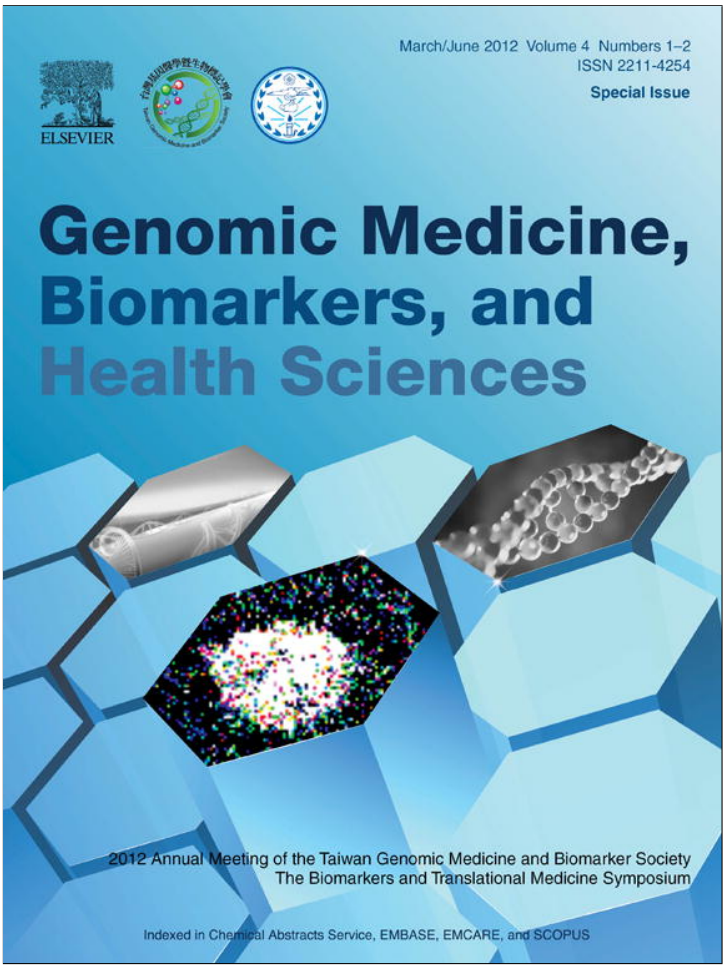

Genomic Medicine, Biomarkers, and Health Sciences (2012) 4, 57–60

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SHORT COMMUNICATION

Proteomic analysis of mitochondrial proteins in doxorubicin-resistant cancer cells

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Received 11 March 2012; accepted 30 March 2012
Available online 10 May 2012

KEYWORDS
uterine cancer;
doxorubicin;
drug resistance;
mitochondrial proteins

Abstract Human uterine cancer occurs most often in menopause women between the ages of 55 and 70 years. In the initial stage, the symptoms of uterus have an abnormal vaginal bleeding or abnormal discharge, and the common treatment is to remove the uterus or treat with chemotherapeutic drugs. For most type of cancers, doxorubicin is frequently prescribed for treating a wide range of various types of cancers, including hematologic malignancies, lymphoma, myeloma, sarcoma, and uterine cancer. However, most cancer cells will become drug insensitivity or resistance via unknown mechanisms. Since mitochondria play a crucial role in the induction apoptosis, it is intriguing to clarify whether doxorubicin-resistance is mediated through mitochondrial protection. In this study, a highly doxorubicin-resistant cell line has been established by continuously culturing the MES-SA/Dx5, a commercial doxorubicin-resistant human uterine cancer cell line, in the presence of doxorubicin for up to 2 years. Mitochondrial proteins from nondrug-resistant MES-SA, low resistant MES-SA/Dx5 (MES-SA/Dx5^{low}), and high resistant MES-SA/Dx5 (MES-SA/Dx5^{high}) were enriched, and mitochondrial proteome is characterized by two dimensional-differential gel electrophoresis and matrix-assisted laser desorption/ ionization time of flight mass spectrometer (MALDI-TOF MS).

Introduction

Uterine cancer is one of the ten leading causes of death of cancer and the most commonly diagnosed gynecologic cancer. The most common type of uterine cancer is called endometrial cancer because it forms in the lining of uterus called the endometrium. Most endometrial cancers are

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2211-4254/$36 Copyright © 2012, Taiwan Genomic Medicine and Biomarker Society. Published by Elsevier Taiwan LLC. All rights reserved.
doi:10.1016/j.gmbhs.2012.04.008

adenocarcinomas. Endometrial cancer is often diagnosed using an endometrial biopsy or dilation and curettage (D&C) when a woman presents with symptoms. Medicines, such as doxorubicin used alone or in combination with cisplatin, paclitaxel, and so on, may be given after surgery for endometrial cancer depending on the stage of the cancer and the risk for the cancer to spread or recur. However, chemotherapeutic uterine cancer is a major impediment to cancer therapy because long-term drug use can affect drug efficacy through changes in cell cycle, apoptosis pathway, metabolism, DNA repair, and drug efflux.[1] Mitochondria play important roles in cellular energy metabolism, free-radical generation, and apoptosis. Defects in mitochondrial function have been proposed to contribute todrug-resistance pathway activation.[2,3,4,5] Herein, we tested the hypothesis that mitochondrial alteration and their led to drug-resistance.

Materials and methods

Cell line and culture

Cell lines used in this study are MES-SA, MES-SA/Dx5^{low} and MES-SA/ Dx5^{high}. Human uterine sarcoma cell line MES-SA and its doxorubicin selected drug resistant cell line MES-SA/ Dx5^{low} are purchased from ATCC (Washington, DC, USA).[5] MES-SA/ Dx5^{high}, derived from MES-SA/Dx5^{low}, is a multi-drug resistant (MDR) variant that possess both self-renewal capabilities and the expression of p-glycoprotein (MDR-1) by constitutively selection with doxorubicin (data not shown). MES-SA/Dx5^{low} and MES-SA/Dx5^{high} were cultured in McCoy' 5A with 1.5 g/L sodium bicarbonate containing 10% (v/v) fetal bovine serum and 1 IU/mL penicillin/ streptomycin at 37 °C and 5% CO_2.

Sample preparation

Firstly, 5 × 10^7 of MES-SA, MES-SA/Dx5^{low} and MES-SA/ Dx5^{high} cells were collected and washed with ice-cold buffer solution phosphate buffered saline (PBS). Mitochondria-enriched fractions are isolated using the mitochondria/cytosol fractionation kit (Millipore, MA, USA), 1 ml of 1 × cytosol extraction buffer containing redox

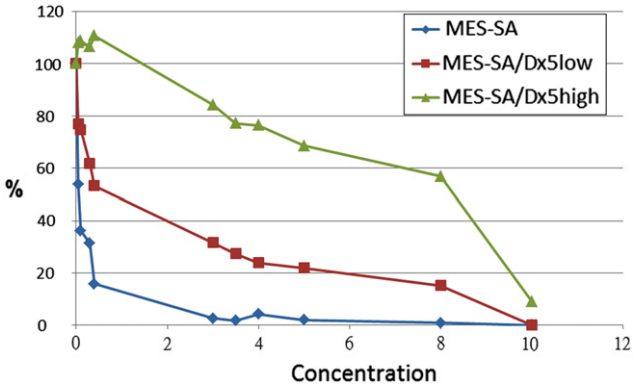

Figure 1 Effect of doxorubicin on the viability of uterine cancer cells.

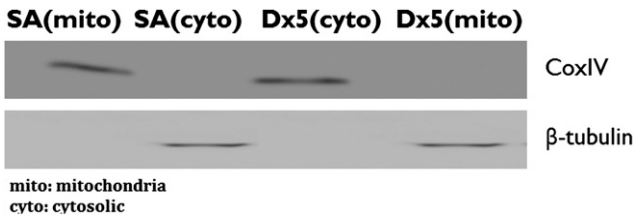

Figure 2 Western blot analysis of Tubulin-beta and COX IV in cytosolic and mitochondrial fractions of uterine cancer cells.

reagent dithiothreitol (DTT) and protease inhibitors on ice for 10 minutes. Later, cells were homogenized in an ice-cold of tissue grinder, and then centrifuged at 700× g for 10 minutes at 4 °C. The supernatant was centrifuged at 10,000× g for 30 minutes at 4 °C. The cell pellet was mitochondrial-enriched fraction.[6]

Doxorubicin dose response curve

Uterine sarcoma cells were seeded in 96-well plates and treated with various concentrations of doxorubicin for 24 hours. MTT cell viability assay was performed to measure the activity of mitochondrial succinate dehydrogenase that reduce MTT [3- (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to formazan dyes. After removal of the medium, 50 μl of MTT working solution was added to the cells in each well followed by incubation at 37 °C for 4 hours. The supernatant was carefully removed. One hundred microliters of dimethyl sulfoxide (DMSO) was added to each well and the plates were shaken for 20 minutes. The absorbance of the samples was then measured at a wavelength of 540 nm in a multiwell-plate reader.

Two dimensional-differential gel electrophoresisanalysis

Before performing two dimensional-differential gel electrophoresis (2D-DIGE), the samples were labeled with the

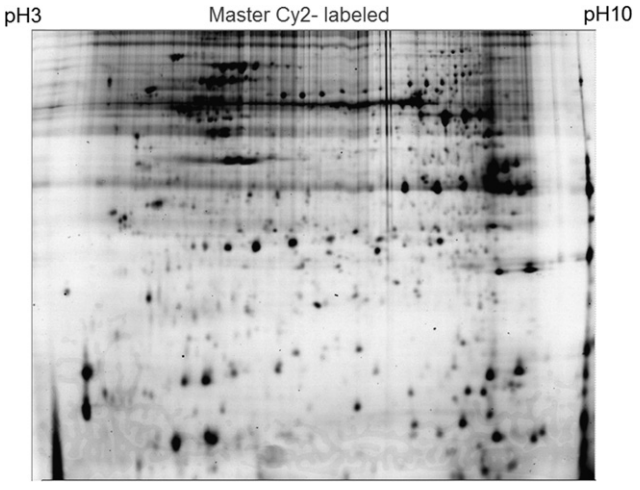

Figure 3 Representative 2D-DIGE image showing the separation of mitochondrial proteins of uterine cancer cells.

Table 1 Protein identification of differentially expressed mitochondrial proteins between doxorubicin-sensitive and doxorubicin-resistant cells.

Spot number	Protein name	isoelectric point (pI)	Molecular weight	Number of matched peptides	Coverage (%)	Score	Matched peptides
434	1	4.29	48283	8/20	17	79/56	EQFLDGDGWTSR
482	2	7.96	58470	5/9	15	72/56	NTGIICTIGPASR
528	3	6.3	55454	5/14	10	61/56	QAFQIGSPWR
593	4	5.06	53676	8/24	18	62/56	TYSLGSALRPSTSR
628	5	7.71	56251	6/17	58	58/56	LLWNWWK
653	6	6.3	55454	9/24	19	82/56	QAFQIGSPWR
661	7	5.06	53676	7/40	15	63/56	SYVTTSTR
666	8	6.07	6756	4/19	20	66/56	MQCQLFR
667	9	6.02	78265	6/22	10	57/56	QQISGIQPHGLPNALDDR
669	10	5.43	35806	5/25	15	61/56	APLDLDKYVEIAR
671	11	5.75	161030	8/19	4	61/56	EQIEHHPR
680	12	5.84	46161	4/10	12	60/56	HEFPFR
681	13	5.84	46161	4/13	12	58/56	MGGEAGCAAAVGAEGR
691	14	6.1	10544	5/20	44	68/66	EVGQLAETQR
706	15	4.87	74959	7/26	14	56/56	VYGAQNEMCLATQQLSK
717	16	6.3	55454	11/23	23	81/56	QAFQIGSPWR
748	17	6.3	55454	9/24	17	76/56	QAFQIGSPWR
748	18	6.3	55454	10/19	20	105/56	QAFQIGSPWR
774	19	5.26	56525	5/12	12	64/56	LVLEVAQHLGESTVR
785	20	6.56	104912	10/26	10	56/56	EEIVKAGLK
804	21	6.56	104912	10/31	11	57/56	KLAQALLEQGGR
818	22	7.01	47481	8/23	20	89/56	AAVPSGASTGIYEALELR
829	23	7.01	47481	10/36	20	83/56	AAVPSGASTGIYEALELR
829	24	5.99	42199	5/25	15	57/56	VYADGIFDLFHSGHAR
840	25	5.59	223946	15/31	8	58/56	IHFGTTGK
981	26	8.3	44985	6/21	16	68/56	NNQITNNQR
1138	27	6.28	64887	8/33	15	57/56	YFGAHERLEETK
1160	28	8.91	33863	7/31	25	58/56	SWDPNPVPRTLR
1173	29	8.54	65916	6/21	11	58/56	MQWLMR
1339	30	8.56	61634	7/25	15	58/56	MTTSLQDGQSAASRAAAR
1509	31	9.63	17104	5/22	33	58/56	MELVQVLKR
1511	32	9.01	66808	7/40	12	57/56	MGTTGLESLSLGDR
1527	33	4.87	74959	6/17	12	57/56	VYGAQNEMCLATQQLSK
1600	34	5.7	27815	4/15	20	63/56	ELPAAVAPAGPASLAR
1719	35	5.46	95729	7/15	7	59/56	EAEFLQK
1734	36	4.87	74959	6/17	12	64/57	VYGAQNEMCLATQQLSK
1854	37	8.93	83611	6/10	7	60/56	GHVHRLVSGK
1857	38	8.27	22324	8/16	38	92/56	IGHPAPNFK
1858	39	8.27	22324	5/11	18	62/56	ADEGISFR
1882	40	8.16	66149	5/14	12	58/56	SLNNQFASFIDK
1933	41	5.66	22049	5/13	23	56/56	EGGLGPLNIPLLADVTR
2053	42	5.62	74033	6/18	9	56/56	LENLIIK
2058	43	5.43	35806	4/13	10	57/56	MAPLDLDK
2107	44	5.34	15048	6/22	58	69/56	ACGLVASNLNLKPGECLR

cyanine dyes containing Cy2, Cy3, and Cy5. Briefly, the protein sample was individual labeled with Cy3 and Cy5. Separated all samples were labeled with Cy2, which was an internal standard to all gels. The labeled samples were incubated in the dark for 30 minutes on ice, and then the reaction was stopped by the addition of L-lysine. The Cy3 and Cy5 labeled samples were combined and mixed with an equal volume with Cy2-labeled internal standard. After the addition of DTT and carrier ampholyte IPG (immobilized pH gradient) buffer, the final volume was adjusted to 450 ml with 2D-lysis buffer. Nonlinear strips (pH3-10, 24 cm) were rehydrated by CyDye-labeled samples in the dark at room temperature overnight. 2D-DIGE was performed and gel analysis was used DeCyder (GE Healthcare, Uppsala, Sweden) to 2D differential analysis.

Protein analysis

The interested proteins on 2D gel were subjected to in-gel digestion. The peptide samples were dissolved in 0.1% trifluoroacetic acid (TFA), spot on an anchor chip target plate, cover matrix α- cyano-4-hydroxy-cinnamic acid (HCCA) after samples become dried. The peptide mass fingerprint was analyzed by matrix-assisted laser desorption/ ionization time of flight mass spectrometer (MALDI-TOF-MS) (Bruker Daltonics Inc., Bremen, Germany), and Mascot search software (Matrix Science, London, UK) was used to identify these proteins.

Results

As shown in Fig. 1, doxorubicin different induced death of MES-SA and multidrug-resistant cell lines MES-SA/Dx5^{low} and MES-SA/Dx5^{high} cells. Compared with MES-SA/Dx5^{low} and MES-SA/Dx5^{high} cells, MES-SA cells were more sensitive to the cytotoxicity of doxorubicin. Mitochondrial-enriched fractions were successfully isolated as evidenced by Western blot analysis of tubulin-beta (cytosolic marker) and cytochrome c oxidase IV (COX IV) (mitochondrial marker). This is shown in Fig. 2. The mitochondrial proteomic profiles of MES-SA, MES-SA/Dx5 ^{low}, and MES-SA/Dx5 ^{high} were conducted by three replicates of the different mitochondrial extracts. A representative 2D gel is shown in Fig. 3. The results of MALDI-TOF MS analyses on gel-to-gel variation of spots identified alterations in mitochondrial protein expression in MES-SA, MES-SA/Dx5^{low}, and MES-SA/Dx5^{high} cells (Table 1). Several differentially expressed proteins may merit study further and identify their contribution to doxorubicin-resistant phenotype.[7]

Discussion

Uterine cancer is notoriously known for its resistant to doxorubicin treatment, but themechanisms responsible for doxorubicin resistance remain to be clarified. Proteomic analysis on mitochondrial proteins may elucidate their importance in tumor growth, angiogenesis, metastasis, and cancer progression. Extensive studies on differentially expressed proteins may lead to find novel targets for uterine cancer therapy.

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