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

Acknowledgements
This work was supported by the Japanese Government, Kakken-B No. 213901832.

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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.

Received: 5 July 2011 Accepted: 15 February 2012
Published: 15 February 2012

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doi:10.1186/1743-422X-9-40
Cite this article as: Kamihira *et al.*: 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. *Virology Journal* 2012 9:40.

Tropical Medicine and Health Vol. 40 No. 2, 2012, pp. 55-58
doi:10.2149/tmh.2012-02

Original article

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

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Received 20 January, 2012 Accepted 5 June 2012 Published online 4 August, 2012

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

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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].

ROLE OF FUNDING SOURCE

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20390186, 221S0001, 23659354, 23590800), Cooperative Research Grant (2009-E-1) of the Institute of Tropical Medicine, Nagasaki University and by the Global Center of Excellence Program at Nagasaki Uni-

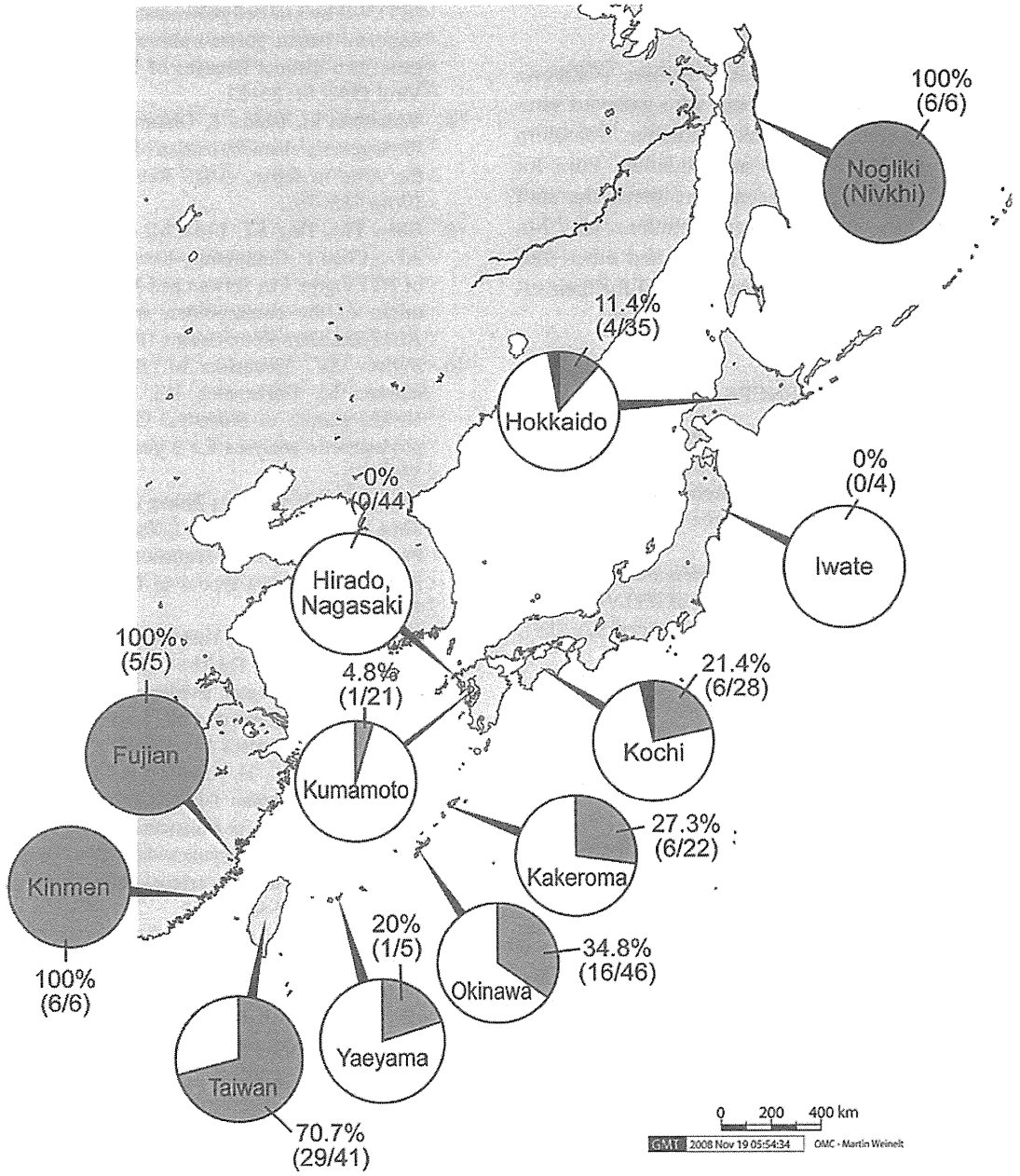

Fig. 1. Ratio of the transcontinental subgroup (grey) to the Japanese subgroup (white) of HTLV-1 cosmopolitan subtype in various localities of East Asia. The data of Nogliki, Kakeroma, Taiwan, Kinmen and Fujian were cited from Syrtsev et al. [10], Eguchi et al. [13], Yang et al. [7], Chen et al. [9]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

ACKNOWLEDGEMENTS

We gratefully thank Dr. Osamu Ikehara (Okinawa Prefectural Yaeyama Hospital, Japan) who gathered samples on Ishigaki I., and Drs. Junko Okumura, Masahiro Hashizume, Toshihiko Sunahara and Hidefumi Fujii for their important suggestions. The authors thank the staff members in all the collaborating institutions and Mr. Makoto Nakashima, Ms. Takako Akashi, and other staff members in the central office of the JSPFAD for their efforts in sample processing and biologic assays.

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

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Received March 2, 2012; Accepted September 10, 2012

DOI: 10.3892/etm.2012.709

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.

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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%).

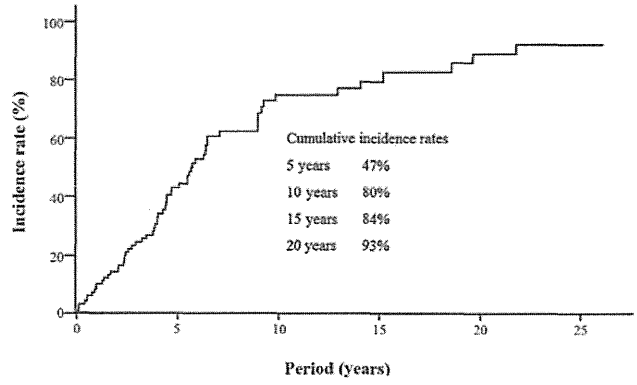

Figure 1. Cumulative incidence rates of hepatocellular carcinoma (HCC) in all patients.

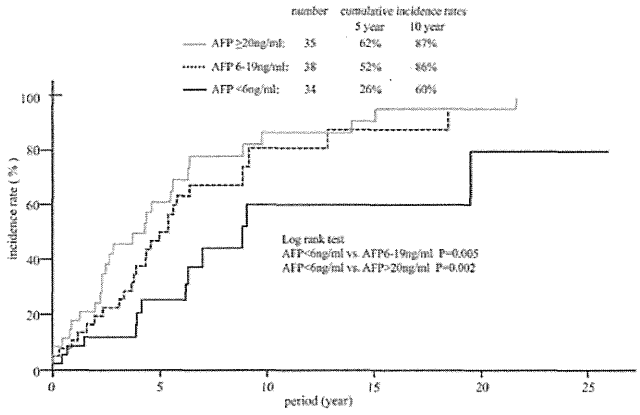

Figure 2. Cumulative incidence rates of hepatocellular carcinoma (HCC) according to α-fetoprotein (AFP) levels.

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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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2012, Article ID 317580, 7 pages
doi:10.1155/2012/317580

Clinical Study

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

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Received 16 May 2012; Accepted 24 September 2012

Academic Editor: Edoardo Giovanni Giannini

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 ($10^4/\mu$L)	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

Table 2: Factors associated with response to peginterferon alfa-2b and ribavirin therapy.

	SVR	(Range or %)	Non-SVR	(Range or %)	*P* value
Total	74		26		
Age (y.o.)	57	(24–72)	57	(31–78)	NS
Sex (%)					
Male	33	(45)	13	(50)	
Female	41	(55)	13	(50)	NS
BMI (kg/m^2)	23.1	(15.4–30.9)	21.0	(18.4–26.0)	NS
WBC (/μL)	5100	(2100–9730)	5145	(3000–8300)	NS
Hemoglobin (g/dL)	14.1	(10–16)	14.0	(10–16)	NS
Platelet (10^4/μL)	21.7	(6.9–26.5)	11.5	(7.3–21.1)	NS
AST (IU/L)	39	(17–377)	44	(17–140)	NS
ALT (IU/L)	51	(11–751)	53	(14–169)	NS
TC (mg/dL)	183	(106–269)	163	(127–248)	NS
<177 mg/dL (%)	31	(42)	19	(73)	
≥177 mg/dL (%)	43	(58)	7	(27)	0.005
TG (mg/dL)	98	(56–262)	83	(74–176)	NS
<88 mg/dL (%)	33	(44)	17	(67)	
≥88 mg/dL (%)	41	(56)	9	(33)	NS
LDL-C (mg/dL)	109	(30–167)	88	(64–117)	0.015
<98 mg/dL (%)	30	(40)	20	(77)	
≥98 mg/dL (%)	44	(60)	6	(23)	0.020
HCV RNA (KIU/mL)	1000	(20–40900)	1850	(37–24200)	NS
Distribution of stage of fibrosis (%)					
1	31	(42)	12	(46)	
2	14	(19)	3	(12)	
3	6	(8)	5	(19)	
4	3	(4)	1	(4)	
Unknown	20	(27)	5	(19)	NS
Distribution of grade of inflammation (%)					
1	27	(36)	12	(46)	
2	25	(34)	9	(35)	
3	2	(3)	0	(0)	
Unknown	20	(27)	5	(19)	NS

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

type 2 diabetes who is at low risk [21]. Furthermore, Miller et al. reported that American type 2 diabetic patients had an average cholesterol level of 179 mg/dL [22].

The reason for SVR improvement in patients with elevated serum cholesterol levels is unknown. In patients with chronic hepatitis B and hepatitis C, serum lipid levels have been reported to be correlated with specific cytokines that may have antiviral activity, including tumor necrosis factor-alpha and interleukin-6 [23]. This hyperlipidemia-induced increase in cytokine levels may have a favorable and potentially additive effect on antiviral treatment in patients with chronic hepatitis C. Another proposed mechanism may be related to a possible regulatory effect of cholesterol in HCV binding to cell surface receptors, which in turn may be relevant to viral clearance [24]. The LDL receptor, a membrane glycoprotein, has been shown to be involved in HCV entry into hepatocytes, and data suggest that HCV RNA levels correlate with LDL receptor expression [25, 26]. Elevated serum concentrations of LDL may decrease the number of LDL receptors located on hepatocytes.

Recent studies have shown that single nucleotide polymorphisms located in the gene region encoding interleukin 28b (IL28B) are strongly associated with the response to PEG-IFN and ribavirin therapy [17, 27, 28]. Total cholesterol, LDL cholesterol, and ApoB concentrations are significantly higher in chronic hepatitis C patients carrying a second IL28B major allele (CC in rs 12979860) compared with those possessing minor alleles (CT or TT) [29]. Therefore, the association between serum LDL cholesterol concentration and SVR may be reflected by the underlying link

Table 3: Univariate and multivariate analysis of the factors associated with SVR to peginterferon alfa-2b and ribavirin therapy.

		Univariate analysis		Multivariate analysis	
		P	RR (95% CI)	*P*	RR (95% CI)
Age	<57 years	0.646	1.24 (0.50–3.09)		
Sex	Female	0.634	0.80 (0.33–1.97)		
BMI	≥23 kg/m^2	0.221	1.86 (0.69–5.02)		
Underlying liver disease					
	CH	0.872	1.15 (0.21–6.32)		
WBC	≥5100 /μL	0.827	0.75 (0.37–2.22)		
Hb	≥14.0 g/dL	0.317	0.62 (0.25–1.58)		
Plt	≥20 × 10^4/μL	0.112	2.10 (0.84–5.24)		
AST	<40 IU/L	0.429	1.44 (0.58–3.55)		
ALT	<52 IU/L	0.649	1.23 (0.50–3.02)		
γ-GTP	<35 IU/L	0.525	0.75 (0.30–1.83)		
TC	≥177 mg/dL	0.008	3.77 (1.41–10.05)	0.015	18.59 (1.78–193.65)
TG	≥88 mg/dL	0.101	2.60 (0.83–8.13)		
LDL-C	≥98 mg/dL	0.028	4.91 (1.19–20.23)	0.800	1.25 (0.22–7.01)
Stage	F 3-4	0.419	0.60 (0.17–2.07)		
Grade	A 2-3	0.809	1.13 (0.41–3.18)		
HCV RNA					
	<1000 KIU/mL	0.310	1.65 (0.63–4.31)		

Relative risk (RR); 95% confidence interval (95% CI).

Table 4: Comparison between HCV patients with high and low serum TC.

TC	<177 mg/dL	(Range or %)	≥177 mg/dL	(Range or %)	*P* value
Total	50		50		
Age (y.o.)	57	(24–78)	57	(36–69)	NS
Sex (%)					
Male	34	(68)	20	(40)	
Female	16	(32)	30	(60)	0.005
BMI (kg/m^2)	21.5	(18.4–26.8)	23.5	(15.4–30.6)	0.027
WBC (/μL)	5100	(2100–9730)	5100	(3000–8300)	NS
Hemoglobin (g/dL)	14.2	(10–16)	13.9	(10–16)	NS
Platelet (10^4/μL)	17.6	(7.3–26.5)	21.7	(6.9–26.1)	NS
AST (IU/L)	48	(17–377)	33	(18–199)	0.033
ALT (IU/L)	67	(16–751)	40	(11–283)	NS
TG (mg/dL)	83	(46–203)	111	(43–262)	NS
<88 mg/dL (%)	32	(63)	19	(38)	
≥88 mg/dL (%)	18	(37)	31	(62)	0.045
LDL-C (mg/dL)	84	(43–118)	121	(30–167)	<0.001
<98 mg/dL (%)	40	(79)	9	(19)	
≥98 mg/dL (%)	10	(21)	41	(81)	<0.001
HCV RNA (KIU/mL)	1000	(20–24200)	2670	(20–40900)	0.029
Distribution of stage of fibrosis (%)					
1	18	(36)	25	(50)	
2	7	(14)	10	(20)	
3	8	(16)	3	(6)	
4	2	(4)	2	(4)	
Unknown	15	(30)	10	(20)	NS
Distribution of grade of inflammation (%)					
1	20	(40)	19	(38)	
2	14	(28)	20	(40)	
3	1	(2)	1	(2)	
Unknown	15	(30)	10	(20)	NS

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

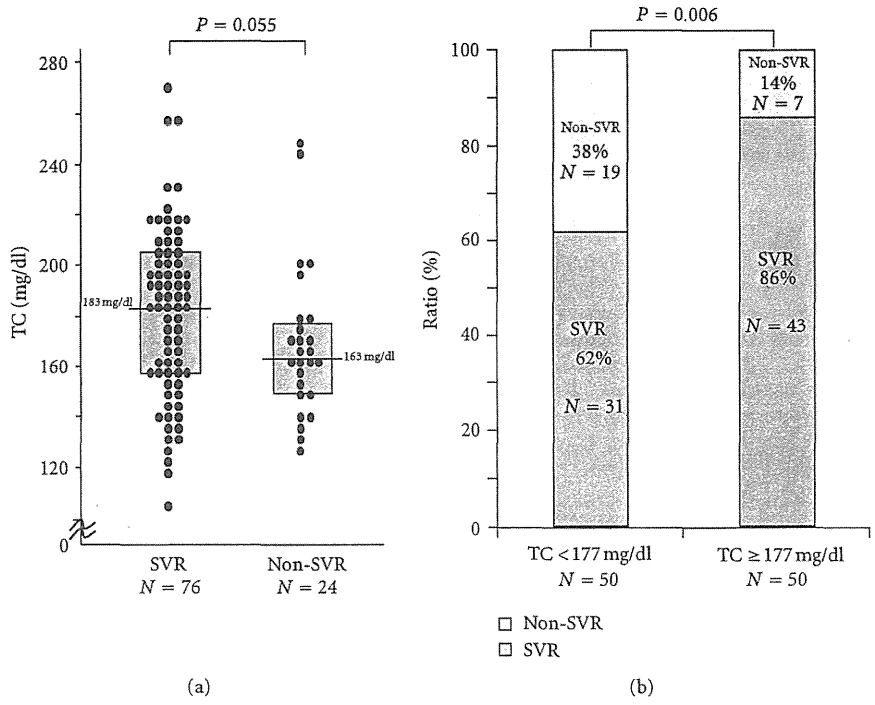

FIGURE 1: Comparison between SVR rate in patients with high serum TC levels (≥177 mg/dL) and patients with low serum TC levels (<177 mg/dL) in HCV genotype 2 patients.

between IL28B genotypes and LDL cholesterol concentrations. As discussed above, we cannot exclude the possibility that high cholesterol levels in patients with HCV only reflect the presence of the IL28B major allele. It may simply reflect the wild-type sequence at core amino acid 70 because substitution in the core protein correlated significantly with a low concentration of LDL cholesterol [30, 31]. However, we could not identify IL28-B and core amino acid 70 as predictive for our patients with HCV genotype 2 because our sample population was limited.

Petta and Craxì reported that age, sex, stage of fibrosis, and baseline viral load were important predictive factors SVR in patient with HCV genotype 2 [32]. However, our study was not significantly differente in these factors for SVR. Furthermore, there was no significant difference in the serum TC between SVR and non-SVR by the Mann-Whitney U test. The reason may be explained as follows: severe stage of fibrosis (F3-4) was recruited only 15%, and 25% was stage unknown in this study. HCV RNA in high TC group was significant higher than low TC group. Finally, this was the limitation small sample size and retrospective study. The discrepancies of the observation from different reports need further investigation.

In conclusion, our data suggest that baseline serum total cholesterol levels should be considered when assessing the likelihood of sustained treatment response following PEG-IFN and ribavirin therapy in patients with chronic HCV genotype 2 infection. However, this finding requires further analysis.

Conflict of Interests

The authors declare that they have no conflict of interests and financial support.

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J Gastroenterol
DOI 10.1007/s00535-012-0656-2

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Accumulation of platelets in the liver may be an important contributory factor to thrombocytopenia and liver fibrosis in chronic hepatitis C

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Received: 3 April 2012 / Accepted: 25 July 2012

Abstract
Background Thrombocytopenia is a marked feature of chronic liver disease and cirrhosis. We tried to clarify whether an accumulation of platelets in the liver contributes to thrombocytopenia and liver fibrosis in chronic liver disease.
Methods Thirty-eight patients who underwent hepatectomy for hepatocellular carcinoma (HCC) with hepatitis C virus infection were included. The locations of platelets and Kupffer cells and the expression of platelet-derived growth factor (PDGF) receptor-β and smooth muscle actin (SMA) were identified by immunohistochemistry. Perisinusoidal mesenchymal cells that express PDGF receptor-β and SMA were interpreted as transformed hepatic stellate cells (HSCs).
Results Patients with cirrhosis had a more extensive platelet area in the liver compared to controls (5601 ± 5611 vs. 564 ± 361 μm^2, $p = 0.02$), although the blood platelet count significantly decreased along with the progression of liver fibrosis. In cirrhotic liver, most platelets were present in the sinusoidal space of the periportal area with inflammation, where HSCs expressing PDGF receptor-β were frequently observed. In addition, the platelet and Kupffer cell areas were significantly smaller in cancerous tissue than those in noncancerous tissues (platelet area: 492 ± 823 vs. 3643 ± 4055 μm^2, $p = 0.001$; Kupffer cell area: 450 ± 841 vs. 3012 ± 3051 μm^2, $p = 0.001$).
Conclusions The accumulation of platelets in the liver with chronic hepatitis may be involved in thrombocytopenia and liver fibrosis through the activation of HSCs. In addition, our findings also indicate that both platelets and Kupffer cells decrease in HCC tissues.

Keywords Platelet · PDGFR-β · Hepatic stellate cells · Sinusoidal endothelial cells

Introduction

Blood platelets, besides hemostatic properties, have the features of inflammatory cells. Blood platelets, while activated in inflammatory processes, release active compounds: platelet-derived growth factors (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, and so forth [1]. Platelets transport these active compounds to the target cells [2, 3]. There are many reports presenting multipotential properties of blood platelets, such as angiogenesis [4–6], wound healing [7, 8], liver regeneration [9], and metastasis in cancer [6, 10, 11]. In acute viral hepatitis, platelets mediate cytotoxic T lymphocyte-induced liver damage [12]. After virus infection, platelets were recruited to the liver, delaying virus elimination and increasing immunopathological liver cell damage [13].

In chronic hepatitis, the blood platelet level gradually decreases, which is reflected in liver fibrosis. Blood

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Published online: 22 August 2012

platelets and chronic liver disease are also closely related. Thrombocytopenia is a marked feature of chronic liver disease and cirrhosis. Cirrhosis is a major cause of morbidity and mortality in many countries, and results in liver failure, portal hypertension, and increased risk of carcinogenesis [14]. The main causes of liver fibrosis include chronic hepatitis C virus infection, alcohol abuse, and nonalcoholic steatohepatitis [15]. The role of platelets in the pathogenesis of liver damage and the exact mechanisms leading to thrombocytopenia in cirrhosis are not yet clear [16]. The thrombocytopenia that occurs in cirrhosis is most likely due to various processes, including increased splenic platelet breakdown [17–19], splenic pooling [20, 21], and the inability of the bone marrow to increase platelet production adequately [22]. According to previous reports assessing the feasibility of platelet scintigraphy, an accumulation of platelets in the liver was observed in patients presenting with thrombocytopenia [17–20, 23–25]. Based on these findings, thrombocytopenia with chronic hepatitis and cirrhosis may be caused by hypersplenism, as well as by the capture of platelets by the liver. However, the platelet kinetics of patients with chronic liver disease are not well characterized. Therefore, the aim of the study described in the present paper was to clarify the histopathological findings of platelets in human liver tissue and to elucidate the role of platelets in the pathogenesis of chronic liver disease.

Methods

Tissues

We studied 38 patients who underwent hepatectomy for hepatocellular carcinoma (HCC) with hepatitis C viral infection at the Kurume University Hospital; their clinical backgrounds are shown in Table 1. These cases did not receive preoperative anticancer therapies. Both cancerous tissues and adjacent noncancerous liver tissues were obtained by surgical operation.

Five specimens of liver tissues obtained from patients who underwent hepatectomy for hepatic cavernous hemangioma without chronic hepatitis were used as controls.

The study was performed with informed consent obtained from patients for the use of their liver tissues in the investigation, and was approved by the ethical committee at Kurume University (approved ID number: 11200).

Histopathology

Each tissue was fixed with 10 % formalin, embedded in paraffin, cut into 5 μm sections, and then used for histological and immunohistological analyses. The specimens were stained with hematoxylin and eosin and examined under a light microscope. The histological liver damage of these specimens was evaluated for fibrosis and inflammation according to the classification proposed by the International Association for the Study of the Liver [26, 27]. The severity of fibrosis (stage of disease) was classified as none (stage 0), mild (portal fibrosis; stage 1), moderate (periportal fibrosis; stage 2), severe (bridging fibrosis with lobar distortion; stage 3), and cirrhosis (stage 4), and the inflammatory activity (grade of disease activity) was classified as none (grade 0), minimal (grade 1), mild (grade 2), moderate (grade 3), or severe (grade 4). The pathological features of HCC were evaluated according to the World Health Organization classification [28].

Histopathological diagnosis and classification were performed by four pathologists (R.K., H.Y., O.N., and M.K.).

Table 1 Clinical features of the patients studied in this work

Stage	1	2	3	4
No. of subjects	10	10	8	10
Sex (male/female)	8/2	9/1	6/2	5/5
Age (years)[a]	74 ± 5.0	71 ± 6.9	69 ± 9.9	74 ± 4.2
Grade (1/2)	7/3	3/7	1/7	1/9
Platelet count ($\times 10^4$)[a]	15.8 ± 3.4	13.5 ± 2.5	11.8 ± 5.1	10.6 ± 2.5
HCC (well/moderate/poor)	0/10/0	1/8/1	1/7/0	0/10/0

The severity of fibrosis was classified as none: stage 0, portal fibrosis: stage 1, periportal fibrosis: stage 2, bridging fibrosis with lobar distortion: stage 3, and cirrhosis: stage 4. The inflammatory activity was classified as none: grade 0, minimal: grade 1, mild: grade 2, moderate: grade 3, or severe: grade 4

HCC hepatocellular carcinoma, *well* well differentiated HCC, *moderate* moderately differentiated HCC, *poor* poorly differentiated HCC

[a] Mean ± SD

Immunohistochemical analysis

The avidin–biotin peroxidase complex method was used for immunohistochemistry. We used monoclonal antibodies against CD41 (1:500, Beckman Coulter, Brea, CA, USA), CD68 (1:200, DAKO, Glostrup, Denmark), PDGF receptor-β (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and smooth muscle actin (1:200, DAKO). CD41 (glycoprotein IIb/IIa complex) is a specific marker for platelets, so a CD41-positive reaction was taken to indicate the presence of platelets. CD68, an anti-human macrophage antibody, is expressed not only in residential macrophages such as Kupffer cells, but also in migrating macrophages. Among

the CD68-positive cells, those in the sinusoidal space or blood space of cancerous tissues with spindle or stellate-shaped cytoplasm, and those partly adhering to the sinusoidal endothelial cells, were evaluated as Kupffer cells. Perisinusoidal mesenchymal cells express PDGF receptor-β as transformed hepatic stellate cells (HSCs) [29–31]. These cells were evaluated as activated HSCs.

Measurement of cells

The area of platelets and Kupffer cells in each specimen was measured using the WinROOF software package (version 6.1, Mitani Corporation, Fukui, Japan). In noncancerous liver tissues, the areas were measured in five randomly selected periportal regions. In cancerous tissues, five visual fields were randomly selected.

Transmission electron microscopy

The liver was cut into small pieces (approximately 1 mm^3), the specimens were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and they were then post-fixed in 1 % O_8O_4 in 0.1 M phosphate buffer. Next, the specimens were dehydrated through a graded series of ethanol, passed through propylene oxide, and embedded in Epok 812. Ultrathin sections mounted on copper grids were stained with uranyl acetate and lead citrate and observed in a Hitachi (Tokyo, Japan) H-7650 transmission electron microscope.

Statistical analysis

The arithmetic means and standard deviations of our data were calculated using the JMP software package (release 9.0, SAS Institute, Cary, NC, USA). All data are expressed as mean $\pm$ SD, and p values of less than 5 % were considered to indicate significance.

Results

Histological findings

Noncancerous liver tissues of all patients with hepatitis C virus infection showed various degrees of fibrosis and chronic inflammation. The severity of fibrosis was mild (stage 1) in ten patients, moderate (stage 2) in ten patients, and severe (stage 3) in eight patients, while ten patients had cirrhosis (stage 4). The inflammatory activity was minimal (grade 1) in 12 patients and mild (grade 2) in 26 patients. In control tissues, there were a few lymphocytes, but only in the portal area, and neither necroinflammatory reactions nor fibrosis were noted (grade 0, stage 0).

Among the cancerous tissues in 38 cases, 35 cases were moderately differentiated HCCs, two cases were well-differentiated HCCs, and one case was poorly differentiated HCC. In terms of the diameters of the HCCs, there were two cases with an HCC diameter of $\leq$1.0 cm, 11 cases with a diameter of 1.1–2.0 cm, 14 cases with 2.1–3.0 cm, and 11 cases with $\geq$3.0 cm. Comparing the histological differentiation of cancerous tissues, the mean tumor size was 2.6 $\pm$ 0.6 cm in the well-differentiated HCCs, 3.0 $\pm$ 1.8 cm in the moderately differentiated HCCs, and 6.3 cm in the poorly differentiated HCCs. Among the 37 nodular-type HCCs, 24 specimens had clear fibrous capsules at the tumor and nontumor boundary, while 13 cases had no fibrous capsules.

Platelets in noncancerous liver tissues

In all noncancerous liver tissues, including patients with chronic hepatitis or cirrhosis and in the controls, there were platelets but no megakaryocytes in the sinusoidal space. Patients with cirrhosis had a more extensive platelet area in the noncancerous liver tissue than in controls (5601 $\pm$ 5611 vs. 564 $\pm$ 361 μm^2, $p = 0.02$, $p < 0.05$, Fig. 1a). In patients with chronic hepatitis or cirrhosis, the platelet area in noncancerous liver tissues increased along with an increase in histological liver damage ($p = 0.02$, $p < 0.05$), although the blood platelet count significantly decreased (Fig. 1b). In noncancerous liver tissues with chronic hepatitis or cirrhosis, platelets were present predominantly in the sinusoidal space of the periportal area with inflammation. In high-stage cases, platelets were observed along the destroyed limiting plate of the expanded fibrous portal area with inflammation, and in the sinusoidal space of the periportal area (Fig. 2).

Relationship among platelets, HSCs, and Kupffer cells in non-cancerous liver tissues

Immunohistochemical studies of noncancerous liver tissues with cirrhosis revealed that most platelets were present in the periportal area with inflammation, where HSCs expressing PDGF receptor-β were frequently observed (Fig. 3a, b). Most smooth muscle actin stained cells were identical to those expressing PDGF receptor-β (Fig. 3c). In noncancerous liver tissues of controls and cases at the lower stage of chronic hepatitis, only a few HSCs expressing PDGF receptor-β were seen.

In noncancerous liver tissues, including patients with chronic hepatitis or cirrhosis and in controls, CD68-positive Kupffer cells were seen in the sinusoidal spaces of both periportal and lobular areas diffusely.

Platelets and Kupffer cells in cancerous tissues

In all cancerous tissues, a few platelets and Kupffer cells were present in the blood spaces of cancerous tissues.