stress inducible genes, we carried out an *in vivo* microarray study specifically investigating liver tissue in B6C3F1 mice administered with seven well-characterized genotoxins (GTXs) and three non-genotoxins (NGTXs). Compounds with all negative data in regulatory genotoxicity assays (including Ames test, *in vitro* chromosome aberration test, mouse lymphoma assay and *in vivo* micronucleus test) were chosen as non-genotoxins. The dosage used for GTXs was selected based on data from *in vivo* transgenic mouse mutation assays, where significantly higher mutant frequencies were observed in liver tissue. The mutant frequency was determined as described previously [23]. While the dosage used for NGTXs was 1/2 LD_{50} (Table 1). To study both early and late or sustained genotoxic stress responses, time points at 4 h, 20 h, 2 weeks and 4 weeks after treatment were chosen. To select genotoxic stress responsive genes, we adopted a self-defined weight scoring approach. Candidate genes were scored based on their specificity, sensitivity (including average ratio, positive condition, positive chemical and reverse change), statistical *P* value, basal expression level, and coefficient of variation (CV). A total score, considering all the above parameters, was finally calculated (Table 2). Further analysis of the top ranked 50 genes by hierarchical clustering showed clear gene sets, whose expression could distinguish GTXs from NGTXs (Fig. 1A). These included some well-known DNA damage inducible genes e.g. p21$^{WAF1/Cip1}$ [24] and ccng1 [25]. The highest scoring gene was an unknown gene BC005512 (identified by probe set 1426936_at, Gene symbol: BC005512, official full name: cDNA sequence BC005512). Its expression was specifically induced by GTXs, but not by NGTXs, which was further confirmed by quantitative real-time PCR (Fig. 1B and 1C).

2. BC005512 is a member of the GLN family of murine endogenous retrovirus

To characterize BC005512 in the mouse database, we performed bioinformatics analysis. A nucleotide BLAST search using the genechip probe sequence (Fig. S1) as the query identified two cDNAs (GenBank accession no. BC005512 and BC062922). Analysis of both sequences using ORF finder (http://www.ncbi. nlm.nih.gov/gorf/gorf.html) and protein BLAST revealed that the putative proteins encoded by these sequences have great similarity to retrovirus related proteins encoded by *env* genes of mouse ERV (data not shown) [10]. To determine the relationship of BC005512 with mouse ERVs, we analyzed BC005512 and BC062922 sequences using an online CENSOR program (http://www. girinst.org/censor/index.php), which screens query sequences for interspersed repeats [26]. The sequence analysis classified them into ERV-Class I, and revealed strong similarities to MMERGLN_I, a sequence submitted as one copy of GLN family in Repbase (Fig. 2). These findings suggested that the sequence identified in the above microarray study, i.e. BC005512 (abbreviated as BC in the following text), was a member of the GLN family of murine ERV.

3. Expression of BC was specifically induced by genotoxins in NIH/3T3 cells

The microarray study showed that BC was induced by genotoxic stress *in vivo*. To further characterize the responsiveness of BC to GTXs *in vitro*, we tested another seven GTXs with different genotoxic mechanisms and two NGTXs (Table 3). For each GTX, 24 h IC_{50} was determined and used to study the effects of treatment on BC expression. NIH/3T3, a well-characterized mouse embryonic fibroblast cell line, was used as the *in vitro* model system. In accordance with results obtained *in vivo*, all seven GTXs

up-regulated expression of BC to varying degrees, while all three NGTXs tested had no obvious effects on BC expression in NIH/3T3 cells (Fig. 3). Similar results were also obtained in a mouse hepatoma cell line, Hepa 1–6 cells (Fig. S2).

4. Induced expression level of BC correlated with DNA damage in NIH/3T3 cells

Inducing DNA damage is one of many genotoxicity mechanisms. To further study the relationship between BC expression and DNA damage, we compared transcriptional expression level of BC and the extent of DNA damage using methyl methanesulfonate (MMS) as a DNA-reactive model compound [27]. MMS was chosen as it gave the strongest response in the *in vitro* BC induction assay (Fig. 3) and has been used extensively as a DNA damaging model agent. MMS modifies both guanine (to 7-methylguanine) and adenine (to 3-methyladenine) causing base mispairing and replication blocks, respectively [27]. DNA damage was indicated by olive tail moment in the alkaline comet assay [28]. As shown in Fig. 4A and 4B, MMS induced a concentration-dependent increase in both BC expression and DNA damage.

In addition to DNA-reactive chemicals, aneugens that directly target spindles but not DNA during chromosome segregation were another class of GTXs. To determine whether BC was responsive to aneuploidy, we examined the effects of two aneugens, colchicine [29] and paclitaxel [30], on BC expression. Chromosome abnormality was determined using the micronucleus test [31]. The highest concentration of paclitaxel or colchicine was limited to 48 h IC_{50}. As expected, both colchicine and paclitaxel induced a dose-dependent increase in micronucleus formation (Fig. 4F and 4I) but not in DNA damage except at very high concentrations, possibly due to nonspecific effects under cytotoxic conditions (bar graph in Fig. 4D and 4G, 4E and 4H). In accordance with MMS treatment, quantitative PCR analysis showed that expression level of BC was in parallel with DNA damage i.e., BC induction only occurred when DNA damage was observed, regardless of whether aneuploidy was induced (line graph in Fig. 4D and 4G). Furthermore, the linear regression analysis of data obtained from MMS, colchicine and paclitaxel revealed a strong correlation between expression level of BC and the extent of DNA damage (Fig. 4C).

5. Expression of BC could not be induced by GTXs and did not correlate with DNA damage in L5178Y cells

In addition to NIH/3T3 cells, L5178Y, a widely used mouse lymphoma cell line in *in vitro* genotoxicity assays was also adopted to investigate the effects of GTXs on BC expression and the relationship between BC expression and DNA damage. For each GTX, 24 h IC_{50} was determined and used in L5178Y cells. Surprisingly, GTXs that substantially induce BC expression in NIH/3T3 cells did not show similar effects in L5178Y cells (Fig. 5A and Fig. S3). Concordantly, expression level of BC did not correlate with DNA damage induced by MMS (Fig. 5B, 5C and 5D). Together, these results suggested that expression of BC could not be induced by GTXs and did not correlate with DNA damage in L5178Y cells.

6. Down-regulating BC expression suppressed cell growth in several mouse cell lines

DNA damage triggers a variety of biological responses including the transcriptional activation of genes regulating DNA repair, cell cycle checkpoint and cell death [32]. The observation that BC was responsive to DNA damage raised the question whether BC was involved in these processes.

Table 1. Model compounds selected in the *in vivo* microarray study.

Compounds	Abbr[1]	CAS[2] No.	Dosage (mg/kg)	Classification
Dimethylnitrosamine	DMN [23]	62-75-9	5	Genotoxins
Diethylnitrosamine	DEN [46]	55-18-5	80	
Ethyl nitrosourea	ENU [47]	759-73-9	150	
Dipropylnitrosamine	DPN [48]	621-64-7	250	
o-Aminoazotoluene	AAT [49]	97-56-3	300	
Dibenzo[a,l]pyrene	DBP [50]	191-30-0	6	
7,12-Dimethylbenz[a]anthracene	DMBA [48]	57-97-6	20	
Ethanol	EtOH	64-17-5	1000	Non-genotoxins
Phenobalbital sodium	PB	57-30-7	30	
Diethylhexylphthalate	DEHP	117-81-7	2000	

[1]Abbr: Abbreviation;
[2]CAS: Chemical Abstracts Service.
doi:10.1371/journal.pone.0035010.t001

Table 2. Weight score for genotoxic stress responsive gene selection in the *in vivo* microarray study (liver, B6C3F1).

Systematic	Specificity		Ave ratio		Positive condition		Positive chemical		P value		Basal		Reverse change		CV (%)		TOTAL SCORE	Common
	V[1]	S[2]	V	S	V	S	V	S	V	S	V	S	V	S	V	S		
1426936_at	1.00	5	10.62	5	11.5	5	6	5	0.14	5	0.92	0	0.0	2	64	2	**32.0**	BC005512
1417185_at	1.00	5	3.98	2	14.5	5	6	5	0.07	5	2.35	3	0.0	2	27	4	**31.5**	Ly6a
1433691_at	1.00	5	3.31	2	5.0	2	5	4	0.21	4	2.64	4	2.0	5	24	4	**28.5**	Ppp1r3c
1449002_at	1.00	5	4.40	2	12.0	5	6	5	0.33	2	1.80	1	0.0	2	26	4	**27.5**	Phlda3
1421040_a_at	1.00	5	2.49	2	5.5	2	5	4	0.18	4	2.63	4	0.0	2	33	4	**27.0**	Gsta2
1424638_at	0.83	3	24.58	5	15.0	5	7	5	0.19	4	1.51	0	0.0	2	70	2	**27.0**	Cdkn1a
1450016_at	1.00	5	3.26	2	11.0	5	5	4	0.35	1	2.13	2	0.0	2	12	5	**26.5**	Ccng
1450017_at	1.00	5	4.55	2	11.0	5	5	4	0.29	2	1.79	1	0.0	2	23	4	**26.5**	Ccng
1420827_a_at	0.92	4	7.88	4	11.0	5	5	4	0.33	2	1.75	1	0.0	2	43	3	**26.0**	Ccng
1416578_at	1.00	5	2.88	2	5.0	2	5	4	0.17	4	1.92	1	0.0	2	35	4	**25.5**	Rbx1
1424744_at	1.00	5	2.42	0	6.5	3	6	5	0.46	0	3.01	5	2.0	5	33	4	**25.0**	Sds
1425631_at	1.00	5	3.72	2	4.0	1	4	2	0.15	5	2.09	2	4.0	5	38	3	**25.0**	Ppp1r3c
1416125_at	1.00	5	2.49	2	5.0	2	4	2	0.12	5	2.28	3	0.0	2	71	2	**24.5**	Fkbp5
1419874_x_at	0.90	4	5.77	3	9.0	4	5	4	0.15	5	1.37	0	−1.0	1	107	0	**24.5**	AI467657
1427422_at	0.83	3	3.15	2	10.0	5	6	5	0.27	3	1.99	1	1.0	3	46	3	**24.5**	BM122014
1442026_at	0.83	3	3.70	2	10.0	5	5	4	0.11	5	1.62	0	0.0	2	50	3	**24.5**	AI467657
1416926_at	0.86	3	3.37	2	12.0	5	6	5	0.31	2	2.25	2	0.0	2	24	4	**24.0**	Stinp
1448265_x_at	1.00	5	2.55	2	7.5	3	6	5	0.36	1	2.02	2	−1.0	1	52	3	**24.0**	Eva
1455892_x_at	0.91	4	2.52	2	10.5	5	5	4	0.48	0	2.92	5	1.0	3	80	2	**24.0**	BB794742
1418787_at	1.00	5	1.89	0	5.0	2	5	4	0.31	2	3.16	5	0.0	2	13	4	**23.5**	Mbl2

[1]"V" represents values.
[2]"S" represents score. Only the top 20 genes are shown. A full list is attached in Table S3.
Detailed scoring rules are described in the supporting information. "Pink cells" represent up-regulation and "blue cells" represent down-regulation.
Specificity = (number of total pink cells in GTXs)/(number of total pink cells in GTXs and NGTXs); **Ave ratio** = average of ratios of all pink cells in GTXs; **Positive condition** = number of total pink cells in GTXs. Since DEN was duplicated, each pink cell was considered 0.5; **Positive chemical** = number of GTXs with at least one pink cell; *P* value was calculated by *t* test of signal intensity between GTXs and NGTXs in GeneSpring software; **Basal** represents basal expression level, equals to $\log_{10}$ value of signal intensity of control animals; **Reverse change** reflects opposite change of gene expression in different treatment groups. Reverse change = number of blue cells in NGTXs - number of blue cells in GTXs; **CV%** = 100×SD/MEAN% based on the signal intensity of all control animals. **Total score** = Score of 2× Specificity + Ave ratio + Positive condition + Positive chemical + *P* value + 0.5× Basal + 0.5× Reverse change + 0.5× CV%.
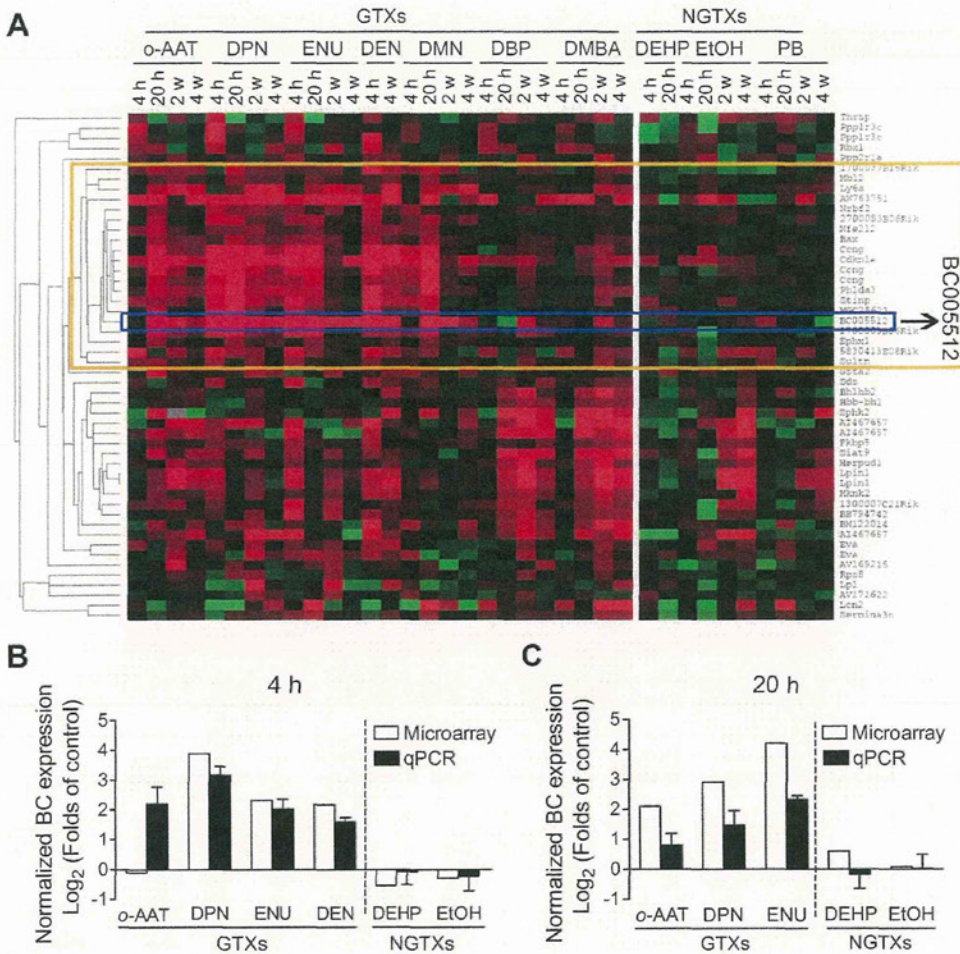doi:10.1371/journal.pone.0035010.t002

Figure 1. Selection of sensitive and specific genotoxic stress responsive genes. (A) Hierarchical clustering of top 50 scored up-regulated genes shown in gene symbol. Red and green indicate up-regulation and down-regulation, respectively. The orange box represents genes whose expression could distinguish GTXs from NGTXs. The blue box represents the gene with the highest score, BC005512. (B and C) Microarray and quantitative PCR (qPCR) data showing BC expression levels in livers of mice dosed with indicated chemicals at 4 h or 20 h after administration. Microarray data represented pooled samples from 4 animals per group. Quantitative PCR data were mean ± s.d. (n=4).
doi:10.1371/journal.pone.0035010.g001

We first examined the effects of knocking-down BC expression on cell growth by RNA interference. Two BC siRNAs were designed; one nonsense siRNA and two corresponding scrambled siRNAs were used as negative controls. Quantitative PCR showed that at 48 h after siRNA transfection, the level of BC mRNA was markedly reduced in cells transfected with BC siRNAs (siBC.1 and siBC.2) compared with cells transfected with nonsense (Fig. 6A) or scrambled siRNAs (Fig. S4A). Due to the lack of appropriate antibody against BC, to confirm the knock-down efficiency of BC siRNAs at the protein level, we generated a myc-tagged BC clone. Following co-transfection of the clone and BC siRNA into NIH/3T3 cells, the protein expression level of BC was monitored using
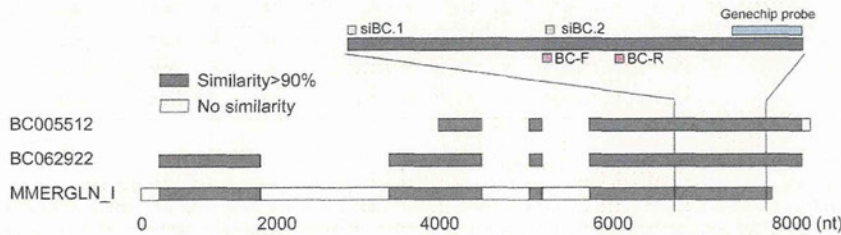

Figure 2. BC005512 is a member of the GLN family of murine endogenous retrovirus. Sequence alignment between BC005512, BC062922 and MMERGLN_I. Locations of the genechip probe, quantitative PCR primers (BC-F and BC-R) and BC siRNAs are shown.
doi:10.1371/journal.pone.0035010.g002

Table 3. Model compounds selected in the *in vitro* BC induction study.

Compounds	Abbr.	CAS No.	Primary modes of action	Concentration		Classification
				NIH/3T3	L5178Y	
Camptothecin	CPT	7689-03-4	Topoisomerase I poison [51]	25 µM	2.87 µM	Genotoxins
tert-Butyl hydroperoxide	TBHP	75-91-2	Reactive oxygen species [52]	50 µM	——	
γ-ray	IR	——	Ionizing radiation	8 Gy	10 Gy	
Mitomycin C	MMC	50-07-7	Bi-functional cross-link alkylating agent [53]	30 µM	6 µM	
Etoposide	ETOP	33419-42-0	Topoisomerase II poison [54]	50 µM	1.7 µM	
Cisplatin	CDDP	15663-27-1	Crosslink agent [55]	12.5 µM	——	
Methyl methanesulfonate	MMS	66-27-3	Mono-functional alkylating agent [27]	0.5 mM	——	
Acetonitrile	ACN	75-05-8		10 mM	10 mM	Non-genotoxins
Ethanol	EtOH	64-17-5		10 mM	10 mM	
Sucrose	SUC	57-50-1		10 mM	10 mM	

doi:10.1371/journal.pone.0035010.t003

a myc-tag antibody. Meanwhile, we introduced two point mutations into the siBC.2 target region to generate a mutant clone. By co-transfecting this mutant clone and siBC.2 into NIH/3T3 cells, we further examined the specificity of siBC.2. As shown in Fig. S5, siBC.2 decreased the wild-type BC, but not the mutant BC protein levels, indicating that siBC.2 worked specifically and effectively at the protein level.

Cell growth analysis of NIH/3T3 cells transfected with BC siRNAs showed that the total cell number was markedly reduced compared with cells transfected with nonsense (Fig. 6B) or scrambled siRNAs (Fig. S4B). Similar results were also obtained in a mouse hepatoma cell line Hepa 1–6 (Fig. 6C and 6D), and a mouse glomerular mesangial cell line SV40 MES 13 (Fig. 6E and 6F). These results suggested that knock-down of BC expression suppressed cell growth in several mouse cell lines.

7. Knock-down of BC induced G1/S phase arrest and inhibited cell proliferation in NIH/3T3 cells

To investigate whether BC affected cell growth due to its effect on cell proliferation or cell survival, we performed an EdU incorporation assay, which is similar to BrdU incorporation assay. As shown in Fig. 7A and 7B, fewer EdU-positive cells were found in BC siRNAs-transfected cells (30.3% ± 1.2% for siBC.1, and 19.0% ± 1.9% for siBC.2) than in nonsense siRNA-transfected cells (39.5% ± 2.0%), indicating that knock-down of BC suppressed NIH/3T3 cells proliferation.

To elucidate how knock-down of BC can suppress cell proliferation, we examined the effects of knocking-down BC on cell cycle. At 48 h after transfection, the percentage of cells in G0/G1 phase was substantially increased (60.5 ± 0.3 for nonsense siRNA, 69.7 ± 1.0 for siBC.1 and 78.7 ± 1.3 for siBC.2) with a concomitant reduction of cells in S phase (25.9 ± 0.5 for nonsense siRNA, 20.8 ± 0.7 for siBC.1 and 11.7 ± 1.4 for siBC.2, Fig. 7C, 7D and Fig. S4C).

To determine whether down-regulation of BC expression affected cell death, we examined apoptosis in NIH/3T3 cells by flow cytometry using Annexin V and propidium iodide double staining. Knock-down of BC did not induce apoptosis at 48 h after siRNA transfection (Fig. S6A). Consistently, no obvious sub-G1 peak was observed (Fig. 7C), indicating that down-regulation of BC did not induce apoptosis directly in NIH/3T3 cells. Nevertheless, the obvious apoptosis observed at 96 h after siRNA transfection (Fig. S6B) might be caused by cell cycle arrest, occurring at 48 h after transfection.

Taken together, these results suggested that down-regulation of BC induced G1/S arrest, and thus inhibited cell proliferation in NIH/3T3 cells.

Discussion

In this work, we have shown that BC005512, a member of the GLN family of murine ERV, was responsive to DNA damage. This conclusion was supported by several observations. Multiple well-characterized GTXs with a diversity of genotoxicity mechanisms induced elevated expression of BC both *in vivo* (mouse liver) and *in vitro* (NIH/3T3 cells). More specifically, a strong correlation
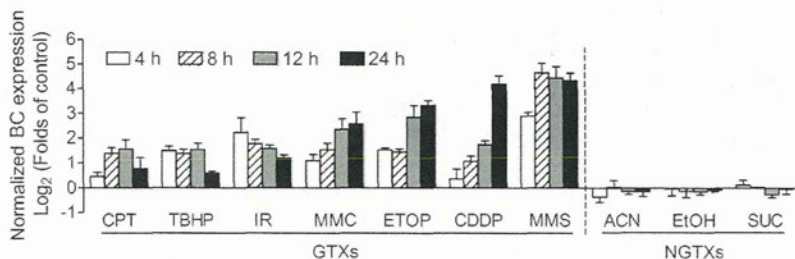

Figure 3. Expression of BC was specifically induced by GTXs in NIH/3T3 cells. Data from quantitative PCR showing transcriptional expression of BC in NIH/3T3 cells treated with genotoxic or non-genotoxic chemicals for indicated time. Data were mean ± s.d. of three independent experiments.
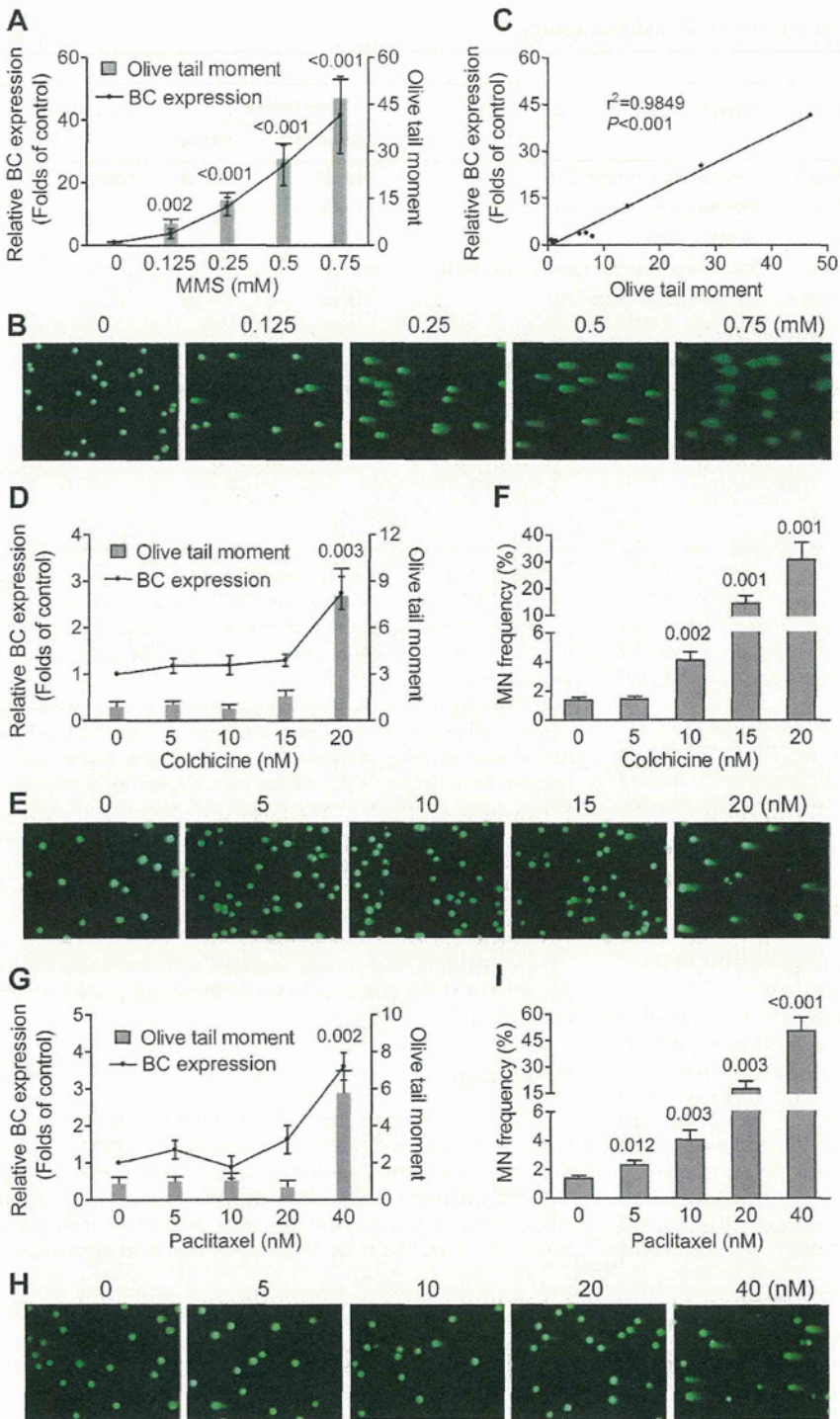doi:10.1371/journal.pone.0035010.g003

Figure 4. Induced expression level of BC correlated with DNA damage in NIH/3T3 cells. (A, B, D, E, G and H) Comparison between expression level of BC and DNA damage in NIH/3T3 cells exposed to MMS (A, B) for 8 h, or to colchicine (D, E) or paclitaxel (G, H) for 24 h. DNA damage was measured by olive tail moment (tail length × percentage of DNA in tail) in an alkaline comet assay (representative figures are shown in B, E and H). Data were mean±s.d. of three independent experiments. (**C**) Linear regression analysis between expression level of BC and DNA damage, reflected by olive tail moment. Each dot represents the mean of data shown in (A), (D) and (G). (**F and I**) Micronucleus frequency in NIH/3T3 cells exposed to colchicine or paclitaxel for 24 h. Data were mean ± s.d. of three independent experiments. Values shown on top of bars are P values vs control.
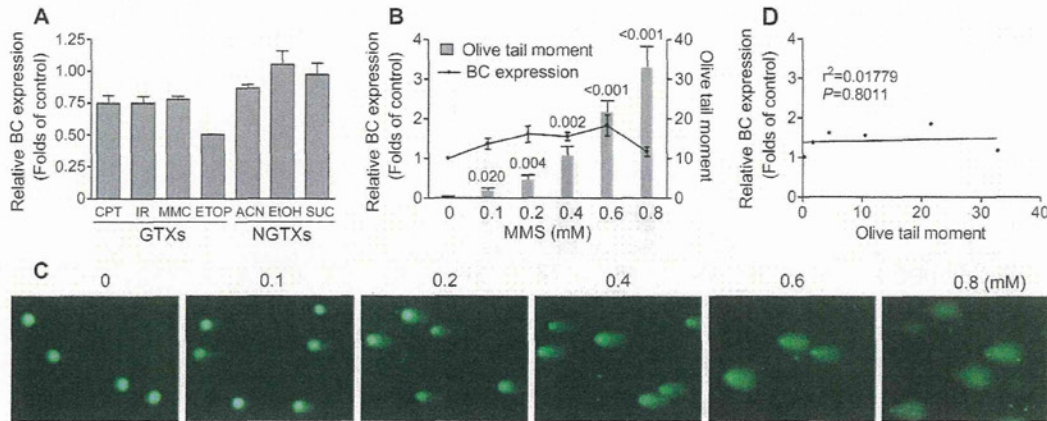
Figure 5. Expression of BC could not be induced by GTXs and did not correlate with DNA damage in L5178Y cells. (A) Quantitative PCR data showing transcriptional expression of BC in L5178Y cells treated with indicated chemicals for 4 h. Data were mean ± s.d. of three independent experiments. **(B and C)** Comparison between expression level of BC and DNA damage in L5178Y cells exposed to MMS for 8 h. DNA damage was measured by olive tail moment (tail length × percentage of DNA in tail) in an alkaline comet assay (representative figures are shown in C). Data were mean ± s.d. of three independent experiments. **(D)** Linear regression analysis between expression level of BC and DNA damage, reflected by olive tail moment. Each dot represents the mean of data shown in (B). Values shown on top of bars are P values vs control.
doi:10.1371/journal.pone.0035010.g005

was found between expression level of BC and the extent of DNA damage. Besides the above findings, we provide the first evidence that BC was involved in cell growth regulation, suggesting that BC was biologically relevant to the DNA damage response.

Recent studies have reported elevated expression of ERVs in many different types of human cancers or tumor cell lines [17]. UVB irradiation was reported to induce transcriptional activation of ERV sequences in human epidermal keratinocytes [33]. However, the relationship between GLN and DNA damage was not clear. This is the first report that demonstrates the involvement of a GLN family member in genotoxic stress, particularly in the DNA damage response. This quantitative DNA damage-responsive property of BC could be potentially useful in genotoxicity prediction.

In addition to BC, we identified another ERV with a high score (1455892_x_at, BB794742, mouse endogenous murine leukemia virus mRNA), whose expression was induced by GTXs (Table 2). A great number of human ERVs have been reported to have perfect p53 binding sites that not only regulate adjacent gene expression, but may also give ERVs the advantage of exiting the host cell for their own survival under stress conditions [14]. As discussed later, we also found that the long terminal repeat (LTR) of GLN has a putative p53 binding site. Combined with our findings, it would be of great interest to study the relationship between ERVs and stress responses, and determine whether ERVs have intrinsic advantages in becoming potential genotoxicity biomarkers.

In the microarray study, BC was initially identified using self-defined weight scoring. In our scoring system, well-known DNA damage responsive genes such as p21 [24] and ccng1 [25] were also assigned high scores, indicating that this method worked effectively in identifying genotoxic stress inducible genes. Although the basal expression level was low, BC had priority over other candidate genes in its high specificity and sensitivity (Table 2). However, it should be noted that this would not exclude the possibility that other genes could become potential genotoxicity biomarkers.

Transcriptional expression of BC was induced by GTXs in NIH/3T3 cells, but not in L5178Y cells. We inferred that this discrepancy might be due to a lack of functional p53 in L5178Y cells [34]. A search for transcriptional factor binding sites revealed that GLN LTR has a putative p53 binding site (Fig. S7A). In a

preliminary study on the mechanisms of BC expression regulation in NIH/3T3 cells, we found that the wild-type LTR had great promoter/enhancer activity, even stronger than the positive control using luciferase reporter gene assay (Fig. S7B). However, when the putative p53 binding site was deleted, the promoter/enhancer activity dropped significantly (Fig. S7B). To address the role of p53 in BC induction, we examined the effects of treatment with pifithrin-alpha (PFT-α), a widely used p53 inhibitor [35], on BC expression in NIH/3T3 cells. Consistent with our expectations, treatment with PFT-α decreased ETOP or MMS induced BC expression (Fig. S8). These results indicated that p53 might play a role in BC induction following GTXs treatment.

There are many genotoxicity mechanisms and the model compounds selected in this work could not be expected to cover all of them. More model compounds with diverse genotoxicity mechanisms should be tested to further examine the sensitivity and specificity of BC expression. An interesting finding in this study was that BC correlated with DNA damage but not chromosomal lesion, indicating that BC might not be appropriate in aneugen genotoxicity prediction. Given both the *in vivo* (Fig. 1 and Table 2) and *in vitro* data (Fig. 3), BC has the intrinsic ability to become a sensitive biomarker of DNA damage. Although BC induction was not obvious in response to colchicine and paclitaxel treatment, this would not compromise the sensitivity of BC as a potential biomarker of DNA damage, since colchicine and paclitaxel mainly target microtubules rather than DNA. Additionally, it should be noted that compounds that require S9 activation were excluded from the *in vitro* study, in order to avoid potential S9 effects on BC expression. It would be of great interest to examine the effects of indirect genotoxic chemicals on BC expression in future studies. Moreover, we are currently developing a GLN LTR driven luciferase reporter gene system in NIH/3T3 cells, to facilitate toxicity screening in early drug development [36,37,38,39].

The GLN family was recently reported to be potentially active in the mouse genome [21], thus uncovering its cellular functions would be of great significance. To the best of our knowledge, this is the first report showing the involvement of a GLN family member in cell growth regulation, thus providing basic information towards a deeper understanding of its biological roles. Several studies have
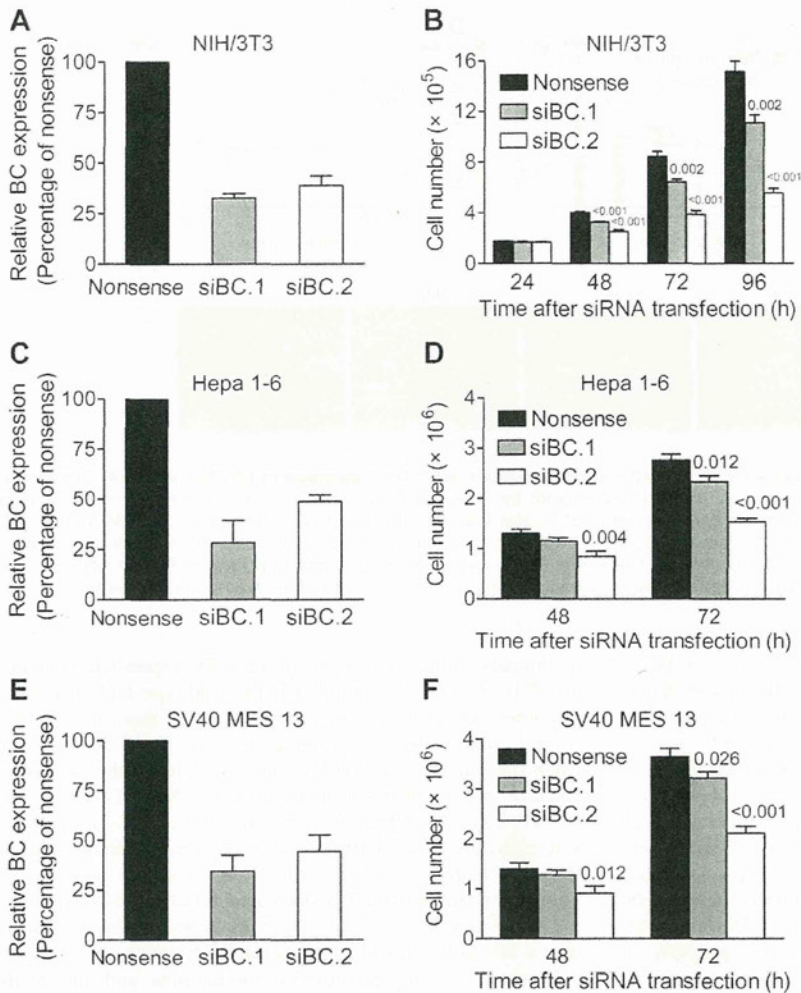
Figure 6. Down-regulating BC expression suppressed cell growth in several mouse cell lines. (A, C and **E)** Quantitative PCR results showing knock-down efficiency of BC siRNAs in NIH/3T3, Hepa 1–6 or SV40 MES 13 cells at 48 h after siRNA transfection. Data were mean ± s.d. of at least three independent experiments. **(B, D** and **F)** Cell numbers of NIH/3T3, Hepa 1–6 and SV40 MES 13 cells at indicated times after siRNA transfection. Data were mean ± s.d. of at least three independent experiments performed in triplicate. Values shown on top of bars are *P* values *vs* nonsense.
doi:10.1371/journal.pone.0035010.g006

reported the mitogenic effects of exogenous retroviruses (XRVs) on certain cell lines [40,41]. One suggested mechanism was the interaction of glycoproteins encoded by retroviral *env* genes with cellular cytokine receptors, such as interleukin-2 receptor or erythropoietin receptor. One study also suggested a role for *env* of mink cell focus-forming (MCF) ERV in regulating pluripotent hemopoietic progenitor proliferation. Given previous reports that GLN was potentially active and might have an extracellular life cycle similar to XRVs, it would be of great interest to study whether GLN regulates cell growth through similar mechanisms. Such studies will not only assist in a deeper understanding of its biological function, but also provide theoretical support for its application in genotoxicity prediction.

In summary, this is the first study to investigate the relationship between ERVs and genotoxicity. We showed that expression level of BC005512, a member from GLN family of murine ERV, was elevated by multiple GTXs both *in vivo* and *in vitro*, and correlated with the extent of DNA damage. It is thus possible that BC might

be useful as a potential molecular biomarker for assessment of DNA damaging effects. Additionally, we first demonstrated that BC was involved in cell growth regulation. This contributes to a deeper understanding of the biological functions of BC005512 and GLN, and provides fundamental support for their application in genotoxicity predictions.

Materials and Methods

Ethics Statement

Animal-use protocols were approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Materia Medica (Shanghai, China) with IACUC No. 2010-10-RJ-05.

Animal treatment in microarray study

Two-month-old male B6C3F1 mice were given a single intraperitoneal injection of model compounds or solvent control (corn oil or saline) as listed in Table 1. *O*-AAT, DBP, DMBA and
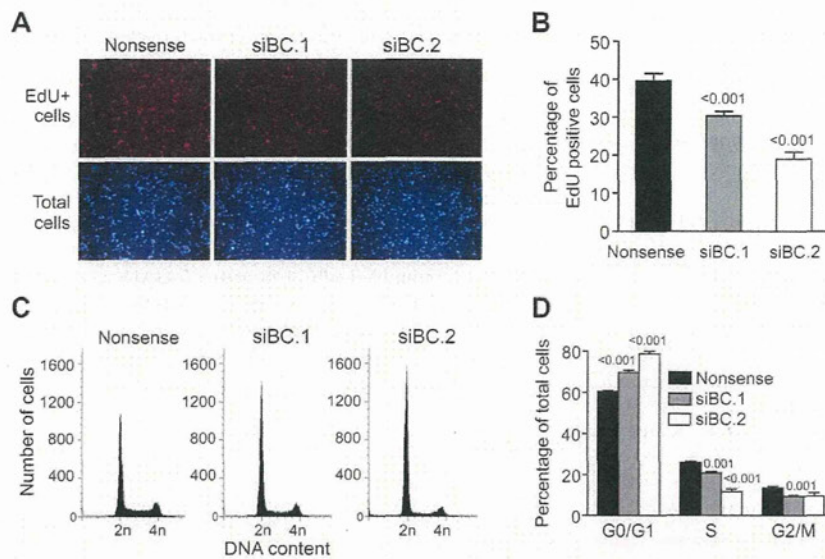
Figure 7. Knock-down of BC induced G1/S phase arrest and thus inhibited cell proliferation in NIH/3T3 cells. (A) Representative fluorescent images of EdU incorporation assay. Blue nuclei represent total cells visualized by UV excitation, while red nuclei represent EdU-positive cells visualized by green light excitation. (B) Quantitative analysis of EdU-positive cells (shown in A). A total of >4000 cells were counted for each group. Data were mean ± s.d. of three independent experiments. (C) Flow cytometric analysis of cell cycle in NIH/3T3 cells transfected with nonsense or BC siRNAs at 48 h after siRNA transfection. 2n = cells in G0/G1 phase, and 4n = cells in G2/M phase. (D) Quantitative analysis of cell cycle phase distribution (shown in C). Data were mean ± s.d. of at least three independent experiments performed in triplicate. Values shown on top of bars are *P* values *vs* nonsense.
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DEHP were dissolved in corn oil while other chemicals were dissolved in saline. Mice were sacrificed at 4 h, 20 h, 14 days or 28 days after administration and liver samples were collected immediately. Animal administration was the same in the quantitative PCR confirmation experiment except that B6C3F1 mice were substituted with C57BL/6 mice (supplied by Shanghai Slac Laboratory Animal, Shanghai, China). DEHP and EtOH were purchased from Sinopharm Chemical Reagent (Shanghai, China), and other chemicals were from Sigma (St Louis, MO, USA).

Microarray and data analysis

Five µg pooled total RNA of liver from 4 animals per group was used as starting material. For each time point of each compound, only one sample pooled from 4 animals was tested. cDNA synthesis, cRNA labeling, and cRNA fragmentation were conducted according to the manufacturer's instructions (Affymetrix Inc., Santa Clara, CA, USA). The hybridization mixture was hybridized to an Affymetrix Mouse Genome 430 2.0 array. Hybridized arrays were washed and stained, and fluorescence signals were detected using the Affymetrix GeneChip Scanner 3000. The image files were converted into expression data by the Microarray Suite Software (Affymetrix) and the data was imported into GeneSpring software (Silicon Genetics, Redwood City, CA, USA). Signal intensity was normalized by per-gene and per-chip. Ratio was calculated by normalizing treatment samples to solvent control samples. The procedure was in compliance to MIAME guidelines. The raw data has been deposited in GEO database (GSE33248).

We used a step-wise selection criterion (set different significance levels according to the intensity) in selecting differentially expressed genes. For each time point of each chemical, genes that met with either criterion listed in Table S1 were considered as up-regulated genes and the corresponding cells in the Table S3 were marked with pink. While genes that met with either criterion listed in Table S2 were considered as down-regulated genes and the corresponding cells in the Table S3 were marked with blue.

Those selected up-regulated genes were further analyzed by self-defined weight scoring based on several important parameters in identifying genotoxic stress responsive genes (including specificity, sensitivity, statistical P value, basal expression level, and coefficient of variation) Detailed scoring rules are described in supporting information (Text S1). Top 50 genes were further analyzed by hierarchical clustering (average linkage clustering) by using Gene Cluster and TreeView programs [42].

Cell lines and culture

NIH/3T3, Hepa 1–6 and SV40 MES 13 cells were purchased from Type Culture Collection of Chinese Academy of Sciences (http://www.cellbank.org.cn/index.asp). L5178Y cells were kindly provided by Dr. M. Honma (NIHS, Japan) [43]. NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% calf serum (Sijiqing Biological Engineering Materials, Hangzhou, China). L5178Y cells were cultured in RPMI 1640 medium with 10% horse serum. Hepa 1–6 and SV40 MES 13 cells were cultured in DMEM with 10% fetal bovine serum (FBS, Gibco) for maintenance or in DMEM with 2.5% FBS for cell growth assay. All cells were maintained at 37°C in 5% CO_2.

Chemicals treatment in *in vitro* BC expression assay

Compounds that require metabolic activation were excluded to avoid potential effects of S9 fraction on BC expression. The seven selected genotoxins represent different mechanisms of action. NIH/3T3 or L5178Y cells were treated with indicated concentration of model compounds or irradiated with γ-ray by

Gammacell 3000 Elan (MDS Nordion, Ottawa, ON, Canada) as listed in Table 3. After 4, 8, 12 and 24 h treatment or irradiation for NIH/3T3, 4 h for L5178Y, cells were harvested and subjected to RNA isolation and qPCR. MMC was purchase from Kyowa (Tokyo, Japan), ACN from Merck (Darmstadt, Germany), SUC from Sinopharm, and other chemicals were from Sigma.

Quantitative real-time PCR (qPCR)

Total RNA of mouse liver, NIH/3T3, L5178Y, Hepa 1–6 or SV40 MES 13 cells was extracted by using the UNIQ-10 total RNA isolation kit (Sangon Biotech, Shanghai, China). The remnant genomic DNA in total RNA was digested by RNase-free DNase I (Fermentas, Burlington, Canada). DNase-digested RNA was further reverse transcribed into cDNA by using the PrimeScript RT reagent kit (TaKaRa, Otus, Shiga, Japan). DNase-digested RNA without reverse transcription was used as a negative control. qPCR was carried out by using the SYBR Premix Ex Taq (TaKaRa) with BC primers (forward: 5'-ATCACCCTGCATCCAGTTTAG -3', reverse: 5'-TATTGCCGCTAGGTCTTCATT -3') or GAPDH primers (forward: 5'-GGCTACACTGAGGACCAGGTT-3', reverse: 5'-TGCTGTAGCCGTATTCATTGTC-3'). The qPCR conditions were as follows: 95°C, 10 s; (95°C, 5 s, 60°C, 34 s) 40 cycles, with a melting-curve process. Amplification process was performed on 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and data was analyzed by using $2^{-\Delta\Delta Ct}$ with the Sequence Detection Software.

Alkaline comet assay

The alkaline comet assay was performed as previous described with slight modifications [28]. Briefly, NIH/3T3 cells were exposed to MMS for 8 h or to paclitaxel (Sigma) or colchicine (Sigma) for 24 h. Then cells were trypsinized, resuspended in PBS, mixed with 0.5% low-melting agarose and applied to glass slides pre-coated with 1% normal-melting agarose. Slides mounted with cells were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH = 10; the last two compounds were added fresh) for 2.5 h. After lysis, slides were immersed in alkaline buffer (300 mM NaOH, 1 mM EDTA, pH = 13) for 20 min to allow DNA unwinding and DNA breakage at alkali-labile sites. Then electrophoresis was performed at 300 mA for 20 min. After electrophoresis, slides were neutralized in 0.4 M Tris-HCl, dipped in ethanol and air-dried. Cells were stained with SYBR Green and observed by a fluorescent microscope (Olympus BX 51). At lease 100 cells were pictured for each group, and olive tail moment (tail length×percentage of DNA in tail) was determined by image analysis software Komet 5.5 (Kinetic Imaging, Liverpool, UK).

Micronucleus test

The micronucleus test was performed as described previously with some modifications [44] [31]. Briefly, NIH/3T3 cells were exposed to paclitaxel or colchicine for 24 h. Then the attached cells were trypsinized, incubated in 0.075 M KCl hypotonic solution, fixed in methanol-acetic acid (3:1) and finally suspended in methanol containing 1% acetic acid. A drop of cell suspension was placed on a clean glass slide and air-dried. Cells were stained by mounting with acridine orange and immediately observed by a fluorescent microscope (Olympus BX 51). The micronucleus frequency was determined in 1000 total cells according to published criteria [44], and cells with irregular shape of nucleus were also counted as micronucleated cells. Cytochalasin B was not used since it has been reported that the alteration of two

cytoskeletal elements, microtubules and microfilaments, concomitantly could influence the formation of micronucleated cells [45].

Cell transfection

Transfection of siRNA into NIH/3T3, Hepa 1–6 or SV40 MES cells was conducted by using RNAiMAX (Invitrogen) with 50 pmol siRNA per 35 mm dish. BC siRNA-1 (siBC.1) targeting 5'-CAGGUACCUCUAACUAUUAdTdT-3', BC siRNA-2 (siBC.2) targeting 5'-CCAGUUUAGAAGAAAGCUAdTdT-3', nonsense siRNA targeting 5'- GCGACGAUCUGCCUAA-GAUdTdT-3', scrambled-siBC.1 targeting 5-GAUCGAUAACC-CAUCUUUAdTdT-3 and scrambled-siBC.2 targeting 5-GCUAAUACUAGGCAAUGAAdTdT-3 were synthesized by GenePharma (Shanghai, China). All siRNAs were chemically modified with 2'-fluoro-dU and 2'-fluoro dC.

Cell growth assay

At 24, 48, 72 or 96 h after siRNA transfection, NIH/3T3, Hepa 1–6 or SV40 MES cells were trypsinized and cell numbers were determined by using a Z1 Coulter counter (Beckman Coulter, Fullerton, CA, USA).

EdU incorporation assay

EdU incorporation assay was performed by using Cell-Light EdU DNA imaging kit (RiboBio, Guangzhou, China) according to the manufacture's instructions. Briefly, at 48 h after siRNA transfection, NIH/3T3 cells were cultured in medium containing 50 µM EdU for 3.5 h. After EdU incorporation, cells were fixed in 4% paraformaldehyde and permeabilized by 0.5% Triton X-100. After a click reaction between Apollo dye and ethynyl group in EdU, EdU positive cells were visualized with a green light excitation (550 nm), while total cells were visualized by Hoechst 33342 staining with UV excitation. At least a total of 4000 cells were pictured for each group. EdU positive and total cells were counted by using software Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA).

Cell cycle analysis

At 48 h after siRNA transfection, NIH/3T3 cells were trypsinized, fixed in 70% ethanol, incubated with RNaseA, stained with propidium iodide (Sigma) and analyzed by a FACSCalibur (BD, Franklin Lakes, NJ, USA) instrument. Analysis of cell cycle phase distribution was performed by using the Modfit software (Verity Software House, Topsham, ME, USA).

Statistical analysis

Data were analyzed by Student's unpaired t-test in Excel. Differences were considered significant at $*P<0.05$, $**P<0.01$. For BC expression study, the raw data was divided by that of control, generating the normalized BC expression (folds of control). For linear regression analysis, each dot in Fig. 4C represents the mean of data shown in Fig. 4A, 4D and 4G. The linear regression analysis was performed by using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Supporting Information

Text S1 Supporting materials and methods.
(DOC)

Figure S1 Probe sequence (1426936_at) in Affymetrix Mouse Genome 430 2.0 array.
(TIF)

Figure S2 Expression of BC was induced by GTXs in Hepa 1–6 cells. Quantitative PCR data showing transcriptional expression of BC in Hepa 1–6 cells treated with CPT (3 μM), MMC (100 μM), ETOP (50 μM) or MMS (0.5 mM) for indicated times. Dose was 24 h IC_{50}. Data were mean ± s.d. of three independent experiments.
(TIF)

Figure S3 Effects on BC expression in L5178Y cells following MMS treatment at various time points. L5178Y cells were treated with indicated concentrations of MMS. At various time points after MMS incubation, cells were harvested and expression levels of BC were analyzed by quantitative PCR. Data were mean ± s.d. of three independent experiments.
(TIF)

Figure S4 Specific effects of BC siRNAs on cell growth and cell cycle progression. Expression level of BC (**A**), cell number (**B**) and cell cycle phase distribution (**C**) in NIH/3T3 cells transfected with indicated siRNAs at 48 h after transfection. Data were mean ± s.d. of at least three independent experiments performed in triplicate. Values shown on top of bars are the P values vs corresponding scrambled siRNA.
(TIF)

Figure S5 Representative western blot results showing protein level knock-down efficiency and specificity of siBC.2. NIH/3T3 cells were co-transfected with wild-type or mutant myc tagged BC clone with nonsense siRNA or siBC.2. Cell lysates were collected at 24 h after co-transfection. β-Actin served as a loading control. Primary antibody against Myc tag was from Cell Signaling (Danvers, MA, USA).
(TIF)

Figure S6 Flow cytometric analysis of apoptosis in NIH/3T3 cells. NIH/3T3 cells were transfected with indicated siRNAs. At 48 or 96 h after transfection, apoptosis was determined using Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen) and a FACSCalibur (BD Pharmingen) instrument. The lower right quadrant cells indicate early apoptotic cells, while the upper right quadrant cells indicate late-apoptotic or dead cells.
(TIF)

Figure S7 The promoter/enhancer activity of wild-type and p53 binding site deleted LTRs of GLN. (**A**) LTR sequence of GLN. The sequence of the putative p53 binding site is shown in bold and is consistent with the consensus p53 sequence (5′-RRRCWWGYYY-3′, R = purine, Y = pyrimidine, W = A or T). This putative p53 binding site was composed of two half-site RRRCWWGYYY with no spacers. Right arrows indicate RRRCW and left arrows indicate WGYYY. (**B**) Luciferase assay of the GLN LTRs in NIH/3T3 cells. "Basic" and "Control" represents negative and positive control respectively. LTR-D represents LTR with the putative p53 binding site deletion. LTR-WT represents wild-type LTR. Data were mean ± s.d. of at least three independent experiments.
(TIF)

Figure S8 Effects of pifithrin-alpha (PFT-α) treatment on GTX-induced BC expression in NIH/3T3 cells. NIH/3T3 cells were treated with ETOP or MMS in combination with indicated concentrations of PFT-α. Cells were harvested after 24 h and BC expression was analyzed by quantitative PCR. Data were mean ± s.d. of three independent experiments. *$P<0.05$, **$P<0.01$ vs PFT-α 0 μM.
(TIF)

Table S1 Step-wise criterion in selecting up-regulated genes.
(DOC)

Table S2 Step-wise criterion in selecting down-regulated genes.
(DOC)

Table S3 Raw data and detailed scoring rule of up-regulated genes.
(XLS)

Acknowledgments

Author Contributions

Conceived and designed the experiments: YL JR. Performed the experiments: YW XQ YL. Analyzed the data: YW XQ YL LG. Contributed reagents/materials/analysis tools: GX MC LM JY TS CF. Wrote the paper: YW XQ YL.

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Discrimination of genotoxic and non-genotoxic hepatocarcinogens by statistical analysis based on gene expression profiling in the mouse liver as determined by quantitative real-time PCR[☆]

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ARTICLE INFO

ABSTRACT

The general aim of the present study is to discriminate between mouse genotoxic and non-genotoxic hepatocarcinogens via selected gene expression patterns in the liver as analyzed by quantitative real-time PCR (qPCR) and statistical analysis. qPCR was conducted on liver samples from groups of 5 male, 9-week-old B6C3F$_1$ mice, at 4 and 48 h following a single intraperitoneal administration of chemicals. We quantified 35 genes selected from our previous DNA microarray studies using 12 different chemicals: 8 genotoxic 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, di(2-ethylhexyl)phthalate and furan). A considerable number of genes exhibited significant changes in their gene expression ratios (experimental group/control group) analyzed statistically by the Dunnett's test and Welch's t-test. Finally, we distinguished between the genotoxic and non-genotoxic hepatocarcinogens by statistical analysis using principal component analysis (PCA) of the gene expression profiles for 7 genes (Btg2, Ccnf, Ccng1, Lpr1, Mbd1, Phlda3 and Tubb2c) at 4 h and for 12 genes (Aen, Bax, Btg2, Ccnf, Ccng1, Cdkn1a, Gdf15, Lrp1, Mbd1, Phlda3, Plk2 and Tubb2c) at 48 h. Seven major biological processes were extracted from the

gene ontology analysis: apoptosis, the cell cycle, cell proliferation, DNA damage, DNA repair, oncogenes and tumor suppression. The major, biologically relevant gene pathway suggested was the DNA damage response pathway, resulting from signal transduction by a p53-class mediator leading to the induction of apoptosis. Eight genes (Aen, Bax, Btg2, Ccng1, Cdkn1a, Gdf15, Phlda3 and Plk2) that are directly associated with Trp53 contributed to the PCA. The current findings demonstrate a successful discrimination between genotoxic and non-genotoxic hepatocarcinogens, using qPCR and PCA, on 12 genes associated with a Trp53-mediated signaling pathway for DNA damage response at 4 and 48 h after a single administration of chemicals.

1. Introduction

Based on their mechanisms of action, chemical carcinogens are classified as genotoxic or non-genotoxic carcinogens [1,2]. Genotoxic carcinogens induce positive genotoxic responses which can occur through any of a number of relevant processes, including direct DNA damage, delayed or inhibited repair, interferences with repair processing enzymes such as topoisomerase, and so forth [3]. Non-genotoxic carcinogens, however, do not induce positive genotoxic responses. According to Waters et al. although the number of presumed 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 [4].

Mathijs et al. hypothesized that genotoxic and non-genotoxic carcinogens induce distinct gene expression profiles, which consequently may be used for a mechanism-based classification of unknown compounds as either genotoxic carcinogens or non-genotoxic carcinogens [2]. The DNA microarray is a powerful technology for characterizing gene expression on a genomic scale [5], although issues of reliability, reproducibility and correlation of data produced across different DNA microarrays are still being addressed [6]. The combination of toxicogenomics data on chemical carcinogens coupled with DNA microarrays has gradually become more common and suggests their usefulness [3]. However, the published studies on in vivo rodent livers are limited.

Quantitative real-time PCR (qPCR) is generally considered the "gold-standard" assay for measuring gene expression and is often used to confirm DNA microarray data [7]. qPCR is the most sensitive technique for the detection and quantification of mRNA targets [8]. It has been suggested that qPCR may be a simpler, more reliable and more reproducible method than DNA microarray [9], although it requires more time for a large number of genes and samples; more recently though, a high-density qPCR technique has appeared [10]. There are only a few papers that have examined selected genes by qPCR in rodent livers in vivo.

Previously, we examined differential gene expression using DNA microarrays upon 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 up to 28 days following a single intraperitoneal administration. Many candidate genes were identified to discriminate the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogens; the results were reported in part [11] and registered to the GEO database (GEO accession GSE33248). Notably, the changes at 4 h were much greater than those at 20 h, 14 days and 28 days. Additionally, dose-dependent alterations in the gene expression were

demonstrated in 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, 1/40–1/2 of lethal dose 50% (LD50) and ENU (6, 17, 50 and 150 mg/kg bw, 1/80–1/3 of LD50) as determined by qPCR [12].

In the present study, we evaluated the gene expression profiles of 12 genotoxic and non-genotoxic mouse hepatocarcinogens, using qPCR on 34 genes selected from our previous DNA microarray studies. The chemicals were intraperitoneally injected into 9-week-old male B6C3F$_1$ mice and analyzed at 4 and 48 h after administration. We speculated that the period at 4 h post-hepatocarcinogen administration in liver would be the time of DNA damage determined by in vivo unscheduled DNA synthesis test [13–15] and Comet assay [16] and that the period at 48 h would be the period of DNA replication after damage determined by replicative DNA synthesis test [13–15,17]. We examined genotoxic hepatocarcinogens, which are positive in the Ames test and in in vivo genotoxicity tests in the mouse liver (transgenic mouse mutation assay or micronucleus assay) and exhibit various chemical properties (summarized in Table 1), and non-genotoxic hepatocarcinogens, which are negative in the Ames test and in in vivo genotoxicity tests (micronucleus assay, unscheduled DNA synthesis assay or Comet assay) and exhibit various chemical properties (summarized in Table 1). Finally, we succeeded in discriminating the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogens by statistical analysis using PCA. We showed that the major biologically relevant gene pathway of PCA contributed genes is a Trp53-mediated signaling pathway for the DNA damage response resulting in the induction of apoptosis.

2. Materials and methods

2.1. Chemicals

All chemical names, abbreviations, genotoxic vs. non-genotoxic; CAS numbers; makers; doses; LD50; in vivo mouse genotoxic test and Ames test are summarized in Table 1. The solvents; olive oil (CAS 8001-25-0) and saline were obtained from Wako Pure Chemical Industries; Ltd.; Osaka; Japan.

2.2. Animal treatment

Male B6C3F$_1$ mice were obtained at 8 weeks of age from Charles River Japan, Inc. (Yokohama, Japan) and Japan SLC, Inc. (Shizuoka, Japan) and were kept in plastic cages with wood chip bedding and access to food (Oriental MF, Oriental Yeast Co., Tokyo) and water ad libitum in an air-conditioned room (12 h light, 12 h dark; 23 ± 2 °C; 55 ± 5% humidity) at the Biosafety Research Center, Foods, Drugs, and Pesticides in Shizuoka and the National Institute of Health Sciences in Tokyo. All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee in the Biosafety Research Center, Foods, Drugs, and Pesticides and in the National Institute of Health Sciences. Groups of 5 mice at 9 weeks of age were injected i.p. with 8 genotoxic and 4 non-genotoxic mouse hepatocarcinogens. DIPN and URE were dissolved in saline, and the other chemicals were suspended in olive oil. The control animals received plain saline or olive oil. As shown in Table 1, the doses for the genotoxic hepatocarcinogens were similar to the positive doses used in previous in vivo mouse liver genotoxic studies (transgenic mouse studies (2AAF [18], DAT [19], DIPN; T. Suzuki unpublished data, NNM; T. Suzuki unpublished data, NNK [20], QN [21] and URE [22])) and in the micronucleus test (DAB [23]). Doses for the non-genotoxic hepatocarcinogens were 1/3–1/2 of the LD50 or similar to the doses used for previous in vivo genotoxic studies (DCB [24], DDT [25] and FUR [26]). The dose of DEHP (2000 mg/kg bw) constituted the highest recommended dose for

Table 1
Chemicals.

No.	G vs NG	Name	Abbrev.	CAS no.	Maker	Dose (mg/kg)	LD50 (mg/kg)	Genotoxicity test (in vivo)	(mg/kg)	Ames test
1	G	2-acetylaminofluorene	2AAF	CAS 53-96-3	Nacalai	300	2,200 (m, ip)	TG+	100	+
2	G	2,4-diaminotoluene	DAT	CAS 95-80-7	Wako	200	380 (m, po)	TG+	200	+
3	G	diisopropanolnitrosamine	DIPN	CAS 53609-64-6	Nacalai	500	5160 (m, sc)	TG+	500	+
4	G	4-dimethylaminoazobenzene	DAB	CAS 60-11-7	Sigma	100	230 (m, ip)	MN+	165	+
5	G	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone	NNK	CAS 64091-91-4	Wako	250	1000 (m, ip)	TG+	250	+
6	G	*N*-nitrosomorpholine	NNM	CAS 59-89-2	TCI	32	100 (rat, ip)	TG+	16×4	+
7	G	quinoline	QN	CAS 91-22-5	Nacalai	100	331 (rat, po)	TG+	100	+
8	G	urethane	URE	CAS 51-79-6	TCI	1000	2500 (m, po)	TG+	900	+
9	NG	1,4-dichlorobenzene	DCB	CAS 106-46-7	TCI	1000	2000 (m, ip)	MN−	2500	−
10	NG	dichlorodiphenyltrichloroethane	DDT	CAS 50-29-3	TCI	50	135 (m, po)	Comet−	75	−
11	NG	di(2-ethylhexyl)phthalate	DEHP	CAS 117-81-7	TCI	2000	14,000 (m, ip)	UDS−	500	−
12	NG	furan	FUR	CAS 110-00-9	TCI	30	7 (m, ip)	UDS−	50	−

G: genotoxic, NG: non-genotoxic, No.1: polycyclic aromatic compound, No.2: aromatic compound, No. 3, 5, 6: *N*-nitroso compounds, No. 4: aromatic azo compound, No. 7, 12: hetero cyclic compounds, No. 8: compound with the functional group RO(CO)NHR, No. 9, 10: chlorinated aromatic compounds, No. 11: phthalate, peroxisome proliferator.
Nacalai: Nacalai Tesque, Inc., Wako: Wako Pure Chemical Industries, Ltd., Sigma: Sigma Co., TCI: Tokyo Chemical Industry Co., Ltd.
LD50: m: mouse, TG: transgenic rodent mutation assay, MN: micronuclei assay, Comet: comet assay.
DEHP: genotoxicity test in vivo [27].

an in vivo short-term test. At 4 and 48 h time points after treatment, the animals were sacrificed and the main lobe of the liver was collected, cut into small pieces (~3 mm × 3 mm × 3 mm), placed in RNAlater (Applied Biosystems, Foster City, CA, USA) and stored at −20 to −30 °C until further use.

2.3. RNA isolation and relative quantification by real-time PCR

To isolate the total RNA, approximately 30 mg from each liver (main lobe) was placed into LRT buffer (FUJIFILM Corp., Tokyo, Japan) and immediately homogenized using a MicroSmash (TOMY SEIKO CO., LTD. Tokyo, Japan). The total RNA was then purified with QuickGene 800 (FUJIFILM Corp., Tokyo, Japan). Complimentary DNA (cDNA) was produced from the total RNA using the SuperScript III First Strand Synthesis System for RT-PCR kit (Invitrogen Corp., Carlsbad, CA, USA). The RNA isolation and cDNA preparation were conducted at Aoyama Gakuin University, and the cDNA was distributed to collaborative laboratories.

The qPCR amplifications were performed in triplicate by the SYBR Green I assay in an Opticon II (MJ Research, Inc., Waltham, MA, USA) and Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reactions were carried out in a 96-well plate in 20-μl reactions containing 2X SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 2 pmol each of the forward and reverse primers and a cDNA template corresponding to 400 pg of total RNA. We quantified 35 genes based on our previous DNA microarray and qPCR results [19,20]. The symbol, gene name and accession number of the 35 genes are summarized in Table 2. Each primer sequence and the Ct value of the 35 genes are shown in Table 3. The SYBR Green PCR conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 50 s and 72 °C for 20 s. Each 96-well reaction plate was composed of 5 standard samples, diluted up to 1/5, 1/25, 1/125, 1/625 and 1/3,125 of the selected standard liver cDNA for each gene, and a negative control. The relative quantitative values of each sample were determined with 1/25-diluted cDNA and were normalized to *Gapdh* [12].

2.4. Statistical analysis

For the 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 qPCR data for each animal were statistically analyzed by the Dunnett's test, and the results of the experimental groups were compared to those of a control group. The statistical significance for each gene between the genotoxic and non-genotoxic hepatocarcinogens, at 4 and 48 h, was assessed by the Welch's t-test.

Discrimination of the genotoxic and non-genotoxic hepatocarcinogens was achieved by statistical analysis using PCA. PCA involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called "principal components". The first principal component (PC1) accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. The mathematical formula of PC1 (z_1) for 4 h is presented as the following:

$$z_{1(4h)} = a_{11}x_1 + a_{12}x_2 + - - - + a_{1p}x_p,$$

where a_{1p} is the eigenvector and x_p is the canonicalized logarithmic ($\log_2$)-transformed gene ratios (exp/cont). PCA was performed using the PCA programs in GeneSpringGX11.0.1 (Agilent Technologies, Santa Clara, CA, USA). Initially PCA was applied to all 34 logarithmic ($\log_2$) transformed ratios (exp/cont) and subsequently tried with various candidate gene sets until the optimal discrimination was

achieved. The candidate genes were selected primarily using the Welch's t-test from the results at 4 h, 48 h and a combination of both time points.

2.5. Gene ontology, pathways and network analysis

Gene ontology analysis was performed with Gene Ontology (http://geneontology.org/) and Ingenuity Pathways Analysis 7.0 (IPA) (http://www.Ingenuity.com). The results were confirmed with references in PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Gene pathways and networks were generated with GeneSpringGX11.0.1 and IPA, which enables the visualization and analysis of biologically relevant networks to allow for discovery, visualization, and exploration of therapeutically relevant networks, as described previously [12].

3. Results

3.1. Changes in the gene expression 4 and 48 h after chemical administration and statistical analysis by the Dunnett's test and the Welch's t-test

The gene expression ratio (experimental group/control group; exp/cont) was calculated individually for each group (5 mice in triplicate assays) from the qPCR results. The mean ± SD was determined and the statistical significance was assessed by the Dunnett's test. Thirty-four genes exhibited statistically significant changes in their gene expression, at least once, at 4 h and/or 48 h, as computed by the Dunnett's test (Tables 4.1–4.4). The statistical significance between the genotoxic and non-genotoxic hepatocarcinogens for each gene was assessed by the Welch's t-test at 4 and 48 h after chemical administration (Tables 4.1–4.4). Different sets of 17 genes at 4 h and 19 genes at 48 h showed statistical significance between the genotoxic and non-genotoxic hepatocarcinogens, as analyzed by the Welch's t-test (Tables 4.2 and 4.4). In Tables 4.1–4.4, the results of *Gapdh*, a housekeeping gene, are shown. This gene was used to normalize the gene expression ratio, as this gene did not show changes in expression.

The changes in the gene expression of 14 major genes (*Aen, Bax, Btg2, Ccnf, Ccng1, Cdkn1a, Egfr, Gdf15, Lrp1, Mbd1, Phlda3, Plk2, Ppp1r3c* and *Tubb2c*) are shown in Fig. 1. *Aen, Bax, Btg2, Ccnf, Ccng1, Cdkn1a, Gdf15, Phlda3, Plk2, Ppp1r3c* and *Tubb2c* all showed increases in their gene expression, while *Egfr, Lrp1* and *Mbd1* showed decreases. No single gene completely discriminate the genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens.
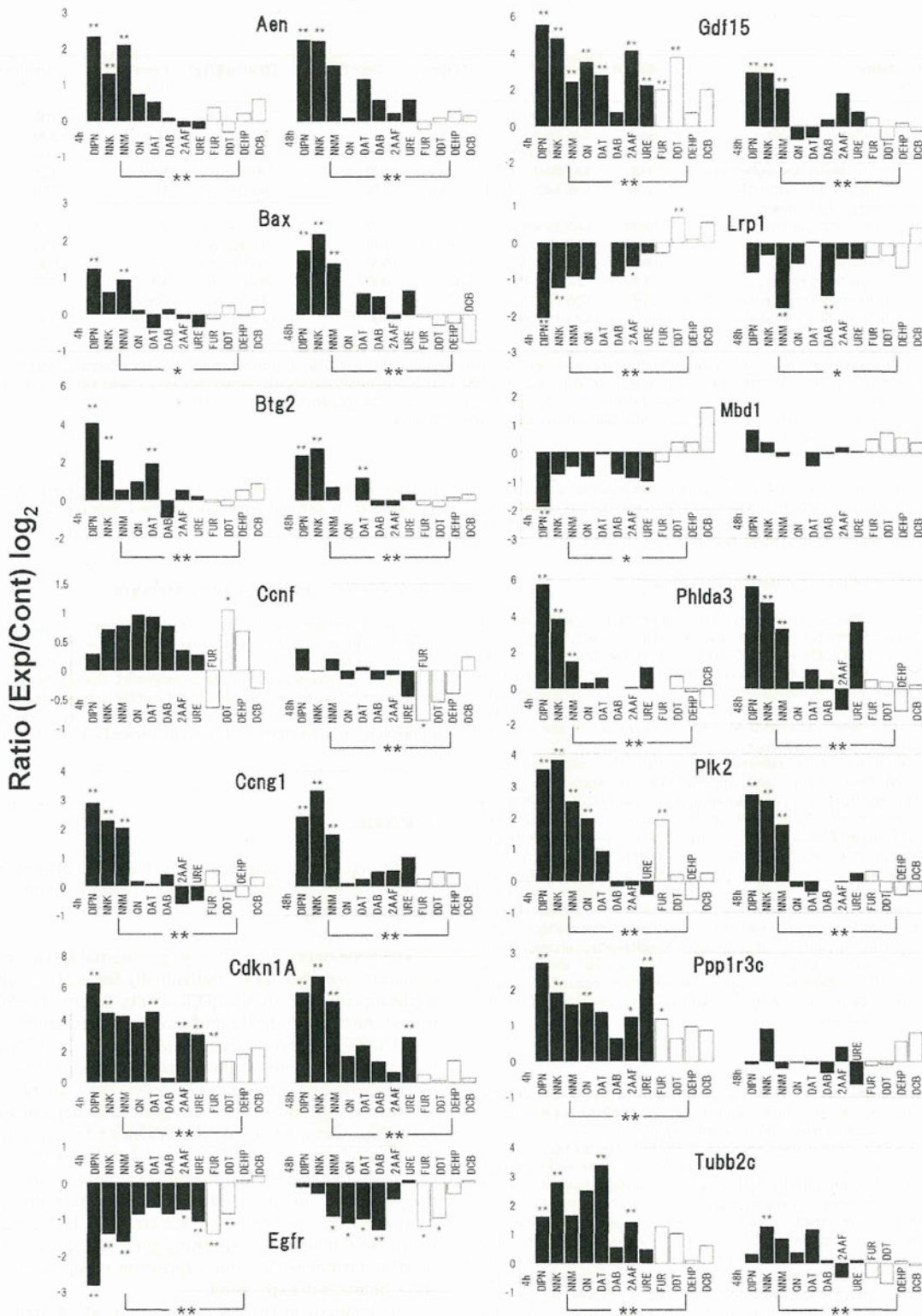
Fig. 1. Changes in the gene expression of 14 major genes (*Aen*, *Bax*, *Btg2*, *Ccnf*, *Ccng1*, *Cdkn1a*, *Egfr*, *Gdf15*, *Lrp1*, *Mbd1*, *Phlda3*, *Plk2*, *Ppp1r3c* and *Tubb2c*) as quantified by qPCR at 4 and 48 h. DIPN: diisopropanolnitrosamine, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, 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 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].
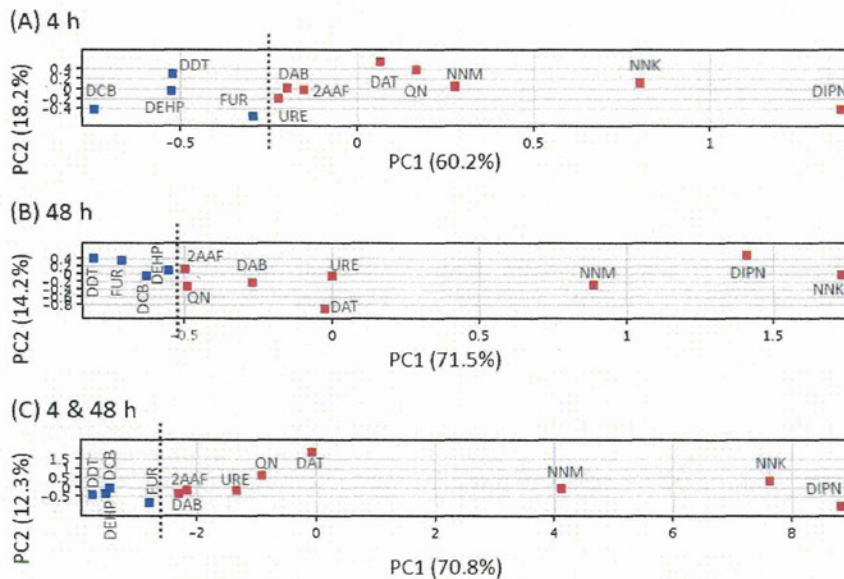

Fig. 2. Principal component analysis (PCA) of the gene expression levels between genotoxic and non-genotoxic hepatocarcinogens as quantified by qPCR. The mean values of triplicate qPCR assays for each chemical were statistically analyzed using PCA programs in GeneSpringGX11.0.1. The results of the PCA 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: dichlorodiphenyl-trichloroethane, 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 onco-genes 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 net-work 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 ± 0.43	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 $P < 0.05$).
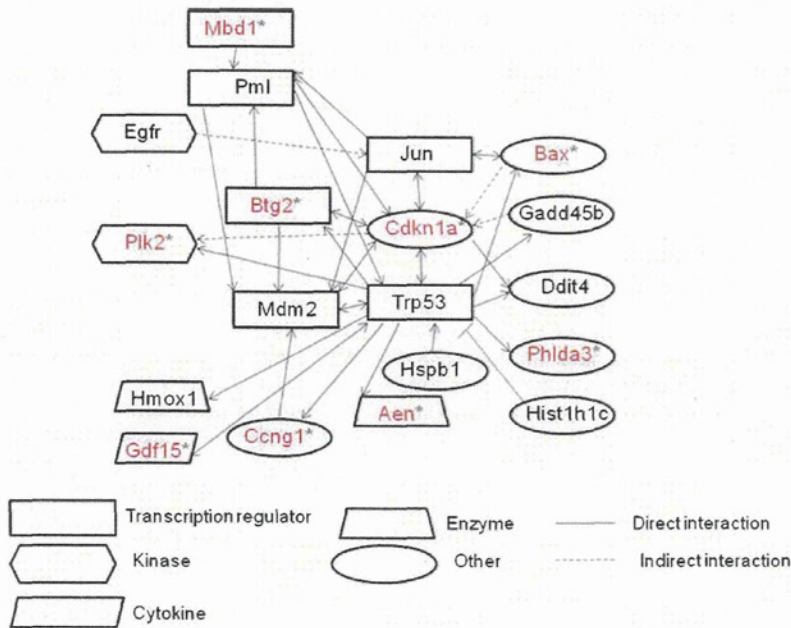

Fig. 3. The gene networks and pathways of 19 genes as determined by qPCR. The network was combined from the results of Ingenuity Pathways Analysis, GeneSpring software and references from PubMed. The 9 red-colored genes indicated by "*" mark genes that significantly contributed to the discrimination 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].