High levels of HCV core +1 antibodies in HCV patients with hepatocellular carcinoma

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The core region of the hepatitis C virus (HCV) genome possesses an overlapping ORF that has been shown to encode a protein, known as the alternate reading frame protein (ARFP), F or core +1. The biological role of this protein remains elusive, as it appears to be non-essential for virus replication. However, a number of independent studies have shown that the ARFP/F/core +1 protein elicits humoral and cellular immune responses in HCV-infected individuals and interacts with important cellular proteins. To assess the significance of the core +1 humoral response in HCV-infected patients, we examined the prevalence of anti-core +1 antibodies in sera from patients with hepatocellular carcinoma (HCC) in comparison with chronically HCV-infected individuals without HCC. We produced two HCV core +1 histidine-tagged recombinant proteins for genotypes 1a (aa 11–160) and 1b (aa 11–144), as well as a non-tagged highly purified recombinant core +1/S protein (aa 85–144) of HCV-1b. Using an in-house ELISA, we tested the prevalence of core +1 antibodies in 45 patients with HCC in comparison with 47 chronically HCV-infected individuals without HCC. We produced two HCV core +1 histidine-tagged recombinant proteins for genotypes 1a (aa 11–160) and 1b (aa 11–144), as well as a non-tagged highly purified recombinant core +1/S protein (aa 85–144) of HCV-1b. Using an in-house ELISA, we tested the prevalence of core +1 antibodies in 45 patients with HCC in comparison with 47 chronically HCV-infected patients without HCC and 77 negative-control sera. More than 50% of the serum samples from HCC patients reacted with all core +1 antigens, whereas 26% of the sera from the non-HCC HCV-infected individuals tested positive. No core +1-specific reactivity was detected in any of the control samples. In conclusion, the high occurrence of anti-core +1 antibodies in the serum of HCC patients suggests a role for the ARFP/F/core +1 protein in the pathogenesis of HCC.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease that frequently leads to liver fibrosis, cirrhosis and eventually to hepatocellular carcinoma (HCC) (Di Bisceglie, 2000; Hoofnagle, 2002; Ikeda et al., 1993). An estimated 170 million people are infected worldwide and, despite many efforts, there is no vaccine or broadly effective therapy for this viral infection (Houghton & Abrignani, 2005). The enormous genetic variability of the virus has resulted in the classification of HCV into six different genotypes (1–6) and more than a hundred subtypes, and it also manifests itself in patient sera or tissues with the appearance of quasispecies (Simmonds, 1995; Simmonds et al., 2005).

HCV is an enveloped positive-strand RNA virus, and is classified in the genus Hepacivirus of the family Flaviviridae (van Regenmortel et al., 2000). The virus genome consists of a single-stranded RNA molecule, composed of an ORF encoding a polypeptide of approximately 3000 aa that is flanked by 5′ and 3′ non-coding regions (NCRs) (Bartenschlager et al., 2004; Moradpour et al., 2007). Translation of the polypeptide is mediated by an internal ribosome entry site (IRES) embedded within the 5′ NCR, and the individual viral proteins are produced upon cleavage of the polypeptide by host and viral proteases.
These include three structural proteins (core, E1 and E2), the p7 protein and six non-structural proteins (NS2, NS3, NS4A–B, and NS5A–B), which are involved in virion assembly and viral RNA replication (Bartenschlager et al., 2004; Moradpour et al., 2007).

Recently, an additional protein was shown to be synthesized by an alternative ORF overlapping the core coding sequences in the +1 frame (core +1 ORF), which is conserved in all HCV genotypes (Branch et al., 2003; Vassilaki & Mavromara, 2009). This new protein is known as ARFP (alternative reading frame protein) (Walewski et al., 2001), F (frameshift protein) (Xu et al., 2001) or core +1 protein (to indicate the location of the novel ORF) (Varakioti et al., 2002). Based on the first reports on HCV genotype 1a, translation of the core +1 ORF is mediated by ribosomal frameshifting within ten consecutive adenines present at core codons 9–11 (prototype HCV-1a strain). This led to the synthesis of a chimeric ARFP/F/core +1 protein, containing the first 10 aa from the amino terminus of the core protein (Varakioti et al., 2002; Xu et al., 2001). However, the majority of HCV strains lack this sequence motif and they fail to synthesized this form of ARFP/F/core +1 protein (Baril & Brakier-Gingras, 2005; Boulant et al., 2003; Vassilaki & Mavromara, 2003). Still, alternative shorter forms of ARFP/F/core +1 protein can be synthesized independently of the HCV polyprotein by a novel mechanism that involves internal translation initiation (Baril & Brakier-Gingras, 2005; Vassilaki et al., 2007, 2008a, c; Vassilaki & Mavromara, 2003). The conserved initiation (AUG) codons at positions 85/87, and, to a lesser extent, the non-AUG codon at position 26 may serve as translation initiation sites (Baril & Brakier-Gingras, 2005; Vassilaki & Mavromara, 2003). In support of this model, the Branch group has recently reported the presence of core isoforms that are produced independently of the full-length core by an internal translation initiation mechanism. In fact, core codon 91, which is proximal to core +1 codon 85/87, represents a dominant internal translation initiation site for the synthesis of mini core(s) (Eng et al., 2009).

Since the discovery of the ARFP/F/core +1 protein(s) eight years ago, its biological role has remained elusive as it does not appear to be essential for productive HCV replication in the Huh-7-based cell-culture system or in animal models, nor has it been directly detected in native tissue (McMullan et al., 2007; Vassilaki et al., 2008b). However, since the HCV core protein has unusual properties for a nucleocapsid protein, it has been hypothesized that core +1 may be responsible for some of the biological functions attributed to the core protein. It is interesting to note that, in transfected mammalian cells, the expression of the ARFP/F/core +1 protein has been associated with the suppression of cellular p21 (Basu et al., 2004), enhancement of c-myc activity (Wu et al., 2007), modulation of p53 expression (Wu et al., 2007), perturbation of the tubulin cytoskeleton (Tsao et al., 2006) and modulation of cytokine or chemokine expression (Fiorucci et al., 2007), thus suggesting a possible involvement of core +1 in core functions related to liver cancer. This is further supported by recent findings reporting the presence of distinct core and ARFP/F/core +1 quasispecies in cancerous versus non-cancerous liver (Sobesky et al., 2007).

Nevertheless, the strongest evidence supporting the expression of the core +1 ORF during natural infection is based on independent reports from various laboratories worldwide that indicate the presence of specific humoral and cellular immune responses against the ARFP/F/core +1 protein in HCV-infected patients (Bain et al., 2004; Chuang & Allain, 2008; Cohen et al., 2007; Komurian-Pradel et al., 2004; Morice et al., 2009; Pawlotsky et al., 2005; Troesch et al., 2005; Varakioti et al., 2002; Walewski et al., 2001; Wu et al., 2007). However, the prevalence of anti-core +1 antibodies in patients with hepatocellular carcinoma (HCC) has never been published, even though a role for the ARFP/F/core +1 protein in liver cancer has been suggested (Branch et al., 2005; Vassilaki & Mavromara, 2009).

The aim of the present study was to characterize the prevalence of the anti-core+1 antibody response in chronic HCV-infected patients with or without HCC. Our results showed that the prevalence of anti-core+1 antibodies was significantly increased in the patients with HCC, suggesting a possible role for the protein in carcinogenesis.

RESULTS

Experimental design

To detect anti-core+1 antibodies in human sera, we developed a sensitive and specific ELISA protocol based on recombinant core+1 proteins or peptides. Two carboxy-terminal 6 × histidine-tagged recombinant core+1 proteins, corresponding to amino acids 11–160 from genotype 1a (core+1a) and amino acids 11–144 from 1b (core+1b), were produced in Escherichia coli. In addition, a highly purified untagged core+1 antigen corresponding to amino acids 85–144 from genotype 1b (core+1b/S) was used (Boumlic et al., 2010). It is important to note that none of the core+1 antigens are core/core+1 chimeric proteins, as they lack any amino acids from the amino terminus of the core protein. Furthermore, tagging the core+1 ORF at the carboxy-terminus was essential to avoid any potential translation of core amino acids because of the −1 frameshifting events that have been detected in E. coli (Komurian-Pradel et al., 2004; Varakioti et al., 2002; Vassilaki & Mavromara, unpublished data). The size and identity of the recombinant antigens was verified by Western blot analysis using anti-core+1 [Fig. 1b(i)] or anti-histidine-tag polyclonal or monoclonal antibodies [Fig. 1b(ii)]. The core+1b/S antigen was purified under native conditions and was subjected to extensive biochemical and structural analyses (Boumlic et al., 2010). The ELISA was developed as described in Methods. The
dilution factor for the patient sera was set at 1:40, based on the sera titrations shown in Fig. 1(c).

Detection of anti-core+1 antibodies in human sera of chronically HCV-infected patients with and without HCC

A total of 45 HCV-positive human serum samples from patients with HCC [the chronic hepatitis C (CHC)-HCC group] and 47 samples from patients with chronic HCV infection without HCC (the CHC-nonHCC group) were examined for the presence of antibodies against the ARFP/F/core+1 protein (Table 1). In parallel, 77 serum samples obtained from healthy blood donors (control) and five serum samples obtained from patients with non-HCV related liver disease (HBV positive) were used to assess the specificity of the method. ELISA tests were carried out by using the recombinant core+1a and core+1b proteins. Results were further confirmed using a peptide corresponding to core+1 amino acids 90–106 of genotype 1a, named R2 (Varaklioti et al., 2002) (Supplementary Fig. S1, available in JGV Online).

As is shown in Fig. 2, we observed a significantly higher percentage (>50%) of anti-core+1 antibodies in the HCV-associated HCC patients (CHC-HCC) than in the control group, using both core+1 antigens. On the other hand, the antigenic prevalence of anti-core+1 antibody was dramatically lower (<25%) in chronically HCV-infected patients without HCC (CHC-nonHCC), as compared with patients with HCC. No reactivity was detected in the control and the non-HCV liver-disease serum samples (HBV positive). There was a statistically significant difference between the HCV-positive and -negative sera against the respective antigens, with \( P<0.05 \) in all cases. Specifically, an anti-core+1a response was obtained in 58% of serum samples from HCC as compared with 21% of non-HCC patients. Similarly, 73% of HCC as compared with 26% of non-HCC serum samples reacted with the core+1b antigen (Table 2). The Pearson \( \chi^2 \) test verified that the prevalence of anti-core+1 antibodies was significantly higher in the HCC patients compared with the non-HCC HCV patients for all the core+1 antigens tested (\( P<0.001 \) for the core+1a and \( P<0.002 \) for the core+1b antibody). As expected, almost 100% of the HCV patient sera were positive for core antibodies (data not shown). Next, we sought to investigate the possible impact of patient age and HCC genotype on core+1serum positivity by applying a binary logistic regression model. Core+1b positivity was correlated principally to HCC status (\( P<0.05 (0.044) \), OR (odds ratio)=5.1, 95% confidence interval (CI)=1.04–25.2), irrespective of the age of the patient at the time of diagnosis (\( P=0.270, \text{OR}=0.972, 95 \% \text{ CI}=0.92–1.02 \)) and HCV genotype (\( P=0.898, \text{OR}=1.05, 95 \% \text{ CI}=0.53–2.04 \)). In the case of core+1a antigen, however, we failed to detect such a correlation with HCC status (\( P=0.132, \text{OR}=3.46, 95 \% \text{ CI}=0.69–17.43 \)), irrespective of the age of the patient at the time of diagnosis (\( P=0.213, \text{OR}=0.968, 95 \% \text{ CI}=0.92–1.02 \)) and HCV genotype (\( P=0.441, \text{OR}=0.710, 95 \% \text{ CI}=0.30–1.70 \)).

These results were verified independently by the use of the synthetic peptide R2 (aa 90–106) (Varaklioti et al. 2002). The anti-R2 response showed a significantly higher reactivity (55.5%, \( P<0.05 \)) in sera samples from the HCC patients as compared with those from the non-HCC patients (21.3%, \( P<0.005 \)). Taken together, these results indicate, importantly, a higher prevalence of anti-core+1 response in chronic HCV patients with HCC, which is likely to associate with the disease rather than with the longer incubation time of infection in the HCC patients.

Seroprevalence of different epitopes for core+1 antibodies in HCV-infected patients

Results from our laboratory have previously described a short form of core+1 in genotype 1a, corresponding to amino acids 85–160, as the predominant ARFP/F/core+1-protein form expressed in transfected mammalian cells (Vassilaki et al., 2007, 2008a). Therefore, it was of interest to compare the levels of specific anti-core+1 antibodies reacting with the long and the short forms of the core+1b proteins.

As shown in Table 2, when the full-length 6 x histidine-tagged core+1b antigen (aa 14–144) was compared with the short untagged core+1b antigen (aa 85–144), 32 of 44 (73%) samples from HCC patients reacted with the recombinant core+1b protein, whereas 22 of 42 (52%) reacted with the short form of core+1b. Despite the lower reactivity rate of the core+1b/S antigen, by using McNemar’s \( \chi^2 \) test with a 2 x 2 contingency table, there was no statistically significant difference between the reactivity rate of the sera against core+1b and core+1b/S (\( P=0.123 \)). In contrast, we noticed a significantly lower reactivity of the core+1b/S (9%) when compared with the longer core+1b antigen (26%) (\( P<0.005 \)) among the chronically infected HCV patients (Table 2). Also, by applying a binary logistic regression model, the core+1b/S positivity was correlated with HCC status (\( P<0.05 (0.013), \text{OR}=15.48, 95 \% \text{ CI}=1.77–135.35 \)), irrespective of the age of the patient at the time of diagnosis (\( P=0.583, \text{OR}=0.983, 95 \% \text{ CI}=0.923–1.05 \)) and HCV genotype (\( P=0.338, \text{OR}=1.445, 95 \% \text{ CI}=0.68–3.07 \)). Most importantly, the difference in reactivity between the long and short forms of core+1 protein suggest that longer forms of core+1 protein may be synthesized during natural infection. In fact, recent studies in cultured cells have suggested the presence of additional internal translation initiation events upstream of codon 85/87 for the core+1 protein of HCV genotype 1b (Boumlic et al., 2011).

DISCUSSION

It has previously been reported that HCV-infected patients develop specific humoral and cellular responses against the
Proteins were detected using a polyclonal core + 1 antibody (i). Blotting was performed using 1 μg ml⁻¹ of recombinant proteins. Sizes of the protein markers are indicated on the left. (c) Optimization of serum dilution factors for the ELISA experiments against core + 1a (i) and core + 1b (ii) recombinant proteins. Titration of patient sera were carried out by using the chequerboard serial dilution method. The cut-off is represented by the black horizontal line. Each patient serum sample with HCC (ca) is represented by a different number, which is shown in the inset panel.

In this study, we examined the prevalence of anti-core + 1 antibodies in the sera of chronically HCV-infected Greek patients with HCC in comparison with those without. Our results showed that the prevalence of anti-core + 1 antibody levels were significantly higher in patients with HCC (>50 %) than those without HCC (<26 %), suggesting a possible association between the core + 1 protein and HCC.

Previous studies have shown that the titres of core + 1 antibodies in the sera of chronically HCV-infected patients varies considerably, ranging from 12.5 % to approximately 80 %, a finding that has been correlated with the nature of the core + 1 antigen used in the in-house ELISA tests (reviewed by Vassilaki & Mavromara, 2009). Studies using a core + 1 antigen corresponding to a chimeric core + 1 protein that contained the first ten amino acids from core fused in-frame with core + 1 amino acid sequences (F protein) gave the highest reactivity (Komurian-Pradel et al., 2004). Conversely, the anti-core + 1 responses were substantially reduced when a recombinant core + 1 protein lacking the first ten amino acids from core, or core + 1 oligopeptides, was used (Komurian-Pradel et al., 2004; Varaklioti et al., 2002; Walewski et al., 2001). It is likely that core + 1 oligopeptides may lack important epitopes that are present in the full-length, tagged core + 1 proteins. Alternatively, the chimeric core/core + 1 protein may adopt a conformation that enhances the exposure of core + 1 epitopes on it which react with human sera in a non-specific way due to the presence of the first ten amino acids of core. In our study, we developed an ELISA based on two full-length recombinant 6×histidine-tagged antigens, corresponding to amino acids 11–160 and 11–144 of genotypes 1a and 1b, respectively. These antigens contained all core + 1 amino acid sequences downstream of core codon 11 but lacked any amino-terminal core amino acid sequences. In addition, an untagged, highly purified short form of the core + 1 antigen corresponding to amino acids 85–144 from HCV genotype 1b (core + 1b/S) was used.

**Fig. 1.** Expression and production of core + 1 antigens. (a) Schematic representation of core + 1 antigens. Core + 1a (pHPI120) and core + 1b (pHPI1901) recombinant proteins were expressed from the pET20b(+) vector. Recombinant proteins were expressed and purified as described in Methods. (b) Immunoblot of the purified core + 1 proteins. Recombinant proteins were detected using a polyclonal core + 1b antibody (i) and a polyclonal anti-polyhistidine antibody (ii).
Our results showed that the positivity rates in sera from HCC patients were 58 and 73%, whereas for the non-HCC patients the positivity rates for each antigen were only 21 and 26% for the core +1a and core +1b, respectively, indicating a dramatic increase in the anti-core +1 antibody level in sera from HCC patients. Furthermore, it is of interest to note that a major difference in the reactivity of sera from chronic patients without HCC, compared with those with HCC, was observed with core +1b/S, suggesting a possible association of the short form of core +1 with HCC. This is of particular importance in light of the fact that different forms of the core +1 protein may be produced based on the presence of different translation initiation mechanisms. Notably, and as shown in Table 1, the two groups of patients were not perfectly matched in terms of age (CHC-HCC is associated with the elderly) and genotype (HCV-3 is prevalent in the CHC-nonHCC group). Usually, HCV-associated HCC develops in persons with chronic infection over a period of 20–30 years and the prevailing genotype for Greek patients is 1b. Nevertheless, results from multivariate analyses with the two core +1b antigens showed that anti core +1b antibody is correlated with disease rather than age (duration of infection) or genotype. Interestingly, this analysis worked only for the core +1b antigen and not the core +1a antigen, suggesting that inherent mismatches between the two groups are indeed significant. Further studies with larger and better matched groups of patients are needed to confirm these findings.

HCV genotypes 1b and 3a are the most prevalent in Greece, and their prevalence is linked to intravenous drug use. In agreement with previous studies (Komurian-Pradel et al., 2004), we found that the positive samples from different genotypes exhibited a great deal of cross-reactivity to core +1 antigens from 1a, 1b and 1b/S (Table 3).

The core protein is not only a viral structural component but also a pathogenic factor, since its expression has been strongly linked to the development of liver steatosis and HCC (Lemon et al., 2000; Lerat et al., 2002). To this end, it is intriguing to speculate that the ARFP/F/core +1 protein may contribute to some of the functions attributed to core that are related to the development of HCC. Long-term studies on the genomic heterogeneity of HCV have shown a markedly higher variability within the core gene for isolates from HCC patients than for those isolated from individuals with chronic HCV infection (Fishman et al., 2009; Pavio et al., 2005; Smith & Simmonds, 1997). However, the role of these mutations in liver cancer remains unclear. These polymorphisms, apart from their putative effect on protein stability and function, might alter the RNA structures and regulate processes that contribute to oncogenesis. Interestingly, a recent study by Branch and associates has identified seven polymorphisms within the core/core +1 region as being clearly associated with HCC (Fishman et al., 2009). Of these, mutation at position 309 (309 A/C) is predicted to modify the RNA structure of stem–loop 248 (SL248). Interestingly, predictions of the RNA structure based on using the MFOLD program suggested a size increase in the loop that contains the AUG codon at position 87, a proposed codon for internal translation initiation of core +1 protein (Supplementary Fig. S2, available in JGV Online). As loop structures may facilitate the docking of ribosomes at internal translation initiation sites, it is tempting to speculate that mutation

### Table 1. Characterization of HCV patients

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<th>2–3</th>
<th>≥ 3</th>
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<td>13</td>
<td>6</td>
<td>11</td>
<td>45</td>
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ND, Not determined.
309 A/C may favour the expression of core +1/S protein and that this could correlate with the increased prevalence of anti-core +1 antibodies in HCC patients. Further investigations are needed to determine the significance of mutation 309 A/C for core +1/S synthesis and antibody prevalence. Notably, a second polymorphism at nucleotide 271 (271 uracil—cytosine) is also located within SL248 and has been associated with the expression of mini-core (Eng et al., 2009).

In conclusion, the significant increase of anti-core +1 antibodies in sera from HCV-infected Greek patients with HCC suggests a role for the ARFP/F/core +1 protein in HCV-induced hepatocellular carcinoma.

**METHODS**

The E. coli BL21(DE3) cells were purchased from Novagen. The rabbit polyclonal anti-histidine-tag antibody was purchased from Santa Cruz Biotechnology and used at a dilution of 1:1000. The purified goat anti-human IgG antibody was purchased from DakoCytomation and used at a dilution of 1:2000. The Ni–NTA agarose beads and purification kit solutions were acquired from Qiagen. All other chemicals and solutions were from Sigma.

**Patient sera.** Serum samples from 92 patients with chronic HCV infection (obtained from Hippokration or Henry Dunant Hospitals, Athens, Greece) were used in this study. Of these, 45 samples originated from patients who had HCV-associated hepatocellular carcinoma (HCV-HCC) and were designated as the CHC-HCC group. The remaining 47 samples consisted of the chronic HCV patients with various stages of liver disease and were designated as the CHC-nonHCC group (Table 1). The HBV-positive group comprised five samples from patients with HBV-associated liver disease. The control group consisted of 70 samples from healthy blood donors who tested negative for anti-HCV, anti-HBV and anti-HIV antibodies (kindly provided by Dr Mentis, Hellenic Pasteur Institute). All serum samples were centrifuged and stored at −20 °C prior to use.

**Construction of recombinant protein expression vectors.** The core/core +1-encoding sequences from HCV genotype 1b were derived from plasmid pRSV-BT, kindly provided by Dr C. Brechot (INSERM U785, Paris, France) (Pavio et al., 2005). The cDNA fragment between nt 44–425 was amplified using primers 5′-CATGCGATCCGCAACCGCGCGACAG-3′ (sense) and 5′-CCCAGTGGGCGGCCGACAGACCGCA-3′ (antisense) (Nol and Hin III sites are shown in italics) and the fragment was cloned into the HindIII site of the pUC19 vector, yielding plasmid pHPI1900. For the expression of the ARFP/F/core +1 hybrid protein in E. coli cells, the Ncol–HindIII core +1 fragment from pHPI1900 was cloned into the Ncol–HindIII site of the plasmid pET-20b (+) expression vector upstream of the 6 × histidine tag, yielding plasmid pHPI1901. The recombinant ARFP/F/core +1 protein of prototype-1a (GenBank accession no. M67463) was expressed from plasmid pHPI8120. To construct this plasmid, the cDNA fragment between nt 44–487 was amplified using primers 5′-ATGCGATCCGCAACCGCGACAGACCGCA-3′ (sense) and 5′-CCCAGTGGGCGGCCGACAGACCGCA-3′ (antisense) (Nol and HindIII sites are shown in italics) and was cloned into pET20b (+) as described above. The results of all plasmid constructions were confirmed by DNA sequencing. Finally, the fragment corresponding to amino acids 85–144 of short core +1b was obtained by PCR, using pRSV-BT plasmid and the primers 5′-ATCCGGGGTGCTCTGGACACATGGCCCGCTTGGGGGTG-3′ (sense) and 5′-ATCCGGGGTGCTCTGGACACATGGCCCGCTTGGGGGTG-3′ (antisense) and 5′-ATCCGGGGTGCTCTGGACACATGGCCCGCTTGGGGGTG-3′ (antisense) and 5′-ATCCGGGGTGCTCTGGACACATGGCCCGCTTGGGGGTG-3′ (antisense)...
(antisense) (BsaI sites are shown in italics). The PCR fragment was digested with Bsal and cloned into the Ncol and AccrSI sites of pETm-60 at the carboxy terminus of the NusA tag (a gift from Gunter digested with BsaI, EMBL, Heidelberg, Germany), yielding plasmid pHPI1904 (Boumlnic et al., 2010).

**Production of recombinant proteins and polyclonal antibodies.**

BL21(DE3) cells were transformed with plasmids pHPI8120 and pHPI1901. Cultures in the exponential phase of growth (OD600=0.5) were induced for 3 h with 0.5 mM IPTG. For the 6× histidine-tagged core +1a and core +1b proteins, the bacteria were pelleted by centrifugation at 3800 g for 10 min, resuspended in 8 M urea buffer (8 M urea, 0.1 M NaH2PO4, 10 mM Tris pH 8.0), disrupted by sonication and incubated at 4 °C overnight on a shaking platform. Next, the lysate was centrifuged at 10 000 g at 4 °C for 1 h. The supernatant was bound to Ni–NTA agarose beads and the 6× histidine-tagged proteins were purified under denaturing conditions, essentially according to the manufacturer’s instructions (Qiagen), with additional stringent washing steps to improve purity. The histidine-tagged ARFP/F/core +1 proteins were eluted from the column with 8 M urea buffer (pH 4.5), and the eluted fractions were stained with Coomassie blue and analysed on a 15% polyacrylamide gel.

The purification of the short core +1b protein (core +1S) was performed under native conditions and has been described in detail previously (Boumlnic et al., 2010). Briefly, pHPI1904-transformed BL21 cells, grown at 37 °C to OD600=0.7 were induced by the addition of IPTG overnight at 15 °C. Pelleted cells were resuspended in 20 mM sodium phosphate (pH 6.8), 400 mM NaCl, supplemented with 50 mM arginine, 50 mM glutamic acid, 2.5 μM DNsA 1 m1−1, 2.5 μM RNase A 1 m1−1 and protease inhibitors (complete, Mini Protease Inhibitor Cocktail; Roche). Purification was performed at 4 °C by ion-metal affinity chromatography. The protein was eluted with buffer B (Boumlnic et al., 2010) containing 250 mM imidazole and mixed with recombinant tobacco etch virus protease to cleave core +1S from NusA. The truncated, untagged short core +1b protein was further purified by size exclusion chromatography to produce a highly purified protein. All purified proteins were subjected to Western blot analysis as described previously (KalavHKvi et al., 2006) by using an anti-histidine-tag antibody and an in-house polyclonal core +1b antibody, at dilutions of 1:1000, and 1:200, respectively.

To produce an in-house polyclonal antibody against the HCV core +1 (genotype 1b) protein, 100 μg of purified core +1b recombinant protein were mixed with 750 μl of complete Freund’s adjuvant and injected into a New Zealand white rabbit. The rabbit was boosted three times with the same antigen mixed with incomplete Freund’s adjuvant at intervals of approximately 2 weeks. The antiserum was collected 2 weeks after the last boost and used in the Western blot analysis and ELISA.

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

Microplate wells were coated with 100 μl of a 5 μg ml−1 solution of either core +1a, core +1b or short core +1b, or a 2 μg ml−1 solution of the R2 peptide in 50 mM sodium carbonate (pH 9.6), overnight at room temperature. Wells were blocked with PBST [0.1% (v/v) Tween-20 in PBS (pH 7.4)] containing 5% (w/v) non-fat dried milk for 1 h at 37 °C and washed three times with washing buffer [PBST +1% (w/v) milk]. For all antigens, 100 μl of serum at a dilution of 1:40 was added in duplicate wells and the plates were incubated at 37 °C for 1.5 h. The plates were then washed three times with washing buffer and further incubated with 100 μl of peroxidase-conjugated affinity-purified goat anti-human IgG whole antibody for 1 h at 37 °C. Following three washes with washing buffer and two with PBST, the substrate tetramethyl benzidine (TMB Substrate kit; Thermo Scientific) was added, incubated at room temperature for 15 min in the dark and stopped with 2 M H2SO4. The absorbance was measured at 450 nm with a microplate reader.

**Assembly of sequences and analysis.**

The RNA secondary structural model was based on the 1b reference sequence Con1 with GenBank accession number AJ238799 and was generated using MOFOLD with specific default parameters (Choi et al., 2003; McMullan et al., 2007). The following base-pairing parameters were modified for our

<table>
<thead>
<tr>
<th>Patients’ genotype</th>
<th>n</th>
<th>Core +1a positive response: n (%)</th>
<th>Core +1b positive response: n (%)</th>
<th>Short core +1b positive response: n (%)</th>
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</thead>
<tbody>
<tr>
<td>HCC – 1a/b</td>
<td>29</td>
<td>18/29 (62%)</td>
<td>20/29 (69%)</td>
<td>14/27 (51.8%)</td>
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<td>1/1</td>
<td>1/1</td>
<td>0/1</td>
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<tr>
<td>HCC – 3a</td>
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<td>2/7 (28.5%)</td>
<td>5/7 (71.4%)</td>
<td>5/7 (71.4%)</td>
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<tr>
<td>HCC – 4</td>
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<td>2/3 (75%)</td>
</tr>
<tr>
<td>HCC – ND</td>
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<td>3/4 (75%)</td>
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<tr>
<td>non-HCC – 1a/b</td>
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<td>2/15 (13.3%)</td>
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<tr>
<td>non-HCC – 3a</td>
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<tr>
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<td>2/6 (33.3%)</td>
<td>1/6 (16.6%)</td>
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</tbody>
</table>
purposes: F 48 83 2, F 87 167 2, F 332 406 4, F 248 325 4, F 444 474 1, P 217–219 415–417 (Supplementary Fig. S2). The MFOLD program is freely available at http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form.

**Statistical analysis.** Statistical analysis was carried out using EXCEL 2003 (Microsoft) and SPSS 16.0 (SPSS). For each experiment, the cut-off value was determined as being the mean value of a group of control sera plus 2 SD. A serum sample was considered positive when the absorbance measurement was above the cut-off. The Shapiro–Wilks test was used to evaluate whether the data fitted the normal distribution. The Mann–Whitney U test for non-parametric data was used for the evaluation of statistical significance between samples testing positive and negative for each antigen. Pearson’s $r^2$ test was used for ‘between groups’ comparison of reactivity for each antigen, based on $2 \times 2$ contingency tables. This test was used to correlate positivity rates with disease state. McNemar’s $\chi^2$ test was used to evaluate whether the reactivity percentages of the core + 1b and short core + 1b/S antigens were statistically different. Finally, binary logistic regression was carried out to determine whether the age of patients and HCV genotype was independently associated with the positivity rate of core + 1 antibodies in all serum samples. In any case, $P$ values $<0.05$ were considered statistically significant.

**ACKNOWLEDGEMENTS**

We thank Dr U. Georgopoulou and Dr P. Lyberi for stimulating scientific discussions and technical advice. We also thank R. Kanzipa for administrative support.

**REFERENCES**


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