Epigenetic repression of the estrogen-regulated Homeobox B13 gene in breast cancer

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Several studies have reported that a high expression ratio of HOXB13 to IL7BR predicts tumor recurrence in hormone-negative, estrogen receptor (ER) α-positive breast cancer patients treated with tamoxifen. The molecular mechanisms underlying this dysregulation of gene expression remain to be explored. Our epigenetic analysis has found that increased promoter methylation of one of these genes, HOXB13, correlates with the decreased expression of its transcript in breast cancer cell lines (P < 0.005). Transcriptional silencing of this gene can be reversed by a demethylation treatment. HOXB13 is suppressed by the activation of estrogen signaling in ERα-positive breast cancer cells. However, treatment with 4-hydroxytamoxifen (4-OHT), an antiestrogen, abrogates the ERα-mediated suppression in cancer cells. The notion that this transcriptional induction of HOXB13 occurs in vitro with simultaneous exposure to both estrogen and 4-OHT may provide a biological explanation for its aberrant expression in many node-negative patients undergoing tamoxifen therapy. Interestingly, promoter hypermethylation of HOXB13 is more frequently observed in ERα-positive patients with increased lymph node metastasis (P = 0.031) and large tumor sizes (>5 cm) (P = 0.008). In addition, this aberrant epigenetic event is associated with shorter disease-free survival (P = 0.029) in cancer patients. These results suggest that hypermethylation of HOXB13 is a late event of breast tumorigenesis and a poor prognostic indicator of node-positive cancer patients.

Introduction

Breast tumorigenesis and cancer progression are directly related to the effects of the hormone estrogen (1). Tamoxifen, an antiestrogen, is widely used in the treatment of hormone-dependent breast cancers, but up to 40% of estrogen receptor (ER) α-positive breast cancer patients fail to respond or develop resistance to the drug. Intensive efforts have been undertaken to identify molecular biomarkers for predicting response (or resistance) to antiestrogen treatment. Recent reports identify a two-gene expression ratio HOXB13:IL7BR, derived from a genome-wide microarray analysis of ERα-positive breast tumors that is prognostic for disease-free survival (DFS) and/or relapse-free survival in lymph node-negative patients receiving tamoxifen (2–5).

The HOXB13 gene is related to a large Homeobox superfamily whose members take part in establishing cell fate and identity throughout embryonic development. This group of genes also plays an important role in maintaining cellular homeostasis in adult cells (6). Alterations of Homeobox gene expression have been identified in many solid tumors including cancers of the endometrium, cervix, ovary and prostate that overexpress HOXB13 (7–10). For HOXB13, mice homozygous for its functional mutation show overgrowth in all major structures derived from the tail bud (11). Furthermore, overexpressed HOXB13 is a proliferative modulator of ovarian cancer cell growth and may play a role in the development of tamoxifen resistance in patients with ERα-positive ovarian cancer (12). In contrast, the gene is subject to epigenetic control and may function as a tumor suppressor in renal cell carcinoma and melanoma (13,14).

Because loss of HOXB13 may lead to a deregulation of cellular processes that promote neoplasia and its expression is undetectable in 41% of ERα-positive tumors (5), we hypothesize that this gene may be epigenetically repressed in a subset of breast cancers and that detection of its methylation may have a prognostic value. Herein, we report that the HOXB13 promoter CpG island is frequently hypermethylated in ERα-positive breast tumors and cell lines. Further analysis confirms that HOXB13 is an ERα-responsive gene and suggests that its upregulation may be the result of tamoxifen’s modulation on estrogen signaling. In addition, hypermethylation of HOXB13 may be an adverse prognostic factor in ERα-positive breast cancer patients.

Materials and methods

Reagents and antibodies

17β-Estradiol (E2), 4-hydroxytamoxifen (4-OHT) and 5-aza-2'-deoxycytidine were purchased from Sigma (St Louis, MO). Culture media and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). The monoclonal HOXB13 and β-actin antibodies used in western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Clinical samples and cell lines

Frozen tissues were collected by the tissue procurement service in accordance with the protocols approved by the Institutional Review Board of the Ohio State University. Clinical and pathological data, including age at surgery, lymph node status, ER status, progesterone receptor (PR) status, lymphovascular invasion, the presence of ductal carcinoma in situ component, tumor grade and tumor size, were collected from pathology reports. Clinical follow-up data were available for 57 ERα-positive breast cancer patients. The time in months from the definitive breast surgery until the last known disease-free follow-up or first cancer recurrence was collected. Genomic DNAs were isolated from tissues with the QIamp DNA mini kit according to the manufacturer’s protocol (Qiagen, Valencia, CA). Genomic DNAs for 38 breast cancer cell lines were obtained from the laboratory of Dr Joe Gray at the Lawrence Berkeley National Laboratory, Berkeley, CA.

Cell culture

MCF7 breast cancer cell line was maintained in minimum essential medium with 2 mM l-glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, 6 ng/ml insulin and 10% FBS. T47D cells were maintained in RPMI 1640 with 2 mM l-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 6 ng/ml insulin and 10% FBS. BT474, MCF7 (a second, independent MCF7 cell line) and SKBR3 breast cancer cell lines (gifts from Dr Kay Huebner, Columbus, OH) were maintained in Dulbecco’s modified Eagle’s medium with 50 units/ml penicillin, 50 μg/ml streptomycin and 10% FBS. MDA-MB-453, MDA-MB-134, MDA-MB-435S and MDA-MB-231 breast cancer cell lines were maintained in our laboratory under conditions recommended by the American Type Culture Collection (Manassas, VA).

In E2 stimulation experiments, T47D cells were hormone deprived for 72 h in phenol red-free RPMI 1640 media supplemented with 4% charcoal dextran-treated FBS prior to treatment with 10 nM E2. In combined ligand experiments, T47D cells were treated with vehicle or 1 μM 4-OHT during the final 48 h of serum deprivation. MCF7 cells were hormone deprived for 72 h in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 4% charcoal dextran-stripped FBS prior to treatment with 10 nM E2. In order to restore HOXB13 expression, MCF7 and MDA-MB-231 cells were treated every 24 h with 5 μM 5-aza-2’-deoxycytidine for 5 days.

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Bisulfite conversion and quantitative methylation-specific polymerase chain reaction

Approximately 300 ng of genomic DNA was bisulfite modified with EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer’s protocol. Bisulfite-converted DNA was subjected to real-time quantitative methylation-specific polymerase chain reaction (qMSP) to determine the methylation status of HOXB13 using the SYBR Green-based detection technology (Applied Biosystems, Foster City, CA). All polymerase chain reactions (PCRs) were assayed in triplicate with melt curve analysis and performed on an ABI 7500 (Applied Biosystems). The HOXB13 MSP primers interrogate five CpG dinucleotides spanning –40 to +57 bp relative to the HOXB13 transcriptional start site. To normalize the amount of methylated DNA among test samples, total input DNA was assayed by a separate PCR amplification using primers devoid of CpG dinucleotides targeting a region of COL2A1. Primer sequences for test genes and COL2A1, as well as PCR conditions, are available on request. Experimental methylated and reference DNA copy number was determined by six-point absolute standard curves (125 000 to 40 copies) of cloned PCR products amplified from SssI-treated DNA (Chemicon, Temecula, CA). Cloned products were verified by sequencing. Methylation levels were expressed as the ratio of the methylated target copy number to COL2A1 copy number in the same sample and calibrated by the ratio in amplified SssI-treated DNA, giving percentage of methylated reference (PMR). Methylation events were classified by applying a PMR threshold value of >4% in accordance with established protocols (15).

Bisulfite sequencing

Bisulfite-modified DNA was subjected to PCR with primers that interrogate 25 CpG dinucleotides spanning upstream of the HOXB13 transcriptional start site and downstream into the first exon. The HOXB13 qMSP primers interrogated CpG positions 5, 6, 7, 14 and 15 of the bisulfite sequencing reaction. PCR products were cloned and 8–10 clones were sequenced for each sample. Bisulfite sequencing data were analyzed by BiQ Analyzer (16).

Quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated by Trizol (Invitrogen) and further purified by RNAeasy kit (Quiagen) following a modified protocol. Reverse transcription reactions were performed with oligo dT using Superscript III (Invitrogen). RT–PCR primers for HOXB13 were designed using Oligo v6.59 (Molecular Biology Insights, Cascade, CO) and are available by request. Primers for the reference gene TATA binding protein were described previously (17). Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was performed using Power SYBR Green master mix (Applied Biosystems, Foster City, CA). All polymerase chain reactions were performed with oligo dT using Superscript III (Invitrogen). RT–PCR primers for HOXB13 were designed using Oligo v6.59 (Molecular Biology Insights, Cascade, CO) and are available by request. Primers for the reference gene TATA binding protein were described previously (17). Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was performed using Power SYBR Green master mix (Applied Biosystems). Expression copy number was determined using absolute standard curves (125 000 to 40 copies) of cloned PCR products. All PCRs were assayed in triplicate and included melting curve analysis. Reactions were performed on an ABI 7500 (Applied Biosystems).

Immunoblotting

After exposing to E2 (10 nM), T47D cells were harvested at various periods of time and lysed in presence of proteinase inhibitors. Twenty micrograms of protein was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with mouse anti-HOXB13 (sc-28333; Santa Cruz Biotechnology) and labeled secondary antibody (sc-2030; Santa Cruz Biotechnology). Beta-actin (AB8227; Abcam, Cambridge, MA) was used as loading control with labeled secondary antibody (sc-2031; Santa Cruz Biotechnology). Proteins were detected by enhanced chemiluminescence using an Amersham Biosciences ECL kit (GE Healthcare, Piscataway, NJ).

Statistical analysis

Published Affymetrix expression data for breast cancer cell lines by Neve et al. (18) were downloaded from http://cancer.lbl.gov/breastcancer/data.php. Cell lines were categorized by the PMR threshold and differences in HOXB13 expression were measured by Mann–Whitney U-test. Signmap plot 10 (Systat Software, San Jose, CA) was used for linear regression of HOXB13 methylation and expression in breast cancer cell lines. For the analysis, qMSP and qRT–PCR values were transformed by arcsine transformation. Statistical analyses were performed in Minitab 15 (Minitab, State College, PA) unless otherwise specified. In graphs of qRT–PCR and qMSP experiments, data are presented as mean ± SD. Differences in DNA methylation were assessed by Mann–Whitney U-test. Differences in expression after exposure to estrogen were assessed by paired t-test. All tests were two-tailed and P-values of <0.05 were considered significant.

Stat Ana 9 (StatCorp, College Station, TX) was used for univariate and multivariate analysis of clinicopathological and HOXB13 methylation data for the 120 patient cohort. The PMR threshold was used to delineate HOXB13 methylation as a categorical variable. Logistic regression analysis was used to predict the probability of ERa-positive cell lines. (A) Levels of HOXB13 methylation in 17 ERa-positive cell lines. (B) Levels of HOXB13 methylation in 21 ERa-negative cell lines. Published Affymetrix U133A gene expression data for breast cancer cell lines analyzed for HOXB13 DNA methylation were obtained from Neve et al. (18). (C) Box plot of HOXB13 expression array data. The 38 cell lines were categorized by HOXB13 methylation status (4% PMR threshold) and centered RNA HOXB13 expression values were plotted. Box represents interquartile range, inset horizontal line represents median, vertical lines represent 95% CIs.
measure the association of HOXB13 methylation with ERα status. For multivariate logistic regression analysis, all the other covariates were included in the model already containing ERα status one by one and the variables with P-value < 0.20 were included in the final model. Three different models were obtained. Interactions were tested among the two variables in each model. R was used to perform Cox proportional hazards regression for univariate and multivariate analyses of patients’ DFS and HOXB13 methylation data (19). The PMR threshold was used to delineate HOXB13 methylation as a categorical variable. The hazard ratio (HR) correlating HOXB13 methylation status with patient DFS was estimated. Cox proportional hazards regression models were used for the multivariate analysis of methylation status with the following patient/tumor characteristics: PR state, tumor size, number of nodes and age at time of surgery. Based on the PMR threshold, DFS curves were generated using the Kaplan-Meier method, and a log-rank test was used to test for differences.

**Results**

*Hypermethylation of the HOXB13 promoter CpG island is associated with gene silencing in ERα-positive breast cancer cells*

A qMSP assay was developed to determine the methylation status of five CpG dinucleotides spanning −40 to +57 bp relative to the transcription start site of HOXB13. Methylation status was classified by applying a PMR threshold value of >4% in accordance with...
established protocols (15). Promoter methylation levels were measured by this qMSP in 38 breast cancer cell lines recently characterized by global expression and genomic analyses (18). HOXB13 hypermethylation was detected in 26 (68%) of these cell lines and was significantly greater in ERα-positive than ERα-negative cell lines ($P = 0.0033$) (Figure 1A–B). When comparing the expression level of HOXB13 for each cell line derived from the published Affymetrix expression data, hypermethylation was strongly associated with reduced HOXB13 expression ($P = 0.0163$) (Figure 1C). The methylation status of IL17BR was also determined in these breast cancer cell lines by qMSP. There was no significant difference in methylation with respect to hormone receptor status (supplementary Figure S1 is available at Carcinogenesis Online).

Bisulfite sequencing of MCF7T (the MCF7 line routinely cultured by our laboratory) confirmed that this cell line exhibited a high level (95%) of methylation in the HOXB13 promoter CpG island (Figure 2). T47D cells displayed substantially lower levels (50%) of methylation downstream of the transcription start site (Figure 2A). Virtually no methylation was detected in normal, non-transformed mammary epithelial cells (Figure 2A). Treatment with 5-aza-2'-deoxycytidine, a demethylating agent, led to a reduction of DNA methylation levels and partial restoration of HOXB13 expression in MCF7T cells (Figure 2B and C). We used linear regression to investigate the significance of the relationship between promoter hypermethylation and HOXB13 expression in 10 breast cancer cell lines and found an inverse association ($R^2 = 0.7817, P < 0.005$) between DNA methylation and reduced gene expression (Figure 2D).

The expression of HOXB13 is differentially repressed through estrogen signaling in ERα-positive cancer cell lines, MCF7H and T47D

The above finding and a recent study (20) implicate that the methylation-associated silencing of HOXB13 may be regulated in an ERα-dependent manner. We therefore investigated the effects of estrogen on the expression of HOXB13 in the MCF7H cell line, which is independent of that (MCF7T) routinely analyzed in our laboratory. It was initially chosen because a low level of HOXB13 expression was detected, despite the presence of moderate methylation. We treated hormone-deprived MCF7H cells with 10 nM E2 and measured its HOXB13 messenger RNA level by qRT–PCR (Figure 3A). No effect was observed after 30 min of E2 treatment (data not shown). After 3 h, estrogen repressed HOXB13 expression 1.75-fold ($P < 0.005$). Next, we used a second cell line, T47D, for more detailed studies because HOXB13 was partially methylated and expressed at higher levels than that of MCF7H (Figure 2D). Similarly, hormone-deprived T47D cells were treated with 10 nM E2, and HOXB13 expression was measured by both qRT–PCR and western blotting. The results showed significant estrogen-induced changes in HOXB13 messenger RNA levels, increasing after 30 min of the treatment ($P < 0.005$) and decreasing below the control after 3 h ($P < 0.05$) (Figure 3B). We also observed a similar trend in estrogen’s effect on HOXB13 protein expression (Figure 3C). In support of our evidence that it is an estrogen-responsive gene, bioinformatics analysis identified a potential ERα cis-regulatory region upstream of the HOXB13 transcriptional start site (supplementary Table SI is available at Carcinogenesis Online).

**Fig. 3.** Expression of HOXB13 is differentially regulated via estrogen signaling in breast cancer cells. (A–B) Absolute expression levels of HOXB13 were assessed by qRT–PCR in ERα-positive MCF7H and T47D cells as described in Figure 2. Cells were hormone deprived for 72 h followed by treatment with 10 nM E2 or vehicle (dimethyl sulfoxide) for the indicated times. Data presented relative to 0 h time points. Results are the mean ± SD of two independent experiments. Significance of messenger RNA (mRNA) level changes relative to 0 time point were measured by paired t-test; *$P < 0.05$ and **$P < 0.005$. (A) Effect of estrogen on HOXB13 expression in MCF7H cells. (B) Effect of estrogen on HOXB13 expression in T47D cells. (C) Effect of estrogen on HOXB13 protein expression in T47D cells. Hormone-deprived T47D cells were treated with 10 nM E2 or dimethyl sulfoxide for the indicated times. HOXB13 protein levels in whole-cell lysates were assayed by western blotting. Beta-actin protein was used as a loading control. (D) Effect of 4-OHT and estrogen on HOXB13 expression in T47D cells. Hormone-deprived T47D cells were treated with vehicle or 1 μM 4-OHT for 48 h prior to E2 treatment. Cells were then coadministered with 1 μM 4-OHT and 10 nM E2 for the indicated times. Absolute expression levels of HOXB13 were assessed by qRT–PCR as described in Figure 2. Results are presented relative to vehicle (data not shown). Columns, mean (n = 3); bars, standard deviation.
Interestingly, treatment with 4-OHT abrogated the ability of E2 to repress HOXB13 expression in T47D cells; as a result of this derepression, its messenger RNA levels steadily increased over a 24 h period (Figure 3D).

**Hypermethylation of the HOXB13 promoter CpG island frequently occurs in ERα-positive breast tumors**

To determine whether promoter hypermethylation of HOXB13 occurred in vivo, we performed qMSP in 120 primary breast tumors (supplementary Table SII is available at Carcinogenesis Online). Hypermethylation was detected in 36 (30%) of the tumors analyzed, 30 (38%) of which were ERα-positive tumors, whereas only 6 (14%) receptor-negative tumors were methylated (Figure 4A). No HOXB13 methylation was detected by qMSP in seven paired tumor adjacent normal (Figure 4B) or in 10 normal reduction mammoplasty tissues (data not shown). An analysis of IL17BR DNA methylation was also performed by qMSP in a subset of primary tumors (supplementary Figure S1 is available at Carcinogenesis Online).

Hypermethylation of HOXB13 was strongly associated with patients’ ERα status [odds ratio (OR) = 3.75, 95% confidence interval (CI) 1.41–9.96; P = 0.008] in a univariate analysis. Multivariate logistic regression models were performed to correlate this aberrant epigenetic event with other clinicopathological variables (Table I). Hypermethylation of HOXB13 was independently associated with tumors >5 cm (OR = 6.34, 95% CI 1.63–24.58, P = 0.008), having more than three positive lymph nodes (OR = 2.99, 95% CI 1.11–8.11, P = 0.031), and with PR-negative status (OR = 0.27, 95% CI 0.07–0.99, P = 0.048). Interactions were tested among the two variables in each model, but none were significant.

**Hypermethylation of HOXB13 is associated with poor DFS in ERα-positive breast cancer patients**

We used Cox proportional hazard analysis to test for a relationship between hypermethylation of HOXB13 and clinical outcome in 57 of the ERα-positive patients (Table II). In univariate analysis, hypermethylation of HOXB13 was significantly associated with poor DFS (HR = 3.25, 95% CI 1.13–9.37, P = 0.029). As expected, tumor size, lymph node metastasis and PR status were each associated with DFS (Table II).

In conjunction with the methylation status of HOXB13, tumor sizes >5 cm were associated with poor DFS (HR = 4.43, 95% CI 1.18–16.62, P = 0.027) as was PR-negative status (HR = 0.26, 95% CI 0.07–0.91, P = 0.035) (Table II). After controlling for PR status, hypermethylation of HOXB13 remained significantly associated with poor DFS (HR = 4.01, 95% CI 1.16–13.38, P = 0.028). The sample size was insufficient to provide a power of 80% for models including greater than two patient covariates. The predictive value of HOXB13 hypermethylation for DFS was visualized by Kaplan–Meier curve (Figure 4C).

**Discussion**

The present finding supports a recent study by Wang et al. (20) that the expression of HOXB13 is regulated by estrogen signaling. Using a lower concentration (1 nM) of E2, these authors observed a direct

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**Fig. 4.** DNA methylation profiles of HOXB13 in primary tumors and adjacent normal tissues. Bisulfite-converted DNA samples were analyzed by qMSP as described in Figure 1. (A) Analysis of HOXB13 methylation in 78 ERα-positive and 42 ERα-negative primary breast cancers. The solid horizontal line represents 4% PMR, the threshold level for classifying a significant DNA methylation event. (B) HOXB13 methylation analysis of paired tumor (T) and tumor adjacent normal (N) tissues from seven breast cancer patients. Columns, mean (n = 3); bars, standard deviation. (C) Relationship between HOXB13 methylation status and DFS curves analyzed in 57 ERα-positive breast cancer patients. Kaplan–Meier curves were plotted based on methylation status (PMR >4%).
repressive effect of estrogen on HOXB13 in MCF7 and T47D cells. However, we observed a biphasic response of HOXB13, similar to the condition of other estrogen-repressive genes (21,22), when ERα-positive breast cancer cells were treated with a higher dose of E2 (10 nM). The biphasic regulation of HOXB13 in T47D cells may result from either non-genomic estrogen signaling or differential ERα recruitment mediated by a cooperating transcription factor. Bioinformatics analysis identified a potential ERα cis-regulatory region upstream of the HOXB13 locus consisting of three known binding motifs: AP-1, Forkhead and estrogen response element half site (supplementary Table S1 is available at Carcinogenesis Online). The hormone-responsive corepressor NRP1 mediates late, direct gene repression through ERα binding to AP-1 motifs (23). We speculate that early estrogen-induced HOXB13 upregulation may occur downstream of a membrane-initiated non-genomic signal transduction pathway or via direct ERα recruitment to the estrogen response element half site. Subsequent downregulation of HOXB13 may involve an NRP1-associated ERα complex recruited to the AP-1 site. Further studies are warranted to test these hypotheses.

We demonstrated that administration of 4-OHT abolished E2-mediated repression in T47D cells and actually led to a sustained induction of HOXB13 in MCF7 and T47D cells. Furthermore, we found that HOXB13 induction is enhanced in ERα-positive breast cancer patients undergoing tamoxifen therapy. As discussed earlier, over-expressed HOXB13 may disrupt cellular homeostasis, resulting in further promotion of tumor cell proliferation (12). Therefore, this aberrant upregulation could potentially account for the high expression ratio of HOXB13 to IL17BR observed in ERα-positive breast cancer patients who develop subsequent tamoxifen resistance (2–5).

Our further analysis suggests that epigenetic mechanisms play a role in this estrogen-mediated repression. Increased density of methylation in the HOXB13 promoter CpG island correlates with the reduction of gene expression in ERα-positive breast cancer cells (Figure 2D). In a scenario with persistent activation of estrogen signaling, DNA methyltransferases and other epigenetic components are recruited to methylate CpG sites within the HOXB13 promoter CpG island, leading to permanent gene silencing (13,14). This epigenetic effect is also a potential mechanism for the absence of HOXB13 expression in vivo. In a large clinical study of 1252 primary tumors, HOXB13 expression was undetectable in 41% of ERα-positive tumors (5). These results are consistent with the current study showing that HOXB13 hypermethylation occurs in 38% of ERα-positive tumors (Figure 4A).

Because this study investigated HOXB13 only as an epigenetic marker in patients, its findings must be interpreted separately from four HOXB13:IL17BR expression ratio studies primarily for early stage, node-negative and ERα-positive patients (2–5). Compared with these four cohorts, our studied population has a lower percentage of both node-negative patients and tumors ≤2 cm, whereas at the same time a higher percentage of patients with three or more positive lymph nodes and tumors >5 cm. In addition, nearly half of our studied tumors are poorly differentiated. The multivariable models (Table I) indicate that HOXB13 hypermethylation correlates with established predictors of poor prognosis such as PR-negative status (24,25), increasing lymph node metastases (26,27) and increasing tumor sizes (28,29). This hypermethylation is also associated with poor DFS in ERα-positive breast cancer patients (Figure 4C). The relatively small sample size makes the 95% CI surrounding the point estimates of both the ORs and HRs large (see Tables I and II). This sample size limits our ability to construct more comprehensive models for Cox proportional hazard analysis. However, suggested by these data, the robustness of HOXB13 hypermethylation as a potential prognostic factor merits future investigation in a large cohort.

In conclusion, dysregulation of the estrogen-regulated HOXB13 may have a dual role in breast tumorigenesis. On one hand, the expression of HOXB13 may be enhanced in ERα-positive tumors exposed to the long-term tamoxifen treatment. This drug-induced overexpression may signify the establishment of endocrine resistance
in the treated patients. On the other hand, *HOXB13* may already undergo epigenetic silencing, which is triggered by estrogen signaling in other ERβ-positive tumors. In this case, *HOXB13* hypermethylation probably is a later event of tumor progression and can be a prognostic indicator for advanced breast cancer.

**Supplementary material**

Supplementary Figure S1 and Tables SI and SII can be found at http://carcin.oxfordjournals.org/

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**References**


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