

Expression of Hepatitis C Virus E2 Ectodomain in *E. coli* and Its Application in the Detection of Anti-E2 Antibodies in Human Sera

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Abstract The second envelope glycoprotein (E2) of hepatitis C virus has been shown to bind human target cells and has become a major target for the development of anti-HCV vaccines. Anti-E2 antibodies have been suggested to be of clinical significance in diagnosis, treatment and prognosis of hepatitis C. However, large-scale expression and purification of E2 proteins in mammalian cells is difficult. As an alternative, E2 fragment (aa 385–730) with a four-amino-acid mutation (aa 568–571 PCNI to RVTS) was expressed as hexa-histidine-tagged full length protein [E2N730(m)] in *E. coli* and purified to over 85% purity. Purified E2N730(m) was specifically recognized by homologous hepatitis C patient serum in Western blot, suggesting that it displayed E2-specific antigenicity. Rabbit antiserum raised against E2N730(m) recognized E2 glycoproteins expressed in mammalian cells in Western blot. Purified E2N730(m) was used to detect anti-E2 antibodies in human sera and showed better specificity and sensitivity than previously reported C-terminally truncated E2 fragment (aa 385–565). Association between anti-E2 antibodies in patient sera and HCV RNA status was also demonstrated using this *E. coli*-derived protein. E2N730(m) might serve as an inexpensive alternative to mammalian cell-expressed E2 proteins in clinical and research applications.

Key words hepatitis C virus; envelope protein; E2; expression and purification; *Escherichia coli*

Hepatitis C virus (HCV) is the major etiological agent of both community-acquired and post-transfusion non-A, non-B hepatitis [1]. In 1998, it was estimated that 3% of the world population (about 170 million) was infected with HCV [2]. Prognosis of HCV infection is poor, with approximately 85% of patients developing chronic infection, and about 20% of the chronic cases progressing onto cirrhosis and/or hepatocellular carcinoma [3]. Lack of effective vaccines and satisfactory treatments makes HCV a global health threat.

HCV is an enveloped plus-strand RNA virus and has been classified as the sole member of the hepacivirus genus of the *Flaviviridae* family [4]. Of the viral struc-

tural proteins, E1 and E2 are predicted to be glycosylated type I membrane proteins and generally believed to constitute the protein components of virion membrane [5, 6]. E2 encompasses aa 384–746 of the HCV polyprotein, with the extremely hydrophobic aa 718–746 region as its putative transmembrane domain (TMD) [7]. In addition to the TMD, C-terminal aa 662–717 region of E2 ectodomain (aa 384–717) is also highly hydrophobic.

E2 has been suggested to play an important role in HCV binding and entering into target cells [8–10]. Vaccination studies in chimpanzees using E2-based glycoprotein or DNA vaccines have shown that limited but measurable protection could be achieved [11]. Data from natural infection cases have also associated natural resolution of infection with certain types of anti-E2 antibodies [12,13]. Therefore, E2 has become a major target in anti-HCV vaccine research. Humoral immune responses against E2 also have diagnostic significance, since there have been studies showing that testing for antibodies against E2 could

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improve the performance of current HCV EIA kits, which do not incorporate any forms of envelope proteins [14–17].

In this work, E2 fragment (aa 385–730) covering the E2 ectodomain and the upstream half of TMD was expressed as hexa-histidine-tagged protein [E2N730(m)] in *E. coli*. A four-residue mutation was introduced to enhance full-length expression. Purified E2N730(m) displayed E2-specific antigenicity and rabbit antiserum raised against E2N730(m) was able to recognize E2 glycoproteins expressed in mammalian cells in Western blot. Anti-E2 antibodies in human sera could be detected in EIA with E2N730(m) and association between the presence of anti-E2 antibodies and serum HCV RNA status was demonstrated. E2N730(m) might serve as an inexpensive alternative to mammalian cell-expressed E2 proteins in clinical and research applications, whereas rabbit anti-E2N730(m) could be a useful tool in biochemical and vaccinological studies of E2.

Materials and Methods

Plasmids and bacterial host

pUC18/CE1E2 containing C, E1 and E2 coding sequences of HCV (subtype 1b) was provided by Professor WANG Yu of Peking University, Beijing, China (GenBank accession No. D10934) [18]. pQE8 is a N-terminal hexa-histidine fusion expression vector from Qiagen GmbH, Hilden, Germany. pQE8/E2C'730(m) is a previously described plasmid expressing E2 aa 567–730 fragment in pQE8 background. E2 coding sequences of pQE8/E2C'730(m) were mutated from PCNI to RVTS at aa 568–571, due to a spontaneous frameshift by one nucleotide affecting 36 basepairs [19]. There was also an additional hexa-histidine tag at the C-terminal of E2 coding sequences in pQE8/E2C'730(m). *E. coli* strain TG-1 was used as cloning and expression host.

Construction of expression plasmid pQE8/E2N730(m)

DNA sequences encoding E2 aa 385–700 were amplified from pUC18/CE1E2 using the primer set: 5'-GCGTTGACGGATCCACCTACGTG-3' (upstream); 5'-GCGAAGCTTGCACGTCCACGATG-3' (downstream). Amplified fragment was cloned between the *Bam*HI and *Hind*III sites on pQE8 to create pQE8/E2N (designated pQE8/E2-316 in reference [20]). Coding sequences for mutated aa 567–730 were amplified from pQE8/E2C'730(m) [19] using the primer set: 5'-GTCGGGCCCCGTGTAACATCG-3' (upstream); 5'-CAAGCTAGCTTGATCTCACCC-3' (downstream), and then used to replace coding sequences for aa 567–700 on pQE8/E2N. The obtained recombinant plasmid was designated

pQE8/E2N730(m), which carries the coding sequences for E2 aa 385–730 with aa 568–571 mutated from PCNI to RVTS [19]. All PCR and recombinant cloning steps were performed according to standard protocols. Sequences of plasmids used for expression were confirmed by automatic sequencing.

Expression and purification of recombinant E2 protein

Freshly saturated recombinant TG-1 culture was inoculated into fresh LB media at 1:100. Two hours after inoculation, expression was induced by adding IPTG to a final concentration of 1 mmol/L. Cells were harvested 6 hours later by centrifugation and stored at –20 °C.

Solubility analysis and purification of expression products were performed as previously described [19,20]. Briefly, harvested bacteria were resuspended in PBS, sonicated on ice-bath, and centrifuged. The soluble and insoluble fractions were analyzed for the presence of expression products. Insoluble recombinant E2 proteins were extracted with 6 mol/L Gu•HCl/100 mmol/L β -ME/PBS (pH 8.0), centrifuged, and diluted four fold with 6 mol/L Gu•HCl/PBS (pH 8.0) before loading onto preequilibrated Ni²⁺-NTA agarose (Qiagen). The gel matrices were sequentially washed with 6 mol/L Gu•HCl/20 mmol/L β -ME/PBS (pH 6.3) and 8 mol/L urea/20 mmol/L β -ME/PBS (pH 6.3), and then eluted with 8 mol/L urea/20 mmol/L β -ME/PBS (pH 4.3).

E2 proteins expressed in mammalian cells

Expression of the same E2 gene in recombinant vaccinia virus system was done by co-infecting HeLa cells with vTT7 and vCEH-2 as previously described [21]. Recombinant vaccinia virus vCEH-2 contained coding sequences of HCV polyprotein aa 1–730 under the control of T7 promoter, whereas vTT7 encoded the T7 polymerase required for expression. Briefly, HeLa cells were coinfecting with vTT7 and vCEH-2 at a multiplicity of infection of 4:4:1 (vTT7 : vCEH-2 : cell) and cultured for 48 hours. Cells were collected by scraping, washed with 4 °C PBS and stored at –20 °C.

Protein analysis

SDS-PAGE under reducing conditions and Western blot were conducted according to standard protocols. In Western blot, first antibody was diluted 1:100 or 1:500 for human sera and 1:1000 for rabbit sera, and second antibody [HRP-labeled protein A (Sigma) or swine anti-rabbit Ig (DaKo)] was diluted 1:1000. Blots were developed using the ECL method (PerfectBio).

Animal and human sera

One 1.5 kg female rabbit (Shanghai Laboratory Animal Center) was immunized subcutaneously on the back with

300 µg purified recombinant E2 protein emulsified in complete Freud's adjuvant and boosted 4 and 8 weeks later with the same amount of antigen emulsified in incomplete Freud's adjuvant. One week after the last boosting, total blood was collected through the carotid artery and serum was prepared according to standard procedures.

Human serum S94 was collected from a Chinese patient with chronic hepatitis C and provided by Professor Yu WANG. The HCV cDNA used in this work was cloned from the same patient. Other human sera were collected from Chinese hepatitis patients and healthy blood donors.

Detection of anti-HCV antibodies and HCV RNA

Anti-HCV antibodies in human sera were detected using UBI HCV EIA 4.0 (United Biomedical Inc.) according to manufacturer's instructions. For the detection of anti-E2 antibodies in human sera, polystyrene microplates (Nalge Nunc International) were coated with purified recombinant E2 at 0.15 µg/hole in 100 µl 50 mmol/L carbonate buffer at 4 °C for 18 h. The microplates were then blocked with 1% BSA/2% inactivated new-born calf serum/PBS at 37 °C for 2 h. Human sera were 1:20 diluted and secondary antibody (HRP-labeled goat anti-human-Ig) was 1:150 diluted in blocking buffer. Incubation was continued for 30 minutes at 37 °C followed by thorough washing with PBST. Color was developed using TMB substrate and developing was stopped by adding 1 mol/L HCl according to standard protocols. A_{450} was measured using a microplate reader. The mean A_{450} of 100 healthy blood donors' sera multiplied by 2.1 was set as cut-off value for determining positivity. Anti-E2 antibodies in post-immune rabbit sera were detected and titrated with similar method using HRP-labeled swine anti-rabbit Ig as secondary antibody. HCV RNA in human sera was detected using HCV Gene Detection Kit from Shanghai Forward Biomedical Ltd. based on RT-PCR/DNA-EIA methodology. All EIA tests were done in duplicates and the mean absorbance value was used.

Results and Discussion

Construction of recombinant plasmid expressing E2 ectodomain

In our efforts to express different fragments of HCV E2 protein ectodomain in *E. coli*, we found that aa 566–622 region of E2 had a negative effect on expression in *E. coli*, resulting in low or no production of full-length protein [19,20]. However, we identified a spontaneous mutation affecting aa 568–571 of E2 which could counteract such a negative effect and significantly enhance full-length expression of fragments containing this region [19]. This mutation is a frameshift of a single nucleotide,

changing aa 568–571 of E2 from PCNI to RVTS. Sequences encoding aa 567–730 of E2 harboring this mutation were amplified by PCR from pQE8/E2C'730(m), which expressed mutated aa 567–730 of E2 [19]. The amplified sequences were used to replace aa 567–700 coding sequences in pQE8/E2N, which expressed aa 385–700 of E2 [20]. The resultant expression plasmid was designated pQE8/E2N730(m) and carried coding sequences for aa 385–730 of E2 fused to hexa-histidine tags at both termini, with aa 568–571 mutated from PCNI to RVTS. Comparison of E2 coding sequences in aa 568–571 region between pQE8/E2N and pQE8/E2N730(m) is shown in Fig. 1.

	562						568				571					576	
	K	T	F	G	G	P	P	C	N	I	G	G	A	G	N		
pQE8/E2N	AAGACGTCG <u>GGG</u> GcCccCGTGTAAcATC-GGGGGGGcCGcAAC																
pQE8/E2N730(m)	AAGACGTCG <u>GGG</u> GcCcc- <u>CGTGTAAcATcGGGGGGcCGcAAC</u>																
	K	T	F	G	G	P				R	V	T	S	G	A	G	N
	562									568				571			576

Fig. 1 E2 aa 568–571 sequences in pQE8/E2N and pQE8/E2N730(m)

Amino acid residues affected by mutation were shown in italic. *Apa*I site used for cloning is underlined.

Expression and purification of recombinant E2 fusion protein

Expression from pQE8/E2N730(m) was induced with IPTG. A prominent band of approximately 42 kD was observed after induction [Fig. 2(A), lane 2], matching the predicted molecular weight of 41.8 kD of full-length product. In contrast, several similar constructs lacking the aa 568–571 mutation only expressed barely detectable level of full-length protein (data presented in reference [19]). Densitometric scanning showed that this band constituted over 10% of total bacterial protein. This 42 kD protein was designated E2N730(m). After sonication, E2N730(m) was almost exclusively found in the insoluble fraction [Fig. 2(A), compare lane 3 and 4].

Insoluble E2N730(m) was solubilized with high concentration of strong chaotropic agent (6 mol/L Gu•HCl) in the presence of high concentration of reducing agent (100 mmol/L β-ME) and purified under denaturing conditions on Ni²⁺-NTA agarose. Purified E2N730(m) appeared as a fairly homogenous band of 42 kD on SDS-PAGE, with minor amounts of lower molecular weight bands, most likely non-full-length products as a result of premature translational termination or protease degradation [Fig. 2(A), lane 5]. The 42 kD full-length E2N730(m)

constituted over 85% of purified proteins and its final yield was higher than 1 mg/L initial *E. coli* culture.

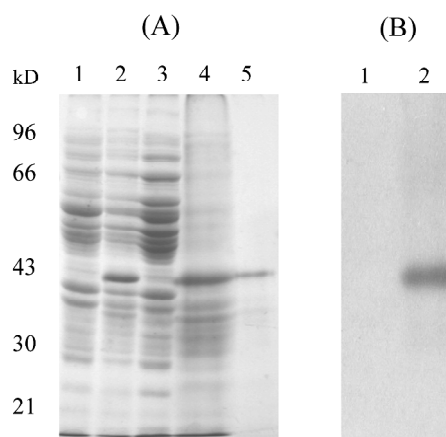


Fig. 2 Expression and purification of E2N730(m) fusion protein and Western blot analysis using homologous patient serum

(A) SDS/PAGE stained with Coomassie blue (12% gel). 1, 2, whole-cell lysates of induced TG-1(pQE8) and TG-1[pQE8/E2N730(m)] respectively; 3, soluble fraction after sonication of induced TG-1[pQE8/E2N730(m)]; 4, insoluble fraction after sonication of induced TG-1[pQE8/E2N730(m)]; 5, purified E2N730(m). (B) Western blot using homologous HCV patient serum S94 as first antibody (12% gel). 1, mock purification products from induced TG-1(pQE8); 2, purified E2N730(m).

Antigenicity and immunogenicity analysis of E2N730(m)

Reactivity of E2N730(m) against human sera in Western blot was analyzed. Homologous hepatitis C patient serum S94 specifically recognized E2N730(m) [Fig. 2(B)], whereas sera from healthy blood donor or hepatitis B patient showed no specific recognition (data not shown). This result demonstrated that, despite the presence of four-residue mutation, *E. coli*-derived E2N730(m) still displayed HCV E2-specific antigenicity. Also, it was clear that at least some of the anti-E2 antibodies present in infected patients' sera were directed towards E2 polypeptide backbone, suggesting the possibility of using bacterially expressed E2N730(m) for the clinical detection of anti-E2 antibodies in human sera.

A rabbit subcutaneously immunized with E2N730(m) three times produced anti-E2 antibodies with a titer of 1 : 32000. The animal was sacrificed and the obtained sera was designated R_{E2N730(m)} which was used to detect E2 proteins expressed in *E. coli* and mammalian cells in Western blot. R_{E2N730(m)} not only specifically recognized E2N730(m) [Fig. 3(A)], but also showed specific recognition of glycosylated E2 proteins expressed in

recombinant vaccinia virus system [Fig. 3(B)]. In addition to the major 42 kD full-length band, R_{E2N730(m)} also reacted with heterogeneous bands of higher and lower mobility rates in purified E2N730(m), although with much lower intensity [Fig. 3(A)]. Some of these bands were also faintly observable in purified E2N730N(m) after staining or blotting with homologous serum S94 [Fig. 2(A), lane 5 and Fig. 2(B), lane 2]. Since these minor bands were not observed in pQE8 transformed *E. coli* cells [Fig. 3(A), lane 1], they are most likely non-full-length and polymeric forms of E2N730(m). In reacting with R_{E2N730(m)}, glycosylated E2 appeared as two heterogeneous species with apparent molecular weights of approximately 50 and 70 kD, respectively [Fig. 3(B), lane 2]. The difference in mobility rate of these two species of E2 glycoprotein most likely reflected differences in the degree and type of glycosylation. The same recognition pattern of E2 glycoproteins was also observed with rabbit sera raised against aa 450–565 fragment of E2 expressed in *E. coli* [20].

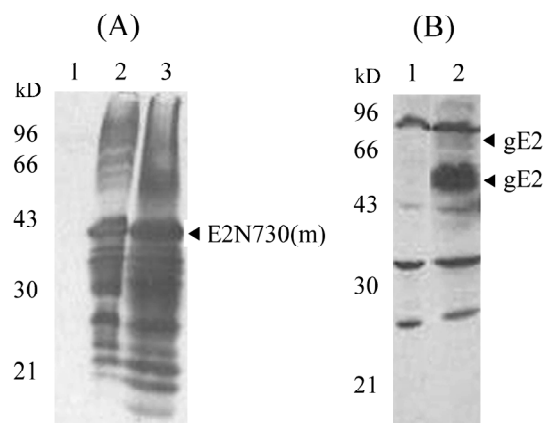


Fig. 3 Western blot detection of E2 protein expressed in various systems using R_{E2N730(m)}

(A) E2 expressed in *E. coli* (15% gel). 1, 2, whole-cell lysates of induced TG-1(pQE8) and TG-1[pQE8/E2N730(m)] respectively; 3, purified E2N730(m). (B) E2 (aa 384–730) expressed in recombinant vaccinia virus system (15% gel). 1, HeLa cells infected with vvT7 alone; 2, HeLa cells co-infected with vvT7 and vCEH-2. gE2 stands for glycosylated E2 proteins.

This result indicated that E2N730(m) was able to present E2-specific immunogenicity *in vivo* and elicit antibodies directed against epitopes shared by glycosylated and unglycosylated E2 proteins. The fact that R_{E2-116R} was reactive against E2 proteins expressed in various prokaryotic and eukaryotic systems regardless of glycosylation status suggested that it could be used to detect different types of E2 proteins in biochemical, virological and vaccinological studies of E2.

Application of E2N730(m) in the detection of anti-E2 antibodies in human sera

Some reports have suggested that including anti-E2 antibody testing into current EIA kits could improve their performance [14–17]. Our results shown here and elsewhere [19,20] demonstrated that *E. coli*-derived E2 fragments reacted specifically with infected patient sera in Western blot and elicited antibodies in rabbits against glycosylated E2 expressed in mammalian cells, indicating E2 proteins expressed in bacteria shared antigenic/immunogenic epitopes with mammalian E2 glycoproteins. Previously, we developed an anti-E2 EIA using aa 385–565 fragment of E2 expressed in *E. coli* to detect anti-E2 antibodies in human sera and obtained a positivity rate of 40% in Chinese anti-HCV EIA-positive patients [20]. Since E2N730(m) almost covered the whole length of E2 ectodomain and the upstream half of TMD, and the four-residue mutation at aa 568–571 did not affect its reactivity with homologous patient sera [Fig. 2(B)], we further tested its ability to detect anti-E2 antibodies in human sera in EIA.

Sera from 100 healthy blood donors, 20 hepatitis B patients, 20 non-A-to-E hepatitis patients and 158 hepatitis C patients were reacted with E2N730(m) in EIA under optimized conditions. All hepatitis patients presented corresponding serological as well as pathological symptoms. The results are summarized in Table 1. Readings from 100 healthy blood donor samples were used to calculate the cut-off value for positivity and three out of the one hundred samples (3%) gave positive readings. In order to rule out the false-positive reactions caused by *E. coli* host protein contaminations in E2N730(m) preparation causing false-positive reactions, these three samples were confirmed using purified E2N730(m) (Fig. 4). Two samples reacted specifically with the 42 kD E2N730(m) band, although the intensity of the band was lower than that obtained with hepatitis C patient sera (Fig. 4, compare lane 2 and 4 with lane 1). The third sample showed negative reaction with E2N730(m) (Fig. 4, lane 3). The two donors positive for anti-E2 in both EIA and Western blot might have been exposed to HCV. Unfortunately, because of limited sample availability and difficulty in tracing the individual donors, we were unable to ascertain the nature of this anti-E2 response in these subjects.

None of the hepatitis B and non-A-to-E hepatitis patients' sera reacted with E2N730(m) (Table 1). This result and the result with healthy blood donors demonstrated that E2N730(m) displayed high specificity and sensitivity in EIA.

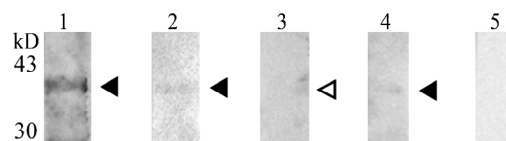


Fig. 4 Confirmatory Western blot analysis of anti-E2 antibodies in healthy blood donor sera

Purified E2N730(m) was run on 12% SDS/PAGE gel, transferred to nitrocellulose membrane, and blotted against human sera as described in Materials and Methods. 1, hepatitis C patient sera positive for anti-E2N730(m) in EIA as positive control; 2–4, three healthy blood donors sera positive for anti-E2N730(m) in EIA; 5, healthy blood donor sera negative for anti-E2N730(m) in EIA as negative control. Solid and open arrows indicate positive and negative signals, respectively.

Table 1 Detection of anti-E2 antibodies in human sera using E2N730(m) as capture antigen

Serum sample (number of samples tested)	Antibodies			
	Anti-HCV		Anti-E2	
	Number of positive samples	Positivity rate (%)	Number of positive samples	Positivity rate (%)
Healthy blood donors (100)	0	0	3	3
Hepatitis B patients (20)	0	0	0	0
Non-A-to-E patients (20)	0	0	0	0
Hepatitis C patients (158)	158	100	89	56

Anti-E2 antibodies in human sera were detected using *E. coli*-derived E2N730(m) as coating antigen. Anti-HCV antibodies were detected using UBI HCV EIA 4.0, which does not incorporate any E2 components.

Eighty-nine samples out of 158 hepatitis C patient sera (56%) were positive for anti-E2N730(m). This positivity rate is higher than that we obtained with *E. coli*-derived aa 385–565 fragment of E2 which lacked the C-terminal half of E2N730(m) [20]. Some reports in the literature have also used E2 proteins expressed in bacteria to detect anti-E2 antibodies in hepatitis C patient sera, either in EIA or in Western blot [22–25]. The reported positivity rate for anti-E2 varies from study to study between 17% and 73%. Conceivably, fragment selection, antigen formulation, assay format, and patient selection all played a role in creating such discrepancies. Our EIA employed an E2 antigen encompassing the largest amount of E2 amino acid residues reported so far for *E. coli* expression, and also used the largest number of patient samples

reported for such an assay. It is our opinion that the 56% positivity rate obtained with E2N730(m) largely reflected the prevalence of glycosylation- and conformation-independent anti-E2 antibodies in Chinese hepatitis C patient sera.

Theoretically, E2 glycoproteins purified from mammalian cells would be ideal for the detection of anti-E2 antibodies in human sera, because they could best mimic the immunological properties of natural E2 glycoproteins on HCV virions. However, high-level and large-scale expression/purification of E2 in mammalian cells is difficult and very expensive. It is worth comparing *E. coli*-derived E2N730(m) and mammalian cell-expressed E2 glycoproteins in EIA using the same patient group and under the same conditions, to see whether the difference in positivity rate would be small enough to justify using E2N730(m) as an inexpensive alternative to E2 glycoproteins. We are now probing such a possibility.

Using mammalian cell or insect cell-derived E2 glycoproteins, some researchers have also linked the presence of anti-E2 antibodies in chronic hepatitis C patients and interferon therapy recipients with on-going HCV replication indicated by RT-PCR positivity [26–28]. Therefore, anti-E2 testing has been suggested to be a useful indicator for clinical diagnosis of HCV infection and monitoring the outcome of interferon therapy. One hundred and forty-three out of the 158 hepatitis C patient sera were subjected to anti-E2N730(m) and HCV RNA detection to find out whether an association between anti-E2 positivity and HCV RNA positivity could also be established using this *E. coli*-derived E2 protein. The results are summarized in Table 2. In a total of 143 samples, 86 were HCV RNA positive, of which 64 (74%) were also anti-E2 positive. In the 57 HCV RNA negative samples, however, only 24 (42%) were anti-E2 positive. The difference was statistically significant (χ^2 test, $P < 0.05$), indicating an association between the presence of anti-E2N730(m) antibodies and HCV viremia. In other words, patients positive for anti-E2N730(m) have a higher likelihood of being actively infected by HCV. This result suggested that anti-E2 EIA based on E2N730(m) might be of clinical value for both blood donor screening and hepatitis C diagnosis/therapy monitoring.

In summary, despite a four-residue mutation and no glycosylation or three-dimensional conformation, E2N730(m) expressed in *E. coli* displayed E2-specific antigenicity and immunogenicity shared by mammalian E2 glycoproteins. After further evaluation and optimization, E2N730(m) could be used for preliminary clinical detection of anti-E2 antibodies in human sera, as a substitute for expensive mam-

Table 2 Association between anti-E2 antibodies in hepatitis C patient sera and viremia

HCV RNA status (number of samples)	Number of anti-E2 positive samples	Anti-E2 positivity rate (%)
Positive (86)	64	74
Negative (57)	24	42

A total of 143 hepatitis C patient sera samples positive for anti-HCV by UBI HCV EIA 4.0 were analyzed for RNA positivity. Anti-E2 antibodies were detected in EIA using *E. coli*-derived E2N730(m) as capture antigen and HCV RNA was detected using a commercially available HCV Gene Detection Kit as described in Materials and Methods.

malian cell-derived E2 glycoproteins. In addition, E2N730(m) would be useful for the detection of anti-E2 immune response in post-immune animals in E2-based HCV vaccine research. On the other hand, rabbit antisera against E2N730(m) (R_{E2-116R}) could be used to detect E2 proteins expressed in different systems, and possibly, to detect E2 antigen in liver biopsy samples. Results presented in this report provide a possible inexpensive alternative to the cumbersome and costly route of expressing E2 glycoproteins in mammalian cells, and might result in the development of new diagnostic, therapeutic and prophylactic measures against HCV.

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