

SHORT COMMUNICATION

Reactivation of epigenetically silenced *HER4/ERBB4* results in apoptosis of breast tumor cellsPM Das¹, AD Thor², SM Edgerton², SK Barry³, DF Chen³ and FE Jones¹¹Department of Cell and Molecular Biology, Tulane University, New Orleans, LA, USA; ²Department of Pathology, University of Colorado Denver, Aurora, CO, USA and ³School of Medicine, Tulane University, New Orleans, LA, USA

Experimental and clinical data support a growth inhibitory role for HER4 in breast cancer. Clinically HER4 expression is extinguished during breast tumorigenesis supporting a tumor suppressor function for HER4, however, a molecular mechanism to explain the selective loss of HER4 expression has remained elusive. Epigenetic mechanisms, for example, aberrant gene promoter hypermethylation, have been shown to ablate tumor suppressor gene expression in breast carcinomas. We identified a CpG island within the HER4 promoter and show by pyrosequencing of bisulfite-treated DNA an inverse correlation between HER4 expression and the extent of promoter methylation. Treatment of the HER4-negative BT20 cell line with the DNA demethylating agent 5-aza-2'-deoxycytidine (DAC)-enhanced HER4 expression, confirming a role for DNA methylation in suppressed HER4 expression. DAC treatment to reactive HER4 expression in combination with the HER4 ligand heregulin- β 1 (HRG) resulted in apoptosis of BT20 cells providing a novel therapeutic strategy for triple-negative tumors. The BT20 cells were rescued from apoptosis when preincubated with HER4 small interfering RNA, thereby confirming a role for HER4 in DAC/HRG-induced apoptosis. We verified HER4 promoter methylation in primary breast carcinomas and detected a significant increase in HER4 promoter methylation in HER4-negative breast tumors ($P < 0.001$). Furthermore, increased levels of HER4 promoter methylation were significantly associated with worse patient prognosis ($P = 0.0234$). Taken together, our data support a tumor suppressor function for HER4, which is epigenetically suppressed in breast tumors through promoter hypermethylation.

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Introduction

The HER4/ERBB4 receptor tyrosine kinase belongs to the epidermal growth factor receptor-family of potent

breast oncogenes. However, HER4 expression in breast carcinomas independently predicts improved patient survival (Thor *et al.*, 2009) while inversely correlating with tumor grade, metastasis and disease recurrence (Sundvall *et al.*, 2008; Kreike *et al.*, 2009). Furthermore, when examined in the context of the recently defined molecular subtypes of breast tumors, HER4 expression is most prevalent in the luminal subtypes associated with the best overall patient survival while HER4 is rarely expressed in the HER2-positive or triple-negative tumor subtypes with the worst patient prognosis (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Hoadley *et al.*, 2007). When expressed in tumor subtypes associated with poor prognosis, HER4 predicts improved patient response to multiple therapeutic interventions (Naresh *et al.*, 2008; Sassen *et al.*, 2009).

Experimental models provide mechanistic support for these clinical observations and further suggest that HER4 has potent anti-tumor activity in the breast. For example, HER4 activation inhibits proliferation of multiple breast tumor cell lines by promoting differentiation and/or apoptosis (Naresh *et al.*, 2006; Muraoka-Cook *et al.*, 2006b; Jones, 2008). In preclinical xenograft models, HER4 apoptotic activity is essential for tamoxifen-mediated tumor cell killing and patients with tumor expression of HER4 respond to tamoxifen therapy with no failures after 14 years (Naresh *et al.*, 2008). Ligand-activated HER4 has also been shown to cooperate with BRCA1 to induce a G2/M delay during breast tumor cell cycle progression (Muraoka-Cook *et al.*, 2006a). Although under some experimental conditions HER4 has also been shown to promote breast tumor cell proliferation (Zhu *et al.*, 2006; Jones, 2008; Muraoka-Cook *et al.*, 2009) these results require clinical verification.

Although loss of HER4 expression during breast tumor progression to higher grade and metastatic carcinomas is supported clinically, (Sundvall *et al.*, 2008) a mechanism for the selective loss of HER4 activity during breast tumorigenesis remains elusive. Independent laboratories have identified somatic mutations, which potentially attenuate HER4 activity (Soung *et al.*, 2006; Rokavec *et al.*, 2007; Tvorogov *et al.*, 2008). These mutations, however, were only observed in a small percentage (1–5%) of breast tumors and therefore cannot account for the absence of HER4 expression observed in 32% of all breast tumors (Thor *et al.*, 2009) and 90% of the most aggressive breast carcinomas

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(Hoadley *et al.*, 2007). Epigenetic mechanisms of gene suppression are commonly observed in breast tumors and one epigenetic event involving hypermethylation of gene promoter CpG sites has been shown to abolish expression of multiple tumor suppressors in the breast including BRCA1 (Esteller, 2007). In this study, we show that hypermethylation of a CpG island in the *HER4* promoter is associated with suppressed HER4 expression in breast tumors. Reactivation of HER4 expression results in tumor cell apoptosis supporting a tumor suppressor function for HER4 in the breast.

Results and discussion

The HER4 promoter is hypermethylated in HER4-negative breast tumor cell lines

In breast cancer, aberrant hypermethylation of CpG islands within gene promoters is a common mechanism of tumor suppressor inactivation (Esteller, 2007). To determine if promoter hypermethylation regulates HER4 expression in breast tumor cells, we first analyzed the *HER4* gene for the presence of CpG islands using UCSC Genome Bioinformatics. One CpG island was identified positioned at -580 to +1107 relative to the *HER4* transcriptional start site (Figure 1). Extension of the CpG island into the first exon-intron sequence is potentially significant because these CpG sites can affect chromatin structure and gene regulatory transcription factor binding (Strathdee *et al.*, 2004). In addition, exon-intron CpG sites may serve as 'seed' sequences for upstream CpG methylation and gene silencing (Nguyen *et al.*, 2001). An analysis of *HER4* promoter methylation by pyrosequencing of bisulfite-treated DNA isolated from multiple breast tumor cell lines revealed a striking inverse correlation between HER4 expression and the extent of promoter methylation (Figures 2a-c).

To determine the methylation status of each CpG site between -274 and +43, we analyzed DNA from three representative cell lines by direct bisulfite DNA sequencing. For each cell line, multiple DNA strand copies

were analyzed by cloning the bisulfite PCR product and sequencing at least 10 individual clones. None of the 42 CpG sites was methylated in the 11 individual DNA isolates analyzed from HER4-expressing T47D cells (Figure 2d). In concordance with decreasing levels of HER4 expression, the extent of CpG site methylation increased within MDAMB468, and was even greater within BT20 cells (Figure 2d). Taken together, our data suggest that HER4 expression is suppressed in breast tumor cells through hypermethylation of multiple CpG sites within the *HER4* promoter.

Reactivated expression of HER4 promotes breast tumor cell apoptosis

We have previously shown that HER4 functions as a pro-apoptotic BH3-only protein of the BCL-2 family and when ligand-activated HER4 induces apoptosis of multiple tumor cell lines (Vidal *et al.*, 2005; Naresh *et al.*, 2006). This apoptotic activity may not be unique to HER4. For example, HER2, another epidermal growth factor receptor-family member, undergoes caspase cleavage to generate a HER2 intracellular domain with pro-apoptotic BH3-only protein activity (Strohecker *et al.*, 2008). Epigenetic silencing of HER4 expression may provide a mechanism for breast tumor cells to evade HER4-induced apoptosis. Indeed, evasion of apoptosis is one of the obligate hallmarks of tumorigenesis (Hanahan and Weinberg, 2000). To determine if reactivated HER4 expression can suppress tumorigenesis, we reactivated HER4 expression by treating BT20 cells with the demethylating agent 5-aza-2'-deoxycytidine (DAC). This cell line had the highest levels of HER4 promoter methylation and resembles triple-negative breast tumors. Patients with advanced triple-negative breast tumors have extremely poor prognosis and because these tumors lack targetable molecular markers therapeutic options for these patients are limited to cytotoxic therapy. DAC treatment of BT20 cells resulted in increased HER4 expression detectable at both the RNA and protein levels (Figures 3a and c). These results further confirm that HER4 expression is suppressed in these breast tumor cell lines through a reversible DNA methylation mechanism.

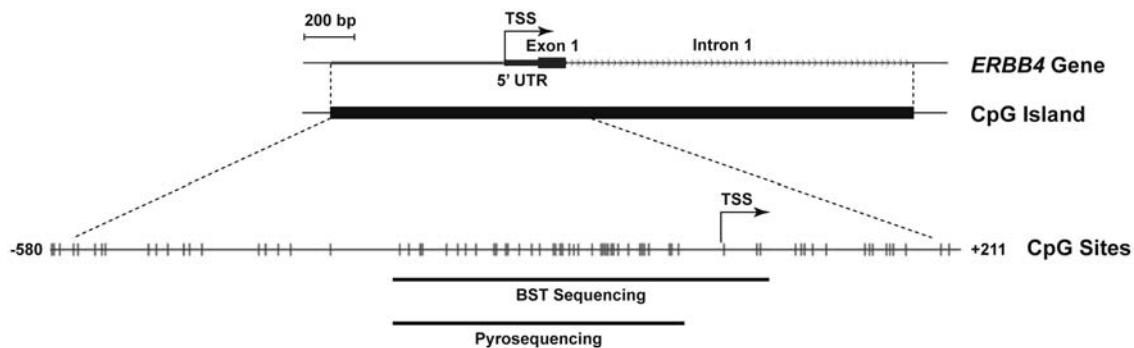


Figure 1 Identification of an *ERBB4* CpG island. A CpG island was identified within the *ERBB4* locus between -580 and +1107 relative to the transcriptional start site (TSS) using UCSC Genome Bioinformatics software. The lower portion shows a schematic of the CpG island between -580 and +211 relative to the TSS with CpG sites indicated by vertical black lines. Regions amplified by PCR for BST sequencing and pyrosequencing are indicated.

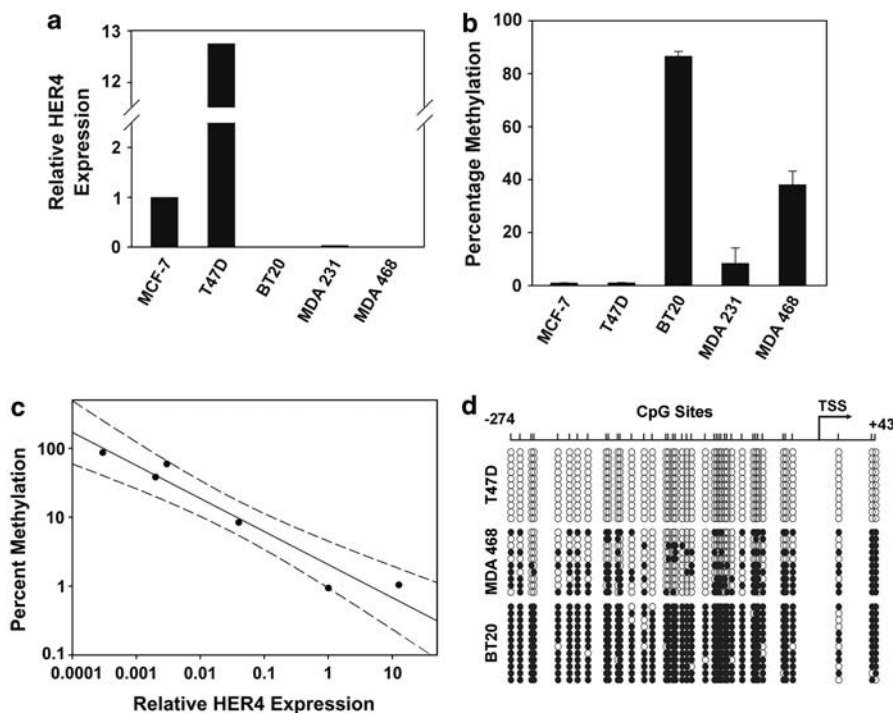


Figure 2 The *HER4* promoter is hypermethylated in breast tumor cells. (a) Relative HER4 expression determined by quantitative reverse transcriptase (qRT)-PCR with each cell line normalized to MCF-7 cells. Total RNA was isolated from cell lines using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturers' instructions. Superscript III (Invitrogen Life Technologies) was used to generate complementary DNA and HER4 expression was determined using ERBB4 TaqMan PCR primers and probe (assay ID Hs00171783_m1) with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a ABI Prism 7000 (Applied Biosystems). HER4 expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (assay ID Hs99999905_m1) and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative HER4 expression levels. (b) The CpG sites between -274 and -26 were analyzed by pyrosequencing and the mean methylation was determined. Data represent the mean and standard error of three independent DNA purifications. For pyrosequencing, the region of the *ERBB4* CpG island between -130 and -62 (CpG sites 24-38) was amplified from bisulfite-treated DNA using primers 5'-GTTYGTTTTGGGAGTYGTTATAT-3' and biotinylated 5'-ATCCAAATAACATATCCCCCTTT-3'. The sequencing primer 5'-GTTTTGGGAGTYGTTATA-3' was used for pyrosequencing at EpigenDx (Worcester, MA, USA). (c) Simple regression plot with 95% confidence interval in which each point represents one breast tumor cell line. (d) Bisulfite sequencing analysis of HER4 CpG sites between -274 and +43 relative to the transcriptional start site (TSS) from 10 independent DNA clones for each cell line. Open boxes indicate unmethylated CpG sites and closed boxes indicate methylated CpG sites. DNA was isolated from cultured cells and bisulfite treated using EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. A region of the *ERBB4* CpG island between -274 and +43 relative to the transcriptional start site (TSS) was PCR amplified using primers designed for bisulfite-modified DNA (5'-AGTGAGAGAGAGAGAAAGTGAGGAG-3' and 5'-CACCCAAACCCAAAATCCTA-3') and PCR products were cloned into TOPO TA Cloning Kit (Invitrogen Life Technologies). DNA was purified from 10 or 11 clones for each cell line and sequenced.

We have previously shown that heregulin- β 1 (HRG) stimulation of endogenous HER4 results in apoptosis of breast tumor cell lines (Naresh *et al.*, 2006), however, non-malignant cells appear to be resistant to HER4 apoptotic activity (Vidal *et al.*, 2007). In non-transformed cells, the HER4 intracellular domain and BH3-only protein, 4ICD, is sequestered to the nucleus in which it cooperates with STAT5A to regulate gene expression (Long *et al.*, 2003; Williams *et al.*, 2004). To determine if the apoptotic activity of reactivated HER4 can be induced in the BT20 cell line, we combined DAC treatment with HRG stimulation of the BT20 cell line. Treatment of BT20 cells with increasing concentrations of DAC resulted in an insignificant increase in cell death. Concurrent treatment with HRG, however, resulted in dramatic cell killing after 48 h of treatment (Figure 3b). Although DAC treatment typically affects expression of <1% of the human transcriptome, we

cannot rule out the possibility that the DAC/HRG combination induces apoptosis independent of HER4. To confirm a role for reactivated HER4 in DAC/HRG-mediated cell killing, we suppressed reactivated HER4 expression by concurrent HER4 RNA interference treatment. The DAC/HRG combination failed to promote significant levels of tumor cell apoptosis when HER4 expression was suppressed thereby demonstrating an obligate role for HER4 expression in DAC/HRG-induced apoptosis (Figures 3b and c). Taken together, these data suggest that breast tumor cells selectively suppress HER4 expression through promoter hypermethylation to evade the cell-killing activity of HER4. Interestingly, the *HRG* gene is also epigenetically silenced in breast carcinomas (Chua *et al.*, 2009; Fernandez *et al.*, 2010). Our observation that HRG stimulation of reactivated HER4 results in breast tumor apoptosis serves as an important proof-of-principle for

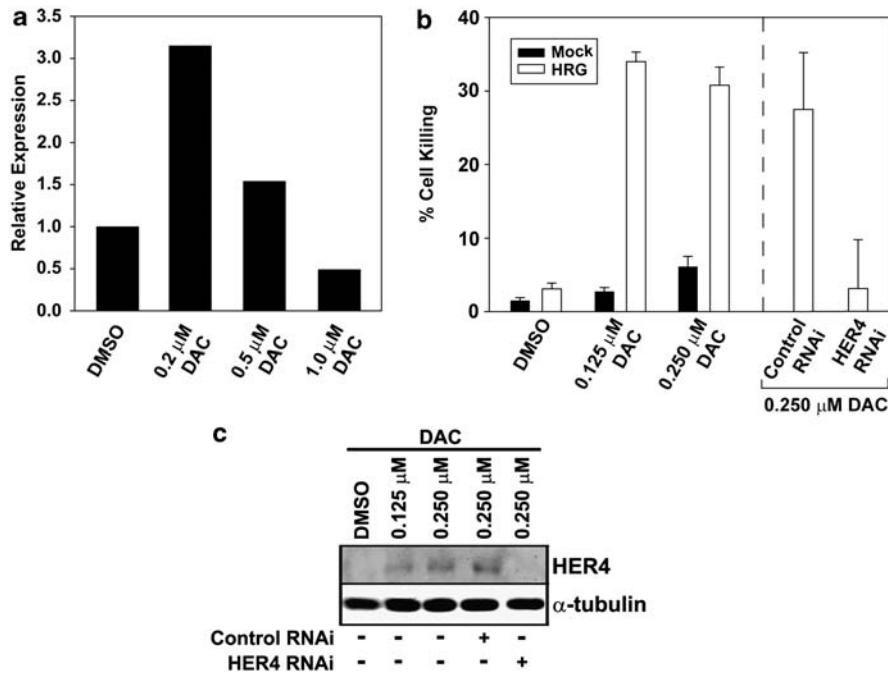


Figure 3 Reactivation of HER4 expression results in tumor cell apoptosis. (a) The BT20 breast tumor cell line was treated with the indicated amount of DAC for 48 h. HER4 expression after DAC treatment relative to dimethylsulphoxide (DMSO) vehicle control was determined by quantitative reverse transcriptase (qRT)-PCR as described in Figure 2a. (b) BT20 cells were treated with the indicated amount of DAC in combination with 50 ng/ml of HRG and analyzed for apoptosis after 48 h. The percentage of cells undergoing apoptosis was determined using a combination of 4',6-diamidino-2-phenylindole (DAPI) staining for condensed chromatin and TUNEL assay as described elsewhere (Naresh *et al.*, 2008). In some experiments, HER4 expression was suppressed using erbB-4/HER4 small interfering RNA (siRNA) SMARTpool or a nonspecific negative control pool (Upstate Biotechnology, Lake Placid, NY, USA) exactly as described elsewhere (Naresh *et al.*, 2008). BT20 cells were pretreated with HER4 siRNA 24 h before DAC and HRG treatments. Results represent the mean and standard error of at least three independent experiments. (c) Western blot analysis of HER4 expression in BT20 cells treated with DMSO or DAC in the presence or absence of control RNA interference (RNAi) or HER4 RNAi. Treatments were performed as described in panel (b). Total cell lysates were prepared and analyzed by western blot exactly as described elsewhere (Jones *et al.*, 1999). Primary antibodies used for western blot analysis included ErbB4 E200 (Abcam, Cambridge, MA, USA) and α -tubulin #05829 (Upstate Biotechnology). Secondary antibodies were IRDye 680 goat anti-rabbit (Li-Cor Biosciences, Lincoln, NE, USA) or IRDye 800 goat anti-mouse (Li-Cor Biosciences) detected using an Odyssey Infrared Imaging System (Li-Cor Biosciences).

marshaling HER4 apoptotic activity as a therapeutic approach to breast cancer.

HER4 promoter hypermethylation in primary breast tumors is associated with decreased patient survival

We next determined the levels of *HER4* promoter methylation in a cohort of 25 primary breast tumors previously analyzed for *HER4* expression by immunohistochemistry (Thor *et al.*, 2009). In the 13 *HER4*-positive tumors, the levels of *HER4* promoter methylation ranged from 0.5 to 4.3% with a mean of 2.4% (Figures 4a and b). *HER4* promoter methylation in 12 *HER4*-negative tumors had a significantly higher mean of 11.5% (Figures 4a and b; $P < 0.001$). These results implicate *HER4* promoter hypermethylation as an important mechanism promoting suppression of *HER4* expression in primary breast carcinomas. We expanded our cohort to 35 patients and determined the effect of *HER4* promoter methylation on patient survival using a cutoff of 3% methylation. We found that patients whose tumors had *HER4* promoter methylation levels $\geq 3\%$ had significantly shorter disease-specific survival

(Figure 4c; $P = 0.0234$). Strikingly, a failure in the low *HER4* methylation population was not observed until nearly 15 years after diagnosis. Taken together, our results provide an important clinical correlate implicating *HER4* promoter hypermethylation as an epigenetic event promoting breast tumor progression and reduced patient survival.

In summary, we have identified a molecular mechanism to explain the selective suppression of *HER4* expression observed clinically during breast tumorigenesis. Accordingly, we show that extinguished *HER4* expression is associated with promoter hypermethylation in both breast tumor cell lines and primary carcinomas. Furthermore, breast tumor *HER4* promoter methylation $> 3\%$ was significantly associated with poor patient prognosis. In fact, all patients in our cohort with tumor *HER4* promoter methylation $< 3\%$ survived for at least 15 years after diagnosis. Significantly, reactivation of epigenetically silenced *HER4* followed by HRG stimulation results in tumor cell apoptosis supporting a tumor suppressor function for *HER4* in breast cancer. Our results, therefore, provide a proof-of-principle therapeutic rationale for marshaling *HER4*

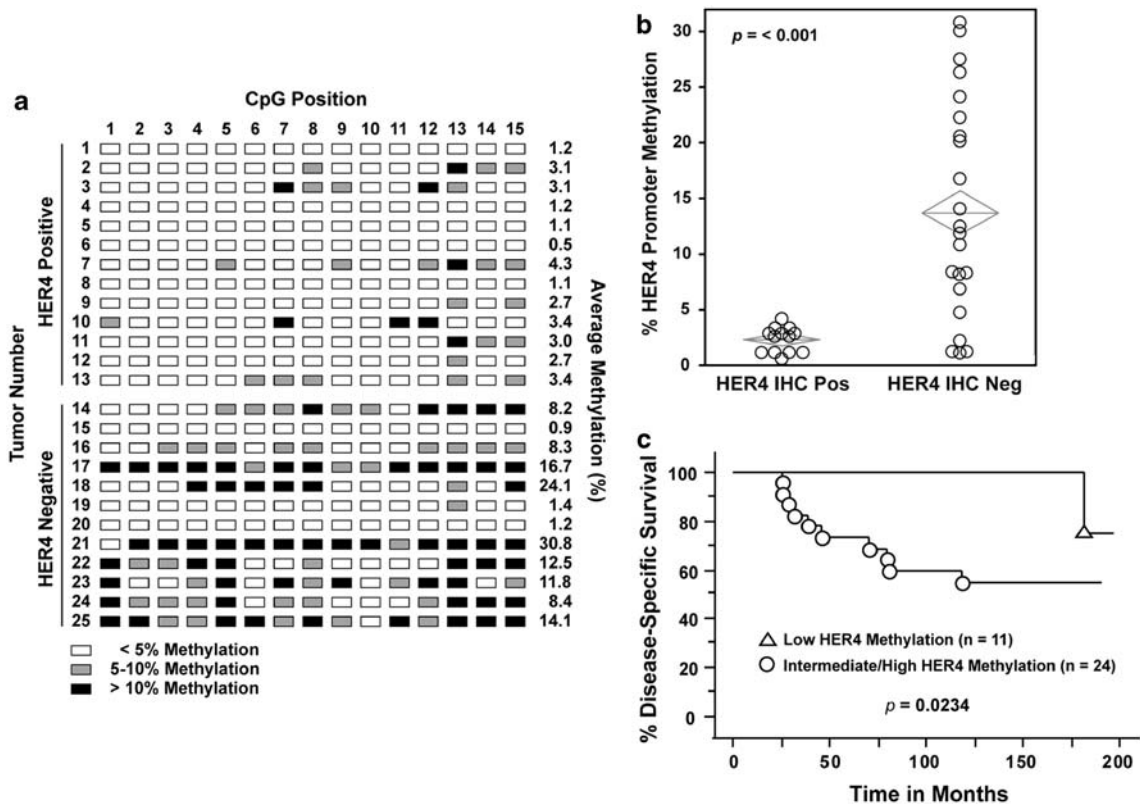


Figure 4 Tumor hypermethylation of the *HER4* promoter predicts breast cancer patient outcome. The patient population and *HER4* expression analysis by immunohistochemistry (IHC) are described elsewhere (Thor *et al.*, 2009). (a) Bisulfite-treated primary breast tumor DNA was analyzed by pyrosequencing as described in Figure 2d. DNA was isolated from 50 μ m sections of formalin fixed and paraffin embedded (FFPE) primary breast tumors and bisulfite treated at EpigenDx. The position and extent of methylation for each site in 13 *HER4*-positive and 12 *HER4*-negative tumors is indicated. (b) Box plot of *HER4* promoter methylation in *HER4*-positive ($n = 13$) and *HER4*-negative ($n = 22$) primary breast tumors ($P < 0.001$). (c) Kaplan–Meier survival curves for disease-specific survival (DSS). *HER4* methylation $< 3\%$ (triangles; $n = 11$) vs $\geq 3\%$ (circles; $n = 24$) ($P = 0.0234$). For Kaplan–Meier survival curves *HER4* methylation determined by pyrosequencing was dichotomized at $< 3\%$ vs $\geq 3\%$. Outcomes included DSS defined as the number of months between the diagnosis date and the date of death from breast cancer.

tumor cell-killing activity in breast carcinomas associated with the worst patient prognosis.

Conflict of interest

The authors declare no conflict of interest.

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