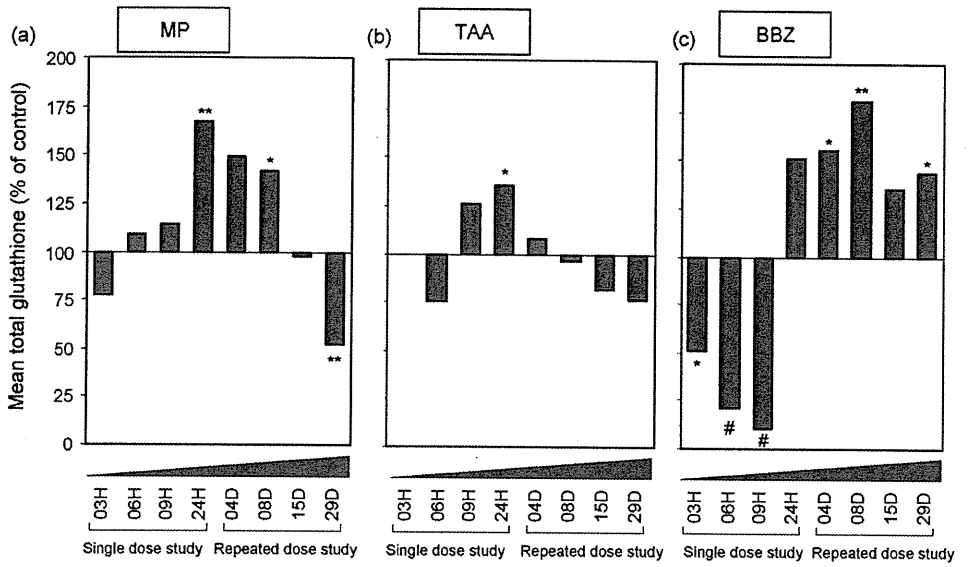

Fig. 5. Effects of MP, TAA or BBZ on glutathione contents in rat liver. Hepatic total glutathione was measured in the liver of rats receiving a high dose of MP (a), TAA (b) or BBZ (c), and their corresponding controls. Measurements were performed for five rats per group using Glutathione Quantification Kit. The results were expressed as percent of control at each time point. Statistical analysis was done by an unpaired two-tailed Student's *t*-test or Welch's *t*-test as appropriate. $^{*}p < 0.05$, $^{**}p < 0.01$, by Student's *t*-test, $\#p < 0.05$ by Welch's *t*-test.

The gene list selected as a marker for predicting hepatic carcinogenicity contained oxidative stress-, oxidative DNA damage-, and cell cycle regulation-related genes, which were changed in the early stage of administration. The oxidative stress is due to the production of reactive oxygen species more than the anti-oxidant capability of the target cells. Unregulated or prolonged production of cellular oxidants has been thought to lead to mutation as a result of oxidant-induced DNA damage, thought to participate in non-genotoxic carcinogenesis (Klaunig et al., 1998; Klaunig and Kamendulis, 2004). The observed expression changes in these genes is in accordance with previous reports that the repetitive cycle of DNA damage (initiation) and reproduction (promotion) caused by sustained oxidative stress is closely related to the carcinogenic process of non-genotoxic carcinogens. This does not mean that the classifier detects any compounds causing oxidative stress. Of the compounds used as negative sets, APAP is known as a prototypic oxidative stressor, which induces glutathione depletion in liver when overdosed (James et al., 2003; Kiyosawa et al., 2004). ASA was reported to induce some antioxidant enzymes and components (Cai et al., 1995), and stimulates some beta-oxidation enzymes, bringing about an overproduction of H_2O_2 (Rivero et al., 1994). PhB was reported to accelerate glutathione oxidation and it induces lipid peroxidation of microsomes (Miura et al., 2002). All of these were successfully classified as negative, suggesting that the classifier discriminates non-carcinogens causing oxidative stress.

The validity of the presently developed discriminator for carcinogenesis was examined on our large-scale database, and all of the 20 chemicals except BBZ (selected as a non-carcinogen) were judged as negative at any time points. Of the eight chemicals classified as non-genotoxic carcinogens, CMA, ET, CCl4 and WY showed positive prediction and increase in the PAM prediction scores in repeated administrations, whereas enzyme inducers such as PB and HCB, and other peroxisome proliferators were all judged as negative.

For CMA (Lake et al., 2002; National Toxicol Program, 1993), ET (Ogiso et al., 1990; Svardal et al., 1988), and CCl4 (Castro et al., 1989; Natarajan et al., 2006), oxidative stress was reported as being involved in their hepatotoxicity and carcinogenesis. It could be con-

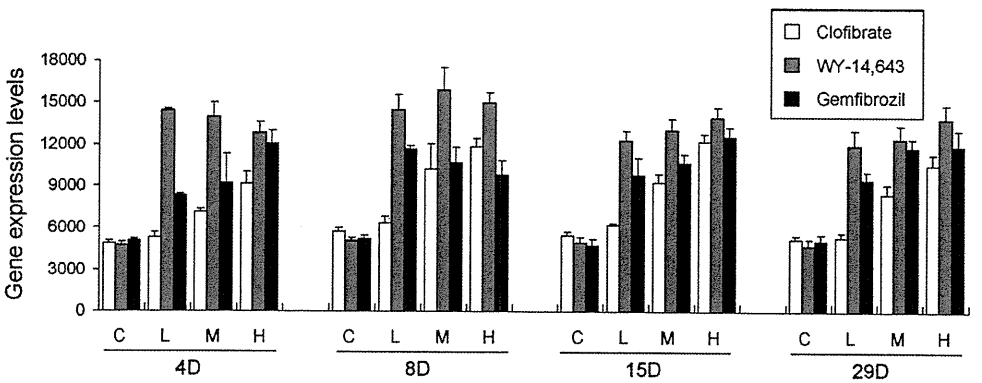

Fig. 6. Effects of repeated administration of CFB, WY or GFZ on expression of acyl-CoA oxidase-1. Expression of acyl-CoA oxidase-1, a gene directly regulated by PPARα, was measured by GeneChip, and the intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization). The results were expressed as mean ± S.D. ($n = 3$). For each panel, C: control, L: low dose, M: middle dose, H: high dose, for CFB: 30, 100, 300 mg/kg; WY: 10, 30, 100 mg/kg; GFZ: 30, 100, 300 mg/kg, respectively.

cluded that sustained oxidative stress plays an important role in their carcinogenesis, as in MP and TAA.

The induction of PPARα in rodents treated with peroxisome proliferators was considered to be related to hepatocarcinogenesis (Holden and Tugwood, 1999). Moreover, increased levels of H_2O_2 generation, hydroxyl free-radical formation and lipid peroxidation were found in the liver of rats following long-term treatment with peroxisome proliferators. It was also reported that 8-hydroxydeoxyguanosine was found in the liver DNA of rats chronically treated with a PPARα (Reddy and Lalwai, 1983; Reddy and Rao, 1989). In the present study, our discriminator designated WY as positive among the PPARα agonists, CFB, GFZ, and WY. This result suggests that either the discriminator could predict the carcinogenesis of PPARα agonists (although its sensitivity is relatively low) or that WY had an additional carcinogenicity differing from other PPARα agonists. The latter would be more likely since the low and middle doses of WY (by which the induction of acyl-CoA oxidase 1 reached a maximum) did not classify as positive and since the highest doses of CFB and GFZ induce acyl-CoA oxidase 1 to almost the same extent as WY. It was also suggested that WY might share a carcinogenic mechanism with MP and TAA apart from its PPARα agonist's activity.

The P450 enzymes generate oxygen free radicals in the process of metabolizing xenobiotic chemicals (Parke and Ioannides, 1990), including PB (Utley and Mehendale, 1991) and HCB (Smith and De Matteis, 1990). Kinoshita et al. (2002) reported that PB-induced reversible alteration to nuclear 8-hydroxydeoxyguanosine by oxidative stress in rat liver after several days of continuous application. Furthermore, Elrick et al. (2005) provided evidence for the relationship between oxidative stress and PB-induced non-genotoxic hepatic carcinogenesis. On the other hand, HCB exposure induces long-term alterations in intercellular communication via gap junction in rat liver. This effect is thought to be a critical mechanism of HCB-induced non-genotoxic hepatocarcinogenesis and tumor promotion (Plante et al., 2002). However, these chemicals were classified as non-carcinogens based on gene expression profiling. There are likely to be numerous mechanisms involved in non-genotoxic rodent hepatic carcinogenesis. Therefore, it is thought that these chemicals induce non-genotoxic hepatocarcinogenesis through chemical-specific mechanisms.

For the evaluation of these results of prediction, we developed a PAM prediction score based on the positive/negative class probability. In the present study, we compared the score with the hepatic glutathione contents in order to examine the validity of the prediction. In association with the largest decrease of hepatic glutathione contents at 3H (MP), 6H (TAA) and 9H (BBZ), the PAM prediction score increased with the peak at 6H (MP), 9H (TAA) and 24H (BBZ). This could be explained as follows: hepatic glutathione was rapidly consumed to detoxify the oxidants produced by these toxicants, and in the subsequent glutathione-depleted state the expression of these marker genes was up-regulated. The excess production of glutathione for homeostasis tended to decrease in MP or TAA, whereas its high value was maintained in BBZ during their repeated administrations. It is known that some reactive intermediates are conjugated with glutathione to be excreted from the cell. The hepatotoxicity of the acute dose of BBZ was significantly reduced by prior sub-chronic exposure to BBZ. Therefore, the enhanced BBZ excretion by glutathione conjugation could partly explain such potential tolerance against its acute hepatotoxicity (Chakrabarti and Brodeur, 1984). It would be reasonable to speculate that BBZ, which causes transient hepatic and DNA damage by oxidative stress at the early stage of dosing, does not result in hepatic cancer since metabolic protection against oxidative stress does not allow the sustained stressful condition up to 28 days of administration, whereas a breakdown of protection occurs in the case of MP and TAA suggested by the glutathione contents. There was a close correlation between the pattern of change in glutathione and PAM scores, supporting the usefulness of the present marker genes. The present scoring system also enables us to make a prediction based on important toxicological points, *e.g.*, dose- and time-dependency and it would be a quite convenient way for evaluation of the results of discriminant analysis.

In summary, we showed that the expression profile of 112 genes selected by the PAM method could make a prediction of oxidative stress-related hepatocarcinogenicity with high precision at the early stage of administration. The possibility of non-genotoxic carcinogenicity is suggested as early as 24 h after the single dosing. Although pseudo-positives are included in the chemicals selected by the single dose experiments, these can be discriminated by the prediction based on repeated administration up to 28 days. At present, tests for carcinogenicity using rats takes at least 2 years. The present study has suggested a possibility to enable it to take as short as 28 days with high precision. Although neither a single gene nor a single pathway is sufficient to predict non-genotoxic hepatocarcinogens at present, it is evident that combinations of biomarker gene sets appeared to be useful for prediction of carcinogenesis. Further study is clearly necessary to clarify the pathophysiological roles of the genes included in the marker gene list for the process of carcinogenesis.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2008.05.013.

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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),

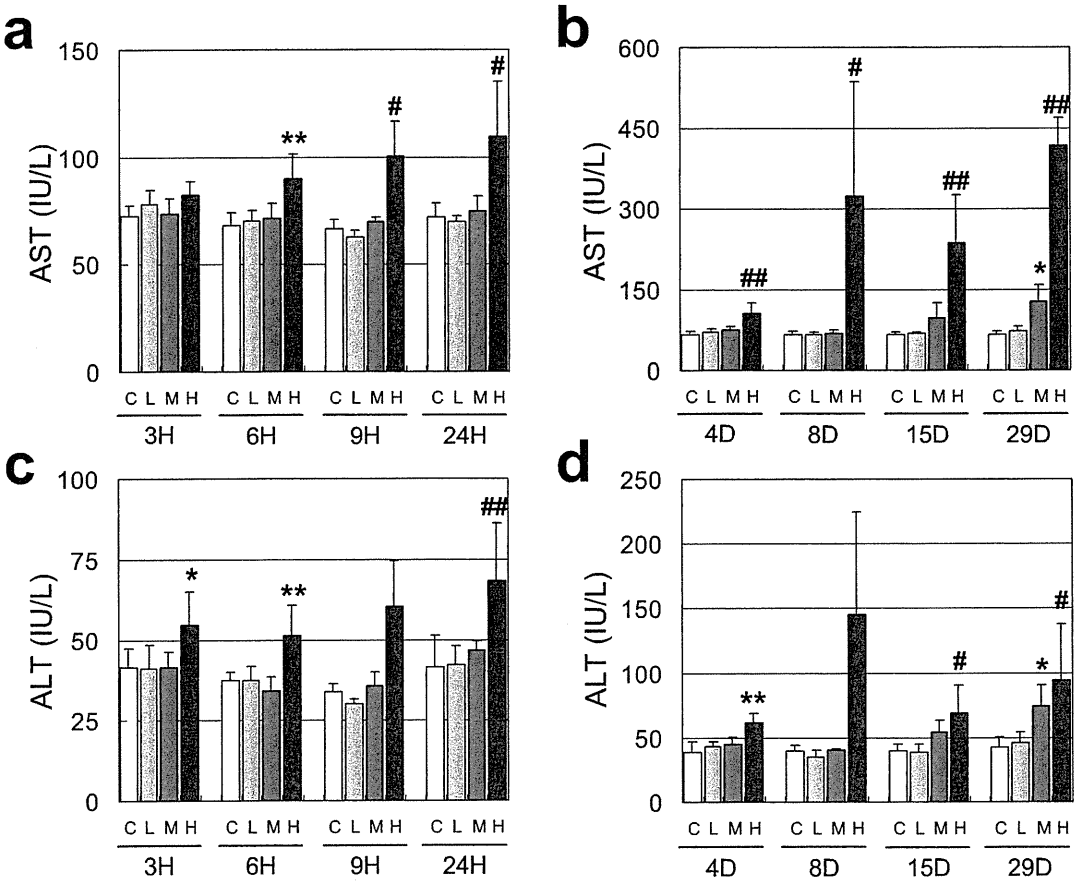

Fig. 1. Serum AST (a and b) and ALT activities (c and d) in rats treated with 10, 30 and 300 mg/kg MP in single and repeated dose studies.
Data are expressed as mean± S.D. (n = 5). *, **Significant difference from the control group, $p < 0.05$, 0.01, by Dunnett's multiple comparison test. #, ##Significant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

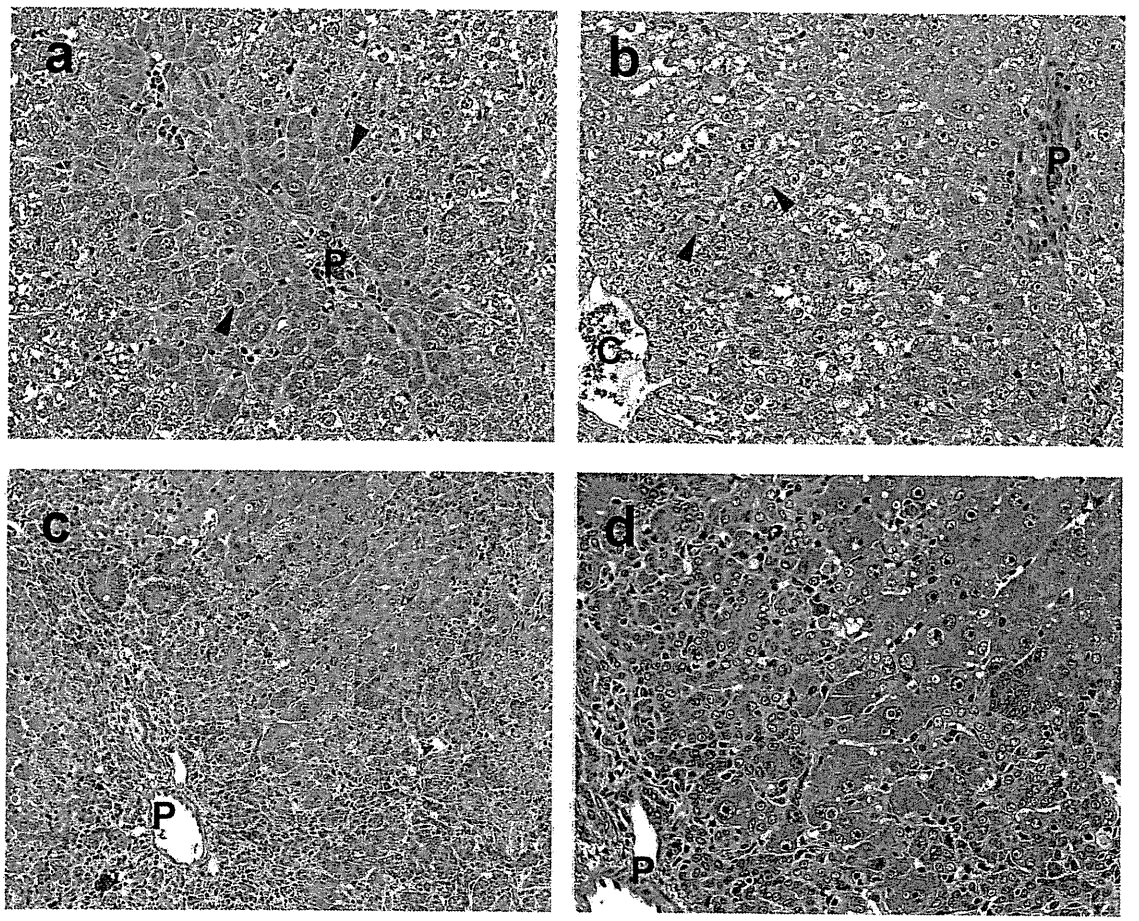

Fig. 2. Histopathological changes of liver treated with 100 mg/kg MP.
a: Hepatocellular hypertrophy and single cell necrosis (arrow head) in the periportal region (P) are observed at early time point, 24 hr after single dosing. b: Additional regenerative changes, such as increased mitosis, bile duct 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	Gqt1	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	1.8	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	8.8	8.5
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	1.8	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	0.8	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
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	0.8	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
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	1.6	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
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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Species-specific differences in coumarin-induced hepatotoxicity as an example toxicogenomics-based approach to assessing risk of toxicity to humans

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One expected result from toxicogenomics technology is to overcome the barrier because of species-specific differences in prediction of clinical toxicity using animals. The present study serves as a model case to test if the well-known species-specific difference in the toxicity of coumarin could be elucidated using comprehensive gene expression data from rat in-vivo, rat in-vitro, and human in-vitro systems. Coumarin 150 mg/kg produced obvious pathological changes in the liver of rats after repeated administration for 7 days or more. Moreover, 24 h after a single dose, we observed minor and transient morphological changes, suggesting that some early events leading to hepatic injury occur soon after coumarin is administered to rats. Comprehensive gene expression changes were analyzed using an Affymetrix GeneChip® approach, and differentially expressed probe sets were statistically extracted. The changes in expression of the selected probe sets were further examined in primary cultured rat hepatocytes exposed to coumarin, and differentially expressed probe sets common to the in-vivo and in-vitro datasets were selected for further study. These contained many genes related to glutathione metabolism and the oxidative stress response. To incorporate human data, human hepatocyte cultured cells were exposed to coumarin and changes in expression of the bridging gene set were examined. In total, we identified 14 up-regulated and 11 down-regulated probe sets representing rat–human bridging genes. The overall responsiveness of these genes to coumarin was much higher in rats than humans, consistent with the reported species difference in coumarin toxicity. Next, we examined changes in expression of the rat–human bridging genes in cultured rat and human hepatocytes treated with another hepatotoxicant, diclofenac sodium, for which hepatotoxicity does not differ between the species. Both rat and human hepatocytes responded to the marker genes to the same extent when the same concentrations of diclofenac sodium were exposed. We conclude that toxicogenomics-based approaches show promise for overcoming species-specific differences that create a bottleneck in analysis of the toxicity of potential therapeutic treatments.

Key words: coumarin; hepatocyte; hepatotoxicity; human; liver; rat; toxicogenomics

Introduction

The Toxicogenomics Project (TGP) is a 5-year collaborative project of the National Institute of Health Sciences, the National Institute of Biomedical Innovation, and 15 pharmaceutical companies in Japan that began in 2002.[1] The aim was to construct a large-scale toxicology database of transcriptomes useful to predict the toxicity of new chemical entities in early stages of drug development. About 150 chemicals, primarily medicinal compounds, were selected and gene expression in the rat liver (also kidney in some cases) or rat and human hepatocytes was comprehensively analyzed by using the Affymetrix GeneChip®.[2] In 2007, the project was completed and the whole system, consisting of a database, an analysis system, and a prediction system, was completed and named TG-GATEs (for Genomics Assisted Toxicity Evaluation System developed by Toxicogenomics Project in Japan).

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The main purpose of creating the system was to facilitate analysis of the mechanisms of toxicity and prediction of chronic toxicity from acute data in preclinical studies, and the consensus response to the project is that toxicogenomics-based technologies provide a useful tool. However, the final goal of a preclinical study should be prediction of clinical toxicity based on animal data. Toward this end, overcoming species-specific differences has proved to be the most difficult problem. We expect that elucidation of mechanisms of toxicity using toxicogenomics-based tools should lead to an improved ability to use animal data to make reasonable predictions of toxicity in humans.[3] However, there have been few reports of species-specific differences in the toxicological response at the level of changes in gene expression.[4]

We obtained gene expression data from rat primary hepatocytes as well as human frozen hepatocytes (in addition to rat in-vivo liver) to build an informational bridge between the two species. In the present study, we analyzed the effects of coumarin, a representative hepatotoxicant with a known species-specific difference in toxicity, as a model case for determining if species-specific differences in hepatotoxicity can be accurately predicted using a toxicogenomics-based approach.

Materials and methods

Chemicals

Coumarin and diclofenac sodium (DFNa) were obtained from Tokyo Chemical Industry (Tokyo, Japan).

Animal Treatment

All experimental protocols using animals were reviewed and approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences. The experimental protocols using human hepatocytes were reviewed and approved by both the Ethics Review Committees for Experimentation on Human Subjects of the National Institute of Health Sciences and of the National Institute of Biomedical Innovation.

Five-week-old male Sprague-Dawley rats were obtained from Charles River Japan Inc. (Kanagawa, Japan). After a 7-day quarantine and acclimatization period, 6-week-old animals were assigned to dosage groups (five 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 h (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., Ltd., Tokyo, Japan).

Either vehicle (corn oil), or 15, 50, or 150-mg/kg coumarin was administered orally to rats once daily on day 1, 3, 7, 14, and 28, and the animals were euthanized 24 h after the last dosing by exsanguination from the abdominal aorta under ether anesthesia. Liver samples were obtained from the left lateral lobe of the liver of each animal immediately after sacrifice. For light microscopy, liver samples were fixed in 10% neutral-buffered formalin, dehydrated in alcohol, and embedded in paraffin. Paraffin sections were prepared and stained using standard methods for hematoxylin and eosin staining. Histopathological findings were graded into four categories: very slight, slight, moderate, and severe. For electron microscopy, a piece of tissue from the liver was fixed in 2.5% glutaraldehyde solution. Ultra-thin sections, stained with Mayer's hematoxylin and lead citrate after standard tissue processing, were observed under a Hitachi electron microscope (H-7650; Hitachi High-Technologies Corporation Tokyo, Japan).

Hepatocyte treatment

Hepatocytes were isolated from 6-week-old male Sprague-Dawley rats under sodium pentobarbital (120 mg/kg, i.p.) and anesthetized using a modified two-step collagenase perfusion method. The liver was perfused via the portal vein for 10 min with divalent, cation-free, EGTA (ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid) (0.5 mM)-supplemented HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered Hank's balanced salt solution followed by a 10-min perfusion with HEPES (10 mM)-buffered normal Hank's balanced salt solution containing soybean trypsin inhibitor (0.05 g/L, T-2011; Sigma Aldrich, St Louis, Missouri, USA) and collagenase (0.5 g/L, 034-10533; Wako Pure Chemical Industries, Osaka, Japan) at a flow rate of 10–30 mL/min. The isolated cells were washed three times and centrifugated at 50 × *g* for 1 min to obtain a parenchymal cell-enriched pellet. Hepatocytes were not used when their viability was <70% (as assessed by trypan blue exclusion). Cell samples that passed the threshold for viability were seeded into collagen-coated six-well plates (BD BioCoat™ Collagen I Cellware; BD Bioscience, Bedford, Massachusetts, USA) at a density of 1×10^6 cells/well in 2 mL HMC Bulletkit medium (Cambrex,

Walkersville, Maryland, USA) supplemented with 10% fetal bovine serum.

For human hepatocytes (Tissue Transformation Technologies Inc., presently BD Biosciences, San Jose, California, USA), the frozen cells were thawed, washed twice with medium (L15 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum), and then seeded as described for rat hepatocytes except that the cell density was 1.2×10^6 cells/well.

Following an attachment period of 3 h, the medium was replaced and kept overnight before exposure to the drug at 37 °C in an atmosphere of 5% CO_2. The test compounds were added to the medium directly or as a 1000× stock solution in dimethylsulfoxide (DMSO). After 2, 8, or 24-h exposure, cells were dissolved with RLT buffer (Qiagen, Valencia, California, USA) and collected for expression profiling. GeneChip analysis was performed in duplicate for each concentration.

Cell viability was assessed by monitoring leakage of lactate dehydrogenase (LDH). To do this, both the culture medium and the cell lysate (lysis with 0.1% Triton X-100) were analyzed using an automatic biochemical analyzer (TBA-200FR; Toshiba, Tokyo, Japan) and the rate of survival relative to a control was calculated as follows: $LDH_{cell}/(LDH_{cell} + LDH_{medium})$.

The appropriate concentrations of test drugs were determined in a preliminary experiment. For our general protocol, the highest concentration was set to 10–20% of the lethal concentration as estimated by LDH leakage over 24 h. When the cells could tolerate as much as 10 mM or the level equal to the solubility limit of the compound in DMSO (allowed to add up to 0.1% in the final concentration), the highest concentration was set to either value. Exposures were performed at two different concentrations, 1/5 and 1/25 of the highest concentration. In case of coumarin, no LDH leakage was observed for either rat or human hepatocytes after treatment with concentrations of up to 300 μM, which was the solubility limit. Thus, concentrations of 12, 60, and 300 μM coumarin were used in subsequent assays. For DFNa, 400 μM was set as the maximum both in rats and humans. Thus, concentrations of 16, 80, and 400 μM DFNa were used in subsequent assays.

GeneChip analysis

For analysis of rat livers, microarray analysis was conducted on three of five samples from each single dose group (24-h post-dose) using GeneChip® RAE 230A probe arrays (Affymetrix, Santa Clara, California, USA). Liver samples were homogenized with buffer RLT supplied with the RNeasy Mini Kit (Qiagen) and total RNA was isolated according to the manufacturer's instructions.

For hepatocytes, GeneChip analysis was performed in duplicate for each concentration using RAE230 2.0 probe arrays for rat hepatocytes and U133 Plus 2.0 arrays for human hepatocytes (Affymetrix). Cells were dissolved with RLT buffer and collected for expression profiling. Different versions were used for the in-vitro versus the in-vivo study because RAE230 2.0 was released after the in-vivo experiments were completed. The procedure was conducted basically as described in the manufacturer's instructions using Superscript Choice System (Invitrogen, Carlsbad, California, USA) and T7-(dT)24-oligonucleotide primer (Affymetrix) for cDNA synthesis, cDNA Cleanup Module (Affymetrix) for purification, and BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, New York, USA) for synthesis of biotin-labeled cRNA. Ten micrograms of fragmented cRNA was hybridized to a RAE230A probe array for 18 h at 45 °C at 60 rpm, after which the array was washed and stained with streptavidin–phycoerythrin using Fluidics Station 400 (Affymetrix) and scanned with a Gene Array Scanner (Affymetrix). The digital image files were processed using Affymetrix Microarray Suite version 5.0. Microarray image data were analyzed with GeneChip Operating Software (Affymetrix). All microarray data were scaled by global normalization with the mean signal intensity of all data adjusted to 500.

Gene expression data analysis

To identify genes that are differentially expressed after in-vivo coumarin treatment, the Student's *t*-test was applied with a *P* value cut-off of 0.05 in combination with fold changes of 2.0 or greater and 0.5 or less using Spotfire® DecisionSite for Functional Genomics (Spotfire, Göteborg, Sweden). Probe sets designated as absent by an Affymetrix detection call in any of six samples (three each for control and treated) were excluded from further analysis.

To extract genes that changed in response to coumarin in both the in-vivo and in-vitro sample groups, the changes in expression of the above-mentioned probe sets were examined in rat hepatocytes treated with the high dose (300 μM) of coumarin. Probe sets showing 1.5-fold or greater (up-regulated) and 0.6-fold or less (down-regulated) were selected.

In the next step, the genes in common to the in-vivo and in-vitro rat assays were compared in rat versus human hepatocytes. To do this, we first examined public data on human orthologs of the