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Distinct patterns of gene expression in hepatocellular carcinomas and adjacent non-cancerous, cirrhotic liver tissues in rats fed a choline-deficient, L-amino acid-defined diet

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Gene expression profiles of HCC and surrounding non-cancerous tissues in rats fed a CDAA diet for 70 weeks, as well as normal liver tissues, were explored using an oligonucleotide microarray for 3757 genes. A total of 146 genes were identified as differentially expressed; the affected functions including metabolism, apoptosis, cell cycling, RNA splicing, Wnt signaling, reactive oxygen species-induced stress, and fibro/cirrhogenesis. The genes were found to fit into four distinct expression patterns after classification by hierarchical and *k*-means clustering procedures. Notably, genes within the same functional category tended to be found within the same cluster, thus gene functions appeared to be related to their expression patterns. For example, genes encoding receptors (Fisher's exact test, $P < 0.01$) and cytokines (Fisher's exact test, $P < 0.05$) were both enriched in a cluster characterized by low expression in HCC compared to their surrounding tissues. While some of the receptors in this cluster had cell-proliferative potential, others are known to be growth-suppressive. It was noted, however, that four of the 10 receptor genes encode G-protein-coupled receptors, for which growth-suppressive potential has been reported. The seven growth factors in the same cluster included two fibroblast growth factors. The current findings suggest the possibility that genes differentially expressed in this multistep carcinogenic model may be classified into relatively few clusters according to their expression patterns, and that these clusters may be associated with gene functional categories. (*Cancer Sci* 2005; 96: 414–424)

Hepatocellular carcinomas are common in Asia and Africa, and the incidence is increasing in Europe and North America. The prognosis for HCC is extremely poor. In human HCC, both genetic and epigenetic alterations have been detected with regard to particular genes such as *p53*, cyclin D, $p16^{Ink4}$, $p21^{Waf1/Cip1}$, *Rb*, β-catenin, mannose-6-phosphate/insulin-like growth factor II receptor (*M6P/IGF2R*), E-cadherin, cyclo-oxygenase (*COX*)-*2*, and telomerase reverse transcriptase (*hTERT*).[1–3] In addition to studies on individual genes, microarray technology has allowed the exploration of comprehensive changes in expression during HCC development. To gain insight into the overall picture, suitable animal models are necessary so that samples reflecting various stages of carcinogenesis can be collected. It should be noted, however, that in animal models featuring the use of chemical carcinogens, unavoidable carcinogen-specific molecular alterations may mask generic and essential changes. In addition, the major risk factor in human HCC has been established as being continuous chronic liver injury, which occurs in hepatitis virus infections.[1–3] Thus we have chosen to use an animal model that employs the administration of a CDAA diet, which induces HCC on a background of continuous hepatic injury and cirrhosis. It has been confirmed that this model resembles human carcinogenesis caused by chronic viral hepatitis, hemochromatosis, and Wilson's disease in many respects.[1–4]

The CDAA diet is hepatocarcinogenic in male rats of Fischer 344 and Wistar strains.[4] As with other diets deficient in choline and low in methionine (CMD diets), HCC are induced in the absence of chemical carcinogens. In the CDAA model, HCC occur at a high rate through the induction and growth of preneoplastic hepatocellular lesions, which are followed by progression to hepatocellular adenomas and subsequent conversion to malignancy.[4] Chronic feeding of CMD diets in rats has also been accepted as an animal model for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis (NAFLD/NASH), a condition in which a substantial number of patients develop HCC.[5] Here we have performed microarray analyses to obtain gene-expression profiles for HCC and surrounding non-cancerous tissues in CDAA treated rats, as well as normal liver samples, so as to gain insight into the mechanisms of multistep hepatic carcinogenesis.

Materials and Methods

Ethical considerations. The experimental protocols were approved by the Animal Experimentation Committee of the Sasaki Institute prior to their execution. The experiment was conducted under monitoring by the committee in accordance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105, and Japanese Government Notification on Feeding and Safekeeping of Animals Number 6.

Animals, diets and animal treatment. A total of 10 male 5-week-old Fischer 344 rats were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). They were divided into two groups of five animals, housed in plastic cages with white-flake

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Abbreviations: CDAA, choline-deficient, L-amino acid-defined; HCC, hepatocellular carcinoma; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RT-PCR, reverse transcription polymerase chain reaction.

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bedding in an airconditioned room (25 ± 3°C temperature, 55 ± 8% relative humidity, 10–12 times/h ventilation and 12-h dark/light cycle). They were used after a 1-week acclimation on basal diet (CRF-1, Oriental Yeast Corporation, Itabashi, Tokyo, Japan) and allowed free access to food and tap water throughout the acclimation and experimental periods. Bodyweight, food consumption, and water intake were monitored weekly. The CDAA diet was obtained from Dyets (Bethlehem, PA, USA).

After acclimation, group 1 rats were administered the basal diet, while group 2 rats were placed on the CDAA diet for 70 weeks and then killed. The livers were then macroscopically examined. Portions of group 1 livers, and the macroscopic tumors and surrounding non-cancerous tissues of group 2 livers were fixed in 10% neutrally buffered-formalin for 24 h, embedded in paraffin, processed for the routine HE staining procedure, and histologically examined. Remaining portions of these three types of liver tissues were immediately frozen in liquid nitrogen and stored at −80°C. Group 1 liver tissues, group 2 liver tissues from non-cancerous areas, and group 2 liver tissues from tumors (after being histologically diagnosed as HCC) were employed as normal liver (CON), surrounding non-cancerous liver (NC), and HCC samples (CA). The microarray and RT-PCR experiments and the subsequent data analysis were performed using five CON samples from individual group 1 rats and five matched pairs of NC and CA samples from individual group 2 animals.

RNA isolation and probe labeling. Total RNA from liver tissues was isolated using an RNeasy Midi kit (QIAGEN, Hilden, Germany) and its integrity checked by electrophoresis on 1% agarose-formaldehyde gels. Five micrograms of total RNA from individual samples were then labeled with Cy3 (Amersham Biosciences, Uppsara, Sweden) using a BD Atlas PowerScript Fluorescent Labeling kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's protocols.

Hybridization, scanning and quantification. Cy3-labeled probes were hybridized to Atlas Rat 3.8 I microarrays (BD Biosciences Clontech) containing 3757 genes for 16 h at 50°C. After hybridization, microarrays were washed, dried and scanned using a GMS 418 confocal laser scanner (Genetic MicroSystems, Woburn, MA, USA). Fluorescence intensities of the Cy3 channels were quantified using ImaGene 4.0 software (BioDiscovery, El Segundo, CA, USA).

Microarray analysis and annotation of gene function. Data analysis was performed using GeneSpring software, version 5.1 (Silicon Genetics, Redwood City, CA, USA), including appropriate statistics. Dividing by the median calculated from all of the signal intensities for the Cy3 in a given sample, the fluorescence signal for each gene was normalized and the expression ratio was then calculated by dividing the normalized signal by the median for each gene to offset differences in expression levels between genes. Values were displayed as average of five animal samples for the three groups and were then logged (base 10) for further analyses. Standard deviations of the normalized values were calculated for the three groups, and genes in which standard deviation was more than two for any of the three groups were excluded. Genes differentially expressed among the three tissue types were detected by one-way ANOVA ($P = 0.02$).

The functions of the genes were assigned referring to a table attached to Atlas Rat 3.8 I microarrays (BD Biosciences Clontech), and classified into 21 categories: cell surface antigens, transcription factors, cell cycle-related factors, cell adhesion receptors/proteins, extracellular transport/carriers, stress response proteins, membrane channels/transporters, extracellular matrix proteins, trafficking/targeting proteins, metabolism-related factors, post-translational modification/protein folding-related factors, translation-related factors, apoptosis-related factors, RNA processing/turnover/transport-related factors, DNA binding and chromatin proteins, cell receptors, cell signaling/extracellular communicating factors, intracellular transducers/effectors/modulators, protein turnover-related factors, cytoskeleton/motility proteins, and others. Statistical significance for frequencies of genes of each functional category in each cluster was assessed as follows: values of the other categories in the relevant cluster, values of the other clusters in the relevant category, and values of the other clusters in the other categories were all combined, and the resultant combined values were compared with the relevant value by Fisher's exact test for the 2×2 table.

Semi-quantitative RT-PCR. Semi-quantitative RT-PCR was performed for seven genes, at least one gene from each cluster. They were endothelin receptor B (*EDNRB*), zinc finger protein 265 (*ZNF265*), neurofibromatosis type 1 (*NF1*), hepatic nuclear factor 1/transcription factor 1 (*HNF1/TCF1*), leukemia/lymphoma related factor (*LRF*), cyclin L (*CCNL*), and lamin A (*LMNA*). cDNA was synthesized from 3 μg of total RNA using a First-Strand cDNA Synthesis kit (Amersham Biosciences) and oligo$(dT)_{18}$ primer according to the manufacturer's instructions. RT-PCR was carried out using the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) and cycling conditions were 2 min at 94°C, followed by 30 cycles (25 cycles for β-*actin*) of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Forward and reverse primer sequences were: *EDNRB*, 5′-TTGATGTGATTACGTCGGAC-3′ and 5′-GGACTGTTTTTCCTCAAACG-3′; *ZNF265*, 5′-AGAAGTACTACATCTGCTAG-3′ and 5′-TTCCCAGTTGTCAGCTTTGC-3′; *NF1*, 5′-GTACACCAAATACCATGAGC-3′ and 5′-ATGAAGAGGGTGTTGTTGGC-3′; *HNF1/TCF1*, 5′-TGCACTAGAAAGGCTGCTTC-3′ and 5′-GGTTTCTTGCAGTACCGAGG-3′; *LRF*, 5′-TGGGCCGCCTGAATGTAGCG-3′ and 5′-GTATGTCAGTGGTGGCCATG-3′; *CCNL*, 5′-TAATAGGCGAAGTCGATCTG-3′ and 5′-CATCGTCACACTGCATATGG-3′; *LMNA*, 5′-ATGAGAGCAGGTCTGAAGCC-3′ and 5′-AAGCATGGCAGATTTGCCTC-3′; β-*actin* (used as control), 5′-TTGAACACGGCATTGTAACC-3′ and 5′-ATCTCTTGCTCGAAGTCTAG-3′. PCR products were analyzed on 2% agarose gels with ethidium bromide and subsequently underwent densitometry. The obtained values were then normalized to those for β-*actin*.

Western blotting. Western blotting was conducted using the liver samples. Liver tissues were homogenized in 5 volumes of extraction buffer (10 mM Tris-HCl, pH 6.8, 1% SDS) using a polytron homogenizer at setting 7 for 90 s. The homogenates were heated on boiling water for 5 min and centrifuged at 13 000 × *g* for 30 min. The supernatants were used for western blotting. Protein concentrations of the lysates were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Samples containing 5 μg of protein were separated on 7.5% SDS polyacrylamide gel electrophoresis and transferred to Hybond-P PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). The blots were blocked with 5% dried milk in PBS for 1 h, incubated with 1:200 dilution of rabbit anti-EDNRB polyclonal antibody (Chemicon International, Temecula, CA, USA), washed in 0.1% Tween 20 in PBS, and incubated with 20 000-fold diluted antirabbit donkey IgG conjugated with peroxidase (Amersham Biosciences). Both primary and secondary antibodies were diluted with 0.1% Tween 20 in PBS, and incubation was at room temperature for 1 h. The immunoreactive bands were detected using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences).

Results

General findings. All rats survived until their scheduled killing in relatively healthy conditions, but the mean bodyweight of group 2 animals was lighter than that of group 1 animals (Table 1). Group 1 livers showed no particular pathological changes either macroscopically or histologically. All of group 2 livers were macroscopically yellowish-white and appeared cirrhotic with 1 or 2 large tumoral nodules with a dark color. The mean relative liver weight was greater in group 2 compared

Table 1. Final body and relative liver weights

Group	Treatment	Animal	Final bodyweight (g)	Relative liver weight (g/100 g bodyweight)
1	Control	1	456	3.26
		2	448	3.04
		3	462	2.78
		4	460	2.88
		5	450	3.12
		Mean	455	3.02
		Standard deviation	6	0.19
2	CDAA	6	416	5.12
		7	422	4.88
		8	418	4.66
		9	408	5.38
		10	404	4.72
		Mean	414*	4.95*
		Standard deviation	7	0.3

*Significantly different from the group 1-value ($P = 0.0079$ by the two-tailed Mann–Whitney non-parametric test).

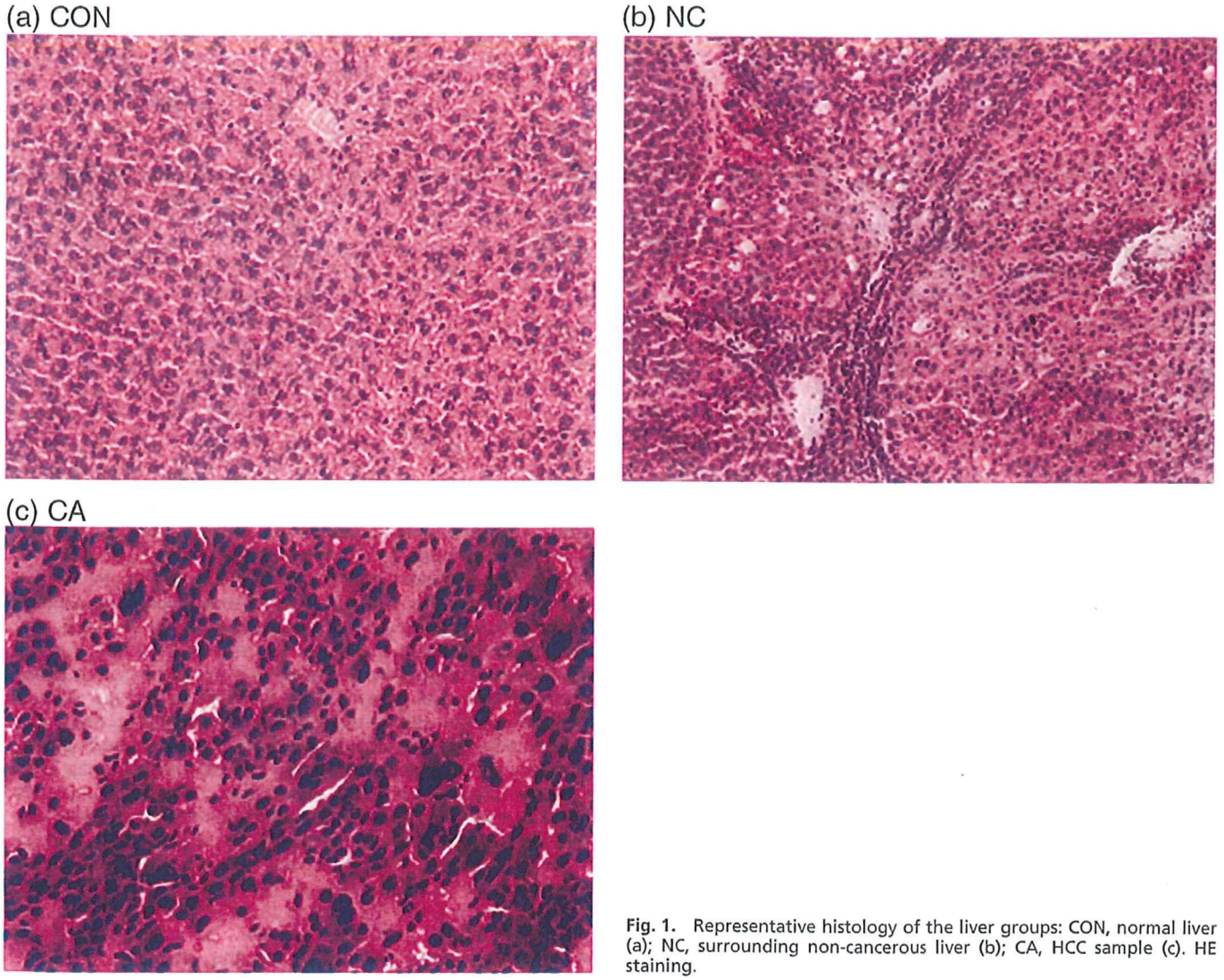

Fig. 1. Representative histology of the liver groups: CON, normal liver (a); NC, surrounding non-cancerous liver (b); CA, HCC sample (c). HE staining.

to group 1 animals (Table 1). Tumors were histologically diagnosed as relatively well-differentiated HCC, chiefly showing a trabecular pattern. The surrounding non-cancerous areas were cirrhotic, featuring intrahepatocellular fat accumulation, frequent hepatocellular apoptosis, and nuclear divisions of hepatocytes. These findings are in accordance with our previous results.[4] Representative histology of the three groups of liver samples is shown in Figure 1.

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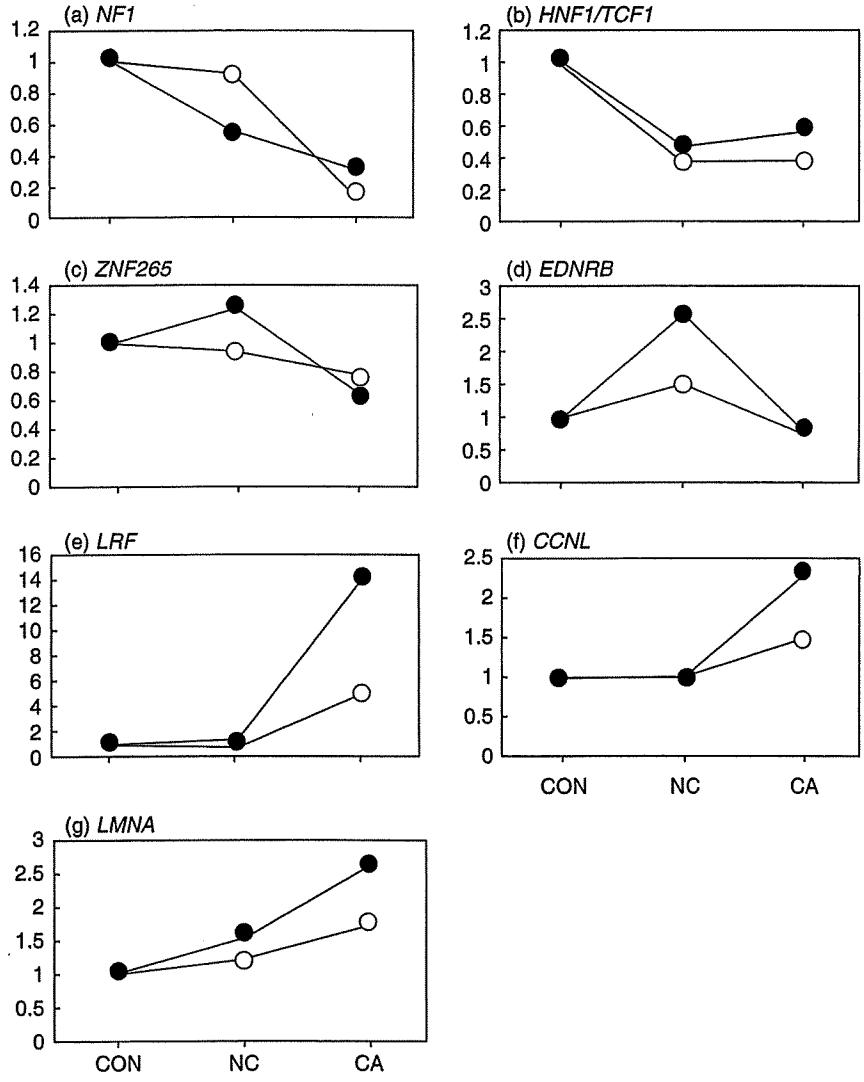

Fig. 2. Comparison of results obtained with microarray and semiquantitative RT-PCR for relative mRNA expression levels among normal liver (CON), surrounding non-cancerous liver (NC), and HCC samples (CA). The average expression levels of CON samples were set at 1. (○), data from RT-PCR; (●), data from microarray analysis. The indicated data for RT-PCR are from a single representative experiment, which was reproduced at least three times. (a), neurofibromatosis 1 (*NF1*); (b), hepatic nuclear factor 1/transcription factor 1 (*HNF1/TCF1*); (c), zinc finger protein 265 (*ZNF265*); (d), endothelin receptor B (*EDNRB*); (e), leukemia/lymphoma related factor (*LRF*); (f), cyclin L (*CCNL*); (g), lamin A (*LMNA*).

Changes in gene expression during carcinogenesis. We compared the gene expression profiles among CON, NC and CA using oligonucleotide microarrays containing 3757 genes. A total of 146 genes were detected as differentially expressed among the three tissue types. To verify the results of microarray analysis, expression of a total of seven genes (at least one gene from each cluster) was investigated by semiquantitative RT-PCR. These genes are involved in cell functions important in tumor progression, such as transcriptional regulation (*HNF1/TCF1* and *LRF*), RNA splicing (*ZNF265* and *CCNL*), signal transduction (*NF1* and *EDNRB*), and nuclear structure (*LMNA*) (Fig. 2). For comparison, the relative expression levels of CON were set to one. The expression patterns among the three sample groups were similar for these genes, although there are some discrepancies (Fig. 2). We considered that the results of microarray analysis were generally reproducible in RT-PCR, but the causes of the discrepancies are yet to be investigated.

Western blotting. In some cases, expression intensities at mRNA level and protein levels are not in parallel. So, we have investigated the expression levels of *EDNRB* by western blotting for comparison with data obtained by microarray and semiquantitative RT-PCR. At protein levels, the tendency of *EDNRB* expression was as follows: CON < NC > CA (Fig. 3a,b). So, in the case of *EDNRB*, changes in mRNA levels appear to be accompanied by changes in protein levels. High expression levels of *EDNRB* in rat[6] and human[7] liver cirrhosis compared to normal liver tissues have already been reported. The activated endothelin system can increase tonality of the hepatic microvasculature, which may contribute to the formation of liver cirrhosis.[6,7]

Detection of multiple patterns of expression with CON, NC and CA. Only the 146 genes that showed differential expressions were subjected to further analysis to prevent invariant genes from negatively affecting the clustering results. We first used a hierarchical clustering procedure and a dendrogram for classification of genes based on expression patterns (Fig. 4a). The genes were thereby divided along two major branches and then subdivided into two branches (Fig. 4a). Accordingly, we chose four for the total number of clusters in the subsequent *k*-means clustering analysis. The gene members contained in the four clusters obtained by *k*-means clustering were identical to those obtained by hierarchical clustering. We named them clusters 1–4, containing 18, 54, 45, and 29 members shown in Tables 2, 3, 4 and 5, respectively. Each of the four clusters had its own different pattern of expression profiles, with tendencies as follows: Cluster 1, CON > NC ≈ CA; cluster 2, CON < NC > CA [≈ CON]; cluster 3, CON ≥ NC < CA [> CON]; cluster 4, CON < NC < CA (Fig. 4b).

Gene function category of differentially expressed genes. In cluster 2, genes categorized as 'cell receptors' ($P < 0.01$) and 'cell signaling/extracellular communications' ($P < 0.05$) were significantly enriched. Genes in the categories of 'intracellular transducers' ($P < 0.05$) and 'cell receptors' ($P < 0.01$) were significantly low in clusters 1 and 3, respectively (Fig. 5).

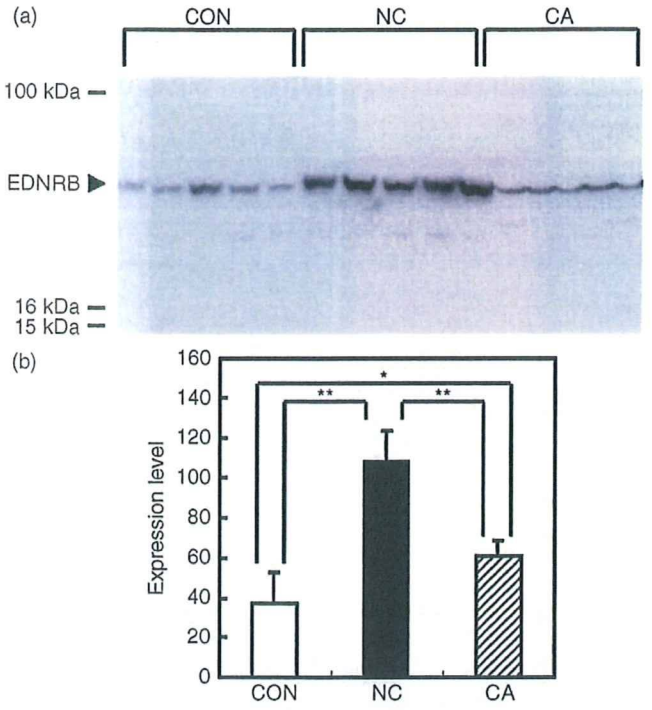

Fig. 3. (a) Expression of endothelin receptor B (*EDNRB*) detected in rat-liver samples by western blotting. (b) Protein levels of *EDNRB* compared between normal liver (CON), surrounding non-cancerous liver (NC), and HCC sample (CA). Data are expressed as mean ± SD in arbitrary units. There was a significant difference in expression levels among the three groups by ANOVA ($P < 0.01$). *$P < 0.05$ and **$P < 0.01$ by Student–Neuman–Keuls' test. The indicated data for western blotting are from a single representative experiment that was reproduced three times.

Discussion

Significance of the four clusters in hepatocarcinogenesis. The present results revealed that the gene expression profiles were differentially altered in HCC and their adjacent non-cancerous, cirrhotic liver tissues in rats. The differentially expressing genes could be divided into four clusters according to their expression patterns. Although we need to be cautious with the interpretation of these clusters, we speculate on the possible meanings of the expression patterns observed in the current study as follows. Genes belonging to cluster 1 were down-regulated in NC and also stayed low in CA, suggesting that the aberrant function of these genes may be involved in continuous liver injury, including fibro/cirrhogenesis, and early stage hepatocarcinogenesis. We have explored the expression of the cluster 1 gene *HNF1/TCF1* also by RT-PCR, and the roles of this gene in hepatocarcinogenesis are discussed further. Genes belonging to cluster 2 were upregulated in NC and then downregulated to the control level in CA, suggesting that the aberrant function of these genes is involved both in liver injury and hepatocarcinogenesis but with opposite influences. Alternatively, it is possible that these genes are necessary to be upregulated for liver injury but no longer have to be aberrantly expressed for carcinogenic processes. Genes belonging to cluster 3 were normally expressed in NC but then upregulated in CA, suggesting that the function of these genes may not be involved in liver injury but mainly in hepatocarcinogenesis. Genes belonging to cluster 4 were upregulated in NC and further upregulated in CA, suggesting that the function of these genes is positively involved both in liver injury and in hepatocarcinogenesis. However, to fully understand the roles of differentially expressed genes, exploration of the sequential expression of these genes at more time points may be needed.

Genes in the category of 'cell receptors' enriched in cluster 2. The present study showed that genes differentially expressed among CON, NC and CA fit into relatively few clusters, each showing distinct expression patterns. Further analyses revealed that certain functionally categorized genes are enriched in some clusters (Fig. 5). Notably, genes categorized as 'cell receptors' and 'cell signaling/extracellular communication proteins' were both enriched in cluster 2 (Table 3), characterized by high gene expression levels in NC compared with CON or CA (Fig. 4b). The precise significance of this expression pattern is not clear at present, but at least four of the 10 receptors in this cluster,

Table 2. Genes of cluster 1

Category	Gene name	GenBank accession number	Expression ratio		
			CON	NC	CA
Cell surface antigens	CD48 antigen	X13016	1.59	1.13	0.74
Transcription factors	D site albumin promoter binding protein	J03179	1.28	0.61	0.46
	Hepatic nuclear factor 1/*TCF1*	J03170	1.66	0.62	0.66
Cell cycle-related factors	Synaptonemal complex protein 3	X75785	1.84	0.99	0.56
Cell adhesion receptors/proteins	Milk fat globule membrane protein/ O-acetyltransferase	D84068	1.57	0.44	0.63
Extracellular matrix proteins	Collagen type X α1	AJ131848	1.41	0.91	0.99
Metabolism-related factors	α-1,3-Fucosyltransferase	U58860	1.82	0.99	0.71
	UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase T5	AF049344	1.15	0.82	0.81
	Glucose-6-phosphatase	D78592	1.77	0.50	0.46
	Branched chain aminotransferase 1	AF165887	1.29	0.76	0.90
Post-translational modification/ protein folding-related factors	FK506-binding protein 1	D86641	1.95	0.57	1.12
	Ubiquitin-conjugating enzyme E2D 3	AB006852	1.41	0.76	0.64
Translation-related factors	Ribosomal protein S15a	X77953	1.47	0.79	1.10
Cytoskeleton/motility proteins	Keratin complex 1, acidic, gene 18	U67992	1.09	0.55	0.94
Others	Ras homolog enriched in brain	U08227	1.05	1.00	0.65
	Peptidyl arginine deiminase, type 3	D88034	2.05	0.42	0.59
	Prostatic binding protein	X05034	1.69	1.01	0.48
	Scrapie responsive gene 1	AJ132434	1.17	0.63	0.83

CON, normal liver; NC, surrounding non-cancerous liver; CA, HCC sample.

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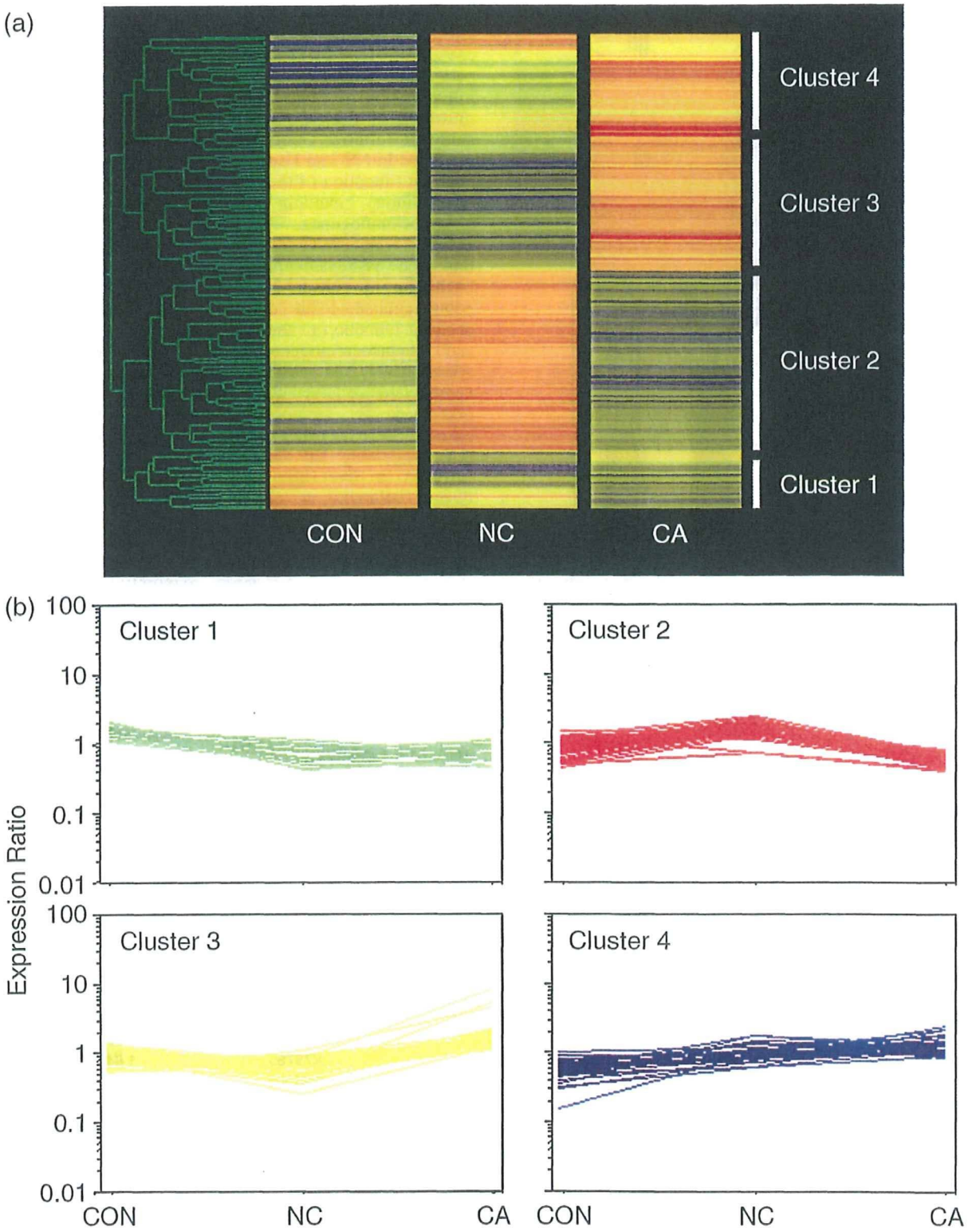

Fig. 4. Gene expression profiles among normal liver (CON), surrounding non-cancerous liver (NC), and HCC samples (CA). One hundred and forty-six genes were identified as demonstrating differential expressions among the three tissue types and subjected to further analysis. (a) Hierarchical clustering. A dendrogram is shown on the left side of the diagram. Tissue types are represented by columns and genes in rows. Red, yellow and blue represent the higher, equal and lower mRNA levels, respectively, relative to that of the median of each gene. The dendrogram indicates that data are divided along two main branches, each divides further into two branches. (b) Expression patterns of the genes included in the four clusters. The 146 genes were classified by hierarchical and *k*-means clustering procedures. Expression ratios of the genes are indicated.

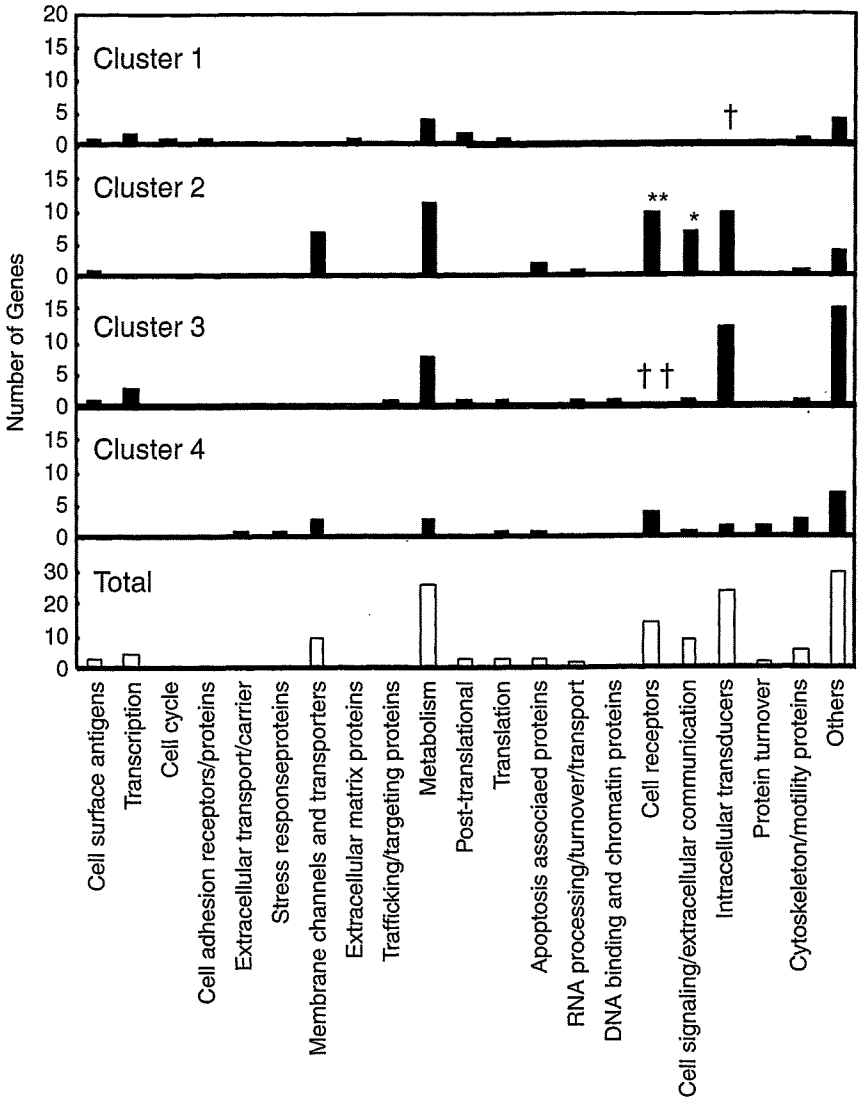

Fig. 5. Distribution of functionally categorized genes in each cluster. Numbers of genes within each functional category are shown. Frequency of each category in each cluster was evaluated by Fisher's exact test. Significantly elevated ($^{*}P < 0.05$, $^{**}P < 0.01$) and lowered ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$) frequencies for genes in each cluster are indicated.

namely, opioid receptor κ1 (*OPRK1*), somatostatin receptor 1 (*SSTR1*), endothelin receptor A (*EDNRA*), and endothelin receptor B (*EDNRB*), are known for their inhibitory effects on cell growth.[8–11] This is in theoretical accordance with the downregulation of these genes observed between NC and CA (Fig. 4b, Table 3), as this would give cells a growth advantage. These four genes also belong to the same family, namely, G-protein-coupled receptors. In fact, the somatostatin and opioid systems are the main inhibitory systems in mammals, and relevant molecules including *OPRK1* and *SSTR1* have been under intense investigation in terms of their roles in tumorigenesis.[8–11] Moreover, downregulation of *EDNRB* has been studied in human prostate and nasopharyngeal cancer in relation to its abnormal methylation in CpG islands.[12,13] Another receptor gene, platelet derived growth factor receptor α (*PDGFRA*), however, is known for its growth-stimulating activity in certain types of cells. *TrkB*, a neurotrophic tyrosine kinase receptor, also found in this cluster, has recently been identified as a potent suppressor of anoikis and inducer of metastasis in epithelial cells.[14] *TrkB* and its ligand brain-derived neurotrophic factor are frequently coexpressed in human cancers, especially those with aggressive phenotypes,[15] suggesting the formation of an autocrine signaling pathway. So, while the expression patterns of the receptor genes in cluster 2 appear similar to each other, the cell signals related to each gene are diverse and should be individually explored.

Genes in the category of 'cell signaling/extracellular communications' enriched in cluster 2. Genes in the category of 'cell signaling/extracellular communications' were also enriched in cluster 2 (Fig. 5, Table 3). The seven genes included in this cluster encode polypeptide cytokines with various functions and related signal transduction systems after binding to their cognate receptors. It should be noted that fibroblast growth factor 5 (*FGF5*) and *FGF9*, both members of the FGF family, were found in this cluster. For FGF members stimulate growth and differentiation in a variety of cell types, and their roles in carcinogenesis have been investigated.[16] Hu *et al.* reported high expression levels of FGF1 in rat HCC induced by the Solt-Farber protocol.[17] In their results, FGF1 levels were elevated at early stages, decreased after one month, and remained low until the development of tumors. The reason why FGF5 and FGF9 were higher in NC rather than CA in the current study is not clear at present. However, there have been at least 23 FGF discovered thus far, and the roles of individual FGF in carcinogenesis have not been exactly elucidated.[16] Sequential expression analysis of these molecules during carcinogenesis may also help fully understand the roles of individual FGF in hepatocarcinogenesis.

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Table 3. Genes of cluster 2

Category	Gene name	GenBank accession number	Expression ratio		
			CON	NC	CA
Cell surface antigens	CD36 antigen-like 2	M68965	0.73	2.10	0.67
Membrane channels/transporters	Inositol 1,4,5-triphosphate receptor 3	L06096	0.87	1.56	0.63
	X transporter protein 2	U12973	0.87	1.67	0.45
	ATPase, H^+K^+ transporting, non-gastric, α polypeptide	M90398	0.69	1.65	0.55
	Dopa decarboxylase	M27716	0.87	1.41	0.73
	Synaptotagmin 2	M64488	1.19	1.63	0.55
	Fatty acid binding protein 2	M35992	0.74	1.78	0.56
	Interleukin-2 receptor, β chain	M55050	1.15	1.44	0.55
Metabolism-related factors	Cytochrome P450 1B1	U09540	1.32	2.48	0.70
	Sucrose isomaltase	X15546	1.03	2.22	0.69
	Propionyl coenzyme A carboxylase, β polypeptide	M14634	1.30	1.56	0.62
	Mevalonate kinase	M29472	0.45	1.36	0.64
	Hexokinase 2	M68971	0.56	1.51	0.61
	Plasma glutathione peroxidase precursor	D00680	1.34	1.19	0.52
	Testis lipid binding protein	U07870	1.03	1.86	0.68
	Lactate dehydrogenase 3, C chain	U07177	1.01	1.41	0.71
	Solute carrier family 18, member 2	M97381	0.56	1.71	0.68
	Glycerol kinase	D16102	1.05	1.97	0.64
	Cytochrome P450 3A9	U60085	1.11	1.25	0.56
Apoptosis-related factors	Caspase 3	U49930	1.01	1.33	0.69
	α-Inhibin	M36453	1.18	1.50	0.83
RNA processing/turnover/ transport-related factors	Zinc finger protein 265	AF013967	1.18	1.47	0.78
Cell receptors	Opioid receptor, κ1	D16829	0.82	1.55	0.78
	Neural receptor protein-tyrosine kinase *TrkB*	M55291	0.51	1.42	0.67
	Somatostatin receptor subtype 1	X62314	1.01	2.15	0.58
	Platelet-derived growth factor receptor α	M63837	0.63	1.88	0.75
	Diphtheria toxin receptor	L05489	0.79	1.84	0.67
	Glutamate receptor, ionotropic, δ1	U08255	0.95	1.72	0.72
	Interleukin-1 receptor-like 1	U04317	0.72	1.70	0.45
	Endothelin receptor type A	X57764	0.60	1.53	0.50
	Interleukin-1 receptor, type I	M95578	0.80	1.47	0.56
	Endothelin receptor type B	M60786	0.93	1.56	0.54
Cell signaling/extracellular communicatimg factors	β-Nerve growth factor	M36589	0.54	1.72	0.81
	Cytokine-induced neutrophil chemoattractant-2	D21095	1.29	1.45	0.46
	Placental growth factor	L40030	0.96	1.90	0.42
	Neurotrophin-3	M34643	1.23	1.36	0.74
	Fibroblast growth factor-5	D64085	1.18	2.43	0.57
	c-fos induced growth factor	AF014827	0.97	1.82	0.66
	Fibroblast growth factor-9	D14839	0.76	1.92	0.65
Intracellular transducers/ effectors/modulators	Annexin 1/*p35*/lipocortin 1	M19967	1.04	1.37	0.61
	Insulin receptor substrate 1	X58375	1.03	1.67	0.77
	Neurofibromatosis type 1	D45201	1.32	0.75	0.40
	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase, A	AB032551	0.84	1.48	0.47
	Endothelin converting enzyme-like 1	AB026293	0.85	1.79	0.79
	Src related tyrosine kinase	U09583	0.77	1.65	0.61
	S6 kinase	M58340	0.99	1.79	0.51
	Guanine nucleotide binding protein, γ7	L23219	1.11	1.44	0.78
	Polo-like kinase homolog	U10188	1.02	1.40	0.47
	Protein tyrosine phosphatase, non-receptor type 5	S49400	1.04	1.46	0.61
Cytoskeleton/motility proteins	Troponin 1, type 2	M73701	0.56	1.89	0.77
Others	P-glycoprotein/multidrug resistance 1	M81855	1.04	1.53	0.57
	Secretory zymogen granule membrane glycoprotein *GP2*	M58716	1.05	1.53	0.56
	Probasin	M27156	0.80	1.54	0.42
	Palmitoyl-protein thioesterase	L34262	0.51	2.30	0.62

CON, normal liver; NC, surrounding non-cancerous liver; CA, HCC sample.

Table 4. Genes of cluster 3

Category	Gene name	GenBank accession number	Expression ratio		
			CON	NC	CA
Cell surface antigens	MHC class II antigen RT1.B-1 β-chain	X56596	0.85	0.49	1.44
Transcription factors	Hepatic nuclear factor 4	D10554	1.09	0.60	1.56
	Mini chromosome maintenance deficient 6	U17565	1.04	0.71	1.46
	Leukemia/lymphoma related factor	D88450	0.62	0.83	8.57
Trafficking/targeting proteins	Clathrin, heavy polypeptide	J03583	1.25	0.60	1.58
Metabolism-related factors	Peroxiredoxin 6	AF110732	1.23	0.68	1.79
	Superoxide dismutase 3	Z24721	1.19	0.67	1.16
	Glutamate dehydrogenase	X14044	0.95	0.82	1.51
	Prostaglandin-endoperoxide synthase 1/cyclo-oxygenase 1	NM017043	0.73	0.63	1.12
	Dimethylglycine dehydrogenase precursor	X55995	1.01	0.49	1.72
	Hydroxyacid oxidase 3/glycolate oxidase 3	X67156	1.33	0.68	1.14
	Glycerol-3-phosphate dehydrogenase 2	X78593	1.27	0.45	1.55
	β-4N-acetylgalactosaminyltransferase	D17809	0.57	0.53	1.44
Post-translational modification/ protein folding-related factors	Peptidylglycine α-amidating monooxygenase	M25732	0.55	1.14	4.71
Translation	Eukaryotic translation initiation factor 2B, subunit 2	U31880	0.91	0.78	1.48
RNA processing/turnover/ transport-related factors	Cyclin L	AF030091	0.75	0.75	1.71
DNA binding and chromatin proteins	Histone H10	U49737	1.25	0.68	1.11
Cell signaling/extracellular communicating factors	Endothelin-2	U64949	1.03	0.55	1.17
Intracellular transducers/ effectors/modulators	Insulin receptor substrate 3	U93880	0.95	0.91	1.35
	PCTAIRE3	AB005541	0.81	0.37	2.07
	Calbindin 1	M31178	0.79	0.75	1.85
	Arrestin, β1	M91589	0.90	0.93	1.55
	Adenylyl cyclase 2	M80550	0.83	0.68	1.34
	Homeodomain-interacting protein kinase 3	AF036959	0.93	0.66	1.65
	Tuberous sclerosis 2	U24150	1.04	0.75	1.66
	AMP-activated protein kinase	Z29486	1.35	0.74	1.49
	Sialyltransferase 5	X76988	1.02	0.62	1.46
	Thyroid hormone receptor interactor 10	AB006914	0.58	0.67	1.81
	Peroxiredoxin 5	Y17295	1.02	0.87	1.47
	Sialyltransferase 8	U55938	1.10	0.68	2.16
Cytoskeleton/motility proteins	Kinesin heavy chain member 2	AF155824	0.91	0.76	1.21
Others	Cell growth regulatory with EF-hand domain	U66470	1.36	0.61	1.33
	Homeobox protein *R3*	M37567	1.01	0.55	2.28
	ADAMTS-1/METH-1	AF149118	0.94	0.67	1.29
	Outer mitochondrial membrane receptor *rTOM20*	U21871	1.01	0.94	1.92
	Coronin, actin-binding protein, 1B	AJ006064	1.41	0.53	1.40
	Unconventional myosin *Myr2* I heavy chain	X74800	0.98	0.86	1.41
	Replication factor C 2	AF208499	0.81	0.76	1.26
	Solute carrier family 29, member 1/*ENT1*	AF015304	0.94	0.55	1.47
	Aspartyl-tRNA synthetase/*DRS1*	U30812	1.16	0.50	1.16
	Quinoid dihydropteridine reductase	J03481	1.18	0.40	1.44
	Fibrinogen, γ-polypeptide	J00734	0.63	0.48	5.35
	Myosin light chain kinase 2	J03886	0.98	0.46	1.51
	Hyperpolarization-activated cyclin nucleotide-gated cation channel 1	AF247450	1.00	0.27	1.20
	ECL	X56190	0.79	0.50	1.57

CON, normal liver; NC, surrounding non-cancerous liver; CA, HCC sample.

Other deregulated genes associated with carcinogenesis. Metabolism-related factors found to be differentially expressed are mainly enzymes with a variety of functions and their genes did not show any significant enrichment in any of the four clusters (Fig. 5). Glucose-6-phosphatase is a negative phenotype marker of putatively preneoplastic foci of cellular alteration in the liver of rats,[18] and its activity is frequently lost also in human HCC.[19] CYP1B1 and CYP3A9 are both related to estrogen metabolism, and CYP enzymes are known as target genes of hepatic transcription factor 4 (HNF4). In human HCC, CYP enzymes are also generally down-regulated compared to surrounding tissues.[20] Aberrant expression of β-4N-acetylgalactosaminyl-transferase, observed here, is frequently detected in human HCC and other cancers.[21] Potassium voltage-gated channels play an important role in the proliferation and metastasis of HCC cells.[22]

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Table 5. Genes of cluster 4

Category	Gene name	GenBank accession number	Expression ratio		
			CON	NC	CA
Extracellular transportors/carriers	Apolipoprotein A-V	AF202887	0.45	1.50	1.30
Stress response proteins	Solute carrier family 22, member 2/*OCT2*	D83044	0.52	0.64	0.95
Membrane channels/transporters	Solute carrier family 6, member 13/*GAT2*	M95762	0.73	1.38	1.14
	Chloride channel 1, skeletal muscle	X62894	0.60	1.77	1.39
	Potassium voltage-gated channel, subfamily H, member 2	Z96106	0.31	0.78	2.54
Metabolism-related factors	Protease 28 subunit, β/prosome/macropain	NM017257	0.33	0.66	2.28
	Protease, cysteine, 1/legumain	AF154349	0.51	1.04	1.32
	Malate dehydrogenase-like enzyme	AF093773	0.65	0.89	1.67
Translation-related factors	Ribosomal protein S2	U92698	0.78	1.10	1.13
Apoptosis-related factors	Lifeguard/neural membrane protein 35	AF044201	0.16	1.05	2.19
Cell receptors	Galanin receptor 3	AF073798	0.84	1.05	1.90
	Fc receptor, IgG, low affinity III	M32062	0.44	1.41	0.89
	Polymeric immunoglobulin receptor	X15741	0.40	0.74	1.33
	Neuromedin B receptor	U37058	0.36	1.76	1.17
Cell signaling/extracellular communicating factors	Glucose-dependent insulinotropic peptide	L08831	0.65	0.93	1.34
Intracellular transducers/ effectors/modulators	Phospholipase C, β3	M99567	0.67	0.86	1.32
	Thiol-specific antioxidant-like protein	AF053093	0.51	0.82	1.69
Protein turnover-related factors	Serine protease inhibitor	X16359	0.57	1.13	1.45
	Calpain, small subunit 1	U53859	0.55	0.94	1.65
Cytoskeleton/motility proteins	Actinin α1	AF115386	0.69	0.73	1.13
	Lamin A	X66870	0.56	0.87	1.47
	β-Spectrin 3	AB001347	0.64	0.85	1.15
Others	Thymosin, β10	M58404	0.55	1.42	1.07
	Solute carrier family 19, member 1/*RFC1*	AF099010	0.70	0.87	1.37
	Vitronectin	U44845	1.04	1.22	1.78
	Dendrin	X96589	0.91	0.96	1.05
	HLA-B-associated transcript 3	AB018791	0.75	0.97	1.08
	Rhodopsin/retinitis pigmentosa 4, autosomal dominant	Z46957	0.48	0.64	1.14
	Myosin heavy chain *Myr 8*	AF209114	0.59	0.95	0.98

CON, normal liver; NC, surrounding non-cancerous liver; CA, HCC sample.

In the CDAA diet model, repeated apoptotic death and proliferation of hepatocytes and fibro/cirrhogenesis have been shown to be important in the processes underlying hepatocarcinogenesis.[4,23] Genes with possible relation to fibro/cirrhogenesis included *PDGFRA*, diphtheria toxin receptor/heparin-binding epidermal growth factor receptor, *FGF5*, *FGF9*, interleukin *(IL)-1* receptor-like 1, *IL-1* receptor type *B*, *IL-2* receptor β-chain, *EDNRA*, *EDNRB*, endothelin converting enzyme-like 1, annexin 1/*p35*/lipocortin 1, insulin receptor substrate 1, *Src* related tyrosine kinase, and *S6* kinase.[24–28] Differentially expressed genes in the current study included both pro-apoptotic factors such as caspase 3 and α-inhibin,[29,30] and antiapoptotic factors such as calbindin 1, arrestin β1, homeodomain-interacting protein kinase 3, lifeguard, and lamin A.[31–35] Cyclin L[36] and ZNF265[37] are regulatory factors for the RNA splicing machinery so that their aberrant expressions may result in abnormal RNA splicing.

Reactive oxygen species-induced stress has been shown to be involved in human hepatocarcinogenesis,[1–3] NAFLD/NASH[5] and the rat CDAA diet model.[4,22] The present study detected the altered expression of several genes related to both anti- and pro-oxidative machinery, such as plasma glutathione peroxidase precursor, peroxiredoxins 5 and 6, superoxide dismutase 3, prostaglandin endoperoxide synthase 1/*COX-1*, phospholipase C β3, *and* thiol-specific antioxidant-like protein.

Our results have also shown aberrant expression of *HNF1/TCF1* and kinesin heavy chain member 2, two molecules involved in the Wnt signaling pathway, a major system involved in human hepatocarcinogenesis.[38] Biallelic inactivation of *HNF1/TCF1*, detected in human hepatic adenoma and HCC, is suspected to be important in early stages of liver tumor development.[39]

Regarding tumor-suppressor genes, the expression of both tuberous sclerosis 2 (*TSC2*) and its molecular target ras homolog enriched in brain (*Rheb*) was found to be altered in the current experiment. Neurofibromatosis type 1 (*NF1*) also exhibited variation between tissue types, and this may be the first report to suggest a potential involvement of *NF1* in HCC development. Other altered genes, fibrinogen γ-polypeptide, actinin α1 and vitronectin, have previously been reported to demonstrate corresponding changes in human hepatocarcinogenesis.[40,41]

Conclusion. Among the differentially expressed genes found in the present study, many have already been reported to have involvement in human and animal hepatocarcinogenesis. Statistical methods have proved useful for classifying these genes into relatively few clusters according to their expression patterns. Based on the functional classification of the differentially expressed genes, it was further indicated that these clusters may be associated with gene functional categories. For example, genes encoding the two major components of signal-transducing systems, cell receptors and cytokines, were significantly enriched in a particular cluster. Data have recently been accumulated regarding behaviors of individual tumor-related genes,[1–3] but mechanisms for global regulation of particular groups of genes are not fully understood. Several factors are supposed to affect overall behaviors of multiple genes. These include concordant methylation of CpG islands, which can result in suppression of multiple genes as described

in human cancers including HCC.[42] Although the causation of the association between expression patterns of genes and gene functional categories suggested in the current study is yet to be elucidated, these findings may give insight into underlying mechanisms for the evolution of HCC.

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Dietary indole-3-carbinol promotes endometrial adenocarcinoma development in rats initiated with *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, with induction of cytochrome P450s in the liver and consequent modulation of estrogen metabolism

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Indole-3-carbinol (I3C), found in cruciferous vegetables, has been shown to suppress or promote carcinogenesis depending on various animal models. Regarding its preventive effects, I3C acts as an anti-estrogen and can induce apoptosis, but precise mechanisms remain to be determined. Since I3C induces cytochrome P450 enzymes in the liver, it affects hydroxylation of estrogens and might therefore be expected to influence endometrial adenocarcinoma development. The present study was performed to clarify the effects of I3C using a rat two-stage endometrial carcinogenesis model, focusing on induction of cytochrome P450s and other estrogen-metabolic enzymes in the liver. First, to determine the estrogenic or anti-estrogenic activity, an uterotropic assay was conducted using ovariectomized Donryu rats (experiment 1). Second, to elucidate the effects on endometrial carcinogenicity, female Donryu rats initiated with a single dose of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine into a uterine horn were fed 0 or 500 p.p.m. I3C in diets for 12 months (experiment 2). In experiment 3, similarly initiated animals received 0 or 2000 p.p.m. I3C in their diet, or 1 μg/kg 17β-estradiol (E2) or 5 μg/kg 4-hydroxyestradiol (4HE) subcutaneously twice a week for 12 months. In the uterotrophic assay, neither 500 nor 2000 p.p.m. of I3C showed any estrogenic or anti-estrogenic activity. In the two uterine carcinogenicity studies, I3C and 4HE increased incidences of uterine adenocarcinomas and/or multiplicities of uterine proliferative lesions, E2-treatment being associated with a tendency for promotion. In the liver, I3C treatment consistently elevated estradiol 2- and 4-hydroxylase activities, in particular the latter, but without effects on estradiol 16α-hydoxylase activity. mRNAs for CYP 1A1, 1A2 and 1B1 were increased by I3C treatment, with translation confirmed immunohistochemically. These results suggest that induction of the CYP 1 family in the liver and sequential modulation of estrogen metabolism to increase 4HE might play a crucial role in promoting the effects of dietary I3C on endometrial adenocarcinoma development.

Introduction

Indole derivatives are contained in cruciferous vegetables such as cabbage, broccoli, brussels, sprout and cauliflower (1). Indole-3-carbinol (I3C) is known to be an anti-estrogenic (2–4) or apoptosis-inducing compound (5), and has shown anticarcinogenic activity in a number of animal studies such as DMBA-induced rat mammary tumorigenesis (6) and spontaneous rat uterine adenocarcinoma development (7). I3C also has chemopreventive activity against benzo[*a*]pyrene-induced mouse forestomach carcinogenicity (8). It is neither cytotoxic, nor mutagenic *in vitro* (9,10), and thus I3C is a promising candidate for a chemopreventive agent against various tumors, especially estrogen-related examples. However, the compound has been documented to promote development of colon proliferative lesions in an animal model (11), and in a multi-organ rat model both inhibition and promotion were apparent, depending on the organ (12).

As for a hypothesized mechanism of chemopreventive effects of I3C, an anti-estrogenic and/or apoptosis-inducing effect have been widely accepted. In addition, the compound induces hepatic cytochrome P450s (CYPs) such as 1A1 and/or 1A2 (13–15), and increased activity of some phase I drug-metabolizing enzymes, including the CYP 1 family, can protect in some instances by increasing the rate of oxidation to less toxic metabolites (16–19). Recently I3C treatment was reported to also induce CYP 1B1 in the liver and/or other organs (20,21). In most animal species, it is well established that estradiol is metabolized by microsomal P450s in the liver and other organs/tissues, and that these enzymes therefore have the ability to modulate its effects (22–24).

In rats, CYP 1A2, 2B1/2B2 and 3A catalyze 2- or 4-hydroxylation of estradiol, mainly in the liver (24,25). In addition, evidence has recently been presented that CYP 1B1 is a major enzyme catalyzing 17β-estradiol (E2) to 4-hydroxyestradiol (4HE) (26). In the rat liver, E2 is metabolized by estradiol 2- and 4-hydroxylases into two types of catechol estrogens, 2-hydroxyestradiol (2HE) and 4HE, respectively. 2-Hydroxylation of estradiol is the dominant pathway for catechol estrogen formation (22,24), and 2HE can bind to the classical estrogen receptors, but with a markedly reduced binding affinity. This metabolite possesses much weaker hormonal potential than the parent hormone (27,28), and is not a carcinogenic agent (7,24,29). In contrast, 4HE, produced only in small amounts in the liver compared with 2HE, is hormonally active and can stimulate uterine growth by strong binding to estrogen receptors when injected into animals (24,26, 29–31). In addition, this catechol estrogen causes tumor development in the kidney in hamsters (23), and also has been implicated in uterine and mammary tumor development in human beings (32,33).

Much attention has been paid to modulation of estrogen metabolism by chemicals such as phenobarbital, dexamethasone, 3-methylcholanthrene and environmental pollutants via

Abbreviations: CYP, cytochrome; E2, 17β-estradiol; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; 2HE, 2 hydroxyestradiol; 4HE, 4-hydroxyestradiol; I3C, indole-3-carbinol; PE, persistent estrus.

induction of cytochrome P450 enzymes, especially of the CYP 1 family, in the liver or other organs (26,34,35). Thus, it is hypothesized that chemicals exerting no estrogenic activity themselves but inducing CYP 1 might also modify estrogen-dependent tumor development. However, solid evidence in animal models is limited, although Kojima *et al.* (7) reported previously that dietary I3C inhibited spontaneous uterine adenocarcinoma development by increasing estradiol 2-hydroxylation activity.

Cancers of the uterine corpus, most of them being histologically endometrial adenocarcinomas, have recently been increasing in many countries of the economically developed world. The tumor development is strongly related to estrogen statement in women. In rats, spontaneous endometrial adenocarcinomas are generally very rare but Maekawa and his co-workers have described high incidences of such lesions with morphological and biological similarities to human tumors in aged Donryu rats, and shown that this is due to an age-related ovarian hormonal imbalance resulting in an increase of the serum estrogen/progesterone ratio (36–38). In addition, they have established a two-stage uterine carcinogenesis model using this rat strain to detect promotive or preventive effects of test-chemicals (39–42). The present study was conducted to clarify effects of I3C on uterine carcinogenesis using this rat model, focusing on modulation of estrogen-metabolic enzymes in the liver. In addition, estrogenic or anti-estrogenic activity of I3C on ovariectomized rat uteri was also investigated.

Materials and methods

Animals and housing conditions

236 female Crj:Donryu rats at 8 weeks of age were purchased from Charles River Japan (Kanagawa, Japan). The animals were maintained in an air-conditioned animal room under constant conditions of 24 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle (light, 08:00–20:00; dark, 20:00–08:00), housed three or four to a cage. Commercial powder diet (CRF-1, Oriental Yeast, Kanagawa, Japan) and drinking water were available *ad libitum* for the acclimatizing period. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Chemicals

I3C, 4HE, 2HE and 16α-hyroxyestradiol (16αHE) were purchased from Sigma-Aldrich (MO), *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) from Nacalai Tesque (Kyoto, Japan), and E2 and dimethylsulfoxide (DMSO) from Wako Pure Chemicals (Osaka, Japan).

Selection of dosing of I3C

2000 p.p.m. of I3C in diet has been reported as an effective dose in a multi-organ tumorigenesis model in rats (12) and 500 p.p.m. is known to induce cytochrome P450s in the rat liver (20).

Uterotrophic assay (experiment 1)

To assess estrogenic or anti-estrogenic activity of I3C, 38 female Donryu rats were ovariectomized under ether anaesthesia at 9 weeks of age, and starting 2 weeks thereafter were assigned to nine groups receiving: only ovariectomy (controls); daily s.c. treatment of E2 at a dose of 1 μg/kg; 4HE at a dose of 5 μg/kg; 2HE at a dose of 5 μg/kg; 16αHE at dose of 1 μg/kg: daily administration of 500 or 2000 p.p.m.-I3C in basal diets (I3C500 or I3C2000, respectively); or daily s.c. treatment of 1 mg/kg E2 plus I3C500 or I3C2000 for 2 weeks. After 2 weeks treatment, all animals were killed and the uteri were weighed. The uteri and livers were fixed in 10% neutral-buffered formaldehyde solution, routinely processed, sectioned and stained with hematoxylin and eosin. The uteri were measured for the height of the luminal epithelium with an image analyzer, IPAP-Win (Sumika-techno Service Co., Osaka, Japan).

Uterine carcinogenesis (experiments 2 and 3)

To clarify the effects of I3C on rat uterine endometrial adenocarcinoma development, female rats were treated with a single dose of 20 mg/kg ENNG into a unilateral uterine horn via the vagina using a stainless catheter at 11 weeks of age. This is known to exert no carcinogenic effects except in the uteri (39). After the initiation, in experiment 2, 30 animals were fed dietary I3C500 up to 15 months of age (for 12 months), and compared with 24 control rats fed powder basal diet without I3C. At 15 months of age, all surviving animals were necropsied (experiment 2).

For experiment 3 to elucidate sequential changes regarding the effects of I3C on uterine carcinogenesis and hepatic metabolism of E2, 144 females were allocated to the following four groups after the ENNG initiation: control (39 females); dietary I3C2000 (39 females); and twice weekly s.c. treatment with 1 μg/kg E2 (E2, 30 females) or 5 μg/kg 4HE (4HE, 36 females). At 6, 9 and 12 months of age, four to nine animals per group were examined, and all survivors were terminated at 15 months of age. After macroscopic examination, the reproductive system and related organs, including the ovaries, uteri and vagina, endocrine system organs and any macroscopical abnormalities, were fixed in 80% cold ethanol solution (uteri), or 10% neutral-buffered formaldehyde solution (other organs). These tissues and/or organs fixed were routinely processed for histopathological examination.

In both experiments, the upper, middle and lower parts of each uterine horn and the cervix were cut into three pieces in cross-section to evaluate uterine proliferative lesions, classified into three degrees of atypical hyperplasia (slight, moderate or severe) and adenocarcinomas, according to the criteria described previously (37,38). Briefly, slight hyperplasia was used when the numbers of glands with no or slight cellular atypia were increased within the endometrium. Moderate hyperplasia referred to increased numbers of glands with slightly to moderately atypical cells in focal and/or diffuse areas of the endometrium. Severe hyperplasia was composed of irregular proliferations of atypical glands in diffuse area of the endometrium. Adenocarcinomas were diagnosed on the basis of invasion of tumor cells into the muscularis. In addition, adenocarcinomas were subdivided into well, moderately and poorly differentiated types, and also classified as to the degree of invasion: limited to the uterus, invading into the serosa and/or surrounding adnexae, and with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers (43). Animals found dead or killed when moribund were also examined in the same manner. Throughout the two experiments, body weights were measured at regular intervals and clinical signs were checked daily for all animals.

Estrous cyclicity

Vaginal cytology was observed in all animals to investigate estrous cyclicity throughout the study (experiments 2 and 3).

Preparation of livers

At 6, 9, 12 and 15 months of age in experiment 3, right and median lobes of selected livers of each group were frozen in liquid nitrogen for analysis of enzyme activities related to estrogen metabolism (6, 9, 12 and 15 months of age) or mRNA expression of cytochrome P450s by reverse transcription PCR (RT-PCR) (15 months of age), and stored at −80°C until use.

mRNA expression of cytochrome P450 enzymes in the liver

Small pieces of the liver (~200 mg) were obtained from three control and four I3C2000-treated animals in experiment 3 at 15 months of age. The samples were homogenized in 4 ml RLT buffer, mixed with 40 μl β-mercaptoethanol, and RNA was isolated using an RNeasy Midi extraction Kit (QIAGEN, Germany) and stored at −80°C until RT-PCR analysis.

RT-PCR and PCR primers of cytochrome P450 1A1, 1A2, 1B1 and GAPDH mRNA transcription in the present study were done as reported previously (26,44,45). Aliquots (500 ng) of total liver RNA were used for the RT-PCR. The primers were synthesized and purified by Takara Bio (Shiga, Japan). Levels of cytochrome P450s mRNA expression relative to GAPDH mRNA expression were calculated as ratios using an image analyzer (NIH image, Bethesda, MD).

Immunohistochemical distribution of cytochrome P450 enzymes in the liver

Cytochrome P450 protein amounts in the liver were examined immunohistochemically using paraffin-embedded sections from animals in experiments 2 and 3. After blocking endogenous peroxidase by incubation with hydrogen peroxidase (3%, v/v) in methanol, deparaffinized liver sections were incubated with anti-rat CYP 1A1, 1A2, 2B1 or 3A2 (Daiichi Pure Chemicals, Tokyo, Japan), diluted 1:100 in Tris-buffered solution (Takara Bio) with 1% skim milk at 37°C for 1 h. After the incubation, the sections were exposed to secondary antibodies and linked with streptavidin peroxidase using a DAKO LSAB+ kit (DAKO cytomation, CA). Binding was visualized by incubating sections with 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals), and counterstaining with hematoxylin for histopathological examination. Immunohistochemical distribution of CYP 1B1 could not be examined in the present study, because no anti-rat CYP 1B1 antibody was available for immunohistochemistry using formalin-fixed and paraffin-embedded sections.

Enzyme activities related to estrogen metabolism in the liver

Estradiol 2- and 4-hydroxylase and 16α-hydroxylase activities in liver (median lobe) samples obtained from four or five rats in the control-, I3C2000-, E2- or 4HE-treated groups at 6, 9, 12 and 15 (except 16α-hydroxylase activity) months of age in experiment 3 were determined by SRL (Tokyo, Japan), as for previous reports (7,22).

Statistical analysis

Values for incidences including data of uterine proliferative lesions and estrous cyclicity were analyzed statistically using the Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between the treated and control groups were made with the Dunnett's *t*-test. *P* values < 0.05 were considered to be statistically significant. In the uterotrophic assay, the uterine weights and heights in treated groups were compared with those in the control (only ovarietomized rats) and positive control (E2-treated) groups.

Results

Estrogenic or anti-estrogenic activities of I3C (experiment 1)

Uterine weights and heights of the luminal epithelium are shown in Figure 1. Neither dose of I3C affected parameter in ovariectomized rats, with or without E2 replacement. The uterine weights and heights with 5 μg/kg 4HE treatment were comparable with those with 1 μg/kg E2 treatment, while 16αHE and 2HE treatments had much lower and no estrogenic activity, respectively.

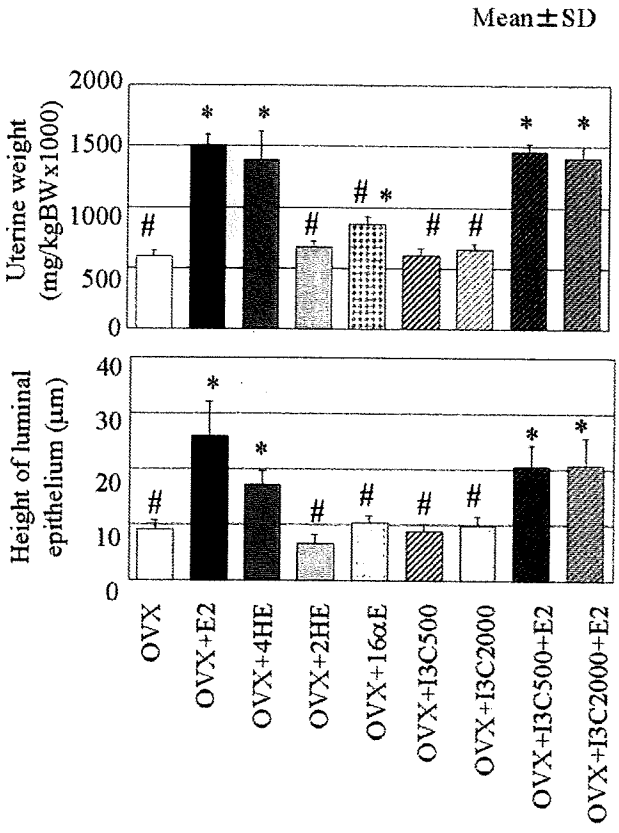

Fig. 1. Relative uterine weights and heights of uterine luminal epithelium in experiment 1. **P* or *#P* refer to significant differences from the control (ovariectomy only) and E2-treated groups, respectively, at 1% or below.

Body weights, clinical signs, survival curves and estrous cyclicity (experiments 2 and 3)

Body weights were depressed by I3C treatment with both doses (data not shown). During experiments 2 and 3, no treatment-related clinical signs were observed and survival curves in all treated groups were comparable with those of the relevant control groups (data not shown). In both experiments, I3C and E2 treatment did not increase persistent estrus (PE) status up to 15 months of age, while subcutaneous treatment of 4HE in experiment 3 significantly increased PE status after 5 months of age (Figure 2).

Effects of I3C on uterine carcinogenesis (experiments 2 and 3)

Incidences of uterine proliferative lesions and data for their multiplicity are shown in Table I. In experiment 2, the incidence of adenocarcinomas in the group treated with I3C500 was significantly elevated compared with the control group. In experiment 3, the incidence of adenocarcinomas was significantly increased in the 4HE group, compared with that of the control group. I3C2000 and E2 treatments also increased the incidences (44 and 50%, respectively) as compared with the control value (22%), but not significantly. Multiplicities of the uterine proliferative lesions were significantly increased by both I3C and 4HE treatments, whereas only a tendency for increase was evident with E2. Histologically, almost all uterine adenocarcinomas were of well-differentiated type, and morphological or biological malignancy was not influenced by the I3C treatment. In sequential observation of uterine tumor development, slight atypical hyperplasias had already appeared in the 4HE- and E2-treated groups at 6 months of age when no proliferative lesions were detected in controls. Development profiles for uterine proliferative lesions for I3C-treated and control animals were comparable up to 12 months of age.

Pathological examination of other organs

At all examined times in experiment 3, the relative liver weights were consistently elevated in the I3C2000 treated group (data no shown). Microscopically, centrilobular hypertrophy of hepatocytes was observed in all I3C-treated groups of experiments 1, 2 and 3. Most ovaries in all groups were atrophic with small cystic atretic follicles and lacking corpus lutea at termination of experiments 2 and 3. In these two experiments, various non-neoplastic and neoplastic lesions were observed in representative organs and other endocrine tissues; however, all lesions were similar to those detected

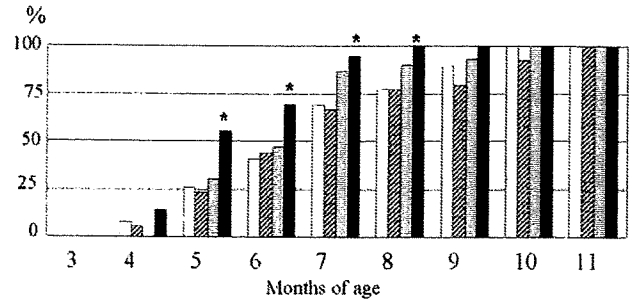

Fig. 2. Percentage incidences of animals showing PE from vaginal cytology in experiment 3. White, stripe, gray and black columns indicate the control, I3C2000-, E2- and 4HE-treated groups, respectively. **P* refers to significant differences from control incidences at 5% or below.

Table I. Incidence of uterine proliferative lesions[a] and their multiplicities in experiments 2 and 3

	No. of rats with no abnormalities	Hyperplasia			Adenocarcinoma	Multiplicities[b]
		Slight	Moderate	Severe		
Experiment 2						
15 months of age						
Control (n = 24)	4	2	5	7	6	1.04 ± 0.62
I3C500 (n = 30)	1	2	3	7	17*	1.50 ± 0.63*
Experiment 3						
15 months of age						
Control (n = 18)	2	2	7	3	4	1.17 ± 0.62
I3C2000 (n = 18)	1	2	5	2	8	1.78 ± 0.73**
E2 (n = 16)	0	3	2	3	8	1.50 ± 0.52
4HE (n = 16)	0	0	5	1	10*	1.69 ± 0.60**

[a]Uterine proliferating lesions include slight to severe atypical hyperplasia and adenocarcinomas, these criteria referred to Nagaoka *et al.* (37,38).
[b]Multiplicities are calculated average number of uterine proliferative lesion per rats, and indicated mean ± SD.
Values in parentheses show the number of rats examined.
*,**Significantly different from relevant control group at $P < 0.05$ and $P < 0.01$, respectively.

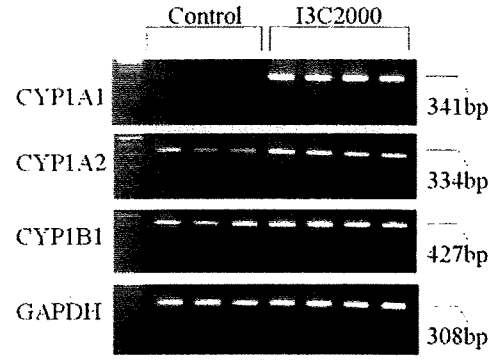

Fig. 3. mRNA expression for CYPs 1A1, 1A2, 1B1 and GAPDH in the livers of control and I3C2000-treated groups at 15 months of age in experiment 3.

spontaneously in this rat strain (36), and there were no differences in these lesions among the groups. Necropsy of animals found dead or killed when moribund also did not reveal any treatment-related changes.

mRNA expression of cytochrome P450s in the liver

Findings for mRNA expression of CYP 1A1, 1A2 and 1B1 in the liver of experiment 3 are demonstrated in Figures 3 and 4. In the control group, CYP 1A1 was not detectable. I3C treatment significantly increased CYP 1A1, 1A2 and 1B1 mRNA expression compared with the control group, with induction of 1A1 expression being the most prominent.

Immunohistochemical staining of cytochrome P450s

CYP 1A1 and 1A2 were clearly demonstrable in the hepatocytes of centrilobular areas in all I3C-treated groups in experiments 2 and 3 up to 12 months of age, while very weak expression of 1A2 was observed in relevant controls (Figure 5). At 15 months of age, 1A1 expression in the I3C-treated group was similar to that at the other examined times, whereas 1A2 expression was too varied to detect any differences from relevant controls in experiments 2 and 3. Results for other CYPs such as 2B1 or 3A2 were comparable among the livers in the control and treated groups up to 15 months of age.

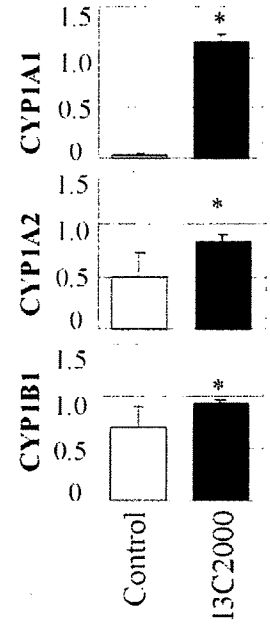

Fig. 4. Levels of expression of CYPs 1A1, 1A2 and 1B1 mRNAs relative to GAPDH mRNA in the liver, as for Figure 3. The intensities of P450s are relative to GAPDH mRNA levels (calculated as 1.0%).

Enzyme activities related to estrogen metabolism in the liver

Estadiol 2-, 4- and 16α-hydroxylase activities in the liver (experiment 3) are shown in Table II. The estradiol 2-hydroxylase activities in the I3C-, E2- and 4HE-treated groups showed increasing trends compared with the control group at most of the examined points. However, there were no significant differences among them due to great variation except 15 months of age, when a significant increase was increased by I3C treatment. The 4-hydroxylase activities demonstrated significant increases in the I3C- and 4HE-treated groups at 9 and 15 months of age, or tendencies for increase in all treated groups at all examined times, except the 4HE-treated group at 12 months of age. At all examined points, 16α-hydroxylase activities showed neither significant differences nor any tendency for change with the treatments.

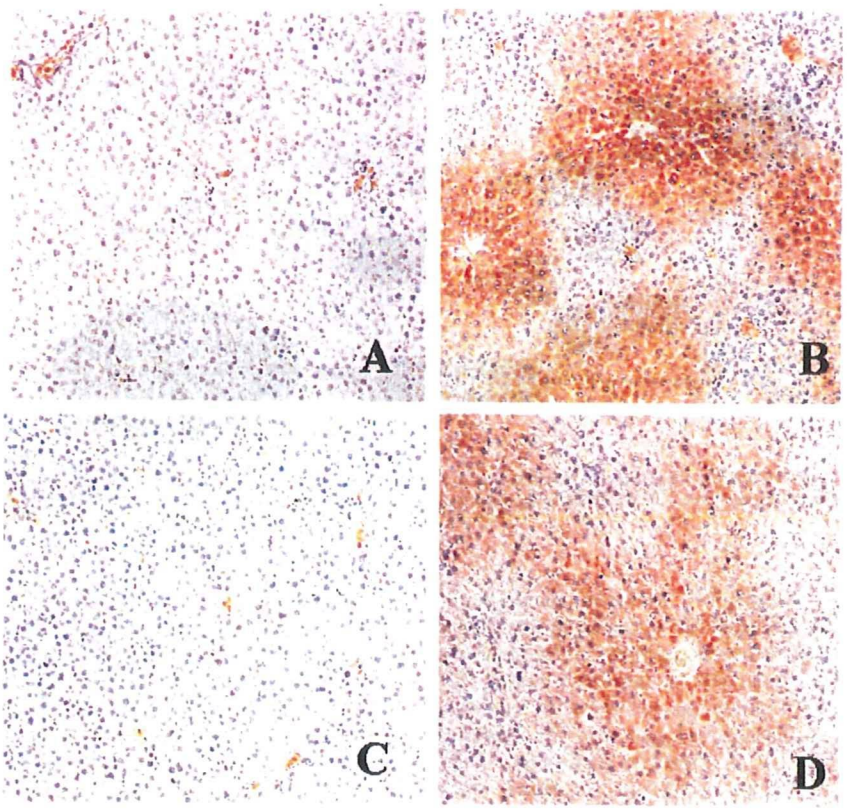

Fig. 5. Immunohistochemical staining of CYP 1A1 and 1A2 in the livers of animals at 15 months of age in experiment 3. (**A** and **B**) CYP 1A1 expression in control and I3C2000-treated animals. (**C** and **D**) CYP 1A2 expression in control and I3C2000-treated animals. Hematoxylin was used for counterstaining.

Table II. Enzyme activity related to estrogen metabolism in the liver (experiment 3)

	Enzyme activities related to estrogen metabolism (pmol/min/mg protein)		
	Estradiol 2-Hydroxylase	Estradiol 4-Hydroxylase	Estradiol 16α-Hydroxylase
6 months of age			
Control (5)	66.76 ± 23.50	2.97 ± 1.18	2.88 ± 1.00
I3C2000 (5)	139.72 ± 83.72	5.24 ± 2.39	2.65 ± 1.31
E2 (3)	104.88 ± 23.44	5.46 ± 2.40	3.34 ± 0.59
4HE (4)	108.14 ± 5.84	5.66 ± 0.58	4.37 ± 0.90
9 months of age			
Control (5)	61.82 ± 29.35	1.78 ± 0.73	1.30 ± 0.21
I3C2000 (5)	88.30 ± 25.79	4.19 ± 1.63*	1.75 ± 0.62
E2 (4)	92.72 ± 24.47	3.44 ± 1.66	2.18 ± 0.34
4HE (3)	110.22 ± 31.36	4.60 ± 1.54*	1.99 ± 0.85
12 months of age			
Control (4)	78.05 ± 29.45	3.52 ± 2.52	1.24 ± 0.36
I3C2000 (4)	149.80 ± 51.88	5.14 ± 2.52	1.05 ± 0.13
E2 (4)	86.37 ± 64.90	5.06 ± 3.16	1.47 ± 0.64
4HE (4)	78.57 ± 8.63	3.20 ± 0.49	1.42 ± 0.64
15 months of age			
Control (4)	75.08 ± 15.23	3.94 ± 1.18	NE
I3C2000 (4)	205.32 ± 51.92**	7.14 ± 1.36**	NE
E2 (4)	67.79 ± 10.55	3.01 ± 0.87	NE
4HE (4)	108.16 ± 7.32	5.97 ± 0.75*	NE

Values in parentheses mean number of rats examined. Values mean average ± SD. NE, not examined.
*Significantly different from control group, $P < 0.05$.
**Significantly different from control group, $P < 0.01$.

Discussion

In the present study using rat uterine cancer model, dietary treatment with I3C clearly demonstrated promoting effects on endometrial adenocarcinoma development. I3C can act both as an inhibitor and promoter of carcinogenesis, and our data are in line with the promoting results observed earlier with several animal carcinogenesis models (11,12). As for a cause of the complex effects, I3C is unstable under the acid condition and a number of acid-catalyzed metabolites such as 3,3′-diindolylmethane and indolcarbazole are produced in the gut (46). The acid condensation product has shown to be a potent aryl hydrocarbon receptor agonist, providing anti-estrogenic and antitumorigenic activity (47). In the present study, the activity of each acid-catalyzed metabolite of I3C to the rat uteri was not investigated; however, the dietary treatment with I3C at doses of 500 or 2000 p.p.m. did not show any estrogenic- or anti-estrogenic activity in the rat uteri, indicating that the promoting effect did not result from direct binding of I3C to estrogen receptor α in the rat uteri as estrogenic or anti-estrogenic agents.

I3C is widely accepted to induce CYPs 1A1, 1A2 and/or 1B1 in the liver and other organs (13–15,20,21). In rats, CYPs 1A1 and 1A2 catalyze mainly E2 into 2HE, the dominant product of catechol estrogen with weak hormonal potency and no carcinogenic effects (7,22,24,25,31), by hepatic 2-hydroxylation of estradiol (24,25), whereas CYP 1B1 is a major catalyzing enzyme of E2 to 4HE, a strongly carcinogenic and toxic metabolite (23,26,32,33,48).

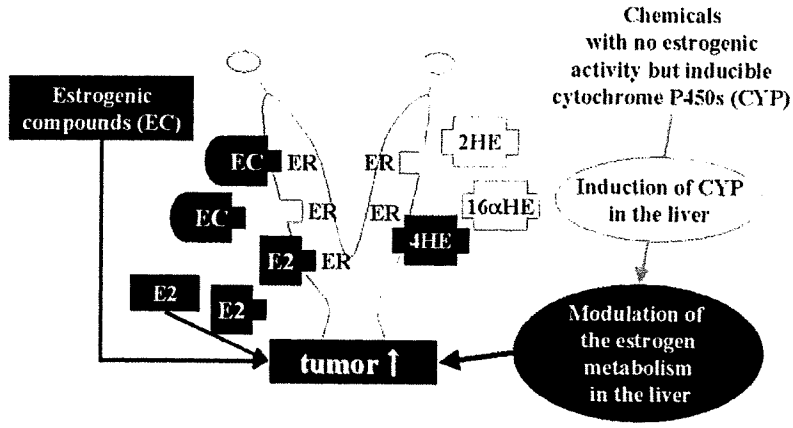

Fig. 6. Hypothesized mechanisms of promoting effects of chemicals with or without estrogenic activity on uterine carcinogenesis in rats. 16αHE, 16α-hydroxyestradiol; ER, estrogen receptor α. Black blocks indicate promotion of uterine carcinogenesis, while white blocks indicate weak or no promoting activity.

In the present study, dietary I3C treatment increased the induction of CYPs 1A1, 1A2 and 1B1 enzymes in the liver at either the mRNA level or its producing proteins. In the assays of estradiol hydroxylase activities in the liver, dietary I3C increased both 2- and 4-hydroxylase activities, in particular the latter. These results strongly suggest that the induction of the CYP 1 family by I3C is linked to modulation of E2 metabolism. In this study we could not determine which enzyme in the CYP 1 family was most effective in this regard. The present finding that 4HE treatment increased uterine adenocarcinoma development provides the evidence that it possesses stronger carcinogenic effects on rat uterus than E2, whereas uterotrophic activity of 4HE was weaker, in line with previous reports (23,32,33,48).

Endometrial adenocarcinoma development is strongly related to estrogen exposure in women and the Donryu rat features endocrinological similarities to the human case, ovarian hormonal imbalance leading to elevation of the serum estrogen/progesterone ratio, manifested as atrophic ovary with small polycystic atretic follicles and lack of corpora lutea and a long-term PE status as indicated by vaginal cytology (36–38). Using the two-stage uterine carcinogenesis model in this rat strain (39), continuous stimulation by estrogens or estrogenic compounds, which directly bind to estrogen receptor in the uteri or induction of early occurrence of the PE status enhanced uterine carcinogenesis (49,50).

In the present study, dietary I3C enhanced uterine carcinogenesis without affecting estrous cyclicity or showing estrogenic activity in the uteri. Induction of CYPs 1A1, 1A2 and 1B1 in the liver by dietary I3C and sequential modulation of estrogen metabolism therefore should be nominated as crucial to the promoting effects. The modulation, in particular the continuous increase of 4HE level, by I3C treatment might be important as part of the hypothesized pathway described schematically in Figure 6.

A number of chemicals and environmental pollutants induce CYP 1 family enzymes in the liver or other organs (26,34,51,52). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin or diesel exhaust is reported to up-regulate CYP 1B1 (45,51–54). In most mammalian species, the main estradiol metabolites were generated by hepatic microsomal P450s in the liver and other tissues (22,23) and the functions and regulation of CYPs 1A1 and 1A2 appear to be highly conserved (55). Therefore, animal data concerning modulation of estrogen metabolism via induction of CYP 1 family may provide useful information for human risk assessment, although further investigations are required to detail their exact significance.

The precise reason for the discrepancy between the promotion observed here and the previous report that dietary I3C inhibited spontaneous uterine tumor development in Donryu rats (7) could not be determined. The differences might be due to the dietary doses applied, or resultant variation in the ratios of E2 to 2HE and 4HE, especially the latter, in addition to the difference in the uteri with or without initiation of ENNG. Several reports proposed that the ratio of 2HE/4HE formation was important as a marker of estrogen-dependent tumor development (26,33).

Acknowledgements

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