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cDNA Microarray Analysis of Macroregenerative and Dysplastic Nodules in End-Stage Hepatitis C Virus-Induced Cirrhosis

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Hepatocellular carcinoma is a common malignancy causing significant morbidity and mortality worldwide. In this study we use expression microarray technology to identify novel genes that consistently displayed altered expression levels in the earliest identifiable precursors to hepatocellular carcinoma, dysplastic and macroregenerative nodules. The gene expression profiles from nine patients with end-stage hepatitis C cirrhosis that contained a combined 11 dysplastic or macroregenerative nodules were compared to the patient's matched cirrhotic liver tissue. A total of 53 genes were consistently dysregulated in the patient liver specimens. Six of seven genes were validated by quantitative real-time reverse transcriptase-polymerase chain reaction, or by immunohistochemical studies performed on an independent set of lesions. The novel genes, including caveolin-1, semaphorin E, and *FMS*-like tyrosine kinase 3 ligand, have putative roles in carcinogenesis but have not been reported in hepatocellular carcinogenesis. Microarray expression analysis of dysplastic and macroregenerative liver nodules provide insight into the earliest changes in hepatocellular carcinogenesis. *(Am J Pathol 2003, 162:991–1000)*

Hepatocellular carcinoma (HCC) is among the most common malignancies throughout the world and causes significant medical expense and mortality.[1,2] Hepatocellular carcinogenesis is not well understood. Risk factors for the development of HCC include toxin exposure, chronic viral infection, and cirrhosis. It is now recognized that the etiology of cirrhosis has a strong influence on the molecular pathway leading to HCC.[3]

One of the most common causes of cirrhosis is the hepatitis C virus (HCV), which infects 170 million people worldwide.[4,5] Approximately 75% of people exposed to HCV develop chronic infection with ~33% of them developing cirrhosis.[6,7] The increased turnover of hepatocytes and the inflammatory cell infiltrate seen in chronic HCV hepatitis and cirrhosis is thought to lead to accumulation of genetic alterations, which ultimately can result in the development of HCC. In HCC, as with most solid tumors, there are usually numerous chromosomal and genetic changes, which may obscure critical early genetic events.

Studies of adenomatous polyps, the precursor lesions of colonic adenocarcinoma, have played an important role in establishing the sequence of genetic alterations important in colonic carcinogenesis. Similarly, the premalignant precursors to HCC may exhibit critical early genetic alterations important to the understanding of hepatic carcinogenesis. Dysplastic nodules (DNs) and macroregenerative nodules (MRNs) are recognized morphological precursors to HCC.[8–11] They occur with the highest frequency in the chronic liver diseases most strongly associated with the development of HCC.[12–14] In addition, malignant transformation has been reported in nodule-within-nodule lesions.[15,16] MRNs and DNs both show changes in their apoptotic rate and evidence of clonal expansion, making them distinct from the surrounding cirrhotic nodules.[17,18]

We wanted to test the hypothesis that MRNs and DNs exhibit altered gene expression profiles distinct from that of cirrhotic tissue. Because MRNs and DNs are precursor lesions with malignant potential, we subjected them to expression microarray analysis to identify novel genes altered in the earliest stages of hepatocellular carcinogenesis.

Because the etiology of cirrhosis can strongly influence the events leading to HCC, we examined the gene expression profile of macroregenerative and dysplastic liver nodules in the setting of a single etiology of cirrhosis, namely chronic HCV hepatitis. We found 53 genes with expression levels that were significantly and consistently changed in a series of patient samples. Six of these genes were further investigated with quantitative real-

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time reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry. We report several novel genes whose dysregulation might play a role in the earliest stages of hepatocellular carcinogenesis and extend the findings of previously reported genetic alterations identified in HCCs to the setting of HCV. We also placed genes previously reported as altered in HCC at an early point in the multistep transformation process.

Materials and Methods

Clinical Samples

This study was subject to the University of Chicago Institutional Review Board and approved as protocol number 11084A in accordance with the Helsinki Accord on Human Rights guidelines for human research. Explanted livers from nine patients undergoing orthotopic liver transplantation for end-stage hepatitis C cirrhosis were carefully sectioned and grossly examined for MRNs and DNs. Samples from nine nodules and paired samples of distant cirrhotic liver from the same patient were freshly snap-frozen. The remainder of each nodule was submitted for histological examination. Histological criteria for the diagnosis of MRNs and DNs were those outlined in a working statement.[19] MRNs are recognized grossly because they are larger than the surrounding ordinary regenerative nodules, bulge from the cut surface, and differ in color and/or texture. Microscopically portal tracts can be identified within the lesion, and there is no significant cytological or architectural atypia. DNs are similar grossly to MRNs. Microscopically, however, they exhibit cytological and/or architectural atypia (eg, small cell change, increased numbers of unpaired arteries, small clone-like foci, and so forth).

Microarray Analysis

Total RNA was isolated from liver samples after mechanical disruption and extraction following the TRIzol procedure (Invitrogen, Carlsbad, CA). The precipitated products were treated with DNase (Invitrogen) and further purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was converted to cDNA with oligo d(T) primers using the 3DNA Submicro Expression Array Detection Kit (Genisphere, Montvale, NJ). This system is detailed at *http://www.genisphere.com/about.html* but briefly entails generating cDNA that hybridize to fluorescent reagents. The cDNA 5′ end is tagged with an oligo d(T) primer that also contains a capture sequence that is complementary to a sequence linked to multiple fluorescent Cy3 or Cy5 molecules. The capture sequence-tagged cDNA is preincubated with a fluorescent reagent that is linked to the complementary capture sequence. A specific capture sequence is used for each Cy3 and Cy5 molecule. The result is an indirect linkage of Cy3 or Cy5 to a cDNA pool resulting in significant signal amplification. For each patient sample 1 μg of total RNA (determined using Ribogreen; Molecular Probes, Eugene, OR) was reverse-transcribed (Invitrogen) with 0.2 pmol of oligo(dT) primer at 42°C for 2 hours. The reaction was stopped in 0.2 mol/L of NaOH and 20 mmol/L of ethylenediaminetetraacetic acid and DNA/RNA hybrids were broken by incubation at 65°C for 10 minutes. After neutralization of the reaction with 0.25 mol/L of Tris-HCl (pH 7.5) the reaction mixture was precipitated in 0.6 mol/L of ammonium acetate and 80% ethyl alcohol at −20°C for 30 minutes. Samples were spun at 10,000 × *g* and the resulting pellet was washed with 70% ethyl alcohol. The precipitated cDNA was prehybridized to the fluorescent reagents by incubation with 2.5 μl of Cy3 and Cy5 dendrimer reagents (Genisphere), 10 mmol/L of dithiothreitol, and 35 μl of Glass Hybridization Solution (Clontech, Palo Alto, CA) at 55°C for 15 minutes. The GeneMap-Cancer Array (Genomic Solutions, Ann Arbor, MI), which includes 1152 human cDNAs spotted in duplicate, was used in these studies (for a full gene list see *http://www.genomicsolutions.com/genemap/humanarray.html*).

The cDNA microarrays were hybridized with 1.8 ml of Atlas Glass Human Hybridization Solution in a Clontech Hyb Chamber (Clontech) at 55°C for 30 hours. The arrays were washed with Clontech GlassHyb Wash Solution, 1× standard saline citrate with one tenth Clontech GlassHyb Wash Solution, and 0.1× standard saline citrate each with 2 μm dithiothreitol and for 10 minutes at room temperature. The arrays were dried and fluorescent intensities scanned with the Genomic Solutions GeneTAC 2000 scanner (Genomic Solutions) at Cy3 and Cy5 wavelengths using acquisition times that minimized saturation of the maximum fluorescent levels. Fluorescent intensities were acquired from image files using IP Lab Microarray Suite (Scanalytics, Fairfax, VA).

Microarray Data Analysis

Data analysis was performed using GeneSpring version 4.1.5 (Silicon Genetics, Redwood City, CA) analysis software. Dividing by the 50% median calculated from all of the signal intensities for either the Cy3 or Cy5 channel normalized the fluorescent signal for each gene. The expression ratio for each gene was then calculated by dividing the normalized signal for a given gene in a patient's MRN or DN tissue by the normalized signal for the same gene in the patient's distant cirrhotic tissue. Because each gene is represented twice on each array, the expression ratios for each gene were averaged and a percentage coefficient of variation (%CV) was calculated. The mean expression ratios were transformed by log base 2 for all subsequent analyses. Because there were duplicate spots for each gene, quality control could be performed for each chip hybridization. The mean %CV was calculated from the entire %CV for each gene on the array. This gives a global measure of reproducibility between two identically spotted genes on the same chip and takes into account the hybridization and signal acquisition steps, both of which might harbor significant sources of variation and contribute to the random variation of the system. For all of the reported hybridizations the mean %CV was 27% (range, 12 to 35%). This level of intra-array reproducibility (%CV) was similar to that seen

in duplicate runs within a competitive (non-real time) RT-PCR reaction and was considered to represent very good performance.[20]

Two methods were used to identify differentially expressed gene transcripts. In both methods, expression profiles were generated as histograms of the gene expression ratios (nodule/cirrhosis). First, individual expression profiles were generated for each patient sample. The genes with expression ratios at least two standard deviations from the mean in at least five of the samples were regarded as significant (1.8- to 2.2-fold). Second, aggregate expression profiles were generated as a combined histogram for all 11 samples. A gene with an expression ratio at least two standard deviations from the mean was regarded as significant (±1.9-fold). These two methods produced similar but slightly different results. The first method detects genes that were consistently up- or down-regulated in multiple samples, whereas the second method was influenced by extreme outliers in a few samples. Hierarchical clustering and construction of a dendrogram was performed using the "Tree" function in GeneSpring software.[21,22]

Quantitative Real-Time RT-PCR

Total RNA, 500 ng, was converted to cDNA with oligo-dT primers using the MMULV reverse transcriptase protocol (Invitrogen). The cDNA reaction mixture was diluted 1:10 and used in real-time PCR reaction conditions: 200 μmol/L each dNTP, 5.5 mmol/L MgCl, 200 μmol/L each primer, 100 μmol/L probe, 0.75 U Hotstart *Taq* polymerase (Qiagen) under the following reaction conditions: 95°C for 900 seconds followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds (optics on) using the Cephid SmartCycler Version 1.2b (Cepheid, Inc., Sunnyvale, CA). The fold change was calculated by dividing the ratio of the normalized copies of the experimental gene in a nodule by the normalized copies of the experimental gene in cirrhotic tissue. The normalized copies were calculated in standard manner (Applied Biosystems ABI Prism 7700, Bulletin 2, Dec 11 1997). We used the equation Nc = (1 + E)deltaCt where Nc = normalized copies of experimental gene per copies of c-*ABL*, E is the efficiency of the PCR reaction (nearly always 1.0) and delta Ct is the difference in the cycle threshold for the experimental gene and c-*ABL*. The PCR reaction efficiency is determined by the difference between the slope of a standard curve generated for each gene [Ct *versus* log (total RNA)] from the expected slope in an ideal PCR reaction. The c-*ABL* gene was used to normalize patient samples to each other because it has been shown to be stably expressed, whereas other putative normalizing genes such as *GAPDH* have been shown to be strongly induced in HCC.[23] All runs were performed in duplicate. The sequences (5′ to 3′) for each primer (Integrated DNA Technologies Coralville, IA) and probe (Synthegen, Houston, TX) are *ILGFBP3*: 136F GCTCTATGCAGCGTGTGTCC, 201R CAAAGTCAGGCTCAGGGAGACT, probe-157 (FAM)CACCGAGGTCTGCAGCAGGGC(TAMRA); *FLT 3L*: 130F AGCCTGCGGAGAGAGTAGCC, 205R GGAATGTCCTCACACCTACCAAA, probe-154 (FAM)ATCCATCTCTCTGCTGAAAGGTCGCCTG(TAMRA); c-*ABL*: ABL-F AAAATGACCCCAACCTTTTCG, ABL-R CCATTCCCCATTGTGATTATAGC, ABL-probe (FAM)TCTAAGCATAACTAAAGGTGAAAAGCTCCGGGTCTT(TAMRA); *SEMA-E*: 163F GAAAGACAGAATGTCTCCTCATATTGTTT, 271R CTGATTTTTAAGTCAACACTATATATTTTATGATCT, probe-193 (FAM) TCTTACTTTCTAGACATAACAAGCACCTTGCCATCAC (TAMRA).

Tissue Arrays and Immunohistochemistry

A tissue array containing MRNs, DNs, and HCCs from patients not included in the cDNA microarray analysis was constructed. Paraffin-embedded tissue from each case was cored with a 1.5-mm needle using a Beecher machine and placed into a predrilled recipient block. Tissue array blocks were sectioned at 7 μm and prepared for immunohistochemistry. Antibodies used included caveolin-1 (1:200; Becton, Dickinson and Co., San Diego, CA), Retinoblastoma (RB) (2 μg/ml, Becton, Dickinson and Co.), and collagen IV (1:100; DAKO, Indianapolis, IN). Antigen retrieval, consisting of 95°C for 10 minutes (caveolin-1) in 0.01 mol/L citrate buffer, pH 6.0, DAKO target retrieval buffer, pH 6.0, 27 minutes in a bench top pressure cooker (pRB), or 15 minutes 99°C (collagen IV) was performed. pRB and collagen IV were stained on the automated Ventana system as per the manufacturer's directions with rabbit anti-mouse secondary antibody and biotin/streptavidin/diaminobenzidine detection reagents (Pierce, Rockford, IL). For caveolin-1 a 3% H_2O_2 quenching solution, 10% normal serum blocking solution, and incubation overnight at 4°C with the primary antibody was used. The secondary biotinylated antibody (Zymed Labs., San Francisco, CA) was followed with streptavidin horseradish peroxidase, developed with diaminobenzidine and counterstained with hematoxylin. The arrays were examined and the intensity of staining for each core was graded on a 0 (absent), 1+ (weak), 2+ (moderate), and 3+ (strong staining) scale. The number of positive nuclei and intensity of staining was scored for pRB while the intensity of sinusoidal staining was graded for collagen IV and caveolin. Tissue cores that were missing from a section were not graded. Percentages summarizing the staining grades for each array were calculated as the number of cores with a particular staining grade divided by the total number of cores present.

Results

Microarray Results

To assess the biological relevance of the microarray results we examined clinical subsets to determine whether they yielded similar expression patterns. Each paired sample was placed into a dendrogram based on the Pearson's correlation coefficient calculated for each sample using all of the gene expression ratios for each paired sample. As expected, patients with two samples from the

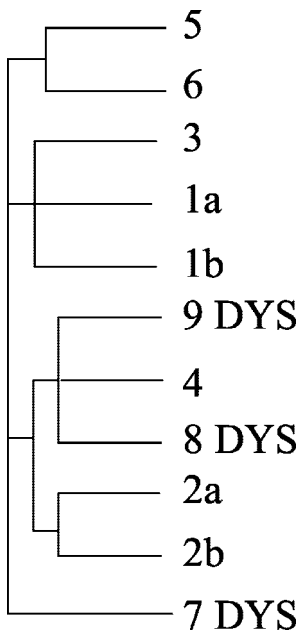


Figure 1. The dendrogram shows the relationship of the patient samples containing either MRNs (number), dysplastic nodules (DYS), or with more than one sample (1a and 1b, 2a and 2b) based on the expression levels of all of the 1152 genes represented on the cDNA microarray. The relationships were determined by the Pearson's correlation coefficient calculated using the global expression pattern for pairs of patient samples.

same explanted liver had expression profiles with similar correlation coefficients and therefore are located near each other in the dendrogram (Figure 1).

The nine patient samples consisting of MRNs and DNs and paired distant cirrhotic tissue from the same patient were analyzed with the cDNA arrays for significant changes in gene expression levels. Fifty-three genes met the criteria for significant up- or down-regulation as defined in the Materials and Methods section (Table 1). When the data were treated in aggregate with all patient data averaged together (see Materials and Methods), 45 genes were determined to be significant (changed 1.9-fold in either direction). Alternatively, treating the data as individual samples identified 34 genes as significant (changed 1.8- to 2.2-fold in either direction) in more than half of the paired patient samples (Figure 2). Although there was some variation in the magnitude of each individual gene's change in expression level (fold change), the genes identified as significant were consistently changed in the same direction (same color). Moreover, there was significant overlap between those identified from the aggregate and individual methods, with 26 genes identified by both methods.

Quantitative Real-Time RT-PCR Validation Studies

To validate the microarray data we performed quantitative real-time RT-PCR on a set of five genes thought to be novel to hepatocellular carcinogenesis. The FMS-like tyrosine kinase (flt) receptor system that participates in hematopoiesis has also been demonstrated in hepatocytes.[24,25] Our cDNA microarray data demonstrated a consistent decrease in flt-3 ligand (flt-3l) mRNA in all MRN and DN samples relative to paired cirrhotic tissue (Figure 3A). Quantitative real-time RT-PCR confirmed the cDNA microarray result, with 9 of 11 samples showing decreased flt-3l mRNA in nodules compared to paired sample of cirrhotic tissue.

Semaphorin E (Sema E) is a member of a family of secreted proteins that are involved in the guidance of axons.[26] Our cDNA microarray analysis identified this gene as overexpressed in all of the MRN and DN samples. Quantitative real-time RT-PCR confirmed this result with all but a single nodule showing an increase in *Sema E* expression (Figure 3B, sample 9). The *Sema E* gene belongs by sequence homology to class III semaphorins.[27] It is possible other semaphorin members are responsible for cross-hybridization on the cDNA microarray.

PAX5 is involved in organogenesis through *HOX* gene expression. In the cDNA microarray analysis this gene was up-regulated in 5 of 11 of the patient MRN and DN samples. Quantitative real-time RT-PCR was able to detect transcripts in 6 of 11 nodules without detectable product in any of the cirrhotic tissue (data not shown). Therefore, *PAX5* appears to be significantly up-regulated in MRNs and DNs.

A second transcription factor, the Wilms tumor 1 gene (*WT1*) was up-regulated according to our cDNA microarray analysis, however, this was not confirmed by quantitative real-time RT-PCR as 7 of the 11 samples showed a down-regulation and the other 4 showed no change in expression level. The *WT 1* gene product is comprised of 10 exons and is known to have a complex assortment of alternative splicing products, which might explain the lack of a correlation between quantitative real-time RT-PCR and cDNA microarray results for this particular gene.[28]

Although the insulin-like growth factor (IGF) pathway has been implicated in HCC development, the contribution of IGF-binding proteins is not clear.[29–31] Our cDNA microarray results showed an increase in IGF-BP3 in MRNs and DNs in the aggregate analysis (Figure 3C, filled bars). We validated this finding by quantitative real-time RT-PCR, confirming an increase in expression in 10 of 11 samples (Figure 3C, open bars). There was a single discordant result between the cDNA microarray and quantitative real-time PCR methods (Figure 3C, patient 1b).

Immunohistochemistry Validation Studies

We further validated the data by examining expression levels of selected genes at the protein level. We also wished to validate our findings using samples from patients other than those used for the cDNA microarray and quantitative real-time PCR experiments. To this end, we constructed a tissue microarray from an independent set of patients with HCV cirrhosis. The tissue array consisted of 36 MRNs, 18 DNs, and 18 HCCs with paired cirrhotic tissue as controls. We performed immunohistological stains of tissue microarray with antibodies to three proteins identified as outliers by the cDNA microarray experiments.

Table 1. Up- and Down-Regulated Genes Grouped by Known Function

Gene no.	Accession no.	Gene name	Ag	Ind	Validation
		Down-regulated			
Oncogene					
1	AA457097	v-akt homolog 2	X	X	Lit
2	AA922309	Kangai 1, CD82	X		Lit
DNA repair					
3	N62586	DNA excision repair protein ERCC5	X		NI
Growth factors and cytokines					
4	AI150354	Flt-3	X	X	Q-PCR
5	R35665	Epidermal growth factor receptor	X		IHC
Cell adhesion					
6	AA746392	pax5	X		Q-PCR
Signal transduction					
7	AA030029	Protein kinase C, alpha	X		Lit
8	AA485523	Protein kinase, cAMP dependent, type I, beta	X		NI
Cell-cell interactions					
9	AA078778	Tyrosine protein kinase CSK	X	X	Lit
10	R89615	Human protocadherin	X		NI
Invasion regulators					
11	N71159	Human metastasis associated MTA1	X		NI
Transcription factor/DNA binding					
12	AA417562	ESTs, similar to zinc finger protein	X	X	NI
13	AA120823	fra1	X	X	NI
14	AA026102	Transcription factor 3	X	X	NI
15	AA496576	Transcription factor 11 basic leucine zipper		X	NI
16	AA932311	HOXC6		X	NI
17	AA811084	TRT	X		NI
18	AA482079	ESTs, similar to zinc finger protein XLCGF8.2DB	X		NI
19	N94321	ESTs, similar to zinc finger protein 84	X		NI
20	N80235	Human GC box binding protein	X		NI
21	AA45587	Human TFIID subunits TAF20 and TAF15	X		NI
22	AA406269	Nuclear factor I/X	X		NI
Translation					
23	W81684	RNA polymerase II elongation factor SIII, p15	X	X	NI
24	H56918	Eukaryotic translation factor 4A	X		NI
Housekeeping					
25	R91078	Cytochrome P450 IIIA7		X	Lit
26	AI420192	H4 histone D		X	NI
27	H90219	Myoinositol-1-monophosphatase	X		NI
28	R83355	ESTs, similar to NK tumor recognition protein	X		NI
Cell cycle					
29	AA676387	Cell cycle progression 2 protein (CRP2)	X		NI
		Up-regulated			
Oncogene/tumor suppressor					
30	AA045192	Retinoblastoma 1	X	X	Lit
31	T50699	Homo sapiens cancer associated surface antigen (RCAS1)	X	X	Lit
32	N24699	v-erb2 viral homolog 3	X	X	NI
33	AA130187	Wilms tumor 1		X	Dis
Growth factor/cytokines					
34	AA598601	Insulin like growth factor binding protein 3	X	X	Q-PCR
35	AA489602	Tumor necrosis factor type 1 receptor associated protein	X	X	Lit
36	AA452627	Endothelin receptor type A	X	X	Lit
37	T58932	FOS related antigen 2		X	Lit
38	H16854	CD 86		X	Lit
Cell adhesion, motility and invasion					
39	AA487560	Caveolin 1	X	X	IHC
40	AA042990	Semaphorin E	X	X	Q-PCR
41	AA150402	Collagen, type IV	X	X	NI
42	N51018	ESTs, similar to proteoglycan precursor	X	X	Lit
Other					
43	R53935	P glycoprotein 3/multiple drug resistance 3	X		Lit
Transcription factor and DNA binding					
44	H29557	Helix loop helix protein 2	X	X	NI
45	AA013268	Homo sapiens leucine zipper bearing kinase	X	X	NI
46	AA043503	Down regulator of transcription TBP 1	X	X	NI
47	AA013268	Human transcriptional coactivator PC4	X	X	NI
48	N50513	KRAB zinc finger protein	X	X	NI
49	R91570	Signal transducer and activator of transcription 4	X	X	NI
Housekeeping					
50	AA668425	Amylo1,6 glucosidase, 4 alpha glucanotransferase	X	X	NI
51	AA683578	Adenosine deaminase	X	X	NI
52	AA598814	ATPase, Na+/K+ transporting, beta 1		X	NI
53	AA676466	Argininosuccinate synthetase	X	X	NI

Significantly up- or down-regulated genes with accession number are listed and grouped by known function. Significant genes were determined to have an expression ratio (normalized signal in a patients nodule/cirrhosis) that was two standard deviations from the mean in an aggregate (Ag) analysis or in 5 of 11 individual patient pairs (Ind). The validation column indicates genes that were independently validated by quantitative real-time RT-PCR (Q-PCR), immunohistochemistry (IHC), agree with published literature (Lit) regarding hepatocellular carcinoma or with no additional information (NI). Genes with Q-PCR in disagreement (Dis) with the cDNA microarray findings are also indicated.

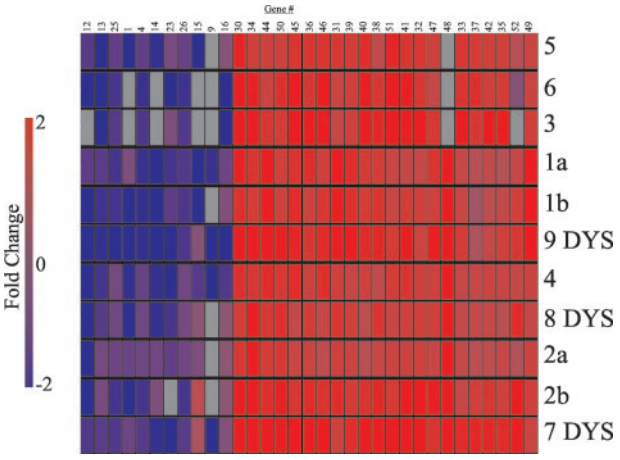


Figure 2. Overview of the expression profiles for genes identified by the cDNA microarray. Each **row** represents an individual patient sample pair with a MRN (number), dysplastic nodule (DYS), or with multiple samples (1a and 1b, 2a and 2b). Each **column** represents an individual gene with a number that refers to the gene number listed in Table 1. The relative expression level for each gene is indicated by a color gradient from red (high expression in a nodule relative to cirrhosis) to blue (low expression in nodule relative to cirrhosis); gray indicates no information.

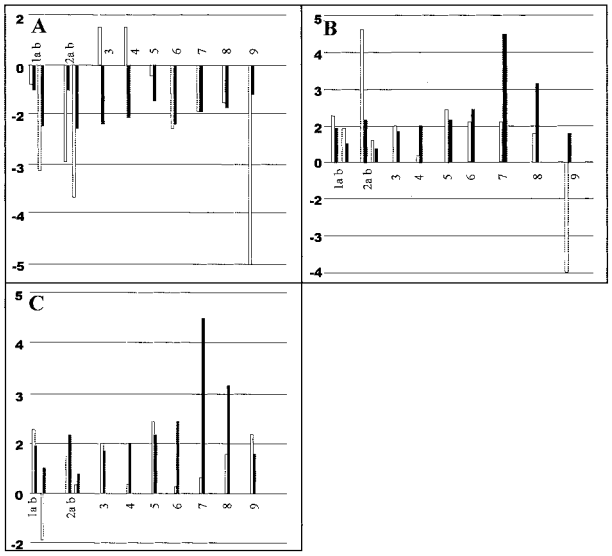


Figure 3. Quantitative real-time RT-PCR summary. The expression for flt-3l (**A**), Semaphorin E (**B**), and ILGF BP3 (**C**) was determined by cDNA microarray (**filled bars**) or quantitative real-time RT-PCR (**open bars**) and reported as the fold change in the MRNs or DNs compared to the patients (1 to 9) matched cirrhotic liver.

Collagen IV is a component of the basement membrane that has been shown to have an altered expression pattern in cirrhosis.[32–34] The cDNA microarray analysis demonstrated that collagen IV mRNA levels were increased in MRNs and DNs. An increase in collagen IV protein was confirmed by immunohistological stains of the tissue array (Figure 4; A to C). More than half (52%) of the MRNs and DNs showed more intense (2+ to 3+) collagen IV reactivity than the cirrhotic tissue (20% 2+ to 3+). Reactivity was seen along sinusoids in both the cirrhotic and MRN/DN samples, but was more intense in the MRNs and DNs. Interestingly, 15 of 17 (88%) of the HCCs showed a uniform decrease (0 to 1+) of this intense sinusoidal staining (Figure 4D).

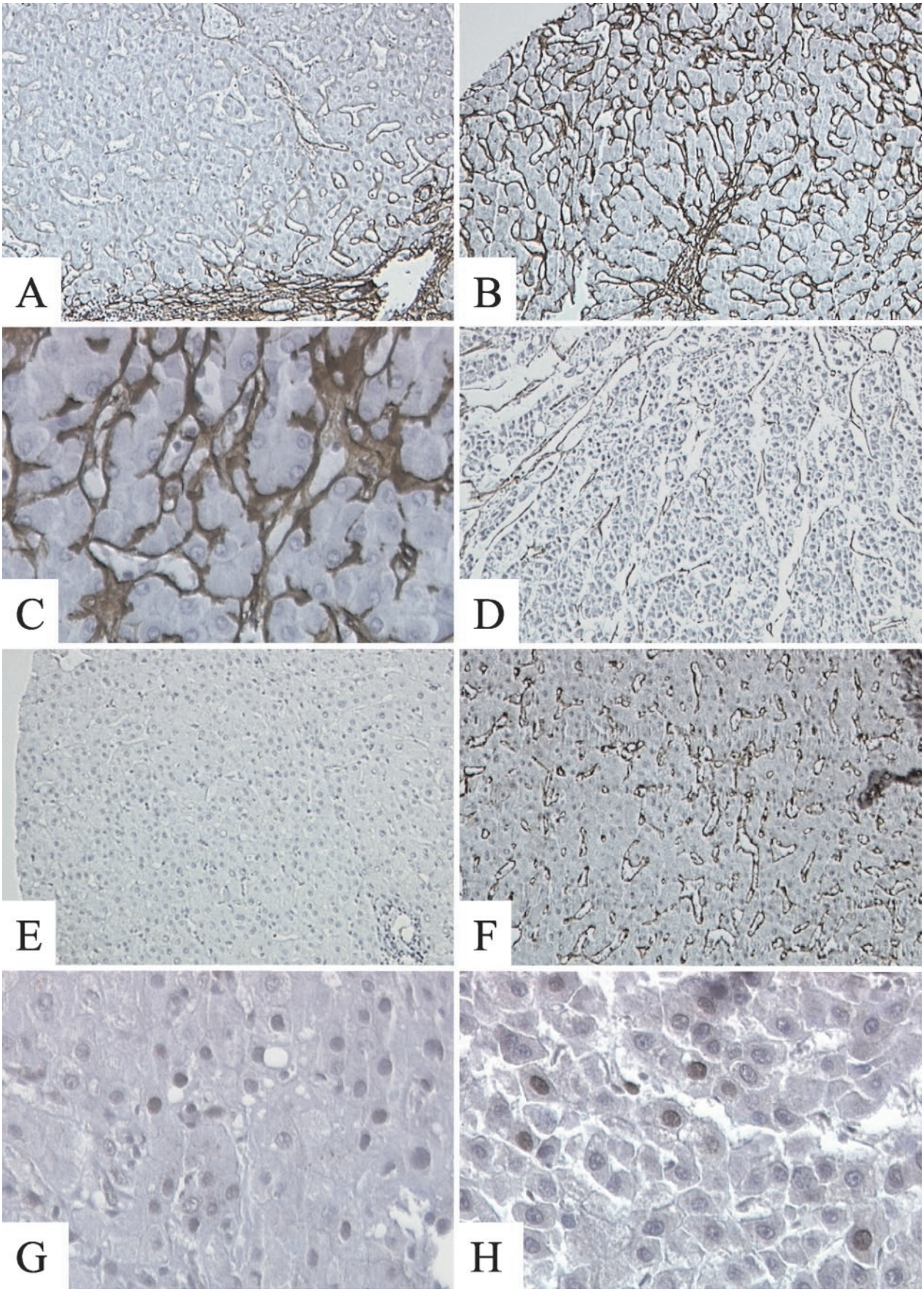
Caveolin-1 has recently been reported to have altered expression in a variety of tumors.[35–37] The cDNA microarray analysis showed increased expression of this gene in MRNs and DNs when compared to cirrhotic tissue. Immunohistochemistry performed on the tissue array with caveolin-1 revealed moderate to strong (2+ to 3+) reactivity in sinusoidal endothelial cells of 90% of the MRNs and DNs and absent or weak (0 to 1+) reactivity in 71% of the cirrhotic tissue samples (Figure 4, E and F).

Retinoblastoma (*Rb*) is a tumor suppressor gene whose expression is altered in several tumors and been shown to be altered in hepatic carcinogenesis.[38,39] *Rb* mRNA was markedly increased according to the cDNA microarray experiment. Immunohistochemical staining for Rb protein revealed nuclear staining in rare hepatocytes in the cirrhotic tissue samples. The MRN and DN samples showed weak reactivity for Rb protein in hepatocyte nuclei (Figure 4H), compared to frequent nuclear reactivity in HCC samples (Figure 4G). The lack of concordance between the mRNA level and protein level for Rb in liver has been reported in transgenic mouse studies.[40] In these studies *Rb* tumor suppressor function correlated better with increased mRNA measured by reverse transcription PCR than with protein detection by immunohistochemistry. The high level of Rb protein expression detected in HCC samples supports the suggestion that *Rb* mRNA expression in MRNs and DNs is increased.

Discussion

The early steps in hepatocellular carcinogenesis are not well defined. As with many solid tumors, complex genetic changes occur in established HCCs. The number of genetic and gross chromosomal changes that occurs late in tumor progression make sorting out primary and secondary changes difficult. The critical early steps of hepatocellular carcinogenesis have not been studied extensively in humans, primarily because tissue from the precursor lesions (MRNs and DNs) has not been easy to obtain. Investigations of the earliest precursors to adenocarcinoma, adenomatous polyps, has been key to understanding the sequence of genetic alterations important in colon carcinogenesis. To understand the earliest changes in hepatocellular carcinogenesis, we examined a single background of liver disease, hepatitis C related cirrhosis, and the earliest possible malignant precursors to HCC, namely MRNs and DNs. MRN and DN samples were obtained from patients undergoing hepatectomy for orthotopic liver transplant and were analyzed by cDNA

Figure 4. Immunohistochemical staining of tissue array containing cirrhotic, MRN/DN, and HCC tissue. **A:** Weak to absent staining for collagen IV along the sinusoids in cirrhotic tissue. **B** and **C:** Strong staining highlights the hepatic sinusoids in MRNs. **D:** HCCs show a loss of collagen IV staining. Caveolin-1 antibodies do not stain cirrhotic tissue (**E**) but do stain MRN (**F**) endothelial cells. The retinoblastoma protein shows light nuclear staining in the MRN (**G**) and more intense nuclear staining in HCC (**H**). Original magnifications: ×10 (**A**, **B**, **D–F**); ×40 (**C**, **G**, **H**).


A
B
C
D
E
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H

microarrays. The main goal of the analysis was to identify genes whose expression was consistently altered in comparison to the surrounding cirrhotic liver. Although comparison of gene expression between cirrhotic tissue and normal liver would also be of interest, the focus of these experiments was the genetic alterations important during the progression from an abnormal, yet nonmalignant state (cirrhosis) to a premalignant one (MRNs and DNs). We used nine patient samples containing 11 nodules (three DNs and eight MRNs) that were paired with the patient's own cirrhotic tissue. Stringent criteria were applied to generate a list of outlier genes. The cDNA microarrays used contained 1152 genes that were spotted in duplicate, which added a measure of reproducibility within a given array hybridization. Furthermore, we required outlier genes to be significantly altered in at least half of the paired patient samples. We identified 29 up-regulated and 24 down-regulated genes, many of which are novel to the understanding of hepatocellular carcinogenesis. The findings were validated in six of seven selected genes with quantitative real-time RT-PCR and by immunohistochemistry.

The classes of genes identified as altered (Table 1) fit well with the current understanding of carcinogenesis and hepatic biology. Transcription factors and housekeeping genes constituted the largest classes of altered genes. As would be expected, hepatic nodules are metabolically distinct from the surrounding cirrhotic liver and these differences are likely to be represented by alterations in expression levels of transcription factor and metabolic genes.

The next largest class of genes identified was growth factors and cytokines. They are responsible for paracrine and autocrine regulation of many cellular processes including cell growth and apoptosis. Because our experiments were performed on patient material rather than hepatocyte cell lines, we identified genetic alterations not only in hepatocytes but also in endothelial, fibroblast, and inflammatory cell population. Thus, our list of growth factors and cytokines reflect the microenvironment that likely promotes progression to HCC.

Among the smallest classes of genes identified were the cell-cycle and signal transduction genes. Signal transduction and the cell cycle are not necessarily transcriptionally regulated and therefore might not be identified in mRNA-based microarray experiments.

Flt-3l has been shown to cause hematopoietic and bile duct cell proliferation and differentiation.[41,42] We found the levels of flt-3l to be reduced in 9 of 11 MRNs and DNs compared to surrounding cirrhotic tissue. Interestingly, two samples show an inverse correlation between the cDNA microarray and quantitative real-time RT-PCR. The quantitative real-time RT-PCR method interrogates a smaller (76 bp) cDNA region than the cDNA placed on the microarray (480 bp). Splice variants have been documented in the 3′ region of flt 3l that result in either a long transmembrane or short soluble form. The cDNA microarray would not necessarily distinguish between these two forms, whereas quantitative real-time RT-PCR would.[43,44] Because flt-3l has been shown to be expressed in inflammatory cells, a decrease in flt-3l might indicate a unique inflammatory milieu within the nodules.[25,45] It has been established that the inflammatory cell populations in cirrhotic livers are quantitatively and qualitatively different from those in peripheral blood.[25,45]

Sema E was originally identified through its role in controlling axon migration.[46] It has been implicated in chemotherapy resistance independent of multidrug resistance and is overexpressed in metastatic lung adenocarcinoma cell lines.[26,27,46,47] Our analysis indicated an increase in both multidrug resistance and *Sema E* expression, and this duel expression might explain the relative chemoresistance of HCC. *Sema E* has been found to be overexpressed in lung adenocarcinoma cell lines and in synovial cells from chronic inflammatory disease patients.[27,48] *Sema E* might play a role not only in the tumor progression in the liver but might also be related to the chronic inflammation seen in HCV-induced cirrhosis. The understanding of the functions of the semaphorin family of proteins is expanding to neoplastic processes and our results suggest *Sema E* is involved early in hepatocellular carcinogenesis.[27]

The caveolin family of proteins are the principal components of caveolae, plasma membrane invaginations involved in endocytosis and signal transduction. Caveolin has been suggested to act as a putative oncogene and may be involved in drug resistance.[49] Caveolin-1 mRNA was up-regulated according to our cDNA microarray analysis and immunohistochemical studies validated a marked increase in caveolin-1 expression in MRNs and DNs. The immunohistochemistry studies provided additional information by localizing caveolin-1 expression to endothelial cells lining the hepatic sinusoids. Overexpression of caveolin-1 was recently demonstrated in colonic adenocarcinoma and transitional cell carcinoma but not in corresponding normal epithelia, suggesting caveolin-1 is associated with tumor progression.[35,36] Taken together these data suggest that alterations in caveolin expression maybe involved in malignant progression.

A variety of changes in the IGF axis occur in HCC. On the one hand IGF-binding proteins (IGF-BPs) have been shown to inhibit proliferation by sequestering IGFs, while on the other hand it has been suggested that they potentiate proliferation by stabilizing IGF receptor binding.[50] Conflicting reports regarding the change in expression of IGF ligands, receptors, and binding proteins might in part reflect differences between *in vivo* and *in vitro* systems.[29] Studies with human liver samples have shown IGF-BP3 to be methylated in HCCs.[51] Loss of expression of IGF-BP3 might be an important step in hepatic tumor progression because we found it to be markedly up-regulated in MRNs and DNs. Overexpression of IGF-BP3 might be a gatekeeper for the IGF axis which, when lost, leads to tumor progression. However several IGF-BPs are likely to be important in hepatocellular carcinogenesis, because changes in multiple IGF-BPs have been documented in preneoplastic foci in rat livers.[52] Furthermore, the source of IGF-BP3 is unclear because it appears to be secreted from Kupffer, endothelial, and hepatic stellate cells.[53,54]

We also found increased expression of collagen IV, a principal component of the basement membrane, in DNs

and MRNs. Immunohistochemical stains revealed deposition of collagen IV in the basement membrane of the hepatic sinusoidal endothelial cells of MRNs and DNs, beyond that which was evident in cirrhotic tissue. Using electron microscopy, thickening of the basement membrane has been documented in carcinogen-treated rat liver nodules.[55] Thickening of the basement membrane results in alterations in endothelial cell permeability by decreasing endothelial cell fenestrae. This offers another potential mechanism to explain chemoresistance in HCC. In addition, basement membrane thickening also has been associated with changes in the vascularity or arterialization of nodules. Neovascularization has been shown to be an early marker of tumor progression, particularly in oral cancers.[56] Our findings suggest alterations in the vascularity of MRNs and DNs also correlates with early changes in hepatocellular carcinogenesis. Interestingly immunohistological staining for collagen IV decreased in the HCC samples in the tissue arrays. Loss of the basement membrane has been shown to be associated with the late stage of tumor invasion in other carcinomas.[57–59]

Finally, we identified a number of genes belonging to pathways previously implicated in hepatocellular carcinogenesis. Our results extend earlier observations to the earliest changes in hepatitis C-induced premalignant lesions. Epidermal growth factor, retinoblastoma, and IGF pathways have been implicated in hepatocellular carcinogenesis. A change in the IGF pathway members is documented in three other studies of hepatitis C-infected livers using expression microarrays. Two studies document specific change in IGF-BP3 levels similar to our results.[60,61] Loss of expression of the EGF receptor, such as we report, was also identified in another HCC expression microarray study.[62] Other reported altered genes, such as the multidrug resistance protein and human cancer-associated surface antigen (RCAS1) agree with our results and have specifically been shown to be markedly up-regulated in established HCC.[63–65]

We also performed a hierarchical analysis (Figure 1) primarily to determine whether the expression profiles of the paired patient samples grouped into clinically important groups. Reassuringly, duplicate patient samples clustered together. However, the three DNs did not all cluster closely together. Instead two of the DNs grouped with a MRN suggesting molecular homogeneity between DNs and MRNs. Review of the histology of these nodules supports their initial morphological distinction. These nodules seem to have greater morphological differences than biological. A third DN (patient 7) was isolated from all other patient cases, suggesting that it is biologically distinct although not morphologically different from the other DNs.

In conclusion, we identified genes with marked and consistent alteration in expression levels in MRNs and DNs that arose in a single etiological background of hepatitis C-induced end-stage liver disease. Because MRNs and DNs are the earliest identifiable morphological precursors with malignant potential, the identified genes provide insight into to the understanding of the earliest stages in hepatocellular carcinogenesis. The alteration in expression of a selected subset of genes was used to validate the cDNA microarray findings.

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References

1. Hussain SA, Ferry DR, El-Gazzaz G, Mirza DF, James ND, McMaster P, Kerr DJ: Hepatocellular carcinoma. Ann Oncol 2001, 12:161–172
2. Feitelson MA, Sun B, Satiroglu Tufan NL, Liu J, Pan J, Lian Z: Genetic mechanisms of hepatocarcinogenesis. Oncogene 2002, 21:2593–2604
3. Laurent-Puig P, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, Monges G, Thomas G, Bioulac-Sage P, Zucman-Rossi J: Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. Gastroenterology 2001, 120:1763–1773
4. Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA, Margolis HS: The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med 1999, 341:556–562
5. Lauer GM, Walker BD: Hepatitis C virus infection. N Engl J Med 2001, 345:41–52
6. Komanduri S, Cotler S: Hepatitis C. Clin Perspect Gastroenterol 2002, (March/April):91–99
7. Seeff LB, Hollinger FB, Alter HJ, Wright EC, Cain CM, Buskell ZJ, Ishak KG, Iber FL, Toro D, Samanta A, Koretz RL, Perrillo RP, Goodman ZD, Knodell RG, Gitnick G, Morgan TR, Schiff ER, Lasky S, Stevens C, Vlahcevic RZ, Weinshel E, Tanwandee T, Lin HJ, Barbosa L: Long-term mortality and morbidity of transfusion-associated non-A, non-B, and type C hepatitis: a National Heart, Lung, and Blood Institute collaborative study. Hepatology 2001, 33:455–463
8. Terada T, Terasaki S, Nakanuma Y: A clinicopathologic study of adenomatous hyperplasia of the liver in 209 consecutive cirrhotic livers examined by autopsy. Cancer 1993, 72:1551–1556
9. Furuya K, Nakamura M, Yamamoto Y, Togei K, Otsuka H: Macroregenerative nodule of the liver. A clinicopathologic study of 345 autopsy cases of chronic liver disease. Cancer 1988, 61:99–105
10. Sun M, Eshleman JR, Ferrell LD, Jacobs G, Sudilovsky EC, Tuthill R, Hussein MR, Sudilovsky O: An early lesion in hepatic carcinogenesis: loss of heterozygosity in human cirrhotic livers and dysplastic nodules at the 1p36–p34 region. Hepatology 2001, 33:1415–1424
11. Thung SN, Hytiroglou P, Fiel I, Theise N: Preneoplastic lesions in chronic hepatitis C. Princess Takamatsu Symp 1995, 25:171–178
12. Hytiroglou P, Theise ND, Schwartz M, Mor E, Miller C, Thung SN: Macroregenerative nodules in a series of adult cirrhotic liver explants: issues of classification and nomenclature. Hepatology 1995, 21:703–708
13. Ferrell L, Wright T, Lake J, Roberts J, Ascher N: Incidence and diagnostic features of macroregenerative nodules vs. small hepatocellular carcinoma in cirrhotic livers. Hepatology 1992, 16:1372–1381
14. Takano S, Yokosuka O, Imazeki F, Tagawa M, Omata M: Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. Hepatology 1995, 21:650–655
15. Matsuno Y, Hirohashi S, Furuya S, Sakamoto M, Mukai K, Shimosato Y: Heterogeneity of proliferative activity in nodule-in-nodule lesions of small hepatocellular carcinoma. Jpn J Cancer Res 1990, 81:1137–1140
16. Oda T, Tsuda H, Sakamoto M, Hirohashi S: Different mutations of the p53 gene in nodule-in-nodule hepatocellular carcinoma as a evidence for multistage progression. Cancer Lett 1994, 83:197–200
17. Sakamoto M, Hirohashi S, Shimosato Y: Early stages of multistep hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular carcinoma. Hum Pathol 1991, 22:172–178
18. Thomas RM, Berman JJ, Yetter RA, Moore GW, Hutchins GM: Liver cell dysplasia: a DNA aneuploid lesion with distinct morphologic features. Hum Pathol 1992, 23:496–503
19. International Working Party: Terminology of nodular hepatocellular lesions. Hepatology 1995, 22:983–993
20. Gerard CJ, Olsson K, Ramanathan R, Reading C, Hanania EG: Improved quantitation of minimal residual disease in multiple myeloma using real-time polymerase chain reaction and plasmid-DNA complementarity determining region III standards. Cancer Res 1998, 58: 3957–3964
21. Eisen MB, Spellman PT, Brown PO, Botstein D: Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998, 95:14863–14868

22. Nguyen U: GeneSpring Advanced Analysis Techniques, chapter 2. Redwood City, CA, Silicon Genetics, 2000
23. Gong Y, Cui L, Minuk GY: Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28s-ribosomal RNA gene expression in human hepatocellular carcinoma. Hepatology 1996, 23:734–737
24. Omori M, Omori N, Evarts RP, Teramoto T, Thorgeirsson SS: Coexpression of flt-3 ligand/flt-3 and SCF/c-kit signal transduction system in bile-duct-ligated SI and W mice. Am J Pathol 1997, 150:1179–1187
25. Monga SP, Tang Y, Candotti F, Rashid A, Wildner O, Mishra B, Iqbal S, Mishra L: Expansion of hepatic and hematopoietic stem cells utilizing mouse embryonic liver explants. Cell Transplant 2001, 10:81–89
26. Tessier-Lavigne M, Goodman CS: The molecular biology of axon guidance. Science 1996, 274:1123–1133
27. Martin-Satue M, Blanco J: Identification of semaphorin E gene expression in metastatic human lung adenocarcinoma cells by mRNA differential display. J Surg Oncol 1999, 72:18–23
28. Hirose M: The role of Wilms' tumor genes. J Med Invest 1999, 46: 130–140
29. Scharf JG, Dombrowski F, Ramadori G: The IGF axis and hepatocarcinogenesis. Mol Pathol 2001, 54:138–144
30. Norstedt G, Levinovitz A, Moller C, Eriksson LC, Andersson G: Expression of insulin-like growth factor I (IGF-I) and IGF-II mRNA during hepatic development, proliferation and carcinogenesis in the rat. Carcinogenesis 1988, 9:209–213
31. Cariani E, Lasserre C, Seurin D, Hamelin B, Kemeny F, Franco D, Czech MP, Ullrich A, Brechot C: Differential expression of insulin-like growth factor II mRNA in human primary liver cancers, benign liver tumors, and liver cirrhosis. Cancer Res 1988, 48:6844–6849
32. Hahn E, Wick G, Pencev D, Timpl R: Distribution of basement membrane proteins in normal and fibrotic human liver: collagen type IV, laminin, and fibronectin. Gut 1980, 21:63–71
33. Milani S, Herbst H, Schuppan D, Hahn EG, Stein H: In situ hybridization for procollagen types I, III and IV mRNA in normal and fibrotic rat liver: evidence for predominant expression in nonparenchymal liver cells. Hepatology 1989, 10:84–92
34. Jezequel AM, Ballardini G, Mancini R, Paolucci F, Bianchi FB, Orlandi F: Modulation of extracellular matrix components during dimethylnitrosamine-induced cirrhosis. J Hepatol 1990, 11:206–214
35. Fine SW, Lisanti MP, Galbiati F, Li M: Elevated expression of caveolin-1 in adenocarcinoma of the colon. Am J Clin Pathol 2001, 115:719–724
36. Rajjayabun PH, Garg S, Durkan GC, Charlton R, Robinson MC, Mellon JK: Caveolin-1 expression is associated with high-grade bladder cancer. Urology 2001, 58:811–814
37. Wiechen K, Diatchenko L, Agoulnik A, Scharff KM, Schober H, Arlt K, Zhumabayeva B, Siebert PD, Dietel M, Schafer R, Sers C: Caveolin-1 is down-regulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. Am J Pathol 2001, 159:1635–1643
38. Hsia CC, Di Bisceglie AM, Kleiner Jr DE, Farshid M, Tabor E: RB tumor suppressor gene expression in hepatocellular carcinomas from patients infected with the hepatitis B virus. J Med Virol 1994, 44:67–73
39. Zheng L, Lee WH: The retinoblastoma gene: a prototypic and multifunctional tumor suppressor. Exp Cell Res 2001, 264:2–18
40. Ichihara T, Komagata Y, Yang XL, Uezato T, Enomoto K, Koyama K, Miyazaki J, Sugiyama T, Miura N: Resistance to fulminant hepatitis and carcinogenesis conferred by overexpression of retinoblastoma protein in mouse liver. Hepatology 2001, 33:948–955
41. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR: A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell 1991, 65:1143–1152
42. Rosnet O, Marchetto S, deLapeyriere O, Birnbaum D: Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. Oncogene 1991, 6:1641–1650
43. Lyman SD, Stocking K, Davison B, Fletcher F, Johnson L, Escobar S: Structural analysis of human and murine flt3 ligand genomic loci. Oncogene 1995, 11:1165–1172
44. Lyman SD, James L, Escobar S, Downey H, de Vries P, Brasel K, Stocking K, Beckmann MP, Copeland NG, Cleveland LS: Identification of soluble and membrane-bound isoforms of the murine flt3 ligand generated by alternative splicing of mRNAs. Oncogene 1995, 10:149–157
45. Smith JR, Thackray AM, Bujdoso R: Reduced herpes simplex virus type 1 latency in Flt-3 ligand-treated mice is associated with enhanced numbers of natural killer and dendritic cells. Immunology 2001, 102:352–358
46. Chen H, He Z, Tessier-Lavigne M: Axon guidance mechanisms: semaphorins as simultaneous repellents and anti-repellents. Nat Neurosci 1998, 1:436–439
47. Yamada T, Endo R, Gotoh M, Hirohashi S: Identification of semaphorin E as a non-MDR drug resistance gene of human cancers. Proc Natl Acad Sci USA 1997, 94:14713–14718
48. Mangasser-Stephan K, Dooley S, Welter C, Mutschler W, Hanselmann RG: Identification of human semaphorin E gene expression in rheumatoid synovial cells by mRNA differential display. Biochem Biophys Res Commun 1997, 234:153–156
49. Timme TL, Goltsov A, Tahir S, Li L, Wang J, Ren C, Johnston RN, Thompson TC: Caveolin-1 is regulated by c-myc and suppresses c-myc-induced apoptosis. Oncogene 2000, 19:3256–3265
50. Clemmons DR: Insulin-like growth factor binding proteins and their role in controlling IGF actions. Cytokine Growth Factor Rev 1997, 8:45–62
51. Hanafusa T, Yumoto Y, Nouso K, Nakatsukasa H, Onishi T, Fujikawa T, Taniyama M, Nakamura S, Uemura M, Takuma Y, Yumoto E, Higashi T, Tsuji T: Reduced expression of insulin-like growth factor binding protein-3 and its promoter hypermethylation in human hepatocellular carcinoma. Cancer Lett 2002, 176:149–158
52. Scharf JG, Ramadori G, Dombrowski F: Analysis of the IGF axis in preneoplastic hepatic foci and hepatocellular neoplasms developing after low-number pancreatic islet transplantation into the livers of streptozotocin diabetic rats. Lab Invest 2000, 80:1399–1411
53. Villafuerte BC, Koop BL, Pao CI, Phillips LS: Glucocorticoid regulation of insulin-like growth factor-binding protein-3. Endocrinology 1995, 136:1928–1933
54. Scharf JG, Knittel T, Dombrowski F, Muller L, Saile B, Braulke T, Hartmann H, Ramadori G: Characterization of the IGF axis components in isolated rat hepatic stellate cells. Hepatology 1998, 27:1275–1284
55. Yamamoto T, Kaneda K, Hirohashi K, Kinoshita H, Sakurai M: Sinusoidal capillarization and arterial blood supply continuously proceed with the advance of the stages of hepatocarcinogenesis in the rat. Jpn J Cancer Res 1996, 87:442–450
56. Macluskey M, Chandrachud LM, Pazouki S, Green M, Chisholm DM, Ogden GR, Schor SL, Schor AM: Apoptosis, proliferation, and angiogenesis in oral tissues. Possible relevance to tumour progression. J Pathol 2000, 191:368–375
57. Lee CS, Redshaw A, Boag G: Abnormalities in type IV collagen immunoreactivity in human laryngeal cancers. Pathology 1996, 28:135–138
58. Sheela S, Barrett JC: Degradation of type IV collagen by neoplastic human skin fibroblasts. Carcinogenesis 1985, 6:173–179
59. Zeng ZS, Cohen AM, Guillem JG: Loss of basement membrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumorigenesis. Carcinogenesis 1999, 20:749–755
60. Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y, Nakamura Y: Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 2001, 61:2129–2137
61. Honda M, Kaneko S, Kawai H, Shirota Y, Kobayashi K: Differential gene expression between chronic hepatitis B and C hepatic lesion. Gastroenterology 2001, 120:955–966
62. Xu XR, Huang J, Xu ZG, Qian BZ, Zhu ZD, Yan Q, Cai T, Zhang X, Xiao HS, Qu J, Liu F, Huang QH, Cheng ZH, Li NG, Du JJ, Hu W, Shen KT, Lu G, Fu G, Zhong M, Xu SH, Gu WY, Huang W, Zhao XT, Hu GX, Gu JR, Chen Z, Han ZG: Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding noncancerous liver. Proc Natl Acad Sci USA 2001, 98:15089–15094
63. Noguchi K, Enjoji M, Nakamuta M, Nakashima M, Nishi H, Choi I, Taguchi K, Kotoh K, Shimada M, Sugimachi K, Tsuneyoshi M, Nawata H, Watanabe T: Expression of a tumor-associated antigen RCAS1 in hepatocellular carcinoma. Cancer Lett 2001, 168:197–202
64. Ng IO, Liu CL, Fan ST, Ng M: Expression of P-glycoprotein in hepatocellular carcinoma. A determinant of chemotherapy response. Am J Clin Pathol 2000, 113:355–363
65. Kato A, Miyazaki M, Ambiru S, Yoshitomi H, Ito H, Nakagawa K, Shimizu H, Yokosuka O, Nakajima N: Multidrug resistance gene (MDR-1) expression as a useful prognostic factor in patients with human hepatocellular carcinoma after surgical resection. J Surg Oncol 2001, 78:110–115