A Two-Gene Expression Ratio of Homeobox 13 and Interleukin-17B Receptor for Prediction of Recurrence and Survival in Women Receiving Adjuvant Tamoxifen

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Abstract

Purpose: In the adjuvant treatment of estrogen receptor (ER) – positive breast cancer, additional markers are needed to identify women at high risk for recurrence.

Experimental Design: We examined the association between the ratio of the homeobox 13 (HOXB13) to interleukin-17B receptor (IL-17BR) expression and the clinical outcomes of relapse and survival in women with ER-positive breast cancer enrolled onto a North Central Cancer Treatment Group adjuvant tamoxifen trial (NCCTG 89-30-52).

Results: Tumor blocks were obtained from 211 of 256 eligible patients, and quantitative reverse transcription-PCR profiles for HOXB13 and IL-17BR were obtained from 206 patients. The cut point for the two-gene log 2(expression ratio) that best discriminated clinical outcome (recurrence and survival) was selected and identified women with significantly worse relapse-free survival (RFS), disease-free survival (DFS), and overall survival (OS), independent of standard prognostic markers. The cut point differed as a function of nodal status [node negative (59th percentile) versus node positive (90th percentile)]. In the node-positive cohort (n = 86), the HOXB13/IL-17BR ratio was not associated with relapse or survival. In contrast, in the node-negative cohort (n = 130), a high HOXB13/IL-17BR ratio was associated with significantly worse RFS [hazard ratio (HR), 1.98; P = 0.031], DFS (HR, 2.03; P = 0.015), and OS (HR, 2.4; P = 0.014), independent of standard prognostic markers.

Conclusion: A high HOXB13/IL-17BR expression ratio is associated with increased relapse and death in patients with resected node-negative, ER-positive breast cancer treated with tamoxifen and may identify patients in whom alternative therapies should be studied.

In the adjuvant treatment of estrogen receptor (ER) – positive breast cancer, hormonal therapy reduces the risk of breast cancer recurrence and decreases mortality. Tamoxifen, one of the most commonly used medications in the adjuvant treatment of ER-positive breast cancer, is a selective ER modulator that competes with estrogen for binding to the ER. When administered to women with surgically treated ER-positive breast cancer, tamoxifen reduces the risk of recurrence and death when taken for 5 years (1).

The ER and progesterone receptor are the most important tumor markers that predict response to tamoxifen (2). However, because a significant proportion of ER-positive breast cancers fail to respond or eventually develop resistance to tamoxifen, additional prognostic markers, including tumor size, tumor grade, and nodal status, are commonly used by physicians to make treatment decisions. Clinical studies have shown, however, that even in "good-prognosis" tumors (e.g., estrogen positive, lymph node negative), up to 20% of women will experience recurrence despite 5 years of adjuvant tamoxifen therapy (3, 4). These findings indicate the need for additional markers that will identify women at high risk for recurrence.

Recent studies have shown that the gene expression signature of a tumor is a means to predict recurrence and survival in women with surgically treated breast cancer (5 – 11). Paik et al. selected 16 cancer-related genes from a panel of 250 candidate genes, and based on their performance in three independent studies, developed a score that was predictive of recurrence in women with node-negative, ER-positive breast cancer enrolled

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onto the National Surgical Adjuvant Breast and Bowel Project clinical trial B-14 (9) but not in a separate retrospective study of ER-positive, node-negative women who did not receive adjuvant therapy (12).

Independently, Ma et al. suggested that a two-gene expression ratio derived from a genome-wide, 22,000-gene oligonucleotide microarray analysis of ER-positive, invasive breast cancers from unselected patients treated only with tamoxifen was predictive of relapse and survival (10). The homeobox gene *HOXB13* was overexpressed in patients who experienced disease recurrence, whereas the interleukin-17B receptor (*IL-17BR*) was overexpressed in those with no evidence of recurrence. The expression ratio of HOXB13 over IL-17BR provided the best composite predictor of recurrence in a multivariate analysis.

In an effort to better evaluate the value of the HOXB13/IL-17BR ratio, we assessed the association between the ratio and relapse-free survival (RFS), disease-free survival (DFS), and overall survival (OS) in a carefully followed and validated cohort of postmenopausal women with ER-positive breast cancer treated only with tamoxifen from North Central Cancer Treatment Group (NCCTG) adjuvant tamoxifen trial (NCCTG 89-30-52; ref. 13).

Materials and Methods

Patients. The NCCTG conducted a randomized phase III clinical trial in postmenopausal women with resected ER-positive breast cancer to assess the value of adding 1 year of fluoxymesterone to 5 years of tamoxifen adjuvant therapy (NCCTG 89-30-52; ref. 13). Postmenopausal women with node-negative disease were required to have a stage T_{1c} or $T_2N_0M_0$ and could be any age, whereas women with node-positive disease were required to be at least 65 years of age with a tumor stage T_1-$T_2N_1M_0$. A woman was classified as postmenopausal if one of the following held: (*a*) her last menstrual cycle was >12 months before diagnosis; (*b*) her last menstrual cycle was 4 to 12 months before diagnosis and her follicle-stimulating hormone level in the postmenopausal range; (*c*) she had a bilateral oophorectomy at least 2 months before diagnosis; or (*d*) she had a hysterectomy without oophorectomy and was either >60 years old or her follicle-stimulating hormone was in the postmenopausal range. Patients were surgically treated with either a modified radical mastectomy or breast conservative therapy, including lumpectomy, axillary nodal dissection, and radiation therapy. The axillary dissection must have involved at least levels I and II and the examination of at least six axillary nodes. Patients who underwent lumpectomy must have had a primary tumor no larger than 5 cm, and the surgical margins must have been microscopically free of tumor. Post-lumpectomy radiation therapy consisted of a total cumulative breast dose of 5,040 cGy in 28 fractions, and those with axillary nodal involvement also received radiation to the axilla and supraclavicular regions. Patients were classified as ER positive if $\geq$10 fmol/mg cytosol protein or positive by an immunohistochemical assay. All patients were randomized within 6 weeks of definitive surgery.

A total of 541 patients were randomized to either oral tamoxifen, 20 mg daily for 5 years (256 eligible) or tamoxifen, 20 mg daily for 5 years plus oral fluoxymesterone, 10 mg twice daily for 1 year (258 eligible). Patients were stratified based on axillary lymph node status (0, 1-3, 4-9 versus $\geq$10), age (<65 versus >65 years), primary tumor size (<3 versus >3 cm), ER status (10-49 versus $\geq$50 fmol versus positive by immunohistochemical assay), and extent of surgery (mastectomy versus breast conservation therapy).

Clinical evaluations including history, physical examination, blood and chemistry groups, chest X-ray, and toxicity assessments were done every 4 months for the first year, every 6 months for years 2 to 5, and then yearly. Mammograms and pelvic examinations were done annually. Toxicities were graded using the National Cancer Institute Common Toxicity Criteria version 1.0 and the NCCTG supplement.

Within 30 days of registration, a paraffin-embedded tumor block was submitted to the NCCTG Operations Office for future research purposes. The current study was approved by the institutional review board of Mayo Clinic Rochester and the individual NCCTG sites that enrolled patients onto the clinical trial. The need for additional informed consent was waived by the institutional review boards.

Tissue preparation and RNA amplification. Using the available 227 paraffin-embedded tissue blocks for the women enrolled in the tamoxifen only arm, one H&E slide and two 7-μm sections were cut from each paraffin-embedded block, mounted on PEN membrane glass slides (Microdissect Gmbh, Germany), and stained using the Histogene Staining kit (Arcturus Bioscience, Mountain View, CA). Two breast cancer pathologists independently verified the presence of invasive breast cancer in 211 of the 227 paraffin blocks and provided Nottingham tumor grade for both invasive ductal and lobular carcinomas. In the case of assigning a score for tubule formation, the pathologist automatically assigned lobular carcinomas a score of 3 (<10% tubule formation). Tumor regions were isolated using laser cutting and subsequently captured by using the Arcturus Veritas Laser Microdissection System (Arcturus Bioscience) and immediately placed in proteinase K buffer (Paradise Reagent System, Arcturus Bioscience). The proteinase K lysates were incubated for 16 hours at 50°C, and total RNA was purified and subjected to two rounds of linear amplification as described by manufacturer (Paradise Reagent System) to obtain amplified RNA.

Real-time PCR. Taqman primers and probes were designed using Primer Express (Applied Biosystems, Foster City, CA). The primer sequences for HOXB13 were 5′-GCCATGATCGTTAGCCTCATATT-3′ (forward primer) and 5′-CAATTCATGAAAGCGGTTTCTAAAG-3′ (reverse primer) with a minor groove binder (MGB) probe sequence VIC-TCTATCTAGAGCTCTGTAGAGC-MGB; the primer sequences for IL-17BR were 5′-GGCTTCCTATCCCACCAATT-3′ and 5′-AGGCTGTTTG-TAGGCTGCA-3′ with an MGB probe sequence VIC-CAGGGAAAAAA-CGTGTGATG-MGB. An aliquot (200-500 ng) of the amplified RNA from each tumor sample was converted into cDNA via reverse transcription using the Paradise Reagent System. Taqman assays using 1/30th of the reverse transcribed material were done in duplicate in 20 μL in a 384-well plate using the ABI 7900HT instrument (Applied Biosystems). The samples were heated to 50°C for 2 minutes, 95°C for 10 minutes, followed by then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. For each gene, a standard curve with cDNA dilutions derived from amplified human universal total RNA (Stratagene, La Jolla, CA) was constructed to obtain relative expression levels (i.e., quantities) of HOXB13 and IL-17BR. The HOXB13/IL-17BR ratio was obtained as the difference of log 2–transformed quantities of HOXB13 and IL-17BR. No control genes were measured as the direct ratio calculation does not require a normalization factor. To control for plate-to-plate variation of PCR reaction, standard curves were run on each plate.

Study design and end points. The primary objective of this study was to examine the relationship between the HOXB13/IL-17BR expression ratio and clinical outcomes of RFS, DFS, and OS. RFS was defined as the time from randomization to documentation of the first of the following events: any recurrence (local, regional, or distant) of breast cancer, a contralateral breast cancer, or death. When estimating the distribution of RFS, patients who developed a non-breast second primary cancer (other than squamous or basal cell carcinoma of the skin, carcinoma *in situ* of the cervix, or lobular carcinoma *in situ* of the breast) before the diagnosis of a breast event were censored on the day their second primary was diagnosed. Patients who were alive without a breast recurrence, contralateral breast cancer, or a second non-breast primary cancer were censored at the date of their last disease evaluation. DFS was defined as the time from randomization to documentation of the first of the following events: any recurrence (local, regional, or distant)

of breast cancer, a contralateral breast cancer, a second primary cancer, or death due to any cause. Patients who were alive without any of these events were censored at the date of their last disease evaluation. OS was estimated as the time from registration to death due to any cause.

To assess whether clinical outcome differed with respect to the HOXB13/IL-17BR expression ratio, a minimum *P* approach was used to identify a cut point for the HOXB13/IL-17BR expression ratio that best discriminates between those patients with a poor clinical outcome and those patients with a better clinical outcome. For each clinical outcome, this "optimal" cut point was sought from among the observed values of the expression ratio above the 10th percentile and below the 90th percentile of the expression ratio distribution. To account for multiple testing a correction to the *P* value associated with the optimal cut point was employed as proposed by Lausen and Schumacher (14) and modified by Altman et al. (15). The resulting *P* value, denoted as P_{cor}, and uncorrected log-rank *P* value are reported. Because inclusion of a biomarker dichotomized at its optimal cut point in a Cox regression analysis may inflate the effect (14–18), the 2-fold cross-validation approach of Faraggi and Simon was used to establish whether different HOXB13/IL-17BR expression ratio risk groups exist and obtain an interval estimate of the hazard of each clinical outcome for those with a high HOXB13/IL-17BR expression ratio relative to those with a low HOXB13/IL-17BR expression ratio (18). This cross-validation approach was repeated 100 times, and the median value of the resulting hazard ratios (HR) for the HOXB13/IL-17BR expression ratio and its corresponding risk limits is reported. Faraggi and Simon recommend that the cut point associated with the smallest log-rank *P* value in the entire data set be selected for future use. As such, the variable estimates of the multivariate Cox models associated with this optimal cut point are presented.

Log-rank tests and univariate Cox proportional hazard models were used to assess whether the distributions of RFS, DFS, or OS differed with respect to any one of the following factors: age ≥65 years (yes versus no), extent of surgery (mastectomy versus breast conserving), estrogen receptor status (10-49 versus ≥50 fmol versus positive by immunohistochemistry), number of positive nodes (represented as three indicator variables for 1-3, 4-9, and ≥10 positive nodes), tumor size ≥3 cm (yes versus no), Nottingham grade (3 versus 1 or 2), HER2 expression (3+ versus 0, 1+, or 2+), and prior exposure to exogenous estrogens (yes versus no). For each clinical outcome, multivariate Cox proportional hazard modeling was done to obtain a subset of the potential prognostic factors, which provided an adequate fit to the data. Residual plots were examined. The likelihood ratio test was then applied to assess whether HOXB13/IL-17BR expression ratio dichotomized at its optimal cut point made a significant contribution to the model. We then used the cross-validation approach (18) to assess the effect of expression ratio on RFS, DFS, or OS after known prognostic factors have been accounted for. Finally, the prognostic value of the two-gene expression ratio was assessed in the node-negative and node-positive breast disease cohorts separately, using the same analysis approach.

Table 1. Preregistration characteristics of the patients randomized to the tamoxifen arm that did and did not have expression ratio data

	Women with expression ratio data (*n* = 206)	Women without expression ratio data (*n* = 50)
Race		
Caucasian	92%	91%
Age		
Median (range)	68 (42-84)	68 (48-87)
Operative procedure		
Mastectomy	83%	74%
Breast conservation	17%	26%
No. positive nodes		
0	63%	62%
1-3	26%	15%
4-9	7%	15%
≥10	4%	6%
Tumor size (cm)		
<3	76%	90%
≥3	24%	10%
ER status		
10-49 fmol	20%	20%
≥50 fmol	69%	56%
Positive	11%	24%
HER2		
0	11%	Not determined
1	36%	
2	34%	
3	18%	
Unknown	<1%	
Histology		
Ductal	86%	Not determined
Lobular	10%	
Other	4%	
Nottingham tumor grade		
Grade 1	26%	Not determined
Grade 2	55%	
Grade 3	18%	
Unknown	<1%	

Results

Characteristics of the patients. Of the 256 eligible women enrolled to the tamoxifen only arm, 211 paraffin-embedded tumor blocks were available for RNA extraction. The relative expression levels of HOXB13 and IL-17BR were obtained for 206 of these 211 patients. Table 1 presents the preregistration characteristics for patients with and without gene expression data. The overall patient characteristics were similar, although a higher percentage of patients with HOXB13/IL-17BR expression ratio data had a tumor size of >3 cm (24%) compared with the group without HOXB13/IL-17BR expression ratio data (10%).

For the group of patients with gene expression data available, the first documented event was as follows: local, regional, or distant breast recurrence (39 patients); contralateral breast cancer (12 patients); a second non-breast primary cancer (13 patients); both a breast recurrence and a second non-breast primary cancer (1 patient); and death without a breast recurrence or second primary cancer (37 patients). At last follow-up, 104 women are alive without evidence of a breast event or second primary, 25 are alive following a breast event or second primary cancer, 29 died with disease recurrence, 8 died having developed a second primary cancer, 29 died of other causes, and 8 died of unknown causes. The Kaplan-Meier estimates for the 5-year RFS, DFS, and OS were as follows: 75.6% [95% confidence interval, 69.1-80.9%], 74.3% (67.7-79.7%), and 78.2% (71.9-83.3%). The median length of follow-up among the 129 patients still alive is 11.0 years (range, 5.7-13.6 years).

HOXB13/IL-17BR expression ratio cut point. The cut point for the log (HOXB13/IL-17BR expression ratio) that best

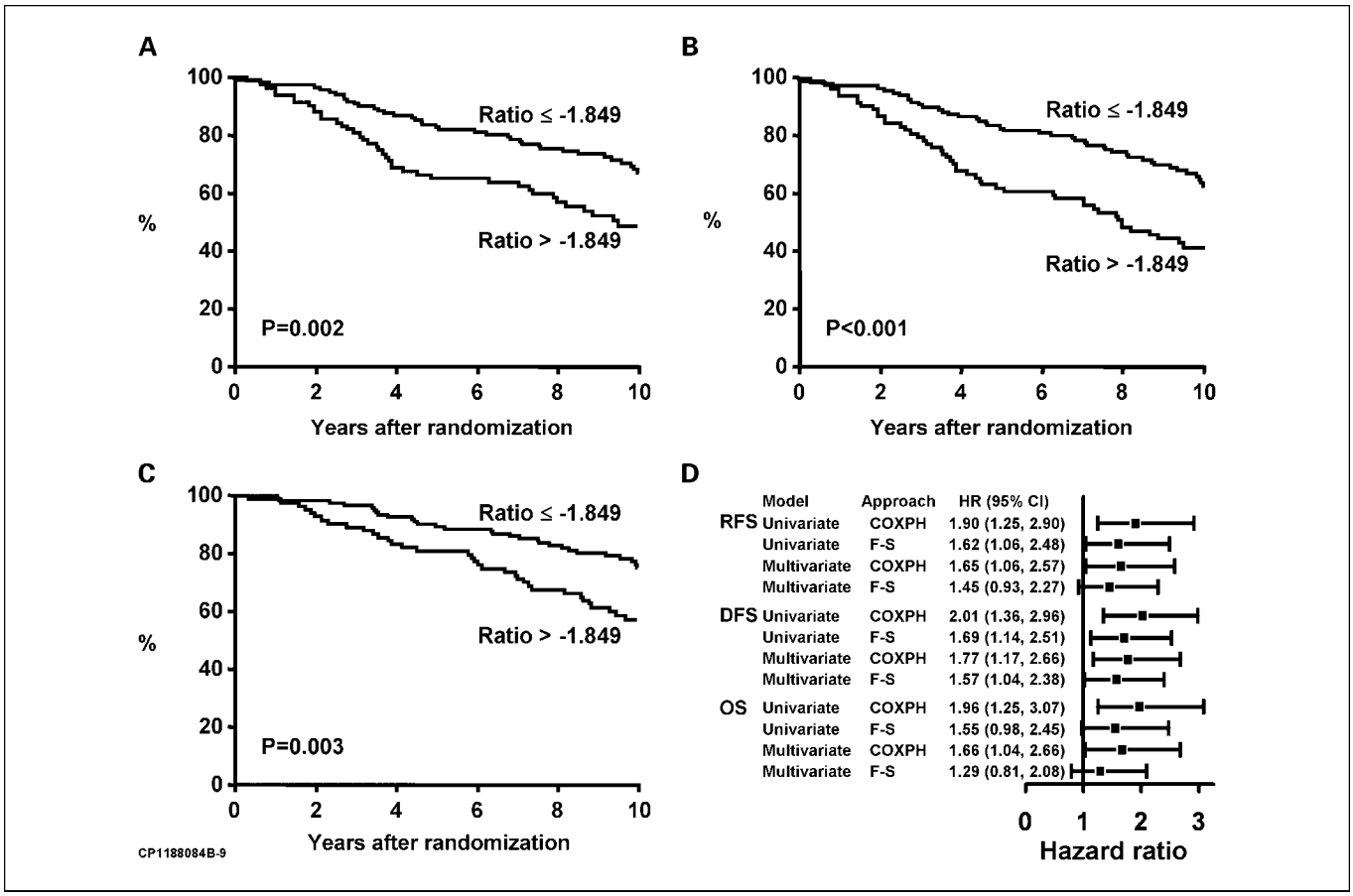

Fig. 1. Kaplan-Meier estimates of RFS (*A*), DFS (*B*), and OS (*C*) in all patients by HOXB13/IL-17-BR (› −1.849 versus ‹ −1.849). *D*, HR for RFS, DFS, and OS in all patients by univariate and multivariate Cox proportional hazards modeling and univariate and multivariate Faraggi-Simon cross-validation using HOXB13/IL-17BR (› −1.849 versus ‹ −1.849).

discriminated clinical outcome (recurrence and survival) fell at the 58th percentile of the observed HOXB13/IL-17BR expression ratio distribution (−1.849). This cutoff provided a classification that divided the women into two groups with significantly different DFS (P_{unc} < 0.001), RFS (P_{unc} = 0.002), and OS (P_{unc} = 0.001). The Kaplan-Meier curves for RFS, DFS, and OS using the cut point of −1.849 are shown in Fig. 1A-C respectively. After applying the Altman method to correct for multiple testing, RFS (P_{cor} = 0.044), DFS (P_{cor} < 0.001), and OS (P_{cor} = 0.025) still differed with respect to HOXB13/IL-17BR expression ratio cut point of −1.849. When using the Faraggi and Simon cross-validation method in the univariate Cox model, RFS (median HR_{FS}, 1.62; 95% CI, 1.06-2.48; P_{FS} = 0.027), DFS (median HR_{FS}, 1.69; 95% CI, 1.14-2.51; P_{FS} = 0.009), but not OS (median HR_{FS}, 1.55; 95% CI, 0.98-2.45; P_{FS} = 0.060) differed with respect to HOXB13/IL-17BR expression ratio (less than or greater than −1.849).

Assessing the added value of HOXB13/IL-17BR expression ratio. For each end point (RFS, DFS, and OS), Cox proportional hazard modeling was done using traditional patient and tumor prognostic factors. Nodal status (positive versus negative), tumor size (≥3 versus <3 cm), and Nottingham grade (3 versus 0 or 1) were significantly associated with each of these end points. When adjusting for these factors, women with a HOXB13/IL-17BR expression ratio greater than −1.849 disease had significantly worse RFS (HR, 1.63; 95% CI, 1.05-2.53; *P* = 0.030), DFS (HR, 1.75; 95% CI, 1.16-2.63; *P* = 0.008), and OS (HR, 1.63; 95% CI, 1.02-2.60; *P* = 0.041), independent of tumor size, nodal status, and tumor grade, than women with a HOXB13/IL-17BR ratio less than −1.849 (Table 2).

We then applied the Faraggi and Simon cross-validation method in the multivariate analysis and found that although women with a high HOXB13/IL-17BR expression ratio disease had significantly worse DFS (median HR, 1.57; 95% CI, 1.04-2.38; *P* = 0.03) compared with a low HOXB13/IL-17BR expression ratio, there were no significant differences with respect to RFS (median HR, 1.45; 95% CI, 0.93-2.27; *P* = 0.100) or survival (median HR, 1.29; 95% CI, 0.81-2.08; *P* = 0.284) when tumor size, nodal status, and tumor grade were accounted for in this model. Figure 1D shows a forest plot for each of the statistical analyses showing the HR and corresponding 95% CIs for RFS, DFS, and OS using the HOXB13/IL-17 cut point of −1.849.

HOXB13/IL-17BR expression ratio and nodal status. We assessed the distribution of the HOXB13/IL-17BR ratio by nodal status and found that the median ratio was similar when comparing node-positive patients (median, −3.81; range, −10.15 to 9.37) with node-negative patients (median, −2.73; range, −11.04 to 7.79), indicating that the assay performed well for both populations. Because the *HOXB13* gene was previously determined to affect cell migration and invasion (10), we

Table 2. Results of Cox modeling of RFS, DFS, and OS: HR (95% CI)

Factor	Clinical outcome		
	RFS	DFS	OS
Entire patient cohort			
Positive nodes	2.31 (1.50-3.54)	2.22 (1.49-3.31)	2.41 (1.54-3.79)
Tumor size	1.93 (1.23-3.03)	1.98 (1.31-3.00)	2.01 (1.26-3.21)
Tumor grade	1.88 (1.13-3.14)	1.69 (1.04-2.75)	1.88 (1.11-3.18)
HOXB13/IL-17BR expression ratio	1.63 (1.05-2.53)	1.75 (1.16-2.63)	1.63 (1.02-2.60)
Node-negative patient cohort			
Tumor size	1.83 (0.92-3.65)	2.38 (1.30-4.36)	2.48 (1.22-5.06)
HOXB13/IL-17BR expression ratio	1.98 (1.07-3.68)	2.03 (1.15-3.59)	2.40 (1.19-4.84)
Tumor grade	1.40 (0.67-2.89)	1.14 (0.57-2.27)	1.54 (0.71-3.37)

hypothesized that the cut point that best discriminated clinical outcome would differ as a function of nodal status. Therefore, we separately determined the optimal cut point in the node-negative (n = 130) and node-positive (n = 96) cohorts.

Node-negative disease. Among the 130 patients diagnosed with node-negative disease, the optimal cut point fell at the 59th percentile of the expression ratio distribution (−1.339). This cutoff provided a classification that divided the women into two groups with significantly different DFS (P_{unc} = 0.001), RFS (P_{unc} = 0.007), and OS (P_{unc} < 0.001). The Kaplan-Meier curves are shown in Fig. 2A-C and show that node-negative patients with a HOXB13/IL-17BR ratio greater than −1.339 have significantly worse RFS, DFS, and OS compared with patients with a ratio less than −1.339. After applying the Altman method to correct for multiple testing, we found that DFS (P_{cor} = 0.025), OS (P_{cor} = 0.003), but not RFS (P = 0.282) differed with respect to HOXB13/IL-17BR expression ratio cut point of −1.339. However, when the Faraggi and Simon

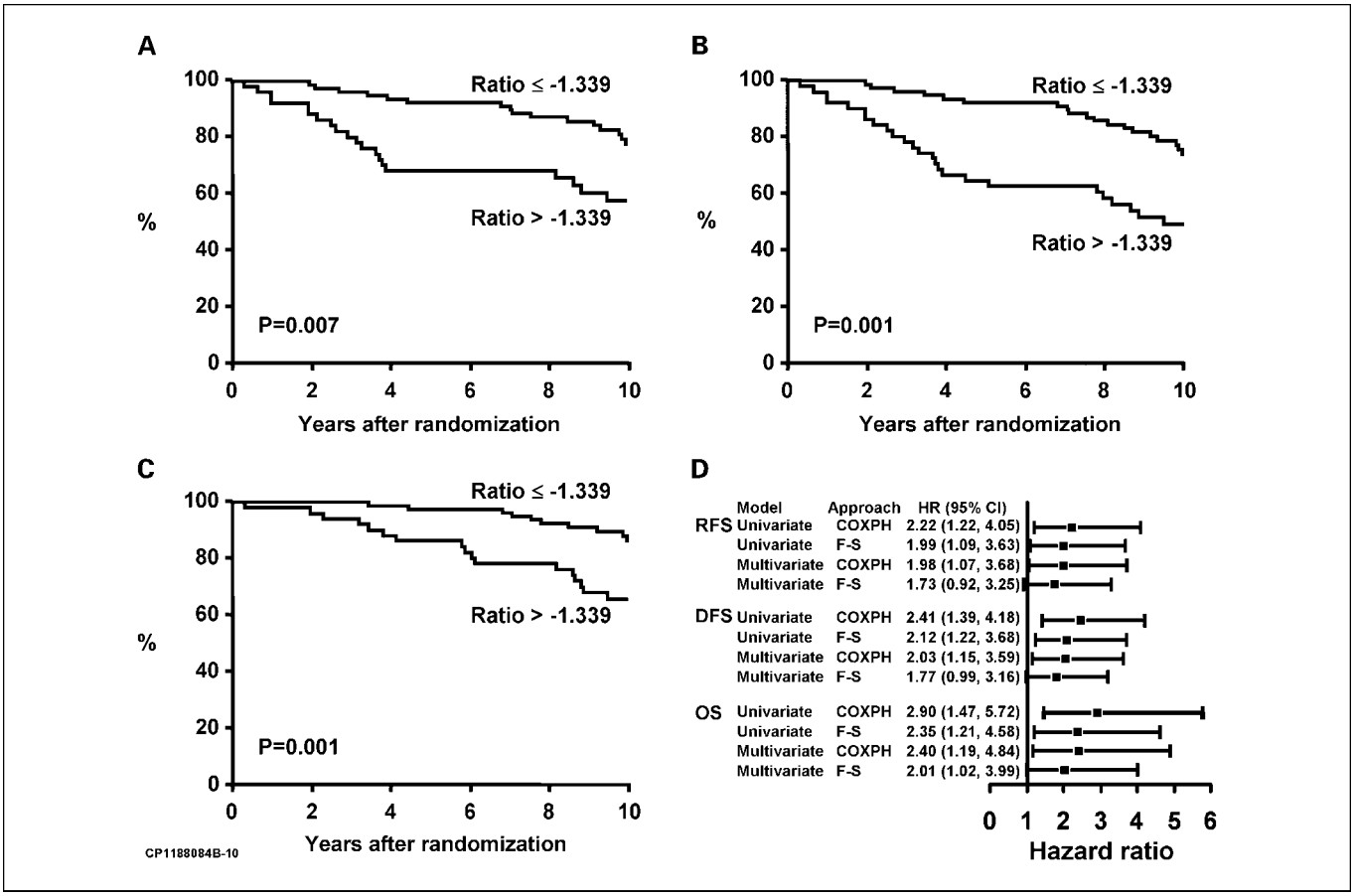

Fig. 2. Kaplan-Meier estimates of RFS (*A*), DFS (*B*), and OS (*C*) in node-negative patients by HOXB13/IL-17-BR (› −1.339 versus ‹ −1.339). *D*, HR for RFS, DFS, and OS in node-negative patients by univariate and multivariate Cox proportional hazards modeling and univariate and multivariate Faraggi-Simon cross-validation using HOXB13/IL-17BR (› −1.339 versus ‹ −1.339).

cross-validation method was applied using a univariate Cox model, we found that that all three end points, including RFS (median HR_{FS}, 1.99; 95% CI, 1.09-3.63; $P_{FS} = 0.025$), DFS (median HR_{FS}, 2.12; 95% CI, 1.22-3.68; $P_{FS} = 0.008$), and OS (median HR_{FS}, 2.35; 95% CI, 1.21-4.58; $P_{FS} = 0.012$), still differed with respect to HOXB13/IL-17BR expression.

For each end point, Cox proportional hazard modeling was done using traditional patient and tumor prognostic factors in the node-negative cohort. Univariately, tumor size of ≥3 cm was associated with decreased RFS, DFS, and OS; Nottingham grade 3 was associated with decreased OS and a tendency towards decreased RFS. For each of the clinical outcomes (RFS, DFS, and OS), multivariate Cox's modeling found that once tumor size was accounted for, none of the other traditional patient or tumor prognostic factors under investigation (including Nottingham grade) made a significant contribution to explaining the variability in these clinical outcomes.

We then used the HOXB13/IL-17BR expression ratio of −1.339 and applied the likelihood ratio test to assess whether the expression ratio was significantly associated with RFS, DFS, or OS. Patients with a HOXB13/IL-17BR expression ratio greater than −1.339 had significantly worse RFS (HR, 1.98; 95% CI, 1.07-3.68; $P = 0.031$), DFS (HR, 2.03; 95% CI, 1.15-3.59; $P = 0.015$), and OS (HR, 2.4; 95% CI, 1.19-4.84; $P = 0.014$), independent of tumor size, compared with patients with a HOXB13/IL-17BR expression ratio less than −1.339. As tumor grade has been found in previous studies to be significantly associated with patient outcome, we further explored Nottingham grade in terms of whether it made a significant contribution to explaining the variability in each clinical outcome, independent of tumor size and HOXB13/IL-17BR expression ratio. Nottingham grade was not found to be significantly associated with RFS (HR, 1.40; 95% CI, 0.67-2.89; $P = 0.369$), DFS (HR, 1.14; 95% CI, 0.57-2.27; $P = 0.707$), or OS (HR, 1.54; 95% CI, 0.71-3.37; $P = 0.276$) when tumor size and HOXB13/IL-17BR expression ratio were accounted for.

Finally, we applied the Faraggi and Simon cross-validation method in the multivariate analysis and found that women with a high HOXB13/IL-17BR expression ratio tended to have worse RFS (median HR, 1.72; 95% CI, 0.92-3.25; $P = 0.088$), DFS (median HR, 1.77; 95% CI, 0.99-3.16; $P = 0.054$), and statistically significantly worse OS (median HR, 2.01; 95% CI, 1.02-3.99; $P = 0.045$), compared with patients with a low expression ratio. Figure 2D shows a forest plot for each of the statistical analyses, showing the HR and corresponding 95% CI for RFS, DFS, and OS using the HOXB13/IL-17BR cut point of −1.339.

Node-positive disease. Among the 96 patients diagnosed with node-positive disease, the optimal cut point was at the far right of the expression ratio distribution [i.e., the 90th percentile (4.4)]. Both the Altman approach and Faraggi and Simon procedure led to the conclusion that there was no evidence to suggest that RFS ($P_{cor} = 0.217$, $P_{FS} = 0.120$), DFS ($P_{cor} = 0.148$, $P_{FS} = 0.069$), or OS ($P_{cor} = 0.148$, $P_{FS} = 0.324$) differs with respect to the HOXB13/IL-17BR expression ratio.

Discussion

By studying a cohort of postmenopausal women with tamoxifen-treated breast cancer, we have shown that the HOXB13/IL-17BR gene expression ratio is associated with breast cancer recurrence and survival, independent of standard clinical and pathologic prognostic markers. Furthermore, we have shown that this marker may be most useful in the node-negative breast cancer patient population. Using statistical cross-validation, we showed that only in the node-negative cohort was a high HOXB13/IL-17BR ratio associated with worse survival in the univariate ($P < 0.0001$), the univariate Cox cross-validation model ($P = 0.012$), multivariate (HR, 2.4; 95% CI, 1.19-4.84; $P = 0.014$), and multivariate cross-validation analysis (HR, 2.01; 95% CI, 1.02-3.99; $P = 0.045$).

Our finding that a high HOXB13/IL-17BR expression ratio is associated with a greater risk of relapse and death in node-negative ER-positive breast cancer but not node-positive breast cancer suggests that this biomarker may be a marker of early invasion and metastatic potential. This notion is further supported by the fact that the recurrences seen in patients with a high HOXB13/IL-17BR ratio occurred quickly, within the first 4 years of beginning tamoxifen followed by a plateau until year 8, at which time further relapses were seen in both arms (Fig. 2A). In women with negative lymph nodes and a HOXB13/IL-17BR ratio of less than −1.339, there were no events (recurrence or death) within the first 2 years after randomization. Therefore, the two gene expression ratio may identify the biological underpinnings for the early peak in the hazard rate for relapse, typically seen 18 to 24 months following initiation of hormonal therapy (19).

The HOX families of genes are known regulators of morphogenesis and cell differentiation during embryogenesis; however, multiple studies have also implicated the HOX gene family in tumor invasion and metastases (reviewed in ref. 20). Ma et al. showed that HOXB13 expression was frequently up-regulated in breast cancer cells relative to normal breast epithelial cells (10), and that ectopic expression of HOXB13 in MCF10A cells (a nontransformed human mammary epithelial cell line) in the presence of epidermal growth factor exhibited increased cell migration and invasion (10). Recent studies have also shown overexpression of HOXB13 in endometrial carcinoma and cell lines (21) as well as in melanoma, wherein the expression levels of four different HOX genes (including *HOXB13*) were significantly higher in melanomas with distant metastases as opposed to melanomas without distant metastases (22).

IL-17 is a proinflammatory cytokine with a total of six family members (A-F), which induce the expression of mediators of inflammation (reviewed in ref. 23). IL-17 has been implicated in a variety of inflammatory mediated diseases, such as rheumatoid arthritis (24) and organ transplant rejection (25). Although the IL-17 family members and their receptors have not been implicated in the pathogenesis or outcome of breast cancer, IL-17 has been shown to inhibit the growth of hematopoietic tumors in mice through the increased generation of specific cytolytic T lymphocytes (26). This finding is notable given multiple previous studies that have found that the presence of lymphocytic tumor infiltration was associated with improved clinical outcomes in breast cancer patients (27–30). An additional role of IL-17B in tamoxifen recurrence may involve cross-talk between IL-17B and the extracellular signal-regulated kinase pathway. You et al. showed IL-17B expression in mouse embryonic limb buds, and down-regulation of IL-17B by fibroblast growth factor via the extracellular signal-regulated kinase pathway (31). Therefore, IL-17

BR may play a role in tamoxifen recurrence either through the induction of antitumor immunity, or in mediating the response to growth factors involved in breast epithelial tumor proliferation. This latter hypothesis is supported by studies that suggest that tamoxifen may interact with membrane ER, leading to growth factor receptor tyrosine kinase activation (e.g., epidermal growth factor receptor and HER-2; refs. 32, 33).

The demonstration that the two-gene assay is associated with poorer outcomes in node-negative ER-positive breast cancer but not node-positive breast cancer illustrates the complexity of performing biomarker studies in patients with breast cancer. Multiple gene expression profiling studies have been published, which correlate a specific profile with breast cancer outcomes; however, some of these profiles were derived from patients treated with multiple different therapies (e.g., either chemotherapy alone or with/without hormonal therapy) for varying stages (I-III) of premenopausal and postmenopausal estrogen-positive and estrogen-negative breast cancer (7–9). In contrast, the two-gene profile was discovered and tested in patients with ER-positive breast cancer treated with tamoxifen monotherapy. This point is important, because for ER-positive breast cancers, it is less likely that clinicians will use gene expression profiling to exclude patients from hormonal therapy, given that hormonal therapy not only reduces the risk of distant recurrence but also prevents the development of contralateral breast cancer (34). However, in the case of node-negative, ER-positive breast cancer, the two-gene biomarker may identify a high-risk group of patients for which upfront aromatase inhibitors and/or chemotherapy may prevent some of the immediate recurrences seen within the first 5 years with tamoxifen monotherapy. Although 5 years of tamoxifen remains the standard of care for the adjuvant treatment of premenopausal breast cancer, for postmenopausal women, the role of tamoxifen priming before the use of aromatase inhibitors is still being resolved. Further study of the effect of the HOXB13/IL-17BR ratio in these clinical settings is indicated.

It is important to note that the "cut point" generated and studied is different than that developed by Ma et al. (10) because of two reasons. First, new primers were designed for both HOXB13 and IL-17BR to improve PCR efficiency and precision using formalin-fixed, paraffin-embedded samples that had been collected from 1989 to 1994. Second, in contrast to the PCR values of HOXB13 and IL-17BR originally generated by Ma et al. which were z-transformed before taking the ratio and which required input normalization, the current data (derived from the NCCTG paraffin tumor blocks) were obtained without normalization genes and z-transform was not done. The resultant calculated ratio of HOXB13 over IL-17BR is therefore on a different scale than the ratio obtained by Ma et al. (10). Based on these differences in assay methodology and data analyses, a new cut point needed to be established and "cross-validated" using the statistical methods that were chosen.

Recently, Reid et al. published their analysis of the effect of the HOXB13/IL-17BR ratio on a retrospective cohort of 58 patients treated with tamoxifen for 5 years (35). This retrospective cohort was comprised of predominantly node-positive patients (77%). Reid et al. showed that the HOXB13/IL-17BR ratio was not statistically associated with relapse or survival. In contrast, the results from our study were generated from a prospective cooperative group trial, in which >250 women were randomized to 5 years of tamoxifen and in which the median follow-up was >10 years. Like Reid et al., we found no statistically significant association between the HOXB13/IL-17BR ratio and disease outcome in the 96 patients that were node positive; however, we showed that the HOXB13/IL-17BR ratio was associated with relapse and survival in node-negative patients. Our findings are supported by data recently presented by Erlander et al., wherein the HOXB13/IL-17BR ratio was associated with relapse and death in node-negative but not node-positive, ER-positive patients derived from a large cohort (n = 852) of both untreated and tamoxifen-treated patients from the Baylor College of Medicine (36). We believe that the findings of Reid et al. illustrate the potential problem with using a small retrospective cohort of patients for the validation of a biomarker wherein patient populations are often biased toward patients with available tumor specimens and specimen availability may be related to tumor size and patient outcome (37).

In summary, we have shown that the HOXB13/IL-17BR gene expression ratio is associated with relapse and survival in node-negative but not node-positive breast cancer. Further studies are needed in untreated breast cancer patients to determine whether the two-gene expression ratio represents a prognostic marker, and whether alternative hormonal therapy (aromatase inhibitors) or chemotherapy will improve the outcomes of women identified to be at high risk by means of the HOXB13/IL-17BR ratio.

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