

Use of arrays to investigate the contribution of ATP-binding cassette transporters to drug resistance in cancer chemotherapy and prediction of chemosensitivity

Jian-Ting Zhang¹

¹*Department of Pharmacology and Toxicology, Walther Oncology Center/Walther Cancer Institute and IU Cancer Center, Indiana University School of Medicine, 1044 W. Walnut Street, R4-166, Indianapolis, IN 46202, USA*

Multidrug resistance (MDR) is a major problem in cancer chemotherapy. One of the best known mechanisms of MDR is the elevated expression of ATP-binding cassette (ABC) transporters. While some members of human ABC transporters have been shown to cause drug resistance with elevated expression, it is not yet known whether the over-expression of other members could also contribute to drug resistance in many model cancer cell lines and clinics. The recent development of microarrays and quantitative PCR arrays for expression profiling analysis of ABC transporters has helped address these issues. In this article, various arrays with limited or full list of ABC transporter genes and their use in identifying ABC transporter genes in drug resistance and chemo-sensitivity prediction will be reviewed.

Keywords: genomics, MDR, drug resistance, ABC transporter, microarray, real time quantitative PCR

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Introduction

Multidrug resistance (MDR) is a major problem in cancer chemotherapy. One of the best known mechanisms of MDR is the elevated expression of ATP-binding cassette (ABC) transporters such as ABCB1 (MDR1, Pgp) [1-3], ABCC1 (MRP1) [4], and ABCG2 (BCRP, MXR, ABCP) [5] (Figure. 1). ABC transporters comprise a superfamily of more than 1000 members from bacteria to human. Human alone has 49 members of the superfamily which are divided into 7 subfamilies, ABCA through ABCG (Table 1), and they transport a wide variety of substrates including drugs, lipids, metabolites, and ions. These human transporters are expressed in various tissues and some of them are known to function as drug efflux pumps to cause drug resistance by actively extruding multiple anticancer drugs with expenses of ATP. However, two of these human

proteins (ABCE1 and ABCF1) do not contain any known putative transmembrane domain and, therefore, may not function as transporters by themselves (see http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=mono_001).

While some members of human ABC transporters have been shown to cause drug resistance with elevated expression, it is not yet known whether over-expression of other members could also contribute to drug resistance. Many model cancer cell lines selected by anticancer drugs are also available for investigating the mechanisms of drug resistance. However, dissecting which ABC transporter is activated in a particular case is not an easy task. Knowledge of expression profiles of ABC transporters and other genes involved in MDR will likely help therapeutic optimization for cancer patients in clinics. The recent development of genomic approaches such as microarrays and quantitative PCR arrays (Table 2) for expression profiling analyses has helped address these issues. In this paper, I will review recent progress in the application of these genomic tools to identify ABC transporter genes likely responsible for MDR in model cancer cell lines and their potential use as predictors of chemo-sensitivity. However, it should also be noted

Correspondence: Jian-Ting Zhang
Tel: +1-317-278-4503; Fax: +1-317-274-8046;
E-mail: jianzhan@iupui.edu

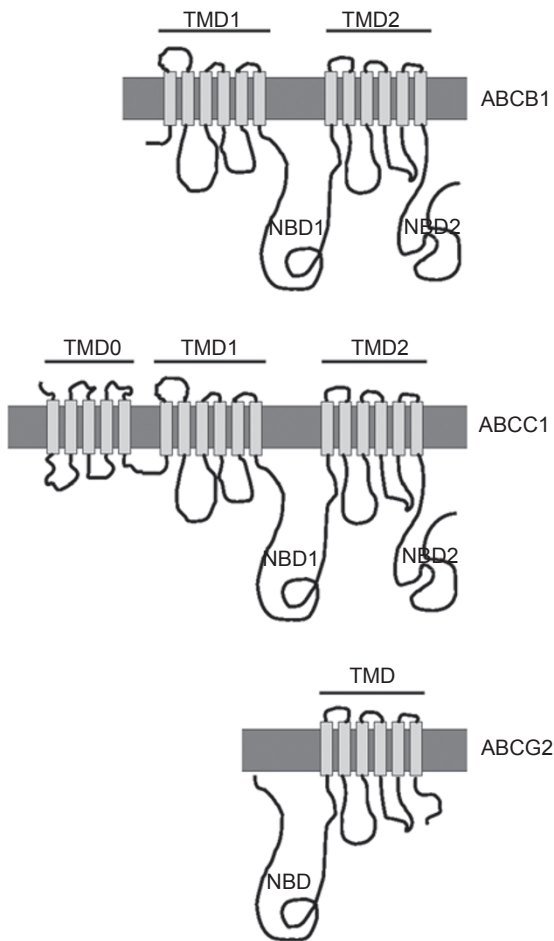


Figure 1 Schematic topological structure of representative ABC transporters: ABCB1, ABCC1, and ABCG2. ABC transporters consist of transmembrane (TMD) and nucleotide-binding (NBD) domains. The topological structures shown are putative ones and have not yet been proven. However, evidence arguing against the putative topological folding of ABCB1 and ABCC1 has been published elsewhere [51-55].

Table 1 Human ABC Transporters

Subfamily	Members
ABCA	ABCA1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A12, A13
ABCB	ABCB1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11
ABCC	ABCC1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13
ABCD	ABCD1, D2, D3, D4
ABCE	ABCE1
ABCF	ABCF1, F2, F3
ABCG	ABCG1, G2, G4, G5, G8

For nomenclature of human ABC transporters, see <http://nutrigene.4t.com/humanabc.htm>.

that in some cases, the findings from these profiling studies await further validation for the expression at the protein level, and the functional role of the implicated transporters in drug resistance remains to be verified.

Generation of drug sensitivity predictors using high-density microarrays

Many high density microarrays have been used to profile gene expression patterns of a large number of cell lines with known responses to multiple drugs and compounds. Correlation between the expression profiles and the drug sensitivity data of these cell lines helped generate a number of predictors for specific drugs or compounds. Some of the 49 human ABC transporters found in these predictors are highlighted in Table 2. However, further testing is needed to determine if these predictors are useful in clinics.

The earliest studies of drug sensitivity involved cDNA microarray analysis of the NCI-60 cell lines [6, 7]. In these studies, a microarray containing approximately 8 000 cDNA clones (Table 2) that represent unique human genes including a limited number of ABC transporters such as ABCA3, ABCB1, ABCB2, ABCB7, ABCC1, ABCC6, and ABCF1, was used. Analysis of gene expression profiles of the NCI-60 cell lines and clustering the cell lines on the basis of their responses to 1400 compounds or drugs revealed many gene-drug response relationships. It appears that the cell lines with elevated expression of ABCB1 clustered on the same branch for drug responses. This is also true for ABCC1, suggesting that the expression of these ABC transporter genes likely contribute to the poor drug sensitivity of these cancer cell lines (Table 3). Both ABCB1 and ABCC1 are well known genes involved in resistance to multiple anticancer drugs. In addition to these ABC transporter genes, this study also revealed correlative gene-drug relationships for other 1376 genes. One of these genes, dihydropyrimidine dehydrogenase, was found to have a significant negative correlation between its expression and 5-FU potency in the 60 cell lines, suggesting that its over-expression may cause resistance to 5-FU.

A similar study of NCI-60 cell lines was also conducted, but instead used the Affymetrix oligonucleotide microarray Hu6800 (Table 2) containing 6 817 human genes including ABC transporters such as ABCA3, ABCA4, ABCB1, ABCB2, and ABCB3 [8]. The correlation of drug sensitivity to gene expression was generated from a training set of data and this was then used to predict the sensitivity of cell lines to any particular drug using data in a testing set. Expression-based classifiers containing multiple genes for each of the 232 compounds were generated to predict drug sensitivity. Eighty-eight such classifiers performed accurately to predict drug sensitivity. Unfortunately, no

Table 2 Hybridization and PCR-based arrays for ABC transporter expression profiling

Arrays	Type	No. of genes	Probes (size)	No. of ABC genes ^a	References
High Density Arrays					
ABC-ToxChip	Hybridization	20 000	cDNA/oligos (70 mer)	36	[30]
Affymetrix Hu6800	Hybridization	6 817	oligonucleotides (25mer)	?	[8]
Affymetrix HG-U95Av2	Hybridization	9 600	oligonucleotides (25mer)	14	[19]
Affymetrix HG-U133	Hybridization	47 000	oligonucleotides (25mer)	49	[12, 25]
Affymetrix MU11	Hybridization	34 000 mouse	oligonucleotides (25mer)	6	[17]
Affymetrix MuU74v2	Hybridization	36 000 mouse	oligonucleotides (25mer)	49	[29]
Agilent Human 1 microarray	Hybridization	16 281	cDNAs	45	[22]
Clontech 1.2 II Atlas array	Hybridization	1 176	cDNAs	1	[18]
Clontech Atlas Rat array	Hybridization	588	cDNAs	4	[14]
In-house cDNA microarray	Hybridization	8 000	cDNAs	7	[6, 7]
In-house cDNA microarray	Hybridization	9 216	cDNAs	?	[9]
IntelliGene Chip	Hybridization	557	cDNAs	2	[20]
Transportome array	Hybridization	632	oligonucleotides (70mer)	40	[13]
Low Density Arrays					
DualChip human ABC	Hybridization	49	cDNAs/oligos ^a	38	[24]
PCR Arrays, Low Density					
AmpArray	PCR	47	oligonucleotides	47	[40]
Real time PCR	PCR	48	oligonucleotides	48	[36]
TaqMan PCR	PCR	47	oligonucleotides	47	[38, 39]

^a For some arrays this information is not available in the publication nor could it be found on the internet.

Table 3 Highlight of ABC transporters identified as a predictor for poor chemo response using multiple cancer cell lines

ABC transporters	No. of cell lines used	Drugs	References
ABCA1	30 cancer cell lines	Mitoxantrone	[12]
ABCB1	NCI-60 cancer cell lines	Multiple	[6, 7] ^b
	NCI-60 cancer cell lines	Multiple	[13] ^c
ABCB5	NCI-60 cancer cell lines	Multiple	[13] ^c
ABCB6 ^a	39 cancer cell lines	Multiple	[9]
ABCC1	NCI-60 cancer cell lines	Multiple	[6, 7] ^b
	30 cancer cell lines	Etoposide	[12]
	NCI-60 cancer cell lines	Multiple	[13] ^c
ABCC2	30 cancer cell lines	5-FU	[12]
ABCC3	NCI-60 cancer cell lines	Multiple	[13] ^c
ABCD3	30 cancer cell lines	Topotecan	[12]

^a ABCB6 expression was reported to have positive correlation with drug sensitivity in this study

^b For more information see <http://genome-www.stanford.edu/nci60/>

^c For information see <http://www.r-project.org>

For additional information see <http://dtp.nci.nih.gov/>

particular analyses on ABC transporters and their roles in drug sensitivity prediction were reported in this study.

Dan *et al.* [9] performed a similar study using a cDNA

microarray containing 9216 human genes (Table 2), but with only 39 cell lines. Of these 39 cell lines, 9 were not included in the original set of NCI-60 collection used in

Table 4 Highlights of ABC transporters identified to have increased expression and potential contributions to drug resistance (to be continued)

ABC transporters	Cell lines/samples	Drugs	Functional validation ^a	References
ABCA1	Breast Clinical samples	5-FU/Paclitaxel		[25]
ABCA2	ALL Samples		Yes	[35]
	HL60/Ar	Adriamycin		[24]
	GLC4-MITO	Mitoxantrone	Yes	[42]
ABCA3	AML Samples		Yes	[33]
	ALL Samples		Yes	[35]
	HT-29/ETP	Etoposide		[10]
	K562/etop80	Etoposide		[10]
ABCA4	HL60/Ar	Adriamycin		[24]
	MCF7/AdVp3000	Adriamycin		[40]
ABCA5	MCF7/AdVp3000	Adriamycin		[40]
ABCA7	CEM/Adr5000	Adriamycin		[24]
	HL60/Ar	Adriamycin		[24]
ABCA12	Breast Clinical samples	5-FU/Paclitaxel		[25]
ABCB1	TRL1215	Arsenic	Yes	[14, 15]
	SKOV3 _{TX}	Paclitaxel		[19]
	KF28 _{TX}	Paclitaxel		[20]
	HCT-15/FK228	FK228		[21]
	IGROV1/FK228	FK228		[21]
	MCF7/FK228	FK228		[21]
	K562/FK228	FK228		[21]
	TC/ET	ET-743	Yes	[22]
	CCRF-CEM/ADR5000	Adriamycin		[24]
	KB-8-5	Colchicine		[30]
	SK3/VP16	VP-16		[10]
	KB-3-1	NSC73306		[36, 37]
ABCB2	HKCI-DOX	Adriamycin		[23]
	HL60/Ar	Adriamycin		[24]
ABCB3	CEM/ADR5000	Adriamycin		[24]
	HL60/Ar	Adriamycin		[24]
ABCB4	TRL1215	Arsenic		[14, 15]
	TC/ET	ET-743		[22]
ABCB6	MCF7/AdVp1000 ^b	Adriamycin		[24]
	A549/CPT	Camptothecin		[10]
	Breast Clinical samples	5-FU/Paclitaxel		[25]
ABCB8	CEM/ADR5000	Adriamycin		[24]
	T24/cDDP10	Cisplatin		[10]
ABCB9	HKCI-DOX	Adriamycin		[23]
	CEM/ADR5000	Adriamycin		[24]
ABCB10	MCF7/AdVp3000	Adriamycin		[40]
	HT-29/CPT	Camptothecin		[10]
ABCB11	A549/CPT	Camptothecin		[10]
ABCC1	TRL1215	Arsenic	Yes	[14, 15]
	HL60/AR	Adriamycin		[24]
	HT-29/ETP	Etoposide		[10]
	K562/etop20	Etoposide		[10]
	K562/etop80	Etoposide		[10]

Table 4 Highlights of ABC transporters identified to have increased expression and potential contributions to drug resistance (continued)

ABC transporters	Cell lines/samples	Drugs	Functional validation ^a	References
ABCC2	RCO.1	9-nitro-camptothecin		[30]
	MCF7/AdVp3000	Adriamycin		[40]
ABCC3	MCF7/AdVp1000	Adriamycin		[24]
	MCF7/AdVp3000	Adriamycin	Yes	[40]
ABCC4	HL60/AR	Adriamycin		[24]
	KK47/cDDP10	Cisplatin		[10]
ABCC5	MCF7/AdVp1000	Adriamycin		[24]
	MCF7/AdVp3000	Adriamycin		[40]
ABCC6	Breast Clinical samples	5-FU/Paclitaxel		[25]
	HL60/AR	Adriamycin		[24]
ABCC8	MCF7/AdVp3000	Adriamycin		[40]
ABCC9	SK3/VP16	VP-16		[10]
	SK3/VP	VP-16		[10]
ABCC11	Breast Clinical samples	5-FU/Paclitaxel		[25]
ABCC13	Breast Clinical samples	5-FU/Paclitaxel		[25]
ABCD3	SK3/VP	VP-16		[10]
ABCD4	HT-29/ADM	Adriamycin		[10]
	KK47/cDDP10	Cisplatin		[10]
	K562/etop80	Etoposide		[10]
ABCE1	CEM/ADR5000	Adriamycin		[24]
	HT-29/CPT	Camptothecin		[10]
ABCF2	CEM/ADR5000	Adriamycin		[24]
	T24/cDDP10	Cisplatin		[10]
ABCF3	MCF7/AdVp1000	Adriamycin		[24]
ABCG1	MCF7/AdVp1000	Adriamycin		[24]
ABCG2	LY/NR2	NB-506	Yes	[17]
	MCF7/AdVp1000	Adriamycin		[24]
	MCF7/AdVp3000	Adriamycin		[40]

^aThe functional validation was conducted following identification by array analyses to verify the functional role of the ABC transporter in resistance to corresponding drugs tested. Some ABC transporters have known roles in resistance to the corresponding drugs from prior studies, which are not counted as functional validation in this table. ^bThis cell line was also named as MCF7/CH1000 in the original publication.

previous studies [6-8]. The drug database was also smaller with only 55 anticancer drugs. Fifty genes were found to have a significant positive (e.g., aldose reductase) or negative (e.g., LIM domain kinase 2) correlation between their expression and sensitivity to ≥ 10 of the 55 drugs tested. Interestingly, ABCB6, the only ABC transporter gene among the 50 genes, was found to have a significant positive correlation with sensitivity to 20 anticancer drugs including Adriamycin, camptothecin, and mitoxantrone, suggesting that over-expression of ABCB6 may contribute to cellular sensitivity to these drugs (Table 3). ABCB6 is a mitochondrial ABC transporter known for its role in mitochondrial porphyrin uptake and possibly in controlling

haem biosynthesis [11]. It should be noted, however, that in the study by Yasui *et al.*, it was found that the ABCB6 gene was amplified in the camptothecin-resistant A549 cells [10]. Currently, there is no direct evidence regarding whether over-expression of ABCB6 causes resistance or sensitivity to anticancer drugs. Clearly, further studies are needed to more directly test the role of ABCB6 in drug responses.

Most recently, Gyorffy *et al.* [12] analyzed 30 cancer cell lines for their responses to 11 common anticancer agents and their gene expression profiles using the Affymetrix HG-U133 microarray containing all 49 human ABC transporter genes (Table 2). They found that the elevated expression of

ABCA1 correlates with mitoxantrone resistance, ABCC1 with etoposide resistance, ABCC2 with 5-FU resistance, and ABCD3 with topotecan resistance (Table 3). In addition, another 67 genes were found by these authors to have correlation with responses to multiple anticancer drugs. One of the notable genes is tripartite motif-containing 2 of which the expression correlates with cellular resistance to 8 different anticancer drugs.

A 70-mer oligonucleotide array containing probes for 640 transporter genes (transportome) including 40 ABC transporters (Table 2) has also been engineered to investigate the potential contribution of membrane transporters to drug responses of cancer cell lines [13]. Using this array, many genes that encode ABC transporters and transporters of nucleosides, amino acids, glucose, folate, channels for calcium and water, Ca^{++} -ATPase and Na^+/K^+ -ATPase were found to significantly correlate with either drug resistance or sensitivity of the NCI-60 cell lines. Highly statistically significant negative correlations with drug sensitivity, indicative of a possible role in chemoresistance, were found for ABCB1, ABCB5, ABCC1, and ABCC3 (Table 3). The differential expression of these genes, except for ABCC1, was also confirmed using real time RT-PCR. While ABCB1, ABCC1, and ABCC3 were known to cause drug resistance, the expression of ABCB5, which was not previously known to affect drug responses, was found to negatively correlate with camptothecin and 1,2,4,5-Tetramethylbenzene sensitivity. The follow-up study using siRNA to down-regulate ABCB5 expression in melanoma SK-MEL-28 cells caused sensitization to several anticancer drugs including camptothecin, 10-OH camptothecin, and 5-FU. These findings suggest that ABCB5 over-expression likely also causes MDR and may serve as a predictor along with ABCB1, ABCC1, and ABCC3 for poor drug sensitivity.

In addition to ABC transporters, other transporters have also been found to have significant correlations with drug responses by using the transportome array. While some of them such as the folate carrier are known to affect sensitivity to drugs such as methotrexate, others such as amino acid transporters have not been implicated in such a process. However, the sufficient structural similarity between amino acids and their analogue drugs may warrant the involvement of these transporters in drug responses of these cancer cells. This speculation awaits verification from future studies.

Identification of ABC transporter genes in drug-selected cell lines using high density microarrays

Comparative gene expression profiling has been conducted by using the Clontech Atlas Rat cDNA array (Table 2) containing 588 genes, including 4 ABC

transporters (ABCB1, ABCB4, ABCC1, and ABCC9), to investigate genes potentially responsible for arsenic tolerance in chronically exposed rat liver epithelial cell line TRL1215 [14]. Compared with the parental arsenic sensitive TRL1215 cells, the arsenic resistant TRL1215 has ~80 genes with altered expressions. Of these genes, ABCB1, ABCB4, and ABCC1 were among the genes with increased expression (Table 4). In a follow-up study, Liu *et al.* verified the altered expression of these three ABC transporters by using RT-PCR and western blot analyses [15]. They further demonstrated that inhibition of ABCB1 and ABCC1 function using their respective inhibitors could significantly increase arsenic sensitivity of the arsenic tolerant TRL1215 cell line, suggesting that over-expression of ABCB1 and ABCC1 may contribute to arsenic tolerance. However, it is not yet known if arsenic or its metabolites are directly effluxed by these ABC transporters. Furthermore, because the rat array contains only a limited number of ABC transporter genes, its usefulness is likely limited as it can not identify the potential roles of other ABC transporters in arsenic tolerance.

The anticancer drug NB-506, a Topo I inhibitor, has been shown not to be a substrate of the known drug transporter ABCB1 or ABCC1 [16], although NB-506 resistant cell lines showed decreased intracellular accumulation of NB-506 [17]. To discover genes that could be involved in NB-506 resistance and elimination, Komatani *et al.* performed comparative genomic analyses using Affymetrix arrays (Mu11K and Mu19K, Table 2) containing over 34 000 mouse genes [17]. They found that ABCG2, in addition to 11 other genes, had the most prominent elevation in expression in NB-506 resistant mouse fibroblast cell line LY/NR2 compared with the parental cell line LY (Table 4). The role of ABCG2 in cellular resistance to NB-506 was validated subsequently by ectopic over-expression of ABCG2 in the lung cancer cell line PC-13 [17].

In a similar study to discover resistance mechanisms of Adriamycin-selected acute myeloid leukemia cell line K562/A02 using the Clontech Human 1.2 II Atlas array (Table 2) containing only 1 ABC transporter gene (ABCD1), Tan *et al.* found 12 differentially expressed genes between K562/A02 and its parental sensitive line K562 [18]. Because of the limited ABC transporter genes on the array, no ABC transporter gene would have been found here despite the fact that ABCB1 is known to be strongly over-expressed in the K562/A02 cell line. Thus, the usefulness of this array for ABC transporter analysis is very limited.

A regular Affymetrix HG-U95Av2 array (Table 2) containing 9 600 known human genes including 14 ABC transporters was used to identify molecular signatures that are possibly responsible for paclitaxel resistance in 3 stepwise

selected SKOV3 ovarian cancer cell lines [19]. Of the 14 ABC transporter genes collected on this array, only ABCB1 was found to have increased expression correlative with the sequential increase in drug resistance of the 3 stepwise selected cell lines. Interestingly, the expression of ABCC3 and ABCC4 was decreased in the drug resistant cell lines. In a similar study of ovarian cancer cell line KF28_{TX} resistant to paclitaxel using an IntelliGene microarray (Table 2) containing 557 human cancer-related cDNAs including ABCB1 and ABCC3, ABCB1 was also found to be over-expressed and likely contribute to paclitaxel resistance [20]. Similar to the Clontech Rat cDNA array (see above), the usefulness of these arrays in ABC transporter profiling, however, is also limited.

In the study using Affymetrix HG-U95Av2 array [19], some other genes in addition to ABC transporters, were also found to have increased expression either in the early cell line with low paclitaxel resistance level or in the later cell line with intermediate or high level of resistance; and they were clustered into 3 categories based on their emerged increase in the 3 stepwise selected cell lines. The early respondent genes are primarily inflammatory response genes such as IL-8. The intermediate genes include carrier and primary active transporter genes. The late respondent genes include tumor and cell surface antigens and signal transducers. Still many other genes were also found to have altered expression in the cell line either with low or higher level of resistance. However, the expression of these genes did not follow the evolution trend with increased expression correlating with the increased drug resistance in the 3 stepwise selected cell lines. Therefore, their contributions to paclitaxel resistance in ovarian cancer cells may be limited.

Using the same transportome array developed by Huang *et al.* [13] (see above), Xiao *et al.* analyzed the underlying mechanism of acquired resistance of cancer cell lines HCT-15, IGROV1, MCF7, and K562 to the histone deacetylase inhibitor FK228 [21]. These authors found that ABCB1 expression is dramatically up-regulated in all four FK228-resistant derivative cell lines (Table 4). It was also found that ABCB1 expression was induced by FK228 likely via alterations in histone acetylation at the ABCB1 promoter. However, ABCB1-mediated efflux of FK228 and its direct involvement in FK228 resistance have not yet been established.

ET-743, a new anticancer agent derived from marine products, is currently under clinical trial; and variation in responses to this drug has been observed, suggesting that clinical resistance to ET-743 may develop. Using the Human 1 cDNA microarray containing 16,281 cDNA probes with 45 ABC transporter genes from Agilent Technologies (Table 2), Manara *et al.* profiled the differential gene expression between two ET-743 resistant Ewing's

sarcoma cell lines, TC/ET 6 nM and TC/ET 12 nM, and their parental sensitive cell line TC-71 [22]. The expressions of sixty-five genes were found commonly changed in the two resistant sublines compared with the sensitive cells. Among these genes, ABCB1 and ABCB4 were up-regulated most prominently in the resistant cells (Table 4). Using comparative genomic hybridization, ABCB1, but not ABCB4, was also found to have been amplified in the resistant cells. Since ABCB1 is the gene with most prominent increases in expression, it is likely very important for ET-743 resistance. Indeed, inhibition of ABCB1 activity by PSC-833 dramatically reduced ET-743 resistance level [22]. However, the potential contribution of the remaining 64 genes including ABCB4 to ET-743 resistance has not yet been rigorously examined.

The mechanism of Adriamycin resistance in five pairs of hepatocarcinoma cell lines has also been examined using a 19 K EST microarray [23] (Table 2). The expression of over 60 genes including ABCB2 and ABCB9 was found to have commonly increased in the 5 resistant sublines compared with their sensitive parental hepatocellular carcinoma cells. While ABCB2 participates in peptide transport in ER for antigen presentation, the function of ABCB9 was not yet known. Neither of these two genes has been shown previously to cause drug resistance. However, in the profiling study by Gillet *et al.* using their low density DualChip human ABC microarray (see below), ABCB2 and ABCB9 were also found to be over-expressed in Adriamycin-resistant HL-60 and CCRF-CEM cell lines, respectively [24] (see below). It is currently unknown if the over-expression of ABCB2 and ABCB9 causes Adriamycin resistance.

The high density Affymetrix arrays have also been used for focused analysis of ABC transporter gene expression despite the fact that they contain many more genes. One of these studies was performed using Affymetrix HG-U133 Plus 2.0 GeneChip which contains all 49 human ABC transporter genes (Table 2). ABC transporter expression profiles were generated using this array from breast cancer patients who received neoadjuvant chemotherapy including 5-FU and paclitaxel [25]. Comparison between patients who had complete response and those who had residual disease revealed that the expression of ABCA1, ABCA12, ABCB6, ABCC5, ABCC11, ABCC13 was elevated in the residual disease group, suggesting that their expression may contribute to the incomplete response of these patients to neoadjuvant therapy. Of these transporters, ABCC5 was found to be the elevated gene with the highest significance and highest expression level (Table 4). ABCC5 has been shown previously to cause resistance to several drugs including 5-FU [26, 27]. Thus, its high expression level in breast cancers may contribute to clinical resistance to drugs such as 5-FU. Gillet *et al.* also performed an analysis of

16 breast cancer samples using their low density DualChip human ABC microarray (see below) and found 23 ABC transporters including ABCC1 and ABCC5 were expressed in the majority (≥ 10 of 16) of the breast cancer samples tested [28]. Unlike the study by Park *et al.* [25], however, the Gillet study [28] did not have the correlation analysis between patient response to chemotherapy and their ABC transporter expression level. Thus, the implication of ABC transporter expression in this study is limited.

The Affymetrix MuU74v2 GeneChip with 33 000 mouse genetic elements contains annotated oligonucleotide probes for 43 of the 51 mouse ABC transporter genes (Table 2). Using this array, Mutch *et al.* [29] conducted a focused analysis of ABC transporter expression profiles along the intestinal tract of mice. While most ABC transporters do not show differential expressions in the duodenum, jejunum, and colon, 8 ABC transporter genes (ABCB2, ABCB3, ABCB9, ABCC3, ABCC6, ABCD1, ABCG5, and ABCG8) show significant alterations in expression along the intestinal tract. The alterations in expression of these 8 genes have also been validated using real-time PCR. These findings suggest that the Affymetrix MuU74v2 GeneChip may also be used for a focused analysis to investigate differential ABC transporter gene expression in drug resistant mouse cell lines compared with their parental sensitive lines.

Gottesman's laboratory has developed a high density ABC-ToxChip for microarray analysis of toxicological response genes [30]. On this microarray, in addition to 36 human ABC transporter genes, a comprehensive set of detoxifying genes were also included. By comparing the KB-3-1 cell line with its corresponding resistant derivative cell line selected using colchicine (KB-8-5), they showed that ABCB1 had dramatic over-expression in the KB-8-5 cell line (Table 4), the known mechanism of resistance in this cell, thus validating the usefulness of the array. Using this array to analyze DU-145 cell line and its corresponding resistant derivative cell line (RCO.1) selected using 9-nitro-camptothecin revealed that RCO.1 over-expresses ABCC2 (Table 4), suggesting that ABCC2 may contribute to 9-nitro-camptothecin resistance. However, the notion that over-expression of ABCC2 causes 9-nitro-camptothecin resistance has not yet been validated. Furthermore, RCO.1 also over-expresses many other genes of the 20 000 genes tested such as the EGF-like repeats and discoidin I-like domain protein (U70312), which may also contribute to 9-nitro-camptothecin resistance.

Identification of ABC transporter genes in drug-selected cell lines using low density microarrays

Use of high-density arrays to profile only ABC transporter expression is apparently not cost-effective. Recently, low

density arrays containing only ABC transporter genes have been developed which can be used more cost-effectively for focused studies of ABC transporters in drug resistant versus sensitive cells (Table 2). The first of such arrays contains 38 human ABC transporter genes (DualChip human ABC low-density microarray) and it has been validated using three MDR cell lines, CEM/ADR5000, HL60/AR, and MCF7/AdVp1000 in comparison with their respective parental CEM, HL60, and MCF7 cells [24]. Using this array it was found that CEM/ADR5000, HL60/AR, and MCF7/AdVp1000 over-expressed ABCB1, ABCC1, and ABCG2, respectively, as previously demonstrated [5, 31, 32], thus validating the usefulness of this low density array. In this study, several other ABC transporters were also found to be over-expressed in the drug resistant cell lines (Table 4), suggesting that they may also contribute to the resistance selected in these cells. However, none of these additional ABC transporter genes was validated for their functional role in drug resistance.

The same array was also used to profile ABC transporter expression and its correlation to clinical responses of 42 childhood acute myeloid leukemia (AML) patients [33]. It was found that, of all 38 human ABC transporters examined, only ABCA3 had significant correlation of over-expression to poor response (Table 4), suggesting that ABCA3 may contribute to clinical drug resistance in AML. Knocking down ABCA3 expression in the osteosarcoma cell line 143B with siRNA caused a statistically significant increase in sensitivity to Adriamycin. Nevertheless, this increase in sensitivity is very small ($<20\%$) [33]. Because ABCA3 is normally located at intracellular membranes [34], it seems unlikely to mediate drug resistance by directly effluxing drugs as do the traditional ABC drug transporters such as ABCB1 and ABCC1.

In another study, the same research group analyzed 21 childhood T cell acute lymphoblastic leukemia (ALL) samples using the same DualChip human ABC low-density microarray and found consistent over-expression of ABCA2 and ABCA3, similar to the findings in childhood myeloid leukemia [35]. However, correlation analysis between ABC transporter expression and the clinical outcome did not generate any statistically significant differences in the ALL. Although ABCA3 knock-down in the osteosarcoma cell line 143B by siRNA appeared to generate a significant increase in cellular sensitivity to Adriamycin [33, 35], it did not result in any survival effect in response to vinblastine and methotrexate. Knock-down of ABCA2 alone in 143B cells did not appear to have any effect on survival in response to Adriamycin, vinblastine, or methotrexate. However, the combined knock down of ABCA2 and ABCA3 resulted in a significant increase in cell death in response to all three anticancer drugs [35],

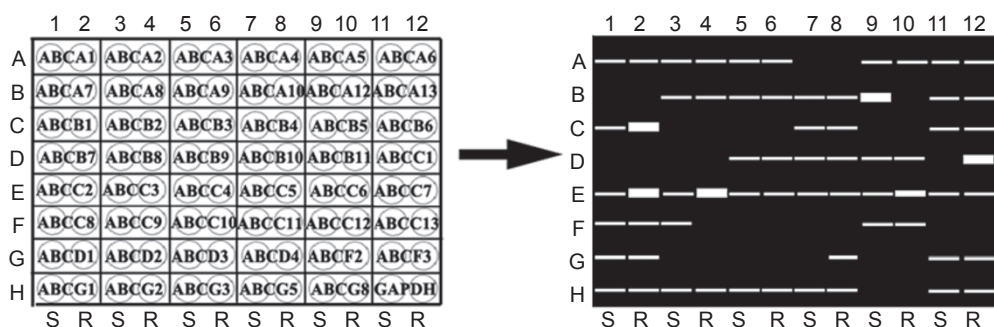


Figure 2 Schematic presentation of AmpArray template and its application. The template of AmpArray for 47 ABC transporters in a 96-well plate format is shown on the left. The right image is a schematic presentation of a theoretical agarose gel image demonstrating the comparative level of each ABC transporter between sensitive (S) and resistant (R) cells.

suggesting a potential compensatory effect between the two ABC transporters.

Quantitative PCR arrays to identify ABC transporter genes

The same 60 NCI cell lines discussed above were also examined for the correlation of their drug sensitivities with the expression of 48 human ABC transporters using a focused real-time RT-PCR approach [36] (Table 2). Correlation between the expression of ABC transporter genes and resistance to candidate drugs was analyzed. While there were interesting positive correlations between ABC transporter expression and resistance to drugs, along with the discovery of some previously unknown substrates for certain ABC transporters such as ABCB1, negative correlations between ABC transporter expression and drug resistance were also observed. One of the novel compounds that have positive correlations in resistance with ABCB1 expression is NSC363997. MTT assay using KB-3-1 and the drug-selected KB-V1 cells, which over-express ABCB1, showed that NSC363997 is likely an ABCB1 substrate [36]. In contrast, NSC73306 is an example for compounds that have negative correlations in resistance with ABCB1 expression. Validation studies showed that cells over-expressing ABCB1 are less resistant to NSC73306 [36]. More recently, Ludwig *et al.* followed up on the observation of NSC73306 and found that NSC73306 toxicity is actually mediated by ABCB1 over-expression [37]. Inhibiting the function of ABCB1 using inhibitors or decreasing its expression using siRNA both decreased cellular sensitivity to NSC73306. Interestingly, long term selection of several cancer cell lines with NSC73306 also resulted in NSC73306-resistant derivative cells, which apparently have decreased expression of ABCB1. These

findings are interesting because ABCB1 over-expression has previously been recognized only to cause resistance but not sensitivity to drugs. Nevertheless, the mechanism of ABC-transporter-mediated cytotoxicity is currently unknown and further studies are needed to interpret this phenomenon and investigate the mechanism of action.

The TaqMan real-time RT-PCR approach involving 47 ABC transporters has been developed by another group [38] to profile ABC transporter expression in human tissues. The expression profiles of ABC transporters in human tissues appear to be consistent with their known ubiquitous distribution. However, some ABC transporters show restricted expression patterns such as ABCG5 and ABCG8 in liver and intestine. The utility of this approach for analyzing drug resistant cells has yet to be tested.

While the above real-time PCR approach represents a focused assay on ABC transporters, performing this assay routinely on all ABC transporter genes is time-consuming. Recognizing this problem, Langmann *et al.* [39] recently improved their TagMan real-time RT-PCR approach by developing a low-density array of 47 ABC transporters based on an Applied Biosystems 7900HT Micro Fluidic Card, which allows simultaneous PCR analysis of 47 ABC transporters (Table 2). Although this array has not been tested for analyzing drug resistant cells, it was used to analyze the expression profiles of ABC transporters in primary human monocytes, differentiated macrophages, and macrophages treated with LXR and RXR agonist T0901317 and retinoic acid. It was found that 30 ABC transporters were expressed in human primary monocytes. Following differentiation into macrophages, the number of expressed ABC transporters decreased to 26. Although the expression level of most of these ABC transporters is lower than that in monocytes, ABCB1, ABCB11, and ABCG2 are the new genes detected while the expression of ABCB9 and ABCC5 is increased

in macrophages. Treatment of macrophages with LXR and RXR agonist T0901317 and retinoic acid further decreased the number of expressed ABC transporter genes to 12. It may be too premature to conclude that ABCB1, ABCB9, ABCB11, ABCC5, and ABCG2 expression plays important roles in differentiation. However, future detailed studies should help answer the question regarding the relationship between these ABC transporters and differentiation.

Another simpler and inexpensive AmpArray approach (Figure 2, Table 2) has also been designed recently and tested for investigating the expression profiles of 47 ABC transporters using RT-PCR on a 96-well plate [40]. This comparative assay is quick, convenient, and economical, as 47 ABC transporters are assayed at the same time on a regular thermal cycler that houses a 96-well plate format. This approach was tested using parental cell line MCF7 and its drug resistant derivative subline MCF7/AdVp3000. It was found that ABCG2 expression was dramatically increased in the resistant MCF7/AdVp3000 cells, which are consistent with previous findings that increased ABCG2 expression is a major cause of resistance in MCF7/AdVp3000 cells [5]. Interestingly, several other ABC transporters were also found to show altered expression levels. Particularly, the expression of ABCA4 and ABCC3 was drastically increased in the drug resistant subline and then reversed completely along with ABCG2 in the revertant MCF7/AdVpRev cells as determined by real-time PCR. The altered expression and possible contribution of ABCC3 to Adriamycin and mitoxantrone resistance were also validated by using western blot and SRB assays, respectively. However, the altered expression of ABCA4 could not be confirmed at the protein level. These findings suggest that more than one ABC transporters could be up-regulated to cause resistance in drug selected cell lines and that the altered expression at the mRNA level as determined by arrays or PCR does not necessarily correlate with the level of the corresponding protein. Compared to the real-time PCR low-density arrays, which require the use of specialized instrumentation and reagents, the AmpArray analysis is less expensive and simpler to perform. However, AmpArray suffers from its semi quantitative nature.

Comparative genomic approaches to investigate ABC transporter gene amplifications and their contribution to drug resistance

In addition to microarrays and real time quantitative PCRs to investigate the potential involvement of ABC transporters in drug resistance, other approaches have recently been developed. One of these approaches is subtractive comparative genomic hybridization of metaphase DNA on slides [41]. By using this approach to profile ABC

transporter gene amplifications in 23 drug resistant cancer cell lines, Yasui *et al.* recently found that ABCA3, ABCB1, ABCB6, ABCB8, ABCB10, ABCB11, ABCC1, ABCC4, ABCC9, ABCD3, ABCD4, ABCE1, and ABCF2 were amplified in various drug resistant cancer cell lines relative to their parental sensitive ones (Table 4). The amplified genes of ABCA3, ABCC1, and ABCC9 were also shown to have increased expression in the drug resistant cells at the RNA level using northern blot or RT-PCR analyses. However, it is currently unknown if amplifications of other ABC transporter genes also cause their over-expression in the drug resistant cells. Such a potential problem was better addressed in another similar study by Boonstra *et al.* who also used the similar subtractive comparative genomic hybridization to analyze a mitoxantrone resistant small cell lung cancer cell line GLC4-MITO [42]. Of the 7 amplified ABC transporter genes (ABCA2, ABCB2, ABCB3, ABCB6, ABCC4, ABCC10 and ABCF1) detected using comparative genomic hybridization, only ABCA2 was found to show corresponding over-expression at RNA and protein levels, and its over-expression likely causes mitoxantrone resistance (Table 4). This observation suggests that caution should be taken when the comparative genomic hybridization approach is used and validations using other approaches to determine the expression level should be performed.

Concluding remarks and perspectives

Both high and low density microarrays and quantitative PCR approaches with limited or full list of ABC transporter genes have been developed and tested. These arrays clearly can be used for a variety of studies to profile ABC transporter gene expression in drug resistant cancer cell lines or clinical tissues, which could not have been accomplished in the past. Predictors for drug sensitivity can also be built from the results of ABC transporter expression profiling which may assist the design of individualized therapies.

It is clear from the studies reviewed here that any given drug resistant cancer cell line likely has multiple ABC transporters selected for contribution to resistance (Table 4). Although the most frequently selected ABC transporters in various drug resistant cell lines appeared to be ABCB1 and ABCC1, consistent with their known roles in MDR, new roles in drug resistance previously unknown for some ABC transporters were also found using these profiling analyses. For example, ABCC3 and ABCC5 were both found up-regulated in the Adriamycin-selected MCF7/AdVp1000 [24] and MCF7/AdVp3000 [40] cell lines using two different arrays, strongly suggesting that these transporters may contribute to Adriamycin resistance. Although these transporters may be selected by verapamil that was present

during the selection of these cell lines, the role of ABCC3 in Adriamycin resistance has been validated functionally by the siRNA knock down strategy [40]. Clearly, further studies are still needed to more definitively demonstrate that the over-expression of these transporters causes Adriamycin resistance.

The findings that drug resistant cancer cells may over-express multiple ABC transporters simultaneously impose a challenge for successful clinical cancer chemotherapy. Sensitization of the drug resistance phenotype to optimize cancer chemotherapy may require considerations of inhibiting multiple drug efflux ABC transporters. It is noteworthy that in some studies members of ABC transporters were found to have decreased expression in drug resistant cells. It is currently unknown whether the decreased expression of these ABC transporters contributes to drug resistance. In other studies, it has been found that the over-expression of ABC transporter mediates cytotoxicity of some compounds. Again, the mechanism of action is unknown. Clearly, more studies are needed to investigate if decreased expression of some ABC transporters causes drug resistance and how certain ABC transporter over-expression mediates cytotoxicity of some drugs.

Based on the studies reviewed here, it is clear that while mass information could be produced from the array profiling studies for potential drug resistance contributors, most of the studies are correlative and lack functional validation of the implicated genes that may be responsible for the drug resistance/sensitivity observed. Of course, simultaneous analyses of cell lines with intermediate drug resistance generated during stepwise selections and revertant cell lines that have lost resistance as demonstrated in the studies by Lamendola *et al.* [19] and Liu *et al.* [40] would help identify genes that likely contribute to the resistance of the cells. Further functional validation of these genes in drug resistance (Table 3) using siRNA-mediated silencing, ectopic over-expression, and known inhibitors would be very fruitful and could help verify the new drug resistance functions of these ABC transporters.

Another problem associated with ABC transporter gene profiling using microarrays or quantitative PCRs is that these approaches only detect the changes in expression at the mRNA level. The protein level of the corresponding transporter may or may not be changed. For example, the expression of ABCA4 in the AmpArray study by Liu *et al.* [40] was increased at the mRNA level in the drug resistant MCF7/AdVp3000 cells, as detected by the array profiling and confirmed by real time RT-PCR. However, the change of ABCA4 protein was not detected using several antibodies. Rather than validating the gene expression using real time PCR, as shown in most of the studies discussed above, western blot analyses would be preferred if antibodies are

available. Furthermore, immunohistochemistry using tissue microarrays has been developed to examine the expression of ABC transporters, which would also facilitate the study using clinical human tissue samples and eliminate the potential problems from detection of mRNAs as mentioned above [43, 44]. Proteomic approaches have also been developed to investigate drug resistance mechanisms and may help solve the aforementioned problem of gene profiling studies. However, the proteomic approach can be problematic and limited in separating and identifying polytopic membrane proteins including ABC transporters (Zhang and Liu, manuscript submitted).

Because ABC transporters share high homology, there is a potential problem of cross hybridization associated with microarrays, although the oligonucleotide arrays contain multiple probes targeting different regions of each target. As suggested by Lee *et al.* [45], performing similar studies using both cDNA and oligonucleotide microarrays, which are subjected to different artifactual influences during hybridization, may help minimize this potential problem. For quantitative PCR approaches, the use of certain reference genes such as GAPDH can also be problematic. It has been found that GAPDH expression was increased in the Adriamycin-resistant MCF7/Adr cells [46, 47]. In a metabolic marker-focused proteomic profiling analysis of 101 breast cancer tissues, Isidoro *et al.* [48] also found that the expression of GAPDH was significantly increased in patients with poor survival. These studies suggest that GAPDH may not be an ideal internal reference gene for the profiling studies using PCR. Most recently, Calcagno showed that plasma membrane calcium-ATPase 4 (PMCA4) is a better reference gene for real time PCR quantification of ABC transporter genes [49]. PMCA4 is a ubiquitously expressed house keeper plasma membrane protein and its expression in several cancer cell lines appeared to be unaffected by treatment with various agents including anticancer drugs.

Some arrays are limited in the number of ABC transporter genes (Table 1), and thus, their usefulness in profiling ABC transporter expression is likely also limited. The past studies using these arrays would likely have missed some ABC transporter genes that have *bona fide* roles in drug resistance. Furthermore, using the low density arrays and quantitative PCRs also likely would miss information about other potential mechanisms of drug resistance when profiling drug resistant and sensitive cancer cells, although the low density arrays are more cost effective. Taken together, it is clear that comprehensiveness, cost effectiveness, simplicity of operation, and fidelity should all be considered when choosing any of these comparative genomic profiling approaches. At least two independent approaches are recommended to validate the findings.

Furthermore, it is known that ABC transporter polymorphism such as that in ABCG2 [50] could affect substrate specificity and, thus, resistance to certain anticancer drugs. These polymorphisms of ABC transporters have not yet been considered in the ABC transporter arrays developed thus far. Future studies to develop ABC transporter arrays with consideration of polymorphisms would be important for studies of MDR and drug responses.

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