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Prognostic utility of HOXB13:IL17BR and Molecular Grade Index in early stage breast cancer patients from the Stockholm trial

Running title: Prognostic Utility of the Breast Cancer Index

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Abstract

Background A dichotomous index combining two gene expression assays, HOXB13:IL17BR (H:I) and molecular grade index (MGI), was developed to assess risk of recurrence in breast cancer patients. The study objective was to demonstrate the prognostic utility of the combined index in early stage breast cancer.

Methods In a blinded retrospective analysis of 588 ER-positive tamoxifen-treated and untreated breast cancer patients from the randomized prospective Stockholm trial, H:I and MGI were measured using real-time RT-PCR. Association with patient outcome was evaluated by Kaplan-Meier analysis and Cox proportional hazard regression. A continuous risk index was developed using Cox modeling.

Results The dichotomous H:I+MGI was significantly associated with distant recurrence and breast cancer death. The >50% of tamoxifen-treated patients categorized as low-risk had < 3% 10-year distant recurrence risk. A continuous risk model (Breast Cancer Index (BCI)) was developed with the tamoxifen-treated group and the prognostic performance tested in the untreated group was 53% of patients categorized as low-risk with an 8.3% 10-year distant recurrence risk.

Conclusion Retrospective analysis of this randomized, prospective trial cohort validated the prognostic utility of H:I+MGI and was used to develop and test a continuous risk model that enables prediction of distant recurrence risk at the patient level.

Keywords: Breast Cancer Index, recurrence, risk assessment, gene expression profiling, prognosis

Introduction

Conventional criteria used for risk prediction of breast cancer patients include clinicopathological characteristics such as lymph node involvement, tumor size, histological tumor grade and hormone receptor status (Carter *et al*, 1989; Galea *et al*, 1992; Press *et al*, 1997). However, even with utilization of these prognostic and treatment predictive factors under- and overtreatment can occur (van't Veer & Bernards, 2008). Using traditional risk classifications, a large proportion of patients are classified as intermediate risk, which is uninformative for choosing optimal treatment strategies for the individual patients. Currently, the selection criteria for using or withholding chemotherapy are coarsely defined; however, this selection is of major importance in order to avoid unnecessary toxic side effects associated with therapy (Goldhirsch *et al*, 2009). Adjuvant treatment decisions based on clinicopathological characteristics as recommended by guidelines, such as the St Gallen breast cancer consensus guidelines and the Adjuvant! Online tool, can be used for assessing risks and benefits associated with adjuvant therapy (Ravdin *et al*, 2001; Goldhirsch *et al*, 2006; Goldhirsch *et al*, 2009).

With the development of prognostic and predictive gene expression signatures, clinicians can be provided with information beyond the traditional criteria to guide treatment selection. Adjunctive to other standard risk factors, such as tumor size, tumor grade, hormone receptor status and nodal status, integration of gene expression signatures in the clinic provides the potential to more accurately identify low and high risk patients for better informed treatment decision making. Despite a lack of overlapping genes in different predictive gene signatures, the risk

classification of different tumors can be equivalent between different signatures (Desmedt & Sotiriou, 2006; Fan *et al*, 2006), whereas it is evident that using conventional criteria, as compared to these novel risk stratification tools, a significant proportion of the patients are misclassified (van de Vijver *et al*, 2002; Goldstein *et al*, 2008).

In a previous study, it was demonstrated that the combination of *HOXB13:IL17BR* (H:I) and molecular grade index (MGI), two independent prognostic markers, outperformed either index alone in predicting risk of recurrence in breast cancer patients (Ma *et al*, 2004; Ma *et al*, 2008). H:I is an independent prognostic factor for patients with estrogen receptor (ER) positive and node negative disease that has also been shown to be a negative predictive factor of tamoxifen benefit (Ma *et al*, 2004; Goetz *et al*, 2006; Ma *et al*, 2006; Jerevall *et al*, 2008). HOXB13 separately can also identify patients with limited benefit of endocrine treatment. This has been shown for gene expression as well as protein levels (Jerevall *et al*, 2008; Jerevall *et al*, 2010). MGI is a gene expression assay, comprised of five genes related to histological grade and tumor progression, which recapitulates tumor grade and can predict clinical outcome with high performance (Ma *et al*, 2008). With the combinatorial approach of H:I+MGI, it was shown that breast cancer patients could be stratified into three risk groups with better risk prediction of distant metastasis in ER positive, lymph node negative patients.

Herein, we have further validated the prognostic utility of the dichotomous H:I+MGI index retrospectively in a large patient cohort from the prospective randomized Stockholm trial of low risk, ER positive, node negative, tamoxifen-treated or untreated

patients (Rutqvist & Johansson, 2007). In addition, in order to facilitate individualized risk assessment in the clinical setting, a continuous predictor based on the H:I and MGI referred to as the Breast Cancer Index (BCI) was developed and tested. Within this cohort from the Stockholm trial, BCI is shown to predict risk of recurrence at the individual level.

Patients and methods

Patients and tumor samples

The Stockholm Breast Cancer Study Group conducted a randomized tamoxifen trial during 1976 through 1990 within a total of 2 738 postmenopausal women with invasive early stage disease (Rutqvist & Johansson, 2007). The trial included a lymph node negative low-risk group comprising 1 780 patients with tumors ≤ 30 mm in diameter, randomized to 2 years of adjuvant tamoxifen (40 mg daily) versus control. The control patients were systemically untreated and did not receive any chemotherapy. In 1983, a new trial was initiated in which recurrence-free patients, after 2 years of tamoxifen treatment, were randomized to 3 more years of tamoxifen or no further therapy. As a result of the new trial, the patients in the tamoxifen arm were treated either for 2 years or 5 years. In the Stockholm cohort, the benefit from tamoxifen was largely independent of treatment duration (Rutqvist & Johansson, 2007).

For the present study, tumor blocks from 808 patients were received (tamoxifen treated (2-5 years) and untreated). Since tumor grade was not determined during the actual trial, it was determined retrospectively, by one pathologist blinded to outcome.

The tumors were graded according to the Nottingham system. After pathology review, 37 cases were excluded due to insufficient number of tumor cells in the sample, or only containing carcinoma in situ. The remaining subset (771 tumor blocks) was well balanced to the original low risk cohort regarding the tumor characteristics, such as tumor size of ≤ 20 mm (78% vs. 81%), positive ER status (78% vs. 80%) and tamoxifen treatment (52% vs. 50%).

The standard procedure for tissue collection was fixation in 4% phosphate-buffered formalin and embedment in paraffin. Follow-up data was collected from regional population registers and the Swedish Cause of Death Registry. The mean follow-up period for patients in the present investigation was 17 years. The retrospective investigation of the collected tumor samples was approved by the ethical committee at the Karolinska University Hospital. According to the approval, informed consent from the patients was not required.

Hormone receptor status

Status of ER, PR (progesterone receptor) and Her2 (human epidermal growth factor receptor 2) was assessed retrospectively with immunohistochemistry. ER and PR were examined using the Ventana® automated slide stainer (Ventana Medical Systems, S.A., Cedex, France). Primary monoclonal antibodies (CONFIRM from Ventana® Medical Systems) were mouse anti-ER antibody (clone 6F11) and mouse anti-PR antibody (clone 16). Cut-off level was set to 25% positively stained tumor cell nuclei. In cases when immunohistochemical data for ER was missing (12%), ER status as determined in clinical routine practice at time of diagnosis was used

(Wrange *et al*, 1978), with a cut-off level of 0.05 fmol/µg DNA. For Her2 status, tissue was stained and scored as previously described (Jerevall *et al*, 2010).

Gene expression analysis by real time RT-PCR and calculation of gene expression indices

MGI, H:I and BCI analysis were performed blinded to outcome. The genes analyzed were *HOXB13, IL17BR* (*HOXB13:IL17BR* index or H:I), *BUB1B*, *CENPA*, *NEK2*, *RACGAP1*, *RRM2 (*Molecular Grade Index), *ACTB*, *HMBS*, *SDHA*, and *UBC* (reference genes). Primer and probe sequences for these genes were the same as previously described (Ma *et al*, 2006; Ma *et al*, 2008). From each sample, 10 µm tissue sections were cut. To enrich for tumor content, all sections were subject to manual macrodissection prior to RNA extraction. RNA extraction from formalin-fixed paraffin-embedded (FFPE) sections was performed as before (Ma *et al*, 2006). Prior to TaqMan RT-PCR, total RNA was reverse transcribed, and the resulting cDNA was pre-amplified by performing 10 rounds of PCR using the PreAmp Master Mix Kit per manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA). The pre-amplified products were analyzed by TaqMan RT-PCR as previously described (Ma *et al*, 2008). H:I and MGI were calculated as previously described (Ma *et al*, 2006; Ma *et al*, 2008).

Development of a continuous risk model

Previously, we reported the categorical combination of binary H:I (cut-off = 0.06) and MGI (cut-off = 0) into three risk groups as follows: low risk, low MGI; intermediate risk, low H:I and high MGI; and high risk, high H:I and high MGI. Here a continuous risk model was built by combining H:I and MGI as continuous variables, using the ER positive patients in the tamoxifen arm of the trial (n = 314). We first checked linearity

of these two variables by fitting a Cox proportional hazard regression model with restricted cubic splines, and H:I demonstrated significant nonlinearity. We used a polynomial function of H:I to approximate the restricted cubic spline, and the final model was selected by comparing Cox regression models using Akaike Information Criterion. The resulting predictor from the final Cox regression model was then re-scaled into the range of 0 to 10, which we refer to as the Breast Cancer Index (BCI). We further categorized BCI into three levels: low risk, BCI < 5; intermediate risk, 5 ≤ BCI < 6.4; high risk, BCI ≥ 6.4. These cut-offs were chosen such that the resulting proportions of low, intermediate and high risk groups were similar to those formed by the three categorical combination groups of H:I+MGI. The endocrine untreated arm in the Stockholm randomized trial was used as a testing cohort for BCI.

Clinicopathological Risk Assessment

The St. Gallen's guidelines were used to assess the risk of recurrence in the ER positive tamoxifen-treated and untreated patients, while Adjuvant! Online (http://www.adjuvantonline.com) was used to assess 10-year risk of recurrence and survival for the ER positive, node negative patients using the following information: tumor grade, tumor size and age. Multivariate Cox proportional hazard regression models were used to assess the prognostic performance of Adjuvant! Online and BCI.

Statistical analysis

The primary clinical endpoint used in data analysis was time to distant metastasis. Distant metastasis-free survival (DMFS) was defined as the time from diagnosis to first distant metastasis. Local/regional recurrences prior to distant metastasis were censored at the time of relapse. For analysis, data was censored at 15 years, since

>90% of the distant metastatic events occurred before this time point. Association of gene expression indices with the clinical endpoint was assessed by the Kaplan-Meier method with the use of log-rank test and Cox proportional hazard regression. The proportional hazard assumption was verified by scaled Schoenfeld residuals. Multivariate Cox proportional hazard regression models were used to assess whether gene expression indices provided prognostic information independent of traditional clinical and histopathological parameters. The hazard ratio for the continuous BCI score was calculated relative to a 5-unit increment except for in the multivariate analysis of BCI and Adjuvant! Online. To more accurately compare BCI to Adjuvant! Online, hazard ratios were calculated relative to an increment of their inter-quartile ranges (2.484 for BCI; 8 for Adjuvant! Online). All statistical procedures are conducted in the statistical software Statistica 9.1 (StatSoft Scandinavia AB, Uppsala, Sweden) and the free software environment R (version 2.11.1, http://www.r-project.org).

Results

Patient and tumor characteristics

The randomized Stockholm trial conducted during 1976 through 1990 examined the efficacy of adjuvant tamoxifen compared to no adjuvant treatment among postmenopausal women with early stage breast cancer. From the “low risk” patient group (negative lymph nodes and tumor size ≤ 3 cm) in this trial, a total of 808 FFPE tumor blocks were retrieved for molecular analysis. After pathological review, 37 cases had to be excluded due to insufficient tumor cells or only containing carcinoma in situ. Reportable gene expression data by real time RT-PCR were obtained for all

but two samples, leaving a total of 769 cases in the final analysis (Fig 1). This corresponds to a success rate of 99.7% among the samples assayed. The ER-positive tamoxifen-treated and untreated patients were examined in this study (n=588). Further tumor characteristics of this cohort are summarized in Table 1.

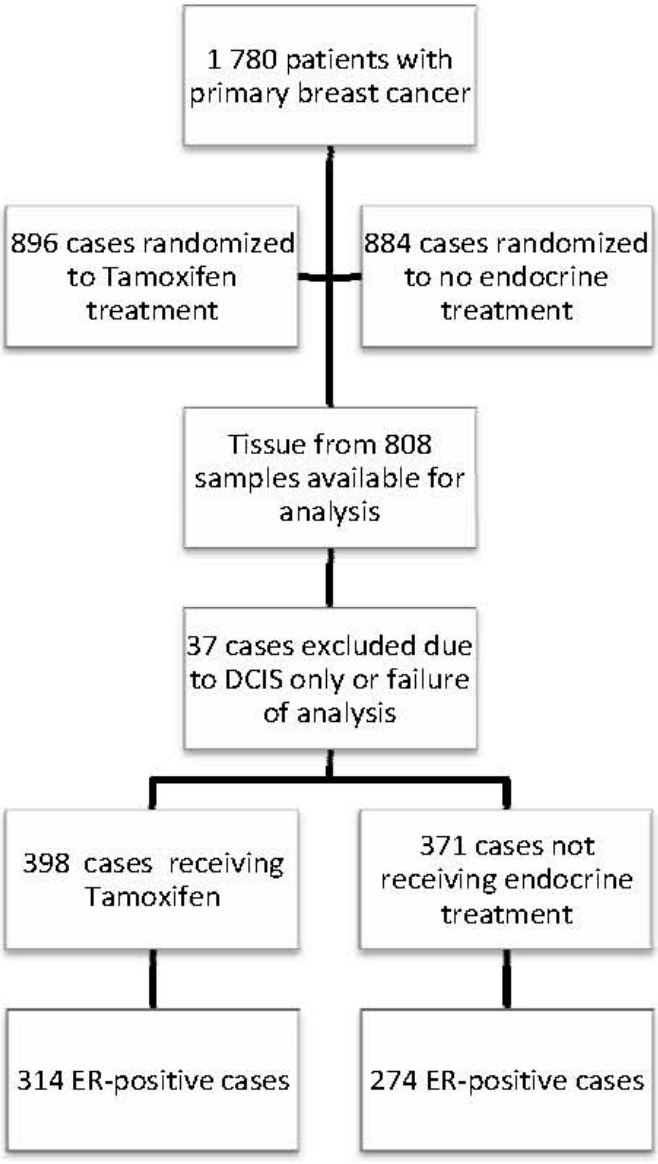

Figure 1: CONSORT diagram.

Table 1.

Tumor characteristics for the 588 early-stage postmenopausal ER-positive breast cancer patients included in the present study.

	Tamoxifen treated		Untreated	
	No.	**%**	**No.**	**%**
Tumor size (mm)				
≤ 20	256	82	223	81
> 20	55	18	49	18
Unknown	3	1	2	1
Tumor grade				
1	67	21	67	24
2	209	67	172	63
3	38	12	35	13
PR status				
Negative	109	35	107	39
Positive	180	57	139	51
Unknown	25	8	28	10
HER2 status				
Negative	272	87	238	87
Positive	14	4	13	5
Unknown	28	9	23	8

Abbreviations: HER2 = human epidermal growth factor receptor 2; PR = progesterone receptor.

Association of the combined H:I+MGI with distant metastasis and breast cancer specific death

The dichotomous H:I+MGI index was defined for each patient using pre-specified cut-off values (H:I 0.06 and MGI 0) and a previously described combination algorithm (low risk: low MGI; intermediate risk: low H:I and high MGI; and high risk: high H:I and high MGI) (Ma *et al*, 2008). Using this approach to estimate risk among the ER-positive tamoxifen-treated and untreated patients, the index was significantly associated with both time to first distant metastasis and breast cancer-specific death within these independent cohorts. In the tamoxifen-treated patients, more than 50%

of the patients were classified as having a low risk of recurrence. Kaplan-Meier analysis demonstrated that tamoxifen-treated patients assigned to the low risk group had a rate of distant recurrence or death at 10 years of less than 3% (distant recurrence rate: 2.9%, 95% CI 0.4-5.4 (Fig 2A); rate of death: 2.3%, 95% CI 0.1-4.5 (data not shown)). The dichotomous H:I+MGI index also identified 23% of the tamoxifen-treated patients as intermediate risk and 18% as high risk. In the intermediate and high risk groups, the Kaplan-Meier estimates of the rate of distant recurrence were 16.9% (95% CI 7.2-25.6) and 16.3% (95% CI 6.0-25.5; Fig 2A) and estimates of the 10-year rate of breast cancer-specific death were 13.8% (95% CI 5.0-21.9) and 11.0 (95% CI 2.3-18.9).

A multivariate Cox regression model including tumor grade, tumor size and the dichotomous H:I+MGI index confirmed that this risk classification was associated with distant recurrence independent of tumor size, grade, Her2 status and PR status in the tamoxifen-treated patients (Table 2). The dichotomous H:I+MGI index was also prognostic of breast cancer specific death independent of tumor size, grade, Her2 status and PR status (Supplementary Table 1).

The dichotomous H:I+MGI index also demonstrated prognostic utility in the ER-positive untreated arm of the trial (p=0.0004; Fig 2B). Of the untreated cohort, 50% were classified as low risk, 27% as intermediate risk and 23% as high risk. The prediction of outcome for the untreated patients appeared similar with higher recurrence rates and death rates than in the tamoxifen-treated patients. In univariate Cox regression analysis, risk assessment with the dichotomous H:I+MGI index showed a statistical significance both for distant recurrence as well as death due to

the disease (data not shown). The rate of breast-cancer specific death for the low, intermediate and high risk groups in the untreated arm of the trial was 5.3% (95% CI 1.4-9.0), 19.3% (95% CI 9.3-28.3) and 26.3% (95% CI 14.4-36.7).

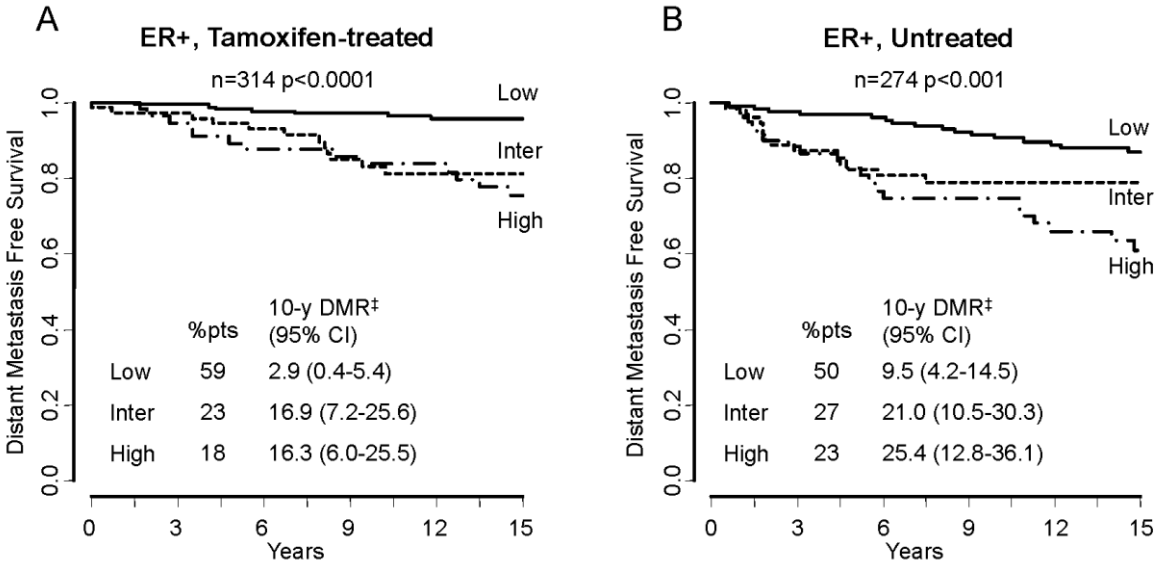

Figure 2: Kaplan-Meier estimates of likelihood of distant metastasis, stratified by the combined index of HI and MGI. (A) ER positive, tamoxifen treated subcohort. (B) ER positive, untreated subcohort. ‡: DMR denotes distant metastasis rate.

In a multivariate analysis, the dichotomous H:I+MGI index was prognostic of distant recurrence (Table 3) and breast cancer specific death (Supplementary Table 2) independent of tumor size, grade, HER2 status and PR status in the untreated cohort.

Table 2.

Multivariate Cox regression analysis of distant metastasis for tamoxifen-treated patients with estrogen receptor positive disease.

	Patients (n)	Hazard Ratio (95% CI)	P value
Multivariate Analysis without H:I + MGI			
Tumor grade			
NHG I	54		
NHG II	183	2.6 (0.6-11.4)	0.20
NHG III	36	5.7 (1.2-27.3)	0.03
Tumor size			
≤ 20 mm	225		
> 20 mm	48	1.0 (0.4-2.5)	0.98
HER2			
Negative	259		
Positive	14	2.1 (0.7-6.4)	0.22
PR			
Negative	104		
Positive	169	0.8 (0.4-1.7)	0.56
Multivariate Analysis with H:I + MGI			
Tumor grade			
NHG I	54		
NHG II	183	1.5 (0.3-7.0)	0.59
NHG III	36	2.0 (0.4-10.7)	0.44
Tumor size			
≤ 20 mm	225		
> 20 mm	48	1.0 (0.4-2.5)	0.96
HER2			
Negative	259		
Positive	14	1.6 (0.5-5.3)	0.42
PR			
Negative	104		
Positive	169	0.8 (0.4-1.8)	0.62
H:I + MGI			0.02
Low	155		
Intermediate	67	4.2 (1.5-12.1)	0.007
High	51	4.4 (1.4-13.7)	0.01

Abbreviations: HER2 = human epidermal growth factor receptor 2; MGI = molecular grade index; NHG = Nottingham grade; PR = progesterone receptor.

Table 3

Multivariate Cox regression analysis of distant metastasis for untreated patients with estrogen receptor positive disease.

	Patients (n)	Hazard Ratio (95% CI)	P value
Multivariate Analysis without H:I + MGI or BCI			
Tumor grade			
NHG I	53		
NHG II	149	1.2 (0.5-2.6)	0.74
NHG III	34	1.6 (0.6-4.4)	0.37
Tumor size			
≤ 20 mm	190		
> 20 mm	46	3.0 (1.6-5.6)	0.0005
HER2			
Negative	223		
Positive	13	3.0 (1.1-8.3)	0.03
PR			
Negative	101		
Positive	135	1.3 (0.7-2.4)	0.41
Multivariate Analysis with H:I + MGI			
Tumor grade			
NHG I	53		
NHG II	149	0.9 (0.4-2.1)	0.79
NHG III	34	0.9 (0.3-2.8)	0.84
Tumor size			
≤ 20 mm	190		
> 20 mm	46	2.8 (1.5-5.3)	0.001
HER2			
Negative	223		
Positive	13	2.8 (1.0-7.7)	0.053
PR			
Negative	101		
Positive	135	1.3 (0.7-2.4)	0.39
H:I + MGI			0.048
Low	116		
Intermediate	62	1.8 (0.8-3.9)	0.17
High	58	2.6 (1.2-5.6)	0.01
Multivariate Analysis with BCI Categories			
Tumor grade			
NHG I	53		
NHG II	149	0.8 (0.3-1.8)	0.55
NHG III	34	0.6 (0.2-1.9)	0.39
Tumor size			
≤ 20 mm	190		
> 20 mm	46	3.0 (1.5-5.6)	0.001
HER2			
Negative	223		
Positive	13	3.5 (1.2-9.8)	0.019
PR			
Negative	101		
Positive	135	1.4 (0.7-2.6)	0.30
BCI			0.001
Low	122		
Intermediate	66	2.3 (1.1-5.0)	0.03
High	48	4.7 (2.1-10.8)	0.0003
Multivariate Analysis with Continuous BCI Score			
Tumor grade			0.89
NHG I	53		

NHG II	149	1.3 (0.4-3.8)	0.69
NHG III	34	1.1 (0.3-4.4)	0.91
Tumor size			
≤ 20 mm	190		
> 20 mm	46	2.1 (1.1-4.2)	0.03
HER2			
Negative	223		
Positive	13	2.1 (0.7-6.4)	0.18
PR			
Negative	101		
Positive	135	1.0 (0.5-1.9)	0.91
BCI[a]	236	7.5 (2.4-23.6)	0.0006
Multivariate Analysis with BCI and Adjuvant! Online			
Adjuvant! Online[‡]	246	1.4 (1.0-1.8)	0.03
BCI[b]	246	2.0 (1.3-3.1)	0.001

Abbreviations: BCI = Breast Cancer Index; HER2 = human epidermal growth factor receptor 2; MGI = molecular grade index; NHG = Nottingham grade; PR = progesterone receptor [a]Analysis of BCI as a continuous variable. The hazard ratio for BCI is calculated relative to a 5-unit increment. [b]To more accurately compare BCI to Adjuvant! Online, the calculated hazard ratios are relative to an increment of their inter-quartile ranges, 2.484 for BCI, 6 for Adjuvant! Online.

Development and testing of H:I+MGI as a continuous index (Breast Cancer Index)

To enable individual risk assessment of the risk of recurrence, we developed a continuous algorithm based on the dichotomous H:I+MGI index. Using the ER positive tamoxifen-treated patients in this cohort as a training set, we developed a polynomial function to compute a continuous risk index from H:I and MGI, which we henceforth refer to as the Breast Cancer Index or BCI. BCI provides an individual risk score on the scale of 0 to 10 for each patient, which has a continuous relationship with the rate of distant metastasis at 10 years (Fig 3A). For patient stratification using BCI, we also defined three risk groups using two cut-off points: BCI < 5, low risk; BCI ≥ 5 and < 6.4, intermediate risk; BCI ≥ 6.4, high risk. BCI classified 59.6% of the tamoxifen-treated patients as having a low risk of recurrence (Kaplan-Meier estimates of the rate of distant recurrence: 1.7%, 95% CI 0-3.5; rate of death: 1.1%,

95% CI 0-2.6 (Fig 3A; data not shown)). BCI also identified 22.0% of the tamoxifen-treated patients as intermediate risk and 18.4% as high risk. In the intermediate and high risk groups, the Kaplan-Meier estimates of the rate of distant recurrence were 17.8% (95% CI 7.6-26.8) and 20.0% (95% CI 8.7-30.0) and estimates of the 10-year rate of breast cancer-specific death were 14.5% (95% CI 5.2-22.9) and 14.7% (95% CI 4.7-23.6) (data not shown).

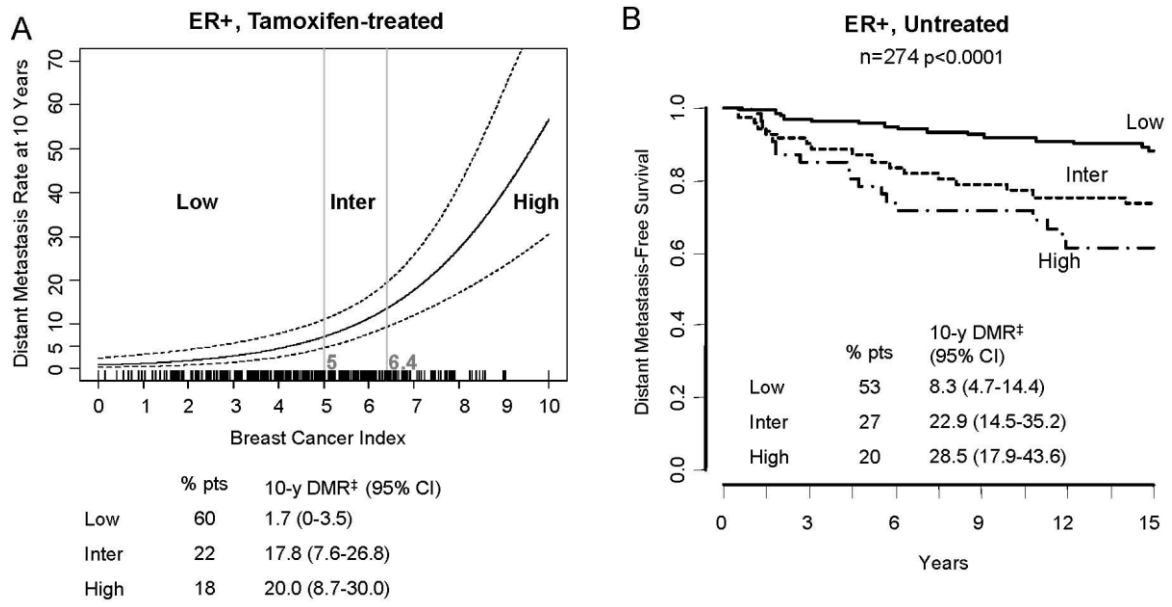

Figure 3: BCI predicts distant metastasis. A patient is in the low risk group if BCI < 5, intermediate group if 5 ≤ BCI < 6.4, and high risk group if BCI ≥ 6.4. (A) Kaplan-Meier estimates of 10-year distant recurrence stratified by categorical BCI and distant metastasis rate at 10 years as a function of BCI based on ER positive tamoxifen-treated patients from the Stockholm trial. (B) Kaplan-Meier estimates of likelihood of distant metastasis stratified by categorical BCI in the ER positive untreated patients from the Stockholm trial. ‡: DMR denotes distant metastasis rate.

To test the performance of the BCI model, the ability of BCI to predict distant metastasis in the ER positive patients in the untreated arm of the Stockholm trial was

examined (n = 274). In these patients, 53%, 27% and 20% were classified as low, intermediate and high risk. The rate of distant metastasis at 10 years in these risk groups was 8.3% (95% CI 4.7-14.4), 22.9% (95% CI 14.5-35.2) and 28.5% (95% CI 17.9-43.6), respectively (Fig 3B) and the rate of breast cancer-specific death was 5.1% (95% CI 1.3-8.7), 19.8% (95% CI 10.0-28.6) and 28.8% (95% CI 15.3-40.2). BCI was a strong prognostic factor for distant recurrence independent of tumor size, grade, PR status and Her2 status, although tumor size did contribute prognostic value (Table 3). BCI was also predictive of breast cancer specific death applying a similar multivariate model (Supplementary Table 2).

Clinicopathological risk assessment

The risk of recurrence was also assessed in both ER positive cohorts from the Stockholm trial using the St. Gallen's guidelines. In the tamoxifen-treated cohort, 22% were classified as low risk and 78% as intermediate risk with a rate of recurrence of 5.2% (95% CI -0.5-10.9) and 8.5% (95% CI 4.9-12.1), respectively. In the untreated cohort, 19% were classified as low risk and 81% as intermediate risk with a rate of recurrence of 8.8% (95% CI 1.4-16.2) and 17.0% (95% CI 11.7-22.3), respectively.

The prognostic utility of BCI was also assessed in comparison to Adjuvant! Online, a web-based tool used to assess risk of recurrence and breast cancer specific death based on clinicopathological information. In multivariate analyses, both BCI and Adjuvant! Online were significant predictors of distant recurrence and death (Table 3 and Supplementary Table 2).

Correlation of BCI with traditional prognostic factors

Comparisons between BCI and classic prognostic factors showed a correlation to tumor size and tumor grade, as well as HER2 status (Supplementary Table S3). Significantly more patients categorized into the low risk group than in the high risk group had tumors that were ≤20 mm in size, of a low grade and Her2 negative.

Discussion

It was previously demonstrated that the combination of H:I and MGI into a dichotomous index outperforms either index alone in predicting the risk of recurrence in ER positive, node negative breast cancer patients (Ma *et al*, 2004; Ma *et al*, 2008). In the present study, the prognostic performance of the dichotomous H:I+MGI index was validated in a large retrospective analysis of patients from the randomized Stockholm trial and a continuous risk model of H:I+MGI (BCI) was developed and tested.

Consistent with previous reports, analysis in this cohort of 588 early stage, postmenopausal ER positive breast cancer patients demonstrated that the combination of H:I+MGI was strongly associated with the risk of distant metastasis and death due to breast cancer (Ma *et al*, 2008). The combined H:I+MGI identified more than 50% of the patients to have a low 10-year recurrence risk with fewer than 3% of the patients relapsing during this period of time. Additionally, the results from analysis of the ER positive patients not treated with endocrine therapy suggested that the combined H:I+MGI also has prognostic utility in untreated patients. H:I+MGI was shown to outperform tumor grade and PR status in a multivariate analysis with tumor size still contributing significant prognostic value only in the untreated patient

population. The results in the present study thus confirmed the previous findings that the combined index performs well for prediction of breast cancer outcome, both in treated and untreated patients.

The tamoxifen-treated ER positive, node negative cohort from the Stockholm trial was utilized to develop the BCI algorithm. The BCI model was developed to assign a different index score for each patient, each associated with a different level of individualized risk of distant recurrence. The BCI scores were categorized into three levels of recurrence risk (low, intermediate and high) by using proportional values established with the dichotomous H:I+MGI index. This entire cohort was used to train the algorithm in order to retain the entirety of the prognostic information available with this large, prospective trial and to maximize the accuracy of BCI to predict a distant metastatic recurrence. An initial test of the prognostic ability of BCI was performed in the ER positive patient cohort that was not treated with endocrine therapy and not used to develop BCI. In this untreated cohort, BCI provided similar prognostic utility compared to that of the combined H:I+MGI in the untreated cohort. Specifically, BCI classified 53% of the patients into the low risk group with a 8.3% risk of distant recurrence, while H:I+MGI stratified 50% of the untreated, ER positive patients into the low risk group with a 9.5% risk of recurrence. In addition, these results also suggest that the prognostic utility of BCI extends into untreated patients.

In this study, BCI classified 53%, 27% and 20% as low, intermediate and high risk. In contrast, traditional risk classification based on clinicopathological criteria, such as suggested by the St Gallen recommendations from 2005 (Goldhirsch *et al*, 2006), classifies a majority of ER positive, node negative breast cancer patients as being of

intermediate risk, with few patients in the low or high risk group. For the low risk group with endocrine responsiveness, endocrine therapy remains the primary treatment, but for the corresponding intermediate group the treatment of choice is not completely clear, likely resulting in both over- and under-treatment. With BCI, the intermediate risk group was reduced, and a significantly larger proportion of patients were predicted to have low risk of recurrence, suggesting additional chemotherapy to be unnecessary.

The prognostic performance of BCI was also compared to Adjuvant! Online. In a multivariate analysis, both BCI and Adjuvant! Online, which estimates risk based on clinicopathological information, retained predictive significance suggesting that they provide complementary information in the assessment of risk of recurrence and overall survival.

There are at least two gene expression profiling assays that were developed that identify breast cancer patients with a low risk of recurrence. The 70-gene profile MammaPrint (the Amsterdam signature; Agendia, BV; Amsterdam, Holland), which is based on a microarray platform, was developed as a predictor of 5-year risk of distant metastasis and stratifies patients into low or high risk. It was reported that MammaPrint classifies approximately 40% of node negative, ER positive and negative patients into the low risk group with a 10-year risk of recurrence of 13% in this cohort that was predominantly untreated (<7% received chemotherapy and or hormonal therapy) (van de Vijver *et al*, 2002). Oncotype DX (Genomic Health, Inc., Redwood City, CA) is a quantitative RT-PCR assay of a panel of 21-genes, which employs a continuous Recurrence Score for risk assessment. For decision-making in

the clinical setting, three risk groups are defined, with risk assessment in the ER positive, node negative, tamoxifen-treated arm of the NSABP-B14 trial ranging from 7% in the low-risk to 31% in the high-risk group (Paik *et al*, 2004). Risk assessment in the validation cohort for the dichotomous H:I+MGI index was consistent with these results with 59% of the tamoxifen-treated and 50% of the untreated classified into the low risk group with a 10-year risk of recurrence of 3% and 10%, respectively.

In this study we demonstrate that the combination of H:I and MGI, either as dichotomous or as a continuous variable (i.e. BCI), is a significant prognostic for early breast cancer. Studies determining the prognostic and/or predictive properties of the individual components of BCI are also ongoing. For example, within the same cohort, HOXB13 protein expression was demonstrated to be associated with patient benefit for tamoxifen treatment (Jerevall et al, 2010). This suggests that in addition to the strong performance of BCI as a prognostic, its components may also have predictive properties. Further studies are warranted to determine whether these findings will extend to current standard of care of ER+ patients receiving 5 to 10 years of aromatase inhibitors.

Conclusions

Taken together, this study validates the predictive performance of the dichotomous H:I+MGI index in a retrospective analysis of postmenopausal early stage breast cancer patients randomized to tamoxifen or no endocrine treatment. We have also developed and tested a continuous risk index of H:I+MGI, called BCI, for estimation of recurrence risk at the individual level. The results from this study suggest that BCI has significant prognostic utility in an untreated population. BCI has the ability to

identify a large fraction (>50%) of patients with a low risk of distant recurrence at 10 years more accurately than using traditional risk assessment. These results suggest that BCI may help clinicians to make better informed treatment decisions and spare toxic chemotherapy for a large group of breast cancer patients.

List of abbreviations

BCI, Breast Cancer Index; DMFS, distant metastasis-free survival; ER, estrogen receptor; FFPE, formalin-fixed paraffin-embedded; H:I, *HOXB13:IL17BR*; Her2, human epidermal growth factor receptor 2; MGI, Molecular Grade Index; PR, progesterone receptor.

Competing interests

HL, RS, NCK and MGE are employees of and stockholders in bioTheranostics, Inc. X-JM was an employee of bioTheranostics, Inc. X-JM, MGE and DCS are named inventors on a patent for the technology described herein.

Authors' contributions

X-JM and MGE conceived of the study. P-LJ, X-JM, MGE and OS participated in the study design and coordination. LS, TF and BN provided the study material and clinical information, and collected laboratory data on the study patients. P-LJ, RS, DCS and BH performed the laboratory work. P-LJ, X-JM, HL, MGE and OS performed the statistical analyses. P-LJ, X-JM, HL and NCK participated in interpreting results and drafting the manuscript. MGE and OS provided critical revision of the manuscript. All authors read and approved the final manuscript.

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Supplementary Table S1. Online only.
Multivariate Cox regression analysis of breast cancer specific death for tamoxifen-treated patients with estrogen receptor positive disease.

	Patients (n)	Hazard Ratio (95% CI)	P value
Multivariate Analysis without H:I + MGI			
Tumor grade			0.21
NHG I	54		
NHG II	183	1.6 (0.4-7.3)	0.53
NHG III	36	3.5 (0.7-18.0)	0.14
Tumor size			
≤ 20 mm	225		
> 20 mm	48	2.2 (0.9-5.7)	0.09
HER2			
Negative	259		
Positive	14	1.2 (0.3-5.3)	0.84
PR			
Negative	104		
Positive	169	1.0 (0.4-2.4)	0.96
Multivariate Analysis with H:I + MGI			
Tumor grade			0.93
NHG I	54		
NHG II	183	1.0 (0.2-4.7)	0.95
NHG III	36	1.2 (0.2-7.3)	0.87
Tumor size			
≤ 20 mm	225		
> 20 mm	48	2.2 (0.9-5.7)	0.10
HER2			
Negative	259		
Positive	14	0.9 (0.2-4.2)	0.89
PR			

Negative	104		
Positive	169	1.0 (0.4-2.5)	0.98
H:I + MGI			0.05
Low	155		
Intermediate	67	3.9 (1.2-12.9)	0.03
High	51	4.3 (1.2-15.5)	0.03

Supplementary Table S2. Online only.

Multivariate Cox regression analysis of breast cancer specific death for untreated patients with estrogen receptor positive disease.

	Patients (n)	Hazard Ratio (95% CI)	P value
Multivariate Analysis without H:I + MGI or BCI			
Tumor grade			0.098
NHG I	53		
NHG II	149	2.0 (0.7-5.9)	0.19
NHG III	34	3.7 (1.1-12.2)	0.04
Tumor size			
≤ 20 mm	190		
> 20 mm	46	2.5 (1.3-4.9)	0.0087
HER2			
Negative	223		
Positive	13	1.9 (0.7-5.7)	0.24
PR			
Negative	101		
Positive	135	1.0 (0.5-1.9)	0.95
Multivariate Analysis with H:I + MGI			
Tumor grade			0.85
NHG I	53		
NHG II	149	1.3 (0.4-3.9)	0.65
NHG III	34	1.5 (0.4-5.3)	0.57
Tumor size			
≤ 20 mm	190		
> 20 mm	46	2.3(1.2-4. 7)	0.016
HER2			
Negative	223		
Positive	13	1.9 (0.6-5.8)	0.27
PR			
Negative	101		

Positive	135	1.0 (0.5-2.0)	0.99
H:I + MGI			0.005
Low	116		
Intermediate	62	3.9 (1.5-10.1)	0.005
High	58	4.5 (1.8-11.6)	0.002
Multivariate Analysis with BCI Categories			
Tumor grade			0.86
NHG I	53		
NHG II	149	1.4 (0.5-4.1)	0.59
NHG III	34	1.3 (0.3-5.1)	0.71
Tumor size			
≤ 20 mm	190		
> 20 mm	46	2.5 (1.2-5.1)	0.01
HER2			
Negative	223		
Positive	13	2.1 (0.7-6.4)	0.20
PR			
Negative	101		
Positive	135	1.0 (0.5-1.9)	1.00
BCI			0.003
Low	122		
Intermediate	66	2.4 (1.0-5.7)	0.054
High	48	5.1 (2.0-12.7)	0.0006
Multivariate Analysis with Continuous BCI Score			
Tumor grade			0.64
NHG I	53		
NHG II	149	0.8 (0.3-1.7)	0.50
NHG III	34	0.6 (0.2-1.8)	0.34
Tumor size			
≤ 20 mm	190		

	> 20 mm	46	2.4(1.3-4.6)	0.006
HER2				
	Negative	223		
	Positive	13	3.1 (1.2-8.6)	0.026
PR				
	Negative	101		
	Positive	135	1.3 (0.7-2.5)	0.40
BCI[†]		236	5.7 (2.1-15.4)	0.0006
	Multivariate Analysis with BCI and Adjuvant! Online			
Adjuvant! Online[‡]		246	1.4 (1.0-1.8)	0.04
BCI[‡]		246	2.3 (1.5-3.7)	<0.001

[†]Analysis of BCI as a continuous variable. The hazard ratio for BCI is calculated relative to a 5-unit increment. [‡]To more accurately compare BCI to Adjuvant! Online, the calculated hazard ratios are relative to an increment of their inter-quartile ranges, 2.484 for BCI, 6 for Adjuvant! Online.

Supplementary Table S3. Online only.
Correlation between BCI and tumor characteristics.

	BCI						
	Low		**Intermediate**		**High**		**P value**
	No	%	No	%	No	%	
Tumor size							
≤ 20 mm	327	83	142	78	128	70	
> 20 mm	67	17	39	22	56	30	0.00040
Tumor grade							
1	124	31	18	10	6	3	
2	240	60	123	67	88	47	
3	36	9	42	23	92	49	<0.00001
HER2 status							
Negative	331	95	151	88	141	82	
Positive	18	5	21	12	30	18	<0.00001