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Redox-proteomic analysis of doxorubicin resistance-induced altered thiol activity in uterine carcinoma

Szu-Ting Lin[a,1], Yi-Wen Lo[b,1], Shing-Jyh Chang[c,d], Wen-Ching Wang[e], Margaret Dah-Tsyr Chang[e], Ping-Chiang Lyu[a], Yi-Wen Chen[a], Hsiu-Chuan Chou[b,**], Hong-Lin Chan[a,*]

[a] Department of Medical Science and Institute of Bioinformatics and Structural Biology, National Tsing Hua University, Hsinchu, Taiwan
[b] Department of Applied Science, National Hsinchu University of Education, Hsinchu, Taiwan
[c] Department of Obstetrics and Gynecology, MacKay Memorial Hospital Hsinchu Branch, Hsinchu, Taiwan
[d] Department of Nursing, Yuanpei University, Hsinchu, Taiwan
[e] Department of Medical Science and Institute of Molecular and Cellular Biology, National Tsing Hua University, Hsinchu, Taiwan

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ABSTRACT

Doxorubicin is an anticancer drug used in a wide range of cancer therapies; however, doxorubicin-induced drug resistance is one of the most serious obstacles of cancer chemotherapy. Recent studies have indicated that reduced oxidative stress levels in cancer cells induce drug resistance. However, the redox-modifications of resistance – associated cellular targets are largely unknown. Thus, the current study employed cysteine-labeling based two-dimensional differential gel electrophoresis (2D-DIGE) combined with MALDI-TOF mass spectrometry (MALDI-TOF MS) to analyze the effect of doxorubicin resistance on redox regulation in uterine cancer and showed 33 spots that were significantly changed in thiol reactivity. These proteins involve cytoskeleton regulation, signal transduction, redox-regulation, glycolysis, and cell-cycle regulation. The current work shows that the redox 2D-DIGE-based proteomic strategy provides a rapid method to study the molecular mechanisms of doxorubicin-induced drug resistance in uterine cancer. The identified targets may be used to further evaluate their roles in drug-resistance formation and for possible diagnostic or therapeutic applications.

1. Introduction

Drug resistance is one of the main obstacles during the process of cancer chemotherapy. The exact mechanism of drug resistance is complex and poor understood. The most potential factors for resistance include over-expression of ABC transporter, reduced drug uptake, enhanced drug detoxification, decreased apoptosis and increased DNA repair system [1]. Doxorubicin is one of the anti-cancer drug has been used clinically for decades to treat a number of cancers, such as breast cancer, lung cancer and many other carcinoma types [2–5]. However, some of the side effects of doxorubicin treatment have been reported and one of these is the doxorubicin-induced drug resistance.

Reactive oxygen species (ROS) are species of oxygen which are in a more reactive state than molecular oxygen, and in which the oxygen is reduced to varying degrees. ROS comprise several species such as hydrogen peroxide, the hydroxyl radical, superoxide and singlet oxygen. $O2^-$ can be generated by the action of such enzymes as NADPH oxidase, lipoxygenase, cyclooxygenase, cytochrome P450 or through UV irradiation and can be converted into H_2O_2 and O_2 by the action of superoxide dismutases. H_2O_2 can be also further converted to· OH in the presence of Fe^{2+} [6,7]. In general, high concentrations of ROS have been observed in most cancers, in which these ROS promote cancer progression and development. Numerous anticancer drugs including doxorubicin work by further increasing cellular concentrations of ROS to overcome the detoxification and anti-oxidant ability of the cancer cells [8]. Recent studies indicated that adaptation of the concentrations of intracellular anti-oxidants could result in drug resistance. For example, reduced glutathione levels are increased in numerous cancers that show elevated resistance against chemotherapeutic drugs [9,10]. These processes are mediated by intracellular redox-regulation enzymes such as alpha-glutamycysteine synthetase [11], catalase [12] and glutathione reductase [13].

Abbreviations: 2D-DIGE, two-dimensional differential gel electrophoresis; CCB, colloidal coomassie blue; ICy dyes, iodoacetyl cyanine dyes; MALDI-TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; mPR, membrane-associated progesterone receptor component 1; ROS, reactive oxygen species.

* Corresponding author at: Institute of Bioinformatics and Structural Biology & Department of Medical Sciences, National Tsing Hua University, No. 101, Kuang-Fu Rd. Sec. 2, Hsinchu 30013, Taiwan. Tel.: +886 3 5742476; fax: +886 3 5715934.
** Corresponding author. Tel.: +886 3 5213132x2721; fax: +886 3 5257178.
E-mail addresses: chouhc@mail.nhcue.edu.tw (H.-C. Chou), hlchan@life.nthu.edu.tw (H.-L. Chan).
[1] These authors contributed equally to this work.

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Proteomics is a powerful tool to monitor protein expression and post-translational modification of proteins in response to specific treatment. 2-DE remains an important technique in proteomics for global protein profiling within biological samples and plays a complementary role to LC–MS-based analysis. However, reliable quantitative comparison between gels remains the primary challenge in 2-DE analysis. A significant improvement in gel-based protein detection and quantification was achieved by the introduction of 2D-DIGE, where several samples can be co-detected on the same gel using differential fluorescent labeling. This approach alleviates gel-to-gel variation and allows comparison of the relative amount of resolved proteins across different gels using a fluorescently-labeled internal standard. Moreover, the 2D-DIGE technique has the advantages of a broader dynamic range of detection, higher sensitivity and greater reproducibility than traditional 2-DE [14]. Recently, a cysteine labeling version of 2D-DIGE was developed, using ICy dyes (iodoacetyl cyanine dyes) which react with the free thiol group of cysteines *via* alkylation. The paired of ICy dyes (ICy3 and ICy5) have been used to monitor redox-dependent protein thiol modifications in model cell systems [15,16].

In our previous publication, 37 proteins have been reported to show differentially expressed between uterine cancer cell and its derived resistant line. In which, asparagine synthetase and membrane-associated progesterone receptor component 1 (mPR) are both evidenced to be essential for the formation of doxorubicin-induced drug resistance [5]. Followed study demonstrated that decreased oxidative stress levels were observed in doxorubicin resistance cancer cells; however, the redox-modifications of resistance-associated cellular targets have not been reported in our knowledge. Accordingly, the aim of this investigation was to conduct an *in vitro* investigation into doxorubicin-induced drug resistance using quantitative redox-proteomic strategies including ICy dyes-based labeling and MALDI-TOF MS to monitor redox-dependent protein thiol modifications, to increase the understanding of the molecular processes involved, and to identify potential drug resistance biomarkers with possible diagnostic or therapeutic applications.

2. Materials and methods

2.1. Chemicals and reagents

Generic chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), while reagents for 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden). The synthesis of the ICy3 and ICy5 dyes has been previously reported in previous publication [17]. All primary antibodies were purchased from Genetex (Hsinchu, Taiwan) and anti-mouse, and anti-rabbit secondary antibodies were purchased from GE Healthcare. All the chemicals and biochemicals used in this study were of analytical grade.

2.2. Cell lines and cell culture

The uterine sarcoma cancer line MES-SA was purchased from American Type Culture Collection, (Manassas, VA, USA). The doxorubicin resistance line MES-SA/DxR cell was cultured in McCoy's 5a modified medium containing 10% fetal bovine serum, L-glutamine (2 mM), streptomycin (100 μg/mL), penicillin (100 IU/mL) (all from Gibco-Invitrogen Corp., Paisley, UK) and maintained with 0.6 μM doxorubicin. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged at 80–90% confluence by trypsinization according to standard procedures.

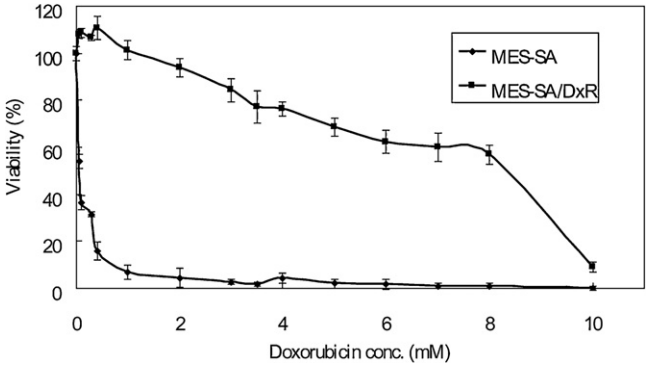

Fig. 1. Dose-dependent kinetics of doxorubicin-induced loss of cell viability in MES-SA and MES-SA/DxR cells. MES-SA and MES-SA/DxR cells grown overnight were treated with a range of doses of doxorubicin and cell viability was determined by MTT assay.

2.3. Assay for endogenous reactive oxygen species using DCFH-DA

MES-SA and MES-SA/DxR cells (10,000 cells/well) were incubated with the indicated concentrations of doxorubicin for 20 min. After two washes with PBS, cells were treated with 10 μM of 2,7-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes) at 37 °C for 20 min, and subsequently washed with PBS. Fluorescence was recorded at an excitation wavelength 485 nm and emission wavelength at 530 nm with Gemini fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. MTT cell viability assay

MES-SA and MES-SA/DxR cells growing exponentially were trypsinized, counted using a haemocytometer and 10,000 cells/well were seeded into 96-well plates. The culture was then incubated for 24 h before pre-treatment with the indicated concentrations of doxorubicin for 20 min or left untreated. After removal of the medium, 50 μL of MTT working solution (1 mg/mL) was added to the cells in each well, followed by a further incubation at 37 °C for 4 h. The supernatant was carefully removed. 100 μL of DMSO was added to each well and the plates shaken for 20 min. The absorbance of samples was then measured at 540 nm in a multi-well plate reader. Values were normalized against the untreated samples and were averaged from 4 independent measurements.

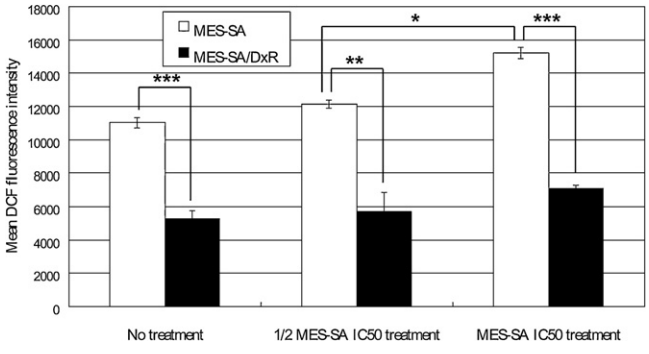

Fig. 2. Effect of doxorubicin-resistance on MES-SA and MES-SA/DxR ROS levels. 100,000 MES-SA and MES-SA/DxR cells were used for DCFH-based intracellular ROS production assays. The cells were treated with the indicated concentrations of doxorubicin for 20 min followed by treated with 10 μM of DCFH-DA at 37 °C for 20 min and the levels of cellular ROS were determined with fluorescence reader to record at excitation and emission wavelengths of 485 nm and 530 nm, respectively. All of statistic comparisons used in this study were performed with paired Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences between the experiments.