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Genome-wide differences in hepatitis C- *vs* alcoholism-associated hepatocellular carcinoma

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Abstract

AIM: To look at a comprehensive picture of etiology-dependent gene abnormalities in hepatocellular carcinoma in Western Europe.

METHODS: With a liver-oriented microarray, transcript levels were compared in nodules and cirrhosis from a training set of patients with hepatocellular carcinoma (alcoholism, 12; hepatitis C, 10) and 5 controls. Loose or tight selection of informative transcripts with an abnormal abundance was statistically valid and the tightly selected transcripts were next quantified by qRTPCR in the nodules from our training set (12 + 10) and a test set (6 + 7).

RESULTS: A selection of 475 transcripts pointed to significant gene over-representation on chromosome 8 (alcoholism) or -2 (hepatitis C) and ontology indicated a predominant inflammatory response (alcoholism) or changes in cell cycle regulation, transcription factors and interferon responsiveness (hepatitis C). A stringent selection of 23 transcripts whose differences between etiologies were significant in nodules but not in cirrhotic tissue indicated that the above dysregulations take place in tumor but not in the surrounding cirrhosis. These 23 transcripts separated our test set according to etiologies. The inflammation-associated transcripts pointed to limited alterations of free iron metabolism in alcoholic *vs* hepatitis C tumors.

CONCLUSION: Etiology-specific abnormalities (chromosome preference; differences in transcriptomes and related functions) have been identified in hepatocellular carcinoma driven by alcoholism or hepatitis C. This may open novel avenues for differential therapies in this disease.

Key words: Alcoholism; Chromosome; Cirrhosis; Hepatitis C; Transcriptomes; Protein function

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary liver cancer, the main causes of which are viral hepatitis (HBV; HCV), alcoholism or aflatoxin B1 intoxication. In most instances HCC develops in the setting of chronic hepatitis and/or cirrhosis. Numerous HCC-associated genomic and/or epigenomic alterations result in a dysregulated expression of genes and proteins[1,2]. Liver transcriptome analysis by microarray has resulted in the identification of numerous genes with an aberrant expression in HCC as compared to the surrounding cirrhosis[2-6]. Although mRNA down-regulation predominates in this context[7], HCC-associated gene expression profiles largely vary between patient

subgroups[6,8]. This feature is of prognostic interest as different profiles are associated with the evolution rate or the occurrence of metastasis and relapse[2-4,9-11]. However, a comprehensive picture of altered gene regulation in HCC remains elusive[2,5,12]. Notably, the variety of etiologies with their associated abnormalities at the genome level is likely to promote distinct gene dysregulations and hence creates further complexity. For instance, the HBV- or HCV-induced genetic alterations are known to be different[9] and the associated transcriptomes have proven to vary significantly[13,14]. Therefore, deciphering the transcriptome patterns as a function of HCC etiology is of critical importance. However, the gene dysregulations in the context of alcohol abuse are poorly understood[15] and the associated transcriptome has seldom been studied. *A fortiori*, a comparison of liver transcriptomes in HCV virus- *vs* alcoholism-associated HCC has never been done. We now report that, among various findings, these transcriptomes differ in the cancerous nodules whereas, unexpectedly, they remain similar in the surrounding cirrhosis. This points to etiology-dependent mechanisms that take place at a relatively late stage of tumoral transformation.

MATERIALS AND METHODS

Human subjects and RNA sources

Liver fragments were obtained under strict anonymity from the digestive surgery unit of Charles Nicolle Hospital (Rouen, France). A fragment of a cancerous nodule as well as distant cirrhotic tissue were taken whenever an HCC resection was performed. In multinodular livers, only 1 nodule was studied, provided the tumor grade of this nodule was known. Control human livers were non-tumorous tissue from patients operated for benign liver tumor. Histopathology was carried out by a trained pathologist (AF). According to the current French rules and ethical guidelines, neither an informed consent nor advice from an ethical committee are requested prior to analysis of RNA in resected tissues that would otherwise be disposed off. Tissue storage and RNA extraction were done as described[16].

HCV- vs HBV-infection vs chronic alcohol abuse

Chronic alcohol abuse was defined as a regular, daily consumption of > 80 g or > 60 g ethanol in men or women, respectively, as estimated from three cumulated criteria, namely: (1) alcohol consumption, as indicated by the patient, (2) alcohol dependency, as evaluated from a specific interview and (3) blood level of several hepatic proteins (alanine aminotransferase, aspartate aminotransferase, gammaglutamyl transpeptidase). HBV infection was serologically assessed with the HBs antigen and anti-HBc antibodies (AxSYM kits from Abbott Laboratories). HCV infection was serologically determined by enzyme immunoassay (AxSYM HCV-3.0 kit from Abbott Laboratories). Both infections were further searched at the nucleic acid level in all patients of this study. HBV DNA was detected as described[17] in genomic DNA extracted from paraffin-embedded liver samples (DEXPAT Kit from TaKaRa Laboratories). HCV RNA was detected in 2 μg hepatic RNAs with the Abbott real-time HCV kit (Abbott Laboratories, France). Serological and genomic determinations were consistent in all cases.

Transcriptome analysis and real-time qRTPCR

Our set of human cDNA probes dubbed *Liverpool* that is tailored to a complete coverage of the human transcriptome in healthy or cancerous liver (*ca.* 10^4 genes), the associated *LiverTools* database, as well as the procedures from array preparation to final data handling have all been detailed[16]. In brief, every RNA sample was subjected to 3 hybridization replicates. The resulting, normalized values were used for selection of significantly up- or down-regulated mRNA in cirrhosis *vs* paired nodule, using a statistically validated, funnel-shaped confidence interval ($P < 0.05$) calculated from every mRNA detected per hybridization. This resulted in a false discovery rate (FDR) that is below 10% of the total number of regulated mRNAs, in agreement with an FDR estimate obtained from other, cumulated analyses in liver[5]. Abnormal mRNA ratio in cirrhosis *vs* paired nodule was defined from a statistically different abundance in at least 2 out of 3 replicates. A control of every cDNA probe was done by DNA re-sequencing with an ABI3100 capillary sequencer (Applied Biosystems). Real-time qRTPCR of mRNAs was done as described[16] with primers designed with the Primer3 software (http://frodo.wi.mit.edu), and normalized with the 18S RNA level. The primers were: *AGXT*, forward CGCTGGCTATGACTGGAGAG, reverse GTCACGCGGTCCACATTCT, amplicon size 150 bp; *APCS*, TGGGAGAGATTGGGGATTTG, CCACAC CAAGGGTTTGATGA, 158 bp; *APOC3*, ACTGAG-CAGCGTGCAGGAG, TCACGGCTGAAGTTG-GTCTGA, 154 bp; *ATP6-V0D1*, TACCTC AACCTGGTGCAGTG, GTCTAGGAAGCTGGCGA-GTG, 198 bp; *C4A*, TTGATC-ATGGGTCTGGATGG, CCTGGAGGA-AGTCGT-TGAGC, 157 bp; *CES1*, GGGTGCCTCAGAAG-AGGAGA, CTGGGTGTT-GGCACCAATCT, 154 bp; *CLU*, ATGTTC-CAGCCCTTCCTTGA, TCGTCGCCT-TCTCGTATGAA, 112 bp; *COBL*, TGGCATCCTCTGCTTCTGAG, CGT CCTTGGTGCAGAGAGAG, 161 bp; *CRP*, TCG TATGCCACCAAGAGACA, CTTCTGCCCCCAC AGTGTAT, 235 bp; *CYP2E1*, TCAAGCCATTTTC CACAGGA, CGATATCCTTTGGGTCAACGA, 129 bp; *FGL1*, GAAATTCAG-CACGTGGGACA, CCATTGTCT GTTTTAGCCGTGT, 150 bp; *HP*, AACTGCGCAC AGAAGGAGAT, TGGTGGGAAACCATCTTAGC, 202 bp; *HPR*, AGGGCGTGTGGGTTATGTTT, TTCTTTTCGGGGACTGTGCT, 141 bp; *HPX*, TGTGGATGCGGCCTTTATCT, GGCCAAG GGACTTTTCCATA, 167 bp; *IDH2*, AAAGATGGC-AGT-GGTGTCAAG, TCATGTACAGCGGCCATTTC, 151 bp; *MAGI1*, GGCAATGCATGTGTGGCTAT, CATCCATTTACTGCCAAGATCC, 113 bp; *NNMT*, CCCTCGGGATTACCTAGAAAAA, AGAGCCG ATGTCAATCAGCA,145 bp; *PDIA3*, AGAACTC ACGGACGACAACTTC, GCAGTGCAAT CAACCTTTGC, 177 bp; *PSMD10*, GCATCCACA-AACATCCAAGA, TACTTGCTCCTTGGGACACC, 106 bp; *RBP4*, GATGGCACCTGTGCTGACA, TCGCAGTAACCGTTGTGGAC, 149 bp; *SAA*,

TTTTCTGCTCCTTGGTCCTG, GAATGAGG GGTGCTCTTTCA, 161 bp; *SCARB2*, TTTGATCA TCACCAACATACCC, ATCATAGTTCCCCCG-AGCAT, 134 bp; *18S*, GTGGAGCGATTTGTCTGGTT, CGCTGAGCCAGTCAGTGTAG, 200 bp.

Data mining

Our raw data are deposited in the GEO repository (GSE3632). The TIGR Multiexperiment viewer (Tmev version 2.2, http://www.tm4.org) was used for (1) unsupervised hierarchical clustering (UHC) using the average dot product and complete linkage options, (2) evaluation of sample re-assignment by a jacknife procedure (1000 iterations), and (3) supervised classification with the Significance Analysis of Microarrays (SAM) tool that selects discriminating transcripts[18] (our parameters were adjusted to an FDR < 1%). Supervised classification by Support Vector Machine (SVM) was done as indicated (http://svm.sdsc.edu/). The Onto-Express program (http://vortex.cs.wayne.edu) and the FatiGO program set to level 4 or more (http://fatigo.bioinfo.cnio.es/) were used to categorize mRNA/protein function(s) by ontology. Detailed protein functions were retrieved with the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and/or OMIM (http://www3.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) tools, and protein networks were identified with Bibliosphere (www.genomatix.de). Gene locations on chromosomes were found in Onto-Express. Statistical analysis was carried out with the GraphPad Instat software, version 3 (http://www.graphpad.com/). Differences in transcript levels between groups were evaluated with Mann and Whitney's non parametric test. Significant differences in the numbers of functionally-related mRNAs that were differently regulated in tumor *vs* cirrhosis as a function of etiology were evaluated from 2 × 2 tables (number of mRNAs in a given functional subset *vs* number of all other mRNAs with other functions, in HCV *vs* alcoholic patients) by Chi square test (with Yates' correction, when required). Likewise, significant differences in the chromosomal locations of dysregulated genes as a function of etiology were evaluated from a 2 × 2 table per chromosome (number of dysregulated genes on this chromosome *vs* total number of other dysregulated genes, in HCV *vs* alcoholic patients) or arm (number of dysregulated genes on this arm *vs* number of all other *Liverpool* genes on this arm, in HCV *vs* alcoholic patients).

RESULTS

Some genomic and functional features of HCC/cirrhosis are etiology-dependent

In Table 1 various features are detailed for 35 patients with HCC (18 alcoholic patients A2-A34; 17 HCV patients V1-V35; no HBV-positive patients), and 5 HCC-free controls (C1 to C5). No clinical parameter differed between our alcoholic *vs* HCV groups. These groups were randomly separated into training (V1-A22) and test set (V23-A34). The number of mRNAs detected by microarray in the training set was 7617 ± 1270 (mean ± SD in controls), 7225 ± 1586 (tumors), or 6955 ± 1644 (cirrhosis), in keeping with a trend to down-regulation in HCC[7]. A comparison of transcript levels between tumor and surrounding cirrhosis selected a number of transcripts that could separate 3 major clusters by UHC, namely tumors, cirrhosis, and controls, as expected[2,4-6,10] (Figure S1, available on the wjg website).

We sought for transcripts with etiology-associated differences and this was first done regardless of the source, i.e. tumor *vs* cirrhosis. Using a pair-wise ratio (transcript level in tumor/transcript level in cirrhosis) resulted in 2730 transcripts with an abnormal ratio in at least 1 patient. Dysregulated transcripts were then limited to those with an abnormal ratio in at least 3 patients from at least 1 etiology group. This empirically determined cut-off was a compromise between a lower figure that provided many non-informative transcripts of a higher figure that selected too few transcripts (not detailed), and this resulted in 475 dysregulated transcripts. Because clusters of tissue-dependent genes can be co-regulated by chromosomal co-localization[19], we investigated whether some of these 475 genes were co-localized on given chromosome(s). Indeed, a higher number of dysregulated genes on chr.2 along with a lower number of dysregulated genes on chr.8 (supplemental Table S1 available on the wjg website) were found in HCV *vs* alcoholic patients (Figure 1). These etiology-dependent differences in gene location were still found when separately considering the p arm ($P < 0.01$) or q arm ($P < 0.01$) of chr.2 and -8. Remarkably, abnormalities on chr.2 and -8 have been previously associated with HCC (see Discussion), which indirectly supports our cut-off above and etiology-dependent findings.

Within the above 475 transcripts we next searched for prominent protein functions as identified by ontology, and 283 transcripts with such functional information could be retrieved (further details in Table 2, footnote 3). As shown in Table 2, a significantly increased frequency and expression [(tumor/cirrhosis) ratio] of dysregulated transcripts coding for cell cycle regulation or transcription factors was found in the HCV patients. Proteins of the cell cycle were mainly activators of cyclin-dependent kinase phosphorylation (CDC37, CKS2), microtubule-associated proteins (CCT4, MAPRE1), a negative regulator of the G1/S transition (CUL1), a proliferation-associated c-myc activator (NME1) and a tumor suppressor (TSC1). Several transcription factors were directly relevant to a defence of the tumoral hepatocyte following HCV infection. Although the difference between tumor and cirrhosis was not always significant, in HCV patients the tumor/cirrhosis ratios for IRF3 and SPIB, two interferon alpha and beta activators, were increased whereas the ratio for IRF6, an as yet unclear regulator of interferon production, was decreased and that of IRF2, a repressor of interferon synthesis, remained close to 1. Also, the ratio for the repressor ATF3 that targets many viral promoters was increased.

On the opposite, an increased frequency of dysregulated transcripts for plasma proteins of the acute phase response was found in the alcoholic patients. In these patients, the tumor/cirrhosis ratio of these transcripts was indicative of an inflammatory condition as it was increased (CRP, ORM1, SAAs) or decreased

Table 1 Clinical data from patients with HCC or controls

Patient[1]	Sex	Age	Pathology	Etiology[2]	Tumor grade[3]	Number of nodules[4]	Nodule size (cm)	Vascular invasion	Lymphocyte infiltration[5]
V1	F	71	HCC	HCV	3	2	4; 5	Yes	0
V3	F	67	HCC	HCV	3	1	2.5	No	+++
V4	F	72	HCC	HCV	3	1	4	No	+++
V8	M	66	HCC	HCV	3	1	4	Yes	++
V9	M	65	HCC	HCV	3	2	1.5; 3	No	+
V14	M	63	HCC	HCV	2	1	2	No	0
V15	M	70	HCC	HCV	1	1	3.5	No	+++
V17	M	69	HCC	HCV	3	1	2.5	No	+++
V20	F	73	HCC	HCV	3	1	1.5	Yes	++
V21	M	65	HCC	HCV	2	1	2	No	+
V23	F	68	HCC	HCV	2	2	1; 5.5	Yes	0
V24	M	80	HCC	HCV	1; 3	2	2.5; 3.5	No	0
V25	M	64	HCC	HCV	3	2	2; 5.5	Yes	+++
V26	M	46	HCC	HCV	3	1	2.5	Yes	+
V27	M	55	HCC	HCV	3	2	3; 3.5	Yes	0
V28	M	55	HCC	HCV	2	2	4; 4	No	++
V35	M	53	HCC	HCV	2; nd; nd	3	7; nd; nd	Yes	+
A2	M	68	HCC	ALC	2	1	11	No	0
A5	M	79	HCC	ALC	2	1	6	No	++
A6	M	63	HCC	ALC	2	1	3	No	+
A7	M	49	HCC	ALC	1	1	2	No	0
A10	M	73	HCC	ALC	2	1	5	No	+
A11	M	50	HCC	ALC	2	1	4.5	No	+
A12	M	64	HCC	ALC	1	1	2.5	No	+
A13	M	72	HCC	ALC	3	1	2.5	Yes	++
A16	M	56	HCC	ALC	3	1	8.5	Yes	++
A18	M	70	HCC	ALC	2	1	3.5	No	+
A19	M	70	HCC	ALC	3	1	2.5	No	+++
A22	M	78	HCC	ALC	1	1	1.7	No	+
A29	M	66	HCC	ALC	2	1	7	No	+
A30	M	55	HCC	ALC	2	1	4.5	No	0
A31	M	56	HCC	ALC	2	1	4	Yes	0
A32	F	66	HCC	ALC	3	1	8	No	0
A33	M	55	HCC	ALC	3	1	15	Yes	0
A34	M	69	HCC	ALC	3	1	2.2	Yes	0
C1	M	74	AD[6]	-					
C2	F	45	AD	-					
C3	F	68	AD	-					
C4	F	43	AD+FNH[6]	-					
C5	F	30	FNH	-					

[1]V1 to A34, patients with cirrhosis and HCC, A or V refers to the alcoholic or viral etiology. C1 to -5, control patients. Patients V1 to V21 and A2 to A22 were first studied by microarray, and next used as a training set for SVM. [2]HCV: Hepatitis C virus infection; ALC: Alcohol abuse; -: None. [3]Differentiation grade; nd: Not determined. [4]In multinodular HCCs, only 1 nodule was studied. [5]Nodular infiltration; semi-quantitative appraisal done by a trained anatomopathologist (AF). [6]Histologically normal liver taken away from a benign adenoma (AD) or a focal nodular hyperplasia (FNH).

(AHSG) in keeping with known regulation in an acute phase response[16]. Surprisingly, the ratio for the anti-inflammatory glucocorticoid receptor NR3C1 was concomitantly increased, possibly as an attempt to limit the extent of this inflammation. Conversely, in HCV patients the ratios for APCS and CRP (acute phase plasma proteins), FOSL2 (a member of the Jun/Fos family that regulates some acute phase genes) and ETS2 (an up-regulator of inflammation) were decreased and the ratio for DSIPI (an anti-inflammatory transcription factor) was increased. Taken together, our data argued for a significant inflammatory condition in tumor as compared to adjacent cirrhosis in alcoholic but not HCV patients.

Finally, a tight SAM selection made from the 2730 transcript ratios above identified 23 non-redundant transcripts (29 probes) whose higher ratio in alcoholic *vs* HCV patients was statistically significant (red dots in Figure 2A). No transcript with a decreased ratio could be identified, but such an imbalance in informative transcripts is not unusual with SAM[18]. Using these 23 transcripts in UHC separated the 22 HCV and alcoholic patients into two etiology-associated groups (Figure 2B). Only 2 patients were misclassified (A18, V15) which was further evaluated by a jacknife procedure (Figure 2B legend).

The etiology-dependent transcriptomes are found in the HCC nodules

We next investigated whether the etiology-associated differences in transcript levels depended on the transcript source, i.e. tumor *vs* cirrhosis. When using the (transcript level in tumor/mean transcript level in controls) ratio in nodules, a total of 2641 transcripts had a significantly abnormal ratio in at least 1 of our patients 1-22 (black and red dots in Figure 3A). SAM identified 18 non-redundant transcripts whose (tumor/controls) ratio was significantly higher in alcoholic *vs* HCV patients (red dots in Figure 3A).

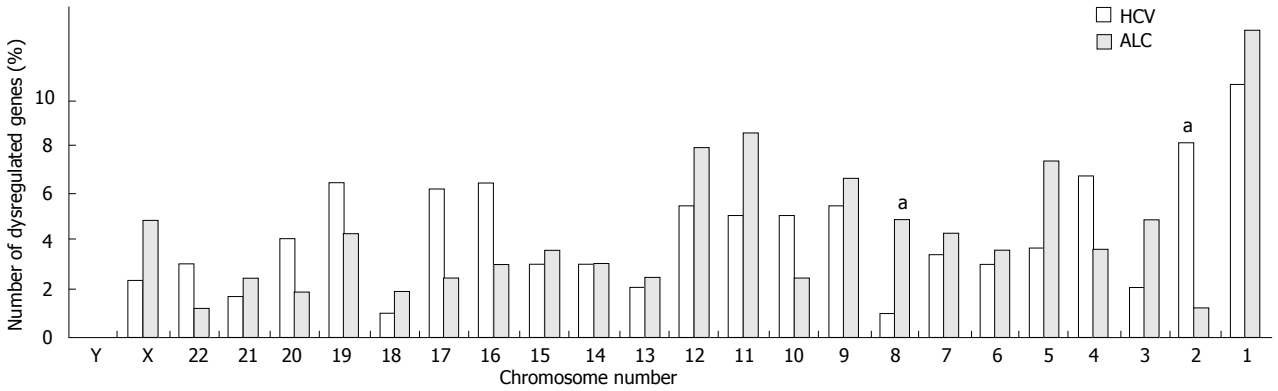

Figure 1 Etiology-dependent location of dysregulated genes. A total of 475 genes (HCV, 301 genes; alcoholism, 174 genes) with a dysregulated transcript were studied. Dysregulated transcripts were defined by an abnormal (tumor/cirrhosis) ratio in at least 3 patients of at least one etiology group (see details in Table 2, footnote 3). The number of dysregulated genes per chromosome is expressed as a percentage of the total number of dysregulated genes per etiology. Significant differences of gene frequency on a given chromosome in HCV *vs* alcoholic patients are: chr 2, $P = 0.004$; chr 8, $P = 0.02$ (Chi square test with Yates' correction), $^{a}P \leqslant 0.02$.

Table 2 Etiology-dependent frequency of dysregulated transcripts within functionally defined subsets

	HCV patients (n = 10)			Alcoholic patients (n = 12)		
			Tumor/cirrhosis[1]			Tumor/cirrhosis
Subset: Regulation of cell cycle (GO 0000074)[2]						
CCT4[3]	Hs.421509[4]	1.38 (7)[a]				
ZAK	Hs.444451	1.44 (8)				
CUL1	Hs.146806	1.69 (7)				
MAPRE1	Hs.472437	1.78 (10)	χ^2			
CLK1	Hs.433732	1.80 (7)	$P < 0.05$			
CKS2	Hs.83758	1.92 (8)				
NME1	Hs.463456	1.97 (9)				
CDC37	Hs.160958	2.05 (6)				-
TSC1	Hs.370854	2.60 (6)[a]				
Subset: Transcription factor (GO 0003700)						
IRF6	Hs.355827	0.55 (10)[b]		*RUNX1*	Hs.149261	0.67 (12)[b]
FOSL2	Hs.220971	0.58 (10)[a]		*NR3C1*	Hs.122926	1.68 (12)[b]
ETS2	Hs.592158	0.79 (10)[b]				
IRF2	Hs.374097	0.83 (9)				
DSCR1	Hs.282326	1.53 (10)				
NR4A3	Hs.279522	1.63 (7)[a]				
ZNF397	Hs.84307	1.72 (10)	χ^2			
HMGA1	Hs.518805	1.75 (9)	$P < 0.05$			
SPIB	Hs.437905	1.77 (10)				
NME1	Hs.463456	1.97 (6)[a]				
ATF3	Hs.460	2.22 (6)				
MSRB2	Hs.461420	2.32 (6)				
DSIPI	Hs.420569	2.51 (7)				
IRF3	Hs.75254	2.70 (7)				
Subset: Acute phase response (GO 0006953)						
CRP[5]	Hs.76452	0.46 (10)[a]		*AHSG*	Hs.324746	0.84 (12)
APCS[5]	Hs.507080	0.84 (10)[a]	χ^2	*ORM1*	Hs.567311	1.24 (12)
			$P < 0.05$	*SAA2*[5]	Hs.1955	1.84 (11)[a]
				SAA1[5]	Hs.632144	1.90 (11)[a]
				CRP[5]	Hs.76452	2.17 (12)

[1]Average ratio of mRNA levels in tumor and cirrhotic tissue in patients with a detectable expression of this mRNA. The number of such patients is indicated in brackets and a significant difference between tumors and paired cirrhotic tissues per etiology is indicated ([a]$P < 0.05$; [b]$P < 0.01$; Wilcoxon's non parametric, paired test). [2]Subset of mRNAs coding for proteins with a shared function as identified by Gene Ontology (GO) number. [3]Dysregulated mRNAs, as defined by an abnormal tumor/cirrhosis ratio found in at least 3 patients of at least one etiology group (total 475 mRNAs; HCV, 301; alcoholism, 174). The dysregulated mRNAs with an ontology-defined function (HCV, 183; alcoholism, 100) were a subset of the 475 mRNAs. [4]Hs. number as a unique mRNA identifier. [5]Only 5 of the 23 mRNAs listed in Figure 2B appear herein because the other mRNAs in Figure 2B belonged to functional classes in which the number of mRNAs was not different in alcoholic *vs* HCV patients.

As shown in Figure 3B, UHC of our 22 patients as based upon these 18 ratios provided two major clusters of HCV- or alcohol-associated HCCs. Only 2 samples were misclassified (V-TU14, V-TU15), which was confirmed by a jacknife procedure (Figure 3B legend). Interestingly, all controls were clustered with the alcoholic tumors (right

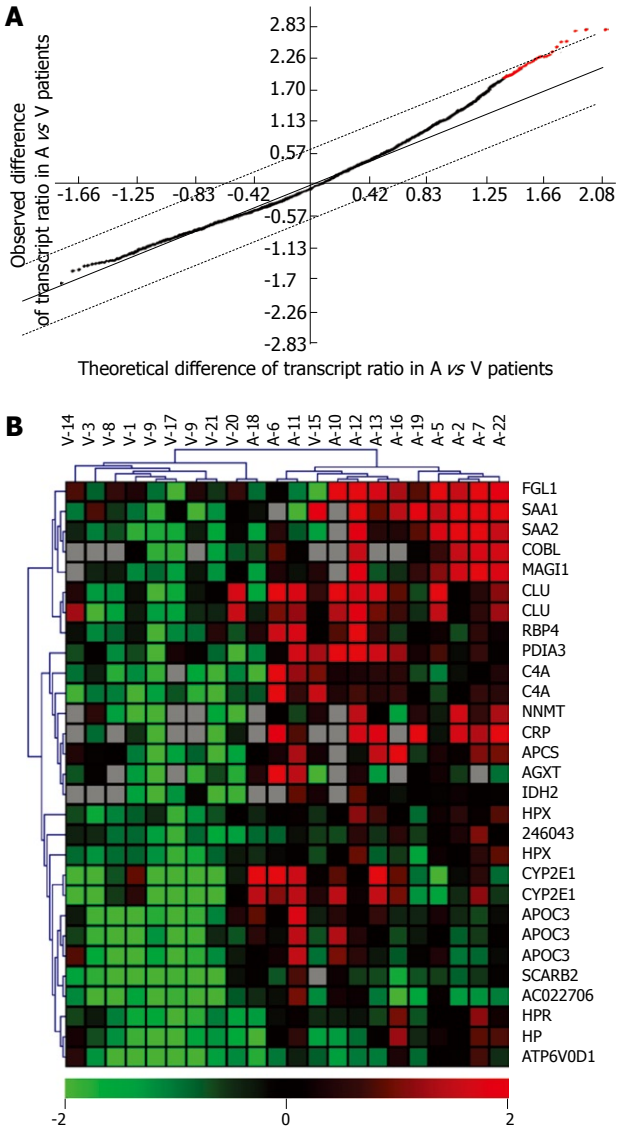

Figure 2 Etiology-dependent clustering of patients with (tumor/cirrhosis) ratios. **A**: Selection of transcripts with a significantly abnormal ratio of abundance in (tumor/paired cirrhosis). Black + red dots: 2730 transcripts with an abnormal ratio in at least 1/22 patients. Red dots: 23 informative transcripts (29 probes) with a significantly increased ratio in alcoholic *vs* HCV patients as selected by SAM with an FDR < 1%; **B**: UHC of 22 HCC patients with the above 23 transcripts. Note that the misclassification of two samples (A18, V15) was further evaluated by a jacknife procedure (1000 iterations) which supported cluster assignment to a variable extent (A18, < 50%; V15, 100%). Note that several transcripts were each detected with 2 or more different cDNA probes. Bottom scale bar: decreased (green), increased (red) or identical ratio (black). Gray squares are missing values. All data given on a $\log_2$ scale.

side of Figure 3B), thus suggesting that alcoholism may alter expression of these 18 transcripts to a lesser extent than HCV infection.

Because the etiology-dependent transcripts identified from (nodule/cirrhosis) ratios (Figure 2) or from (nodule/controls) ratios (Figure 3B) largely overlapped (15/18 transcripts; 83%; stared in Figure 3B), this suggested that the nodules could be responsible for transcript abnormalities. Indeed, SAM made with the ratios from cirrhotic samples, i.e. (transcript level in cirrhosis/mean transcript level in controls), failed to identify a series of etiology-discriminant transcripts (no red dots in Figure 3C). Moreover, and as shown in Figure 3D, no etiology-dependent clustering of patients V1-A22 was obtained when using the (cirrhosis/controls) ratios for these 18 stared transcripts. We concluded that such transcript levels now appear to be an etiology-dependent variable in nodules, but not in cirrhosis.

Taken together, our data indicated that a subset differently dysregulated following HCV infection *vs* alcoholism can be identified in cancerous nodules whereas it cannot be detected at an earlier stage of hepatic dysplasia, namely the surrounding cirrhosis.

Data validation

Our data above were validated with a conceptually different tool (qRTPCR). After excluding 3 transcripts whose gene structure was unknown (AC022706, FLJ33814, 246043) all transcripts previously found to vary significantly with etiology (i.e. cumulated lists from Figure 2B and 3B, total 23 transcripts) were quantified in every HCC patient in this study. Given our previous observation that cirrhotic samples were not informative, these transcripts were measured in tumors only. First, the informativeness of each of the above 23 transcripts was tested in our entire population of 35 HCC samples. As shown in Figure 4A, most transcripts (18/23, 78%) were significantly overexpressed in alcoholic tumors, as expected from Figure 3A. Next, UHC reproducibly resulted in a perfect and etiology-dependent separation of our test set (V23-V35 and A29-A34) (Figure 4B). Finally, a classification algorithm generated with qRTPCR data from the training set (V1-A22) by SVM separated our test set into etiology-dependent groups with 2 misclassifications (V23, V24).

As for protein functions the increased level of CYP2E1 transcript in alcoholic patients fits its known up-regulation by ethanol[20]. Most other proteins in alcoholic patients were associated with the inflammatory response, as inferred from a comparison with our earlier data[16] as well as putative protein relationships retrieved with Bibliosphere (data not shown). They included acute phase proteins (APCS, APOC3, C4A, CRP, HP, HPX, NNMT, RBP4, SAAs) whose directions of variations indicated a stronger inflammatory condition of the tumor in alcoholic *vs* HCV patients. Remarkably, most of these acute phase proteins are scavengers of endogenous toxicants or protect against membrane peroxidation (CRP, HP, HPX, RBP4, SAAs). Among them, heme detoxicants include two hemoglobin transporters (HP, HPR) and one heme scavenger (HPX), whose variations suggested a higher iron metabolism in alcoholic *vs* HCV tumors. In alcoholism-associated tumors, only two proteins were associated with cell proliferation (FGL1) and apoptosis limitation (CLU).

DISCUSSION

Transcriptome-wide analysis in alcoholism-induced HCC has seldom been studied, which has prevented its detailed comparison with other etiologies. Indeed, transcript alterations in alcoholism-associated HCC have been reported but they focused on a limited number of transcripts or they did not differentiate between cirrhotic and cancerous tissues[21,22]. In contrast, we have now compared transcripts in tumor *vs* paired cirrhosis

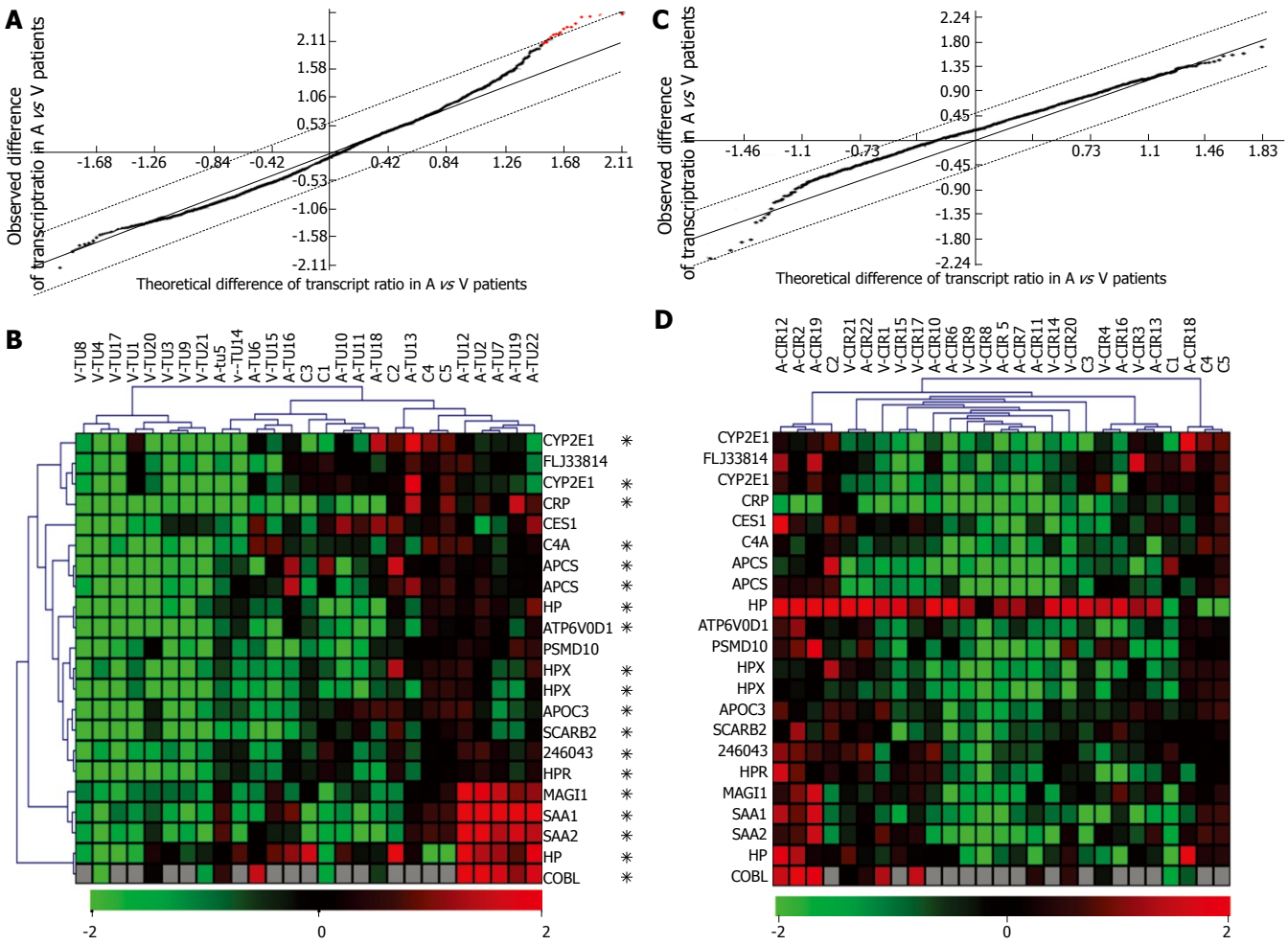

Figure 3 Etiology-dependent clustering of tumors only, with (tumor/controls) ratios. **A**: Selection of transcripts with a significantly abnormal (level in tumor/mean level in controls) ratio. Black + red dots: 2641 transcripts with an abnormal ratio in at least 1/22 patients. Red dots: SAM selection of 18 informative transcripts (22 probes) with a significantly increased (tumor/controls) ratio in alcoholic *vs* HCV patients. SAM parameters as in Figure 2; **B**: UHC of 5 control livers and 22 tumors from 22 HCC patients, as done with the data of the 18 informative transcripts selected in **A**; The stars point to 15 transcripts (19 probes) that also belonged to the set of 23 transcripts previously selected in Figure 2; **C**: Selection of transcripts with a significantly abnormal (level in cirrhosis/mean level in controls) ratio. Black dots: 2037 transcripts with an abnormal ratio in at least 1/22 patients. No informative transcript was selected by SAM (no red dot); **D**: UHC of 5 control patients and 22 HCC-associated cirrhotic samples from 22 HCC patients, as done with the list of 18 transcripts used in **B**. Other details as in Figure 2.

by searching for abnormal (tumor/cirrhosis) ratios at a genome-wide level. We used a non-stringent selection of 475 transcripts whenever wide groups of transcripts were required (chromosomes; ontology) but we used SAM with a tight FDR to identify a total of 23 transcripts with strong etiology-associated variations. Second, we compared tumors (or cirrhosis) *vs* controls, which identified 18 transcript alterations in tumors only. The combined levels of these 18 transcripts in tumors but not in paired cirrhosis could classify the patients by etiology. Interestingly, most of these transcripts were previously associated with cirrhosis and HCC, this including, for instance, APCS, APOC3, CLU, CRP, CYP2E1, FGL1, HP, HPX, NNMT, RBP4, SAAs, and their down-regulations, mainly seen in an HCV context, were consistent with our present data[14,23-25]. The present lack of an etiology-dependent transcriptome in the cirrhosis surrounding HCC indicates that some etiology-dependent mechanisms take place at a relatively late stage of tumoral transformation. Further analysis of HCC-free cirrhosis will clarify this stepwise process.

As inferred from ontology, cell cycle regulation and a response to interferons appear to predominate in HCV-associated tumors. Our observation that over-expression of interferon-responsive genes in tumor *vs* cirrhosis is restricted to HCV patients also fits this viral etiology, as well as the ethanol-induced down-regulation of interferon gamma signalling in hepatoma cells[26]. HCCs with an alcoholic or viral origin have been subgrouped by others as a function of activation or repression of interferon-regulated genes but etiology influence was not documented[6]. The potent response of the normal hepatocyte to interferons is repressed by an HCV infection[27]. Therefore, the over-expression of interferon-responsive genes in nodule *vs* cirrhosis, as seen herein, suggests that a repression of these genes occurs in cirrhosis but escapes, at least partly, this viral mechanism in nodules.

The iron overload/HCC association is well established. Non transferrin-bound, free iron is carcinogenic and facilitates tumor growth *via* the production of ROS and free radicals, and subsequent lipid peroxidation[28]. Free iron and ROS in hepatocytes are a side effect of chronic

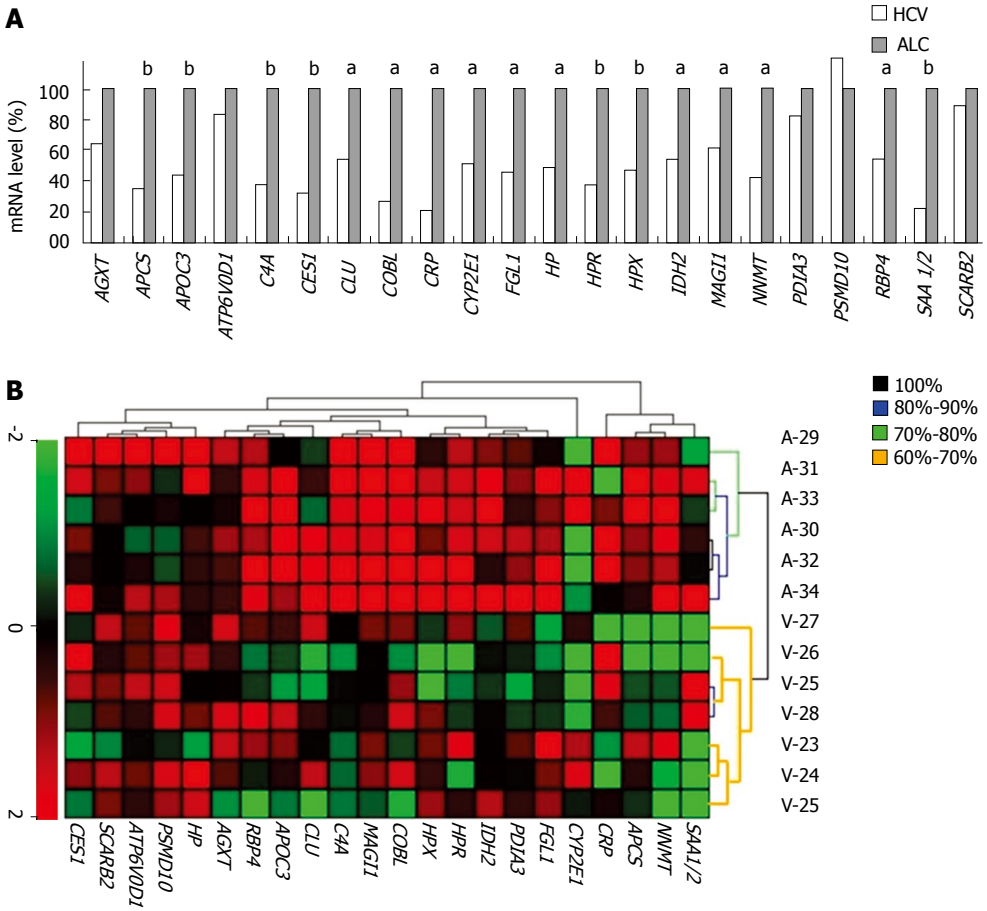

Figure 4 Data validation by qRTPCR of tumor mRNAs. The levels of 23 transcripts (listed from Figures 2A and 3A; SAA1 and -2 are counted as 2 transcripts) were determined in tumors and normalized with the 18S RNA level. **A**: Every histogram depicts the mean transcript level in all patients from Table 1 (n = 35) and is expressed as a percentage of the mean level in alcoholic patients (100%). Mann and Whitney's test ($^aP < 0.05$; $^bP < 0.01$). **B**: UHC made with qRTPCR data from our test set (n = 13). The colors in dendrogram indicate the percentage of iterations reproducibly providing the same separation. Note the significant separation of 2 major, etiology-related branches (black branches found in 100% of 10^3 iterations).

alcoholism[29]. Our ontology-based data, up-regulations and functions of SAM-selected transcripts indicated that in alcoholic nodules an acute phase response is a prominent event. Therefore, a high extent of inflammation could participate in an etiology-dependent antitumoral response of the hepatocyte. However, this view is now challenged when considering (1) the induction of an inflammatory response in liver following both alcoholism and HCV infection[6,30,31], (2) the similar extent of lymphocyte infiltration in both etiologies in our patients, (3) the limited apoptosis in alcoholic tumors and, most importantly, (4) the restricted functions of the afore mentioned set of acute phase transcripts. Indeed, in alcoholic patients the up-regulated levels of acute phase transcripts point to acceleration of iron metabolism (HP, HPX), a detoxication mediated by the haemoglobin degradation pathways (HP, HPR, HPX), and a protection against membrane peroxidation (CLU, CRP, RBP4, SAAs), and hence they strongly suggest accelerated exchange of free- *vs* bound iron in nodules. If so, the increase in proteins that prevent membrane peroxidation (CLU, CRP, RBP4, SAAs) represents a concomitant against free iron. Overall, we propose a set of transcripts as indicators of a detrimental iron metabolism whose extent and control are etiology-dependent. Alteration of this metabolism in alcoholism-induced tumors, as now suggested by the high levels of relevant transcripts, could participate in the free iron limitation noticed in HCC nodules[32].

An increasing number of chromosome amplifications, mutations, deletions and transpositions develop during the transition from preneoplasia to HCC[1]. Such events on chr.8 have allowed discrimination of patients with beta-catenin mutations and an allelic loss of chr.8p only from patients with a heterogeneous series of gains/losses of various other chromosome segments[9]. In contrast, chr.2 abnormalities are infrequent in HCCs[9]. Our present work now establishes a link between etiology and an abnormal expression of various genes on chr.2 (HCV) or -8 (alcoholism). This conclusion based upon transcript levels will require further investigations of etiology-dependent structural or epigenomic alterations on these chromosomes. As our data indicate that abnormal gene expressions are spread on both arms of chr.2 and -8, aberrant methylation of a series of promoters along a chromosome segment, long range epigenetic silencing and/or abnormal copy numbers of a chromosome[33-35] could explain our observations.

Overall, our data point to major etiology-associated differences in HCC. Given that HCC therapies have not yet considered any etiology-dependent mechanisms of carcinogenesis, our observations open new avenues for therapies that should take into account HCC etiology.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection and alcoholism are two important causes for hepatocellular carcinoma (HCC). Liver transcriptome analysis has resulted in the identification of genes with an aberrant expression according to different physiopathological states. In the present work, we performed a comparison of liver transcriptomes in HCV virus- *vs* alcoholism-associated HCC.

Research frontiers

Table S1 Etiology-dependent location of dysregulated genes on chromosomes

HCV			Alcohol		
gene	**Hs. Number**	**chr.**	**gene**	**Hs. Number**	**chr.**
Trans. Locus	Hs.597833	2	MAP3K2	Hs.145605	2q14.3
VAMP5	Hs.172684	2p11.2	WDFY1	Hs.642721	2q36.1
KCMF1	Hs.345694	2p11.2			
LOC56902	Hs.262858	2p13.3			
UGP2	Hs.516217	2p14-p13			
CCT4	Hs.421509	2p15			
KIAA1387	Hs.516182	2p16.1			
MRPL33	Hs.515879	2p21			
FNDC4	Hs.27836	2p23.3			
FOSL2	Hs.220971	2p23.3			
CKAP2L	Hs.434250	2q13			
CXCR4	Hs.421986	2q21			
ZAK	Hs.444451	2q24.2			
LEREPO4	Hs.368598	2q32.2			
CLK1	Hs.433732	2q33			
MAP2	Hs.368281	2q34-q35			
CPS1	Hs.149252	2q35			
NHEJ1	Hs.225988	2q35			
WDFY1	Hs.642721	2q36.1			
AGXT	Hs.144567	2q36-q37			
TNRC15	Hs.565319	2q37.1			
DGKD	Hs.471675	2q37.1			
STK25	Hs.516807	2q37.3			
LONRF1	Hs.180178	8p23	PLAT	Hs.491582	8p12
EXOSC4	Hs.632041	8q24.3	CLU	Hs.436657	8p21-p12
TIGD5	Hs.71574	8q24.3	FGL1	Hs.491143	8p22-p21.3
			MCM4	Hs.460184	8q11.2
			FAM92A1	Hs.125038	8q22.1
			EXOSC4	Hs.632041	8q24.3
			TIGD5	Hs.71574	8q24.3
			RPL8	Hs.178551	8q24.3

The genes shown here are those located on the significant chrs in Figure 1. They are referred to by an Hs. number as a unique identifier. Every such gene had an mRNA level that was significantly altered in tumor vs cirrhosis in at least 25% of the patients within at least one etiology.

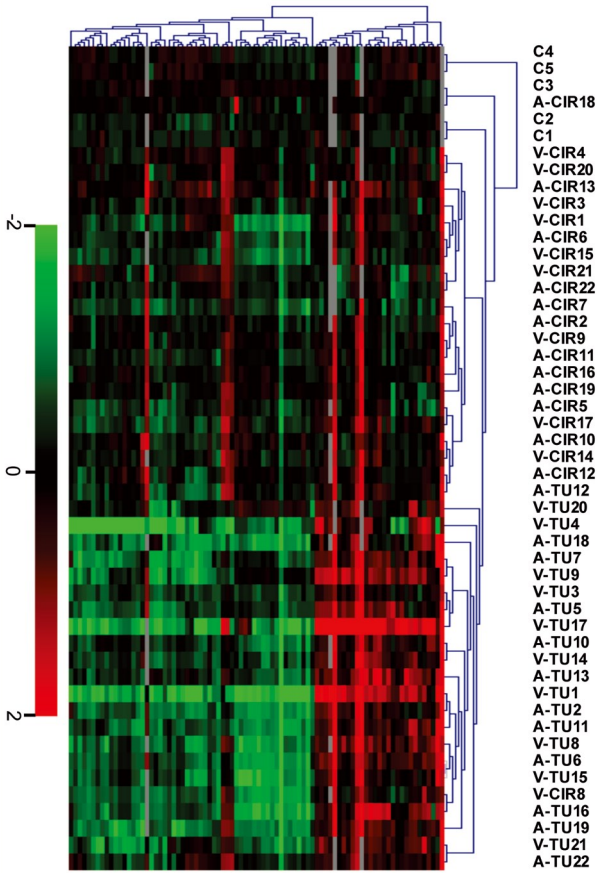

Figure S1 Sample clustering: Tumor *vs* cirrhosis. Unsupervised hierarchical clustering of 5 control livers (C) and paired HCC nodule (TU) and surrounding cirrhosis (CIR) (44 samples from patients 1 to 22, see clinical data in Table 1) shown from left to right was based upon 81 transcripts (84 probes) shown rom top to bottom. Transcript levels were expressed as a ratio [level in sample/mean level in controls] and 81 transcripts were next selected as informative transcripts by SAM. The patients are listed on top (V, HCV; A, alcoholism). Scale bar (log2 ratio): decreased (green), increased (red) or identical mRNA level (black) in any sample *vs* controls. Gray squares are missing values.

The hepatitis B virus (HBV)- or HCV-induced genetic alterations are known to be different and the associated transcriptomes have proven to vary significantly. Therefore, deciphering the transcriptome patterns as a function of HCC etiology is of critical importance. However, the gene dysregulations in a context of alcohol abuse are poorly understood and the associated transcriptome has seldom been studied. *A fortiori*, a comparison of liver transcriptomes in HCV virus- *vs* alcoholism-associated HCC has never been done.

Applications

Given that HCC therapies have not yet considered any etiology-dependent mechanisms of carcinogenesis, our observations open new avenues for therapies that should take into account HCC etiology.

Peer review

The manuscript by Derambure *et al.* describes a study that compared microarray data from hepatocellular carcinoma as a result from alcohol or hepatitis C. Interestingly, the authors found etiology-specific alterations in gene expression between the two HCC. These data would lead to a better understanding of the molecular basis of these disease states.

REFERENCES

1 **Laurent-Puig P**, Zucman-Rossi J. Genetics of hepatocellular tumors. *Oncogene* 2006; **25**: 3778-3786

2 **Thorgeirsson SS**, Lee JS, Grisham JW. Molecular prognostication of liver cancer: end of the beginning. *J Hepatol* 2006; **44**: 798-805

3 **Iizuka N**, Oka M, Yamada-Okabe H, Mori N, Tamesa T, Okada T, Takemoto N, Hashimoto K, Tangoku A, Hamada K, Nakayama H, Miyamoto T, Uchimura S, Hamamoto Y. Differential gene expression in distinct virologic types of hepatocellular carcinoma: association with liver cirrhosis. *Oncogene* 2003; **22**: 3007-3014

4 **Ye QH**, Qin LX, Forgues M, He P, Kim JW, Peng AC, Simon R, Li Y, Robles AI, Chen Y, Ma ZC, Wu ZQ, Ye SL, Liu YK, Tang ZY, Wang XW. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med* 2003; **9**: 416-423

5 **Choi JK**, Choi JY, Kim DG, Choi DW, Kim BY, Lee KH, Yeom YI, Yoo HS, Yoo OJ, Kim S. Integrative analysis of multiple gene expression profiles applied to liver cancer study. *FEBS Lett* 2004; **565**: 93-100

6 **Breuhahn K**, Vreden S, Haddad R, Beckebaum S, Stippel D, Flemming P, Nussbaum T, Caselmann WH, Haab BB, Schirmacher P. Molecular profiling of human hepatocellular carcinoma defines mutually exclusive interferon regulation and insulin-like growth factor II overexpression. *Cancer Res* 2004; **64**: 6058-6064

7 **Coulouarn C**, Derambure C, Lefebvre G, Daveau R, Hiron M, Scotte M, Francois A, Daveau M, Salier JP. Global gene repression in hepatocellular carcinoma and fetal liver, and suppression of dudulin-2 mRNA as a possible marker for the cirrhosis-to-tumor transition. *J Hepatol* 2005; **42**: 860-869

8 **Chen X**, Cheung ST, So S, Fan ST, Barry C, Higgins J, Lai KM, Ji J, Dudoit S, Ng IO, Van De Rijn M, Botstein D, Brown PO. Gene expression patterns in human liver cancers. *Mol Biol Cell*

2002; **13**: 1929-1939
9 **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
10 **Iizuka N**, Oka M, Yamada-Okabe H, Nishida M, Maeda Y, Mori N, Takao T, Tamesa T, Tangoku A, Tabuchi H, Hamada K, Nakayama H, Ishitsuka H, Miyamoto T, Hirabayashi A, Uchimura S, Hamamoto Y. Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection. *Lancet* 2003; **361**: 923-929
11 **Lee JS**, Chu IS, Heo J, Calvisi DF, Sun Z, Roskams T, Durnez A, Demetris AJ, Thorgeirsson SS. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology* 2004; **40**: 667-676
12 **Llovet JM**, Wurmbach E. Gene expression profiles in hepatocellular carcinoma: not yet there. *J Hepatol* 2004; **41**: 336-339
13 **Delpuech O**, Trabut JB, Carnot F, Feuillard J, Brechot C, Kremsdorf D. Identification, using cDNA macroarray analysis, of distinct gene expression profiles associated with pathological and virological features of hepatocellular carcinoma. *Oncogene* 2002; **21**: 2926-2937
14 **Iizuka N**, Oka M, Yamada-Okabe H, Mori N, Tamesa T, Okada T, Takemoto N, Tangoku A, Hamada K, Nakayama H, Miyamoto T, Uchimura S, Hamamoto Y. Comparison of gene expression profiles between hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma by oligonucleotide microarray data on the basis of a supervised learning method. *Cancer Res* 2002; **62**: 3939-3944
15 **Morgan TR**, Mandayam S, Jamal MM. Alcohol and hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S87-S96
16 **Coulouarn C**, Lefebvre G, Derambure C, Lequerre T, Scotte M, Francois A, Cellier D, Daveau M, Salier JP. Altered gene expression in acute systemic inflammation detected by complete coverage of the human liver transcriptome. *Hepatology* 2004; **39**: 353-364
17 **Wong DK**, Yuen MF, Tse E, Yuan H, Sum SS, Hui CK, Lai CL. Detection of intrahepatic hepatitis B virus DNA and correlation with hepatic necroinflammation and fibrosis. *J Clin Microbiol* 2004; **42**: 3920-3924
18 **Tusher VG**, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 2001; **98**: 5116-5121
19 **Mijalski T**, Harder A, Halder T, Kersten M, Horsch M, Strom TM, Liebscher HV, Lottspeich F, de Angelis MH, Beckers J. Identification of coexpressed gene clusters in a comparative analysis of transcriptome and proteome in mouse tissues. *Proc Natl Acad Sci USA* 2005; **102**: 8621-8626
20 **Dey A**, Cederbaum AI. Alcohol and oxidative liver injury. *Hepatology* 2006; **43**: S63-S74
21 **Edamoto Y**, Hara A, Biernat W, Terracciano L, Cathomas G, Riehle HM, Matsuda M, Fujii H, Scoazec JY, Ohgaki H. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *Int J Cancer* 2003; **106**: 334-341
22 **Kim JW**, Ye Q, Forgues M, Chen Y, Budhu A, Sime J, Hofseth LJ, Kaul R, Wang XW. Cancer-associated molecular signature in the tissue samples of patients with cirrhosis. *Hepatology* 2004; **39**: 518-527
23 **Kim MY**, Park E, Park JH, Park DH, Moon WS, Cho BH, Shin HS, Kim DG. Expression profile of nine novel genes differentially expressed in hepatitis B virus-associated hepatocellular carcinomas. *Oncogene* 2001; **20**: 4568-4575
24 **Xu L**, Hui L, Wang S, Gong J, Jin Y, Wang Y, Ji Y, Wu X, Han Z, Hu G. Expression profiling suggested a regulatory role of liver-enriched transcription factors in human hepatocellular carcinoma. *Cancer Res* 2001; **61**: 3176-3181
25 **Hu L**, Lau SH, Tzang CH, Wen JM, Wang W, Xie D, Huang M, Wang Y, Wu MC, Huang JF, Zeng WF, Sham JS, Yang M, Guan XY. Association of Vimentin overexpression and hepatocellular carcinoma metastasis. *Oncogene* 2004; **23**: 298-302
26 **Osna NA**, Clemens DL, Donohue TM Jr. Ethanol metabolism alters interferon gamma signaling in recombinant HepG2 cells. *Hepatology* 2005; **42**: 1109-1117
27 **Geiss GK**, Carter VS, He Y, Kwieciszewski BK, Holzman T, Korth MJ, Lazaro CA, Fausto N, Bumgarner RE, Katze MG. Gene expression profiling of the cellular transcriptional network regulated by alpha/beta interferon and its partial attenuation by the hepatitis C virus nonstructural 5A protein. *J Virol* 2003; **77**: 6367-6375
28 **Deugnier Y**, Turlin B. Iron and hepatocellular carcinoma. *J Gastroenterol Hepatol* 2001; **16**: 491-494
29 **De Feo TM**, Fargion S, Duca L, Cesana BM, Boncinelli L, Lozza P, Cappellini MD, Fiorelli G. Non-transferrin-bound iron in alcohol abusers. *Alcohol Clin Exp Res* 2001; **25**: 1494-1499
30 **Nagy LE**. Recent insights into the role of the innate immune system in the development of alcoholic liver disease. *Exp Biol Med* (Maywood) 2003; **228**: 882-890
31 **Iizuka N**, Oka M, Yamada-Okabe H, Mori N, Tamesa T, Okada T, Takemoto N, Sakamoto K, Hamada K, Ishitsuka H, Miyamoto T, Uchimura S, Hamamoto Y. Self-organizing-map-based molecular signature representing the development of hepatocellular carcinoma. *FEBS Lett* 2005; **579**: 1089-1100
32 **Holmstrom P**, Gafvels M, Eriksson LC, Dzikaite V, Hultcrantz R, Eggertsen G, Stal P. Expression of iron regulatory genes in a rat model of hepatocellular carcinoma. *Liver Int* 2006; **26**: 976-985
33 **Kawaguchi K**, Honda M, Yamashita T, Shirota Y, Kaneko S. Differential gene alteration among hepatoma cell lines demonstrated by cDNA microarray-based comparative genomic hybridization. *Biochem Biophys Res Commun* 2005; **329**: 370-380
34 **Plentz RR**, Schlegelberger B, Flemming P, Gebel M, Kreipe H, Manns MP, Rudolph KL, Wilkens L. Telomere shortening correlates with increasing aneuploidy of chromosome 8 in human hepatocellular carcinoma. *Hepatology* 2005; **42**: 522-526
35 **Stransky N**, Vallot C, Reyal F, Bernard-Pierrot I, de Medina SG, Segraves R, de Rycke Y, Elvin P, Cassidy A, Spraggon C, Graham A, Southgate J, Asselain B, Allory Y, Abbou CC, Albertson DG, Thiery JP, Chopin DK, Pinkel D, Radvanyi F. Regional copy number-independent deregulation of transcription in cancer. *Nat Genet* 2006; **38**: 1386-1396

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