Internal Medicine
The Japanese Society of Internal Medicine
doi: 10.2169/internalmedicine.7132-21
Intern Med 60: 3239-3243, 2021
http://internmed.jp

【 CASE REPORT 】

Discrepant Diagnostic Results of Nested Polymerase Chain Reaction-based Genotyping in a Patient with Hepatitis C Virus and Human Immunodeficiency Virus Coinfection

Masami Minemura[1,2], Kazuto Tajiri[1], Yuka Hayashi[1], Naoki Takahashi[1], Kasumi Watanabe[1], Tatsuyuki Hanaoka[1], Yasuhiro Araki[1], Kosuke Takahashi[1], Terumi Takahara[1], Shigeyuki Kojima[3] and Ichiro Yasuda[1]

Abstract:
Accurate genotyping is important to improve the treatment of hepatitis C virus (HCV) infection. We herein report a 44-year-old Japanese man with hemophilia A and coinfection of HCV and human immunodeficiency virus (HIV) who was diagnosed with HCV genotype 4 by direct sequencing. Two genotyping tests based on the nested polymerase chain reaction method that we used misdiagnosed his genotype as 2b and 1b. Although several HCV genotyping tests are available in Japan, it is important to recognize that some cannot detect genotype 4. Care should be taken when genotyping HCV patients who have received non-heated coagulation factor preparations or were infected abroad.

Key words: hepatitis C virus, direct-acting antiviral agent, genotyping, genotype 4

(Intern Med 60: 3239-3243, 2021)
(DOI: 10.2169/internalmedicine.7132-21)

Introduction

Hepatitis C virus (HCV) infection is a worldwide health problem because it frequently leads to liver cirrhosis and hepatocellular carcinoma. Recent advances in direct-acting antiviral agents (DAAs) have markedly improved the antiviral efficacy in patients with HCV infection (1). Although the latest combination therapy with an HCV protease inhibitor [glecaprevir (GLE)] and an HCV NS5A inhibitor [pibrentasvir (PIB)] is available for several genotypes (2), it is important to determine the genotypes or serotypes of HCV in patients before DAA treatment, as the effects of DAAs differ among genotypes.

Several methods for HCV typing are clinically available, but only serotyping (3), not genotyping, is approved for coverage by the public health insurance program in Japan. The serotyping method can distinguish type 1 (group 1) and type 2 (group 2) with high accuracy (4), but there are a few discrepancies between serotypes and genotypes in some patients that can lead to treatment failure (5-7).

We herein report a patient with HCV and human immunodeficiency virus (HIV) co-infection who initially showed a mismatch between his serotype and the HCV genotype. This is a very rare case, as genotype 2b was first determined using one of the nested polymerase chain reaction (PCR)-based assays, whereas genotype 1b was determined by another genotyping assay. We eventually confirmed the patient's genotype to be genotype 4 by direct sequencing. We discuss the cause of the discrepancy in genotyping results.

Case Report

A 44-year-old Japanese man was diagnosed with hemophilia A and co-infected with HIV and HCV by the administration of non-heat concentrated coagulation factor preparations in Japan. Although a liver biopsy was not performed because of the patient's hemophilia, chronic hepatitis C was

[1]Department of Gastroenterology, Toyama University Hospital, Japan, [2]Department of Community Medical Support, Toyama University Hospital, Japan and [3]Division of Advanced Technology and Development, BML Inc, Japan
Received: January 20, 2021; Accepted: March 15, 2021; Advance Publication by J-STAGE: April 26, 2021
Correspondence to Dr. Masami Minemura, minemura@med.u-toyama.ac.jp

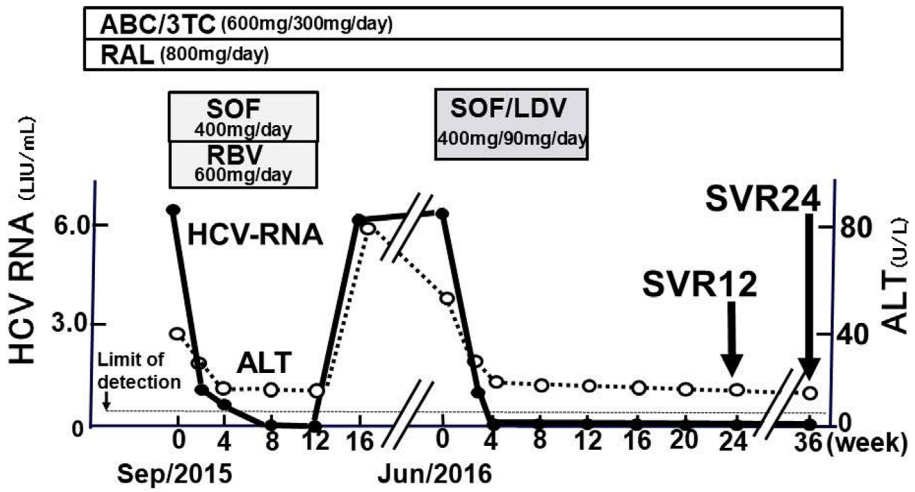

Figure 1. **The patient's clinical course. ABC: abacavir, ALT: alanine aminotransferase, HCV: hepatitis C virus, 3TC: lamivudine, LDV: ledipasvir, RAL: raltegravir, RBV: ribavirin, SOF: sofosbuvir**

clinically diagnosed due to liver dysfunction with a persistent elevation of alanine aminotransferase (ALT). He had no splenomegaly and had a liver fibrosis-4 (FIB-4) index of 1.22 (8), so the liver fibrosis was considered non-progressive.

At the patient's first diagnosis in August 2008, HCV serotyping (3) and HCV ribonucleic acid (RNA) quantitative test results led to a diagnosis of serogroup 1, and the HCV RNA titer was 6.40 log IU/mL. In February 2010, we started antiviral treatment with pegylated interferon alfa 2a (Peg-IFNα2a; 180 μg/day) and ribavirin (RBV; 600 mg/day). However, even at 16 weeks after the initiation of this treatment, there was no significant decrease in the patient's HCV-RNA titer (5.90 logIU/mL), and the treatment was discontinued as a non-response case. The patient's IL28B genetic polymorphism turned out to be heterozygous (rs 8099917 T/G, rs11881222 A/G, rs8103142 T/C) in 2012 (9).

In January 2012 the patient received a combination antiretroviral therapy with abacavir (ABC; 600 mg/day)/lamivudine (3TC; 300 mg/day) and raltegravir (RAL; 800 mg/day) (10). His HIV viral load remained undetectable, and his CD4-positive (CD4+) cell count was over 500/μL.

We considered introducing IFN-free treatment with DAA, and we first used the core region nested PCR method (11) (HCV Genotype Primer Kit; Institute of Immunology, Tokyo, Japan) to determine the patient's HCV genotype; this method indicated genotype 2b. Although the possibility of a mixed infection of multiple HCV genotypes could not be completely ruled out, we treated the patient with sofosbuvir (SOF; 400 mg/day) and RBV (600 mg/day) for 12 weeks beginning in February 2016. He tested negative for HCV RNA at the end of the 12-week period, but he was positive for HCV RNA at 4 weeks following that treatment's completion (Fig. 1).

At the relapse, the patient's HCV genotype was determined again by the same method, and the result was again genotype 2b. To confirm the genotype, we performed HCV genotyping using both pre- and post-treatment serum samples with another genotyping system (HCV RNA Core Genotype Kit; SRL, Tokyo, Japan) (12). Surprisingly, the genotype was genotype 1b in both samples with this test, differing from the previously identified genotype.

To investigate the cause of the discrepancy in HCV genotype test results, we performed direct sequencing of the 5'-untranslated region (5'-UTR) and core gene region (-116 to +343; 459 bp) using the following primers: sense 5'-CTCCCGGGAGAGCCA TAG-3' and antisense 5'-ATGTACCCCATGAGRTCGGC-3'. A molecular phylogenetic tree analysis for the viral genotype led to a diagnosis of genotype 4 (Fig. 2). In June 2016 we treated the patient with SOF (400 mg/day) and ledipasvir (LDV; 90 mg/day) for 12 weeks with the patient's fully informed consent, since SOF/LDV was reported to be effective for patients with HCV genotype 4 (13, 14), and pan-genotype DAAs, such as GLE/PIB, were not available at that time. The patient tested negative for HCV RNA at 4 weeks of SOF/LDV treatment, and he achieved a sustained virological response (SVR).

Discussion

We treated a patient with a rare HCV and HIV co-infection for whom it was difficult to determine the HCV genotype by two different nested PCR-based genotyping methods; the genotype was finally determined as genotype 4 by direct sequencing. Since pan-genotype DAAs, such as GLE/PIB, have become available, the importance of genotype determination has diminished. However, accurate genotyping is still useful for determining the appropriate duration of treatment for chronic hepatitis in Japan, as the duration differs between genotype 1/2 and other types according to the current guidelines for the treatment of HCV issued by the Japanese Society of Hepatology (15). Accurate genotyping of HCV can achieve a high SVR.

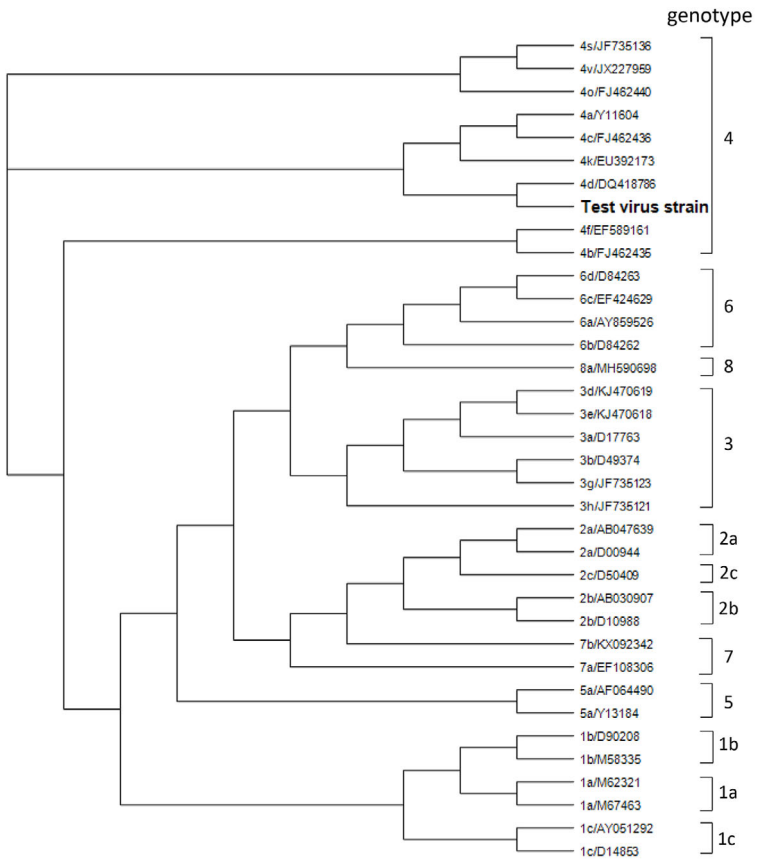

Figure 2. A molecular phylogenetic tree that analyzes whether or not a test virus strain belongs to a known hepatitis C virus genotype strain. The genetic distance was estimated using the Kimura two-parameter method, and the tree was constructed using a neighbor-joining method.

In Japan, an HCV serotyping assay using non-structural protein 4 (NS 4)-specific antibodies is commonly used (3), as an HCV genotyping assay based on the nested PCR method is not approved for coverage by the national health insurance program. When determining HCV genotypes, the frequency of discrepancy between the serotype and genotype is reported to be low (4, 16, 17), but some clinically problematic cases have been reported (5-7). Such discrepancies may be caused by coinfection of different genotypes of HCV (6) or infection with an intergenotypic recombinant virus (7).

The genotype reportedly cannot be accurately determined even when performing genotyping using nested PCR methods targeting the core region, such as genotyping with an HCV genotype primer kit (5). In the present case, we used two types of genotyping test kits based on nested PCR targeting the core region: an HCV genotype primer kit based on Okamoto's method (11), and an HCV RNA core genotype kit based on Ohno's method (12). The first kit can detect genotypes 1a, 1b, 2a, 2b, and 3a, but not 4, as the kit does not include the setting of PCR primers for genotype 4. With this kit, our patient was misidentified as genotype 2b. The HCV genotyping system that was originally reported by Ohno et al. can detect genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a (12), but the commercially usable diagnostic kit (HCV RNA Core Genotype Kit) has no setting of PCR primers for genotypes 4, 5a, and 6a. This assay also provided an incorrect result (genotype 1b) for our patient.

Direct sequencing performed to determine the patient's exact genotype revealed genotype 4, which was not initially expected (Fig. 2, 3). The mistaken results with the first two methods may have been due to the corresponding site of this HCV strain having a relatively high homology for each specific primer (Table) and there being no suitable primer for genotype 4. Because ultra-deep and whole genome sequencing was not performed, the possibility of a mixed HCV genotype infection or an intergenotypic recombinant viral infection could not be completely ruled out. This is a limitation of this case report.

HCV genotype 4 accounts for up to 13% of all HCV infections worldwide and is found frequently in North and Central Africa and the Middle East (18). Hayashi et al. reported that the prevalence of genotype 4 was very low in Japan (0.4%) and that all of the individuals with genotype 4 were male hemophiliacs who received concentrated coagulation factor preparations from abroad (19). Our patient was also diagnosed with hemophilia A and had previously been given non-heat concentrated coagulation factor preparations.

The guidelines from the European Association for the Study of the Liver (EASL) recommend several DAA combination therapy regimens as anti-HCV therapy for patients with HCV genotype 4 infection (1). Since SOF/LDV has

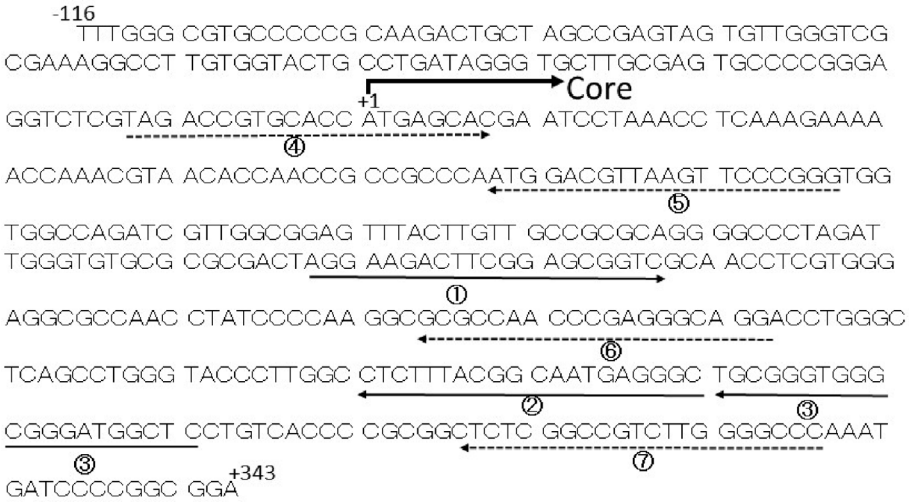

Figure 3. **Nucleotide sequence of the 5'-UTR and core gene region of the test virus strain determined by direct sequencing. The arrows indicate target regions of the genotype-specific primers for the nested PCR. Region ①: the second-round PCR sense primer, ②: the anti-sense primer for genotype 2b, and ③: genotype 1b of Okamoto's method (11). Region ④: the second-round PCR sense primer, ⑤: the anti-sense primer for genotype 4, and ⑥: genotype 1b and ⑦: genotype 2b of Ohno's original method (12).**

Table. **Comparison of Nucleotide Sequences of Nested PCR Primers and the Test Virus Strain.**

Second-round PCR primer	Sequence (5'- 3')	Nucleotide position
Okamoto's method		
① sense primer for genotyping the test virus strain	5'-AGGAAGACTTCGGAGCGGTC-3' - - - - - - - - - - -C- - - - - - - -	148 - 167
② anti-sense primer for genotype 2b the test virus strain	5'-GAGCCATCCTGCCCACCCCA-3' - - - - - - - - -C- - - - - - - -G-	270 - 257
③ anti-sense primer for genotype 1b the test virus strain	5'-ACCCTCGTTTCCGTACAGAG-3' G - - - - -A- -G - - - - -A- - - -	291 - 271
Ohno's method		
④ sense primer for genotyping the test virus strain	5'-AGACCGTGCACCATGAGCAC-3' -	- 12 - 8
⑤ anti-sense primer for genotype 4 the test virus strain	5'-CCCGGGAACTTAACGTCCAT-3' -	87 - 58
⑥ anti-sense primer for genotype 1b the test virus strain	5'-CCTGCCCTCGGGTTGGCTA-3' - - - - - - - - - - - - - - - - - -GC	222 - 204
⑦ anti-sense primer for genotype 2b the test virus strain	5'-GGCCCCAATTAGGACGAGAC -3' - - - - - - - -GAC- - C- - - - -G	325 - 306

PCR: polymerase chain reaction

been reported to be effective in patients with HCV genotype 4 (13), we have administered SOF/LDV to patients who relapsed after treatment with SOF/RBV.

In conclusion, we reported a rare case of a patient with an HCV genotype 4 infection in whom two usable nested PCR genotyping assays failed to determine the exact genotype. Several HCV genotyping assays are currently available in Japan, but it is important to recognize that some testing kits cannot detect genotype 4. Care should be taken when genotyping HCV-infected patients who have been given non-heated coagulation factor preparations or were infected abroad.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

We thank Megumi Minemura for her technical support.

References

1. European Association for Study of the Liver. EASL recommendations on treatment of hepatitis C 2018. J Hepatol **69**: 461-511, 2018.
2. Asselah T, Kowdley KV, Zadeikis N, et al. Efficacy of glecaprevir/pibrentasvir for 8 or 12 weeks in patients with hepatitis C virus genotype 2, 4, 5, or 6 infection without cirrhosis. Clin Gastroenterol Hepatol **16**: 417-426, 2018.
3. Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, et al. Significance of specific antibody assay for genotyping of hepatitis C virus. Hepatology **19**: 1347-1353, 1994.
4. Mizoguchi N, Mizokami M, Orito E, Shibata H, Shibata H. Serologically defined genotypes of hepatitis C virus among Japanese patients with chronic hepatitis C. J Virol Methods **58**: 71-79, 1996.
5. Ishikawa T, Abe S, Kojima Y, Kojima S, Yoshida T. Discrepant diagnostic results using two genotyping methods in a chronic hepatitis C patient in serogroup 1. Hepatol Res **46**: 354-356, 2016.
6. Sohda T, Yamauchi E, Anan A, et al. Non-response to daclatasvir and asunaprevir therapy in patients co-infected with hepatitis C virus genotypes 1 and 2. Hepatol Res **47**: 364-367, 2017.
7. Kurata H, Uchida Y, Kouyama J, et al. Chronic hepatitis caused by hepatitis C virus showing a discrepancy between serogroup and genotype because of intergenotypic 2b/1b recombination: a pitfall in antiviral therapy with direct-acting antivirals. Hepatol Res **48**: E 372-E378, 2018.
8. Vallet-Pichard A, Mallet V, Nalpas B, et al. FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. Comparison with liver biopsy and fibrotest. Hepatology **46**: 32-36, 2007.
9. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet **41**: 1105-1109, 2009.
10. Lennox JL, DeJesus E, Lazzarin A, et al. Safety and efficacy of raltegravir-based versus efavirenz-based combination therapy in treatment-naive patients with HIV-1 infection: a multicentre, double-blind randomised controlled trial. Lancet **374**: 796-806, 2009.
11. Okamoto H, Tokita H, Sakamoto M, et al. Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. J Gen Virol **74**: 2385-2390, 1993.
12. Ohno O, Mizokami M, Wu RR, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol **35**: 201-207, 1997.
13. Kohli A, Kapoor R, Sims Z, et al. Ledipasvir and sofosbuvir for hepatitis C genotype 4: a proof-of-concept, single-centre, open-label phase 2a cohort study. Lancet Infect Dis **15**: 1049-1054, 2015.
14. Naggie S, Cooper C, Saag M, et al. Ledipasvir and sofosbuvir for HCV in patients coinfected with HIV-1. N Engl J Med **373**: 705-713, 2015.
15. Hepatitis C treatment guidelines (8th edition). Jpn Soc Hepatol [Internet]. 2020 [cited 2021 Feb 2021]. Available from: http://www.jsh.or.jp/medical/guidelines/jsh_guidlines/hepatitis_c.
16. Hoshino H, Hino K, Miyakawa H, Takahashi K, Akbar SM, Mishiro S. Inter-genotypic recombinant hepatitis C virus strains in Japan noted by discrepancies between immunoassay and sequencing. J Med Virol **84**: 1018-1024, 2012.
17. Inoue J, Kanno A, Wakui Y, et al. Identification of genotype 2 HCV in serotype-1 hepatitis C patients unresponsive to daclatasvir plus asunaprevir treatment. Tohoku J Exp Med **241**: 21-28, 2017.
18. Hathorn E, Elsharkawy AM. Management of hepatitis C genotype 4 in the directly acting antivirals era. BMJ Open Gastroenterol **3**: e000112, 2016.
19. Hayashi K, Fukuda Y, Nakano I, et al. Prevalence and characterization of hepatitis C virus genotype 4 in Japanese hepatitis C carriers. Hepatol Res **25**: 409-414, 2003.

The Internal Medicine is an Open Access journal distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License. To view the details of this license, please visit (https://creativecommons.org/licenses/by-nc-nd/4.0/).

© 2021 The Japanese Society of Internal Medicine