Translational Oncology
www.transonc.com

Volume 10 Number 4 August 2017 pp. 589–598 589

Evaluation of the Prognostic Value of RANK, OPG, and RANKL mRNA Expression in Early Breast Cancer Patients Treated with Anthracycline-Based Adjuvant Chemotherapy

Eleni Timotheadou[*,1], Konstantine T. Kalogeras[†,‡,1], Georgia-Angeliki Koliou[§], Ralph M. Wirtz[||], Flora Zagouri[¶], Angelos Koutras[#], Elke Veltrup[||], Christos Christodoulou[**], George Pentheroudakis[††], Aris Tsiftsoglou[‡‡], Pavlos Papakostas[§§], Gerasimos Aravantinos[||||], Vasilios Venizelos[¶¶], Dimitrios Pectasides[##], Paris Kosmidis[***], Charisios Karanikiotis[†††], Christos Markopoulos[‡‡‡], Helen Gogas[§§§] and George Fountzilas[†,||||||]

*Department of Medical Oncology, Papageorgiou Hospital, Aristotle University of Thessaloniki, School of Health Sciences, Faculty of Medicine, Thessaloniki, Greece; [†]Laboratory of Molecular Oncology, Hellenic Foundation for Cancer Research/ Aristotle University of Thessaloniki, Thessaloniki, Greece; [‡]Translational Research Section, Hellenic Cooperative Oncology Group, Athens, Greece; [§]Section of Biostatistics, Hellenic Cooperative Oncology Group, Data Office, Athens, Greece; [||]STRATYFIER Molecular Pathology GmbH, Cologne, Germany; [¶]Department of Clinical Therapeutics, Alexandra Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece; [#]Division of Oncology, Department of Medicine, University Hospital, University of Patras Medical School, Patras, Greece; [**]Second Department of Medical Oncology, Metropolitan Hospital, Piraeus, Greece; [††]Department of Medical Oncology, Ioannina University Hospital, Ioannina, Greece; [‡‡]Department of General Surgery, Breast Division, St Luke's Hospital, Thessaloniki, Greece; [§§]Oncology Unit, Hippokration Hospital, Athens, Greece; [||||]Second Department of Medical Oncology, Agii Anargiri Cancer Hospital, Athens, Greece; [¶¶]Breast Unit, Metropolitan Hospital, Piraeus, Greece; [##]Oncology Section, Second Department of Internal Medicine, Hippokration Hospital, Athens, Greece; [***]Second Department of Medical Oncology, Hygeia Hospital, Athens, Greece; [†††]Department of Medical Oncology, 424 Army General Hospital, Thessaloniki, Greece; [‡‡‡]Second Department of Prop. Surgery, Laiko General Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece; [§§§]First Department of Medicine, Laiko General Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece; [||||||]Aristotle University of Thessaloniki, Thessaloniki, Greece

Address all correspondence to: Prof. George Fountzilas, Hellenic Cooperative Oncology Group, 18 Hatzikostanti Street, 115 24, Athens, Greece.
E-mail: fountzil@auth.gr

[1] The first two authors (E.T. and K.T.K.) have contributed equally to this work.

Received 21 April 2017; Revised 17 May 2017; Accepted 22 May 2017

1936-5233/17
http://dx.doi.org/10.1016/j.tranon.2017.05.006

Abstract

BACKGROUND: Prevention of bone metastases is a major issue for breast cancer patients, as it would improve quality of life in a population where long survival is anticipated. *PATIENTS AND METHODS:* Early breast cancer patients, who had been treated with anthracycline-based chemotherapy within two randomized trials, were included in the study. We evaluated, by quantitative reverse transcription–polymerase chain reaction, 819 formalin-fixed paraffin-embedded tumor tissue samples for mRNA expression of RANK, OPG, and RANKL, as well as their ratios, for potential prognostic significance for the development of bone metastases and also for disease-free survival (DFS) and overall survival. *RESULTS:* Median age was 52.7 years, whereas 54.2% of the patients were postmenopausal and 78.3% estrogen receptor/progesterone receptor positive. After a median follow-up of 119.9 months, 226 patients (27.6%) had died and 291 patients (35.5%) had disease progression. Low mRNA expression of RANKL was associated with postmenopausal status and greater number of positive lymph nodes ($P = .002$ and $P < .001$, respectively). In the univariate analysis, low RANKL mRNA expression was found to be an unfavorable factor for DFS [hazard ratio (HR) = 1.33, 95% confidence interval (CI) 1.05-1.68, Wald's $P = .018$] and bone metastasis–free survival (HR = 1.67, 95% CI 1.09-2.56, $P = .018$), although it did not retain its significance in the multivariate analysis. *CONCLUSIONS:* Low RANKL mRNA expression in early breast cancer patients is of prognostic significance for increased risk for relapse and bone metastases and might potentially guide clinical decision-making for the use of anti-RANKL agents in the treatment of early breast cancer patients at high risk for metastatic spread, provided that our findings are validated in independent cohorts.

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Introduction

Bone metastases are common events in breast cancer, affecting 65% to 75% of patients that relapse [1,2]. The microenvironment of the host tissue and its interactions with tumor cells play a critical role in the development of metastases. More specifically in the bone, the malignant cells take advantage of the normal mechanism of bone remodeling and induce interactions with bone and stromal cells. These may occur either directly or via soluble mediators; they can be produced by any type of cells involved, malignant or not, and lead to a "vicious cycle" of development and maintenance of bone metastases [3].

Receptor activator of nuclear factor–kB (RANK) is a homotrimeric protein from the tumor necrosis factor (TNF) receptor family. It is the receptor activator of nuclear factor–kB and is expressed on the surface of osteoclasts and also on lymphocytes and dendritic cells. It binds to the RANK ligand (RANKL), which is expressed in osteoblasts and bone marrow stromal cells; is secreted by activated T cells and promotes osteoclast differentiation and maturation; inhibits osteoclast apoptosis; and consequently increases bone resorption [4]. RANKL also stimulates the migration of RANK-expressing tumor cells, primary breast epithelial cells, and osteoclasts [5]. RANKL activity is monitored by a variety of cytokines and hormones, known as regulators of the immune system and calcium homeostasis.

Osteoprotegerin (OPG) is also a member of the TNF receptor family and a decoy receptor for RANKL. It is secreted by osteoblasts and other cell types and appears to prevent bone destruction by blocking RANKL from binding to its receptor [6]. OPG is not expressed in normal epithelial and lobular breast tissue [7], but it has been shown to be expressed in 40% to 55% of breast cancer cells, being positively associated with estrogen receptor (ER) expression and negatively so with ascending histological tumor grading [8].

TNF, 1,25-dihydroxyvitamin D3, parathyroid hormone, parathyroid hormone–related protein, prostaglandin E2, interleukin-1, interleukin-6, and corticosteroids promote bone resorption, whereas estrogens, calcitonin, transforming growth factor-β, platelet-derived growth factor, and calcium induce OPG expression that blocks RANKL activity and inhibits osteoclastogenesis and bone resorption [9].

It has been demonstrated in animal models that RANK and RANKL-deficient mice had reduced or missing osteoclast differentiation, severe osteopetrosis, and serious defects like missing lymph nodes [10,11]. Furthermore, continuous RANKL inhibition in OPG-overexpressing rats resulted in higher bone density and normal immune responses and lymphatics [12]. On the contrary, RANKL overexpression in mice led to reduced bone density and osteoporosis [13].

When the RANK/OPG/RANKL system functions normally, it maintains homeostasis of bone remodeling. Dysregulations caused by neoplastic cells are responsible for the osteolysis observed in malignant tumors and the development of bone metastases. These factors are active in cells of primary cancers, such as breast, prostate, and hepatocellular carcinomas, and also in their bone metastases [5,14–16]. It has been shown in primary breast cancer tissues that increased RANK protein expression is associated with the development of bone metastases and shorter skeletal disease-free survival (DFS) [17]. Also, there is an association between high RANK mRNA expression and negative prognostic factors like tumor size >2 cm, histological grade III, and lack of ERs. Interestingly, when the authors

divided their population into poor and good- prognosis groups- on the basis of their microarray signature, RANK mRNA expression was significantly higher in the poor-prognosis group, whereas OPG and RANKL mRNA expression was higher in the good-prognosis group [17].

The identification of patients with breast cancer most likely to develop bone metastases has long been pursued, but biomarkers with predictive value as to who would develop such metastases and who might benefit from prophylactic treatment with agents such as bisphosphonates are still lacking. The data from the Adjuvant Zoledronic Acid to Reduce Recurrence trial [18] have shown that adjuvant use of bisphosphonates does not offer any benefit to the DFS of patients with breast cancer.

We designed this study to retrospectively evaluate RANK, OPG, and RANKL mRNA expression, as well as their ratios, for potential prognostic significance for the development of bone metastases and also for DFS and overall survival (OS) in an early breast cancer patient population treated with anthracycline-based adjuvant chemotherapy.

Patients and Methods

Patient Population

This was a retrospective translational research study among 1681 early breast cancer patients enrolled in two prospective phase III adjuvant trials. The HE10/97 trial [19] was a randomized phase III trial (ACTRN12611000506998) in patients with intermediate-/high-risk operable breast cancer comparing four cycles of epirubicin (E) followed by four cycles of intensified CMF (E-CMF) with three cycles of E followed by three cycles of paclitaxel (T, Taxol; Bristol Myers-Squibb, Princeton, NJ) and followed by three cycles of intensified CMF (E-T-CMF). The current definition of high-risk breast cancer is based on the "International expert consensus on the primary therapy of early breast cancer 2007" [20]. Specifically, high-risk patients were node-positive patients with one to three involved lymph nodes and ER and progesterone receptor (PgR) absent, or HER2/neu gene overexpressed or amplified; or node-positive patients with four or more involved lymph nodes. The cycles were given every 2 weeks with G-CSF support. Dose intensity of all drugs in both treatment arms was identical, but cumulative doses and duration of chemotherapy period differed. In total, 595 eligible patients entered the study in a period of 3.5 years (1997-2000).

The HE10/00 trial [21,22] was a randomized phase III trial (ACTRN12609001036202) in which patients were treated with E-T-CMF (exactly as in the HE10/97 trial) or with four cycles of epirubicin/paclitaxel (ET) combination (given on the same day) every 3 weeks followed by three cycles of intensified CMF every 2 weeks (ET-CMF). By study design, the cumulative doses and the chemotherapy duration were identical in the two arms, but dose intensity of epirubicin and paclitaxel was double in the E-T-CMF arm. A total of 1086 eligible patients with node-positive operable breast cancer were accrued in a period of 5 years (2000-2005).

HER2-positive patients received trastuzumab upon relapse, as previously described [23]; no anti-HER2 treatment was given in the adjuvant setting. Treatment schedules for the two studies are shown in Table S1. Baseline characteristics and clinical outcomes of both trials have already been described [19,21,22,24]. Primary tumor diameter, axillary nodal status, and tumor grade were obtained from the pathology report. Clinical protocols were approved by local regulatory authorities, whereas the present translational research study was approved by the "Papageorgiou" Hospital Institutional Review Board (July 15, 2013) and the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (December 18, 2013). All patients signed a study-specific written informed consent before randomization, which in addition to giving consent for the trial allowed the use of biological material for future research purposes. All clinical investigations related to the present study have been conducted according to the principles expressed in the Declaration of Helsinki.

Tissue Microarray (TMA) Construction

Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples from 975 patients (58.0% of 1681 randomized patients) were obtained during the initial breast surgery, before the initiation of adjuvant chemotherapy, and were collected retrospectively in the first trial (HE10/97) and prospectively in the second (HE10/00). The REMARK diagram [25] for the study is shown in Figure 1. Hematoxylin-eosin–stained sections from the tissue blocks were reviewed by two experienced breast cancer pathologists, and the most representative tumor areas were marked for the construction of the TMA blocks with the use of a manual arrayer (Model I; Beecher Instruments, San Prairie, WI), as previously described [26,27]. Each case was represented by two tissue cores, 1.5 mm in diameter, obtained from the most representative areas of primary invasive tumors or in some cases (9.6%) from synchronous axillary lymph node metastases and reembedded in 51 microarray blocks. Each TMA block contained 38 to 66 tissue cores from the original tumor tissue blocks, whereas cores from various neoplastic, nonneoplastic, and reactive tissues were also included, serving as orientation controls for slide-based assays. Cases not represented, damaged, or inadequate on the TMA sections were recut from the original blocks, when material was available, and these sections were used for protein expression analysis.

Immunohistochemistry (IHC)

Immunohistochemical labeling was performed according to standard protocols on serial 2.5 μm–thick sections from the original blocks or the TMA blocks. To assure optimal reactivity, immunostaining was applied 7 to 10 days after sectioning at the Laboratory of Molecular Oncology of the Hellenic Foundation for Cancer Research, Aristotle University of Thessaloniki School of Medicine. The staining procedures for HER2 (A0485 polyclonal antibody, dilution 1:200; Dako, Glostrup, Denmark), ER (clone 6F11, dilution 1:70; Novocastra, Leica Biosystems, Newcastle, UK), PgR (clone 1A6, dilution 1:70; Novocastra, Leica Biosystems), and Ki67 (clone MIB-1, dilution 1:70; Dako) were performed using a Bond Max autostainer (Leica Microsystems, Wetzlar, Germany), as previously described in detail [28–32].

Interpretation of the IHC Results

The evaluation of all IHC sections was done by two experienced breast cancer pathologists, blinded as to the patients' clinical characteristics and survival data, according to existing established criteria, as previously described [23]. Briefly, HER2 protein expression was scored in a scale from 0 to 3+, the latter corresponding to uniform, intense membrane staining in >30% invasive tumor cells [33]; ER and PgR were considered positive if staining was present in ≥1% of tumor cell nuclei [34]; and for Ki67, the expression was defined as low (<20%) or high (≥20%) based on the percentage of stained/unstained nuclei from the tumor areas [35]. If one of the tissue cores was lost or damaged, the overall score was determined

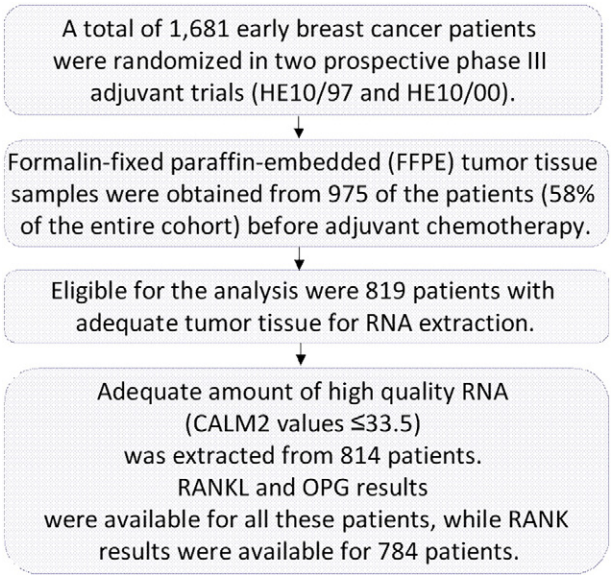

Figure 1. Consort diagram.

from the remaining one. When whole tissue sections were used, the entire tumor area was evaluated.

Fluorescence In Situ Hybridization (FISH)

TMA sections or whole tissue sections (5 μm thick) were used for FISH analysis using the ZytoLight SPEC *HER2*/*TOP2A*/CEP17 triple-color probe (Z-2073; ZytoVision, Bremerhaven, Germany), as previously described [36]. FISH was performed according to the manufacturer's protocol with minor modifications in all cases, not only the HER2 IHC 2+ cases.

Digital images were constructed using specifically developed software for cytogenetics (XCyto-Gen; ALPHELYS, Plaisir, France). Processed sections were considered eligible for FISH evaluation according to the American Society of Clinical Oncology/College of American Pathologists criteria [33]. For the evaluation of the *HER2* gene status, nonoverlapping nuclei from the invasive part of the tumor were randomly selected, according to morphological criteria using DAPI staining, and scored. Twenty tumor nuclei were counted according to Press et al. [37]. The *HER2* gene was considered to be amplified when the *HER2*/CEP17 ratio was >2.2 [33] or the mean *HER2* copy number was >6 [38]. In cases with values at or near the cutoff (1.8-2.2), 20-40 additional nuclei were counted, and the ratio was recalculated. In cases with a borderline ratio, additional FISH assays were performed in whole sections [39]. The data from the evaluation of *TOP2A* gene status were neither analyzed nor presented in the present manuscript.

RNA Isolation and Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR) Assessment

Prior to RNA isolation, macrodissection of tumor areas was performed in most (69%) of the FFPE sections (all sections with <50% tumor cell content). More than one FFPE section (2-8 sections, 10 μm thick) was used for RNA extraction when the tumor surface of a given sample was less than 0.25 cm^2. From each FFPE section or macrodissected tissue fragments, RNA was extracted using a standardized fully automated isolation method for total RNA from FFPE tissue based on germanium-coated magnetic beads (XTRAKT kit; STRATIFYER Molecular Pathology GmbH, Cologne, Germany) in combination with a liquid handling robot (XTRAKT XL; STRATIFYER Molecular Pathology GmbH), as previously described in detail [29,31,32,40,41]. The method involves extraction-integrated deparaffinization and DNase I digestion steps. The quality and quantity of RNA were checked by measuring CALM2 expression as a surrogate for amplifiable mRNA by qRT-PCR. CALM2 was used as endogenous reference because it had previously been identified as being highly and stably expressed among breast cancer tissue samples. Of the 975 FFPE tumor tissue samples collected, 819 (84.0%) had enough material left for RNA isolation needed for the present study.

qRT-PCR primers and labeled hydrolysis probes were selected using Primer Express Software, Versions 2.2 and 3 (Applied Biosystems/Life Technologies, Karlsruhe, Germany), according to the manufacturer's instructions, and were controlled for single nucleotide polymorphisms. All primers, probes, and amplicons were checked for their specificity against nucleotide databases at NCBI using Basic Local Alignment Search Tool. Primers and probes were purchased from Eurogentec S.A. (Seraing, Belgium). For each primer/probe set, the amplification efficiency was tested, aiming to reach comparable efficiency of >90% (efficiency range from 97.7 to 99.7%). Primers and hydrolysis probes were diluted to 100 μM using a stock solution with nuclease-free water (Life Technologies GmbH, Darmstadt, Germany) [31,32,41]. qRT-PCR was applied for the relative quantification of RANK, OPG, and RANKL. The Primer/Probe (YakimaYellow/FAM-labeled) sets used for amplification of the target and reference genes are shown in Table 1.

For PCR, 0.5 μM of each primer and 0.25 μM of each probe were used. All qRT-PCRs were performed in triplicates using the SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen/Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. Experiments were performed on a Stratagene Mx3005p (Agilent Technologies, Waldbronn, Germany) with 30 minutes at 50°C and 2 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. The lengths of the amplicons detected by the RANK, OPG, RANKL, and CALM2 assays were 106 bp, 83 bp, 72 bp and 72 bp, respectively, with PCR efficiencies [$E = 1^{(10\ -\ slope)}$] of 93.5%, 101.6%, 101.7%, and 99.7%, respectively. Samples were considered eligible for further investigation ($N = 814$, Figure 1) when the cycle threshold (CT) values of the housekeeping gene were ≤33.5 (triplicate mean values). Relative expression levels (relative quantification) of the target transcripts were calculated as 40 − DCT values (DCT = mean CT target gene − mean CT housekeeping gene) to yield positively correlated numbers and to facilitate comparisons [31,32,41]. OPG and RANKL results were available for all 814 eligible samples, whereas RANK results were available for 784 patients because of inadequate amount of RNA extract in 30 samples, in which only OPG and RANKL were evaluated. A commercially available human reference RNA (Stratagene qPCR Human Reference Total RNA; Agilent Technologies) was used as positive control. No-template controls were assessed in parallel to exclude contamination.

Statistical Analysis

DFS was defined as the time from study entry to first tumor recurrence, secondary neoplasm, or death from any cause [42]. Patients alive and without recurrence at the date of last contact were censored. OS was also measured from study entry until death from any cause, whereas bone metastasis–free survival was defined from study entry to first bone metastasis. Surviving patients and patients free of bone metastases were censored at the date of last contact.

Table 1. Primer and Probe Sequences Used for qRT-PCR

Gene Symbol	NM_Number	Probe Name	Probe Sequence	Forward Name	Forward Sequence	Reverse Name	Reverse Sequence
RANK	NM_003839	MP660	ACGGTGCTGTAACAAATGTGAACCAGGAAAGTACAT	MP660_For	CCAGTGAGAAGCATTATGAGCATCT	MP660_Rev	GGCAGACATACACTGTCAGAGGTAGT
OPG	NM_002546	MP628	AATGTGGAATAGATGTTACCCTGTGTGAGGAGG	MP628_For	CCGGAAACAGTGAATCAACTCAA	MP628_Rev	TGTAGGAACAGCAAACCTGAAGAA
RANKL	NM_003701	MP630	TCAGAGCGCAGATGGATCCTAATAGA	MP630_For	GCGTCGCCCTGTTCTTCTAT	MP630_Rev	TGCAGTGAGTGCCATCTTCTG
CALM2	NM_001743	MP501	TCGCGTCTCGGAAACCGGTAGC	MP501_For	GAGCGAGCTGAGTGGTTGTG	MP501_Rev	AGTCAGTTGGTCAGCCATGCT

The prognostic value of RANK, OPG, and RANKL mRNA expression was examined in terms of DFS, OS, and bone metastasis–free survival using the 50th percentile (median value) as the optimal cutoff, and if this was not significant, the upper and lower quartiles of the mRNA distribution were to be examined as possible thresholds. Chi-square tests were used for group comparisons of categorical data, whereas Wilcoxon rank-sum tests were performed to detect

Table 2. Selected Patient and Tumor Characteristics

Characteristics	(*N* = 819)
Age	
Mean (SD)	53.2 (11.2)
Median	52.7
Min-max	22-79
Ki67	
Mean (SD)	30.7 (24.2)
Median	25
Min-max	0-98
	***N* (%)**
Age (in years)	
<50	348 (42.5)
≥50	471 (57.5)
Treatment group	
E-CMF	123 (15.0)
E-T-CMF	392 (47.9)
ET-CMF	304 (37.1)
Menopausal status	
Premenopausal	375 (45.8)
Postmenopausal	444 (54.2)
Breast surgery	
Modified radical mastectomy	579 (70.7)
Breast-conserving surgery	240 (29.3)
ER/PgR status	
Negative	165 (21.7)
Positive	596 (78.3)
Histological grade	
I-II	406 (49.6)
III-undifferentiated	413 (50.4)
Tumor size	
≤2 cm	181 (22.8)
2-5 cm	517 (65.2)
>5 cm	95 (12.0)
Positive lymph nodes	
1-3 nodes	331 (40.5)
≥4 nodes	487 (59.5)
Adjuvant radiotherapy	
No	184 (23.2)
Yes	609 (76.8)
Adjuvant hormonal therapy	
No	154 (18.9)
Yes	661 (81.1)
Bone metastases	
No	711 (88.4)
Yes	93 (11.6)
HER2 status	
Negative	593 (76.5)
Positive	182 (23.5)
Subtypes	
Luminal A	245 (32.3)
Luminal B	248 (32.8)
Luminal-HER2	99 (13.1)
HER2-enriched	79 (10.4)
Triple-negative	86 (11.4)

differences in continuous variables. Kaplan-Meier curves and log-rank tests were used to compare time-to-event distributions.

Prognostic significance of RANK, OPG, and RANKL mRNA expression was evaluated by hazard ratios (HRs) estimated with univariate and multivariate Cox proportional hazards models. Cox regression analyses were also performed to estimate the predictive significance of RANK, OPG, and RANKL by interaction tests between RANK, OPG, and RANKL mRNA expression and chemotherapy treatment with paclitaxel (yes versus no), hormonal therapy (yes versus no), and radiation therapy (yes versus no). In multivariate analysis, a backward selection procedure with a removal criterion of $P > .15$ based on the likelihood ratio test was performed to identify significant variables among the following: age (≥50 vs <50), nodal status (≥4 vs 1-3 positive lymph nodes), tumor size (2-5 cm vs ≤2 cm and >5 cm vs ≤2 cm), hormonal therapy (yes versus no), type of operation (breast-conserving surgery versus modified radical mastectomy), subtypes (TNBC versus HER2-enriched, luminal A versus HER2-enriched, luminal B versus HER2-enriched, luminal HER2 versus HER2-enriched), treatment group (E-T-CMF versus E-CMF, ET-CMF versus E-CMF), as well as each of the markers that were found to be significant or revealed a trend in the univariate analysis.

Results of this study were presented according to reporting recommendations for tumor marker prognostic studies [25]. This study is prospective-retrospective as described in Simon et al. [43]. All tests were two-sided at an alpha 5% level of significance. No adjustment for multiple comparisons was performed. Analyses were conducted using the SAS software (version 9.3; SAS Institute Inc., Cary, NC).

Results

Patient Characteristics

Among the 819 patients included in the analysis, mRNA expression data for RANK, OPG, and RANKL were available for 784 (95.7%), 814 (99.9%), and 814 (99.9%) patients, respectively. Basic patient and tumor characteristics are presented in Table 2. Median age at study entry was 52.7 years, although most patients were older than 50 years. The majority of patients were postmenopausal (54.2%) and ER/PgR positive (78.3%).

The distribution of tumor samples based on the normalized expression of mRNA encoding for the three examined markers is presented in Figure 2. The median value of RANK, OPG, and RANKL mRNA expression was 30.7, 33.3, and 32.9, with a range of 9.3, 12.3, and 14.4, respectively.

Association of Markers with Clinicopathological Parameters

No statistically significant associations were found between age and RANK mRNA expression (using the median value as a cutoff) (chi-square, $P = .56$). Similarly, no significant association was observed between age and OPG mRNA expression ($P = .84$), whereas a statistically significant association was demonstrated between patient age and expression of RANKL (using the median value as a cutoff), with patients younger than 50 years presenting higher expression of RANKL mRNA ($P = .016$). Low expression of OPG mRNA (using the median value as a cutoff) was significantly associated with modified radical mastectomy and higher histological grade ($P = .008$ and $P = .001$, respectively). High expression of RANK, OPG, and RANKL mRNA was associated with luminal A subtype ($P = .008$, $P = .002$, and $P = .005$, respectively), whereas low expression of RANK and RANKL was associated with adjuvant hormonal therapy ($P = .015$ and $P < .001$, respectively). In addition, patients who received E-CMF treatment were more likely to have low expression of RANK, OPG, and RANKL ($P < .001$, $P = .025$, and $P < .001$, respectively). Finally, low expression of RANKL was associated with postmenopausal status and greater number of positive lymph nodes ($P = .002$ and $P < .001$, respectively).

RANK/OPG and RANKL/OPG ratios were found to be significantly associated with adjuvant hormonal therapy, with patients receiving hormonal therapy presenting lower RANK/OPG and RANKL/OPG ratios (using the median value as a cutoff) ($P = .005$ and $P = .002$, respectively). Low RANK/OPG ratio was also associated with ER/PgR presence ($P = .005$), whereas low RANKL/OPG ratio was associated with ER positivity and postmenopausal status ($P = .028$ and $P = .015$, respectively). Postmenopausal status was also associated with low RANKL/RANK ratio (using the median value as a cutoff) ($P = .011$). Low RANKL/RANK was found to be associated with age older than 50 years and luminal B subtype ($P = .023$ and $P = .003$, respectively). Low RANKL/OPG ratio (using the median value as a cutoff) was associated with a larger number of

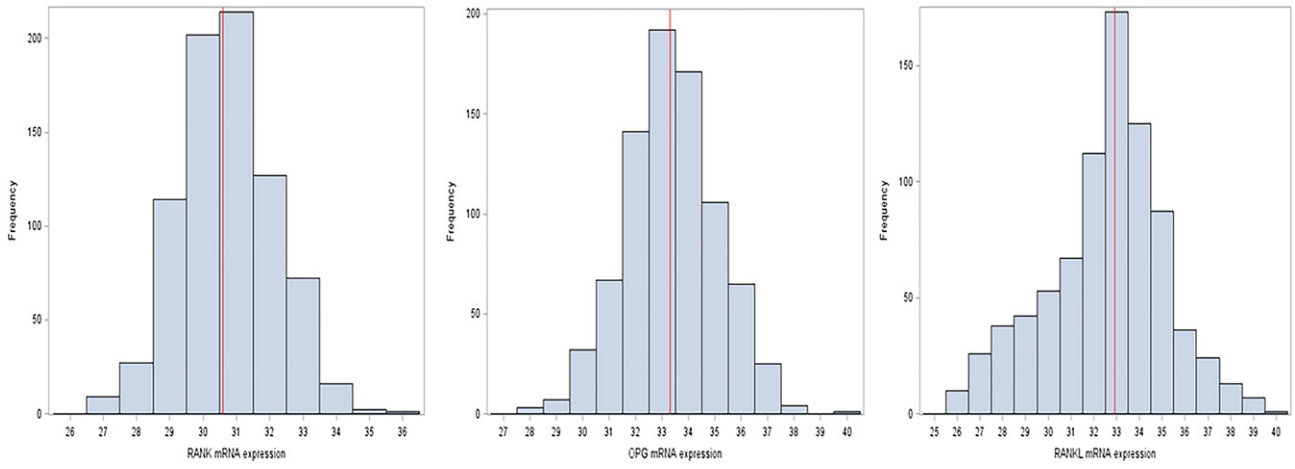

Figure 2. Histograms of RANK, OPG, and RANKL mRNA expression (40 − DCT values). Red line represents the 50th percentile (median).

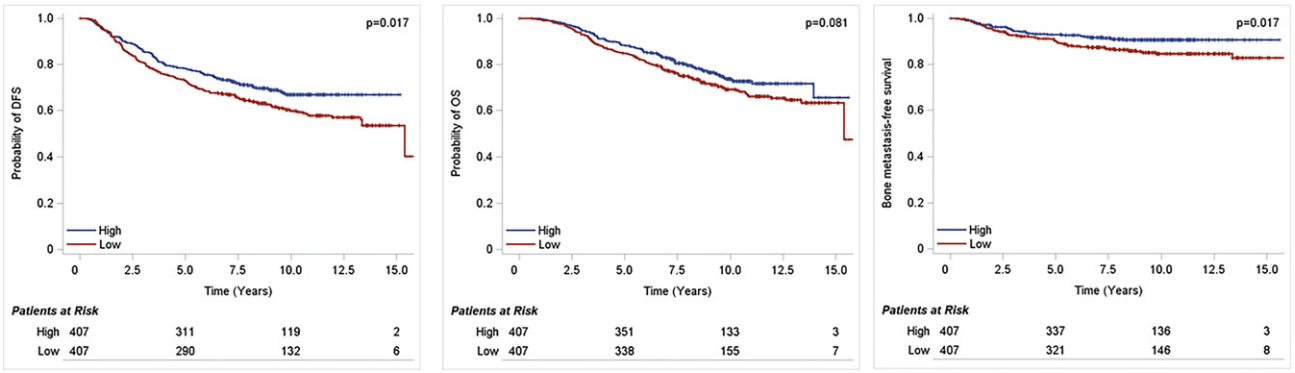

Figure 3. Kaplan-Meier plots for RANKL mRNA expression (using the median as a cutoff point) with regard to DFS, OS, and bone metastasis–free survival.

involved lymph nodes and radiotherapy (P = .015 and P = .010, respectively). There were no other significant associations between the examined markers and the selected clinicopathological parameters.

Association of Markers with Clinical Outcome

Survival status of all patients was updated in June 2014. The median follow-up time was 119.9 months (range, 0.1-191.9). It is of note that, during this period, there were 226 (27.6%) documented deaths, whereas 291 (35.5%) patients had reported disease progression.

In the univariate analysis, with respect to DFS, low RANKL mRNA expression (using the median as a cutoff point) was an unfavorable factor for DFS (HR = 1.33, 95% CI 1.05-1.68, Wald's P = .018), whereas mRNA expression of RANK and OPG was not found to be significant for DFS (P = .53 and P = .46, respectively). None of the examined markers, using all quartile cutoffs, were significantly associated with OS in the univariate analysis. Low RANKL mRNA expression (using the median value as a cutoff) was associated with increased risk for bone metastases (HR = 1.67, 95% CI 1.09-2.56, P = .018) in the univariate analysis, whereas no significant association was observed between the rest of the examined markers and the risk for bone metastases. Kaplan-Meier curves for DFS, OS, and bone metastasis–free survival based on RANKL mRNA expression are presented in Figure 3. RANK/OPG, RANKL/RANK, and RANKL/OPG ratios (using the median value as a cutoff) were not associated with DFS, OS, or bone metastasis–free survival. In the multivariate analysis, RANKL mRNA expression did not retain its prognostic significance for either DFS or bone metastasis–free survival (P = .36 and P = .18, respectively).

RANK, OPG, and RANKL were not found to be predictive for benefit from radiotherapy for DFS (interaction P = .58, P = .71, and P = .22, respectively), OS (interaction P = .98, P = .62, and P = .85, respectively), or bone metastasis–free survival (interaction P = .62, P =.22, and P = .96, respectively). In addition, none of the markers under investigation were found to be predictive for benefit from hormonal therapy for OS, whereas a trend for a significant interaction of RANK with hormonal therapy was observed for DFS (interaction P = .095). More specifically, among patients treated with hormonal therapy, low RANK mRNA expression was found to be associated with increased risk for relapse (HR = 1.32, 95% CI 1.01-1.73, Wald's P = .041), whereas no significant difference was observed among patients that did not receive hormonal therapy (P = .31). A trend was also observed for the interaction of OPG mRNA expression with hormonal therapy for bone metastasis–free survival (P = .097). Low OPG mRNA expression was marginally significantly associated with increased risk for bone metastases in the subgroup of patients that did not receive hormonal therapy (HR = 3.30, 95% CI 0.91-12.1, Wald's P = .070), whereas no significant difference was found among patients treated with hormonotherapy (P = .80). RANK, OPG, and RANKL were not predictive for benefit from the addition of paclitaxel to the E-CMF regimen for DFS (P = .55, P = .18, and P = .72, respectively), OS (P = .25, P = .30, and P =.68, respectively), or bone metastasis–free survival (P = .94, P = .40, and P = .71, respectively).

Discussion

The primary objective of the study was to identify potential value of RANK, OPG, and RANKL as prognostic biomarkers regarding the development of bone metastases, whereas the secondary objective was to test their association with DFS and OS in early breast cancer patients. Our results showed that low RANKL mRNA expression was associated with postmenopausal status and greater number of positive lymph nodes, whereas high expression of RANK, OPG, and RANKL was associated with the luminal A subtype. Also, low RANKL mRNA expression was significantly associated with unfavorable DFS and the development of bone metastases, whereas no such associations were observed for RANK or OPG mRNA expression.

The association of postmenopausal status with low RANKL is in agreement with the results of a large analysis that has shown increased expression of RANKL in younger breast cancer patients [44]. They showed that, when adjusted for tumor size, nodal status, histological grade, and subtype, decreasing age was associated with higher expression of RANKL, mammary stem cells, and luminal progenitors. In an effort to apply these findings to clinical practice, a phase II study (D-BEYOND; EudraCT # 2011-006224-21) is soon to be completed. It includes administration of the anti-RANKL antibody denosumab (two 120-mg injections) in premenopausal patients prior to primary breast surgery to study the effect on different biological processes that guide tumor progression and metastases in younger women [45].

High RANK mRNA expression was previously found to be associated with negative prognostic factors, like tumor size >2 cm, histological grade III, and lack of ERs [17]; in our study, however, no such associations were detected. Interestingly, when the authors divided their population into poor- and good-prognosis groups on the basis of their microarray signature, RANK mRNA expression was

significantly higher in the poor-prognosis group, whereas OPG and RANKL mRNA expression was higher in the good-prognosis group [17]. The latter observation of high RANKL mRNA expression being associated with good prognosis is in agreement with the main finding of our study, in a much larger cohort of early breast cancer patients, showing that low RANKL mRNA expression is significantly associated with unfavorable DFS and the development of bone metastases.

RANKL forms stable trimers, and besides high levels in skeletal and lymphoid tissues, RANKL mRNA can be identified in several sites, such as skin keratinocytes, mammary epithelial cells, heart, skeletal muscles, lung, stomach, placenta, thyroid, and brain [46]. Three distinct splice variants have been described, but their significance remains unclear. It has been shown that, *in vitro*, only RANKL1 can induce osteoclastogenesis, whereas RANKL2 is inactive and RANKL3 inhibits osteoclastogenesis when co-expressed with RANKL1 [47]. Soluble RANKL can be cleaved by specific matrix metalloproteinases, like MMP14 among others. *In vitro* and *in vivo* data in mice show that soluble RANKL, shed by MMP14, inhibits osteoclastogenesis. Mice deficient in MMP14 have decreased production of RANKL by osteoblasts, increased numbers of osteoclasts, and an osteoporosis phenotype [48]. All these are indications that soluble RANKL can counteract the activity of membrane-bound RANKL and inhibit osteoclastogenesis. On the contrary, data from a prostate cancer model show that osteoclasts at the tumor-bone interface enhance MMP7 expression, thus increasing soluble RANKL and further promoting osteoclast activation and osteolysis [49]. It seems, therefore, that soluble RANKL can act as a promoter or inhibitor of osteoclastogenesis, depending on the context.

The fact that RANKL function can work by both promoting and inhibiting osteoclastogenesis suggests that local conditions might play a major role on the final outcome of RANKL activity and that the protein itself might not consistently be used as a biomarker in breast cancer. RANKL mRNA expression might be a more useful prognostic factor with regard to bone metastases and individual patient outcomes. At present, it appears that there might be a role for RANKL as a predictive biomarker for response to bone-targeted treatments, as a recently published study suggested [50]. It showed that baseline RANKL mRNA expression was significantly higher in the responders, as compared to the nonresponders, based on the response evaluation criteria of the MD Anderson Cancer Center and the Positron Emission Tomography Response and Evaluation Criteria, a finding that is in agreement with our results.

OPG is expressed in about 55% of breast cancers and breast cancer cell lines [7]. It has been shown that high OPG mRNA expression is associated with low histological grade and ER positivity [8], which is also in agreement with our results. However, the link between OPG and ERs is not yet clear, as data in cell lines showed an inverse correlation between them, where activation of ER reduces OPG expression [51]. The influence of OPG on breast cancer prognosis remains unclear. Data from 127 breast cancer tumor tissues showed that patients with high expression of OPG had a poor outcome [52] compared with patients with low expression. In a different study of 295 patients, high OPG expression was associated with longer OS and DFS [17]. A possible explanation for this discordance might relate to the tumor subtypes, as the analysis from publicly available microarray data found that the positive association of high OPG expression with better outcome was seen in the ER-positive group but was not confirmed for the ER-negative cases [53]. However, no such association was observed in our study.

In a recent project presented at the 2016 San Antonio Breast Cancer Symposium, an analysis for RANK/RANKL expression was performed in breast tissues of BRCA1 carriers undergoing prophylactic mastectomies. Two luminal progenitors were identified, RANKL+ and RANKL−, in histologically normal breast tissue. RANKL+ cells were highly proliferative, with grossly aberrant DNA repair and with a molecular signature similar to that of the basal-like phenotype. In addition, established BRCA1-related tumors were found to have high levels of RANK [54]. Based on these results, a clinical trial with the prophylactic use of denosumab in BRCA mutation carriers is currently under way.

The RANK/OPG/RANKL system appears to be involved in many intracellular pathways that are associated with survival, proliferation, and formation of metastases. Breast cancer cells are able to modify the function of the system, together with the existing tumor microenvironment, especially tumor-infiltrating lymphocytes (TILs), hence the conflicting results in the literature regarding the association of the RANK/OPG/RANKL system with prognosis and the development of bone metastases. Therefore, there is currently no adequate documentation that any of these factors may be safely used as prognostic or predictive biomarkers, at least until the results of ongoing studies become available. In an effort to shed more light in these issues, our group is in the process of evaluating, in the same cohort, percent of TILs; protein expression of RANK and RANKL; and protein and mRNA expression of T-cell markers, such as CD3, CD8, and forkhead box P3 (FOXP3).

In conclusion, our study showed that low RANKL mRNA expression was significantly associated with unfavorable DFS and the development of bone metastases in early breast cancer patients, a finding that needs to be validated in independent cohorts. Further investigations for the potential prognostic value of RANK, RANKL, and T-cell markers, assessed by immunohistochemistry, are currently under way. More information on the function of the RANK/OPG/RANKL system, in conjunction with number of TILs and the expression of T-cell markers, might allow for further development of prognostic or predictive tools that can guide clinical decision-making and potentially establish the use of anti-RANKL agents or OPG analogs in the treatment of early breast cancer patients at high risk for metastatic spread, provided that our current results are validated in independent cohorts.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2017.05.006.

Acknowledgements

The authors are indebted to all patients and their families for their trust and participation in the HE10/97 and HE10/00 trials and for the provision of biological material for research purposes.

The authors also wish to thank all Hellenic Cooperative Oncology Group personnel (data managers, research assistants, and monitors) for their dedication and especially Maria Moschoni for data coordination.

The study was supported by a research grant from Amgen Ltd. and by an internal Hellenic Cooperative Oncology Group translational research grant (HE TRANS_BR).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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