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Paradoxical expression of *IL-28B* mRNA in peripheral blood in human T-cell leukemia virus Type-1 mono-infection and co-infection with hepatitis C Virus

Shimeru Kamihira[1*], Tetsuya Usui[2], Tatsuki Ichikawa[3], Naoki Uno[1,2], Yoshitomo Morinaga[1,2], Sayaka Mori[2], Kazuhiro Nagai[2], Daisuke Sasaki[1,2], Hiroo Hasegawa[1,2], Katsunori Yanagihara[1,2], Takuya Honda[3], Yasuaki Yamada[1,2], Masako Iwanaga[4], Takashi Kanematu[5] and Kazuhiko Nakao[3]

Abstract

Background: Human T-cell leukemia virus type-1 (HTLV-1) carriers co-infected with and hepatitis C virus (HCV) have been known to be at higher risk of their related diseases than mono-infected individuals. The recent studies clarified that IL-28B polymorphism rs8099917 is associated with not only the HCV therapeutic response by IFN, but also innate immunity and antiviral activity. The aim of our research was to clarify study whether IL-28B gene polymorphism (rs8099917) is associated with HTLV-1/HCV co-infection.

Results: The genotyping and viral-serological analysis for 340 individuals showed that IL-28B genotype distribution of rs8099917 SNP did not differ significantly by respective viral infection status. However, the IL-28B mRNA expression level was 3.8 fold higher in HTLV-1 mono-infection than HTLV-1/HCV co-infection. The high expression level was associated with TT (OR, 6.25), whiles the low expression was associated with co-infection of the two viruses (OR, 9.5). However, there was no association between down-regulation and ATL development (OR, 0.8).

Conclusion: HTLV-1 mono-infection up-regulates the expression of IL-28B transcripts in genotype-dependent manner, whiles HTLV-1/HCV co-infection down-regulates regardless of ATL development.

Keywords: IL-28B, IL-λ3, HTLV-1, HCV, SNP

Introduction

A retrovirus, human T-cell leukemia virus type-1 (HTLV-1), and a positive-strand RNA virus, hepatitis C virus (HCV), are completely different in terms of virologic characteristics. Nevertheless, they play a similar role in the pathogenesis of viral-induced malignant neoplasms, such as adult T-cell leukemia (ATL) in HTLV-1- infected individuals, and hepatocellular carcinoma (HCC) and B-cell lymphoma in HCV- infected individuals, during long-term chronic infections.

Furthermore, it is known that co-infection with HCV and HTLV-1 is frequently observed in an area endemic for HTLV-1. HCV/HTLV-1 co-infected individuals have been reported to be at higher risk for developing HCC than those infected with HCV alone [1-3]. Although the pathologic mechanism of the co-infection remains to be elucidated, it is thought that the impaired immunity due to HTLV-1 infection may contribute to HCV infection and HCV-related disorders, which is suggested by previous reports. Kohno et al. reported that the severe immunodeficiency and anergic state in patients with ATL may be associated with a functional property of leukemic cells originating from regulatory T-cells expressing CD4, CD25, CCR4, GITR and Foxp3 [4]. Kishihara et al. also reported that impairment of the immune response by HTLV-1 could explain the reduced effectiveness of interferon (IFN) treatment in patients co-infected with HTLV-1 and HCV [5].

* Correspondence: kamihira@nagasaki-u.ac.jp
[1]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501, Japan
Full list of author information is available at the end of the article

Recently, genome-wide association studies of patients with HCV have made great advances in viral clearance associated with *IL-28B* single nucleotide polymorphisms (SNP) [6,7]. IL-28B is a type III Lambda interferon (IFN-λ) and a cytokine similar to IL-10 with IFN-like activities [8]. This new IFN-λ family includes IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) [9]. Although the IFN-λ genomic structure resembles that of the IL-10 family [10], the amino acid and functional level of IFN-λs are more closely related to type I IFNs than IL-10. The IFN-λs are induced by stimulation with several single-strand RNAs (ssRNA) and several kinds of viruses. The IL-28B SNPs, such as rs8099917, rs12979860, and 12980275, have been reported to be associated with spontaneous clearance [10], innate HCV immunity [9], HCV-related disease chronicity, and therapeutic response to pegIFN-α and ribavirin (RBV) [6,7].

From these observations, we hypothesized that IFN-λ3 encoded from the *IL-28B* gene would be associated with HTLV-1 infection. The aim of the present study was to examine the mutual association between IL-28B polymorphism (rs8099917 SNP) and mono-infected-HTLV-1 and co-infected HTLV-1 with HCV subjects.

Materials and methods

Clinical subjects

All subjects were of Japanese origin living in Nagasaki City, an endemic area for HTLV-1 in Japan. For genomic specimens, 340 blood samples were randomly collected from patients who visited a liver clinic and liver transplantation center from April 2009 to March 2011 from the departments of Hepatology and a Hematology Clinic. One hundred and twenty-four of the 340 samples were also available for total RNA tests. Accordingly, most patients had either chronic liver disease (CLD) or adult T-cell leukemia (ATL). This study was done under informed consent after the approval of the Nagasaki University hospital IRB (IRB Approval No.10050). Since the samples used here were un-linked materials, patient information was restricted.

Cell lines

Eight HTLV-1-infected T-cell lines, Hut 102, MT-1, MT-2, ST1, KK1, KOB, OMT, and LMY-1, were used for IL-28B mRNA quantification. The first three were purchased and latter five were established in our laboratory [11].

Serological and genetic tests for HCV and HTLV-1

HCV and HTLV-1 infections were mainly serologically detected using commercially available kits, CLEIA-anti-HTLV-1, Lumipulse-II Ortho HCV (Fujirebio-INC, Tokyo, Japan). The confirming examination was genetically performed by the Cobas TaqMan HCV test (TaqMan HCV; Roche Tokyo INC, Tokyo, Japan) for HCV and in-house HTLV-1 proviral real-time RT quantifiable PCR [12]. Genomic DNA and total RNA were extracted from peripheral blood mononuclear cells (PBMC) using commercially available QuickGene DNA Whole blood kits (FUJIFILM Corp., Tokyo, Japan) and PureLink RNA Micro Kits (Invitrogen Corp., Carlsbad, Ca, USA). The extraction protocol was performed according to the manufacturer's instructions.

Genotyping for SNPs

SNP genotyping was performed using multiplex PCR amplification and Pyrosequencing technology. To amplify target regions, newly designed biotinylated-primers were employed: sense and anti-sense for rs8099917, 5'-TCCTCCTTTTGTTTTCCTTTCTG-3' and 5'-AAAAAGCCAGCTACCAAACTGT-3'. Then, the amplicon was sequenced according to the manufacturer's instructions based on Pyrosequence technology (Qiagen, Hilden, Germany). Biotin-labeled amplicons from the 1st PCR were captured by binding to streptavidin-coated Sepharose beads, and DNA was denatured to produce an ssDNA template for the Pyrosequencing assay. The ssDNA was released and combined with the sequencing primer, which was extended during the Pyrosequencing reaction to provide the sequence of the template DNA. Pyrosequencing data were produced in the form of Pyrograms, and genotypes were assigned by the peak pattern presented in the Pyrogram.

Real-time reverse transcription (RT) quantifiable PCR for IL-28B mRNA

mRNA for IL-28B transcribed into cDNA by a GoScript™ RT System (Promega, Madison, WI, USA) was quantified by a LightCycler System (Rosche, Mannheim, Germany) using newly designed sense and anti-sense primers, 5'-AAGGACTGCAAGTGCCGCT-3' and 5'-GCTGGTCCAAGACATCCC-3' (AY129149). A standard curve was generated using a tenfold dilution method with a reference material derived from pTAC-1.2735 inserted with 166 base fragments including the target. The amplicon was assayed by the Cyber green method. The raw data were normalized by *abl* mRNA density and evaluated as the relative value for *abl* gene expression calculated by *IL-28B* data/*abl* data $\times 10^4$, modified from our previous mRNA real time RT qPCR method [12].

Statistical analysis

The minor-allele frequency (MiAF) was set as the less frequent allele in a population for SNPs analyzed. Viral infectious status was divided into 4 groups of HTLV-1 mono-infection, HCV mono-infection, HTLV-1/HCV-co-infection, and non-infection (double negative; DN).

Differences in the genotype distribution of IL-28B SNPs among groups were compared using the Chi-square or Fisher exact test. The level of mRNA expression was compared using the Mann Whitney U test. Correlation analysis was performed by the Nonparametric Spearman's rank correlation method. The relationship between a factor and an outcome was estimated the magnitude of the association by the odds ratio with 95% confidence intervals (95%CI). Statistical analysis was performed using SAS 9.1. The statistical significance level was set at 0.05.

Results

IL-28B genotypes and the sero-status

Three hundred and forty samples were genotyped on IL-28B rs8099917 SNP and were serologically examined for viral infection of HTLV-1 and HCV. As summarized in Table 1. They consisted of 263 (77.4%) major TT homozygotes, 171 (20.9%) minor TG heterozygotes, and 6 (1.8%) minor GG homozygotes. The virus tests revealed that 59 were negative for both HTLV-1 and HCV, 73 were positive for HTLV-1 alone, 179 were positive for HCV alone and 29 were positive for both viruses. The genotypic distributions, as well as minor allele frequency (MAF) of the IL-28B gene, did not significantly differ among each viral infection status as a control of no-infection.

Since the HTLV-1 mono-infection group consisted of 47 ATL patients and 26 HTLV-1 carriers, we stratified the two groups of ATL patients and carriers and the minor allele frequencies of the two groups were compared; the difference between that of ATL and carriers was not statistically significant (p = 0.21). The prevalence of TT was not different statistically either (p = .495).

Next, the expression levels of IL-28B were quantified using 124 samples randomly collected during this study period.

Table 1 IL-28B genetic distribution and allele frequency in stratification based on the combination of HTLV-1 and HCV infection

		Genotype r(rs8099917)			Allele fequency		
	No	TT	TG	GG	T	G	
All cases	340	263 (77.4%)	71 (20.9%)	6 (1.8%)	0.86	0.14	
1) non-Infection	59	45(76.3)	10 (16.9)	4(6.8)	0.84	0.15	
2) HTLV-1 mono	73	55(75.3)	17 (23.3)	1(1.9)	0.87	0.13	P = 0.90
ATL patients	47	37(78.7)	10 (21.3)	0(0.0)	0.89	0.11	P = 0.11
carriers	26	18(69.2)	7(26.9)	1(3.8)	0.82	0.18	P = 0.46
3) HCV-mmono	179	141 (78.7)	37 (20.7)	1(1.0)	0.89	0.11	P = 0.68
4) co-infection	29	22(75.9)	7(24.1)	0(0.0)	0.88	0.14	P = 0.9

There was no significant difference in the genetic distribution and allele frequency among respective infectious states

P values were compared with non infection

IL-28B mRNA expression level and HCV/HTLV-1 co-infection

The expression levels of IL-28B mRNA were generally low in most cases with a median value of 5.2 in no-infection, 10.6 in HTLV-1 mono-infection, 3.9 in HCV mono-infection, and 2.8 in HTLV-1/HCV co-infection (Figure 1a). Notably, a small number of measurement values shown as open circles was high, and they were distributed only within the HTLV-1 mono-infected and HCV mono-infected groups. Moreover, all of those who had high values were exclusively TT homozygous, as shown in Figure 1b (samples marked by $^{A\text{-}E)}$ were the same in Figure 1(a) and Figure 1(b)). Surprisingly, the median value was the highest in HTLV-1 mono-infection and the lowest in the co-infection group (10.6 versus 2.8; p = 0.013). Therefore, to clarify whether ATL cells directly affect the expression of IL-28B mRNA, we compared the mRNA expression levels in mainly HTLV-1 carriers, ATL patients with ATL cells, and ATL cell lines. As shown in Figure 2, the median values were significantly higher in mono HTLV-1 carriers with TT (17.9 vs 5.6, $P < 0.05$) and ATL patients with TT having ATL cells than those of non-infected individuals (13.4 vs 5.6, $p < 0.05$). No high expression level was observed in two ATL or 16 carriers with HTLV-1/HCV co-infection. Surprisingly, these data were lower rather those from TG/GG. On the other hand, IL-28B mRNA expression in 8 HTLV-1-infected T-cell lines was undetectable in all but one (Hut 102). The genotype was TT in all cell lines.

In addition, there was no correlation between the IL-28B mRNA levels and HCV-RNA levels (non-parametric Spearman's rank correlation, R^2 = 0.0543, Figure 3).

Assessment by odds ratio analysis for an outcome if a risk factor is present

As shown in Figure 2, HTLV-1 was revealed to have an association similar to HCV and IL-28B mRNA. However, the up-regulated-action of HTLV-1 was nullified if the virus was co-infected with HCV. The prevalence of a major TT and minor TG/GG was similar among individuals infected with either HTLV-1 or HCV, as well as the allele frequency, indicating that there is no specific correlation between IL-28B and HTLV-1. Thus, to approach a causative clue, assessment by odds ratio (OR) analysis was performed (Table 2). Only the high mRNA level besides 3 states of HTLV-1 mono-infection,

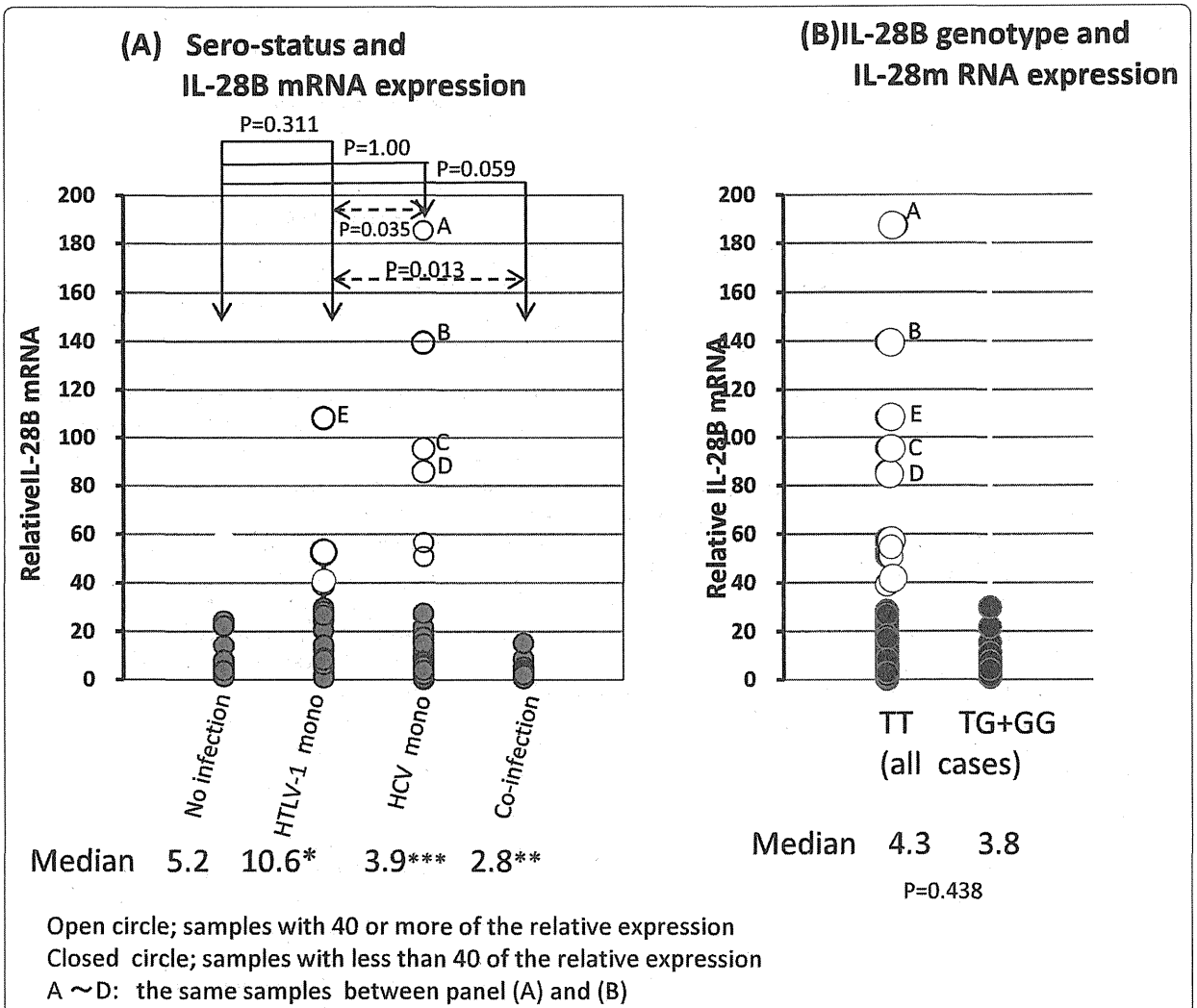

Figure 1 Relative individual IL-28B mRNA levels in peripheral blood mononuclear cells among viral infectious groups (a) and among the TT and TG/GG genotypes (b). The median values were significantly different between * vs ** and *** vs **. Open circles; those with high IL28BmRNA with TT homozygotes.

co-infection with HCV and ATL was associated with TT genotype (OR = 6.25). On the other hand, down-regulation of the mRNA density was defined as HTLV-1/HCV co-infection (OR = 9.5 p = 0.004), but low expression was not associated with ATL development (OR = 0.8, p = 0.81).

Discussion

Although co-infection with HTLV-1 and HCV has been shown to result in higher rates of cirrhosis and increased death from liver diseases [1,2], the caustic mechanism by which the co-infection affects HCV pathogenesis remains to be elucidated. Some clue to the mechanism may be found by studying the relation between IL-28B genotypes and co-infection, because IL-28B encoding IFN-λs are categorized as type 3 IFNs and are potent endogenous anti-viral cytokines. They signal via JAK/STAT intracellular pathways and up-regulated transcription of IFN-stimulated genes (ISGs) that are required to control viral infection [13]. Here, we investigated whether IL-28B polymorphism rs8099917 is associated with co-infection status.

The present study is the first to reveal that the IL-28B genotype is not associated with stratification based on the combination of HTLV-1 and HCV infection; no infection for both (double negative; DN), HTLV-1 mono-infection, HCV mono-infection and HTLV-1/HCV co-infection. Similarly, the frequency of the major TT homozygotes was not associated among ATL patients and HTLV-1 carriers (Table 2). These two

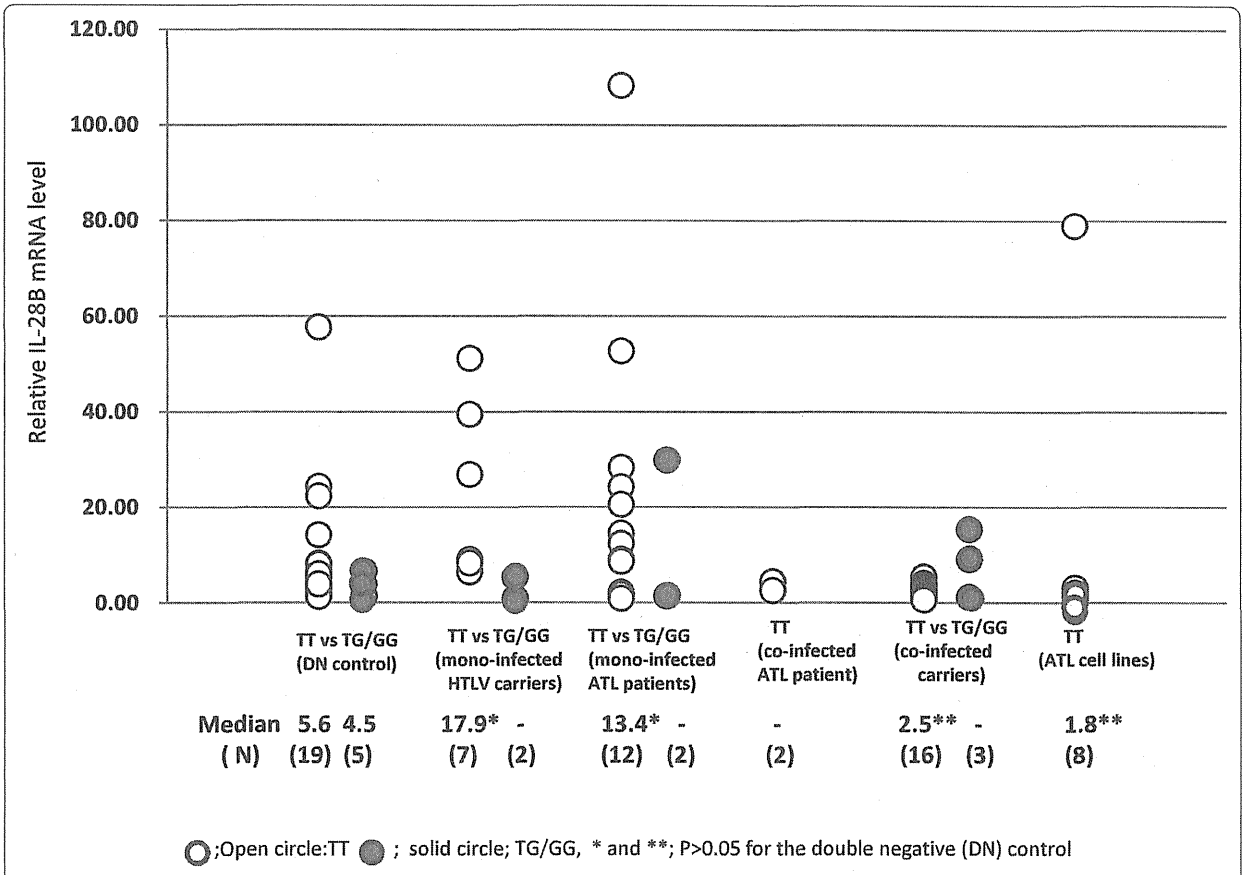

Figure 2 Individual relative expression density of IL-28B mRNA in TT vs TG/GG derived from 24 double-negative (DN) controls consisting of 19TT and 5TG/GG, 9 mono- infected HTLV-1 carriers, 14 mono-infected and 2 co-infected ATL patients, 19 co-infected carriers and 8 ATL cell lines samples.

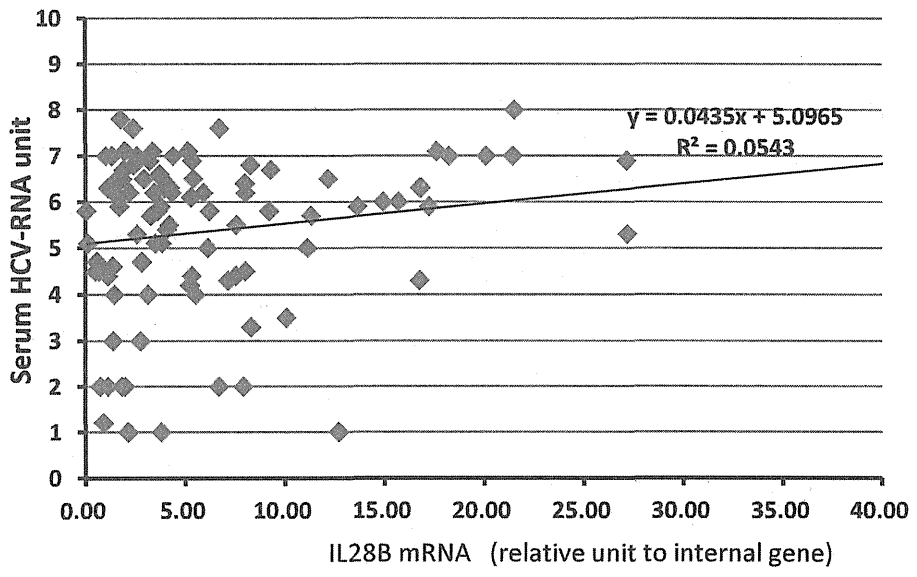

Figure 3 Correlation between serum HCV-RNA level and IL-28B mRNA. No-correlation (r^2) = 0.0543.

Table 2 Assessment by odds ratio analysis for an outcome if a risk factor is present

	factor			
(A) Outcome	**dependent independent**	**Odds ratio**	**95%CI**	**P**
1) HTLV-1 mono-infection	TT vs TG/GG	1.11	0.62-1.99	0.72
2) Co-infection	TT vs TG/GG	0.54	0.04-6.88	1.00
3) High mRNA Expression*	TT vs TG/GG	6.25	1.16-33.75	0.04
4) ATL	TT vs TG/GG	1.50	0.60-3.75	0.39
(B)				
5) Low mRNA Expression	HTLV-1 mono vs DN	0.34	0.06-2.04	0.24
6) Low mRNA Expression	HCV mono vs DN	0.29	0.07-2.23	0.15
7)) Low mRNA Expression	Co-Inf** vs HTLV-1-mono**	9.5	2.06-43.76	0.004
8) ATL	low expression or not	0.8	0.14-4.74	0.81

(A) Upper 4 lines; assessing the risk of 1) HTLV-1 persistent infection, 2) super-imposed HTLV-1 infection with HCV (co-infection), 3) high IL-28B mRNA expression, and 4) ATL development when the genotype is a risk factor (B) Lower 4 lines; assessing the risk factors described in the outcome, the IL-28B mRNA expression level in peripheral blood (5, 6, and 7), and ATL development (8). Consequently, similarly to HCV, HTLV-1 is associated with up-regulation of IL-28B mRNA along with the TT homozygote, and co-infection with HTLV-1 and HCV paradoxically down-regulates the mRNA level

*; IL-28B Expression level, Co-inf = co-infection with HTLV-1 and HCV, mono = mono-infection

findings suggest that the SNP rs8099917 is not associated with susceptibility to HTLV-1 infection or the development of ATL. On the other hand, all of ATL cell- or HTLV-1-infected T-cell- lines examined were exclusively TT homozygous, implying that HTLV-1-infected cells carrying TT homozygotes may immortalize easily in vitro.

Next, we found a strange phenomenon that the IL-28B mRNA expression levels in peripheral blood were lower in samples with HTLV-1/HCV co-infection than in samples with either HTLV-1 or HCV alone, especially significantly for HTLV-1 mono-infection. In particular, samples carrying TT homozygotes were strongly down-regulated, more than the minor TG hetero- and GG-homozygotes. Why are the mRNA expression levels different in mono- and dual-infection? Although it is not known how rs8099917 affects the action of IL-28B, presumably it alters the immune function to viruses. In addition to a common anti-viral IFN-stimulating signal pathway, HTLV-1 may use an alternative anti-viral pathway like HBV [14], because the HTLV-1 provirus is integrated into host genomic DNA and replicates in distinctive life cycle kinetics. Moreover, ATL originates from Treg cells, which play a central role in suppressing immunity [15]. However, this cannot fully explain the impairment in the HTLV-1 carrier's immunity because no ATL cells are present during the carrier period. Thus, we noted IFN-λ(IL-29, IL-28), which was recently discovered as a type III IFNs with anti-virus ability, anti-tumor and immune responses [16-18].

From our results, the IL-28B expression level was higher in HTLV-1 mono-infected individuals including ATL patients. IFN-λis usually up-regulated through activation of the NF-kappaB pathway after viral infection. Actually, the Sendai virus, an influenza A virus, and others have been demonstrated to activate the NF-kappa B pathway, resulting in up-regulated IL-28B expression [19,20]. Accordingly, the highest up-regulation of IFN-λ3 in HTLV-1 mono-infection may be explained by virtue of a viral protein of HTLV-1 having strong NF-kB activating ability. Moreover, it is instructive that IFN-λhas a potent function to expand Treg cells [21], which are mainly infected with HTLV-1, predisposing development of ATL. However, there has not yet been evidence that co-infection with HCV damages Tax action.

Of IL-28B producing cells in the literature, most cells in the blood are described as having a weak or absent expression under the steady state conditions. Li et al. [9] reported that IL-28B mRNA is not always expressed in virally infected cells. Actually, our findings in HTLV-1-infected cases also showed that at least the main producing cells are likely to be cells other than ATL cells because most cell lines from ATL and some blood samples containing ATL cells were expressed faintly. At present, plasmacytoid dendritic cells are indicated to be the most potent producers of IFN-λs [19]. On the other hand, IFN-λ3 reportedly has the functions of proliferating Treg cells which are the origin of ATL cells, suggesting that HTLV-1 is associated with up-regulation via Treg cells infected with HTLV-1.

In conclusion, we found an unusual phenomenon in that the expression of IL-28B mRNA was affected by not only the IL-28B rs8099917 genotype, but also co-infected HTLV-1 with HCV. This will contribute to a better understanding the enigmatic impairment of immunity in the HTLV-1 carrier state, including co-infection with HTLV-1 and HCV.

Abbreviations

HTLV-1: Human T-cell leukemia virus type -1; HCV: Hepatitis C virus; SNP: Single nucleotide polymorphism; IFN: Interferon; PBMC: Peripheral blood

mononuclear cell; PCR: Polymerase chain reaction; MAF: Minor-allele frequency.

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Author details
[1]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501, Japan. [2]Central Diagnostic Laboratory of Nagasaki University Hospital, Nagasaki, 852-8501, Japan. [3]Department of Gastroenterology and Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501, Japan. [4]Faculty of Wellness Studies, Kwassui Women's University, Nagasaki, 850-8515, Japan. [5]Division of Surgical Oncology, Department of Translational Medical Science, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501, Japan.

Authors' contributions
SK designed the study and wrote the manuscript, and SM, TU, KN, DS HH, KY, NU, YM analyzed the genotype, TK collected samples, and TK, KN, MI and SK organized and assessed the data. All authors interpreted the data and were financially supported. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Original article

Distribution of Two Subgroups of Human T-Lymphotropic Virus Type 1 (HTLV-1) in Endemic Japan

Masashi Otani[1], Noritaka Honda[2], Pin-Cang Xia[3], Katsuyuki Eguchi[3], Tatsuki Ichikawa[1], Toshiki Watanabe[4], Kazunari Yamaguchi[5], Kazuhiko Nakao[1] and Taro Yamamoto[3]*

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Abstract: Endemic areas of human T-lymphotropic virus type 1 (HTLV-1) have been reported in Japan as well as tropical Africa, Central and South America and Melanesia. The existence of two subgroups, i.e., the transcontinental and Japanese subgroups, was reported in Japan. In the present study, we provide data on the ratio of the two subgroups in each endemic area and infection foci and examine the distribution of HTLV-1 in Japan and neighboring areas. A 657 bp fragment of env region of HTLV-1 proviral genome was successfully amplified for 183 HTLV-1 positive DNA samples. The subgroup determination was done by RFLP reactions using endonucleases *HpaI* and *HinfI*. The northern part of mainland Kyushu, represented by Hirado and Kumamoto, was monopolized by the Japanese subgroup, while the transcontinental subgroup ranged from 20 to 35% in the Pacific coast areas of Shikoku (Kochi), the Ryukyu Archipelago (Kakeroma and Okinawa) and Taiwan. An interesting finding in the present study is the presence of the transcontinental subgroup in Kochi, suggesting the endemicity of the transcontinental subgroup along the Kuroshio Current.

Key words: Japanese subgroup, transcontinental subgroup, human migration, Kuroshio Current

INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) was first isolated in 1980 [1] and has been identified as a causative agent of adult T cell leukemia (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 has three major transmission routes: from mother to infant through breast milk, from male to female through semen, and to blood recipients through the lymphocytes of HTLV-1 carriers. These transmission routes, especially mother-to-child transmission, allow HTLV-1 to pass from generation to generation and localize within family, community and ethnic groups. Thus, the elucidation of the geographical distribution of HTLV-1 has important ethnoepidemiological implications [2].

In view of this unique fact, a large number of phylogeographycal and epidemiological studies have been conducted within and beyond the borders of Japan, and valuable results have been obtained. Firstly, endemic areas were reported in tropical Africa, the Caribbean basin, Central and South America, Papua New Guinea and other islands of Melanesia, as well as Japan [3, 4]. Secondly, there are three major lineages existing worldwide: the Melanesian subtype, the Central African subtype, and the cosmopolitan subtype, ubiquitous in endemic areas around the world [5, 6]. Thirdly, the cosmopolitan subtype is further divided into three major subgroups: A, B, and C, which correspond to the transcontinental subgroup, the Japanese subgroup, and the West African subgroup, respectively [7, 8]. Fourthly, within Japan, endemicity is found in Kyushu and Okinawa, and small infection foci are seen in coastal islands of the Japan Sea and the Pacific side of Shikoku, Kii and Tohoku, while most of Honshu is HTLV-1-free [3]. Furthermore, a few endemic areas have been found in areas neighboring Japan: Nogliki of Sakhalin, Kinmen, Fujian and Taiwan [7, 9–11]. Fifthly, the existence of two different subgroups of

[1] Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
[2] School of Medicine, Nagasaki University
[3] Department of International Health, Institute of Tropical Medicine (NEKKEN), the Global Center of Excellence, Nagasaki University
[4] Department of Medical Genome Sciences, Graduate School of Frontier Sciences, University of Tokyo, Chiba, Japan
[5] Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, Tokyo, Japan
*Corresponding author:
Department of International Health, Institute of Tropical Medicine, Nagasaki University, Sakamoto 1-12-4, Nagasaki, Japan
Tel/Fax: +81-95-819-7869
E-mail: y-taro@nagasaki-u.ac.jp

HTLV-1, i.e., the transcontinental and Japanese subgroups, in Japan and clusters of the former subgroup in Kyushu and the Ryukyu islands were reported [12].

In the present study, we provide data on the ratio of the two subgroups in each endemic area and infection foci within Japan and use that data to elucidate the distribution of HTLV-1 in Japan and neighboring area.

MATERIALS AND METHODS

DNA samples from a total of 197 anonymous HTLV-1 positive donors were obtained from the Joint Study on Predisposing Factors of ATL Development (JSPFAD) and used in the present study. Of the 197 samples, 40 were gathered in Hokkaido (Hokkaido University Hospital), four in Iwate (Iwate Medical University), 30 in Kochi (Kochi Medical School Hospital), 50 in Hirado (Nagasaki University Hospital), 23 in Kumamoto (Kumamoto University Hospital) and 50 in Okinawa (Okinawa Kyodo Hospital).

Furthermore, DNA was extracted from peripheral blood donated by five anonymous HTLV-1 carriers on Ishigaki Island, Japan (Yaeyama County, Ishigaki City, Okinawa Prefecture). The analysis of samples donated by the Yaeyama residents was approved by the ethics committee of the Institute of Tropical Medicine, Nagasaki University, Japan (Approval No. 10012147).

A 657 bp fragment of env region was amplified by nested PCR. The first reactions were performed in 20 μl volumes containing 1 μl (ca. 50 ng) of the extracted DNA, 200 μM (final conc.) of dNTP mixture, 0.25 μM (final conc.) of the primer sets, 2 μl of 10 × Ex Taq Buffer and 0.5U TaKaRa Ex Taq HS (TAKARA BIO Inc., Shiga, Japan). The external primers were TAATAGCCGCCAGTGGAA AG (nucleotide positions according to the J02029 sequence: 5027–5046) and AGTCCTTGGAGGCTGAACG (6786–6768). The thermal conditions were as follows: 5-min denature at 94°C, 40 cycles of 40 sec at 94°C, 30 sec at 61°C and 40 sec at 72°C, and 10-min final extention at 72°C. The second reactions were performed in 40 μl volumes containing 2 μl of the first PCR product, 200 μM (final conc.) of dNTP mixture, 0.25 μM (final conc.) of the primer sets, 4 μl of 10 × Ex Taq Buffer and 1U TaKaRa Ex Taq HS. The internal primers were CTCCCTTCTAGTCGACGCTCCAGG (5685–5708) and CGTCTGTTCTGGGCAGCATA (6341–6322). The thermal conditions were as follows: 2-min denature at 95°C, 35 cycles of 20 sec at 95°C, 20 sec at 58°C and 30 sec at 72°C, and 2-min final extention at 72°C.

All of the 35 samples from Hokkaido, all of the four from Iwate, 28 of 30 from Kochi, 44 of 50 from Hirado, 21 of 23 from Kumamoto, 46 of 50 from Okinawa and all of the five from Yaeyama were well amplified. RFLP reactions were performed using endonucleases *HpaI* and *HinfI* as designed by Yang et al. [7]. The digested DNA fragments were electrophoresed on 2% agarose gel pre-stained with ethidium bromide and visualized.

RESULTS AND DISCUSSION

All except one of the HTLV-1 isolates from Iwate, Hirado and Kumamoto were determined as the Japanese subgroup, while 20–35% of the isolates from Hokkaido, Kochi, Okinawa and Yaeyama were determined as the transcontinental subgroup (Fig. 1). The electrophoresis profile of two isolates (Hokkaido and Kochi) was consistent with neither the Japanese nor the transcontinental subgroup but similar to the West African/Caribbean subgroup shown by Yang et al. [7]. Thus, these were tentatively treated as "undetermined" in the present paper.

The uneven distribution of the transcontinental and Japanese subgroups in the endemic areas of Japan was clarified in the present study, whereas only the transcontinental subgroup was reported from neighboring areas such as Nogliki of Sakhalin, Kinmen, and Fujian [9–11].

The northern part of mainland Kyushu, represented by Hirado and Kumamoto, seems to be monopolized by the Japanese subgroup. On the other hand, the presence of the transcontinental subgroup ranges from 20 to 35% in the Pacific coast areas of Shikoku (Kochi), the Ryukyu Archipelago (Kakeroma [13] and Okinawa) and Taiwan [7]. An interesting finding in the present study is the presence of the transcontinental subgroup in Kochi, suggesting the endemicity of the transcontinental subgroup along the Kuroshio Current.

A north-flowing ocean current on the west side of the Pacific Ocean, the Kuroshio Current has played the role of an aorta for migration and transportation along the Pacific coast of southwestern Japan since prehistoric times. The endemicity of the transcontinental subgroup along the Kuroshio Current might reflect this human movement. If so, we need to pay more attention to the date and mode of local human movements which may have implications in the epidemiology of HTLV-1 and other infectious agents such as hepatitis B virus [14].

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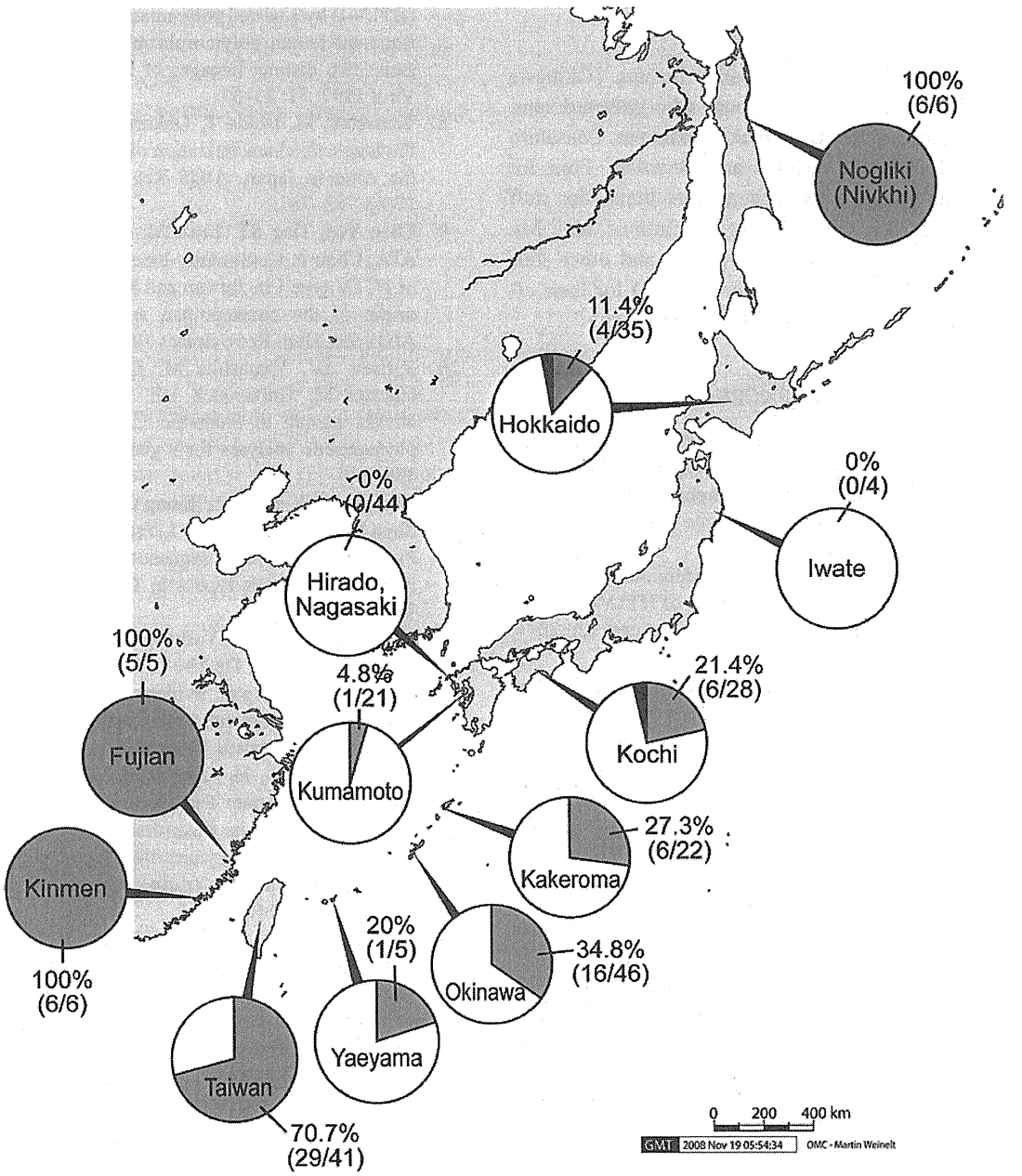

Fig. 1. Ratio of the transcontinental subgroup (grey) to the Japanese subgroup (white) of HTLV-1 cosmopolitan subtype in various localities of East Asia. The data of Nogliki, Kakeroma, Taiwan, Kinmen and Fujian were cited from Syrtsev et al. [10], Eguchi et al. [13], Yang et al. [7], Chen et al. [9] and Wang et al. [11], respectively.

versity. No sponsor, however, participated in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

AUTHOR DISCLOSURE STATEMENT

Drs. Otani, Yamamoto and Eguchi have full access to all the data in the study and hold final responsibility for the decision to submit for publication. All authors declare that they have no conflict of interest.

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Relationship of α-fetoprotein levels and development of hepatocellular carcinoma in hepatitis C patients with liver cirrhosis

NAOTA TAURA[1], SACHIKO FUKUDA[1], TATSUKI ICHIKAWA[1], HISAMITSU MIYAAKI[1], HIDETAKA SHIBATA[1], TAKUYA HONDA[1], TOHEI YAMAGUCHI[1], YOKO KUBOTA[1], SHINJIRO UCHIDA[1], YASUHIRO KAMO[1], EMI YOSHIMURA[1], HAJIME ISOMOTO[1], TAKEHIRO MATSUMOTO[1], FUMINAO TAKESHIMA[1], TAKUYA TSUTSUMI[2], SHOTARO TSURUTA[3] and KAZUHIKO NAKAO[1]

[1]Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8501; [2]Department of Gastroenterology and Hepatology, Nagasaki Municipal Hospital, Nagasaki 850-8555, [3]Department of Gastroenterology and Hepatology, Japanese Red Cross Nagasaki Genbaku Hospital, Nagasaki 852-8511, Japan

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Abstract. α-fetoprotein (AFP) is a tumor marker of hepatocellular carcinoma (HCC) and has also been reported to reflect the effectiveness of long-term low-dose interferon (IFN) therapy in hepatitis C virus (HCV)-infected patients with chronic liver disease. The correlation between AFP levels and the incidence of HCC has been discussed over a long period. We investigated whether high levels of AFP at the time of diagnosis were associated with an increased incidence of HCC in patients with HCV. A total of 107 HCV patients with liver cirrhosis without other risks were evaluated for the predictive value of non-invasive risk factors for HCC, including age, gender, alcohol intake, aspartate and alanine aminotransferase levels, bilirubin, albumin, platelet count and AFP levels at study entry, as well as the IFN therapy received. During the follow-up period, HCC developed in 68 (63.6%) patients. Kaplan-Meier estimates were made to assess the cumulative risk of HCC. The 10-year cumulative incidence rate of HCC was 80%. Cox regression analysis was performed on several variables, including age, gender, alcohol consumption, experience of IFN therapy and biochemical parameters. The following factors were identified as exhibiting an increased risk of HCC by univariate analysis: aspartate transaminase (AST) ≥71 IU/l, alanine transaminase (ALT) ≥60 IU/l, AFP ≥6 ng/ml and IFN therapy. Multivariate analysis identified that the AFP level [6-19 ng/ml: hazard ratio (HR), 2.22; P=0.006 and ≥20 ng/ml: HR, 2.09; P=0.003] was an independent and significant risk factor for the development of HCC. A slightly elevated (6-19 ng/ml) AFP level may be a risk factor for HCC in certain cases. By contrast, AFP levels <6 ng/ml indicate a low risk of HCC development in HCV patients with liver cirrhosis.

Correspondence to: Dr Naota Taura, Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Sakamoto 1-7-1, Nagasaki 852-8501, Japan
E-mail: ntaura-gi@umin.ac.jp

Key words: hepatocellular carcinoma, hepatitis C virus, α-fetoprotein

Introduction

Primary liver cancer is the most common primary cancer of the liver, accounting for approximately 6% of all human cancers. It is estimated that half a million cases are diagnosed worldwide annually, making primary liver cancer the fifth and ninth most common malignancy in males and females, respectively (1-6). Hepatocellular carcinoma (HCC) accounts for 85-90% of primary liver cancers (7) and the age-adjusted HCC mortality rate has increased in recent decades in Japan (8). Similarly, a trend of increasing rates of HCC has been reported in several developed countries in North America, Europe and Asia (9,10). HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, excessive alcohol consumption or non-alcoholic fatty liver disease. Of the hepatitis viruses that cause HCC, HCV is predominant in Japan (11-14).

α-fetoprotein (AFP) is a tumor marker of HCC and is also reported to reflect the effectiveness of long-term low-dose interferon (IFN) therapy in HCV patients with chronic liver disease (15). The correlation between AFP levels and the incidence of HCC has been discussed over a long period. We investigated whether high levels of AFP at the time of diagnosis were associated with an increased incidence of HCC in patients with HCV.

Patients and methods

Study population. Between 1976 and 2010, 107 patients were diagnosed with liver cirrhosis due to HCV infection at the Department of Gastroenterology and Hepatology, Nagasaki

University Hospital (Nagasaki, Japan). The diagnosis of liver cirrhosis was based on biopsy and/or clinical findings. Sera were stored at -80˚C until they were used for assays. The diagnosis of chronic HCV infection was based on the presence of anti-HCV antibodies (HCV Abs; microparticle enzyme immunoassay; Abbott Laboratories, Chicago, IL, USA) and HCV RNA, as detected by the polymerase chain reaction. The diagnosis of chronic HBV infection was excluded on the basis of the presence of hepatitis B surface antigen (HBsAg; enzyme-linked immunosorbent assay; Abbott Laboratories). Serum AFP was measured using a radioimmunoassay (Abbott Laboratories). The patient alcohol intake histories were obtained from their medical records. Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g of pure ethanol for a period of >10 years.

Follow-up of patients and diagnosis of HCC. Following the initial diagnosis of patients with liver cirrhosis and HCV infection, the patients underwent measurement of AFP levels and liver function biochemistry every 1 to 3 months during the follow-up period and ultrasonography (USG) was performed every 3 to 6 months. The diagnosis of HCC was based on imaging techniques, including USG, computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG) and/or liver biopsy. The diagnostic criteria for HCC included confirmative liver biopsy, neovascularization in HAG and/or CT.

The end date of the present study was December 2010, detection of HCC or the time of patient mortality. If a patient had not been monitored in the hospital for >1 year, the patient was considered lost to the follow-up. The median observation period was 3.8 years (IQR, 5.0).

IFN therapy. During the observation period, 43 (40%) of the 107 patients received IFN monotherapy, PEGylated (PEG)-IFN monotherapy or combination therapy with IFN and ribavirin or PEG-IFN and ribavirin. A sustained virological response (SVR) was defined as the absence of detectable HCV RNA at the end of treatment persisting for >6 months, while a failure to meet these criteria was defined as non-SVR. There were no relapses of viremia in the SVR patients after 6 months.

Statistical analysis. The HCC development rate was analyzed using the Kaplan-Meier technique and differences in the curves were studied using the log-rank test. Independent risk factors associated with the rate of HCC development were identified using the stepwise method of non-time-dependent Cox regression analysis. Parametric comparisons were performed using analysis of variance (ANOVA). The significance of individual differences was evaluated using the Scheffe test. Data analysis was performed with SPSS version 16.0 for Windows. $P<0.05$ was considered to indicate a statistically significant result.

Results

Clinical features of the studied patients. Patient characteristics at the time of the cirrhosis diagnosis are shown in Table I. There were 54 male (51%) and 53 female (49%) cirrhosis patients (median age, 62.5 years). Habitual drinkers and diabetic patients were 10% (11 of 107) and 44% (41 of 107) of all cases, respectively. Child-Pugh grade A was recorded in 52% (56 of 107) of patients, grade B in 41% (44 of 107) and grade C in 7% (7 of 107). Of the studied patients, 40% (43 of 107) underwent IFN therapy and 60% (64 of 107)

Table I. Characteristics of 107 studied hepatitis C patients with liver cirrhosis.

Characteristic	Value
Number of patients	107
Age (years), median (IQR)	62.5 (13.3)
Gender, n (%)	
Male	54 (51)
Female	53 (49)
Height (m), median (IQR)	1.58 (0.2)
Weight (kg), median (IQR)	56.4 (13.3)
BMI (kg/m^2), median (IQR)	22.6 (4.2)
Alcohol consumption, n (%)	
Excessive	11 (10)
Not excessive	96 (90)
Diabetes mellitus, n (%)	
+	44 (41)
-	63 (59)
Diagnosis, n (%)	
Histological	80 (75)
Clinical	27 (25)
Child-Pugh grade, n (%)	
A	56 (52)
B	44 (41)
C	7 (7)
Platelet count ($10^3/\mu$l), median (IQR)	100 (6.5)
AST (IU/l), median (IQR)	71 (64)
ALT (IU/l), median (IQR)	60 (61)
γ-GTP (IU/l), median (IQR)	45 (58)
Bilirubin (mg/dl), median (IQR)	1.0 (0.9)
Albumin (mg/dl), median (IQR)	3.8 (0.9)
TC (mg/dl), median (IQR)	152 (44)
TG (mg/dl), median (IQR)	92 (57
AFP (ng/ml), median (IQR)	11 (24)
<6, n (%)	34 (32)
6-19, n (%)	38 (35)
≥20, n (%)	35 (33)
BCAA, n (%)	
+	39 (36)
-	68 (64)
Interferon therapy, n (%)	
SVR	11 (10)
Non-SVR	32 (30)
No therapy	64 (60)

Data are median (IQR) or frequency (%). BMI, body mass index; AST, aspartate transaminase; ALT, alanine transaminase; TC, total cholesterol; TG, triglyceride; BCAA, branched-chain amino acids; AFP, α-fetoprotein; SVR, sustained virological response.

Table II. Factors increasing the risk of hepatocellular carcinoma (HCC) determined by univariate analysis.

Parameters	Hazard ratio	P-value
Age (years)		
>62	1.29	0.291
Gender		
Male	0.80	0.360
BMI (kg/m^2)		
>25	0.88	0.636
Alcohol consumption		
Excessive	1.40	0.211
Diabetes mellitus (%)		
+	1.10	0.712
Child-Pugh grade		
A	1	-
B	1.20	0.474
C	0.94	0.925
Platelet ($10^3/\mu$l)		
<100	1.07	0.788
AST (IU/l)		
≥71	1.83	0.016
ALT (IU/l)		
≥60	1.80	0.020
γ-GTP (IU/l)		
≥45	1.25	0.970
Bilirubin (mg/dl)		
≥1.0	0.72	0.189
Albumin (mg/dl)		
<3.8	0.85	0.520
TC (mg/dl)		
≥152	0.66	0.095
TG (mg/dl)		
≥92	0.76	0.269
AFP (ng/ml)		
<6	1	-
6-19	2.54	0.006
≥20	2.71	0.003
BCAA		
+	1.59	0.063
Interferon therapy (%)		
No therapy	1	-
Non-SVR	0.77	0.366
SVR	0.26	0.031

BMI, body mass index; AST, aspartate transaminase; ALT, alanine transaminase; TC, total cholesterol; TG, triglyceride; BCAA, branched-chain amino acids; AFP, α-fetoprotein; SVR, sustained virological response.

were followed closely without receiving IFN treatment. The proportion of IFN-treated patients exhibiting an SVR was 25.6% (11/43). The patients were classified into 3 categories according to the level of AFP. The AFP levels were <6 ng/ml in 34 (32%) patients, between 6 and 19 ng/ml in 38 (35%) and ≥20 ng/ml in 35 (33%).

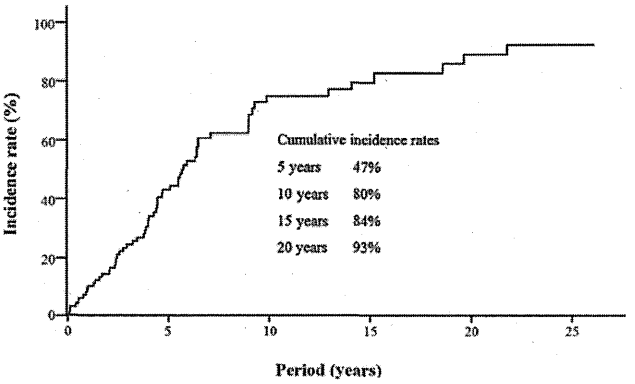

Figure 1. Cumulative incidence rates of hepatocellular carcinoma (HCC) in all patients.

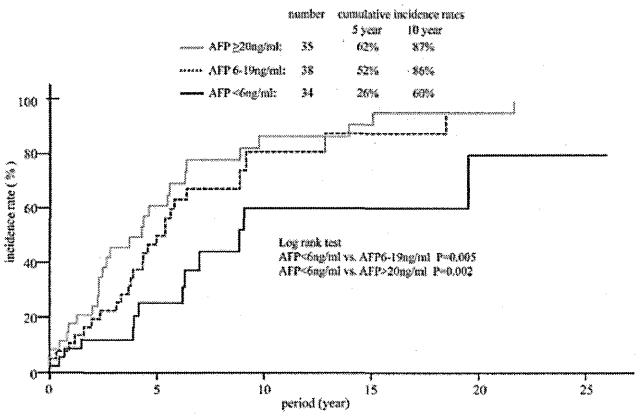

Figure 2. Cumulative incidence rates of hepatocellular carcinoma (HCC) according to α-fetoprotein (AFP) levels.

Risk factors for HCC. Cox regression analysis was performed on variables, including age, gender, alcohol consumption, experience of IFN therapy and biochemical parameters. The following factors were identified as exhibiting an increased risk of HCC by univariate analysis: aspartate transaminase (AST) ≥71 IU/l, alanine transaminase (ALT) ≥60 IU/l, AFP ≥6 ng/ml and IFN therapy (Table II). Multivariate analysis identified the etiology of the AFP level [6-19 ng/ml: hazard ratio (HR), 2.22; P=0.006 and ≥20 ng/ml: HR, 2.09; P=0.003] as independent and significant risk factor for the development of HCC (Table III).

Development of HCC. During the follow-up period, HCC developed in 68 (63.6%) patients. Kaplan-Meier estimates of the cumulative risk of HCC are shown in Fig. 1. The 10-year cumulative incidence rate of HCC was 80%. The cumulative incidence of HCC in patients with various AFP levels is shown in Fig. 2. The 10-year cumulative risk of HCC was 60% in the 34 patients with AFP levels <6 ng/ml at study entry, 86% in the 38 patients with AFP levels between 6 and 19 ng/ml and 87% in the 34 patients with AFP levels ≥20 ng/ml. Significant differences were observed in the HCC incidence between those with AFP level <6 ng/ml and those with an AFP level between 6 and 19 ng/ml and ≥20 ng/ml.

Table III. Factors increasing the risk for hepatocellular carcinoma (HCC), determined by multivariate analysis.

Parameters	Hazard ratio	95% CI	P-value
AST (IU/l)			
≥71	1.27	0.72-2.26	0.411
ALT (IU/l)			
≥60	1.40	0.81-2.43	0.229
AFP (ng/ml)			
<6	1	-	-
6-19	2.22	1.13-4.38	0.006
≥20	2.09	1.03-4.23	0.003
Interferon therapy (%)			
No therapy	1	-	-
Non-SVR	0.99	0.55-1.80	0.989
SVR	0.46	0.14-1.57	0.218

CI, confidence interval; AST, aspartate transaminase; ALT, alanine transaminase; AFP, α-fetoprotein; SVR, sustained virological response.

Discussion

In the present study, the AFP level was identified as a risk factor for HCC in HCV patients with liver cirrhosis. Notably, patients with high (≥20 ng/ml) and elevated AFP levels (between 6 and 19 ng/ml) had an increased risk of HCC development. This deviated slightly from the serum AFP levels of healthy adults reported to range between 0.1 and 5.8 ng/ml (16). In the present study, analyses were performed by setting various AFP cut-off levels to evaluate their performance as risk factors. In HCV patients with cirrhosis, an AFP level ≥6 ng/ml was observed to be associated with the development of HCC in the multivariate analysis.

AFP is used as a serological marker of HCC and employed in combination with USG for HCC screening (17,18). Numerous studies have demonstrated an elevated AFP level to be a risk factor for the development of HCC in HCV patients (19-26). There is extensive evidence demonstrating that AFP is functionally an embryonic and fetal carrier/transport molecule for a number of of ligands, including fatty acids, bilirubin, heavy metals, steroids, retinoids, drugs, dyes and antibiotics (27). However, the biological and pathophysiological roles of the association of AFP with an increased risk of HCC development remain unclear. Tateyama *et al* reported that AFP levels were elevated in parallel with advanced fibrosis stages and correlated well with the fibrosis stage (26). Since the patients with slightly elevated AFP levels (between 6 and 19 ng/ml) had moderately advanced liver fibrosis stages, these AFP levels may indicate an elevated risk of HCC in patients with chronic HCV infection. Li *et al* identified a functional link between cytoplasmic AFP and the PTEN/AKT signalling pathway and provided further evidence for the understanding of the novel role of cytoplasmic AFP in the maintenance of tumor cell growth (28). The silencing of AFP expression by a knockdown of its gene may play a role in growth arrest and apoptosis in human HCC cells (28-31).

IFN has been used to treat patients with HCV infection. A failure to achieve an SVR with IFN-based therapies, pre-existing advanced hepatic fibrosis and/or cirrhosis are major predictors of HCC (20,32-35). Numerous Japanese cohort studies have demonstrated that IFN therapy reduces the incidence of HCC, not only in sustained virological responders but also in transient responders in whom the elimination of HCV has failed (32,36-40). In cirrhotic patients, Nishiguchi *et al* reported that the relative risk of patients receiving IFN-α treatment developing HCC was 0.067 in comparison with the control group (34). By contrast, Valla *et al* were unable to demonstrate any significant benefit for the prevention of HCC in patients with or without IFN treatment (41). Cammà *et al* suggested a slight preventive effect of IFN on HCC development in patients with HCV-related cirrhosis (42). Shiffman *et al* reported that continuous IFN therapy led to a decline in hepatic fibrosis despite the persistence of viremia (43). In addition, Nomura *et al* reported that the AFP level was significantly decreased at 3 months following the start of low-dose long-term IFN treatment (15). Murashima *et al* demonstrated that IFN therapy, but not Stronger Neo-Minophagen C (SNMC), universally reduced basic AFP levels (44). In an *in vitro* study of the effects of IFN on an HCC cell line, IFN exhibited antitumor effects (45). Taken together, these findings suggest that AFP levels may aid the prediction of the development of HCC during IFN-based treatments, including long-term low-dose IFN therapy.

In conclusion, AFP is a non-invasive predictive marker of the development of HCC in HCV patients. The present study indicates that high (≥20 ng/ml), and slightly elevated (between 6 and 19 ng/ml) AFP levels, may suggest a substantial risk of HCC development, complementing the fibrosis stage. By contrast, AFP levels <6 ng/ml indicate a low risk of HCC development.

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Clinical Study

Baseline Serum Cholesterol Is Associated with a Response to Pegylated Interferon Alfa-2b and Ribavirin Therapy for Chronic Hepatitis C Genotype 2

Naota Taura,[1] Tatsuki Ichikawa,[1] Hisamitsu Miyaaki,[1] Yoshiko Kadokawa,[2] Takuya Tsutsumi,[3] Shotaro Tsuruta,[4] Yuji Kato,[5] Osami Inoue,[6] Noboru Kinoshita,[7] Kazuo Ohba,[8] Hiroyuki Kato,[9] Kazuyuki Ohata,[10] Junichi Masuda,[11] Keisuke Hamasaki,[12] Hiroshi Yatsuhashi,[13] and Kazuhiko Nakao[1]

[1] *Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Sakamoto 1-7-1, Nagasaki 852-8501, Japan*
[2] *Department of Gastroenterology and Hepatology, Sasebo City General Hospital, Hirase-machi 9-3, Sasebo 857-8511, Japan*
[3] *Department of Gastroenterology and Hepatology, Nagasaki Municipal Hospital, Shinchi-machi 6-39, Nagasaki 850-8555, Japan*
[4] *Department of Gastroenterology and Hepatology, Japanese Red Cross Nagasaki Genbaku Hospital, Mori-machi 3-15, Nagasaki 852-8511, Japan*
[5] *Department of Gastroenterology and Hepatology, Oita Prefectural Hospital, Bunyo 467, Oita 870-8511, Japan*
[6] *Digestive Organ Center, Japan Labour and Welfare Organization, Nagasaki Labour Welfare Hospital, Setogoe 2-12-5, Sasebo 857-0134, Japan*
[7] *Department of Gastroenterology and Hepatology, Sasebo Chuo Hospital, Yamato-machi 15, Sasebo 857-1195, Japan*
[8] *Department of Internal Medicine, Goto Central Hospital, Nagasaki Prefectural, Yoshikugichou 205, Goto 853-0031, Japan*
[9] *Department of Internal Medicine, National Hospital Organization Saga National Hospital, Hinode 1-20-1, Saga 849-8577, Japan*
[10] *Department of Internal Medicine, Kouseikai Hospital, Hayama1-3-12, Nagasaki 852-8053, Japan*
[11] *Department of Gastroenterology and Hepatology, Medical Inc. Kosei-kai Nijigaoka Hospital, Nijigaoka-machi 1-1, Nagasaki 852-8055, Japan*
[12] *Department of Internal Medicine, Caritas Clinic, Nishiizuru-machi 65-7, Nagasaki 851-2322, Japan*
[13] *Clinical Research Center, National Hospital Organization Nagasaki Medical Center, Kubara 2-1001-1, Omura, Nagasaki 856-8562, Japan*

Correspondence should be addressed to Naota Taura, ntaura-gi@umin.ac.jp

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Background. HCV infection is associated with lipid disorders because this virus utilizes the host lipid metabolism to sustain its life cycle. Several studies have indicated that higher concentrations of serum cholesterol and LDL before treatment are important predictors of higher rates of sustained virological response (SVR). However, most of these studies involved patients infected with HCV genotype 1. Thus, we performed a multi-institutional clinical study to evaluate the impact of lipid profiles on SVR rates in patients with HCV genotype 2. *Methods.* A total of 100 chronic hepatitis C patients with HCV genotype 2 who received peg-IFN alfa-2b and ribavirin therapy were consecutively enrolled. The significance of age, sex, BMI, AST level, ALT level, WBC, hemoglobin, platelet count, gamma-glutamyltransferase, total cholesterol level (TC), LDL level, HCV RNA, and histological evaluation was examined for SVR using logistic regression analysis. *Results.* The 100 patients infected with HCV genotype 2 were divided into 2 groups, an SVR group and a non-SVR group. Characteristics of each group were subsequently compared. There was no significant difference in the level of HCV RNA, BMI, platelet, TG, or stage of fibrosis between the groups. However, there were significant differences in the levels of TC and LDL-C. In multivariate logistic regression analysis using baseline characteristics, high TC level was an independent and significant risk factor (relative risk 18.59, $P = 0.015$) for SVR. *Conclusion.* Baseline serum total cholesterol levels should be considered when assessing the likelihood of sustained treatment response following the course of peg-IFN and ribavirin therapy in patients with chronic HCV genotype 2 infection.

1. Introduction

Hepatitis C virus (HCV) causes acute and chronic hepatitis as well as liver cirrhosis and hepatocellular carcinoma [1]. A single-stranded RNA genome encodes 1 large open reading frame that is processed into at least 10 proteins by host and viral enzymes [2]. Some viral proteins are known to affect the outcome of pegylated interferon (PEG-IFN) and ribavirin combination therapy, which is the current standard for treating chronic hepatitis [3, 4].

HCV infection is associated with lipid disorders because this virus utilizes the host lipid metabolism to sustain its life cycle [5, 6]. Accordingly, understanding lipid metabolism in HCV infection is necessary for developing new strategies for complete eradication of this virus. Characteristic lipid disorders observed in chronic hepatitis C patients include steatosis and hypocholesterolemia, which are primarily caused by abnormal triglyceride (TG) and cholesterol metabolism, respectively [7]. The metabolic pathways of these 2 lipids are closely related to each other.

Several studies have indicated that higher concentrations of serum cholesterol and LDL before treatment are important predictors of high rates of sustained virological response (SVR) [8–10]. However, most of these studies involved patients who were infected with HCV genotype 1. Prognostic factors are likely to differ considerably between genotypes 1 and 2. For example, two studies have shown that total PEG-IFN and ribavirin doses are independent predictive factors of an SVR to the HCV genotype 1, whereas another found that dosages of PEG-IFN and ribavirin on SVR are not related to the genotype 2 [11, 12]. Total dosages of PEG-IFN and ribavirin may similarly influence the SVR to genotypes 1 and 2. Identifying factors involved in the responses of patients infected with HCV genotype 2 to PEG-IFN and ribavirin is important when considering treatment strategies. Fewer patients are infected with HCV genotype 2 than genotype 1. Thus, we performed a multi-institutional clinical study to evaluate the impact of lipid profiles on SVR rates in patients with HCV genotype 2.

2. Patients and Methods

2.1. Patients. A total of 685 patients with chronic hepatitis C diagnosed between 2004 and 2008 in the Nagasaki Association for the Study of Liver Disease (NASLD) were recruited for this study. All patients were included if they were positive for HCV antibodies and serum HCV RNA. One hundred patients with HCV genotype 2 who received pegylated interferon alfa-2b (PEG-INF) and ribavirin therapy were consecutively enrolled. Exclusion criteria were as follows: (1) positive for serum hepatitis B virus surface antigen, (2) abnormal thyroid and kidney functions, (3) decompensated liver disease, (4) presence of human immunodeficiency virus type I infection, and (5) ever received specific antiviral therapy prior to referral.

2.2. Study Protocol. This study is retrospective study. Response to antiviral treatment was assessed in patients based on HCV viremia and aminotransferase levels. Patients treated with a combination of PEG-IFN alfa-2b (product by MSD) and ribavirin received 1.0–1.5 μg/kg and 600–800 mg daily of each drug, respectively. SVR was defined as both normal aminotransferase levels and undetectable serum HCV RNA 24 weeks after the end of antiviral therapy. The remaining patients were considered nonvirus responders (non-SVR).

Fasting serum samples were obtained in the early morning for biochemical analysis. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of the height in meters (kg/m^2). Liver biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut to a thickness of 4 μm, and stained with hematoxylin-eosin and Azan. All liver tissue specimens were evaluated by one pathologist who was unaware of patient clinical conditions. Liver histology was evaluated according to the degree of fibrosis and necroinflammatory activity [13]. The extent of fibrosis (staging) was classified as follows: F1 (periportal expansion), F2 (portoportal septa), F3 (portocentral linkage or bridging fibrosis), and F4 (cirrhosis). Necroinflammatory activity (grading) was classified as follows: A1 (mild), A2 (moderate), and A3 (severe). In order to define the cutoff parameter for total cholesterol level (TC), LDL, and TG for the SVR of PEG-IFN alfa-2b and ribavirin in HCV patients, we used the ROC curve. The area under the curve was 62% (CI 95%: 51%–75%), 72% (CI 95%: 59%–86%), and 61% (CI 95%: 46%–76%), respectively. The ideal cutoff point for the TC, LDL, and TG was calculated to be 177 with sensitivity equal to 58% and specificity equal to 77%, 98 with sensitivity equal to 57% and specificity equal to 77%, and 88 with sensitivity equal to 56% and specificity equal to 67%, respectively.

The protocol was approved by the Ethical Committee of the Nagasaki University School of Medicine.

2.3. Statistical Analysis. Descriptive summaries of study groups are reported as the median (range) and number (%). Data were analyzed using the Mann-Whitney U test for continuous ordinal data, and the chi-square test with Yates' correction and Fisher's exact test were performed for intergroup comparisons to determine the association between 2 qualitative variables. P-values <0.05 were considered statistically significant. Variables achieving statistical significance according to univariate analysis were subsequently included in the multivariate analysis using a logistic regression model and were described as relative risk (RR) with 95% confidence intervals (CI). Coefficients were calculated from the linear discriminating function of the variables. Data analysis was performed using SPSS version 16.0 for Windows.

3. Results

3.1. Patient Clinical Features. Baseline characteristics of the 100 patients infected with HCV genotype 2 are shown in Table 1. There were 54 male (54%) and 46 female (46%) patients, with a median age of 57 years.

The 100 patients infected with HCV genotype 2 were then divided into 2 groups, an SVR group (74 patients) and Non-SVR group (26 patients). Characteristics of each group were subsequently compared (Table 2). There was no

Table 1: Characteristics of 100 studied patients with HCV genotype 2.

All	100	
Age	57.0	(24–76)
Sex (%)		
Male	54	(54)
Female	46	(46)
Height (cm)	162	(138–186)
Weight (kg)	58	(37–87)
BMI (kg/m^2)	22.7	(18.4–30.8)
Clinical finding (%)		
Chronic hepatitis	93	(93)
Cirrhosis	7	(7)
WBC (/μL)	5100	(2100–9730)
Hemoglobin (g/dL)	14.0	(10–16)
Platelet (10^4/μL)	20.4	(6.9–26.5)
AST (IU/L)	42	(17–157)
ALT (IU/L)	52	(11–280)
TC (mg/dL)	177	(106–269)
<177 mg/dL (%)	50	(50)
≥177 mg/dL (%)	50	(50)
TG (mg/dL)	88	(56–262)
<88 mg/dL (%)	50	(50)
≥88 mg/dL (%)	50	(50)
LDL-C (mg/dL)	98	(30–167)
<98 mg/dL (%)	50	(50)
≥98 mg/dL (%)	50	(50)
HCV RNA (KIU/mL)	1000	(20–40900)
Distribution of stage of fibrosis (%)		
0-1	43	(43)
2	17	(17)
3	11	(11)
4	4	(4)
Unknown	25	(25)
Distribution of grade of inflammation (%)		
0-1	39	(39)
2	34	(34)
3	2	(2)
Unknown	25	(25)
Treatment period (week) (%)		
<24	10	(10)
24	83	(83)
25–48	5	(5)
>48	2	(2)
Therapeutic efficacy (%)		
SVR	74	(74)
Non-SVR	26	(26)

Data are median (range) or frequency (%).

significant difference in the level of HCV RNA, BMI, platelet, TG, or stage of fibrosis between the groups. However, there were significant differences in the level of TC and LDL-C.

3.2. Univariate and Multivariate Analysis of Factors Associated with SVR to Pegylated Interferon Alfa-2b and Ribavirin Therapy. Univariate and multivariate analysis in 100 patients infected with HCV genotype 2 was performed to identify independent factors relevant to an SVR (Table 3). In univariate analysis, the following 2 factors significantly influenced the SVR: TC (≥177 mg/dL; relative risk, 3.77; 95% confidence interval (95% CI), 1.41–10.05; $P = 0.008$) and LDL-C (≥98 mg/dL; relative risk, 4.91; 95% CI, 1.19–20.23; $P = 0.028$). However, in multivariate analysis, TC was the only independent factor for SVR (relative risk, 18.59; 95% CI, 1.78–193.65; $P = 0.015$).

3.3. Association of SVR Rate to Combination Therapy and TC Level. The 100 patients infected with HCV genotype 2 were then divided into 2 groups, a high serum TC level group (≥177 mg/dL) and a low serum TC level group (<177 mg/dL). Characteristics of each group were subsequently compared (Table 4). There was no significant difference in age, the level of ALT, WBC, hemoglobin, platelet, TG, stage of fibrosis or grade of inflammation between the groups. However, there were significant differences in sex, BMI, the level of AST, TG, LDL-C, and HCV RNA.

We examined the differences in the 4 indices related to SVR rate between high serum TC level and low serum TC level in HCV genotype 2 patients (Figure 1). The SVR rate in low serum TC level patients was 62% (31 of 50), whereas 86% of patients (43 of 50) had serum high TC levels. The significantly higher SVR rate of serum high TC level than low serum TC levels was observed in 100 patients infected with HCV genotype 2.

4. Discussion

In this retrospective study, we showed a significant association of treatment response with baseline characteristics of patients infected with HCV genotype 2, including HCV viral load, BMI, and serum cholesterol level. Several baseline predictors for SVR have been identified in earlier studies [14–17]. Notably, among pretreatment features in the present study, serum TC levels appeared to discriminate responders from nonresponders independently of different treatment schedules. The response rate to standard treatment for patients with HCV genotype 2 using a combination of PEG-IFN and ribavirin is approximately 80% and remains a major concern in patient care. Our findings confirm serum high TC level as a good predictor of SVR in genotype 2. In patients with genotype 2, the SVR rate in patients with low serum TC levels was 62%, whereas 86% had high serum TC levels. Serum cholesterol as a predictor of SVR in patients with chronic hepatitis C is in accordance with the results of previous studies [8–10, 18–20]. However, our study design included only patients with HCV genotype 2.

A cutoff value of total cholesterol of 177 mg/dL in this study represented the best value in terms of sensitivity and specificity for SVR. Our cutoff total cholesterol level was lower than other previous studies [8–10, 18–20]. However, American Diabetes Association guidelines suggest that a goal should be a total cholesterol of <160 mg/dL in patient with