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ORIGINAL ARTICLE

# Gene expression analysis of drug-resistant MCF-7 cells: implications for relation to extracellular matrix proteins

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**Abstract**
*Purpose* Since multidrug resistance is a multifactorial phenomenon, a large-scale expression analysis of drug-resistant cells by using high-density oligonucleotide microarrays may provide information about new candidate genes contributing to resistance. Extracellular matrix (ECM) is responsible for many aspects of proliferation and invasive/metastatic behavior of tumor cells. This study demonstrates alterations in gene expression levels of several ECM components, matrix metalloproteinases (MMPs), adamalysins (ADAMs and ADAMTSs) and tissue inhibitors of metalloproteinases (TIMPs) in paclitaxel, docetaxel, vincristine and doxorubicin-resistant MCF-7 cells.
*Methods* Resistant MCF-7 cells were developed by stepwise selection of cells in increasing concentrations of drugs. Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array was used for hybridizations. Statistical significance was determined by independent sample *t* test. The genes having altered expression levels in drug-resistant sublines were selected and filtered by volcano plots.
*Results* Genes up/downregulated more than twofolds were selected and listed. Expression of 25 genes encoding ECM proteins (including collagen, finronectin and syndecan) and integrin receptor subunits were found to be upregulated in drug-resistant cells. In addition, expression levels of, 13 genes encoding MMPs, ADAMs, ADAMTSs and TIMPs (including MMP1, MMP9, ADAM9 and TIMP3) were found to be altered in drug-resistant sublines when compared with sensitive MCF-7.
*Conclusions* Based on the expression analysis profiles, this report provides a preliminary insight into the relationship between drug resistance and ECM components, which are related to invasion and metastasis. Correlation of each specific ECM component with drug resistance requires further analysis.

**Keywords** Multidrug resistance · cDNA microarray · ECM · Integrin · MMP · ADAM

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## Introduction

Multidrug resistance (MDR) describes a complex phenotype whose predominant feature is resistance to wide range of structurally unrelated anticancer agents and is a serious limitation to the effective chemotherapeutic treatment [1]. There are several mechanisms by which cancer cells develop resistance to cytotoxic agents involving the alterations in various biochemical pathways. Mechanisms of drug resistance include decreased intracellular drug levels, which could result from increased drug efflux (ATP-binding cassette transporters such as MDR1/P-glycoprotein), decreased conversion of drug to an active form, altered amount of target enzyme or receptor, decreased affinity of target enzyme or receptor for drug, enhanced repair of the drug-induced defect, decreased activity of an enzyme required for apoptosis, altered expression of genes for survival and altered target type [2]. Genome wide expression analysis techniques may provide additional information on contribution of novel candidate genes to drug

resistance. Particularly, large-scale expression microarray analysis using high-density oligonucleotide cDNA microarrays enable researcher's assessment of expression profiles of drug-resistant cells.

Extracellular matrix (ECM) made up of collagens, fibronectin, laminins, proteoglycans and other macromolecules, controls many aspects of cells such as gene expression, differentiation and proliferation, and invasive/metastatic phenotype of tumor cells. Altered expression levels of these molecules in tumor cells was reported to affect their sensitivity to drug-induced apoptosis and drug resistance through activation of survival pathways of cells [3, 4]. Tumor cells remodel their microenvironment for survival and invasion. Differential expression of ECM components, matrix metalloproteinases (MMPs) and adamalysins (a disintegrin and metalloproteinases; ADAMs and a disintegrin and metalloproteinase with thrombospondin motifs; ADAMTSs) may contribute the remodeling of ECM, cancer invasion and metastasis. According to a previous report [5] on invasive/metastatic behavior of drug-resistant tumor cells, carboplatin-resistant mouse epithelial cells had increased levels of ECM components (fibronectin, laminin, collagen type IV) with enhanced MMP-2 activity. Remodeling of ECM due to drug exposure has also been related to drug resistance that it can affect tumor cell survival by preventing the penetration of the drug into the tumor in vivo. In addition, changes in ECM can also affect the sensitivity of the tumor cells to apoptosis through growth factors dependent on cell-matrix and cell–cell interactions through ECM–integrin signaling [6] as well as development of cell adhesion-mediated drug resistance (CAM-DR).

This study demonstrates alterations in gene expression levels of ECM components, MMPs, ADAMs, ADAMTSs and tissue inhibitors of metalloproteinases (TIMPs) in paclitaxel (MCF-7/Pac), docetaxel (MCF-7/Doc), vincristine (MCF-7/Vinc) and doxorubicin (MCF-7/Dox)-resistant MCF-7 cells.

## Materials and methods

### Cell lines

MCF-7 cell line, which is a model cell line for human mammary carcinoma, was used as model cell line. The cell line exhibits some features of differentiated mammary epithelium and was donated by ŞAP Institute, Ankara, Turkey. 400 nM paclitaxel (MCF-7/Pac), 120 nM docetaxel (MCF-7/Doc), 120 nM vincristine (MC-F-7/Vinc) and 1,000 nM doxorubicin (MCF-7/Dox)-resistant MCF-7 cell lines were developed from sensitive MCF-7 cells (MCF-7/S) as previously described [7] and the cells were 150-, 47-, 30- and 161-folds resistant to their selective drugs, respectively [8], as determined by Cell Proliferation Kit (Biological Industries, Kibbutz Beit Haemek, Israel).

### RNA isolation, cDNA synthesis and target preparation

RNA isolation form MCF-7/S, and all resistant sublines were performed using TRI Reagent (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Absorbance values (260, 280 nm) were measured for RNA quantification by spectrophotometry. RNA intactness was checked by 1% w/v denaturing agarose gel electrophoresis at 70 V in MOPS (3-N-morpholinopropanesulfonic acid) buffer. RNA concentration in each sample was adjusted to at least 2.5 μg/μL with $OD_{260}/OD_{280}$ ratio of 1.8–2.0. All RNA samples were prepared as duplicates. cDNA was synthesized from total RNA by One-Cycle Target Labelling Assay® (Affymetrix, Santa Clara, CA, USA) according to manufacturer's instructions. Second strand cDNA synthesis, biotin-labeled cRNA synthesis (IVT Labeling) and cRNA fragmentation were performed by Affymetrix GeneChip® kit reagents according to procedure as described in the Affymetrix GeneChip® Expression Analysis Technical Manual.

### Target hybridization and scanning

Biotin-labeled and fragmented target cRNA samples were loaded into 49/64 format type Affymetrix GeneChip® (Human Genome U133 Plus 2.0 Array) together with control cRNAs and oligo B2. Target hybridization and scanning procedures were performed in Molecular Biology and Biotechnology R&D Center (METU-Central Laboratory, Ankara, Turkey). Hybridization procedure was conducted at 45°C, 60 rpm for 17 h in Affymetrix GeneChip® Hybridization Oven 640. Washing and staining procedure was performed in Affymetrix GeneChip® Fuidics Station 450 with Euk Ge-WS2v5 fluidics script according to the instructions in the technical manual. Affymetrix GeneChip® Scanner 3000 was used for scanning the arrays.

### Data analysis and preparation of gene lists

Preliminary analysis of the scanned chips was performed using Affymetrix GeneChip® Operating Software. The quality of gene expression data was checked according to quality control criteria [9]. Then, GeneSpring GX 7.3.1 Software (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for further data analysis and evaluation. Agilent GeneSpring GX is a powerful visualization and analysis solution designed for use with genomic expression data. The GeneSpring GX platform is designed to break through bottlenecks in the analysis process and to help

identify genes/pathways that are truly relevant to the biological question by comparing analysis results from expression, genotyping, protein, metabolite and other data types. The program allows identifying targets quickly and reliably, covering statistically meaningful results, displaying expression data with customizable visualization tools, and creating complex experiments that link trends in expression data to a variety of test parameters.

The data was initially normalized by RMA normalization algorithms. Statistically significant data were selected by independent sample $t$ test ($\alpha = 0.05$) between duplicate data for resistant and sensitive cell lines. Significantly altered genes between resistant sublines and MCF-7/S were listed and gene trees were constructed from these lists by standard correlation (Fig. 1). Upregulated and downregulated genes were selected from gene trees. The genes were filtered by volcano plots (with a cut off value of 0.1, i.e., in Fig. 2) and genes upregulated and downregulated more than twofold were considered in preparing gene lists. The gene lists were classified with respect to "biological processes" (i.e., cell growth and maintenance, etc.) and "molecular functions" (i.e., apoptosis regulator activity, binding activity, catalytic activity, etc.) of the proteins that are encoded by the genes. Finally, the genes that encode proteins related to ECM were selected from the gene lists and new lists were generated to evaluate the relationship between MDR phenotype.

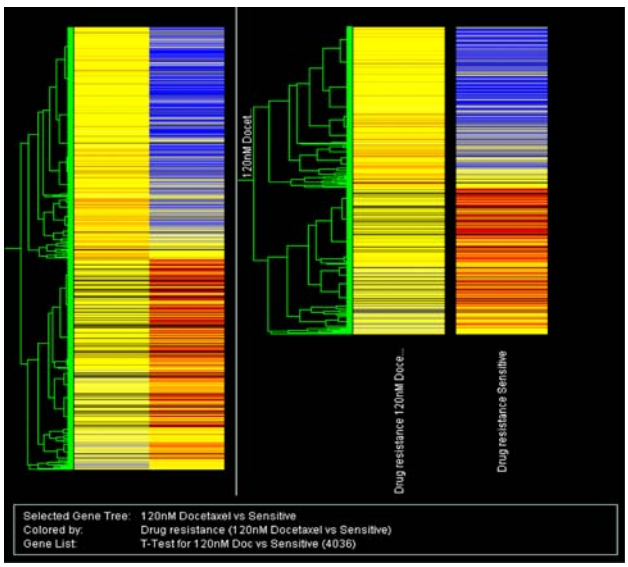


**Fig. 1** Gene tree that compares docetaxel resistant and sensitive MCF-7. *Red color* represents upregulated genes, *blue color* represents down regulated genes, and *yellow color* represents the genes that did not change

## Results

### Gene chip scanning and preliminary analysis

The quality of all GeneChip expression data were in "good sample" limits according to preliminary data analysis parameters such as background and noise averages, percentage of present calls, presence of internal hybridization controls in increasing signals, presence of poly-A controls as decreasing signals and GAPDH to beta actin 3′/5′ signal ratios.

### Data analysis, gene lists and evaluation

The genes of interest that are related with ECM structure and metastatic property of the cancer cells were selected. Although in vitro developed drug-resistant sublines do not have microenvironment or ECM, the evaluation of expression levels of ECM related genes in drug-resistant sublines will provide some information about invasive/metastatic property of drug-resistant mammary tissue. Tables 1 and 2 summarizes the alterations in gene expression levels in drug-resistant sublines. Significant changes greater than 2-fold and less than 0.5-fold were considered for evaluation of their contribution to drug resistance. The genes whose expression level changed in between 2- and 0.5-fold alteration were considered as 'not significant (NS)' in constructing gene lists.

Collectively, expression of 25 genes encoding ECM proteins and integrin receptor subunits (Table 1) were found to be upregulated in drug-resistant cells. Eighteen of these genes (72%) were upregulated in MCF-7/Vinc, 19 of them (76%) were upregulated in MCF-7/Pac and MCF-7/Doc cells where 22 genes (88%) were upregulated in MCF-7/Dox cells. Twelve of these 25 genes were significantly upregulated in all drug-resistant sublines. These were *ITGA6*, *COL4A1, COL4A2, COL6A1, COL6A2, LAMA1, FN1, CLDN1, GPC6, SDC2, FBN1* and *FBLN1* genes, which encode key components of ECM (Table 1). In addition, other genes encoding integrin, collagen and laminin subunits were also upregulated in different sublines.

According to Table 2, representing the fold changes in expression levels of MMPs, ADAMs, ADAMTSs and TIMPs, thirteen genes were found to be altered in drug-resistant sublines when compared with sensitive MCF-7. Nine genes (70%) were upregulated in MCF-7/Vinc where six (46%), seven (54%) and three (23%) genes have altered expression levels in MCF-7/Pac, MCF-7/Doc and MCF-7/Dox cells, respectively. Interestingly, four genes (31%) were only upregulated in MCF-7/Vinc cells. One of them is *MMP1* that was overexpressed very dramatically (around 1,000-fold). Similarly, *MMP9*, *ADAM17* and ADAMTS6

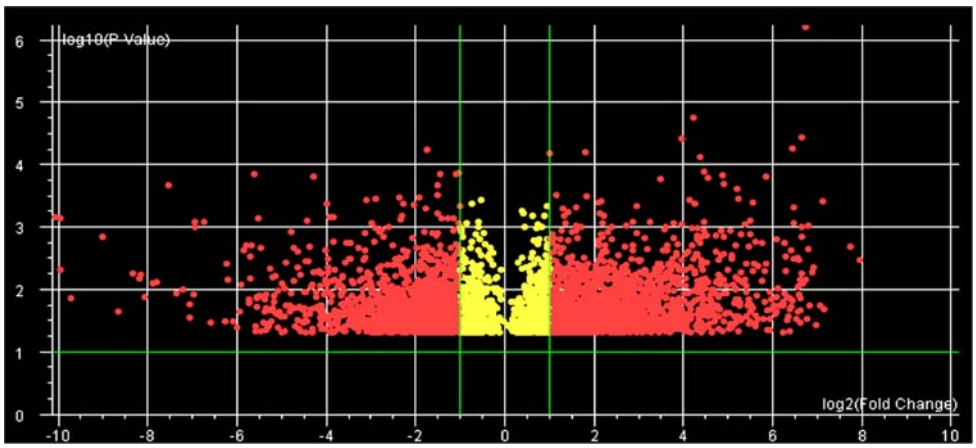


**Fig. 2** Volcano plot displaying twofold and more up/downregulated genes (*red dots*) of MCF-7/Doc with respect to sensitive MCF-7. The plot filters the genes having a fold change value in between 0.5- and 2-fold (*yellow dots*)

**Table 1** The gene list that represents the fold change of genes encoding extracellular matrix related proteins in drug-resistant MCF-7 sublines with respect to MCF-7/S

| Gene name | Gene symbol | Description | Fold change | | | |
|---|---|---|---|---|---|---|
| | | | MCF-7/Vinc | MCF-7/Pac | MCF-7/Doc | MCF-7/Dox |
| 215177_s_at | ITGA6 | Integrin, alpha 6 | 39.68 | 23.96 | 24.66 | 27.92 |
| 201389_at | ITGA5 | Integrin, alpha 5 (fibronectin receptor, alpha polypeptide) | 5.591 | NS | 4.914 | 4.039 |
| 1553678_a_at | ITGB1 | Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) | 2.55 | 3.013 | NS | 2.762 |
| 211964_at, 211966_at | COL4A2 | Collagen, type IV, alpha 2 | 77.66 | 76.95 | 54.65 | 114.7 |
| 211981_at | COL4A1 | Collagen, type IV, alpha 1 | 17.47 | 16.39 | 11.99 | 27.97 |
| 213428_s_at, 212937_s_at | COL6A1 | Collagen, type VI, alpha 1 | 17.58 | 7.673 | 19.1 | 12.03 |
| 209156_s_at, 213290_at | COL6A2 | Collagen, type VI, alpha 2 | 8.124 | 7.15 | 6.193 | 5.343 |
| 231879_at | COL12A1 | Collagen, type XII, alpha 1 | NS | 14.35 | NS | 4.574 |
| 204345_at | COL16A1 | Collagen, type XVI, alpha 1 | 9.57 | 14.29 | 2.73 | NS |
| 227048_at | LAMA1 | Laminin, alpha 1 | 140.5 | 72.65 | 84.3 | 86.86 |
| 210990_s_at | LAMA4 | Laminin, alpha 4 | 4.24 | 5.781 | NS | NS |
| 211651_s_at | LAMB1 | Laminin, beta 1; laminin, beta 1 | 11 | NS | 16.72 | 14.58 |
| 209270_at | LAMB3 | Laminin, beta 3 | NS | NS | 7.755 | NS |
| 219407_s_at | LAMC3 | Laminin, gamma 3 | NS | 8.87 | 9.479 | 6.381 |
| 214701_s_at | FN1 | Fibronectin 1 | 88.28 | 30.34 | 33.94 | 11.23 |
| 222549_at | CLDN1 | Claudin 1 | 88.29 | 88.77 | 42.79 | 75.12 |
| 227059_at | GPC6 | Glypican 6 | 28.36 | 67.36 | 78.43 | 41.77 |
| 212236_x_at, 205157_s_at | KRT17 | Keratin 17 | NS | 8.197 | NS | 32.71 |
| 212158_at | SDC2 | Syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan) | 2.669 | 25.03 | 48.98 | 27.15 |
| 213905_x_at, 201261_x_at, 201262_s_at | BGN; SDCCAG33 | Biglycan; serologically defined colon cancer antigen 33 | NS | NS | 19.87 | 11.91 |
| 202766_s_at, 202765_s_at | FBN1 | Fibrillin 1 (Marfan syndrome) | 19.99 | 20.16 | 37.19 | 43.44 |
| 203184_at, 203886_s_at | FBN2 | Fibrillin 2 (congenital contractural arachnodactyly) | NS | 48.24 | NS | 79.54 |
| 202995_s_at, 201787_at | FBLN1 | Fibulin 1 | 6.478 | 6.299 | 5.36 | 7.065 |
| 203088_at | FBLN5 | Fibulin 5 | NS | NS | NS | 4.725 |
| 209365_s_at | ECM1 | Extracellular matrix protein 1 | 11.7 | NS | 21.56 | 14.29 |

*NS* fold change values in between 0.5 and 2.00 or insignificant alterations

**Table 2** The gene list that represents the fold changes of matrix metallopeptidases and related genes in drug-resistant MCF-7 sublines with respect to MCF-7/S

| Gene name | Gene symbol | Description | Fold change | | | |
|---|---|---|---|---|---|---|
| | | | MCF-7/Vinc | MCF-7/Pac | MCF-7/Doc | MCF-7/Dox |
| 204475_at | MMP1 | Matrix metallopeptidase 1 (interstitial collagenase) | 1,013 | NS | NS | NS |
| 203936_s_at | MMP9 | Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase) | 2.368 | NS | NS | NS |
| 202827_s_at | MMP14 | Matrix metallopeptidase 14 (membrane-inserted) | NS | 5.048 | 3.493 | NS |
| 207118_s_at | MMP23B; MMP23A | Matrix metallopeptidase 23B; matrix metallopeptidase 23[a] | 15.94 | 15.07 | 11.71 | 14.38 |
| 1555326_a_at, 202381_at | ADAM9 | ADAM metallopeptidase domain 9 (meltrin gamma) | 3.13 | 4.517 | 5.751 | 5.505 |
| 217007_s_at | ADAM15 | ADAM metallopeptidase domain 15 (metargidin) | NS | 0.383 | 0.465 | NS |
| 213532_at | ADAM17 | ADAM metallopeptidase domain 17 (tumor necrosis factor, alpha, converting enzyme) | 2.92 | NS | NS | NS |
| 208227_x_at | ADAM22 | ADAM metallopeptidase domain 22 | NS | NS | 2.134 | NS |
| 222162_s_at | ADAMTS1 | ADAM metallopeptidase with thrombospondin type 1 motif, 1 | 11.79 | 5.799 | NS | NS |
| 214913_at | ADAMTS3 | ADAM metallopeptidase with thrombospondin type 1 motif, 3 | 5.949 | NS | 13.64 | NS |
| 1570351_at | ADAMTS6 | ADAM metallopeptidase with thrombospondin type 1 motif, 6 | 9.276 | NS | NS | NS |
| 201150_s_at | TIMP3 | TIMP metallopeptidase inhibitor 3 | NS | NS | 0.0103 | 0.00917 |
| 206243_at | TIMP4 | TIMP metallopeptidase inhibitor 4 | 12.07 | 6.733 | NS | 8.366 |

*NS* fold change values in between 0.5 and 2.00 or insignificant alterations

were also upregulated only in MCF-7/Vinc. Only *MMP23* and *ADAM9* (15%) represent common expression patterns in resistant sublines. On the other hand, *ADAM15* and particularly, *TIMP3* were significantly downregulated in resistant cells. Fold changes in expression levels of all genes are summarized in Tables 1 and 2.

## Discussion

This report represents the correlation between mRNA levels of the genes encoding ECM, some tumor microenvironment-related proteins and drug resistance phenotype. Since genes having fold change values in between 2- and 0.5-fold were not considered as significant, their relation to drug resistance will not be discussed. In this report, a particular group of genes from the extensive microarray data was evaluated. The microarray data was confirmed with the qPCR results of the representative genes (*MDR1, MRP1*) of MDR phenotype (data not shown). Additionally, western blot results of P-gp and MRP1 proteins were also correlated with the alterations in expression levels of the genes encoding the proteins (data not shown).

The adhesion of cells to each other or to the ECM is responsible for stimulating signals that regulate migration of immune cells, invasion/metastasis and angiogenesis in tumor cells. Previous reports demonstrated the association between MDR phenotype with P-gp expression and increased invasive ability [10, 11]. The proteins involved in intercellular adhesion and ECM including collagens, laminin, fibronectin, vitronectin, aggrecan, enactin, tenascin, elastin and proteoglycans, are likely to be important in mediating drug resistance. The genes encoding ECM components and integrins had elevated levels of expression in drug-resistant MCF-7 sublines (Table 1). Eighty-eight percent of these genes were upregulated in MCF-7/Dox where 72% and 75% of them were upregulated in MCF-7/Vinc and MCF-7/Pac, MCF-7/Doc, respectively. Concordantly, among these genes 48% were found to have increased expression levels in all of the resistant sublines. These genes are encoding particularly, integrin, collagen IV and VI, laminin, fibronectin, claudin, glypican, syndecan, fibrillin and fibulin. Upon growth factor signaling, these proteins are able to modulate gene expression regulation, cell death/proliferation, angiogenesis and invasion/metastasis.

Laminins, collagens and fibronectins are among attachment proteins that mediate cell survival via integrin interactions [6, 12, 13]. Among genes encoding the integrins, gene encoding $\alpha$6 integrin (*ITGA6*) was highly overexpressed in all resistant sublines. To a lesser extent, genes encoding $\alpha$5 integrin (*ITGA5;* except in MCF-7/Pac) and $\beta$1 integrin (*ITGB1;* except in MCF-7/Doc) were also upregulated. Several reports have demonstrated differential expression of integrin subunits in drug-resistant variants of tumor cell lines exhibiting altered binding efficiencies to

ECM ligands such as fibronectin, laminin and collagen IV [14–16]. In addition, Aoudjit et al. [17] identified the inhibitory role of $\beta$1 integrin signaling in paclitaxel- and vincristine-induced apoptosis of breast carcinoma cells, which was related to integrin-mediated inhibition of cytochrome c release from mitochondria dependent activation of the PI3K (phosphatidylinositol 3-kinase)/Akt pathway.

Among collagens *COL4A2, COL4A1, COL6A1* and *COL6A2* were overexpressed in all drug-resistant sublines. Among them *COL4A2* was highly overexpressed in resistant cells. Collagen IV and laminin are the main components of the basement membrane. They are important ECM remodeling proteins and contribute to the invasive behavior of tumor cells. *LAMA1* gene encoding the $\alpha$1 chain of laminin was drastically overexpressed in drug-resistant cells (between 70- and 140-folds in different sublines). The laminin $\alpha$1 is known to interact with ECM compounds and integrin receptors with its implications in cellular signaling pathways and assembly of the basement membrane. Other genes encoding laminin subunits were also significantly upregulated in resistant cells, demonstrating a collective increase in expression level of laminin. *FN1* gene encoding the type I domain of fibronectin was upregulated in drug-resistant cells. Fibronectin was shown to upregulate MMP9 expression, activity, and invasiveness in a concentration dependent manner in different types of tumor cells [18, 19]. It was also found to enhance activation of Ras and downstream Erk and Akt pathways [12].

Claudins are tight junction proteins that regulate paracellular transport. In addition, overexpression of *CLDN1* gene, which was also highly upregulated in all drug-resistant cells, was also found to have a contribution to melanoma cell invasion as it increased MMP2 secretion and activation in a PKC regulated manner [20].

Increase in expression levels of *GPC6*, encoding the glypican 6, was common in resistant cells. Although there is evidence that some glypicans have critical role in the regulation of cell proliferation and survival based on their capacity to modulate the activity of various growth and survival factors, *GPC6* has not been correlated with these functions, yet. *SDC2* gene encodes the syndecan 2 protein. It was highly upregulated in paclitaxel, docetaxel and doxorubicin-resistant cells and to a lesser extent in vincristine-resistant cells. Reports on syndecan 2 are contradicting. Modrowski et al. [21] reported that overexpression of syndecan 2 induced osteoblastic cell apoptosis through the JNK/Bax pathway. They have also demonstrated the sensitization of the human osteosarcoma cells to chemotherapy-induced apoptosis upon overexpression of syndecan 2 supporting tumor suppressor function [22] of the gene. However, syndecan-2 interacts with cytokines and growth factors that stimulate angiogenesis (e.g., interleukin-8, VEGF, bFGF and TGF) and these factors with MMPs may also stimulate syndecan-2 shedding, which in turn promotes angiogenic processes [23] and TGF-$\beta$ induced increased matrix deposition in fibroblasts [24]. Collectively, molecular interactions of syndecan 2 and downstream effects can differ with cell type, i.e., it may have an apoptotic effect on particular cell types dependent on tissue specific expression of interacting molecules or expression profiles of other cell surface and ECM molecules.

Fibrillins and fibulins are the microfibrils of ECM. Fibrillin 1 (*FBN1*) was upregulated in all resistant MCF-7 cells (Table 1) where fibrillin 2 (*FBN2*) was highly upregulated in MCF-7/Pac and MCF-7/Dox. Although fibrillins have not been correlated to proliferation of tumor cells before, being key components of ECM they may function in remodeling of ECM as well as growth factor shedding [25]. Fibulins upon binding to other ECM molecules functions as the bridges in the organization of ECM. *FBLN1* gene encoding the fibulin 1 had increased mRNA levels in drug-resistant cells. Pupa et al. [26] reported that it can promote survival of breast cancer cells during doxorubicin treatment although its modulation of expression was result of doxorubicin-induced stress rather than development of chemoresistance. Similarly, tumor-promoting function for fibulin 5 was identified in developing and progressing breast cancers, which might be related to its ability to enhance MMP expression correlated with *FBLN5* overexpression [27]. The upregulation of *FBLN5* in only MCF-7/Dox cells may be correlated to doxorubicin-induced cytotoxicity.

*ECM1* gene, upregulated in resistant cells (except for the MCF-7/Pac), encodes the ECM protein 1. Stimulatory effect of ECM1 on proliferation of endothelial cells and angiogenesis of breast carcinoma cells with its increased expression was identified before [28].

Likewise ECM components, invasive/metastatic phenotypic alterations of cancer cells are also related to enzymes that degrade and modulate ECM. Extracellular proteolysis by zinc proteases is responsible for ECM degradation and remodeling. Matrixins (MMPs) and adamalysins (ADAMs) are among proteins of zinc endopeptidase family [29]. These proteinases and their inhibitors control a variety of cellular functions in ECM formation and remodeling as well as growth factor shedding. MMPs are also responsible for cleavage of cell surface receptors, release of apoptotic ligands, and chemokine in/activation. They are thought to play a major role on cell behavior such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defense. In our study, 13 genes encoding the MMPs, ADAMs, ADAMTSs and TIMPs were found to have altered expression levels (Table 2). Overexpression of *MMP1, MMP9, ADAM17* and *ADAMTS6* in MCF-7/Vinc cells address the

question if these genes may be related specifically to vincristine-induced cytotoxicity or resistance. In addition, 46 and 54% of the altered gene expression levels were determined in MCF-7/Pac and MCF-7/Doc cells where only 23% were altered in MCF-7/Dox cells. It is interesting to see a higher alteration in gene expression levels of matrix endoproteinases encoding genes in cells resistant to microtubule disturbing agents (vincristine, paclitaxel and docetaxel). MMP1 and MMP14 are able to degrade interstitial fibrillar collagens I, II and III to form gelatin for further degradation by gelatinase MMPs [30]. They have also been known to enhance cancer cell migration. *MMP1* gene is drastically overexpressed in MCF-7/Vinc cells (1,000-fold) and increase in this gene may be correlated to invasive ability of these cells. In a previous report on gene expression analysis of drug-resistant cells by cDNA microarray, overexpression of *MMP1* was also demonstrated [31] and correlated to invasive property of drug-resistant cells. Among MMPs, two gelatinases MMP2 and MMP9 are distinguished both structurally and functionally. MMP9 is rapidly available upon activation for any remodeling events [32]. It has been proposed that cleavage of collagen type IV (major component of basement membranes) by MMP2/9 exposes a cryptic site which displays affinity for $\alpha v\beta 3$ integrin, thereby leading to enhancement of angiogenesis [33]. Additionally, knockout mice lacking MMP9 have shown decreased incidence of skin and pancreatic carcinogenesis and metastasis [34, 35]. Interestingly, *MMP14* was five- and fourfold upregulated in both paclitaxel- and docetaxel-resistant cells. *MMP14* is expressed in fibroblast cells during both wound healing and human cancer progression.

Through binding to integrin, ADAMs modulate ECM–integrin interactions, and thus they can indirectly promote proliferation through integrin signaling. Collectively, the ADAMs family has been implicated in the control of membrane fusion, cytokine and growth factor shedding, and cell migration [29]. ADAM9 was overexpressed in all drug-resistant sublines (Table 2). It can modulate EGF receptor activity upon shedding of heparin-binding-EGF, cause transactivation of EGFR, which in turn activates pathways to promote cell survival [36]. So besides playing role in ECM remodeling, invasion and metastasis, modulation of growth factor activity ADAM9 by seems to be directly related to cell survival. Unlike other metalloproteases, *ADAM15* was down regulated in paclitaxel and docetaxel-resistant sublines (0.4- and 0.5-fold, respectively). The protein encoded by *ADAM15* is unique among ADAM proteins since it contains the integrin binding motif Arg-Gly-Asp (RGD) within its disintegrin region [37]. Trochon-Joseph et al. [38] previously identified ADAM15 as a potent intrinsic inhibitor of angiogenesis, tumor growth and metastasis in breast carcinoma cells and mice. Furthermore, they have concluded that it could be a promising tool for use in anticancer treatment. Moreover, Beck et al. [39] reported ADAM15 as a natural binding partner of integrin $\alpha v\beta 3$, which loosens tumor cell adhesion to the underlying matrix and regulated tumor cell migration and invasion.

ADAM17 is the TNF-α converting enzyme (TACE). TNF-α is synthesized in its precursor, membrane anchored form, activated and solubilized through ADAM17 shedding. Upon binding to EGFR, it promotes cell proliferation and survival. *ADAM17* gene is upregulated in MCF-7/Vinc cells and it may be one of the survival factors of these cells.

The adamalysins also contain the ADAMTS family proteinases; members having a variable number of thrombospondin-like (TS) motifs [40]. *ADAMTS1* gene was upregulated in vincristine and paclitaxel-resistant sublines. Liu et al. [41] have demonstrated that overexpression of full length ADAMTS1 in mammary carcinoma cells promoted tumor angiogenesis and invasion, shedding of the transmembrane precursors of heparin-binding EGF and activation of the EGFR leading to cell proliferation and survival. *ADAMTS3* was upregulated in vincristine and docetaxel-resistant cells. Although its function still remains unclear, it is one of the ECM-degrading enzymes of procollagen type [40]. On the other hand, *ADAMTS6* was upregulated in MCF-7/Vinc cells. The protein function of this gene product has not been well characterized yet. However, Porter et al. [42] reported an association of cDNA expression between *ADAMTS6* and *MMP9*, which is also upregulated only in MCF-7/Vinc cells.

The TIMPs regulate ECM turnover and tissue remodeling by forming tight-binding inhibitory complexes with the MMPs and ADAMs directly interacting with their active sites [32]. *TIMP3* was drastically downregulated in MCF-7/Doc and MCF-7/Dox cells. In addition to its inhibitor activity, its proapoptotic function was also demonstrated by Ahonen et al. [43]. It was reported that overexpression of TIMP3 promoted apoptosis through stabilization of TNF-α receptors on the cell surface [44]. It is also known to inhibit ADAM17 [45]. However, *TIMP4* was upregulated in MCF-7/Vinc, MCF-7/Pac and MCF-7/Dox cells. TIMP4 has been shown to have antiapoptotic activity and tumor-stimulating effect in breast cancer cells and upregulate Bcl-2 and Bcl-$X_L$ proteins [46].

Proteins encoded by the genes presented here are also important modulators and contributors of invasive phenotype of tumor cells. However, the relationship between invasion and drug resistance is complicated. Whether invasive/metastatic phenotype enhances development of drug resistance or drug-resistant tumor cells are more invasive/metastatic still remains to be solved. The expression profiles demonstrated here provide a preliminary and the first comprehensive insight to the differential

expression of ECM components and related genes in drug resistant and sensitive breast carcinoma cells. Significance of correlation of these genes to drug resistance requires further functional analysis.

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## References

1. Deuchars KL, Ling V (1989) P-glycoprotein and multidrug resistance in cancer chemotherapy. Semin Oncol 16:156–165
2. Young AM, Allen CE, Audus KL (2003) Efflux transporters of the human placenta. Adv Drug Deliver Rev 55:125–132
3. Hoyt DG, Rusnak JM, Mannix RJ, Modzelewski RA, Johnson CS, Lazo JS (1996) Integrin activation suppresses etoposide-induced DNA strand breakage in cultured murine tumor-derived endothelial cells. Cancer Res 56:4146–4149
4. Kraus AC, Ferber I, Bachmann SO, Specht H, Wimmel A et al (2002) In vitro chemo- and radio-resistance in small cell lung cancer correlates with cell adhesion and constitutive activation of AKT and MAP kinase pathways. Oncogene 21:8683–8695
5. Mitsumoto M, Kamura T, Kobayashi H, Sonoda T, Kaku T, Nakano H (1998) Emergence of higher levels of invasive and metastatic properties in the drug-resistant cancer cell lines after the repeated administration of cisplatin in tumor-bearing mice. J Cancer Res Clin Oncol 124:607–614
6. Morin PJ (2003) Drug resistance and the microenvironment: nature and nurture. Drug Resist Updat 4:169–172
7. Kars MD, Işeri OD, Gündüz U, Ural AU, Arpaci F, Molnar J (2006) Development of rational in vitro models for drug resistance in breast cancer and modulation of MDR by selected compounds. Anticancer Res 26:4559–4568
8. Iseri ÖD, Kars MD, Eroglu S, Gündüz U (2009) Cross-resistance development and combined applications of anticancer agents in drug resistant MCF-7 cell lines. Int J Hem Oncol 1(19):1–8
9. Yilmaz R, Yücel M, Öktem HA (2008) Quality assessment of gene expression data for an affymetrix platform with the two sample t-tests statistical analysis. Int J Biotechnol Biochem 4:101–108
10. Li QQ, Wang WJ, Xu JD, Cao XX, Chen Q, Yang JM, Xu ZD (2007) Up-regulation of CD147 and matrix metalloproteinase-2, -9 induced by P-glycoprotein substrates in multidrug resistant breast cancer cells. Cancer Sci 98(11):1767–1774
11. Weinstein RS, Jakate SM, Dominguez JM, Lebovitz MD, Koukoulis GK, Kuszak JR et al (1991) Relationship of the expression of the multidrug resistance gene product (P-glycoprotein) in human colon carcinoma to local tumor aggressiveness and lymph node metastasis. Cancer Res 51(10):2720–2726
12. Ahmed N, Riley C, Rice G, Quinn M (2005) Role of integrin receptors for fibronectin, collagen and laminin in the regulation of ovarian carcinoma functions in response to a matrix microenvironment. Clin Exp Metastasis 22(5):391–402
13. Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C et al (1999) Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nat Med 6:662–668
14. Nista A, Leonetti C, Bernardini G, Mattioni M, Santoni A (1997) Functional role of alpha4beta1 and alpha5beta1 integrin fibronectin receptors expressed on adriamycin-resistant MCF-7 human mammary carcinoma cells. Int J Cancer 72(1):133–141
15. Narita T, Kimura N, Sato M, Matsuura N, Kannagi R (1998) Altered expression of integrins in adriamycin-resistant human breast cancer cells. Anticancer Res 18(1A):257–262
16. Liang Y, Meleady P, Cleary I, McDonnell S, Connolly L, Clynes M (2001) Selection with melphalan or paclitaxel (Taxol) yields variants with different patterns of multidrug resistance, integrin expression and in vitro invasiveness. Eur J Cancer 37(8):1041–1052
17. Aoudjit F, Vuori K (2001) Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. Oncogene 20(36):4995–5004
18. Shibata K, Kikkawa F, Nawa A, Suganuma N, Hamaguchi M (1997) Fibronectin secretion from human peritoneal tissue induces Mr 92,000 type IV collagenase expression and invasion in ovarian cancer cell lines. Cancer Res 57(23):5416–5420
19. Esparza J, Vilardell C, Calvo J, Juan M, Vives J, Urbano-Márquez A et al (1999) Fibronectin upregulates gelatinase B (MMP-9) and induces coordinated expression of gelatinase A (MMP-2) and its activator MT1-MMP (MMP-14) by human T lymphocyte cell lines A process repressed through RAS/MAP kinase signaling pathways. Blood 94(8):2754–2766
20. Leotlela PD, Wade MS, Duray PH, Rhode MJ, Brown HF, Rosenthal DT et al (2007) Claudin-1 overexpression in melanoma is regulated by PKC and contributes to melanoma cell motility. Oncogene 26(26):3846–3856
21. Modrowski D, Orosco A, Thévenard J, Fromigué O, Marie PJ (2005) Syndecan-2 overexpression induces osteosarcoma cell apoptosis: implication of syndecan-2 cytoplasmic domain and JNK signaling. Bone 37(2):180–189
22. Orosco A, Fromigué O, Bazille C, Entz-Werle N, Levillain P, Marie PJ, Modrowski D (2007) Syndecan-2 affects the basal and chemotherapy-induced apoptosis in osteosarcoma. Cancer Res 67(8):3708–3715
23. Fears CY, Gladson CL, Woods A (2006) Syndecan-2 is expressed in the microvasculature of gliomas and regulates angiogenic processes in microvascular endothelial cells. J Biol Chem 281(21):14533–14536
24. Chen L, Klass C, Woods A (2004) Syndecan-2 regulates transforming growth factor-beta signaling. J Biol Chem 279(16):15715–15718
25. Chaudhry SS, Cain SA, Morgan A, Dallas SL, Shuttleworth CA, Kielty CM (2007) Fibrillin-1 regulates the bioavailability of TGFbeta1. J Cell Biol 176(3):355–367
26. Pupa SM, Giuffré S, Castiglioni F, Bertola L, Cantú M, Bongarzone I et al (2007) Regulation of breast cancer response to chemotherapy by fibulin-1. Cancer Res 67(9):4271–4277
27. Lee YH, Albig AR, Maryann R, Schiemann BJ, Schiemann WP (2008) Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-$\beta$ in mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis 29(12):2243–2251
28. Han Z, Ni J, Smits P, Underhill CB, Xie B, Chen Y et al (2001) Extracellular matrix protein 1 (ECM1) has angiogenic properties and is expressed by breast tumor cells. FASEB J 15(6):988–994
29. Seals DF, Courtneidge SA (2003) The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev 17:7–30
30. Lovejoy B, Welch AR, Carr S, Luong C, Broka C, Hendricks RT et al (1999) Crystal structures of MMP-1 and -13 reveal the structural basis for selectivity of collagenase inhibitors. Nat Struct Biol 6:217–221
31. Turton NJ, Judah DJ, Riley J, Davies R, Lipson D, Styles JA et al (2001) Gene expression and amplification in breast carcinoma

cells with intrinsic and acquired doxorubicin resistance. Oncogene 20(11):1300–1306
32. Somerville RPT, Oblander SA, Apte SS (2003) Matrix metalloproteinases: old dogs with new tricks. Genome Biol 4(6):216
33. Stefanidakis M, Koivunen E (2006) Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression. Blood 108(5):1441–1450
34. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K et al (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2(10):737–744
35. Coussens LM, Tinkle CL, Hanahan D, Werb Z (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell 103(3):481–490
36. Fischer OM, Hart S, Gschwind A, Prenzel N, Ullrich A (2004) Oxidative and osmotic stress signaling in tumor cells is mediated by ADAM proteases and heparin-binding epidermal growth factor. Mol Cell Biol 24(12):5172–5183
37. Krätzschmar J, Lum L, Blobel CP (1996) Metargidin, a membrane-anchored metalloprotease-disintegrin protein with an RGD integrin binding sequence. J Biol Chem 271(9):4593–4596
38. Trochon-Joseph V, Martel-Renoir D, Mir LM, Thomaïdis A, Opolon P, Connault E et al (2004) Evidence of antiangiogenic and antimetastatic activities of the recombinant disintegrin domain of metargidin. Cancer Res 64(6):2062–2069
39. Beck V, Herold H, Benge A, Luber B, Hutzler P, Tschesche H et al (2005) ADAM15 decreases integrin alphavbeta3/vitronectin-mediated ovarian cancer cell adhesion and motility in an RGD-dependent fashion. Int J Biochem Cell Biol 37(3):590–603
40. Mochizuki S, Okada Y (2007) ADAMs in cancer cell proliferation and progression. Cancer Sci 98(5):621–628
41. Liu YJ, Xu Y, Yu Q (2006) Full-length ADAMTS-1 and the ADAMTS-1 fragments display pro- and antimetastatic activity, respectively. Oncogene 25(17):2452–2467
42. Porter S, Scott SD, Sassoon EM, Williams MR, Jones JL, Girling AC, Ball RY, Edwards DR (2004) Dysregulated expression of adamalysin-thrombospondin genes in human breast carcinoma. Clin Cancer Res 10(7):2429–2440
43. Ahonen M, Baker AH, Kähäri VM (1998) Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. Cancer Res 58(11):2310–2315
44. Smith MR, Kung H, Durum SK, Colburn NH, Sun Y (1997) TIMP-3 induces cell death by stabilizing TNF-alpha receptors on the surface of human colon carcinoma cells. Cytokine 9(10):770–780
45. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ et al (1998) TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett 435(1):39–44
46. Jiang Y, Wang M, Celiker MY, Liu YE, Sang QX, Goldberg ID, Shi YE (2001) Stimulation of mammary tumorigenesis by systemic tissue inhibitor of matrix metalloproteinase 4 gene delivery. Cancer Res 61(6):2365–2370