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ORIGINAL ARTICLES

Transient and etiology-related transcription regulation in cirrhosis prior to hepatocellular carcinoma occurrence

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Abstract

AIM: To search for transcription dysregulation that could (1) differentiate hepatocellular carcinoma (HCC)-free from HCC-related cirrhosis (2) differentiate HCC-free cirrhosis related to HCV from that related to alcohol intake.

METHODS: Using microarray analysis, we compared transcript levels in HCC-free cirrhosis (alcoholism: 7; hepatitis C: 7), HCC-associated cirrhosis (alcoholism: 10; hepatitis C: 10) and eight control livers. The identified transcripts were validated by qRT-PCR in an independent cohort of 45 samples (20 HCC-free cirrhosis; 15 HCC-associated cirrhosis and 10 control livers). We also confirmed our results by immunohistochemistry.

RESULTS: In HCC-free livers, we identified 70 transcripts which differentiated between alcoholic-related cirrhosis, HCV-related cirrhosis and control livers. They mainly corresponded to down-regulation. Dysregulation of Signal Transduction and Activator of Transcription-3 (STAT-3) was found along with related changes in STAT-3 targets which occurred in an etiology-dependent fashion in HCC-free cirrhosis. In contrast, in HCC, such transcription dysregulations were not observed.

CONCLUSION: We report that transcriptional dysregulations exist in HCC-free cirrhosis, are transiently observed prior to detectable HCC onset and may be appear like markers from cirrhosis to HCC transition.

Key words: Liver; Pathology; Alcoholism; Hepatitis C virus; Gene expression; Carcinogenesis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prominent, primary liver cancer. Its main etiologies are viral hepatitis B or C (HBV; HCV), alcoholism or aflatoxin B1 intoxication, with both HCV and alcoholism currently increasing in incidence in Western countries and predominating as etiologies[1,2]. In most instances, HCC develops in the setting of chronic hepatitis and/or cirrhosis[3]. Cirrhosis is the end stage of a chronic liver disease which results in regenerating nodules surrounding by fibrous septa and, ultimately, may lead to cancerous nodules. HCC has a poor prognosis but,

apart from surgery, no major improvements in disease therapies have been recently reported[4], most likely because the heterogeneity of the disease and its various etiologies prevent any progress in our understanding of HCC development and mechanisms[5].

Numerous genome-wide analyses of abnormal gene expression in HCC as compared to normal, control livers, have resulted in identification of gene sets with altered expression[2,6,7], part of which result from underlying gene mutations and/or chromosome alterations[8-10] and account for a limited number of altered pathways[9,11]. A few similar studies have been done in HCC-free cirrhosis and they mostly considered markers for a pre-HCC condition[12], or selected pathways[13], or mixed etiologies[12,14]. In fact, the number of comparative studies devoted to HCC etiology has remained scarce, whether this was done in a clinical setting[15-19], cell lines[20] or animal models with oncogene overexpression[21]. In particular, the viral etiologies have been considered[17,18,20,22] whereas abnormal gene expression in alcoholism-dependent HCC has received very little attention. Therefore, the impact of etiology still remains an important issue[7,23]. We recently reported that a number of genome-wide abnormalities in alcoholism-associated *vs* HCV-associated HCC are etiology-dependent and some of them are of pathological relevance[24]. Remarkably, the abnormal transcription levels that differentiate HCC nodules in an alcoholism-dependent *vs* HCV-dependent fashion can no longer discriminate between both etiologies when transcripts are measured in the surrounding cirrhosis[24]. Yet, any etiology-dependent abnormalities that could be observed in HCC-free cirrhosis would be of interest. We investigated whether some transcription dysregulations could be found in HCC-free cirrhosis in an etiology-dependent fashion. Furthermore, we searched and found transcript dysregulations that differentiate HCC-free cirrhosis from peritumoral cirrhosis. We now report that such transcription dysregulations do exist in HCC-free cirrhosis and are observed prior to detectable HCC onset.

MATERIALS AND METHODS

Human subjects and tissue sampling

Non-alcoholic steatohepatitis, primary biliary cirrhosis and infant biliary atresia were excluded from this study. Chronic alcohol abuse was estimated as detailed[24]. HBV and HCV infections were serologically determined in every patient and any HBV-positive patient was excluded. Patients with an HCC-free or HCC-associated cirrhosis were histologically diagnosed by trained pathologists (AF, PBS). Liver fragments came to our laboratory from the digestive surgery unit of Charles Nicolle Hospital (Rouen, France) or the pathology unit of Pellegrin Hospital (Bordeaux, France) under strict anonymity. HCC-free, cirrhotic tissue was obtained from transplanted patients. Peri-tumoral, cirrhotic tissue was taken at a distance from HCC resection whenever the latter was excised for curative purposes. Control, non-cirrhotic human liver (CL) was obtained from patients operated on for a benign liver tumor or metastasis of a non-hepatic cancer. According to the current French rules and ethical guidelines, neither informed consent nor advice from an ethical committee were requested prior to RNA analysis in tissues that would otherwise be disposed of. Various clinical features in a total of 69 cirrhosis without HCC, (n = 34) or with HCC (n = 35), as well as in a set of 18 histologically normal CLs are summarized in Table 1. A METAVIR score from the combined extents of inflammation (A0-A3) and fibrosis (F0-F4) were histologically diagnosed by trained pathologists (AF, PBS).

Transcriptome analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA extraction from tissues stored at -80℃ was done with Trizol. Our set of human cDNA probes dubbed *Liverpool* and tailored to a complete coverage of the human liver transcriptome under healthy or pathological conditions (ca. 10^4 genes), the associated *LiverTools* database, as well as the procedures from array preparation to data handling have all been detailed[25]. In brief, every RNA sample was subjected to three rounds of hybridization and the resulting signals were normalized from the average signal of every spot (mean grey) on the matching hybridization image. The mean signal per transcript was used for selections of significantly regulated transcripts. Probe re-sequencing was done with an ABI3100 capillary sequencer (Applied Biosystems, Foster City, USA). Real-time qRT-PCRs of transcripts were done with a Light Cycler (Roche Diagnostics, Manheim, Germany). Transcript normalization was done with the 18S RNA. The primers designed with the Primer3 software (http://frodo.wi.mit.edu) are listed in a Table 2.

Data mining

Our raw data are deposited in the GEO repository under accession number GSE10356. The TIGR Multiexperiment viewer (Tmev version 2.2, http://www.tm4.org) was used for (1) unsupervised hierarchical clustering (UHC) using the Manhattan distance and complete linkage options; (2) supervised analyses such as the *t*-test or ANOVA adjusted with Bonferroni's correction or K-nearest neighbour classification (KNNC) and (3) evaluation of sample re-assignment by a random procedure (jackknife-10^6 iterations). Another, supervised classification was done by Support Vector Machine (SVM) (http://svm.sdsc.edu/). The Gene Ontology Tree Machine (GOTM) program (http://bioinfo.vanderbilt.edu/gotm/) was used to categorize 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://www.ncbi.nlm.nih.gov/sites/entrez) tools. Protein networks were identified with Bibliosphere (www.genomatix.de). Statistics were carried out with the GraphPad Instat software, version 3 (http://www.graphpad.com/).

Table 1 Biological and clinical data from patients with cirrhosis alone, HCC-associated cirrhosis and controls

Patient[1]	Number	Male/Female	Age[2]	Pathology[3]	Etiology[4]	Metavir[5]			
						A0	A1	A2	A3
A1 to A7	7	3/4	48.4 ± 3.2	CIR	ALC	0	4	1	2
A15 to A24	10	7/3	50.4 ± 7.5	CIR	ALC	0	8	2	0
PA1 to PA10	10	8/2	67.1 ± 8.0	CIR + HCC	ALC	2	5	3	0
PA21 to PA29	9	9/0	60.9 ± 8.2	CIR + HCC	ALC	1	7	1	0
V8 to V14	7	6/1	57.4 ± 8.9	CIR	HCV	0	2	4	1
V25 to V34	10	5/5	55.7 ± 16.0	CIR	HCV	0	5	3	2
PV11 to PV20	10	6/4	71.5 ± 5.1	CIR + HCC	HCV	0	1	6	3
PV30 to PV35	6	2/4	66.2 ± 9.5	CIR + HCC	HCV	0	1	3	2
CL1 to CL8	8	3/5	59.9 ± 15.9	No CIR, no HCC[6]	--	7	1	0	0
CL9 to CL18	10	2/8	49.5 ± 14.0	No CIR, no HCC[6]	--	9	1	0	0

[1]HCC-free alcoholic cirrhosis; PA: Peritumoral alcoholic cirrhosis; V: HCC-free HCV cirrhosis; PV: Peritumoral HCV cirrhosis; CL: Control without any detectable fibrosis. Underlined samples were studied by microarray and qRT-PCR; No underlining, independent cohort of samples studied by qRT-PCR only. [2]mean ± SD. [3]CIR: Cirrhosis; CIR + HCC: Peritumoral cirrhosis. [4]HCV: Hepatitis C virus infection; ALC: Alcoholism; --: None. [5]From left to right, number of patients with a given score of inflammation A0-A3. Difference in METAVIR score in HCC-free cirrhosis between all V *vs* A patients, $P = 0.17$ (Mann and Whitney's test); In peritumoral cirrhosis between all PV *vs* PA patients, $P = 0.005$. [6]Histologically normal liver sampled at a distance from a benign liver tumor or from a metastasis of non-hepatic cancer.

Table 2 Oligonucleotides for qRT-PCR

Oligonucleotides	Forward	Reverse	Amplicon size (bp)
ACSM2	AAATCCCGACAAGACAGCAG	CTGATCACAGCCGTCTCAAC	201
GSTA1/2	TCTGCAGAAGATTTGGACAAG	TCAATCAGGGCTCTCTCCTT	170
ADH4	GTCTGCTTGGATGTGGGTTT	TGATTCTGGAAGCTCCTGCT	150
HSCARG	GAAACTGGTGGTGGTTTTCG	CATCTTGGTCTCCCTGCACT	170
AHNAK	CAAAGGGAAACACACCGACT	GCTCTCAGCAGTCAATGCAA	207
HSD17B6	TCTGGGGACTGGTGAACAAT	GTGCTCTCCTCACCAAAGGA	150
APOH	CCGAGGAGGGATGAGAAAGT	AGAATCAGCGCCATTCAGAT	193
IFI27	CCAAGCTTAAGACGGTGAGG	AAAACTACGGCAGAGCCAGA	196
ARID1A	CTACGCTGCCACGTGTGTAT	GTACAGCATCGCACCAAGAG	187
MT1G	TCCTGCAAGTGCAAAGAGTG	ACTTCTCCGATGCCCCTTT	118
ARL2BP	AGGATGAAGTGGCTGGTGAC	GGAAGCTGGCAGAGAAGATG	170
ORM2	TTATATCGCATCGGCCTTTC	CCGCTGGACATTCAGGTAAC	172
ATP5G2	TTGTCTCCACTCCCTCCTTG	TGTGTCGATGTCCCTTGAAA	191
PLG	GTAGGTGGTCCCTGGTGCTA	CCTACAACCCTTCCAGGACA	137
CYP3A4	CTTTGGAAGTGGACCCAGAA	CGGGTTTTTCTGGTTGAAGA	164
STAT3	CCCCATACCTGAAGACCAAG	CTCCGAGGTCAACTCCATGT	185
CYP2E1	TCAAGCCATTTTCCACAGGA	CGATATCCTTTGGGTCAACGA	129
TIMP1	AATTCCGACCTCGTCATCAG	GTTGTGGGACCTGTGGAAGT	195
DPF2	CTCCTGGCTCACTCTTACGG	AAGGGGATTTTGGAGGTAGG	211
18S	GTGGAGCGATTTGTCTGGTT	CGCTGAGCCAGTCAGTGTAG	200
DPM1	GCAGTCCACGACAGAACAAA	CATCTGGGCTTCCATCATCT	150

Immunohistochemistry

D4 zinc and double PHD fingers family 2 (DPF2) and plasminogen (PLG) protein levels were assessed in formaldehyde-fixed, paraffin-embedded, 5 mm thick liver section samples giving a total of 40 other cirrhosis without or with HCC (10 alcoholic and 10 HCV in each group), as well as a set of 10 other histologically normal CLs. This assessment was effected by immunohistochemistry using the ultraView™ Universal DAB Detection Kit following the manufacturers instructions (Ventana Medical systems) with mouse anti-DPF2 IgGs (Abnova corporation) at 1 μg/mL or mouse anti-PLG IgGs (Interchim) at 38 μg/mL.

The percentage of positive cells was evaluated on the whole surface of the histological section and the staining intensity was estimated. Two scores from 0 to 4 were given as two independent visual scores by a trained pathologist and were evaluated using a LEICA DMR microscope equipped with a camera. The number of positive cells or P score was: 0, no positivity; 1, < 25%; 2, 25%-50%; 3, 50%-75% and 4, > 75%-100%. The determination of immunostaining intensity or I score was: 0, no staining; 1, very weak staining only seen at magnification × 10; 2, staining obviously seen at magnification × 10; 3, moderate staining seen at magnification × 2.5; 4, strong staining seen at magnification × 2.5. The IP score was obtained from the additional combination of the two parameters I + P.

RESULTS

Different transcriptome alterations in HCC-free or peritumoral cirrhosis vs CLs

First, with microarray data from 14 HCC-free cirrhosis samples (A1-A7, V8-V14) and eight CLs (CL1-CL8), we identified 30 transcripts

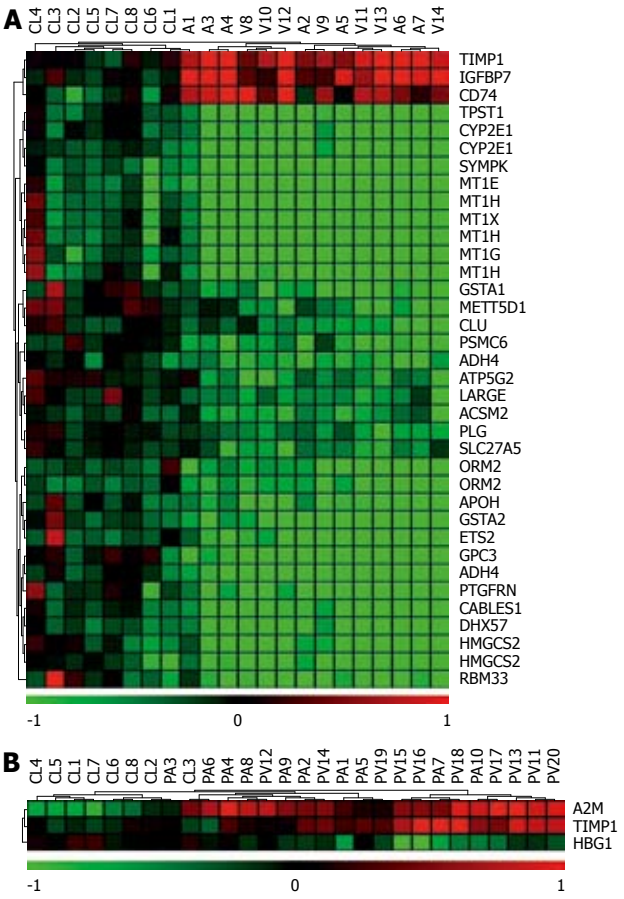

Figure 1 Clustering of cirrhosis from comparisons of transcript levels *vs* CIs. Every transcript level expressed as a [level per patient/median level in CLs] was measured by microarray. The samples are shown as a dendrogram on top and the transcripts are listed vertically. Bottom scale bar (log2 scale): decreased (green), increased (red) or unchanged (black) transcript level. A: UHC was made in 14 HCC-free cirrhosis samples and 8 CLs, from 30 transcript levels first identified as cirrhosis markers in an HCC-free context. B: UHC was made with 20 peritumoral cirrhosis samples and 8 CLs, from 3 transcript levels identified as cirrhosis markers in an HCC context.

whose levels differed between [A + V] cirrhosis *vs* CLs (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 1A). In contrast, similar data from 20 peritumoral cirrhosis samples (PA1-PA10, PV11-PV20) and eight CLs identified only three transcripts, but they did not distinguish [PA-PV] from CLs (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 1B). This HCC-dependent difference in transcript number was significant (30 *vs* 3, Fisher's test, $P < 0.0001$). Furthermore, the expression levels of the 30 transcripts, which distinguished HCC-free cirrhosis, from CLs, did not distinguish [PA-PV] from CLs (data not shown). Thus, we identified transcripts which were dysregulated in HCC-free cirrhosis but not in peritumoral cirrhosis.

Different transcriptome alterations in alcoholic- vs HCV cirrhosis vs CLs

Next, the comparison of transcript levels in alcoholic HCC-free cirrhosis *vs* CLs identified 10 dysregulated transcripts (*t*-test adjusted by Bonferroni's correction, $P < 0.05$). Likewise, we identified 49 dysregulated transcripts in HCV HCC-free cirrhosis *vs* CLs. We also found 33 transcripts that were differentially expressed when directly comparing alcoholic *vs* HCV HCC-free cirrhosis. Overall, we obtained a non-redundant list of 70 transcripts whose levels were able to completely distinguish between the three groups by UHC: alcoholic, HCV HCC-free cirrhosis and CLs (Figure 2A). This was supported by a jackknife procedure (100% success). In contrast, in HCC five transcripts failed to properly distinguish between these three groups (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 2B). This HCC-related difference was significant (70 *vs* 5, Fisher's test, $P < 0.0001$). Furthermore, the expression levels of these 70 transcripts, which separated alcoholic, HCV and CLs in an HCC-free context, did not distinguish between them in an HCC context (data not shown).

Table 3 Performance of various, unsupervised or supervised classification tools for HCC-free cirrhosis samples

Samples[2]	Tool[1]		
	UHC (%)	SVM (%)	KNNC (%)
A1-A7 + A15-A24	65[3]	100	100
V8-V14 + V25-V34	71	80	70
CL1-CL18	100	70	80
All test samples	79	83	83

[1]Unsupervised hierarchical clustering (UHC) was done with 52 cirrhotic or CL samples. Supervised training/testing procedures (KNNC; SVM) were each done by first separating these 52 samples into 22 training (A1-A7, V8-V14 and CL1-CL8) and 30 test samples (A15-A24, V25-V34 and CL9-CL18). [2]20 transcript levels were measured in total RNA from every sample by qRT-PCR. These transcripts had the most significant difference in levels (Bonferroni-corrected ANOVA, $P < 0.05$) between alcoholism *vs* HCV *vs* CLs (ACSM2, ADH4, AHNAK, APOH, ARID1A, ARL2BP, ATP5G2, CYP2E1, CYP3A4, DPF2, DPM1, GSTA 1-2, HSCARG, HSD17B6, IFI27, MT1G, ORM2, PLG, STAT3, TIMP1). [3]% of properly classified test samples.

From our list of 70 transcripts obtained from our microarray data, we measured by real-time qRT-PCR the 20 most discriminant transcripts (as listed in Table 3, footnote 2) which were differentially expressed according to etiology. Using both the 22 training samples (A1-A7, V8-V14 and CL1-CL8) and 30 further independent test samples (A15-A24, V25-V34 and CL9-CL18) in order to classify HCC-free cirrhosis by unsupervised (UHC) or supervised training/testing procedures (KNNC and SVM),we found that these 20 transcripts resulted in a classification accuracy of 79%-83% test samples (Table 3).

Most transcriptome alterations in HCC-free cirrhosis are a transient event

The abnormalities in transcript levels seen in HCC-free cirrhosis, but not in peritumoral cirrhosis, were further evaluated timewise. As shown in Figure 3A (upper left star) the expression levels in CLs ($n = 18$), HCC-free cirrhosis ($n = 34$) and peritumoral cirrhosis ($n = 35$) were measured by qRT-PCR. When comparing the above 20 transcript levels between HCC-free *vs* CLs, their mean level in HCC-free cirrhosis was up-regulated (TIMP1), unchanged (3/20 transcripts, 15%, DPF2, IFI27, STAT3),

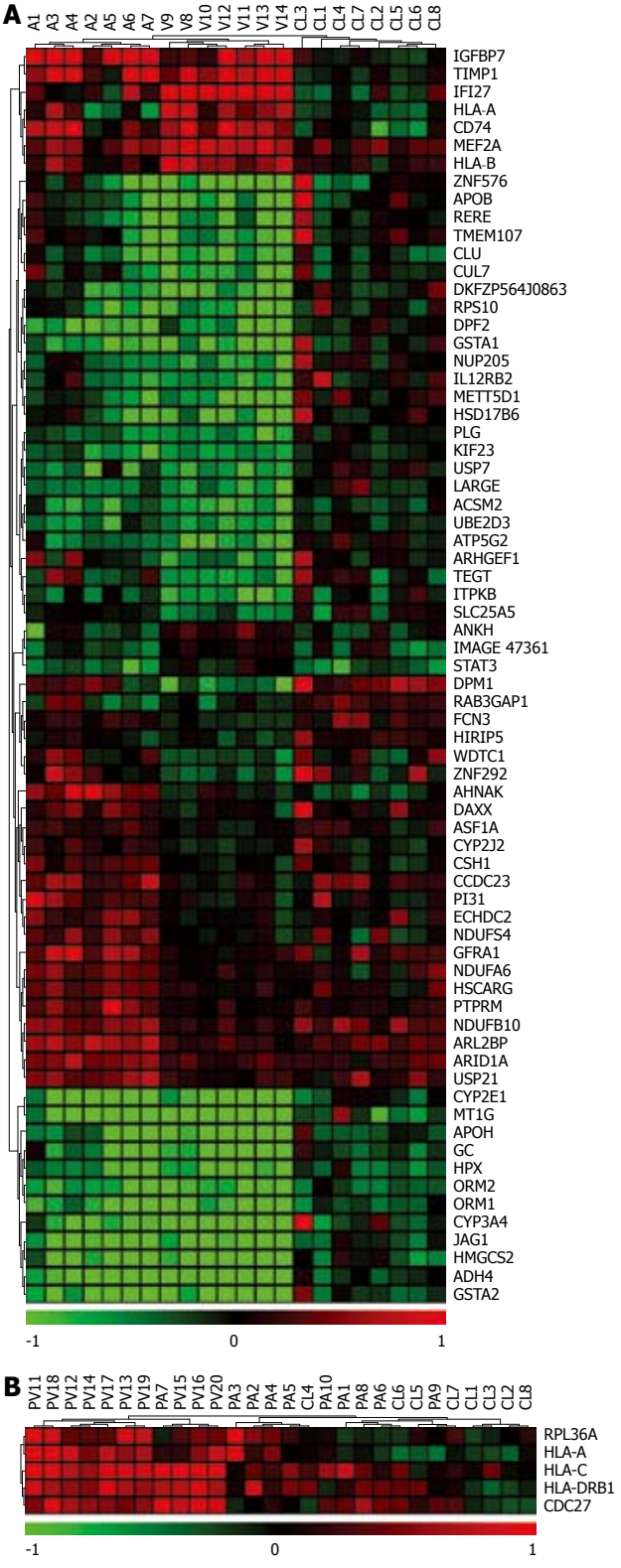

Figure 2 Clustering of cirrhosis samples from transcript levels. Every transcript was measured by microarray and expressed as a [level per patient/median level in CLs]. The samples are shown as a dendrogram on top and the transcripts are listed vertically. Bottom scale bar (log2 scale): decreased (green), increased (red) or unchanged (black) transcript level. A: UHC was made in 14 HCC-free cirrhosis samples and eight CLs, from 70 altered transcript levels first identified as markers of HCC-free cirrhosis. A or V, alcoholism-related or HCV-related etiology B: UHC was made in 20 HCC-associated cirrhosis samples and eight CLs, from five transcript levels identified by *t*-test adjusted by Bonferroni's correction. PA or PV, perinodular cirrhosis, with an alcoholism-related or HCV-related etiology, respectively.

or mostly down-regulated (16/20, 80%). In contrast, this down-regulation was not found when cirrhosis was associated with HCC. Indeed, in peri-tumoral cirrhosis a significant return to the CL level or even an up-regulation was observed (Figure 3A, upper right star). Moreover, as shown in Figure 3B, 9/20 (45%) transcript levels further displayed etiology-dependent differences found (1) only in HCC-free cirrhosis (alcoholism *vs* HCV, lower left star, 6/9 transcripts, 66%), or (2) only in peritumoral cirrhosis (lower right star, 2/9 transcripts, 22%, ARID1A, ORM2), or (3) in both (IFI27). Overall, these transcript dysregulations were mostly seen in HCC-free cirrhosis, often resulted from a transient down-regulation, and half of them were etiology-dependent in agreement with the initial selection.

Semi-quantitative immunodetection of DPF2 and PLG in liver samples

Among the nine genes mentioned above which displayed etiology-dependent differences, DPF2 and PLG, whose antibodies were marketed for immunohistochemistry use, were selected. We quantified their protein levels in a total of 40 other cirrhosis without or with HCC (10 alcoholic and 10 HCV in each group), as well as in a set of 10 other histologically normal CLs.

The DPF2 protein level was significantly higher in HCC-free cirrhosis, and then decreased in HCC-associated cirrhosis to return to a level similar to that observed in CLs, but this level regulation was mild (data not shown).

The PLG protein level was also significantly different in CLs, HCC-free and HCC-associated cirrhosis. Indeed, the PLG level was significantly lower in HCC-free cirrhosis as compared to that observed in CLs and then increased in HCC-associated cirrhosis to return to a level quite similar to that observed in CLs (Figure 4A). The immunohistochemical pattern for CLs with a strong hepatocellular staining was shown in Figure 4D). The PLG protein level also displayed etiology-dependent differences. Indeed, the decrease of the staining was significantly higher in HCC-free HCV cirrhosis (Figure 4E) than in HCC-free alcoholic cirrhosis (Figure 4B) and in HCC-associated cirrhosis the increase with a return to the baseline was the same whatever the etiology (HCV or alcohol) (Figure 4C and F). Thus, the PLG protein level confirmed our results obtained at the transcriptional level.

Functional differences in HCC-free vs peritumoral cirrhosis

We first investigated whether transcript variations could point to functional dysregulation in HCC-free cirrhosis. By comparing our list of 70 transcripts and our *Liverpool*, a different frequency of dysregulated transcripts in eight functional subsets was found (detailed as a Table 4): (1) cell proliferation ($P = 0.01$); (2) regulation of cell migration ($P = 0.009$); (3) blood vessel development ($P = 0.007$); (4) lipid metabolism ($P = 0.007$); (5) antigen processing and presentation

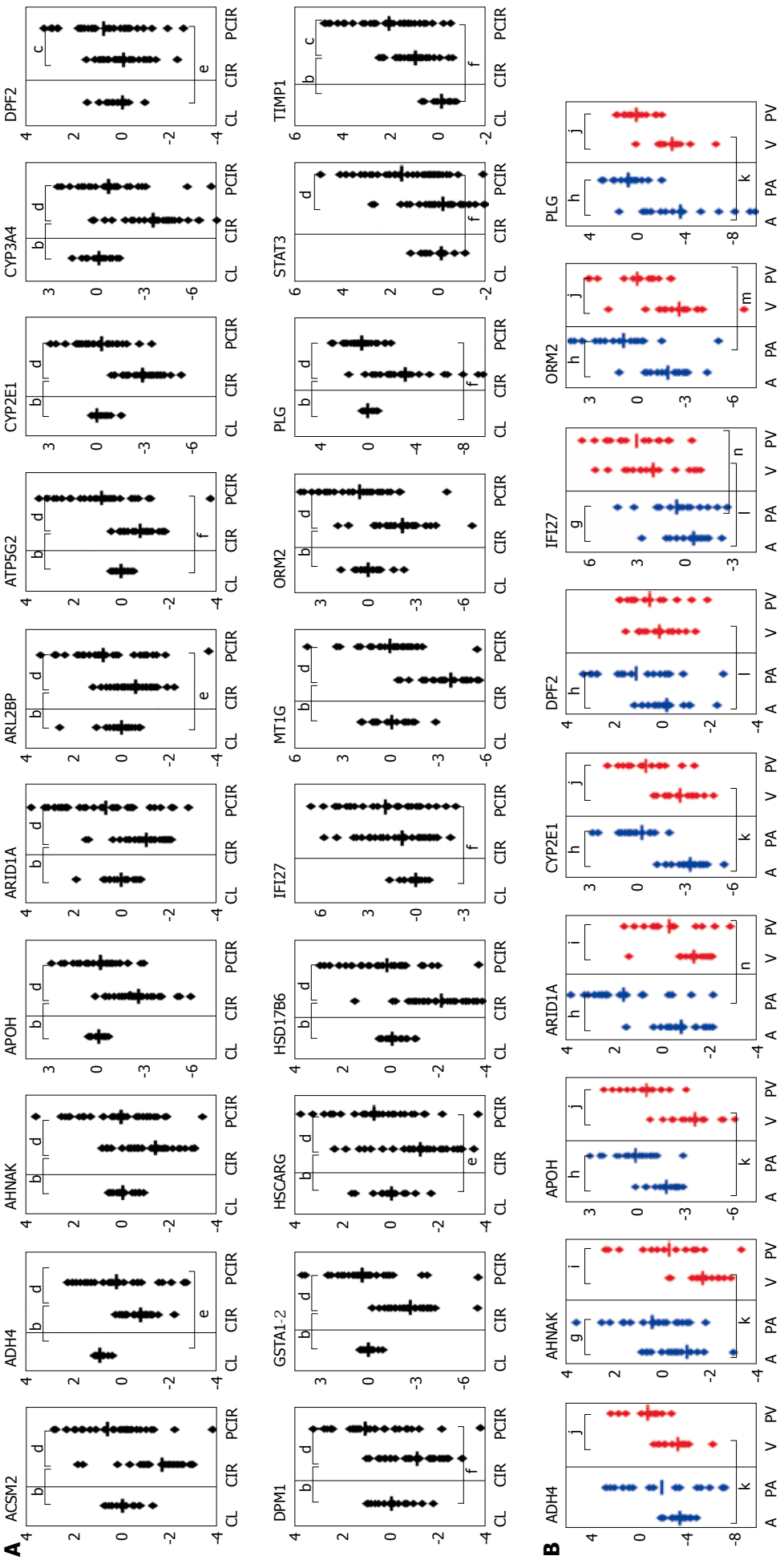

Figure 3 Changes in transcript levels in cirrhosis. The transcripts are those listed in Table 3, footnote 2F. Their levels were determined by real-time qRT-PCR in training and testing samples (18 CLs, 34 HCC-free cirrhosis and 35 peritumoral cirrhosis). Every transcript name is noted on top and its level per cirrhosis type is expressed on the ordinate as a log2 [level in type/median level in CLs]. The mean value of transcripts is shown as an horizontal bar. A : CL: control; CIR: HCC-free cirrhosis (regardless of etiology); PCIR: perinodular cirrhosis (regardless of etiology). Significant difference between CIR and CL ($^{a}P < 0.05$, $^{b}P < 0.01$, Mann-Whitney *U* test), between CIR and PCIR ($^{c}P < 0.05$, $^{d}P < 0.01$) and between PCIR and CL ($^{e}P < 0.05$, $^{f}P < 0.01$). B: Transcripts separated per etiology : alcoholism (blue), HCV (red); A or V: alcoholic or viral, HCC-free cirrhosis; PA or PV: perinodular, alcoholic or viral cirrhosis. Significant difference between A and PA ($^{g}P < 0.05$, $^{h}P < 0.01$), between V and PV ($^{i}P < 0.05$, $^{j}P < 0.01$), between A and V ($^{k}P < 0.05$, $^{l}P < 0.01$) and between PA and PV ($^{m}P < 0.05$, $^{n}P < 0.01$).

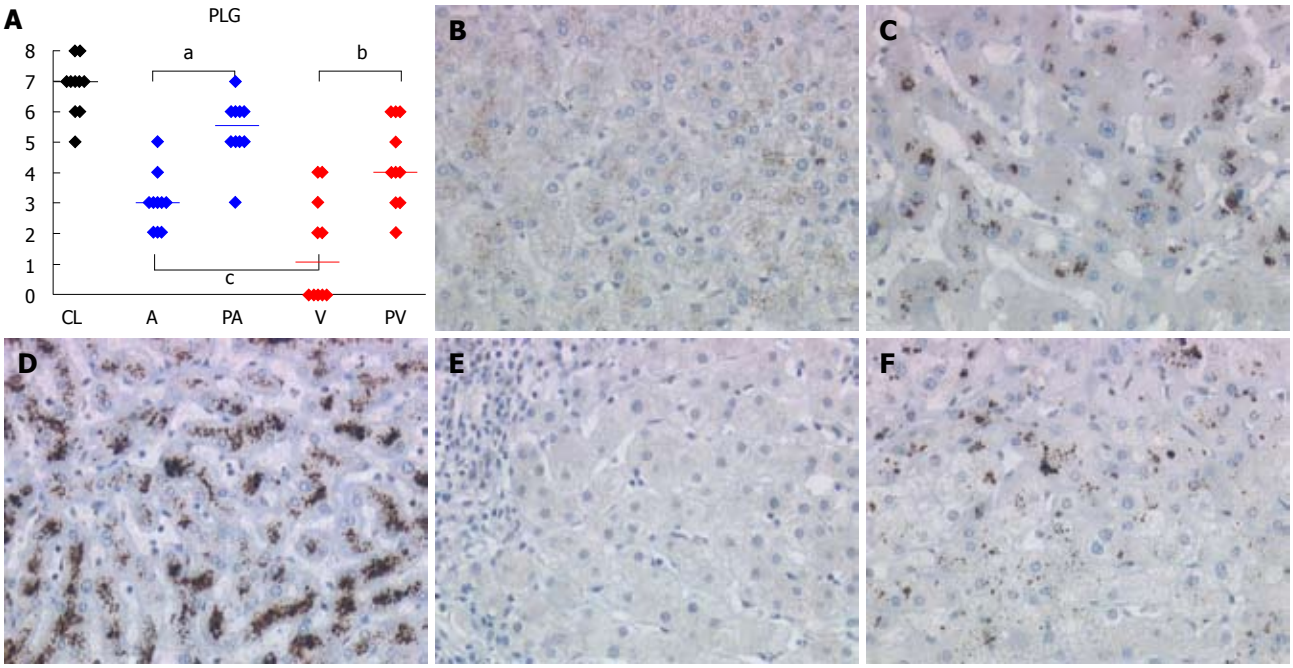

Figure 4 PLG protein expression in control liver, HCC-free and HCC-associated cirrhosis. The PLG protein levels were determined by immunohistochemistry (magnification x 20). A: Every protein level (IP score) per cirrhosis type is expressed on the ordinate. The mean value of protein level is shown as an horizontal bar. The samples abbreviations are the same as for Figure 3. Significant differences between A and PA ($^aP < 0.05$), between V and PV ($^bP < 0.05$), between A and V ($^cP < 0.05$). B-F: PLG immunostaining of hepatic sections corresponding to alcoholic HCC-free cirrhosis (B), alcoholic HCC-associated cirrhosis (C), control liver (D), HCV-related HCC-free cirrhosis (E) and HCV-related HCC-associated cirrhosis (F).

Table 4 Over-representation of functional subsets in our list of 70 transcripts

Cell proliferation[1] (O = 7; E = 2.66; *P* = 0.01[2])	Regulation of cell migration (O = 2; E = 0.15; *P* = 0.009)	Blood vessel development (O = 3; E = 0.41; *P* = 0.007)	Lipid metabolism (O = 9; E = 3.55; *P* = 0.007)	Antigen processing and presentation (O = 3; E = 0.25; *P* = 0.001)	Acute inflammatory response (O = 4; E = 0.58; *P* = 0.002)	NADH dehydrogenase activity (O = 3; E = 0.36; *P* = 0.005)	Oxidoreductase activity (O = 3; E = 0.25; *P* = 0.001)
JAG1 (Hs.590881[3])	JAG1 (Hs.590881)	JAG1 (Hs.590881)	CLU (Hs.436657)	HLA-A (Hs.181224)	CLU (Hs.436657)	NDUFA6 (Hs.274416)	CYP2E1 (Hs.12907)
IGFBP7 (Hs.479808)	PLG (Hs.143436)	PLG (Hs.143436)	CYP2J2 (Hs.152096)	HLA-B (Hs.77961)	ORM1 (Hs. 567311)	NDUFB10 (Hs.513266)	CYP2J2 (Hs.152096)
IL12RB2 (Hs.479347)		CUL7 (Hs.520136)	CYP3A4 (Hs.567254)	CD74 (Hs.591258)	ORM2 (Hs.522356)	NDUFS4 (Hs.528222)	CYP3A4 (Hs.567254)
PLG (Hs.143436)			HMGCS2 (Hs.59889)		STAT3 (Hs.463059)		
TIMP1 (Hs.522632)			APOB (Hs.120759)				
ARHGEF1 (Hs.438429)			HSD17B6 (Hs.524513)				
CD74 (Hs.591258)			DPM1 (Hs.301898)				
			LARGE (Hs.474667)				
			CD74 (Hs.591258)				

[1]This transcript subset coding for proteins with a related function was identified by gene ontology with the GOTM tool; [2]Significance of enrichment for the GO category between transcript number observed (O) and expected (E) in this cate gory; [3]Hs. number: Unique transcript identifier.

($P = 0.001$); (6) acute inflammatory response ($P = 0.002$); (7) NADH dehydrogenase activity ($P = 0.005$) and (8) oxidoreductase activity ($P = 0.001$).

Within our set of 70 transcripts, 23 transcripts with available information from Bibliosphere exhibited an etiology-associated level variation in HCC-free cirrhosis (Figure 5), but not in peritumoral cirrhosis (data not shown). This resulted from a variable extent of down-regulation (17/23 transcripts, 74%) in a single etiology or both. In turn, this down-regulation resulted, at least partly, from variable, etiology-dependent regulation of STAT-3 and its target genes (lower right area of Figure 5).

DISCUSSION

Our search first focused on the significance of

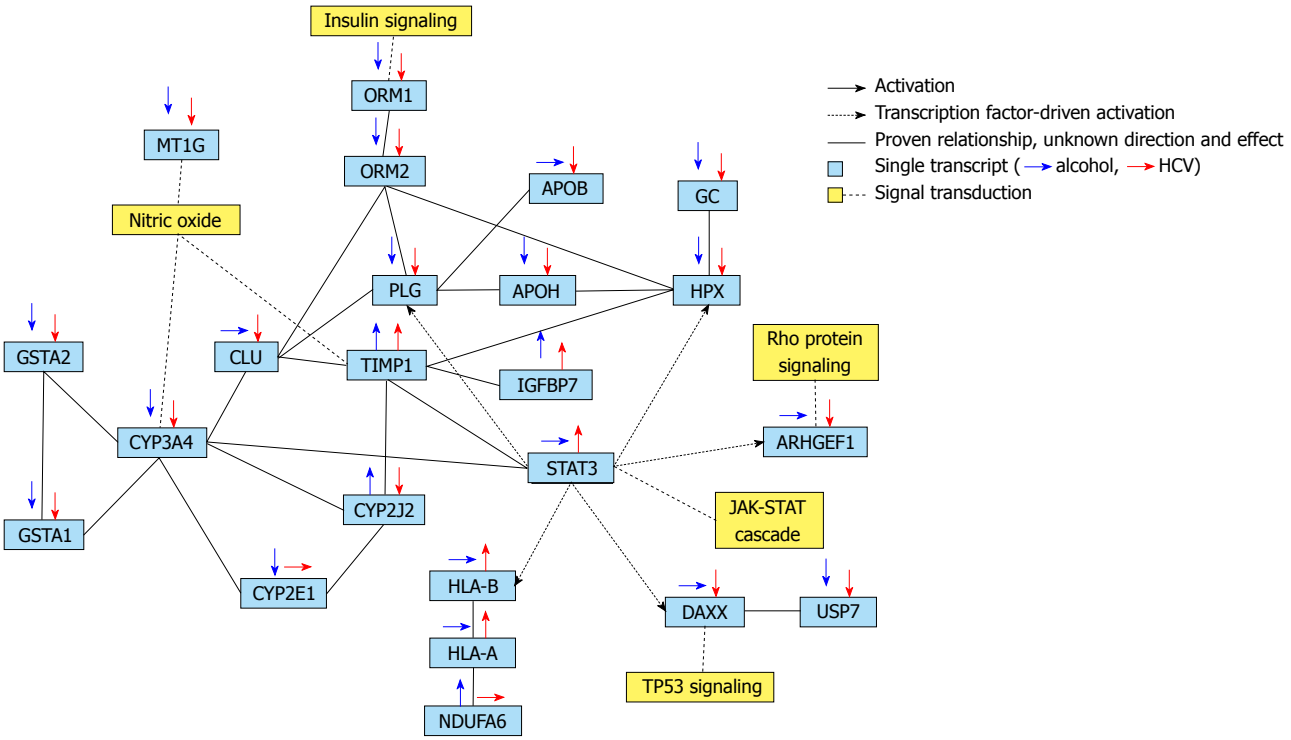

Figure 5 Networks of etiology-independent or dependent transcript regulations in HCC-free cirrhosis. From 70 transcripts with a significantly different expression between alcoholic-associated *vs* HCV-associated cirrhosis *vs* CLs ($P < 0.05$, as in Figure 3), 23 transcripts (boxes) associated with different pathways were identified in Bibliosphere. In a context of HCC-free cirrhosis, etiology-specific arrows (alcoholism, blue; HCV, red) above the transcript indicates the direction of regulation: upward, downward or horizontal arrow: up-regulation, down-regulation or unchanged regulation. For a transcript with an up- (down-) regulation in both etiologies, the highest (lowest) arrow indicates the most marked dysregulation.

dysregulations found in HCC-free cirrhosis. We investigated the influence of an HCC-free *vs* peritumoral environment of cirrhosis and we searched for early markers of HCC onset. The ideal study design would be to compare cirrhosis that develops into HCC and cirrhosis that does not develop into HCC in the follow-up of the disease, but such samples were very scarce because the follow-up among cirrhotic patients would be extremely long and difficult. So, we compared HCC-free and HCC-associated cirrhosis and we used a stringent selection of informative transcripts because sharp, timewise variations of potential markers of HCC occurrence were to be identified. We have shown that transcription dysregulation does exist in HCC-free cirrhosis. This is observed before any histologically detectable HCC nodule is seen, and hence supports the "field cancerization" model. Specifically, from our GOTM results, it seems plausible that malignant transformation of cirrhosis could be favored by abnormal expression of factors regulating cell migration, cell proliferation and blood vessel development. These dysregulations in cirrhosis mainly correspond to down-regulations, and they usually re-normalize or are even up-regulated in peritumoral cirrhosis. It remains to be determined how such transient down-regulations, which have been as yet unreported, contribute to HCC initiation.

We next carried out a similar search, further integrating alcoholism and HCV etiologies. A few, etiology-dependent markers for a high *vs* low risk of HCC development have been previously identified by others, but they relied on an unproven assignment of alcoholism or HCV patients to either risk group and they could not predict HCC occurrence in a timewise fashion[12]. We had previously reported that abnormalities of some transcript levels are observed to a different extent in HCC developed on alcoholic-associated cirrhosis *vs* HCV-associated cirrhosis, whereas they remain similar in peritumoral cirrhosis, thus indicating that these abnormalities are etiology-related in HCC tumors only[24]. In the same way, in the present study, we found transcript dysregulation only in HCC-free cirrhosis and not in peritumoral cirrhosis. We now document that histochemical evaluation of the PLG protein level confirms our results obtained at the transcriptional level. In contrast, for DPF2, dysregulation observed at both transcript and protein levels were in opposite directions, but discrepancies between transcript levels and protein levels have been previously noticed[26,27]. As markers of HCC occurrence are still very scarce[4,28], our observation on transcripts and proteins is of strong interest in early HCC diagnosis. This will need to be further evaluated in HCC-free/cirrhosis-free, fibrotic livers, with selection of marker combinations.

Some up- or down-regulations of transcription factors in HCC have already been documented[29], but the facts that dysregulation of STAT-3 and a related gene network take place in HCC-free cirrhosis, and in an etiology-dependent fashion, are novel findings. The JAK-STAT pathway is critical in the proinflammatory

cytokine-driven inflammatory response provided by hepatocytes[30] and it is tempting to speculate that a weakening of this defense in early cirrhosis may participate in HCC development. However, this mechanism appears unlikely. Indeed, our data were obtained by comparison of alcoholic *vs* HCV cirrhosis samples whose extent of inflammation was similar and still had different STAT-3 regulation. The HCV has a clear effect on the activity of STAT-3, but the meaning of this is controversial. Some studies show inhibition of STAT-3 activity[31] while others show activation of STAT-3[32-34]. Our data are in keeping with documented HCV proteins/STAT-3 interferences and STAT-3 activation in HCV-induced liver disease. STAT-3 directly affects cell proliferation, cell differentiation[35] and angiogenesis[32]. Moreover, STAT-3 and its targets are regulated in some cancers, such as breast and prostate cancer[34]. Thus, the dysregulation of the STAT-3 pathway which follows HCV infection may participate in HCC development at an early stage of hepatocyte dysplasia. In addition, recent reports have highlighted the potential of STAT-3 as a therapeutic target in different neoplasms[36,37].

In conclusion, our data point to major transcription dysregulations in HCC-free cirrhosis. These dysregulations often result from a transient dysregulation, and half are etiology-dependent. Our observations open new avenues for the follow-up of HCC-free cirrhosis because dysregulated transcripts or proteins may be appear like markers for the cirrhosis to HCC transition. In order to complement these results, studies performed at an earlier state before cirrhosis, i.e. on fibrosis samples are now under investigation.

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COMMENTS

Background

Chronic viral hepatitis C (HCV) infection and alcoholism are two important causes of cirrhosis and hepatocellular carcinoma (HCC). Liver transcriptome analysis has resulted in the identification of genes with an aberrant expression according to different pathophysiological states. In the present work, we investigated whether some transcription dysregulations could be found in HCC-free cirrhosis in an etiology-dependent fashion. Furthermore, we searched and found transcription dysregulations that differentiate HCC-free cirrhosis from peritumoral cirrhosis.

Research frontiers

Numerous, genome-wide analyses of abnormal gene expression in HCC as compared to normal, control liver, have resulted in identification of gene sets with altered expression. Few similar studies have been done in HCC-free cirrhosis. In fact, viral etiologies have often been considered whereas abnormal gene expression in alcoholism-dependent HCC has received very little attention. Therefore, the impact of etiology still remains an important issue. We recently reported that a number of genome-wide abnormalities in alcoholism-associated *vs* HCV-associated HCC are etiology-dependent and some of them are of pathological relevance. Remarkably, the abnormal transcript levels that differentiate HCC nodules in an alcoholism-dependent *vs* HCV-dependent HCC can no longer discriminate between the two etiologies when transcripts are measured in the surrounding cirrhosis. Yet, any etiology-dependent abnormalities that could be observed in HCC-free cirrhosis would be of interest.

Applications

These data point to major transient transcription dysregulations in HCC-free cirrhosis. These observations open new avenues for the follow-up of HCC-free cirrhosis because dysregulated transcripts or proteins may be appear like markers for the cirrhosis to HCC transition.

Peer review

The aims of the study were to identify genes that were differentially expressed between HCC-free and HCC-related cirrhosis. The differentially expressed genes were further investigated to see if they were associated with alcoholism and HCV etiologies. The authors suggested that genes that were deregulated in HCC-free cirrhosis might serve as markers for the cirrhosis to HCC transition.

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