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CLINICAL TRIAL

Receptor activator of nuclear factor kappa B (RANK) expression in primary breast cancer correlates with recurrence-free survival and development of bone metastases in I-SPY1 (CALGB 150007/150012; ACRIN 6657)

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Abstract
Purpose The receptor activator of nuclear factor kappa B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) axis may contribute to the development of bone metastases (BM). We studied gene expression in this pathway in primary breast cancer (BC) to determine correlations with clinical characteristics and outcomes in the neoadjuvant I-SPY1 study.
Methods We evaluated RANK/RANKL/OPG expression using expression microarrays in I-SPY1 ($n = 149$). Associations with clinical features were determined using *t* test and ANOVA. Associations between biomarker high versus low groups (dichotomized at an optimal cutpoint) and recurrence-free survival (RFS) were evaluated using the log-rank test and in a multivariate Cox proportional hazard model. A pooled external neoadjuvant cohort with gene expression data (GSE25066) (Hatzis et al. in JAMA 305(18):1873–1881, 30) ($n = 425$) was used for validation. Associations with site-specific relapse were evaluated using the *t*-test and multivariate logistic regression adjusting for hormone receptor (HR) status.
Results RANK was significantly higher in HR negative versus HR positive ($p = 0.027$), in basal versus non-basal disease ($p = 0.004$), and in those achieving pathologic complete response ($p = 0.038$); the associations with HR negative and basal BC were also significant in GSE25066. In both datasets, higher RANK associated with significantly worse RFS (I-SPY1: $p = 0.045$, GSE25066: $p = 0.044$). However, this association did not remain significant after adjusting for HR status. In I-SPY1 patients with recurrence, higher RANK correlated with BM versus non-BM ($p = 0.045$), even after adjusting for HR status ($p = 0.035$).
Conclusions RANK is increased in HR negative and basal BC, and correlates with worse RFS and risk of BM. The RANK pathway is a potential therapeutic target in BC.

Keywords Receptor activator of nuclear factor kappa B (RANK) · RANK ligand (RANKL) · Osteoprotegerin (OPG) · Bone metastases · Breast cancer · Recurrence-free survival

Introduction

Breast cancer remains the most common cancer diagnosis in women, accounting for 40,000 deaths in the U.S. annually [1]. While many breast cancers are diagnosed at early stages, distant recurrence can occur despite standard treatment [2]. Up to 20% of patients diagnosed with early-stage breast cancer will experience distant disease recurrence including metastases to bone [2]. Historically, risk of recurrence has been associated with clinicopathologic features such as tumor size and grade, but the critical impact of tumor biology is now clear [2]. Recent studies have demonstrated that risk of recurrence can be predicted

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based on gene expression profiles from tumor tissue at the time of breast cancer diagnosis [3–5]. Identifying patients at risk for distant recurrence including metastases to bone could help to detect those who may benefit from additional risk-reducing interventions.

Bone is a common site of metastases in breast cancer [6]. The bone marrow is a potential sanctuary site for disseminated tumor cells due to its vascularity and matrix microenvironment [7]. These tumor cells can persist in the bone marrow despite primary tumor resection and systemic therapy, ultimately giving rise to bone metastases.

The interaction between cancer cells and bone in the development of bone metastases is a complex process that likely involves multiple signal transduction pathways including receptor activator of nuclear factor kappa B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) [8–10], SDF/CXCR4 [11, 12], and TGFβ/SMAD [13], among others. The RANK/RANKL/OPG pathway is intricately involved in normal bone remodeling. RANK is a cytokine receptor generally expressed on osteoclasts and osteoclastic precursors; the interaction of RANK with RANKL on osteoblasts causes osteoclastogenesis and bone remodeling [14]. OPG is an endogenous decoy receptor to RANKL which prevents osteoclastogenesis by inhibiting the interaction of RANK and RANKL.

In preclinical studies, RANK expressed in breast tumors has been shown to contribute to altered mammary cell differentiation leading to breast carcinogenesis. Eliminating RANK from the mammary epithelium or blocking RANKL decreased the development of breast cancer in animal models [15, 16]. RANKL may promote chemotaxis between RANK-expressing tumor cells and the bone marrow [17]. In vitro, RANKL has been shown to stimulate cell migration and invasion as well as osteolysis [18, 19]. In melanoma cells, RANKL promoted the migration of RANK-expressing cancer cells to bone [20]. In an animal model, RANKL induced pulmonary metastases of RANK-expressing tumor cells, and treatment with a RANK inhibitor decreased the incidence of these metastases [21]. An upregulated ratio of RANKL to OPG may be seen in bone metastases, leading to increased bone destruction [14].

Preclinical data suggest that deregulation of the RANK/RANKL/OPG pathway may lead to the development of bone metastases from breast cancer [19, 22–24], although the role of this pathway in the clinical setting as well as associated clinical and pathologic factors is less well defined with conflicting data [25–29]. In this study, we analyzed the gene expression of the RANK/RANKL/OPG pathway in patients enrolled in the I-SPY1 clinical trial and a pooled external neoadjuvant cohort, GSE25066 [30], and correlated this gene expression with tumor and clinical features, as well as outcomes. We also evaluated correlations between other genes that may be involved in the development of metastases and RANK [11, 31–48].

Methods

Study population

The I-SPY1 trial (Investigation of serial studies to predict your therapeutic response with imaging and molecular analysis) was a large multicenter study performed in collaboration with the American College of Radiology Imaging Network (ACRIN), Cancer and Leukemia Group B (CALGB), and Specialized Programs of Research Excellence. The trial design has been previously described; and the trial schema is shown in Fig. 1 [4, 49]. Briefly, patients with at least 3.0 cm of invasive breast cancer with no distant metastases were eligible for participation. Treatment included four cycles of anthracycline-based neoadjuvant chemotherapy (NAC) with or without taxane (given before or after the anthracycline) followed by surgery. Trastuzumab was given to patients with HER2-amplified disease starting in 2005. Patients underwent serial core needle biopsies before, during, and after NAC, as well as serial breast MRI scans. Post surgical treatment including additional chemotherapy, hormonal therapy, or radiation was determined by the individual treating physician; all patients with HR-positive disease received adjuvant hormone therapy.

I-SPY1 gene expression dataset

High-quality pre-treatment biopsy gene expression data, assayed using Agilent 44 K arrays, were available for 149 I-SPY1 patients (GSE22226). The clinical characteristics of this patient subset did not differ significantly from the overall set of 221 evaluable patients [4].

The methods for microarray data generation, processing, and molecular profiling (including intrinsic subtype classification) have previously been described [4]. In this study, genes represented by multiple probes were collapsed by taking the average across probes. Specifically, the expression of OPG was computed as the average across two probes (A_23_P71530 and A_24_P192485), while RANK and RANKL expression were measured by probes A_23_P390518 and A_23_P99386, respectively.

GSE25066 gene expression dataset

Expression data generated on Affymetrix U133A arrays from pre-treatment biopsies of 508 patients who underwent NAC (sequential taxane and anthracycline regimens) were obtained from the GEO database (GSE25066) [30]. Raw

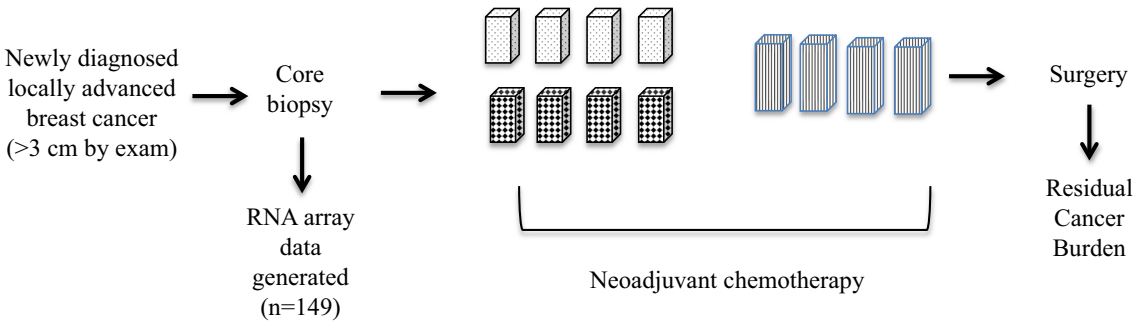

Fig. 1 I-SPY1 Clinical Trial Design. Patients who had locally advanced breast cancer (>3 cm) were eligible for study enrollment. They underwent core biopsies prior to treatment, and RNA array data were collected. The patients then underwent neoadjuvant chemotherapy with adriamycin ( ) and cyclophosphamide ( ). Some patients also received a taxane ( ). Residual cancer burden (RCB) was calculated based on surgical resection pathology

Affymetrix.cel files were RMA-normalized, and the normalized data were adjusted for source site bias using the ComBat algorithm. I-SPY1 samples were removed, resulting in a fully external expression dataset of 425 cases with clinical and molecular characteristics, NAC response, and distant recurrence-free survival annotations. A summary of the clinical characteristics of this external cohort (in comparison to the 149 I-SPY1 cases) is provided in Table 1. Once again, genes represented by multiple probes were assessed using the average across probes. Specifically, OPG expression was computed as the average across probes 204932_at and 204933_s_at, and RANKL expression was determined as the average of probes 210643_at and 211153_s_at. RANK expression was measured by the expression of probe 207037.

Association between RANK, RANKL, and OPG primary tumor expression and tumor characteristics

In each dataset, we assessed the association between RANK, RANKL, and OPG expression in primary tumor with patient age (≤50 or >50), clinical stage (stage I/II vs. stage III or inflammatory), tumor hormone receptor (HR) and human epidermal growth factor receptor 2 (HER2) status, and intrinsic subtype (basal vs. non-basal) using the *t*-test. We also evaluated the associations with tumor grade using analysis of variance (ANOVA).

Association between RANK, RANKL, and OPG expression with chemotherapy response and outcome

In each dataset, we used the *t*-test to assess associations between RANK, RANKL, or OPG expression with chemotherapy response as assessed by pathologic complete response (pCR). We also evaluated whether RANK, RANKL, or OPG expression could be used to dichotomize

Table 1 Clinical and pathologic characteristics of patients in I-SPY1 and GSE25066 datasets

Characteristic	I-SPY1 $n = 149$ (%)	GSE25066 $n = 425$ (%)
Age at diagnosis		
≤50 years	84 (56.3%)	224 (52.7%)
>50 years	65 (43.7%)	201 (47.3%)
Menopausal status		
Pre-menopausal	73 (49.0%)	N/A
Post-menopausal	49 (32.9%)	N/A
Indeterminate	27 (18.1%)	N/A
Hormone receptor (HR) status		
HR positive	86 (57.7%)	254 (59.8%)
HR negative	63 (42.3%)	170 (40.0%)
HER2 status		
HER2 positive	44 (29.5%)	2 (0.5%)
HER2 negative	97 (65.1%)	421 (99%)
Unknown	8 (5.4%)	2 (0.5%)
Clinical stage (at the time of diagnosis)		
I	3 (2.0%)	8 (1.9%)
II	70 (47.0%)	233 (54.8%)
III	64 (43.0%)	184 (43.3%)
Inflammatory	11 (7.4%)	0 (0%)
Unknown	1 (0.7%)	0 (0%)
Histology grade		
I	10 (6.7%)	26 (6.1%)
II	63 (42.3%)	155 (36.5%)
III	75 (50.3%)	229 (53.9%)
Unknown	1 (0.7%)	15 (3.5%)
Intrinsic subtype		
Luminal A	43 (28.9%)	136 (32.0%)
Luminal B	28 (18.8%)	69 (16.2%)
Basal	48 (32.2%)	151 (35.5%)
HER2	22 (14.8%)	33 (7.8%)
Normal	8 (5.4%)	36 (8.5%)

N/A not applicable

the datasets into subsets with significant differences in recurrence-free survival (RFS). For each biomarker, using the I-SPY1 cohort, we determined an optimal percentile cut-off point that yielded the most significant Kaplan–Meier (KM) curve separations between subsets (i.e., minimum log-rank test p-value) while maintaining a minimum subset size of 20% of samples. This percentile cut-off point was then used to dichotomize the GSE25066 cohort, and the significance in KM curve separation was assessed with the log-rank test. Multivariate Cox proportional hazard modeling was used to assess the association between these optimal biomarker-dichotomized groups with RFS after adjustment for HR status. In addition, in the I-SPY1 dataset where relapse site information is available, we evaluated the association between these genes with site-specific relapse (any bone metastases (BM) vs. non-bone metastases (non-BM)) using the t-test. We also assessed these associations in a multivariate logistic regression adjusting for HR status.

Determination of external gene correlations with RANK expression

We investigated possible correlations between genes that have been described in the literature as potential contributors to the development of metastases from cancer and RANK expression [11, 31–48], using both the I-SPY1 and GSE25066 cohorts. Altogether, 44 genes that could be mapped to both datasets were identified (Supplemental Table 1). Pearson correlations were determined to identify those genes with a significant ($p < 0.05$) and consistently positive (or negative) correlation with RANK expression in both the I-SPY and GSE25066 datasets.

Results

Patient demographics and tumor characteristics

Table 1 displays the clinical characteristics of patients enrolled in I-SPY1 who provided data for this study ($n = 149$), and of the GSE25066 validation cohort of 425 patients receiving NAC. The two cohorts are similar in that the majority of patients were younger than 50 years of age, had HR-positive disease, high-grade tumors, and stage II or III disease. However, almost all the patients in the GSE25066 cohort had HER2-negative disease, whereas 29.5% of patients in I-SPY1 had HER2-positive disease.

Association between RANK, RANKL, and OPG expression and primary tumor characteristics

In the I-SPY1 dataset, RANK was more highly expressed in HR-negative than HR-positive tumors (Fig. 2a, $p = 0.027$), in basal versus non-basal tumors (Fig. 2b, $p = 0.004$), as well as in stage III or inflammatory breast cancer relative to stage I/II disease ($p = 0.012$). RANK expression was not associated with tumor grade ($p = 0.176$) or patient age ($p = 0.698$). Significant associations between RANK expression and HR status, as well as basal subtype, were also observed in the GSE25066 cohort (Fig. 2c, d).

In I-SPY1, RANKL expression was higher in non-basal versus basal tumors ($p = 0.043$) and stage I/II versus III/inflammatory disease ($p = 0.028$). In addition, OPG was higher in HER2− versus HER2+ tumors ($p = 0.002$), stage I/II versus III/inflammatory tumors ($p = 0.026$), and in grade I versus II/III tumors ($p = 0.002$). However, these associations did not achieve significance in the GSE25066 cohort.

Association between RANK and OPG expression with patient chemotherapy response and outcome

RANK expression was higher in patients achieving pCR following neoadjuvant chemotherapy in the I-SPY1 dataset ($p = 0.038$), but not in the GSE25066 cohort ($p = 0.194$). To assess the association between RANK and RFS, an optimal cut-off point of 63% was derived from the I-SPY1 dataset to dichotomize patients into those with high versus low RANK expression. Higher RANK expression (greater than 63%) in primary tumor was found to be associated with worse RFS in both the I-SPY1 dataset (Fig. 3a, log-rank $p = 0.045$) and the GSE25066 dataset (Fig. 3b, log-rank $p = 0.044$). However, this association did not remain significant after adjusting for HR status (I-SPY1: Wald test $p = 0.078$, GSE25066: Wald test $p = 0.23$).

In contrast, OPG expression was not associated with chemotherapy response in either dataset ($p = 0.83$ and 0.58 for I-SPY1 and GSE25066, respectively). Using an optimal cut-off point of 79% to dichotomize OPG expression, no significant difference in RFS was seen between the OPG-high or OPG-low groups in either I-SPY1 ($p = 0.078$) or GSE25066 ($p = 0.8$) (KM curves not shown).

RANKL expression was not associated with chemotherapy response in I-SPY1 ($p = 0.87$), but did correlate with absence of chemotherapy response in GSE25066 ($p = 0.012$). Paradoxically, at an optimal cut-off point of 34%, higher RANKL expression correlated with better RFS in I-SPY1 ($p = 0.014$), but worse RFS in the GSE25066 cohort ($p = 0.049$) (KM curves not shown).

Correlation of RANK, RANKL, and OPG primary tumor expression with site-specific relapses

In I-SPY1, 41 patients developed recurrent disease, of which 12 patients had BM, 22 patients had non-BM

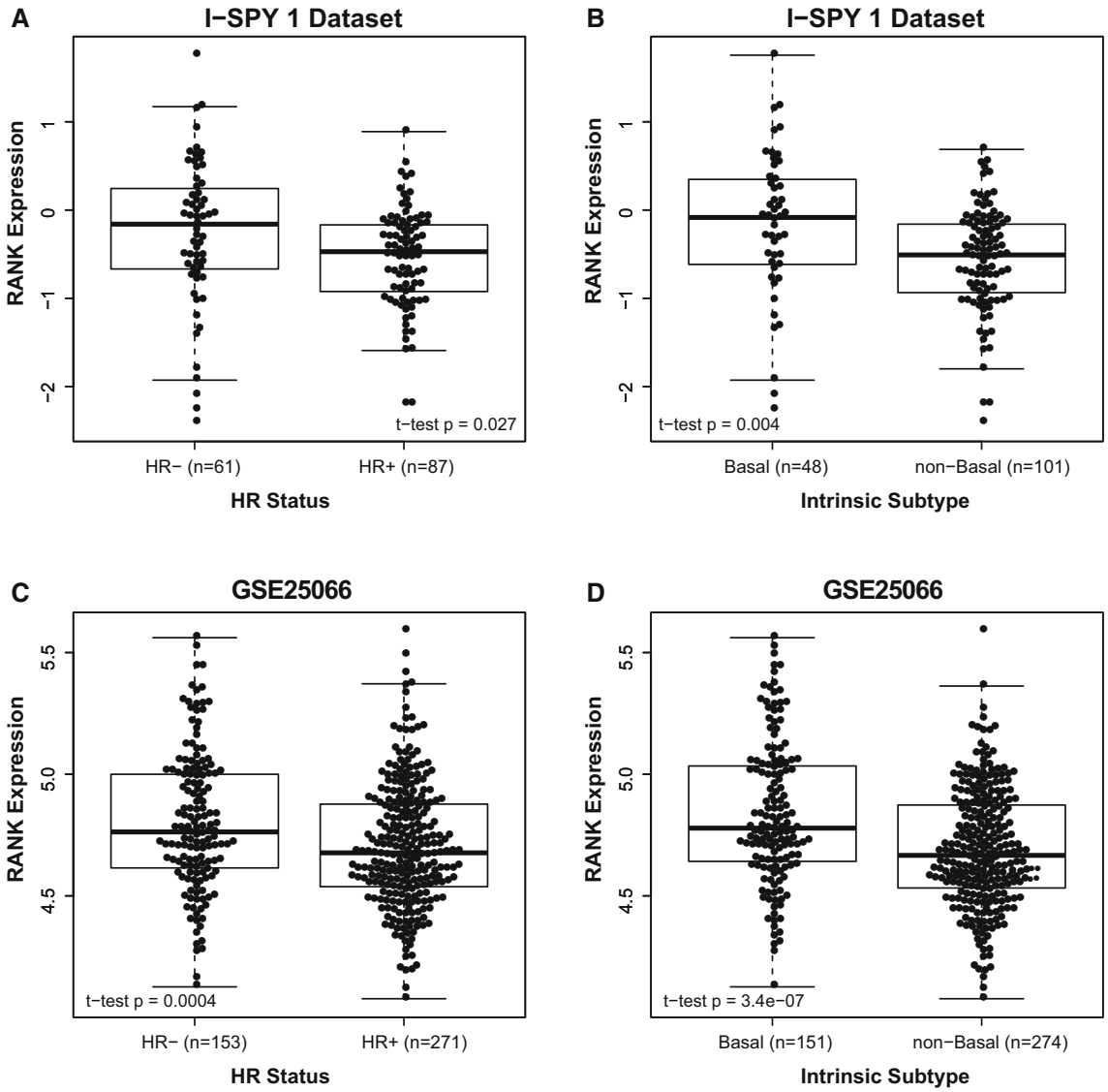

Fig. 2 RANK expression according to hormone receptor (HR) status and intrinsic subtype. In both I-SPY 1 (**a**) and the GSE25066 cohort (**c**), RANK expression was significantly higher in HR-negative (−) versus HR-positive (+) tumors. Similarly, in both I-SPY 1 (**b**) and the GSE25066 cohort (**d**), RANK expression was significantly higher in basal versus non-basal tumors

including liver, lung, brain, lymph node, and contralateral breast cancer, and seven patients were not evaluable (i.e., missing or ambiguous annotations). Of the 12 patients with BM, five had bone-only disease, and seven patients had additional sites of involvement primarily in lung and liver, with rare cases of scalp and leptomeningeal disease, brain metastasis, and bowel involvement. The median RFS for patients with BM was 804 days in comparison with 550 days for those patients with non-BM.

Figure 4a shows the median-centered expression levels of RANK, RANKL, and OPG in primary tumors in patients who remained recurrence free, developed BM, or developed Non-BM. RANKL and OPG expression were not associated with BM (Fig. 4a). In contrast, RANK expression in patients with BM was significantly greater than those patients with Non-BM (Fig. 4a, b, *t*-test $p = 0.045$). This association remained significant in a multivariate logistic model adjusting for HR status (Fig. 4c, Wald test $p = 0.035$).

Gene correlations with RANK expression

We determined correlations between the expression of other genes which may be involved in the development of metastases and RANK expression in primary tumor, using both the I-SPY1 and GSE25066 cohorts. Altogether, 44 genes were found which could be mapped to both datasets. A total of nine genes common to both datasets (BMP1,

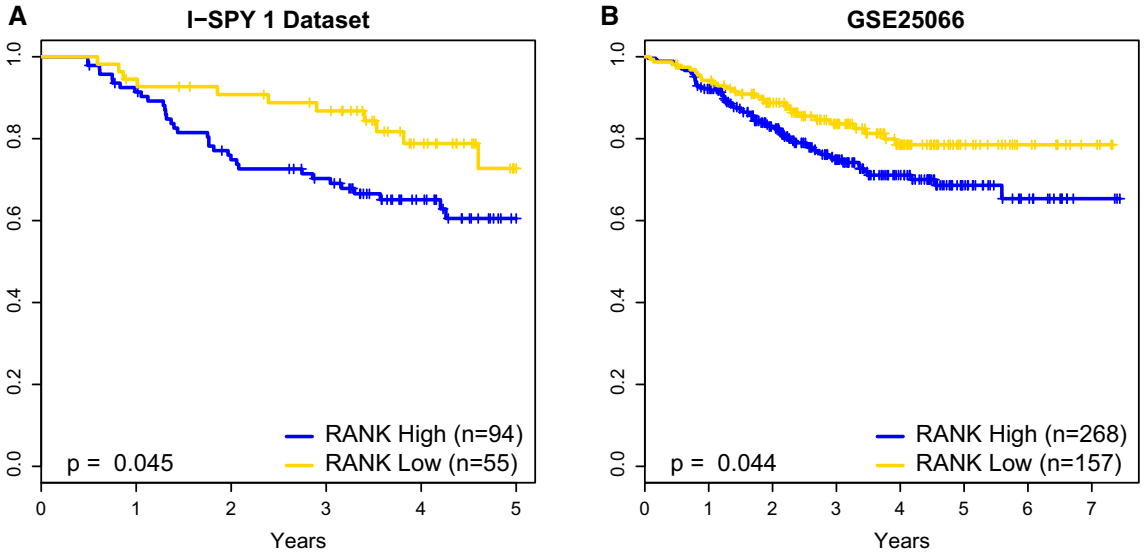

Fig. 3 Association of RANK with recurrence-free survival (RFS). In both I-SPY 1 (**a**) and the GSE25066 cohort (**b**), at an optimal cut-off point of 63% to dichotomize RANK expression, higher RANK expression (>63%) was significantly associated with worse RFS

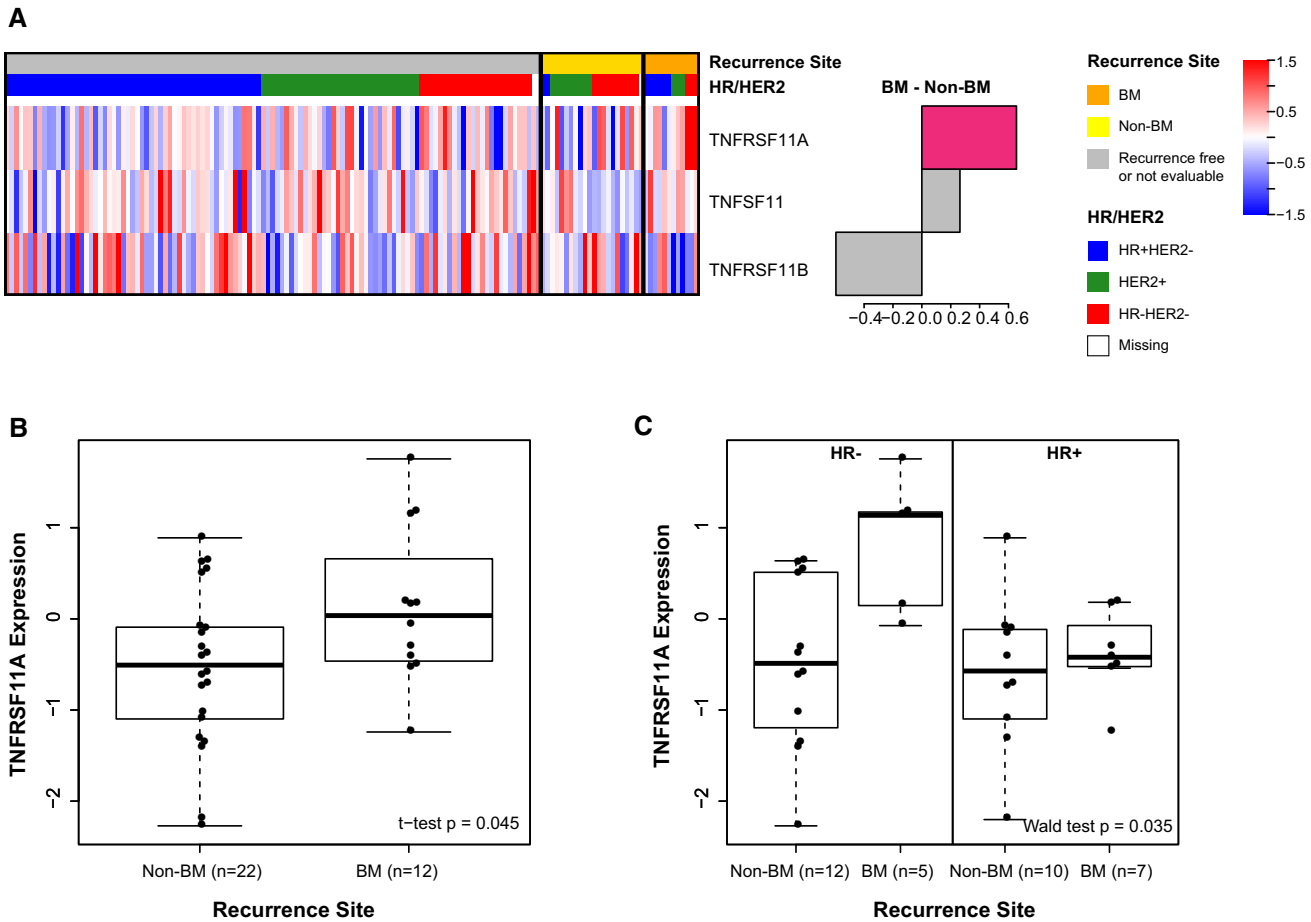

Fig. 4 RANK, RANKL, and OPG primary tumor expression in patients who remained recurrence free, developed any bone metastases (BM), or non-bone metastases (non-BM). Higher RANK (TNFRSF11A) expression, but not RANKL (TNFSF11) or OPG (TNFRSF11B), was associated with BM (**a**). RANK expression was significantly higher in patients with BM than Non-BM (**b**), and this association remained significant in a multivariate logistic model adjusting for hormone receptor (HR) status (**c**)

HGF, CSF1, SIRPA, IL11, EDNRB, EDN1, TRAF6, and SPP1) were found to correlate positively with RANK expression, and two genes (ESR1 and PGR) had a negative correlation with RANK expression.

Discussion

The RANK/RANKL/OPG pathway plays an important role in bone remodeling and recent studies suggest that this pathway may also be a significant contributor to cancer recurrence, especially the development of skeletal metastases [27, 50].

In this study, we analyzed gene expression in this pathway in patients enrolled in the I-SPY1 trial, using the GSE25066 dataset for validation. In both cohorts, we determined that RANK expression is higher in HR-negative and basal breast cancer. Our findings that higher RANK expression in primary breast cancer correlates with high-risk clinicopathologic features including a HR-negative status and the basal subtype are similar to Pfitzner [26] and Santini's [27] findings.

In both I-SPY1 and GSE25066, higher RANK expression correlated with worse recurrence-free survival, concordant with prior studies [25–27, 51]. Pfitzner's study [26] is similar to ours in that they evaluated patients undergoing NAC, using the GeparTrio cohort. However, prior studies evaluated RANK expression using immunohistochemistry analysis [25–27, 52], whereas our study used microarray gene expression, which may have less variability than immunohistochemistry. Santini et al. [27] evaluated RANK mRNA expression in the NKI cohort ($n = 295$), and noted a correlation with increased RANK expression and worse survival outcomes; in our study, we were able to demonstrate similar findings in two publically available databases, including a large database (GSE25066) of 425 patients.

In I-SPY1, although we found a correlation between higher RANK expression and complete pathologic response, tumors with higher RANK expression were associated with worse survival outcomes. These findings suggest confounding of RANK with HR status, where RANK is higher in HR negative breast cancer, which has a higher pathologic complete response rate, but worse outcomes [4, 49]. Similarly, Pfitzner et al. [26] determined that tumors with a higher RANK expression had a higher pathologic complete response rate, but worse survival outcomes.

Similar to our findings in I-SPY1 where patients with bone metastasis have RANK expression levels higher than those with non-bone recurrences, other studies have demonstrated a correlation between the development of bone metastases with expression of RANK [27, 51]. Limitations to our analysis include a relatively small sample size ($n = 149$) and short median follow-up of 3.5 years. Although this time period is reasonable for triple-negative and most HER2-positive disease, those patients with HR-positive cancers still have a substantial risk of distant recurrence [51]. Nevertheless, these are intriguing findings that merit further study.

The development of bone metastases from breast cancer is likely to involve additional genes outside of the RANK/RANKL/OPG pathway. We identified several genes that correlated with RANK expression in both datasets, including HGF (61), BMP1 (26), and CSF1 (62) that may play a role in the development of metastases (32, 56, 66). Concordant with our finding that RANK expression is higher in HR-negative breast cancer, a negative concordance with ESR1 and PGR was seen with RANK in both datasets in our study.

The reproducibility of findings in expression array-based studies is a well-recognized issue; and thus, in our study, we have employed an external dataset to validate our findings in I-SPY1, with the goal of identifying the most reproducible signals. Indeed, some clinicopathologic associations seen in I-SPY1, including some that were consistent with previous studies such as higher RANK expression in cancers achieving a pathologic complete response, were not validated by the GSE25066 dataset. Differences in array platforms (Agilent 44 K vs. Affymetrix U133A) used to generate the expression data between the two cohorts likely play a major role in these discrepancies. As well, population differences between the I-SPY1 and external pooled cohorts, such as a significantly higher proportion of HER2+ patients in the I-SPY1 cohort (29.5 vs. 0.5%), and the absence of patients with inflammatory breast cancer in GSE25066, may also contribute.

Recent studies suggest that the molecular characteristics of both cancer cells and the target tissue microenvironment may determine the organotropism of metastases, a modern version of the "seed and soil theory," initially presented by Stephen Paget in 1889, where tumor cells (seed) grow preferentially in select organs (soil) [41, 53, 54]. Consistent with this notion, our study suggests that the RANK/RANKL/OPG axis plays a role in the development of bone metastases in patients with breast cancer.

Targeting the RANK/RANKL/OPG pathway could therefore be a promising strategy to prevent the development of bone metastases in high-risk patients with breast cancer. Preclinical studies have shown that inhibiting RANKL may decrease mammary tumorigenesis, delay the onset of skeletal metastases, and decrease tumor burden in the bone [55, 56]. The monoclonal antibody to RANKL, denosumab, decreases the time to onset of skeletal events in patients with metastatic breast cancer, which may occur due to osteoclastogenesis [57, 58]. In addition, denosumab decreases the risk of fractures in post-menopausal patients

treated with aromatase inhibitors in the adjuvant setting [59]. Denosumab delayed the onset of bone metastases in patients with prostate cancer [55]. In the adjuvant setting, the phase III ABCSG-18 trial demonstrated borderline improvement in disease-free survival in patients with HR-positive breast cancer receiving aromatase inhibitors and denosumab versus placebo (HR 0.86, $p = 0.05$) [59]. Follow-up is ongoing to evaluate longer-term results and potential differences in sites of metastases. D-CARE (Study of denosumab as adjuvant treatment for women with high-risk early breast cancer receiving neoadjuvant or adjuvant therapy, NCT01077154) is an ongoing phase III trial investigating the use of denosumab as adjuvant treatment for the prevention of skeletal metastases in patients with high-risk early-stage breast cancer [60].

In conclusion, our study suggests that the RANK/RANKL/OPG axis may play a role in breast cancer recurrence, particularly in bone, and has prognostic implications. Therapies directed towards targeting the RANK/RANKL/OPG pathway are an appealing treatment approach, with early encouraging supportive clinical data.

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Compliance of ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This research study complied with standard research and ethical practices in the USA.

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