



Mechanism-Based Evaluation System for Hepato- and Nephrotoxicity or Carcinogenicity Using Omics Technology

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Abstract. We have been developing a carcinogenicity prediction system based on gene expression profiles focusing on omics technology to enable mechanism-based evaluations of toxicity to reduce the numbers of animals and toxicological endpoints required by animal studies. Here, we report the development of a mechanism-based evaluation system focused on chemically induced hepato- and nephrotoxicity or hepatic and renal carcinogenicity using a gene expression analysis with a DNA microarray. As a case study, the mode-of-action (MoA)/adverse outcome pathway (AOP) was constructed from the gene expression profiles and histopathological findings of carbon tetrachloride and cisplatin for hepatotoxicity and nephrotoxicity, respectively. Consequently, we developed an advanced toxicity evaluation system for hepato- and nephrotoxicity or hepatic and renal carcinogenicity based on the toxicity mechanisms. We also developed a new prediction system named “CARCINOscreen[®]” for evaluating the carcinogenic potentials of chemicals using the gene expression profiles of liver and kidney tissues from rats after a 28-day repeated administration. The prediction system could predict the carcinogenicity potential of a training chemical set including carcinogens and non-carcinogens with an accuracy of more than 90%. The marker genes established in this study are promising for the development of new effective in vitro testing methods in the future.

Keywords: Adverse outcome pathway (AOP) · Gene expression profiles · Hepatotoxicity · Nephrotoxicity · Carcinogenicity
CARCINOscreen[®]

Introduction

Of the more than 80,000 chemicals in commerce, rigorous safety testing and risk assessment has been carried on relatively few. As an example, rodent carcinogenicity test data available for less than 1,000 compounds in the US National Toxicology Program database. The carcinogenicity of chemicals in our environment is an important health hazard to humans. Carcinogenicity studies using rodents have long been the standard for evaluating the carcinogenic potential of chemicals [1]; however, such studies are time-consuming, expensive, and require large numbers of experimental animals. Therefore, the carcinogenic potential of many important chemicals remains

untested. In addition to the carcinogenic potential, the hepatotoxicity and nephrotoxicity of xenobiotics, which include classical drugs, herbal medicines, and chemical products, represents a significant cause of liver and kidney diseases [2, 3]. To evaluate hazards of a compound, various toxicity studies are needed, leading to problems such as a high cost and long test period in regulatory sciences. The test guideline known as the “repeated dose 28-day oral toxicity study in rodents” (TG 407) adopted by the Organization for Economic Co-operation and Development (OECD) is used mainly in Japan and Europe as a screening toxicity test. If an initial response, such as a change in a gene expression level associated with toxic effects, could be detected, a single animal study might be capable of predicting various toxicity endpoints, including long-term toxicity. Under these circumstances, the development of an efficient hazard assessment system for chemicals is needed. Moreover, the promotion of a “3Rs” policy and the development of promising *in vitro* alternative test methods, are both progressing in toxicological studies.

Omics technology, such as gene expression analyses, can be used effectively for the identification and prediction of hazards. Toxicogenomics has been established as a powerful tool for elucidating the mechanisms of chemical toxicity, such as carcinogenicity [4–6], hepatotoxicity [7, 8] and nephrotoxicity [9, 10]. However, numerous unknown pathways or gene networks that lead to toxicity exist. For a better understanding of adverse outcome pathways (AOPs) and the expansion of mode of action (MoA) applications, the elucidation of pathways/networks or biomarkers to detect or predict *in vivo* toxicity is needed.

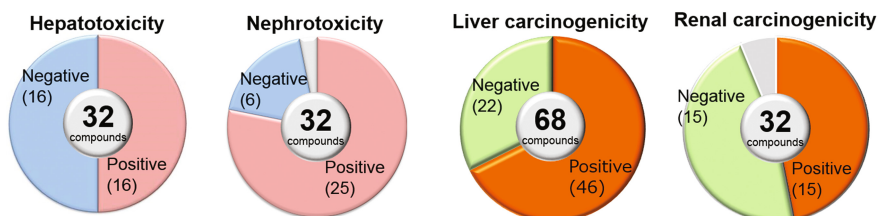
We participated in a 5-year ARCH-Tox project conducted by the Ministry of Economy, Trade and Industry (METI) in Japan with the aim of developing a new testing approach that would enable the evaluation of multiple endpoints (hepatotoxicity/nephrotoxicity, carcinogenicity and neurotoxicity) in a single 28-day repeated dose toxicity study using sets of marker genes selected based on toxicity mechanism such as MoAs or AOPs. Mechanism-based analysis using omics technology is expected to reveal new MoAs or AOPs, leading to the development of new *in vitro* assays.

Chemicals, Animal Test and Microarray Analysis

A total of 100 chemicals, consisting of 68 chemicals used in prediction systems examining hepatic carcinogenicity and 32 chemicals commonly used in prediction systems examining renal carcinogenicity and detection systems for hepatotoxicity and nephrotoxicity, were selected from among chemicals used in previous studies [11–13]. The number of test compounds used in each experiment is shown in Fig. 1a.

Four-week-old specific-pathogen-free (SPF) male Crl:CD (SD) rats and Fischer 344 (F344) rats were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The rats were treated with the test compounds in a suitable vehicle by gavage for 28 days. The animals were then sacrificed by exsanguination under anesthesia with CO₂–O₂ (4:1) or isoflurane gas inhalation 24 h after the final administration, and the livers were immediately excised and weighed. Then, the left lateral lobe of the liver was sliced and immediately placed in RNeasy[®] (Ambion, Austin, TX, USA) for RNA extraction; the remaining liver sample was submitted for histopathological examination. All the animals were treated in compliance with the applicable animal

(a) Test compounds



(b) Animal study

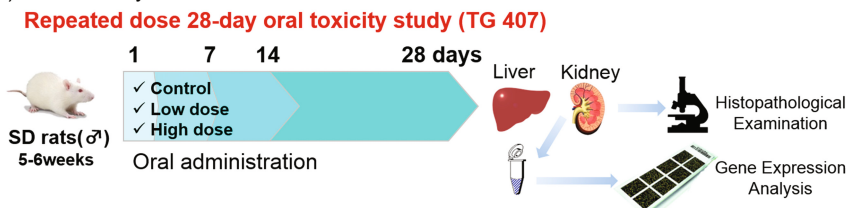


Fig. 1. Number of test compounds used in each experiment and animal study design **a** Test compounds: Sixty-eight chemicals were used to develop a prediction system for hepatic carcinogenicity, and 32 chemicals were used to develop prediction systems for renal carcinogenicity and detection systems for hepatotoxicity and nephrotoxicity. **b** Animal study: The gene expression profiles of liver and kidney tissues were detected after a 28-day repeated dose toxicity study in male Crl:CD (SD) rats

Table 1. Histopathological findings of liver (CCl₄)

Findings	Grade	0 mg/kg/day				20 mg/kg/day				100 mg/kg/day			
		D1	D7	D14	D28	D1	D7	D14	D28	D1	D7	D14	D28
Fatty degeneration, /Hepatocyte/ Centrilobular	—	4	4	4	5	3	0	0	0	0	0	0	0
	±	0	0	0	0	1	1	0	0	4	0	0	0
	+	0	0	0	0	0	1	4	5	0	1	2	2
	++	0	0	0	0	0	2	0	0	0	3	2	2
	+++	0	0	0	0	0	0	0	0	0	0	1	1
Hydropic degeneration, Hepatocyte/ Centrilobular	—	4	4	4	5	4	4	4	5	3	1	0	0
	±	0	0	0	0	0	0	0	0	1	0	0	0
	+	0	0	0	0	0	0	0	0	0	3	3	3
	++	0	0	0	0	0	0	0	0	0	0	1	2
Microgranuloma	—	4	4	4	5	4	0	0	0	4	0	0	1
	+	0	0	0	0	0	1	2	5	0	4	2	0
	++	0	0	0	0	0	3	2	0	0	0	2	4
	+++	0	0	0	0	0	0	0	0	0	0	0	1
Mitosis, increased	—	4	4	4	5	4	4	2	3	4	2	0	1
	+	0	0	0	0	0	0	1	2	0	0	3	1
	++	0	0	0	0	0	0	1	0	0	2	1	3
	+++	4	4	4	5	4	4	4	5	1	4	4	5
Single cell necrosis, Hepatocyte/ Centrilobular	—	4	4	4	5	4	4	4	5	1	4	4	5
	+	0	0	0	0	0	0	0	0	3	0	0	0

—: no remarkable, ±: mild, +: slight, ++: moderate, +++: marked.

Numbers indicate the number of animals whose histopathological findings of liver were observed in each treatment group (4 animals on days 1, 7, and 14, and 5 animals on days 28).

welfare regulations (Declaration of Helsinki [2000] and guidelines for animal experiments at CERl according to LABORATORY ANIMAL SCIENCE [1987] published by the American Association for Laboratory Animal Science). The experimental design and the results of histopathological findings is shown in Fig. 1b and Tables 1 and 2, respectively.

Table 2. Histopathological findings of kidney (cisplatin)

Findings	Grade	0 mg/kg/day				0.15 mg/kg/day				0.75 mg/kg/day			
		D1	D7	D14	D28	D1	D7	D14	D28	D1	D7	D14	D28
Single cell necrosis / Renal tube/ Cortico-medullary junction	—	4	4	4	5	4	4	4	5	4	3	2	1
	+	0	0	0	0	0	0	0	0	0	1	3	2
	++	0	0	0	0	0	0	0	0	0	0	0	2
Single cell necrosis / Renal tube/ inner medullar	—	4	4	4	5	4	4	4	5	4	4	4	2
	+	0	0	0	0	0	0	0	0	0	0	0	2
	++	0	0	0	0	0	0	0	0	0	0	0	1
Degeneration / Renal tube/ Cortex, Cortico-medullary junction	—	4	4	4	5	4	4	4	5	4	3	1	5
	+	0	0	0	0	0	0	0	0	0	1	3	0
	++	0	0	0	0	0	0	0	0	0	0	0	0
Karyomegaly / Renal tube/ Cortico-medullary junction	—	4	4	4	5	4	4	4	5	4	4	4	0
	+	0	0	0	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0	0	0	5
Karyomegaly / Renal tube/ inner medullar	—	4	4	4	5	4	4	4	5	4	4	4	2
	+	0	0	0	0	0	0	0	0	0	0	0	3
	++	0	0	0	0	0	0	0	0	0	0	0	0
Dilation / Renal pelvis	—	4	4	4	5	4	4	4	5	4	3	3	5
	+	0	0	0	0	0	0	0	0	0	0	1	0
	++	0	0	0	0	0	0	0	0	0	1	0	0
Dilation / Renal tube/ Cortico-medullary junction	—	4	4	4	5	4	4	4	5	4	4	4	0
	+	0	0	0	0	0	0	0	0	0	0	0	1
	++	0	0	0	0	0	0	0	0	0	0	0	2
Regeneration / Renal tube/ Cortico-medullary junction	—	4	4	4	5	4	4	4	4	4	4	0	0
	+	0	0	0	0	0	0	0	1	0	0	1	0
	++	0	0	0	0	0	0	0	0	0	0	3	0
Regeneration / Renal tube/inner medullar	—	4	4	4	5	4	4	4	5	4	4	4	0
	+	0	0	0	0	0	0	0	0	0	0	0	3
	++	0	0	0	0	0	0	0	0	0	0	0	2

—: no remarkable, +: slight, ++: moderate, +++: marked.

Numbers indicate the number of animals whose histopathological findings of kidney were observed in each treatment group (4 animals on days 1, 7, and 14, and 5 animals on days 28).

Total RNA was extracted from the liver samples using QIAzol (Qiagen, Hilden, Germany) and the RNeasy Mini Kit or miRNeasy Mini Kit (Qiagen), in accordance with the manufacturer’s protocol. The quality of the RNA samples was examined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and undegraded RNA samples were used for the experiments; for this study, we used RNA samples with RIN values of > 7.0 as an index of the high purity and integrity of the RNA samples.

Microarray analysis was performed as described previously [12]. Briefly, three types of custom arrays, Toxarray III ver.2 and Agilent Whole Rat Genome Microarrays 8 × 60 K Toxplus ver.1 and ver.2, and the gene-expression-based carcinogenicity prediction system CARCINOscreen® were used for the microarray analysis. Global normalization was applied to one-color microarray data using GeneSpring GX 10 (Agilent Technologies). Lowess normalization was applied to two-color microarray data using Feature Extraction Software 9.5.3.1 (Agilent Technologies). The signal log₂ ratio of the administration group vs. the vehicle control group was calculated using the mean normalized signal intensity in each group. The pathway or functional analysis for the DNA microarray data was performed using Ingenuity Pathways Analysis (IPA) software (Qiagen).

AOP-Based Mechanism of Hepatotoxicity Suggested by Case Study with Carbon Tetrachloride

The liver has long been considered the major target organ for most of the chemicals implicated in eliciting toxic effects following environmental exposure. Hepatotoxicity represents a major regulatory issue, and the pathophysiological mechanisms of hepatotoxicity are still being explored and include both hepatocellular and extracellular mechanisms. We investigated the mechanism of hepatotoxicity induced by carbon tetrachloride (CCl₄), which is a well-known hepatotoxin. CCl₄ reportedly damages liver cell mitochondria and causes the failed transport of fatty acids as phospholipids [14]. We attempted to create an AOP for liver fibrosis induced by CCl₄ using gene expression data and histopathological data obtained in our studies as well as previously reported information [14]. A previous study reported that CCl₄ was biotransformed by the cytochrome P450 system in the endoplasmic reticulum to produce trichloromethyl free radical (CCl₃·) [15]. This CCl₃· then combined with cellular lipids and proteins to form trichloromethyl peroxy free radical, which attacks lipids on the membrane of the endoplasmic reticulum as a molecular initiating event (MIE). Thus, trichloromethyl peroxy free radical is thought to lead to lipid peroxidation [15]. In the results of our case study using CCl₄, Cyp2c12 and Cyp4f5 were upregulated and cholesterol biosynthesis appeared to be activated, while fatty acid β-oxidation appeared to be downregulated in association with a 1-day treatment with CCl₄. A functional analysis using IPA software of significantly downregulated genes in the liver after the administration of CCl₄ showed that these genes were strongly correlated with fatty acid metabolism, transport of lipid and cleavage of lipid became with the severity depending on the administration period (Fig. 2). After 7 days of administration or thereafter, the significantly upregulated genes were strongly correlated with increases in the synthesis of DNA, DNA replication and chromosomal congression as well as the p63 signaling pathway and the G2/M DNA damage checkpoint pathway (data not shown). Histopathologically, fatty degeneration and centrilobular hydropic degeneration were observed by macroscopic examination on the first day of administration. Furthermore,

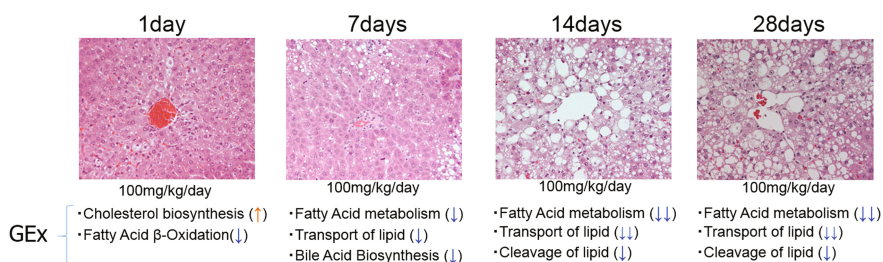


Fig. 2. Histopathological changes and functional analysis of DNA microarray data obtained after the oral administration of carbon tetrachloride (CCl₄). The *red* and *blue* arrows indicate a significant functional analysis using IPA software for the up- and downregulated genes, respectively. The number of arrows shows the degree of relevance of these function and gene expression changes

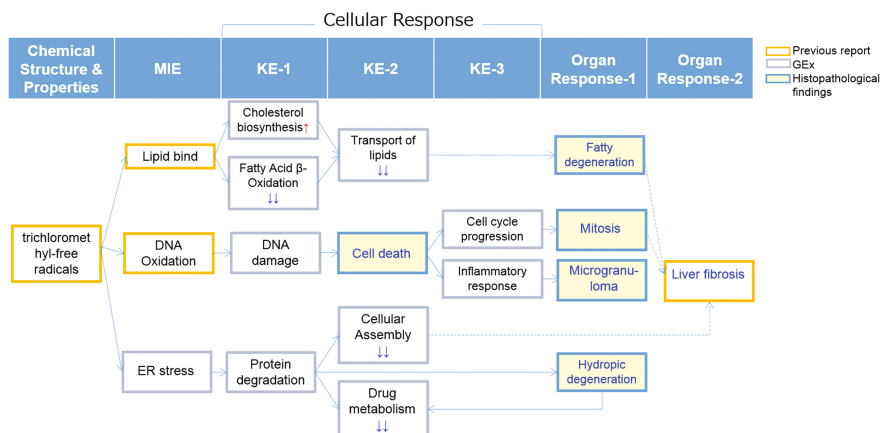


Fig. 3. AOP-based mechanisms of hepatotoxicity of carbon tetrachloride (CCl₄). MIE: molecular initiating event, KE: key event, GEX: Gene expression data. The red and blue arrows indicate the significance of a functional analysis using IPA software for the up- and downregulated genes, respectively. The number of arrows shows the degree of relevance of these function and gene expression changes

microgranuloma, mitosis and single cell necrosis in the centrilobular area were observed, and the degree of severity increased with the dose and administration period (Table 1). We constructed AOP-based hepatotoxicity mechanisms of CCl₄ using these multifaceted considerations, and the mechanism map is shown in Fig. 3.

AOP-Based Mechanism of Nephrotoxicity Suggested by Case Study with Cisplatin

Recent studies have demonstrated that the kidney is also an important target of injury after chemical exposure, although substantial gaps in knowledge remain regarding the effects of environmental chemicals on specific aspects of kidney function [16, 17]. Cisplatin is a potent anticancer drug that is widely used in chemotherapy. However, adverse effects in normal tissues and organs, notably nephrotoxicity in the kidneys, limit the use of cisplatin and related platinum-based therapeutics. Recent research has shed significant new light on the mechanism of cisplatin nephrotoxicity, especially on the signaling pathways leading to tubular cell death and inflammation [18]. As a case study of nephrotoxicity, we administered cisplatin to male rats for 28 days; kidney samples were then obtained and anatomically separated into the papilla, inner medulla, outer medulla, and cortex, which have different structures and functions, and gene expression analyses were performed for each of these renal anatomic regions, since the marked morphological, functional and biochemical heterogeneity of the kidney accounts for the site-specific toxicity of several drugs and xenobiotics [19]. In our

previous DNA microarray study, no significant variations for each renal anatomic region were seen between the left and right kidneys or among individuals (data not shown). Nevertheless, the gene expression profiles differed in each renal anatomic region, and the DNA microarray data of the outer medulla and cortex were used to analyze the nephrotoxicity of cisplatin. The major focus in renal damage research is on proximal tubule toxicity, where the majority of the reabsorption of drug metabolites occurs, and the proximal tubules of the nephron in animals including the proximal convoluted tubules, which are situated in the cortical labyrinth and are connected directly to the proximal straight tubules in the inner cortex and outer stripe of the outer medulla [19]. Cisplatin has been suggested to produce reactive oxygen species (ROS) via NADPH oxidase activation [20]. ROS are highly reactive molecules that can damage cell structures such as carbohydrates, nucleic acids, lipids, and proteins and alter their functions [21]. In this gene expression data, *Nrf2*, *Gpx2*, *Ho-1*, *Scarb1*, *Gstm3*, and *Mgst2*, which are concerned with the NRF2-mediated oxidative stress response, were significantly upregulated, supporting the MIE of cisplatin, *i.e.* the oxidation of DNA, proteins, lipids, and co-factors (data not shown). A functional analysis using IPA software of significantly upregulated genes in the outer medulla of the kidney after the administration of cisplatin showed that these genes were strongly correlated with cell death and survival, inflammatory disease, cellular growth and proliferation, organismal injury and abnormalities, and apoptosis (data not shown). The downregulated genes were involved in amino acid metabolism, lipid metabolism, vitamin and mineral metabolism, drug metabolism, and molecular transport, which is involved in basic renal function (data not shown). In particular, many genes expressed in the outer medulla and cortex related to oxidative phosphorylation, were downregulated, resulting in mitochondrial dysfunction (Fig. 4). The proximal tubule of the kidney has three morphologically distinct segments, S_1 , S_2 , and S_3 , which can be distinguished as the *pars convoluta* and the *pars recta* of the proximal tubule [22]. Epithelial cells in the S_1 segments possess a tall brush border, a well-developed vacuolar lysosomal system, and many long mitochondria that fill the basal portion of the cell. The S_2 segments are not as tall as the S_1 segments. The S_3 cells have rare apical vacuoles and fewer and smaller mitochondria than the S_1 and S_2 cells [22]. These observations suggest that the administration of cisplatin leads to kidney injury and abnormalities. Histopathologically, after 7 days of administration or longer, single cell necrosis of the proximal tubule in the cortico-medullary junction or the inner medulla was observed microscopically. Furthermore, degeneration, karyomegaly, dilation, and regeneration were observed, and the degree of severity increased with the dose and administration period (Table 2). We constructed an AOP-based hepatotoxicity mechanism for cisplatin using these multifaceted considerations, as shown in Fig. 5.

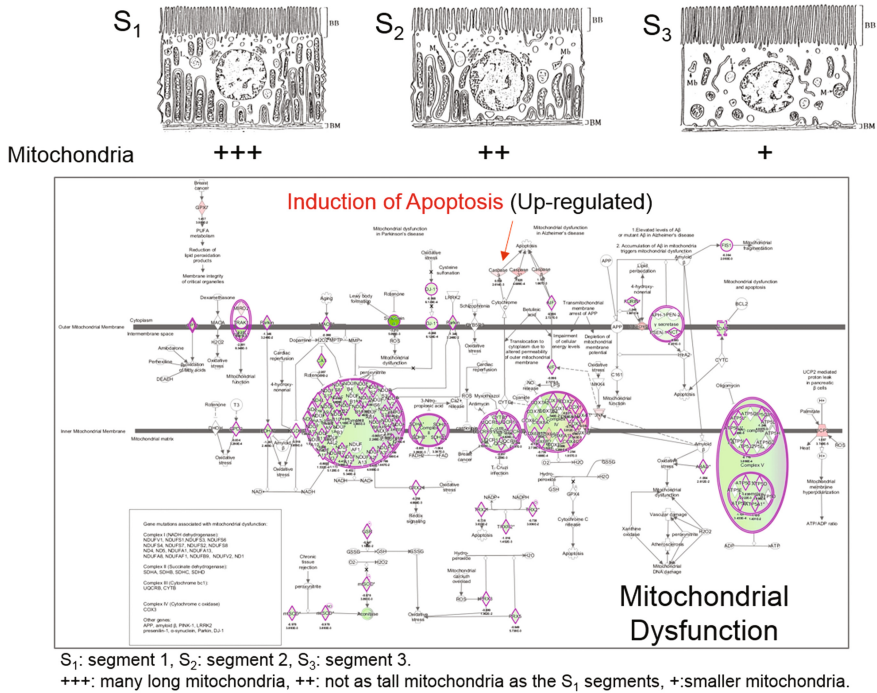


Fig. 4. An example of a pathway analysis of DNA microarray data obtained after the intraperitoneal administration of cisplatin. The red and green colored objects indicate the up- and downregulated genes, respectively

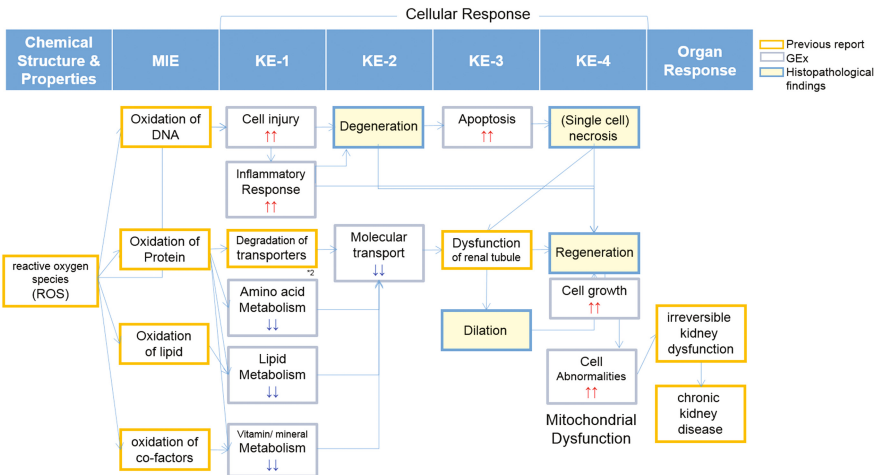


Fig. 5. AOP-based mechanisms of nephrotoxicity of cisplatin. MIE: molecular initiating event, KE: key event. The red and blue arrows indicate the significance of a functional analysis using IPA software for the up- and downregulated genes, respectively. The number of arrows shows the degree of relevance of these function and gene expression changes

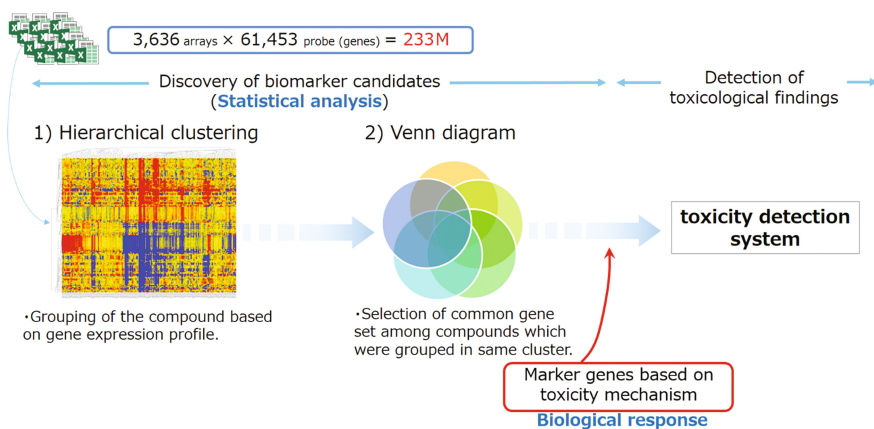


Fig. 6. Strategy of a detection system for hepato- and nephrotoxicity. To discover biomarker candidates, microarray data was analyzed using hierarchical clustering to group compounds based on gene expression profiles, and common gene sets among the compounds that were grouped in the same cluster were selected and used in a Venn diagram. Furthermore, marker genes based on toxicity mechanisms were selected based on the results of an AOP-based mechanisms analysis of hepato- and nephrotoxicity, and toxicity detection systems were constructed for each toxicological finding

Table 3. Toxicological findings and number of detection genes for hepato- and nephrotoxicity

Organ	Toxicological findings		Detection genes
Liver	Centrilobular	Fatty degeneration	8
	Periportal		36
	–	Cell death	14
	Centrilobular	Hypertrophy	24
	Diffuse		18
Kidney	Proximal tubule	Vacuolization	3
	Proximal tubule	Anisonucleosis	4
	Proximal tubule	Pyknosis	4
	Proximal tubule	Cell death	10
	Papilla	necrosis	9

Detection System for Hepato- and Nephrotoxicity

We attempted to develop a detection system for hepato- and nephrotoxicity using DNA microarray data; the strategy used to construct the detection system is shown in Fig. 6. We focused on frequently listed toxicity findings in the Hazard Evaluation Support System Integrated Platform database (HESS-DB: <http://www.nite.go.jp/en/chem/qsar/hess-e.html>), which contains information on toxicity and metabolism released in Japan. We then chose five toxicological findings for each toxicity: centrilobular fatty

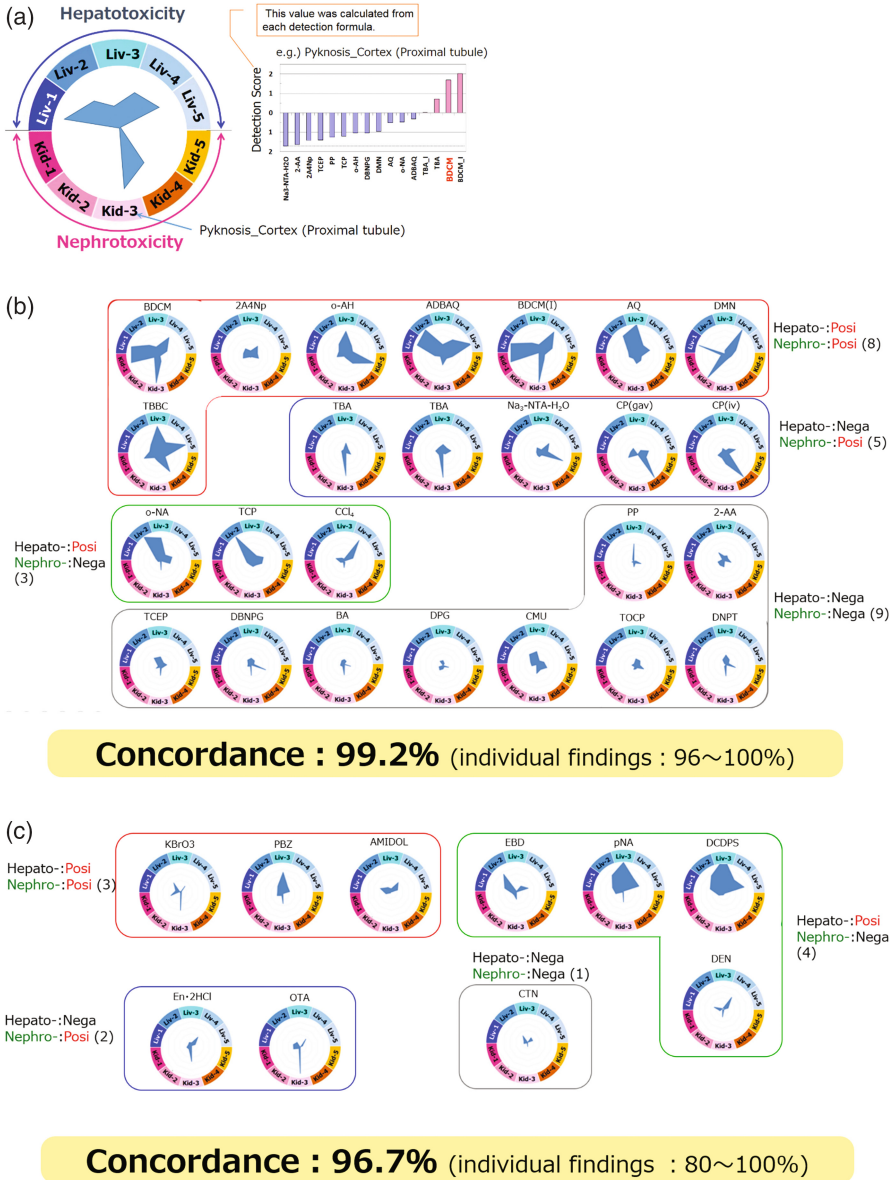


Fig. 7. Detection results for hepato- and nephrotoxicity. **a** Radar chart model of the detection system: The detection score was calculated using a support vector machine with the detection genes. Each detection score for the five toxicological findings in the liver and kidney was plotted in the upper and lower areas of the radar chart, respectively. **b** Results of training data (25 tests/22 compounds), **c** Results of validation data (10 tests/10 compounds): Liv-1: centrilobular fatty degeneration, Liv-2: periportal fatty degeneration, Liv-3: cell death, Liv-4: centrilobular hypertrophy, Liv-5: hypertrophy (diffuse). Kid-1: vacuolization of proximal tubule, Kid-2: anisonucleosis of proximal tubule, Kid-3: pyknosis of proximal tubule, Kid-4: cell death of proximal tubule, Kid-5: necrosis of papilla

degeneration, periportal fatty degeneration, cell death, centrilobular hypertrophy, and hypertrophy (diffuse) for hepatotoxicity, and vacuolization of the proximal tubule, anisonucleosis of the proximal tubule, pyknosis of the proximal tubule, cell death of the proximal tubule, and necrosis of the papilla for nephrotoxicity. The detection formula was generated using a support vector machine with detection genes selected from 22 training chemicals (25 tests) datasets, and a predictive score was then calculated to detect the hepato- or nephrotoxicity potentials of the tested chemicals. The detection genes were selected for each toxicological finding: 8–36 genes for hepatotoxicity and 3–10 genes for nephrotoxicity (Table 3). The potential score for each toxicological finding was shown in a radar chart model that allowed the visualization of multiple toxicity findings at a glance (Fig. 7a). In the training data, the potential score for each toxicological finding was 96%–100%, resulting in a 99.2% total concordance (Fig. 7b). In the validation data for ten chemicals, the potential score for each toxicological finding was 80%–100%, resulting in a 96.7% total concordance (Fig. 7c).

Prediction System for Hepatic and Renal Carcinogenicity: CARCINOscreen[®]

Carcinogenicity is one of the most serious toxic effects of chemicals, and highly accurate methods for predicting carcinogens are strongly desired for the assessment on human health. We previously developed a prediction system named “CARCINOscreen[®]” for evaluating the carcinogenic potentials of chemicals using the gene expression profiles of liver tissues from rats after a 28-day repeated dose toxicity study [12]. The prediction formula was generated using a support vector machine with predictive genes selected from 68 training chemical datasets; a predictive score was then calculated to predict the carcinogenic potentials of the tested chemicals. To ensure the accuracy of the prediction system, the chemicals were divided into three groups (Groups 1 to 3) according to the resulting hepatic gene expression profiles, and a prediction formula was generated for each group. The prediction system was capable of predicting the carcinogenicity of the training carcinogens and the non-carcinogens with an accuracy of 92.9%–100%. The final prediction result was determined based on the maximum prediction value obtained with three independent prediction formulas to establish the CARCINOscreen[®]. The system was able to accurately predict carcinogenicity in rats in 94.1% of the 68 training chemicals [12]. Furthermore, we attempted to develop a quantitative PCR (qPCR)-based system as an alternative to the microarray-based CARCINOscreen[®] [23]. The prediction accuracies of the qPCR-based alternative for training- and validation-phase trials were 82.8% and 86.4%, respectively [23].

Recently, we reported a renal carcinogenicity prediction system to predict chemical carcinogenicity in rats; a 28-day repeated-dose test was performed using male Crl:CD (SD) rats with 12 carcinogens and 10 non-carcinogens as the training dataset and five carcinogens and five non-carcinogens as the validation dataset [13]. In this prediction system, the prediction accuracies for the training and the validation datasets were calculated to be 100% and 90%, respectively, while 4-hydroxy-*m*-phenylenediammonium dichloride (AMIDOL), a known non-renal carcinogen, was judged as being positive. Among the predictive genes, *Hamp* and *Ranbp1* are known to be important

for cell growth and cell cycle regulation, which are important events in carcinogenesis. Given our current limited knowledge of the genes responsible for renal carcinogenesis, the identification of candidate genes for chemical-induced renal carcinogenicity using this gene expression-based prediction method represents a promising advance in renal carcinogen identification [13].

Concluding Remarks

In hepatotoxicity and nephrotoxicity, marker genes can be selected based on toxicity mechanisms such as MoA or AOP, enabling a detection accuracy of more than 90% for five kinds of toxicity findings in both the liver and kidney. For carcinogenicity, the CARCINOscreen[®] system predicted the carcinogenic potential of a training compound set that included non-carcinogens with a more than 90% accuracy for the liver and kidney. Furthermore, we developed a qPCR-based prediction system as an alternative to the microarray-based CARCINOscreen[®] for rat liver carcinogenicity. The prediction performance of the qPCR-based CARCINOscreen[®], as well as its user-friendliness and cost effectiveness, suggests that this method is promising for application in primary health hazard assessments. These results suggested that omics technology, such as gene expression analysis, can be used effectively for hazard identification and prediction. From now on, the application of urine and blood samples, which are non- or semi-invasive to animals, might be more important as a contribution to the 3Rs policy. Blood and urine samples are used in metabolomics and proteomics approaches with a high frequency, and these techniques may also be powerful tools for the identification of toxicity mechanisms and to resolve issues in which changes in gene expression levels are not always correlated with the phenotypes.

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