Adriamycin resistance-associated prohibitin gene inhibits proliferation of human osteosarcoma MG63 cells by interacting with oncogenes and tumor suppressor genes

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Abstract. The resistance of cancer cells to chemotherapeutic agents is a major obstacle for successful chemotherapy, and the mechanism of chemoresistance remains unclear. The present study developed an adriamycin-resistant human osteosarcoma MG-63 sub-line (MG-63/ADR), and identified differentially expressed proteins that may be associated with adriamycin resistance. Two dimensional gel electrophoresis, matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis and a protein identification assay were performed. Western blot analysis was used to examine the prohibitin (PHB) levels in the MG-63/ADR cells. Quantitative polymerase chain reaction was utilized to detect adriamycin resistant-associated genes. Laser-scanning confocal microscope was employed to examine the colocalization of PHB with v-myc avian myelocytomatosis viral oncogene homolog (c-myc), FBJ murine osteosarcoma viral oncogene homolog (c-fos), tumor protein p53 and retinoblastoma 1 (Rb). In addition, the full length of the open reading frame of human PHB was subcloned into a lentiviral vector pLVX-puro. The proliferative rate of MG-63 cells was also investigated. The overall protein expression in MG-63/ADR cells was clearly suppressed. Three notable protein regions, representing high mobility group box 1, Ras homolog gene family, member A, and PHB, were identified to be significantly altered in MG-63/ADR cells when compared with its parental cells. Therefore, PHB modulated the chemoresistance of MG-63/ADR cells by interacting with multiple oncogenes or tumor suppressor genes (c-myc, c-fos, p53 and Rb). In addition, overexpression of PHB decreases the proliferative rate of MG-63 cells. In conclusion, PHB is an adriamycin resistance-associated gene, which may inhibit the proliferation of human osteosarcoma MG-63 cells by interacting with the oncogenes or tumor suppressor genes, c-myc, c-fos, p53 and Rb.

Introduction

It has been ~70 years since chemotherapy was introduced into clinical practice to treat malignant tumors (1). A variety of chemotherapeutic agents have been developed to interfere with the metabolism of cancer cells, including osteosarcoma, colon carcinoma, liver carcinoma and breast carcinoma cells, and a clinician may improve therapeutic outcomes by dose escalation, alterations in the combination of chemotherapy and the addition of irradiation therapy (1,2). However, the overall survival rate of osteosarcoma, colon carcinoma, liver carcinoma and breast carcinoma patients has not improved with the chemotherapeutic agents as much as expected (1,2). Intrinsic and acquired resistance to chemotherapeutic agents is the major obstacle for successful chemotherapy (2). Clinical practice has revealed that the profile of intrinsic gene expression varies greatly in patients that respond poorly to chemotherapy (3). Several genes, including multi-drug resistant protein 1 (4), cluster of differentiation 117 and ATP binding cassette subfamily G member 2 (5), have been identified as drug resistance genes in human osteosarcoma; however, there is no consensus regarding biomarkers for the detection of cancer resistance to a certain chemotherapy.

Two dimensional gel electrophoresis (2-D PAGE) is a powerful method for analyzing complex protein samples, and previous studies have successfully employed 2-D PAGE for the identification of chemoresistance-associated genes (6). In the present study, an adriamycin-resistant human osteosarcoma MG-63 sub-line was established, and adriamycin resistance-associated proteins were identified by comparing the adriamycin-resistance sub-line with its parental cell line, with the aid of 2-DE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALI-TOF-MS). Out of all the genes that were aberrantly expressed in the resistant
sub-line, prohibitin (PHB) was demonstrated to be capable of interacting with multiple oncogenes and tumor suppressor genes. This suggests that it has the potential to be a biomarker for chemotherapy resistance.

Materials and methods

Cell cultures. Human osteosarcoma cell line MG-63 was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were routinely maintained in complete growth medium (RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.)) at 37°C in a humidified 5% CO₂ incubator. Unless specified, all reagents and materials for cell culture were from Gibco (Thermo Fisher Scientific, Inc.) or Corning Incorporated (Corning, NY, USA).

Establishment of adriamycin resistant MG-63 sub-line. MG-63 cells were routinely maintained in complete growth medium. The method for establishing drug resistant cell lines was performed as previously reported (7). Briefly, the resistant MG-63 cells were established by exposure to increasing concentrations of adriamycin, starting from 4 ng/ml with a 25% increase each time. Subsequent to 6 months of continuous cultivation, the subcultures that capable of growing exponentially in the presence of the highest concentration of adriamycin (44 ng/ml) were designated as the adriamycin-resistant sub-line (MG-63/ADR).

2-D-polyacrylamide gel electrophoresis (PAGE). MALDI-TOF-MS analysis and protein identification. 2-D-PAGE was conducted as previously described (8). Briefly, the protein samples from the adriamycin-resistant sub-lines and parental MG-63 cells were diluted in sample buffer with 2% IPG buffer (pH 3-10; GE Healthcare Life Sciences, Chalfont, UK). The samples were applied to 18-cm, immobilized pH gradient strips (Immobiline Drystrip pH 3-10; GE Healthcare Life Sciences). Upon completion of isoelectric focusing, the strips were equilibrated and the second dimension was run at 20°C. Triplicate sets of silver-stained gels were scanned using a UMAX POWER LOOK III flat-bed scanner (UMAX Technologies, Dallas, TX, USA) and analyzed with the PDQuest 2-D Analysis software, version 8.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The digitalized 2-DE gel images were compared using an electronic alignment method PDQuest 2-D analysis software version 8.0; Bio-Rad Laboratories, Inc.) (6). Differentially expressed spots were identified and annotated.

The spots were cut into pieces and digested with 12.5 ng/μl trypsin (Promega Corporation, Madison, WI, USA) in 50 mM ammonium bicarbonate (pH 8.0; Sigma-Aldrich, St. Louis, MO, USA). Following elution with 2 μl matrix solution consisting of 10 mg/ml α-cyano-3-hydroxy-cinnamic acid (Sigma-Aldrich), the remaining liquid was submitted to a mass spectrometer (MALDI-TOF III; Bruker Corporation, Billerica, MA, USA). The spectra were internally calibrated using the trypsin autobiography products as controls [842.51 m/z (M+H) and 2,211.11 m/z (M+H)] by flexImaging software version 2.0 (Bruker Corporation) and blasted against Swiss-Prot (www.uniprot.org/) and National Center for Biotechnology Information BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) databases using the Proteomics Mascot software version 2.0, which was purchased from Matrix Science, Inc. (Boston, MA, USA). All searches were analyzed with a 50 ppm mass tolerance.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of adriamycin resistance-associated genes. The cDNA samples were prepared using a 1st Strand cDNA Synthesis kit (catalog no., D6110S; Takara Bio, Inc.), according to the manufacturer’s protocol. cDNA samples were prepared using the Premix Ex Taq II kit (catalog no., RR820A; Takara, Bio, Inc.) in ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems®, Thermo Fisher Scientific, Inc.). Hot-start PCR was performed as follows: 30 sec at 95°C; 45 cycles, with 1 cycle of 5 sec at 95°C and 44 cycles of 30 sec at 60°C. All samples were read in triplicate, and values were normalized to β-actin expression. The relative expression data were calculated according to the 2−ΔΔCq method and presented as fold-change (7).
cells were incubated in the mixture of two primary antibodies [mouse anti-human PHB (monoclonal antibody; catalogue no. sc-377037; 1:2,000; Santa Cruz Biotechnology, Inc.)/rabbit anti-human FBJ murine osteosarcoma viral oncogene homolog (c-fos; polyclonal antibody; catalogue no. sc-52; 1:2,000; Santa Cruz Biotechnology, Inc.); mouse anti-human PHB (monoclonal antibody; catalogue no. sc-377037; 1:2,000; Santa Cruz Biotechnology, Inc.)/rabbit anti-human v-myc avian myelocytomatosis viral oncogene homolog (c-myc; polyclonal antibody; catalogue no. sc-788; 1:2,000; Santa Cruz Biotechnology, Inc.); mouse anti-human PHB (monoclonal antibody; catalogue no. sc-377037; 1:2,000; Santa Cruz Biotechnology, Inc.)/rabbit anti-human tumor protein p53 (polyclonal antibody; catalogue no. sc-6243; 1:2,000; Santa Cruz Biotechnology, Inc.); and mouse anti-human PHB (monoclonal antibody; catalogue no. sc-377037; 1:2,000; Santa Cruz Biotechnology, Inc.)/rabbit anti-human retinoblastoma 1 (Rb; monoclonal antibody; catalogue no. sc-1538; 1:2,000; Santa Cruz Biotechnology, Inc.)] in 1% BSA in PBST in a humidified chamber for 1 h at room temperature. Following washing 3 times in PBS for 5 min, the cells were incubated with the mixture of two secondary antibodies [cyanine dye (Cy®3)-conjugated goat anti-mouse IgG (polyclonal antibody; catalogue no. 115-165-164; 1:2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA)/Cy3-conjugated goat anti-rabbit IgG (polyclonal antibody; catalogue no. 111-165-003; 1:2,000; Jackson ImmunoResearch Laboratories, Inc.)] in 1% BSA for 1 h at room temperature in dark. The cells were washed in PBS for 5 min in the dark. For counter staining, the cells were incubated in DAPI (Sigma-Aldrich) for 5 min at 37°C. Following rinsing in PBS, the cells were mounted with a drop of mounting medium (Gibco; Thermo Fisher Scientific, Inc.) and sealed with nail polish to prevent movement under the microscope. Image acquisition was performed by laser confocal scanning microscopy (TCS-SP2 MP; Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Overexpression of PHB in MG-63/ADR sub-line. The full length of open reading frame of human PHB was subcloned into lentiviral vector pLVX-puro (Invitrogen; Thermo Fisher Scientific, Inc.). The lentivirus was subsequently generated by cotransfection of human embryonic kidney 293T cells with the recombinant lentiviral expression vector and Lenti-X™ Packaging System from Clontech Laboratories, Inc. (Mountainview, CA, USA). For probing the effects of PHB on cell growth, the resistant MG-63/ADR sub-line was transduced with lentivirus bearing PHB (Invitrogen; Thermo Fisher Scientific, Inc.), and a stable clone were screened by limiting dilution under the selective pressure of puromycin (2.5 µg/ml; Sigma-Aldrich). Cell growth curve analysis was performed as described previously (9).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were cultured in 96-well tissue culture plates. At 24 and 48 h after transfection, MTT (Sigma-Aldrich) was added to each well to a final concentration of 5 mg/ml in culture medium, and incubated at 37°C for 4 h. The reaction was terminated by removal of the supernatant and addition of 150 µl dimethyl sulfoxide (Sigma-Aldrich) to dissolve the formazan product. The plates were read at 405 nm on an MK3

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Table I. Differentially expressed proteins in adriamycin-resistant human osteosarcoma MG-63 and parental MG-63 cells.

Genes were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. HMGB1, high mobility group box 1 protein; RhoA, Ras homolog gene family, member A; NCBI, National Center for Biotechnology Information; Mw, molecular weight.

Figure 1. Representative image of silver-stained two dimensional gel electrophoresis map of human osteosarcoma parental MG-63 and adriamycin-resistant MG-63 cells. S1, high mobility group box 1 protein; S2, Ras homolog gene family, member A; S3, prohibitin.
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Each assay was performed in duplicates of 10 wells.

Statistical analysis. SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) was used to perform statistical analysis. Data are represented as the mean, median, minimum and maximum values. Student's t test was used to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

Results

Proteomic analysis of MG-63 cells pre- and post-chemo-resistance. The whole cell lysates from MG-63 and MG-63/ADR cells were separated using 2-DE, and the gel was visualized by silver-staining. The procedure was independently repeated 3 times and a representative gel image is shown in Fig. 1. The number of protein spots in the MG-63/ADR sub-line markedly decreased when compared with the parental MG-63 cells. The spots differentiated by intensity were excised and digested with trypsin, and were subsequently identified by mass spectrometry. The identified proteins are listed in Table I.

Analysis of aberrant expression of PHB by qPCR and western blot analysis. To additionally verify the aberrant alterations identified by 2D-PAGE, western immunoblotting and RT-qPCR were employed to confirm expression levels. The results showed that PHB in MG-63/ADR cells was much lower compared with parental MG-63 cells, suggesting the expression of PHB in the MG-63/ADR cells was significantly inhibited (Fig. 2; P<0.01). These results were consistent with the 2-DE analysis.

Colocalization of PHB with c-myc, c-fos, p53 and Rb staining. The MG-63 and MG-63/ADR cells were immunostained with primary antibodies for PHB, c-myc, c-fos, p53 and Rb. The anti-PHB antibody and other antibodies were labeled with red fluorescence Cy3. Laser confocal scanning microscopy was used to observe the alteration of colocalization of PHB with other proteins. The colocalized region was yellow or orange.
Colocalization of PHB with c-myc. The green fluorescence representing PHB was distributed throughout the MG-63 cells. The fluorescent density in the nucleus was clearer compared with the cytoplasm. The red fluorescence representing c-myc was markedly distributed in the nucleus and the density was not uniform. In images where the red and green fluorescence has been merged together, PHB was observed to be colocalized with c-myc in the nucleoplasm region, particularly in the nucleolus region (Fig. 3A). However, in the MG-63/ADR cells, the PHB expression was primarily distributed in nucleus, and the red fluorescence of c-myc was decreased and uniformly distributed in the nucleus. The merged images demonstrated that the colocalization of PHB with c-myc in the cytoplasm was not clear, suggesting the colocalized region of PHB with c-myc was not in the cytoplasm (Fig. 3A).

Colocalization of PHB with c-fos. In MG-63 parental cells, PHB and c-fos were primarily expressed in the cytoplasm and nucleoplasm expression was extremely weak. PHB and c-fos colocalized together in the cytoplasm; however, the overall fluorescent intensity in MG-63/ADR cells was much lower compared with parental MG-63 cells, indicating that PHB and c-fos were downregulated when the MG-63 cells were conferred with adriamycin resistance. Nevertheless, the colocalization region was not altered pre- and post-chemoresistance (Fig. 3B).

Colocalization of PHB with p53. Cytoplasmic p53 was dominant in parental MG-63 cells. The fluorescence intensity of PHB and p53 was relatively weak in the nucleus. The expression of PHB and p53 was entirely attenuated in MG-63/ADR cells, and the fluorescence in the nucleus was almost completely depleted. However, the colocalization of PHB with p53 was present in cells pre- and post-chemoresistance (Fig. 3C).

Colocalization of PHB with Rb. The green fluorescence representing PHB was distributed in the nucleus region of parental MG-63 cells. The fluorescent density in the nucleolus was relatively clear, while the fluorescence in the cytoplasm was much weaker. The red fluorescence representing Rb was distributed throughout the whole cell. The merged image indicated that PHB colocalized with Rb in the nucleoplasm region, particularly in the nucleolus region (Fig. 3D). Adriamycin treatment did not affect the colocalization of PHB with Rb, but did attenuated their expression levels.

Overexpression of PHB in MG-63/ADR cells. To additionally elucidate the function of PHB in modulating the sensitivity of MG-63 cells to chemotherapeutic drugs, the expression of PHB was artificially increased in the MG-63/ADR cells using a lentiviral expression vector (Fig. 4A). The cell proliferation assay revealed that overexpression of PHB decreased the proliferative rate of MG-63/ADR cells (Fig. 4B; P<0.01), indicating its pivotal role in mediating chemoresistance in human osteosarcoma cells.

Discussion

Chemotherapy has prolonged the life span of patients with localized osteosarcoma (10,11). However, almost one third of patients with localized osteosarcoma suffer from recurrence or progressive disease due to the development of drug resistance (10,11). The present study developed an adriamycin-resistant MG-63 sub-line, and employed the 2-DE method to identify differentially expressed genes in the resistant sub-line compared with the parental MG-63 cells. All these differentially expressed genes are directly associated with chemoresistance, and one of these, PHB, is involved in the evolution of osteosarcoma resistance to adriamycin.

RhoA is a member of the Ras superfamily, which regulates cytoskeletal dynamics; therefore participating in multiple cellular activities, including cell motility and polarity (12). The Rho subfamily includes three isoforms RhoA, RhoB and RhoC, which share 84% homology in sequence differing near the C terminus (13). When overexpressed, RhoA, RhoB and RhoC induce stress fibers and induce terminal morphological alterations during apoptosis (14). However, several studies have indicated that the three isoforms have distinct functions. RhoA is localized to the plasma membrane, while RhoB is directed to endosomal membranes, due to its unique C-terminal lipid modifications, and manipulates the endosomal trafficking of membrane receptors (15). Furthermore, RhoA inhibits cancer cell invasion in vitro, whereas RhoC often enhances cancer metastasis (16). Depletion of RhoA promotes cell invasion (17) and constitutive overexpression in T cells induces the expression of anti-apoptotic protein B-cell lymphoma-2, which protects cells from apoptosis (18). By contrast, knockdown of RhoA results in the apoptosis of T cells (19). The present data demonstrates that the expression levels of RhoA were markedly increased in the MG-63/ADR sub-line, which may have a role in overcoming cytotoxic drug-induced apoptotic cell death.
High-mobility group box 1 (HMGB1) is a highly conserved nuclear protein, which is a damage-associated molecule that interacts with receptors for advanced glycation end products and toll-like receptors (20-22). A number of studies have revealed its pivotal role in mediating autophagy in cancer development and therapy (23,24). Endogenous HMGB1 may negatively regulate apoptosis of tumor cells, and manipulating HMGB1 expression may alter the sensitivity of cancer cells to chemotherapeutic drugs (25,26). Various anticancer agents, including doxorubicin, cisplatin and methotrexate, upregulate HMGB1 expression in human osteosarcoma cells, while suppression of its expression using RNA interference-mediated knockdown restores the chemosensitivity of osteosarcoma cells in vivo and in vitro (27,28). The present data demonstrated that HMGB1 levels were increased in the MG-63/ADR sub-line, which was consistent with the results from another study (28), indicating it may serve as a candidate gene for monitoring osteosarcoma chemoresistance.

PHB is known as a tumor suppressor and is ubiquitously expressed in multiple tissues with antiproliferative properties (29). It controls the transition from G1 to S phase in cycling cells (29). High levels of PHB are commonly observed in various human cancer solid tumor cell lines (30,31). In the nucleus, PHB interacts with EZF transcription factor 1 (32), p53 and phosphorylated Rb (33) to regulate the expression of genes that are associated with cell proliferation and differentiation. The present data demonstrated that the level of PHB in the MG-63/ADR sub-line was decreased compared to parental cells. In addition, the present data from laser confocal microscopy revealed that PHB colocalized with c-myc, c-fos, p53 and Rb in the parental MG-63 cells; however, the regions where colocalization was observed was distinct from colocalization regions observed in the MG-63/ADR sub-line. Furthermore, the fluorescence intensity representing PHB staining was attenuated in the resistant sub-line compared with the parental MG-63 cells. Overexpression of PHB in the MG-63/ADR sub-line decreased the proliferative rate of cells in the present study. Previously, it was observed that a deletion of the PHB gene led to an 80% reduction of mitochondrial potential (34), and subsequently triggered the release of apoptogenic factors, indicating that PHB-regulated mitochondria potential may also affect chemotherapeutic effects.

Overall, the present study employed 2-DE and MALDI-TOF-MS methods and identified notable genes that respond to adriamycin resistance in human osteosarcoma cells. The functions of these genes were associated with apoptotic signaling pathways. Of all the identified genes, PHB was demonstrated to be a promising target for novel therapeutic strategies, as it interacted with c-myc, c-fos, p53 and Rb, and an overexpression of PHB modulated the proliferative rate of adriamycin-resistant MG-63 cells. However, additional study is required to elucidate how these chemoresistance-associated genes interfere with the adriamycin-activated pathway leading to adriamycin resistance in human osteosarcoma.

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References