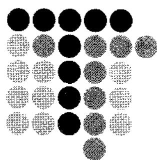

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Analysis of Gene Expression in Carcinogen-induced Acute Hepatotoxicity

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Accepted 29 January 2009

Abstract

The 2-year rodent carcinogenicity test involves long-term, repetitive dosing of animals that is both time consuming and expensive. Alternative approaches have been attempted using specific transgenic or knockout mice or toxicogenomics to predict carcinogenicity without conducting a 2-year rodent test. In addition, toxicogenomic analysis of carcinogen-treated animals could also enhance our understanding of molecular mechanisms and aid in the diagnosis of acute toxicity induced by carcinogens. Therefore, we investigated transcription profiles after administering the carcinogens 4,4-dimethylformamide (DMF) and 4-biphenylamine (ABP). BALB/c male mice were treated once with DMF (650 mg/kg i.p.) or ABP (120 mg/kg p.o.). Standard blood biochemistry and histological changes were observed. Gene expression profiles in the livers of mice treated with either vehicle or the carcinogens were analyzed using the Affymetrix GeneChip® assay. In all, 1,474 differentially expressed genes in DMF- or ABP-treated mice were identified as being either up- or down-regulated over 1.5-fold ($P<0.01$), and these genes were analyzed using hierarchical clustering and Ingenuity Pathways Analysis. Of these, 107 genes were consistently regulated in both carcinogen-treated groups. Genes associated with cancer were upregulated (*Por, S100a10, Tes, Ctcf, Ddx21, Eapp, Ncl,* and *Pa2g4*) or downregulated (*Cbs* and *Gch1*). Toxicological function analysis also identified genes involved in organ toxicity, including hepatotoxicity. These data may help to identify molecular markers for acute hepatotoxicity induced by carcinogens.

Keywords: 4,4-Dimethylformamide, 4-Biphenylamine, Carcinogen, Gene expression profiling

To assess the carcinogenicity of pharmaceutical agents in non-clinical studies, the 2-year rodent carcinogenicity test is usually performed[1]. Due to its expensive and time-consuming nature, the 2-year rodent test acts as a rate-limiting step in non-clinical studies for drug development. Furthermore, the increasing amounts of chemical carcinogens in the environment have become a national health concern. Consequently, efforts have been made to develop alternative assays to assess carcinogenicity, such as toxicogenomic approaches.

To date, many investigations have attempted to assess toxicity or elucidate the mechanism of toxicity using microarray analysis[2-5], and several studies have reported the ability to predict the carcinogenicity of genotoxic or non-genotoxic carcinogens in rodent models[6-8]. Ellinger-Ziegelbauer *et al.*[7,8] identified genes regulated by four non-genotoxic carcinogens (methapyrilene, diethylstilbestrol, Wy-14643, and piperonylbutoxide) and four genotoxic carcinogens [dimethylnitrosamine, 2-nitrofluorene, aflatoxin B1, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone] in short-term *in vivo* studies lasting 14 days. Although several attempts have been made to identify early molecular markers of carcinogens using microarrays, little is known regarding the early effects of carcinogens on gene expression.

In this study, we described global gene expression patterns in the liver in response to treatment with 4,4-dimethylformamide (DMF) or 4-biphenylamine (ABP). Our objectives were to identify acute molecular markers of carcinogens and understand the molecular mechanism of carcinogen-induced hepatotoxicity. DMF is a common solvent that is used in the manufacture of fibers and plastics, and in the development of pesticides and pharmaceutical products. Chronic exposure to DMF is reported to lead to hepatotoxicity[9-11], gastric irritation[12], and carcinogenesis[13,14]. ABP is a tobacco

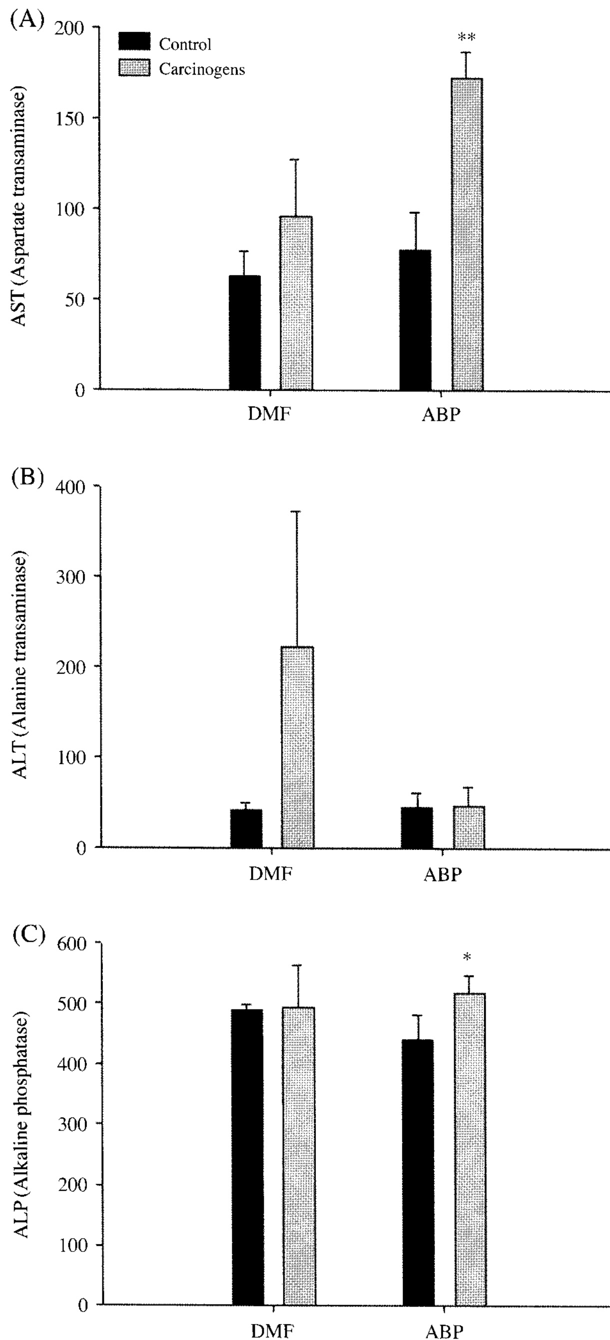

Figure 1. Serum biochemical data for the DMF- and ABP-treated mice. (A) AST, aspartate transaminase; (B) ALT, alanine transaminase; (C) ALP, alkaline phosphatase. Values represent the means ± standard deviation (SD); $n=3$ (* $P<0.05$; ** $P<0.01$).

smoke constituent and an environmental contaminant. ABP is a genotoxic carcinogen that forms DNA adducts; in contrast, the genotoxicity of DMF remains controversial. Humans can be exposed to ABP via smoking, diesel exhaust, or cooking oil fumes, and several studies have reported that DNA adducts cause cancers, such as bladder cancer[15-18]. However, little is known regarding the early transcriptional changes induced by DMF and ABP in mouse models. Here, we analyzed hepatic gene expression profiles in mice treated with DMF or ABP, with the objective of identifying molecular markers related to carcinogen-induced acute hepatotoxicity.

Blood Biochemistry and Histopathology

As shown in Figure 1, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities were measured in serum collected from DMF- and ABP-treated mice. DMF treatment caused 1.5- and 5.2-fold increases in AST and ALT levels, respectively, whereas no change was observed in ALP activity. After ABP treatment, AST levels increased significantly, ALP levels increased slightly, and ALT levels remained unchanged. Histopathological analysis showed that the DMF and ABP treatments produced discernable hepatic pathology (Figure 2). In the livers of DMF-treated mice, cytoplasmic basophilia and centrilobular hypertrophy were observed, whereas hepatocyte vacuolation was observed in ABP-treated mice. These results indicate that the administration of DMF and ABP caused acute hepatotoxicity in mice.

Microarray Analysis

To elucidate the molecular mechanism of carcinogen-induced acute toxicity, gene expression profiles in the livers of DMF- and ABP-treated mice were analyzed using the Affymetrix GeneChip® system and compared to an untreated control group. Each dataset consisted of an expression array from three animals. In total, 1,474 genes, including 823 genes for DMF and 758 genes for ABP, showed greater than 1.5-fold changes in expression ($P<0.01$, two-tailed, unpaired Welch's t-test) compared to the control. Hierarchical clustering showed that samples corresponding to each group clustered closely (Figure 3). Using these gene expression patterns, gene sets could be classified as DMF-specific, ABP-specific, or a group regulated in common. We focused on the genes regulated in common in both the DMF- and ABP-treated groups.

In a classification based on hierarchical clustering, 107 genes with altered expression profiles were identified in both the DMF- and ABP-treated groups. For the 48 upregulated and 59 downregulated genes, the top 20 most markedly modulated genes were selected and are shown in Tables 1 and 2. The genes *Tnfrsf12a, Tes, S100a10, Crp2b10,* and *Pgd* were upregulated (Table 1), whereas the genes *Upp2, 2010305C02Rik,*

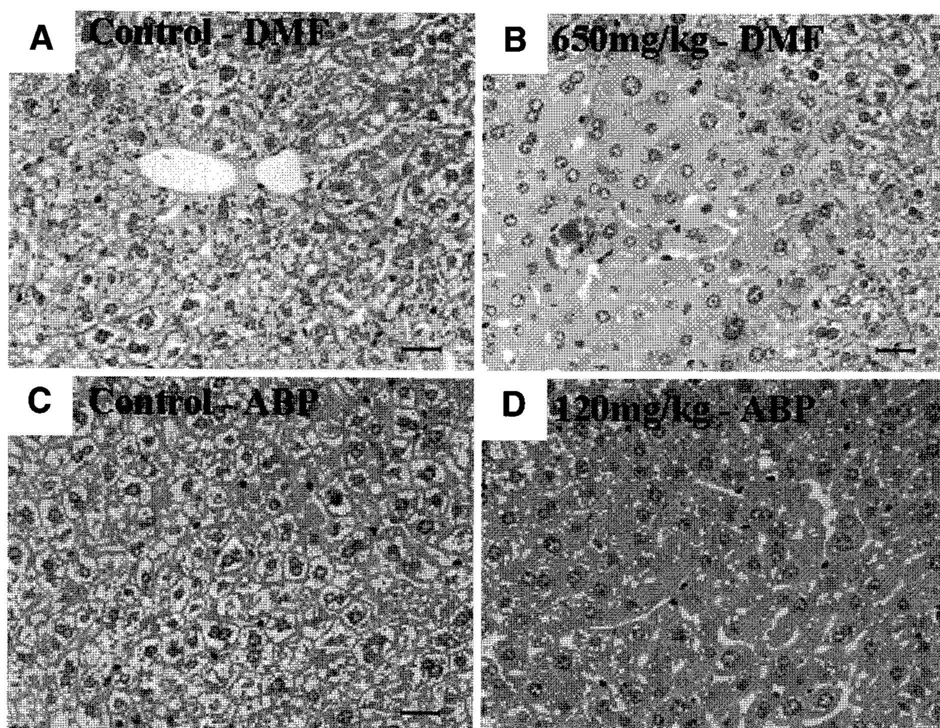

Figure 2. Light microscopic observation of the livers from DMF- and ABP-treated mice. (A) Control, saline; (B) DMF, 600 mg/kg; (C) Control, corn oil; (D) ABP, 120 mg/kg.

Aldoc, and *Otc* were downregulated in the carcinogen-treated groups (Table 2).

Functional Classification of Differentially Expressed Genes

Biological functional analysis of the 107 genes modulated in response to either carcinogen revealed that genes involved in small molecule biochemistry, cellular growth and proliferation, the cell cycle, cell death, free radical scavenging, and DNA replication were both up- and downregulated, whereas genes involved in amino acid metabolism, cell signaling, and protein synthesis were downregulated only (Figure 4A). Within these categories, small molecule metabolism included the upregulated genes *Cyp2b10, Eif6, Pgd, Pik3c2*, and *Ppap2c*, and the downregulated genes *BC089597, Cbx, Cxcl12, Cyb5r3, Dpyd, Gch1*, and *Il18*. In the cell death category, *Por* was upregulated and *Il18, Cyb5r3*, and *Cxcl12* were downregulated in the carcinogen-treated groups.

Toxicological functional analysis identified genes involved in hepatotoxicity (*i.e.*, liver proliferation and hepatocellular carcinoma), nephrotoxicity (*i.e.*, renal thrombosis, renal damage, and kidney failure), and cardiotoxicity (*i.e.*, cardiac hypertrophy, cardiac hemorrhage, and cardiac stenosis; Figure 4B). Many of the downregulated genes were involved in organ toxicity. Looking at specific organs, *Tnfrsf12a* and *Dpyd* were involved in hepatotoxicity, *C2* and *Il18* were involved in nephrotoxicity, and *S100a10, Il18, F7, Proz*, and *Klf15* were involved in cardiotoxicity.

Validation of Gene Expression Changes using Quantitative Real-time PCR

To validate the transcriptional level of differentially expressed genes, we selected highly deregulated genes in both of the carcinogen-treated groups and performed real-time RT-PCR on the liver total RNA used in the microarray analysis. To analyze changes in gene expression in cancer-related genes during carcinogen-induced acute toxicity, we selected differentially expressed cancer-related genes in both carcinogen-treated groups using the functional analysis mode within the Ingenuity Pathways Analysis software application (Table 3).

As shown in Figure 5A, all of the selected genes (*Tnfrsf12a, Tes, S100a10, Cyp2b10, Ppap2c, Ktn1, Vnn1, Apoa4, Itgb4bp*, and *Tbrg1*) were upregulated in the DMF- and ABP-treated groups. *Tnfrsf12a* and *Tes* were predominantly upregulated in the DMF-treated group compared to the ABP-treated group, whereas *Cyp2b10* and *Apoa4* were more markedly upregulated in the ABP-treated group. The expression of downregulated genes (*Upp2, Otc, Lvd, Klf15, Agxt, Ethe1, Sult1c2, Acss2, Cml1*, and *Amdhd1*) was low in both carcinogen-treated groups (Figure 5B). As shown in

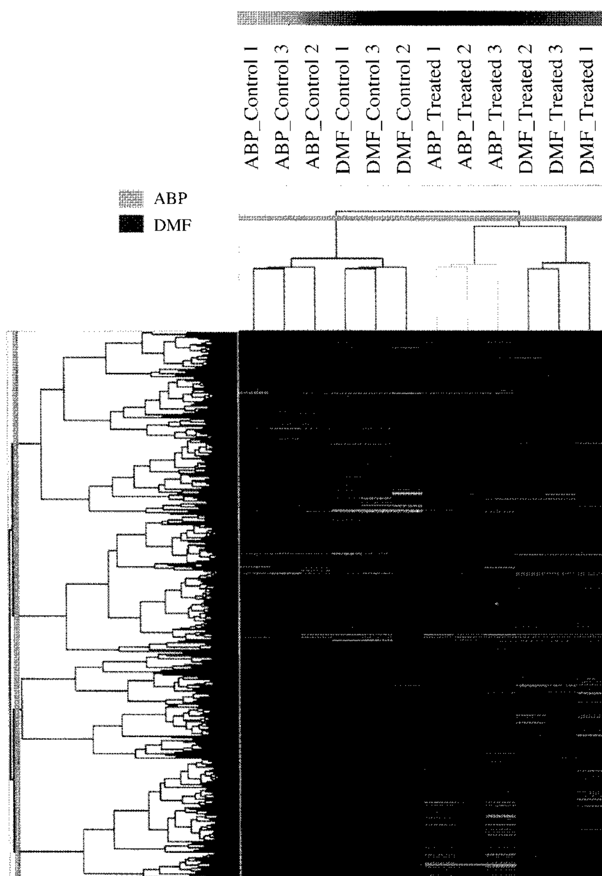

Figure 3. Hierarchical clustering of differentially expressed genes in the livers of DMF- and ABP-treated mice.

Figure 5C, *Por, Ctcf, Ddx21, Eapp, Ncl*, and *Pa2g4* were upregulated, whereas *Cbs* and *Gchl* were downregulated in the DMF- and ABP-treated groups. The cancer-related genes *Tes* and *S100a10* were also upregulated (Figure 5A).

Discussion

Recently, toxicological mechanisms and drug safety have been investigated using toxicogenomic tools[19-22]. Several studies have shown that the prediction of toxicity is possible using toxicogenomics, especially hepatotoxicity[23-25]. In the assessment of carcinogenicity, the toxicogenomic approach has also been used to interpret the mode of action of carcinogens and predict their toxic effects[6-8]. We examined the acute effects of two hepatotoxic carcinogens, DMF and ABP, on gene expression in the mouse liver.

Some studies have reported that DMF treatment elevates serum markers of hepatotoxicity, showing that the liver is a primary target organ[9,10]. Biochemical and histopathological analysis indicated that mild hepatotoxicity occurs 24 h after administering DMF and ABP. These results showed that a single dose of DMF (650 mg/kg) or ABP (120 mg/kg) is sufficient to induce acute hepatotoxicity, although the pathological observation of carcinogenicity requires long-term exposure to these chemicals. In the microarray analysis, hierarchical clustering distinguished clusters that were chemical-specific and regulated in common. Genes belonging to a chemical-specific cluster are likely involved in chemical-specific metabolism or toxicity in the liver. Among all differentially expressed genes, we focused on the transcriptional modulation of genes regulated in both the DMF- and ABP-treated groups. Functional analysis showed that genes related to small molecule metabolism were upregulated in both carcinogen-treated groups. *Cyp2b10*, a member of the cytochrome P450 family, was upregulated in both the DMF- and ABP-treated groups. DMF is metabolized *in vitro* by the P450 2E1 isozyme[26], although our microarray analysis showed that the level of *Cyp2e1* transcription was unchanged. ABP is oxidized primarily by the cytochrome P450 1A2 isozyme in the liver and then metabolized sequentially by many enzymes to form DNA adducts[27]. *Cyp2b10* is a non-specific monooxygenase belonging to the *Cyp2b* family that catalyzes xenobiotics, such as cyclophosphamide[28], in the process of drug metabolism. Its involvement in the metabolism of DMF or ABP has not been reported. In contrast, *Tnfrsf12a*, which is related to hepatotoxicity, was highly upregulated in both the DMF- and ABP-treated groups. *Tnfrsf12a* is involved in angiogenesis, cell motility, and apoptosis, and causes the proliferation of oval cells in the liver[29]. Among the downregulated genes, *Upp2* was markedly downregulated in both carcinogen-treated groups. *Upp2* is a member of the uridine phosphorylase family and is involved in pyrimidine metabolism (*i.e.*, nucleoside metabolism). The function of *Upp2* related to metabolism induced by carcinogens has not been reported.

The functional analysis of differentially expressed genes identified cancer-related genes such as *Tes, Por, S100a10, Ctcf, Ddx2l, Eapp, Ncl, Pa2g4, Cbs*, and *Gch1*. The verification of the transcriptional levels of selected genes showed that the expression patterns detected via microarray analysis corresponded well with those determined via quantitative real-time PCR. Studies using *Tes* knockout mice showed that mutant *Tes* expression is associated with the increased incidence of tumors[30]. Although *Tes* upregulation in response to carcinogens or toxins has not been reported, residual *Tes* expression has been noted in several cancer cell lines. *Por* is involved in cytotoxicity and cell

Table 1. Genes upregulated in the livers of both DMF- and ABP-treated mice.

Gene symbol	Gene title	Fold change (Log2)	
		ABP	DMF
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a	2.3	6.3
Tes	testis derived transcript	1.1	3.3
S100a10	S100 calcium binding protein A10 (calpactin)	1.1	2.5
Cyp2b10	cytochrome P450, family 2, subfamily b, polypeptide 10	3.1	2.3
Pgd	phosphogluconate dehydrogenase	0.9	2.1
Lrrfip1	leucine rich repeat (in FLII) interacting protein 1	0.8	1.8
Ppap2c	phosphatidic acid phosphatase type 2c	1.3	1.8
Ncl	nucleolin	0.9	1.6
Ktn1	kinectin 1	1.1	1.5
Ncl	nucleolin	0.8	1.5
Vnn1	vanin 1	1.6	1.5
Nola2	nucleolar protein family A, member 2	0.6	1.5
Apoa4	apolipoprotein A-IV	2.7	1.4
Itgb4bp	integrin beta 4 binding protein	1.3	1.4
Xpo1	exportin 1, CRM1 homolog (yeast)	0.6	1.4
Pkp2	plakophilin 2	0.7	1.4
Ddx39	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	0.6	1.2
Tbrg1	transforming growth factor beta regulated gene 1	1.2	1.2
Tmem62	transmembrane protein 62	1.1	1.2
Gas5	growth arrest specific 5	1.2	1.2

The fold change was calculated as the relative average value of three arrays in each group compared to controls, and the values are reported as the natural logarithm.

Table 2. Genes downregulated in the livers of both DMF- and ABP-treated mice.

Gene symbol	Gene title	Fold change (Log2)	
		ABP	DMF
Upp2	uridine phosphorylase 2	−3.5	−4.7
2010305C02Rik	RIKEN cDNA 2010305C02 gene	−2.2	−2.7
Aldoc	aldolase 3, C isoform	−2.0	−2.4
Otc	ornithine transcarbamylase	−2.0	−2.4
Ivd	isovaleryl coenzyme A dehydrogenase	−1.8	−1.9
Klf15	Kruppel-like factor 15	−1.8	−1.9
Agxt	alanine-glyoxylate aminotransferase	−1.8	−1.9
Ethe1	ethylmalonic encephalopathy 1	−1.6	−1.8
2810439F02Rik	RIKEN cDNA 2810439F02 gene	−1.6	−1.7
Sult1c2	sulfotransferase family, cytosolic, 1C, member 2	−1.6	−1.7
Acss2	acyl-CoA synthetase short-chain family member 2	−1.6	−1.7
Ropn1l	ropporin 1-like	−1.6	−1.7
Qdpr	quininoid dihydropteridine reductase	−1.5	−1.7
Hrasls3	HRAS like suppressor 3	−1.5	−1.7
Cml1	camello-like 1	−1.5	−1.6
Ivd	isovaleryl coenzyme A dehydrogenase	−1.4	−1.6
Cyp4b1	cytochrome P450, family 4, subfamily b, polypeptide 1	−1.4	−1.5
Amdhd1	amidohydrolase domain containing 1	−1.4	−1.5
Aldh1l1	aldehyde dehydrogenase 1 family, member L1	−1.4	−1.5
Cbs	cystathionine beta-synthase	−1.5	−1.3

The fold change was calculated as the relative average value of three arrays in each group compared to controls, and the values are reported as the natural logarithm.

survival. The anticancer drug doxorubicin, which has been tested in phase II clinical trials for leukemia and gastric and ovarian cancers, inhibits human POR protein[31]. In our study, *Por* was upregulated soon after the administration of DMF and ABP. The expression of *Pa2g4* was also upregulated in the carcinogen-treat-

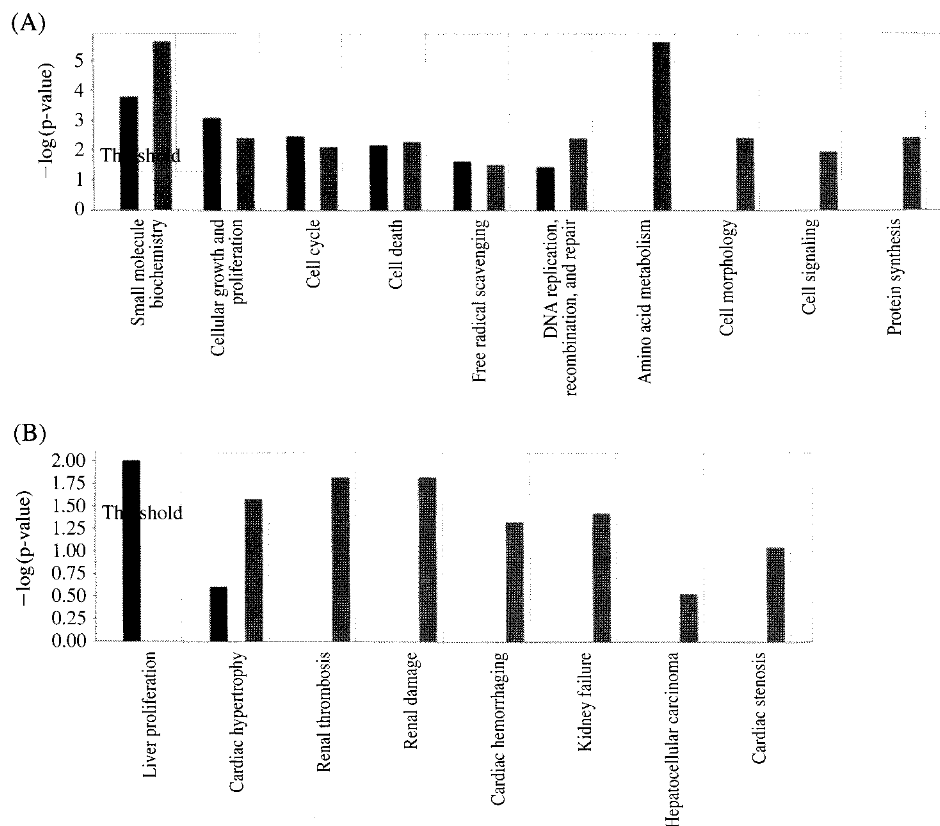

Figure 4. Functional analysis of 107 genes deregulated in both carcinogen-treated groups. (A) Biological functional analysis showing categories of interest. (B) Toxicological analysis showing genes involved in hepatotoxicity, nephrotoxicity, and cardiotoxicity. The dotted orange line indicates the threshold of statistical significance ($P<0.05$). The dark blue and light blue bars in the histogram indicate the up- and downregulated genes in the carcinogen-treated groups, respectively.

Table 3. Cancer-related genes in the livers of DMF- and ABP-treated mice.

Gene symbol	Gene title	Fold change (Log2)	
		ABP	DMF
Por	P450 (cytochrome) oxidoreductase	1.8	1.1
S100a10	S100 calcium binding protein A10 (calpactin)	1.1	3.3
Tes	testis derived transcript	1.1	2.5
Ctcf	CCCTC-binding factor	0.7	0.7
Ddx21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	0.8	1.1
Eapp	E2F-associated phosphoprotein	0.6	0.8
Ncl	nucleolin	0.8	1.5
Pa2g4	proliferation-associated 2G4	0.6	0.6
Cbs	cystathionine beta-synthase	−1.9	−1.1
Gch1	GTP cyclohydrolase 1	−1.6	−1.2

The fold change was calculated as the relative average value of three arrays in each group compared to controls, and the values are reported as the natural logarithm.

ed groups. *Pa2g4* plays a role in growth regulation and *Pa2g4* mutation is associated with gastric cancer in humans[32]. Using microarray analysis, we compiled a comprehensive gene expression profile demonstrating the changes in gene expression that occur soon after the administration of DMF and ABP. We identified several genes involved in carcinogenicity and acute toxicity in the liver. This information may provide insight into the toxicological mechanisms of early carcinogenesis and assist in cancer diagnosis.

Materials & Methods

Animals

Before the experiment, approximately 8-week-old BALB/c male mice (SLC, Japan) were kept under a 12-h light/dark cycle at a controlled temperature and

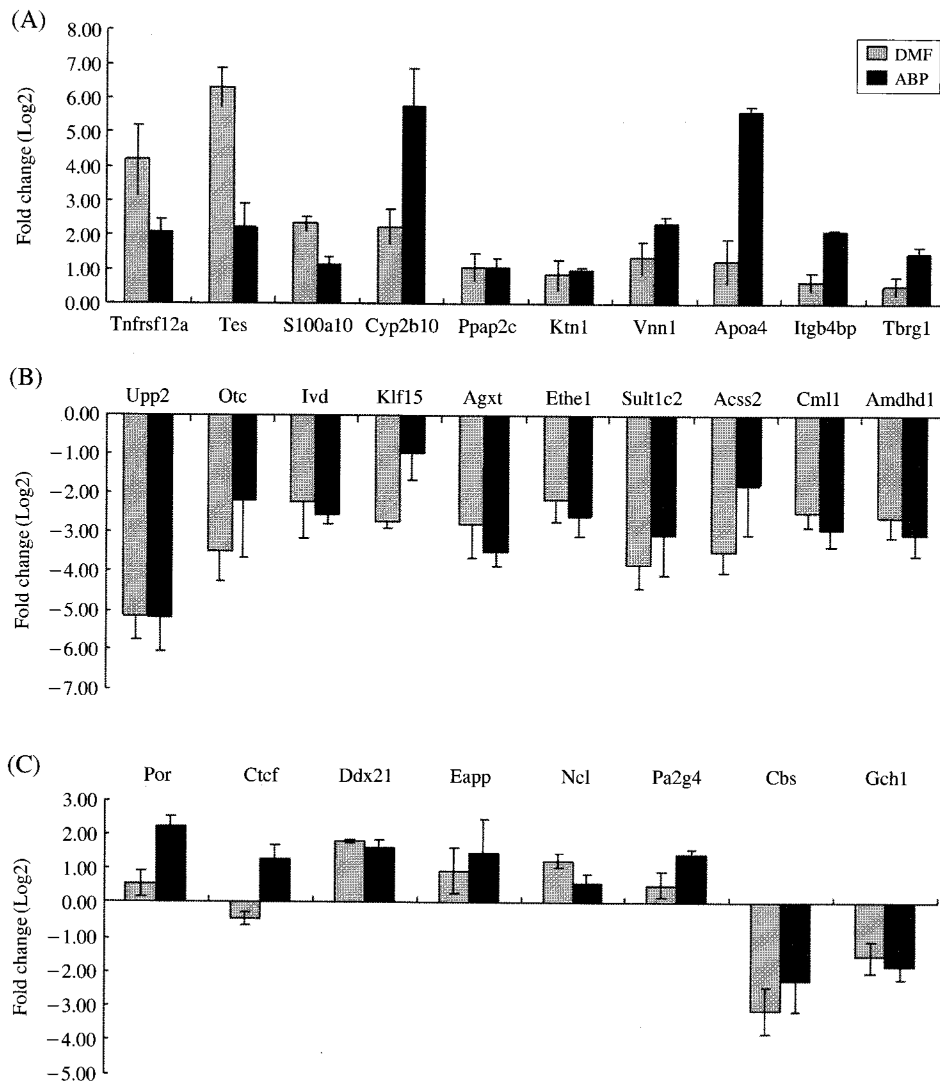

Figure 5. Real-time PCR verification of the gene expression changes observed in microarray analyses in both the DMF- and ABP-treated groups. The expression patterns of selected genes detected in the microarray experiments were analyzed using real-time PCR. (A) Top 10 upregulated genes, (B) top 10 downregulated genes, and (C) cancer-related genes in the carcinogen-treated groups.

humidity for 2 weeks in an animal room. The mice were fed standard food pellets and water *ad libitum*. DMF dissolved in saline was injected intraperitoneally at 650 mg/kg body weight and ABP dissolved in corn oil was administered orally at 120 mg/kg body weight. The control mice were given corresponding volumes of vehicle. The mice were sacrificed 24 h after treatment and blood and liver samples were collected. All experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with Association for Assessment and Accreditation of Laboratory Animal Care international guidelines.

Blood Biochemistry and Histopathology

The levels of AST, ALT, and ALP, biochemical markers of hepatotoxicity, were measured using an Automated Biochemical Analyzer (Fuji Dri-Chem 3500s, Fujifilm, Japan). The average values were given and statistical significance was calculated using a two-tailed, unpaired *t*-test for comparison between groups. For histopathology, formalin-fixed liver tissue was embedded in paraffin, cut into 4-μm sections, stained with hematoxylin and eosin (H & E), and analyzed using light microscopy.

RNA Extraction

At necropsy, each mouse was anesthetized with isoflurane and the liver tissue was dissected out and submerged in an appropriate volume of RNAlater (QIAGEN, Germany) for RNA extraction. The liver tissues were stored overnight in RNAlater at 4°C, after which the reagent was discarded and the tissue frozen at −80°C until RNA extraction. The liver samples were homogenized in TRIzol (Invitrogen, USA) and total RNA was isolated using TRIzol and purified using an

RNeasy mini kit (QIAGEN, Germany) according to the manufacturers' instructions. Total RNA was quantified using NanoDrop (NanoDrop, USA) and the quality of the RNA was evaluated using a 2100 Bioanalyzer (Agilent Technologies, USA).

Microarray Analysis

The Affymetrix GeneChip® 430A 2.0 assay, which contains over 22,600 probes, was used for the microarray analysis. Sample labeling, microarray hybridization, washing, and scanning were performed according to the manufacturer's protocols (Affymetrix, USA). The pre-processing procedure, including the resultant cell intensity files (CEL), and subsequent microarray analysis was performed using GenPlex software (Istech, Korea). The data were normalized using global scale normalization. The differentially expressed genes were selected based on >1.5-fold changes and Welch's *t*-test ($P<0.01$). The selected differentially expressed genes were analyzed using a hierarchical clustering algorithm. Based on hierarchical clustering, 107 genes were deregulated in both carcinogen-treated groups were selected. Functional analysis of differentially expressed genes was performed using Ingenuity Pathways Analysis (IPA).

Quantitative Real-time RT-PCR

Gene transcripts were detected and quantified using SYBR Green (QuantiTect SYBR Green PCR Master Mix; QIAGEN, USA), according to the manufacturer's instructions, on a Rotor Gene 6000 real-time rotary analyzer (Corbett Research, Australia). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/). To ensure the specificity and integrity of the PCR products, melting curve analyses were performed for all amplified products. The *Actin* level was used as an internal control, and fold changes were calculated using the $2^{-\Delta\Delta CT}$ method[33].

Acknowledgements

This work was supported by the Ministry of Science and Technology as part of the 2006 Project for Developing a Toxicological Evaluation System for Specific Toxicity-inducing Chemicals (KK-0604) at the Korea Institute of Toxicology.

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