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Construction and characterization of infectious hepatitis C virus chimera containing structural proteins directly from genotype 1b clinical isolates

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ABSTRACT

HCV genotype is a major determinant of clinical outcome, and GT1b HCV infection is the most difficult to treat and also the predominant genotype in East Asia and Europe. We developed 1b/JFH-1 inter-genotypic recombinants containing the structural genes (Core, E1, E2), p7 and the 1stTMD of NS2 directly from GT1b clinical isolates. Through a cloning selection strategy, we obtained 4 functional clones from 3 cases of GT1b patients' sera, which could produce infectious viruses in Huh7.5.1 cells. Sequencing analysis of recovered viruses from serial passage and reverse genetics revealed that adaptive mutations in the GT1b-originated region were enough for the enhancement of infectivity. A monoclonal antibody to E2 and original patient sera could efficiently block 3 of the viruses (26C3mt, 52B6mt and 79L9) while had little effect on 26C6mt viruses. The availability of 1b/JFH-1 chimeric viruses will be important for studies of isolate-specific neutralization and useful in evaluating antiviral therapies.

Introduction

About 3% of the world's population is infected with hepatitis C virus (HCV), and chronic HCV infection is a strong indicator of end-stage liver disease, such as cirrhosis, liver failure and hepatocellular carcinoma (HCC). No vaccine is available yet. The current standard treatment, combining pegylated interferon-α and ribavirin, was recently supplemented with small-molecule inhibitors targeting HCV encoded NS3/4A serine protease (Sarrazin et al., 2012). The responsiveness to the therapy is affected by viral and host factors, including virus genotype, viral load and host genetics such as single nucleotide polymorphism (SNP) in *IL28B* loci (Feld and Hoofnagle, 2005; Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009).

HCV is an enveloped, positive-stranded RNA virus belonging to the *Flaviviridae* family. Its genome is 9.6 kb in length and consists of a 5′ untranslated region (UTR), an open reading frame (ORF), and a 3′ UTR. The ORF encodes a single polyprotein, which is cleaved into the structural proteins (Core, E1 and E2), p7, and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Bartenschlager et al., 2004). Due to the rapid replication rate and poor fidelity of the RNA-dependent RNA polymerase (RdRp), HCV has a high degree of genetic and antigenic heterogeneity with six major genotypes and numerous subtypes. Genotypes have more than 30% variation at the nucleotide level; subtypes typically differ from each other by 20–25%. Furthermore, 5–8% sequence divergence is present between individual strains (variants) of HCV within a given subtype (Simmonds et al., 2005). In China, Japan and Europe, genotype 1b is the predominant genotype. With the significant variations at the nucleotide and amino acid levels among HCV genotypes, biological and clinical differences are expected: Genotype 1b is the least sensitive to IFNα-based treatment, with the sustained virological response rate around only 50% (Feld and Hoofnagle, 2005). Meanwhile, HCV exists in host as quasispecies, which is characterized as a heterogeneous population of highly related but genetically distinct variants and may play a role in disease progression and treatment response.

The progress in HCV field was greatly hindered by the lack of *in vitro* culture models until the development of infectious cell culture systems (HCVcc) based on genotype 2a isolate JFH-1 (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Since then, JFH-1-based intra- and inter-genotypic recombinants

Abbreviations: HCVcc, HCV cell culture-derived; HCVpp, HCV pseudotype particles; FFU, focus forming units; TMD, trans-membrane domain; ORF, open reading frame; UTR, untranslated region; GT, genotype.

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harboring structural proteins (Core, E1, E2), p7, and the complete or partial NS2, were developed and efficient growth depended on various adaptive mutations (Gottwein et al., 2007, 2009; Jensen et al., 2008; Pietschmann et al., 2006; Scheel et al., 2008; Yi et al., 2007), allowing the study of vaccines and entry inhibitors for all genotypes. However, these studies are all based on a few laboratory optimized prototype strains (established HCV consensus molecular clones) (Bukh et al., 2010, 2002; Gottwein et al., 2010; Kato et al., 2008; Kolykhalov et al., 1997; Lohmann et al., 1999; Sakai et al., 2007; Yanagi et al., 1997, 1998, 1999). Though the establishment of these well characterized virus strains has greatly facilitated the development of new experimental *in vitro* systems (Gottwein et al., 2011; Imhof and Simmonds, 2010; Li et al., 2011a, 2011b, 2012b, 2012c; Scheel et al., 2011a, 2011b) and antiviral therapies, they may not reflect the heterogeneous viral population that exists in HCV-infected patients. No full-length or chimeric HCVcc system has been developed directly from a clinical isolate.

To investigate the possibility of constructing HCV recombinants directly from clinical isolates, we selected three Chinese GT1b hepatitis C patients, amplified the region harboring Core-1st transmembrane domain (TMD) of NS2 from the patient sera, and construct a library of JFH-1 based recombinants. After transfecting the library of synthesized chimeric HCV RNA into Huh7.5.1 cells, we were able to select the cDNA clones from each clinical isolate that produce infectious viruses in cell culture. The infectivity of these chimeric viruses can be significantly enhanced by continuous passaging of the viruses. Reverse genetic study demonstrated the mutations in the Core-NS2 (1st TMD) region are responsible for the enhanced infectivity. These established chimeric HCVcc displayed different sensitivity to neutralization by an anti-E2 monoclonal antibody or patient sera. Our study not only offers a new strategy to construct HCVcc directly from clinical isolates, but also provides a useful tool for the isolate-specific studies, including studies of virus assembly, neutralizing antibodies and vaccine development.

Results

Development and cell-culture adaptation of 1b/JFH-1 inter-genotypic recombinants

The recombinant 1b/JFH-1 chimeric virus was constructed in a manner analogous to that used to generate JC1 (Pietschmann et al., 2006). The region harboring Core-NS2 (1st TMD) (amino acid 1-842) (Yamaga and Ou, 2002) was amplified from HCV patient sera and used to substitute the JFH-1's counterpart (Fig. 1A). Three genotype 1b patients (PR26, PR52, PR79) were selected for this study. Patient sera were collected prior to the interferon therapy, and HCV RNA levels in the sera were determined (Table 1). The consensus sequence of the GT1b isolates were obtained by bulk PCR sequencing of six overlapping fragments (Xiang et al., 2011). The intact ORFs were assembled and deposited to the NCBI Genbank with accession number HQ912956 (PR26), HQ912958

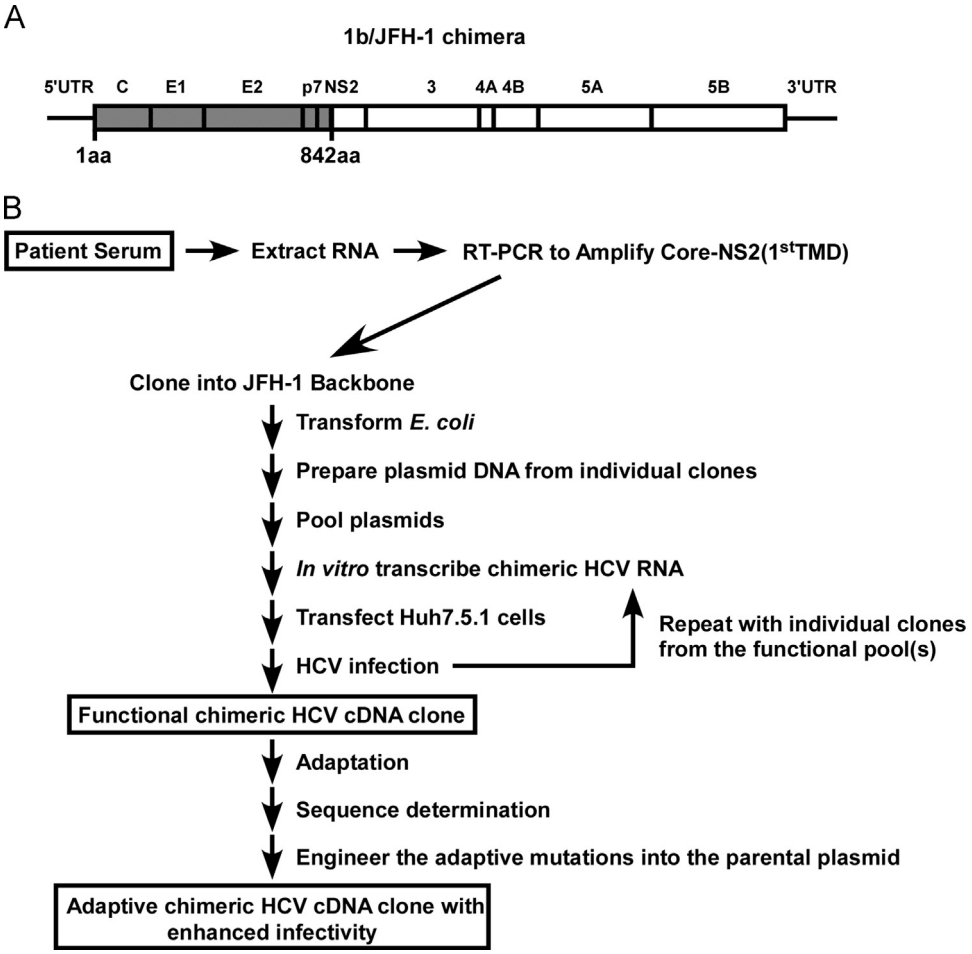

Fig. 1. Construction of inter-genotypic 1b/JFH-1 chimeras. (A) Schematic diagram of 1b/JFH-1 chimera. The region harboring Core-1st TMD of NS2 derived from GT1b isolates (black dotted) was cloned into JFH-1 backbone (white box). The inter-genotypic junction is located at nucleotide position 2866/2867 (con1 reference [AJ238799] 2866/2867), or at amino acid residues 842/843. (B) Protocol for the construction of infectious 1b/JFH-1 chimeric cDNA clone.

(PR52) and HQ912959 (PR79). The protein sequence comparison between GT1b isolates and a reference strain con1 (AJ238799) is summarized in Table 1 and Supplementary Fig. S1. The sequence homology of entire ORF or the core-NS2 (1st TMD) region between PR26, PR52, PR79 and con1 is about 92–93% at the amino acid level.

The common strategy of constructing inter-genotypic HCV recombinants is based on established prototype strains with consensus sequence. However, the consensus sequence results from the predominant nucleotide at each position, and may not exist in nature. Taking this into account, we designed a functional screening strategy to select the most viable clones from the quasispecies population (Fig. 1B) instead of using the consensus sequence to make a prototype cDNA clone by direct DNA synthesis. The Core-NS2 (1st TMD) PCR amplification products, containing a pool of variants, were fused with JFH-1 backbone. We randomly picked colonies from *E. coli* library transformants. After confirming the correct size of the inserted Core-NS2 (1st TMD) region, we pooled 20–30 clones and separated them into several groups for HCV RNA synthesis by *in vitro* transcription. After transfecting the pools of RNAs into Huh7.5.1 cells, we passaged the cells and monitored HCV-positive cells percentage and supernatant infectivity. When a certain group produced detectable infectious particles in the culture supernatant, we then repeated the above experiment to test individual plasmids within this group to identify the viable candidate(s).

For PR26 and PR52, we transfected 4 groups of RNA transcripts from a total of 24 plasmids. For PR79, we transfected 6 groups of RNA transcripts from a total of 30 plasmids. We could find HCV-positive cells in almost all groups at D2 post-transfection by anti-NS3 immuno-fluorescence staining (data not shown). We kept passaging the transfected cells as long as HCV-positive cells were detected. During the long-term passaging, HCV-positive cells in most groups became fewer and ultimately disappeared, while in certain groups HCV-positive cells finally spread to almost the entire cell population (over 80%). To identify individual functional clones responsible for virus expansion, we synthesized HCV RNA from each plasmid in the selected positive pool, and transfected them individually into Huh7.5.1 cells. By this way, we selected 2 viable clones from PR26 (named 26C3 and 26C6), 1 from PR52 (named 52B6) and 1 from PR79 (named 79L9). Sequence comparison of these single clones with their consensus sequence from the original sera is shown in Fig. 2. Clones 26C3 and 26C6 are selected from the same patient's serum, and only differ in 2 residues in E1 region: at residue 290, 26C3 is Leu while 26C6 is Pro; at residue 364, 26C3 is Thr while 26C6 is Met.

26C3 chimeric virus reached a peak titer of 1.8×10^4 FFU/mL at 74 days post-transfection (DPT); 26C6 had a peak titer of 6.5×10^3 FFU/mL at 74 DPT; the peak infectivity titer of 52B6 was 3.2×10^4 FFU/mL at 41 DPT; 79L9 could produce relatively high titers of viruses at earlier days after transfection compared with the others: it was 10^3 FFU/mL at 5 DPT, and reached the peak titer of 1.3×10^4 FFU/mL at 21 DPT.

Since it took long time for 26C3, 26C6 and 52B6 to reach the peak titers, we speculated that the viruses may have acquired adaptive mutations to enhance the viral infectivity. Thus, we sequenced the Core-NS2(1st TMD) region of 26C3, 26C6 and 52B6 viruses collected at their peak titers. In the viral supernatants of 26C3 recovered at 75 DPT, two nucleotide changes were found in E1 and E2 (G1097T and C1903T), resulting in amino acid change in E1 (V253F) (numbering throughout is according to the con1 reference sequence AJ238799). In 26C6, we found two nucleotide changes in E2 and NS2 (G1816A and A2844G), resulting in amino acid change in NS2 (Y835C) (Fig. 2A). In the viral supernatants of 52B6 recovered at 39 DPT, three nucleotide changes occurred in E2 (T1529C, G1533A, and A1586G) resulting in three amino acid changes (W397R, G398D and T416A) (Fig. 2B). In the case of 79L9, the infectivity titers at earlier days post-transfection were high (10^3 FFU/mL at 5 DPT, and 1.3×10^4 FFU/mL at 21 DPT). It is therefore not surprising that no

Table 1
Similarity between GT1b isolates and con1 reference sequence at amino acid level. The sequence homology was calculated with BIOEDIT Clustal Multiple Alignment software.

Patient Id	Genotype	RNA titer at collection (IU/mL)	Amino acid identity with con1 (AJ238799) (%)	
			Core-NS2 (1st TMD)	ORF
PR26	1b	2.7×10^7	93	93
PR52	1b	3.2×10^6	92	92
PR79	1b	4.3×10^6	93	93

A

Residues*	253	290	364	476	531	835
Protein	E1	E1	E1	E2	E2	NS2
PR26	V	L	M	E	E	Y
26C3	V	L	T	T	G	Y
26C3mt	F	L	T	T	G	Y
26C6	V	P	M	T	G	Y
26C6mt	V	P	M	T	G	C

B

Residues*	180	397	398	416	837
Protein	Core	E2	E2	E2	NS2
PR52	A	W	G	T	V
52B6	T	W	G	T	L
52B6mt	T	R	D	A	L

C

Residues*	38	183	184	217	365	405	415	532	785	827
Protein	Core	Core	Core	E1	E1	E2	E2	E2	p7	NS2
PR79	P	S	C	A	V	T	N	N	G	M
79L9	S	P	R	T	A	L	S	S	E	V

Fig. 2. Sequence comparison of core-NS2 (1st TMD) between (A) clone 26C3/26C3mt, 26C6/26C6mt and PR26 consensus sequence, (B) clone 52B6/52B6mt and PR52 consensus sequence and (C) clone79L9 and PR79 consensus sequence. *The position of amino acid (single-letter code) is according to con1 reference polyprotein sequence.

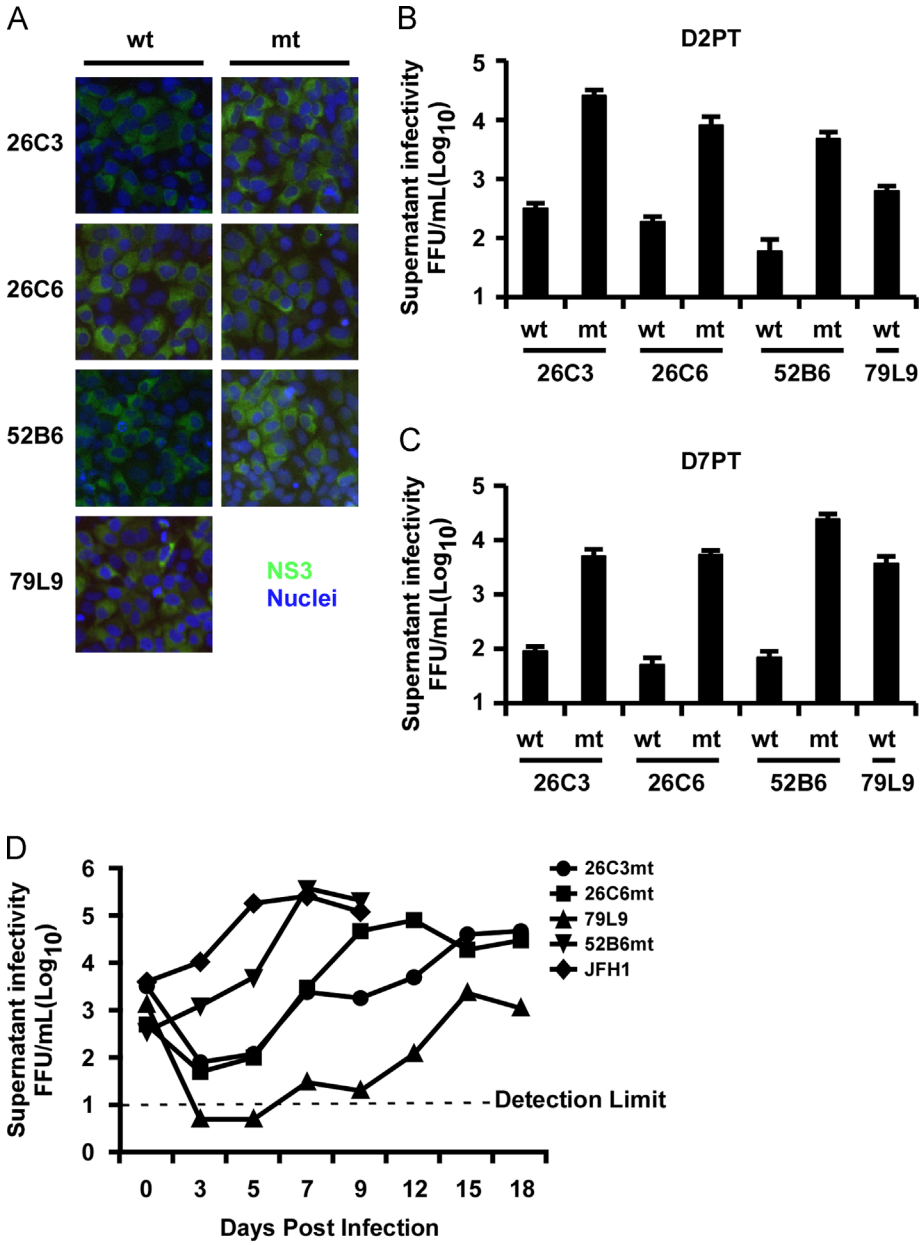

Fig. 3. Adaptive mutations enhance virus production of the selected clones. (A) *In vitro* transcribed RNA synthesized from wild-type clones (26C3wt, 26C6wt, 52B6wt and 79L9wt) and clones with adaptive mutations (26C3mt, 26C6mt, 52B6mt) were electroporated into Huh7.5.1 cells. Immuno-fluorescence staining of HCV NS3 protein at day 2 post-transfection showed the transfection efficiencies were comparable between the wild-type and mutant RNAs. NS3 staining in green; nuclei stained with Hoechst (blue). Cell culture supernatants were collected at day 2 (B) and day 7 (C) post-transfection and analyzed for the infectivity titers by a titration assay. (D) Infection kinetics of adapted viruses. The naïve Huh7.5.1 cells were inoculated with viruses at an moi∼0.02. Supernatants from the inoculated cells were collected at the time points indicated and analyzed for infectivity titers as above. 10 FFU/mL is the detection limit.

nucleotide change was detected at the end of culture (30 DPT). It is noteworthy that other adaptive mutations may exist outside the region of Core-NS2 (1st TMD) and are beyond of our current study interest.

Identification of adaptive mutations leading to efficient growth of 1b/JFH-1 viruses

To identify adaptive mutations that are responsible for the enhanced infectivity, we engineered the non-synonymous amino acid changes into the original clones, and transfected Huh7.5.1 cells with the *in vitro* synthesized RNA of 26C3 wild-type (wt), 26C3-V253F (26C3mt), 26C6 wt, 26C6-Y835C (26C6mt), 52B6 wt, 52B6-W397R-G398D-T416A (52B6mt) and 79L9. The anti-NS3 immuno-fluorescence staining at 2 DPT showed the percentage of HCV-positive cells were comparable among all groups, indicative of similar transfection efficiencies (Fig. 3A). At day 2 and day 7, the cell culture supernatants were collected and infectivity was measured (Fig. 3B, C). Adaptive mutations greatly enhanced the virus titers: the V253F mutation enhanced the infectivity titers of 26C3 virus at 2 DPT for 78 folds; the Y835C mutation enhanced the infectivity titers of 26C6 virus at 2 DPT for 43 folds; the three mutations W397R-G398D-T416A introduced to the N-terminus of its E2 protein of 52B6 virus enhanced the infectivity titers at 7 DPT for 342 folds. 79L9 had an infectivity of 610 FFU/mL and 3700 FFU/mL at 2 DPT and 7 DPT, respectively. Direct sequencing of the intracellular HCV RNA confirmed no other mutations occurred at 7 DPT (data not shown).

Next we compared the growth kinetics of these rescued 1b/JFH1 viruses. Naïve Huh7.5.1 cells were infected at low multiplicity of infection (moi~0.02) with viral supernatants collected from

the transfection experiment described above, along with JFH1 viruses as a positive control (Fig. 3D). The culture supernatants at the various days post infection were collected for measuring the infectivity titers. For 52B6mt and JFH1, the infectivity plateaued on 7 days post infection (DPI) and the titers were over 10^5 FFU/mL. Due to severe cytopathological effect, we could not continue the passage of JFH-1 and 52B6mt after 9 DPI. 26C3mt and 26C6mt propagated more slowly than 52B6mt, both of which had titers of around 10^3 FFU/mL at 7DPI and finally reached peak titers of above 10^4 FFU/mL at 15 DPI (26C3mt) and 12 DPI (26C6mt), respectively. 79L9 had an eclipse phase before achieving titers of over 10^3 FFU/mL at 15DPI.

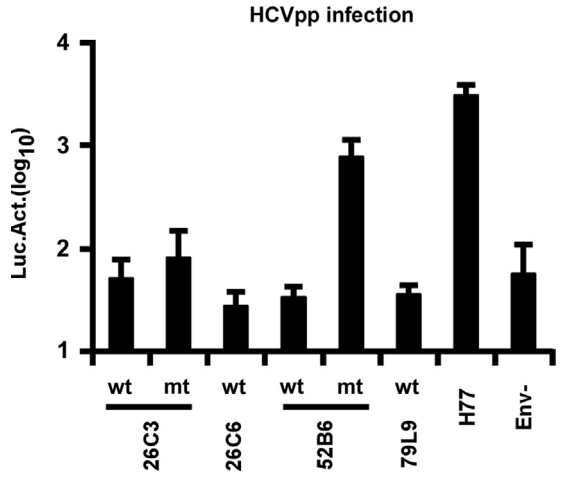

Fig. 4. The infection of Huh7.5.1 cells with HCVpp bearing the E1–E2 regions of 26C3wt, 26C3mt, 26C6wt, 52B6wt, 52B6mt and 79L9 clones. H77 and env- was positive and negative controls, respectively. HCVpp produced from transfection of HEK293T cells were used to infect Huh7.5.1 cells for 3 days. The lysates of infected cells were assayed for the luciferase activities. The error bar represented triplicates.

Pseudotype retroviral particles bearing the HCV envelope glycoproteins (HCVpp) have provided a practical approach to HCV entry studies. To test whether adaptive mutations enhanced the infectivity by improving the entry efficiency of HCV particles, we compared the infection of HCVpp bearing the wild type or mutant E1–E2 sequence (Fig. 4). For 26C6, since the mutation is located in the NS2 protein, the HCVpp bearing E1–E2 from 26C6wt and 26C6mt is identical and thus only 26C6wt was included in the HCVpp assay. H77 (GT1a reference clone, GenBank AF009606) E1–E2 and env- served as positive and negative controls for the experiment, respectively. As shown in Fig. 4, the infection of HCVpp bearing the envelope proteins of 26C3wt, 26C3mt, 26C6wt and 79L9 was similar to that of the env- negative control, suggesting these envelope proteins are not functional in the context of HCVpp infection. However, the three mutations in its N-terminus of E2 (W397R, G398D and T416A) in 52B6 greatly enhanced the HCVpp infection, indicating these mutation may improve the HCV entry efficiency.

Blockade of 1b/JFH-1 viruses by anti-E2 neutralizing antibody

The existence of broadly conserved neutralizing antibodies to HCV has been confirmed with HCVpp (Bartosch et al., 2003). These antibodies differ in their breadth and mechanism of neutralization (Edwards et al., 2012). To investigate whether the 1b/JFH-1 chimeric viruses could be blocked by neutralizing antibodies, we performed the neutralization assay with a previously reported human anti-E2 monoclonal antibody (clone AR3A) (Law et al., 2008). Due to the low infectivity of 26C3, 26C6 and 52B6 wild-type viruses, we used the mutant virus with the adaptive mutations to perform this assay. HCV E2 protein has been reported to contain 3 discontinuous regions (residues 396–424, 436–447, 523–540) important for the binding with its monoclonal antibody AR3A (Law et al., 2008). The sequence comparison of the E2 epitopes between 26C3mt, 26C6mt, 52B6mt, 79L9 and H77 reference strain is shown in Fig. 5A. For the neutralization assay, the infectious HCV supernatants were pre-incubated with

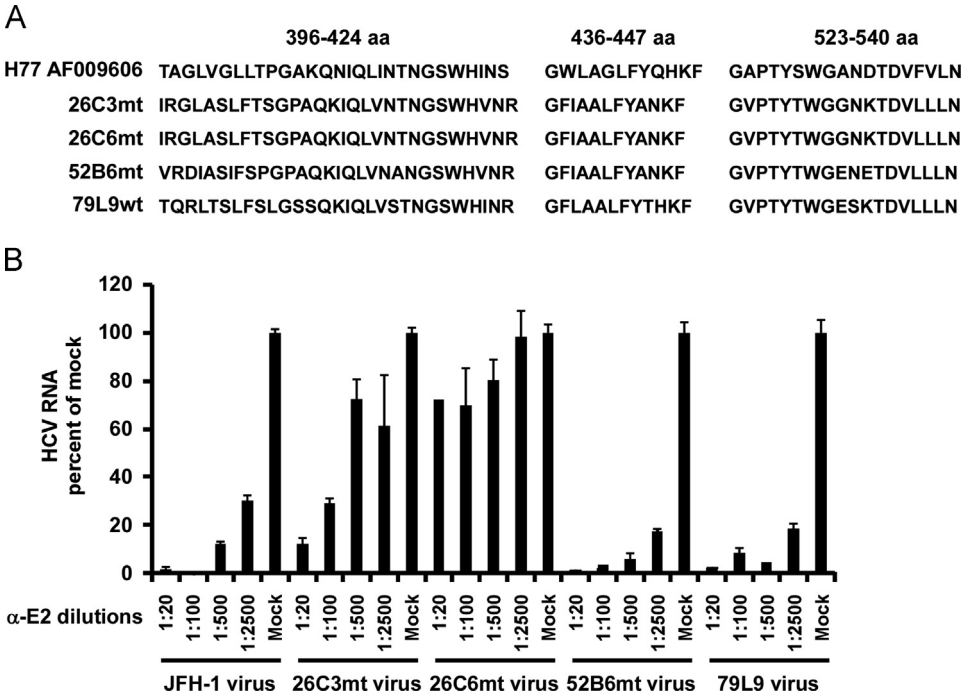

Fig. 5. Mean ± SE neutralization of 1b/JFH-1 viruses by E2 monoclonal antibody. (A) Alignment of deduced E2 epitopes. The residues 396–424, 436–447 and 523–540 are regions important for E2 binding with its monoclonal antibody (AR3A clone) (Law et al., 2008). The corresponding amino acids sequence of 26C3mt, 26C6mt, 52B6mt and 79L9 clones were aligned. (B) ~250 FFU viruses were incubated with serial 5-fold dilutions (1:20–1:2500) of E2 monoclonal antibody for 1 h and then infected Huh7.5.1 cells pre-seeded in 48-well plate for 3 days. The intracellular HCV RNA was quantified by RT-qPCR and expressed as the percentage of the culture medium control. Each assay was performed twice and representative data were shown. The error bars indicate standard deviation from the mean.

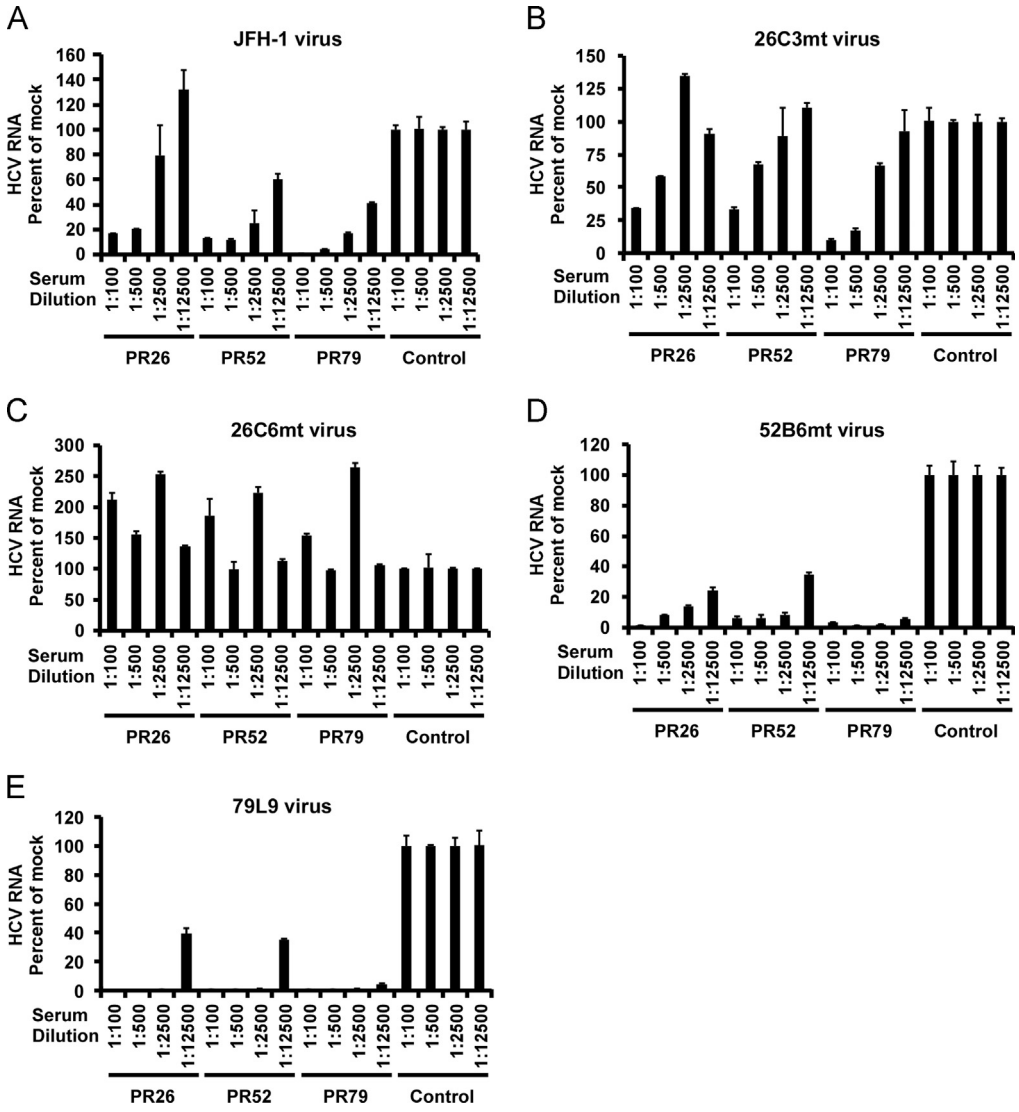

Fig. 6. Neutralization of a panel of viruses by PR26, PR52, PR79 and control sera: (A) JFH-1 viruses; (B) 26C3mt viruses; (C) 26C6mt viruses; (D) 52B6mt viruses; and (E) 79L9 viruses. ~250 FFU viruses were incubated with serial 5-fold dilutions (1:100–1:12,500) of each serum prior to inoculation into Huh7.5.1 cells. The intracellular HCV RNA was quantified by RT-qPCR and expressed as the percentage of the control serum of the same dilution. The error bars indicate standard deviation from the mean.

a serial dilution of anti-E2 antibody prior to the addition to the Huh7.5.1 cells. The neutralization of HCV infection was measured by intracellular HCV RNA levels. As shown in Fig. 5B, the HCV infection of JFH-1, 52B6mt, 79L9 viruses was efficiently inhibited by pre-incubation with the anti-E2 antibody in a dose-dependent manner. For JFH-1, a ~90% inhibition was observed at 1:500 dilution of anti-E2 antibody. For both 52B6mt and 79L9 virus, a >90% inhibition was achieved with 1:500 dilution of anti-E2 antibody. 26C3mt virus was moderately inhibited by the anti-E2 antibody: about 70% of virus was neutralized at 1:100 dilution of anti-E2 antibody. As for 26C6mt virus, the anti-E2 antibody had barely any effect on its infection: even at the highest concentration of E2 antibody (1:20), 70% of the virus infection remained. Given the fact that the E2 sequence of 26C3mt and 26C6mt is identical (Figs. 2A and 5A), it is intriguing to study the mechanism underlying the difference in their sensitivity to E2 antibody.

Neutralization of 1b/JFH-1 viruses by GT1b patient sera

We investigated whether the GT1b sera where the structural proteins were originally derived from could neutralize the homologous or heterologous 1b/JFH-1 virus. We first used JFH-1 virus to test whether the patient sera contained neutralizing antibodies. As shown in Fig. 6A, the neutralizing ability in the patient sera against JFH-1 virus was different: we observed a >50% inhibition of JFH-1 infection by PR26 serum at 1:500 dilution, by PR52 serum at 1:2500 dilution and by PR79 serum at 1:12,500 dilution, which indicated the PR79 serum had the highest neutralizing activity. Next we tested how these patient sera neutralized the 1b/JFH-1 viruses. 26C3mt virus can be blocked by the sera of PR26, PR52 and PR79 in a dose-dependent manner (Fig. 6B). With a 1:500 dilution of PR26 serum, 1:500 dilution of PR52 serum and 1:2500 dilution of PR79 serum, the neutralization of 26C3mt virus was about 40%, 30% and 30%, respectively. 26C6mt virus is very unique in that it cannot be neutralized by any patient sera at even the highest serum dosage (Fig. 6C). 52B6mt virus and 79L9 virus are both sensitive to patient sera treatment (Fig. 6D, E): at 1:12,500 dilution of PR26 serum and PR52 serum, >50% inhibition of both viruses were observed. When using PR79 serum to neutralize 52B6mt virus and 79L9 virus, >90% inhibition was observed at 1:12,500 dilution.

Discussion

JFH-1 based intra- or inter-genotypic recombinant viruses have been constructed with well characterized prototype strains

(1a (H77, H77C, DH6), 1b (con1, J4, DH1, DH5), 2a (J6), 2b (J8), 3a (S52, 452, DBN), 4a (ED43), 5a (SA13), 6a (HK6a), 7a (QC69)) (Gottwein et al., 2007, 2011, 2009; Imhof and Simmonds, 2010; Jensen et al., 2008; Li et al., 2011; Lindenbach et al., 2005; Pietschmann et al., 2006; Scheel et al., 2011, 2008; Yi et al., 2007). Most of the prototype strains have been optimized and validated functional *in vivo* (Bukh et al., 2010).

To develop novel cell culture system for uncharacterized clinical HCV variants is still of few reports. The common way is to generate a consensus sequence from hepatitis C patient serum and construct cDNA clone by *in vitro* DNA synthesis, the infectivity of which usually relies on adaptive mutations (Scheel et al., 2011). Single-genome sequencing results revealed enormous genetic diversity of HCV quasispecies in chronically infected subjects (Li et al., 2012a). How to make a representative master sequence for these subjects will be of difficulty. Thus, the use of a consensus sequence for cell culture model without validation of function *in vivo* is of high risk.

To overcome the obstacle, we designed a functional screening method to select viable clones from a pool directly constructed from patient sera. We have identified clones 26C3 and 26C6 from PR26-origin serum, clone 52B6 from PR52-origin serum and clone 79L9 from PR79-origin serum, all of which could produce infectious virus albeit at a low efficiency. Passage of the viruses enhanced the infectivity titers, and reverse genetic studies showed that 26C3-V253F, 26C6-Y835C, 52B6-W397R/G398D/T416A yielded robust production of infectious viruses (Fig. 3B, C). It is interesting to note that most of these mutations are found within the structural proteins. This is in contrast to the reported 1b/JFH-1 chimeric viruses J4/JFH-1 (Gottwein et al., 2009), DH-1/JFH-1 and DH5/JFH-1 (Scheel et al., 2011), all of which need adaptive mutations in JFH1's non-structural proteins. It is still possible that we may have missed some mutations in JFH-1-origin sequence since we only sequenced the first 842 residues of 1b-origin, although this possibility seems slim given the observations that the recombinant chimeric viruses containing only these mutations in the 1b-origin region can almost recapitulate the phenotype of the adapted viruses in which these mutations were identified.

The retroviral HCVpp system could be used to study HCV entry in a single round infection (Bartosch et al., 2003; Drummer et al., 2003; Hsu et al., 2003). To our surprise, 26C3wt, 26C3mt, 26C6wt and 79L9 had similar entry efficiency as the env- control; the only improvement was observed in 52B6mt: it had more than 1 log increase in luciferase activity compared with 52B6. This result suggests that HCVpp may not be an ideal *in vitro* system to study HCV entry because HCVpp lacks lipoprotein association, an important character found in HCVcc and clinical HCV isolates.

Sequence comparison between con1, 26C3, 26C6, 52B6 and 79L9 showed the highest heterogeneity in the N-terminus of E2 protein (data not shown). When we used an E2 monoclonal antibody to check its neutralizing effect against 26C3mt, 26C6mt, 52B6mt and 79L9 viruses, an efficient inhibition of 52B6mt and 79L9 infection was observed, while 26C3mt viruses were less responsive and 26C6mt viruses were almost resistant to AR3A neutralization (Fig. 5B). Since 26C3mt and 26C6mt have identical E2 sequence, their different susceptibility to the neutralization might be due to the altered epitope conformations or exposure. Our previous studies showed that a single-point mutation in E2 could reduce the association of viral particles to host lipoproteins, exposing the epitopes on the viral glycoproteins to neutralizing antibodies (Grove et al., 2008; Tao et al., 2009). It would be interesting to test whether 26C3mt and 26C6mt viruses have a different lipoprotein association. We found that these two viruses had different buoyant density distribution profiles in the sucrose gradient ultracentrifugation assay (data not shown). The infectivity of 26C6mt was enriched in lower density fractions compared with that of 26C3mt virus. Further studies are needed to investigate whether and how this altered density distribution contributes to the neutralization sensitivity.

In summary, we have developed a method to construct inter-genotypic 1b/JFH-1 viruses directly from clinical isolates using functional cloning and selection strategy. A total of 4 chimeric viruses have been constructed. Infectivity can be improved by adaptive mutations. Recombinant viruses with adaptive mutations in the Core-NS2 region yielded infectivity titers of above 10^4 FFU/mL. Infection with these viruses was inhibited by pre-incubation with anti-E2 monoclonal antibody or genotype 1b patient sera to different extent. The availability of 1b/JFH-1 viruses from clinical strains not only permits an effective tool in screening drug candidates, but also enables isolate-specific studies for virus assembly and neutralizing antibodies.

Materials and methods

Source of HCV and patient serum

The sources of the genotype 1b isolates PR26, PR52 and PR79 were three hepatitis C patients who received the treatment of Peg-IFN-α plus Ribavirin from July 2007 to July 2008 at the Department of Infectious Diseases of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The study was approved by the Ethics Committee of Shanghai Ruijin Hospital in accordance with the Helsinki Declaration, and written informed consents were obtained from all patients. HCV RNA quantification was performed using a one-step quantitative HCV RT-PCR kit (PG Biotech, Shenzhen, China). HCV genotyping was performed using the HCV genotyping gene chip kit (Realchip Biotech, Ningbo, China) according to the manufacturer's instructions. The HCV RNA titers of the 3 patients are listed in Table 1.

HCV ORF amplification and sequence determination

The amplification and sequence determination of GT1b HCV isolates was based on serum from treatment-naïve state. The HCV consensus genome was amplified by nested RT-PCR with primers described as described elsewhere (Xiang et al., 2011; Yao and Tavis, 2005). Briefly, total RNA was extracted from 140 μl of serum samples, using QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany), and dissolved in 40 μl of nuclease-free water and stored at −80 °C until use. HCV RNA was reverse-transcribed (RT) into cDNA using TaKaRa AMV XL reverse transcriptase (TaKaRa, Dalian, China) with HCV specific primers. HCV cDNA was divided into six overlapping fragments and amplified by RT-PCR. All amplified products were purified and sequenced directly. The consensus sequence of HCV ORF was obtained by aligning overlapping sequences of PCR products. A total of three HCV genotype 1b sequences generated in this study have been deposited in GenBank under the accession numbers of HQ912956 (PR26), HQ912958 (PR52) and HQ912959 (PR79), respectively. Sequence homology of these genomes with con1 reference sequence (AJ238799) is listed in Table 1.

Construction of JFH-1-based inter-genotypic recombinants and HCVpp genesis

The HCV region covering Core-NS2 (1st TMD) was amplified by RT-PCR from patient serum RNA and then fused with 5′UTR and remaining NS2 to NS3 from JFH-1 by overlapping PCR. This fusion PCR product was digested by *EcoR* I and *Spe* I and then ligated into pUC-vJFH-1 plasmid. The randomly-selected and sequence-confirmed correct clones were divided into 4–6 groups (with each

group containing 6–8 plasmids) and plasmids in each group were pooled together for HCV RNA synthesis and transfection as described previously (Zhong et al., 2005). After transfection, the HCV expression was monitored by NS3 immuno-fluorescence staining and supernatant infectivity. When most of the cells were infected (≥80% HCV-positive cells of total population) we stopped passage and sequenced the region of Core-NS2 (1st TMD) from cell culture supernatants. Then we individually transfected clones that had the highest sequence identity with the supernatant HCV sequence. If adaptive mutations in Core-NS2 (1st TMD) region occurred, we engineered mutations back into the parental clone by point mutation with Quick Change II XL Site-Directed Mutagenesis Kit (Stratagene). Primers for the Core-NS2 amplification and fusion PCR are listed in Supplementary Table S1.

To make the HCV E1–E2 expression constructs for HCV pseudotype particles (HCVpp), the E1 and E2 sequences were amplified by PCR from wild-type or mutant 1b/JFH-1 chimeric constructs and inserted into the *Kpn* I and *EcoR* I sites of pCDNA3.1. H77 E1–E2 was sub-cloned similarly as positive control for HCVpp assay. Empty vector was included as negative control (envelop$^-$, env$^-$). Final DNA stocks were prepared with Tiangen Plasmid Mini kit and the HCV sequence was confirmed by sequencing. HCVpp were generated as previously described (Tao et al., 2009). Briefly, 293T cells were transfected with expression vectors encoding the HCV E1 E2, retroviral Core packaging component, and luciferase. The medium was replaced 6 h after transfection. Supernatants were harvested 48 h later, filtered through 0.45 μm-pore membranes, and used to infect Huh7.5.1 cells, which had been seeded the day before at a density of 1×10^4 cells per well in 96-well plate. Three days later, the Huh7.5.1 cells were lysed and measured for luciferase activity (Promega).

HCV cell culture, RNA transfection and immuno-staining

The cell culture conditions and protocols for *in vitro* transcription and HCV RNA electroporation have been described previously (Zhong et al., 2005). Anti-NS3 immuno-fluorescense staining was done with mouse anti-HCV NS3 protein monoclonal antibody (Abmart, Shanghai, China) as primary antibody and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) as secondary antibody.

Neutralization of virus infection with E2 antibody or patient serum

Approximately 250 focus-forming-units (FFU) viruses were incubated for 1 h at 37 °C with serial dilutions of heat-inactivated (56 °C for 30 min) patient sera, healthy control serum or anti-E2 monoclonal antibody (AR3A) (Law et al., 2008) in final dilutions as indicated. The virus-serum mixture was added to 2×10^4 of pre-seeded Huh7.5.1 cells in each well of 48-well plate and incubated for 3 days. The efficiency of infection was determined by quantitative RT-PCR analysis. The percentage of neutralization for each serum sample was calculated by comparison with the control serum of the same dilution. The percentage of neutralization of anti-E2 antibody was compared with the cell culture medium.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.04.030.

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