Wilms’ Tumor Suppressor Gene (WT1) Is Expressed in Primary Breast Tumors Despite Tumor-specific Promoter Methylation

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Abstract

We analyzed Wilms’ tumor suppressor 1 (WT1) expression and its regulation by promoter methylation in a panel of normal breast epithelial samples and primary carcinomas. Contrary to previous reports, WT1 protein was strongly expressed in primary carcinomas (27 of 31 tumors) not in normal breast epithelium (1 of 20 samples). Additionally, the WT1 promoter was methylated in 6 of 19 (32%) primary tumors, which nevertheless expressed WT1. The promoter is not methylated in normal epithelium. Thus, although tumor-specific methylation of WT1 is established in primary breast cancer at a low frequency, other transcriptional regulatory mechanisms appear to supercede its effects in these tumors. Our results demonstrate expression of WT1 in mammary neoplasia, and that WT1 may not have a tumor suppressor role in breast cancer.

Introduction

WT1 encodes a transcriptional regulatory protein that binds DNA via four Cys2-His2 zinc fingers (1). WT1 mRNA undergoes two independent splicing events, leading to the expression of at least four predominant isoforms (2). These splices result in the inclusion or omission of exon 5 (51 bases) and the presence or absence of a nine-base insert (encoding three amino acids, KTS) between the third and fourth zinc finger domains. Lack of expression has been observed in some Wilms’ tumors, leading to its classification as a tumor suppressor gene. However, WT1 is overexpressed in 75% of cases of acute leukemia and is up-regulated as chronic myeloid leukemia progresses into blast crisis (3). Thus, WT1 can apparently behave either as a tumor suppressor or as an oncogene.

The role of WT1 in breast cancer remains unclear. Using immunohistochemistry, Silberstein et al. demonstrated WT1 expression in normal breast tissue, particularly in the myoepithelial cells that overlie the polygonal cells lining the ductal lumen. Reduced or absent WT1 staining was seen in 60% of breast tumors, leading these authors to conclude that loss of WT1 expression might be correlated with tumorigenesis. Furthermore, by RT-PCR analysis, they reported that WT1 is associated with >80% of epithelial cells, and frozen at –70°C until used (8). We also used highly purified (95–99%) myoepithelial and luminal epithelial cells isolated by differential centrifugation and fluorescence-activated cell sorting of enzymatically digested normal mammary explants (9).

RT-PCR for WT1 mRNA. Methods for RNA extraction and RT-PCR have been described previously (7). The sequences of the primers used are as follows: for amplifying the 555-bp region surrounding WT1 exon 5, 5'-GGCGGCGAGTTCTCCCCAACA-3' (sense, nucleotides 882–901) and 5'-ATGTGTTTCTACCGATGTGCTT-3' (antisense, nucleotides 1416–1437); for amplifying the 382-bp region surrounding the KTS insert, 5'-GCTTCT-GAACCAGTGAGAA-3' (sense, nucleotides 1320–1339) and 5'-TTCTGAGTCATTTG-3' (antisense, nucleotides 1685–1702). Amplification was performed using a hot-start protocol; samples were heated to 94°C for 4 minutes and then cooled to 80°C prior to the addition of Taq polymerase (RedTag; Sigma Chemical Co., St. Louis, MO). Samples were then heated to 94°C for 30 s, followed by either 50°C for 30 s (for the KTS primers) or 56°C for 30 s (for the exon 5 primers) and then 72°C for 1 min for 40 cycles. PCR products were resolved by electrophoresis, using a 2% agarose gel for the exon 5-splice variants and a 12% polyacrylamide gel to resolve the KTS insert variants. Coamplification of the ribosomal RNA 36B4 was performed as an internal control using the following primers: 5'-GATTTGGCTACCC-3' (antisense, nucleotides 1320–1339) and 5'-CAGGGCGAGCAGCCACACAAGGC-3' (sense) using highly purified (95–99%) myoepithelial and luminal epithelial cells.

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Northern Blots. Total RNA was extracted as described (7). After electrophoresis through a 1.5% agarose gel in 4-morpholinepropanesulfonic acid buffer with 6.7% formaldehyde, RNA was transferred to nitrocellulose. Blots were probed with a PCR product corresponding to the WT1 zinc finger region, amplified using the primers described above, and labeled with [-32P]dCTP by random priming using standard techniques.

Methylation-specific PCR. Genomic DNA was isolated using standard techniques and treated with sodium bisulfite as described (10). Methylation-
specific PCR was performed using the following primers: to detect methylated promoter DNA, 5’-TCTTGGTTAAGTGGCTACGTCG-3’ (sense, nucleotides −267 to −243) and 5’-ACACTACTCCTCCTGACTGACCG-3’ (antisense, nucleotides +33 to +59); to detect unmethylated promoter DNA, 5’-TCTTGGTTAAGTGGCTACGTCG-3’ (sense) and 5’-ACACTACTCCTCCTGACTGACCG-3’ (antisense); to detect methylated intron 1 DNA, 5’-CTTGTTGGTTAAGTGGCTACGTCG-3’ (sense) and 5’-ACACTACTCCTCCTGACTGACCG-3’ (antisense); to detect unmethylated intron 1 DNA, 5’-CTTGTTGGTTAAGTGGCTACGTCG-3’ (sense) and 5’-ACACTACTCCTCCTGACTGACCG-3’ (antisense). The PCR reaction was as above, except that the annealing temperature was 59°C, and the extension time was 45 s.

Western Blots. Total protein from cell lines was obtained from material harvested in TriReagent (Molecular Research Center, Cincinnati, OH) and initially used for RNA isolation. Protein purification was according to the manufacturer’s protocol. After separation by SDS-PAGE and electrophoretic transfer to nitrocellulose membranes, proteins were incubated with an anti-WT1 antibody [WT (C-19); Santa Cruz Biotechnology, Santa Cruz, CA], diluted 1:1000 in the blocking solution. Horseradish peroxidase-conjugated antibody against rabbit IgG (Amersham, Arlington Heights, IL) was used at 1:1000, and binding was revealed using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Results and Discussion

Expression of WT1 mRNA in Mammary Epithelial and Breast Cancer Cell Lines. To evaluate WT1 expression in the breast, we analyzed mRNA expression by RT-PCR in a panel of normal and transformed cell lines. We were unable to detect WT1 mRNA in three independently derived, finite lifespan mammary epithelial strains: HMEC 166372, 1-26, and 3-14 (Fig. 1A). Among the three immortal breast epithelial cell lines, WT1 expression was observed in HMECs HBL-100 and MCF-10A but not in H16N (Fig. 1A). WT1 mRNA expression was examined in nine breast cancer cell lines; expression was easily detectable in five: HS578T, T47D, MDA-MB-468, 21MT, and 21PT, and undetectable in the remaining four: SKBR3, MDA-MB-435, MCF-7, and MDA-MB-231 (Fig. 1B).

The specific expression of WT1 isoforms lacking the 5th exon and lacking the KTS insert has been reported to occur in breast cancer (4). To determine whether differential expression of WT1 splice variants is seen in breast cancer, we designed PCR primers spanning the 5th exon such that mRNA encoding the isoform containing exon 5 yielded a 555-bp PCR product, whereas if exon 5 were missing, a 504-bp PCR product was generated. We also used PCR primers spanning the region of the KTS insert, such that mRNA containing the insert would yield a 382-bp product, whereas mRNA lacking the insert would generate a 373-bp product. Contrary to the findings in the published report (4), in the five WT1-expressing breast cancer cell lines and in the WT1-expressing immortalized HMECs, all four splice variants, the two exon 5 isoforms (Fig. 1B) and the two KTS isoforms (Fig. 1C), were present.

To confirm the results of our RT-PCR experiments, Northern blot analysis was performed using total RNA isolated from a number of breast cancer cell lines. Similar to the results obtained by RT-PCR (Fig. 1A, A−C), WT1 mRNA expression was readily detected in HBL-100, HS578T, T47D, and MDA-MB-468 cells but was not detected in MDA-MB-435, MDA-MB-231, SKBR3, or MCF-7 cells (Fig. 1D).

Thus, WT1 mRNA expression was undetectable in finite lifespan primary breast epithelial cell cultures but was easily detectable in the neoplastic and immortalized HMECs and in 7 of 12 breast cancer cell lines. Also, the striking correlation between results of our Northern blot and RT-PCR experiments validated the RT-PCR protocol as an accurate reflection of WT1 mRNA expression in the breast cancer cells.

Methylation of the WT1 Locus in Breast Cancer Cell Lines. The promoter and first intron of the WT1 gene contain dense CpG islands. These sequence elements are frequently sites of DNA methylation and play a role in transcriptional silencing (11, 12). To determine whether methylation silences gene expression in the WT1-negative cell lines, we investigated the status of the WT1 promoter in the breast cancer cell lines. We found that the promoter was methylated in the four cell lines that did not express WT1 (Fig. 2A) but not in the five cell lines that did (Fig. 2A and data not shown), consistent with the idea that methylation is a critical determinant of WT1 expression. There was one exception to this correlation. T47D cells contained methylated, as well as unmethylated, WT1 sequences but nevertheless expressed WT1 mRNA, suggesting that in this case, methylation alone is insufficient to silence expression.

Promoter methylation is postulated to silence transcription, at least in part, by recruitment of HDAC to hypermethylated loci (11, 12). To assess the functional significance of WT1 promoter methylation, MDA-MB-231 and MCF-7 cells were treated with 5-aza-deoxycytidine, an inhibitor of DNA methyltransferases, or with TSA, an inhibitor of HDAC. As demonstrated before (6), treatment with 5-aza-deoxycytidine reinitiated WT1 expression in MDA-MB-231 cells (Fig. 2B). Interestingly, this treatment did not cause WT1 expression in MCF-7 cells (Fig. 2B), nor did TSA restore expression in either cell line (Fig. 2C).

In the same samples, these treatments restored expression of another gene, 14-3-3σ, frequently hypermethylated in breast cancers (7). These findings suggest that although promoter methylation correlates...
contain a layer of myoepithelial cells that overlie the luminal epithelium. To ensure that our normal samples contained luminal epithelial cells, we used three different types of epithelial cell preparations including (a) three short-term cultures of HMECs; (b) nine organoid preparations of mammary ducts; and (c) eight samples of highly purified luminal and myoepithelial cells (isolated from four patient samples).

WT1 expression was undetectable by RT-PCR in three HMEC samples (Fig. 1A) in eight of nine breast organoid preparations (Fig. 3A) nor in any of eight purified epithelial cell preparations (Fig. 3B). By Western blotting, WT1 protein was not detected in three organoid samples or in two HMECs (Fig. 3C). In contrast, WT1 expression was easily detectable by Western blotting in 27 of 31 (87%) primary breast carcinomas (Fig. 3C and data not shown).

Silberstein et al. (4) reported the specific expression of the WT1 isoforms lacking exon 5 in primary tumors but not in normal epithelial cells. Contrary to their findings, our HMECs did not express WT1; however, RT-PCR demonstrated the expression of exon 5 (+) and exon 5 (−) isoforms in five of seven tumors, whereas the remaining two expressed only the exon 5 (+) isoform (Fig. 4A). We also detected KTS (+) and KTS (−) isoforms in all nine tumors examined (Fig. 4B). Thus, in agreement with the published data, a majority of the tumors expressed both exon 5 splice variants of WT1, and all of the tumors expressed both splice variants involving the KTS insert. Interestingly, the sole breast organoid sample that expressed WT1 (B44; Fig. 3A) expressed all four splice variants as well (data not shown).

Methylation of WT1-associated CpG Islands in Normal and Malignant Breast Tissue. Because methylation of the promoter-associated CpG island correlated with a lack of WT1 expression in breast cancer cell lines, we investigated the methylation status of the promoter and 1st intron CpG islands in our panel of breast organoids and carcinomas. Prior studies demonstrating tumor-specific methylation of the CpG islands associated with the WT1 gene have used methylation-sensitive restriction enzymes (5, 6, 13). Although this technique is a reliable way to identify individual methylated sites, it is unable to assess large-scale methylation patterns. There is growing agreement that it is the density of methylation, rather than methylation of any specific CpG dinucleotide, that is responsible for gene silencing (14). For this reason, we chose to evaluate the CpG islands using MSP. This method allows the direct evaluation of several methylation

Fig. 2. A, methylation of the WT1 promoter in breast cancer cell lines. Sodium bisulfite-treated genomic DNA was used for methylation-specific PCR. U, samples amplified with primers that recognized the modified (unmethylated) sequence; M, samples amplified with primers that recognize the unmethylated (modified) sequence; B, expression of WT1 in 5-aza-deoxycytidine (Aza)-treated samples. RT-PCR was performed on RNA isolated from MCF-7 and from MDA-MB-231 cells treated with 5-aza-deoxycytidine (+) or with vehicle alone (−) for the indicated number of days using primers that amplify the region surrounding exon 5, with 36B4 as an internal control. B, expression of WT1 in MCF-7 and from MDA-MB-231 cells treated with TSA (+) or with vehicle alone (−) for the indicated number of days. RT-PCR was performed using the same primer sets as in B, which served as the positive control.

Expression of WT1 in Primary Breast Tissue. We expanded our findings from cell lines to patient samples, including normal breast epithelium and primary breast tumors. Breast carcinomas arise from luminal epithelial cells in the mammary duct. Normal breast ducts also

with gene silencing in breast cancer cells, it may not play a causal role.

Fig. 3. A, WT1 is not expressed in the majority of organoids. RT-PCR was performed on RNA from mammary organoid preparations from women with no (N) or benign (B) abnormalities and from K562 cells using primers that amplify the region surrounding exon 5, with 36B4 as an internal control. B, WT1 is not expressed in highly purified myoepithelial or luminal epithelial cells. RT-PCR using primers that amplify the region surrounding the KTS insert was performed on RNA from highly purified myoepithelial and luminal epithelial cells, with 36B4 as an internal control. C, WT1 protein is not expressed in normal breast tissue but is expressed in most breast tumors. Total cellular protein from three mammary organoid samples (N33, N44, and B54), two strains of normal human mammary epithelial cells (1-26 and 3-14), and several breast tumors (as indicated) was subjected to Western blotting using an antibody against WT1. The 32D c13 murine myeloblast cell line was used as a negative control, and 32D c13 cells transfected with a WT1 cDNA served as a positive control. Blots were stripped and reprobed with an antibody against β-actin to control for protein loading.
sites per PCR reaction, and choosing a variety of sequences for PCR primers allows the rapid assessment of many CpG dinucleotides (10).

Methylation-specific PCR was performed using DNA extracted from 19 primary tumors and 9 breast organoid preparations. The WT1 promoter CpG island was unmethylated in DNA from all nine organoid samples (Fig. 5A and data not shown). In contrast, 6 of 19 tumors contained methylated DNA, and the remaining 13 were completely unmethylated (Fig. 5B and data not shown). This rate of promoter methylation (32%) is similar to the 25% incidence reported by Laux et al. (6). Thus, methylation of the WT1 promoter is a tumor-specific phenomenon. Contrary to expectation, however, each of the six tumors that contained methylated WT1 also expressed WT1 protein (Table 1). Specifically, tumor 7103 clearly expresses WT1, as judged by Western blotting (Fig. 3C), despite the presence of methylated CpG dinucleotides in the promoter (Fig. 5B). WT1 promoter methylation, therefore, was not effective in silencing gene expression.

Next, we examined the CpG island in the first intron of the WT1 gene, a region where tumor-specific methylation has also been reported previously (5). We detected methylation of WT1 in three of three breast organoid preparations (Fig. 5C) and in 9 of 10 tumor samples evaluated (Fig. 5D and data not shown). Thus, the 1st intron of WT1 is methylated in both normal and malignant breast tissue, and this methylation is unrelated to tumorigenesis.

This is the first report of experiments aimed at correlating methylation of the WT1 promoter with gene expression in normal and neoplastic breast tissue. Consistent with previous reports, we demonstrated that methylation of the CpG island associated with the WT1 promoter correlates with gene silencing in several breast cancer cell lines (6). Although treatment of MDA-MB-231 cells with the methyltransferase inhibitor 5-aza-deoxyC results in reexpression of the gene, this was not seen in MCF-7 cells. Additionally, treatment with the HDAC inhibitor TSA had no effect on WT1 expression, suggesting that DNA methylation and histone acetylation play only minor roles in the regulation of WT1 expression in mammary epithelium.

We also found evidence of tumor-specific methylation of the CpG islands. Surprisingly, we detected expression of WT1 mRNA and protein in the majority of breast cancer samples that we evaluated, including in every sample that contained methylated DNA (Table 1). Our finding that breast carcinomas express WT1 despite tumor-specific gene methylation emphasizes the importance of evaluating methylation and gene expression concurrently in the same tissue.

Although at first glance our findings seem to contradict those of Silberstein et al. (4), who reported that breast tumors underexpress WT1, in fact they are probably in agreement. These authors stated that 15 of 21 carcinomas were WT1 negative by immunohistochemistry. However, they defined as negative a tumor with less than half of the cells staining for WT1. Six of their 15 WT1-negative tumors had between 10 and 50% positive cells and would certainly be positive by either of our assays. The other nine tumors may have had up to 10% of their cells expressing WT1, and these, too, may have been WT1

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**Table 1 Methylation of the WT1 promoter does not silence expression**

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. methylated</th>
<th>No. expressing WT1 mRNA/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast carcinomas</td>
<td>6/19</td>
<td>19/19</td>
</tr>
<tr>
<td>Normal breast epithelial cells</td>
<td>19/20</td>
<td>1/20</td>
</tr>
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**Fig. 4.** A, tumors express different exon 5 isoforms of WT1. RT-PCR was performed on RNA from primary breast tumors with primers spanning the region containing exon 5. 36B4 served as an internal control. B, tumors express both KTS isoforms. RT-PCR with primers that span the region surrounding the KTS insert. 36B4 was coamplified as an internal control, and RNA from K562 cells and from 32D c3 cells served as positive and negative controls.

**Fig. 5.** A, schematic of the CpG island surrounding the WT1 promoter. Vertical tick marks, the locations of CpG dinucleotides. Arrows, the positions of the primers used for MSP. The translation start site is also shown. B, the WT1 promoter is unmethylated in organoids. Sodium bisulfite-treated genomic DNA was used in MSP reactions. C, the WT1 promoter is methylated in some breast tumors. Sodium bisulfite-treated genomic DNA was used in MSP reactions. D, the first intron of WT1 is methylated in organoids. Sodium bisulfite-treated genomic DNA was used in MSP reactions with primers complementary to sequences within the first intron. E, intron 1 of WT1 is methylated in breast tumors. Sodium bisulfite-treated genomic DNA was used in MSP reactions. For all panels: U, unmethylated; M, methylated.
positive in our RT-PCR assays but negative by Western analysis. However, we were unable to replicate their finding of WT1 expression in the majority of nontransformed mammary epithelial samples. Interestingly, their paper reports the use of nested PCR to detect WT1 mRNA. We readily detected WT1 mRNA in tumor samples using a single-step PCR protocol. Although it is possible that we would have detected WT1 expression in normal epithelium using a nested PCR, this would not alter our basic finding that the gene is overexpressed in tumors compared with normal tissue, a finding strongly borne out by data obtained by Western blotting of normal and breast tumor samples. This finding suggests that WT1 may play a functional role in breast cancer.

Our data also reveal a discrepancy between gene regulation in tissue culture and in vivo. We found that methylation of the WT1 promoter is associated with gene silencing in breast cancer cell lines. In contrast, the promoter-associated CpG island was methylated in 32% of the tumors we examined; contrary to expectation, these tumors express WT1. These findings are reminiscent of those of Costello et al. (15), who reported data suggesting that there is no correlation between methylation and expression of WT1 in human gliomas and in glioma cell lines. Very similar results were also reported recently for the CpG island associated with the human telomerase gene promoter. Dessain et al. (16) reported that methylation silences expression of hTERT in some cell lines, and that treatment with 5-aza-deoxycytidine induces expression, but that the CpG island is methylated in many tumors that are telomerase positive. These data, and ours, highlight the fact that there are multiple mechanisms for gene silencing, of which hypermethylation of a CpG island is only one. More importantly, these findings emphasize the idea that cell lines do not necessarily reflect the situation in vivo. They also serve to point out that hypermethylation of a CpG island may be insufficient to silence expression, demonstrating the importance of assessing gene expression as well as promoter methylation status when evaluating the role of a particular gene in a particular tumor type.

The precise function of WT1 outside of the genitourinary system is unclear. A number of WT1 target genes have been identified, some of which may be relevant for tumorigenesis, such as E-cadherin (17), expression of which has been associated with improved cell survival in metastatic foci of breast cancer. Another group demonstrated that WT1 can transcriptionally activate bcl-2 (18), a gene associated with resistance to apoptosis. Our laboratory has identified cyclin E as another WT1 target, and there is accumulating evidence implicating this gene in mammary carcinogenesis as well (19). The growing number of identified WT1 target genes implicated in mammary carcinogenesis strengthens the concept that this gene may play a crucial role in this disorder.

In summary, our data demonstrate that WT1 is not expressed in normal breast epithelium and is overexpressed in the majority of primary breast tumors. Tumor-specific methylation of the CpG island occurs in breast cancer but appears to be inconsequential to gene expression.

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References

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