Inactivation of Wnt inhibitory factor-1 (WIF1) expression by epigenetic silencing is a common event in breast cancer

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The Wnt signaling pathway is a powerful and prominent oncogenic mechanism dysregulated in numerous cancer types. While evidence from transgenic mouse models and studies of human tumors clearly indicate that this pathway is of likely importance in human breast cancer, few clues as to the exact molecular nature of Wnt dysregulation have been uncovered in this tumor type. Here, we show that the Wnt inhibitory factor-1 (WIF1) gene, which encodes a secreted protein antagonistic to Wnt-dependent signaling, is targeted for epigenetic silencing in human breast cancer. We show that cultured human breast tumor cell lines display absent or low levels of WIF1 expression that are increased when cells are cultured with the DNA demethylating agent 5-aza-2'-deoxycytidine. Furthermore, the WIF1 promoter is aberrantly hypermethylated in these cells as judged by both methylation-specific PCR and bisulfite genomic sequencing. Using a panel of patient-matched breast tumors and normal breast tissue, we show that WIF1 expression is commonly diminished in breast tumors when compared with normal tissue and that this correlates with WIF1 promoter hypermethylation. Analysis of a panel of 24 primary breast tumors determined that the WIF1 promoter is aberrantly methylated in 67% of these tumors, indicating that epigenetic silencing of this gene is a frequent event in human breast cancer. Using an isogenic panel of cell lines proficient or deficient in the DNA methyltransferases (DNMTs) DNMT1 and/or DNMT3B, we show that hypermethylation of the WIF1 promoter is attributable to the cooperative activity of both DNMT1 and DNMT3B. Our findings establish the WIF1 gene as a target for epigenetic silencing in breast cancer and provide a mechanistic link between the dysregulation of Wnt signaling and breast tumorigenesis.

Introduction

Tumorigenesis is a multi-step process in which the activity of cellular growth-promoting genes, termed oncogenes, is increased. Conversely, the activity of genes that normally serve to constrain growth, termed tumor suppressors, is lost or diminished. Collectively, the mechanisms that drive upregulation of oncogene activity and downregulation of tumor suppressor activity stem from genetic and epigenetic changes in the genome (1). Wnt was originally identified as a putative proto-oncogene activated by viral insertion in mouse mammary tumors (2). Since this seminal discovery, it is now well documented that Wnt proteins form a family of secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis (3). Binding of Wnt proteins to the frizzled receptors activates the intracellular Dishevelled, which inhibits glycogen synthase kinase 3β (GSK3β) and allows the cytoplasmic accumulation of stabilized β-catenin. Consequently, β-catenin translocates to the nucleus where it engages transcription factors such as T-cell factors (TCFs) and lymphoid-enhancing factors (LEFs). Several growth-promoting genes are transcriptionally activated through this mechanism, including the oncogenic transcription factor c-myc (4). C-myc, in turn, activates a variety of genes involved in cell cycle regulation such as cyclins D1, D2 and E and the phosphatase cdc25A (5,6). Further, c-myc also transcriptionally activates the hTERT gene that encodes the catalytic subunit of telomerase (7). This is probably a prominent feature stemming from dysregulation of Wnt signaling since telomere stabilization is an important step in cell immortalization (8).

While no direct evidence linking Wnt to cancer has been uncovered, several of the downstream molecules in the Wnt signaling pathway (i.e. β-catenin, APC, Axin) are dysregulated in a variety of human tumors (9). Moreover, Wnt transgensics have proven to be a powerful model for the study of breast cancer since these mice display high-penetrance mammary adenocarcinomas early in life (10). Additionally, transgensics expressing activated β-catenin display a very similar phenotypic tumor (11). Such observations clearly imply that Wnt signaling is of importance in breast tumorigenesis in mice, and evidence collected on human breast tumors (12,13) supports the concept that dysregulation of Wnt signaling is of importance in human breast disease as well. However, mutations in key regulatory molecules of the Wnt signaling cascades are rather infrequent in breast cancer, leaving open the question as to the mechanism(s) that underlie dysregulation of Wnt signaling in this tumor type.

Wnt inhibitory factor-1 (WIF1) is a secreted protein that binds to Wnt proteins and inhibits their activity (14). Recently, the WIF1 gene was shown to be downregulated in human lung (15), gastrointestinal (16), and breast, prostate and bladder tumors (17). The former two groups observed that decreased WIF1 expression correlated with methylation of CpG dinucleotides within the WIF1 promoter. Numerous tumor

Abbreviations: 5-azaC, 5-aza-2'-deoxycytidine; BGS, bisulfite genomic sequencing; DKO, double knockout; DNMT, DNA methyltransferase; KO, knockout; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MSP, methylation-specific PCR; RT–PCR, reverse transcriptase–polymerase chain reaction; TSA, trichostatin A; WIF1, Wnt inhibitory factor-1.
suppressor or growth regulatory genes have been shown to undergo aberrant de novo methylation and transcriptional silencing in human tumors, and epigenetic silencing of tumor suppressor genes is now widely recognized as either a causative or a correlative event in tumor development (18). Thus, it is unsurprising that a list of well-characterized tumor suppressors including BRCA1, p16INK4a, 14–3–3σ, E-cadherin and ATM are known to be targets for epigenetic silencing in breast cancer (19–24). Site-specific DNA methylation is often an early event, as demonstrated in tumor types with a well-defined pattern of progression, such as colon cancer, and is now widely regarded as one of the ‘hits’ in the Knudson hypothesis leading to tumor suppressor gene inactivation.

While the WIF1 gene is a target for epigenetic silencing in some tumor types and its expression is downregulated in breast cancer, it is currently unknown if this gene is subject to epigenetic silencing in breast cancer. In order to determine the frequency and mechanism of WIF1 downregulation in breast tumors, we have analyzed the expression and DNA methylation status of the WIF1 promoter in a panel of cultured human breast tumor cell lines and surgically obtained human breast tumors. Our results demonstrate that WIF1 inactivation is a frequent event in breast cancer and suggests that dysregulation of Wnt signaling is an important contributor to human breast tumorigenesis.

Materials and methods

Cell culture and drug treatments

The breast tumor cell lines MCF-7, T47D, MDA-MB-231, MDA-MB-435, MDA-MB-468, BT549, ZR75-1 and SK-BR3 were purchased from the American Type Culture Collection. The YYC-B1 cell line was provided by Dr Sun Young Rha (Yonsei Cancer Center, Korea) and the HCT116 colorectal carcinoma cell line and its isogenic derivatives in which the ATM and TP53 genes were knocked out (25) were provided by Dr Bert Vogelstein (The Johns Hopkins University). All cell lines were maintained in McCoy’s 5-A media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (both from Invitrogen). For drug treatments, 5-aza-2′-deoxycytidine (5-azaCdC) was added to a final concentration of 5 μM every 24 h. Trichostatin A (TSA) treatments were performed for 24 h using a concentration of 100 nM. All drugs were purchased from Sigma.

Tumor specimens

Fresh-frozen breast tumors were obtained from the University of Florida Shands Cancer Center Molecular Tissue Bank. All specimens and pertinent patient information were treated in accordance with policies of the Institutional Review Board of the University of Florida Health Sciences Center. Tumors analyzed in this study were examined by a surgical pathologist and identified as invasive breast adenocarcinoma (Stages II or III). All patients were negative for hormone receptor status.

Reverse-transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was used in first-strand cDNA synthesis reactions using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers (GE Healthcare). WIF1 expression was subsequently analyzed by PCR. WIF1-specific primers are (F) 5′-CCG AAA TGG AGG CTT TA-3′ and (R) 5′-GAC CTC CGC CCG CAA-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a control for RNA integrity and was amplified using primers (F) 5′-CTG ACC CAC CAA CTG AG-3′ and (R) 5′-AGG TCC ACC ACT GAC AGC TT-3′. PCR thermocycling conditions were: 9 min, 94 °C; 1 cycle; 94 °C, 30 s; 59 °C, 1 min; 72 °C, 1 min; 32–35 cycles for WIF1. PCR conditions for GAPDH were the same as for WIF1 except that an annealing temperature of 58 °C was used and PCR was conducted for 20 cycles.

A PCR reaction substituting dH2O for cDNA was conducted as a negative control.

Bisulfite genomic sequencing (BGS) and MSP

BGS was performed as described previously (26) using PCR primers designed to amplify a 500 bp segment of the WIF1 promoter. Primer sequences are (F) 5′-TTA TTA GTA TTA TTT AGT GTA TTT T-3′ and (R) 5′-ACC TAA ATA CAA AAA AAC CTA-3′ and were used under the following thermocycling conditions: 9 min, 94 °C, 1 cycle; 94 °C, 30 s; 50 °C, 1 min; 72 °C, 1 min for 35 cycles and using Taq Gold DNA Polymerase (Applied Biosystems). Following PCR, products were cloned using the TOPO-TA cloning kit (Invitrogen), and recombinants were identified by restriction analysis and subsequently sequenced at the University of Florida Center for Mammalian Genetics DNA sequencing facility using a vector-encoded primer (M13-Forw).

WIF1 MSP primers are as follows: unmethylated (U) allele-specific primers (F) 5′-TGGT ATT TAG GTT GGG AGG TGA TGT-3′ and (R) 5′-AAC TTC CAC CCA CAA TAC CAA-3′, methylated (M) allele-specific primers (F) 5′-ATT TAG GTG GGG AGG CGA CGC-3′ and (R) 5′-GAC TTC CAC CCG CCA TAC CAA-3′. PCR conditions for MSP are 95 °C, 15 min, 1 cycle; 94 °C, 30 s; 65 °C (M primers) or 56 °C (U primers), 30 s; 72 °C, 45 s, 40 cycles. PCR reactions were resolved on a 2% agarose gel.

Results

WIF1 downregulation and promoter hypermethylation in breast tumor cell lines

To initially examine if the WIF1 gene is targeted for epigenetic silencing in breast cancer, we cultured a panel of four breast tumor cell lines and used in RT–PCR reactions using WIF1-specific primers (Figure 1A). We observed in all four lines (MCF-7, MDA-MB-231, MDA-MB-468 and BT549) that cells cultured in the presence of 5 μM 5-azaCdC display elevated levels of WIF1 transcripts, suggesting that genome methylation was repressing WIF1 expression in these cells. To directly test WIF1 promoter methylation, we designed a set of PCR primers for use in MSP assays (27). When used in PCR reactions containing normal human sperm DNA (generally highly hypomethylated) pre-incubated in either the presence or absence of SssI (CpG) methylase and S-adenosyl-L-methionine prior to bisulfite modification, results using designed methylated-specific (M) and unmethylated-specific (U) primers indicate that these MSP primers amplify DNA in a manner that clearly ascertain the methylation status of the WIF1 promoter (Figure 1B). Sequence analysis confirmed that this amplicon corresponds to the targeted region of the WIF1 promoter (data not shown). When MSP analysis was used on bisulfite-modified genomic DNA harvested from the panel of breast tumor cell lines, we observed amplification with the methylated-specific primer set in all four lines, indicating CpG methylation within the WIF1 promoter (Figure 1C). Further, genomic DNA harvested from cells cultured with 5-azaCdC display either complete, or markedly increased, methylation of the WIF1 promoter as judged by increases in MSP amplification with unmethylated-specific primers and decreases in amplification with methylation-specific primers. MCF-7 cells, which displayed low levels of WIF1 transcription before 5-azaCdC treatment, also displayed amplification with the unmethylated DNA-specific primers. Two of the cell lines (MDA-MB-468 and BT549) showed a lack of WIF1 expression but some amplification of unmethylated alleles by MSP (Figure 1A and C). This is most probably due to differences in sensitivity of the RT–PCR and the MSP reactions. Increasing the number of PCR cycles for RT–PCR does reveal low-level expression of WIF1 in these cell lines (data not shown).
We next examined WIF1 expression in five additional breast cancer cell lines. RT–PCR analysis showed that MDA-MB-435, YYC-B1, ZR75-1 and SK-BR3 cells display diminished or a complete absence of expression of WIF1 when compared with normal human breast tissue from an individual without cancer (Figure 2A). Further, MSP analysis indicated that each of these cell lines contains detectable levels of methylated WIF1 promoter. In contrast, T47D cells, which express WIF1 at levels comparable with the normal breast, did not contain detectable methylated WIF1 promoter (Figure 2B).

As an independent means of assessing WIF1 promoter methylation, bisulfite-modified genomic DNA from several breast tumor lines was analyzed by sequencing a 500 bp region of the promoter following PCR with primers that amplify the WIF1 promoter in a methylation-independent manner. Specifically, PCR primers were designed for regions of the WIF1 promoter devoid of CpG dinucleotides; thus, amplification proceeds in a manner unbiased by promoter methylation status. Amplicons were subsequently subcloned, and the recombinants were identified and subjected to automated DNA sequencing. Resulting sequences were compared with non-modified WIF1 promoter sequence and the methylation status of the 40 CpG dinucleotides within this amplicon was determined by characteristic chemical changes associated with cytosines existing in either a methylated or an unmethylated state prior to bisulfite treatment (28). This BGS analysis was conducted on the MCF-7, MDA-MB-231, MDA-MB-468 and BT549 breast tumor cell lines (Figure 2C). In ZR75-1, MDA-MB-231, YYC-B1 and BT549 cells before (−) and after (+) 5-azadC treatment was bisulfite-modified as described in Materials and methods. Subsequently, MSP analysis was conducted on these samples using primers and conditions outlined in Materials and methods and validated in part B.

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WIF1 downregulation and promoter methylation in primary breast tumors

To determine if epigenetic silencing of the WIF1 gene occurs in primary breast tumors, we obtained a panel of four surgically removed, snap-frozen breast adenocarcinoma samples and adjacent normal breast tissue from the same patient. Breast tumors and normal tissues were obtained from the University of Florida Shands Cancer Center Molecular Tissue Bank according to Institutional Review Board-approved procedures.
We extracted total RNA from these tissues and subsequently performed RT–PCR to examine WIF1 transcript abundance. In this panel of matched tumor and normal samples, we observed detectable WIF1 expression in all normal breast tissue samples (Figure 3A). However, WIF1 expression was not detected in three of the matched tumors (BrCa-11, 12, and 13) while tumor BrCa-14 showed expression of this transcript at levels consistent with matched normal tissue. We also extracted genomic DNA from these tissue samples, subsequently bisulfite-modified this material and conducted MSP analysis. In concordance with RT–PCR results, the WIF1 promoter was found to be methylated in tumors BrCa-11, 12 and 13, and not detectable in tumor BrCa-14. Of note, MSP revealed that these tumors display methylation of the WIF1 promoter. Conversely, tumors BrCa-2, 22 and 24 do not display this aberrant event. Collectively, analysis of 24 primary breast tumors determined that 16 (67%) display aberrant methylation of the WIF1 promoter as judged by MSP, indicating that this is a common event in breast malignancies.

To independently confirm our MSP results and determine how densely the WIF1 promoter is methylated in primary breast tumors, we subjected three tumors to BGS analysis. Specifically, we analyzed the methylation-positive tumors BrCa-6 and 20, and the methylation-negative tumor BrCa-2. We observed hypermethylation of the majority of the CpG dinucleotides within the amplified 500 bp region of the WIF1 promoter in tumors BrCa-6 and 20 (90% methylation across all CpG sites and all clones for both tumors; Figure 4B). In BrCa-2, only a low-level of CpG methylation was observed (7% methylation). BGS analysis of the WIF1 promoter from normal breast tissue (from an individual without cancer) shows that this region is essentially methylation-free (Figure 4B) and

Fig. 2. WIF1 downregulation and promoter hypermethylation are frequent events in breast tumor cell lines. (A) RT–PCR analysis of WIF1 (top) and GAPDH (bottom) expression was conducted on the breast tumor cell lines MDA-MB-435, YYC-B1, T47D, ZR75-1 and SK-BR3 and one normal breast tissue sample from an individual without cancer. (B) MSP analysis was conducted on bisulfite-modified genomic DNA harvested from MDA-MB-435, YYC-B1, T47D, ZR75-1 and SK-BR3 cells. (C) BGS was conducted on MCF-7, ZR75-1, MDA-MD-231, YYC-B1 and BT549 breast tumor cell lines. Tick marks along the horizontal line at the top represent the location of CpG sites within the region of the WIF1 promoter analyzed [numbering is relative to the transcription start site defined using NCBI Map Viewer (www.ncbi.nlm.nih.gov/mapview)]. Each row of circles represents the sequence analysis of a single clone. Closed circles indicate that the CpG site is methylated; open circles indicate that the CpG site is unmethylated. The percent methylation across all CpG sites and all clones is shown in parenthesis at the left. The horizontal bar with asterisks indicates the location of a putative Sp1 binding site.
further supports the notion that the DNA methylation we observe in the tumor samples results from the disease and is not part of the normal biology of breast tissue. The results of this experiment clearly indicate that large regions of the \textit{WIF1} promoter are subject to dense methylation in primary breast tumors consistent with epigenetic silencing of this gene during breast tumorigenesis.

\textbf{DNMT1 and DNMT3B cooperate to methylate the \textit{WIF1} promoter}

Increased expression of one or more of the DNA methyltransferases (DNMTs) has been reported to occur in breast (31) as well as other tumor types (32) and probably contributes to some degree to the aberrant hypermethylation of the \textit{WIF1} promoter we have observed in breast tumors. To assess which of the DNMTs may mediate this aberrant methylation, we employed a model cell line system, namely the HCT116 colon cancer cell line and its isogenic derivatives in which the \textit{DNMT1}, \textit{DNMT3B} and \textit{DNMT1} and \textit{DNMT3B} genes have been genetically disrupted (KO, knockout) (33). We found that untreated parental HCT116 cells, like many of the breast cancer cell lines and tumors, demonstrate a complete lack of \textit{WIF1} expression that is upregulated following 5 \text{mM} 5\text{-azadC} treatment (Figure 5A). Furthermore, BGS analysis determined that this cell line contains a hypermethylated \textit{WIF1} promoter (Figure 5B), supporting our view that these cells are, in general, a valid model for studying methylation of the \textit{WIF1} promoter in cell lines and tumors. Upon disruption of the \textit{DNMT1} gene, and to a lesser extent the \textit{DNMT3B} gene, weak re-expression of \textit{WIF1} was detected (weak expression is observable in the \textit{DNMT3B}-deficient cells upon additional PCR cycles; data not shown). In contrast, disruption of both DNMTs (DKO, double knockout), treatment of cells with 5-azadC alone, or a combination of 5-azadC and the histone
deacetylase inhibitor, TSA, resulted in robust re-expression of the WIF1 gene (Figure 5A). DNA methylation was clearly the dominant silencing mechanism since TSA treatment alone did not mediate re-expression of WIF1. BGS DNA methylation analysis of each of the cell lines and drug treatments was highly consistent with the expression data (Figure 5B). Interestingly, the pattern of DNA methylation in the HCT116 DNMT1 KO cells was most similar to that of the 5-azadC treated cells, in that the demethylation was largely an all-or-none event (i.e. all CpG sites demethylated on a DNA molecule or none changed from the parental pattern). In contrast, the HCT116 DNMT3B KO cells displayed a more limited and random pattern of demethylation across most of the region analyzed. The WIF1 promoter in DKO cells was almost totally hypomethylated and 5-azadC-treated HCT116 cells displayed a significant fraction of completely hypomethylated clones. Collectively, these results indicate that DNMT1 and DNMT3B work in a cooperative fashion to methylate the WIF1 promoter.

Discussion

Here we demonstrate, using both breast tumor cell lines and primary human breast tumor samples, that the WIF1 gene is subject to DNA hypermethylation-mediated downregulation in this tumor type. WIF1 expression was highly correlated with promoter DNA methylation levels as monitored by both MSP and BGS. BGS analysis indicated that DNA methylation spanned a large region of the WIF1 promoter, with the exception of a small area in the upstream region. Interestingly, this region, which was hypomethylated in all cell lines, primary tumors and the HCT116 colon cancer cell line, contained a consensus Sp1 binding site. Other studies have shown that the binding of Sp1 may be able to block regions of the genome from de novo methylation (29) although it remains possible that other DNA binding proteins or aspects of chromatin structure may mediate the hypomethylation of this small region. Lastly, we used the HCT116 cell line and its DNMT-KO derivatives to demonstrate that the enzymatic activity of both DNMT1 and DNMT3B contribute to aberrant WIF1 promoter DNA methylation.

As a result of this work, we have established the WIF1 gene as a target for epigenetic silencing in breast adenocarcinoma. Similarly, numerous genes have been characterized in a diverse array of tumor types as subject to silencing through aberrant CpG methylation within their proximal promoter regions (18,34). Breast cancer is certainly no exception as many bona fide tumor suppressors have been found to undergo...
Epigenetic silencing of WIF1 in breast cancer

Studies using the HCT116 parental and DNMT-KO derivatives revealed that both DNMT1 and DNMT3B must be inactivated in order to achieve efficient WIF1 promoter demethylation and robust re-expression of the gene. We observed low-level re-expression of WIF1 in the DNMT1 KO cells, and consistent with this we found a 21% reduction in DNA methylation levels by BGS. This is in contrast to the 5-azadC-treated and DKO (DNMT1 + DNMT3B) HCT116 cells, where high-level WIF1 re-expression and 34 and 90% reductions in methylation levels, respectively, were observed. Interestingly, the pattern of demethylation in the DKO, 5-azadC-treated and DNMT1 KO cells was similar in that the demethylation was largely all-or-none on an individual DNA strand. This is probably due to DNMT1 operating at replication foci in a processive manner (46). DNMT3B KO HCT116 cells displayed only a low level of DNA demethylation and very limited re-expression of WIF1 (visible with a higher number of PCR cycles than used in Figure 5A, not shown). The limited demethylation in the DNMT3B KO cells was, however, more random and no completely demethylated strands were observed. Our findings that both DNMT1 and DNMT3B are needed to mediate full WIF1 promoter hypermethylation is consistent with recent findings that the maintenance function of DNMT1 is less efficient than previously thought, and that continued de novo activity from DNMT3B is probably necessary to maintain the full complement of cellular DNA methylation patterns (47,48). Our results also indicate that any therapy aimed at pharmacologically reactivating expression of WIF1 in tumors would have to target both DNMTs in order to be effective.

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Conflict of Interest Statement: None declared.

References


