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Blood gene expression markers to detect and distinguish target organ toxicity

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Abstract The purpose of this study was to investigate whether the expression of specific genes in peripheral blood can be used as surrogate marker(s) to detect and distinguish target organ toxicity induced by chemicals in rats. Rats were intraperitoneally administered a single, acute dose of a well-established hepatotoxic (acetaminophen) or a neurotoxic (methyl parathion) chemical. Administration of acetaminophen (AP) in the rats resulted in hepatotoxicity as evidenced from elevated blood transaminase activities. Similarly, administration of methyl parathion (MP) resulted in neurotoxicity in the rats as evidenced from the inhibition of acetyl cholinesterase activity in their blood. Administration of either chemical also resulted in mild hematotoxicity in the rats. Microarray analysis of the global gene expression profile of rat blood identified distinct gene expression markers capable of detecting and distinguishing hepatotoxicity and neurotoxicity induced by AP and MP, respectively. Differential expressions of the marker genes for hepatotoxicity and neurotoxicity were detectable in the blood earlier than the appearance of the commonly used clinical markers (serum transaminases and acetyl cholinesterase). The ability of the marker genes to detect hepatotoxicity and neurotoxicity was further confirmed using the blood samples of rats administered additional hepatotoxic (thioacetamide, dimethylnitrobenzene, and carbon tetrachloride) or neurotoxic (ethyl parathion and malathion) chemicals. In summary, our results demonstrated that blood gene expression markers can detect and distinguish target organ toxicity non-invasively.

Keywords Blood gene expression · Hepatotoxicity · Neurotoxicity · Toxicity markers

Christina Umbright and Rajendran Sellamuthu contributed equally to this work.

Data deposition The microarray data have been deposited in the Gene Expression Omnibus (GEO) Database, www.ncbi.nlm.nih.gov/geo (accession number GSE13442).

Disclaimer The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Introduction

Occupational and environmental exposure to chemicals frequently occurs, and various organs in the body have been identified as targets for toxicity resulting from such exposures. Several serum biomarkers expected to detect chemical-induced damage to these organs have been developed [1, 2]. Many such markers, however, have limitations including lack of adequate sensitivity and specificity, as well as inadequate potential to predict toxicity at an early stage.

Recent developments in toxicogenomics offer promise in developing toxicity markers based on differential gene expression profiles induced by chemicals. For example, gene expression profiling in target organs has been successfully employed to detect, classify, and to predict toxicity [3–7]. Furthermore, gene expression profiling can provide significant insight regarding the mechanism(s) underlying chemical toxicity [8–10]. To date, most of the toxicogenomics data obtained have been derived from experiments that employed target organs of experimental animals [5, 6, 8, 11]. This is a major drawback for their applicability to human exposure as many of the organs and tissues which are targets for chemical toxicity are inaccessible and, therefore, not readily available for examination.

Easily accessible biospecimens have been employed as surrogate tissues primarily to gain insight into the effects of toxicants on target tissues and organs [12]. Among the various surrogate tissues available, blood appears to be the most suitable for toxicogenomics studies to identify systemic toxic responses [13]. Recently, Bushel et al. [14] and Lobenhofer et al. [15] have reported that peripheral blood gene expression profiles can be employed to detect hepatotoxicity induced by classic hepatotoxic chemicals. At present, we have conducted rat experiments to investigate whether blood gene expression markers can be employed to detect as well as to distinguish target organ toxicity induced by chemicals. Microarray analysis of the global gene expression profile was initially determined using RNA isolated from leukocytes of rats administered either a model hepatotoxic chemical—acetaminophen (AP), or a neurotoxic chemical—methyl parathion (MP). Results from these studies suggested that blood gene expression can potentially be employed as a non-invasive, surrogate marker not only to detect but also to distinguish target organ toxicity induced by chemicals in the rat. A few more studies with known hepatotoxic (thioacetamide, carbon tetrachloride, and dimethylnitrosamine) and neurotoxic (ethyl parathion and malathion) chemicals helped in supporting and confirming these findings.

Materials and methods

Animals and treatments

Approximately 4-month-old, male Fisher 344 rats (Charles River Laboratories, Wilmington, MA) were housed in the animal facility at the National Institute for Occupational Safety and Health (NIOSH), Morgantown, WV. The animals were kept under controlled lighting (12-h light–dark cycle), temperature (72° ± 5° F), and humidity (50 ± 20%) with free access to food and tap water. All the animal experiments were conducted according to protocols approved by the Animal Care and Use Committee (ACUC), NIOSH, Morgantown, WV. The rats were randomly assigned to the various control and treatment groups and were administered intraperitoneally a single dose of AP (1200 mg/kg b.w., Sigma Chemical Company, St. Louis, MO) or MP (15 mg/kg b.w., Supelco, Bellefonte, PA) prepared as homogeneous suspensions in vegetable oil. Selection of doses employed for administration of the model chemicals in the rat was based on previous reports [14, 16], and the selected doses were expected to result in overt toxicity at later time intervals (12-h and beyond) and no detectable toxicity at the earliest time interval of 4-h. The control rats received an equivalent volume of vegetable oil used as the vehicle to prepare the chemicals. At time intervals of 4-, 12-, and 24-h, and 1 week after administration of the chemicals, four rats each from the control and treated groups were sacrificed using a head-only, focused microwave irradiation (power level of 3 kilowatts for 1.5 s) generated by a microwave applicator (Muromachi Kikai, Inc, Tokyo, Japan, Model TMW 4012C, 10 kilowatts). The microwave irradiation procedure was preferred over euthanasia after the administration of anesthetic agents simply to avoid any effect these agents may have on peripheral blood gene expression profile. Blood was collected immediately and directly from the heart and placed into Vacutainer tubes (Becton Dickinson and Company, Franklin Lakes, NJ) containing EDTA as the anticoagulant for use in hematology, clinical chemistry, and gene expression studies. The cardiac puncture procedure facilitated the rapid collection of at least 5 ml of unclotted blood per rat that was required to isolate sufficient quantities of high quality, globin mRNA-free, RNA from leukocytes required for microarray and real-time PCR analysis to determine gene expression profile.

Hematology and clinical chemistry

An aliquot of each of the unclotted blood samples collected was used to perform hematological analyses (platelets, white and red blood cell counts, differential white blood cell counts, and the determination of hematocrit and hemoglobin content). Leukocytes were separated from the blood samples for isolation of RNA (see details elsewhere in this article) and the remaining sample was centrifuged at 3000 RPM for 10 min to collect plasma. Activities of aspartate aminotransferase (AST, E.C.2.6.1.1), alanine aminotransferase (ALT, E.C.2.6.1.2), and acetyl cholinesterase (AChE, E.C.3.1.1.7) were determined spectrophotometrically in each of the plasma samples using commercially available diagnostic kits (TECO Diagnostics, Anaheim, CA).

RNA isolation and microarray analysis to identify global gene expression profiles

Total RNA, with significantly depleted globin mRNA, was isolated from the leukocytes collected from the unclotted blood samples using the LeukoLOCK™ Total RNA Isolation System (Ambion, Inc, Austin, TX). The RNA was quantitated by UV-spectrophotometry, and the integrity was determined using a Bioanalyzer (Agilent Technology, Palo Alto, CA).

The Rat Genome Survey Microarray (Applied Biosystems, Foster City, CA) containing 26,857 gene expression probes was used to determine global gene expression profiles in RNA samples isolated from the blood samples. The RNA samples were used to generate targets for hybridization to the microarray. All procedures of target labeling, array hybridization, washing, chemiluminiscence detection and image analysis were done at the Vanderbilt Microarray Shared Resources (Vanderbilt University, Nashville, TN) using Applied Biosystem's Chemiluminiscence Kit and AB1700 Chemiluminiscent Microarray Analyzer according to the procedures established by Applied Biosystems (Foster City, CA). In brief, one microgram total RNA was reverse transcribed to synthesize double-stranded cDNA using the NanoAmp RT-IVT Probe Labeling Kit (Applied Biosystems, Foster City, CA). The cDNA samples were purified and in vitro transcribed to generate DIG labeled cRNA. Subsequently, the cRNA samples were purified and quantitated using UV-spectrophotometry and assessed for quality on a Bioanalyzer (Agilent Technologies, Palo Alto, CA). The cRNA samples meeting the ABI criteria in terms of quantity and size of targets produced were fragmented and then hybridized to the rat genome microarray for 16 h, 100 RPM at 55°C. The hybridized arrays were washed and incubated with the Anti-Dig-AP antibody for 20 min. The arrays were washed and incubated with the Chemilumenescence Enhancing Solution (Applied Biosystems, Foster City, CA), washed, and incubated with the substrate for chemiluminescence reaction. The arrays were immediately imaged on the AB1700 Chemiluminescent Analyzer, and the hybridization signal intensities were determined.

Microarray data were analyzed using the ABarray package written in Bioconductor for R (http://www.bioconductor.org/packages/1.9/bioc/html/ABarray.html). Raw data were processed for quality control by evaluating signal distribution ranges, MA plots, and CV plots for variation among hybridization replicates. Data were transformed into log base 2 units and underwent a quantile normalization procedure, and these data were analyzed for differential expression using ANOVA and subsequent *t*-tests. Only genes with a signal-to-noise ratio greater than 3 and a quality flag value less than 5000 were included in the analysis. Gene expression changes ranging from 1.5-fold [17] to 2-fold [18] or a P value <0.05 [17] compared with the corresponding controls have been commonly employed as the selection criteria for differentially expressed genes. Selection of the significantly differentially expressed genes in this study was done more stringently and was based on either an ANOVA P value ≤ 0.05 and a ≥ 1.8-fold difference compared to the controls or by applying a multiple-comparison correction for type 1 error to the ANOVA results ($P \leq 0.05$). The significantly differentially expressed genes identified based on both criteria were independently validated by quantitative real-time PCR analysis of a subset of 39 genes and subsequent statistical analysis of the data. The results of the QRT-PCR analysis correlated better with the selection criterion based on the ANOVA P value ≤ 0.05 and ≥ 1.8-fold difference than those of the multiple-comparison correction method. Therefore, genes selected based on the former criterion were employed in all the subsequent studies. The significantly differentially expressed genes were used as input for bioinformatic analysis using PANTHER software (Applied Biosystems, Foster City, CA).

Validation of hepatotoxicity and neurotoxicity marker genes

On the basis of the microarray results, 39 genes were selected to further validate their potential use as hepatotoxicity and neurotoxicity marker genes. In order to select these genes, we first identified 4763 AP and 2129 MP candidate genes whose expression levels were considered significantly different from those of the control samples based on a $P \leq 0.05$ and an absolute fold change ≥ 1.8. The list was then filtered to genes meeting the same ($P \leq 0.05$ and a fold change ≥ 1.8) significance criteria in at least two out of the four time points tested for each chemical, resulting in 2428 AP and 665 MP genes. On removing the 84 genes present in both the AP and MP gene pools, the numbers of potential markers were reduced to 2344 and 581 for AP and MP, respectively. From these two lists, 31 AP and 8 MP genes were chosen. For AP, nine genes (C3d, Cd86, RT1-DOa, Bcl2l1, Hspa1a, Alox5rap, Ccr1, Gng8, and Gprk5) were selected because they function in specific pathways such as apoptosis, inflammation, and T-cell activation which are known to be involved in AP-induced hepatotoxicity [19–21]. In this study, these pathways were found significantly affected in the AP-administered rats. Three genes (Il1r2, Cd79b, and Rnase6) were selected because they were significantly differentially expressed at all the four time points examined. The remaining hepatotoxicity genes selected were significantly differentially expressed at least in three out of the four time intervals examined. Similarly, five MP genes (Gabrr2, St14, Add3,

P2rx1, and Sfrs5) where chosen because they were significantly differentially expressed at all the four time points examined, while the remaining three MP genes (Gp1bb, Cck, and Timp3) chosen were significantly differentially expressed at three out of the four time points examined (a complete list of the marker genes selected is provided in Supplemental Tables 9 and 10).

The capability of the hepatotoxicity and neurotoxicity marker genes to detect the corresponding target organ toxicity was tested by determining their expressions in the blood (leukocytes) of rats treated with additional hepatotoxic or neurotoxic chemicals. Rats were intraperitoneally administered a single dose of one of the selected hepatotoxic chemicals—thioacetamide (TA, 200 mg/kg b.w.), carbon tetrachloride (CCl4, 0.5 ml/kg b.w.), and dimethylnitrosamine (DMN, 10 mg/kg b.w.) or neurotoxic chemicals—malathion (100 mg/kg b.w.), and ethyl parathion (0.75 mg/kg b.w.). All the test chemicals were purchased from Sigma Chemical Co., (St Louis, MO), and the doses were selected based on earlier publication [18] and data presented in the Material Safety Data Sheet (MSDS). At 24-h after administering the toxic chemicals, the rats were sacrificed and unclotted blood samples were collected, and RNA isolated using the LeukoLOCK^{TM} Total RNA Isolation System. Activities of transaminases and acetylcholinesterase were determined in the blood samples as described above. Expressions of the selected hepatotoxicity and neurotoxicity signature genes were determined by quantitative real-time PCR (QRT-PCR) analysis of the RNA isolated from the leukocytes. The PCR amplification, detection of the PCR amplified gene products, and their quantitations were performed with the 7900HT Fast Real-Time PCR machine and the Taqman Universal PCR Master Mix with UNG (Applied Biosystems, Foster City, CA). The expression levels of the genes were normalized to that of the housekeeping gene, 18S, and the fold changes in expressions compared to the control were calculated using the formula 2^(−(ΔCt_target − ΔCt_18S)).

In vitro experiments using leukocytes

An in vitro experiment using rat blood leukocytes was conducted to investigate whether the differential expressions of the hepatotoxicity marker genes identified in response to exposure of rats to AP is due to the direct interaction between the chemical and the blood (leukocytes) or mediated indirectly, such as through the target organs (experiments using MP could not be conducted due to its precipitation in the cell culture medium). Blood was collected directly from the heart of control, untreated rats after euthanasia into Vacutainer tubes containing EDTA as described above. The leukocytes, isolated from the blood samples by Ficoll-hypaque method [22], were resuspended in RPMI 1640 medium (ATCC, Manassas, VA,) containing 10% FBS, 100U/ml penicillin G, and 100 μg/ml streptomycin and were cultured under standard cell culture conditions (37°C, 5% carbon dioxide). Cells were treated with 10 mM AP for 6 h in the culture medium. The concentration of AP and the treatment duration were determined based on the results of a preliminary cytotoxicity study and was found to result in less than 10% cell death. At the end of the incubation period, cells were washed once with phosphate buffered saline, and total RNA was isolated using RNeasy Mini Kit (Qiagen Inc, Valencia, CA). A subset of seven hepatotoxicity marker genes was selected at random for determining their expression in the RNA samples by QRT-PCR analysis.

Statistical analysis of data

Measures such as clinical chemistry including WBC and lymphocyte counts; circulating clinical enzymatic biomarkers including AST, ALT, and AChE; and PCR validation of gene probes were analyzed using ANOVA in JMP 5.1 (SAS, Cary, NC).

Results

Toxicity symptoms

Administration of AP and MP resulted in a loss in body weight of 8 and 12%, respectively, among the rats (data not presented). In addition, rats from both chemical treatment groups consumed less water and food for the first 48-h after dose administration. Furthermore, the MP-administered rats alone exhibited slight nasal discharge for the first 48 h after the dose administration.

Hematology

Administration of MP resulted in a significant decrease in the total WBC and lymphocytes counts at the 12- and 24-h post-exposure time intervals (Supplemental Table 1). The only hematotoxicity seen among the AP-administered rats was a statistically significant reduction in the number of lymphocytes at 12-h post-exposure to the chemical (Supplemental Table 2).

Clinical markers of hepatotoxicity and neurotoxicity

Administration of AP and MP to rats resulted in increased levels of serum transaminases and decreased level of AChE, respectively (Fig. 1a–c). Significant increases in AST and ALT activities, indicative of hepatotoxicity, were observed in the AP-administered rats only at 24-h after

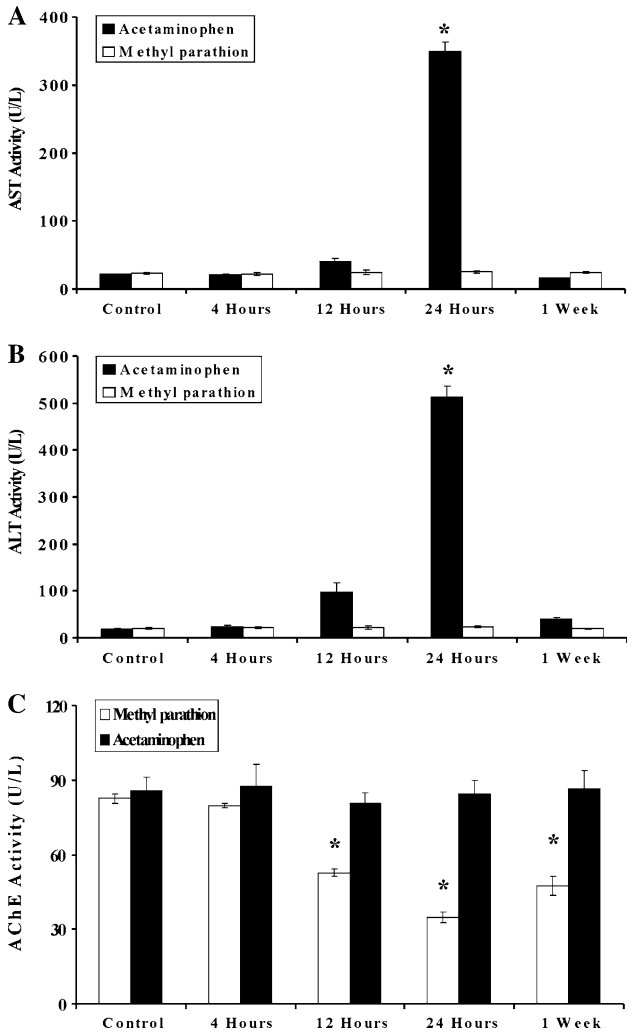

Fig. 1 **a–c** Blood transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) and acetyl cholinesterase (AChE) activities in the blood of rats administered acetaminophen (AP) or methyl parathion (MP). Groups of rats were administered a single, acute, intraperitoneal dose of AP (1200 mg/kg b.w.) or MP (15 mg/kg b.w.) or vegetable oil, the vehicle used to prepare the test chemicals for administration. At time intervals of 4-, 12-, and 24-h, and 1-week after administration of the chemicals, the rats were euthanized and the activities of AST (**a**), ALT (**b**) and AChE (**c**) were determined in the blood samples by procedures as described in the "Methods" section. The data is presented as mean ± S.E. ($n = 4$). The statistically significant differences ($P \leq 0.05$) in enzyme activities in the rats administered the chemicals compared with the controls are indicated by an asterisk

treatment (Fig. 1a, b). At time intervals of 4- and 12-h and 1 week post-AP exposure, there was no indication of any significant hepatotoxicity as evidenced from the near normal AST and ALT levels. A significant inhibition of AChE activity indicating neurotoxicity was observed at time intervals of 12-, 24-h, and 1 week after MP administration, with the highest inhibition seen at the 24-h time interval (Fig. 1c). There were no change in AChE activity in the blood of the rats administered AP or in AST and ALT

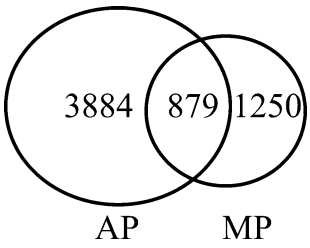

Fig. 2 Venn diagram of differentially expressed genes in the blood of rats administered acetaminophen (AP) or methyl parathion (MP). Groups of rats were administered a single, acute, intraperitoneal dose of AP (1200 mg/kg b.w.) or MP (15 mg/kg b.w.) or vegetable oil, the vehicle used to prepare the test chemicals for administration. At time intervals of 4-, 12-, and 24-h, and 1-week after administration of the chemicals, the rats were euthanized and global gene expression profile was determined in the blood samples using microarray as described in the "Methods" section. The number of significantly differentially expressed genes ($P \leq 0.05$ and ≥1.8-fold change) observed in each of the four time intervals are combined and the results are presented

activities in the blood of the rats administered MP compared with the corresponding controls suggesting that administration of AP and MP did not result in neurotoxicity and hepatotoxicity, respectively.

Differential blood gene expression in response to hepatotoxicity and neurotoxicity

Of the 26,857 genes represented on the microarray, the expression of 13,974 and 14,296 genes each were detected in the RNA isolated from the blood samples of rats treated with AP and MP, respectively (data not presented). Those genes which exhibited ≥1.8-fold differential expression and a P value ≤0.05 in the rats administered the toxic chemicals compared with the controls in at least one of the four time intervals studied were considered significantly differentially expressed. Using this criteria, administration of AP and MP resulted in the significant differential expression of 4763 (34%) and 2129 (15%) genes, respectively (Fig. 2). Of the 4763 genes which were differentially expressed in the AP-administrated rats, the expressions of 3884 (82%) genes were specific for AP, i.e., expressions of these genes were not significantly different in the MP-administrated rats. Similarly, out of the 2129 differentially expressed genes in the MP-administrated rats, 1250 (59%) genes were specific for MP, i.e., their expressions were not significantly different in the AP-administrated rats. Eight hundred and seventy-nine genes were significantly differentially expressed in both the AP and MP-administrated rats.

Unlike the biochemical toxicity markers in the blood, microarray analysis after administration of AP or MP, identified the differential up- and down-regulation of expression of a large number of genes at all time intervals studied (Fig. 3a–d). For example, at 4- and 12-h after

administration of AP (time intervals at which significant hepatotoxicity was undetectable based on the blood transaminases' activities), hundreds of genes were found significantly differentially expressed in the rats (Fig. 3a, b). Similarly, in spite of the normal AChE activity at the 4-h time interval after administration of MP, more than 200 genes were found differentially expressed in the blood of the rats (Fig. 3c, d). In the case of AP- and MP-administrated rats, 29 and 11 genes, respectively, were found differentially expressed at all four time intervals analyzed.

Classification of the differentially expressed genes into pathways, biological processes, and molecular functions (PANTHER analysis) demonstrated certain differences in the gene expression profiles between AP-induced hepatotoxicity and MP-induced neurotoxicity (Supplemental Tables 3–8). In general, more pathways, molecular functions, and biological processes were found significantly altered in the AP-administrated rats compared with those administered MP at all time intervals studied.

QRT-PCR confirmation of microarray data

QRT-PCR resulted in the amplification of all of the selected marker genes. The differential expressions of all the 31 hepatotoxicity marker genes and eight neurotoxicity marker genes were confirmed by QRT-PCR analysis (Supplemental Table 9 and 10).

Validation of hepatotoxicity and neurotoxicity marker genes

Administration of additional hepatotoxic chemicals (CCl4, DMN, and TA) in the rats resulted in hepatotoxicity as evidenced from significant elevations in serum transaminase (AST and ALT) activities (Table 1). Similarly, the administration of additional neurotoxic chemicals (ethyl parathion and malathion) resulted in significant inhibition of AChE activity (Table 2). Out of the 31 candidate hepatotoxicity marker genes which were selected for

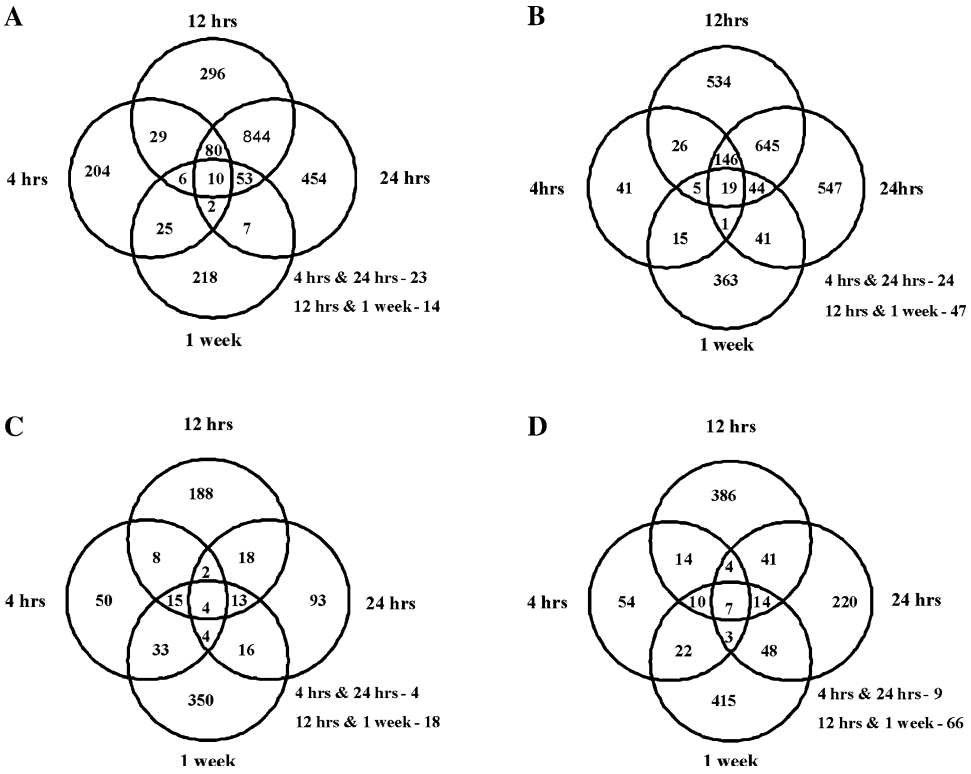

Fig. 3 **a–d** Venn diagrams of differentially expressed genes in the blood of rats administered acetaminophen (AP) or methyl parathion (MP). Groups of rats were administered a single, acute, intraperitoneal dose of AP (1200 mg/kg b.w.) or MP (15 mg/kg b.w.) or vegetable oil, the vehicle used to prepare the test chemicals for administration. At time intervals of 4-, 12-, and 24-h, and 1-week after administration of the chemicals, the rats were euthanized and global gene expression profile was determined in the blood samples by microarray analysis. The number of significantly ($P \leq 0.05$ and $\geq$1.8-fold change) differentially expressed (up- and down-regulated) genes in the rats administered the chemicals compared with the controls are presented. **a** Significantly up-regulated by AP., **b** significantly down-regulated by AP., **c** significantly up-regulated by MP., and **d** significantly down-regulated by MP. The significantly differentially expressed genes which were common between 4 and 24 h and 12 h and 1 week could not be presented in the Venn diagram and are presented separately

Table 1 Activities of blood transaminases (ALT and AST) in rats at 24-h after administration of the selected hepatotoxic chemicals

Serum transaminases	Hepatotoxic chemicals			
	None (vehicle only)	Thioacetamide	Carbon tetrachloride	Dimethylnitrobenzene
AST (U/L)	21.35 ± 1.06	333.69 ± 10.85*	329.14 ± 9.90*	304.84 ± 11.80*
ALT (U/L)	18.13 ± 1.63	474.79 ± 2.93*	603.43 ± 11.55*	546.95 ± 14.70*

* Significantly different ($P \leq 0.05$) compared with the control rats. Values are presented as mean ± S.E. ($n = 4$)

Table 2 Acetyl cholinesterase activity in the blood of rats at 12-h after the administration of various neurotoxic chemicals

Acetyl cholinesterase activity (U/L)	Neurotoxic chemicals			
	None (vehicle only)	Methyl parathion	Ethyl parathion	Malathion
	139 ± 9.1	52.7 ± 1.42*	69.8 ± 8.35*	93.6 ± 4.35*

* Significantly different ($P \leq 0.05$) compared with the control rats. Values are presented as mean ± S.E. ($n = 4$)

validation, 17 were found significantly differentially expressed in the RNA samples isolated from the blood of all the rats administered CCl4, DMN, or TA (Supplemental Table 11). Furthermore, the differential expressions of 11 hepatotoxicity marker genes were found to be specific for hepatotoxicity in that they were differentially expressed in rats treated with all four hepatotoxic chemicals (including AP) but not in those administered any of the neurotoxic chemicals including MP (Table 3). It was noticed that the fold changes in the expression of most of the candidate marker genes in the rats administered the additional hepatotoxic chemicals were much lower compared to those administered AP, and the reason(s) for this difference is(are) neither known nor further investigated. Further functional analysis of the 11 hepatotoxicity specific genes using Ingenuity Pathway Analysis (IPA, Ingenuity Pathway Analysis, Mountainview, CA) demonstrated that most of them (9 genes) belonged to cellular networks related to amino acid metabolism, nucleic acid metabolism, and small molecule biochemistry (data not presented). Similarly, three out of eight neurotoxicity marker genes tested were able to detect neurotoxicity induced by the two additional neurotoxic chemicals (Supplemental Table 12). However, the differential expression of only one gene, Add3, was found to be specific for neurotoxicity since its expression was significantly different in the rats

Table 3 Expression of hepatotoxicity and neurotoxicity marker genes in the blood of rats administered various hepatotoxic and neurotoxic chemicals

Gene symbol[a]	Control (vehicle only)	Hepatotoxic chemicals				Neurotoxic chemicals		
		AP	CCl_4	DMN	TA	MP	EP	Malathion
Alp1ra1	22.03 ± 1.32	15.42 ± 0.30*	19.57 ± 0.20*	18.34 ± 0.29*	19.32 ± 0.29*	21.16 ± 0.40	21.59 ± 0.28	21.97 ± 0.43
Ddah1	21.51 ± 0.31	17.20 ± 0.35*	19.42 ± 0.24*	18.77 ± 0.09*	19.35 ± 0.17*	20.30 ± 0.42	22.78 ± 0.28	22.57 ± 0.42
Ggt1	19.86 ± 0.23	14.04 ± 0.06*	18.77 ± 0.31*	16.32 ± 0.15*	18.27 ± 0.28*	19.22 ± 0.68	20.62 ± 0.45	20.76 ± 0.47
HK3	20.32 ± 0.23	15.68 ± 0.17*	18.42 ± 0.18*	17.14 ± 0.10*	18.27 ± 0.23*	19.37 ± 0.60	18.91 ± 0.39	20.60 ± 0.54
Il1rap	17.54 ± 0.18	13.10 ± 0.33*	16.33 ± 0.20*	16.18 ± 0.51*	15.50 ± 0.21*	16.91 ± 0.80	17.59 ± 0.43	17.72 ± 0.54
Mmp9	17.41 ± 0.21	12.00 ± 0.22*	15.46 ± 0.09*	13.10 ± 0.11*	14.83 ± 0.08*	16.75 ± 0.38	18.41 ± 0.30	18.10 ± 0.39
Nos2	17.63 ± 0.30	13.02 ± 0.12*	14.65 ± 0.09*	15.73 ± 0.14*	14.61 ± 0.09*	17.08 ± 0.48	17.98 ± 0.42	18.59 ± 0.56
Socs3	16.71 ± 0.19	12.27 ± 0.29*	15.94 ± 0.13*	14.72 ± 0.25*	15.82 ± 0.28*	16.01 ± 0.45	16.47 ± 0.39	16.52 ± 0.47
Tgm1	21.26 ± 0.33	13.83 ± 0.32*	19.18 ± 0.27*	17.25 ± 0.30*	18.60 ± 0.29*	19.82 ± 0.48	21.95 ± 0.48	22.20 ± 0.88
Ccr1	15.14 ± 0.25	11.28 ± 0.21*	14.15 ± 0.15*	13.41 ± 0.14*	13.62 ± 0.13*	14.31 ± 0.37	15.72 ± 0.41	16.03 ± 0.40
Gprk5	17.54 ± 0.21	15.48 ± 0.09*	16.64 ± 0.16*	16.15 ± 0.07*	16.35 ± 0.22*	17.32 ± 0.26	19.02 ± 0.54	18.09 ± 0.40
Add3	11.14 ± 0.14	10.95 ± 0.06	11.29 ± 0.07	11.41 ± 0.10	11.34 ± 0.05	12.13 ± 0.15*	14.14 ± 0.28*	12.22 ± 0.16*

[a] More details about the genes including their full nomenclature and gene bank accession number are available at http://www.ncbi.nlm.nih.gov/geo (Accession number GPL2996)

* Significantly differentially expressed ($P \leq 0.05$). The values represent CT (threshold cycle) and are mean ± S.E. ($n = 4$)

AP Acetaminophen, *CCL_4* Carbon tetrachloride, *DMN* Dimethyl nitrobenzene, *TA* Thioacetamide, *MP* Methyl parathion, *EP* Ethyl parathion

Table 4 Fold change in the expression of selected hepatotoxicity marker genes in the blood of rats administered acetaminophen in vivo and in the leukocytes treated in vitro with acetaminophen

Gene symbol	Fold change in expression	
	Rat (in vivo)	Leukocytes (in vitro)
Alp1ra1	57.45*	10.52*
Ddah1	14.33*	36.27**
Bcl211	6.33*	7.71*
Nrp1	3.23*	6.34*

* Statistically significant ($P \leq 0.05$); ** statistically significant ($P \leq 0.1$). The values are the means of four independent samples. The cT values were used to determine statistical significance of the differences between the control and treated samples

Out of the seven hepatotoxicity marker genes analyzed, the expressions of three genes—Crisp1d2, Ccr1, and Mmp9, were significantly different only in the leukocytes of rats administered AP (in vivo) and not in the leukocytes treated in vitro with acetaminophen and are not presented

administered all three neurotoxic chemicals, and not in the rats which were administered any of the four hepatotoxic chemicals (Table 3).

Expression of toxicity marker genes in leukocytes treated in vitro with AP

Analysis of RNA samples isolated from leukocytes treated in vitro with AP resulted in a significant differential expression of four out of seven selected hepatotoxicity marker genes (Table 4).

Discussion

The findings of this study support our research hypothesis that blood gene expression markers may serve as surrogate biomarkers to non-invasively detect as well as distinguish target organ toxicities. By employing a model hepatotoxic or neurotoxic chemical—AP or MP, respectively, we were able to identify genes in the blood of rats that were uniquely expressed with respect to target organ toxicity. These surrogate gene expression markers identified in the blood were also capable of detecting and distinguishing overt hepatotoxicity and neurotoxicity induced by the additional toxic chemicals tested in the study. In addition, the novel surrogate blood gene expression markers were capable of detecting hepatotoxicity and neurotoxicity at earlier time points than the currently available and widely used clinical markers.

The toxic chemicals AP and MP, selected as model chemicals in this study, are known for their potential to cause hepatotoxicity and neurotoxicity, respectively [14, 16, 18, 23]. Global gene expression profiling of RNA isolated from the blood leukocytes of the rats revealed that administration of either model chemical at overtly toxic doses resulted in alterations in the expression of thousands of genes. Furthermore, analysis of the differentially expressed genes demonstrated significant qualitative differences in the blood gene expression profiles of the rats exhibiting hepatotoxicity and neurotoxicity. Taken together, these results demonstrated that blood gene expression profiling, similar to assaying the activities of toxicity marker enzymes, was capable of detecting and distinguishing target organ toxicities induced by the model chemicals.

Human exposure to toxic chemicals, either from the environment or from the workplace, is a serious problem and responsible for many illnesses. Inaccessibility of organs and tissues that are often the targets for chemical toxicity, as well as the lack of available techniques have been a major obstacle in efficiently monitoring humans for toxicity after environmental and/or occupational exposure to toxic chemicals. Recent developments in toxicogenomics have demonstrated the usefulness of global gene expression profiling by employing microarray and other techniques to study the toxicity of chemicals. The differential gene expression profile induced by MP, the model neurotoxic chemical employed in this study, has not been reported in the literature. However, AP, the model hepatotoxic chemical employed in this study, has been the subject of several previous toxicogenomics studies [5, 18, 24–27]. In spite of the differences in experimental designs, microarray platforms, and computational programs employed in the gene expression analysis, some similarities were observed between the blood gene expression profile (results of this study) and that of the primary target organ, liver, after AP administration [5, 24–27]. For example, many genes involved in cellular functions such as inflammation, immune response, stress response, energy metabolism, etc., were found differentially expressed in the liver after AP administration [5, 24–27] and in the blood of rats (results of this study). These findings, therefore, suggest that global gene expression profiling in blood may be an alternative approach to detect chemical toxicity in inaccessible target organs.

A major drawback with the majority of the existing biomarkers of toxicity is their lack of sensitivity. Ideally, a biomarker for toxicity should be able to detect the potential for toxicity prior to frank target organ damage and well before the onset of any clinical manifestations. Histopathology, a well-established technique used to determine target organ toxicity, may detect toxicity before clinical manifestation, but require sacrifice of the animals. Results of this study demonstrated that blood gene expression profiling was capable of detecting hepatotoxicity and neurotoxicity induced by the model chemicals earlier than

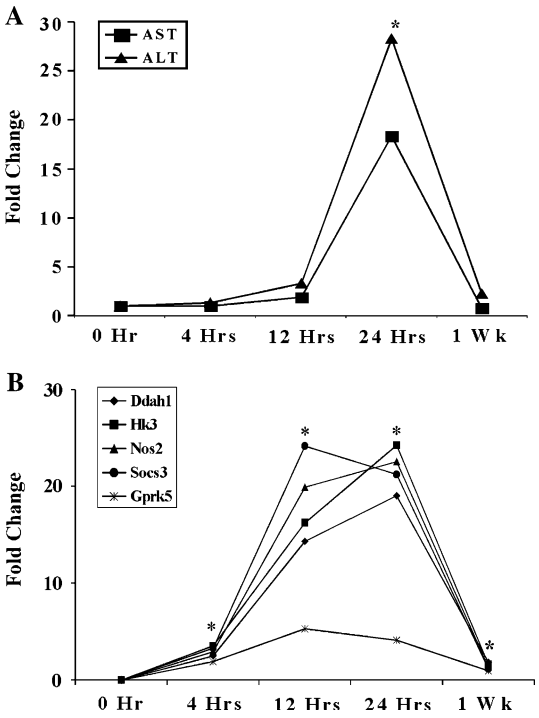

Fig. 4 **a**, **b** Transaminases activities and expression of hepatotoxicity marker genes in the blood of rats administered acetaminophen. Groups of rats were administered intraperitoneally a single dose of acetaminophen (1200 mg/kg b.w.) or vegetable oil—the vehicle that was used to prepare the chemicals for administration. At time intervals of 4-, 12-, and 24-h, and 1-week after the chemical administration, the rats were euthanized, and the blood samples were collected. Activities of transaminases (AST and ALT) and expressions of hepatotoxicity marker genes were determined in the blood samples by procedures as described in the "Methods" section. The mean ($n = 4$) fold changes in activities of the enzymes (**a**) or expressions of the genes (**b**) compared with the controls are presented. The microarray data were used to calculate fold changes in expression of the individual genes. The time intervals at which statistically significant ($p \leq 0.05$) differences are observed for all the selected genes and enzymes in the AP-administrated rats compared with the controls are indicated by an asterisk

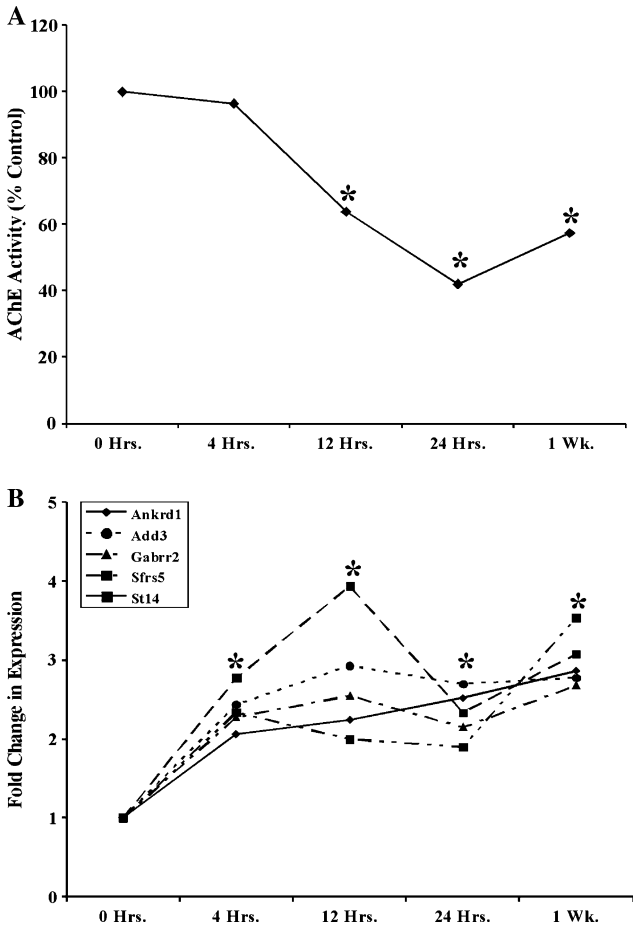

Fig. 5 **a**, **b** Acetyl cholinesterase (AChE) activity and expression of neurotoxicity marker genes in the blood of rats administered methyl parathion. Groups of rats were administered intraperitoneally a single dose of methyl parathion (15 mg/kg b.w.). At time intervals of 4-, 12-, and 24-h, and 1-week after the chemical administration, the rats were euthanized, and the blood samples were collected. Acetyl cholinesterase activity and expressions of neurotoxicity marker genes were determined in the blood samples by procedures as described in the "Methods" section. The fold changes in AChE activity (**a**) or expressions of the genes (**b**) in the methyl parathion-administrated rats compared with the controls are presented. The mean ($n = 4$) fold change in expression of the individual genes was calculated using the microarray data. The time intervals at which statistically significant ($P \leq 0.05$) differences are observed for all the selected genes and enzyme in the MP-treated rats compared with the controls are indicated by an asterisk

those detected by employing the most widely used biochemical markers of toxicity. There was no indication of either hepatotoxicity or neurotoxicity at the earliest 4-h time interval after administration of the chemicals when the blood levels of transaminases and acetyl cholinesterase—the most widely used and generally considered to be sensitive markers of hepatotoxicity and neurotoxicity, respectively, were tested as the toxicity markers (Figs. 4 and 5). At later time intervals, for example, 12- and 24-h, and 1 week after MP administration, and 24-h after AP administration, significant changes in the blood levels of the biochemical markers (AChE and transaminases, respectively) were seen suggesting "the onset of target organ toxicity" in the rats. In contrast, differential expressions of some of the hepatotoxicity and neurotoxicity marker genes in the blood were seen as early as 4 h after the intraperitoneal administration of the model toxic chemicals (Figs. 4 and 5). The neurotoxicity and hepatotoxicity marker genes that were significantly altered as early as 4-h post-administration exhibited even more significant alterations at the 12- and 24-h time intervals compared with the 4-h time interval. With respect to the superior sensitivity of the blood gene expression profiles as surrogate biomarkers of target organ toxicity, our data support those of Bushel et al. [14] who have recently demonstrated that blood gene expression data

outperformed hepatotoxicity markers based on hematology, histopathology, and clinical chemistry in the rat. At present, we are investigating the potential application of blood gene expression profiling to non-invasively detect and distinguish target organ toxicity induced by low level, repeated exposure to toxic chemicals such as those taking place in workplaces and the environment.

Another desirable feature required for toxicity biomarkers is specificity. A hepatotoxicity-specific marker gene, for example, should be able to detect hepatotoxicity induced by any and all hepatotoxic chemicals, but it should not respond to toxicity targeting organs other than the liver. Even though the specificity issue was not thoroughly investigated, the limited data obtained from this study indicated that the blood gene expression markers identified exhibit considerable specificity for target organ toxicity. Out of the 31 candidate hepatotoxicity marker genes which were tested in this study, 11 (35%) were found to be specific for liver toxicity—they were differentially expressed in the blood samples of the rats administered each of the four hepatotoxic chemicals while retaining normal expression in the blood of the rats administered the neurotoxic organophosphorus chemicals (Table 3). On the contrary, only one out of the eight (12.5%) neurotoxicity marker genes tested was found to be specific for neurotoxicity (Table 3). It is worth mentioning that only a small number of genes (31 and 8 for hepatotoxicity and neurotoxicity, respectively) was tested as potential markers of target organ toxicity in this study. It might have been possible to identify additional marker genes for hepatotoxicity and neurotoxicity if all differentially expressed genes as identified by the microarray screening were further tested for their potential specificity. However, it needs to be emphasized that a full validation of the hepatotoxicity and neurotoxicity marker genes would require additional studies involving a large number of compounds, both positive (preferably those inducing target organ toxicity by different mechanisms) and negative, at a variety of doses ranging from non-toxic to overtly toxic.

At present, we attempted to determine the role of target organs, if any, in the differential gene expression taking place in the blood leukocytes of rats administered AP in vivo. Neither the total leukocyte count nor that of any particular leukocyte population changed in the AP-administered rats compared to the control suggesting that the differential gene expression seen in the AP-administrated rats is not due to change in the number of leukocyte(s). In vitro exposure of leukocytes to AP resulted in the differential expression of four out of seven hepatotoxicity marker genes whose expressions were analyzed. One of the most likely explanations for the differences noticed with respect to the differential expression of the hepatotoxicity marker genes in the leukocytes treated in vivo (rat experiment) and in vitro with AP may be related to the metabolism of the chemical. Acetaminophen, after its entry into the body, is activated, predominantly, by cytochrome P450 into a reactive metabolite, N-acetyl-p-benzoquinonimine (NAPQI) [28, 29]. NAPQI, in the absence of detoxification, binds with cellular macromolecules and results in toxicity. Even though the ability of leukocytes to metabolize AP is not clearly known, it is reasonable to assume that liver is capable of metabolizing AP at a significantly higher rate than the leukocytes due to the relatively higher activity of cytochrome P450 enzymes in the liver. The amount of NAPQI and other reactive metabolites of AP generated and, therefore, available in the blood to interact with the leukocytes might have been more in the case of rats administered AP in vivo compared to the leukocytes treated with AP in vitro, and this might account for the increased number of significantly differentially expressed genes observed in the leukocytes of the in vivo experiment. With respect to the metabolism of AP, it may be important to pay attention to the route of administration of AP in the rats. At present, AP was administered intraperitoneally; and dosing through mouth, for example, might result in a difference in the metabolism of the chemical possibly resulting in a corresponding difference in the blood gene expression profile. In addition to metabolism, inflammatory factors released by liver in response to AP-induced hepatotoxicity [30] might have played a role in influencing the gene expression profile of leukocytes in vivo. Therefore, even though, at present, we could not precisely determine the actual contribution of the target organs in the differential expression of the toxicity marker genes in the leukocytes, it appears that the differential gene expression observed in the leukocytes of rats treated in vivo with the toxic chemicals might be a combination of the direct effect of the chemical on the leukocytes and those mediated directly or indirectly by the target organs.

The findings of this study further support and, thereby, confirm the recent reports by Bushel et al. [14] and Lobenhofer et al. [15] who have demonstrated that the toxicity induced by several classic hepatotoxic chemicals can be detected by blood gene expression profiling in the rat. In addition, we have demonstrated that a similar approach can be employed to detect neurotoxicity induced by selected organophosphorus chemicals. Furthermore, we were able to distinguish hepatotoxicity and neurotoxicity induced by several additional chemicals based on the blood gene expression markers in the rat. However, whether a similar approach can be employed to detect and distinguish toxicity targeting organs other than the liver and nervous system remains to be investigated. Our findings along with those of Bushel et al. [14] and Lobenhofer et al. [15] are promising and may be of potential value to develop simple, sensitive, and non-invasive assays to detect toxicity arising

from human exposure to toxic chemicals such as those taking place from the environment and workplace. In this study, a PCR assay, developed based on microarray analysis of blood gene expression profiling, was employed to detect and distinguish target organ toxicity in the rat. Bushel et al. [14] were able to detect acute liver toxicity induced by AP in humans by employing human orthologs of rat blood gene expression markers. Therefore, it may be feasible to develop simple PCR assays based on the results of microarray analysis of rat blood gene expression profile that may eventually be employed to monitor human exposure to toxic chemicals resulting in target organ toxicity. However, any potential application of the findings of this study as well as those of Bushel et al. [14] and Lobenhofer et al. [15] to monitor human exposure to chemicals resulting in toxicity would require further validation. It needs to be determined, for example, whether peripheral blood gene expression profiling is sensitive enough to detect low level chronic exposure to toxic chemicals and the resulting toxicity with significant specificity in a preclinical stage in human beings. It is, however, worth mentioning that there are some articles suggesting an association between alterations in peripheral blood gene expression profile and exposure to toxic chemicals in human beings [17, 31].

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