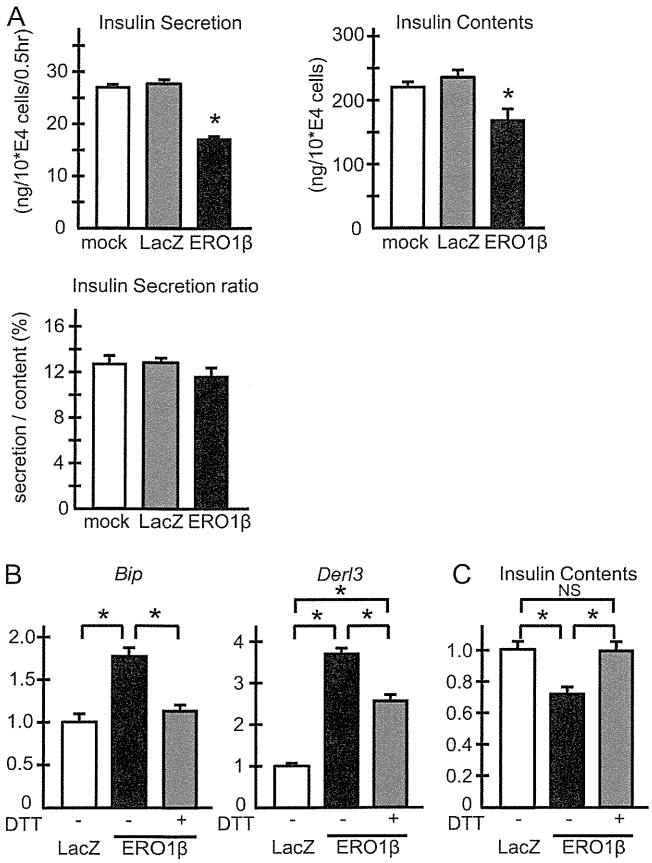

FIG 8 Adenoviral overexpression of *ERO1B* in MIN6 cells. (A) Insulin secretion rate of MIN6 cells with human *ERO1B* overexpression under high-glucose conditions. MIN6 cells infected with adenovirus as indicated were incubated with KRB buffer with 2.8 mM glucose for 60 min at 37°C, and then the medium was changed to KRB buffer with 22.4 mM glucose for a further 30 min of incubation. The medium was then subjected to insulin concentration measurement (left upper panel). Insulin was extracted from the cells by overnight incubation with acid ethanol at −20°C for the measurement of insulin content (right upper panel). Insulin concentrations were measured with an insulin radioimmunoassay kit. Insulin secretion was determined as the ratio of secreted insulin to the insulin content of the cells (lower panel). Representative results of two independent experiments are shown. $n = 4$; *, $P < 0.05$. (B) UPR gene expression of MIN6 cells with human *ERO1B* overexpression. MIN6 cells infected with the adenovirus indicated were incubated for 4 h with or without 0.5 mM DTT. Total mRNA was extracted from the cells and subjected to RT-PCR analysis of *Bip* and *Derl3*. $n = 4$; *, $P < 0.05$. (C) Restored insulin contents via mild DTT treatment under *ERO1B* overexpression. MIN6 cells infected with adenovirus as indicated were incubated for 12 h with or without 0.1 mM DTT. Insulin was extracted from the cells by overnight incubation with acid ethanol at −20°C and subjected to insulin measurement. $n = 9$; *, $P < 0.05$. The data shown are means ± the standard errors of the means.

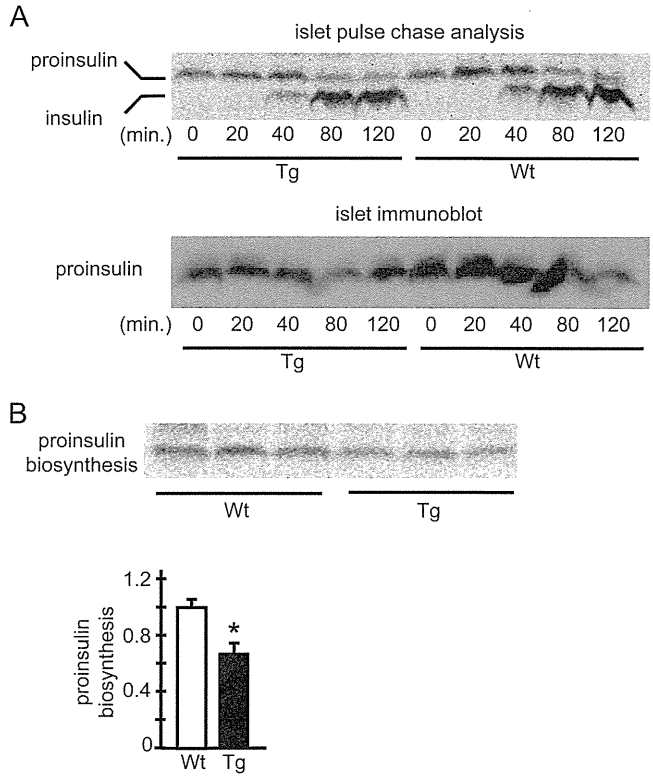

FIG 9 Analysis of insulin synthesis in hERO1βTg islets by pulse-chase experiments. Pancreatic islets were isolated from 12-week-old hERO1βTg (Tg) or control Wt mice. On the day after isolation, the islets were subjected to pulse-labeling with [^{35}S]methionine-cysteine for 30 min. (A) Subsequently, the islets were incubated in radiolabel-free medium for the indicated periods. The lysates were immunoprecipitated with anti-insulin/proinsulin antibody. Immunoprecipitated proteins were resolved by Tricine-SDS-PAGE and detected by autoradiography (top). The same membrane was subjected to immunoblotting for proinsulin with a C-peptide antibody (bottom). (B) The islets were directly collected. The lysates were immunoprecipitated with anti-insulin/proinsulin antibody. (Top) Immunoprecipitated proteins were resolved by Tricine-SDS PAGE and detected by autoradiography. (Bottom) Quantification ($n = 3$; *, $P < 0.05$). The data are means ± the standard errors of the means.

The exact mechanisms whereby ERO1β overexpression caused ER stress in β cells were uncertain. The ER stress caused by ERO1β overexpression seems to be due to the oxidizing actions of ERO1β, instead of being a nonspecific artifact, as DTT treatments reversed UPR gene upregulation, as well as reduced insulin contents by ERO1β overexpression in MIN6 β cells. One possible mechanism is that inappropriately high oxidizing conditions in the ER created by ERO1β overexpression resulted in aberrant disulfide formation within client proteins and thus led to the accumulation of misfolded proteins. Another possibility is that ERO1β overexpression caused ER stress by impairing the ERAD system. Given that the reduction of protein disulfides is required within misfolded proteins before they are dislocated and successfully degraded (36, 37), ERO1β overexpression could have hampered the reduction of misfolded proteins and thereby interfered with the ERAD system. Although we could not demonstrate delayed insulin maturation or compromised ERAD in the pulse-chase analysis, these two possibilities are still not excluded as causes of the increased ER stress in our model. Importantly, one of the most upregulated UPR genes under ERO1β overexpression was *Derl3*, which is essential to the machinery of the ERAD system (39). Therefore, it is tempting to suspect that ERAD functions were enhanced in our ERO1β overexpression model as a compensatory response by which healthy insulin handling was, if impaired by EROβ overexpression, successfully restored.

ERO1β overexpression led to impaired glucose tolerance due to impaired insulin secretion. Insulin staining in mice showed that there was a tendency toward reduction of the insulin-positive areas of hERO1βTg islets only at 36 weeks under HFD feeding, which did not reach statistical significance because of their large variations. At earlier time points of HFD feeding, ERO1β overex-

pression did not lead to changes in the insulin-positive areas, when impairment of insulin secretion was already observed upon a glucose challenge, indicating that the mechanism of impaired insulin secretion could be explained not by the changes in β cell mass but only by the altered functions of β cells. A GSIS study with isolated islets suggested that the functional impairment of ERO1β Tg islets was associated with a reduction of islet insulin contents. We observed no consistent changes in basal insulin secretion under low-glucose status in our experimental settings, including GTT of mice or GSIS of islets or MIN6 cells. Although the reason for this is unclear, there might be a specific mechanism whereby ERO1β overexpression preferentially affected the insulin granules responsible for phase 1 and 2 insulin release or, more plausibly, with the relatively small decrease in the insulin contents in any of our models, it might be due to a mere lack of enough sensitivity to detect the difference in the basal states. In fact, previous models with a more pronounced decrease in islet insulin contents do not consistently show a decrease in basal insulin secretion at low glucose concentrations (40, 41).

There could be more than one mechanism whereby ERO1β overexpression caused the reduction of islet insulin contents. *Ins1* and *Ins2*, as well as *Pdx1*, gene expression was significantly downregulated, while the magnitude of the reduction was relatively smaller than the magnitude of the insulin content reduction. Considering that ERO1β overexpression led to ER stress and that one of the fundamental ER stress responses is to downregulate protein synthesis (42), it is tempting to speculate that global repression of protein synthesis is taking place as well. In fact, the pulse-chase analysis showed a significant decrease in insulin biosynthesis in ERO1βTg islets, the magnitude of which was greater than the decrease in *Ins1* and *Ins2* mRNA levels and comparable to the decrease in insulin contents. However, the exact mechanism of insulin synthesis suppression, as well as the mechanism of decreased *Ins1* and *Ins2* mRNA levels, during ERO1β overexpression is unclear and remains to be further investigated and clarified.

So, how is ERO1β involved in the pathogenesis of diabetes mellitus? Here we have shown that ERO1β expression gradually decreases with age in the islets of *db/db* mice in parallel with the progression of glucose intolerance and that ERO1β expression was also decreased in the islets of Akita mice. The reductions in ERO1β expression are in a sharp contrast to the expression of other UPR genes, which were all upregulated in the islets of these model mice possibly because of the increased ER stress. These results indicate that ERO1β has a special place among the UPR components in the islets of diabetic model mice and that ERO1β regulation during diabetes progression is subject to mechanisms distinct from those of UPR, which are currently unknown and need to be clarified by further research. Given that ERO1β suppression leads to decreased insulin content and increased susceptibility to ER stress in β cells (24), we speculate that the reduced expression of ERO1β, or its paradoxical response to ER stress during diabetes progression, could be associated with β cell dysfunction and the inability to synthesize adequate insulin to compensate for peripheral insulin resistance. However, as we have reported here, simply upregulating ERO1β in β cells would not benefit β cell homeostasis and, on the contrary, could worsen ER stress and lead to the suppression of insulin synthesis. Although there remains the possibility that the overexpressed levels of ERO1β in our Tg models are beyond the physiological range and pathologically damaged β cell homeostasis, these results nevertheless clearly illustrate the importance of the fine-tuning of ERO1β regulation required in the maintenance of ER homeostasis, the disturbance of which compromises β cell function for insulin synthesis and could contribute to the pathogenesis of diabetes mellitus.

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REFERENCES

1. **Taniguchi A, Nakai Y, Fukushima M, Kawamura H, Imura H, Nagata I, Tokuyama K.** 1992. Pathogenic factors responsible for glucose intolerance in patients with NIDDM. Diabetes **41**:1540–1546.
2. **Rhodes CJ.** 2005. Type 2 diabetes—a matter of β-cell life and death? Science **307**:380–384. http://dx.doi.org/10.1126/science.1104345.
3. **Eizirik DL, Cardozo AK, Cnop M.** 2008. The role for endoplasmic reticulum stress in diabetes mellitus. Endocr. Rev. **29**:42–61. http://dx.doi.org/10.1210/er.2007-0015.
4. **Scheuner D, Kaufman RJ.** 2008. The unfolded protein response: a pathway that links insulin demand with β-cell failure and diabetes. Endocr. Rev. **29**:317–333. http://dx.doi.org/10.1210/er.2007-0039.
5. **Zhang L, Lai E, Teodoro T, Volchuk A.** 2009. GRP78, but not protein-disulfide isomerase, partially reverses hyperglycemia-induced inhibition of insulin synthesis and secretion in pancreatic β-cells. J. Biol. Chem. **284**:5289–5298. http://dx.doi.org/10.1074/jbc.M805477200.
6. **Song B, Scheuner D, Ron D, Pennathur S, Kaufman RJ.** 2008. Chop deletion reduces oxidative stress, improves β cell function, and promotes cell survival in multiple mouse models of diabetes. J. Clin. Invest. **118**:3378–3389. http://dx.doi.org/10.1172/JCI34587.
7. **Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M.** 2002. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J. Clin. Invest. **109**:525–532. http://dx.doi.org/10.1172/JCI14550.
8. **Szegezdi E, Logue SE, Gorman AM, Samali A.** 2006. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep. **7**:880–885. http://dx.doi.org/10.1038/sj.embor.7400779.
9. **Ron D, Walter P.** 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. **8**:519–529. http://dx.doi.org/10.1038/nrm2199.
10. **Freedman RB, Hirst TR, Tuite MF.** 1994. Protein disulphide isomerase: building bridges in protein folding. Trends Biochem. Sci. **19**:331–336. http://dx.doi.org/10.1016/0968-0004(94)90072-8.
11. **Tu BP, Weissman JS.** 2004. Oxidative protein folding in eukaryotes: mechanisms and consequences. J. Cell Biol. **164**:341–346. http://dx.doi.org/10.1083/jcb.200311055.
12. **Sevier CS, Kaiser CA.** 2008. Ero1 and redox homeostasis in the endoplasmic reticulum. Biochim. Biophys. Acta **1783**:549–556. http://dx.doi.org/10.1016/j.bbamcr.2007.12.011.
13. **Zito E, Melo EP, Yang Y, Wahlander Å, Neubert TA, Ron D.** 2010. Oxidative protein folding by an endoplasmic reticulum-localized peroxiredoxin. Mol. Cell **40**:787–797. http://dx.doi.org/10.1016/j.molcel.2010.11.010.
14. **Frand AR, Kaiser CA.** 1998. The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. Mol. Cell **1**:161–170. http://dx.doi.org/10.1016/S1097-2765(00)80017-9.
15. **Pollard MG, Travers KJ, Weissman JS.** 1998. Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. Mol. Cell **1**:171–182. http://dx.doi.org/10.1016/S1097-2765(00)80018-0.
16. **Pagani M, Fabbri M, Benedetti C, Fassio A, Pilati S, Bulleid NJ,**

Cabibbo A, Sitia R. 2000. Endoplasmic reticulum oxidoreductin 1-Lbeta (ERO1-Lbeta), a human gene induced in the course of the unfolded protein response. J. Biol. Chem. 275:23685–23692. http://dx.doi.org/10.1074/jbc.M003061200.

17. Gess B, Hofbauer K-H, Wenger RH, Lohaus C, Meyer HE, Kurtz A. 2003. The cellular oxygen tension regulates expression of the endoplasmic oxidoreductase ERO1-Lα. Eur. J. Biochem. 270:2228–2235. http://dx.doi.org/10.1046/j.1432-1033.2003.03590.x.
18. May D, Itin A, Gal O, Kalinski H, Feinstein E, Keshet E. 2005. Ero1-L alpha plays a key role in a HIF-1-mediated pathway to improve disulfide bond formation and VEGF secretion under hypoxia: implication for cancer. Oncogene 24:1011–1020. http://dx.doi.org/10.1038/sj.onc.1208325.
19. Dias-Gunasekara S, Gubbens J, van Lith M, Dunne C, Williams JAG, Kataky R, Scoones D, Lapthorn A, Bulleid NJ, Benham AM. 2005. Tissue-specific expression and dimerization of the endoplasmic reticulum oxidoreductase Ero1{beta}. J. Biol. Chem. 280:33066–33075. http://dx.doi.org/10.1074/jbc.M505023200.
20. Van Lommel L, Janssens K, Quintens R, Tsukamoto K, Vander Mierde D, Lemaire K, Denef C, Jonas J-C, Martens G, Pipeleers D, Schuit FC. 2006. Probe-independent and direct quantification of insulin mRNA and growth hormone mRNA in enriched cell preparations. Diabetes 55:3214–3220. http://dx.doi.org/10.2337/db06-0774.
21. Schuit FC, Kiekens R, Pipeleers DG. 1991. Measuring the balance between insulin synthesis and insulin release. Biochem. Biophys. Res. Commun. 178:1182–1187. http://dx.doi.org/10.1016/0006-291X(91)91017-7.
22. Goodge KA, Hutton JC. 2000. Translational regulation of proinsulin biosynthesis and proinsulin conversion in the pancreaticβ-cell. Semin. Cell Dev. Biol. 11:235–242. http://dx.doi.org/10.1006/scdb.2000.0172.
23. Zito E, Chin K-T, Blais J, Harding HP, Ron D. 2010. ERO1-beta, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. J. Cell Biol. 188:821–832. http://dx.doi.org/10.1083/jcb.200911086.
24. Khoo C, Yang J, Rajpal G, Wang Y, Liu J, Arvan P, Stoffers DA. 2011. Endoplasmic reticulum oxidoreductase-1-like β (ERO1lβ) regulates susceptibility to endoplasmic reticulum stress and is induced by insulin flux in β-cells. Endocrinology 152:2599–2608. http://dx.doi.org/10.1210/en.2010-1420.
25. Awazawa M, Ueki K, Inabe K, Yamauchi T, Kaneko K, Okazaki Y, Bardeesy N, Ohnishi S, Nagai R, Kadowaki T. 2009. Adiponectin suppresses hepatic SREBP1c expression in an AdipoR1/LKB1/AMPK dependent pathway. Biochem. Biophys. Res. Commun. 382:51–56. http://dx.doi.org/10.1016/j.bbrc.2009.02.131.
26. Ueki K, Yballe CM, Brachmann SM, Vicent D, Watt JM, Kahn CR, Cantley LC. 2002. Increased insulin sensitivity in mice lacking p85beta subunit of phosphoinositide 3-kinase. Proc. Natl. Acad. Sci. U. S. A. 99:419–424. http://dx.doi.org/10.1073/pnas.012581799.
27. Ueki K, Okada T, Hu J, Liew CW, Assmann A, Dahlgren GM, Peters JL, Shackman JG, Zhang M, Artner I, Satin LS, Stein R, Holzenberger M, Kennedy RT, Kahn CR, Kulkarni RN. 2006. Total insulin and IGF-I resistance in pancreatic [beta] cells causes overt diabetes. Nat. Genet. 38:583–588. http://dx.doi.org/10.1038/ng1787.
28. Kulkarni RN, Brüning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR. 1999. Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 96:329–339. http://dx.doi.org/10.1016/S0092-8674(00)80546-2.
29. Koike M, Shibata M, Waguri S, Yoshimura K, Tanida I, Kominami E, Gotow T, Peters C, von Figura K, Mizushima N, Saftig P, Uchiyama Y. 2005. Participation of autophagy in storage of lysosomes in neurons from mouse models of neuronal ceroid-lipofuscinoses (Batten disease). Am. J. Pathol. 167:1713–1728. http://dx.doi.org/10.1016/S0002-9440(10)61253-9.
30. Szöcs K, Lassègue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD, Griendling KK. 2002. Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. Arterioscler. Thromb. Vasc. Biol. 22:21–27. http://dx.doi.org/10.1161/hq0102.102189.
31. Wang J, Takeuchi T, Tanaka S, Kubo S-K, Kayo T, Lu D, Takata K, Koizumi A, Izumi T. 1999. A mutation in the insulin 2 gene induces diabetes with severe pancreatic β-cell dysfunction in the Mody mouse. J. Clin. Invest. 103:27–37. http://dx.doi.org/10.1172/JCI4431.
32. Cabibbo A, Pagani M, Fabbri M, Rocchi M, Farmery MR, Bulleid NJ, Sitia R. 2000. ERO1-L, a human protein that favors disulfide bond formation in the endoplasmic reticulum. J. Biol. Chem. 275:4827–4833. http://dx.doi.org/10.1074/jbc.275.7.4827.
33. Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD, Ron D. 2001. Diabetes mellitus and exocrine pancreatic dysfunction in Perk−/− mice reveals a role for translational control in secretory cell survival. Mol. Cell 7:1153–1163. http://dx.doi.org/10.1016/S1097-2765(01)00264-7.
34. Francisco AB, Singh R, Li S, Vani AK, Yang L, Munroe RJ, Diaferia G, Cardano M, Biunno I, Qi L, Schimenti JC, Long Q. 2010. Deficiency of suppressor enhancer Lin12 1 Like (SEL1L) in mice leads to systemic endoplasmic reticulum stress and embryonic lethality. J. Biol. Chem. 285:13694–13703. http://dx.doi.org/10.1074/jbc.M109.085340.
35. Haynes CM, Titus EA, Cooper AA. 2004. Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. Mol. Cell 15:767–776. http://dx.doi.org/10.1016/j.molcel.2004.08.025.
36. Fagioli C, Mezghrani A, Sitia R. 2001. Reduction of interchain disulfide bonds precedes the dislocation of Ig-μ chains from the endoplasmic reticulum to the cytosol for proteasomal degradation. J. Biol. Chem. 276:40962–40967. http://dx.doi.org/10.1074/jbc.M107456200.
37. Ushioda R, Hoseki J, Araki K, Jansen G, Thomas DY, Nagata K. 2008. ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER. Science 321:569–572. http://dx.doi.org/10.1126/science.1159293.
38. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell 11:619–633. http://dx.doi.org/10.1016/S1097-2765(03)00105-9.
39. Oda Y, Okada T, Yoshida H, Kaufman RJ, Nagata K, Mori K. 2006. Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. J. Cell Biol. 172:383–393. http://dx.doi.org/10.1083/jcb.200507057.
40. Eizirik DL, Welsh M, Strandell E, Welsh N, Sandler S. 1990. Interleukin-1 beta depletes insulin messenger ribonucleic acid and increases the heat shock protein hsp70 in mouse pancreatic islets without impairing the glucose metabolism. Endocrinology 127:2290–2297. http://dx.doi.org/10.1210/endo-127-5-2290.
41. Marchetti P, Lupi R, Federici M, Marselli L, Masini M, Boggi U, Del Guerra S, Patane G, Piro S, Anello M, Bergamini E, Purrello F, Lauro R, Mosca F, Sesti G, Del Prato S. 2002. Insulin secretory function is impaired in isolated human islets carrying the Gly(972)→Arg IRS-1 polymorphism. Diabetes 51:1419–1424. http://dx.doi.org/10.2337/diabetes.51.5.1419.
42. Brostrom CO, Brostrom MA. 1998. Regulation of translational initiation during cellular responses to stress. Prog. Nucleic Acid Res. Mol. Biol. 58:79–125.

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Utility of Detection of Telaprevir-Resistant Variants for Prediction of Efficacy of Treatment of Hepatitis C Virus Genotype 1 Infection

Norio Akuta,[a] Fumitaka Suzuki,[a] Taito Fukushima,[a] Yusuke Kawamura,[a] Hitomi Sezaki,[a] Yoshiyuki Suzuki,[a] Tetsuya Hosaka,[a] Masahiro Kobayashi,[a] Tasuku Hara,[a] Mariko Kobayashi,[b] Satoshi Saitoh,[a] Yasuji Arase,[a] Kenji Ikeda,[a] Hiromitsu Kumada[a]

Department of Hepatology, Toranomon Hospital, and Okinaka Memorial Institute for Medical Research, Tokyo, Japan[a]; Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan[b]

The clinical usefulness of detecting telaprevir-resistant variants is unclear. Two hundred fifty-two Japanese patients infected with hepatitis C virus (HCV) genotype 1b received triple therapy with telaprevir–peginterferon (PEG-IFN)–ribavirin and were evaluated for telaprevir-resistant variants by direct sequencing at baseline and at the time of reelevation of the viral load. An analysis of the entire group indicated that 76% achieved a sustained virological response. Multivariate analysis identified a PEG-IFN dose of <1.3 μg/kg of body weight, an *IL28B* rs8099917 genotype (genotype non-TT), detection of telaprevir-resistant variants of amino acid (aa) 54 at baseline, nonresponse to prior treatment, and a leukocyte count of <5,000/mm^3 as significant pretreatment factors for detection of telaprevir-resistant variants at the time of reelevation of the viral load. In 63 patients who showed nonresponse to prior treatment, a higher proportion of patients with no detected telaprevir-resistant variants at baseline (54%) achieved a sustained virological response than did patients with detected telaprevir-resistant variants at baseline (0%). Furthermore, 2 patients who did not have a sustained virological response from the first course of triple therapy with telaprevir received a second course of triple therapy with telaprevir. These patients achieved a sustained virological response by the second course despite the persistence of very-high-frequency variants (98.1% for V36C) or a history of the emergence of variants (0.2% for R155Q and 0.2% for A156T) by ultradeep sequencing. In conclusion, this study indicates that the presence of telaprevir-resistant variants at the time of reelevation of viral load can be predicted by a combination of host, viral, and treatment factors. The presence of resistant variants at baseline might partly affect treatment efficacy, especially in those with nonresponse to prior treatment.

New strategies have been introduced recently for the treatment of chronic hepatitis C virus (HCV) infection based on the inhibition of protease in the nonstructural 3 (NS3)/NS4 region of the HCV polyprotein. Of the new agents currently available, telaprevir (VX-950) is used for the treatment of chronic HCV infection (1). Three studies (PROVE1, PROVE2, and a Japanese study [2–4]) showed that a 24-week regimen of triple therapy (telaprevir, peginterferon [PEG-IFN], and ribavirin) for 12 weeks followed by dual therapy (PEG-IFN and ribavirin) for 12 weeks (also called the T12PR24 regimen) achieved sustained virological response (SVR) (negative for HCV RNA for >24 weeks after the withdrawal of treatment) rates of 61%, 69%, and 73%, respectively, in patients infected with HCV genotype 1 (HCV-1). However, another study (PROVE3) found lower SVR rates to the T12PR24 regimen (39%) in nonresponders to previous PEG-IFN–ribavirin therapy infected with HCV-1 who did not achieve HCV RNA negativity during or at the end of the initial triple therapy course (5).

Telaprevir-based therapy is reported to induce resistant variants of HCV (6, 7). A recent report indicated that resistant variants are observed in most patients after failure to achieve an SVR by telaprevir-based treatment and that they tend to be replaced with wild-type viruses over time, presumably due to the lower fitness of those variants (8). However, the clinical usefulness of detecting telaprevir-resistant variants is still unclear. First of all, pretreatment factors associated with the detection of telaprevir-resistant variants at the time of reelevation of viral load have not been investigated. Furthermore, it is not clear at this stage whether the detection of telaprevir-resistant variants at baseline is useful for predicting the efficacy of telaprevir-based treatment and whether a history of the emergence of telaprevir-resistant variants affects treatment efficacy with the second course of telaprevir-based treatment.

Based on the above background, there is a need to investigate the clinical usefulness of detecting telaprevir-resistant variants. The aim of this study was to determine the pretreatment factors associated with the subsequent detection of telaprevir-resistant variants at the time of reelevation of viral load and the importance of telaprevir-resistant variants for predicting the efficacy of telaprevir-based treatment in patients infected with HCV-1b.

MATERIALS AND METHODS

Study population. From May 2008 through August 2013, 340 consecutive patients infected with HCV were selected for triple therapy with telaprevir (MP-424 or Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan), PEG-IFN-α2b (PegIntron; MSD, Tokyo, Japan), and ribavirin (Rebetol; MSD, Tokyo) at the Department of Hepatology, Toranomon Hospital (located in metropolitan Tokyo, Japan). Subsequently, 252 of these patients received the triple therapy based on the following inclusion and exclusion criteria: (i) diagnosis of chronic hepatitis C, (ii) HCV-1b confirmed by sequence analysis, (iii) HCV RNA level of ≥5.0 log IU/ml as determined

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Address correspondence to Norio Akuta, akuta-gi@umin.ac.jp.

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by the Cobas TaqMan HCV test (Roche Diagnostics, Tokyo, Japan), (iv) follow-up duration of ≥24 weeks after the completion of triple therapy, (v) no history of treatment with NS3/4A protease inhibitors, (vi) absence of decompensated liver cirrhosis and hepatocellular carcinoma (HCC), (vii) negative for hepatitis B surface antigen (HBsAg), (viii) no evidence of human immunodeficiency virus infection, (ix) negative history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, and chronic liver disease other than chronic hepatitis C, (x) negative history of depression, schizophrenia, or suicide attempts, angina pectoris, cardiac insufficiency, myocardial infarction, severe arrhythmia, uncontrolled hypertension, uncontrolled diabetes, chronic renal dysfunction, cerebrovascular disorders, thyroidal dysfunction uncontrolled by medical treatment, chronic pulmonary disease, allergy to medication, or anaphylaxis at baseline, and (xi) pregnant or breastfeeding women or those willing to become pregnant during the study and men with a pregnant partner were excluded. The study protocol was in compliance with the guidelines for good clinical practice and the 1975 Declaration of Helsinki and was approved by the institutional review board of the Toranomon Hospital. Each patient received ample information about the goals and potential side effects of the treatment and their right to withdraw from the study at any time. Each patient provided a signed consent form before participating in this trial.

The efficacy of treatment was evaluated by the presence or absence of an HCV RNA-negative result at 24 weeks after the completion of therapy (i.e., SVR), as determined by the Cobas TaqMan HCV test (Roche Diagnostics). Furthermore, failure to achieve an SVR was classified as nonresponse (HCV RNA detected during or at the end of treatment) or relapse (at the time of reelevation of viral load after the end of treatment, even when HCV RNA result was negative at the end of treatment).

Twenty patients (8%) were assigned to a 12-week regimen of triple therapy (the T12PR12 group) and were randomly divided into two groups (10 patients each) treated with either 1,500 mg/day or 2,250 mg/day of telaprevir to evaluate the treatment efficacy during 12 weeks on treatment. Sixty patients (24%) were allocated to a 24-week regimen of the same triple therapy described above followed by dual therapy of PEG-IFN and ribavirin for another 12 weeks (the T12PR24 group) to evaluate treatment efficacy according to the response to prior treatment, and they were treated with 2,250 mg/day of telaprevir. Another group of 172 patients (68%) was treated as described above for the T12PR24 group except for the dosages of telaprevir; this group was divided into two groups treated with either 1,500 mg/day (111 patients) or 2,250 mg/day (61 patients) of telaprevir, as selected by the attending physician. Table 1 summarizes the profiles and laboratory data of the entire group of 252 patients at the commencement of treatment. They included 155 males and 97 females 21 to 73 years of age (median, 58 years). At the start of treatment, telaprevir was administered at a median dose of 30.8 mg/kg of body weight (range, 14.1 to 59.2 mg/kg) daily. One hundred thirty-one patients (52%) were treated with 2,250 mg/day of telaprevir, while the other 121 patients (48%) were treated with 1,500 mg/day of telaprevir. PEG-IFN-α2b was injected subcutaneously at a median dose of 1.5 μg/kg (range, 0.7 to 1.8 μg/kg) once a week. Ribavirin was administered at a median dose of 10.9 mg/kg (range, 4.3 to 15.8 mg/kg) daily. Each drug was discontinued or its dose reduced as required per the judgment of the attending physician, in response to a fall in hemoglobin level, leukocyte count, neutrophil count, or platelet count, or the appearance of side effects. The triple therapy was discontinued when the leukocyte count decreased to $<1{,}000/mm^3$, the neutrophil count decreased to $<500/mm^3$, the platelet count decreased to $<5.0 \times 10^4/mm^3$, or when hemoglobin decreased to <8.5 g/dl.

Follow-up. Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. They were performed every week in the initial 12 weeks of treatment. Adverse effects were monitored clinically by careful interviews and a medical examination at least once every month. Compliance with treatment was evaluated by a questionnaire.

TABLE 1 Profile and laboratory data at commencement of telaprevir, peginterferon, and ribavirin triple therapy in patients infected with HCV genotype 1b

Variable	Patient data
Patient demographics	
No. of patients	252
Sex (no. of males/no. of females)	155/97
Median age (yr) (range)	58 (21–73)
Median body mass index (kg/m^2) (range)	22.8 (16.0–36.7)
Laboratory data (median [range])	
Level of viremia (log IU/ml)	6.7 (5.0–7.8)
Aspartate aminotransferase (IU/liter)	37 (15–624)
Alanine aminotransferase (IU/liter)	42 (11–525)
Albumin (g/dl)	3.9 (2.5–4.7)
Gamma-glutamyl transpeptidase (IU/liter)	34 (3–319)
Leukocyte count ($/mm^3$)	4,700 (2,000–8,400)
Hemoglobin (g/dl)	14.3 (12.1–17.6)
Platelet count ($10^4/mm^3$)	16.5 (8.5–33.8)
Treatment	
Median PEG-IFN-α2b dose (μg/kg) (range)	1.5 (0.7–1.8)
Median ribavirin dose (mg/kg) (range)	10.9 (4.3–15.8)
Median telaprevir dose (mg/kg) (range)	30.8 (14.1–59.2)
No. of patients with telaprevir dose of 1,500/2,250 mg/day	121/131
No. of patients on T12PR12/T12PR24 treatment regimen	20/232
Response to prior treatment	
No. of treatment-naive patients/no. of patients with relapse to prior treatment/no. of patients with nonresponse to prior treatment (IFN monotherapy/ribavirin combination therapy)/unknown	79/109/63 (16/47)/1
Amino acid substitutions in HCV genotype 1b	
Core aa 70 (arginine/glutamine [histidine]/ND[a])	162/88/2
Core aa 91 (leucine/methionine/ND)	139/111/2
ISDR of NS5A (wild type/non-wild type/ND)	199/24/29
IRRDR of NS5A (≤5/≥6/ND)	180/69/3
V3 of NS5A (≤2/≥3/ND)	64/185/3
IL28B genotype	
rs8099917 genotype (TT/non-TT/ND)	181/69/2
ITPA genotype	
rs112735 genotype (CC/non-CC)	186/65/1
NS3/4A protease inhibitor-resistant variants by direct sequencing[b]	
V36/T54/Q80/R155/A156/D168/V170	1/7/55/1/2/26/0

[a] ND, not determined.

[b] The NS3/4A protease inhibitor-resistant variants detected by direct sequencing included V36A/C/M/L/G, T54A/S, Q80K/R/H/G/L, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, D168A/V/E/G/N/T/Y/H/I, and V170A (19, 20).

Measurement of HCV RNA. The antiviral effects of the triple therapy on HCV were assessed by measuring blood plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. HCV RNA concentrations were determined using the Cobas TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2 to 7.8 log IU/ml, and undetectable samples were defined as negative.

Determination of *IL28B* and *ITPA* genotypes. The *IL28B* rs8099917 and *ITPA* rs112735 genotypes have been reported as predictors of treatment efficacy and side effects to PEG-IFN–ribavirin dual therapy, and they were genotyped by using the Invader assay, TaqMan assay, or direct sequencing, as described previously (9–13).

Detection of amino acid substitutions in core and NS5A regions of HCV-1b. With the use of HCV-J (GenBank accession no. D90208) as a reference type (14), the sequence of amino acids (aa) 1 to 191 in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed in a previous study to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and at aa 91 of leucine (Leu91) or methionine (Met91) (15). The sequence of aa 2209 to 2248 in the NS5A of HCV-1b (the interferon sensitivity-determining region [ISDR]) reported by Enomoto and coworkers (16) was determined, and the number of amino acid substitutions in the ISDR was defined as wild type ($\leq$1) or non-wild type ($\geq$2) compared to that of HCV-J. Furthermore, the sequence of aa 2334 to 2379 in the NS5A region of HCV-1b (the IFN/ribavirin resistance-determining region [IRRDR]) reported by El-Shamy and coworkers (17), including the sequence of aa 2356 to 2379 referred to as the variable region 3 (V3), was determined and then compared with the consensus sequence constructed in a previous study. The numbers of amino acid substitutions in the IRRDR and V3 regions were divided into two groups for analysis (those with $\leq$5 and $\geq$6 aa substitutions in the IRRDR, and those with $\leq$2 and $\geq$3 aa substitutions in the V3). In the present study, the amino acid substitutions of the core region and the NS5A-ISDR/IRRDR/V3 of HCV-1b were analyzed by direct sequencing.

Assessment of NS3/4A protease inhibitor-resistant variants. The genome sequence of 609 nucleotides (203 amino acids) in the N terminal of the NS3 region of HCV isolates from the patients was examined. HCV RNA was extracted from 100 μl of blood serum sample, and the nucleotide sequences were determined by direct sequencing and deep sequencing. The primers used to amplify the NS3 region were NS3-F1 (5′-ACA CCG CGG CGT GTG GGG ACA T-3′, nucleotides 3295 to 3316) and NS3-AS2 (5′-GCT CTT GCC GCT GCC AGT GGG A-3′, nucleotides 4040 to 4019) as the first (outer) primer pair and NS3-F3 (5′-CAG GGG TGG CGG CTC CTT-3′, nucleotides 3390 to 3407) and NS3-AS2 as the second (inner) primer pair (18). Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 s at 95°C, annealing of primers for 1 min at 63°C, extension for 1 min at 72°C, and final extension at 72°C for 7 min. The PCR-amplified DNA was purified after agarose gel electrophoresis and then used for direct sequencing and ultradeep sequencing.

Patients were examined for NS3/4A protease inhibitor-resistant variants by direct sequencing at baseline and at the time of reelevation of viral loads. Furthermore, patients who did not have an SVR with the first course of triple therapy with telaprevir and received the second course of the triple therapy with telaprevir were analyzed for telaprevir-resistant variants by ultradeep sequencing at baseline and at the time of reelevation of viral loads. NS3/4A protease inhibitor-resistant variants included V36A/C/M/L/G, T54A/S, Q80K/R/H/G/L, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, D168A/V/E/G/N/T/Y/H/I, and V170A. Telaprevir-resistant variants (at aa 36, aa 54, aa 155, aa 156, and aa 170) and TMC435-resistant variants (at aa 80, aa 155, and aa 168) were evaluated (19, 20).

Direct sequencing was analyzed by the Dye Terminator method. Dideoxynucleotide termination sequencing was performed with the BigDye deoxy terminator version 1.1 cycle sequencing kit (Life Technologies, Carlsbad, CA) (18). The sequence data were deposited in GenBank. Also, ultradeep sequencing was performed using the Ion Personal Genome Machine (PGM) sequencer (Life Technologies). An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus fragment library kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick translated and PCR amplified for a total of 8 cycles. Subsequently, the library was purified using AMPure beads (Beckman Coulter, Brea, CA) and the concentration determined using the StepOnePlus real-time PCR (Life Technologies) and Ion Library quantitation kit, according to the instructions provided by the manufacturers. Emulsion PCR was performed using the Ion OneTouch (Life Technologies) in conjunction with the Ion OneTouch 200 template kit version 2 (Life Technologies). Enrichment for templated Ion Sphere particles (ISPs) was performed using the Ion OneTouch enrichment system (Life Technologies) according to the instructions provided by the manufacturer. Templated ISPs were loaded onto an Ion 314 chip and subsequently sequenced using 130 sequencing cycles according to the Ion PGM 200 sequencing kit user guide. The total output read length per run was >10 Mb (0.5 million tags, 200-base read) (21). The results were analyzed with the CLC Genomics Workbench software (CLC bio, Aarhus, Denmark) (22).

We also included a control experiment to validate the error rates in ultradeep sequencing of the viral genome. In this study, the amplification products of the second-round PCR were ligated with a plasmid and transformed in *Escherichia coli* by using a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived NS3 sequence was determined as the template, in a control experiment. The fold coverages evaluated per position for aa 36, aa 54, aa 155, aa 156, and aa 170 in the NS3 region were 359,379×, 473,716×, 106,435×, 105,979×, and 49,058×, respectively. Thus, using the control experiment based on a plasmid carrying the HCV NS3 sequence, amino acid mutations were defined as amino acid substitutions at a frequency of >0.2% among the total coverage. This frequency ruled out putative errors caused by the ultradeep sequence platform used in this study (23).

Statistical analysis. Nonparametric variables were compared between the groups by the chi-square and Fisher's exact probability tests. Univariate and multivariate analyses for factors affecting the presence of telaprevir-resistant variants by direct sequencing at the reelevation of viral load were performed by the chi-square test and logistic regression, respectively. Patients who achieved an SVR were said to have no detection of resistant variants at the reelevation of viral load. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to determine the reliability of the predictors of the response to therapy.

Nucleotide sequence accession numbers. The N-terminal sequences of the NS3 regions of the telaprevir-resistant variant isolates were deposited in GenBank under accession numbers AB709241, AB709263, AB709264, AB709276, AB709279, AB709283, AB709286, AB709289, AB709295, AB709296, AB709300, AB709303, AB709307, AB709310, AB709311, AB709312, AB709317, AB709319, AB709321, AB709322, AB709345, AB709348, AB709352, AB709353, AB709354, AB709356, AB709357, AB709358, AB709360, AB709370, AB709377, AB709382, AB709383, AB709384, AB709388, AB709390, AB709392, AB709396, AB709398, AB709399, AB709401, AB709405, AB709409, AB709410, AB709414, AB709418, AB709422, AB709426, AB709437, AB709444, AB709445, AB709451, AB709456, AB709461, AB709474, AB709476, AB709481, AB709484, AB709485, AB709486, AB709488, AB709489, AB709490, AB709491, AB709492, AB709493, AB709502, AB709507, AB709508, AB709514, AB709515, AB709525, AB709526, AB709527, and AB826566 to AB826684.

RESULTS

Virological response to therapy. An analysis of the entire group showed that 76% (192 of 252 patients) achieved an SVR. According to the treatment regimen, an SVR was achieved by 45% (9 of 20 patients) and 79% (183 of 232 patients) of the T12PR12 and T12PR24 groups, respectively. Taking into consideration the response to prior treatment, an SVR was achieved by 86% (68 of 79 patients), 84% (91 of 109 patients), and 35% (32 of 63 patients) of the treatment-naive patients, patients who showed relapse following prior treatment, and nonresponders to prior treatment, respectively. In the 231 patients of the T12PR24 group, an SVR was achieved by 88% (61 of 69 patients), 85% (89 of 105 patients), and

TABLE 2 Frequencies of the subjects in whom NS3/4A protease inhibitor-resistant variants were detected by direct sequencing at baseline and at the time of reelevation of viral loads[a]

Time of variant detection	% (*n*) by aa position[b]:						
	36	54	80	155	156	168	170
Baseline	0.4 (1)	3 (7)	22 (55)	0.4 (1)	0.8 (2)	10 (26)	0 (0)
Reelevation of viral load	7 (18)	12 (30)	5 (11)	0.4 (1)	4 (10)	1.2 (3)	0.4 (1)

[a] NS3/4A protease inhibitor-resistant variants included V36A/C/M/L/G, T54A/S, Q80K/R/H/G/L, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, D168A/V/E/G/N/T/Y/H/I, and V170A (19, 20).

[b] The data represent the percentages (*n*) of patients in whom NS3/4A protease inhibitor-resistant variants were detected by direct sequencing. Patients who achieved a sustained virological response were said to have no detection of resistant variants by direct sequencing at the time of reelevation of the viral load.

56% (32 of 57 patients) of the treatment-naive patients, patients who showed relapse following prior treatment, and nonresponders to prior treatment, respectively. Furthermore, an SVR was achieved by 86% (12 of 14 patients) and 47% (20 of 43 patients) of the nonresponders to prior IFN monotherapy and ribavirin combination therapy, respectively.

NS3/4A protease inhibitor-resistant variants detected by direct sequencing at baseline and at the time of reelevation of viral loads. All of the 252 patients were evaluated for resistant variants by direct sequencing at baseline. Sixty patients who did not achieve an SVR were also analyzed for resistant variants by direct sequencing at the time of reelevation of viral load. One hundred ninety-two patients who achieved SVR were said to have no detection of resistant variants as determined by direct sequencing at the reelevation of viral load.

As a whole, the frequency of the subjects in whom telaprevir-resistant variants were detected increased from 5% (12 of 252 patients) at baseline to 18% (45 of 252 patients) at the time of reelevation of viral load. On the other hand, the frequency of the subjects in whom TMC435-resistant variants were detected decreased from 31% (78 of 252 patients) at baseline to 6% (14 of 252 patients) at the time of reelevation of viral load. Table 2 shows the frequencies of subjects in whom resistant variants were detected at baseline and at the time of reelevation of viral load per position for aa 36, aa 54, aa 80, aa 155, aa 156, aa 168, and aa 170 in the NS3 region.

Pretreatment factors associated with detection of telaprevir-resistant variants by direct sequencing at the time of reelevation of viral load. Univariate analysis of the data of the entire group identified eight pretreatment factors that were significantly associated with the detection of telaprevir-resistant variants by direct sequencing at the time of reelevation of viral load: *IL28B* rs8099917 genotype (genotype non-TT) ($P < 0.001$), nonresponse to prior treatment ($P < 0.001$), PEG-IFN dose of <1.3 μg/kg ($P = 0.001$), detection of variants at aa 54 at baseline ($P = 0.002$), Gln70/His70 substitution of aa 70 ($P = 0.003$), gamma-glutamyl transpeptidase (GGT) level of ≥50 IU/liter ($P = 0.006$), leukocyte count of <5,000/mm^3 ($P = 0.026$), and ribavirin dose of <8.0 mg/kg ($P = 0.026$). Multivariate analysis that included the above variables identified five pretreatment factors that were independently associated with the detection of telaprevir-resistant variants at the time of reelevation of viral load: PEG-IFN dose of <1.3 μg/kg (odds ratio [OR], 9.71; $P < 0.001$), *IL28B* rs8099917 genotype (genotype non-TT) (OR, 8.61; $P < 0.001$), detection of variants at aa 54 at baseline (OR, 33.4; $P = 0.002$), nonresponse to prior treatment (OR, 2.66, $P = 0.018$), and leukocyte count of <5,000/mm^3 (OR, 2.46; $P = 0.042$) (Table 3).

Prediction of treatment efficacy by the combination of response to prior treatment and presence of telaprevir-resistant variants by direct sequencing at baseline. The SVR rates based on the combination of response to prior treatment and the presence of telaprevir-resistant variants by direct sequencing at baseline are shown in Fig. 1. In 79 treatment-naive patients, the SVR rates were not different between those patients in whom there were no detected telaprevir-resistant variants (86% [65 of 76 patients]) and those in whom variants were detected (67% [2 of 3 patients]). In 109 patients who showed relapse following prior treatment, the SVR rates were not different between those patients in whom there were no detected variants (83% [86 of 104 patients]) and those in whom variants were detected (100% [5 of 5 patients]). In contrast, in 63 patients who showed nonresponse to prior treatment, a higher proportion of patients with undetected telaprevir-resistant variants (54% [32 of 59 patients]) achieved an SVR than did patients in whom telaprevir-resistant variants were detected (0% [0 of 4 patients]) ($P = 0.053$). Thus, with the combination of nonresponse to prior treatment and detection of telaprevir-resistant variants, the sensitivity, specificity, PPV, and NPV for those with non-SVR were 7% (4 of 60 patients), 100% (191 of 191 patients), 100% (4 of 4 patients), and 77% (191 of 247 patients), respectively. These results indicated that the use of the combination of the above two factors has high specificity and PPV for the prediction of a non-SVR.

TABLE 3 Multivariate analysis of factors associated with detection of telaprevir-resistant variants by direct sequencing at the reelevation of viral load, to telaprevir, peginterferon, and ribavirin triple therapy in patients infected with HCV genotype 1b

Detection factors	Category	Odds ratio (95% CI[a])	*P*[b]
PEG-IFN-α2b dose (μg/kg)	≥1.3	1	
	<1.3	9.71 (3.23–29.4)	<0.001
IL28B rs8099917 genotype	TT genotype	1	
	Non-TT genotype	8.61 (3.48–21.3)	<0.001
Variants of aa 54 at baseline	No detection	1	
	Detection	33.4 (3.77–295)	0.002
Response to treatment	Naive or relapse	1	
	Nonresponse	2.66 (1.18–5.96)	0.018
Leukocyte count (/mm^3)	≥5,000	1	
	<5,000	2.46 (1.03–5.85)	0.042

[a] CI, confidence interval.

[b] Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression analysis are shown.

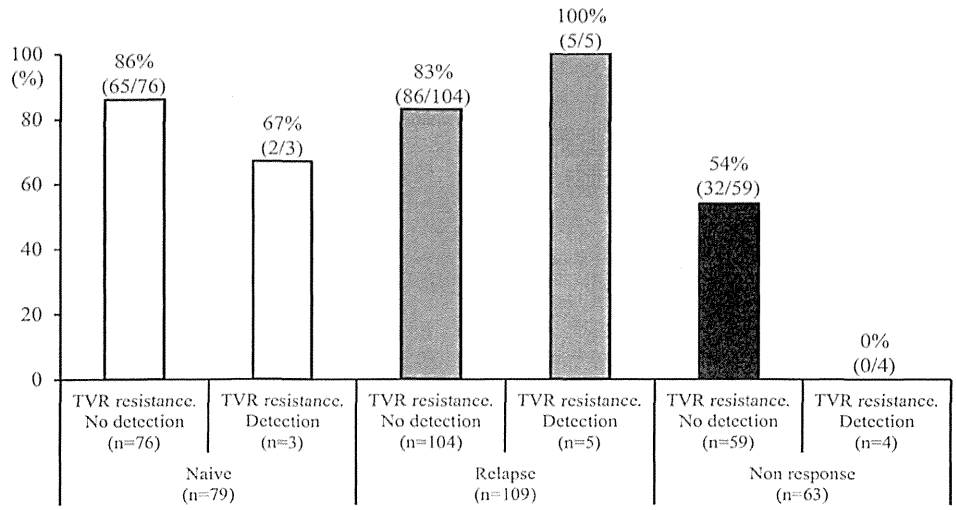

FIG 1 The rates of sustained virological response by the combination of response to prior treatment and presence of telaprevir (TVR)-resistant variants by direct sequencing at baseline are shown. Of those who showed nonresponse to prior treatment, a higher proportion of patients with undetected TVR-resistant variants (54%) achieved a sustained virological response than patients with detected TVR-resistant variants (0%) ($P = 0.053$).

Table 4 summarizes the profiles of 4 patients with nonresponse to prior treatment and in whom telaprevir-resistant variants were detected by direct sequencing at baseline. All of these 4 patients did not achieve an SVR with triple therapy. Interestingly, both T54S as a telaprevir-resistant variant and Q80L as a TMC435-resistant variant (19) were detected by direct sequencing at baseline.

Evolution of telaprevir-resistant variants over time as investigated by ultradeep sequencing in patients who received the second course of triple therapy. Two of 60 patients who did not achieve an SVR with the first course of triple therapy with telaprevir received the second course of triple therapy with telaprevir. They were analyzed for telaprevir-resistant variants by ultradeep sequencing at baseline and at the time of reelevation of viral loads.

Figure 2A shows the clinical course of case 1. In the first course of triple therapy with telaprevir (T12PR24) in a 57-year-old, V36C (0% of 32,413× coverage) was not detected by ultradeep sequencing at baseline of the first course, but very-high-frequency variants of V36C (97.2% of 36,757× coverage) were detected at the time of reelevation of viral loads. In the second course of triple therapy with telaprevir (T12PR54) when the patient was 59 years old, very-high-frequency variants of V36C (98.1% of 94,547× coverage) persisted at baseline of the second course, despite the passing of 2 years after cessation of the first therapy course. Case 1 achieved HCV RNA-negative status at 20 weeks after the start of the second course (late virological response), so PEG-IFN and ribavirin therapy was extended to 54 weeks. In conclusion, case 1 achieved an SVR after the second course of triple therapy with telaprevir, despite the persistence of very-high-frequency variants.

Figure 2B shows the clinical course of case 2. In the first course of triple therapy with telaprevir (T12PR24) in a 61-year-old patient, R155Q (0% of 23,751× coverage) and A156T (0% of 16,040× coverage) were not detected by ultradeep sequencing at baseline of the first course, but very-low-frequency variants of R155Q (0.2% of 11,572× coverage) and A156T (0.2% of 16,040× coverage) were detected at the time of reelevation of viral loads. In the second course of triple therapy with telaprevir (T12PR20) when the patient was 64 years old, R155Q (0% of 80,572× coverage) and A156T (0% of 87,686× coverage) were not detected by ultradeep sequencing at baseline of the second course, which was 2 years after cessation of the first course. In conclusion, case 2 achieved an SVR by the second course of triple therapy with telaprevir, despite the history of the emergence of variants.

TABLE 4 Profiles of 4 patients with nonresponse to prior treatment and detection of telaprevir-resistant variants by direct sequencing at baseline

Case no.	Sex	Age (yr)	Response to prior treatment[a]	Amino acid detected at aa position: 36	54	80	155	156	168	170	Time of HCV RNA-negative result during treatment (wks)	Efficacy of triple therapy
1	Male	70	Nonresponse to IFN monotherapy	V	S	L	R	A	D	I	2	Non-SVR
2	Male	47	Nonresponse to IFN monotherapy	V	S	L	R	A	D	I	4	Non-SVR
3	Male	61	Nonresponse to RBV combination therapy	V	S	L	R	A	D	I	3	Non-SVR
4	Female	60	Nonresponse to RBV combination therapy	V	S	L	R	A	D	I	4	Non-SVR

[a] RBV, ribavirin.

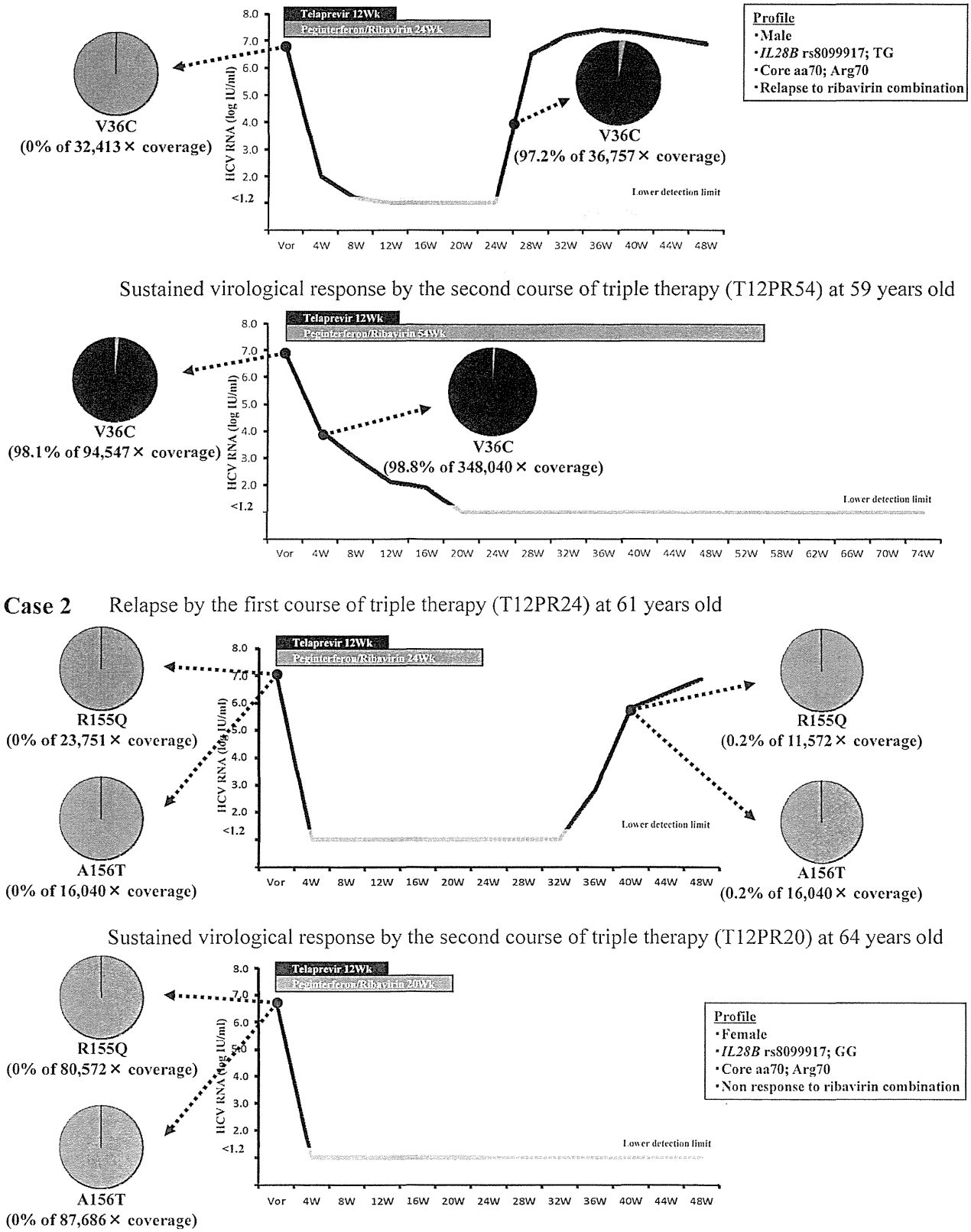

FIG 2 Two patients who did not achieve a sustained virological response with the first course of triple therapy with telaprevir received the second course of the triple therapy with telaprevir. They were analyzed for telaprevir-resistant variants by ultradeep sequencing at baseline and at the time of reelevation of viral loads. (A) Case 1 achieved a sustained virological response with the second course of therapy despite the persistence of very-high-frequency variants. (B) Case 2 achieved a sustained virological response with the second course of therapy despite the history of the emergence of variants.

DISCUSSION

Patients who fail to achieve an SVR to triple therapy need to be identified to avoid unnecessary side effects, high costs, and the emergence of telaprevir-resistant variants. Host genetic factors (e.g., *IL28B* genotype), and viral factors (e.g., amino acid substitutions in the core/NS5A region) have often been used as pretreatment predictors of poor virological response to PEG-IFN–ribavirin dual therapy (9–11, 15, 17) and telaprevir–PEG-IFN–ribavirin triple therapy (24–26). However, the pretreatment factors associated with the detection of telaprevir-resistant variants at the time of reelevation of viral load are still unknown. The present study identified that the detection of telaprevir-resistant variants at the time of reelevation of viral load can be predicted by a combination of host (*IL28B* rs8099917 genotype and leukocyte count), viral (variants of aa 54 at baseline), and treatment factors (PEG-IFN dose). All of the 4 patients with nonresponse to prior treatment and in whom telaprevir-resistant variants were detected at baseline did not achieve an SVR with triple therapy, and the use of the combination of nonresponse to prior treatment and the detection of telaprevir-resistant variants at baseline had high specificity and PPV for the prediction of a non-SVR. This finding suggests that there is a complex relationship between host susceptibility to IFN and viral sensitivity to NS3/4A protease inhibitors in determining treatment efficacy. Interestingly, in all of the 4 patients, both T54S as a telaprevir-resistant variant and Q80L as a TMC435-resistant variant (19) were detected by direct sequencing at baseline. This result suggests that patients with the above two factors should be carefully introduced to NS3/4A protease inhibitors besides telaprevir because of the high risk of the emergence of resistant variants. However, the present study was performed with a small number of patients, so further studies based on a larger number of patients should be performed.

In the present study employing ultradeep sequencing technology, 2 patients who did not achieve an SVR with the first course of triple therapy with telaprevir received the second course of the triple therapy with telaprevir. They achieved an SVR with the second course, despite the persistence of very-high-frequency variants (case 1, 98.1% for V36C) or a history of the emergence of variants (case 2, 0.2% for R155Q and 0.2% for A156T) as determined by ultradeep sequencing. This finding may be due to one or more reasons. One reason is probably related to the high susceptibility of telaprevir-resistant variants to IFN. One previous study indicated that mice infected with the resistant strain (A156F [99.9%]) developed only low-level viremia, and the virus was successfully eliminated with IFN therapy (27). In the other clinical report, telaprevir-resistant variants that emerged during 24-week telaprevir monotherapy were eliminated by the combination therapy of PEG-IFN plus ribavirin (28). Furthermore, this finding probably suggests that a small number of mutant-type viral RNAs may be incomplete or defective, since a large proportion of viral genomes are thought to be defective due to their high replication and mutation rates (29). Further studies employing ultradeep sequencing should be performed to evaluate whether a history of the emergence of NS3/4A protease inhibitor-resistant variants, besides telaprevir-resistant variants, affects the efficacy of a second course of NS3/4A protease inhibitor-based treatment.

The results of the present study should be interpreted with caution, since the study was performed with a small number of Japanese patients infected with HCV-1b. Any generalization of the results should await confirmation by a multicenter randomized trial based on a larger number of patients, including patients of other races and those infected with HCV-1a. Furthermore, the other limitation of the present study is that the loss of telaprevir-resistant variants was not investigated long after the cessation of therapy. Further large-scale studies should be performed to investigate the impacts of telaprevir-resistant variants on the response to treatment using new drugs, including direct-acting antiviral agents.

In conclusion, this study based on Japanese patients infected with HCV-1b indicates that telaprevir-resistant variants at the time of reelevation of viral load can be predicted by a combination of host, viral, and treatment factors. In those patients with no response to prior treatment, the present results suggest that telaprevir-resistant variants at baseline might partly affect the efficacy of triple therapy treatment. This finding indicates the clinical utility of detecting telaprevir-resistant variants to predict treatment efficacy, and it suggests a complex relationship between host susceptibility to IFN and viral sensitivity to NS3/4A protease inhibitors in determining treatment efficacy. Further large-scale prospective studies are needed to investigate the clinical usefulness of telaprevir-resistant variants and to develop more effective therapeutic regimens in patients infected with HCV-1.

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REFERENCES

1. Lin C, Kwong AD, Perni RB. 2006. Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease. Infect. Disord. Drug Targets 6:3–16. http://dx.doi.org/10.2174/187152606776056706.
2. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J, Muir AJ, PROVE1 Study Team. 2009. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N. Engl. J. Med. 360:1827–1838. http://dx.doi.org/10.1056/NEJMoa0806104.
3. Hézode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourlière M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S, PROVE2 Study Team. 2009. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N. Engl. J. Med. 360:1839–1850. http://dx.doi.org/10.1056/NEJMoa0807650.
4. Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. 2012. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J. Hepatol. 56:78–84. http://dx.doi.org/10.1016/j.jhep.2011.07.016.
5. McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, Heathcote EJ, Zeuzem S, Reesink HW, Garg J, Bsharat M, George S, Kauffman RS, Adda N, Di Bisceglie AM, PROVE3 Study Team. 2010. Telaprevir for previously treated chronic HCV infection. N. Engl. J. Med. 362:1292–1303. http://dx.doi.org/10.1056/NEJMoa0908014.
6. Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, Frantz JD, Lin K, Ma S, Wei YY, Perni RB, Kwong AD. 2005. *In vitro* studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. J. Biol. Chem. 280:36784–36791. http://dx.doi.org/10.1074/jbc.M506462200.
7. Kieffer TL, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, Kwong AD, Zeuzem S. 2007. Telaprevir and pegylated interferon-

alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. Hepatology 46:631–639. http://dx.doi.org/10.1002/hep.21781.

8. Sullivan JC, De Meyer S, Bartels DJ, Dierynck I, Zhang EZ, Spanks J, Tigges AM, Ghys A, Dorrian J, Adda N, Martin EC, Beumont M, Jacobson IM, Sherman KE, Zeuzem S, Picchio G, Kieffer TL. 2013. Evolution of treatment-emergent resistant variants in telaprevir phase 3 clinical trials. Clin. Infect. Dis. 57:221–229. http://dx.doi.org/10.1093/cid/cit226.
9. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401. http://dx.doi.org/10.1038/nature08309.
10. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat. Genet. 41:1100–1104. http://dx.doi.org/10.1038/ng.447.
11. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of *IL28B* with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat. Genet. 41:1105–1109. http://dx.doi.org/10.1038/ng.449.
12. Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. 2001. A high-throughput SNP typing system for genome-wide association studies. J. Hum. Genet. 46:471–477. http://dx.doi.org/10.1007/s100380170047.
13. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M, Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K. 2003. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat. Genet. 34:395–402. http://dx.doi.org/10.1038/ng1206.
14. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U. S. A. 87:9524–9528. http://dx.doi.org/10.1073/pnas.87.24.9524.
15. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2005. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48:372–380. http://dx.doi.org/10.1159/000086064.
16. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N. Engl. J. Med. 334:77–81. http://dx.doi.org/10.1056/NEJM199601113340203.
17. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48:38–47. http://dx.doi.org/10.1002/hep.22339.
18. Suzuki F, Sezaki H, Akuta N, Suzuki Y, Seko Y, Kawamura Y, Hosaka T, Kobayashi M, Saito S, Arase Y, Ikeda K, Kobayashi M, Mineta R, Watahiki S, Miyakawa Y, Kumada H. 2012. Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. J. Clin. Virol. 54:352–354. http://dx.doi.org/10.1016/j.jcv.2012.04.024.
19. Romano KP, Ali A, Royer WE, Schiffer CA. 2010. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. Proc. Natl. Acad. Sci. U. S. A. 107:20986–20991. http://dx.doi.org/10.1073/pnas.1006370107.
20. Barbotte L, Ahmed-Belkacem A, Chevaliez S, Soulier A, Hézode C, Wajcman H, Bartels DJ, Zhou Y, Ardzinski A, Mani N, Rao BG, George S, Kwong A, Pawlotsky JM. 2010. Characterization of V36C, a novel amino acid substitution conferring hepatitis C virus (HCV) resistance to telaprevir, a potent peptidomimetic inhibitor of HCV protease. Antimicrob. Agents Chemother. 54:2681–2683. http://dx.doi.org/10.1128/AAC.01796-09.
21. Elliott AM, Radecki J, Moghis B, Li X, Kammesheidt A. 2012. Rapid detection of the ACMG/ACOG-recommended 23 CFTR disease-causing mutations using ion torrent semiconductor sequencing. J. Biomol. Tech. 23:24–30. http://dx.doi.org/10.7171/jbt.12-2301-003.
22. Vogel U, Szczepanowski R, Claus H, Jünemann S, Prior K, Harmsen D. 2012. Ion torrent personal genome machine sequencing for genomic typing of *Neisseria meningitidis* for rapid determination of multiple layers of typing information. J. Clin. Microbiol. 50:1889–1894. http://dx.doi.org/10.1128/JCM.00038-12.
23. Akuta N, Suzuki F, Seko Y, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2013. Emergence of telaprevir-resistant variants detected by ultradeep sequencing after triple therapy in patients infected with HCV genotype 1. J. Med. Virol. 85:1028–1036. http://dx.doi.org/10.1002/jmv.23579.
24. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Chayama K, Nakamura Y, Kumada H. 2010. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. Hepatology 52:421–429. http://dx.doi.org/10.1002/hep.23690.
25. Chayama K, Hayes CN, Abe H, Miki D, Ochi H, Karino Y, Toyota J, Nakamura Y, Kamatani N, Sezaki H, Kobayashi M, Akuta N, Suzuki F, Kumada H. 2011. IL28B but not ITPA polymorphism is predictive of response to pegylated interferon, ribavirin, and telaprevir triple therapy in patients with genotype 1 hepatitis C. J. Infect. Dis. 204:84–93. http://dx.doi.org/10.1093/infdis/jir210.
26. Akuta N, Suzuki F, Fukushima T, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2013. Prediction of treatment efficacy and telaprevir-resistant variants after triple therapy in patients infected with hepatitis C virus genotype 1. J. Clin. Microbiol. 51:2862–2868. http://dx.doi.org/10.1128/JCM.01129-13.
27. Hiraga N, Imamura M, Abe H, Hayes CN, Kono T, Onishi M, Tsuge M, Takahashi S, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, Yoshizato K, Matsui H, Kanai A, Inaba T, Tanaka S, Chayama K. 2011. Rapid emergence of telaprevir resistant hepatitis C virus strain from wild type clone *in vivo*. Hepatology 54:781–788. http://dx.doi.org/10.1002/hep.24460.
28. Ozeki I, Akaike J, Karino Y, Arakawa T, Kuwata Y, Ohmura T, Sato T, Kamiya N, Yamada I, Chayama K, Kumada H, Toyota J. 2011. Antiviral effects of peginterferon alpha-2b and ribavirin following 24-week monotherapy of telaprevir in Japanese hepatitis C patients. J. Gastroenterol. 46:929–937. http://dx.doi.org/10.1007/s00535-011-0411-0.
29. Bartenschlager R, Lohmann V. 2000. Replication of hepatitis C virus. J. Gen. Virol. 81:1631–1648.

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Evolution of Simeprevir-Resistant Variants Over Time by Ultra-Deep Sequencing in HCV Genotype 1b

Norio Akuta,[1]* Fumitaka Suzuki,[1] Hitomi Sezaki,[1] Yoshiyuki Suzuki,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Mariko Kobayashi,[2] Satoshi Saitoh,[1] Kenji Ikeda,[1] and Hiromitsu Kumada[1]

[1]*Department of Hepatology, Toranomon Hospital and Okinaka Memorial Institute for Medical Research, Tokyo, Japan*

[2]*Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan*

Using ultra-deep sequencing technology, the present study was designed to investigate the evolution of simeprevir-resistant variants (amino acid substitutions of aa80, aa155, aa156, and aa168 positions in HCV NS3 region) over time. In Toranomon Hospital, 18 Japanese patients infected with HCV genotype 1b, received triple therapy of simeprevir/PEG-IFN/ribavirin (DRAGON or CONCERT study). Sustained virological response rate was 67%, and that was significantly higher in patients with *IL28B* rs8099917 TT than in those with non-TT. Six patients, who did not achieve sustained virological response, were tested for resistant variants by ultra-deep sequencing, at the baseline, at the time of re-elevation of viral loads, and at 96 weeks after the completion of treatment. Twelve of 18 resistant variants, detected at re-elevation of viral load, were de novo resistant variants. Ten of 12 de novo resistant variants become undetectable over time, and that five of seven resistant variants, detected at baseline, persisted over time. In one patient, variants of Q80R at baseline (0.3%) increased at 96-week after the cessation of treatment (10.2%), and de novo resistant variants of D168E (0.3%) also increased at 96-week after the cessation of treatment (9.7%). In conclusion, the present study indicates that the emergence of simeprevir-resistant variants after the start of treatment could not be predicted at baseline, and the majority of de novo resistant variants become undetectable over time. Further large-scale prospective studies should be performed to investigate the clinical utility in detecting simeprevir-resistant variants. ***J. Med. Virol. 86:1314–1322, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: HCV; ultra-deep sequence; simeprevir; resistant variants

INTRODUCTION

New strategies have been introduced for the treatment of chronic hepatitis C virus (HCV) infection based on inhibition of protease in the NS3/NS4 of the HCV polyprotein. Of the first-generation NS3/4A protease inhibitors, telaprevir had been approved for the treatment of patients infected with HCV genotype 1. The inclusion of this agent in HCV treatment regimens had led to large improvements in sustained virological rates, though this agent requires dosing three times daily and the use is associated with increased incidence and, in some cases, severity of adverse events such as anemia and rash [Hézode et al., 2009; McHutchison et al., 2009, 2010; Hayashi et al., 2012; Kumada et al., 2012].

Simeprevir (TMC435) is an investigational, once-daily oral NS3/4A protease inhibitor currently under clinical development globally. Phase II trials of simeprevir in combination with peginterferon (PEG-IFN) and ribavirin for patients infected with HCV genotype 1 demonstrated that simeprevir was generally well tolerated, had a pharmacokinetic profile that supports once-daily dosing and resulted in high virologic response rates. Treatment-naive patients, who received simeprevir for 12 or 24 weeks (SMV12 or SMV24) with PEG-IFN/ribavirin for 24 or 48 weeks (PR24 or PR48) (according to response-guided therapy), could achieve sustained virological response rates of 74–86%

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*Correspondence to: Norio Akuta, MD, Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo, 105-0001, Japan.
E-mail: akuta-gi@umin.ac.jp

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(PILLAR study) [Fried et al., 2013] and 77–92% (DRAGON study) [Hayashi et al., 2014]. Furthermore, treatment-experienced patients, who received simeprevir for 12, 24, or 24 weeks (SMV12, SMV24, or SMV24) with PEG-IFN/ribavirin for 48 weeks (PR48), could achieve sustained virological response rates of 61–80% (ASPIRE study) [Zeuzem et al., 2014].

Along with a high sustained virological response, NS3/4A protease inhibitor-based therapies are reported to induce resistant variants in patients, who could not achieve sustained virological response [Hézode et al., 2009; McHutchison et al., 2010; Fried et al., 2013; Hayashi et al., 2014; Zeuzem et al., 2014]. Hence, there is a need to predict at baseline the appearance of NS3/4A protease inhibitor-resistant variants. Previous reports have described the advantages of ultra-deep sequencing technology, including faster processing and large-scale sequencing, in addition to providing a better understanding of the dynamics of variants in HCV quasispecies [Hiraga et al., 2011; Nasu et al., 2011; Ninomiya et al., 2012]. Recent study based on telaprevir-based therapy showed that it was difficult to predict at baseline the emergence of telaprevir-resistant variants during triple therapy, even with the use of ultra-deep sequencing [Akuta et al., 2013]. However, it is not clear at this stage whether such technology is useful for the prediction of the emergence of simeprevir-resistant variants during or after the administration of simeprevir-based therapy.

The aim of this study using ultra-deep sequencing technology was to investigate the evolution of simeprevir-resistant variants over time after commencement of triple therapy of simeprevir/PEG-IFN/ribavirin, in adult Japanese patients infected with HCV genotype 1.

PATIENTS AND METHODS

Study Design

The Dose and duration Ranging study of Antiviral agent TMC435 in Genotype One HCV treatment-Naive patients (DRAGON) was a Phase II study conducted across Japan to evaluate the efficacy, safety, and pharmacokinetics of simeprevir and PEG-IFNα-2a/ribavirin in treatment-naive patients infected with HCV genotype 1. Simeprevir dose of 50 or 100 mg once-daily was administered orally for evaluation in this dose-ranging study. Patients were randomized to one of five treatment groups (SMV12/PR24 50 mg, SMV24/PR24 50 mg, SMV12/PR24 100 mg, SMV24/PR24 100 mg, and PR48) [Hayashi et al., 2014]. Furthermore, Clinical Optimization of New treatment strategy with TMC435 in Combination with pEginterferon plus Ribavirin for Treatment-naive and treatment-experienced patients infected with HCV genotype 1 (CONCERT) was a Phase III study conducted across Japan to evaluate the efficacy and safety of simeprevir and PEG-IFNα-2a/ribavirin in treatment-naive patients (CONCERT-1), simeprevir and PEG-IFNα-2a/ribavirin in treatment-experienced patients (CONCERT-2 for prior non-response, and CONCERT-3 for prior relapse), and simeprevir and PEG-IFNα-2b/ribavirin in treatment-naive and experienced patients (CONCERT-4). In CONCERT-1, patients were randomized to one of two treatment groups (SMV12/PR24 100 mg and PR48). In CONCERT-2, patients were randomized to one of two treatment groups (SMV12/PR24 100 mg and SMV24/PR24 100 mg). In CONCERT-3, patients were randomized to one treatment group (SMV12/PR24 100 mg). In CONCERT-4, treatment-naive and relapse patients were assigned to one treatment group (SMV12/PR24 100 mg), and patients of prior non-response were assigned to one treatment group (SMV12/PR48 100 mg) [Drafting Committee for Hepatitis Management Guidelines, the Japan Society of Hepatology, 2014]. In DRAGON and CONCERT-1,2,3, at week 24, patients either stopped or continued treatment with PEG-IFN/ribavirin up to week 48, according to response-guided therapy criteria. These studies protocols were in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and were approved by Toranomon Hospital Ethics Committee. Each patient gave an informed consent before participation in these trials.

In DRAGON and CONCERT-1,2,3, PEG-IFNα-2a (Pegasys®; Chugai, Tokyo, Japan) was administered as a subcutaneous injection (180 μg once weekly) and ribavirin (Copegus®; Chugai, Tokyo, Japan) as oral tablets (600–1,000 mg total daily dose, depending on body weight) in accordance with the manufacturer's prescribing information for both medication. In CONCERT-4, PEG-IFNα-2b (PegIntron®; MSD, Tokyo, Japan) was administered as a subcutaneous injection (1.5 μg/kg once weekly, depending on body weight), and ribavirin (Rebetol®; MSD, Tokyo, Japan) as oral tablets (600–1,000 mg total daily dose, depending on body weight) in accordance with the manufacturer's prescribing information for both medication.

The efficacy of treatment was evaluated by HCV-RNA negativity at 24 weeks after the completion of therapy (sustained virological response), based on the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). Failure to achieve sustained virological response was classified as non-response (HCV-RNA detected during or at the end of treatment), viral breakthrough (re-elevation of viral loads before the end of treatment, even when HCV-RNA was temporarily negative during treatment), and relapse (re-elevation of viral loads after the end of treatment, even when HCV-RNA was negative at the end of treatment). Especially, non-response was defined as null response (a reduction of less than 2 $\log_{10}$ in HCV RNA at 12 weeks after the start of therapy) or partial response (a reduction of 2 $\log_{10}$ or more in HCV RNA at 12 weeks).

Study Patients

Between October 2009 and June 2011, 20 patients infected with HCV met the following inclusion and exclusion criteria in one of these two studies (DRAGON or CONCERT) at the Department of Hepatology, Toranomon Hospital: (1) HCV genotype 1 confirmed by sequence analysis. (2) HCV RNA levels of ≥5.0 log IU/ml determined by the COBAS TaqMan HCV test (Roche Diagnostics). (3) Age at study entry of 20–70 years. (4) Absence of liver cirrhosis or hepatic failure, or other liver disease. (5) No infection/co-infection with HIV-1, HIV-2, hepatitis B, or HCV non-genotype 1. (6) No treatment for malignancy within 5 years prior to study. (7) No history of hepatocellular carcinoma. (8) No conditions that required caution with PEG-IFN or ribavirin treatment. (9) No history of any clinically significant disease. (10) No history of organ transplant. Absence of defined laboratory abnormalities during screening. In DRAGON, four treatment-naive patients were randomized to one of five treatment groups (two patients of SMV12/PR24 50 mg, none of SMV12/PR24 100 mg, one of SMV24/PR24 100 mg, and one of PR48). In CONCERT-1, six treatment-naive patients were randomized to one of two treatment groups (five patients of SMV12/PR24 100 mg, and one of PR48). In CONCERT-2, four patients of prior non-response were randomized to one of two treatment groups (three patients of SMV12/PR24 100 mg, and one of SMV24/PR24 100 mg). In CONCERT-4, three patients of prior relapse were assigned to one treatment group (SMV12/PR24 100 mg), and three patients of prior non-response were assigned to one treatment group (SMV12/PR48 100 mg).

The present study based on the 18 patients, who were assigned to triple therapy of simeprevir and PEG-IFN/ribavirin, was performed to investigate the evolution of simeprevir-resistant variants over time. Especially, patients, who did not achieve sustained virological response, was investigated whether the presence of low frequency resistant variants at baseline could predict the emergence of simeprevir-resistant variants after the start of triple therapy, using ultra-deep sequencing.

Measurement of HCV RNA

The antiviral effects of the triple therapy on HCV were assessed by measuring plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. HCV RNA concentrations were determined using the COBAS TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2–7.8 log IU/ml, and the undetectable samples were defined as negative.

Determination of *IL28B* Genotype

IL28B rs8099917 were genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described previously [Ohnishi et al., 2001; Suzuki et al., 2003].

Detection of Amino Acid Substitutions in Core Regions of HCV Genotype 1

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of HCV genotype 1b was determined and then compared with the consensus sequence constructed in a previous study to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005, 2007, 2010].

Assessment of Simeprevir-Resistant Variants

The genome sequence of the N-terminal 609 nucleotides (203 amino acids) in the NS3 region of HCV isolates from the patients was examined. HCV RNA was extracted from 100 μl of serum, and simeprevir-resistant variants were determined by ultra-deep sequencing. The primers used to amplify the NS3 region were NS3-F1 (5′-ACA CCG CGG CGT GTG GGG ACA T-3′; nucleotides 3295–3316) and NS3-AS2 (5′-GCT CTT GCC GCT GCC AGT GGG A-3′; nucleotides 4040–4019) as the first (outer) primer pair and NS3-F3 (5′-CAG GGG TGG CGG CTC CTT-3′; nucleotides 3390–3407) and NS3-AS2 as the second (inner) primer pair [Suzuki et al., 2012]. Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 sec at 95°C, annealing of primers for 1 min at 63°C, extension for 1 min at 72°C, and final extension was performed at 72°C for 7 min. The PCR-amplified DNA was purified after agarose gel electrophoresis, and then used for ultra-deep sequencing. Patients, who did not achieve sustained virological response, were tested for simeprevir-resistant variants by ultra-deep sequencing, at the baseline, at the time of re-elevation of viral loads, and at 96 weeks after the completion of treatment. simeprevir-resistant variants included Q80K/R/H/G/L, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, and D168A/V/E/G/N/T/Y/H/I [Romano et al., 2010].

Ultra-deep sequencing was performed using the Ion Personal Genome Machine™ (PGM™) Sequencer (Life Technologies, Carlsbad, CA). An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick-translated and PCR-amplified for a total of eight cycles. Subsequently, the library was purified using AMPure beads (Beckman Coulter, Brea, CA) and the concentration determined using the StepOne Plus RealTime PCR (Life Technologies) and Ion Library Quantitation Kit, according to the instructions provided by the manufacturer. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with Ion OneTouch 200 Template Kit v2 (Life Technologies).

Enrichment for templated Ion spheres particles (ISPs) was performed using Ion OneTouch Enrichment System (Life Technologies), according to the instructions provided by the manufacturer. Templated ISPs was loaded onto an Ion 314 chip, and subsequently sequenced using 130 sequencing cycles according to the Ion PGM 200 Sequencing Kit user guide. Total output read length per run is over 10 Mbase (0.5M-tag, 200 base read) [Elliott et al., 2012]. The results were analyzed with the CLC Genomics Workbench software (CLCbio, Aarhus, Denmark) [Vogel et al., 2012].

A control experiment was also included to validate the error rates in ultra-deep sequencing of the viral genome. In this study, amplification products of the second-round PCR were ligated with plasmid and transformed in *Escherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived NS3 sequence was determined as the template, by the control experiment. The fold coverage evaluated per position for aa 80, aa 155, aa 156, and aa 168 in NS3 region, were 332,062×, 106,435×, 105,979×, and 33,725×, respectively. Thus, using the control experiment based on plasmid encoding HCV NS3 sequence, amino acid mutations were defined as amino acid substitutions at frequency of more than 0.2% among the total coverage. This frequency ruled out putative errors caused by ultra-deep sequence platform used in this study [Akuta et al., 2013].

Statistical Analysis

Non-parametric tests (chi-squared test and Fisher's exact probability test) were used to determine those factors that significantly contributed to sustained virological response. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL).

RESULTS

Virological Response to Therapy

Table I summarizes the profiles and laboratory data of the 18 patients at commencement of triple therapy, treatment regimen, treatment duration, and efficacy. They included 6 males and 12 females, aged 38–70 years (median, 60 years). The sustained virological response rate was 66.7% (12 of 18 patients). Taking into consideration the response to prior treatment, sustained virological response was achieved by 75.0% (6 of 8 patients), 100% (3 of 3 patients), and 42.9% (3 of 7 patients) by treatment-naive patients, patients who showed relapse to prior treatment, and non-responders to prior treatment, respectively. Of the six patients who did not show sustained virological response, the relapse, breakthrough, and non-response rates were 66.7% (Cases 2,3,11,17), 16.7% (Cases 12), and 16.7% (Cases 18), respectively. Cases 1 stopped simeprevir before the completion of 12-week treatment, due to the increase of total bilirubin levels (48-week PEG-IFN and ribavirin continued), and Case 5 stopped PEG-IFN and ribavirin before the completion of 24-week treatment, due to a fall in Hb concentration. Case 18 stopped the triple therapy at 12 weeks before the completion of the 48-week regimen, due to null response.

The sustained virological response rate was significantly higher in patients with *IL28B* rs8099917 TT (100% [8 of 8 patients]) than in those with non-TT (40% [4 of 10]) ($P = 0.013$). However, the present study based on the small numbers of patients did not identify the other factors that significantly contributed to sustained virological response.

Simeprevir-Resistant Variants Detected by Ultra-Deep Sequencing at Re-Elevation of Viral Load

Table II indicates the evolution of simeprevir-resistant variants over time by ultra-deep sequencing in patients, who did not achieve sustained virological response.

In Case 3,11,18, very low frequency variants at baseline persisted during treatment as very low frequency variants, but de novo resistant variants also emerged during treatment. In Case 3 (relapse), very low frequency variants of Q80R (0.2% of 141,326× coverage) at baseline persisted during treatment as very low frequency variants of Q80R (0.2% of 174,968× coverage), but de novo resistant variants (D168V [0.8% of 200,756× coverage] and D168G [0.2% of 200,756× coverage]) also emerged during treatment. In Case 11 (relapse), very low frequency variants of R155Q (0.2% of 29,454× coverage) at baseline persisted during treatment as very low frequency variants of R155Q (0.4% of 34,796× coverage), but de novo resistant variants (D168V [77.4% of 80,708× coverage]) also emerged during treatment. In Case 18 (null response), very low frequency variants of Q80R (0.3% of 143,435× coverage) at baseline persisted during treatment as very low frequency variants of Q80R (0.4% of 152,564× coverage), but de novo resistant variants (D168V [99.5% of 131,749× coverage] and D168E [0.3% of 131,749× coverage]) also emerged during treatment.

In Case 2 (relapse), very high frequency variants of Q80L (99.8% of 213,853× coverage) at baseline persisted during treatment as very high frequency variants of Q80L (99.9% of 188,910× coverage), but de novo resistant variants (D168G [61.3% of 137,720× coverage] and R155Q [2.4% of 165,980× coverage]) also emerged during treatment.

In Case 12 (breakthrough), very low frequency variants (Q80L [1.0% of 151,968× coverage] and Q80R [0.2% of 151,968× coverage]) at baseline increased during treatment (Q80L [76.0% of 158,451× coverage] and Q80R [1.7% of 158,451× coverage]), and de novo resistant variants (D168V [25.8% of 184,963× coverage] and D168E [2.0% of 184,963× coverage]) also emerged during treatment.

TABLE I. Profile at Commencement of Triple Therapy, Treatment Regimen, Treatment Duration, and Efficacy

Case	Sex	Age	HCV genotype	*IL28B* rs8099917	Core aa70	BMI (kg/m^2)	Previous response	Treatment regimen					Treatment duration		Efficacy
								Study	SMV (mg)	PEG type	PEG dose (μg)	RBV/BW (mg/kg)	SMV (wk)	PEG/RBV (wk)	
1[a]	M	60	1b	TT	Arg70	26.3	Naive	DRAGON	50	α2a	180	10.9	3	48	SVR
2	F	46	1b	TG	Arg70	20.9	Naive	DRAGON	50	α2a	180	10.6	12	24	Relapse
3	F	63	1b	TG	Gln70	20.9	Naive	DRAGON	100	α2a	180	11.4	24	24	Relpase
4	M	69	1b	TT	Arg70	23.2	Naive	CONCERT1	100	α2a	180	13.2	12	24	SVR
5[a]	F	63	1b	TT	Arg70	21.4	Naive	CONCERT1	100	α2a	180	11.2	12	22	SVR
6	F	52	1b	TT	Arg70	21.3	Naive	CONCERT1	100	α2a	180	11.2	12	24	SVR
7	M	53	1b	TT	Arg70	24.2	Naive	CONCERT1	100	α2a	180	11.2	12	24	SVR
8	F	55	1b	TG	Arg70	19.5	Naive	CONCERT1	100	α2a	180	12.5	12	24	SVR
9	F	70	1b	TG	Arg70	19.6	Partial	CONCERT2	100	α2a	180	12.9	12	24	SVR
10	F	61	1b	TG	Gln70	22.5	Partial	CONCERT2	100	α2a	180	10.5	24	24	SVR
11	M	45	1b	TG	Gln70	21.1	Null	CONCERT2	100	α2a	180	13.2	12	24	Relapse
12	F	67	1b	TG	Gln70	23.4	Null	CONCERT2	100	α2a	180	10.9	12	24	Breakthrough
13	F	59	1b	TT	Arg70	21.6	Relapse	CONCERT4	100	α2b	80	11.2	12	24	SVR
14	F	43	1b	TT	Arg70	22.4	Relapse	CONCERT4	100	α2b	80	10.0	12	24	SVR
15	M	38	1b	TT	Gln70	27.5	Relapse	CONCERT4	100	α2b	120	11.7	12	24	SVR
16	F	51	1b	TG	Gln70	22.9	Partial	CONCERT4	100	α2b	80	11.3	12	24	SVR
17	F	64	1b	TG	Arg70	20.1	Partial	CONCERT4	100	α2b	60	13.6	12	48	Relapse
18[a]	M	62	1b	TG	Gln70	26.2	Partial	CONCERT4	100	α2b	100	12.2	12	12	Null

[a]Cases 1 stopped simeprevir before the completion of 12-week treatment, due to the increase of total bilirubin levels (48-week PEG-IFN and ribavirin continued), and Case 5 stopped PEG-IFN and ribavirin before the completion of 24-week treatment, due to a fall in Hb concentration. Case 18 stopped the triple therapy at 12 weeks before the completion of the 48-week regimen, due to null response.

TABLE II. Evolution of Simeprevir-Resistant Variants Over Time by Ultra-Deep Sequencing in Patients, Who Did Not Achieve Sustained Virological Response

		At point of baseline			At point of re-elevation of viral loads			At point of 96Wk after the stop of therapy			
Case	Position	Frequencies (%)	Coverage	Viral loads	Frequencies (%)	Coverage	Viral loads	Frequencies (%)	Coverage	Viral loads	Efficacay
2	aa80	L (99.8%)	213,853×	6.0	L (99.9%)	188,910×	5.3	L (99.8%)	266,418×	6.1	Relapse
	aa155	—	197,250×		Q (2.4%)	165,980×		—	187,727×		
	aa156	—	176,208×		—	159,755×		—	179,884×		
	aa168	N (0.2%)	174,675×		G (61.3%)	137,720×		—	192,342×		
3	aa80	R (0.2%)	141,326×	6.8	R (0.2%)	174,968×	3.8	R (0.2%)	138,981×	6.7	Relapse
	aa155	—	114,101×		—	145,934×		—	92,640×		
	aa156	—	129,256×		—	165,294×		—	99,197×		
	aa168	—	166,778×		V (0.8%)·G (0.2%)	200,756×		G (0.2%)	115,136×		
11	aa80	—	141,147×	6.8	—	122,613×	7.3	—	86,092×	6.8	Relapse
	aa155	Q (0.2%)	29,454×		Q (0.4%)	34,796×		Q (0.2%)	20,149×		
	aa156	—	46,117×		—	47,303×		—	30,053×		
	aa168	—	91,622×		V (77.4%)	80,708×		—	65,050×		
12	aa80	L (1.0%)·R (0.2%)	151,968×	7.3	L (76.0%)·R (1.7%)	158,451×	7.3	R (0.2%)	189,759×	7.3	Breakthrough
	aa155	—	177,405×		—	194,176×		—	184,982×		
	aa156	—	189,503×		—	202,505×		—	194,138×		
	aa168	—	176,868×		V (25.8%)·E (2.0%)	184,963×		—	182,671×		
17	aa80	—	175,466×	7.0	R (0.2%)	156,143×	7.1	—	231,206×	6.9	Relapse
	aa155	—	135,348×		Q (2.2%)	161,895×		—	149,245×		
	aa156	—	150,908×		—	196,537×		—	178,579×		
	aa168	—	86,237×		V (79.0%)	92,466×		—	96,975×		
18	aa80	R (0.3%)	143,435×	6.4	R (0.4%)	152,564×	4.0	R (10.2%)	153,700×	6.8	Null
	aa155	—	88,537×		—	85,458×		Q (0.2%)	89,740×		
	aa156	—	109,437×		—	106,712×		—	114,061×		
	aa168	—	120,870×		V (99.5%)·E (0.3%)	131,749×		E (9.7%)	116,052×		

In Cases 17 (relapse), resistant variants were not detected at baseline, but de novo resistant variants (D168V [79.0% of 92,466× coverage], R155Q [2.2% of 161,895× coverage], and Q80R [0.2% of 156,143× coverage]) were detected at re-elevation of viral load.

Thus, the present study using ultra-deep sequencing indicates that the majority of resistant variants detected at re-elevation of viral load was de novo resistant variants (12 of 18 variants), and that the emergence of variants after the start of treatment could not be predicted at baseline.

Simeprevir-Resistant Variants Detected by Ultra-Deep Sequencing at 96-Week after the Cessation of Treatment

At 96-week after the cessation of treatment, de novo resistant variants detected at re-elevation of viral load, were undetected (R155Q of Case 2,17) (D168G of Case 2) (D168V of Case 3,11,12,17,18) (D168E of Case 12) (Q80R of Case 17), or detected as very low frequency variants of 0.2% (D168G of Case 3). However, very high (≥99.0%) or low (0.2%) frequency variants at baseline persisted as very high or low frequency variants at 96-week after the cessation of treatment (Q80L of Case 2) (Q80R of Case 3,12) (R155Q of Case 11).

In Case 18, variants of Q80R at baseline (0.3% of 143,435× coverage) persisted as very low frequency variants at re-elevation of viral load (0.4% of 152,564× coverage), but increased at 96-week after the cessation of treatment (10.2% of 153,700× coverage). Furthermore, de novo resistant variants of D168E (0.3% of 131,749× coverage) were detected at re-elevation of viral load, but increased at 96-week after the cessation of treatment (9.7% of 116,052× coverage).

Thus, the present study using ultra-deep sequencing indicates that the majority of de novo resistant variants become undetectable over time (10 of 12 variants), and that the majority of resistant variants detected at baseline persisted over time (5 of 7 variants).

DISCUSSION

Previous study based on telaprevir-based therapy showed that de novo telaprevir-resistant variants emerged regardless of variants frequencies at baseline, and that the emergence of variants after the start of triple therapy could not be predicted at baseline [Akuta et al., 2013]. The present study also indicated that the majority of simeprevir-resistant variants at re-elevation of viral load was de novo resistant variants, and that the emergence of variants could not be predicted at baseline. Hence, patients, who failed to achieve sustained virological response to simeprevir/PEG-IFN/ribavirin triple therapy, need to be identified to avoid the emergence of simeprevir-resistant variants. *IL28B* genotype have been useful as pretreatment predictors of poor virological response to telaprevir/PEG-IFN/ribavirin triple therapy [Akuta et al., 2010]. The present study based on the small numbers of patients indicated that sustained virological response rate was significantly higher in patients with *IL28B* rs8099917 TT than in those with non-TT, but did not identify the other factors that significantly contributed to sustained virological response. Recently, a polymorphism (ss469415590) within the gene that encodes a novel IFN-lambda 4 (*IFNL4*) protein has been found to be strongly associated with outcome of PEG-IFN/ribavirin dual therapy or telaprevir/PEG-IFN/ribavirin triple therapy [Fujino et al., 2013; Kawakami et al., 2013; Prokunina-Olsson et al., 2013]. In this study, the effect of *IFNL4* genotype could be assessed in 8 of 18 patients. Interestingly, sustained virological response rate for patients with the *IFNL4* TT/TT of the treatment-sensitive genotype was higher than patients with non-TT/TT of the treatment-resistant genotype in preliminary study based on the small numbers of patients (100% and 20%). Furthermore, one patient with the *IFNL4* TT/TT of the treatment-sensitive genotype, could achieve sustained virological response, regardless *IL28B* rs8099917 TG of the treatment-resistant genotype. Further studies of larger number of patients should be performed to investigate the pretreatment predictive factors of sustained virological response to simeprevir-based therapy, including host genetic factors (e.g., *IL28B* and *IFNL4* genotype) and viral factors (e.g., amino acid substitutions in the core region) [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Akuta et al., 2010; Fujino et al., 2013; Kawakami et al., 2013; Prokunina-Olsson et al., 2013].

It is not clear at this stage whether the emergence of NS3/4A protease inhibitors-resistant variants might affect the second course of NS3/4A protease inhibitors-based treatment. The present study indicates that the majority of simeprevir-resistant variants become undetectable over time. However, in Case 18, variants of Q80R at baseline (0.3%) increased at 96-week after the cessation of treatment (10.2%), and de novo resistant variants of D168E (0.3%) also increased at 96-week after the cessation of treatment (9.7%). Recent report based on telaprevir-based therapy showed that one patient, who did not achieve sustained virological response by the first course of telaprevir/PEG-IFN/ribavirin, could achieve sustained virological response by the second course of telaprevir/PEG-IFN/ribavirin despite the persistence of de novo telaprevir-resistant variants (98.1% for V36C) [Akuta et al., 2014]. This finding may be due to one or more reasons. One reason is probably related to the high susceptibility of telaprevir-resistant variants to IFN. One previous study indicated that mice infected with the resistant strain (A156F [99.9%]) developed only low-level viremia and the virus was successfully eliminated with IFN therapy [Hiraga et al., 2011]. In the other clinical report, telaprevir-

resistant variants, which emerged during telaprevir monotherapy for 24 weeks, could be eliminated by PEG-IFN/ribavirin [Ozeki et al., 2011]. Furthermore, this finding probably suggests that a small number of mutant type viral RNA may be incomplete or defective, since a large proportion of viral genomes are thought to be defective due to the high replication and mutation rates of the virus [Bartenschlager and Lohmann, 2000]. However, recent report based on simeprevir-based therapy indicated persistence of simeprevir-resistant variants in patients infected with HCV genotype 1 at approximately 1.5 years after the cessation of simeprevir monotherapy, and which might affect response to re-treatment with simeprevir/PEG-IFN/ribavirin [Lenz et al., 2012]. Further studies of larger number of patients should be performed to evaluate whether the emergence of NS3/4A protease inhibitors-resistant variants affects treatment efficacy by the second course of NS3/4A protease inhibitors-based treatment.

In conclusion, the present study indicates that the emergence of simeprevir-resistant variants after the start of treatment could not be predicted at baseline, and the majority of de novo resistant variants become undetectable over time. One limitation in the present Japanese study is that the significance of preexisting resistant variants, especially HCV genotype 1a with variants of Q80K, could not be investigated. Patients with variants of Q80K at baseline indicated the lower rates of sustained virological response (46.7%) in PROMISE phase III trial with simeprevir/PEG-IFN/ribavirin [Forns et al., 2013]. Another limitation is that it could not be investigated at this stage whether the emergence of simeprevir-resistant variants (especially, variants of aa168) might affect the interferon-free regimens, including direct-acting antiviral agents, in future (e.g., Oral dual therapy of daclatasvir and asunaprevir, without PEG-IFN/ribavirin [Chayama et al., 2012; Karino et al., 2013]). Further large-scale prospective studies should be performed to investigate the clinical utility in detecting simeprevir-resistant variants on the response to treatment, and to help in the design of more effective therapeutic regimens.

REFERENCES

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2005. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48:372–380.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 46:403–410.

Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Chayama K, Nakamura Y, Kumada H. 2010. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. Hepatology 52:421–429.

Akuta N, Suzuki F, Seko Y, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2013. Emergence of telaprevir-resistant variants detected by ultra-deep sequencing after triple therapy in patients infected with HCV genotype 1. J Med Virol 85:1028–1036.

Akuta N, Suzuki F, Fukushima T, Kawamura Y, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Hara T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2014. Utility of detection of telaprevir-resistant variants for prediction of efficacy of treatment of hepatitis C virus genotype 1 infection. J Clin Microbiol 52:193–200.

Bartenschlager R, Lohmann V. 2000. Replication of hepatitis C virus. J Gen Virol 81:1631–1648.

Chayama K, Takahashi S, Toyota J, Karino Y, Ikeda K, Ishikawa H, Watanabe H, McPhee F, Hughes E, Kumada H. 2012. Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. Hepatology 55:742–748.

Drafting Committee for Hepatitis Management Guidelines, the Japan Society of Hepatology. 2014. JSH Guidelines for the Management of Hepatitis C Virus Infection: A 2014 Update for Genotype 1. Hepatol Res 44:59–70.

Elliott AM, Radecki J, Moghis B, Li X, Kammesheidt A. 2012. Rapid detection of the ACMG/ACOG-recommended 23 CFTR disease-causing mutations using ion torrent semiconductor sequencing. J Biomol Tech 23:24–30.

Forns X, Lawitz E, Zeuzem S, Gane EJ, Bronowicki J, Andreone P, Horban A, Brown AS, Peeters M, Lenz O, Ouwerkerk-Mahadevan S, Scott JA, Kalmeijer R, De La Rosa G, Sinha R, Beumont-Mauviel M. 2013. Simeprevir (TMC435) with peginterferon α-2a/ribavirin for treatment of chronic HCV genotype 1 infection in patients who relapsed after previous interferon-based therapy: Efficacy and safety in patient subpopulations in the PROMISE phase III trial. Poster 1092 presented at the 64st Annual Meeting of American Association for the Study of Liver Diseases (AASLD), Washington, DC. USA. 1–5 November, 2013.

Fried MW, Buti M, Dore GJ, Flisiak R, Ferenci P, Jacobson I, Marcellin P, Manns M, Nikitin I, Poordad F, Sherman M, Zeuzem S, Scott J, Gilles L, Lenz O, Peeters M, Sekar V, De Smedt G, Beumont-Mauviel M. 2013. Once-daily simeprevir (TMC435) with pegylated interferon and ribavirin in treatment-naïve genotype 1 hepatitis C: The randomized PILLAR study. Hepatology 58:1918–1929.

Fujino H, Imamura M, Nagaoki Y, Kawakami Y, Abe H, Hayes CN, Kan H, Fukuhara T, Kobayashi T, Masaki K, Ono A, Nakahara T, Honda Y, Naeshiro N, Urabe A, Yokoyama S, Miyaki D, Murakami E, Kawaoka T, Hiraga N, Tsuge M, Hiramatsu A, Hyogo H, Aikata H, Takahashi S, Miki D, Ochi H, Ohishi W, Chayama K. Hiroshima Liver Study Group. 2013. Predictive value of the *IFNL4* polymorphism on outcome of telaprevir, peginterferon, and ribavirin therapy for older patients with genotype 1b chronic hepatitis C. J Gastroenterol [Epub ahead of print].

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in *IL28B* predicts hepatitis C treatment-induced viral clearance. Nature 461:399–401.

Hayashi N, Okanoue T, Tsubouchi H, Toyota J, Chayama K, Kumada H. 2012. Efficacy and safety of telaprevir, a new protease inhibitor, for difficult-to-treat patients with genotype 1 chronic hepatitis C. J Viral Hepat 19:e134–e142.

Hayashi N, Seto C, Kato M, Komada Y, Goto S. 2014. Once-daily simeprevir (TMC435) with peginterferon/ribavirin for treatment-naïve hepatitis C genotype 1-infected patients in Japan: The DRAGON study. J Gastroenterol 49:138–147.

Hézode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourlière M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S. PROVE2 Study Team. 2009. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. N Engl J Med 360:1839–1850.

Hiraga N, Imamura M, Abe H, Hayes CN, Kono T, Onishi M, Tsuge M, Takahashi S, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, Yoshizato K, Matsui H, Kanai A, Inaba T, Tanaka S, Chayama K. 2011. Rapid emergence of telaprevir resistant hepatitis C virus strain from wildtype clone in vivo. Hepatology 54:781–788.

Karino Y, Toyota J, Ikeda K, Suzuki F, Chayama K, Kawakami Y, Ishikawa H, Watanabe H, Hernandez D, Yu F, McPhee F, Kumada H. 2013. Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir. J Hepatol 58:646–654.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 87:9524–9528.

Kawakami Y, Suzuki F, Karino Y, Toyota J, Kumada H, Chayama K. 2013. Telaprevir is effective given every 12 hours at 750 mg with peginterferon-alfa-2b and ribavirin to Japanese patients with HCV-1b *IL28B* rs8099917 TT. Antivir Ther [Epub ahead of print].

Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. 2012. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. J Hepatol 56:78–84.

Lenz O, de Bruijne J, Vijgen L, Verbinnen T, Weegink C, Van Marck H, Vandenbroucke I, Peeters M, Simmen K, Fanning G, Verloes R, Picchio G, Reesink H. 2012. Efficacy of re-treatment with TMC435 as combination therapy in hepatitis C virus–infected patients following TMC435 monotherapy. Gastroenterology 143:1176–1178.

McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J, Muir AJ. PROVE1 Study Team. 2009. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med 360:1827–1838.

McHutchison JG, Manns MP, Muir AJ, Terrault NA, Jacobson IM, Afdhal NH, Heathcote EJ, Zeuzem S, Reesink HW, Garg J, Bsharat M, George S, Kauffman RS, Adda N, Di Bisceglie AM. PROVE3 Study Team. 2010. Telaprevir for previously treated chronic HCV infection. N Engl J Med 362:1292–1303.

Nasu A, Marusawa H, Ueda Y, Nishijima N, Takahashi K, Osaki Y, Yamashita Y, Inokuma T, Tamada T, Fujiwara T, Sato F, Shimizu K, Chiba T. 2011. Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. PLoS ONE 6:e24907.

Ninomiya M, Ueno Y, Funayama R, Nagashima T, Nishida Y, Kondo Y, Inoue J, Kakazu E, Kimura O, Nakayama K, Shimosegawa T. 2012. Use of illumina deep sequencing technology to differentiate hepatitis C virus variants. J Clin Microbiol 50:857–866.

Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. 2001. A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 46:471–477.

Ozeki I, Akaike J, Karino Y, Arakawa T, Kuwata Y, Ohmura T, Sato T, Kamiya N, Yamada I, Chayama K, Kumada H, Toyota J. 2011. Antiviral effects of peginterferon alpha-2b and ribavirin following 24-week monotherapy of telaprevir in Japanese hepatitis C patients. J Gastroenterol 46:929–937.

Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, Hergott D, Porter-Gill P, Mumy A, Kohaar I, Chen S, Brand N, Tarway M, Liu L, Sheikh F, Astemborski J, Bonkovsky HL, Edlin BR, Howell CD, Morgan TR, Thomas DL, Rehermann B, Donnelly RP, O'Brien TR. 2013. A variant upstream of *IFNL3* (*IL28B*) creating a new interferon gene *IFNL4* is associated with impaired clearance of hepatitis C virus. Nat Genet 45:164–171.

Romano KP, Ali A, Royer WE, Schiffer CA. 2010. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. Proc Natl Acad Sci USA 107:20986–20991.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. *IL28B* is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100–1104.

Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M, Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K. 2003. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginie deminase 4, are associated with rheumatoid arthritis. Nat Genet 34:395–402.

Suzuki F, Sezaki H, Akuta N, Suzuki Y, Seko Y, Kawamura Y, Hosaka T, Kobayashi M, Saito S, Arase Y, Ikeda K, Kobayashi M, Mineta R, Watahiki S, Miyakawa Y, Kumada H. 2012. Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. J Clin Virol 54:352–354.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of *IL28B* with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105–1109.

Vogel U, Szczepanowski R, Claus H, Jünemann S, Prior K, Harmsen D. 2012. Ion torrent personal genome machine sequencing for genomic typing of Neisseria meningitidis for rapid determination of multiple layers of typing information. J Clin Microbiol 50:1889–1894.

Zeuzem S, Berg T, Gane E, Ferenci P, Foster GR, Fried MW, Hezode C, Hirschfield GM, Jacobson I, Nikitin I, Pockros PJ, Poordad F, Scott J, Lenz O, Peeters M, Sekar V, De Smedt G, Sinha R, Beumont-Mauviel M. 2014. Simeprevir increases rate of sustained virologic response among treatment-experienced patients with HCV genotype-1 infection: A phase IIb trial. Gastroenterology 146:430–441.