Original Article

Detection of Specific Antibodies to HCV-ARF/CORE+1 Protein in Cirrhotic and Non-Cirrhotic Patients with Hepatitis C: A Possible Association with Progressive Fibrosis

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Abstract

Background: The role of different viral proteins in the progression of the disease to cirrhosis is not completely understood. The ARFP/F protein is a newly described protein synthesized from the +1 or -2 reading frames of the core protein gene, which its function remains unknown.

The purpose of this study is to detect specific antibodies to HCV-ARF/Core+1 protein in cirrhotic and non-cirrhotic patients with HCV and investigate any possible association.

Methods: ARF/Core+1 recombinant proteins from HCV genotype 1a were expressed in Escherichia coli, and purified. Using an enzyme-linked immunosorbent assay, we assessed the prevalence of anti-ARF/Core+1 antibodies in 50 cirrhotic and 50 non-cirrhotic hepatitis C patients.

Results: All 50 cirrhotic patients were positive for anti-ARF/Core+1 antibody, while only 80% positive samples among non-cirrhotic patients were detected. The titer of anti-ARF/Core+1 antibody was also significantly higher in patients with cirrhosis than in non-cirrhotic patients.

Conclusion: Compared to 80% positive samples among non-cirrhotic patients all 50 cirrhotic patients were positive for anti-ARF/Core+1 antibody and titer of anti-ARF/Core+1 antibody was significantly higher in patients with cirrhosis than in non-cirrhotic. These results suggest that ARF/Core+1 protein is associated with cirrhosis. A possible causative association between ARF/Core+1 and cirrhosis as well as the mechanism of this association needs to be further investigated.

Keywords: ARFP/F protein, cirrhotic, hepatitis C virus, non-cirrhotic

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Introduction

Hepatitis C virus (HCV) is a major causative agent of blood-transmitted hepatitis, chronic hepatitis, and cirrhosis.[1] The pathogenesis of HCV has been associated with the interaction between host immune response and viral factors.[2] HCV is a leading cause of liver diseases including acute and chronic hepatitis, as well as cirrhosis and hepatocellular carcinoma (HCC).[3] In spite of the recent advances, the treatment of HCV is not optimal and a proper vaccine is not available yet.[3] The genome of HCV is about 9.6 kb with positive polarity and contains a large open reading frame (ORF) encoding a polyprotein of about 3000 amino acids that produce viral proteins after cleavage by the host and virus-encoded proteases.[4] Translation of the HCV polyprotein is regulated by an internal ribosomal entry site (IRES) at the 5' non-coding region of the genome.[5] The coding region of the genome encodes the classical HCV proteins including Core, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.[6] There is an evidence that the Core-encoding region of HCV expresses an additional protein of about 16 – 17 kDa named alternative reading frame shift protein (ARFP) or the frame shift (F) protein.[7,8] The Core sequence contains an adenosine-rich region located at the codon 8 – 14 in which -2/+1 ribosomal frame shifting can take place.[9,10] ARFP ends at different stop codons for various genotypes.[11] In the case of genotype 1a, F protein contains 161 amino acids. However, alternative forms of this protein such as ARFP/DF (double-frame shift) in genotype 1b, and ARFP/S (short form of core+1) were recently described.[9,10]

Many studies have shown that the ARF/Core+1 protein is recognized by antibodies[10] and memory T cells in HCV infected patients.[12] It has been reported that the ARF/Core+1 ORF is produced in 42 – 89 percent of non-cirrhotic HCV-infected people.[12–14] Higher expression of anti- ARF/Core+1 antibodies has been detected in specific stages of HCV infection such as advanced stages of cirrhosis and hepatocellular carcinoma.[15,16] However, the prevalence of anti- ARF/Core+1 antibody in cir-

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rhotic compared with chronic patients was not evaluated. The ARF/Core+1 protein may play some roles in pathogenesis and progress of the disease, however the exact function of this protein is still unknown.[17,18] Furthermore, ARF/Core+1 protein increases cell cycle growth through interference with p21.[18] In this study the prevalence and titer of anti-ARF/Core+1 antibody among cirrhotic and non-cirrhotic HCV patients has been studied.

Patients and Methods

Production of recombinant ARF/Core+1 protein

HCV-ARF/Core+1 gene of genotype 1a was amplified by Hotstart PCR amplification with introducing a deletion mutation in the forward primer of core gene. The PCR product was digested using restriction enzymes and ligated into the pET28a (+) expression vector (Novagen).

The constructed plasmid was transformed into BL21 *E. coli* cells. The expressed protein was verified with 15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

The bacterial pellet was resuspended in lysis buffer, lysed by sonication, centrifuged and inclusion body in the pellet was treated with 8M urea. The 6x His-tagged proteins in the supernatant were purified with Ni-NTA agarose column (QIAgen). The purified proteins were analyzed by 15% SDS-PAGE.[14]

Subjects

Fifty serum samples were collected from cirrhotic patients infected with HCV genotype 1a (the stage of fibrosis in these patients was between F3 and F4) and 50 samples were collected from non-cirrhotic treatment-naïve patients infected by genotype 1a. Study subjects were selected from patients referring to the HCV clinic of Shariati Hospital (Tehran University of Medical Sciences, Iran). Genotyping process had already been performed for the samples using RT-PCR RFLP method in Digestive Disease Research Center (DDRC) of Shariati Hospital. Patients discovered to have cirrhosis either by liver histology or clinical characteristics such as findings in abdominal ultrasonography (ascites, splenomegaly, coarse nodular small liver, and portal vein dilatation) or laboratory data (increased prothrombin time, decreased albumin level and etc). Patients with active hepatitis B virus (HBV) or human immunodeficiency virus (HIV) infection were excluded from this study. The inclusion criteria were Child-Pugh class a cirrhosis without prior decompensation, defined as a history of ascites, visceral bleeding, and hepatic encephalopathy, at inclusion and no other concomitant liver diseases. Non-cirrhotic samples were obtained from 38 males and 12 females. In this group the minimum and the maximum level of ALT and AST were (13 – 293 IU/mL) and (7 – 191 IU/mL), and the average of them were 132 IU/mL and 160 IU/ml respectively. Cirrhotic samples were taken from 44 males and 6 females. The average ALT and AST were 52 IU/ml and 43 IU/mL respectively. Furthermore, 30 samples were collected from non-HCV infected healthy blood donors referring to the Iranian Blood Transfusion Organization as negative controls and for cut-off determination. The average of ALT and AST in this group was 14 IU/mL and 12 IU/mL respectively. This study was approved by the hospital's ethics committee of Teheran University of Medical Science.

Enzyme-linked immunosorbent assay (ELISA)

Recombinant ARF/Core+1 protein was coated on the wells as described by Hashempour, et al.[14] The cut-off value was determined by adding three standard deviations to the average OD 450 nm of healthy individuals. After cut-off determination, patient's serum samples were added to the wells in 2 fold serial dilutions from 1/1000 to 1/32000. A 1/1000 dilution of a serum sample of healthy individual was added to the wells of negative controls. The appropriate controls were also included in each plate to check the stages of the process. Then, 1/10000 dilution of the whole human IgG conjugated with horseradish proxidase (Sigma-Aldrich) was added to all wells. The 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added to the wells and the optical density of each well was measured at OD 450 nm. All samples were tested in duplicates and to determine the reproducibly of the developed ELISA, titration of some samples were repeated in three different runs. Since there are about 10 shared aa between the N-terminal of core and ARF/Core+1 proteins, it is critical to determine possible cross reaction between core and recombinant ARF/Core+1 proteins. One of the major antigenic sites of the core protein is located within amino acids 11 – 45, therefore no cross-reactivity was reported between the detection of anti-F-antibodies and that of anti-core antibodies.[19,20] In addition to, several samples that were negative for F antibody, were also tested for core antibody with ELISA that all of them became positive.

Statistical analysis

Comparison between the presence of antibody in cirrhotic and non-cirrhotic groups was done using the Fisher's test. For analysis of mean antibody titers between two groups, *t*-test was used. All of the analysis was done by statistical software SPSS ver.15.

Results

The cut-off value was defined as the average OD 450 nm of sera from healthy individuals plus 3 standard deviation. In this study, the average OD 450nm of control group was obtained as 0.22 with a standard deviation of plus 3, thus the cut-off was determined to be 0.4. All 50 cirrhotic patients were positive for anti-ARF/Core+1 antibody, whereas only 80% of non-cirrhotic patients had positive results (Figure 1). Titration of positive sera showed that 98% of cirrhotic patients compared to 30% of non-cirrhotic group had a titer of 1/2000 or greater. Positive sera in 70% of non-cirrhotic patients had a titer of less than 1/2000. In the cirrhotic group, except for one sample, all had titers above 1/2000 (Figure 2). The mean of antibody titers between two groups was compared that the difference was statistically significant ($P < 0.001$).

In order to ascertain the absence of cross-reactivity, anti-ARF/Core+1 negative samples were tested for reaction with core antigen; all of which did react.

Discussion

There is some evidence that the core-encoding region of HCV expresses an additional protein by ribosomal frame shift mechanism; the ARF/Core+1 protein. Although the exact function of ARF/Core+1 protein is unknown, multiple studies have shown that it is recognized by humoral and cellular immune system.[10,15] There are some evidences showing that immunization of mice with ARF/Core+1 protein is able to induce a humoral response.[21] Karamitros, et al. found that the specific ARF/Core +1 antibodies were persistently present in chronic patients who were treated

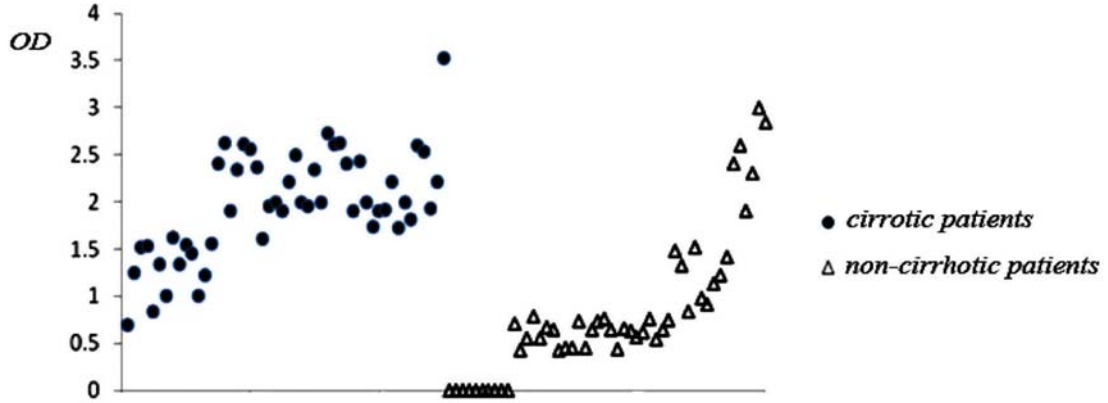

Figure 1. Prevalence of anti-F antibody in cirrhotic and non-cirrhotic patients. The circles represent the cirrhotic group while triangles represent the non-cirrhotic group. All 50 cirrhotic patients were positive for anti-ARF/Core+1 antibody whereas only 80% with positive result detected among non-cirrhotic patients (Circles: cirrhotic patients. Triangles: non-cirrhotic patients).

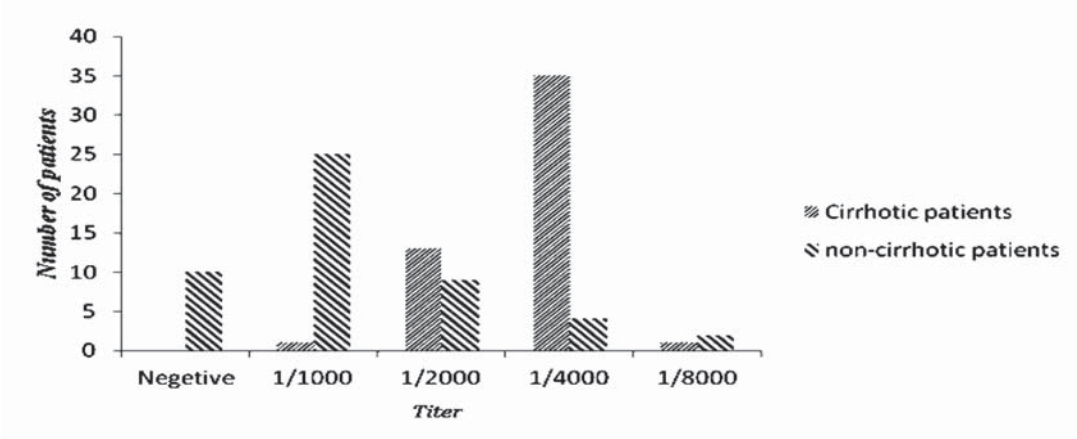

Figure 2. Titration of the antibody against F protein in the serum of cirrhotic HCV compared to non-cirrhotic patients.

with Peg-IFN and ribavirin during treatment follow-up in accordance with levels of HCV-RNA.[22]

Some researchers have suggested that ARF/Core+1 protein may have a role in the progress of hepatic disease to cirrhosis or HCC.[17,18] Results of the present study showed that the prevalence of anti- ARF/Core+1 antibody in non-cirrhotic patients to be 80%, which is in accordance with previous reports ranging from 62 to 89%.[13,16] In addition, it was observed that the prevalence of anti-ARF/Core+1 antibody and its titer was significantly higher in cirrhotic patients. Development of cirrhosis is mainly due to persistence of hepatic injury leading to failure in liver regeneration and induction of fibrosis.[16,23] Thus, the immune system of cirrhotic patients has been often exposed to hepatitis C antigens for a longer period than non-cirrhotics. This longer period of exposure might explain why anti-ARF/Core+1 antibodies are more frequent in cirrhotics. Alternatively, the higher titer and prevalence of ARF/Core+1 protein among cirrhotics might indicate a causative relation. Further studies are required to settle this issue.

The presence of anti-ARF/Core+1 antibodies in the serum of HCC patients suggests that ARF/Core+1 protein might also be involved in HCV-induced hepatocellular carcinoma.[24]

Although the complete molecular mechanism of progression toward fibrosis, cirrhosis and HCC in HCV infected livers needs to be further studied, there is a circumstantial evidence suggesting that immune-mediated mechanisms are crucial in establishment of cirrhosis. The dynamics of ARF/Core+1 protein antibody titration as well as the relationship of ARF/Core+1 antibody and immune response to HCV infection may provide some clues. Inflammation and related cytokines play a pivotal role in all stages of the disease. In addition, activation of cytotoxic T cells and continued induction of apoptosis pathways will result in persistent liver damage. ARF/Core+1 protein might contribute to persistence of HCV infection and hepatic damage[25–29] by modulation of cellular factors involved in hepatic damage. It has been reported that ARF/Core+1 protein can stimulate T cells and also transactivate some genes involved in cell apoptosis.[30] ARF/Core+1 protein also regulates p21 expression which in turn modulates apoptosis.[30,31]

Although the role of ARF/Core+1 protein in stimulation of inflammatory cytokines and progress to cirrhosis needs further clarification, our data suggest that the presence of ARF/Core+1 protein is associated with more advanced liver fibrosis in patients with hepatitis C. Whether this is a causative association or merely the result of longer duration of infection in fibrotic, cirrhotic and HCC patients remains to be determined. Both HCV core and F antigens could enhance liver cell proliferation[32] and subsequently might progress to cirrhosis. It has been suggested that F protein overproduction may lead to a loss of apoptosis, cell cycle arrest, differentiation and antiangiogenesis activity in the host. Also the

increase of cell proliferation in response to growth promoting signals,[11] suggest a possible role of F protein in the transforming ability of HCV in cases of HCV associated cirrhosis.

This study is the first survey that was done to determine the role of ARF protein in cirrhotic patients in Iran. In the future studies use of truncated ARF and Core proteins are recommended.

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Conflict of interest

The authors of this article declare that they have no conflict of interest related to the material in the manuscript.

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