Table 2 *(continued)*

Affymetrix probe set ID	*Gene symbol*	*Gene description*	*Fold change*		
			Dose (mg/kg)		
			15	*50*	*150*
1369693_a_at	Slc1a2	Solute carrier family 1, member 2	1.81	1.96	2.07
1370541_at	Nr1d2	Nuclear receptor subfamily 1, group D, member 2	0.91	1.01	2.06
1389004_at	Josd2_predicted	Josephin domain containing 2 (predicted)	1.12	0.95	2.06
1370030_at	Gclm	Glutamate cysteine ligase, modifier subunit	1.06	0.81	2.06
1370000_at	Thra	Thyroid hormone receptor alpha	1.79	1.66	2.06
1370663_at	Wee1	Wee 1 homolog	1.92	1.00	2.05
1390321_at	RGD1304693_predicted	Similar to CG14803-PA (predicted)	1.68	1.15	2.03
1389209_at	RGD1306274	Similar to hypothetical protein BC002942	1.82	1.17	2.03
1388750_at	Tfrc	Transferrin receptor	1.32	0.87	2.03
1373935_at	Pold2	Polymerase (DNA directed), delta 2, regulatory subunit	1.35	1.18	2.03
1390579_at	RGD1305222_predicted	Similar to RIKEN cDNA 1810029B16 (predicted)	0.84	0.70	2.02
1389889_at	RGD1306404_predicted	Similar to mKIAA1402 protein (predicted)	1.21	1.03	2.02
1373499_at	Gas5	Growth arrest specific 5	1.15	1.03	2.02
1373386_at	Gjb2	Gap junction membrane channel protein beta 2	0.81	1.08	2.02
1376001_at	Praf1_predicted	Polymerase (RNA) I associated factor 1 (predicted)	1.17	0.98	2.01
1373200_at	Eef1e1_predicted	Eukaryotic translation elongation factor 1 epsilon 1 (predicted)	1.19	1.25	2.01
1380854_at	R3hdm1	R3H domain containing 1	1.20	1.28	2.00

Probe sets are sorted by fold change. Shaded probe sets, those selected as in-vivo–in-vitro bridging probes (see Figure 3).

subunit", "glutamate-cysteine ligase, modifier subunit", "aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)", "NAD(P)H dehydrogenase, quinone 1", "thioredoxin reductase 1", and "metallothionein"; genes related to the heat shock response: "crystallin, lamda 1", "DnaJ (Hsp40) homolog, subfamily B, member 11", "heat shock 70kD protein 1A/1B", and "protein kinase inhibitor p58"; genes responsive to hypoxia: "hypoxia up-regulated 1" and "ischemia/reperfusion inducible protein"; and genes related to DNA repair and the cell cycle: "DNA-damage-inducible transcript 4-like", "cyclin E", "growth arrest specific 5", and "wee 1 homolog". Changes in expression of these genes in hepatocytes can be interpreted as a reflection of the adaptive response to oxidative stress and cellular damage. Among the extracted genes, the following genes appeared to be the most sensitive to coumarin, "aldo-keto reductase family 7, member A3", "NAD(P)H dehydrogenase, quinone 1", "glutathione reductase", "glutathione-S-transferase, pi 1/2", and "glutathione S-transferase Yc2 subunit", as they were remarkably mobilized at the lowest dose of coumarin-treatment (15 mg/kg).

Comparison between in-vivo and in-vitro rat hepatocyte responses

Primary cultured rat hepatocytes were exposed to 12, 60, and 300 μM coumarin for 24 h. No obvious cytotoxicity was detected by LDH release (100.5, 97.7, and 95.1% of control, respectively). In case of the in-vitro system, statistical filtering was not appropriate because the data were the duplicate measurements from a single rat. We then extracted the significant genes according to the gene list obtained from in-vivo study, that is, the genes showing significant up- (136 probe sets) or down-regulation (79 probe sets) in livers treated with 150 mg/kg coumarin. As shown in Figure 3a, a similar trend was observed between in-vivo and in-vitro cell responses, although the extent of the response (i.e., fold change) was generally smaller, and fewer genes showed a measurable change in the in-vitro cell assay. Probe sets showing changes of 1.5-fold or more and 0.6-fold or less than that of control at the highest concentration (300 μM) in rat hepatocytes were selected as those reflecting the toxicological mechanism of coumarin *in vivo*, namely, "in-vivo–in-vitro bridging probes". For the selected genes (37 up-regulated and 29 down-regulated; see shading in Tables 2 and 3), clear dose-dependent changes in expression were observed (Figure 3b), and the observation enabled us to assess hepatotoxicity of coumarin using the in-vitro data.

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Comparison between rat and human hepatocytes

Cultured human hepatocytes were also exposed to 12, 60, and 300 μM coumarin for 24 h. No obvious cytotoxicity was detected by LDH release (100.6, 100.9, and 102.0% of control, respectively). The in-vivo–in-vitro bridging probes were assigned to their human ortholog genes to form a set of "rat–human bridging probes" and changes in their expression were compared in rat versus human hepatocytes. In total, 14 up-regulated and 11 down-regulated probe sets were identified and their relative expres-

Table 3 Genes down-regulated in the rat liver 24 h after administration of coumarin

Affymetrix probe set ID	*Gene symbol*	*Gene description*	*Fold change*		
			Dose (mg/kg)		
			15	*50*	*150*
1386977_at	Ca3	Carbonic anhydrase 3	0.64	1.01	0.13
1370778_at	LOC259245	Alpha-2u globulin	0.87	0.84	0.16
1386474_at	—	Transcribed locus	1.53	0.59	0.19
1393902_at	Akr1c6	Aldo-keto reductase family 1, member C6	0.42	0.53	0.20
1375900_at	LOC500590	Similar to T-cell antigen 4-1BB precursor – mouse	0.56	0.52	0.21
1371412_a_at	Nrep	Neuronal regeneration related protein	0.86	0.88	0.25
1387491_at	Gyk	Glycerol kinase	0.76	0.72	0.28
1376637_at	—	Transcribed locus	0.62	1.03	0.29
1373722_at	Kif20a_predicted	Kinesin family member 20A (predicted)	1.12	0.91	0.29
1367896_at	Ca3	Carbonic anhydrase 3	0.80	0.98	0.29
1385247_at	Ugt2b	UDP glycosyltransferase 2 family, polypeptide B	0.68	0.04	0.31
1387852_at	Thrsp	Thyroid hormone–responsive protein	1.06	0.87	0.32
1393221_at	RGD1564865_predicted	Similar to 20-alpha-hydroxysteroid dehydrogenase (predicted)	0.67	0.97	0.32
1387665_at	Bhmt	Betaine-homocysteine methyltransferase	0.81	1.18	0.33
1387185_at	Apbb3	Amyloid beta (A4) precursor protein-binding, family B, member 3	0.84	0.84	0.33
1387053_at	Fmo1	Flavin containing monooxygenase 1	0.62	0.79	0.34
1398286_at	Csad	Cysteine sulfinic acid decarboxylase	1.07	0.92	0.34
1387655_at	Cxcl12	Chemokine (C-X-C motif) ligand 12	0.67	0.45	0.34
1368458_at	Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	0.78	0.69	0.36
1388583_at	Cxcl12	Chemokine (C-X-C motif) ligand 12	0.77	0.67	0.37
1387243_at	Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2	0.78	0.90	0.37
1387139_at	Hao2	Hydroxyacid oxidase 2 (long chain)	0.79	1.15	0.37
1373006_at	Prp2	Proline-rich protein PRP2	0.70	0.79	0.37
1370026_at	Cryab	Crystallin, alpha B	1.01	0.71	0.37
1390443_at	—	Transcribed locus	0.88	0.75	0.38
1375144_at	—	Transcribed locus	1.02	0.52	0.39
1369044_a_at	Pde4b	Phosphodiesterase 4B	0.59	0.64	0.39
1370057_at	Csrp1	Cysteine and glycine-rich protein 1	0.93	0.90	0.39
1369450_at	Ust5r	Integral membrane transport protein UST5r	0.74	0.94	0.39
1367729_at	Oat	Ornithine aminotransferase	0.63	1.00	0.39
1374677_at	LOC684425	Similar to adenylosuccinate synthetase isozyme 1	0.80	1.00	0.40
1388038_at	Atrn	Attractin	0.88	0.88	0.40
1388031_x_at	LOC259245/Mup5	Alpha-2u globulin	0.70	0.95	0.40
1370150_a_at	Thrsp	Thyroid hormone–responsive protein	1.03	0.97	0.41
1390450_a_at	Ogn_predicted	Osteoglycin (predicted)	0.63	0.53	0.41
1369664_at	Avpr1a	Arginine vasopressin receptor 1A	0.88	0.87	0.41
1388433_at	Krt1-19	Keratin complex 1, acidic, gene 19	1.09	0.71	0.42
1371400_at	Thrsp	Thyroid hormone–responsive protein	1.04	0.98	0.42
1369296_at	Sult1c1	Sulfotransferase family, cytosolic, 1C, member 1	0.86	1.13	0.42
1389728_at	—	—	0.77	0.56	0.42
1389188_at	Gpr108	G protein-coupled receptor 108	0.69	0.53	0.42
1380546_at	LOC298250	Similar to hypothetical protein FLJ10986	0.87	0.89	0.42
1376427_a_at	Gldc_predicted	Glycine decarboxylase (predicted)	0.85	0.88	0.42
1372685_at	Cdkn3_predicted	Cyclin-dependent kinase inhibitor 3 (predicted)	1.03	1.14	0.42
1369546_at	Bbox1	Butyrobetaine (gamma), 2-oxoglutarate dioxygenase 1	0.90	0.82	0.42
1389566_at	Ccnb2	Cyclin B2	1.13	1.11	0.43
1374157_at	Rgs8	Regulator of G-protein signaling 8	0.76	0.61	0.43
1398282_at	Kynu	Kynureninase (L-kynurenine hydrolase)	0.73	0.96	0.44
1387816_at	Igfals	Insulin-like growth factor binding protein, acid labile subunit	1.09	0.77	0.45
1387528_at	Mbl2	Mannose binding lectin 2, protein C	0.77	0.86	0.45
1387372_at	Slc6a13	Solute carrier family 6, member 13	0.89	0.75	0.45
1376311_at	RGD1563465_predicted	Similar to netrin G1 (predicted)	0.79	0.29	0.45
1374072_at	LOC689898	Hypothetical protein LOC689898	0.97	0.62	0.45
1370355_at	Scd1	Stearoyl-coenzyme A desaturase 1	1.56	1.13	0.45
1368627_at	Rgn	Regucalcin	0.85	0.91	0.45
1387203_at	Gckr	Glucokinase regulatory protein	0.79	1.01	0.46
1388425_at	RGD1305890	Similar to RIKEN cDNA D130038B21	0.83	0.78	0.46
1377412_at	—	Transcribed locus	0.91	0.67	0.46
1377375_at	Aass_predicted	Aminoadipate-semialdehyde synthase (predicted)	0.98	0.80	0.46
1374760_at	—	Transcribed locus	0.76	1.07	0.46
1373967_at	—	Transcribed locus	0.87	0.77	0.46
1367979_s_at	Cyp51	Cytochrome P450, subfamily 51	0.92	0.77	0.46
1367939_at	Rbp1	Retinol binding protein 1, cellular	0.62	0.90	0.46

(continued)

Table 3 *(continued)*

Affymetrix probe set ID	*Gene symbol*	*Gene description*	*Fold change*		
			Dose (mg/kg)		
			15	*50*	*150*
1394068_x_at	Klf2_predicted	Kruppel-like factor 2 (lung) (predicted)	0.74	0.70	0.47
1389681_at	Pvrl2	Poliovirus receptor-related 2	0.88	0.97	0.47
1387307_at	Hal	Histidine ammonia lyase	0.87	0.76	0.47
1373814_at	RGD1310066	Similar to mKIAA1002 protein	0.80	0.84	0.47
1390672_at	Rprm	Candidate mediator of the p53-dependent G2 arrest	0.83	0.79	0.48
1367857_at	Fads1	Fatty acid desaturase 1	1.06	0.93	0.48
1387328_at	Cyp2c	Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)	0.80	1.06	0.48
1386975_at	Pdk2	Pyruvate dehydrogenase kinase, isoenzyme 2	0.61	0.65	0.48
1376785_at	Sycp3	Synaptonemal complex protein 3	0.70	0.85	0.48
1367804_at	Apcs	Serum amyloid P-component	0.70	0.78	0.48
1398759_at	Tgfb1i4	Transforming growth factor beta 1 induced transcript 4	0.72	0.83	0.49
1386041_a_at	Klf2_predicted	Kruppel-like factor 2 (lung) (predicted)	0.83	0.63	0.49
1375599_at	Ddx31_predicted	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 31 (predicted)	0.86	0.57	0.49
1388300_at	Mgst3_predicted	Microsomal glutathione S-transferase 3 (predicted)	0.69	1.00	0.50
1368733_at	Ste	Sulfotransferase, estrogen preferring	0.88	0.98	0.50
1368227_at	Slc28a2	Solute carrier family 28, member 2	1.05	0.84	0.50

Probe sets are sorted by fold change. Shaded probe sets are the ones selected as in-vivo–in-vitro bridging probes (see Figure 3).

sion levels are shown as a heatmap in Figure 4. It appears that the pattern of changes in gene expression is similar in rat and human cells but that the extent of the changes is more prominent in rat cells than in human cells. Among them, "protein kinase inhibitor p58", "DnaJ (Hsp40) homolog, subfamily B, member 11", "crystallin, lamda 1", "hypoxia up-regulated 1", and "aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)", which showed remarkable expression changes both in rat *in vivo* and *in vitro*, did not show any significant changes in human hepatocytes. As for the genes such as "ischemia/reperfusion inducible protein", "glutathione reductase", "glutamate-cysteine ligase, catalytic subunit", "NAD(P)H dehydrogenase, quinone 1", and "DNA-damage-inducible transcript 4-like", these were up-regulated in both species, but the extent of up-regulation was much less in human cells than in the rat.

In the next step, changes in expression of these were examined in cells treated with another hepatotoxicant, DFNa, which is known to elicit a similar response as does coumarin, that is, oxidative stress and glutathione depletion.[8,9] Although not all the genes showed changes in common with those observed after coumarin treatment, the trend appeared similar, suggesting that both compounds share the same toxicological pathway(s).

To make a quantitative comparison of responsiveness of the marker genes between species, the mean value of the effect size of the probe sets (TGP2 score) was calculated (Figure 5). It is obvious from the results presented in Figure 5a that the score shows a good dose-dependency, suggesting that the score successfully expresses the responsiveness of cells to the toxicant. Moreover, in the case of coumarin, the score of human hepatocytes to the marker genes is a much lower value than the score observed for rat cells, supporting the known species-specific difference. However, both rat and human cells responded to the markers to the same extent at the same concentration of DFNa. For genes such as "ischemia/reperfusion inducible protein" and "hypoxia up-regulated 1", these were up-regulated in both species at a high-dose DFNa exposure (data not shown). This clearly indicates that the marker genes respond similarly in rat and human hepatocytes when a drug with a similar level of toxicity in each species is applied.

Discussion

Coumarin is a toxin found in many plants, including the tonka bean, and it has clinical value as the precursor for several anticoagulants, especially warfarin. Although coumarin has a sweet scent, its use as a food additive is restricted because of its hepatotoxicity. It is well known that coumarin is a non-genotoxic hepatocarcinogen in rats, whereas such a property has not been probed in other species.[10] The mechanism of coumarin toxicity has been extensively studied and elucidated; it produces oxidative stress leading to glutathione depletion.[11–13] The species-specific difference between rat and human responses to coumarin has been explained as a difference in detoxification after metabolic activation.[14,15]

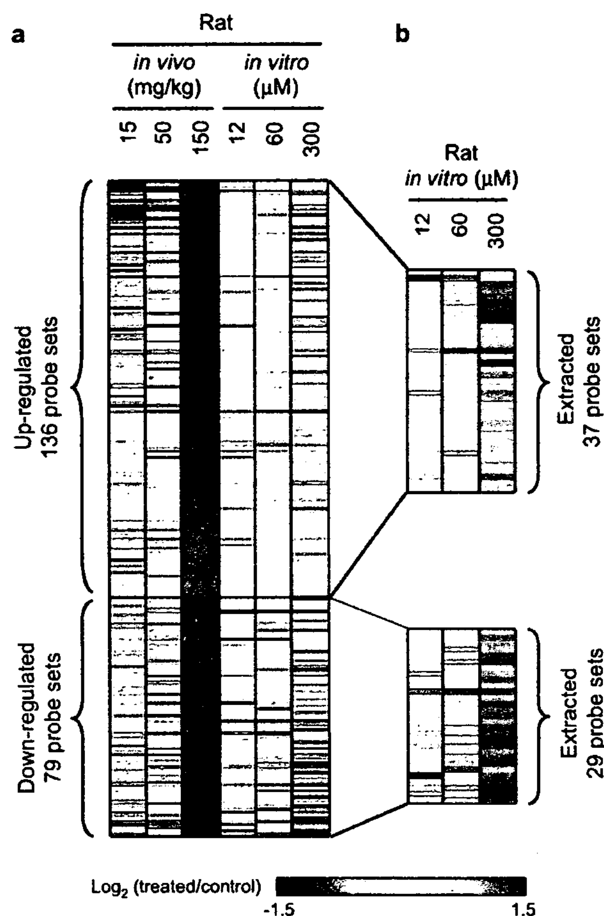

Figure 3 Heatmap of the expression profiles of probe sets in rat liver and rat hepatocytes treated with coumarin. (a) Heatmap of the changes in gene expression induced by coumarin treatment in the in-vivo rat liver (15, 50, 150 mg/kg) and in-vitro hepatocytes (12, 60, and 300 μM). Probes sets were statistically extracted from the data presented in Tables 2 and 3. b: Probe sets with >1.5-fold or <0.6-fold change in rat hepatocytes (the specific sub-set grouped as in-vivo–in-vitro bridging probes are indicated by shading in Tables 2 and 3).

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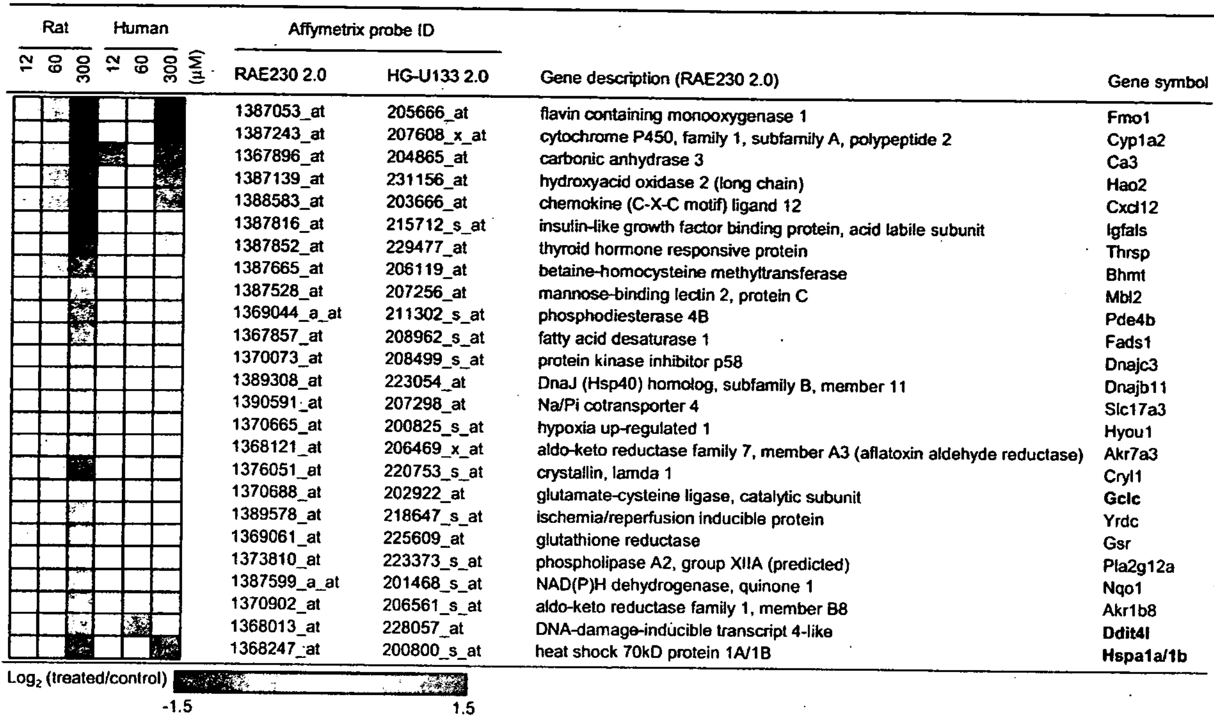
In most species, including humans, coumarin is hydroxylated by CYP2A to 7-hydroxycoumarin (7-HC), a non-toxic metabolite. In rats, however, CYP2A has a stronger affinity for testosterone than for coumarin, such that 7HC levels are extremely low in this species. Another influencing factor is that in rats, coumarin is converted to coumarin 3,4-epoxide (CE), a reactive intermediate that is detoxified via glutathione conjugation and excreted as a conjugate. When the amount of the active metabolite exceeds the cellular capacity for glutathione conjugation, cell injury may occur. However, there is some evidence to suggest that this pathway is of minor importance to hepatotoxicity, as mouse-liver microsomes show hepatic clearance of coumarin via the epoxide intermediate at levels four times greater than that in rats,[16] but hepatotoxicity is not induced by coumarin treatment in mice. CE is spontaneously converted to another toxic compound, *o*-hydroxyphenylacetaldehyde (*o*-HPA). It has been found that *o*-HPA is rapidly detoxified to *o*-hydroxyphenylacetic acid in mice and humans, whereas this pathway works little in rats.[16,17] Therefore, the difference in *o*-HPA detoxification is currently considered to be the main cause of species-specific differences in sensitivity to coumarin.

The main purpose of the present study was to explore a possible strategy for overcoming the problem of species-specific differences in toxicity that affect testing of potential toxins and therapeutic treatments. Specifically, we were interested to test a toxicogenomics-based approach to address species-specific differences in response to toxins. In the livers of rats treated with coumarin, changes in gene expression were observed in various known genes, possibly reflecting a response to oxidative stress, cell injury, and glutathione depletion, and these coumarin-responsive genes seem likely to be related to the hepatotoxic mechanism of coumarin. Of the coumarin-responsive gene identified in the in-vivo rat liver assay, not all but a considerable numbers of the genes were found to be common to those that were coumarin-responsive in the in-vitro assay, with an observable dose-dependency. The present results suggest that whole-transcriptome analysis of the response can be used to estimate the hepatotoxicity of coumarin using the in-vitro rat hepatocytes. In our experience with other compounds, we found that some chemicals showed a considerably different gene expression profile *in vivo* and *in vitro*,[6] whereas the results with coumarin suggest that it is possible to build a reasonable bridge between rat and human responses using an in-vitro cell assay system.

The responsive genes in common to the in-vivo and in-vitro assay datasets were used to identify human ortholog genes useful for making a comparison between rat and human responses. As it is obvious from the results presented in Figure 4, the trend in changes in expression was similar in both species, but the extent of the changes was generally smaller in human cells than in rat cells in accordance with the known species-specific difference in hepatotoxicity. The observation that induction of stress-related genes and glutathione metabolism–related genes was more robust in rat cells than in human cells could be a direct reflection of the extent of stress and subsequent damage caused by coumarin in each species. The genes "Protein kinase inhibitor p58", "DnaJ (Hsp40) homolog, subfamily B, member



RAE230 2.0	HG-U133 2.0	Gene description (RAE230 2.0)	Gene symbol
1387053_at	205666_at	flavin containing monooxygenase 1	Fmo1
1387243_at	207608_x_at	cytochrome P450, family 1, subfamily A, polypeptide 2	Cyp1a2
1367896_at	204865_at	carbonic anhydrase 3	Ca3
1387139_at	231156_at	hydroxyacid oxidase 2 (long chain)	Hao2
1388583_at	203666_at	chemokine (C-X-C motif) ligand 12	Cxcl12
1387816_at	215712_s_at	insulin-like growth factor binding protein, acid labile subunit	Igfals
1387852_at	229477_at	thyroid hormone responsive protein	Thrsp
1387665_at	206119_at	betaine-homocysteine methyltransferase	Bhmt
1387528_at	207256_at	mannose-binding lectin 2, protein C	Mbl2
1369044_a_at	211302_s_at	phosphodiesterase 4B	Pde4b
1367857_at	208962_s_at	fatty acid desaturase 1	Fads1
1370073_at	208499_s_at	protein kinase inhibitor p58	Dnajc3
1389308_at	223054_at	DnaJ (Hsp40) homolog, subfamily B, member 11	Dnajb11
1390591_at	207298_at	Na/Pi cotransporter 4	Slc17a3
1370665_at	200825_s_at	hypoxia up-regulated 1	Hyou1
1368121_at	206469_x_at	aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	Akr7a3
1376051_at	220753_s_at	crystallin, lamda 1	Cryl1
1370688_at	202922_at	glutamate-cysteine ligase, catalytic subunit	Gclc
1389578_at	218647_s_at	ischemia/reperfusion inducible protein	Yrdc
1369061_at	225609_at	glutathione reductase	Gsr
1373810_at	223373_s_at	phospholipase A2, group XIIA (predicted)	Pla2g12a
1387599_a_at	201468_s_at	NAD(P)H dehydrogenase, quinone 1	Nqo1
1370902_at	206561_s_at	aldo-keto reductase family 1, member B8	Akr1b8
1368013_at	228057_at	DNA-damage-inducible transcript 4-like	Ddit4l
1368247_at	200800_s_at	heat shock 70kD protein 1A/1B	Hspa1a/1b

Figure 4 Heatmap of the expression profile of probe sets in rat and human hepatocytes treated with coumarin. Among the set of in-vivo–in-vitro bridging probes for rats, 14 up-regulated and 11 down-regulated probe sets were assigned human orthologs (species bridging markers) and their expression is shown as a heatmap that includes the expression profiles in rat and human hepatocytes treated with 12, 60, or 300 μM coumarin. Note that each probe set responded dose-dependently to coumarin in both species, whereas the extent of the changes appears to be more prominent in rat than in human cells.

11", "crystallin, lamda 1", "hypoxia up-regulated 1", and "aflatoxin aldehyde reductase" were extensively up-regulated in rats both *in vivo* and *in vitro*, whereas almost no change was observed in these genes in human hepatocytes. It will likely be interesting to determine if the gene sets include genes involved in the cause of the species-specific responses that lead to differences in hepatotoxicity and those genes not involved in the response to coumarin. Clearly, it will be necessary to perform additional experiments to address this question.

We next explored the utility of a score, the TGP2-score that is aimed at quantifying responsiveness of a set of marker genes. The TGP2-score is the average of the effect size on gene expression. When species-specific differences in drug-induced gene expression changes are examined, we often encounter clusters of genes, possibly related to toxicological function, that are affected by the drug in both species tested but in different directions (i.e., up-regulated in one but down-regulated in the other). If responsiveness is quantified by taking account of the direction of changes, we might underestimate the extent to which the set of genes affected are similar. Using the TGP2-score, we estimate responsiveness of a given species when expression of a gene in the analysis set is mobilized in either direction. In the present case, however, the direction of expression change was in common between two species in most or all cases, such that the factor did not contribute much to the scores. The score is also clearly useful to visualize quantitative responsiveness of a set of genes to a toxicant (Figure 5) and a prediction of species-specific difference can also be represented (i.e., the higher toxicity of coumarin in rat than in humans or the lack of a species-specific difference in the case of toxicity of DFNa; Figure 5). It also follows that the genes selected in this study may be useful in-vitro markers of oxidative stress–related hepatocyte injury in both rats and humans and that differential responses in these marker genes are indicative of a species-specific difference.

In conclusion, we successfully used a toxicogenomics approach to reproduce the known species-specific difference in hepatotoxicity of coumarin between rats and humans using an in-vitro hepatocyte culture system and microarray analysis. The application of this approach to other chemicals in our database should reveal other examples that can build bridges between species or suggest other strat-

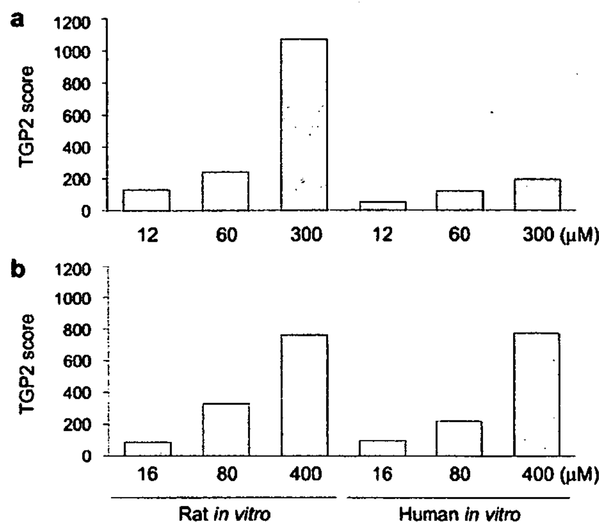


Figure 5 The responsiveness of rat and human hepatocytes expressed as a score based on the effect size (TGP2 score). The expression of each probe set in the set of rat–human bridging probes (Figure 4) was converted to a TGP2 score as described in "Materials and methods" to quantify the responsiveness of each species. (a) Responsiveness to coumarin. Note that the score shows a clear dose-dependency and the expected species-specific difference, that is, rat hepatocytes are more sensitive than human hepatocytes. (b) Responsiveness to diclofenac sodium, a known hepatotoxicant that causes oxidative stress but does not show a species-specific difference in hepatotoxicity. Again, a dose-dependent increase in the score is observed but in this case, as expected, no species-specific difference is observed.

egies for bridging information among species. The most important mission for a toxicologist interested in drug development is to make a precise prediction of the potential clinical toxicity based on animal studies. Toxicogenomics-based approaches are emerging as a promising new avenue of study for making the most of the results of animal studies.

Acknowledgement

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Original Article

Gene expression profiling of methapyrilene-induced hepatotoxicity in rat

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ABSTRACT — The present study was conducted as a model case of the toxicogenomics approach for analyzing toxicological mechanisms and toxicity assessments in the early stage of drug development by comparing with classical toxicology data. Methapyrilene (MP) 100 mg/kg produced obvious histopathological changes in liver of rats by single or repeated dose up to 28 days with significant elevation of ALT and AST. In the middle dose groups (30 mg/kg MP), no apparent changes were noted in blood biochemical data by single dosing or repeated dosing up to one week, and no obvious histopathological changes were observed except a slight hypertrophy in the hepatocytes. Comprehensive gene expression changes were analyzed using Affymetrix GeneChip® and differentially expressed probe sets were statistically extracted. These contained many genes related to "glutathione metabolism", "apoptosis", "MAPK signaling pathway" and "regulation of cell cycle", which were all thought to be involved in the development of presently observed phenotypes. In the high dose groups, TGP1 scores (developed in our system in order to overview the responsiveness of drugs to multiple marker gene lists) for these categories were markedly increased from the early time point after single dose and kept their high expression throughout the repeated dose period. In the middle dose groups, the increment of the scores were noted not only at the time points when apparent pathological changes emerged, but also at the earlier stage of repeated dosing and even after single dosing. We conclude that toxicogenomics would enable a more sensitive assessment at the earlier time point than classical toxicology evaluation.

Key words: Methapyrilene; Rat; Liver; Toxicogenomics; Microarray

INTRODUCTION

The toxicogenomics approach has attracted attention in the field of drug safety assessment as a promising tool in predicting the toxicity of chemicals and analyzing the mechanism of toxicity. Classical toxicology works to characterize the emerged toxic changes, but it is not always powerful in predicting potential toxicity that has not emerged at the point of assessment but might occur in the future or to detect serious disease without apparent change in observation of the classical phenotype. On the other hand, extraction of toxicologically meaningful information from comprehensive gene expression analysis is expected to be useful since these changes precede toxicity and occur in the lower dose range.

The toxicogenomics project was a 5-year collaborative project conducted by the National Institute of Health Sciences, the National Institute of Biomedical Innovation and 15 pharmaceutical companies in Japan that started in 2002 (Urushidani and Nagao, 2005). Its aim was to construct a

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large-scale toxicology database of transcriptome for prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medicinal compounds, were selected, and gene expression in the liver (also the kidney in some cases) was comprehensively analyzed by Affymetrix GeneChip®. In 2007, the project was finished and the whole system, consisting of the database, the analyzing system and prediction system, was completed and named as TG-GATEs (Genomics Assisted Toxicity Evaluation System developed by the Toxicogenomics Project, Japan).

In the present study, we selected methapyrilene, a prototypic hepatotoxicant (Lijinsky *et al.*, 1980), as a model case of the toxicogenomics approach for analyzing the toxicological mechanism and toxicity assessment in the early stage of drug development by comparing with classical toxicology data.

MATERIALS AND METHODS

Chemical

Methapyrilene (MP) was obtained from Sigma Chemical Company (St. Louis, MO, USA) and a suspension formulation was prepared by mixing with 0.5% methylcellulose (MC) solution.

Animal and experimental design

Five-week old male Sprague-Dawley rats were obtained from Charles River Japan Inc. (Kanagawa, Japan). After a 7-day quarantine and acclimatization period, the animals (6-week old) were assigned to dosage groups (5 rats per group) using a computerized stratified random grouping method based on individual body weight. The animals were individually housed in stainless-steel cages in an animal room that was lighted for 12 hr (7:00–19:00) daily, ventilated with an air-exchange rate of 15 times per hour and maintained at 21–25°C with a relative humidity of 40–70%. Each animal was allowed free access to water and pellet diet (CRF-1, sterilized by radiation, Oriental Yeast Co., Tokyo, Japan).

According to the standard protocol in our project, rats received single or repeated doses of MP by gavage at doses of 0 (vehicle only), 10, 30 or 100 mg/kg. For the single-dose study, rats were sacrificed at 3, 6, 9 and 24 hr after dosing. For the repeated dose study, the animals were treated daily for 3, 7, 14 and 28 days, and they were sacrificed 24 hr after the last dosing. The animals were euthanized by exsanguination from the abdominal aorta under ether anesthesia after blood sampling, and liver samples were obtained from the left lateral lobe of the liver in each animal immediately after sacrifice for the following examinations.

The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences.

Blood chemical examination

Blood samples were collected upon sacrifice in tubes containing heparin lithium, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using an auto analyzer (Hitachi 7080).

Histopathological examination

For light microscopic examination, liver samples were fixed in 10% neutral buffered formalin, dehydrated in alcohol and embedded in paraffin. Paraffin sections were prepared and stained by a routine method using hematoxylin and eosin (H&E).

Microarray gene expression analysis

Microarray analysis was conducted on 3 out of 5 samples for each group. Total RNA was isolated from RNAlater® (Ambion, Austin, TX, USA)-preserved samples using RNeasy kit by Bio Robot 3000 (Qiagen, Valencia, CA, USA). Homogenization was conducted by Mill Mixer (Qiagen) and zirconium beads. Purity of RNA was checked by gel electrophoresis confirming that the 260/280 nm ratio was between 2.2 and 3.0. Gene expression analysis was carried out using GeneGhip® RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA) containing 15,923 probe sets. The procedure was basically conducted according to the manufacture's instructions as previously reported. Microarray Analysis Suite 5.0 (MAS; Affymetrix) was used to quantify microarray signals and the intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization).

Microarray data analysis

To determine differentially expressed genes between high and time-matched control sample groups, Welch's *t* test was applied with a p cut off value of 0.01 in combination with a 1.5-fold regulation-ratio of means using GeneSpring software (Agilent Technologies, Inc., Santa Clara, CA, USA). Probe sets, which were labeled as absent by Affymetrix detection call in any of the 48 samples in single or repeated dose study, were excluded from further analysis. For the extracted probe sets, showing significant changes for at least one time point of either single or repeated administrations, pathway and GO analysis was performed using David 2.1 beta (http://david.abcc.ncifcrf.gov/) to identify overrepresented gene categories in

each gene list, and a p value of < 0.05 determined by Fisher's exact test was considered statistically significant.

Scoring of the marker gene sets (TGP1 score)

To facilitate the analytical procedures for large-scale microarray data, we developed a simple one-dimensional score, named TGP1, which is useful to overview the trend of the changes in expression of multiple biomarker gene lists all at once (Kiyosawa *et al.*, 2006). For each gene list, the signal log ratio was calculated by dividing the mean signal value of the chemical-treated group by that of the corresponding control. First, the sum of the signal log ratios for the used probe sets was calculated, and then divided by the number of probe sets used (Index 1). Next, the sum of squared signal log ratios for the used probe sets was calculated, and then divided by the number of probe sets used (Index 2). Finally, the TGP1 score was calculated by multiplying Index 1 with Index 2.

Statistical analysis

For blood chemical parameters, ANOVA followed by Dunnett's multiple comparison test or Kruskal-Wallis mean rank test followed by Mann-Whitney's U test was used as appropriate (Snedecor and Cochran, 1989).

RESULTS

Conventional toxicological parameters

Measurements of AST and ALT, representative markers of hepatotoxicity, are shown in Fig. 1 and histopathological findings are summarized in Tables 1 and 2. In the highest dose groups, both AST and ALT were elevated 3 or 6 hr after treatment, and they kept increasing with time. They also showed histopathological changes at every time point, such as hepatocellular hypertrophy and single cell necrosis in the peripheral lobular region, and obvious inflammation and anisonucleosis were noted at 24 hr after dosing (Fig. 2a). Furthermore, these lesions were aggravated and additional regenerative changes such as increased mitosis, bile duct proliferation, and hyperplasia, during repeated administrations were evident (Fig. 2b). In the middle dose groups, no apparent changes were noted in blood biochemical data by single dosing or repeated dosing up to one week, and no obvious histopathological changes were observed except a slight hypertrophy in the hepatocytes. At the time of the 15th and 29th days, single cell necrosis and infiltration of mononuclear cells were noted as in the highest group, though their severity was low. In the lowest dose groups, no obvious changes were noted either in blood biochemistry or in histopathology except for one case with hepatocyte hypertrophy in each of the 8th and 15th days.

Gene expression changes

Using the highest dose group showing obvious hepatotoxicity in terms of both blood biochemistry and histopathology, we extracted genes that showed statistically significant changes at least once in any time point in single and repeated dose experiments. For single and repeated dosing, up-regulated probe sets were 399 and 2509, respectively, and down-regulated probe sets were 235 and 876, respectively, namely, expression changes occurred in many genes in repeated dosing where obvious pathophysiological changes emerged.

Extracted genes were categorized by pathway and GO analysis and the results are summarized in Tables 3 (up-regulated) and 4 (down-regulated). Among the genes up-regulated in single dose experiments, gene ontologies or pathways related to "regulation of cell cycle", "MAPK signaling pathway" and "glutathione metabolism" were still significantly up-regulated in repeated dosing. The genes related to "apoptosis" or "ribosome" were clearly up-regulated in repeated dosing, while they were not affected by single dosing.

In single dose experiments, the number of down-regulated genes was quite a few (Table 4). Significant suppression of gene expression by repeated administration was noted in various categories, including "starch and sucrose metabolism", "steroid metabolism", "complement activation" and "complement and coagulation cascades".

We considered the pathways and GO categories of "glutathione metabolism", "apoptosis", "MAPK signaling" and "regulation of cell cycle" as most important in the toxicological mechanisms of MP. In order to overview the effects of MP on these pathways, we calculated the TGP1-score for each (Table 5). Upon calculation of the score, redundant probe sets were unified based on their reliability and dose-dependency. It is obvious from Table 5 that the marker scores of these categories markedly increased in the early stage of single dosing of the highest dose and they kept increasing during repeated dosing. In case of middle dosing, an obvious increase of scores for glutathione metabolism and apoptosis was detected not only at the 15th and 29th days with obvious pathological changes but also at the 4th and 8th days of repeated dosing and 6 hr after single dosing. In the lowest dose groups, the only detectable change was a tendency of increment in the score of apoptosis at the 8th day.

Up-regulated genes involved in glutathione metabolism, apoptosis, MAPK signaling pathway, and regulation of cell cycle

The individual gene expression change (ratio to control) in each category was depicted as heatmap (Fig. 3 to 6) for "glutathione metabolism", "apoptosis", "MAPK signaling" and "regulation of cell cycle", respectively. In general, most of the genes were dose-dependently mobilized and characteristic changes were noticed in single and repeated dosing groups. As for genes involved in glutathione biosynthesis: glutamate cysteine ligase, modifier subunit (Gclm) and glutamate-cysteine ligase, catalytic subunit (Gclc) (Fig. 3); those involved in the regulation of apoptosis: v-akt murine thymoma viral oncogene homolog 1 (Akt1) and programmed cell death 6 interacting protein (Pdcd6ip) (Fig. 4), and those belonging to heat shock proteins: heat shock 70 kD protein 1A/1B (Hspa1a/1b) and heat shock protein 8 (Hspa8) (Fig. 5), these were markedly up-regulated in the early stage of single dose, whereas little or no changes were noted in repeated dosing. Excluding these genes, the extent of up-regulation increased with repeated administration in most of the genes. Especially, those involved in glutathione metabolism: glucose-6-phosphate dehydrogenase (G6pdx), glutathione *S*-transferase M4 (Gstm4) and glutathione *S*-transferase Yc2 subunit (Yc2) (Fig. 3), those involved in regulation of apoptosis: nucleolar protein 3 (Nol3), rhoB gene (Rhob) and tribbles homolog 3 (Drosophila) (Trib3) (Fig. 4), those belonging to MAPK signaling and known as cell cycle regulators: myelocytomatosis viral oncogene homolog (avian) (Myc),

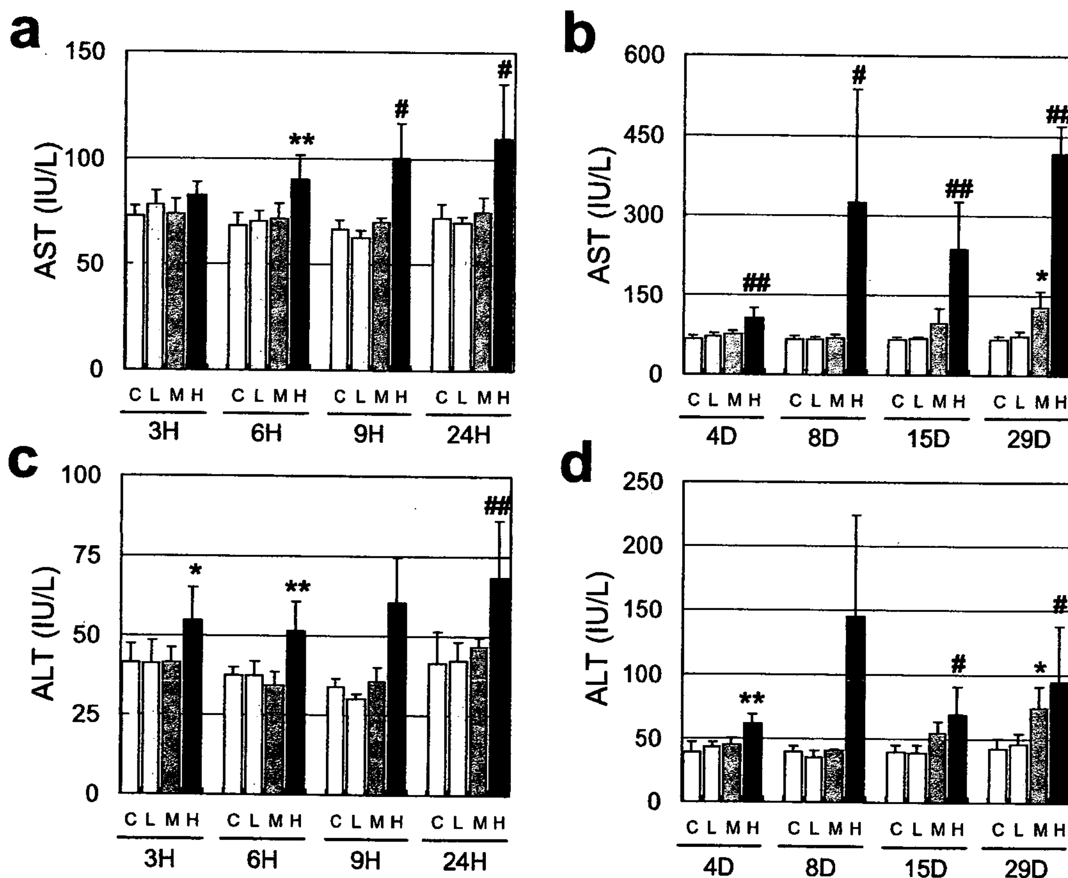

Fig. 1. Serum AST (a and b) and ALT activities (c and d) in rats treated with 10, 30 and 300 mg/kg MP in single and repeated dose studies.
Data are expressed as mean± S.D. (n = 5). *, **Significant difference from the control group, $p < 0.05, 0.01$, by Dunnett's multiple comparison test. #, ##Significant difference from the control group, $p < 0.05, 0.01$, by Mann-Whitney's U test.

Table 1. Histopathological findings in rat liver treated with MP in single dose study.

Morphology	Time Point (hrs)	3			6			9			24		
	Dose (mg/kg)	10	30	100	10	30	100	10	30	100	10	30	100
	Number of animals examined	5	5	5	5	5	5	5	5	5	5	5	5
Hepatocyte / Anisonucleosis		0	0	0	0	0	0	0	0	0	0	0	5
slight													5
Hepatocyte / Hypertrophy		0	0	1	0	0	3	0	0	4	0	1	5
slight				1			3			4		1	5
Hepatocyte / Single cell necrosis		0	0	1	0	0	3	0	0	5	0	0	5
slight				1			3			5			5
Periportal / Cellular infiltration, mononuclear cell		0	0	0	0	0	0	0	0	5	0	0	5
slight										5			5

Vehicle alone, or MP 10, 30, or 100 mg/kg was administered orally to rats, and the animals were euthanized at 3, 6, 9 and 24 hr after dosing (n = 5). The histopathological change in liver was graded into 4 categories: very slight, slight, moderate, and severe. The number of animals affected at each grade is shown.

Table 2. Histopathological findings in rat liver treated with MP in repeated dose study.

Morphology	Time Point (days)	4			8			15			29		
	Dose (mg/kg)	10	30	100	10	30	100	10	30	100	10	30	100
	Number of animals examined	5	5	5	5	5	5	5	5	5	5	5	4[a]
Hepatocyte / Alteration, cytoplasmic		0	0	0	0	0	0		0	0	0	0	1
slight													1
Hepatocyte / Anisonucleosis		0	0	3	0	0	4		0	5	0	0	4
slight				3			4			5			4
Hepatocyte / Hyperplasia		0	0	0	0	0	0		0	0	0	0	4
slight													4
Hepatocyte / Hypertrophy		0	2	5	1	2	5		3	5	1	5	4
slight			2	5	1	2	5		3	5	1	5	4
Hepatocyte / Increased mitosis		0	1	4	0	0	3		1	0	0	3	3
slight			1	4			3		1			3	3
Hepatocyte / Single cell necrosis		0	0	5	0	0	5		3	5	0	3	4
slight				5			5		3	5		3	4
Interlobular / Proliferation, bile duct		0	0	5	0	0	5		1	5	0	0	4
slight				5			5		1	4			
moderate										1			4
Periportal / Cellular infiltration, mononuclear cell		0	1	4	0	0	4		5	5	0	2	4
slight			1	4			4		5	2		2	4
moderate										3			
Periportal / Deposit, pigment		0	0	0	0	0	0		0	0	0	0	3
slight													3

Vehicle alone, or MP 10, 30, or 100 mg/kg was administered orally to rats once daily for 1, 3, 7, 14, and 28 days, and the animals were euthanized at 24 hr after dosing, namely, on 2, 4, 8, 15, and 29 days (n = 5). [a]One of the 5 rats died and was not examined histopathologically due to advanced autolysis. For more detailed information, see Table 1.

FBJ murine osteosarcoma viral oncogene homolog (Fos), v-jun sarcoma virus 17 oncogene homolog (avian) (Jun) and fibroblast growth factor 21 (Fgf21) (Fig. 5), and those related to DNA damage: growth arrest and DNA-damage-inducible 45 alpha (Gadd45a) and DNA-damage inducible transcript 3 (Ddit3) (Fig. 6), these kept up-regulated throughout the repeated dosing periods.

DISCUSSION

Methapyrilene hydrochloride is an antihistamine drug and had been used in the 1970s, but was removed from the market once it was known to be carcinogenic in rat liver (Lijinsky *et al.*, 1980; Fischer *et al.*, 1983). It is now considered to be a rat-specific carcinogen since hepatocellular carcinoma and cholangiocarcinoma were induced by administration of MP at 1000 ppm for 64 weeks, whereas no such findings were observed either in Syrian hamsters, Guinea-pigs, B6C3F1 mice, or humans (Mirsalis, 1987). As for its genotoxicity, the Ames test, DNA addition test, chromosome abnormality test (NTP, 2000) and irregular DNA synthesis test in rat and mouse (Steinmetz *et al.*, 1988) were all negative, whereas the cell transformation assay and L5178Y/TK+/− mouse lymphoma assay were positive (Turner *et al.*, 1987). Based on these observations, hepatocarcinogenicity of MP in rat has been considered to be non-genotoxic, whereas the involvement of its initiation activity cannot be completely excluded (Althaus *et al.*,

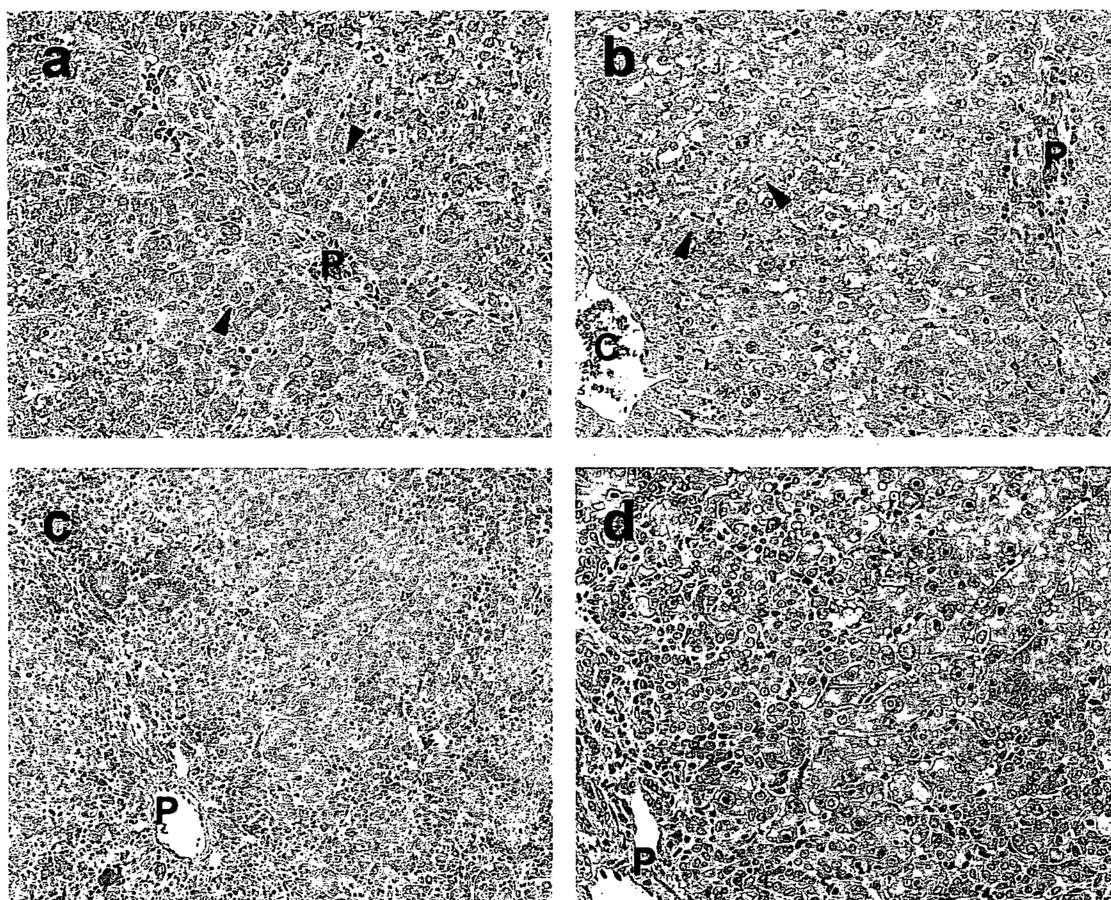

Fig. 2. Histopathological changes of liver treated with 100 mg/kg MP.
a: Hepatocellular hypertrophy and single cell necrosis (arrow head) in the periportal region (P) are observed at early time point, 24 hr after single dosing. b: Additional regenerative changes, such as increased mitosis, bile duct proliferation, and hyperplasia are evident by repeated administration.

Table 3. Gene ontology and pathway classification of extracted probe sets (up-regulation).

Exp. type	TERM[a]	Count[b]	p value[c]
Single dose study			
	GOTERM_BP_5		
	REGULATION OF NUCLEOBASE, NUCLEOSIDE, NUCLEOTIDE AND NUCLEIC ACID METABOLISM	18	6.41E-2
	TRANSCRIPTION	18	7.48E-2
	MACROMOLECULE BIOSYNTHESIS	**13**	**1.92E-2**
	PROTEIN BIOSYNTHESIS	**11**	**3.84E-2**
	REGULATION OF CELL CYCLE	**11**	**5.95E-4**
	INTRACELLULAR TRANSPORT	**9**	**8.5E-2**
	AMINO ACID METABOLISM	6	2.75E-2
	AMINE BIOSYNTHESIS	5	9.11E-3
	CELL GROWTH	5	4.86E-2
	NUCLEAR TRANSPORT	5	2.6E-3
	NUCLEOCYTOPLASMIC TRANSPORT	5	6.12E-3
	PROTEIN KINASE CASCADE	5	4.18E-2
	REGULATION OF CELL SIZE	5	4.86E-2
	RNA METABOLISM	5	4.63E-2
	POSITIVE REGULATION OF NUCLEOBASE, NUCLEOSIDE, NUCLEOTIDE AND NUCLEIC ACID METABOLISM	4	8.74E-2
	PROTEIN IMPORT	4	1.49E-2
	RNA PROCESSING	4	6E-2
	GOTERM_CC_5		
	NUCLEUS	34	7.29E-3
	KEGG PATHWAY		
	MAPK SIGNALING PATHWAY (Rattus norvegicus)	**10**	**3.73E-2**
	GAP JUNCTION (Rattus norvegicus)	6	5.15E-2
	TGF-BETA SIGNALING PATHWAY (Rattus norvegicus)	5	8.22E-2
	ARGININE AND PROLINE METABOLISM (Rattus norvegicus)	4	3.69E-2
	GLUTATHIONE METABOLISM (Rattus norvegicus)	**4**	**2.42E-2**
Repeated dose study			
	GOTERM_BP_5		
	CELLULAR PROTEIN METABOLISM	189	2.86E-8
	MACROMOLECULE BIOSYNTHESIS	**89**	**4.92E-17**
	PROTEIN BIOSYNTHESIS	**85**	**2.13E-19**
	INTRACELLULAR TRANSPORT	**60**	**1.22E-9**
	PROTEIN TRANSPORT	47	1.48E-6
	INTRACELLULAR PROTEIN TRANSPORT	44	4.84E-7
	APOPTOSIS	38	3.07E-4
	REGULATION OF CELL CYCLE	**32**	**6.48E-4**
	REGULATION OF APOPTOSIS	31	2.99E-4
	REGULATION OF PROGRAMMED CELL DEATH	31	3.48E-4
	GOTERM_CC_5		
	VESICLE-MEDIATED TRANSPORT	30	1.48E-2
	CYTOSKELETON	64	6.02E-2
	RIBOSOME	60	1E-13
	MICROTUBULE CYTOSKELETON	41	2.59E-3
	KEGG_PATHWAY		
	MICROTUBULE ASSOCIATED COMPLEX	29	6.25E-2
	CYTOSOLIC RIBOSOME (SENSU EUKARYOTA)	26	2.79E-11
	RIBOSOME (Rattus norvegicus)	40	1.79E-24
	FOCAL ADHESION (Rattus norvegicus)	36	6.68E-2
	MAPK SIGNALING PATHWAY (Rattus norvegicus)	**33**	**7.61E-2**
	TIGHT JUNCTION (Rattus norvegicus)	27	4.25E-3
	GLUTATHIONE METABOLISM (Rattus norvegicus)	**8[d]**	**4.55E-2**

Pathway and GO analysis was performed using David 2.1 beta. Statistical significant terms are listed (Fisher's exact test, $p < 0.05$; threshold counts: greater than 10% of the number of probe sets involved in the examined gene list). Bold terms were commonly affected in both single and repeated dose studies. Shaded terms were further analyzed by scoring based on the TGP1-score.

Table 4. Gene ontology and pathway classification of extracted probe sets (up-regulation).

Exp. type	TERM[a]	Count[b]	p value[c]
Single dose study			
	GOTERM_BP_5		
	REGULATION OF NUCLEOBASE, NUCLEOSIDE, NUCLEOTIDE AND NUCLEIC ACID METABOLISM	13	9.72E-2
	REGULATION OF TRANSCRIPTION	13	9.51E-2
	RESPONSE TO CHEMICAL SUBSTANCE	4	8.4E-2
	CHEMOTAXIS	3	5.47E-2
	STEROL METABOLISM	3	6.27E-2
	KEGG_PATHWAY		
	STARCH AND SUCROSE METABOLISM (Rattus norvegicus)	3	4.97E-2
Repeated dose study			
	GOTERM_BP_5		
	CARBOXYLIC ACID METABOLISM	45	2.26E-16
	ELECTRON TRANSPORT	37	2.19E-8
	CELLULAR LIPID METABOLISM	34	7.06E-8
	IMMUNE RESPONSE	27	5.94E-2
	RESPONSE TO PEST, PATHOGEN OR PARASITE	21	4.78E-5
	AMINO ACID METABOLISM	20	1.32E-7
	CELLULAR CARBOHYDRATE METABOLISM	18	1.36E-3
	LIPID BIOSYNTHESIS	15	2.41E-3
	STEROID METABOLISM	15	1.6E-5
	WOUND HEALING	14	4.27E-5
	BLOOD COAGULATION	13	2.03E-6
	FATTY ACID METABOLISM	13	2.59E-3
	MONOSACCHARIDE METABOLISM	13	3.96E-3
	AMINO ACID DERIVATIVE METABOLISM	12	2.04E-4
	COENZYME METABOLISM	12	1.31E-2
	COFACTOR BIOSYNTHESIS	11	1.7E-2
	COMPLEMENT ACTIVATION	11	1.28E-7
	HUMORAL IMMUNE RESPONSE	11	3.22E-6
	AMINE CATABOLISM	10	1.37E-5
	RESPONSE TO CHEMICAL SUBSTANCE	10	2.22E-2
	INFLAMMATORY RESPONSE	9	3.47E-2
	GOTERM_CC_5		
	MITOCHONDRION	38	2.07E-5
	ENDOPLASMIC RETICULUM	26	8.26E-6
	MICROSOME	18	5.64E-7
	KEGG_PATHWAY		
	TRYPTOPHAN METABOLISM (Rattus norvegicus)	22	5.32E-12
	COMPLEMENT AND COAGULATION CASCADES (Rattus norvegicus)	17	7.4E-7
	FATTY ACID METABOLISM (Rattus norvegicus)	17	3.65E-7
	GLYCINE, SERINE AND THREONINE METABOLISM (Rattus norvegicus)	11	5.69E-7
	BUTANOATE METABOLISM (Rattus norvegicus)	9	7.11E-4
	GAMMA-HEXACHLOROCYCLOHEXANE DEGRADATION (Rattus norvegicus)	9	1.29E-3
	LYSINE DEGRADATION (Rattus norvegicus)	9	1.26E-5
	PYRUVATE METABOLISM (Rattus norvegicus)	9	3.61E-4
	STARCH AND SUCROSE METABOLISM (Rattus norvegicus)	**9**	**1.08E-4**
	VALINE, LEUCINE AND ISOLEUCINE DEGRADATION (Rattus norvegicus)	9	3.61E-4

1982).

The analysis of hepatotoxicity of MP has been repeatedly performed by various techniques including the toxicogenomics approach (Hamadeh *et al.*, 2002). This compound induces marked and reproducible hepatic injury in rodents, and was used to assess the validity of toxicogenomics analyses among the multicenter platform (Waring *et al.*, 2004; Chu *et al.*, 2004). In the former study, there was a pessimistic interpretation that microarrays never supply highly reliable measures because of too large variance between research facilities. In this case, samples from the same animal were analyzed in multiple facilities but there were almost no genes that were detected as commonly changed in all the facilities. However, the latter study revealed that the robustness of the results regarding the movement of certain toxicological pathways was sufficient although the fitness of each gene was somewhat questionable. In other words, when we have a reasonable list of genes with certain toxicological significance, the reliability would be highly improved. The strategy of our project follows this idea, i.e., the results are interpreted as a trend for a set of functional genes.

Presently extracted genes from the group receiving the highest dose (showing obvious phenotypes) were categorized and this revealed that genes related to the regulation of cell cycle, MAPK signaling, and the glutathione metabolism were all involved in the development of the presently observed phenotypes. As for the down-regulated genes in repeated dosing, it could be a reflection of the failure of hepatic functions, *i.e.*, metabolism of sugar and sterols, and production of functional proteins such as complements and blood coagulation.

To facilitate the analytical procedures for our large-scale microarray database, we developed two types of the one-dimensional score, named as TGP1 and TGP2, which express the trend of the changes in expression of biomarker genes as a whole. The former is based on the signal log ratio (Kiyosawa *et al.*, 2006) and is convenient to compare the responsiveness of many drugs to a marker gene list. The disadvantages of this scoring system are that it overestimates the responsiveness when the list contains a gene where the induction is extreme (such as CYP1A1) and it

Table 5. Time course changes of TGP-1 scores in selected MP-responsive gene lists.

MP-RESPONSIVE GENE LISTS	03H			06H			09H			24H		
	L	M	H	L	M	H	L	M	H	L	M	H
GLUTATHIONE METABOLISM	23	2	7	2	39	607	–2	24	498	–35	–1	409
APOPTOSIS	6	7	103	3	24	342	8	10	128	0	2	195
MAPK SIGNALING PATHWAY	3	9	190	–2	7	114	–5	–26	15	–5	–2	57
REGULATION OF CELL CYCLE	3	2	108	–2	5	133	–3	–1	33	–3	–2	21
MP-RESPONSIVE GENE LISTS	04D			08D			15D			29D		
	L	M	H	L	M	H	L	M	H	L	M	H
GLUTATHIONE METABOLISM	2	118	476	2	170	3466	0	235	2285	5	712	2865
APOPTOSIS	2	54	286	93	227	1172	3	154	1360	–4	115	1396
MAPK SIGNALING PATHWAY	13	3	34	13	60	295	3	68	354	7	29	378
REGULATION OF CELL CYCLE	10	4	7	13	20	219	1	15	247	4	28	470

AFFYMETRIX PROBE ID	SYMBOL	03H			06H			09H			24H			04D			08D			15D			29D		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
1367856_at	G6pdx	1.1	0.9	0.9	1.2	1.4	2.5	1.1	1.5	4.2	1.0	1.4	2.9	1.4	0.8	1.4	0.6	0.9	1.8	1.1	1.2	3.9	1.0	0.8	5.0
1368374_a_at	Ggt1	1.1	1.0	0.9	1.1	0.9	0.7	1.0	0.7	0.9	1.0	1.2	1.3	1.0	1.0	1.3	1.3	1.4	2.8	1.1	1.3	4.5	0.9	1.3	8.0
1369061_at	Gsr	1.2	1.1	1.2	1.1	1.5	1.8	1.2	1.8	2.7	0.9	1.2	1.7	0.9	1.2	1.2	1.0	1.2	2.1	0.8	1.0	1.6	0.9	1.2	1.9
1369921_at	Gstm4	1.1	1.1	1.1	1.2	2.0	12	0.7	1.1	6.9	0.4	0.7	6.6	1.2	4.6	7.7	1.1	1.0	63	0.7	5.0	30	1.3	6.8	6.5
1369926_at	Gpx3	1.1	1.0	0.9	1.2	1.0	1.1	0.8	0.9	0.8	0.9	0.9	0.9	1.0	1.0	0.8	0.9	0.9	2.0	1.0	1.1	2.9	1.1	1.3	9.1
1370030_at	Gclm	1.1	1.2	1.3	1.2	1.4	2.5	1.2	1.8	2.1	0.9	0.7	1.0	1.1	1.3	1.0	1.1	1.0	1.6	1.0	1.2	1.3	0.9	0.8	1.0
1370365_at	Gss	1.4	1.1	1.2	1.0	1.3	1.2	0.9	1.0	1.2	1.1	1.1	2.1	0.9	1.1	1.6	0.7	0.9	2.0	1.0	1.1	2.7	1.0	1.2	2.4
1371089_at	Yc2	2.4	1.3	1.4	1.0	1.8	2.8	0.9	1.4	3.9	0.7	1.2	5.6	0.9	1.9	6.7	1.7	5.9	30	1.4	4.6	20	1.7	11	34
1372523_at	Gclc	1.4	1.4	1.7	1.4	2.4	3.6	1.4	2.2	3.8	1.0	0.9	1.4	0.9	1.2	1.3	1.0	1.1	1.1	1.0	1.1	1.0	0.9	1.0	1.0
1374070_at	Gpx2	1.3	0.9	1.0	1.2	1.0	1.8	0.7	0.6	1.5	0.9	1.0	1.3	1.4	1.2	2.0	1.4	1.1	3.7	1.2	1.3	3.4	1.3	1.6	13

The number in each column expresses the ratio to control (N=3).

Fig. 3. Heatmap of individual gene expression change in category of "glutathione metabolism".

also underestimates the responsiveness when the genes in the list are mobilized to either direction. To overcome these disadvantages, we employed another score, TGP2, based on the effect size. In the present study, we employed the TGP1 score for assessment of the responsiveness to the gene lists, *i.e.*, "regulation of cell cycle", "MAPK signaling" and "glutathione metabolism" since the direction of expression changes was uniform. In the highest dose group, the scores for these categories markedly increased from the early time point after single dose and kept their high expression throughout the repeated dose period. In the middle dose groups, the increment of the scores were noted not only at the time points when apparent pathological changes emerged, but also at the earlier stage of repeated dosing and even after single dosing. This indicates that the toxicogenomics approach enables more sensitive assessment at the earlier time point than classical toxicology evaluation. Among the responding genes, glutathione-related: glucose-6-phosphate dehydrogenase (G6pdx), glutathione *S*-transferase M4 (Gstm4) and glutathione *S*-transferase Yc2 subunit (Yc2), apoptosis related: nucleolar protein 3 (Nol3), rhoB gene (Rhob) and tribbles homolog 3 (Drosophila) (Trib3), MAPK signaling-related: myelocytomatosis viral oncogene homolog (avian) (Myc), FBJ murine osteosarcoma viral oncogene homolog (Fos), v-jun sarcoma virus 17 oncogene homolog (avian) (Jun) and fibroblast growth factor 21 (Fgf21), and DNA damage-related: growth arrest and DNA-damage-inducible 45 alpha (Gadd45a) and DNA-damage inducible transcript 3 (Ddit3), these were markedly up-regulated from the early point of dosing. Especially, Trib3, which showed typical changes in the present study, would be one

AFFYMETRIX PROBE ID	SYMBOL	03H			06H			09H			24H			04D			08D			15D			29D		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
1367827_at	Ppp2cb	1.1	1.0	1.0	0.9	0.9	1.0	1.1	1.0	1.0	0.9	0.9	1.1	0.9	1.0	1.1	1.0	1.1	1.8	0.9	1.2	2.1	1.0	1.2	2.2
1367831_at	Tp53	1.1	1.2	1.2	0.9	0.8	1.0	1.0	0.9	0.9	0.9	1.0	1.1	1.3	1.0	1.3	1.3	1.3	2.0	1.3	1.0	2.4	0.8	1.2	2.0
1367856_at	G6pdx	0.9	1.2	4.8	1.1	1.6	13	1.3	1.5	2.8	1.0	1.0	1.1	1.4	0.8	1.4	0.6	0.9	1.8	1.1	1.2	3.9	1.0	0.8	5.0
1367890_at	Casp2	1.0	0.8	0.8	1.0	0.8	0.9	0.9	1.1	0.8	0.9	1.0	1.1	1.1	1.0	1.0	1.1	1.2	1.3	0.9	1.0	1.2	1.1	1.1	1.9
1367922_at	Adam17	1.0	1.1	0.9	1.0	1.1	1.5	1.1	1.2	1.8	1.0	1.0	1.1	1.1	0.9	1.0	1.1	1.1	1.0	1.1	0.9	1.1	1.1	1.1	1.7
1368118_at	Bcl10	0.9	0.9	1.0	1.0	0.9	0.8	1.0	0.8	0.9	0.9	1.1	1.1	1.1	0.9	1.0	0.9	0.9	1.6	0.9	1.0	1.6	1.1	1.3	1.8
1368305_at	Casp6	1.1	1.0	1.0	0.7	0.9	0.8	0.8	0.8	0.9	0.9	0.9	1.0	1.3	1.0	1.4	1.0	0.9	1.1	1.0	1.1	1.1	0.9	1.1	1.8
1368544_a_at	Nol3	1.2	1.8	3.1	2.2	3.2	13	1.2	1.0	2.5	2.2	1.5	4.3	1.4	1.4	1.4	1.5	1.0	2.9	1.3	1.5	7.5	0.6	2.5	10
1368856_at	Jak2	1.1	1.1	1.0	1.1	0.9	1.1	1.0	1.1	1.1	1.3	1.2	1.3	1.3	1.0	1.1	1.0	1.1	1.6	1.0	0.8	2.4	0.9	1.1	3.9
1368862_at	Akt1	0.5	0.7	0.9	0.8	1.4	1.6	1.9	3.7	4.5	1.1	1.2	1.4	0.9	0.8	0.9	1.0	0.9	1.3	1.0	1.0	1.6	0.8	1.0	1.4
1368888_a_at	Rtn4	1.1	1.0	1.1	0.9	1.2	1.9	1.1	1.3	1.9	1.1	0.9	1.1	1.3	0.9	1.0	0.9	1.8	2.5	1.3	1.8	2.7	0.8	1.7	4.2
1369104_at	Prkaa1	1.2	1.3	1.8	0.9	1.3	2.6	1.4	1.2	1.3	1.0	0.7	1.0	0.9	1.1	1.3	0.7	0.8	1.6	0.9	1.4	2.1	1.0	1.5	2.0
1369122_at	Bax	1.1	1.1	1.1	0.9	1.1	1.1	1.0	0.9	1.1	1.0	1.0	1.2	1.0	0.9	1.2	1.2	1.4	3.7	0.8	1.1	3.3	0.8	1.6	4.3
1369948_at	Ngfrap1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.3	0.9	1.0	1.4	1.3	1.7	1.7	0.7	1.1	12	1.5	1.7	37	0.9	4.5	63
1369958_at	Rhob	1.0	1.4	2.9	1.2	1.2	3.4	1.2	1.0	2.2	1.1	1.1	2.7	0.9	1.0	1.1	1.0	1.1	2.0	1.2	1.3	3.0	1.0	1.5	3.9
1369995_at	Faf1	0.9	0.9	0.9	1.0	1.0	1.0	1.0	1.1	1.1	1.1	0.9	1.1	1.0	1.0	1.0	1.0	1.0	1.3	0.9	0.9	1.2	1.0	1.0	1.6
1370080_at	Hmox1	0.8	0.9	1.0	1.0	1.0	1.2	1.0	0.8	0.8	1.1	0.8	1.3	1.2	1.0	1.0	1.1	1.1	1.7	1.2	1.4	2.1	0.9	1.1	2.5
1370113_at	Birc3	1.0	1.0	1.1	0.8	1.2	1.1	1.2	0.9	1.2	1.1	1.1	1.2	1.3	0.9	1.2	0.9	0.9	1.1	0.7	0.9	1.4	1.0	1.2	1.9
1370141_at	Mcl1	0.9	1.0	1.0	1.0	1.0	1.1	1.0	0.9	0.9	1.0	1.1	1.3	1.1	1.1	1.2	1.0	1.2	1.6	1.1	1.2	1.4	0.9	1.0	1.6
1370226_at	Cstb	0.9	1.0	1.7	1.0	1.0	3.9	1.2	1.1	1.9	0.8	0.7	0.9	1.2	1.0	1.1	0.9	1.2	1.4	1.0	1.2	2.0	1.0	1.1	2.4
1370243_a_at	Ptma	0.9	0.8	0.9	1.1	1.0	0.9	0.9	0.7	0.8	1.0	1.2	1.2	1.0	0.9	1.0	1.0	1.0	1.3	0.9	1.0	1.5	0.9	1.1	1.7
1370290_at	Tubb5	1.0	1.1	1.2	0.9	1.1	1.4	1.0	1.1	1.1	0.9	0.9	1.0	1.1	1.1	1.1	0.8	0.8	1.3	0.9	0.9	1.8	1.1	1.2	2.6
1370695_s_at	Trib3	0.9	1.1	0.9	1.2	1.5	3.7	1.1	1.3	3.1	1.3	1.5	1.9	1.0	6.5	27	9.8	21	137	1.1	13	77	2.6	8.0	40
1371572_at	App	1.1	0.9	0.9	1.2	1.4	2.5	1.1	1.5	4.2	1.0	1.4	2.9	1.1	1.0	1.4	1.1	1.4	5.0	1.1	1.7	8.0	1.0	2.3	8.9
1373733_at	Bok	0.9	0.9	0.9	0.9	1.0	1.1	1.1	1.2	1.2	0.9	1.0	1.4	1.2	1.0	1.1	1.2	1.0	1.1	1.0	1.0	2.2	1.0	1.1	3.5
1386866_at	Ywhag	1.1	1.0	1.1	1.0	1.0	1.1	1.1	1.0	1.1	1.0	1.0	1.2	1.0	1.0	1.2	1.1	1.2	1.7	1.0	1.1	1.8	1.0	1.2	2.2
1387021_at	Wig1	1.0	1.1	1.1	1.1	1.1	1.7	1.1	1.1	1.6	0.9	0.9	1.2	1.1	1.0	1.1	0.9	1.0	1.5	1.1	1.1	2.3	1.2	1.3	3.4
1387087_at	Cebpb	0.9	1.0	1.0	1.2	1.3	0.7	0.6	0.8	0.7	0.6	0.8	1.7	1.3	1.5	1.3	1.5	1.2	1.6	0.9	1.3	1.6	0.7	1.1	0.8
1387502_at	Stk17b	1.1	1.3	1.4	0.9	1.0	1.9	1.3	1.1	1.1	0.8	1.0	1.1	1.2	1.1	1.2	1.2	1.2	1.6	1.0	1.0	1.5	0.9	1.1	2.0
1387605_at	Casp12	1.0	1.1	1.0	0.9	1.0	1.4	1.1	0.9	1.2	0.9	0.6	1.0	1.3	1.0	1.6	1.2	1.1	1.7	1.8	2.6	5.3	0.4	0.9	1.6
1387818_at	Casp11	2.5	2.3	1.3	1.2	1.0	1.0	0.8	0.9	0.7	1.3	1.7	2.9	1.1	0.9	1.5	1.1	1.2	3.3	1.4	1.7	7.4	0.6	1.6	4.5
1388099_a_at	Tfpt	0.8	0.9	0.9	1.0	1.0	1.0	1.1	0.9	1.0	1.3	1.8	1.6	0.9	0.8	1.1	0.9	0.9	1.4	0.9	1.1	1.9	1.1	1.3	2.6
1388120_at	Pdcd6ip	0.7	2.0	8.0	0.9	3.9	14	2.7	2.9	8.5	1.4	0.6	16	1.0	1.0	1.2	0.9	1.0	1.3	1.0	1.0	1.5	0.9	1.0	1.6
1388674_at	Cdkn1a	1.0	1.1	1.0	0.9	1.0	1.0	0.9	1.0	1.5	1.0	1.3	2.0	1.1	1.0	1.5	0.9	1.5	2.1	1.2	1.8	2.1	0.8	1.9	1.7
1388805_at	Ppp2ca	0.9	0.8	0.9	0.9	1.0	1.1	0.9	0.9	1.1	0.9	1.1	1.1	1.2	1.1	1.5	0.9	1.0	1.7	0.8	1.0	2.7	1.1	1.3	3.6
1388867_at	MGC112830	0.9	1.1	1.2	1.2	1.3	1.7	1.0	1.2	1.6	0.8	0.8	1.0	1.0	1.1	1.1	0.9	1.1	1.3	1.0	1.0	1.2	0.9	1.0	1.7
1389170_at	Casp7	1.0	1.1	1.2	0.9	1.0	0.9	1.0	0.8	1.1	1.3	1.0	1.0	1.0	1.0	1.1	1.0	1.1	1.3	1.0	1.0	1.5	1.1	1.2	1.8
1398948_at	Tax1bp1	0.8	0.8	0.8	1.2	1.0	0.9	1.3	1.0	1.3	1.3	1.8	2.1	1.0	1.1	1.2	1.1	1.2	1.5	1.0	1.1	1.5	1.1	1.1	1.7

The number in each column expresses the ratio to control (N=3).

Fig. 4. Heatmap of individual gene expression change in category of "apoptosis".

of the promising candidates of biomarker genes for oxidative stress-mediated DNA damage, since it was reported to be up-regulated specifically by stress-inducing DNA damage (Corcoran *et al.*, 2005).

It was reported that hepatotoxicity of MP was due to its active metabolite(s) and that oxidative stress was involved (Ratra *et al.*, 1998). However, these authors excluded the involvement of glutathione depletion followed by oxidative stress in the later paper (Ratra *et al.*, 2000). We measured hepatic glutathione contents in rats treated with MP in a separate study (Uehara *et al.*, submitted). Immediately after MP dosing, a transient decrease, not statistically significant, was noted and a rebound-like increase was evident at 24 hr after dosing, which persisted for one week. The increment of glutathione contents disappeared till 2 weeks and it turned to a marked decrease after 4 weeks. These results suggest that MP causes oxidative stress in consuming glutathione while the hepatocytes defend against it by gene expression changes to keep a high glutathione level. Finally, glutathione depletion occurs when the toxicity of MP persists for a long period. We have extracted marker genes for hepatic glutathione depletion using a glutathione depletor, phorone (Kiyosawa *et al.*, 2007). Also in this work, phorone caused a transient decrease of glutathione with a peak at 3 to 6 hr after dosing followed by a rebound-like increase 24 hr after dosing. Taken together, the key of hepatotoxicity of MP is considered to be oxidative damage of DNA followed by changes in MAPK signaling and cell cycle induced by excess production of active metabolites. Sustained oxidative damage of DNA and stimulation of cell proliferation is closely related to hepatocarcinogenesis of MP.

The main purpose of the toxicogenomics approach was to analyze the mechanism of toxicity and predict chronic toxicity from acute data in the preclinical study. In the present study, we simulated the prediction of the toxicity

AFFYMETRIX PROBE ID	SYMBOL	03H			06H			09H			24H			04D			08D			15D			29D		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
1367577_at	Hspb1	1.0	0.9	0.6	1.3	1.4	1.4	0.6	0.8	1.0	1.2	1.3	2.0	1.2	1.5	1.1	1.4	1.3	2.9	0.9	1.3	3.1	0.9	1.6	2.9
1367624_at	Atf4	1.0	1.2	1.8	0.9	1.3	2.2	1.5	1.4	2.2	1.0	0.9	1.3	1.1	1.1	1.3	1.2	1.2	1.6	0.9	1.1	2.0	1.1	1.0	2.0
1367760_at	Map2k1	1.0	1.1	1.1	1.0	1.0	1.1	0.9	0.9	1.0	1.0	1.1	1.2	1.0	1.0	1.3	1.0	1.0	2.0	1.0	1.1	3.1	0.9	1.2	3.7
1367831_at	Tp53	1.0	0.9	0.9	1.0	1.0	1.0	1.0	1.1	1.3	1.1	1.3	1.2	1.3	1.0	1.3	1.3	1.3	2.0	1.3	1.0	2.4	0.8	1.2	2.0
1367890_at	Casp2	1.1	1.1	1.1	0.9	1.1	1.1	1.0	0.9	1.1	1.0	1.0	1.2	1.1	1.0	1.0	1.1	1.2	1.3	0.9	1.0	1.2	1.1	1.1	1.9
1368247_at	Hspa1a / 1b	0.9	1.2	1.4	1.4	1.4	2.8	1.1	1.1	1.5	1.5	1.7	1.8	1.2	1.1	1.8	1.5	1.5	1.4	0.8	1.2	1.2	0.7	0.6	0.4
1368273_at	Mapk6	1.1	1.2	1.2	1.0	1.3	1.6	1.0	1.1	1.4	1.1	1.0	1.0	0.9	0.9	0.9	0.9	0.9	1.1	1.0	0.9	1.1	0.9	1.0	1.0
1368277_at	Ppp3ca	1.1	0.9	1.0	0.9	0.9	1.2	1.2	1.2	1.2	1.1	1.1	1.2	1.0	1.1	1.1	1.0	1.3	1.3	1.1	1.0	1.3	1.2	1.1	1.6
1368305_at	Casp6	0.9	0.9	0.9	0.9	1.0	1.1	1.1	1.2	1.2	0.9	1.0	1.4	1.3	1.0	1.4	1.0	0.9	1.1	1.0	1.1	1.1	0.9	1.1	1.8
1368308_at	Myc	2.0	2.1	3.9	0.7	0.8	3.6	0.7	1.1	1.8	0.8	0.8	1.4	2.0	1.5	1.8	1.5	1.6	2.8	1.9	2.4	5.1	0.9	1.4	3.4
1368862_at	Akt1	1.0	0.8	0.8	1.0	0.8	0.9	0.9	1.1	0.8	0.9	1.0	1.1	0.9	0.8	0.9	1.0	0.9	1.3	1.0	1.0	1.6	0.8	1.0	1.4
1368871_at	Map3k1	1.3	1.0	1.0	0.8	0.7	0.7	0.8	1.2	1.2	0.8	0.7	0.7	0.9	0.9	0.8	0.9	1.0	2.0	1.0	1.2	2.4	1.1	1.0	3.0
1368947_at	Gadd45a	1.3	1.3	5.3	0.6	0.7	2.5	0.8	0.9	1.2	0.6	0.9	1.8	1.1	0.8	1.4	1.1	1.3	3.4	0.6	1.3	3.7	1.5	2.1	7.6
1369590_a_at	Ddit3	1.0	1.3	4.3	1.1	1.1	3.0	1.1	0.9	1.3	[illegible]	1.1	1.2	0.9	1.0	1.2	0.7	1.0	3.0	0.9	1.2	5.3	1.1	1.3	7.1
1369653_at	Tgfbr2	1.0	1.0	0.9	0.6	0.9	0.7	0.8	1.7	1.5	1.1	1.1	1.0	1.3	1.1	1.4	1.1	1.2	6.5	0.8	2.0	3.2	0.8	1.7	3.2
1369932_a_at	Raf1	1.0	1.1	1.1	1.0	1.2	1.6	1.2	1.2	1.6	0.9	0.9	1.0	1.0	1.1	1.0	0.9	0.9	1.1	1.0	0.9	1.2	0.9	0.9	1.2
1370035_at	Kras2	0.9	0.9	0.8	1.1	1.2	1.5	1.1	1.2	1.5	1.0	0.9	1.1	1.0	1.1	1.0	1.0	1.0	1.3	0.9	1.0	1.2	0.9	1.0	1.3
1370265_at	Arrb2	0.8	0.9	1.2	1.2	1.1	1.1	0.6	1.0	0.8	0.8	0.8	1.2	1.2	1.1	1.1	1.0	1.4	2.4	1.0	0.8	1.6	0.8	1.2	3.2
1370427_at	Pdgfa	0.9	0.8	0.9	1.5	2.4	4.2	1.0	0.9	2.0	0.6	0.7	1.2	1.2	1.1	1.1	2.6	2.5	5.4	0.8	2.0	4.5	0.9	1.0	4.1
1370585_a_at	Prkcb1	0.9	1.0	1.1	1.0	0.9	1.2	1.3	1.0	1.0	0.8	0.9	1.3	1.5	0.9	0.9	0.9	1.0	1.4	1.0	1.0	1.4	1.1	1.2	2.2
1370825_a_at	Cdc42	1.0	0.9	1.0	1.0	1.1	1.0	1.1	1.1	1.1	1.0	1.0	1.1	1.0	1.1	1.1	1.0	1.0	1.3	1.0	1.1	1.5	1.0	1.1	1.5
1370968_at	Nfkb1	1.0	0.9	1.0	1.0	1.0	1.2	1.0	1.0	1.2	1.0	0.8	1.1	1.1	0.9	1.0	1.0	1.0	1.3	1.2	1.1	1.9	1.0	1.2	2.0
1372982_at	Ppp3r1	0.9	1.3	1.3	1.1	1.3	1.5	1.4	1.2	1.2	0.9	1.0	1.2	1.0	1.1	1.0	0.9	0.9	1.5	0.9	0.7	1.1	1.1	1.2	2.2
1375043_at	Fos	0.4	0.3	3.6	0.5	1.0	3.4	2.8	1.7	3.0	0.2	0.2	0.2	0.8	0.8	0.6	1.5	5.5	14	0.5	1.0	6.8	1.8	1.7	15
1376425_at	Tgfb2	1.2	1.1	1.0	1.1	1.4	1.0	1.2	1.0	1.0	1.2	1.3	1.2	1.1	1.0	1.0	1.0	0.9	1.6	1.1	1.1	2.1	1.1	1.3	2.8
1386935_at	Nr4a1	1.1	1.0	1.4	1.1	1.0	1.5	0.7	0.8	0.8	1.1	1.0	1.0	1.2	1.0	1.0	0.9	0.9	1.4	1.0	1.0	1.5	1.1	1.5	2.7
1387377_a_at	Pak1	0.7	0.8	0.9	1.6	1.3	1.5	0.5	0.3	0.6	0.5	0.8	0.9	1.4	0.8	1.2	1.5	0.8	1.4	0.9	2.2	5.1	0.4	0.7	2.8
1387498_a_at	Fgfr1	1.0	0.8	1.0	0.7	0.9	0.7	0.9	1.0	0.9	1.0	1.0	0.9	1.0	1.1	0.9	1.5	1.4	1.9	1.0	1.0	1.3	0.8	1.0	2.3
1387643_at	Fgf21	1.2	2.1	8.8	0.6	1.2	3.6	0.7	1.0	1.6	1.0	1.1	5.1	1.1	1.5	3.9	1.1	5.0	7.5	0.9	4.8	6.4	3.1	3.5	6.6
1387771_a_at	Mapk3	1.2	1.2	1.1	1.1	1.0	0.9	0.9	1.1	0.9	1.1	0.9	1.0	1.2	1.2	1.0	0.9	1.1	1.2	0.9	0.9	1.3	0.9	0.9	2.1
1387806_at	Rap1b	1.0	1.0	1.0	1.0	1.0	1.2	1.0	1.0	1.1	0.9	0.9	1.1	1.1	1.0	1.0	1.0	1.1	1.3	1.0	1.0	1.4	1.0	1.1	1.6
1389170_at	Casp7	1.0	0.9	0.9	0.9	1.0	0.8	0.9	1.0	1.0	1.0	1.0	1.2	1.0	1.0	1.1	1.0	1.1	1.3	1.0	1.0	1.5	1.1	1.2	1.8
1389528_s_at	Jun	1.3	1.3	3.4	0.9	0.6	2.0	0.8	0.8	1.5	0.9	0.5	0.9	1.5	1.5	1.3	1.7	1.4	2.3	0.7	1.4	1.6	1.4	1.4	2.8
1398240_at	Hspa8	0.9	1.3	1.2	1.0	1.1	1.6	1.2	1.2	1.5	1.2	1.2	1.2	1.0	1.0	1.2	1.0	1.1	1.1	0.9	0.9	1.2	0.9	0.9	1.1
1398256_at	Il1b	0.7	0.6	0.6	0.8	0.6	0.8	0.9	0.9	1.1	0.6	0.7	1.1	2.3	0.8	1.5	1.2	1.1	1.5	1.0	1.1	1.8	1.2	1.0	2.1

The number in each column expresses the ratio to control (N=3).

Fig. 5. Heatmap of individual gene expression change in category of "MAPK signaling".

AFFYMETRIX PROBE ID	SYMBOL	03H			06H			09H			24H			04D			08D			15D			29D		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
1367590_at	Ran	1.1	0.9	0.9	1.0	1.1	1.5	1.0	1.1	1.8	1.0	1.3	1.7	0.9	1.0	1.3	1.1	1.2	2.4	1.0	1.2	1.9	1.0	1.4	1.9
1367764_at	Ccng1	0.9	1.0	1.0	1.0	1.2	2.4	1.0	0.9	1.9	1.3	1.3	2.2	0.8	0.9	1.3	1.0	1.1	2.4	1.0	1.0	3.4	1.3	2.2	6.2
1367827_at	Ppp2cb	1.0	1.1	0.9	1.0	1.1	1.5	1.1	1.2	1.8	1.0	1.0	1.1	0.9	1.0	1.1	1.0	1.1	1.8	0.9	1.2	2.1	1.0	1.2	2.2
1367831_at	Tp53	1.0	0.9	0.9	1.0	1.0	1.0	1.0	1.1	1.3	1.1	1.3	1.2	1.3	1.0	1.3	1.3	1.3	2.0	1.3	1.0	2.4	0.8	1.2	2.0
1368076_at	Vhl	1.0	1.1	1.1	1.0	1.0	1.1	1.0	0.9	1.1	1.0	0.9	1.0	1.1	1.0	1.0	1.0	1.1	1.4	1.0	1.0	1.6	1.0	1.0	1.9
1368308_at	Myc	2.0	2.1	3.9	0.7	0.8	3.6	0.7	1.1	1.8	0.8	0.8	1.4	2.0	1.5	1.8	1.5	1.6	2.8	1.9	2.4	5.1	0.9	1.4	3.4
1368947_at	Gadd45a	1.3	1.3	5.3	0.6	0.7	2.5	0.8	0.9	1.2	0.8	0.9	1.8	1.1	0.8	1.4	1.1	1.3	3.4	0.6	1.3	3.7	1.5	2.1	7.6
1369590_a_at	Ddit3	1.0	1.3	4.3	1.1	1.1	3.0	1.1	0.9	1.3	1.0	1.1	1.2	0.9	1.0	1.2	0.7	1.0	3.0	0.9	1.2	5.3	1.1	1.3	7.1
1369932_a_at	Raf1	1.0	1.1	1.1	1.0	1.2	1.5	1.2	1.2	1.6	0.9	0.9	1.0	1.0	1.1	1.0	0.9	0.9	1.1	1.0	0.9	1.2	0.9	0.9	1.2
1369950_at	Cdk4	1.0	1.0	0.9	1.1	1.1	1.1	1.1	1.2	1.3	1.0	1.0	1.2	1.0	1.0	1.1	0.9	1.2	2.0	1.0	1.1	1.9	1.0	1.3	2.2
1369958_at	Rhob	0.9	1.0	1.7	1.0	1.0	3.9	1.2	1.1	1.9	0.8	0.7	0.9	0.9	1.0	1.1	1.0	1.1	2.0	1.2	1.3	3.0	1.0	1.5	3.9
1370035_at	Kras2	0.9	0.9	0.8	1.1	1.2	1.5	1.1	1.2	1.5	1.0	0.9	1.1	1.0	1.1	1.0	1.0	1.0	1.3	0.9	1.0	1.2	0.9	1.0	1.3
1370361_at	Cgref1	1.1	0.6	1.1	0.7	1.0	1.1	0.7	0.9	1.4	0.5	0.6	1.4	1.6	1.9	1.6	1.3	2.0	5.4	0.7	1.2	3.3	1.5	2.3	6.5
1370427_at	Pdgfa	0.9	0.8	0.9	1.5	2.4	4.2	1.0	0.9	2.0	0.8	0.7	1.2	1.2	1.1	1.1	2.6	2.5	5.4	0.8	2.0	4.5	0.9	1.0	4.1
1370504_a_at	Pmp22	1.2	1.5	1.2	0.8	0.9	1.1	0.7	0.7	0.6	0.8	0.8	1.0	1.2	1.4	1.3	1.3	1.6	1.6	1.1	1.3	1.5	1.8	1.4	2.7
1370809_at	Tubg1	1.1	1.0	0.9	1.0	1.0	1.1	1.0	0.9	1.0	1.0	1.0	1.4	1.1	1.2	1.1	1.1	1.0	1.5	0.9	1.2	2.2	1.2	1.5	2.6
1371308_at	Rps4x	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	1.1	1.0	1.1	1.2	1.0	1.0	1.1	1.0	1.1	1.6	1.1	1.3	1.6	1.2	1.3	1.6
1374956_at	Pcm1	1.2	0.9	1.1	0.9	0.9	0.9	1.3	1.1	1.2	1.0	1.1	1.0	1.0	0.9	0.9	1.2	1.2	1.4	1.0	0.9	1.3	1.1	0.9	1.6
1375630_at	RGD:1303103	1.0	0.8	1.0	1.0	1.1	1.3	1.1	1.0	1.4	1.0	1.1	1.3	0.9	1.0	1.0	1.2	1.2	1.6	1.1	1.2	1.9	1.0	1.2	1.8
1376425_at	Tgfb2	1.2	1.1	1.0	1.1	1.4	1.0	1.2	1.0	1.0	1.2	1.3	1.2	1.1	1.0	1.0	1.0	0.9	1.6	1.1	1.1	2.1	1.1	1.3	2.8
1379375_at	Pdgfa	1.1	1.1	1.1	1.0	1.2	2.0	0.9	0.9	1.6	0.7	0.8	1.1	1.2	1.1	1.3	0.8	1.0	1.9	0.9	0.9	2.1	1.1	1.3	3.0
1386866_at	Ywhag	1.1	1.0	1.1	0.9	1.2	1.9	1.1	1.3	1.9	1.1	0.9	1.1	1.0	1.0	1.2	1.1	1.2	1.7	1.0	1.1	1.8	1.0	1.2	2.2
1387391_at	Cdkn1a	0.9	1.1	0.9	1.1	1.5	3.9	0.6	0.7	2.8	1.2	2.2	2.9	1.2	1.1	1.4	1.9	2.5	5.9	1.1	2.1	3.1	0.9	2.8	2.5
1387616_at	Pdgfc	1.0	1.0	0.9	1.0	0.9	1.2	1.1	1.0	0.8	1.0	0.9	0.8	1.2	0.8	1.1	1.3	1.3	1.6	0.9	0.9	1.2	0.8	0.9	2.2
1387644_at	Btc	1.0	1.1	1.0	1.1	0.9	0.9	1.0	0.9	0.7	0.9	1.1	1.1	1.3	1.0	1.4	1.3	0.8	1.0	0.9	1.0	1.6	1.0	0.8	1.9
1387788_at	Junb	1.2	1.0	1.6	1.1	0.7	1.3	0.7	0.9	1.5	1.6	1.4	1.4	1.4	0.9	1.4	1.1	1.0	1.3	1.0	1.1	2.1	1.1	1.0	4.0
1388154_at	E2f5	1.0	1.0	1.1	1.0	1.4	1.7	1.0	1.1	1.7	1.2	1.1	1.2	1.0	1.0	1.1	1.1	1.1	1.4	1.0	1.0	1.5	0.9	1.1	1.7
1388805_at	Ppp2ca	1.1	1.0	1.0	1.1	1.0	1.4	1.1	1.0	1.4	0.9	1.0	1.5	1.2	1.1	1.5	0.9	1.0	1.7	0.8	1.0	2.7	1.1	1.3	3.6
1388867_at	MGC112830	1.0	1.1	1.2	0.9	1.1	1.4	1.0	1.1	1.1	0.9	0.9	1.0	1.0	1.1	1.1	0.9	1.1	1.3	1.0	1.0	1.2	0.9	1.0	1.7
1389101_at	Ccnc	0.7	0.7	0.5	0.9	1.1	1.2	1.2	1.0	0.8	1.2	1.1	1.4	1.2	1.1	1.1	1.1	1.3	2.0	1.3	1.6	2.5	1.5	1.6	2.5
1389528_s_at	Jun	1.3	1.3	3.4	0.9	0.6	2.0	0.8	0.8	1.5	0.9	0.5	0.9	1.5	1.5	1.3	1.7	1.4	2.3	0.7	1.4	1.6	1.4	1.4	2.8
1398240_at	Hspa8	0.9	1.3	1.2	1.0	1.1	1.6	1.2	1.2	1.5	1.2	1.2	1.2	1.0	1.0	1.2	1.0	1.1	1.1	0.9	0.9	1.2	0.9	0.9	1.1
1398256_at	Il1b	0.7	0.6	0.6	0.8	0.6	0.8	0.9	0.9	1.1	0.6	0.7	1.1	2.3	0.8	1.5	1.2	1.1	1.5	1.0	1.1	1.8	1.2	1.0	2.1

The number in each column expresses the ratio to control (N=3).

Fig. 6. Heatmap of individual gene expression change in category of "regulation of cell cycle".

of MP by focusing on the toxicological pathway drawn from transcriptome analysis. Genes up-regulated from the early stage described above would be promising candidates of biomarkers for hepatotoxicity. However, the present analysis focused on one chemical, MP. It is necessary to analyze other chemicals causing glutathione depletion/oxidative stress and nongenotoxic hepatocarcinogenesis, such as thioacetamide, coumarin and ethionine, in order to establish a useful and precise prediction system based on the toxicogenomics approach.

The greatest advantage of toxicogenomics in toxicology is that various toxicity mechanisms can be elucidated at once compared with the conventional strategy where many experiments are performed one by one. This strategy is so powerful that comprehensive seizure of what happens for the mechanism in the target organ is possible. Toxicogenomics enables one to supply supporting data for any conventional toxicological changes and suggests the appropriate toxicological mechanism behind them.

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