Identification of Markers of Taxane Sensitivity Using Proteomic and Genomic Analyses of Breast Tumors from Patients Receiving Neoadjuvant Paclitaxel and Radiation

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Abstract

Purpose: To identify molecular markers of pathologic response to neoadjuvant paclitaxel/radiation treatment, protein and gene expression profiling were done on pretreatment biopsies.

Experimental Design: Patients with high-risk, operable breast cancer were treated with three cycles of paclitaxel followed by concurrent paclitaxel/radiation. Tumor tissue from pretreatment biopsies was obtained from 19 of the 38 patients enrolled in the study. Protein and gene expression profiling were done on serial sections of the biopsies from patients that achieved a pathologic complete response (pCR) and compared to those with residual disease, non-pCR (NR).

Results: Proteomic and validation immunohistochemical analyses revealed that α-defensins (DEFA) were overexpressed in tumors from patients with a pCR. Gene expression analysis revealed that MAP2, a microtubule-associated protein, had significantly higher levels of expression in patients achieving a pCR. Elevation of MAP2 in breast cancer cell lines led to increased paclitaxel sensitivity. Furthermore, expression of genes that are associated with the basal-like, triple-negative phenotype were enriched in tumors from patients with a pCR. Analysis of a larger panel of tumors from patients receiving presurgical taxane-based treatment showed that DEFA and MAP2 expression as well as histologic features of inflammation were all statistically associated with response to therapy at the time of surgery.

Conclusion: We show the utility of molecular profiling of pretreatment biopsies to discover markers of response. Our results suggest the potential use of immune signaling molecules such as DEFA as well as MAP2, a microtubule-associated protein, as tumor markers that associate with response to neoadjuvant taxane–based therapy. Clin Cancer Res; 16(2); 681–90. ©2010 AACR.
Materials and Methods

Patients and neoadjuvant treatment. Women with high-risk (stages IIA-IIIB), operable breast cancer were treated with three cycles of paclitaxel (175 mg/m² every 3 wk), followed by twice weekly paclitaxel (30 mg/m²) and concurrent radiation. Patients underwent definitive surgery after completion of chemoradiation (19). Tissue samples were taken from individuals treated at Vanderbilt University or New York University Medical Centers with institutional review board approval. All patients signed a protocol-specific consent. Pretreatment core biopsies were analyzed for ER, PR, and HER2 as previously described (19). HER2 amplification was confirmed by fluorescence in situ hybridization when immunohistochemistry score was 2+. pCR was defined as the absence of any invasive cancer and NR was defined as any viable tumor in breast or lymph nodes (partial response) or those with progressive disease. pCR and NR were determined from the primary pathologic slide at the time of surgery by a breast pathologist. Median follow-up time for surviving patients was 51 mo (range, 40-73 mo).

Histology-directed MALDI-MS and proteomic data analysis. For MALDI-MS profiling, serial sections from each frozen biopsy were H&E-stained or thaw-mounted and fixed onto a MALDI plate. Photomicrographs of H&E-stained sections were annotated to mark areas (~200 μm, minimum of 10 spots) of interest for both tumor and stroma by a breast pathologist for matrix spotting. All normal, dysplastic or necrotic tissue, areas of inflammation, potential contamination (e.g., blood), and edges of tumor sections were avoided. Annotated H&E images were overlaid with MALDI plate images to extract coordinates for robotic spotting. An acoustic robotic spotter (LabCyte) placed crystalline matrix (20 mg/mL sinapinic acid in 1:1 acetone/methanol solution, washed, and incubated in 3% hydrogen peroxide. Blocking was continued using Dako Protein Block (Dako). Sections were incubated with α-defensin (DEFA) antibody (AbD Serotec) diluted 1:1,000 for 1 h at 25°C followed by exposure to a labeled streptavidin-biotin system (Dako). Scoring for immunohistochemical staining of DEFA and MAP2 and inflammation are described in the Supplemental Materials and Methods.

Gene expression profiling and data analysis. Of the 38 patients, 14 had sufficient tissue (4 with pCR, 10 with NR) for quality RNA. Invasive tumor cells from serial sections were captured onto polymeric caps using the PixCell II laser capture microdissection system (Arcturus). Areas of ductal carcinoma in situ and normal breast tissue were excluded and areas of inflammation were avoided. Total RNA was isolated from captured cells, quantified, integrity analyzed, and microarray analyses done as previously described using Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix; ref. 22). Data from duplicate RNA samples from each biopsy (n = 28) were imported into GeneSpring GX (version 10.0.2) software (Agilent). Probe level analysis was done using Guanine Cytosine Robust Multi-Array analysis that includes background correction, quantile normalization, and probe summarization. Probe sets were log-transformed and averaged among duplicate samples. An unpaired t test was used to identify differential expression of genes between pCR and NR samples. A FDR multiple testing t test (Benjamini-Hochberg) was used to generate corrected P values for each probe (23). A heat map was generated to show differentially expressed probes. An enrichment-based statistical test was used to generate P values for gene ontology categories using GeneSpring.

Cell culture and cell engineering. MCF-7 and MDA-MB-468 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 10 μg/mL insulin (MCF-7 cells), and 1% penicillin-streptomycin as previously described (24). All cells were cultured at 37°C with 5% CO₂. For three-dimensional culture models, cells were seeded on growth
factor–reduced Matrigel (BD Biosciences) as previously described (25–27). Paclitaxel (Sigma-Aldrich) was added 24 h after cell seeding and replaced every 3 d. Mammospheres were digested, trypsinized, and single cells counted using a hemocytometer.

To generate stable cell lines, MAP2 cDNA was cloned into the FG12 lentiviral vector. Viral production and gene transduction were done as previously described (28).

**Immunoblotting.** Cell lysates were immunoblotted as previously described using a MAP2 antibody, dilution 1:500 (Lab Vision/NeoMarkers), and a GAPDH antibody at a dilution of 1:1,000 (Chemicon International; ref. 21).

**Biomarker analysis and taxane-neoadjuvant patients.** For biomarker analysis, 64 pretreatment biopsies were obtained from patients that received taxane-based neoadjuvant chemotherapy. Patients were classified into those that received taxane-based neoadjuvant treatments including (a) paclitaxel/radiation, (b) dose-dense docetaxel alone, or (c) Adriamycin/cyclophosphamide followed by paclitaxel. Clinical variables (e.g., age, ER/PR, etc.) for patient and treatment comparisons are shown in Supplementary Table S1. Weighted index of MAP2 expression by immunohistochemical scoring was summarized using the median and interquartile range (25th to 75th percentile). Linear pair-wise correlations among variables were estimated using Spearman’s correlation. The odds of a pCR for a unit change in DEFA expression or inflammation and an interquartile change in MAP2 expression were estimated using logistic regression. $P < 0.05$ was considered statistically significant.

**Results**

**Neoadjuvant paclitaxel/radiation.** Patients with high-risk, operable breast cancer were treated with three cycles of paclitaxel followed by concurrent paclitaxel/radiation. Previously, we reported that mitotic index was a significant prognostic marker of response (pCR = 34%) to this presurgical therapy (19). For this study, we obtained sufficient tumor tissue from the same patient cohort to perform proteomic and gene expression analyses on 19 and 14 patients, respectively (Table 1).

**Proteomic tumor profiling.** We performed histology-directed MALDI-MS to identify protein markers of response to neoadjuvant paclitaxel/radiation from 19 of the 38 patients (6 with pCR) for which sufficient tissue was available. This technique allows for cellular specificity (200 μm diameter) and the generation of protein profiles for biomarker discovery (20). MALDI-MS profiles from each annotated tumor and stroma area were collected and averaged for each biopsy. Average tumor spectra from the 6 pCR and 13 NR patients were compared using two statistical methods (see Materials and Methods) to identify differentially expressed features (i.e., peak intensity of each $m/z$). Average NR spectra intensity was offset ($y = +400$) to visually compare differences in average pCR spectra (Fig. 1). Three features

| Table 1. Patient profiles and tumor features |
|-----------------|-----------------|----------------|-----------------|-----------------|
| **Patient no.** | **ER** | **PR** | **HER2** | **pCR** | **Outcome** | **Analyses** |
| **Proteomics** | **Microarray** |
| 1 | + | + | – | No | AWD | Yes | Yes |
| 2 | + | – | – | No | NED | Yes | Yes |
| 3 | + | + | + | No | NED | Yes | Yes |
| 4 | + | + | – | No | NED | Yes | Yes |
| 5 | – | – | – | No | AWD | Yes | Yes |
| 6 | – | – | + | No | DOD | Yes | No |
| 7 | + | – | – | No | DOD | Yes | Yes |
| 8 | – | – | + | No | DOD | Yes | Yes |
| 9 | + | + | – | No | DOD | Yes | Yes |
| 10 | – | – | + | No | DOD | Yes | Yes |
| 11 | – | – | + | No | DOD | Yes | No |
| 12 | – | – | – | No | DOD | Yes | No |
| 13 | – | – | + | No | DOD | Yes | Yes |
| 14 | – | – | + | Yes | NED | Yes | No |
| 15 | – | – | – | Yes | NED | Yes | Yes |
| 16 | – | – | + | Yes | NED | Yes | Yes |
| 17 | – | – | – | Yes | NED | Yes | No |
| 18 | – | – | – | Yes | NED | Yes | Yes |
| 19 | – | – | – | Yes | NED | Yes | Yes |

Abbreviations: DOD, dead of disease; NED, no evidence of disease; AWD, alive with disease.

*HER2* determined both by immunohistochemistry (≥2+) and fluorescence in situ hybridization (≥2 copies).
linear mixed effects model. 
P values were generated using the linear mixed effects model.

(m/z = 3371, 3442, and 3485) were overexpressed >30-fold (P < 0.05) and four features (m/z = 5677, 6955, 7007, and 15348) were underexpressed >2-fold (P < 0.05) in patients with pCR. No statistically significant, differentially expressed features were observed in the stroma profiles (n = 19) of pCR and NR biopsies (data not shown).

The three most significant peaks, m/z = 3371, 3442, and 3485 (Fig. 2A), have previously been identified as α-DEFA1, α-DEFA2, and α-DEFA3, respectively (29-31). DEFAs are a family of microbicidal and cytotoxic peptides involved in phagocyte-mediated host defense and are abundant in neutrophil granules (32). To eliminate the possibility that DEFA peptides were present as an artifact of contaminating blood in the biopsy material (33), we first evaluated the tumor specimens for the presence of histone H4 peptides, which are abundant in nucleated cells (Fig. 1; peaks m/z = 11,307 and 11,349). More significantly, our tumor MALDI spectra lacked hemoglobin peaks (m/z = 7,564 and 7,835 or 15,127 and 15,668; Fig. 1), which are highly abundant in blood-contaminated specimens (33).

To validate our proteomic profiling-based findings and to identify the source of DEFA expression, we analyzed pre-treatment biopsies by immunohistochemistry with a DEFA-specific antibody. Several patients who achieved pCRs (nos. 14 and 19) showed robust DEFA expression in tumor cells and tumor stromal areas, whereas there was little to no DEFA expression in NR tumors (nos. 9 and 11; Fig. 2B). Of note, infiltrating neutrophils stained positive for DEFA expression in all sections indicating the specificity of the antibody.

To further ensure that there was no bias to detect DEFA expression based on placement of matrix spots, MALDI-MS imaging was done on the entire tissue section to allow visualization of the spatial distribution of DEFAs across a biopsy section. One representative NR and pCR sample showed that DEFA1 and DEFA2 were present in both tu-

mor and stroma of patients who achieved a pCR, but was absent in patients that did not (NR: Fig. 2C). Of note, DEFA peptides were only identified in areas of tumor (compare H&E and MALDI-MS images in Fig. 2C), suggesting that DEFAs are specifically expressed in tumor cells or surrounding stroma and not just present due to whole blood contamination. Nonetheless, any future consideration of DEFA as a biomarker would need to take into account the use of tissue that is not contaminated with tumor-associated blood as we have done in this study. Also, we cannot rule out the possible contribution of neutrophils to DEFA expression in areas of tumors with high levels of neutrophil infiltrate. Consistent with our results, DEFA expression has been shown to be prevalent in squamous cell carcinomas (30). These profiling and imaging data show for the first time that histology-directed MALDI-MS could accurately identify differential features in pre-treatment biopsies that correlate with response and show the use of proteomics for future biomarker analyses.

**Gene expression profiling.** Gene expression profiling was used to identify differential expression patterns that correlated with response to neoadjuvant paclitaxel/radiation treatment in 14 patients (4 with pCR). Laser capture microdissection was done because there was significant heterogeneity in the biopsies with tumor cellularity ranging from 20% to 95%. RNA isolated from captured tumor cells was hybridized to Affymetrix GeneChips. We first annotated pCR and NR gene expression data and did an unpaired t test on all probes to identify differentially expressed genes. A heat map was generated for a 101-probe set that represents genes with a >3-fold change in expression (P < 0.05) between pCR and NR samples (Fig. 3A). MAP2, microtubule-associated protein 2, which had a >4-fold change in expression as assessed by two probes was a highly significant, differentially expressed gene (FDR-corrected t test, P < 0.03) in tumors from patients with pCR versus NR (Fig. 3B).

Because MAP2 binds and stabilizes microtubules, and paclitaxel treatment enhances this interaction, which may contribute to drug sensitivity, we chose to further study this gene as a marker of paclitaxel sensitivity (34). MAP2 overexpression has been observed in several types of cancers (35-39). We obtained basal MAP2 mRNA expression from five breast cancer cell lines and the concentration of paclitaxel at which their growth was inhibited by 50% (GI_{50} values; see Supplementary Materials and Methods). The expression of MAP2 in the breast cancer cell lines is highly correlated (R^2 > 0.99) with paclitaxel sensitivity in vitro (Supplementary Fig. S1). These data suggest that MAP2 expression is a critical determinant of taxane sensitivity in breast cancers.

To determine if a mechanistic link exists between levels of MAP2 protein and taxane sensitivity, we engineered two breast cancer cell lines, MCF-7 and MDA-MB-468, to overexpress MAP2. Both cell lines were transduced with a vector containing MAP2 cDNA or empty vector. Stable overexpression of MAP2 was confirmed in MCF-7 and MDA-MB-468 cells by immunoblot analysis (Fig. 4A). The cells were
Fig. 2. A, average pCR (green) and NR (red) spectra peaks for DEFA1-3. B, immunohistochemical analysis for DEFAs from two representative pCR and NR tumors. C, MALDI-MS imaging of DEFA1 and DEFA2 was done by overlaying spectral files that were reconstructed into ion density images with an H&E image (left) of an NR and pCR for visualization.
grown as three-dimensional mammospheres, a culture method that has been well characterized and recapitulates breast epithelial function and morphology (25–27). Paclitaxel treatment reduced mammosphere formation and overall cell number by 53.7% and 46.4% in MCF-7 and MDA-MB-468 cells overexpressing MAP2 relative to vector control, respectively (Fig. 4B). We also examined the sensitivity of these engineered breast cancer cell lines to paclitaxel when grown as two-dimensional colonies and observed a similar phenomenon (data not shown).

Having identified individual biomarkers that influence response to paclitaxel, we used our gene expression data to explore clinical subgroups of tumors associated with response. Gene expression profiling has resulted in the molecular classification of breast cancers into five distinct subtypes: normal breast, HER2+, basal-like, luminal-A, and luminal-B.
and luminal-B (40–42). The basal-like subtype is predominantly composed of triple-negative breast cancers (43, 44). Cross-referencing the gene signature that defines these molecular subtypes to our set of probes that had >3-fold change in expression in pCR versus NR (P < 0.05) resulted in subtype classification of these tumors. For example, we observed the overexpression of 9 of 19 (ACTG2, SLPI, ANXA8, KRT5, TRIM29, KRT17, GAPRP, FOXC1, and CHI3L2) known genes in the basal-like signature and the downregulation of 5 of 13 genes (TFF3, ESR1, GATA3, ACADS, and RERG) in the luminal signature as previously defined (43), in patients that experienced a pCR (Supplementary Table S2). Similarly, expression of ER-regulated genes (e.g., GATA3 and RERG) was found in ER+ patients.

Fig. 4. (A) Western analyses showing MAP2 and GAPDH protein levels in MCF-7 and MDA-MB-468 cells transduced with vector control or MAP2. (B) Control and MAP2-expressing cells were grown as mammospheres ± paclitaxel. Arrows, nonadherent or apoptotic cells. The number of viable cells was determined after 10 d ± paclitaxel and represented relative to untreated control.
that lacked a response (NR). These observations are also in concordance with our finding that five of six patients with a pCR had triple-negative tumors (Table 1). Of note, the number of triple-negative tumors molecularly analyzed herein was similarly proportioned to those enrolled overall in the study, eliminating potential bias.

An enrichment-based statistical test was used for gene ontology categories for probes that were differentially expressed between patients with pCR versus NR (>3-fold; \( P < 0.05 \)). There was a significant enrichment for immune signaling categories, including defense response, immune response, MHC complexes, response to stimulus, chemokine activity, and immune cell activation (Supplementary Table S3).

**Biomarker validation in another patient cohort receiving taxane-based neoadjuvant treatment.** To further validate if the expression of the two markers, DEFA and MAP2, was associated with pCR in an independent patient cohort, we obtained tissue from 47 additional pretreatment biopsies from patients treated with neoadjuvant taxane–based chemotherapy and evaluated DEFA and MAP2 expression by immunohistochemistry (Supplementary Table S1). Eleven of 47 (23%) of the patients in the second cohort which received taxane-based neoadjuvant therapy achieved a pCR. In addition, because DEFA is a marker of neutrophils and our gene expression and gene ontology analyses of tumors from patients with pCR revealed a link to host immune response, we also scored the biopsy specimens for immune cell infiltrate surrounding the tumor as a marker of inflammation as well as counted neutrophils within the infiltrate (as described in Materials and Methods). We found that six tumors (9% of total) had high levels of neutrophil infiltrate (>10/high-power field) of which three tumors were from patients that achieved a pCR (Supplementary Fig. S2B; data not shown).

Univariate logistic regression of pCR indicated statistically significant associations with all three markers, DEFA \(( P = 0.007 \)) , inflammation \(( P = 0.031 \) ), and MAP2 \(( P = 0.037 \) ) as assessed by immunohistochemistry. Increases in DEFA expression and levels of inflammation were associated with an increase in the odds of achieving a pCR of 3.4 (95% confidence interval, 1.4-8.1) and 2.3 (95% confidence interval, 1.1-4.9), respectively (Table 2). Of note, DEFA, a neutrophil marker, had correlation with inflammation in tumor samples \(( r = 0.36, P = 0.007, \text{Spearman's correlation} \) ). pCR was 3.2 times (95% confidence interval, 1.1-9.3) more likely in a patient with MAP2 at the 75th percentile than a patient at the 25th percentile (Table 2). Statistical significance was not reached in a multivariable analysis of all three markers (data not shown), likely due to the limited number of pCRs in this patient cohort. Definitive multivariate evaluation of these markers is warranted in larger patient cohorts that have received neoadjuvant taxane–based therapy.

**Discussion**

We used proteomic and genomic analyses to identify molecular markers of pCR to neoadjuvant paclitaxel/radiation treatment. A marker of inflammation, DEFA, higher levels of tumor-infiltrating immune cells, and a microtubule-associated protein 2 (MAP2), were all found to be differentially expressed between pCR and NR tumor biopsies. To validate our findings, we showed that these markers were significantly associated with response in a separate cohort of patients receiving taxane-based neoadjuvant therapy. Of the 38 patients enrolled in our study, 12 were triple-negative and of those 58% (7 patients) had a pCR. Only 23% of patients with non–triple-negative tumors achieved pCR. Thus, patients with triple-negative disease are twice as likely to respond to this treatment regimen. Likewise, in two independent studies, the basal-like (triple negative) and HER2+ subgroups were found to be associated with higher rates of pCR (27-45%), versus luminal pCR rates (6-7%), in patients receiving taxane-based neoadjuvant treatment (45, 46). Of note, with further follow-up, patients that achieved

<table>
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<tr>
<th>Score</th>
<th>NR, n (% of score)</th>
<th>pCR, n (% of score)</th>
<th>OR (95% CI)</th>
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<tr>
<td>DEFA</td>
<td></td>
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<tr>
<td>0</td>
<td>8 (100%)</td>
<td>0 (0%)</td>
<td>3.4 (1.4-8.1), ( P = 0.007 )</td>
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<tr>
<td>+1</td>
<td>17 (81.0%)</td>
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<td>+2</td>
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<td>10 (43.5%)</td>
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<tr>
<td>+3</td>
<td>2 (40.0%)</td>
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<tr>
<td>Inflammation</td>
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<tr>
<td>0</td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>2.3 (1.1-4.9), ( P = 0.031 )</td>
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<tr>
<td>+3</td>
<td>7 (63.6%)</td>
<td>4 (36.4%)</td>
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<tr>
<td>MAP2*</td>
<td>P50 (P25-P75)</td>
<td>P50 (P25-P75)</td>
<td>3.2 (1.1-9.3), ( P = 0.037 )</td>
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<tr>
<td></td>
<td>33.3 (18.3-38.3)</td>
<td>35.0 (31.7-40.0)</td>
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</tbody>
</table>

Abbreviations: P50, median (P25, 25th percentile; P75, 75th percentile); OR, odds ratio; 95% CI, 95% confidence interval.

*Weighted index of MAP2 expression (Materials and Methods).
Markers of Taxane Sensitivity in Breast Cancer

a pCR have remained disease-free (median follow-up of 59 months) to date, indicating the importance of identifying molecular markers of response.

Proteomic analysis identified DEFAs as predictors of pCR. DEFAs play an important role in innate immune defense against inflammatory-related diseases, including epithelial tumors (30, 47). DEFA expression was also associated with high levels of inflammation and immune cell infiltrate. Furthermore, from gene expression profiling and statistical testing to analyze differentially expressed genes between pCR versus NR patients, we observed a significant enrichment for immune response categories. Of note, DEFAs were not differentially expressed by gene profiling, likely because DEFAs are regulated at the posttranslational level of granule processing (32).

Using gene expression profiling, a recent study of triple-negative tumors found that tumors with increased lymphocytic infiltrate and high expression of IFN-regulated and immunoglobulin genes are associated with improved metastasis-free survival (44). The IFN-regulated gene (CXCL10) and two immunoglobulin genes (IGHG1 and IGHG3) that cluster with triple-negative tumors in the study were found to be increased (>3-fold) in tumors from patients with pCR (data not shown). These data suggest that expression of immunologically related proteins, such as DEFAs, and the presence of immune cell infiltrate in triple-negative breast tumors might be predictive of response to neoadjuvant paclitaxel/radiation treatment, and that additional molecular insights could be gained from the integrated use of both genomic and proteomic tumor profiling.

From our microarray analysis, we found that MAP2 was expressed at a higher level in pCR versus NR tumors (P < 0.03). MAP2 plays a critical role in neurite outgrowth and dendrite development through its microtubule-stabilizing function (48), and is expressed in non–small cell (38), neuroendocrine (35, 37), and oral squamous cell carcinomas (36). Expression of MAP2 in metastatic melanoma cells leads to microtubule stabilization, G2–M cell cycle arrest, and growth inhibition in vitro and in vivo (39). Primary melanomas with high MAP2 expression have significantly better metastatic disease-free survival than those with little or no expression. MAP2-related peptides are highly expressed in docetaxel-sensitive pancreatic ductal adenocarcinoma compared with docetaxel-refractory pancreatic cancers (49). Paclitaxel treatment enhances the interaction between tubulin and MAP2, which may contribute to taxane sensitization in cells with high MAP2 expression (34). Supporting this theory, we found that MAP2 overexpression in two breast cancer cell lines correlated with paclitaxel sensitivity. Pretreatment expression of MAP2 in breast tumor biopsies may serve as a marker of response to taxane-based neoadjuvant treatment.

Taxane treatment remains one of the most effective therapies for breast cancer in the neoadjuvant, adjuvant, and metastatic settings as well as improving both disease-free and overall survival (8, 50). DEFA and MAP2 expression and immune infiltration were significantly associated with pCR in a large spectrum of patients that received neoadjuvant taxane–based therapy. These biomarkers may have future utility for the selection of breast cancer patients that receive taxanes as part of their treatment regimen.

Disclosure of Potential Conflicts of Interest

A.B. Chakravarthy: commercial research grants for the support of the clinical trials from Aventis and BMS. The other authors disclosed no potential conflicts of interest.

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