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 17β-hydroxysteroid dehydrogenase, an enzyme that mainly degrades active 17β-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 Mr 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-positiveHR (hormone receptor)-positive tumors are less responsive to tamoxifen treatment than are patients with HER-2/neu-negativeHR-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 chemotheraphy 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
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). 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′. 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 TFIIID) 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...
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 -negative) groups.

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.

Figure 2. Example of extracted tumor epithelium and tumor stroma before and after laser-assisted microdissection and laser-captured cells.
HSD17B4 expression. \( P = 0.04 \), indicating that DNA methylation in HER-2/neu-positive cancers creates (or reflects) 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-\( \beta \)-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 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...
DNA Methylation and HER-2/neu Status

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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References
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