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Prevalence of Amino Acid Mutation in Hepatitis C Virus Core Region Among Japanese Volunteer Blood Donors

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It is not known whether there is a trend of increasing or decreasing incidence of new hepatitis C virus (HCV) infections in Japan. From the treatment point of view, it is important to verify HCV genotypes and the prevalence of treatment-resistant clones of HCV. At the Japanese Red Cross blood centers, all blood samples obtained from blood donation have been screened using serological methods and the minipool nucleic acid amplification testing. One hundred and fourteen donors have been identified over the past 10 years to be HCV RNA-only positive without detectable anti-HCV and were considered to be in the acute phase of HCV infection. There was a trend of decreasing incidence of such new infections among the blood donors. HCV RNA-only-positive samples were examined further for genotyping and HCV RNA quantitation. Genotype 2 (2a plus 2b) was predominant (78.2%) among them followed by genotype 1b (21.2%). Direct sequencing was carried out to detect the possible treatment-resistant mutant clones 70Q and 91M, clones with amino acid substitutions at positions 70 and 91 of the HCV core protein, respectively. 70Q and 91M were found regularly in donors with genotype 1b, but not in those with other genotypes. No particular endemic areas for the mutant clones were identified. There was no significant difference in the mean viral titer between the 70Q mutant type and the non-70Q wild-type. Even in newly infected people, the mutant clone 70Q was detected frequently.
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KEY WORDS: HCV core mutation; HCV genotype; acute HCV infection; window period; blood donors

INTRODUCTION

The hepatitis C virus (HCV; *Flaviviridae, Hepacivirus, Hepatitis C virus*) infects approximately 170 million people worldwide and is a major causative agent of chronic liver diseases including liver cirrhosis and hepatocellular carcinoma [Liang et al., 2000]. Although strategies focusing on education and increasing awareness to prevent HCV infection have been developed, it is reported that 3–4 million people are newly infected worldwide with HCV [Kim, 2002].

After entry into a human host, HCV is exposed to pressures exerted by innate/acquired immunity including endogenous interferon (IFN), which could lead to viral eradication. However, only about 30% of infected persons can clear the virus and the remainder progress to chronic infection. The inherent sequence diversity of HCV in conjunction with host immunological impairments contributes to viral persistence. In the presence of immune selection pressures exerted by cytotoxic T cells against the virus, a genomic diversity could facilitate the preferential expansion of mutant progeny [Bowen and Walker, 2005]. Recently, amino acid substitution at position 70 of the HCV core [arginine (R)-to-glutamine (Q)] (70Q) has been reported to be associated significantly with a poor response to current treatments in patients with HCV-1b [Akuta et al., 2005]. These clones may resist the current treatments, whereas, IFN-sensitive clones are eradicated by either spontaneous responses or the treatment.

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In view of the development of treatment regimens for hepatitis C, it is important to determine both the distribution pattern of HCV genotypes and the prevalence of treatment-resistant HCV clones among recently infected patients who would require treatments in the near future. In this study, Japanese volunteer blood donors who were found to be positive for HCV RNA and negative for anti-HCV were examined. These donors were considered to have acquired infection within a few months of their blood donation. It is expected that the investigation of these people will verify the current trend of new HCV infection in the Japanese general population [Murokawa et al., 2005] and that such HCV RNA-only-positive blood donors have only naive viral clones without the influence of immunological pressures or endogenous IFN.

MATERIALS AND METHODS

Screening Algorithm and Blood Samples

The Japanese Red Cross (JRC) blood centers are the sole facilities that deal with blood collection, processing, testing, and delivery. In these centers, blood donations are screened for the hepatitis B surface antigen (HBsAg), anti-HCV, anti-human immunodeficiency virus types 1 and 2 (HIV-1, 2), anti-human T lymphotropic virus type 1 (HTLV-1), anti-*Treponema pallidum*, and human parvovirus B19 antigen. Blood donations with an elevated serum alanine aminotransferase level (>60 IU/ml) are also rejected. Anti-HCV is screened by passive hemagglutination (PHA; Abbott Japan, Tokyo, Japan) or particle agglutination (PA; Fujirebio, Tokyo, Japan). A PHA titer $\geq 2^5$ and a PA titer $\geq 2^4$ indicate positivity for anti-HCV. In the current study, HCV Dynapack II (Abbott Japan) was used to confirm the presence of anti-HCV.

Screening by the nucleic acid amplification testing (NAT) was implemented nationwide in 1999, in which only seronegative blood samples were screened further by the AmpliNAT multiplex test (Roche Diagnostics Japan, Tokyo, Japan), targeting HCV RNA, hepatitis B virus DNA, and HIV-1 RNA [Mine et al., 2003]. It started with a pool size of 500 that was soon reduced to 50 in 2000 and to 20 in 2004. In this study, HCV RNA-positive samples without detectable anti-HCV were subjected to further analysis.

As a comparative baseline, a group of asymptomatic HCV carrier donors was also included in this study. Blood samples positive for both anti-HCV and HCV RNA were selected from the sample registry that had been prepared for the establishment of a standard HCV plasma archive. The archive consists of consecutive samples obtained from 400 ml whole blood donations in 2006 and 2007. Samples for this study were selected at random from those with sufficient plasma volume.

Genotyping of HCV and Determination of Core Amino Acid Sequence

HCV RNA was extracted from 265 μl of a human plasma sample using a QIAamp Virus BioRobot MDx kit (Qiagen, Tokyo, Japan) and reverse-transcribed to complementary DNA using SuperScript II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). The target sequence of the core region (nt. 500–695, 196 bp) was amplified by nested polymerase chain reaction (PCR) using primers (Table I) on the basis of the methods of Okamoto et al. [1993] and Lole et al. [2003]. The polynucleotides obtained were sequenced directly using a BigDye Terminator v1.1 Cycle Sequencing kit and an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). To analyze sequences, SEQUENCHER WIN version 4.8 (Hitachi Software Engineering, Tokyo, Japan) was used. Amino acid sequences were deduced and aligned using GENETYX WIN version 9.0 (Software Development, Tokyo, Japan). The HCV genotype was determined by comparing the deduced amino acid sequences with those of strains available in the GenBank database (genotype 1a: D10749 and M62321; genotype 1b: D13558 and M58335; genotype 2a: AB047641 and D00944; genotype 2b: AB030907 and D10988; genotype 2c: D50409; genotype 3a: D17763 and D28917; genotype 3b: D49374). HCV RNA was determined quantitatively by TaqMan RT-PCR using a QuantiTect Probe RT-PCR kit (Qiagen) and specific primers and a probe (Table I).

RESULTS

Among 51,744,475 consecutive blood samples obtained from blood donations between January 2000 and December 2009, 114 were found positive for HCV RNA and negative for serological markers including anti-HCV. Of the 114 samples, 85 (52 from male and 33 from female) were available for further

TABLE I. Primers Used for Nested PCR and Quantitative PCR and a Probe Used for Quantitative PCR

	Name	Polarity	Sequence	Position
Nested PCR	186	Antisense	5′-ATGTACCCCATGAGGTCGGC-3′	nt. 742–723
	256	Sense	5′-CGCGCGACTAGGAAGACTTC-3′	nt. 471–490
	104	Sense	5′-AGGAAGACTTCCGAGCGGTC-3′	nt. 480–499
	CC4	Antisense	5′-CACGTTAGGGTATCGATGAC-3′	nt. 715–696
Quantitative PCR	TCF-2	Sense	5′-AGACTGCTAGCCGAGTAGTGTTG-3′	nt. 235–257
	TCR-2	Antisense	5′-TGCACGGTCTACGAGACCTC-3′	nt. 330–311
	TP-03 (Probe)	Sense	FAM-AAGGCCTTGTGGTACTGCCTGATAGGG-TAMRA	nt. 266–292

The positions refer to the sequence of prototype HCV (GenBank accession no. M58335).

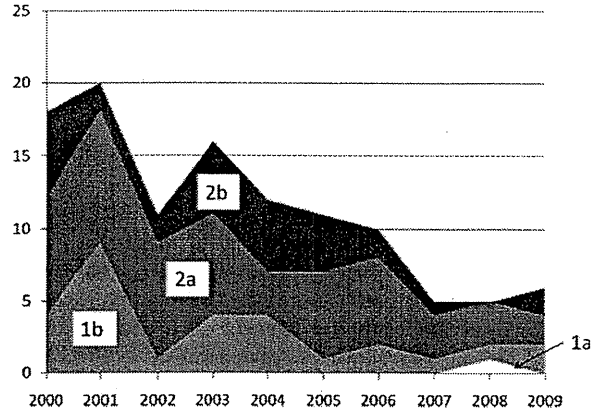

Fig. 1. Changes in number of donors in acute phase HCV infection over past decade. Y-axis shows the number of donors in the acute phase for each HCV genotype.

examination. It is considered that the donors of these blood samples were in the acute phase of infection or in the window period when they donated blood. Their mean ages were 34 ± 13 (18–69) years for male and 29 ± 11 (16–56) years for female. The number of acute phase donations peaked in 2001 and has been decreasing over the years (Fig. 1), despite the increased sensitivity of NAT during that time along with the decrease in NAT pool size as well as the increase in sample volume for nucleic acid extraction. Table II shows the genotypes of HCV that infected the donors in the acute phase over the past 10 years. Genotype 2 (2a plus 2b) and genotype 1b accounted for 77.6% and 21.2% of HCV infections among blood donors, respectively. Genotype 1b has historically been considered to be the major genotype in Japan accounting for approximately 70% of all HCV infections among chronic hepatitis C patients. It has been extremely rare to identify genotype 1a in Japan.

Direct sequencing of HCV RNA extracted from the acute phase samples revealed that the amino acid substitution to glutamine at position 70 (70Q) of the HCV core protein was observed in 7 of the 18 samples with genotype 1b (Table II). There was no significant difference in the mean viral titer among the 70Q mutant type (3.8×10^7 copies/ml, n = 7), the non-70Q wild-type of genotype 1b (6.5×10^7 copies/ml, n = 11), and the non-70Q wild-type of all genotypes (4.9×10^7 copies/ml, n = 78, Table III). 70Q was first detected among acute phase donors in 2003 and has been identified frequently in the following 6 years (Table III). The identification of these clones was reported from various regions in Japan, suggesting that there is no particular specific endemic area for

TABLE II. HCV Genotypes and Amino Acid Substitution 70Q and 91M Found in HCV RNA-Positive and Anti-HCV-Negative Blood Donors

	Genotype				
	1a	1b	2a	2b	Total
Number of donors	1 (1.2%)	18 (21.2%)	42 (49.4%)	24 (28.2%)	85

		Genotype				
Position[a]	Amino acid substitution	1a	1b	2a	2b	Total
70	R	1	11	42	24	78
	Q/R		1			1
	Q		6			6
91	L or C	1	11	42	24	78
	M		7			7

[a]Amino acid position of HCV core protein

TABLE III. Characteristics of Blood Donors With Amino Acid Substitution at Position 70 of HCV Core of Genotype 1b

Gender	Age	ALT	Viral titer	70[a]	91[b]	Year reported
F	25	35	5.3×10^6	Q	M	2003
F	22	40	1.1×10^8	Q	M	2003
M	37	56	2.0×10^7	Q	M	2004
F	43	40	9.3×10^7	Q	L	2005
M	59	18	5.7×10^6	Q	M	2006
F	25	21	1.5×10^7	Q/R	L	2006
M	48	52	1.9×10^7	Q	L	2008
Mean viral titer (n = 7)			3.8×10^7			

[a]Amino acid at position 70 of HCV core protein.
[b]Amino acid at position 91 of HCV core protein.

them, although the sample number was too small to draw a conclusion. Amino acid substitution to methionine at position 91 (91M) was also observed in 7 samples, which were all genotype 1b (Table II). Four samples had both 70Q and 91M. Neither 70Q nor 91M was detected in other genotypes.

Eighty-five blood samples positive for both HCV RNA and anti-HCV were collected from the blood archive of HCV RNA-positive standard plasma (Table IV). The donors of these samples were either asymptomatic chronic hepatitis C patients or otherwise healthy HCV carriers not knowing their infection status. It is considered that the time between HCV acquisition and blood donation was at least 3–4 months for these donors; they were in the post-acute phase. Male donors predominate in this group with 68 of 85 being males (Table IV), which could be partly explained by the fact that these samples were collected from 400 ml whole-blood donations, more than 70% of which were obtained from male donors in Japan. As these samples were selected from the blood archive without using strict randomizing measure, it may be inappropriate to conduct a statistical evaluation regarding the significance of the difference in the mean age of the donors or HCV genotype distribution between the donor groups. There is still a trend that the mean ages of the donors in the acute phase (34 ± 13 years for male and 29 ± 11 years for female) are younger than those of donors in the post-acute phase (42 ± 12 years for male and 46 ± 12 years for female; Table IV). The proportion of genotype 1b among acute phase donors is 21.2%, much lower than that among donors (44.7%) after the acute phase. Accordingly, the proportion of genotype 2 (2a plus 2b) is higher among the donors in the acute phase than among donors after the acute phase (77.6% vs. 54.1%; Table IV).

In this category of asymptomatic carrier donors, the 70Q mutant clone was found in 6 of 38 donors with genotype 1b (Table IV), three of which were over 50 years of age. It is possible that some of these donors might have long-term infection. The 91M mutant clone was also detected in the genotype 1b group. Neither 70Q nor 91M substitutions were detected in other genotypes.

TABLE IV. Profile of Donors of Anti-HCV-Negative and -Positive Blood With HCV RNA and Genomic Profile of HCV Found Among Them

	Anti-HCV	
	Negative (acute phase)	Positive
N	85	85
Male: Female	52:33	68:17
Mean ages		
Male	34 ± 13	42 ± 12
Female	29 ± 11	46 ± 12
HCV-1a	1 (1.2%)	1 (1.2%)
HCV-1b total	18 (21.2%)	38 (44.7%)
70Q[a]	7	6
91M[b]	7	9
HCV-2a	42 (49.4%)	28 (32.9%)
HCV-2b	24 (28.2%)	18 (21.2%)

[a]Glutamine at amino acid position 70 of HCV core protein.
[b]Methionine at amino acid position 91 of HCV core protein.

DISCUSSION

In the present study, the number of donors who were HCV RNA-only-positive was verified to have decreased over the past 10 years. Because RNA-only-positive donation implies that the donor acquired infection within approximately 3 months, it is considered that the mode of infection observed among such donors reflects the current mode of infection in the general population. It is thus speculated that there is indeed a tendency toward decreasing number of new HCV infections in the general population. It should, however, be noted that there are limitations in a blood donor-based study in terms of estimation of disease prevalence in the general population. The blood donor population could be a skewed one because, for example, people donate blood with diverse motivations or because donors are prescreened by questionnaire about at risk behavior, all of which would bias donor population in terms of previous opportunity of exposure to infectious risks. It may still be possible to speculate reasons for the trend of decreasing incidence of new HCV infections. One of them would be the decrease in the number of nosocomial infections owing to the strengthening of measures for the prevention of such infections among medical staff members. It is also possible that the educational program for the prevention of HCV infection for the general population has been effective, although it cannot be ruled out that blood donors with at risk behavior self-defer from donating. Indeed, JRC has implemented rigorous measures to prevent window period blood donations, for example, an advertising campaign against blood donation after at risk behavior.

The present study also showed that 77.6% of blood donors, who acquired HCV infection during the past few months, were infected by genotype 2. This contrasts with the genotype distribution among Japanese patients with chronic hepatitis C, among which genotype 1b is predominant (approx. 70%) followed by genotypes 2a (20%) and 2b (10%) [Tanaka et al., 1995; Ohno et al., 1997]. The age and genotype distribution in the blood donor population positive for anti-HCV as well as HCV RNA were between those for newly infected donors and those for chronic hepatitis C patients. This would indicate the gradual transition of HCV genotypes among infected people from those in elderly patients with chronic hepatitis C to those in asymptomatic carriers and those in newly infected young people. It is thus speculated that the predominant viral genotype currently causing HCV infection among the general Japanese population is genotype 2 (2a plus 2b). The shift of the prevailing genotype from 1b to 2 correlated with the decrease in the mean age

of the groups studied. Although the reasons for the shift are not clear, one possible explanation is that there was a skewed distribution of genotypes in Japan with regard to the transmission mode; genotype 1b was maintained mainly through medical-related transmission, whereas, genotype 2 was maintained through nonmedical-related transmission. As a result of the development of preventive measures for HCV transmission through medical procedures, the incidence of iatrogenic HCV infection decreased, hence the decrease in the incidence of infection by genotype 1b HCV. It is speculated that there has been little progress in the strategies to decrease the incidence of HCV transmission by means of unhygienic practice, for example, tattooing or drug injection, and this may have led to the predominance of genotype 2.

HCV core mutant clones, particularly 70Q, were observed in blood donors who recently acquired infection. Interestingly, this amino acid substitution was only observed in HCV-1b with a frequency of 38.9% (7/18). It has recently been reported that a treatment regimen of pegylated IFN plus ribavirin (Peg-IFN + Rib) could induce amino acid mutation in the core region of HCV [Kurbanov et al., 2010]. A study of chimeric mouse carrying human hepatocytes indicated that HCV core mutations appeared after Peg-IFN + Rib treatment [Kurbanov et al., 2010]. In this regard, the frequent identification of 70Q among RNA-only-positive blood donors who acquired infection very recently was somewhat unexpected. This finding could indicate that treatment induced mutant clones are entering rapidly the Japanese general population. However, such a high incidence of transmission involving blood or body fluid between the healthy general population and hepatitis C patients who received treatment is improbable. Moreover, JRC blood centers do not accept any donations from donors with a history of treatment for hepatitis C. On evaluation, it is more likely that mutant clones found among RNA-only-positive blood donors were present long before the implementation of IFN treatment in the Japanese population. These clones may have developed as a result of immune pressures over a long period of HCV carriage. Although several studies have verified the association between HCV amino acid substitution and IFN response, it should be noted that in such studies the HCV sequences that had been present before treatment were dealt with [Enomoto et al., 1996; Akuta et al., 2005]. By estimating serial alterations of selective pressure against HCV throughout the phylogenetic tree, sequential reduction of both the extent of positive selection pressure such as innate/acquired immunity or endogenous IFN and the degree of liver injury during the long course of chronic HCV infection was found [Hanada et al., 2007]. These findings indicate that the viruses mutate to an immune-resistant sequence to escape from innate/acquired immune pressures during long-term HCV infection. Furthermore, virus sequence changes associated with transmission probably represent selective transmission or the outgrowth of particular clones [Kuntzen et al., 2007] among a complex mixture of different but closely related genomes known as quasispecies [Martell et al., 1992]. It would thus not be surprising to identify mutant clones such as 70Q in newly infected blood donors.

Recently, it has been reported that the number of mutations in the pretreatment sequences of IFN + Rib-resistance-determining region (IRRDR) and IFN-sensitivity-determining region (ISDR) of HCV has substantial effects on the outcome of the Peg-IFN + Rib treatment of chronic HCV hepatitis patients with genotype 1b [El-Shamy et al., 2011]. It will be interesting to see the difference in the number of those mutations between HCV derived from RNA-only positive blood donors and that from chronic HCV hepatitis patients, which would provide another clue to the understanding of the relative role of innate/acquired immune pressure and the treatment effect on the development of mutation. It has also been verified that human IL28B polymorphism significantly influences the response to Peg-IFN + Rib treatment. If the treatment regimen is determined on the basis of the patient's IL28B allele, the distribution of HCV mutants among HCV hepatitis patients will be altered after treatment.

In this study, HCV RNA-only positive blood donors were examined. There is, however, another group of donors that should be included in this category of people; people newly infected with HCV do not show a HCV RNA or specific antibody in their peripheral blood for approximately 1 month after acquiring infection and were not included in this study. Another category of people that underwent self-limited HCV infection was also not considered in this study. If the number of donors and the distribution of genotypes or mutant clones in these categories show patterns quite different from those in the acute phase discussed above, the conclusion of this study on the current trend of new infection would have to be revised.

In conclusion, it is considered that the incidence of new HCV infection is decreasing among the Japanese population. The predominant genotype causing the new infection is genotype 2, which is more responsive to current treatment regimens than other genotypes. The treatment-resistant clone 70Q was identified at a high frequency even among people who acquired new HCV infection and was found exclusively in genotype 1b.

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新時代のウイルス性肝炎学

—基礎・臨床研究の進歩—

II. C型肝炎

C型肝炎ウイルス感染とその予防対策

輸血，血液製剤によるHCV感染の現状とその予防対策

高橋雅彦　　内田茂治

C型肝炎ウイルス感染とその予防対策

輸血，血液製剤によるHCV感染の現状とその予防対策

Present status of transfusion-associated hepatitis C virus infection and efficiency after implementation of nucleic acid amplification testing

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Key words：核酸増幅検査，輸血，ウインドウ期，HCV感染

はじめに

1989年，C型肝炎ウイルス(HCV)はウイルス粒子の発見[1]に先駆けてウイルス遺伝子の断片として発見された[2]．更に，HCV抗体の測定として遺伝子組換え技術により酵母で発現させたC100-3タンパクを抗原とした系が使用可能となった[3]．これにより，輸血後肝炎の調査は堰を切ったように進み，その主因がHCVであることが明らかとなった[4]．その後，輸血後肝炎の征圧を目指して，献血血液に対してHCV抗体スクリーニング検査(第一世代)が導入され，1992年には第二世代の試薬に変更された．同スクリーニング検査の導入により，輸血によるC型肝炎は激減し，主な残存リスクはウインドウ期(WP)の献血血液によるC型肝炎となった．現在，WPの献血血液を排除するために，B型肝炎ウイルス(HBV)，HCVおよびヒト免疫不全ウイルス(HIV)に対する核酸増幅検査(NAT)を導入し，血清学的検査を補っている．これにより，輸血の安全性は格段に向上した．一方，HCVの感染経路については，静注用薬物乱用者，針刺し事故，透析，性感染，出産時，医原性など，今なお議論されているが[1]，過去には非加熱血液製剤や輸血での感染も問題となっていた．

本稿では主に輸血によるHCV感染の現状とその予防策を中心に解説した．

1. 輸血によるHCV感染の予防対策の歩みと輸血後肝炎の変遷

日本赤十字社が行ってきた輸血の安全性確保対策と輸血後肝炎の変遷を示す(表1，図1)．1960年代前半までの売血時代には輸血を受けた患者の50％以上が肝炎を発症していたが，1960年代後半に献血制度への切り替えにより，輸血後肝炎は16％に減少した．1972年にHBs抗原検査を開始し，1986年の400mL採血，成分採血の導入により高単位製剤の供給が可能になり患者への献血者曝露数が減り，更に輸血後肝炎は8.7％になった．1989年にはHBV感染既往献血者の排除のためにHBc抗体検査(導入時期はHI法で2^6以上不適→現在は，輸血感染症の解析結果からCLEIA法でs/co値が12以上(HIで2^5相当)を不適としている)を導入した．今でもその出庫基準は変わっておらず，HBV感染既往献血者の血液(HBc抗体がCLEIAでs/co値が<12)による感染が問題となっている．HCV(当時は非A非B)に対する安全性確保対策は，献血制度および高単位製剤の供給を第一歩

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表1 献血血液に対する感染症スクリーニング導入の変遷

導入年	月	HCV関連検査項目	その他検査項目
1952(昭和27)年	4		梅毒血清学的検査
1964(昭和39)年	8		献血の推進について閣議決定
1970(昭和45)年	10		肝機能検査(GPT, GOT)
1972(昭和47)年	1		HBs抗原検査(DRID法)
	4		HBs抗原検査変更(ES法)
1975(昭和50)年	10		肝機能検査変更(GOT)
1978(昭和53)年	4		HBs抗原検査変更(RPHA法)
1981(昭和56)年	1		肝機能検査変更(GPT)
1986(昭和61)年	11		HIV抗体検査(EIA法)
			HTLV-1抗体検査(PA法)
1987(昭和62)年	10		HIV抗体検査変更(PA法)
1989(平成1)年	12	HCV抗体検査(EIA法)	HBc抗体検査(HI法)
1992(平成4)年	2	HCV抗体検査変更(PHA法：第二世代試薬)	
1993(平成5)年	9	HCV抗体検査変更(PHA法またはPA法：第二世代試薬)	
1994(平成6)年	3		HIV-2抗体検査(PA法)
1996(平成8)年	9		検体保管開始
	10		HIV1/2抗体検査変更(PA法：コンビネーション試薬)
1997(平成9)年	9		ヒトパルボウイルスB19抗原検査(RHA法)
1998(平成10)年	2		梅毒血清学的検査変更(TPPA法)
1999(平成11)年	10	HBV, HCV, HIV核酸増幅検査(NAT)導入 検体プールサイズ500本	
2000(平成12)年	2	HBV, HCV, HIV核酸増幅検査(NAT)検体プールサイズを500から50本	
2004(平成16)年	8	HBV, HCV, HIV核酸増幅検査(NAT)検体プールサイズを50本から20本	
2008(平成20)年	8	・HBV, HCV, HIV-1/2核酸増幅検査(NAT)試薬・機器変更 検体プールサイズ20本 ・HCV抗体検査変更(CLEIA法)	HBs抗原, HBs抗体, HBc抗体, HIV-1/2抗体, HTLV-1抗体, 梅毒トレポネーマ抗体, ヒトパルボウイルスB19抗原検査(CLEIA法)

に，1970年10月，献血血液のスクリーニング検査に肝機能検査を非A非B肝炎の代替え検査として導入したのが輸血によるHCV感染に対する征圧の第一歩であった．その後，1989年12月に，輸血後肝炎の主因を成すウイルスとして特定されたHCVを検出するための抗体検査(c100-3: 第一世代)が導入され，1992年2月には，コアタンパクを追加し感度と特異性を向上させた第二世代の試薬に変更した．更に，HCV抗体検査のWPを短縮するために，1999年10月より500人分の献血者検体をプールし，それを検体としたNATが開始された(血清学的検査に合格した血液のみに対して実施)．2000年2月にNATの検出感度を高めるためにプー

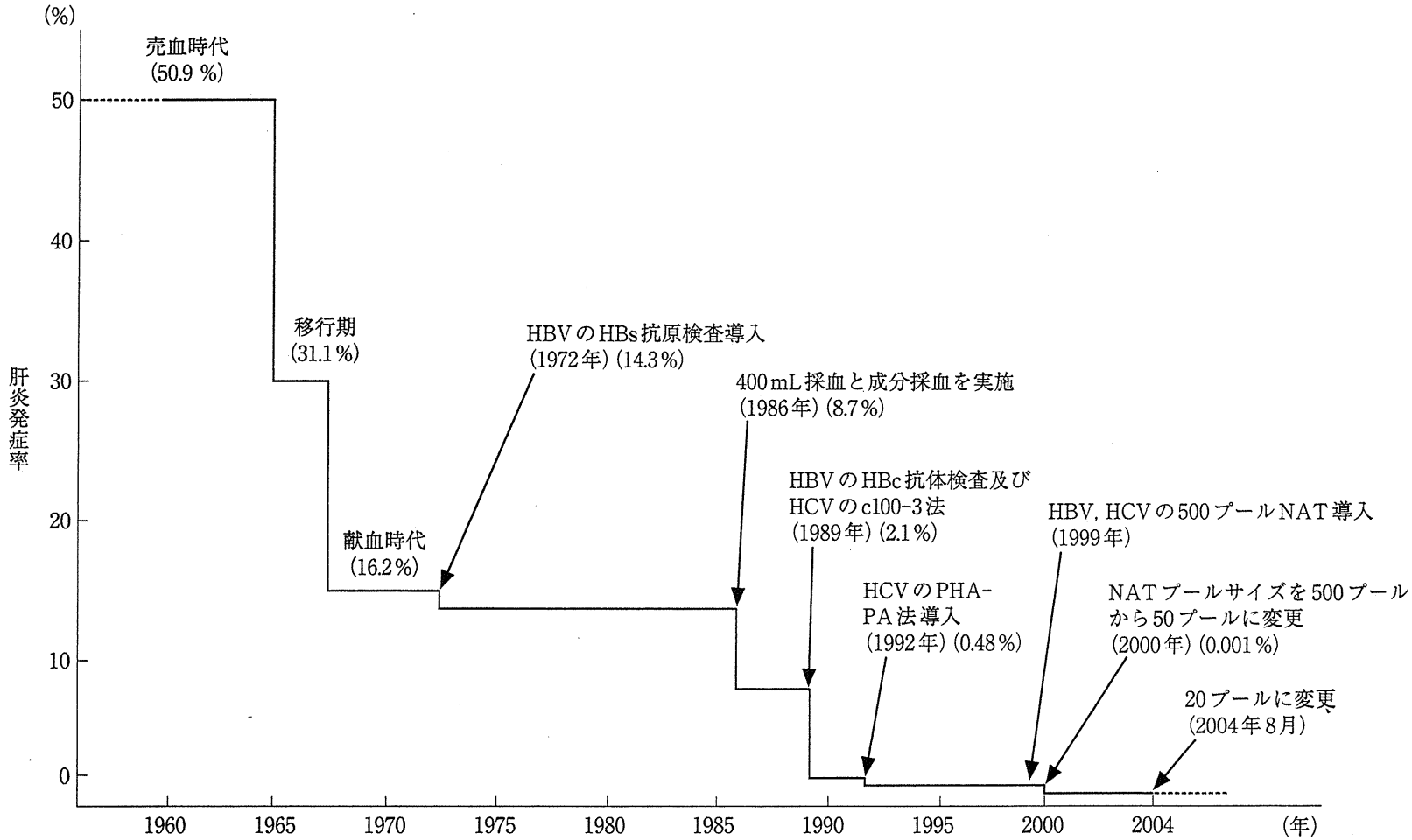

図1　輸血後肝炎の変遷

'日本赤十字社輸血後肝炎の防止に関する特定研究班' 研究報告書(1993.4-1996.3)一部改変を基に厚生労働省作成

ルサイズを50人分に縮小し，2004年8月にプールサイズを20とした．2008年には第二世代の試薬・機器へ変更となり，検査に使用する検体量を200 μLから850 μLへと4.25倍増量し検出感度の向上を図った．また，第二世代の試薬ではHIV-1だけでなくHIV-2も捕捉が可能となり，輸血用血液の安全性が強化され現在に至っている．HCVに対するNATの検出感度は33.5 copies/mL(12.4 IU/mL)である．また，輸血後感染症報告などの科学的解析を可能にするために，1996年9月から全国血液センターは献血血液の検体を保管するシステムを整備した．

2. HCV抗体スクリーニング検査の導入効果

HCV抗体スクリーニング検査(第一世代)の導入効果について，導入前後の輸血後肝炎の発生頻度を2群(1-10単位輸血群と11-20単位輸血群)で解析した結果，それぞれ，導入前4.9％と16.3％の発生頻度であったのに対して，導入後は1.9％と3.3％に減少した[5]．しかし，HCV抗体スクリーニング検査(第一世代)導入後も，輸血後肝炎の発生が認められ，かつ症例報告としても抗c100-3抗体陰性の輸血によるC型肝炎が報告された[6]．このことから，c100-3タンパクを抗原とした第一世代のHCV抗体スクリーニング検査は感度と特異性が低く，HCV感染例を十分に検出できないことが示唆された．その後，コアタンパクを加えた第二世代の試薬に変更された．変更後の輸血後肝炎の発生頻度を6,458例の輸血患者を対象に追跡調査を行ったところ，輸血後肝炎は0.48％まで減少し，C型肝炎の確診例は1例も確認されなかった[7]．HCVには数種の遺伝子型が存在することが知られているが，コアタンパクをコードする遺伝子領域の塩基配列は遺伝子型間でよく保存されている．つまり，コアタンパクを加えた第二世代の抗体測定系はHCV遺伝子型に関係なくHCV感染例を効率よく検出でき，スクリーニング検査としては極めて有用であることが確認された．しかし，1994年，HCV-RNA陽性，HCV抗体陰性のWPが疑われる献血血液によるC型肝炎が1992年2月(第二世代の試薬に変更)以降の輸血で初めて報告された[8]．一方，1993年から日本赤十字社は輸血感染症の実態調査として医療機関から報告される輸血後感染症報告の解析を開始した．当初は献血血液の検体が保管されていなかったことから，報告症例に対しては，当該献血者の理解と協力を求め，原因究明用の検体を再採血し，その血液で精査する調査(追跡調査)であった．症例にもよるが1本の輸血症例から100本を超える輸血症例まで多彩で，全報告例の追跡調査は困難であり，報告症例のうち，約25％が解析可能例にすぎなかった．また，当該輸血用血液を直接検査できないことからWPの献血血液を特定できないという難点があった．このような要因から1994-95年の調査では，輸血によるC型肝炎を見いだすことはできなかった．医療機関から報告された症例の中で，輸血によるC型肝炎が初めて特定されたのは1996年であった．これは，都内の血液センターで先行的に開始(1994年4月より)した検体保管システムが功を奏したことによる．感染の原因となった輸血用血液はHCV抗体陰性，HCV-RNA陽性で遺伝子型が2a型(患者も2a型)で，当該献血者を追跡調査した結果，HCV抗体が陽転していた．WPの献血血液によるC型肝炎として国内で科学的に証明された初の症例である[9]．1996年9月，献血血液の検体保管が全国の血液センターで開始され，1997年，1998年，1999年に輸血によるC型肝炎が，それぞれ1例，7例，5例と複数確認された(**表2，3**)．NAT導入後の輸血によるHCV感染は，2000年から2004年まで確認されず，2005年，2006年に50プールNATのWPに採血された血液で各々1例が確認され，2007年に20プールに感度を上げてからの1例を確認したのが最後で，2008年以降，2010年10月までにNATシステムの改良に伴い輸血によるHCV感染は確認されていない．つまり，現在の輸血によるHCV感染の残存リスクは1<10,000,000で輸血リスクとして許容できるものである．仮にNATを導入していなければ，約10年間に120例以上の患者が不幸にも輸血に

表2 輸血後HCV感染として医療機関から報告された数と輸血との関連性

年	報告数	関連性が高い	関連性が低い	不明例
1994	23	0	6	17
1995	25	0	17	8
1996	33	1	17	15
1997	29	1	24	4
1998	51	7	42	2
1999	60	5	54	1
2000	56	0	56	0
2001	51	0	51	0
2002	33	0	32	1
2003	58	0	57	1
2004	70	0	70	0
2005	64	1	62	1
2006	45	1	44	0
2007	33	1	32	0
2008	29	0	29	0
2009	18	0	18	0

よりHCVが感染した可能性があったことになる．逆をいえば，NAT導入前にも同様に1年間に10例以上の感染例を見逃していたことになる．

3. 感染原因となった血液の特徴

原因血液はすべて，当該献血時はHCV抗体陰性で医療機関に供給されたものであった．しかし，医療機関から輸血後感染症として報告を受け，事後に保管検体を精査したところ17例(献血者は16例)すべてにHCV-RNAが検出された．再検査でもHCV抗体は陰性であった．追跡できた献血者はすべて，HCV抗体が陽転しており(データ省略)，WPの献血血液であることが確認された．ウイルスの遺伝子型は解析できた15例のうち1b型が8例，2a型が6例，2b型が2例であった．年齢は，平均31歳(19-49歳)と若年齢層で，男女比は6：9であった(表4)．献血者への感染経路は特定されていない．アメリカでも25歳以下の若年齢者層の感染リスク行為が問題となっているが[10]，我が国でも軽視できない状況にある．一方，NATが開始されてから2010年7月までにHCV抗体陰性，HCV-RNA陽性の献血血液が122例確認されている．検出頻度は，27-70万回の献血に1回となる．当然ながらこれらの血液は輸血には使用されていないが，その血液中のHCVの特徴を次にまとめた．

4. NATから検出されたWPに献血された血液の特徴

NATのみ陽性の113検体を測定した結果，遺伝子型別では1a: 1例，1b: 30例，2a: 53例，2b: 29例と2a型が半数近くを占め，1b型と2b型がほぼ同数を占めていた．HCVによる慢性肝疾患患者では1b型が約70％，2a型が約20％で2b型が約10％を占めると報告されており，NATで検出された遺伝型割合とは大きく乖離する．慢性肝疾患患者では少なくとも10年以上前(多くは第二次世界大戦後の混乱期と考えられている)の感染であり，現在の献血者のスクリーニングNATで検出された113例はすべてHCV抗体陰性の感染初期例で，感染経路の違いによる差であると考えられる．HCV-RNA検出献血者の性別は男性71例(62.8％)，女性42例(37.2％)と男性の方が多かったが，平成18年度の全献血者の男女比とほぼ一致しており，性別による差は認められなかった．年代別では10歳代13人(11.5％)，20歳代48人(42.5％)，30歳代29人(25.7％)，40歳代13人(11.5％)，50歳代5人(4.4％)，60歳代5人(4.4％)と，20歳代，30歳代で陽性者が多く認められた．しかし，これを平成18年度の献血者年代別構成比と比較すると，10歳代，20歳代で陽性比率が構成比を上回っており，30歳代では構成比とほぼ一致した．40歳代，50歳代では逆に構成比を下回っており，10歳代，20歳代の若年齢層で新たなHCV感染が多く発生しているのではないかと考えられた．遺伝子型と年代との間には明確な傾向は認められなかったが，遺伝子型2bの比率が男性で有意に高いことがわかった．これらの例を採血地域別に遺伝子型を

表3 血液センターに報告された輸血感染症

	HBV	HCV	HIV	PVB19	HEV	バベシア	マラリア	細菌
1991							1	
1992								
1993	1							
1994	1							
1995	1							
1996	1	1						
1997	12	1	1					
1998	22	7						
1999	21	5	2(1)			1		
2000	5			1				2
2001	7							
2002	8			3	1			
2003	12		1	1				
2004	20				2			
2005	11	1		3	1			1&
2006	6	1		1	1			3
2007	13	1*						
2008	4				2			2
2009	7				1			2

*: 20プールWP
(1): 献血者は1名
&: 医療機関と日赤とでの解析結果乖離例

比較すると，遺伝子型1bは中部以西の西日本で多く認められ，関東・東北ではやや低く北海道では検出されていない．2aは中部で一番高かく北海道ではやや低かったが，その他の地域では差が認められなかった．逆に2bは東北・中部で検出されておらず，九州でやや低めであったがその他の地域ではあまり差が認められなかった．

5. NAT導入後の輸血によるC型肝炎の発生状況と残存リスク

感染初期のHCVのramp-up期間での倍加速度は約5.8-21時間とHBVの4日に比して著しく早く[11,12]，特に，HCVに対するNATはHCV抗体検査のWPを大幅に短縮させ，その導入効果は絶大なものであった．このような理由から，NAT導入以来，輸血によるC型肝炎はわずか3例しか確認されておらず．20プールサイズのWPが原因となったのは1例のみであるが，現行のNATシステムでは，感染例は特定されていない．しかし，海外ではNATウインドウの理論的リスクが報告されている(数百万回の献血に1回)[13]し，個別NAT陰性の輸血用血液によるC型肝炎が報告されている[14]．また，HCVの感染価はチンパンジー接種実験から50％感染率が10 copies/mL[15]と少なく，我が国においても決してゼロリスクとはいえない[16]．しかし，このリスクは前述したようにリスクという概念からは許容できるものと著者は認識している．毎年，輸血によるHCV感染の疑い症例として

表4 原因血液と患者の背景

症例	報告年	患者成績				献血者成績			
		年齢(性)	原疾患	ALT(IU/L) MAX.	genotype	個別NAT	genotype	HCV抗体	年齢(性)
1	1996	46(男)	S状結腸癌	581	2a	+	2a	−	23(女)
2	1997	70(女)	急性リンパ性白血病	254	1b	+	1b	−	26(男)
3	1998	63(男)	骨髄異型成症候群	123	1b	+	1b	−	19(女)
4	1998	60(男)	出血性胃潰瘍	81	2a	+	2a	−	34(男)
5	1998	56(男)	胃癌	221	2a	+	2a	−	34(男)
6	1998	32(男)	広範囲熱傷	245	N.T	+	1b	−	23(女)
7	1998	65(男)	十二指腸出血	211	2a	+	2a	−	48(男)
8	1998	78(女)	急性骨髄性白血病	107	1b	+	1b	−	29(女)
9	1998	63(男)	右大腿骨頸部骨折	308	1b	+	1b	−	49(女)
10	1999	48(男)	多発性骨髄腫	11	2b	+	2b	−	32(女)
11	1999	75(男)	肝細胞癌	298	2b	+	2b	−	38(男)
12	1999	38(女)	再生不良性貧血	46	1b	+	1b	−	20(女)
13	1999	48(男)	胃潰瘍	903	1b	+	1b	−	27(男)
14	1999	69(男)	多発性骨髄腫	69	N.T	+	N.T	−	37(男)
15	2005	81(女)	骨髄異型成症候群	407	1b	+	1b	−	44(女)
16	2006	35(女)	常位胎盤早期剥離	12	2a	+	2a	−	21(女)
17	2007	54(女)	再生不良性貧血	26	2a	+	2a	−	22(男)

症例4，5の献血者は同一
上記以外に初流血除去・保存前白血球除去も導入済み

医療機関から報告されてくるが，特定される例は極めて少なく，輸血とは別経路のHCV感染を疫学的に調査することが必要ではないかと思われる．

6. イギリスでの現状(SHOTより)

輸血による肝炎は2004年にHEVの1例，2005年にHBVの1例が同定されているが，それ以降は，細菌感染が主な原因となっている．

(from http://www.shotuk.org/shot-reports/reports-and-summaries-2001-2002/)

7. 血液製剤によるHCV感染

血液製剤でHCV感染が問題となったのは，非加熱製剤である．最近では，過去にフィブリノゲン製剤，血液凝固因子製剤を投与された患者で問題となった．免疫グロブリン製剤でもバクスター社のGammagardに関連したHCV感染が報告され，HCVを不活化するS/D処理が製造工程に導入された経緯もある[17]．その後，血漿分画製剤についてはウイルスの除去・不活化工程を2種以上導入することが義務付けられ，少なくとも，HBV・HCV・HIVの製造工程中のウイルスクリアランス指数が10^9以上を担保しなくてはいけないことになっている．また，国内原料血漿については，NAT導入によりウイルス混入量が極限まで少なくなっている．更に，2010年度中にはHIV，HBV，HCVを検出するNATの感度がHBV-DNA(2,000 IU/mL)，HCV-RNA(2,000 IU/mL)，HIV-RNA(4,000 IU/mL)と規定される予定であるが(血液製剤のウイルスに対する安全性確保を目的としたNATに必要とされる検出限界値について．平成22年10月6日付厚生労働省医薬食品局血液対策課よ

り), 日赤のNATはこれを上回る感度で実施しているので問題はない. つまり, 現在, 市場に流通している血液製剤によってHCV感染は起こらない.

8. まとめ

献血血液に対するNATの導入と改善によって輸血によるC型肝炎は激減し, 許容できるリスクとなった. しかし, ゼロリスクではなく現行のNATシステムでもすり抜けるWPの血液は否定できない. 医療機関側では, 輸血とは別の感染経路を調査すると供に, HCV感染の早期検出のためにも抗体検査からc抗原へ変更することが肝要である.

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Hepatic Medicine: Evidence and Research Dovepress

ORIGINAL RESEARCH

Type I interferon receptor in peripheral blood mononuclear cells may predict response to intra-arterial 5-fluorouracil + interferon therapy for advanced hepatocellular carcinoma

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Background: Type 1 interferon alpha receptor 2 (IFNAR2) in the liver has been reported to be a predictive factor for the response to intra-arterial 5-fluorouracil (5-FU) + systemic interferon (IFN)-alpha combination therapy in patients with advanced hepatocellular carcinoma. We tested whether IFNAR2 expression in peripheral blood mononuclear cells could predict the response to 5-FU + IFN.

Methods: Predictive factors for survival and response to therapy were determined in 30 patients with advanced hepatocellular carcinoma who underwent treatment with 5-FU + IFN. IFNAR2 expression in peripheral blood mononuclear cells was measured in 11 of the 30 patients.

Results: With a mean number of 4.2 courses of combination therapy, one patient (3%) showed a complete response, eight (27%) showed partial responses, 13 (43%) had stable disease, and eight (27%) showed progressive disease. The median survival time of responders (complete response/partial response) was 12.7 months and that of nonresponders (stable disease/progressive disease) was 7.5 months. The one-year and two-year cumulative survival rates of responders and nonresponders were 87/69% and 40/11%, respectively ($P = 0.019$). Multivariate analysis identified response to therapy ($P = 0.037$) as the sole independent determinant of survival. The expression level of IFNAR2 in peripheral blood mononuclear cells was significantly ($P = 0.012$) higher in responders (6.5 ± 2.4) than in nonresponders (2.4 ± 0.6), even though no clinical factors were identified as being associated with the response to the combination therapy.

Conclusion: IFNAR2 expression in peripheral blood mononuclear cells may predict the response to 5-FU + IFN therapy in patients with advanced hepatocellular carcinoma, although these data are preliminary.

Keywords: interferon, 5-fluorouracil, hepatocellular carcinoma, receptor

Introduction

Hepatocellular carcinoma is the third leading cause of cancer-related death globally, behind lung and stomach cancers.[1] Its incidence has been increasing in Japan in the last 30 years[2] and also in the US more recently.[3] Intensive management of patients at high risk for hepatocellular carcinoma and advances in diagnostic techniques have facilitated the detection of hepatocellular carcinoma in the early stage.[4–7] Simultaneously, several therapeutic modalities, including hepatic resection, liver transplantation, radiofrequency ablation, percutaneous ethanol injection, and transcatheter arterial chemoembolization have substantially improved the prognosis of patients with hepatocellular carcinoma.[8–11] Nevertheless, we still sometimes see patients with advanced hepatocellular carcinoma

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at their first visit or after repeated treatment due to the frequent recurrence of the disease.

The prognosis of patients with advanced hepatocellular carcinoma, especially if complicated by portal venous invasion, is extremely poor.[12,13] Recently, sorafenib, an oral multikinase inhibitor of the vascular endothelial growth factor receptor, the platelet-derived growth factor receptor, and Raf, has been shown to prolong median survival time and the time to progression by nearly three months in patients with advanced hepatocellular carcinoma as compared with those given placebo.[14] However, no complete responses and only a few partial responses (2%) were found in the same study. Although sorafenib can be used for the treatment of patients with advanced hepatocellular carcinoma, its clinical effectiveness is still controversial in Japan. According to the consensus-based clinical manual proposed by the Japan Society of Hepatology,[15] arterial infusion chemotherapy using an implantable drug delivery system is recommended as one of the treatments for advanced hepatocellular carcinoma with portal venous invasion, based on the favorable results of combination therapy with intra-arterial 5-fluorouracil (5-FU) + systemic interferon (IFN)[16–19] or another combination of low-dose cisplatin + 5-FU.[20,21] To improve the effects of these combination therapies and to increase the response rates, it is important to find a practical and useful predictor of the response to therapy. Hepatic expression of type I interferon alpha receptor 2 (IFNAR2) has been shown to correlate with the response to 5-FU + IFN in patients with advanced hepatocellular carcinoma and portal venous invasion.[17] However, liver biopsy is sometimes difficult to perform before combination therapy in patients with advanced hepatocellular carcinoma because of a bleeding tendency with a low count platelet and/or decreased activity of prothrombin. In this pilot study, we tested whether IFNAR2 expression in peripheral blood mononuclear cells could predict the response to 5-FU + IFN in patients with advanced hepatocellular carcinoma.

Materials and methods

Patients

A single-arm, open-label study of intra-arterial combination therapy was conducted in patients with advanced hepatocellular carcinoma. Eligibility criteria were as follows: hepatocellular carcinoma with tumor thrombi invading at least one of the major branches of the portal vein (Vp3 or Vp4, according to the criteria of the Liver Cancer Study Group of Japan [LCSGJ]) or multiple intrahepatic metastases in more than three segments, irrespective of the degree of portal venous invasion (Vp1, Vp2, Vp3, or Vp4); tumor staging III or IVA based on the TNM (tumor node metastasis) staging system of the LCSGJ; absence of extrahepatic metastases; Child–Pugh A or B liver function; an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1;[22] leukocyte count >2000/μL; platelet count >50,000/μL; unresectable disease or not suitable for local ablation therapy; and unlikelihood of effectiveness with transcatheter arterial chemoembolization. All patients provided written informed consent for this study, which was approved by the institutional review board of Kawasaki Medical University.

Implantation of intra-arterial catheter

An indwelling intra-arterial catheter (Piolax W spiral catheter, Piolax Medical Devices Inc, Kanagawa, Japan) was inserted through the femoral artery by the Seldinger method, and its tip was put in the proper hepatic artery or common hepatic artery, embolizing the right gastric and gastroduodenal arteries to avoid efflux of chemotherapeutic agents into the stomach and duodenum. The other end of the catheter was connected to the injection port (Vital-Port, Cook Japan, Tokyo, Japan) subcutaneously implanted in the lower abdomen.

Evaluation of response to therapy

The response to therapy was assessed with contrast-enhanced computed tomography after each therapeutic cycle. The response was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST)[23] as: complete response (complete disappearance of all target lesions); partial response (at least a 30% decrease in the sum of the longest diameter of the target lesions), taking as reference the baseline sum of the longest diameter; progressive disease (at least a 20% increase in the sum of the longest diameter of target lesions or the appearance of one or more new lesions); or stable disease (neither partial response nor progressive disease criteria). The best response to therapy was defined as the response to therapy when a different response, such as partial response or stable disease, was found in the same patient during multiple treatment cycles. Adverse reactions were assessed using the National Cancer Institute Common Toxicity Criteria (NCI-CTC, version 3.0, [http://ctep.cancer.gov/reporting/ctc.html]).

Treatment protocol

One cycle of treatment consisted of four weeks in which 5×10^6 U (5 MU) of IFN-alpha (OIF; Otsuka Pharmaceutical, Tokyo, Japan) was administered intramuscularly

on days 1, 3, and 5 of each week, resulting in a total dose of 60 MU per cycle. 5-FU (Kyowa Hakko, Tokyo Japan) 500 mg/day was administered into the hepatic artery over five hours using a portable infusion pump on days 1–5 of the first and second weeks (5 g per cycle). The combination therapy was discontinued in patients who did not meet the eligibility criteria and also in those with progressive disease or NCI-CTC Grade 3 adverse reactions, otherwise the treatment was repeated after a 2–4-week rest period without treatment.

Measurement of IFNAR2 expression in peripheral blood mononuclear cells

Peripheral blood mononuclear cells were separated from 10 mL of heparinized blood by density gradient centrifugation using Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden), washed three times with RPMI 1640 culture medium, and stored at −80°C until use. RNA was extracted from the homogenized peripheral blood mononuclear cells using a High Pure RNA kit (Roche Diagnostics Ltd, Germany), and its integrity was confirmed by spectrophotometry. The IFNAR2 mRNA expression level was quantified using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and real-time reverse transcription polymerase chain reaction (RT-PCR), as described previously.[24] Briefly, a RT-PCR assay was performed on a 25 μL reaction mixture containing 20 ng of sample cDNA, 100 nM sense primer, 100 nM antisense primer, and 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems). The following specific primers were designed to amplify their respective genes; IFNAR2, sense; 5′-GAAGGTGGTTAAGAACTGTGC-3′, antisense; 5′-CCCGCTGAATCCTTCTAGGACGG-3′; β2-microglobulin, sense; 5′-ACCCCCACTGAAAAAGATGA-3′, antisense; 5′-ATCTTCAAACCTCCATGATG-3′. The PCR was carried out for 45 cycles at 95°C for 15 seconds and 60°C for one minute. A standard curve for each mRNA expression was generated using five-fold dilutions of a control RNA sample (25×, 5×, 1×, 0.2×, and 0.04×). The mRNA expression levels of the target genes (IFNAR2) were presented as a ratio to that of β2-microglobulin, and the relative expression levels were calculated.

Statistical analysis

Quantitative values were expressed as the mean ± standard deviation. Cumulative survival was calculated using the Kaplan–Meier method, and the differences between the groups were analyzed using the log-rank test. Univariate and multivariate analyses of predictors of survival were assessed by the Cox proportional hazards model. Univariate and multivariate analyses of predictors for the response to therapy were assessed by the logistic regression test. Differences between the two groups were examined for statistical significance using the Mann–Whitney U test. A P value < 0.05 was considered to be statistically significant. All analyses described above were performed using SPSS software (version 11, SPSS Inc, Chicago, IL).

Results

Patient profile

Forty-five patients with advanced hepatocellular carcinoma fulfilled the eligibility criteria for 5-FU + IFN therapy. Among them, 30 patients (24 men and six women) with an average age of 64.7 ± 1.8 (range 48–84) years provided written informed consent to receive the combination therapy. Patient characteristics at baseline are shown in Table 1. Eight patients were positive for both hepatitis B (HBV) surface antigen and HBV DNA, and 18 for both anti-hepatitis C virus (HCV) and HCV RNA. The remaining four patients were negative for both hepatitis B surface antigen and anti-HCV. Liver disease stage was Child–Pugh A and tumor stage was IV in 23 patients (76.7%). The integrated staging scores for the Japan Integrated Staging[25] and Cancer of the Liver Italian Program (CLIP)[13] were ≥3 in 23 (76.7%) and 17 patients (56.7%), respectively. Twelve patients (40%) had portal venous invasion at a major branch (Vp3) or in the main trunk (Vp4).

Table 1 Patient characteristics

Number of patients	30
Age, years, mean ± SD (range)	64.7 ± 1.77 (48–84)
Gender, male/female	24/6
Etiology (HBV/HCV/NBNC)	8/18/4
Total bilirubin (mean ± SD, mg/dL)	1.1 ± 0.1
Albumin (mean ± SD, g/dL)	3.5 ± 0.08
Prothrombin time (mean ± SD, %)	77.2 ± 2.2
Platelet count (mean ± SD, ×10⁴/μL)	14.7 ± 1.4
AFP (mean ± SD, ng/mL)	33,715 ± 13,255
AFP-L3 (mean ± SD, %)	23.1 ± 4.6
DCP (mean ± SD, mAU/mL)	37,905 ± 17,417
Child–Pugh status (A/B/C)	23/7/0
TNM staging by LCSGJ (III/IVA)	7/23
JIS score (1, 2/3, 4, 5)	7/23
CLIP score (1, 2/3, 4, 5)	13/17
Portal vein invasion (Vp1 or Vp2/Vp3 or Vp4)	18/12

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-HBV non-HCV; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; TNM, tumor node metastasis; LCSGJ, Liver Cancer Study Group of Japan; JIS, Japan integrated staging; CLIP, Cancer of the Liver Italian Program; SD, standard deviation.

Response to combination therapy and survival

Thirty patients with advanced hepatocellular carcinoma completed 5-FU + IFN therapy, with a mean treatment cycle number of 4.2 (range 2–12). The median survival time was 7.5 months, and the one-year and two-year cumulative survival rates were 53% and 33%, respectively. Of these 30 patients, one (3%) had a complete response, eight (27%) had a partial response, 13 (43%) had stable disease, and 8 (27%) had progressive disease, ie, nine (30%) had objective responses (complete response or partial response). The median survival time of responders (complete response/partial response) was 12.7 months and that of nonresponders (stable disease/progressive disease) was 7.5 months. The one-year and two-year cumulative survival rates for responders and nonresponders were 87%/69% and 40%/11%, respectively. Thus, there was a significant difference in the overall survival rate between responders and nonresponders ($P = 0.019$, Figure 1).

Factors associated with survival

We investigated the predictors of survival in patients who underwent 5-FU + IFN therapy. Univariate analysis identified total bilirubin concentration ($P = 0.005$), CLIP score ($P = 0.019$), and response to therapy ($P = 0.033$) as factors associated with survival (Table 2). Among these factors, multivariate analysis identified the response to therapy ($P = 0.037$) as a significant and independent determinant of survival (Table 3).

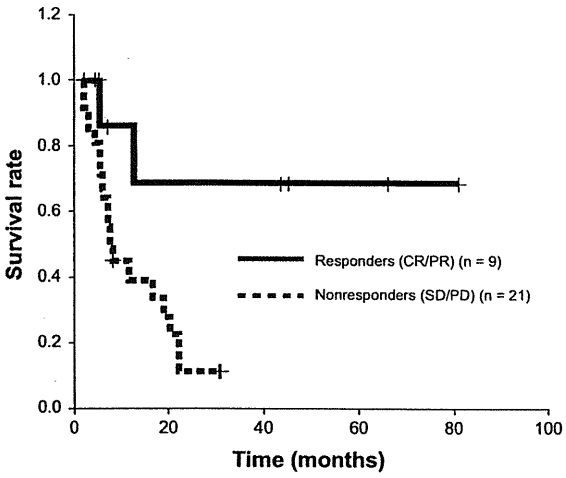

Figure 1 Comparison of overall survival rates of responders (complete response or partial response) and nonresponders (stable disease or progressive disease) to 5-FU + IFN therapy. The survival rate was significantly higher in responders than in nonresponders (log-rank test, $P = 0.019$).
Abbreviations: 5-FU, 5-fluorouracil; IFN, interferon.

Table 2 Univariate analysis of predictors for survival

Variable	Hazards ratio	95% CI	*P* value
Age	0.956	0.907–1.007	0.091
Male	2.675	0.694–10.311	0.153
HBsAg positive	0.460	0.169–1.249	0.128
Anti-HCV positive	1.503	0.604–3.735	0.381
Total bilirubin (mg/dL)	3.222	1.420–7.313	0.005
Albumin (g/dL)	0.413	0.143–1.193	0.102
Prothrombin time (%)	0.964	0.917–1.014	0.160
Platelet count ($\times 10^4/\mu L$)	0.976	0.918–1.036	0.976
AFP (<100 ng/mL)	1.372	0.551–3.416	0.497
AFP-L3 (<20%)	1.509	0.610–3.731	0.373
DCP (<100 mAU/mL)	0.445	0.101–1.954	0.283
Child–Pugh status A	2.549	0.950–6.843	0.063
Tumor stage III	2.995	0.858–10.460	0.086
JIS score (<3)	2.995	0.858–10.460	0.086
CLIP score (<3)	3.421	1.222–9.576	0.019
Portal vein invasion (<Vp3)	2.288	0.871–6.010	0.093
Response to therapy (CR or PR)	4.960	1.136–21.668	0.033

Abbreviations: CI, confidence interval; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; NBNC, non-HBV non-HCV; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; TNM, tumor node metastasis; LCSGJ, Liver Cancer Study Group of Japan; JIS, Japan Integrated Staging; CLIP, Cancer of the Liver Italian Program; CR, complete response; PR, partial response.

Factors associated with response to combination therapy

We examined factors associated with the response to 5-FU + IFN therapy, because response to therapy was found to be the only independent factor associated with survival in patients who underwent treatment with this combination. However, univariate and multivariate analyses did not identify any significant factors associated with response to the combination therapy (Table 4).

IFNAR2 in peripheral blood mononuclear cells and response to 5-FU + IFN

To explore factors associated with the response to the combination treatment, we next measured IFNAR2 mRNA expression in peripheral blood mononuclear cells in 11 patients from whom peripheral blood mononuclear cells were

Table 3 Multivariate analysis of predictors for survival

Variable	Hazards ratio	95% CI	*P* value
Total bilirubin (mg/dL)	1.076	0.484–3.711	0.574
CLIP score (<3)	3.434	0.907–13.000	0.069
Response to therapy (CR or PR)	5.478	1.108–27.093	0.037

Abbreviations: CLIP, Cancer of the Liver Italian Program; CR, complete response; PR, partial response; CI, confidence interval.

Table 4 Univariate and multivariate analyses of predictors for the response to 5-FU + IFN therapy

Variable	Univariate analysis			Multivariate analysis		
	Odds ratio	95% CI	*P* value	Odds ratio	95% CI	*P* value
Age	1.034	0.951–1.125	0.431	–	–	–
Male	0.333	0.053–2.115	0.244	0.266	0.036–1.966	0.194
HBsAg positive	0.250	0.026–2.416	0.231	0.204	0.019–2.216	0.191
Anti-HCV positive	1.818	0.357–9.272	0.472	–	–	–
Total bilirubin (mg/dL)	0.607	0.132–2.796	0.552	–	–	–
Albumin (g/dL)	1.139	0.206–6.297	0.882	–	–	–
Prothrombin time (%)	0.976	0.912–1.045	0.486	–	–	–
Platelet count ($\times 10^4/\mu L$)	1.012	0.915–1.118	0.820	–	–	–
AFP (<100 ng/mL)	0.880	0.183–4.226	0.873	–	–	–
AFP-L3 (<20%)	0.727	0.151–3.493	0.691	–	–	–
DCP (<100 AU/mL)	0.750	0.067–8.363	0.815	–	–	–
Child–Pugh status A	3.198	0.326–31.39	0.318	–	–	–
Tumor stage III	0.914	0.142–5.902	0.925	–	–	–
JIS score (<3)	0.914	0.142–5.902	0.925	–	–	–
CLIP score (<3)	2.031	0.417–9.886	0.380	–	–	–
Portal vein invasion (<Vp3)	2.031	0.417–9.886	0.380	–	–	–

Abbreviations: HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; NBNC, non-HBV non-HCV; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; TNM, tumor node metastasis; LCSGJ, Liver Cancer Study Group of Japan; JIS, Japan integrated staging; CLIP, Cancer of the Liver Italian Program.

available before therapy, because the effect of 5-FU + IFN has been demonstrated to depend significantly on hepatic IFNAR2 expression,[17] and there is a significant correlation between IFNAR2 expression in the liver and peripheral blood mononuclear cells.[26] Seven of the 11 patients were responders (complete response/partial response) and the remaining four patients were nonresponders (stable disease/progressive disease). The expression level of IFNAR2 in peripheral blood mononuclear cells was significantly ($P = 0.012$) higher in responders (6.5 ± 2.4) than in nonresponders (2.4 ± 0.6, see Figure 2).

Adverse reactions and complications

Most patients complained of flu-like symptoms, including fever, nausea, and loss of appetite, but the degree of these adverse reactions was NCI-CTC Grade 1 or 2. Among patients with NCI-CTC Grade 3 adverse reactions, stomatitis was observed in two patients, diarrhea in one, leukopenia in one, thrombocytopenia in one, and hemorrhagic gastric ulcer in another. None of the patients required administration of granulocyte colony-stimulating factor or blood transfusion. There were five complications resulting from the arterial catheter, ie, occlusion in two patients, infection associated with the indwelling catheter in two patients, and dislocation in a further patient.

Additional therapy

Three patients each were treated with transcatheter arterial chemoembolization and intra-arterial 5-FU + cisplatin, respectively, after identification of progressive disease. Three patients assessed to exhibit a partial response had additional therapy, one of whom underwent partial hepatectomy because of downstaging of hepatocellular carcinoma, and the other two were repeatedly treated with transcatheter arterial chemoembolization because of dislocation of an indwelling intra-arterial catheter or downstaging of

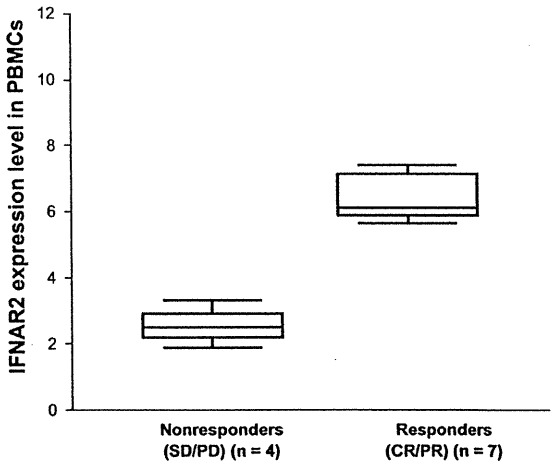

Figure 2 Expression levels of IFNAR2 in peripheral blood mononuclear cells in responders (complete response or partial response) and nonresponders (stable disease or progressive disease) to 5-FU + IFN therapy. The relative quantities of IFNAR2 mRNA in peripheral blood mononuclear cells were normalized to β-actin mRNA. The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Median values are shown by the lines within the boxes. IFNAR2 expression was significantly higher in responders than in nonresponders (Mann–Whitney *U* test, $P = 0.012$).
Abbreviations: IFNAR2, Type I interferon alpha receptor 2; 5-FU, 5-fluorouracil; IFN, interferon.