ORIGINAL ARTICLE

Expression of receptor activator of nuclear factor κ B ligand (RANKL) and tumour necrosis factor related, apoptosis inducing ligand (TRAIL) in breast cancer, and their relations with osteoprotegerin, oestrogen receptor, and clinicopathological variables

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Background: Receptor activator of nuclear factor κ B ligand (RANKL) has an important role in bone remodelling, and tumour necrosis factor related, apoptosis inducing ligand (TRAIL) can induce apoptosis in cancer cells. Their functions are linked by their interactions with osteoprotegerin (OPG).

Objective: To investigate the expression of RANKL and TRAIL in a large series of unselected breast cancers and to analyse the relations between these expressions and the expression of OPG, oestrogen receptor, and clinicopathological variables.

Methods: 395 breast cancers were sampled into tissue microarrays and immunohistochemistry undertaken for RANKL and TRAIL.

Results: There was strong expression of RANKL in 14% of the cancers and strong expression of TRAIL in 30%. Expression of RANKL had a negative association with expression of oestrogen receptor ($p=0.036$). Expression of TRAIL had a negative association with the Nottingham Prognostic Index ($p=0.021$). There was a significant negative relation between expression of RANKL and TRAIL ($p<0.005$). Unsupervised cluster analysis produced a dendrogram that showed a clear division into two groups, and the expression of oestrogen receptor was significantly higher in one of those groups ($p=0.012$).

Conclusions: There is apparent loss of expression of RANKL in 86% of breast cancers; those tumours that retain expression tend to be oestrogen receptor negative and of a high histological grade. There is strong expression of TRAIL in 30% of breast cancers and these tend to be of better prognostic type. These results may be important in the processes of metastasis to bone and the apoptotic cell death pathway in cancer.

Receptor activator of nuclear factor κ B ligand (RANKL), together with osteoprotegerin (OPG), is involved in the control of bone remodelling. Because of this its role in cancer metastasis to bone has been investigated. In bone, RANKL is expressed by osteoblasts and stromal cells, and binding of RANKL to its receptor RANK on the surface of pre-osteoclasts and osteoclasts stimulates osteoclast maturation and activity. OPG is a soluble receptor for RANKL which is expressed by various different cell types, including osteoblasts, and binding to RANKL prevents RANK–RANKL associations and inhibits osteoclast development.¹ Recent studies have shown that both OPG and RANKL are expressed by a variety of different cell types, including tumour cells, suggesting that these molecules may have functions in addition to their established role in bone turnover. In vitro studies have shown that breast cancer cell lines can produce RANKL and stimulate osteoclast differentiation when co-cultured with bone marrow stromal cells.² It has also been shown that RANKL is expressed in most prostate cancers and this expression increases in bony metastases from these tumours.³

The function of tumour necrosis factor related, apoptosis inducing ligand (TRAIL) is linked to OPG as OPG is a decoy receptor for it. Normally TRAIL binds to death receptors DR4 and DR5 and induces apoptosis in cancer cells⁴; recombinant TRAIL protein is being evaluated as a therapeutic agent because its apoptosis inducing actions are relatively selective for cancer cells.^{4–6} However, this function can be inhibited by the presence of OPG which, by binding to TRAIL, prevents its

binding to the death receptors.^{7–8} It has been shown that TRAIL is endogenously produced by cutaneous melanoma,⁹ hepatocellular carcinoma,¹⁰ and 91% of non-small-cell lung cancers,¹¹ but the functional significance of this has not been defined.

There are very few published studies of RANKL and TRAIL expression in breast cancer. Reinholz *et al*¹² looked at RANKL expression in 24 primary breast cancers using quantitative reverse transcriptase polymerase chain reactions. They found the gene expression of RANKL (and OPG) was not altered in different stages of breast carcinogenesis from normal, through ductal carcinoma in situ, to invasive disease.

We previously investigated the expression of OPG, TRAIL, and RANKL in non-neoplastic breast tissue and a small series of breast cancers.¹³ This showed that each protein localised to a different cellular compartment in non-neoplastic breast—TRAIL to the myoepithelial cells, RANKL to the luminal surface of epithelial cells, and OPG was only expressed in epithelial cells which showed columnar cell change. In the 40 cancers in that study there was strong expression of each protein in about a half the cases but the numbers were insufficient for any analysis of the relations with clinicopathological variables apart from oestrogen receptor status. We have investigated the expression of OPG in a larger series of breast cancers and have shown it is negatively correlated

Abbreviations: ER, oestrogen receptor; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κ B ligand; TRAIL, tumour necrosis factor related, apoptosis inducing ligand

with increasing tumour grade and positively correlated with oestrogen receptor expression.¹⁴ However, to the best of our knowledge, there are no published studies investigating the expression of RANKL and TRAIL protein in a large series of unselected breast cancers. As RANKL, TRAIL, and OPG have all been proposed as therapeutic agents or targets, it is increasingly important to determine the expression levels and functions of these molecules in different cancers.

METHODS

Study population

Four hundred unselected cases of invasive breast cancer, from both screening detected and symptomatic presentations, were retrieved from the surgical archives of the department of histopathology, Royal Hallamshire Hospital, Sheffield, UK. The study received appropriate ethical approval. All slides were reviewed by a specialist breast pathologist (SSC) and a block containing well preserved tumour was selected from each case. The grade of the breast cancers was reviewed at this point. Four 0.6 mm cores of tissue were sampled from each tumour and put into separate tissue array blocks using a tissue microarrayer (Beecher Instruments Inc, Sun Prairie, Wisconsin, USA). Haematoxylin and eosin stained sections were taken from the arrays to verify the sampling. The pertinent clinicopathological information was abstracted from the pathology and medical records. After arraying, there were 395 assessable breast cancers—296 ductal no special type (NST), 41 lobular, 20 tubular, 16 mucinous, and 22 other special types; and these had tumour gradings (by the Elston modification of the Bloom and Richardson system¹⁵) of 83 grade 1, 188 grade 2, 114 grade 3, and 10 ungraded. Follow up data were available on 286 cases, with a median follow up of 50.3 months (25th centile, 36.6 months; 75th centile, 69.4 months). During the follow up period there were 32 deaths from a breast cancer related cause.

Immunohistochemistry

Immunohistochemical studies were carried out on paraffin blocks from tissue fixed in 10% neutral buffered formalin. Tissue sections of 4 µm thickness were prepared, mounted on polylysine coated slides. Sections were dewaxed and hydrated to 70% EtOH, and incubated in 3% hydrogen peroxide in methanol for 15 minutes, followed by three washes in phosphate buffered saline (PBS). The sections were then microwaved for 10 minutes in 0.1 M Tris-HCl at full power, washed in PBS, and blocked with Casein (Vector Laboratories Ltd, Peterborough, UK) for 30 minutes. The slides were incubated either with mouse monoclonal anti-OPG 1:250 (MAB 8051, R&D Systems, Abingdon, UK), or with mouse monoclonal anti-TRAIL 1:100 (MAB 687, R&D Systems), goat polyclonal anti-RANKL 1:100 (SC-7627, St Cruz, California, USA) or mouse monoclonal anti-oestrogen receptor 1:20 (clone 6F11 Vector Laboratories) for one hour, washed, and incubated for 30 minutes with anti-mouse/goat biotin conjugated secondary antibody (30 µl/2 ml of casein solution diluted 1:50) (Vector Laboratories). Slides were washed and developed using ABC Elite kit (Vector Laboratories), followed by DAB and a 20 second counterstain with haematoxylin. Negative controls (no primary antibody) were included in all runs.

The immunohistochemical staining was analysed by an experienced pathologist (SSC). For RANKL, TRAIL, and OPG the intensity of staining of each core was graded as negative (0), weak (1), moderate (2), or strong (3). There was no heterogeneity of staining within individual tissue cores so estimation of the percentage of cells staining was not required. The scores for the triplicate cores for each case were averaged; the fourth array was used to provide results for cores which were missing from one of the first three. For oestrogen receptor the cores were evaluated using the McCarty H score¹⁶ and then classified into 0 (score less than 50), 1 (score ≥50, <100), 2 (score ≥100, <200), or 3 (score ≥200). For binary division into oestrogen receptor positive

Figure 1 Examples of immunohistochemical staining of the breast cancers in the tissue microarray. Row A: a breast cancer that shows no expression of OPG, strong expression of RANKL and TRAIL, and weak to moderate expression of oestrogen receptor. Row B: a breast cancer that shows strong expression of OPG, no expression of RANKL and TRAIL, and strong expression of oestrogen receptor. OPG, osteoprotegerin; ER, oestrogen receptor; RANKL, receptor activator of nuclear factor κ B ligand; TRAIL, tumour necrosis factor related, apoptosis inducing ligand.

Figure 2 The dendrogram of the unsupervised cluster analysis of the expression of OPG, TRAIL, and RANKL in the breast cancers in the tissue microarray. The three rows of coloured squares running across the whole dendrogram represent expression of the proteins, OPG being the top row, TRAIL the middle row, and RANKL the bottom row. An absence of expression is represented by a bright green square, strong expression by a bright red square, and intermediate expression by darker squares. Each column represents one breast cancer, and annotated labels are visible in the enlarged sections below the main dendrogram. (A) The whole dendrogram showing the primary bifurcation into the two groups used for analysis, the first group is the smaller group on the right hand side indicated by the green line beneath, the second group is the large group on the left hand side indicated by the red line beneath. (B) A subsample of group 2 showing a group of tumours which strongly expressed RANKL (shown by the red squares in the bottom row) but which showed weak expression of TRAIL and OPG (the darker squares in the row above). (C) A subsample of group 1 showing a group of tumours which did not express RANKL (shown by the green squares in the bottom row) and showed either weak or moderate expression of OPG and TRAIL (the darker squares in the rows above, the dark red squares indicating moderate expression). OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κ B ligand; TRAIL, tumour necrosis factor related, apoptosis inducing ligand.

(ER+) and oestrogen receptor negative (ER-), a threshold of 50 was applied as this is the threshold that is employed in the clinical therapeutic context in our institution.

Data analysis

The data were not normally distributed so appropriate non-parametric tests were used to investigate the relations between expression of the proteins and clinicopathological variables (Mann–Whitney U test for two independent groupings, Kruskal–Wallis test for more than two independent groupings, and Jonckheere–Terpstra test for ordinal categorical groupings). Survival analysis was carried out for each protein using Cox regression with the Nottingham Prognostic Index¹⁷ as the sole co-factor, as this is a summated measure of tumour grade, size, and axillary lymph node status and the most widely used prognostic index.

The Cluster 3.0 software package¹⁸ was used, which developed from the original Cluster programme¹⁹ to carry out (unweighted) average linkage hierarchical clustering analysis on the expression of TRAIL, RANKL, and OPG. This method was chosen because it is relatively robust and offers some advantages over the methods of single, complete, or centroid linkage also available. Here only samples with less than one third missing values were included (348 of 395).

Table 2 Distribution of tumour grade in the two groups derived by cluster analysis

Cluster analysis group	Grade 1	Grade 2	Grade 3	Grade not assessable
1	20	48	18	0
2	51	120	85	6

χ^2 test, $p=0.141$.

The data were centred about their median values and clustering was carried out on both samples and proteins using “uncentered correlation” as a measure of similarity. The resulting dendrogram was displayed in the Java TreeView²⁰ program. The primary bifurcation was used to divide the cases into two groups and then the distributions of clinicopathological variables between these groups were compared using χ^2 tests.

RESULTS

The results are summarised in tables 1 to 4 and figs 1 and 2.

There was strong expression of RANKL in 14% of the cancers and strong expression of TRAIL in 30%. RANKL was

Table 1 Univariate relations between protein expression and clinicopathological variables

	Grade (Jonckheere–Terpstra test)	Size (Jonckheere–Terpstra test)	Lymph node status (Mann–Whitney U test)	Vascular invasion (Mann–Whitney U test)	Histological type (Kruskal–Wallis test)	NPI (Jonckheere–Terpstra test)	ER status (Jonckheere–Terpstra test)
ER	<0.005*	0.149	0.830	0.416	0.007	0.002*	–
OPG	0.003*	0.136	0.334	0.582	0.349	0.028*	0.007†
TRAIL	0.159	0.147	0.088	0.567	0.770	0.021*	0.154
RANKL	0.050†	0.808	0.934	0.062	0.367	0.263	0.036*

*Negative relation; †positive relation.

OPG, osteoprotegerin; ER, oestrogen receptor; RANKL, receptor activator of nuclear factor κ B ligand; TRAIL, tumour necrosis factor related, apoptosis inducing ligand.

Table 3 Distribution of nodal status in the two groups derived by cluster analysis

Cluster analysis group	N0	N1	Nx
1	51	31	4
2	167	77	18

 χ^2 test, $p=0.298$.

expressed homogeneously in the cytoplasm of the breast cancer cells in those tumours that were positive, with no discernable nuclear staining. There was no expression of RANKL in the stromal cells surrounding the cancers. TRAIL was also expressed homogeneously in the cytoplasm of the breast cancer cells in positive tumours, again with no nuclear or stromal staining.

Cox regression survival analysis with Nottingham Prognostic Index (NPI) as the sole co-factor showed that none of the proteins studied provided any additional prognostic value: oestrogen receptor, $p=0.220$; OPG, $p=0.938$; TRAIL, $p=0.635$; RANKL, $p=0.801$. Investigating co-expression of the proteins, there was a significant negative association between expression of TRAIL and RANKL ($p<0.005$, Jonckheere–Terpstra test) but no significant relation between expression of OPG and TRAIL ($p=0.511$) or OPG and RANKL ($p=0.347$).

DISCUSSION

This is the first published study that investigates the expression of RANKL and TRAIL protein in a large series of breast cancers. The most important findings are that there was an apparent loss of expression of RANKL in the majority of breast cancers and that expression of TRAIL was associated with breast cancers of a generally better prognostic type.

We have previously shown that RANKL is expressed in normal breast tissue at the luminal surface of all epithelial cells in lobules and ducts.¹³ As most breast cancers appear to arise from such cells it would be expected that the majority of breast cancers would express RANKL. In this study we have shown that expression of RANKL is only retained in 14% of these breast cancers. This is much lower than the 60% we reported in our previous study¹³ but that study only included 40 cases and those were selected to be half OR+ and half OR–. In contrast, this extended study included 243 OR+ and 94 OR– tumours, and gave a much better estimate of the true level of expression of RANKL in breast cancers. Expression of RANKL showed a significant negative association with oestrogen receptor expression and was very close ($p=0.050$) to a significant positive association with tumour grade. It thus appears that RANKL expression is retained in a small percentage of breast cancers which are more likely to be OR– and of higher histological grade. The functional significance of the loss of RANKL can only be speculated from the results of this study. It has been shown that OPG can act as a decoy receptor for TRAIL, leading to an inhibition of TRAIL induced apoptosis in various tumour cells including breast.^{7–8} As RANKL is a high affinity binding molecule for OPG, expression of RANKL could reduce the effectiveness of OPG as a decoy receptor of TRAIL, allowing TRAIL induced tumour cell apoptosis to occur. We have shown this with in vitro studies.²¹ It is possible that loss of expression of RANKL could increase overall function of OPG as a decoy receptor of TRAIL. Further in vitro studies are required to investigate the effect of underexpression or overexpression of RANKL in breast cancer cells to clarify its role, if any, in tumour progression. The mechanism of the loss of RANKL expression also requires further investigation. It is, however, unlikely to

Table 4 Distribution of oestrogen receptor status in the two groups derived by cluster analysis

Cluster analysis group	ER positive	ER negative	ER not assessable
1	68	14	4
2	175	80	7

 χ^2 test, $p=0.012$.
ER, oestrogen receptor.

be a somatic mutation and may result from either chromosomal loss or hypermethylation of the promoter region.²² The observation that RANKL is expressed in normal breast tissue, not expressed in the majority of breast cancer, but is expressed in a small group of OR– higher grade tumours could be explained by two epigenetic/genetic events in the sequence from normal to high grade OR– tumours.

The expression of TRAIL in 30% of breast cancers raises some interesting questions. Although there was no significant relation between expression of TRAIL and tumour grade, size, and lymph node status individually, there was a negative association between the expression of TRAIL and the Nottingham Prognostic Index, suggesting a multivariate relation with these individual prognostic indices. There was no correlation with survival when the NPI was a co-factor, so there is no additional independent prognostic value provided by TRAIL expression in these breast cancers. TRAIL can induce apoptosis by binding to the death receptors DR4 and DR5, and this is thought to be the main effector of macrophage induced tumour cell death.²³ It has also been shown that interferon can induce the production of TRAIL by tumour cells and this then causes TRAIL mediated apoptosis in an autocrine or paracrine manner.²³ Our findings of TRAIL expression in breast cancers with a lower NPI support this, as TRAIL mediated apoptosis would be likely to produce a comparative reduction in tumour size and lessen the probability of lymph node metastasis. Functional in vitro studies are needed to investigate the effect of TRAIL produced by breast cancer cells.

An advantage of tissue microarrays is that they allow the expression of many different proteins to be investigated in the same set of tumours.²⁴ In this study we have only looked at three proteins (excluding oestrogen receptor) but as all three have some statistically significant relations with clinicopathological variables an unsupervised cluster analysis was justified to investigate whether their collective expression produced any useful classification. The dendrogram in fig 2 shows that clustering does occur, with some clearly distinguishable subgroups, group 1 containing the majority of OPG+/RANKL– tumours. Taking the primary bifurcation as the divisor the two groups do show a significant difference in oestrogen receptor expression, with group 1 containing a greater proportion of oestrogen receptor positive tumours. However, this is to be expected given the significant relations already demonstrated by univariate statistical analysis between oestrogen receptor and both OPG and RANKL. The current dataset seems to be insufficiently rich to produce any new classification of breast cancers based on protein expression, but if future studies on the same arrays produce more data then new patterns may emerge.

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