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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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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Rat liver

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

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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).

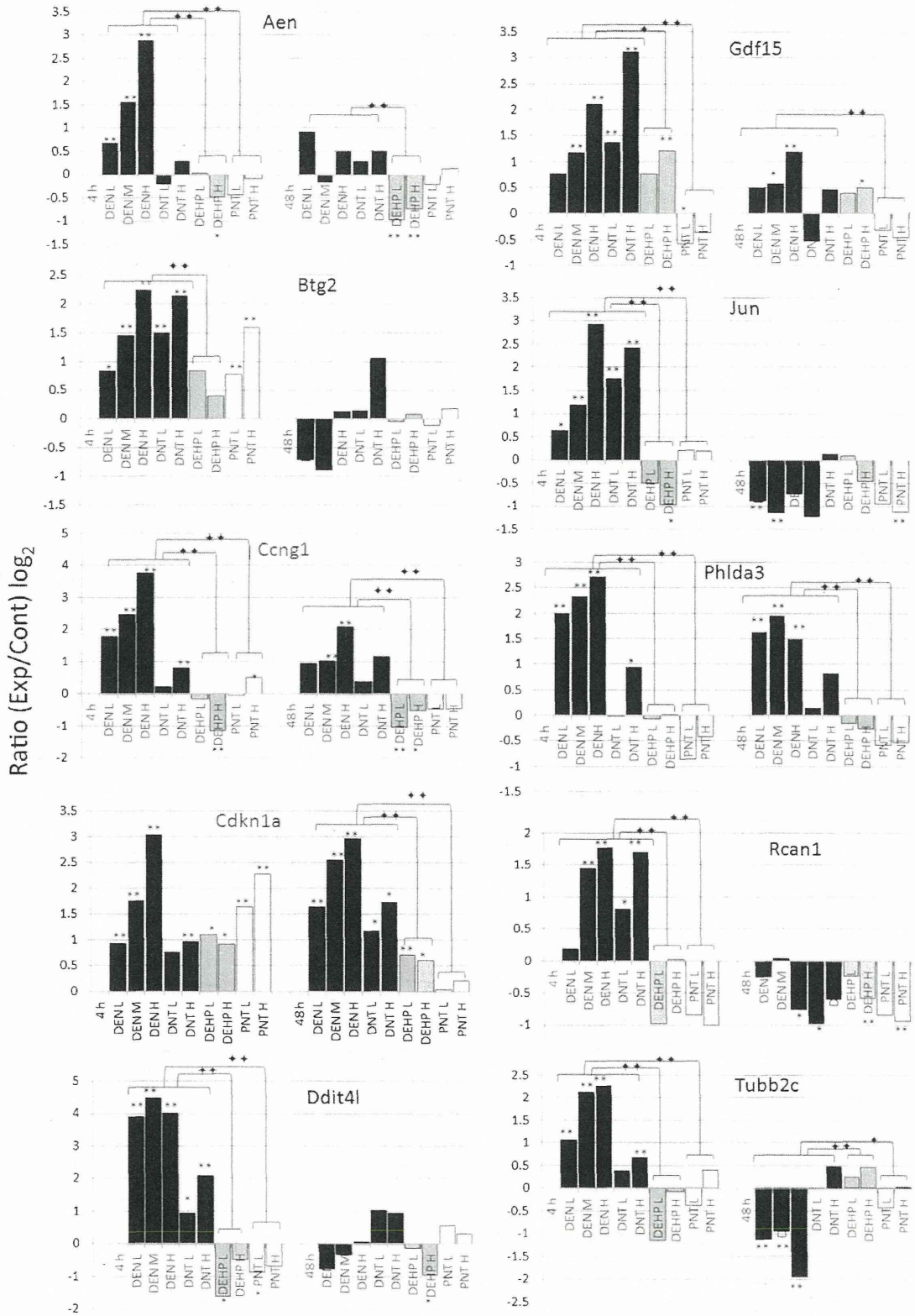

Fig. 1. Changes in the gene expression of 10 genes (*Aen*, *Btg2*, *Ccng1*, *Cdkn1a*, *Ddit4l*, *Gdf15*, *Jun*, *Phlda3*, *Rcan1* and *Tubb2c*) as quantified by qPCR at 4 h and 48 h. DEN L: DEN low dose, DEN M: DEN middle dose, DEN H: DEN high dose, DNT L: DNT low dose, DNT H: DNT high dose, DEHP L: 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.

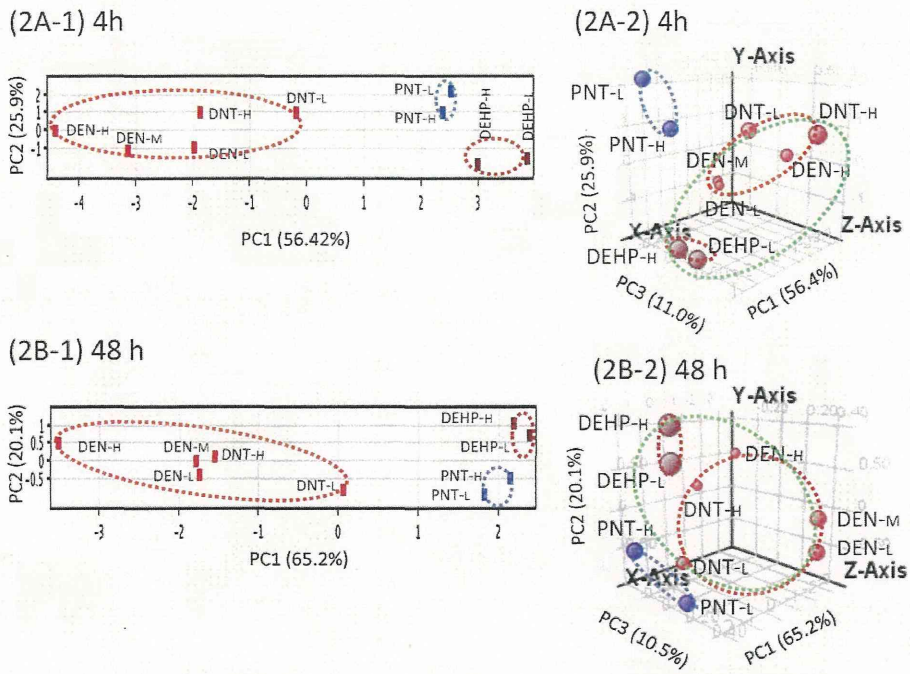

Fig. 2. Principal component analysis (PCA) of the gene expression levels under treatment with 3 types of carcinogens as quantified by qPCR. Genotoxic hepatocarcinogens (red-colored, DEN-L: DEN low dose, DEN-M: DEN middle dose, DEN-H: DEN high dose, DNT-L: DNT low dose and DNT-H: DNT high dose), a non-genotoxic 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.)

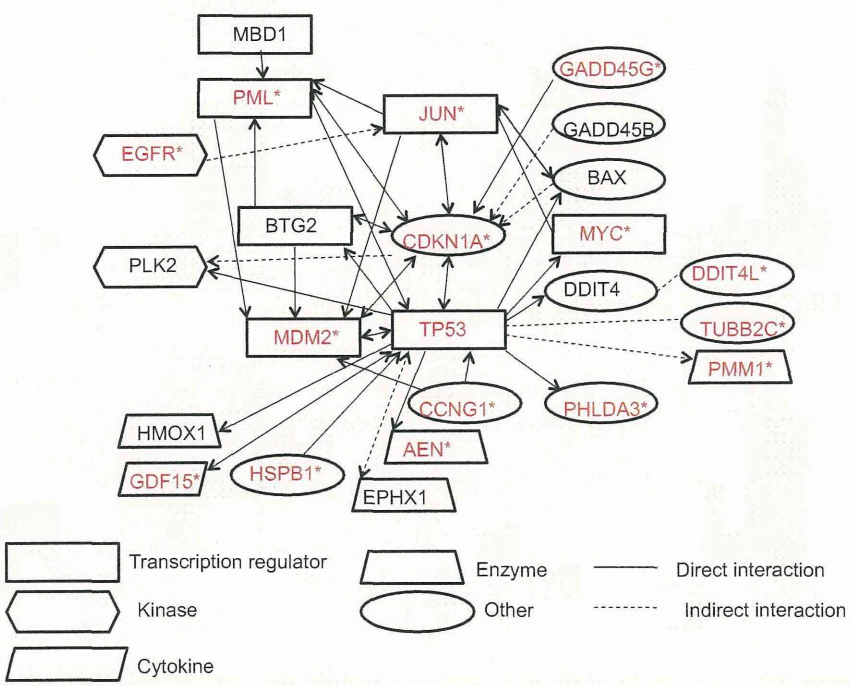

Fig. 3. The gene networks and pathways of 24 genes as determined by qPCR. The network was constructed from the results of Ingenuity Pathways Analysis, GeneSpring software and references from PubMed. The 15 red-colored genes indicated with an asterisk are genes that significantly contributed to the discrimination 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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再生医療における細胞・組織加工製品の品質・安全性の評価

Safety and quality assessment of human cell/tissue-engineered products in regenerative medicine

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1　はじめに

『再生医療』とは，加齢，疾病，損傷，または先天的傷害により組織・器官が失った機能を修復ないし置換することを目的に，機能的かつ生きている組織を作り出すプロセスのことである。再生医療・細胞治療のツールとして利用することを目的とし，細胞または組織を加工することによって製造される生細胞を含んだ医薬品または医療機器を『細胞・組織加工製品』と呼ぶ。ヒトの臓器の確保は難しいこともあり，細胞・組織加工製品を用いた再生医療は，治療法に乏しく，重篤・致死的ないしQOLを著しく損なう疾病・損傷に対して極めて有効な治療法になると期待されている。

2　細胞・組織加工製品の安全性評価における問題点

細胞・組織加工製品の原料となる細胞・組織は，複雑な構造と動的な特性をもつため，従来の化学薬品やタンパク質性医薬品などに適用された品質管理，非臨床試験，臨床試験に必要とされる事項が，必ずしも適用できるとは限らない。細胞・組織加工製品に特有な問題として，①細胞の形質は置かれる（微小）環境に依存する，②細胞は周囲の環境に対して薬理的・免疫学的・物理的に作用する，③培養により均一性が低下する可能性がある，④脱分化する可能性がある，⑤遊走する可能性がある，⑥壊れやすい・寿命が有限である場合が多い，⑦高度な精製，ウイルス活性化・除去が困難，が挙げられる。これらの問題点の程度や重みは製品の種類により様々であることから，細胞・組織加工製品の品質・安全性の確保には特別な配慮が必要となる。最近注目を浴びているiPS細胞をはじめとする幹細胞は，多分化能（multipotency）または多能性（pluripotency）と自己複製能という特徴を持つ。従って，幹細胞を加工した製品は，加工内容や適用部位によっては，たとえ自己に由来するものであっても元来の細胞そのものではなく，また，存在していた，あるいは存在すべきであった（微小）環境とは異なる状態の下に臨床適用される可能性が高い点で注意を要する。

3　リスク・ベース・アプローチ

細胞・組織加工製品の品質マネジメントの原則は，リスク・ベース・アプローチ（Risk-Based Approach）とするのが妥当とされている。リスク・ベース・アプローチとは，対象となる各製品に固有，かつその品質・安全性・有効性に関連するリスク要因を探り当てることをベースにし，その影響の度合いを科学的に評価することにより品質確保の方針・内容をケース・バイ・ケースで柔軟に

定めるアプローチ方法である。ここで言うリスクとは，ある目的（有効性・安全性など）の達成する上での阻害要因を指す。細胞・組織加工製品の安全性面でのリスクは，「ウイルス等の感染性因子の伝搬」，「細胞の遺伝的不安定性（染色体異常）と造腫瘍性」，「血清等の不純物混入」，「望まない免疫応答」，「非細胞成分による不必要な免疫応答，炎症反応，毒性」，「製品の意図しない生物応答」などが挙げられる。これらの細胞・組織加工製品のリスクの度合いは，細胞の生物学的特性と由来，細胞培養・加工技術，臨床における具体的な使用方法などによって大きく変化する。

例えばヒト自己由来細胞・組織とヒト同種由来細胞・組織とを比較した場合，自己由来製品は感染因子の混入は同種由来ほど気にする必要はなく，免疫拒絶の懸念も少ないという利点を持つが，品質のばらつきを最小限に抑える厳重な品質管理が必要で，しかも品質の特性評価に利用できる検体の量が限られているという欠点を持つ。一方で，同種由来製品は，バンク化と徹底した特性解析により一定の品質を確保しやすく，異常発生時には免疫抑制剤中止により移植細胞を除去できる可能性があるという利点を持つが，感染因子混入に関する厳重な管理が必要なのと，免疫反応を抑制する必要があるという欠点を持つ。

細胞・組織加工製品は非常に多様性が高く，製品ごとに高リスク製品 / 低リスク製品といったような製品リスクの程度を段階的にクラス分けするような，確固とした評価系は不可能である。科学的知見に基づき，各々の細胞・組織加工製品で品質・安全性の面からリスクとリスク要因を同定し，リスクを低減・管理する方策を見出して行くことが重要である。

4　おわりに

細胞・組織加工製品の品質・安全性の評価は，多様なリスクとリスク要因を考慮したリスク・ベース・アプローチによりケース・バイ・ケースで考えることが原則である。したがって開発者にも審査側にも常に合理的なリスク分析が要求され，科学的に合理性・妥当性のある品質・安全性評価試験が要求される。また細胞・組織加工製品を用いた再生医療は，重篤・致死的ないしQOLを著しく損なうような難治性疾患に対して有効性の高い治療法として期待されており，従来の治療法が適用できない患者の「新たな治療機会を失うことにより被るかも知れないリスク」も考慮することが当然のことながら必要である。

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2. 再生医療における細胞・組織加工製品の治験とレギュレーション

草川森士，佐藤陽治

わが国では，再生医療に利用される細胞・組織加工製品の実用化には主に，治験を経て薬事承認を受けるルートと，「ヒト幹細胞を用いる臨床研究」を経て先進医療・高度医療等に向かうルートがあるが，国民のアクセシビリティと産業化という面からは前者を採ることが必要になる．細胞・組織加工製品の治験に関しては，患者数の少ない難病を対象とすることが多い点や，挑戦的な研究開発である点などから医師主導治験の環境整備が期待される．本稿ではこれらとともに，治験開始にあたっての細胞・組織加工製品の品質・安全性確保や治験の実施における規制等について概説する．

はじめに

ヒト由来の細胞を用いた再生医療は，これまで治療が困難であった疾患・損傷の新たな突破口として熱い期待を集めている．再生医療においてヒトに投与することを目的に，生きた細胞または組織を加工して製造される製品は「細胞・組織加工医薬品/医療機器」（細胞・組織加工製品）と呼ばれる．現在までに世界で実用化されている細胞・組織加工製品は，皮膚や軟骨などの体細胞を培養・加工したものがほとんどであるが，近年，体性幹細胞，ES細胞，iPS細胞を原材料とした新たな製品の開発も進んでいる．これら幹細胞に関する研究は日進月歩で進んでおり，新たな細胞の樹立，培養法，分化誘導技術など，急速に進展するバイオサイエンスの医療への応用は，治療を待つ患者のためという側面だけでなく新たな産業の創生という面でも高い関心を集めており，世界的にも激しい開発競争が展開されている．しかし，原材料や製造技術が何であれ，新たな細胞・組織加工製品の実用化が本当に可能かどうかを判断するには，実際にヒトで試し，科学的妥当

[キーワード&略語]
再生医療，細胞・組織加工製品，医師主導治験，ガイドライン

ES細胞：embryonic stem cells（胚性幹細胞）
GCP：good clinical practice
GLP：good laboratory practice
GMP：good manufacturing practice
GTP：good tissue practice
ICH：The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
iPS細胞：induced pluripotent stem cells（人工多能性幹細胞）

The Japanese regulatory framework for clinical trials of cell/tissue-engineered products

Shinji Kusakawa[1]/Yoji Sato[2]：Foundation for Biomedical Research and Innovation[1]/Division of Cellular and Gene Therapy Products, National Institute of Health Sciences[2]（先端医療振興財団[1]/国立医薬品食品衛生研究所遺伝子細胞医薬部[2]）

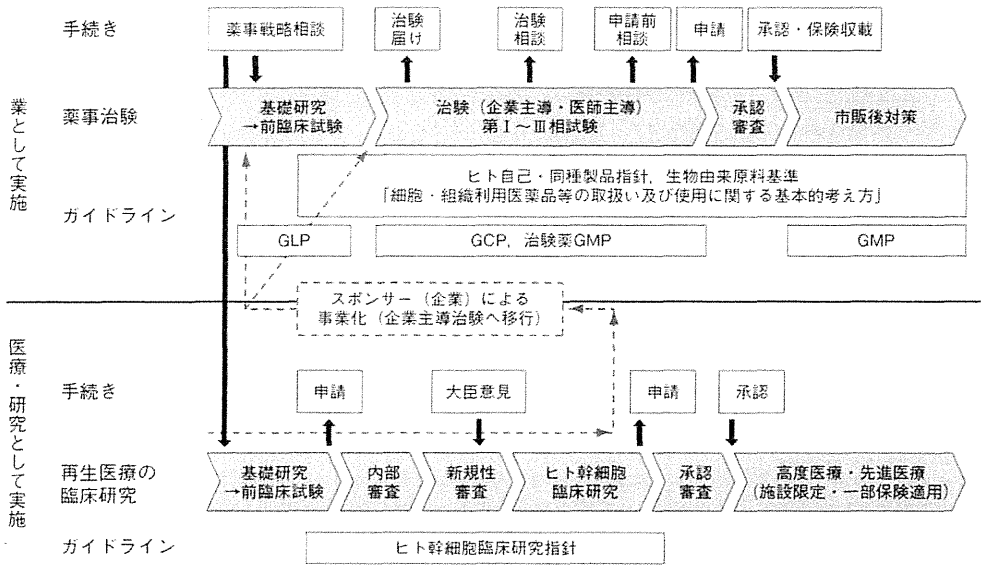

図1　日本における細胞・組織加工製品の開発から使用まで
再生医療を目的とした新規の細胞・組織加工製品の国内実用化には主に，治験を行い厚生労働省の製造販売承認を受けて保険適用医療として実現するルートと，厚生労働省「ヒト幹細胞を用いる臨床研究に関する指針」に則った臨床研究（ヒト幹細胞臨床研究）の成果に基づくルートが存在する．それぞれに係る手続き，ガイドラインを示している

性のある有効性・安全性および品質の検証がなされなければならない．

1 細胞・組織加工製品の国内実用化の道筋

再生医療を目的とした新規の細胞・組織加工製品の国内実用化には，主に2つのルートがある（**図1**）．1つは，治験を行った上で厚生労働省の製造販売承認を受けて保険適用医療として実現するルート，言い換えれば薬事法上の「業」としての実用化である．もう1つは，厚生労働省「ヒト幹細胞を用いる臨床研究に関する指針」に則った臨床研究（ヒト幹細胞臨床研究）の成果に基づく，先進医療[※1]・高度医療[※2]評価制度による医療，もしくは保険適用外医療としての実用化であり，これらは医療法・医師法の下で行われる「医療行為」として実施される．ただし先進医療・高度医療評価制度による医療の場合，実施可能な医療機関が限られると同時に製品の品質にばらつきが生じる可能性があり，また，開発に多くの投資を要する新規製品を用いた保険適用外医療は高額となりやすいため，いずれの場合も多くの国民が簡単には享受できない恐れがある．したがって，国民が広くアクセスできるという観点からすれば，治験を通じて薬事法上の承認を得る必要がある．また，ヒト幹細胞臨床研究は手続きや費用などの面で治験よりも実施が比較的容易であるものの，治験の国際ガイドライン（ICH-GCP）に沿った国内GCP（good clinical practice）ガイドライン（後述）の準拠が義務ではなく，得られたデータを製品の薬事承認申請資料としてそのまま使用できない場合が多い．つまり，新規の細胞・組織加工製品に関して，ヒト幹細胞臨床研究で有効性・安全性を確認してから産業化をめざして薬事承認を得ようとしても，多くの

※1　先進医療
未だ保険診療の対象に至らない先進的な医療技術について，安全性，有効性等を確保するために一定の施設基準を設定し，保険診療との併用を認められるもの．

※2　高度医療（第3項先進医療）
高度医療評価制度の制定において定められた，薬事法の承認等が得られていない医薬品・医療機器の使用を伴う先進的な医療技術を，一定の要件の下に「高度医療」として認め，保険診療と併用できる．

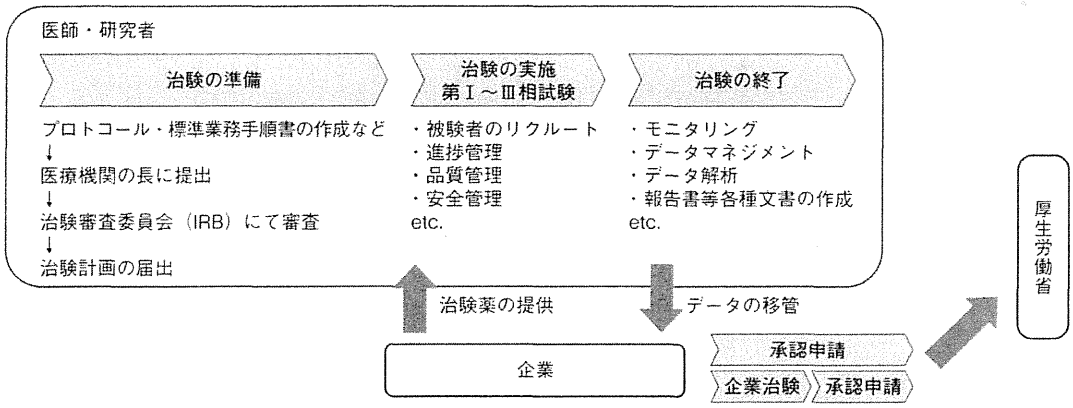

図2　医師主導治験の流れ
医師主導治験は，医師・研究者や医療機関が主体となって企画・実施する治験である．医療機関は企業から未承認の医薬品または医療機器の提供を受け治験を実施し，そのデータを企業に提供し承認申請に進んでいく

場合には，GCPに則った治験をやり直さなければならない．（ちなみに，欧米ではわが国のような「治験」（商業目的の臨床試験）と「臨床研究」（医療・研究目的の臨床試験）という区別はなく，すべての臨床試験はICH-GCPに準じた各国の規制に従う必要がある．したがって，大学等における非商業的な臨床試験にも多くの資金・労力が必要となるものの，企業への技術移転が日本よりスムーズに進みやすいしくみだといわれている）

2 医師主導治験

わが国では10年ほど前まで，治験を企画・実施する主体は企業のみということになっていたが，平成14年7月公布の改正薬事法により，医師・歯科医師も自ら主体となって治験を企画・実施することが可能となった（医師主導治験；**図2**）．医師主導治験が可能となった背景としては，海外ですでに承認済み，あるいはすでに標準的な製品として確立されている医薬品・医療機器について，わが国の臨床現場においても必要性があるが，採算性等の問題から国内企業が治験を実施しない場合があるという実態があった．そうした製品について，医師自らが治験を実施することが可能となり薬事承認への道が開かれたことで，状況が改善されることが期待されている．再生医療および細胞・組織加工製品に関しては，患者数の少ない難病の治療に向けた研究開発や，治療方法の概念が新しい挑戦的な研究開発になるケースがほとんどであり，また，国内外ですでに承認されている製品について適応拡大を目的とする場合も考えられ，さらに製品の有効性・安全性が未知数で企業が着手しにくい故に国内開発が出遅れるという懸念もある．わが国における再生医療および細胞・組織加工製品の開発の多くは，大学等の研究機関の研究者（の臨床研究）により行われる場合が多いという事情も考えると，医師主導治験の積極的実施は，医師が開発した治療法・製品を一般に普及するための効果的な方策となると考えられる．医師主導治験の実施には，医師のデータ・技術の企業への橋渡しのしくみ（実施医療機関の体制整備費，治験薬の製造，プロトコール作成，データ管理業務，治験相談等の費用を補助する等の支援，研究費など）をさらに充実させる必要もあり，平成19年度から開始されている「新たな治験活性化5カ年計画」[1]等の中で，文部科学省・厚生労働省は日本医師会治験促進センターや中核病院・拠点医療機関等と協力し，医師主導治験を含めたわが国の治験実施の環境整備に努めている．

3 治験開始に関する規制・制度

新規の細胞・組織加工製品を治験でヒトに使用する際には，開発者は治験実施に適うだけの安全性と品質をあらかじめ示す必要がある．先ごろまでわが国では，