The Prognostic Biomarkers \textit{HOXB13}, \textit{IL17BR}, and \textit{CHDH} Are Regulated by Estrogen in Breast Cancer

Zuncai Wang, Sonika Dahiya, Heather Provencher, Beth Muir, Erin Carney, Kathryn Coser, Toshi Shioda, Xiao-Jun Ma, and Dennis C. Sgroi

\textbf{Abstract} Purpose: We previously identified three genes, \textit{HOXB13}, \textit{IL17BR}, and \textit{CHDH}, that strongly predict clinical outcome in estrogen receptor (ER)–positive breast cancer patients receiving tamoxifen monotherapy. The biological mechanisms linking these genes to estrogen signaling and tamoxifen response in breast cancer remain to be determined.

\textbf{Experimental Design:} In a consecutive series of 148 ER-positive and ER-negative breast cancers, \textit{HOXB13}, \textit{IL17BR}, and \textit{CHDH} gene expression was measured by quantitative real-time PCR and correlated with \textit{ER}, \textit{PR}, and \textit{HER2} expression. The role of estrogen and ER in the regulation of these three genes was assessed in several ER-positive and ER-negative breast cancer cell lines.

\textbf{Results:} In primary breast tumors, \textit{HOXB13} expression correlated negatively, and \textit{IL17BR} and \textit{CHDH} expression correlated positively, with \textit{ER} status, and all three genes exhibited an \textit{ER}-dependent correlation pattern with \textit{HER2} status that differs from \textit{PR} and \textit{PS2}, two canonical estrogen-regulated genes. Results using breast cancer cell lines show that these genes are regulated by estradiol in an \textit{ER}-dependent manner, and that this regulation is abrogated by tamoxifen.

\textbf{Conclusions:} \textit{HOXB13}, \textit{IL17BR}, and \textit{CHDH} are estrogen-regulated genes, but their pattern of correlation with known positive (\textit{ER}, \textit{PR}) and negative (\textit{HER2}) predictors of tamoxifen response differs from canonical \textit{ER} signature genes. These results provide a biological rationale for the prognostic utility of these three genes in early-stage ER-positive breast cancer and for their potential to predict anti-estrogen resistance.
HOXB13, IL17BR, and CHDH is regulated by estrogen in an ER-dependent manner, which, in turn, is abrogated by tamoxifen. The estrogen-regulated pattern of HOXB13, IL17BR, and CHDH expression differs from canonical ER signature genes, suggesting that these genes may serve as a biomarker for dysfunctional ER signaling.

### Materials and Methods

#### Patients.

The Massachusetts General Hospital institutional review board approved this study in accordance to NIH guidelines. To avoid bias, formalin-fixed, paraffin-embedded (FFPE) tumor samples from 75 consecutive ER-positive and 73 consecutive ER-negative invasive breast carcinomas diagnosed at the Massachusetts General Hospital (Boston, MA) between 2005 and 2006 were prospectively collected and processed for RNA extraction and real-time quantitative PCR. Receptor status had been determined by immunohistochemistry using the BenchMark XT automated system (Ventana Medical Systems Inc.). Only cases with more than 10% positivity of tumor cells were considered as ER positive. HER2 amplification was determined by fluorescence in situ hybridization using the PathVysion HER-2 DNA probe kit (Vysis). For each case, the ratio of ≥2 HER2 to CEP 17 signals in at least 60 interphase tumor cell nuclei was considered as amplification of HER2. Clinicopathologic characteristics are described in Table 1.

#### RNA isolation from FFPE tumor samples and real-time quantitative PCR.

Total RNA was isolated from two to three 5-μm tissue sections for each sample using Optimum FFPE RNA isolation kit from Ambion Diagnostics according to the manufacturer’s instructions and reverse-transcribed into cDNA using gene-specific reverse priming using Powerscript Reverse Transcriptase (Clontech Laboratories) Reagent System. TaqMan primers and probes were designed using Primer Express (Applied Biosystems) for 10 genes (Table 2). The three reference genes (ACTB, HMBS, and UBC) were selected by assessing 10 commonly used housekeeping genes in an independent breast cancer cohort, as described previously (13). Gene expression was quantitated by TaqMan reverse transcription-PCR (RT-PCR) in triplicate in 96-well plate using an ABI 7900HT (Applied Biosystems) as described.

### Table 1. Clinicopathologic characteristics of 148 patients

<table>
<thead>
<tr>
<th>Factor description</th>
<th>ER-positive patients (n = 75), n (%)</th>
<th>ER-negative patients (n = 73), n (%)</th>
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<tbody>
<tr>
<td>Age</td>
<td>&lt;50 23 (31) 33 (45)</td>
<td>&gt;50 52 (69) 40 (55)</td>
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<tr>
<td>Histologic type</td>
<td>IDC 61 (81) 69 (95)</td>
<td>ILC 12 (16) 2 (3)</td>
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<td></td>
<td>Mixed 2 (3) 1 (1)</td>
<td>Metaplastic 0 (0) 1 (1)</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>1 18 (24) 0 (0)</td>
<td>2 45 (60) 14 (19)</td>
</tr>
<tr>
<td></td>
<td>3 12 (16) 58 (81)</td>
<td>&gt;5 1 (1) 4 (5)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>&lt;2 55 (73) 44 (60)</td>
<td>2-5 19 (25) 25 (34)</td>
</tr>
<tr>
<td></td>
<td>&gt;5 1 (1) 4 (5)</td>
<td>Lymph node</td>
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<tr>
<td></td>
<td>Negative 38 (51) 43 (59)</td>
<td>Positive 23 (31) 26 (36)</td>
</tr>
<tr>
<td></td>
<td>Not sampled 34 (45) 4 (5)</td>
<td>American Joint Committee I</td>
</tr>
<tr>
<td></td>
<td>on Cancer stage*</td>
<td>II-A 23 (38) 22 (32)</td>
</tr>
<tr>
<td></td>
<td>II-B 7 (12) 10 (14)</td>
<td>III-A 1 (2) 3 (4)</td>
</tr>
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|                   | III-B 0 (0) 1 (1)             | *Cases without lymph-node sampling are excluded from staging.

Fig. 1. Real-time quantitative PCR analysis of HOXB13, IL17BR, and CHDH expression according to ER status. A-C, box plots of HOXB13, IL17BR, and CHDH mRNA levels (Y-axis) versus ER status (X-axis). P values are from Wilcoxon two-sample test. D, bimodal distribution of HOXB13 expression. Histograms of HOXB13 mRNA levels (X-axis) in ER-positive and ER-negative tumors. Using a cutoff of -9 (vertical dotted line), high HOXB13 expression was seen in 50% ER-positive and 67% ER-negative tumors (P = 0.047).
previously (4). For each gene, a standard curve with cDNA dilutions derived from human universal total RNA (Stratagene) was constructed and was run on each plate to control for the interplate variability.

**Cell culture and reagents.** The T47D cell line was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FCS (DMEM-FCS). The MDA-MB-436, -453, and -468 human breast cancer cells were a generous gift from Daphne Bell (Massachusetts General Hospital, Boston, MA). MCF-7 cells (BUS stock) were maintained as previously described (15, 16). MDA-MB-231-neo and MDA-MB-231-ER8 cells were generously provided by Robert Weinberg (Massachusetts Institute of Technology, Cambridge, MA) and maintained as described (17). Charcoal/dextran-treated fetal bovine serum (cFBS) and fetal bovine serum (FBS) were obtained from Hyclone Laboratories, Inc. Tamoxifen and 17β-estradiol were obtained from Sigma Chemical Co.

**Data analysis.** All statistical analysis was done in the R statistical software. Association of continuous variables with binary factors (e.g., ER/HER2 status) was tested using nonparametric Wilcoxon two-sample test. Pair-wise gene-gene correlations were examined using the Pearson correlation coefficients. Relative gene expression levels in the cell line studies were compared between two conditions using the Student’s t test with unequal variance. All P values are two-sided, and P values <0.05 were considered significant.

**Results**

**Correlation of HOXB13, IL17BR, and CHDH expression with the estrogen and progesterone receptor.** Using a population-based cohort of 75 consecutive ER-positive and 73 ER-negative invasive breast cancers, we investigated the correlation of HOXB13, IL17BR, and CHDH gene expression with the estrogen and progesterone receptors, two well-known positive biomarkers of tamoxifen response. First, we correlated the mRNA levels of HOXB13, IL17BR, and CHDH with ER status as determined by immunohistochemistry (Fig. 1). The relative

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<table>
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<th>Table 2. Real-time PCR primer and probe design</th>
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<td><strong>Gene</strong></td>
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</tr>
<tr>
<td>ACTB</td>
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<td>CHDH</td>
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<td>ESR1</td>
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**Fig. 2.** Heat map of pair-wise correlation coefficients of gene expression values after hierarchical clustering.
expression of HOXB13 mRNA was higher in ER-negative tumors as compared with ER-positive tumors (Fig. 1A), whereas the relative expression of IL17BR and CHDH was significantly higher in ER-positive tumors as compared with ER-negative tumors (Fig. 1B and C). Using a Gaussian model–based clustering technique, HOXB13 mRNA levels were found to be bimodal, with 67% of ER-negative tumors and 50% of ER-positive tumors expressing HOXB13 (Fig. 1D). Next, we correlated the expression of HOXB13, IL17BR, and CHDH with ER mRNA levels as well as two other well-characterized estrogen-regulated genes, PR and pS2. Clustering of all pairwise correlation coefficients among these genes indicated that HOXB13 correlated negatively with ER, whereas IL17BR and CHDH correlated positively with each other and with ER (Fig. 2 and Table 3). As expected, the expression of PR and PS2 (Trefoil factor 1, TFF1), two canonical ER signature genes, correlated most strongly with ER. However, the positive correlation of IL17BR and CHDH with ER was less than that of PR and PS2 (Table 3).

**Correlation of HOXB13, IL17BR, and CHDH expression with HER2.** Using the same cohort described above, we next investigated the correlation of HOXB13, IL17BR, and CHDH mRNA expression with HER2 status, a known negative predictor of tamoxifen response. Notably, these three genes exhibited an ER-dependent correlation pattern with HER2 status (Fig. 3). More specifically, HOXB13 expression positively correlated with HER2 status in ER-positive but not in ER-negative tumors, and IL17BR and CHDH negatively correlated...
with HER2 status more strongly in ER-positive tumors than in ER-negative tumors. Interestingly, this ER-dependent correlative pattern for these genes with HER2 is distinct from that for PR and PS2 in that neither of these latter genes showed significant correlation with HER2 status in ER-positive tumors (Fig. 3).

Expression of HOXB13, IL17BR, and CHDH is regulated by estradiol. The observation that IL17BR and CHDH expression directly correlates and HOXB13 expression inversely correlates with ER expression raises the possibility that these three genes are targets of estrogen signaling. To address this issue, we exposed several ER-positive and ER-negative breast cancer cell lines to estradiol (E2) and measured transcript levels of all three genes by real-time quantitative-PCR (QRT-PCR). As expected, E2 stimulation resulted in elevated expression of PS2 in the ER-positive MCF7 and T47D cells, but in none of the three ER-negative cell lines (Fig. 4A). HOXB13 expression was significantly reduced, and IL17BR and CHDH expression was significantly increased by E2 in the ER-positive MCF7 and T47D cell lines, but not in the ER-negative cell lines (MDA-MB-436, MDA-MB468, and MDA-MB-453; Fig. 4A). Therefore, these data suggest that HOXB13, IL17BR, and CHDH are estrogen-responsive genes, consistent with their respective correlations with ER status in primary breast tumors described above. Furthermore, this estrogen response is not seen in ER-negative cells, suggesting a requirement for the ER.

To further test whether the E2 response of HOXB13, IL17BR, and CHDH requires functional ER, we evaluated the ability of ectopically expressed ER to modulate HOXB13, IL17BR, and CHDH expression. We measured transcript levels of these three genes in ER-negative MDA-MB-231 cells engineered to stably express a functional ER (17, 18). As compared with the parental MDA-MB-231 cell line, the ER-expressing MDA-MB-231 cells (MDA-MB-ER8) in the presence of E2 show dramatically elevated expression of IL17BR and CHDH and a near-complete suppression of HOXB13 expression (Fig. 4B). These results suggest that E2 regulation of HOXB13, IL17BR, and CHDH is mediated through the ER.

Because HOXB13, IL17BR, and CHDH were initially discovered as prognostic biomarkers in ER-positive patients treated with tamoxifen monotherapy (4), we next determined whether the estrogen response of these genes could be modulated by tamoxifen, a partial anti-estrogen. We stimulated MCF7 cells with two doses (0.1 and 1 nmol/L) of 17β-estradiol (E2) or ethanol vehicle (control). After 24 h of E2 exposure, the cells were harvested and processed for real-time PCR. B, real-time quantitative PCR analysis of HOXB13, IL17BR, CHDH, and PS2 expression in ER-negative breast cancer cells expressing a control vector (MDA-MB231-neo) or an ER expression vector (MDA-MB231-ER8). MDA-MB231-neo and MDA-MB231-ER8 cells were grown in medium containing non-charcoal–stripped FBS. Gene expression values were normalized to β-actin. Columns, means from triplicate experiments; bars, SE. *, P = 0.05; **, P = 0.01; ***, P = 0.001.
E2 resulted in 15-fold increase in PS2 expression, which was blocked by tamoxifen (Fig. 5). Notably, the enhancement of IL17BR and CHDH expression and the suppression of HOXB13 expression by E2 were abrogated by tamoxifen (Fig. 5).

Discussion

Estrogen plays an important role in breast cancer pathogenesis, and selective interference of the estrogen/ER-mediated signaling cascade is the most effective means of treating ER-positive breast cancer patients. High-throughput gene expression technologies have revealed a robust gene expression signature that readily distinguishes ER-positive tumors from ER-negative tumors (6, 19). Comparative analysis of ER-positive breast tumor gene expression profiles with those generated from E2-stimulated breast cancer cell lines has shown a remarkable overlap of genes (20). However, despite the identification of such ER gene expression signatures, it remains to be determined which gene or subset of genes regulated by estrogen is critical for breast tumorigenesis.

Previously, through genome-wide expression profiling, we identified a simple gene expression pattern that sub-stratifies ER-positive breast cancer patients into good and poor prognosis categories (4). More specifically, we have shown that a distinct breast cancer gene expression pattern characterized by higher levels of HOXB13 and lower levels IL17BR and CHDH gene expression predicts for distant tumor recurrence in tamoxifen-treated patients with ER-positive breast cancer (4, 11, 13). More recently, data generated by Jansen et al. (12, 14) suggest that HOXB13 and IL17BR expression is predictive of response to first-line tamoxifen monotherapy in ER-positive breast cancer patients with metastatic disease.

To begin to understand the biological mechanisms underlying their prognostic and predictive potential, we investigated whether estrogen signaling regulates HOXB13, IL17BR, and CHDH expression. Our results provide two lines of evidence indicating that these genes are regulated by estrogen. First, in a population-based, prospectively collected, consecutive series of ER-positive and ER-negative breast tumors, HOXB13 expression correlated negatively, and IL17BR and CHDH expression correlated positively, with ER status. Second, using a panel of ER-positive and ER-negative breast cancer cell lines, we showed...
that all three genes are estrogen responsive in an ER-dependent manner. Although these experiments do not formally establish that these genes are direct targets of the ER, this possibility is supported by results from a recent genome-wide location analysis of ER binding sites by chromatin immunoprecipitation combined with microarrays (ChIP-on-chip) in MCF7 cells upon E2 stimulation (21). In this published ChIP-on-chip database, an ER binding site exists within the second intron (2.4 kb from the transcriptional start site) of \( \text{IL17BR} \) (21). Due to the head-to-head genomic arrangement of \( \text{CHDH} \) and \( \text{IL17BR} \), this same ER binding site is also just 2.8 kb upstream of \( \text{CHDH} \), likely explaining their co-regulation by ER. In addition, this database reveals an ER-binding site that maps 76 kb upstream of the \( \text{HOXB13} \) transcriptional start site; this long distance does not rule out its functional relevance to \( \text{HOXB13} \) because long-range, non-proximal-promoter regulation may be important in many ER-regulated genes (21). The functional relevance of these ER binding sites needs direct experimentation in future studies.

The bimodal expression of \( \text{HOXB13} \) in breast cancer is notable. The promoter region and the first exon of \( \text{HOXB13} \) contain a CpG island, and it has been shown to be hypermethylated in renal cell carcinoma as a mechanism of shutting down its expression (22). Perhaps the bimodal expression pattern of \( \text{HOXB13} \) in breast cancer reflects this epigenetic regulation, where promoter methylation status serves as an on-off switch for transcription.

Furthermore, our findings suggest that \( \text{HOXB13}, \text{IL17BR}, \) and \( \text{CHDH} \) are regulated by ER in a manner different from other well-known ER-regulated genes such as \( \text{PR} \) and \( \text{pS2} \) based on the following observations. First, \( \text{HOXB13} \) belongs to a class of genes negatively regulated by ER, likely through a different mechanism than gene activation (21). Secondly, although \( \text{IL17BR} \) and \( \text{CHDH} \) are both positively correlated with \( \text{ER} \) expression, their extent of correlation is less than that for \( \text{PR} \) and \( \text{pS2} \). Thirdly, all three genes showed an ER-dependent correlation pattern with \( \text{HER2} \) status not seen with \( \text{PR} \) and \( \text{pS2} \). Previously, the \( \text{HOXB13}:\text{IL17BR} \) index was found to be higher in \( \text{HER2} \)-positive tumors than that in \( \text{HER2} \)-negative tumors (13). The relationship of these three E2-regulated genes to \( \text{HER2} \) is notable because recent data from preclinical models indicate that crosstalk between the HER2 and ER signaling pathways directly contributes to the development of tamoxifen resistance (23, 24).

Taken together, our current results suggest a biological framework to understand the proposed \( \text{HOXB13}:\text{IL17BR} \) index as a novel prognostic factor in early-stage ER-positive breast cancer patients and its potential for predicting tamoxifen resistance. Because ER functions to suppress \( \text{HOXB13} \) and augment \( \text{IL17BR} \) expression, either directly or indirectly, hormone-responsive tumors will have a low \( \text{HOXB13}:\text{IL17BR} \) index. When estrogen signaling is impaired by either loss of \( \text{ER} \) (25, 26), aberrant expression of certain cofactors (27), or by \( \text{HER2} \) signaling (24), suppression of \( \text{HOXB13} \) and induction of \( \text{IL17BR} \) expression is lost, resulting in a high \( \text{HOXB13}:\text{IL17BR} \) index. Therefore, the \( \text{HOXB13}:\text{IL17BR} \) index may serve as a biomarker for dysregulation of gene expression by ER and, thus, for tamoxifen resistance. Moreover, due to their distinctive patterns of correlation with \( \text{ER} \) and \( \text{HER2} \) as noted above, \( \text{HOXB13} \) and \( \text{IL17BR} \) may serve to report a subset of ER signaling targets different from canonical ER signature genes such as \( \text{ER}, \text{PR}, \) and \( \text{pS2} \), thus providing distinct prognostic or predictive information. Intriguingly, a possible interplay between growth factor and estrogen signaling in the regulation of \( \text{HOXB13} \) may be particularly relevant because we have observed synergistic action of epidermal growth factor and ectopic \( \text{HOXB13} \) overexpression in stimulating MCF10A cell motility and invasion (4). This interrelationship between \( \text{HOXB13}:\text{IL17BR}, \text{ER}, \) and \( \text{HER2} \) may also be important in the context of tamoxifen resistance because a high \( \text{HOXB13}:\text{IL17BR} \) index may indicate impaired ER signaling and increased growth factor signaling, both known to predict tamoxifen resistance (28, 29).

In summary, \( \text{HOXB13}, \text{IL17BR}, \) and \( \text{CHDH} \) are estrogen-regulated genes, and their prognostic utility is likely to reflect their complex regulation through both \( \text{ER} \)- and \( \text{HER2} \)-dependent pathways. Future work will be directed toward elucidating the pathways and cofactors used by ER to regulate these genes, understanding the functional interrelationship of these genes to the \( \text{HER2} \) signaling pathway, and determining whether aberrant expression of these genes contributes directly to the clinical behavior of breast cancer.

Acknowledgments

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