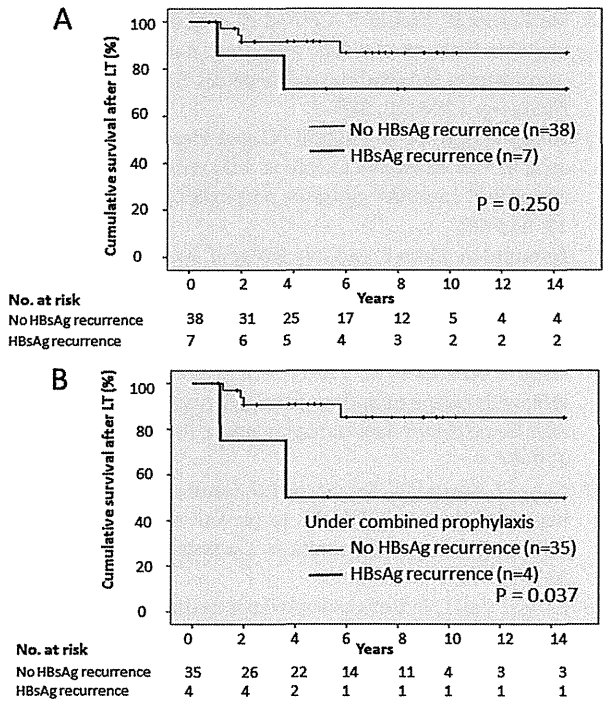

Figure 4 Cumulative survival in liver transplant recipients: (a) with and without hepatitis B surface antigen (HBsAg) recurrence; (b) with and without HBsAg recurrence under combined prophylaxis. LT, liver transplant.

higher genetic barrier to resistance, resulting in lower resistance rates in HBV-related liver disease patients. Further studies are needed to establish an optimal prophylactic regimen.

Except for a few studies that suggested a higher incidence of HBV recurrence in transplanted patients with HCC,[10–12] previous studies had not shown any association between HCC and a higher risk of HBV recurrence.[7,26,5,27,28] In 2008, Faria *et al.* reported an association between HCC recurrence and HBV re-infection.[10] In their study, the presence of HCC at transplantation and HCC recurrence after LT were independent risk factors associated with HBV recurrence. The authors demonstrated the presence of cccDNA in both HCC cells and in non-tumor cells in explanted livers, suggesting that HBV replication may also occur in tumor cells. In 2009, Saab *et al.* reported that pre-LT HCC and HCC recurrence after transplantation were associated with HBV reinfection and with decreased patient survival.[11] HCC recurrence itself is suggested to be a product of any breakthrough of the host immunity, and active cell proliferation due to malignant transformation can induce active replication of the HBV in the liver.[29] In addition, Yi *et al.* reported that chemotherapy and a high corticosteroid dose used for HCC were risk factors for HBV recurrence.[9] From that point of view, the differences in the virological kinetics in our three cases with HCC recurrence (Table 4, cases 4–6) are interesting, and may be explained by the condition of HCC and the use of systemic chemotherapy. These results require confirmation by further investigations.

Saab *et al.* reported decreased cumulative survival for patients with HBV recurrence.[11] Our present study showed no significant effect of HBV recurrence on the overall survival (Fig. 4a). However, if the analysis was limited to the patients with combined prophylaxis, the cumulative survival rates were significantly reduced in the HBV recurrence group compared with the HBV non-recurrence group (Fig. 4b). This result is consistent with previous studies,[11,30] but it should be noted that both of the two death cases in the HBsAg recurrence group (Fig. 4b) were the cases of HCC recurrence (Table 4, cases 4 and 5). The remaining two cases were another case of HCC recurrence and the case from a HBsAg positive donor (Table 4, cases 6 and 7). It is difficult to reach a definite conclusion because of small sample size, but HCC recurrence may be a strong prognostic factor for survival in a HBsAg recurrence group. Whether HBV recurrence itself is truly affecting prognosis or not should be confirmed in further studies.

Although the limitations of this study include its retrospective design and relatively small sample size, our results demonstrated the importance of combined prophylaxis, and confirmed that there is a relationship between HBV and HCC recurrence.

In conclusion, cessation of HBIG and post-LT HCC recurrence were independent risk factors for HBV recurrence in LDLT patients. Despite the improvements achieved in HBV prophylaxis following LDLT, clinicians should remain cautious concerning the risk of HBV recurrence, particularly in these groups.

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First report of human immunodeficiency virus transmission via a blood donation that tested negative by 20-minipool nucleic acid amplification in Japan

The Japanese Red Cross (JRC) blood centers screen donated blood for infectious agents using serologic assays and nucleic acid amplification testing (NAT). A multiplex NAT for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus Type 1 (HIV-1) with a minipool (MP) format comprising 50 seronegative samples was started in 2000.[1] During the implementation of the 50-MP-NAT in 2003, HIV-1 was transmitted through fresh-frozen plasma (FFP) from one blood donor during the window period. To reinforce NAT screening, the pool size was decreased to 20 in 2004. Since 20-MP-NAT implementation in 2004, we have found 19 donations that were seronegative but positive for HIV in the 20-MP-NAT. The rate of HIV-infected donations that were positive only in the NAT was approximately 1 in 2.7 million. No transfusion-transmitted HIV infection (TT-HIV) has been reported in Japan since the 20-MP-NAT was introduced.

In November 2013, anti-HIV was detected in a blood sample from a repeat male blood donor aged in his 40s. Western blotting (New LAV Blot 1, Bio-Rad, Hercules, CA), real-time reverse transcription–polymerase chain reaction assay (Cobas TaqScreen HIV, Roche, Basel, Switzerland), and transcription-mediated amplification assay using a kit (Procleix Ultrio ABD, Novartis Diagnostics, Emeryville, CA) confirmed HIV-1 infection. A qualitative NAT for HIV-1 (Cobas TaqMan, Roche) detected a plasma HIV-1 viral load of 4.7×10^4 copies/mL. A cryopreserved sample of plasma from his previous donation in February 2013 was retested in accordance with the Japanese guidelines for lookback studies on blood products. Using individual donation (ID-) NAT, the Cobas TaqScreen HIV (plasma input volume, 850 μL; 95% limit of detection [LOD], 24.3 IU/mL) detected HIV-1 RNA in an archived blood sample from his previous donation, whereas the Procleix (plasma input volume, 500 μL; 95% LOD, 19.6 IU/mL) did not. Each of these NAT assays was performed as a single test. The low plasma volume in the archival sample did not allow for repeat analysis.

Red blood cell (RBC) and FFP components were prepared from the previous donation and transfused into two recipients. The RBCs were transfused to a female patient in her 80s. A pretransfusion sample and a posttransfusion sample collected 9 months after transfusion were HIV seronegative. The latter sample was also negative for HIV RNA. The FFP was transfused 8 months after donation to a male patient in his 60s, from whom a pretransfusion sample was seronegative for HIV. Serologic tests and NAT assay identified HIV-1 infection in this recipient at 34 days after transfusion, and the plasma HIV-1 viral load was 1.1×10^6 copies/mL (Fig. 1).

The viral sequences determined in blood samples from both the donor (postseroconversion donation) and the FFP recipient differed by only one among 341 nucleotides in the *env* region (99.7% identity) and by four of 2800 nucleotides in the *pol* region (99.9% identity). Such high genetic similarity among the sequences supported the notion that HIV had been transferred from the donor to the FFP recipient. Isolates of HIV-1 from the donor and recipient were Subtype B, which is the most common among individuals infected with HIV-1 in Japan. Major antiretroviral drug-resistant mutations were not detected in either the donor or the recipient. Sequencing the HIV-1 5′-long terminal repeat, which was the target region of our NAT screen, did not detect HIV-1 mutations that caused false-negative NAT results.[2]

To estimate the HIV-1 viral load in the implicated blood, the sensitivity of both the Cobas TaqScreen HIV and the Procleix was reassessed by probit analysis using serial threefold dilutions (four replicates per dilution) of postseroconverted plasma (4.7×10^4 copies/mL) from the donor, which revealed that the 95% LOD of both NAT screens was 10 copies/mL. The archived blood sample from the implicated donation was reactive in the Cobas, but not in the Procleix screen; therefore, we speculated that the viral load in the donor plasma was approximately at the detection limit of the two NATs. Thus, the estimated total amount of HIV-1 in the FFP (containing

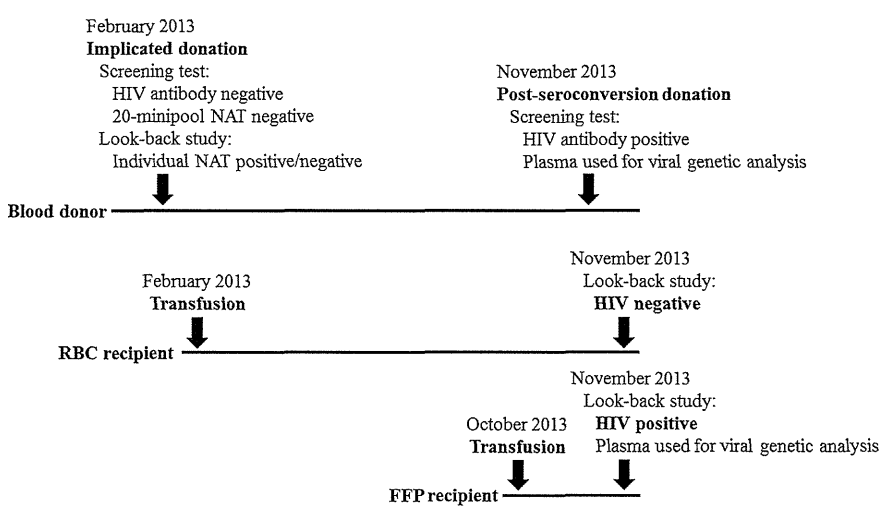

Fig. 1. Timeline of blood donations, transfusions, and infections.

approximately 240 mL of plasma) that caused TT-HIV was fewer than 2400 copies.

The prevalence of HIV infections detected by NAT or antibody screening among blood donors during 2012 was 1.3 per 100,000 donations, whereas the prevalence among first-time blood donors has recently been five to six per 100,000 donations. This figure exceeded the frequency of individuals who were newly diagnosed with HIV infection among the general population aged 15 to 64 years in 2012 (1.2 per 100,000).[3] The donor described herein was probably in the very early stage of HIV-1 infection at the time of the implicated donation. That blood donation is being used for testing individuals with high-risk behaviors for HIV transmission is a concern. Questions given to blood donors about behaviors that confer risk for HIV are probably not being answered precisely. Methods of confirming whether or not donors understand the questions and the need to answer them precisely need to be improved.

This report describes the first known case of TT-HIV through a 20-MP-NAT–negative blood component in Japan. Our results indicate that ID-NAT using even the most sensitive methods available today might not detect HIV-1 in window period donations. Transfusion with FFP, but not RBC, components resulted in HIV-1 transmission in the TT-HIV case described herein. Similar cases with differential HIV transmission via blood components have been reported in other countries.[4] However, TT-HIV arising via an ID-NAT–negative blood component has not been identified in Japan or in any other country where donated blood is screened by NAT for HIV. We estimated that the risk of collecting a unit during the ID-NAT–negative HIV-1 window period is 2.75 per 5.5 million donations in Japan.[5] The JRC will introduce ID-NAT screening in August 2014, which should further reduce window period transmissions. However, even ID-NAT might not completely eliminate the window period of HIV-1 infection.

In the era of very high-sensitive ID-NAT or MP-NAT, TT-HIV will arise mainly in patients transfused with components with larger plasma volumes such as FFP and apheresis platelets, because these components contain a larger amount of HIV. Although pathogen inactivation technologies might help to reduce residual risk, the introduction of pathogen inactivation technology should be determined after carefully considering the balance among benefit, risk, and cost.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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N-Acetylcysteine for thrombotic thrombocytopenic purpura: is a von Willebrand factor–inhibitory dose feasible in vivo?

We read with interest the recent report by Li and colleagues[1] describing the first apparent successful use of *N*-acetylcysteine (NAC) in refractory thrombotic thrombocytopenic purpura (TTP). As we had previously described a case of NAC failure in a refractory TTP case that subsequently responded to bortezomib,[2] we sought to clarify whether our NAC dosing protocol differed from that utilized by Li and coworkers.[1]

As the only precedent for NAC dosing in TTP was preclinical,[3] we too adapted a clinical acetaminophen overdose protocol.[4] Our patient received 150 mg/kg NAC as a 1-hour bolus, followed by 50 mg/kg over 4 hours and then 100 mg/kg over the next 16 hours for the first day of dosing (with a cumulative exposure of 15 g in the first 24 hr). Twice-daily plasma exchange could not be interrupted

Establishment of culture systems for Genotypes 3 and 4 hepatitis E virus (HEV) obtained from human blood and application of HEV inactivation using a pathogen reduction technology system

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BACKGROUND: It has been demonstrated that the hepatitis E virus (HEV) can be transmitted via blood transfusion, and the risk of HEV transmission via transfusion has become a major global concern. An HEV culture system for blood-derived HEV has been sought to obtain valuable knowledge of the virus and the risk of HEV infection through blood products.

STUDY DESIGN AND METHODS: We endeavored to establish an HEV culture system using RNA-positive blood specimens for Genotypes (G) 3 and 4 and applied this system to evaluate tissue culture infectious dose (TCID). We applied this method to investigate the potential of the Mirasol pathogen reduction technology (PRT) system (Terumo BCT) to inactivate live HEV in contaminated platelet samples (PLTs). PLTs were spiked with cultured HEV G3 or G4 and then treated with the Mirasol PRT system. PLTs were examined before and after the treatment for HEV load using TCID titration.

RESULTS: We successfully established two strains for HEV production: the JRC-HE3 strain for G3 and the UA1 strain for G4. The Mirasol PRT system expressed more than 3 log inactivation for JRC-HE3 and more than 2 log inactivation for UA1.

CONCLUSION: The Mirasol PRT system inactivated greater than 2 to 3 logs of live HEV in PLTs and can potentially be used to lower the possibility of blood-borne HEV transmission. The G3 and G4 HEV inocula identified in this study and the hepatoma cell culture system provide a new means to assess HEV infectious titer and to evaluate other pathogen reduction strategies.

The hepatitis E virus (HEV), the causative agent of hepatitis E, was once believed to be transmitted orally and to be particularly localized in developing countries and regions.[1,2] However, a recent report demonstrated that HEV is spreading throughout the world, including industrialized nations.[3,4] HEV can also be transmitted by blood transfusion, and occasionally causes severe hepatitis,[5,6] so the risk of HEV infection via transfusion has become a major global concern.

HEV is roughly classified into four genotypes (G1-G4). Among these, G3 is the most widely distributed worldwide, while G4 has a tendency to cause severe hepatitis and is localized mainly in Asia.[7,8] For these reasons, the establishment of an HEV culture system, particularly for G3 and G4, has long been attempted, because it would be applicable to the assessment of technologies for blood product safety and as a tool to elucidate the HEV infection mechanism. Recently, two HEV cultivation systems using two cell lines—human hepatoma cells (PLC/PRF/5 cells) and human lung adenocarcinoma cells (A549 cells)—as the hosts, with HEV specimens derived from the feces or

ABBREVIATIONS: cDNA = complementary DNA; G = genotype; ORF = open reading frame; PRT(s) = pathogen reduction technology (-ies); TCID = tissue culture infectious dose.

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serum of HEV-infected individuals, have been established.[9,10] However, recent studies have demonstrated that the viruses obtained from the blood have an envelope-like structure that differs from the structure of those obtained from feces.[11] Therefore, in our view, it was reasonable to establish HEV cultivation systems using G3 and G4 HEV commonly isolated in Japan, from HEV RNA–positive blood specimens. Our aim was also to apply this system to perform an assay of HEV infectious titer, referred to as a tissue culture infectious dose (TCID), to evaluate the infection risk of HEV through blood or plasma products.

Although the risk of HEV infection via transfusion is now being recognized all over the world, an assay for viral infectivity using HEV proliferated in a cultured cell line originally isolated from HEV RNA–positive blood specimens did not exist. In addition, an assessment of the ability of a pathogen reduction technology (PRT) system to inactivate HEV virus in blood samples had never been performed before. We investigated the effect of the Mirasol PRT system on cultured G3 and G4 HEV originally derived from blood specimens and hereby report it.

MATERIALS AND METHODS

Establishment of HEV culture systems

HEV specimens

Ten plasma specimens and four serum specimens containing G3 or G4 HEV were employed. Plasma and serum were obtained from blood donors and patients who had been confirmed to be HEV RNA positive. The viral concentration of the specimens ranged from $10^{5.6}$ (HRC-HE21) to $10^{7.5}$ (JRC-HE3) copies/mL. The accession numbers of some specimens are also described in Table 1.

Cell culture and virus inoculation

The procedure for cell culture, HEV infection, and the maintenance of HEV-infected cells has been discussed in a previous report by Okamoto.[8] Briefly, human hepatoma cells (PLC/PRF/5 cells) and human lung adenocarcinoma cells (A549 cells; Health Science Research Resources Bank, Tokyo, Japan) were used in this assay. The cells were then infected with 2.5 mL of viral specimens for 2 hours at 37°C in a 5% CO_2 incubator. HEV-infected cells were maintained in a medium (maintenance medium) consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and medium 199 (Invitrogen, Tokyo, Japan), containing 2% heat-inactivated fetal calf serum, 30 mmol/L Mg^{2+} (Wako Pure Chemical Industries, Ltd, Osaka, Japan), and 2.5 μg/mL amphotericin B (Wako Pure Chemical Industries, Ltd), and were cultured at 37°C in a humidified 5% CO_2 atmosphere using 10 mL of maintenance medium. Whole media were recovered weekly and replaced with fresh maintenance media. HEV RNA copies in the recovered media were quantified using real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis. The method for HEV RNA quantification is summarized below.

Confirmation of HEV infectivity against cells and method for quantifying HEV TCID

The acquisition of HEV infectivity was confirmed by detection of the viral progeny in the recovered cell culture supernatant. Given the establishment of HEV infection, the total number of viral RNAs existing in the recovered medium was overwhelmingly larger than the originally inoculated number. Viral TCID was investigated by limiting dilution analysis. The original viral solution for which the concentration of viral RNA (copies/mL) was already determined was serially diluted tenfold. The maximum dilution ratio against the original viral solution expressing infectivity on host cells was examined. The number of copies of HEV RNA required to express infectivity against host cells was calculated using both the original viral RNA concentration and the ratio of dilution.

TABLE 1. HEV specimens used for the establishment of the culture system

HEV strain	Source	Genotype	Log copies/mL (original concentration of specimen)	GenBank Accession Number for 412 bp (full genome)
HRC-HE21	Plasma	3	5.6	AB670957
HRC-HE22			5.8	AB670958
HRC-HE30			5.6	AB670966
HRC-HE80			6.0	AB671016
HRC-HE104			6.7	AB602891 (AB630970)
HRC-HE121			6.6	AB671054
HRC-HE159			5.7	AB671092
JRC-HE1			6.8	AB434144
JRC-HE3			7.5	AB434146 (AB630971)
JRC-HE8			5.9	AB434151
UA1	Serum	4	7.2	Not determined
UA2			6.9	
UA3			5.8	
SA1		Not determined	6.2	

Quantitation of HEV RNA

Quantitation of HEV RNA was performed following a previously reported method with minor modifications.[5] Briefly, RT-PCR was used to determine the number of copies of HEV RNA by targeting 75 nucleotides of a highly conserved sequence in the open reading frame (ORF) 2 region. The conditions for the RNA amplification stage were as follows: 25 μL of extracted RNA was incubated at 50°C for 30 minutes and at 95°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds and 60°C for 1 minute. Real-time RT-PCR was then performed using PCR and a Sequence Detection System (TaqMan and PRISM 7900, respectively, Applied Biosystems, Tokyo, Japan). The sensitivity of this system was determined to be 25 copies/mL, with a 95% confidence interval of 13 to 166 copies/mL by logistic analysis. The quantitation standard for HEV RNA was generated by transcribing the HEV complementary DNA (cDNA) of the HEV ORF2 region cloned into a plasmid (pCRII-TOPO, Invitrogen).

Full genome sequencing of HEV RNA

We referred to a previous report by Urayama and colleagues[12] for the method of full genome sequencing of HEV RNA. Total RNA was extracted either from 300 μL of HEV-positive blood specimens or from 200 μL of recovered cell culture supernatant. RT of HEV RNA was performed at 42°C for 1 hour. Consequently, nearly the full length of the cDNA was constructed. This cDNA was divided into two fragments: a 3′ fragment (3960 nucleotides) and a 5′ fragment (3160 nucleotides).

Phylogenetic analysis of HEV isolates

Complete or nearly complete sequences of HEV isolates were determined as previously described.[12] Sequences were aligned with the reported HEV strains using a computer program (ClustalW, Version 1.8, http://www.genome.jp/tools/clustalw/).

Photochemical inactivation of HEV in platelet samples

Preparation of platelets

Rejected platelets (PLTs), which are not clinically suitable to be used as blood derivatives because the alanine aminotransferase value exceeds the acceptance criteria, were used in this assay. All PLTs were collected by apheresis methods using equipment of the component collection system of Haemonetics (Braintree, MA), as well as TRIMA and TERUSYS of Terumo BCT (Lakewood, CO). All procedures were accomplished according to the manufacturers' protocols. The basic criteria for the volume and concentration of PLTs were 190 to 250 mL/bag and 1.9×10^{11} to 4.7×10^{11}/bag, respectively.

Treatment of PLTs with the Mirasol PRT system

Mirasol PRT treatment of PLTs was performed as previously described.[13] Briefly, 35 mL of riboflavin solution was added to a Mirasol PRT system kit to obtain a final concentration of 50 μmol/L, the unit was then exposed to a dose of UVB light (6.24 J/mL) and then left to rest for 30 minutes at an ambient temperature to prevent activation of the PLTs.

Sampling of HEV specimens from PLTs

Three milliliters of the pre-Mirasol-treated HEV PLT specimens was collected after the PLTs were thoroughly mixed with the virus in the dedicated bags, and the same volume of posttreated HEV specimens was likewise acquired. Titration samples for HEV infectivity were obtained from the supernatants of PLTs by centrifugation at 3000 rpm ($1750 \times g$) for 15 minutes under ambient atmosphere.

Confirmation of the noncytotoxicity against a cultured cell line caused by plasma from both pre- and post-Mirasol-treated PLTs

A cytotoxicity test against A549 cells was performed using virus-free control PLTs. This experimental condition was the same as the HEV infectivity assay, as described in the paragraph, "Cell culture and virus inoculation." Both pre- and post-Mirasol PRT-treated plasma obtained from the supernatant of PLTs were laid onto A549 cells for 2 hours. After the plasma was removed, the cells were cultured to confirm if any positive cell death or expression of a negative impact on cell growth were seen.

RESULTS

Establishment of the HEV culture systems

Selection of an HEV strain to establish a culture system using A549 cells

Fourteen G3- and G4-containing HEV specimens from plasma or serum (Table 1) were examined. All samples were diluted to $10^{4.8}$ copies/mL (total amount, $10^{5.2}$ copies in 2.5 mL) and then inoculated into A549 cells (Fig. 1). Consequently, only two HEV strains (JRC-HE3 for G3 and UA1 for G4) were found to be infectious. The HEV load of JRC-HE3 reached a plateau at an approximate concentration of 10^6 copies/mL (total amount, 10^7 copies in 10 mL of recovered medium) at around 42 days (6 weeks) postinoculation. On the other hand, UA1 reached a plateau at $10^{3.3}$ copies/mL at 60 days postinoculation.

HEV cultivation of JRC-HE3 using PLC/PRF/5 cells to procure a higher load of viral progeny

JRC-HE3 was diluted to $10^{5.5}$ copies/mL ($10^{5.9}$ copies in 2.5 mL) and then inoculated into alternative cells, namely, PLC/PRF/5 cells (Fig. 2). The viral concentration increased until 91 days (13 weeks) postinoculation and reached a plateau at an approximate concentration of 10^8 copies/mL (10^9 copies in 10 mL of recovered medium;

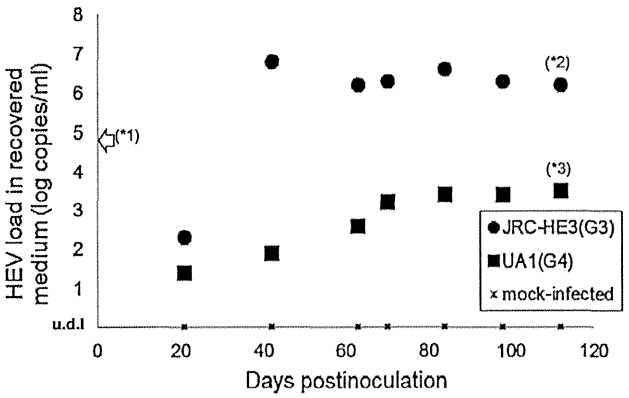

Fig. 1. Selection of HEV strains for the establishment of a culture system using A549 cells. The initial concentrations of the HEV strains used were all $10^{4.8}$ copies/mL ($10^{5.2}$ copies in 2.5 mL; *1). The concentrations of JRC-HE3 for G3 and UA1 for G4 in the recovered culture medium at 112 days postinoculation were $10^{6.2}$ copies/mL ($10^{7.2}$ copies in 10 mL) (*2) and $10^{3.3}$ copies/mL ($10^{4.3}$ copies in 10 mL; *3), respectively. A549 cells were utilized as the host for this assay. u.d.l. = under the detection limit.

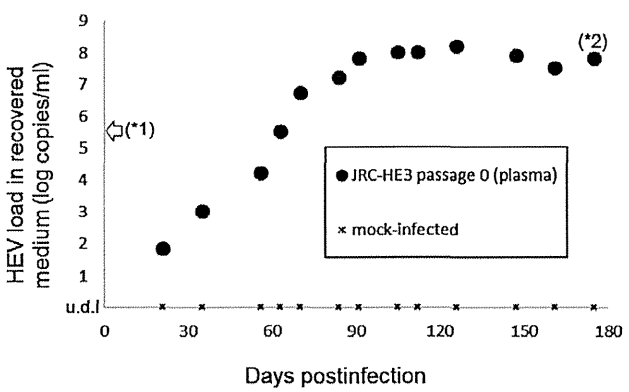

Fig. 2. Confirmation of a higher load of HEV progeny using PLC/PRF/5 cells. A high concentration of HEV progeny was obtained when PLC/PRF/5 cells were used. The HEV JRC-HE3 strain was inoculated at $10^{5.5}$ copies/mL ($10^{5.9}$ copies in 2.5 mL; *1). The load of JRC-HE3 in recovered culture medium at 175 days postinoculation was $10^{7.9}$ copies/mL ($10^{8.9}$ copies in 10 mL; *2). No viral progeny were detected in the mock-infected samples.

Fig. 2). It was possible to obtain a higher load of JRC-HE3 with PLC/PRF/5 cells than with A549 cells for virus proliferation.

Serial culture passage of JRC-HE3 using PLC/PRF/5 cells

HEV production resulting from different JRC-HE3 culture passages was examined using PLC/PRF/5 cells. For the first cultivation, an original HEV-positive-plasma specimen was used (Fig. 2, Passage 0). This means that the viral progeny referred to as Passage 1 was obtained from the result of Passage 0 infection. Each viral progeny from the specimens at 70 days (10 weeks) postinoculation was diluted to $10^{5.7}$ copies/mL before the next infection step (Fig. 3). The growth curves obtained for viral production were identical for all passages (Passages 1-4).

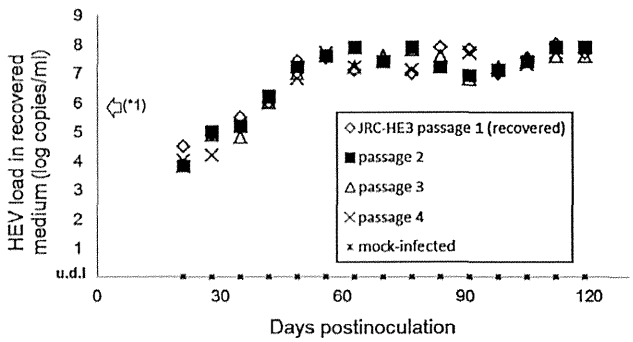

Fig. 3. JRC-HE3 serially passaged using PLC/PRF/5 cells. HEV samples in recovered culture media were diluted to $10^{5.7}$ copies/mL ($10^{6.1}$ copies in 2.5 mL; *1). Each viral sample from specimens at 70 days (10 weeks) postinoculation was inoculated into PLC/PRF/5 cells for each successive passage. There were no significant differences between the viral production curves from each passage. No viral progeny were detected in the mock-infected samples.

Genomic analysis of JRC-HE3 associated with culture passage and longer incubation of the virus

Full genome sequencing of JRC-HE3 was carried out on the original plasma (Passage 0) and progeny after long-term incubation (119 days [17 weeks], Passage 4), and genomic substitutions and amino acid alterations were investigated (Table 2). Consequently, only point substitutions at five bases in the ORF1 region and duplicate substitution at two bases in the ORF2 and ORF3 regions were found, respectively. These substitutions were demonstrated to result in alterations at four amino acid sites. Note that, due to triplet code degeneracy, some of the observed genomic base substitutions did not result in amino acid alteration.

Infectious titration of JRC-HE3 and UA1 using A549 cells and PLC/PRF/5 cells

Limited dilution assays were performed to determine the minimum HEV RNA load for infectivity against two cell lines. High loads of JRC-HE3 ($10^{9.4}$ copies/2.5 mL or $10^{8.5}$ copies/2.5 mL) were serially diluted 10-fold and inoculated into A549 cells and PLC/PRF/5 cells, respectively. Viral progeny were quantitatively investigated at 3, 6, and 9 weeks postinfection. Consequently, JRC-HE3 expressed its infectivity against A549 cells (Fig. 4A) and PLC/PRF/5 cells (Fig. 4B) even at 10^4- and 10^3-fold dilutions,

TABLE 2. HEV RNA and amino acid substitution associated with in vitro passaging and the longer incubation of HEV JRC-HE3 obtained during Passage 4 (Fig. 3)

ORF 1, five bases		Methyl transferase G137G [GGU(G) ⇒ GGC(G)] Papain-like cysteine protease D452D [GAU(D) ⇒ GAC(D)] Domain X W741P [UGG(W) ⇒ CGG(P)] Domain X E871K [GAG(E) ⇒ AAG(K)] Helicase F1124S [UUC(F) ⇒ UCC(S)]
ORF 3 and ORF 2 (duplicative two bases)	ORF3 C20C [UGU(C) ⇒ UGC(C)] ORF3 P98P [CCG(P) ⇒ CCA(P)]	ORF2, Capsid L8L [UUG(L) ⇒ CUG(L)] ORF2, Capsid A119T [GCU(A) ⇒ ACU(T)]

respectively. This indicates that $10^{5.4}$ and $10^{5.5}$ copies of JRC-HE3 are necessary to achieve infectivity against A549 cells and PLC/PRF/5 cells (Table 3). Thus, for both cell lines, 1 TCID of JRC-HE3 was estimated to be approximately $10^{5.5}$ copies. A similar experiment was performed using UA1 ($10^{7.5}$ copies/2.5 mL) along with A549 cells (Fig. 4C). Consequently, 1 TCID of UA1 was found to be approximately $10^{5.5}$ copies, although infectivity against PLC/PRF/5 cells could not be confirmed (data not shown). Based on the above-mentioned results, A549 cells may have to be used as the hosts when titration of both JRC-HE3 and UA1 are performed.

Photochemical inactivation of HEV in PLTs

The assays of HEV inactivation of both G3 (JRC-HE3) and G4 (UA1) in PLTs were performed thrice. The mean concentration of each strain in PLTs was consequently $10^{9.8}$ and $10^{7.9}$ copies per total volume (approx. 200 mL) of PLTs, respectively. HEV specimens collected from PLTs were serially diluted 10^n-fold (n = 0-6). Consequently, JRC-HE3 was found to express infectivity even if the original specimen (10^0-fold) was diluted 10^3-fold (Fig. 5A). This was clearly demonstrated by the fact that the viral progeny was found in the cell culture supernatant when the 10^3-fold–diluted specimens were applied. On the other hand, UA1 showed infectivity to a level of 10^2-fold dilution (Fig. 5C). No infectivity was recognized in any specimens after treatment with the Mirasol PRT system at the ninth or 10th week postinfection (Figs. 5B and 5D). In some cases, HEV RNA was detected in the cell culture supernatant in posttreated samples (Figs. 5B and 5D, white arrows). However, it would be reasonable to understand that these HEV RNA samples are derived from originally challenged HEV, not from virus progeny. All the data described in Fig. 5 are representative data of the results of each assay that was performed thrice. There was little difference between the assays. Based on these results, it was concluded that the Mirasol PRT system lowers the infectivity of G3 and G4 of live HEV, by more than 3 log (99.9%) and more than 2 log (99%), respectively (Table 4). In this experiment, A549 cells were utilized for HEV titration. The effect of HEV-free plasma treated or not treated with the Mirasol PRT system on the cells was also investigated. Consequently, no positive factors for cell death nor a negative impact on cell growth was observed.

DISCUSSION

Since HEV was recently categorized as a transfusion-transmittable infectious disease, the risk of HEV infection via blood transfusion has become a major global concern in transfusion medicine.[5,6] Hence, there has been a demand to establish an HEV culture system, especially for G3 and G4, which are frequently detected in Japan, China, and southeastern Asia,[7,8] as well as for a method to assay the HEV infectious titer, TCID. We therefore attempted to establish a culture system for G3 and G4 HEV and have successfully obtained two positive systems: the JRC-HE3 strain for G3 and the UA1 strain for G4. The concentrations of both viral strains in plasma or serum originally exceeded 10^7 copies/mL; thus, it is plausible that the two strains may easily replicate and grow both in vivo and in vitro.

Although JRC-HE3 can infect both PLC/PRF/5 cells and A549 cells, the production efficiencies of the virions differed. A higher load of JRC-HE3 was inoculated into PLC/PRF/5 cells, and a higher concentration of approximately 10^8 copies/mL of virus progeny was confirmed. In contrast, A549 cells generated progeny at a concentration of approximately 10^6 copies/mL, with inoculations ranging from $10^{5.4}$ to $10^{9.4}$ copies in 2.5 mL. Since the PLC/PRF/5 cells are derived from human hepatoma, this cell line may be more suitable for HEV production than the A549 cells.

We examined the genomic alterations and amino acid substitutions of JRC-HE3 derived from PLC/PRF/5 cells after long-term culture. Full genome sequencing of JRC-

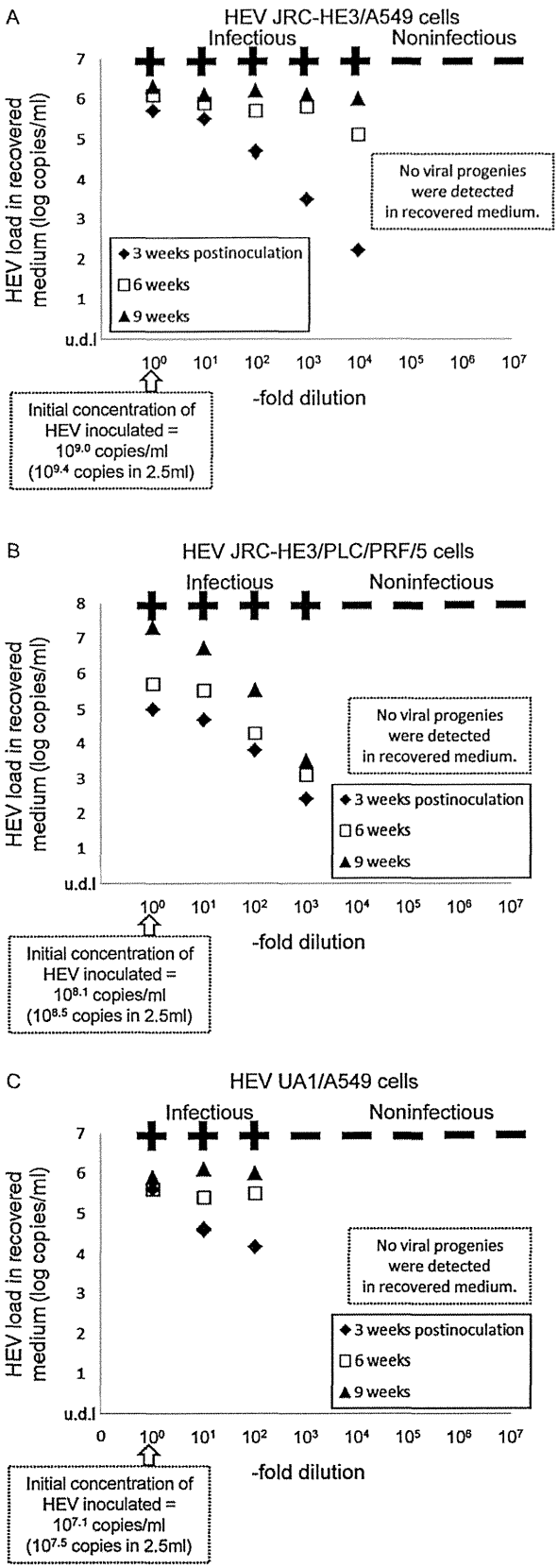

Fig. 4. (A) Titration of infectious HEV JRC-HE3 using A549 cells. A high HEV load of $10^{9.0}$ copies/mL was obtained by ultracentrifugation. The original HEV samples were serially diluted tenfold. Each diluted HEV sample was inoculated into A549 cells. The viral progeny in the recovered culture medium were examined every 3 weeks. Even the original HEV samples, diluted at 10^4, were found to be infectious. (B) Titration of infectious HEV JRC-HE3 using PLC/PRF/5 cells. A high HEV load of $10^{8.1}$ copies/mL was obtained by ultracentrifugation. Even the original HEV samples, diluted at 10^3, were found to be infectious. (C) Titration of infectious HEV UA1 using A549 cells. A high HEV load of $10^{7.1}$ copies/mL was obtained by ultracentrifugation. Even the original HEV samples, diluted at 10^2, were found to be infectious. u.d.l. = under the detection limit.

TABLE 3. Numerical relationship between two viral units: copies and TCID

Host cells	HEV strain	Copies per TCID
A549	HEV JRC-HE3 G3	$10^{5.4}$
PLC/PRF/5		$10^{5.5}$
A549	HEV UA1 G4	$10^{5.5}$
PLC/PRF/5		Could not be determined

HE3 revealed few variations. It may be concluded that JRC-HE3 has easily become habituated to in vitro cultivation. If the sequences of the viral genome would drastically change under long-term incubation, the efficacy of HEV production and viral infectiousness (infectious titer) may be negatively impacted. Based on these data, it could be concluded that HEV can stably and consistently be produced by this culture system, and its infectivity may remain almost the same in all situations. This consideration can be supported by the fact that the obtained growth curves demonstrating viral production were identical at a high level up to 120 days postinfection, regardless of the passage of the virus. Currently, a similar trial is being performed using UA1 produced by A549 cells.

In the first half of this article, we conclusively established an HEV culture system using HEV RNA–positive plasma or serum sample obtained from G3 or G4 HEV-infected individuals. Using the system established here, we attempted to undertake photochemical inactivation of HEV in PLTs using the Mirasol PRT system.

Log reduction values (LRVs) of more than 3 log for JRC-HE3 and more than 2 log for UA1 were achieved despite the fact that this agent is a nonenveloped virus, which are traditionally resistant to chemical inactivation. For the inactivation assay of JRC-HE3, a relatively higher concentration of the sample was obtained using PLC/PRF/5 cells as the virus producer. On the other hand, UA1 was not propagated in PLC/PRF/5 cells, but only in A549 cells. Therefore, more than 2-log reduction was marginally demonstrated due to the lower titer of the applied HEV.

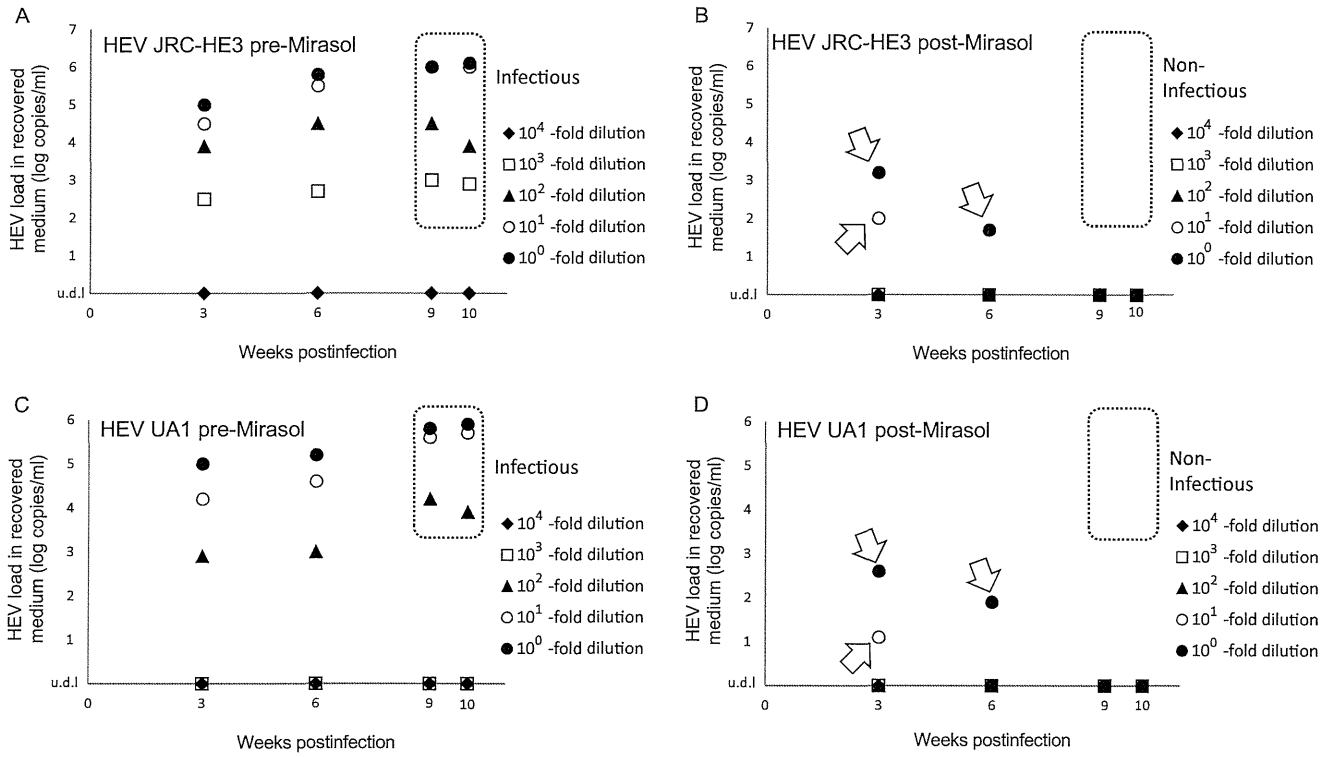

Fig. 5. (A and B) Inactivation of the live HEV JRC-HE3 in PLTs by the Mirasol PRT system. The change in the HEV load in the supernatant of A549 cell culture was investigated. The assayed HEVs were (A) Mirasol pretreated and (B) Mirasol posttreated HEV, respectively. The establishment of HEV infection was consequently confirmed at 9 and 10 weeks postinfection. The concentration of JRC-HE3 in PLTs was consequently $10^{9.8}$ copies per total volume of PLTs. JRC-HE3 expressed infectivity even when the original specimens were 10^3-fold diluted (A). No infectivity (viral progeny) was recognized in any Mirasol PRT system posttreated specimens of (B). HEV-RNAs detected in (B, white arrows) are derived from the originally challenged HEV, because the HEV loads in recovered medium were gradually decreasing. The Mirasol PRT system lowered the infectivity of JRC-HE3 more than 3 log (99.9%). These are the representative data of triplicate assays. (C and D) Inactivation of the live HEV UA1 in PLTs by the Mirasol PRT system. The HEVs assayed were (C) Mirasol pretreated and (D) Mirasol posttreated HEV, respectively. The concentration of UA1 in PLTs was consequently $10^{7.9}$ copies per total volume of PLTs. UA1 expressed infectivity even when the original specimens were 10^2-fold diluted (C). No infectivity (viral progeny) was recognized in any Mirasol PRT system posttreatment specimens (D). The Mirasol PRT system lowered the infectivity of UA1 more than 2 log (99%). These are the representative data of triplicate assays.

TABLE 4. Inactivation of the live HEV and HAV in PLTs by the Mirasol PRT system

HEV and HAV strain	Log reduction value (% of reduction value)
HEV JRC-HE3 G3	More than 3 log (more than 99.9%)
HEV UA1 G4 (Reference data)	More than 2 log (more than 99%)
HAV VR-1402 GIB	1.8 log (98.4%)

The limited load of HEV obtained from in vitro cultivation precluded evaluation of the robustness of the measured LRV. If higher efficacy of viral infection against the hosts were attained, it could also be possible to acquire more robust data.

Of note, a 1.8-log (98.4%) reduction of LRV for the hepatitis A virus (HAV) strain of VR-1402 was previously obtained in our laboratory and an identical value was also reported by Prowse.[14] Thus, HEV inactivation is more efficient than that for HAV. VR-1402 has frequently been used for inactivation assays of HAV and is the usual model viruses for nonenveloped and relatively smaller-sized RNA viruses including HEV, because it is easy to titrate. We believe that the system we described here may be applied widely for infectivity studies of small, nonenveloped viruses, instead of VR-1402 as a model.

The Intercept blood system, similar to the Mirasol PRT system, is also well known as a photochemical method for PRT of blood products. Recently, two cases of HEV transmission caused by the transfusion of plasma treated with the Intercept were reported by Hauser and coworkers.[15] The primary mechanism of viral inactivation by the Intercept process has been described to involve an intercalation of amotosalen (the photochemical agent

employed in this system) into virus genome. This psoralen derivative is excited by UVA light, and the photochemical reaction that ensues subsequently induces a covalent bond between amotosalen and the DNA or RNA of the pathogen, which disrupts viral replication processes. Although the viral load in the transfused plasma that caused HEV infection was not described in the literature, these cases may prove that this inactivation mechanism by the Intercept process may be ineffective for HEV inactivation. It is also possible that the Mirasol system might inhibit certain step(s) essential for the viral life cycle and eventually induce the reduction of HEV infectious titer by at least 2 to 3 log. To fully determine which technology is more efficient for inactivating HEV more information regarding factors associated with HEV pathogenicity, such as the maximum HEV RNA concentration found in donated plasma and the minimum viral load required for the establishment of transfusion-transmitted HEV infection, would be needed. In relation to this, a concentration of more than 10^7 copies/mL has been detected in plasma collected for blood screening.[16] It is therefore possible that transmissions with either technology could still occur if viral titers exceed the capacity of the respective processes to inactivate all infectious particles that are present.

In conclusion, our results show that the Mirasol system has a possibility for the reduction of the risk of HEV transmission caused by blood transfusion. Further studies to determine the relationship between viral load and the pathogenesis of hepatitis E are necessary to establish whether the Mirasol system is indeed effective for preventing HEV transmission under clinical conditions. Since the concern about HEV transmission by blood transfusion has been rapidly increasing worldwide, the effect of PRTs on HEV should be further investigated.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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第１部 論文集
原著

日本国内のＢ型およびＣ型肝炎ウイルス感染者数は？ －献血者スクリーニングデータを補正して－

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要 旨

【目的】 国内のＢ型およびＣ型肝炎ウイルス（以下、HBV、HCV）感染者数を正確に推計することは、肝炎対策基本法に基づく効果的な対策を推進し、肝癌の予防対策を充実させる上で、重要である。

【方法】 全国の９つの地域で、献血歴のある7,000人と、献血経験のない１万人（いずれも40～59歳）を対象に、インターネット調査を行い、献血経験の状況別に、HBV、HCVの感染既往リスク比を計測した。そして、このリスク比と、ネット調査対象者における献血経験状況の分布割合から、初回献血者における肝炎ウイルス感染者割合を一般国民のそれに外挿する際の補正係数を求めた。次に、1995～2000年に、初めて献血した全国3,485,648人のスクリーニングデータから、１出生年代別（1931～1984年生まれ）に、HBs抗原およびHCV抗体陽性割合を得た。この出生年代別陽性割合を2010年の日本人人口に当てはめてHBV、HCV感染者数を求めた。最後に、この感染者数に、上記補正係数を乗じ、推計値を得た。

【結果】 ネット調査結果から、①1995～2000年に、初回献血をした人を基準とした時の、HBV、HCVの感染既往リスク比は、②初回献血時期がそれ以外の者でＢ型1.31（95%CI：0.38-4.04）とＣ型1.24（95%CI：0.38-4.04）、③献血歴のない者で、Ｂ型1.55（95%CI：0.72-3.32）とＣ型1.61（95%CI：0.5-5.13）であった。①,②,③の人口割合は、男で3.5%、57.2%、39.3%、女で2.8%、49.6%、47.6%であった。以上から、上記補正係数は、HBs抗原陽性率で男1.39、女1.42、HCV抗体陽性率で男1.38、女1.41となった。これを上記献血者データ由来の感染者数に乗じ、2010年時点の26～79歳のHBVキャリア数を、男714,600人、女564,600人、HCVキャリア数を、男632,900人、女664,300人と推計した。

【結論】 2010年時点の肝細胞癌に移行した者を除くHBVとHCV感染者数の合計（26～79歳）は、合計で約250万人と推計された。

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Association of serum IFN-λ_3 with inflammatory and fibrosis markers in patients with chronic hepatitis C virus infection

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Abstract

Background Hepatitis C virus (HCV) is one of the major causes of liver cancer. The single nucleotide polymorphisms within the *IFNL3* gene, which encodes interferon (IFN)-λ_3, are strongly associated with the response to pegylated IFN-α (PEG-IFN-α) plus ribavirin (RBV) therapy in chronic hepatitis C (C-CH) patients. However, the roles of IFN-λ_3 in chronic HCV infection are still elusive. In this study, we aimed to identify clinical and immunological factors influencing IFN-λ_3 and evaluated whether serum IFN-λ_3 levels are involved or not involved in the response to PEG-IFN-α plus RBV therapy.

Methods We enrolled 119 C-CH patients with HCV genotype 1 infection who underwent 48 weeks of PEG-IFN-α plus RBV therapy. As controls, 23 healthy subjects and 56 patients with non-HCV viral hepatitis were examined. Serum IFN-λ_3 was quantified by chemiluminescence enzyme immunoassay, and 27 cytokines or chemokines were assayed by the multiplexed BioPlex system.

Results Serum IFN-λ_3 levels were higher in C-CH patients or acute hepatitis E patients than in healthy volunteers. Such levels did not differ between the *IFNL3* genotypes. In C-CH patients, serum IFN-λ_3 was positively correlated with aspartate aminotransferase, alanine aminotransferase, α-fetoprotein, histological activity, fibrosis index, IFN-γ-inducible protein 10, and platelet-derived growth factor. Multivariate analysis showed that *IFNL3* single nucleotide polymorphisms, fibrosis score, and macrophage inflammatory protein 1α were involved in the

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sustained viral clearance in PEG-IFN-α plus RBV therapy; however, serum IFN-λ_3 levels were not involved.
Conclusion Serum IFN-λ_3 levels are increased in C-CH patients regardless of the *IFNL3* genotype. IFN-λ_3 is a biomarker reflecting the activity and fibrosis of liver disease, but is not correlated with the responsiveness to PEG-IFN-α plus RBV therapy.

Keywords Hepatitis C virus · IL-28B · Interferon-λ_3 · Chemokine · Pegylated interferon-α plus ribavirin

Abbreviations

APRI	Aspartate aminotransferase platelet ratio index
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
B-CH	Chronic hepatitis B
C-CH	Chronic hepatitis C
FIB-4	Fibrosis-4
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HV	Healthy volunteer
IFN	Interferon
IP-10	Interferon-γ-inducible protein 10
MIP	Macrophage inflammatory protein
PDGF-BB	Platelet-derived growth factor BB
PEG-IFN-α	Pegylated interferon-α
RANTES	Regulated on activation, normally T cell expressed, and secreted
RBV	Ribavirin
SNP	Single nucleotide polymorphism
SVR	Sustained virological response

Introduction

Hepatitis C virus (HCV) is one of the leading causes of liver cirrhosis and hepatocellular carcinoma (HCC), with nearly 170 million people infected worldwide [1]. A combination therapy with pegylated interferon (IFN)-α (PEG-IFN-α) and ribavirin (RBV) has been used for chronic hepatitis C (C-CH) patients as the standard of care, achieving sustained virological response (SVR) in 42–52 % of genotype 1 patients [2]. Even in the coming era of all oral and IFN-free regimens for the treatment of C-CH patients [3–5], PEG-IFN-α plus RBV therapy could hold promise for elderly patients with advanced fibrosis and high risk of HCC.

Genome-wide association studies, including ours, have demonstrated that single nucleotide polymorphisms (SNPs) upstream of the promoter region within the *IFNL3* gene (also known as *IL28B*), which encodes a type III IFN (IFN-λ_3), are strongly associated with the response to PEG-IFN-α plus RBV therapy in C-CH patients [6–9]. Although such significant impact of the *IFNL3* genotype on the outcome of the combination therapy is well acknowledged, the biological and clinical roles of IFN-λ_3 in chronic HCV infection are still elusive. Furthermore, it is controversial if patients with the *IFNL3* major genotype are capable of producing larger amounts of IFN-λ_3 than those with the minor genotype.

The IFN-λ family consists of several subtypes, such as IFN-λ_1 (IL-29), IFN-λ_2 (IL-28A), and IFN-λ_3 (IL-28B), which are biologically active for the suppression of HCV replication [10, 11]. On initial exposure to HCV, primary human hepatocytes in vitro produced IFN-λ and subsequently induced antiviral IFN-stimulated genes [12]. It is thus rational to consider that the more IFN-λ family members are produced in the exposed hosts, the more likely they are to protect the hosts from HCV virulence in the primary infection. However, in chronically HCV-infected patients, it has not been proven that such a scenario could be applicable for the outcome of the disease.

To gain insight into the role of IFN-λ_3 in chronic HCV infection, we aimed to clarify the factors influencing serum IFN-λ_3 levels, including *IFNL3* genotype, clinical parameters, and various cytokines and chemokines. For application in clinical practice, we evaluated whether serum IFN-λ_3 levels are associated or not associated with the response to PEG-IFN-α plus RBV therapy for C-CH patients.

Materials and methods

Study subjects

One hundred nineteen Japanese patients with C-CH (genotype 1b and high viral load) were enrolled in the study. All patients were negative for hepatitis B virus (HBV) and human immunodeficiency virus (HIV) and did not have any other chronic liver diseases, such as alcoholic, autoimmune, and fatty liver disease. The presence of HCC was ruled out by ultrasonography or computed tomography examinations. The patients had been followed at the National Center for Global Health and Medicine Kohnodai Hospital, the National Hospital Organization Nagasaki Medical Center, Shin-Kokura Hospital, and Musashino Red Cross Hospital. They were treated with PEG-IFN-α_{2b} (subcutaneously once a week; 1.5 μg/kg body weight) or PEG-IFN-α_{2a} (180 μg once a week) plus RBV (600–1,000 mg daily depending on body weight) for 48 weeks according to the guidelines of the Japan Society of Hepatology [13]. Virological response to the combination therapy was defined according to the practical

guidelines of the American Association for the Study of Liver Diseases [14]. All patients attained adherence to PEG-IFN-α plus RBV therapy exceeding 80 % of the estimated total dose. Liver biopsy was performed before the start of the therapy. Histological activity and fibrosis were determined according to the METAVIR scoring system [15]. Serum samples were collected from the patients before PEG-IFN-α plus RBV treatment started and were stored at −80 °C. In some patients, the samples were obtained 24 weeks after the cessation of the therapy (at the end of follow-up).

As controls, serum was obtained from 23 healthy subjects without HCV, HBV, and HIV infection (male-to-female ratio, 5:5, mean age ± standard deviation, 45 ± 12 years). In the comparison of serum IFN-λ levels between C-CH patients and patients with other types of liver diseases, 11 patients with chronic HBV infection (three HBeAg-positive patients and eight HBeAg-negative patients) were examined as well. They were not treated with IFN or nucleot(s)ide analogues for HBV infection. In addition, we compared serum IFN-λ_3 levels among patients with acute viral hepatitis of various causes, such as acute hepatitis A, acute hepatitis B, or acute hepatitis E, the diagnosis of which was determined by serological examinations at Teine Keijinkai Hospital and Kurume University Hospital. The serum samples were obtained from the patients at the time of active liver inflammation [alanine aminotransferase (ALT) levels more than two times the upper limit of the normal range]. As representatives for noninvasive fibrosis markers, the fibrosis-4 (FIB-4) score and the aspartate aminotransferase (AST) platelet ratio index (APRI) were calculated as reported previously [16, 17].

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board at the National Center for Global Health and Medicine (approval ID and date, NCGM-G-001379-00, March 14, 2013) and the ethical committee of each institute. Written informed consent was obtained from all patients.

IFNL3 genotyping

The subjects were evaluated for SNPs near the *IFNL3* gene (rs8099917) using the Invader Plus assay (Invader Chemistry, Madison, WI, USA) as previously reported [18]. The TT, TG, and GG genotypes were determined accordingly.

Measurement of serum IFN-λ_3

Serum levels of IFN-λ_3 were evaluated by the newly developed chemiluminescence enzyme immunoassay system as reported previously [19]. The system enables one to quantify serum IFN-λ_3 specifically without any overlap from IFN-λ_1 and IFN-λ_2. The threshold of the assay is 10 pg/mL and its range is 10–1,000 pg/mL.

Simultaneous measurement of multiple chemokines and cytokines

To quantify multiple chemokines and cytokines simultaneously in the limited volume of the samples, we used the BioPlex 3D system (BioPlex Pro Human GI 27Plex; Bio-Rad, Hercules, CA, USA) for the study. In this system, 27 chemokines and cytokines were measurable, such as basic fibroblast growth factor, eotaxin, granulocyte colony stimulating factor, granulocyte–macrophage colony stimulating factor, IL-1β, IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, IFN-γ-inducible protein 10 (IP-10), monocyte chemotactic protein 1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor BB (PDGF-BB), regulated on activation, normally T cell expressed, and secreted (RANTES), TNF-α, and vascular endothelial growth factor. The detection range and thresholds are given in Table S1. For the measurement of IP-10, ELISA (R&D Systems, MN, USA) was performed as well.

Statistical analyses

Continuous variables were compared between groups using the Wilcoxon signed-rank test and the Mann–Whitney *U* test, and categorical data were compared using the χ^2 test or Fisher's exact test. The correlations between cytokines, chemokines, and clinical markers were evaluated by Spearman's correlation coefficient. A p value below 0.05 was considered to be significant. Logistic regression was used for multivariate analyses. All statistical analyses were performed with PRISM and SPSS.

Results

Serum IFN-λ_3 levels are increased in patients with chronic HCV infection

The clinical backgrounds of C-CH patients are shown in Table 1. First, we compared serum IFN-λ_3 levels among patients with C-CH or chronic hepatitis B (B-CH) and uninfected healthy volunteers (HVs). Such levels in the C-CH group were significantly higher than those in the B-CH group or the HV group (Fig. 1a). The levels in the B-CH group were increased, but the significance of this was much less than in the C-CH group (Fig. 1a). When we compared serum IFN-λ_3 levels in B-CH patients between

Table 1 Clinical backgrounds of the patients with chronic hepatitis C virus (*HCV*) infection

Factors	Values
Number	119 (69 male, 50 female)
Age (years)	56.5 ± 10.1
WBC (/mm^3)	5,120 ± 1,575
Hb (g/dL)	14.4 ± 1.5
Plt (×10^4/mm^3)	17.7 ± 5.2
TP (g/dL)	7.5 ± 0.5
Alb (g/dL)	4.2 ± 0.4
AST (U/L)	54.7 ± 38.3
ALT (U/L)	71.5 ± 54.2
T-bil (mg/dL)	0.8 ± 0.3
T-chol (mg/dL)	176.6 ± 37.0
AFP (ng/mL)	9.7 ± 13.4
HCV RNA (log IU/mL)	6.3 ± 0.6
Activity (A0/A1/A2/A3)	1/68/33/2
Fibrosis (F1/F2/F3/F4)	48/36/16/4
IFNL3 rs8099917 (TT/non-TT)	100:19

Alb albumin, *AFP* α-fetoprotein, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *Hb* hemoglobin, *Plt* platelets, *T-bil* total bilirubin, *T-chol* total cholesterol, *TP* total protein, *WBC* white blood cells

HBeAg-positive and HBeAg-negative patients, we found no difference between them (2.5 ± 0.9 pg/mL vs 1.8 ± 1.7 pg/mL, respectively). Next, we compared serum IFN-λ$_3$ levels between patients with the *IFNL3* TT genotype and those with the TG/GG (non-TT) genotype in the C-CH group. Although some patients in the TT group showed relatively higher levels of IFN-λ$_3$ than those in the non-TT group, this difference between the TT and non-TT groups did not reach significance (Fig. 1b). Third, we compared serum IFN-λ$_3$ levels before and after the combination therapy in the relevant cases. In patients who successfully eradicated HCV (SVR), serum IFN-λ$_3$ levels were significantly decreased at 24 weeks after the therapy. In contrast, such levels did not change in those patients who failed to eradicate HCV (transient virological response and no virological response groups, respectively) (Fig. 1c). Fourth, we compared serum IFN-λ$_3$ levels among patients with various causes of acute viral hepatitis. Unfortunately, serum samples from acute hepatitis C patients were not available in this study. The IFN-λ$_3$ levels in the acute hepatitis E group were higher than those in the HVs (Fig. 1d). The IFN-λ$_3$ levels in the acute hepatitis B group tended to be higher than those in the HVs; however, statistical analysis was not performed because of the limited number of samples ($N = 2$). No significant difference was observed between the acute hepatitis A and HV groups. These results indicate that serum IFN-λ$_3$ levels are increased in patients with C-CH or acute hepatitis E.

Serum IFN-λ$_3$ levels may be related to liver inflammation or fibrosis in patients with C-CH

To explore the clinical significance of IFN-λ$_3$ in chronic HCV infection, we simultaneously examined 27 chemokines and cytokines in serum by means of the BioPlex system, which allows one to measure multiple factors at high sensitivity in a small volume of samples (10 μL per sample). In comparison with the results for HVs, we found that the levels of some chemokines in the C-CH group were higher than those in the HV group, such as IP-10, MIP-1α, MIP-1β, RANTES, and PDGF-BB (Figs. 2, S1).

Next, we examined whether serum IFN-λ$_3$ levels are correlated or not correlated with clinical parameters or immunological markers in the C-CH group. The IFN-λ$_3$ levels were weakly and positively correlated with AST, ALT, and α-fetoprotein levels and histological activity (Table 2). These results indicate that the increase of serum IFN-λ$_3$ levels in patients with C-CH is related to liver inflammation. The FIB-4 score and the APRI are representatives of noninvasive markers of liver fibrosis. The levels of serum IFN-λ$_3$ were positively correlated with the APRI, but not with the FIB-4 score (Table 2). With regard to the chemokines displaying higher values in the C–CH group, the levels of IP-10 and PDGF-BB were positively correlated with the IFN-λ$_3$ levels (Table 2). Such chemokines are reported to be involved in the early stage of liver fibrosis [20–22]. Thus, serum levels of IFN-λ$_3$ may be related to the fibrotic markers as well. To clarify the mechanisms causing the increase of serum IFN-λ$_3$ levels in B-CH patients, we examined the correlations between serum IFN-λ$_3$ levels and clinical markers and fibrosis indices. Serum IFN-λ$_3$ levels were correlated with the levels of AST ($r = 0.64$, $p = 0.03$) and total cholesterol ($r = -0.76$, $p = 0.03$), FIB-4 score ($r = 0.65$, $p = 0.03$), and APRI ($r = 0.76$, $p = 0.007$) (Table S2). In addition, serum IFN-λ$_3$ levels tended to be higher in HBV-positive patients with liver cirrhosis or HCC (3.0 ± 3.1 pg/mL in liver cirrhosis patients and 4.1 ± 4.7 pg/mL in HCC patients, respectively) (Fig. S2). These results show that serum IFN-λ$_3$ levels are related to liver inflammation and fibrosis not only in C-CH patients but also in B-CH patients.

Pretreatment serum IFN-λ$_3$ is not related to SVR to PEG-IFN-α plus RBV therapy in patients with C-CH

Because the *IFNL3* genotype is a strong predictor of the efficacy of PEG-IFN-α plus RBV therapy for C-CH, we sought to examine the clinical value of serum IFN-λ$_3$ in patients who underwent the combination therapy. In a comparison of the clinical and immunological factors between the SVR and non-SVR groups, univariate analysis

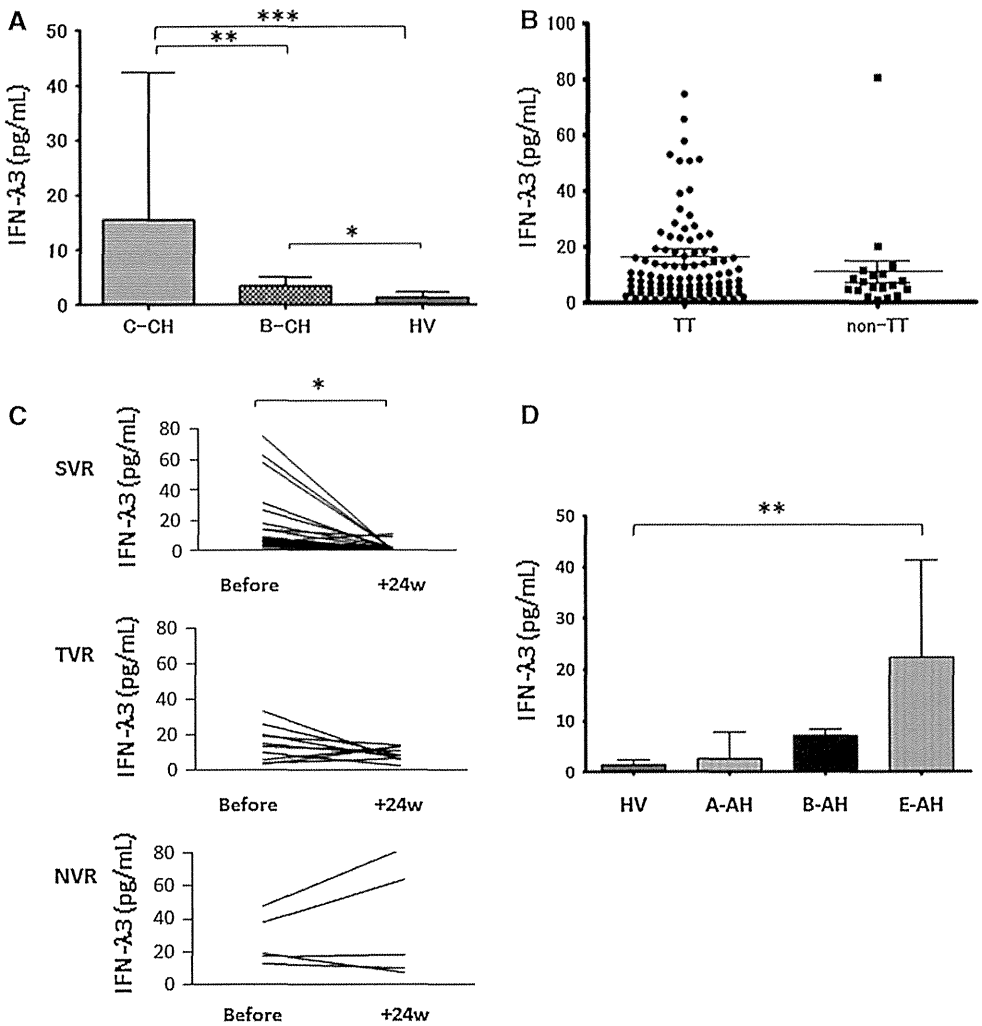

Fig. 1 Serum interferon-λ_3 (*IFN-λ3*) levels are increased in patients with chronic hepatitis C virus infection or acute hepatitis E virus infection. **a** Serum IFN-λ_3 levels in patients with chronic hepatitis C (*C–CH*; $N = 119$), patients with chronic hepatitis B (*B-CH*; $N = 11$), and healthy volunteers (*HV*; $N = 23$) were quantified by the chemiluminescence enzyme immunoassay (CLEIA) method as described in "Materials and methods." *One asterisk* $p < 0.05$, *two asterisks* $p < 0.01$, *three asterisks* $p < 0.0001$ by the Mann–Whitney *U* test. **b** Serum IFN-λ_3 levels in the C-CH group were compared between the patients with the *IFNL3* TT (rs8099917) genotype ($N = 100$) and those with non-TT (TG/GG) genotype ($N = 19$). **c** Serum IFN-λ_3 levels in C-CH patients were compared before and 24 weeks after the pegylated interferon-α plus ribavirin therapy. *SVR* sustained virological response ($N = 21$), *TVR* transient virological response ($N = 10$), *NVR* nonvirological response ($N = 5$), *one asterisk* $p < 0.05$ by Wilcoxon's signed-rank test. **d** Serum IFN-λ_3 levels in acute hepatitis patients of various causes were quantified by CLEIA as described in "Materials and methods." All samples were collected from patients whose alanine aminotransferase levels were two times higher than the upper limit of the normal range. *HV* healthy volunteers ($N = 23$), *A-AH* acute hepatitis A patients ($N = 34$), *B-AH* acute hepatitis B patients ($N = 2$), *E-AH* acute hepatitis E patients ($N = 9$), *two asterisks* $p < 0.0001$ by the Mann–Whitney *U* test

revealed that AST, *IFNL3* genotype, fibrosis score, and MIP-1α were associated with the SVR (Table 3). However, serum IFN-λ_3 or IP-10 levels were not different between the SVR and non-SVR groups (Table 3). Subsequently, multivariate analysis including such factors of significance ($p < 0.05$ by univariate analysis) showed that *IFNL3* SNPs, fibrosis score, and MIP-1α were involved in the SVR (Table 3). These results suggest that serum IFN-λ_3 fails to be a predictive marker for SVR in PEG-IFN-α plus RBV therapy.

Discussion

In this study, we demonstrated that serum IFN-λ_3 levels were higher in patients with C-CH than in uninfected or HBV-positive patients, the levels in whom did not differ regardless of the *IFNL3* genotype. Serum IFN-λ_3 levels were correlated with clinical and immunological markers of liver inflammation and fibrosis, suggesting that the production of IFN-λ_3 may be regulated by not only the presence or absence of HCV but also by the status of liver

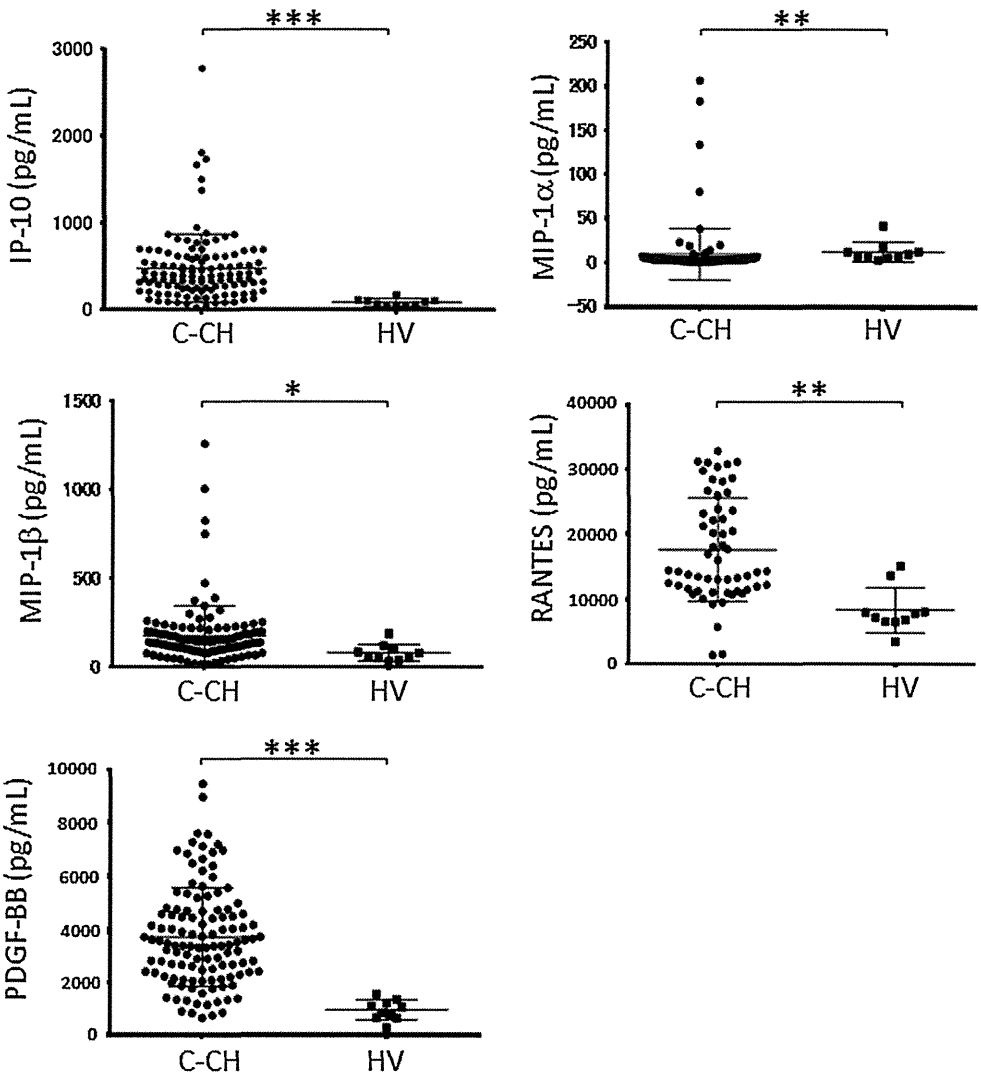

Fig. 2 The levels of several chemokines are increased in patients with chronic hepatitis C virus infection. Twenty-seven chemokines and cytokines in serum from chronic hepatitis C patients (*C-CH*) and healthy volunteers (*HV*) were assayed by means of the BioPlex method. Interferon-γ-inducible protein 10 (*IP-10*) was measured by ELISA. Representative results for chemokines that showed statistical significance between the groups are shown, such as IP-10, macrophage inflammatory protein 1α (*MIP-1α*), macrophage inflammatory protein 1β (*MIP-1β*), regulated on activation, normally T cell expressed, and secreted (*RANTES*), and platelet-derived growth factor BB (*PDGF-BB*). * $p < 0.005$, ** $p < 0.001$, *** $p < 0.0001$ by the Mann–Whitney *U* test

disease. It is well acknowledged that *IFNL3* genotype is a strong predictor of SVR in PEG-IFN-α plus RBV therapy for C-CH [7–9]. However, serum IFN-λ_3 fails to be a surrogate marker for *IFNL3* genotype in the combination therapy.

On primary HCV infection, IFN-λ is produced by hepatocytes that subsequently induce antiviral IFN-stimulated genes [23]. Parallel reduction of serum IFN-λ_3 levels in C-CH patients who attained SVR by PEG-IFN-α plus RBV treatment indicates that the presence of HCV is involved in the production of IFN-λ_3. In addition to hepatocytes, dendritic cells or macrophages are capable of producing IFN-λ in response to HCV [24]. For sensing HCV, hepatocytes and BDCA3$^+$ dendritic cells mainly utilize Toll-like receptor 3 and retinoic acid inducible gene I, and plasmacytoid dendritic cells utilize Toll-like receptor 7 [24, 25]. It is yet to be clarified which cells—hepatocytes or dendritic cells—have stronger potential to secrete IFN-λ at the single-cell level. However, it is rational to consider that serum IFN-λ_3 levels in patients are determined by the sum of IFN-λ_3 sporadically released from both types of cells. Therefore, it is plausible that the amount of IFN-λ released from hepatocytes or dendritic cells is influenced by the environment of the producers, such as inflammation and fibrosis. A positive correlation observed between serum IFN-λ_3 levels and AST levels, FIB-4 score, and APRI in B-CH patients may support such a possibility. In this study, serum IFN-λ_3 levels in the B-CH group were higher than those in HVs. However, this difference was slim compared with the difference between the C-CH group and HVs, suggesting that the difference in their genome structure, either RNA or DNA virus, may influence IFN-λ_3 production by infected cells. Of interest is the finding that serum IFN-λ_3 levels were higher in patients with acute hepatitis E than in patients with acute hepatitis A. It is reported that dendritic cells localized in the

Table 2 Correlation of interferon-λ_3 (*IFN-λ_3*) with clinical or immunological parameters in patients with chronic hepatitis C

Factors	CC with IFN-λ_3	*p*
Age (years)	−0.10	–
WBC (/mm^3)	−0.05	–
Hb (g/dL)	0.07	–
Plt ($\times 10^4$/mm^3)	−0.09	–
TP (g/dL)	0.07	–
Alb (g/dL)	−0.01	–
AST (U/L)	0.34	<0.0001
ALT (U/L)	0.34	<0.0001
T-bil (mg/dL)	0.03	–
T-chol (mg/dL)	−0.22	0.02
AFP (ng/mL)	0.30	0.001
HCV RNA (log IU/mL)	−0.05	–
Fibrosis score	0.07	–
Histological activity score	0.25	0.01
FIB-4 score	0.10	–
APRI	0.29	0.001
IP-10 (pg/mL)	0.53	<0.0001
MIP-1α (pg/mL)	−0.08	–
MIP-1β (pg/mL)	−0.18	–
RANTES (pg/mL)	0.26	–
PDGF-BB (pg/mL)	0.40	<0.0001

Alb albumin, *AFP* α-fetoprotein, *ALT* alanine aminotransferase, *APRI* aspartate aminotransferase platelet ratio index, *AST* aspartate aminotransferase, *CC* correlation coefficient by Spearman's analysis, *FIB-4* fibrosis-4, *Hb* hemoglobin, *HCV* hepatitis C virus, *IP-10* interferon-γ-inducible protein 10, *MIP-1α* macrophage inflammatory protein 1α, *MIP-1β* macrophage inflammatory protein 1β, *PDGF-BB* platelet-derived growth factor BB, *Plt* platelets, *RANTES* regulated on activation, normally T cell expressed, and secreted, *T-bil* total bilirubin, *T-chol* total cholesterol, *TP* total protein, *WBC* white blood cells

intestine are capable of producing IFN-λ in response to rotavirus to protect the host from infection [26]. Although both hepatitis E virus and hepatitis A virus are RNA viruses that are transmissible by the enterofecal route, the difference in serum IFN-λ_3 levels suggests that there are distinct mechanisms of recognition of hepatitis E virus and hepatitis A virus by the hosts. Further investigation is needed to disclose which pattern recognition receptors are utilized in hepatocytes or immune cells for the recognition of such viruses to produce IFN-λ.

The regulatory mechanisms of transcription and translation of IFN-λ_3 have not been well documented. The *IFNL3* SNPs (rs8099917) are located 8.9 kb upstream of the promoter region of the *IFNL3* gene [8, 9, 11]. Because of such localization, it is less likely that the genetic variation has some impact on the transcriptional level of *IFNL3*. With regard to the relationship between the *IFNL3* genotype and its transcripts, controversial results have been reported thus far. Some groups reported that IFN-λ_3 messenger RNA levels in peripheral blood mononuclear cells were higher in patients with the *IFNL3* major genotype than in those with the minor genotype [9]. In contrast, others showed that in hepatocytes such levels were comparable regardless of *IFNL3* SNPs. In the search for some genetic factors influencing *IFNL3* transcription, Sugiyama et al. [27] reported the existence of variable-length TA repeats in the promoter of the *IFNL3* gene. Other investigators showed that a certain structure of the 3′ untranslated region in the *IFNL3* gene is involved in the durability/stability of the gene [28]. Nevertheless, the contribution of such factors is not enough to fill in the gap, suggesting that certain other regulatory factors for *IFNL3* are still to be revealed.

Reports concerning serum IFN-λ in C-CH patients are limited. Langhans et al. [29] showed that serum levels of IFN-λ, which includes IFN-λ_2 and IFN-λ_3, were higher in patients with the *IFNL3* major genotype than in those with the minor genotype. One of the limitations of their study seems to be the lack of specificity for the measurement of IFN-λ_3. Since the homology of *IFNL2* (which encodes IFN-λ_2) and *IFNL3* is quite high, it is difficult to quantify specifically IFN-λ_3 by excluding contamination by IFN-λ_2. To exclude such a possibility, we used the newly developed chemiluminescence enzyme immunoassay for IFN-λ_3, which enables one to quantify IFN-λ_3 without any influence from IFN-λ_2 in the range from 0 to 1,000 pg/mL. By means of this system, we found that serum levels of IFN-λ_3 are not statistically different between patients with the *IFNL3* major genotype and those with the minor genotype.

On primary HCV exposure, the significance of IFN-λ family members as an antiviral protein is evident. However, such impact of IFN-λ_3 in chronically HCV-infected patients is still elusive. Langhans et al. [29] reported that serum IFN-λ levels in patients who had spontaneously cleared HCV were higher than in patients with chronic HCV infection, implying that a higher level of IFN-λ somewhat contributed to HCV eradication. In this study, we aimed to clarify the significance of IFN-λ_3 in patients with chronic HCV infection with different approaches. Firstly, we searched for the factors influencing serum IFN-λ_3 quantity by correlation analysis with clinical markers and multiple cytokines/chemokines. We found that AST, ALT, and α-fetoprotein levels and histological activity were positively correlated with serum IFN-λ_3 levels. In addition, one of the noninvasive fibrosis markers, APRI, was weakly correlated with serum IFN-λ_3 levels. Among the chemokines examined in this study, serum IFN-λ_3 was positively correlated with IP-10 and PDGF-BB. IP-10 (CXCL10) is induced in HCV-infected hepatocytes as one of the IFN-stimulated genes, and attracts CXCR3-positive T cells and natural killer cells and subsequently activates inflammation. IP-10 is also reported to be involved in the early stage of liver fibrosis [30, 31]. A similar fibrotic