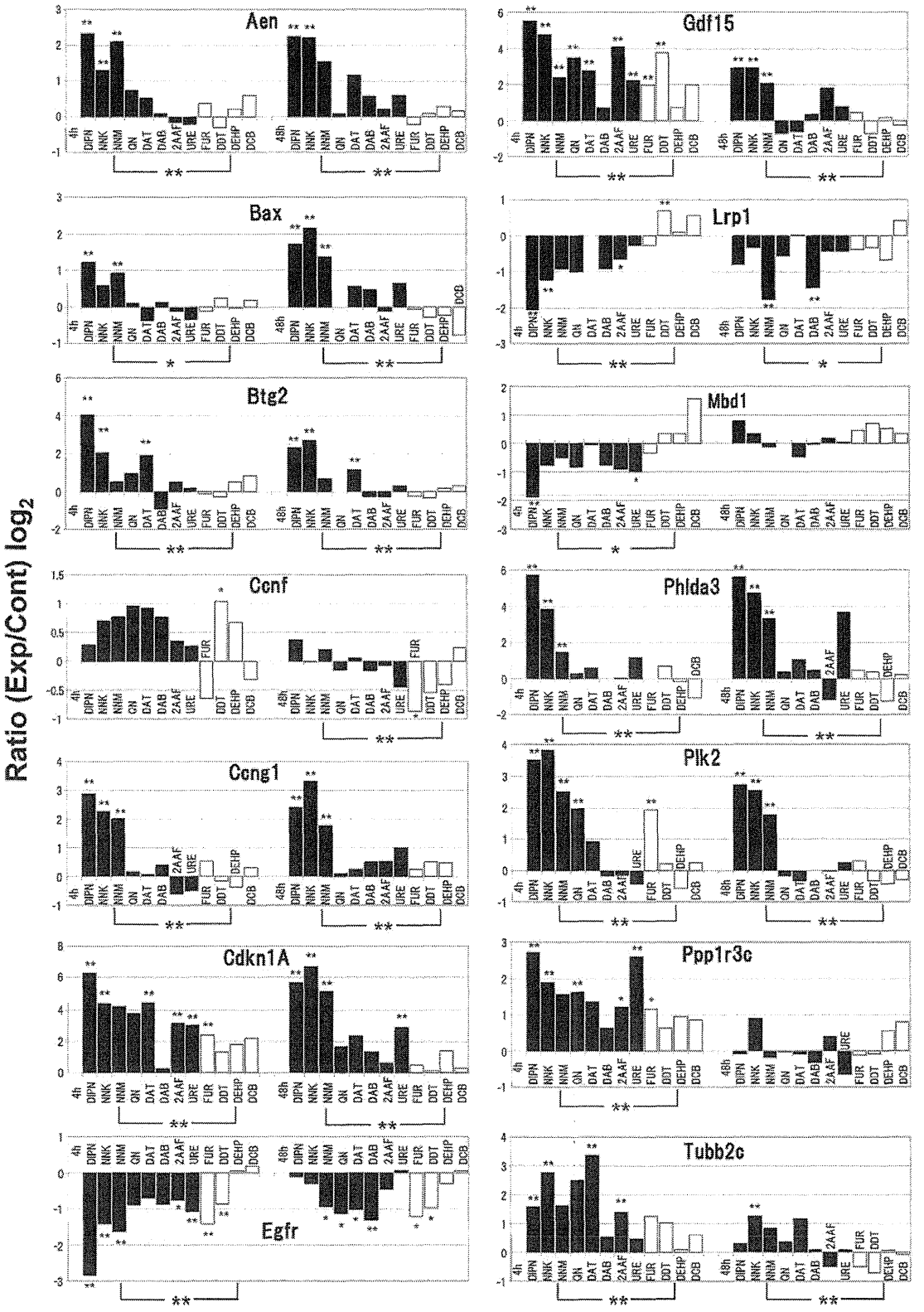

Fig. 1. Changes in the gene expression of 14 major genes (*Aen*, *Bax*, *Btg2*, *Ccnf*, *Ccng1*, *Cdkn1a*, *Egfr*, *Gdf15*, *Lrp1*, *Mbd1*, *Phlda3*, *Plk2*, *Ppp1r3c* and *Tubb2c*) as quantified by qPCR at 4 and 48 h. DIPN: diisopropanolnitrosamine, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNM: *N*-nitrosomorpholine, QN: quinoline, DAT: 2,4-diaminotoluene, DAB: 4-dimethylaminoazobenzene, 2AAF: 2-acetylaminofluorene, URE: urethane, FUR: furan, DDT: dichlorodiphenyltrichloroethane, DEHP: di(2-ethylhexyl)phthalate, DCB: 1,4-dichlorobenzene. The statistical significance for each chemical was analyzed by the Dunnett's test. ⋆: $P < 0.05$, ⋆⋆: $P < 0.01$ at each bar. The statistical analysis for each gene between the genotoxic and non-genotoxic carcinogens was paerformed using the Welch's t-test. ⋆: $P < 0.05$, ⋆⋆: $P < 0.01$ outside the framework. ■: Genotoxic hepatocarcinogen, □: non-genotoxic hepatocarcinogen. Total RNA was extracted from individual livers (5 mice/group) and reverse-transcribed into cDNA. Changes in gene expression were determined in triplicate by qPCR.

Table 2
Thirty-five genes quantified in the present study.

No.	Symbol	Gene name	Accession no.
1	Aen	Apoptosis enhancing nuclease	NM_026531
2	Bax	BCL2-associated X protein	NM_007527
3	Bhlhe40	Basic helix-loop-helix domain containing, Class B2	NM_011498
4	Btg2	B-cell translocation gene 2, anti-proliferative	NM_007570
5	Ccnf	Cyclin F	NM_007634
6	Ccng1	Cyclin G1	NM_009831
7	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	NM_007669
8	Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2	NM_009993
9	Ddit4	DNA-damage-inducible transcript 4	NM_029083
10	Ddit4l	DNA-damage-inducible transcript 4-like	NM_030143
11	Egfr	Epidermal growth factor receptor	NM_207655
12	Ephx1	Epoxide hydrolase 1, microsomal	NM_010145
13	Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	NM_008655
14	Gapdh	glyceraldehyde-3-phosphate dehydrogenase	NM_008084
15	Gdf15	Growth differentiation factor 15	NM_011819
16	Hist1h1c	H1 histone family, member 2	NM_015786
17	Hmox1	Heme oxygenase (decycling) 1	NM_010442
18	Hspb1	Heat shock protein 1	NM_013560
19	Igfbp1	Insulin-like growth factor binding protein 1	NM_008341
20	Jun	Jun oncogene	NM_010591
21	Lrp1	Low density lipoprotein receptor-related protein 1	NM_008512
22	Ly6a	Lymphocyte antigen 6 complex, locus A	NM_010738
23	Mbd1	Methyl-CpG binding domain protein 1	NM_013594
24	Mdm2	Transformed mouse 3T3 cell double minute 2	NM_010786
25	Phlda3	Pleckstrin homology-like domain, family A, member 3	NM_013750
26	Plk2	Polo-like kinase 2; serum-inducible kinase	NM_152804
27	Pml	Promyelocytic leukemia	NM_008884
28	Pmm1	Phosphomannomutase 1	NM_013872
29	Ppp1r3c	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	NM_016854
30	Psma3	Proteasome (prosome, macropain) subunit, alpha type 3	NM_011184
31	Rad52	RAD52 homolog (S. cerevisiae)	NM_011236
32	Rcan1	Regulator of calcineurin 1	NM_001081549
33	St3gal5	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	NM_011375
34	Trp53	Tranformation related protein 53	NM_011640
35	Tubb2c	Tubulin, beta 2c	NM_146116

The genes were selected to discriminate genotoxic carcinogens from non-genotoxic carcinogens based on our previous studies [11,12].

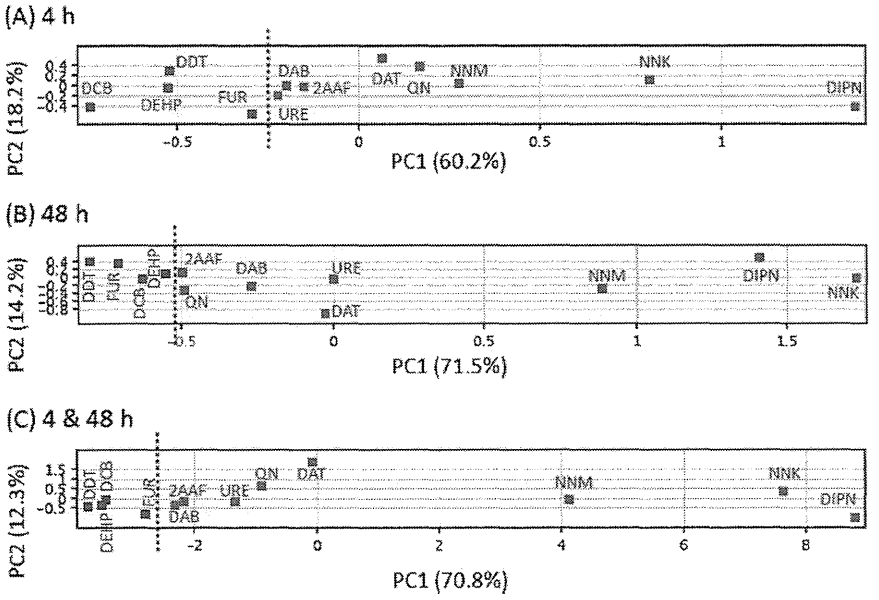

Fig. 2. Principal component analysis (PCA) of the gene expression levels between genotoxic and non-genotoxic hepatocarcinogens as quantified by qPCR. The mean values of triplicate qPCR assays for each chemical were statistically analyzed using PCA programs in GeneSpringGX11.0.1. The results of the PCA are shown in the two-dimensional contribution scores for component numbers 1 and 2 (PC1 and PC2). The contribution scores were produced by conversion from each eigenvector value. A: 4 h with 7 genes (*Btg2*, *Ccnf*, *Ccng1*, *Lrp1*, *Mbd1*, *Phlda3* and *Tubb2c*), B: 48 h with 12 genes (*Aen*, *Bax*, *Btg2*, *Ccnf*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Lrp1*, *Mbd1*, *Phlda3*, *Plk2* and *Tubb2c*) and C: both 4 and 48 h with 12 genes (*Aen*, *Bax*, *Btg2*, *Ccnf*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Lrp1*, *Mbd1*, *Phlda3*, *Plk2* and *Tubb2c*). Genotoxic hepatocarcinogens (red-colored, DIPN: diisopropanolnitrosamine, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNM: *N*-nitrosomorpholine, QN: quinoline, DAT: 2,4-diaminotoluene, DAB: 4-dimethylaminoazobenzene, 2AAF: 2-acetylaminofluorene, URE: urethane) and non-genotoxic hepatocarcinogens (bleu-colored, FUR: furan, DDT: dichlorodiphenyltrichloroethane, DEHP: di(2-ethylhexyl)phthalate, DCB: 1,4-dichlorobenzene). Dashed line is added between genotoxic and non-genotoxic hepatocarcinogens. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 3
Primer sequences of the 35 genes quantified in the preset study.

No.	Symbol	Left	Right	Ct
1	Aen	TTGAAGGGCAAGGTGGTGGTG	GAGCAGGTTTGGGACATAAGTG	27–30
2	Bax	CCAGGATGCGTCCACCAAGAAG	GGAGTCCGTGTCCACGTCAGC	29–33
3	Bhlhe40	CCAGGCCTCAACACCTCAGCTG	CCGAAGAGTCGAGGGACGAATG	24–28
4	Btg2	ACGGGAAGAGAACCGACATGC	ATGATCGGTCAGTGCGTCCTG	24–28
5	Ccnf	AGCACAAAGCCTTGCCACCATC	AAGCCAGGTGCGTGTCCTTGTC	27–31
6	Ccng1	TGGCCGAGATTTGACCTTCTGG	GTGCTTCAGTTGCCGTGCAGTG	27–33
7	Cdkn1a	TCCCGTGGACAGTGAGCAGTTG	CGTCTCCGTGACGAAGTCAAAG	25–35
8	Cyp1a2	GATGCTCTTCGGCTTGGGAAAG	CCATAGTTGGGTGTCAGGTCCAC	23–30
9	Ddit4	GCACCTGTGTGCCAACCTGATG	TGTATGCCAGGCGCAGGAGTTC	34–44
10	Ddit4l	ACCAGCTTGGCTGGGACAAATG	CGTGCTCATTGGAACAGTGATG	33–36
11	Egfr	AGAGCGCCTTCCACAGCCAC	ACTCTCGGAACTTTGGGCGG	24–29
12	Ephx1	CATTGTCTCCTCCCAGCGCTTC	GGGCATGCAGGATCTCAGAAGG	20–26
13	Gadd45b	TGTACGAGGCGGCCAAACTG	TGTCGCAGCAGAACGACTGG	23–28
14	Gapdh	GCTCTCAATGACAACTTTGTCAAG	TCCTTGGAGGCCATGTAGGC	24–27
15	Gdf15	AGCTGGAACTGCGCTTACGGG	CTCCAGCCCAAGTCTTCAAGAG	25–30
16	HistH1	CGAGCTCATCACCAAGGCTGTG	CCCTTGCTCACCAGGCTCTTC	27–31
17	Hmox1	AAGACCGCCTTCCTGCTCAAC	CGAAGTGACGCCATCTGTGAGG	24–45
18	Hspb1	CGGTGCTTCACCCGGAAATAC	GCTGACTGCGTGACTGCTTTGG	23–29
19	Igfbp1	GATCAGCCCATCCTGTGGAACG	TTCTCGTTGGCAGGGCTCCTTC	24–28
20	Jun	GCCAAGAACTCGGACCTTCTC	AGTGGTGATGTGCCCATTGCTG	22–29
21	Lrp1	GGGCCATGAATGTGGAAATTGG	GTGGCATACACTGGGTTGGTG	21–36
22	Ly6a	CTTGTGGCCCTACTGTGTGCAG	GGGCAGGTAATTGATGGGCAAG	27–33
23	Mbd1	GGATCCTGACACTCAAGAATGG	GTTTGGGCTAACACAGGAAGAG	21–24
24	Mdm2	TTGATCCGAGCCTGGGTCTGTG	AAGATCCTGATGCGAGGGCGTC	26–32
25	Phlda3	TGGCTGGAACGCTCAGATCAC	TTAGGACACAAGGGTCCCAGTC	22–29
26	Plk2	CTGTTGAGAGCGTCTTCAGTTG	CCATAGTTCACAGTTAAGCAGC	28–32
27	Pml	GGCAAGAAGCGTCCTTACCTTC	GGACAGCAACAGCAGTTCAGTC	26–31
28	Pmm1	TGTCCCGAGGAGGCATGATAAG	CAAAGTCATTCCCGCCAGGAC	25–29
29	Ppp1r3c	TGGAAACCTGACGGAGTGCAG	GCAAGCCTTGGACTGCCAAAG	24–28
30	Psma3	GATCGACCCATCAGGTGTTTC	CACGGCAAGTCATTTCCTTCATCTG	24–28
31	Rad52	TGACGCCACTCACCAGAGGAAG	GCTGGAAGTACCGCATGCTTGG	31–33
32	Rcan1	GGTCCACGTGTGTGAGAGTG	TGGATGGGTGTGTACTCCGG	28–32
33	St3gal5	GCAGGTCATGCACAATGTGACC	CTGGGTGAGGTTTGCCGTGTTC	23–30
34	Trp53	TTGGACCCTGGCACCTACAATG	GCAGACAGGCTTTGCAGAATGG	25–30
35	Tubb2c	TTGGCAACAGCACCGCTATTC	TCGGACACCAGGTCGTTCATG	29–33

The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold under the present experimental condition.

3.2. Discrimination of the gene expression between genotoxic and non-genotoxic hepatocarcinogens by PCA

Discrimination of the gene expression profile between the genotoxic and non-genotoxic hepatocarcinogens was achieved by statistical analysis using PCA. PCA of all 34 genes was unable to discriminate the genotoxic from the non-genotoxic hepatocarcinogens. Therefore, we selected specific genes to obtain an optimal separation between the two types of hepatocarcinogens using PCA. PCA of 7 genes (*Btg2*, *Ccnf*, *Ccng1*, *Lrp1*, *Mbd1*, *Phlda3* and *Tubb2c*) at 4 h (Fig. 2A) and of 12 genes (*Aen*, *Bax*, *Btg2*, *Ccnf*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Lrp1*, *Mbd1*, *Phlda3*, *Plk2* and *Tubb2c*) at 48 h (Fig. 2B) successfully discriminated the genotoxic from the non-genotoxic hepatocarcinogens. The genotoxic hepatocarcinogens exhibited a first principal component (PC1) greater than −0.23 and the non-genotoxic hepatocarcinogens exhibited a PC1 less than −0.30 at 4 h (Fig. 2A). At 48 h, the genotoxic hepatocarcinogens exhibited a PC1 greater than −0.50 and the non-genotoxic hepatocarcinogens exhibited a PC1 less than −0.55 (Fig. 2B). When the results at 4 and 48 h were combined, PCA of the same 12 genes discriminate the genotoxic from the non-genotoxic hepatocarcinogens (Fig. 2C). The Genotoxic hepatocarcinogens exhibited a PC1 greater than −2.3 and the non-genotoxic hepatocarcinogens exhibited a PC1 less than −2.8 when the 4 and 48 h time points were combined (Fig. 2C).

Additionally, the 3 *N*-nitroso genotoxic hepatocarcinogens, NNK, DIPN and NNM, were distinguished from the 5 other genotoxic hepatocarcinogens by PCA with the present genes (Fig. 2A, B and C).

3.3. Gene ontology and the biologically relevant gene networks

We analyzed the gene ontology of the examined genes using Gene Ontology (in Mus musculus), and the results are shown in Table 5. Seven major biological processes were extracted from this analysis. The first process, containing 16 genes, was associated with apoptosis; the second was associated with the cell cycle and contained 10 genes; the third was associated with cell proliferation, containing 10 genes; the fourth process, containing 13 genes, was associated with DNA damage; the fifth was associated with DNA repair, containing 3 genes; the sixth was associated with oncogenes and contained 2 genes; and the seventh was associated with tumor suppression and contained 3 genes. Fourteen genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Ddit4*, *Gdf15*, *Hist1h1c*, *Hmox1*, *Hspb1*, *Mdm2*, *Phlda3*, *Plk2* and *Pml*) from the present study were reported to be associated with *Trp53*. Among these, 8 genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Phlda3* and *Plk2*) contributed to the discrimination of the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogens by PCA. The DNA damage response, which works via signal transduction by a p53-class mediator and results in the induction of apoptosis, was characteristically suggested as an associated biological process.

To further understand the biological networks of the examined genes, we subsequently analyzed their biological interactions using IPA and GeneSpring. Three similar gene networks were extracted for each chemical from the 4 and 48 h data points when analyzed by IPA using the numerical data [Ratio log2 (experimental group/control group)] for all 34 genes. The associated gene network functions, as determined by IPA, are shown in Table 6, with

Table 4.1
Gene expression ratio (Exp/Cont) and Dunnett's test of genotoxic hepatocarcinogens at 4 h.

No.	Symbol	Mean ± SD and Dunnett's test							
		DIPN	NNK	NNM	QN	DAT	2AAF	DAB	URE
1	Aen	5.00 ± 2.18**	2.45 ± 0.74**	4.28 ± 3.05**	1.67 ± 0.98	1.44 ± 0.90	0.90 ± 0.19	1.06 ± 0.59	0.86 ± 0.05
2	Bax	2.33 ± 0.19**	1.48 ± 0.24	1.90 ± 0.37**	1.08 ± 0.16	0.77 ± 0.08	0.91 ± 0.10	1.09 ± 0.41	0.79 ± 0.15
3	Bhlhe40	1.01 ± 0.21	0.78 ± 0.08	1.25 ± 0.22	1.51 ± 0.48	1.90 ± 0.41**	1.44 ± 0.38	1.00 ± 0.50	0.77 ± 0.15
4	Btg2	16.5 ± 4.93**	4.13 ± 0.44**	1.43 ± 0.48	1.88 ± 0.77	3.76 ± 1.43**	1.43 ± 0.34	0.52 ± 0.28	1.13 ± 0.30
5	Ccnf	1.21 ± 0.36	1.62 ± 0.26	1.70 ± 0.62	1.93 ± 0.43	1.90 ± 0.83	1.28 ± 0.41	1.70 ± 0.78	1.20 ± 0.59
6	Ccng1	7.22 ± 2.38**	4.76 ± 0.99**	4.04 ± 3.14*	1.11 ± 0.35	1.05 ± 0.52	0.66 ± 0.07	1.33 ± 0.47	0.71 ± 0.15
7	Cdkn1a	77.2 ± 3.91**	20.3 ± 2.15**	18.4 ± 14.5	13.6 ± 8.33	21.4 ± 19.3*	8.71 ± 4.08**	1.23 ± 0.83	8.23 ± 6.82**
8	Cyp1a2	0.70 ± 0.13	0.37 ± 0.17**	1.25 ± 0.31	0.95 ± 0.16	1.16 ± 0.20	0.85 ± 0.22	1.03 ± 0.23	0.86 ± 0.13
9	Ddit4	7.85 ± 0.72**	4.73 ± 0.98**	1.77 ± 1.91	3.17 ± 1.43	1.76 ± 0.98	5.69 ± 1.27**	1.19 ± 0.91	2.61 ± 1.17*
10	Ddit4l	25.7 ± 4.50**	3.42 ± 0.87**	1.66 ± 0.73	1.32 ± 0.18	0.71 ± 0.10	0.48 ± 0.16	0.75 ± 0.42	3.95 ± 5.65
11	Egfr	0.14 ± 0.03**	0.37 ± 0.06**	0.32 ± 0.17**	0.54 ± 0.23	0.62 ± 0.52	0.59 ± 0.26*	0.55 ± 0.20	0.47 ± 0.16**
12	Ephx1	0.57 ± 0.36	0.80 ± 0.17	1.33 ± 0.99	1.05 ± 0.47	1.11 ± 0.47	0.74 ± 0.20	1.19 ± 0.47	0.66 ± 0.32
13	Gadd45b	3.37 ± 0.66*	14.3 ± 6.41**	1.31 ± 0.80	1.96 ± 0.81	1.12 ± 0.45	18.5 ± 12.4**	0.83 ± 0.26	3.49 ± 2.39*
14	Gdf15	46.5 ± 16.5**	27.7 ± 6.21**	5.28 ± 2.65	6.8 ± 3.91*	11.3 ± 4.51**	17.2 ± 6.90**	1.62 ± 0.60	4.71 ± 2.50**
15	Hist1h1c	1.22 ± 0.32	0.68 ± 0.07	1.82 ± 0.92	1.98 ± 0.96	2.22 ± 1.07	0.57 ± 0.16*	1.02 ± 0.30	0.99 ± 0.34
16	Hmox1	2.11 ± 0.60*	0.65 ± 0.27	1.64 ± 1.42	9.78 ± 2.99**	2.39 ± 2.02	1.31 ± 0.60	1.78 ± 1.14	1.88 ± 0.64
17	Hspb1	1.71 ± 0.19**	1.18 ± 0.44	1.43 ± 0.82	12.4 ± 12.6*	2.69 ± 1.55	0.49 ± 0.05	1.31 ± 0.57	0.47 ± 0.27
18	Igfbp1	4.45 ± 2.06**	1.73 ± 1.11	0.60 ± 0.83	2.40 ± 1.77	5.27 ± 2.99**	1.60 ± 0.39	0.27 ± 0.24	10.8 ± 5.42**
19	Jun	9.32 ± 2.15**	14.2 ± 3.37**	1.67 ± 1.25	11.3 ± 17.6	8.44 ± 8.21	2.24 ± 0.51**	0.82 ± 0.50	1.50 ± 0.77
20	Lrp1	0.24 ± 0.04**	0.42 ± 0.03**	0.53 ± 0.24	0.50 ± 0.43	0.99 ± 0.97	0.63 ± 0.14*	0.53 ± 0.40	0.82 ± 0.23
21	Ly6a	1.29 ± 0.32	1.11 ± 0.27	1.46 ± 1.06	1.38 ± 0.41	1.11 ± 0.61	0.86 ± 0.41	1.24 ± 0.43	0.92 ± 0.40
22	Mbd1	0.27 ± 0.05**	0.59 ± 0.31	0.70 ± 0.21	0.56 ± 0.27	0.96 ± 0.42	0.54 ± 0.49	0.59 ± 0.34	0.50 ± 0.29*
23	Mdm2	6.22 ± 2.96**	1.98 ± 0.83*	3.39 ± 0.65**	4.2 ± 1.63**	2.52 ± 0.28*	0.93 ± 0.12	0.98 ± 0.29	0.98 ± 0.16
24	Phlda3	54.1 ± 8.11**	13.9 ± 5.53**	2.75 ± 1.54*	1.24 ± 0.26	1.50 ± 0.83	1.01 ± 0.34	1.00 ± 0.48	2.19 ± 2.21
25	Plk2	11.4 ± 1.14**	14.2 ± 2.26**	5.73 ± 1.58**	3.88 ± 1.99**	1.91 ± 0.35	0.91 ± 0.26	0.89 ± 0.10	0.75 ± 0.42
26	Pml	0.76 ± 0.26	1.02 ± 0.15	1.47 ± 0.60	1.00 ± 0.29	0.88 ± 0.31	1.09 ± 0.19	0.71 ± 0.20	0.77 ± 0.19
27	Pmm1	0.96 ± 0.20	0.88 ± 0.22	1.39 ± 0.33	2.25 ± 0.37**	1.71 ± 0.67*	1.43 ± 0.10	0.98 ± 0.37	0.81 ± 0.29
28	Ppp1r3c	6.55 ± 2.84**	3.70 ± 0.42**	2.97 ± 1.74	3.08 ± 1.07*	2.53 ± 1.59	2.33 ± 0.51*	1.53 ± 0.68	6.07 ± 4.34**
29	Psma3	0.90 ± 0.25	0.55 ± 0.09*	1.04 ± 0.58	1.14 ± 0.21	1.53 ± 0.52	0.80 ± 0.33	1.05 ± 0.60	1.04 ± 0.63
30	Rad52	1.46 ± 0.33	0.56 ± 0.50	0.98 ± 0.19	0.97 ± 0.11	0.91 ± 0.06	0.45 ± 0.24	0.85 ± 0.22	1.22 ± 0.27
31	Rcan1	2.00 ± 1.05	4.43 ± 1.29**	0.73 ± 0.63	7.68 ± 5.77*	7.00 ± 4.65*	1.63 ± 0.43	1.15 ± 1.05	0.87 ± 0.14
32	St3gal5	0.79 ± 0.20	1.02 ± 0.11	1.08 ± 0.79	1.54 ± 0.52	2.79 ± 0.98**	2.21 ± 0.57**	1.03 ± 0.46	2.21 ± 0.41**
33	Trp53	1.13 ± 0.17	1.29 ± 0.17	1.04 ± 0.31	1.33 ± 0.39	0.72 ± 0.15	0.82 ± 0.42	0.78 ± 0.25	0.82 ± 0.15
34	Tubb2c	3.01 ± 0.51**	6.85 ± 0.15**	3.12 ± 1.01	5.72 ± 2.60	10.3 ± 7.57**	2.61 ± 0.77**	1.46 ± 0.64	1.38 ± 0.51
35	Gapdh	0.88 ± 0.13	0.84 ± 0.33	1.18 ± 0.41	0.73 ± 0.09	0.93 ± 0.34	0.87 ± 0.16	1.16 ± 0.37	1.16 ± 0.39

Total RNA was extracted from the individual liver and cDNA was prepared. The expression of the 35 genes was quantified by quantitative real-time PCR and the gene expression ratio (exp/cont) was calculated. The results were analyzed by Dunnett's test (**: significant by $P<0.01$. *: significant by $P<0.05$).

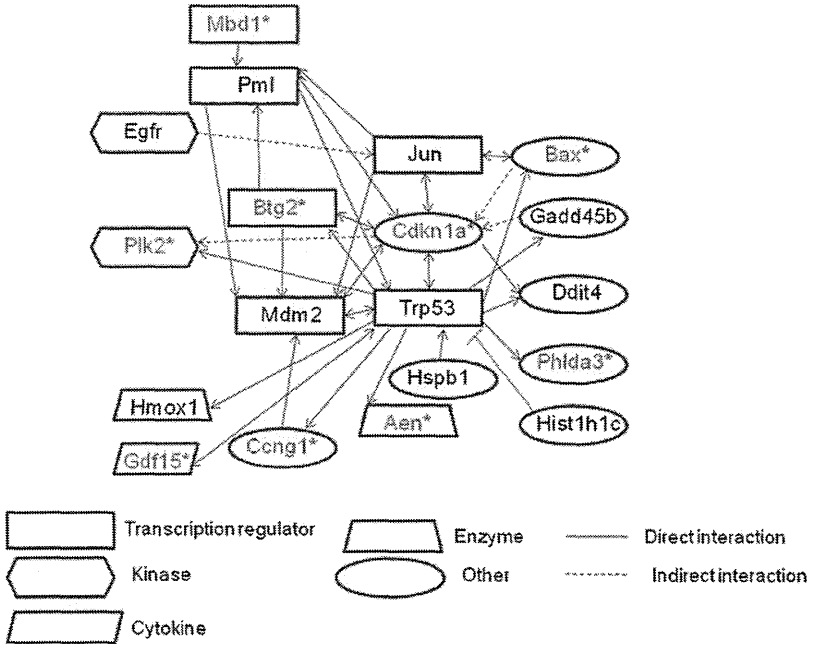

Fig. 3. The gene networks and pathways of 19 genes as determined by qPCR. The network was combined from the results of Ingenuity Pathways Analysis, GeneSpring software and references from PubMed. The 9 red-colored genes indicated by "*" mark genes that significantly contributed to the discrimination of the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogens by PCA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 4.2
Gene expression ratio (Exp/Cont) and Dunnett's test of non-genotoxic hepatocarcinogens and Welch's t-test at 4 h.

No.	Symbol	Mean ± SD and Dunnett's test				Welch's test
		FUR	DDT	DEHP	DCB	G vs NG
1	Aen	1.30 ± 0.16	0.81 ± 0.20	1.16 ± 0.23	1.51 ± 0.61	$P<0.01$
2	Bax	0.91 ± 0.08	1.17 ± 0.09	0.98 ± 0.13	1.14 ± 0.35	$P<0.05$
3	Bhlhe40	0.83 ± 0.14	2.68 ± 0.84**	0.93 ± 0.27	1.67 ± 0.33	
4	Btg2	0.90 ± 0.22	0.81 ± 0.12	1.44 ± 0.38	1.80 ± 0.41	$P<0.01$
5	Ccnf	0.64 ± 0.17	2.05 ± 0.63*	1.59 ± 0.32	0.80 ± 1.00	
6	Ccng1	1.44 ± 0.32	0.90 ± 0.18	0.77 ± 0.08	1.22 ± 0.27	$P<0.01$
7	Cdkn1a	5.37 ± 0.94**	2.48 ± 4.19	3.35 ± 0.98	4.5 ± 2.97	$P<0.01$
8	Cyp1a2	0.69 ± 0.48	0.80 ± 0.13	0.83 ± 0.27	1.65 ± 0.49	
9	Ddit4	1.93 ± 1.12	3.20 ± 2.19	4.29 ± 1.76**	6.70 ± 3.59	
10	Ddit4l	1.17 ± 0.56	0.43 ± 0.23	0.66 ± 0.40	2.82 ± 2.33	
11	Egfr	0.37 ± 0.05**	0.55 ± 0.24**	1.03 ± 0.11	1.13 ± 0.59	$P<0.01$
12	Ephx1	1.41 ± 0.31	0.74 ± 0.30	0.61 ± 0.07	1.02 ± 0.40	
13	Gadd45b	2.81 ± 2.08	1.10 ± 1.10	1.51 ± 0.84	0.89 ± 0.32	
14	Gdf15	3.98 ± 1.18**	13.5 ± 3.64**	1.67 ± 0.70	4.04 ± 2.56	$P<0.01$
15	Hist1h1c	0.32 ± 0.15**	0.64 ± 0.12	0.41 ± 0.06**	2.12 ± 1.55	$P<0.01$
16	Hmox1	8.19 ± 2.78**	1.81 ± 0.70	0.94 ± 0.34	14.9 ± 11.4*	
17	Hspb1	1.91 ± 0.55*	0.63 ± 0.21	0.81 ± 0.36	0.42 ± 0.36	
18	Igfbp1	1.92 ± 0.35	0.75 ± 1.12	1.04 ± 0.44	5.84 ± 4.36	
19	Jun	6.75 ± 1.67**	1.64 ± 0.37	1.42 ± 0.41	2.75 ± 0.81*	$P<0.05$
20	Lrp1	0.82 ± 0.10	1.60 ± 0.10**	1.06 ± 0.43	1.45 ± 0.59	$P<0.01$
21	Ly6a	1.05 ± 0.26	1.05 ± 0.72	0.84 ± 0.07	0.84 ± 0.32	
22	Mbd1	0.79 ± 0.35	1.27 ± 0.89	1.26 ± 0.73	2.92 ± 2.87	$P<0.05$
23	Mdm2	2.67 ± 0.50**	0.83 ± 0.17	0.97 ± 0.21	1.58 ± 0.12	$P<0.01$
24	Phlda3	0.99 ± 0.36	1.57 ± 0.29	0.88 ± 0.27	0.46 ± 0.45	$P<0.01$
25	Plk2	3.84 ± 1.21**	1.16 ± 0.38	0.67 ± 0.26	1.20 ± 0.49	$P<0.01$
26	Pml	1.07 ± 0.12	1.45 ± 0.48	1.28 ± 0.14	0.83 ± 0.16	$P<0.05$
27	Pmm1	0.71 ± 0.16	1.19 ± 0.16	1.16 ± 0.25	0.78 ± 0.49	
28	Ppp1r3c	2.22 ± 0.65*	1.55 ± 1.23	1.91 ± 0.73	1.75 ± 0.39	$P<0.01$
29	Psma3	0.68 ± 0.24	0.80 ± 0.21	0.97 ± 0.14	1.67 ± 0.79	
30	Rad52	1.12 ± 0.11	1.05 ± 0.45	0.91 ± 0.88	0.90 ± 0.16	
31	Rcan1	5.05 ± 0.46**	0.99 ± 0.49	1.07 ± 0.33	1.06 ± 0.68	
32	St3gal5	0.50 ± 0.05**	1.28 ± 0.48	1.62 ± 0.18	3.21 ± 0.90	
33	Trp53	1.25 ± 0.21	1.26 ± 0.21	1.19 ± 0.33	0.77 ± 0.16	
34	Tubb2c	2.42 ± 0.41**	2.03 ± 0.22**	1.08 ± 0.22	1.53 ± 1.33	$P<0.01$
35	Gapdh	1.31 ± 0.24	1.13 ± 0.16	1.04 ± 0.21	1.10 ± 0.93	

Total RNA was extracted from the individual liver and cDNA was prepared. The expression of the 35 genes was quantified by quantitative real-time PCR and the gene expression ratio (exp/cont) was calculated. The results were analyzed by Dunnett's test (**: significant by $P<0.01$. *: significant by $P<0.05$). The results of genotoxic hepatocarcinogens (G) were compared to non-genotoxic hepatocarcinogens (NG) by Welch's t-test.

2AAF at 4 h as a representative. The top functions of network 1 and 3 were cellular growth and proliferation and the cell cycle, respectively. Those of network 2 were the cell cycle, cell death and cellular growth and proliferation. The summarized gene networks are shown in Fig. 3. The major gene pathway suggested by the network was the *Trp53*-mediated DNA damage response pathway.

4. Discussion

In the present study, we used qPCR to quantify the expression levels of 35 genes selected from our previous DNA microarray studies upon exposure to 12 different chemicals to discriminate genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens in the mouse liver at 4 and 48 h after a single intraperitoneal injection. In effect, we were able to distinguish the 8 genotoxic hepatocarcinogens from the 4 non-genotoxic hepatocarcinogens by statistical analysis using PCA (Fig. 2). The PCA discrimination was successful for 7 genes from the gene expression profiles (*Btg2*, *Ccnf*, *Ccng1*, *Lpr1*, *Mbd1*, *Phlda3* and *Tubb2c*) at the 4 h time point and for 12 genes (*Aen*, *Bax*, *Btg2*, *Ccnf*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Lrp1*, *Mbd1*, *Phlda3*, *Plk2* and *Tubb2c*) at the 48 h time point. When the results for both time points were combined, the genotoxic hepatocarcinogens were distinguished from the non-genotoxic hepatocarcinogens by the same 12 genes, including the 7 genes observed at 4 h. Moreover, the 12 genes showed similar changes at both 4 and 48 h. We were also able to use the same 12 genes to distinguish the genotoxic from the non-genotoxic hepatocarcinogens at the 4 h time point (data not shown), but the discrimination was less than that obtained with the aforementioned 7 genes. Six of the 7 genes selected at 4 h (excluding *Ccnf*) and 11 of the 12 genes selected at 48 h (excluding *Mbd1*) exhibited statistically significant differences between the genotoxic and non-genotoxic hepatocarcinogens determined by the Welch's t-test (Tables 4.1–4.4).

Each gene was associated with multiple biological processes based on their Gene Ontology classifications. Six of the 12 selected genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a* and *Phlda3*) were classified as DNA damage-associated genes, and 7 genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Lrp1* and *Phlda3*) were classified as apoptosis-associated genes (Table 5). The major biologically relevant gene pathway that resulted from the network analysis was a *Trp53*-mediated signaling pathway (Fig. 3) associated with the DNA damage response. Nine of the 12 PCA-contributed genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Mbd1*, *Phlda3* and *Plk2*) are known to be associated with the *Trp53*-mediated signaling pathway, as shown in Fig. 3. The DNA damage response, through signal transduction by a p53 class mediator resulting in the induction of apoptosis, was characteristically suggested for the genes that contributed to the discrimination of the genotoxic from the non-genotoxic hepatocarcinogens. Fourteen of the genes identified in this study have been reported to be directly associated with *Trp53* (*Aen* [28], *Bax*, *Cdkn1a*, *Mdm2* [29], *Btg2* [30], *Ccng1* [31], *Ddit4* [32], *Gdf15* [33], *Hist1h1c*: [34], *Hmox1* [35], *Hspb1* [36], *Phlda3* [37], *Plk2* [38], *Pml* [39]) (Fig. 3). Among these, 11 genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Hist1h1c*, *Mdm2*, *Phlda3*, *Plk2* and *Pml*) showed statistical significance between the genotoxic and non-genotoxic hepatocarcinogens when analyzed by the Welch's t-test at 4 and/or 48 h (Tables 4.2 and 4.4). The PCA results

Table 4.3
Gene expression ratio (Exp/Cont) and Dunnett's test of genotoxic hepatocarcinogens at 48 h.

No.	Symbol	Mean ± SD and Dunnett's test							
		DIPN	NNK	NNM	QN	DAT	2AAF	DAB	URE
1	Aen	4.69 ± 0.75**	4.68 ± 1.32**	2.89 ± 1.17	1.06 ± 0.72	2.21 ± 2.06	1.17 ± 0.20	1.50 ± 0.75	1.53 ± 0.31
2	Bax	3.25 ± 0.98**	4.47 ± 0.53**	2.57 ± 1.25**	1.00 ± 0.32	1.45 ± 0.47	0.92 ± 0.09	1.38 ± 0.62	1.55 ± 0.34
3	Bhlhe40	0.58 ± 0.09	0.48 ± 0.17**	0.53 ± 0.18*	0.97 ± 0.52	0.62 ± 0.17	0.43 ± 0.10**	0.59 ± 0.12	0.99 ± 0.48
4	Btg2	5.01 ± 1.55**	6.55 ± 1.16**	1.61 ± 0.88	1.00 ± 0.29	2.2 ± 0.44**	0.81 ± 0.15	0.83 ± 0.27	1.21 ± 0.47
5	Ccnf	1.29 ± 0.55	1.64 ± 0.40	1.15 ± 0.20	0.90 ± 0.21	1.04 ± 0.12	0.94 ± 0.13	0.89 ± 0.16	0.74 ± 0.17
6	Ccng1	5.23 ± 1.35**	9.76 ± 1.83**	3.4 ± 1.06**	1.07 ± 0.58	1.17 ± 0.15	1.46 ± 0.37	1.40 ± 0.35	2.01 ± 0.72
7	Cdkn1a	51.2 ± 14.5**	103 ± 10.8**	34.5 ± 8.72**	3.10 ± 2.24	4.97 ± 2.59	1.55 ± 0.35	2.50 ± 1.00	7.22 ± 2.44**
8	Cyp1a2	0.50 ± 0.09*	0.34 ± 0.16**	0.47 ± 0.27	0.96 ± 0.34	0.34 ± 0.24*	1.23 ± 0.17	0.91 ± 0.66	0.81 ± 0.73
9	Ddit4	1.05 ± 1.42	2.32 ± 0.46*	3.35 ± 1.84**	0.94 ± 0.38	2.39 ± 1.07	0.84 ± 0.24	1.33 ± 0.52	1.94 ± 0.99
10	Ddit4l	4.59 ± 1.15**	3.71 ± 1.18**	2.31 ± 1.07	1.25 ± 0.83	1.61 ± 1.19	0.32 ± 0.14	1.21 ± 0.47	1.29 ± 0.46
11	Egfr	0.92 ± 0.23	0.81 ± 0.26	0.52 ± 0.38*	0.45 ± 0.24*	0.49 ± 0.26*	0.73 ± 0.09	0.40 ± 0.12**	1.04 ± 0.10
12	Ephx1	3.47 ± 2.95	2.43 ± 0.40**	2.45 ± 0.79**	0.98 ± 0.40	0.61 ± 0.27	0.96 ± 0.22	1.82 ± 0.36	1.42 ± 0.44
13	Gadd45b	2.27 ± 1.52	12.3 ± 4.91**	1.52 ± 1.00	2.26 ± 1.29	4.24 ± 3.17*	1.24 ± 0.60	1.01 ± 0.25	0.98 ± 0.34
14	Gdf15	7.40 ± 4.85**	7.54 ± 4.22**	4.11 ± 2.26**	0.61 ± 0.27	0.65 ± 0.31	3.51 ± 6.00	1.29 ± 0.66	1.74 ± 0.71
15	Hist1h1c	3.01 ± 0.89**	1.19 ± 0.28	1.19 ± 0.36	0.64 ± 0.18	0.86 ± 0.56	0.99 ± 0.23	1.02 ± 0.77	0.88 ± 0.13
16	Hmox1	0.61 ± 0.20	1.34 ± 0.26	0.93 ± 0.46	1.92 ± 1.70	2.11 ± 1.44	1.06 ± 0.10	1.69 ± 0.91	0.73 ± 0.06
17	Hspb1	1.03 ± 0.12	3.47 ± 1.36**	1.26 ± 1.09	0.52 ± 0.30	1.16 ± 1.29	0.83 ± 0.27	0.54 ± 0.36	1.10 ± 0.56
18	Igfbp1	1.15 ± 0.43	10.9 ± 1.56	0.92 ± 0.46	1.44 ± 0.91	1.98 ± 1.08	0.78 ± 0.45	0.76 ± 0.31	0.71 ± 0.29
19	Jun	2.23 ± 0.43**	3.01 ± 1.30**	2.35 ± 1.50	0.85 ± 0.32	2.09 ± 2.12	1.33 ± 0.80	1.24 ± 0.38	0.84 ± 0.26
20	Lrp1	0.57 ± 0.31	0.80 ± 0.17	0.29 ± 0.24**	0.67 ± 0.15	1.01 ± 0.46	0.74 ± 0.28	0.36 ± 0.22**	0.74 ± 0.10
21	Ly6a	5.62 ± 1.74**	1.36 ± 0.27	2.99 ± 0.44**	0.93 ± 0.42	1.38 ± 0.51	0.51 ± 0.12	1.30 ± 0.48	0.8 ± 0.35
22	Mbd1	1.71 ± 1.03	1.26 ± 0.68	0.91 ± 0.73	1.01 ± 0.44	0.73 ± 0.68	1.14 ± 0.30	0.98 ± 0.79	1.04 ± 0.33
23	Mdm2	4.24 ± 0.63**	3.75 ± 1.02**	2.38 ± 1.10*	0.75 ± 0.22	1.18 ± 1.27	1.14 ± 0.13	0.68 ± 0.30	1.21 ± 0.34
24	Phlda3	49.9 ± 15.4**	26.8 ± 10.5**	9.58 ± 3.63**	1.26 ± 0.71	2.02 ± 1.87	0.44 ± 0.10	1.36 ± 0.26	12.7 ± 9.55
25	Plk2	6.60 ± 1.19**	5.76 ± 0.33**	3.44 ± 1.20**	0.88 ± 0.28	0.79 ± 0.18	0.99 ± 0.16	1.01 ± 0.20	1.18 ± 0.56
26	Pml	1.31 ± 0.44	4.04 ± 0.72**	1.16 ± 0.45	0.67 ± 0.17	0.91 ± 0.24	1.66 ± 0.12	0.82 ± 0.31	0.83 ± 0.26
27	Pmm1	3.82 ± 1.26**	10.9 ± 3.15**	0.80 ± 0.17	0.65 ± 0.35	0.64 ± 0.33	1.11 ± 0.20	0.56 ± 0.42	1.05 ± 0.09
28	Ppp1r3c	0.94 ± 0.24	1.86 ± 0.55	0.88 ± 0.12	0.97 ± 0.37	0.94 ± 0.32	1.30 ± 0.53	0.80 ± 0.24	0.64 ± 0.13
29	Psma3	0.93 ± 0.35	2.68 ± 0.73**	0.61 ± 0.43	0.73 ± 0.46	0.38 ± 0.20	1.30 ± 0.06	0.82 ± 0.25	0.86 ± 0.35
30	Rad52	0.96 ± 0.34	2.34 ± 0.73**	0.79 ± 0.14	0.70 ± 0.21	1.24 ± 0.56	1.19 ± 0.40	0.93 ± 0.18	0.59 ± 0.10
31	Rcan1	0.52 ± 0.36	1.22 ± 0.06	1.70 ± 0.86	0.98 ± 0.52	1.71 ± 1.37	0.79 ± 0.43	1.35 ± 0.97	1.27 ± 0.26
32	St3gal5	1.80 ± 0.18	2.18 ± 1.23**	1.89 ± 0.87	1.20 ± 0.40	1.72 ± 0.48	1.08 ± 0.34	1.11 ± 0.49	1.19 ± 0.11
33	Trp53	1.32 ± 0.23	1.89 ± 0.27**	1.15 ± 0.25	0.70 ± 0.23	1.14 ± 0.34	1.16 ± 0.10	0.86 ± 0.28	0.87 ± 0.22
34	Tubb2c	1.22 ± 0.14	2.39 ± 1.04**	1.78 ± 0.74	1.28 ± 1.05	2.24 ± 1.08	0.70 ± 0.19	1.06 ± 0.66	1.07 ± 0.53
35	Gapdh	0.88 ± 0.16	1.06 ± 0.46	0.72 ± 0.14	0.85 ± 0.12	0.81 ± 0.41	0.93 ± 0.11	1.20 ± 0.25	1.33 ± 0.19

Total RNA was extracted from the individual liver and cDNA was prepared. The expression of the 35 genes was quantified by quantitative real-time PCR and the gene expression ratio (exp/cont) was calculated. The results were analyzed by Dunnett's test (**: significant by $P<0.01$. *: significant by $P<0.05$).

further confirmed that 8 of these genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Phlda3* and *Plk2*) contributed to the discrimination of the genotoxic from the non-genotoxic hepatocarcinogens. When we analyzed the expression of *Trp53* itself, we identified a significant increase only with the NNK injection at the 48 h time point (Tables 4.1–4.4), though the basal expression of *Trp53* in the control animals may already have been sufficient for DNA damage under the present experimental conditions. Little is known about the acute expression changes of *Trp53* in the rodent liver after exposure to hepatocarcinogens; only a few reports have suggested the activation of a *Trp53*-mediated signaling pathway following the administration of hepatocarcinogens [40]. In one study [40], male F344 rats were dosed daily via gavage, up to 28 days, with 73 test chemicals, including 23 hepatocarcinogens. The paper suggested a possible gene network that included *Trp53*, *Bax*, *Btg2* and *Mdm2*. Our extracted network of the *Trp53*-mediated signaling pathway includes these genes (Fig. 3), however, it is much more extensive. *Mbd1* has been found to play a role in *Pml-Rara*-induced acute promyelocytic leukemia [41] and is associated with the *Trp53*-mediated signaling pathway via *Pml* (Fig. 3).

Some of the other identified PCA-contributed genes have been reported to be associated with cancer. Researchers have identified the associations of *Ccnf*, *Lrp1* and *Tubb2c* with cancer [42–44]. *Ccnf* is known to be associated with the cell cycle, cell division and mitosis; *Lrp1* is associated with apoptotic cell clearance, cell proliferation and the positive regulation of anti-apoptosis; and *Tubb2c* is associated with the G/M transition of the mitotic cell cycle. Little is currently known about the direct relationship between these genes and the *Trp53*-mediated signaling pathway.

The expression of *Ccng1* was remarkably increased by the injection of DIPN, NNK and NNM at both the 4 and 48 h time points. This increase has also been shown to be induced by other *N*-nitroso hepatocarcinogens, such as diethylnitrosamine, ethylnitrosourea [12] and dipropylnitrosamine [11]. Thus, *Ccng1* was suggested to be a characteristic gene that is amplified by *N*-nitroso hepatocarcinogens shortly after administration. Interestingly, *Ccng1* has been reported to be involved in growth inhibition, which is mechanistically linked to the ARF-p53 and pRb tumor suppressor pathways [31].

In total, 3 gene networks were extracted by IPA. The top functions of networks 1 and 3 were cellular growth and proliferation and the cell cycle, respectively, and the top functions of network 2 were the cell cycle, cell death and cellular growth and proliferation (Table 6). According to current understanding, these networks are assumed to be associated with carcinogenesis. Among the 12 PCA-identified genes, *Aen*, *Ccnf*, Gdf15, *Phlda3*, *Plk2* and *Tubb2c* belong to gene network 1. *Bax*, *Ccng1*, *Cdkn1a* and *Gdf15* belong to network 2; and *Btg2*, *Gdf15*, *Lrp1* and *Mbd1* belong to gene network 3.

Few time-course-based differential gene expression profiles of genotoxic and non-genotoxic hepatocarcinogens in rodents have been reported using DNA microarray and real-time PCR. Ellinger-Ziegelbauer et al. used the Affymetrix RG_U34 microarray system to examine the differential gene expression between 4 genotoxic (dimethylnitrosamine, 2-nitrofluorene, aflatoxin B1 and 4-(methylnitrosamino)1-(3-pyridyl)-1-butanone) and 4 non-genotoxic hepatocarcinogens (methapyrilene, diethylstilbestrol, Wy-14643 and piperonylbutoxide) in the livers of rats that had been given doses of the chemicals for 1, 3, 7 and 14 days [45].

Table 4.4
Gene expression ratio (Exp/Cont) and Dunnett's test and Welch's t-test of non-genotoxic hepatocarcinogens at 48 h.

No.	Symbol	Mean ± SD and Dunnett's test				Welch's test
		FUR	DEHP	DDT	DCB	G vs NG
1	Aen	0.87 ± 0.25	1.22 ± 0.31	1.06 ± 0.23	1.11 ± 0.58	$P<0.01$
2	Bax	0.95 ± 0.08	0.85 ± 0.27	0.82 ± 0.08	0.59 ± 0.11	$P<0.01$
3	Bhlhe40	0.39 ± 0.16*	0.47 ± 0.15**	0.48 ± 0.15**	1.11 ± 0.44	
4	Btg2	0.85 ± 0.08	1.09 ± 0.32	0.79 ± 0.27	1.23 ± 0.62	$P<0.01$
5	Ccnf	0.55 ± 0.14**	0.76 ± 0.19	0.68 ± 0.08	1.18 ± 0.30	$P<0.01$
6	Ccng1	1.19 ± 0.07	1.37 ± 0.45	1.40 ± 0.33	1.00 ± 0.27	$P<0.01$
7	Cdkn1a	1.37 ± 0.23	2.57 ± 1.92	1.12 ± 0.28	1.22 ± 0.46	$P<0.01$
8	Cyp1a2	0.51 ± 0.17	0.74 ± 0.16	1.01 ± 0.18	0.41 ± 0.25	
9	Ddit4	1.00 ± 0.39	1.02 ± 0.48	0.88 ± 0.16	1.23 ± 0.47	
10	Ddit4l	0.45 ± 0.38	0.85 ± 0.37	0.67 ± 0.16	1.47 ± 0.73	$P<0.05$
11	Egfr	0.43 ± 0.22*	0.81 ± 0.30	0.51 ± 0.14*	1.02 ± 0.39	
12	Ephx1	1.63 ± 0.17	1.13 ± 0.48	0.93 ± 0.34	0.79 ± 0.40	$P<0.01$
13	Gadd45b	1.20 ± 0.52	1.37 ± 0.91	0.58 ± 0.20	0.57 ± 0.41	$P<0.01$
14	Gdf15	1.37 ± 0.30	1.14 ± 0.56	0.61 ± 0.27	0.84 ± 0.23	$P<0.01$
15	Hist1h1c	0.71 ± 0.15	1.27 ± 0.28	0.71 ± 0.15	1.48 ± 0.40	
16	Hmox1	1.08 ± 0.28	0.86 ± 0.27	1.21 ± 0.36	0.65 ± 0.32	
17	Hspb1	2.17 ± 0.58*	2.74 ± 0.94**	1.25 ± 0.29	0.66 ± 0.23	
18	Igfbp1	1.28 ± 0.36	3.12 ± 2.59	0.63 ± 040	2.99 ± 1.49*	
19	Jun	1.59 ± 0.22	1.36 ± 0.59	1.71 ± 0.33	1.04 ± 0.56	
20	Lrp1	0.76 ± 0.11	0.62 ± 0.18	0.80 ± 0.16	1.34 ± 1.08	$P<0.05$
21	Ly6a	0.19 ± 0.05**	0.13 ± 0.05**	0.28 ± 0.11**	1.28 ± 0.60	$P<0.01$
22	Mbd1	1.36 ± 0.53	1.43 ± 1.60	1.62 ± 1.01	1.28 ± 1.27	
23	Mdm2	0.87 ± 0.23	1.26 ± 0.55	1.15 ± 0.24	1.38 ± 0.77	$P<0.01$
24	Phlda3	1.38 ± 0.51	0.42 ± 0.14	1.29 ± 0.60	1.12 ± 1.48	$P<0.01$
25	Plk2	1.25 ± 0.16	0.74 ± 0.16	0.80 ± 0.21	0.82 ± 0.27	$P<0.01$
26	Pml	1.28 ± 0.14	1.19 ± 0.46	1.21 ± 0.26	1.13 ± 0.40	
27	Pmm1	1.06 ± 0.22	1.11 ± 0.60	0.71 ± 0.08	1.00 ± 0.53	$P<0.05$
28	Ppp1r3c	0.92 ± 0.34	1.47 ± 0.86	0.93 ± 0.13	1.72 ± 0.49	$P<0.01$
29	Psma3	1.10 ± 0.09	1.03 ± 0.42	1.04 ± 0.37	0.59 ± 0.13	
30	Rad52	1.00 ± 0.45	1.12 ± 0.20	1.34 ± 0.37	0.97 ± 0.45	
31	Rcan1	0.97 ± 0.19	0.56 ± 0.31	0.93 ± 0.38	3.05 ± 1.59	
32	St3gal5	0.74 ± 0.21	1.27 ± 0.79	0.85 ± 0.18	1.08 ± 0.63	$P<0.01$
33	Trp53	1.21 ± 0.23	1.10 ± 0.43	0.80 ± 0.20	1.32 ± 0.53	
34	Tubb2c	0.70 ± 14	1.05 ± 0.29	0.62 ± 0.20	0.95 ± 0.63	$P<0.01$
35	Gapdh	1.30 ± 0.39	1.12 ± 0.28	1.32 ± 0.30	0.65 ± 0.60	

Total RNA was extracted from the individual liver and cDNA was prepared. The expression of the 35 genes was quantified by quantitative real-time PCR and the gene expression ratio (exp/cont) was calculated. The results were analyzed by Dunnett's test (**: significant by $P<0.01$. *: significant by $P<0.05$). The results of genotoxic hepatocarcinogens (G) were compared to non-genotoxic hepatocarcinogens (NG) by Welch's t-test.

They reported 477 deregulated genes in 18 categories. A total of 9 out of our 34 genes agreed with their candidates, namely 5 genes that are involved in the DNA damage response (*Bax*, *Btg2*, *Ccng1*, *Cdkn1a* and *Mdm2*), 2 genes that are involved in the oxidative stress response (*Ephx1* and *Hmox1*) and 2 genes that are involved in cell survival/proliferation (*Gdf15* and *Igfbp1*). Kang et al. examined the genotoxic hepatocarcinogen MeIQx at weeks 4, 16 and 102 in rat livers using the Affymetrix Gene Chip, Rat Genome 230 2.0 Array and observed no major differences at weeks 4 and 16 but found a few differentially expressed genes in tumors at 102 weeks [46].

There are very few reports on the acute gene expression changes within 48 h in mouse or rat liver after the administration of hepatocarcinogens. We speculated that carcinogens at high doses would induce various acute changes including general toxic effects in their target organs. Some changes might be associated with immediate response to exposure to DNA damaging agents and which are likely to reflect genotoxic insult and therefore associated with initiation (presumably due to mutagenesis). However, most cells would be repaired rapidly and some cells might be induced to undergo apoptosis. Only a few initiated cells may continue to develop into tumors. In previous studies, we have observed that

Table 5
Gene ontology of genes examined in the present study.

Biological processes	Genes
Apoptosis	Aen*, Bax*, Btg2*, Ccng1*, Cdkn1a*, Ddit4, Egfr*, Gadd45b*, Hmox1, Hspb1, Jun, Lrp1*, Mdm2*, Phlda3*, Pml*, Trp53
Cell cycle	Ccnf*, Ccng1*, Cdkn1a*, Egfr*, Gadd45b*, Jun, Mdm2*, Plk2*, Pml*, Trp53
Cell proliferation	Ccnf*, CCng1*, Cdkn1a*, Egfr*, Gdf15*, Jun, Lrp1*, Mdm2*, Pml*, Trp53
DNA damage	Aen*, Bax*, Btg2*, Ccng1*, Cdkn1a*, Ddit4, Gadd45b*, Hmox1, Mdm2*, Phlda3*, Pml*, Rad52, Trp53
DNA repair	Egfr*, Rad52, Trp53
Oncogene	Jun, Mdm2*
Tumor suppression	Pml*, Ppp1r3c*, Trp53

Gene ontology of examined genes, as referred by Gene Ontology (http://www.geneontology.org/) and references. The red-colored genes indicated by "*" mark showed statistically significant differences in expression between genotoxic and non-genotoxic hepatocarcinogens at 4 and/or 48 h.

Table 6
Associated gene network functions (2AAF, at 4 h).

Gene network	Molecules within the network	Score	Focus molecule	Top functions
1	Abl1, **Aen**, Aspm, Bub1, Cables1, **Ccnf**, Cdc7, Cdkn3, Ddb2, **Ddit4**, **Ddit4l**, **Ephx1**, Gadd45, **Gdf15**, Hprt1, hydrogen peroxide, Mk167, Mtor, P4HA1, **Phlda3**, **Plk2**, **Pmm1**, **Ppp1r3c**, Prc1, **Rad52**, retinoic acid, Rfc4, ***St3gal5***, *Tcn2*, Tgfb1, **Trp53**, Tprkb, Tsc1-Tsc2, **Tubb2c**, Ube2c	31	14	Cellular growth and proliferation Cell cycle
2	14-3-3, Ahr, Akt, **Bax**, **Bhlhe40**, caspase, Cbp, **Ccng1**, Ccng2, **Cdkn1a**, CyclinA, Cytochrome c, E2f, Estrogen receptor, **Gadd45b**, **Gdf15**, hCG, **Mdm2**, Mek, Hhex, **Hspb1**, Hspb2, **Jun**, Ldl, Map2K1/2, NFkB, **Plk2**, **Pml**, Pp2a, Proteasome, **Psma3**, Rb, **Rcan1**, Ubiquitin	30	13	Cell cycle Cell death Cellular growth and proliferation
3	Ap1, **Btg2**, Calpain, Ck2, **Cyp1a2**, **Egfr**, Erk, Erk1/2, Fsh, **Gdf15**, Histone h3, Histone h4, **Hmox1**, Ifn beta, **Igfbp1**, IgG, Il1, Insulin, interferon alpha, Jnk, Lpp, **Lrp1**, Mapk, **Mbd1**, P38, Mapk, Pdgf, Pi3k, Pks, Pkc, Ras, RNA polymerase II, Stat, Tgf beta, Vegf	18	8	Cellular growth and proliferation Cell cycle

Associated gene network functions, as determined by ingenuity pathways analysis 7.0 (IPA), a web-based application (http://www.Ingenuity.com) are shown for 2AAF at 4 h as a representative. Boldface genes were examined in the present study. The score indicates the likelihood of the focus genes in a given network being found together due to random chance. A score of >2 indicates that there is a <1 in 100 chance that the focus genes were assembled randomly into a network due to random chance.

the initial changes seen at 4 h were much greater than those at 16, 20, 24 and 48 h and 14 and 28 days (published in part: [11,12]). Therefore, in the present study, we attempted to detect the specific acute changes that occur within the first 48 h. At the 48 h mark, we expected to find changes in the expression of genes that are responsible for evaluating cell proliferation. However, no genes were identified that were specific to cell proliferation at 48 h. Essentially, we observed similar changes at both 4 and 48 h, with a few exceptions.

In our previous mouse studies [11,12] and in additional unpublished work, we compared the results of DNA microarray (Affymetrix GeneChip and 45-mer oligonucleotide in-house microarray) and qPCR. The qPCR findings generally coincided with those of the DNA microarray, and the qPCR was more sensitive at detecting low levels of gene expression. Ten-fold greater amounts of total RNA and more procedural steps are required for a DNA microarray. qPCR experiments are simpler, and the resultant data are highly reliable and reproducible. In summary, DNA microarray technology is helpful for identifying candidate genes across the whole genome in the preliminary step, but qPCR is more useful for routine studies on selected genes when evaluating genotoxic and non-genotoxic mouse hepatocarcinogens.

We are interested in short-term in vivo genotoxicity tests in the mammalian liver because the effects of chemicals are not necessarily the same between a single cell and a mammalian body. Previously, we studied various short-term in vivo genotoxicity tests in rodent livers [13–15,47–50]. Recently we attempted gene expression profiling in short-term in vivo genotoxicity tests [11,12].

In summary, we have shown that qPCR and PCA are effective methods for distinguishing between genotoxic and non-genotoxic hepatocarcinogens in the mouse liver at the early time points of 4 and 48 h after administration, when analyzing the 12 genes selected from our preliminary DNA microarray studies.

Conflict of interest

We do not have any conflicts of interest, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations and grants or other funding.

The authors declare that there are no conflicts of interest.

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References

[1] J. Ashby, Use of short-term tests in determining the genotoxicity or nongenotoxicity of chemicals, IARC Sci. Publ. 116 (1992) 135–164.
[2] K. Mathijs, K.J. Brauers, D.G. Jennen, A. Boorsma, M.H. van Herwijnen, R.W. Gottschalk, J.C. Kleinjans, J.H. van Delft, Discrimination for genotoxic and nongenotoxic carcinogens by gene expression profiling in primary mouse hepatocytes improves with exposure time, Toxicol. Sci. 112 (2009) 374–384.
[3] B. Mahadevan, R.D. Snyder, M.D. Waters, R.D. Benz, R.A. Kemper, R.R. Tice, A.M. Richard, Genetic toxicology in the 21st century: reflections and future directions, Environ. Mol. Mutagen. 52 (2011) 339–354.
[4] M.D. Waters, M. Jackson, I. Lea, Characterizing and predicting carcinogenicity and mode of action using conventional and toxicogenomics methods, Mutat. Res. 705 (2010) 184–200.
[5] Z. Wu, A review of statistical methods for preprocessing oligonucleotide microarrays, Stat. Methods Med. Res. 18 (2009) 533–541.
[6] C.L. Yauk, M.L. Berndt, Review of the literature examining the correlation among DNA microarray technologies, Environ. Mol. Mutagen. 48 (2007) 380–394.
[7] A.M. Calcagno, S.V. Ambudkar, Analysis of expression of drug resistance-linked ABC transporters in cancer cells by quantitative RT-PCR, Methods Mol. Biol. 637 (2010) 121–132.
[8] E. Nardon, M. Donada, S. Bonin, I. Dotti, G. Stanta, Higher random oligo concentration improves reverse transcription yield of cDNA from bioptic tissues and quantitative RT-PCR reliability, Exp. Mol. Pathol. 87 (2009) 146–151.
[9] M. Provenzano, S. Mocellin, Complementary techniques: validation of gene expression data by quantitative real time PCR, Adv. Exp. Med. Biol. 593 (2007) 66–73.
[10] G. Fabian, N. Farago, L.Z. Feher, L.I. Nagy, S. Kulin, K. Kitajka, T. Bito, V. Tubak, R.L. Katona, L. Tiszlavicz, L.G. Puskas, High-density real-time PCR-based in vivo toxicogenomic screen to predict organ-specific toxicity, Int. J. Mol. Sci. 12 (2011) 6116–6134.
[11] T. Watanabe, K. Tobe, Y. Nakachi, Y. Kondoh, M. Nakajima, S. Hamada, C. Namiki, T. Suzuki, S. Maeda, A. Tadakuma, M. Sakurai, A. Arai, A. Hyogo, M. Hoshino, T. Tashiro, H. Ito, H. Inazumi, Y. Sakaki, H. Tashiro, C. Furihata, Differential gene expression induced by two genotoxic *N*-nitroso carcinogens, phenobarbital and ethanol in mouse liver examined with oligonucleotide microarray and quantitative real-time PCR, Gene Environ. 29 (2007) 115–127.
[12] T. Watanabe, G. Tanaka, S. Hamada, C. Namiki, T. Suzuki, M. Nakajima, C. Furihata, Dose-dependent alterations in gene expression in mouse liver induced by diethylnitrosamine and ethylnitrosourea and determined by quantitative real-time PCR, Mutat. Res. 673 (2009) 9–20.
[13] S. Sawada, C. Furihata, T. Matsushima, In vivo short-term assays of repair and replication of rat liver DNA, J. Cancer Res. Clin. Oncol. 115 (1989) 345–350.
[14] S. Asakura, S. Sawada, H. Daimon, T. Fukuda, K. Ogura, K. Yamatsu, C. Furihata, Effects of dietary restriction on induction of unscheduled DNA synthesis (UDS) and replicative DNA synthesis (RDS) in rat liver, Mutat. Res. 322 (1994) 257–264.
[15] S. Sawada, S. Asakura, H. Daimon, C. Furihata, Comparison of autoradiography, liquid scintillation counting and immunoenzymatic staining of 5-bromo-2′-deoxyuridine for measurement of unscheduled DNA synthesis and replicative DNA synthesis in rat liver, Mutat. Res. 344 (1995) 109–116.

[16] S. Tsuda, N. Matsusaka, H. Madarame, S. Ueno, N. Susa, K. Ishida, N. Kawamura, K. Sekihashi, Y.F. Sasaki, The comet assay in eight mouse organs: results with 24 azo compounds, Mutat. Res. 465 (2000) 11–26.
[17] Y. Uno, H. Takasawa, M. Miyagawa, Y. Inoue, T. Murata, M. Ogawa, K. Yoshikawa, In vivo-in vitro replicative DNA synthesis (RDS) test using perfused rat livers as an early prediction assay for non-genotoxic hepatocarcinogens: II. assessment of judgement criteria, Toxicol. Lett. 63 (1992) 201–209.
[18] T.M. Brooks, M. Szegedi, P. Rosher, S.W. Dean, The detection of gene mutation in transgenic mice (Muta Mouse) following a single oral dose of 2-acetylaminofluorene, Mutagenesis 10 (1995) 149–150.
[19] D. Kirkland, C. Beevers, Induction of LacZ mutations in Muta Mouse can distinguish carcinogenic from non-carcinogenic analogues of diaminotoluenes and nitronaphthalenes, Mutat. Res. 608 (2006) 88–96.
[20] K. Hashimoto, K. Ohsawa, M. Kimura, Mutations induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the lacZ and cII genes of Muta Mouse, Mutat. Res. 560 (2004) 119–131.
[21] T. Suzuki, Y. Miyata, K. Saeki, Y. Kawazoe, M. Hayashi, T. Sofuni, In vivo mutagenesis by the hepatocarcinogen quinoline in the *lacZ* transgenic mouse: evidence for its in vivo genotoxicity, Mutat. Res. 412 (1998) 161–166.
[22] C.V. Williams, K. Fletcher, H. Tinwell, J. Ashby, Weak mutagenicity of ethyl carbamate to lacZ- transgenic mice, Mutagenesis 13 (1998) 133–137.
[23] S.J. Biswas, A.R. Khuda-Bukhsh, Cytotoxic and genotoxic effects of the azo-dye p-dimethylaminoazobenzene in mice: a time-course study, Mutat. Res. 587 (2005) 1–8.
[24] K. Tegethoff, B.A. Herbold, E.M. Bomhard, Investigations on the mutagenicity of 1,4-dichlorobenzene and its main metabolite 2,5-dichlorophenol in vivo and in vitro, Mutat. Res. 470 (2000) 161–167.
[25] K. Sekihashi, A. Yamamoto, Y. Matsumura, S. Ueno, M. Watanabe-Akanuma, F. Kassie, S. Knasmüller, S. Tsuda, Y.F. Sasaki, Comparative investigation of multiple organs of mice and rats in the comet assay, Mutat. Res. 517 (2002) 53–75.
[26] D.M. Wilson, T.L. Goldsworthy, J.A. Popp, B.E. Butterworth, Evaluation of genotoxicity, pathological lesions, and cell proliferation in livers of rats and mice treated with furan, Environ. Mol. Mutagen. 19 (1992) 209–222.
[27] T. Smith-Oliver, B.E. Butterworth, Correlation of the carcinogenic potential of di(2-ethylhexyl)phthalate (DEHP) with induced hyperplasia rather than with genotoxic activity, Mutat. Res. 188 (1987) 21–28.
[28] T. Kawase, H. Ichikawa, T. Ohta, N. Nozaki, F. Tashiro, R. Ohki, Y. Taya, p53 target gene AEN is a nuclear exonuclease required for p53-dependent apoptosis, Oncogene 27 (2008) 3797–3810.
[29] F. Lanza, S. Bi, Role of p53 in leukemogenesis of chronic myeloid leukemia, Stem Cells 13 (1995) 445–452.
[30] J.P. Rouault, N. Falette, F. Guéhenneux, C. Guillot, R. Rimokh, Q. Wang, C. Berthet, C. Moyret-Lalle, P. Savatier, B. Pain, P. Shaw, R. Berger, J. Samarut, J.P. Magaud, M. Ozturk, C. Samarut, A. Puisieux, Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway, Nat. Genet. 14 (1996) 482–486.
[31] L. Zhao, T. Samuels, S. Winckler, C. Korgaonkar, V. Tompkins, M.C. Horne, D.E. Quelle, Cyclin G1 has growth inhibitory activity linked to the ARF-Mdm2-p53 and pRb tumor suppressor pathways, Mol. Cancer Res. 1 (2003) 195–206.
[32] M. Zemskova, M.B. Lilly, Y.W. Lin, J.H. Song, A.S. Kraft, p53-dependent induction of prostate cancer cell senescence by the PIM1 protein kinase, Mol. Cancer Res. 8 (2010) 1126–1141.
[33] Y. Zhou, Y. Zhong, Y. Wang, X. Zhang, D.L. Batista, R. Gejman, P.J. Ansell, J. Zhao, C. Weng, A. Klibanski, Activation of p53 by MEG3 non-coding RNA, J. Biol. Chem. 282 (2007) 24731–24742.
[34] M. Nishiyama, K. Oshikawa, Y. Tsukada, T. Nakagawa, S. Iemura, T. Natsume, Y. Fan, A. Kikuchi, A.I. Skoultchi, K.I. Nakayama, CHD8 suppresses p53-mediated apoptosis through histone H1 recruitment during early embryogenesis, Nat. Cell Biol. 11 (2009) 172–182.
[35] A. Meiller, S. Alvarez, P. Drané, C. Lallemand, B. Blanchard, M. Tovey, E. May, p53 dependent stimulation of redox-related genes in the lymphoid organs of gamma-irradiated–mice identification of Haeme-oxygenase 1 as a direct p53 target gene, Mol. Cancer Res. 8 (2010) 1126–1141.
[36] K. Vedam, Y. Nishijima, L.J. Druhan, M. Khan, N.I. Moldovan, J.L. Zweier, G. Ilangovan, Role of heat shock factor-1 activation in the doxorubicin-induced heart failure in mice, Am. J. Physiol. Heart Circ. Physiol. 298 (2010) H1832–H1841.
[37] T. Kawase, R. Ohki, T. Shibata, S. Tsutsumi, N. Kamimura, J. Inazawa, T. Ohta, H. Ichikawa, H. Aburatani, F. Tashiro, Y. Taya, PH domain-only protein PHLDA3 is a p53-regulated repressor of Akt, Cell 136 (2009) 535–550.
[38] K. Strebhardt, Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy, Nat. Rev. Drug Discov. 9 (2010) 643–660.
[39] H. Shen, C.G. Maki, p53 and p21(Waf1) are recruited to distinct PML-containing nuclear foci in irradiated and Nutlin-3a-treated U2OS cells, J.Cell Biochem. 111 (2010) 1280–1290.
[40] H. Matsumoto, Y. Yakabe, K. Saito, K. Sumida, M. Sekijima, K. Nakayama, H. Miyaura, F. Saito, M. Otsuka, T. Shirai, Discrimination of carcinogens by hepatic transcript profiling in rats following 28-day administration, Cancer Inform. 7 (2009) 253–269.
[41] R. Villa, L. Morey, V.A. Raker, M. Buschbeck, A. Gutierrez, F. De Santis, M. Corsaro, F. Varas, D. Bossi, S. Minucci, P.G. Pelicci, L. Di Croce, The methyl-CpG binding protein MBD1 is required for PML-RARalpha function, Proc. Natl. Acad. Sci. U S A. 103 (2006) 1400–1405.
[42] P.M. de Angelis, B. Fjell, K.L. Kravik, T. Haug, S.H. Tunheim, W. Reichelt, M. Beigi, O.P. Clausen, E. Galteland, T. Stokke, Molecular characterizations of derivatives of HCT116 colorectal cancer cells that are resistant to the chemotherapeutic agent 5-fluorouracil, Int. J. Oncol. 24 (2004) 1279–1288.
[43] S. Dedieu, B. Langlois, J. Devy, B. Sid, P. Henriet, H. Sartelet, G. Bellon, H. Emonard, L. Martiny, LRP-1 silencing prevents malignant cell invasion despite increased pericellular proteolytic activities, Mol. Cell Biol. 28 (2008) 2980–2995.
[44] Z.Y. He, H. Wen, C.B. Shi, J. Wang, Up-regulation of hnRNP A1, Ezrin, tubulin β-2C and Annexin A1 in sentinel lymph nodes of colorectal cancer, World J. Gastroenterol. 16 (2010) 4670–4676.
[45] H. Ellinger-Ziegelbauer, B. Stuart, B. Wahle, W. Bomann, H.J. Ahr, Comparison of the expression profiles induced by genotoxic and non-genotoxic carcinogens in rat liver, Mutat. Res. 575 (2005) 61–84.
[46] J.S. Kang, H. Wanibuchi, T. Murai, K. Morimura, A. Kinoshita, S. Fukushima, Analysis of gene expression in different stages of MeIQx-induced rat hepatocarcinogenesis, Oncol. Rep. 17 (2007) 747–752.
[47] S. Sawada, H. Daimon, S. Asakura, T. Kawaguchi, K. Yamatsu, C. Furihata, T. Matsushima, Cumulative effects of chromosome aberrations and sister chromatid exchanges in rat liver induced in vivo by heterocyclic amines, Carcinogenesis 15 (1994) 285–290.
[48] M. Nakajima, M. Kikuchi, K. Saeki, Y. Miyata, M. Terada, F. Kishida, R. Yamamoto, C. Furihata, S.W. Dean, Mutagenicity of 4-nitroquinoline 1-oxide in the Muta Mouse, Mutat. Res. 444 (1999) 321–336.
[49] S. Hamada, S. Sutou, T. Morita, A. Wakata, S. Asanami, S. Hosoya, S. Ozawa, K. Kondo, M. Nakajima, H. Shimada, K. Osawa, Y. Kondo, N. Asano, S. Sato, H. Tamura, N. Yajima, R. Marshall, C. Moore, D.H. Blakey, L.M. Schechtman, J.L. Weaver, D.K. Torous, R. Proudlock, S. Ito, C. Namiki, M. Hayashi, Evaluation of the rodent micronucleus assay by a 28-day treatment protocol: Summary of the 13th Collaborative Study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Environmental Mutagen Society of Japan (JEMS)-Mammalian Mutagenicity Study Group (MMS), Environ. Mol. Mutagen. 37 (2001) 93–110.
[50] A. Rothfuss, M. O'Donovan, M. De Boeck, D. Brault, A. Czich, L. Custer, S. Hamada, U. Plappert-Helbig, M. Hayashi, J. Howe, A.R. Kraynak, B.J. van derLeede, M. Nakajima, C. Priestley, V. Thybaud, K. Saigo, S. Sawant, J. Shi, R. Storer, M. Struwe, E. Vock, S. Galloway, Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies, Mutat. Res. 702 (2010) 40–69.

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Differential gene expression profiling between genotoxic and non-genotoxic hepatocarcinogens in young rat liver determined by quantitative real-time PCR and principal component analysis☆

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ABSTRACT

We recently successfully discriminated mouse genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens *via* selected gene expression profiling in the mouse liver based on quantitative real-time PCR (qPCR) and statistical analysis using principal component analysis (PCA). In the present study, we applied these candidate marker genes to rat hepatocarcinogens in the rat liver. qPCR analysis of 33 genes was conducted on liver samples from groups of 4 male 4-week-old F344 rats at 4 and 48 h after a single oral administration of chemicals [2 genotoxic hepatocarcinogens: diethylnitrosamine and 2,6-dinitrotoluene; a non-genotoxic hepatocarcinogen: di(2-ethylhexyl)phthalate; and a non-genotoxic non-hepatocarcinogen: phenacetin]. Thirty-two genes exhibited significant changes in their gene expression ratios (experimental group/control group) according to statistical analysis using the Williams' test and the Dunnett's test. The changes appeared to be greater at 4 h than at 48 h. Finally, statistical analysis *via* PCA successfully differentiated the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen at 4 h based on 16 genes (*Ccnf, Ccng1, Cyp4a10, Ddit4l, Egfr, Gadd45g, Gdf15, Hspb1, Igfbp1, Jun, Myc, Net1, Phlda3, Pml, Rcan1* and *Tubb2c*) and at 48 h based on 10 genes (*Aen, Ccng1, Cdkn1a, Cyp21a1, Cyp4a10, Gdf15, Igfbp1, Mdm2, Phlda3* and *Pmm1*). Eight major biological processes were extracted from a gene ontology analysis: apoptosis, the cell cycle, cell proliferation, DNA damage, DNA repair, oxidative stress, oncogenes and tumor suppression. The major, biologically relevant gene pathway suggested was the DNA damage response, which signals through a *Tp53*-mediated pathway and leads to the induction of apoptosis. Immunohistochemical analyses for the expression of Cdkn1a and Hmox1 proteins and the level of apoptosis measured by the TUNEL assay in the liver confirmed the aforementioned results. The present results showed that mouse candidate marker genes are applicable for differentiating genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens examined in this paper in the rat liver.

1. Introduction

For risk assessment purposes, there is general agreement that chemicals acting through genotoxic and non-genotoxic mechanisms of carcinogenesis should be distinguished [1]. According to Waters et al., although the number of presumably non-genotoxic rodent carcinogens has dramatically increased over the past two decades, the fact remains that ~90% of the known, probable and possible human carcinogens, classified by the International Agency for Research on Cancer, are detected in conventional short-term tests for genotoxicity and induce tumors at multiple sites in rodents [2]. Mathijs et al. [3] hypothesized that genotoxic and non-genotoxic carcinogens induce distinct gene expression profiles, which may therefore be used to classify the mechanisms of compounds as either genotoxic carcinogens or non-genotoxic carcinogens. DNA microarrays are a powerful technology for characterizing gene expression on a genome-wide scale [4], although issues regarding the reliability, reproducibility and correlation of

☆ This work was the 4th collaborative study by Toxicogenomics/JEMS·MMS.

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the data produced across the different DNA microarray technologies are still being addressed [5]. qPCR is the field standard for measuring gene expression and is often used to confirm DNA microarray data [6] because qPCR is the most sensitive technique for the detection and quantification of mRNA targets [7].

Previously, we (the collaborative study group of toxicogenomics of the Japanese Environmental Mutagen Society/Mammalian Mutagenicity Study Group; Toxicogenomics/JEMS·MMS) examined differential gene expression using DNA microarrays following the application of 13 different chemicals, including 8 genotoxic hepatocarcinogens [*o*-aminoazotoluene, chrysene, dibenzo[*a*,*l*]pyrene, diethylnitrosamine (DEN), 7,12-dimethylbenz[*a*]anthracene, dimethylnitrosamine, dipropylnitrosamine and ethylnitrosourea (ENU)], 4 non-genotoxic hepatocarcinogens [carbon tetrachloride, di(2-ethylhexyl)phthalate (DEHP), phenobarbital and trichloroethylene] and a non-genotoxic non-hepatocarcinogen [ethanol]. DNA microarray analysis was conducted on 9-week-old male mouse liver samples at 4 h and for up to 28 days following a single intraperitoneal administration of these chemicals. A considerable number of candidate genes were extracted to differentiate the genotoxic hepatocarcinogens from the other chemicals (the non-genotoxic hepatocarcinogens and a non-genotoxic non-hepatocarcinogen). The results were reported in part [8] and registered in the NCBI Gene Expression Omnibus (GEO) database (GEO accession GSE33248). Notably, the changes in gene expression observed at 4 h were much greater than those at 20 h, 14 days and 28 days. Additionally, dose-dependent alterations in gene expression were demonstrated for 31 out of 51 of the examined candidate genes at 4 h and 28 days after the administration of DEN (3, 9, 27 and 80 mg/kg bw) and ENU (6, 17, 50 and 150 mg/kg bw), as determined by qPCR [9]. More recently, we demonstrated successful discrimination between 8 genotoxic mouse hepatocarcinogens (2-acetylaminofluorene, 2,4-diaminotoluene, diisopropanolnitrosamine, 4-dimethylaminoazobenzene, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, *N*-nitrosomorpholine, quinoline and urethane) and 4 non-genotoxic hepatocarcinogens (1,4-dichlorobenzene, dichlorodiphenyltrichloroethane, DEHP and furan) using qPCR analysis and PCA for 12 genes associated with a *Trp53*-mediated signaling pathway involved in the DNA damage response; this discrimination was demonstrated at 4 and 48 h after a single administration of the chemicals [10].

Rats, as well as mice, are often used for the study of experimental chemical carcinogenesis and *in vivo* genotoxicity tests. However, studies published on the gene expression profiles induced by genotoxic hepatocarcinogens in the *in vivo* rat livers are still limited; studies on the changes in gene expression profiles within a few hours after the administration of genotoxic hepatocarcinogens are particularly scarce. In the present study, we applied our mouse candidate marker genes [8–10] to rat hepatocarcinogens in an established rat liver genotoxicity test system. We evaluated the gene expression profiles in the rat liver treated with the 4 chemicals [DEN, 2,6-dinitrotoluene (DNT), DEHP and phenacetin (PNT)] that were previously examined previously using the liver micronucleus assay by the collaborative study group of micronucleus test of the Japanese Environmental Mutagen Society/Mammalian Mutagenicity Study Group (CSGMT/JEMS·MMS) [11,12]. DEN and DNT exhibited positive results [12] and DEHP [11] and PNT [12] exhibited negative results in the liver micronucleus assay. DEN [13] and DEHP [14] induce hepatocellular carcinoma in mice and rats. DNT induces hepatocellular carcinoma in rats, but not in mice [15]. Whether the mouse candidate marker genes will also be responsive to DNT in the rat liver is a topic of interest. PNT has been shown to induce tumors of the urinary tract in mice and rats and tumors of the nasal cavity in rats [16]. The induction of liver tumors in mice and rats by PNT has not previously been reported, although the addition of N-hydroxyphenacetin, a PNT metabolite, has been shown to induce liver tumors in rats [17]. DEN [13] and DNT [18] are positive and DEHP [14] and PNT [19] are negative in the Ames test using rat S9 mix.

The chemicals were administered orally by gavage into 4-week-old male F344 rats, which were analyzed at 4 and 48 h after administration using the qPCR analysis of 33 genes. We speculated that the period of 4 h post-hepatocarcinogen administration in the liver would be the time by which DNA damage would occur, as determined by the *in vivo*/*in vitro* unscheduled DNA synthesis (UDS) assay [20–22] and the *in vivo* Comet assay [23], and that the 48 h time point would represent the period of DNA replication after damage, as determined with a replicative DNA synthesis test [20–22,24].

Finally, we succeeded in differentiating the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogens and the non-genotoxic non-hepatocarcinogen *via* statistical analysis using PCA. We showed that the major biologically relevant gene pathway associated with the PCA-contributed genes is a *Tp53*-mediated DNA damage response signaling pathway, which ultimately results in the induction of apoptosis. We additionally examined both the protein expression level of the DNA damage markers, Cdkn1a and Hmox1, by immunohistochemistry and the level of apoptosis by the TUNEL assay in the rat liver following treatment with the 4 hepatocarcinogens. The results confirmed the expression of Cdkn1a, Hmox1 proteins and an enhanced level of apoptosis in rat liver following treatment with the genotoxic hepatocarcinogens (DEN and DNT).

2. Materials and methods

2.1. Animal treatment

Male F344 rats were obtained at 3 weeks of age from Charles River Japan, Inc. (Yokohama, Japan). They were maintained in plastic cages with wood chips as bedding in an air-conditioned room [12 h light (7 a.m. to 7 p.m.), 12 h dark; 22 ± 3 °C; 55 ± 20% humidity], and they were provided food (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and water provided *ad libitum*. All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Mitsubishi Chemical Medience Corp.

DEN (CAS No. 55-18-5) and PNT (CAS No. 62-44-2) were purchased from Wako Pure Chem. Ind. Ltd., Osaka, Japan. DNT (CAS No. 606-20-2) was obtained from Johnson Matthey Company, London, UK, and DEHP (CAS No. 117-81-7) was purchased from Tokyo Chem. Ind. Co. Ltd., Tokyo, Japan.

Groups of 4-week-old rats (4 rats per group) were dosed by gavage (p.o.) with DEN (12.5, 25 and 50 mg/kg bw) dissolved in sterile water, DNT (125 and 250 mg/kg bw) suspended in olive oil, DEHP (1000 and 2000 mg/kg bw) dissolved in olive oil or PNT (500 and 1000 mg/kg bw) suspended in olive oil. The doses for DEN [12], DNT [12], DEHP [11] and PNT [12] were comparable to the doses used in the previous young rat micronucleus assay. The control animals received only sterile water or olive oil. At 4 and 48 h after treatment, the left lateral lobe of the liver was dissected and stored in either RNAlater (Applied Biosystems/Ambion, Austin, TX, USA) at −20 °C until use in qPCR experiments or in 10% buffered formalin for immunohistochemical and histopathological analyses.

2.2. RNA isolation and relative quantification via real-time PCR

Total RNA was extracted from a liver sample of approximately 30 mg from each rat using Micro Smash MS-100 (TOMY DIGITAL BIOLOGY Co., LTD., Tokyo, Japan) and QuickGene-800 (FUJIFILM Holdings Corp., Tokyo, Japan). Complimentary DNA (cDNA) was prepared from the total RNA using the SuperScript first-strand synthesis system for RT-PCR kit (Invitrogen Corp., Carlsbad, CA, USA). qPCR analyses were performed in triplicate assays using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a DNA Engine Opticon 2 Real-Time Cycler (MJ Research, Inc., Waltham, MA, USA). The reactions were carried out as previously described [10]. We quantified 33 genes based on our previous DNA microarray and qPCR studies performed in the mouse liver. The symbols, gene names and accession numbers of the 33 genes are summarized in Table 1. The sequences of the primers used in our experiments and the cycle threshold (Ct) values that were obtained are shown in Table 2. The primer sequences were determined using Primer3 (http://frodo.wi.mit.edu/). *Gapdh* was selected as a housekeeping gene. Finally, the relative quantitative values for each sample were normalized to the value of the *Gapdh* gene. The variability of the relative *Gapdh* expression in the

Table 1
The 33 genes examined in the present study.

No.	Symbol	Gene name	Accession no.
1	*Aen*	Apoptosis enhancing nuclease	NM_001108487
2	*Bax*	Bcl2-associated X protein	NM_017059
3	*Btg2*	B-cell translocation gene 2, anti-proliferative	NM_017259
4	*Ccnf*	Cyclin F	NM_001100474
5	*Ccng1*	Cyclin G1	NM_012923
6	*Cdkn1a*	Cyclin-dependent kinase inhibitor 1A	NM_080782
7	*Cyp21a1*	Cytochrome P450, subfamily 21A, polypeptide 1	NM_057101
8	*Cyp4a1*	Cytochrome P450, family 4, subfamily a, polypeptide 1	NM_175837
9	*Ddit4l*	DNA-damage-inducible transcript 4-like	NM_080399
10	*Egfr*	Epidermal growth factor receptor	NM_031507
11	*Ephx1*	Epoxide hydrolase 1, microsomal	NM_012844
12	*Gadd45b*	Growth arrest and DNA-damage-inducible 45 beta	NM_001008321
13	*Gadd45g*	Growth arrest and DNA-damage-inducible 45 gamma	NM_001077640
14	*Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008
15	*Gdf15*	Growth differentiation factor 15	NM_019216
16	*Hhex*	Hematopoietically expressed homeobox	NM_024385
17	*Hmox1*	Heme oxygenase (decycling) 1	NM_012580
18	*Hspb1*	Heat shock protein 1	NM_031970
19	*Igfbp1*	Insulin-like growth factor binding protein 1	NM_013144
20	*Jun*	Jun oncogene	NM_021835
21	*Lpp*	LIM domain containing preferred translocation partner in lipoma	NM_001013864
22	*Ly6al*	Lymphocyte antigen 6 complex, locus A-like	NM_001128009
23	*Mdm2*	Mdm2 p53 binding protein homolog (mouse)	NM_001108099
24	*Myc*	*Myelocytomatosis oncogene*	NM_012603
25	*Net1*	Neuroepithelial cell transforming 1	NM_001039023
26	*Phlda3*	Pleckstrin homology-like domain, family A, member 3	NM_001012206
27	*Plk2*	Polo-like kinase 2 (Drosophila)	NM_031821
28	*Pml*	Promyelocytic leukemia	XM_236296
29	*Pmm1*	Phosphomannomutase 1	NM_001008323
30	*Rcan1*	Regulator of calcineurin 1	NM_153724
31	*Tnf*	Tumor necrosis factor (TNF superfamily, member 2)	NM_012675
32	*Tp53*	Tumor protein p53	NM_030989
33	*Tubb2c*	Tubulin, beta 2c	NM_199094

Table 2
Primer sequences of the 33 genes examined in this study.

No.	Symbol	*Left primer*	*Right primer*	*Ct*
1	Aen	GCACTGCACAATGACTTCCAG	GCCAGGTCCTTAAGAGAGACCC	26–30
2	Bax	GGCGAATTGGAGATGAACTGG	GTTGAAGTTGCCATCAGCAAAC	29–33
3	Btg2	GAGAGGTGGCTCAAAGCTCCAG	AGGACCCAACCGCAGGAAAG	23–29
4	Ccnf	CCATAAGCTCCCTGGATGGTG	CATGACTTCTTGGCCTGATGG	26–30
5	Ccng1	TAAGGCAAAGCCTTCTGTGCTG	CTCGGCCACTTATCTTGGAATG	26–33
6	Cdkn1a	TTGTCGCTGTCTTGCACTCTGG	GCGCTTGGAGTGATAGAAATCTG	22–28
7	Cyp21a1	GACATGATTGACTACATGCTCCAG	GTGAAGCAGGAAAGCCACAG	30–35
8	Cyp4a1	TCTGACAAGGACCTACGTGCTGAGG	GTGTGTGGCCAGAGCATAGAAGATC	25–28
9	Ddit4l	CCTGGGAGTCTGCTAAGTGATTTC	CCAAATTCTGGCATGTTGTCTC	28–34
10	Egfr	ACAGCAAGGCTTCTTCAACAGC	GTCTTCTTTGACACGGCAGCTC	26–29
11	Ephx1	TACCGTGAACTGGAGGATGGAG	GAGGAGACAATGGTTCCTGTCG	18–21
12	Gadd45b	GAGCGACAACGCGGTTCAGAAG	TCAGTTTGGCCGCCTCGTACAC	27–34
13	Gadd45g	GAAAGCACAGCCAGGATGCAG	TTCAGGACTTTGGCGGACTCG	27–30
14	Gapdh	ATGGCCTTCCGTGTTCCTACCC	GCCTGCTTCACCACCTTCTTGATG	17–20
15	Gdf15	CTGGAGACTGTGCAGGCAACTC	CATGCAGGCGTGCTTTGATC	27–34
16	Hhex	GGACAGTTTGGACACTTCCTGTG	GGTCGGAATCCTCTGAGATCTC	24–26
17	Hmox1	CAAGCACAGGGTGACAGAAGAGG	TCTGTGAGGGACTCTGGTCTTTGTG	18–28
18	Hspb1	TCCCTGGACGTCAACCACTTCG	TTTCCGGGTGAAGCACCGAGAG	24–28
19	Igfbp1	GACCTCAAGAAATGGAAGGAGCC	CCATTCTTGTTGCAGTTTGGCAG	20–27
20	Jun	AAAGGAAGCTGGAGCGGATCG	CACCTGTTCCCTGAGCATGTTGG	24–29
21	Lpp	CCGTGATTTCCATGTGCACTGC	CTTGGCCGTCAAGACCCTGATG	29–31
22	Ly6a1	CTGCAGACCCTGCTGTGATGTC	AAGGTGTTGCACACCCTACCC	36–40
23	Mdm2	GCCTGGATCAGGATTCAGTTTCTG	GTGACCCGATAGACCTCATCATCC	24–29
24	Myc	AGAGGGCCAAGTTGGACAGTGG	GACGTTGTGTGTCCGCCTCTTG	25–30
25	Net1	ATTGTCTGGCTGAACCAGAGG	TGCAGGTATGAGAAACCAAAGC	24–28
26	Phlda3	CGCATCAAAGCCGTGGAGTG	AGGGTGATCTGAGCGTTCCAG	23–27
27	Plk2	TGGAGGAGAACCTCATGGATGG	CACCTGAAATGTGCCGTCATTG	23–27
28	Pml	TCAAGGCCTTGGATGAGAGCC	CGGAACTTGCTTTCCCGGTTC	27–31
29	Pmm1	GAAGACAGAGTTTGCTGGCAAGG	CTGTCCAGGCAGTAGCGCTTATC	24–29
30	Rcan1	AGAGGGCAGAGGGAGTTTCAAG	AAAGGCACTGTGTCCCTCTAGG	24–30
31	Tnf	GCTGAGCTCAAGCCCTGGTATG	CCCGGACTCCGTGATGTCTAAG	32–35
32	Tp53	ATGGCTTCCACCTGGGCTTC	TGACCCACAACTGCACAGGGC	26–29
33	Tubb2c	GCTAAATGCTGACCTGCGGAAAC	CTGGGTGAGCTCAGGAACTGTC	23–26

The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold under the present experimental conditions.

Table 3.1
Gene expression ratio (Exp/Cont) at 4 h and the results of the Williams' test and Dunnett's test.

No.	Gene symbol	Gene expression ratio (Exp/Cont) and Williams' test									Dunnett's test	
		Genotoxic hepatocarcinogens					Non-genotoxic hepatocarcinogen		Non-genotoxic non-hepatocarcinogen		G/DEHP	G/PNT
		DEN (mg/kg bw)			DNT (mg/kg bw)		DEHP (mg/kg bw)		PNT (mg/kg bw)			
		12.5 mg	25 mg	50 mg	125 mg	250 mg	1000 mg	2000 mg	500 mg	1000 mg		
1	Aen	1.60 ± 0.25**	2.96 ± 1.05**	7.40 ± 1.47**	0.86 ± 0.12	1.22 ± 0.62	1.02 ± 0.13	0.72 ± 0.17*	0.74 ± 0.05	0.94 ± 0.40	$P<0.01$	$P<0.01$
2	Bax	4.63 ± 1.60**	4.67 ± 0.78**	5.08 ± 1.49**	0.87 ± 0.22	0.95 ± 0.22	3.37 ± 0.49**	0.95 ± 0.27	0.75 ± 0.14	0.78 ± 0.08		$P<0.01$
3	Btg2	1.78 ± 0.67*	2.72 ± 0.75**	4.72 ± 1.95**	2.81 ± 1.55**	4.40 ± 0.85**	1.78 ± 1.68	1.32 ± 0.36	1.71 ± 0.33**	3.02 ± 0.85**	$P<0.01$	
4	Ccnf	3.34 ± 0.67**	3.45 ± 0.59**	2.31 ± 0.65**	1.11 ± 0.39	1.38 ± 0.41	0.89 ± 0.29	1.41 ± 0.55	0.73 ± 0.11	0.84 ± 0.05	$P<0.01$	$P<0.01$
5	Ccng1	3.45 ± 1.10**	5.55 ± 2.26**	13.6 ± 3.12**	1.16 ± 0.22	1.75 ± 0.30**	0.90 ± 0.09	0.45 ± 0.02**	0.96 ± 0.11	1.40 ± 0.23*	$P<0.01$	$P<0.01$
6	Cdkn1a	1.90 ± 0.17**	3.38 ± 0.19**	8.20 ± 1.88**	1.69 ± 0.87	1.95 ± 0.24**	2.14 ± 0.67*	1.89 ± 0.41*	3.10 ± 0.57**	4.87 ± 0.28**		
7	Cyp21a1	1.01 ± 0.23	1.08 ± 0.10	2.28 ± 0.80**	0.91 ± 0.38	1.09 ± 0.33	0.94 ± 0.17	0.77 ± 0.29	0.70 ± 0.21	0.66 ± 0.07		
8	Cyp4a1	2.68 ± 1.13	1.44 ± 0.25	1.34 ± 0.61	1.62 ± 0.11**	1.44 ± 0.43	3.53 ± 1.25**	6.75 ± 0.30**	0.60 ± 0.04	1.66 ± 0.34		
9	Ddit4l	15.1 ± 5.10**	22.2 ± 8.85**	16.1 ± 7.37**	1.91 ± 0.55*	4.27 ± 1.82**	0.33 ± 0.09*	0.71 ± 0.55	0.55 ± 0.10*	0.62 ± 0.11	$P<0.01$	$P<0.01$
10	Egfr	1.77 ± 0.91	1.09 ± 0.55	0.87 ± 0.20	1.00 ± 0.35	1.31 ± 1.44	2.02 ± 0.88	3.71 ± 0.91**	0.78 ± 0.10	1.49 ± 0.53		
11	Ephx1	2.73 ± 0.20**	2.33 ± 0.27**	2.48 ± 0.28**	0.97 ± 0.37	1.39 ± 0.30	1.43 ± 0.36	2.12 ± 0.42**	0.81 ± 0.26	0.99 ± 0.21		$P<0.01$
12	Gadd45b	1.08 ± 0.44	1.53 ± 0.87	3.09 ± 1.12**	1.69 ± 0.51*	2.11 ± 0.56**	1.62 ± 0.92	2.50 ± 1.02*	3.97 ± 0.46**	5.41 ± 0.63**		
13	Gadd45g	0.98 ± 0.44	1.17 ± 0.76	1.21 ± 0.39	0.95 ± 0.43	0.75 ± 0.11	14.2 ± 9.08	3.30 ± 1.26**	0.84 ± 0.30	1.96 ± 0.71*	$P<0.01$	
14	Gdf15	1.69 ± 0.45	2.24 ± 0.25**	4.31 ± 1.48**	2.56 ± 0.76**	8.66 ± 1.05**	1.70 ± 0.99	2.30 ± 0.68**	0.67 ± 0.04*	0.78 ± 0.19	$P<0.05$	$P<0.01$
15	Hhex	0.82 ± 0.25	0.54 ± 0.17	1.24 ± 0.37	1.24 ± 0.12*	1.68 ± 0.40*	1.07 ± 0.50	1.20 ± 0.33	0.68 ± 0.25	0.70 ± 0.06*		
16	Hmox1	0.44 ± 0.08	0.61 ± 0.26	1.29 ± 0.35	1.33 ± 0.30	4.79 ± 2.60**	1.26 ± 0.28	0.70 ± 0.15	0.77 ± 0.10	1.33 ± 0.44		
17	Hspb1	2.50 ± 1.27**	2.48 ± 0.35**	1.98 ± 0.42*	1.30 ± 0.33	1.42 ± 0.16*	0.92 ± 0.16	0.59 ± 0.06*	0.86 ± 0.14	0.94 ± 0.19	$P<0.01$	$P<0.01$
18	Igfbp1	1.42 ± 0.84	0.44 ± 0.15	1.04 ± 0.45	2.24 ± 0.99*	2.34 ± 0.96*	0.74 ± 0.31	0.91 ± 0.21	2.59 ± 0.42**	3.94 ± 0.79**		
19	Jun	1.56 ± 0.49*	2.27 ± 0.51**	7.62 ± 3.56**	3.39 ± 0.83**	5.33 ± 1.40**	0.71 ± 0.56	0.51 ± 0.15*	1.15 ± 0.55	1.14 ± 0.20	$P<0.01$	$P<0.01$
20	Lpp	1.58 ± 0.11	1.41 ± 0.30	0.63 ± 0.36	0.89 ± 0.36	1.12 ± 0.71	2.35 ± 0.85*	1.52 ± 0.43	0.78 ± 0.18	0.95 ± 0.14	$P<0.05$	
21	Ly6al	0.98 ± 0.06	1.03 ± 0.09	1.63 ± 0.24**	0.74 ± 0.11	1.02 ± 0.41	1.65 ± 0.50	0.97 ± 0.16	0.81 ± 0.21	0.72 ± 0.04*		
22	Mdm2	0.79 ± 0.12	1.53 ± 0.98	2.05 ± 0.66*	1.25 ± 0.45	2.00 ± 0.31**	1.72 ± 0.27**	1.07 ± 0.19	1.11 ± 0.32	1.12 ± 0.25		
23	Myc	2.33 ± 0.97*	1.02 ± 0.38	10.0 ± 2.40**	5.43 ± 1.48**	12.4 ± 2.67**	1.71 ± 0.95	0.83 ± 0.18	1.12 ± 0.47	2.07 ± 0.40*	$P<0.01$	$P<0.01$
24	Net1	3.04 ± 1.30**	1.71 ± 0.39*	2.62 ± 1.30**	1.29 ± 0.16	1.46 ± 0.30*	0.22 ± 0.07**	1.08 ± 0.64	0.66 ± 0.11	0.85 ± 0.13	$P<0.01$	$P<0.01$
25	Phlda3	3.99 ± 0.64**	5.03 ± 0.81**	6.60 ± 1.68**	0.99 ± 0.31	1.93 ± 0.42*	0.95 ± 0.19	1.01 ± 0.18	0.55 ± 0.12*	0.75 ± 0.20	$P<0.01$	$P<0.01$
26	Plk2	0.57 ± 0.09	1.06 ± 0.71	2.02 ± 0.58*	1.16 ± 0.48	1.70 ± 0.21**	2.42 ± 0.55**	1.50 ± 0.27	0.54 ± 0.26*	0.63 ± 0.09**	$P<0.05$	$P<0.01$
27	Pml	2.01 ± 0.64*	1.86 ± 0.49*	1.71 ± 0.24**	1.18 ± 0.28	0.97 ± 0.25	2.98 ± 0.66**	2.08 ± 0.15**	0.66 ± 0.27	0.94 ± 0.21	$P<0.01$	$P<0.05$
28	Pmm1	2.18 ± 0.36**	2.86 ± 0.72**	2.73 ± 0.47**	0.85 ± 0.07	1.22 ± 0.30	0.77 ± 0.06	1.22 ± 0.23	1.13 ± 0.16	1.08 ± 0.13	$P<0.01$	$P<0.05$
29	Rcan1	1.14 ± 0.41	2.72 ± 0.30**	3.41 ± 0.37**	1.75 ± 0.5*	3.24 ± 0.81**	0.51 ± 0.13	1.02 ± 0.06	0.56 ± 0.34	0.50 ± 0.08	$P<0.01$	$P<0.01$
30	Tnf	0.87 ± 0.17	0.91 ± 0.14	1.44 ± 0.24	1.31 ± 0.33	1.01 ± 0.42	0.74 ± 0.24	0.74 ± 0.23	1.67 ± 0.40*	1.98 ± 0.31**	$P<0.01$	$P<0.01$
31	Tp53	1.12 ± 0.19	1.34 ± 0.34	1.33 ± 0.11	1.63 ± 0.64	1.78 ± 0.63	0.61 ± 0.19	1.43 ± 0.29	0.63 ± 0.19	1.14 ± 0.29		
32	Tubb2c	2.10 ± 0.22**	4.38 ± 1.41**	4.79 ± 1.02**	1.30 ± 0.36	1.59 ± 0.13**	0.45 ± 0.09	0.96 ± 0.16	0.77 ± 0.11	1.33 ± 0.24	$P<0.01$	$P<0.01$
33	Gapdh	0.94 ± 0.12	0.79 ± 0.10	0.52 ± 0.07	0.90 ± 0.16	0.87 ± 0.18	0.84 ± 0.08	0.75 ± 0.06	1.19 ± 0.38	1.13 ± 0.08		

Total RNA was extracted from individual livers, and cDNA was prepared. The expression of the 33 genes was quantified by qPCR, and the gene expression ratio (Exp/Cont) was calculated. The results were analyzed statistically using the Williams' test for each chemical (**significant at $P<0.01$, *significant at $P<0.05$) and the Dunnett's test to compare the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen (DEHP) or the non-genotoxic non-hepatocarcinogen (PNT).

experimental groups (experimental group/control group; Exp/Cont) was within the range of 0.52 to 1.58, as shown in Tables 3.1 and 3.2.

2.3. Statistical analysis

For statistical analysis, we performed a logarithmic (log_2) transformation of the data to stabilize the variance, and the gene expression profiles were normalized to the median gene expression level for the entire sample set.

The significance of dose-dependent increases or decreases in the individual qPCR data was statistically determined using the Williams' test at 4 and 48 h. The experimental groups were compared to a control group. The statistical significance for each gene between the genotoxic hepatocarcinogens, the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen was assessed with the Dunnett's test at 4 and 48 h. The statistical significance between the control water group and olive oil group was assessed using Welch's *t*-test.

Differentiation of the gene expression profiles associated with genotoxic hepatocarcinogens from those associated with the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen was achieved through statistical analysis using PCA. PCA involves a mathematical procedure that transforms a number of potentially correlated variables into a smaller number of uncorrelated variables referred to as "principal components". The first principal component (PC1) accounts for as much of the variability in the data as possible, and each subsequent component accounts for as much of the remaining variability as possible. PCA was performed using the PCA programs in GeneSpringGX11.0.1 (Agilent Technologies, Santa Clara, CA, USA). Initially, PCA was applied to all 32 logarithmically (log_2) transformed ratios (Exp/Cont), with the exception of *Gapdh* and was subsequently tested with various candidate gene sets until the optimal discrimination was achieved. The optimal candidate genes were primarily selected based on the results of Dunnett's test at 4 h and 48 h. The results are presented in two-dimensional (PC1 and PC2) and three-dimensional figures (PC1, PC2 and PC3).

2.4. Gene ontology, pathways and network analysis

Gene ontology analysis was performed using the Gene Ontology Database (http://geneontology.org/) and Ingenuity Pathways Analysis 7.0 (IPA) (http://www.Ingenuity.com). The results were confirmed using the references available in PubMed (http://www.ncbi.nlm.nih.gov/pubmed). The gene pathways and networks were generated with GeneSpringGX11.0.1 and IPA, which enable the visualization and analysis of biologically relevant networks to allow for discovery, visualization, and exploration of therapeutically relevant networks, as previously described [9,10].

2.5. Immunohistochemistry

Immunohistochemical staining was performed using monoclonal antibodies against Cdkn1a/p21 [(p21 (F-5): sc-6246), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)] and Hmox1 [(Anti-HO-1), Stressgen Bioreagents (Brussels, Belgium)], as described in the manufacture's protocol, on the livers of 4 rats in each group. The TUNEL method was applied using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Flowgen Bioscience Ltd., Nottingham, UK).

3. Results

3.1. Changes in gene expression determined by qPCR and analyzed with the Williams' test and the Dunnett's test

The individual qPCR gene expression results (Exp/Cont) were calculated for each group (4 rats in triplicate assays), the mean ± SD was determined, and statistical significance was assessed using the Williams' test. All 32 genes, with the exception of *Gapdh*, exhibited statistically significant changes in gene expression at least once, at 4 h and/or 48 h, as calculated using the Williams' test (Tables 3.1 and 3.2). The changes in gene expression were generally greater at 4 h than at 48 h. Furthermore, at 4 h, statistical significance was observed in the Dunnett's test between the genotoxic hepatocarcinogens (DEN and DNT) and the non-genotoxic hepatocarcinogen (DEHP) for 19 genes (*Aen*, *Btg2*, *Ccnf*, *Ccng1*, *Ddit4l*, *Gadd45g*, *Gdf15*, *Hspb1*, *Jun*, *Lpp*, *Myc*, *Net1*, *Phlda3*, *Plk2*, *Pml*, *Pmm1*, *Rcan1*, *Tnf* and *Tubb2c*) and between genotoxic hepatocarcinogens and the non-genotoxic non-hepatocarcinogen (PNT) for 18 genes (*Aen*, *Bax*, *Ccnf*, *Ccng1*, *Ddit4l*, *Ephx1*, *Gdf15*, *Hspb1*, *Jun*, *Myc*, *Net1*, *Phlda3*, *Plk2*, *Pml*, *Pmm1*, *Rcan1*, *Tnf* and *Tubb2c*), as shown in Table 3.1. At 48 h, statistical significance was observed between genotoxic hepatocarcinogens (DEN and DNT) and the non-genotoxic hepatocarcinogen (DEHP) for 14 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Cyp4a1*, *Hhex*, *Igfbp1*, *Ly6al*, *Mdm2*, *Myc*, *Phlda3*, *Pml*, *Pmm1* and *Tubb2c*) and between the genotoxic hepatocarcinogens and the non-genotoxic non-hepatocarcinogen (PNT) for 8 genes (*Ccng1*, *Cdkn1a*, *Cyp4a1*, *Gdf15*, *Igfbp1*, *Mdm2*, *Phlda3 and Plk2*) using the Dunnett's test, as shown in Table 3.2. The results for the housekeeping gene *Gapdh* are also shown in Tables 3.1 and 3.2. This gene was used to normalize the gene expression ratio, as it did not show any changes in expression.

The changes in gene expression detected for 10 major genes (*Aen*, *Btg2*, *Ccng1*, *Cdkn1a*, *Ddit4l*, *Gdf15*, *Jun*, *Phlda3*, *Rcan1* and *Tubb2c*) are shown in Fig. 1. At 4 h, DEN and DNT produced a dose-dependent increase in all of these 10 genes, with the exception of *Aen* under DNT treatment. At 48 h, DEN and DNT produced dose-dependent increases in *Ccng1*, *Cdkn1a* and *Phlda3*. However, DEHP and PNT did not cause dose-dependent increases in these 10 genes at 4 or 48 h. Furthermore, statistical significance (using the Dunnett's test) was observed between the genotoxic hepatocarcinogens and one non-genotoxic hepatocarcinogen (DEHP) and/or the non-genotoxic non-hepatocarcinogen (PNT) for 9 of the genes, with the exception of *Cdkn1a*, at 4 h and for *Aen*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Phlda3* and *Tubb2c* at 48 h. No single gene completely discriminated genotoxic hepatocarcinogens (DEN and DNT) from the non-genotoxic hepatocarcinogen (DEHP) and/or the non-genotoxic non-hepatocarcinogen (PNT).

3.2. Differentiation of the gene expression profiles of the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen by statistical analysis using PCA

Differentiation of the gene expression profile obtained from the genotoxic hepatocarcinogens and from the non-genotoxic hepatocarcinogen and/or from the non-genotoxic non-hepatocarcinogen was achieved *via* statistical analysis using PCA. PCA of all 32 genes was able to differentiate genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen at 4 and 48 h (data not shown). Furthermore, we selected specific genes to obtain optimal separation between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen using PCA. PCA of 16 genes (*Ccnf*, *Ccng1*, *Cyp4a1*, *Ddit4l*, *Egfr*, *Gadd45g*, *Gdf15*, *Hspb1*, *Ighbp1*, *Jun*, *Myc*, *Net1*, *Phlda3*, *Pml*, *Rcan1* and *Tubb2c*) at 4 h and of 10 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Cyp4a1*, *Gdf15*, *Igfbp1*, *Mdm2*, *Phlda3* and *Pmm1*) at 48 h optimally differentiated the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen as well as the non-genotoxic non-hepatocarcinogen, with principal component 1 (PC1) (Fig. 2A-1 at 4 h and Fig. 2B-1 at 48 h). At 4 h, the genotoxic hepatocarcinogens exhibited a PC1 of less than −0.24, while the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen exhibited a PC1 of greater than 2.4 (Fig. 2A-1). At 48 h, the genotoxic hepatocarcinogens presented a PC1 less than 0.06, whereas the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen presented a PC1 greater than 1.8 (Fig. 2B-1). The hepatocarcinogens (in the green circle) were distinguished from the non-hepatocarcinogen (PNT, in the blue circle) with PC1, PC2 and PC3 in 3 dimensions at 4 and 48 h (Fig. 2A-2 and B-2).

3.3. Gene ontology and biologically relevant gene networks

We analyzed the gene ontology of the examined genes using the Gene Ontology Database (in *Rattus norvegicus*) to clarify which categories of genes contributed to the differentiation between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen; the

Table 3.2
Gene expression ratio (Exp/Cont) at 48 h and the results of the Williams' test and Dunnett's test.

No.	Gene symbol	Gene expression ratio (Exp/Cont) and Williams' test									Dunnett's test	
		Genotoxic hepatocarcinogens					Non-genotoxic hepatocarcinogen		Non-genotoxic non-hepatocarcinogen		G/DEHP	G/PNT
		DEN (mg/kg bw)			DNT (mg/kg bw)		DEHP (mg/kg bw)		PNT (mg/kg bw)			
		12.5 mg	25 mg	50 mg	125 mg	250 mg	1000 mg	2000 mg	500 mg	1000 mg		
1	Aen	1.90 ± 0.40	0.88 ± 0.13	1.43 ± 0.21	1.22 ± 0.52	1.42 ± 1.11	0.51 ± 0.10**	0.60 ± 0.06**	0.87 ± 0.27	1.10 ± 0.21	$P<0.01$	
2	Bax	0.61 ± 0.10**	0.71 ± 0.15*	0.53 ± 0.22*	1.34 ± 0.57	1.41 ± 0.59	0.77 ± 0.12	0.62 ± 0.11**	0.81 ± 0.23	0.82 ± 0.21		
3	Btg2	0.61 ± 0.37	0.54 ± 0.09	1.10 ± 0.13	1.11 ± 0.31	2.07 ± 0.94	0.97 ± 0.15	1.06 ± 0.34	0.93 ± 0.15	1.13 ± 0.16		
4	Ccnf	0.67 ± 0.09*	0.60 ± 0.18*	0.52 ± 0.11**	1.63 ± 0.65	2.30 ± 1.19*	0.97 ± 0.20	0.69 ± 0.32	0.80 ± 0.31	1.10 ± 0.31		
5	Ccng1	1.90 ± 1.01	2.04 ± 0.54**	4.22 ± 0.45**	1.30 ± 0.78	2.22 ± 2.34	0.49 ± 0.04**	0.70 ± 0.16*	0.72 ± 0.26	0.73 ± 0.17	$P<0.01$	$P<0.01$
6	Cdkn1a	3.12 ± 0.42**	5.88 ± 0.93**	7.79 ± 1.51**	2.26 ± 0.79*	3.31 ± 2.04*	1.63 ± 0.27**	1.53 ± 0.29*	1.03 ± 0.14	1.16 ± 0.21	$P<0.01$	$P<0.01$
7	Cyp21a1	1.32 ± 0.44	1.14 ± 0.42	1.18 ± 0.37	0.91 ± 0.08	1.32 ± 0.26	2.04 ± 0.61**	2.68 ± 0.66**	0.93 ± 0.20	1.73 ± 0.56*	$P<0.01$	
8	Cyp4a1	0.56 ± 0.11**	0.50 ± 0.14**	0.29 ± 0.09**	0.72 ± 0.20	0.70 ± 0.36	5.43 ± 2.30**	9.66 ± 3.13**	1.04 ± 0.42	0.91 ± 0.35	$P<0.01$	$P<0.01$
9	Ddit4l	0.59 ± 0.21	0.80 ± 0.22	1.05 ± 0.26	2.04 ± 1.41	1.93 ± 1.01	0.92 ± 0.13	0.52 ± 0.04**	1.48 ± 0.21**	1.25 ± 0.25		
10	Egfr	0.65 ± 0.18*	0.73 ± 0.15*	0.73 ± 0.24	1.10 ± 0.41	1.02 ± 0.36	1.03 ± 0.18	0.66 ± 0.12*	0.97 ± 0.34	1.01 ± 0.44		
11	Ephx1	0.85 ± 0.13	1.09 ± 0.16	2.05 ± 0.20**	1.80 ± 0.75	1.32 ± 0.16	1.15 ± 0.32	0.91 ± 0.12	0.98 ± 0.15	1.12 ± 0.32		
12	Gadd45b	0.58 ± 0.16	1.18 ± 0.07	0.62 ± 0.24	0.60 ± 0.08	1.00 ± 0.57	1.07 ± 0.16	0.68 ± 0.31	0.72 ± 0.19	0.89 ± 0.17		
13	Gadd45g	1.05 ± 0.05	1.69 ± 0.35*	2.14 ± 0.53**	1.62 ± 0.49	1.42 ± 0.29	0.63 ± 0.17	3.03 ± 4.49	0.83 ± 0.19	2.46 ± 1.77		
14	Gdf15	1.42 ± 0.48	1.49 ± 0.36*	2.29 ± 0.51**	0.70 ± 0.19	1.38 ± 0.79	1.31 ± 0.33	1.41 ± 0.26*	0.81 ± 0.19	0.73 ± 0.16		$P<0.01$
15	Hhex	0.31 ± 0.06**	0.38 ± 0.11**	0.35 ± 0.07**	0.85 ± 0.11	1.02 ± 0.25	1.20 ± 0.42	1.30 ± 0.43	0.57 ± 0.15*	0.84 ± 0.41	$P<0.01$	
16	Hmox1	0.77 ± 0.19	0.58 ± 0.10	1.29 ± 0.28	1.16 ± 0.38	1.63 ± 0.98	0.74 ± 0.11	1.05 ± 0.07	0.95 ± 0.04	1.02 ± 0.09		
17	Hspb1	0.55 ± 0.22**	0.53 ± 0.12**	0.55 ± 0.15**	1.56 ± 0.37*	1.54 ± 0.69	1.33 ± 0.25	0.93 ± 0.25	0.91 ± 0.19	0.97 ± 0.19		
18	Igfbp1	0.27 ± 0.03**	0.35 ± 0.16**	0.44 ± 0.17**	0.59 ± 0.24	0.45 ± 0.23*	0.73 ± 0.16	0.77 ± 0.25	0.62 ± 0.30	0.97 ± 0.18	$P<0.01$	$P<0.01$
19	Jun	0.54 ± 0.02**	0.45 ± 0.07**	0.60 ± 0.20	0.43 ± 0.15	1.10 ± 0.90	1.06 ± 0.20	0.73 ± 0.11	0.52 ± 0.15*	0.46 ± 0.09**		
20	Lpp	0.51 ± 0.09**	0.47 ± 0.22*	0.38 ± 0.11**	1.23 ± 0.16	1.42 ± 0.35	0.59 ± 0.15*	0.37 ± 0.04**	0.54 ± 0.16	0.66 ± 0.14		
21	Ly6al	1.33 ± 0.34	1.17 ± 0.44	1.24 ± 0.23	0.67 ± 0.40	0.92 ± 0.18	1.51 ± 0.26	1.87 ± 0.52*	0.66 ± 0.28	1.45 ± 0.24	$P<0.01$	
22	Mdm2	1.99 ± 0.63*	2.29 ± 0.35**	4.40 ± 0.99**	1.77 ± 0.82	2.33 ± 2.11	1.16 ± 0.19	1.75 ± 0.22**	1.10 ± 0.29	1.05 ± 0.30	$P<0.05$	$P<0.01$
23	Myc	0.17 ± 0.05**	0.37 ± 0.09**	0.48 ± 0.15	1.04 ± 0.61	1.02 ± 0.34	1.18 ± 0.48	0.85 ± 0.52	0.66 ± 0.26	0.73 ± 0.56	$P<0.05$	
24	Net1	0.69 ± 0.10	0.79 ± 0.25	0.40 ± 0.10**	1.15 ± 0.29	1.15 ± 0.36	0.81 ± 0.11	0.65 ± 0.09*	0.90 ± 0.26	1.84 ± 0.94		
25	Phlda3	3.10 ± 1.21**	3.86 ± 1.00**	2.80 ± 0.32**	1.11 ± 0.61	1.76 ± 1.74	0.90 ± 0.28	0.83 ± 0.19	0.67 ± 0.22	0.69 ± 0.17	$P<0.01$	$P<0.01$
26	Plk2	2.16 ± 1.04**	2.91 ± 0.40**	3.14 ± 0.65**	1.01 ± 0.23	1.07 ± 0.38	1.09 ± 0.30	1.75 ± 0.80*	0.86 ± 0.16	0.81 ± 0.18		$P<0.01$
27	Pml	1.02 ± 0.42	1.01 ± 0.28	1.61 ± 0.47	1.01 ± 0.30	1.14 ± 0.60	0.92 ± 0.29	0.57 ± 0.08**	0.76 ± 0.21	1.20 ± 0.27	$P<0.05$	
28	Pmm1	0.90 ± 0.13	0.98 ± 0.03	3.06 ± 0.63**	1.50 ± 0.82	2.98 ± 2.32	1.22 ± 0.16	1.12 ± 0.15	0.76 ± 0.17	1.11 ± 0.26	$P<0.05$	
29	Rcan1	0.84 ± 0.24	1.03 ± 0.17	0.59 ± 0.06*	0.51 ± 0.26*	0.66 ± 0.25	0.85 ± 0.20	0.67 ± 0.07**	0.56 ± 0.13**	0.52 ± 0.13**		
30	Tnf	0.99 ± 0.42	0.59 ± 0.12	1.15 ± 0.19	0.57 ± 0.28	1.01 ± 0.57	1.19 ± 0.16	1.21 ± 0.07	0.57 ± 0.07	1.07 ± 0.57		
31	Tp53	1.29 ± 0.39	1.18 ± 0.16	1.60 ± 0.19**	0.99 ± 0.38	0.90 ± 0.48	1.14 ± 0.21	0.92 ± 0.16	1.02 ± 0.44	0.68 ± 0.36		
32	Tubb2c	0.46 ± 0.09**	0.52 ± 0.07**	0.26 ± 0.07**	0.99 ± 0.19	1.39 ± 0.70	1.18 ± 0.08	1.37 ± 0.29	0.75 ± 0.16	1.01 ± 0.21	$P<0.01$	
33	Gapdh	1.03 ± 0.12	1.00 ± 0.16	1.08 ± 0.15	1.58 ± 0.15	1.55 ± 0.05	0.73 ± 0.14	0.76 ± 0.08	1.08 ± 0.15	1.07 ± 0.20		

Total RNA was extracted from individual livers, and cDNA was prepared. The expression of the 33 genes was quantified by qPCR, and the gene expression ratio (Exp/Cont) was calculated. The results were analyzed statistically using the Williams' test for each chemical (**significant at $P<0.01$, *significant at $P<0.05$) and the Dunnett's test to compare the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen (DEHP) or the non-genotoxic non-hepatocarcinogen (PNT).

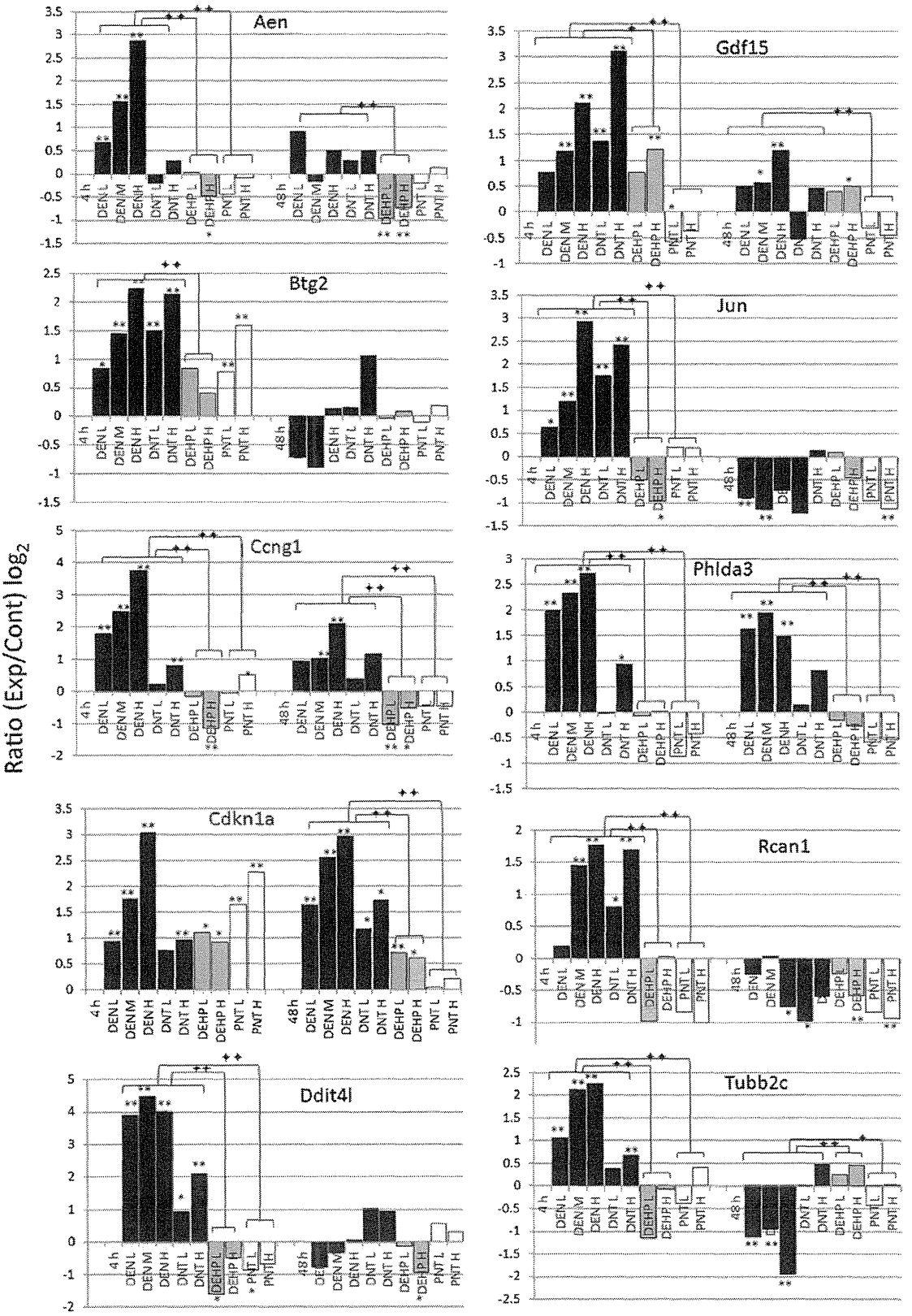

Fig. 1. Changes in the gene expression of 10 genes (*Aen*, *Btg2*, *Ccng1*, *Cdkn1a*, *Ddit4l*, *Gdf15*, *Jun*, *Phlda3*, *Rcan1* and *Tubb2c*) as quantified by qPCR at 4 h and 48 h. DEN L: DEN low dose, DEN M: DEN middle dose, DEN H: DEN high dose, DNT L: DNT low dose, DNT H: DNT high dose, DEHP L: DEHP low dose, DEHP H: DEHP high dose, PNT L: PNT low dose and PNT H: PNT high dose. The statistical significance for each chemical was analyzed using the Williams' test. $^{*}P < 0.05$; $^{**}P < 0.01$. The statistical significance between genotoxic hepatocarcinogens and non-genotoxic hepatocarcinogens or the non-genotoxic non-hepatocarcinogen was analyzed using the Dunnett's test, ♦$P < 0.05$, and ♦♦$P < 0.01$ outside the framework. ■: Genotoxic hepatocarcinogen, ▒ : non-genotoxic hepatocarcinogen, □: non-genotoxic non-hepatocarcinogen. Total RNA was extracted from individual livers (4 rats/group) and reverse-transcribed into cDNA. Changes in gene expression were determined in triplicate by qPCR.

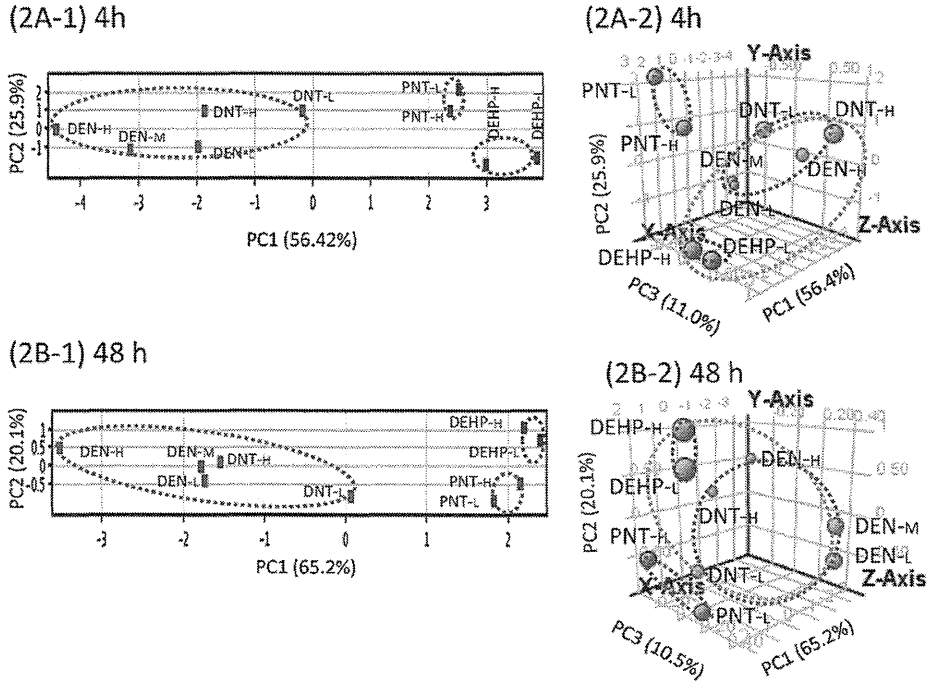

Fig. 2. Principal component analysis (PCA) of the gene expression levels under treatment with 3 types of carcinogens as quantified by qPCR. Genotoxic hepatocarcinogens (red-colored, DEN-L: DEN low dose, DEN-M: DEN middle dose, DEN-H: DEN high dose, DNT-L: DNT low dose and DNT-H: DNT high dose), a non-genotoxic hepatocarcinogen (brown-colored, DEHP-L: DEHP low dose and DEHP-H: DEHP high dose) and a non-genotoxic non-hepatocarcinogen (blue-colored, PNT-L: PNT low dose and PNT-H: PNT high dose). The mean values of triplicate qPCR assays for each sample were analyzed statistically using the PCA program in GeneSpringGX11.0.1. The results of the PCA are shown as the two- or three-dimensional contribution scores for component numbers 1, 2 and 3 (PC1, PC2 and PC3). The contribution scores were produced by conversion from each eigenvector value. A: 4 h, with 16 genes (*Ccnf*, *Ccng1*, *Cyp4a1*, *Ddit4l*, *Egfr*, *Gadd45g*, *Gdf15*, *Hspb1*, *Ighbp1*, *Jun*, *Myc*, *Net1*, *Phlda3*, *Pml*, *Rcan1* and *Tubb2c*), B: 48 h, with 10 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Cyp4a1*, *Gdf15*, *Igfbp1*, *Mdm2*, *Phlda3* and *Pmm1*). PCA successfully differentiated the genotoxic hepatocarcinogen (red circle) from the non-genotoxic hepatocarcinogen (brown circle) and non-genotoxic non-hepatocarcinogen (blue circle) with principal component 1 at 4 and 48 h (A-1 and B-1). The hepatocarcinogens (green circle) were distinguished from the non-hepatocarcinogen (blue circle) with PC1, PC2 and PC3 at 4 and 48 h (A-2 and B-2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

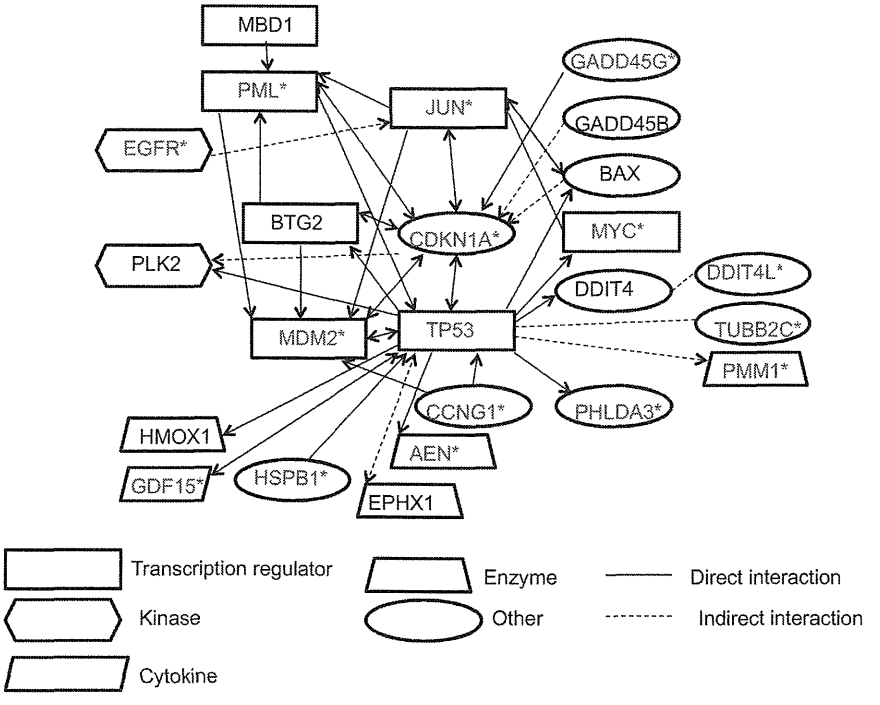

Fig. 3. The gene networks and pathways of 24 genes as determined by qPCR. The network was constructed from the results of Ingenuity Pathways Analysis, GeneSpring software and references from PubMed. The 15 red-colored genes indicated with an asterisk are genes that significantly contributed to the discrimination of the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen by PCA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

results are shown in Table 4. Eight major biological processes were extracted from this gene ontology analysis. The first process, which included 18 genes, was associated with apoptosis; the second was associated with the cell cycle and included 14 genes; the third was associated with cell proliferation and included 11 genes; the fourth process, which included 10 genes, was associated with DNA damage; the fifth was associated with DNA repair and included 1 gene; the sixth was associated with oxidative stress and included 3 genes; the seventh was oncogenes and included 2 genes; and the eighth process was tumor suppressors and included 1 gene. A considerable number of genes classified in the apoptosis, cell cycle, cell proliferation and DNA damage categories exhibited differential gene expression between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen as well as the non-genotoxic non-hepatocarcinogen. The DNA damage response, which functions *via* signal transduction through a p53 class mediator and results in the induction of apoptosis, was characteristically suggested as an associated biological process. Sixteen genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Ephx1*, *Gdf15*, *Hmox1*, *Hspb1*, *Mdm2*, *Myc*, *Phlda3*, *Plk2*, *Pmm1*, *Pml* and *Tbb2c*) from the present study were reported to be associated with *Tp53*. Among these, 9 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Gdf15*, *Hspb1*, *Mdm2*, *Myc*, *Pml* and *Phlda3*) contributed to the differentiation of the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen in the PCA. The summarized gene networks are shown in Fig. 3. The major gene pathway suggested by the network was the *Tp53*-mediated DNA damage response.

3.4. The expression of Cdkn1a and Hmox1 proteins, the level of apoptosis and histological changes

Changes in the expression of Cdkn1a and Hmox1 proteins, the level of apoptosis measured by the TUNEL assay and histology were observed in the genotoxic hepatocarcinogen-treated rats at 48 h (Table 5) but were nearly undetectable at 4 h (results not shown) in all groups. Cdkn1a-positive cells and TUNEL-positive cells were observed in 2 of 4 and all 4 DEN-treated rats at the highest doses, respectively. Cdkn1a-positive cells, Hmox1-positive cells and TUNEL-positive cells were observed in all 8, 6 of 8 and 4 of 8 DNT-treated rats, respectively. An increase in the number of mitotic cells was observed in all 4 DEN-treated rats at the highest dose and 2 of the 4 DNT-treated rats at the highest dose, as determined by HE staining.

3.5. Relative gene expression ratio between the control olive oil and water groups

In the present study, DEN was dissolved in sterile water, while the other chemicals were dissolved or suspended in olive oil. Although olive oil is often used as a non-toxic solvent in animal studies, its effect on gene expression has rarely been examined. Table 6 shows the relative gene expression in the liver in the control olive oil and water groups at 4 and 48 h. Although statistically significant differences were observed in 18 genes based on Welch's *t*-test, the differences in 9 genes did not exceed 2-fold, which could be considered within normal variations, while only 2 genes (*Myc* and *Pml*) showed a 3-fold difference at 48 h. These differences did not appear to affect the results regarding the gene expression ratio (Exp/Cont) (Tables 3.1 and 3.2).

4. Discussion

In the present study, we applied our selected candidate marker genes, which were previously demonstrated to discriminate genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens in the mouse liver [8–10], to rat hepatocarcinogens in the young rat liver. Consequently, we suggest that the selected genes are also useful for differentiating genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen examined (in the present study) in the young rat liver; these differences were determined by qPCR and PCA at 4 and 48 h after a single administration of these chemicals. Although we did not examine nitroaromatic compounds in our previous experimental method in the mouse, our selected candidate marker genes were also useful for discriminating DNT from the non-genotoxic hepatocarcinogen in the young rat liver. Present results were also congruent with the results of micronucleus assay in young rats [11,12].

In the present study, 32 genes, with the exception of *Gapdh*, exhibited statistically significant changes in gene expression (Exp/Cont) at least once, at 4 and/or 48 h, as detected using the Williams' test (Tables 3.1 and 3.2). The changes in gene expression were generally greater at 4 h than at 48 h. Furthermore, statistical significance was observed, using the Dunnett's test, between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen (DEHP) and/or the non-genotoxic non-hepatocarcinogen (PNT) for 29 genes (with the exceptions being *Egfr*, *Hmox1*, *Tp53* and *Gapdh*) at 4 and/or 48 h (Tables 3.1 and 3.2). In PCA, the optimal differential gene expression was detected for 16 genes (*Ccnf*, *Ccng1*, *Cyp4a1*, *Ddit4l*, *Egfr*, *Gadd45g*, *Gdf15*, *Hspb1*, *Ighbp1*, *Jun*, *Myc*, *Net1*, *Phlda3*, *Pml*, *Rcan1* and *Tubb2c*) at 4 h and 10 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Cyp4a1*, *Gdf15*, *Igfbp1*, *Mdm2*, *Phlda3* and *Pmm1*) at 48 h. Seven of these candidate genes (*Aen*, *Ccng1*, *Cdkn1a*, *Mdm2*, *Myc*, *Phlda3* and *Pml*) were classified as DNA damage-associated genes in the Gene Ontology analysis (Table 4), while 11 genes (*Aen*, *Ccng1*, *Cdkn1a*, *Gadd45g*, *Hspb1*, *Jun*, *Mdm2*, *Myc*, *Net1*, *Phlda3* and *Pml*) were classified as apoptosis-associated genes. Fifteen genes (*Aen*, *Ccng1*, *Cdkn1a*, *Ddit4l*, *Egfr*, *Gadd45g*, *Gdf15*, *Hspb1*, *Jun*, *Mdm2*, *Myc*, *Phlda3*, *Pml*, *Pmm1* and *Tubb2c*) were associated with a *Tp53*-mediated signaling pathway (Fig. 3). These genes were characteristically suggested to be induced in the DNA damage response.

Table 4
Gene ontology analysis of the rat genes examined in the present study.

Biological process	Genes
Apoptosis	**Aen***, **Bax***, **Btg2***, **Ccng1***, **Cdkn1a***, Egfr, **Gadd45g***, Hmox1, **Hspb1***, **Jun***, **Mdm2***, **Myc***, **Net1***, **Phlda3***, **Plk2***, **Pml***, **Tnf***, Tp53
Cell cycle	**Bax***, **Ccnf***, **Ccng1***, **Cdkn1a***, Egfr, Gadd45b, **Gadd45g***, **Hhex***, **Jun***, **Mdm2***, **Myc***, **Plk2***, **Pml***, Tp53
Cell proliferation	**Bax***, **Ccng1***, **Cdkn1a***, Egfr, **Hhex***, Hmox1, **Jun***, **Myc***, **Pml***, **Tnf***
DNA damage	**Aen***, **Bax***, **Btg2***, **Ccng1***, **Cdkn1a***, Hmox1, **Mdm2***, **Myc***, **Phlda3***, **Pml***
DNA repair	Egfr
Oxidative stress	Egfr, Hmox1, **Pml***
Oncogene	**Jun***, **Myc***
Tumor suppressor	Tp53

Gene ontology analysis of the examined genes, based on Gene Ontology annotation (http://www.geneontology.org/) and references. Boldface with an asterisk (*) indicates differential gene expression between the genotoxic hepatocarcinogens and the non-genotoxic hepatocarcinogen and/or the non-genotoxic non-hepatocarcinogen at 4 and/or 48 h that was statistically significant based on the Dunnett's test.

Table 5
Immunohistochemistry and histopathological findings in the liver 48 h after treatment with the test chemicals.

Chemical	Vehicle	DEN			DNT		DEHP		PNT	
Dose (mg/kg bw)	0	12.5	25	50	125	250	1000	2000	500	1000
Animal no.	1234	1234	1234	1234	1234	1234	1234	1234	1234	1234
Description of immunohistochemistry										
Anti-Cdkn1a	----	----	----	--11	2111	3111	----	----	----	----
Anti-Hmox1	----	----	----	----	111-	211-	----	----	----	----
TUNEL	--1-	----	----	2222	---1	1-11	----	----	----	----
Test chemical-related histopathological finding										
Cell infiltration, inflammatory, Glisson's sheath	----	----	----	----	11--	2---	----	----	----	----
Hypertrophy, hepatocyte, diffuse	----	----	----	----	1---	2---	----	----	----	----
Single-cell necrosis	----	----	----	----	----	1---	----	----	----	----
Increase, mitosis	----	----	----	2222	----	--11	----	----	----	----

The liver was dissected and examined immunohistochemically. Vehicle: olive oil or sterile water. Observations were graded from 0 (-) to 3 semiquantitatively. -: no findings, 1: minimal, 2: mild, 3: severe.

When we analyzed the expression of *Tp53* itself, we identified a statistically significant but less than 2-fold increase only at 48 h post-injection of DEN (50 mg/kg bw) (Table 3.2), although the basal expression of *Tp53* in the control animals may already have been sufficient for DNA damage to occur under the experimental conditions.

In this paragraph, we compare the dose-dependent alterations in the gene expression induced by 4 h of DEN treatment in the 9-week-old male mouse livers using intraperitoneal injection [9] and the 4-week-old rat livers with oral administration. We observed generally similar changes between mice and rats. Specifically, 18 of the examined genes (*Aen*, *Bax*, *Btg2*, *Ccng1*, *Cdkn1a*, *Cyp21a1*, *Gadd45b*, *Gdf15*, *Hspb1*, *Jun*, *Mbd1*, *Mdm2*, *Myc*, *Net1*, *Plk2*, *Pmm1*, *Rcan1* and *Tubb2c*) showed similar dose-dependent alterations or positive alterations in gene expression in the rat liver at 4 h after DEN administration in the present study (Table 3.1). Among these genes, 7 (*Ccng1*, *Gdf15*, *Hspb1*, *Jun*, *Myc*, *Rcan1* and *Tubb2c*) contributed to the PCA in distinguishing the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen.

Table 6
Relative gene expression ratio between the control olive oil and water groups at 4 h and 48 h and the results of Welch's *t*-test.

No.	Gene symbol	Ratio (olive/water)	
		4 h	48 h
1	Aen	0.81 ± 0.13	1.24 ± 0.14
2	Bax	0.75 ± 0.11	**1.48 ± 0.27***
3	Btg2	1.01 ± 0.39	1.34 ± 0.21
4	Ccnf	1.10 ± 0.16	1.24 ± 0.46
5	Ccng1	0.98 ± 0.19	**1.89 ± 0.17****
6	Cdkn1a	0.58 ± 0.38	0.80 ± 0.19
7	Cyp21a1	0.89 ± 0.23	1.23 ± 0.41
8	Cyp4a1	**1.40 ± 0.25***	1.23 ± 0.56
9	Ddit4l	1.08 ± 0.21	0.79 ± 0.07
10	Egfr	1.26 ± 0.64	**1.81 ± 0.46****
11	Ephx1	1.52 ± 0.71	1.27 ± 0.33
12	Gadd45b	1.41 ± 0.59	0.93 ± 0.12
13	Gadd45g	1.10 ± 1.52	0.50 ± 0.17
14	Gdf15	0.78 ± 0.16	**0.46 ± 0.06***
15	Hhex	1.35 ± 0.51	**0.41 ± 0.11***
16	Hmox1	**0.48 ± 0.09****	**2.06 ± 0.16****
17	Hspb1	0.99 ± 0.14	0.76 ± 0.23
18	Igfbp1	**2.47 ± 1.09***	0.85 ± 0.27
19	Jun	1.11 ± 0.41	0.66 ± 0.29
20	Lpp	0.86 ± 0.17	**1.84 ± 0.18****
21	Ly6al	1.19 ± 0.11	1.60 ± 0.52
22	Mdm2	**0.63 ± 0.07***	**1.64 ± 0.14****
23	Myc	1.09 ± 1.26	**0.32 ± 0.03****
24	Net1	1.10 ± 0.26	0.84 ± 0.22
25	Phlda3	1.05 ± 0.38	**2.82 ± 0.81****
26	Plk2	1.16 ± 0.13	**1.30 ± 0.04***
27	Pml	**0.55 ± 0.25***	**3.48 ± 0.56****
28	Pmm1	**2.86 ± 0.31****	**1.58 ± 0.13****
29	Rcan1	1.04 ± 0.37	0.87 ± 0.22
30	Tnf	**2.58 ± 0.91***	1.23 ± 0.30
31	Tp53	0.90 ± 0.44	**1.35 ± 0.19***
32	Tubb2c	**0.60 ± 0.17***	1.02 ± 0.30
33	Gapdh	1.05 ± 0.14	1.20 ± 0.22

Total RNA was extracted from individual livers, and cDNA was prepared. The expression of the 33 genes was quantified by qPCR and the gene expression ratio (olive/water) was calculated. The results were analyzed statistically using Welch's *t*-test (boldface with **significant at $P < 0.01$, boldface with *significant at $P < 0.05$).

In this paragraph, we compare the gene expression changes induced by 2000 mg/kg bw DEHP at 4 and 48 h after administration between the 9-week-old male mouse livers [10] and the 4-week-old rat livers examined in the present study. The gene expression changes induced by DEHP were rather different between the mouse liver and the rat liver under the present experimental conditions. Specifically, we observed statistically significant changes in the gene expression induced by DEHP in the mouse liver in only 2 genes (*Ddit4 and Hist1h1c*) at 4 h and in 3 genes (*Bhlhe40*, *Hspb1* and *Ly6a*) at 48 h; however, we observed changes in gene expression in a greater number of genes in the rat liver induced by DEHP treatment at a dose of 2000 mg/kg bw in the present study. Statistically significant changes in gene expression were induced in the rat liver by treatment with 2000 mg/kg bw DEHP in 12 genes at 4 h and 16 genes at 48 h: however, only 3 genes (*Cyp41a*, *Egfr* and *Gadd45g*) at 4 h and only 1 gene (*Cyp41a*) at 48 h exhibited changes of greater than 3-fold in response to DEHP; these genes were not associated with DNA damage, and other genes presented rather minor changes.

We examined both the levels of protein expressions by immunohistochemistry using commercially available antibodies (anti-Cdkn1a and anti-Hmox1) and the levels of apoptosis by the TUNEL assay. Slight changes in the protein expression of Cdkn1a and Hmox1 and in the number of TUNEL-positive cells were only observed in the DEN- and DNT-treated rats at 48 h (Table 6), but not in the DEHP- and PNT-treated rats. However, dose-dependent alterations in the expression of proteins or in the level of apoptosis were not observed with DEN and DNT treatment. Although 1 of 4 rats in the vehicle control group showed positive results in the TUNEL assay, it was at a minimal grade, and it has been reported that the TUNEL assay is not necessarily completely negative in the rat liver of vehicle control groups [25]. The present results suggested the moderate induction of apoptosis in DEN-treated rats at a dose of 50 mg/kg bw and weak induction of apoptosis in DNT-treated rats at a dose of 250 mg/kg bw. The immunohistochemical results generally agreed with the results of the gene expression analyses for these proteins and with the apoptotic gene expression.

Few time-course-based differential gene expression profiles of genotoxic and non-genotoxic hepatocarcinogens in rodents have been published based on DNA microarray and real-time PCR analyses. Ellinger-Ziegelbauer et al. used the Affymetrix RG_U34 microarray system to examine the differential gene expression produced by 4 genotoxic (dimethylnitrosamine, 2-nitrofluorene, aflatoxin B1 and 4-(methylnitrosamino)1-(3-pyridyl)-1-butanone) and 4 non-genotoxic hepatocarcinogens (methapyrilene, diethylstilbestrol, Wy-14643 and piperonylbutoxide) in the livers of rats that had been administered doses of the chemicals for 1, 3, 7 and 14 days [26]. They reported the detection of 477 deregulated genes in 23 categories. A total of 9 out of our 33 genes agreed with their candidates, specifically, 5 of these genes were involved in the DNA damage response (*Bax*, *Btg2*, *Ccng1*, *Cdkn1a* and *Mdm2*), 2 genes were involved in the oxidative stress response category (*Ephx1* and *Hmox1*) and 2 genes were involved in cell survival/proliferation (*Gdf15* and *Igfbp1*). These authors proposed that there was a prominent induction of the p53 target genes (*Cdkn1a*, *Bax*, *Btg2*, *Ccng1* and *Mdm2*) by genotoxic carcinogens and of genes involved in cell cycle progression, oxidative protein damage and a regression response by the non-genotoxic carcinogens. We extracted a network associated with the *Tp53*-mediated signaling pathway, which includes these 5 p53 target genes (Fig. 3); however, our network is much more extensive.

In summary, based on our analysis of the 21 genes selected from our mouse DNA microarray and qPCR studies, we suggest that qPCR and PCA are effective methods for distinguishing genotoxic hepatocarcinogens from a non-genotoxic hepatocarcinogen and a non-genotoxic non-hepatocarcinogen in the 4-week-old male F344 rat liver at the early time points of 4 and 48 h after a single administration. The changes in gene expression were greater at 4 h than at 48 h for genotoxic hepatocarcinogens. We recommend the 4 h time point for the first experiment. We analyzed a nitroso compound (DEN) and a nitroaromatic compound (DNT) as genotoxic hepatocarcinogens, a peroxisome proliferator (DEHP) as a non-genotoxic hepatocarcinogen, and an aromatic amide (PNT) as a non-genotoxic non-hepatocarcinogen. Further analysis using a greater number of rat hepatocarcinogens with different chemical properties are required for a final selection of marker genes for discrimination of genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens as well as non-genotoxic non-hepatocarcinogens in the young rat liver.

Conflict of interest

We do not have any conflicts of interest, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent application/registrations or grants or other sources of funding.

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References

[1] H.M. Bolt, A. Huici-Montagud, Strategy of the scientific committee on occupational exposure limits (SCOEL) in the derivation of occupational exposure limits for carcinogens and mutagens, Arch. Toxicol. 82 (2008) 61–64.
[2] M.D. Waters, M. Jackson, I. Lea, Characterizing and predicting carcinogenicity and mode of action using conventional and toxicogenomics methods, Mutat. Res. 705 (2010) 184–200.
[3] K. Mathijs, K.J. Brauers, D.G. Jennen, A. Boorsma, M.H. van Herwijnen, R.W. Gottschalk, J.C. Kleinjans, J.H. van Delft, Discrimination for genotoxic and nongenotoxic carcinogens by gene expression profiling in primary mouse hepatocytes improves with exposure time, Toxicol. Sci. 112 (2009) 374–384.
[4] Z. Wu, A review of statistical methods for preprocessing oligonucleotide microarrays, Stat. Methods Med. Res. 18 (2009) 533–541.
[5] C.L. Yauk, M.L. Berndt, Review of the literature examining the correlation among DNA microarray technologies, Environ. Mol. Mutagen. 48 (2007) 380–394.
[6] A.M. Calcagno, S.V. Ambudkar, Analysis of expression of drug resistance-linked ABC transporters in cancer cells by quantitative RT-PCR, Methods Mol. Biol. 637 (2010) 121–132.
[7] E. Nardon, M. Donada, S. Bonin, I. Dotti, G. Stanta, Higher random oligo concentration improves reverse transcription yield of cDNA from bioptic tissues and quantitative RT-PCR reliability, Exp. Mol. Pathol. 87 (2009) 146–151.
[8] T. Watanabe, K. Tobe, Y. Nakachi, Y. Kondoh, M. Nakajima, S. Hamada, C. Namiki, T. Suzuki, S. Maeda, A. Tadakuma, M. Sakurai, Y. Arai, A. Hyogo, M. Hoshino, T. Tashiro, H. Ito, H. Inazumi, Y. Sakaki, H. Tashiro, C. Furihata, Differential gene expression induced by two genotoxic *N*-nitroso carcinogens, phenobarbital and ethanol in mouse liver examined with oligonucleotide microarray and quantitative real-time PCR, Gene Environ. 29 (2007) 115–127.
[9] T. Watanabe, G. Tanaka, S. Hamada, C. Namiki, T. Suzuki, M. Nakajima, C. Furihata, Dose-dependent alterations in gene expression in mouse liver induced by diethylnitrosamine and ethylnitrosourea and determined by quantitative real-time PCR, Mutat. Res. 673 (2009) 9–20.
[10] T. Watanabe, T. Suzuki, M. Natsume, M. Nakajima, K. Narumi, S. Hamada, T. Sakuma, A. Koeda, K. Oshida, Y. Miyamoto, A. Maeda, M. Hirayama, H. Sanada, H. Honda, W. Ohyama, E. Okada, Y. Fujiishi, S. Sutou, A. Tadakuma, Y. Ishikawa, M. Kido, R. Minamiguchi, I. Hanahara, C. Furihata, Discrimination of genotoxic and non-genotoxic hepatocarcinogens by statistical analysis based on gene expression profiles in mouse liver as determined by quantitative real-time PCR, Mutat. Res. 742 (2012) 164–173.
[11] H. Suzuki, N. Ikeda, K. Kobayashi, Y. Terashima, Y. Shimada, T. Suzuki, T. HagiwaraT., S. Hatakeyama, K. Nagaoka, J. Yoshida, Y. Saito, J. Tanaka, M. Hayashi, Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats. A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)-Mammalian Mutagenicity Study Group (MMS), Mutat. Res. 583 (2005) 133–145.
[12] H. Takasawa, H. Suzuki, I. Ogawa, Y. Shimada, K. Kobayashi, Y. Terashima, H. Matsumoto, C. Aruga, K. Oshida, R. Ohta, T. Imamura, A. Miyazaki, M. Kawabata, S. Minowa, M. Hayashi, Evaluation of a liver micronucleus assay in young rats (III): a study using nine hepatotoxicants by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)-Mammalian Mutagenicity Study Group (MMS), Mutat. Res. 698 (2010) 30–37.
[13] U.S. Environmental Protection Agency, N-Nitrosodiethylamine, CASRN 55-18-5 in Integrated Risk Information System, http://www.epa.gov/iris/subst/0042.htm
[14] U.S. Environmental Protection Agency, Di(2-ethylhexyl)phthalate (DEHP), CASRN 117-81-7 (03/01/1997) in Integrated Risk Information System, http://www.epa.gov/iris/subst/0014.htm
[15] U.S. Environmental Protection Agency, 2,4-/2,6-Dinitrotoluene mixture in Integrated Risk Information System, http://www.epa.gov/iris/subst/0397.htm
[16] National Toxicology Program, Department of Health and Human Services, Phenacetin and Analgesic Mixtures Containing Phenacetin in Report on Carcinogens, Twelfth Edition, http://ntp.niehs.nih.gov/ntp/roc/twelfth/profiles/PhenacetinAndAnalgesicMixtures.pdf (2011).
[17] I.C. Calder, D.E. Goss, P.J. Williams, C.C. Funder, C.R. Green, K.N. Ham, J.D. Neoplasia in the rat induced by N-hydroxyphenacetin, a metabolite of phenacetin, Pathology 8 (1976) 1–6.
[18] H. Suzuki, T. Imamura, A. Koeda, K. Morimoto, Y. Wakasa, Y. Takei, R. Amemiya, H. Hatakeyama, H. Satoh, S. Sato, Genotoxicity studies of 2,6-dinitrotoluene (2,6-DNT), J. Toxicol. Sci. 36 (2011) 499–505.
[19] A.M. Camus, M. Friesen, A. Croisy, H. Bartsch, Species-specific activation of phenacetin into bacterial mutagens by hamster liver enzymes and identification of N-hydroxyphenacetin O-glucuronide as a promutagen in the urine, Cancer Res. 42 (1982) 3201–3208.
[20] S. Sawada, C. Furihata, T. Matsushima, In vivo short-term assays of repair and replication of rat liver DNA, J. Cancer Res. Clin. Oncol. 115 (1989) 345–350.
[21] S. Asakura, S. Sawada, H. Daimon, T. Fukuda, K. Ogura, K. Yamatsu, C. Furihata, Effects of dietary restriction on induction of unscheduled DNA synthesis (UDS) and replicative DNA synthesis (RDS) in rat liver, Mutat. Res. 322 (1994) 257–264.
[22] S. Sawada, S. Asakura, H. Daimon, C. Furihata, Comparison of autoradiography, liquid scintillation counting and immunoenzymatic staining of 5-bromo-2′-deoxyuridine for measurement of unscheduled DNA synthesis and replicative DNA synthesis in rat liver, Mutat. Res. 344 (1995) 109–116.
[23] S. Tsuda, N. Matsusaka, H. Madarame, S. Ueno, N. Susa, K. Ishida, N. Kawamura, K. Sekihashi, Y.F. Sasaki, The comet assay in eight mouse organs: results with 24 azo compounds, Mutat. Res. 465 (2000) 11–26.
[24] Y. Uno, H. Takasawa, M. Miyagawa, Y. Inoue, T. Murata, M. Ogawa, K. Yoshikawa, In vivo-in vitro replicative DNA synthesis (RDS) test using perfused rat livers as an early prediction assay for non-genotoxic hepatocarcinogens: II. Assessment of judgement criteria, Toxicol. Lett. 63 (1992) 201–209.
[25] B.L. Copple, C.M. Rondelli, J.F. Maddox, N.C. Hoglen, P.E. Ganey, R.A. Roth, Modes of cell death in rat liver after monocrotaline exposure, Toxicological Sciences 77 (2004) 172–182.
[26] H. Ellinger-Ziegelbauer, B. Stuart, B. Wahle, W. Bomann, H.J. Ahr, Comparison of the expression profiles induced by genotoxic and non-genotoxic carcinogens in rat liver, Mutat. Res. 575 (2005) 61–84.