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RAPID COMMUNICATION

Liver *insulin-like growth factor 2* methylation in hepatitis C virus cirrhosis and further occurrence of hepatocellular carcinoma

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Abstract

AIM: To assess the predictive value of the insulin-like growth factor 2 (*Igf2*) methylation profile for the occurrence of Hepatocellular Carcinoma (HCC) in hepatitis C (HCV) cirrhosis.

METHODS: Patients with: (1) biopsy-proven compensated HCV cirrhosis; (2) available baseline frozen liver sample; (3) absence of detectable HCC; (4) regular screening for HCC; (5) informed consent for genetic analysis were studied. After DNA extraction from liver samples and bisulfite treatment, unbiased PCR and DHPLC analysis were performed for methylation analysis at the *Igf2* locus. The predictive value of the *Igf2* methylation profile for HCC was assessed by Kaplan-Meier and Cox methods.

RESULTS: Among 94 included patients, 20 developed an HCC during follow-up (6.9 ± 3.2 years). The methylation profile was hypomethylated, intermediate and hypermethylated in 13, 64 and 17 cases, respectively. In univariate analysis, two baseline parameters were associated with the occurrence of HCC: age (P = 0.01) and prothrombin (P = 0.04). The test of linear tendency between the three ordered levels of *Igf2* methylation and probability of HCC occurrence was significant (Log Rank, P = 0.043; Breslow, P = 0.037; Tarone-Ware, P = 0.039).

CONCLUSION: These results suggest that hypomethylation at the *Igf2* locus in the liver could be predictive for HCC occurrence in HCV cirrhosis.

Key words: Liver cancer; Cirrhosis; Insulin-growth factor 2, DNA methylation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignant neoplasms worldwide[1], and its incidence has increased in the past decade in Europe and the USA[2-5]. In Western countries, HCC mostly develops in the presence of cirrhosis. Chronic Hepatitis C virus (HCV) infection plays an important role in the increased incidence of HCC in the western world[6] where HCC is presently the leading cause of death

of patients with HCV related cirrhosis[7]. In these patients, the annual incidence of HCC varies from 2% to 6%[8-13]. The main predictive factors of HCC in patients with HCV-cirrhosis are age over 50[14-17], male gender[12,14,15,17], increased serum alpha-fetoprotein (AFP) baseline levels[12,18,19], symptoms of portal hypertension, such as thrombopenia[17,18] or esophageal varices[17,19], obesity[20,21] and diabetes[22,23]. Identification of molecular abnormalities associated with an increased risk of HCC is particularly important to improve knowledge of both the pathways of liver carcinogenesis and the outcomes.

Insulin-like growth factor 2 (IGF2) is a fetal growth peptide produced by the liver which is structurally and functionally closely related to insulin[24]. It is over expressed in a wide variety of neoplasms[25,26] and is involved in experimental liver carcinogenesis. *In vitro*, a pathophysiological link between IGF2 over expression and hepatocyte proliferation was demonstrated by Lin *et al*[27], who found high concentrations of IGF2 in human hepatoma cell lines HuH7 and HepG2 and showed that antisense oligonucleotides complementary to *Igf2* mRNA reduced both *Igf2* mRNA and protein, in association with decreased cell proliferative activity. *In vivo*, Rogler *et al*[28] reported an increased frequency of HCC in *Igf2* transgenic mice and serum IGF2 has been recently proposed as a marker for human HCC to improve the diagnostic accuracy and sensitivity in patients with low serum AFP level[29].

Various epigenetic alterations have been reported in human cancers, including global DNA hypomethylation, gene hypomethylation and promoter hypermethylation, and *Igf2* loss of imprinting[30]. The *Igf2* gene is controlled by genomic imprinting, a non-Mendelian inherited epigenetic process that leads to the silencing of either a maternal or paternal allele[31,32]. In the liver, unlike in other tissues, its expression is monoallelic (maternally imprinted) during the fetal period and becomes biallelic thereafter. Early observations showed over expression of the *Igf2* gene in liver tumors and preneoplastic hepatic foci in different animal models as well as in human HCC[33,34]. This over expression is associated with re-expression of the fetal pattern of *Igf2* transcripts and restoration of monoallelic *Igf2* expression in preneoplastic hepatic foci[35] as well as in HCC[36], and with re-expression of monoallelic fetal promoters P2-P4[37] and loss of activity of the adult biallelic promoter P1[38]. One key factor of these epigenetic changes is the alteration of the genomic methylation pattern within regulatory Differentially Methylated Regions (DMRs) of imprinted genes, which inappropriately leads to loss of imprinting in the *Igf2* gene[39] and to transcriptional activation of the normally silent maternal allele. Hypomethylation at the *Igf2* locus has been found in many type of cancers, including ovarian, lung and colon[40]. In a previous study analyzing the methylation status of *Igf2* DMR2 in 71 liver samples from mostly viral HCC compared to 6 normal liver samples, we observed a hypomethylated profile at the *Igf2* locus in 89% of cases of HCC in contrast with the pattern observed in normal livers[41]. In addition, Cui *et al*[42] showed that hypomethylation of the *Igf2* gene in peripheral blood lymphocytes (PBL) is associated with a predisposition to colorectal cancer, suggesting that the epigenetic alteration of *Igf2* could be an early event in colorectal carcinogenesis.

The aim of the present study was to investigate whether hypomethylation at the *Igf2* locus in the liver is a predisposing factor for HCC in patients with HCV-related cirrhosis. Thus, we analyzed the methylation status of the *Igf2* gene spanning the 11p15 imprinted domain in patients with compensated HCV-related cirrhosis who were prospectively followed-up with periodic HCC screening.

MATERIALS AND METHODS

Patients

Ninety-four patients were retrospectively selected for this study among all patients hospitalized for liver biopsy between January, 1989 and December, 2000 in our department, based on the following criteria: (1) compensated (Child-Pugh A) HCV-related cirrhosis with presence of serum HCV RNA; (2) absence of viral co-infection by hepatitis B virus or human immunodeficiency virus; (3) regular follow-up until death with periodic HCC screening by liver ultrasonography and test of serum AFP levels every 6 mo at least; (4) absence of detectable HCC at enrollment; (5) available baseline frozen (-80℃) liver biopsy specimen for genetic study; (6) informed consent for genetic analysis obtained from the patient according to French guidelines.

Baseline demographic, clinical, biological and histological data (at time of liver biopsy) were recorded. All patients were prospectively followed-up. Complete physical examination, standard biochemical tests, serum AFP determination and abdominal ultrasonography were repeated every 6 mo. When a focal liver lesion or increased AFP levels were detected, tomodensitometry and, whenever possible, fine needle guided liver biopsy were performed. Diagnostic criteria for HCC were: (1) histological and (2) clinical, in patients with AFP value greater than 400 ng/mL and evidence of focal liver lesion at imaging techniques. After 2002, the HCC diagnosis was based on the guidelines of the European Association for the Study of the Liver[43].

Twenty-five histopathologically normal liver samples were also studied as control cases.

DNA extraction, bisulfite treatment of DNA and methylation analysis

DNA from frozen liver biopsies was extracted and treated with sodium bisulfite. Unbiased PCR amplification and Denaturing High Performance Liquid Chromatography (DHPLC) analysis were

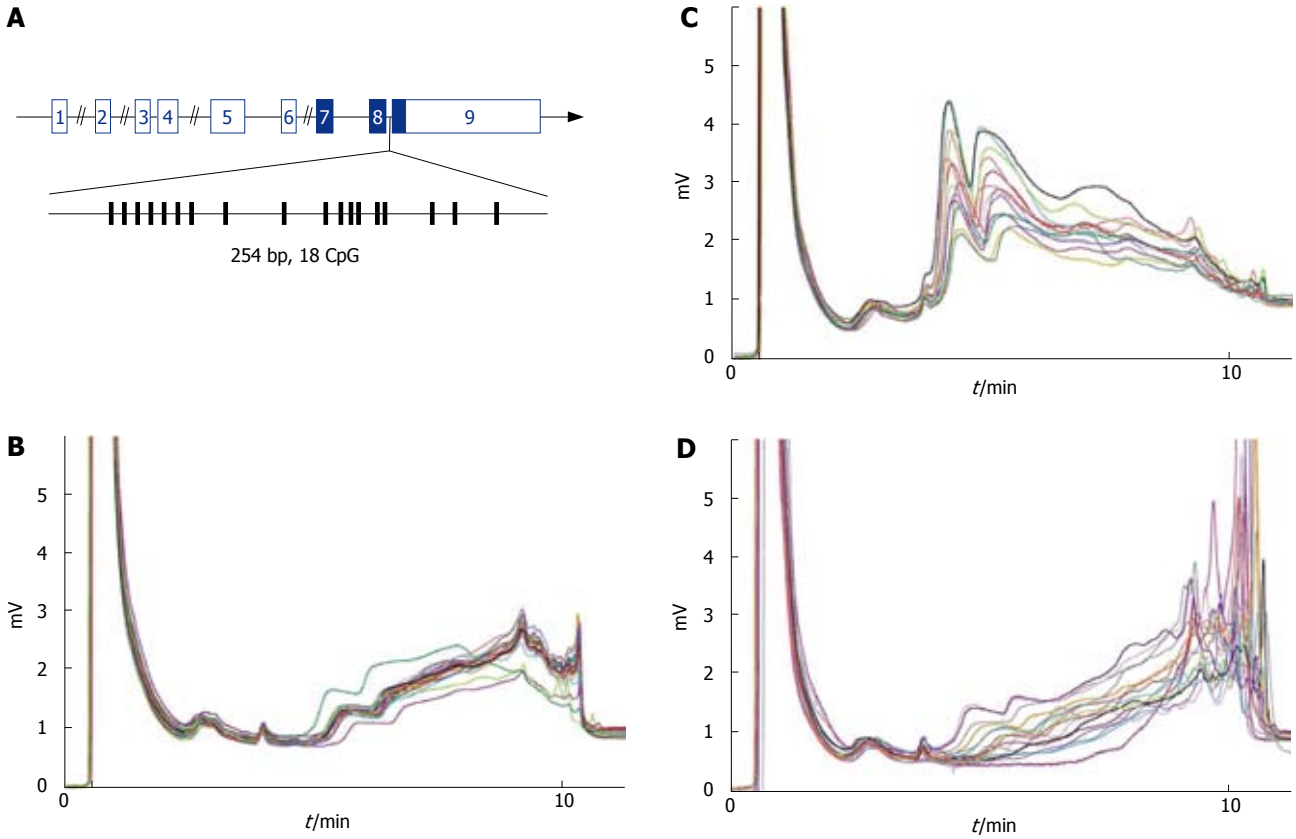

Figure 1 Methylation analysis of *Igf2* DMR2 in normal and HCV related cirrhosis livers. **A**: Exon-intron structure of *Igf2* gene. Exons are shown as numbered boxes (plain are coding). The 254bp fragment of *Igf2* DMR2 amplified for methylation analysis is enlarged below. Vertical lines indicate CpG positions. **B**: DHLPLC chromatograms of PCR products from normal liver samples. Twenty-two out of 25 are superimposable, and this major profile was used to assess hypermethylated profiles (ie more methylated than normal liver). **C** and **D**: DHPLC chromatograms of PCR products from HCV-related cirrhosis. Among 94 samples, 13 (**C**) and 17 (**D**) samples show respectively hypomethylated and hypermethylated profiles.

used for methylation analysis at the DMR of exon 8 and 9 of the *Igf2* imprinted gene (Figure 1A), as previously reported[42]. We amplified a DNA fragment encompassing 18 CpGs of the *Igf2* gene (Accession number AC005809; nt 43058-43312; 254 bp) by nested-PCR using the following primers: forward external 5'-GTTAAGAGGTTTTAGAGGTTATAGG-3', reverse external 5'-CCTTCCAAAACCTAACCTAAAAACA-3', forward internal 5'-GGGAAAGGGGTTTAGGAT-TTTTAT-3', reverse internal 5'-ATAATTTACTCCCCC-TTCAACCTC-3'. PCRs were performed in 3 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.5 μmol/L of each primer and 1.25 U of AmpliTaq Gold® DNA polymerase (Perkin Elmer, Norwalk, CT) under the following conditions: 94℃ for 10 min followed by 40 cycles of 94℃ for 45 s, 62℃ for 45 s, 72℃ for 1 min and a final extension step of 10 min at 72℃. After the first round of DNA amplification, a 1 μL aliquot of the PCR solution was used for the nested PCR.

Methylation profiles were studied by a newly developed DHPLC-based method, as previously described[44]. Briefly, DHPLC scanning was performed on an automated DHPLC instrument (WAVE®, Hitachi model D-7000. Chromatography Data Station Software, Transgenomic LTD Cheshire, UK); the column used was a DNAsep® Cartridge (Transgenomic, Santa Clara, CA) and the running temperature experimentally evaluated was 57℃. In a second step, methylation profiles of PCR products from liver biopsies were analyzed in comparison with PCR products from reference 100% methylated and 0% methylated control alleles, and with PCR product from normal liver biopsies. Fifteen microliter aliquots of the PCR products were eluted within a linear acetonitrile gradient. Because of the difference in retention times, the methylation patterns could be assessed by DHPLC independently of sequencing information by overlaying the DHPLC profiles with those of reference fragments. Methylation profiles were objectively classified in three categories as follows: samples displaying a higher proportion of methylated alleles than normal liver were considered as hypermethylated (M); samples which showed less methylation than normal liver were sorted according to the maximal absorbances of their first (4 min < retention time < 5 min, demethylated alleles) and last (10 min < retention time < 11 min, methylated alleles) elution specific peaks, by calculating R = [Abs (first peak) - Abs (baseline)]/[Abs (last peaks) - Abs (baseline)]; samples with R>2 (high proportion of demethylated alleles) were considered hypomethylated (U), the others being intermediate (UM). Reproducibility of the method was checked by double testing of randomly chosen

Table 1 Baseline characteristics in 94 patients with Child-Pugh A hepatitis C-related cirrhosis and defined methylation profile at the *insulin growth factor 2* gene: distribution and prognostic value for the occurrence of hepatocellular carcinoma at 10 years in univariate analysis

		Patients (n = 94)	No HCC (n = 74)	HCC (n = 20)	HR (95% CI)	P
Gender	Female	39	32	7	1	
	Male	55	42	13	1.38 (0.55-3.48)	0.49
Age (yr)		57.7 ± 13.7	56.0 ± 14.4	63.9 ± 8.0	1.05 (1.01-1.09)	0.01
BMI (kg/m^2)		25.0 ± 4.8	25.1 ± 4.8	24.7 ± 4.8	1.00 (0.90-1.11)	0.96
BMI ⩾ 30 kg/m^2	No	80	64	16	1	
	Yes	14	10	4	2.16 (0.72-6.51)	0.17
Diabetes	No	73	57	16	1	
	Yes	21	17	4	1.00 (0.33-2.98)	0.99
Oesophageal	0 or Ⅰ	78	63	15	1	
Varices grade	Ⅱ or Ⅲ	16	11	5	2.47 (0.89-6.83)	0.08
Bilirubin (μmol/L)		15.1 ± 8.8	14.6 ± 8.3	17.2 ± 10.4	1.05 (1.00-1.10)	0.05
Albumin (g/L)		41.1 ± 5.7	41.1 ± 5.6	41.0 ± 6.3	0.98 (0.90-1.06)	0.59
Prothrombin (%)		84.1 ± 16.2	85.2 ± 15.9	79.7 ± 16.9	0.97 (0.95-1.00)	0.04
Platelets (× 10^3/mm^3)		151 ± 61	155 ± 64	138 ± 51	1.00 (0.99-1.00)	0.29
ALAT (× ULN)		3.2 ± 2.5	3.2 ± 2.6	3.2 ± 1.8	0.97 (0.80-1.19)	0.80
ASAT (× ULN)		2.9 ± 2.1	2.8 ± 2.0	3.2 ± 2.5	1.10 (0.91-1.33)	0.33
GGT (× ULN)		2.8 ± 2.7	2.8 ± 2.9	2.7 ± 1.7	0.99 (0.84-1.16)	0.86
AFP (ng/mL)		16.4 ± 26.9	17.2 ± 29.2	13.3 ± 15.7	1.01 (0.99-1.02)	0.54
Knodell score		10.8 ± 2.2	10.6 ± 2.4	11.7 ± 2.4	1.23 (1.00-1.51)	0.06
Serum IGF2 (ng/mL)[1]		279.6 ± 114.3	291.4 ± 117.3	234.1 ± 92.1	0.99 (0.98-1.01)	0.08
Liver Igf2	U	13	9	4	7.64 (0.85-68.62)	0.07
Methylation profile	UM	64	49	15	3.98 (0.53-30.14)	0.18
(3 classes)	M	17	16	1	1	

Continuous values are used for quantitative parameters. HR: Hazard ratio; CI: Confidence interval; HCC: Hepatocellular carcinoma; BMI: Body mass index; ALAT: Alanine amino-transferase; GGT: Gamma glutamyl transferase; ULN: Upper limit of normal; AFP: Alpha-foetoprotein; IGF2: Insulin-growth factor 2. [1]Performed in 63 patients.

samples. To rule out interpretation bias, clinical database including outcome of the patients, especially in relation to the occurrence of HCC, was kept by clinicians (NG, PN, JCT, MB) and not available for molecular biologists (PC, AK, AM, JC).

IGF2 serum quantification

Frozen serum collected at enrollment and stored at -25℃ was available in 63 (67%) of the 94 included patients. In these patients, serum IGF2 was quantified using an enzymatic amplified "two step" sandwich-type immunoassay (active IGF2 ELISA, Diagnostic Systems Laboratories, Webster, USA). Each sample was duplicated and tested blindly.

Statistical analysis

Data were expressed as mean ± SEM and percentages. All means were compared using the Mann-Whitney rank-sum test or the Kruskal-Wallis nonparametric analysis of variance. Furthermore, continuous variables were transformed into binary information according to median and cut-off points. Associations were tested in 2 × 2 cross tabulations using the Fisher's exact test. In case of larger cross tabulations, and as appropriate according to the validity conditions, liaisons were tested by the Pearson's Chi-square, or by computing either the exact probability value or the Monte Carlo estimate of the exact probability value. The basic non parametric Kaplan-Meier method[45] was used to search for heterogeneity of time-dependent cumulative probabilities of HCC according to levels of methylation and a linear trend between HCC probability and ordered methylation levels. From then on and practically, we used a series of tests, the Log Rank (Mantel-Cox) test, the Breslow test (Generalized Wilcoxon), and Tarone-Ware test in the two situations in which we attempted to test the heterogeneity of HCC occurrence or a linear trend between HCC probability and ordered methylation levels[46]. As regards to heterogeneity of risk according to *Igf2* levels, the Cox regression[47] was used for the estimation of the Hazard Ratios and 95% CI intervals. The 0.05 probability level was used for all statistical significance. Statistical analyses were performed using SPSS software (SPSS 10.05, SPSS Inc., Chicago, IL) and STATXACT (StatXact, CYTEL Software Corporation, Cambridge, MA).

In Table 1, the expression HR = 1.05 (1.01-1.09) is linguistically awkward, henceforth, 1.05 indicates that with each extra year in age the estimated hazard is 1.05 times that for subjects one year younger. Another way to express this variation is to convert it into a percentage difference in hazard by using the expression 100 × (HR - 1). Then, 100 × (1.05 - 1) = 5% tells us that the HR of HCC is 5% higher for each additional year of age.

RESULTS

Characterization and interpretation of methylation profiles

Methylation profiles of normal liver samples were highly

Table 2 Baseline characteristics according to the methylation profile at the *Igf2* locus (U, UM, and M, respectively, for hypomethylated, normal and hypermethylated patterns) in patients with Child-Pugh A hepatitis C-related cirrhosis

	U (*n* = 13)	UM (*n* = 64)	M (*n* = 17)	Asymptotic global *P*-value
Male gender (%)	7 (53.8%)	38 (59.4%)	10 (58.8%)	0.934
Age (yr)	58.15 (16.71)	57.55 (13.00)	57.89 (14.41)	0.985
Alcohol (g/d)	43.08 (64.21)	22.28 (45.97)	38.24 (77.48)	0.551
Tobacco (Pack, yr)	3.8 (7.1)	6.1 (13.1)	5.7 (11.5)	0.981
BMI (kg/m^2)	26.32 (5.18)	25.41 (4.97)	23.86 (4.04)	0.244
Diabetes (%)	2 (15.4%)	14 (21.9%)	4 (23.5%)	0.883
Platelets (× 10^3/mm^3)	147.28 (64.46)	152.32 (63.71)	177.30 (127.43)	0.958
Prothrombin (%)	79.46 (17.81)	84.58 (16.59)	85.59 (13.28)	0.433
Albumin (g/L)	40.45 (4.96)	40.77 (6.27)	42.76 (3.40)	0.437
Bilirubin (μmol/L)	14.58 (9.66)	15.16 (8.85)	15.50 (8.45)	0.731
ALAT (× ULN)	3.23 (2.13)	3.23 (2.61)	3.14 (2.16)	0.961
AFP (ng/mL)	13.58 (11.24)	13.88 (18.57)	28.21 (51.52)	0.530
Serum IGF2 (ng/mL)[1]	249.64 (81.23)	276.32 (116.97)	321.55 (129.24)	0.393
OV grade Ⅱ or Ⅲ (%)	5 (38.5%)	9 (14.1%)	2 (11.8%)	0.084
Knodell score (mean, SD)	11.6 (1.8)	10.6 (2.3)	10.7 (2.5)	0.414
HCV genotype 1[2] (%)	7 (63.6%)	41 (78.8%)	8 (53.3%)	0.125

Quantitative variables are expressed as means (SD). BMI: Body mass index; ALAT: Alanine amino-transferase; ULN: Upper limit of normal; AFP: Alpha-foetoprotein; IGF2: Insulin growth factor 2; OV: Esophageal varices; HCV: hepatitis C virus. [1]Performed in 63 patients, [2]known in 78 patients (11, 52, 15 patients respectively in U, UM and M groups).

similar (Figure 1B) and 22 of 25 were superimposable. Among 94 tested patients, 13 (14%) were considered as hypomethylated (U), 64 (68%) as intermediate (UM) and 17 (18%) as hypermethylated (M) (Figure 1C). All double tested samples showed similar results in both experiments (data not shown).

Baseline patient characteristics

The main characteristics of patients at enrollment were not significantly different according to the methylation profile at the *Igf2* locus as shown in Table 2. All patients but 2 were Caucasians (1 from Africa with M profile and 1 from Asia with UM profile). In addition, the proportion of patients who received antiviral treatment during the study (72.7% in U, 58.8% in UM and 61.7% in M; $P = 0.738$), the proportion of sustained responders (22.2% in U, 31.4% in UM and 40.0% in M; $P = 0.707$), the mean follow-up (5.20 ± 3.63 years in U, 7.21 ± 3.09 years in UM, 7.22 ± 3.04 years in M; $P = 0.198$), were not statistically different between the three groups.

IGF2 serum quantification

Each IGF2 serum level measurement was duplicated and results were reproducible in 98% of cases. The mean serum value was 279.6 ng/mL (range, 36-640) without any significant difference between patients with U, UM or M methylation profiles at the *Igf2* locus (Table 2).

Predictive value for HCC

During a mean follow-up of 6.9 ± 3.2 years, 20 patients developed an HCC (4, 15 and 1 cases, in patients with U, UM and M methylation profile, respectively). The cumulative incidence of HCC at 10 years reaches 30.8% in patients with a U profile, 23.4% in patients with a UM profile, and 24.7% in patients with either a U or a UM profile in contrast with 5.9% only in patients with a M profile.

In the Cox analysis testing successively each of the 17 baseline studied variables, two were predictive for the occurrence of HCC: age at liver biopsy ($P = 0.01$; HR, 1.05; 95% CI, 1.01-1.09) and prothrombin time ($P = 0.04$, HR, 0.97; 95% CI, 0.95-1.00; Table 1). Moreover, a clear trend ($0.05 < P < 0.1$) was observed for 5 baseline variables: bilirubin, esophageal varices, Knodell score, serum IGF2 level and liver *Igf2* hypomethylation.

When patients with a U profile were compared in a paired way to those with M profile, the Log-Rank test was significant ($P = 0.047$). Moreover, the test for linear tendency between the 3 ordered levels of *Igf2* methylation and cumulative probability of HCC occurrence was significant (Log Rank, $P = 0.043$; Breslow, $P = 0.037$; Tarone-Ware, $P = 0.039$; Figure 2).

DISCUSSION

A growing body of evidence underlines that both DNA hypomethylation, leading to genomic instability, and regional CpG hypermethylation, leading to silence tumor suppressor gene, are dominant events during HCC development[48]. Calvisi *et al* recently showed that the extent of genome-wide hypomethylation progressively increased from non-neoplastic surrounding liver to fully malignant HCC[49], indicating that genomic hypomethylation is an important prognostic factor in HCC and opens the possibility of using molecular targets for chemoprevention or

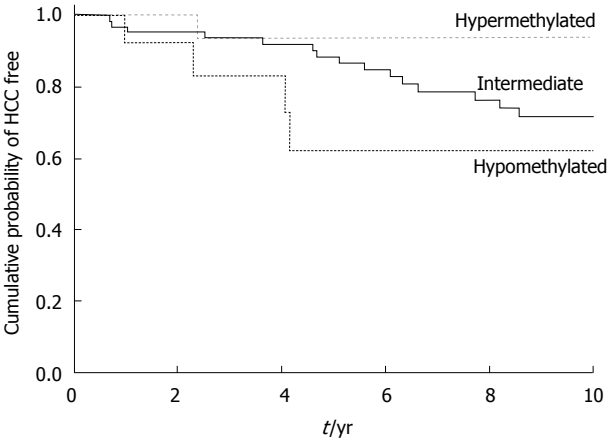

Figure 2 Occurrence of hepatocellular carcinoma at 10 years according to the methylation profile at the *Igf2* gene in 94 patients with Child-Pugh A hepatitis C-related cirrhosis (Kaplan-Meier method). Test of heterogeneity of HCC distributions: (log-rank test) $P = 0.13$. Test of the linear trend between levels of *Igf2* methylation and corresponding survival functions: Breslow (Generalized Wilcoxon) $P = 0.037$.

treatment of HCC. Regarding *Igf2* locus, we have observed hypomethylation at *Igf2* exon 8-9 in 90% (28/31) of HCV associated HCC, in contrast to the normal methylation pattern of two other genes located in the same area, the 11p15 locus[41]. This indicates that alterations in the IGF2 pathway are a pivotal event in hepatocarcinogenesis, at least in patients with HCV-related cirrhosis.

Our results suggest a possible link between *Igf2* hypomethylation in the liver of caucasians with uncomplicated and compensated HCV cirrhosis and the further occurrence of HCC. We observed a significant increased cumulative incidence of HCC at 10 years in patients with a hypomethylated pattern compared with those with a hypermethylated profile (30.8% *versus* 5.9%, $P = 0.047$) and a significant linear tendency between the ordered levels of *Igf2* methylation and the probability of HCC occurrence. The other variables identified in our patients were 2 well-known predictive factors for the occurrence of HCC: age[14-17] and prothrombin time related to liver failure[12,16-18] (Table 1). Due to a low number of patients, we could not show significant link with other known predictive factors (esophageal varices and bilirubin serum levels $0.05 < P < 0.08$). Conversely, as previous studies in patients with HCV-cirrhosis, we did not identify male gender[16,19], high AFP serum level[14,17], low platelet count[14,16,19] and diabetes[21] as predictive factors for the occurrence of HCC. These results, observed in Caucasians, may be different in other ethnic groups. However, IGF2 overexpression in HCC, which is mainly due to aberrant activation of the epigenetically regulated *Igf2* promoters, seems to be independent of ethnic origins. In addition to hypermethylation of promoters of several tumor suppressing genes found even in premalignant conditions, *Igf2* hypomethylation could thus contribute to the multistep process leading to malignant transformation[50].

This link between *Igf2* methylation and HCC occurrence should be validated in an external independent cohort. It may be underestimated in this study for several reasons. First, although the cumulative incidence was as high as previously reported in HCV-cirrhosis, the relative number of patients who further developed HCC ($n = 20$) is low. Secondly, being given cirrhotic liver heterogeneity and the small size of liver samples obtained by fine needle percutaneous biopsy, the extent of *Igf2* hypomethylation could have been underestimated. In addition, as samples were not microdissected, we analyzed not only hepatocytes, but also a variable amount of other minority hepatic cell types, such as sinusoidal cells and Kupffer cells, which may not share the same methylation pattern. Lastly, *Igf2* hypomethylation could be a late event in hepatocarcinogenesis, present to a low extent in uncomplicated cirrhosis and occurring later with the onset of liver failure and/or portal hypertension.

If there is a true link, whether these altered methylation patterns at *Igf2* locus lead to significant changes in expression profile and the function of genetic networks, or whether these changes just indicate severe epigenetic disturbances, remains to be investigated. A link between increased IGF2 expression and HCV infection has already been reported, showing that IGF2 over expression is significantly associated with HCV replication in patients with HCV-related cirrhosis[51]. The persistent process of hepatocyte damage and regeneration in HCV chronic hepatitis could provoke uncontrolled growth of hepatocytes and lead to malignant transformations due to disruption of growth regulation or mitogenic factors. However, whether HCV plays a direct or indirect role in IGF2 deregulation remains unknown. One could wonder if the link between Igf2 and HCC could be mediated by diabetes and metabolic syndrome. Indeed, epidemiological association between diabetes mellitus and HCC has been corroborated by molecular studies related to IGF1 or Igf Binding Protein 3. However, conversely to IGF1, IGF2 is mainly a fetal protein and its insulin-like metabolic effects in the post-natal period remains uncertain[52].

In these experiments, epigenetic changes in *Igf2*, potentially leading to re-expression of its fetal pattern, could be considered in parallel with AFP over expression as the hallmark of some fetal characteristics in the cirrhotic liver. The lack of correlation between the *Igf2* intron 8-9 methylation profile and IGF2 serum levels may be explained by an IGF2 local over expression leading to an autocrine effect, as previously suggested by Cariani *et al*[53].

Our observation that hypomethylation at the *Igf2* exon 8-9 is present in 14% of patients (13/94) with uncomplicated HCV-related cirrhosis and associated with a trend of overrisk of cancer are comparable to recent studies in the field of colorectal cancer[42,54]. Cui

et al[42] observed *Igf2* hypomethylation in normal colonic mucosa in 30% of patients with colorectal cancer in contrast to 10% in healthy patients. Moreover, *Igf2* hypomethylation is present in mesoderm-derived PBL and abnormal methylation profiles in this tissue are also highly correlated with both familial and personal histories of colorectal cancer. The prevalence of abnormal methylation patterns in PBL increases from 6.5% in patients with no personal history of colorectal cancer to 23% and 28%, respectively in patients with a personal history of adenoma or a family history of colorectal cancer, and to 56% in patients with colorectal cancer. These facts support a possible role of epigenetic changes of *Igf2* in the early steps of colorectal carcinogenesis. The most obvious unanswered question is whether the *Igf2* hypomethylation profile in PBL could be constitutive, resulting from inherited genetic mutations, or due to environmental events leading to epigenetic alterations. To try to answer this question in the field of HCC, further studies are ongoing in the PBL of a large cohort of patients with HCV-related cirrhosis screened for HCC and in healthy controls. If the results are comparable to those in patients with colorectal cancer, this may have clinical implications for defining high risk patients for HCC eligible for intensive screening and/or to reduce their risk with the use of dietary and/or therapeutic agents developed to reverse the epigenetic alterations such as methylated-oligonucleotides[55-57].

COMMENTS

Background

Hepatocellular carcinoma (HCC) is considered the fifth most frequent malignant neoplasm worldwide. In high incidence areas, it is strongly associated with viral hepatitis B and C and liver cirrhosis. Identification of molecular abnormalities associated with an increased risk of HCC is particularly important to improve knowledge of both the pathways of liver carcinogenesis and the outcomes.

Research frontiers

Insulin-like growth factor 2 (IGF2) is a fetal growth peptide produced by the liver, which is over expressed in a wide variety of neoplasms including HCC and is involved in experimental liver carcinogenesis. In a previous work analyzing the methylation status of *Igf2* in 71 HCC liver samples, we observed an hypomethylated profile in 89% of HCC.

Innovation and breakthroughs

Not only can *Igf2* hypomethylation be observed in HCC liver samples, but also in premalignant hepatitis C cirrhotic livers. In this case, *Igf2* hypomethylation is associated with a higher risk of HCC occurrence than *Igf2* hypermethylation.

Applications

Studies examining the Igf2 methylation status in hepatitis C cirrhotic liver could help identify patients with a high risk and patients with a low risk of HCC occurrence.

Peer review

This is a well designed study with interesting results. These results suggest that hypomethylation at the *Igf2* locus in the liver could be predictive for HCC occurrence in HCV cirrhosis.

REFERENCES

1 **Bosch FX**, Ribes J, Borras J. Epidemiology of primary liver cancer. *Semin Liver Dis* 1999; **19**: 271-285
2 **De Vos Irvine H**, Goldberg D, Hole DJ, McMenamin J. Trends in primary liver cancer. *Lancet* 1998; **351**: 215-216
3 **Deuffic S**, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. *Lancet* 1998; **351**: 214-215
4 **El-Serag HB**, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**: 745-750
5 **Taylor-Robinson SD**, Foster GR, Arora S, Hargreaves S, Thomas HC. Increase in primary liver cancer in the UK, 1979-94. *Lancet* 1997; **350**: 1142-1143
6 **El-Serag HB**, Mason AC. Risk factors for the rising rates of primary liver cancer in the United States. *Arch Intern Med* 2000; **160**: 3227-3230
7 **Benvegnu L**, Gios M, Boccato S, Alberti A. Natural history of compensated viral cirrhosis: a prospective study on the incidence and hierarchy of major complications. *Gut* 2004; **53**: 744-749
8 **Bolondi L**, Sofia S, Siringo S, Gaiani S, Casali A, Zironi G, Piscaglia F, Gramantieri L, Zanetti M, Sherman M. Surveillance programme of cirrhotic patients for early diagnosis and treatment of hepatocellular carcinoma: a cost effectiveness analysis. *Gut* 2001; **48**: 251-259
9 **Colombo M**, de Franchis R, Del Ninno E, Sangiovanni A, De Fazio C, Tommasini M, Donato MF, Piva A, Di Carlo V, Dioguardi N. Hepatocellular carcinoma in Italian patients with cirrhosis. *N Engl J Med* 1991; **325**: 675-680
10 **Kobayashi K**, Sugimoto T, Makino H, Kumagai M, Unoura M, Tanaka N, Kato Y, Hattori N. Screening methods for early detection of hepatocellular carcinoma. *Hepatology* 1985; **5**: 1100-1105
11 **Cottone M**, Turri M, Caltagirone M, Parisi P, Orlando A, Fiorentino G, Virdone R, Fusco G, Grasso R, Simonetti RG. Screening for hepatocellular carcinoma in patients with Child's A cirrhosis: an 8-year prospective study by ultrasound and alphafetoprotein. *J Hepatol* 1994; **21**: 1029-1034
12 **Zoli M**, Magalotti D, Bianchi G, Gueli C, Marchesini G, Pisi E. Efficacy of a surveillance program for early detection of hepatocellular carcinoma. *Cancer* 1996; **78**: 977-985
13 **Oka H**, Kurioka N, Kim K, Kanno T, Kuroki T, Mizoguchi Y, Kobayashi K. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. *Hepatology* 1990; **12**: 680-687
14 **Bruno S**, Silini E, Crosignani A, Borzio F, Leandro G, Bono F, Asti M, Rossi S, Larghi A, Cerino A, Podda M, Mondelli MU. Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a prospective study. *Hepatology* 1997; **25**: 754-758
15 **Chiba T**, Matsuzaki Y, Abei M, Shoda J, Aikawa T, Tanaka N, Osuga T. Multivariate analysis of risk factors for hepatocellular carcinoma in patients with hepatitis C virus-related liver cirrhosis. *J Gastroenterol* 1996; **31**: 552-558
16 **Fattovich G**, Giustina G, Degos F, Diodati G, Tremolada F, Nevens F, Almasio P, Solinas A, Brouwer JT, Thomas H, Realdi G, Corrocher R, Schalm SW. Effectiveness of interferon alfa on incidence of hepatocellular carcinoma and decompensation in cirrhosis type C. European Concerted Action on Viral Hepatitis (EUROHEP). *J Hepatol* 1997; **27**: 201-205
17 **Degos F**, Christidis C, Ganne-Carrie N, Farmachidi JP, Degott C, Guettier C, Trinchet JC, Beaugrand M, Chevret S. Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; **47**: 131-136
18 **Ikeda K**, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, Tsubota A, Arase Y, Murashima N, Chayama K, Kumada H. Long-term interferon therapy for 1 year or longer reduces

the hepatocellular carcinogenesis rate in patients with liver cirrhosis caused by hepatitis C virus: a pilot study. *J Gastroenterol Hepatol* 2001; **16**: 406-415

19 **Gramenzi A**, Andreone P, Fiorino S, Camma C, Giunta M, Magalotti D, Cursaro C, Calabrese C, Arienti V, Rossi C, Di Febo G, Zoli M, Craxi A, Gasbarrini G, Bernardi M. Impact of interferon therapy on the natural history of hepatitis C virus related cirrhosis. *Gut* 2001; **48**: 843-848

20 **N'Kontchou G**, Paries J, Htar MT, Ganne-Carrie N, Costentin L, Grando-Lemaire V, Trinchet JC, Beaugrand M. Risk factors for hepatocellular carcinoma in patients with alcoholic or viral C cirrhosis. *Clin Gastroenterol Hepatol* 2006; **4**: 1062-1068

21 **Ioannou GN**, Splan MF, Weiss NS, McDonald GB, Beretta L, Lee SP. Incidence and predictors of hepatocellular carcinoma in patients with cirrhosis. *Clin Gastroenterol Hepatol* 2007; **5**: 938-945, 945.e1-945.e4

22 **Lai MS**, Hsieh MS, Chiu YH, Chen TH. Type 2 diabetes and hepatocellular carcinoma: A cohort study in high prevalence area of hepatitis virus infection. *Hepatology* 2006; **43**: 1295-1302

23 **Veldt BJ**, Chen W, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, de Knegt RJ, Zeuzem S, Manns MP, Hansen BE, Schalm SW, Janssen HL. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. *Hepatology* 2008; **47**: 1856-1862

24 **Le Roith D**. Seminars in medicine of the Beth Israel Deaconess Medical Center. Insulin-like growth factors. *N Engl J Med* 1997; **336**: 633-640

25 **LeRoith D**, Roberts CT Jr. The insulin-like growth factor system and cancer. *Cancer Lett* 2003; **195**: 127-137

26 **Scharf JG**, Braulke T. The role of the IGF axis in hepatocarcinogenesis. *Horm Metab Res* 2003; **35**: 685-693

27 **Lin SB**, Hsieh SH, Hsu HL, Lai MY, Kan LS, Au LC. Antisense oligodeoxynucleotides of IGF-II selectively inhibit growth of human hepatoma cells overproducing IGF-II. *J Biochem* 1997; **122**: 717-722

28 **Rogler CE**, Yang D, Rossetti L, Donohoe J, Alt E, Chang CJ, Rosenfeld R, Neely K, Hintz R. Altered body composition and increased frequency of diverse malignancies in insulin-like growth factor-II transgenic mice. *J Biol Chem* 1994; **269**: 13779-13784

29 **Tsai JF**, Jeng JE, Chuang LY, You HL, Ho MS, Lai CS, Wang LY, Hsieh MY, Chen SC, Chuang WL, Lin ZY, Yu ML, Dai CY. Serum insulin-like growth factor-II and alpha-fetoprotein as tumor markers of hepatocellular carcinoma. *Tumour Biol* 2003; **24**: 291-298

30 **Thorgeirsson SS**, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002; **31**: 339-346

31 **Falls JG**, Pulford DJ, Wylie AA, Jirtle RL. Genomic imprinting: implications for human disease. *Am J Pathol* 1999; **154**: 635-647

32 **Reik W**, Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001; **2**: 21-32

33 **Sohda T**, Yun K, Iwata K, Soejima H, Okumura M. Increased expression of insulin-like growth factor 2 in hepatocellular carcinoma is primarily regulated at the transcriptional level. *Lab Invest* 1996; **75**: 307-311

34 **Cariani E**, Lasserre C, Seurin D, Hamelin B, Kemeny F, Franco D, Czech MP, Ullrich A, Brechot C. Differential expression of insulin-like growth factor II mRNA in human primary liver cancers, benign liver tumors, and liver cirrhosis. *Cancer Res* 1988; **48**: 6844-6849

35 **Aihara T**, Noguchi S, Miyoshi Y, Nakano H, Sasaki Y, Nakamura Y, Monden M, Imaoka S. Allelic imbalance of insulin-like growth factor II gene expression in cancerous and precancerous lesions of the liver. *Hepatology* 1998; **28**: 86-89

36 **Takeda S**, Kondo M, Kumada T, Koshikawa T, Ueda R, Nishio M, Osada H, Suzuki H, Nagatake M, Washimi O, Takagi K, Takahashi T, Nakao A, Takahashi T. Allelic-expression imbalance of the insulin-like growth factor 2 gene in hepatocellular carcinoma and underlying disease. *Oncogene* 1996; **12**: 1589-1592

37 **Uchida K**, Kondo M, Takeda S, Osada H, Takahashi T, Nakao A, Takahashi T. Altered transcriptional regulation of the insulin-like growth factor 2 gene in human hepatocellular carcinoma. *Mol Carcinog* 1997; **18**: 193-198

38 **Li X**, Nong Z, Ekstrom C, Larsson E, Nordlinder H, Hofmann WJ, Trautwein C, Odenthal M, Dienes HP, Ekstrom TJ, Schirmacher P. Disrupted IGF2 promoter control by silencing of promoter P1 in human hepatocellular carcinoma. *Cancer Res* 1997; **57**: 2048-2054

39 **Cui H**, Onyango P, Brandenburg S, Wu Y, Hsieh CL, Feinberg AP. Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2. *Cancer Res* 2002; **62**: 6442-6446

40 **Feinberg AP**. Imprinting of a genomic domain of 11p15 and loss of imprinting in cancer: an introduction. *Cancer Res* 1999; **59**: 1743s-1746s

41 **Poirier K**, Chalas C, Tissier F, Couvert P, Mallet V, Carrie A, Marchio A, Sarli D, Gicquel C, Chaussade S, Beljord C, Chelly J, Kerjean A, Terris B. Loss of parental-specific methylation at the IGF2 locus in human hepatocellular carcinoma. *J Pathol* 2003; **201**: 473-479

42 **Cui H**, Cruz-Correa M, Giardiello FM, Hutcheon DF, Kafonek DR, Brandenburg S, Wu Y, He X, Powe NR, Feinberg AP. Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. *Science* 2003; **299**: 1753-1755

43 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodes J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430

44 **Couvert P**, Poirier K, Carrie A, Chalas C, Jouannet P, Beldjord C, Bienvenu T, Chelly J, Kerjean A. DHPLC-based method for DNA methylation analysis of differential methylated regions from imprinted genes. *Biotechniques* 2003; **34**: 356-362

45 **Kaplan E**, Meier P. Nonparametric Estimation from Incomplete Observations. *J Am Stat Assoc* 1958; **53**: 457-481

46 **Tarone RE**. Tests for trend in life table analysis. *Biometrika* 1975; **62**: 679-682

47 **Cox DR**. Regression models and life tables (with discussion). *J R Stat Soc* 1972; **34**: 187-220

48 **Teufel A**, Staib F, Kanzler S, Weinmann A, Schulze-Bergkamen H, Galle PR. Genetics of hepatocellular carcinoma. *World J Gastroenterol* 2007; **13**: 2271-2282

49 **Calvisi DF**, Ladu S, Gorden A, Farina M, Lee JS, Conner EA, Schroeder I, Factor VM, Thorgeirsson SS. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. *J Clin Invest* 2007; **117**: 2713-2722

50 **Tischoff I**, Tannapfe A. DNA methylation in hepatocellular carcinoma. *World J Gastroenterol* 2008; **14**: 1741-1748

51 **Tanaka S**, Takenaka K, Matsumata T, Mori R, Sugimachi K. Hepatitis C virus replication is associated with expression of transforming growth factor-alpha and insulin-like growth factor-II in cirrhotic livers. *Dig Dis Sci* 1996; **41**: 208-215

52 **Bach LA**, Rechler MM. Insulin-like growth factors and diabetes. *Diabetes Metab Rev* 1992; **8**: 229-257

53 **Cariani E**, Seurin D, Lasserre C, Franco D, Binoux M, Brechot C. Expression of insulin-like growth factor II (IGF-II) in human primary liver cancer: mRNA and protein analysis. *J Hepatol* 1990; **11**: 226-231

54 **Cruz-Correa M**, Cui H, Giardiello FM, Powe NR, Hylind L, Robinson A, Hutcheon DF, Kafonek DR, Brandenburg S, Wu Y, He X, Feinberg AP. Loss of imprinting of insulin growth factor II gene: a potential heritable biomarker for colon neoplasia predisposition. *Gastroenterology* 2004; **126**: 964-970

55 **Yao X**, Hu JF, Daniels M, Shiran H, Zhou X, Yan H, Lu H, Zeng Z, Wang Q, Li T, Hoffman AR. A methylated oligonucleotide inhibits IGF2 expression and enhances survival in a model of hepatocellular carcinoma. *J Clin Invest* 2003; **111**: 265-273

56 **Santini V**, Kantarjian HM, Issa JP. Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. *Ann Intern Med* 2001; **134**: 573-586

57 **Esteller M**. Relevance of DNA methylation in the management of cancer. *Lancet Oncol* 2003; **4**: 351-358

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