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.

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

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignant neoplasms worldwide¹, and its incidence has increased in the past decade in Europe and the USA²⁻⁴. 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 world⁵ where HCC is presently the leading cause of death
of patients with HCV related cirrhosis. 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,[17,38] 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 vivo, 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 vitro, 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[29] in association with decreased cell proliferative activity. Unpublished, Rogler et al[29] 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°C) 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[40].

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
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\'-GGGAAAGGGGTTTAGGATTTTTAT-3\', reverse internal 5\'-ATAATTTACTCCCCCCTCAACCTC-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°C for 10 min followed by 40 cycles of 94°C for 45 s, 62°C for 45 s, 72°C for 1 min and a final extension step of 10 min at 72°C. 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°C. 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
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°C 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[102] 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[104]. As regards to heterogeneity of risk according to IGF2 levels, the Cox regression[105] 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

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**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/m²)                      | 25.0 ± 4.8 | 25.1 ± 4.8 | 24.7 ± 4.8 | 1.00 (0.90-1.11) | 0.96 |
| BMI > 30 kg/m²                   | No         | 80         | 64 | 1 | 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 grade                | 0 or I     | 78         | 63 | 15 | 1 |
|                                 | 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 (× 10³/mm³)            | 151 ± 61   | 155 ± 64   | 138 ± 51  | 1.00 (0.99-1.00) | 0.29 |
| ALT (× 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)               | 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. *Performed in 63 patients.*
sequential (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...
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\[16\]. 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.131 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 50% in patients with colorectal cancer. These facts support a possible role of genetic 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.

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