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Identification of Marker Genes Related to Cardiovascular Toxicity of Doxorubicin and Daunorubicin in Human Umbilical Vein Endothelial Cells (HUVECs)

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Abstract

Doxorubicin and daunorubicin are excellent chemotherapeutic agents utilized for several types of cancer but the irreversible cardiac damage is the major limitation for its use. The biochemical mechanisms of doxorubicin- and daunorubicin- induced cardiotoxicity remain unclear. There are many reports on toxicity of doxorubicin and doxorubicin in cardiomyocytes, but effects in cardiovascular system by these drugs are almost not reported. In this study, we investigated gene expression profiles in human umbilical vein endothelial cells (HUVECs) to better understand the causes of doxorubicin and doxorubicin-induced cardiovascular toxicity and to identify differentially expressed genes (DEGs). Through the clustering analysis of gene expression profiles, we identified 124 up-regulated common genes and 298 down-regulated common genes changed by more than 1.5-fold by all two cardiac toxicants. HUVECs responded to doxorubicin and doxorubicin damage by increasing levels of apoptosis, oxidative stress, EGF and lipid metabolism related genes. By clustering analysis, we identified some genes as potential markers on apoptosis effects of doxorubicin and doxorubicin. Six genes of these, BBC3, APLP1, FAS, TP53INP, BIRC5 and DAPK were the most significantly affected by doxorubicin and doxorubicin. Thus, this study suggests that these differentially expressed genes may play an important role in the cardiovascular toxic effects and have significant potential as novel biomarkers to doxorubicin and doxorubicin exposure.

Keywords: Doxorubicin, Daunorubicin, Cardiovascular toxicity, HUVECs, Apoptosis

Anthracycline antibiotics, particularly doxorubicin and daunorubicin, have been used extensively in the treatment of human malignancies[1]. Anthracyclines are a class of antitumor drugs widely used for the treatment of a variety of malignancy, including leukemias, lymphomas, sarcomas and carcinomas. Different mechanisms have been proposed for anthracycline antitumor effects including free radical generation, DNA intercalation/binding, activation of signaling pathways, inhibition of topoisomerase II and apoptosis[2]. Their usefulness has been limited to a degree, however, by their long-known associated cardiotoxicity. The antitumor activity of the anthracyclines damages not only the neoplastic but also the normal cells of the body, especially the myocytes. Increased free radical production and decreased myocardial endogenous antioxidants are believed to be the important mechanisms responsible for myocyte damage[3-5].

The vascular endothelium plays a fundamental role in maintenance of organ function by forming a barrier regulating water and solute distribution between blood and tissues. Dysfunction results in movement of water and proteins from the vascular system into tissues, causing edema and compromising organ function. Endothelial dysfunction is a common finding in diseases such as diabetes, Alzheimer's disease, atherosclerosis and ischemia-reperfusion injury. A common thread among these diseases is oxidative stress, a preponderance of oxidant generation over antioxidant defenses-a condition that is known to cause toxicity to Endothelial Cells (EC), leading to loss of barrier function[6]. Drugs, particularly anti-cancer drugs, also cause oxidant stress. The primary aim of cancer chemotherapy is to kill tumor cells by inhibiting cell proliferation. However, more recently, toxicity to vascular endothelium has also received attention because tumor cells require a functioning endothelium for growth and proliferation. Indeed, some cancer chemotherapeutic agents are more toxic to EC than to tumor cells[7], and endothelial damage may contribute to the cardiotoxicity and side-effects of chemotherapy.

Although most studies on the mechanism of action of doxorubicin and daunorubicin have focused on damage to target tumor cells, Kalayanaraman and

colleagues[8] have shown that doxorubicin-induced, metal-catalyzed production of H_2O_2, even at submicromolar concentrations of doxorubicin, contributes to the toxicity of doxorubicin to both EC and cardiomyocytes and probably underlies the cardiotoxicity associated with anthracyclines therapy.

The purpose of the present study was to identify the potential gene-based cardiovascular toxic markers and to investigate the effects of doxorubicin and daunorubicin on endothelial dysfunction in an Human Umbilical Vein Endothelial Cells (HUVECs). We examined global gene expression in a small number of well-matched exposed-control subject pairs. Genes with differential expression were then ranked and selected for further examination using several forms of statistical analysis. The identification of Differentially Expressed Genes (DEGs) may assist in the identification of potential biomarker and may understand molecular toxicological mechanisms of doxorubicin and daunorubicin in human umbilical vein endothelial.

Cytotoxicity of Doxorubicin and Daunorubicin in HUVECs

Relative survival of HUVECs following exposure to a range of concentrations of doxorubicin and daunorubicin was determined by MTT assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of Optical Density (OD) value measured after treatment. Based on the results of MTT assay, 20% inhibitory concentration (IC_{20}) of each compound was calculated. Dose dependent cell viability curves were obtained after 48 hrs of exposure to doxorubicin and daunorubicin in HUVECs as shown in Figure 1. Doxorubicin and daunorubicin reduced cell viability gradually at increasing concentrations. The IC_{20} for doxorubicin and daunorubicin were 82.97 ng/mL and 191.00 ng/mL, respectively (Figure 1).

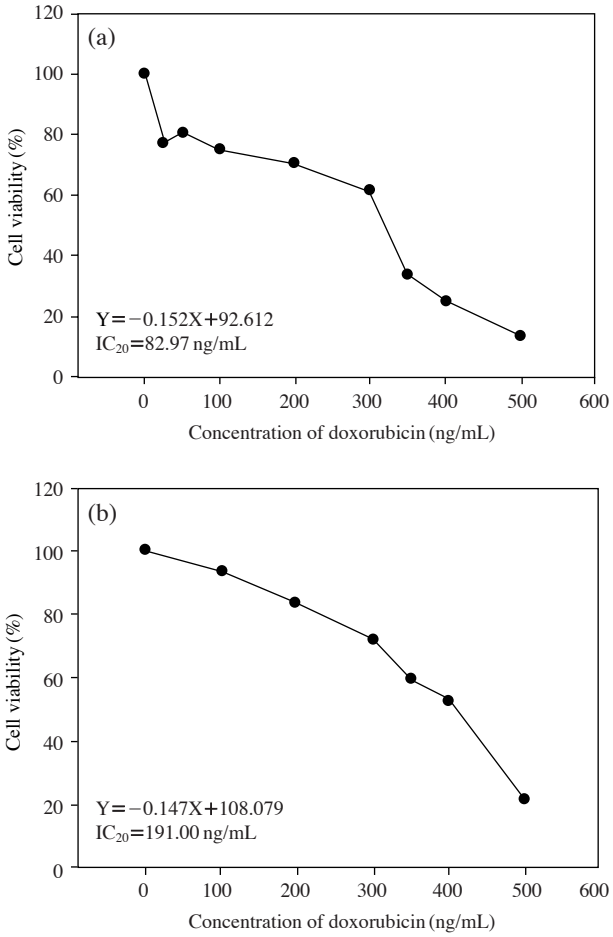

Figure 1. Dose-response curves were assessed by the MTT assay in HUVECs after 48 hrs doxorubicin and daunorubicin treatment. IC_{20} values were calculated as (a) 82.97 ng/mL doxorubicin and (b) 191.00 ng/mL daunorubicin.

Gene Expression Profiles Altered by Doxorubicin and Daunorubicin

HUVECs were treated with 82.97 ng/mL doxorubicin and 191.00 ng/mL daunorubicin for 48 hrs, and the RNA was subjected to microarray analysis. For each treatment, genes with statistically significant expression changes were identified by microarray. Three independent experimental samples for each treatment group were analyzed to determine RNA transcript levels. Only those genes, which displayed either greater than or equal to a 1.5 fold up- and down-regulation, have been considered for this study. Hierarchical clustering was used to aid in visualization and biological interpretation of this extensive data set, and in particular, to identify correlated expression patterns. Hierarchical clustering was applied across the two drugs, using a combined list of genes identified to be altered statistically significant in at least one of the sample studied relative to control (Figure 2). From the clustering analysis, we can assume that the gene expression patterns of doxorubicin and daunorubicin were similar. Oligonucleotide microarray analysis was employed to characterize the cells response to doxorubicin and daunorubicin. The gene expression profiles in HUVECs exposed to these two anthracyclines at one dose and one time point were analyzed. Venn diagram shows the gene expression profiles; doxorubicin and daunorubicin were up-regulated 249 and 697 genes and also down-regulated 893 and 1,130 genes, respectively (Figure 3).

There were 125 common up-regulated genes and 298 common down-regulated genes through doxoru-

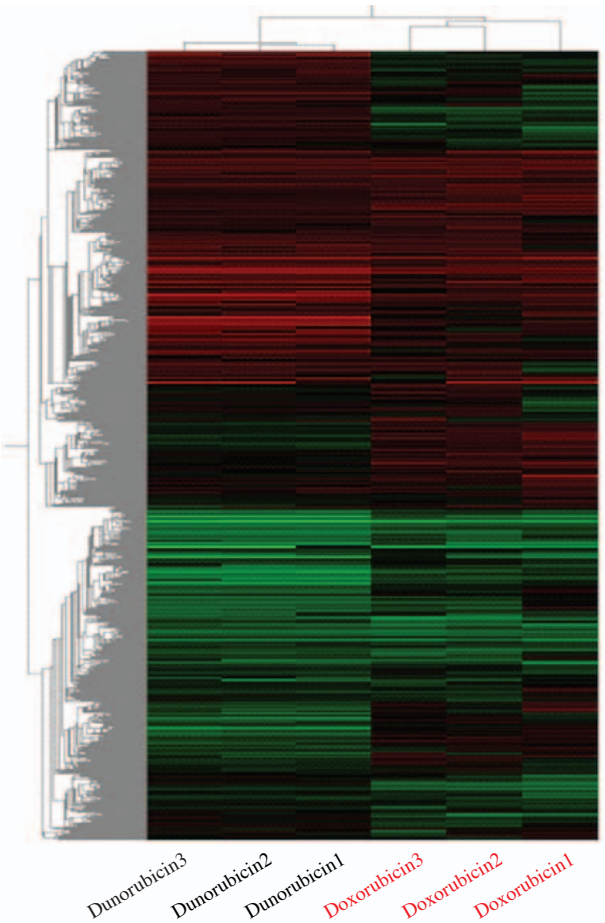

Figure 2. Hierarchical cluster image showing the differential gene expression profiles of doxorubicin and daunorubicin exposed in HUVECs.

bicin and daunorubicin exposure (Figure 3). Table 1 showed the list of the up-regulated common genes and Table 2 showed some down-regulated genes, which statistically significantly changed their expression profiles, along with their function.

Discussion

Very few data is available about the alteration of gene expression with anthracyclines exposure. In this study, we have used genomic analysis to identify differentially expressed significant genes induced by doxorubicin and daunorubicin in HUVECs using 44 k whole human oligonucleotide microarray. Triplicate assays were performed for each chemical to avoid the error. From microarray study in conjunction with statistical analysis, doxorubicin and daunorubicin were up-regulated 249 and 697 genes and also down-regulated 893 and 1,130 genes, respectively (Figure 3).

Toxicogenomic study has been widely used to characterize toxicological properties of disease and to develop and validate of biomarkers that permit the prediction of the risk of disease. Through previous studies in our laboratory, we confirmed toxicogenomic effects of several chemicals involved in inducing adverse or toxic effects[9-11]. In this paper, we subjected to anthracyclines for observing their cardiovascular toxic effects on genes and evaluating the associated mechanisms using toxicogenomic tools.

Amyloid beta (a4) precursor-like protein 1 (APLP1) is processing of the amyloid beta protein precursor

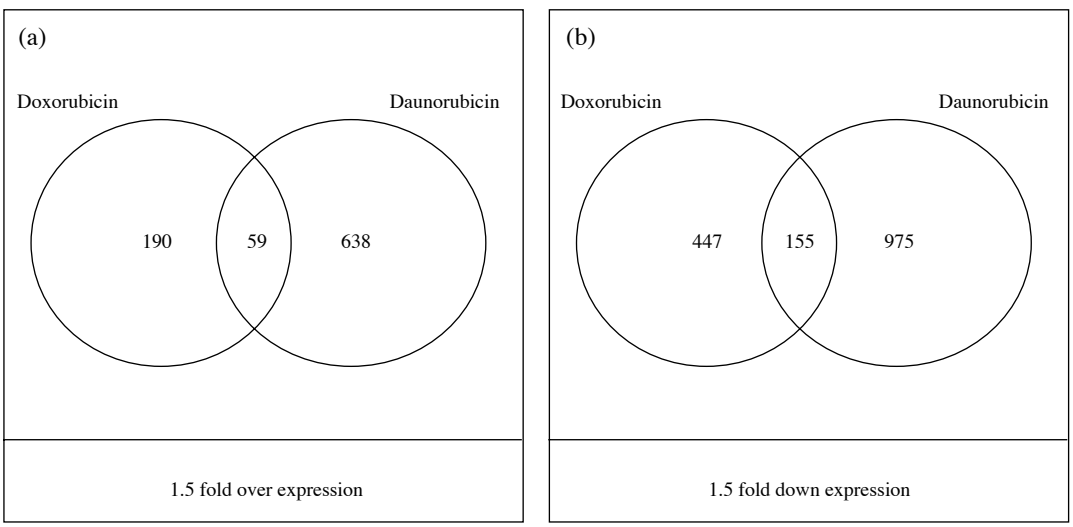

Figure 3. Venn diagram showed the differentially expressed genes by doxorubicin and daunorubicin in HUVECs. The diagrams were generated from the list of up (a) and down (b) regulated genes that were >1.5 fold with microarray analysis.

Table 1. List of up-regulated common genes by doxorubicin and daunorubicin in HUVECs.

Accession no.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)	
			Doxorubicin	Daunorubicin
Apoptosis				
AB209361	FAS	fas (tnf receptor superfamily, member 6)	2.88	10.76
NM_078467	CDKN1A	cyclin-dependent kinase inhibitor 1a (p21, cip1)	2.58	8.10
AF332558	BBC3	bcl2 binding component 3	2.69	3.20
Cell cycle				
BC040303	PTP4A1	protein tyrosine phosphatase type iva, member 1	3.03	4.25
NM_001759	CCND2	cyclin d2	2.42	4.75
NM_003620	PPM1D	protein phosphatase 1d magnesium-dependent, delta isoform	2.03	2.28
CR590366	MDK	midkine (neurite growth-promoting factor 2)	2.20	2.07
AF033122	SESN1	sestrin 1	2.42	2.79
CR623038	ID2	inhibitor of dna binding 2, dominant negative helix-loop-helix protein	3.63	5.77
BC060797	PARD6G	par-6 partitioning defective 6 homolog gamma (c. elegans)	2.85	4.98
NM_078467	CDKN1A	cyclin-dependent kinase inhibitor 1a (p21, cip1)	2.58	8.70
AY123223	SESN2	sestrin 2	2.16	2.63
DNA metabolism				
CR608156, NM_003512	H2AC	histone 1, h2ac	2.37	4.35
NM_003516	H2AA	histone 2, h2aa	2.85	4.12
NM_001012716	TYMS	thymidylate synthetase	2.22	3.03
NM_004628	XPC	xeroderma pigmentosum, complementation group c	2.13	5.55
NM_080593	H2BK	histone 1, h2bk	2.24	8.32
BU603483	H1E	histone 1, h1e	3.04	2.41
AB209274	POLD4	polymerase (dna-directed), delta 4	2.10	2.38
NM_006763	BTG2	btg family, member 2	2.46	2.18
Development				
AY358720	PCDHB10	protocadherin beta 10	3.46	2.68
CR590366	MDK	midkine (neurite growth-promoting factor 2)	2.20	2.07
BC035939	MRAS	muscle ras oncogene homolog	3.26	2.02
AK122796	ETV7	ets transcription factor tel-2b	2.41	2.96
NM_006426	DPYSL4	dihydropyrimidinase-like 4	4.74	2.20
CR623416	PHYH	phytanoyl-coa 2-hydroxylase	2.11	2.01
NM_004235	KLF4	kruppel-like factor 4 (gut)	2.17	2.24
AB191264	AGRN	agrin	2.07	5.56
BC040303	PTP4A1	protein tyrosine phosphatase type iva, member 1	3.03	4.25
NM_001553	IGFBP7	insulin-like growth factor binding protein 7	2.14	2.20
AY268890	TPP1	tripeptidyl peptidase i	2.02	2.00
NM_005940	MMP11	matrix metallopeptidase 11 (stromelysin 3)	2.65	2.63
NM_000899	KITLG	kit ligand	2.44	2.48
NM_001024807	APLP1	amyloid beta (a4) precursor-like protein 1	5.53	6.35
AK128769	CACNLB2	calcium channel, voltage-dependent, beta 2 subunit	2.15	2.06
NM_018935	PCDHB15	protocadherin beta 15	2.19	2.78

(A beta PP) family member. Functions identified for APLP1 include its ability to trigger apoptosis and a role in regulating gene transcription, in combination with the A beta PP binding protein Fe65[12]. The tumor suppressor p53 is a key modulator of the cellular stress response, inducing cell-cycle arrest, apoptosis, senescence and cell differentiation. In particular, APLP1 is induced in senescent cells in a p53-dependent manner[13]. Fas is a cell surface receptor and member of the tumor necrosis factor receptor superfamily which mediates apoptosis in sensitive cells upon oligomerization by specific antibodies or by its ligand (FasL)[14]. The Fas ligand (FasL, CD95/Apo-1 ligand, CD178 and TNFSF6) molecules belong to the TNF family and potently induce cell death in Fas (CD95/Apo-1/TNFRSF6) receptor-expressing cells[15]. BBC3, a transcriptionally regulated proapoptotic member of the BCL2 family, was the most highly induced proapoptotic gene[16]. BBC3 gene expression is activated by at least three apoptotic stimuli, including DNA damage, glucocorticoid treatment and growth factor deprivation, constituting a broad transcriptio-

Table 2. List of down-regulated common genes by doxorubicin and daunorubicin in HUVECs.

Accession no.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)	
			Doxorubicin	Daunorubicin
Apoptosis				
NM_002982	CCL2	chemokine (c-c motif) ligand 2	0.47	0.39
NM_007313	ABL1	v-abl abelson murine leukemia viral oncogene homolog 1	0.33	0.18
BM471410, NM_002156	HSPD1	heat shock 60kda protein 1 (chaperonin)	0.49	0.41
NM_004938	DAPK1	death-associated protein kinase 1	0.42	0.19
NM_001012271	BIRC5	baculoviral iap repeat-containing 5 (survivin)	0.04	0.09
AB209660	CD40	cd40 antigen (tnf receptor superfamily member 5)	0.47	0.18
AY358868	TNFSF18	tumor necrosis factor (ligand) superfamily, member 18	0.34	0.20
D79987	ESPL1	extra spindle poles like 1 (s. cerevisiae)	0.21	0.10
Carboxylic acid metabolism				
XM_371677	LOC389173	similar to phosphoserine aminotransferase isoform 1	0.49	0.47
NM_147161	THEA	acyl-coa thioesterase 11	0.45	0.26
AK093306	PHGDH	phosphoglycerate dehydrogenase	0.43	0.20
NM_005063	SCD	stearoyl-coa desaturase (delta-9-desaturase)	0.32	0.21
AK123190	GCAT	glycine c-acetyltransferase (2-amino-3-ketobutyrate coenzyme a ligase)	0.41	0.42
BX640945	FADS2	fatty acid desaturase 2	0.44	0.15
NM_198839	ACACA	acetyl-coenzyme a carboxylase alpha	0.34	0.22
X66610	ENO1B	enolase alpha, lung-specific	0.44	0.33
NM_005504	BCAT1	branched chain aminotransferase 1, cytosolic	0.46	0.42
NM_003046	SLC7A2	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	0.45	0.24
AB209172	SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	0.45	0.23
NM_030919	C20orf129	chromosome 20 open reading frame 129	0.13	0.09
AL833741	ENO1	enolase 1, (alpha)	0.45	0.46
NM_003486	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	0.26	0.20
Double-strand break repair				
NM_006904	PRKDC	protein kinase, dna-activated, catalytic polypeptide	0.36	0.25
NM_004111	FEN1	flap structure-specific endonuclease 1	0.34	0.19
AF360549	BRIP1	brca1 interacting protein c-terminal helicase 1	0.39	0.30
DNA metabolism				
L08238	JRK	suppressor of variegation 3-9 homolog 1 (drosophila)	0.37	0.45
NM_001254	PHF19	cdc6 cell division cycle 6 homolog (s. cerevisiae)	0.28	0.28
NM_003258	MAZ	thymidine kinase 1, soluble	0.45	0.46
DQ097177	INCENP	hect, uba and wwe domain containing 1	0.34	0.42
AK074377	CDT1	ubiquitin-like, containing phd and ring finger domains, 1	0.38	0.30
NM_001274	KIF4A	chk1 checkpoint homolog (s. pombe)	0.18	0.06
NM_144678	Spc25	target of myb1-like 2 (chicken)	0.31	0.26
NM_006739	OIP5	mcm5 minichromosome maintenance deficient 5, cell division cycle 46 (s. cerevisiae)	0.18	0.09
NM_001067	KIF23	topoisomerase (dna) ii alpha 170kda	0.17	0.07
NM_006904	OSM	protein kinase, dna-activated, catalytic polypeptide	0.42	0.33
AK056803	CDKN3	h2a histone family, member z	0.15	0.07
NM_012415	KIF2C	fibrinogen silencer binding protein	0.05	0.03
NM_003579	MELK	rad54-like (s. cerevisiae)	0.26	0.22
AF360549	AQP1	brca1 interacting protein c-terminal helicase 1	0.29	0.04
U81234	TRIP13	chemokine (c-x-c motif) ligand 6 (granulocyte chemotactic protein 2)	0.14	0.06
AK123010	CXCL5	ribonucleotide reductase m2 polypeptide	0.42	0.42
CR600021	HOXB4	high-mobility group box 2	0.43	0.29
AY032677	E2F2	polymerase (dna directed), theta	0.34	0.31
NM_005915	MYOHD1	mcm6 minichromosome maintenance deficient 6 (mis5 homolog, s. pombe) (s. cerevisiae)	0.47	0.45
AB053172	C18orf34	dna replication factor	0.45	0.45

Table 2. Continued.

Accession no.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)	
			Doxorubicin	Daunorubicin
BU675073	HAPLN3	pituitary tumor-transforming 1	0.49	0.32
NM_003504	MCM7	cdc45 cell division cycle 45-like (s. cerevisiae)	0.48	0.22
NM_182776	THRAP3	mcm7 minichromosome maintenance deficient 7 (s. cerevisiae)	0.49	0.45
AF095289	CCL14	pituitary tumor-transforming 3	0.37	0.17
D80008	IMPDH1	dna replication complex gins protein psf1	0.28	0.18
NM_007313	ASF1B	v-abl abelson murine leukemia viral oncogene homolog 1	0.2	0.06
NM_002915	LOC401127	replication factor c (activator 1) 3, 38 kda	0.45	0.42
NM_015895	RACGAP1	geminin, dna replication inhibitor	0.17	0.10
BC067848	GABBR1	karyopherin alpha 2 (rag cohort 1, importin alpha 1)	0.30	0.30
NM_004111	MGC5395	flap structure-specific endonuclease 1	0.45	0.27
NM_001618	KNTC2	poly (adp-ribose) polymerase family, member 1	0.03	0.03

nal response thus far unique among mammalian cell death regulatory genes[17]. BIRC5 has been described for surviving, which has been implicated in both inhibition of apoptosis and regulation in mitosis in many tumor types. Another selected protein was the human death-associated protein kinase 1 (DAPK1), that functions as a positive mediator of apoptosis[18]. Significant up-regulation of APLP1, FAS and BBC3 genes and down-regulation of survidin and DAPK1 genes reveal that doxorubicin and daunorubicin exposure to human vascular cell system may hamper apoptosis-mediated toxicity.

In conclusion, although this data is not enough to say the mechanistic inside of doxorubicin and daunorubicin toxicity, we have identified apoptosis related genes including BBC3, APLP1, FAS, TP53INP, BIRC5 and DAPK which differentially expressed in HUVECs. These genes could be a promising biomarker to detect other anthracyclines inducing the cardiovascular toxicity.

Methods

Chemicals and Reagents

Doxorubicin, daunorubicin and dimethylsulphoxide (DMSO) were obtained from Sigma-Aldrich Chemical Company (USA). RPMI-1640, Dulbecco's Phosphate Buffered Saline (PBS) and Fetal Bovine Serum (FBS) were the products of GIBCO™ (USA). All other chemicals used were of analytical grade or the highest grade available.

Cell Lines and Culture

HUVECs (human umbilical vein endothelial cells) used throughout this study, were purchased from Clonetics (San Diego,USA) . The cells were grown in EGM-2 medium (CAMBREX, USA) supplemented with 5% inactivated FBS, hydrocortisone 0.2 mL, H-FGF-b 2 mL, v-EGF 0.5 mL, ascdrbic acid 0.5 mL, H-BGF 0.5 mL, GA-1000 0.5 mL and heparin 0.5 mL at 37˚C in 5% CO_2 atmosphere. For cell growth, the medium was renewed every two or three days at a density of 4×10^5 cells/mL in 100 mm culture dish (Falcon Becton Dickinson, USA).

Determination of Cell Viability

MTT assay was performed for the detection of cell viability[19]. For anthracycline drugs, 24 well plate was used for cytotoxicity assay. 0.5 mL of HUVECs were seeded at a seeding density of 10×10^4 cells/mL. Cells were exposed to various concentrations of doxorubicin and daunorubicin in culture medium at 37˚C for 48 hrs exposure time. MTT (5 mg/mL in PBS) solution was added to each well and incubated for 3 hrs. DMSO solution was added to each tube and transfer in 96 well plate. The OD of the purple formazan product was measured at a wavelength of 540 nm. The IC_{20} of cell proliferation in a particular drug was defined as the concentration that causes a 20% reduction in the cell viability versus the solvent treated control. The IC_{20} values were directly determined from the linear dose-response curves.

RNA Extraction

Total RNA was extracted from the HUVECs treated to 82.973 ng/mL and 191.00 ng/mL for doxorubicin and daunorubicin, respectively, for 48 hrs using the Trizol reagent (Invitrogen, USA) and purified using RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. Genomic DNA was removed using RNase-free DNase set (Qiagen, USA) during RNA purification. The amount of each total RNA concentration was quantified using SmartSpec

3000 (Bio-Rad, Hercules, USA). Only samples with an A260/A280 ratio between 1.9 and 2.2 were considered for suitable use and its quality was checked by agarose-gel electrophoresis.

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on the RNA samples using 44 k whole human genome microarray (Agilent Biotechnologies, USA). Triplicate analysis was performed for each chemical simultaneously. Labelling and hybridization were performed by instruction of Platinum Biochip Reagent Kit (GenoCheck Co. Ltd, Korea). This was followed by the coupling of the Cy3 dye for the controls (DMSO) or Cy5 dye for the treated samples. Hybridization was performed in a hybridization oven at 62°C for 12 h. After washing (2 × SSC/0.1% SDS for 2 min at 58°C, 1 × SSC for 2 min at RT and 0.2 × SSC for 3 min at RT), the slide was dried by centrifugation at 800 rpm for 3 min at RT. Hybridization images on the slides were scanned by ScanArray Lite (PerkinElmer Life Sciences, USA). Scanned images were analyzed with GenePix 3.0 software (Axon Instruments, USA) to obtain gene expression ratios.

Data Analysis

The fluorescent intensity of each spot was calculated by local median background subtraction. The robust scatter-plot smoother LOWESS function was used to perform intensity dependent normalization for the gene expression. Scatter plot analysis was made by Microsoft Excel 2000 (Microsoft, WA, USA). Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes[20]. Computing a q-value for each gene assessed the statistical significance of the differential expression of genes. To determine the q-value, a permutation procedure was used and for each permutation, two-sample t statistics were computed for each gene. Genes were considered differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 1.5 or less than 0.50, i.e., 1.5-fold difference in expression level, and when the q-values were < 5.

Acknowledgements

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