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Analysis of ABC transporter expression in drug-selected cell lines by a microarray dedicated to multidrug resistance

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Running Title: Microarray platform to study drug resistant cells

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ABBREVIATIONS: MDR, Multidrug Resistance, MRP2 Multidrug resistance associated protein 2; BSO, L-buthionine-[S,R]-sulfoximine; DTNB, 5,5'-Dithiobis 2-nitrobenzoic acid; CPT, Camptotecin; 9NC, 9-nitro-Camptothecin; SAGE, Serial Analysis of Gene Expression; GO, Gene Otology; HUGO, Human Genome.

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ABSTRACT

The discovery of the multidrug resistance protein 1 (MDR1), an ATP-binding cassette transporter able to transport many anticancer drugs, represents a clinically relevant breakthrough in multidrug resistance. Although the overexpression of ABC transporters such as P-gp/ ABCB1, MRP1/ABCC1 or MXR/ABCG2 appears to be a major cause of failure in the treatment of cancer, acquired resistance to multiple anticancer drugs may also be multifactorial, involving alteration of detoxification processes, apoptosis, DNA repair, drug uptake, and overexpression of further ABC transporters. We created a microarray platform to evaluate relative levels of transcriptional activation among genes involved in various mechanisms of resistance. In the ABC-ToxChip, a comprehensive set of genes important in toxicological responses (2,200 cDNA probes together with ~18,000 oligonucleotide probes) are complemented with probes specifically matching ABC transporters as well as oligos representing 18,000 unique human genes. By comparing the transcriptional profiles of KB-3-1 and DU-145 cells to resistant derivatives selected in colchicine (KB-8-5), and 9-nitro-camptothecin (RCO.1), respectively, we demonstrate that ABC transporters (ABCB1/MDR1 and ABCC2/MRP2, respectively) show dramatic overexpression, whereas the glutathione S-transferase gene (GST-Pi) shows the strongest decrease among the 20,000 genes studied. The results were confirmed by quantitative RT-PCR and immunohistochemistry. These results suggest that custom-made, dedicated microarrays will be helpful to elucidate mechanisms leading to anticancer drug resistance.

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INTRODUCTION

Mechanisms leading to multidrug resistance (MDR) include increased drug efflux (Shen et al., 1986a; Gottesman et al., 2002), decreased drug uptake (Shen et al., 2000), recruitment of drug-processing and metabolizing enzymes (Schuetz et al., 1996), conjugating enzymes (Goto et al., 2002), alteration of the DNA repair activity, reduction of cell susceptibility to apoptosis (Reinhold et al., 2003), and mutation of specific drug targets (Urasaki et al, 2001). *In vitro* selected cell lines most likely utilize more than just one of the pathways cited above (Gottesman et al., 2002).

Tools, such as the oligo-based, cDNA-based microarrays or SAGE (Serial Analysis of Gene Expression, Velculescu et al., 1995) are relevant methodologies to screen for multifactorial mechanisms of drug resistance. However, microarray technology has several inherent problems that hamper definitive interpretation. Typical problems with the current technology include the necessity of validation by complementary technology such as further microarray analyses or RT-PCR (Lee et al., 2003). The lack of sensitivity could also prevent the detection of infrequent transcripts (Evans et al., 2002) and lead to biased conclusions. In research aiming at the elucidation of mechanisms underlying acquired drug resistance, the shortcomings of arrays available at the time of our study originated from the low representation of genes of interest and the general lack of specificity of the probes. This is especially true for the ABC (ATP Binding Cassette) superfamily, which has 48 members sharing high sequential similarity with highly divergent functions and/or specificities.

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Like endogenous and exogenous toxic compounds, anti-cancer drugs diffuse through the cell membrane. One of the most interesting features of ABC transporters is that these proteins can transport many substrates across the membrane, including anti-cancer drugs, ions and peptides. MDR1 or P-gp was the first ABC transporter found to confer MDR in resistant tumor samples and *in vitro* selected cell lines (Shen et al., 1986b). Using cell lines selected in various anti-cancer drugs, overexpression of further ABC transporters, such as ABCC1-MRP1 (Cole et al., 1992), and ABCG2- MXR-BCRP (Miyake et al., 1999; Doyle et al., 1998) were clearly shown to be associated with drug export and resistance. In experiments performed *in vitro*, other members, such as ABCA2 and ABCB4 (MDR3) were shown to actively transport cytotoxic drugs (Laing et al., 1998; Smith et al., 2000). Among the members of the MRP (ABCC) subfamily, MRP2 (ABCC2, cMOAT) transports GSH-*S*-conjugates (e.g., leukotriene C4 and 2,4-dinitrophenyl-*S*-GSH), oxidized GSH (GSSG), glucuronide conjugates (e.g., glucuronidated bilirubin and bile salts), and sulfate conjugates of certain bile acids (e.g., 3-sulfatolithocholytaurine), (Muller et al., 1994). By their ability to transport nucleoside analogues, ABCC3-MRP3 and ABCC5-MRP5 are proteins that are thought to cause certain forms of drug resistance (Reid et al., 2003). Recently, ABCC11-MRP8 has also been associated with anti-cancer drug export (Guo et al., 2003; Szakács et al., 2004).

Although microarrays make possible the screening of thousands of genes in the same matrix, attempts aiming to address anticancer drug resistance have been biased in their interpretation by microarrays bearing a low number of ABC transporter superfamily probes (Lamendola et al., 2003). Here, we report the design and the application of a dedicated ABC-ToxChip, in which we complemented a comprehensive

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set of detoxifying genes with probes specifically matching ABC transporters. Instead of using the short 25-mer probe-technology (Affymetrix, Santa Clara, CA), not optimized for detecting infrequent transcripts, we printed a combination of longer 70-mer oligo probes and 200-500 bp fragments, offering higher sensitivity due to the longer complementary sequences.

Camptothecin (CPT) derivatives such as CPT-11 (Irinotecan) or Hycamtin (Topotecan) are increasingly used in anti-tumor therapy against colon or lung cancers (Kudoh et al., 1998). 9-Nitro-camptothecin (9NC) has recently been used in phase II studies for pancreatic cancer and is now in phase 3 clinical trials (Pantazis et al., 2003). Many factors, such as specific mutations in topoisomerase I, complemented by a general alteration of apoptotic regulation, have been proposed to explain the phenotype of campthotecin resistance in a prostate cancer cell line (RCO.1) selected for resistance to 9NC (Reinhold et al., 2003; Urasaki et al., 2001; Chatterjee et al., 2001). To specifically address the role of detoxifying enzymes and ABC transporters in campthotecin resistance, we compared the transcriptional profiles of the parental prostate cancer DU145 cells and their 9NC-selected derivative cell line, RCO.1. Here, we show that in RCO.1 cells, 9NC resistance is accompanied by the differential expression of ABCC2-MRP2 and enzymes regulating glutathione metabolism.

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Materials and Methods

Taq polymerase and RT-PCR reagents were from Invitrogen (Carlsbad, CA). Reagents for quantitative RT-PCR were from Roche. Inc. (Indianapolis, IN). High-density microarrays were printed in the NIEHS facility. 9NC (Ref. C0156) was purchased from LKT laboratories (St. Paul, MN).

Cell Lines and Cell Culture. DU-145 and its 9NC-selected derivative, RCO.1 cell lines were a generous gift from Dr. P. Pantazis (University of Miami, Coral Gables, FL). Cells were cultured in RPMI (Invitrogen) supplemented with 10% tetracycline-approved FBS (Hyclone Inc. Logan, UT), and 2mM L-glutamine by Quality Biological, Inc., (Gaithersburg, MD) at 37°C with 5% CO_2. Resistance to camptothecin analogs of RCO.1 was maintained with passage in camptothecin-containing media (0.1 μM) every three months.

RNA Preparation. Total RNA was purified as described in Reinhold et al. (2003). For the preparation of the RNA used in the microarray analysis or RT-PCR, cells were cultured in non-selective media.

Microarray Design. A 20K Human Oligo/cDNA hybrid Chip, printed at NIEHS (NIEHS Microarray Group, National Institute of Environmental Health Sciences) was used for gene expression profiling experiments. The chip contained three categories of probes:

1. 96 ABC-specific probes (matching 36 ABC transporters), consisting of either cDNA probes developed in-house or oligo-probes obtained from Operon Technologies

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(Qiagen, Valencia, CA). To create the cDNA-probes, we performed PCR amplifications using specific primers on full-length cDNA sequences (ABCB1-MDR1, ABCC1-MRP1, ABCC2-MRP2, ABCG2-MXR, ABCC7-CFTR and ABCB11) or on cDNA prepared from cells rich in a given transporter (unpublished data). We used a blast-based algorithm to increase specificity: first, we aligned the targeted transporter with members of its own subfamily to locate a candidate region representing minimal overlap. Specificity was then verified by blasting the candidate sequence against the whole non-redundant human database. The probes synthesized by RT-PCR were TA cloned (Invitrogen), as detailed in Table 1. ABC transporters are named using the HGNC nomenclature (http://nutrigene.4t.com/humanabc.htm). When more than one probe was used for an ABC transporter, the probe name was chosen to match the closest Ref-Seq entry from the NCBI (i.e., ABCC3 (NM-003786, AJ294547.1, AJ294559.1, AJ294558.1). The closest exon matching the probe sequence is indicated in Table 1.

2. 2,200 probes matching genes important in toxicological responses (cDNA clones from the NIEHS ToxChip version 3.0 (Nuwaysir et al., 1999). The NIEHS set contains approximately 2,200 known human genes involved in pathways such as response to estrogens, polycyclic aromatic hydrocarbons, peroxisome proliferators, DNA damage, and oxidant stress, as well as genes involved in apoptosis, cell cycle, tumor suppression, signal transduction and transcription.

3. 18,000 genome-wide 70mer probes obtained from Operon Technologies (Qiagen, Valencia, CA). A complete listing of the two sets (Human Oligo gene-set and the Human TOX V1.0-set) printed on the ABC-ToxChip is available at http://dir.niehs.nih.gov/microarray/annereau/home.htm.

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Gene Representation on the Microarray. The gene ontology classification (http://www.geneontology.org/#ontologies) annotates each gene in three categories: Molecular Function, Biological Process and Cellular Component. To search the Gene Ontology (GO) reference for each gene represented on our microarray, we used the human genome annotation (HUGO) reference converted from the Gene Accession number by the Source data base (http://genome-www5.stanford.edu/cgi-bin/SMD/source/sourceSearch) and the MatchMiner database (http://discover.nci.nih.gov/matchminer/html/index.jsp). Extraction of the GO annotation starting keyword entry was performed on the QuickGO server at EBI (http://www.ebi.ac.uk/ego/).

Microarray Spotting. The oligonucleotides were resuspended in ArrayIt Spotting Solution Plus buffer (Telechem, San Jose, CA) and spotted at a concentration of 40 □M onto poly-L-lysine coated glass slides using a modified, robotic DNA arrayer (Beecher Instruments, Bethesda, MD). The spotting was performed in an environmentally controlled chamber with a temperature of 25°C at 40% relative humidity. After printing, the arrays were cross linked in a Stratalinker at a power of 300 millijoules and blocked with succinic anhydride/1-methyl-2-pyrrolidinone (protocol available at: http://dir.niehs.nih.gov/microarray/methods.htm)

Microarray Hybridizations. Each total RNA sample (25 µg) was labeled with Cyanine 3 (Cy3) or Cyanine 5 (Cy5)-conjugated dUTP (Amersham, Piscataway, NJ) by

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a reverse transcription reaction using the reverse transcriptase, SuperScript II (Invitrogen, Carlsbad, CA), and the primer, Oligo dT (Amersham, Piscataway, NJ). The fluorescently-labeled cDNAs were mixed and hybridized simultaneously on the microarray chip. Each RNA pair was hybridized to a total of 4 arrays employing a fluor reversal accomplished by labeling the control sample with Cy3 in 2 hybridizations and with Cy5 in the other 2 hybridizations. After hybridization, arrays were washed with Telechem wash buffers A, B, and C (Telechem International Inc., Sunnyvale, CA) for 2, 5, and 5 minutes respectively. The hybrid chips were scanned with an Agilent Scanner (Agilent Technologies, Wilmington, DE) using independent laser excitation of the two fluors at 532 and 635 nm wavelengths for the Cy3 and Cy5 labels, respectively. Results are available at http://dir.niehs.nih.gov/microarray/annereau/home.htm.

Microarray Outlier Filtering. Raw pixel intensity images were analyzed using ArraySuite v2.0 extensions of the IPLab's image processing software package (Scanalytics, Fairfax, VA). This program uses methods that were developed and previously described by Chen et al. (2002) to locate targets on the array, measure local background for each target and subtract it from the target intensity value, and to identify differentially expressed genes using a probability-based method. The data were filtered with a cut-off at the intensity level just above the buffer blank measurement values to remove genes having one or more intensity values in the background range. After pixel intensity determination and background subtraction, the ratio of the intensity of the treated cells to the intensity of the control was calculated. The ratio intensity data from all probes on the Human Oligo chip was used to fit a probability distribution to the ratio

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intensity values. The resulting probability distribution was used to calculate a 95% confidence interval for the ratio intensity values. Genes having normalized ratio intensity values outside this interval were considered to show significant differential expression.

Statistical Analysis and Filtering the Outliers. For each of the 4 replicate arrays for each sample, lists of differentially expressed genes at 95% confidence levels were created and deposited into the NIEHS MAPS database (Bushel et al., 2001). Genes that indicated fluorescence bias or high variation were not considered for further analysis. Assuming that the replicate hybridizations are independent, a calculation using the binomial probability distribution indicated that the probability of a single gene appearing on this list when there was no real differential expression was <0.00048. The entire dataset is available at http://dir.niehs.nih.gov/microarray/annereau/home.htm.

Identification of Relevant Biological Processes. The GoMiner software, which can be downloaded at the LMB/NCI web site (discover.nci.nih.gov) offers a newly implemented feature—the ability to point out a biological process that has been altered. The algorithm uses Gene Ontology entries found in the outlier list as well as all the GO entries available on the chip. The Gene Ontology database lists three structured, controlled vocabularies (ontologies) that describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner. The GO entries are hierarchically linked, thus allowing pooling and construction of cluster genes of crossed pathways. The bias in the gene

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representation (only a part of the genome is represented on the chip) could potentially lead to misleading conclusions. To evaluate the statistical weight of each emerging cluster, we have used the updated version of GoMiner that is now able to process multiple comparison analyses (Zeeberg et al., 2003, http://discover.nci.nih.gov). The statistical relevance of a candidate process found to be altered with drug resistance is therefore calculated in comparison to the overall processes that could be theoretically identified.

Quantitative RT-PCR. Real time PCR was performed with a Light Cycler RNA SYBR Green kit (Roche Biochemicals, Indianapolis, IN). The reaction was in a 20 µl final volume with 0.150 µg of purified total RNA, 4 µl PCR mix provided by the manufacturer, 4 µl $MgCl_2$ (25 mM), 2.5 µl of each primer (2 µM), 0.4 µl of enzyme mix and DEPC-H_2O. Optimized and specific primers were designed to produce a unique band for the 47 ABC transporters (Szakács et al., 2004). The reverse transcriptase (RT) reaction was performed at 55°C for 20 min. cDNA generated by the RT step was denatured at 95°C for 20 sec. Amplification of the cDNA was achieved in 45 cycles of 95°C, 5 sec; 58°C, 10 sec; 72°C, 13 sec. Fluorescence was recorded during the elongation phase at 72°C.

Western Blots. Following SDS-Page electrophoresis on 6% acrylamide SDS-Page gels (Invitrogen, Carlsbad, CA), samples (50 µg) were electro-blotted on nitrocellulose membranes (Invitrogen). Membranes were saturated with a 10%, fat-free milk solution (Giant Foods, Inc., Landover, MD), and then incubated overnight in the presence of an

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anti-MRP2 primary antibody (M-8316, Sigma, St-Louis, MO) at 1:1000 dilution. An HRP-conjugated secondary antibody (anti-rabbit Goat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied for 1 hour at 1:2000 dilution. ECL was purchased from Amersham (Pharmacia Biotech, Inc., Little Chalfond Bucks, UK).

For GAPDH and GST-Pi, 12% SDS-Page gels were used (Invitrogen). Primary antibodies were anti-GAPDH RDI-TRK-5G4-6C5 (Research Diagnostic Inc., Flanders, NJ) at 1:1000 dilution and anti-GST-P IgG1 at 1:1000 dilution (BD Biosciences Pharmingen, San Diego, CA). The secondary antibody was Goat-IgG anti-IgG Mouse HRP-conjugated (Jackson ImmunoResearch Laboratories, # IR-115-035-164) at 1:2000 dilution.

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Results

Creation of a Hybrid cDNA/Oligo ABC-ToxChip to Study Drug Resistance.

The ABC-ToxChip is a dedicated microarray to study the drug resistance of cancer cells. The platform contains three distinct sets of probes (Fig. 1A). The first set of probes targets 36 ABC transporters (70-mer oligonucleotides, and cDNA probes). Specificity and sensitivity was ensured by targeting each transporter with multiple probes (Table 1). Since multidrug resistance can be regarded as a detoxification problem, we also applied the TOX-probes (Nuwaysir et al., 1999) that cover known detoxification genes involved in response to estrogens, polycyclic aromatic hydrocarbons, peroxisome proliferators, DNA damage and oxidant stress, as well as genes involved in apoptosis, cell cycle, tumor suppression, signal transduction and transcription. To reduce the problem of sequence reliability encountered in commercially available cDNA clones (Knight et al, 2001), the probes (200-500 bp) of the TOX-set were derived from sequence-verified plasmids originally compiled at the National Institute of Environmental Health Sciences (NIEHS). To avoid the risk of a biased analysis inherent to highly specialized microarrays, we completed the chip with 18,000 probes (GEN-set) targeting the human transcriptome (Operon Inc., Alameda, CA). To overcome the qualitative errors found on various platforms and to rule out possible cross-hybridization, we verified the specificity of the cDNA and 70-mer oligo sequences by extensive *in silico* analysis (not shown).

Of the 18,398 probes represented by 16,329 oligo probes and 2069 cDNA probes matching a valid gene reference, 11,813 were annotated with HUGO references,

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using the Source / Matchminer databases (Zeeberg et al., 2003). The cDNA, or detoxification set has 1,394 probes with valid GO annotations, of which 1,152 are also represented in the GEN-set. The distribution of gene ontology entries proves that the cDNA set is indeed enriched in genes related to detoxification processes. For example, genes related to drug stress and DNA repair are highly represented in the cDNA TOX set (1.3% and 3.3% of the total genes, respectively), as compared to the GEN-set (0.3% and 0.6%, respectively).

Validation of the ABC-ToxChip Using an Established Model of Resistance. To identify markers of resistance or sensitivity to colchicine, we compared two cell lines using the ABC-ToxChip. KB-8-5 cells were derived from parental KB-3-1 cells by selection in colchicine (Akiyama et al., 1985). RNA samples from the two cell lines were reverse transcribed, labeled with Cy3/Cy5 and hybridized to 4 ABC-ToxChip slides. After filtering the data for the most reliably differentially expressed genes with a statistical confidence of 95%, the comparison revealed 241 statistically significant variations (Table S1). Among the four cross-labeled experiments, 98 hits (40.6%) appeared in all 4 replicate scans. The rest (59.4%) were significant in only 3 of the 4 replicate slides.

In total we identified 13 differentially expressed genes associated with colchicine resistance with a ratio above 2.7 (Fig. 1C). Among the 20,000 genes, ABCB1/P-gp showed the highest variation (10-fold fluorescence ratio) associated with colchicine resistance. Closely related members of the same subfamily (ABCB2-3-4) showed only moderate (about 2-fold) changes, presumably due to cross-hybridization, confirming the improved specificity of our dedicated microarray for ABC transporters. To verify the

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patterns of ABC-expression detected by the microarray analysis, we measured the expression of 47 ABC transporters by real time RT-PCR. The analysis confirms that ABCB1 (MDR1) shows the largest variation among the ABC transporters, while the expression of the other members is virtually unchanged (Fig. 1B). Comparison of KB-3-1 cells to KB-C1, a derivative showing extreme resistance (1 ug/ml) of colchicine showed the highest change in the expression of the same genes (log2ratio of 6.32 and –4.2 for ABCB1/MDR1 and GST-Pi, respectively, data not shown).

Increased expression of MDR1 (ABCB1) and the glycoprotein hormone subunit (GPH) was revealed by several independent probes (#M29447, #M37723 and #S70585, #W86681 for GPH and ABCB1, respectively). The list of further upregulated genes include Interleukin 8, MHC-I, COX-2 and the Interferon stimulated protein, while GST-Pi, Calcium-binding protein A10, Voltage gated sodium channel and EGF-like domain 5 are markedly downregulated in KB-8-5 cells as compared to the sensitive cell line. We used the information provided by HUGO to process the *ab initio* and hypothesis free GoMiner algorithm to detect biological processes that are represented by the differentially expressed genes and could possibly explain mechanisms leading to colchicine resistance.

Among 241 statistically significant outlier genes (see Materials and Methods), we identified 125 unique HUGO entries (52%). Since the dedicated design of the microarray can introduce a bias in analysis, with, for example, an overrepresented class of detoxifying genes printed, we evaluated the significance of the results in the light of the global representation of the GO ontology annotation entries. The GoMiner analysis indicates that expression of genes found to be altered by selection for colchicine

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resistance is linked to antigen presentation and carbohydrate metabolism (P<0.0001). In Table S2, we present the statistically relevant biological processes that can be associated with colchicine resistance. In Table S3, we present the genes that belong to the altered gene ontology categories related to antigen presentation, carbohydrate metabolism, transport and oxydoreduction.

Application of the ABC-ToxChip to Study 9-nitro-camptothecin Resistance. We next turned our attention to RCO.1, a prostate cell line selected in 9NC, where mechanisms leading to the resistant phenotype have not been fully understood. This cell line was created by selecting parental DU-145 cells in 0.1 µM 9-nitro-camptothecin (Urasaki et al., 2001). RCO.1 presents a unique profile of multidrug resistance with a selective resistance to camptothecin analogs, NB-506 (an inhibitor of topoisomerase I) and cisplatin, while being sensitive to drugs known to be exported by ABCB1-MDR1 or ABCC1-MRP1 (Chatterjee et al., 2001). Several laboratories have attempted to elucidate this atypical profile of resistance: a mutation of topoisomerase I, preventing the binding of camptothecin analogs (Chatterjee et al., 2001) and an alteration of the apoptotic pathway (Chatterjee et al., 2001; Reinhold et al., 2003) have been proposed as explanations. However, an exhaustive analysis of ABC transporters or genes involved in detoxification has not been reported to date. To explore the role of ABC transporters and other detoxifying genes in 9NC resistance, we compared the mRNA expression profiles of DU-145 and RCO.1 cells using the ABC-ToxChip. Total cellular RNA was extracted from both cell lines. Since the phenotype of acquired 9NC resistance is stable without the pressure of selection, RNA was collected from cells cultured in drug-free medium. Microarray experiments were performed as described

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previously for the KB cells. To control for labeling differences, reactions were carried out in quadruplicate, and the fluorescent dyes were switched.

The list of the most significant differentially expressed genes is shown in Figure 1C and in Table S4. Among the 20,000 genes, ABCC2-MRP2 shows one of the highest variations associated with 9NC resistance. First named the canalicular multispecific organic anion transporter (cMOAT), ABCC2-MRP2 is a 190-kDa phosphoglycoprotein localized in the canalicular (apical) membrane of hepatocytes. It is involved in the transport of organic anions, including sulfated and glucuronidated bile salts. Overexpression of MRP2 has been suggested to confer resistance to anti-cancer drugs such as cisplatin, anthacyclines, and methotrexate, and animal models have shown reduced hepatic transport of camptothecins (Horikawa et al., 2002).

Gamma-glutamylcysteine synthetase (GCS), also known as glutamate cysteine ligase, a key enzyme in glutathione metabolism, was coordinately up-regulated with MRP2, in keeping with the findings that MRP2 exports glutathione conjugates (Paulusma et al., 1996). In contrast, despite its general up-regulation in cancer cells, the expression of GST-Pi, catalyzing the conjugation of glutathione to electrophilic carcinogens, was significantly reduced in RCO.1 cells, as it was in the colchicine-selected KB cells.

Additional changes which might contribute to the pattern of drug resistance in RCO.1 cells were also observed. The up-regulation of various histones in the selected cells may provide means for the cells to adapt to the 9NC-mediated DNA insult. Further changes associated with drug resistance include reduction in the tumor-associated antigen L6 (transmembrane super family 4 or TM6). TM6 is also found to be down-

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regulated in cisplatin-resistant cells (KB/cDDP) (Higuchi et al., 2003), indicating that the loss of TM6 expression is potentially associated with resistance to camptothecin and cisplatin. Of the genes presented in Table S4, the decreased expression of GST-Pi, NKT4 and Interleukin-4 in RCO.1 cells was also observed by an independent microarray analysis (Table 2), which however failed to detect the overexpression of ABCC2-MRP2.

Although 85 of the 125 outliers (68%) have a HUGO reference, the GoMiner algorithm did not suggest specific biological processes to be significantly linked to camptothecin resistance. (See tables S5 and S6 for the biological processes and their associated genes linked to camptothecin resistance.)

We next sought to determine whether the differential expression of ABCC2-MRP2 and GST-Pi could be confirmed by quantitative RT-PCR. The results show that among the 47 ABC transporters, ABCC2-MRP2 is overexpressed in the resistant cell line, while the expression of the other members is unchanged (Fig. 2A). Since mRNA levels may not reflect protein expression, due to modulation of translation or inhibition of protein processing, we analyzed the expression of MRP2 and GSTPi at the protein level. Fig. 2C shows that the protein expression of these two genes follows the pattern predicted by the RNA analyses: In the RCO.1 cells, GST-Pi is down-regulated and ABCC2-MRP2 is up-regulated as compared to the parental line.

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Discussion

In this report, we present a new microarray design and analysis to study multifactorial drug resistance. We have designed and synthesized probes to match 36 of the 48 ABC transporters to print them on a platform enriched in genes involved in detoxification, as well as a general set of 18,000 human gene probes (the ABC-ToxChip). To increase at the same time sensitivity and specificity for genes involved in drug resistance, we combined on the same chip both cDNA and oligos probes. This approach provides an example of how existing microarray platforms may be modified to target a gene family with high sensitivity and specificity to study multidrug resistance of cancer. In this study, we confirm that overexpression of ABCB1 is a principal component of the genetic changes underlying colchicine resistance. KB-8-5 cells express ABCB1-MDR1 at a moderate level, comparable to that found in clinical samples. We had made several earlier attempts to identify the molecular signature of colchicine resistance, using a 9K cDNA microarray platform (UniGemV2, Advanced Technology Center, NIH, Gaithersburg, MD). The UniGemV2 chip failed to identify the overexpression of ABCB1-MDR1 and suggested, incorrectly, that the main outlier is ABCB2, a protein closely related to ABCB1 (not shown). ABCB2-TAP1 is a component of the ER transport system for peptide antigen presentation and is not believed to play a role in the efflux of cytotoxic compounds, and, in fact, was not actually overexpressed in KB-8-5 cells.

Analysis of a prostate cancer cell line (RCO.1) indicated the potential role of ABCC2/MRP2 in 9NC resistance. Previous studies have shown that the resistance of RCO.1 cells was also due to a mutation in the Topoisomerase 1 gene (Urasaki et al.,

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2001) and to a defect in apoptosis pathways (Reinhold et al, 2003; Chatterjee et al., 1996), suggesting that resistance to camptothecin is multifactorial. Previous attempts to elucidate mechanisms leading to campthotecin resistance included microarray analyses of parental DU145 cells and RCO.1 cells (Reinhold et al., 2003), using a UniGem microarray. Despite the high expression of ABCC2/MRP2 in the resistant cells (Fig. 1C), it's overexpression wasn't detected in these earlier studies. Taken together, these findings suggest that the specific targeting of ABC transporters in dedicated, custom-made arrays may improve the specificity and sensitivity of earlier generation microarrays. Typical shortcomings of microarray platforms may be attributed to imperfect clone annotation as well as the lack of specificity of probes targeted at overlapping or homologous sequences of closely related proteins, such as ABCB1 and ABCB2. These considerations prompted us to create our own, dedicated microarray platform. In order to ensure specificity, we designed probes uniquely matching the target transporters. The ABC-ToxChip analysis of cells selected in colchicine and 9NC demonstrate the elevated expression of ABCB1 and ABCC2, respectively. Since gene expression levels obtained by even the most carefully designed microarrays must be validated by independent methods, we also designed specific primers for the human ABC transporters (Szakács et al, 2004). The quantitative RT-PCR data confirmed the pattern of ABC transporter expression and suggested that there are no further ABC transporters differentially expressed in the cells analyzed in this study. Through the systematic analysis of ABC transporters and other genes of detoxification, our data provide novel information about the effect of 9NC selection on gene expression.

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While the GoMiner algorithm did not identify major biological processes linked to 9NC resistance, the list of differentially expressed genes may provide some insight into mechanisms underlying (or accompanying) resistance. An interesting example is Radixin, which is overexpressed in the 9NC selected cell line. Radixin belongs to the ERM (Ezrin-Radixin-Myosin) protein complex, and is involved in localization of integral membrane proteins. Radixin -/- mice have Dubin-Johnson-like symptoms (Kikuchi et al, 2002), because MRP2 is not properly localized to the plasma membrane.

The coordinated expression of phase II (conjugating) and phase III (efflux) systems has been shown to improve cellular detoxification (Morrow et al., 2000). We found significant changes in the expression of two glutathione metabolism-related genes. GST-Pi is involved in coupling electrophilic drugs to reduced glutathione, and GCS is the rate-limiting enzyme for glutathione synthesis. Neither GST-Pi mRNA nor the protein is detectable in the resistant cell line. This observation is striking given the association of high GST-Pi levels found in several resistant tumors and cell lines (Liu et al., 2001; Tew, 1994). In contrast, gamma glutamate cysteine synthetase (GCS) is overexpressed in the camptothecin resistant cells, as shown both by oligo and cDNA probes (see Fig. 1C). The changes in GST-Pi, GCS and ABCC2 expression suggest a putative “metabolic switch” necessary for resistance. As shown in Fig. 2B, these enzymes play a role in the pathway of glutathione-mediated detoxification. Consistently, two further genes belonging to the “Cysteine metabolism” ontology group (Table S4) show differential expression (cystathioneine gamma-lyase and glutamate cyteine-ligase, Table S6).

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Camptothecin resistance has been associated with the elevated expression of ABCG2-MXR (Brangi et al., 1999), which was observed in a sub-line of the human ovarian cancer cell line, A2780, selected against an analog of camptothecin: DX-8951f (van Hattum et al., 2002). Our results do not show elevated ABCG2 expression in RCO.1 cells, and suggest that ABCC2 may be involved in part of the resistant phenotype. Consistent with our findings, previous studies have reported MRP2 as a detoxifying transporter for camptothecin in animal models (Arimori et al., 2003) and cell lines transfected with antisense MRP2 (Koike et al., 1997). Since resistance of RCO.1 against 9NC was shown to be partially mediated by mutations in topoisomerase I, we speculate that the elevated expression of ABCC2 may play a role during the initial steps of the selection process. This early adaptation may provide the background for the evolution of further resistance mechanisms, such as mutation of topoisomerase 1 or loss of the expression of other topoisomerase(s) 1 genes (Urasaki et al., 2001).

Retroviral transfer of ABCC1 (MRP1) has been shown to result in decreased intracellular glutathione levels and increased sensitivity to BSO (Rappa et al., 2003). Furthermore, *in vitro* reversal of MRP1-mediated resistance and *in vivo* potentiation of the cytotoxicity of doxorubicin in MRP1-overexpressing tumors by BSO were previously reported (Rappa et al., 2003). In an analogous fashion, consistent with the capacity of ABCC2 to export glutathione conjugates (Konig et al., 1999), RCO.1 cells also proved hypersensitive to BSO treatment (data not shown). It is conceivable that the coordinated expression of MRP2 and GCS occur as the cells adapt to the cytotoxic stress. Since the maintenance of glutathione levels is critical for the survival of the

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cells, overexpression of GCS may represent an adaptive response compensating for the loss of intracellular glutathione in the RCO.1 cells.

In conclusion, we have used a novel ABC-ToxChip to show the overexpression of two ABC transporters in two different cellular models of drug resistance. In both cases, ABC transporter overexpression occurs at the level of transcription and is prominent when compared to the pattern of expression of 18,000 other genes. Our results reinforce our interest in following the expression of ABC transporters as specific markers of acquired drug resistance. The ABC-ToxChip should be a helpful tool to assess the role of ABC transporters, and how their expression is linked to other detoxification genes in various pathophysiological processes, such as drug resistance, not only in tissue culture models, but in clinical samples as well.

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TABLE 1

ABC probes printed on the ABC-ToxChip

Probe[a]	ABC transporter[b]	Gene bank identifier[c]	Matching exon
OLIGO	ABCA1	NM-005502.1	
CDNA	ABCA1	XM-050009.5	
OLIGO	ABCA2	AF178941.1	
OLIGO	ABCA3	NM-001089.1	
cDNA	ABCA3	XM-028843.2	
OLIGO	ABCA4	NM-000350.1	
cDNA	ABCA4	Y15644.1	exon 10
cDNA	ABCA4	Y15676.1	exon 42
cDNA	ABCA5	NM-018672.1	
cDNA	ABCA5	XM-057257.3	
OLIGO	ABCA8	NM-007168.1	
cDNA	ABCA12	NM-015657.1	
OLIGO	ABCB1	NM-000927.2	
cDNA	ABCB1	M29426.1	exon 4
cDNA	ABCB1	M37723.1	exon 6
cDNA	ABCB1	XM-029059.2	
cDNA	ABCB1	M29447.1	exon 28
OLIGO	ABCB2	NM-000593.2	
cDNA	ABCB2	U07198.1	exon 4,
cDNA	ABCB2	S70260.1	
cDNA	ABCB2	NM-000593.3	
OLIGO	ABCB3	NM-000544.2	
cDNA	ABCB3	XM-165824.1	
OLIGO	ABCB4	NM-018850.1	
cDNA	ABCB4	XM-167466.1	
cDNA	ABCB5	XM-166496.1	
OLIGO	ABCB6	NM-005689.1	
cDNA	ABCB6	BC000559.1	
cDNA	ABCB6	XM-050891.4	
OLIGO	ABCB7	NM-004299.2	
cDNA	ABCB7	XM-032877.2	
OLIGO	ABCB8	XM-032165.2	
cDNA	ABCB8	XM-032165.5	
OLIGO	ABCB10	NM-012089.1	
cDNA	ABCB10	XM-001871.4	
OLIGO	ABCB11	NM-003742.1	
OLIGO	ABCC1	NM-019902.1	
cDNA	ABCC1	AF022824.1	exon 2
cDNA	ABCC1	AF022826.1	exon 4
cDNA	ABCC1	AF022830.1	exon 8
cDNA	ABCC1	AF022853.1	exon 31
cDNA	ABCC1	AJ003198.1	
cDNA	ABCC1	NM-019902.1	
cDNA	ABCC1	AF022830.1	exon 8
OLIGO	ABCC2	XM-050760.1	
cDNA	ABCC2	AJ132306.1	exon 23
cDNA	ABCC2	AJ245627.1	exon 26
cDNA	ABCC2	U63970.1	
OLIGO	ABCC3	NM-003786.2	
cDNA	ABCC3	AJ294547.1	exon 3
cDNA	ABCC3	AJ294559.1	exons 20-23
cDNA	ABCC3	AJ294558.1	exon 19
cDNA	ABCC3	NM-020038.1	
OLIGO	ABCC4	NM-005845.1	
cDNA	ABCC4	XM-036453.1	
cDNA	ABCC4	AF071203.1	
cDNA	ABCC4	U66686.1	
cDNA	ABCC4	AF071202.1	
OLIGO	ABCC5	NM-005688.1	
cDNA	ABCC5	BC007229.1	
cDNA	ABCC5	AF146074.1	
cDNA	ABCC5	AB005659.1	
cDNA	ABCC5	XM-002914.6	
OLIGO	ABCC6	NM-001171.2	
cDNA	ABCC6	AF076622.1	
cDNA	ABCC6	AF168791.1	
cDNA	ABCC6	NM-001171.2	
OLIGO	ABCC7	NM-000492.2	
cDNA	ABCC7	M55109.1	exon 4
cDNA	ABCC7	M55032.1	exon 19
cDNA	ABCC7	XM-004980.4	
OLIGO	ABCC8	NM-000352.2	
OLIGO	ABCC9	NM-005691.1	
cDNA	ABCC11	NM-032583.2	
cDNA	ABCC12	NM-033226.1	
OLIGO	ABCD1	NM-000033.2	
cDNA	ABCD1	BC025358.1	
OLIGO	ABCD2	NM-005164.1	
OLIGO	ABCD3	NM-002858.2	
cDNA	ABCD3	BC009712.1	
OLIGO	ABCD4	NM-005050.1	
cDNA	ABCD4	NM-020326.1	
OLIGO	ABCE1	NM-002940.1	
cDNA	ABCE1	XM-003555.9	
OLIGO	ABCF1	NM-001090.1	
cDNA	ABCF1	BC016772.1	
OLIGO	ABCF2	XM-039075.1	
cDNA	ABCF2	BC001661.1	
cDNA	ABCF3	NM-018358.1	
OLIGO	ABCG1	NM-004915.2	
cDNA	ABCG1	XM-032950.3	
OLIGO	ABCG2	NM-004827.1	
cDNA	ABCG2	XM-032424.1	
cDNA	ABCG2	AF098951.2	
cDNA	ABCG4 lo	XM-012099.8	
cDNA	ABCG8	NM-022437.1	

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[a]Oligo indicates that the probe is a single-stranded, 70 mer oligonucleotide; cDNA indicates the probe is a fragment of double-stranded DNA amplified by PCR.

[b]ABC transporters are named using the HGNC nomenclature (http://nutrigene.4t.com/humanabc.htm).

[c] The probes are named after the closest matching RefSeq entries (National Center for Biotechnology Information (NCBI), e.g., C3 (NM-003786, AJ294547.1, AJ294559.1, AJ294558.1). The exon most closely matching the sequence is indicated.

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TABLE 2

Differentially expressed genes identified by two independent microarray analyses (ATC-DU-145 versus RCO.1)

Clone ID	HCNG reference	Gene description	Ratio R/S[a] Reinhold et al., 2003	Ratio R/S[a] Present study[b]
266146	CYP24A1	Cytochrome P450 XXIV	12.66	NA (1.91)
429091	H2A1	Histone 2A like protein (H2A-l)	6.93	NA (2.70)
376370		EGR-Early Growth response protein	5.22	1.94(NA)
209156	DAD1	Defender against cell death	2.29	NA (1.91)
774710	GSTP1	Glutathione-S-tranferase Pi	0.12	0.12 (0.03)
810859	NK4	Natural killer cells protein-4	0.13	NA (0.25)
328692	IL8	Interleukin 8	0.13	NA (0.26)
840683	CIN	Cytokine inducible nuclear protein	0.23	NA (0.45)

[a] Relative expression, resistant versus sensitive (R/S)

[b] Relative expression based on oligo and cDNA probes. Results based on cDNA probes are shown in parentheses. NA, not applicable.

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Figure Legends

Fig. 1. Creation and application of the ABC-ToxChip. A, Details of a scan of the ABC-ToxChip . The section shown is one of the 32 blocks printed. The platform has 4 x 8 such blocks, each containing 625 (25 x 25) slots, yielding in total 20,000 probe-slots available for printing. The upper part of the block contains the oligo-set, and the lower part bears the cDNA probes matching the detoxification set. The probes specifically matching the ABC transporters are printed in the lowest row. B**,** Quantitative RT-PCR to follow transcriptional changes of ABC transporters and GST-Pi. Crossing point values of amplification for 47 ABC transporters and GST-Pi in the two samples are shown in a scatter plot. Deviations greater than 2 cycles are considered significant. Results demonstrate activation of MDR1 and downregulation of GST-Pi with colchicine resistance. C, Significant outliers (>2.7 fold) identified by the microarray experiments. ABC transporters appear as major outliers in both analyses, suggesting their strong contribution to the MDR phenotype. Genes where the variation was confirmed by more than one probe are indicated in grey. Arrows point out the most drastic changes discussed in this paper (i.e. GST and ABC transporters). OL and cD refer to oligo and cDNA probes, respectively. n defines the number of experiments (out of 4 replicates) where gene expression is found significantly altered. C.R. (calibrated ratio) indicates the fold of difference of expression between the resistant cell line versus the parental cell line.

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Fig. 2. Overexpression of ABCC2 and downregulation of GST-Pi with 9NC selection. A, Confirmation of the overexpression of ABCC2, and down-regulation of GST-Pi with 9NC selection by quantitative RT-PCR. Crossing point values of amplification for 47 ABC transporters and GST-Pi in the two samples are shown in a scatter plot. B, Illustration of the role of ABCC2, GST and GCS in the glutathione-related detoxification pathway. The concentration of glutathione is regulated by its metabolism (GSH–GSX, GSH-GSSG) and biosynthesis, catalyzed by GST and GCS, respectively, and the activity of ABCC2. C, Correlation between the mRNA and protein levels. The symbols S and R correspond to the sensitive (ATC-DU-145) and resistant cell line (RCO.1), respectively. The signal for GST-Pi in the resistant cell line was below the level of detection.

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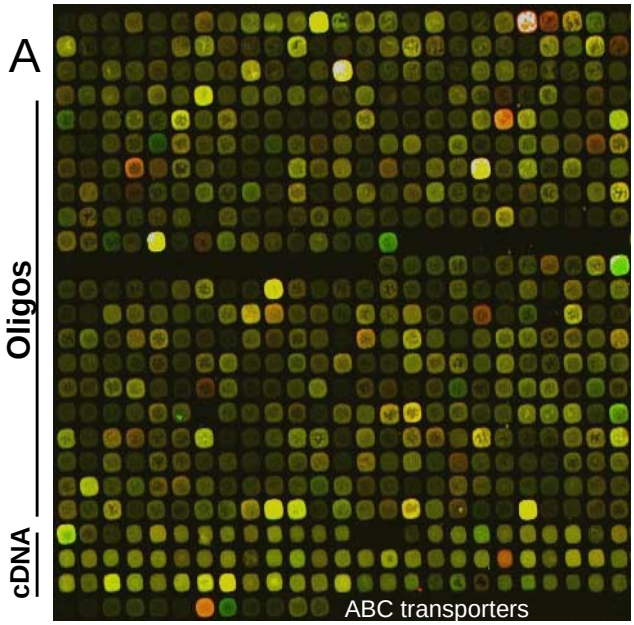

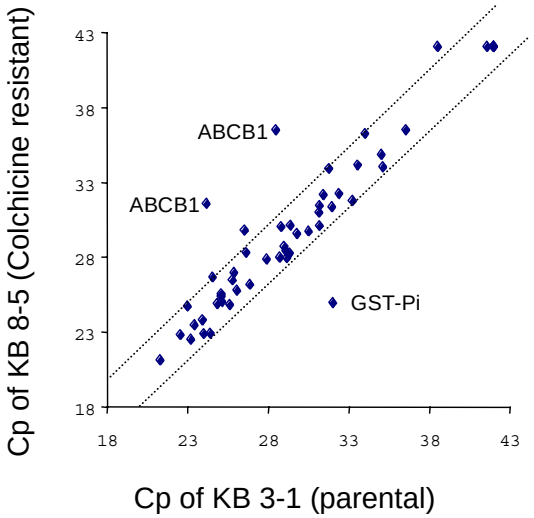

C

KB-3-1 versus KB-8-5

	Gene ID	n	Description of up-regulated genes	C.R.
OL	M29447	3	Human P-glycoprotein [MDR1] gene, exon 28	14.8
OL	S70585	4	Glycoprotein hormone, alpha polypeptide	13.5
cD	W86681	4	Glycoprotein hormone, alpha polypeptide	13
OL	M37723	3	Human MDR1/P-glycoprotein gene, exon 6	11.3
OL	M29426	3	Human P-glycoprotein [MDR1] gene, exon 4	9.39
cD	W45324	4	Interleukin 8	7.19
OL	X15422	3	Mannose-binding lectin 2	4.5
OL	X58536	4	Major histocompatibility complex, class I, C	3.86
OL	AF071596	4	Immediate early response 3	3.83
OL	M80469	4	MHC class I HLA-J gene, exons 1-8	3.74
OL	M12758	4	MHC class I HLA-A24 gene, exons 4,6-8	3.19
cD	R80217	4	Cyclo oxygenase 2	2.99
OL	M13755	4	Interferon stimulated protein	2.98

	Gene ID	n	Description of down-regulated genes	C.R.
OL	M38591	3	S100 calcium-binding protein A10	0.37
cD	AA040702	4	Phosphoglyceratemutase 1 [brain]	0.36
OL	AF225985	3	Sodium channel, voltage-gated, type I	0.36
cD	R33755	4	Glutathione-S-transferase-Pi	0.35
OL	AB011542	3	EGF-like-domain, multiple 5	0.35
OL	U12472	3	Glutathione-S-transferase-Pi	0.06

ATC-DU-145 versus RCO.1

	Gene ID	n	Description of up-regulated genes	C.R.
OL	U70312	3	EGF-like repeats and discoidinI-like domains 3	12.50
OL	AJ132306	4	HSA132306 MRP2 gene, exon 23""	12.32
cD	376184	4	Insulin-like growth factor binding protein 3	6.88
OL	M38591	4	S100 calcium-binding protein A10	5.79
cD	308366	3	Folate receptor 1	5.13
OL	J02763	4	S100 calcium-binding protein A6	4.65
OL	X60484	3	H4 histone family, member E	4.37
OL	U05598	4	Aldo-keto reductase family 1, member C2	4.27
OL	AB032261	4	Stearoyl-CoAdesaturase	4.07
OL	U28369	3	Sema domain, immunoglobulin domain 3B	3.90
OL	M30704	4	Amphiregulin	3.35
cD	203721	4	Glutamate cysteine ligase , catalytic	3.01
OL	M76231	3	Sepiapterin reductase	2.96
OL	X05908	4	Annexin A 1	2.82

	Gene ID	n	Description of down-regulated genes NCBI	C.R.
OL	Y11307	3	Cysteine-rich, angiogenic inducer, 61	0.36
OL	X04741	3	Ubiquitin carboxyl-terminal esterase L1	0.35
cD	110503	4	Fos Related Antigen 1	0.35
cD	484963	4	Metallothionein from cadmium treated cells	0.33
OL	M24283	3	Intercellular adhesion, human rhinovirus receptor	0.32
OL	M90657	4	Transmembrane 4 super family member 1	0.32
OL	S52784	3	Cystathionase	0.31
cD	328692	4	Interleukin 8	0.26
OL	AJ270993	4	Homeobox B6	0.25
OL	M59807	3	Natural killer cell transcript 4	0.25
OL	X16172	4	Human HOX-2.5 gene for homeodomain protein	0.17
cD	136235	4	Glutathione-S-transferase-Pi	0.12
OL	U12472	4	Glutathione-S-transferase-Pi	0.03

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