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

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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.

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

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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.

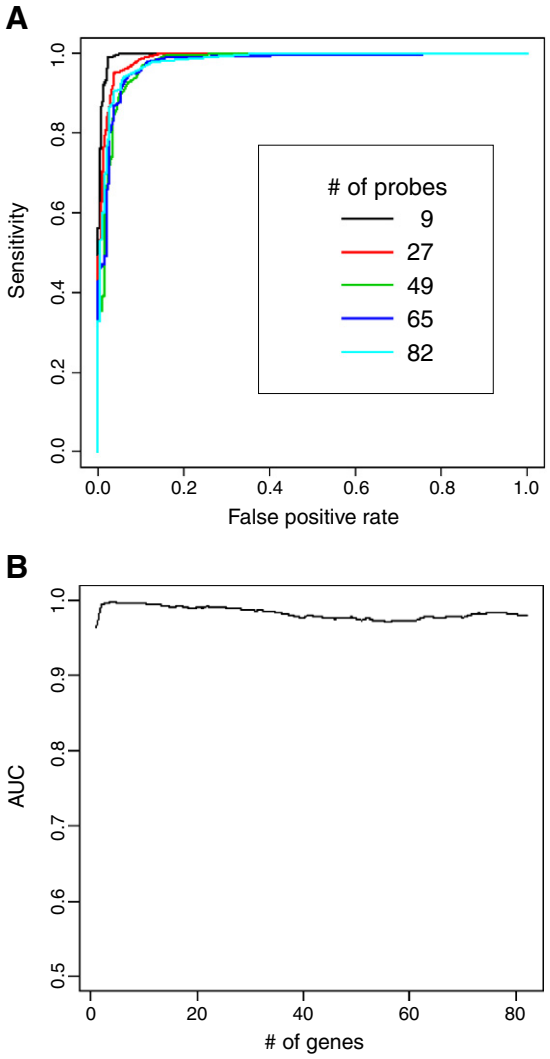

Fig. 1. Receiver operating characteristic analysis of prediction models. ROC curve (A) and area under the ROC curve (B) of prediction models are plotted. A model consisting of 9 probes was selected as the best model in our training data set.

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 (*Pl3k*)-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

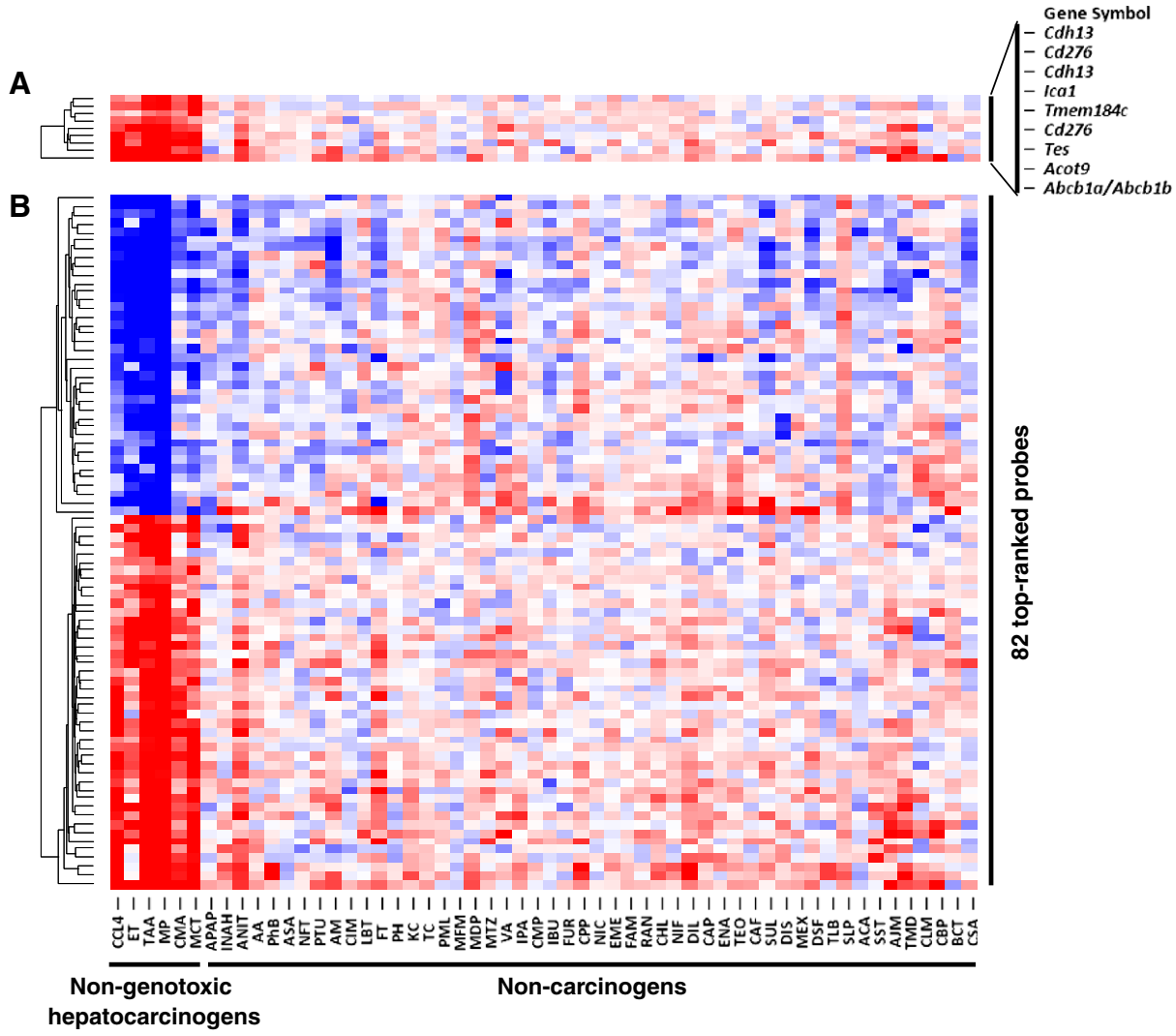

Fig. 2. Hierarchical clustering analysis of expression changes of selected feature genes. Gene expression changes of the 9 (A) and 82 top-ranked probes (B) were analyzed by hierarchical clustering. Clustering method: UPGMA (unweighted average); similarity measure: Euclidean distance; ordering function: 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).

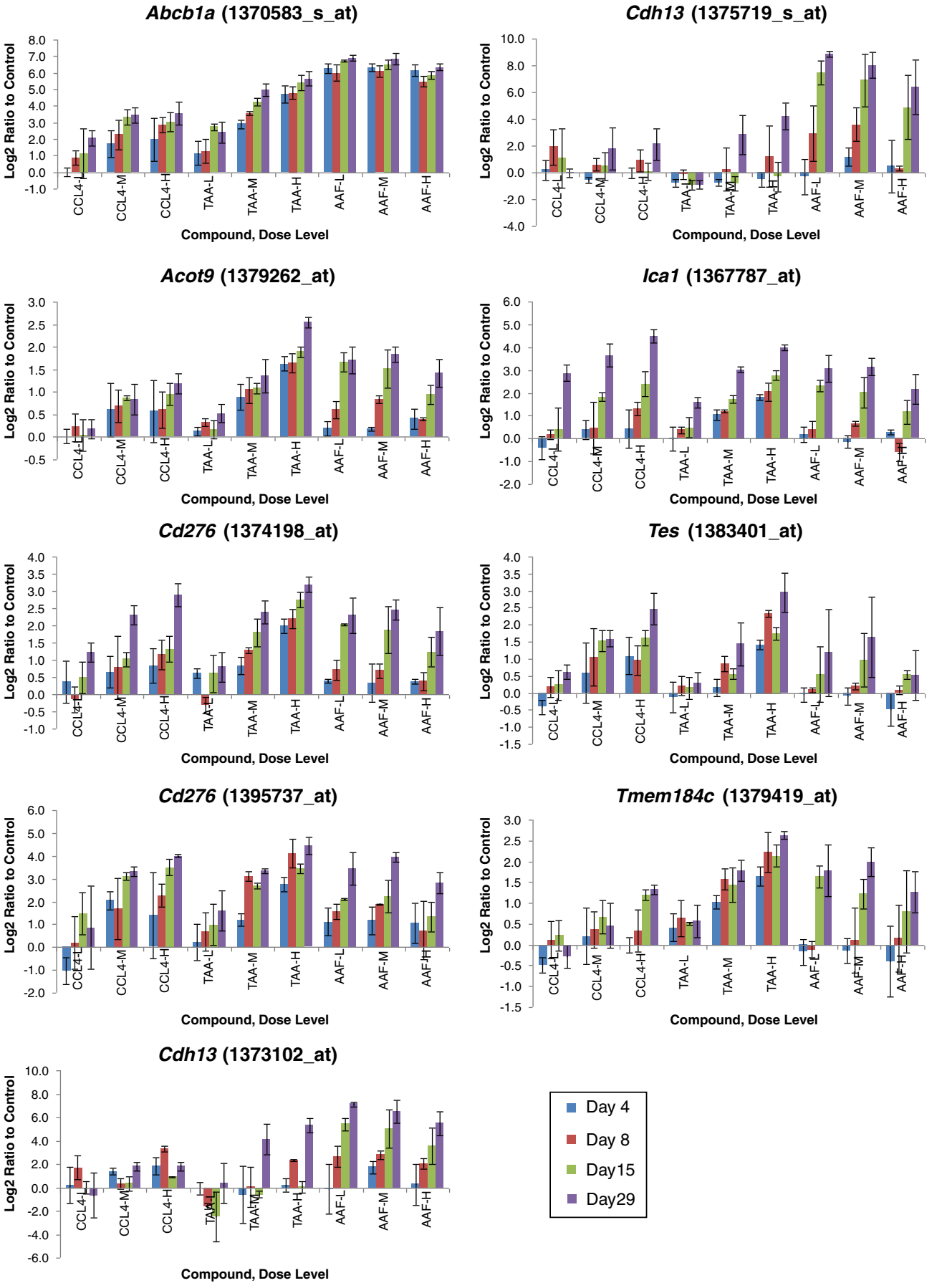

Fig. 3. Effect of dose and exposure duration on the expression of candidate biomarker genes for hepatocarcinogenesis. Log-ratio of base 2 to the means of the corresponding control groups with standard deviation are shown in 3 representative hepatocarcinogens, carbon tetrachloride, thioacetamide and acetamidofluorene.

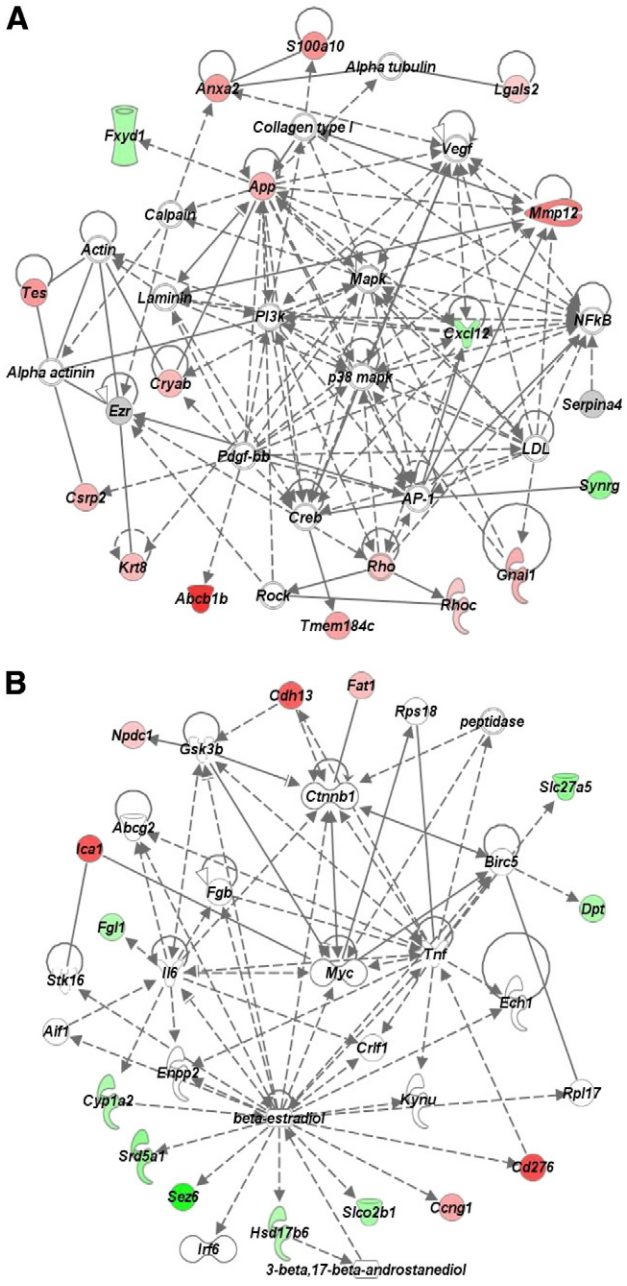

Fig. 4. Molecular networks representing selected feature genes. *p38 Mapk*- (A) and *Myc*-centered interactomes (B) were selected as significant network in the selected 82 genes. Red and green represent molecules upregulated or downregulated in positive compounds compared to negative compounds, respectively. Molecules incorporated into the network are shown in white. Ellipse, square, triangle, trapezoid, lozenge and circle represent transcription regulator, cytokine, kinase, transporter, enzyme and other molecules, respectively. Arrows connecting molecules indicate one molecule acts on another, and lines indicate one molecule binds to another. Dashed arrows or lines indicate indirect interactions of 2 molecules.

Furthermore, unsupervised analysis, PCA, was also performed based on the expression profiles of 53 probes in all of the NEDO data set. The scores of the first and second principal components (PC1 and PC2) at 4, 15 and 29D were separately plotted in Fig. 5. Several genotoxic hepatocarcinogens, such as nitrosodiethylamine, N-nitrosomorpholine, 2-nitropropane, furan and N-nitrosopiperidine, which show positive findings in in vitro genotoxicity assays and in vivo carcinogenicity assays in rats, were separately plotted from non-hepatocarcinogens toward the PC1 direction. In addition, non-genotoxic hepatocarcinogens, methapyrilene, thioacetamide and carbon tetrachloride, which are also included in our data set, showed clear separations in the PC1 direction. In almost all of these compounds, a distinct separation was observed in the data set after long-term repeated dosing.

Predictions using published biomarker genes. ROC curves of all models are plotted in Fig. 6. Although there was no large overlap of genes among the models, all models showed high prediction performance in our data set. Among all models, our new model achieved the best classification accuracy under the 5-fold cross-validation (Table 3).

Discussion

The goal of the present study was to develop a robust toxicogenomic model for the early assessment of potential hepatocarcinogenicity of chemicals based on gene expression changes stored in our toxicogenomics database, TG-GATEs. Carcinogenesis occurs via multiple mechanisms. Based on the properties of DNA damage, carcinogens are classified as genotoxins or non-genotoxins. Genotoxic carcinogens induce direct DNA damage, that is detectable by in vitro genotoxicity assays (e.g., Ames test and chromosomal aberration assays) and in vivo micronucleus test. Non-genotoxic carcinogens act through various modes of action that do not involve direct DNA damage (e.g., hepatocellular necrosis and regenerative proliferation, xenobiotic receptor agonists, peroxisome proliferation, or hormonal-mediated processes). Although it would be useful to detect the potential hepatocarcinogenicity of these different classes of non-genotoxic hepatocarcinogens by a single model, it is generally believed that a mechanism-based approach would be a promising strategy for robust toxicogenomic modeling since different classes of compounds generally show different gene expression profiles. In the present study, we have tried to establish reliable candidate gene biomarkers to assess the potential risks of hepatocarcinogenicity in the early stage of drug development with a particular focus on liver necrogenic compounds. Consequently, we have successfully identified robust biomarker genes specifically upregulated in necrogenic compounds with hepatocarcinogenicity. In contrast, no positive predictions were observed in the other class of non-genotoxic hepatocarcinogens involved in hepatic enzyme inducers, peroxisome proliferators and hormonal modulators. Therefore, these characteristics of prediction profile might help to elucidate the mechanisms of action involved in non-genotoxic hepatocarcinogenesis.

In contrast to our previous classifier (Uehara et al., 2008), our current model successfully achieved robust detection of non-genotoxic hepatocarcinogenicity of compounds by using hepatic gene expression data after 28 days of repeated dosing. There were some differences in the prediction properties of these 2 models. Namely, the current model enables us to detect robust gene expression changes possibly related to hepatocarcinogenic process following chronic doses in contrast to the previous model, which is useful to detect early temporal signals after a single dose as well as repeated doses; therefore, the combined use of both models for comprehensive judgment is thought to be the best strategy for sensitive and robust detection of hepatocarcinogenicity in short-term repeated dosing studies.

Interestingly, our current classifier as well as the previous classifier (data not shown) provided positive prediction results for not only non-genotoxic hepatocarcinogens but genotoxic hepatocarcinogens as well. This observation was further confirmed by the external test data set from the NEDO project (Matsumoto et al., 2009). It is generally believed that genotoxic compounds directly induce DNA damage by themselves or their reactive metabolites and that sufficient initiation is achieved after a single dose or short-term repeated doses due to the strong potency of their genotoxic stimulus. In contrast, non-genotoxic carcinogens generally require repeated dosing for sufficient initiation. Although the mechanisms involved in hepatocarcinogenic process are not the same between genotoxic and non-genotoxic hepatocarcinogens, our selected genes showed similar

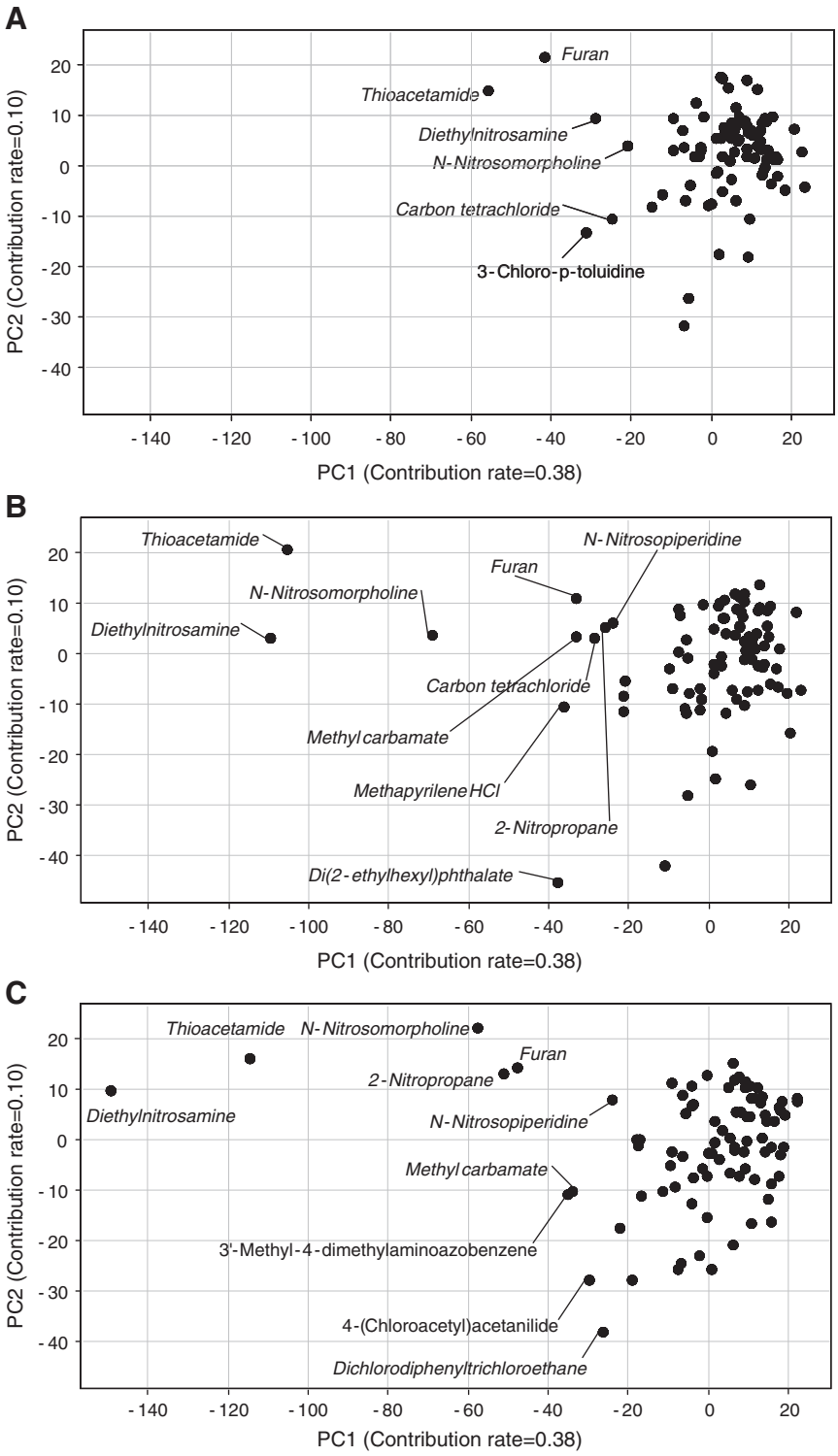

Fig. 5. Principal component analysis in independent validation. An external microarray data set obtained from the NEDO project was used for independent validation of our model. PCA was performed on all compounds by using the expression data at 3 different time points of 4 (A), 15 (B) and 29D (C). Italic font indicates hepatocarcinogens. Several hepatocarcinogens were separated from other chemicals including non-carcinogens toward the direction of PC1.

expression profiles after repeated dosing. Our results indicate that the expression profiles of our newly selected candidate biomarker genes might be common characteristics in the early stage of hepatocarcinogenic process, regardless of the type of carcinogens.

Among the test compounds, ethionamide and etambutol showed positive predictions in the current model, although there is no direct evidence supporting the hepatocarcinogenicity of these compounds in rodents. In our experiments, repeated doses of ethionamide caused anisokaryosis of centrilobular hepatocytes. In the etambutol-treated liver, karyomegaly was observed in hepatocytes with distinct nucleoli. Although it is hard to conclude that these morphological changes in the nucleus of hepatocytes are directly connected to hepatocarcinogenicity, these morphological changes might be early indicative changes of hepatocarcinogenesis and reflect nuclear DNA damage caused by these compounds. Thus, gene expression changes after further long-term repeated dosing as well as a 2-year bioassay study would be of particular

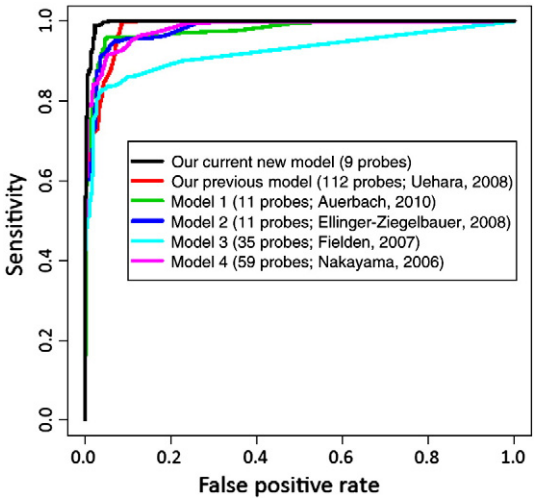

Fig. 6. Receiver operating characteristic analysis of previously published prediction models. ROC curves of prediction models are plotted. Our current new model: prediction results obtained by the best model consisting of 9 probes are shown. Model 1: a model consisting of 11 probes in the 90-day optimal-model, which showed the best prediction performance in our data set among their models, was used for this analysis. Model 2: among their models, commonly selected 101 probes were used for modeling. Models 3 and 4: 35 and 56 probes were used for modeling. Lists of genes used for each modeling are shown in Supplemental Table 5.

interest to determine the carcinogenic potentials of these compounds as well as to confirm the biological significance of these gene expression changes.

This study also provided the novel biological information that modulation of *p38 Mapk*- and *Myc*-centered networks is a common characteristic of gene expression in the early stage of hepatocarcinogenesis. There is increasing evidence that *p38 Mapk*, which is a stress-activated kinase, also participates in cell-cycle regulation, functioning as a suppressor of cell proliferation and tumorigenesis (Campbell et al., 2011). *p38* plays essential roles in modulating chronic inflammation-related cytokine expression, such as tumor necrosis factor α, interleukin 6 and 12, which might act as promoters of cancer growth and progression (Karin et al., 2006; Kumar et al., 2003; Naugler et al., 2007). *p38alpha*-deficient mice are susceptible to carcinogens, and *p38alpha* negatively regulates cell proliferation by antagonizing the *JNK-c-Jun* pathway in multiple cell types and in liver cancer development (Hui et al., 2007a, 2007b). Therefore, upregulation of genes involved in the *p38 Mapk*-network might be a tumor-suppressive action against DNA damage stimulus in the liver following repeated doses of carcinogens. Furthermore, these genes might be useful indicators in the early stage of hepatocarcinogenesis. The *Myc* oncogene is a transcription factor that plays an important role in the pathogenesis of hepatocellular carcinoma (Calvisi and Thorgeirsson, 2005; Thorgeirsson and Grisham, 2002). *Myc* overexpression is believed to exert its neoplastic function via several mechanisms, such as inducing autonomous cell proliferation and growth, blocking differentiation, and causing genomic destabilization (Dang, 1999; Felsher and Bishop, 1999; Grandori et al., 2000; Oster et al., 2002; Pelengaris et al., 2002). While the detailed biological significance should be determined in further studies, several genes were up or downregulated in this network. Among the genes involved in these networks, there is increasing evidence supporting their involvement in carcinogenesis. *Cdh13* (T-cadherin), an atypical member of the cadherin family, is thought to affect cellular biological processes largely via its signaling properties. It is often downregulated in cancerous cells, which is associated with a poor prognosis in various human carcinomas (Andreeva and Kutuzov, 2010). It is also reported that *Cdh13* re-expression in cancer cell lines inhibits cell proliferation and invasiveness, increases susceptibility to apoptosis, and reduces tumor growth (Andreeva and Kutuzov, 2010). Approximately 50% of human hepatocellular carcinomas overexpress *Cdh13* (Riou et al., 2006). While *Cdh13* expression is restricted to endothelial cells from large blood vessels in normal livers, it is upregulated in sinusoidal endothelial cells in invasive hepatocellular carcinomas (Riou et al., 2006). B7-H3 (*Cd276* protein), a surface immunomodulatory glycoprotein, inhibits the functions of natural killer cells and T cells. Whereas the B7-H3 transcript is ubiquitously expressed in various types of human solid tumors as well as normal tissues, the B7-H3 protein is preferentially expressed only in tumor tissues (Xu et al., 2009). While additional biological studies would be required for further confirmation of the biological significance of the modulation of genes involved in these networks, these gene expression changes might play pivotal roles in the early stage of hepatocarcinogenesis.

It is well-known that carcinogenicity is dependent on total exposure levels of compounds; therefore, we have compared total exposure levels of compounds between our experimental conditions and the 2-year bioassay for a better understanding of the toxicological significance of positive results by our prediction model. Due to the abundant literature on carcinogenesis studies, we selected 2 representative hepatocarcinogens, nitrosodiethylamine and acetamidofluorene, for detailed discussions. In our experimental condition, positive predictions for the nitrosodiethylamine and acetamidofluorene groups were observed at cumulative total doses of 240 to 450 mg/kg for nitrosodiethylamine and 420 to 8400 mg/kg for acetamidofluorene. Williams et al. (2004) performed a series of dose-response investigations with these 2 compounds to characterize differences in hepatocarcinogenic effects with respect to exposures. In their two-stage hepatocarcinogenesis study of rats, nitrosodiethylamine was weekly dosed at 40.9 mg/kg for 10 weeks, and a cumulative nitrosodiethylamine dose of 409 mg/kg yielded an 80% hepatocellular tumor incidence followed by 4 weeks of recovery and 24 weeks of promotion with phenobarbital. In contrast, another study demonstrated that a cumulative dose of 409 mg/kg induced a 45% hepatic tumor incidence after 130 weeks of exposure in drinking water (Peto et al., 1991a, 1991b). Regarding acetamidofluorene, a 100% incidence of hepatic tumors was reported at cumulative doses of 282 mg/kg (3.4 mg/kg/day for 12 weeks) followed by 24 weeks of promotion with phenobarbital, while the dose of 1772 mg/kg (3.3 mg/kg/day for 76 weeks) was needed without the promotion (Williams et al., 1991). Although there were some differences in the susceptibility to tumor occurrence in these 2 reports, the cumulative doses of nitrosodiethylamine and acetamidofluorene in our present study were sufficiently above the cumulative dose needed for hepatocarcinogenesis in rats. Taken together, it might be reasonable to conclude that sufficient initiation effects have been achieved under our current dosing conditions of hepatocarcinogens with positive prediction. For building

Table 3
Prediction performance of previously published models.

Models	# of probes	AUC	Sensitivity	False positive rate	Specificity	Correct classification rate
Our current new model	9	0.996	0.990	0.033	0.967	0.978
Our previous model	112	0.983	0.997	0.087	0.913	0.955
Model 1 (Auerbach)	11	0.977	0.960	0.053	0.947	0.953
Model 2 (Ellinger)	101	0.980	0.937	0.057	0.943	0.940
Model 3 (Fielden)	35	0.923	0.830	0.040	0.960	0.895
Model 4(Nakayama)	56	0.982	0.917	0.053	0.947	0.932

models, we have anchored on available carcinogenicity results from 2-year rat bioassays of each compound. Our toxicogenomics project does not only focus on the carcinogenicity of compounds; therefore, SD rats were used as experimental animals in our project, while carcinogenicity tests generally use F344 rats. In addition, rats received orally or intravenously administered compound at the 1-month maximum tolerated doses. However, other dosing methods, generally in the diet or water, are also used for repeated exposure during the 2-year lifespan of rats. Therefore, differences in experimental conditions should be taken into account for a precise comparison. Since we hypothesized that expression changes in our feature genes might reflect the initiated condition of the liver following large doses of carcinogens for up to 1 month, we are conducting further biological studies by using a 2-step carcinogenicity study model in rats. Further data will be published in the near future.

In conclusion, we constructed a new toxicogenomic model for early prediction for both genotoxic and non-genotoxic hepatocarcinogens by using comprehensive gene expression data stored in our large-scale toxicogenomics database. The usefulness and robustness of our predictor were further confirmed in an independent validation data set obtained from a public database. Our toxicogenomic model might be useful for the prospective screening of hepatocarcinogenicity of compounds and prioritization of compounds for carcinogenicity testing. Furthermore, these characteristics of gene expression changes would help to elucidate the mechanisms of action involved in hepatocarcinogenesis.

Conflict of interest statement

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.taap.2011.07.001.

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