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DNA-methylation of the homeodomain transcription factor PITX2 reliably predicts risk of distant disease recurrence in tamoxifen-treated, node-negative breast cancer patients – Technical and clinical validation in a multi-centre setting in collaboration with the European Organisation for Research and Treatment of Cancer (EORTC) PathoBiology group

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ABSTRACT

Our aim was to identify and validate DNA-methylation markers associated with very good outcome in node negative, hormone receptor positive breast cancer patients after adjuvant endocrine therapy which might allow identifying patients who could be spared the burden of adjuvant chemotherapy. Using a methylation microarray, we analysed 117 candidate genes in hormone receptor-positive tumours from 109 breast cancer patients treated by adjuvant tamoxifen. Results were validated in an independent cohort ($n = 236$, 5 centres). Independent methodological validation was achieved by a real-time polymerase chain reaction (PCR)-based technique. DNA methylation of PITX2 showed the strongest correlation with distant recurrence. Its impact on patient outcome was validated in the independent cohort: 86% of patients with low PITX2 methylation were metastasis-free after 10 years, compared to 69% with elevated PITX2 methylation. Moreover, PITX2 methylation added significant independent information to established clinical factors. All clinical and

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technical findings were confirmed by quantitative DNA-methylation PCR. These results provide strong evidence that DNA-methylation analysis allows clinically relevant risk assessment in tamoxifen-treated primary breast cancer. Based on PITX2 methylation, about half of hormone receptor-positive, node-negative breast cancer patients receiving adjuvant tamoxifen monotherapy can be considered low-risk regarding development of distant recurrences and may thus be spared adjuvant chemotherapy. In addition, these low-risk postmenopausal patients seem to respond sufficiently well to tamoxifen so that they may not require up-front aromatase inhibitor therapy.

1. Introduction

Current guidelines recommend adjuvant chemotherapy followed by endocrine therapy for most women with node-negative, steroid hormone receptor-positive breast cancer.[1] This recommendation is based on a significant reduction of the risk of disease recurrence by chemotherapy in this population, independent of the risk reduction by endocrine therapy.[2] However, these patients have a rather good prognosis and generally derive significant benefit from endocrine treatment alone.[2] Hence, after endocrine treatment, the majority will never suffer recurrence and thus would have been adequately treated by tamoxifen alone. Unfortunately, traditional prognostic factors are not adequate to identify those patients at low risk who can be spared over-treatment by chemotherapy, as is true in the majority of hormone receptor-positive, node-negative patients. In postmenopausal hormone receptor-positive patients, aromatase inhibitors have become an important treatment option.[3] Yet, it is still unclear which patients will be sufficiently treated by adjuvant tamoxifen and which will benefit more from aromatase inhibitors – a rather important question given the lack of information on long-term side effects and the increased costs for aromatase inhibitors.

A common and early event in cancer is aberrant DNA methylation of cytosine phosphoguanine dinucleotides (CpG) within gene regulatory regions.[4,5] Frequently, hypermethylation of promoters is associated with suppression of gene expression.[5,6] DNA-methylation patterns are tumour-specific and can be used for molecular subclassification of tumours.[7–9] Additionally, several studies have demonstrated the potential of DNA-methylation as prognostic or predictive markers in a variety of cancers.[10–12] Recently, some studies have suggested that methylation of certain genes may correlate with tamoxifen response and survival in breast cancer.[13,14]

Our aim was to identify and validate, for the first time, DNA-methylation markers associated with a low risk of distant recurrence in breast cancer patients receiving adjuvant tamoxifen monotherapy. Using a previously described microarray approach[7,14], we analysed DNA methylation of 117 candidate genes in primary tumours of 109 hormone receptor-positive breast cancer patients, all of whom had received tamoxifen as their sole adjuvant systemic treatment. The candidate genes were selected because of their potential function in breast tumourigenesis or metastasis, because of their presumed role in resistance to endocrine therapy or steroid hormone regulation, or because they had been described as being methylated in cancer. In the subsequent validation study, 33 candidates were selected and analysed in an independent cohort encompassing 236 node-negative, hormone receptor-positive patients from five clinical centres.

2. Materials and methods

2.1. Patient and tumour characteristics

DNA-methylation measurements were performed on DNA isolated from snap-frozen primary breast cancers. The unicentre marker discovery study included 39 node-negative and 70 node-positive breast cancer patients (Department of Obstetrics and Gynecology, Technical University of Munich, Germany) who underwent surgery in 1998 or earlier. In the multi-centre validation study, 236 independent node-negative breast cancer patients were analysed (Departments of Obstetrics and Gynecology, Technical University of Munich and University Hospital Eppendorf, Hamburg, Germany; Stiftung Tumorbank Basel, Switzerland; Clinical Experimental Oncology Laboratory, National Cancer Institute, Bari, Italy; and Laboratoire d'Oncogénétique, Centre René Huguenin, St. Cloud, France). Inclusion criteria were availability of frozen tumour specimens, T1–3, oestrogen receptor (ER) and/or progesterone receptor (PR) expression, no lymph node involvement (validation phase only), age > 35 years at diagnosis, surgery by 1998, adjuvant tamoxifen monotherapy (indicated duration 5 years), availability of follow-up data and written informed patient consent. Clinical patient characteristics are summarised in Table 1. Note that due to the lack of material (in most cases, only cellular nuclei available), tumour grade and oestrogen receptor protein levels could not be confirmed centrally, but data from medical records of each individual centre were used. Furthermore, tumour cell content could not be determined. However, since the cellular nuclei originated from specimens used for clinical determination of oestrogen receptor protein levels, the specimens were considered appropriate for the reported studies. Median follow-up in patients still alive at the time of analysis was 5.5 years for both studies. Follow-up data were obtained regularly according to local guidelines. Ethical approval for the study was obtained from local ethics committees of each participating centre.

2.2. Determination of ER and PR expression

All tumours were ER and/or PgR positive (either $\geqslant$10 fmol/mg of cytosolic protein by enzyme immunoassay (EIA) or dextrane charcoal assay (DCC), or immunohistochemical Remmele Score > 0 on a scale ranging from 0 to 12).

Table 1 – Patient characteristics of marker discovery and validation study

	Marker discovery study	Validation study
Number	109	236
Grade		
G1	5	38
G2	60	109
G3	42	82
G4	0	1
Unknown	2	6
Tumour stage		
T1	28	83
T2	54	139
T3	10	6
T4	17	5
Unknown	0	3
Nodal status		
N0	39	236
N+	70	0
Age at diagnosis		
40 and younger	0	3
41–50	4	15
51–60	29	75
61–70	39	79
71–80	34	56
81 and older	3	8
Menopausal status		
Post	105	200
Pre	4	26
Unknown	0	10

2.3. DNA extraction for methylation profiling

Genomic DNA was extracted (QIAamp Blood Kit, QIAGEN, Hilden, Germany) either from deep-frozen 100,000*g* pellets containing cellular nuclei obtained after tumour tissue cytosol preparation, or from snap-frozen tumour tissue.

2.4. Bisulphite treatment, polymerase chain reaction (PCR) amplification, and microarray hybridisation

Bisulphite treatment, PCR amplification and microarray hybridisation were performed as previously described.[7,14] Briefly, regulatory regions of candidate genes were amplified from bisulfite-treated genomic DNA in multiplex PCR reactions labeled with fluorochrome Cy5. Primers were designed ensuring unbiased amplification of methylated and unmethylated alleles. PCR products were hybridezed to microarrays, carrying pairs of immobilized oligonucleotides reflecting the methylated (CG) and non-methylated (TG) status for each position of 1–3 CpG dinucleotides. Microarrays included 499 probe pairs to 117 candidate genes in the marker discovery study and 151 probe pairs representing 33 genes in the validation study. Fluorescent images of the hybridized microarrays were obtained using a GenePix 4000 microarrys scanner (Axon Instruments, Union. City, CA). Median spot intensities of detection oligonucleotides were taken to calculate the methylation score (log [CG/TG]). The methylation score for further analyses was calculated as median over four spot repetitions per chip and over on average four hybridization repetitions (for the gene list, see Supplementary material).

2.5. Quantitative DNA-methylation analysis by real-time PCR (QM-PCR)

Bisulphite-converted DNA from all node-negative patients (of both studies) with sufficient remaining DNA ($n = 245$) was analysed for PITX2 methylation by real-time PCR. The assay format has been described previously.[14] Briefly, in order to ensure unbiased amplification of both methylated and unmethylated states, primers were designed to exclude any CpG dinucleotides. TaqMan probes, specific for methylated and unmethylated states, respectively, were designed to probe the same sequence as covered by the DNA microarray in a duplex probe assay. Threshold cycles obtained on 7900 real-time PCR System (Applied Biosystems) from both probes were used to calculate the methylation score as follows:

$$\text{methylation score} = 100/[1 + 2\{\text{Ctm} - \text{Ctu}\}],$$

where Ctm and Ctu denote threshold cycles of the probe representing methylated and unmethylated state, respectively.

2.6. Statistical analyses

DNA-methylation scores were measured for each probe pair – either from microarray or real-time PCR. The relation between metastasis-free survival times (MFS) and the standardised methylation score of the CpG sites from the microarrays was analysed by linear univariate Cox Proportional Hazard Models.[15,16] Likelihood ratio (LR) tests were performed to test for a significant impact of methylation score at a CpG site on metastasis-free survival. Survival curves were depicted according to the Kaplan–Meier method.[17] Log-rank tests were performed to test for differences between dichotomised survival curves. Multivariable regression analysis was performed by linear Cox Proportional Hazard Models. The models included one of the clinical factors (nodal status (discovery study only), tumour stage, nuclear grade and standardised age at time of diagnosis) and the methylation score of a particular CpG site. Wald tests were performed to test the hypothesis that the CpG site in question provides significant information to the model.

For the technical validation study, the Pearson correlation coefficient between transformed microarray (methylation score: (100*CG/(CG+TG)) and QM-PCR assay data was calculated.

3. Results

3.1. Marker discovery study

Using a methylation microarray, we assessed DNA-methylation scores of 117 different candidate genes for association with metastasis-free survival. The marker discovery study set consisted of 109 primary tumour tissues from consecutive cases of breast cancer patients treated at one clinical centre (Technical University of Munich, Germany). All patients had hormone receptor-positive tumours and received tamoxifen as the only adjuvant therapy. Postmenopausal patients were

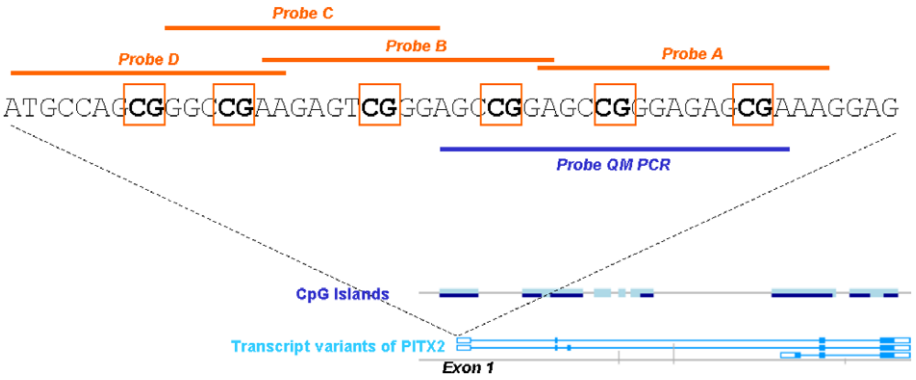

Fig. 1 – Analysed region of the PITX2 gene with details of sequence and detection probes for microarray (A–D, orange) and Quantitative DNA-methylation analysis by real-time PCR (QM-PCR) (blue), respectively. Organisation of PITX2 gene and cytosine phosphoguanine dinucleotides (CpG) Islands (according to NCBI, http://www.ncbi.nlm.nih.gov) is shown at the bottom of the figure.

somewhat over-represented in our data set, most likely due to the fact that premenopausal patients were preferentially treated by chemotherapy at the time our patients had been diagnosed. For this exploratory first step, we included breast cancer patients irrespective of their nodal status (N0: 39; N+: 70). Since nodal status is a strong prognostic factor in breast cancer, lymph node involvement was included as multivariate factor when associating the single CpG sites with survival. Among all candidate genes, DNA methylation of the PITX2 gene showed the strongest correlation to metastasis-free survival. For probe A (covering 2 CpG dinucleotides in the regulatory region of PITX2, Fig. 1), hypermethylation was significantly associated with poor outcome, with a hazard ratio of 2.1 ($p = 0.0014$, Table 2A). For probes B and C, covering CpG dinucleotides in the vicinity, similar results were obtained (Table 2A).

3.2. *Validation study*

To validate our findings, we determined methylation scores of PITX2 in an independent cohort of 236 patients from five clinical centres, using the same microarray technology. Only PITX2 DNA methylation was a significant predictor of outcome in the marker discovery study. However, since potential markers might have been missed in the discovery study due to the low power, we included 32 additional candidates from the discovery step, who had shown a trend for correlation, in the validation study. These additional genes were selected based on the following criteria: first, the ranking of the single CpG sites in the discovery step, second, the degree of co-methylation of several CpG sites within the gene or promoter. Furthermore, we included those genes which had previously been identified as best outcome predictors in advanced breast cancer.[14] Again, all patients had hormone receptor-positive breast cancer and had received tamoxifen as the only systemic adjuvant therapy. Since over-treatment is clinically most relevant in node-negative breast cancer patients, we only included breast cancer patients without nodal involvement in the validation step.

The validation study confirms the significant impact of PITX2 methylation on metastasis-free survival. The CpG site of PITX2 (covered by probe A), which had been identified during the marker discovery step as the best marker for metastasis-free survival, again showed the strongest impact on outcome (univariate Cox regression analysis: hazard ratio = 2.5, $p = 0.000033$, Table 2B). Confirming our previous results, hypermethylation of this site was associated with

Table 2B – Metastasis-free survival: validation study, univariate analysis

	p-Value	Hazard ratio (95% confidence interval (CI))
CpG site A of PITX2	0.000033	2.5 (1.5, 4.1)
CpG site B of PITX2	0.00045	2.1 (1.3, 3.3)
CpG site C of PITX2	0.000021	2.3 (1.4, 3.8)
CpG site D of PITX2	0.11	1.3 (0.9, 1.9)
Tumour stage	0.72	1.1 (0.6, 2.3)
Tumour grade	0.83	0.9 (0.5, 1.9)
Age at diagnosis	0.27	1.2 (0.9, 1.8)

Table 2A – Metastasis-free survival: marker discovery study

CpG sites of PITX2	p-Values of LR-test for complete model	*p*-Values for CpG sites of PITX2	Hazard ratios for CpG sites of PITX2	*p*-Values for N stage	Hazard ratios for N stage
A	0.000036	0.0014	2.1 (1.3, 3.4)	0.0044	5.8 (1.7, 20.0)
B	0.00013	0.0069	2.0 (1.2, 3.3)	0.0057	5.5 (1.6, 18.0)
C	0.00039	0.016	1.8 (1.1, 3.0)	0.0059	5.5 (1.6, 19.0)
D	0.011	1	1.0 (0.6, 1.6)	0.013	4.6 (1.4, 15.0)

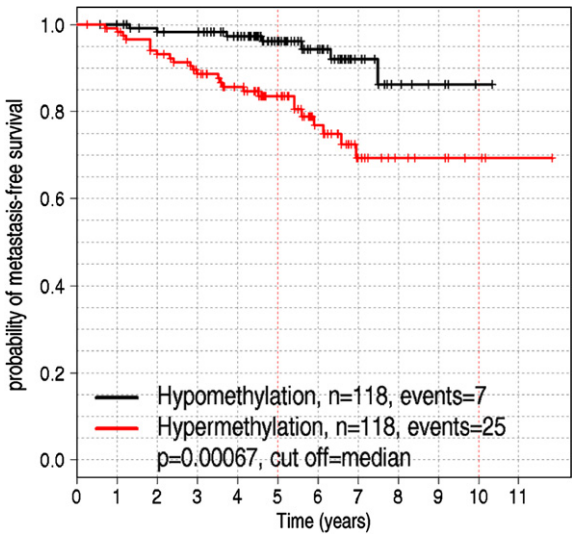

Fig. 2 – Kaplan–Meier curves for metastasis-free survival stratified by DNA-methylation scores of the PITX2 gene (Probe A) in validation study (n = 236; N0 patients only). Patients with PITX2 methylation score above median (*hyper*methylation) are depicted in the red (lower) curve; patients with methylation score below median (*hypo*methylation) are indicated in the black (upper) curve. *p*-Value according to Logrank-test.

Table 2C – Metastasis-free survival: validation study, multivariate analysis

	p-Value CpG site	Hazard ratio CpG site (95% CI)	*p*-Value clinical factor	Hazard ratio clinical factor (95% CI)
Tumour stage				
A	0.00024	2.5 (1.5, 4.2)	0.71	1.1 (0.6, 2.4)
B	0.0017	2.1 (1.3, 3.3)	0.72	1.1 (0.6, 2.4)
C	0.0012	2.3 (1.4, 3.8)	0.69	1.2 (0.6, 2.4)
D	0.1	1.3 (0.9, 1.9)	0.65	1.2 (0.6, 2.4)
Tumour grade				
A	0.00024	2.6 (1.5, 4.2)	0.76	1.1 (0.5, 2.3)
B	0.0017	2.1 (1.3, 3.3)	0.86	1.1 (0.5, 2.2)
C	0.0012	2.3 (1.4, 3.8)	0.87	1.1 (0.5, 2.2)
D	0.11	1.3 (0.9, 1.9)	0.98	1.0 (0.5, 2.0)
Age at diagnosis				
A	0.00029	2.5 (1.5, 4.1)	0.41	1.2 (0.8, 1.7)
B	0.0021	2.0 (1.2, 3.2)	0.45	1.1 (0.8, 1.6)
C	0.0015	2.2 (1.4, 3.7)	0.46	1.1 (1.0, 1.6)
D	0.12	1.3 (0.9, 1.9)	0.31	1.2 (0.8, 1.7)

poor metastasis-free survival. Using the median methylation ratio at this site as a cut-off, we defined a low-risk group in which 86% of the patients were metastasis-free at 10 years after surgery compared to only 69% in the high-risk group (Fig. 2). In each of several multivariate models of MFS, including tumour size, grade, or patient age, PITX2 methylation contributed significantly to risk prediction (Table 2C). We also observed a trend towards association of higher PITX2 methylation scores with poor overall survival, although the finding was not statistically significant. In the group with low PITX2 methylation (probe A), 81% of patients were alive at 10 years, compared to 68% in the group with high PITX2 methylation (data not shown). Similar results were obtained for probes B and C.

3.3. *Technical validation of PITX2 by independent DNA-methylation detection technology*

To confirm the PITX2 DNA-methylation scores obtained by microarray, we re-analysed all node-negative tumour specimens from both studies with sufficient remaining DNA (n = 245), using a newly developed real-time PCR-based, quantitative DNA-methylation assay (QM assay), covering the same region of PITX2 as the microarray (Fig. 1). DNA-methylation scores calculated from the QM assay were compared with those obtained from the microarray analyses. These PITX2 methylation scores correlated well with those obtained by the QM assay (Pearson correlation coefficient = 0.87, Fig. 3a). Kaplan–Meier curves calculated from the QM assay data provided clinical information comparable to the curves calculated based on the microarray data (Fig. 3b). Thus, the significant correlation of PITX2 DNA-methylation score with outcome is confirmed by an independent method.

4. Discussion

This paper for the first time gives strong evidence in larger patient cohorts that DNA methylation provides clinically relevant information with regard to patient outcome in primary breast cancer. PITX2 methylation was identified as a strong marker for assessment of distant recurrence risk in a cohort of tamoxifen-treated patients, and then successfully clinically validated in a larger, independent cohort. In addition, technical validation was obtained using an independent methodology; moreover, this independent methodology produces entirely consistent clinical implications. Thus, PITX2 constitutes the first independently validated DNA-methylation marker for outcome prediction in primary breast cancer. The results suggest that PITX2 tumour methylation is a clinically useful marker to identify node-negative, hormone receptor-positive patients who are sufficiently treated by adjuvant tamoxifen alone and thus suited to guide physicians and patients in their decision for or against adjuvant chemotherapy in addition to adjuvant tamoxifen. Moreover, these low-risk patients may derive sufficient benefit from adjuvant tamoxifen and – if postmenopausal – may not require *up-front* aromatase inhibitor therapy.

The role of the PITX2 protein in breast carcinogenesis and progression is unclear. We included PITX2 in our list of candidate genes based on a report that described PITX2 methylation in acute myeloid leukaemia.[18] PITX2 encodes a homeodomain transcription factor and is regulated by the WNT/DVL/beta-catenin and hedgehog/TGFβ pathways.[19] The protein is required for morphogenesis of anterior structures such as eyes, teeth and anterior pituitary; it plays a role in left/right patterning[20,21] and is differentially expressed in pituitary adenomas .[20,22,23] Germ-line mutations cause Rieger's syndrome, which is characterised by eye and teeth

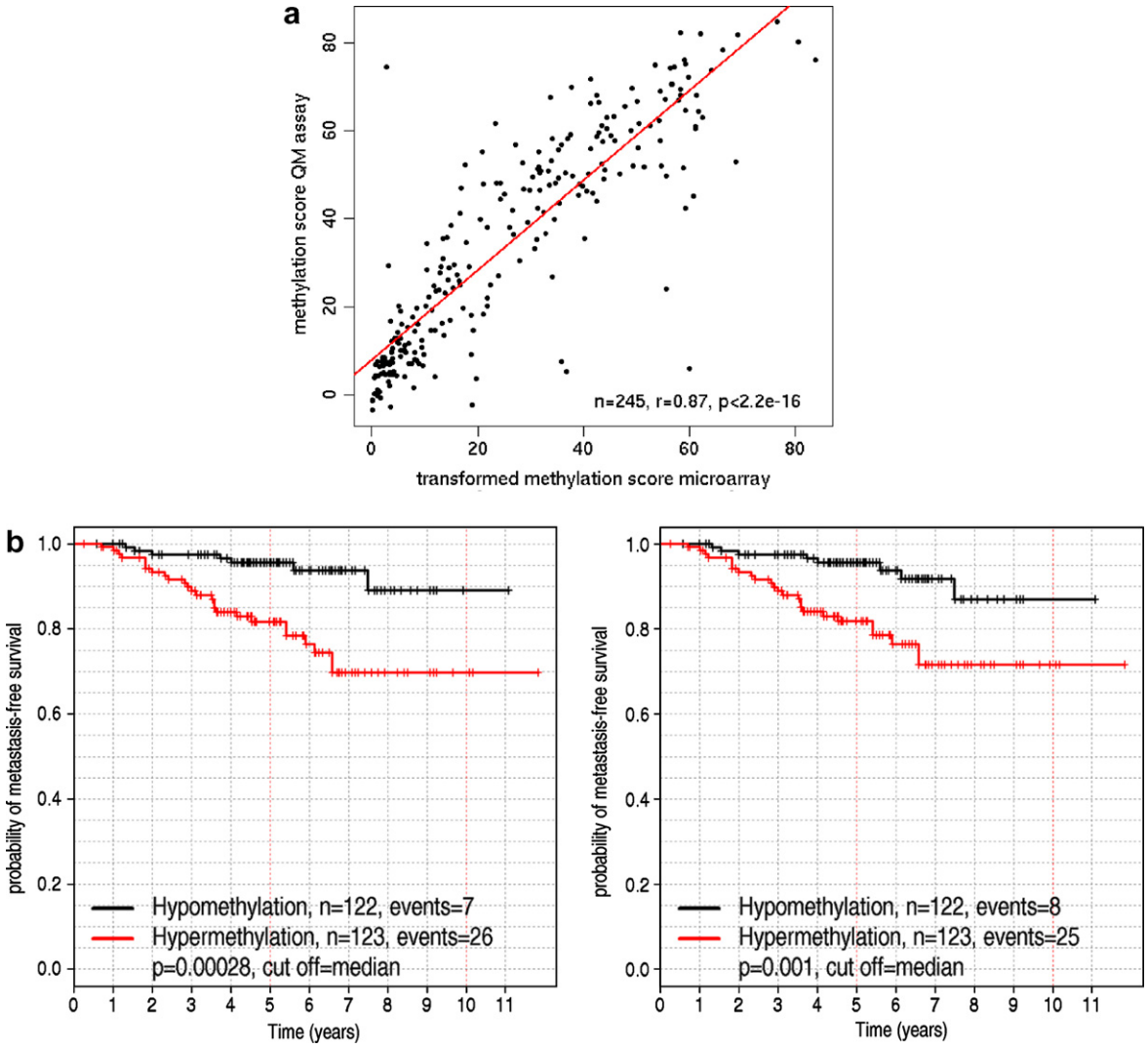

Fig. 3 – Technical (a) and clinical (b) consistency of PITX2 methylation determination by microarray and quantitative methylation analysis by real-time PCR (QM assay). (a) Correlation of DNA methylation scores of the most significant capture probe A of microarray analysis (*x*-axis) versus QM-assay (*y*-axis) in 245 node-negative breast cancer patients. (b) Kaplan–Meier curves for metastasis-free survival stratified by DNA-methylation scores of the PITX2 gene, as determined by two different technologies (245 node-negative breast cancer patients from marker discovery and validation study). (Left) Data derived from microarray analysis. (Right) Data derived QM-analysis. Patients with PITX2 methylation scores above median (*hyper*methylation) are depicted in the red (lower) curve; patients with methylation scores below median (*hypo*methylation) are indicated in the black (upper) curve. *p*-Values are according to the Logrank-test.

malformations.[24,25] Interestingly, no association with neoplastic disorders has been reported for Rieger's syndrome. Collectively, these data imply a growth or differentiation control function for PITX2, which could contribute to malignancy when out of equilibrium.

Our results are consistent with an association of PITX2 methylation either with tumour aggressiveness or with tamoxifen response, both of which could contribute to clinical outcome in breast cancer patients treated with adjuvant tamoxifen. In rats, Schausi et al. demonstrated that PITX2 activates the promoter of the truncated ER product-1 (TERP-1),[26] an ER variant which has been shown to be specifically expressed in lactotropic cells of the pituitary gland, thus suggesting that a possible link between PITX2 methylation and endocrine responsiveness. On the other hand, we found that PITX2 methylation was not associated with clinical benefit from first-line tamoxifen monotherapy in endocrine-naïve, metastatic breast cancer,[14] suggesting it may primarily be a marker of tumour aggressiveness and treatment in the adjuvant setting is still early enough to counteract this particular tumour biology whereas treatment in the metastatic setting may be too late. Preliminary results from tumours of untreated patients do suggest that the PITX2 methylation marker also has a prognostic component.[35] These studies will also further explore the relation of PITX2 with other established clinical factors; in the present study, we found that

PITX2 predicts outcome independent of tumour stage, grade and patient age.

The relationship between PITX2 methylation and mRNA expression is unknown. The PITX2 gene generates three major transcripts. Transcripts A and B use one common promoter, whereas transcript C is regulated via an alternative downstream promoter. In our study, we investigated a CpG Island in exon 1, i.e. in the regulatory region of transcripts A and B. Following current assumptions[27] DNA methylation in this area is expected to lead to loss of expression of PITX2 isoforms A and B. Studies testing this hypothesis are currently ongoing and will help to clarify the biological role of PITX2 methylation in breast cancer. Yet, such a deeper understanding is helpful but not a prerequisite for the clinical use of PITX2 as a marker for outcome prediction.

Recently, in endocrine-naïve, metastatic breast cancer, we identified several genes whose methylation score was associated with clinical benefit from first-line tamoxifen monotherapy.[14] Interestingly, none of the genes associated with response in *advanced* breast cancer were a good outcome predictor in the *adjuvant* setting, possibly due to differences in the underlying tumour biology of early and advanced breast cancer, or because markers with a (additional) prognostic component may prevail in the current (adjuvant) study.

Widschwendter et al.[13] described an association of ESR1 hypermethylation with favourable outcome in patients receiving adjuvant tamoxifen. Since high ER levels predict response to tamoxifen,[28] and since ESR1 gene expression is negatively regulated by DNA methylation, [29–31] this finding is quite unexpected. We analysed ESR1 methylation in the same genomic region as Widschwendter et al. in both our discovery and validation study, but did not find a correlation to patient outcome (data not shown). However, in contrast to our study, some of the patients analysed by Widschwendter et al. received additional therapy (i.e. chemotherapy) besides endocrine treatment, complicating the interpretation of their results. In our study, only ER- and/or PR-expressing tumours were included; thus ER expression and consequently DNA methylation of ESR1 is not expected to be a major outcome predictor.

Several groups have identified mRNA expression patterns associated with outcome in breast cancer.[32–34] Van't Veer et al.[32] and Wang et al.[33] identified prognostic signatures in patients without adjuvant systemic therapy. Paik et al.[34] developed a recurrence score for tamoxifen-treated patients based on 16 genes and 5 controls. The score assigns 51% of patients to a low-risk group (10-year-MFS 93.2%), 22% of patients to an intermediate-risk group (10-year-MFS 85.7%), and 27% to a high-risk group (10-year-MFS 69.5%). The performance of their recurrence score and our single methylation marker seems comparable, although caution has to be applied when comparing clinical performance of markers measured in cohorts that are not completely identical (e.g. the 10-year-MFS of our whole cohort was 77.5% versus 85.2% in the study of Paik et al.[34]). Furthermore, the recurrence score of Paik et al.[34] was optimised and grouped into three categories prior to validation, whereas we used a median cut-off to assign patients into two risk groups to avoid potential over-fitting at an early phase in marker development. For eventual routine clinical application, measurement of a single, DNA-based marker may be more practical than a larger, mRNA-based panel. Although the data indicate that mRNA quantification from paraffin-embedded tissue is feasible,[34] DNA-based markers are generally expected to be more robust in a clinical environment.

5. Conclusions

We have described the identification and independent clinical and technical validation of a DNA-methylation marker, PITX2, for outcome prediction in primary breast cancer. The results provide strong evidence that, based on PITX2 methylation alone, up to half of the patients with hormone receptor-positive, node-negative breast cancer can be considered low-risk for distant recurrence if treated by adjuvant tamoxifen and thus may be candidates for being spared adjuvant chemotherapy. Moreover, PITX2 methylation may help to determine a subgroup of postmenopausal patients who do sufficiently well on tamoxifen as their endocrine therapy and may not require up-front aromatase inhibitors. These promising results warrant further studies to determine a clinically optimal cut-off and to address the performance of the marker in larger cohorts in order to allow more detailed subgroup analyses, e.g. in premenopausal patients.

Conflict of interest statement

SM, IN, TK, AK, IS, AM, and RL were employed by Epigenomics AG, a company, which is in the business of commercialising diagnostic products. NH and MS received research support from Epigenomics AG. NH and CT received consulting fees and/or research support from Roche Diagnostics. The other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2007.04.025.

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