Epigenetic Regulation of WTH3 in Primary and Cultured Drug-Resistant Breast Cancer Cells

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Abstract

Previous studies showed that the WTH3 gene functioned as a negative regulator during multidrug resistance (MDR) development in vitro. To understand whether this gene is also involved in clinical drug resistance, hypermethylation at its promoter region observed in cultured MDR MCF7/AdrR cells was examined in primary drug-resistant breast cancer epithelial cells isolated from effusions of breast cancer patients. The results showed that this event also occurred in drug-resistant breast cancer epithelial cells and a newly induced drug-resistant cell line, MCF7/inR. Interestingly, we found that a CpG (CpG 23) that was close to the TATA-like box was constantly methylated in the WTH3 promoter of drug-resistant breast cancer epithelial and cultured MDR cells. Mutagenic study suggested that this CpG site had a functional effect on promoter activity. We also discovered that MCF7/AdrR cells treated with trichostatin A, a histone deacetylase inhibitor, exhibited higher WTH3, but lower MDR1, expression. A reverse correlation between WTH3 and MDR1 gene expression was also observed in MCF7/AdrR, and its non-MDR parental cell line, MCF7/WT. This result indicated that both DNA methylation and histone deacetylation could act in concert to inhibit WTH3 and consequently stimulate MDR1 expression. This hypothesis was supported by data obtained from introducing the WTH3 transgene into MDR cell lines, which reduced endogenous MDR1 expression. Therefore, our studies suggested that the behavior of WTH3 in primary drug-resistant breast cancer epithelial cells was similar to that in a model system where epigenetic regulation of the WTH3 gene was linked to the MDR phenotype. (Cancer Res 2005; 65(21): 10024-31)

Introduction

Developing multidrug resistance (MDR) during clinical chemotherapy is a fatal occurrence for cancer patients. Several genes, such as MDR1 and MRP, have been identified as being directly involved in MDR, and their detailed biological pathways are currently under investigation (1–6). It is common knowledge that multiple elements and mechanisms are involved in MDR development. In the past, we used the methylation-sensitive representation difference analysis technique to study DNA hypermethylation events in a human MDR breast cancer cell line, MCF7/AdrR, compared with its parental line, MCF7/WT. This led to the discovery of the WTH3 gene (7), a homologue of the Rab6 and Rab6c genes that belong to the ras superfamily (8–14). Similar to its homologues, WTH3 binds to GTP. However, differing from the Rab6 proteins, it contains an elongated 46-amino-acid COOH terminus with no cysteine for posttranslational modification (geranylgeranlyation). WTH3 is primarily located in the cytosol instead of the Golgi apparatus, where Rab6 and Rab6c reside. These findings indicate that WTH3 could have diverse biological use compared with its homologues. Previous studies showed that WTH3 plays a negative role in MDR development in vitro because it was down-regulated in MDR cell lines and its introduction into those lines reversed the MDR phenotype to various anticancer drugs (7). Earlier research also identified several mechanisms that could be involved in the down-regulation of WTH3 in MCF7/AdrR cells, such as epigenetic modification (the distal WTH3 promoter region was heavily methylated in MCF7/AdrR cells but not methylated in MCF7/WT cells; ref. 15), and trans-regulator modulation (transcriptional factors associated with a repeat motif, RR28, were altered in MCF7/AdrR relative to that in MCF7/WT cells; ref. 15). These observations indicated that the WTH3 gene played an important role in cells with the MDR phenotype that was artificially induced. A recent study showed that the copy number of WTH3 varied in the normal population; among 20 healthy individuals tested, one exhibited a hemizygous deletion in chromosome 2q14 containing the WTH3 gene, indicating a possible relationship between its susceptibility and health problems (16). On the other hand, another investigation implied that its micro-RNA was able to regulate protein production of target transcripts by yet to be determined mechanisms (17). Apparently, others and we provided evidence that indicated that WTH3 could possess important biological functions. The focus of this study was to explore potential epigenetic roles involved in the regulation of WTH3 in primary breast cancer epithelial cells with drug-resistant phenotypes and in a newly induced drug-resistant cell line, MCF7/inR. In addition, the possible relationship between WTH3 and the MDR1 gene at the transcription level was examined.

Drug-resistant breast cancer epithelial cells were selected from primary breast cancer epithelial cells that were prepared from the effusions of cancer patients, and the drug-resistant cell line, MCF7/inR, was established. The methylation status of the distal WTH3 promoter region in drug-resistant breast cancer epithelial and MCF7/inR cells was analyzed compared with MCF7/AdrR versus MCF7/WT cells. To determine the influence of the most frequently methylated CpGs on promoter activity, site-directed mutagenesis and the luciferase reporter system were used. Because histone deacetylase, as a corepressor, is often physically related to various CpG methyl-binding proteins (18–20), MCF7/AdrR cells were treated with trichostatin A to assess if this procedure could boost WTH3 gene expression in the host cells. Due to a reverse correlation between WTH3 and MDR1 gene expression observed in paired MCF7 cells and trichostatin A–treated MCF7/AdrR cells,
the possibility that the WTH3 gene regulates MDR1 gene expression was examined by introducing it into MCF7/AdrR and MES-SA/Dx5 (a MDR human uterine sarcoma line; ref. 7). Taken together, our results confirmed that the behavior of the WTH3 gene behavior in drug-resistant breast cancer epithelial cells, with regard to DNA methylation, was similar to that observed in the working model system (7, 15) and could also be involved in the transcription regulation pathway of the MDR1 gene. Therefore, WTH3 functioned as a negative regulator during the onset of MDR in both in vitro and primary drug-resistant breast cancer epithelial cells.

Materials and Methods

Cell lines, multidrug resistance induction, and trichostatin A treatment. The MDR cell line, MCF7/Adr, and its sensitive parental cell line, MCF7/WT, as well as MES-SA/Dx5 (American Type Culture Collection, Rockville, MD), were grown under the conditions described (1, 7). The MCF7/inR cell line was established by inducing the MDR phenotype in MCF7/WT cells when they were treated with gradually increasing concentrations of doxorubicin. The initial concentration of doxorubicin for the induction was 20 nmol/L. Established surviving cells were then treated with a higher dose of the drug. This cycle was repeated for increased drug concentrations of 40, 60, 80, 100, 120, 150, 200, and 250 nmol/L. The IC50 value for MCF7/inR was ~0.5 μmol/L as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (7, 11), which was 25 times higher than the original IC50 (20 nmol/L). Duration for the induction was ~8 months. The MCF7/AdrR cells were incubated with different trichostatin A concentrations of 0, 100, 300, and 500 ng/mL. After 48 hours, the cells were collected for RNA preparations.

Primary breast cancer epithelial cells. Primary breast cancer epithelial cells were prepared following the previous protocol (21). Cancer epithelial cells were isolated from the effusions obtained from breast cancer patients who were at the metastasis stage. Briefly, breast cancer epithelial cells were isolated using a differential centrifugation method and then cultured in RPMI medium (Invitrogen, Carlsbad, CA) with 20% FCS (21). Usually, breast cancer epithelial cells adhered in 3 to 5 days. Nonadherent cells were discarded. Cells were monitored regularly for their morphology and epithelial character. When adequate growth appeared, the cells were trypsinized and passed at 1:3 ratios. The criteria used to ascertain culture success were as follows: (a) attachment and growth of phenotypic epithelial-like cells, mostly with polyploidy; (b) continued growth of up to 6 weeks with an approximate doubling time of 3 to 6 days, with no evidence of fibroblastic infiltration; (c) expression of epithelial cell biomarkers, cytokeratin-19 (K19), and epithelial glycoprotein-2 (EGP-2) (EGP-2; refs. 21–23). Generally, in ~4 to 6 weeks, breast cancer epithelial cells were sustained, and cells that reached 70% to 90% purity were used to select drug-resistant breast cancer epithelial cells. The drug resistance phenotype was defined by measuring their MDR1 reexpression by reverse transcription-PCR (RT-PCR) and carrying out drug-induced cell death assay (MTT assay) to evaluate IC50 to doxorubicin.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. MTT assays were carried out as described (7, 11). Briefly, 3 × 104 cells/well were seeded in a 96-well plate and grown overnight. The cells were treated with serial concentrations of doxorubicin (10 nmol/L to 10 μmol/L). In 4 days, they were then treated with MTT that stains living cells. IC50 was quantitatively measured at 595 nm in a microplate spectrophotometer (Bio-Rad, Hercules, CA).

Reverse transcription-PCR and semiquantitative reverse transcription-PCR. Total RNA was prepared from primary cells and cell lines using the Pure RNA Isolation kit (Roche, Indianapolis, IN) following the instructions of the manufacturer. Expression of the epithelial biomarkers (K19 and EGP-2) in drug-resistant breast cancer epithelial cells was examined by RT-PCR. The sense and antisense primers for K19 were 5′-GCTGGCCTACCTGAAAGAAG-3′ and 5′-CCCGTGGTACTCCTGATC-3′, whereas the sense and antisense primers for EGP-2 were 5′-GAAACATGTGCGTCTTATGA-3′ and 5′-TGAGAATTGCTGCTTCTTTT-3′. To evaluate the MDR1 and WTH3 gene expression levels in drug-resistant breast cancer epithelial cells and established cell lines, semiquantitative RT-PCR was done using the Titan One Tube RT-PCR System (Roche) as previously described (7, 15), whereas the MCF7/AdrR and MCF7/WT RNAs were used as controls. The sense and antisense primers for MDR1 were 5′-CTCTATCA-TTGCAATAGACCG-3′ and 5′-GTCCAACACTGCTGTCCTGTA-3′. The sense and antisense primers for WTH3 were 5′-GATGGAACAACTCGGGGCTT-3′ and 5′-GCTCTACAGCTGGAAGAGCC-5′ (7). In addition, the sense and antisense primers for β-actin (internal control) were 5′-GACGACATGGAGAGTATCGTGG-3′ and 5′-ATCAGGGCACTGTAAGCTCCTCT-3′ (7, 11). The length of the MDR1, WTH3, and β-actin PCR product was 167, 341, and 495 bp, respectively. Thirty cycles of PCR were done to obtain a linear quantitative correlation between WTH3 and MDR1 fragments. Detailed information about the PCR assay and quantification of PCR products were previously described (7, 11).

Bisulfite genomic DNA sequencing. Genomic DNAs were isolated from drug-resistant breast cancer epithelial cells, paired MCF7, and MCF7/inR cell lines by the Genomic Purification kit (Qiagen, Valencia, CA). Bisulfite genomic DNA sequencing assays were done as previously described (15). Briefly, the purified genomic DNAs were treated with bisulfite using the CpG Genomic DNA Modification kit (Chemicon, Temecula, CA) following the protocol of the manufacturer. The treated DNAs were used as templates for PCR amplification. The primers for amplifying the distal WTH3 promoter region were 5′-TTTCTTTCAGTGTAATTTTGTTTTA-3′ and 5′-CCAAAAATATCCTTACCTCCCACAAC-3′, respectively. The amplified sequence was from ~568 to ~272. The PCR products were purified and cloned into pGEM-T Easy (Promega, Madison, WI) and transformed into DH5α cells. Ten to 15 plasmids with inserts representing each genome were randomly picked and sequenced (Genewiz, North Brunswick, NJ).

Site-directed mutagenesis and luciferase assay. Bisulfite genomic DNA sequencing assays indicated that CpG 23 was the most popular site for hypermethylation in most of the cells examined, which included drug-resistant breast cancer epithelial cells and MCF7/AdrR. To test whether this particular CpG could play a regulatory role in WTH3 gene expression, its C was replaced by a T. The substitution was accomplished by using the Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), where pGL/WTH3P containing the original promoter was used as the template. The resulting construct, pGL/WTH3P/C-T, was used to evaluate the influence of the mutation on promoter activity. The LipofectAMINE Plus Transfection kit was used for the transient transfection assays. The host cell lines for determining the activity of the wild-type and mutated WTH3 promoter were parental MCF7 cell lines. In brief, 0.2 μg of the empty vector (pGL3, negative control), pGL/WTH3P, or the pGL/WTH3P/C-T construct were transfected along with 0.2 μg of pCMV/β-galactosidase (transfection efficiency control) when the cells (seeded onto 24-well plates) reached 50% to 70% confluence. After 24 hours of transfection, luciferase activity was measured using the Steady-Glo Luciferase Assay System (Promega) according to the instructions of the manufacturer. β-galactosidase activity was evaluated using the Beta-Glo Assay System (Promega). The luciferase activity of the transfectants was compared after normalizing their β-galactosidase activity and protein concentrations (15). The experiments were done thrice.

Transient transfection. To test whether exogenous WTH3 gene expression could repress endogenous MDR1 gene expression in MCF7/AdrR and MES-SA/Dx5 cells, the WTH3 gene was cloned into the mammalian expression vector, pcDNA3.1, to obtain pcDNA3.1/WTH3 (7). This plasmid, the empty vector (negative control), and pcDNA3.1/β-galactosidase were temporarily transfected into the host cell lines as previously described (7, 15). After 30 hours of transfection, the cells were harvested for total RNA preparation. MDR1 expression was determined by semiquantitative RT-PCR, where β-actin served as a quantitative control. The quantifications of PCR products were done as previously described (7, 11).

Results

Preparation of primary breast cancer epithelial cells. Because of the size difference between malignant epithelial cells
and lymphocytes, the first step in separating these two cell types was to carry out low-speed centrifugation. The enriched epithelia were then transferred to a 25 cm² flask where adherent cells were retained, whereas those that floated were discarded. The content of breast cancer epithelial cells in the effusions varied—some were contaminated with a relatively high numbers of fibroblasts, whereas others were rich in breast cancer epithelial cells. Due to the growth potential and relative insensitivity to trypsin treatment of malignant epithelial cells, normal fibroblasts and other cells, such as lymphocytes, were further reduced during a short period of cell culturing (∼4-6 weeks). The resulting breast cancer epithelial cells showed typical epithelial morphology (Fig. 1A) and their purity varied from 70% to 95%. In addition, some of the cells expressed malignant features, such as enlarged cell size and tumors, and K19, a primitive keratin expressed in all epithelial cells.

MDR1 was expressed in drug-resistant breast cancer epithelial cells. Usually, MDR1 gene expression is not detectable in non-MDR cells (7), whereas its reexpression is considered an indicator for drug resistance development in vitro and in vivo. Thus, reexpression of MDR1 in breast cancer epithelial cells was analyzed by carrying out semiquantitative RT-PCR where MCF7/AdrR and MCF7/WT cells served as positive and negative controls, respectively. β-actin gene expression was used as a quantitative control. The results showed that the MDR1 gene was activated in most samples tested. The expression level was highest in MCF7/AdrR but not detectable in MCF7/WT (Fig. 2A). Therefore, breast cancer epithelial cells expressing MDR1 were determined to be drug resistant. As a result, EK4, EK5, EK151, EK237, EK245, EK259, EK596, and EK628, which represented eight patients, were considered drug-resistant breast cancer epithelial cells. To further verify the results, IC₅₀ values of some drug-resistant breast cancer epithelial cells to doxorubicin were measured.

IC₅₀ values were increased in drug-resistant breast cancer epithelial cells. IC₅₀ values of four drug-resistant breast cancer epithelial cell samples (EK4, EK5, EK151, and EK259), which contained 90% to 95% epithelial cells, were estimated by MTT assays when they were treated with different concentrations of doxorubicin. The non-MDR cell line, MCF7/WT, was used as a negative control (15). The results showed that the IC₅₀ values of the four samples ranged from 0.8 to 2.8 µmol/L, which was ~40 to 140 times higher than MCF7/WT whose IC₅₀ was ~20 nmol/L (Fig. 2B). Therefore, the drug-resistant feature of this group of breast cancer epithelial cells was further verified.

WTH3 expression was relatively low in drug-resistant breast cancer epithelial cells. Prior studies discovered that the WTH3 gene was down-regulated in MCF7/AdrR cells. To evaluate the expression level of WTH3 in drug-resistant breast cancer epithelial cells, semiquantitative RT-PCR was done using RNA prepared from eight drug-resistant breast cancer epithelial cells and paired MCF7 cells (quantitative controls). β-actin expression served as the endogenous control. The results showed that WTH3 expression was lower in most drug-resistant breast cancer epithelial cell samples than that in MCF7/WT cells (Fig. 2A). Because MDR1 transcripts reappeared in drug-resistant breast cancer epithelial cells, we investigated the possibility of a proportional reverse correlation between WTH3 and MDR1 expression in those cells. We found that this correlation was present in EK4, EK151, EK237, EK259, EK596, and EK628 cells, but was not observed in EK5 and EK245 cells. Consequently, no general conclusion could be reached concerning a proportional reverse correlation between WTH3 and MDR1 expression in the patient’s cells that were studied (Fig. 2B). Because earlier investigations showed that DNA methylation in the distal WTH3 promoter region contributed to the down-regulation of the gene in MCF7/AdrR cells, we were interested in determining if it is also a factor for reduced expression in drug-resistant breast cancer epithelial cells. To answer this question, bisulfite genomic DNA sequencing was done to analyze the distal WTH3 promoter region in some drug-resistant breast cancer epithelial cells.

Methylation was found in the distal WTH3 promoter region of drug-resistant breast cancer epithelial, MCF7/inR, and MCF7/AdrR cells. Bisulfite genomic sequencing assays were done in the past to examine the methylation status of CpG islands in the WTH3 promoter of MCF7/AdrR versus MCF7/WT cells. Detailed information about the promoter and CpG island was given in our previous publication (15). It was found that 22 CpGs in the distal WTH3 promoter region were highly methylated in MCF7/AdrR, but none were methylated in MCF7/WT cells (15). To see if the same phenomenon could also be observed in drug-resistant breast cancer epithelial cells, genomic DNAs isolated from four drug-resistant breast cancer epithelial cell samples (EK151, EK237, EK245, and EK259) were treated with bisulfite. The treated DNAs were used as templates for PCR amplification. The amplified sequence (from −568 to −272) was extended to Cpg 23, a site that was closest to the TATA box and not previously analyzed in paired MCF7 cells (15). The PCR products were cloned into the pGEM-T vector and >10 inserts, representing each sample, were randomly picked for sequencing. The results showed that DNA methylation was observed in three of four samples (75%). Among 12 sequences derived from EK151, five contained methylated CpGs (a single

Figure 1. A, morphology of EK259 cells compared with MCF7/AdrR cells. B, RT-PCR results for EGP-2 and K19 gene expression in drug-resistant breast cancer epithelial cell sample was listed on top. Line M, molecular weight markers.
methylation at CpG 23 was observed in four colonies, whereas multiple methylations at CpGs 7, 8, and 10 to 23 were seen in the fifth colony, which also included a 3 bp deletion that eliminated CpG 17). In 11 sequences obtained from EK237, five contained methylated CpGs (four had a single methylation at CpG 23 and one contained two methylated sites at CpGs 20 and 23). Five of 13 sequences derived from EK245 showed single methylation at CpG 23. However, methylation was not detected in all clones obtained from EK259 (Fig. 3 A and B). The methylation status of CpG 23 in 13 colonies derived from MCF7/AdrR and 15 from MCF7/WT was also examined and served as a control. Consequently, we found that it was an all or none outcome, with the former cell line being methylated whereas the latter was not. To further confirm that the hypermethylation event was correlated with the MDR phenotype, a freshly induced MDR cell line, MCF7/inR, was developed using MCF7/WT cells. Methylation analysis of 14 sequences showed that five colonies contained methyl CpGs. Among them, two were at position 23 whereas the other three were at sites 22, 13, and 10. Thus, DNA methylation was induced during MDR development. Because CpG 23 was the most frequently methylated site, it might play an important role in down-regulating gene promoter activity. To test this hypothesis, a corresponding mutation was created and its influence on the promoter was analyzed using the luciferase reporter system.

Figure 2. A, semiquantitative RT-PCR for MDR1 and WTH3 gene expressions in drug-resistant breast cancer epithelial cells compared with that in MCF7/AdrR and MCF7/WT cells. B, quantitative information of MDR1 and WTH3 gene expression in drug-resistant breast cancer epithelial cells as well as their IC50 values.

Table 1. Methylation status of the distal WTH3 promoter region in drug-resistant breast cancer epithelial and MCF7/inR cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>EK151</th>
<th>EK237</th>
<th>EK245</th>
<th>EK259</th>
<th>MCF7/AdrR</th>
<th>MCF7/WT</th>
<th>MCF7/inR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DNA fragments sequenced</td>
<td>12</td>
<td>11</td>
<td>13</td>
<td>10</td>
<td>13</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>DNA fragments methylated</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Percent methylated</td>
<td>42</td>
<td>46</td>
<td>38</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Position of methylated CpGs</td>
<td>(4)* 23**</td>
<td>(1) 7,8</td>
<td>(1) 20,23</td>
<td>(5) 23</td>
<td>(13)23</td>
<td>–</td>
<td>(2)*23</td>
</tr>
</tbody>
</table>

* Number of fragments that were methylated at a given CpG site.
** Position of the methylated CpG.

Figure 3. A, detailed information of the distal WTH3 promoter region sequence. The first half of the CpG island was marked in bold type, TATA- and CCAAT-like boxes were delineated by boxes. The CpG sites were numbered. ●, methylated CpGs; ○, nonmethylated CpGs. The minideletion in EK151 was underlined and the bisulfite PCR primer positions are presented in italics. The first letter of the start codon is counted as +1. B, methylation status of the distal WTH3 promoter region in drug-resistant breast cancer epithelial and MCF7/inR cells.
CpG 23 had a positive effect on the WTH3 promoter function. To generate a single point mutation at CpG 23, the site-directed mutagenesis technique was used to obtain pGL/WTH3P/C-T from the pGL/WTH3P construct. The plasmid pGL/WTH3P/C-T, pGL/WTH3P (positive control), the empty vector pGL3 (negative control), and pCMV/β-galactosidase were transiently introduced into MCF7/WT and MCF7/AdrR cells. The enzyme activities driven by the mutated and original promoters in those host cell lines were measured and compared with justification of protein concentrations and transfection efficiency. It was discovered that the activity of the mutated promoter in MCF7/WT cells was decreased by ~30% relative to the wild-type promoter (100%), whereas it was reduced 10% in MCF7/AdrR (Fig. 4). Clearly, CpG 23 is a cis-activator with regard to WTH3 gene promoter activity.

Histone deacetylase was involved in regulating WTH3 expression. Discovery of differential DNA methylation in the distal WTH3 promoter region indicated that methyl-binding proteins and histone deacetylase, which is a potential associate to a majority of known methyl-binding proteins (18–20), could be involved in regulating WTH3. It has been well documented that if a methylation repression event is histone deacetylase dependent, a histone deacetylase inhibitor, such as trichostatin A, could erase the negative effect of epigenetic modification on the expression of a given gene. To test whether WTH3 expression was influenced by trichostatin A, MCF7/AdrR cells were treated with different doses of the inhibitor. The WTH3 transcripts were then evaluated by semiquantitative RT-PCR. The results showed that the transcripts of interest were increased compared with the nontreated control and the elevation was trichostatin A dose dependent (Fig. 5A). This finding indicated that histone deacetylase was directly or indirectly involved in regulating the WTH3 gene. In addition, we measured MDR1 gene expression levels to see if there was a reverse correlation between those two genes in the same trichostatin A–treated cell line. We observed that MDR1 and WTH3 expression was proportionally decreased or increased depending on trichostatin A concentration, respectively (Fig. 5A and B). Therefore, WTH3 could play a role in regulating the MDR1 gene. To test this possibility, the WTH3 transgene was introduced into two MDR cells lines to see if it could inhibit MDR1 gene activity.

The WTH3 transgene repressed endogenous MDR1 gene expression. To understand whether overexpression of the WTH3 gene could influence endogenous MDR1 gene expression, pcDNA3.1, pcDNA3.1/WTH3, and pCMV/β-galactosidase were transiently introduced into MCF7/AdrR and MES-SA/Dx5 cells, which expressed the MDR1 gene to different degrees (MCF7/AdrR > MES-SA/Dx5). After 36 hours, the transfectants were collected to prepare total RNA. The MDR1 transcripts in each group of cells were measured by semiquantitative RT-PCR where the β-actin gene served as quantitative control. The results showed that the amount of MDR1 mRNA in the host cell lines containing the WTH3 transgene was reduced compared with the corresponding controls that were transfected with the empty vector (Fig. 6A and B). Densitometry analysis determined that the expression level of the MDR1 gene in MCF7/AdrR or MES-SA/Dx5 was 3.5 or 8 times less than that in the corresponding control (Fig. 6C). This suggests that the WTH3 gene could be a component in the transcriptional regulation pathway of the MDR1 gene.

Discussion

It is well documented that many mechanisms are involved in the development of MDR during chemotherapy (24–28). By concentrating our efforts on understanding this fatal event, we discovered a MDR-related gene, WTH3, via the methylation-sensitive representative difference analysis technique (7, 11, 29). Information about this gene and its relationship to MDR obtained during its characterization using a working model system has been previously reported (7, 15). This includes the discovery of a reverse correlation between WTH3 and MDR1 gene expression, as well as two
regulatory machineries involved in its down-regulation in MDR cells: DNA methylation (an epigenetic machinery that repress gene expression in mammals; refs. 30–34) and transcriptional modulation. To further comprehend the significance of WTH3 in MDR development, its behavior in primary drug-resistant breast cancer epithelial cells and its relationship with the MDRI gene were studied.

First, breast cancer epithelial cells were isolated from the effusions of breast cancer patients and drug-resistant cells were selected from these cells. Second, WTH3 gene expression levels in drug-resistant breast cancer epithelial and paired MCF7 cells were compared. We found lower WTH3 expression in primary drug-resistant breast cancer epithelial cells than in MCF7/WT, but higher compared with MCF7/AdrR. These results were consistent with the strength of their drug resistance (7, 15). However, a proportional inverse correlation between WTH3 and MDRI expression in the primary samples remains to be determined. Third, because the distal WTH3 promoter region was hypermethylated in MCF7/AdrR compared with its parental cell line, bisulfite genomic DNA sequencing assays were done to analyze its methylation status in four drug-resistant breast cancer epithelial cell samples. It was discovered that hypermethylation events occurred in three samples and the concurrent rate among colonies was relatively high [EK151 (42%), EK237 (46%), and EK245 (38%)]. The most methylated colonies contained a single methyl CpG. A few colonies contained two methylated CpGs simultaneously. Only one, derived from EK151, displayed an extensively methylated pattern, which was similar to that in MCF7/AdrR cells. However, we did not find any methylated CpGs in EK259 cells. This result was consistent with the observation that the level of WTH3 expression in EK259 was similar to that in MCF7/WT where the distal WTH3 promoter region was also not methylated and exhibited the lowest IC50 compared with other primary cells. We also noticed that the most frequently methylated site was CpG 23 in drug-resistant breast cancer epithelial cells. Its methylation situation was also analyzed in paired MCF7 cells because it had not been tested earlier. Consequently, we found that CpG 23 was methylated in all the sequences derived from MCF7/AdrR but in none of the sequences obtained from MCF7/WT cells. This result further confirmed the importance of CpG 23. Because drug-resistant breast cancer epithelial cells exhibited lower drug resistance relative to MCF7/AdrR, it is possible that methylation of CpG 23, which was in close proximity to a TATA-like box, was an initial site for epigenetic modification in those cells. To test this possibility, the distal WTH3 promoter region methylation pattern was analyzed in a newly induced MDR cell line, MCF7/inR, which expressed a much weaker MDR phenotype than MCF7/AdrR. We found that 36% of the colonies examined contained a methylated CpG in the distal WTH3 promoter region. Two were located at CpG 23, whereas the other three were situated at different positions. Therefore, the methylation patterns observed in MCF7/inR cells were relatively random compared with that in MCF7/AdrR and drug-resistant breast cancer epithelial cells. The reason for such disparity is currently unclear. However, the limited scope of methylation in MCF7/inR (IC50, 0.5 μmol/L) compared with MCF7/AdrR (IC50 >10 μmol/L) could be explained by their MDR strength. Whether MDR development is accompanied by progressive methylation of the WTH3 promoter remains to be determined. We plan to treat MCF7/inR cells with increasing concentrations of doxorubicin and sequentially collect cell populations during the induction process. By analyzing their methylation status in the distal WTH3 promoter region, we could determine a possible relationship between progressive DNA methylation and MDR development.

Because CpG 23 was most frequently methylated, it seemed to exhibit a regulatory role on gene promoter activity. This hypothesis was tested by carrying out site-directed mutagenesis and luciferase assays. Results showed that the enzyme activity driven by the mutated promoter was significantly decreased in the MCF7/WT host line, which indicated that CpG 23 was a cis-acting element for the promoter. However, the mutation exerted a minor influence on the promoter in MCF7/AdrR cells. This could be due to drug treatment that possibly stimulated epigenetic modification or altered transcriptional factors. Whether this speculation is true remains to be determined. Another positive element, DMR45, that was located at the 3′ end of CpG 23 was identified previously (15), and diverse nuclear protein complexes obtained from MCF7/AdrR and MCF7/WT bound to methylated DMR45 (15). Possible nuclear proteins targeting CpG 23 was also explored by electrophoretic mobility shift assay (data not shown). However, we failed to define a protein complex that bound specifically to this site, although the flanking sequence was attacked by the same proteins existing in both MCF7 cell lines. The negative outcome could be because this particular site was isolated from other CpGs, and, as a result, possessed a low binding affinity for a potential methyl-binding protein. To find a remedy, we applied another strategy to indirectly
test the possible involvement of methyl-binding proteins. Trichostatin A, an inhibitor of histone deacetylase that was a common co-repressor of several known methyl-binding proteins, including MBD1, MBD2, and Mcp2 (19, 35–38), was used to treat MCF7/AdrR cells to determine WT3H gene expression. The result showed that WT3H expression was elevated in cells treated with trichostatin A, which suggested that histone deacetylase, and perhaps known and/or unknown methyl-binding proteins, participated as suppressors for its expression. It is important to also mention that histone modification is another complex factor of epigenetic gene expression regulation. This is because the core components of histatins are histones H2A, H2B, H3, and H4, which are major targets for covalent modifications (acetylation, phosphorylation, methylation, and ubiquitination) by specific chromatin-modifying enzymes (39, 40). The numerous combination patterns of these modifications have been called "the histone code," and many reports indicate a correlation between a defined histone code and particular gene expression states (41, 42). It will be interesting to determine which type(s) of histone modification and what methyl-binding proteins are involved in WT3H gene regulation in MDR cells. Furthermore, MDR1 transcript levels were measured in trichostatin A–treated MCF7/AdrR cells to see if there was a reverse correlation between MDR1 and WT3H gene expression, a constant observation in MCF7/AdrR versus MCF7/WT (15) and MES-SA versus MES-SA/Dx5 cells (data not shown). The findings confirmed that the expression of the two genes went at opposite directions. Based on information supplied by other scientific groups, the MDR1 gene promoter was found to be demethylated in MDR cells (43, 44). As a result, it seems unlikely that this gene was subjected to epigenetic regulation. Therefore, trichostatin A treatment might not directly influence MDR1 gene expression. Instead, trichostatin A–stimulated WT3H gene expression could, in turn, down-regulate MDR1. To explore whether WT3H participates along the transcription regulation pathway of MDR1, it was introduced into two MDR cell lines, MCF7/AdrR and MES-SA/Dx5, both expressing high levels of MDR1. The results showed that the transgene was capable of suppressing endogenous MDR1 gene activity in both cell lines. The degree to which MDR1 expression decreased in MES-SA/Dx5 was greater than that in MCF7/AdrR cells, the cause of which could be attributed to the better transcription efficiency of the former cell line. The detailed mechanisms for such regulation are currently under investigation.

In summary, our present study discovered data supporting the notion that the WT3H functions as a negative regulator for the development of MDR in vitro and in primary drug-resistant breast cancer epithelial cells. Its down-regulation in those cells was through epigenetic modification at the DNA methylation level where histone deacetylase participated. We are currently focusing our efforts on generating detailed information about how methyl-binding protein(s) and corepressors are involved in down-regulation of WT3H in MDR cells. Hopefully, this will help us understand the etiology of MDR development.

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References


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