Functional Genomics Identifies ABCC3 as a Mediator of Taxane Resistance in HER2-Amplified Breast Cancer

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Abstract
Breast cancer is a heterogeneous disease with distinct molecular subtypes characterized by differential response to targeted and chemotherapeutic agents. Enhanced understanding of the genetic alterations characteristic of different subtypes is needed to pave the way for more personalized administration of therapeutic agents. We have taken a functional genomics approach using a well-characterized panel of breast cancer cell lines to identify putative biomarkers of resistance to antimitotic agents such as paclitaxel and monomethyl-auristatin-E (MMAE). In vitro studies revealed a striking difference in sensitivity to these agents between cell lines from different subtypes, with basal-like cell lines being significantly more sensitive to both agents than luminal or HER2-amplified cell lines. Genome-wide association studies using copy number data from Affymetrix single nucleotide polymorphism arrays identified amplification of the chromosome 17q21 region as being highly associated with resistance to both paclitaxel and MMAE. An unbiased approach consisting of RNA interference and high content analysis was used to show that amplification and concomitant overexpression of the gene encoding the ABC3 drug transporter is responsible for conferring in vitro resistance to paclitaxel and MMAE. We also show that amplification of ABCC3 is present in primary breast tumors and that it occurs predominantly in HER2-amplified and luminal tumors, and we report on development of a specific fluorescence in situ hybridization assay that may have utility as a predictive biomarker of taxane resistance in breast cancer. [Cancer Res 2008;68(13):5380–9]

Introduction
A key goal of modern molecular oncology is identifying the underlying genetic and genomic abnormalities that characterize a given tumor so that the patient can receive targeted and chemotherapeutic agents likely to provide the most benefit. Breast cancer is the most common form of cancer among women in the Western World, with an estimated 1 million new diagnoses and 400,000 deaths per year worldwide (1). The advent of targeted therapies such as tamoxifen for estrogen receptor (ER)–positive cancer (2) and Herceptin for tumors harboring amplification of the HER2 oncogene (3) has had significant effect on patient survival; yet, various chemotherapy regimens still form an important component of breast cancer treatment (4). Although chemotherapy is a successful treatment regimen in many cases, an estimated 50% of patients fail to benefit due to intrinsic or acquired multidrug resistance (MDR; ref. 1). MDR refers to the resistance of cancer cells to multiple classes of chemotherapeutic drugs that can be structurally and mechanistically unrelated and is related to the overexpression of a variety of proteins that act as ATP-dependent efflux pumps (5). Understanding the molecular alterations that contribute to MDR in breast cancer will be a crucial first step in enabling the development of diagnostic tests capable of predicting resistance to a given therapy and rationally selecting more efficacious therapeutic agents.

One means of elucidating the relationship between genomic features and therapeutic response is through pharmacogenomics, or the study of how inherited and spontaneous genetic variation affects sensitivity to pharmacologic agents (6). Efforts have been directed at identifying pharmacogenomic correlates of MDR in tumor cell lines, notably in the 60 cancer cell lines used by the National Cancer Institute to screen for anticancer activity (the NCI-60). At least 100,000 compounds have been screened through the NCI-60 over the past 15 years (7), including a recent study that profiled mRNA expression of the 48 known human ABC transporters in the NCI-60 and correlated the results with growth-inhibitory profiles of 1,429 candidate anticancer drugs. This study identified several candidate transporters likely to confer resistance to specific agents (8) and provided insights into the general mechanisms of resistance across different tumor types, paving the way for follow-up studies to investigate mechanisms specific to individual tumor types.

Pharmacogenomic studies must also be carried out in the context of the emerging picture of breast cancer as a heterogeneous disease with distinct molecular subtypes. A number of studies over the past several years have shown that primary breast tumors may be classified into at least three major subtypes by gene expression profiling and that the subtypes have different prognostic outcomes in terms of patient survival (9). Luminal breast cancers are typically ER positive and characterized by coordinate expression of a number of epithelial specific genes, a relatively good prognosis, and good response rates to targeted hormonal therapies. HER2-positive breast cancers are characterized by high-level gene amplification of the HER2 oncogene, relatively poor prognosis if untreated, and significant clinical benefit from the HER2-targeting monoclonal antibody trastuzumab (Herceptin, Genentech; ref. 3). Basal-like breast cancers typically lack expression of HER2, ER, and progesterone receptor (PR) and hence are sometimes referred to as “triple-negative” tumors (10).
Basal-like breast cancers have a relatively poor prognosis and currently have not been shown to respond to any targeted therapy (11). It has recently been shown that the subtypes display differential response to preoperative chemotherapy regimens (12); however, for the most part, the drug resistance mechanisms underlying these differences have yet to be determined.

Recent studies have revealed that large collections of breast cancer cell lines reflect many of the genetic and genomic changes characteristic of human breast tumors and hence may serve as a model system for a population of molecularly heterogeneous breast cancers (13). For instance, cells may be classified into basal-like and luminal subtypes based on gene expression profiling signatures and they retain most of the high-level amplifications and deletions that are associated with poor outcome in primary tumors (13, 14). We have used a panel of 31 breast cancer cell lines that we have molecularly characterized as a model for pharmacogenomic analysis to identify resistance mechanisms and subtype differences in response to two antimitotic-based therapeutics, monomethyl-auristatin-E (MMAE) and paclitaxel. MMAE is structurally related to dolastatin 10, a pentapeptide natural product that has been the subject of several human clinical trials for cancer therapy, and exhibits potent antitumor activities by inhibiting tubulin polymerization and thus destabilize cellular microtubules (15). Auristatin-monoclonal antibody conjugates have been developed with the rationale that targeted delivery of the drug through specific antigen recognition by the antibody will lead to enhanced chemotherapeutic efficacy while sparing nontarget-expressing tissues from toxicity (15). Paclitaxel and the related compound docetaxel are anticancer cytotoxic drugs that stabilize microtubules and are widely used in the treatment of breast cancer (16).

Although most in vitro profiling efforts directed at understanding drug resistance to date have focused on gene expression analyses, here we sought to identify DNA copy number alterations that were associated with altered drug sensitivity through analyses of Affymetrix high-density SNP array profiles. Recent studies have shown that high-density single nucleotide polymorphism (SNP) arrays, in addition to their intended application in genotyping, can be used to detect genome-wide DNA copy number changes and loss of heterozygosity in human cancers (17). These arrays have been shown to have applications in the identification of tumor suppressor and oncogene loci by pinpointing recurrently deleted or amplified chromosomal regions (18); we show here that they can be used to identify amplified regions harboring genes that may modulate the activity of therapeutic drugs.

The major finding of this study is that amplification of a region of chromosome 17 (17q21) is strongly associated with in vitro resistance to taxanes and auristatins. The region of amplification harbors at least 100 genes; therefore, to identify the relevant gene, we used an unbiased approach consisting of RNA interference (RNAi) and high content analysis. These studies show that amplification and concomitant overexpression of the ABCG3 gene is most likely responsible for conferring resistance to paclitaxel and MMAE. We also show that this amplicon is present in primary breast tumors, that it occurs predominantly in HER2-amplified tumors, and we report on development of a specific fluorescence in situ hybridization (FISH) assay that may have utility as a biomarker of taxane response in breast cancer. The results also suggest that genome-wide copy number changes assessed by SNP arrays are a valuable tool for preclinical biomarker discovery when used in conjunction with pharmacologic data.

Materials and Methods

Cell Lines and Viability Experiments

Breast cancer cell lines AU565, BT-474, BT-549, CAMA-1, DU4475, HCC1143, HCC1419, HCC1928, HCC2218, HCC70, Hs578T, KPL-1, MCF-7, MDA-MB-231, MDA-MB-361, MDA-MB-435S, MDA-MB-453, MDA-MB-468, T-47D, UACC-812, ZR-75-1, and ZR-75-30 were obtained from the American Type Culture Collection. The cell lines CAL-120, CAL-51, CAL-85-1, EFM-19, EFM-192A, EVSA-T, and MT-3 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. All cell lines were maintained in RPMI 1640 or DMEM supplemented with 10% fetal bovine serum (Sigma), nonessential amino acids, and 2 mmol/L L-glutamine. Although annotated as breast lines, MDA-MB-435S may actually be of melanoma origin and MT-3 of colorectal origin based on molecular and genetic criteria (19, 20). These findings do not affect the conclusions of this study. For MMAE and paclitaxel IC50 determination, cells were plated in quadruplicate at a density of 3,000 per well in 384-well plates in normal growth medium and allowed to adhere overnight. Paclitaxel (Sigma) or MMAE (Seattle Genetics) were added in 10 concentrations based on a 3-fold dilution series (1 μmol/L maximal paclitaxel or 0.1 μmol/L maximal for MMAE). Cell viability was measured 72 h later using the Celltiter-Glo Luminescent Cell Viability Assay (Promega). The concentration of drug resulting in the 50% inhibition of cell viability (IC50) was calculated from a four-parameter curve analysis (XLfit, IDBS software) and was determined from a minimum of three experiments. Cell lines that did not show 50% reduction in cell viability in response to drug treatment in the majority of experiments conducted were considered to not have reached an IC50 by definition and are listed as having an IC50 of >100 nmol/L. (MMAE) or >1,000 nmol/L (paclitaxel). For ABC33 overexpressing clones of the EVSA-T cell line that did not achieve IC50, we calculated the half-maximal effect concentration, or EC50 in GraphPad Prism software (GraphPad Software, Inc.).

Breast Tumor Samples

Primary breast tumors from 145 independent breast cancer patients were used to make genomic DNA for Agilent Array comparative genomic hybridization analysis (aCGH; ref. 21). All the tumors were fresh frozen and found to have >70% tumor content, and all were classified as infiltrating ductal carcinoma. ABC33 FISH studies were conducted on 61 additional independent primary breast tumor samples from the Genentech tumor bank.

Gene Expression Microarray Studies

Gene expression analysis of breast cancer cell lines was carried out on RNA extracted from subconfluent cell cultures using Qiagen RNAeasy kits. RNA quality was verified by running samples on an Agilent Bioanalyzer 2100 and samples of sufficient quality were profiled on Affymetrix HGU133-Plus_2.0 chips. Preparation of complementary RNA, array hybridizations, scanning, and subsequent array image data analysis were done using the manufacturer's specified protocol.

For overall unsupervised hierarchical clustering analysis of breast cancer cell lines, gene expression data were filtered to remove probe sets that showed little variation across the cell lines. Briefly, probes that did not show at least a 5-fold variation across the samples (max/min >10) and an absolute intensity difference of at least 250 (max-min >250) were excluded from hierarchical clustering analysis. Data preprocessing involved log-transforming and median-centering gene expression values, after which average linkage clustering was carried out using Cluster and TreeView software (22).

SNP Array and Agilent aCGH Copy Number Studies

Cell line copy number analysis was carried out on genomic DNA extracted from subconfluent cell cultures using Qiagen DNAeasy kits. For each cell line, 500 ng of genomic DNA were hybridized to Genochip 100 K mapping arrays (Affymetrix, Inc.) according to the manufacturer's instructions. These arrays contain probe sets for >116,000 SNP loci derived from all human chromosomes (except the Y chromosome), with a mean marker distance of 26 kb (23). SNP calls and signal quantification were obtained with Gene Chip Operating System. Agilent Human Genome 244A
CGH microarrays and Agilent feature extraction software were run according to the manufacturer’s instructions and genome-smoothed analysis DNA copy number (GSA_CN) was calculated based on the hybridization intensity (the sum of both allele intensities) for each SNP probe with the Affymetrix Chromosome Copy Number Analysis Tool 3.0 (CNAT 3.0). Copy number data were segmented with the GLAD segmentation algorithm (24). Genome-wide analysis of copy number gains and losses shown in Supplementary Fig. S2 were determined using the Genomic Identification of Significant Targets in Cancer algorithm (25).

Associations between GSA_CN copy number and drug sensitivity were identified with Matlab software (The MathWorks, Inc.) using a version of the maxT procedure (26). For each drug, a test statistic was calculated for each SNP reflecting the difference between log-transformed copy number in sensitive and resistant cell lines. The statistic was calculated as the absolute value of a standard t statistic (two sample, unequal variance), except that it was set to zero for those SNPs with <1.75-fold difference in mean copy number between sensitive and resistant classes. Then, the null distribution of maximum test statistics across all SNPs was estimated in 10,000 random permutations of the sensitivity labels. The P value for each SNP was calculated as the fraction of permutations in which the maximum test statistic was greater than or equal to the observed statistic for that SNP. The resulting P values control the family-wise error rate and take into account the number of SNPs tested.

HER2 Copy Number Determination by Quantitative Reverse Transcription-PCR

Quantitative PCR was done using ABI Prism 7900 Sequence Detection System (Applied Biosystems) on genomic DNA prepared as described above. Quantitative reverse transcription-PCR (qRT-PCR) was done using primers CACTGTCTGCACCTTGCTTTG and GCTCTGCAGCTATTGAAT for HER2 and AAAGCCGTCAACTACATGG and TGCTTTGAATGGTGCCAGAG for line-1 repetitive elements. Line-1 is a repetitive element with similar copy numbers per haploid genome between human normal and neoplastic cells (27). Quantification was based on standard curves from a serial dilution of human normal genomic DNA. The relative target copy number level was also normalized to normal human genomic DNA as calibrator. Copy number change of target gene relative to the line-1 and the calibrator were determined using the formula: $E = (CP_{target} - CP_{control}) - (CP_{target} - CP_{control})$ as described by Kindich and colleagues (28). Conditions for quantitative PCR reaction were as described in the Invitrogen Platinum SYBR Green qPCR SuperMix-UDG w/ROX package insert.

Fluorescence In situ Hybridization Analysis

Probes. A bacterial artificial chromosome contig comprising of two overlapping clones, RP11-2605A1 and CTD-3006C13, covering the entire ABC3 locus and adjoining areas (based on the UCSC Genome Browser March 2006 assembly) were used as a probe for the FISH experiments. Commercially available probes for HER2/CEP17 (Pathvisiohn, Vysis/Abbott Laboratories) and CEP17 (Vysis/Abbott Laboratories) were also used for the FISH experiments.

FISH analysis. Cell lines were prepared for cytogenetic analysis by incubation with 0.1 µg/mL colcemid (Invitrogen) for 30 min, followed by osmotic swelling in KCl (0.075 mol/L) and fixation in methanol/acetic acid (3:1), as previously described (29). DNA from the bacterial artificial chromosome clones was extracted by standard methods. The extracted bacterial artificial chromosome DNA was directly labeled with Spectrum Orange or Spectrum Green (Vysis/Abbott Laboratories) by nick translation using the Vysis Nick Translation Kit (Vysis/Abbott Laboratories) according to the manufacturer’s instructions. FISH to normal human metaphases (Abbott Laboratories) confirmed the genomic location of the bacterial artificial chromosome clones. Approximately 300 ng of labeled probes were precipitated in excess Human Cot-1 DNA (Invitrogen) and sonicated salmon sperm DNA (Sigma) and resuspended in a 50% formamide, 10% dextran sulfate, and 2× SSC hybridization buffer (Vysis/Abbott Laboratories) for the FISH experiments. FISH on cytogenetic preparations and formalin-fixed paraffin-embedded tissue was performed as described previously (30), with some modifications. After an overnight incubation at 56°C, the slides were deparaffinized in three washes of CitroSolv for 5 min each, followed by two washes in alcohol. After air drying, the slides were incubated in a 1 mol/L solution of sodium sulfocyanate for 30 min at 80°C and then were treated with pepsin before additional washes in water and a series of ethanol. Dried slides were then codenatured (76°C for 6 min) with the probe and were hybridized overnight at 37°C (ThermoBrite; Vysis). Posthybridization washes and counterstaining were done in a manner similar to those previously described. The slides were visualized using an Olympus BX61 microscope and analyzed using FISHview software (Applied Spectral Imaging). The copy number analysis and ratio of HER2/ABC3 to CEP17 was performed as per the manufacturer’s instructions.

Functional Validation Experiments

High-content screening assays were carried out on an Arrayscan VTI (Cellomics, Inc.). Cells were transfected in 96-well format using small interfering RNA (siRNA) “Smartpool” oligonucleotides purchased from Dharmacon, Inc., and Oligofectamine transfection reagents. To prioritize genes for functional studies, two- or three-color Wilcoxon rank sum tests using the R programming language4 were done to identify genes with a significant difference in gene expression in cell lines with >4 copies compared with those with <4 copies of the 17q21.3 amplicon. This analysis combined with availability of reagents for RNA interference experiments led to the selection of the following 24 genes for functional studies in the EFM-192A cell line: ABC3, COL1A1, COX17, DDX5, FLJ13855, FLJ20990, HOXB7, LOC201191, ITGB3, KIAA0924, KPNB1, LOC400604, LOC81558, MGC1224, MGC15396, NDKJ2, PDK2, PHB, PPI19B, SLCT3B1, SPO1, TOP1, and WNT3. Follow-up studies with ABC3-specific siRNA were conducted in the additional cell lines ZR75-30, MDAMB-453, and HCC-1428. A nontargeting control siRNA that does not show significant homology to any sequence in the human genome was used as a negative control in all RNAi experiments (as described in technical notes online).

After 48 or 72 h incubation at 37°C, cells were fixed in 3.7% formaldehyde and permeabilized in 0.1% Triton X-100, followed by labeling with a 1:500 dilution of anti-phospho-histone H3 (pH3, Upstate) and subsequent 1:250 dilution of Alexa-fluor 488 (Molecular Probes) goat anti-rabbit secondary antibody. Cells were counterstained with Hoechst-33258 to allow identification of nuclei and the percentage of cells positive for nuclear pH3 immunofluorescence, also known as the mitotic index (31), was then quantitated for at least 1,000 cells per well using Cellomics Target Activation software. All experiments were repeated at least twice. Quantitative PCR to assess ABC3 transcript levels after siRNA transfection was performed using ABC3-specific primers (5’ primer GATCCAGCCGCTCTAGTT, 3’ primer CCTGGCTGGTCTCACA CTT).

For ABC3 overexpression experiments, a full-length ABC3 cDNA cloned in the cytomegalovirus (CMV) promoter containing vector pCMV5 (Invitrogen, Carlsbad, CA) was verified by sequencing the entire coding sequence. The construct was transfected into EVSA-T cells and stable clones were selected by growth in 1 mg/mL geneticin (Invitrogen). Overexpression of ABC3 in stable cells was confirmed by qRT-PCR on cDNA derived from lines containing pCMV5-ABC3, pCMV5 vector alone, or the parental EVSA-T strain.

Results

Molecular characterization of cell lines. Affymetrix gene expression profiling was performed on cDNA prepared from total mRNA and Affymetrix 100 K SNP array profiling was done on DNA from 44 breast cancer cell lines. Unsupervised analysis with the 11,000 most differentially expressed genes across the cell line panel was used to classify the cell lines into luminal and basal-like subtypes based on gene expression (Supplementary Fig. S1). Cell

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4 http://www.r-project.org
5 http://www.dharmacon.com
lines classified as luminal expressed high levels of ERα and many of the target genes regulated by ER, including GATA3, HNF3A, IGF-IR, and XBP1. Cell lines classified as basal-like expressed high levels of some or all of the well-described basal markers vimentin, caveolin, MFGE8, and the basal cytokeratins such as KRT5 (Supplementary Fig. S1; ref. 32). Because amplification of the HER2 oncogene clearly defines a separate disease subtype that is not apparent from overall gene expression classification in cell lines (13), we determined HER2 copy number by qRT-PCR on genomic DNA and normalization to line 1 repetitive elements for all cell lines (Supplementary Table S1). The molecular subtype in Fig. 1 is a classification derived from both the overall gene expression results as well as the HER2 copy number analysis. Our findings agree with previous reports (13) and suggest that this collection of breast cancer cell lines reflects to some degree the major transcriptional distinctions that define breast cancer subtypes and to some extent are representative as models of subtypes as luminal, basal-like, and HER2-amplified tumors. Genome-wide patterns of copy number gain and loss in the cell lines (13), we determined HER2 copy number alterations (e.g., MYC, CCND1, HER2 gain p16, PTEN loss) that have been described in tumors (Supplementary Fig. S2 as well as discussed in detail by Hu and colleagues, but a finding relevant to this study is that amplification at 17q21.3 is common in HER2-amplified and luminal cell lines but not in basal-like cell lines (Supplementary Fig. S2).

**In vitro sensitivity to antimitotic drugs.** We screened 31 breast cancer cell lines for in vitro sensitivity to paclitaxel and MMAE as assessed by the IC50 value for each compound in a standard luciferin-based viability assay (Supplementary Table S1; Supplementary Fig. S3). Notably, there was significant correlation between the relative sensitivity to each agent across the panel of cell lines (Spearman rank-order correlation coefficient, r = 0.55). In addition, Fig. 1 shows that cell lines with the basal-like gene expression signature had lower average IC50 values and were more sensitive to each agent than luminal or HER2-amplified cell lines as determined by Kruskal-Wallis rank sum test (P = 0.002 for MMAE, P = 0.005 for paclitaxel).

**Identification of genomic alterations that correlate with in vitro sensitivity.** We next sought to identify regions of chromosomal gain or loss that correlated with sensitivity to paclitaxel or MMAE through supervised analysis of SNP array copy number data. First, cell lines were classified into either sensitive (IC50 <10 nmol/L) or resistant (MMAE IC50 >100 nmol/L, paclitaxel IC50 >1,000 nmol/L) groups based on the sensitivity data. We then used the maxT algorithm (26) to analyze data from ~115,000 SNPs and identify individual SNPs where the mean copy number differed between sensitive and resistant classes with genome-wide significance. In the case of paclitaxel, a group of SNPs on chromosome 17 starting at chromosome position 44,303,217 and ending at position 44,724,301 (17q21.21 to 17q21.23) showed statistically significant copy number differences between sensitive and resistant classes (P for rs2411377 = 0.04). The same group of markers also showed significant association between copy number and MMAE sensitivity (P for rs2411377 = 0.05). Figure 2 shows the relationship between paclitaxel sensitivity and genomic DNA copy number in this part of chromosome 17 and that a significant number of cell lines (8 of 14) that showed resistance to paclitaxel have at least four gene copies within the region.

**Identification of candidate genes in the interval.** The chromosomal region from 17q21.31 to 17q21.33 encodes ~100 expressed transcripts according to the University of California Santa Cruz Genome Browser. Based on the principle that functionally relevant genes in regions of amplification should exhibit a concomitant increase in mRNA expression, we filtered this list down to 24 genes that showed significant overexpression upon amplification and had reagents available to conduct RNAi studies. An example of significantly higher expression of the candidate gene ABCC3 in amplified cell lines compared with nonamplified cell lines is shown in Supplementary Fig. S4. These 24 genes were selected for subsequent functional analysis to identify the locus responsible for conferring resistance to taxanes and auristatins.

**Functional validation of ABCC3 by RNA interference.** We used an RNA interference strategy to identify the gene responsible for mediating resistance to taxanes and auristatins in amplified cell lines. The assay utilized made use of the fact that treatment of cells with paclitaxel or MMAE results in a block of cell cycle progression at the M phase that can be assayed by the presence of the mitotic

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7 http://genome.ucsc.edu
marker pH3 (33). Phosphorylation at Ser10 of histone H3 is tightly correlated with chromosome condensation during the M phase, and the percentage of cells that are positive for pH3 staining, or mitotic index, can be determined through an immunofluorescence assay. We reasoned that cellular knockdown of the gene-mediating resistance should increase sensitivity of cell lines harboring the amplification to paclitaxel and MMAE and hence result in an accumulation of arrested cells and a higher mitotic index relative to control-treated cells at a given drug concentration. Higher mitotic index correlates with reduction in viability and proliferation determined by other assays but is a more specific readout of the antimitotic effects of these drugs. We found that RNAi of 23 of the 24 candidate genes did not reproducibly result in accumulation of arrested cells and increased mitotic index in EFM-192A cells (data not shown), but that RNAi of ABCC3/MRP3 resulted in a 2- to 3-fold increase in mitotic index relative to control treatment with a nontargeting control siRNA in the cell lines EFM-192A and ZR75-30 (Fig. 3A and B). In contrast, ABCC3 RNAi did not appreciably alter the mitotic index in nonamplified cell lines HCC-1428 and MDA-MB-453 (Fig. 3C and D). siRNA knockdown of ABCC3 at the transcript level was confirmed by qRT-PCR (Supplementary Fig. S5). Similar results were obtained with MMAE (data not shown). ABCC3/MRP3 (henceforth referred to as ABCC3) is a member of the MDR-associated protein (MRP) subfamily of ATP-dependent drug efflux pumps (34).

**Overexpression of ABCC3 causes in vitro MDR.** Given that ABCC3 knockdown increased sensitivity to paclitaxel and MMAE, we tested whether ABCC3 overexpression in taxane- and MMAE-sensitive cells with low levels of ABCC3 would render cells resistant to these agents. EVSA-T cells were selected as a model to generate ABCC3-overexpressing lines because they do not show ABCC3 amplification and express low levels of ABCC3 transcripts. Three independently derived lines were confirmed to overexpress ABCC3 transcripts (Supplementary Fig. S5) and screened for in vitro sensitivity to paclitaxel and MMAE using an ATP-based luminescence assay. All three overexpressing cell lines were at least 20-fold less sensitive to paclitaxel and MMAE based on EC_{50} values and also showed markedly less inhibition of cell growth compared with a vector-alone control stable cell line in an ATP-based luminescence assay (Fig. 4).

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* C. O’Brien and M.R. Lackner, unpublished observations.

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Amplification of ABCC3 occurs in breast tumors. Analysis of the region of chromosome 17 encompassing HER2 and ABCC3 in the cell line 100K SNP array data suggested that the ABCC3 amplicon was most commonly associated with the HER2-amplified subtypes and was not seen in the cell lines classified as luminal or basal-like (Fig. 5, top two panels).

To ensure that ABCC3 amplification was not a cell line–specific phenomenon, we also characterized copy number data at the ABCC3 locus using Agilent aCGH arrays on DNA from 145 primary breast tumors. These tumor samples were also classified into luminal, basal-like, and HER2 subtypes using a predictor based on expression levels of ER, PR, and HER2 as described by Hu and colleagues. ABCC3 copy number gains (>3.5 copies) are present in 25% of HER2-amplified and 11% of luminal tumors but were not present in basal-like tumors (Fig. 5, bottom two panels).

Finally, to confirm the cytogenetic basis of the apparent copy number gains observed by SNP and aCGH arrays, we developed a FISH assay using a bacterial artificial chromosome clone (see Materials and Methods) spanning the ABCC3 locus and performed FISH analysis on select cell lines and 61 primary tumors that had been classified as overexpressing HER2 based on the HerceptTest (immunohistochemistry assay, reviewed in ref. 35; data not shown). The FISH results from cell lines corroborated the data obtained from the SNP array and qPCR analyses. As depicted in Fig. 6, cell line EFM-192A predicted from SNP arrays to have elevated ABCC3 copy number indeed exhibited a high-level amplification of ABCC3, which is manifested as homogeneously staining regions with single or multiple integration into various chromosomes while maintaining single copies of HER2 and ABCC3 on chromosome 17 (Fig. 6A and B). Cell lines predicted to be diploid for ABCC3 based on SNP array analysis were confirmed to be diploid based on FISH analysis with CEP17 and ABCC3 (data not shown). FISH analysis of the 61 HER2-positive primary tumors that were screened for ABCC3 amplification confirmed that elevated copy number at ABCC3 is common in HER2-positive breast tumors (Fig. 6C and D; Supplementary Table S2). High-level gene amplification (>2.2 ratio of ABCC3/CEP17; see Fig. 6C) was seen in 25% of the tumors, whereas an additional 11% of the tumors showed moderate increases for ABCC3 (3–7 copies of ABCC3, see Fig. 6D) for ABCC3. Interestingly, a number of tumors show evidence of heterogeneity and exhibit cells with both very high-level amplification of ABCC3 alongside cells with diploid copy number of ABCC3.

Discussion

Molecular classification of breast cancers into subtypes with shared features and similar prognostic outcomes provides a framework to begin efforts to individualize cancer therapy. We have shown here that a large collection of breast cancer cell lines reflects many of the genetic and genomic alterations characteristic of human breast tumors and that subtypes show clear differences in response to antimitotic agents, with the basal-like subtype being the most sensitive. We present functional evidence that one...
mechanism for this differential response is amplification of the ABCC3 drug efflux pump in a subset of luminal and HER2-amplified cell lines but not in basal-like cell lines. Future functional genomic efforts in this cell line panel should help identify targeted agents likely to specifically show efficacy in one or more of the subtypes, a particularly pressing need for the basal-like or triple-negative class of tumors. The detailed understanding of the molecular alterations in the cell lines also raises the likelihood of identifying biomarkers of efficacy that are not only correlated with response but also linked to the biology of the target pathway being modulated.

ABCC3 overexpression has been implicated in acquired MDR in cancer cell lines in previous studies. For instance, Liu and colleagues report 459-fold overexpression of ABCC3 relative to the parental in a cell line, MCF-7/AdVp3000, that was derived by selection for growth in the presence of doxorubicin (36). In addition, it has recently been shown that treatment of carcinoma cell lines with vincristine results in significant up-regulation of ABCC2 and ABCC3 transcripts in these cells (37). The closely related pumps ABCC2 (MRP2) and ABCC10 (MRP7) have both been shown to confer paclitaxel resistance when overexpressed (37,38), and moreover ABCC2 has been shown to be an important determinant of paclitaxel pharmacokinetics in vivo in mouse models (39). Paclitaxel has not been previously shown to be a substrate for ABCC3 and indeed studies of ectopic overexpression of ABCC3 in Madin-Darby canine kidney cells or NIH-3T3 cells have failed to show increased resistance to paclitaxel (40, 41). Notably, one of these studies also found (40) that ABCC3 overexpression does not confer resistance to doxorubicin in long-term assays despite other published reports of functional studies suggesting a role for ABCC3 in transporting this agent (36). Taken together, these findings suggest that either the ability of ABCC3 to transport paclitaxel is context or cell type dependent, or that differences in assay format or duration account for the apparent role for ABCC3 in transporting paclitaxel in breast cancer suggested by our studies.

An unusual aspect of our findings is that resistance may be derived from an amplification event present in the primary tumor independent of prior selection due to drug treatment because a number of the samples profiled are mostly either primary tumors or cell lines derived from such tumors or pleural effusions collected at the time of diagnosis. Therefore, it may be postulated that Chr17q21.31-21.33 amplification is selected as part of tumor evolution/homeostasis and that ABCC3 amplification is coamplified as a passenger gene that confers serendipitous chemotherapy resistance. As such, ABCC3 amplification may represent a mechanism of intrinsic resistance present in the tumor due to amplification of adjacent sequences that are presumably being selected for during the process of tumorigenesis. One possible candidate for such selection of Chr17q21.31-21.33 amplification is the nearby gene Myst2, which encodes a histone acetyltransferase that has been shown by Hu and colleagues (36, 37) to result in transformation in soft agar when overexpressed in cell lines that do not normally have amplification or overexpression of Myst2. Other examples of amplification of ABC transporters have been reported, but usually in the context of the tumor-evolving
resistance. For instance, resistant variants of the T-cell leukemia cell line CCRF-CEM selected by growth in increasing levels of the anthracycline epirubicin were shown to have acquired amplification of the ABCC1/MRP1 gene (42).

Other studies have also addressed resistance mechanisms to paclitaxel in breast cancer and have hinted at a multiplicity of mechanisms. For instance, a recent report suggested that in primary cell cultures derived from breast carcinomas, expression of PR was negatively correlated with response to paclitaxel in the adjuvant setting (43). This finding is consistent with the observation here that basal-like breast cancer cell lines are more sensitive to paclitaxel on average than luminal or HER2-amplified lines. Another potential mechanism that has been identified is paclitaxel-triggered phosphorylation of the protein caveolin-1, which has been shown to sensitize cells to apoptosis by regulating cell cycle progression and activation of the apoptotic signaling molecules p53 and p21 (44). Because caveolin-1 expression is highest in basal-like breast cancers (45), this is another potential mechanism to explain the differential antimitotic response observed across subtypes. Finally, gene expression studies on biopsies obtained before adjuvant chemotherapy followed by functional studies in vitro have suggested that low expression of the microtubule-binding protein tau is a potential biomarker for pathologic response to paclitaxel-containing regimens (46). The results described in this article affirm that even well-defined subtypes such as HER2-positive breast cancer may harbor unexpected heterogeneity that may affect response to therapeutics, in this case amplification of the ABCC3 transporter in 25% to 35% of primary HER2-amplified breast tumors. All of these results taken together highlight the fact that breast cancer is a heterogeneous disease that can evade chemotherapy through multiple mechanisms and highlight the need for panels of biomarkers that can be used to predict response in individual tumors.

The results presented here also illustrate the utility of high-density SNP arrays for biomarker discovery when used in conjunction with pharmacologic data in cell lines. A key advantage of this approach is that gene amplification events are relatively stable and can ultimately be assayed by FISH on archival samples from clinical trials. FISH assays are already part of routine clinical practice in the diagnosis of HER2-positive MBC (47). A recent retrospective analysis of 1,500 women with node-positive breast cancer showed that expression or amplification HER2 in a breast cancer is associated with enhanced clinical benefit from the addition of paclitaxel after adjuvant treatment with doxorubicin compared with patients with HER2-negative, ER-positive, and node-positive breast cancer (48). However, a significant fraction of women with HER2-positive tumors failed to show a survival benefit in this study, suggesting the possibility that paclitaxel resistance mechanisms are also present in a proportion of HER2-positive breast tumors. Utilizing a FISH assay to stratify patients by

![Figure 5](https://www.aacrjournals.org/doi/figure/10.1158/0008-5472.CAN-07-3107)

**Figure 5.** Presence of HER2 and ABCC3 amplification determined by SNP array (cell lines) or array CGH (primary tumors) with copy number gains (red) and losses (green) represented by heat map. The ideogram to the left indicates the location of the areas of detail in the heat map. Each column represents a separate DNA sample and each row an independent SNP (cell lines) or probe (tumor samples). Arrows, positions of HER2 and ABCC3. Samples were classified into three major molecular subtypes of breast cancer as described in the text.
ABCC3 amplification status in this setting would allow determination as to whether ABCC3 amplification correlates with lack of clinical benefit from taxane-containing regimens and hence whether ABCC3 amplification could serve as a biomarker to identify patients who should receive other chemotherapeutic agents in addition to taxanes.

Future studies with this large panel of well-characterized cell lines should help to identify additional genomic alterations that predict response to chemotherapeutics as well as novel targeted agents and lead to the development of companion diagnostic tests that can be validated in clinical studies and ultimately to match patients with appropriate therapies.

**Disclosure of Potential Conflicts of Interest**


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**References**


Figure 6. FISH analysis of ABCC3 amplification in cell lines and tumors. A, FISH probes for ABCC3 (green) and HER2 (red) show high-level amplification of both loci in the well-established breast cancer cell line EFM-192A. Multiple homogenously staining regions are found integrated in various chromosomes and HER2 and ABCC3 seem to colocalize (yellow staining). The bright green signal (arrows) indicates the CEP17 centromeric probe. B, HER2 and ABCC3 sequences are highly amplified and are present in a 1:1 ratio in this cell line. C and D, ABCC3 amplification in two independent primary breast tumors. FISH probes for ABCC3 (red) and CEP17 (green) show (C) amplification in tight clusters, indicative of very high level amplification in this tumor. The tumor sample in D shows evidence of multiple copies of ABCC3 and polysomy for chromosome 17 as indicated by multiple copies of CEP17 in each nucleus.


Correction: ABCC3 Amplification and Taxane Resistance

In the article on ABCC3 amplification and taxane resistance in the July 1, 2008 issue of Cancer Research (1), there is an error in Fig. 2. The corrected figure appears below.

Figure 2.


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Functional Genomics Identifies ABCC3 as a Mediator of Taxane Resistance in HER2-Amplified Breast Cancer

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