

Epigenetic profiling of multidrug-resistant human MCF-7 breast adenocarcinoma cells reveals novel hyper- and hypomethylated targets

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Abstract

The successful treatment of cancer requires a clear understanding of multiple interacting factors involved in the development of drug resistance. Presently, two hypotheses, genetic and epigenetic, have been proposed to explain mechanisms of acquired cancer drug resistance. In the present study, we examined the alterations in epigenetic mechanisms in the drug-resistant MCF-7 human breast cancer cells induced by doxorubicin (DOX) and cisplatin (cisDDP), two chemotherapeutic drugs with different modes of action. Despite this difference, both of the drug-resistant cell lines displayed similar pronounced changes in the global epigenetic landscape showing loss of global DNA methylation, loss of histone H4 lysine 20 trimethylation, increased phosphorylation of histone H3 serine 10, and diminished expression of Suv4-20h2 histone methyltransferase compared with parental MCF-7 cells. In addition to global epigenetic changes, the MCF-7/DOX and MCF-7/cisDDP drug-resistant cells are characterized by extensive alterations in region-specific DNA methylation, as indicated by the appearance of the number of differentially methylated DNA genes. A detailed analysis of hypo- and hypermethylated DNA sequences revealed that the acquisition of drug-resistant phenotype of MCF-7 cells to DOX and cisDDP, in addition to specific

alterations induced by a particular drug only, was characterized by three major common mechanisms: dysfunction of genes involved in estrogen metabolism (sulfatase 2 and estrogen receptor α), apoptosis (p73, α -tubulin, BCL2-antagonist of cell death, tissue transglutaminase 2 and forkhead box protein K1), and cell-cell contact (leptin, stromal cell-derived factor receptor 1, activin A receptor E-cadherin) and showed that two opposing hypo- and hypermethylation processes may enhance and complement each other in the disruption of these pathways. These results provided evidence that epigenetic changes are an important feature of cancer cells with acquired drug-resistant phenotype and may be a crucial contributing factor to its development. Finally, deregulation of similar pathways may explain the existence and provide mechanism of cross-resistance of cancer cells to different types of chemotherapeutic agents. [Mol Cancer Ther 2007;6(3):1089–98]

Introduction

The development of cancer cells that are resistant to chemotherapeutic agents is a major clinical obstacle to the successful treatment of cancer, including breast cancer (1, 2). Acquired drug resistance is a multifactorial phenomenon, involving multiple mechanisms (1–3). At present, several major mechanisms involved in the resistance of cancer cells to chemotherapeutic drugs are being considered, including the decreased uptake of water-soluble drugs, which require transporters to enter cells (4); various cellular changes, including alterations in cell cycle and signal transduction pathways, increased repair of DNA damage, reduced apoptosis, and altered metabolism of drugs that diminish the capacity of cytotoxic drugs to kill cancer cell (5–8); increased energy-dependent efflux of hydrophobic drugs (4, 9); and increased DNA tolerance to DNA-damaging drugs through the inactivation of DNA mismatch repair pathways (10). The successful treatment of cancer requires a clear understanding of multiple interacting factors involved in the development of drug resistance (11). Resistance to individual chemotherapeutic agents usually occurs by alterations in the targets for these drugs, but can also develop more broadly to a variety of diverse unrelated antitumor drugs with different chemical structures and different mechanisms of action (11). The existence of this cross-resistance suggests that many chemotherapeutic agents are likely to share some common mechanisms of resistance (12).

Presently, two hypotheses, genetic and epigenetic, have been proposed to explain mechanisms of acquired cancer drug resistance (13–16). "Genetic" is defined as a heritable change in the DNA sequence, and according to this

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mechanism, the occurrence of random drug-induced mutational events leads to the formation of drug-resistant cells from sensitive cells (13, 14). "Epigenetic" refers to the information contained in chromatin rather than in the actual DNA sequence (17) and consists of DNA methylation and histone modifications. According to epigenetic hypothesis, the induction of epigenetic changes results in resistance to cytotoxic drugs (15, 16). The absence of convincing evidence that genetic changes have a role in acquired clinical resistance following anticancer therapy undermines the genetic hypothesis (16). In contrast, a number of studies have indicated substantial alterations of epigenetic mechanisms in drug-resistant cancer cells, including changes in DNA methylation and histone modification patterns. It is well documented that epigenetic dysregulation of multidrug-resistant 1 gene (*MDR1*), characterized by prominent hypomethylation and altered histone modifications at *MDR1* promoter region, directly correlates with *MDR1* up-regulation and is associated with increased resistance of cancer cells, including breast cancer cells, to chemotherapy (15, 18–21). Increased expression of the glutathione *S*-transferase π (*GST* π), *O*⁶-methylguanine DNA methyltransferase (*MGMT*), and breast cancer resistance protein (*BCRP*) genes, which are regulated by cytosine methylation in drug-resistant cancer cells, clearly points toward the epigenetic mechanisms of drug resistance (22–24). In contrast to DNA hypomethylation, a number of recent studies have suggested a direct role of promoter CpG island hypermethylation in epigenetic inactivation of genes in the development of drug resistance (16, 25). For instance, hypermethylation of CpG-islands in promoters of *MLH1* and *WTH3* genes and the associated down-regulation of their gene expression are also involved in drug resistance (26, 27). These results indicate that two opposing processes, hypomethylation and hypermethylation of certain genes, may be associated with cancer drug-resistant phenotype. Additionally, recent evidence from cell lines (19) and *in vivo* studies (28) shows prominent changes in chromatin structure associated with drug resistance. Furthermore, it has been suggested that epigenetic changes may be a crucial driving force leading to the acquisition of drug resistance (16, 25).

In view of these considerations, the present study was undertaken to define the precise role of epigenetic changes in the development of breast cancer cell resistance to the chemotherapeutic agents doxorubicin (DOX) and cisplatin (cisDDP) and to identify the mechanisms contributing to this resistance.

Materials and Methods

Cell Lines and Cell Culture

The human breast adenocarcinoma MCF-7 cell line and its variants resistant to DOX (MCF-7/DOX) and to cis-dichlorodiammine platinum(II) (cisDDP; MCF-7/cisDDP) were cultured using Dulbecco IS-OV (Sigma, St. Louis, MO) containing 10% newborn calf serum (HyClone, Logan, UT) and 40 μ g/mL gentamicin at 37°C in a 5% CO₂

atmosphere. The drug-resistant variants of MCF-7 cell lines were established by stepwise selection after prolonged (>6 months) treatment of original MCF-7 cells to increasing concentrations of DOX or cisDDP at a range of 0.5 to 15 μ g/mL in the medium. After 6 months of culturing in the presence of drugs, the IC₅₀ (inhibitory concentration to produce 50% cell death) values were 19.2 and 3.6 mg/L for the MCF-7/DOX and MCF-7/cisDDP cells, respectively.

Cells were seeded at a density of 0.5×10^6 viable cells per 100-mm plate, and the medium was changed every other day for 6 days. Trypsinized cells were washed in PBS and immediately frozen at –80°C for subsequent analyses. The experiments were repeated twice, and each cell line was tested in triplicate.

Immunocytochemistry

Expression of P-glycoprotein (Pgp) and GST π in the MCF-7 and MCF-7/DOX and MCF-7/cisDDP cells was detected by immunocytochemistry as described by Chekhun et al. (21). Cells were cultured on glass coverslips for 24 h and fixed in PBS containing 0.4% paraformaldehyde. The fixed cells were then rinsed with PBS and incubated at room temperature for 60 min with either primary mouse anti-human Pgp monoclonal (clone C494) or mouse anti-human GST π monoclonal (clone 353-10) antibodies (DAKO, Carpinteria, CA) diluted 1:100 and 1:50, respectively. Horseradish peroxidase-coupled secondary antibodies and DAKO EnVision System were used for visualization.

Flow Cytometry Analysis

Detection of Pgp and GST π expression in the MCF-7 and MCF-7/DOX, and MCF-7/cisDDP cells was done by flow cytometry as described by Robey et al. (29). Single-cell suspensions were obtained by trypsinization. Cells were washed with PBS, fixed in 1% paraformaldehyde for 15 min, followed by incubation in 70% methanol at –20°C for 1 h. Cells were rinsed with PBS, resuspended in Dulbecco's PBS and incubated with either anti-Pgp monoclonal (clone C494) or anti-GST π monoclonal (clone 353-10) antibodies (DAKO) at 4°C for 30 min. Incubation with CD3 monoclonal PC3/188 (DAKO) antibody served as a negative control. After incubation with primary antibodies, cells were washed in PBS and subsequently incubated with a FITC-labeled goat anti-mouse immunoglobulin polyclonal secondary antibody (DAKO). Stained cells were analyzed with a Coulter Epics XL flow cytometer equipped with a 488-nm argon laser (Beckman Coulter, Fullerton, CA) and a 530-nm band-pass filter allowing detection of FITC fluorescence. Appropriate isotypic controls were used in all experiments. A minimum of 5,000 events was collected. Pgp or GST π fluorescence was expressed as a ratio of the mean channel value of the Pgp or GST π histogram to the mean channel value of the isotype control histogram.

Global DNA Methylation Analysis

The extent of the global DNA methylation was evaluated with a radiolabeled [³H]-dCTP extension assay as described previously (30). Briefly, 1 μ g of genomic DNA was digested with 20 units of methylation-sensitive *Hpa*II restriction endonuclease (New England Biolabs, Beverly, MA) for 16 to 18 h at 37°C. A second DNA aliquot (1 μ g) was digested

with methylation-insensitive isoschizomer MspI, which cleaves CCGG sites in DNA regardless of CpG methylation status, to serve as a control for the digestion efficiency. Undigested DNA served as a background control. The single nucleotide extension reaction was done in a 25- μ L reaction mixture containing 1.0 μ g DNA, 1 \times PCR buffer II, 1.0 mmol/L MgCl₂, 0.25 unit AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 0.1 μ L of [³H]dCTP (57.4 Ci/mmol; Perkin-Elmer Life and Analytical Sciences, Boston, MA) and incubated at 56°C for 1 h. Samples were applied to DE-81 ion-exchange filters and washed thrice with 0.5 mol/L sodium phosphate buffer (pH, 7.0) at room temperature. The filters were dried and processed for scintillation counting. [³H]dCTP incorporation into DNA is expressed as mean disintegrations per minute per microgram of DNA after subtraction of the disintegrations-per-minute incorporation in undigested samples (background).

Analysis of Histone H3 and Histone H4 Modifications

Acidic cell extracts were prepared as described previously (31). An equal amount of total histones (40 μ g) was mixed with two volumes of gel loading buffer [250 mmol/L Tris-HCl (pH, 8.0), 20% β -mercaptoethanol, 40% glycerol, 8% SDS, and 1.2 mg/mL bromophenol blue], heated for 5 min at 95°C, and resolved on 15% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (GE Healthcare Biosciences, Piscataway, NJ). The membranes were blocked for 4 h in TBS containing 5% nonfat dry milk and 0.1% Tween 20. Primary antibodies against trimethyl-histone H3 lysine 9 (H3K9), acetyl-histone H3 lysine 9 (H3K9ac), phospho-histone H3 serine 10 (H3S10ph), and trimethyl-histone H4 lysine 20 (H4K20) were diluted 1:1,000, 1:1,000, 1:400, and 1:2,000, respectively, according to manufacturer's recommendations (Upstate, Charlottesville, VA). Primary antibody binding was done at 4°C overnight with constant shaking. A secondary donkey anti-rabbit antibody, labeled with alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA), was applied at 1:5,000 dilutions, and binding was carried out at room temperature for 1.5 h. Chemifluorescence detection was done with the ECF Substrate for Western blotting (GE Healthcare Biosciences) and measured directly by Storm Imaging System (Molecular Dynamics, Sunnyvale, CA). Images are representative of three independent immunoblots and were analyzed by ImageQuant software. All membranes were stained with Coomassie blue and with anti-histone H3 and anti-histone H4 antibodies to confirm equal protein loading.

Immunoblotting

Levels of Suv39h1, Suv4-20h2, PRDM2/RIZ1, HAT1, p73, and sulfatase 2 were measured by immunoblot analysis. Total cellular extracts were prepared by homogenization of 3 to 5 \times 10⁶ cells in 500 μ L of lysis buffer [50 mmol/L Tris-HCl (pH, 7.4); 1% NP40; 0.25% sodium deoxycholate; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride; 1 μ g/mL each of aprotinin, leupeptin, and pepstatin; 1 mmol/L Na₃VO₄; 1 mmol/L NaF], sonication, and incubation at 4°C for 30 min, followed by centrifugation at 10,000 \times g at 4°C for 20 min.

Extracts containing equal quantities of proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were probed with antibodies specific for Suv39h1 (Upstate), Suv4-20h2 (Abcam, Cambridge, MA), PRDM2/Riz1 (Abcam) histone methyltransferases, HAT1 histone acetyltransferase (Abcam), p73 protein (Abcam), and sulfatase 2 (Cosmo Bio Co., Tokyo, Japan) at 1:1,000, 1:1,500, 1:500, 1:500, 1:1,000, and 1:250 dilutions, respectively. Alkaline phosphatase-coupled donkey anti-rabbit secondary antibodies were used for visualization. Chemifluorescence detection was done with the ECF Substrate for Western blotting and measured directly by Storm Imaging System. Images are representative of three independent immunoblots and were analyzed by ImageQuant software. All membranes were stained with Coomassie blue and with anti- β -actin antibodies to confirm equal protein loading.

Determination of Differentially Methylated DNA Regions by McrBC-Methylation – Sensitive Arbitrarily Primed PCR Method

The determination of differentially methylated DNA fragments was done by a McrBC-methylation-sensitive arbitrarily primed PCR (McrBC-msAP-PCR) method (32). This technique allows the detection of differentially methylated sites within unmethylated DNA domains enriched by regulatory sequences and CpG islands. Briefly, 2 μ g of genomic DNA were digested overnight at 37°C with 10 units/ μ g DNA of McrBC endonuclease (New England Biolabs). McrBC is a methylation-specific endonuclease, which, as opposed to methylation-sensitive restriction endonucleases, cleaves DNA containing 5-methylcytosine on one or both strands but will not act on unmethylated DNA. Additionally, it will not recognize *Hpa*II sites (CCGG) in which the internal cytosine is methylated. McrBC-digested DNA fragments were separated on a 1% agarose gel, and DNA fragments larger than 1 kb were excised from the gel and purified by using a QIAquick Gel Extraction kit according to manufacturer's protocol (Qiagen, Valencia, CA). These fragments, which were enriched for unmethylated DNA, were then digested overnight with 20 units/ μ g DNA of methylation-sensitive restriction endonuclease *Sma*I (New England Biolabs), followed by digestion with 20 units/ μ g DNA of *Hpa*II (New England Biolabs).

The digested DNA was amplified by PCR using a single MLG2 primer containing 5'-AACCCCTACCCTAACCCCGG-3' (33). Each PCR reaction contained 0.2 μ g of digested DNA, 50 pmol of primer, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L Mg Cl₂, 150 μ mol/L of each deoxynucleotide triphosphate, 5 μ Ci of [³³P]dCTP (Perkin-Elmer Life and Analytical Sciences), and 1.2 units of AmpliTaq DNA polymerase (Applied Biosystems) in a total volume of 25 μ L. The cycling conditions consisted of five cycles at low-stringency conditions of 95°C for 30 s, 40°C for 60 s, 72°C for 90 s, followed by 30 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 60 s (33). PCR products were resolved on a 5% polyacrylamide 7 mol/L urea-sequencing gel. Gel resolution of amplified PCR products resulted in the generation of methylation-sensitive

fingerprints. After electrophoresis, the gel was analyzed by Cyclone Storage Phosphor Screen system (Packard Instrument Company, Meriden, CT) for differences in the intensity of bands between cancer cells. Bands appearing in resistant cancer cells (MCF-7/DOX and MCF-7/cisDDP) but not in the sensitive cells (MCF-7) were interpreted as hypermethylated. Conversely, the disappearances of bands in resistant cells compared with sensitive cells were considered as a loss of methylation.

Differentially methylated bands were excised from the polyacrylamide gel, purified by ethanol precipitation, and amplified using the same primer and with the same PCR protocol as in the original PCR. PCR products were cloned into plasmid vectors using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated from several independent clones by using a QIAprep Spin Miniprep kit (Qiagen), and the nucleotide sequences of the inserts were determined by automated sequencing (Retrogen, San Diego, CA). Sequence homologies were searched using the BLAST program of the National Center for Biotechnology Information.⁴

Methylation-Specific PCR

Analysis of methylation patterns within the CpG island of the *p73* gene was determined after the standard bisulfite modification of genomic DNA and methylation-specific PCR (MSP) as previously described (34).

Real-Time Methylation-Sensitive McrBC-PCR Assay

The methylation status of sulfatase 2 gene was determined by methylation-sensitive McrBC-PCR assay as described previously (32). Genomic DNA (1 μ g) was digested with 20 units of McrBC endonuclease (New England Biolabs) overnight at 37°C. Cleavage of methylated DNA by McrBC induces DNA strand breaks and abrogates PCR amplification. Conversely, the presence of unmethylated cytosines in DNA prevents enzyme cleavage and can be detected by PCR amplification product recovery. Undigested DNA was served as control. Following the McrBC treatment, subsequent real-time PCR was used to amplify promoter of sulfatase 2 gene. Methylated DNA sequences have decreased amounts of PCR product after McrBC digestion. The results are presented as the ratio of PCR product recovery after the digestion of DNA with McrBC relative to undigested DNA.

Statistical Analyses

Results are presented as mean \pm SE. Statistical analyses were conducted by one-way ANOVA using SigmaStat software (Jandel Scientific, San Rafael, CA).

Results

Expression of Pgp and GST π in Drug-Resistant Breast Cancer Cells

Transcriptional activation of Pgp and GST π has been implicated in the acquisition of drug resistance by cancer cells to various chemotherapeutic drugs (9, 12, 19). To determine whether or not the development of drug

resistance of MCF-7 cells is associated with increased expression of Pgp and GST π , we measured the level of these proteins by immunocytochemistry and flow cytometry in MCF-7 cells and their MCF-7/DOX and MCF-7/cisDDP drug-resistant variants. Development of drug resistance of MCF-7 cells to DOX was characterized by intense Pgp staining (Fig. 1A-b) and a low level of GST π staining in MCF-7/DOX cells (Fig. 1A-d). In contrast, acquisition of drug resistance of MCF-7 cells to cisDDP was associated with increased level of GST π (Fig. 1A-c) and absence of Pgp staining (Fig. 1A-a). The parental MCF-7 cells did not express Pgp or GST π (data not shown). Quantitative analysis of Pgp or GST π expression in the MCF-7/DOX, MCF-7/cisDDP and parental MCF-7 cells was determined by flow cytometry. Figure 1B shows that the level of Pgp in MCF-7/DOX cells was 1.6-fold higher than in MCF-7 and MCF-7/cisDDP cells, whereas MCF-7/cisDDP cells displayed a 1.8- and 1.7-fold increase in GST π expression compared with MCF-7 and MCF-7/DOX, respectively.

The differential expression of Pgp and GST π proteins indicates that acquisition of drug resistance of MCF-7 cells to DOX and cisDDP is characterized by two different mechanisms: resistance to DOX via the increased efflux of hydrophobic drugs (as indicated by an increased expression of Pgp), whereas resistance to cisDDP via the increased elimination of drug through conjugation reactions with thiol-containing molecules (as indicated by an increased expression of GST π).

Global Genome Hypomethylation in Drug-Resistant Breast Cancer Cells

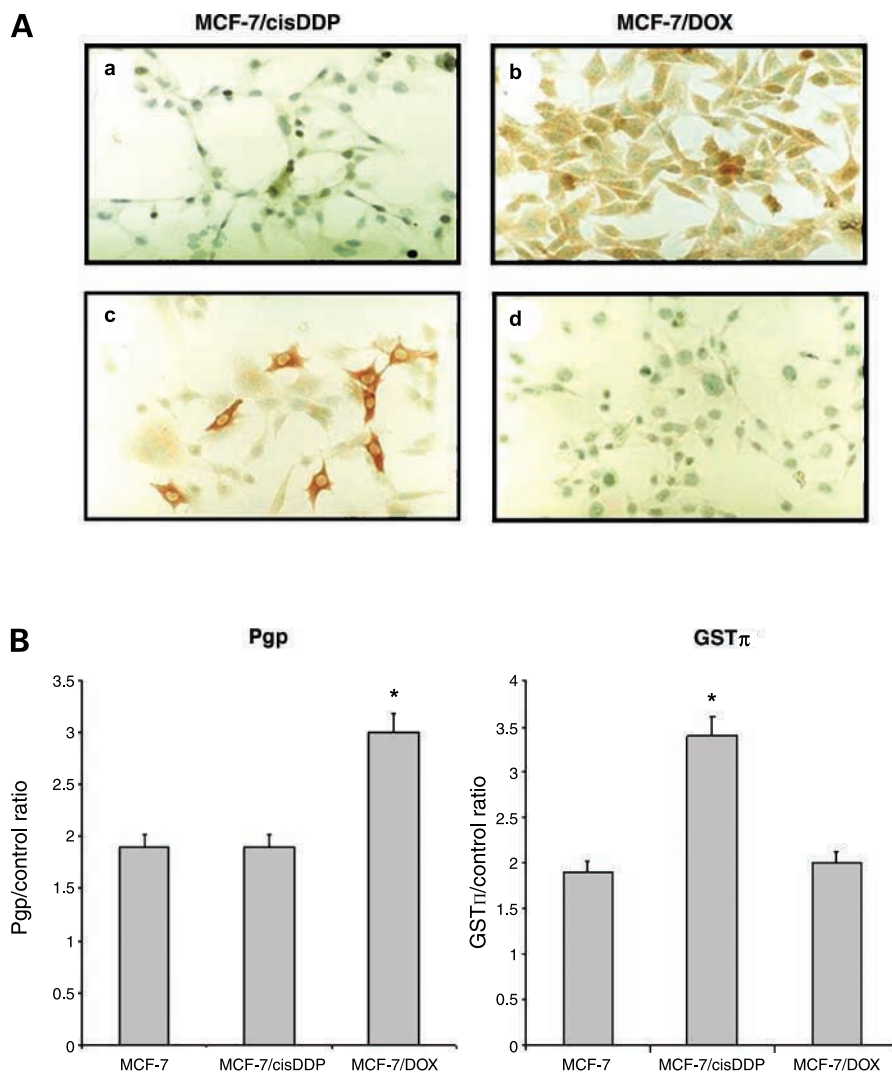
DNA methylation status was assessed with a sensitive cytosine extension assay that measures the proportion of unmethylated CpG sites in DNA. The assay is based on the ability of the *Hpa*II methylation-sensitive restriction enzyme to cleave unmethylated CCGG sequences and leave a 5' guanine overhang that can be used for the subsequent single nucleotide extension with labeled [³H]dCTP (30). The extent of [³H]dCTP incorporation is directly proportional to the number of unmethylated CpG sites. Figure 2A shows that incorporation of [³H]dCTP into *Hpa*II-digested DNA isolated from MCF-7/DOX and MCF-7/cisDDP cancer cells was ~47% and 26% greater than into DNA isolated from MCF-7 cells. The greater extent of [³H]dCTP incorporation indicates that the MCF-7/DOX and MCF-7/cisDDP cells have a more extensive loss of DNA methylation compared with the parental MCF-7 cells.

Status of Histone H3 and Histone H4 Modifications and Protein Expression of Histone-Modifying Enzymes in Drug-Resistant Breast Cancer Cells

Considering the tight link between DNA methylation and global modifications of histones (35) and the results of recent studies showing that aberrant histone modifications play an important role in cancer (36, 37), we examined alterations in the trimethylation of histones H3K9 (H3K9me3) and H4K20 (H4K20me3), acetylation of H3K9 (H3K9ac), and phosphorylation of H3S10 (H3S10ph) in the MCF-7, MCF-7/DOX, and MCF-7/cisDDP cells. The extent

⁴ Available at <http://www.ncbi.nlm.nih.gov>.

Figure 1. Expression of P-glycoprotein and GST π protein in MCF-7, MCF-7/cisDDP, and MCF-7/DOX human breast adenocarcinoma cells as detected by immunocytochemistry (A) and by flow cytometry (B). A, fixed MCF-7/cisDDP and MCF-7/DOX-resistant cells were incubated with primary mouse anti-human Pgp monoclonal (clone C494; a and b) or mouse anti-human GST π monoclonal (clone 353-10) antibodies (c and d) and visualized by incubation with horseradish peroxidase-coupled secondary antibodies and DAKO EnVision System (DAKO). B, Pgp or GST π fluorescence in MCF-7, MCF-7/cisDDP, and MCF-7/DOX cells was expressed as a ratio of the mean channel value of the Pgp or GST π histogram to the mean channel value of the isotype control histogram. Representative results from two independent experiments. Each cell line was tested in triplicate.



of H3K9me3 did not differ between the three cell lines (Fig. 2B). In contrast, the level of H3K9ac decreased and the level of H3S10ph increased in drug-resistant cells compared with the parental MCF-7 cells. This finding is in good correspondence with the observation that alteration of one histone modification may affect the other modifications on the same amino-terminal tail of histones (38). Indeed, it has been shown that alteration in H3S10ph affects methylation, acetylation, or both of H3K9 (38). The level of H4K20me3 in the MCF-7/DOX and MCF-7/cisDDP cells was substantially decreased compared with the MCF-7 cells and was strongly correlated with the changes in protein expression of histone methyltransferase Suv4-20h2, the primary function of which is trimethylation of histone H4K20 (Fig. 2C). The protein level of HAT1 acetyltransferase decreased in drug-resistant cells, and the level of H3K9 methyltransferase PRDM2/RIZ1 was slightly lower in MCF-7/cisDDP cells than in MCF-7 cells and did not differ between MCF-7/DOX and MCF-7 cells.

Region-Specific DNA Methylation Changes in Drug-Resistant Breast Cancer Cells

The development of full drug-resistant phenotype of cancer cells induced by chemotherapeutic drugs is not likely to be due to the differential expression of one gene alone, but rather due to altered expression of many different genes (11, 39). Recent studies have indicated that chemotherapeutic drugs can influence the function of a number of genes by actively inducing epigenetic changes enhancing multidrug resistance MDR, especially by changing the DNA methylation patterns (11, 15, 16, 40). In view of this, we used McrBC-msAP-PCR to identify region-specific DNA methylation changes that are associated with the acquisition of a cancer drug resistance phenotype. McrBC-msAP-PCR allows the detection of differentially methylated sites within unmethylated DNA domains enriched by CpG islands-associated regulatory sequences (32). Figure 3 shows McrBC-msAP-PCR fingerprints of MCF-7 breast cancer cells and MCF-7/DOX and

MCF-7/cisDDP-resistant cancer cells. Genomic DNA from MCF-7/DOX and MCF-7/cisDDP cells exhibited novel new fragments (hypermethylated sequences) and the disappearance of fragments (hypomethylated sequences) compared with their parental MCF-7 cells. These differentially methylated fragments were excised from the gel, re-amplified, cloned, and sequenced. A DNA homology search of the hyper- and hypomethylated fragments,

using the BLAST program, revealed 93% to 99% of homology to different genes that play important roles in many cellular processes (Table 1). Additionally, by using the CpG Island Searcher program (41),⁵ we determined that according to defined parameters (42), 10 hypermethylated genes and hypomethylated genes contained CpG islands within or near the amplified fragments (Table 1).

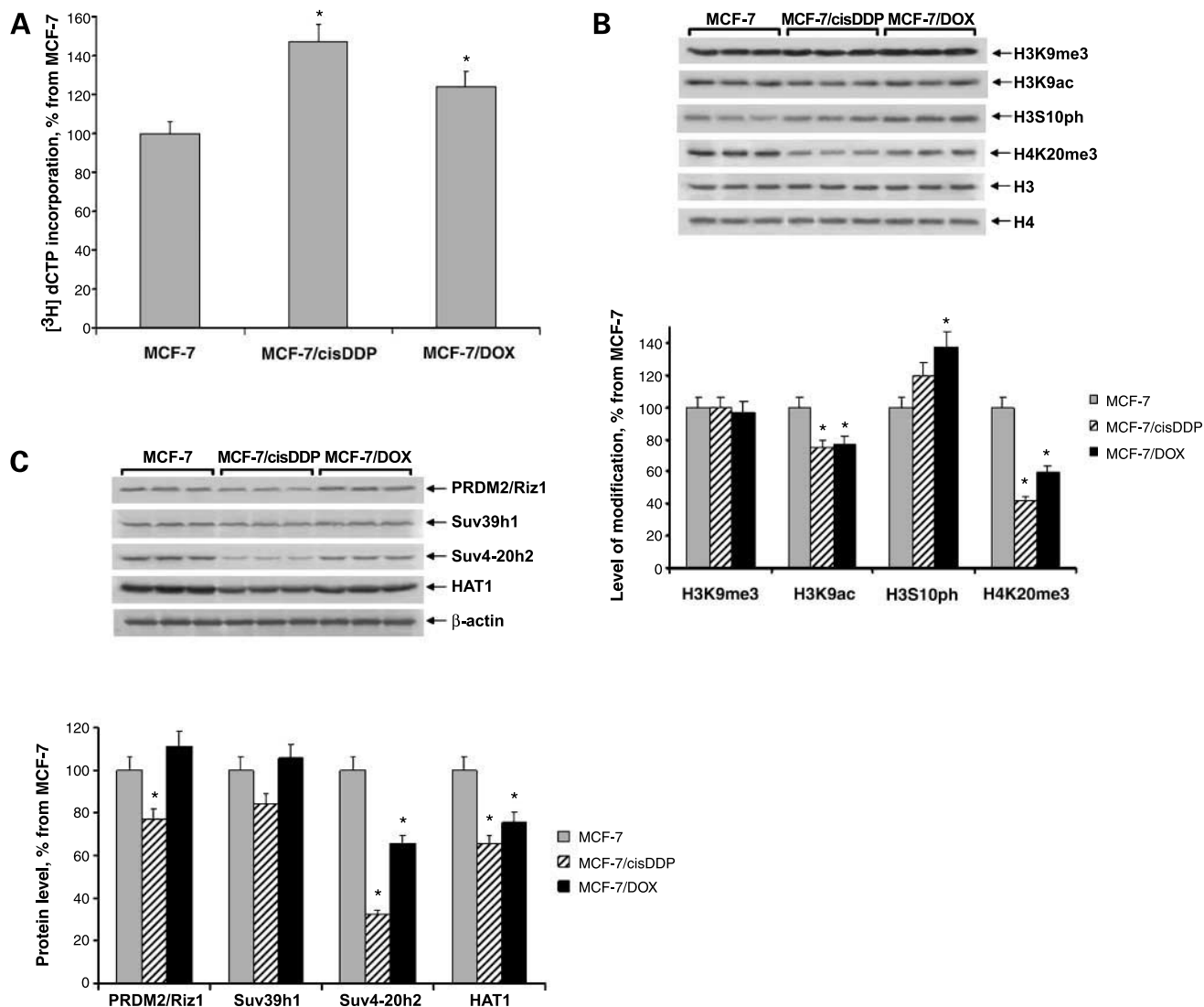


Figure 2. Level of global DNA methylation (**A**), histone H3 and histone H4 modifications (**B**), and protein level of histone-modifying enzymes (**C**) in MCF-7, MCF-7/cisDDP, and MCF-7/DOX human breast adenocarcinoma cells. **A**, DNA methylation in MCF-7, MCF-7/cisDDP, and MCF-7/DOX cells was measured by the cytosine extension assay after treatment of DNA with methylation-sensitive restriction endonuclease *HpaII* that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands. **B**, Western blot analysis of histone H4K20me3, H3K9me3, H3K9ac, and H3S10ph in MCF-7, MCF-7/cisDDP, and MCF-7/DOX cells. Acid extracts of total histones were separated by SDS-PAGE and subjected to immunoblotting using specific antibodies against histone H3K9me3, H4K20me3, H3K9ac, and H3S10ph. Equal sample loading was confirmed by immunostaining against histone H3 and histone H4. *Top*, representative Western immunoblot images from two independent experiments; *bottom*, a quantitative evaluation of H3K9me3, H4K20me3, H3K9ac, and H3S10ph in MCF-7/cisDDP and MCF-7/DOX cells relative to those in MCF-7 cells. **C**, expression of PRDM2/Riz1, Suv39h1, Suv4-20h2 histone methyltransferases and HAT1 histone acetyltransferase in MCF-7, MCF-7/cisDDP, and MCF-7/DOX cells. Cell lysates were separated by SDS-PAGE and subjected to immunoblotting using specific antibodies against PRDM2/Riz1, Suv39h1, and Suv4-20h2. Equal sample loading was confirmed by immunostaining against β-actin. *Top*, representative Western immunoblot images from two independent experiments; *bottom*, a quantitative evaluation of the PRDM2/Riz1, Suv39h1, Suv4-20h2, and HAT1 expressions in MCF-7/cisDDP and MCF-7/DOX cells relative to those in MCF-7 cells. *Columns*, mean percent fractions of MCF-7 cells; *bars*, SE. *, significantly different from MCF-7 ($n = 3$).

Status of the *p73* and *Sulfatase 2* Promoter Methylation and Protein Expression in Drug-Resistant Breast Cancer Cells

The hypermethylated state of 5' CpG island of the *p73* gene in MCF-7/DOX and MCF-7/cisDDP cells was further confirmed by an independent MSP assay (Fig. 4A). By using a MSP assay, we found that promoter region of the *p73* gene was unmethylated in parental MCF-7 cells and was highly methylated in the MCF-7/DOX and MCF-7/cisDDP drug-resistant variants. Recent studies have shown an inverse correlation between the methylation state and expression of *p73* gene (34, 43) and have suggested that altered function of *p73* protein may be involved in sensitivity to chemotherapeutic drugs (12). Therefore, we examined whether or not the hypermethylation of the *p73* gene in the MCF-7/DOX and MCF-7/CIS cells was accompanied by the loss of *p73* protein. As shown in Fig. 4C, *p73* protein was expressed in MCF-7 cells but was markedly down-regulated in the MCF-7/DOX and MCF-7/CIS cells.

In contrast, McrBC methylation-sensitive analysis confirmed that promoter region of *sulfatase 2* gene underwent hypomethylation in MCF-7/DOX and MCF-7/cisDDP cells (Fig. 4B) accompanied by the increased level of sulfatase 2 protein in drug-resistant cells (Fig. 4C).

Discussion

In this study, we examined the impact of epigenetic dysregulation on the resistance of human breast cancer cells exposed to chemotherapeutic agents. The results showed that the acquisition of resistance in the cancer cells was associated with profound deregulation of cellular epigenetic landscape as characterized by global DNA hypomethylation, altered histone H3K9 and histone H4K20 trimethylation, aberrant expression of histone methyltransferases Suv4-20h2, Suv39h1, and PRDM2/RIZ1, and the appearance of drug-induced regional hypo- and hypermethylation. In a previous study, we showed that a DOX-resistant variant of MCF-7 cells was characterized by the high expression of P-glycoprotein and promoter hypomethylation of *MDR1*, *GSTπ*, and *MGMT* genes (21). Similar findings were also reported by David et al. (20) and Harbottle et al. (22). However, the results of recent studies have shown clearly that the acquired drug resistance of cancer cells is a multifactorial phenomenon rather than the result of only one mechanism misregulation (11). Additionally, the existence of cross-resistance to different chemotherapeutic agents suggests that many of them may share common mechanisms of resistance (12). In the present study, we examined the alterations in epigenetic mechanisms in drug resistance induced by DOX and cisDDP MCF-7 human breast cancer cells. The MCF-7 cells with acquired drug resistance to DOX were characterized by increased expression of P-glycoprotein, whereas MCF-7 cells resistant to cisDDP were characterized by increased expression of *GSTπ* (Fig. 1). Despite this difference, both of the drug-

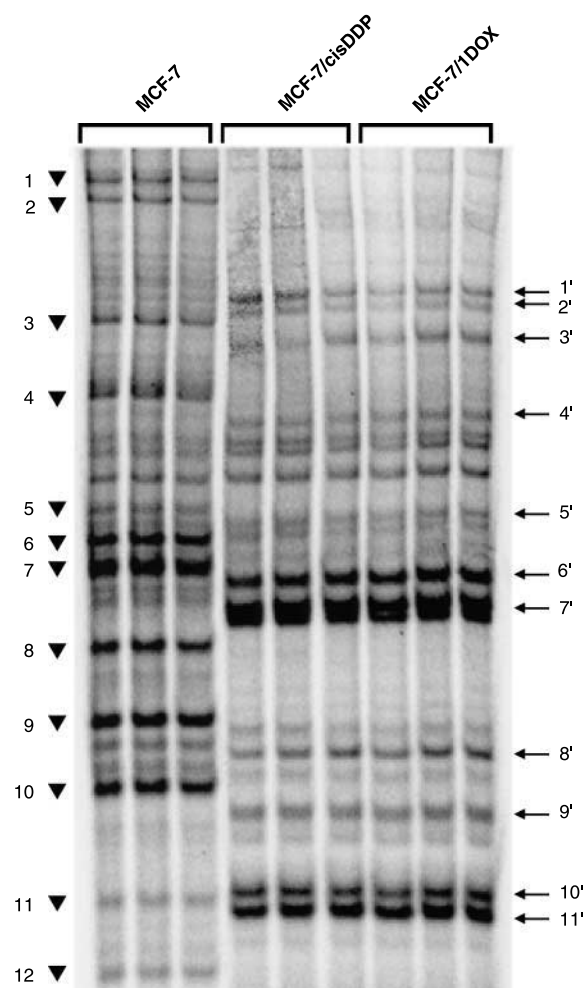


Figure 3. McrBC-msAP-PCR fingerprints of MCF-7, MCF-7/cisDDP, and MCF-7/DOX cells. Bands that appeared in MCF-7/cisDDP and MCF-7/DOX drug-resistant cells but not in the MCF-7 cells were interpreted as being hypermethylated (arrows). Disappearance of bands in MCF-7/cisDDP and MCF-7/DOX drug-resistant cells compared with MCF-7 cells was considered as loss of methylation (▼). Differentially methylated bands were excised from the gel, re-amplified, cloned, and sequenced.

resistant cell lines displayed similar pronounced changes in the global epigenetic landscape as exemplified by the loss of global DNA methylation, loss of histone H4K20 trimethylation, loss of histone H3K9 acetylation, increased histone H3S10 phosphorylation and diminished expression of Suv4-20h2 histone methyltransferase compared with parental MCF-7 cells. These results are consistent with other *in vitro* and *in vivo* studies demonstrating extensive drug-induced changes in DNA methylation and in chromatin structure (11, 15, 16, 19, 28).

In addition to global epigenetic changes, the MCF-7/DOX and MCF-7/cisDDP drug-resistant cells are characterized by extensive alterations in region-specific DNA methylation, as indicated by the appearance of a number of differentially methylated DNA fragments (Table 1). A detailed analysis of hypo- and hypermethylated DNA sequences revealed that

⁵Available at <http://www.cpgislands.com>.

the acquisition of drug-resistant phenotype of the MCF-7 cells to DOX and cisDDP, in addition to specific alterations induced by a particular drug (e.g., expression of Pgp in the MCF-7/DOX cells and expression of GST π in the MCF-7/cisDDP cells; Fig. 1), was characterized by three major common mechanisms: dysfunction of genes involved in estrogen metabolism, apoptosis, and cell-cell contact. Furthermore, the results showed that two opposing hypo- and hypermethylation processes enhance and complement each other in the disruption of these pathways. For example, the combination of altered functions of sulfatase 2 (44) and estrogen receptor α (ER α , refs. 45, 46) induced by hypomethylation of the sulfatase 2 precursor gene and by hypermethylation of ER α gene indicates a profound deregulation of estrogen metabolism in MCF-7 drug-resistant cells resulting in the loss of estrogen responsiveness. Promoter hypermethylation and the associated silencing of the proapoptotic *p73*, α -tubulin genes (12, 47–49) accompanied by hypomethylation and activation of antiapoptotic and cell survival tissue transglutaminase 2 and forkhead box protein K1 genes (50, 51), may result in an inhibition of apoptosis and enhanced cell survival. Additionally, based on the observations of hypermethylation of 5' CpG island of BCL2 antagonist of cell death (BAD) gene (Table 1) and that inhibition of BAD expression prevents apoptosis (52), it may be possible that hypermethylation of the BAD gene in the MCF-7/DOX and MCF-7/cisDDP drug-resistant cells further abrogates apoptosis and promotes survival of these cells. These findings provide further support and show the importance of "permissive apoptosis resistance" as a major route by which cancer cells acquire multidrug resis-

tance to chemotherapeutic agents (8). On the other hand, the hypomethylation and activation of leptin, stromal cell-derived factor receptor 1 and activin A receptor genes (53–55) and hypermethylation and inhibition of E-cadherin gene (54) in MCF-7 drug-resistant cells may increase their invasion potential.

Importantly, observed transcriptional down-regulation (via promoter hypermethylation) of the *p73* or transcriptional up-regulation (via promoter hypomethylation) of the sulfatase 2 in MCF-7 drug-resistant cells suggests the role of altered epigenetic mechanisms in the acquisition of cancer cell drug resistance (56, 57).

The results reported in this paper clearly show that MCF-7/DOX and MCF-7/cisDDP cancer cells with acquired drug-resistant phenotype are characterized by profound alterations in cellular epigenetic landscape. Furthermore, the similarity between epigenetic dysfunction of genes involved in estrogen metabolism, resistance to apoptosis, and invasion observed in the MCF-7/DOX and MCF-7/cisDDP cells indicate that despite the different mechanisms for the induction of drug resistance, these cells are characterized by common epigenetic alterations of crucial cellular pathways. Deregulation of similar pathways may explain the existence of cross-resistance of cancer cells to different types of chemotherapeutic agents and may be a basis for its development. The results of the study show that epigenetic changes are an important feature of cancer cells with acquired drug-resistant phenotype and may be a crucial contributing factor to its development (16, 25). Finally, it should be noted that a recent study by Chen et al. (58) has provided evidence of the importance of interplay between

Table 1. Sequence analysis of differentially methylated DNA fragments in MCF-7 cells and MCF-7/DOX and MCF-7/cisDDP drug-resistant cells

Clone	BLAST homology	Gene	CpG island
Hypermethylated			
1	X15610	Proliferating cell nuclear antigen P40	+
2	NM_006912	5'end of RIT1 gene	+
3	Y11416	<i>p73</i> gene	+
4	NM_00600	α -Tubulin gene	+
5	X62462	Estrogen receptor α	+
6	NM_004723	Rho/rac guanine nucleotide exchange factor (GEF) 2	+
7	X03131	Interleukin 2 receptor	+
8	AL592449	Novel FLJ12505 gene	+
9	AF283508	Cell death regulator Aven	+
10	NM_004322	BCL2-antagonist of cell death (BAD)	+
Hypomethylated			
1	NT 086910	Sulfatase 2 precursor	+
2	X95523	Utrophin	+
3	NM_001033886	Stromal cell-derived factor receptor 1	+
4	D63710	Leptin	+
5	AY310398	Sidekick 2	+
6	U13920	Tissue transglutaminase 2	+
7	NM_014674	ER degradation enhancer, mannosidase α -like 1	+
8	AK122663	Forkhead box protein K1	+
9	D28463	Ferritin heavy chain	+
10, 11	NM_001105	Activin A receptor	+

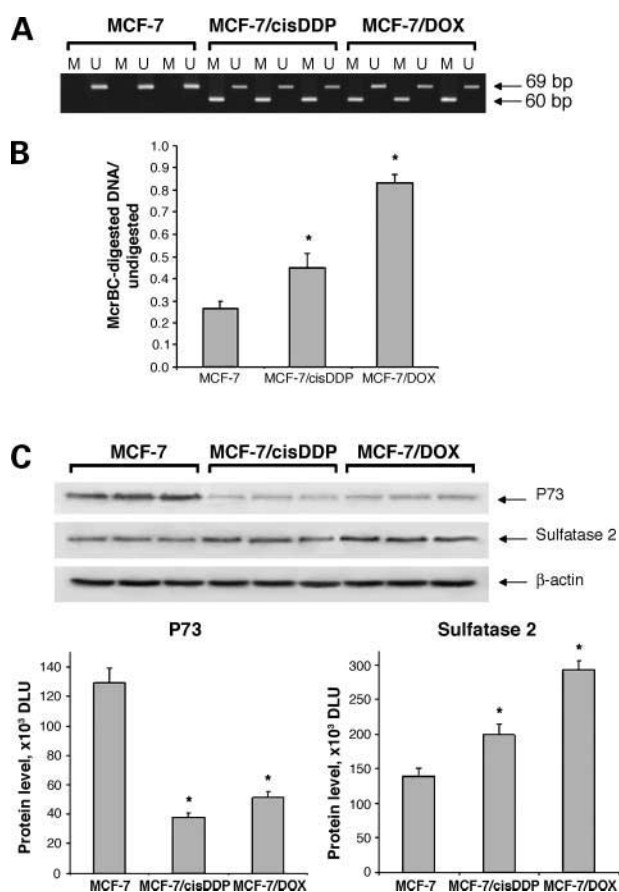


Figure 4. Methylation of the *p73* (A) and *sulfatase 2* (B) promoter region CpG islands, and level of *p73* and *sulfatase 2* proteins (C) in MCF-7, MCF-7/cisDDP, and MCF-7/DOX cells. Methylation-specific PCR analysis of *p73* promoter methylation. Presence of PCR product: *U* lanes, unmethylated state of *p73* promoter; *M* lanes, occurrence of *p73* promoter methylation. **B**, methylation status of *sulfatase 2* promoter as detected by methylation-sensitive MspI PCR assay. For this assay, genomic DNA was digested with methylation-sensitive endonuclease MspI. Cleavage of methylated DNA by MspI induces DNA strand breaks and abrogates PCR amplification. Conversely, the presence of unmethylated cytosines in DNA prevents enzyme cleavage and can be detected by PCR amplification product recovery. Undigested DNA served as control. *Columns*, mean ratio of PCR product recovery after digestion of DNA with MspI relative to undigested DNA; *bars*, SE. *, significantly different from MCF-7 cells ($n = 3$). **C**, Western blot analysis of *p73* and *sulfatase 2* proteins in MCF-7, MCF-7/cisDDP, and MCF-7/DOX cells. Cell lysates were separated by SDS-PAGE and subjected to immunoblotting using specific antibodies against *p73* and *sulfatase 2* proteins. Equal sample loading was confirmed by immunostaining against β -actin. *Top*, representative Western immunoblot images from two independent experiments; *bottom*, quantitative evaluation of *p73* and *sulfatase 2* protein expressions in MCF-7/cisDDP and MCF-7/DOX cells relative to those in MCF-7 cells. *, significantly different from MCF-7 cells ($n = 3$, mean \pm SE).

drug-induced genetic and epigenetic mechanisms for the origin of acquired drug resistance, indicating that the synergism between genetic and epigenetic abnormalities play a crucial role in the development of cancer drug resistance.

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