Methylation of the TWIST1 Promoter, TWIST1 mRNA Levels, and Immunohistochemical Expression of TWIST1 in Breast Cancer

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Abstract

TWIST1, an antiapoptotic and prometastatic transcription factor, is overexpressed in many epithelial cancers including breast. Only little is known regarding the regulation of TWIST1 in these cancers. Recently, an increase in the TWIST1 promoter methylation has been shown in breast cancers. To correlate the percentage of TWIST1 promoter methylation to the protein levels, we analyzed simultaneously the methylation status as well as the mRNA and the percentage of cells expressing TWIST1 in normal breast tissue and 76 invasive breast cancers. We found that TWIST1 promoter methylation is significantly more prevalent in malignant compared with healthy breast tissue. Furthermore, the percentage of cells expressing TWIST1 was greater in breast malignancy compared with matched healthy tissue from the same patients. There was no correlation, however, between TWIST1 promoter methylation and TWIST1 protein or RNA expression. This indicates that although TWIST1 CpG methylation is useful as a biomarker in breast cancer diagnosis, there is no direct correlation with TWIST1 expression. (Cancer Epidemiol Biomarkers Prev 2008;17(12):3325–30)

Introduction

TWIST1 has recently emerged as an interesting breast cancer biomarker (1, 2). It belongs to the basic-helix-loop-helix family of transcription factors and is implicated in lineage-specific cellular differentiation and survival (3, 4). TWIST1 function in vertebrates governs early mesodermal patterning and osteogenesis (5). Individuals with germ-line haploinsufficiency of the TWIST1 gene suffer from the hereditary disorder Saethre-Chotzen syndrome (acrocephalosyndactyly type III) characterized by premature craniosynostosis and limb, head, and face anomalies (6). In cancer development, TWIST1 functions as a prometastatic oncogene. Expression of TWIST1 protein counteracts the proapoptotic effects of N-MYC by repression of p19ARF, and thereby hampers Tp53 function (4). In a metastatic breast cancer mouse model, TWIST1 was necessary for onset of metastasis (2). TWIST1 induces epithelial to mesenchymal transition (EMT). EMT is a developmental program that enables epithelial cells to undergo a mesenchymal cell fate (7). This is characterized by differential regulation of several epithelial and mesenchymal marker genes, such as E-cadherin, N-cadherin, vimentin, α-smooth muscle actin, and β-catenin. Loss of E-cadherin, a common phenomenon in breast cancer, is an important hallmark of EMT and a predictor of poor prognosis in various cancers (7). EMT is orchestrated by transcriptional activation and repression by a group of transcription factors, including SNAIL, E-CADHERIN, ZEB1A, ZEB1B, and TWIST1 (8). TWIST1 regulates EMT by repression of E-cadherin and induction of N-cadherin (7, 9). In addition to its antiapoptotic and prometastatic function, TWIST1 overexpression induces angiogenesis and chromosomal instability (10).

At the DNA level, cancer development is characterized by genetic and epigenetic events. Methylation of CpG islands in promoter regions of tumor suppressor genes is a common epigenetic event (11). Methylation abrogates proper TATA-binding protein binding to the promoter, leading to reduced expression of genes that play important roles in the cell cycle, cell adherence, cell signaling, DNA repair, and apoptosis (12). Human breast carcinomas exhibit TWIST1 promoter hypermethylation at high frequency, ranging from 16% to 77% (1, 13-18). Moreover, methylation of the TWIST1 promoter is a good predictor of human breast cancer presence (1). These findings postulate methylation of the TWIST1 promoter as an interesting breast cancer biomarker, but its functional significance remains unknown. Whereas its role in promoting EMT and metastasis and suppressing apoptosis suggests TWIST1 functions as an oncogene, TWIST1 promoter methylation may also suggest a role for TWIST1 silencing in breast cancer development.

To further elucidate the putative function of TWIST1 as a biomarker in breast carcinogenesis, we studied the relation between TWIST1 promoter methylation and...
TWIST1 expression in normal and malignant breast tissue specimens.

Materials and Methods

Patients. Seventy-six invasive breast carcinomas were obtained from the Pathology Department of the University Medical Center Utrecht. Use of anonymous or coded leftover material for scientific purposes is part of the standard treatment contract with patients in our hospital (19). H&E-stained slides of the paraffin blocks were reviewed by a pathologist (P.J.V.D.) to confirm the presence of malignancy in tumor samples. Histologic type was assessed according to the WHO. Grade was assessed according to the Nottingham system, and the estrogen, progesterone, and HER-2/neu receptors were assessed by standard immunohistochemistry (20). We selected 34 patients with invasive ductal breast cancer supplemented with a set of 42 patients with invasive lobular cancer, as these usually show loss of E-cadherin expression. Table 1 shows baseline clinicopathologic features of 76 breast cancers studied for TWIST1 promoter methylation and TWIST1 expression.

Table 1. Baseline clinicopathologic features of 76 breast cancers

<table>
<thead>
<tr>
<th>Clinicopathologic features of breast carcinoma patients</th>
<th>Ductal (n = 34), n (%)</th>
<th>Lobular (n = 42), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5 (15)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>II</td>
<td>13 (38)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>III</td>
<td>16 (47)</td>
<td>4 (67)</td>
</tr>
<tr>
<td>Lymph node status positive</td>
<td>16/34 (47)</td>
<td>17/35 (49)</td>
</tr>
<tr>
<td>Estrogen receptor positive</td>
<td>25/34 (74)</td>
<td>34/36 (94)</td>
</tr>
<tr>
<td>Progesterone receptor positive</td>
<td>20/34 (59)</td>
<td>21/35 (60)</td>
</tr>
<tr>
<td>HER-2/neu positive</td>
<td>8/30 (27)</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>Age (y), mean (range)</td>
<td>57 (40-85)</td>
<td>64 (37-88)</td>
</tr>
<tr>
<td>Mitotic index, mean</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>Tumor size (cm), mean</td>
<td>2.6</td>
<td>3.9</td>
</tr>
</tbody>
</table>

DNA Isolation, RNA Isolation, and Quantitative Reverse Transcription-PCR. For isolation of DNA from paraffin-embedded specimen, a 10 μm unstained section was deparaffinized by treatment of 2 × 5 min xylene, and the relevant tissue was scraped from the slide. TONES (50 μL; 10 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% SDS) extraction buffer containing 250 ng salmon sperm DNA (Invitrogen) and 100 μg proteinase K (Invitrogen) was added to the tissue. After 4 h rotation at 52°C, samples were heat inactivated for 5 min at 99°C and stored at 4°C.

RNA and DNA were isolated from frozen material by dissolving three 10 μm thick sections in 1 mL Trizol reagent (Invitrogen) followed by chloroform/phenol extraction. The aqueous phase was used to precipitate RNA. The interphase was used to precipitate DNA. cDNA was prepared from 1 μg RNA using reverse transcriptase (Roche) and a 1:1 oligo(dT)/hexamer primer mix (Invitrogen). Quantitative PCR was done using commercially available TaqMan assays for TWIST1 (Hs00361186) and the housekeeping gene hydroxymethylbilane synthase (Hs00609297_m1) (Applied Biosystems) using an ABI7900 analyzer (Applied Biosystems). Data were analyzed using the SDS2.1 program (Applied Biosystems) using hydroxymethylbilane synthase for normalization.

Quantitative Multiplex Methylation-Specific PCR. Quantitative multiplex methylation-specific PCR was done as described previously (1, 22). Briefly, 13.5 μL isolated DNA was heated at 99°C for 10 min and quick chilled on ice after which 1.5 μL freshly prepared 2 mol/L NaOH was added. Sodium bisulfite (35 μL; 4.5 mol/L; Sigma) containing 1 mmol/L hydroquinone (Sigma; both freshly prepared; mixed just before adding) was added to the sample, after which it was kept at 55°C in the dark under oil for 4 h.

Microspin ion-exchange columns (Amersham Biosciences) were used for purification according to the manufacturer's directions. After 5 min incubation, a mixture of 212 μL water, 130 μL of 10 mol/L ammonium acetate, 3 μL glycerol, and 1 mL absolute ethanol was used for precipitation (at −20°C overnight). The next day, after 30 min centrifuge for 13,000 rpm at 4°C and drainage and washing with 75% ethanol, the pellet was dissolved in 5 μL water. Dissolved DNA (5 μL) was multiplexed in a 50 μL PCR using methylation-specific PCR buffer (16.6 mmol/L NH4SO4; 67 mmol/L Tris [pH 8.8]; 6.7 mmol/L MgCl2, 10 mmol/L L-mercaptoethanol, 0.1% DMSO; 0.0625 mmol/L deoxynucleoside triphosphate, 0.1 μmol/L MgCl2, 10 units Platinum Taq (Invitrogen), and 100 ng of each reverse and forward primer for 11 genes. The external (non-CpG-dependent) primers for TWIST1 were TWIST Ext F gagagtagatatatttgg and TWIST Ext R ctcctccacatttga. Primers and probes for the other genes were as described previously (1, 22). PCR conditions were as follows: 95°C for 5 min, 36 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s followed by an extension cycle of 7 min.
72°C for 5 min. Human sperm DNA was used as a negative control. Ss1-treated MDA-MB-231 cells served as a positive control. The PCR products were diluted 1:5 with water and stored at −20°C.

Diluted multiplexed DNA (2 μL; 1:5) were used for real-time PCR in a final volume of 25 μL using 2.5 μL of 10× methylation-specific PCR buffer (as above), 200 μmol/L deoxynucleoside triphosphate, 1.25 units Ramp Taq (Denville Scientific), and 1× ROX (Invitrogen). A gene-specific primer and probe set containing 600 nmol/L each of two primers (forward and reverse) and 200 nmol/L labeled probe (Applied Biosystems) was added to the reaction mix. The internal primers and probes for TWIST1 were TWIST RT-FM gttagggttcgggggcgttgtt, TWIST RT-RM cggtgccttcctccgacgaa, TWIST RT-FUM ggtttgggggtgttgtttgtatg, TWIST RT-RUM cccacctcctaac-ttttttttttttttttttgg, TWIST M probe aaacgatttccttccccgccgaaa, and TWIST RT-RUM ccacacctcaac-accccttc, TWIST M probe aaacgatttccttccccgccgaaa, and TWIST UM probe aaacattacctccccacaaaaaa. PCR conditions were 95°C for 7 min followed by 40 cycles of 95°C for 15 s and 65°C for 1 min. A standard curve (dilutions 10−2, 10−4, 10−6, and 10−8) and a 80K copy number control were included for extrapolating percentage methylation from the U and M curves as described previously (1). Percentage methylation was calculated as 100 × [amount of methylated DNA] / [amount of methylated DNA + unmethylated DNA].

Immunohistochemistry. For TWIST1 immunohistochemistry, paraffin slides (5 μm thick) were deparaffinized with xylene and serial ethanol dilutions. Endogenous peroxidase activity was blocked for 30 min with a buffer solution containing peroxide (0.5% H2O2 in phosphate citrate) followed by antigen retrieval [boiling in citrate buffer (pH 6.0), 20 min]. Slides were incubated overnight with the anti-TWIST1 antibody (1:100) followed by the secondary antibody (Powervision; poly-HRP anti-Ms/Rb/Rt IgG biotin-free). All slides were developed with diaminobenzidine followed by hematoxylin counterstaining. Before the slides were mounted, all sections were dehydrated in alcohol and xylene. Percentage of positively stained nuclei was estimated by an experienced pathologist (P.J.v.D.) in all cancers. In addition, in those cases where normal breast tissue was present, the nuclear staining intensity of cancer cells was scored in comparison with normal breast cells as higher, similar, or lower.

Statistical Analysis. The Mann-Whitney test was used for comparing medians between groups. The χ2 test was used for comparing frequency distributions. Spearman correlations were used for assessing the association between continuous variables. Wilcoxon and Sign tests were used to compare paired data. SPSS 12.0.1 for Windows was used for statistical analysis. Statistical tests were considered statistically significant at two-sided P < 0.05.

Results

TWIST1 Promoter Methylation. The mean percentage methylation of the TWIST1 promoter in paraffin-embedded specimens of the 76 invasive cancers was 34% (median, 17%). There was no difference in mean percentage of methylation of the TWIST1 promoter between ductal (34%, n = 34) and lobular cancers (33%, n = 42). TWIST1 promoter methylation showed a weak correlation with age (Spearman correlation coefficient = 0.34; P = 0.003) as has been described previously for other genes. TWIST1 promoter methylation was not associated with mitotic index, tumor size, nor grade or with lymph node, HER2/neu, estrogen receptor, or progesterone receptor status. TWIST1 methylation values were higher in 12 available fresh frozen tumors (median, 49%) than in adjacent normal breast tissue (median, 1%; P = 0.004, Wilcoxon test; Fig. 1). Methylation in paraffin-embedded malignant specimens of these 12 patients (median, 51%) was not significantly different from the matched frozen specimens (P = 0.59, Wilcoxon test).

Other genes that were simultaneously multiplexed by the quantitative multiplex methylation-specific PCR method showed very low values of methylation (e.g., mean, 2% for BRCA2 and 3% for CDKN2A), indicating that the high abundance of methylation of the TWIST1 promoter is not due to contamination or an artifact of the method.

Immunohistochemical Analysis of TWIST1. To study expression of TWIST1 in breast cancer specimens, a previously described polyclonal TWIST1 antibody was used (10). This antibody was validated by Western analysis of MCF-7 cells overexpressing exogenous TWIST1 and immunofluorescence analysis of endogenous TWIST1 in HeLa cancer cells, displaying nuclear staining (Fig. 2A and B). TWIST1 immunohistochemistry of breast cancers revealed a nuclear staining in malignant cells (Fig. 2C-F). In addition, normal breast regularly showed nuclear TWIST1 staining. Mean percentage of stained malignant nuclei was 51% (median, 50%; n = 71). More than 10% staining was present in 82% of tumors (cutoff was chosen analogously with estrogen receptor and progesterone receptor positivity). There was no difference in percentage of TWIST1-positive nuclei between ductal and lobular cancers (P = 0.97, Mann-Whitney test). No association between TWIST1 expression and clinicopathologic data was found. Of the

Figure 1. Comparison of TWIST1 promoter methylation in breast cancer and normal breast in matched frozen tissue specimens. Every matched sample (n = 12) is indicated by a dot. The line indicates the equivalence.

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65 patients with normal and malignant cells in the same slide, TWIST1 expression was unchanged in 44 cases, enhanced in malignant cells in 17 patients, and decreased in the tumor in 4 cases ($P = 0.007$, Sign test).

Relation between TWIST1 Promoter Methylation Status and Expression. The percentage of TWIST1-positive nuclei did not correlate to TWIST1 promoter methylation (Spearman correlation coefficient $= -0.01$, $P = 0.93$). To analyze the relation between TWIST1 promoter methylation and TWIST1 expression in more detail, we analyzed TWIST1 mRNA of healthy tissue samples and malignant tissue from the same 7 patients. Results were normalized to TWIST1 mRNA expression levels in HeLa cell lysates. Relative mRNA expression in normal tissue (median, 0.65) was not significantly different from the mean relative RNA expression in malignant tissue (median, 0.82; $P = 1.00$, Wilcoxon test). mRNA analysis of 30 available frozen malignant specimens revealed a mean relative TWIST1 mRNA expression of 0.95 (median, 0.72), which was also not significantly different from normal tissue ($P = 0.58$, Mann-Whitney test). Furthermore, no correlation between TWIST1 promoter methylation status and mRNA expression could be shown in malignant specimens (Spearman correlation coefficient $= -0.24$; $P = 0.22$).

Discussion

In this study, TWIST1 expression and promoter methylation was studied in breast carcinoma specimens and adjacent normal breast tissue. TWIST1 expression has been correlated to invasive lobular carcinoma in association with E-cadherin loss (2). TWIST1 promoter methylation has been suggested to occur less frequently in lobular compared with ductal breast carcinomas (15, 16). However, in another study, Bae et al. described a higher frequency of TWIST1 promoter hypermethylation in lobular compared with ductal cancer (13). Our
study does not support differential TWIST1 promoter methylation and patterns of protein expression between ductal and lobular breast carcinomas. TWIST1 promoter methylation was found to be enhanced in breast cancer specimen compared with normal breast tissue from reduction mammoplasty and prophylactic mastectomy samples (18). By direct comparison, the present study shows that in the same patient healthy breast epithelium is less frequently methylated for TWIST1 compared with the malignant tissue it surrounds. These findings might be explained by a selective advantage of breast epithelial cells with methylation of the TWIST1 promoter during breast carcinogenesis. Interestingly, mean methylation of the TWIST1 promoter in healthy breast tissue adjacent to a malignant tumor was still higher than the percentages described in completely normal breast tissue from unmatched samples in our previous study (18). Although a role for age-related methylation cannot be ruled out in this comparison, it might also point to an early contribution of TWIST1 promoter methylation to breast cancer development and perhaps a role in field cancerization as it has been described previously for other genes (23, 24). In this model, TWIST1 CpG island methylation might prime an apparently healthy-looking population of breast epithelial cells for clonal outgrowth and the accumulation of additional mutations.

We found frequent protein expression of TWIST1 in invasive breast cancers, in accordance with previous studies (2, 10, 16, 25). TWIST1 protein expression was also observed in ductal carcinoma in situ in the present study (data not shown), which supports a role for TWIST1 expression in breast cancer development, maybe even in an early stage. TWIST1 levels were similar in ductal and lobular cancers, although lobular cancers with their loss of E-cadherin expression might have a higher degree of EMT. Interestingly, TWIST1 nuclear expression was also observed in normal ducts in some patients, implying that TWIST1 may have a physiologic role in normal breast epithelium. However, when comparing paired normal and malignant tissues, TWIST1 was significantly higher expressed in malignant nuclei, in accordance with its role in promoting breast cancer.

The fact that high frequency and degree of promoter methylation of TWIST1 in the present study paralleled the increased expression remains puzzling. Furthermore, besides the lack of an inverse correlation between promoter methylation and the percentage of nuclei expressing TWIST1, TWIST1 mRNA levels between normal and malignant tissues were not clearly different. Therefore, the difference in TWIST1 protein expression may be explained by post-transcriptional regulation of TWIST1.

The CpG island investigated in this study is located ~250 bp upstream of the TWIST1 TATA box. This region has been implicated in regulation of TWIST1 by STAT3 and hypoxia-inducible factor-1α (26, 27). Indeed, TWIST1 expression is regulated by hypoxia, a common feature in solid cancer, via 3′ enhancer sequences (21, 24).

An interesting possibility is that the methylation observed in the proximal part of the TWIST1 promoter does not relate to expression of TWIST1 but of other genes in genomic proximity [e.g., HDAC9 and FERD3L (N-TWIST)]. Alternatively, TWIST1 promoter methylation might be an early event, which precedes compensatory TWIST1 overexpression. Indeed, TWIST1 haploinsufficiency predisposes to breast cancer in Saethre-Chotzen syndrome (28). To further elucidate these mechanisms, the relation between methylation of the TWIST1 promoter and TWIST1 expression should be addressed in vitro models. Previous studies have shown that increased TWIST1 expression is enhanced in lymph node metastases and predicts survival in breast and other cancers (10, 16, 24, 29-31). Although TWIST1 expression did not correlate to lymph node status in the present study, it will be interesting to further study TWIST1 in relation to clinical outcome or response to therapy (32).

We conclude that TWIST1 promoter methylation is not related to TWIST protein expression in the normal and malignant breast. TWIST1 promoter methylation is, however, a strong biomarker for breast cancer. The often increased TWIST1 protein level in breast cancer cells fits with the proposed oncogenic role of TWIST1 in breast carcinogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
18. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. For. BMJ 2002;325:648–51.
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