High prevalence of antibodies to core+1/ARF protein in HCV-infected patients with advanced cirrhosis

Katerina Kassela, Ioannis Karakasiliotis, Stefanos Charpentidis, John Koskinas, Theodora Mylopoulou, Konstantinos Mimidis, Christoph Sarrazin, Georgios Grammatikos and Penelope Mavromara

Abstract
Hepatitis C virus (HCV) possesses a second open reading frame (ORF) within the core gene encoding an additional protein, known as the alternative reading frame protein (ARFP), F or core+1. The biological significance of the core+1/ARFP protein remains elusive. However, several independent studies have shown the presence of core+1/ARFP antibodies in chronically HCV-infected patients. Furthermore, a higher prevalence of core+1/ARFP antibodies was detected in patients with HCV-associated hepatocellular carcinoma (HCC). Here, we investigated the incidence of core+1/ARFP antibodies in chronically HCV-infected patients at different stages of cirrhosis in comparison to chronically HCV-infected patients at earlier stages of disease. Using ELISA, we assessed the prevalence of anti-core+1 antibodies in 30 patients with advanced cirrhosis [model for end-stage liver disease (MELD) ≥15] in comparison with 50 patients with mild cirrhosis (MELD <15) and 164 chronic HCV patients without cirrhosis. 28.7% of HCV patients with cirrhosis were positive for anti-core+1 antibodies, in contrast with 16.5% of non-cirrhotic HCV patients. Moreover, there was significantly higher positivity for anti-core+1 antibodies in HCV patients with advanced cirrhosis (36.7%) compared to those with early cirrhosis (24%) (P<0.05). These findings, together with the high prevalence of anti-core+1 antibodies in HCV patients with HCC, suggest that core+1 protein may have a role in virus-associated pathogenesis, and provide evidence to suggest that the levels of anti-core+1 antibodies may serve as a marker for disease progression.

INTRODUCTION
Hepatitis C virus (HCV) infection is a major cause of chronic liver disease that often leads to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [1]. HCV affects approximately 150 million people worldwide and is the main cause of liver transplantation in developed countries [2, 3]. The considerable genetic variability of HCV has led to its classification into seven genotypes and several subtypes [4]. No vaccine is yet available, and pegylated interferon and ribavirin regimes, which are still applied in countries with limited health-care resources, result in a sustained virological response (SVR) in only half of the patients infected with the most common genotypes [5, 6]. Recently, the approval of new anti-HCV drugs (direct antiviral agents, DAAAs) has shown significant effectiveness in all HCV genotypes, but access to such treatment remains limited due to its high cost [7].

HCV is an enveloped virus of the Flaviviridae family with a single-stranded positive-sense RNA genome encoding a polyprotein precursor of approximately 3000 aa. An internal ribosome entry site (IRES) located in the 5′ UTR mediates the translation of the polyprotein, which is cleaved by cellular and viral proteases to yield at least 10 structural and non-structural proteins [8, 9].

Unexpectedly, the HCV genome contains a second functional open reading frame (ORF) within the core region, encoding a protein known as ARFP (alternative reading frame protein), F (frameshift) or core+1 [10–12]. The biological role of this protein remains elusive, since its expression is not required for
Here, we investigated the presence of anti-core +1 antibodies in HCV patients with mild and advanced cirrhosis. Our results demonstrate an increased prevalence of anti-core +1 antibodies in HCV patients with advanced cirrhosis [model for end-stage liver disease (MELD) ≥15] compared to cirrhotic patients at earlier stages (MELD <15) or non-cirrhotic patients, and suggest that the presence of anti-core +1 antibodies in HCV-infected patients may present a marker for the progression of liver disease.

RESULTS

Prevalence of anti-core +1 antibodies in HCV infected patients with and without cirrhosis

To investigate the prevalence of anti-core+1 antibodies, we developed a sensitive ELISA protocol using a recombinant core+1 protein corresponding to amino acids 14–160 from genotype 1a, produced in Escherichia coli as described in the Methods section. A total of 80 HCV-positive serum samples from patients with cirrhosis and 164 samples from patients with chronic HCV infection without cirrhosis were examined for the presence of anti-core+1 antibodies. The patients’ group with cirrhosis consisted of 50 patients at an earlier stage of the disease and 30 patients at an advanced stage, based on their MELD score. In parallel, 51 serum samples obtained from healthy blood donors (control group) and 26 serum samples obtained from patients with non-HCV cirrhosis (HBV-positive) were used to assess the specificity of the method.

As shown in Fig. 1(a), the prevalence of anti-core+1 antibodies was significantly increased in the HCV patients with cirrhosis (28.7%) as compared to chronic HCV patients without cirrhosis (16.5%) (P=0.0026). Furthermore, when we only considered genotype 1 patients, the positivity rate changed to 35% for HCV cirrhotic patients and 18.6% for HCV patients without cirrhosis (P=0.0001). This could be related to the use of genotype 1a core +1 as the antigen for the ELISA test, as the core+1/ARFP protein is not well conserved among HCV genotypes [28]. Moreover, Student’s t-test analysis verified that the prevalence of anti-core+1 antibodies was significantly higher in HCV patients with advanced cirrhosis (MELD ≥15) as compared to those with mild cirrhosis (MELD <15) (P<0.05) (Fig. 1b). Specifically, anti-core+1 reactivity was detected in 36.7% of serum samples from cirrhotic patients with a MELD score ≥15, whereas 24% of patients with a MELD score <15 were positive for antibodies against the core +1 protein. No or weak reactivity was detected in healthy blood donors and the HBV-positive group, respectively (Fig. 1a).

Correlation of core +1 positivity rate with patients’ baseline characteristics and transaminases in cirrhotic HCV patients

In order to further dissect the possible relationship between core +1 positivity and other disease parameters we
performed correlation tests with patients' baseline characteristics. No difference was detected in the median age between the different disease groups (Table 1). In addition, the anti-core+1 response in cirrhotic patients was also analysed against the levels of transaminases. As indicated in Fig. 2(a), the levels of alanine transaminase (ALT) were significantly reduced in cirrhotic patients who were positive for anti-core+1 antibodies ($P=0.0145$). Furthermore, the levels of gamma-glutamyltransferase (GGT) and aspartate transaminase (AST) were also decreased in cirrhotic patients who responded to core+1 protein, but this was without statistical significance ($P=0.2744$ and 0.1434, respectively) (Fig. 2b, c). The serum levels of various other markers (creatinine, bilirubin, leukocytes, albumin, etc.) were also assessed. No correlation was identified between these markers and anti-core +1 positivity (Fig. S1, available with the online Supplementary Material).

**Analysis of the core/core +1 coding region of HCV isolates derived from patients with cirrhosis**

In order to investigate whether seroconversion to core+1 was associated with specific mutations in the core/core +1 region that could impair protein expression, we analysed core/core+1 sequences obtained from 23 HCV-1a/1b serum samples from patients with cirrhosis. Eleven samples were anti-core+1-positive and 12 were anti-core+1-negative. Interestingly, the amino acid alignment of the core +1 region showed a significant statistical difference in the conservation of codons 85/87, the potential translation initiation codons for the short core+1/ARFP isoform [29]. At position 87, a methionine codon was only highly conserved among isolates derived from the anti-core+1-positive cirrhotic patients ($P=0.0113$). That is, of the 12 isolates of the anti core+1-negative group, only 3 had an AUG codon at position 87, whereas 9 out of 11 isolates of the anti-core+1-positive group had an AUG codon at the same position. Codon 85 was highly conserved among all cirrhotic patients. A strong conservation was also detected for the potential translation initiation codon 26, the proposed start codon for the long core +1/ARFP isoform. All of the analysed sequences had either the amino acid valine (GTG) or the amino acid alanine (GCG) at this position. (Fig. S2). Further, it has been suggested that mutations at nucleotide level could affect the secondary structure of RNA in a way that favours or inhibits core+1 protein synthesis [30]. Thus, analysis of the predicted RNA secondary structure in the core/core +1 coding region of the same isolates was also performed. We analysed the predicted secondary structures of the stem loops SL47 (nt 388–423) and SL248 (nt 588–665), where codons 26 and 85/87 are located, respectively. Concerning the SL248 (nt 588–665) structure, no profound differences were detected between isolates from patients who were positive or negative for anti-core+1 antibodies. On the other hand, an important difference was observed at nucleotide 418 within SL47 (nt 66 of core sequence), where a trend for C rather than U was observed among the isolates from the negative anti-core +1 patients ($P=0.068$). (Fig. S3).

**DISCUSSION**

HCV-associated cirrhosis and HCC represent a major health problem worldwide, since they are responsible for about 1 million deaths every year [31]. According to the World Health Organization, 15–30 % of patients with chronic HCV infection evolve to cirrhosis, and 1–4 % of them progress to HCC annually [3]. Despite the significant effectiveness of the new anti-HCV drugs, treatment of HCV patients, especially those who suffer from advanced or decompensated cirrhosis, is still a challenge [3]. Thus, it is of major importance to identify novel and reliable biomarkers that can predict disease progression. The MELD score is currently used to evaluate the severity of liver cirrhosis and is widely applied in order to stratify patients eligible for liver transplantation [32]. However, despite its high efficacy in predicting short-term mortality, the MELD scoring system also has some weaknesses [33]. Over the years, various modifications have been proposed to improve MELD’s predictive accuracy, such as the incorporation of serum Na concentration or patient’s age into its formula [33, 34].

In this study, we investigated the prevalence of anti-core+1 antibodies in HCV patients with mild and advanced cirrhosis. Specifically, we assessed the prevalence of anti-core +1 antibodies in 30 patients with advanced cirrhosis (MELD $\geq 15$) compared to that in 50 patients with mild cirrhosis (MELD $<$ 15) and 164 chronic HCV patients without cirrhosis. Our results indicated that anti-core +1 antibodies were detected in 28.7 and 16.5 % of cirrhotic and non-cirrhotic patients, respectively ($P=0.0026$), while positivity for anti-core +1 antibodies was significantly higher in HCV patients at advanced (36.7 %) as opposed to early stages of cirrhosis (24 %) ($P<0.05$). These findings, combined with previous results indicating a high prevalence of anti-core +1 antibodies in HCV patients with HCC [16], provide strong evidence that the presence of anti-

**Table 1. Characterization of HCV-infected patients with cirrhosis**

<table>
<thead>
<tr>
<th>Anti-core+1 positivity (n)</th>
<th>Stage of disease</th>
<th>MELD $\geq 15$, n=30</th>
<th>MELD $&lt;$ 15, n=50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive response n=11</td>
<td>Negative response n=19</td>
<td>Positive response n=12</td>
</tr>
<tr>
<td>Age</td>
<td>Median age</td>
<td>48</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>40–71</td>
<td>41–76</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4</td>
<td>12</td>
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Core +1 antibodies in HCV patients may indicate the progression of liver disease. Thus, evaluating the presence of anti-core +1 antibodies may improve the stratification of patients eligible for liver transplantation.

Core +1/ARFP is a newly discovered protein of HCV. According to the first reports, the core +1/ARF protein was shown to be synthesized by a -2/+1 ribosomal frameshift occurring in an A-rich (10 consecutive adenines) region within core codons 8–11, present in the HCV-1 prototype isolate [11, 12]. However, the majority of HCV strains cannot support the suggested frameshift mechanism, as they lack the cluster of 10 As [11, 35, 36]. Instead, subsequent studies from our laboratory and others have shown that the internal translation initiation at methionine codons at positions 85/87 or at the non-AUG codon 26, in the +1 ORF, of HCV-1a and HCV-1b, serves as the main mechanism for core +1/ARF protein production [35–39]. In addition, our laboratory recently demonstrated the expression of core +1/ARF protein from HCV-2a for the first time in the context of a bicistronic JFH1-based replicon [29]. This study demonstrated the synthesis of two isoforms, core +1/L (long) and core +1/S (short), with different expression kinetics during the replication of the JFH1 replicon [29]. The ribosomal frameshift at codons 8–11 was not detected in the replicon system [29].

A number of mutations in the core/core +1 region have been associated with increased HCC risk in HCV-infected patients [40]. However, to date respective data are lacking in cirrhotic patients. In order to further elucidate the fact that anti-core +1 antibodies were only detected in some patients, we analysed the core/core +1 sequences of 11 anti-core +1-positive and 12 negative patients. Sequence analysis of this region revealed a remarkable fluctuation between two amino acids in codon 87, a potential translation initiation site for the short isoform of core +1/ARFP. The presence of an AUG codon at position 87 was more prevalent among isolates from anti-core +1-positive patients (P=0.0113). This is in contrast to the strong conservation observed at codons 26 and 85, which are considered to be alternative translation initiation sites for the core +1 ORF. In addition, the predicted RNA secondary structure analysis in the core/core +1 coding region revealed that a C-U
variation at position 418 within the SL47 loop region had a significant trend towards C among anti-core +1-negative patients ($P=0.068$). On the other hand, no significant differences were observed for the SL248 structure. Genetic variability within core/core +1 region of HCV clinical isolates from cirrhotic patients, taken together with anti-core +1 antibody positivity, might reflect differences in core +1/ARF protein expression. However, the number of available samples remains small and additional studies are required.

A significant number of independent studies have reported the presence of anti-core +1 antibodies in the sera of chronically infected HCV patients. However, the reported prevalence of anti-core +1 antibodies varies significantly, ranging from 11.5 to 89% [15, 17–22]. Such differences could be attributed to several factors, including the nature of the core +1 antigen and the sample cohort. In fact, the use of a recombinant core +1 protein that shared the first 10 amino acids with the core protein (F protein) led to higher anti-core +1 positivity (62%), whereas the reactivity was significantly reduced (25%) when smaller core +1 peptides were used [28]. Similarly, Ajjorloo et al. recently reported a 100% anti-core +1-positive response in HCV cirrhotic patients, and 80% in chronic HCV patients without cirrhosis, using a recombinant F protein synthesized in E.coli [19]. This is in contrast to our data, in which we detected anti-core +1 antibodies in 28.7% of HCV cirrhotic patients using a full-length core +1 protein lacking any core amino acid sequences. Alternatively, the purification process for recombinant core +1 protein from E.coli may differ between our study and that of Ajjorloo et al.

ALT levels in cirrhotic patients positive for anti-core+1 antibodies were significantly reduced compared to those in anti-core+1-negative cirrhotic patients ($P=0.0145$). This observation was not related to the extent of liver damage, as no significant correlation was observed between ALT levels and MELD scores in our cohort ($P=0.35$) (Fig. S4). Interestingly, a recent study by Rao et al. that included 1000 treatment-naïve HCV patients from China suggested that low levels of ALT might be a risk factor for the development of cirrhosis [41]. Other factors, most likely related to the biological function(s) of core +1/ARFP, such as its ability to modulate liver immune responses, may also be important.

In conclusion, the prevalence of anti-core+1 antibodies was significantly increased in HCV patients with advanced cirrhosis, as well as in cirrhotic patients with HCC [16], supporting the possible association of core +1/ARFP protein with the progression of HCV-induced cirrhosis and carcinogenesis. Additional studies are required to clarify the significance of anti-core+1 antibodies and evaluate their prevalence as a marker for disease progression.

**METHODS**

**Patients**

Serum samples from 244 HCV-infected patients (obtained from Goethe University Hospital, Frankfurt am Main, Germany, Hippokration Hospital, Athens, Greece, and University Hospital of Alexandroupolis, Thrace, Greece) were used in this study. Of these, 80 samples [HCV gen1=48 (including 1a: 27, 1b: 15 and not determined: 6), HCV gen2=3, HCV gen3=16, not determined=13] originated from patients with HCV-associated cirrhosis, whereas the remaining 164 samples [HCV gen1=113 (including 1a: 42 and 1b: 71), HCV gen2=14, HCV gen3=24 and not determined=13] were obtained from patients with chronic HCV infection without cirrhosis. The patient group with cirrhosis consisted of 50 patients at earlier stages of disease and 30 patients at advanced stages. Fifty-one serum samples from healthy blood donors (kindly provided by Agoritsa Varaklioti, Blood Center, Laiko General Hospital, Athens, Greece) constituted the control group. Further, 26 serum samples from patients with HBV-associated hepatic cirrhosis were also included in the study.

**Expression and purification of recombinant core +1 protein**

The pHPl8120 vector, constructed as described earlier [30], was used for the expression of core +1 protein corresponding to amino acids 14–160 from genotype 1a. The recombinant core +1 protein was expressed in E.coli BL21 (DE3) cells. Expression of recombinant protein was induced for 3 h with 0.2 mM IPTG. The bacteria were pelleted by centrifugation at 5000 r.p.m. for 10 min and lysed in 8 M urea buffer [8 M urea, 0.1 M NaH$_2$PO$_4$, 250 mM NaCl, 10 mM Tris (pH 8.0)], with this followed by sonication (10×30 s) and then incubation at 4°C overnight on a rotary shaker. Subsequently, the lysate was centrifuged at 8000 r.p.m. for 1 h and the supernatant was boiled to Ni–NTA agarose beads. The 6×His-tagged core +1 protein was purified under denaturing conditions, essentially according to the manufacturer’s instructions (Qiagen, Germany). Recombinant protein was eluted from the column five times, with 0.5 ml of elution buffer [8 M urea, 0.1 M NaH$_2$PO$_4$, 250 mM NaCl, 10 mM Tris (pH 4.5)]. In order to improve the purity of the eluted protein, the elution fractions were pooled and purified further using fresh Ni–NTA agarose beads under native conditions. Briefly, the protein elution fractions were applied onto an Ni–NTA column in 30 mM imidazole binding buffer [0.1 M NaH$_2$PO$_4$, 0.3 M NaCl, 30 mM imidazole, 10 mM Tris (pH 7.4)] and incubated for 3 h at 4°C with thorough mixing. Next, the beads were washed five times with binding buffer and the core +1 protein was eluted four times with 0.5 ml of 500 mM imidazole buffer [0.1 M NaH$_2$PO$_4$, 0.3 M NaCl, 500 mM imidazole, 10 mM Tris (pH 7.4)]. The purity of the recombinant antigen was evaluated by Coomassie Brilliant Blue staining of a 15% (v/v) polyacrylamide SDS-PAGE gel. The expression of His-tagged core +1 protein was validated by Western blot analysis using an in-house rabbit polyclonal core +1 antibody (genotype 1b) [16] at a dilution of 1:500.

**ELISA for the detection of anti-core +1 antibodies**

Microplate wells were coated with 100 μl of a 1 μg ml$^{-1}$ solution of core +1 antigen in 50 mM sodium carbonate (pH
9.6) overnight at 4 °C. Next, the wells were washed with washing buffer [0.1% (v/v) Tween-20 in PBS+1% (w/v) non-fat dried milk] and blocked with PBST [0.1% (v/v) Tween-20 in PBS] containing 5% (w/v) milk for 1 h at 37 °C. The wells were washed three times with washing buffer and then 100 µl of serum diluted to 1:200 in washing buffer was added in triplicate wells and the plates were incubated overnight at 4 °C. After incubation, the wells were washed three times with washing buffer and then incubated with 100 µl of peroxidase-conjugated rabbit anti-human IgG antibody (Dako) at a dilution of 1:4000 (in washing buffer) for 1 h at 37 °C. After three more washes with washing buffer and two with PBST, the reaction was developed by adding 100 µl tetramethyl benzidine substrate (TMB substrate kit; Thermo Scientific) and incubating the plate for 9.6 h overnight at 4 °C. The absorbance was measured at 450 nm with a microplate reader (Bio-Rad). The cutoff value was determined as the absorbance measurement was above the cutoff value.

**CORE/CORE+1 sequence analysis**

In order to detect mutations in core/core +1 sequences, viral RNA was extracted from 150 µl of patients’ serum using the NucleoSpin Dx virus kit (Macherey-Nagel) and reverse-transcribed to complementary DNA using M-MLV reverse transcriptase (Promega). In brief, after 3 min of denaturation at 90 °C, the viral RNA was reverse-transcribed at 42 °C for 1 h using 200 U of M-MLV reverse transcriptase and 1 pmol of antisense primer CORE_R (5′ CAGTTCA TCATCATATCCCAAGCCAT 3′). Sense primer CORE_F (5′ GGCCTGATAGGTTGTCGCGATGCC 3′) and anti-sense primer CORE_R were used to amplify a 1028 bp PCR product, including a sequence from 5′ UTR to E1 from genotype 1a/1b isolates. The 50 µl reaction mixture for the PCR contained 1 µl cDNA, 2.5 U TaKaRa LA Taq polymerase, 1× TaKaRa GC buffer, 0.4 mM dNTPs mix and 0.5 pmol of each primer. The thermal profile of the PCR included 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 40 s and elongation at 72 °C for 2 min. A final elongation step was performed at 72 °C for 5 min. The PCR products were purified using NucleoSpin Gel and PCR clean-up purification kit (Macherey-Nagel), and then the sequencing reaction was carried out using sense CORE_F primer. A total of 23 core sequences (P1–23) from patients with cirrhosis and genotype 1 were analysed. Twelve were obtained from patients who were negative for anti-core +1 antibodies (P1–6/GenBank accession numbers KY707313–KY707318 and P13–18/GenBank accession numbers MF039290–MF039295) and 11 were obtained from patients who were positive for anti-core +1 antibodies (P7–12/GenBank accession numbers KY707319–KY707324 and P19–23/GenBank accession numbers MF039296–MF039300). The nucleotide and amino acid sequences were aligned using MEGA 7 software [42]. Prediction analysis for the RNA secondary structure in the core/core +1 coding region was performed using Mfold web server [43].

**Statistical analysis**

Student’s t-test analysis was used to evaluate the presence of an association between the prevalence of anti-core +1 antibodies and the stage of liver disease (chronic HCV infection, advanced or mild cirrhosis). Student’s t-test analysis was also used to access the presence of a correlation between the serum transaminase levels and anti-core +1 positivity or MELD score. The odds ratio was calculated to analyse core/core +1 sequences from patients who were positive or negative for anti-core +1 antibodies. In general, P value<0.05 was considered statistically significant.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all the patients. The study was approved by the ethics committees of the Universitätssklinikum Frankfurt and the Department of Medicine of Democritus University of Thrace.

**References**


