Multiple pathways are involved in drug resistance to doxorubicin in an osteosarcoma cell line

Thangarajan Rajkumar and Manoharan Yamuna

Drug resistance continues to be a stumbling block in achieving a better cure rate in several cancers, including osteosarcoma. To understand this, we developed a doxorubicin drug-resistant osteosarcoma cell line (143B-DR-DOX). This cell line had an IC_{50} of 75 μmol/l compared with the parental 143B cell line's IC_{50} of 0.4 μmol/l. Using a 22 000 70-mer oligomicroarray, gene expression studies were performed in four replicates. Data analysis was done using the TIGR Microarray suite. Seventy-four genes were found to be either upregulated (21) or downregulated (53). Real time quantitative-PCR was done on 21 genes, which confirmed the gene expression data for 11 genes. Choosing the significant fold change criteria of greater than 2-fold upregulation or downregulation, four genes including multidrug resistance 1, interleukin-8, Krüppel-like factor 2 and MGC4175 were found to be upregulated and seven genes including epidermal growth factor receptor-coamplified and overexpressed protein, uridine phosphorylase 1, a disintegrin and metalloproteinase domain 19, cytochrome C_1, SEC, *S*-adenosyl homocysteine hydrolase and p53 were found to be downregulated. The data suggest that apart from the known gene alterations in doxorubicin resistance (multidrug resistance 1, topoisomerase IIβ), others can also contribute to the drug-resistance phenotype. The involvement of interleukin-8 and Krüppel-like factor 2 suggests that the peroxisome proliferator-activated receptors γ pathway may also be involved in doxorubicin drug resistance in the 143B-DR-DOX cell line. *Anti-Cancer Drugs* 19:257–265 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Department of Molecular Oncology, Cancer Institute (WIA), Adyar, Chennai, India

Correspondence to Dr Thangarajan Rajkumar, Professor and Head, Department of Molecular Oncology, Cancer Institute (WIA), Adyar, Chennai 600020, India
Tel: +91 44 22201121; fax: +91 44 24912085;
e-mail: rajkumart@yahoo.com

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Introduction

Osteosarcomas are highly aggressive bone cancers, commonly seen in adolescents and children. Before intensive chemotherapy, cure rates were only around 18% and often needed amputation or disarticulation of a limb. With the introduction of intensive chemotherapy with cisplatinum, doxorubicin, ifosphamide, and high-dose methotrexate, the outlook, however, has changed, with more than 60% of the patients being cured and in a substantial number of these patients it is now possible also to conserve their limb. Nearly 30–40% of the patients, however, fail, usually owing to the emergence of metastasis, commonly in the lung [1]. Response to preoperative (neoadjuvant) chemotherapy, measured as the grade of necrosis, has been shown to be a strong predictor of outcome [2,3].

Drug resistance has been a stumbling block in achieving better cure rates. Drug resistance can be classified as primary or innate or intrinsic drug resistance seen in chemotherapy-naive cells and acquired drug resistance seen after exposure to chemotherapeutic drugs. Acquired drug resistance has been considered to be due to host-related and tumor cell-related factors [4]. Multidrug resistance (MDR) has been one of the mechanisms for the development of resistance to not only the drug the patient has been exposed to, but also to other seemingly chemically unrelated drugs [5]. MDR phenotype has been associated with MDR1, MRP, LRP, etc. genes and is a common mechanism for drug resistance in doxorubicin [6]. Other genes involved in drug resistance include those involved in DNA repair, alteration in the drug target through mutation, reduced uptake of the drug, etc. [7–9].

It is likely that in addition to MDR1's role there are other genes, which could also play a significant role in osteosarcomas. Overcoming drug resistance will need a better understanding of the mechanisms involved in drug resistance. To this end, we have developed a doxorubicin drug-resistant osteosarcoma cell line (143B-DR-DOX) and used it in conjunction with the parental doxorubicin-sensitive 143B cells in an oligomicroarray experiment, to identify genes which are differentially expressed and likely to be related to drug resistance. Twenty-one genes were further evaluated by real-time quantitative-PCR (RT Q-PCR) and 11 genes were found to be either upregulated or downregulated, with some likely to be associated with novel pathways.

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Materials and methods

Establishment of drug-resistant cell line

The 143B osteosarcoma cell line was obtained from the National Center for Cell Science, Pune, India and grown in Dulbecco's-modified Eagle medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, California, USA), antibiotics penicillin (10 000 U/ml) and streptomycin (10 mg/ml). To establish a doxorubicin-resistant cell line, the 143B cell line ($IC_{50} = 0.4$ μmol/l) was exposed intermittently to gradually increasing concentration of doxorubicin. After each exposure of the drug for 3 h, medium containing the drug was removed, fresh medium was added and cells were allowed to recover. The drug-resistant cell line was exposed to up to 100 μmol/l of doxorubicin. Cell viability was assessed using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit as per the manufacturer's protocol (Promega Corporation, Madison, USA).

Microarray slides

The 70-mer oligomicroarray slides were purchased from the Genomics and Microarray Laboratory, University of Cincinnati, USA. These slides have 22 000 genes spotted on them.

RNA extraction and labeling

RNA extraction from both the cell lines was done using the Qiagen RNeasy kit including the removal of DNA by on-column DNAse digestion using Qiagen's RNAse-free DNAse, as per the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). RNA was assessed by formaldehyde agarose gel electrophoresis and quantitated by UV absorbance and checked for DNA contamination using RT-PCR for c-ABL.

Approximately, 25 μg of total RNA was used for indirect amino-allyl labeling using the Cyscribe postlabeling kit (Amersham GE, Little Chalfont, UK), with either cyanine 3 or cyanine 5 (Cy3/Cy5) fluorescent dyes, as per the manufacturer's protocol. Four biological replicates were done, two experiments with the 143B cells labeled with Cy3 and 143B-DR-DOX cells labeled with Cy5 and two with the dyes swapped. Cy-dyes incorporation was measured with UV–Vis spectrophotometer.

A prehybridization step consisted of incubation of the array chip in filter sterilized prehybridization solution [5 × SSC, 0.1% sodium dodecyl sulfate (SDS) and 1% bovine serum albumin] for 60 min at 48°C, after which the slides were rinsed and dried. Equal picomoles (minimum of 30 picomoles) of denatured, Cy3-labeled and Cy5-labeled cDNA was resuspended in a solution of 50% formamide hybridization mix and allowed to hybridize for 16 h at 42°C in the Luceidea Pro hybridization chamber (Amersham GE). After hybridization, slides were washed for 5 min each in the following series of solutions: 1 × SSC, 0.2% SDS; 0.1 × SSC, 0.2% SDS; and a final 0.1 × SSC wash, followed by drying by centrifugation. Slides were immediately scanned at 10-μm resolution using the Perkin Elmer (Shelton, Connecticut, USA) ProScanArray scanner.

Microarray image analysis

Processing of 16-bit TIFF images from the hybridized arrays was performed using the TIGR-TM4 package (TIGR, Boston, Massachusetts, USA) [10]. Intensity values for Cy3 and Cy5 channels were obtained using TIGR-Spotfinder software. QC filter for background correction was set at the threshold of 1 background median + 3 background standard deviation. LOWESS normalization, standard deviation regularization and flip dye consistency checking (for paired analysis) was performed with the algorithms available in TIGR-MIDAS software.

Four hybridizations were performed from each of four biological replicate cell line pairs (control and treatment). Half of the biological replicate dye labelings were dye swaps (flip dyes). The four biological replicates were analyzed individually (for flip dye experiments, Spotfinder was used to swap the images) and in pairs [Experiment 1 = 143B–Cy3, 143B-DR-DOX–Cy5; Experiment 2 = 143B–Cy5, 143B-DR-DOX–Cy3; Experiment 3 = 143B–Cy5, 143B-DR-DOX–Cy3; Experiment 4 = 143B–Cy3, 143B-DR-DOX–Cy5. Experiments 1 vs. 2, 1 vs. 3, 4 vs. 2 and 4 vs. 3 were analyzed using the MIDAS software and the MEV format file generated was then loaded into Multi-Experiment Viewer (MEV) software].

To determine genes whose expression was significantly different from zero, significance analysis of microarray (SAM) and *t*-test were used for statistical analysis. Hierarchical clustering with average linkage and Euclidean distance metric was done, in the MEV component of the TIGR suite. SAM was employed using the one-class response with unique 16 permutations. Again, the MIDAS generated mev files for individual experiment analysis and paired experiment analysis were loaded independently into the MEV and analyzed, using both SAM and *t*-test.

All the image files and raw data files have been submitted to the GEO web site, with the accession no. GSE3362.

Real-time quantitative-PCR

For the RT Q-PCR experiments, mRNA was isolated afresh from 143B and 143B-DR-DOX cell lines as described earlier. Template cDNAs were synthesized using 1 μg of total RNA using 200 U of MMLV reverse transcriptase. The RT-PCR relative quantification assay was performed according to the fluorescent TaqMan methodology (Applied Biosystems, Foster City, California,

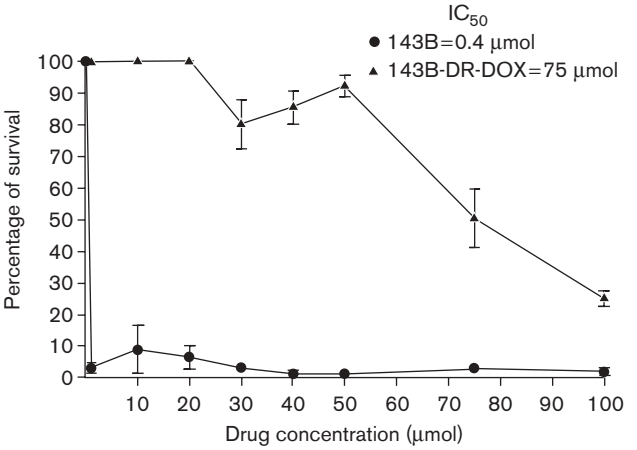
USA). The primers and Taqman probes targeting adenosine triphosphate (ATP)-binding cassette-subfamily B (ABCB1), hypoxia upregulated 1 (HYOU1), Krüppel-like factor 2 (KLF2), DNAJB11, dysadherin (FXYD5), ferredoxin reductase (FDXR), C7ORF23, cytochrome c_1 (CYC1), tumor protein p53, topoisomerase IIβ (TOP2B), calreticulin (CALR), cytochrome b_5 (CYB5), SEC61 γ subunit (SEC61G), epidermal growth factor receptor (EGFR)-coamplified and overexpressed protein (ECOP), a disintegrin and metalloproteinase domain 19 (ADAM19), GK003, interleukin-8 (IL-8), DAP4, *S*-adenosyl homocysteine hydrolase (AHCY), uridine phosphorylase 1 (UPP1) and keratin 18 (KRT18) were purchased as Assay by Demand products for gene expression (Applied Biosystems). The target gene-specific Taqman MGB probe was labeled with fluorescent reporter dye FAM. Our study involving northern hybridization with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe followed by densitometric analysis showed no differential expression of the GAPDH gene between parental and drug-resistant cells (data not shown). Thus, the GAPDH gene that was amplified with VIC-labeled Taqman MGB probe and primers was selected as an endogenous control for RNA input and reverse transcription efficiency (Applied Biosystems). Twenty-five micro liters of PCRs mixture consisted of 1 × final concentration of 20 × primer and probe mix, 1 × final concentration of 2 × Universal PCR Master Mix (Applied Biosystems) and 50 ng of cDNA. The amplification and detection was performed with ABI Prism 7000 Sequence Detection System (Applied Biosystems) under universal thermal cycler conditions that included 2 min at 50°C, 10 min at 95°C and each cycle of 95°C for 30 s and 60°C for 1 min for 40 cycles. All PCRs were done in triplicates for both target gene and internal control, along with NTC in duplicates for each target gene. The assay was repeated once or twice for each target gene. The minus RT control was set in duplicates for each total RNA sample to check for the amount of DNA contamination. The relative fold difference in expression of the target gene between parental versus drug-resistant cells was analyzed with the $2^{\Delta\Delta C_T}$ method using QBASE software.

Results

Development of the doxorubicin drug-resistant cell line

With intermittent exposure to doxorubicin as described in the methodology, the 143B-DR-DOX cells were found to have an IC_{50} of 75 µmol/l in comparison with the IC_{50} of 0.4 µmol/l seen in the parental 143B cells, indicating that more than 100-fold resistance was achieved (Fig. 1).

Fig. 1



MTS assay using the drug-sensitive 143B and the drug-resistant 143B-DR-DOX cell lines.

Microarray experiment results

The microarray analysis was done as described in the Materials and methods. The complete list of genes found to be significant by SAM and the *t*-test with both methods of analysis (as individual experiments and as paired experiments) is given in Supplementary Tables 1–4. Of the 485 differentially expressed significant genes identified by SAM (paired analysis), 74 genes were found to have an observed score of either ≥ 3 or ≤ -3 and of these 21 were upregulated and 53 downregulated in the drug-resistant 143B-DR-DOX cells relative to the expression levels in the parental 143B osteosarcoma cells (Supplementary Table 5). Among the top 10 genes that were upregulated, nine were common in at least three methods of analysis. Twenty-one genes were taken up for validation with the RT-PCR technique (Table 1).

Real-time quantitative-PCR validation of microarray expression results

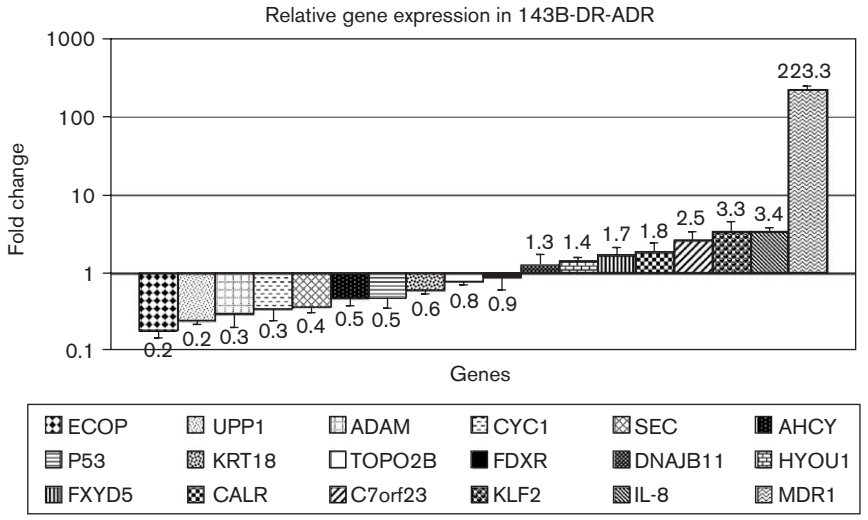
Differential expression of the genes identified in microarray experiments were further validated using RT Q-PCR and freshly isolated total RNA. The integrity, quantity and quality of total RNA isolated from the 143B and 143-DR-DOX cell lines were verified and cDNA synthesized by reverse transcription was used in the RT Q-PCR. Differential expression of the 21 genes was validated using RT Q-PCR. Although the validation with RT Q-PCR confirmed the upregulation ($n = 8$) and downregulation ($n = 10$) of 18 out of the 21 significant genes, only 11 genes demonstrated significant fold changes (Fig. 2). Choosing the significant fold change criteria of greater than twofold upregulation or downregulation, four genes including MDR1, IL-8, KLF2 and MGC4175 were found to be upregulated and seven genes including ECOP, UPP1, ADAM 19, CYC1, SEC, AHCY and p53 were found to be downregulated. The comparison of gene expression results with microarray and RTQ-PCR is given in Table 2.

Table 1 List of 21 significant genes taken up for validation with real time PCR and their scores

Description	Paired SAM analysis (dExp)	Paired SAM analysis [observed score (d)]	Individual experiment SAM analysis (dExp)	Individual experiment SAM analysis [observed score (d)]	Paired analysis, *t*-test, $P=0.009$ gene mean	Individual experiment analysis, *t*-test, $P=0.009$ gene mean
ATP-binding cassette, subfamily B (MDR/TAP), member 1	−0.667	7.946	−0.407	4.069	4.520	4.856
Interleukin 8	0.294	9.999	0.161	3.098	2.646	2.694
Kruppel-like factor 2 (lung)	0.148	6.020	0.082	2.390	2.351	2.381
Hypoxia upregulated 1	0.303	7.043	0.166	1.899	1.955	2.163
DNAJ (Hsp40) homolog, subfamily B, member 11	0.304	6.531	0.166	1.780	1.839	–
Cytochrome b_5	0.301	6.412	0.165	1.856	2.048	–
GK003 protein	−0.394	5.577	−0.242	1.820	2.041	–
FXYD domain-containing ion transport regulator 5	0.544	5.293	0.320	1.745	1.849	1.720
Hypothetical protein MGC4175	−0.517	4.756	−0.316	1.482	1.644	–
KIAA0964 protein	−0.366	4.074	−0.227	1.502	1.897	–
Calreticulin	0.392	2.989	0.224	1.302	–	–
Topoisomerase (DNA) II β (180 kDa)	−0.268	−2.991	–	–	−0.917	−0.835
Tumor protein p53 (Li–Fraumeni syndrome)	−0.009	−3.438	−0.017	−1.139	−1.012	1.082
S-adenosylhomocysteine hydrolase	0.249	−4.141	0.134	−1.436	−1.269	−1.413
Hypothetical protein DKFZp564K0822	−0.124	−4.227	−0.088	−1.533	−1.697	–
Uridine phosphorylase	0.889	−4.350	0.560	−1.627	−1.923	–
SEC61 γ	0.846	−4.357	0.523	−1.364	−1.262	–
A disintegrin and metalloproteinase domain 19 (meltrin β)	0.382	−4.387	0.217	−1.545	1.726	−1.495
Ferredoxin reductase	0.407	−4.412	0.232	−1.415	−1.375	−1.375
Cytochrome c_1	−0.033	−4.777	0.036	−1.483	−1.339	−1.437
Keratin 18	−0.440	−5.979	0.231	−1.601	−2.296	−1.588

A dash denotes that the analysis did not identify the gene as significant.
MDR, multidrug resistance; SAM, significance analysis of microarray.

Fig. 2



Real-time quantitative-PCR validation of microarray results.

Discussion

Doxorubicin is one of the widely used chemotherapeutic agents in the treatment of cancers including osteosarcoma. Cytotoxic effects of doxorubicin on malignant cells involve (i) DNA base pair intercalation; (ii) interaction with topoisomerase II that induce the formation of DNA-cleavable complexes; and (iii) interaction of drug molecule with the cells electron transport chain resulting in the generation of superoxide anion radicals [11]. In tumor cells, the common mechanisms involved in doxorubicin resistance include (i) overexpression of membrane-associated efflux pump P-glycoprotein mediating MDR; (ii) altered expression of topoisomerase II and integrins; and (iii) changes in glutathione levels.

Table 2 RT Q-PCR fold change values for the 21 genes

Sl. no.	Gene symbol	Gene description	Microarray	RT Q-PCR fold change (mean ± SD)
			Paired SAM analysis [observed score (d)]	
1	ABCB1	ATP-binding cassette, subfamily B (MDR/TAP), member 1	**7.946**	**223.2 ± 24.8**[b]
2	IL-8	Interleukin-8	**9.999**	**3.4 ± 0.5**[b]
3	KLF2	Kruppel-like factor 2 (lung)	**6.020**	**3.3 ± 1.2**[b]
4	HYOU1	Hypoxia upregulated 1	7.043	1.4 ± 0.2
5	DNAJB11	DNAJ (Hsp40) homolog, subfamily B, member 11	6.531	1.3 ± 0.5
6	CYB5	Cytochrome b_5	6.412	0.7 ± 0.1[c]
7	GK003	GK003 protein	5.577	0.5 ± 0.1[c]
8	FXYD5	FXYD domain-containing ion transport regulator 5	5.293	1.7 ± 0.5
9	MGC4175	Hypothetical protein MGC4175	**4.756**	**2.5 ± 0.8**[b]
10	CALR	Calreticulin	2.989	1.8 ± 0.6
11	KIAA0964	KIAA0964 protein	4.074	0.4 ± 0.08[c]
12	TP53	Tumor protein p53 (Li–Fraumeni syndrome)	**−3.438**	**0.5 ± 0.05**[a]
13	AHCY	*S*-adenosylhomocysteine hydrolase	**−4.141**	**0.5 ± 0.09**[a]
14	DKFZP564K082 (ECOP)	Hypothetical protein DKFZp564K0822	**−4.227**	**0.2 ± 0.04**[a]
15	UP	Uridine phosphorylase	**−4.350**	**0.2 ± 0.03**[a]
16	SEC61G	SEC61 γ	**−4.357**	**0.4 ± 0.06**[a]
17	ADAM19	A disintegrin and metalloproteinase domain 19 (meltrin β)	**−4.387**	**0.3 ± 0.1**[a]
18	FDXR	Ferredoxin reductase	−4.412	0.9 ± 0.3
19	CYC1	Cytochrome c_1	**−4.777**	**0.35 ± 0.1**[a]
20	KRT18	Keratin 18	−5.979	0.6 ± 0.08
21	TOP2B	Topoisomerase (DNA) IIβ (180 kDa)	−2.991	0.8 ± 0.09

ECOP, epidermal growth factor receptor-coamplified and overexpressed protein.
[a]Significant downregulation.
[b]Significant upregulation.
[c]False positve result in microarray.

Emergence of multidrug resistance posses a significant obstacle to the success of doxorubicin chemotherapy in osteosarcoma. Therefore, in view of the complex array of genetic factors contributing to drug resistance, we used oligomicroarray technology to study the expression profiles of doxorubicin-resistant versus -sensitive 143B osteosarcoma cells.

Microarray studies showed 485 differentially expressed genes by SAM (paired analysis). After an arbitrary cutoff value of an observed score of either ≥ 3 or ≤ -3, 74 genes were found to be either upregulated or downregulated. From the 74 differentially expressed significant genes, 21 were chosen for validation using RT Q-PCR, which confirmed the differential expression for 18 genes, of which 11 genes demonstrated a significant fold change (Table 2). RT Q-PCR did not confirm the levels of change seen in the microarray with regard to KIAA0964, GK003, cytochrome b_5 and FDXR genes, suggesting that this could be due to a false-positive result of the microarray.

Acquired drug resistance in osteosarcoma

Multidrug resistance mediated by ABCB1 (MDR1)

Earlier studies have shown that doxorubicin is involved in acquired MDR mediated by the expression of P-glycoprotein in drug-resistant tumor cells of osteosarcoma [12]. P-glycoprotein, a product of MDR1 gene, is an ATP-dependent drug efflux pump that extrudes drugs from cells leading to drug resistance. In-vitro studies had reported the establishment of multidrug-resistant variants of human osteosarcoma cell lines including: (i) U2 OS and SAOS-2 [13]; and (ii) MNNG/HOS and MG63 [14] through selection of cells on exposure to doxorubicin, which showed increased expression of MDR1 resulting in an in-vitro model for the classical MDR phenotype. In this context, our gene expression profiling study had also showed induced overexpression (223.3-fold) of MDR1 mRNA in doxorubicin-resistant 143-DR-DOX cells.

Interleukin-8

IL-8 has been implicated in various functions involving growth potentiation, angiogenesis and metastasis. Other investigators have demonstrated overexpression of IL-8 in drug-resistant tumor cells, including paclitaxel-resistant ovarian cancer cell line (SKOV-3TR), doxorubicin-resistant human breast cancer (MCF-7ADR) and multiple myeloma cell line (8226/DOX40) [15] and dacarbazine-resistant melanoma cancer cell lines (SB2-D and MeWo-D) [16]. The latter had demonstrated the reversal of drug resistance by blockade of IL-8 function in melanoma tumor cells revealing direct evidence for its involvement in the mediation of drug resistance. In addition, their study indicated the clinical relevance of IL-8 overexpression, wherein it acts as an angiogenesis inducer and survival factor resulting in enhanced tumor growth and protection of tumor-associated endothelial cells against cytotoxicity, respectively. It was shown that increased extracellular signal-regulated kinase activity in melanoma tumor cells contributed to transcriptional upregulation of IL-8, thus enhancing its function as survival factor through mitogen-activated protein kinase pathway [16]. The expression profiling study [15] using

MDR1 overexpressing paclitaxel-resistant SKOV-3TR cells that demonstrated MDR to other antitumor drugs including doxorubicin and vincristine also showed upregulation of IL-8. Our study showed a 3.4-fold level of IL-8 mRNA overexpression in drug-resistant (143-DR-DOX) cells relative to the level of expression in parental cells. Since the significance of IL-8 coexpression with MDR1 has not been elucidated and hypoxic tumor microenvironment is known to stimulate IL-8 activity through increased transcription and prolonged half life, our microarray data suggest that secretion of IL-8 may either play a role in the drug-resistance phenotype or in the stress response of tumor cells exposed to an antineoplastic agents leading to drug resistance.

MGC4175

Earlier in-vitro gene expression studies using high-density Affymetrix microarray identified the overexpression of a novel gene MGC4175 in human cancer cell line resistant to taxol and doxorubicin drugs. Sequence analyses of MGC4175 cDNA identified an open reading frame of 356 bp encoding a protein product of 118 amino acids. Since then, studies have shown (i) the coexpression of MGC4175 with MDR1 as both genes are located at chromosome position 7q21, (ii) its role in taxol resistance and (iii) its intracellular localization in mitochondria. The transcript of the MGC4175 gene was given the name MDR1 and mitochondrial taxol resistance associated gene [17]. The study showed varied levels of MGC4175 overexpression, ranging between 0.65- and 6.5-fold after treatment with doxorubicin in different human cancer cell lines. Our study using microarray and validation with RT Q-PCR had demonstrated a 2.5-fold level of upregulation of mRNA in 143-DR-DOX cells, when compared with the parental 143B osteosarcoma tumor cells, suggesting a role in drug-resistant phenotype. The mechanism of interaction between the products of these two genes (MDR1 and mitochondrial taxol resistance associated gene) is yet to be studied.

Genes associated with apoptosis

Krüppel-like factor 2

KLF2 is a member of the KLF family of zinc-finger proteins, which constitute an important class of transcriptional regulators. The promoter region, between –138 and –111 base pairs of KLF2 gene has been shown to be a highly evolutionary conserved region, which is required for its transcription. Studies suggest that the transcription factor KLF2 may negatively regulate FasL promoter in quiescent single positive T lymphocytes resulting in cell survival through resistance to Fas-mediated apoptosis [18].

Krüppel-like factor 2 coexpressed with Interleukin-8

In-silico expression analysis have identified a few genes coexpressed with IL-8 in tumor tissues and that included the gene KLF2 [19]. Studies have suggested that IL-8 may also mediate induction of the KLF2 expression in tumor cells leading to tumor cell survival by resistance to apoptosis [19,20]. Similarly, our microarray data and RT Q-PCR analysis had also revealed an almost equal level of increased coexpression of IL-8 (3.4-fold) and KLF2 (3.3-fold) in the doxorubicin-resistant 143-DR-DOX osteosarcoma cell line compared with the parental 143B cell line.

Our observation of the increased coexpression of these two genes suggests inactivation of a novel molecular pathway involving the peroxisome proliferator-activated receptors γ (PPARγ) to be involved in the mediation of drug resistance and represents a novel target to the treatment of doxorubicin resistance. The PPARs are members of a superfamily of nuclear hormone receptors, which functions through transcriptional regulation of gene expression in response to its ligand-activated extracellular stimulus. Three isoforms of PPAR include PPARα, PPARβ/δ and PPARγ. As PPARγ is found mainly in adipocytes, the two isoforms of PPARγ including γ1 and γ2 have been suggested to play a key role in regulation of lipid homeostasis and energy metabolism. The PPARγ receptors are activated by several lipophilic endogenous ligands and also have been shown to be activated by synthetic agents that belong to a class of antidiabetic drug thiazolidinedione or glitazones. PPARγ ligands induced activation of its receptors results in the induction of both transcriptional transactivation and transrepression of genes in cells and that leads to the activation of several antineoplatic mechanisms including antiproliferation, proapoptotic mechanisms, cellular differentiation and inhibition of angiogenesis. Grommes *et al.* [21] had shown that troglitazone can induce antineoplastic effects in several tumor cell lines through its activation of the PPARγ.

KLF2 was shown to be constitutively overexpressed in preadipocyte cells, acting as a negative regulator of adipocyte differentiation through inhibition of the expression of PPARγ, a central regulator of adipogenesis. KLF2 mediated its effect through (i) inhibition of factors such as C/EBP and ADD1/SREBP 1c that positively regulated PPARγ expression and function, and (ii) inhibition of PPARγ2 promoter activity [20]. Hence, our finding of the KLF2 overexpression in 143-DR-DOX cells suggests that KLF2 may mediate resistance to doxorubicin-induced apoptosis and can cause drug resistance through suppression of the proapoptotic activity of PPARγ. On the basis of these earlier studies [19,20,22] and our study that showed overexpression of IL-8 in drug-resistant cells, we suggest a hypothesis that the IL-8 may have induced the expression of KLF2 which in turn inhibited the expression of PPARγ-mediated apoptosis.

Moreover, PPARγ ligands including thiazolidinediones have demonstrated the antiproliferative effects independent of the PPARγ pathway in tumor cells by blocking G_1–S transition through mechanisms involving the inactivation of eukaryotic initiation factor 2 resulting in an inhibition of the initiation of translation [23]. These studies suggest that the PPARγ ligands can be evaluated for reversing doxorubicin drug resistance in tumor cells.

A disintegrin and metalloproteinase domain 19

ADAMs are a family of type I transmembrane glycoproteins that contain a disintegrin and metalloprotease domain. These membrane-anchored glycoproteins are involved in proteolysis, adhesion, fusion and intracellular signaling. Hence, they plays a role in biological and cellular process including fertilization, heart development, neurogenesis and protein ectodomain shedding. Protein ectodomain shedding catalyzed by ADAM19 involves proteolytic release of numerous membrane-anchored proteins including cytokines, growth factors, receptors, adhesion molecules and enzymes from the plasma membrane. Chesneau *et al.* [24] had reported three potential substrates for the proteolytic enzyme ADAM19 and these include tumor necrosis factor (TNF) α, TNF-related activation-induced cytokine and kit ligand-1 (KL-1). The study revealed an increased shedding of TNF-related activation-induced cytokine in COS-7 cells overexpressing ADAM19 and suggested that ADAM19 can negatively regulate KL-1 shedding, a ligand for the kit receptor tyrosine kinase, in both COS-7 and mouse embryonic fibroblast cells.

c-Kit is a transmembrane protein tyrosine kinase and acts as a receptor for mast cell growth factor. The signal transduction of the protooncogene c-kit plays a central role in regulating normal cell differentiation, maturation and proliferation. Enhanced shedding of KL-1 in mouse embryonic fibroblast cells that had null expression of ADAM19 was reported earlier [24]. Our data has shown a downregulation of ADAM19 mRNA (3.3-fold) in the drug-resistant osteosarcoma (143-DR-DOX) cells, which could result in enhanced shedding of the KL-1. Kit-mediated intracellular signaling through Akt pathway has been shown to suppress apoptosis and confer resistance to antitumor drugs including alkylating agents [25].

Epidermal growth factor receptor-coamplified and overexpressed protein

A novel protein ECOP (EGFR-coamplified and overexpressed protein) was found to play a role in the regulation of NF-κB activity. ECOP activates the nuclear translocation and DNA binding of NF-κB, thus increasing the transcriptional activity of NF-κB and suppression of apoptosis. In ECOP knockdown cells, degradation of IκB α was delayed and that leads to the inhibition of transcriptional activity of the NF-κB and enhanced cellular susceptibility to apoptosis [26].

Our microarray study has demonstrated fivefold lower level of mRNA expression of ECOP in the doxorubicin-resistant (143-DR-DOX) cell line compared with the parental 143B osteosarcoma cell line. Hence, our study suggests that ECOP may play a role in drug resistance through other drug-resistance pathways, which are yet to be identified.

p53

p53 plays a central role in the activation of either of the two separate cellular responses including apoptosis and cell cycle arrest in response to the cytotoxic effects induced by chemotherapeutic drugs. The loss of functional p53 owing to gene deletions and mutations in tumor cells of osteosarcoma had been found to be associated with drug resistance. An in-vitro study using SAOS-2 osteosarcoma cell line transfected with mutant p53 had demonstrated resistance to the antitumor drug cisplatin [27]. Chen *et al.* [28] have shown that higher levels of p53 protein are required for the activation of apoptosis than those required for cell cycle arrest. Our study suggests that the 2-fold decrease in the p53 mRNA expressed in drug-resistant 143-DR-DOX tumor cells may not be able to induce apoptosis, potentially leading to drug resistance.

Ferredoxin reductase

FDXR is a 50 000-kDa mitochondrial flavoprotein, located on the matrix side of the inner mitochondrial membrane and involved in cellular biochemical process of lipid metabolism. It transports electron from nicotinamide adenine dinucleotide phosphate to a membrane-integrated cytochrome P450 enzyme (CYP11A1) via the electron shuttle ferredoxin in mitochondria. Studies had shown that ferredoxin protein contributes to p53-mediated apoptosis through the generation of oxidative stress in mitochondria. An in-vitro study had shown that ferredoxin protein increased the sensitivity of tumor cell lines including H1299 and HCT116 cells to the reactive oxygen species (ROS)-mediated apoptosis induced by antitumor drugs including 5-fluorouracil and doxorubicin [29]. Their study supports a model of a feed-forward loop for p53 activity in which the oxidative stress caused by ROS activates p53 and this induces the expression of its downstream target FDXR, which in turn sensitizes the cells to ROS-mediated apoptosis. Partial disruption of the FDXR gene was shown to suppress the response to apoptosis induced by 5-fluorouracil in colon cancer cells [30]. Our data showed a decreased FDXR mRNA levels (1.1-fold) in 143-DR-DOX cells as compared with parental 143B cells. In our study, p53 mRNA was downregulated (twofold) in the drug-resistant osteosarcoma tumor cell line, suggesting that the down-regulated p53 may play a role in the decreased expression of FDXR leading to inhibition of apoptosis and that in turn may protect the cells from the cytotoxic effects of oxidative stress generated by doxorubicin.

Structural molecule

Keratin 18

KRT18 encodes the type I intermediate filament chain keratin 18, which together with keratin 8, forms an intermediate filament that provides structural integrity in epithelial cells. A differential gene expression study had reported lower levels of KRT18 expression in cisplatin-resistant ovarian cancer (2008/C13) cell line and also had demonstrated the reversal of resistance by the transfection of KRT18 cDNA in cisplatin-resistant cells [31]. In this context, our study showed a decreased expression of KRT18 mRNA (1.7-fold) in the doxorubicin-resistant cell line compared with the drug-sensitive 143B cell line.

Genes involved in mitochondrial electron transfer

Cytochrome c_1

CYC1 is one of the subunits of cytochrome bc_1 complex of the mitochondrial electron-transfer chain. It was found to be downregulated in the drug-resistant 143B-DR-DOX cells.

Metabolic genes

Uridine phosphorylase

Uridine phosphorylase (UPase) catalyzes the catabolism of uridine to uracil. It also catalyzes salvage pathway involving the anabolic reverse reaction forming uridine from uracil in the presence of ribose-1-phosphate. The downregulation of uridine phosphorylase was shown to be associated with 5-fluorouracil resistance in gastric cancer cell lines [32]. Our study has shown fivefold decreased expression of the UPP1 mRNA level in the doxorubicin-resistant 143-DR-DOX cells.

S-adenosyl homocysteine hydrolase (AdoHcyase)

AHCY belongs to the family of adenosyl homocysteinase and it catalyzes the reversible hydrolysis of *S*-adenosyl homocysteine (AdoHcy) to adenosine (Ado) and L-homocysteine (Hcy). Thus, AdoHcyase regulates the intracellular concentration of *S*-adenosylhomocysteine (SAH), which in turn plays a role in the regulation of transmethylation reactions. As there exists a structural similarity of polypeptide folding pattern at the catalytic domain between AdoHcyase and DNA methyl transferases, the SAH also acts as a substrate for DNA methyl transferases. Deficiency in SAH hydrolase causes hypermethioninemia and also results in excess accumulation of SAH, which binds to methyltransferases and that inhibits DNA methylation reactions leading to DNA hypomethylation [33]. DeAngelis *et al.* [34] demonstrated downregulation of the gene AHCY in 5-flurouracil-resistant colorectal cancer cell lines. Our study showed that the AHCY mRNA expression was twofold downregulated in doxorubicin-resistant 143B cell line compared to parental 143B osteosarcoma cell line. Drug-resistance pathways mediated by decreased expression of AHCY in doxorubicin-resistant tumor cells are yet to be studied.

Endoplasmic reticulum genes

SEC61G

Sequence information for the SEC61G gene indicates that this gene encodes a γ-subunit protein of the SEC61 complex, which consists of two other membrane proteins, α and β. The SEC61 complex is a central component of the protein translocation apparatus of the endoplasmic reticulum (ER) membrane and it forms a transmembrane channel where proteins are translocated across and integrated into the ER membrane. Our microarray study has shown the SEC61G mRNA level to be 2.5-fold downregulated in the doxorubicin-resistant 143-DR-DOX cell line. The pathway through which SEC61G mediates drug resistance is yet to be studied.

Endoplasmic reticulum stress response genes

The ER plays several important roles in folding, export and processing of newly synthesized proteins. Various conditions can interfere with ER function leading to accumulation of unfolded proteins and which results in ER stress. The ER stress induces functionally distinct cellular responses including (i) upregulation of genes encoding ER chaperone proteins; (ii) translational attenuation; and (iii) apoptosis [35]. In our study, we found an increased expression of ER stress response genes that encoded chaperone proteins including DNAJB11, HYOU1 and CALR which could be involved in resistance to the chemotherapeutic drug doxorubicin in the cell line.

Gene associated with metastasis

FXYD5 (dysadherin)

A cancer-associated cell membrane glycoprotein that downregulates E-cadherin and promotes cancer metastasis has been named as dysadherin. Our data showed a 1.7-fold level of increased FXYD mRNA expression in 143-DR-DOX cells as compared with the parental 143B cells.

Alteration in drug target

Topoisomerase IIβ

DNA topoisomerases are nuclear enzymes that regulate DNA topology and are recognized as the primary targets of effective antitumor drugs including doxorubicin. Two genetically distinct isoforms of DNA topoisomerase II including α and β are expressed by human cells. In-vitro studies using human leukemic cell lines including ALL-C and HL-60/MX2 have shown an association between a low level of altered expression of TOP2B and resistance to antitumor drugs including doxorubicin, etoposide and mitoxantrone [36,37]. In this context, our study had also showed a reduced mRNA expression of TOP2B (1.25-fold) in the 143-DR-DOX cell line, when compared with the parental 143B cell line. Thus, similar to the lines of evidence shown by previous studies, our study also demonstrated alteration in the level of drug target as a resistance mechanism to doxorubicin.

Conclusion

Drug resistance in cancer therapy is a major issue preventing better cure rates. Although MDR1 and TOP2B have been known to be associated with doxorubicin drug resistance, our study using the 143B-DR-DOX cell line suggests that additional pathways could be involved and may offer potential targets for circumventing drug resistance to doxorubicin.

Supplementary data

Supplementary data are available at the *Anti-Cancer Drugs* journal online (*www.anti-cancerdrugs.com*).

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