SUPPORTING INFORMATION

PCR-based *in vitro* synthesis of HCV NS3 protease for rapid phenotypic resistance testing of protease inhibitors

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Supplementary Methods

NS5b subtyping of HCV

All the clinical samples were subtyped by a NS5b subtyping method published by French National Research Agency on HIV/AIDS and viral hepatitis (ANRS)[1]. Briefly, HCV RNA was extracted from 140 μL of serum using a QIAamp Viral RNA Kit (Qiagen Inc., Valencia, CA). Then, the NS5b sequences of all the clinical samples were amplified using a hemi-nested PCR. The first step of RT-PCR with Pr3 and Pr4 was carried out at 50 °C for 30 min, then 5 cycles at 93 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min followed immediately by 35 cycles at 93 °C for 30 s, 60 °C with a drop of - 0.3 °C between each cycle and elongation at 72 °C for 1 min. A final elongation at 72 °C for 5 min was also included. The second amplification step with Pr3 and Pr5 was carried out on 2 μL of first PCR products and performed as follows: 95 °C for 5 min, then 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final elongation at 72 °C for 10 min. Finally the PCR products were sent for direct sequencing (Invitrogen Inc. ,Shanghai, China). Phylogenetic analysis was performed after alignment of the NS5b sequences (381bp), compared with the reference

sequences of genotypes 1-6 using the Neighbor-Joining method in MEGA4 software. The reliability of the phylogenetic clustering was evaluated using bootstrap analysis with 1000 replicates. The type and subtype reference sequences were obtained from the National Center for Biotechnology Information (NCBI)[2].

Reverse transcription-PCR (RT-PCR) and expression PCR for preparing DNA templates for *in-vitro* synthesis of HCV NS3 protease

To prepare the DNA template for in vitro synthesis of NS3 protease, we used a nested PCR. The first round PCR was a one-step RT-PCR with primers designed from the upstream and the downstream regions of NS3 protease on HCV genome in order for the PCR amplicons to cover all possible mutations in the open reading frame (ORF) of NS3 protease. The RT-PCR reactions were done by using the subtype-specific primers (0.4 μM each) shown in Table 2 and the extracted RNA (10 μL) as the templates in a 50 μL reaction volume using PrimeScript® One Step RT-PCR Kit Ver. 2 as recommended by the manufacturer (Takara Bio Inc., Otsu, Japan). The RT-PCR was run on a thermal cycler (Biometra, Germany) using the following program: 50 °C for 30 min, 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a final 10 min elongation. The RT-PCR amplicon was used further as the template both in the DNA sequencing to find mutations on the NS gene and in the subsequent expression PCR for introducing expression elements for *in-vitro* synthesis of NS3 protease.

The expression PCR using primers F-Ptac-NS3 and R-NS3 shown in Table 1 was performed to introduce Tac promoter, lac operator and SD sequence required for the *E. coli* S30 Extract System for Linear Templates (Promega Corporation, Madison, USA) to express NS3 protease, as well as further amplify the NS3 protease gene fragment. After 30 s initial denaturation time at 98 °C, the PCR reaction was cycled 38 times at 95 °C for 10s , 59 °C for 1min and 72 °C for 30s with a final 10 min elongation. Each reaction contained 1 U of Phusion®High-Fidelity DNA Polymerase (NEB, UK), 10 μL 5×GC buffer, 200 μM of each dNTP, 20 μM of each primer, 3% (v/v) Dimethyl-sulfoxide and 20 ng DNA template. The amplified products were analyzed

by electrophoresis using 1.5% agarose gel with ethidium bromide and gel-purified by E.Z.N.A. Gel Extraction Kit (Omega Biotek, Doraville, GA, USA). Then the purified DNA was concentrated by DNAmate (Takara Bio Inc., Otsu, Japan) following the manufacturer's instruction. The concentration and the quality of the concentrated DNAs were evaluated by a Nanodrop 2000 spectrophotometer (Thermo, USA) before adding into the *E. coli* S30 Extract System for in vitro synthesis of NS 3 protease.

***In vitro* synthesis of NS 3 protease**

The *in vitro* synthesis of NS3 protease was carried out at 24 °C for 4 h after mixing the lysate of ***E. coli* S30 Extract System** with 8 μg DNA purified from the product of the ePCR. At the same time, lysate of *E. coli* S30 Extract System in another tube without adding the DNA template was also incubated at 24 °C for 4 h as a negative control.

Susceptibility assay of NS3 protease to telaprevir and boceprevir

Susceptibility of the synthesized NS3 protease to telaprevir and boceprevir was determined with HCV Protease FRET Substrate RET S1 (Anaspec Inc., San Jose, CA), which is a 4A/4B peptide substrate (Ac-DED(EDANS)EE Abuψ[COO]ASK (DABCYL)-NH2), in 96-well microtiter plates (PerkinElmer, California, USA) as described previously[3]. Briefly, 20 μL of the S30 lysate with *in vitro* synthesized NS3 protease was used directly and pre-incubated with 10 μM co-factor peptide KK4A (KKGSVVIVGRIVLSGK) in a proteolytic buffer (50 mM HEPES with 100 mM NaCl, 20% glycerol and 5mM dithiothreitol, pH 7.8) for 10min at room temperature first. Then, either 1 μL of DMSO or 1 μL of telaprevir (or boceprevir) dissolved in DMSO with different concentrations (100nM, 1μM, 10μM and 100μM) was added into the lysate and the mixture was incubated for 20 min at 30 °C. After adding 10 μL of 2.5 μM substrate RET-S1 into the mixture, the fluorescence (λex = 355 nm, λem = 500 nm) of each well was monitored continuously at 30 °C for 1 h by a Synergy H1 Hybrid Reader (BioTek, USA). All reactions were done in duplicate wells. Each sample was performed in three independent tests.

References

1. Sandres-Saune, K., et al., *Determining hepatitis C genotype by analyzing the sequence of the NS5b region.* J Virol Methods, 2003. **109**(2): p. 187-93.
2. *http://www.ncbi.nlm.nih.gov/projects/genotyping/formpagex.cgi*.
3. Sarrazin, C., et al., *Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir.* Gastroenterology, 2007. **132**(5): p. 1767-77.

Supplementary figures

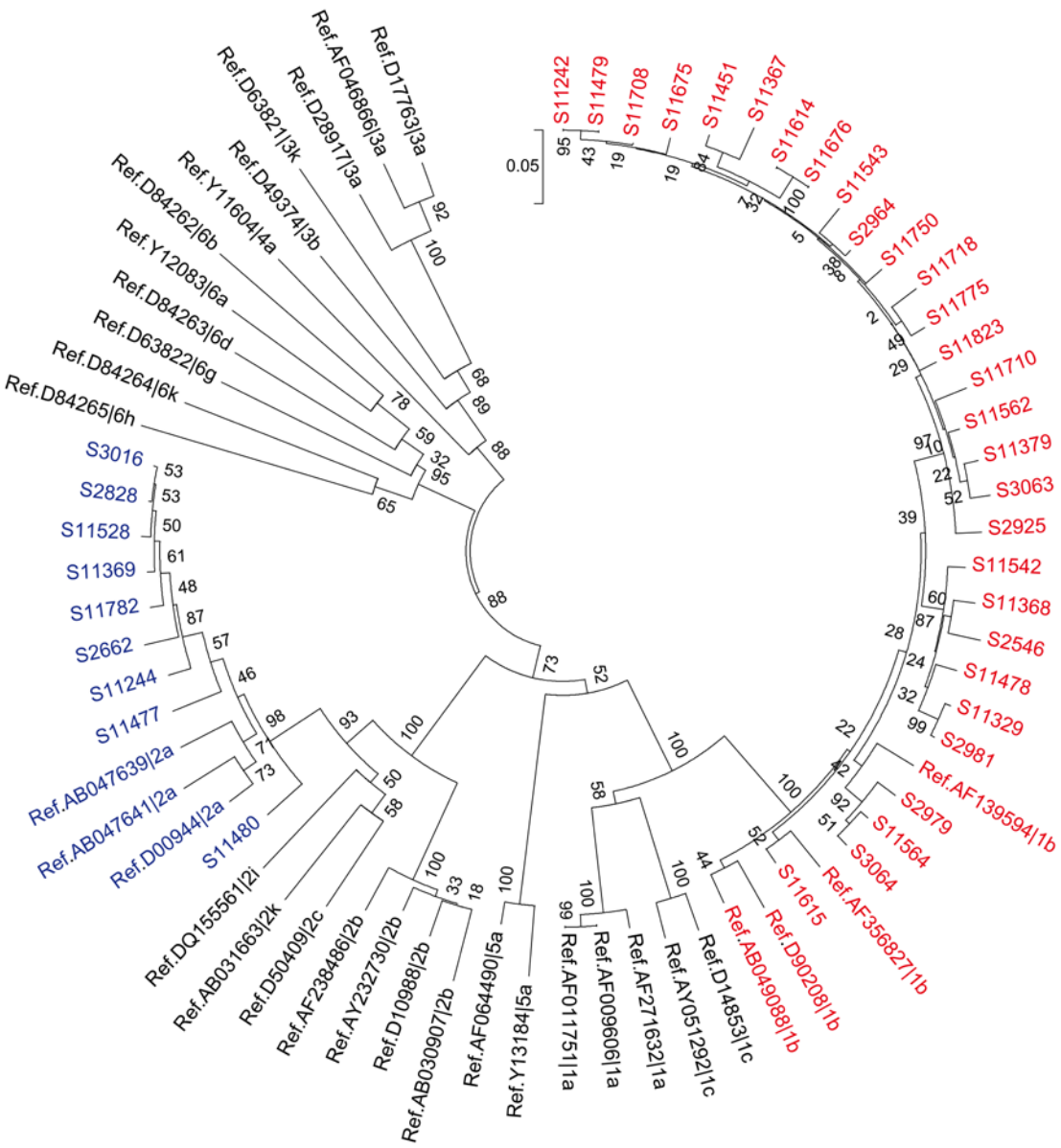

Fig S1 NS5B phylogenetic analysis of clinical serum. The phylogenetic tree was drawn by comparing nucleotide sequences of 38 HCV clinical samples with subtype reference sequences from the GenBank.

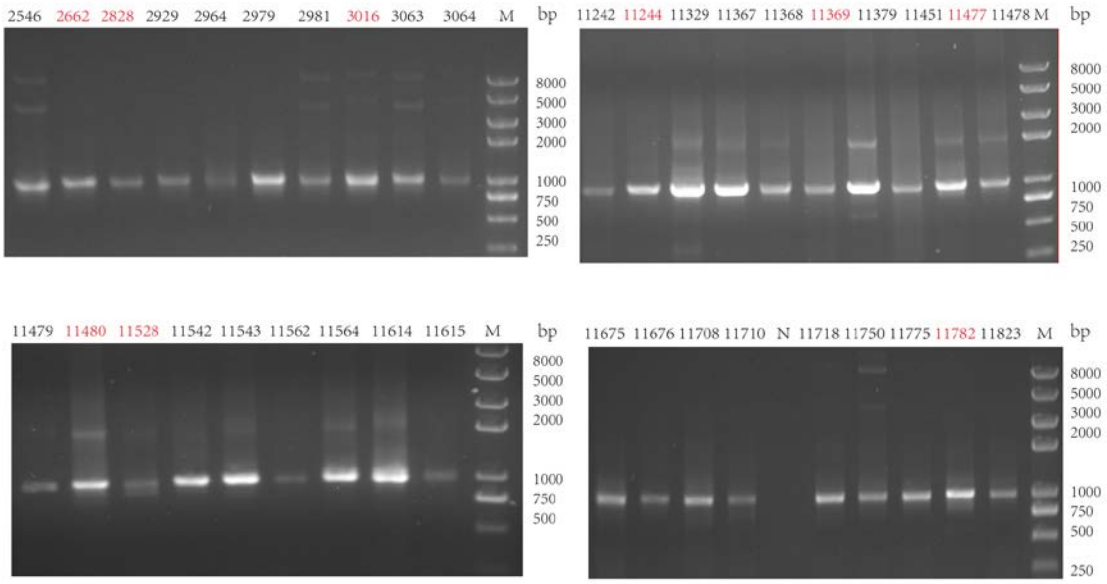

Fig S2 Gel electrophoresis of RT-PCR products of HCV clinical samples. Lane M, DNA markers; lane N, negative control.

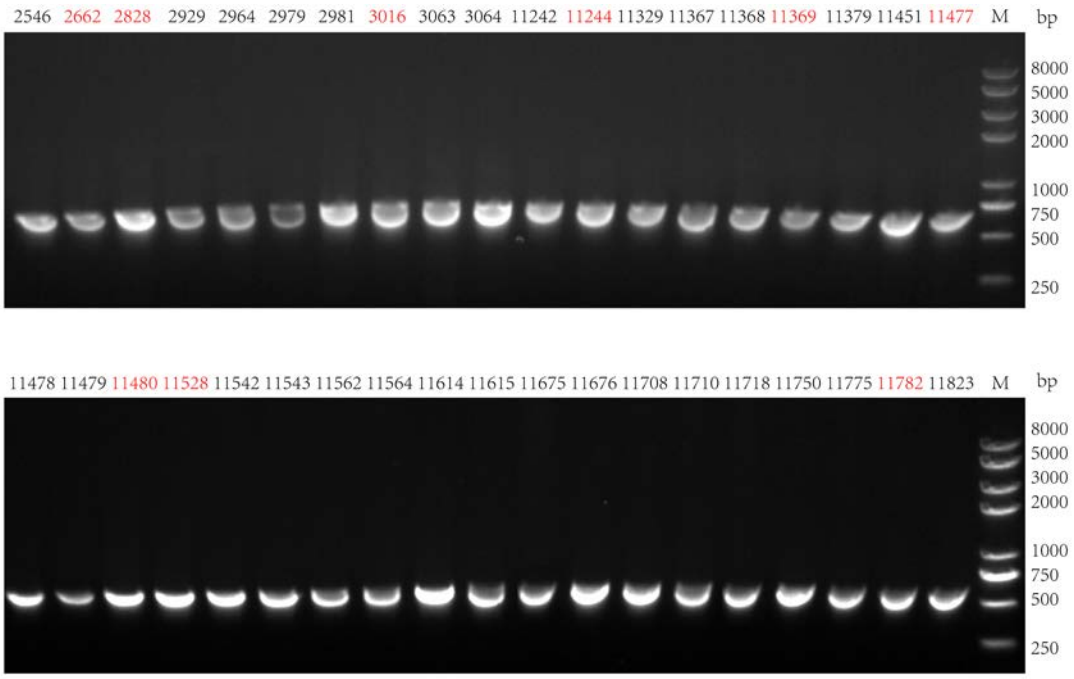

Fig S3 Gel electrophoresis of the ePCR products of HCV clinical samples. Lane M, DNA markers.

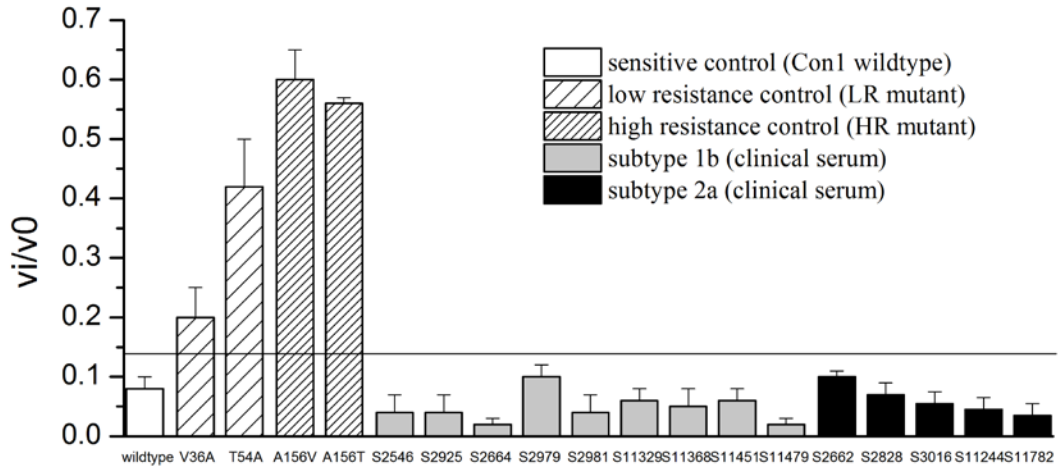

Fig S4 The v_i/v_0 ratios of the *in-vitro* synthesized NS3 proteases determined under 100 nM boceprevir. The cut-off value is the mean plus 3 standard deviations of the v_i/v_0 ratio of Con1 (wildtype control). Test of each control and sample was repeated thrice.