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Single Strain Isolation Method for Cell Culture-Adapted Hepatitis C Virus by End-Point Dilution and Infection

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Abstract

The hepatitis C virus (HCV) culture system has enabled us to clarify the HCV life cycle and essential host factors for propagation. However, the virus production level of wild-type JFH-1 (JFH-1/wt) is limited, and this leads to difficulties in performing experiments that require higher viral concentrations. As the cell culture-adapted JFH-1 has been reported to have robust virus production, some mutations in the viral genome may play a role in the efficiency of virus production. In this study, we obtained cell culture-adapted virus by passage of full-length JFH-1 RNA-transfected Huh-7.5.1 cells. The obtained virus produced 3 log-fold more progeny viruses as compared with JFH-1/wt. Several mutations were identified as being responsible for robust virus production, but, on reverse-genetics analysis, the production levels of JFH-1 with these mutations did not reach the level of cell culture-adapted virus. By using the single strain isolation method by end-point dilution and infection, we isolated two strains with additional mutations, and found that these strains have the ability to produce more progeny viruses. On reverse-genetics analysis, the strains with these additional mutations were able to produce robust progeny viruses at comparable levels as cell culture-adapted JFH-1 virus. The strategy used in this study will be useful for identifying strains with unique characteristics, such as robust virus production, from a diverse population, and for determining the responsible mutations for these characteristics.

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Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality [1,2]. HCV is a positive-stranded RNA virus belonging to the Flaviviridae family. Its genome, about 9.6-kb long, consists of an open reading frame (ORF) encoding a large polyprotein that is cleaved by cellular and viral proteases into at least 10 structural and non-structural (NS) proteins [3,4]. The structural proteins include core, E1 and E2, which form virus particles. The NS proteins include p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B, which are associated with viral replication.

For research into the HCV life cycle and development of antivirals, *in vitro* models of this virus are indispensable. First, an HCV subgenomic replicon system was used to examine HCV replication in cell culture [5,6]. The HCV infectious step has been assessed by an HCV pseudo-particle (HCVpp) system harboring E1 and E2 glycoproteins [7,8]. This system enabled us to identify several HCV receptors. Finally, to investigate other steps in the HCV life cycle, an HCV cell culture system was developed with a unique genotype 2a strain, JFH-1 [9]. This strain is able to replicate efficiently in culture cells, and its characteristics enabled us to observe the whole life cycle of this virus in cell culture by using cell-culture generated HCV (HCVcc) [10–12].

By modifying this system with CD81-lacking HuH-7-derived cells, we established a novel system designated the single cycle virus production assay, and this enabled us to estimate the efficiency of each step of viral replication, infectious virus production, secretion and infection [13–16]. However, virus production levels of wild-type JFH-1 (JFH-1/wt) in these systems are limited, and this shortage sometimes leads to difficulties in experiments that require high viral concentrations. To overcome these shortcomings, recent studies have identified several adaptive or compensatory mutations that enhance viral production of JFH-1 [17–24]. The contributions of these mutations to the viral life cycle are not well defined. In this study, we isolated the cell culture-adapted JFH-1 virus, which that can efficiently produce progeny viruses by serial passaging of JFH-1 transfected Huh-7.5.1 cells, and evaluated the affected steps in the viral life cycle.

Materials and Methods

Cell Culture

The HuH-7-derived cell lines Huh-7.5.1, provided by Francis Chisari (Scripps Research Institute, La Jolla, CA), and Huh7-25, which lacks CD81 expression, were cultured at 37°C in a 5% CO2 environment using Dulbecco's Modified Eagle's Medium contain-

ing 10% fetal bovine serum [11,25]. 293T cells were also kept under the same conditions.

Plasmid Construction and RNA Transfection

Mutation-introduced JFH-1 variants were prepared by site-directed mutagenesis with appropriate primers. The methods of *in vitro* RNA synthesis and electroporation were described previously [26,27].

Quantification of HCV RNA and Core Antigen

Total RNA was extracted from 140 μL of culture medium or from harvested cell pellets, and the real-time quantitative RT-PCR was performed to determine the HCV RNA titer as described previously [28]. The concentration of total RNA in the cells was also measured. The concentration of HCV core antigen (Ag) in culture medium and cell lysates were measured by the Lumipulse Ortho HCV Ag kit (Ortho Clinical Diagnostics, Tokyo, Japan) [29].

Titration of HCV Infectivity

The infectivity titers of HCV were measured by indirect immunostaining as described previously [27]. The infectivity titer was expressed as focus-forming units (FFU) per mL. The intracellular infectivity and specific infectivity titer were determined as described previously [14].

HCV Pseudo-Particles Assay

HCV pseudo-particles (HCVpp) containing E1 and E2 glycoproteins of wild-type or mutation-introduced JFH-1 were produced as described previously [7,8]. To adjust the amount of virus, copy number of packaged luciferase reporter RNA was quantified by real-time detection PCR with primers and probe as reported previously [30].

HCV Trans-complemented Particles Assay

Generation and infection of HCV trans-complemented particles (HCVtcp) has been reported elsewhere [31–33]. Briefly, the RNA polymerase I-driven JFH-1 reporter replicon plasmid (pHH/SGR-Luc) and the CAG promoter-driven JFH-1 core – NS2 expression plasmid (pCAGC-NS2_JFH1) or T416N mutation in the E2 region introduced pCAGC-NS2/JFH1 (pCAGC-NS2_JFH1/T416N) were co-transfected into Huh-7.5.1 cells. Culture medium was harvested at 6 days after transfection, and was passed through a 0.45- μM pore-size filter for infection. To adjust the amount of virus, RNA in culture medium was extracted with the QIAamp Viral RNA kit, treated with DNase (TURBO DNase; Ambion, Austin, TX), and purified with an RNeasy Mini kit using on-column DNase digestion (QIAGEN). Copy number of HCV was then measured as described previously [34]. Generated viruses were infected into naïve Huh-7.5.1 cells, and cells were harvested at 72 h for analysis of luciferase activity.

HCV Sequencing

Total RNA was extracted from culture medium, and cDNA was synthesized using Superscript III (Invitrogen, Carlsbad, CA) with random 6-mer primer. Synthesized cDNA was subsequently amplified by nested-PCR covering almost the entire open reading frame and part of the 5′-untranslated region with TaKaRa LA *Taq* DNA polymerase (Takara Bio, Shiga, Japan), as described previously [14], and the sequence of amplified fragments was determined directly.

Density gradient analysis

The culture medium of JFH-1 and variants -transfected cells were layered on top of 10–40% iodixanol gradient and centrifuged for 16 h at 40,000 rpm, 4°C in an SW-41 rotor. Fractions were collected from the top of gradient, and the density, HCV core Ag and infectivity titer in each fraction was measured.

Statistical Analysis

Experiments were performed in triplicate, and obtained data are expressed as means ± standard deviation. Statistical analysis was performed by Student's t-test. The p values of less than 0.05 are considered to be statistically significant.

Results

Isolation of Cell Culture-adapted JFH-1

In order to obtain cell culture-adapted JFH-1, we passaged full-length JFH-1 RNA-transfected Huh-7.5.1 cells and monitored extra- and intra-cellular HCV RNA and infectivity of culture medium. At 25 days after transfection, HCV RNA and infectivity titer in culture medium peaked (Figure 1A). To assess cell-culture adaptation, we compared the progeny virus production levels by infection with the same amount of viruses harvested at day 5 (JFH-1/day5) and at day 25 (JFH-1/day25). The intra- and extra-cellular HCV RNA titers of JFH-1/day25-infected cells were $1.42\times10^7\pm3.49\times10^6$ copies/ μg RNA and $7.66\times10^7\pm3.61\times10^7$ copies/mL, respectively, which was 3 log-fold higher than those of JFH-1/day5-infected cells (Figure 1B).

Responsible Mutations in Cell Culture-adapted JFH-1 virus

In order to identify the responsible mutations introduced in the cell culture-adapted virus (JFH-1/day25), we directly sequenced the virus ORF. As indicated in Table 1, we identified 3 non-synonymous mutations at E2 (T416N), NS3 (K1122R) and NS5B (L2525F). To assess the effects of these mutations on HCV propagation, we generated the JFH-1 full-genome constructs with these mutations solely (JFH-1/T416N, JFH-1/K1122R and JFH-1/L2525F) or in combination (JFH-1/3mut). In the transfection assay with full-length HCV RNAs transcribed from these constructs, HCV core Ag in culture medium of JFH-1/K1122R and JFH-1/3mut transfected cells was approximately 1 log-fold higher than that of JFH-1/wt and other variants transfected cells (Figure 2A). HCV core Ag in cells was highest in JFH-1/3mut RNA-transfected cells, followed by JFH-1/K1122R. In the infection study of these variants (multiplicity of infection (MOI) = 0.1), the HCV RNA titer in culture medium of JFH-1/3mut infected cells was highest among these variants and JFH-1/wt, but was approximately 2 log-fold lower than that of cell culture-adapted JFH-1 virus, JFH-1/day25. The intra-cellular HCV RNA titer of culture-adapted JFH-1 virus infected cell was also higher than that of JFH-1/3mut or other variants infected cells (Figure 2B).

In order to assess the function of these mutations on steps of the virus lifecycle, we used the single cycle virus production assay using Huh7–25 cells, which lacks surface expression of CD81. We compared the intra-cellular HCV RNA titer of these variants transfected Huh7–25 cells in order to assess the effects of mutations on HCV replication. The intra-cellular HCV RNA titer of JFH-1/L2525F was lower than that of other variants and JFH-1/wt (Figure 3A). To assess the effects of mutations on infectious virus production in culture-cells and the efficiency of infection, we compared the specific infectivity of these variants in transfected Huh7–25 cells. The mutations K1122R and L2525F

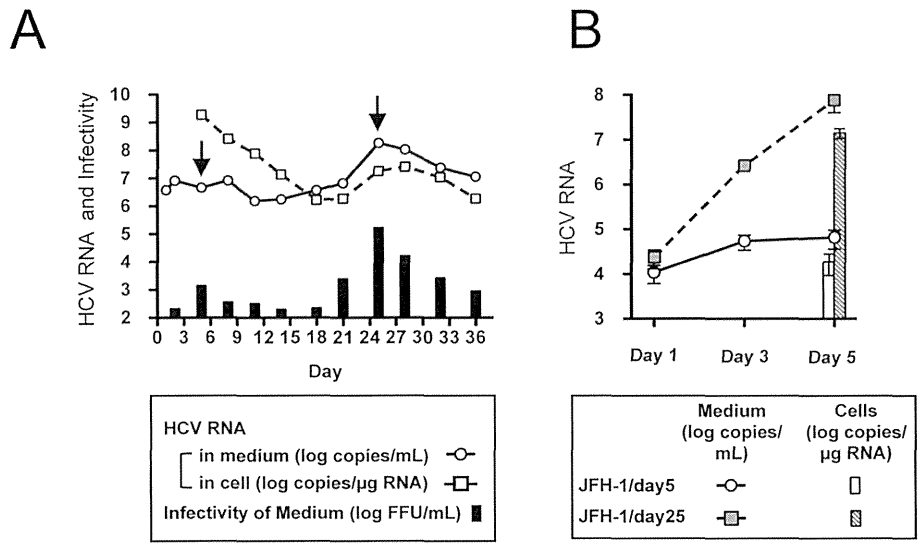

Figure 1. Isolation of cell culture-adapted JFH-1. (A) Long-term culture of JFH-1-transfected cells. The full-genome JFH-1 RNA was transfected into Huh-7.5.1 cells, and transfected cells were passaged for 3 to 4 days. HCV RNA titers in culture medium and cells were monitored. Arrows indicate the harvest points used for the infection study. (B) Production of progeny by infection with viruses harvested at day 5 and day 25 after transfection. The same amount of virus was used for infection at a multiplicity of infection (MOI) of 0.5, and HCV RNA titer was monitored.
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enhanced intra-cellular specific infectivity by 7.2- and 3.7-fold, respectively, although extra-cellular specific infectivity was not affected, thus suggesting their contribution to intra-cellular infectious virus production. JFH-1/3mut containing 3 mutations also showed an 8.5-fold increase in intra-cellular specific infectivity (Figure 3B). On the other hand, JFH-1/T416N-transfected cells showed 2-fold higher intra- and extra-cellular infectivity, as compared with JFH-1/wt and other variants. JFH-1/3mut also showed enhanced extra-cellular infectivity in addition to the effects of K1122R and L2525F (Figure 3B). These data suggest that

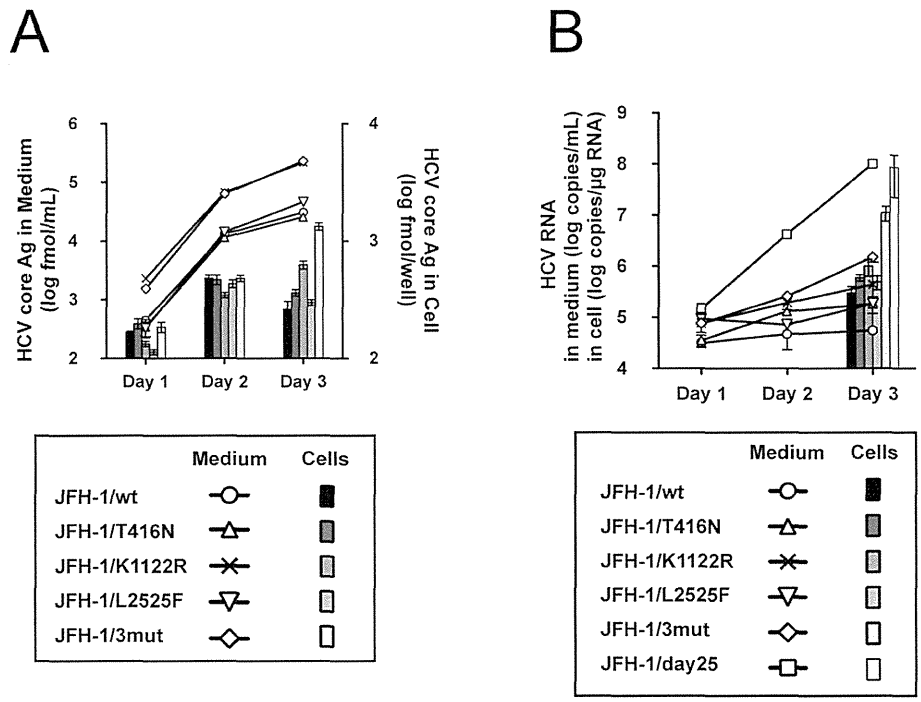

Figure 2. Effects of cell culture-adapted mutations on virus propagation. (A) One million cells were transfected with 2 μg of *in vitro*-transcribed RNA from JFH-1/wt, JFH-1/T416N, JFH-1/K1122R, JFH-1/L2525F and JFH-1/3mut. HCV propagation was monitored by measuring HCV core Ag. (B) The same amounts of JFH-1/wt, JFH-1/T416N, JFH-1/K1122R, JFH-1/L2525F, JFH-1/3mut and JFH-1/day25 viruses were used for infection of naïve Huh-7.5.1 cells (MOI = 1.0), and HCV RNA titers were subsequently monitored.
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Table 1. Mutations Detected in Cell Culture-adapted JFH-1 Variants.

Region	Identified mutation		JFH-1/day25	2G	6B
	Nucleotide	Amino Acid[a]			
E1	C1198T	–	+	+	+
E2	C1587A	T416N	+	+	+
p7	T2612G	L758V		+	
	T2641A	H767Q			+
NS3	A3631G	–			+
	A3705G	K1122R	+	+	+
	G3715A	–			+
	A4294G	I1318M			+
	C5182T	–			+
NS5A	G7069A	–			+
	G7658C	V2440L		+	+
NS5B	C7913T	L2525F	+	+	+
	G8458C	–		+	
	C8932T	–	+	+	+
	A9235G	–	+	+	+

[a]'–' means synonymous mutation.

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T416N enhances the infection step. To assess the biophysical properties of particles with T416N, we analyzed the culture medium of JFH-1/wt- and JFH-1/T416N- transfected cells in the density gradient. However, the density gradient profiles of these strains were similar and we could not detect the mutation specific peak of infectivity in the density gradient of JFH-1/T416N. The peak density of infectivity titer of JFH-1/T416N (1.05 g/mL) was almost identical with that of JFH-1/wt (1.07 g/mL), but the peak infectivity titer of JFH-1/T416N was 1.75-fold higher than that of JFH-1/wt (Figure 4). To confirm the advantage of T416N in the infection step, we exploited the HCVpp system. T416N was introduced into JFH-1 E1 and E2 glycoprotein-expressing vector and generated HCVpp harboring envelope proteins of JFH1/wt and JFH1/T416N. To adjust the amount of HCVpp, the copy number of packaged luciferase reporter RNA was measured. The same copy numbers of HCVpp JFH-1/wt and JFH-1/T416N were infected into naïve Huh-7.5.1 cells and luciferase activities were compared. In contrast to expectations, luciferase activity in JFH-1/T416N HCVpp-infected cells was lower than in JFH-1/wt HCVpp-infected cells (Figure 5A). We also examined the effects of T416N using the recently developed HCVtcp system. This HCVtcp contains the HCV subgenomic replicon and supports single-round infection. In contrast to the HCVpp system, we were able to observe consistent results with single cycle virus production assay in the HCVtcp system. Luciferase activity in JFH-1/T416N HCVtcp-infected cells was 2.8-fold higher than in JFH-1/wt HCVtcp-infected cells (Figure 5B).

Single Strain Isolation Method of Cell Culture-adapted Virus by End-point Dilution and Infection

In order to isolate the JFH-1 variants that can produce more progeny viruses, the JFH-1/day25 virus was diluted and infected into naïve Huh-7.5.1 cells seeded in a 96-well plate at a concentration of 1 FFU per well. After 72-h culture, media were kept in another plate and cells were fixed and stained with anti-HCV core antibody to visualize the foci. Culture media were then harvested from wells that contained a single focus and were used to re-infect naïve Huh-7.5.1 cells. The production of progeny viruses were compared by measuring the HCV RNA titer of infected cells (Figure 6). Inoculation with harvested media resulted in varied progeny virus production. HCV RNA titers in culture medium were 4 to 7 log copies/mL on day 3 after infection.

Among the assessed variants, we selected two strains, 2G and 6B, which showed the highest virus production. Direct sequencing of these isolated strains revealed that they possessed more non-synonymous mutations, in addition to those observed in JFH-1/3mut. The 2G strain had 2 additional mutations; L758V at p7 and V2440L at NS5A. The 6B strain had 3 additional mutations; H767Q at p7, I1318M at NS3 and V2440L at NS5A (Table 1). We generated JFH-1 variants with these mutations and designated them as JFH-1/2G and JFH-1/6B. When transfected with full-genome RNA, extracellular HCV core Ag was approximately 50-fold and 10-fold higher when compared with JFH-1/wt and JFH-1/3mut, respectively, and intracellular HCV core Ag was approximately 10-fold and 2-fold higher when compared with JFH-1/wt and JFH-1/3mut, respectively (Figure 7A).

In an infection study at MOI = 0.1, the variants JFH-1/2G and JFH-1/6B produced more progeny viruses than JFH-1/wt and JFH-1/3mut, and the production levels were comparable to that of JFH-1/day25 (Figure 7B). To assess the effects of additional introduced mutations in these variants on the virus life cycle, we used a single cycle virus production assay. After transfection of full-length RNA from JFH-1/wt, JFH-1/3mut, JFH-1/2G and JFH-1/6B into Huh7–25 cells, the intra-cellular HCV RNA titer was compared. We found that JFH-1/2G-transfected cells showed 1.35-fold higher intracellular HCV RNA titer, thus suggesting enhanced viral replication (Figure 8A).

Next, specific infectivities were compared. Consistent with previous data, JFH-1/3mut showed enhanced intra- and extra-cellular specific infectivity, as compared with JFH-1/wt, and was approximately 8-fold and 2-fold, respectively (Figure 8B). The strains JFH-1/2G and JFH-1/6B also indicated enhanced intra-cellular specific infectivity, 27.8- and 43.7-fold higher when

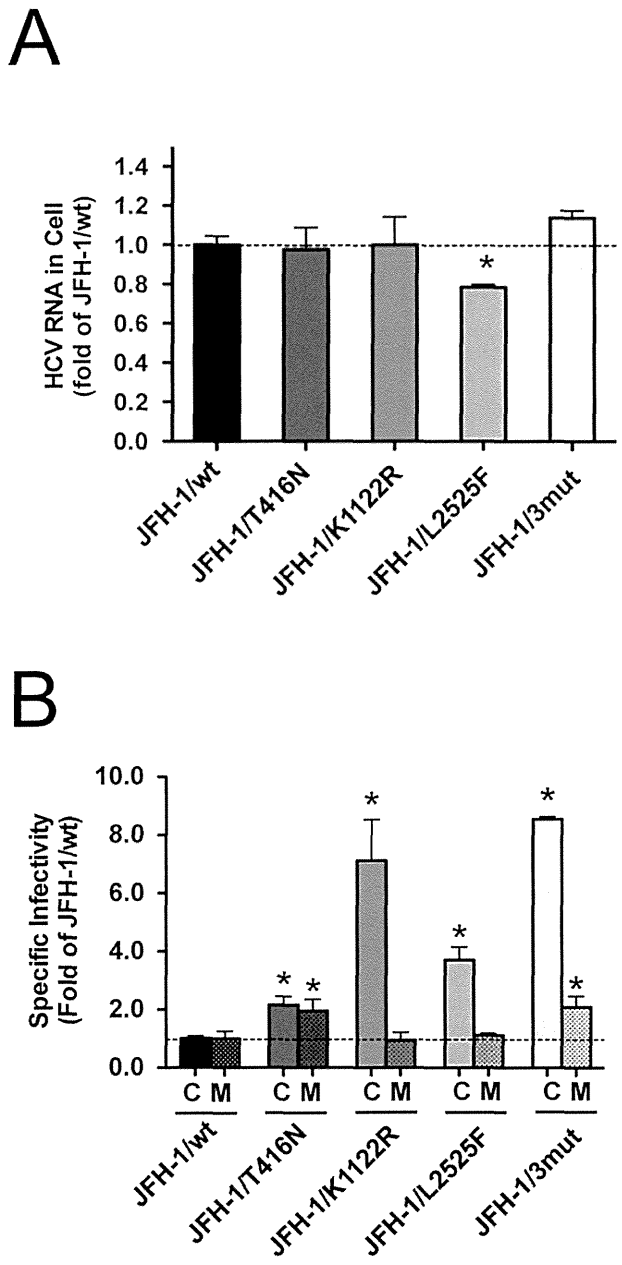

Figure 3. Single cycle virus production assay to assess *contribution of mutations on viral life cycle.* (A) Intra-cellular HCV RNA titers were assessed in full-length RNA of JFH-1/wt and its variants transfected into Huh7–25 cells. Data are given as fold change vs. JFH-1/wt. (B) Intra- and extra-cellular specific infectivity of JFH-1/wt and its variants transfected into Huh7–25 cells were calculated. Data are given as fold change vs. JFH-1/wt. C; intracellular specific infectivity, M; extracellular specific infectivity, *$p<0.05$.
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compared with JFH-1/wt, respectively (Figure 8A). These enhancements were much higher than in the case of JFH-1/3mut. These strains also showed enhanced extra-cellular specific infectivity (4.92- and 5.83-fold, respectively). To assess the biophysical properties of particles with strains of JFH-1/2G and JFH-1/6B, we analyzed the culture medium of these strains transfected cells in the density gradient (Figure 9). The density

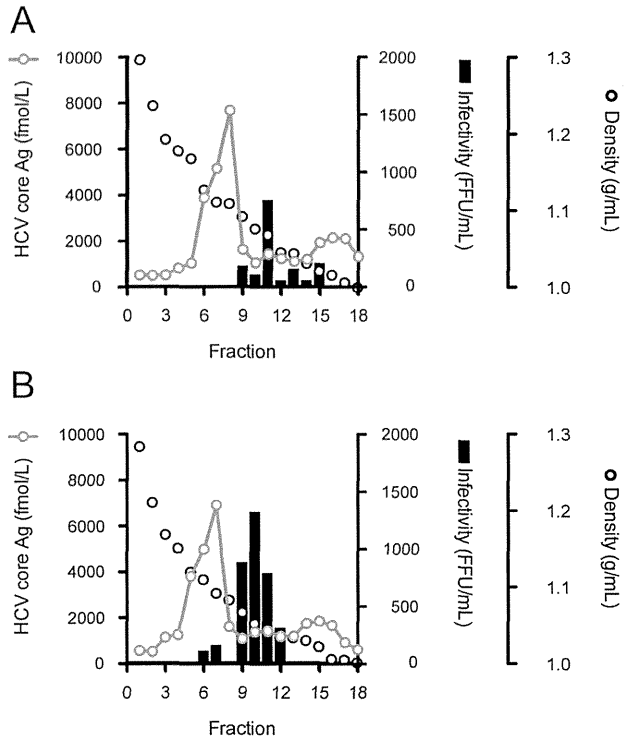

Figure 4. Iodixanol density gradient analysis of JFH-1/wt and JFH-1/T416N. Huh7.5.1 cells were transfected with full-length RNAs of JFH-1/wt and JFH-1/T416N. Culture medium of each strain was collected and analyzed by 10%–40% of iodixinol density gradient. Fractions were collected, and HCV core Ag and infectivity titers JFH-1/wt (A) JFH-1/T416N (B) were measured.
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gradient profiles of these strains were similar to that of JFH-1/wt. The peak densities of infectivity titer of JFH-1/2G (1.06 g/mL) and JFH-1/6B (1.05 g/mL) were almost identical with that of JFH-1/wt (1.07 g/mL), but the peak infectivity titers of these strains were 3 log-fold higher than that of JFH-1/wt.

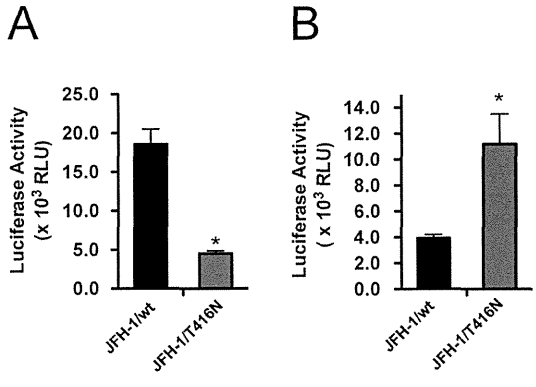

Figure 5. Assessment of T416N on infection step by HCVpp and HCVtcp. (A) Infectivity of JFH-1/wt and JFH-1/T416N with HCVpp envelopes was assessed. Luciferase activity was measured in HCVpp-infected cells. *$p<0.05$. (B) Infectivity of HCVtcp with the structural regions of JFH-1/wt and JFH-1/T416N was assessed. Luciferase activity was measured in HCVtcp-infected cells. *$p<0.05$.
doi:10.1371/journal.pone.0098168.g005

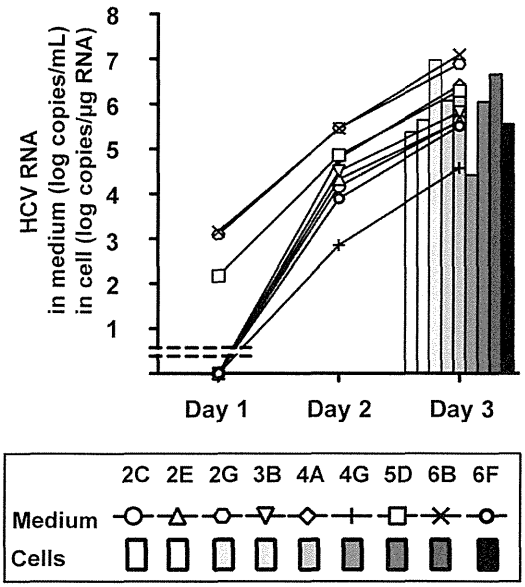

Figure 6. Production of progeny virus after infection of culture media harvested in wells that have a single focus. HCV RNA titers were measured in culture medium and cells.
doi:10.1371/journal.pone.0098168.g006

Discussion

In this study, we obtained cell culture-adapted virus by serial passage of full-length JFH-1 RNA-transfected Huh-7.5.1 cells for 25 days after transfection. The obtained virus produced 3 log-fold more progeny viruses as compared with JFH-1/wt infected the same amount of FFU. On sequence analysis of this cell culture-adapted virus, 3 amino acid mutations (T416N at E2, K1122R at NS3 and L2525F at NS5B) were identified. On assessment of JFH-1 constructs with these mutations, they were revealed to have advantages in various steps of the viral life cycle. Mutations in K1122R at NS3 and L2525F at NS5B are considered to contribute to efficient infectious virus production. The JFH-1 variants with each mutation, JFH-1/K1122R and JFH-1/L2525F, produced more infectious virus (7.2- and 3.7-fold, respectively), although K1122R slightly reduced intra-cellular viral replication. The mutation T416N at E2 is associated with enhancement of the infection step. JFH-1/T416N showed 2-fold higher efficiency of infection when compared with JFH-1/wt. T416 at E2 is located at the epitope of neutralizing monoclonal antibodies of AP33 and 3/11 and is conserved among genotypes [35–37]. The mutation at this site has been detected in cell culture-adapted J6/JFH-1 chimeric virus, but enhanced infectivity has not been observed in the mutation-introduced chimeric virus [20]. Moreover, substitution of T416A was reported to abolish infectivity of HCVpp with envelopes of H77 strain, genotype 1a [38]. In accordance with these data, we found that mutation T416N also reduces the infectivity in the HCVpp system. In contrast, we confirmed that this mutation enhances infectivity in HCVcc and HCVtcp systems. Such discrepancies between HCVpp and HCVcc on the HCV infection step have been reported previously [33]. Because HCVpp is generated in non-hepatic 293T cells, it is likely that the cell-derived components of HCVpp are different from those of HCVcc and HCVtcp. Thus, we believe that the HCVpp system does not reflect the characteristics of mutations in HCV envelopes and may not be suitable for assessing the effects of mutations in the HCV infection step. On the other hand, the

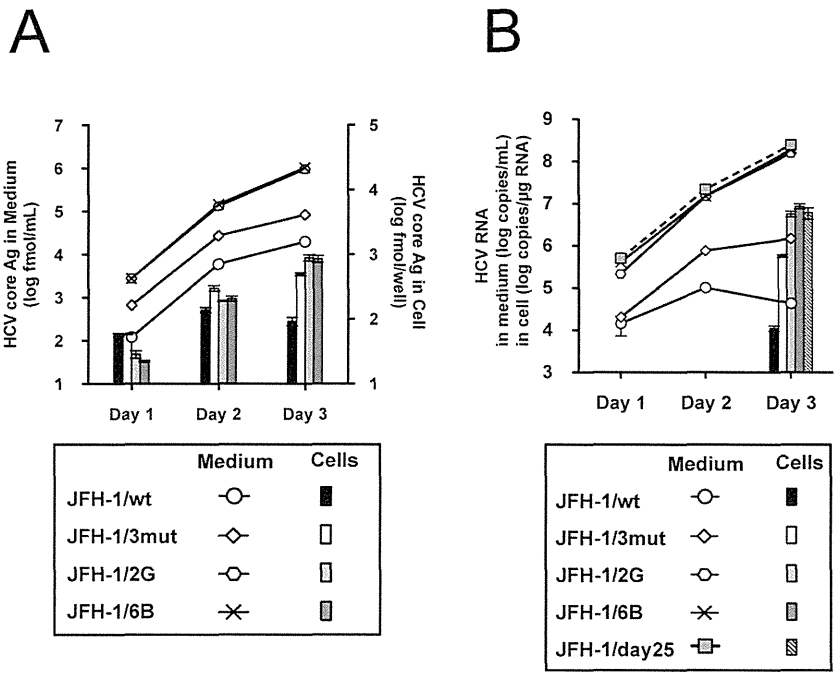

Figure 7. Effects of mutations detected after single-strain isolation method. (A) One million cells were transfected with 2 μg of *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/3mut, JFH-1/2G and JFH-1/6B. HCV propagation was monitored by measuring HCV core Ag. (B) The same amount of JFH-1/wt, JFH-1/3mut, JFH-1/2G, JFH-1/6B and JFH-1/day25 viruses were infected into naïve Huh-7.5.1 cells (MOI = 1.0), and HCV RNA titers were monitored.
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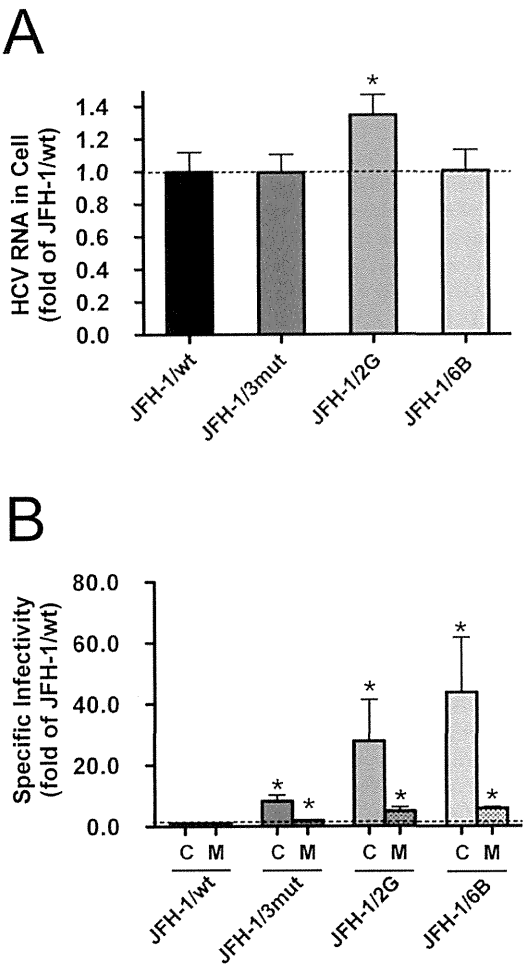

Figure 8. Single cycle virus production assay to assess the contribution of introduced mutations on viral life cycle. (A) Intra-cellular HCV RNA titers were assessed in full-length RNA of JFH-1/wt-, JFH-1/3mut-, JFH-1/2G- and JFH-1/6B-transfected Huh7–25 cells. Data are presented as fold change vs. JFH-1/wt. (B) Intra- and extracellular specific infectivity of JFH-1/wt-, JFH-1/3mut-, JFH-1/2G- and JFH-1/6B-transfected Huh7–25 cells were calculated. Data are given as fold change vs. JFH-1/wt. C; intracellular specific infectivity, M; extracellular specific infectivity, $*p<0.05$.
doi:10.1371/journal.pone.0098168.g008

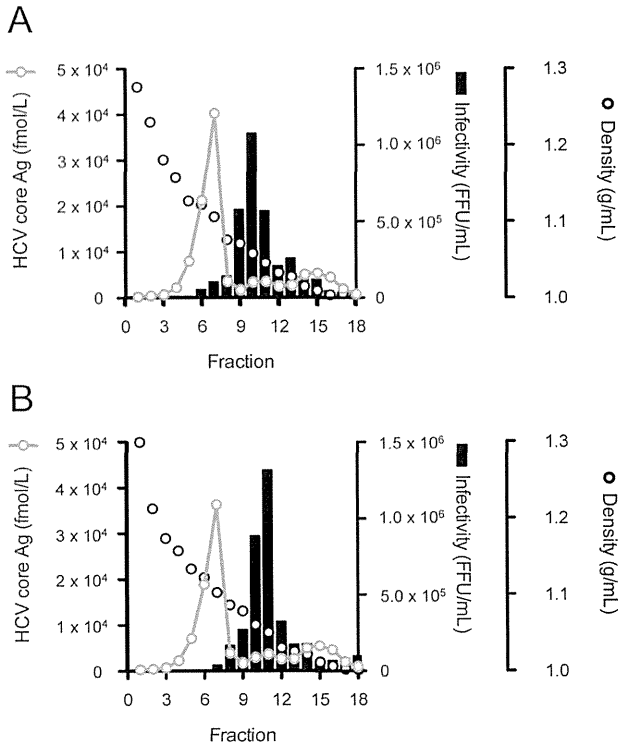

Figure 9. Iodixanol density gradient analysis of JFH-1/2G and JFH-1/6B. Huh7.5.1 cells were transfected with full-length RNAs of JFH-1/2G and JFH-1/6B. Culture medium of each strain was collected and analyzed by 10%–40% of iodixinol density gradient. Fractions were collected, and HCV core Ag and infectivity titers of JFH-1/2G (A) and JFH-1/6B (B) were measured.
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T416N mutation in the HCVtcp system indicated consistent data with HCVcc. Therefore, we conclude that this mutation enhances the infectivity of HCV.

The variant JFH-1/3mut with all three mutations, T416N, K1122R and L2525F, has advantages in infectivity and infectious virus production, and results in the highest efficiency of progeny virus production. However, in the infection study, this JFH-1/3mut could not reach the virus production level of the obtained cell culture-adapted JFH-1 virus, JFH-1/day25. We were puzzled by this, and speculated that JFH-1/day25 was not monoclonal, as the direct sequence method is unable to identify responsible mutations associated mixed viruses. Thus, we exploited another strategy. We isolated a single JFH-1 variant that can produce more progeny virus, and we used a method for single virus isolation by infection with a diluted mixture of cell culture-adapted virus. By infection with cell culture-adapted virus at a concentration of 1 FFU/well, we were able to isolate two variants that showed the highest virus production among the tested strains.

The isolated variants, 2G and 6B strains, have additional mutations at p7, NS3 and NS5A. A reverse-genetics analysis revealed that these variants could produce progeny virus more efficiently than JFH-1/wt and JFH-1/3mut after transfection with full-length RNA. In the infection study, the production of progeny virus of these variants was also superior to the levels of JFH-1/wt and JFH-1/3mut, and was comparable to JFH-1/day25. In order to identify the advantages of these variants in the virus life cycle, we used the single cycle virus production assay. JFH-1/2G was able to replicate 1.35-fold more efficiently in culture cells. Both strains have advantages in the steps of infectious virus production and infection. The intra-cellular specific infectivity of JFH-1/2G and JFH-1/6B was 27.8- and 43.7-fold higher, and the extracellular specific infectivity was 4.92- and 5.83-fold higher than that of JFH-1/wt. This suggests that enhancement of infectious virus production is a major advantage in these strains. These strains included the additional adaptive mutation V2440L. We examined this sequence in JFH-1/day25 retrospectively, and found a mixture of nucleotide G/C at nucleotide 7658 (data not shown). Thus, there may be many strains with mutations other than V2440L and they are able to propagate efficiently as like as clones, JFH-1/2G and 6B, but we might not be able to isolate such strains in this experiment. This mutation, V2440L, has already been reported in cell culture-adapted JFH-1 virus, and to contribute to slow cleavage at the NS5A-NS5B site, increasing the production of

infectious virus [17]. The ability of efficient virus production of JFH-1/2G and JFH-1/6B may be attributable to this mutation.

In conclusion, we were able to successfully isolate 2 cell culture-adapted variants that can produce 3 log-fold more progeny viruses than JFH-1/wt, and identified the responsible mutations. The strategy of single virus isolation by end-point dilution and infection used in this study may be useful for identifying strains with unique characteristics, such as robust virus production, from diverse populations, and for identifying the responsible mutations for these characteristics.

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Author Contributions

Conceived and designed the experiments: TJL TW TK. Performed the experiments: NS AM RS NW MS TK. Analyzed the data: NS AM TK. Wrote the paper: NS AM MS TK.

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CASE REPORT

Acute hepatitis B of genotype H resulting in persistent infection

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Abstract

A 47-year-old man presented with general fatigue and dark urine. The laboratory data showed increased levels of hepatic transaminases. The patient was positive for hepatitis B virus (HBV) markers and negative for anti-human immunodeficiency virus. The HBV-DNA titer was set to 7.7 log copies/mL. The patient was diagnosed with acute hepatitis B. The HBV infection route was obscure. The serum levels of hepatic transaminases decreased to normal ranges without any treatment, but the HBV-DNA status was maintained for at least 26 mo, indicating the presence of persistent infection. We isolated HBV from the acute-phase serum and determined the genome sequence. A phylogenetic analysis revealed that the isolated HBV was genotype H. In this patient, the elevated peak level of HBV-DNA and the risk alleles at human genome single nucleotide polymorphisms s3077 and rs9277535 in the human leukocyte antigen-DP locus were considered to be risk factors for chronic infection. This case suggests that there is a risk of persistent infection by HBV genotype H following acute hepatitis; further cases of HBV genotype H infection must be identified and characterized. Thus, the complete determination of the HBV genotype may be essential during routine clinical care of acute hepatitis B outpatients.

Key words: Acute hepatitis; Chronic hepatitis; Genotyping; Hepatitis B virus; Single nucleotide polymorphisms

Core tip: Hepatitis B virus (HBV) genotype H infection is rare in Asia, particularly in Japan. Here, we report a case of acute hepatitis B caused by a genotype H strain with persistent infection, although most adult cases of acute hepatitis B are self-limiting in Japan. This case suggests that the HBV genotype H infection can be a risk factor for persistent infection. Therefore, it is necessary to investigate the characteristics of genotype H infection in an accumulation of cases. Thus, the

complete determination of the HBV genotype may be essential in the routine clinical care of acute hepatitis B patients.

Yamada N, Shigefuku R, Sugiyama R, Kobayashi M, Ikeda H, Takahashi H, Okuse C, Suzuki M, Itoh F, Yotsuyanagi H, Yasuda K, Moriya K, Koike K, Wakita T, Kato T. Acute hepatitis B of genotype H resulting in persistent infection. *World J Gastroenterol* 2014; 20(11): 3044-3049 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i11/3044.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i11.3044

INTRODUCTION

Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV); it represents a major global health problem. HBV can cause chronic liver diseases and increases the risk of death from cirrhosis and liver cancer. Worldwide, an estimated two billion people have been infected with HBV and more than 240 million have chronic infections[1]. The HBV genome consists of approximately 3200-nucleotides of DNA; the virus replicates using a reverse transcriptase enzyme that lacks proofreading ability. Therefore, HBV possesses diverse genetic variability, and the viral population is classified into at least eight genotypes that are designated A -H[2-6]. In Japan, genotypes B and C are prevalent among patients with chronic infections. However, in the last decades, the prevalent genotype in acute HBV infections has shifted from genotype C to A[7-9]. There are some differences in the clinical features and outcomes among the genotypes[10-13]. It has been reported that the persistent infection from acute hepatitis is prevalent in adults that are infected with genotype A HBV. Thus, determining the HBV genotype is of increasing importance even in routine clinical practice, although a reliable kit for determination of all HBV genotypes is still uncommon and is not yet covered by insurance. The host factors associated with persistent infection by HBV have also been reported, such as single nucleotide polymorphisms (SNPs) or genotypes in the human leukocyte antigen-DP locus. It may also be useful for identifying the patients who are prone to develop chronic hepatitis.

In this report, we describe a case of acute hepatitis B resulting from infection by a genotype H strain of HBV. Although the laboratory data and symptoms were not distinguishable from acute hepatitis B with other genotypes, this patient developed persistent infection.

CASE REPORT

A 47-year-old man living in Kawasaki, Japan, presented at our hospital with general fatigue and dark urine. Approximately 1 wk before visiting the hospital, the patient developed nausea, loss of appetite, and a feeling of fullness in the abdomen. Four days later, he noted darkening of his skin and urine. Upon admission, the patient's laboratory data revealed elevated serum aspartate aminotransferase, alanine aminotransferase (ALT), lactate dehydrogenase, alkaline phosphatase, γ-glutamylt ranspeptidase, and total bilirubin (T-Bil) levels (Table 1). The prothrombin activity was within the normal range (95%). Test for hepatitis B surface antigen (HBsAg; HISCL-2000i, Sysmex, Kobe, Hyogo, Japan), hepatitis B e-antigen (HBeAg; ARCHITECT® CLIA, Abbott Japan, Tokyo, Japan) and anti-hepatitis B core antigen (anti-HBc) IgM (ARCHITECT® CLIA) were positive. A test for HBV-DNA was also positive, exhibiting a titer of 7.7 log copies/mL (COBAS TaqMan HBV Test v2.0, Roche Diagnostics, Tokyo, Japan). HBsAg had not been detected 2 years previously when the patient had been admitted to another hospital for treatment of acute enterocolitis. Other hepatitis virus markers were negative. Therefore, the patient was diagnosed with acute hepatitis B. The genotype of the infecting HBV, as assessed by the Immunis HBV Genotype Immunis® HBV Genotype EIA Kit (Institute of Immunology, Tokyo, Japan), was determined as genotype C. The patient had not been abroad in the past 12 mo; he had no history of receiving blood or blood-related products, transfusions, or drug injections, and he reported no personal or family history of liver disease. The man was unmarried and declared that he was heterosexual, with no history of sexual contact with commercial sex workers or strangers. Anti-human immunodeficiency virus (HIV) was not detected. In the absence of medication, the patient's condition and elevated ALT level improved within a month. Anti-HBe became detectable, and HBeAg disappeared 2 mo after onset of the symptoms. HBsAg became undetectable at 5 mo, but the patient still tested positive for HBV-DNA, a status that persisted for at least 26 mo following his presentation at our hospital (Figure 1). We are now preparing to administer anti-viral medication.

For further analysis of the HBV infecting this patient, HBV-DNA was extracted from the acute-phase serum using a QIAamp DNA Blood Mini kit (QIAGEN, Valencia, CA). The entire HBV genome sequence was determined after polymerase chain reaction (PCR) amplification using the following primers [the number of nucleotides (nt) added to the primers were deduced from the prototype HBV/C clone, with accession no. AB246344]. For the amplification of half of the HBV genome, the outer primers were 5'-ATTCCACCAAGCTCTGCTAGATCCCAGAGT-3' (nt 10-39) and 5'-GGTGCTGGTGAACAGACCAATTTATGCCTA-3' (nt 1813-1784), and the inner primers were 5'-CCTATATTTTCCTGCTGGTGGCTCCAGTTC-3' (nt 46-75) and 5'-TAGCCTAATCTCCTCCC CCAACTCCTCCCA-3' (nt 1760-1731). For the other half of the HBV genome, the outer primers were 5'- ACGTCGCATGGAGACCACCGTGAACGCCCA-3' (nt 1601-1630) and 5'-AAGTCCACCACGAGTCTAGACTCTGTGGTA-3' (nt 266-237), and the inner primers were 5'-CCAGGTCTTGCCCAAGGTCTTACATAAGAG-3' (nt 1631-1660) and 5'-CCCGCCTGTAACACGAGCAGGGGTCCTAGG-3' (nt 207-178). The PCR was performed in a thermal cycler for 30 cycles

Table 1 Laboratory findings at first visit to our hospital

Hematology		Blood chemistry		Viral markers		Immunology		Coagulation	
WBC	7400/μL	TP	7.4 g/dL	Anti-HA IgM	(-)	IgA	183 mg/dL	PT%	95%
Neutrophil	72.0%	Albumin	4.5 g/dL	Anti-HCV	(-)	IgG	1168 mg/dL	APTT	36.4 s
Eosinophil	1.0%	T-Bil	11.1 mg/dL	HBsAg	(+) 197333	IgM	220 mg/dL		
Basophil	0.0%	D-Bil	8.0 mg/dL	Anti-HBc IgM	(+) 25.5 C.O.I	ANA	× 40, homogeneous		
Monocyte	10.0%	AST	1942 IU/L	HBeAg	(+) 253 C.O.I				
Lymphocyte	17.0%	ALT	2963 IU/L	Anti-HBe	(-) 0.0 %				
RBC	457/μL	ALP	612 IU/L	HBV-DNA	7.7 log copies/mL				
Hemoglobin	16.0 g/dL	γGTP	756 IU/L	Anti-HIV	(-)				
Hematocrit	46.4%	LDH	739 IU/L	RPR	(-)				
Platelet	36.6×10^4/μL	BUN	8.2 mg/dL	TPHA	(+)				
		Creatinine	0.64 mg/dL	Anti-CMV IgG	(+)				
		T-Chol	225 mg/dL	Anti-CMV IgM	(-)				
				Anti-EBV EBNA	(+)				
				Anti-EBV EA IgG	(-)				
				Anti-EBV VCA IgG	(+)				
				Anti-EBV VCA IgM	(-)				

WBC: White blood cells; RBC: Red blood cells; ANA: Antinuclear antibody; TP: Total protein; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; γGTP: γ-glutamyltranspeptidase; LDH: Lactase dehydrogenase; BUN: Blood urea nitrogen; T-Chol: Total cholesterol; PT: Prothrombin activity; APTT: Activated partial thromboplastin time; C.O.I: Cutoff index; HA: Hepatitis A; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; HBc: Hepatitis B core; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; RPR: Rapid plasma regain; TPHA: Treponema pallidum hemagglutination assay; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; EBNA: Epstein-Barr virus nuclear antigen; EA: Early antigen; VCA: Viral capsid antigen.

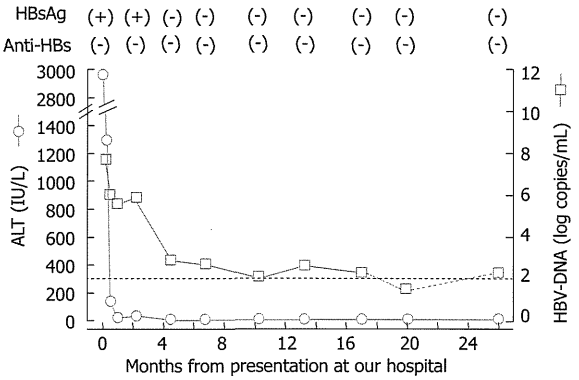

Figure 1 Clinical course of the patient infected with the genotype H strain. The dotted line indicates the detection limit of HBV-DNA (2.1 log copy/mL); the titer of the HBV-DNA was below the lower limit at 18 mo. HBsAg: Hepatitis B surface antigen; Anti-HBs: Antibody to hepatitis B surface antigen; ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

(94 ℃, 30 s; 60 ℃, 30 s; 72 ℃, 30 s) with TAKARA LA Taq® DNA polymerase (TAKARA, Shiga, Japan). The amplified fragments were sequenced directly with an automated DNA sequencer (3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, United States).

The genome of the infecting HBV (designated as B-MHJ9014) was 3215 bases in size. A phylogenetic analysis was performed with this strain and several database reference strains. B-MHJ9014 sorted with the genotype-H branch of the tree and clustered with the genotype-H strains previously isolated from Japanese patients (Figure 2). The substitutions at nt 1762 and nt 1764 (the basal core promoter region) and at nt 1896 (the precore region) were not observed. The length of the deduced amino acid sequences of the S, X, Core, and P proteins were identical to those encoded by other genotype H strains in the databases. The α determinant region of the S protein of B-MHJ9014 harbored an amino acid polymorphism (phenylalanine to leucine) at residue 134. The predicted B-MHJ9014 reverse transcriptase did not include any of the amino acid substitutions known to be associated with nucleotide analog resistance. To assess the complexity of the infecting virus, S region sequences from 51 clones in acute phase serum were determined. The detected sequences were genotype H and were closely related to the consensus sequence determine by direct sequencing with 1-3 amino acids polymorphisms (data not shown).

To assess the presence of human genome SNPs in the HLA-DP locus that are associated with persistent infection by HBV[14,15], a blood specimen was obtained from the patient (who had previously provided informed consent). Genomic DNA was extracted from buffy coat samples with the QIAamp DNA Mini kit (QIAGEN); DNA for SNPs rs3077 and rs9277535 were amplified with the appropriate primers and TAKARA LA Taq® DNA polymerase and were sequenced directly. The patient was homozygous (G/G) at both of these SNPs; these alleles are considered to be risk alleles for persistent infection.

DISCUSSION

HBV genotype H was first reported in 2002[5]. Infections by this genotype have been found mainly in Nicaragua, Mexico, and California; this genotype is considered to be rare in Asia, particularly in Japan[5,16-18]. However, since the first recognition of genotype H in Japan in 2005, eight strains have been isolated from Japanese patients (Table 2)[18-25]. All reported genotype H strains were isolated from male patients aged 35 to 65 years old, and the major route of infection was sexual transmission (5/8, 62.5%). Four cases (50%) represent transmissions that

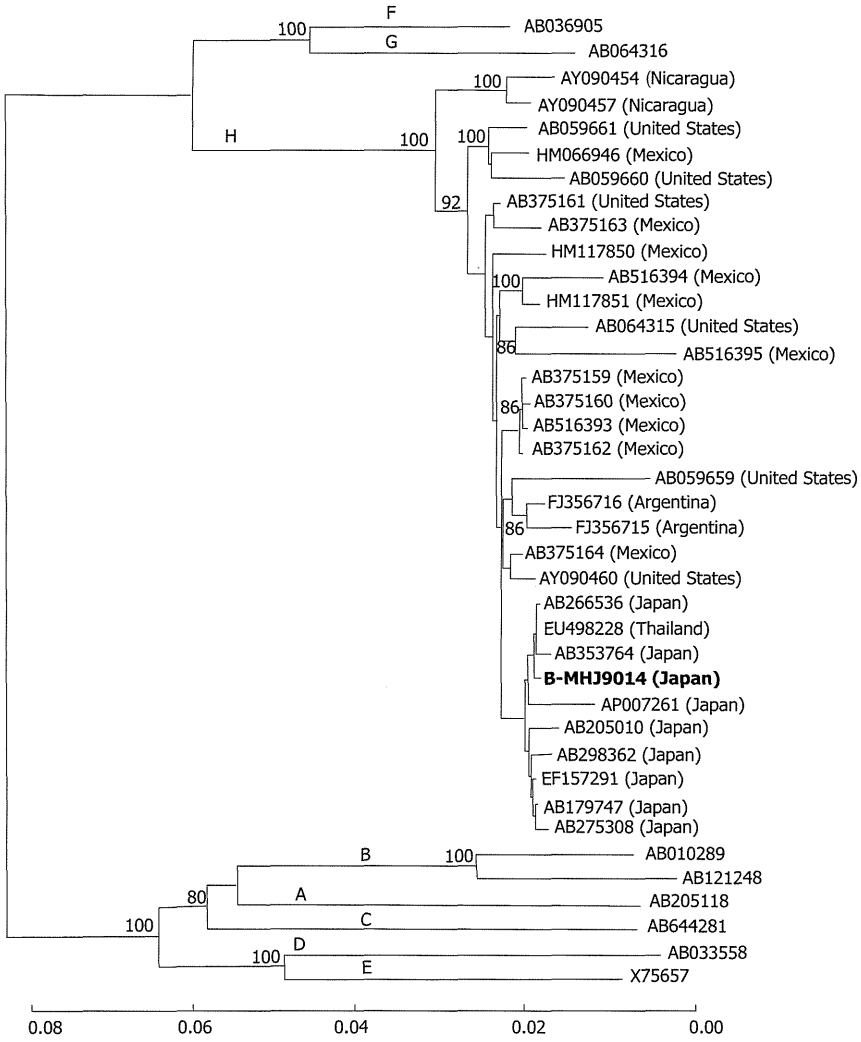

Figure 2 A phylogenetic trees constructed using the neighbor-joining method with the full hepatitis B virus genome sequence of the isolated and reference strains. The strain isolated in this case (B-MHJ9014) is shown in bold. The horizontal bar indicates the number of nucleotide substitutions per site. The reference sequences are shown with the DDBJ/EMBL/GenBank accession numbers. The HBV genotypes are indicated on each branch. The bootstrap values (> 80%) are indicated at the nodes as a percentage of the data obtained from 1000 resamplings. HBV: Hepatitis B virus.

Table 2 Genotype H strains reported in Japan

No.	Patient		Hypothesized source of infection		HIV infection[1]	Clinical feature	Accession number (Ref.)
	Age	Gender	Route	Place			
1	52	Male	Unknown	Japan	NA	Unknown blood donor	AB179747, [18]
2	61	Male	Sexual contact (heterosexual)	Thailand	NA	Chronic	AB205010, [19]
3	46	Male	Sexual contact (bisexual)	South America	(+)	Chronic	AP007261, [20]
4	38	Male	Sexual contact (homosexual)	Unknown	NA	Chronic	AB298362, [21]
5	65	Male	Unknown	Japan	NA	Acute	EF157291, [22]
6	35	Male	Unknown	Japan	NA	Acute	AB266536, [23]
7	60	Male	Sexual contact (homosexual)	Japan	(-)	Acute	AB275308, [24]
8	60	Male	Sexual contact (heterosexual)	Unknown	(+)	Chronic	AB353764, [25]
9	47	Male	Unknown	Japan	(-)	Acute to chronic	AB846650, this paper

[1]NA: Not available; HIV: Human immunodeficiency virus.

occurred in Japan. Co-infection with HIV was not common (2/8, 25%). These characteristics were similar to the case described here. All isolated strains from Japanese patients clustered together as a branch on the phylogenetic tree; therefore, it is possible that a specific strain of genotype H has emerged and spread in Japan. Presumably, the infrequent use of a reliable and convenient detection kit for genotype H infection has hampered the

correct diagnosis of genotype H infection; some cases may be misdiagnosed and considered to be infections by other genotypes. In fact, in the current case, our HBV isolate was originally identified as genotype C by the commercial kit that is covered by insurance in Japan. This kit was developed before the discovery of genotype H; thus, such a misidentification is a potential risk, as noted in the kit's instruction manual. The clinical features of genotype H infection remain obscure. There is a growing need for an accumulation of genotype H infection cases. To this end, the use of a reliable HBV genotyping kit that can correctly distinguish all genotypes is essential for routine clinical practice.

In Japan, most cases of acute hepatitis B are self-limiting, but some cases have been reported to have progressed to persistent infections[9,26-29]. Among the reported cases of genotype H infection, 4 strains were isolated from chronic hepatitis patients; in all cases, the infection was ascribed to sexual contact (Table 2)[19-21,25]. In our case, the HBV-DNA persisted for at least 26 mo. To our knowledge, this report represents the only case of genotype H infection in which chronic hepatitis was observed following acute infection. HBsAg was no longer detected at 4 mo from onset by HISCL-2000i. This disappearance was also confirmed by ARCHITECT® HBsAg (CMIA, Abbott Japan, Tokyo, Japan). In the S protein analysis, we found an amino acid polymorphism in the α determinant region. This polymorphism may affect the sensitivity for detecting HBsAg. HIV infection, a well-known risk factor for prolonged HBV infection[30], was not detected in our patient. Recently, the risk factors for HBV persistent infection have been reported in an analysis of a cohort that excluded patients co-infected with HIV[29]. In that report, infection with genotype A, elevated peak levels of HBV-DNA, and attenuated peak levels of ALT were suggested as risk factors for chronic infection. In the case described here, the peak level of HBV-DNA was 7.7 log copy/mL, which was consistent with increased risk for chronic infection. However, our patient exhibited a peak level of ALT of 2963 IU/L, which is a value that would classify this individual in the self-limiting group. Therefore, the clinical features of this case did not completely fit the risk factors associated with the establishment of chronic infection in the previous analysis[29]. Another reported risk factor for chronic HBV infection is the presence of certain SNP alleles. Specifically, selected SNPs around the HLA-DP locus have been reported to be associated with chronic hepatitis B in Asians[14,15]. With the informed consent of our patient, we determined the sequences for these SNPs (rs3077 and rs9277535) and found that this patient harbored risk alleles at both polymorphisms. This factor may have contributed to the establishment of chronic infection in this case.

In conclusion, we report a case of acute hepatitis B caused by a genotype H strain of HBV. This patient exhibited persistent infection. Our finding suggests that the infection of HBV genotype H can be a risk factor for persistent infection. We believe that it is necessary to use kits that are capable of accurate genotyping to permit an accumulation of cases and to investigate the clinical features of genotype H infection in routine clinical practice.

COMMENTS

Case characteristics

The main symptoms were nausea, loss of appetite, and a feeling of fullness in the abdomen.

Clinical diagnosis

The patient was a case of acute hepatitis B caused by a genotype H strain with persistent infection.

Differential diagnosis

The hepatitis B virus (HBV) genotype was considered to be important to predict the outcome and clinical features.

Laboratory diagnosis

To diagnose this patient, the detection of HBV markers and the complete determination of the HBV genotype were essential.

Treatment

The anti-viral treatment was not administered because we expected this case was self-limiting. Authors are now preparing medication.

Experiences and lessons

The infection of HBV genotype H can be a risk factor for persistent infection and the complete determination of HBV genotype is important.

Peer review

To conclude the association between HBV genotype H and chronic infection, the accumulation of cases of genotype H infection is essential.

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HCV Infection Enhances Th17 Commitment, Which Could Affect the Pathogenesis of Autoimmune Diseases

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Abstract

Background: Various kinds of autoimmune diseases have been reported to have a significant relationship with persistent hepatitis c virus (HCV) infection and Th17 cells. Previously, our group reported that the existence of HCV in T lymphocytes could affect the development of $CD4^+$ helper T cells and their proliferation, in addition to the induction of immunoglobulin hyper-mutation.

Methods: Therefore, we analyzed the relationship between persistent infection of HCV and the mechanism of Th17 cell induction *ex vivo* and *in vitro*.

Results: The prevalence of autoimmune-related diseases in chronic hepatitis c patients (CH-C) was significantly higher than in other types of chronic hepatitis (hepatitis B and NASH). A significantly higher frequency of IL6 and TGF-β double-high patients was detected in CH-C than in other liver diseases. Moreover, these double-high patients had significantly higher positivity of anti-nuclear antibody, cryoglobulinemia, and lymphotropic HCV and higher amounts of IL1-β, IL21, IL23. In addition to the previously reported lymphotropic SB-HCV strain, we found a novel, genotype 1b lymphotropic HCV (Ly-HCV), by deep sequencing analysis. Lymphotropic-HCV replication could be detected in the lymphoid cells with various kinds of cytokine-conditions including IL1β, IL23, IL6 and TGF-β in vitro. Infection by HCV could significantly enhance the development of Th17 cells. The HCV protein responsible for inducing the Th17 cells was HCV-Core protein, which could enhance the STAT-3 signaling and up-regulate the expression of RORγt as a Th17 master gene.

Conclusion: Infection by lymphotropic HCV might enhance the Th17 development and contribute to understanding the pathogenesis of autoimmune-related diseases.

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Introduction

Cellular and humoral immune responses to HCV play an important role in the pathogenesis of chronic hepatitis, HCC and B-lymphocyte proliferative disorders including mixed cryoglobulinemia, a disorder characterized by the oligoclonal proliferation of B cells [1–5]. B cell activation and/or dis-regulation could originate as a result of HCV binding to CD81 tetraspanin molecule or as a consequence of its ability to replicate in B lymphocytes[6]. It has been reported that HCV could infect B lymphocytes[7–9]. We previously reported that HCV-replication in B lymphocytes could induce immunoglobulin hypermutation and reduce the affinity and neutralizing activities of antibodies against HCV envelope protein[5]. On the other hand, the hypermutation of immunoglobulin might induce autoantibodies that contribute to the immunopathogenesis of autoimmune diseases, since various kinds of autoimmune diseases were reported to have a significant relationship with persistent HCV infection [10–12].

Previously, our group reported that the existence of HCV in T lymphocytes could affect the development and proliferation of type 1 T helper (Th1) cells[3,4,13]. Other groups have also reported the existence of HCV in T lymphocytes[14,15]. HCV replication in T lymphocytes could suppress Interferon-γ (IFN-γ)/signal transducers and activators of transcription factor 1 (STAT-1) signaling that might affect signal transducers and activators of transcription factor 3 (STAT-3) signaling[4,13].

It has been reported that a subset of type 17 T helper (Th17) cells might be involved in various kinds of autoimmune diseases[16–19]. The orphan nuclear receptor RORγt (RORγt) is the key transcription factor that induces the transcription of the genes encoding Interleukin (IL)-17 in naïve $CD4^+$ T helper cells[20]. Moreover, the activation of STAT-3 signaling could contribute to the induction of Th17 development[21–23]. Previously, Machida et al. reported that HCV replication in B lymphocytes could enhance the production of IL-6 from B lymphocyte[24]. In addition to TGF-β1, the existence of IL-6 could enhance the development of Th17 cells. IL17A-producing T lymphocytes have been recently shown to comprise a distinct lineage of pro-inflammatory T helper cells, termed Th17 cells, that are major contributors to autoimmune disease[20]. IL17A stimulates the secretion of a wide range of proinflammatory chemokines and cytokines. As its receptor is widely expressed, various kinds of immune cells as well as other cell types can respond to it[25]. Recently, we reported that the frequency of Th17 cells was remarkably high in a difficult-to-treat case of pyoderma gangrenosum-like lesion in a patient with lymphotropic HCV infection[26].

In this study, we clarified the relationship between Th17 cells and the biological significance of lymphotropic HCV.

Material and Methods

Study design and Patients

Two hundred-fifty patients with HCV persistent infection who were treated in Tohoku University Hospital were enrolled in this study. None of the patients had liver disease due to other causes, such as alcohol, drug, or congestive heart failure. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2006–194) following ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all the participants enrolled in this study. Participants were monitored for 6 months and peripheral blood samples were obtained from selected patients. We collected the peripheral blood before the treatment (treatment naïve). The concurrent diseases were diagnosed by specialized physicians belonging to the department of hematology and rheumatology. Patients were evaluated for serum levels of HCV-RNA, blood chemistry and hematology.

Quantification of IL1β, IL6, Transforming growth factor 1 (TGF-β1) and IL17A, IL21, IL23 in the serum

The amounts of IL1β, IL6, TGF-β1, IL17A, IL21 and IL23 were quantified using IL1β, IL6, TGF-β, IL17A, IL21 and IL23 enzyme-linked immunosorbent assay (ELISA) kits (eBioscience). The serum samples from patients were collected at sampling points and stored at −20°C. The ELISA procedure was performed according to the manufacturer's protocol.

Isolation of peripheral blood mononuclear cells (PBMCs), $CD4^+$ cells, $CD19^+$ cells and $CD45RA^+$ naïve $CD4^+$ cells

PBMCs were isolated from fresh heparinized blood by means of Ficoll-Paque (Amersham Bioscience) density gradient centrifugation. $CD4^+$ T cells and $CD19^+$ B cells were positively isolated by dynabeads (Dynal) to carry out the analysis of strand-specific HCV RNA detection. Naïve $CD4^+$ cells were isolated by the MACS beads system (Miltenyi Biotec).

Strand-specific intracellular HCV RNA detection

Strand-specific intracellular HCV RNA was detected using a recently established procedure that combined previously published methods [27,28] with minor modifications [4,13]. Positive- and negative-strand-specific HCV RNAs were detected by a nested polymerase chain reaction (PCR) method. Semi-quantification was achieved by serial fourfold dilutions (in 10 μg/ml of *Escherichia coli* tRNA) of an initial amount of 200 ng of total RNA. The relative titer was expressed as the highest dilution giving a visible band of the appropriate size on a 2% agarose gel stained by ethidium bromide. For the internal control, semi-quantification of β-actin mRNA was performed using the same RNA extracts. To rule out false, random, and self-priming, extracted HCV RNA was run in every RT–PCR test without the addition of an upstream HCV primer.

The deep-sequencing analysis of Ly-HCV

Serum samples and PBMCs were collected from a patient with para-aortic lymph node enlargement with chronic HCV infection. Serum samples were stored at −20°C until testing. Total RNA was extracted from 800 μl of serum and 1.0×10^7 of PBMC using Trizol LS (Invitrogen). Each library was prepared using TruSeq RNA sample preparation kits v2 (Illumina). Libraries were clonally amplified on the flow cell and sequenced on an Illumina HiSeq 2000 (HiSeq Control Software 1.5, Illumina) with a 101-mer paired end sequence. Image analysis and base calling were performed using Real Time Analysis (RTA) 1.13. In the first mapping analysis, sequence reads not of human origin were aligned with 27675 reference virus sequences registered at the Hepatitis virus database server (HVDB) (http://s2as02.genes.nig.ac.jp/index.html) and the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using bwa (0.5.9-r26) and allowing mismatches of within 10 nucleotide bases. Based on the highest homology to the reference virus genome in the first mapping analysis, the tentative consensus HCV full genome sequence was created. The second mapping analysis was conducted using the tentative consensus HCV full genome sequence and bwa, allowing mismatches of within 5 nucleotide bases. The result of the analysis was displayed using Integrative Genomics Viewer (IGV; 2,0,17). Sequence analysis was performed using Genetyx-Mac ver.12. A phylogenetic tree was constructed by the unweighted pair group method with the arithmetic mean. The reliability of the phylogenetic results was assessed using 100 bootstrap replicate.

Inoculation of lymphotropic HCV strains in various kinds of lymphoid cell lines and human primary lymphocyte with stimulation

We used two different lymphotropic HCV strains. One was the SB-HCV strain that was previously reported by Sung et al[29]. The other one was Ly-HCV that was identified in this study by our group. The almost full-length sequence (95.9% coverage) of Ly-HCV was determined using a deep-sequence Hi-Seq 2000 system (illumina) (Fig S1A and B). These two-lymphotropic HCV strains were used for the experiments of HCV infection into lymphoid cells. Previously, we reported Raji, Molt-4 and primary human lymphoid cells were susceptible to the SB-HCV strain. In addition to these cells, we used miR122-transduced RIG-1/MDA-knock-down Raji cells provided by Machida K et al, since this cell line was most susceptible to SB-HCV replication (ongoing project, data not shown). These lymphotropic HCV strains were inoculated at day 0. SB cell culture supernatant and diluted serum from the patient with Ly-HCV, which contained 2×10^5 copies/ml of HCV-

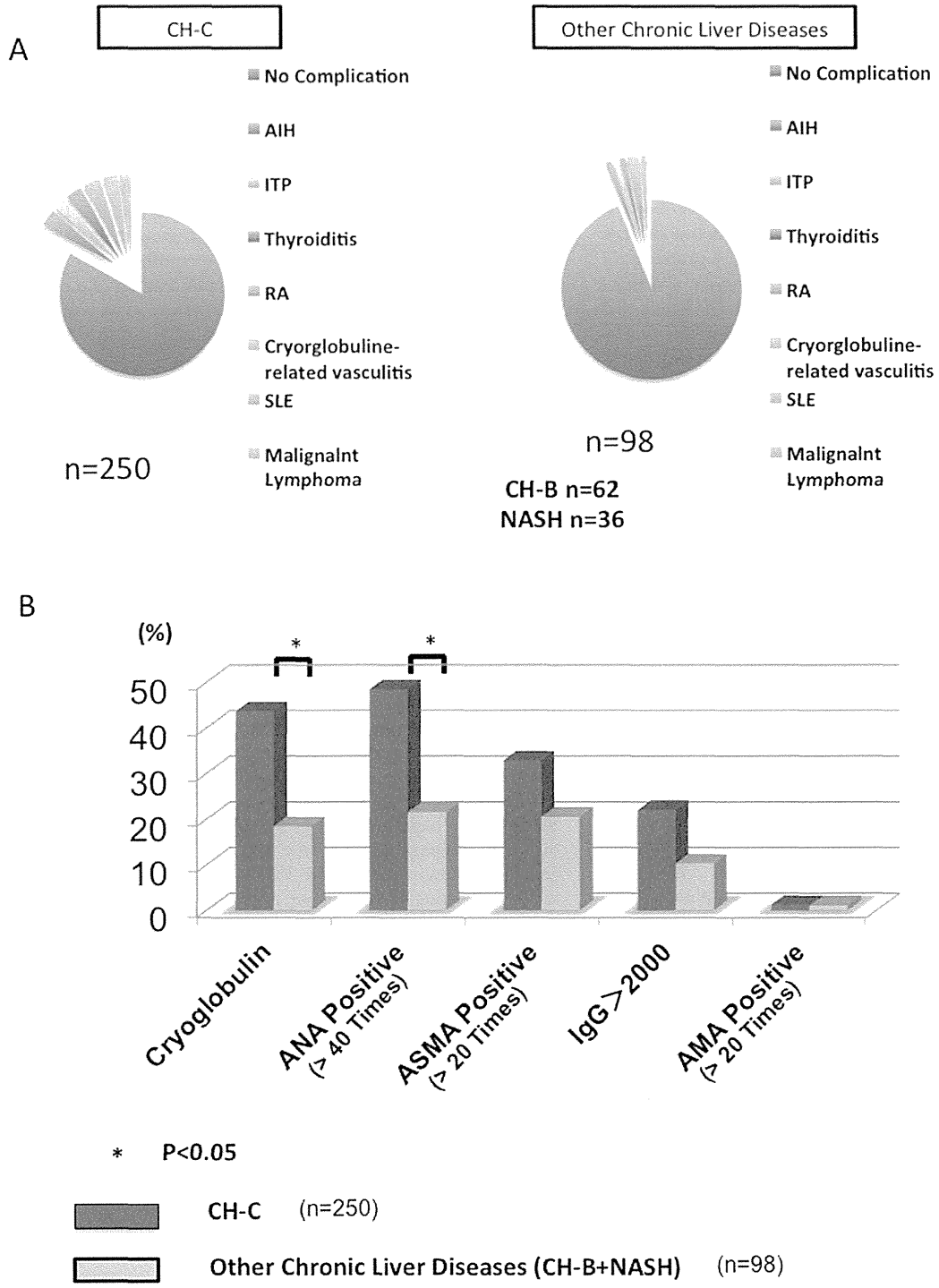

Figure 1. The relation between CH-C and the phenotype of autoimmune- diseases. The prevalence of these diseases in CH-C (n = 250) was significantly higher than in other chronic liver diseases (n = 98) (p = 0.0011) (A). The prevalence of these diseases and the positive rate of cryoglobulin, ANA (>40 times), ASMA (>20 times) and AMA (>20 times), and the amount of IgG (>2000mg/dl) are shown (B).
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RNA, were used for the infection of several kinds of human primary lymphoid cells (1×10^5 cells). A control infection with UV-irradiated HCV was included in every experiment. The supernatant of Huh-7 cells transfected with JFH-1 strains at 10 days post-transfection was used for several control experiments. The HCV-1T strain obtained from a CH-C patient without extrahepatic diseases and lymphoproliferative diseases was also used for several control experiments.

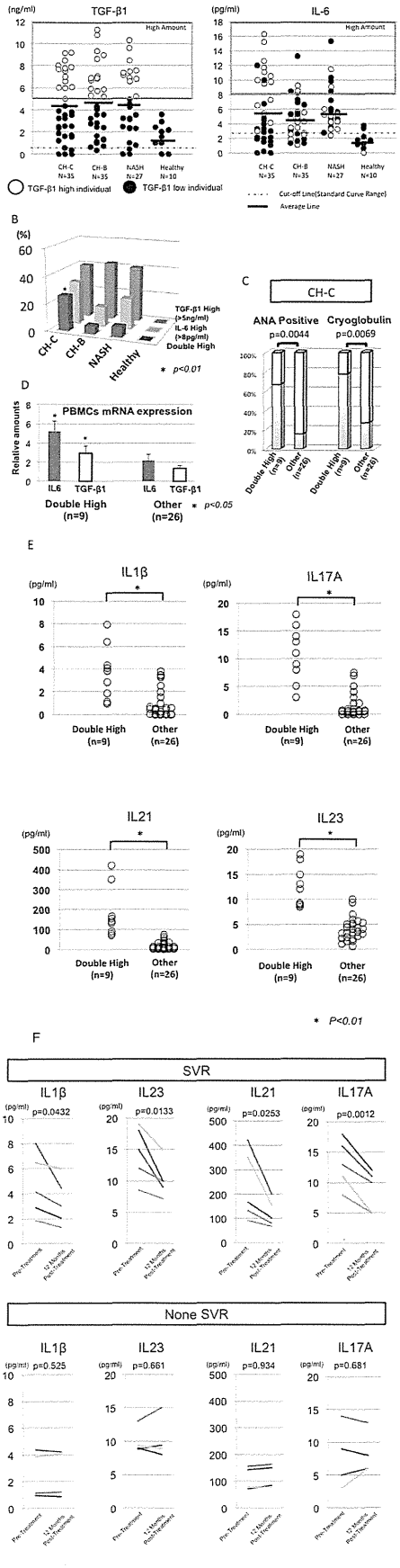

Figure 2. The cytokine conditions affecting the positivity of ANA and Cryoglobulin, and Th17 development. A comparison of the amounts of IL6 and TGF-β among the CH-C, CH-B, NASH and healthy subjects is shown (A). The bar indicates the mean cytokine amounts. The frequency of TGF-β1 high, IL6 high, and TGF-β1 and IL6 double high patients among the 4 groups (CH-C, CH-B, NASH, and healthy subjects) is shown (B). The positive rate of ANA and Cryoglobulin in the double high CH-C patients (n = 9) and the other CH-C patients (n = 26) is shown(C). The IL6 and TGF-β1 mRNA expression of PBMCs in the double-high patients (n = 9) and other patients (n = 26) is shown in the bar graphs (D). The amounts of IL1β, IL17A, IL21 and IL23 in the serum were compared between double high CH-C patients (n = 9) and the other CH-C patients (n = 26) (E). The comparisons of serum cytokines before and after the Peg-interferon/ Ribavirin treatment are shown (F). Serum samples were collected at just before the treatment and twelve month after the end of treatment. SVR indicates sustained virological treatment (n = 5).
doi:10.1371/journal.pone.0098521.g002

The analysis of IL17-secreting CD4$^+$ T cells

Naïve CD4$^+$ cells were negatively isolated by using a naïve CD4$^+$ T cells isolation kit II (Miltenyi Biotec). Isolated naïve CD4$^+$ cells were exposed to SB-HCV, Ly-HCV, UV-irradiated-SB-HCV, UV-irradiated-Ly-HCV or Mock. Then, CD3$^+$CD28$^+$ coated beads and various kinds of cytokines were added to the culture medium to analyze the Th17 commitment and development (Table S1). The cytokine conditions for Th17 commitment and development included IL-1β (10 ng/ml), and IL23 (1 ng/ml), which are important for the Th17 development in human, because the differentiation of Th17 cells is very difficult without these cytokines when using human PBMCs[30]. The cells were harvested at 7 days post-inoculation and IL17A-secreting cells were analyzed by MACS cytokine secretion assay (Miltenyi Biotec).

Transwell co-culture system

The trans-membrane with 0.4 um pore size was used for the analysis of soluble factor-inducing Th17 cells, especially IL6 and TGF-β1. The upper chamber included PBMCs (2×10^6 cells/ml) of CH-C patients (Ly-HCV or HCV-1T). The lower chamber included naïve CD4$^+$ cells (2×10^5 cells/ml) of a healthy individual and CD3CD28 coated beads with or without IL6 (40 ng/ml)(abcam) and TGF-β1 (40 ng/ml)(abcam) neutralizing antibodies. After five days incubation, the total RNA was isolated from cells of the lower chamber. The expression levels of RORγt were analyzed by real time PCR.

Construction of Lenti-virus expressing HCV-Core antigen

HCV core cDNA cloned in pcDNA3 was kindly provided by Dr. K. Takeuchi [31]. The full length HCV core cDNA was cloned into lentiviral vector, pCSII-EF plasmid, to create the pCSII-EF-HCV core[32]. The pCSII-EF-HCV core or control pCSII-EF-IRES-GFP plasmid was transfected into HEK293T cells together with two packaging plasmids, pCAG-HIVgp and pCMV-VSV-G/RSV-Rev (provided by the RIKEN Bio-resource Center), using the calcium phosphate method. The supernatants containing the recombinant lenti-virus were used for the infection of human primary lymphocyte.

Transfection of HCV individual protein expression plasmids

Various expression plasmids were constructed by inserting HCV-core, E1, E2, NS3, NS4B, NS5A and NS5B cDNA of genotype 1a behind the cytomegalovirus immediate-early promoter in pCDNA3.1 (Invitrogen). Primary CD4$^+$ cells were transfect-

Table 1. The frequency of Strand specific-HCV-RNA positive CD4+ T cells and CD19+ B cells.

		Negative-st-positive		Positive-st-positive	
		%(n: positive/total)		%(n: postive/total)	
	Double High (n=9)	33.3 (3/9)		44.4 (4/9)	
CD4+ T cell			p=0.0166		p=0.033
	Other (n=26)	3.8 (1/26)		11.5 (3/26)	
	Double High (n=9)	44.4 (4/9)		66.6 (6/9)	
CD19+ B cell			p=0.0027		p=0.0003
	Other (n=26)	3.8 (1/26)		7.6 (2/26)	

St-specific HCV-RNA were detected by nested PCR with rTth polymerase.
Double high indicates that the amount of IL6 and TGF-β are high.
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ed using 4D-Nucleofector II (Amaxa, Gaithersburg, Washington DC, USA) with a human T cell nucleofector kit (Amaxa), and various plasmids were purified using the EndFree plasmid kit (QIAGEN, Valencia, CA, USA). Viable transfected cells were isolated by Ficoll-Paque centrifugation (Amersham Bioscience) at 24 hour post-transfection. The transfection and expression efficiencies were analyzed using intracellular staining of individual proteins of HCV and flow cytometry analysis.

Real-time PCR analysis

Cells were collected before the inoculation of lenti-virus and 10 days after the inoculation of lenti-virus. Total RNA was isolated using a column isolation kit (QIAGEN). After the isolation of RNA, one-step real-time PCR using a TaqMan Chemistry System was carried out. The ready-made set of primers and probe for the amplification of IL-6 (Hs00985639_m1), TGF-β1 (Hs00998133_m1), T-bet (Hs00203436_m1), GATA-3 (Hs00231122_m1), RORC (Hs01076112_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs03929097_g1) were purchased from Applied Biosystems. The relative amount of target mRNA was obtained using a comparative threshold cycle (CT) method. The expression level of mRNAs of the non-stimulation sample of mock infected $CD4^+$ cells was represented as 1.0 and the relative amounts of target mRNA were calculated according to the manufacturer's protocol.

The analysis of STAT-1 and STAT-3 signaling

STAT-1 and STAT-3 signaling was analyzed by phospho-STAT-1 (Tyr701) and phosphor-STAT-3 (Tyr705) sandwich ELISA kit (Cell Signaling Technology). Briefly, naïve $CD4^+$ cells transfected with or without HCV-core expressing plasmid were incubated with IL6 and TGF-β1. The cells were harvested at various time points. Then, the cell lysates were used for the quantification of phosphor-STAT-1 and phosphor-STAT-3.

Statistical analysis

The data in Figure 1A, 1B, 2B and 2C were analyzed by χ^2 test. The data in Figure 2D and 2E were analyzed by independent Students t test. Figure 3A, 3C, 4A, 4B and 4C were analyzed by Mann-Whitney U test. All statistical analyses were carried out using JMP Pro version 9.

Accession Numbers

Accession number EntryID
AB779562 51027b2b6a8011fb860007e4.LyHCVserumSR
Accession number EntryID
AB779679 51029c6f6a8011fb8600093e.LyHCVpbmcSR

Results

Prevalence of autoimmune-related diseases in the CH-C patients

The prevalence of autoimmune-related disease in the CH-C patients was significantly higher than in the subjects with other chronic liver diseases in Tohoku University Hospital (p = 0.0011) (Fig.1A). In addition to the prevalence of autoimmune-related diseases, we analyzed the immunological laboratory tests including cryoglobulin, anti-nuclear antibody (ANA), anti-smooth muscle antibodies (ASMA), Immunoglobulin G (IgG), anti-mitochondrial antibody (AMA). The frequency of ANA positive or cryoglobulin positive patients in CH-C patients was significantly higher than in those with other chronic liver diseases ($p<0.05$) (Fig.1B).

The amount of IL6 and TGF-β1 in the peripheral blood of CH-C patients

The average amounts of IL6 and TGF-β1 were comparable among healthy subjects, CH-C, CH-B and NASH (IL6: 1.77, 5.83, 4.84 and 5.99 pg/ml), (TGF-β: 1.45, 4.18, 4.68 and 4.5 mg/ml), (average amount) (Fig. 2A). However, the frequency of patients with high amounts of IL6 (over 8 pg/ml) and TGF-β1 (over 5 ng/ml) (double-high) was significantly higher than in those with other chronic liver diseases ($p<0.05$)(Fig. 2B). The cut-off levels of high amount of IL6 (over 8 pg/ml) and TGF-β1 (over 5 ng/ml) were determined by the appearance of two clusters(high and low) in the CH-C samples. Interestingly, Most of the TGF-β1 high CH-C patients had high amounts of IL6 (Fig. 2B). Moreover, the amount of IL6 were significantly correlated with the amount of TGF-β1(data not shown). The serum amounts of IL6 and TGFβ1 were analyzed at 6 months after the sampling points. The serum amount of IL6 and TGF-β1 in the high amount of IL6 and TGF-β1 both (double-high) patients remained doubly high (data not shown). It has been reported that the combination of IL6 and TGF-β1 cytokines could induce Th17 cells[20]. Therefore, we compared the frequency of ANA-positive or cryoglobulin-positive patients between double-high patients and the other patients with HCV persistent infection. The frequency of ANA-positive or cryoglobulin-positive patients among the double-high patients was significantly higher than among the other CH-C patients ($p<0.01$)(Fig. 2C). The expression of IL-6 and TGF-β1-mRNA in PBMCs of double-high patients was significantly higher than in other CH-C patients ($p<0.05$)(Fig. 2D). Moreover, the serum amounts of IL1-β, IL17A, IL21 and IL23 in the double-high