Association of DNA Methylation of Phosphoserine Aminotransferase with Response to Endocrine Therapy in Patients with Recurrent Breast Cancer

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Abstract

To understand the biological basis of resistance to endocrine therapy is of utmost importance in patients with steroid hormone receptor–positive breast cancer. Not only will this allow us prediction of therapy success, it may also lead to novel therapies for patients resistant to current endocrine therapy. DNA methylation in the promoter regions of genes is a prominent epigenetic gene silencing mechanism that contributes to breast cancer biology. In the current study, we investigated whether promoter DNA methylation could be associated with resistance to endocrine therapy in patients with recurrent breast cancer. Using a microarray-based technology, the promoter DNA methylation status of 117 candidate genes was studied in a cohort of 200 steroid hormone receptor–positive tumors of patients who received the antiestrogen tamoxifen as first-line treatment for recurrent breast cancer. Of the genes analyzed, the promoter DNA methylation status of 10 genes was significantly associated with clinical outcome of tamoxifen therapy. The association of the promoter hypermethylation of the strongest marker, phosphoserine aminotransferase (PSAT1) with favorable clinical outcome was confirmed by an independent quantitative DNA methylation detection method. Furthermore, the extent of DNA methylation of PSAT1 was inversely associated with its expression at the mRNA level. Finally, also at the mRNA level, PSAT1 was a predictor of tamoxifen therapy response. Concluding, our work indicates that promoter hypermethylation and mRNA expression of PSAT1 are indicators of response to tamoxifen-based endocrine therapy in steroid hormone receptor–positive patients with recurrent breast cancer. (Cancer Res 2005; 65(10): 4101-7)

Introduction

Endocrine therapy is the most common and most effective treatment in breast cancer patients with tumors that express estrogen and/or progesterone receptors (1). Although the estrogen receptor-α (ER-α) is the prime target for endocrine therapy, failure or success of this preferred therapy is poorly understood. Systemic therapy in patients with recurrent disease in distant sites is palliative treatment and endocrine therapy in this setting accomplishes a clinical benefit in about 50% to 60% of the patients. Yet, progression is inevitable in these patients because recurrent breast cancer in distant metastatic sites is not a curable disease (2, 3). From a biological point of view, however, first-line single agent endocrine therapy in patients with recurrent breast cancer is an excellent setting to study response to therapy because it is less subject to prognostic influences unavoidably present when a similar study would be done in the adjuvant setting.

Altered DNA methylation of cytosine phosphoguanine dinucleotide (CpG) sites is an early and common alteration in cancer, including breast cancer (4, 5). Many tumor suppressor genes are inactivated by hypermethylation (6), and often aberrant DNA methylation occurs in a tumor type–specific manner (7). With the aid of a high-throughput DNA methylation profiling method that uses a standard microarray read-out technology, we have shown recently that differentially methylated CpG islands correctly distinguish normal from malignant tissue specimens (8). In addition, subclassification of tumor types was possible (8, 9).

In the current study, we combined the candidate gene approach with our high-throughput DNA methylation profiling technology. Our aim was to identify genes whose promoter DNA methylation status was associated with clinical benefit of tamoxifen therapy. In the screening process, 117 candidate genes were included which were selected based on (i) their function in breast tumorigenesis or metastasis, (ii) their presumed role in resistance to endocrine treatment, or (iii) their role in steroid hormone regulation or action. The effect of endocrine therapy on size of the metastatic lesions was used as the main clinical end point of the study. In total, 499 CpGs in the promoter regions of these 117 genes (two to six CpGs per gene) were analyzed using a microarray-based DNA methylation detection assay (8) in a series of 200 steroid hormone receptor–positive tumor samples from hormone-naive recurrent breast cancer patients, treated first-line with the antiestrogen tamoxifen as a single agent.

Note: John W.M. Martens and Inko Nimmrich contributed equally to this study.

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

Conflict of interest: I. Nimmrich, T. Koenig, F. Model, A. Kluth, C. Piepenbrock, A. Olek, and S. Maier are employees and stockholders of Epigenomics AG (Berlin, Germany), a company that aims to commercialize DNA methylation markers. J. Martens, I. Nimmrich, T. Koenig, M. Look, N. Harbeck, F. Model, M. Schmitt, S. Maier, and J. Foekens are inventors of a patent on the DNA methylation markers identified (WO 2004 035803). The patent is filed by Epigenomics AG.

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Materials and Methods

Patients and treatment. The present study, in which coded tumor tissues were used, was done in accordance to the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl). The study design was approved by the institutional Medical Ethical Committee (MEC no. 02.953). This retrospective study was done on fresh-frozen tumor tissue specimens from 200 patients. Clinical and follow-up data are presented in Table 1. In the patients included in the study, primary operable invasive breast cancer was diagnosed between 1978 and 1993. Furthermore, they were selected based on a disease recurrence and treated with the antiestrogen tamoxifen (40 mg daily until progression) as first-line systemic therapy without any prior (neo)-adjuvant endocrine therapy. Patients were routinely followed at the outpatient clinic, generally once every 3 weeks during the first 6 months, and in case of objective response approximately every 6 weeks later on. Skin metastases were assessed clinically by palpation and documented by photography; lymph node metastases were assessed by palpation and sometimes if necessary by ultrasound; lung metastases were routinely followed by X-thorax (once every 6-12 weeks), and by CT-thorax where applicable; liver metastases were always followed by CT of the liver, in general once every 12 weeks; brain metastases were assessed by magnetic resonance imaging if indicated; and bone metastases were followed by X-rays (every 6-12 weeks) and bone scan (every 6-12 months) as a standard, and by magnetic resonance imaging if indicated. Furthermore, plasma tumor marker levels (CA15.3 and/or CA125) were regularly measured. Response to therapy was defined by standard Union International Contre le Cancer criteria as having complete remission (CR) or partial remission (PR), both response types taken together were classified as objective response (10). Patients with stable disease (SD) were defined as those with no disease change for >6 months, those with a short stable disease as those with no disease change for ≤6 months, and patients were considered having progressive disease (PD) when progression occurred immediately after the start of treatment. Objective response and SD (>6 months) were combined and considered as clinical benefit, as commonly done before (11) because patients with SD have a progression-free survival time (PFS) comparable with patients with a PR.

Table 1. Clinical data of patients, follow-up information

Clinical characteristics	*n* (%)
No. cases	200
Age at primary surgery, y (range)	56 (26-81)*
Type of recurrence	
Local regional relapse	28 (14)
Distant recurrence	172 (86)
Dominant site of recurrence	
Soft tissue	32 (16)
Bone	103 (52)
Visceral site	65 (32)
Type of response	
CR	9
PR	36
No change for >6 mos (SD)	59
No change for ≤6 mos	18
PD	78
Menopausal status (% postmenopausal) †	140 (70)
Overall follow-up, mos (range)	106 (30-174)*
Follow-up after tamoxifen therapy, mos (range)	46 (6-135)*
Tumor progression at end of follow-up	197 (99)
Deceased at the end of follow-up	172 (86)
Adjuvant chemotherapy	42 (21)
CMF	26 (13)
Anthracycline-based	16 (8)
Additional treatments after tamoxifen for metastatic disease	
Hormonal therapy(ies)	150 (75)
(Poly)chemotherapy	129 (65)

*Median and range is given.
† At start of therapy.

Tumors and DNA extraction for DNA methylation profiling. Cytosolic ER and progesterone receptor levels were measured as described before (12, 13). All tumors included were either ER and/or progesterone receptor positive (≥10 fmol/mg of cytosolic protein). Genomic DNA was extracted (QIAamp Blood Kit, Qiagen, Hilden, Germany) from 100,000 × *g* pellets obtained after cytosol extraction as described earlier (12).

Bisulfite treatment, PCR amplification, and microarray hybridization. CpG sites from regulatory regions of 117 candidate genes were amplified in multiplex PCR reactions (7 or 8 primer pairs; one of each pair labeled with fluorochrome Cy5) from bisulfite-treated genomic DNA (14). Primers were designed (15) ensuring unbiased amplification of methylated and unmethylated alleles from successfully bisulfite-converted genomic DNA alone. All Cy5-labeled PCR products obtained from one individual sample were mixed and hybridized to glass slides (8), carrying pairs of immobilized oligonucleotides reflecting the methylated (CG) and non-methylated (TG) status for each CpG position. The hybridization conditions allowed the detection of the single nucleotide differences. The microarrays included 499 CpG sites from 117 genes. Gene names and other specifics are available in supplementary information (Supplementary Table S1). Fluorescent images of the hybridized slides were obtained using a GenePix 4000 microarray scanner (Axon Instruments, Union City, CA). From those images, median spot intensities of the CG and TG detection oligonucleotides of each CpG position were taken to calculate the log methylation ratio (log [CG / TG]). The median log methylation ratio over four spot repetitions per chip and over on average four hybridization repetitions were used for further analyses. The mean ratios determined for the 499 CpG sites analyzed in the 200 samples are available in the supplementary data sheet (Supplementary Table S2).

Quantification of DNA methylation of PSAT1 by PCR using LightCycler probes. To determine the DNA methylation of PSAT1 quantitatively, real-time PCR (QM-PCR) for PSAT1 on bisulfite-treated genomic DNA was conducted on the LightCycler (Roche Diagnostic, Mannheim, Germany) by using a competitive probe hybridization approach with methylation state–specific detection probes detecting simultaneously the methylated status (CG) and the unmethylated status (TG) of the locus in one sample. Technical details of this assay format are described separately.[6] The relative signals as exemplified by the cycle threshold (C_t) value specific for the methylated state (C_{tm}) and the unmethylated state (C_{tu}) were used for calculation of methylation rates (methylation rate = $100 / [1 + 2^{\{Ctm - Ctu\}}]$).

Quantification of mRNA of PSAT1. Total RNA was extracted with RNAzol B (Campro, Veenendaal, the Netherlands) and reverse transcribed (16). To quantify the messengers, real-time specific PCR products were generated in 35 cycles from copy DNA (30 ng) in an ABI Prism 7700 apparatus (Applied Biosystems, Foster City, CA) in a mixture containing 330 nmol/L primers (Table 2), and SYBR green (Applied Biosystems; ref. 16). SYBR green fluorescent signals were used to generate C_t values (16) from which PSAT1 mRNA levels were calculated, normalized against the average of three housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase, porphobilinogen deaminase, and β-2-microglobulin. Comparable results were obtained when the PSAT1 mRNA levels were quantified using the PSAT1 mRNA Taqman quantitation kit (Hs.00185582) from Applied Biosystems.

Statistics. Initial marker selection was done on a per gene basis using Hotelling's T^2 test (17) on patients that showed extreme types of response (CR + PR versus PD). The Hotelling's T^2 test computes the Euclidean distance between the mean methylation vectors of the two response classes.

[6] A. Kluth et al, in preparation.

Table 2. Intron-spanning primers used for quantitative real-time PCR

Gene	Forward primer	Reverse primer	Product size (bp)
PSAT1	CCTCGGTCCTGGAATACAAG	GCCAGCTTCTGAACGTCTTC	400
B2M	CTTTGTCACAGCCCAAGATAG	TGAGTGCTGTCTCCATGTTTG	139
HPRT	TATTGTAATGACCAGTCAACAG	GGTCCTTTTCACCAGCAAG	192
PBGD	CATGTCTGGTAACGGCAATG	GTACGAGGCTTTCAATGTTG	85

In analogy to Student's univariate t test, this mean distance is then set in relation to the sample covariance matrix and the probability of observing a more extreme observation under the null hypothesis is computed. Because multiple markers were evaluated at the same time, we used the false discovery rate (FDR) correction to limit the expected number of false discoveries (18). To analyze the relation of a particular gene with the clinical benefit to therapy, a multivariable logistic regression analysis was used treating the measurements of single CpG dinucleotides as independent variables and calculating a score per gene (between two and six CpGs per gene) from the regression coefficients by exponentiated linear prediction. We used Spearman rank correlation to correlate DNA methylation of the individual CpG dinucleotides of the *PSAT1* gene with its mRNA levels (n = 111). The relation of the mRNA expression of PSAT1 with clinical benefit to tamoxifen therapy was analyzed in univariate analysis with logistic regression. PSAT1 mRNA levels dichotomized at the median value were subsequently used in univariate logistic regression analysis for clinical benefit (n = 111). Odds ratios (OR) were calculated and presented with their 95% confidence interval (95% CI). The Cox proportional hazards model was used to calculate the hazard ratios (HR) and their 95% CI in the analyses of PFS. Kaplan and Meier (19) survival curves were generated and the log-rank test was used to test for differences. All Ps are two sided. Computations were done with the STATA statistical package, release 8.2 (STATA Co., College Station, TX).

Results

Association of promoter DNA methylation with the clinical benefit to endocrine treatment with tamoxifen. To identify potential markers associated with therapy success or failure, we did our initial analysis on those 123 patients who showed the extreme types of response to tamoxifen treatment; either an objective response (CR + PR, 45 patients) or progressive disease right from the start of treatment (PD, 78 patients; Table 1). The DNA methylation status of the individual CpGs measured per gene were simultaneously tested using Hotelling's T^2 statistics (17). After correcting for multiple comparison using a FDR of 25% (18), the promoter DNA methylation status of 10 genes was significantly different in patients with objective response compared with those having PD (Fig. 1*A*). For the majority of these genes, CpG hypermethylation was correlated with favorable outcome to tamoxifen treatment, the strongest being PSAT1 (P < 0.0001), whereas for stathmin 1/oncoprotein 18 (STMN1) and stratifin/14-3-3 protein δ, hypomethylation was associated with tumor remission (Fig. 1*B*).

To determine whether the identified genes retained their association with response in a representative group of metastatic

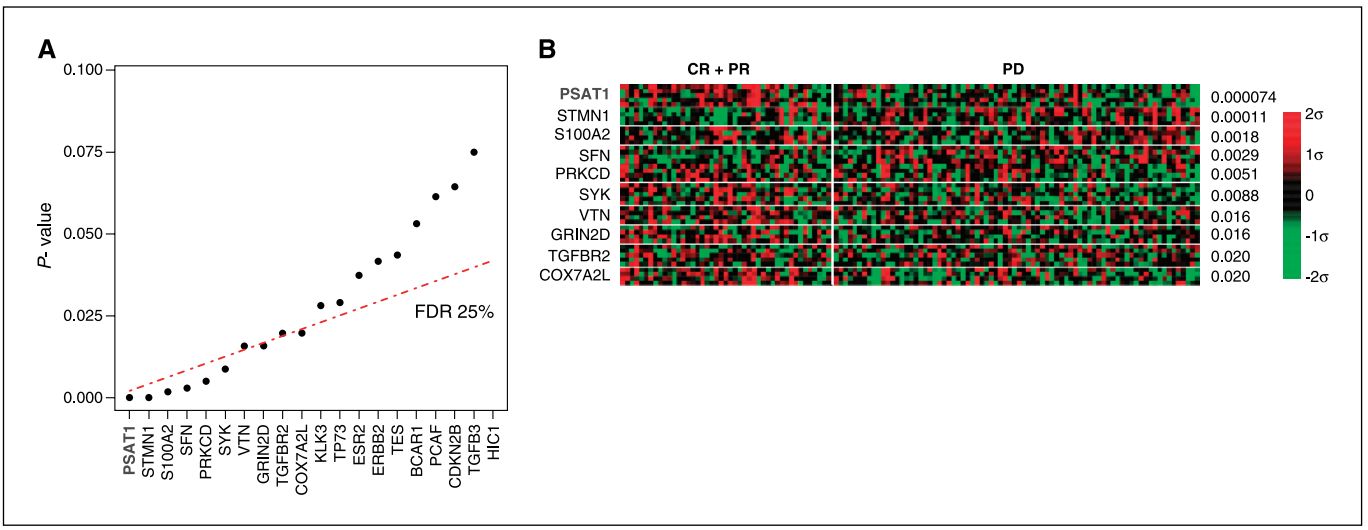

Figure 1. Promoter DNA methylation in primary breast cancer tissues and the patient's response to tamoxifen. *A,* correlation of promoter DNA methylation status of 117 candidate genes to the type of response to endocrine treatment with tamoxifen in 123 patients with recurrent breast cancer. DNA methylation status of 499 CpG dinucleotides in promoter regions of 117 genes (Supplementary Table S1) was determined in tumor tissues of 45 objective responders (CR + PR) versus 78 patients with PD as described in Materials and Methods. The CpG dinucleotides measured per gene were grouped and their correlation with objective response was determined by Hotelling's T^2 statistics (17). *Black dots, P* of the indicated gene. The 20 most informative genes ranked from left to right with increasing *P*. Significance after FDR correction of 25% (*dotted red line*; ref. 18). All genes with a *P* smaller or equal to the gene with the largest *P* that is below the dotted line (in this case until COX7A2L) are considered significant. The FDR correction chosen implies that the identified genes are with 75% chance true discoveries. *B,* raw CpG dinucleotide methylation data of the 10 genes that significantly correlated with the type of response. Tumors from objective responders (CR + PR; *left*); tumors from patients with PD (*right*). *Column,* one tumor; *row,* one CpG dinucleotide. CpG dinucleotides (4 or 5 per gene) are grouped per gene. The indicated genes are ordered from top to bottom according to decreasing significance. DNA methylation data are centered and normalized to one standard deviation (σ) for every individual CpG dinucleotide. The color represents the relative distance of the CpG dinucleotide methylation status from the mean value. *Green,* a hypomethylated CpG dinucleotide; *red,* a hypermethylated CpG dinucleotide.

patients treated with tamoxifen, all 200 patients were subsequently analyzed including those with an intermediate type of response (59 patients with no change of disease for >6 months (SD) and 18 patients with no change of their disease for ≤6 months; Table 1). We used logistic regression analysis with clinical benefit of tamoxifen therapy (objective response + SD) as the end point, an end point used in the clinic nowadays to determine whether a treatment is effective or not. For each of the 10 selected genes, we combined per gene the estimates from the logistic regression for clinical benefit of the different CpG sites per gene measured. In the whole cohort, for all 10 genes, the clinical benefit of tamoxifen treatment was ~10% to 30% higher for patients whose tumors had a favorable DNA methylation status than those with an unfavorable status. In concordance, for the 10 genes, the median PFS in the former group of patients was on average 1.8 times longer than that in the latter group. Thus, also in the complete cohort these 10 genes were associated with disease outcome after the start of tamoxifen treatment.

Independent confirmation of predictive value of PSAT1. To explore the significance of the DNA methylation data with regard to resistance to tamoxifen, we selected the strongest marker identified, PSAT1, and did additional studies.

First, we measured the DNA methylation status of PSAT1 using an independent assay. For this, we used a quantitative methylation detecting PCR (QM-PCR). The method also relies on a bisulfite conversion of epigenetic into genetic information and unbiased amplification of the regulatory region of interest. Instead of an array, however, the detection occurs, similar to quantitative reverse transcription-PCR, during each round of PCR amplification using detection specific oligonucleotide probes measuring either the methylated (CG) or the unmethylated (TG) status of the CpG site position of interest in the same sample simultaneously. In this confirmatory study, an unbiased subset of the samples [CR + PR (n = 19) and PD (n = 19)] was selected and analyzed for the DNA methylation status of the most informative CpG site in the promoter of PSAT1 according to microarray analysis (Oligo 3880:1813; Supplementary Table S2). Representative results of 16 samples (8 CR + PR and 8 PD) analyzed in triplicates, are graphically presented in Fig. 2. It shows that different levels of methylated and unmethylated DNA

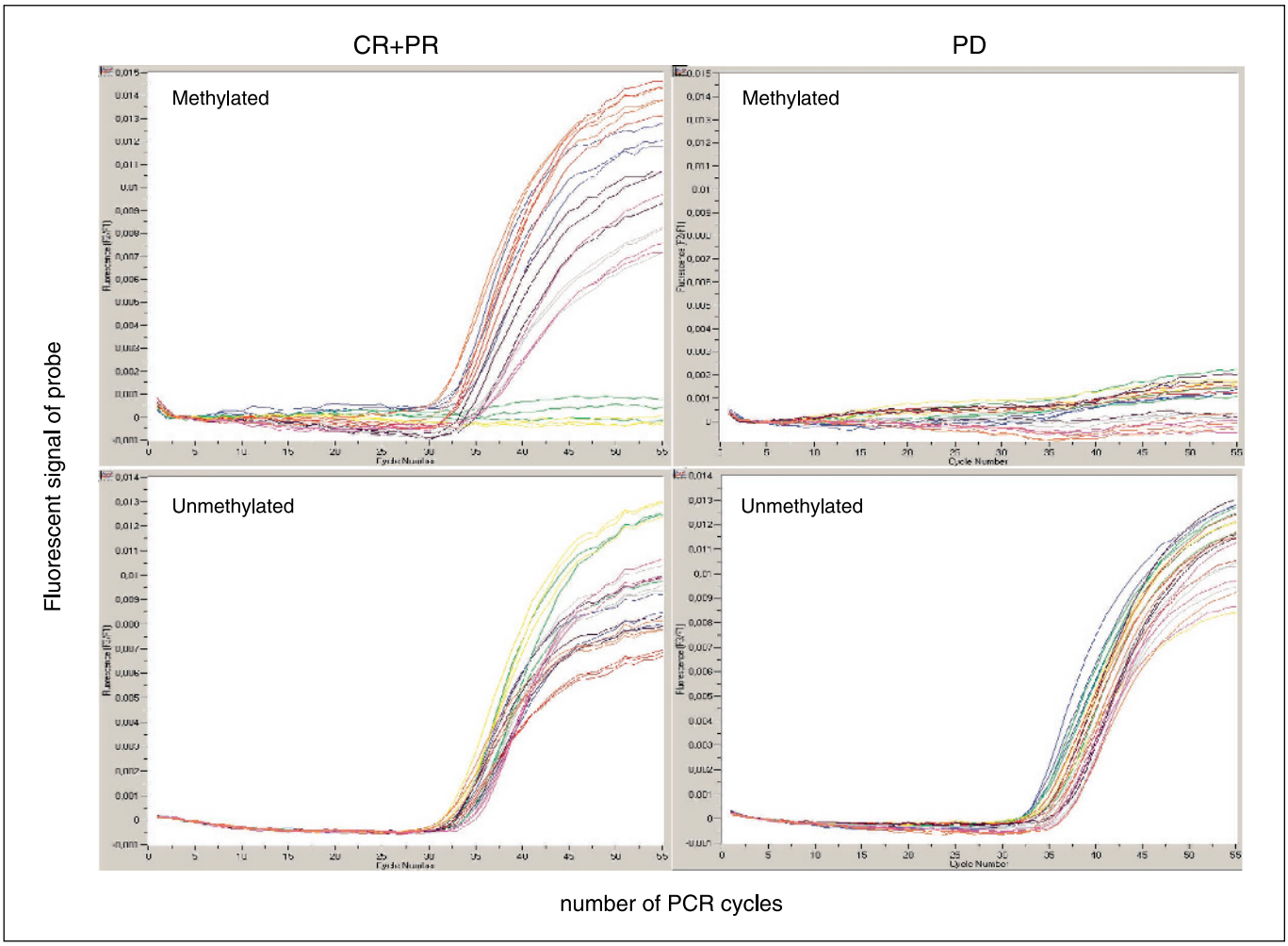

Figure 2. DNA methylation analysis of PSAT1 by quantitative PCR using methylation-specific probes. To confirm the DNA methylation array results, the methylation status of the most informative CpG site (CpG site 3880:1813; Supplementary Table S2) in the PSAT1 promoter was analyzed by QM-PCR using probes specifically recognizing the methylated or the unmethylated status of the CpG site of interest. A random subset of the samples was selected (n = 38). Amplification plots of representative results for some samples (8 CR+PR and 8 PD). Each sample has its own color and triplicate analyses of the same samples are shown in the same color. *Top,* QM-PCR results using detecting probes for methylated DNA (CG); *bottom,* signals retrieved using the oligo specific for unmethylated DNA (TG). Amount of amplicon detected (*y-axis*); number of PCR cycles (*x-axis*).

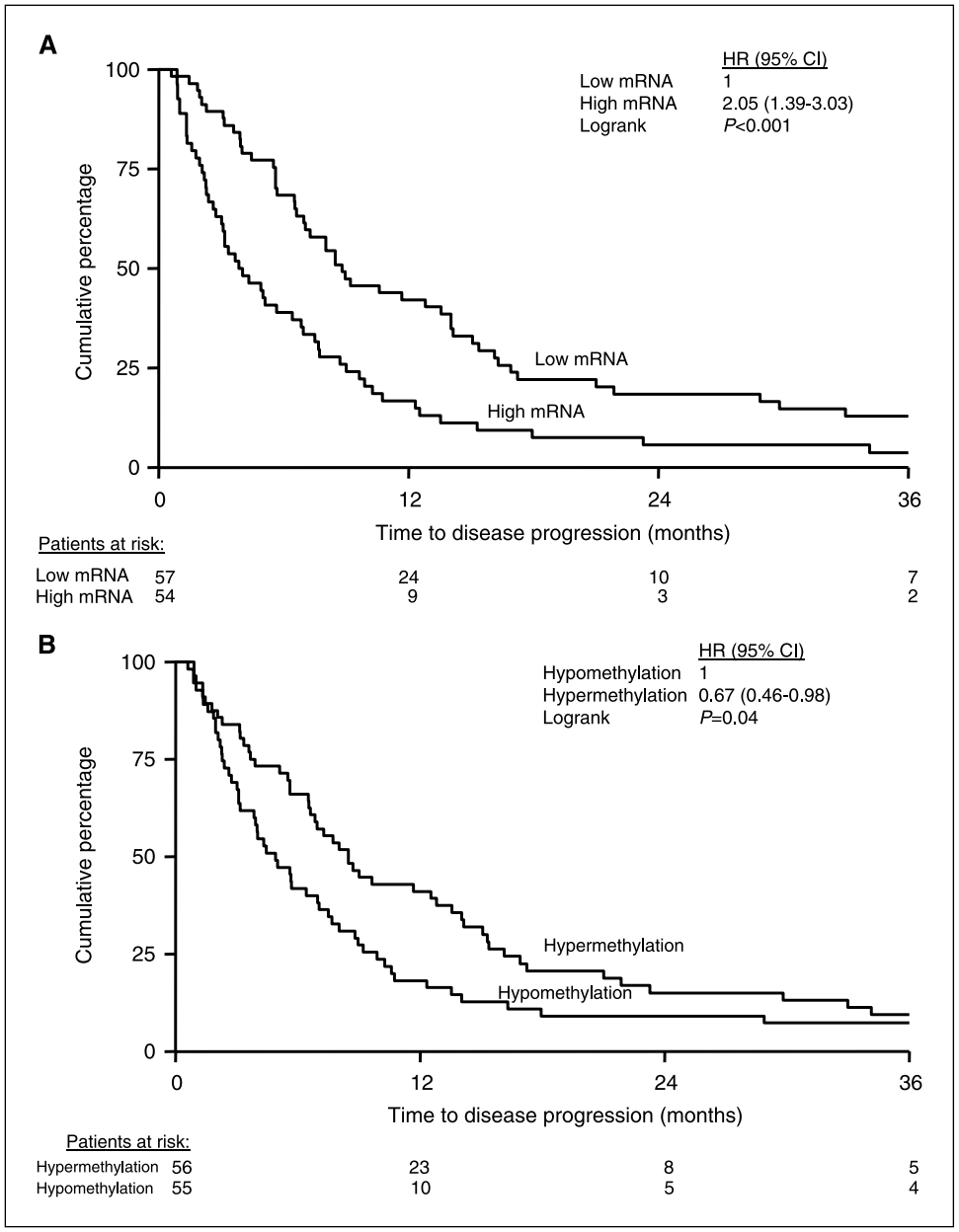

Figure 3. PFS curves as a function of PSAT1 mRNA and DNA methylation status. Kaplan-Meier PFS curves after start of tamoxifen treatment based on PSAT1 mRNA expression or DNA methylation (*B*) are shown for patients in which PSAT1 mRNA levels were available ($n = 111$). The patients in this subgroup had a median age of 57 years, were mostly postmenopausal (66%), and showed in 49.6% of the cases clinical benefit to tamoxifen therapy. The patients are dichotomized using median mRNA and DNA methylation levels. Survival curves were generated and the significance between the subgroups calculated. The numbers below the *x*-axis are the number of patients at risk in each group at the indicated time points of 0, 12, 24, and 36 months.

can be measured reproducibly and that measuring the methylated copies is more discriminative than measuring unmethylated molecules. The latter is probably due to the unavoidable presence of normal breast tissue in the nonmicrodissected samples used in this study, which in general has unmethylated promoter regions. More important for this study is that the QM-PCR DNA methylation ratios significantly correlated with the results of the array ($r_s = 0.75$, $n = 38$, $P < 0.001$). Also based on the QM-PCR, the DNA methylation status of PSAT1 was significantly different between patients with CR + PR versus those with PD ($P = 0.0041$) and comparable to the chip data ($P = 0.0005$ for the same CpG site in the same samples).

Second, we determined the consequence of the differential DNA methylation of the promoter of PSAT1 for the downstream gene. For this purpose, we used quantitative RT-PCR to measure the mRNA levels of PSAT1 in a subset of tumors included in this study from which high quality RNA was available ($n = 111$). In line with the view that DNA methylation silences a downstream gene, we observed an inverse association between the DNA methylation status of four of the five CpG islands in the promoter of PSAT1 and its mRNA expression level (r_s for CpG1 = −0.18, $P = 0.05$; r_s for CpG2 = −0.45, $P < 0.001$; r_s for CpG3 = −0.09, $P = 0.33$; r_s for CpG4 = −0.24, $P = 0.01$; and r_s for CpG5 = −0.34, $P < 0.001$). In addition, we found that high mRNA expression levels of PSAT1 dichotomized at the median value were associated with a poor clinical benefit to tamoxifen therapy (OR, 0.27; 95% CI, 0.12-0.59; $P = 0.001$) and a shorter PFS (Fig. 3*A*; $P < 0.001$). The association with clinical benefit was independent of the traditional predictive factors, age, dominant site of first relapse, disease-free interval, and log ER levels ($\Delta\chi^2 = 8.84$; $df = 1$; $P = 0.003$). As expected in this same group of patients, hypermethylation of the PSAT1 promoter was associated with good clinical benefit (OR, 2.92; 95% CI, 1.35-6.31)

and longer PFS (Fig. 3*B*; $P = 0.04$). Thus, besides the DNA methylation status of PSAT1, its mRNA expression levels are also informative with regard to endocrine therapy response in steroid hormone receptor–positive breast cancer patients treated with first-line tamoxifen monotherapy.

Discussion

In this study, using a high-throughput DNA methylation detection microarray, we show that the DNA methylation status of CpG sites in the regulatory regions of several genes measured in the primary tumor tissue is associated with the likelihood of therapy response in patients with steroid hormone receptor–positive recurrent breast cancer treated by first-line tamoxifen monotherapy. In addition, for the most significant marker, PSAT1, we confirmed the findings using an independent quantitative DNA methylation detection assay. This shows that DNA methylation profiling in clinical samples is feasible and that differential DNA methylation levels of specific genes can be associated with tamoxifen therapy response in steroid hormone receptor–positive patients who in general are candidates for endocrine therapy.

Based on recent advances in breast cancer management, endocrine therapy with aromatase inhibitors may become the treatment of choice for postmenopausal women, a subgroup of the patients studied here (20–22). Because both aromatase inhibitors and tamoxifen aim to deprive the estrogen receptor from estrogens, it would be interesting to learn whether our epigenetic markers can also be linked to clinical benefit of aromatase inhibitors. In addition, as the majority of patients receive adjuvant treatment today, it will be important to learn whether these DNA methylation markers are also informative for early stage patients.

Just recently, using quantitative DNA methylation detection assays, Widschwendter et al. (23) have studied in 148 human breast tumors the DNA methylation status for 35 genes. Although their initial aim was to link epigenetic markers to hormone receptor status they found that methylation of ER-α was associated with clinical response in patients treated with the antiestrogen tamoxifen. Furthermore, in the same article, a significant differential association of DNA methylation of ARHI and CYP1B1 to outcome was seen in the tamoxifen-treated versus the not tamoxifen-treated cohort. In our study, CYP1B1 was not analyzed, whereas for ARHI we did not find a correlation to the rate of response nor did we find a significant association to tamoxifen response for ER-α, although ER-β was of borderline significance. The reasons for the difference between that study and ours could be manifold including difference in study design (adjuvant versus first-line treatment), in the CpG sites analyzed, in the technology used, or in size or composition of the tissue collections used. Due to the heterogeneity of the cohorts and the likely confounding influence of steroid hormone receptor status, and different treatment modalities, the results of the study of Widschwendter et al. are difficult to interpret. Our study focused deliberately on patients with steroid hormone receptor–positive tumors, who were all treated with first-line tamoxifen monotherapy. In any case, proper validation studies will be needed in uniformly defined patient and treatment collectives to determine the clinical significance of DNA methylation ER-α, ARHI and CYP1B1 identified by Widschwendter (23) and of the 10 DNA methylation markers identified in our present study. Nonetheless, both studies show that an epigenetic signature containing several DNA methylation markers may become a competitive alternative to tedious assessment of mRNA profiles.

Besides the clinical significance, it will be of interest to learn whether and how the current candidate genes, showing an altered CpG methylation status, are causally involved in the process of tamoxifen resistance in recurrent breast cancer. In general, hypermethylation of CpG islands is correlated with gene silencing. For PSAT1, we have shown this in this set of tumors. For some of the genes, *S100A2* (24), stratifin (*SFN*; ref. 25), and spleen tyrosine kinase (*SYK*; ref. 26), this has earlier been shown in breast tumor tissues or in breast cancer cell lines. For the others, however, this remains to be established. With regard to function, several of the identified genes code for signaling molecules that may act on different checkpoints of tumor biology. As such, *GRIN2D*, *SYK*, *S100A2*, and protein kinase C (*PRKCD*) are involved in cell survival (27); *SYK* acts on the cell cycle (28); *STMN1* controls cell division (29); and *SYK* and *SFN* modulate breast tumor growth (30, 31); whereas vitronectin (*VTN*) promotes metastasis (32). On the other hand, some of the candidate genes play a crucial role in catabolism like *PSAT1*, which is catalyzing the second step in the biosynthesis of the amino acid serine (33), which in turn is the crucial carbon source for purine nucleotides, phosphatidylcholine and phosphatidylserine, and other cellular metabolites (33). Another identified gene, *COX7A2L*, codes for the cytochrome *c* oxidase subunit VIIA subunit 2L that is involved in energy expenditure (34). Finally, expression of two of the identified genes, *S100A2* and *SFN*, marks a myoepithelial-like breast (cancer) cell type (35). Myoepithelial-like tumor cells tend to be ER negative and more aggressive (36). The fact that the DNA methylation pattern of S100A2 that predicts response is associated with ER expression ($r_s = 0.37$; $P < 0.001$) is in line with the view that we might be dealing with a myoepithelial-like phenotype among the tamoxifen-resistant tumors. Further studies are required to substantiate this. For most of the DNA methylation markers detected here, the exact molecular mechanism that links them to endocrine resistance in clinical samples remains unresolved. Nevertheless, it is clear, from our work, that the current DNA methylation markers are connected to the breast tumor's escape from antiestrogen action leading in the long run to a better understanding of endocrine resistance in recurrent breast cancer.

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