REVIEW

The Japanese toxicogenomics project: Application of toxicogenomics

Takeki Uehara[1,2], Atsushi Ono[2,3], Toshiyuki Maruyama[1], Ikuo Kato[1], Hiroshi Yamada[2], Yasuo Ohno[2,3] and Tetsuro Urushidani[2,4]

[1] Developmental Research Laboratories, Shionogi & Co., Ltd., Futaba-cho, Toyonaka, Osaka, Japan
[2] Toxicogenomics Informatics Project, National Institute of Biomedical Innovation, Asagi, Ibaraki, Osaka, Japan
[3] National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo, Japan
[4] Department of Pathophysiology, Faculty of Pharmaceutical Sciences, Doshisha Women's College of Liberal Arts, Kodo, Kyotanabe, Kyoto, Japan

Received: April 13, 2009
Revised: July 29, 2009
Accepted: August 6, 2009

Biotechnology advances have provided novel methods for the risk assessment of chemicals. The application of microarray technologies to toxicology, known as toxicogenomics, is becoming an accepted approach for identifying chemicals with potential safety problems. Gene expression profiling is expected to identify the mechanisms that underlie the potential toxicity of chemicals. This technology has also been applied to identify biomarkers of toxicity to predict potential hazardous chemicals. Ultimately, toxicogenomics is expected to aid in risk assessment. The following discussion explores potential applications and features of the Japanese Toxicogenomics Project.

Keywords:
Genomic biomarker / Hepatotoxicity / Microarray / Toxicogenomics Project / Toxicogenomics

1 Introduction

Today, in the post-genomic era, there have been remarkable advances in the technology of drug development. Drug development in the previous century was usually based on screening the effects of chemicals in model animals with artificially created diseases; subsequently, it sometimes happened that an excellent drug was produced not for humans, but for rats. In recent years, however, it has been possible to start the development process by targeting disease-related genes whose molecular functions are well elucidated, and indeed, human genes are always available. Therefore, it is now easy to select a chemical that is effective on the human molecule on at least the *in vitro* level. Even with this advantage, many candidate drugs have been eliminated because of toxicity that could not be found in preclinical tests in the early stage of drug development; rather, the toxicity became apparent at the later stage of drug development, such as during long-term toxicity studies for animal models and after several stages of clinical trials [1]. In extreme cases, serious adverse effects emerge even after the drugs are widely distributed on the world market. A top priority should be the solution of this paradox; *i.e.* how to predict ''unpredictable'' toxicity. The response of the organism to the toxicant that subsequently causes pathological changes in certain organs with a low dose should be detectable as changes in the expression of genes, protein synthesis, and metabolism. Of these changes, the expression of genes, or the amount of mRNA, is the most sensitive measure and one of the largest advantages in the technology of genomics. Therefore, toxicogenomics, which enables us to comprehensively analyze gene expression changes caused by an external stimulus in a specific organ, is considered to

Correspondence: Dr. Takeki Uehara, Developmental Research Laboratories, Shionogi & Co., Ltd., 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan
E-mail: takeki.uehara@shionogi.co.jp
Fax: +81-6-6332-6385

Abbreviations: BSO, L-buthionine-S,R-sulfoximine; **PAM,** prediction analysis of microarray; **PCA,** principal component analysis; **PPARα,** peroxisome proliferator-activated receptor alpha; **PSTC,** Predictive Safety Testing Consortium; **SVM,** support vector machine; **TGP,** Toxicogenomics Project

be one of the most powerful strategies. In particular, the identification of predictive biomarkers for drug-induced toxicity at or before the pre-clinical stages of drug development is of great importance to pharmaceutical companies.

2 Current status of worldwide toxicogenomics database creation

To appropriately interpret the microarray data, it is desirable to perform comparative analyses with data obtained from prototypical toxicants. Moreover, toxicogenomics studies are built on standard toxicology studies, and one goal of toxicogenomics is to detect relationships between changes in gene expression and toxicological end-point data, such as histopathology, clinical chemistry, and other toxicity data. Therefore, a large-scale, high-quality, and well-designed toxicogenomics database of gene expression information and standard toxicological data are essential. Several public toxicogenomic database efforts have been initiated, such as Gene Expression Omnibus [2, 3] (GEO; National Center for Biotechnology Information, National Institutes of Health; www.ncbi.nlm.nih.gov/geo), ArrayExpress [4, 5] (European Bioinformatics Institute; www.ebi.ac.uk/microarray-as/ae/), Center for Information Biology Gene Expression [6] (CIBEX; DNA Data Bank of Japan, National Institute of Genetics; http://cibex.nig.ac.jp/), EDGE (McArdle Laboratory for Cancer Research [7], University of Wisconsin-Madison; http://edge.oncology.wisc.edu/edge3.php), Chemical Effects in Biological Systems [8] (CEBS; National Institute of Environmental Health Sciences; http://cebs.niehs.nih.gov/cebs-browser/), dbZach [9] (Department of Biochemistry & Molecular Biology, Michigan State University; http://dbzach.fst.msu.edu), and Comparative Toxicogenomics Database [10, 11] (CTD; Mount Desert Island Biological Laboratory; http://ctd.mdibl.org).

In addition to these public microarray databases, public consortia provide a forum to address questions requiring more resources than one organization alone could provide and to engage many sectors of the scientific community. InnoMed PredTox [12] is a joint Industry and European Commission collaboration to improve drug safety. The consortium is a collaborative project of 15 research groups from 12 pharmaceutical companies, three academic institutions and two technology providers. The goal of this consortium is to assess the value of combining results from ''omics'' technologies (transcriptomics, proteomics, metabolomics) with results from more conventional toxicology methods for more informed decision making in pre-clinical safety evaluation. Genedata (http://www.genedata.com/), a company that offers expertise in research informatics combined with open and scalable computational solutions, has provided the computational infrastructure for InnoMed PredTox, in particular the software for data management and analysis.

The Liver Toxicity Biomarker Study [13] is a collaborative pre-clinical research effort in molecular systems toxicology between the National Center for Toxicological Research and BG Medicine and it is supported by seven pharmaceutical companies and three technology providers. The Liver Toxicity Biomarker Study is an innovative approach to investigate drug-induced liver injury because it compares molecular events produced *in vivo* by compound pairs that (i) are similar in structure and mechanism of action, (ii) are associated with few or no signs of liver toxicity in pre-clinical studies, and (iii) show marked differences in hepatotoxic potential.

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 TGP in Japan]. Several genomic candidate biomarkers to predict the toxicity of chemicals have been successfully identified by using our database. The work and results reviewed here focuses on our efforts in toxicogenomics research and highlights recent progress in the application of toxicogenomics.

3 The TGP in Japan

3.1 Features of the project

The Ministry of Health, Labour and Welfare, National Institute of Health Sciences (NIHS), and the working group of Japan Pharmaceutical Manufacturers Association planned the TGP, a collaborative project of the government and private companies. The TGP was a 5-year project (2002–2007) performed by National Institute of Health Sciences, 15 pharmaceutical companies (Astellas, Chugai, Daiichi, Dainippon-Sumitomo, Eisai, Kissei, Mitsubishi, Mochida, Ono, Otsuka, Sankyo, Sanwa, Shionogi, Takeda, Tanabe) and the National Institute Biomedical Innovation (NIBIO), which was the core institute where the actual work was performed. Half of the budget was provided by a grant from the Ministry of Health, Labour and Welfare, and the other half was provided by the pharmaceutical companies.

The primary goal of the TGP was to create a gene expression database by using the Affymetrix GeneChip® of 150 chemicals, mainly medical drugs (Table 1), and the main target organ was the liver. Most clinically serious adverse effects occur in the liver, and the cell-type composition of the liver is relatively homogenous; thus, the expected variation based on sampling differences would be minimal. For these reasons, the liver was selected as the target organ to accumulate know-how regarding the toxicogenomics technique. Nephrotoxicity was also considered to be important; therefore, the kidney, in addition to the liver, was sampled for microarray analysis and pathologically examined in all the animals.

The TGP was completed in 2007. The entire system consists of a database, an analysis system, and a prediction system and is named as TG-Genomics-Assisted Toxicity

Table 1. Chemicals selected (in total 150 compounds) in TGP

Acetaminophen	Doxorubicin hydrochloride	Nitrofurantoin
Acetazolamide	D-Penicillamine	Nitrofurazone
Ajmaline	Enalapril maleate	N-nitrosodiethylamine
Allopurinol	Erythromycin ethylsuccinate	N-phenylanthranilic acid
Allyl alcohol	Ethambutol dihydrochloride	Omeprazole
Alpha-naphthyl isothiocyanate	Ethanol	Papaverine hydrochloride
Amiodarone hydrochloride	Ethionamide	Pemoline
Amitriptyline hydrochloride	Etoposide	Perhexiline maleate
Aspirin	Famotidine	Phenacetin
Azathioprine	Fenofibrate	Phenobarbital sodium
Bendazac	Fluphenazine dihydrochloride	Phenylbutazone
Benzbromarone	Flutamide	Phenytoin
Benziodarone	Furosemide	Promethazine hydrochloride
Bromobenzene	Gemfibrozil	Propylthiouracil
Bucetin	Gentamicin sulfate	Puromycin aminonucleoside
Caffeine	Glibenclamide	Quinidine sulfate
Captopril	Griseofulvin	Ranitidine hydrochloride
Carbamazepine	Haloperidol	Rifampicin
Carbon tetrachloride	Hexachlorobenzene	Simvastatin
Carboplatin	Hydroxyzine dihydrochloride	Sodium valproate
Cephalothin sodium	Ibuprofen	Sulfasalazine
Chloramphenicol	Imipramine hydrochloride	Sulindac
Chlormadinone acetate	Indomethacin	Tacrine hydrochloride
Chlormezanone	Iproniazid phosphate	Tamoxifen citrate
Chlorpromazine Hydrochloride	Isoniazid	Tannic acid
Chlorpropamide	Ketoconazole	Terbinafine hydrochloride
Cimetidine	Labetalol hydrochloride	Tetracycline hydrochloride
Ciprofloxacin hydrochloride	Lomustine	Theophylline
Cisplatin	Lornoxicam	Thioacetamide
Clofibrate	Mefenamic acid	Thioridazine hydrochloride
Clomipramine hydrochloride	Meloxicam	Ticlopidine hydrochloride
Colchicine	Metformin hydrochloride	Tiopronin
Coumarin	Methapyrilene hydrochloride	Tolbutamide
Cyclophosphamide monohydrate	Methimazole	Triamterene
Cyclosporine A	Methotrexate	Triazolam
Danazol	Methyldopa	Trimethadione
Dantrolene sodium Hemiheptahydrate	Methyltestosterone	Vancomycin hydrochloride
Diazepam	Mexiletine hydrochloride	Vitamin A
Diclofenac sodium	Monocrotaline	WY-14,643
Diltiazem hydrochloride	Moxisylyte hydrochloride	(±)-Chlorpheniramine maleate
Disopyramide	Naproxen	(±)-Sulpiride
Disulfiram	Nicotinic acid	17-alpha-Ethinyl estradiol
DL-ethionine	Nifedipine	2-Acetamidofluorene
Doxepin hydrochloride	Nimesulide	2-Bromoethylamine hydrobromide

Drug candidates supplied from the member companies, which were withdrawn in various stages of drug development, were excluded.

Evaluation System. The database will be available to the public in the near future.

3.2 Contents of the database

Our standard study protocol is summarized in Table 2.

In vivo study: The rat was selected as the species for analysis. Rats were very frequently used in non-clinical examinations, and toxicological information for the rat has been accumulated. Both a single-dose study, consisting of multiple time points with multiple dose levels, and a repeated-dose study, consisting of multiple dose periods with multiple dose levels, were performed. Data obtained from each animal included body weight, general symptoms, hematology, blood biochemistry, organ weight, and a histopathological examination of the liver and kidney. Gene expression in the liver and kidney was comprehensively analyzed by using Affymetrix GeneChip® arrays.

In vitro study: A modified two-step collagenase perfusion method was used to isolate liver cells from 6-week-old male Sprague–Dawley rats. A comprehensive gene expression analysis was performed on the primary cultured cells at multiple time points after treatment with various concen-

Table 2. The standard study protocol in TGP

In vivo	
Animal	Sprague–Dawley rat (6 wk old, $N = 5$ for each group)
Vehicle	0.5% Methylcellulose or corn oil (oral dose) Saline or 5% glucose solution (intravenous dose)
Dose	Low, middle, and high (mainly 1:3:10)
Route	Oral (intravenous in a few cases)
Sacrifice	3, 6, 9, and 24 h after a single administration 24 h after the last dose of repeated administration for 3, 7, 14 and 28 days
Sampling	Liver, kidney, and plasma
Microarray analysis	Affymetrix GeneChip ($N = 3$ for each group)
Items examined	Histopathology: liver and kidney Body weight, organ weight (liver and kidney), food consumption, hematology, and blood biochemistry
In vitro: rat	
Animal	Sprague–Dawley rat (6 weeks old)
Cell	Hepatocyte isolated by collagenase digestion
Vehicle	Culture medium or DMSO
Concentration	Low, middle, high (1:5:25)
Treatment	2, 8, 24 h
Microarray analysis	Duplicate
Items examined	Cell viability (LDH release and DNA contents)
In vitro: human	
Cell	Human frozen hepatocytes
Vehicle	Culture medium or DMSO
Concentration	Low, middle, and high (1:5:25, low is omitted in some cases)
Treatment	2, 8, 24 h (2 h is omitted in some cases)
Microarray analysis	Duplicate
Items examined	Cell viability (LDH release and DNA contents)

trations of each of the 150 compounds. The same gene expression analysis was also performed with human liver cultured cells obtained from Tissue Transformation Technologies.

3.3 Analysis and prediction systems

Analysis system: Since microarray data consist of large amounts of numerical data, statistical knowledge and computational skills are required to interpret the results. Multivariate analysis methods are utilized for both data mining and pattern recognition, such as hierarchical clustering, K-means clustering, self-organizing map (SOM), and principal component analysis (PCA). PCA is a convenient tool for the qualitative classification of compounds against a list of genes. As a prediction system, however, some quantitative data would be favorable for the final output. Therefore, in our system, when the user specifies a principal component with high contribution, the compounds are sorted by value, and the genes with large eigenvector values are easily obtained. This analysis provides the relative position of the test drug among the drugs in the database and supports to generate a candidate gene list for further investigation.

Prediction system: Discriminant analysis is a powerful technique that can be used when a phenotype that can be judged as positive or negative is available [14]. Prediction analysis of microarray (PAM) [15] and support vector machine (SVM) [16] have been employed in our systems. The sample size and appropriate selection of the training data set are crucial for establishing reliable classifiers. In our system, by a semi-automatic system of training and validation, the efficiency improves for the creation of classifiers.

BaseView system: When an assessment or prediction of toxicity is made by a list of multiple measures, it is necessary to summarize or quantify these measurements. Ideally, the quantification process should be optimized for each marker gene list. However, because this approach is practically difficult, a uniform system has to be created. In our system, a new scoring system was developed in one trial. The TGP1 score is calculated based on the ratio to control value (log 2) for each gene in the marker list and expressed as a heat map [17, 18]. This scoring system makes it easy to summarize the assessments of a target compound against many marker lists and to summarize the assessments of many compounds against a particular marker list. However, this system has some problems; the score is biased when the list contains a gene whose expression change is extremely large (*e.g.*, CYP1A1), and changes are canceled when up- and down-regulated genes coexists in the list. Therefore, another scoring system, the TGP2 score, is available in our system. The TGP2 score is based on the effect size and calculated as the absolute value of the difference between means divided by the covariance [19].

4 Application of toxicogenomics

Our strategy is to prepare a large set of genomic biomarkers that are related to toxicological phenotypes, pathways, or any other biologically meaningful factor. Until now, several potential genomic biomarkers to predict the toxicity of chemicals have been successfully identified. In this article, we provide several applications of toxicogenomics by using our database.

4.1 Glutathione depletion [20]

The hepatotoxicity of acetaminophen is caused by the excessive production of active metabolite that exceeds the detoxification capacity of intracellular glutathione [21]. Therefore, drugs that

have the potential to deplete hepatocyte glutathione carry the risk of causing acetaminophen-type hepatotoxicity with overdosage. In a previous report, a list of marker genes for glutathione depletion was extracted by using BSO, a glutathione biosynthesis inhibitor [22]. However, phorone is considered to be superior to L-buthionine-S,R-sulfoximine (BSO) as a model system, since its mechanism of glutathione depletion is similar to that of acetaminophen-type hepatotoxicants (*i.e.* it covalently binds to glutathione and is excreted from the cell). Phorone (40, 120, or 400 mg/kg) was administered according to the same protocol as the regular single-dose experiments, and the glutathione content was measured. Phorone caused a marked but transient depletion of glutathione with maximal depletion occurring at 3 h. Then, the glutathione level recovered, and it was increased at 24 h as a rebound effect. A total of 161 probe sets was identified with signal levels that were inversely correlated with the hepatic glutathione content (Fig. 1). PCA of the chemicals in the database with these probe sets revealed that chemicals with a risk of glutathione depletion, such as bromobenzene and coumarin, in addition to acetaminophen, were clearly separated from other chemicals or controls toward the direction of principal component 1, suggesting that the list was useful as a genomic biomarker for risk assessment of glutathione depletion.

4.2 Phospholipidosis [23]

In toxicity studies, phospholipidosis is often observed in various tissues including the liver. Despite efforts to establish methods to predict the phospholipidosis of drugs, sensitive diagnostic markers, and effective prognostic markers were still desired. To identify a genomic biomarker for the prediction of hepatic phospholipidosis, we extracted 78 probe sets of rat hepatic genes based on data from five drugs (amiodarone, amitriptyline, clomipramine, imipramine, and ketoconazole) that induce this phenotype. A PCA was performed, and the possible induction of phospholipidosis was predictable by the expression of these genes 24 h after a single administration.

4.3 Cholestasis [24]

Cholestatic hepatitis is the most common type of drug-induced cholestasis and is more frequent than cholestatic viral hepatitis. Cholestasis is caused by a functional defect in bile formation at the level of the hepatocyte or by an impairment in bile secretion and flow at the level of the bile ductules or ducts. To identify a biomarker for the diagnosis of elevated total and direct bilirubin, we extracted 59 probe sets of rat hepatic genes based on data from seven drugs (gemfibrozil, phalloidin, colchicine, bendazac, rifampicin, cyclosporine A, and chlorpromazine) that induce cholestatic hepatitis after 3–28 days of repeated administration. PCA with these probe sets clearly separated dose- and time-dependent clusters in the treated groups from the control groups. Although further work is required to improve and generalize the candidate for a marker suggested in this study, these identified probe sets should be useful to diagnose the cause of elevated total and direct bilirubin.

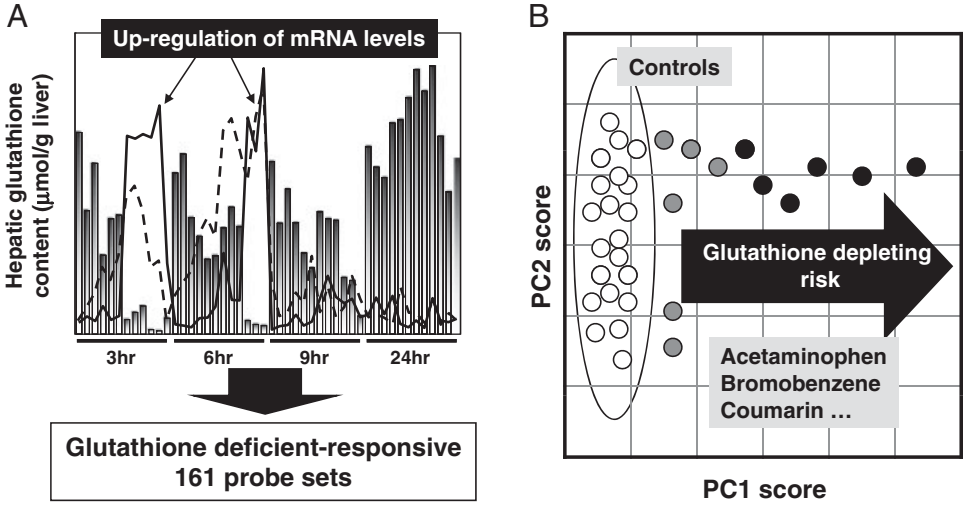

Figure 1. Identification and application of genomic biomarkers for assessing glutathione depletion. A model case for identifying the candidate genomic biomarker associated with glutathione depletion-type liver injury is presented. Rats were treated with a glutathione depletor, phorone, and microarray analysis was performed on the liver tissue. (A) A total of 161 probe sets had signal levels that were inversely correlated with the hepatic glutathione content. (B) The validity of these probe sets as biomarkers for the evaluation of glutathione depletion risk was evaluated by PCA. This evaluation revealed that chemicals with a risk of glutathione depletion, such as bromobenzene and coumarin, in addition to acetaminophen, were clearly separated from other chemicals or controls toward the direction of PC1.

4.4 Non-genotoxic hepatocarcinogenicity [25]

Assessing carcinogenicity in animals is difficult and costly; therefore, an alternative strategy is desired. Genotoxic compounds are usually identified and removed early from compound pipelines. However, the discovery of unexpected, presumed non-genotoxic, carcinogenicity late in drug development may prevent potentially good medicines from reaching patients for years while the human risk is qualified. Microarrays and expression profiling have been used to make classifiers for the early prediction of non-genotoxic carcinogenicity in the liver [26–29]. The goal of these studies was to extract common gene sets coordinately deregulated by different classes of non-genotoxic hepatocarcinogenesis. These publications confirm that multiple genes are required for accurate classification due to the multiple mechanisms of action that must be included in the prediction model. Therefore, the effects of chemicals with similar mechanisms are likely to be reflected in similar gene expression profiles in the early stage of non-genotoxic carcinogenesis [28]. Arguably more important than the identification of potential carcinogenicity of a compound is the identification of the mechanism of action [30]. Our strategy was to focus on one important mechanism, cytotoxic oxidative stress, responsible for non-genotoxic hepatocarcinogenesis.

We selected thioacetamide and methapyrilene as prototypic oxidative stress-mediated, non-genotoxic hepatocarcinogens and performed PAM discriminant analysis. A PAM classifier containing 112 probe sets that yielded an overall success rate of 95% was successfully obtained from the training procedure. Based on gene ontology, the content of genes related to cellular metabolism, including anti-oxidative metabolism, cell proliferation, cell cycle, and response to DNA damage stimulus, was significantly high. The validity of this classifier was checked for 30 chemicals. The classification results showed characteristic time-dependent increases by treatment with several non-genotoxic hepatocarcinogens, including thioacetamide, methapyrilene, coumarin, and ethionine (Fig. 2). Although all of the carbon tetrachloride-treated groups were predicted as negative, the score tended to increase with repeated dosing. On the other hand, the enzyme inducers with carcinogenic activity, phenobarbital and hexachlorobenzene, and peroxisome proliferators other than Wy-14 643 (*i.e.* clofibrate and gemfibrozil) had negative scores for all time points. Of the non-carcinogenic samples, bromobenzene had a transient score increase at 24 h but returned to negative during repeated dosing. Almost all of the non-carcinogens were correctly predicted as negative, but it was not possible to completely eliminate false positives. This work suggested that the possibility of lowering the days of repeated administration to less than 28, at least for a category of non-genotoxic hepatocarcinogens causing oxidative stress.

The carcinogenicity working group of the C-Path Predictive Safety Testing Consortium (PSTC) has selected genes from published toxicogenomics research that were

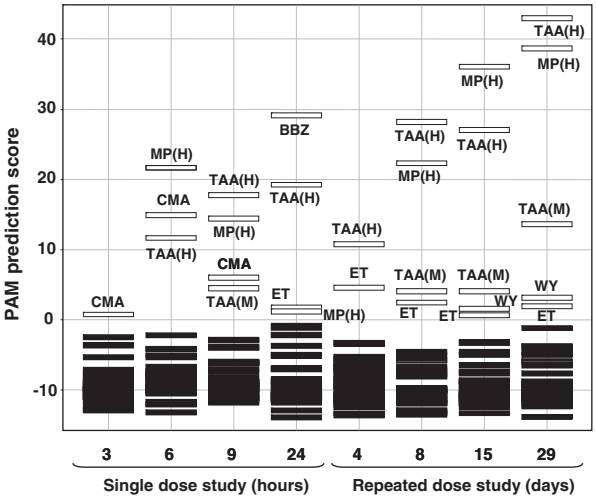

Figure 2. Time-course changes in prediction results for non-genotoxic hepatocarcinogenicity of chemicals. A model case for identifying the candidate genomic biomarkers associated with non-genotoxic hepatocarcinogenicity is presented. The PAM class probability was converted to a score to enable quantitative comparison. The PAM score showed characteristic time-course changes for several non-genotoxic hepatocarcinogens. For methapyrilene, thioacetamide and other carcinogens, such as the ethionine and coumarin, the scores transiently increased at an early time point after a single dosing. In the case of repeated dosing, the scores increased with the repeated doses. The following samples were classified as positive: methapyrilene 100 mg/kg (high dose, H); thioacetamide 15 mg/kg (middle dose, M) and 45 mg/kg (H); coumarin 150 mg/kg; ethionine 250 mg/kg; Wy-14 643 100 mg/kg; and bromobenzene 300 mg/kg. Each box indicates the PAM score. Black boxes indicate samples that are predicted as negative.

determined to be of high predictive value in the early recognition of non-genotoxic hepatocarcinogenicity. The group consolidated this list for refinement and qualification as a gene signature to predict a compound's potential to be a non-genotoxic hepatocarcinogen. To ensure the independence and cost effectiveness of the platform, mRNA for these genes was assayed by real-time quantitative PCR, and a final signature was re-derived from genes with confirmed expression. The robustness and potential utility of this new quantitative PCR-based signature will be discussed in future reports.

4.5 Bridging between *in vivo* and *in vitro*: Peroxisome Proliferator-Activated Receptor alpha-mediated response [31]

Data from three ligands of peroxisome proliferator-activated receptor alpha (PPARα) – *i.e.* clofibrate, WY-14 643, and gemfibrozil – in our database were analyzed. Many of the β-oxidation-related genes were commonly induced *in vivo* and *in vitro*, whereas expression changes in genes related to cell proliferation and apoptosis were detected *in vivo* but not

in vitro (Fig. 3). By using the genes commonly up-regulated both *in vivo* and *in vitro*, PCA was performed for 32 compounds, and principal component 1 was identified as a convenient parameter to extract PPARα agonists from the database (Fig. 3). This study is one of the first to create an *in vivo–in vitro* bridge for the validation of a genomic biomarker.

4.6 Bridging between the rat and human: Coumarin-induced hepatotoxicity [19]

A system that perfectly predicts hepatotoxicity in the rat would not necessarily improve the prediction of hepatotoxicity in humans. The final goal must be the prediction of hepatotoxicity in humans for drug development. The extrapolation of toxicity data from rodent to human is not sufficient. However, if general toxic mechanisms or toxicological pathways are conserved over species, they would be useful bridges between animal models and clinical events. One expected result from toxicogenomics technology is to overcome the barrier due to species difference in the prediction of clinical toxicity.

We investigated the possibility of an informational bridge connecting transcript responses between rat and human hepatocytes, and rat liver *in vivo* after the administration of coumarin. In this experiment, primary cultured rat hepatocytes were exposed to 12, 60, and 300 μM coumarin for 24 h. No obvious cytotoxicity was detected by LDH release (100.5, 97.7, and 95.1% of control, respectively). Then, we extracted the significant genes according to the gene list obtained from *in vivo* study; the extracted genes showed

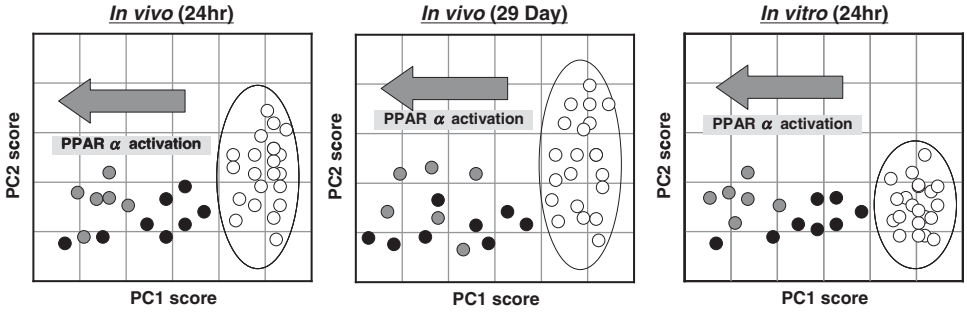

Figure 3. An *in vivo – in vitro* bridge for genomic biomarkers to assess PPARα agonistic action. A model case for creating an *in vivo – in vitro* bridge for genomic biomarkers is presented. The data from three agonists of PPARα in our database (clofibrate, WY-14 643 and gemfibrozil) were analyzed, and 41 commonly up-regulated probe sets between *in vivo* and *in vitro* were extracted. The validity of these probe sets as biomarkers for the evaluation of PPARα agonistic activity was evaluated by PCA. These plots show the principal separation of samples due to putative PPARα agonistic activity toward the negative direction on the *x*-axis, PC1.

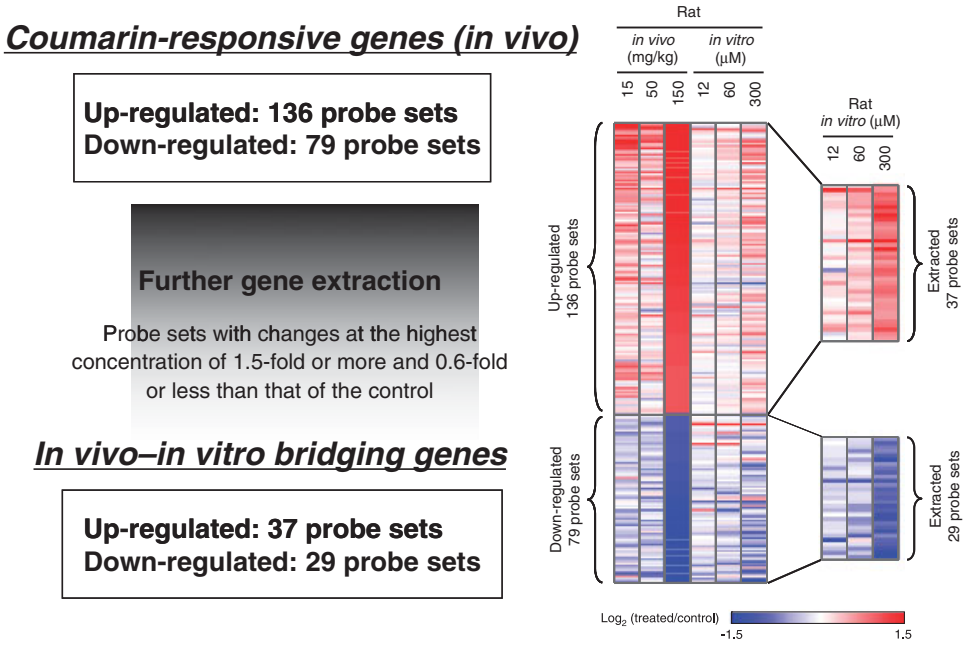

Figure 4. Heat map of the expression profiles of probe sets in rat liver and rat hepatocytes treated with coumarin. A considerable number of the *in vivo*-selected probe sets show similar profiles between *in vivo* and *in vitro* assays. The selected genes, namely the *in vivo – in vitro* bridging probes, had clear dose-dependent changes in expression.

significant up-regulation (136 probe sets) or down-regulation (79 probe sets) in livers treated with 150 mg/kg coumarin. A similar trend was observed between *in vivo* and *in vitro* cell responses, although the extent of the response (the fold change) was generally smaller, and fewer genes showed a measurable change in the *in vitro* cell assay (Fig. 4). Probe sets showing changes of 1.5-fold or more or 0.6-fold or less than that of the control at the highest concentration (300 μM) in rat hepatocytes were selected as *in vivo–in vitro* bridging probes that reflect the toxicological mechanism of coumarin *in vivo*. The selected genes (37 up-regulated and 29 down-regulated) had clear dose-dependent changes in expression that enabled us to assess the hepatotoxicity of coumarin by using the *in vitro* data (Fig. 4).

Next, cultured human hepatocytes were exposed to 12, 60, and 300 μM coumarin for 24 h. No obvious cytotoxicity was detected by LDH release (100.6, 100.9, and 102.0% of control, respectively). The *in vivo–in vitro* bridging probes were assigned to their human ortholog genes to form a set of rat–human bridging probes, and changes in their expression were compared in rat *versus* human hepatocytes. In total, 14 up-regulated probe sets and 11 down-regulated probe sets were identified; their relative expression levels are shown in Fig. 5. The pattern of changes in gene expression was similar in rat and human cells, but the extent of the changes was more prominent in rat cells than in human cells, in accordance with the known species-specific difference in hepatotoxicity [32–38]. In the case of diclofenac, which is a hepatotoxicant without species difference, there was no evidence of a species-specific difference in gene expression between rat and human cells. The observation that the induction of stress-related genes was more robust in rat cells than in human cells could be a direct reflection of the extent of stress and subsequent damage caused by coumarin in each species. Although more data are needed to connect species and model systems with human risk assessment, this approach is an important step in bridging the differences between species.

5 Future perspectives

This review focuses on our efforts in toxicogenomics research and highlights recent progress in the application of toxicogenomics. In the early stage of drug development, genomic biomarkers are used to identify and optimize lead compounds among several candidates. As full-scale toxicity testing is quite costly, safety assessment of candidate drugs is usually performed just before the clinical trial. If serious toxicity emerges at this stage, it might be necessary to return to the screening of seed compounds, because toxicity is often inherent to the basic structure and is thus never eliminated by minor modification. If the potential phenotype (when repeatedly dosed) is predictable in the early stage

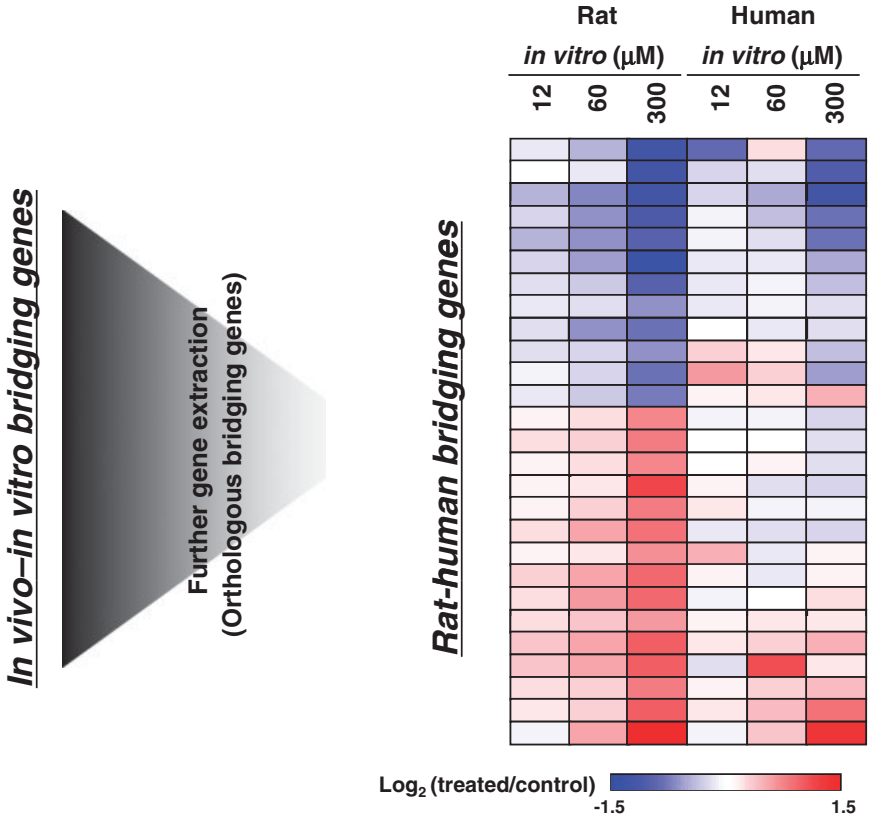

Figure 5. Heat map of the expression profile of probe sets in rat and human hepatocytes treated with coumarin. Among the *in vivo – in vitro* bridging probes for rats, 14 up-regulated and 11 down-regulated probe sets were assigned to human ortholog (species bridging marker), and their expression is shown as a heat map of the expression profile in rat and human hepatocytes treated with coumarin (12, 60 and 300 μM). Each probe set dose-dependently responded to coumarin in both species, whereas the extent of the changes appears to be more prominent in rats than in humans.

of drug development by gene expression data from a small number of experimental animals, it would effectively cut the time and cost of drug development. The use of genomic biomarkers in the early stage of drug development will strengthen the safety screening of drug candidates before they are administered to humans. The use of genomic biomarkers will also reduce the number of animals sacrificed during drug development. However, the candidate biomarkers reviewed here have not necessarily been evaluated with large independent test sets and are rarely validated across laboratories. Further definitive validation studies are absolutely essential for judging the acceptability of candidate genomic biomarkers in pre-clinical safety assessments. Furthermore, regulatory agencies, the pharmaceutical industry and academia must establish guidelines for the integration of ''omics'' data, including toxicogenomics and genomic biomarkers, into drug safety assessment. We are currently in the project's second stage, known as the TGP2. Our goals are as follows: (i) establishment of genomic biomarkers to predict the toxicity of drug candidates in the early stage of drug development, (ii) bridging of species differences, and (iii) application of toxicogenomic data for regulatory science. These efforts will contribute to the accelerated development of more effective and safer drugs.

The PSTC also represents a next important step in the validation and regulatory use of new pre-clinical biomarker tests with the initiative of the C-Path Institute. The novel biomarkers are internally developed and used by each individual pharmaceutical company and consortium are of limited value for regulatory use because the methods used have not been validated by an independent party. To resolve these issues, there is a growing need for a large and cross-institutional study on a global scale. The PSTC is a public–private partnership, led by the C-Path Institute, which brings together pharmaceutical companies to share and validate each other's safety testing methods under advisement of the Food and Drug Administration (FDA) and its European counterpart, the European Medicines Evaluation Agency (EMEA). The aim of the PSTC is to identify and qualify safety biomarkers for regulatory use as part of the Food and Drug Administration's Critical Path Initiative. The 17 corporate members of the consortium share internally developed pre-clinical safety biomarkers in five workgroups: carcinogenicity, kidney, liver, muscle, and vascular injury. Consortium members are sharing their new pre-clinical biomarker tests for examination and cross-validation by other members of the consortium. Candidate genomic biomarkers reviewed here will need a similar validation process through collaborative research like that of PSTC. These processes are expected to enable the regulatory agencies to write new guidelines for industry that identify more accurate methods to predict drug safety.

The authors have declared no conflict of interest.

6 References

[1] Ismail, K., Landis, J., Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discov.* 2003, *3*, 711–715.

[2] Edgar, R., Domrachev, M., Lash, A. E., Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* 2002, *30*, 207–210.

[3] Barrett, T., Troup, D. B., Wilhite, S. E., Ledoux, P. *et al.*, NCBI GEO: archive for high-throughput functional genomic data. *Nucleic Acids Res.* 2009, *37*, D885–D890.

[4] Brazma, A., Parkinson, H., Sarkans, U., Shojatalab, M. *et al.*, ArrayExpress – a public repository for microarray gene expression data at the EBI. *Nucleic Acids Res.* 2003, *31*, 68–71.

[5] Parkinson, H., Kapushesky, M., Kolesnikov, N., Rustici, G. *et al.*, ArrayExpress update – from an archive of functional genomics experiments to the atlas of gene expression. *Nucleic Acids Res.* 2009, *37*, D868–D872.

[6] Ikeo, K., Ishi-i, J., Tamura, T., Gojobori, T. *et al.*, CIBEX: center for information biology gene expression database. *C. R. Biol.* 2003, *326*, 1079–1082.

[7] Hayes, K. R., Vollrath, A. L., Zastrow, G. M., McMillan, B. J. *et al.*, EDGE: a centralized resource for the comparison, analysis, and distribution of toxicogenomic information. *Mol. Pharmacol.* 2005, *67*, 1360–1368.

[8] Waters, M., Stasiewicz, S., Merrick, B. A., Tomer, K. *et al.*, CEBS Chemical Effects in Biological Systems: a public data repository integrating study design and toxicity data with microarray and proteomics data. *Nucleic Acids Res.* 2008, *36*, D892–D900.

[9] Burgoon, L. D., Zacharewski, T. R., dbZach toxicogenomic information management system. *Pharmacogenomics* 2007, *8*, 287–291.

[10] Mattingly, C. J., Colby, G. T., Forrest, J. N., Boyer, J. L., The Comparative Toxicogenomics Database (CTD). *Environ. Health Perspect.* 2003, *111*, 793–795.

[11] Mattingly, C. J., Rosenstein, M. C., Colby, G. T., Forrest, Jr, J. N. *et al.*, The Comparative Toxicogenomics Database (CTD): a resource for comparative toxicological studies. *J. Exp. Zoolog. A Comp. Exp. Biol.* 2006, *305*, 689–692.

[12] Mulrane, L., Rexhepaj, E., Smart, V., Callanan, J. J. *et al.*, Creation of a digital slide and tissue microarray resource from a multi-institutional predictive toxicology study in the rat: an initial report from the PredTox group. *Exp. Toxicol. Pathol.* 2008, *60*, 235–245.

[13] McBurney, R. N., Hines, W. M., Von Tungeln, L. S., Schnackenberg, L. K. *et al.*, The liver toxicity biomarker study: phase I design and preliminary results. *Toxicol. Pathol.* 2009, *37*, 52–64.

[14] Porter, M. W., Castle, A. L., Orr, M. S., Mendrick, D. L., in: Burczynski, M. E. (Ed.), *Predictive Toxicogenomics: An Introduction to Toxicogenomics*, CRC Press, Boca Raton 2003, pp. 225–260.

[15] Tibshirani, R., Hastie, T., Narasimhan, B., Chu, G., Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc. Natl. Acad. Sci. USA* 2002, *99*, 6567–6572.

[16] Brown, M. P., Grundy, W. N., Lin, D., Cristianini, N. *et al.*, Knowledge-based analysis of microarray gene expression data by using support vector machines. *Proc. Natl. Acad. Sci. USA* 2000, *97*, 262–267.

[17] Kiyosawa, N., Shiwaku, K., Hirode, M., Omura, K. *et al.*, Utilization of a one-dimensional score for surveying chemical-induced changes in expression levels of multiple biomarker gene sets using a large-scale toxicogenomics database. *J. Toxicol. Sci.* 2006, *31*, 433–448.

[18] Uehara, T., Kiyosawa, N., Hirode, M., Omura, K. *et al.*, Gene expression profiling of methapyrilene-induced hepatotoxicity in rat. *J. Toxicol. Sci.* 2008, *33*, 37–50.

[19] Uehara, T., Kiyosawa, N., Shimizu, T., Omura, K. *et al.*, Species-specific differences in coumarin-induced hepatotoxicity as an example toxicogenomics-based approach to assessing risk of toxicity to humans. *Hum. Exp. Toxicol.* 2008, *27*, 23–35.

[20] Kiyosawa, N., Uehara, T., Gao, W., Omura, K. *et al.*, Identification of glutathione depletion-responsive genes using phorone-treated rat liver. *J. Toxicol. Sci.* 2007, *32*, 469–486.

[21] James, L. P., Mayeux, P. R., Hinson, J. A., Acetaminophen-induced hepatotoxicity. *Drug Metab. Dispos.* 2003, *31*, 1499–1506.

[22] Kiyosawa, N., Ito, K., Sakuma, K., Niino, N. *et al.*, Evaluation of glutathione deficiency in rat livers by microarray analysis. *Biochem. Pharmacol.* 2004, *68*, 1465–1475.

[23] Hirode, M., Ono, A., Miyagishima, T., Nagao, T. *et al.*, Gene expression profiling in rat liver treated with compounds inducing phospholipidosis. *Toxicol. Appl. Pharmacol.* 2008, *229*, 290–299.

[24] Hirode, M., Horinouchi, A., Uehara, T., Ono, A. *et al.*, Gene expression profiling in rat liver treated with compounds inducing elevation of bilirubin. *Hum. Exp. Toxicol.* 2009, *28*, 231–244.

[25] Uehara, T., Hirode, M., Ono, A., Kiyosawa, N. *et al.*, A toxicogenomics approach for early assessment of potential non-genotoxic hepatocarcinogenicity of chemicals in rats. *Toxicology* 2008, *250*, 15–26.

[26] Ellinger-Ziegelbauer, H., Gmuender, H., Bandenburg, A., Ahr, H. J., Prediction of a carcinogenic potential of rat hepatocarcinogens using toxicogenomics analysis of short-term *in vivo* studies. *Mutat. Res.* 2008, *637*, 23–39.

[27] Ellinger-Ziegelbauer, H., Stuart, B., Wahle, B., Bomann, W., Ahr, H. J., Comparison of the expression profiles induced by genotoxic and nongenotoxic carcinogens in rat liver. *Mutat. Res.* 2005, *575*, 61–84.

[28] Fielden, M. R., Brennan, R., Gollub, J., A gene expression biomarker provides early prediction and mechanistic assessment of hepatic tumor induction by nongenotoxic chemicals. *Toxicol. Sci.* 2007, *99*, 90–100.

[29] Nie, A. Y., McMillian, M., Parker, J. B., Leone, A. *et al.*, Predictive toxicogenomics approaches reveal underlying molecular mechanisms of nongenotoxic carcinogenicity. *Mol. Carcinog.* 2006, *45*, 914–933.

[30] Fielden, M. R., Nie, A., McMillian, M., Elangbam, C. S., Interlaboratory evaluation of genomic signatures for predicting carcinogenicity in the rat. *Toxicol. Sci.* 2008, *103*, 28–34.

[31] Tamura, K., Ono, A., Miyagishima, T., Nagao, T. *et al.*, Profiling of gene expression in rat liver and rat primary cultured hepatocytes treated with peroxisome proliferators. *J. Toxicol. Sci.* 2006, *31*, 471–490.

[32] Vassallo, J. D., Hicks, S. M., Daston, G. P., Lehman-McKeeman, L. D., Metabolic detoxification determines species differences in coumarin-induced hepatotoxicity. *Toxicol. Sci.* 2004, *80*, 249–257.

[33] Felter, S. P., Vassallo, J. D., Carlton, B. D., Daston, G. P., A safety assessment of coumarin taking into account species-specificity of toxicokinetics. *Food Chem. Toxicol.* 2006, *44*, 462–475.

[34] Vassallo, J. D., Hicks, S. M., Daston, G. P., Lehman-McKeeman, L. D., Metabolic detoxification determines species differences in coumarin-induced hepatotoxicity. *Toxicol. Sci.* 2004, *80*, 249–257.

[35] Felter, S. P., Vassallo, J. D., Carlton, B. D., Daston, G. P., A safety assessment of coumarin taking into account species-specificity of toxicokinetics. *Food Chem. Toxicol.* 2006, *44*, 462–475.

[36] Born, S. L., Hu, J. K., Lehman-McKeeman, L. D., o-Hydroxyphenylacetaldehyde is a hepatotoxic metabolite of coumarin. *Drug Metab. Dispos.* 2000, *28*, 218–223.

[37] Born, S. L., Caudill, D., Smith, B. J., Lehman-McKeeman, L. D., *In vitro* kinetics of coumarin 3,4-epoxidation: application to species differences in toxicity and carcinogenicity. *Toxicol. Sci.* 2000, *58*, 23–31.

[38] Lake, B. G., Investigations into the mechanism of coumarin-induced hepatotoxicity in the rat. *Arch. Toxicol.* 1984, *7*, 16–29.

