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Characterization of liver-cirrhosis nodules by analysis of gene-expression profiles and patterns of allelic loss

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Abstract To disclose genetic mechanisms involved in development or progression of hepatocellular carcinoma (HCC), we used a genome-wide cDNA microarray consisting of 8,448 genes to compare gene-expression profiles among 12 liver-cirrhosis nodules (LCNs) and five specimens of HCC excised from a single patient and carefully prepared by laser-capture microdissection (LCM). The expression patterns enabled us to identify 72 genes that were frequently upregulated and 57 that were downregulated specifically in the LCN specimens as compared to the HCCs. We also documented upregulation of 31 genes and downregulation of seven others in both HCC and LCN tissues. Several types of intracellular kinase, including receptor-type kinase, were upregulated in LCNs. Expression patterns of HCCs and LCNs generally represented two genetically distinct groups when subjected to a hierarchical clustering analysis, although expression profiles of two of the LCNs resembled the HCC pattern. Analysis of allelic losses at microsatellite loci revealed that LCNs showed frequent loss of heterozygosity (LOH) (33%) in chromosomal regions 6q and 22q; over half of the LCNs had lost an allele for at least one of the 28 loci examined. The presence of early genetic changes among LCNs, with

additional genetic changes occurring during formation of HCCs, suggests that hepatocellular carcinogenesis follows the multistep model established for colon cancers and that some LCNs may be precancerous lesions.

Keywords Hepatocellular carcinoma · Liver-cirrhosis nodule · Loss of heterozygosity · Microsatellite instability · Hepatitis B virus · Hepatitis C virus · cDNA microarray

Introduction

Hepatocellular carcinoma (HCC) is the most common type of malignancy in Northern Africa, Southeast Asia, Japan, and China, and it is the most common cause of cancer death worldwide (Nagai and Buendia 1998). Etiological risk factors for development of HCC include infection with hepatitis B or C viruses, alcohol consumption, and dietary exposure to aflatoxin B1. Inactivation of the p53 gene occurs late in tumor progression among patients exposed to aflatoxin B1, and its loss late in transformation of liver cells is a common observation (Oda et al. 1994). Early molecular events in liver carcinogenesis remain to be elucidated.

Viral hepatitis is an important epidemiological factor, since chronic infection forces hepatocytes to regenerate with consequently excessive replication. Moreover, accumulation of viral products in hepatocytes and subsequent immune responses by lymphocytes are suspected of causing death of liver cells (Chisari et al. 1985). However, cirrhosis is the most important clinicopathological background of hepatocellular carcinomas, regardless of epidemiology.

Our experiments for this study focused on genetic changes that might be present in liver-cirrhosis nodules (LCNs). Recent reports have implicated certain early genomic alterations in such nodules (Yamada et al. 1997); for instance, microsatellite instability (MI), which

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is driven by defects of mismatch-repair genes and is considered a malignant phenotype, is found in some LCNs (Salvucci et al. 1999). Furthermore, allelic losses at certain tumor-suppressor loci have been observed in LCNs, reflecting early clonal changes in chronic liver disease (De Souza et al. 1995; Roncalli et al. 2000). However, previous studies have fallen short of comprehensive comparisons between HCCs and LCNs that might reveal the LCN as a precancerous lesion. Therefore, we compared LCNs with HCCs by examining expression profiles of 8,448 genes in specimens of those tissues, as well as allelic status at 28 microsatellite marker loci.

Materials and methods

Patients and tissue samples

Specimens of LCNs and primary HCCs were obtained postmortem from a 51-year-old man with multiple HCCs and histologically macronodular liver cirrhosis. Informed consent was obtained according to guidelines approved by the Institutional Research Board. Tissues were frozen immediately and stored at -80°C . Serological tests were positive for hepatitis B virus (HBV) and C antigens but negative for HCV antibodies. A total of 12 LCNs and five HCCs, which were well separated by intertumoral septa, were dissected by micromanipulation under microscopy at the same time as routine histopathological examinations were performed. Non-cancerous liver tissues were obtained from operatively dissected, nonaffected liver of another patient whose primary colon cancer had metastasized to liver; that patient gave informed consent for participation in our research. Integration of HBV was examined by Southern blotting using a ^{32}P -labeled HBV DNA probe. Serum antihepatitis C virus (HCV) antibody was measured by an enzyme-linked immunosorbent assay (Miyamura et al. 1990).

Laser-capture microdissection

After rapid staining with hematoxylin and eosin, the tissue sections were microdissected under a PixCell laser-capture microscope with an infrared diode laser (Arcurus Engineering, Santa Clara, CA, USA) as described previously (Bonner et al. 1997; Okabe et al. 2001; Ono et al. 2000). Briefly, tissue section was prepared on a specific polymer film mounted on optically transparent caps designed to fit 500-ml microcentrifuge tubes (CapSure, Arcurus Engineering). Cells were captured under visual control by focal melting of membranes by the laser beam. The caps with captured single nodules were fixed to blank 500-ml microcentrifuge tubes, labeled, and either stored at -80°C until use or processed immediately by adding denaturing solution to the microcentrifuge tubes.

Fabrication of cDNA microarrays

We fabricated a genome-wide cDNA microarray with 8,448 cDNAs selected from the UniGene database of the National Center for Biotechnology Information. The cDNAs were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using poly(A) + RNA isolated from various human organs as templates. Lengths of the amplicons ranged from 200 to 1,100 bp without repetitive or poly(A) sequences. The products were spotted in duplicate on type-7 glass slides (Amersham Pharmacia Biotech, Buckinghamshire, UK) using an Array Spotter Generation III (Amersham) (Okabe et al. 2001; Ono et al. 2000).

T7-based RNA amplification

Total RNAs were extracted from the frozen materials using Trizol (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocols and purified by RNeasy mini kits (Qiagen, Hilden, Germany). T7-based amplification was carried out as described previously (Okabe et al. 2001; Ono et al. 2000). Using 2 μg of total RNA from each tissue sample as starting material, we performed two rounds of amplification; the amount of each amplified RNA (aRNA) was measured by a spectrophotometer, and its quality was checked by agarose gel electrophoresis. We had previously shown by RT-PCR that aRNAs reliably reflected the proportions present in the original total RNA samples (Ono et al. 2000).

Probe synthesis, hybridization, and washing

The cDNA probes were prepared from aRNA as described previously (Ono et al. 2000). Ten-microgram aliquots of aRNA from normal liver tissues and from nodules or tumor tissues were labeled, respectively, with Cy5-dCTP or Cy3-dCTP (Amersham). The labeled probes were mixed with microarray hybridization solution version 2 (Amersham) and formamide (Sigma, St Louis, MO, USA) to a final concentration of 50%. The slides and probes were hybridized at 40°C for 15 h. After hybridization, the slides were washed in $1\times$ SSC and 0.2% SDS for 10 min at 55°C , washed in $0.1\times$ SSC and 0.2% SDS for 10 min at 55°C , and washed in $0.1\times$ SSC for 1 min at room temperature.

Microarray analysis and validation of data

Hybridized slides were scanned for Cy3 and Cy5 using the GenePix 4000A Microarray Scanner (Amersham). The signal intensity of each spot was evaluated photometrically by GenePix Pro version 3.0 software (Amersham). Each slide contained 52 housekeeping genes to normalize the signal intensities of the different fluorescent dyes according to the Lucidea Microarray Score

Card (Amersham). Reproducibility of the normalized intensity ratios was assessed by comparing the log₂ Cy3: Cy5 intensity ratios of the 52 housekeeping genes between different slide sets. Fluorescence intensities of Cy5 (nontumor) and Cy3 (tumor) for each target spot were normalized by positive and negative controls using the Lucidea Microarray Score Card (Amersham) so that the mean Cy5 and Cy3 intensities for each slide were equalized. The Cy3: Cy5 ratio for each sample was calculated by averaging spots. A cut-off value for each expression level was determined according to the degree of background fluctuation, essentially as described previously (Ono et al. 2000).

Statistical analysis

For comparisons of expression levels, the relative expression of each gene was recorded in one of four categories: (1) over-expressed in nodule/tumor cells (Cy3: Cy5 ratio 2.0), (2) under-expressed in nodule/tumor cells (Cy3: Cy5 ratio 0.5), (3) unchanged from normal in nodule/tumor cells, or (4) not expressed in normal cells or nodule or cancer cells. The number of items in each category was recorded within each group. In principle, we used these categories to detect changes in expression that were common among samples as well as specific to a certain subgroup. To detect differentially expressed genes, we recorded the number of samples in each category within each subgroup for each gene. Then, we calculated the *U* values of Mann-Whitney tests, which measure how sample distributions between subgroups overlap; i.e., the number of samples within each group is counted and, according to the order of the category, the number of overlapped samples is incorporated into the *U* value (Okabe et al. 2001; Ono et al. 2000). Using these data, we applied a hierarchical clustering algorithm using Genespring version 4.04 software (Silicon Genetics, Redwood, CA, USA).

Loss of heterozygosity analysis by microsatellite markers

Sixteen CA-dinucleotide markers were selected for analyzing loss of heterozygosity (LOH) (Dib et al. 1996): D1S199 (1p36), D1S235 (1q43), D1S248 (1p21), D2S338 (2q37), D3S1276 (3p12), D4S1538 (4q27), D4S3051 (4q35), D6S305 (6q27), D8S277 (8p23), D8S281 (8q23.3–8q24), D9S273 (9q13), D10S587 (10q26), D13S1325 (13q14), D16S420 (16p13), D17S1791 (17p13), and D22S420 (22q11). Twelve intronic microsatellites at tumor-suppressor loci (Piao et al. 1997) were also examined, including EXT1 (8q24.1), APC (5q21), NF1 (17q11.2, GDB acc. no. 63848), VHL (3p25), ATM (11q22, D11S1778), Rb1 (13q14), p53 (17p13), BRCA1 (17q21), DCC (18q21), TBP (6q27, GDB acc. no. 138768), CYL (16q12, D16S771), and IGFIIr (6q26, GDB acc. no. 62001). All primer sequences were

downloaded from the genome database (<http://www.gdb.org>). Genomic DNA was obtained from single regenerating nodules of approximately similar size with DNA isolation kits (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. DNA extracted from the patient's leukocytes served as a control.

All microsatellites were amplified by the polymerase chain reaction (PCR) using 20 ng of genomic DNA in a Gene Amp PCR 9600 System (Perkin Elmer Cetus, Norwalk, CT, USA) as described elsewhere (Iida et al. 1997). PCR products were electrophoresed at 2,000 V for 2–4 h in 0.2-mm thick, 6% polyacrylamide denaturing gels containing 36% formamide and 8 M urea. Gels were transferred to filters, dried at 80°C, and exposed to autoradiographic film at room temperature for 16–20 h (Iida et al. 1997). Signal intensities of polymorphic alleles were quantified by a scanning densitometer; peak areas corresponding to each signal were calculated by electronic integration (Hoefer Scientific Instruments, San Francisco, CA, USA). The signal intensities of alleles of tumor-tissue DNA were compared to those of corresponding leukocyte DNA. We judged a reduction in signal intensity greater than 50% to be LOH. We distinguished LOH from chromosome multiplication by normalizing each signal to the signal obtained when the same DNA was analyzed with markers for loci on other chromosomes, as described elsewhere (Iida et al. 1997).

Results

Genes differently expressed between LCNs and HCCs

A total of 12 LCNs and five HCCs, well separated by intertumoral septa, were dissected under microscopy and carefully isolated by laser-capture microdissection (LCM). We examined gene-expression profiles of both types of tissue on a cDNA microarray after applying T7-based RNA amplification to obtain sufficient quantities of RNA for analysis. Total RNAs isolated from freshly frozen normal, nodule, or tumor specimens were amplified several 100-fold; sizes of the amplified products ranged from 0.3 to 1.0 kb (data not shown). Each cDNA to be used as a probe was labeled with dyes conjugated to either Cy3 (nodule or tumor cells) or Cy5 (normal cells) and hybridized to microarrayed cDNAs or ESTs representing 8,448 genes. Scatter plots of the two fluorescent signals revealed different gene-expression patterns between nodules and tumors (data not shown). Comparisons of the expression patterns enabled us to identify 72 genes that were frequently upregulated and 57 that were specifically downregulated in the LCN specimens (Table 1). We also noted 31 genes that were upregulated in both HCCs and LCNs and seven that were downregulated in both types of tissue. Characteristic expression profiles of LCNs included genes encoding several types of intracellular kinases including receptor kinases, transcription factors, or products

Table 1 Genes differently regulated in liver-cirrhosis nodules (LCNs) and hepatocellular carcinomas (HCCs). Samples indicate the number of samples differently regulated among 12 LCNs and five HCCs

Genbank no.	Samples	Genes
Genes upregulated in LCNs ^a		
L16794	11	Source: Human transcription factor (MEF2) mRNA, complete cds.
J04046	11	Source: Human calmodulin mRNA, complete cds.
L11706	10	Source: Human hormone-sensitive lipase (LIPE) gene, complete cds.
M12824	10	Source: Human T-cell differentiation antigen Leu-2/T8 mRNA, partial cds.
L34041	10	Source: Homo sapiens L-glycerol-3-phosphate:NAD oxidoreductase mRNA, complete cds.
M76125	10	Source: Human tyrosine kinase receptor (axl) mRNA, complete cds.
AF006464	9	Source: Homo sapiens muscle specific tyrosine kinase receptor (MUSK) mRNA, complete cds.
U14650	9	Source: Human platelet-endothelial tetraspan antigen three mRNA, complete cds.
M15395	9	Source: Human leukocyte adhesion protein (LFA-1/Mac-1/p150,95 family) beta subunit mRNA.
M33906	9	Source: Human MHC class II HLA-DQA1 mRNA, complete cds.
M62402	9	Source: Human insulin-like growth factor binding protein six (IGFBP6) mRNA, complete cds.
J03260	9	Source: Human transducin alpha-subunit (GNAZ) mRNA, complete cds.
M86826	9	Source: Human IGF binding protein complex acid-labile subunit a mRNA, complete cds.
X60188	9	Source: Human ERK1 mRNA for protein serine/threonine kinase.
U83411	9	Source: Homo sapiens carboxypeptidase Z precursor, mRNA, complete cds.
AF005038	9	Source: Homo sapiens secretory carrier membrane protein (SCAMP2) mRNA, complete cds.
U51477	9	Source: Human diacylglycerol kinase zeta mRNA, complete cds.
AF039442	8	Source: Homo sapiens colon cancer antigen NY-CO-45 mRNA, partial cds.
AF038421	8	Source: Homo sapiens GPI-linked anchor protein (GFRA1) mRNA, complete cds.
L07555	8	Source: Homo sapiens early activation antigen CD69 mRNA, complete cds.
AF068760	8	Source: Homo sapiens MAD3-like protein kinase mRNA, complete cds.
HSMIG2GNA	8	Source: H. sapiens mitogen inducible gene mig-2, complete CDS.
AF023917	8	Source: Homo sapiens protein tyrosine phosphatase PIR1 mRNA, complete cds.
AB014582	8	Source: Homo sapiens mRNA for KIAA0682 protein, complete cds.
X73502	8	Source: H. sapiens mRNA for cytokeratin 20.
D50487	8	Source: Human mRNA for RNA helicase (HRH1), complete cds.
Y12226	8	Source: H. sapiens mRNA for gamma-adaptin.
S62908	8	Source: gamma-aminobutyric acidA receptor alpha 3 subunit (human, fetal brain, mRNA, 1,637 nt).
U10302	8	Source: Human glutamate receptor flop isoform (GluR3-flop) mRNA, complete cds.
D26069	7	Source: Human mRNA for KIAA0041 gene, partial cds.
X69490	7	Source: H. sapiens mRNA for titin.
V00572	7	Source: Human mRNA encoding phosphoglycerate kinase.
Z96932	7	Source: Homo sapiens mRNA for NA14 protein.
Z68228	7	Source: H. sapiens mRNA for plakoglobin.
M65105	7	Source: Human noradrenaline transporter mRNA, complete cds.
M21304	7	Source: Human glutathione peroxidase (GPX1) mRNA, complete cds.
L19161	7	Source: Human translation initiation factor eIF-2 gamma subunit mRNA, complete cds.
L03840	7	Source: Human fibroblast growth factor receptor 4 (FGFR4) mRNA, complete cds.
X82321	7	Source: H. sapiens mRNA for thiol-specific antioxidant.
U19977	7	Source: Human preprocarboxypeptidase A2 (proCPA2) mRNA, complete cds.
AB010427	7	Source: Homo sapiens mRNA for NORI-1, complete cds.
AF028828	7	Source: Homo sapiens Tax interaction protein 43 mRNA, partial cds.
AB001895	7	Source: Homo sapiens mRNA for B120, complete cds.
AF058922	7	Source: Homo sapiens GLE1 (GLE1) mRNA, complete cds.
AF071884	7	Source: Homo sapiens p21 activated kinase 1B (PAK1B) mRNA, complete cds.
AF035625	7	Source: Homo sapiens serine threonine kinase 11 (STK11) mRNA, complete cds.
AF047826	7	Source: Homo sapiens cadherin-7 (CDH7) mRNA, partial cds.
U16996	6	Source: Human protein tyrosine phosphatase mRNA, complete cds.
D49742	6	Source: Human mRNA for HGF activator like protein, complete cds.
U61262	6	Source: Human neogenin mRNA, complete cds.
U26209	6	Source: Human renal sodium/dicarboxylate cotransporter (NADC1) mRNA, complete cds.
S68805	6	Source: L-arginine:glycine amidinotransferase (human, kidney carcinoma cells, mRNA, 2,330 nt).
D50310	6	Source: Human mRNA for cyclin I, complete cds.
S67970	6	Source: ZNF75 = KRAB zinc finger (human, lung fibroblast, mRNA, 1,563 nt).
X75593	6	Source: H. sapiens mRNA for rab 13.
D38441	6	Source: Human mRNA for acylamino acid-releasing enzyme, complete cds.
X96586	6	Source: H. sapiens mRNA for FAN protein.
AI275846	6	Source: ql68a06.x1 Soares_NhHMPu_S1 Homo sapiens cDNA clone IMAGE: 1877458 3', mRNA sequence.
D30612	6	Source: Homo sapiens mRNA for repressor protein, partial cds.
L24158	6	Source: Human integrin alpha 9 protein mRNA, 3' end.
L22846	6	Source: Homo sapiens transcription factor E2F-2 mRNA, complete cds (clone 9).
AF011468	6	Source: Homo sapiens serine/threonine kinase (BTAK) mRNA, complete cds.
L39211	6	Source: Homo sapiens mitochondrial carnitine palmitoyltransferase I mRNA, complete cds.
L34155	6	Source: Homo sapiens laminin-related protein (LamA3) mRNA, complete cds.

Table 1 (Continued)

Genbank no.	Samples	Genes
K02765	6	Source: Human complement component C3 mRNA, alpha and beta subunits, complete cds.
J05081	6	Source: Human endothelin 3 (EDN3) mRNA, complete cds.
AF045161	6	Source: Homo sapiens cyclin T mRNA, complete cds.
L14076	6	Source: Human pre-mRNA splicing factor SRp75 mRNA, complete cds.
AB008549	6	Source: Homo sapiens mRNA for type 1 procollagen C-proteinase enhancer protein, complete cds.
AB011083	6	Source: Homo sapiens mRNA for KIAA0511 protein, partial cds.
HSU1RNPA	6	Source: Human mRNA for U1 small nuclear RNP-specific A protein.
M64722	6	Source: Human TRPM-2 mRNA, complete cds.
Genes downregulated in LCNs ^b		
AJ006701	9	Source: Homo sapiens mRNA for putative serine/threonine protein kinase, partial.
S80343	8	Source: ArgRS = arginyl-tRNA synthetase (human, ataxia-telangiectasia patients, EBV-lymphoblastoid cells, mRNA, 2,120 nt).
X54232	8	Source: Human mRNA for heparan sulfate proteoglycan (glypican).
U46920	8	Source: Human metaxin (MTX) gene, complete cds.
U79734	8	Source: Human huntingtin interacting protein (HIP1) mRNA, complete cds.
Z50115	8	Source: H. sapiens mRNA for thimet oligopeptidase (metalloproteinase).
HUMB180AA	8	Source: Human Bullous pemphigoid autoantigen BP180 gene, 3' end.
Z97029	8	Source: Homo sapiens mRNA for ribonuclease H I large subunit.
M16328	7	Source: Human glucocerebrosidase mRNA, complete cds.
M26393	7	Source: Human short chain acyl-CoA dehydrogenase mRNA, complete cds.
AF072860	7	Source: Homo sapiens protein activator of the interferon-induced protein kinase (PACT) mRNA, complete cds.
AB002376	7	Source: Human mRNA for KIAA0378 gene, partial cds.
U35373	7	Source: Human DXS8237E mRNA, partial cds.
M98343	7	Source: Homo sapiens amplexin (EMS1) mRNA, complete cds.
D87437	7	Source: Human mRNA for KIAA0250 gene, complete cds.
D78012	7	Source: Homo sapiens mRNA for dihydropyrimidinase related protein-1, complete cds.
Y11588	7	Source: H. sapiens mRNA for apoptosis specific protein.
X97324	7	Source: H. sapiens mRNA for adipophilin.
U92314	7	Source: Homo sapiens hydroxysteroid sulfotransferase SULT2B1a (HSST2) mRNA, complete cds.
X51630	7	Source: Human Wilms tumor WT1 mRNA for zinc finger protein, Krueppel-like.
X03747	7	Source: Human mRNA for Na/K-ATPase beta subunit.
M86400	6	Source: Human phospholipase A2 mRNA, complete cds.
X53296	6	Source: H. sapiens mRNA for IRAP.
X76732	6	Source: H. sapiens mRNA for NEFA protein.
X79444	6	Source: H. sapiens endonuclease G (ENDOG) mRNA.
M88282	6	Source: Human tactile protein mRNA, complete cds.
M60091	6	Source: Homo sapiens galactose-1-phosphate uridyl transferase (GALT) mRNA, complete cds.
M60974	6	Source: Human growth arrest and DNA-damage-inducible protein (gadd45) mRNA, complete cds.
Z22555	6	Source: H. sapiens encoding CLA-1 mRNA.
M34338	6	Source: Human spermidine synthase mRNA, complete cds.
M77640	6	Source: Homo sapiens L1 cell adhesion molecule (L1CAM) mRNA, complete cds.
X92814	6	Source: H. sapiens mRNA for rat HREV107-like protein.
M73746	6	Source: Homo sapiens lutropin/choriogonadotropin receptor (LHCGR) mRNA, complete cds.
M75106	6	Source: Human prepro-plasma carboxypeptidase B mRNA, complete cds.
U78678	6	Source: Human thioredoxin mRNA, nuclear gene encoding mitochondrial protein, complete cds.
U51678	6	Source: Homo sapiens small acidic protein mRNA, complete cds.
U40671	6	Source: Human DNA ligase III mRNA, complete cds.
U35451	6	Source: Homo sapiens heterochromatin protein p25 mRNA, complete cds.
U66346	6	Source: Homo sapiens cAMP phosphodiesterase (PDE4C) mRNA, 4C-791 isoform, partial cds.
U20157	6	Source: Human platelet-activating factor acetylhydrolase mRNA, complete cds.
U20285	6	Source: Human Gps1 (GPS1) mRNA, complete cds.
X00474	6	Source: Human pS2 mRNA induced by estrogen from human breast cancer cell line MCF-7.
X80590	6	Source: H. sapiens PHKG1 mRNA.
U82668	6	Source: Homo sapiens shox gene, alternatively spliced products, complete cds.
S59049	6	Source: BL34 = B cell activation gene (human, mRNA, 1,398 nt).
D14134	6	Source: Human mRNA for RAD51, complete cds.
D21238	6	Source: Homo sapiens mRNA for glutaredoxin, complete cds.
J03870	6	Source: Human cystatin SA-I mRNA, complete cds.
K03515	6	Source: Human neuroleukin mRNA, complete cds.
M12623	6	Source: Human non-histone chromosomal protein HMG-17 mRNA, complete cds.
D50929	6	Source: Human mRNA for KIAA0139 gene, complete cds.
HSINT2	6	Source: Human int-2 proto-oncogene.
J03779	6	Source: Human common acute lymphoblastic leukemia antigen (CALLA) mRNA, complete cds.
AF033095	6	Source: Homo sapiens testis enhanced gene transcript protein (TEGT) mRNA, complete cds.
AF035299	6	Source: Homo sapiens clone 23863 mRNA, partial cds.
AF073920	6	Source: Homo sapiens regulator of G-protein signaling 6 (RGS6) mRNA, complete cds.

Table 1 (Continued)

Genbank no.	Samples	Genes
M12625	6	Source: Human lecithin-cholesterol acyltransferase mRNA, complete cds, with 5' and 3' flanking DNA sequences.
Genes upregulated commonly to both LCNs and HCCs ^c		
X60188	-	Source: Human ERK1 mRNA for protein serine/threonine kinase.
U83411	-	Source: Homo sapiens carboxypeptidase Z precursor, mRNA, complete cds.
AF045161	-	Source: Homo sapiens cyclin T mRNA, complete cds.
AA348274	-	Source: EST54719 Hippocampus I Homo sapiens cDNA 3' end, mRNA sequence.
M76125	-	Source: Human tyrosine kinase receptor (axl) mRNA, complete cds.
M86826	-	Source: Human IGF binding protein complex acid-labile subunit a mRNA, complete cds.
D38441	-	Source: Human mRNA for acylamino acid-releasing enzyme, complete cds.
U10302	-	Source: Human glutamate receptor flop isoform (GluR3-flop) mRNA, complete cds.
U16996	-	Source: Human protein tyrosine phosphatase mRNA, complete cds.
L34041	-	Source: Homo sapiens L-glycerol-3-phosphate:NAD oxidoreductase mRNA, complete cds.
M12824	-	Source: Human T-cell differentiation antigen Leu-2/T8 mRNA, partial cds.
M62402	-	Source: Human insulin-like growth factor binding protein 6 (IGFBP6) mRNA, complete cds.
M65105	-	Source: Human noradrenaline transporter mRNA, complete cds.
M15395	-	Source: Human leukocyte adhesion protein (LFA-1/Mac-1/p150,95 family) beta subunit mRNA.
V00572	-	Source: Human mRNA encoding phosphoglycerate kinase.
X69490	-	Source: H. sapiens mRNA for titin.
S68805	-	Source: L-arginine:glycine amidinotransferase (human, kidney carcinoma cells, mRNA, 2,330 nt).
U19977	-	Source: Human preprocarboxypeptidase A2 (proCPA2) mRNA, complete cds.
AF011468	-	Source: Homo sapiens serine/threonine kinase (BTAK) mRNA, complete cds.
AF035625	-	Source: Homo sapiens serine threonine kinase 11 (STK11) mRNA, complete cds.
AB008549	-	Source: Homo sapiens mRNA for type 1 procollagen C-proteinase enhancer protein, complete cds.
J03260	-	Source: Human transducin alpha-subunit (GNAZ) mRNA, complete cds.
J04046	-	Source: Human calmodulin mRNA, complete cds.
L11706	-	Source: Human hormone-sensitive lipase (LIPE) gene, complete cds.
L16794	-	Source: Human transcription factor (MEF2) mRNA, complete cds.
L34155	-	Source: Homo sapiens laminin-related protein (Lama3) mRNA, complete cds.
L07555	-	Source: Homo sapiens early activation antigen CD69 mRNA, complete cds.
D49742	-	Source: Human mRNA for HGF activator like protein, complete cds.
D50310	-	Source: Human mRNA for cyclin I, complete cds.
D26069	-	Source: Human mRNA for KIAA0041 gene, partial cds.
D30612	-	Source: Homo sapiens mRNA for repressor protein, partial cds.
Genes downregulated commonly to both LCNs and HCCs ^d		
AJ006701	-	Source: Homo sapiens mRNA for putative serine/threonine protein kinase, partial.
D78012	-	Source: Homo sapiens mRNA for dihydropyrimidinase related protein-1, complete cds.
X03747	-	Source: Human mRNA for Na/K-ATPase beta subunit.
M60091	-	Source: Homo sapiens galactose-1-phosphate uridyl transferase (GALT) mRNA, complete cds.
Z22555	-	Source: H. sapiens encoding CLA-1 mRNA.
M75106	-	Source: Human prepro-plasma carboxypeptidase B mRNA, complete cds.
M12623	-	Source: Human non-histone chromosomal protein HMG-17 mRNA, complete cds.

^a Shown are genes plus 107 ESTs^b Shown are genes plus 89 ESTs^c Shown are genes plus 50 ESTs^d Shown are genes plus 10 ESTs

involved in the extracellular matrix, intracellular signal-transduction pathways, or the antiviral action of cytokines. We also identified multiple uncharacterized ESTs that were frequently upregulated or downregulated in LCNs, an indication that many uncharacterized genes also play roles in development of LCNs.

Classification of LCNs and HCCs according to specific gene-expression profiles

A hierarchical clustering method was applied to the microarray data in order to classify LCNs and HCCs into specific groups on the basis of characteristic gene-expression profiles. We carried out semiquantitative RT-

PCR for the majority of genes that displayed stably high levels of expression as well as relatively altered gene expression between the two nodule groups. Then, we selected the top 37 genes from the RT-PCR results that exhibited significantly low *p* values by *t* test. These 37 genes had sufficient discriminating power to distinguish between the two nodule groups in the cluster analysis. Representative results of overall expression patterns across five HCC and 12 LCN specimens are shown in Fig. 1. This analysis resulted in the clustering of most specimens of the same histopathological status, indicating that distinctive cDNA profiles characterize each group; however, two nodules that had been diagnosed as LCNs (#10 and #12) fell into the HCC group on the basis of their expression profiles.

Fig. 1 Hierarchical analysis of 12 liver-cirrhosis nodules (LCNs) and five hepatocellular carcinomas (HCCs) from a single patient according to their gene-expression profiles. The dendrogram at the *top* indicates the Genebank accession numbers of 37 representative genes (see “Results”) used for the clustering analysis. The dendrogram on the *right* indicates the degree of similarity between samples, demonstrating that they could be divided into an HCC group (*upper column*) and an LCN group (*lower column*). “HCC mix” indicates the expression profile of all the HCC RNAs mixed together (C1–C5). LCNs 10 and 12 were classified into the HCC group. Changes in relative expression are represented in graduated colors: *red*, upregulated; *green*, downregulated; *yellow*, unchanged

Allelic losses in LCNs and HCCs

We examined the LCM-dissected LCN nodules and HCC tumors for allelic losses of 28 microsatellite markers selected either to represent regions where genetic alterations were frequent in HCCs (Nagai et al. 1992) or intronic regions of known tumor-suppressor genes (Fig. 2). Seven of the 12 LCNs (58%) had lost an allele for at least one of the 28 loci examined, implying a high incidence of clonal growth of hepatocytes in cirrhotic nodules and suggesting that some of those nodules might represent precancerous lesions. Previously, a clonal pattern of integration of HBV DNA into the host genome was described (Yasui et al. 1992) in 31% of LCNs examined, which also implied clonal proliferation of hepatocytes in the nodules. In the present experiments, LOH analysis revealed frequent allelic losses (4/12, 33%) at chromosomes 6q and 22q in LCNs. MI was observed in one LCN (N1 at *BRCA1*) and one HCC (C5 at D13S1325). The cirrhotic nodules that showed LOH or MI were no different from other LCNs in histological features.

Discussion

In the present study, we compared LCNs with HCCs at the molecular level by examining both cDNA-expression

Fig. 2 Schematic representation of allelic loss and microsatellite instability (MI) analysis in five hepatocellular carcinomas (HCCs) (C1–5) and 12 liver-cirrhosis nodules (LCNs) (N1–12). Identification numbers are at the top of each column. *Solid, shaded and unfilled circles* indicate loss, MI, and retention of heterozygosity, respectively; *gaps* reflect uninformativeness for specific markers

profiles and allelic losses in multiple nodule specimens from a single patient that were carefully prepared by LCM. We identified 72 genes that were frequently

upregulated and 57 genes that were downregulated specifically in the LCNs, as well as 31 genes that were upregulated and seven that were downregulated in both types of aberrant tissue.

Genes that displayed characteristic activation in LCNs included some encoding intracellular kinases, indicating that this system plays a major role in maintaining hepatocytes in a hyperproliferative state. Receptor-tyrosine kinases MUSK and AXL are critical for the growth and differentiation of hepatocytes (DeChiara et al. 1996; O'Bryan et al. 1991). Another cellular-kinase gene that was upregulated in LCNs was *STK15/BTAK*, encoding a centrosome-associated enzyme that is amplified and over-expressed in multiple types of human tumors (Zhou et al. 1998). This kinase is involved in the induction of centrosome duplication-distribution abnormalities and aneuploidy in mammalian cells.

Genes encoding transcription factors also displayed characteristic upregulation in LCNs. MEF2 binds dsDNA and controls gene expression during differentiation of neural tissues (Breitbart et al. 1993); MEF2 is phosphorylated and activated by p38 MAP kinase. Mitogens for hepatocytes were also activated in the LCN nodules we examined. Hepatocyte growth factor/scatter factor (HGF/SF) is a cytokine associated with migration and invasion of cancer cells when it is activated by HGF activator (Parr and Jiang 2001). Insulin-like growth factors (IGFs) bind to one of the six IGF-binding proteins (IGFBPs); we found both IGFBP3 and IGFBP6 to be upregulated in LCNs. IGFBP3 controls circulating IGF levels and is inversely related to the risk of cancers, being upregulated by antiproliferating agents such as antiestrogen and retinoic acids (Deal et al. 2001). In addition, cyclins I and T were activated in both LCNs and HCCs in our experiments.

Some of the genes specifically downregulated in LCNs are involved in intracellular signal transduction pathways, such as PACT/PRKRA and Gb5 (Patel and Sen 1998; Levay et al. 1999). PACT/PRKRA is a mediator of interferon and activator of PKR, a dsRNA-activated protein kinase that phosphorylates eIF-2 to inhibit protein synthesis. In virus-infected cells, viral dsRNA can serve as a PKR activator; thus, PKR mediates the antiviral actions of interferon (Levay et al. 1999). The impairment of this system may allow overgrowth of hepatocytes in association with viral replication.

Outstanding in our list of downregulated genes are several encoding mismatch-repair enzymes such as GADD45, RAD51, and DNA ligase III (Chen et al. 1995; Papathanasiou et al. 1991). Suppression or impairment of the repair system may relate to the hypermutable state of the LCN genome we detected in this study. Many of the other genes downregulated in LCNs were associated with cellular metabolism, such as adipophilin, CLA1, PHKG1, ArgRS, NaK-ATPase and Calmodulin, a feature likely to reflect dysregulation in

LCNs of normal cellular function among differentiated hepatocytes (Calvo and Vega 1993; Kawakami et al. 1986).

More than half of the LCNs in the patient studied here had lost heterozygosity for at least one of the 28 marker loci we examined. MI was observed in one LCN and one HCC, but the histology of LCNs that showed LOH or MI was not different from other cirrhotic nodules. MI appears to be rare, reportedly 3%, in HCCs. Although MI has been observed at an early stage of carcinogenesis in hereditary nonpolyposis colonic cancer (HNPCC) patients, it would be important to confirm that MI is not present in the normal tissue of individuals in HNPCC families (Lothe et al. 1993). The mutator phenotype in cirrhosis nodules is rarely associated with carcinogenesis in human liver either, but it might play a role in the genesis of multiple primary HCCs (Takagi et al. 1998). We found no neoplastic foci in any cirrhotic nodules examined in the present study, and a microsatellite analysis of 15 samples of healthy liver from three individuals showed no alterations (data not shown). These results indicated that events causing genomic instability tend to occur at the LCN stage.

Over-expression of mRNA was examined by semi-quantitative RT-PCR experiments using RNAs from in nodules/tumors for confirmation. Gene amplification, transcriptional activation through hypomethylation of promoter regions, or trans-activation by other cellular factors are speculated to underlie mRNA over-expression. We have shown here presence of LOH or MI in some genome regions in LCNs, suggesting that other as yet unidentified mechanisms may underlie to account for mRNA over-expression in some pathological conditions. Further systematic approach from both gene-expression analysis and genome alteration analysis is warranted to clarify such mechanism.

Expression profiles of LCNs 10 and 12 fell into the HCC group in our hierarchical analysis, although their histopathology was clearly that of LCNs. The phenomenon of clonally growing cells with apparently noncancerous morphology is called "field cancerization," which is thought to result occasionally in multiple primary tumors arising in a localized region (Garcia et al. 1999). This hypothesis predicts that independent epithelial tumors form when carcinogenic exposure affects multiple cells in the field (Garcia et al. 1999). This concept originated from the observation that 11% of individuals with oral cancer have multiple upper aerodigestive-tract tumors and the fact that multiple invasive foci are sometimes associated with overlying areas of in situ squamous carcinomas (Garcia et al. 1999).

HCCs are often multifocal; whether these foci arise from independent clones or as a result of intrahepatic metastasis of a single origin is controversial. Results of the present study, involving a comprehensive analysis of geneexpression and allelic losses in LCNs, appear to suggest that hepatocellular tumors undergo development

and progression according to a multistep carcinogenetic model and that "field cancerization" may occur in the cirrhotic liver. The data reported here should allow a more precise estimation of genetic changes in LCNs and may shed light on the importance of cirrhotic nodules as precancerous lesions. It is clear also that LCNs and HCCs possess characteristic features at the molecular level and that expression profiling might uncover early-stage HCCs that have been histopathologically diagnosed as LCNs.

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