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Original Article

Development of a Recombinant Based ELISA using Specific Antibodies to F Protein in HCV Chronically Infected Patients-A Seroprevalence Study

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Abstract

Background and Aims: The hepatitis C virus (HCV) F protein is a recently described, frameshift product of HCV core encoding sequence. Its function and antigenic properties are unknown. In order to assess the presence of antibodies specific for F protein we characterized specific anti-F antibodies in patients with chronic HCV infection.

Methods: The F protein was cloned from the HCV genome. The recombinant protein was expressed in Escherichia coli and purified by immunoaffinity chromatography. An enzyme-linked immunosorbent assay was developed using the purified recombinant HCV F protein.

Results: Serum samples were collected from 72 patients with chronic HCV infection and from 30 healthy controls. Eighty-two percent of chronic HCV patients had evidence of anti-F antibodies. 59 samples out of the 72 HCV infected patients exhibited a positive anti-F reaction, showing significant difference from the controls with no HCV infection ($P < 0.01$).

Conclusion: Based on these findings, HCV F protein elicits a specific antibody response, so frameshift could occur in the core-coding sequence in HCV genotype 1a in Iranian patients. As the first report, the prevalence of anti-F antibodies in chronic hepatitis C in Iran is of the order of 82%.

Keywords: Hepatitis C virus; HCV F protein; Alternative reading frame protein

Introduction

Hepatitis C virus (HCV) is a small, enveloped positive-stranded RNA virus with a genome size of approximately 9,600 bases long, classified within the genus Hepacivirus, family Flaviviridae. HCV is a major cause of chronic liver disease which chronically infects more than 170 million persons worldwide. The prevalence of HCV infection varies throughout the world affecting an estimated 3% of the world's population, with rates reaching more than 10% in some countries (1-3). In Iran, the prevalence of anti-HCV was reported to be 0.5% in general population (4). The HCV genome contains a large open reading frame flanked by nontranslated regions. The coding region encodes a polyprotein of approximately 3000 amino acids that is cleaved by host and virus-encoded proteases producing four structural proteins (core, E1, E2 and P7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (4-7).Subsequent

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studies by several independent groups provided evidence of a 14- and 17-kD protein designated as F (for "frameshift") or ARFP (for "alternative reading frame protein") (8-10). This protein encodes during core translation from the first nucleotide of core-encoding region and by a -2/+1 ribosomal frameshift ends at different stop codons for various genotypes (11, 12).

For HCV genotype 1a the frameshifting takes place at or near codon 11 where an adenosine-rich region located, causing a 161 aa protein (13, 14). The function of ARFP in virus lifecycle and its role in pathogenesis are still unknown (12). Recent studies have suggested probable roles for F protein in pathogenesis of HCV. It has been shown that F is expressed at high level in advanced stages of disease and cancer (15). Although it has known that specific antibody and memory T cells (11,16, 17) are induced against F protein, the level of immune system activation varies in different population which may be due to unknown frequency of the frameshift event. In other word, specific flanking regions of the frame shift point may influence the frequency of F production. Regarding the probable role of F protein in viral pathogenesis it is of great importance to determine the level of F protein by quantitation of anti-F antibody in HCV patients which may vary in different communities on the circulating HCV isolates and different virus- host interactions. In the present study, the prevalence of specific anti-F antibodies in the Iranian patients with well-characterized chronic HCV infection was investigated.

Methods

HCV F gene cloning

A cDNA clone of Core gene of genotype 1a was a gift from Dr. M Baril (Departement of Biochimie, University of Montreal) (3) which used as template for amplification of F gene. A 485-bp fragment encoding F gene was amplified using the specific sense primer:

5-TACATATGATGAGCACGAATCCTAAA CCTCAAAGAAAACCAAAC-3.

5-ATATAAGCTTTAATTCACGCCGTCCT CCAGAACC-3

The primers were designed by GeneRunner software and the appropriate restriction sites were included at the 5′ ends of both primers. The Hot-start PCR amplification with introducing a deletion mutation in nt 31 was carried out using 25 µl of reaction mixture containing 0.5-1 µg of the cDNA clone, 1 picomol of each primer, 0.2 mmol/L deoxynucleotide triphosphate (dNTPs) mix, PCR buffer l0×, 1.5 mM of MgCl2, and 0.2-0.3 µl of Taq DNA polymerase for 30 cycles. The cycling conditions were 96 °C for 5 min, 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min.

The PCR product was digested using NdeI and HindIII enzymes and ligated into the pET28a(+) expression vector (Novagen). The constructed plasmid was verified by restriction enzyme analysis and sequencing.

Production of recombinant F protein

The constructed plasmid was transformed into BL21 E. coli cells and cultured overnight at 37◦C in LB medium containing 50 μg/ml kanamycine. Afterwards, the bacteria were subcultured in diluted cultures until an optical density at 600 nm (OD600) of 0.6-0.8 was reached. The culture was induced with 1 mmol/ml isopropyl-β-D-thiogalactopyranosole *(IPTG)* at 37◦C for 7h, and centrifuged at 12,000 rpm for 5min at 4◦C. The lysis buffer (2mM Imidasole, 50mM Tris-base, 300mM NaCl, 1mM PMSF with pH:8) was add to the pelleted bacteria. After sonication and centrifugation at 12,000 rpm for 30min at 4◦C, the lysis buffer was re-added to the pellet. The expressed protein was verified with 15% SDS-PAGE (sodium dodecyl sulfate- polyacryl-amide gel electrophoresis).

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Protein purification

The pelleted bacteria was resuspended in lysis buffer containing 50mM Tris, 300mM NaCl, 1% Triton X-100, 1.5% 2ME, with pH 8, and were lysed by sonication. Then, centrifuged at 12000rpm for 30 minutes at 4◦C. Inclusion body in the pellet were treated with 8M urea. The 6x His-tagged proteins in the supernatant were purified on Ni-NTA agarose column (QIAgen) using elution buffer containing 50mM Tris, 300mM NaCl, 250mM Imidasole with pH 8.

The purified proteins were analyzed in 15% SDS-PAGE.

Enzyme-linked immunosorbent assay (ELISA)

Microplates were coated with 5 μg/ml of purified F protein in bicarbonate buffer pH 9.6 at 4◦C. After overnight incubation, wells were washed with PBS-Tween 20 and tapped 6-7 times for three rounds. Then, wells blocked with 1% Gelatin in PBS for 2h at 37◦C. The coated wells were washed three times with PBS-Tween 20 as mentioned before and serum samples were added in dilution of 1:500 in duplicate for 1h at 37◦C. The wells were washed and incubated with whole human IgG conjugated with horseradish proxisade (Sigma, UK) for 1h at 37ºC. After washing, 3, 3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma, UK) was added to wells for 10-15 minutes in darkness at room temperature. The reaction was stopped by the stop solution (H_2SO_4, 2N) and the absorbancy was measured at 450 nm.

The cutoff was determined as mean of OD of 30 negative sera + 3SD.

Patients

Serum samples from 72 patients with chronic HCV infection of genotype 1a obtained from Shariati Hospital and 30 samples from healthy individuals (Blood Transfusion Center, Tehran) were analyzed for anti-F antibodies. The size sample was calculated by the following formula:

$$n = \frac{(Z_{1-\frac{\alpha}{2}})^2 \times pq}{d^2}$$

Reproducibility Assay

To determine the reproducibly of the developed ELISA, the optimized test was repeated twice for many samples. In each plate the sample was tested in duplicate. Eleven positive samples and twenty negative samples were tested by two independent assays.

Statistical analysis

Comparison between positive and negative groups was done using unpaired *t*-test.

Results

To evaluate the prevalence of anti-F Antibody in Iranian chronically HCV infected individual, an Enzyme-linked immunosorbent assay (ELISA) was performed with F-1a recombinant protein:

To produce the recombinant protein, the specific sense primer was designed in which according to the +1 ribosomal frame shift at the codon 11 one adenosine at position 372 of whole genome was deleted. The PCR product was analyzed in agarose gel (Fig. 1A).

After cloning F gene into the pET28a(+) expression vector and confirming the constructed plasmid (Fig. 2A), the plasmid was transformed into E.coli strain BL21,and the protein was expressed in different concentrations and times of induction with IPTG. The maximum expression was attained where 1mM IPTG was added for 7 h. Since the expressed protein formed inclusion bodies, the protein was unfolded with 8M Urea and the 6x His-tagged protein was purified on Ni-NTA agarose column (Fig. 2).

The purified 24 kD protein was used as antigen in ELISA assay for detection of anti-F antibody in Iranian chronically HCV infected individual.

Several strategies were experienced in order to develop an ELISA with optimal signal/noise ratio. Alternative reagents were tested for each step. For all steps, different dilutions were tested and the optimal dilution was used in all subsequent experiments.

The maximum signal/noise ratio was attained with bicarbonate buffer pH 9.6 as coating buffer, and 1% gelatin in PBS as blocking reagent.

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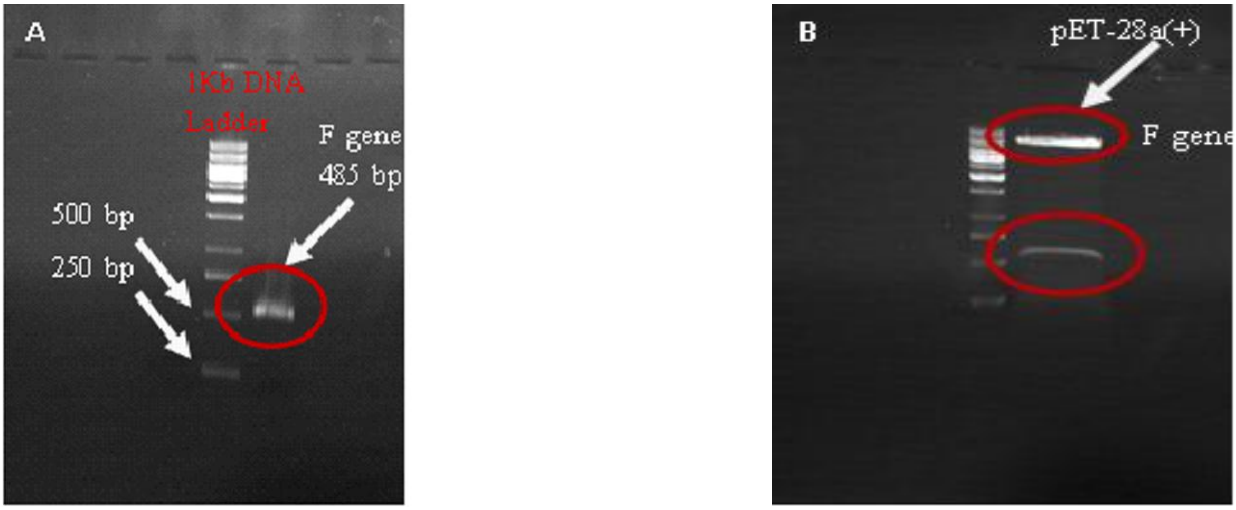


Fig. 1. (A): Agarose gel electrophoresis of the amplified F gene from Core template by PCR. (B): Confirmation of cloned F gene in Pet32 plasmid by restriction enzyme digestion.

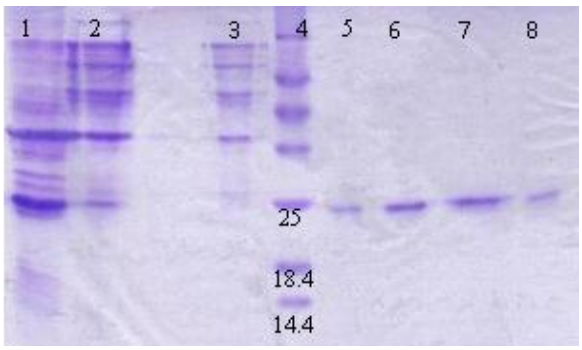


Fig. 2. Expression and purification of the HCV recombinant proteins F with SDS-PAGE analysis. 1) The supernatant of induced bactria after treatment with urea. 2) The pellet of bactria after treatment with urea. 3) Non-specific proteins washed from column. 4) Protein Molecular Weight Marker. 5-8) Different fractions of the eluted F protein.

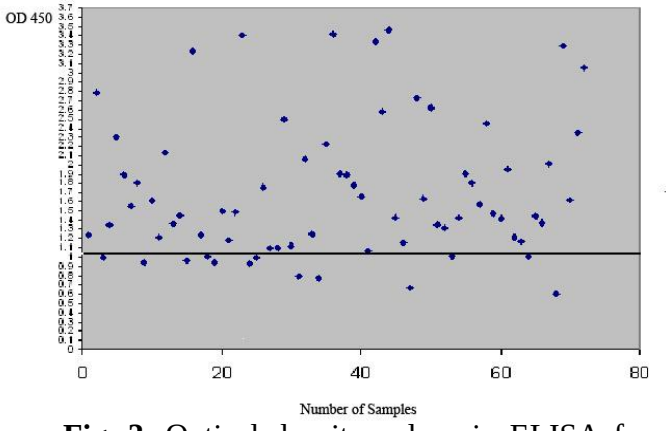


Fig. 3. Optical density values in ELISA for HCV F antibody in chronic patients.

The cut off value was obtained as the mean OD450 nm of sera from healthy individuals and 3SD, which was found to be 0.93. Sera were considered positive for anti-F antibodies if the optical density was above the cut off value. All the control groups were negative for anti-F antibodies and of the 72 patients, 59 (82 %) were positive for anti F antibodies (Fig. 3). To determine the reproducibly of the developed ELISA, the optimized test was repeated twice for each sample.

Discussion

HCV F protein is a newly identified HCV gene product that is produced from the core encoding region by a translational ribosomal frame shift (18). For HCV genotype 1a-derived ARFP, the frameshifting occurs at codons 9 to 11 of the core protein sequence (19). Little is known about the biological properties of this protein. F protein may play a role in viral entry and/or viral morphogenesis or regulate the cellular function mediating in the viral life cycle (20).

It has been suggested that the protein may have a role in viral pathogenesis (21). In order to study the prevalence of anti-F antibodies in patients with chronic HCV infection, HCV F recombinant protein from HCV genotype 1a was expressed in E. coli. The results showed

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that 59 samples out of the 72 (82%) HCV infected patients exhibited a positive anti-F reaction, showing significant difference from the controls with no HCV infection ($P < 0.01$).
This fact that the major antigenic domain of Core protein is located in amino acids 11-45, and the shared part of F and Core is the first 10 amino acids, indicates the absence of cross-reactivity between core and F antigenic domains. Furthermore, to investigate the lack of cross reactivity between core and F some samples were tested for both Core and F antibody in which there were some samples reacting with Core without any significant detection of F antibody (data not shown).
The seroprevalence of anti-F antibodies in our study is in accordance with the results reported by Cohen et al who investigated specific anti-F antibodies in 89% of the 1b hepatitis C patients in Israel (18), but are higher than those reported by Miladi *et al*. who observed anti-F antibodies only in an estimated 10% of HCV patients infected with genotype 1b in France (17). To our knowledge there is only one report of 62% seroprevalance of anti F in 1a chronically infected patients in France (20). The different races of the patients, different HCV genotypes and isolates in various geographical regions might be the reasons leading to the different reported data, the same as reported in previous studies that the geographical distribution of each HCV genotype influence clinical implications, such as response to therapy and disease progression (22).
The complete mechanisms applied with Hepatitis C Virus to cause a chronic infection are still poorly understood (23). Persistent infection with HCV is associated with the expression of proteins that can inhibit viral elimination by the immune system. Among the HCV proteins, NS5A, E2, and NS3 have been shown to interfere with IFN-α or $-\beta$ treatment, causing antiviral resistance (16, 24). The HCV core protein perturbs the function of cellular proteins involved in T-cell proliferation and induces apoptosis, thereby leading to immune suppression and liver damage (18). Our results confirm the expression of F protein during natural HCV infection, but the role of F protein in the HCV life cycle and the significance of anti-F antibodies in chronic HCV infection have yet to be elucidated. Further studies are needed to determine if immune response has potential value for diagnostic tests.

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