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Research paper

Association of thrombogenic genes polymorphisms with hepatocellular carcinoma in HCV Egyptian patients

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ABSTRACT

The rate of development of fibrosis varies among HCV patients and affected by many variables. We aimed to investigate the association between mutations in Factor V, prothrombin gene and thrombospondin 1 polymorphisms with hepatic fibrosis progression rate and development of HCC in patients infected with HCV and if they are potential markers for early prediction of disease progression. A total of 280 HCV-infected patients (70 with mild fibrosis, 70 with advanced fibrosis, 70 cirrhotic patients and 70 HCC patients) and 100 healthy controls were included. Factor V Leiden G1691A, prothrombin G20210A and thrombospondin 1 mutations were analyzed by restriction fragment length polymorphism. We observed that there were no significant differences between Factor V Leiden (G1691A) or TPS-1 (A2210G) polymorphisms in the four patient subgroups and control group. In HCC patients, the frequencies of GA genotype were significantly increased compared with control subject. HCV patients carrying GA genotype were more likely to develop hepatocellular carcinoma (OR = 5.4, 95% CI = 1.09–27.05; P = 0.026).We concluded that the risk of HCC was increased 5-fold in subjects carrying GA genotype of prothrombin G20210A gene. However, there was no evidence for a significant association between thrombogenic genes polymorphisms and progression of fibrosis in HCV Egyptian patients.

1. Introduction

The final consequence of chronic hepatitis c virus (HCV) is the development of advanced fibrosis or cirrhosis in significant number of cases; about 70% of HCV patients develop chronicity, and 25% of them develop cirrhosis in about 20 years. 4% of cirrhotic patients develop decompensation with annual death rate of 15–30%, and 1.6% of cirrhotic patients develop HCC (Hoofnagle, 1997). The rate of development of fibrosis varies among HCV patients and affected by many variables including male gender, age at the onset of infection, alcohol consumption, and hepatic steatosis (Marcellin et al., 2002) and non-O blood group (Tripodi et al., 2005).

There is a debate about the role of hypercoaguable states in the progression of liver fibrosis; the mechanisms by which hypercoaguable states cause liver fibrosis include microthrombi within branches of the hepatic vein and portal vein (Tripodi et al., 2005). Portal vein thrombosis (PVT) occurring in cirrhosis may aggravate portal hypertension related complications of cirrhosis and is important in this context (Seligsohn and Lubetsky, 2001).

The generation of thrombin which is a stellate cell mitogen and the activation of protease activated receptor-1 (PAR-1) by thrombin lead to rapid stellate cell activation and secretion of extracellular matrix proteins, tissue remodeling and fibrogenesis (Fiorucci et al., 2004).

Among hereditary causes of venous thrombosis, Factor V Leiden (FVL) G1691A polymorphism, prothrombin gene G20210A, thrombospondin-1 (TSP-1) gene polymorphisms are the three most common (Ashjazadeh et al., 2012).

The gene that codes the Factor V Leiden protein is referred to as F5. Mutation of this gene is a single nucleotide polymorphism (SNP) located on chromosome 1 in exon 10. As a missense substitution of base A to base G, it changes the protein's amino acid from arginine to glutamine, the mutation prevents efficient inactivation of Factor V (Voorberg et al., 1994).

Prothrombin gene mutation G → A transition at nucleotide 20210 leading to elevation of plasma prothrombin levels. Prothrombin is the precursor to thrombin, which plays a key role in blood clotting (Poort et al., 1999).

Thrombospondin-1 (TSP-1) is a multifunctional matrix protein, influencing tumor growth through its inhibition of angiogenesis. It inhibits vascular endothelial cell migration and adhesion in vitro and also inhibits basic fibroblast growth factor induced angiogenesis (Wessel et al., 2004). TSP-1 polymorphism might play an additional role in liver fibrogenesis by stimulating angiogenesis and could be a

Abbreviations: ANOVA, Analysis of variance; CIs, Confidence intervals; ORs, Odds ratios; PCR, Polymerase chain reaction; RFLP, Restricted fragment length polymorphism; HCV, Hepatitis c virus; HCC, Hepatocellular carcinoma; FVL, Factor V Leiden; TSP-1, Thrombospondin-1.

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potential target to prevent fibrogenesis in chronic inflammatory diseases of the liver (Pek et al., 2008).

The ability to predict the risk of progressive liver disease has important prognostic and therapeutic intentions. The aim of this work is to investigate the association of mutations in Factor V, prothrombin gene and thrombospondin-1 polymorphisms with hepatic fibrosis progression rate and development of HCC in patients infected with HCV and if they are potential markers for early prediction of disease progression.

2. Patients and methods

2.1. Patients

The clinical data of 280 HCV-infected patients were retrospectively evaluated. The patients were recruited during their preparation for combined antiviral therapy at the Hepatology Clinic, Internal Medicine Department, Zagazig University Hospital, Egypt, which is a tertiary referral hospital. The data of HCC patients were collected during their follow-up at the Hepatology Clinic, Zagazig University hospital. The subject included the patients with chronic active HCV proved by positive HCV antibodies, HCV RNA positivity by PCR with elevated liver enzymes, and histological staging of HCV on liver biopsy.

Patients were classified into mild fibrosis (F1–F2, n = 70), advanced fibrosis (F3, N = 70), and cirrhosis (F4, N = 70); this classification was based on liver biopsy performed within 6 months before enrollment, and HCC (n = 70) which were diagnosed by abdominal ultrasonography (USG), pathognomonic rise of AFP > 400 ng/ml and confirmation by triphasic CT. In the HCC group 30 patients had mild to moderate ascites (42.9%) who responded to diuretic therapy.

A questionnaire regarding the medical history including duration of acquisition of infection and drug history was obtained. Clinical signs of portal hypertension and liver cell failure were evaluated.

Patients were excluded if they had definitive liver disease due to any other cause as autoimmune hepatitis, alcoholic liver disease, or if they had positive serology for hepatitis B or HIV. Patients for whom the date of infection was unknown were also excluded.

The control group which comprised 100 healthy volunteer subjects was matched for ethnicity, age and same geographical region.

The ethical committee of Zagazig University approved this study, and a written informed consent was obtained from all subjects prior to their inclusion in this work.

2.2. Abdominal ultrasonography

The patients were examined after 6 h fast. Criteria of liver cirrhosis and portal hypertension were evaluated. The presence of ascites and HCC was documented.

2.3. Liver biopsy

Liver biopsy specimen of at least 2 cm in length was taken and fixed in 10% formalin buffer. Biopsy samples were stained with hematoxylin–eosin to elucidate histological grading based on histological activity index (HAI) of Knodell et al. (1981).

Staging of liver histology into F0–4 according to the Metavir scoring systems: F0 = none, F1 = portal expansion, F2 = bridging fibrosis, F3 = bridging fibrosis with lobular distortion, and F4 = cirrhosis (The METAVIR cooperative group, 1991).

2.4. Biochemical measurements

Blood samples were drawn from all subjects after an overnight fast.

Routine investigations included liver function tests, prothrombin time, prothrombin concentration (%), complete blood count (CBC), kidney function tests, fasting blood sugar, HCV antibodies, and serum AFP. For each patient Child Turcotte Pugh was calculated.

Real-time quantitative PCR was done by COBAS Ampliprep/TaqMan HCV monitor, with detection limit of 15 IU/ml; Roche Diagnostic Systems.

2.4.1. Isolation of DNA

Genomic DNA was isolated from white blood cells isolated from a 5 ml sample of whole blood collected into EDTA by a spin column method according to the protocol (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany). The extracted DNA was stored at −20 °C until the time of use.

2.4.2. Genetic polymorphism detection.

The subjects were genotyped for Factor V Leiden G1691A (rs 6025), prothrombin G20210A (rs1799963) and thrombospondin-1 (rs7057) mutations by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to Ashjazadeh et al. (2012) and Zhou et al. (2004). The sequence of the primers and band size for PCR product as stated in Table 1. PCR amplified products were run on a 3% ethidium bromide-stained agarose gel and visualized under a UV transilluminator.

2.5. Statistical analysis

Categorical variables were presented using frequency counts and descriptive parameters are presented as mean ± SD. Chi-square test (χ^2) was used to compare categorical variables between the groups. Genotype frequencies in cases and controls were tested for Hardy–Weinberg equilibrium, and any deviation between the observed and expected frequencies was tested for significance using the χ^2 test. Comparisons between groups of means were performed using ANOVA. A difference was considered significant at $P < 0.05$. All data were evaluated using SPSS version 10.0 of windows.

3. Results

3.1. Clinical characteristics of the study subjects (Table 2)

There was no difference between subgroups and control group in gender (p = 0.27). However, there was a highly significant statistical difference among the 4 patient subgroups and the control group in other demographic and laboratory data.

The incidence of blood group non-O in the subgroups mild, significant fibrosis, cirrhosis, HCC and controls was [51 (72.98%), 57 (81.4%), 67 (95.7%), 66 (94.3%), 63 (63%) respectively], and that difference was statistically highly significant ($\chi^2 = 40.017$, p = 0.000).

3.2. Distribution of studied genotypes in the studied groups (Table 3)

Distributions of resultant genotypes in the studied groups were in agreement with Hardy–Weinberg equilibrium in all groups. There were no significant differences in Factor V Leiden (G1691A) or TSP-1 (A2210G) polymorphism between the 4 patient subgroups and control group.

3.3. Association between prothrombin (G20210A) and development of hepatocellular carcinoma (Table 4)

In HCC patients, the frequencies of GA genotype of prothrombin gene were significantly increased compared with control subject. HCV patients carrying GA genotype were more likely to develop hepatocellular carcinoma (OR = 5.4, 95% CI = 1.09–27.05; P = 0.026).

4. Discussion

In this study conducted on 280 Egyptian patients infected with HCV who were selected and grouped into mild fibrosis, advanced fibrosis,