Research Article

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Impact of cytokine gene variants on the prediction and prognosis of hepatocellular carcinoma in patients with cirrhosis

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Background & Aims: Genetic polymorphisms modulate the expression of proinflammatory cytokines. We prospectively assessed the influence of 6 single nucleotide polymorphisms (SNPs) in *TNFα*, *IL6*, and *IL1β* genes on the risk of hepatocellular carcinoma (HCC) in patients with cirrhosis.

Methods: *TNFα* (G-238A, C-863A, G-308A), *IL6* (C-174G), and *IL1β* (C-31T, C-511T) SNPs were assessed in 232 alcoholics and 253 HCV-infected patients with biopsy-proven cirrhosis, prospectively followed-up and screened for HCC. Their influence on HCC development was assessed using the Kaplan-Meier method.

Results: These variants did not influence the risk of HCC in alcoholic patients. Conversely, two variants influenced the risk of HCC occurrence in patients with HCV-related cirrhosis, namely the *TNFα*-308 (A) allele (HR = 2.4 [1.6–3.7], Log-rank <0.0001) and the *IL1β*-31 (T) allele (HR = 1.5 [1.1–2.1], Log-rank = 0.004). When stratifying HCV-infected patients into four genotypic associations expected to progressively increase TNFα and IL1β production, we observed increasing risk of HCC occurrence (Log-rank <0.0001) from group 1 to 4. The *TNFα*-308 (A) allele was the only genetic trait independently associated with risk of HCC in these patients, along with older age, male gender, BMI, and platelet count. These variables led to construction of a predictive score able to separate patients with HCV-related cirrhosis into three subgroups with progressively increasing 5-year cumulative incidences of 4.7%, 14.1%, and 36.3%, respectively (Log-rank <0.0001).

Conclusions: Genetic heterogeneity in the *TNFα* and *IL1β* gene promoters influences the risk of HCC in patients with HCV-induced cirrhosis. These genetic data, when incorporated into clinical scores, are able to refine selection of risk classes of HCC.

Keywords: Inflammation; Cytokines; Polymorphisms; Liver cancer.
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Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IL, Interleukin; SNP, single nucleotide polymorphism; SVR, sustained virological response; TNF, tumour necrosis factor.

Introduction

Hepatocellular carcinoma (HCC) is the main cause of death in patients with cirrhosis and its recognition at an early stage by periodical screening is recommended. Prediction of the occurrence of liver cancer in these patients is based on the association of simple epidemiological and biological features that can be combined into HCC risk assessment models [1]. However, in clinical practice all cirrhotic patients are subjected to the same management, namely 6-month HCC screening, without any specific preventive measures undertaken. Such attitude needs to be questioned when considering that the proportion of patients diagnosed with liver cancer ineligible for curative procedures can reach 20% in surveillance trials [2]. In this setting, wide inter-individual variations in liver cancer risk raise the question of whether recommendations concerning the efficiency and periodicity of HCC screening, as well as initiation of personalized therapeutic management such as chemoprevention, can be adjusted to individual risk [3]. These new approaches incorporating host parameters to develop risk prediction models for HCC are currently emerging and have excellent prediction accuracy as well as discriminatory ability [4].

As in many tumour types, a link between chronic inflammation and HCC development has been demonstrated [5]. The implication of a large network of cytokines in cancer-related immune reaction is also well documented [6]. In particular, the signalling pathway involving TNFα-IL-6/JAK/STAT has been recently highlighted in the onset of hepatocarcinogenesis [7]. Among

variants of genetic heterogeneity thought to be implicated in the emergence of cancer, several SNPs that modulate production of proinflammatory cytokines have been reported to be associated with the risk of HCC. Initially based on reports conducted in small sample-size cohorts, genetic polymorphisms influencing TNF-α, IL-1β, or IL-6 secretion were recently incorporated into large meta-analyses, leading to confirmation of plausible associations between these variants and HCC risk (Supplementary Table 1) [8–10].

These findings now warrant replication and validation of their clinical usefulness at the longitudinal level [11]. For this purpose, we selected two large prospective cohorts of cirrhotic patients representing the most frequent causes of chronic liver diseases in Europe (alcoholic and HCV-related), in whom frozen serum and DNA samples are available [12]. These cohorts present the advantage of a long follow-up that allowed to record a large number of incidental cases of HCC developed in these patients. Furthermore, the quality of recorded clinical data at baseline and during follow-up of these patients provides the opportunity to perform complex multivariate analyses taking into account competitive risks of death and to control for other important HCC risk factors such as features of the metabolic syndrome or the influence of viral treatments. Thus, the main aim of this study was to investigate the influence of the 6 most frequently studied genetic variants modulating *TNFα*, *IL1β*, and *IL6* expression (either individually or collectively combined) and their corresponding circulating levels upon the risk of HCC. A second goal was to assess their potential contribution toward distinguishing patients according to individual prognosis, as a supplement to the usual components included in clinical HCC risk assessment models.

Patients and methods

Patients

We considered all patients consecutively referred to the Jean Verdier Hospital Liver Unit for diagnosis and management of cirrhosis between January 1999 and December 2007, and who fulfilled the following inclusion criteria: (1) histologically proven cirrhosis; (2) no infection from HIV or HBV; (3) no evidence of HCC at the time of inclusion; (4) residence in France; (5) acceptance of regular follow-up; (6) Caucasian origin; (7) written informed consent for use of frozen DNA and serum samples.

Patients were divided into two distinct cohorts according to the cause of cirrhosis. The first cohort consisted of patients with alcoholic cirrhosis, defined as: (1) daily excessive alcohol consumption (>80 g per day in males and >60 g per day in females for at least 10 years); and (2) no HCV infection, as defined by negative serum HCV antibodies. The second cohort included patients with HCV-related cirrhosis defined as: (1) absence of past or present daily alcohol intake; (2) chronic infection by HCV defined by positive serum HCV-RNA. A total of 532 patients were initially considered. Among them, 47 were chronically infected by HCV as well as daily alcohol consumers and were not further considered for enrolment. Thus, a total of 485 cirrhotic patients (232 alcoholics and 253 HCV-infected) were included in the present study. The study protocol was in conformity with ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee. All patients gave their written consent for blood sampling and genotyping.

For each patient, date of inclusion was the date of the first liver biopsy showing cirrhosis. Daily alcohol intake was recorded at inclusion and at each visit by interviewing all patients. Interviews were performed by different physicians, either during hospitalization or in the setting of their regular follow-up in our unit. In case of discordances, medical files were reviewed by the medical staff. Virus genotype was assessed in patients with HCV-related cirrhosis. After inclusion, all patients were followed and evaluated at minimum 6-month intervals by physical examination and liver ultrasonography. If these investigations suggested possible HCC, computed tomodensitometry, magnetic resonance imaging and/or guided liver biopsy was performed according to recommendations of the Barcelona Conference [13].

The main endpoint was occurrence of HCC during follow-up. Follow-up ended at the date of death or liver transplantation (considered as death) or at the last recorded visit (or information gathering) within the 6 months prior to August 31st 2012. This date was set as the final time limit for upgrading the patient file, using either our computerized database, departmental certificates for patients who died outside our liver unit or contacting patients, their relatives or their general practitioner. All included patients were screened for at least two years; patients lost to follow-up after this period were included in the analysis and censored at the date of the last recorded information. For patients with HCV-related cirrhosis, antiviral treatment and a sustained virological response (SVR) were recorded at endpoint, with SVR defined as persistence of negative serum HCV-RNA at 6 months after the end of therapy. Daily alcohol intake was again recorded at endpoint by medical interview in patients with alcoholic cirrhosis.

DNA extraction and genotyping

DNA samples were prepared from frozen blood samples stored in the Liver Biobank of "Hôpitaux Universitaires Paris-Seine-Saint-Denis", Jean Verdier Hospital, APHP, University Paris 13, Bondy, France.

SNPs within each gene were selected according to review of the medical literature, using the following criteria: (1) each polymorphism had some degree of likelihood to alter the expression of the gene in a biologically relevant manner, as detailed in Supplementary Table 1 displaying differences in cytokine productions according to the different alleles; (2) the association with HCC was documented in well-designed published studies. According to these criteria, 6 SNPs affecting *TNFα* [G-238A (rs361525), C-863A (rs1800630), G-308A (rs1800629)], *IL1β* [C-31T (rs1143627), C-511T (rs16944)] or *IL6* (C-174G) production were selected (Supplementary data).

Serum cytokine level assessment

Serum TNF-α, IL-1β, and IL-6 levels were determined using the Human TNF alpha ELISA Ready-SET-Go, Human IL-1β ELISA Ready-SET-Go and Human IL-6 ELISA Ready-SET-Go ELISA kits (eBioscience, Paris, France), respectively.

Statistical analyses

Qualitative variables were compared using Fisher's exact test, the χ^2 test or the χ^2 trend test with one degree of freedom, whereas quantitative variables were compared using the non-parametric Kruskal–Wallis test. The Kaplan–Meier method was used to estimate the occurrence of HCC for each parameter noted at enrolment; death was considered as an outcome in the experiment. The distribution of death and HCC was compared with the Log-rank test. A significant level <0.10 was used to select variables for the Cox proportional hazards model using a stepwise procedure with a threshold of $\alpha = 0.05$. Variables associated with risk of death or HCC, based on knowledge and findings from previous studies, were also selected for multivariate analyses (age, gender, BMI, diabetes, Child–Pugh score, platelet count, HCV genotype, SVR). Statistical analyses were performed using the SAS System Package version 8.02 (SAS Institute, Cary, NC). All reported *p* values are uncorrected and two-tailed. Associations were considered statistically significant at a two-tailed α of 0.05.

Results

Baseline characteristics and clinical outcome of patients

A total of 485 patients were enrolled in this study (Table 1). Nearly one-third of this population developed HCC and about the same proportion died (27 patients were transplanted). Causes of death or transplantation were related to HCC development in half of the cases, while the others were related to progression of liver disease (42%) or, less frequently (n = 11), were non liver-related (extrahepatic cancers: 4, cardiovascular diseases: 3, others/unknown: 4).