Expression Profile of Receptor Activator of Nuclear-κB (RANK), RANK Ligand (RANKL) and Osteoprotegerin (OPG) in Breast Cancer

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Abstract. *Background: Breast cancer, the most common cancer affecting women in the USA and UK, is known to have a high frequency of osteolytic bone metastasis. Receptor activator of nuclear-κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) are a group of important regulators for osteoclast differentiation and activation. These molecules have been implicated in bone metastasis. Since the discovery of the triad of RANK, RANKL and OPG in healthy bone turnover, a better understanding of these factors in bone metastasis has been sought. Materials and Methods: Using our clinical breast cancer cohort, the transcript levels of RANK, RANKL and OPG were examined using real-time quantitative-polymerase chain reaction (qPCR). Expression of these molecules in the immortalised breast cancer cell lines MCF-7 and MDA-MB-231 was also analyzed using qPCR following treatment with β-oestradiol in a concentration-dependent manner. Results: RANK, RANKL and OPG were all shown to be expressed in the breast cancer cell lines examined. Transcript levels were shown to be reduced in tumour samples when compared with normal tissue. Reduced RANK expression was associated with a worse clinical outcome and levels were significantly reduced in patients with general metastasis, bone metastasis and those who had died of the disease. Patients with reduced RANKL expression were more likely to develop local recurrence, bone metastasis or die from the disease. Using Kaplan-Meier survival analysis, lower expression levels of OPG were found to be associated with significantly better overall patient survival in our cohort. Conclusion: The corresponding prognostic and therapeutic potential is yet to be further investigated. Our data suggest that RANK, RANKL and OPG may potentially be used as novel prognostic markers for bone metastasis and provide new therapeutic targets in the treatment of breast cancer.*

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Breast cancer is the most common cancer affecting women in the UK and the USA and is associated with a high frequency of bone metastasis, especially in the advanced stages of the disease (1). Histological evidence shows that the majority of bone metastases are osteolytic in radiographic phenotype, accompanied by an increased number of osteoclasts (2). Bone metastases clinically result in intractable pain, pathological fractures and reduced quality of life with the main course of action being palliative care (3). The identification of the triad of receptor activator of NF-κB (RANK), its ligand (RANKL) and osteoprotegerin (OPG) of the tumour necrosis factor receptor (TNFR) superfamily of molecules involved in healthy bone remodelling has been a major advance in our understanding over bone biology and pathophysiology (4). Bone is constantly being remodelled in small patches with a dynamic equilibrium between bone resorption and bone formation (5). This delicate balance is controlled by osteoblasts and osteoclasts through the binding of RANK and RANKL (6). These two molecules have also been shown to have a role in mammary gland development, pregnancy and lactation with various cytokines and growth hormones (7, 8). OPG as a secreted member of the TNFR superfamily is a potent inhibitor of osteoclast maturation and activity by acting as a decoy receptor of RANKL (9). RANK is a type I transmembrane protein, approximately 616 amino acids in length with a 28-amino-acid signal peptide, sharing several characteristics which are common in the TNFR superfamily (10). RANKL is the only ligand which is known to bind to the extracellular portion of RANK. RANKL, a 317-amino- acid type II transmembrane protein, is primarily expressed on the surface of bone marrow stromal cells, osteoblasts and activated T-cells (11). The binding of the osteoblastic RANKL to RANK on preosteoclasts is necessary for osteoclast maturation, function and survival which leads to osteoclastogenesis, and subsequential bone absorption (12). RANKL has also been shown to stimulate the migration of RANK-expressing tumour cells, primary breast epithelial cells

and osteoclasts (13). OPG, identified in 1997 as a 401- amino-acid propeptide, is cleaved to a mature form of 380 amino acids (14). By acting as a natural inhibitor of RANKL, its main role in healthy bone biology is to control the bone cycle in favour of osteoblasts and the formation of new bone (15). There is also evidence that OPG can stimulate cell survival, particularly of tumour cells, by acting as a receptor for TNF-related apoptosis-inducing ligand (TRAIL) (16, 17). This is supported by the fact that OPG has a weak affinity for TRAIL (18).

The present study examined the transcript expression levels of RANK, RANKL and OPG in a breast cancer cohort in order to assess the implication of these molecules in disease progression as well as their prognostic potential. The study also aimed to assess the impact of β-oestradiol treatment on the expression of these molecules in the immortalised breast cancer cell lines MCF-7 and MDA-MB-231.

Materials and Methods

Breast tissue sample collection. Ethical approval was obtained from the South East Wales Ethics Subcommittee. Primary breast cancer tissues (n=127) and matching non-neoplastic mammary tissue (from the same mastectomy specimens) (n=31) were collected immediately after surgery and stored at –80˚C. All tissues were randomly numbered and details of histology, grade and Nottingham prognostic index (NPI) were only made known during experimental data analysis. Medical notes and histology reports were used for extraction of clinical and pathological data at the time of surgery and during the post-operative follow-up to monitor clinical outcomes (Table I).

Table I. *Patients' clinicopathological information*.

Clinical information	Patient numbers
Grade	
Well-differentiated	20
Moderately-differentiated	37
Poorly-differentiated	7
TNM stage	
TNM 1	2
TNM 2	35
TNM 3	7
TNM 4	4
NPI staging	
NPI <3.4	55
NPI 3.4-5.4	37
NPI >5.4	15
ER status	
Negative	67
Positive	34
Clinical outcome	
Disease-free	78
Metastasis	7
Local recurrence	5
Died of breast cancer	14
Bone metastasis	8
Died with metastasis	21

TNM: Tumour, nodes and metastasis; NPI: Nottingham prognostic index; ER: oestrogen receptor status.

RNA extraction from cells and reverse transcription PCR. Human breast cancer cell lines ZR-75-1, MCF-7 and MDA-MB-231 were obtained from the European Collection of Animal Cell Culture (Salisbury, UK). Cells were maintained routinely in Dulbeccos modified Eagle's medium nutrient mixture -F12 medium (Sigma, Dorset, UK) supplemented with 10% foetal calf serum (Sigma) and antibiotics. RNA was extracted from cells using TRI-reagent (Sigma), in line with the manufacturer's instructions. Reverse transcription (RT) was carried out using 0.5 μg of total RNA for each 20 μl RT reaction. Conventional PCR was carried out using GoTaq Green master mix (Promega, USA) and specific primers for *GAPDH*, *OPG*, *RANK* and *RANKL* were designed using the Beacon designer software and synthesised by Sigma (see Table II for full sequences). PCR conditions were as follows: 94˚C for 5 min, followed by 34-38 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 40 seconds with a final extension step of 72˚C for 10 minutes. Subsequently samples were separated electrophoretically on a 0.8% agarose gel. Agarose gels were stained using SYBR safe DNA gel stain (Invitrogen, Paisley, UK) as described in the manufacturer's instructions, after which they were then visualised using Syngene gel doc system (U:Genius version 3.0.2.0) on a blue-light transilluminator.

RNA extraction from tissues and qPCR. Sections were mixed and homogenised using a hand-held homogeniser in ice-cold RNA extraction solution (Sigma, Dorset, England, UK). The concentration of RNA was determined using UV spectrophotometry. Reverse transcription was carried out using high capacity cDNA reverse transcription kits from Applied Biosystems (Carlsbad, CA, USA). PCR primers are detailed in Table II. Real-time qPCR master mix was bought from ABgene (Epsom, Surrey, UK).

Levels of RANK and RANKL transcripts were determined using real time quantitative PCR based on the Amplifluor technology, modified from a method reported previously (19). An additional Z sequence (5'-ACTGAACCTGACCGTACA-3), complementary to the universal Z probe (Intergen, Oxford, UK), was added to the reverse primer. Each reaction was carried out using Hotstart q-master mix (ABgene), 10 pmol of forward primer, 1pmol of reverse primer with the additional Z sequence, 10 pmol of FAM-tagged probe and cDNA from 50 ng of RNA. The reaction was carried out using an IcyclerIQ (BioRad, Surrey, UK) equipped with optimised real time detection conditions of 94˚C for 12 minutes and 90 cycles of 94˚C for 15 seconds, 55˚C for 40 seconds and 72˚C for 15 seconds. The levels of RANK, RANKL and OPG transcripts are shown here as the number of copies per 50ng RNA generated from an internal standard which was run simultaneously in each qPCR.

Breast cancer cell lines treated with β-oestradiol. Concentration dependent treatment: Breast cancer cells were seeded into a 6 well plate (1×10^6 cells/well) and left to settle for 24 h. Wells were individually treated with different concentrations (0, 10^{-7} M, 10^{-8} M, 10^{-9} M and 10^{-10} M) of β-oestradiol (Sigma) for a 2-h period. Following incubation, RNA was extracted from cells as described above. Transcript levels of *RANK*, *RANKL* and *OPG* were then measured using real-time qPCR as described above.

Statistical analysis. Statistical analysis was performed using the Minitab (Minitab Ltd, Coventry, UK) statistical software package (version 14). Non-normally distributed data were assessed using the

Table II. *Primer sequences used in reverse transcriptase PCR and qPCR.*

Gene symbol	Forward	Reverse
GAPDH (Glyeraldehyde 3-phospate dehydrogenase)	5'-AGCTTGTCATCAATGGAAAT	5'-CTTCACCACCTTCTTGATGT
OPG (Oestoprotegerin)	5'-GAACCCCAGAGCGAAATACA	5'-CGGTAAGCTTTCCATCAAGC
RANK (Receptor activator of nuclear κB)	5'-TTGCAGCTCAACAAGGACAC	5'-CGTAGGGACCACCTCCTACA
RANKL (RANK Ligand)	5'-TGGTTCCCATAAAGTGAGAGTC	5'-AACTTTAAAAGCCCCAAAGT
hOPG	5'-GTTCTGCTTGAAACATAGGAG	5'-ACTGAACCTGACCGTACACGTCTCATTTGAGAAGAACC
hRANK	5'-TCTGATGCCTTTTCCTCCAC	5'-ACTGAACCTGACCGTAACATGGCAGAGAAGAACTGCAAA
hRANKL	5'-CGCGCCAGCAGAGACTAC	5'-ACTGAACCTGACCGTACACCGAGCCACGCAGGTACT
hCK19 (Cytokeratin 19)	5'-CAGGTCCGAGGTTACTGAC	5'-ACTGAACCTGACCGTACACCGTTTCTGCCAGTGTGTCTTC

Mann-Whitney test, whilst normally distributed data were assessed using the two-sample *t*-test. Kaplan Meier survival analysis and Pearson correlation were performed using the SPSS statistical software (version 11; SPSS, Chicago, IL, USA), Differences were considered to be statistically significant at $p<0.05$.

Results

Expression of RANK, RANKL and OPG in breast cancer. Expression of *RANK*, *RANKL* and *OPG* were examined in breast cancer cell lines and in the breast cancer cohort. The initial expression profile of each molecule in the breast cancer cell lines was determined using RT-PCR (Figure 1). *RANK* expression appears to be consistent amongst the breast cancer cell lines, demonstrating expression in all lines tested. *RANKL* expression differs in the breast cancer cell lines, with *RANKL* expression appearing to be lower in ZR-75-1 and MCF-7 cells than in MDA-MB-231cells. *OPG* mRNA expression also demonstrates variability in the breast cancer cell lines, where ZR-75-1 cells seem to have the weakest expression when compared with MCF-7 and MDA-MB-231 cells.

Quantitative analysis of the breast cancer cohort indicated a reduced transcript expression of *RANK* (Figure 1B), *RANKL* (Figure 1C) and *OPG* (Figure 1D) in tumour samples, when compared to normal tissue samples from the same patients, although none of these reductions were found to be statistically significant ($p>0.05$).

Oestrogen receptor (ER) status. ER status is a critical aspect in the management and prognosis of breast cancer. Levels of ER are well-known for affecting the personalised therapy and the clinical outcome of patients with breast cancer. *RANK*, *RANKL* and *OPG* transcript levels were analyzed against patient ER status. *RANK* transcript expression was significantly reduced in ERβ-positive samples compared to ERβ-negative samples ($p=0.026$) (Figure 2A). *RANKL* transcript expression was significantly reduced in both ERα-positive ($p=0.039$) and ERβ-positive ($p=0.036$) samples compared to ERα- and ERβ-negative samples (Figure 2B/C). *OPG* transcript expression was reduced in both ERα- and ERβ-positive samples when compared to ERα- and ERβ-negative samples, although these reductions were not found to be statistically significant ($p>0.05$).

Taking this into consideration, MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) cells were treated with differing concentrations of β-oestradiol for 2 h. *RANK*, *RANKL* and *OPG* transcript levels were measured using qPCR. Over the concentration gradient tested, RANK, RANKL and OPG transcript levels in MCF-7 cells were significantly reduced at various concentrations when compared to the untreated cells (Figure 3A, C and E). In contrast *RANK*, *RANKL* and *OPG* transcript levels in MDA-MB-231 cells were significantly increased at different oestrogen concentrations (Figure 3 B, D and F).

Prognostic relevance of RANK, RANKL and OPG in breast cancer. At the patients' final follow-up (June 2004), they were divided into the following categories: disease-free, with metastasis, with local recurrence, and death due to breast cancer. The *RANK* transcript levels were found to be significantly reduced in patients who had presented with metastasis, including both bone metastasis and other distant metastases ($p=0.039$) or had died of the disease ($p=0.0052$), when compared to those who had remained disease-free (Figure 4A). Patients who were diagnosed with bone

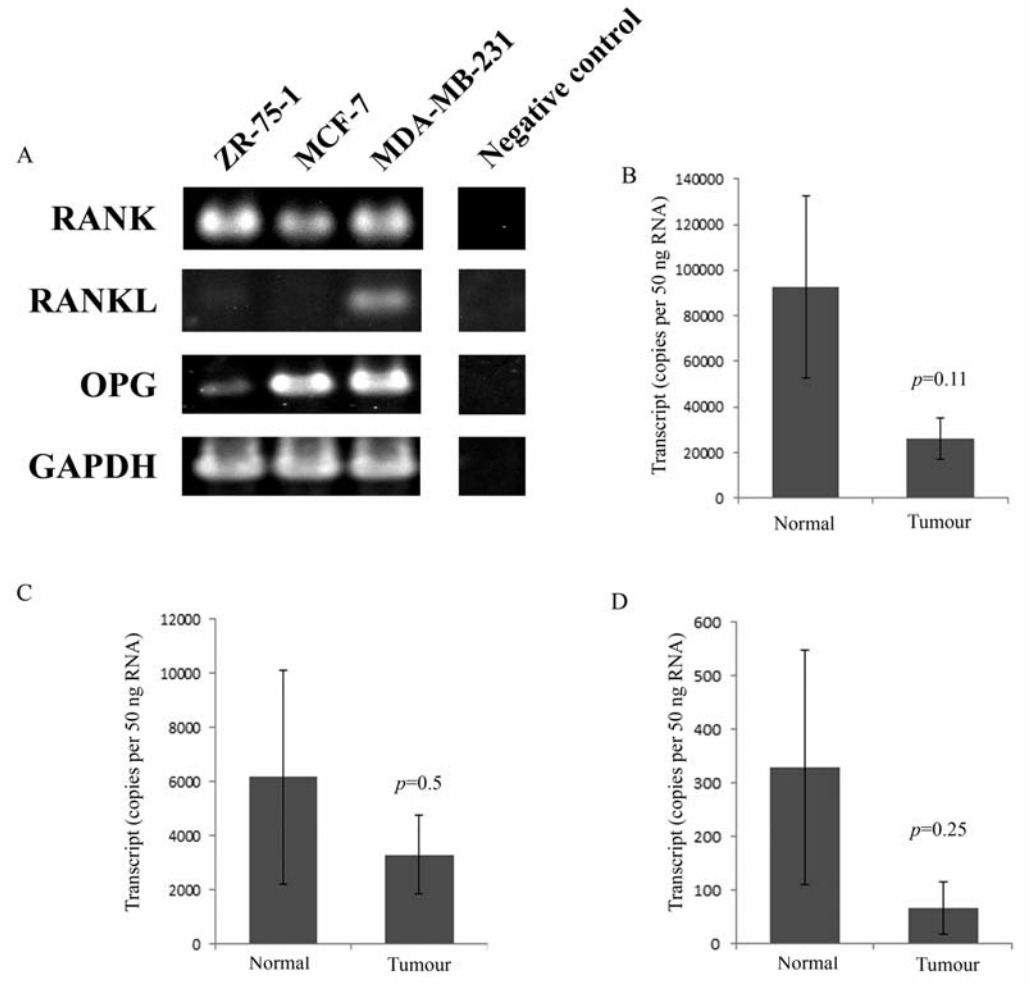

Figure 1. *Expression of receptor activator of nuclear-κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) in breast cancer. A: The expression of RANK, RANKL and OPG mRNA in breast cancer cell lines using reverse transcriptase polymerase chain reaction. B: RANK transcript level is decreased in human breast cancer using quantitative polymerase chain reaction (qPCR). C: RANKL transcript level is decreased in human breast cancer using q-PCR. D: OPG transcript level is decreased in human breast cancer using q-PCR. Normalized against GAPDH. Data are mean±SEM. For n= see Table I.*

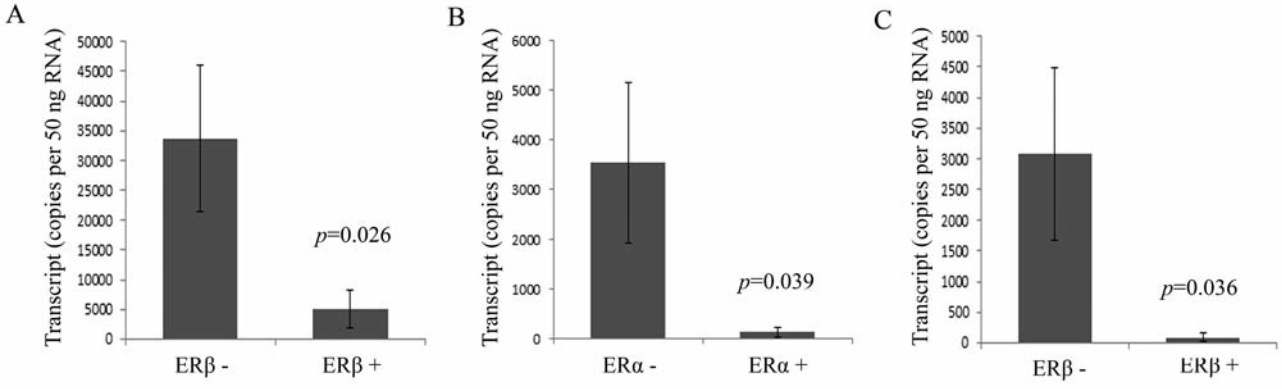

Figure 2. *Oestrogen receptor (ER) status in relation to receptor activator of nuclear-κB (RANK) and RANK ligand (RANKL) expression. A: Decreased RANK transcript level in positive compared to ERβ-negative cases. B: Decreased RANKL transcript level in positive compared to ERa-negative cases. C: Decreased RANKL transcript level in ERβ-positive compared to ERβ-negative cases.*

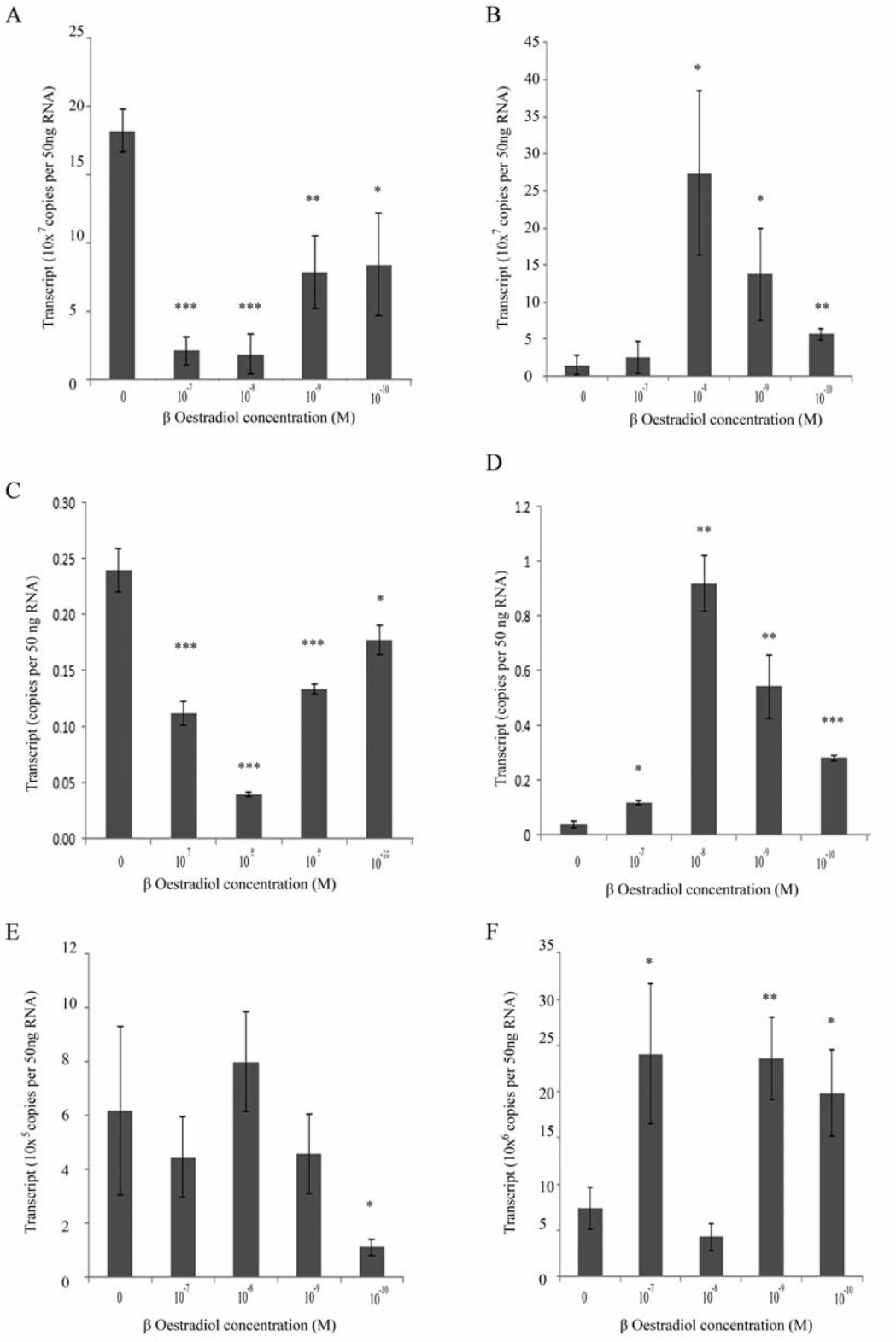

Figure 3. *Expression of receptor activator of nuclear-κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) transcript levels in breast cancer cell lines after treatment with β-oestradiol. RANK transcript levels in MCF-7 (A) and MDA-MB-231 (B) after treatments with different concentrations of β-oestradiol. RANKL transcript levels in MCF-7 (C) and MDA-MB-231 (D) after treatments with different concentrations of β-oestradiol. OPG transcript levels after β-oestradiol treatments in MCF-7 (E) and MDA-MB-231 (F). No symbol p>0.05, *p≤0.05, **p≤0.01, ***p≤0.001.*

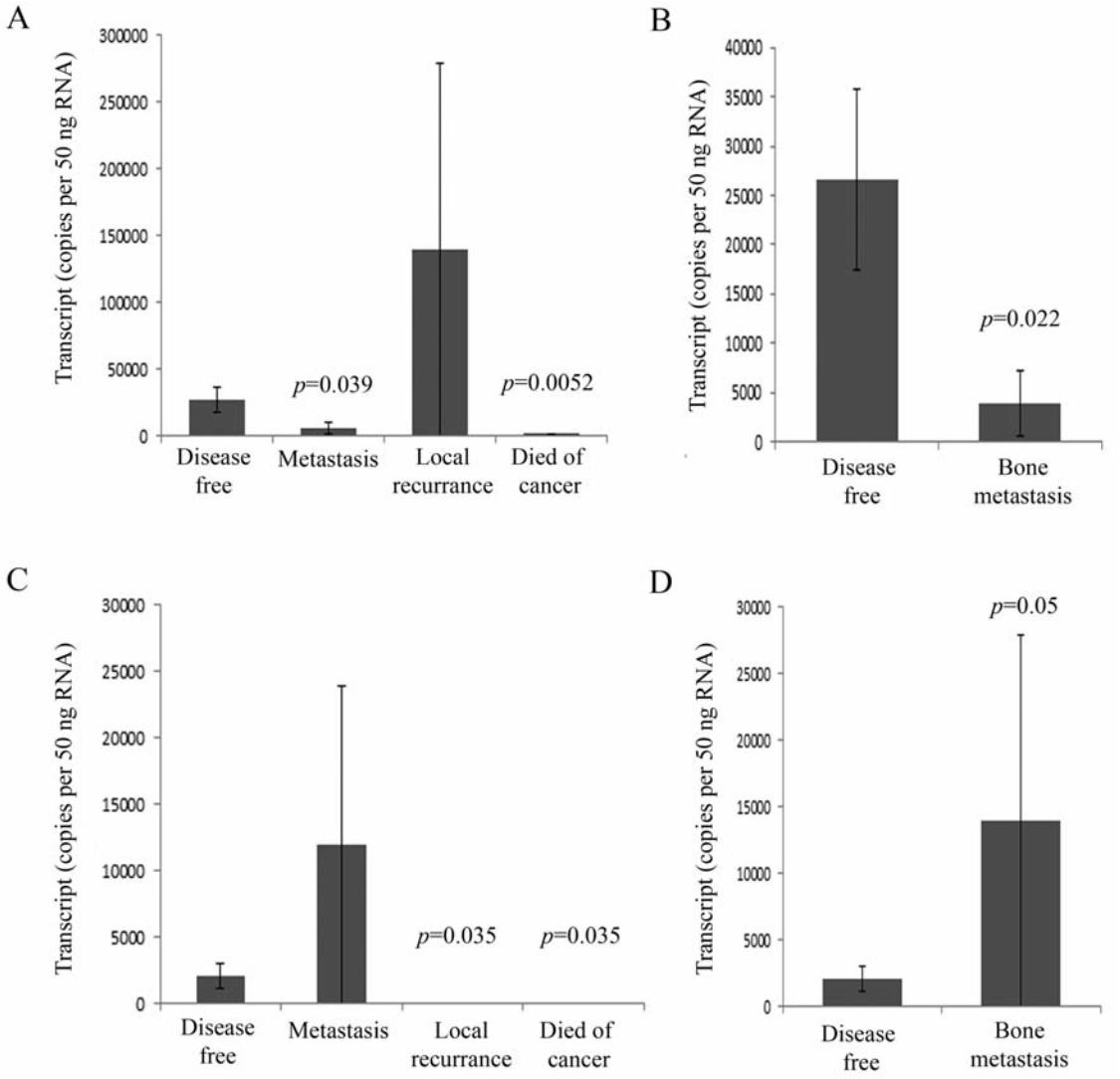

Figure 4. *Expression of receptor activator of nuclear-κB (RANK) and RANK ligand (RANKL) associated with clinical outcomes. A: RANK and overall clinical outcomes. B: RANK expression was significantly lower in patients with bone metastases. C: RANKL and overall clinical outcomes. D: RANKL expression was significantly increased in patients with bone metastases.*

metastasis were also shown to have a significantly lower levels of *RANK* (p=0.022) than those who were disease-free (Figure 4B).

RANKL transcript expression was significantly reduced in patients with local recurrence and those who had died from the disease when compared to those who were disease-free (p=0.035) (Figure 4C). Patients with bone metastasis were shown to have a significantly increased *RANKL* transcript expression (p=0.05) (Figure 4D).

Kaplan Meier survival analysis showed that patients' median survival with higher expression levels of *RANK* 140 months (95% CI=131-148 months) and of *RANKL* 147 months (95% CI=138-156 months), had significantly better overall survival *versus* those patients with lower expression levels (*RANK* 125 months (95% CI=110-139 months), (*RANKL* 117 months (95% CI=102-132 months) (Figure 5A and B). In contrast patients with higher expression levels of *OPG* were found to have significantly poorer overall survival, 108 months (95% CI=84-132 months) than patients who had lower expression levels, 142 months (95% CI=132-151 months) (Figure 5C).

Discussion

RANK, RANKL and OPG have been shown to be involved in the homeostasis of healthy bone turnover. We recently examined the expression profiles of these molecules in our clinical breast cancer cohort and in immortalised breast cancer

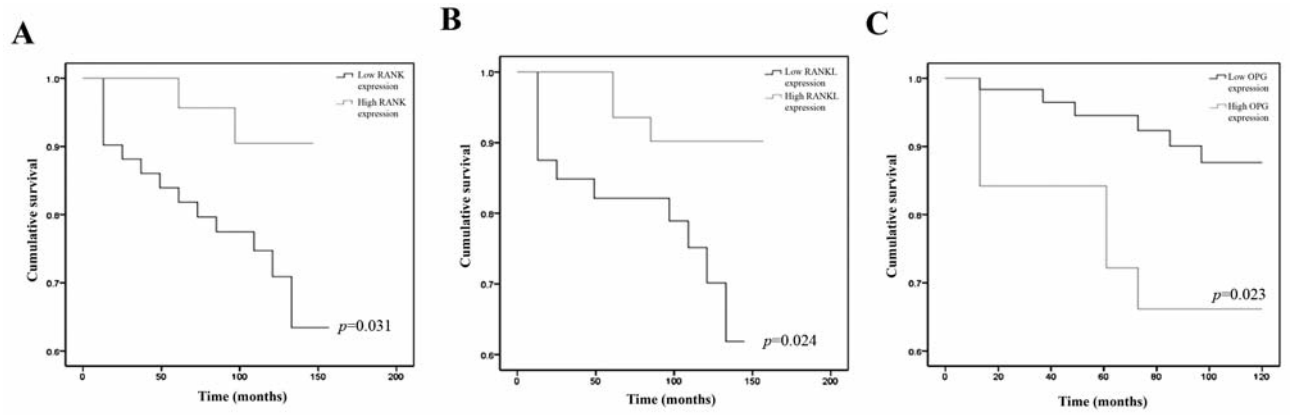

Figure 5. *Effect of expression of receptor activator of nuclear-κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) on prognosis. A: Lower levels of RANK expression correlated with poorer overall survival. B: Lower levels of RANKL expression correlated with poorer overall survival. C: Lower levels of OPG expression correlated with longer overall survival.*

cell lines. RT-PCR screening of immortalised breast cancer cell lines suggests that higher levels of *RANKL* and *OPG* are seen in the poorly-differentiated, ER-negative breast cancer cell line MDA-MB-231, compared to the ZR-75-1 and MCF-7 ER-positive cell lines. Reinholz *et al*. (20), also reported that *RANKL* mRNA transcripts were detected in breast tumours, whilst Thomas *et al*. (21) reported that *OPG* mRNA transcripts could be detected in primary breast tumours. It is well-known that breast carcinomas have the ability to metastasise to the bone; however, the establishment mechanism remains unknown. RANKL, vital for normal mammary gland development, has been demonstrated to aid breast cancer cell migration into the bone *in vitro* (13). The clinical cohort data indicates that RANK, RANKL and OPG all demonstrate reduced expression in tumour samples *versus* normal breast tissues. MDA-MB-231 already expresses RANKL, with higher RANKL expression associated with increased likelihood of bone metastasis (21, 22). This evidence supports the suggestion that RANK and RANKL expression may crucially direct breast cancer cells to metastasise to the bone. RANK is also expressed on the surface of various cancer cells and RANKL is thought to act as a chemokine directing cancer cells to preferentially migrate to the bone (23).

In the clinical cohort, reduced expressions of *RANK* and *RANKL* were found to be significantly correlated with poor overall survival. In contrast to this, higher levels of *OPG* expression correlated with a poorer overall survival. ER levels are well-known to affect the clinical outcome of breast cancer patients. Transcript levels for *RANK* and *RANKL* were shown to be significantly lower in patients whose tumours were ERβ-positive. Using the expression profile of these molecules, the results in relation to ER status were examined. *In vitro* work was carried out to examine the effects that oestrogen treatment may have on immortalised breast cancer cell lines. *RANK* and *RANKL* mRNA expression levels were examined in response to β-oestradiol in a concentration-dependent treatment. In MCF-7 cells *RANK* and *RANKL* expression levels were shown to be reduced. However, in contrast, expression levels in MDA-MB-231 cells were shown to increase with concentration-dependent exposure to oestrogen. RUNX2 is a Runt homology domain transcription factor which has been shown to be highly expressed in breast cancer cell lines that metastasise to the bone (25, 26 and 27), with aberrant expression only being detected in primary breast tumour cells (28). RANKL production has also been linked to RUNX2, therefore possibly contributing to osteolytic metastasis (29). Given the changes seen in the mRNA expression levels of *RANK* and *RANKL*, there may be a possible change in the transcription levels of Runx2 which could be influenced by oestrogen exposure. Further investigation into the mechanism of bone metastasis associated with breast cancer is needed, potentially exploring the influence the RUNX2 transcription factor has on the expression of RANK and RANKL in breast cancer cells, both in the primary tumours and in the bone and other distant metastatic sites.

Our data suggest *RANK* and *RANKL* expression might be independent and novel prognostic markers for clinical breast cancer, particularly for those with bone metastasis or with a predisposition to develop them. There may also be potential for OPG administration to re-adjust the balance between the RANKL/OPG cytokine system especially in ER-positive cancer.

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