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Association between the epidermal growth factor rs4444903 G/G genotype and advanced fibrosis at a young age in chronic hepatitis C

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ABSTRACT

Background: The epidermal growth factor (EGF) rs4444903 A > G polymorphism has been associated with the development of liver cancer, which commonly complicates cirrhosis of viral origin; however, whether this polymorphism might be associated with fibrosis progression in chronic viral hepatitis is unknown. The present study was performed to assess the allelic and genotypic frequencies of the rs4444903 A > G polymorphism in patients with chronic hepatitis C virus HCV infection and to ascertain whether this polymorphism might be an independent predictor of the degree of fibrosis.

Methods: An RFLP–PCR technique was used to genotype 645 patients (211 with cirrhosis); 528 were referred for the diagnosis and treatment of chronic hepatitis C, and 117 were transplanted for HCV-related end stage liver disease. A group of 428 healthy subjects served as a control. All the subjects were of Caucasian ethnicity.

Results: The EGF rs4444903 A > G polymorphism genotype frequencies in HCV chronic infected patients were as follows: A/A = 227 (35.3%), A/G = 328 (50.9%), and G/G = 90 (14.8%). Genotype frequencies were found to differ between patients with an Ishak staging score $\leqslant 2$ (A/A = 117, A/G = 157, G/G = 34) and patients with a score > 2 (A/A = 110, A/G = 171, G/G = 56, $p = 0.038$). A highly significant linear relationship between increasing stage scores and EGF genotype was detected in younger patients (A/A: 2.02 ± 0.18, A/G: 2.55 ± 0.17, G/G: 3.00 ± 0.32, $p = 0.008$). However, no significant association was detected between the stage score and EGF genotype in older patients (A/A: 3.79 ± 0.19, A/G: 3.64 ± 0.15, G/G: 3.98 ± 0.30 $p = 0.579$).

Conclusions: The EGF rs4444903 A > G polymorphism may facilitate liver fibrosis progression in Caucasian patients with chronic hepatitis C, especially in younger patients.

1. Introduction

The natural history of hepatitis C virus (HCV) infection varies greatly; spontaneous viral clearance occurs in a minority of cases, whereas the majority of patients progress to chronic disease [1]. During chronic hepatitis C, progressive fibrosis occurs, but it results in cirrhosis only in 20–30% of chronic HCV carriers. The remaining HCV-infected patients never progress beyond a mild disease stage [2]. Several predictors of progression to advanced fibrosis have been identified [2,3], including male gender [2], ethnicity [4], insulin resistance [5] and older age [6]. However, other factors (including genetic predisposition) are likely to play a role.

The epidermal growth factor (EGF) rs4444903 A > G polymorphism, involving an A > G transition at position 61 of the 5′ untranslated region of the EGF gene, is associated with several types of cancer [7], including malignant melanoma [8,9], oesophageal cancer [10], gastric cancer [11], breast cancer [12] and colorectal cancer [13]. Carriers of the G allele, especially those with the G/G genotype, may have an increased risk of developing hepatocellular carcinoma (HCC), as shown in a series of European [14], American [15] and Asian studies [16,17]. The association with HCC, however, was not confirmed by all studies [18].

EGF up-regulation is characteristic of the cirrhotic liver [19]; moreover, the production of non-structural HCV viral proteins may interfere with the EGF signalling pathway, representing a

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mechanism by which HCV could prevent the apoptosis of infected cells and facilitate its replication [20–22]. Very recently, EGF receptor (EGFR) has been found to be involved in the efficient entry of HCV into its target cells. EGFR ligands such as EGF and transforming growth factor alpha (TGF-α) were found to enhance HCV infection, while EGFR-specific antibodies were found to inhibit it [23]. However, whether the rs4444903 A > G polymorphism might be associated with progression to advanced fibrosis in chronic hepatitis C is unknown.

The aims of this study were as follows: (a) to assess the allelic and genotypic frequencies of the rs4444903 A > G polymorphism in patients with chronic HCV infection in comparison with controls and (b) to verify whether this polymorphism is an independent predictor of progression to advanced fibrosis in chronic hepatitis C.

2. Materials and methods

2.1. Patients

The study included 645 consecutive Italian patients of Caucasian ethnicity with chronic hepatitis C. The majority of the patients (*N* = 528, 81.9%) had been referred to our regional liver and transplant unit for the diagnosis and treatment of chronic hepatitis C, while the remainder (*N* = 117, 18.1%) had been transplanted for HCV-related end stage liver disease. Approximately two thirds of patients had chronic hepatitis (*N* = 434), defined at liver biopsy as histologically mild (Ishak staging score ⩽2; *N* = 308) or more advanced (Ishak staging score 3–4; *N* = 126). All were naïve to antiviral treatment. The diagnosis was confirmed in all cases by the histological evaluation of a liver biopsy specimen. The remaining 211 patients had liver cirrhosis and 55 of these cases were complicated by HCC. In the 117 patients from this group who underwent liver transplantation, the diagnoses of liver cirrhosis and HCC (*N* = 39, 33.3%) were confirmed by histological evaluation of the explanted liver. In the 94 untransplanted patients, liver cirrhosis was diagnosed clinically based on the presence of signs of portal hypertension, pertinent imaging features and laboratory findings such as hypo-albuminemia, INR increase and low platelet count. In 54 cases (57.4%), the diagnosis was indicated by liver histology based on a percutaneous liver biopsy. The diagnosis of HCC (*N* = 16, 17.0%) in this group of patients was largely based on the results of dynamic imaging studies in accordance with the AASLD practice guidelines [24]. A liver biopsy was performed in 2 cases in which imaging studies were not conclusive, and the results confirmed the presence of HCC. The main demographic and clinical characteristics of the patients are reported in Table 1. The control group consisted of 428 healthy Italian blood donors of Caucasian ethnicity, 314 males (73.4%) and 114 females (26.6%); the median age was 49 years, with a range of 18–77 years. Control subjects did not have any clinical and/or laboratory evidence of liver disease or of any other major pathological condition, such as diabetes mellitus. Informed consent to participate in the study was obtained from each subject, in accordance with the Declaration of Helsinki and the local ethics committee guidelines. All study participants approved the storage of their frozen DNA specimens in our laboratory for research purposes.

2.2. Histological evaluation

Liver fibrosis was assessed using the Ishak staging score [25], as follows: 0 = no fibrosis, 1 = fibrous expansion of some portal areas, 2 = fibrous expansion of most portal areas, 3 = fibrous expansion of most portal areas with occasional portal to portal bridging, 4 = fibrous expansion of portal areas with marked bridging, 5 = marked bridging with occasional nodules (incomplete cirrhosis) and 6 = cirrhosis (probable or definite).

Table 1
Demographic and clinical characteristics of the studied population (*N* = 645). Continuous variables are reported as the median (range), categorical variables as frequencies (%).

Clinical variables	
Male gender, *N*	369 (57.2)
Age (years)	53 (17–86)
Body mass index (kg/m^2)	24.0 (15.1–42.6)
Presence of diabetes mellitus, *N*	74 (11.5)
Concomitant alcohol consumption, N	
No	529 (82.0)
Yes	116 (18.0)
HCV genotype, N	
1	386 (59.9)
2	148 (22.9)
3	81 (12.6)
5-Apr	30 (4.6)
Patient presentation, N	
HCV-related chronic liver disease	528 (81.9)
Follow-up after liver transplantation for HCV-related liver disease	117 (18.1)
Liver disease, N	
Mild (Ishak staging score 0–2)	308 (47.8)
Moderate (Ishak staging score 3–4)	126 (19.5)
Severe (Ishak staging score 5–6)	211 (32.7)
Presence of hepatocellular carcinoma, *N*	55 (8.5)
Child–Pugh score (only in patients with cirrhosis)	7 (5–13)

HCV = hepatitis C virus.

2.3. Molecular biology

Genotyping of the EGF rs4444903 A > G polymorphism was performed using a polymerase chain reaction-based restriction fragment length polymorphism assay. Genomic DNA was extracted from whole blood samples using the QIAamp DNA blood mini kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. A 260 base pair (bp) product was obtained with the forward primer 5′-CATTTGCAAACAGAGGCTCA-3′ and the reverse primer 5′-GTTTAACAGCCCTGCTCTGG-3′, which were designed using NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR amplification was carried out in a total volume of 10 μL containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, tween-20 0.01%, 0.2 mM deoxyribonucleotides, 2–4 pmol of each primer, 2.0 mM $MgCl_2$ and 0.5 units hot-start Taq DNA polymerase (@Taq, Euroclone, Milan, Italy). Samples containing 10 ng of genomic DNA were subjected to 40 cycles of denaturation (at 95 °C for 30 s), annealing (at 61 °C for 30 s) and elongation (at 72 °C for 45 s) using a Techne TC-412 thermal cycler. In a total volume of 20 μL, 10 μL of the amplicons were digested with 1 unit of the Alu-I restriction endonuclease (New England Biolabs, Hitchin, UK) at 37 °C overnight. The digested fragments were 94, 91, 60 and 15 bp for the A allele and 185, 60 and 15 bp for the G allele variant. The fragments were resolved by electrophoresis on 3.5% agarose gels after staining with ethidium bromide. The genomic region encompassing the EGF rs4444903 A > G polymorphism was sequenced in 20 patients, with all the results confirming those obtained using the RFLP assay.

2.4. Statistical analysis

Statistical analysis of the data was performed using the BMDP dynamic statistical software package 7.0 (Statistical Solutions, Cork, Ireland). Continuous variables are presented as the median (range) or mean ± standard error, while categorical variables are expressed as frequencies (%). The chi-square G test for goodness of fit was used to verify whether the proportions of the polymorphism were distributed in both the controls and the patients in accordance with the Hardy–Weinberg equation. The existence of