

Reassessment necessity of patients with serogroup 2 of hepatitis C virus and undetectable serum

HCV RNA

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List of abbreviations:

HCV, hepatitis C virus; CAP/CTM, Roche AmpliPrep/Cobas TaqMan HCV Assay; ART, Abbott RealTime HCV assay

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ABSTRACT

Background: We encountered a patient with positive anti-hepatitis C virus (HCV) whose serum HCV RNA was undetectable with the Roche AmpliPrep/Cobas TaqMan HCV Assay (CAP/CTM) version 1, but in high viral load with the Abbott RealTime HCV assay (ART).

Methods: Discrepancies in the detectability of serum HCV RNA were investigated among 891 consecutive patients who were positive for anti-HCV. Specific nucleotide variations causing the undetectability of HCV RNA were determined, and confirmed by synthesizing RNA coding those variations. Serum samples with the discrepancies were also reassessed by CAP/CTM version 2.

Results: Among the 891 anti-HCV-positive patients, 4 patients had serum HCV RNA that was undetectable by CAP/CTM version 1 despite being >5 log IU/ml by ART. All four patients had HCV genotype 2a with high titers of anti-HCV. Sequencing of the HCV 5' non-coding regions revealed 2 common variations: A at nt 145 and T at nt 151. Synthesized RNAs of the HCV 5' non-coding region with standard (NCR145G151C) and variant nucleotides at nt 145 and nt 151 were quantified with CAP/CTM. RNAs of NCR145G151C and NCR145G151T were quantifiable with CAP/CTM version 1, while those of NCR145A151T and NCR145A151C went undetected. The

substitution from G to A at nt 145 specifically conferred this undetectability, while synthesized HCV RNA in correcting this variation will revert this undetectability. Reassessment of these samples by CAP/CTM version 2 resulted in similar levels of HCV RNA by ART.

Conclusions: HCV patients with undetectable HCV RNA by CAP/CTM version 1, should be reassessed for viral quantification.

INTRODUCTION

Hepatitis C virus (HCV) infection causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1, 2]. More than 170 million people worldwide are infected with HCV, creating a serious global health problem. Monitoring of serum HCV RNA levels during antiviral therapy is essential for its management [3]. Sustained virological response is generally evaluated by whether HCV RNA can be detected in the serum 12 or 24 weeks after the cessation of the treatment. Recent monitoring of serum HCV RNA has mostly been done by real-time PCR methods, because real-time PCR methods are sensitive with low limits of detection, and have broad dynamic ranges of quantification [4, 5].

The Roche AmpliPrep/Cobas TaqMan HCV Assay (CAP/CTM; Roche Molecular Systems, Pleasanton, CA) version 1 may underestimate or overestimate HCV RNA levels in a number of patients infected with HCV genotypes 2 and 4, because of mismatch of the primers or probes with the viral sequence [6]. The undetectability due to sequence mismatch in CAP/CTM version 1 has been overcome for the HCV genotype 4 by CAP/CTM version 2 [7]. The superiority of the Abbott RealTime HCV assay (ART; Abbott Molecular, Des Plaines, IL) or CAP/CTM version 2 to CAP/CTM version 1 in the sensitivity and accuracy has been also reported.

The present study is the first to report on patients with HCV genotype 2a whose serum HCV RNA was undetectable with CAP/CTM version 1, despite a high viral load by ART. We clarified the cause

of the undetectability of HCV, and estimated the prevalence of this discrepancy among patients with positive results on the anti-HCV test. The serum samples with the discrepancies were also reassessed by CAP/CTM version 2, resulting in similar levels of HCV RNA by ART.

METHODS

Patients

The present study enrolled consecutive patients who had positive results on the anti-HCV test (Lumipulse Presto Ortho HCV, Fujirebio, Tokyo, Japan), admitted for further examination or therapy for liver cirrhosis, esophageal and gastric varices, or hepatocellular carcinoma to the gastrointestinal unit of Okayama University Hospital between 2008 and 2012. Liver histology was evaluated according to the criteria of Desmet et al. [8]. HCV serogroup was assessed by the HCV serogrouping assay (HCV Gr, Sysmex International Reagents, Kobe, Japan), which can subgroup the patients in HCV serogroups 1 and 2, corresponding to HCV genotypes 1 and 2 respectively, with HCV group specific anti-non-structural region 4 antibodies. This assay is available not only for the patients with chronic HCV infection, but for those with resolved HCV. This study was performed in accordance with the Helsinki Declaration, and the protocol was approved by the ethics committee of the institute. This study was registered for University Hospital Medical Information Network

Clinical Trials Registry (UMIN 000001031). All patients provided informed consent before enrollment in the study.

Quantification of HCV RNA

Serum HCV RNA quantification was performed by RT-qPCR system of CAP/CTM version 1 with an automated sample preparation on the Cobas AmpliPrep extractor from 850 μ L of serum, and the Cobas TaqMan 48 analyzer was used for automated real-time PCR amplification and the detection of PCR products, according to the manufacturer's instructions. When the HCV RNA was undetectable, additional quantification of HCV RNA was done within a month by ART with serum samples stored at -80°C. The results of HCV RNA were also compared with the levels of HCV core antigens (Architect HCV Ag, Abbott, Tokyo, Japan), and the reassessed results of HCV RNA by CAP/CTM version 2.

Analysis of sequence in the HCV 5' non-coding region

Serum RNA was extracted by means of a QIAamp Viral RNA Mini Kit according to the manufacturer's protocol (Qiagen, Tokyo, Japan). HCV RNA was amplified by RT-PCR with primers corresponding to the HCV 5' non-coding region for HCV genotype 2a as modified from the previous report [6]. The primers were 5'NCRS (5'-GGGCGACACTCCGCCATGAA-3', nt 17 to

36) and 5'NCRAS (5'-CCCTGCGCGGCAACAAGTA-3', nt 462 to 444). The first and second PCR rounds included an initial denaturation step at 95°C for 1 minute, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and elongation at 70°C for 30 seconds, followed by a final elongation step at 70°C for 5 minutes. Direct sequencing was carried out by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

RNA synthesis of the HCV 5' non-coding region

Two vectors expressing the HCV 5' non-coding region between nt 17 and 462 were constructed, whose sequences were identical except for G at nt 145 and C at nt 151 for NCR145G151C (the detectable standard), A at nt 145 and T at nt 151 for NCR145A151T, G at nt 145 and T at nt 151 for NCR145G151T, and A at nt 145 and C at nt 151 for NCR145A151C. NCR145A151T was the undetectable variant obtained from the serum of Patient 1. NCR145G151C, NCR145G151T, and NCR145A151C were constructed from NCR145A151T by PCR mutagenesis with primers containing base alterations. The PCR amplicons were cloned into pCR2.1 by means of a TA Cloning Kit according to the manufacturer's protocol (Life Technologies, Tokyo, Japan). The sequences of these inserts were confirmed by Big Dye termination cycle sequencing (Applied Biosystems). The plasmids were utilized for RNA synthesis by means of a MEGAscript Kit according to the

manufacturer's protocol (Life Technologies). Synthesized RNA was quantified with an automated sample preparation from 850 μL of the RNA sample by CAP/CTM version 1 and 500 μL of the RNA sample by CAP/CTM version 2 respectively on the Cobas TaqMan instruments, according to the manufacturer's instructions.

Immunohistochemistry of HCV core

The presence of HCV core protein was assessed by immunohistochemistry. The liver tissues of the patients were fixed in 10% buffered formalin and embedded in paraffin. The sections were treated with citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6.0) for 12 min at room temperature for antigen retrieval. The sections underwent a reaction with a monoclonal antibody for HCV core protein (CP-9; Institute of Immunology, Tokyo, Japan) after nonspecific binding was blocked with casein. The intrinsic peroxidase activity was blocked by immersing sections in a methanol solution containing 0.3% hydrogen peroxide. The Envision detection system was applied with DAB (Dako, CA, US). Two negative controls were applied: one without the first monoclonal antibody and the other from the HCV seronegative patients.

Results

Our first patient in whom serum HCV RNA was undetectable with CAP/CTM version 1 despite a high viral load with ART

A male patient at forty-nine years of age (Patient 1) suffered from chronic hepatitis due to HCV genotype 2a with an abnormal level of alanine aminotransferase >100 IU/ml (Figure 1A). He received antiviral therapy of pegylated interferon and ribavirin. His therapeutic response was only partial, and his liver function test remained abnormal thereafter. Two years later, an abrupt decrease of serum HCV RNA was observed, and every 3 month-quantification with CAP/CTM version 1 for 2 years detected no serum HCV RNA, although his level of alanine aminotransferase remained abnormal. Liver biopsy was performed for further examination for abnormal liver function test, revealing that his liver disease had advanced to liver cirrhosis with interface hepatitis (Figure 1B). The existence of HCV core antigens in both the serum (Table 1) and the liver (Figure 1C) was confirmed. Furthermore, reassessment of HCV RNA with ART showed a high viral load in his serum.

Characteristics of patients with HCV RNA detectable by ART, but not by CAP/CTM

Among the 891 consecutive patients with positive results on the anti-HCV test who were admitted to the gastrointestinal unit for liver related disorders due to HCV, CAP/CTM version 1 detected no

HCV RNA in 69; of these, 38 patients were sustained viral responders to previous interferon therapy, and 31 patients had not undergone antiviral therapies. Among those patients, 3 patients other than Patient 1 had serum HCV RNA >5 log IU/ml by HCV RNA quantification with ART (~~0.45%~~). The characteristics of these patients are summarized in Table 1. All four patients were males in their forties or fifties. Three patients other than Patient 1 had not undergone antiviral therapies. Three had histological liver cirrhosis. They commonly had high titers of anti-HCV antibody and HCV genotype 2a. Co-infection with hepatitis B virus or human immunodeficiency virus was ruled out. Although we doubted that some sustained viral responders might still have HCV because of false negative results by CAP/CTM version 1, serum HCV RNA was not detected by ART among the sustained viral responders.

Comparison of sequences of the 5' non-coding region of HCV

We hypothesized that undetectable HCV RNA with CAP/CTM version 1 might occur due to the mismatch of the primers or probe with the sequence of the target regions. Therefore, the sequences of the HCV 5' non-coding regions were determined by direct sequencing for all 4 patients whose HCV RNA was undetectable in the serum with CAP/CTM version 1, and their sequences were compared with the sequences of HCV genotypes 1a, 1b, 2a, 2b, and 3a using H77 as a reference (Figure 2). Their sequences commonly showed genotype 2a-specific features in the nt 218–224, and

nt 240–243 ranges, according to the report of the HCV genotyping [9, 10]. It is noteworthy that all 4 patients had common variations of G to A at nt 145, C to T at nt 151, and G to A at nt 241. These variations are not seen in other sequences of various genotypes. Therefore, we synthesized RNAs coding the HCV 5' non-coding region with different nucleosides at nt 145 and nt 151 (standard, NCR145G151C and variants, NCR145A151T, NCR145G151T, and NCR145A151C). The nucleoside type at nt 241 was A in the both RNAs, as the region around it commonly shows different sequences among the HCV genotypes [9]. The RNAs synthesized in various copy numbers/ml were quantified with CAP/CTM version 1. Synthesized RNAs of NCR145G151C and NCR145G151T at 10^{10} copies/ml could be quantified as 3.9 Log IU/ml and 3.7 Log IU/ml respectively with CAP/CTM version 1, while the RNAs of NCR145A151T or NCR145A151C were undetectable. It was reported that the specific types of nucleoside at nt 107, nt 165, and nt 206 are associated with underestimation of HCV RNA levels as for HCV genotype 2 [6]. However, those features were not observed in the sequences of the present patients.

Reassessment of serum HCV RNA by CAP/CTM version 2

The levels of HCV RNA were also reassessed by CAP/CTM version 2 for the serum samples with discrepant quantification results. The samples resulted in equivalent levels of HCV RNA by both CAP/CTM version 2 and ART.

Analysis of nucleotide variation at nt 145 in the HCV 5' non-coding region retrieved from the Genbank

Total of 1090 HCV sequences were retrieved from the Genbank, exclusive of repetitive sequences, including 472 of genotype 1a, 466 of genotype 1b, 73 of genotype 2, 34 of genotype 4, and 3 of genotype 5. Nucleotide type at nt145 is G in most sequences and C in 17 sequences among HCV genotype 1a. Only one sequence among HCV genotype 1b had specific nucleotide variation type of A at nt 145. All the sequences among HCV genotypes 2 to 5 in the Genbank had G at nt 145.

Discussion

The present study is the first report of patients with HCV genotype 2a, in whom CAP/CTM version 1 failed to detect HCV RNAs despite detection of a high viral load by ART. If such patients are erroneously considered to have spontaneous viral clearance, or autoimmune hepatitis with positive anti-HCV antibody, the opportunity of their receiving critical antiviral therapy will be lost. Occult HCV infection, defined as detection of HCV RNA in liver tissue or peripheral blood mononuclear cells with constantly undetectable serum HCV RNA [11], might involve such cases in whom HCV RNA is undetectable with CAP/CTM version 1. Reassessment of HCV RNA with ART or

quantification of HCV core antigen is desirable in cases in which HCV is not detected by CAP/CTM version 1. CAP/CTM version 2 is also helpful for the reassessment, if available.

The present study investigated the characteristics of the patients whose serum HCV RNA was undetectable by CAP/CTM version 1. They were all male patients in their forties or fifties. We focused on the sequences of the HCV 5' non-coding region, targeted by CAP/CTM and ART, and compared their sequences with those of the representative strains of HCV genotypes 1a, 1b, 2a, 2b, and 3a. It is noteworthy that they all had genotype 2a HCV and exhibited the same variations at nt 145 and nt 151. Quantification of synthesized RNA with these specific variations at nt 145 and nt 151 confirmed that the variation at nt 145 caused the undetectability of HCV RNA by CAP/CTM version 1.

For searching the patient whose serum HCV RNA was undetectable by CAP/CTM version 1, the present study enrolled the consecutive patients who were positive for anti-HCV, admitted for further examination or therapy for liver related disorders. It was because these patients were supposed to suffer from chronic liver diseases due to HCV, although their HCV RNAs were not routinely assessed. Assuming from the genotype distribution among Japanese patients with chronic hepatitis C, two third of them might be infected with genotype 1b of HCV, one third with genotype 2, and a few with genotype 3. The present study had not determined the HCV genotypes of all the patients enrolled, and the frequency of these specific variations was 0.45% among those patients. By the

search in the Genbank, only one sequence of HCV genotype 1b had specific nucleotide variation type of A at nt 145 among the sequences of HCV genotypes 1 to 5 (0.092%), suggesting very low frequency of this specific variation.

In conclusion, the present study reported on patients with HCV genotype 2a, whose serum HCV RNA was undetectable with CAP/CTM version 1 despite detection of a high viral load by ART. Specific nucleotide variation at nt 145 was found to be responsible for the undetectability of HCV RNA by CAP/CTM version 1. Reassessment of HCV RNA with ART or quantification of HCV core antigen is desirable in cases in which HCV is not detected by CAP/CTM version 1. CAP/CTM version 2 is also helpful for the reassessment if available.

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Figure legends**Figure 1: Clinical course and histology of Patient 1.**

The changes of serum HCV RNA and alanine aminotransferase are shown in Figure 1A. Liver histology indicates liver cirrhosis (Figure 1B, AZAN staining, 4-fold of original magnification). Immunohistochemical staining with a monoclonal antibody specific for HCV core demonstrates granular signal in the hepatocytes (Figure 1C, 400-fold of original magnification).

Figure 2: Sequence alignment of HCV 5' non-coding region of various HCV genotypes and the patients in this study.

The sequence of HCV-H77 (accession# AF009606) was used as a reference; position numbers are given at the top. Dots indicate identical nucleosides. Sequence alignment of HCV 5' non-coding regions contains genotype 1A of HCV-1 (M62321) and HCV-H (M67463), genotype 1B of HCV-J (D90208) and HCV-N (AF139594), genotype 2A of JCH-1 (AB047640), and HC-J6 (D00944), genotype 2B of HCV-MA (AB030907) and HCV-J8 (D10988), and genotype 3A of HCV-NZL1 (D17763) and HCV-K3A (D28917). Sequences of Patients 1 to 4 are aligned at the bottom.


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ACCESSION #      37                                     140
AF009606      TCACCTCCCCTGTGAGGAACTATTGTCTTCACGCAGAAAAGCGTCTAGCCATGGCGTTAGTATGAGTGTGCGTGCGAGCCTCCAGGACCCCCCTCCCGGGAGAGCCA
M62321      -----C-----
M67463      -----C-----
D90208      -----C-----T-----
AF139594      -----C-----
AB047640      -----C-----A-----C-----
D00944      -----C-----A-----C-----
AB030907      -----C-----A-----C-----
D10988      -----C-----A-----C-----
D17763      -----TC-----G-----C-----C-----
D28917      -----TC-----G-----C-----C-----

Patient 1      C-----C-----T-A-----C-----
Patient 2      C-----C-----A-----C-----
Patient 3      C-----C-----A-----C-----
Patient 4      -----C-----A-----C-----

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ACCESSION #      141                                     240
AF009606      TAGT  G  GTCTG  C  GGAACCGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTGGATAAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCC
M62321      ---  -  ---  -  -----C-----
M67463      ---  -  ---  -  -----C-----
D90208      ---  -  ---  -  -----C-----
AF139594      ---  -  ---  -  -----C-----
AB047640      ---  -  ---  -  -----G--A--T-----A--T---C--CC-----
D00944      ---  -  ---  -  -----G--A--T-----A--T---C--TC-----
AB030907      ---  -  ---  -  -----G-A-A--T-----A--T---T-C--TC-----
D10988      ---  -  ---  -  -----A-G-A-A--T-----A--T---T-C--TC-----AC-----
D17763      ---  -  ---  -  -----C-TG-GT-----GC-----A-CA-A-
D28917      ---  -  ---  -  -----C-TG-GT-----AC-----A-CA-A-

Patient 1      ---  A  ---  T  -----G--A--T-----A--T---C--CC-----
Patient 2      ---  A  ---  T  -----G--A--T-----A--T---C--CC-----
Patient 3      ---  A  ---  T  -----G--A--T-----A--T---C--CC-----
Patient 4      ---  A  ---  T  -----A--T-----A--T---C--CC-----

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ACCESSION #      241                                     341
AF009606      GCAAGACTGCTAGCCGAGTAGTGTGGGTCCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCCTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACC
M62321      -----
M67463      -----
D90208      --G-----T-----
AF139594      --G-----
AB047640      -----C-----T-----
D00944      -----C-----T-----
AB030907      -----C-----T-----C-----
D10988      -----C-----T-----
D17763      --G-----A-----
D28917      --G-----C-----T-A-----

Patient 1      A-----C-----T-----T-----
Patient 2      A-----C-----T-----
Patient 3      A-----C-----T-----T-----
Patient 4      A-----C-----T-----T-----

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Figure 2