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Treatment schedule and estrogen receptor-status influence acquisition of doxorubicin resistance in breast cancer cells

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ABSTRACT

Breast cancer is the most common cancer in women for which doxorubicin is still the mainstay treatment. However, chemotherapy resistance is a major limitation in breast cancer treatment. Role of treatment schedule and estrogen receptor (ER) status in subtypes of breast cancers in acquired resistance development is not clear. Therefore, objective of this study was to evaluate whether the treatment schedule and ER status in breast cancer cells influence the doxorubicin resistance. To address these questions, ER-positive MCF-7 and triple-negative MDA-MB-231 breast cancer cell lines were given either continuous or intermittent exposure with clinically relevant concentration of doxorubicin and the influence of these two treatment strategies on resistance to drug sensitivity was evaluated. Results revealed that intermittent treatment but not the continuous treatment induced resistance in breast cancer cells against doxorubicin. MCF-7 cells developed relatively earlier and high level of resistance when compared to MDA-MB-231 cells. Acquisition of epithelial to mesenchymal transition (EMT) and cancer stem cell-like phenotype was also observed during resistance development in MCF-7 cells. Changes in the expression of selected marker genes including drug transporters confirmed doxorubicin resistance in these cells. In summary, this study suggests that acquisition of resistance against doxorubicin depends on the treatment schedule of this drug as well as the estrogen receptor-based subtypes of breast cancer. Additionally, acquisition of EMT and stem cell-like phenotype further provided a molecular basis for breast cancer subtype-dependent chemotherapeutic resistance development. Findings of this study will have significant clinical implications in optimizing the chemotherapy schedule to minimize chemoresistance in breast cancer patients.

1. Introduction

Breast cancer is the most commonly diagnosed invasive cancer in women globally (ACS, 2017). Breast cancers are being treated with either local treatment such as surgery, radiation therapy or with systemic treatments such as chemotherapy, hormone therapy, targeted therapy and bone-directed therapy. However, they are not successful due to various limitations including acquired resistance. Advanced new therapeutic strategies such as high-dose chemotherapy with stem cell transplant have been tried with no significant positive outcome (Berry et al., 2011), whereas immunotherapies with vaccines are still being tested (Ernst and Anderson, 2015; Mittendorf and Peoples, 2016).

Chemotherapy, either as single or in combination therapy is the only choice of treatment for triple negative and/or basal-like breast cancer (TNBC/BLBC), and treatment of choice for estrogen receptor positive (ER +) breast cancer. Despite advanced therapeutic modalities, patients with metastatic breast cancer are failed to clinical treatment owing to rapid resistance development, leading to disease progression and death. Thus chemoresistance development is a major limitation in clinical treatment (Longley and Johnston, 2005). Chemoresistance may be of innate or acquired during treatment, and is inevitable in treatment of most of the cancers especially solid cancers such as breast cancer. Multiple factors have been identified in inducing chemoresistance (Gatti and Zunino, 2005; Kovalev et al., 2013; O'Reilly et al., 2015). For example, the levels of antioxidants such as glutathione (GSH) is known to play an important role in sensitivity to chemotherapy and induction of chemoresistance (Batrakova and Kabanov, 2008). Induction of apoptosis through generation of reactive oxygen species (ROS) by doxorubicin is one among various known mechanisms of action of this anticancer drug (Tsang et al., 2003; Mizutani et al., 2005; Trachootham et al., 2009). Reduced levels of GSH can enhance cellular sensitivity to chemotherapy-induced apoptosis (Batrakova and Kabanov, 2008). In contrast, elevated levels of antioxidant defense system can confer resistance to drug-induced ROS and that may lead to chemotherapy resistance in cancer cells (Landriscina et al., 2009; Trachootham et al., 2009; Ponnusamy et al., 2016). Though, these

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reports provide some mechanistic basis for doxorubicin resistance development in cancer cells, further studies are needed to fully understand the precise mechanism underlying the doxorubicin resistance development in cancer cells and identify the molecules that can be targeted to inhibit resistance development.

Chemotherapy is being administered in either dose-dense schedule or standard schedule involving multiple cycles of chemotherapy with intermittent recovery period. While single agents are being administered as continuous therapy, combination therapy is administered in an intermittent schedule. Clinically, patients are treated with doxorubicin either as single agent or in combination with either paclitaxel, docetaxel, cyclophosphamide, 5-Flurouracil, trastuzumab or pertuzumab depending on the subtype of the breast cancer (NCCN, 2016). As a single agent for metastatic or recurrent breast tumor, doxorubicin is either being employed at 20 mg/m^2 or 60–75 mg/m^2 with repeated administration either weekly or once in 21 days respectively. In case of combination therapy, depending upon the combination agents, doxorubicin is either given as dose-dense schedule where standard dose of doxorubicin (60 mg/m^2) is repeated every 2 weeks for 4 cycles to achieve maximal killing of cancer cells or by standard schedule where standard dose is repeated once in 3–4 weeks for 6 cycles to allow patients to recover from chemotherapeutic damage (NCCN, 2016). Regardless of its effectiveness, acquired resistance to doxorubicin limits it therapeutic efficacy.

It is not clear how the clinical treatment schedule with either continuous or intermittent exposure influence acquired drug resistance. Despite reports suggesting that treatment phase is unimportant based on existence of inherent resistance (Komarova and Wodarz, 2005), dynamic selection of acquired resistant tumor cell population during the multiple cycles of therapy emphasizes the influence of chemotherapy dosing schedule on the resistance induction (De Souza et al., 2011). Chemotherapy at the maximum tolerated dose necessitates recovery period in between treatment schedules, and thus favor cancer cells to repair the damage to induce chemoresistance. In contrast, administering continuous, low dose chemotherapy might eliminate the need for recovery period thus induce more cytotoxicity (Browder et al., 2000). However, clinical resistance development has been reported in both treatment strategies, and these strategies are similar to high-dose and chronic low-dose selection based resistance development in the laboratory settings. Since various treatment schedules and strategies being used in clinic, studying how cancer cells acquire chemoresistance *in vitro* should complement the clinical relevancy.

In vitro models are being commonly used to understand the mechanism of drug resistance. Depending on the cancer type and drug employed, development of *in vitro* drug resistance cell model takes anywhere between 10 weeks to 2 years (McDermott et al., 2014). In the case of breast cancer, it varies with type of parental cell lines selected, type of selecting agent and the dose, and optimization of treatment interval/schedule. In addition, resistance may persist after prolonged storage (Davies et al., 2009) or may decrease by continuous culturing in drug free medium (Twentyman et al., 1986; Watson et al., 2007; Xu et al., 2016). Multiple researchers defined their resistance cell model by either selecting survived clones following single exposure (Smith et al., 2006) to single or combination of chemotherapeutic drugs or by following exposure to incremental dose for a week long (Chekhun et al., 2007; Davies et al., 2009) or months (AbuHammad and Zihlif, 2013) and year-long exposure (Kars et al., 2006; Tegze et al., 2012). In addition, previous models reportedly used range of doxorubicin concentrations with either dose incremental or time incremental based selection demonstrating different levels of resistance index (Park et al., 2004; Kars et al., 2006; Chekhun et al., 2007; Tegze et al., 2012; Braunstein et al., 2016; Xu et al., 2016). However, strategies followed for resistance development in these studies are not consistent, therefore, conclusion drawn from these studies may not be relevant to clinical resistance observed in patients.

In addition to treatment schedule, differential effectiveness of chemotherapy in ER + and basal-like triple negative breast cancer subtypes further question the role of receptor status in the response as well as resistance development to chemotherapy. There are no studies to differentially evaluate the doxorubicin resistance in ER + and basal-like breast cancer cell lines using clinically followed treatment strategies as well as clinically relevant concentration. Since chemoresistance being major obstacle for successful clinical treatment, *in vitro* resistance cell models reflecting clinical treatment strategies could compliment the understanding of acquired resistance in patients. Therefore, objective of this study was to evaluate the impact of different treatment strategies and estrogen receptor status on doxorubicin resistance using *in vitro* cell model. To address this objective, most commonly used representative of luminal, non-invasive estrogen receptor positive breast cancer cell line MCF-7, and a basal, aggressive triple negative breast cancer cell line MDA-MB-231 were used in this study. As representative of clinically relevant treatment schedules, cells were treated with two different strategies, (a) continuous exposure by maintaining cells all the time in drug containing media, and (b) intermittent exposure in which cells were first grown in media with drug, and then after a passage grown in media without drug and this pattern of drug exposure followed by a recovery period without drug was repeated.

2. Materials and methods

2.1. Reagents

Doxorubicin HCl, and 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Trypan blue dye, Cell cycle reagent (Guava), RIPA lysis buffer (1 ×) and PCR reagents were procured from Amresco LLC. (Solon, Ohio, USA), Millipore (Hayward, California, USA), Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA) and BioRad, Inc. (Hercules, California, USA) respectively. DMEM/F12 medium, Cancer stem cell medium-Premium, trypsin/EDTA and Trizol reagents were acquired from Hyclone laboratories, Inc. (Logan, Utah, USA), ProMab Biotechnologies, Inc. (Richmond, California, USA), and Invitrogen Inc. (Carlsbad, California, USA) respectively. Fetal bovine serum (FBS), and antibiotic/anti-mycotic solution were obtained from Life technologies (Carlsbad, California, USA).

2.2. Cell lines and culture conditions

Human breast cancer MCF-7, luminal, non-invasive estrogen receptor positive cell line and MDA-MB-231, a basal, aggressive triple negative breast cancer cell lines were obtained from American Type Culture Collection (ATCC). After an initial procurement, subsequent *in vitro* expansion was followed and cell lines were maintained in DMEM/F12 medium supplemented with 5% FBS and 1% antibiotic and antimycotic solution at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Doxorubicin concentration, treatment strategies and development of in vitro doxorubicin resistant breast cancer cells

Doxorubicin resistant MCF-7 and MDA-MB-231 cell lines were established from the sensitive parental cell lines using doxorubicin at the concentration of 100 nM. Previous studies on doxorubicin resistance breast cancer cell models have used range of doxorubicin concentrations with dose incremental and/or time incremental strategies (Kars et al., 2006; AbuHammad and Zihlif, 2013; Felipe et al., 2014; Kim et al., 2014; Xu et al., 2016). In the present study, doxorubicin at concentration of 100 nM that has been shown as clinically relevant concentration (de Bruijn et al., 1999; Kars et al., 2006; Pritchard et al., 2012) was used without any dose increment to develop/establish *in vitro* model of doxorubicin resistance. Concentra-