervous system. Less common sites are the adrenals, ovaries, pericardium, thyroid and bone marrow (Valagussa *et al.*, 1978).

In patients of primary breast cancer, PTHrP was identified in 60% of the tumours. However, PTHrP was found in 92% of metastatic bone lesions in breast cancer (Powell *et al.*, 1991; Yoshida *et al.*, 2000). Another study has shown that PTHrP is produced by 40 to 60% of breast tumours and is elevated in the serum of up to 50% of patients with breast cancer metastases to bone (Tabuenca *et al.*, 1995). Along with the skeleton, the kidney appears to be another specific target for PTHrP, in which PTHrP found to be expressed in vessels, glomeruli, tubules and regulates vascular tone, glomerular filtration rate, as well as cell growth and differentiation in the organ.

The current study aimed to investigate the level of the expression of PTHrP and PTHrP-R transcripts in breast cancer and normal background breast tissues. Also, we made an attempt to assess the staining and location of PTHrP and PTHrP-R in both malignant and benign mammary tissues in a portion of the primary tumours. We found the expression of PTHrP appears to be positively correlated to the poor prognosis of breast tumour, when we investigated the level of the expression against the clinical/pathological parameters.

MATERIALS AND METHODS

Tissue Collection and Preparation

Breast tissue samples (tumour and matched background, Table 1) were collected and immediately frozen in liquid nitrogen before being Processed for Quantitative-PCR (Q-PCR) analysis. Clinical details were obtained from routine followup data base and histology of the tissues was assessed by Dr. Anthony Douglas-Jones, consultant pathologist (Cardiff University) A portion of the tissues (30 pairs) were also frozen sectioned for immunohistochemical analysis, as we recently reported (Martin *et al.*, 2005). RNA was isolated from tissue samples using standard RNazol procedures. For RT-PCR, cDNA was synthesised in a 20 μL reaction mixture using 0.5 ng RNA, as described in the protocol (AB Gene Reverse Transcription System, Surrey, UK).

Table 1: Clinical information for breast tumour tissues analysed (n = 124)

Tissue type	No.	Grade	No.	NPI	No.	TNM	No.	Histology	No.	Outcome	No.
Background	30	1	24	1	68	1	70	Ductal	94	Healthy	85
Tumour	124	2	42	2	38	2	40	Lobular	14	Metastasis	7
		3	58	3	16	3	7	Other	16	Local recurrence	5
					Unknown	2	4		4	Mortality	15
								Unknown	3	Unknown	12
										All poor outcomes	27

NP = INottingham Prognostic Indicator, TNM = Tumour/Nodal Status

Table 2: Primer sequence

	Sense primer F1(5' 3')	Antisenes primer ZR (5'-3')
PTHrP	ggtgttctctgctgagctac	actgaacctgacctacacgtaaatcttgatggactt
PTHrP-R	ggtgttctctgctgagctac	actgaacctgacctacagatgatccacttttggctcc
β actin	atgatatcgccgctcg	cgctcggatgaggatctca

Quantitative-PCR

The Q-PCR system used the Amplofluor™ Uniprimer™ probe system (Intergen Company Oxford, UK) and Thermo-Start customer master mix (ABgene, Epsom, Surrey, UK). PTHrP and PTHrP-R-specific primer pairs were designed by the authors using Beacon Designer software and manufactured by Invitrogen (Invitrogen Life Technologies, Paisley, Scotland, UK), each amplifying a region that spans at least one intron, generating an approximately 100-base pair product from both the control plasmid and cDNA. Using the Icyler IQ system (Bio-Rad), which incorporates a gradient thermocycler and a 96-channel optical unit, the plasmid standards and breast cancer cDNA were simultaneously assayed in duplicated reactions using a standard hot-start Q-PCR master mix. Q-PCR conditions were as follows: enzyme activation 95°C for 12 min, 1 cycle, followed by 60 cycles of denaturing: 95°C for 15 s; annealing: 55°C for 40 s; extension: 72°C for 25s. The primers used were as show in Table 2. Purified plasmids used as internal standards, the level of cDNA (copies/50 ng RNA) in the breast cancer samples were calculated. Q-PCR for β -actin was also performed on the same samples, to correct for any residual differences in the initial level of RNA in the specimens (in addition to spectrophotometry). The products of Q-PCR were verified on agarose gels.

Immunohistochemistry

Thirty frozen breast cancer tissues together with 30 normal background tissues (from the same patients) were collected immediately after mastectomy and stored at -80°C until ready for use. All tissues were collected with the approval of local ethical committee. Cancer and normal background tissues were verified and confirmed by a pathologist (Dr. A Douglas-Jones).

Briefly, staining for PTHrP and PTHrP-R was performed on sequential frozen-sectioned tissues (cancer tissues paired with normal background tissue from the same patient). Frozen sections were cut, air dried and fixed in 50% methanol and 50% acetone for 15 min. The sections were then air dried once more for 10 min and stored at -20°C in foil-wrapped slide trays. Immediately before staining specimens were then placed in PBS (Optimax wash buffer) for 5 min. The slides were incubated with primary rabbit polyclonal antibodies against PTHrP or PTHrP-R antibody (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) or positive control at 1:50 dilution for 1 h. After 4 washes with PBS, the slides were placed in universal multi-link biotinylated (Dako). Secondary antibody at (1:100) dilution and incubated for 30 min. This was followed by 4 washes with PBS. Slides were then placed in avidin biotin complex (ABC-Vector Labs) for 30 min. The bound antibody complex was detected using diaminobenzidine tetrahydrochloride (3,3'-diaminobenzidine)-DAB (Sigma), chromogen for 5 min. The slides were washed with H₂O for 5 min and placed in Mayer's haematoxylin for 1 min, followed by differentiation in H₂O for 10 min. This was followed by dehydration in methanol (3 times) and clearing in 2 changes of xylene before mounting under cover slip and examined on the microscope

negative controls (using PBS buffer instead of the primary antibody) and positive controls were used in this study. The complete procedure was carried out at room temperature.

RESULTS

Expression of PTHrP and its Receptor PTHrP-R Transcripts in Tumour and Normal Background Tissues

We first analysed the overall levels of the PTHrP and PTHrP-R transcripts in the cohort of mammary tissues. As shown in Fig. 1a, breast tumour tissues had higher levels of the PTHrP transcript than normal mammary tissues (64.2 ± 32 (mean \pm SD) vs. 19.4 ± 8.5 copies, respectively, $p = 0.05$). However, PTHrP-R showed no significant difference in the level of expression between tumour and normal breast tissues (2.1 ± 0.7 vs 1.8 ± 0.5 copies, respectively $p = 0.73$) (Fig. 1b).

Levels of the PTHrP and PTHrP-R Transcripts and Their Association with the Nodal Involvement in Patients with Breast Cancer

As shown in Fig. 2a, there was a higher level of the PTHrP transcript in node positive tumours (Mean \pm SD 117 ± 74) compared with node negative (24.8 ± 8.7 copies) tumours, but not reached statistical significance ($p = 0.23$). Moreover its receptor PTHrP-R transcript levels was marginally higher in node positive tumours than node negative tumours (2.08 ± 0.67 vs 1.05 ± 0.25 , respectively $p = 0.15$) (Fig. 2b).

PTHrP and PTHrP-R Expression in Relation to Breast Tumour Prognosis

Here, we used the Nottingham Prognostic Index (NPI) as a tool to predict the prognosis of the patients, in that $NPI < 3.4$ was regarded as having a good predicted prognosis (referred to as NPI-1

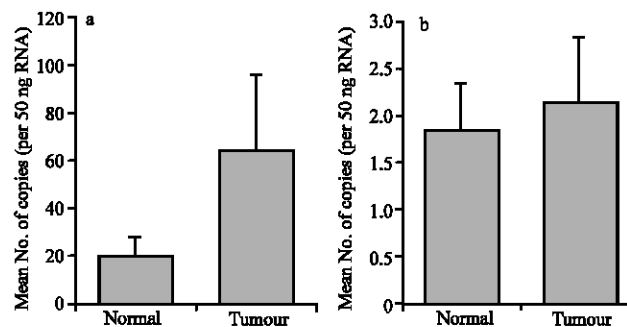


Fig. 1: PTHrP (a) and PTHrP-R (b) transcripts levels in normal and tumour tissues

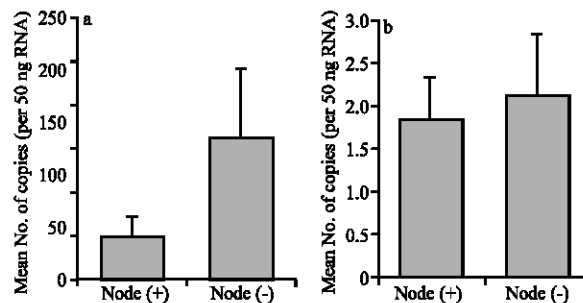


Fig. 2: Graphs showing (a) increased transcript number of PTHrP and its receptor (b) PTHrP-R in node negative and node positive tumours

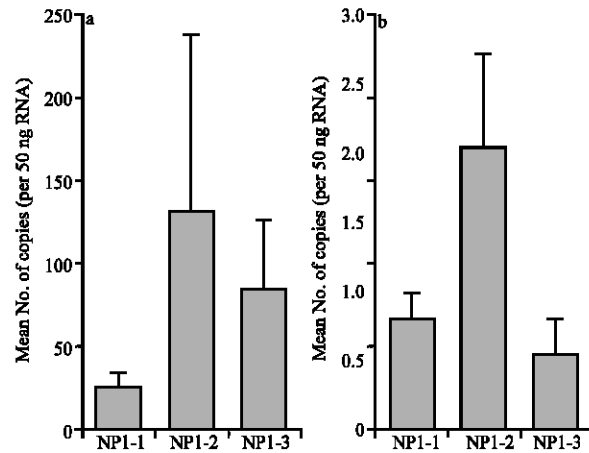


Fig. 3: Levels of PTHrP (a) and PTHrP-R (b) and their relationship with predicted clinical prognosis, using NPI as an indicator

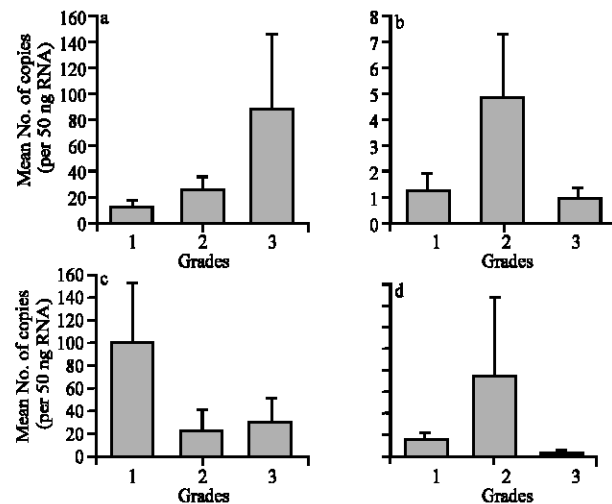


Fig. 4: PTHrP and PTHrP-R transcript levels tumours with different grade (A and B) and in different histological types (C and D)

here), 3.4-5.4 moderate (NPI-2) and >5.4 poor prognosis (NPI-3). As shown in Fig. 3a, patients with moderate (131 ± 107) and poor prognosis (85 ± 41) had higher levels of the PTHrP transcript than patients with good prognosis (24.8 ± 8.5). However, this correlation between PTHrP and prognosis did not reach statistical significance in these instances ($p = 0.33$ and $p = 0.17$, respectively).

Figure 3b shown higher levels of PTHrP-R transcripts in NPI-2 (2.7 ± 0.9 copies) than a NPI-1 (0.7 ± 0.35) and NPI-3 (0.7 ± 0.35) and these values failed to reach a statistical significant ($p = 0.09$ and $p = 0.41$, respectively).

PTHrP and PTHrP-R and Their Correlation with Breast Tumour Grade

PTHrP values in the moderate grade 2 (25.4 ± 9.4 $n = 41$) and poorly differentiated grade 3 (88.5 ± 57 $n = 55$) tumours were elevated compared with well-differentiated grade-1 tumours (11.1 ± 4.6 , $n = 23$). This trend linking PTHrP to a poor patient outlook did not reach statistical

significance with the current sample number (grade 1 versus grade 2, $p = 0.22$; grade 1 versus grade 3, $p = 0.18$) (Fig. 4a). The highest level of PTHrP-R was observed in Grade 2 (4.9 ± 2.4 copies; $p = 0.16$) tumours, compared with Grade 1 (1.3 ± 0.6) and Grade 3 (1 ± 0.3 ; $p = 0.73$) tumours (Fig. 4b). In addition, More malignant ductal breast cancer had the highest level of PTH-rP transcripts (75 ± 39 copies), compared with less malignant lobular (17 ± 12) and other less malignant varieties (22 ± 15) (Fig. 4c), While PTHrP-R shows highest level in lobular breast cancer (7.6 ± 7) compared to ductal (1.7 ± 0.4) and other (0.36 ± 0.09) (Fig. 4d).

Association Between the PTHrP Transcript and Clinical Outcomes of the Patients

The current cohort had a median 10-year follow-up. The patients were accordingly divided into the following four groups: those who remained disease free, who developed metastatic disease, who had a local recurrence and those who died of breast cancer (Table 1). As shown in Fig. 5a, although patients died from breast cancer had higher levels of the PTHrP transcript than those who remained disease free (306 ± 23.5 vs disease free: 25.9 ± 8), the difference was not statistically significant $p = 0.26$. In addition, tumours from patients with metastasis 66.5 ± 64 had marginally higher levels of the PTHrP transcript, than those who remained disease free ($p = 0.56$). On other hand, patients with local recurrence had a low level of expression of PTHrP (17 ± 16 ; $p = 0.64$) than diseases free. More over, Patients who were disease free post treatment for breast cancer had low level (1.2 ± 0.2 copies) along with patients who developed local recurrence (1.3 ± 1 ; $p = 0.94$). Contrary to this, patients who developed distant metastases showed high levels of PTHrP-R transcript (4.5 ± 3 ; $p = 0.44$), with the highest levels observed in patients who died of breast cancer (6.2 ± 5 ; $p = 0.35$) with distant metastases (Fig. 5b).

PTHrP Expression and Survival Status

We assessed the survival status of the breast cancer patients in association with PTHrP and its receptor levels, with an average of a 10-year follow-up period (Fig. 6a). Patients were divided into two groups; those who remained disease free were assigned to the good prognosis group ($n = 89$), whereas, the patients who had recurrence, metastasis to a distant site or had died as a result of breast cancer were allocated to the poor prognosis group ($n = 30$). The quantity of PTHrP from each tumour specimen was assessed and we reveal that the patients with a poor prognosis had dramatically elevated levels of PTHrP (123 ± 2.4) compared with good prognosis group (4.8 ± 2.3) ($p < 0.05$). Our results also show that the good prognosis group had low levels of PTHrP-R (1.2 ± 0.5) compared with the higher levels observed in the poor prognosis group (4.7 ± 2.45) and statistically not reached a significant (Fig. 6b).

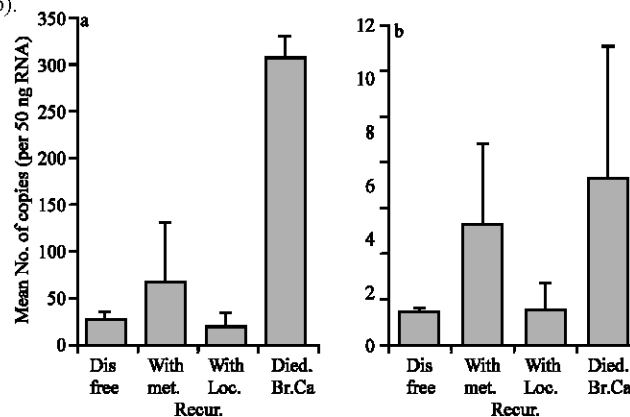


Fig. 5: PTHrP (a) and PTHrP-R (b) in relation to clinical outcome. Patients who had died from breast cancer within the 10-year follow-up period had higher levels of transcripts of PTHrP and its receptor PTHrP-R compared to those who remained alive and well or had metastatic disease

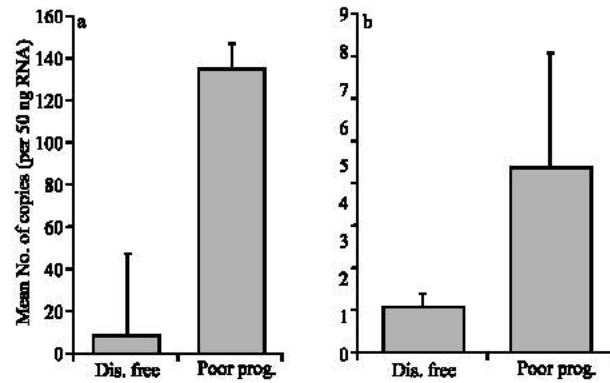


Fig. 6: Patients who were alive and well with no recurrence were allocated to the good prognosis group, whereas those who had recurrence, metastasis to distant areas or had died as a result of breast cancer were allocated to the poor prognosis group. Patients with a poor prognosis had dramatically elevated levels of PTHrP (a) and PTHrP-R (b). ($p = 0.017$)

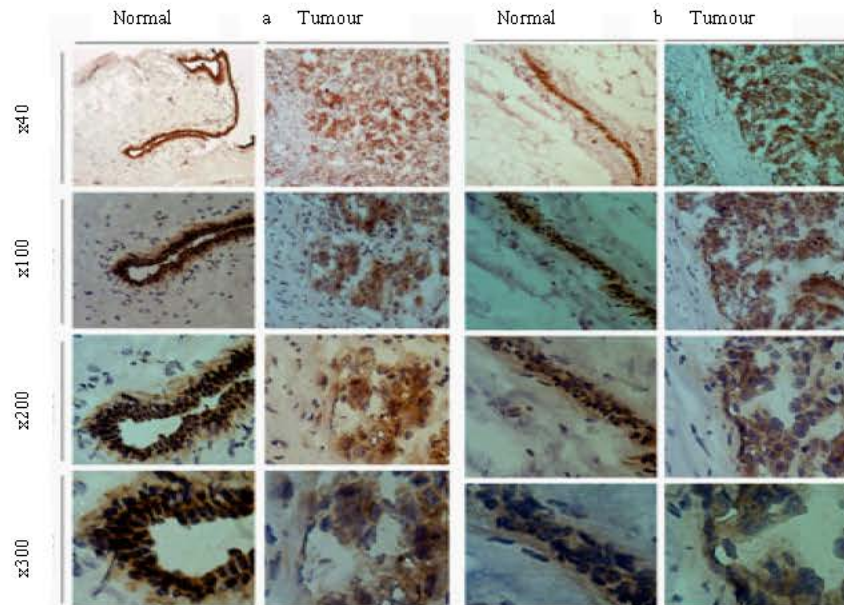


Fig. 7: (a) Immunohistochemical staining of PTHrP in normal (left panel) and tumour (right panel) tissues. Both normal epithelial cells and cancer cells stained PTHrP in the cytoplasmic region of the cells. (b) Immunohistochemical staining of PTHrP receptor (PTHrP-R) in normal (left panel) and tumour (right panel) tissues from different sample sets. Both normal epithelial cells and cancer cells stained PTHrP in the cytoplasmic region of the cells

Immunohistochemical Detection of PTHrP and its Receptor PTHrP-R in Mammary Breast Cancer and Normal Background Tissues

The staining and location of PTHrP and PTHrP-R in mammary tissues were assessed using immunohistochemistry on frozen sections. Figure 7a and b (from different sample sets), show PTHrP and PTHrP-R in both normal epithelial cells (left panel) and in breast cancer cells (right panel). On the left, mammary ducts are seen lined by normal epithelial cells. The staining was mostly confined to the

cytoplasmic regions in both cancer cells and normal background tissues. However, breast cancer cells had PTHrP and PTHrP-R present in their nuclei as well. In addition, breast cancer cells were scattered in the field of view, with some PTHrP and PTHrP-R staining of the stromal tissue between cancer cells. Over all, it is apparent that PTHrP and PTHrP-R were more abundant in breast cancer tissues in contrast to normal background tissues.

DISCUSSION

Breast cancer is the most common malignancy in women, with a worldwide prevalence of 1.5 million throughout the industrialized countries. Its mortality rate is second only to lung cancer in the USA and Europe (Martin and Cultul., 2002). Up to one-third of women with early stage breast cancer will eventually succumb to the disease and most of these will develop bone metastases during the course of the disease. Bone metastasis cause increased morbidity and mortality in patients with advanced breast cancer and lead to hypercalcaemia, severe bone pain, pathological fractures, which are in need for radiotherapy or surgery and nerve compression (Body, 2001; Guise *et al.*, 2005; Kozlow and Guise, 2005).

Our study has shown that PTHrP and PTHrP-R were present in both normal epithelial cells and in breast cancer cells, however the staining was mostly confined to the cytoplasm regions in both these tissues. This confirmed the current thinking from recent studies that PTHrP and PTHrP-R are mostly confined to the cytoplasm of most cells. Interestingly, we found that breast cancer cells had some PTHrP and PTHrP-R staining present in their nuclei as well. Importantly, it was observed that PTHrP and PTHrP-R stained more abundantly in breast cancer tissues in contrast to normal background tissues. In addition, this observation was supported further by Q-RT-PCR which showed that PTHrP transcripts were significantly low in normal breast tissues in comparison with breast tumours.

In one study PTHrP was identified immunohistochemically in 60% of breast carcinoma and in 92% of breast cancer metastases in bone (Vargas *et al.*, 1992). In another study PTHrP was localized by immunohistology in 92% breast cancer metastases in bone and in 17% metastases in non-bone sites (Powell *et al.*, 1991). A recent study has suggested that PTHrP is a potential autocrine growth factor in primary breast carcinoma as both PTHrP and PTHrP-R were found to be present in normal breast epithelium as well as breast tumour tissues (Cataisson *et al.*, 2000; Cross *et al.*, 2002; Devys *et al.*, 2001; Downey *et al.*, 1997).

A higher expression of PTHrP is positively correlated with an invasive tumour phenotype and the development of bone metastases (Devys *et al.*, 2001; Sugimoto *et al.*, 1999). Our results lend support to these observations as we found out that breast cancer patient with NPI-2 and NPI-3 tumours showed high levels of PTHrP transcripts as compared with patients who had NPI-1 tumours. Furthermore, we found low levels of PTHrP and PTHrP-R transcripts in node negative breast tumours as compared with node positive cancers. In direct contrast to these observations, a recent study has shown that increased production of PTHrP by breast cancers confers on them a less invasive phenotype (Henderson *et al.*, 2006).

Nevertheless, PTHrP may mediate local bone destruction in the absence of increased circulating PTHrP (Rong *et al.*, 1999). Recent work indicates that PTHrP exerts its effects on cell adhesion, migration, invasion and integrin expression via an intracrine pathway (Shen and Falzon, 2004). PTHrP-R is frequently expressed in breast cancer skeletal metastasis and indicates that receptor overexpression drives proliferation via autocrine signals (Hoey *et al.*, 2003).

PTHrP-R is expressed in skeletal metastasis and is associated with poor clinical outcome and survival (Linforth *et al.*, 2002). There are two well-described syndromes caused by tumour production of PTHrP, namely osteolytic bone disease associated with breast cancer and Humoral Hypercalcaemia of Malignancy (HHM) that occurs with or without bone metastasis (Gallwitz *et al.*, 2002).

It is suggested that the growth-promoting effect of PTHrP has a direct impact on tumour growth at both skeletal and non skeletal sites (Rabbani *et al.*, 2005). Other tumour-bone interactions, such as stimulation of tumour-homing through the CXCR4 chemokine receptor by its bone-derived ligand stromal cell derived factor-1alpha (SDF-1), may be responsible for the site-specific predilection of breast cancer for bone (Kozlow, 2005). The overexpression of PTHrP may be a potential prognostic factor for human breast carcinoma (Yoshida *et al.*, 2000). In addition, transient oestrogen or tamoxifen-induced hypercalcaemia in patients with breast carcinoma may be a PTHrP-mediated effect that is a marker of ER positivity (Funk and Wei, 1998).

As PTHrP is expressed by many normal tissues, its diverse biologic activities in these tissues are mediated by the PTH/PTHrP receptor through autocrine and paracrine mechanisms and this autocrine effector system is also present in the great majority of invasive breast carcinomas (Iezzoni *et al.*, 1998). This was supported by our findings that high levels of PTHrP and PTHrP-R transcripts were observed in Grade 2 and 3 tumours as compared with Grade 1 tumours and more malignant ductal breast cancers had the highest level of PTHrP and PTHrP-R transcripts as compared with less malignant lobular varieties. One aspect of the current study is the weakness of statistical power, in which a few differences failed to reach statistical significance. This was due largely to two main reasons: First the natural variance of gene transcript, as it has been recognized that gene transcripts (mRNA) are highly regulated and volatile which would have an impact on the uniformity of the analysis. Second, the sample size. A larger sized cohort would undoubtedly help to address the statistical issues.

The growth-factor like properties, together with the complex modulation of its expression by a number of growth and angiogenic factors such as interleukins, tumour derived-growth factor- β (TGF- β), Platelet-Derived Growth Factor (PDGF) and vascular endothelial-derived growth factor (VEGF), point towards potential roles for PTHrP in the regulation of tumour growth and invasion.

In conclusion, the current study and recent reports have clearly shown the important role of PTHrP in the breast cancer. These data suggest that PTHrP is not only a highly useful prognostic indicator in breast cancer, it may also be a valid therapeutic target in cancer.

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