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Differential Gene Expression in Estradiol-3-Benzoate-Treated Liver and Chemically-Induced Hepatocellular Carcinoma

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Abstract In a previous study by the current authors, hepatocellular carcinoma (HCC) was determined to be epidemiologically sex-dependent, and the incidence and multiplicity of HCC found to decrease in estradiol-3 benzoate (EB)-treated F344 rats. Therefore, to ascertain the anticancer mechanism of EB, a commercially available cDNA microarray, with a total of 14,815 cDNA rat gene clones, was used to determine the differentially expressed genes in nontreated livers, EB-treated livers, and diethynitrosolamine (DEN)-induced HCC. In the sequenced experiment, a total of 85 genes were differentially expressed at either two or more times the rate of the normal expression, where 33 genes were downregulated by EB, and 52 genes upregulated. Candidate genes were selected according to significant changes observed in the mRNA expression in the EB-treated livers compared with the nontreated livers, then these genes were filtered according to their different expression patterns in the DEN-induced tumors compared to the estrogen-treated livers. To confirm the microarray data, a real-time PCR analysis was performed for ten selected genes: the H-ras revertant protein 107 (H-rev107), insulin-like growth factor binding protein (IGFBP), parathyroid hormone receptor (PTHR), SH3 domain binding protein (SH3BP), metallothionein, src-suppressed C-kinase substrate (SSeCK) gene, phosphodiesterase I, CD44, epithelial membrane protein 3 (EMP3), and estrogen receptor α (ERα). The SSeCK and phosphodiesterase I genes were both upregulated in the DEN-induced hepatocarcinomas, yet their possible carcinogenic functions remain unknown. Meanwhile, the other genes were downregulated, including the genes related to growth regulation (IGFBP, H-rev107, ERα), adipogenesis inhibition (PTHR), and tumor suppression (metallothionein).

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Estrogens are a family of structurally related hormones that function in many different tissue types, and are relevant in both male and female physiology. However, the highest amounts of estrogen receptors (ERs) are found in reproductive tissues, including such organs as the mammary glands, ovaries, vagina, and uterus, where estradiol stimulates cell proliferation and the biosynthesis of the progesterone receptor [12]. Meanwhile, in the male physiology, ER can be detected in the epididymis and prostate. Estrogens also function in other, less expected tissues, including the brain, bones, cardiovascular system, kidneys, immune system, and liver. Furthermore, estrogen deficiency would seem to be involved in many pathological processes, such as arteriosclerosis [10], osteoporosis [30], and the degenerative processes in the CNS [9], whereas elevated estrogen levels are believed to support the development and promotion of tumors [4].

The liver contains enzymes that are essential for hormonal biotransformation and the regulation of numerous metabolic reactions that control hormone metabolism. The liver also manufactures several proteins that carry circulating hormones to their respective effector sites. Yet, the endocrine system exerts tight control of the metabolic reactions within the liver; hence the liver can often be disturbed by endocrine disorders.

Hepatocellular carcinoma (HCC) can be attributed to a clinical history of liver cirrhosis [15]. However, the incidence of progression from hepatic fibrosis to liver cirrhosis in female rats treated with CCl_4 has been found to be significantly lower than that observed in male rats. Estradiol reduces

CCl_4-induced liver cirrhosis, while tamoxifen, an estrogen antagonist, has the opposite effect. Therefore, the antifibrogenic role of estrogen in the liver may be one reason for the sex-associated differences in the progression from hepatic fibrosis to cirrhosis [33]. HCC accounts for more than 90% of all primary liver cancers, ranks fifth in frequency among all malignancies worldwide, and causes nearly one million deaths annually [34]. In general, men are two to four times more frequently afflicted with HCC than women. Although both men and women have a higher incidence of HCC in cirrhosis, the development of HCC in cirrhotic livers occurs with much greater frequency in men than in women [8]. Moreover, carcinomas experimentally induced using carcinogens as well as the appearance of spontaneous neoplasms occur at a higher incidence in male rats and mice [14, 25]. As such, these reports suggest that there are marked sex-associated differences in hepatocarcinogenesis, and also that sex hormones may be involved. In other experiments, androgen was found to enhance the growth of liver tumor cells [7], while the removal of androgen inhibited the growth of liver tumor cells [18]. Conversely, estrogen inhibited malignant transformations and reduced the occurrence of preneoplastic lesions in the liver [19, 27]. Consequently, these facts indicate that androgens act as promoting factors, whereas estrogen acts as an inhibiting factor in hepatocarcinogenesis.

Accordingly, in the current study, an EB treatment experiment verified the progressive modification of estrogen receptor expression in the liver during diethylnitrosamine (DEN)-induced hepatocellular carcinogenesis. Using cDNA microarray technology, the genes that were differentially regulated in response to EB treatment were then identified to delineate the mechanisms of sex-associated hepatocellular carcinogenesis. The feasibility of this approach was also validated by a real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting.

MATERIALS AND METHODS

Animals and Chemical Treatment

This study used male F344 rats that were supplied by the National Institute of Toxicological Research (Korea). The rats were allowed access to distilled water and pellet chow diets (CRF-1, Charles River Japan, Tokyo, Japan) *ad libitum*.

To observe the role of estrogen, an estradiol-3-benzoate (EB) pellet was prepared by mixing 1.32 mg of EB, 2 g of cholesterol, and 1.7 ml of olive oil and inserted into a silastic tube (Silicon Medical Tube No. 2; Kaneka Medix Corp., Japan). The EB content in each 1 cm tube was approximately 10 μg. The estradiol benzoate (EB) pellet was implanted subcutaneously at 6 weeks, then changed every 4 weeks for 5 months. To chemically induce liver carcinogenesis, the rats received diethynitrosamine (DEN; Sigma, St Louis, MO, U.S.A.) dissolved in dimethyl sulfoxide (DMSO) at a dose of 47.5 mg at 6 weeks of age using mini-osmotic pumps (Durect, Cupertino, CA, U.S.A.), which provided a continuous infusion (0.5 μl/h for 2 weeks) and were inserted into the abdominal cavity of each animal under ether anesthesia. The rats were sacrificed at 32 weeks.

Protein Extraction and Western Immunoblotting

The liver tissue samples were homogenized with a tissue grinder (Pyrex corning glass, Corning, NY, U.S.A.) in 5 volumes of a buffer containing 600 mM Tris-HCl, 1 mM EDTA (pH 7.4), and four protease inhibitors (leupeptin 1 μM, aprotinin 0.2 μM, PMSF 1 mM, and pepstatin 1 μM) per 50 ml at 4°C. The homogenates were then centrifuged for 1 h at 105,000 ×*g*, and the protein content of the supernatant fractions purified using Vivapure (Vivascience, Hannover, Niedersachsen, Germany) and measured based on a DC Protein Assay (Bio Rad, Hercules, CA, U.S.A.), with BSA as the standard. One hundred μg of proteins were resolved on 10% SDS-polyacrylamide gels in a Tris-glycine buffer, and electrotransferred to PVDF membranes (Bio Rad) using an Xcell II blot (NOVEX, Carlsbad, CA, U.S.A.). ERα and ERβ were visualized by incubation with primary antibody solutions (1:1,000) of ERα (Santa Cruz sc-7207, Santa Cruz, CA, U.S.A.) and ERβ (Santa Cruz sc-8974) overnight and anti-rabbit IgG streptavidin-HRP (1:2,000, Santa Cruz) for 30 min. The control protein used anti-beta actin (Santa Cruz sc-1616), and the bands were visualized based on their reaction with luminol (Santa Cruz).

RNA Extraction and cDNA Microarray

The total mRNA was isolated from the liver with RNAwiz (Ambion, Inc. Austin, TX, U.S.A.) according to the manufacturer's instructions. To anneal the primer, the oligo-dT primer was mixed with 50 μg of each total RNA, then the mixture was heated to 65°C for 10 min and cooled on ice. The reverse transcription procedure was performed using Superscript II (200 U/ul, Gibco BRL, Carlsbad, CA, U.S.A.), plus Cy3 and Cy5 fluorescence dye was added to each reaction group, and the reactions incubated at 42°C for 2 h. The Cy5 probe and Cy3 probe were mixed in the same Microcon YM-30 column (Millipore, Billerica, MA, U.S.A.), which was spun for 7–8 min at 14,000 rpm and the flow-through removed. The microcon was then inverted into a clean tube and spun briefly at 14,000 rpm to recover the probe.

The Agilent rat cDNA microarray, consisting of 14,815 cDNA clones, was used for this study. As such, the preparation of the rat microarrays, probe labeling, hybridization, microarray scanning, normalization, and ratio determination were all performed according to the protocols available from

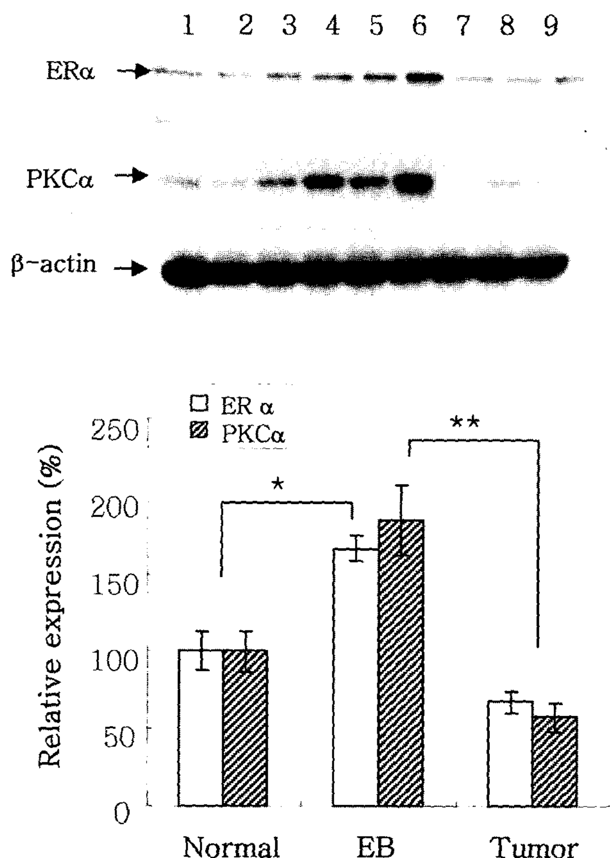

Fig. 1. Western blot analysis of the expression pattern for estrogen receptor α and protein kinase Cα in liver.

Upper figure is normal liver (1-3), EB-treated liver (4-6), and HCC tumor (7-9). The graph below shows the counted density. * Means significantly different from normal and EB ($P<0.05$). **Means significantly different from EB and tumor ($P<0.01$). The expression levels in each tissue sample were normalized with respect to the β-actin signal, and are expressed as a percentage of the control. Values show the mean±SEM.

Agilent Technologies. (Palo Alto, CA, U.S.A.). Each hybridization was performed in duplicate and the duplicate gene expression profiles compared for three different cell types; EB-treated liver, normal liver, and DEN-induced HCC (Fig. 1). The microarray slide was scanned using a Scan Array 5000 (GSI Lumomics, Ottawa, Japan) at a laser setting that preserves linearity and minimizes background. The images acquired by the scanner were analyzed using GenePix Pro 3.0 software, the image analysis (grid generation and dye quantification) of the scanned slides was performed using ImaGene v4.2 (Bio-Discovery), and the data mining was conducted using GeneSpring v4.2 (Silicon Genetics), in which 3 normalization steps were performed: per spot, intensity-dependent, and per chip (slide). Several spots were found with scores of -1.00 or +1.00, where only normalized ratios were found in the replicate slides for the set 1 or set 2 comparative experiments [13].

Real-Time PCR

The real-time PCR analysis was performed using an I-cycler (Bio-rad, Hercules, CA, U.S.A.) and SYBR Green DNA PCR Core Reagent Kit according to manufacturer's instructions. For each time point, the cDNA was analyzed from the total liver RNA of five control rats and five rats from each treatment group (i.e. EB, EB plus DEN, and DEN-only). All reactions were performed in triplicate. The thermal cycling conditions for the real-time PCR were 10 min at 95°C to activate the Amplitaq Gold, followed by 40 cycles of denaturation for 15 sec at 95°C, and annealing/extension for 30 sec at 60°C. The β-Actin expression was quantified to normalize the amount of cDNA in each sample, then the specificity of the amplified product was monitored based on its melting curve. The PCR primers were designed using the primer3 website (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi), and are shown in Table 1. A non-reverse transcription control was run with every assay to assess the contamination of the RNA by genomic DNA. A Student's t-test was also used to compare the expression of each gene (relative to β-actin) in the livers of the 5 rats from each treatment group [2, 11].

Statistical Analysis

The density data for the immunoblotting tests are presented as the mean±standard error (SEM), and were analyzed by

Table 1. Primers used in real-time PCR verification.

	Primer set (5'→3')	
Gene	Forward	Reverse
Phosphodiesterase I	TCATTCAGAAAGGAATGGAG	ATTTCCGAATAGCTACGGCT
Rat insulin-like growth-factor-binding protein	CCTAAGTCAGGCATGAAGGA	GTGGTTTACTGCACCCTTTG
EMP3	GGACTCTCCCAGAGAAGGAG	AGGACAGACAGCAGAGGATG
SH3-binding protein	GATGAAGTTCCTGGCTCTGC	TGGCAACTCCTCTGAGACCT
Parathyroid hormone receptor	ACTTGCACAGCCTCATCTTC	TTCCTAATCTCTGCCTGCAC
Estrogen receptor α	TGACCAACCTGGCAGACAGG	GCCTTTGTTACTCATGTGCC
Rat (Sprague-Dawley) H-rev107 mRNA	CAGGCTGACCAGTGAGAACT	AGGTCCTGAGTTCAAATCCC
Rat metallothionein-2 and metallothionein-1	TCTTGAGTTCAAGGCCAGAC	CTAAGACTGCTGGCTTCTCG
Rat mitogenic regulation SSeCKS (322) gene	CGGCTACTGCTCAGAAAGAG	TTCCTGCTCACCTTCTTCAC
CD44	GGAGTTAGCCCTGAGAAAGG	GGATGAGTCTCCATCACTGG

unpaired t-tests using the Prism 3.0 program. The means were considered statistically different if P<0.05.

RESULTS

Previous studies have demonstrated that EB interferes with the tumor-promoting effect of DEN [as yet unpublished]. Thus, to determine whether EB also upregulates ERα protein expression, a Western blotting analysis was performed on rat livers treated with EB and DEN-induced tumors. As shown in Fig. 1, the ERα protein expression in the tumor

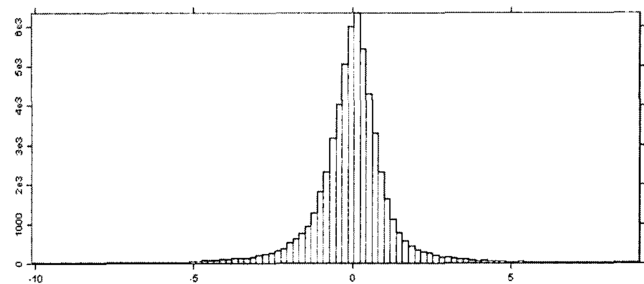

Fig. 2. Histogram displaying median expression profiles of all genes represented in the cDNA microarray, based on four replicate slides.

Table 2. Representative list of differentially expressed genes in estrogen-treated liver, for comparison with normal liver and DEN-induced hepatocellular carcinoma.

GenBank access no.	Gene name	Mean fold change (log)
	Genes Upregulated by EB treatment	
AF079846	Mouse dematin 48 kDa subunit (Epb4.9)	6.9578
J05035	Rat steroid 5 alpha-reductase	3.9916
M31672	Rat insulin-like growth-factor-binding protein	3.8253
U09583	Rat Sprague-Dawley src-related tyrosine kinase	3.8247
AB041537	Mouse brain cDNA, clone MNCb-4931, similar to X96994 BR-1 protein (Helix pomatia)	3.5466
U94828	Mouse retinally abundant regulator of G-protein signaling mRGS-r (RGS-r)	3.5237
AF180471	Mouse Kiaa0188	2.9797
AB009372	Rat mRNA for lysophospholipase	2.9425
X96553	Rat mRNA for hepatocyte nuclear factor 6 alpha	2.9193
D10728	Rat mRNA for lymphocyte antigen CD5	2.8744
X76453	Rat (Sprague-Dawley) H-rev107	2.771
AB010711	Mouse (Mus sp.) mRNA for MB20	2.5526
AF201285	Mouse TSC22-related inducible leucine zipper 1b (Tilz1b)	2.342
AF106658	Rat deubiquitinating enzyme Ubp45 (ubp45)	2.3073
Z11690	Rat mRNA for V1a arginine vasopressin receptor	2.2967
U17607	Rat CCAAT binding transcription factor CBF subunit C	2.2744
AF221952	Rat mu-protocadherin	2.1935
U88630	Rat Sp1-like zinc finger protein (TIEG)	2.1694
M59742	Rat GABA transporter protein	2.0738
AF081825	Rat sodium-dependent high-affinity dicarboxylate transporter (NADC3)	2.0698
AAD39140	Xenobiotic/medium-chain fatty acid:CoA ligase form XL-III	2.0345
M29084	Rat T-kininogen (T-KG) gene, exon 2	1.9591
M58308	Rat histidase	1.9212
AF186092	Rat transducin-like enhancer 3 (Esp3)	1.888
U93880	Rat insulin receptor substrate-3 (IRS-3)	1.8855
U85259	Estrogen-related receptor alpha	1.8779
D85036	Mouse AK4 mRNA for adenylate kinase 4	1.8759
X62295	Rat mRNA for vascular type-1 angiotensin II receptor	1.8747
CAA55956	NADP+-dependent malic enzyme	1.8651
X87671	3BP-1, an SH3 domain-binding protein	1.8527
CAC11110	RNA polymerase III subunit	1.8109
X07365	Rat mRNA for glutathione peroxidase	1.7729
AB021967	Mouse mRNA for adhesion protein RA175N	1.7464
U66723	Rat purine-selective sodium/nucleoside cotransporter (rCNT2)	1.6509
AF033186	Mouse WSB-1	1.6433
AAF59581	Contains similarity to TR:O23191	1.6081

Table 2. Continued.

GenBank access no.	Gene name	Mean fold change (log)
L23088	Rat P-selectin	1.5538
AAC39669	Putative seven pass transmembrane protein	1.5384
AF180801	Mouse peroxisomal long-chain acyl-CoA thioesterase Ib (Pte1b) gene, exon 3	1.5013
L14441	Rat phosphotidylethanolamine N-methyltransferase	1.4642
CAB81951	Integral membrane transporter protein	1.4577
M11794	Rat metallothionein-2 and metallothionein-1 genes	1.4532
Y07711	Mouse mRNA for zyxin	1.4496
AF004017	Rat electrogenic Na+ bicarbonate cotransporter (NBC)	1.3906
AB012933	Rat mRNA for acyl-CoA synthetase 5	1.3643
BAA24419	Alpha-antitrypsin-like protein	1.3534
CAC15920	Microtubule-actin crosslinking factor	1.2702
M77184	Rat parathyroid hormone receptor mRNA	1.2453
U78875	Rat zinc finger transcription factor homolog CPG20 (cpg20)	1.2412
AF124429	Mouse claudin-14	1.2378
AF021345	Mouse plasma selenoprotein P (SELP) gene	1.2277
BAA31202	UDP-N-acetylglucosamine pyrophosphorylase	1.1382
U19485	Rat spp-24 precursor	1.119
	Genes Downregulated by EB Treatment	
X63744	Rat mRNA for glutamate/aspartate transporter protein	-7.3722
X15313	Mouse MPO mRNA for myeloperoxidase (EC 1.11.7)	-6.3123
X89264	Mouse mRNA for zinc-finger protein Zfp-37	-5.6992
U88984	Mouse NIK	-4.5764
BAA33019	Kinesin-like DNA-binding protein	-4.2306
U49062	Rat heat-stable antigen CD24	-4.0743
U23146	Rat mitogenic regulation SSeCKS (322) gene	-4.0426
U69262	Mouse matrilin-2 precursor	-3.9963
AF144756	Rat adipocyte lipid-binding protein (ALBP)	-3.6702
AAC82536	GC-rich sequence DNA-binding factor	-3.5025
M61875	Rat glycoprotein CD44 (CD44) mRNA	-3.4931
X60767	Rat mRNA for cdc2 promoter region	-3.2883
S65555	Gamma-glutamylcysteine synthetase light chain [rats, kidney, mRNA, 1380 nt]	-2.9832
X55660	Rat pcRF104 mRNA for furin	-2.9682
J04632	Mouse glutathione S-transferase class mu (GST1-1)	-2.7556
Y10889	Rat mRNA for EMP3 homolog	-2.7437
AAC97955	Putative dimethyladenosine transferase	-2.5451
AC004807	Mouse chromosome 11, clone mCIT.268_P_23	-2.4421
X65026	Mouse mRNA for GTP-binding protein	-2.4014
AB036747	Mouse Mporc-a mRNA for porcupine-A	-2.3696
AF079366	Mouse Dlgh3 protein (Dlgh3)	-2.3667
AF023621	Rat sortilin	-2.2868
AF245448	Mouse sphingosine kinase type 2 isoform	-2.1444
CAB07297	Contains similarity to Pfam domain: PF01603 (Protein phosphatase 2A regulatory B subunit (B56 family))	-1.9885
AJ132661	Rat brain mRNA for cysteine-sulfinate decarboxylase	-1.9084
AF311213	Mouse BAC 171m12 MESDC1 (Mesdc1) and MESDC2 (Mesdc2) genes	-1.8403
AF084569	Mesocricetus auratus cph proto-oncogene product (cph)	-1.7652
D50410	Mouse mRNA for meltrin beta	-1.7254
CAB94756	Axonemal dynein heavy chain	-1.7194
AF135059	Rat fibrillin-1	-1.4954
D14014	Rat mRNA for cyclin D1	-1.4223
AC007665	Mouse chromosome 18 clone mgsriii-p1-3084 strain RIII fibroblast cell line C127	-1.4097
D28560	Rat mRNA for phosphodiesterase I	-1.37278
AF224494	Mouse arsenite-inducible RNA-associated protein (Airap)	-1.3925

Table 3. Comparative analysis of real-time RT-PCR of selected genes. The real-time data represent the average fold change for each gene in three independent experiments.

Gene ID	Control	EB	EB+DEN	DEN tumor
Rat insulin-like growth-factor-binding protein (IGFBP)	1	4.09±0.85	7.52±0.77	0.08±0.02
Rat (Sprague-Dawley) H-rev107 mRNA (H-rev107)	1	3.31±0.64	10.84±0.75	1.30±0.07
Parathyroid hormone receptor (PHR)	1	1.51±0.18	1.62±0.18	0.06±0.01
SH3-binding protein (SH3BP)	1	1.51±0.17	2.03±0.34	0.62±0.12
Rat metallothionein-2 and metallothionein-1	1	1.30±0.15	1.53±0.16	0.36±0.02
Estrogen receptor α (ERα)	1	1.21±0.11	1.58±0.25	0.68±0.13
Phosphodiesterase I (PD)	1	0.47±0.08	0.39±0.05	1.27±0.07
EMP3	1	0.23±0.03	0.61±0.03	3.31±0.12
Rat mitogenic regulation SSeCKS (322) gene (SseCK)	1	0.30±0.02	0.51±0.02	2.41±0.09
CD44	1	1.22±0.12	1.39±0.23	6.40±0.06

tissue decreased significantly, yet the EB-treated liver showed a higher expression level of ERα than in the control. As regards other signaling, protein kinase C is rapidly upregulated in an ER-independent manner when treated with estradiol [23, 29]. However, in the current study, PKCα exhibited the same expression pattern as ERα (Fig. 1). Thus, the current results indicate that the EB signaling in the rat livers was mediated by both ERα and PKCα.

Since the anticarcinogenic effect of EB could be mediated through modulated gene expression in rat HCC, the gene expression profiles of the rat livers in response to EB were obtained using a cDNA microarray containing 14,800 rat genes. Sequential hybridizations were performed using different cDNA probes, and liver tissue samples collected from normal livers, EB-treated livers, and DEN-induced HCC. Figure 2 shows a histogram representing all the slides from the calibration experiments, where the horizontal axis is labeled with class intervals or subsets used to partition the data, while the height of the horizontal axis reflects the frequency of the gene expression values by subset.

As such, the results demonstrated that the 1260 and 2304 genes were both differentially expressed in the EB-treated tissue and DEN-induced tumors. Meanwhile, in the cDNA microarray data, ERα was observed to be downregulated in the tumors, compared to the normal liver tissue, as shown by immunoblotting.

Among the downregulated genes in the tumors, 50 genes were highly expressed in the EB-treated livers, when compared with the normal livers. Conversely, among the upregulated genes in the tumors, 30 genes were less expressed in the EB-treated livers than in the normal livers. As such, these genes may have been influenced by EB, and related to the inhibition of carcinogenesis in the rat livers. Lists of the upregulated and downregulated genes in the EB-treated livers are shown in Table 2.

To validate the microarray data, a quantitative real-time RT-PCR was performed, and each gene normalized with respect to GAPDH using the same cDNA pool. From the list of genes in the EB-treated livers, the insulin-like growth-factor-binding protein (IGFBP), H-rev107, parathyroid receptor (PTHR), metallothionein (MT), SH3 domain-binding protein (SH3BP), and ERα were selected as the upegulated genes, while phosphodiesterase I (PDI), epithelial membrane protein 3 (EMP3), glycoprotein CD44, and the mitogen regulator SSeCKS were selected as the downregulated genes (Table 3).

To further identify the effect of EB on mRNA expression, livers treated with both EB and DEN were also used. The RT-PCR results, as shown in Table 3, demonstrated identical expression patterns for all the selected genes, as revealed by the microarray analysis. The upregulated genes in the EB-treated tissue were shown to significantly enhance the mRNA expression, as revealed by the microarray analysis. Moreover, the EB- and DEN-treated samples also showed a higher mRNA expression level. However, the DEN-treated samples revealed lower expression levels of the upregulated genes than in the normal samples, whereas the genes downregulated by EB were more highly expressed in the DEN-treated livers than in the normal livers. Therefore, the real-time quantitative PCR on selected representative genes confirmed the validity of the cDNA microarray as regards the appropriate identification of differentially expressed genes in the normal and EB-treated livers, and in HCC.

DISCUSSION

Preliminary data have shown that estrogenization inhibits preneoplastic liver cells [27] and the incidence of hepatocellular carcinomas. Therefore, the goal of this study was to gain a better understanding of the mechanisms of rat liver anticarcinogenesis with EB treatment. To identify the important effects of estrogen-related protein expression changes, the expression patterns of ERα and protein kinase C were examined first. ERα is generally known to function as a ligand-dependent transcription factor, which

increases gene transcription from promoters by the direct binding of the receptor to specific DNA target sequences designated as estrogen response elements (EREs) [26]. In the current study, when treated with carcinogenic chemicals, the expression level of ERα in the estrogenized rat livers remained relatively high with a reduced tumor incidence. In addition, estrogen stimulates protein kinase C (PKC) in an ERα-independent manner, via mechanisms that are independent of new gene expression [29]. Here, the ERα and PKCα expression in the EB-treated livers was consistent with classical mechanisms of ligand-dependent estrogenic action, and appeared to be linked to intracellular signal transduction proteins, a nongenomic effect of estrogen, indicating that the liver can be influenced by estrogenic signals through any pathway.

To identify the genes regulated by EB, this study used cDNA microarray systems to comprehensively analyze the differential expression of the genes in the EB-treated livers and DEN-induced HCC, and the expression signals of 85 genes were found to be significantly altered. The expression changes in eight genes were then selected for verification by a real-time PCR analysis, and an agreement rate of 78% (eleven of fourteen) was obtained. The current data indicated that the genes involved in the estrogenic signals and anticarcinogenesis tended to be upregulated by EB, rather than downregulated, suggesting a possible induction of these cell functions by EB. The verified upregulated genes were H-rev107, SH3BP, IGFBP, PHR, metallothionein, and ERα. The class II tumor suppressor H-rev107 is a ubiquitously expressed gene encoding a 16 kDa protein, and is localized in both the cytoplasm and cell membrane [24]. H-rev107, associated with growth arrest, is also downregulated in many carcinomas and tumor cell lines. 3BP-1, an SH3 domain-binding protein (SH3BP), exhibits GTP activating protein (GAP) activity for Rac and inhibits growth factor-induced membrane ruffling in fibroblasts [3]. Oral estrogen replacement therapy seems to enhance the plasma levels of IGFBP-1, plus, an elevated IGFBP-1 level itself may also contribute to estrogenic effects, by inhibiting the cellular growth-promoting effects of IGF-I [21]. Metallothionein (MT) is downregulated in HCC, and other studies have shown very little staining for MT in human primary HCC and metastatic adenocarcinoma, while adjacent normal hepatocytes are MT-positive [1, 6]. Estrogen-stimulated messenger RNA (mRNA) upregulates the expression of parathyroid hormone/parathyroid hormone-related protein receptors (PTH/PTHrP-Rs), decreases adipocyte numbers, and downregulates the mRNA expression of peroxisome proliferator-activated receptor-gamma (PPARgamma) 2, adipocyte protein 2 (aP2), and lipoprotein lipase (LPL) in bone marrow [5]. The PTHR protein and mRNA have been found in the liver [31]. Accordingly, the above studies and current results suggest that estrogen inhibits adipogenesis in the liver, and also inhibits sclerosis.

Meanwhile, the SSeCKS, PD-1, EMP3, and CD44 genes were verified to be downregulated in the EB-treated livers, and upregulated in the DEN-induced tumors. EMP3 genes have been associated with oncogene ERBB2 (HER2/neu) overexpression in human mammary luminal epithelial cells [17]. CD44 is a multifunctional cell surface glycoprotein involved in lymphocyte homing and activation, tumor growth, and metastasis [32, 35]. In estrogen-treated samples, CD44 has not been associated with estrogenic signaling, while in HCC, it has been related with malignancy as a tumorigenic factor. Phosphodiesterase-I, an autophosphorylating protein kinase, has been described as a membrane protein plasma-cell-differentiation antigen 1 (PC-1) [28]. A polymorphism of the human glycoprotein PC-1 gene, the coding region is strongly associated with insulin resistance [22]. SSeCKS, isolated as a G13S inhibitor that is downregulated in src- and ras-transformed cells, is a major cytoskeleton-associated PKC substrate with tumor-suppressing and kinase-scaffolding activities [16, 20]. However, this study showed that SSeCKS was expressed at higher levels in HCC than under normal conditions, plus SSeCK and PKC expression exhibited the opposite pattern, suggesting that these genes may have unique individual signaling cascades; however, this idea requires further study.

In conclusion, the data from this study indicate that EB causes differences in the pathways used for estrogenic signals and hepatocellular carcinogenesis. Based on cDNA microarray technology, it was shown that EB induced the upregulation of genes involved in growth inhibition, tumor suppression, adipogenesis inhibition, and classical signaling, in association with ERα; whereas, ERα was downregulated in HCC, and associated with the downregulation of genes containing oncogenic signals and tumorigenic factors. These techniques are complementary, and the combined data may help explain the estrogenic action in the liver, most notably how it prevents HCC in estrogenized male rats. Further analysis of the other known genes in this study is expected to shed more light on the onset of hepatocarcinogenesis. Also, a future challenge is to identify the numerous unknown ESTs and proteins associated with antihepatocarcinogenesis due to EB.

References

1. Cai, L., C. J. Wang, Z. L. Xu, D. X. Deng, S. Chakrabarti, and M. G. Cherian. 1998. Metallothionein and apoptosis in primary human hepatocellular carcinoma from northern China. *Anticancer Res.* **18:** 4667-4672.
2. Choi, J. Y., M. Kim, and J. H. Lee. 2002. Reevaluation of the change of leuconostoc species and *Lactobacillus plantarum* by PCR during kimchi fermentation. *J. Microbiol. Biotechnol.* **12:** 166-171.

3. Cicchetti, P., A. J. Ridley, Y. Zheng, R. A. Cerione, and D. Baltimore. 1995. 3BP-1, an SH3 domain binding protein, has GAP activity for Rac and inhibits growth factor-induced membrane ruffling in fibroblasts. *EMBO J.* **14:** 3127-3135.
4. Colditz, G. A., K. M. Egan, and M. J. Stampfer. 1993. Hormone replacement therapy and risk of breast cancer: Results from epidemiologic studies. *Am. J. Obstet. Gynecol.* **168:** 1473-1480.
5. Dang, Z. C., R. L. van Bezooijen, M. Karperien, S. E. Papapoulos, and C. W. Lowik. 2002. Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis. *J. Bone Miner. Res.* **17:** 394-405.
6. Deng, D. X., S. Chakrabarti, M. P. Waalkes, and M. G. Cherian. 1998. Metallothionein and apoptosis in primary human hepatocellular carcinoma and metastatic carcinoma. *Histopathology* **32:** 348-355.
7. Erdstein, J., S. Wisebord, S. Y. Mishkin, and S. Mishkin. 1989. The effect of several sex steroid hormones on the growth rate of three Morris hepatoma tumor lines. *Hepatology* **9:** 621-624.
8. Even, C., G. Launoy, T. Collet, O. Duval, M. A. Piquet, A. Rougereau, J. C. Verwaerde, and T. Dao. 1997. Epidemiology of hepatocellular carcinoma in the department of Calvados. *Gastroenterol. Clin. Biol.* **21:** 450-458.
9. Fillit, H., H. Weinreb, I. Cholst, V. Luine, B. McEwen, R. Amador, and J. Zabriskie. 1986. Observations in a preliminary open trial of estradiol therapy for senile dementia-Alzheimer's type. *Psychoneuroendocrinology* **11:** 337-345.
10. Henderson, B. E., R. K. Ross, A. Paganini-Hill, and T. M. Mack. 1986. Estrogen use and cardiovascular disease. *Am. J. Obstet. Gynecol.* **154:** 1181-1186.
11. Hoe, H. S., I. G. Jo, H. J. Shin, H. J. Jeon, H. K. Kim, J. S. Lee, Y. S. Kim, D. S. Lee, and S. T. Kwon. 2003. Cloning and expression of the gene for inorganic pyrophosphatase of *Thermus caldophilus* GK24 and properties of the enzyme. *J. Microbiol. Biotechnol.* **13:** 139-145.
12. Horwitz, K. B., Y. Koseki, and W. L. McGuire. 1978. Estrogen control of progesterone receptor in human breast cancer: Role of estradiol and antiestrogen. *Endocrinology* **103:** 1742-1751.
13. Jeoung, D., J. H. Kim, Y. H. Lee, M. Baek, S. Lee, N. I. Baek, and H. Y. Kim. 2002. cDNA microarray analysis of transcriptional response to hyperin in human gastric cancer cells. *J. Microbiol. Biotechnol.* **12:** 664-668.
14. Kemp, C. J. and N. R. Drinkwater. 1989. Genetic variation in liver tumor susceptibility, plasma testosterone levels, and androgen receptor binding in six inbred strains of mice. *Cancer Res.* **49:** 5044-5047.
15. La Vecchia, C., E. Negri, L. Cavalieri d'Oro, and S. Franceschi. 1998. Liver cirrhosis and the risk of primary liver cancer. *Eur. J. Cancer Prev.* **7:** 315-320.
16. Lin, X., P. Nelson, and I. H. Gelman. 2000. SSeCKS, a major protein kinase C substrate with tumor suppressor activity, regulates G13S progression by controlling the expression and cellular compartmentalization of cyclin D. *Mol. Cell. Biology* **20:** 7259-7272.
17. Mackay, A., C. Jones, T. Dexter, R. L. Silva, K. Bulmer, A. Jones, P. Simpson, R. A. Harris, P. S. Jat, A. M. Neville, L. F. Reis, S. R. Lakhani, and M. J. O'Hare. 2003. cDNA microarray analysis of genes associated with ERBB2 (HER2/neu) overexpression in human mammary luminal epithelial cells. *Oncogene* **22:** 2680-2688.
18. Matsuura, B., Y. Taniguchi, and Y. Ohta. 1994. Effect of antiandrogen treatment on chemical hepatocarcinogenesis in rats. *J. Hepatol.* **21:** 187-193.
19. Mishkin, S. Y., E. Farber, R. K. Ho, S. Mulay, and S. Mishkin. 1983. Evidence for the hormone dependency of hepatic hyperplastic nodules: Inhibition of malignant transformation after exogenous 17 beta-estradiol and tamoxifen. *Hepatology* **3:** 308-316.
20. Nelson, P. and I. H. Gelman. 1997. Cell-cycle regulated expression and serine phosphorylation of the myristylated protein kinase C substrate, SSeCKS: Correlation with cell confluency, G0 phase and serum response. *Mol. Cell. Biochem.* **175:** 233-241.
21. Paassilta, M., A. Karjalainen, K. Kervinen, M. J. Savolainen, J. Heikkinen, A. C. Backstrom, and Y. A. Kesaniemi. 2000. Insulin-like growth factor binding protein-1 (IGFBP-1) and IGF-I during oral and transdermal estrogen replacement therapy: Relation to lipoprotein(a) levels. *Atherosclerosis* **149:** 157-162.
22. Pizzuti, A., L. Frittitta, A. Argiolas, R. Baratta, I. D. Goldfine, M. Bozzali, T. Ercolino, G. Scarlato, L. Iacoviello, R. Vigneri, V. Tassi, and V. Trischitta. 1999. A polymorphism (K121Q) of the human glycoprotein PC-1 gene coding region is strongly associated with insulin resistance. *Diabetes* **48:** 1881-1884.
23. Ramirez, V. D. and J. Zheng. 1996. Membrane sex-steroid receptors in the brain. *Front. Neuroendocrinol.* **17:** 402-439.
24. Roder, K., K. H. Kim, and H. S. Sul. 2002. Induction of murine H-rev107 gene expression by growth arrest and histone acetylation: Involvement of an Sp1/Sp3-binding GC-boxq. *Biochem. Biophys. Res. Commun.* **294:** 63-70.
25. Sawaki, M., K. Enomoto, H. Takahashi, Y. Nakajima, and M. Mori. 1990. Phenotype of preneoplastic and neoplastic liver lesions during spontaneous liver carcinogenesis of LEC rats. *Carcinogenesis* **11:** 1857-1861.
26. Segars, J. H. and P. H. Driggers. 2002. Estrogen action and cytoplasmic signaling cascades. Part I: Membrane-associated signaling complexes. *Trends Endocrinol. Metab.* **13:** 349-354.
27. Shimizu, I., M. Yasuda, Y. Mizobuchi, Y. R. Ma, F. Liu, M. Shiba, T. Horie, and S. Ito. 1998. Suppressive effect of oestradiol on chemical hepatocarcinogenesis in rats. *Gut* **42:** 112-119.
28. Stefan, C., W. Stalmans, and M. Bollen. 1996. Threonine autophosphorylation and nucleotidylation of the hepatic membrane protein PC-1. *Eur. J. Biochem.* **241:** 338-342.
29. Sylvia, V. L., T. Hughes, D. D. Dean, B. D. Boyan, and Z. Schwartz. 1998. 17beta-estradiol regulation of protein kinase C activity in chondrocytes is sex-dependent and involves nongenomic mechanisms. *J. Cell Physiol.* **176:** 435-444.

30. Turner, R. T., B. L. Riggs, and T. C. Spelsberg. 1994. Skeletal effects of estrogen. *Endocr. Rev.* **15:** 275-300.
31. Watson, P. H., L. J. Fraher, G. N. Hendy, U. I. Chung, M. Kisiel, B. V. Natale, and A. B. Hodsman. 2000. Nuclear localization of the type 1 PTH/PTHrP receptor in rat tissues. *J. Bone Miner. Res.* **15:** 1033-1044.
32. Weber, G. F., R. T. Bronson, J. Ilagan, H. Cantor, R. Schmits, and T. W. Mak. 2002. Absence of the CD44 gene prevents sarcoma metastasis. *Cancer Res.* **62:** 2281-2286.
33. Xu, J. W., J. Gong, X. M. Chang, J. Y. Luo, L. Dong, Z. M. Hao, A. Jia, and G. P. Xu. 2002. Estrogen reduces CCL_4-induced liver fibrosis in rats. *World J. Gastroenterol.* **8:** 883-887.
34. Yu, M. C., J. M. Yuan, S. Govindarajan, and R. Ross. 2000. Epidemiology of hepatocellular carcinoma. *Can. J. Gastroenterol.* **14:** 703-709.
35. Yue, S. Q., Y. L. Yang, K. F. Dou, and K. Z. Li. 2003. Expression of PCNA and CD44 mRNA in colorectal cancer with venous invasion and its relationship to liver metastasis. *World J. Gastroenterol.* **9:** 2863-2865.