日本人を対象にしたゲノム・メタボローム解析によるバイオマーカー探索

Biomarker exploration by genomic and metabolomic analyses in Japanese

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1　はじめに

バイオマーカーは医薬品の開発効率の改善や，安全性の向上等に役立つことが期待されている。このため米国では，バイオマーカーを利用した臨床試験が，この 10 年間で実に約 30 倍に増加している。さらに，本邦でも市販後に添付文書の改訂が行われ，バイオマーカーに関する記載が追加される例が増加しており，特にゲノムバイオマーカーに関しては，順調に増えている[1]。

内閣府・総合科学技術会議の平成 24 年度科学技術重要施策アクションプランには，「科学的根拠に基づいたバイオマーカーを開発，利用することで，客観的，確度の高い診断と予測，治療の実現を目指すことが可能となる。そのため，「先制医療（早期医療介入）の実現による発症率の低下」を課題として選択した」と記載されている。バイオマーカーを早期の臨床指標として用いる取組，また医薬品の安全対策に用いる取組は，今後も加速すると考えられている。

本稿では，日本人を対象にした医薬品の薬効・副作用を予測するためのゲノム・メタボローム解析によるバイオマーカー探索に関し，筆者らの成果を中心に述べる。

2　薬効・副作用に関するゲノムマーカー探索

2.1　薬効

(1) ワルファリン

経口抗凝固剤ワルファリンは，安定投薬量に至るまでに時間がかかること，また投薬量に個人差が大きいことが知られている。ワルファリンは薬物代謝酵素 CYP2C9 で主として代謝されるが，本酵素には活性低下をもたらす遺伝子多型 *CYP2C9*3*（I359L，日本人での染色体別頻度：約 3%）が知られている。また標的酵素であるビタミン K エポキシド還元酵素複合体（サブユニット 1，*VKORC1*）にも発現量の低下と関連する多型（−1639G ＞ A，頻度：約 90%）が知られている。

国際ワルファリン薬理遺伝学コンソーシアム（IWPC）は，上記の遺伝子多型情報に加えて，患者背景情報（年齢，身長，体重，人種，併用薬等）を考慮した投薬量計算式を発表した[2]。日本人についても従来の投薬量算出法より，IWPC の方法が適切に投薬量を予測できることが報告されている[3]。

(2) タモキシフェン

抗乳癌剤タモキシフェンは，エストロゲン受容体拮抗作用により薬効を示すとされる。原薬の CYP2D6 および CYP3A4 による代謝で生成する 4-ヒドロキシ体やエ

ンドキシフェンは，原薬に比して強い薬効を有する。

CYP2D6では，日本人で頻度の高い（頻度：約38%）酵素活性低下をもたらす遺伝子多型 *10*（P34S，他）および酵素活性の消失をもたらす *5*（頻度：約6%）が主として知られている。多型群を有する日本人患者では，無再発生存期間が有意に短いことが報告されている[4]。

2.2 副作用

(1) 好中球減少症

抗がん剤イリノテカンは，多くの消化器癌等に，また抗がん剤ゲムシタビンは膵臓癌等に用いられる。これらの抗がん剤では，副作用として骨髄抑制が知られているが，その発症に遺伝子多型の関与が報告されている。イリノテカンに関しては，活性代謝物SN-38を解毒代謝するグルクロン酸転移酵素UGT1A1の活性低下型遺伝子多型である *6*（G71R，頻度：約16%）と *28*（−54_−39A(TA)$_6$TAA ＞ A(TA)$_7$TAA，頻度：約11%）が，重篤な好中球減少症の発現に関連しており，添付文書での注意喚起に加えて，多型診断用の対外診断薬も販売されている[5]。またゲムシタビンに関しては，解毒代謝酵素であるシチジンデアミナーゼ（CDA）の活性低下型遺伝子多型である *3*（A70T，頻度：約4%）が，重篤な好中球減少症の発現に関連している[6]。

(2) 重症薬疹

重症薬疹は医薬品による健康被害被害救済制度において常に上位を占めている。中でも重篤なのが，スティーブンス・ジョンソン症候群（SJS）及び中毒性表皮壊死症（TEN）である。SJSとTENは，多くの医薬品が発症原因となり，皮膚・粘膜部の発疹・びらん，発熱等を主症状とし，表皮の水疱・剥離面積等によりSJSとTENに分類される。致死率も比較的高く，重い後遺症が残ることがある。近年，その発症とヒト白血球抗原（HLA）遺伝子の特定のタイプとの間の強い関連が明らかとなっている。

抗てんかん薬カルバマゼピン誘因性SJS/TEN発症に関し，漢民族・タイ人等で，*HLA-B*1502* との非常に強い関連が報告されているが，白人や日本人では認められない。一方で，韓国人と日本人では，*HLA-B*1502* と同じ血清型B75に属する *HLA-B*1511*（日本人での頻度：約1%）との関連が報告されている[7]。さらに最近，白人，日本人において，*HLA-A*3101*（日本人での頻度：約9%）との相関が認められている[8]。本邦では，*HLA-B*1502* および *HLA-A*3101* に関する注意喚起が添付文書でなされている。

また，高尿酸血症薬アロプリノールによるSJS/TEN等の発症と *HLA-B*5801*（日本人での頻度：約1%）との関連が，台湾の漢民族でまず報告され，日本人を含めた諸民族（韓国人，白人等）でも，関連が報告されている[9]。日本では添付文書における注意喚起がなされている。

(3) 薬物性肝障害

薬物性肝障害は中毒性と特異体質性に大別され，さらに特異体質性はアレルギー性と代謝性に分類される。

抗血小板薬チクロピジンは，主に胆汁うっ滞型肝障害を誘因することが知られている。日本人を対象にした遺伝子解析の結果，*HLA-A*3303* との間に強い関連が認められた[10]。その発症頻度は白人よりも日本人において高いことが知られているが，日本人母集団における *HLA-A*3303* の染色体別頻度（約7%）が，白人（約0.7%）より高いことが一因と考えられる。

トログリタゾンは，肝障害により市場撤退した経口糖尿病薬である。日本人につき遺伝子解析が行われた結果，グルタチオン *S*-転移酵素である *GSTM1* および *GSTT1* 両遺伝子の欠損との関連が示された[11]。日本人における *GSTM1/T1* 両欠損型の頻度は約25%である。

3 薬効・副作用に関するメタボロームマーカーの探索

メタボロームは，アミノ酸，糖，脂質等の内在性代謝物を網羅的に測定する手法である。日本人を対象にした薬効に関するマーカーの報告例はないが，副作用に関しては散見される。薬物性肝障害に関して，電荷を有する内在性代謝物の解析を行った結果，ALT＋γ-グルタミルシトルリンがマーカーとなりうることが報告されている[12]。筆者らは，6カ所のナショナルセンターおよび慶応大学と，腎がん，肥満症，大動脈瘤，脊柱管狭窄症

等の13疾患に関し，日本人患者を対象としたメタボローム解析を行っており，その成果である疾患バイオマーカーは薬効の指標となりうると考えている。また別途，バイオマーカー探索・検証用試料の品質要件に関する研究を開始した。

4 おわりに

最近では，予測が困難であった重篤副作用に関しても，次々とバイオマーカーが発見されている。しかし現段階での報告の多くは，市販後に見出されたものである。有用なバイオマーカーが，本邦にて創薬段階で見出され，市販後にも継続して適正使用に用いられることを祈念したい。

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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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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

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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.

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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.

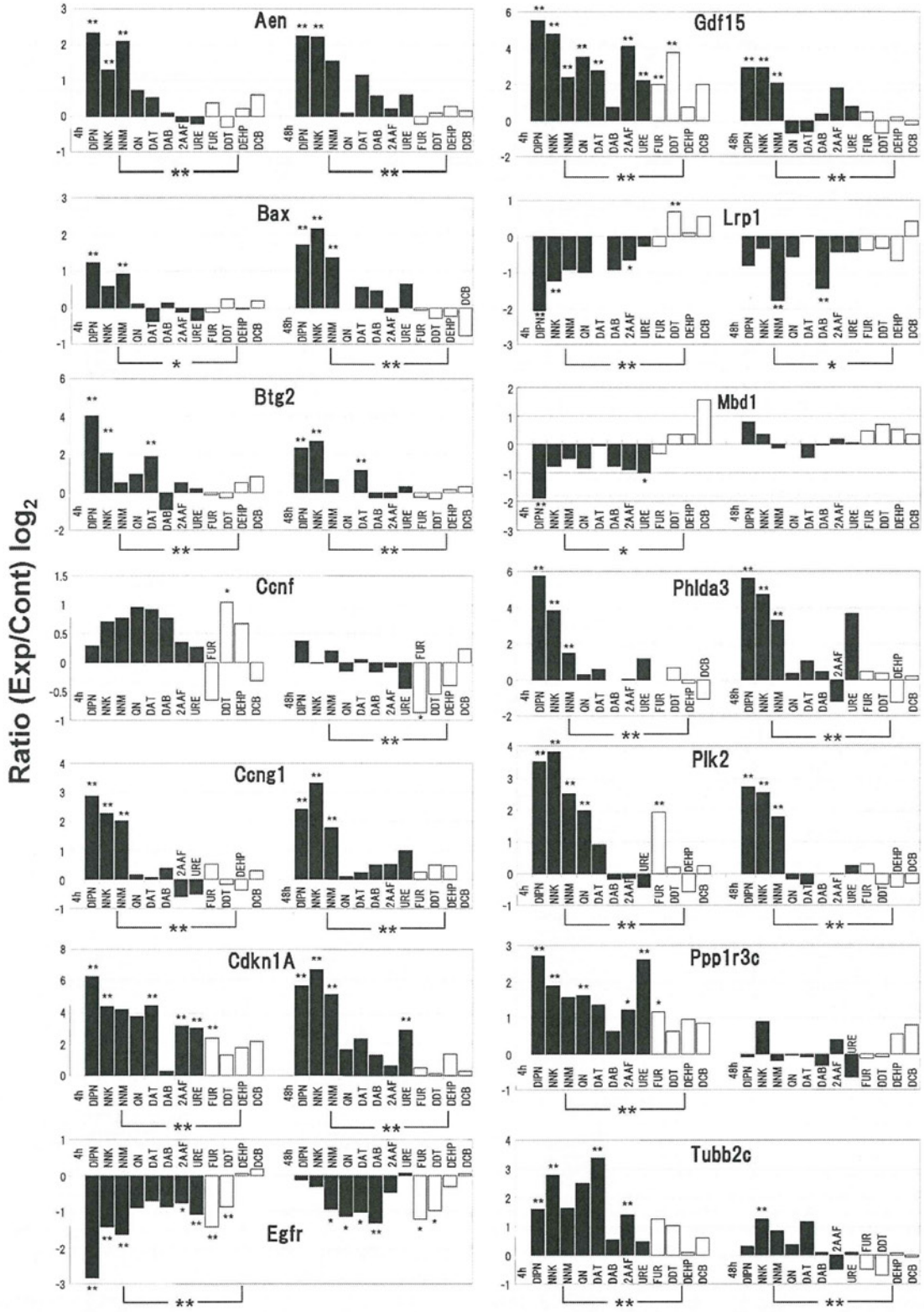

Fig. 1. Changes in the gene expression of 14 major genes (*Aen*, *Bax*, *Btg2*, *Ccnf*, *Ccng1*, *Cdkn1a*, *Egfr*, *Gdf15*, *Lrp1*, *Mbd1*, *Phlda3*, *Plk2*, *Ppp1r3c* and *Tubb2c*) as quantified by qPCR at 4 and 48 h. DIPN: diisopropanolnitrosamine, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, 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-like domain, family A, member 3	NM_013750
26	Plk2	Polo-like kinase 2; serum-inducible kinase	NM_152804
27	Pml	Promyelocytic leukemia	NM_008884
28	Pmm1	Phosphomannomutase 1	NM_013872
29	Ppp1r3c	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	NM_016854
30	Psma3	Proteasome (prosome, macropain) subunit, alpha type 3	NM_011184
31	Rad52	RAD52 homolog (S. cerevisiae)	NM_011236
32	Rcan1	Regulator of calcineurin 1	NM_001081549
33	St3gal5	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	NM_011375
34	Trp53	Tranformation related protein 53	NM_011640
35	Tubb2c	Tubulin, beta 2c	NM_146116

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

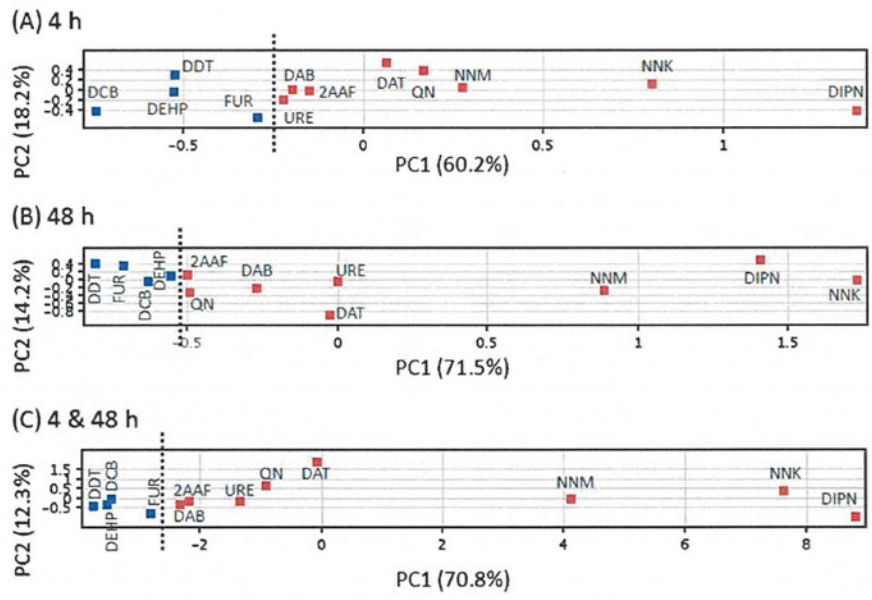

Fig. 2. Principal component analysis (PCA) of the gene expression levels between genotoxic and non-genotoxic hepatocarcinogens as quantified by qPCR. The mean values of triplicate qPCR assays for each chemical were statistically analyzed using PCA programs in GeneSpringGX11.0.1. The results of the PCA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

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

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

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

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

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

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

3.3. Gene ontology and the biologically relevant gene networks

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

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

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

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

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

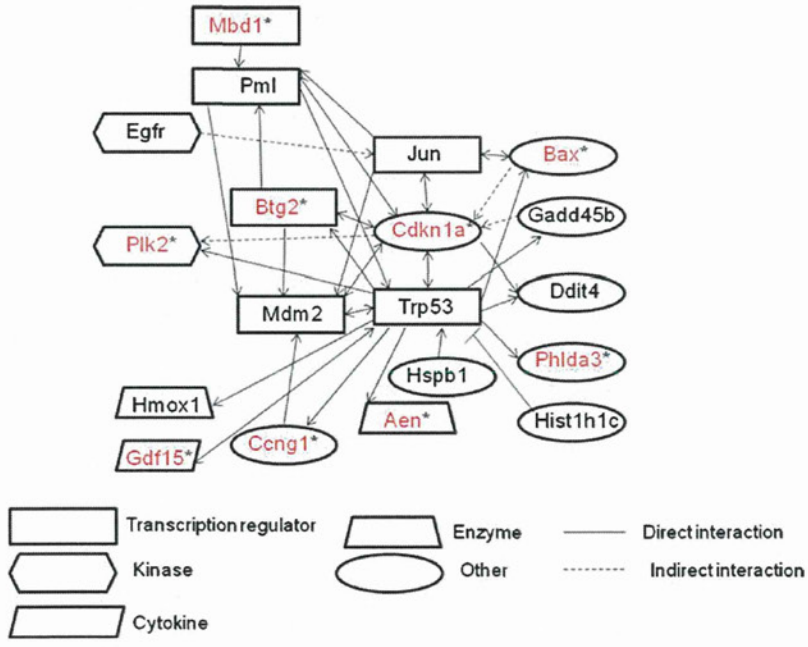

Fig. 3. The gene networks and pathways of 19 genes as determined by qPCR. The network was combined from the results of Ingenuity Pathways Analysis, GeneSpring software and references from PubMed. The 9 red-colored genes indicated by "*" mark genes that significantly contributed to the discrimination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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conflict of interest

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

The authors declare that there are no conflicts of interest.

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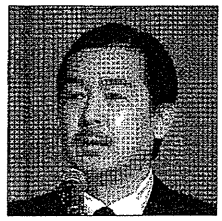
特別講演

早期臨床試験の国際展開の中で日本の進むべき方向性

How should we proceed with clinical trials in Japan given the global tide toward early clinical development?



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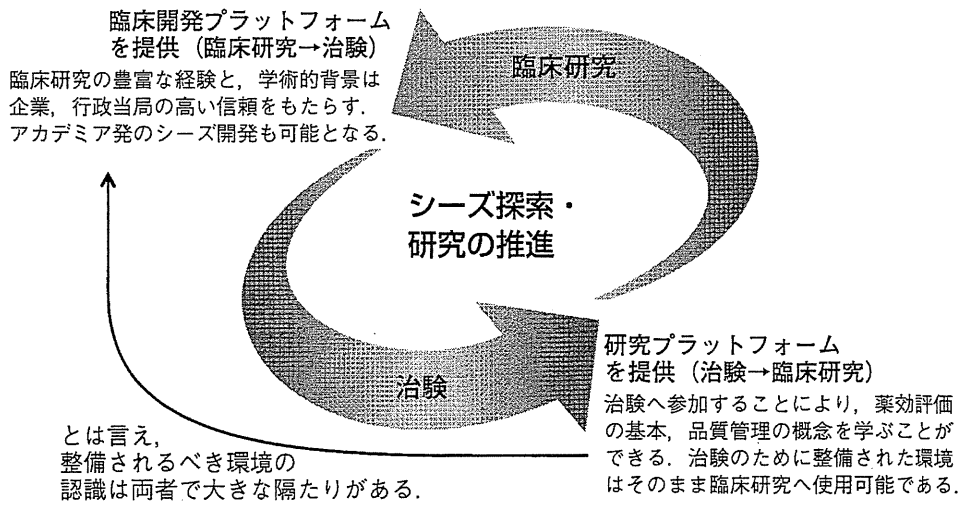
◘略歴◘　臨床薬理学者．大分医科大学を卒業後，同大学大学院において循環器系の臨床薬理学を研究．北里大学へ赴任後は，臨床試験領域を重点的に研究．現在，北里大学医学部附属臨床研究センター（KCRC）教授，北里大学東病院治験管理センターセンター長および北里大学臨床試験事業本部（KitARO）本部長．臨床試験の管理に加え，PK研究，患者のPK/PD研究，first-in-human，microdose研究およびQT研究を含む多くの臨床試験を行なっている．

1. 日本の臨床試験の現状と利点

2011年2月の産経新聞で，当時の厚生労働省の椎葉茂樹氏が韓国ソウルを訪れて，「日本のこの10年間は一体何だったのだろうか」とつぶやいたと報じられました[*1]．臨床試験では日本と韓国，中国がよく比較されます．三大医学誌，*New England Journal of Medicine*，*Lancet*，*JAMA*に発表された日本の論文数は，1993～1997年は12位，1998～2002年は12位，米国，イギリスなどが不動の1位で，2003～2007年には日本が18位に陥落，中国が15位に上がっています．これは国際的な治験に参加していないことにもよります．治験と臨床研究は相補的な関係です（Fig. 1）．治験に携わっていると，薬効評価の基本，特に品質管理の概念を学ぶことができ，そのインフラは臨床研究に使えます．しかし整備された環境は臨床研究に使用可能ですが，臨床研究に費やすお金は十分にありません．また，アカデミア発のシーズ

Fig. 1　臨床試験と治験の相互補完関係



*1　【医薬最前線】第5部　飛躍明日への処方箋（1）「この10年，何を…」気付けば遅れを取る日本．産経新聞．2011 Feb 27.

開発も実際にはかなり難しい．それでも何とかしなければということで早期臨床試験を推進する政策が立ち上がってきています．

日本の治験は，スピードは以前より改善し，品質は無駄といってもよいほどに良く，コストは品質の分だけ高いという状況ですが，国際共同試験への参加はごく普通のことになりました．疾患領域によっては，スピードも海外に勝るものが出てきています．しかし，施設ごとの症例数が足りないことはやはり問題ですし，これは間接的に，モニター等の負担，コスト増加につながっています．

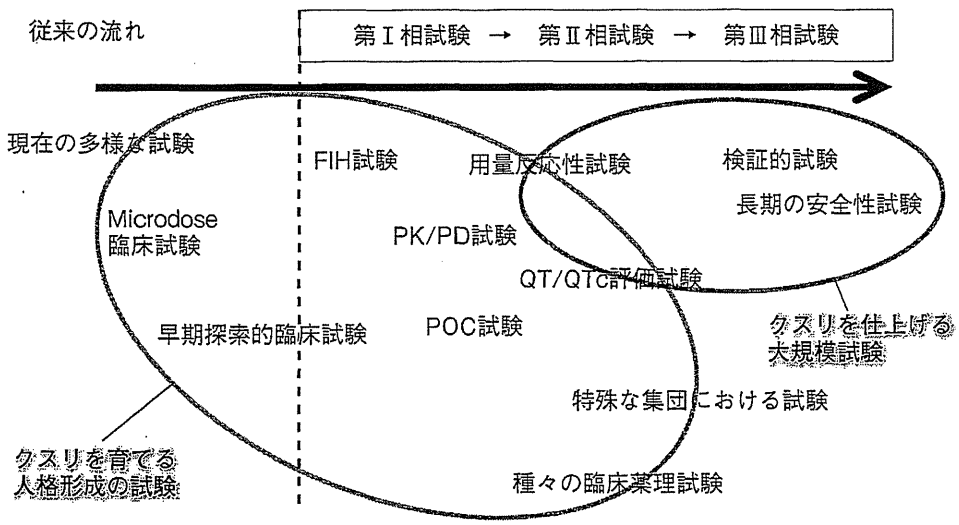
しかし我々は症例数で勝負すべきなのか．言い換えれば，検証的試験，大規模試験で勝負するのか．日本のadvantageを生かすならば，化合物の創成から新薬の申請まで一貫した経験を持つ国は少なく，緻密で真面目という日本人の気質を生かすなら，品質と科学性が基盤となる早期試験の実績を積むことで，海外，外資系企業のheadquarterの信頼を得て，実績のあるPI (principal investigator) の蓄積を経て，大規模検証試験でもイニシアチブを取ることが最終的なゴールではないかと考えられます．

2. 早期臨床試験とは

臨床試験にはⅠ相，Ⅱ相，Ⅲ相という流れがありますが (Fig. 2)，この表現は既に適切でなく，最近はmicrodoseを含めた早期探索的臨床試験，first-in-human (FIH)，PK (pharmacokinetics)/PD (pharmacodynamics)，POC (proof of concept) 試験などがあります．検証的試験，長期の安全性試験，QT/QTc試験などは，大規模試験で薬を仕上げるための人海戦術にあたるものです．早期試験は薬を育てる人格形成の段階にあり，戦略的な意味が強い試験です．開発候補薬物を見極め，go，no goを決める非常に重要な試験です．

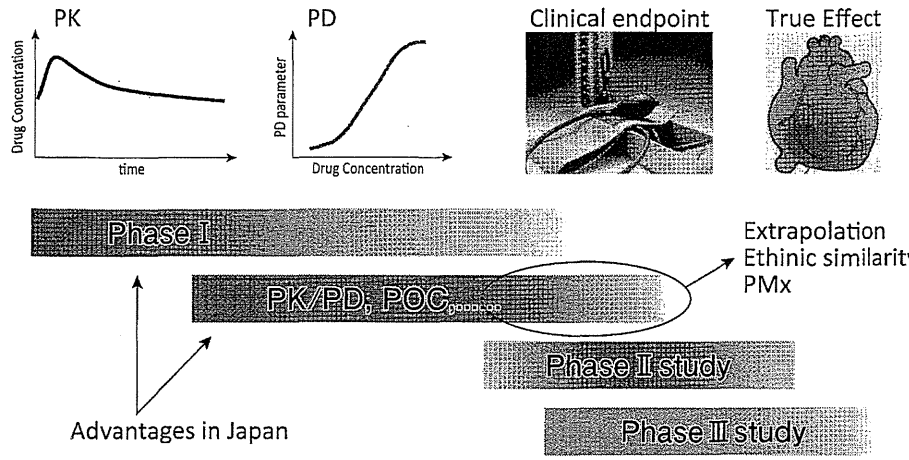
国際共同試験の考え方についてのガイダンス[*2]のQ&Aでは，PK，場合によってはPDの結果によって民族間の差がadjustできないならばglobal dose findingを行うが，もし民族間の相違が説明可能ならそのままグローバルの検証的試験に入ってよいとあります．薬物動態，反応性のデータから，最終的に真の効果を見る．第Ⅰ相試験から第Ⅱ相試験の間のPK/PD，POC試験は，ブリッジ

Fig. 2　臨床試験の種類



[*2] 厚生労働省医薬食品局審査管理課長．国際共同治験に関する基本的考え方について．平成19年9月28日　薬食審査発第0928010号．

Fig. 3　Importance of early stage trials



ング，類似性を見るのに重要です（Fig. 3）．

3. 国際共同試験の戦略

このようなことを踏まえて，世界どの地域でもいいからfirst-in-humanから始めて，POC，Phase Ⅱ，PK/PDなどにより民族差を埋めて，同時開発を目指すのが将来的な戦略です．国際共同試験はfirst-in-humanからというのは少し難しい，POCもできれば1カ国のほうがよい，となるとやはり用量反応，PK/PDあたりからとなります．と言っても，POCを踏まえた国際間のプロトコルのすり合わせ，外挿性の検討を含めた国際開発戦略が重要です．つまりブリッジングを前提としたfirst-in-humanやPOCという国際戦略が重要になってきます．

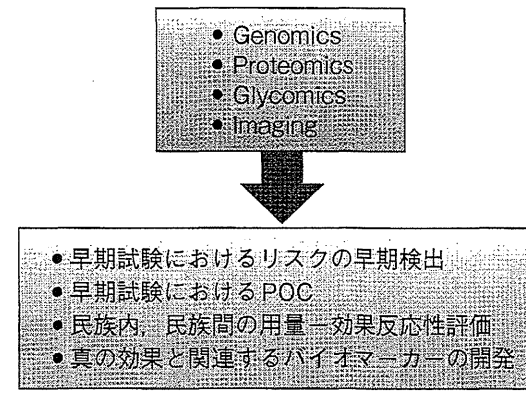
First-in-humanが日本でできるのは当たり前で，問題なのは日本でPOC試験があまり行われていないことです．課題として，バイオマーカーをどう活用するか，症例の集積性，施設の体制，依頼者の体制，さらに得られたデータをどう生かすか，この5つの問題がクリアされなければなりません．

4. バイオマーカー

まずバイオマーカーです（Fig. 4）．効果を見るため，あるいは有害反応を見るためのパラメータはすべてバイオマーカーですが，最近はgenomics，proteomics，glycomics，imagingなどが注目されています．早期臨床では，リスク，有害反応の早期検出に活用されます．臨床検査のAST，ALT，心電図のQT間隔もそうです．あるいはバイオマーカーを用いて，民族内，民族間の用量－効果反応性の評価をする．最終的に，真の効果と関連するバイオマーカーの開発．これが本当に欲しいのですが，非常に難しい．

既にあるバイオマーカーはいいのですが，ない時には開発しなければなりません．そのためにはvalidationをとらなければいけない．治験薬をバイオマーカーと組み合わせて同時に開発するとい

Fig. 4　バイオマーカーの積極的な活用



う手法は，新しいメカニズムの薬剤に関しては当然のことになります．バイオマーカーの開発とvalidationにはアカデミアの積極的な参加が求められます．それは治験でなく臨床研究で，となるかもしれません．

バイオマーカーの測定体制ですが，「海外でないと測れません」とサンプルを海外に送付する試験が多い．日本で技術的にできないわけではなく，海外でvalidationされているからそこで測りたいというだけの話です．これを日本で測定可能な状況にする．あるいは，日本から発信するバイオマーカーの測定の品質保証を行わなければいけない．

アカデミアがすべてではなくても新しい測定に関与するような機関では，GLP，データのvalidationについての認識を十分に持つ必要があります．

また日本の国策として検討すべきことですが，バイオマーカーの特許がとられてしまうために国内での開発が難しいということもあります．やはり情報を共有したい．その第一歩は，まず安全性のバイオマーカーだろうと思います．有効性のバイオマーカーは難しい面もありますが，安全性では既にPSTC (Predictive Safety Testing Consortium) で腎障害のバイオマーカーについて製薬企業十数社が協力してPMDA (医薬品医療機器総合機構) に治験相談を行って，KIM-1 (kidney injury molecule-1)，clusterinのような非常に将来有望なバイオマーカーのデータを検討しました．現在，肝障害のバイオマーカーの検討も進んでいます．こうしたことを日本で進めるべきです．日本でも「QT PRODACT」というプロジェクトで，心電図の安全性のバイオマーカーについて企業で基礎データを持ち寄って論文を書いた実績もあります．日本版コンソーシアムの設立が重要です．それを踏まえて，有効性バイオマーカーの情報共有についても実現させたいところです．

5. 症例の集積性

次に症例の集積性です．企業は必ずしも症例の集積性がいいところばかりを選んではいません．市販後のことを考えて，key opinion leader (KOL) のところに持っていく場合もあります．しかし早期臨床試験に関しては，それは通用しません．1施設当たり20例，30例取れるような施設に頼むべきです．あるいは必ずしも単施設ではなくネットワークであってもよいわけです．「契約したのに1例も出ませんでした」というようなPIでは困るので，医師に対する啓発，市民への啓発も必要です．

6. 治験ネットワーク

2011年3月，治験の効率化に関する報告書の中で，我が国の治験ネットワークの問題点が示されています．「「治験ネットワーク」と称する団体が多く存在し，……」とあります．J-CLIPNET (Japan Clinical Pharmacology Network for Global Trials：グローバル早期臨床試験推進のための大学病院ネットワーク)，ANTCliPh (Academic Network for Trials in Clinical Pharmacology) などもそこに入るのでしょうが，非常に辛口な言い方で，「様々な取組みが行われているが，不明確な部分もある」と書かれました．必要なのは「常に積極的に対応できる複数の医療機関があたかも一つの医療機関のように機能すること」です．そのためには「SOP (standard operating procedures：標準業務手順書) や様式の統一，共同IRB，事務局の積極性」とあります．しかしこれだけでは絶対うまくいきません．POC試験は全く別問題です．ネットワークとしてはこれが重要です．

POCは患者を対象に数施設で完成しなければいけない臨床試験です．これを行うためには，1つのARO (academic research organization) またはネットワークがすべての疾患に対応できるわけがない．ところが，疾患単位ごとにネットワーク