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PITX2 DNA methylation: a potential prognostic or predictive biomarker in early breast cancer

Evaluation of: Nimmrich I, Sieuwerts AM, Meijer-van Gelder ME *et al.*: DNA hypermethylation of *PITX2* is a marker of poor prognosis in untreated lymph node-negative hormone receptor-positive breast cancer patients. *Breast Cancer Res. Treat.* 111, 429–437 (2008).

The ability of established clinical and pathological markers to identify women at high risk of breast cancer recurrence and poor overall survival is limited. In recent years, gene-expression profiling has enabled more accurate identification of women at low-risk of disease recurrence, with the potential of sparing many women from the toxicity of systemic chemotherapy [1,2]. Detection of epigenetic alterations at the promoter regions of genes, such as DNA methylation, appears to be a common and early event in breast carcinogenesis that can lead to gene silencing [3]. Therefore, it is biologically plausible that increased DNA methylation of specific genes is associated with disease recurrence and/or response to therapy in patients with breast cancer.

In the first study, Nimmrich *et al.* evaluated whether paired-like homeodomain transcription factor 2 (*PITX2*) DNA methylation was an independent prognostic marker for disease recurrence and survival in patients with hormone-receptor positive lymph-node negative early breast cancer (T1–3) who received no systemic therapy. The association between *PITX2* methylation and *PITX2* gene expression was also evaluated [4].

PITX2 is a transcription factor involved with embryonal development of anterior structures such as eyes, teeth and the

anterior pituitary. It also plays a role in left–right patterning and is regulated by the WNT/DVL/ β -catenin and hedgehog/TGF- β pathways [5]. The precise role of the *PITX2* proteins in breast cancer initiation and progression is unclear.

To conduct this study, a retrospective cohort (n = 415) with follow up information for a median of 93 months was established from primary breast surgeries performed between 1982 and 1996. *PITX2* DNA methylation was quantitatively assessed using real-time PCR (RT-PCR) in 412 of the breast tumors. The median methylation level was 21.1 (0.006–90.7). Median levels were significantly higher in older, postmenopausal women with larger tumors and higher estrogen receptor (ER) levels. Grade was not associated with methylation levels. Multivariate analysis after adjustment for age, menopausal status, tumor size, tumor grade and ER and progesterone receptor (PR) levels revealed that *PITX2* methylation was independently associated with worse metastasis-free survival (hazard ratio [HR]: 1.74; 95% confidence interval [CI]: 1.26–2.4; $p < 0.01$) and overall survival (HR: 1.46; 95% CI: 1.05–2.01; $p = 0.02$).

In addition, *PITX2* DNA methylation was observed to be inversely associated with *PITX2A* ($p = 0.0004$) and *PITX2B* ($p = 0.0005$) mRNA expression in 32 breast cancer cell lines.

This study, therefore, supports the postulation that DNA methylation of *PITX2* is an independent prognostic biomarker for breast cancer outcome in hormone-receptor positive lymph-node negative early breast cancer (T1–3).

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PITX2 DNA methylation as a marker for outcome prediction in breast cancer patients receiving adjuvant tamoxifen

Evaluation of: Harbeck N, Nimmrich I, Hartmann A *et al.*: Multicenter study using paraffin-embedded tumour tissue testing *PITX2* DNA methylation as a marker for outcome prediction in tamoxifen-treated, node-negative breast cancer patients. *J. Clin. Oncol.* 26(31), 5036–5042 (2008).

In an earlier study by the same group mentioned above, DNA methylation of the *PITX2* gene measured in fresh-frozen breast cancer specimens was observed to be associated with an increased risk of recurrence in women with node-negative hormone-positive breast cancer treated with tamoxifen [1].

The paper by Harbeck *et al.* reports on a follow-up study conducted by these investigators to validate these findings in an independent cohort [2]. First, using breast cancer samples acquired from multiple clinical centers, Harbeck *et al.* demonstrated that *PITX2* DNA methylation could be reliably assessed in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue using quantitative methylation (QM)-PCR [2]. A high concordance ($r_s = 0.81$) was demonstrated between *PITX2* DNA methylation scores from 89 pairs of fresh-frozen tissue and FFPE specimens from the same tumor, providing support for the use of FFPE specimens

in large validation studies. In addition, as fresh-frozen tissue is often more difficult to obtain, the possibility of using FFPE specimens for routine clinical testing is highly attractive.

Replicate measurements of *PITX2* methylation scores by QM-PCR from 30 separate paraffin blocks were also highly correlated ($r_s > 0.95$). Lastly, an assessment of within-tumor heterogeneity was made by evaluating *PITX2* methylation in five samples of tissue from the same tumor. The average standard deviation was 4.3% with a range between 0.3 and 17%, suggesting moderate heterogeneity. Evaluation of more than one slide in future clinical studies assessing *PITX2* methylation would, therefore, be more representative of tumor methylation status.

Having demonstrated the robustness of this assay, Harbeck *et al.* examined the association between *PITX2* DNA methylation and both time to recurrence and overall survival in 427 primary breast cancer patients all aged over 35 years with hormone-receptor positive, node-negative, tamoxifen-treated early breast cancer. Patients were accrued from nine clinical centers in Europe, had their surgery during or prior to 1998 and received no neoadjuvant or adjuvant chemotherapy. Valid *PITX2* DNA methylation scores were obtained by QM-PCR for 93.4% ($n = 399$) of samples.

The primary outcome was time to distant metastases (TDM). Information on standard prognostic factors including tumor size and grade, age at diagnosis, PR status and HER-2 status were also obtained. Increased *PITX2* methylation scores were associated with a shorter TDM (HR = 2.35; 95% CI: 1.2–4.6; $p = 0.013$) after taking into consideration tumor grade, size and age at diagnosis. However, no significant difference in overall survival was observed. Patients were then stratified into two groups, those with a low and high DNA methylation score based on the median methylation score obtained from the initial dataset of tamoxifen-treated, node-negative early breast cancer patients. Using this cutoff, patients with a high *PITX2* methylation score were significantly more likely to recur than patients with a low methylation score. For those with a low methylation score (52%), estimated 10-year metastasis-free survival was 95%. There was, however, no significant difference in overall survival.

This study confirms the findings of the preliminary work by this group suggesting that *PITX2* methylation may be useful in discerning those women with hormone-positive node-negative breast cancer that are likely to experience progression of disease.

Further studies are required to determine whether *PITX2* DNA methylation is an independent prognostic factor for hormone-positive node-negative

breast cancer or a predictor of response to tamoxifen therapy in the same subgroup of women. The next step would be assessment of *PITX2* DNA methylation in tissue collected from a randomized trial that compared adjuvant tamoxifen versus no adjuvant hormonal therapy.

Further elucidation of the biological significance of *PITX2* in breast cancer would also aid in understanding of these observations.

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ABCB1 promoter hypermethylation, transcript expression and gene amplification in docetaxel-resistant breast cancer cell lines

Evaluation of: Reed K, Hembruff SL, Laberge ML *et al.*: Hypermethylation of the *ABCB1* downstream gene promoter accompanies *ABCB1* gene amplification and increased expression in docetaxel-resistant MCF-7 breast tumor cells. *Epigenetics* 3(5), 1–11 (2008).

Multidrug resistance is an important mechanism for failure of response to chemotherapy in patients with malignancy [1]. Drug transporters, such as *ABCB1* (MDR1, P-glycoprotein), are well recognized as mediators of drug resistance by expelling chemotherapy from cancer cells [2]. It has been suggested that epigenetic changes involving methylation of CpG islands within gene promoters can play a significant role in altering *ABCB1* gene expression in the setting of acquired drug resistance.

Reed *et al.* investigated if the increased *ABCB1* expression observed in docetaxel-resistant breast cancer cell lines (MCF-7_{TXT}) was linked to changes in downstream promoter methylation or gene copy-number [3].

The onset of docetaxel drug resistance in MCF-7_{TXT} cells was first identified by exposing these cells to increasing concentrations of docetaxel. Drug resistance of

MCF-7 cells to docetaxel first occurred at dose 9 and then continued to increase until dose 12.

Increased mRNA expression of *ABCB1* was detected in all docetaxel-resistant cells in an incremental fashion. At dose nine and above, a decrease in paclitaxel uptake in the MCF-7 cells was also observed that was reversed with an *ABCB1* inhibitor.

The methylation status of both the upstream and downstream *ABCB1* promoters was then determined by bisulfite sequencing of genomic DNA extracted from cell lines. Hypermethylation of the downstream promoter was identified from dose level 10 and increased at each dose level. Interestingly, *ABCB1* mRNA transcript expression was found by primer extension experiments to derive solely from the upstream promoter, in conflict with previous reports [4]. It was postulated that hypermethylation of the downstream promoter might facilitate activation of the upstream promoter, leading to increased *ABCB1* expression. In support of this, treatment of MCF-7_{TXT} cells with the demethylating agent 5-aza-2'-deoxycytidine resulted in a reduction in *ABCB1* mRNA levels.

The expression of *ABCB1* transcripts in docetaxel-resistant MCF-7 cells appear to be due to a complex interplay between

alterations in promoter methylation status and increased gene amplification. Whether these findings will translate to human breast tumors is not known. Evaluating the effects of both *ABCB1* mRNA expression and methylation on response to therapy in breast tissue from women known to have been treated with taxanes would begin to provide such information. Demethylating agents and histone deacetylase inhibitors may play a role in women who develop resistance to taxanes as a result of methylation of the *ABCB1* promoter.

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