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Breast Cancer DNA Methylation Profiles in Cancer Cells and Tumor Stroma: Association with HER-2/neu Status in Primary Breast Cancer

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Abstract

The *HER-2/neu* gene is amplified and overexpressed in 20% to 30% of invasive breast carcinomas and is associated with increased metastatic potential and less tamoxifen sensitivity. We generated the DNA methylation profiles of 143 human breast tumors and found significant differences in HER-2/neu expression and DNA methylation of five genes. For three of these five genes [*PGR* (coding for the progesterone receptor), *HSD17B4* (coding for type 4 17-β-hydroxysteroid dehydrogenase, an enzyme that mainly degrades active 17-β-estradiol into inactive metabolites), and *CDH13* (coding for H-cadherin)] a higher prevalence of DNA methylation in HER-2/neu-positive cancers was confirmed in an independent set of microdissected primary breast cancers. DNA methylation was not only present in cancer cells but also in the tumor stroma fraction. Of the isolated fractions in HER-2/neu-positive versus -negative cancers, 27.1% versus 10.5%, respectively, showed DNA methylation of the five genes (P = 0.011, Fisher's exact test). In Her-2++/+++ breast cancers, HSD17B4 mRNA expression was inversely associated with *HSD17B4* methylation (P = 0.04). These data support the view that in addition to HER-2/neu-associated signaling, epigenetic changes in cancer as well as in tumor stroma cells might attribute to the specific biological features of HER-2/neu-positive cancers. (Cancer Res 2006; 66(1): 29-33)

Introduction

Breast cancer is the most common malignancy among females in most western countries, where women have an overall lifetime risk of >10% for developing invasive breast cancer (1). The *HER-2/neu* (*erbB-2*) gene encodes a M_r 185,000 transmembrane glycoprotein that is a member of the epidermal growth factor receptor (EGFR or erbB) family of receptor tyrosine kinases. As the preferred heterodimerization partner among ligand-bound EGFR family members, HER-2/neu mediates lateral signal transduction, resulting in mitogenesis, apoptosis, angiogenesis, and cell differentiation (2). The *her-2* gene is amplified and overexpressed in 20% to 30% of invasive breast carcinomas and is associated with increased metastatic potential and decreased overall survival (2, 3). In addition, patients with HER-2/neu-positive:HR (hormone receptor)-positive tumors are less responsive to tamoxifen treatment than are patients with HER-2/neu-negative:HR-positive tumors (4). Letrozole, an aromatase inhibitor, is a more effective neoadjuvant endocrine therapy than tamoxifen for Her-2/neu-positive, estrogen receptor–positive primary breast cancer (5).

Trastuzumab (Herceptin; Genentech, Inc., South San Francisco, CA), a humanized monoclonal antibody directed against the extracellular domain of HER-2/neu, showed an improvement in time to progression, overall response, and duration of response and a favorable effect on survival in phase III randomized trials in combination with standard chemotherapy as compared with the same chemotherapy alone as therapy for metastatic breast cancer overexpressing HER-2/neu (6). Recent evidence suggests that adding trastuzumab to neoadjuvant chemotherapy results in a significantly higher number of complete pathologic remissions (7).

Molecular profiling of HER-2/neu-positive breast cancers has thus far focused primarily on the use of cDNA microarrays (8–11), and its results give rise to the hypothesis that the mammary stroma plays an important role in determining the clinical breast cancer phenotype.

This study explores the use of DNA methylation markers as an alternative approach to molecular profiling. Hypermethylation of promoter CpG islands, frequently observed in breast cancer (12–14), is often associated with transcriptional silencing of the associated gene. We used a moderate-throughput, fluorescence-based, semiautomated quantitative technique called MethyLight (15) to screen a panel of 35 methylation markers in 143 cases of breast cancer with known HER-2/neu status. Of these 35 markers, we identified five genes whose DNA methylation correlated with HER-2/neu status. In an independent set of eight HER-2/neu score +++ and eight HER-2/neu score 0 breast cancer cases, we confirmed the higher prevalence of DNA methylation of three (two of them are involved in estrogen metabolism) of the five genes and found them to also be methylated in the tumor stroma.

We propose that these differences in DNA methylation profile reflect the higher aggressiveness of HER-2/neu tumors and are at least partly responsible for reduced tamoxifen responsiveness.

Materials and Methods

Tissues. Of the 148 tumor samples described earlier (16), 143 samples with known HER-2/neu status were used for this study. Briefly, tumor samples were retrieved from the tissue bank of the Department of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Obstetrics and Gynecology, Innsbruck Medical University Hospital (Innsbruck, Austria). Clinical and pathologic data are stored in a database in accordance with hospital privacy rules and have been published earlier (16). Specimens were brought to the pathologist (E. Müller-Holzner) immediately after resection, and part of the tissue was placed in liquid nitrogen and stored at −80°C until lyophilization.

In addition, 25 nonneoplastic breast specimens from women who had surgery due to benign conditions of the breast (fibroadenoma and fibrocystic disease) have been used. Finally, paraffin-embedded tumor specimens from 16 additional patients without neoadjuvant treatment were also used for this study (for detailed description of clinicopathologic features, see Table 1).

Histopathologic analysis. All breast cancer specimens were reviewed by a single pathologist (E. Müller-Holzner). HER-2/neu status was determined by means of immunohistochemistry using the Dako HercepTest and scored with the Dako scoring system (Dako, Vienna, Austria).

DNA methylation analysis. Genomic DNA isolation, sodium bisulfite conversion, and MethyLight analysis were done as previously described (16).

Analysis of HSD17B4 mRNA expression. RNA extraction, reverse transcription, and real-time PCR amplification were done as described previously (17). Real-time PCR assays were conducted in triplicate for each sample, and the mean value was used for calculation. Primers and probes for *HSD17B4* were determined with the computer program Primer Express (Applied Biosystems, Foster City, CA). Primers for *HSD17B4* were forward primer 5′-ACC AAC TCC TTT GAA GTC CCC-3′, reverse primer 5′-GCC CTG GCT TTT GCA GAA A-3′, and probe 5′-FAM-CCC AAA TCA TTC ACA ACA ACT AAC GCT CCT-3′ TAM. BLASTN searches were conducted to confirm the total gene specificity of the nucleotide sequences chosen for the primers and probes. To prevent amplification of contaminating genomic DNA, the probe was placed at the junction between two exons. Primers and probes for the TATA box-binding protein (a component of the DNA-binding protein complex TFIID) as endogenous RNA control were used as described (17).

Laser-capture microdissection. The PixCell II LCM system (Arcturus Engineering, Mountain View, CA) was used for laser capture microdissection of paraffin-embedded tissues; 10-μm-thick sections from 16 breast cancer patients with invasive ductal cancer were used. For each analyzed fraction, ~1,000 cells were "laser-captured." DNA extraction was done using the Arcturus Pico Pure DNA Extraction Kit according to the manufacturer's instructions. DNA bisulfite modification and MethyLight analysis were done as described (18).

Statistics. The association between gene methylation and HER-2/neu expression was analyzed using the Spearman rank coefficient. Only genes with a significant correlation between the former variables were used for further analysis. The nonparametric Mann-Whitney *U* test was used to assess associations between *HSD17B4* methylation and its expression. $P < 0.05$ was considered statistically significant. All calculations were done using SPSS 10.0 (Chicago, IL).

Results

From the initial set of 148 primary breast cancers (16), we used the 143 cases with known HER-2/neu status to correlate with 35 DNA methylation markers with the HER-2/neu immunohistochemical staining intensity for HER-2/neu. By means of Spearman rank correlation, we identified 5 of 35 genes that showed significant correlation coefficients ($P < 0.05$; Supplemental Data). *CDH13, MYOD1, PGR*, and *HSD17B4* were positively associated with HER-2/neu expression, whereas *BRCA1* was negatively associated with HER-2/neu expression (Fig. 1); methylation of these genes in nonneoplastic breast specimens was either absent or at a low level (Fig. 1). None of the 35 genes yielded a level of significance that would remain significant after multiple test adjustment. To exclude the possibility that these associations were observed only by chance and to study whether the cancer cells and/or the tumor stroma are responsible for this HER-2/neu-specific methylation pattern we used an independent set of 16 primary archival paraffin-embedded breast cancers. Eight tumors were HER-2/neu-positive (score +++) and eight were

Table 1. DNA methylation markers in Her-2/neu-positive and -negative cancers

Her_2	age	tumor size	LN status	HR status	E_CDH13	S_CDH13	E_MYOD1	S_MYOD1	E_PGR	S_PGR	E_BRCA1	S_BRCA1	E_HSD17B4	S_HSD17B4
+++	70	pT2	pos	pos	■	n.d.	■	n.d.		n.d		n.d.		n.d.
+++	51	pT1c	neg	pos	■	n.d.	■	n.d.		n.d.	n.d.	n.d.		
+++	48	pT1c	neg	pos						■				
+++	67	pT2	neg	neg										
+++	64	pT1c	neg	pos	■			■						■
+++	57	pT1c	pos	pos			■	■	■	■				
+++	77	pT1c	pos	neg	■	■	■		■	■			■	■
+++	57	pT1c	pos	pos										
0	58	pT1b	neg	pos	■									
0	63	pT2	pos	pos										
0	77	pT2	neg	neg				■						
0	60	pT1c	pos	pos			■	■						
0	60	pT1b	neg	pos			■	■		■				
0	64	pT1c	neg	pos				■						
0	81	pT2	n.d.	pos										
0	67	pT2	neg	pos		n.d.		n.d.		n.d.		n.d.		

NOTE: Tumor specimens of primary breast cancers [eight HER-2/neu-positive (score +++) and eight -negative (score 0)] were used to analyze five genes in both fractions (E_, cancer epithelium; S_, tumor stroma). n.d., not done (mainly due to an insufficient amount of DNA); black boxes, methylated DNA detected; white boxes, no methylated DNA detected. LN status (lymph node status; pos, positive; neg, negative). HR status (hormone receptor status; pos, positive, estrogen- and/or progesterone receptor–positive, neg, negative).

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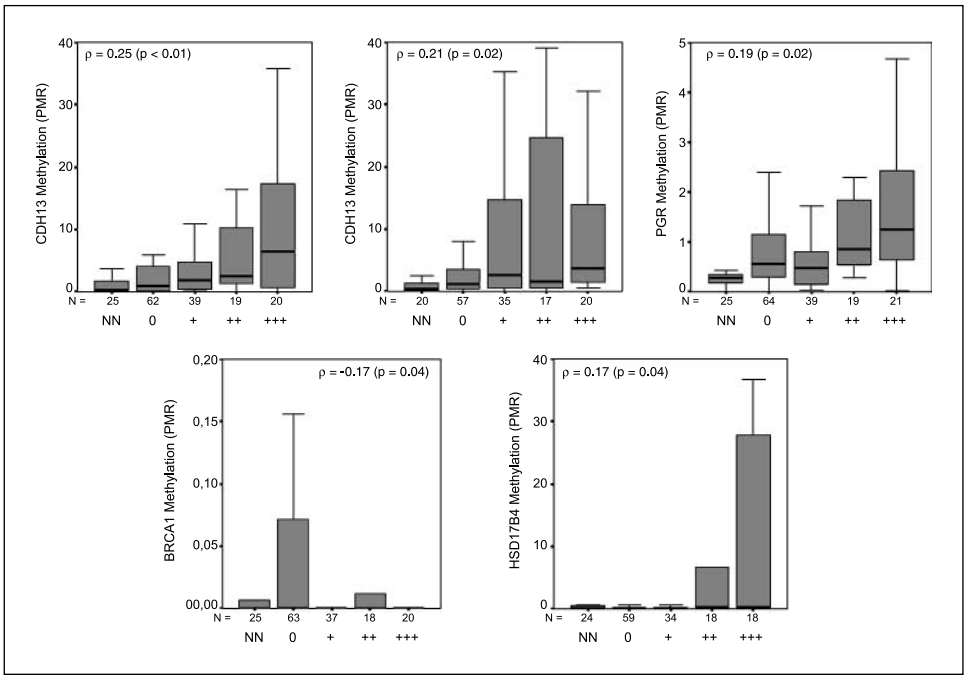

Figure 1. Association between DNA methylation and HER-2/neu expression (0, +, ++, +++) in primary breast cancer specimens. One hundred and forty-three frozen specimens of primary breast cancers were used to analyze DNA methylation of 35 genes (Supplemental Table S1). Box plots of the five genes that yielded significant correlation by means of the Spearman rank test are displayed; 5, 3, 14, and 14 values are missing for *BRCA1, CDH13, MYOD1*, and *HSD17B4* methylation, respectively. Percentage of fully methylated reference (PMR) values of 25 nonneoplastic specimens (NN) are also displayed; 5 and 1 values are missing for *MYOD1* and *HSD17B4*, respectively. For reasons of clarity (scaling of the *y* axis), extreme values were deleted from the blot.

HER-2/neu-negative (score 0). The HER-2/neu-positive and -negative groups did not differ with regard to age, tumor size, lymph node status, or hormone receptor status (Table 1). By means of laser capture microdissection, cancer cells as well as tumor stroma were dissected (examples shown in Fig. 2), and the DNA methylation status of the five genes were analyzed separately in the two tumor compartments.

For three genes (*PGR, CDH13*, and *HSD17B4*) DNA methylation was more prevalent in the HER-2/neu-positive cancers. In 5 of 8 versus 1 of 8 of the cancer cells and in 4 of 6 versus 1 of 7 of the stromal cells of the tumors (HER-2/neu-positive versus -negative) analyzed at least one of these genes was methylated (Table 1). *MYOD1* methylation was detected in 4 of 8 versus 2 of 8, and in 2 of 6 versus 4 of 7 (HER-2/neu-positive versus

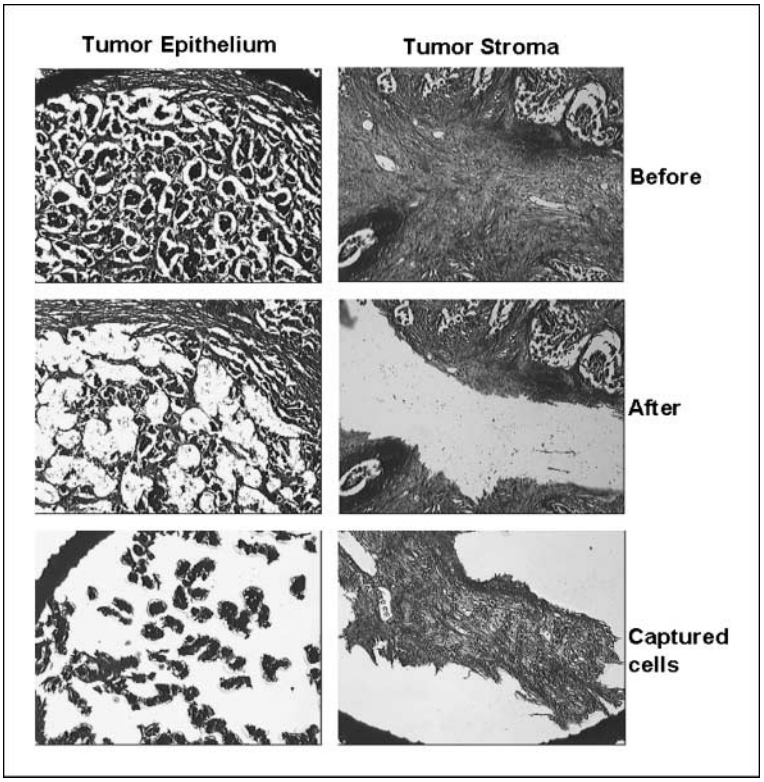

Figure 2. Example of extracted tumor epithelium and tumor stroma before and after laser-assisted microdissection and laser-captured cells.

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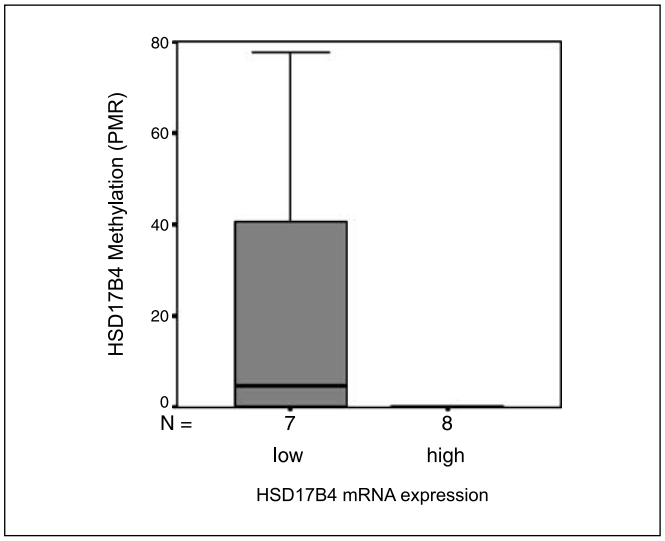

Figure 3. *HSD17B4* methylation and mRNA expression. HSD17B4 mRNA expression was studied in HER-2++/+++ primary breast cancer specimens and dichotomized in low and high expression; box plots show the association with extent of DNA methylation of this gene in the corresponding tumors. For reasons of clarity (scaling of the *y* axis), extreme values were deleted from the blot.

-negative) cancer cells and stromal cells, respectively. BRCA1 methylation was not detected in any of the microdissected cell fractions.

DNA methylation was shown in 19 of 70 (27.1%) and in 8 of 76 (10.5%) DNA preparations analyzed in the HER-2/neu-positive and HER-2/neu-negative cancers, respectively (Table 1). Comparison of overall methylation in HER-2/neu +++ cases versus HER-2/neu score 0 cases showed a significant difference ($P = 0.011$, Fisher's exact test).

HSD17B4 methylation has thus far not been linked to gene suppression. Therefore, we analyzed HSD17B4 mRNA expression in 15 specimens with HER-2/neu score ++/+++ positive primary breast cancers. All tumors expressed HSD17B4 mRNA. Only tumors with low HSD17B4 expression showed significant DNA methylation levels of the corresponding gene (Fig. 3; Mann-Whitney *U* test; $P = 0.04$), indicating that DNA methylation in HER-2/neu-positive breast cancers is at least partly responsible for the suppression of HSD17B4 expression.

Discussion

HER-2/neu positivity defines a subclass of patients with highly aggressive breast cancer resistant to specific treatment strategies. Whether these HER-2/neu-specific properties are direct, tyrosine kinase–mediated effects, or are indirect effects due to HER-2/neu-associated changes in the genome or epigenome, has not yet been clarified. Significant progress has been made in recent years towards the implementation of DNA methylation markers as clinical tools in cancer characterization. We here explore the hypothesis that HER-2/neu breast cancers may be characterized by a specific DNA methylation profile.

Interestingly, we found that most of the genes demonstrating significant correlations between their methylation status and the HER-2/neu status of the corresponding tumor are involved in hormonal regulation (*PGR* and *HSD17B4*) or are members of the cadherin family (*CDH13*). *PGR* codes for the progesterone receptor, and HER-2/neu-positive cancers show a lower level of progesterone receptor in the tumor (19). Recently, we showed a strong association between estrogen receptor and progesterone receptor gene methylation and expression (16): *PGR* methylation indicates the absence of the estrogen receptor. *HSD17B4* gene codes for the type 4 17-β-hydroxysteroid dehydrogenase, an enzyme that mainly degrades 17-β-estradiol into estrone and androst-5-ene-3β (20). Here, we show that significant levels of *HSD17B4* methylation are present only in HER-2/neu-positive (score ++/+++) breast cancers. In these cancers, low HSD17B4 mRNA expression is strongly linked to *HSD17B4* methylation, indicating that in HER-2/neu-positive breast cancers, HSD17B4 mRNA expression is at least partly regulated by DNA methylation. Estrogen deprivation therapy (by means of aromatase inhibition) seems to be more effective in HER-2/neu-positive breast cancers as compared with blocking the receptor for estrogen (by means of tamoxifen; ref. 5). High intratumor estrogen concentrations might prevent antiestrogens from blocking ER action and produce a resistant phenotype (20). Although we had no opportunity to analyze estradiol concentrations in the tumors we studied, we found that *HSD17B4* methylation is prevalent in HER-2/neu-positive cancers and that this is linked to low expression of this gene that is one of the few genes in breast cancers responsible for metabolizing estradiol, the active estrogen, into inactive metabolites. Therefore, we assume that HER-2/neu-positive cancers create (or reflect) an environment that may prevent tamoxifen's antitumor activities: (*a*) low level of functional estrogen receptor (reflected by *PGR* methylation; refs. 16, 21) and (*b*) low expression of 17-β-estradiol metabolizing enzymes (reflected by DNA methylation-mediated low expression of HSD17B4).

Decreased expression of cadherin molecules in invasive carcinomas results in cell scattering and decreased mediated cell-cell adhesion, which may enhance tumor progression and invasion. Although the role of *CDH1* has been studied extensively, there is evidence that *CDH13*, coding for H-cadherin, may also function as a tumor suppressor gene, and it is known to be suppressed by DNA methylation (22).

It is known that breast cancers in patients with *BRCA1* germ line mutations are more often negative for HER-2/neu (23). In concordance with this finding, in frozen tissue, we showed sufficient levels of *BRCA1* methylation (percentage of fully methylated reference values only up to 0.15) only in HER-2/neu-negative tumors (Fig. 1). Probably due to the very low methylation levels of *BRCA1*, we were not able to detect DNA methylation of this gene in any of the microdissected samples. *MYOD1* methylation difference depending on HER-2/neu status primarily found in frozen tissue (Fig. 1) could not be confirmed by analysis of an independent set of microdissected tumor samples (Table 1).

We detected DNA methylation not only in cancer cells but also found DNA methylation changes in the stroma of HER-2/neu-positive cancers. Recent evidence shows that HER-2/neu overexpression in the epithelial fraction of a tumor has a strong effect on the activity of the tumor stroma: in the mouse, mammary tumorigenesis was triggered in a single step by the overexpression of HER-2/neu transgene in the epithelial compartment of the mammary gland. A myofibroblast-like cell line that was derived from this tumor and did not express HER-2/neu transgene was highly aggressive and gave rise to sarcomatoid tumors (24). This indicates that HER-2/neu cancer cell signaling to the surrounding stroma is "memorized" there by means of epigenetic imprints. Genetic alterations have already been described in the tumor

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stroma independently of changes in cancer cells (25). After we finished this study, Hu et al. showed distinct epigenetic changes in cultured epithelial and myoepithelial cells and in stromal fibroblasts from normal breast tissue, and *in situ* and invasive breast carcinomas (26).

Although we used the laser capture microdissection technique, we cannot entirely exclude cross-contamination on a cellular or subcellular level between the two compartments, but the observation of discordant methylation (stroma and epithelium of the same tumor) in at least nine cases is a strong argument against this concern.

In conclusion, we identified DNA methylation changes that are more prevalent in cancer cells and tumor stroma in HER-2/neu-positive breast cancers than in HER-2/neu-negative breast cancers. These alterations could help explain the higher aggressiveness and resistance to antihormonal therapies of HER-2/neu-positive cancers.

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