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Microarray-based Prediction of Cytotoxicity of Tumor Cells to Arsenic Trioxide

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Abstract. *Arsenic has been used since ancient times as a medicinal agent. Currently, arsenic trioxide is experiencing a thriving revival in modern oncology. The aim of this study was to identify the molecular predictors of sensitivity and resistance to arsenic trioxide. We mined the microarray database of the National Cancer Institute (NCI), USA, for genes whose expression correlated with the IC_{50} values for arsenic trioxide of 60 cell lines of different tumor types. By COMPARE analysis, Kendall's τ test, and false discovery rate (FDR) analyses, 47 out of 9706 genes or expressed sequence tags (ESTs) were identified. If the mRNA expression of the 47 genes or ESTs was subjected to hierarchical cluster analysis and cluster image mapping, sensitivity or resistance of the 60 cell lines to arsenic trioxide was predictable with statistical significance ($p=1.01 \times 10^{-5}$). While the proteins encoded by the 47 genes identified differ in their specific functions (signal transducers, transcription factors, proteasome degradation proteins, proliferation-related proteins, regulators of oxidative stress etc.), it is intriguing that many of them are in one way or another involved in the apoptotic machinery, indicating that apoptosis is an important mechanism of arsenic trioxide's cytotoxicity.*

Arsenic is a natural semimetal in soil, water and air. It exists as red arsenic (As_2S_2), yellow arsenic (As_2S_3), white arsenic (As_2O_3, arsenic trioxide), phenylarsine oxide (C_6H_5AsO), and as salts of sodium, potassium and calcium (1). Since ancient times arsenic has been used as a medicinal agent, *i.e.*, in Greek, Roman, and Chinese medicine (2). Arsenic was appreciated as Fowler's Solution for many diseases in the 18th and 19th century, *i.e.*, syphilis, cancer, ulcers, *etc.* (3). In the 20th century, Paul Ehrlich, the founder of modern chemotherapy, found the arsenical salvarsan, which was the standard therapy against syphilis for decades (4). On the other hand, arsenic compounds can be poisonous (5). The revival of arsenic in modern medicine was initiated by Chinese scientists showing dramatic regression rates of acute promyelocytic leukemia by arsenic trioxide (6). These findings were subsequently corroborated in clinical studies in the U.S.A. (7).

Various molecular determinants of the biological effect of arsenic trioxide have been elucidated. It promotes the degradation of the oncogenic fusion protein of the PML and retinoic acid receptor α (RARα) genes which arises from t(15;17) translocation in acute promyelocytic leukemia, resulting in induction of cellular differentiation (7, 8). Apoptosis is selectively induced in malignant cells through enhancement of reactive oxygen species and activation of caspases (9-12). Cells can arrest in the G1- or G2/M- phases of the cell cycle after treatment with arsenic trioxide (12). Tumor angiogenesis is targeted by arsenic trioxide through inhibition of vascular epithelial growth factor production (13). At low concentrations, arsenicals interfere with nucleotide and base excision repair and suppress poly (ADP-ribosyl)ation (14).

As cellular responses of tumor cells to arsenic trioxide seem to be multi-factorial, we aimed to get a comprehensive insight into molecular predictors of sensitivity or resistance to arsenic trioxide. For this reason, we mined the microarray database of the National Cancer Institute (NCI) of the USA (http://dtp.nci.nih.gov). Out of 9706 genes, 47 genes, whose mRNA expression in 60 tumor cell lines correlated with highest correlation coefficients to inhibition concentration 50% (IC_{50}) values, were selected by COMPARE analysis and false discovery rate calculation. These genes were subjected to hierarchical cluster analysis and cluster image mapping to reveal whether the expression profiles of these genes are useful to predict sensitivity or resistance of cell lines to arsenic trioxide.

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Materials and Methods

Cell lines. The panel of 60 human tumor cell lines of the Developmental Therapeutics Program of the NCI consisted of leukemia, melanoma, non-small cell lung cancer, colon cancer, renal cancer, ovarian cancer cells, cells of tumors of the central nervous system, and breast cancer cells. Their origin and processing have been previously described (15).

Sulforhodamine B assay. This assay has been reported in detail (16). The inhibition concentration 50% (IC_{50}) values of the 60 NCI tumor cell lines for arsenic trioxide have been deposited in the database of the Developmental Therapeutics Program of the NCI (http://dtp.nci.nih.gov).

Statistical analyses. The mRNA expression values of genes of interest in 60 cell lines were selected from the database of the NCI, Bethesda, MA, U.S.A. (http://dtp.nci.nih.gov). The mRNA expression has been determined by microarray analyses as reported (17). This database has been searched for genes identified by COMPARE analysis. COMPARE analyses were performed to produce rank-ordered lists of genes expressed in the 60 cell lines of the NCI. Every gene in the database is ranked for similarity of its mRNA expression to the IC_{50} values for arsenic trioxide. To derive COMPARE rankings, a scale index of correlations coefficients (R-values) is created. In the standard COMPARE approach, greater mRNA expression in cell lines correlates with enhanced drug sensitivity, whereas in reverse COMPARE analyses greater mRNA expression in cell lines indicates drug resistance. The methodology has been described (18).

Kendall's τ test was applied to calculate significance values (*p*-values) and rank correlation coefficients (R-values) as a relative measure for the linear dependency of two variables. χ^2 test was used as implement of the WinSTAT program (Kalmia) to prove bivariate frequency distributions for pairs of nominal scaled variables for dependencies.

In addition to the calculation of *p*- and R-values, the problem of multiple hypothesis testing was addressed. Significance tests might be prone to type I errors (false conclusions of significance) (19). Therefore, a step-up re-sampling multi-comparison procedure was applied to control the false discovery rate (FDR) among the significant correlations at a given significance level. This program was developed by Reiner *et al.* (20) and is available on http://www.math.tau.ac.il. The FDR is the expected proportion α of erroneous rejections among all rejections of the null-hypothesis.

Objects were classified by calculation of distances according to the closeness of between-individual distances by means of hierarchical cluster analysis. All objects were assembled into a cluster tree (dendrogram). Cluster analyses applying the complete-linkage method were done with the WinSTAT program (Kalmia) as described earlier by us (21).

Results

We performed COMPARE analyses of the IC_{50} values for arsenic trioxide and the mRNA expression of 9706 genes in the 60 NCI cell lines to produce scale indices of correlation coefficients. First, a standard COMPARE analysis was performed. Cell lines that were most inhibited by arsenic trioxide (lowest IC_{50} values) were correlated with the highest mRNA expression levels of genes. Then, a reverse COMPARE analysis was done which correlated the most inhibited cell lines with the lowest gene expression level. We used each 25 genes or expressed sequence tags (ESTs) with the highest correlation coefficients of both COMPARE analyses for subsequent FDR calculation. Adjusting the significance level to 0.01 revealed an α-value of 0.00522 which means that 47 of the 50 genes or ESTs with $p<0.00522$ have a probability of 1% to correlate erroneously to cellular response to arsenic trioxide. These 47 genes or ESTs are listed in Table I. While the the proteins encoded by the 47 genes differ in their specific functions (signal transducers, transcription factors, proteasome degradation proteins, proliferation-related proteins, regulators of oxidative stress *etc.*), it is intriguing that many of them are, in one way or another, involved in the apoptotic machinery, indicating that apoptosis is an important mechanism of arsenic trioxide's cytotoxicity.

The mRNA expression of these genes were subjected to hierarchical cluster analysis and cluster image mapping. The resulting dendrogram with the cell lines analyzed on the right side of Figure 1 can be divided into three major branches. The dendrogram on the top of Figure 1 shows the genes investigated. By generation of a cluster image map from both dendrograms, areas with different mRNA expression levels became apparent (Figure 1). The distribution of sensitive or resistant cell lines in the right-sided dendrogram was significantly different ($p=1.01 \times 10^{-5}$; χ^2 test) indicating that cellular response to arsenic trioxide is predictable by these genes (Table II).

Discussion

While the specific functions of the proteins encoded by the 47 genes identified by our approach are different, it is intriguing that many of them are, in one way or another, involved in apoptosis (Table I). This may be taken as a clue that arsenic trioxide impairs the apoptotic machinery and that apoptosis is an important mechanism of arsenic trioxide's cytotoxicity. Some of the genes identified in the present investigation and genes closely related to them have previouly been associated with the action of arsenicals.

We found that the mRNA expression of chemokine-like super family member 4 and growth factor receptor-bound protein 7 correlated with the IC_{50} values for arsenic trioxide. Interestingly, using a microarray approach, Wu *et al.* (22) found that several cytokines and growth factors were differentially expressed in arsenic-exposed healthy individuals.

The identification of keratin 8 as a predictor of cellular response to arsenic trioxide corresponds to recent findings

Table I. *Genes identified by standard COMPARE, reverse COMPARE, and FDR analyses whose mRNA expression in 60 NCI cell lines correlated with IC_{50} values for arsenic trioxide.*

Code	Genbank	Name	Function
Standard COMPARE:			
CKLFSF4	W46185	chemokine-like factor super family 4 RNA	General: transcription factor
KRT8	AA052978	keratin 8	General: intermediary filament Apoptosis: phosphorylated upon stimulation of the Fas receptor or UV light (33)
FLJ43380 fis	W74078	*H. sapiens* cDNA FLJ43380 fis, clone OCBBF2004889	General: unknown
NXN	AA047537	nucleoredoxin	General: oxidative stress regulator Apoptosis: regulates NF-kB, AP-1, and CREB activation (34)
KIAA0556	H39852	KIAA0556 protein	General: unknown
TXNRD1	AA055408	thioredoxin reductase 1	General: oxidative stress regulator Apoptosis: inhibited by various anticancer compounds; thioredoxin-regulated apoptosis is partly regulated by p53 and thioredoxin regulates apoptosis-signal-regulating kinase 1 (27, 28)
MRPL37	AA0556668	mitochondrial ribosomal protein L37	General: translational control in protein turnover Apoptosis: L37 shows elevated expression in response to mutant p53 (35); other ribosomal proteins inhibit apoptosis and mediate drug resistance (36, 37)
ALDH3A2	AA004841	aldehyde dehydrogenase 3 family, member 2A	General: converts aldehydes to the carboxylic acids in a NAD(P)(+)-dependent reaction Apoptosis: other ALDHs are induced by p53 and protect against cell damage and mediate drug resistance (38, 39)
DDEF2	N70773	development and differentation enhancing factor 2	General: activates the small GTPases ARF1, ARF5 and ARF6; regulates the formation of post-Golgi vesicles and modulates constitutive secretion.
COX11	W33157	COX11 homologue, cytochrome c oxidase assembly protein (yeast)	General: cytochrome c oxidase synthesis Apoptosis: unknown for COX11. COX-2 promotes cell growth, inhibits apoptosis and enhances cell motility and adhesion (40)
FRING	N62701	fring protein	General: unknown
MGC17528	AA053259	S100 calcium binding protein 100 A16	General: apoptosis regulator?
ANXA3	W45327	annexin A3	General: inhibits phospholipase A2 and anti- coagulant molecule
unknown	T59431	unknown	General: unknown
GCA	R79972	grancalcin, EF-hand calcium-binding protein	General: role in granule-membrane fusion and degranulation Apoptosis: belongs to the EF-hand protein family whose members regulate cell migration, apoptosis, and mobilization of neutrophil effector functions
DPTP6	AA025336	DNA-polymerase transactivated protein 6	General: DNA synthesis
LOC115548	R27977	hypothetical protein014311	General: unknown
SH3BP2	T40608	SH-domain binding protein 2	General: signal transduction, candidate tumor suppressor gene (41)
PIGPC1	N95055	PERP, p53 apoptosis effector	Apoptosis: mediates p53-dependent apoptosis (42)
TJP1	R79560	tight junction protein 1 (zona occludens 1)	General: stabilization of and signal transduction for tight junction assembly
TCF7L2	AA029451	transcription factor 7-like 2 (T cell specific, HMG box)	General: lymphoid transcription factor; participates in the Wnt signaling pathway; master switch that controls proliferation *versus* differentiation by modulation of MYC expression Apoptosis: apoptosis-inducing downstream effector of p53 (43, 44)
SDC1	R00830	syndecan 1	General: receptor for the extracellular matrix signaling the Wnt pathway (45) Apoptosis: induces apoptosis and inhibits cell growth (46)
GRB7	AA053218	growth factor receptor-bound protein 7	General: signal transduction adaptor molecule; involved in cell invasion and metastasis
SMC2L1	H94288	structural maintenance of chromosomes	General: regulates conversion of interphase chromatin

continued on the next page

Table I *continued.*

Code	Genbank	Name	Function
		2-like 1 (yeast)	into mitotic-like condense chromosomes; coordinates of DNA double strand break repair
ACAA2	AA004908	myosin VB	General: myosins interact with actin filaments and hydrolyze ATP to generate mechanical force
Reverse COMPARE:			
C6orf134	H50128	chromosome 6 open reading frame 134	General: unknown
LOC91966	AA039366	hypothetical protein 91966	General: unknown
MGC23937	N35784	hypothetical protein MGC23937 similar to GC4798	General: unknown
ZNF151	AA057307	zinc finger protein 151 (pHZ-67)	General: mediates growth arrest and gene repression by Myc (47) Apoptosis: switches the response to apoptosis after p53 activation (48)
LOC91966	T71548	hypothetical protein 91966	General: unknown
FLJ12586	N32849	hypothetical protein FLJ12586	General: unknown
STMN1	AA055820	stathmin 1	General: tubulin sequestration; signal transduction in proliferation and differentiation (49) Apoptosis: depolymerization of interphase and mitotic microtubules and potentiation of paclitaxel-induced apoptosis (50, 51)
MYL3	N92340	myosin, light polypeptide 3, alkali; ventricular, skeletal, slow	General: myosin light chains stabilize the long α-helical neck of the myosin head Apoptosis: target of caspase-3 (52)
C7orf26	AA047597	chromosome7 open reading frame 26	General: unknown
MEP50	AA034488	MEP50 protein	General: assembly of U snRNP particles as components of the pre-mRNA splicing machinery
NFKB1	T86845	nuclear factor of κ light polypeptide gene enhancer in B-cells (p105)	General: transcription factor that influences tumorigenesis, metastasis, and inflammation Apoptosis: p53 activates NFκB through the RAF/MEK1/p90(rsk) pathway; mediates resistance to standard cancer chemotherapy (53)
FLJ10315	R48272	hypothetical protein FLJ10315	General: unknown
AP4B1	AA002112	adaptor-related protein complex 4, beta 1 subunit	General: mediates neurotransmission
UBE1	AA054312	ubiquitin-activating enzyme E1 (A1S9T and BN75 temperature-sensitive complementing)	General: ubiquitinates proteins for targeted degradation by the 26S proteasome Apoptosis: UBE1 defects induce caspase-independent apoptosis (54)
ARHGEF7	AA042973	rho guanine nucleotide exchanger factor (GEF) 7	General: regulates cytoskeletal architecture, cell growth, metastasis, and membrane ruffling Apoptosis: regulates the apoptotic response to stress-inducing agents (55)
TFDP2	W46792	transcription factor Dp-2 (E2F dimerization partner 2)	General: binds DNA cooperatively with E2F transcription factors to regulate DNA synthesis, cell cycle and apoptosis.
CHL1	R49177	cell adhesion molecule with homology to L1CAM (close homologue of L1)	General: potential neuronal survival factor for neurons of the central nervous system (56)
SF3A1	H45050	splicing factor 3a, subunit 1 (120 kDa)	General: belongs to the spliceosome that splices introns from nuclear pre-mRNA
CDW52	N90866	CDW52 antigen (CAMPATH-1 antigen)	General: glycopeptide that carries carbohydrates on the surface of T and B lymphocytes
TCF19	N80679	transcription factor 19 (SC1)	General: potential trans-activating factor for genes of later stages of cell cycle progression
HSPC182	N66683	HSPC182 protein	General: unknown
FLJ10330	N32972	hypothetical protein FLJ10330	General: unknown

Information on gene functions was taken from the OMIM database, National Cancer Institute, U.S.A. (http://www.ncbi.nlm.nih.gov/Omim/), from the GeneCard database of the Weizman Institute of Science, Rehovot, Israel (http://bioinfo.weizmann.ac.il/cards/index.html), and from the references as indicated.

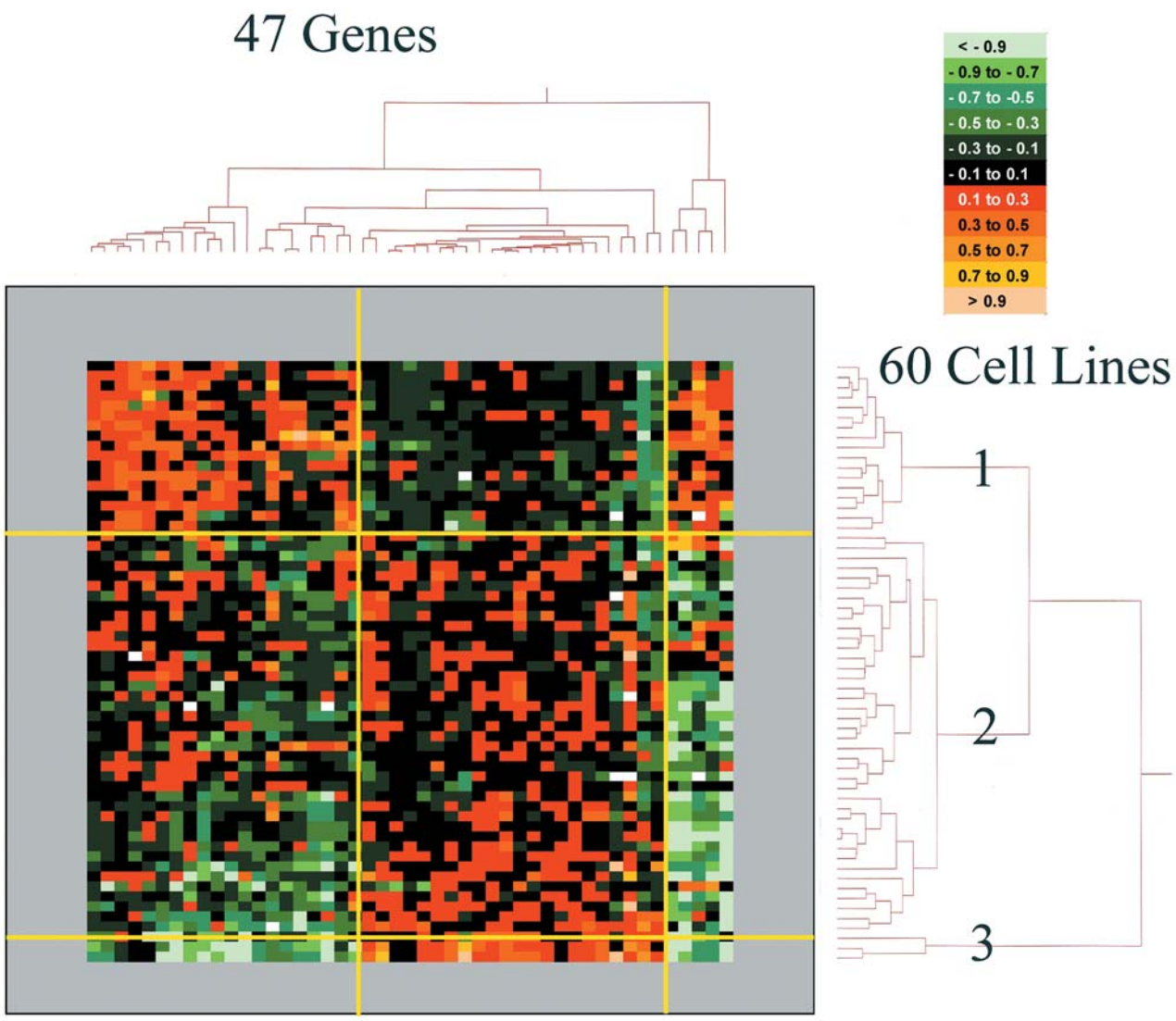

Figure 1. *Dendrograms and cluster image map obtained by hierarchical cluster analysis (complete linkage method) of mRNA expression of 47 genes in 60 NCI cell lines. The genes are listed in Table I. The dendrogram on the right side shows the clustering of cell lines and the dendrogram on the top shows clustering of genes. The cluster image map corresponds to each mRNA expression value obtained by microarray analysis. The expression values have been normalized and color-coded as indicated. For normalization, reference probes were made by pooling equal amounts of mRNA from logarithmically growing HL-60, K562, NCI-H226, COLO205, SNB-19, LOX-IMVI, OVCAR-3, OVCAR-4, CAKI-1, PC-3, MCF7 and Hs578T cell lines. Test and reference probes were combined, denatured and hybridized overnight to Synteni microarrays. Arrays were scanned using a laser-scanning microscope. The ScanAlyze program was used to analyze the microarray images.*

of Ramirez *et al.* (23). These authors observed that arsenite causes DNA-protein crosslinks with cytokeratins due to their high content of sulfhydryl groups. Microarray analyses pointed to various keratins (K8, K15, K18, K19) after treatment of cells with arsenic (24, 25).

Another gene found by our approach, thioredoxin reductase I, is inhibited by arsenicals (26). Thioredoxin is a regulator of reactive oxygen species that also affects apoptosis by apoptosis-signal-regulating kinase I in an at least partially p53-dependent manner (27, 28).

Our result that aldehyde dehydrogenase 3A2 might contribute to resistance to arsenic trioxide is in accord with the alteration of hepatic mitochondrial aldehyde dehydrogenase activity by sodium arsenate (29).

While a relationship of COX11 has not been documented previously, COX2 expression is known to be elevated by

Table II. *Separation of clusters of 60 NCI cell lines obtained by hierarchical cluster analysis shown in Figure 1 in comparison to sensitivity to arsenic trioxide.*

	Sensitive	Resistant
Cluster 1	0	17
Cluster 2	25	14
Cluster 3	3	0

$p = 1.01 \times 10^{-5}$ (χ^2 test)

The median $\log_{10}$ IC_{50} value (-5.468 M) was used as cut-off to separate tumor cell lines as being "sensitive" or "resistant".

arsenite through stimulation of NFκB activity (30). NFÎ B contributes to arsenic-induced apoptosis (31).

The correlation of mRNA expression of the UBE1 gene in the present investigation implies that the proteasome degradation pathway may play a role for resistance to arsenic trioxide. This view is supported by recent miroarray data with arsenic sulfide pointing to the closely related UBE1L gene (32).

In conclusion, the data presented here not only provide expression profiles of genes that predict cellular sensitivities and resistance to arsenic trioxide, but may also serve to generate testable hypotheses on molecular mechanisms of cytotoxicity in tumor cells.

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