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GENOTOXICITY AND CARCINOGENICITY

Identification of biomarkers of chemically induced hepatocarcinogenesis in rasH2 mice by toxicogenomic analysis

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Abstract Toxicogenomic approaches have been applied to chemical-induced heptocarcinogenesis rodent models for the identification of biomarkers of early-stage hepatocarcinogenesis and to help clarify the underlying carcinogenic mechanisms in the liver. In this study, we used toxiciogenomic methods to identify candidate biomarker genes associated with hepatocarcinogenesis in rasH2 mice. Blood chemical, histopathologic, and gene expression analyses of the livers of rasH2 mice were performed 7 and 91 days after the administration of the genotoxic hepatocarcinogens 2-acetylaminofluorene (AAF) and diethylnitrosoamine (DEN), the genotoxic carcinogen melphalan (Mel), and the nongenotoxic noncarcinogen 1-naphthylisothiocynate (ANIT). Histopathologic lesions and a rise in accompanying serum marker levels were found in the DEN-treated rasH2 mice, whereas no neoplastic lesions were observed in the rasH2 mice. However, biological functional analysis using Ingenuity Pathways Analysis (IPA) software revealed that genes with comparable molecular and cellular functions were similarly deregulated in the AAF- and DEN-treated rasH2 mice. We selected 68 significantly deregulated genes that represented a hepatocarcinogen-specific signature; these genes were commonly deregulated in both the AAF- and DEN-treated rasH2 mice on days 7 and 91. Hierarchical clustering analysis indicated that the expression patterns of the selected genes in the hepatocarcinogen (AAF and DEN) groups were distinctive from the patterns in the control, Mel, and ANIT groups. Biomarker filter analysis using IPA software suggested that 28 of the 68 signature genes represent promising candidate biomarkers of cancer. Quantitative real-time PCR analysis confirmed that the deregulated genes, which exhibited sustained up- and down-regulation up to day 91, are likely involved in early-stage hepatocarcinogenesis. In summary, the common and significant gene expression changes induced by AAF and DEN may reflect early molecular events associated with hepatocarcinogenesis, and these "signature" genes may be useful as biomarkers of hepatocarcinogenesis in mice.

Keywords Biomarker · Hepatocarcinogenesis · rasH2 mice · Toxicogenomics · 2-acetylaminofluorene · Diethylnitrosoamine

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Introduction

The conventional two-year rodent bioassay has been widely used during the preclinical stages of drug development as a prerequisite test for defining the carcinogenic

potential of candidate drugs. Although this bioassay has been highly standardized and broadly adopted, there is a clear need to improve the process because it requires a lot of time, money, manpower, and animals to evaluate drug safety and identify environmental carcinogens. For this reason, there have been several attempts to improve the two-year carcinogenicity testing process, including the use of transgenic mouse models (e.g., p53+/−, Tg.AC, and rasH2 mice) to shorten the amount of time necessary to complete the test (Pritchard et al. 2003), and the use of toxicogenomic methods to predict the carcinogenicity of xenobiotics at early time points (Ellinger-Ziegelbauer et al. 2009; Thomas et al. 2007). Transgenic mouse models are considered a promising candidate for short-term studies of carcinogenicity due to the early onset of spontaneous and chemically induced tumors compared with wild-type mice. The possibility of employing a short-term carcinogenicity assay using transgenic mice as a substitute for the two-year rodent bioassay was first introduced in 1996 by the International Committee on Harmonization (ICH) (Contrera and DeGeorge 1998). For more than a decade, regulatory agencies and pharmaceutical associations have evaluated short-term carcinogenicity using transgenic mice, and the consensus is that these models have added value in human cancer hazard identification (Storer et al. 2010).

The rasH2 mouse is a hemizygous transgenic mouse carrying several copies of the human c-Ha-ras oncogene that was established in 1990 at the Central Institute for Experimental Animals (Saitoh et al. 1990). Five to six copies of the prototype human c-Ha-*ras* oncogene with the gene's own promoter and enhancer were inserted in tandem array into the genome of a C57BL/6J × BALB/cByJ mouse embryo by pronuclear microinjection. The founder mouse was backcrossed with C57BL/6 J females to produce transgenic breeding stock. Hemizygous transgenic mice (CB6F1-Tg rasH2mice) for experimentation are produced by breeding transgenic male C57BL/6 J mice with wild-type BALB/cByJ female mice. This model is highly susceptible to genotoxic and nongenotoxic carcinogens through any conventional route of administration (Alden et al. 2002; Morton et al. 2002). Several six-month carcinogenicity studies of rasH2 mice have demonstrated a high incidence and rapid induction of tumors, with sensitivity to various carcinogens (Maronpot et al. 2000; Morton et al. 2002; Yamamoto et al. 1996). Mechanistic studies of tumorigenesis in rasH2 mice have suggested that over-expression of the transgene is the primary cause for accelerated tumor development (Tamaoki 2001).

In addition to the use of transgenic mice, the consideration of gene expression through microarray-based toxicological studies (toxicogenomics) has helped refine carcinogenicity testing. Toxicogenomics has already been employed to elucidate the mechanism of action of toxic compounds in classical toxicological studies (Lord et al. 2006), as well as for the prediction and classification of the carcinogenicity of genotoxic and nongenotoxic compounds (Ellinger-Ziegelbauer et al. 2005; Fielden et al. 2008). The potential importance of toxicogenomics in drug development has been recognized by the US Food and Drug Administration (FDA) and the pharmaceutical industry, and they have established a framework to review toxicogenomic data (Leighton et al. 2006). The accumulated gene expression data resulting from research in the toxicological field over the past few years will enable toxicogenomic methods to more accurately classify genotoxic and nongenotoxic carcinogens and to predict the carcinogenicity of unknown compounds (Waters et al. 2010). Several examples of the use of toxicogenomics in the identification of early marker genes that can be used to predict hepatocarcinogenic potential and in the elucidation of carcinogenic mechanisms in the liver have been reported, including the prediction and classification of genotoxic and nongenotoxic hepatotoxicants (Ellinger-Ziegelbauer et al. 2005, 2008; Kramer et al. 2004). The results of these studies indicate that it may be possible to identify molecular markers for use in predicting hepatic carcinogenicity.

To date, several applications of toxicogenomics to the investigation into candidate genes responsible for enhanced carcinogenesis in the forestomach, lungs, and liver of rasH2 mice have been reported (Okamura et al. 2004, 2006, 2007). However, the use of toxicogenomics for the determination of gene expression patterns or the identification of marker genes for predicting carcinogenicity has not been reported. Here, we investigated the gene expression patterns in the livers of rasH2 mice following short- and long-term exposure to genotoxic hepatocarcinogens to identify marker genes involved in the onset of carcinogenesis.

Materials and methods

Chemicals and animals

Corn oil, 1-naphthylisothiocynate (ANIT), 2-acetylaminofluorene (2-AAF), diethylnitrosoamine (DEN), and melphalan (Mel) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pathogen-free, six-week-old, male rasH2 mice were obtained from CLEA Japan Inc. (Tokyo, Japan). A maximum of five mice were housed in polycarbonate cages with absorbent hardwood bedding in a controlled temperature (23°C ± 3°C) and humidity (50% ± 10%) facility with a 12-h light/dark cycle. Certified rodent chow (Purina, Seoul, Korea) and water were supplied *ad libitum* throughout the experiment. Food was withdrawn 12 h prior to the day of dissection. 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.

Study design

After a one-week acclimatization period, 36 rasH2 mice were randomly divided and allocated to ten groups according to the administered chemicals and the day of killing (Table 1). The four tested chemicals were divided into three categories: genotoxic hepatocarcinogens (AAF and DEN), genotoxic carcinogens (Mel), and nongenotoxic noncarcinogens (ANIT). All tested chemicals were dissolved in corn oil and administered orally at 10 ml/kg body weight. Control mice were administered the corresponding quantity of corn oil (vehicle). The dose of the four administered chemicals was one-fifth the LD_{50} dose for each chemical. The mice in each group were administered their specific chemical daily until the day before killing; however, drug administration to the rasH2 mice in the DEN 91-day (91 d) group was stopped after 7 days of repeated dosing. The relative liver weight was calculated as the percentage of the body weight.

Blood biochemistry and histopathology

All mice were killed on the scheduled date using isoflurane. Blood was collected through the inferior vena cava in Vacutainer® tubes (BD Biosciences, San Jose, CA, USA). Serum was isolated after centrifugation at 3,000 rpm for 10 min. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBIL), and total cholesterol (Tcho) were measured using a DRI-CHEM 3500s (FUJIFILM, Tokyo, Japan). After blood collection, the liver was rapidly removed and fixed in 10% neutral-buffered formalin. The specimens were dehydrated, embedded in paraffin, and sectioned into 4-μm-thick slices onto microscope slides. The sectioned tissues were stained with hematoxylin and eosin and examined by light microscopy (Nikon E-400, Tokyo, Japan).

Gene expression analysis

Total RNA was extracted from a portion of each liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The isolated total RNA was quantified using a spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). For the microarray experiments, the quality of RNA was evaluated using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Changes in gene expression were determined using an Affymetrix Mouse Gene 1.0 ST array (Santa Clara, CA, USA). Three hundred nanograms of total RNA was used for cDNA synthesis. All microarray procedures were performed according to the manufacturer's instructions. Raw image data acquired from a GeneChip Scanner 3000 (Affymetrix) were processed by GeneChip Operating Software (GCOS, Affymetrix). Subsequent data normalization, statistical analysis, differentially expressed gene (DEG) selection, and hierarchical clustering were performed using GenPlex software, version 3.0 (ISTECH Inc., Ilsan, Korea). The criteria for DEG selection in each chemical-treated group were $P < 0.05$ (Student's t test) and a 1.2-fold change in expression relative to that in the corresponding control group. Commonly deregulated genes between the different groups were identified by Venn diagrammatic selection methods. Classification of the selected genes according to their biological and toxicological functions was done using Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, Redwood, CA, USA). The P value, represented as the negative log ratio of the IPA results, is the probability based on Fisher's

Table 1 Experimental design

Group number	Group name	Chemical/vehicle	CAS number	Dose (mg/ml/kg)	Dosing day/(day of killing)	Number of mice
1	Con 7d	–/Corn oil	–	0[a]	7/(8)	3
2	Con 91d				90/(91)	3
3	ANIT 7d	1-Naphthylisothiocynate (ANIT)/corn oil	551–06–4	21	7/(8)	4
4	ANIT 91d				90/(91)	5
5	AAF 7d	2-Acetylaminofluorene (2-AAF)/corn oil	53–96–3	162	7/(8)	4
6	AAF 91d				90/(91)	5
7	DEN 7d	Diethylnitrosoamine (DEN)/corn oil	55–18–5	40	7/(8)	4
8	DEN 91d				7/(91)[b]	5
9	Mel 7d	Melphalan/corn oil	148–82–3	0.6	7/(8)	4
10	Mel 91d				90/(91)	5

[a] Control animals that received vehicle alone

[b] The animals in group DEN 91d stopped receiving a daily dose of diethylnitrosoamine after 7 days and were killed on day 91

exact test. In addition, biomarker filtration analysis of the selected gene set was performed using IPA software. The selected genes were annotated based on the NetAffyx™ and Ingenuity® systems.

Quantitative real-time PCR (RT-qPCR)

cDNA was synthesized from 2 μg of total RNA using random primers and Superscript™ Reverse Transcriptase (Invitrogen) and subsequently diluted to 1:50 with RNA-free water. RT-qPCR was performed using QuantiTect SYBR Green PCR Master Mix (Qiagen) according to the manufacturer's instructions on a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia). Gene-specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/); the sequences are presented in Table 2. Melting curve analyses of all amplified samples were done to ensure the specificity and integrity of the amplified samples. *Gapdh* was used as an internal control. The relative mRNA level for the amplified gene of interest was determined by the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

Survival and body/liver weight

During the experimental period, no animals died prematurely in the ANIT-, AAF-, Mel-treated groups, or control groups; however, 3 unscheduled deaths occurred among the DEN-treated rasH2 mice in the 91-d group. The final body weights and absolute and relative liver weights are shown in Table 3. The seven-day (7d) and 91-d chemical-treated rasH2 mice did not differ significantly in body or liver weight, except for the DEN-treated group. The mean body and liver weights in the DEN 7-d group were markedly reduced compared with those in the control, ANIT, AAF, and Mel 7-d groups; similarly, the relative liver weight was decreased slightly in the DEN 7-d group. The mean liver weights and relative liver weights for the rasH2 mice in the DEN 91-d group were significantly greater than those in the control, ANIT, AAF, and Mel 7-d groups.

Blood biochemistry and histopathology

Serum biochemical analyses to identify liver injury did not reveal significant differences among the control, ANIT-, AAF-, and Mel-treated rasH2 mice. However, the 7-d DEN-treated rasH2 mice showed significantly increased serum levels of AST, ALT, ALP, and TBIL, while the DEN 91-d group showed significantly increased serum AST, ALT, and ALP levels (Fig. 1). The 7- and 91-d DEN-treated mice showed the most dramatic increase in serum ALP (Fig. 1).

No chemically induced or spontaneous neoplastic lesions were observed in the livers of the mice, including those that died prematurely. However, histopathologic examination revealed that treatment with ANIT or DEN induced mild to severe liver injury. Three rasH2 mice in the ANIT 7-d group exhibited mild inflammatory cell infiltration around the portal area, while 4 rasH2 mice in the ANIT 91-d group showed bile duct hypertrophy (data not shown). The livers of the rasH2 mice in the DEN 7-d group showed mild centrilobular hypertrophy (Fig. 2c, e). In the DEN 91-d group, hepatocelluar vacuolation, karyomegaly/cytomegaly, oval cell hyperplasia, bile duct hyperplasia, and mitosis were observed (Fig. 2d, f). No chemically induced histopathologic changes were detected in the 7- or 91-d control, AAF, and Mel groups.

Table 2 Primers used for quantitative real-time PCR analysis

Gene symbol	Forward primer	Reverse primer	Accession no.
Cdkn1a	GTGGCCTTGTCGCTGTCT	CCAATCTGCGCTTGGAGT	NM_007669
Ccng1	AATTGAGTCGGCCCATGA	CCAAGATGCTTCGCCTGT	NM_009831
Trp53inp1	CCAGGGATTGCCACACTC	GGCTGGAAGGAGACAGCA	NM_021897
Mdm2	AGTGACGAAGGGCACGAG	TGTGATGGAAGGGGAGGA	NM_021897
Tnfrsf10b	GAGGGGACAGCCATCCTT	GGAGGCCTGGGGTGTAGT	NM_020275
Tubb2c	TCGGTGTTGGACGTTGTG	GGGTACCCATCCCAGACC	NM_146116
Vcam1	TTGGGAGCCTCAACGGTA	GCCCGTAGTGCTGCAAGT	NM_011693
Serpinf1	TCAAGGGGCAGTGGGTAA	GCTGGGCAATCTTGCAGT	NM_011340
Cdkn1a	GCCTTAGCCCTCACTCTGTG	AGGGCCCTACCGTCCTACTA	NM_007669
Tnfrsf10b	GTCAGAAGGGAACTGCAAGC	GCATCGACACACCGTATTTG	NM_020275
Bcl2L1	TGTGGATCTCTACGGGAACA	AAGAGTGAGCCCAGCAGAAC	NM_009743
Gapdh	TGACCACAGTCCATGCCATC	GACGGACACATTGGGGGTAG	NM_008084

Table 3 Body weight, liver weight, and relative liver weight (g liver/100 g body)

Group	Body weight (g)[a]	Liver weight (g)[a]	Relative liver weight (g liver/100 g body)[a]
7-day			
Con 7d	21.83 ± 0.84	0.99 ± 0.06	4.55 ± 0.31
ANIT 7d	22.30 ± 1.24	0.94 ± 0.05	4.20 ± 0.15
AAF 7d	21.13 ± 1.51	0.86 ± 0.10	4.06 ± 0.42
DEN 7d	17.18 ± 0.94**	0.65 ± 0.06**	3.76 ± 0.19**
Mel 7d	22.20 ± 0.83	0.93 ± 0.05	4.18 ± 0.11
91-day			
Con 91d	29.10 ± 2.12	1.11 ± 0.15	3.81 ± 0.26
ANIT 91d	27.70 ± 3.55	1.06 ± 0.08	3.84 ± 0.26
AAF 91d	24.90 ± 1.66*	1.13 ± 0.13	4.52 ± 0.33*
DEN 91d	27.60 ± 0.21	1.55 ± 0.03*	5.63 ± 0.15**
Mel 91d	24.80 ± 0.69**	1.12 ± 0.10	4.50 ± 0.34*

[a] The values given are the mean ± SD

* Significantly different from the vehicle controls ($P < 0.05$)

** Significantly different from the vehicle controls ($P < 0.01$)

Gene expression analysis

Gene expression analysis was performed using three samples per group. Total RNA samples from the chemical-treated groups were selected based on RNA integrity and blood biochemistry as well as histologic analysis. In the DEN 91-d group, only two samples were used for gene expression analysis due to unscheduled deaths during the experimental period. Each chemical-treated group was compared with the control group at the same time point. Significantly up- and down-regulated genes were identified by *t* tests at each time point ($P < 0.05$) combined with the requirement for at least a 1.2-fold change with respect to the mean intensity of the replicate samples. The number of differentially expressed genes is shown in Table 4. The remarkable increase in the number of differentially expressed genes in the DEN-treated groups is consistent with the elevated levels of serum markers and histopathologic findings. To identify whether the hepatocarcinogen-induced transcriptional effects observed on day 7 persisted until day 91, we profiled the significantly modulated genes in hepatocarcinogen-treated livers taken from mice in the 7- and 91-d groups for each chemical. In the AAF-treated rasH2 mice, 152 genes were identified as significantly deregulated on days 7 and 91, whereas 1,075 genes were so identified in the DEN-treated rasH2 mice.

To elucidate the underlying biological responses regulated by the genes whose expression was modulated by AAF and DEN, we carried out a statistical analysis using IPA software to identify the biological and toxicological functions of the selected gene sets. As shown in Fig. 3a, most of the predicted molecular and cellular functions overlapped between the AAF- and DEN-treated groups, except for amino acid metabolism, cellular response to therapeutics, energy production, free radical scavenging, post-translational modification, and protein synthesis. A toxicological function analysis focusing on hepatotoxicity was also performed for the same gene sets; the results are shown in Fig. 3b. The differentially expressed genes in the DEN 7- and 91-d groups were significantly associated with hepatocellular carcinoma, liver cirrhosis, liver cholestasis, liver necrosis/cell death, liver damage, liver hepatitis, liver hyperplasia, liver inflammation, liver proliferation, and liver steatosis, whereas the AAF-induced gene expression changes were significantly associated with hepatotoxic functions, including hepatocellular carcinoma, liver necrosis/cell death, and liver hepatitis.

Identification of candidate biomarkers

To identify candidate biomarkers that can predict hepatocarcinogenic effects at early time points, we sought to identify genes that displayed common gene expression patterns between the early and late time points in the AAF- and DEN-treated groups. To this end, 52 and sixteen genes were identified as commonly up- and down-regulated in the AAF 7-d, AAF 91-d, DEN 7-d, and DEN 91-d groups, respectively. Gene expression profiles of the selected gene set discriminated the AAF- and DEN-treated groups from the control, ANIT-, and Mel-treated groups by hierarchical clustering analysis (Fig. 4). The expression levels of the genes selected as candidate biomarkers for hepatocarcinogenesis in each group are listed in Table 5. Biomarker filter analysis using IPA revealed that 28 of the 68 genes were feasible cancer biomarkers in humans and mice (Table 5). The 28 selected biomarkers were mainly involved in apoptosis (*Bax*, *Tnfrsf10b*, *Bcl2l1*, *Trp53inp1*, and *Wwox*), transport (*Gria3*, *Nqo1*, and *Selenbp2*), transcriptional regulation (*Nfe2l2*, *Btg2*, and *Mybl1*), the cell cycle (*Cdkn1a* and *Mdm2*), and cell adhesion (*Vcam1*).

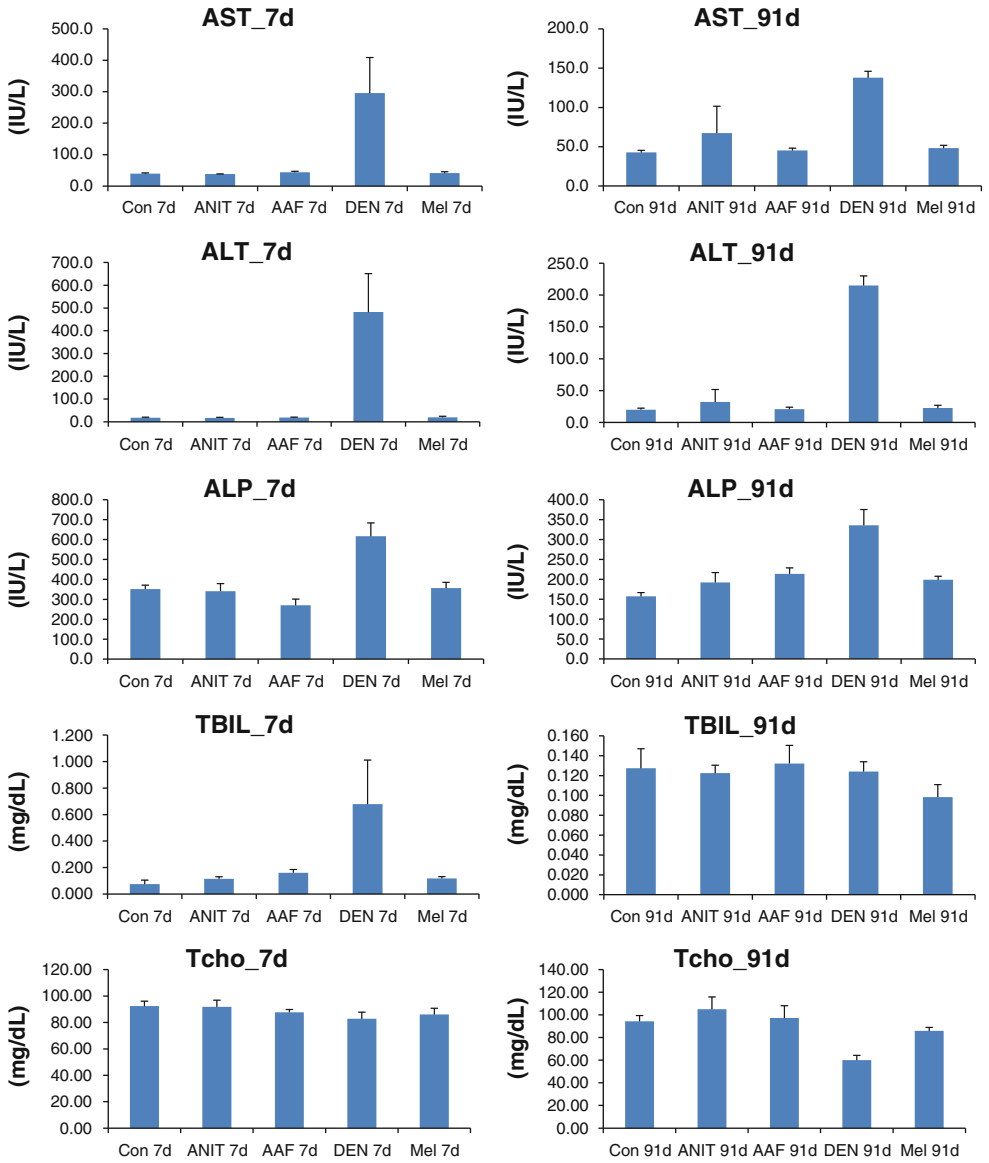

Fig. 1 Blood biochemical analysis of the control and chemical-treated groups. *AST* aspartate transferase; *ALT* alanine transferase; *ALP* alanine phosphatase; *TBIL* total bilirubin; *Tcho* total cholesterol

RT-qPCR Verification

To verify our microarray data, which identified the commonly and significantly deregulated genes (i.e., candidate biomarkers), we selected eleven genes from Table 5 and determined their expression levels using RT-qPCR. The expression values obtained were consistent with the expression levels detected by microarray analysis (Fig. 5). Gene expression changes in the 7-d group were greater than those in the 91-d group in both AAF- and DEN-treated livers. The most prominent changes in expression were observed with cell cycle–related genes such as *Cdkn1a*, *Mdm2*, and *Ccng1*, and apoptosis-related genes such as *Trp53inp1* and *Tnfrsf10b*; expression of these genes was markedly increased (>10-fold) on day 7 in both the AFF- and DEN-treated rasH2 mice and showed slightly reduced expression in the 91-d groups. In contrast, *Nqo1* expression was consistent in all observed groups by microarray and RT-qPCR analyses. Several inconsistencies in expression were observed between the microarray and RT-qPCR analyses, including *Bax* and *Bcl2l1* expression in the AAF and DEN 91-d groups; however, most of the changes observed in the AAF and DEN groups were confirmed by RT-qPCR analysis.

Discussion

The aim of the current study was to investigate gene deregulation in the early stages of carcinogenesis caused by genotoxic hepatocarcinogens at early (7 days) and late

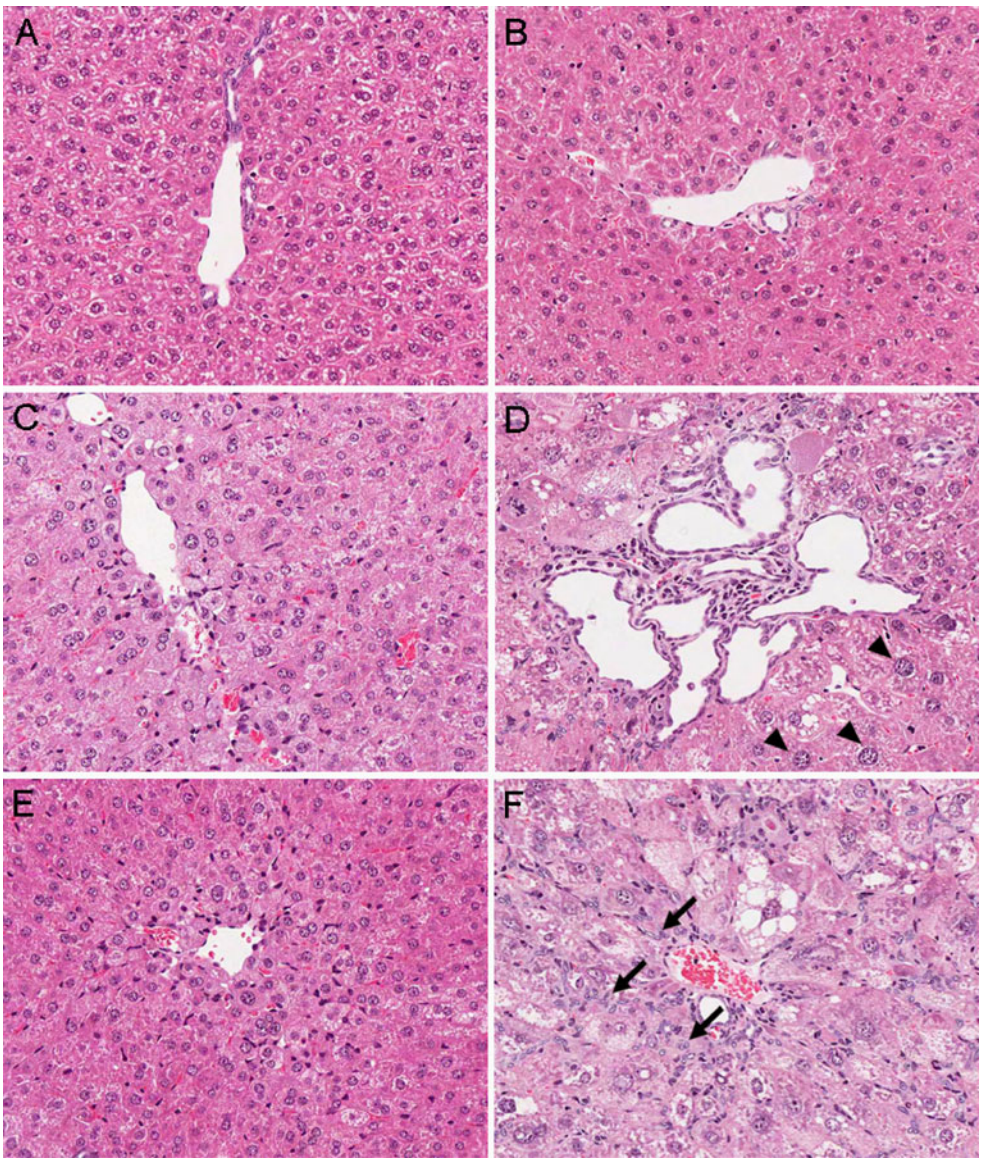

Fig. 2 Histopathologic examination of the livers of vehicle (**a**, **b**) and DEN-treated rasH2 mice (**c**–**f**). **a** Con 7d; **b** Con 91d; (**c**, **e**) DEN 7d, mild centrilobular hypertrophy was observed; (**d**, **f**) DEN 91d, hepatocelluar vacuolation, karyomegaly (*arrowhead*), cytomegaly of hepatocytes, oval cell hyperplasia (*arrow*), bile duct hyperplasia, and hepatocellular mitosis were observed (200×)

(91 days) time points in rasH2 mice and whether this information could be used to discriminate between the hepatocarcinogenic effects of various chemicals and eventually be employed as candidate biomarkers to predict hepatocarcinogenic potential. To accomplish this, we treated rasH2 mice with chemicals from three different well-characterized classes of agents, including AAF and DEN (genotoxic hepatocarcinogens), Mel (genotoxic carcinogen), and ANIT (non-genotoxic noncarcinogen), and we identified commonly deregulated genes in the AAF- and DEN-treated groups (AAF 7d, AAF 91d, DEN 7d, and DEN 91d) using toxicogenomic approaches. AAF and DEN are known to induce heptocarcinogenesis in rodents and humans; the carcinogenicity of the two carcinogens is listed in the Carcinogen Potency Data Base at http://potency.berkeley.edu/ (Gold et al. 1999, 2005). Toxicogenomics as well as cancer studies have been used together with AAF- and DEN-induced hepatocarcinogenesis in rodent models for the prediction of carcinogenic potential (Ellinger-Ziegelbauer et al. 2005; Fielden et al. 2008; Nakayama et al. 2006).

In the present study, treatment of rasH2 mice with DEN was found to affect body and liver weight. Furthermore, chemical-induced histopathologic lesions accompanying elevated serum biomarker levels were observed in DEN-treated rasH2 mice. In these mice, the levels of serum biomarkers indicating liver injury were dramatically

Table 4 Differentially expressed genes in rasH2 mouse liver after treatment with four chemicals

Group	The numbers of significantly deregulated genes		Statistical significance (Welch's *t* test)
	Up-regulation	Down-regulation	
7-day			$P < 0.05$
ANIT 7d	322	204	
AAF 7d	397	462	
DEN 7d	2,474	3,274	
Mel 7d	384	514	
91-day			
ANIT 91d	227	162	
AAF 91d	376	389	
DEN 91d	1,155	1,756	
Mel 91d	484	505	

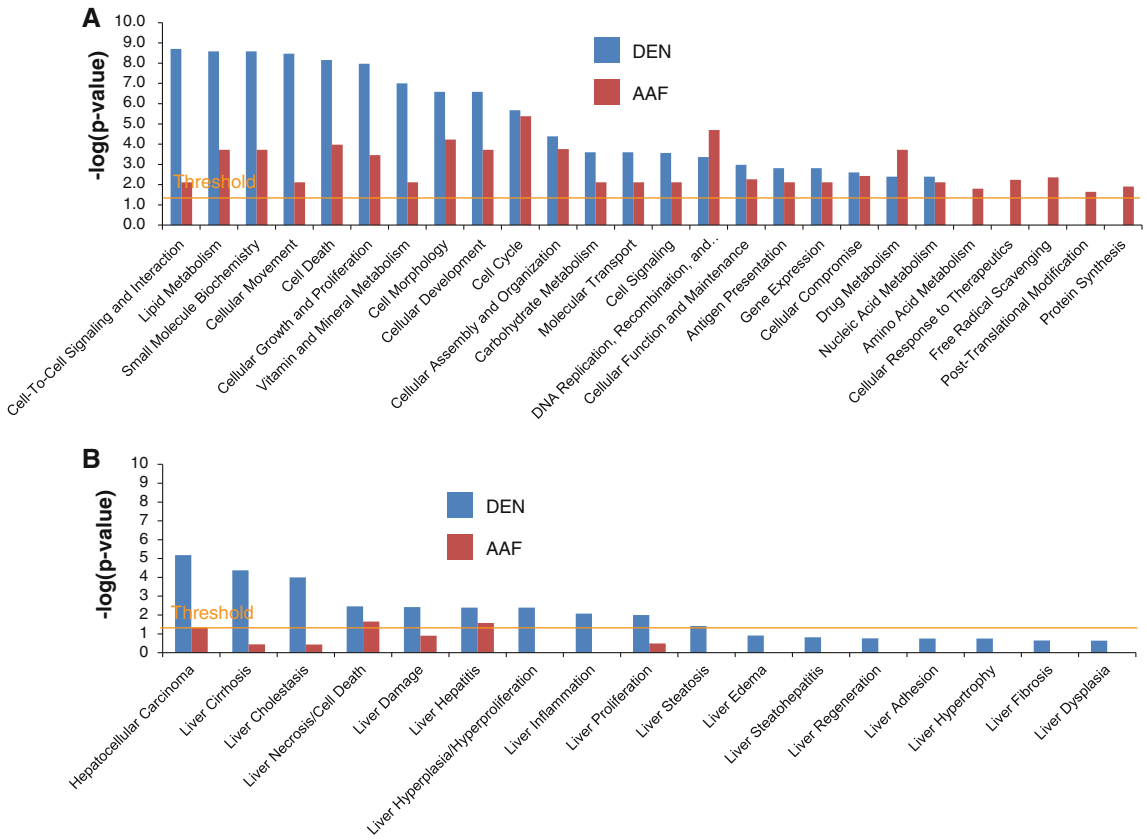

Fig. 3 Biological and toxicological functional analysis using IPA software. **a** Comparison of molecular and cellular functions among commonly deregulated genes in the DEN (7 and 91d) and AAF (7 and 91d) groups. **b** Comparison of the hepatotoxicologic functions among commonly deregulated genes in the DEN (7 and 91 d) and AAF (7 and 91d) groups. The *orange line* indicates the threshold of statistical significance ($P < 0.05$)

elevated, and significant body and liver weight loss was observed in the 7-d group. In the 91-d DEN-treated group, however, various histopathologic lesions with significant liver weight gain were observed. Mild centrilobular hypertrophy was observed in the DEN 7-d group, while hepatocellular vacuolation, karyomegaly/cytomegaly, oval cell hyperplasia, bile duct hyperplasia, and mitosis were observed in the DEN 91-d group. Oval cell hyperplasia is often observed in the livers of rats and mice after treatment with various hepatotoxins or during chemical-induced hepatocarcinogenesis (Factor et al. 1994; Sell and Dunsford 1989). It has been suggested that regeneration of the

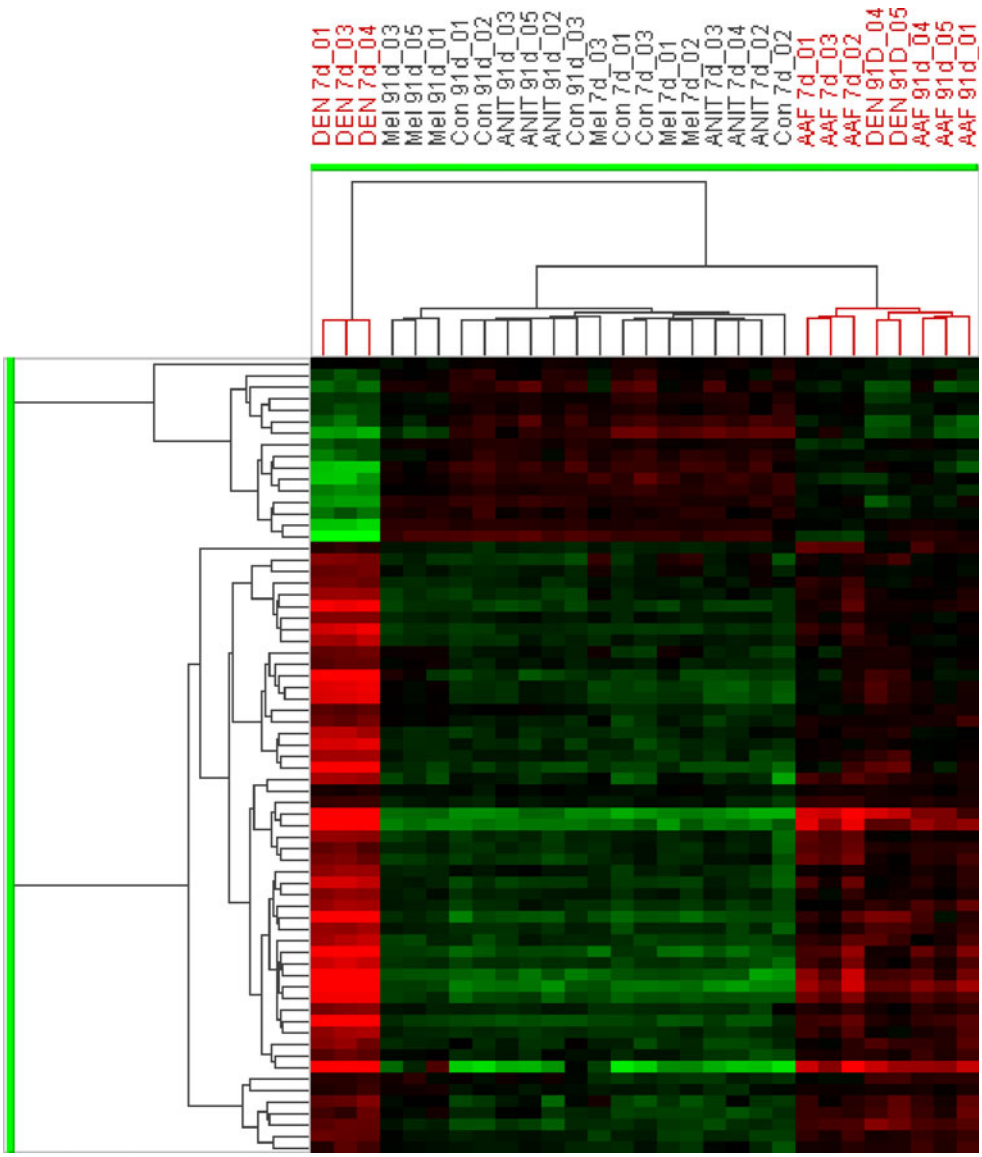

Fig. 4 Hierarchical clustering analysis of commonly and significantly expressed genes in AAF- and DEN-treated rasH2 mice. The gene expression profiles of the AAF- and DEN-treated groups are discriminated from the control-, ANIT-, and Mel-treated groups

hepatic parenchyma after irreversible damage may occur via oval cell hyperplasia and that these cells may eventually differentiate into hepatocytes (Factor et al. 1994). Oval cell hyperplasia has also been observed during AAF- and DEN-induced hepatocarcinogenesis (Becker and Sell 1979; Koen et al. 1983). Karyomegaly and irregularly sized hepatocellular nuclei (i.e., anisonucleosis) were identified in the DEN 91-d group. This histopathologic change has been reported to be associated with toxicity due to chemical hepatocarcinogens such as AAF and DEN (Giri and Das 1996; Svoboda and Higginson 1968). Although DEN-induced preneoplastic lesions such as hepatocellular altered foci were not identified in this study, our data indicate that the DEN-induced histopathologic changes in the rasH2 mouse livers in this study are consistent with the previous findings of chemical carcinogen–induced liver injury in rodents. On the other hand, treatment of rasH2 mice with AAF did not have any significant effect on serum levels of AST, ALT, ALP, TBIL, and hepatic histology. Although AAF has been widely used as a powerful carcinogen, which induces tumor in the liver of rodent, mouse is less susceptible to the hepatocarcinogenicity of AAF (Astrom et al. 1986; Travis et al. 1996). Therefore, it is seemed that the dose of AAF used in this study was not enough to induce histopathologic changes associated with hepatocarcinogenesis. In contrast to our blood biochemical and histopathologic observations, gene expression analysis indicated that the molecular mechanisms underlying the induction of hepatocarcinogenesis by AAF correlated well with DEN. Indeed, the significantly and commonly deregulated genes in the AAF- and DEN-treated groups shared most of their molecular and cellular functions (Fig. 3a).

To date, several toxicogenomic studies employing chemically induced hepatocarcinogenesis in rats have been

Table 5 List of commonly and significantly deregulated genes in the 7- and 91-d AAF and DEN groups

Probe ID	Gene symbol	Fold change				Accession no.
		AAF 7d	DEN 7d	AAF 91d	DEN 91d	
Up-regulation						
10443463	*Cdkn1a**	32.452	135.908	12.410	13.659	NM_007669
10570002	*EG546032*	25.072	60.745	7.277	16.578	XM_486078
10605874	*Eda2r*	9.771	34.665	6.954	5.004	NM_175540
10565570	*4632434I11Rik*	7.010	33.590	4.587	4.111	NM_001080995
10353010	*Mybl1**	3.084	24.385	2.428	5.098	NM_008651
10599348	*Gria3**	11.323	23.446	8.540	5.584	NM_016886
10357875	*Btg2**	2.041	21.804	1.642	3.568	NM_007570
10407126	*Plk2*	3.566	16.701	4.327	2.425	NM_152804
10456400	*Tubb6*	1.523	16.061	1.401	2.514	NM_026473
10385271	*Ccng1*	4.770	14.908	3.417	2.779	NM_009831
10350146	*Phlda3*	2.763	13.582	3.069	2.262	NM_013750
10480628	*Tubb2c**	1.862	11.851	1.524	2.223	NM_146116
10416230	*Tnfrsf10b**	2.085	10.620	2.014	1.869	AK050753
10372668	*Mdm2**	3.331	10.130	2.285	1.732	NM_010786
10432986	*Aaas*	2.374	9.667	1.634	1.621	NM_153416
10411306	*Polk*	3.009	9.579	2.064	1.645	NM_012048
10503259	*Trp53inp1**	4.349	9.462	1.378	1.714	NM_021897
10495316	–	1.567	9.077	1.386	1.576	NM_019976
10496373	*Ddit4 l*	2.435	6.656	1.918	2.649	NM_030143
10540105	*Tmem43*	2.073	6.157	1.955	2.511	NM_028766
10497673	*Zmat3*	1.731	6.039	2.755	2.130	NM_009517
10424404	*Pvt1**	1.616	6.018	1.368	1.837	AK043790
10406817	*Enc1**	1.695	5.674	1.276	2.050	NM_007930
10516932	*Sesn2*	1.832	5.539	1.433	1.255	NM_144907
10554233	*Isg20l1*	2.215	5.245	1.988	1.381	NM_026531
10426016	*Gtse1*	1.577	5.151	1.307	1.676	NM_013882
10574545	*Ces5*	3.072	4.929	1.601	1.437	NM_172759
10489759	*Sulf2*	1.798	4.551	1.703	1.713	NM_028072
10510464	*Lzic*	1.719	4.409	1.468	1.469	NM_026963
10579331	*Gdf15**	1.669	4.264	1.747	1.847	NM_011819
10501608	*Vcam1**	1.702	4.196	1.559	2.491	NM_011693
10561162	*Cyp2a22*	2.261	4.139	2.192	2.581	XM_001480937
10488655	*Bcl2l1**	1.526	3.755	1.300	1.559	NM_009743
10488575	–*	1.456	3.659	1.206	1.378	–
10563303	*Bax**	1.655	3.597	1.712	1.375	NM_007527
10436967	*Cbr1**	1.786	3.578	1.730	1.452	NM_007620
10397763	*9030617O03Rik**	3.157	3.550	2.262	1.685	NM_145448
10537102	*Exoc4*	2.932	3.112	1.983	1.816	NM_009148
10424676	*Ly6e**	2.467	3.085	3.076	4.567	NM_008529
10441270	*Ripk4*	1.323	3.033	1.293	1.248	NM_023663
10481056	*Notch1**	1.777	3.004	1.906	1.581	NM_008714
10472893	*B230120H23Rik**	1.941	2.992	1.813	1.509	NM_023057
10375234	*Nudcd2*	1.638	2.826	1.455	1.234	NM_02602
10581538	*Nqo1**	2.332	2.806	2.251	2.607	NM_008706
10573549	*Fbxw9*	1.298	2.802	1.218	1.293	NM_026791
10393341	*Rhbdf2*	1.346	2.662	1.326	1.561	NM_172572

Table 5 continued

Probe ID	Gene symbol	Fold change				Accession no.
		AAF 7d	DEN 7d	AAF 91d	DEN 91d	
10483809	*Nfe2l2**	1.784	2.425	1.911	1.539	NM_010902
10437639	*Emp2**	1.371	2.073	1.456	1.750	NM_007929
10360684	*Ephx1**	1.956	2.063	1.568	1.478	NM_010145
10462473	*Mbl2*	2.545	1.737	1.950	1.314	NM_010776
10488589	*5430432M24Rik*	1.265	1.608	1.291	1.269	NM_028666
10424781	*Grina*	1.341	1.447	1.232	1.304	NM_023168
Down-regulation						
10515574	*St3gal3*	−1.247	−1.218	−1.294	−1.348	NM_009176
10575706	*Wwox**	−1.680	−2.204	−1.250	−1.260	NM_019573
10584187	–	−1.440	−2.209	−1.292	−1.821	–
10451559	*1700001C19Rik*	−1.304	−2.304	−1.360	−1.529	NM_029296
10493494	*ENSMUSG00000074470*	−1.514	−2.402	−1.479	−1.629	AK140090
10430899	*Cyp2d40*	−1.333	−2.918	−1.507	−1.708	NM_023623
10548931	*9830102E05Rik*	−1.535	−3.124	−1.698	−1.605	NM_177787
10582466	*Sult5a1*	−1.805	−3.357	−2.454	−2.853	NM_020564
10496462	*Adh6-ps1*	−1.935	−3.591	−2.090	−2.720	AK004863
10399801	*Sntg2*	−1.657	−4.466	−1.581	−1.464	NM_172951
10442625	*Igfals*	−1.309	−4.490	−1.593	−2.646	NM_008340
10388430	*Serpinf1**	−1.266	−6.154	−1.243	−1.252	NM_011340
10494085	*Selenbp2**	−1.576	−6.926	−2.513	−1.597	NM_019414
10451818	*Sult1c2**	−2.350	−7.407	−1.444	−1.864	NM_026935
10575685	*Nudt7*	−2.305	−8.766	−2.567	−2.538	NM_024437
10407876	*5033411D12Rik*	−2.136	−22.829	−1.441	−1.756	NM_138654

* The genes were selected as candidate cancer biomarkers using the IPA biomarker filter module

performed to screen for carcinogenicity and to discern the potential mode of action of carcinogens by identifying chemical hepatocarcinogen-specific gene expression signatures (Ellinger-Ziegelbauer et al. 2004, 2005, 2008; Fielden et al. 2008; Nakayama et al. 2006). As reviewed by Waters et al. (2010), these approaches have indicated that distinctive gene expression signatures provide a useful database for the prediction of chemical hepatocarcinogenicity. However, thus far, toxicogenomic approaches have been focused primarily on short-term (≤28 days) effects and have used a limited number of rodent models, especially rats. In order for the biomarker data accumulated thus, far to be of utmost value, distinctive gene signatures resulting from short-term toxicogenomic studies should be validated by long-term studies employing different animal species. In the present study, we focused on genes that demonstrated sustained up- or down-regulation through the duration of the study period following treatment with AAF and DEN and identified 68 genes that were deregulated in both groups and which demonstrated sustained deregulation throughout the study period (up to 91 days). Of the 68 genes, significant alterations were observed mainly in genes involved in cell cycle arrest (*Cdkn1a*, *Mdm2*, and *Ccng1*) and apoptosis (*Tnfrsf10b* and *Trp53inp1*); these changes could be induced by the activation of p53 following DNA damage caused by exposure to AAF and DEN. The DNA damage response, which is one of the major cellular pathways induced by genotoxic carcinogens, involves activation of the ATM-p53 pathway, which can lead to cell cycle arrest, apoptosis, or DNA repair by activating p53 target molecules (Amundson et al. 2001). Several toxicogenomic studies of rat liver have shown that the transcriptional activation of p53 genes by genotoxic carcinogens increased the expression of *Cdkn1a*, *Ccng1*, *Bax*, *Btg2*, and *Mdm2* (Ellinger-Ziegelbauer et al. 2004, 2005, 2008). The prominent hepatic induction of *Cdkn1a*, *Ccng1*, *Bax*, *Btg2*, and *Mdm2* in response to four genotoxic hepatocarcinogens dimethylnitrosamine, 2-nitrofluorene, aflatoxin B1, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone has been observed up to 14 days in Wistar rats (Ellinger-Ziegelbauer et al. 2004, 2005). Moreover, the deregulation of these five genes in DEN- and ethylnitrourea

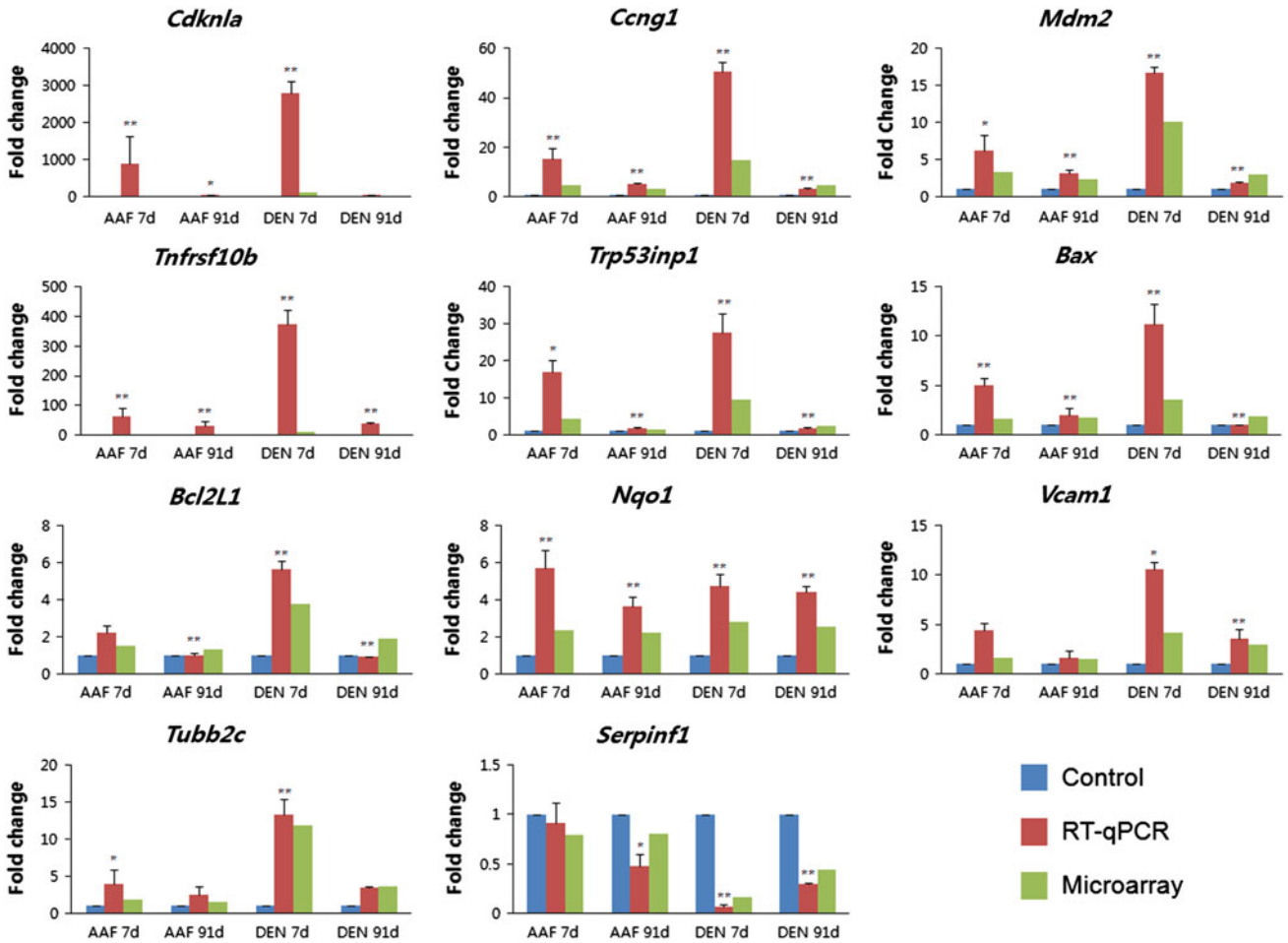

Fig. 5 mRNA expression of 11 selected genes as determined by RT-qPCR and microarray analyses. The gene expression level determined by RT-qPCR was normalized to *Gapdh*. *, **Significantly different from the controls at $P < 0.05$ or 0.01, respectively, according to Student's *t*-test

(ENU)-treated mouse liver was demonstrated by RT-qPCR analysis (Watanabe et al. 2009). Up-regulation of similar p53 target genes induced by genotoxic stress has been also reported in several cell culture models (Amundson et al. 2001; Islaih et al. 2004). CCNG1 is a transcriptional target of p53 that is directly associated with Mdm2 and regulates the accumulation and degradation of p53 in a negative feedback loop (Kimura and Nojima 2002). In response to DNA damage, CCNG1 prevents Mdm2-mediated p53 ubiquitination by associating with the Mdm2-ARF complex, which promotes cell cycle arrest and DNA repair (Amundson et al. 2001). *Cdkn1a*, *Bax*, and *Btg2* have also been shown to be p53-dependent effectors associated with the anti-proliferative pathway triggered in response to DNA damage (Abukhdeir and Park 2008; Rouault et al. 1996). *Cdkn1a* encodes a potent cyclin-dependent kinase inhibitor that mediates the p53-dependent G1 phase arrest by inhibiting cyclin-CDK2 or -CDK4 activity (Abukhdeir and Park 2008). The expression of *Cdkn1a* is tightly controlled by p53 in response to radiation and genotoxic carcinogens (Amundson et al. 2003; Snyder and Morgan 2004). Among the genes selected as candidate biomarkers in the present study, *Cdkn1a* was most prominently induced on day 7 in both the AAF- and DEN-treated mice (Fig. 5). *Cdkn1a* expression was sharply decreased at day 91 but was still up-regulated (≥10-fold) in both the AAF and DEN groups. In addition to *Bax* and *Btg*, two apoptosis- and cell cycle arrest-related genes, *Tp53inp1*and *Tnfrsf10b*, were significantly up-regulated after exposure to AAF and DEN. *Tp53inp1* is a p53 target gene with roles in cellular homeostasis through its anti-proliferative and pro-apoptotic activity response to genotoxic stress (Okamura et al. 2001); moreover, its expression is lost in preneoplastic lesions induced by DEN and AAF in rats (Ogawa et al. 2005). *Tnfrsf10b,* a p53-independent molecule, is known to induce apoptosis by binding to death domain–containing transmembrane receptors, and it preferentially induces apoptosis in cancer cells while exhibiting little or no toxicity in normal cells (LeBlanc and Ashkenazi 2003; MacFarlane et al. 2005). Collectively, the prominent and

sustained up-regulation of p53 target genes including *Cdkn1a*, *Ccng1*, *Bax*, *Btg2*, *Mdm2*, *Tp53inp*, and *Tnfrsf10b* in the present study corresponded with the results of earlier studies, which have been observed in different hepatocarcinogenesis models with a variety of genotoxic stresses (Fig. 5).

In the present study, direct targets of the Ras pathway such as *Raf*, *c-Fos*, *c-Myc*, *JunB*, *Cyclin D1*, and endogenous *Ras* were not examined as part of the 66 gene signature. It has been reported that over-expression of the human c-Ha-ras oncogene plays an important role in the enhanced carcinogenesis in rasH2 mice (Maruyama et al. 2001; Tamaoki 2001). In addition to transgene over-expression, increased levels of endogenous Ha-, N-, and Ki-ras induced by the transgene are thought to be involved in the enhanced carcinogenesis in ENU- and DMBA-treated rasH2 mice (Okamura et al. 2004, 2007). However, McDonald et al. (1994) reported that the over-expression of Ha-, K-, and Ni-ras might mainly be associated with the late stages of tumorigenesis in head and neck cancer in humans. Given this, one could conclude that the expression of endogenous *Ras* genes and target genes downstream of the Ras pathway are unlikely to be affected in the early stages of hepatocarcinogenesis. Furthermore, the fact that the expression of *Ras* genes and downstream effectors is unaffected will facilitate the identification and discrimination of candidate genomic biomarkers capable of predicting the effects of AAF and DEN in rasH2 mice.

In summary, we identified 68 candidate biomarker genes that were deregulated by AAF and DEN and whose expression pattern was clearly discriminated from the control-, Mel-, and ANIT-treated groups through unsupervised hierarchical clustering. This signature, which represents gene expression changes induced by two hepatocarcinogens at early (7 days) and late (91 days) time points, may reflect the early molecular events associated with hepatic carcinogenesis in rasH2 mice and may therefore enable the identification and confirmation of candidate biomarkers of hepatocarcinogenesis.

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