Rapid analysis of gene expression changes caused by liver carcinogens and chemopreventive agents using a newly developed three-dimensional microarray system

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We investigated changes of gene expression in livers of rats treated with carcinogens and tumor promoters using a novel three-dimensional microarray system developed by Olympus Optical Co., Ltd., to assess the feasibility of predicting modifying effects on hepatocarcinogenesis on the basis of changes in the patterns. For this purpose, two genotoxic carcinogens, two non-genotoxic carcinogens (promoters) and seven candidate chemopreventive agents were examined. Six-week-old male F344 rats were treated for 2 weeks with the 11 chemicals (0.05% phenobarbital, 0.3% clofibrate, 0.01% N-diethylnitrosamine (DEN), 0.01% 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 1% catechol, 1% caffeic acid, 0.05% nobiletin, 0.05% garcinol, 0.05% auraptene, 0.05% zermbone and 0.05% 1′-acetoxychavicol acetate (ACA). Test chemicals were mixed in food with the exception of DEN, which was administered in drinking water. RNAs from liver were then analyzed using two kinds of customized microarrays (PamChip® microarray A spotted for 28 genes of drug-metabolizing enzymes in duplicate, and PamChip® microarray B spotted for 131 genes which are known to be up- or down-regulated in hepatocarcinoma cells). Hybridization and subsequent analysis were usually completed within 2 h and the data obtained were highly reproducible. Carcinogens were classified into genotoxic and nongenotoxic substances by clustering analysis. We could also divide test chemicals into carcinogens and chemopreventive agents from their effects on gene expression. In this study, we have thus shown that it is feasible to predict the modifying effects of chemicals on the basis of changes of gene expression patterns after only 2 weeks of exposure, using our novel three-dimensional microarrays. (Cancer Sci 2004; 95: 123–130)

To determine risks of chemicals to humans, rodent bioassays such as carcinogenicity tests are currently essential. However, these assays are very expensive and time-consuming. Therefore, establishment of short-term methods to precisely predict the toxicological effects of chemicals is necessary. Recently, microarray technologies which can simultaneously analyze the expression of large numbers (hundreds to several thousands) of genes have been developed and applied in many scientific areas. Attempts to predict toxicity using microarray techniques have also been initiated as toxicogenomics, defined as the application of knowledge of genes associated with disease states to the study of the toxicology of chemical and physical agents.[1–10] The goals of toxicogenomics are to achieve a better understanding of mechanisms of toxicity and to identify gene expression patterns that are predictive for adverse outcomes more rapidly than with more time-consuming traditional measures.

Oligonucleotide-based or cDNA microarrays, consisting of oligonucleotides or cDNAs spotted on slide glasses or nylon membranes, are currently being utilized to determine changes in gene expression. In contrast to glass or membranes, the PamChip® microarray technology (PamGene B.V., Den Bosch, The Netherlands) employs a solid three-dimensional (3D), multi-porous structure, through which fluids can be repeatedly pumped. With this liquid movement, hybridization efficiency is dramatically increased.[11] Typically, hybridization is complete within 1.5 h, in contrast to overnight incubation for conventional glass or membrane arrays. The 3D-microarray system developed by Olympus Optical Co., Ltd., is an integrated system for solution-driven hybridization, thermal control image capture and image analysis. Real-time signal detection is possible, allowing quantitative detection of the signals. Although a similar method for hybridization on a 3D-biochip was reported,[12, 13] the Olympus 3D-microarray system FD10 is the first available commercially in Japan for gene expression and/or mutation analysis.[14]

In the present study, four liver carcinogens were chosen for gene expression analysis in rat liver. Phenobarbital and clofibrate are nongenotoxic agents which induce drug-metabolizing enzymes in a specific manner.[15–18] N-Diethylnitrosamine (DEN) is a powerful hepatocarcinogen[19] and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) is an example of a carcinogenic heterocyclic amine.[20] In addition to these four liver carcinogens, the effects of the following seven chemopreventive agents were also investigated: caffeic acid; catechol; auraptene isolated from peel of citrus fruit (*Citrus natsudaidai* Hayata)[21, 22]; 1′-acetoxychavicol acetate (ACA), present in the edible plant *Languas galanga* from Thailand[23]; garcinol, isolated from *Garcinia indica* fruit rind[24]; nobiletin isolated from *Citrus unshiu*[25]; and zerumbone isolated from *Zingiber zerumbet*.[26] These compounds have inhibitory effects on rat colon carcinogenesis. Caffeic acid,[27] cathechol[27] and auraptene[28] have inhibitory effects on rat hepatocarcinogenesis. We treated rats with these chemicals for 2 weeks and performed gene expression analysis using a novel 3D-microarray system to assess the feasibility of establishing a bioassay system to predict carcinogenicity and chemopreventive activity of chemicals from effects on gene expression patterns.

Materials and Methods

Animals. Five-week-old male F344 rats were obtained from Charles River, Japan, Inc. (Atsugi). They were randomly divided into groups of three animals per plastic cage with hard

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wood chips as bedding in an air-conditioned room at 22±2°C and 55±5% humidity with a 12 h light/dark cycle. Food (Oriental MF, Oriental Yeast Co., Tokyo) and tap water were available *ad libitum*.

Chemicals. Phenobarbital, clofibrate, caffeic acid and catechol were purchased form Sigma, St. Louis, MO, DEN from Tokyo Kasei Kougyou, Tokyo, and MeIQx from Toronto Research Chemicals, Toronto, ON, Canada. Nobiletin, garcinol and auraptene were kindly provided by Dr. T. Tanaka at the First Department of Pathology, Kanazawa Medical University. Zermbone and ACA were kindly provided by Dr. A. Murakami, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University.

Experiments. Male F344 rats were treated at 6 weeks of age. Test chemicals were mixed in food with the exception of DEN administered in drinking water. After 2 weeks, all rats were killed.

All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University Medical School.

Samples of livers collected at necropsy were routinely fixed in 10% phosphate-buffered formalin for 48 h and then processed for embedding in paraffin. Five-micrometer thick sections were cut and stained with hematoxylin and eosin for histopathological examination. Some portions of livers were frozen in liquid nitrogen for subsequent extraction of total RNA.

Extraction of total RNA, and quantitative RT-PCR. Total RNA extraction was performed according to an ISOGEN protocol (Nippon Gene, Toyama) with DNase treatment using DNase I and the supplied buffer (CLONTECH, Palo Alto, CA). One microgram of the RNA was converted to cDNA with avian myoblastosis virus reverse transcriptase (TaKaRa, Otsu) in a 20-μl reaction mixture. Aliquots of 2 μl of cDNA samples were subjected to quantitative PCR in 20-μl reactions using FastStart DNA Master SYBR Green I and a Light Cycler apparatus (Roche Diagnostics, Mannheim, Germany). Primers used for GST-P were 5′-ATGCCGCCGTACACCA-3′ and 5′-AAAGCCCTAAAGAGCGACCC-3′; for GST-Ya, 5′-AGCCATTCTCAACTACATCG-3′ and 5′-GGCTCTTCAACACCTTTTCA-3′; for GAPDH, 5′-GCATCCTGCACCACCAACTG-3′ and 5′-GCCTGCTTCACCACCTTCTT-3′; for cyclofilin, 5′-TGCTGGACCAAACACAAATG-3′ and 5′-GAAGGGGAATGAGGAAAATA-3′; for CYPs primers for the rat cytochrome P450 Competitive RT-PCR kit (TaKaRa) were used. Initial denaturation at 95°C for 10 min was followed by 35 to 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C (except at 63°C for GST-P, at 64°C for CYP1A1, at 52°C for GST-Ya, and at 60°C for GAPDH) for 5 s, and elongation at 72°C for 30 s. The fluorescence intensity of the double-strand-specific SYBR Green I, reflecting the amount of formed PCR-product, was monitored at the end of each elongation step. GAPDH mRNA levels were employed to normalize the sample cDNA content.

Selection of genes for PamChip® microarrays. For the customized PamChip® microarray A provided by Olympus, 12 cytochrome P450s and 7 glutathione S-transferases, NADPH and NAT2 were chosen. Fatty acid binding protein, α-1-antitrypsin, connexin 32, and PCNA were also included. GAPDH, cyclophilin and β-actin genes were also spotted as internal controls. All spots of the genes were set in duplicate. For selection of genes of the PamChip® microarray B provided by Olympus, cDNA array analysis using Atlas Rat Toxicology 1.2 (CLONTECH) was performed according to the manufacturer's instructions. Gene expression patterns in normal rat liver tissue and MH1C1 hepatoma cells (obtained from ATCC) were compared, and differently expressed genes were selected. A total of 131 genes (Table 1) including phenobarbital- and clofibrate-inducible genes were carried on the PamChip® microarray B. Oligonucleotide DNA probes (60mer) were spotted on both PamChip® microarray A and B.

Hybridization with 3D-microarray system. For sample preparation of fluorochome-labeled cDNA, 30 μg of total RNAs (mixtures of 10 μg from each of 3 rat livers per group) was used, and reverse transcriptase and FITC labeling reactions were performed according to the manufacturer's instructions (RNA Fluorescence Labeling Core Kit; TaKaRa and fluorescein-12-dUTP; NEN, Wellesley, MA). Samples were denatured at 95°C for 5 min and 50 μl of hybridization solution containing 6× SSPE was applied on each test site of the PamChip® microarray. Hybridization, washing and detection of signals were performed with the 3D-microarray system (FD10; Olympus) as follows.

Hybridization was performed at 50°C with recirculation of 150 cycles at a rate of 5 μl/s, and then each test-site was washed with 50 μl of 6× SSPE 3 times. After the washing, fluorescence images were captured automatically by a cooled CCD.

Data analysis. Signals after each treatment were converted to expression ratios relative to the control using the gene expression analysis software integrated in FD10. Normalization was conducted for each gene against GAPDH. Hierarchical cluster analyses were performed with GeneSpring (Silicon Genetics, Redwood City, CA). To illustrate the data as trees, they were subjected to hierarchical clustering, with measurement of similarity in terms of the standard correlation for the gene tree and the Spearman correlation for the experimental tree.

We also applied another statistical approach for prediction analysis, named Class Predictor (contained in GeneSpring). The Class Predictor is designed to predict the value for an individual parameter in an uncharacterized sample or set of samples. It does this in two steps. First, the Class Predictor algorithm examines all genes in the training set individually and ranks them on their power to discriminate each class from all the others. Next it uses the most predictive genes to classify the "test set." The Class Predictor can also be used simply to find genes whose behavior is related to a given parameter by examining the list of predictor genes. The genes that are most accurately segregated by these markers are considered to be the most predictive. A list of the most predictive genes is made for each class and an equal number of genes are taken from each list. To make a prediction, the Class Predictor uses the k-nearest-neighbor method. The class with the lowest *P* value is assigned to the unclassified sample.

Results

Changes of gene expression induced by liver carcinogens in rats. Final body weights did not differ between groups. Relative liver weights were increased by phenobarbital and clofibrate (Table 2). With phenobarbital treatment, CYP2B1, CYP3A1, CYP3A2 and GST-Ya were up-regulated over 2-fold (see Fig.1). After clofibrate administration, expression levels of CYP2B1, CYP4A1 and fatty acid binding protein were increased, and that of α-1-antitrypsin was decreased. CYP1A2 and CYP2C11 were increased by MeIQx. GST-M3 and cyclophilin were up-regulated, whereas 2C11 was down-regulated by DEN (Table 3). Quantitative real-time RT-PCR analysis confirmed these results (data not shown). It is noteworthy that a 45-fold increase of CYP2B1 by phenobarbital treatment was detected by the microarray system as well as by quantitative RT-PCR.

Treatment with chemopreventive agents. Throughout the experimental period, body weights did not differ among groups except for a decrease in the case of catechol (Table 2).

Microarray and cluster analysis. We analyzed 77 obtained genes for clustering and predicting. We allotted the unanalyzable

Table 1. Gene list (PamChip® microarray B)

No.	ACC [1)]	Gene symbol (Abbreviation)	Gene name
1	NM_012488	A2m	alpha-2 macroglobulin
2	U28152	A2UG	α2u-globulin
3	NM_031760	Abcb11	P-glycoprotein sister protein (SPGP)
4	NM_012891	Acadvl	very long chain acyl-CoA dehydrogenase (VLCAD)
5	NM_031144	**ACTB** [2)]	actin beta (ACTB)
6	NM_134432	Agt	angiotensinogen (PAT) gene
7	NM_012898	Ahsg	fetuin
8	NM_031010	Alox12	12-lipoxygenase
9	NM_012738	Apoa1	apolipoprotein AI (APOA1)
10	NM_013112	Apoa2	apolipoprotein AII (APOA2)
11	NM_012737	Apoa4	apolipoprotein AIV (APOA4; APOC4)
12	NM_012501	Apoc3	apolipoprotein CIII (APOC3)
13	NM_138828	Apoe	apolipoprotein E (APOE)
14	NM_017134	Arg1	liver arginase 1 (ARG1)
15	M36708	Ass	argininosuccinate synthetase
16	NM_030850	Bhmt	betaine homocysteine methyltransferase (BHMT)
17	NM_022399	Calr	calreticulin (CALR); calregulin; calcium-binding protein 3 (CABP3); HACBP; ERP60
18	NM_012727	Camk4	Ca-CAM dependent PK
19	NM_012520	Cat	liver catalase (CAT; CAS1)
20	NM_053021	Clu	clusterin (CLU); testosterone-repressed prostate message 2 (TRPM2); apolipoprotein J (APOJ); sulfated glycoprotein 2 (SGP2); dimeric acid glycoprotein (DAG)
21	NM_017202	Cox4a	cytochrome c oxidase, subunit IV
22	NM_053586	Cox5b	cytochrome c oxidase subunit Vb
23	NM_019360	Cox6c	cytochrome c oxidase polypeptide VIc2 (COX6C2)
24	NM_017096	Crp	C-reactive protein
25	U22893	Csda	DNA-binding protein A (DBPA); cold shock domain protein A (CSDA); Y-box-binding protein A (RYBA); muscle Y-box protein 2 (YB2)
26	NM_031315	Cte1	acyl-CoA thioesterase 1, cytosolic
27	NM_013156	Ctsl	cathepsin L
28	NM_017101	**CYCLP**	cyclophilin (CYCLP)
29	NM_012839	Cycs	cytochrome c expressed in somatic tissues (CYCSA)
30	M11251	Cyp2b1	cytochrome P450 IIB1 (CYP2B1)
31	NM_019184	Cyp2c11	cytochrome P450 IIC11 (CYP2C11); P450(M-1); P450H; P450-UT-A; UT2
32	NM_031839	Cyp2c23	cytochrome P-450 2C23, arachidonic acid epoxygenase
33	K03501	Cyp2c9	cytochrome P450 IIC9 (CYP2C9); CYP2C10; CYP2C17; CYP2C18; CYP2C19; S-mephenytoin 4-hydroxylase
34	M10161	Cyp3a1	cytochrome P450 IIIA1 (CYP3A1); P450-PCN1
35	NM_012942	Cyp7a1	cholesterol 7-α-hydroxylase
36	NM_022513	D/T-ST	dopa/tyrosine sulfotransferase
37	NM_031853	Dbi	11-kDa diazepam binding inhibitor (DBI)
38	NM_138877	Dia1	NADH-cytochrome b5 reductase; DIA1
39	NM_053354	Dnmt1	DNA Mtase
40	NM_017245	Eef2	elongation factor 2 (EEF2; EF2)
41	NM_053849	Erp70	endoplasmic reticulum stress protein 72 (ERP72); calcium-binding protein 2 (CABP2)
42	NM_012556	Fabp1	liver fatty acid-binding protein (L-FABP); Z-protein; squalene- & sterol-carrier protein (SCP)
43	NM_145878	Fabp5	fatty acid-binding protein 5 (FABP5); epidermal fatty acid-binding protein (E-FABP); cutaneous FABP (C-FABP)
44	NM_030832	Fabp7	brain fatty acid-binding protein (B-FABP); FABP7; brain lipid-binding protein (BLBP)
45	X05834	FBN	fibronectin
46	NM_012559	Fgg	fibrinogen gamma chain
47	AF014828	Figf	VEGF-D
48	NM_012792	Fmo1	flavin-containing monooxygenase 1
49	NM_024127	Gadd45a	GADD45
50	NM_017008	**GAPDH (IC)** [3)]	glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
51	NM_017251	Gjb1	connexin 32 (CXN32; CX32)
52	NM_019630	Gludins	glucose-dependent insulinotropic peptide
53	NM_012571	Got1	glutamate oxaloacetic transaminase 1 (GOT1); transaminase A; cytoplasmic aspartate aminotransferase
54	NM_013177	Got2	mitochondrial aspartate aminotransferase
55	NM_030826	Gpx1	cellular glutathione peroxidase I (GSHPX1; GPX1)
56	NM_017013	Gsta2	glutathione S-transferase Ya subunit (GST Ya); ligandin subunit 1 alpha
57	NM_177426	Gstm2	glutathione S-transferase Yb2 subunit (GST Yb2); GST mu (GSTM2)
58	NM_138974	Gstp2	glutathione S-transferase P subunit; GST subunit 7 pi (GST7-7)
59	NM_053448	Hdac3	histone deacetylase 3
60	NM_022179	Hk3	ribosomal protein L13A
61	M33648	HMG-CoA	mitochondrial hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase); 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2)

Table 1. (Continued)

No.	ACC [1)]	Gene symbol (Abbreviation)	Gene name
62	NM_013134	Hmgcr	HMG-Co A reductase
63	NM_017080	Hsd11b1	corticosteroid 11-beta-dehydrogenase isozyme 1 (11-DH); 11-beta-hydroxysteroid dehydrogenase 1 (11-beta-HSD1)
64	NM_024392	Hsd17b4	peroxisomal multifunctional enzyme type II
65	S45392	HSP90-beta	heat shock 90-kDa protein beta (HSP90-beta); HSP84; HSPCB
66	NM_013060	Id2	inhibitor of DNA binding 2 (ID2)
67	NM_013144	Igfbp1	insulin-like growth factor-binding protein 1 (IGF-binding protein 1; IGFBP1)
68	S58528	INTA	integrin alpha v subunit
69	NM_031768	Itgae	integrin alpha E1, epithelial-associated
70	NM_019369	Itih4	inter-alpha inhibitor H4 heavy chain (ITIH4)
71	NM_017138	Lamr1	laminin receptor 1
72	NM_022196	Lif	leukemia inhibitory/cholinergic neuronal differentiation factor (LIF/DIF)
73	NM_013136	Mak	Mak; male germ cell-associated kinase; highly expressed at and after meiosis
74	NM_031643	Map2k1	MEK1
75	NM_133283	Map2k2	MAPKK2
76	NM_017246	Map2k5	MAPKK5
77	NM_053887	Map3k1	MEKK1
78	NM_053842	Mapk1	ERK2
79	NM_017347	Mapk3	ERK1
80	NM_031622	Mapk6	MAPK3
81	NM_021846	Mcl1	myeloid cell differentiation protein 1
82	NM_022673	Mecp2	MeCP2
83	X16956	MG	microglobulin; beta-2-microglobulin; prostaglandin receptor F2a
84	NM_012861	Mgmt	O-6-alkylguanine-DNA alkyltransferase.
85	NM_134349	Mgst1	microsomal glutathione S-transferase 1 (MGST1); GST12
86	NM_031051	Mif	macrophage migration inhibitory factor (MIF); glutathione-binding 13-kDa protein
87	L26267	Nfkb1	NF-κB (P105)
88	NM_138548	Nme1	nucleoside diphosphate kinase A (NDP kinase A; NDKA); tumor metastatic process-associated protein; metastasis inhibition factor NM23; NME1
89	NM_012611	Nos2a	iNOS
90	NM_012615	Odc1	ornithine decarboxylase
91	NM_030870	Ogg1	OGG1
92	NM_053288	Orm1	alpha-1 acid glycoprotein
93	NM_012998	P4hb	protein disulfide isomerase (PDI)
94	K03243	PC	phosphoenolpyruvate carboxykinase
95	NM_022381	Pcna	PCNA
96	NM_021766	Pgrmc1	25-Dx (25Dx)
97	NM_031598	Pla2g2a	phospholipase A2
98	NM_080688	Plcd4	phospholipase C-d
99	NM_138895	**POLYU**	polyubiquitin
100	U94856	Pon1	serum paraoxonase/arylesterase 1 (PON1); serum aryldiakylphosphatase 1; aromatic esterase 1 (A-esterase 1)
101	NM_017232	Ptgs2	cyclooxygenase-2 (COX2)
102	NM_021740	Ptma	prothymosin-a (PTMA)
103	NM_031579	Ptp4a1	nuclear tyrosine phosphatase PRL-1
104	AJ006070	Rag1	V (D) J recombination activating protein 1 (RAG1)
105	NM_133525	Rcl	RCL; c-Myc-responsive gene
106	NM_022515	Rpl24	60S ribosomal protein L24 (RPL24)
107	X62166	Rpl3	60S ribosomal protein L3 (RPL3)
108	X51707	**Rps19**	40S ribosomal protein S19 (RPS19)
109	NM_012876	**Rps29**	ribosomal protein S29 (RPS29)
110	NM_138508	Scp2	sterol carrier protein 2 (SCP2); SCPX; nonspecific lipid transfer protein (NSL-TP)
111	NM_013082	Sdc2	ryudocan/syndecan 2
112	NM_022519	Serpina1	alpha-1-antiproteinase; alpha-1-proteinase inhibitor; alpha-1 antitrypsin
113	NM_017047	Slc10a1	solute carrier family 10 member 1 (SLC10A1); sodium/bile acid cotransporter; sodium/taurocholate cotransporting polypeptide (NTCP)
114	NM_019269	Slc22a5	solute carrier family 22 (organic cation transporter), member 5
115	NM_031983	Smarcd2	SWI/SNF
116	NM_017050	Sod1	superoxide dismutase 1, soluble
117	NM_012656	Sparc	secreted acidic cysteine-rich glycoprotein (osteonectin)
118	X69834	SPI2.4	serine protease inhibitor 2.4 (SPI2.4)
119	NM_031531	Spin2c	serine protease inhibitor
120	NM_012881	Spp1	sialoprotein (osteopontin)
121	NM_017055	Tf	serotransferrin (TF); siderophilin; beta-1-metal-binding globulin
122	NM_012671	Tgfa	TGF-a

Table 1. (Continued)

No.	ACC [1)]	Gene symbol (Abbreviation)	Gene name
123	NM_013174	Tgfb3	TGF-b3
124	NM_021261	Tmsb10	thymosin beta 10 (TMSB10; THYB10); PTMB10
125	NM_012675	Tnf	TNF-a
126	NM_139194	Tnfrsf6	Fas LR
127	NM_053331	Txn2	thioredoxin
128	S70360	Ugt	UDP-glucurnosyltransferase
129	NM_031980	Ugt2b12	UDP-glucuronosyltransferase 2B
130	M13506	Ugt-p	UDP-glucurnosyltransferase phenobarbital-inducible type
131	NM_053768	Uox	uricase; urate oxidase (UOX)
Negative control		LAMD	bacteriophage λ DNA
		pRL-TK	PicaGene SeaPansy TK Control Vector DNA

1) GenBank accession number.
2) House keeping genes indicated by bold letters.
3) IC: internal control used for normalization.

Table 2. Body and liver weights

Chemical	Body weight (g)	Liver weight	
		Absolute (g)	Relative (%)
Phenobarbital	201.1±6.18 [1, 4)]	11.0±0.32 [3)]	5.45±0.02 [3)]
Clofibrate	191.3±4.47	14.0±0.23 [2)]	7.31±0.11 [2)]
MeIQx	194.6±2.95	8.51±0.10	4.37±0.12
DEN	166.5±9.75	6.39±0.45	3.84±0.14
Caffeic acid	188.6±6.57	7.92±0.48	4.21±0.31
Catechol	140.9±10.8 [2)]	7.49±1.43	5.30±0.78 [3)]
Nobiletin	199.7±6.13	7.66±0.82	3.84±0.45
Garcinol	188.8±6.70	7.87±0.22	4.17±0.25
Auraptene	196.8±5.03	8.44±0.25	4.29±0.06
Zerunbone	199.5±12.4	8.93±0.54	4.48±0.06
ACA	189.2±18.4	7.59±0.72	4.01±0.01
Control	197.6±9.87	8.16±0.35	4.13±0.13

1) Values are mean±SD.
2) $P<0.0001$.
3) $P<0.01$.
4) Statistically significant when compared to control (Scheffe's test).

spots to "no data" and defined "no expression" spots as those with a low intensity of 50 or less. The minimum intensity was 50 for visible spots. We concluded that a cut-off at 50 is appropriate for this purpose, because the mean+2SD of intensity was 14.7 for λDNA applied as a negative control on each chip. We analyzed all data except those categorized as "no data" through all samples, because they would affect the tree clustering.

Hierarchical clustering analysis of results with PamChip® microarray B revealed that carcinogens could be classified as genotoxic and nongenotoxic (Fig. 2). We could also divide the carcinogens from the chemopreventive agents in terms of their impact on gene expression (Fig. 2). An automatically derived Class Predictor was able to determine the class of these chemicals correctly, except for ACA (Table 4). In this analysis, the numbers of neighbors were 6, predictor genes were 10 (Table 5) and the cut-off value for the *P*-value ratio was 0.2.

Discussion

The present investigation of changes in gene expression in the livers of rats treated with genotoxic and nongenotoxic carcinogens (promoters), as well as chemopreventive agents, using novel 3D-microarrays provided clear evidence that modifying effects on carcinogenesis can be predicted from gene expression patterns.

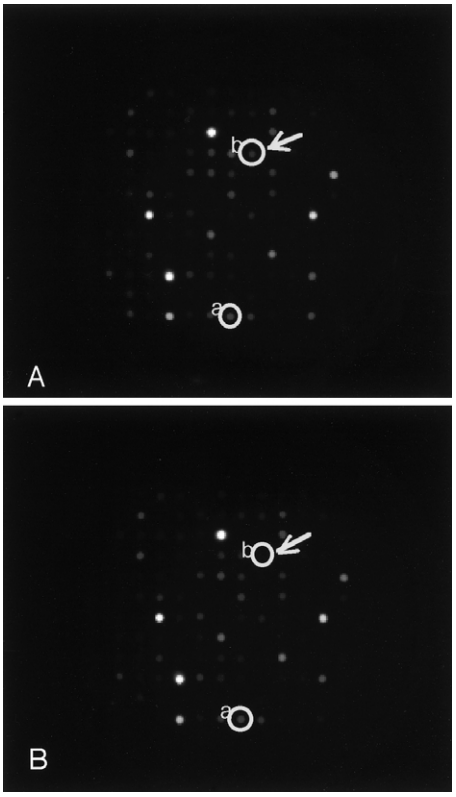

Fig. 1. Representative images of the 3D-microarray system. With phenobarbital treatment (A), an increased signal intensity for CYP 2B1 (spot b, arrow) is apparent compared to the no treatment control (B). Spot a is for GAPDH, an internal control. These chips were PamChip® microarray B.

It has been shown that gene expression of CYPs or GSTs in the liver is changed by treatment with carcinogens. Elevated expression of CYP2B1 and CYP3As, well known to be induced by phenobarbital treatment,[5, 16)] was detected in this new microarray system. By both microarray and RT-PCR methods, a 45-fold increase in expression of CYP2B1 was apparent. These results indicate that this microarray system is appropriate for quantitative analysis and the data are reproducible. Cyclophilin was up-regulated by DEN treatment, although it was categorized as an internal control. However, change of expression lev-

Table 3. Changes of gene expression by liver carcinogens (promoters)

Treatment	Gene name	Up/Down[4)]
Phenobarbital	CYP2B1	UP
	CYP3A1	UP
	CYP3A2	UP
	GST Ya	UP
Clofibrate	CYP2B1	UP
	CYP4A1	UP
	FABP[1)]	UP
	a1AT[2)]	Down
MeIQx	CYP1A2	UP
	CYP2C11	UP
DEN	CYCLP[3)]	UP
	GST M3	UP
	CYP2C11	Down

1) FABP: fatty liver acid binding protein.
2) a1AT: alpha-1-antitrypsin.
3) CYCLP: cyclophilin.
4) Up or Down indicates change over 2-fold compared with the control.

Table 4. The results of cross validation by the class predictor

Treatment	True value[1)]	Prediction	*P*-value ratio[2)]
Caffeic acid	noncarcinogen	noncarcinogen	0.005
Catechol	noncarcinogen	noncarcinogen	0.005
Galcinol	noncarcinogen	noncarcinogen	0.005
Nobiretin	noncarcinogen	noncarcinogen	0.005
Zermbone	noncarcinogen	noncarcinogen	0.120
ACA	noncarcinogen	NP[3)]	0.487
Auraptene	noncarcinogen	noncarcinogen	0.120
DEN	carcinogen	carcinogen	0.167
MeIQx	carcinogen	carcinogen	0.167
Clofibrate	carcinogen	carcinogen	0.167
Phenobarbital	carcinogen	carcinogen	0.167

1) The name of the "class".
2) *P*-value cutoff is 0.2.
3) Not predicted.

Table 5. The list of predictor genes in the class predictor

No.	Gene name
1	cytochrome c oxidase subunit Vb
2	serine protease inhibitor 2.4 (SPI2.4)
3	prothymosin-alpha (PTMA)
4	secreted acidic cysteine-rich glycoprotein (osteonectin)
5	cellular glutathione peroxidase I (GSHPX1; GPX1)
6	cathepsin L
7	connexin 32 (CXN32; CX32)
8	serotransferrin (TF); siderophilin; beta-1-metal-binding globulin
9	C-reactive protein
10	Mak; male germ cell-associated kinase; highly expressed at and after meiosis

Predictive strength=5.799092686.

els of some genes categorized as internal controls by chemical treatments is not unusual. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, cyclophilin and 28S rRNA are commonly used as internal controls to normalize gene expression data, but it has already been reported that their expression levels are not constant and depend on the experimental conditions.[29–31)] Of course it is important to select which gene is best for control purposes under each condition. We con-

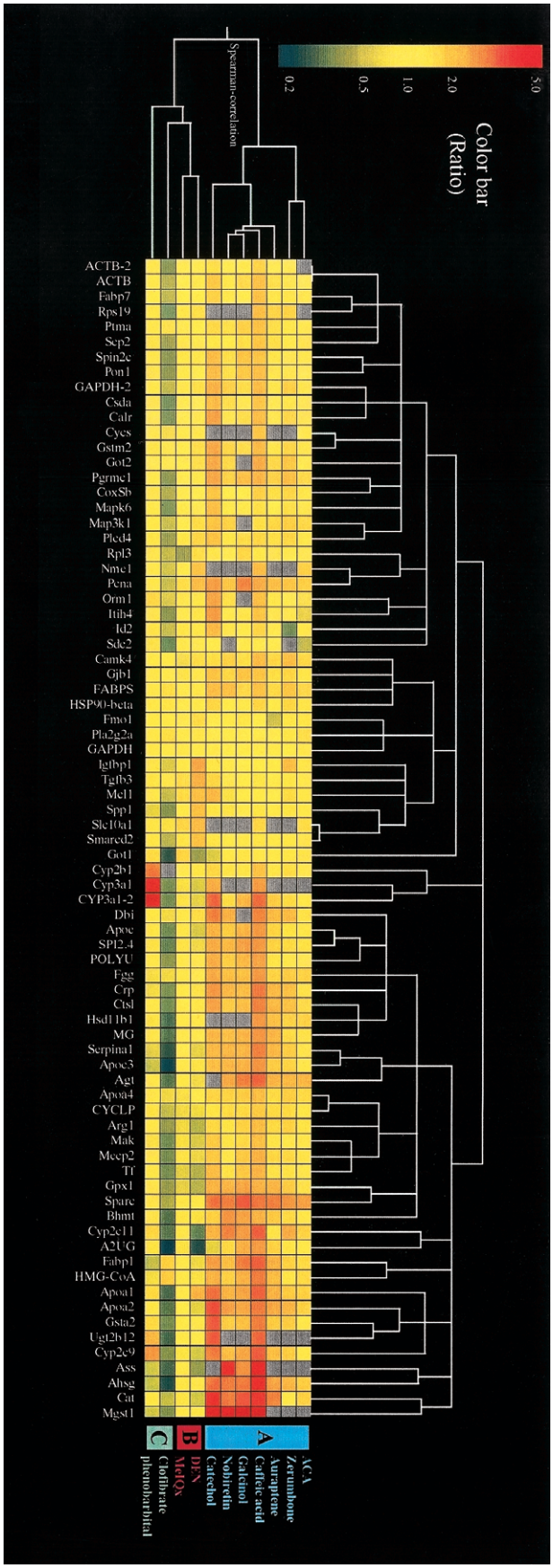

Fig. 2. Clusters displayed as trees. Classifications are non-carcinogen (A) or carcinogen (B, mutagens; C, non-mutagens).

sidered GAPDH to be suitable for this study, because expression levels were almost equal and our data were reasonable.

In this study, chemopreventive agents extracted from fruits and vegetables were selected; most them are known to be antioxidant agents which inhibit development of preneoplastic lesions in the liver or colon.[32–35] Nobiletin[25] and auraptene[22] are constituents of citrus oil. Zermbone[36] and ACA[23] were extracted from plants of the ginger family. Garcinol[24, 35] was obtained from dried peel of *G. indica* fruit rind. These five chemicals suppress aberrant crypt foci or tumor formation in colon carcinogenesis. Chemopreventive effects of caffeic acid and catechol have been previously shown, in terms of inhibition of GST-P preneoplastic foci in rat liver,[27] but these compounds are carcinogens in the stomach.[32]

In the present study there were characteristic weight changes with each of the chemicals, but these were not associated with clear histopathological differences in liver sections. In addition, body weight fluctuation did not appear to be related to differences in carcinogenicity.

In this study, we did not apply new statistical prediction methods. We demonstrated that the gene set used is effective for prediction by two methods. Class Predictor was able to determine the class of these chemicals correctly except for ACA, which it did not assign immediately to the carcinogen class. The results of this prediction may depend on the sample number, and for the Class Predictor, usually over 20 samples are recommended and additional analysis may be necessary to increase reliability, possibly with new oligo probes. However, our present results do suggest that it may be feasible to predict carcinogenicity by investigating changes of gene expression patterns by using novel 3D-microarrays in experiments of only 2 weeks' duration, a great reduction even compared with medium-term bioassays. To detect chemopreventive agents which inhibit preneoplastic foci development, the medium-term liver bioassay is also effective[27, 37] However, agents which inhibit progression of adenomas to carcinomas may not be detectable in the assay. Auraptene is an example which did not show any effect in the medium-term bioassay (unpublished data), but inhibited progression of hepatocellular adenomas to hepatocelllular carcinomas (personal communication from Dr. Akira Hara, Gifu University School of Medicine). In this study, auraptene was classified into the chemopreventive agent category, and this points to an advantage of the present method over the medium-term bioassay.

To conclude, in this study we demonstrated the feasibility of predicting modifying effects of chemical agents by mRNA gene expression profiling using a novel 3D-microarray system. This newly developed approach allows a rapid analysis of gene expression with high reproducibility. We therefore envisage establishing a high-quality, short-term bioassay system in order to identify carcinogens and modifiers.

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