redundancy. In theory, the wrapper model should provide more accurate classification results than the filter model (Langley, 1994). Wrappers use classifiers to estimate the usefulness of feature subsets. The use of optimal feature subsets should provide corresponding classifiers with better classification accuracy because the features are selected according to their contribution to the classification accuracy of the classifiers. The disadvantage of the wrapper approach is its computational expense because the classifier must be repeatedly constructed to evaluate a subset during the CV process. Moreover, wrapper-type feature selection is sensitive to training data and runs the risk of over-fitting, leading to a lack of robustness in the selected gene set.

6 ROBUSTNESS OF THE SELECTED GENE LIST

Although many methods have been developed for conducting feature selection on microarrays, these selection methods produce selected gene lists that are insufficiently robust. If the predictor developed with one selected gene set would work well on data from other studies, then we would not have had to worry about list diversity. However, a lack of transferability of predictive power is often observed as a result of the same reason that causes instability of gene lists. Ein-Dor, Zuk and Domany 2006 found that the gene lists developed from microarrays using different methods in prognostic cancer studies differ significantly, even for different subsets of the same microarray data sets. They concluded that thousands of samples are needed for robust gene selection. As generating a more stable gene list will lead to more robust predictors, we must evaluate not only prediction performance but also the robustness of gene sets in feature gene selection. Assessing the stability of selected gene lists is crucial to guarantee their controlled and reliable utilization.

7 APPLICATION OF FILTER AND WRAPPER METHODS TO DIAGNOSTICS OF DRUG TOXICITY USING TOXICOGENOMICS DATA

Our previous study on concurrent diagnosis of drug-induced tubular injury using TGP data demonstrates the application of filter- and wrapper-type methods (Kondo *et al.*, 2009). Drug-induced renal tubular injury is a major concern in pre-clinical safety evaluations. In this study, we analyzed 33 nephrotoxicants and eight non-nephrotoxic hepatotoxicants to elucidate time- and dose-dependent global gene expression changes associated with proximal tubular toxicity. The compounds were administered orally or intravenously once daily to male Sprague–Dawley rats. The animals were exposed to four different doses of the compounds, and kidney tissues were collected on days 4, 8, 15, and 29. High-dose groups of 23 compounds that caused necrosis, degeneration, or regeneration in the renal tubules during chronic exposure were defined as the positive set (other high-dose groups of 10 nephrotoxicants were used as the external test set). Low-dose groups of all 41 compounds and high-dose groups of the eight hepatotoxicants that had no histopathological findings were defined as the negative set. To perform supervised classification algorithms after selecting differentially expressed genes, the microarray samples treated with nephrotoxicants and hepatotoxicants were divided into positives and negatives of the training set according to their histopathological findings.

Both filter- and wrapper-type gene selection algorithms with SVM-learning algorithms were used to extract biomarker candidates and construct classifiers using the selected genes. RFE methods were used for the wrapper-type gene selection algorithms and intensity-based moderated t-statistics (IBMT; Sartor *et al.*, 2006) was used as a filter-type gene selection algorithm (SVM was used as the classifier in this case).

Fivefold CV was executed for optimization of the classifiers and to calculate their prediction accuracies. First, the whole positive and negative training data sets were randomly divided into five subsets of roughly equal size. The SVM was trained with a selection of optimal genes on four subsets and then applied to the fifth subset as the test data set. Before the SVM was trained, optimal genes were selected from the training sets with both RFE-SVM and IBMT-SVM. The 99 top-ranked genes from each selection strategy were used to construct the classifiers. The prediction model using the top-ranked 99 feature genes exhibited saturated prediction performance (Figures 2 and 3). Thus, by tracing the prediction performance of each constructed classifier for the test sets, we determined an appropriate

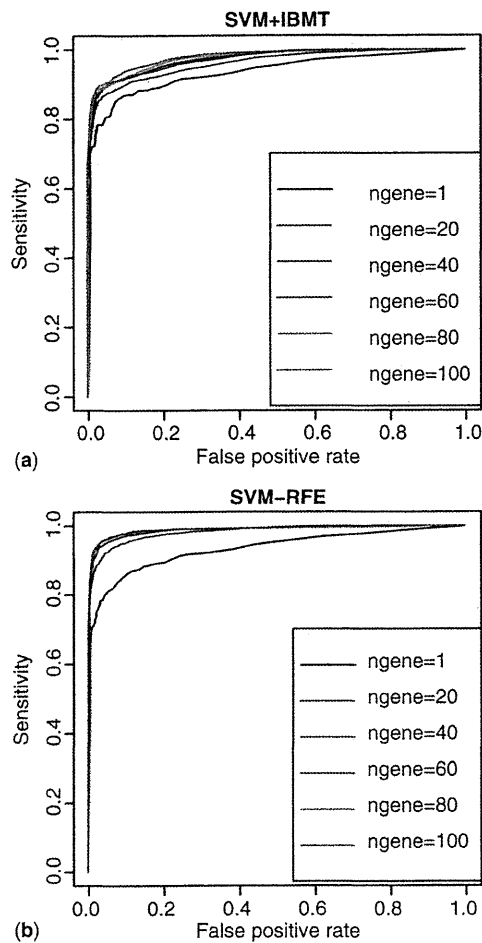


Figure 2. Receiver operating characteristic (ROC) curves for feature gene subsets selected using SVM-IBMT (a) and SVM-RFE (b).

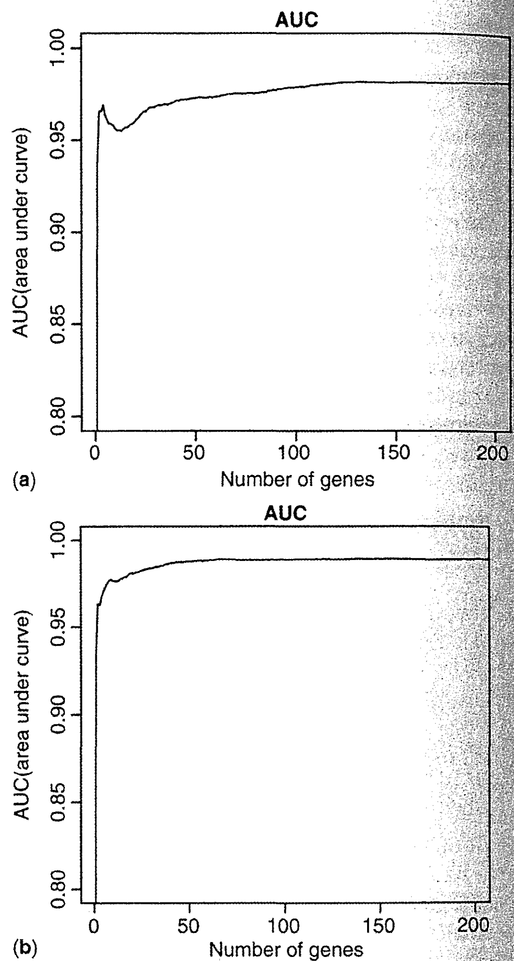
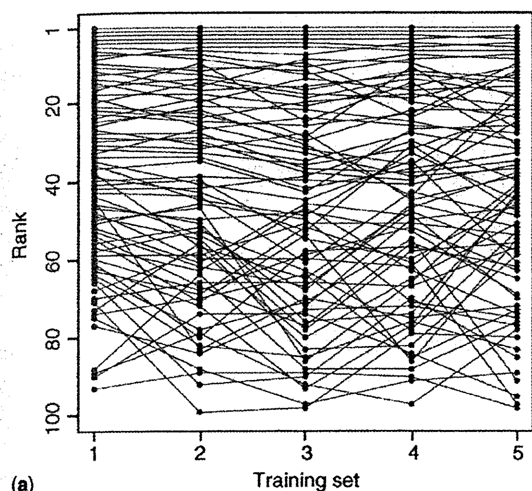


Figure 3. Area under curves (AUC) of the ROC curves by SVM-IBMT (a) and SVM-RFE (b), along with the number of selected feature genes.

threshold number of feature genes, which is one of the challenges in executing search algorithms.

As the result of fivefold CV, we determined the sensitivity of each classifier to be 94% (RFE-SVM; 99 probes) and 93.8% (IBMT-SVM; 99 probes), when we allowed for 10% false positives (Figure 2). Although SVM-RFE exhibited the higher classification accuracy, as we expected, the concordance rate of the feature gene list selected by RFE between the subtraining sets of fivefold CV was lower than that of the filter-type IBMT-SVM (Figure 4). The difference in concordance rate indicates that the RFE-SVM classifier may have over-fitted to the training set, resulting in an insufficiently robust list of biomarkers. In contrast, the feature genes selected by IBMT-SVM were stable between different training data sets generated through fivefold CV. As noted above, in addition to the prediction performance of the constructed classifier, the robustness (stability) of the selected gene sets is critical to successful feature selection. Therefore, in this case using TGP kidney data sets, we concluded that the filter-type IBMT-SVM method was the preferable gene selection and classification algorithm.

Consequently, we determined the appropriate maker gene set using the following criteria: (a) the prediction accuracy was saturated (Figure 3); (b) the



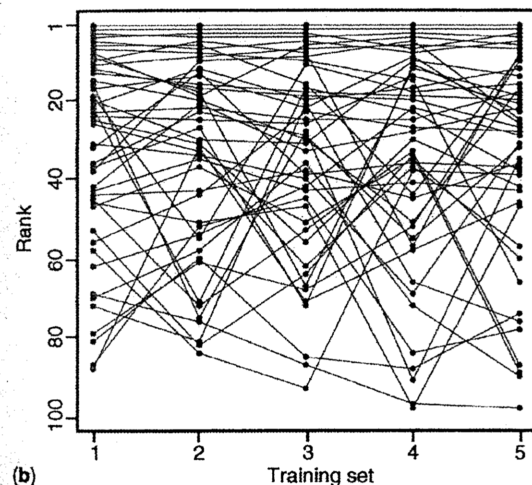
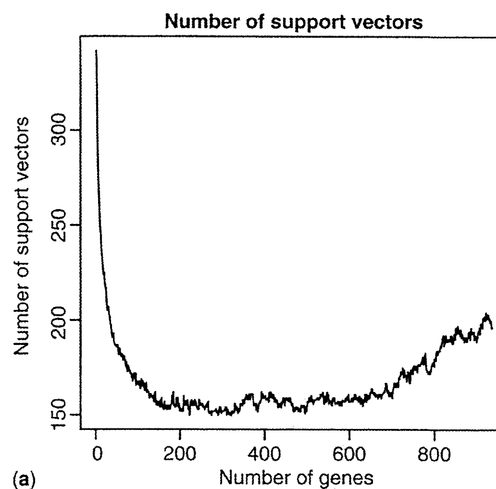


Figure 4. Perturbation of feature gene ranking between different training sets generated during fivefold CV. The horizontal axis indicates the training subsets and the vertical axis indicates the ranking of the feature genes (top 80 probes). Fivefold CV was executed by randomly dividing samples.



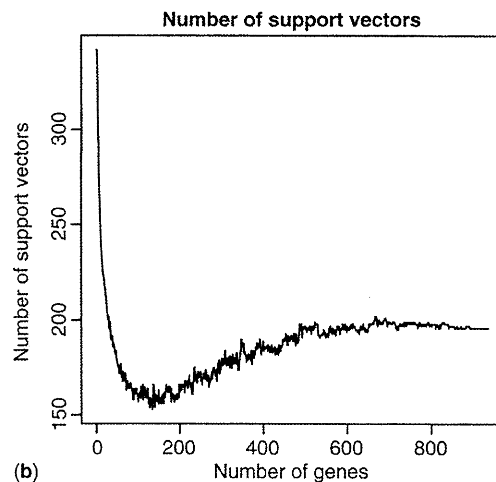


Figure 5. The number of support vectors in the constructed models of SVM-IBMT (a) and SVM-RFE (b), along with the number of selected feature genes.

number of support vectors was adequately low (an excessive number of support vectors could indicate over-fitting of the linear SVM classifier) (Figure 5), and (c) the number of feature genes was substantially lower than the number of samples, to avoid over-fitting.

Also, the selected gene list contained enough key genes to interpret their biological relevance in drug-induced renal tubular injury. The gene list contained well-known biomarkers, such as kidney injury molecule 1, ceruloplasmin, clusterin, and tissue inhibitor of metallopeptidase 1, as well as novel biomarker candidates. Most of the genes involved in tissue remodeling, immune/inflammatory response, cell adhesion/proliferation/migration, and metabolism were predominantly up-regulated. Down-regulated genes participated in cell adhesion/proliferation/migration, membrane transport, and signal transduction. This indicates that the gene list provides us with elaborate knowledge about mechanistic toxicology.

8 CONCLUSIONS

Selecting a small gene set from large microarray data sets is critical from both biological and computational viewpoints, and many algorithms have been developed for feature selection. Most studies on feature selection have found that wrapper methods are superior to filter methods, but many of these studies have over-emphasized prediction accuracy and over-looked the robustness of the selected genes. Prediction reliability assumes the stability of the model. In fact, this study illustrates that IBMT-SVM produces more stable gene lists than RFE-SVM. This finding is adaptable to only this training set. In the case of other training sets, we must evaluate multiple methods and choose the best approach. Therefore, we have to carefully gauge not only prediction performance but also the robustness of gene sets in feature gene selection.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan. This work was also supported in part by the Ministry of Education, Culture, Sports, Science and Technology, Japan; the Japan Society for the Promotion of Science (JSPS); and the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

REFERENCES

Bishop CM. 1995. *Neural Networks for Pattern Recognition*. Oxford University Press: London, UK.

Boverhof DR, Zacharewski TR. 2006. Toxicogenomics in risk assessment: application and needs. *Toxicol. Sci.* **89**: 352–360.

Cunningham ML, Lehman-McKeeman L. 2005. Applying toxicogenomics in mechanistic and predictive toxicology. *Toxicol. Sci.* **83**: 205–206.

Ein-Dor L, Zuk O, Domany E. 2006. Thousands of samples are needed to generate a robust gene list for predicting outcome in cancer. *Proc. Natl Acad. Sci. USA* **103**: 5923–5928.

Hall MA. 2000. Correlation-based feature selection for discrete and numeric class machine learning. *Proceedings of the Seventeenth International Conference on Machine Learning Table of Contents*, San Fransisco, CA: Morgan Kaufmann Publishers Inc. 359–366.

Hayes KR, Vollrath AL, Zastrow GM, McMillan BJ, Craven M, Jovanovich S, Rank DR, Penn S, Walisser JA, Reddy JK, Thomas RS, Bradfield CA. 2005. EDGE: a centralized resource for the comparison, analysis, and distribution of toxicogenomic information. *Mol. Pharmacol.* **67**: 1360–1368.

Kondo C, Minowa Y, Uehara T, Okuno Y, Nakatsu N, Ono A, Maruyama T, Kato I, Yamate J, Yamada H, Ohno Y, Urushidani T. 2009. Identification of genomic biomarkers for concurrent diagnosis of drug-induced renal tubular injury using a large-scale toxicogenomics database. *Toxicology* **265**: 15–26.

Kononenko I. 1994. Estimating attributes: analysis and extensions of RELIEF. *European Conference on Machine Learning*, Catania. Italy: Springer Verlag, 171–182.

Langley P. 1994. Selection of relevant features in machine learning. *Proceedings of AAAI Fall Symposium on Relevance*, New Orleans: AAAI Press, 140–144.

Mattingly CJ, Rosenstein MC, Davis AP, Colby GT, Forrest JN, Jr, Boyer JL. 2006. The comparative toxicogenomics database: a cross-species resource for building chemical-gene interaction networks. *Toxicol. Sci.* **92**: 587–595.

Sartor MA, Tomlinson CR, Wesselkamper SC, Sivaganesan S, Leikauf GD, Medvedovic M. 2006. Intensity-based hierarchical Bayes method improves testing for differentially expressed genes in microarray experiments. *BMC Bioinf.* **7**: 538.

Thomas JG, Olson JM, Tapscott SJ, Zhao LP. 2001. An efficient and robust statistical modeling approach to discover differentially expressed genes using genomic expression profiles. *Genome Res.* **11**: 1227–1236.

Tsai C-A, Chen Y-J, Chen JJ. 2003. Testing for differentially expressed genes with microarray data. *Nucl. Acids Res.* **31**: e52.

Urushidani T, Nagao T. 2005. *Toxicogenomics: the Japanese initiative*. In Handbook of Toxicogenomics – Strategies and Applications, Borlak J (ed). Wiley-VCH: Weinheim; 623–631.

Vapnik VN. 1998. *Statistical Learning Theory*. Wiley, New York.

Waters M, Boorman G, Bushel P, Cunningham M, Irwin R, Merrick A, Olden K, Paules R, Selkirk J, Stasiewicz S, Weis B, Van Houten B, Walker N, Tennant R. 2003. Systems toxicology and the chemical effects in biological systems (CEBS) knowledge base. *EHP Toxicogenomics* **111**: 15–28.

HET

Human and Experimental Toxicology
XX(X) 1–10
© The Author(s) 2011
Reprints and permission:
sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/0960327111399325
het.sagepub.com
SAGE

Effects of DMSO on gene expression in human and rat hepatocytes

Kayo Sumida[1], Yoshinobu Igarashi[2], Naoki Toritsuka[3], Tomochika Matsushita[4], Kaori Abe-Tomizawa[5], Mikio Aoki[6], Tetsuro Urushidani[2,7], Hiroshi Yamada[2] and Yasuo Ohno[8]

Abstract

Dimethyl sulfoxide (DMSO) is a very common organic solvent used for dissolving lipophilic substances, for example for in vitro cell-based assays. At the same time, DMSO is known to be cytotoxic at high concentrations. Therefore, it is important to define threshold concentrations of DMSO for cells but relevant data at the molecular level are very limited. We have focused on conducting microarray analyses of human and rat hepatocytes treated with more than 100 chemicals in attempts to identify candidate biomarker genes. In the present study, the effects of DMSO on gene expression and cytotoxicity were assessed in human cryopreserved hepatocytes and rat primary cultured hepatocytes. A cytotoxicity test with lactate dehydrogenase (LDH) activity demonstrated DMSO to be noncytotoxic up to a concentration of 2% (v/v) in both cases and there were only few effects on the gene expression profiles up to 0.5% (v/v). The observed differences from controls were considered to be of little toxicological importance, but still need to be taken into account in interpretation of findings when DMSO is used at high concentration.

Keywords

gene expression profile, DMSO, human cryopreserved hepatocytes, rat primary cultured hepatocytes, cytotoxicity

Introduction

A large-scale gene expression database, termed TG-GATES (Genomics Assisted Toxicity Evaluation System), has been established by the Toxicogenomics Project in Japan.[1] About 150 chemicals, mainly for medicinal use, were selected, and gene expression in rat liver, rat kidney, rat primary cultured hepatocytes, and human cryopreserved hepatocytes is being comprehensively analyzed using Affymetrix GeneChip system (Santa Clara, CA, USA). In the project, rat and human hepatocytes are treated with toxicological prototype drugs, in three dose-ranges, and samples are collected 2, 8, and 24 hr after a single treatment. One of the main aims of our project is to identify candidate biomarker genes to predict and/or diagnose toxicity.

The actual dose-ranges are set according to dose-finding studies. The maximum concentration was set at 10 mM in the case of a chemical with high solubility. The concentration of a chemical with low solubility is determined with reference to solubility in 0.1%

[1] Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., Konohana-ku, Osaka, Japan
[2] Toxicogenomics Informatics Project, National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan
[3] Applied Pharmacology & New Technologies, Biopharmaceutical Assessments Core Function Unit, Eisai Co., Ltd., Tsukuba, Ibaraki, Japan
[4] Safety Assessment Department, Chugai Pharmaceutical Co. Ltd., Gotemba, Shizuoka, Japan
[5] Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Kawauchi-cho, Tokushima, Japan
[6] Genomic Science Laboratories, Dainippon Sumitomo Pharma Co., Ltd., Konohana-ku, Osaka, Japan
[7] Department of Pathophysiology, Faculty of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts, Kyotanabe, Kyoto, Japan
[8] National Institute of Health Sciences, Setagaya-ku, Tokyo, Japan

Corresponding author:
Kayo Sumida, Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., 3-1-98 Kasugadenaka, Konohana-ku, Osaka 554-8558, Japan
Email: sumida@sc.sumitomo-chem.co.jp

(v/v) DMSO, which is the commonly used concentration for in vitro assays. However, in some cases, this means that the concentration is too low for the effects of the chemical to be adequately reflected in the gene expression profile. It is then necessary to increase the chemical concentration, but this also necessitates increasing the DMSO concentration to obtain a solution. Since DSMO is itself toxic at high concentration, this could result in misleading findings. Since there have been no reports of comprehensive gene expression profiles after treatment with DMSO, the present study was conducted using human cryopreserved hepatocytes and rat primary cultured hepatocytes.

There are several reports about influence of DMSO on expression of genes in cells. For example, it has differentiation-inducing effects on embryonic stem (ES) cells, which are most sensitive towards the cytotoxic effects of DMSO including Oct-4.[2] Klinken et al. also reported induction of differentiation in murine erythroleukemia (MEL) cells with alteration of proto-oncogene levels.[3] Similar findings have been described for HL60 cells.[4] As far as housekeeping genes are concerned, Nishimura et al. examined the effects of DMSO on the expression of beta-actin (Actb), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), beta-glucuronidase (Gusb), phosphoglycerate kinase 1 (Pgk1), peptidylprolyl isomerase A (Ppia), and transferrin receptor (Tfrc) mRNA in cultures of C2C12 myotubes and the mRNA levels of some housekeeping genes were affected by exposure of DMSO concentrations of 0.5% (v/v) or more.[5] The induction of some drug-metabolizing enzyme genes by DMSO has also been reported.[6-8]

Thus, for in vitro assays, it is important to define the threshold concentration of DMSO for cells in accordance with the endpoint. The aim of the present study was to identify the lowest concentration of DMSO at which no significant effects on gene expression profile were observed, with particular attention to candidate biomarker genes. For this purpose, human cryopreserved hepatocytes and rat hepatocytes were examined for effects of DMSO on gene expression with a DNA microarray system and cytotoxicity with a lactate dehydrogenase (LDH) leakage method.

Materials and methods

Cell culture and in vivo studies

Human cryopreserved hepatocytes, purchased from CellzDirect, Inc. (Durham, NC, USA), were recovered in cryopreserved hepatocyte-recovered medium (CellzDirect, Inc.) and seeded in HCM Bullekit® (Cambrex Corp., East Rutherford, NJ, USA) supplemented with 10% FBS (Invitrogen Corp., Carlsbad, CA, USA) in 6-well collagen type-1 coated plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at a concentration of 1.2×10^6 cells/2 mL/well for 4 hours in a humidified atmosphere (37°C, 5% CO_2). After 4-hour culture, medium was replaced with HCM Bullekit® without FBS and human hepatocytes were cultured for 20 more hours.

At day 2, medium was replaced with HCM Bullekit® containing 0% (v/v), 0.1% (v/v), 0.5% (v/v), 0.75% (v/v), 1% (v/v), or 2% (v/v) DMSO (Kanto Chemical Co., Inc., Tokyo, Japan, purity > 99.7%), and the hepatocytes were cultured for 24 more hours in a humidified atmosphere (37°C, 5% CO_2).

At day 3, medium was collected for measurement of LDH activity and total RNA samples were collected for gene expression analysis. Extraction of total RNA was conducted using a RNeasy Mini Kit (Qiagen, Hilden, Germany) before quantification with a spectrophotometer DU-7400 (Beckman Coulter, Fullerton, CA, USA) and assessment of ribosomal RNA integrity using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Rat primary-cultured hepatocytes, prepared from a 5-week-old male Spraque-Dawley rat (Charles River Japan Inc., Kanagawa, Japan), were seeded in HCM Bullekit® supplemented with 10% FBS in 6-well collagen type-1 coated plates at a concentration of 1.0×10^6 cells/2 mL/well for 2-3 hours in a humidified atmosphere (37°C, 5% CO_2). After 2-3 hours culture, medium was replaced with HCM Bullekit® without FBS and rat hepatocytes were cultured for 16 hours.

At days 2 and day 3, rat primary cultured hepatocytes were treated the same as human cryopreserved hepatocytes.

Gene expression analysis

Microarray analysis was conducted on three samples for each group using HGU133 plus 2.0 probe arrays and RG230 2.0 probe arrays (Affymetrix). The procedures were basically conducted following the manufacturer's protocol, as previously reported.[9,10] The obtained image files were analyzed with the Affymetrix data suite system, Microarray Suite 5.0 (MAS 5.0) and derived signal values were globally normalized and targeted to all probe sets equal to 500 before comparative analysis to examine gene expression differences between treatment and control samples.

Quantitative RT-PCR assay validation

To confirm the gene expression data using microarray, we conducted quantitative RT-PCR for some rat genes. Five genes (Ccl20, sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1 [Sult1a1], Cyp1a1, carbonic anhydrase 2 [Car2], and Cyp2c12) were measured by real-time PCR using TaqMan® Gene Expression Assays and the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). cDNAs were generated from 12 ng of total RNA using Invtirogen reverse transcription reagents (SuperScript III Reverse Transcriptase (10,000 units), RNaseOUT, Random Primers, 10 mM dNTP, and 0.1 M DTT). Three replicates were run for each gene for each sample in a 384-well format plate.

Measurement of LDH activity

The collected medium was centrifuged at 1500 × g or more for 5 min at 4°C. About 0.8 mL of the supernatant was applied to a biochemical autoanalyzer TBA-200FR (Toshiba, Tokyo, Japan) with the UV-rate method.

Data analysis

Raw probe intensities in Affymetrix CEL files were normalized using the MAS 5.0 algorithm with default parameters. After the MAS 5.0 process, we selected each probe for which a detection call is "present" among all conditions in this study. The fold change values were calculated as the ratio of gene expression values between DMSO treatment data versus control data. To compare gene expression levels among five different DMSO concentrations, we normalized the expression data using Tukey's biweight method and converted them into signal/control $\log_2$ratios. We used the smoothing spline clustering method[11] to sort and identify gene expression patterns that were dependent on the DMSO concentration. To find significantly and differentially expressed gene sets between control and DMSO treatment data, gene set enrichment analysis (GSEA)[12] was performed. The gene set data for the human and the rat were extracted from the Kyoto encyclopedia of genes and genomes (KEGG) pathway database.[13]

Results

Gene expression analysis

In human cryopreserved hepatocytes, there were only 4 probe sets, which altered in expression ($p < 0.05$ and more than 2-fold) at a concentration of 0.1% (v/v) DMSO compared with control (0% DMSO). There were 16 probe sets altered in expression at 0.5% (v/v) DMSO, 57 probe sets at 0.75% (v/v) DMSO, 133 probe sets at 1% (v/v) DMSO and 752 probe sets at 2% (v/v) DMSO. In rat primary cultured hepatocytes, there were only 1 probe set, which altered in expression ($p < 0.05$ and more than 2-fold) at a concentration of 0.1% (v/v) DMSO compared with control (0% DMSO). There were 10 probe sets altered in expression at 0.5% (v/v) DMSO, 22 probe sets at 0.75% (v/v) DMSO, 67 probe sets at 1% (v/v) DMSO, and 497 probe sets at 2% (v/v) DMSO (Figure 1a-d).

The GSEA detected differentially expressed gene sets in each DMSO treatment data compared to the control data. We analyzed 165 and 195 gene sets for human and rat hepatocytes, respectively. The differentially expressed gene sets were shown in Table 1. In the case of human hepatocytes, the top 10 differentially expressed gene sets at 0.1% (v/v) DMSO were not within the top 10 at 1% (v/v) DMSO. Distinct gene set changes were observed between 0.75% (v/v) DMSO and 1% (v/v) DMSO. In rat primary cultured hepatocytes, of the top 10 gene sets detected at 0.1% (v/v) DMSO, only 2 gene sets were detected at 2% (v/v) DMSO. The data indicate that 0.1% (v/v) DMSO had distinctly less influence than higher DMSO concentration. From 0.5% (v/v) DMSO to 1% (v/v) DMSO, some overlapping gene sets like RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY were observed.

The alterations in expression of Phase I, II, III drug-metabolizing enzymes are summarized in Table 2. Of the total of 728 probe sets (Phase I: 295, Phase II: 325, Phase III: 108), 161 genes demonstrated significantly altered expression levels in human hepatocytes. In rat hepatocytes, of the total of 409 probe sets (Phase I: 170, Phase II: 183, Phase III: 56), 57 genes were significantly altered. The alteration in expression of most drug-metabolizing enzymes was not severe up to 0.75% (v/v) DMSO in both human and rat cases.

Cytotoxicity of DMSO

Human cryopreserved hepatocytes and rat primary cultured hepatocytes were treated with DMSO for 24 hours at five different concentrations in order to examine its cytotoxic effects. In both human and rat hepatocytes, no toxic effects were

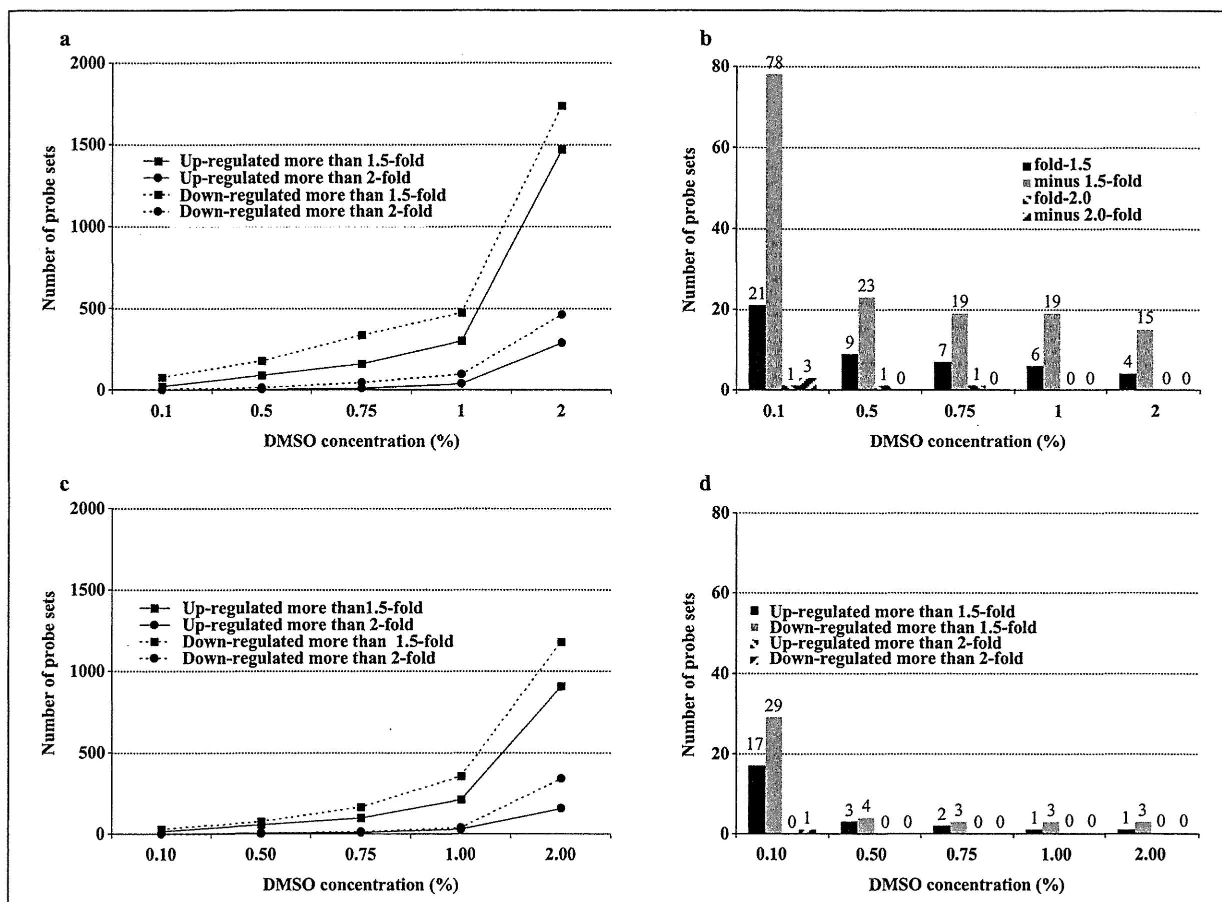


Figure 1. The numbers of differentially expressed genes (probe sets) in human cryopreserved hepatocytes (a) and rat primary cultured hepatocytes (c) are shown. Solid and dashed lines indicate up- and down-regulated genes, respectively. Circles and squares indicate 2.0- and 1.5-fold changes, respectively. The number of the probe sets was significantly increased between 1% (v/v) and 2% (v/v) dimethyl sulfoxide (DMSO). The numbers of differentially expressed genes (probe sets) commonly observed from 0.1% (v/v) DMSO to higher concentrations in human cryopreserved hepatocytes (b) and rat primary-cultured hepatocytes (d) are shown.

morphologically observed up to the concentration of 2% (v/v) DMSO.

In addition, the cytotoxicity of DMSO was examined by the conventional LDH test. LDH activity response curves for DMSO in human and rat hepatocytes are shown in Figure 2. Again, DMSO did not show any toxic effects up to the concentration of 2% (v/v).

Discussion

We have been focusing on identification of candidate biomarker genes to predict and/or diagnose toxicity in our project (TGP2, Toxicogenomics Informatics Project). Gene expression data using rat primary hepatocytes or human cryopreserved hepatocytes as well as gene expression data using rat liver are being analyzed. However, the effects of some chemicals were found to not be adequately reflected in the gene expression profile due to the low concentrations dictated by solubility in the set concentration of DSMO. The necessity to increase the DMSO concentration is the reason for the present study. The fact that there were not many probe sets with altered in expression up to 0.75% (v/v) DMSO is therefore very important.

In particular, there were remarkably few probe sets with altered in expression up to 0.5% (v/v) DMSO. As the concentration of DMSO increased, the number of probe sets with alteration and the magnitude of alteration increased. In human hepatocytes, there was only 1 probe set (histone cluster 1, H2bd; HIST1H2BD), which was upregulated ($p < .05$ and more than 2-fold) at a concentration of 0.1% (v/v) DMSO, 4 probe sets

1

Table 1. The differentially expressed gene sets in human and rat hepatocytes are shown[a]

DMSO Con. (%)	Gene Set (KEGG Pathway ID and Name)	NOM p Value	FDR q Value	FWER p Value
0.1	HSA03010_RIBOSOME	0.00	0.00	0.00
	HSA03410_BASE EXCISION REPAIR	0.00	0.06	0.07
	HSA04110_CELL CYCLE	0.00	0.31	0.47
	HSA03050_PROTEASOME	0.02	0.24	0.48
	HSA03030_DNA REPLICATION	0.03	0.25	0.57
	HSA00760_NICOTINATE AND NICOTINAMIDE METABOLISM	0.03	0.21	0.58
	HSA00480_GLUTATHIONE METABOLISM	0.02	0.54	0.92
	HSA00240_PYRIMIDINE METABOLISM	0.02	0.48	0.92
	HSA03430_MISMATCH REPAIR	0.05	0.57	0.97
	HSA05012_PARKINSON's DISEASE	0.00	0.86	0.99
	HSA04020_CALCIUM SIGNALING PATHWAY	0.03	0.98	1.00
0.5	HSA03010_RIBOSOME	0.00	0.03	0.03
	HSA00590_ARACHIDONIC ACID METABOLISM	0.00	0.27	0.40
	HSA00591_LINOLEIC ACID METABOLISM	0.02	0.27	0.54
	HSA04742_TASTE TRANSDUCTION	0.04	0.20	0.54
	HSA00480_GLUTATHIONE METABOLISM	0.00	0.34	0.85
	HSA05012_PARKINSON's DISEASE	0.05	0.83	1.00
	HSA04080_NEUROACTIVE LIGAND-RECEPTOR INTERACTION	0.05	0.74	1.00
0.75	HSA00590_ARACHIDONIC ACID METABOLISM	0.00	0.02	0.02
	HSA03010_RIBOSOME	0.00	0.20	0.31
	HSA00591_LINOLEIC ACID METABOLISM	0.00	0.14	0.32
	HSA00190_OXIDATIVE PHOSPHORYLATION	0.00	0.16	0.46
	HSA05012_PARKINSON's DISEASE	0.00	0.15	0.50
	HSA00760_NICOTINATE AND NICOTINAMIDE METABOLISM	0.02	0.30	0.85
	HSA03050_PROTEASOME	0.04	0.42	0.95
	HSA05322_SYSTEMIC LUPUS ERYTHEMATOSUS	0.02	0.46	1.00
1	HSA04130_SNARE INTERACTIONS IN VESICULAR TRANSPORT	0.00	0.52	0.35
	HSA05322_SYSTEMIC LUPUS ERYTHEMATOSUS	0.00	0.29	0.38
	HSA05120_EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	0.00	0.44	0.65
	HSA04912_GNRH SIGNALING PATHWAY	0.00	0.37	0.69
	HSA00591_LINOLEIC ACID METABOLISM	0.02	0.39	0.80
	HSA00601_GLYCOSPHINGOLIPID BIOSYNTHESIS - LACTO AND NEOLACTO SERIES	0.02	0.36	0.84
	HSA04142_LYSOSOME	0.00	0.39	0.90
	HSA04010_MAPK SIGNALING PATHWAY	0.03	0.69	1.00
2	HSA04912_GNRH SIGNALING PATHWAY	0.00	1.00	0.76
	HSA05322_SYSTEMIC LUPUS ERYTHEMATOSUS	0.03	0.85	0.85
	HSA04916_MELANOGENESIS	0.00	0.89	0.91
	HSA04070_PHOSPHATIDYLINOSITOL SIGNALING SYSTEM	0.03	0.98	0.98
0.1	RNO03420_NUCLEOTIDE_EXCISION_REPAIR	0.00	0.06	0.04
0.5	RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.52	0.50
	RNO03420_NUCLEOTIDE_EXCISION_REPAIR	0.00	0.41	0.74
	RNO04623_CYTOSOLIC_DNA-SENSING_PATHWAY	0.00	1.00	1.00
	RNO04062_CHEMOKINE_SIGNALING_PATHWAY	0.00	0.83	1.00
	RNO05010_ALZHEIMER's_DISEASE	0.00	0.69	1.00
	RNO05016_HUNTINGTON's_DISEASE	0.00	0.67	1.00
	RNO05012_PARKINSON's_DISEASE	0.02	0.98	1.00
	RNO00190_OXIDATIVE_PHOSPHORYLATION	0.02	1.00	1.00

(continued)

Table 1. (continued)

DMSO Con. (%)	Gene Set (KEGG Pathway ID and Name)	NOM p Value	FDR q Value	FWER p Value
0.75	RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.81	0.49
	RNO05016_HUNTINGTON's_DISEASE	0.00	0.58	0.64
	RNO00071_FATTY_ACID_METABOLISM	0.00	0.44	0.71
	RNO00051_FRUCTOSE_AND_MANNOSE_METABOLISM	0.05	0.60	0.90
	RNO05012_PARKINSON's_DISEASE	0.00	0.60	1.00
	RNO04062_CHEMOKINE_SIGNALING_PATHWAY	0.00	0.55	1.00
	RNO03420_NUCLEOTIDE_EXCISION_REPAIR	0.00	0.50	1.00
	RNO04330_NOTCH_SIGNALING_PATHWAY	0.00	0.46	1.00
	RNO05010_ALZHEIMER's_DISEASE	0.00	0.49	1.00
	RNO04120_UBIQUITIN_MEDIATED_PROTEOLYSIS	0.00	0.45	1.00
	RNO00330_ARGININE_AND_PROLINE_METABOLISM	0.04	0.54	1.00
1	RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.05	0.02
	RNO05016_HUNTINGTON's_DISEASE	0.00	0.06	0.11
	RNO05012_PARKINSON's_DISEASE	0.00	0.10	0.24
	RNO00051_FRUCTOSE_AND_MANNOSE_METABOLISM	0.00	0.26	0.76
	RNO04330_NOTCH_SIGNALING_PATHWAY	0.00	0.28	0.85
	RNO00534_HEPARAN_SULFATE_BIOSYNTHESIS	0.00	0.33	0.92
	RNO04062_CHEMOKINE_SIGNALING_PATHWAY	0.00	0.35	1.00
	RNO05010_ALZHEIMER's_DISEASE	0.00	0.32	1.00
	RNO00190_OXIDATIVE_PHOSPHORYLATION	0.00	0.33	1.00
	RNO04620_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.32	1.00
2	RNO00280_VALINE,_LEUCINE_AND_ISOLEUCINE_DEGRADATION	0.00	0.02	0.00
	RNO04621_NOD-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.03	0.04
	RNO04062_CHEMOKINE_SIGNALING_PATHWAY	0.00	0.02	0.04
	RNO05010_ALZHEIMER's_DISEASE	0.00	0.05	0.13
	RNO04620_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.06	0.13
	RNO00071_FATTY_ACID_METABOLISM	0.00	0.12	0.35
	RNO04330_NOTCH_SIGNALING_PATHWAY	0.00	0.13	0.44
	RNO00600_SPHINGOLIPID_METABOLISM	0.00	0.12	0.53
	RNO04130_SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT	0.00	0.12	0.53
	RNO04622_RIG-I-LIKE_RECEPTOR_SIGNALING_PATHWAY	0.00	0.12	0.56
	RNO04623_CYTOSOLIC_DNA-SENSING_PATHWAY	0.00	0.16	0.76
	RNO00640_PROPANOATE_METABOLISM	0.00	0.17	0.76
	RNO00100_STEROID_BIOSYNTHESIS	0.00	0.17	0.76
	RNO04142_LYSOSOME	0.00	0.16	0.76
	RNO04060_CYTOKINE-CYTOKINE_RECEPTOR_INTERACTION	0.00	0.17	0.81
	RNO04140_REGULATION_OF_AUTOPHAGY	0.00	0.19	0.86
	RNO05215_PROSTATE_CANCER	0.00	0.32	1.00
	RNO04660_T_CELL_RECEPTOR_SIGNALING_PATHWAY	0.00	0.34	1.00

[a]The pathway gene sets were extracted from KEGG pathway database and assessed using GSEA (gene set enrichment analysis). The GSEA can determine differentially expressed gene sets between two biological states or conditions. NOM-*p*val, FDR-*q*val and FWER-*p*val indicate nominal *p*-value, false discovery rate and family-wise error rate *p*-value, respectively.

(HIST1H2BD, BTB [POZ] domain containing 11 [BTBD11], chemokine [C-X-C motif] ligand [CXCL11], and another with no annotation) at 0.5% (v/v) DMSO. And there were 3 probe sets (endoplasmic reticulum aminopeptidase 1 [ERAP1], natural killer-tumor recognition sequence [NKTR], and cleavage and polyadenylation factor subunit, homolog [PCF11]), which were down-regulated ($p < 0.05$ and less than 1/2) at a concentration of 0.1% (v/v) DMSO, 12 probe sets (insulin-like growth factor binding protein 3 [IGFBP3], dehydrogenase/reductase (SDR family) member 9 [DHRS9], LIM and cysteine-rich domains 1 [LMCD1], solute carrier family 13 (sodium-dependent citrate transporter), member 5 [SLC13A5], IGFBP1, thyroid hormone responsive [THRSP], interleukin 1 receptor antagonist [IL1RN],

Table 2. Up- and down-regulated drug metabolizing enzymes in human and rat hepatocytes are shown[a]

Expression	Gene Symbol
Strongly up-regulated	AGXT2L1, BHMT, CYP2C8, CYP39A1, CYP3A4, CYP3A5, CYP4F3, CYP7B1, OAT, TAT
Up-regulated	ABAT, ABCC5, ABCC9, ABCD1, ABCD3, ABCD4, ADH6, ALDH1L1, ALDH2, ALDH3A2, ALDH5A1, ALDH6A1, ALDH7A1, ALDH9A1, BCAT1, BCAT2, BHMT2, COQ5, CYB5A, CYB5R4, CYP1B1, CYP20A1, CYP27A1, CYP2A6, CYP2A7, CYP2B6, CYP2B7P1, CYP2E1, CYP2U1, CYP4A11, CYP4A22, CYP4F11, DPYD, EPHX1, FMO3, FMO5, FTSJD2, GPT2, GPX4, GSTM2, HGSNAT, LRTOMT, MAOB, MSRB2, N6AMT2, NAT12, NAT15, PCMTD1, PCMTD2, PRMT2, PSAT1, SETD7, SLCO4C1, TPMT, TXNDC12, ZADH2
Strongly down-regulated	ABCA1, ABCC3, ADH1B, ADH1C, ADH4, AKR1B10, AKR1D1, ALDH1A1, ALDH1B1, ALPL, CYP2C18, CYP2C9, HNMT, METT10D, METTL12, METTL8, METTL9, NAT11, NAT8, PRMT1, SLCO1B1, SLCO1B3, SULT1A1, SULT1B1, TRMT6, UGT1A6, UGT2A3, UGT2B15, UGT2B28, UGT2B4
Down-regulated	ABCB10, ABCB6, ABCC2, ABCC3, ABCC4, ABCC5, ADH5, AGXT2L2, AKR1A1, AKR1B1, AKR1C1, AKR1C4, AKR7A3, ALDH4A1, AOX1, ARD1A, AS3MT, ASMTL, COMTD1, COQ3, CYB5B, CYB5D1, CYB5R3, CYP51A1, DMAP1, DNMT1, FMO4, GPX1, GSTA1, GSTCD, GSTO1, HEMK1, HNMT, MAOA, METT5D1, METTL1, METTL11A, METTL13, METTL3, METTL5, MGST1, MTR, NAT10, NAT11, NAT13, NAT2, NAT5, NAT9, PEMT, PRMT5, RG9MTD1, RG9MTD2, RNMTL1, RRP8, SETD8, SHMT1, SULT1A1, SULT1A2, SULT2A1, TRDMT1, TRMT1, TRMT11, TRMT5, TRMT6, ZADH2
Up-regulated	Abca4, Abca5, Abcb1a, Aldh1a2, Aldh6a1, As3mt, Bhmt2, Ces3, Coq3, Cyp1a1, Cyp1a2, Cyp4a3, Cyp4f17, Cyp4f5, Gpx3, Gstm7, Gstt1, Gstt2, Nnmt, Pcmtd1, Pcmtd2, Slco3a1, Sult1a1, Tpmt, Trmt12
Down-regulated	Abcb11, Abcc2, Adh1, Akr1b8, Akr1c12, Akr1d1, Akr7a3, Ard1a, Bcat1, Comtd1, Cyp26b1, Cyp2b3, Cyp2c12, Cyp2c22, Cyp3a9, Fmo5, Gpt2, Gstm2, Gstt3, Maoa, Maob, Mett10d, Mettl2, Mgst2, N6amt1, Prmt1, Psat1, Rrp8, Slco1a1, Slco1b2, Ugt2b17, Ugt2b36

[a]All drug metabolizing enzymes are classified using the smoothing spline clustering[11]. This method is able to classify a series data such as time series data. Several genes are shown multiple times due to redundant probe sets in the microarray.

chromosome 10 open reading frame 108 [C10orf108], palmdelphin [PALMD], and choroideremia-like (Rab escort protein 2) [CHML]) at 0.5% (v/v) DMSO. In

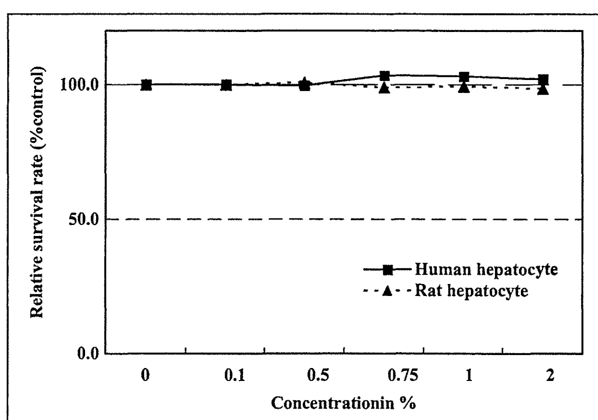


Figure 2. Lactate dehydrogenase (LDH) activity response curves for dimethyl sulfoxide (DMSO) in human cryopreserved hepatocytes and rat primary cultured hepatocytes. The experiments were conducted with hepatocytes in three independent wells (n = 3). Standard error bars are shown.

rat hepatocytes, there was no probe set, which was up-regulated ($p < 0.05$ and more than 2-fold) at a concentration of 0.1% (v/v) DMSO, 6 probe sets (Ccl2, radical S-adenosyl methionine domain containing 2 (Rsad2), Ccl20, Cxcl2, DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 [Ddx60], and Cxcl10) at 0.5% (v/v) DMSO and there was only 1 probe set (with no annotation), which was down-regulated ($p < 0.05$ and less than 1/2) at 0.1% (v/v) DMSO, and 4 probe sets (Car3, D4, zinc and double PHD fingers, family 3 [Dpf3], LOC681825, and another with no annotation) at 0.5% (v/v) DMSO. CXCL11 was up-regulated and IGFBP4 was down-regulated in common, but neither gene is reported to have any relation with DMSO.

To determine whether an a priori defined set of genes shows statistically significant, concordant differences between control and DMSO treatment, GSEA was carried out. In human hepatocytes, a large gap was observed between 0.75% (v/v) and 1% (v/v) DMSO. And only 1 gene set was shared in common. In contrast, there were 3 gene sets in common

between 0.1% (v/v) and 0.5% (v/v) DMSO, 4 gene sets in common between 0.1% (v/v) and 0.75% (v/v) DMSO, and 4 gene sets in common between 0.5% (v/v) and 0.75% (v/v) DMSO. In rat hepatocytes, large gaps were observed between 0.1% (v/v) and 0.5% (v/v) DMSO and between 0.1% (v/v) and 0.75% (v/v) DMSO. There was only 1 gene set in common in each gap. In contrast, 6 gene sets were in common from 0.5% (v/v) to 1% (v/v) DMSO (Table 1).

With regard to Phase I, II, III drug-metabolizing enzymes, DMSO concentration-dependent probe sets were clustered with the smoothing spline clustering method, and 161 genes in human cryopreserved hepatocytes and 57 genes in rat primary cultured hepatocytes were altered in expression level, respectively (Table 2). However, the magnitude of alteration for expression of most drug-metabolizing enzymes was within 1 standard deviation in both cells.

In human hepatocytes, there were 66 enzymes, which were up-regulated, and 95 enzymes, which were down-regulated. Of 66 enzymes, 10 enzymes including CYP3A4 and CYP3A5, were strongly up-regulated enzymes and 56 enzymes including CYP1B1, CYP2A6 and CYP2E1 were moderately up-regulated. Of 95 enzymes, 30 enzymes including UGT1A6 were strongly down-regulated enzymes and 65 enzymes were moderately down-regulated. Nishimura et al. earlier investigated the gene expression of CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2E1, CYP3A4, CYP3A5, CYP3A7, UGT1A6, UGT1A9 and ABCB1 after 24 hours of exposure to 0.1% (v/v), 0.5% (v/v), and 2.5% (v/v) DMSO in primary culture of human hepatocytes.[6] Our results were in excellent agreement with their findings. Wilkening and Bader also reported the response after 24 hours exposure to 0%-1% (v/v) DMSO in primary human hepatocytes.[7] They found CYP3A4 but not CYP3A7 to be induced by DMSO and this also corresponds with our data. Furthermore, Choi et al. reexamined induction of CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D8, CYP3A4, CYP3A5, UGT1A1, UGT1A4, UGT1A6, UGT1A9, and UGT2B7 after exposure to 1% (v/v) DMSO over 20 days in Huh7 cells, which was established from a 57-year-old male with a well-differentiated hepatocellular carcinoma in 1952.[8] In their report, all of the examined enzymes, expect for CYP1A1, showed significant increase in their expression by DMSO treatment. Since their experimental condition was very different from our own, we are not able to make an easy comparison, but CYP2B6, CYP2C8, CYP3A4, and CYP3A5 were induced by DMSO in both data sets. On the other hand, there is a discrepancy between the two concerning CYP2C9 and UGT1A6. In rat hepatocytes, there were 25 enzymes which were up-regulated and 32 enzymes which were down-regulated. Some enzymes including betaine-homocysteine methyltransferase 2 (Bhmt2), protein-L-isoaspartate (D-aspartate) *O*-methyltransferase domain containing 2 (Pcmtd2), aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase; Akr1d1), and methyltransferase 10 domain containing (Mett10D) were up-regulated or down-regulated in both human and rat hepatocytes. The magnitude of change of these transcripts in rat hepatocytes was often larger than that in human hepatocytes.

As for housekeeping genes, Nishimura et al. examined the effects of DMSO on the expression of Actb, Gapdh, Gusb, Pgk1, Ppia, and Tfrc mRNA in cultures of C2C12 myotubes.[5] They reported that Actb, Pgk1 and Tfrc were significantly ($p < 0.05$ and less than 1/2) decreased at 2.5% (v/v) DMSO after 24 hours. At 2% (v/v) DMSO, we found the above 6 genes to show similar expression change as their results.

To confirm the gene expression data using microarray, we conducted quantitative RT-PCR for some rat genes (Ccl20, Sult1a1, Cyp1a1, Car2, and Cyp2c12). Ccl20, which was up-regulated ($p < 0.05$ and 2.13-fold compared with control) in microarray analysis at a concentration of 0.5% (v/v) DMSO, showed up-regulation (dCt = −0.67) in expression in using quantitative RT-PCR. It was also confirmed that Sult1a1 and Cyp1a1 were up-regulated in quantitative RT-PCR analysis (dCt = −0,09 and dCt = −0.26, respectively) as well as in microarray analysis (1.59-fold and 1.84-fold) at 2% (v/v) DMSO. On the other hand, Car2 and Cyp2c12 showed down-regulation in both quantitative RT-PCR analysis and microarray analysis (eTable). The Quantitative RT-PCR data were well coincident with the microarray data.

As regards cytotoxicity, no toxic effects were observed up to the concentration of 2% (v/v) in either of the cells with either of the tests employed.

In conclusion, we showed that there are only very few probe sets altered in expression at doses up to 0.75% (v/v) DMSO in both human and rat hepatocytes. In particular, there are remarkably few probe sets altered in expression up to 0.5% (v/v) DMSO and the magnitude of alteration for expression of most drug-metabolizing enzymes was within 1 standard

deviation in both cells. Alteration of housekeeping genes was also very small. Furthermore, DMSO did not show any toxic effects up to the concentration of 2% (v/v) and there are few differences in effects of DMSO between human and rat hepatocytes with regard to change of transcripts. These results in human cryopreserved hepatocytes and rat primary cultured hepatocytes suggested that a DMSO concentration up to 0.5% (v/v) can be tolerated, although care must be taken in interpretation at higher concentrations.

Funding

This study was supported in part by a grant from the Ministry of Health, Labor and Welfare (H19-Toxico-001).

References

1. Urushidani T. Prediction of hepatotoxicity based on the toxicogenomics database. In: Sahu SC (ed.) *Hepatotoxicity from Genomics to In Vitro and In Vivo Models*. Hoboken: Wiley & Sons, 2007; 507–529.
2. Adler S, Paparella M, Pellizzer C, Hartung T, and Bremer S. The detection of differentiation-inducing chemicals by using green fluorescent protein expression in genetically engineered teratocarcinoma cells. Alternatives to Laboratory Amimals. Chicago: ATLA, 2005; 33: 1–13.
3. Klinken SP, Holmes KL, Morse HC 3rd, and Thorgeirsson SS. Transcriptional and post-transcriptional regulation of c-myc, c-myb, and p53 during proliferation and differentiation of murine erythroleukemia cells treated with DFMO and DMSO. *Exp Cell Res* 1988; 178: 185–198.
4. Jiang G, Bi K, Tang T, Wang J, Zhang Y, Zhang W, et al. Down-regulation of TRRAP-dependent hTERT and TRRAP-independent CAD activation by Myc/Max contributes to the differentiation of HL60 cells after exposure to DMSO. *Int Immunopharmacol* 2006; 6: 1204–1213.
5. Nishimura M, Nikawa T, Kawano Y, Nakayama M, and Ikeda M. Effects of dimethyl sulfoxide and dexamethasone on mRNA expression of housekeeping genes in cultures of C2C12 myotubes. *Biochem Biophys Res Commun* 2008; 367: 603–608.
6. Nishimura M, Ueda N, and Naito S. Effects of dimethyl sulfoxide on the gene induction of cytochrome P450 isoforms, UGT-dependent glucuronosyl transferase isoforms, and ABCB1 in primary culture of human hepatocytes. *Biol Pharm Bull* 2003; 26: 1052–1056.
7. Wilkening S and Bader A. Differential regulation of CYP3A4 and CYP3A7 by dimethylsulfoxide in primary human hepatocytes. *Basic Clin Pharmacol Toxicol* 2004; 95: 92–93.
8. Choi S, Sainz B Jr, Corcoran P, Uprichard S, and Jeong H. Characterization of increased drug metabolism activity in dimethyl sulfoxide (DMSO)-treated Huh7 hepatoma cells. *Xenobiotica* 2009; 39: 205–217.
9. Uehara T, Kiyosawa N, Hirode M, Omura K, Shimizu T, Ono A, et al. Gene expression profiling of methapyrilene-induced hepatotoxicity in rat. *J Toxicol Sci* 2008; 33: 37–50.
10. Uehara T, Kiyosawa N, Shimizu T, Omura K, Hirode M, Imazawa T, et al. Species differences in coumarin-induced hepatotoxicity as an example of how toxicogenomics help assessing risks for human. *Human Exp Toxicol* 2008; 27: 23–35.
11. Ma P, Castillo-Davis CI, Zhong W, and Liu JS. A data-driven clustering method for time course gene expression data. *Nucleic Acids Res* 2006; 34: 1261–1269.
12. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expressionprofiles. *Proc Natl Acad Sci USA* 2005; 102(43): 15545–15550.
13. Kanehisa M, Goto S, Kawashima S, Okuno Y, and Hattori M. The KEGG resource for deciphering the genome. *Nucleic Acids Res* 2004; 32: D277–D280.

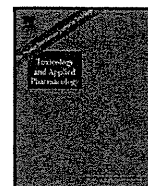
eTable 1. Validation of microarray data using TaqMan® real-time PCR assays

Toxicology and Applied Pharmacology 255 (2011) 297–306

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap



Prediction model of potential hepatocarcinogenicity of rat hepatocarcinogens using a large-scale toxicogenomics database

Takeki Uehara [a,b,*], Yohsuke Minowa [b], Yuji Morikawa [b], Chiaki Kondo [a], Toshiyuki Maruyama [a], Ikuo Kato [a], Noriyuki Nakatsu [b], Yoshinobu Igarashi [b], Atsushi Ono [b], Hitomi Hayashi [c,d], Kunitoshi Mitsumori [c], Hiroshi Yamada [b], Yasuo Ohno [e], Tetsuro Urushidani [b,f]

[a] Drug Developmental Research Laboratories, Shionogi & Co., Ltd., 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan
[b] Toxicogenomics Informatics Project, National Institute of Biomedical Innovation, 7-6-8 Asagi, Ibaraki, Osaka 567-0085, Japan
[c] Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Harumi-cho, Fuchu, Tokyo 183-8509, Japan
[d] Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu, 501-1193 Gifu, Japan
[e] National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[f] Department of Pathophysiology, Faculty of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts, Kodo, Kyotanabe, Kyoto 610-0395, Japan

ARTICLE INFO

Article history:
Received 7 April 2011
Revised 5 July 2011
Accepted 6 July 2011
Available online 19 July 2011

Keywords:
Toxicogenomics
Microarray
Hepatocarcinogenicity
TG-GATEs
Non-genotoxic hepatocarcinogens

ABSTRACT

The present study was performed to develop a robust gene-based prediction model for early assessment of potential hepatocarcinogenicity of chemicals in rats by using our toxicogenomics database, TG-GATEs (Genomics-Assisted Toxicity Evaluation System developed by the Toxicogenomics Project in Japan). The positive training set consisted of high- or middle-dose groups that received 6 different non-genotoxic hepatocarcinogens during a 28-day period. The negative training set consisted of high- or middle-dose groups of 54 non-carcinogens. Support vector machine combined with wrapper-type gene selection algorithms was used for modeling. Consequently, our best classifier yielded prediction accuracies for hepatocarcinogenicity of 99% sensitivity and 97% specificity in the training data set, and false positive prediction was almost completely eliminated. Pathway analysis of feature genes revealed that the mitogen-activated protein kinase p38- and phosphatidylinositol-3-kinase-centered interactome and the v-myc myelocytomatosis viral oncogene homolog-centered interactome were the 2 most significant networks. The usefulness and robustness of our predictor were further confirmed in an independent validation data set obtained from the public database. Interestingly, similar positive predictions were obtained in several genotoxic hepatocarcinogens as well as non-genotoxic hepatocarcinogens. These results indicate that the expression profiles of our newly selected candidate biomarker genes might be common characteristics in the early stage of carcinogenesis for both genotoxic and non-genotoxic carcinogens in the rat liver. Our toxicogenomic model might be useful for the prospective screening of hepatocarcinogenicity of compounds and prioritization of compounds for carcinogenicity testing.

© 2011 Elsevier Inc. All rights reserved.

Introduction

Carcinogenicity is one of the most serious side effects associated with new drug development. Thus, it is especially important for pharmaceutical companies to know as much as possible about the eventual carcinogenic properties of new drugs, even in the early stages of drug development. However, the current "gold standard" for carcinogenicity testing is a bioassay in which mice and rats are treated with a target compound for their entire 2-year lifespan. This carcinogenicity testing cannot be performed in the early stage of drug development because it is time-consuming and expensive and it requires the use of many animals and large amounts of chemicals. Additionally, while this assay provides evidence of carcinogenicity of the target chemicals in rodents, it provides only limited mechanistic information about carcinogenesis. Thus, the current strategy of a 2-year bioassay to evaluate in vivo carcinogenicity is not satisfactory. It should be replaced with better test systems that are cheaper and faster, use fewer animals, and provide the appropriate sensitivity and specificity desired for a screen of carcinogenic potential.

Toxicogenomics has been expected as a powerful approach for elucidating mechanisms underlying toxicological endpoints and a useful strategy for the early detection of potential chemical toxicity (Battershill, 2005; Heinloth et al., 2004; Irwin et al., 2004; Kiyosawa et al., 2009; Searfoss et al., 2005). Important scientific breakthroughs have been achieved by applying toxicogenomics to the early detection of chemical carcinogenicity in vivo. Studies have focused on hepatocarcinogenicity because the liver is the most common target organ for carcinogenesis. Kramer et al. (2004) studied microarray-derived comprehensive gene

* Corresponding author at: Developmental Research Laboratories, Shionogi & Co., Ltd., 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan. Fax: +81 6 6332 6385.
E-mail address: takeki.uehara@shionogi.co.jp (T. Uehara).

0041-008X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2011.07.001

expression data from the livers of rats treated with 10 non-genotoxic hepatocarcinogens and determined that the expression level of NAD(P)H P450 reductase was positively correlated with hepatocarcinogenicity and the expression level of transforming growth factor-β clone 22 was negatively correlated with hepatocarcinogenicity. Nie et al. (2006) demonstrated that 6 biomarker genes predict the carcinogenic potential of non-genotoxic hepatocarcinogens with a prediction accuracy of 88.5% by using expression data from the livers of rats treated with a single dose of 24 non-genotoxic chemicals and 28 non-hepatocarcinogens. Nakayama et al. (2006) successfully separated several isomers with or without hepatocarcinogenicity based on the expression profiles of selected genes, and they identified characteristic gene expression changes in hepatocarcinogenic isomers after up to 28 days of repeated dosing. Ellinger-Ziegelbauer et al. (2008) constructed support vector machine (SVM) prediction models by using gene expression data from rats treated for up to 14 days with 13 different chemicals for the training set and rats treated with 16 independent chemicals for the validation set; the resulting prediction models differentiated between genotoxic hepatocarcinogens, non-genotoxic hepatocarcinogens and non-hepatocarcinogens with up to 88% classification accuracy. Fielden et al. (2007) analyzed hepatic gene expression in rats treated with 25 non-genotoxic hepatocarcinogens and 75 non-hepatocarcinogens for 1 to 7 days. They constructed a SVM model consisting of 37 probes that yielded prediction accuracies for carcinogenicity with 86% specificity and 81% sensitivity. These researchers successfully constructed gene-based prediction models from data obtained from up to 1 month of repeated dosing. In contrast, Auerbach et al. (2010) recently demonstrated further evidence that the dosing period is an important factor in the construction of highly accurate prediction models based on toxicogenomics-derived gene expression profiles, especially in the case of weakly carcinogenic compounds. They concluded that a 90-day exposure period is needed to detect gene expression changes specifically related to carcinogenic compounds.

In Japan, the Toxicogenomics Project (TGP) has established a large-scale toxicogenomics database known as TG-GATEs (Genomics-Assisted Toxicity Evaluation System developed by the Toxicogenomics Project in Japan) (Uehara et al., 2010; Urushidani, 2010). In this project, rats were exposed to 3 different doses of 150 compounds for a period ranging from 1 to 28 days; the gene expression in the livers and kidneys of these animals at 8 different time points was comprehensively analyzed using microarrays. We have used this database to identify several different types of biomarker genes and construct prediction models for hepatotoxicity and nephrotoxicity (Gao et al., 2010; Hirode et al., 2008; Kondo et al., 2009; Uehara et al., 2010). Regarding hepatocarcinogenicity, we previously tried to build a gene-based predictor of non-genotoxic hepatocarcinogenicity in rats (Uehara et al., 2008). Consequently, we have successfully built a model, consisting of 112 probes, for the early detection of hepatocarcinogenesis based on gene expression changes that are commonly induced by compounds with hepatocarcinogenicity in rats. However, since this model was trained to achieve early and sensitive detection of potential carcinogenicity after a single exposure to a compound, false positive predictions occurred in some non-carcinogenic hepatotoxins. Moreover, a limited number of compounds were used for training of the model in the study since our database was under construction. In an effort to make high-quality predictive models based on gene expression data, a fairly extensive data set of several compounds with multiple time points and multiple dose levels is required. Now, our large-scale toxicogenomics database has been completed, and microarray data for all 150 compounds are available. In this research, we hypothesized that our large-scale toxicogenomics database might lead to the construction of a more robust and accurate prediction model of hepatocarcinogenicity in rats. By taking into account the findings of the latest work by Auerbach et al. (2010), we have trained a classifier by using data from our longest dosing period (28 days) to decrease the percentage of false-positive predictions. Consequently, our new SVM-based classifier yielded prediction accuracies for hepatocarcinogenicity with 99% sensitivity and 97% specificity in a training data set, and false-positive predictions were almost completely eliminated. The usefulness and robustness of our predictor were further confirmed in an independent validation data set obtained from a public database. Interestingly, similar positive predictions were obtained for several genotoxic hepatocarcinogens as well as non-genotoxic hepatocarcinogens. In the present report, we provide reliable candidate gene biomarkers in the early stages of the hepatocarcinogenesis that are predictive for both genotoxic and non-genotoxic hepatocarcinogens. Our present toxicogenomic model might be useful to reduce the dependence on 2-year rodent bioassays by instead using a short-term repeated dosing study.

Materials and methods

Animals 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 6-week-old animals were assigned to dosage groups (5 rats per group) by 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 °C–25 °C with a relative humidity of 40%–70%. Each animal was allowed free access to water and a pellet diet (CRF-1, sterilized by radiation, Oriental Yeast Co., Ltd., Tokyo, Japan).

The compounds used in this study are summarized in Table 1 (for detailed experimental conditions, see Supplemental Table 1). A total of 150 compounds were used for training and testing of models. The training data set consisted of 6 positive compounds (necrogenic hepatocarcinogens with no evidence of genotoxicity, namely non-genotoxic hepatocarcinogens) and 54 negative compounds (non-hepatocarcinogens), and the test data set consisted of remaining 90 compounds (for more detailed information, see Supplemental Table 2). According to the standard protocol in our project (Uehara et al., 2010), 5 rats per group were treated with these compounds at 3 different dose levels (low: L, middle: M, and high: H). The maximum tolerated dose of each compound, which was estimated from a preliminary 7-day repeated dosing study, was chosen as the highest dose level. For single-dose studies, rats were euthanized at 3, 6, 9 and 24 h after dosing. For repeated dose studies, the animals were treated daily for 3, 7, 14 and 28 days and euthanized 24 h after the last dosing (4, 8, 15 and 29D). The animals were euthanized by exsanguination from the abdominal aorta under ether anesthesia, and liver samples were collected from the left lateral lobe of the liver immediately after the animals were euthanized. The experimental protocols were carefully reviewed and approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences.

RNA extraction and microarray analysis. An aliquot of the sample (about 30 mg) for microarray analysis was obtained from the left lateral lobe of the liver in each animal immediately after the animals were euthanized. The sample was kept in RNAlater® (Ambion, Austin, TX, USA) overnight at 4 °C and then frozen at −80 °C until use. Liver samples were homogenized with buffer RLT supplied in the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and total RNA was isolated according to the manufacturer's instructions.

Microarray analysis was conducted on 3 of 5 samples for each group by using Affymetrix Rat Genome 230 2.0 arrays (Affymetrix, Santa Clara, CA, USA). The procedure was basically conducted according to the manufacturer's instructions as previously reported (Uehara et al., 2010). The digital image files were preprocessed by Affymetrix Microarray Analysis Suite version 5.0 (MAS5.0). The expression signal values were scaled by the median of each chip sample. The normalized data sets were then converted into the log-ratio of base 2 to the means of the

Table 1
Compounds and carcinogenicity definitions used in this study.

Compound class	Compound name
Training set (positive)	
Non-genotoxic hepatocarcinogen (hepatotoxic oxidative stressor)	Carbon tetrachloride (CCL4), ethionine (ET), thioacetamide (TAA), methapyrilene (MP), coumarin (CMA), monocrotaline (MCT)
Training set (negative)	
Non-hepatocarcinogen	Acetaminophen (APAP), naphthyl isothiocyanate (ANIT), allyl alcohol (AA), theophylline (TEO), trimethadione (TMD), naproxen (NPX), methotrexate (MTX), aspirin (ASA), labetalol (LBT), ketoconazole (KC), tetracycline (TC), metformin (MFM), methyldopa (MDP), vitamin A (VA), chlorpropamide (CPP), nicotinic acid (NIC), famotidine (FAM), ranitidine (RAN), diltiazem (DIL), captopril (CAP), enalapril (ENA), mexiletine (MEX), meloxicam (MLX), lornoxicam (LNX), cyclosporine A (CSA), isoniazid (INAH), phenylbutazone (PhB), nitrofurantoin (NFT), propylthiouracil (PTU), amiodarone (AM), cimetidine (CIM), flutamide (FT), methimazole (MYZ), iproniazid (IPA), chloramphenicol (CMP), furosemide (FUR), chlorpheniramine (CHL), caffeine (CAF), sulpiride (SLP), simvastatin (SST), chlormadinone (CLM), carboplatin (CBP), bucetin (BCT), perhexiline (PH), pemoline (PML), ibuprofen (IBU), erythromycin ethylsuccinate (EME), nifedipine (NIF), sulindac (SUL), disopyramide (DIS), disulfiram (DSF), tolbutamide (TLB), acarbose (ACA), ajmaline (AJM)
Test set	
Genotoxic hepatocarcinogen	Lomustine (LS), acetamidofluorene (AAF), nitrosodiethylamine (DEN)
Non-genotoxic hepatocarcinogen (enzyme inducer)	Phenobarbital (PB), carbamazepine (CBZ), phenytoin (PHE), rifampicin (RIF), hexachlorobenzene (HCB), sulfasalazine (SS)
Non-genotoxic hepatocarcinogen (peroxisome proliferator)	Clofibrate (CFB), WY-14643 (WY), gemfibrozil (GFZ), fenofibrate (FFB)
Non-genotoxic hepatocarcinogen (hormonal modulator)	Ethinylestradiol (EE)
Non-hepatocarcinogen/unknown (non-genotoxicant)	Ethionamide (ETH), indomethacin (IM), bromobenzene (BBZ), ethambutol (EBU), colchicine (COL), clomipramine (CPM), puromycin aminonucleoside (PAN), methyltestosterone (MTS), valproic acid (VPA), chlorpromazine (CPZ), diclofenac (DFNa), benzbromarone (BBr), allopurinol (APL), fluphenazine (FP), thioridazine (TRZ), adapin (ADP), glibenclamide (GBC), chlormezanone (CMN), moxisylyte (MXS), imipramine (IMI), amitriptyline (AMT), hydroxyzine (HYZ), quinidine (QND), mefenamic acid (MEF), tiopronin (TIO), acetazolamide (ACZ), promethazine (PMZ), dantrolene (DTL), triazolam (TZM), terbinafine (TBF), danazol (DNZ), bendazac (BDZ), benziodarone (BZD), bromoethanamine (BEA), nimesulide (NIM), phenylanthranilic acid (NPAA), cephalothin (CLT), ticlopidine (TCP), gentamicin (GMC), vancomycin (VMC), omeprazole (OPZ), diazepam (DZP), haloperidol (HPL), griseofulvin (GF), tamoxifen (TMX), tannic acid (TAN), triamterene (TRI), ethanol (ETN), ciprofloxacin (CPX), tacrine (TAC), nitrofurazone (NFZ), papaverine (PAP), penicillamine (PEN), azathioprine (AZP), doxorubicin (DOX), cyclophosphamide (CPA), etoposide (ETP), cisplatin (CSP), phenacetin (PCT)
Unknown	K01, K02, K03, K04, K05, K06, K07, K08, K09, K10, K11, K12, K13, K14, K15, K16, K17

K01 to K17 were compounds synthesized in member companies.

corresponding control groups. Raw microarray data (CEL files) are available in Open TG-GATEs (http://toxico.nibio.go.jp/).

Gene selection and supervised classification. Among a total of 150 compounds in our database, we have selected 6 compounds consisting of carbon tetrachloride, ethionine, thioacetamide, methapyrilene, coumarin and monocrotaline, as positive training compounds for modeling, which are non-genotoxic hepatocarcinogens with hepatocellular necrosis/degeneration in histopathology following multiple dosing for up to 28 days in our experimental condition. Individual histopathological data of all compounds are available (http://toxico.nibio.go.jp/datalist.html). High-dose 29D groups treated with these compounds were used for the positive training data set, with the exception for monocrotaline; the middle-dose group of monocrotaline at 29D was included in the positive training set because all animals in the high-dose group were dead at 29D in the current experimental condition. High- or middle-dose groups at all time points (3 to 24H for single-dose studies, 4 to 29D for repeated-dose studies) of randomly selected 54 non-hepatocarcinogens were selected as the negative training data set. To exclude genes being transiently regulated by the treatment of non-carcinogenic compounds, data obtained from all time points were used as the negative training set. The remaining compounds were used as the independent test set as follows: (i) genotoxic hepatocarcinogens; (ii) non-genotoxic hepatocarcinogens whose carcinogenic mechanisms are thought to be related to hepatic enzyme induction, peroxisomal proliferation and hormonal modulation; and (iii) non-hepatocarcinogens (for more detailed information, see Supplemental Table 2).

SVM combined with wrapper-type gene selection algorithms was used to build a prediction model, as previously reported (Kondo et al., 2009). First, the probes that were judged as absent in all samples of the training data set using MAS5.0 P/A-call were excluded. Next, the following 3 statistical parameters were calculated for each probe: (i) fold change of gene expression between positive and negative training data sets; (ii) Mann–Whitney U value; (iii) confident margin of SVM classifier (normal margin corrected by classification accuracy). The probes were filtered by following criteria: (i) fold change >2 or <0.5; (ii) p-value <0.01; and (iii) confident margin >0.05. Then, the combined gene ranking based on the 3 parameters was calculated by using a layer ranking algorithm (Chen et al., 2007). To estimate the performance of our classifier, 5-fold cross-validation was executed as described in our previous report (Kondo et al., 2009). Finally, 9 of the 82 top-ranked probes (7 genes) were selected to maximize the classification accuracy and the area under the curve (AUC) of the receiver operating characteristic curve (ROC).

Ingenuity pathways analysis. The 82 top-ranked probes were analyzed by using Ingenuity Pathways Analysis software (v. 7.1; Ingenuity Systems, Redwood City, CA) to determine the biological networks that were enriched in selected feature genes.

Independent validation of our classifier by using an external data set from NEDO. An external microarray data set from the NEDO project, another toxicogenomics consortium in Japan, was used for independent validation of our classifier (Matsumoto et al., 2009). In this project, the NEDO-ToxArray III consists of 6709 genes, and hepatic gene expression data was comprehensively obtained from F344 rats treated with 88 compounds for up to 28 days. All of the microarray data are available in the public microarray database of Gene Expression Omnibus (GEO). SVM modeling and principal component analysis (PCA) were performed by using the expression data at 3 different time points (4, 15 and 29D).

Predictions using published biomarker genes. For comparison of prediction accuracy with previously published models, we built

SVM models with our training data set by using published gene lists and then compared the prediction performance of all models (Auerbach et al., 2010; Ellinger-Ziegelbauer et al., 2008; Fielden et al., 2007; Nakayama et al., 2006; Uehara et al., 2008). To estimate the performance of each model, 5-fold cross-validation was executed using the training data set.

Results

Gene selection and supervised classification. We trained a binary classifier by using an SVM algorithm combined with wrapper-type gene selection to construct a statistically reliable model without over-fitting to the profiles of training samples according to our previous report with minor modifications (Kondo et al., 2009). By applying statistical analysis for feature selection, 82 probes passed current statistical criteria, and these top-ranked probes are summarized in Supplemental Table 3. 1 to 82 of the top-ranked probes were used to construct the classifiers with further feature selection. A ROC curve and its AUC are plotted in Fig. 1. Although there were no big differences in the prediction accuracy of each classifier, a classifier consisting of 9 probes (7 genes; Table 2) achieved the best classification accuracy under the 5-fold cross-validation. The sensitivity and specificity of the prediction of the optimized classifier was 99% and 97%, respectively.

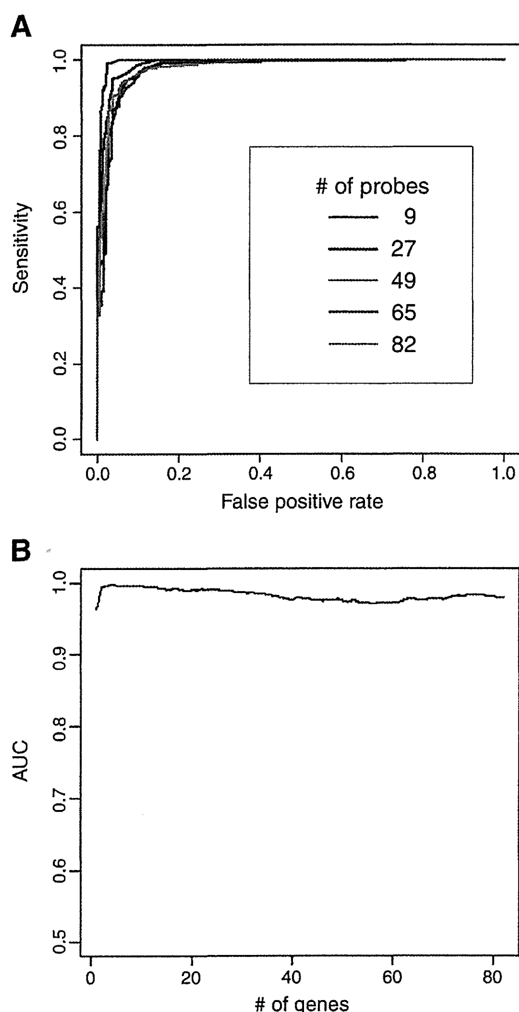


Fig. 1. Receiver operating characteristic analysis of prediction models. ROC curve (A) and area under the ROC curve (B) of prediction models are plotted. A model consisting of 9 probes was selected as the best model in our training data set.

Table 2
The 9 top-ranked probes in the optimized model.

Affymetrix probe ID	Gene title	Gene abbreviation	GO biological process (selected)
1370583_s_at	ATP-binding cassette, sub-family B (MDR/TAP), member 1A/1B	*Abcb1a/1b*	Nucleotide binding/transporter activity/protein binding/ATP binding/ATPase activity/drug transporter activity/hydrolase activity/nucleoside-triphosphatase activity
1395737_at /1374198_at	Cd276 molecule	*Cd276*	Receptor binding/protein binding
1367787_at	Islet cell autoantigen 1	*Ica1*	Protein binding/protein domain specific binding
1379419_at	Transmembrane protein 184C	*Tmem184c*	Unknown
1379262_at	Acyl-CoA thioesterase 9	*Acot9*	Unknown
1383401_at	Testis derived transcript	*Tes*	Zinc ion binding/metal ion binding
1375719_s_at/1373102_at	Cadherin 13	*Cdh13*	Calcium ion binding/protein binding/low-density lipoprotein binding/protein homodimerization activity/cadherin binding/adiponectin binding

Gene expression profiles of selected feature genes. Fig. 2 shows a heat map of the selected 9 probes and all 82 top-ranked probes. Among the 82 probes, 44 probes were upregulated, and 38 probes were downregulated in positive training compounds. All 9 probes involved in the optimized model were upregulated in positive training compounds. The extent of upregulation of genes in positive compounds was clearly higher than that in negative compounds and changed in a time- and dose-dependent manner (Fig. 3).

Ingenuity pathways analysis. To further characterize the biological significance of alterations in gene expression, functional pathway analysis was performed by using the 82 selected probes. The mitogen-activated protein kinase p38 (*p38 Mapk*)- and phosphatidylinositol-3-kinase (*PI3k*)-centered interactome (Fig. 4A) and the v-myc myelocytomatosis viral oncogene homolog (*Myc*)-centered interactome (Fig. 4B) were the 2 most significant networks. Among 9 probes (7 genes) of the best classifier, the following 5 genes were involved in these networks: ATP-binding cassette, sub-family B (MDR/TAP), member 1A/B (*Abcb1a/b*), Cd276 molecule (*Cd276*), islet cell autoantigen 1 (*Ica1*), testis-derived transcript (*Tes*), and cadherin 13 (*Cdh13*).

Prediction results of all compounds. The SVM classification scores of all 150 compounds are summarized in Supplemental Table 2. All 3 dose groups of 90 test compounds that had not been used as the training set and the remaining groups of the 60 training compounds were used as a test data set for external validation of the classifier. The classifier predicted the following samples as positive: thioacetamide (H: 8D and 15D; M: 29D), methapyrilene (H: 8D and 15D), carbon tetrachloride (M: 29D) and monocrotaline (H: 15D). As expected, positive predictions for several hepatocarcinogens were observed only after long-term repeated dosing. There were no positive predictions in the low-dose groups of these positive-training compounds. Among the

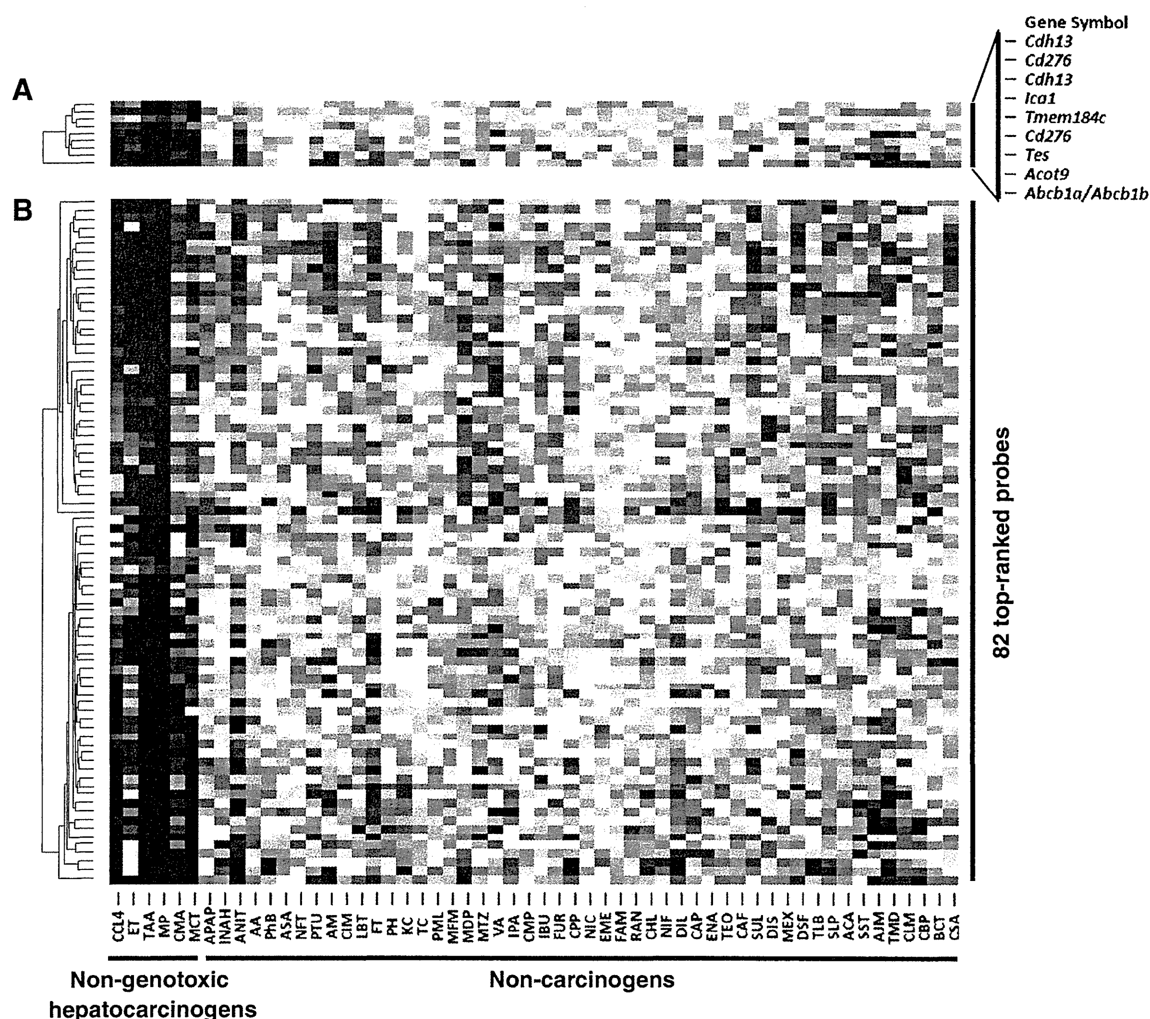


Fig. 2. Hierarchical clustering analysis of expression changes of selected feature genes. Gene expression changes of the 9 (A) and 82 top-ranked probes (B) were analyzed by hierarchical clustering. Clustering method: UPGMA (unweighted average); similarity measure: Euclidean distance; ordering function: average value. Heat map shows log-ratio of base 2 to the means of the corresponding control groups (red: 5-fold higher, blue: -5-fold lower expression of log 2 ratio). Symbols of selected genes are shown on the right side of the heat map.

genotoxins, lomustine (H: 29D), acetamidofluorene (L, M and H: 15 and 29D) and nitrosodiethylamine (M: 29D and H: 8 and 15D), for which the target of carcinogenicity is the liver, were correctly predicted as positive. Several genotoxic compounds that have potential to elicit cancer in other target organs, such as cyclophosphamide, etoposide, cisplatin and carboplatin, colchicine, phenacetin and doxorubicin, were predicted as negative at all time points of the 3 dose groups. Non-genotoxic hepatocarcinogens, including enzyme inducers (phenobarbital, carbamazepine, phenytoin, rifampicin, hexachlorobenzene and sulfasalazine), peroxisome proliferators (clofibrate, Wy-14643, gemfibrozil and fenofibrate) and hormonal modulators (ethinylestradiol), were classified as negative in all groups. The priority focus on this study was to build a prediction model with improved false-positive classification of non-hepatocarcinogens reflecting temporal gene expression changes at early time points after single or short-term repeated dosing. Among the 46 compounds in the negative/unknown test set, almost all compounds were classified as negative except for 2 compounds that were falsely classified as positive, ethionamide (H: 24H, 4D, and 15D; M: 24H) and etambutol (H: 8D).

Further independent validation of the optimized predictor. Since interlaboratory difference is an important issue in the field of toxicogenomics research (Fielden et al., 2008), a further independent validation was performed by using microarray data obtained by independent laboratories. In the present study, we used an external data set obtained in the NEDO project for further validation (Matsumoto et al., 2009). The NEDO study used custom microarrays consisting of 6709 probes, and gene expression data was comprehensively obtained from the livers of rats treated with several compounds for up to 28 days. Among their data set, we used following 14 compounds commonly involved in our data set: carbon tetrachloride, ethionine, thioacetamide, methapyrilene and nitrosodiethylamine (positive test set), and phenobarbital, phenytoin, hexachlorobenzene, clofibrate, ethinylestradiol, indomethacin, acetaminophen, aspirin and tannic acid (negative test set). For the purpose of a comprehensive comparison, we used 82 top-ranked probes for this analysis. Due to differences in the microarray platform, only 53 out of 82 probes were shared by both microarray platforms. By using the gene expression data for 53 probes, a SVM classifier was built without further feature selection using our training data set and then analyzed prediction accuracy of these test chemicals. As a result, all hepatocarcinogens included in the test data set were correctly predicted as positive regardless of using microarray data obtained by the different platform in independent laboratories. The overall sensitivity and specificity of the prediction by this classifier consisting of 53 probes was 100% and 89%, respectively (Supplemental Table 4).

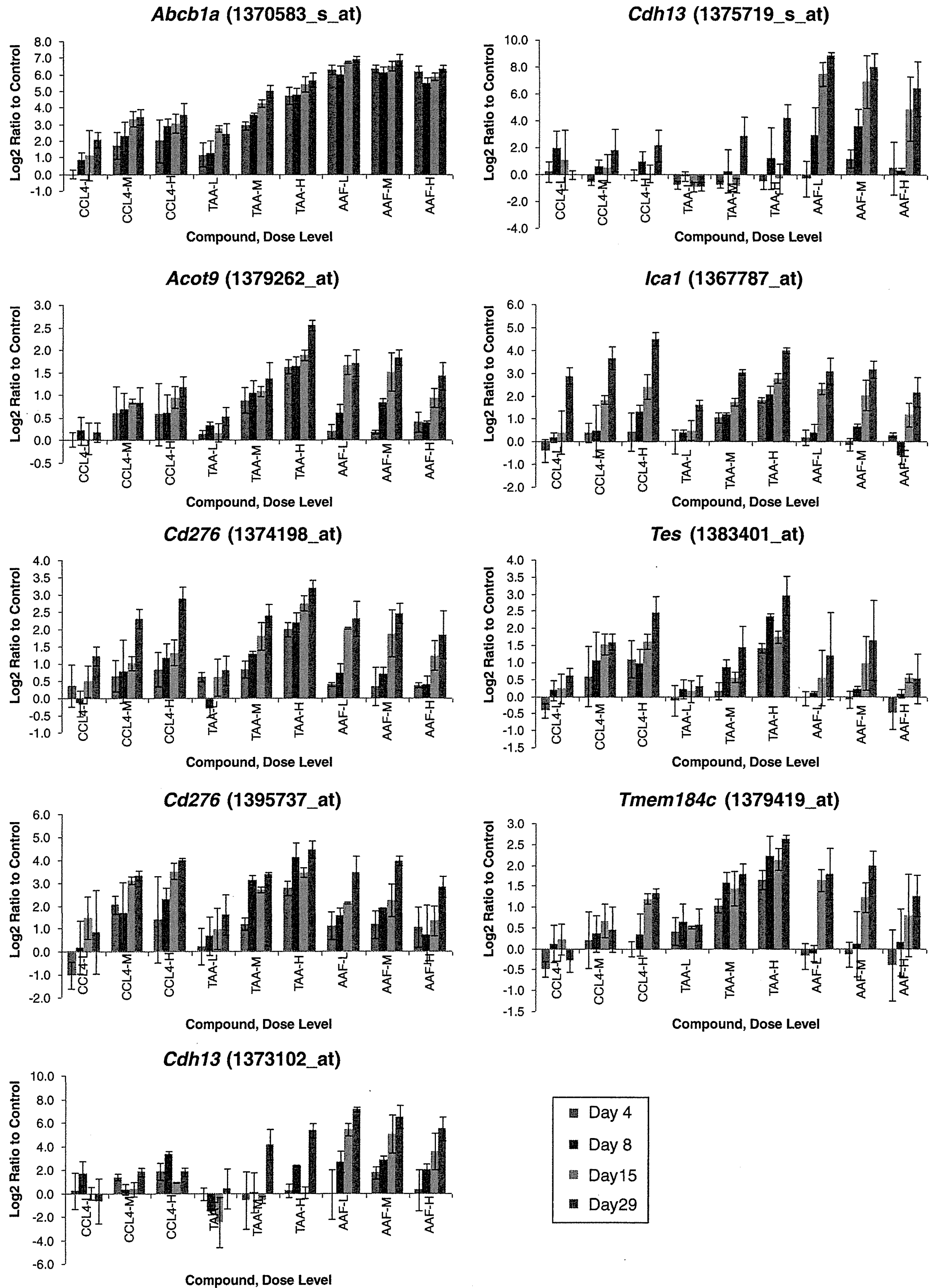


Fig. 3. Effect of dose and exposure duration on the expression of candidate biomarker genes for hepatocarcinogenesis. Log-ratio of base 2 to the means of the corresponding control groups with standard deviation are shown in 3 representative hepatocarcinogens, carbon tetrachloride, thioacetamide and acetamidofluorene.