



Role of DNA hypomethylation in the development of the resistance to doxorubicin in human MCF-7 breast adenocarcinoma cells

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Abstract

The resistance of cancer cells to chemotherapeutic agents is a major clinical problem and an important cause of treatment failure in cancer. Mechanisms that have developed to guard cancer cells against anti-cancer drugs are major barriers to successful anti-cancer therapy. Therefore, the identification of novel mechanisms of cellular resistance holds the promise of leading to better treatments for cancer patients. In the present study, we used human MCF-7 breast adenocarcinoma cell line and its doxorubicin-resistant variant MCF-7/R to determine the role of alterations of DNA methylation of chemoresistance-related genes, such as multidrug resistance 1 (*MDR1*), glutathione-S-transferase (*GSTπ*), O⁶-methylguanine DNA methyltransferase (*MGMT*), and urokinase (*Upa*), in the development of drug resistance. The promoter regions of *MDR1*, *GSTπ*, *MGMT*, and *Upa* genes were highly methylated in MCF-7 cell line but not in its MCF-7/R drug resistant variant. The hypomethylated status of *MDR1* gene was associated with overexpression of P-glycoprotein. We hypothesize that acquirement of doxorubicin resistance of MCF-7 cells is associated with DNA hypomethylation of the promoter regions of the *MDR1*, *GSTπ*, *MGMT*, and *Upa* genes. © 2005 Elsevier Ireland Ltd. All rights reserved.

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1. Introduction

Chemotherapy plays a major role in the treatment of patients with cancer, particularly breast cancer. If the current breast cancer rates remain constant,

a woman born today has one in ten chance of developing breast cancer [1]. The treatment of tumor cells with anti-neoplastic drugs is limited by emergence of cancer cell resistance to anti-cancer agents, which remains a barrier to the establishment of curative chemotherapy for advanced breast cancer. Tumors become resistant to chemotherapy by variety of mechanisms, including alteration in cell cycle checkpoints, failure of apoptotic mechanisms, altered

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DNA repair and scavenging enzymes, and increased drug efflux [2–4]. This latter form of resistance is called multidrug resistance (MDR). One of the most extensively studied mechanisms of multidrug resistance is associated overexpression of transmembrane P-glycoprotein (P-gp) that functions as drug efflux pump to reduce the intracellular concentration of drugs and, thus, cytotoxic activity [3,5].

The human P-glycoprotein is a 170,000-Da-molecular weight phosphoglycoprotein consisting of two ATP binding cassettes and two transmembrane regions, each of which contains six transmembrane domains [3]. P-gp is encoded by the multidrug resistance 1 (*MDR1*) gene [6] and participates in drug resistance against a variety of anti-cancer agents, including anthracyclines (doxorubicin). Amplification of *MDR1* gene, mutations in the *MDR1* gene, and rearrangements at the 5'-flanking region of the *MDR1* cause gene activation during selection of drug-resistant breast cancer cells [5,7].

Recently it has been demonstrated that human *MDR1* promoter is activated by alterations in CpG methylation in several human cancers and cancer cell lines [8–10]. In the repressed state, the *MDR1* promoter is methylated, assembled into chromatin, and enriched with methyl-CpG binding protein 2 (MeCP2) and deacetylated histones. DNA demethylation causes the release of MeCP2, histone acetylation and activation of the *MDR1* gene [10]. The degree of methylation in the *MDR1* promoter region may be closely associated with *MDR1* gene expression [11]. It has been suggested that hypomethylation status of the *MDR1* gene might be a necessary condition for increasing *MDR1* expression and for developing a multidrug resistance phenotype [11]. Additionally, overexpression of genes such as O⁶-methylguanine DNA methyltransferase (*MGMT*) and glutathione-S-transferase (*GSTπ*) may contribute to the development of the drug resistant phenotype of tumor cells to anti-neoplastic drugs [2–4]. *MGMT* is a rapid and error-free DNA repair enzyme that eliminates the alkylating lesion of O⁶-methylguanine in DNA; *GSTπ* catalyzes the interactions between glutathione and alkylating drugs, increases the rate of drug detoxification, and plays a significant role in the resistance of cancer cells to doxorubicin [12]. One of the common important features of these chemoresistance-related genes (*MDR1*, *MGMT*, *GSTπ*) is that all

of them contain mostly unmethylated 5'-CpG islands and, therefore, may be especially prone to regulation by cytosine methylation.

Despite the development of new targeted anti-cancer therapies, mechanisms that have evolved to protect cancer cells against chemotherapeutic agents will continue to act as obstacles to successful treatment of cancer [3]. The elucidation of novel mechanisms of cellular resistance may lead to better treatments for cancer patients [4]. Based on this consideration, in the present study we used human MCF-7 breast adenocarcinoma cell line and its doxorubicin-resistant variant MCF-7/R to determine the role of alterations of DNA methylation of chemoresistance-related genes in the development of the drug resistance of MCF-7 cells to anti-cancer drugs.

2. Materials and methods

2.1. Cell lines and cell culture

Human breast adenocarcinoma MCF-7 cell line and its drug resistant variant MCF-7/R were cultured using Dulbecco's modified–Iscove's medium (Sigma, St Louis, MO) containing 10% newborn calf serum and 40 µg/ml gentamicin at 37 °C in 5% CO₂ atmosphere. The MCF-7/R cell line was established by stepwise selection of original MCF-7 cells exposed to increasing concentration of doxorubicin (DOX) in the medium.

2.2. MTT assay

The drug sensitivity of MCF-7 and MCF-7/R cells was confirmed by MTT cell survival assay. Briefly, MCF-7 or MCF-7/R cells were seeded at density 5000 cells/well in 96-well plates and treated with or without increasing concentrations of doxorubicin at 37 °C for 72 h. After exposure, the medium was replaced with fresh drug-free medium containing 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and the cells were incubated for an additional 3 h. The culture medium was removed, 200 µl of DMSO were added to each well. The absorbance of samples was measured in a microplate reader at 570 nm. The negative control

was conducted using only cell-free culture medium. The experiments were repeated twice, and each concentration was tested in triplicate in each experiment. Viability was expressed as a percent of an untreated control (means \pm SEM).

2.3. Immunocytochemistry

Expression of P-glycoprotein in MCF-7 and MCF-7/R cells was detected by immunocytochemistry. Cells were cultured on glass coverslips for 24 h and fixed in phosphate-buffered saline (PBS) containing 0.4% paraformaldehyde. The fixed cells were then washed with PBS and incubated with 1% bovine serum albumin for 30 min at room temperature to reduce non-specific binding. The cells were incubated with diluted 1:100 primary mouse anti-human P-glycoprotein monoclonal C494 antibodies (DAKO, Carpinteria, CA) at room temperature for 60 min. The immunostaining was visualized by incubation with HRP-labeled secondary antibodies using DAKO EnVision System (DAKO).

2.4. PCR-based assay for MDR1 promoter methylation

A methylation-sensitive PCR was used to analyze the methylation status of the promoter region of *MDR1* gene [8]. DNA was treated with methylation-sensitive restriction endonuclease followed by PCR amplification. Two micrograms of genomic DNA isolated from MCF-7 or MCF-7/R cells was digested with 20 U of *HpaII* restriction endonuclease (New England Biolabs, Beverly, MA) for 16–18 h at 37 °C. *HpaII* is methylation-sensitive restriction endonuclease which cleaves DNA at CCGG sequences when internal cytosine residue is unmethylated on both strands. A second DNA aliquot (2 μ g) was digested with methylation-insensitive isoschizomer *MspI* which cleaves DNA regardless of methylation status and serves as control for digestion efficiency. Two sets of PCR primers 5'-TCTAGAGAGGTGCAACGGAAG-3' and 5'-TCSGCCTCACCACAGATGAC-3' (first set), and 5'-TGAAGTCCTCTGGCAAGTCC-3' and 5'-ATTCTCCCTCCCGGTTCC-3' (second set) were designed to amplify two fragments of *MDR1* promoter that contains *MspI/HpaII* recognition sequences. Each PCR reaction contained 0.25 μ g of

MspI or *HpaII*-digested DNA and 50 pmol of each primer in 25 μ l of 1 \times AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA). The cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 25 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, and polymerization at 72 °C for 30 s. The PCR products were separated on 2% agarose gels, stained with ethidium bromide, and photographed.

2.5. Methylation-specific PCR (MSP)

The methylation status of promoter regions of *MGMT* and *GST π* genes was determined by the modified nested two-step MSP as described by House et al. [13]. Briefly, 2 μ g of DNA were treated with sodium bisulfite according to a previously described protocol [14]. The bisulfite-treated DNA was purified using Wizard DNA Clean-Up system (Promega, Madison, WI), treated with NaOH, and ethanol precipitated. Step one of the nested MSP was performed with primers that flanked the CpG-rich promoter regions of the *MGMT* and *GST π* genes. These primers did not discriminate between methylated and unmethylated alleles. Therefore, a second step MSP was carried out with gene specific primers that were designed for either methylated or unmethylated sequences. The sequence of primers and cycling conditions were those described by House et al. [13]. The methylation status of urokinase (*Upa*) promoter region was determined by MSP. Two sets of MSP primers were used to amplify the methylated 5'-AGCGTTGCGGAAGTACGCGG-3' and 5'-CCG CCGCAGCCCCGCCCAA-3', or unmethylated 5'-AGTGTTGTGGAAGTATGTGG-3' and 5'-CCA CCACAACCCACCCAAA-3' promoter sequence under the following cycle conditions: an initial denaturation at 95 °C for 5 min, followed by 10 cycles of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 30 s, and polymerization at 72 °C for 45 s, additional 20 cycles of denaturation at 95 °C for 30 s, primer annealing at 50 °C for 30 s, and polymerization at 72 °C for 45 s [15]. The MSP products were analyzed on 3% agarose gel. Negative control PCR amplifications were performed by using both sets of modified primers with untreated DNA. The absence of PCR products confirms that unmodified DNA could not be amplified with modified sets of primers.

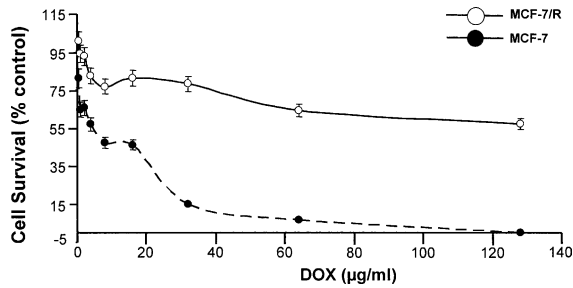


Fig. 1. MTT cell survival assay. MCF-7 or MCF-7/R cells were seeded at density 5000 cells/well in 96-well plates and treated with or without increasing concentration of doxorubicin at 37 °C for 72 h and the viability of cells was determined. The experiments were repeated twice and each concentration was tested in triplicate in each experiment. Viability was expressed as a percent of an untreated control (means \pm SEM).

2.6. Statistical analysis

Results were expressed as the means \pm SEM of at least triplicate measurements, and statistical comparisons were based on the Student's *t*-test analysis using SigmaStat software.

3. Results

3.1. Drug resistance characteristics of MCF-7 and MCF-7/R cells

The MCF-7/R drug-resistant variant of MCF-7 human breast cancer cell line was established by stepwise selection of MCF-7 cells cultured in the growth media containing increasing concentrations of

DOX. To determine the sensitivity of MCF-7 and MCF-7/R cells to DOX, cells were treated with different concentrations of the drug for 72 h. Fig. 1 shows the dose-dependent effect of DOX on MCF-7 and its drug-resistant variant MCF-7/R. DOX is highly cytotoxic to MCF-7 cells. MCF-7/R cells are more resistant to DOX compared to their parental MCF-7 cells. In order to determine whether the development of drug-resistance variant of MCF-7 cells is associated with increased expression of P-gp, we measured P-glycoprotein by immunocytochemistry in MCF-7 and MCF-7/R cells. Fig. 2 shows the expression of P-gp in MCF-7 cells and its MCF-7/R drug-resistant variant. Parental MCF-7 cells do not express P-gp (Fig. 2B). In contrast, P-glycoprotein is highly expressed in MCF-7/R drug-resistant variant (Fig. 2A).

3.2. Methylation status of *MDR1* promoter region

To examine the methylation status of promoter region of *MDR1* gene in MCF-7 human breast cancer cells and its drug-resistant variant MCF-7/R, we used two sets of primers that amplify across the *MspI/HpaII* sites located in *MDR1* gene promoter region (Fig. 3A). Methylated cytosine residues at *HpaII* recognition sequence prevent enzyme cleavage and can be detected by PCR amplification product recovery. Conversely, restriction enzyme cleavage at unmethylated sites induces DNA strand breaks and abrogates PCR amplification. As a control for efficiency of enzyme digestion, we treated DNA with *MspI* endonuclease. *MspI* is an isoschizomer of

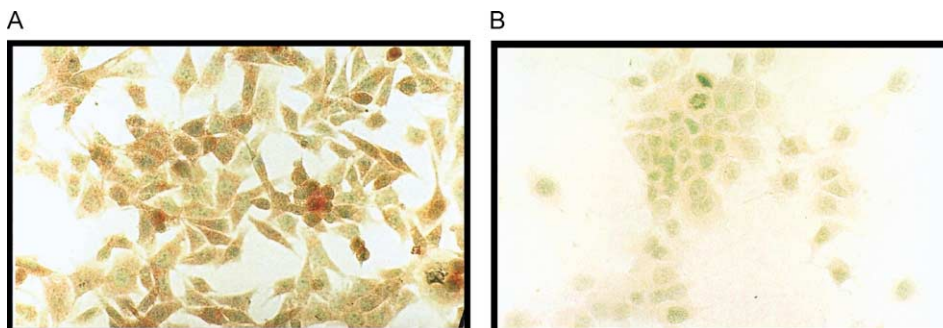


Fig. 2. Detection of P-glycoprotein expression in MCF-7 breast adenocarcinoma cells. Fixed of doxorubicin resistant MCF-7/R (A) and parental MCF-7 breast adenocarcinoma cells were incubated with monoclonal anti-P-glycoprotein antibodies and visualized by incubation with HRP-labeled secondary antibodies using DAKO EnVision System (DAKO).

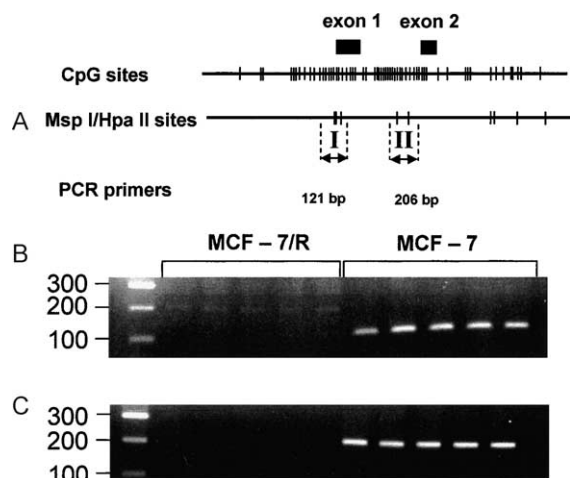


Fig. 3. Methylation status of *MDR1* gene promoter determined by methylation-sensitive PCR analysis in MCF-7 and MCF-7/R cells. (A) The diagram of the promoter of human *MDR1* gene and location of CpG sites, and *HpaII/MspI* sites. For determination of the methylation status of *MDR1* promoter region, we used two primer pairs (fragment I and fragment II) which amplify across the *HpaII/MspI* sites located within *MDR1* promoter. (B) The methylation status of *MDR1* promoter (fragment I, 121 bp) in MCF-7/R and MCF-7 cells. (C) The methylation status of *MDR1* promoter (fragment II, 206 bp) in MCF-7/R and MCF-7 cells. Presence of PCR amplification products after digestion of DNA with *HpaII* methylation-sensitive endonuclease indicates that *HpaII* sites located within regions were predominantly methylated in parental MCF-7 cells. The lack of PCR products after *HpaII* digestion indicates the loss of methylation in doxorubicin resistant MCF-7/R cells.

HpaII that is not sensitive to methylation and cleaves DNA regardless of methylation status. The presence of PCR products after treatment of DNA isolated from MCF-7 cells with *HpaII* methylation-sensitive restriction enzyme indicates that 5' promoter region of the *MDR1* gene is highly methylated in these cells (Fig. 3B, C). The complete absence of PCR products serves as an evidence that *MDR1* promoter is completely unmethylated in MCF-7/R cells.

3.3. Methylation status of the promoter region of *MGMT*, *GSTπ* and *Upa* genes

Fig. 4 shows the status of promoter methylation of *MGMT*, *GSTπ* and *Upa* genes in MCF-7 and MCF-7/R cells as detected by MSP. MCF-7 cells exhibit considerable degree of methylation of analyzed genes,

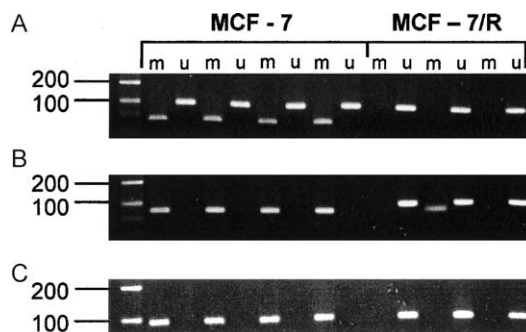


Fig. 4. Status of promoter methylation in *MGMT* (A), *GSTπ* (B) and *Upa* (C) genes determined by the methylation-specific PCR in MCF-7 and MCF-7/R cells. The presence of a visible PCR product in Lane M indicates the presence of methylated alleles of genes, and the presence of PCR product in Lane U indicates the presence of unmethylated alleles.

whereas its MCF-7/R drug resistant variant showed complete loss of methylation.

4. Discussion

In the present study, we have demonstrated that MCF-7 human breast cancer cell line displays high sensitivity to doxorubicin exposure. These cells did not express P-glycoprotein. Exposure of MCF-7 cells to increasing concentrations of DOX resulted in the establishment of MCF-7/R drug-resistant variant with high level of P-gp expression as detected by immunocytochemistry. Based on this finding, we conclude that development of drug resistance of MCF-7 cells to DOX is the result of changes in expression of P-glycoprotein. It is well established that the major mechanism in the development of drug resistance in cancer cells is the expression of an energy-dependent drug efflux pump, known alternatively as P-glycoprotein or the multidrug transporter [3]. In humans this efflux pump is the product of the *MDR1* gene. There is growing evidence that illustrates the presence of CpG methylation in the 5' regulatory region of *MDR1* gene is dominant factor in silencing *MDR1* transcription [10,11]. Recent studies have shown that CpG methylation of *MDR1* promoter is a frequent event in prostate [16] and bladder [11] cancer and is related to progression of the disease [16]. In the present study, we have shown that *MDR1* promoter is completely unmethylated in MCF-7/R cells, and

hypermethylated in the parental MCF-7 cells. This finding and the resulting expression of P-glycoprotein in MCF-7 and MCF-7/R cells clearly points to the inverse correlation between methylation status of *MDR1* promoter and expression of its product P-glycoprotein. It appears that acquisition of multidrug resistant phenotype in MCF-7 cells increases both the level of *MDR1* gene expression and hypomethylation of the 5' regulatory region of *MDR1* gene. The inverse correlation between over-expression of *MDR1* gene and the degree of methylation of promoter CpG sites of the *MDR1* gene after chemotherapeutic treatment provide support for our finding [11]. It has been shown that treatment of MCF-7 human adenocarcinoma cells and human epidermoid KB3-1 cancer cells with DNA methyltransferase inhibitor 5'-aza-2'-cytidine (5-azaC) resulted in hypomethylation of promoter of *MDR1* gene, increased expression of *MDR1*, and in an increase in generation of multidrug resistant clones [9,17]. However, despite the observed correlative evidence between expression of *MDR1* gene and status of its promoter methylation in the development of MDR, the causative role of these changes remains undefined, since changes in promoter methylation could directly contribute to activation of the *MDR1* gene, or be a consequence of expression changes.

P-glycoprotein is an important but not the only factor that contributes to the development of drug resistance. Activation of general response mechanisms that detoxify drugs and repair DNA damage is involved in acquired resistance of cancer cells to chemotherapy [3,4]. For this reason, we measured the status of promoter methylation of *GSTπ* and *MGMT* genes and found that the doxorubicin-resistant variant of MCF-7 cells exhibits loss of methylation within promoters of *GSTπ* and *MGMT* genes.

Another important emerging issue that is closely related to drug resistance is the invasiveness of tumor cells. Urokinase (*Upa*), a member of the serine protease family, plays an important role in several malignancies, including breast cancer. Expression of *Upa* gene is closely associated with breast cancer progression, breast cancer invasion, tumor growth, and metastases [18,19]. The dominant mechanism in the silencing of *Upa* gene is DNA methylation [18]. Highly invasive hormone-insensitive breast cancer cells highly expressed *Upa* gene, which is associated

with demethylation of *Upa* promoter. One of the properties of MCF-7 cells is their low invasiveness which is associated with high degree of methylation of *Upa* promoter region (81.3–90%) [18,19]. In the present study, we detected a high level of *Upa* promoter methylation in MCF-7 cells, and non-existent methylation in MCF-7R. Recently, it has been shown that a multidrug resistant variant of MCF-7 cells displays higher invasive potential compared to its parental counterpart [20], and that treatment of MCF-7 cells with 5-azaC increases its invasiveness [21].

In the present study we show that a doxorubicin resistant variant of MCF-7 cells substantially differs from its parental variant and is characterized by high expression of P-glycoprotein, loss of estrogen responsiveness, and loss of cytosine methylation of *MDR1*, *GSTπ* and *MGMT* chemoresistance related genes. In addition, the promoter region of *Upa* was also hypomethylated in these cells. Taking into consideration the fact of considerable difference in promoter methylation status of *MDR1*, *GSTπ*, *MGMT*, and *Upa* genes in MCF-7 and MCF-7/R cells, we suggest that acquisition of doxorubicin resistance of MCF-7 cells is associated with DNA hypomethylation. It is well known that MDR is not necessarily the result of treatment of cancer cells by anti-neoplastic drugs only; cells may become drug resistant prior to the drug treatment. Genetic, as well as epigenetic, changes may be connected to the development of MDR. Our findings suggest that factors leading to loss of methylation of the chemoresistance related genes may contribute to the development of MDR.

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