Identification of differential expression of genes in hepatocellular carcinoma by suppression subtractive hybridization combined cDNA microarray

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Abstract. The genetic background of hepatocellular carcinoma (HCC) has yet to be completely understood. Here, we describe the application of suppression subtractive hybridization (SSH) coupled with cDNA microarray analysis for the isolation and identification of differential expression of genes in HCC. Twenty-six known genes were validated as up-regulated and 19 known genes as down-regulated in HCC. The known genes identified were found to have diverse functions. In addition to the overexpression of AFP, these genes (increased in the presence of HCC) are involved in many processes, such as transcription and protein biosynthesis (HNRPDL, PABPC1, POLR2K, SRP9, SNRPA, and six ribosomal protein genes including RPL8, RPL14, RPL41, RPS5, RPS17, RPS24), the metabolism of lipids and proteins (FADS1, ApoA-II, ApoM, FTL), cell proliferation (Syndecan-2, and Annexin A2), and signal transduction (LRRC28 and FMR1). Additionally, a glutathione-binding protein involved in the detoxification of methylglyoxal known as GLO1 and an enzyme which increases the formation of prostaglandin E(2) known as PLA2G10 were up-regulated in HCC. Among the under-expressed genes discovered in HCC, most were responsible for liver-synthesized proteins (fibrinogen, complement species, amyloid, albumin, haptoglobin, hemopexin and orosomucoid). The enzyme implicated in the biotransformation of CYP family members (LOC644587) was decreased. The genes coding enzymes ADH1C, ALDH6A1, ALDOB, Arginase and CES1 were also found. Additionally, we isolated a zinc transporter (Zip14) and a function-unknown gene named ZBTB11 (Zinc finger and BTB domain containing 11) which were underexpressed, and seven expression sequence tags deregulated in HCC without significant homology reported in the public database. Essentially, by using SSH combined with a cDNA microarray we have identified a number of genes associated with HCC, most of which have not been previously reported. Further characterization of these differentially expressed genes will provide information useful in understanding the genes responsible for the development of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is a common and highly malignant tumor prevalent in Saharan African and Southeast Asian populations (1). Development of most HCCs results from a multi-step process of carcinogenesis involving expression changes in numerous genes. Many studies have been conducted screening tumor-associated genes in HCC. The results show that changes in the expressed genes, obtained through the same or different methods, were heterogeneous (2,3). However, a comprehensive picture of HCC-specific gene expression has not as yet been outlined. It is essential to identify the undiscovered and unknown expressed genes of HCC to understand its molecular pathogenesis and develop diagnostic markers and new potential therapeutic targets.

Several methods, such as differential display (4), expressed sequence tag analysis (5), suppressive PCR (6), serial analysis of gene expression (7) and microarray (8), have been reported for the detection of differentially expressed genes in tumorous tissues as compared with corresponding non-tumorous tissues. The technique of suppressive subtractive hybridization (SSH) is believed to generate an equalized representation of differentially expressed genes, irrespective

of the relative abundance of the two transcriptomes, and provides a high enrichment of differentially expressed mRNAs (9). The validity of SSH sequences can be confirmed utilizing the high throughput method of microarray hybridization. SSH and microarray have already been successfully used together for studying gene expression profiles in various systems (10,11).

This study describes the application of SSH combined with microarray techniques to identify the differentially expressed genes associated with HCC. In the case of some differential genes, real-time PCR technique was used to validate the difference between cancerous and corresponding non-cancerous tissues. We had to be highly selective in the sourcing of our sample and therefore, owing to the fact that most HCCs develop from liver cirrhosis (12,13), obtained it from a patient with liver cirrhosis and hepatitis B.

Materials and methods

Tissue preparation. HCC samples were collected through collaboration with a local hospital. The samples used for SSH were from a 56-year-old male patient with hepatitis B and liver cirrhosis. The tumor was 5 cm in diameter, with multi-nodules and moderate differentiation. The samples consisted of two sections, including cancer and liver tissue that was at least 3 cm distal from the lesion. All tissues were verified by histological examination. Sample collections were obtained with informed consent, then immediately placed in liquid nitrogen and stored at -80˚C until use.

Isolation of total-RNA and mRNA. Total-RNA was extracted according to standard TRIzol RNA isolation protocol (Life Technologies, Inc., Grand Island, NY). The poly(A)+ mRNA was isolated from the total-RNA using a poly (dT) resin (Qiagen, Hilden, Germany) as per the recommended guidelines. Denaturalization agar gel with formaldehyde and an ultraviolet spectrophotometer were used to detect the quality of the RNA.

Suppressive subtractive hybridization. Poly(A)+ mRNA from cancer tissues was used as the 'tester' while poly(A)+ mRNA from non-cancer tissues served as the 'driver' (forward SSH library). Conversely, poly(A)+ mRNAs from cancer tissues and control non-cancer tissues were also used as driver and tester samples, respectively (reverse SSH library). Differentially expressed cDNAs were present in the tester cDNA, but were either absent or present in only very low levels in the driver cDNAs. Construction of the forward and reverse libraries was performed according to SSH procedure using a PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA).

Equal amounts of poly(A)+ mRNA from each of the tester and driver populations were converted to double-stranded cDNA by reverse transcription, followed by digestion with RsaI to produce shorter blunt-ended fragments. The digested tester cDNA was subdivided into two populations, each of which was ligated with a different adaptor from those provided in the cDNA subtraction kit (Clontech). Ligation efficiency was evaluated by PCR using primers specific to G3PDH mRNA and to the adaptor sequences. Following ligation, two hybridization steps were performed. For the first hybridization, an excess of driver was added to each tester, denatured, and allowed to anneal. The target sequences in the tester were then significantly prepared for identification of differentially expressed genes. In the second hybridization step, the two reaction products from the first hybridization were mixed with each other and with fresh denatured driver cDNA. The populations of normalized and subtracted single-stranded target cDNAs annealed with each other, forming double-stranded hybrids with different adaptor sequences at their 5' ends. The adaptor ends were then filled with DNA polymerase and the subtracted molecules were specifically amplified by 'nested PCR' using adaptor-specific primer pairs.

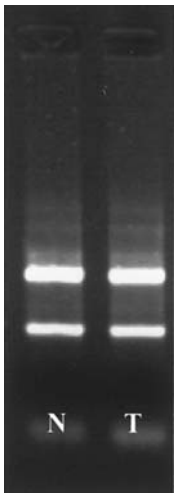

Figure 1. Total-RNA isolated from non-HCC (N) and HCC (T) tissue. Ethidium bromide-stained 1% agarose gel of two RNA samples. There were sharp bands of 18S and 28S ribosomal RNA and minimal degradation, which is critical to expression analysis. Lane 1 represents total-RNA derived from non-HCC; lane 2 represents total-RNA from HCC.

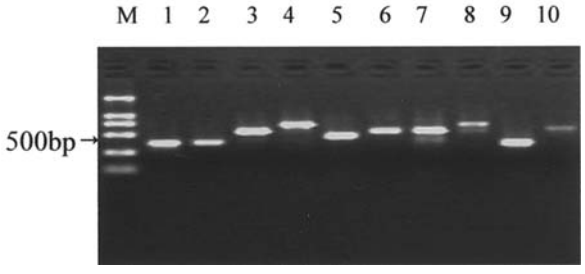

Figure 2. Analysis and identification of SSH library. Ten colonies were randomly picked from the subtraction cDNA library. The inserts were amplified by PCR, then isolated in 1.5% agarose gel. Numbers 1 to 10 are the randomly picked colonies. M stands for marker DL2000.

Construction of subtracted cDNA libraries. The subtracted target cDNAs (the second PCR products) were ligated with the pMD-18T plasmid vector using T4 DNA ligase and transformed into maximum efficiency *Escherichia coli* JM109 cells (Takara). The transformed bacteria were plated onto LB agar plates containing ampicillin, X-gal and IPTG, and were then incubated overnight at 37˚C. pMD-18T plasmid contains LacZ reporter and allows blue-white screening. Recombinant white colonies were randomly selected and cultured in LB broth containing ampicillin. Plasmid extraction was per-

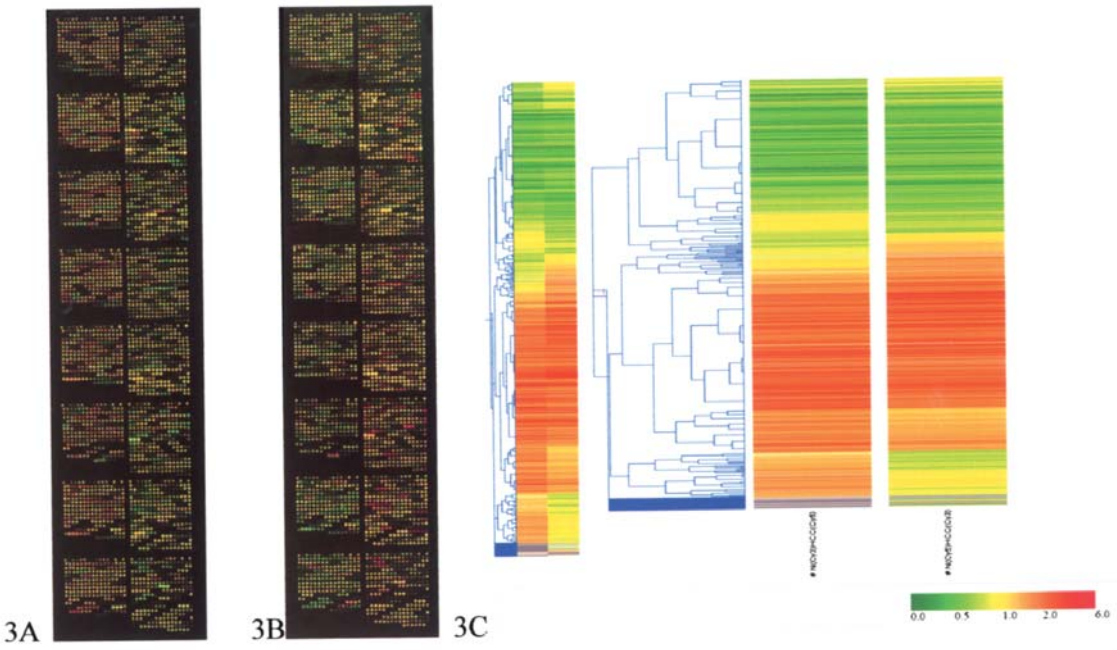

Figure 3. Microarray scanning and tree view graph analyses of cDNA clones enriched in HCC and non-cancer tissue. cDNA clones, 576 enriched in HCC and 760 in non-cancer tissue, were picked after SSH. The PCR products of these clones were spotted on slides for array preparation (triplicate reproduction per PCR product). The negative and positive controls were spotted on the first row from the top in each subarray. The 8 left subarrays were constructed by clones from the forward SSH library, while the 8 right were made by clones from the reverse SSH library. The mRNAs purified from HCC and non-cancer tissue and labeled with Cy5-dUTP and Cy3-dUTP, respectively, were used as probes for the microarray chip hybridization. (A) cDNA probes labeled with non-HCC cy3 and HCC cy5; (B) cDNA probes labeled with non-HCC cy5 and HCC cy3. Pseudo color obtained after microarray analyses indicated that those cDNAs were either up-regulated (red spots in A), down-regulated (green spots in B) or underwent no change (yellow spots in A and B) in the HCC tissue. (C) Microarray results were analyzed by GeneSpring software for a gene expression pattern represented as a tree view graph.

formed with the QIAwell 96-well plasmid purification system. The inserted fragments were amplified by PCR with adaptor-specific primer pairs. These amplified fragments were 150-1000 bp and were purified for the construction of the cDNA chip.

Construction of human cDNA chip, synthesis and hybridization of cDNA probes. A cDNA microarray chip containing cDNA spots representing 1306 SSH clones was constructed. Briefly, 576 forward SSH and 730 reverse SSH clone inserts were amplified by PCR using a primer pair corresponding to the nested adaptor sequences. The PCR products were visualized on 1.5% agarose gel to ensure the quality and quantity of amplification, and were then purified with multiscreen PCR plates (Millipore, Bedford, MA). The PCR products (25 ng/μl, OD_{260}/OD_{280} >1.7, single and significant strip) were printed on poly-L-lysine coated glass slides employing the OmniGrid 100 (GeneMachine), with triplicate reproduction per PCR product. Cotton(1), poly(A), DMSO, vector and cotton(2) were included as negative controls, while a total of 11 cDNAs of human housekeeping genes were included as positive controls. The control elements were spotted on each microarray of the two cDNA chips.

Total-RNAs from cancer and non-cancer tissue were extracted using standard TRIzol RNA isolation protocol (Life Technologies, Inc.) and purified using RNeasy® Mini Kit (Qiagen). The quality of the RNAs was detected by Lab on Chip (Agilent 2100). RNAs thus obtained were reverse transcribed with an oligo(dt)15 primer, Cy3/Cy5 CTP, and Superscript II reverse transcriptase (Invitrogen Life

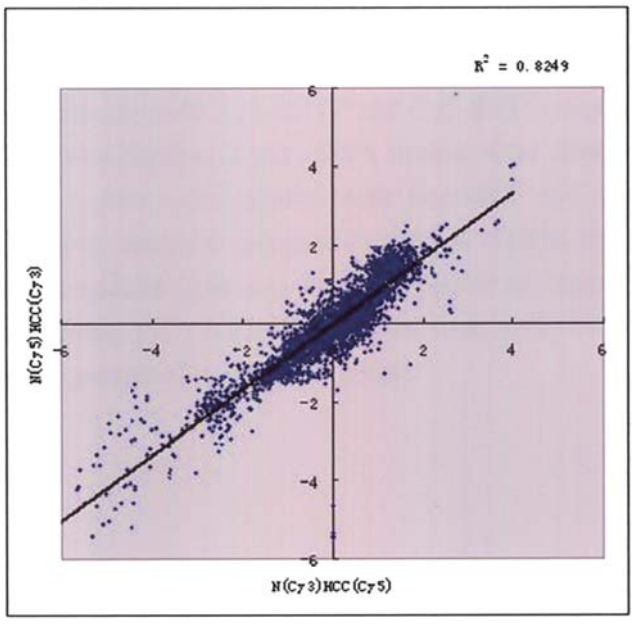

Figure 4. Scatterplot chart of duplicate spot intensities obtained from two independent cDNA array analyses. To eliminate the latent variance resulting from labeling efficiency, cy3 and cy5 dyes were used to label cDNA isolated from cancer and non-cancer tissue samples, respectively, in order to prepare the cDNA probes. Each dot represents a duplicate clone. The X coordinate value is the gene expression level (intensity value) in the test with the cDNA probe labeled as non-HCC-cy3 and HCC-cy5. The Y coordinate value is the other test with the cDNA probe labled as non-HCC-cy5 and HCC-cy3. The trend line shows the mean value of the duplicates. The reproducibility of the data obtained from the two chips was good (R^2=0.8249).

Table I. Sequence analysis of clones isolated from the forward subtracted library HCC cDNA.

UniGene No.	Gene symbol	Gene definition	No. of clones	Ratio of T/N
Hs.518808	AFP	α-fetoprotein	3	16.34
Hs.324746	AHSG	α-2-HS-glycoprotein	50	3.473
Hs.511605	ANXA2	Annexin A2	4	2.102
Hs.237658	APOA2	Apolipoprotein A-II	5	5.19
Hs.534468	APOM	Apolipoprotein M	7	2.7
Hs.503546	FADS1	Fatty acid desaturase 1	3	4.681
Hs.433670	FTL	Ferritin, light polypeptide	36	3.442
Hs.103183	FMR1	Fragile X mental retardation 1	3	2.366
Hs.268849	GLO1	Glyoxalase I	8	2.506
Hs.527105	HNRPDL	Heterogeneous nuclear ribonucleoprotein D-like	2	2.668
Hs.180414	HSPA8	Heat shock 70 kDa protein 8	8	3.086
Hs.459507	LRRC28	Leucine rich repeat containing 28	4	2.339
Hs.387804	PABPC1	Poly(A) binding protein, cytoplasmic 1	3	3.625
Hs.351475	POLR2K	Polymerase (RNA) II (DNA directed) polypeptide K, 7.0 kDa	6	2.187
Hs.89545	PSMB4	Proteasome (prosome, macropain) subunit, ß type, 4	2	2.333
Hs.567366	PLA2G10	phospholipase A2, group X	5	2.454
Hs.178551	RPL8	Ribosomal protein L8	3	2.645
Hs.446522	RPL14	Ribosomal protein L14	4	2.072
Hs.517472	RPL41	Ribosomal protein L41	3	2.201
Hs.433427	RPS17	Ribosomal protein S17	5	2.059
Hs.356794	RPS24	Ribosomal protein S24	39	3.389
Hs.378103	RPS5	Ribosomal protein S5	5	2.05
Hs.511425	SRP9	Signal recognition particle 9 kDa	9	2.078
Hs.466775	SNRPA	Small nuclear ribonucleoprotein polypeptide A	3	2.941
Hs.1501	SDC2	Syndecan-2	10	2.798
Hs.390623	XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	4	2.457
		EL579568 (GenBank_Accn for EST)	3	2.454
		EL579569 (GenBank_Accn for EST)	5	2.521
		EL579570 (GenBank_Accn for EST)	6	2.392
		EL579571 (GenBank_Accn for EST)	2	2.549
	Total		250	

Technologies, Shanghai, P.R. China). In order to prepare the cDNA probes, Cy3 and Cy5 dyes (Amersham, Piscataway, NJ) were used to label cDNA isolated from cancer and non-cancer tissue samples, respectively. The two probes were purified (QIAquick Nucleotide Removal Kit) and later hybridized with the spotted array at 42˚C for 16 h. Images of the hybridized arrays were acquired through laser confocal scanning (Agilent, G2655AA).

Data analysis. Probe synthesis and hybridization to micro-array were repeated twice. The intensities of Cy5 and Cy3 for each spot on the array were determined with Imagene software. The raw data obtained was thus normalized by GeneSpring before being subjected to further analysis. Normalization of the entire data set for both channels was based on subtraction of the local background, fluorescence from the fluorescence (intensity of each of the Cy5 and Cy3 spots) and on the elimination of spots within high background intensity of either dye. The spots that had a Cy5/Cy3 (HCC: non-HCC) ratio ≤0.5 or ≥2 and a signal/noise >2 we strongly believed to be significantly differential in expression according to the average value of replicate spots.

Sequencing and computer analysis of differential expression of cDNA clones. Some of the differential expressed clones were sequenced (Invitrogen Life Technologies) and analyzed with the Basic Local Alignment Search Tool (BLAST) for homology in the public sequence databases.

Validation of differential expression of genes by real-time RT-PCR. The differential expression of selected genes was further validated by real-time PCR with SYBR green-based detection (ABI) using gene-specific primer pairs that were run on an ABI 7000 fluorescent sequence detection system (Perkin-Elmer, Foster City, CA). It is known that only the cDNA of each target gene can be the template, owing to the

Table II. Sequence analysis of clones isolated from the reverse subtracted library non-HCC cDNA.

UniGene No	Gene symbol	Gene definition	No. of clones	Ratio of T/N
Hs.418167	ALB	Albumin	13	0.41
Hs.4	ADH1C	Alcohol dehydrogenase 1C (class I), γ polypeptide	4	0.158
Hs.293970	ALDH6A1	Aldehyde dehydrogenase 6 family, member A1	7	0.484
Hs.530274	ALDOB	Aldolase B, fructose-bisphosphate	5	0.363
Hs.440934	ARG1	Arginase, liver	4	0.183
Hs.558865	CES1	Carboxylesterase 1 (monocyte/macrophage serine esterase1)	2	0.286
Hs.529053	C3	Complement component 3	3	0.394
Hs.458355	C1S	Complement component 1, s subcomponent	3	0.203
Hs.351593	FGA	Fibrinogen α polypeptide	17	0.188
Hs.300774	FGB	Fibrinogen ß chain	3	0.301
Hs.529800	LOC644587	Similar to cytochrome P450, family 4, subfamily F, polypeptide 3	5	0.223
Hs.513711	HP	Haptoglobin	21	0.154
Hs.426485	HPX	Hemopexin	3	0.227
Hs.370504	RPS15A	Ribosomal protein S15a	4	0.0568
Hs.615131	ISCU	IscU iron-sulfur cluster scaffold homolog (*E. coli*)	3	0.297
Hs.522356	ORM2	Orosomucoid 2	8	0.132
Hs.332053	SAA1	Serum amyloid A1	26	0.0152
Hs.491232	SLC39A14	Solute carrier family 39 (zinc transporter), member 14	4	0.438
Hs.545311	ZBTB11	Zinc finger and BTB domain containing 11	5	0.244
		EL579572 (GenBank_Accn for EST)	3	0.453
		EL579573 (GenBank_Accn for EST)	5	0.0811
		EL579574 (GenBank_Accn for EST)	2	0.203
	Total		150	

fact all the primer sequences span one or more introns by design (Gene Runner). The human housekeeping gene ß-actin was used as the control. Quantitative values were obtained from the cycle number (Ct value) at which the increase in fluorescent signal (associated with exponential growth of PCR products) starts to be picked up by the laser detector of the detection system. Results, expressed as N-fold differences in target gene expression between the HCC and non-HCC tissue and termed 'Ntarget', were determined using the formula: $Ntarget = 2^{\Delta Ctsample}$ (while $\Delta Ct_{sample} = \Delta Ct_{HCC}-\Delta Ct_{non-HCC}$), where the ΔCt_{HCC} and $\Delta Ct_{non-HCC}$ value of the sample was determined by subtracting the Ct value of the target gene from the average Ct value of the ß-actin gene.

Results

Isolation of total-RNA and mRNA. The purity and quality of the total-RNA and mRNA was detected using an ultraviolet spectrophotometer and gel electrophoresis. The ratio of OD_{260} to OD_{280} was beyond 1.8 and the total-RNA showed two strips of 28S and 18S. mRNA showed smear mainly between 0.5-12 kb (Fig. 1).

Construction and identification of SSH library. cDNA clones of 672 and 864 were selected from the forward and the reverse subtract library, respectively. The inserted fragments obtained by PCR were mainly distributed between 150-1000 bp (Fig. 2).

Construction of human cDNA chip and synthesis and hybridization of cDNA probe. The 672 and 864 PCR products from the forward and reverse subtract library were purified and identified, with 576 and 730 clones, respectively, qualifying for cDNA chip construction. The 1306 clones were used to prepare the cDNA chips. After hybridization, when the data had been acquired, up-regulated (expression ratio HCC/non-HCC >2.0) and down-regulated (expression ratio of HCC/non-HCC <0.5) differential expressed genes in HCC were screened (Fig. 3). The results from the replicated experiments were almost perfectly concordant with an R^2 (square of Pearson correlation coefficiency, measuring similarity in gene expression level) correlation of 0.8249 in a scatterplot (Fig. 4).

Sequencing and analysis of the homology of differential expressed cDNA clones. A total of 400 clones (250 up-regulated from the forward library and 150 down-regulated from the reverse library) were screened and subjected to nucleotide sequence analysis. The sequence data, obtained by analyzing the homology with reference to the public database, was highlighted. As shown, 26 and 19 unique sequences were acquired as known genes in the forward and reverse libraries,

Table III. Known genes with official names differentially expressed in HCC classified according to their biological functions from literature sources.

Categories	Gene symbol of HCC/non-HCC	Change of expression ratio
Protein metabolism	ALB	↓
	C3	↓
	C1S	↓
	FGA	↓
	FGB	↓
	HPX	↓
	AFP	↑
	PSMB4	↑
	FTL	↑
Lipid metabolism	**PLA2G10**	↑
	FADS1	↑
	ApoA-II	↑
	ApoM	↑
	CES1	↓
Transcription and protein biosynthesis	**RPL8, L14, L41**	↑
	RPS5, S17, S24	↑
	RPS15A	↓
	PABPC1	↑
	POLR2K	↑
	SRP9	↑
	HNRPDL	↑
	SNRPA	↑
Acute phase response	HP	↓
	ORM2	↓
	SAA1	↓
	AHSG	↑
Amino acid metabolism	**XPNPEP1**	↑
	ARG1	↓
Glycogen synthesis	ALDOB	↓
Alcohol metabolism	ADH1C	↓
Metabolic process	ALDH6A1	↓
Detoxification	**GLO1**	↑
Zinc transporter	SLC39A14 (Zip14)	↓
Iron-sulfur cluster assembly	ISCU	↓
Miscellaneous		
Apoptosis/stress response/etc.	**HSPA8**	↑
Cell adhesion/cell proliferation/etc.	**SDC2**	↑
Signal transduction/cell differentiation/etc.	**ANXA2**	↑
Signal transduction/translocation/etc.	**FMR1**	↑
Genes with function inferred		
Signal transduction ?	**LRRC 28**	↑
Biotransformation ?	LOC644587	↓
Gene with unknown function	ZBTB11	↓

Genes in bold indicated as up-regulated in HCC.

Table IV. Primer sequences.

Primer name	Sequence
Annexin II forward	5'AACAGCCATCAAGACCAAAGG3'
Annexin II reverse	5'TGAGCAGGTGTCTTCAATAGGC3'
AFP forward	5'GCCAAAGTGAAGAGGAAGAC3'
AFP reverse	5'GCCCAAAGAAGAATTGTAGGTG3'
RPS24 forward	5'GGTGGTGGCAAGACAACTGG3'
RPS24 reverse	5'AGCACCAACATTGGCCTTTG3'
SAA1 forward	5'TTTCTGCTCCTTGGTCCTGG3'
SAA1 reverse	5'GCATCGCTGTAATCCTGATCAC3'
Albumin forward	5'GGTGTTGATTGCCTTTGCTC3'
Albumin reverse	5'CAGCCATTTCACCATAGGTTTC3'
Alcohol dehydrogenase forward	5'CCTCAGGCTGTGGATTCTCG3'
Alcohol dehydrogenase reverse	5'ATGTCCACCGCAATGATTCTGG3'
ß-actin forward	5'CCCAGAGCAAGAGAGGCATC3'
ß-actin reverse	5'CATGATCTGGGTCATCTTCTCG3'

respectively (Tables I and II). Additionally, 7 expression sequence tags without significant homology to genes within the database were repeatedly isolated.

Genes associated with the metabolism of lipids and proteins, cell proliferation, apoptosis, signal pathway and angiogenesis were found deregulated in hepatocarcinogenesis. Increased expression was observed in genes involved in the process of transcription, protein biosynthesis and antiapoptosis. Cell proliferation was also observed, for example in several ribosomal protein genes (HSPA8, Syndecan-2, and Annexin A2). The enzymes found increased, such as the gene involved in the formation of prostaglandin E(2), known as PLA2G10, and the glutathione-binding protein involved in the detoxification of methylglyoxal known as Glyoxalase I, were up-regulated in HCC. Most of the underexpressed genes were responsible for liver-synthesized functional proteins such as fibrinogen, complement species, amyloid, albumin, haptoglobin, hemopexin and orosomucoid. Enzyme activity implicated in biotransformation, such as LOC644587 (a cytochromes P450 family member), was decreased. Genes coding enzymes such as ADH1C, ALDH6A1, ALDOB, ARG1 and CES1 were discovered as well. A zinc transporter (Zip14) and a function-unknown gene named ZBTB11 were down-regulated in HCC. The functional categories of the differential expressional genes are shown in Table III.

Validation of differential expression of genes by real-time RT-PCR. Differential expression of genes detected through SSH analysis was confirmed in original HCC and non-HCC tissues by real-time RT-PCR, as shown in Fig. 3. Annexin II, AFP and RPS24 were from the forward library, while SAA1, albumin and alcohol dehydrogenase were from the reverse library. The primer sequences for each gene are listed in Table IV. The quality and specificity of the amplified products was confirmed by visualization on a 2% agarose gel. The results confirmed the validity of SSH combined with a cDNA chip. $N_{AFP} = 2^{6.27}$, ($\Delta Ct_{HCC\text{-}AFP} = 5.96$, $\Delta Ct_{non\text{-}HCC\text{-}AFP} = 12.23$); $N_{annexin\ a2} = 2^{1.15}$, ($\Delta Ct_{HCC\text{-}Annexin\ A2} = 2.93$, $\Delta Ct_{non\text{-}HCC\text{-}Annexin\ A2} = 4.08$); $N_{PRS24} = 2^{2.09}$, ($\Delta Ct_{HCC\text{-}RPS24} = 2.17$, $\Delta Ct_{non\text{-}HCC\text{-}RPS24} = 5.26$); $N_{SAA1} = 2^{6.55}$, ($\Delta Ct_{HCC\text{-}SAA1} = 6.61$, $\Delta Ct_{non\text{-}HCC\text{-}SAA1} = 0.06$); $N_{Albumin} = 2^{2.43}$, ($\Delta Ct_{HCC\text{-}Albumin} = 4.32$, $\Delta Ct_{non\text{-}HCC\text{-}Albumin} = 1.89$); $N_{ADH} = 2^{3.26}$, ($\Delta Ct_{HCC\text{-}ADH} = 4.72$, $\Delta Ct_{non\text{-}HCC\text{-}ADH} = 1.46$). The lower the ΔCt value of the target gene, the more mRNA content of the target gene there is in the tissue. The Ct value of ß-actin obtained from HCC and non-HCC tissue is almost identical.

Discussion

Out of the 26 known genes found to be up-regulated in HCC, 11 (RPL8, L14, L41, S5, S17, S24, PABPC1, POLR2K, SRP9, HNRPDL and SNRPA) had an increased involvement in the process of transcription and protein bio-synthesis, which reflects the eugenic growth state of tumor cells. Of these 11 genes, only the overexpression of RPS17 has been reported in HCC (14). The remaining ten had not previously been found up-regulated in HCC, although they might have been detected as increased expression in other cancer cases (15,16).

AFP was the gene with the highest expression ratio of cancer/non-cancer obtained by the cDNA microarray, while AHSG was the most frequent clone present. The expression ratio of HCC/non-HCC of AFP was 16.34, while the serum level of AFP taken from the index case was also reported as high (1000 μg/l). AHSG, a major serum glycoprotein secreted by the liver, was shown to contribute to the early stages of skin tumorigenesis. The absence of AHSG reduced the number of tumors and the tumor burden in AHSG null animals (as compared to wild-type) (17). Additionally, AHSG has been identified and demonstrated to be higher in patients with low-grade glicomas as compared to those in the control group (18).

Several up-regulated genes (FADS1, ApoA-II, ApoM, FTL and PLA2G10) participate in the metabolism of lipids and proteins. PLA2G10 has the most powerful potency in the release of arachidonic acid, leading to COX-2-dependent prostaglandin E(2) formation during colon tumorigenesis (19). Recent research reported that COX-2 itself is directly involved in the proliferation of human HCC cells (20). COX-2 is also involved in the control of HCC-associated angiogenesis (21).

Other up-regulated genes identified, which appear to participate in diverse processes such as apoptosis and proliferation functions, include HSPA8, Glyoxalase I, Annexin A2, and Syndecan-2. HSPs are inducible in response to various stressful conditions, including carcinogenesis (22,23). During the development of HCC, the processes of chronic inflammation and fibrosis act as stressful conditions. Several studies have found that HSPA8 overexpression in malignant tumors protects cells (with protein aggregates) from apoptosis (24-26). Glyoxalase I is a glutathione-binding protein involved in the detoxification of methylglyoxal,

whose experimental overexpression confers resistance to drug-induced apoptosis in human leukemia and lung carcinoma cells (27). Annexin A2 is a Ca^{2+}-dependent RNA-binding protein which interacts with the mRNA of the nuclear oncogene c-myc (28). It is also reported to be a powerful activator of plasminogen and is implicated in normal and pathological processes such as haemostasis and metastasis (29). Furthermore, research in human glicomas shows that Annexin A2 interacts with the actin cytoskeleton, suggesting that it could have a direct link with invasion-associated processes in human gliomas (30). The overexpression of Annexin A2 in HCC and intrahepatic cholangiocarcinoma has been previously reported (31,32). Members of the syndecan family and heparan sulfate proteoglycans can be said to play diverse roles in cell adhesion and cell communication by serving as co-receptors for both cell-signaling and extracellular matrix molecules. Syndecan-2 has been implicated in the formation of specialized membrane domains and functions as a direct link between the extra-cellular environment and the organization of the cortical cytoplasm. Recent studies have also shown that Syndecan-2 is required for angiogenesis, possibly by serving as a co-receptor for the vascular endothelial growth factor (33).

In addition, it was discovered that two genes in particular (LRRC 28 and FMR1), which are believed to be involved in signal transduction, have increased expression in HCC. All proteins containing leucine-rich repeat (LRR, a characterized structural motif used in molecular recognition processes) are thought to be involved in protein-protein interactions, with functions as diverse as signal transduction, cell adhesion, cell development, DNA repair and RNA processing (34,35). For example, the LRR of adenylatecyclase, contributing to its interactions with Ras, participates in the regulation of the signal transduction pathway of the p21Ras family of GTPases (36). In the present study, the overexpression of LRRC28 in HCC was detected for the first time. FMRP, a product of FMR1, plays a role in the regulation of cAMP signal transduction by increasing intracellular cAMP, perhaps through a mechanism involving the binding and enhanced translation of mRNAs for cAMP cascade proteins (37).

The down-regulated genes in HCC were screened when the cut-off intensity of HCC/non-HCC was set at 0.5. The majority of the underexpressed genes encoded hepatocyte-specific gene products (such as fibrinogen, complement species, amyloid, albumin, haptoglobin, hemopexin and orosomucoid). Down-regulation of these genes may reflect the poor differentiation of hepatocytes and the loss of the liver's biological functions in HCC tissues.

Among the down-regulated genes found, five gene-coding enzymes discovered were ADH1C, ALDH6A1, ALDOB, ARG1 and CES1. Most of these had been reported to be associated with tumors. Ethanol is metabolized to acetaldehyde by ADH, while the enzyme responsible for the oxidation of acetaldehyde is ALDH. However, acetaldehyde has previously been considered a carcinogen (38). Research indicates that the growth of cancer cells is dependent on arginine, which is needed for pathways other than protein synthesis. The irreversible conversion of arginine into ornithine by intracellular arginase causes a progressive depletion of arginine from the culture medium, which ultimately inhibits cell protein synthesis and halts growth (39). Decreased expression of arginase 1 in HCC tissues has been reported (40). Cytochromes P450 are key enzymes in cancer formation and treatment. They mediate the metabolic activation of numerous pre-carcinogens and participate in the inactivation and activation of anticancer drugs (41). LOC644587 was among under-expressed genes in the HCC tested here.

We also isolated a function-unknown gene named ZBTB11 underexpressed in HCC. Zinc fingers (ZnFs) are generally regarded as DNA-binding motifs. The broad-complex, tram-track (ttk), bric-a-brac/poxvirus and zinc finger proteins (BTB/POZ) domain is highly conserved in a large family of eukaryotic proteins and is crucial for the latter's diverse roles in mediating interactions among the proteins involved in transcription regulation and chromatin structures. From a fetal brain cDNA library, Chen *et al* isolated a cDNA of 2489 bp encoding a novel human BTB domain-containing protein named BTBD10. The cDNA microarray analysis showed that BTBD10 was down-regulated in all 18 glicoma samples. The expression of BTBD10 in hepatocellular carcinoma was also investigated, with the results revealing no significant differences (42).

In this study, instead of randomly picking clones from the SSH library to sequence, we chose which ones to sequence according to the values of the expression ratio of HCC/non-HCC obtained from the cDNA microarrays. During the preparation of the cDNA chips, we had quality controls at each step to ensure the reliability of the data obtained. The accuracy of repetition between the two chips was indicative of the quality of the processes. Meanwhile, the results from real-time PCR experiments (with representatives from the up-regulated and down-regulated groups) supported the results of our microarray analysis. These sets of data provide useful information, and are potentially crucial to finding the candidate genes responsible for the development of HCC. Further related work is required to conclusively establish the role of genes in HCC.

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