

## **DNA Methylation Markers Predict Outcome in Node-Positive, Estrogen Receptor-Positive Breast Cancer with Adjuvant Anthracycline-Based Chemotherapy**

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**Abstract Purpose:** We have shown that DNA methylation of the PITX2 gene predicts risk of distant recurrence in steroid hormone receptor-positive, node-negative breast cancer. Here, we present results from a multicenter study investigating whether PITX2 and other candidate DNA methylation markers predict outcome in node-positive, estrogen receptor-positive, HER-2-negative breast cancer patients who received adjuvant anthracycline-based chemotherapy.

**Experimental Design:** Using a microarray platform, we analyzed DNA methylation in regulatory regions of PITX2 and 60 additional candidate genes in 241 breast cancer specimens. Using Cox regression analysis, we assessed the predictive power of the individual marker/marker panel candidates. Clinical endpoints were time to distant metastasis, disease-free survival, and overall survival. A nested bootstrap/cross-validation strategy was applied to identify and validate marker panels.

**Results:** DNA methylation of PITX2 and 14 other genes was correlated with clinical outcome. In multivariate models, each methylation marker added significant information to established clinical factors. A four-marker panel including PITX2, BMP4, FGF4, and C20orf55 was identified that resulted in improvement of outcome prediction compared with PITX2 alone.

**Conclusions:** This study provides further evidence for the PITX2 biomarker, which has now been successfully confirmed to predict outcome among different breast cancer patient populations. We further identify new DNA methylation biomarkers, three of which can be combined into a panel with PITX2 to increase the outcome prediction performance in our anthracycline-treated primary breast cancer population. Our results show that a well-defined panel of DNA methylation markers enables outcome prediction in lymph node-positive, HER-2-negative breast cancer patients treated with anthracycline-based chemotherapy.

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In breast cancer, currently available methods are inadequate to determine precisely the aggressiveness of the disease and the likelihood of response to a certain treatment in individual patients. Therefore, biomarkers predicting the tumor's metastatic potential and its response to a specific treatment are urgently needed. This is particularly true for patients for whom current guidelines (1) recommend anthracycline-based chemotherapy. These patients comprise a clinically distinct intermediate- to high-risk subgroup, for which in view of the short and long-term toxicity as well as the availability of new effective anthracycline-free chemotherapy options (2), the widespread use of adjuvant anthracyclines is currently under debate (3). Unfortunately, reliable biomarkers for benefit from anthracycline therapy are still lacking.

DNA methylation of CpG dinucleotides within gene regulatory regions, associated with suppression of gene expression, is a common and early event in cancer (4–6). Specific DNA methylation patterns for tumor subtypes including breast cancer have been reported and associated with clinical outcome (7–16).

### Translational Relevance

Outcome prediction of node-positive patients having received anthracycline-based chemotherapy is of clinical relevance particularly in patients with hormone receptor-positive/HER-2-negative disease. Current guidelines recommend anthracycline-based chemotherapy for such patients, but their clinical outcome remains quite diverse. Patients who have a poor outcome may be recommended for currently available anthracycline-free alternative treatment options, whereas patients with a good outcome on anthracyclines are adequately treated. Reliable biomarkers for benefit from anthracycline therapy for these type of patients are, however, lacking. We have identified a panel of DNA methylation markers that may allow reliable prediction of clinical outcome for these patients. Such a panel, when properly validated in independent multicentric cohorts, may be used to recommend anthracycline-based chemotherapy for node-positive breast cancer patients with hormone receptor-positive, HER-2-negative disease.

We have recently found and validated that DNA hypermethylation of the PITX2 promoter is associated with a high risk of recurrence in node-negative, steroid hormone receptor-positive breast cancer who received tamoxifen as their only systemic adjuvant therapy (13, 17). These studies further showed that DNA methylation can be successfully measured in formalin-fixed, paraffin-embedded breast cancer specimens, which is one of the strengths of DNA methylation technologies and considered a prerequisite for routine clinical application (17). A follow-up study revealed that PITX2 methylation is a strong and pure prognostic factor in patients with node-negative, steroid hormone receptor-positive breast cancer who did not receive any adjuvant systemic treatment (15).

In the present study, we have used a microarray technology to study whether methylation of PITX2 and 60 other candidate genes could be predictors for outcome in breast cancer patients with node-positive, steroid hormone receptor-positive, and HER-2-negative tumors receiving adjuvant anthracycline-based chemotherapy. In our study, we focused on patients with lymph node-positive, estrogen receptor (ER)-positive, and HER-2-negative breast cancer, as these patients comprise a clinically distinct subgroup for which anthracycline-based adjuvant chemotherapy is currently recommended (1). Our study suggests that reliable prediction of clinical outcome for these breast cancer patients may be possible using a well-defined panel of DNA methylation markers.

### Materials and Methods

#### Patients and samples

The study cohort comprised 384 node-positive breast cancer patients whose tumor samples were recruited from four clinical centers: Erasmus Medical Center; Centre René Huguenin; Stiftung Tumorbank; and Department of Obstetrics and Gynecology, Technical University of Munich. Appropriate consent, according to institutional requirements, has been obtained for all patients. The study protocol was approved by the local ethics committees.

To be eligible for this study, patients had to fulfill all of the following criteria: (a) histologically confirmed invasive breast cancer, (b) primary tumor stage pT<sub>1</sub> to pT<sub>3</sub>, (c) histologically confirmed lymph node involvement (pN > 1), (d) surgery before 2002 (potential follow-up of at least 5 years), (e) standard adjuvant anthracycline-based chemotherapy [no dose-dense therapy, no other primary systemic chemotherapy (except hormonal therapy), and no additional taxane], and (f) availability of clinical follow-up data.

Among the 384 patients recruited for the study, 284 were reported as ER-positive. HER-2 status was determined in all samples using the Light Cycler HER-2/*neu* DNA quantification Kit (Roche Applied Science). HER-2-positive patients ( $n = 43$ ) who nowadays would receive trastuzumab treatment were then excluded, leaving a total of 241 node-positive, ER-positive, and HER-2-negative breast cancer patients available for analysis. For the whole cohort, the percentages of patients with distant metastasis at 5 and 10 years were estimated as 70.1% (64.3-76.3%) and 56.1% (48.5-65.0%), respectively; 5- and 10-year disease-free survival (DFS) were 64.5% (58.6-71.0%) and 47.4% (40.0-64.2%), respectively; and 5- and 10-year overall survival (OS) were 85.8% (81.4-90.5%) and 67.7% (59.8-79.1%), respectively. The median follow-up 81.5 months.

#### DNA extraction and bisulfite treatment

Genomic DNA was extracted from snap-frozen tumor tissue or tumor cell nuclei pelleted at  $100,000 \times g$  as described (14). Bisulfite conversion of the DNA was done using the EpiTect Kit (Qiagen).

#### Candidate gene selection

The 61 candidate genes (listed in the Supplementary Material) to be analyzed on the microarray were selected based on (a) genome-wide screens for prognostic markers in ER-positive and ER-negative breast cancer,<sup>6</sup> (b) PITX2 pathway analyses, and (c) literature.

#### PCR amplification and microarray hybridization

PCR amplification and microarray hybridization were done as described previously (7, 14). In total, 64 PCR amplicates representing 61 genes were pooled and hybridized to the microarray on which detection oligonucleotides for methylated (CG) and nonmethylated (TG) gene copies were spotted. This allowed for simultaneous quantitative measurement of unmethylated and methylated copies of the genes. Microarrays included 4 oligonucleotide pairs for each of the 64 PCR amplicates (total of 256 pairs). Each probe pair covered between one and three CpG dinucleotides in the regulatory regions of the respective candidate gene. The methylation score for each CpG site was calculated from the fluorescence intensity values of the methylated (FI<sub>m</sub>) and unmethylated (FI<sub>u</sub>) oligonucleotides. To stabilize the variance, the score was transformed using the generalized log transformation (gLOG): methylation score = gLOG(FI<sub>m</sub> / FI<sub>u</sub>) (18). For statistical analysis, methylation scores for each amplicate were determined by averaging measurements from all probe pairs belonging to one amplicate using the median. Multiple amplicates from the same candidate gene entered data analysis independently. Valid microarray results were obtained for all samples.

#### Clinical endpoints

The primary clinical endpoint in this study was time to distant metastasis (TDM). Secondary endpoints were DFS and OS. For TDM, only distant recurrences were considered. Ipsilateral and locoregional recurrences were not considered as events or censoring events. Contralateral breast cancer, development of other primary tumors, death (from any cause) without observed recurrence, and loss for follow-up were considered censoring events. For DFS, all recurrences were considered as events, whereas death (from any cause) before recurrence, contralateral breast cancer, and development of other

<sup>6</sup> Unpublished data.

primary tumors or loss for follow-up were considered censoring events. For OS, only death (from any cause) was considered as event and patients were censored only when lost for follow-up.

### Statistical analyses

The relation between clinical endpoints and DNA methylation score was analyzed for each amplicate by linear univariate Cox proportional hazards models (19, 20). Here, likelihood ratio tests (LRT) were done to test for a significant relationship of methylation scores of each amplicate with clinical endpoints. Hazard ratios (HR) for continuous variables were calculated relative to an increment of the interquartile range of that variable (for the increment from the 25% quantile to the 75% quantile of the measurements; ref. 20). Multivariate regression analysis, testing the association between clinical endpoint and multiple methylation scores and/or clinical variables, was done by linear Cox proportional hazards models. In that case, LRT was done to test for a significant association of the outcome variables (TDM, DFS, and OS) with the derived model consisting of clinical variables and/or methylation scores. In addition, Wald tests (testing the hypothesis that the variable in question provides significant information to the multivariate model) were calculated. Survival curves were plotted according to Kaplan-Meier (21). Log-rank tests were used to test for differences between survival curves. To describe and compare the predictive performance of each variable and each multivariate model, the concordance index (C index; refs. 20, 22) was calculated. To penalize overfitting by entering multiple factors, the bootstrap-corrected version of the C index is given (20, 22).

To correct for multiple testing in univariate testing (64 amplicates), significance levels were adjusted using false discovery rate correction using the linear step-up procedure proposed in ref. 23.

**Marker panel feature selection.** A nested cross-validation/bootstrap procedure was applied to perform feature selection and panel validation (24, 25). A schematic illustration of the methodology is shown in the Supplementary Material. To identify a marker panel for outcome prediction, the 63 amplicates [all but the amplicate designed in the promoter of transcripts A and B for PITX2 (PITX2P2)] were first ranked by univariate performance (Cox proportional hazards model, LRT). Then, marker panels containing PITX2P2 and increasing numbers of the best 19 single markers were evaluated with respect to their prognostic predictability, which was estimated using the bootstrap-corrected C index with  $B = 200$  bootstrap runs (20). A gene was selected for the final predictor model if the C index of the model including this marker was larger than that of the model excluding the marker. A C index was considered "larger" if at least an increase of 1% over the model containing PITX2P2 was observed. The extensive search was limited to a maximum of 20 amplicates altogether, balancing the critical sample size to build a reasonable regression model (number of distant metastases) on the one side and the request to find markers complementing previously included ones (and therefore not necessarily good univariate markers) on the other.

**Marker panel validation.** The cross-validated prediction score is an unbiased estimate of the performance of the marker panel in a future data set. It corrects for the optimism introduced due to feature selection within the same data set (24, 25). A schematic illustration of the methodology is presented in the Supplementary Material. In brief, the generalization error for the marker panel selection was estimated by repeating the feature selection procedure using a 20-fold cross-validation, starting with all 64 amplicates in each cross-validation run. We trained on 95% of the samples and computed the cross-validated predictive index for the samples in the remaining 5% of samples using the Cox model developed for the training set. To combine results from all runs, the predictive indices for all samples were transformed into risk percentiles within each run. The nested cross-validation procedure itself was replicated 100 times using random permutations of the data set to determine the estimation error of the cross-validated C index. As cross-validated risk percentiles are correlated among cases, the usual null distributions of the test statistics are not

valid. To derive  $P$  values of both the likelihood ratio and Wald tests (Cox model) as well as the log-rank test (Kaplan-Meier analysis) for the cross-validated prediction score, the null distribution was estimated using 100 repetitions of the entire procedure for permutations of survival times and censoring indicators.

To further illustrate the performance of the cross-validated prediction score, the score was analyzed in a similar way as described above for our individual amplicates. All results using a single cross-validated prediction score were generated using results of the first replicate of the nested cross-validation procedure. Statistical analysis was done using R version 2.4.1<sup>7</sup> and the R package "Design" version 2.1.

## Results

**Patient and tumor characteristics.** Patient and tumor characteristics ( $n = 241$ ) are given in Supplementary Material, and these are comparable with previously published cohorts with similar inclusion criteria (26). These clinical standards may differ somewhat from current guidelines as shown by the fact that only 104 (43%) patients received additional adjuvant endocrine treatment, although their tumors had positive steroid hormone receptor status. All patients had received adjuvant anthracycline-based chemotherapy according to clinical standards at the time of their primary therapy.

Tumor size (HR, 1.77;  $P = 0.0225$ ), grade (HR, 2.07;  $P = 0.004$ ), and adjuvant endocrine therapy (HR, 0.49;  $P = 0.0025$ ) were significantly associated with TDM in this cohort (Table 1). Adjuvant endocrine therapy, grade, and progesterone receptor (PgR) were significantly associated with DFS (data not shown). Tumor size, grade, endocrine therapy, and PgR status were significantly associated with OS (data not shown). For all clinical endpoints, risk of recurrence was lower for patients who received adjuvant endocrine therapy.

**Univariate analysis of methylation markers.** Because we have observed DNA methylation of PITX2 as a strong marker in previous studies, we first analyzed the performance of this marker. The methylation score measured with the amplicate designed for the promoter of transcripts A and B of PITX2 (PITX2P2), located upstream of the first transcriptional start, showed that PITX2P2 hypermethylation was associated with a high risk of distant recurrence in this patient cohort [HR, 1.66; 95% confidence interval (95% CI), 1.21-2.28;  $P = 0.002$ ; C index = 0.624; see Table 1 and Fig. 1]. PITX2P2 hypermethylation was also associated with poor DFS (HR, 1.47; 95% CI, 1.11-1.96;  $P = 0.0084$ ) and OS (HR, 2.07; 95% CI, 1.40-3.06;  $P = 0.0003$ ). However, methylation of the amplicate designed for the promoter regulating transcript C of PITX2 (PITX2P1) was not associated with distant recurrence in this patient cohort (HR, 1.23; 95% CI, 0.89-1.68;  $P = 0.21$ ).

Among the other 62 tested amplicates, 15 amplicates, derived from 14 genes, were associated with TDM (false discovery rate < 5%; Table 2). Methylation of BMP4, FOXL2, LMX1A, C20orf55, BARX1, PLAU (both amplicates), FGF4, NR5A1, BRCA1, TLX3, NR2E1, LHX4, ZNF1A1, and CCND2 and BMP4, FOXL2, LMX1A, FGF4, and BRCA1 showed C indices between 0.58 and 0.63 comparable with that of PITX2P2. For DFS, BMP4, FOXL2, and C20orf55 and for OS all 15 amplicates described above were significantly associated with the respective clinical endpoint (false discovery rate < 5%;

<sup>7</sup> <http://www.r-project.org>

**Table 1.** Univariate and multivariate Cox proportional hazards analysis for TDM

	Univariate analysis			Multivariate analysis		
	No. samples	HR (95% CI)	P*	No. samples	HR (95% CI)	P*
PITX2P2 methylation †	241	1.66 (1.21-2.28)	0.002 ‡	200	1.28 (1.03-3.83)	0.026 ‡
Age at surgery †	241	1.00 (0.75-1.32)	0.981	200	1.01 (0.98-1.04)	0.7
T stage (T <sub>2</sub> ,T <sub>3</sub> vs T <sub>1</sub> )§	239	1.77 (1.08-2.91)	0.023 ‡	200	1.47 (0.84-2.59)	0.18
PgR status (positive vs negative)§	241	0.77 (0.43-1.36)	0.367	200	1.11 (0.54-2.31)	0.77
Endocrine treatment (yes vs no)§	239	0.49 (0.30-0.78)	0.003 ‡	200	0.58 (0.33-1.00)	0.052
Grade (1/2 vs 3)§	203	2.07 (1.27-3.83)	0.004 ‡	200	1.68 (1.00-2.81)	0.049 ‡

\*P values refer to LRT for univariate analysis and Wald test for multivariate analysis.

† PITX2P2 methylation and age at surgery were analyzed as continuous variables (95% CI), and HRs were calculated relative to an increment from the lower quartile to the upper quartile.

‡ Significant feature.

§ T stage, endocrine treatment, PgR status, and grade were analyzed as binary variables.

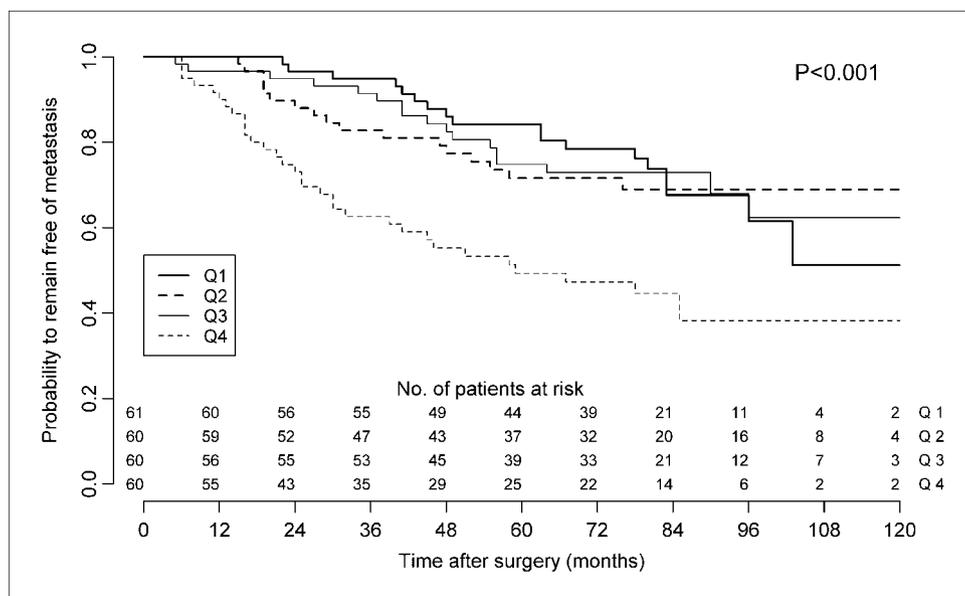
data not shown). For all markers, with the exception of C20orf55, lower methylation scores were associated with better clinical outcome.

**Multivariate analysis of methylation markers and clinical variables.** PITX2P2 methylation was a significant marker in the multivariate analysis including age at surgery, pathologic T stage and grade, PgR status, and adjuvant endocrine therapy ( $P = 0.05$ ; Table 1). To show clinical usefulness of a biomarker, it is necessary that the marker increases the predictive performance of established clinical variables. For the multivariate model including PITX2P2, the bootstrap-corrected C index, which is an unbiased estimate of the effect size for regression models, is larger than that for the model including the clinical variables only (0.647 compared with 0.61, respectively; Table 3). Therefore, PITX2P2 provides additional information for prediction of TDM independent of established clinical variables.

Most of the 15 amplicates identified in univariate analysis were also a significant marker in multivariate analysis when combined with age, pathologic T stage, PgR status, and

adjuvant endocrine therapy. The bootstrap-corrected C indices of the multivariate models (range, 0.620-0.665) were larger than those of the multivariate model using only clinical variables (0.610; Table 2).

**Marker panel selection and performance prediction.** Having established the role of several DNA methylation markers (PITX2 and 15 other markers) for outcome prediction, we assessed if combining these markers into a panel resulted in improved performance compared with that of the individual markers alone. Overfitting becomes a critical issue if feature selection, model building, and performance estimation are done within one data set (27). Therefore, the bootstrapped-corrected C index is used in the feature selection step. To select relevant markers that could improve the performance of our already well-established marker PITX2, we evaluated the gain in estimated effect size (C index) by stepwise adding the best markers from univariate analysis to PITX2P2. Figure 2A illustrates the results of the feature selection step. With PITX2P2 as “anchor,” BMP4, C20orf55, and FGF4 were selected to define a four-marker panel for prediction of TDM in the current cohort.



**Fig. 1.** Prediction of TDM survival probabilities based on PITX2P2 DNA methylation. Kaplan-Meier TDM curves of patients categorized in subgroups based on PITX2P2 DNA methylation scores ( $P = 0.00014$ ). The methylation score was grouped into quartiles. Log-rank test was used to test for significant separation between the groups. C index was 0.624. Patients at risk for the different quartiles are indicated in 12-mo intervals.

**Table 2.** DNA methylation markers in ER-positive, HER-2-negative patients that meet the 5% false discovery rate limit for TDM

No.	Gene	Univariate analysis		Multivariate analysis	
		C index	P*	C index <sup>†</sup>	P*
1	BMP4	0.632	<0.0001	0.680	0.0002
2	FOX2	0.632	0.0001	0.640	0.02
3	LMX1A	0.617	0.0008	0.635	0.068
4	C20orf55	0.592	0.0009	0.637	0.001
5	BARX1	0.613	0.0015	0.624	0.1
6	PLAU Amp2	0.598	0.0017	0.620	0.12
7	FGF4	0.619	0.0019	0.646	0.029
8	NR5A1	0.609	0.0027	0.635	0.059
9	BRCA1	0.631	0.0028	0.647	0.018
10	TLX3	0.583	0.0032	0.634	0.008
11	NR2E1	0.592	0.0054	0.643	0.02
12	LHX4	0.576	0.0076	0.621	0.12
13	PLAU Amp1	0.585	0.0078	0.635	0.051
14	ZNF1A1	0.594	0.008	0.636	0.044
15	CCND2	0.598	0.01	0.631	0.067

NOTE: For all variables, except C20orf55, low methylation scores were associated with long TDM. Multivariate analyses were done including the respective amplicate as well as age at surgery, T stage, endocrine treatment PgR, and grade.

\*P values refer to LRT for univariate analysis and Wald test for multivariate analysis (not corrected for multiple testing).

<sup>†</sup>C index of multivariate model with clinical variables alone (age at surgery, T stage, PgR, endocrine treatment, and grade) was 0.61.

To estimate the performance regarding outcome prediction of the four-marker panel, feature selection was nested within a cross-validation procedure. This corrects for overfitting due to feature selection, and the resulting cross-validated C index is an unbiased estimate of the performance of the marker panel in a future data set. The C index of the cross-validated prediction score is 0.668 compared with 0.624 for PITX2P2 alone (Table 3). We replicated the estimation procedure 100 times, thereby generating a range of C indices for our four-marker panel. As a result, a median C index of 0.654 (range, 0.622-0.676) was obtained for the prediction score, and in 98 of 100 replications, the estimated performance of our four-marker panel was better than that of PITX2P2 alone (Fig. 2B). Based on these results, we conclude that a four-marker panel can indeed improve outcome prediction over a single marker.

To further illustrate the performance of our outcome predictor, we report the results of the first cross-validation

replicate in more detail. The four markers selected for our marker panel represent a stable selection: C20orf55 was selected in all cross-validation runs, FGF4 in 16 of 20 runs, and BMP4 in 14 of 20 runs. Only two other markers were selected in any run: APC and BRCA1 (Fig. 2C). The C index of the cross-validated prediction score for a multivariate model including the cross-validated prediction score as well as age at surgery, pathologic T stage, PgR, and adjuvant endocrine therapy was 0.695 ( $P < 0.01$ , LRT; Table 3). The Wald  $P$  value for the cross-validated prediction score in the multivariate model was  $<0.01$ . Kaplan-Meier estimates illustrate the difference ( $P < 0.01$ , log-rank test; Fig. 2D) between the performance of the identified marker panel (Fig. 3, left) and PITX2P2 alone (Fig. 1). For all patients, the 5-year survival prediction of the quartile with the highest cross-validated prediction scores for the four-marker panel is  $<50%$ , whereas that of the quartile with the lowest cross-validated prediction scores is  $>85%$ . Similar to TDM, significant prediction scores were derived for DFS ( $P = 0.01$ ) and OS ( $P < 0.01$ ). Since today all patients with steroid hormone receptor-positive breast cancer will receive adjuvant endocrine therapy, we performed a subgroup analysis for those patients in our cohort who had received adjuvant endocrine treatment. Although the size of cohort was limited to 104 patients, a highly statistically significant separation of survival groups was observed when stratifying the patients according to the prediction score (Fig. 3, right).

## Discussion

According to current guidelines, adjuvant chemotherapy needs to be considered for the majority of patients with primary breast cancer, with the exception of a small subgroup with very favorable outcome prediction based on clinical factors (1). In node-positive breast cancer, anthracycline-based adjuvant chemotherapy has become the standard of care during the 1990s (28). Moreover, several studies showed that adding a taxane (29), or, more recently, administering dose-dense chemotherapy regimens (including anthracyclines; ref. 30), further improves outcome in node-positive breast cancer. However, not all patients will benefit from such more aggressive therapies and therefore only face their more severe side effects, because these patients either have an excellent prognosis or have tumors that do not respond to certain chemotherapies. Thus, prognostic and predictive markers are urgently needed to tailor the best possible therapy regimen for the individual patients.

**Table 3.** Predictive performance estimated from multivariate Cox proportional hazards analysis for TDM

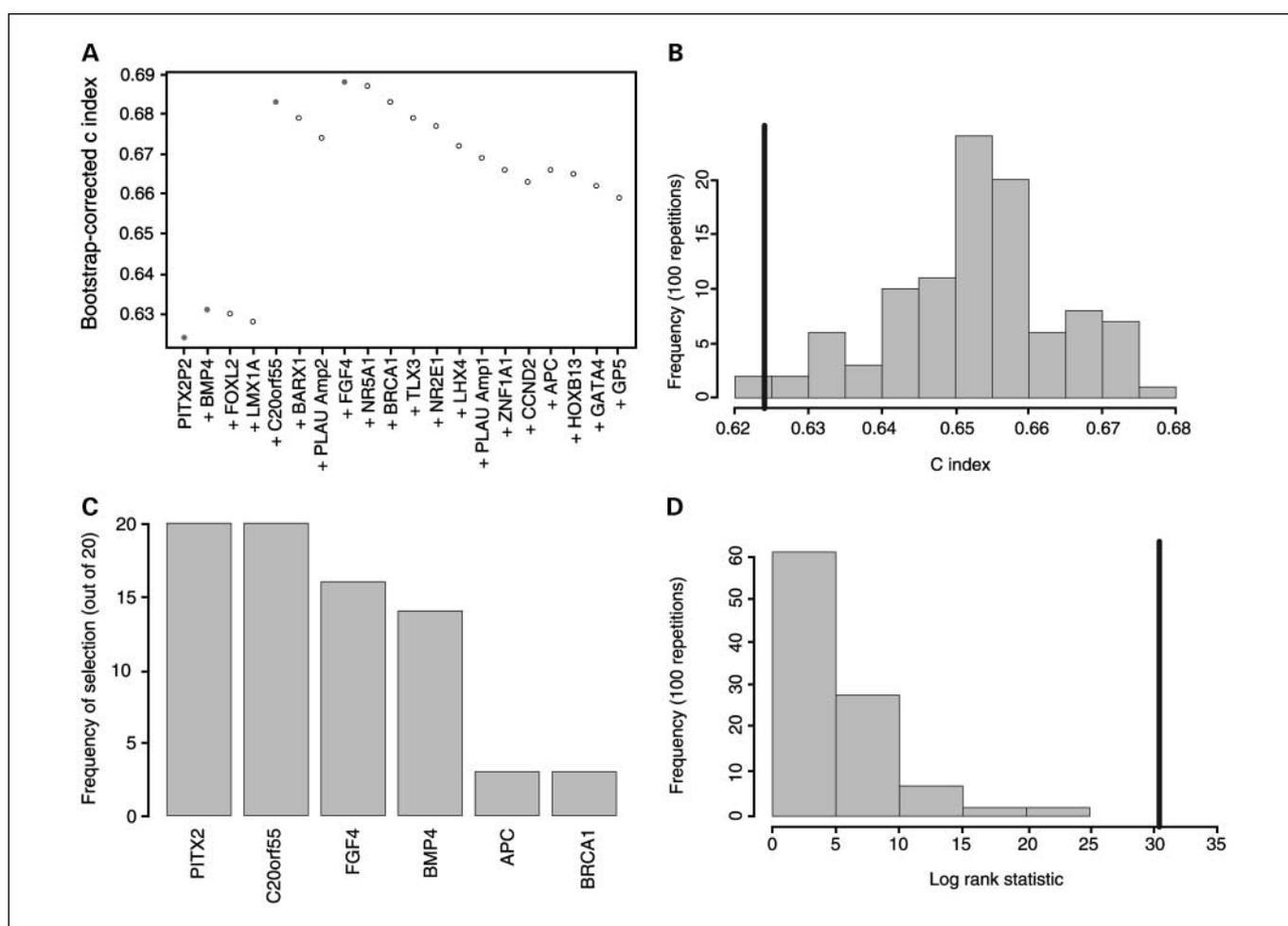
Features in Cox model (TDM)	C index	P (LRT)
Age at surgery, T stage, PgR, endocrine treatment, grade	0.61	0.01
PITX2P2	0.624	0.0019
Age at surgery, T stage, PgR, endocrine treatment, grade, PITX2P2	0.647	0.0029
Four-marker panel	0.668	$<0.00001$
Age at surgery, T stage, PgR, endocrine treatment, grade, four-marker panel	0.69	$<0.00001$

NOTE: T stage, endocrine treatment, PgR status, and grade were analyzed as binary variables. PITX2P2 methylation score and age at surgery were continuous variables. For the four-marker panel, the cross-validated prediction score was used as a continuous variable. LRT  $P$  values as well as the C index (bootstrap corrected) are calculated for each Cox model.

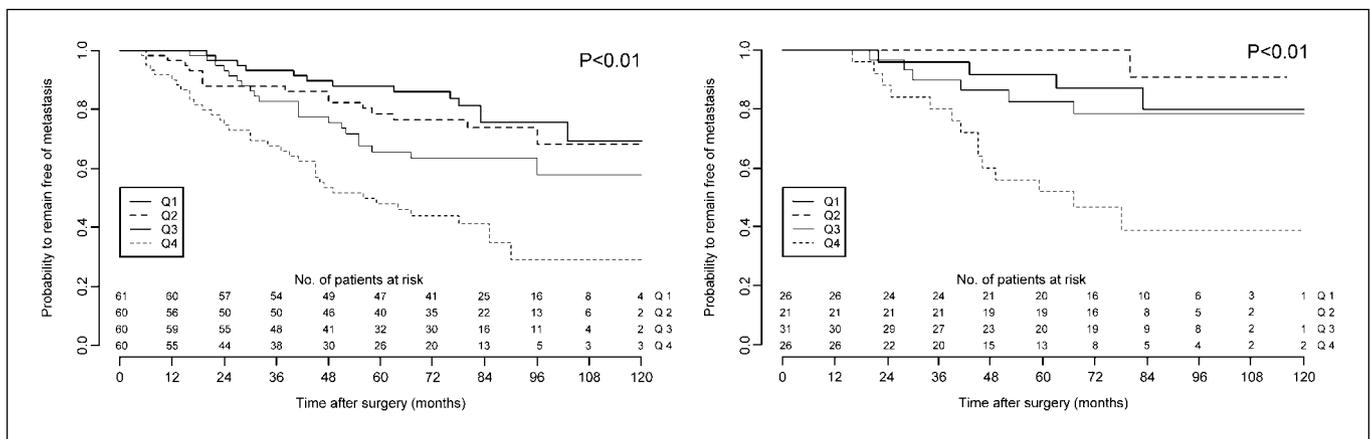
In previous studies, we found that DNA methylation of PITX2 reliably predicts the risk of distant recurrence in node-negative breast cancer (13, 15, 17). These results prompted us to analyze PITX2 methylation in node-positive breast cancer and to measure other DNA methylation markers, with the goal to develop a marker panel for improved outcome prediction. For this purpose, we have analyzed a cohort of 241 patients with lymph node-positive, ER-positive, and HER-2-negative breast cancer, who had all received adjuvant anthracycline-based chemotherapy. We focused on patients with these tumor characteristics, because they constitute a clinically relevant subgroup for which standardized treatment guidelines are available (1, 26). Among the node-positive breast cancer patients, our subpopulation is considered to have a relatively favorable outcome because of their positive steroid hormone receptor status and the absence of HER-2 amplification. Therefore, we hypothesized that it might be possible to further

stratify this group of patients so that a good prognosis group could be defined that would derive sufficient benefit from conventional anthracycline-based therapy and would not require more aggressive therapy such as, for example, dose-dense regimens or addition of taxanes.

In the current study, we were able to show that PITX2 DNA methylation also predicts outcome in node-positive breast cancer. Because clinical factors such as grade, tumor stage, or age have been described previously as outcome predictors in primary breast cancer, we first calculated a base model. The predictive accuracy of the multivariate Cox model with established clinical variables, quantified using the bootstrap-corrected C index as an unbiased measure of the predictive strength of a model, was 0.604. Adding PITX2 to the base model resulted in a significantly improved outcome prediction (C index = 0.651). This confirms the utility of PITX2 as a marker for outcome prediction in primary breast cancer and



**Fig. 2.** Marker panel feature selection and marker panel validation. **A**, marker panel selection. Result of the feature selection step using bootstrap-corrected C index to select markers for the panel to predict TDM. Marker models built using PITX2P2 and the first  $n$  amplificates ( $X$  axis, markers ranked according to univariate performance) are evaluated using the bootstrap-corrected C index ( $Y$  axis). Closed circles, selected markers. These are, from left to right, amplificates for PITX2P2, BMP4, C20orf55, and FGF4. **B**, histogram of cross-validated C index from 100 repetitions. C index for model with PITX2P2 alone was 0.624 (thick vertical black line); 98 of 100 repetitions for the four-marker panel are larger. **C**, stability of marker selection. Frequency of the indicated markers in cross-validation runs. In each of the 20 cross-validation runs, feature selection is repeated on 95% of the samples. C20orf55 was selected in all cross-validation runs, FGF4 in 16 of 20 runs, and BMP4 in 14 of 20 runs. Only two other markers were selected by this procedure: APC and BRCA1, which were both selected in only 3 of 20 runs. **D**, histogram of the null distribution of the log-rank test statistic of our data set. Histogram shows the distribution of log-rank test statistic of 100 data sets, of which survival time and censoring indicator were randomly permuted. This permuted data set was used to derive  $P$  values of the LRT (Cox model) for the cross-validated prediction score. Thick vertical black line, log-rank statistic for the cross-validated prediction score of the Kaplan-Meier TDM curves shown in Fig. 3 (left panel).



**Fig. 3.** Prediction of TDM survival probabilities based on the four-marker panel. Kaplan-Meier TDM curves of patients grouped into quartiles based on the cross-validated prediction score of the four-marker panel. *Left*, results for full data set (C index = 0.668;  $P < 0.01$ ), *right*, subgroup of patients who received adjuvant endocrine therapy.  $P$  value based on log-rank test null distribution as determined in Fig. 2D.

consolidates its role in breast cancer molecular classification. Whether and how PITX2 gene products are involved in breast carcinogenesis and progression is, however, still unclear.

In addition to PITX2, we generated DNA methylation profiles of regulatory regions of 18 candidate genes, which have been earlier linked to the PITX2 signal transduction pathway. Remarkably, eight of these candidate genes (BMP4, LMX1A, BARX1, FGF4, NR5A1, LHX4, ZNF1A1, and CCND2) were, like PITX2, significantly associated with patient outcome in the present study. This observation is in line with our hypothesis that signal transduction pathways related to PITX2 may play a key role in breast cancer progression.

In total, we have identified 15 novel DNA methylation markers, linked to 14 genes, which were significantly associated with TDM in our cohort (Table 2). Furthermore, our study represents the first evidence for DNA methylation of BMP4, FGF4, FOXL2, C20orf55, BARX1, NR5A1, NR2E1, and ZNF1A1 in cancer, whereas DNA methylation for LMX1 was described in the context of colorectal cancer (31) and for TLX3 and LHX4 in prostate cancer cell lines (32) as well as in primary lung cancer and melanoma (33), respectively. DNA methylation of the regulatory region CCND2 has been reported in breast cancer (34) and has been associated with worse outcome before in ovarian and prostate cancers (35, 36). Our finding that increased BRCA1 DNA methylation was associated with worse outcome is in line with the report of Egawa et al. (37) and might hint to a prognostic component of BRCA1 in our population of patients who had all received adjuvant anthracyclines. However, this conclusion remains speculative because our study did not contain an anthracycline-free control arm. For PLAU, for which hypomethylation was observed in more aggressive forms of breast cancer (38–41), we analyzed two fragments within the promoter region and found for both fragments evidence for increased DNA methylation and higher risk of recurrence (data not shown). The differences with previous findings may be explained by the fact that our patients all had received chemotherapy. It was reported previously by us that patients with higher PLAU protein levels benefited more from adjuvant chemotherapy (42). The general observation that patients with higher protein expression are expected to show low promoter DNA methylation might then contribute to our observation.

In conclusion, our data provide further evidence for the relevance of DNA methylation analysis for breast cancer molecular classification and that higher DNA methylation scores are mostly associated with more aggressive disease and that the genes identified may be relevant for breast cancer progression.

Given the strong evidence for PITX2 and several novel DNA methylation markers as outcome predictors, we analyzed whether combining them into a marker panel might improve their predictive performance. To prove that a panel outperforms an individual marker, overfitting has to be accounted for to derive an unbiased estimate of prediction accuracy. We have applied a nested cross-validation approach that uses the available data more efficiently than the usual division into training and test set: both feature selection and performance estimation can be done on the full data set while, at the same time, the performance estimates (C index) are unbiased (24, 25). As PITX2P2 methylation was already proven to be clinically relevant in several studies, we focused our search on models that included this marker. We identified a four-marker panel with improved predictive ability compared with PITX2P2 alone consisting of PITX2P2, BMP4, FGF4, and C20orf55 (Fig. 2A; Table 3). For the four-marker panel, the 5-year estimate for TDM for the quartile with highest scores is <50%, whereas that of the lowest scores is well above 85% (Fig. 3). Like PITX2, the four-marker panel provides information in addition to established clinical variables (Table 3). As mentioned above, BMP4 and FGF4 can be affiliated with developmental pathways shared with PITX2. C20orf55, which we identified in a genome-wide profiling experiment for aberrant methylation in breast cancer (data not shown), was recently characterized as a member of the FAM110 protein family. Members of this family localize to centrosomes and spindle poles, which might hint to a role in tumor pathogenesis (43).

Due to the retrospective nature of our study, the cohort also included patients who have not been treated according to current standards. For example, not all patients with steroid receptor-positive tumors received additional adjuvant endocrine therapy, because they were diagnosed before adjuvant endocrine therapy was introduced as the standard

of care in steroid hormone receptor-positive breast cancer independent of menopausal status in all European countries. Based on this consideration, survival probabilities for patients treated according to current standards can best be estimated using the subgroup of our patients that received adjuvant endocrine therapy. For this subgroup of 104 patients, performance estimates suggest that the four-marker panel might be used to delineate a good prognosis subgroup of patients with a 10-year survival of >90%. For this subgroup of patients, more aggressive combination therapy or dose-dense therapy may not be required; instead, standard anthracycline-based chemotherapy followed by adjuvant endocrine treatment might be the preferable treatment option. In contrast, for those patients with poor outcome according to our marker panel, different adjuvant chemotherapy approaches (e.g., addition of taxanes) or completely anthracycline-free regimens may be warranted because they do not seem to derive substantial benefit from a standard anthracycline-containing regimen. Thus far, a validated marker for anthracycline benefit (topoisomerase IIa) is only available for HER-2-positive disease.<sup>8</sup> Therefore, for the first time, we provide evidence for a clinically useful marker panel

<sup>8</sup> Slamon et al., Role of anthracycline-based therapy in the adjuvant treatment of breast cancer: efficacy analyses determined by molecular subtypes of the disease. SABCS 2007.

that may aid decision-making regarding adjuvant anthracycline therapy, an issue with has recently become increasingly important (3).

We note that ongoing clinical research tries to further optimize adjuvant endocrine therapies and to effectively combine them with other chemotherapy or novel targeted agents. Several clinical trials established the enhanced benefit of aromatase inhibitors regarding DFS compared with tamoxifen for adjuvant treatment of postmenopausal breast cancer (44, 45). These studies indicate that individualization of endocrine therapy may result in further improvement of clinical outcome. With refinement of endocrine therapy, it is conceivable that our four-marker panel may be used to delineate subgroups with a 5- to 10-year survival exceeding 90% as in the cohort described in this study. Therefore, a diagnostic test based on the suggested marker panel could have the potential to reduce overtreatment and avoid unnecessary side effects even in high-risk primary breast cancer.

### Disclosure of Potential Conflicts of Interest

O. Hartmann, D. Dietrich, A. Fassbender, K. Welzel, S. Maier, A. Plum, S. Niemann, and R. Lesche: employees of Epigenomics, a company in the business of commercializing diagnostic tests and which owns the patent to the described work. N. Harbeck, M. Schmitt, S. Eppenberger-Castori, F. Spyrtatos, and J.A. Foekens: research support from Epigenomics.

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## DNA Methylation Markers Predict Outcome in Node-Positive, Estrogen Receptor-Positive Breast Cancer with Adjuvant Anthracycline-Based Chemotherapy

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