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Reversal of epigenetic aberrations associated with the acquisition of doxorubicin resistance restores drug sensitivity in breast cancer cells

Logeswari Ponnusamy, Prathap Kumar S. Mahalingaiah, Yu-Wei Chang, Kamaleshwar P. Singh*

Department of Environmental Toxicology, The Institute of Environmental and Human Health (TIEHH), Texas Tech University, Lubbock, TX 79409, USA

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ABSTRACT

Acquired resistance against doxorubicin is a major limitation in clinical treatment of breast cancer. The molecular mechanism behind the aberrant expression of genes leading to doxorubicin resistance is not clear. Epigenetic changes play an important role in the regulation of gene expression. Therefore, the objective of this study was to identify the epigenetic mechanism underlying acquired doxorubicin resistance in breast cancer cells. Doxorubicin-resistant cells were selected by repeated exposure of MCF-7 and MDA-MB-231 breast cancer cell lines to clinically relevant doses of doxorubicin for 18 months. MTT assay, cell cycle analysis, colony formation, qRT-PCR, and Western blot analyses were used to characterize the epigenetic and molecular mechanism. Pyrosequencing was used to detect MSH2 promoter hypermethylation. Aberrant expression of epigenetic regulatory genes, a significant increase in H3 acetylation and methylation, as well as promoter hypermethylation-mediated inactivation of MSH2 gene were associated with the acquired resistant phenotype. Demethylating agent 5-Aza-deoxycytidine and HDAC inhibitor Trichostatin A significantly re-sensitized resistant cells to doxorubicin. Findings of this study revealed that epigenetic aberrations including promoter hypermethylation-mediated inactivation MSH2 contribute to the acquisition of doxorubicin resistance in breast cancer cells. Additionally, our data suggest that some of these epigenetic aberrations are progressive during resistance development and therefore can potentially be used as biomarkers for early detection of resistance. These epigenetic aberrations, being reversible, can also serve as targets for epigenetic therapy to re-sensitize doxorubicin-resistant breast cancer cells. Epigenetic inactivation of mismatch repair gene MSH2 further suggests that loss of MMR-dependent apoptotic potential could be a novel mechanistic basis for the acquisition of doxorubicin resistance in breast cancer cells.

1. Introduction

Breast cancer is the most commonly diagnosed invasive cancer in women (ACS, 2017). Despite advances in the molecular marker for diagnosis and target characterization for breast cancer treatment, the acquired resistance to chemotherapy is still a major obstacle in successful clinical treatment. Acquired resistance in breast cancer has been reported for all commonly used clinical treatment including radiation (Bensimon et al., 2016), anti-estrogen (Shibata et al., 2017), and chemotherapy (Afghahi et al., 2017) in mono and/or combination therapy. Among the chemotherapeutic options, doxorubicin, an anthracyclic antitumor antibiotic is the mainstay treatment in breast cancer, either as single or in combination therapy (NCCN, 2017). Despite its potential cytotoxicity through multiple mechanisms (Pang et al., 2013; Yang et al., 2015), cancer cells eventually acquire resistance to doxorubicin-induced cytotoxicity. Though significant progress has been made in identifying the aberrantly regulated genes, the mechanism of acquired resistance development to doxorubicin in breast cancer cells is still unclear.

Chemoresistance is multifactorial and involves alteration of multiple genes (Housman et al., 2014). Various pharmacokinetic-based mechanisms such as reduced drug influx, increased drug efflux, drug inactivation, ROS generation as well as exosomes mediated detoxification (Gottesman et al., 2002; Pisco et al., 2014; Patel et al., 2017) have been reported for chemoresistance development. Pharmacodynamic-based mechanisms such as alteration in drug target, repression of

Abbreviations: IC50, concentration that inhibit 50% cell growth; MCF-7 ^{DR}, doxorubicin resistant MCF-7 cells; MDA-MB-231^{DR}, doxorubicin resistant MDA-MB-231 cells; MCF-7 ^{7T}, MCF-7 cells with 7th treatment; MDA-MB-231 ^{7T}, MDA-MB-231 cells with 7th treatment; ER, Estrogen receptor; EMT, Epithelial to Mesenchymal Transition; CSC, cancer stem cell; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); RIPA buffer, Radio immunoprecipitation assay buffer

* Corresponding author.
E-mail address: kamaleshwar.singh@ttu.edu (K.P. Singh).

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tumor suppressor genes, drug-induced senescence, altered DNA damage repair, acquisition of cancer-stem-cell (CSC)-like characteristics and epithelial to mesenchymal transition (EMT), enhanced angiogenesis, and tumor microenvironment have been demonstrated for breast cancer chemoresistance (Achuthan et al., 2011; Housman et al., 2014). Increasingly, altered estrogen receptor status (Jiang et al., 2012) and evasion of apoptosis and/or loss of apoptosis (Mohammad et al., 2015) have been shown to be associated with chemoresistance development. However, the molecular mechanism underlying the aberrant regulation of these pathway genes especially the loss of apoptosis during chemoresistance is not fully understood.

Accumulating evidence suggests that in addition to genetic mechanisms, epigenetic mechanisms such as DNA methylation and histone modifications, play an important role in the regulation of gene expression (Parrella, 2010; Sharma et al., 2010). Epigenetic aberrations resulting in altered expression of genes have been reported to contribute in tumorigenesis, progression/metastasis as well as acquired chemoresistance in breast cancer cells (Wilting and Dannenberg, 2012; Housman et al., 2014). Potential of epigenetic modifying agents in sensitizing resistance cells to radiation or chemotherapy by promoting apoptosis, reducing cancer stemness, and metastasis have been suggested (Candelaria et al., 2007; Munster et al., 2011; Housman et al., 2014; Longacre et al., 2016; Montenegro et al., 2016). Furthermore, DNA demethylating agents in combination with histone modifying agents have been evaluated in various cancers including breast cancer (Frew et al., 2008; Cacan et al., 2014; Lapinska et al., 2016; Pathania et al., 2016). Although some reports suggest the target genes aberrantly regulated by epigenetic mechanism in doxorubicin-resistant breast cancer cells, however, there are some limitations and unanswered questions that warrant further studies. For example, the concentration of doxorubicin or strategies used in those available studies for selecting doxorubicin resistance were clinically not relevant (Chekhun et al., 2007; Calcagno et al., 2008; Boettcher et al., 2010; Luzhna and Kovalchuk, 2010). Previous reports used either ER-positive or ER-negative breast cancer cells and there is no comprehensive analysis of epigenetic aberration in doxorubicin-resistant breast cancer cells of both subtypes parallel in one study (Chekhun et al., 2007; Calcagno et al., 2008; Boettcher et al., 2010). More importantly, the functional significance and contributions of epigenetic alterations in the development of drug-resistant phenotype are not clear. It is in this context, the present study used both estrogen receptor (ER) positive and triple negative breast cancer cell lines in parallel treated with a clinically relevant concentration of doxorubicin to select drug-resistant cells and identify the epigenetic aberrations involved during the acquisition of doxorubicin resistance. The role of epigenetic aberrations in acquired doxorubicin resistance was further confirmed by evaluating the effect of the reversal of epigenetic changes using DNA demethylating agent 5-Aza-2′deoxycytidine (5-Aza-2dC) and histone deacetylase inhibitor Trichostatin A (TSA) in doxorubicin-resistant cells.

2. Materials and methods

2.1. Development of in vitro doxorubicin-resistant breast cancer cells

Breast cancer cell lines, MCF-7, a luminal, non-invasive estrogen receptor positive, and MDA-MB-231, a basal, aggressive triple negative, were obtained from American Type Culture Collection (ATCC). Cells were cultured in DMEM/F12 medium supplemented with 5% FBS and 1% antibiotic and anti-mycotic solution and cultures were maintained in an incubator at 37 °C in a humidified atmosphere containing 5% CO_2.

Doxorubicin-resistant MCF-7 and MDA-MB-231 cell populations were established from the sensitive cell lines using clinically relevant concentration of doxorubicin of 100 nM (Kars et al., 2006; Pritchard et al., 2012) with intermittent exposure. Cells were exposed to 100 nM doxorubicin, the surviving populations were passaged in drug-free media for recovery and then exposed to next treatment. The treatment schedules were followed for 18 months, and resistance development was monitored regularly.

Resistance development to doxorubicin was evaluated based on change (increase) in IC_{50} value compared to corresponding sensitive cells. Sensitive and emerging resistance cells were exposed to different concentrations of doxorubicin (100 nM, 500 nM, 1 μM, 2 μM, and 3 μM) for 48 h, and then cytotoxicity was evaluated by MTT assay. IC_{50} values were estimated from dose-response curves using log-dose 4-parameter sigmoidal non-linear dose-response analysis in Graph Pad Prism software. Corresponding control MCF-7 and MDA-MB-231 cells were treated with 0.001% DMSO vehicle. Cells were maintained in doxorubicin free media for a week before using them for further evaluation.

2.2. 5-Aza-2′-deoxycytidine (de-methylation) and trichostatin A (HDAC inhibition) treatment

Stock solutions of 5-Aza-2′-deoxycytidine (5-Aza-2dC) and Trichostatin A (TSA) were prepared in DMSO and stored at −80 °C for further use. The concentration of 5-Aza-2dC (1 μM) and TSA (25 nM) for treatment of cells was selected based on the commonly used concentration for demethylation and HDAC inhibitory effects in cancer cells. To evaluate the effect of demethylation on doxorubicin sensitivity, resistant MCF-7 and MDA-MB-231 cells were pre-treated with 5-Aza-2dC for 48 h and subsequently co-treated with doxorubicin for additional 48 h. Similarly, resistant MCF-7 and MDA-MB-231 cells were pre-treated with TSA for 48 h and subsequently co-treated with doxorubicin for additional 48 h. Corresponding untreated control MCF-7 and MDA-MB-231 cells were treated with 0.001% DMSO vehicle. After following this treatment schedule, cells were used for cytotoxicity, cell cycle analysis, and in vitro soft-agar colony formation assay. For gene, and protein expression and pyrosequencing studies, resistant MCF-7 and MDA-MB-231 cells were treated either with 5-Aza-2dC or TSA for 72 h and samples were collected for analysis.

2.3. Cell viability/cytotoxicity evaluation by cell count and MTT assay

To evaluate the effect of doxorubicin on MCF-7 and MDA-MB-231 cells viability, the cell count was performed. MCF-7 and MDA-MB-231 sensitive and respective doxorubicin-resistant cells were seeded at a cell density of 1×10^4 cells per well into 12 well plates and allowed to attach and grow for 24 h. Cells were treated with 100 nM doxorubicin for 48 h and trypsinized to prepare a cell suspension. A total number of cells were counted using automated cell counter, and cell viability was calculated. Effect of doxorubicin-induced cytotoxicity on MCF-7 and MDA-MB-231 cells was evaluated by MTT assay as described previously (Ponnusamy et al., 2016). MCF-7 and MDA-MB-231 sensitive and doxorubicin-resistant cells were seeded into 96 well plates at a cell density of 2500 cells per well. The treatment protocol was individually followed for evaluations of cytotoxicity as described in treatment Section 2.2. MTT assay was performed after 48 h of doxorubicin treatment with or without 5-Aza-2dC or TSA. Cells were seeded triplicates, and the experiment was repeated twice.

2.4. Evaluation of doxorubicin-induced apoptosis by flow cytometry

Cell cycle analysis by flow cytometry was performed to examine the effect of doxorubicin on apoptosis induction in acquired resistance development. MCF-7 and MDA-MB-231 sensitive, as well as respective doxorubicin-resistant cells, were treated with either doxorubicin alone or in combination with 5-Aza-2dC or TSA as described in Section 2.2. Cells were harvested and used for cell cycle evaluation as described previously (Ponnusamy et al., 2016). Cell cycle was analyzed in Guava Easy-Cyte HT flow cytometer (Millipore) for a total count of 5000 events. All samples were evaluated in triplicates and the experiment was repeated twice.