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Identification of up- and down-regulated proteins in doxorubicin-resistant uterine cancer cells: Reticulocalbin-1 plays a key role in the development of doxorubicin-associated resistance

Eugenie Wong Soon May[a,1], Szu-Ting Lin[a,1], Chi-Chen Lin[b,c,d,e,1], Jo-Fan Chang[a], Eric Hung[f], Yi-Wen Lo[f], Li-Hsun Lin[a], Ren-Yu Hu[a], Chi-Lun Feng[f], Dai-Ying Lin[a], Shine-Bei Wu[f], Wen-Chi Lee[f], Kevin W. Lyu[g,h], Hsiu-Chuan Chou[f,**], Hong-Lin Chan[a,*]

[a] Institute of Bioinformatics and Structural Biology and Department of Medical Sciences, National Tsing Hua University, Hsinchu, Taiwan
[b] Institute of Biomedical Science, National Chung-Hsing University, Taichung, Taiwan
[c] Institute of Biomedical Science and Rong Hsing Research Center for Translational Medicine, National Chung-Hsing University, Taiwan
[d] Department of Medical Research and Education, Taichung Veterans General Hospital, Taichung, Taiwan
[e] Division of Chest Medicine, Department of Internal Medicine, Changhua Christian Hospital, Changhua, Taiwan
[f] Department of Applied Science, National Hsinchu University of Education, Hsinchu, Taiwan
[g] Lutheran Medical Center, Brooklyn, NY, USA
[h] Global Scholars Program, St. George's University/Northumbria University, Newcastle upon Tyne, United Kingdom

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ABSTRACT

Drug resistance is a frequent cause of failure in cancer chemotherapy treatments. In this study, a pair of uterine sarcoma cancer lines, MES-SA, and doxorubicin-resistant partners, MES-SA/DxR-2 μM cells and MES-SA/DxR-8 μM cells, as a model system to investigate resistance-dependent proteome alterations and to identify potential therapeutic targets. We used two-dimensional differential gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to perform this research and the results revealed that doxorubicin-resistance altered the expression of 208 proteins in which 129 identified proteins showed dose-dependent manners in response to the levels of resistance. Further studies have used RNA interference, H2A.X phosphorylation assay, cell viability analysis, and analysis of apoptosis against reticulocalbin-1 (RCN1) proteins, to prove its potency on the formation of doxorubicin resistance as well as the attenuation of doxorubicin-associated DNA double strand breakage. To sum up, our results provide useful diagnostic markers and therapeutic candidates such as RCN1 for the treatment of doxorubicin-resistant uterine cancer.

Introduction

Doxorubicin, an anticancer drug, is widely used in treating a wide spectrum of cancer types [1–3]. The action mechanism of doxorubicin is complicated; briefly, doxorubicin has been reported to interfere DNA replication by intercalated into DNA molecules, thus inhibiting the biosynthesis of DNA, RNA and protein. In addition, doxorubicin has been shown to inhibit topoisomerase 2 activity and resulted in the breakage of DNA double strands and preventing DNA synthesis. Moreover, doxorubicin directly damages cancer cells by disturbing nuclear function resulting in cell death [4,5]. Nevertheless, doxorubicin-induced chemotherapy resistance has been widely reported in cancers such as osteosarcoma, leukemia, breast cancer, lung cancer, and uterine cancer [6–10], and is a major therapeutic obstacle preventing the successful treatment of patients receiving cancer chemotherapy.

Chemotherapy resistance diminishes the effectiveness of anticancer drugs used in attacking tumors. In clinical research, chemotherapy resistance has been identified as a rigorous problem when the concentrations of chemotherapy drugs reach toxic and harmful doses killing tumors. Previous studies have also revealed that cellular mechanisms associated with drug resistance, including the enhanced activity of plasma membrane-embedded drug efflux

* Corresponding author at: Institute of Bioinformatics and Structural Biology, 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 35213132; fax: +886 3 5257178.
E-mail addresses: hlchan@life.nthu.edu.tw (H.-C. Chou), chouhc@mail.nhcue.edu.tw (H.-L. Chan).
[1] These authors contributed equally to this paper.

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transporters, increased expressions of detoxification enzymes and the modulation of cellular signal transduction pathways that control cell death and cell survival [11]. Nevertheless, chemotherapy resistance cannot be understood completely by these mechanisms. Accordingly, comprehensively investigating additional resistance mechanisms that have not yet been clarified are essential.

In our previous proteomic analysis, we identified numerous proteins, including asparagine synthetase and membrane-associated progesterone receptor component 1, involved in various drug-resistance-forming mechanisms [12]. In this study, to perform an in vitro investigation of doxorubicin-resistance mechanisms in uterine cancer, increase our understanding of the molecular processes involved, and identify potential resistance biomarkers with possible diagnostic or therapeutic applications, we established a pair of uterine sarcoma cancer lines, MES-SA, and doxorubicin-resistant partners, MES-SA/DxR-2 μM cells and MES-SA/DxR-8 μM cells, as a model system for investigating resistance-dependent protein alterations by conducting quantitative proteomic analysis using 2D-DIGE and MALDI-TOF mass spectrometry. In addition, this paper performed studies that used RNA interference against a selected identified protein, reticulocalbin-1 (RCN1), to monitor and evaluate its potency against doxorubicin chemotherapy resistance.

Materials and methods

Chemical and reagents

Generic chemicals were purchased from USB corporation company (Santa Clara, CA, USA), while reagents for 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden). All primary antibodies were purchased from Genetex (Hsinchu, Taiwan) and anti-mouse and anti-rabbit secondary antibodies were purchased from GE Healthcare (Uppsala, Sweden). All the chemicals and biochemicals used in this study were of analytical grade.

Cell lines and cell cultures

The uterine sarcoma cancer line MES-SA was purchased from American Type Culture Collection, (Manassas, VA, USA) and 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). The doxorubicin resistance lines MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cell were both derived from MES-SA through stepwise increasing the doxorubicin concentrations in medium and were cultured in the same medium and supplement with 0.2 μM and 0.8 μM doxorubicin, respectively. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

MTT cell viability assay

The detailed MTT experimental procedure has been described in our previous study [12].

2D-DIGE, gel image analysis, protein staining, in-gel digestion and MALDI-TOF MS analysis

For 2D-DIGE analysis, protein samples were labeled with N-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3 and Cy5. Briefly, 100 μg of protein sample in triplicate was minimally labeled with 250 pmol of either Cy3 or Cy5 for comparison on the same 2-DE gel. To facilitate image matching and cross-gel statistical comparison, a pool of all samples was also prepared and labeled with Cy2 at a molar ratio of 2.5 pmol Cy2 per μg of protein as an internal standard run on all gels. The detailed experimental procedures have been described in our previous study [13–15]. For mass spectrometry analysis, peaks in the mass range of m/z 800–3000 were used to generate a peptide mass fingerprint that was searched against the Swiss-Prot/TrEMBL database (v57.12) with 513,877 entries using Mascot software v2.2.06 (Matrix Science, London, UK). The following parameters were used for the search: *Homo sapiens*; tryptic digest with a maximum of 1 missed cleavage; carbamidomethylation of cysteine, partial protein N-terminal acetylation, partial methionine oxidation and partial modification of glutamine to pyroglutamate and a mass tolerance of 50 ppm. Identification was accepted based on significant MASCOT Mowse scores ($p < 0.05$), spectrum annotation and observed versus expected molecular weight and p*I* on 2-DE as well as at least 5 peptides in each identified protein.

Immunoblotting analysis

Immunoblotting analysis was used to validate the differential abundance of mass spectrometry identified proteins across MES-SA, MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells. The detailed experimental procedure has been described in our previous study [16].

siRNA design, construction and transfection

The siRNA against RCN1 was synthesized by Invitrogen. The targeting sequences 5′-GCC ACU GGA UCC UCC CUC AAG AUU A-3′ against RCN1 were designed and verified to be specific by Blast search against the human genome, and sequences of similar GC contents which do not match any known human coding sequence were used for negative control against RCN1. Transfection was mediated with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction. Briefly, cells were transfected with 30 nM of RCN1 siRNA or the corresponding control (pGCsi-control) in serum free medium containing Lipofectamine RNAiMAX for 4 h followed by recovered in medium containing 10% FCS for 24 h. The efficiency of siRNA knockdown was monitored with immunoblotting by using primary antibodies against RCN1.

Flow cytometry analysis for apoptosis detection

Annexin-V/propidium iodide (PI) double assay was performed using the Annexin V, Alexa Fluor® 488 Conjugate Detection kit (Life technologies). The detailed experimental procedure has been described in our previous study [17].

Statistical analysis

Comparisons of protein expression between resistant and sensitive cells were performed using Student's *t*-test. Statistical analyses of the MTT assays were performed using Student's *t*-test, too. For all tests, a p-value of less than 0.05 was considered to be significant.

Results

Establish of doxorubicin-resistance uterine cancer lines MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells from MES-SA cells

In this study, we grew a doxorubicin-sensitive uterine cancer cell line, MES-SA, in a doxorubicin-free medium. Moreover, the doxorubicin resistance lines MES-SA/DxR-2 μM and MES-SA/DxR-8 μM cells were both derived from MES-SA cells by means of stepwise increasing the doxorubicin concentrations in culture medium followed by stably cultured in the same medium and supplement with 0.2 μM and 0.8 μM doxorubicin, respectively, to keep the doxorubicin resistance phenotype. The doxorubicin-resistant