however, we cannot rule out the possibility of an outside source for genotype III strains, since the HE-JA7 and HE-JI3 strains were interspersed among American swine and human strains in the phylogenetic tree based on the partial ORF2 sequences (Fig. 2).

Anti-HEV IgM and IgA antibodies were detectable in our patient for more than 6 months after the onset of disease. Furthermore, anti-HEV IgG antibodies continued to be detected and remained at high levels for more than 8 years after the onset of disease. Khuroo *et al.* (1993) reported that anti-HEV IgG antibodies were detected in 47 % of patients with HEV infection after 14 years. However, as Krawczynski & Aggarwal (1999) pointed out, it might be difficult to determine the exact duration of persistence of anti-HEV IgG antibodies in endemic areas because of repeated exposure to HEV. On the other hand, in Japan, a country with low endemicity and where clinical HEV infection rarely occurs, there seems to be little or no repeated exposure. The chance of repeated exposure may be negligible in our patient, as supported further by the fact that the patient's husband who lives with the patient was negative for anti-HEV IgG antibodies both in 1993 and 2002. Therefore, we speculate that anti-HEV IgG antibodies persist for more than 10 years after the onset of sporadic acute hepatitis E in industrialized countries with low endemicity as well. Regarding the duration of seropositivity for anti-HEV IgM, it has been reported that, of sera collected from patients during various hepatitis E outbreaks 3–4 and 6–12 months after the onset of jaundice, 50 and 40 %, respectively, were positive for anti-HEV IgM antibodies (Favorov *et al.*, 1996). Our patient continued to be positive for anti-HEV IgM antibodies for more than 9 months during the convalescent phase. Taken together, seropositivity for anti-HEV IgM antibodies can be regarded as the marker of choice as a diagnostic indicator of recent HEV infection in both developing and industrialized countries. As for anti-HEV IgA antibodies, it is unclear whether our assay is detecting both dimeric secretory IgA and monomeric IgA. However, it seems that anti-HEV IgA antibodies can be utilized as an additional confirmatory antibody for recent HEV infection, as suggested by Chau *et al.* (1993) who detected anti-HEV IgA antibodies in serum samples obtained from patients who had acute waterborne hepatitis in southern Somalia and Pakistan.

In conclusion, the finding that genotype III HEV was present in Japan in the early 1990s raises the questions of when variant HEV strains emerged and how widespread they were in industrialized countries. The increasing globalization of the world marketplace and increased overseas travel may facilitate the spread of HEV variants into industrialized countries that were believed to be non-endemic. The reported high prevalence of anti-HEV antibodies in a number of animal species, such as pigs, rats and mice, may suggest that multiple sources of exposure to HEV may exist in the general population in industrialized countries that are not at apparent risk for exposure to HEV (Purcell & Emerson, 2001b). Whether the domestic spread of HEV infection in industrialized countries is via zoonosis and/or food deserves further analysis.

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REFERENCES

Chau, K. H., Dawson, G. J., Bile, K. M., Magnius, L. O., Sjogren, M. H. & Mushahwar, I. K. (1993). Detection of IgA class antibody to hepatitis E virus in serum samples from patients with hepatitis E virus infection. *J Med Virol* **40**, 334–338.

Erker, J. C., Desai, S. M., Schlauder, G. G., Dawson, G. J. & Mushahwar, I. K. (1999). A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. *J Gen Virol* **80**, 681–690.

Favorov, M. O., Khudyakov, Y. E., Mast, E. E. & 7 other authors (1996). IgM and IgG antibodies to hepatitis E virus (HEV) detected by an enzyme immunoassay based on an HEV-specific artificial recombinant mosaic protein. *J Med Virol* **50**, 50–58.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Garkavenko, O., Obriadina, A., Meng, J., Anderson, D. A., Benard, H. J., Schroeder, B. A., Khudyakov, Y. E., Fields, H. A. & Croxson, M. C. (2001). Detection and characterisation of swine hepatitis E virus in New Zealand. *J Med Virol* **65**, 525–529.

Harrison, T. J. (1999). Hepatitis E virus: an update. *Liver* **19**, 171–176.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S. & Shimotohno, K. (1991). Hypervariable regions in the putative glycoprotein of hepatitis C virus. *Biochem Biophys Res Commun* **175**, 220–228.

Hsieh, S. Y., Meng, X. J., Wu, Y. H., Liu, S. T., Tam, A. W., Lin, D. Y. & Liaw, Y. F. (1999). Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. *J Clin Microbiol* **37**, 3828–3834.

Huang, F. F., Haqshenas, G., Guenette, D. K., Halbur, P. G., Schommer, S. K., Pierson, F. W., Toth, T. E. & Meng, X. J. (2002). Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *J Clin Microbiol* **40**, 1326–1332.

Kabrane-Lazizi, Y., Meng, X. J., Purcell, R. H. & Emerson, S. U. (1999). Evidence that the genomic RNA of hepatitis E virus is capped. *J Virol* **73**, 8848–8850.

Khuroo, M. S., Kamili, S., Dar, M. Y., Moecklil, R. & Jameel, S. (1993). Hepatitis E and long-term antibody status. *Lancet* **341**, 1355.

Krawczynski, K. & Aggarwal, R. (1999). Hepatitis E. In *Schiff's Diseases of the Liver*, 8th edn, vol. 1, pp. 849–860. Edited by E. R. Schiff, M. F. Sorrell & W. C. Maddrey. Philadelphia: Lippincott – Raven.

Kwo, P. Y., Schlauder, G. G., Carpenter, H. A., Murphy, P. J., Rosenblatt, J. E., Dawson, G. J., Mast, E. E., Krawczynski, K. & Balan, V. (1997). Acute hepatitis E by a new isolate acquired in the United States. *Mayo Clin Proc* **72**, 1133–1136.

Meng, X. J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. & Emerson, S. U. (1997). A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci U S A* **94**, 9860–9865.

Meng, X. J., Halbur, P. G., Shapiro, M. S., Govindarajan, S., Bruna, J. D., Mushahwar, I. K., Purcell, R. H. & Emerson, S. U. (1998). Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. *J Virol* **72**, 9714–9721.

Meng, X. J., Wiseman, B., Elvinger, F., Guenette, D. K., Toth, T. E., Engle, R. E., Emerson, S. U. & Purcell, R. H. (2002). Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. *J Clin Microbiol* **40**, 117–122.

Meyerhans, A., Cheynier, R., Albert, J., Seth, M., Kwok, S., Sninsky, J., Morfeldt-Manson, L., Asjo, B. & Wain-Hobson, S. (1989). Temporal fluctuations in HIV quasispecies *in vivo* are not reflected by sequential HIV isolations. *Cell* **58**, 901–910.

Mizuo, H., Suzuki, K., Takikawa, Y. & 8 other authors (2002). Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. *J Clin Microbiol* **40**, 3209–3218.

Okamoto, H., Takahashi, M., Nishizawa, T., Fukai, K., Muramatsu, U. & Yoshikawa, A. (2001). Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. *Biochem Biophys Res Commun* **289**, 929–936.

Page, R. D. M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Pina, S., Buti, M., Cotrina, M., Piella, J. & Girones, R. (2000). HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. *J Hepatol* **33**, 826–833.

Purcell, R. H. & Emerson, S. U. (2001a). Hepatitis E virus. In *Fields Virology*, 4th edn, pp. 3051–3061. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Purcell, R. H. & Emerson, S. U. (2001b). Animal models of hepatitis A and E. *ILAR J* **42**, 161–177.

Reyes, G. R., Purdy, M. A., Kim, J. P., Luk, K. C., Young, L. M., Fry, K. E. & Bradley, D. W. (1990). Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. *Science* **247**, 1335–1339.

Saitou, N. & Nei, M. (1987), The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schlauder, G. G. & Mushahwar, I. K. (2001). Genetic heterogeneity of hepatitis E virus. *J Med Virol* **65**, 282–292.

Schlauder, G. G., Dawson, G. J., Erker, J. C., Kwo, P. Y., Knigge, M. F., Smalley, D. L., Rosenblatt, J. E., Desai, S. M. & Mushahwar, I. K. (1998). The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. *J Gen Virol* **79**, 447–456.

Schlauder, G. G., Desai, S. M., Zanetti, A. R., Tassopoulos, N. C. & Mushahwar, I. K. (1999). Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV. *J Med Virol* **57**, 243–251.

Schlauder, G. G., Frider, B., Sookoian, S., Castano, G. C. & Mushahwar, I. K. (2000). Identification of 2 novel isolates of hepatitis E virus in Argentina. *J Infect Dis* **182**, 294–297.

Takahashi, K., Iwata, K., Watanabe, N., Hatahara, T., Ohta, Y., Baba, K. & Mishiro, S. (2001). Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. *Virology* **287**, 9–12.

Takahashi, K., Kang, J. H., Ohnishi, S., Hino, K. & Mishiro, S. (2002a). Genetic heterogeneity of hepatitis E virus recovered from Japanese patients with acute sporadic hepatitis. *J Infect Dis* **185**, 1342–1345.

Takahashi, M., Nishizawa, T., Yoshikawa, A., Sato, S., Isoda, N., Ido, K., Sugano, K. & Okamoto, H. (2002b). Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. *J Gen Virol* **83**, 1931–1940.

Tam, A. W., Smith, M. M., Guerra, M. E., Huang, C. C., Bradley, D. W., Fry, K. E. & Reyes, G. R. (1991). Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* **185**, 120–131.

Wang, Y., Ling, R., Erker, J. C., Zhang, H., Li, H., Desai, S., Mushahwar, I. K. & Harrison, T. J. (1999). A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. *J Gen Virol* **80**, 169–177.

Wang, Y., Zhang, H., Ling, R., Li, H. & Harrison, T. J. (2000). The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. *J Gen Virol* **81**, 1675–1686.

Wang, Y., Levine, D. F., Bendall, R. P., Teo, C. G. & Harrison, T. J. (2001). Partial sequence analysis of indigenous hepatitis E virus isolated in the United Kingdom. *J Med Virol* **65**, 706–709.

Wang, Y., Zhang, H., Xia, N. & 11 other authors (2002). Prevalence, isolation, and partial sequence analysis of hepatitis E virus from domestic animals in China. *J Med Virol* **67**, 516–521.

Weiner, A. J., Brauer, M. J., Rosenblatt, J. & 8 other authors (1991). Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. *Virology* **180**, 842–848.

Worm, H. C., Schlauder, G. G., Wurzer, H. & Mushahwar, I. K. (2000). Identification of a novel variant of hepatitis E virus in Austria: sequence, phylogenetic and serological analysis. *J Gen Virol* **81**, 2885–2890.

Wu, J. C., Chen, C. M., Chiang, T. Y., Tsai, W. H., Jeng, W. J., Sheen, I. J., Kin, C. C. & Meng, X. J. (2002). Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. *J Med Virol* **66**, 488–492.

Zanetti, A. R., Schlauder, G. G., Romano, L., Tanzi, E., Fabris, P., Dawson, G. J. & Mushahwar, I. K. (1999). Identification of a novel variant of hepatitis E virus in Italy. *J Med Virol* **57**, 356–360.

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Circulating KL-6 level at baseline is a predictive indicator for the occurrence of interstitial pneumonia during interferon treatment for chronic hepatitis C

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Abstract

Interstitial pneumonia (IP) is a serious adverse event of interferon alpha (IFNα) treatment for chronic hepatitis C (CH-C). Among 558 CH-C patients who received IFNα treatment with or without ribavirin between January 1992 and June 2002, six patients (1.1%) developed IP, including one patient who developed IP in 1993 and again in 2002. Among the seven cases who contracted IP, at the onset of IP, seven (100%), five (71%), and two cases (29%) had elevated serum levels of KL-6, surfactant protein A (SP-A), and surfactant protein D (SP-D), respectively. Prior to starting IFN treatment (baseline), the serum SP-A and SP-D levels were within the normal range in all seven cases, but the serum KL-6 level was elevated in five of the seven cases, contrasting with that in three of 48 age-adjusted CH-C patients who did not develop IP during IFN treatment (71 vs. 6%; $P = 0.0003$). Furthermore, the circulating KL-6 level at baseline was significantly higher among the seven cases than among the controls (543±105 vs. 304±98 U/ml, $P = 0.0001$). These results indicate that measurement of the circulating KL-6 level in CH-C patients before IFN treatment may be useful for predicting the occurrence of IP during IFN treatment.

Keywords: KL-6; Surfactant protein A (SP-A); Surfactant protein D (SP-D); Interferon treatment; Interstitial pneumonia; Chronic hepatitis C

1. Introduction

Interferon alpha (IFNα), administered with or without ribavirin, is the most widely used antiviral drug in the treatment of chronic hepatitis C (CH-C). It has been reported that interstitial pneumonia (or pneumonitis (IP)) is one of the serious adverse events of IFNα treatment [1,2]; although IFNα-induced IP rarely occurs, it is sometimes fatal [3,4]. It was observed that IFNα-induced IP often developed when IFNα was used concomitantly with the herbal medicine, 'Sho-saiko-to' [3,5]; thus, the concomitant use of IFNα and Sho-saiko-to has been regarded as a contraindication in Japan since 1994. However, IFN-associated IP is still observed among CH-C patients who undergo IFN therapy without the concomitant use of Sho-saiko-to. As described in a review article [6], approximately 60% of patients who contracted IFN-associated IP had not been treated with Sho-saiko-to. Furthermore, a patient with hepatitis C virus (HCV) infection who was receiving low-dose IFNα treatment with ribavirin, developed IP [7]. At present, it is not known how the development of IFN-induced IP can be prevented. Thus, close observation during IFN treatment is important for diagnosing the disease in an early stage, because the disease becomes irreversible as IFN administration is continued [6].

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The serum lactate dehydrogenase (LDH) level has been used for the supplementary diagnosis of IP, although an elevated LDH level is nonspecific for the disease [8]. Recently, the serum levels of lung epithelium-specific proteins such as KL-6 [9] and hydrophilic surfactant proteins A [10] and D [11] (SP-A and SP-D, respectively) have been used for the diagnosis and management of IP. Elevated serum levels of these lung-specific epithelial proteins are thought to be caused by enhanced permeability of the air–blood barrier in the peripheral lungs [12]. KL-6 is a mucin-like high-molecular-weight glycoprotein that is strongly expressed on type 2 alveolar pneumocytes and bronchiolar epithelial cells [9]. An elevated serum KL-6 level has been noted in 70–100% of patients with IP, hypersensitivity pneumonitis, sarcoidosis, or radiation pneumonitis, although it is not seen in patients with noninterstitial lung disease [12]. Furthermore, it has been reported that monitoring of the circulating KL-6 level is useful for predicting the outcome of rapidly progressive idiopathic pulmonary fibrosis [13]. SP-A and SP-D are produced in two types of nonciliated epithelial cells in the peripheral airways, i.e. Clara and type 2 alveolar pneumocytes. Measurement of the serum levels of SP-A and SP-D was also reported to be useful for diagnosing and predicting the outcome of idiopathic IP [10,11,14]. However, to our knowledge, there has been no report on the levels of these serum markers in CH-C patients who developed IFN-induced IP. Here, we report seven cases of IFNα-associated IP, focusing on the clinical usefulness of measuring the serum levels of lung epithelium-specific proteins at baseline prior to IFN treatment.

2. Patients and methods

2.1. Patients

Among the 558 CH-C patients who received IFNα treatment with or without ribavirin in the Department of Gastroenterology at National Tokyo Hospital between January 1992 and June 2002, six patients (1.1%) developed IP during the IFN treatment. One female patient (Patient 5) developed IP during the administration of natural IFNα in 1993. After discontinuation of the IFN monotherapy, she recovered from the IP without any additional therapy, although her serum transaminase levels continued to be elevated. She strongly wanted to receive the combination therapy of IFN and ribavirin when ribavirin became available in Japan. She underwent the combination therapy of recombinant IFNα-2b and ribavirin in 2002, and developed IP again during the second IFN treatment. These two episodes of IFN-induced IP were regarded as independent cases (Case 5 and 6) in the present study, because there was a long interval between the two events (9 years) and she had no respiratory symptoms during the interval. As controls, 48 CH-C patients who did not develop IP during IFNα treatment and whose ages were not lower than the age of the youngest patient who contracted IP (Patients 3 and 6: each 54 years of age), were randomly selected and enrolled in the present study. The standard dosage of IFNα in the monotherapy was 6–10 MU/day for the first 2–8 weeks, followed by 6–10 MU, three times a week, for 16–22 weeks. The standard dosage of IFNα in the combination therapy with ribavirin was 6–10 MU/day for the first 2–4 weeks, followed by 6–10 MU, three times a week, for 20–22 weeks. The diagnosis of CH-C was based on continuous positivity for second-generation antibodies to HCV (Dainabot, Tokyo, Japan) and positivity for HCV RNA [15] in the serum for more than 6 months, and histological findings of hepatitis in liver biopsy specimens. The degree of fibrosis (staging) and necroinflammation (grading) in the liver biopsy specimens obtained from each patient just before the initiation of IFN treatment were evaluated by the scoring system of the New Inuyama classification (staging: F0–F4, grading: A0–A3) [16]. The diagnosis of IFN-induced IP was based on: (1) the absence of any respiratory symptoms before IFN treatment; (2) the appearance of respiratory symptoms during IFN treatment; (3) the emergence of infiltration shadows on bilateral lower or whole lung fields on chest computed tomography (CT) and/or X-ray [1,2,5,7]; (4) abnormalities on pulmonary function tests and arterial blood gas analysis [8]; and (5) negativity for bacteria, fungi, and acid-fast bacilli in sputum cultures. In three cases, the diagnosis of IP was confirmed by the histological findings in lung tissues compatible with IP [8], that had been obtained by autopsy in Case 2 [3] or transbronchial lung biopsy in Case 5 and 6. No CH-C patient had accompanying hepatocellular carcinoma or autoimmune hepatitis, and no patient was co-infected with hepatitis B virus or human immunodeficiency virus type 1. This study conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Ethics Committee at National Tokyo Hospital. Two cases (Case 1 and 2) were previously reported as case reports in a Japanese journal [3].

2.2. Measurement of various serum parameters

Serum samples that had been stored at −80 °C were used for testing the following markers. Quantitation of HCV RNA was carried out using a commercially available kit (Amplicor HCV Monitor assay, version 2.0; Nippon Roche, Tokyo, Japan). The HCV genotype was determined by the method described previously [17]. The serum KL-6 level was measured by enzyme immunoassay (EIA) using a commercially available kit (Eitest KL-6; Eisai, Tokyo, Japan). The serum SP-A

level was measured by the EIA method reported by Kuroki et al. [10]. The serum SP-D level was measured by EIA using a commercially available kit (SP-D EIA kit YAMASA; Yamasa Corp., Choshi, Japan). The upper limit of normal (ULN) for KL-6, SP-A and SP-D was 500 U/ml, 43.8 and 110 ng/ml, respectively.

The ULN for LDH at National Tokyo Hospital was 399 IU/l until September 1993, and then it was changed to 474 IU/l since October 1993. Thus, the LDH level in this study is presented as the ratio of 'measured value/ULN at the time of measurement'.

2.3. Statistical analysis

Data are presented as the mean ± standard deviation (S.D.). Statistical analyses were carried out using the Mann–Whitney *U*-test for comparison of continuous variables between two groups, and Fisher's exact test for comparison of proportions between two groups. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Characterization of seven cases who developed IFN-induced IP

Table 1 shows the background of the seven cases in six patients who developed IFN-induced IP. Of the six IP patients studied, three were male and three were female, and the age at onset of IP in the seven cases was 61 ± 6 year (mean ± S.D.; range, 54–66 year). Four (67%) of the six patients had a past history of blood transfusion. Although the presence of previous lung disease was recorded in three cases (43%), no case had any respiratory symptoms including dry cough or dyspnea just before IFN treatment was started. The chest radiographs of the six cases revealed no specific findings of IP before the initiation of IFN treatment, although that of Case 2 exhibited minor infiltration shadows just over the bilateral diaphragm. Patients 3 and 6 were cigarette smokers and they continued smoking until the onset of IP. Three (50%) of the six patients had a past history of drug allergy: Patient 3 was allergic to antibiotics and analgesics, Patient 5 to antibiotics, and Patient 6 to commercially available medicines for the common cold. The herbal medicine, 'Sho-saiko-to', was given to two cases; it was given during the period from 1 month before the start of IFN treatment to 1 month after the start of IFN treatment in Case 1 and during the period from 3 years before the start of IFN treatment to the onset of IP in Case 2. The remaining five cases had never been treated with Sho-saiko-to. All but one case (Case 6) had no history of previous IFN treatment. No patient had a daily alcohol intake of > 80 g.

In liver biopsy specimens taken just before the administration of IFN treatment, three cases (43%) showed a hepatic fibrosis score of 1 (F1), two cases (29%) showed F2, one case (14%) showed F3, and the remaining one case (14%) showed F4; five cases (71%) showed a hepatic activity score of 2 (A2), and the remaining two cases (29%) showed A3. As to the serological findings in the serum sample obtained just before the initiation of IFN treatment, four cases (57%) had HCV genotype 1b and three cases (43%) had HCV genotype 2b. The HCV viral load in the seven cases was 285.0 ± 285.0 KIU/ml (range, 44.6–843.0 KIU/ml); the serum aspartate aminotransferase level (normal range: 9–31 IU/l) was 129 ± 86 IU/l (range: 52–315 IU/l); and the serum alanine aminotransferase level (normal range: 4–34 IU/l) was 160 ± 141 IU/l (range: 66–467 IU/l). The titer of antinuclear antibody was less than 1:40 in all cases.

Table 1
Profiles of the seven CH-C cases who developed IP during IFN treatment

Patient number	Case number	Age (year)	Sex	Past history				Liver histology[b]
				Lung disease (age)	Smoking	Allergy	Medication of 'Sho-saiko-to'[a]	
1	1	66	Female	None	No	None	2 months	F3/A3
2	2	66	Male	Tuberculosis (22–25 year)	No	None	3 years	F4/A3
3	3	54	Female	None	Yes[c]	Drugs	No	F2/A2
4	4	65	Male	Tuberculosis (26–28 year)	No	None	No	F1/A2
5	5[d]	57	Female	None	No	Drugs	No	F1/A2
	6[d]	66	Female	IFN-associated IP (57 year)	No	Drugs	No	F1/A2
6	7	54	Male	None	Yes[c]	Drugs	No	F2/A2

[a] Sho-saiko-to was administered from 1 month before to 1 month after the start of IFN therapy in Case 1, and from 3 years before the start of IFN therapy to the onset of IP (8 days) in Case 2.
[b] The degree of fibrosis (staging) and necroinflammation (grading) were scored by the New Inuyama classification (staging: F0–F4; grading: A0–A3) [16].
[c] One pack of cigarettes a day for 20 years in Patient 3, and one to 1.5 packs of cigarettes a day for 38 years in Patient 6.
[d] Patient 5 developed IP in 1993 (Case 5) and in 2002 (Case 6) during two separate series of IFN treatments.

3.2. Clinical findings and outcomes of the seven cases with IFN-induced IP

IFN monotherapy or a combination therapy of IFN plus ribavirin was administered in the seven cases who subsequently developed IP (Table 2). In all cases, IFN treatment was discontinued when IP was diagnosed. The mean dosage of IFNα administered until the cessation of therapy was 427±308 (S.D.) MU (range: 102–980 MU). The mean duration from the start of IFN treatment to the onset of IP was 2.5±1.7 (S.D.) months (range: 8 days–5 months). In six cases (86%), IP developed 1 month or longer after the start of IFN treatment. No case showed eosinophilia at the onset of IP.

At the onset of IP, all seven cases experienced dry cough and/or dyspnea. Chest CT scan showed bilateral ground-glass opacities in the whole lung field in three cases (Cases 1, 2 and 4), and bilateral ground-glass opacities localized in the lower lung field in the remaining four cases: no case had bilateral hilar lymphadenopathy. On blood gas analysis performed at the onset of IP, the mean arterial O_2 pressure (normal range: >80.0 mmHg) was 69.0±15.3 (S.D.) mmHg (range: 46.2–87.7 mmHg); mean arterial CO_2 pressure (normal range: 35.0–45.0 mmHg) was 37.4±5.6 (S.D.) mmHg (range: 28.1–43.5 mmHg); and the mean arterial O_2 saturation in room air (normal range: 95.0–98.0%) was 93.5±5.6% (S.D.) (range: 84.2–98.6%). After discontinuation of IFNα therapy, two (29%) of the seven IP cases recovered without any additional therapies, whereas five cases (71%) had to be treated with corticosteroids including high-dose intravenous 'pulse' methylprednisolone. Six cases (86%) recovered from IP, while one case (Case 2) died of respiratory failure.

3.3. Serum levels of KL-6, SP-A, SP-D and LDH

As illustrated in Fig. 1, just before the initiation of IFN treatment, the serum levels of SP-A and SP-D were within the normal range in all seven cases, but, of interest, elevated levels of KL-6 or LDH were noted in five (71%) and four cases (57%), respectively. At the onset of IP, all seven cases had elevated KL-6 and LDH levels, while five (71%) and two patients (29%) had elevated SP-A and SP-D levels, respectively.

At the onset of IP, the serum KL-6 level had increased in all seven cases, the SP-A level had increased in six cases (86%), the SP-D level had increased in six cases (86%), and the LDH level had increased in six cases (86%), in comparison with the respective level immediately before IFN therapy was started.

Table 2
Characteristics of the IP that developed in the seven CH-C cases during IFN treatment

Case number	IFN		IP				Therapy	Outcome
	Kind	Total dose[b] (MU)	Duration from the start of IFN to the onset of IP	Symptoms	Blood gas analysis[a]			
					PaO_2 (mmHg)	$PaCO_2$ (mmHg)		
1	Recombinant α 2a	432	3.5 months	Dry cough, fever	52.5	37.5	Corticosteroids	Improved
2	Natural α	102	8 days	Dry cough, dyspnea	46.2	28.1	Corticosteroids	Dead
3	Recombinant α 2b	560	2.5 months	Dry cough, dyspnea	78.7	38.8	None	Improved
4	Natural α	980	5 months	Dyspnea on exertion	63.4	31.6	Corticosteroids	Improved
5	Natural α	168	1 month	Dry cough	79.1	39.6	None	Improved
6	Recombinant α 2b[c]	560	3 months	Dry cough	75.2	43.5	Corticosteroids	Improved
7	Recombinant α 2b[c]	190	1 month	Dry cough, dyspnea	87.7	42.4	Corticosteroids	Improved

[a] Abbreviations (normal): PaO_2, arterial O_2 pressure (>80.0 mmHg) in room air; $PaCO_2$, arterial CO_2 pressure (35.0–45.0 mmHg) in room air.
[b] Total dose of IFN that was administered until the occurrence of IP in each case.
[c] Combined with ribavirin.

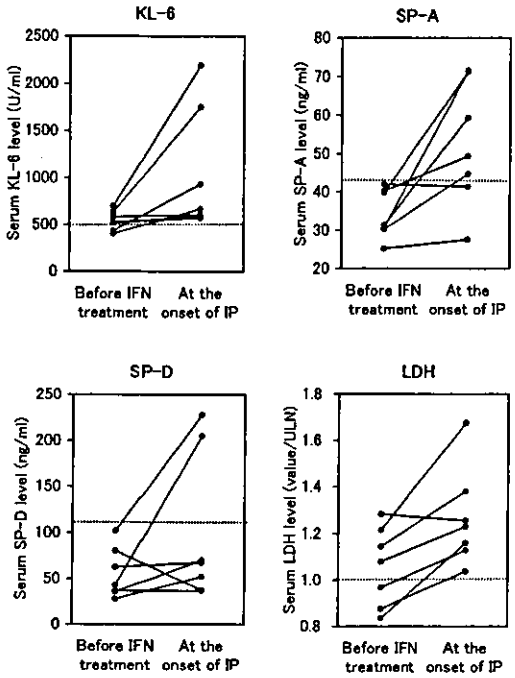

Fig. 1. Comparison of the serum levels of KL-6, SP-A, SP-D and LDH before IFN treatment was started and at the onset of IP in the seven CH-C cases who developed IFN-associated IP. Abbreviations (upper limit of normal) are: SP-A, surfactant protein A (43.8 ng/ml); SP-D, surfactant protein-D (110 ng/ml); LDH, lactate dehydrogenase. The LDH level is expressed as the ratio of the measured value/upper limit of normal at the time of measurement. The upper limit of normal of the serum KL-6 level is 500 U/ml. The dotted line indicates the upper limit of normal.

3.4. Comparison of the serum KL-6 level before IFN treatment between the CH-C cases with IFN-induced IP and those who did not develop IP

To evaluate the clinical significance of the serum KL-6 level immediately before the start of IFN treatment, the serum KL-6 level before IFN treatment was determined in 48 patients selected randomly from 307 CH-C patients who did not develop IP during IFNα treatment and whose age was not lower than that of the youngest case who developed IP (54 year). The mean KL-6 level before IFN treatment in the control subjects was 304±98 U/ml (S.D.), being significantly lower than that in the seven cases who subsequently developed IFN-induced IP (543±105 U/ml, $P=0.0001$) (Fig. 2). Furthermore, the percentage of patients whose serum KL-6 level was higher than the ULN among the controls was only 6% (three of 48 controls), which was significantly lower than that among the seven cases who subsequently developed IFN-induced IP (6 vs. 71%, $P=0.0003$).

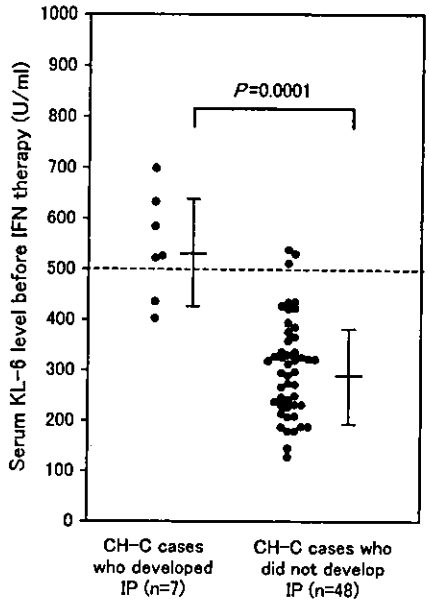

Fig. 2. Comparison of the serum KL-6 level before IFN treatment between the seven CH-C cases who subsequently developed IFN-associated IP and 48 age-adjusted control subjects with CH-C who did not develop IP during IFN treatment. The serum KL-6 level before IFN treatment among the seven cases was significantly higher than that among the controls (543±105 vs. 304±98 U/ml; $P=0.0001$).

4. Discussion

In Japan, IP is one of the well-known adverse reactions associated with natural and recombinant IFNα therapy for chronic hepatitis C [6,18]. In other countries, however, IP is a known but infrequent complication of IFNα when given at a high dose of 6–10 MU/day. The reason for the difference in the incidence of this disease between Japan and other countries is not known, although the dosage of IFN (especially that in the first 2–4 weeks of the therapy), or race (difference in sensitivity to IFNα) may contribute to the difference. However, a recent report [7] in the United States indicated that IFN-induced IP occurs even during low-dose IFNα treatment combined with ribavirin.

The incidence of IFN-induced IP in the present study was 1.1%, which tended to be higher than that in a previous study in Japan (0.4% (3/677)) [18] ($P=0.079$). The age of our seven cases with IFN-induced IP was 61±6 years (range, 54–66 years). As the incidence of idiopathic IP has been reported to increase with older age [8], there may be a relationship between the occurrence of IFNα-induced IP and aging. The Chinese composite herbal preparation, Sho-saiko-to, had been commonly used for the treatment of chronic hepatitis in Japan but it is now known to induce IP with or without concomitant use of IFN. Sho-saiko-to was given before

and during IFN treatment to only two (29%) of the seven cases with IFN-induced IP in our cohort. The remaining five cases had never been treated with Sho-saiko-to. Close relationships between the occurrence of IFN-induced IP and background factors such as sex, liver histology, HCV RNA titer, HCV genotype, or kind of IFNα were not observed in the present study. However, it is of note that past history of drug allergy was observed in 50% of the patients who developed IP during IFN treatment.

It is noteworthy that a CH-C patient (Patient 5) who underwent IFN treatments in 1993 and again in 2002 contracted IP twice, 1 and 3 months, respectively, after the initiation of IFN treatment. In this patient, the first case of IP emerged during treatment with natural IFNα and the second case of IP occurred during combination therapy of IFNα 2b plus ribavirin. Although both IP events which occurred 9 years apart were fortunately reversible in this patient, it may be clinically advisable that IFNα retreatment should be avoided in patients who have a past history of IFNα-induced IP.

At the onset of IFN-induced IP, the serum KL-6 and LDH levels were above the ULN in all cases. However, the serum LDH level is elevated not only in lung diseases but also in many other diseases such as liver diseases, hematological disorders, malignancies, and muscle diseases. Furthermore, an elevated LDH level is not specific for IP even among lung diseases [8]. On the other hand, as mentioned above, KL-6 is a more specific marker than LDH for the diagnosis of IP [9,12]. The results obtained in the present study ascertain that measurement of the serum KL-6 level is useful for the diagnosis of IFN-induced IP.

Of particular interest, the serum KL-6 level before the administration of IFN was above the ULN in five (71%) of the seven CH-C cases. The underlying mechanism of the development of IFNα-induced IP remains unknown. However, IP has been reported as one of the extra-hepatic manifestations of chronic HCV infection [4,19–22]. Therefore, it is tempting to speculate that those patients who develop IP during IFNα treatment have subclinical HCV-related IP before the administration of IFN, and that the administration of IFNα, which has a modulatory effect on the immune response of hosts, deteriorates the lung damage by triggering an immune-mediated response. This speculation is supported by the evidence that the serum KL-6 level was above the ULN in only three (6%) of the 48 age-adjusted control patients who did not develop IP during IFN treatment in the present study. Furthermore, the circulating KL-6 level before IFN treatment was significantly higher among the seven IP cases than among the control subjects ($P = 0.0001$). Even when Case 6 was excluded from the comparison, the mean KL-6 level before IFN treatment was also significantly higher among six IP patients than the control subjects (561 ± 102 vs. 304 ± 98 U/ml, $P = 0.0003$). Similarly, the percentage of patients whose serum KL-6 level was higher than the ULN was significantly higher among six IP patients than the control subjects (83 vs. 6%, $P = 0.0001$).

In the current study, two (29%) out of the seven cases were treated with the combination of IFNα with ribavirin. As this combination therapy has been reported not only to enhance the virus-specific cytotoxic response but also to augment the nonspecific immune response [7], it seems that clinicians should pay further attention to the possibility of development of IP during this combination therapy. Chest CT, especially high-resolution CT, performed just before the start of IFN treatment may be helpful in detecting patients who are likely to develop IFN-associated IP. However, it would not be practical to perform chest CT scanning in all CH-C patients who are scheduled to undergo IFN therapy because of the potential risk of radiation and higher cost. On the other hand, measurement of the serum KL-6 level before IFN treatment is easy, does not pose a potential risk to the patient, and is less expensive.

In conclusion, it is recommended that measurement of the serum KL-6 level be performed in CH-C patients before starting IFN therapy, to avoid a rare, but sometimes fatal, side effect of IFN treatment, that is, IFN-associated IP. Further studies are needed to elucidate the underlying mechanism of the development of IP in patients who undergo IFN therapy with or without ribavirin, in relation to various host and viral factors.

References

[1] Moriya K, Yasuda K, Koike K, et al. Induction of interstitial pneumonitis during interferon treatment for chronic hepatitis C. J Gastroenterol 1994;29:514–7.
[2] Chin K, Tabata C, Satake N, Nagai S, Moriyasu F, Kuno K. Pneumonitis associated with natural and recombinant interferon alfa therapy for chronic hepatitis C. Chest 1994;105:939–41.
[3] Kamitsukasa H, Ohtake M, Kawashima H, Yagura M, Harada H, Katayama T. Two cases of interstitial pneumonia induced by interferon therapy for chronic aggressive hepatitis, type C. Acta Hepatol Jpn 1993;34:478–83 (in Japanese).
[4] Isai H, Takagi S, Sekine K, et al. A prospective study on pulmonary damage during interferon therapy for chronic hepatitis C. Hepatology 1994;19:761.
[5] Ishizaki T, Sasaki F, Ameshima S, et al. Pneumonitis during interferon and/or herbal drug therapy in patients with chronic active hepatitis. Eur Respir J 1996;9:2691–6.
[6] Iino S, Hino K, Yasuda K. Current state of interferon therapy for chronic hepatitis C. Intervirology 1994;37:87–100.
[7] Karim A, Ahmed S, Khan A, Steinberg H, Mattana J. Interstitial pneumonitis in a patient treated with α-interferon and ribavirin for hepatitis C infection. Am J Med Sci 2001;322:233–5.
[8] Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. Am J Respir Crit Care Med 2000;161:646–64.
[9] Kohno N, Akiyama M, Kyoizumi S, Hakoda M, Kobuke K, Yamakido M. Detection of soluble tumor-associated antigens in

sera and effusions using novel monoclonal antibodies, KL-3 and KL-6, against lung adenocarcinoma. Jpn J Clin Oncol 1997;18:203–16.
[10] Kuroki Y, Tsutahara S, Shijubo N, et al. Elevated levels of lung surfactant protein A in sera from patients with idiopathic pulmonary fibrosis and pulmonary alveolar proteinosis. Am Rev Respir Dis 1993;147:723–9.
[11] Honda Y, Kuroki Y, Matsuura E, et al. Pulmonary surfactant protein D in sera and bronchoalveolar lavage fluids. Am J Respir Crit Care Med 1995;152:1860–6.
[12] Kohno N. Serum marker KL-6/MUC1 for the diagnosis and management of interstitial pneumonitis. J Med Invest 1999;46:151–8.
[13] Yokoyama A, Kohno N, Hamada H, et al. Circulating KL-6 predicts the outcome of rapidly progressive idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 1998;158:1680–4.
[14] Takahashi H, Fujishima T, Koba H, et al. Serum surfactant proteins A and D as prognostic factors in idiopathic pulmonary fibrosis and their relationship to disease extent. Am J Respir Crit Care Med 2000;162:1109–14.
[15] Okamoto H, Mishiro S, Tokita H, Tsuda F, Miyakawa Y, Mayumi M. Superinfection of chimpanzees carrying hepatitis C virus of genotype II/1b with that of genotype III/2a or I/1a. Hepatology 1994;20:1131–6.
[16] Ichida F, Tsuji T, Omata M, et al. New Inuyama classification; new criteria for histological assessment of chronic hepatitis. Int Hepatol Commun 1996;6:112–9.
[17] Okamoto H, Tokita H, Sakamoto M, et al. Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. J Gen Virol 1993;74:2385–90.
[18] Okanoue T, Sakamoto S, Itoh Y, et al. Side effects of high-dose interferon therapy for chronic hepatitis C. J Hepatol 1996;25:283–91.
[19] Ueda T, Ohta K, Suzuki N, et al. Idiopathic pulmonary fibrosis and high prevalence of serum antibodies to hepatitis C virus. Am Rev Respir Dis 1992;146:266–8.
[20] Ferri C, La Civita L, Fazzi P, et al. Interstitial lung fibrosis and rheumatic disorders in patients with hepatitis C virus infection. Br J Rheumatol 1997;36:360–5.
[21] Hadziyannis SJ. The spectrum of extrahepatic manifestations in hepatitis C virus infection. J Viral Hepatitis 1997;4:9–28.
[22] Zignego AL, Brechot C. Extrahepatic manifestations of HCV infection: facts and controversies. J Hepatol 1999;31:369–76.

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TT virus of certain genotypes may reduce the platelet count in patients who achieve a sustained virologic response to interferon treatment for chronic hepatitis C

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Abstract

The platelet count increases after a sustained response to interferon (IFN) treatment for chronic hepatitis C (CH-C). However, the extent of the increase differs by patient. We investigated whether concurrent TT virus (TTV) infection interferes with the improvement of thrombocytopenia. Serial serum samples were obtained from 85 noncirrhotic CH-C patients who achieved a sustained virologic response for hepatitis C virus (HCV) upon IFN treatment, and tested for TTV DNA by three polymerase chain reaction (PCR) methods (UTR, N22 and TTV genotype-1). UTR PCR can detect essentially all TTV genotypes, whereas N22 PCR primarily detects four major TTV genotypes (1–4). Eighty-four patients (84/85, 99%) were positive for TTV DNA by UTR PCR, 27 (32%) by N22 PCR and 18 (21%) by TTV genotype-1 PCR just before IFN treatment was started (baseline). A sustained virologic response for TTV was observed in 6% (5/84) by UTR PCR, 52% (14/27) by N22 PCR and 56% (10/18) by TTV genotype-1 PCR. The platelet count was significantly lower in the N22 PCR-positive group than in the N22 PCR-negative group not only at baseline (14.9 ± 3.8 vs. $18.1 \pm 6.4 \times 10^4/\mu l$, $P < 0.05$), but also at the non-HCV-viremic state one year after the completion of IFN treatment (15.5 ± 2.8 vs. $18.6 \pm 5.5 \times 10^4/\mu l$, $P < 0.05$), the differences also being statistically significant by TTV genotype-1 PCR, but not by UTR PCR. These results suggest that certain TTV genotypes including genotype 1 may play a role in aggravating the thrombocytopenia of CH-C patients, either alone or in concert with HCV.

Keywords: Chronic hepatitis C; Interferon treatment; Platelet count; Sustained virologic responder; TT virus (TTV)

1. Introduction

The platelet count tends to decrease according to the severity of chronic liver disease, and has been reported to be an important parameter for

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evaluating the fibrotic stage of chronic hepatitis C (CH-C) [1–3]. Although a reduction in platelet count has also been observed during interferon (IFN) therapy, complete or sustained responders to IFN treatment for CH-C show improvement of their thrombocytopenia [4,5]. However, the extent to which the platelet count increases after the cessation of IFN therapy differs from patient to patient [4,5]. Therefore, there seem to be other factor(s) besides hepatic fibrosis that may be associated with the aggravation of thrombocytopenia in noncirrhotic CH-C patients.

Recently, a novel, unenveloped human virus, named TT virus (TTV), was isolated from the serum of a patient with posttransfusion hepatitis of unknown etiology [6]. The genome of TTV is a circular single-stranded DNA molecule of ~3.8 kilobases (kb) with negative polarity [7–10]. TTV is most closely related to the virus family, *Circoviridae*, among the known animal viruses [11]; however, TTV differs considerably from members of the *Circoviridae* family in physicochemical properties and sequence at both the DNA and amino acid levels. TTV has an extremely wide range of sequence divergence for a DNA virus [12,13], and at least 23 genotypes (1–23) and four major phylogenetic groups (1–4) have been identified [14,15]. Group 1 is represented by the prototype TTV (N22 clone and TA278 isolate) of genotype 1 [6,7], and includes five additional genotypes (2–6) [12]. To date, the exact role of TTV infection in hepatic and extrahepatic diseases remain to be defined [16]. However, among the TTVs of these four genetic groups, TTVs of group 1 detectable by N22 polymerase chain reaction (PCR) or the prototype TTV (TTV genotype 1) has been suggested to influence the thrombocytopenia of patients with chronic liver disease including CH-C [17].

It has been reported that TTV is sensitive to IFN treatment [18–20]. Furthermore, circular double-stranded TTV DNA of replicative intermediate form and TTV mRNAs have been detected in bone marrow cells from infected humans, indicating that TTV replication takes place in the bone marrow where platelets are reproduced [21,22]. Therefore, the aims of the present study were to evaluate the effect of IFN on TTV, and to examine the influence of concurrent TTV viremia on the platelet count before and after IFN therapy, in relation to TTV genotype, among 85 patients who achieved a sustained virologic response (undetectable hepatitis C virus, HCV, RNA at 1 year after completion of IFN therapy) to IFN treatment for CH-C.

2. Materials and methods

2.1. Patients

Eighty-five CH-C patients (51 males and 34 females; age, 55 ± 13 years (mean ± standard deviation, S.D.)) who received IFN treatment in the Department of Gastroenterology, National Tokyo Hospital between January 1992 and September 1999 and achieved a sustained virologic response with regard to HCV, were enrolled in the present study. The following patients were excluded: patients with liver cirrhosis, patients co-infected with hepatitis B virus (HBV), patients with the complication of autoimmune hepatitis, and patients with an alcohol intake of more than 80 g per day. No patient had a history of blood transfusion for the treatment of liver disease, and no patient had concurrent infection of human immunodeficiency virus type 1. The diagnosis of chronic HCV infection was based on continuous positivity for second-generation antibodies to HCV (Dainabot, Tokyo, Japan) and HCV RNA [23] in the serum for more than 6 months before IFN treatment was started. All patients underwent liver biopsy just before IFN treatment was started. The degree of fibrosis (staging) in the liver specimens obtained from each patient just before the start of IFN treatment was evaluated by the scoring system proposed by Desmet et al. [24].

The 85 patients underwent IFN monotherapy for 24 ± 3 weeks. The total dose of IFN was 720 ± 186 million units (MU). Eighty patients received 6–10 MU of IFN-α daily for 2–4 weeks, and the remaining five patients received 6 MU of IFN-β daily for 2–4 weeks, followed by 6–10 MU of IFN-α three times a week. The following parameters were monitored in each patient: presence of HCV RNA and serum levels of aspartate aminotransferase (AST: normal range, 9–31 IU/l), and alanine aminotransferase (ALT: normal

range, 4–34 IU/l), and platelet count (normal range, $15–30 \times 10^4/\mu l$) at baseline (just before IFN therapy was started), and after IFN treatment was completed. The patients were followed for more than 1 year after completion of IFN treatment and did not receive any other treatment for liver disease. In all patients, the sera were negative for HCV RNA at both 6 and 12 months after the cessation of IFN treatment, and therefore, they were considered to be sustained virologic responders for HCV. This study conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Ethics Committee at National Tokyo Hospital. Informed consent was obtained from each patient in this study. There is no overlap between the subjects in the current study and the subjects in our previous study [17].

2.2. Quantitation of HCV RNA and determination of HCV genotypes

From 200 μl of the serum sample that had been stored at −80 °C until testing, RNA was extracted using ISOGEN-LS (Nippon Gene, Tokyo, Japan), and dissolved in 20 μl of nuclease-free distilled water. Detection of HCV RNA in the serum was performed by the previously described method [23] using 10 μl of the RNA solution. Quantitation of HCV RNA was performed on serum samples that had been obtained at baseline, by real-time detection PCR using 5 μl of the RNA solution as a template, primers HC503 [sense: 5′-CTA GCC GAG TAG YGT TGG GT-3′ (Y = T or C)] and HC504 (antisense: 5′-TGC ACG GTC TAC GAG ACC TC-3′), a doubly labeled probe [HC505-P: 5′-(Fam)-AAG GCC TTG TGG TAC TGC CTG ATA GGG-(Tamra)-3′], 20 μg of bovine serum albumin (Roche Diagnostics GmbH, Mannheim, Germany) and the TaqMan EZ RT-PCR Core Reagents kit (PE Applied Biosystems, Foster City, CA). Reverse transcription was performed at 60 °C for 30 min. The cDNAs were denatured at 95 °C for 60 s, and subjected to an initial amplification (1 cycle) of denaturation at 94 °C for 0 s and annealing-extension at 62 °C for 15 s, followed by 48 cycles of denaturation at 90 °C for 0 s and annealing-extension at 62 °C for 15 s and cooling at 40 °C for 30 s. The sequences of the primers and a probe were derived from the well-conserved area in the 5′ untranslated region of the HCV genome [25]. All reactions were performed in a LightCycler System (Roche Diagnostics GmbH). The quantification limit of the system was 3–5 copies per test capillary (20 μl of reaction mixture).

Using the remaining 5 μl of the extracted RNA solution, the genotype of HCV (1a, 1b, 2a, 2b or 3a) was determined according to the previously described method [26] with a slight modification. In brief, the original genotype 1b-specific antisense primer (No. 133) was replaced by primer No. 492 [27].

2.3. Detection of TTV virus DNA

Nucleic acids were extracted from 100 μl of the serum sample using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH), and dissolved in nuclease-free distilled water. The extracted nucleic acids corresponding to 25 μl of the serum served as the template for the detection of TTV DNA by the three distinct PCR methods (UTR, N22 and TTV genotype-1). UTR PCR was carried out in the presence of Perkin-Elmer AmpliTaq Gold (Roche Molecular Systems, Inc, Branchburg, NJ) and nested primers by the method described previously [12], with slight modifications. Briefly, primers NG472 (sense: 5′-GCG TCC CGW GGG CGG GTG CCG-3′ (W = A or T)) and NG352 (antisense: 5′-GAG CCT TGC CCA TRG CCC GGC CAG-3′ (R = A or G)) were used for the first-round PCR, and primers NG473 (sense: 5′-CGG GTG CCG DAG GTG AGT TTA CAC-3′ (D = G, A or T)) and NG351 (antisense: 5′-CCC ATR GCC CGG CCA GTC CCG AGC-3′) were used for the second-round PCR, which were derived from the same well-conserved area in the UTR of the TTV genome as in the original method [12]. The amplification product of the first-round PCR was 91 bp, and that of the second-round PCR was 71 bp.

N22 PCR which detects TTVs in group 1 (essentially the four major TTV genotypes (1–4) distributed in Japan) was performed as previously described [7,28]. Detection of TTV genotype 1

DNA was performed by the PCR method with a genotype 1-specific primer pair (NG162 (sense) and NG165 (antisense)), as described previously [29].

2.4. Statistical analysis

Statistical analysis was performed using the Mann–Whitney U-test for unpaired comparison between two groups; the χ^2 test for comparison of frequency distributions; the Wilcoxon signed rank test for paired comparison between two groups; and the Spearman rank correlation coefficient for the statistical analysis of correlation. Differences were considered to be statistically significant at $P < 0.05$. Data were presented as mean ± S.D.

3. Results

3.1. Characterization of the studied patients

In the 85 enrolled patients who achieved a sustained virologic response to IFN, the HCV RNA titer prior to IFN treatment was 4.1 ± 1.3 log (copies/ml, mean ± S.D.). Thirty-three patients (39%) had HCV genotype 1b, 29 (34%) had HCV genotype 2a, and 23 (27%) had HCV genotype 2b. Two patients (2%) had a hepatic fibrosis score of 0 (F0), 24 (28%) had F1, 35 (41%) had F2, and 24 (28%) had F3 at pretreatment. In all patients, HCV RNA became undetectable at the end of IFN treatment and continued to be undetectable at 6 and 12 months after the completion of treatment. The changes in laboratory data and TTV status before and one year after completion of IFN treatment in the 85 patients are summarized in Table 1. The AST and ALT levels significantly decreased (both $P < 0.0001$). The prevalence of TTV in serum detectable by UTR PCR showed no appreciable difference before and one year after the cessation of IFN therapy (99 vs. 93%). However, the prevalence of TTV detectable by N22 PCR changed from 32 to 15% ($P < 0.05$), and that of TTV genotype 1 changed from 21 to 9% ($P < 0.05$). Conversely, the platelet count increased, the difference between that at baseline and that at 1 year after the cessation of IFN treatment being statistically significant ($P < 0.005$). Specifically, the platelet count improved from 17.1 ± 5.9 × 10^4 to 18.1 ± 5.3 × 10^4/μl, following a sustained response of HCV to IFN.

3.2. Comparison of various features between the patients who were or were not infected with TTV at baseline

Of the 85 CH-C patients, 27 patients (32%) were positive for TTV DNA by N22 PCR and 18

Table 1
Characteristics of the 85 patients who achieved a sustained virologic response to IFN treatment for CH-C

Feature	Data at		*P* value
	Baseline	1 year after the end of treatment	
HCV RNA titer (log (copies/ml))	4.1 ± 1.3	(−)[a]	
AST (IU/l)	82 ± 68	23 ± 6	<0.0001[b]
ALT (IU/l)	112 ± 102	19 ± 9	<0.0001[b]
TTV DNA detectable by UTR PCR	84 (99%)	79 (93%)	NS[c]
TTV DNA detectable by N22 PCR	27 (32%)	13 (15%)	<0.05[c]
TTV DNA of genotype 1	18 (21%)	8 (9%)	<0.05[c]
Platelet count (× 10^4/μl)[d]	17.1 ± 5.9	18.1 ± 5.3	<0.005[b]

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase. Data are expressed as mean ± S.D.

[a] All samples were negative for HCV RNA.

[b] Wilcoxon signed rank test.

[c] χ^2-test.

[d] The correlation coefficient between the platelet count at baseline and that at 1 year after the cessation of IFN treatment was $r = 0.886$ ($P < 0.0001$: Spearman rank correlation coefficient).

Table 2
Comparison of various features at baseline between the patients who were or were not infected with TTV detectable by N22 PCR or genotype 1-specific PCR

Feature[a]	TTV DNA detectable by N22 PCR			TTV DNA of genotype 1		
	Positive ($n=27$)	Negative ($n=58$)	P value	Positive ($n=18$)	Negative ($n=67$)	P value
Age (year)	57 ± 14	54 ± 12	NS[b]	57 ± 14	54 ± 12	NS[b]
Sex (male/female)	19/8	32/26	NS[c]	14/4	37/30	NS[c]
Total dose of IFN (million units)	715 ± 202	722 ± 181	NS[b]	726 ± 207	718 ± 182	NS[b]
Duration of IFN treatment (week)	23 ± 3	24 ± 3	NS[b]	23 ± 3	24 ± 3	NS[b]
Degree of hepatic fibrosis (F0/F1/F2/F3)	1/7/11/8	1/17/24/16	NS[b]	1/3/8/6	1/21/27/18	NS[b]
HCV RNA titer (log (copies/ml))	3.7 ± 1.1	4.2 ± 1.3	<0.05[b]	3.6 ± 1.2	4.2 ± 1.3	NS[b]
HCV genotype (1b/2a/2b)	11/9/7	22/20/16	NS[c]	7/5/6	26/24/17	NS[c]
AST (IU/l)	84 ± 92	81 ± 54	NS[b]	94 ± 110	78 ± 52	NS[b]
ALT (IU/l)	97 ± 100	120 ± 103	NS[b]	107 ± 118	114 ± 98	NS[b]
Platelet count ($\times 10^4/\mu l$)	14.9 ± 3.8	18.1 ± 6.4	<0.05[b]	14.6 ± 3.8	17.8 ± 6.2	<0.05[b]

[a] See Table 1 for abbreviations.
[b] Mann–Whitney U-test.
[c] χ^2-test.

patients (21%) were positive for TTV genotype 1 at baseline. In contrast, only one patient was negative for TTV DNA detectable by UTR PCR. The demographic features including age and sex as well as total dosage of IFN, duration of IFN treatment and degree of hepatic fibrosis, of the TTV DNA-positive and TTV DNA-negative groups did not differ significantly. Table 2 compares various features at baseline according to the detectability of TTV DNA by N22 PCR or genotype 1-specific PCR. The platelet count was significantly lower in the TTV DNA-positive group than in the TTV DNA-negative group ($P < 0.05$, by both PCR methods), which corroborated our previous report [17]. Of interest, the HCV RNA titer was significantly lower in the TTV DNA-positive group than in the TTV DNA-negative group ($P < 0.05$, by N22 PCR), although there were no appreciable differences in the distribution of HCV genotype between those who were positive or negative for TTV DNA by either of the two PCR methods.

3.3. Comparison of various features between the patients who were or were not infected with TTV at 1 year after discontinuation of IFN treatment

The sustained virologic response rate of TTV at 1 year after the completion of IFN treatment was estimated to be 52% (14/27) with regard to TTV detectable by N22 PCR and 56% (10/18) with regard to TTV genotype 1 (Table 3), contrasting with that of TTV detectable by UTR PCR (5/84, 6%). Thirteen patients (48%) were regarded as virologic nonresponders with regard to TTV detectable by N22 PCR and eight patients (44%) as virologic nonresponders with regard to TTV genotype 1, despite the fact that all patients had a complete or sustained response to IFN with regard to HCV. Six patients and four patients responded to IFN with regard to TTV detectable by N22 PCR or TTV genotype 1, respectively; they became negative for TTV DNA at the end of IFN treatment, but TTV DNA reappeared by 1 year after discontinuation of IFN treatment.

At 1 year after the completion of IFN treatment, 13 patients (15%) were positive for TTV DNA by N22 PCR and 8 patients (9%) were positive for TTV genotype 1. At that time, there were no appreciable differences in the AST and ALT levels between the TTV DNA-positive group and the TTV DNA-negative group by either of the two PCR methods (Table 4). On the contrary, the platelet count was significantly lower in the TTV DNA-positive group than in the TTV-DNA-negative group ($P < 0.05$, by both PCR methods). These results suggest that TTV viremia interferes with the improvement of thrombocytopenia in patients who achieve a sustained virologic response to IFN with regard to HCV and even at the state of non-HCV viremia.

3.4. Comparison of various features among the CH-C patients according to the TTV status before and after IFN treatment

In an attempt to evaluate the long-term effect of concurrent TTV infection on the platelet count, various features were compared between the patients who did not respond to IFN with regard to TTV (nonresponders to TTV) and those who were non-TTV-viremic throughout the observation period (Table 5). The platelet count differed significantly between the two groups both at pretreatment and at 1 year after the end of therapy ($P < 0.05$, $P < 0.05$, respectively); the platelet count was significantly lower in the nonresponders to TTV detectable by either N22 PCR or TTV genotype 1-PCR, suggesting that continuous TTV infection worsens the thrombocytopenia.

There were no appreciable differences between the two groups in terms of various other demographic, virological and histological features except for the HCV RNA titer at baseline. The mean HCV viral load was one log lower in the patient group with co-infection of TTV detectable by N22 PCR or TTV genotype 1 than in the non-TTV-viremic group ($P < 0.05$, $P < 0.05$, respectively).

4. Discussion

In the present study, 84 patients (99%) were positive for TTV DNA at baseline by UTR PCR, which can detect essentially all TTV genotypes (1–23), and genetic groups (1–4) thus far identified. Therefore, the influence of TTV infection on the platelet count was investigated longitudinally using the other two distinct PCR methods that can detect TTV of certain genotypes including the prototype TTV (genotype 1 TTV), among 85 noncirrhotic CH-C patients who achieved a sustained virologic response to IFN treatment with regard to HCV. Before IFN therapy was started, the platelet count was significantly lower in the TTV DNA-positive patients than in the TTV DNA-negative patients, corroborating our previous cross-sectional study [17]. A sustained virologic response was observed in 52% of the patients with TTV detectable by N22 PCR and in 56% of the patients with TTV genotype 1, being consistent with previous reports [18–20]. At 1 year after the completion of IFN treatment, the

Table 3
Response of TTV to IFN in 85 patients who achieved a sustained virologic response to IFN treatment for CH-C

TTV DNA at			No. of cases with TTV DNA detectable by N22 PCR	No. of cases with TTV DNA of genotype 1
Baseline	The end of treatment	1 year after the end of treatment		
+	+	+	7	4
+	–	+	6	4
+	–	–	14 (52%)[a]	10 (56%)[a]
–	–	–	58	67

[a] The sustained virologic response rate of TTV to IFN is indicated in parentheses.

Table 4
Comparison of laboratory data between the patients who were or were not infected with TTV detectable by N22 PCR or genotype 1-specific PCR at 1 year after cessation of IFN treatment

Feature[a]	TTV DNA detectable by N22 PCR			TTV DNA of genotype 1		
	Positive ($n = 13$)	Negative ($n = 72$)	P value[b]	Positive ($n = 8$)	Negative ($n = 77$)	P value[b]
AST (IU/l)	26 ± 7	23 ± 6	NS	27 ± 8	23 ± 6	NS
ALT (IU/l)	19 ± 11	19 ± 8	NS	22 ± 13	19 ± 8	NS
Platelet count ($\times 10^4/\mu l$)	15.5 ± 2.8	18.6 ± 5.5	<0.05	14.6 ± 3.0	18.5 ± 5.4	<0.05

[a] See Table 1 for abbreviations.
[b] Mann–Whitney U-test.

platelet count was also significantly lower in the TTV DNA-positive patients i.e. the nonresponders of TTV to IFN treatment, suggesting that continued infection of TTV of certain genotypes can aggravate the thrombocytopenia of hepatitis patients at the state of non-HCV-viremia due to a sustained response to IFN treatment for CH-C.

The mechanism of the development of thrombocytopenia in patients with chronic liver disease has not yet been fully elucidated. Several mechanisms have been proposed, including an increased splenic platelet pool due to hypersplenism based on the underlying chronic liver disease [30]; impaired platelet production in the bone marrow (hypoproliferative) [31]; the involvement of immunological platelet destruction [32–35]; and reduced production of thrombopoietin in the liver [36]. A recent report suggests that advanced hepatic fibrosis, causing an altered production of thrombopoietin, portal hypertension and, consequently, splenomegaly, plays the pivotal role in the pathogenesis of thrombocytopenia in patients with chronic viral hepatitis [37]. Furthermore, thrombocytopenia has recently been reported as one of the extrahepatic manifestations of HCV infection [1,38]; replication of HCV has been suggested to occur in the bone marrow [39,40] and a human megakaryoblastic leukemia cell line [41].

The data obtained in the present study suggest that infection of TTV of certain genotypes including genotype 1 lowers the platelet count in patients with CH-C, either alone or in concert with HCV. The mechanism through which TTV influences the reduced platelet count in patients with CH-C remains unknown. However, the following two mechanisms seem to be likely. First, TTV particles are known to form immune complexes in the circulation, similar to HCV particles in the serum [42–44]. Platelets interact with the immune complexes via the Fc receptor and such interactions may lead to platelet aggregation, suggesting that immunological destruction of platelets, rather than abnormal pooling of platelets in an enlarged spleen, is involved in the reduction of the platelet level in CH-C patients coinfected with TTV. This hypothesis is supported by our previous observation that the platelet-associated IgG level was significantly higher in the N22 PCR-positive group than in the N22 PCR-negative group ($P < 0.05$) [17]. Second, the platelet level in the circulation is mainly regulated by thrombopoietin, which promotes megakaryocytopoiesis and thrombopoiesis [45]. Thrombopoietin is produced predominantly by hepatocytes, but is also synthesized by stromal cells in the bone marrow [46]. As TTV replicates in the liver and bone marrow cells [21,22,47], impaired production of thrombopoietin in the liver and bone marrow may also be involved in the thrombocytopenia in CH-C patients coinfected with TTV. Whether TTV can replicate in megakaryocytes and influence platelet production in the bone marrow, and whether TTV interferes with the synthesis of thrombopoietin in the liver and bone marrow should be clarified in future studies.

The HCV viral load before IFN treatment among the CH-C patients in the present study was significantly lower in the N22 PCR-positive

Table 5
Comparison of various features of the CH-C patients who remained positive for TTV DNA or who remained negative for TTV DNA at 1 year after the cessation of IFN treatment

Feature[a]	TTV DNA detectable by N22 PCR			TTV DNA of genotype 1		
	+/+[b] ($n=13$)	−/− ($n=58$)	P value	+/+ ($n=8$)	−/− ($n=67$)	P value
Age (year)	57 ± 11	54 ± 12	NS[c]	57 ± 11	54 ± 12	NS[c]
Sex (male/female)	9/4	32/26	NS[d]	6/2	37/30	NS[d]
Total dose of IFN (million units)	641 ± 245	722 ± 181	NS[c]	610 ± 252	718 ± 182	NS[c]
Duration of IFN treatment (week)	22 ± 4	24 ± 3	NS[c]	23 ± 4	24 ± 3	NS[c]
Degree of hepatic fibrosis (F0/F1/F2/F3)	0/3/7/3	1/17/24/16	NS[c]	0/0/5/3	1/21/27/18	NS[c]
HCV RNA titer (log (copies/ml))	3.2 ± 1.2	4.2 ± 1.3	<0.05[c]	3.0 ± 1.5	4.2 ± 1.3	<0.05[c]
HCV genotype (1b/2a/2b)	6/4/3	22/20/16	NS[d]	3/3/2	26/24/17	NS[d]
AST (IU/l)						
Before treatment	107 ± 128	81 ± 54	NS[c]	141 ± 155	78 ± 52	NS[c]
1 year after the end of therapy	26 ± 7	23 ± 6	NS[c]	27 ± 8	23 ± 6	NS[c]
ALT (IU/l)						
Before treatment	115 ± 137	120 ± 103	NS[c]	148 ± 166	114 ± 98	NS[c]
1 year after the end of therapy	19 ± 11	20 ± 9	NS[c]	22 ± 13	19 ± 8	NS[c]
Platelet count ($\times 10^4/\mu l$)						
Before treatment	13.6 ± 2.9	18.1 ± 6.4	<0.05[c]	12.8 ± 3.1	17.8 ± 6.2	<0.05[c]
1 year after the end of therapy	15.5 ± 2.8	18.9 ± 6.0	<0.05[c]	14.6 ± 3.0	18.6 ± 5.6	<0.05[c]

[a] See Table 1 for abbreviations.
[b] Positive (+) or negative (−) for TTV DNA at baseline (before slash) and at 1 year after the completion of IFN treatment (after the slash).
[c] Mann–Whitney U-test.
[d] χ^2-test.

group ($P < 0.05$), as previously described for the two groups of CH-C patients who were or were not infected with TTV genotype 1 [48]. Such a reciprocal inverse relationship has also been observed between replications of HBV and HCV [49]. From the virological and clinical points of view, whether coexisting TTV of certain genotypes interferes with the replication of HCV, both of which share replication sites such as the liver and bone marrow, is of great interest and deserves to be further investigated.

In conclusion, infection of TTV of certain genotypes may worsen the thrombocytopenia in CH-C patients, even those who are at the non-HCV-viremic state, due to a sustained virologic response to IFN treatment. The underlying mechanism(s) through which TTV infection influences the hematological manifestations of chronic hepatitis need to be elucidated in future studies.

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References

[1] Hadziyannis SJ. The spectrum of extrahepatic manifestations in hepatitis C virus infection. J Viral Hepat 1997;4:9–28.
[2] Nagamine T, Ohtuka T, Takehara K, Arai T, Takagi H, Mori M. Thrombocytopenia associated with hepatitis C viral infection. J Hepatol 1996;24:135–40.
[3] Ono E, Shiratori Y, Okudaira T, et al. Platelet count reflects stage of chronic hepatitis C. Hepatol Res 1999;15:192–200.

[4] Okanoue T, Itoh Y, Minami M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. J Hepatol 1999;30:653–9.
[5] Shima T, Tada H, Morimoto M, et al. Serum total bile acid level as a sensitive indicator of hepatic histological improvement in chronic hepatitis C patients responding to interferon treatment. J Gastroenterol Hepatol 2000;15:294–9.
[6] Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun 1997;241:92–7.
[7] Okamoto H, Nishizawa T, Kato N, et al. Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. Hepatol Res 1998;10:1–16.
[8] Mushahwar IK, Erker JC, Muerhoff AS, et al. Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. Proc Natl Acad Sci USA 1999;96:3177–82.
[9] Miyata H, Tsunoda H, Kazi A, et al. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J Virol 1999;73:3582–6.
[10] Okamoto H, Nishizawa T, Ukita M, et al. The entire nucleotide sequence of a TT virus isolate from the United States (TUS01): comparison with reported isolates and phylogenetic analysis. Virology 1999;259:437–48.
[11] Todd D, McNulty MS, Mankertz A, et al. Family *Circoviridae*. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, et al., editors. Virus taxonomy. Classification and nomenclature of viruses, seventh report of the international committee on taxonomy of viruses. San Diego: Academic Press, 2000:299–303.
[12] Okamoto H, Takahashi M, Nishizawa T, et al. Marked genomic heterogeneity and frequent mixed infection of TT virus demonstrated by PCR with primers from coding and noncoding regions. Virology 1999;259:428–36.
[13] Khudyakov YE, Cong ME, Nichols B, et al. Sequence heterogeneity of TT virus and closely related viruses. J Virol 2000;74:2990–3000.
[14] Muljono DH, Nishizawa T, Tsuda F, Takahashi M, Okamoto H. Molecular epidemiology of TT virus (TTV) and characterization of two novel TTV genotypes in Indonesia. Arch Virol 2001;146:1249–66.
[15] Tanaka Y, Primi D, Wang RYH, et al. Genomic and molecular evolutionary analysis of a newly identified infectious agent (SEN virus) and its relationship to the TT virus family. J Infect Dis 2001;183:359–67.
[16] Bendinelli M, Pistello M, Maggi F, Fornai C, Freer G, Vatteroni ML. Molecular properties, biology, and clinical implications of TT virus, a recently identified widespread infectious agent of humans. Clin Microbiol Rev 2001;14:98–113.
[17] Tokita H, Murai S, Kamitsukasa H, et al. Influence of TT virus infection on the thrombocytopenia of patients with chronic liver disease. Hepatol Res 2001;20:288–300.
[18] Akahane Y, Sakamoto M, Miyazaki Y, et al. Effect of interferon on a nonenveloped DNA virus (TT virus) associated with acute and chronic hepatitis of unknown etiology. J Med Virol 1999;58:196–200.
[19] Chayama K, Kobayashi M, Tsubota A, et al. Susceptibility of TT virus to interferon therapy. J Gen Virol 1999;80:631–4.
[20] Watanabe H, Saito T, Kawamata O, et al. Clinical implications of TT virus superinfection in patients with chronic hepatitis C. Am J Gastroenterol 2000;95:1776–80.
[21] Okamoto H, Takahashi M, Nishizawa T, et al. Replicative forms of TT virus DNA in bone marrow cells. Biochem Biophys Res Commun 2000;270:657–62.
[22] Okamoto H, Nishizawa T, Tawara A, et al. TT virus mRNAs detected in the bone marrow cells from an infected individual. Biochem Biophys Res Commun 2000;279:700–7.
[23] Okamoto H, Mishiro S, Tokita H, Tsuda F, Miyakawa Y, Mayumi M. Superinfection of chimpanzees carrying hepatitis C virus of genotype II/1b with that of genotype III/2a or I/1a. Hepatology 1994;20:1131–6.
[24] Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–20.
[25] Okamoto H, Kurai K, Okada S, et al. Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology 1992;188:331–41.
[26] Okamoto H, Tokita H, Sakamoto M, et al. Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. J Gen Virol 1993;74:2385–90.
[27] Holland PV, Barrera JM, Ercilla MG, et al. Genotyping hepatitis C virus isolates from Spain, Brazil, China, and Macau by a simplified PCR method. J Clin Microbiol 1996;34:2372–8.
[28] Okamoto H, Akahane Y, Ukita M, et al. Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A-G hepatitis. J Med Virol 1998;56:128–32.
[29] Okamoto H, Fukuda M, Tawara A, et al. Species-specific TT viruses and cross-species infection in nonhuman primates. J Virol 2000;74:1132–9.
[30] Toghill PJ, Green S, Ferguson R. Platelet dynamics in chronic liver disease with special reference to the role of the spleen. J Clin Pathol 1977;30:367–71.
[31] Mayer M, Herrmann I, Kempgens U, Queisser W. In vivo labeling of platelets with ^{75}Se-selenomethionine in patients with hepatic cirrhosis and thrombocytopenia. Thromb Haemost 1977;37:47–52.
[32] Pfueller S, Firkin BG, Kerlero de Rosbo N, Riglar A, Mackay IR. Association of increased immune complexes, platelet IgG and serum IgG in chronic active hepatitis. Clin Exp Immunol 1983;54:655–60.

[33] Kajiwara E, Akagi K, Azuma K, Onoyama K, Fujishima M. Evidence for an immunological pathogenesis of thrombocytopenia in chronic liver disease. Am J Gastroenterol 1995;90:962–6.
[34] Aoki Y, Hirai K, Tanikawa K. Mechanism of thrombocytopenia in liver cirrhosis: kinetics of indium-111 tropolone labelled platelets. Eur J Nucl Med 1993;20:123–9.
[35] Noguchi H, Hirai K, Aoki Y, Sakata K, Tanikawa K. Changes in platelet kinetics after a partial splenic arterial embolization in cirrhotic patients with hypersplenism. Hepatology 1995;22:1682–8.
[36] Kawasaki T, Takeshita A, Souda K, et al. Serum thrombopoietin levels in patients with chronic hepatitis and liver cirrhosis. Am J Gastroenterol 1999;94:1918–22.
[37] Adinolfi LE, Giordano MG, Andreana A, et al. Hepatic fibrosis plays a central role in the pathogenesis of thrombocytopenia in patients with chronic viral hepatitis. Br J Haematol 2001;113:590–5.
[38] Pawlotsky JM, Bouvier M, Fromont P, et al. Hepatitis C virus infection and autoimmune thrombocytopenic purpura. J Hepatol 1995;23:635–9.
[39] Laskus T, Radkowski M, Wang LF, Vargas H, Rakela J. Search for hepatitis C virus extrahepatic replication sites in patients with acquired immunodeficiency syndrome: specific detection of negative-strand viral RNA in various tissues. Hepatology 1998;28:1398–401.
[40] Radkowski M, Kubicka J, Kisiel E, et al. Detection of active hepatitis C virus and hepatitis G virus/GB virus C replication in bone marrow in human subjects. Blood 2000;95:3986–9.
[41] Li X, Jeffers LJ, Garon C, et al. Persistence of hepatitis C virus in a human megakaryoblastic leukaemia cell line. J Viral Hepat 1999;6:107–14.
[42] Hijikata M, Shimizu YK, Kato H, et al. Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. J Virol 1993;67:1953–8.
[43] Nishizawa T, Okamoto H, Tsuda F, et al. Quasispecies of TT virus (TTV) with sequence divergence in hypervariable regions of the capsid protein in chronic TTV infection. J Virol 1999;73:9604–8.
[44] Itoh Y, Takahashi M, Fukuda M, et al. Visualization of TT virus particles recovered from the sera and feces of infected humans. Biochem Biophys Res Commun 2000;279:718–24.
[45] Kaushansky K. Thrombopoietin: the primary regulator of platelet production. Blood 1995;86:419–31.
[46] Sungaran R, Markovic B, Chong BH. Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. Blood 1997;89:101–7.
[47] Okamoto H, Ukita M, Nishizawa T, et al. Circular double-stranded forms of TT virus DNA in the liver. J Virol 2000;74:5161–7.
[48] Nishizawa Y, Tanaka E, Orii K, et al. Clinical impact of genotype 1 TT virus infection in patients with chronic hepatitis C and response of TT virus to α-interferon. J Gastroenterol Hepatol 2000;15:1292–7.
[49] Pontisso P, Ruvoletto MG, Fattovich G, et al. Clinical and virological profiles in patients with multiple hepatitis virus infections. Gastroenterology 1993;105:1529–33.

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High TT Virus Load as an Independent Factor Associated With the Occurrence of Hepatocellular Carcinoma Among Patients With Hepatitis C Virus-Related Chronic Liver Disease

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The TT virus (TTV) load was estimated in sera obtained from 237 patients with hepatitis C virus (HCV)-related chronic liver disease including 42 patients with hepatocellular carcinoma (HCC), by real-time detection PCR using primers and a probe derived from the well-conserved untranslated region of the TTV genome, which can detect all known TTV genotypes. Of the 237 patients studied, 18 (8%) were negative for TTV DNA, 87 (37%) had low TTV viremia (1.3×10^2–9.9×10^3 copies/ml), and 132 (56%) had high TTV viremia (1.0×10^4-2.1×10^6 copies/ml). Various features were compared between the patients with high TTV load (n = 132) and those with no TTV viremia or low viral load (n = 105). High TTV viremia ($\geq 10^4$ copies/ml) was significantly associated with higher age ($P < 0.05$), past history of blood transfusion ($P < 0.001$), complication of cirrhosis ($P < 0.05$) or HCC ($P < 0.0005$), lower HCV RNA titer ($P < 0.05$), and lower platelet count ($P < 0.01$). On multivariate logistic regression analysis, high TTV viral load was a significant risk factor for HCC ($P < 0.05$), independent from known risk factors such as complication of liver cirrhosis ($P < 0.0001$) and high age (≥65 years, $P < 0.05$), among all 237 patients. Furthermore, high TTV viral load was an independent risk factor for HCC among the 90 cirrhotic patients ($P < 0.05$). These results suggest that a high TTV viral load is associated independently with the complication of HCC and may have prognostic significance in patients with HCV-related chronic liver disease, although whether high TTV viremia mediates the progression of HCV-related chronic liver disease remains to be defined. ***J. Med. Virol. 67:501–509, 2002.*** © 2002 Wiley-Liss, Inc.

KEY WORDS: real-time PCR; viremia; untranslated region; genotype; multivariate analysis

INTRODUCTION

TT virus (TTV) was first identified in a patient with posttransfusion hepatitis of unknown etiology [Nishizawa et al., 1997]. It is an unenveloped, single-stranded, circular DNA virus with a diameter of 30–32 nm and a total genomic length of approximately 3.8 kilobases (kb) [Okamoto et al., 1998a, 1999a; Miyata et al., 1999; Mushahwar et al., 1999; Itoh et al., 2000]. Due to its genomic structure, various research groups have placed tentatively TTV within the *Circoviridae* family [Miyata et al., 1999] or in a novel virus family, the *Circinoviridae* [Mushahwar et al., 1999] or the *Paracircoviridae* [Takahashi et al., 2000]; the most closely related known virus is chicken anemia virus (CAV) [Todd et al., 2000]. TTV can replicate in the liver, as evidenced by the findings that circular double-stranded TTV DNA in the replicative form has been detected in the liver tissues [Okamoto et al., 2000a] and TTV DNA has been detected in hepatocytes by in situ hybridization [Rodriguez-Inigo et al., 2000].

For a DNA virus, its genomic sequence shows a great degree of diversity, and at least 40 TTV genotypes or five major phylogenetic groups (1–5) have been identified [Okamoto et al., 1999b; Heller et al., 2001; Muljono et al., 2001; Peng et al., 2002]. Eight genotypes of the SEN virus (SENV: SENV-A to SENV-H) have been classified into TTV group 3, which is represented by the TUS01 and SANBAN isolates [Mushahwar,

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