or core promoter region were present between patients with fulminant hepatitis and self-limited acute hepatitis [12/12 (100%) vs. 14/98 (14%), $P < 0.01$] and between severe acute hepatitis and self-limited acute hepatitis [13/23 (57%) vs. 14/98 (14%), $P < 0.01$]. The frequency of mutation increased proportionately with the severity of disease in patients with acute HBV infection. Our results indicate that mutations in the core promoter and precore regions, together or independently, are associated with fulminant or severe acute hepatitis and that HBV strains with such mutations cannot direct the production of HBeAg. A low prevalence of HBeAg or anti-HBe in patients with fulminant and severe acute hepatitis B has also been noted.

As we had described previously, the G-to-A switch at nt 1896 of the precore region of HBV gene results in a translational stop codon which causes a lack of secretion of HBeAg, and mutations within the core promoter region down-regulate HBeAg expression at the transcriptional level. It is generally believed that immunoelimination of HBV-infected cells occurs following specific targeting of HBeAg on the membrane of liver cells by activated T-lymphocytes [21]. Secretory HBeAg may distract the immune system from eliminating infected hepatocytes, possibly by down-regulation of cellular factors important in recognition by the immune system [21,22]. Therefore, the presence of replicating HBV associated with a lack of circulating HBeAg may result in a higher degree of inflammatory activity and determine an unfavorable clinical outcome.

Baumert et al. [23] demonstrated that two adjacent mutations in the HBV core promoter region were responsible for enhanced replication of the viral genome of a HBV strain associated with an outbreak of fulminant hepatitis (strain FH). Moriyama et al. [24] also demonstrated that mutations at nt 1762 and 1764 in the core promoter were responsible for reduced HBeAg production as well as enhanced replication and core production. Buckwold et al. [25] proved experimentally that HBeAg production was decreased, as a liver-specific transcription factor which normally binds to the basal core promoter was prevented from binding by the mutations and that this mutation did not affect the transcription of the core RNA and protein. This mutation appeared to increase the packaging efficiency of pregenomic RNA and, therefore, increase viral replication. However, there is no direct evidence that the enhanced replication of strain FH HBV contributes to the development of fulminant hepatitis. Studies in an animal model are necessary to demonstrate the biological significance of these mutations in the pathogenesis of HBV infection.

We must discuss the relationship between HBV genotype and precore mutation again. Lindh et al. [26] have demonstrated how the regional differences in precore mutation prevalence have occurred. In patients with genotype A HBV infection, which is a predominant genotype in North America and Europe, a dCTP is at nt 1858. Impaired base-pairing of the pregenomic RNA results when the C1858 tries to pair with a U1858 in the RNA genome. In contrast, among chronic HBV carriers from Asia, Africa or the Middle East, U1858 permits stable basepair formation with a G-to-A mutation at nt 1896 which forms a TAG stop codon. Other studies [27,28] also demonstrated a high prevalence of precore mutation in HBV sequences with genotype D and a low prevalence in sequences with genotype A. Such differences may explain why mutation at nt 1896 is not common in patients with fulminant hepatitis in France or the United States, where HBV of genotype A predominates.

There is still a lack of reliable clinical markers that predict the outcome of acute hepatitis. The most important clinical implication of our findings is that we may be able to predict the outcome of liver disease in patients with acute HBV infection by using PMA for HBV precore and core promoter analysis in patients without HBV genotype A [20].

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Hepatitis B virus of genotype C persistence after recovery from acute hepatitis B virus infection in Japan

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Abstract

Backgroundlaims: this study aimed to determine the viremia status after clinical, biochemical and serological recovery from acute hepatitis B viral (HBV) infection. *Methods:* we detected serum HBV-DNA in 19 patients with acute hepatitis B during followed-up 6–43 months after onset, and analyzed HBV genotypes. *Results:* 13 (72%) of 19 patients had detectable HBV DNA at the point of 6 months after onset, and four (33%) of 12 patients had persisted viremia for more than 1 year although they were recovery with normalization of alanine transaminase (ALT), disappearance of hepatitis B surface antigen (HBsAg) and appearance of antibody against HBsAg (anti-HBs). Eighteen (95%) of 19 patients were infected with HBV genotype C, one (5%) with genotype B. *Conclusions:* these results suggest genotype C of HBV is the predominant genotype of acute hepatitis B in Nagasaki region in Japan. HBV can persist in the serum for more than one year after complete clinical and serological recovery from acute viral hepatitis.
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Keywords: Hepatitis B virus; Acute hepatitis B; Viremia; Genotype; Precore mutation; Core promoter mutation; Direct sequencing

1. Introduction

Hepatitis B virus (HBV) DNA may persist in the serum and liver in patients with chronic hepatitis long after disappearance of hepatitis B surface antigen (HBsAg) and appearance of antibody against HBsAg (anti-HBs) in the natural course of the disease and after antiviral therapy [1–6]. Recent studies using polymerase chain reaction (PCR) have shown long-term persistence of HBV DNA in the serum and peripheral blood mononuclear cells (PBMCs) after serological recovery from acute hepatitis B [7–9].

It was confirmed in countries like Japan, where persistent HBV infection occurs almost exclusively from mother-to-newborn infection, seldom occurs

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after acute infection in adulthood, but in western countries, where HBV chronic carrier after acute infection in adulthood may be commonly observed. The different conditions of persisting HBV after acute HBV infection among those areas may be attributed to the HBV genomic variation. Recent advancement of genomic analysis has revealed at least seven genotypes (A–G) with distinct geographical distribution in the world [10–13]. Overall, genotype A is most prevalent in North Europe, North America and Africa; genotype B and C are mostly found in Asia; genotype D is predominant in the Middle East; genotype E is restricted to Africa; genotype F is found in American natives; genotype G is newly discovered in France and the US from patients chronically infected with HBV. Genotype A is a common genotype in Europe and about 10% of acute hepatitis B patients develop into chronic carrier state [14]. There were reports that adult Japanese patients became HBV carriers after acute hepatitis B; the causal HBV was genotype A[15,16]. These results suggest that genotype A has a tendency to cause persistent HBV infection after acute infection in adulthood.

The mutations in precore region and core promoter region have been studied in relation to HBeAg expression, liver disease severity as well as viral replication [17–24]. The mutations are often present in patients with fulminant hepatitis B, chronic hepatitis B and hepatocellular carcinoma, and less often in asymptomatic carriers and carriers without serological HBV markers. The mutations in preS1 region and preS2 region may contribute to viral persistence due to an evasion of the host immune surveillance [25].

It has been concerned that HBV DNA can persist in patients with resolved acute hepatitis B, but, the factors associated with becoming a HBV carrier after acute HBV infection in adulthood have not been well studied.

In this study, we detected serum HBV–DNA in patients with acute hepatitis B by enzyme-linked mini-sequence assay (ELMA) kit to determine whether HBV might be persistence in recovery stage, and analyzed HBV genotypes and mutations in precore region, core promoter region and preS region by direct sequencing to investigate the probable factors associated with persistent viremia.

2. Patients and methods

2.1. Patients

Nineteen Japanese patients who were admitted to the National Nagasaki Medical Center with acute HBV infection during the period 1990–1999 were studied. The diagnosis of acute hepatitis B was made based on the high titre of immunoglobulin (Ig) M antibody to hepatitis B core antigen (anti-HBc) in the absence of any evidence of acute infection with other hepatitis viruses. The diagnostic criteria for severe hepatitis was prothrombin time less than 40%. These patients were selected based on the availability of two or more serum samples over a follow-up period of at least 6 months. They included eight (42%) males and 11 (58%) females, aged 23–75 years (mean $\pm$ S.D.: 39 ± 18). The biochemical, serological data and serum samples were collected at various times (initiation, 6 months and more than 12 months after onset). Twelve patients were followed up for more than 12 months, seven patients for 6 months. Informed consent to the use of serum samples was obtained from each patient and study was approved by the Ethic Review Committee of Liver disease study in National Nagasaki Medical Center.

2.2. HBV DNA analysis

HBV DNA was extracted from 100 μl serum using a DNA/RNA extraction kit (Smitest EX-R&D, Sumitomo Metal Industries, Tokyo, Japan) according to the manufacturer's instructions. HBV DNA assay was performed with enzyme-linked mini-sequence assay (ELMA) kit (Nippon Roche, Tokyo, Japan), according to the manufacturer's instructions. The sensitivity of ELMA kit was to be reproducible until the HBV DNA concentration of 100 copies/ml [19].

2.3. HBV genotype and subtype assay

2.3.1. PCR amplification

The first PCR was performed on 5 μl extracted DNA in a 50-μl reaction mix containing 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.25 mmol/l of each of the dNTPs, 2.0 U Tag DNA polymerase (Takara Shuzo Co. Ltd. Japan), 0.25 μmol/l of each external primer (Table 1) and sterile water. Amplification was performed in a thermal cycler (GeneAmp PCR system 9600) under the following conditions: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by 38 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min and finally 94 °C for 1 min, 55 °C for 2 min and 72 °C for 10 min. For the second PCR, 2 μl of the first PCR product was added to 48 μl of reaction mix with the same composition as the first PCR reaction, except the internal primers were used. Ten microliters of PCR products was analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide, and visualized with an ultraviolet transilluminator.

2.3.2. HBV DNA sequencing

Amplified PCR products were purified by Qiaquick Spin columns (Qiagen Inc.) according to the manufacturer's instructions. Purified HBV nucleotide was directly sequencing by an automated DNA sequencer ABI PRISM 310 (Perkin

Table 1
Overiew of the amplification and sequencing primers

Purpose	Target region	Primer	Primer sequence 5′-3′	Position
Genotype and subtype	PreS1, PreS2 and S	External		
		HBVgt-S1	TAC ACG CAG TGC CTC ATT CT	2792–2811
		HBVgt-AS1	TAC CCC AAC ATC CAA TTA CA	885–904
		Sequencing		
		HBVgt-SEQ1	TAC ACG CAG TGC CTC ATT CT	2792–2811
		HBVgt-SEQ2	AAT CGG CAG TCA GGA AGA CA	3139–3158
		HBVgt-SEQ3	TCG TGG TGG ACT TCT CTC AAT	254–274
		HBVgt-SEQ4	GTT TCT CCT GGC TCA GTT	660–677
Genotype	PreS1, PreS2	External		
		HBVgt-S2	TGG AAG GCT GGC ATT CTA TA	2760–2779
		HBVgt-AS2	TCC CCC TAG AAA ATT GAG AG	267–286
		Internal		
		HBVgt-S1	TAC ACG CAG TGC CTC ATT CT	2792–2811
		HBVgt-AS3	AGG AAT CCT GAT GTT GTG TT	161–180
		Sequencing		
		HBVgt-SEQ2	AAT CGG CAG TCA GGA AGA CA	3139–3158
Core promoter mutant and precore mutant	Core promoter region and precore region	External		
		HBV1601-S	ACG TCG CAT GGA GAC CAC CG	1601–1620
		HBV1974-AS	GGA AAG AAG TCA GAA GGC AAA	1954–1974
		Internal		
		HBV1653-S	CAT AAG AAG ACT CTT GGA CT	1653–1672
		HBV1959-AS	GGC AAA AAA GAG AGT AAC TC	1959–1978
		Sequencing		
		HBV1698-SEQ	GAG GCA TAC TTC AAA GAC TGT T	1698–1719

Elmer Corp., USA) on the basis of the fluorescence-labelled dideoxynucleotide chain terminating assay using the sequencing primers (Table 1).

2.3.3. Date analysis

The sequence of the PreS2 gene is used for genotyping [26]. Amino acid sequence of the PreS2 region is deduced from the nucleotide sequence, then according to the homologue of amino acid sequence, the genotype is decided by comparing with the reported sequences of the PreS2 region of the different HBV genotypes in the database bank. The subtype is determined by deduced amino acids at codon 122 and 160 in the S region [10].

2.4. Mutation assay of the precore and core promoter regions of HBV

2.4.1. PCR amplification and HBV DNA sequencing

The process of assay was same as genotype and subtype assay described above, except the changes of PCR primers and sequencing primer (Table 1).

2.4.2. Date analysis

The precore mutation is determined by the G to A mutation at nucleotide (nt) 1896 in the precore region. The core promoter mutation is determined by the double mutation, A to T mutation at nt 1762 and G to A mutation at nt 1764, in core promoter region. In addition, the nucleotide at position 1858 is analyzed as well.

2.5. Sequence analysis of preS region after HBsAg seroconversion

2.5.1. PCR amplification and HBV DNA sequencing

The serum samples of patients with HBV persistence more than one year after disappearance of HBsAg or appearance of anti-HBs were used to analyze the sequences of preS regions. The process of assay was same as genotype and subtype assay described above, using primers of the preS1 and preS2 region.

2.6. Statistical analysis

Differences in the proportion of cases infected with core promoter or precore HBV mutants between severe acute hepatitis B and self limited acute hepatitis B were evaluated by the χ^2-test with Yates' correction. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. HBV in serum

Table 2 lists the clinical, biochemical, serological data and HBV DNA detection in individuals convalescent from acute hepatitis B. At the beginning of the disease, all patients exhibited elevations of alanine transaminase (ALT) levels with serum HBV DNA positive and anti-HBs negative, and 17 (89%) of 19 patients were seropositive for HBsAg. At the point of 6 months after disease onset, all of them were recovery with normalization of ALT and disappearance of HBsAg, and 14 (78%) of 18 patients had developed anti-HBs, but 13 (72%) of 18 patients were positive for serum HBV DNA. Four (33%) of the 12 patients remained detectable HBV DNA in serum at the last observation, in which one patient (case 4) had persisted viremia for 43 months (Table 3).

3.2. HBV genotypes and subtypes

According to the homologue of amino acid sequence of the HBV preS2 region, 18 (95%) of 19 patients were infected with genotype C of HBV, 1(5%) with genotype B (Fig. 1). The result suggests that genotype C is the predominant genotype of acute HBV infection in Nagasaki region in Japan.

In addition, the subtypes were analyzed in eight patients who included seven patients with HBV genotype C and 1 with genotype B. Based on the presence of a lysine (K) at S region codon 122 and an arginine (R) at codon 160, all seven of genotype C were associated with subtype adr, based on K at both codon 122 and codon 160, genotype B with adw in our study.

Table 2
Clinical and laboratory data of patients with acute hepatitis B

Patient	Age	Sex	Clinical diagnosis	Time after onset (M)	AST	ALT	HBsAg	Anti-HBs	HBV DNA
1	26	F	SAH	0.1	1802	4026	+	−	+
				6	12	6	−	+	+
				12	10	6	−	+	+
2	25	F	SLAH	0.5	380	947	+	−	+
				6	19	14	−	+	+
				12	17	11	−	+	+
3	40	F	SLAH	0.2	292	784	+	−	+
				6	NT	NT	NT	NT	NT
				12	11	12	−	−	+
4	29	M	SLAH	0.2	965	2849	−	−	+
				6	11	18	−	+	−
				43	19	17	−	+	+
5	46	F	SLAH	0.2	750	1573	+	−	+
				6	16	13	−	+	+
				12	14	12	−	+	−
6	24	F	SAH	0.03	792	3124	+	−	+
				6	10	6	−	+	+
				12	11	6	−	+	−
7	64	M	SLAH	0	69	91	+	−	+
				6	13	7	−	+	+
				12	13	9	−	+	−
8	25	M	SAH	0.1	810	2507	+	−	+
				6	23	17	−	+	+
				12	24	15	−	+	−
9	75	F	SAH	0.07	438	1277	+	−	+
				5	31	29	−	+	+
				12	37	41	−	+	−
10	24	F	SLAH	0.07	666	1552	+	−	+
				6	20	13	−	+	+
				12	20	14	−	+	−
11	71	M	SLAH	3	101	181	−	−	+
				5	44	27	−	+	−
				12	15	11	−	+	−
12	30	M	SLAH	0.2	130	730	+	−	+
				6	9	12	−	−	−
				12	10	10	−	−	−
13	60	F	SLAH	0.1	123	428	+	−	+
				6	23	29	−	+	+
14	32	F	SLAH	0.1	768	1287	+	−	+
				6	13	8	−	+	+
15	41	M	SLAH	0.1	539	1082	+	−	+
				6	14	15	−	+	−
16	23	F	SAH	0.2	123	435	+	−	+
				6	9	7	−	−	+
17	30	M	SLAH	0.5	402	943	+	−	+
				6	17	38	−	−	+
18	37	M	SLAH	0.1	106	476	+	−	+
				6	26	46	−	+	−
19	33	F	SLAH	0.1	1404	2611	+	−	+
				6	16	11	−	+	+

SAH, severe acute hepatitis; SLAH, self-limited acute hepatitis; NT, not tested.

Table 3
Proportion of results of laboratory tests in patients with acute hepatitis B during follow-up

	Onset	6 M	≧12 M
ALT normal	0 (0/19)	100 (18/18)	100 (12/12)
HBsAg positive	89 (17/19)	0 (0/18)	0 (0/12)
HBsAb positive	0 (0/19)	78 (14/18)	83 (10/12)
HBV DNA positive	100 (19/19)	72 (13/18)	33 (4/12)

3.3. Precore mutation and core promoter mutation

All patients, irrespective of who were infected with either HBV genotype C or genotype B, had T1858 in this study (Table 4). Among the patients, two had G to A mutation at nt 1896, three had double A to T mutation at nt 1762 and G to A mutation at nt 1764, one had only G to A mutation at nt 1764. Of the five patients who had severe acute hepatitis B, and treated with corticosteroid, two (40%) had precore mutation, three (60%) had core promoter mutation. Of the other 14 patients who had self-limited acute hepatitis B, none had either precore mutation or core promoter mutation. Interestingly, case 19, who had only nt 1764 mutation, had low PT (46%), albeit disagreed with the diagnosis of severe hepatitis. The results showed that the proportion of the core promoter mutation in severe acute hepatitis B (60%) was significantly higher than in self-limited acute hepatitis B (0%; $P < 0.01$). The proportion of the precore mutation in severe acute hepatitis B (40%) was higher than in self-limited acute hepatitis B (0%) although insignificantly. Among four patients with viremia, one (25%) had precore mutation and core promoter mutation, among eight patients without viremia, two (25%) had precore mutation and/or core promoter mutation. There are no differences of the proportions both in the precore mutation and in the core promoter mutation between group with viremia and group without viremia.

3.4. PreS sequences before and after HBs-seroconversion

Four patients with detectable HBV DNA more than one year were performed to compare in preS sequences before and after HBs-seroconversion. Sequence analysis revealed no differences in preS1 and preS2 regions before and after HBs-seroconversion in three patients with persistent HBV DNA, except one patient (case1) whose HBV preS region after seroconversion to anti-HBs was not able to be amplified by nested PCR.

Patient	Geno-types	Sub-types	Amino Acid Sequence of the PreS2 Region 1 10 20 30 40 50 55
1	C	adr	MQWNSTTFHQALLDPRVRGLYLPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
2	C	adr	MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
3	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
4	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
5	C	adr	MQWNSTTFHHALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
6	C	adr	MQWNSTTFRQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
7	B	adw	MQWNSTTFHQTLQDPRVRALYFPAGGSSSGTVSPAQNTVSAILSILSKTGDPVPN
8	C	adr	MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
9	C	adr	MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
10	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
11	C	adr	MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
12	C		MQWNSTTFHQTLLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
13	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFLRTGDPAPN
14	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
15	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
16	C		MQWNSTTFHQALLDPRVRGLSFPAGGSSSGTVNPVPTTASPISTIFSRPGDPAPN
17	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
18	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN
19	C		MQWNSTTFHQALLDPRVRGLYFPAGGSSSGTVNPVPTTASPISSIFSRTGDPAPN

Fig. 1. Amino acid sequences of the preS2 region products of distinct genotypes and subtypes of the patients with acute hepatitis B. The determinant amino acids of genotypes were highlighted.

Table 4
HBV genotype and precore and core promoter mutation of patients with acute hepatitis B

Patient	Genotype	Precore nt		Core promoter nt		Max. ALT (U/dl)	Max. TB (mg/dl)	PT (%)	CS therapy	HBV DNA	
		1858	1896	1762	1764					6M	≧12 M
1	C	T	A	T	A	4026	6.2	31	+	+	+
2	C	T	G	A	G	1139	2	77.8	−	+	+
3	C	T	G	A	G	797	7.4	98	−	NT	+
4	C	T	G	A	G	2990	1.8	67	−	−	+
5	C	T	G	A	G	2904	6.1	100	−	+	−
6	C	T	A	T	A	3124	15.7	22	+	+	−
7	B	T	G	A	G	1430	1.6	80	−	+	−
8	C	T	G	A	G	3265	7.8	24.5	+	+	−
9	C	T	G	T	A	1277	6	26	+	+	−
10	C	T	G	A	G	1552	1.7	94.8	−	+	−
11	C	T	G	A	G	209	0.9	96	−	−	−
12	C	T	G	A	G	730	4.5	88	−	−	−
13	C	T	G	A	G	558	0.4	70	−	+	NT
14	C	T	G	A	G	1287	0.9	68	−	+	NT
15	C	T	G	A	G	1134	20.9	76	−	−	NT
16	C	T	G	A	G	563	19.2	29	+	+	NT
17	C	T	G	A	G	1261	1.8	95	−	+	NT
18	C	T	G	A	G	476	3.6	92	−	−	NT
19	C	T	G	A	A	2611	3.3	46	−	+	NT

Max. ALT, maximal alanine transaminase during acute phase; Max. TB, maximal total bilirubin during acute phase; PT, prothrombin time; CS, corticosteroid; NT, not tested.

4. Discussion

Seroconversion to anti-HBs or the loss of HBsAg has been considered to be associated with complete elimination of the replicative HBV. However, this study showed that HBV DNA might persist in serum for more than one year after complete clinical and serological recovery from acute viral hepatitis, it was consistent with previous reports [7–9]. HBV must be replicating in the body, because HBV persistent in the blood for a long time. It has been reported that HBV was detected in PBMCs and liver decades after acute viral hepatitis [27], the reverse-transcribed HBV RNA and the covalently closed circular (ccc) HBV DNA, the template for the viral RNA transcription, were also detected in PBMCs or liver [28]. These suggest that, on occasion, HBV may not be in a latent state but undergoing low-level replication.

The rate of HBV DNA persistence by ELMA kit (sensitivity 100 copies/ml) in our study is 33%. Michalak et al. [7] reported that four (80%) of the five patients in Canada were detected HBV DNA in serum by nested PCR (approximate sensitivity 10 to ~100 copies/ml) after recovery from acute hepatitis Yotsuyanagi et al. [9] reported that ten (91%) of 11 patients were positive for HBV DNA in serum by PCR coupled with southern blotting (sensitivity 10 copies/ml). The discrepancy between our results and theirs might be due to the different sensitivity of HBV DNA assays. It is believed that more people in our study would have detectable HBV DNA in serum after serological recovery from acute hepatitis alone with improvement of sensitivity of HBV DNA assay.

In view of the difference in the rate of development of chronic hepatitis from acute hepatitis B in adults between Japanese and Western patients, the rate of HBV DNA persistence may be associated with the viral genetic variations. However, there are very little data on the genotypes associated with HBV DNA persistence after acute HBV infection. Four patients with persistent viremia reported by Michalak et al. [7] were infected with the adw subtype of HBV which is the most common subtype in North America. There are some correlations between subtypes and genotypes, although no specific subtype corresponds to each genotype. In general, genotype A and B were associated with subtype adw, genotype C with adr, and genotype D with ayw [26]. According to HBV genotype with distinct geographical distribution, genotype A is predominant in North America. Hence, we could infer that the four patients were infected with HBV genotype A. While in our study, the patients were mostly infected with HBV genotype C. The variation of HBV genotypes may influence the rate of HBV persistence after acute HBV infection. Lo et al. [29] reported that the prevalence of HBV DNA by nested PCR in liver tissues in HBsAg negative patients showed geographical variation. Two of 18 (11%) of Italian samples and 2/29 (6.9%) of Hong Kong samples were positive for HBV DNA while none of the 70 cases from the United Kingdom was positive. The different prevalence may be contributed to the viral genetic variations.

Our result confirmed that the core promoter mutation and precore mutation are associated with the severity of acute hepatitis. Analysis of HBV core promoter and precore mutation (especially core promoter mutation) may be useful to predict the clinical outcome of live disease in patients with acute HBV infection [19]. The mutation in precore region, a G to A change at nt 1896, which creates a premature stop codon, prevents the translation of the precore protein and abolishes the production of hepatitis B e antigen (HBeAg). The mutation in core promoter region, A to T change at nt 1762 and G to A change at nt 1764, decreases transcription of precore mRNA and secretion of HBeAg. These mutations result in a loss HBeAg and seroconversion to antibody against HBeAg (anti-HBe) with persistence of HBV replication. They may not be associated with HBV persistence after serological recovery from acute hepatitis B, because the proportions of the mutations were low in viral persistent patients in our study.

Mutations in the gene coding for HBsAg may result in infection or viral persistence despite the presence of anti-HBs ('vaccine escape' or 'immune escape') [25]. A recent investigation showed the all HBsAg-negative cases, who were hemodialysis patients and dialysis-unit staff members with

resolved acute hepatitis B, were infected by a mixture of the wild-type virus and a deletion (amino acids 58–118) mutant in the preS1 region [28]. The lack of detection of HBsAg in the presence of low viral levels of replication might be caused by the existence of viral genomes harboring deletions in the preS1 region that affect the S promoter, and produce a reduction of the HBsAg synthesis. However, our study before and after HBs-seroconversion revealed no mutation in preS1and preS2 region in three patients with viral persistence more than 1 year.

The pathogenic significance of low-level viral persistence in the patients, who lost HBsAg or developed anti-HBs, is not clear. In Lo et al.'s study, one HBV DNA positive case had idiopathic chronic active hepatitis, but the other three HBV DNA positive cases did not suggest any aetiological connection between HBV DNA positivity and liver pathology [29]. A study was made to follow up patients with unresolved HBV infection, 25–27 years after the acute infection in Sweden. None of the 100 patients with acute HBV infection who were traced had become chronic carriers. Genotyping of the HBV strains showed that genotype D was the most prevalent [30]. However, some investigations indicated that the persistence of HBV DNA might play an important role in hepatocarcinogenesis in some cases even after serological recovery [31,32]. It is also important to determine whether HBV exists in a free state or immunoglobulin-bound form in the course of acute hepatitis B in the discussion of infectivity, such examination was not done in this study. But there were some documents which reported that immunoglobulin-bound HBV persists as immune complex in the recovery phase [7,9]. After seroconversion to anti-HBs, HBV remaining in circulation is believed to be unlikely to be infectious. The further investigations are necessary to clarify whether the low-level virus has the pathogenic significance.

In conclusion, we demonstrated that HBV DNA may persist in the serum for more than one year after complete clinical and serological recovery from acute hepatitis B. The rate of HBV persistence might be associated with the sensitivity of HBV DNA assay and the variation of HBV genotypes. HBV precore mutation and core promoter mutation are related to the severity of liver disease in patients with acute HBV infection, but no impact on HBV persistence in our study.

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Impact of Aging on the Development of Hepatocellular Carcinoma in Patients with Posttransfusion Chronic Hepatitis C

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BACKGROUND. Hepatitis C virus (HCV) infection is a heterogeneous disease, the natural history of which remains controversial. There is solid evidence that chronic HCV infection is responsible for the occurrence of hepatocellular carcinoma (HCC). The aim of the current cohort study was to determine the rate of the development of HCC from the time of primary HCV infection and to assess the risk factors for the development of HCC in chronic posttransfusion hepatitis C patients.
METHODS. Four hundred sixty-nine patients with clinically compensated HCV, who had undergone a single blood transfusion comprised the current study cohort. Patients with other risk factors for chronic liver disease were excluded. All patients were referred to the liver center at the National Nagasaki Medical Center between December 1980 and December 1998 and were followed prospectively until the end of the analysis (June 2000).
RESULTS. Follow-up data were obtained for 445 patients. The mean duration from HCV infection to the end of the observation was 28 years. Fifty-two patients (11.1%) progressed to HCC. The mean duration from the time of blood transfusion to the diagnosis of HCC was 31 years. Multivariate Cox regression analyses revealed age, fibrosis, duration from HCV infection to study entry, and alcohol consumption to be the independent factors affecting the development of HCC. The risk of developing HCC in patients age $\geq$ 56 years was increased 7.8-fold compared with that in patients age $<$ 56 years. The mean age of patients at the time of HCC diagnosis was 65 years (range, 58–79 years).
CONCLUSIONS. At the time of diagnosis, 92% of the 52 HCC patients were age $>$ 60 years and 38 of the HCC patients (73%) were in their 60s. There was a significantly negative correlation between the duration from HCV infection to the development of HCC and the age of the patient at the time of infection (correlation coefficient = 0.702; $P < 0.0001$; $Y = 61.1-0.82X$), indicating that the age of patients, rather than the duration of HCV infection, is more significant for HCC development in patients with posttransfusion HCV. Moreover, these data may contribute to the design of an optimal follow-up schedule for patients with posttransfusion HCV.
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KEYWORDS: age, chronic hepatitis C virus (HCV), blood transfusion, hepatocellular carcinoma (HCC).

Chronic hepatitis C virus (HCV) infection affects approximately 3% of the world population. There is solid evidence from epidemiologic data that in a large proportion of patients, chronic HCV infection can lead to end-stage liver disease, such as cirrhosis and hepatocellular carcinoma (HCC). However, the exact percentage of infected

individuals who develop these serious complications remains controversial and to our knowledge has not yet been defined precisely.[1,2]

Some authors emphasize the apparently low rate of clinically significant liver disease due to posttransfusion hepatitis C, whereas others focus on the alarming number of patients with end-stage liver disease.[2] This apparent paradox has been only partly resolved. In the patients with posttransfusion hepatitis C, sustained clearance of serum HCV-RNA was observed in 15–20% of patients.[1,3] Among those patients who go on to develop chronicity, the majority remain asymptomatic for years. Some of the patients with chronic hepatitis C were found to have compensated cirrhosis.[1,2] Other patients with chronic HCV infection appear to have persistently normal serum transaminase levels.[4] It is important to prognosticate accurately any given patient's likelihood of developing clinically serious or fatal complications, such as cirrhosis and HCC, for the screening and treatment of these potentially devastating diseases. Several studies have attempted to reveal prognostic factors for this diverse spectrum of diseases. However, it generally is difficult to study this aspect of the prognosis of chronic hepatitis C, because the interval between HCV infection and the development of significant liver disease is very long. Moreover, to our knowledge, the majority of previous studies of the natural history of chronic hepatitis C faced a major limitation, namely inadequate information regarding the duration of infection.[5,6] To overcome this limitation, we studied 469 patients with chronic HCV who received a single blood transfusion by which the infection likely was acquired.

The objective of the current study was to identify predictive risk factors for HCC and to assess determinants of HCC development in patients with posttransfusion hepatitis C in whom the date of blood transfusion was documented, and to examine the effects of the duration of infection and patient age at the time of infection.

MATERIALS AND METHODS

Study Population

The study cohort was comprised of 469 consecutive patients with clinically compensated chronic hepatitis C referred to the liver center at the National Nagasaki Medical Center between December 1980 and December 1998 and followed until the end of the analysis (June 2000).

As previously reported,[3,7] the entry into the study was defined as the time of diagnosis of clinically compensated chronic hepatitis C. Initially, 499 patients who fulfilled the following inclusion criteria were enrolled: 1) positive serologic test for anti-HCV by a second-generation enzyme-linked immunoadsorbent assay that was performed on frozen samples for cases diagnosed before 1992; 2) positive results by polymerase chain reaction (PCR) for HCV-RNA, 3) existence of a documented date of blood transfusion; and 4) 2 years after a blood transfusion.

Thirty patients were excluded from the current study because of multiple blood transfusions (3 patients), an episode of the liver dysfunction before the blood transfusion (11 patients), history of hemophilia (1 patient), intravenous drug abuse (4 patients), history of acupuncture therapy (6 patients), possibility of other liver diseases such as hepatitis B-associated liver diseases (2 patients), autoimmune hepatitis (1 patient), hemochromatosis (1 patient), and the coexistence of HCC (1 patient).

Written informed consent was obtained from each patient.

Histologic Examination

Four hundred thirty-six patients (93%) underwent either peritoneoscopy and/or ultrasonography (US) guided liver biopsy at the time of study entry. All the liver tissue specimens were obtained by needle biopsy and evaluated by one pathologist (O.I.) who was unaware of the patients' clinical condition. The degree of fibrosis and the intensity of inflammatory activity were graded according to the scoring system described by Desmet et al.[8] with slight modification: F0, F1, F2, F3, and F4 were defined as none or minimal fibrosis, fibrous portal expansion, bridging fibrosis (portal-portal or portal-central linkage), bridging fibrosis with lobular distortion, and cirrhosis, respectively. F0, F1, and F2 were defined as "mild fibrosis" and F3 and F4 were termed "severe fibrosis" in the current report.

Alcohol Exposure

Past alcohol exposure was estimated based on the information in the case record concerning drinking patterns over a period of > 5 years. This was supplemented by a prospective survey. The cases were categorized into two groups ("excessive" and "nonexcessive" alcohol consumer) according to the amount of alcohol intake; excessive alcohol consumers had an alcohol consumption of > 50 g/day for 5 years during the time the patient was infected with HCV.

Laboratory Tests for Liver Disease and Virologic Markers

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, bilirubin concentrations, platelets, and prothrombin time were determined at the time of the initial assessment using automated procedures in the clinical pathologic lab-

oratories of the Nagasaki Chuo National Hospital. HCV-RNA was detected by reverse transcriptase-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic System, Basel, Switzerland). The sera that were positive for HCV-RNA were further subjected to a branched DNA signal amplification assay (Quantiplex HCV-RAN; Chiron Corporation, Emeryville, CA) for determination of the amount of HCV-RNA. The detection limit of the assay was 0.5×10^6 genome mega-equivalents (Meq)/mL.

Variables at Study Entry

Various characteristics of the 469 patients were recorded at baseline assessment and included gender; age at entry; age at HCV infection; duration of HCV infection at the time of presentation; past alcohol intake; serum ALT, AST, alkaline phosphatase, and bilirubin concentrations; platelet count; prothrombin time; and the HCV-RNA titer. The prognostic value of interferon treatment also was analyzed. Data were collected in a standardized program and stored in a relational database.

IFN Treatment

IFN therapy was initiated 1–12 months after liver biopsy, and each patient was followed for at least 48 weeks after the completion of IFN therapy. A sustained responder (SR) was defined as a patient who demonstrated negative serum HCV-RNA findings on PCR and normal ATL levels for > 48 weeks after the completion of IFN therapy. A nonresponder (NR) was defined as a patient who demonstrated any other response.

Methods of Follow-Up

All patients were followed at least every 6 months by medical examinations at the outpatient clinic of the study institution or related private hepatology clinics. Imaging diagnosis was performed by US approximately every 6 months for each patient. When the disease was found to have developed into cirrhosis by peritoneoscopy or biopsy, frequent imaging by US and computed tomography (CT) was performed.

Assessment of Outcome

Outcome data were used to assess the development of and time to onset of HCC and death.

HCC

The diagnosis of HCC was based on elevated α-fetoprotein level or abnormal abdominal imaging studies. One or more hepatic spaces occupying lesions observed by US or CT were demonstrated to have vascular patterns typical of HCC by angiography, dual-phase spiral CT, or magnetic resonance imaging. Alternatively, pathologic or histologic examination was performed using liver tissue obtained by fine-needle aspiration or at autopsy. Follow-up was terminated at the time of HCC development, death, or as of June 2000.

Cause of death

The time and cause of death were recorded from the death certificate and medical records or from information obtained from related private hepatology clinics.

Lost to follow-up

When a patient had not been monitored at the study institution or related private hepatology clinics for > 1 year, the patient was considered to be lost to follow-up.

Statistical Analysis

The data were analyzed statistically using the chi-square test and the Student *t* test. The rate of the appearance of HCC was analyzed using the Kaplan–Meier method, and differences in curves were tested using the log-rank test. Univariate and multivariate Cox regression models were used to calculate the hazard ratios and their 95% confidence intervals (95% CIs) for factors potentially associated with HCC. The 15 factors examined included gender, age at infection > 30 years, serum ALT > 50 (IU/L), serum AST > 40 (IU/L), alkaline phosphatase > 87 (IU/L), prothrombin time < 80%, albumin < 3.4 (g/dL), HCV-RNA titer > 1.0 (Meq/L), serum bilirubin > 1.0 (mg/dL), platelet count < 100 (10^3/L), excessive alcohol consumption, lack of IFN treatment, duration from infection to study entry of ≥ 26 years, severe fibrosis, and age at study entry ≥ 56 years. The correlation between the patient age at the time of HCV infection and the duration between HCV infection to HCC development was evaluated by the Pearson correlation coefficient.

RESULTS

Clinical Features of the Patients

Clinical features of the patients at the time of presentation (i.e., at entry to the current study) are shown in Table 1. There were 227 male and 242 female patients, with a mean age of 54.7 years and a mean infection period of 28.1 years (range, 2.5–57.4 years). The median age at the time of infection through blood transfusion was 30.0 years. Biopsy samples were obtained from 436 patients within 6 months. Histologic staging of F0 or F1, F2, F3, and F4 were noted in 144 patients (33%), 87 patients (20.0%), 69 patients (15.8%), and 136 patients (31.2%), respectively, and the mean score

TABLE 1
Clinical Characteristics of 469 Patients with Chronic Hepatitis C

Variable	All patients (n = 469)
Age at entry (yrs)	54.7 (12.2)*
Gender (M/F)	227/242
Age at infection (yrs)	30 (birth–76)
Serum ALT (IU/L)	50 (9–366)
Serum AST (IU/L)	61 (10–491)
Alkaline phosphatase (IU/L)	183 (62–1152)
Prothrombin time (%)	81 (12)*
Serum bilirubin (per mg/dL)	0.7 (0.2–3.2)
Platelets (10^5/L)	132 (63)*
HCV-RNA titer (Meq/mL)	1.4 (0–24)
Fibrosis score	2.4 (1.2)*

HCV: hepatitis C virus; M: male; F: female; ALT: alanine aminotransferase; AST: aspartate aminotransferase.
Data are expressed as the median (range) or * as the mean (standard deviation).

TABLE 2
Outcomes of 469 Patients with Chronic Hepatitis C

Outcome	No. of cases (%)	Follow-up (yrs)
Alive	414 (88.3)	5.6 (3.1)*
Lost to follow-up	24 (5.1)	0.7 (0.2–0.9)
Died nonliver-related	4 (0.9)	6.8 (3–12)
Died liver-related	27 (5.8)	8.6 (1.1–12)
HCC development	52 (11.0)	7.7 (4.7)*

HCC: hepatocellular carcinoma.
Follow-up data are expressed as the median (range) or * the mean (standard deviation).

of fibrosis was 2.4. There were 41 excessive alcohol consumers and 145 patients who received IFN therapy during the follow-up period.

Long-Term Outcomes

Follow-up data were obtained for 445 patients (94.9%) (Table 2). Twenty-four patients (5.1%) were lost to follow-up and 27 (5.8) died of liver-related complications. During an average of 5.6 years (range, 1–20 years) under the current study observation, 52 patients (11.0%) had their disease progress to HCC. Of these 52 HCC patients, 22 died (42.3%).

Cumulative Probability and Predictive Risk Factors for the Development of HCC.

Figure 1 shows the cumulative probability of HCC development, which was 5.2%, 18%, 37%, and 46%, respectively, at 5 years, 10 years, 15 years, and 20 years. The study population was classified into 2 groups according to the median patient age at the time of infection, with those who were age $\geq$ 30 years defined as "older" and those age $<$ 30 years old defined as "younger." In the younger group (n = 243), 21 patients (8.6%) developed HCC, whereas in the older group (n = 226), 31 patients (13.7%) developed HCC. The cumulative progression rates as determined by the Kaplan–Meier method and the probability of HCC did not differ significantly between the 2 groups using the log-rank test (P = 0.12).

With regard to patient age at study entry, we divided the study population into 2 groups according to the median age, with those who were age $\geq$ 56 years defined as "older" and those age $<$ 56 years defined as "younger." In the younger group (n = 224), 9 patients (4.0%) developed HCC, whereas 43 patients in the older group (n = 245) developed HCC (17.6%). The cumulative incidence of HCC was based on the patient age at study entry. The cumulative incidence of HCC in the older group was significantly higher than that in the younger group ($P < 0.0001$).

With regard to fibrosis, the relations between the cumulative incidence of HCC and the histologic staging was examined. The cumulative incidence of HCC in 231 patients with severe fibrosis was found to be significantly higher than that in 205 patients with mild fibrosis ($P < 0.0001$).

Effect of IFN Therapy on the Development of HCC

One hundred forty-five patients received IFN therapy during the follow-up period. Forty-two of these patients (29%) were SRs. One hundred three patients (71%) were NRs. Two SRs and two NRs developed HCC.

Independent Predictors of the Development of HCC

The independent predictors of the development of HCC are summarized in Table 3. Cox proportional hazards regression analysis was performed to determine the factors that affected the development of HCC. According to univariate analysis, 7 of 15 factors (serum bilirubin $>$ 1.0 mg/dL, platelets $<$ 100 [10/L], excessive alcohol consumption, lack of IFN treatment, duration from infection to entry $\geq$ 26 years, severe fibrosis, and age at presentation $\geq$ 56 years) significantly affected the incidence of HCC. However, other factors (gender, age at HCV infection, serum ALT, serum AST, alkaline phosphatase, prothrombin time, albumin, and HCV-RNA titer at study entry) did not appear to affect the development of HCC.

Multivariate Cox regression analyses were performed on the seven significant variables in the model because of the possibility that the variables were correlated mutually. Of these seven variables, four factors (alcohol consumption, duration from HCV infection to entry, fibrosis, and age at study entry) were found to be associated independently with HCC development. The risk of developing HCC in excessive consumers of

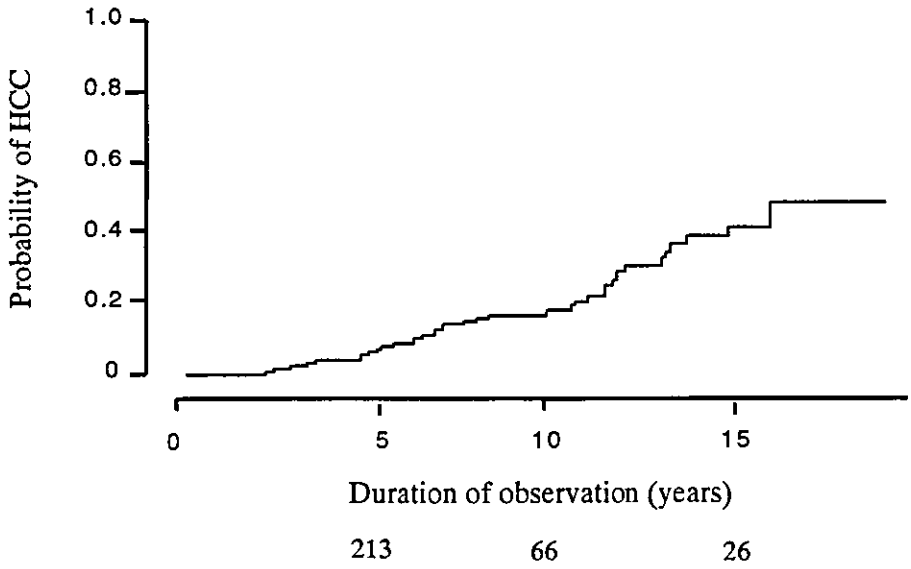

FIGURE 1. Cumulative probability of developing hepatocellular carcinoma (HCC). Number of patients = 469.

TABLE 3
Univariate and Multivariate Analyses of Prognostic Factors for HCC Occurrence

		Univariate analysis		Multivariate analysis	
Variable		*P* value	RR (95% CI)	*P* value	RR (95% CI)
Serum bilirubin (mg/dL)	≤ 1.0				
	> 1.0	0.004	2.21 (1.27–3.86)	0.15	1.59 (0.83–3.26)
Platelets (10^3/L)	> 100				
	≤ 100	< 0.0001	3.30 (1.83–5.94)	0.99	0.99 (0.48–2.04)
Alcohol consumption	Not excessive				
	Excessive	0.008	2.66 (1.28–5.53)	0.04	2.21 (1.00–3.58)
IFN treatment	Yes				
	No	0.003	4.02 (1.89–8.57)	0.06	2.48 (0.93–4.85)
Duration from infection to presentation (yrs)	< 26				
	≥ 26	< 0.0001	8.92 (4.49–17.7)	0.001	4.56 (1.29–6.26)
Fibrosis	Mild				
	Severe	< 0.0001	8.17 (3.48–19.2)	0.001	4.38 (1.72–11.1)
Age at presentation (yrs)	< 56				
	≥ 56	< 0.0001	12.3 (5.68–26.9)	< 0.0001	7.84 (3.26–18.8)

HCC: hepatocellular carcinoma; RR: relative risk; 95% CI: 95% confidence interval; IFN: interferon.

alcohol was 2.21-fold higher than that in nonexcessive alcohol consumers. With regard to the duration of infection, the risk of HCC in patients with a ≥ 26-year duration of HCV infection at study entry was increased 4.56-fold compared with that in patients with a duration of HCV infection < 26 years. The risk of developing HCC in patients with severe fibrosis was 4.38-hold higher than that in patients with mild fibrosis. The risk of developing HCC in patients age ≥ 56 years at the time of study entry was increased 7.84-fold compared with the patients age < 56 years.

Characteristics of HCC Patients

Of the 52 HCC patients, 92% were age ≥ 60 years at the time of diagnosis. The mean age of the patients at the time of HCC diagnosis was 65 years (standard deviation of 4.8 years; range, 58–79 years). Thirty-eight of the 52 HCC patients (73%) were in their 60s and only 4 patients were age < 60 years at the time of diagnosis (Fig. 2).

Figure 3 shows the relations between the period from blood transfusion to the diagnosis of HCC and the age of the patient at the time of blood transfusion.

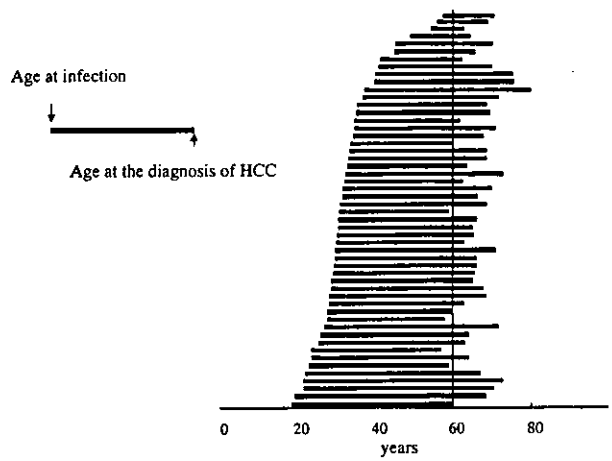

FIGURE 2. The duration between patient age at hepatitis C virus infection and age at diagnosis of hepatocellular carcinoma (HCC) in 52 patients.

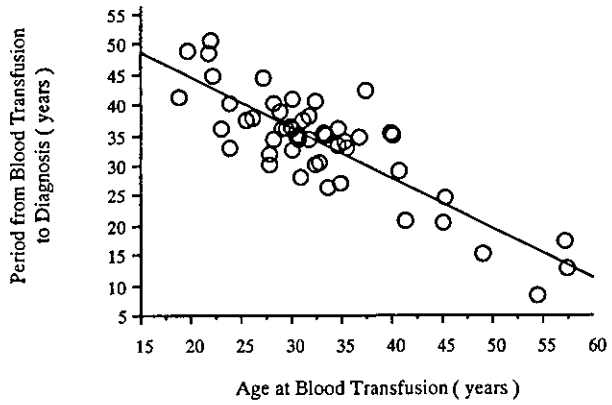

FIGURE 3. The relation between the period from blood transfusion to the diagnosis of hepatocellular carcinoma (HCC) and the age of the patient at the time of blood transfusion. A significant negative correlation between the period between blood transfusion and the diagnosis of HCC and the patient age at blood transfusion was observed (correlation coefficient = 0.702; $P < 0.0001$; Y = 61.1–0.82X).

There was a significantly negative correlation between the period from blood transfusion to the diagnosis of HCC and the age of the patient at the time of the blood transfusion (correlation coefficient = 0.702; $P < 0.0001$; Y = 61.1–0.82X).

DISCUSSION

In the current study, we examined the development of HCC in patients with chronic hepatitis C who had received a blood transfusion in Japan. This study allowed the accurate calculation of timing to various outcomes of hepatitis C, including HCC. The interval between HCV infection and the development of significant liver disease is reported to be very long.[1] Kiyosawa et al. reported that the mean duration between blood transfusion and the diagnosis of HCC was approximately 29 years (standard deviation of 13 years) (n = 21),[5] and Tong et al. reported it to be 28 years (standard deviation of 11 years) (n = 14).[9] This duration has been confirmed further by several investigators in other countries.[1] Our data, which indicated a duration of 31 years (standard deviation of 8 years) (n = 52) between a blood transfusion and the appearance of clinical HCC, are consistent with those found in previous studies.

To our knowledge, there have been a number of reports regarding the cumulative probability of HCC development in HCV-infected patients. In Japan, Ikeda et al. reported that the cumulative probability for HCC development in patients with cirrhosis was 21.5% (n = 349) at 5 years.[10] They reported in another study that the cumulative probability of HCC development in patients with chronic hepatitis C (excluding cirrhosis) was 4.8% at 5 years (n = 1500),[11] whereas Takano et al. reported a probability of approximately 4% (n = 124) in patients with chronic hepatitis C at 5 years,[12] and Aizawa et al. reported a probability of approximately 9% (n = 153) at 5 years in patients with chronic HCV or cirrhosis.[13] In the U.S., Hu et al. reported that the cumulative probability of HCC development in patients with compensated cirrhosis was 10% at 5 years (n = 112).[14] In France, Serfaty et al. reported that the cumulative probability of HCC development in patients with compensated cirrhosis was 11.5% at 4 years (n = 103).[6] The current study data (n = 469) demonstrated a probability of 5.2% at 5 years in all patients. We found the cumulative probability of HCC development to be 3.2% (n = 300) in patients with chronic hepatitis C (F0, F1, F2, and F3) and 10% (n = 136) in patients with cirrhosis (F4) at 5 years after liver biopsy. Our values did not differ significantly from those of other studies but were slightly lower than those found in other Japanese data. One possible reason is that our subjects all had chronic hepatitis C acquired after blood transfusion. Some authors noted that sporadic cases had more liver-related complications or reduced survival compared with cases of HCV acquired from blood transfusion.[15]

Multivariate Cox regression analyses revealed that four factors (i.e., alcohol consumption, duration from HCV infection to study entry, fibrosis, and age at study entry) were associated independently with HCC development. In the current study, IFN therapy appeared at first to reduce the risk of HCC. However, there were significant differences in age and histologic staging in the clinical backgrounds of the patients treated with IFN and those who were

not. Multivariate regression analysis revealed that IFN therapy was not an independent factor. Some multivariate regression studies demonstrated the same outcome as the current study.[13] However, IFN was reported to prevent the development of HCC even in patients in whom the therapy was not effective.[16] Further follow-up studies of IFN-treated patients are essential given that the incidence of HCC in patients with chronic HCV infection increases with its progress over the time of follow-up.

Although alcohol consumption can be classified using different criteria, we used criteria similar to those used in previous studies (i.e., that daily alcohol consumption was 50 g/day for ≥ 5 years' duration after the patient was infected with HCV).[7] The risk of developing HCC in such excessive consumers was 2.21-fold greater than that in nonexcessive consummers. There is solid evidence that excessive alcohol intake is a risk factor for the progression of liver disease caused by HCV.[17,18] The current study data appear to support that view.

Some authors have reported that the longer the duration of exposure, the more severe the liver disease. For example, Niederau et al. noted that the incidence of severe liver disease with a duration of exposure > 14 years was 17.2-fold higher than that with a duration of exposure < 5 years.[19] However, their study population (n = 838) had various etiologic factors for HCV infection in their medical history (e.g., the rate of treatment with blood products or transfusion was 32.3% and the rate of unknown etiology was 40.6%). Thus, the exact duration from the time of HCV infection to HCC development was not clear. The current study data demonstrated that the duration of exposure had a significant effect on the development of HCC.

The current study data also indicated that severe fibrosis is one of the prognostic factors for HCC development.[7,20] Using multivariate Cox regression analyses, some previous studies demonstrated that fibrosis could be a dependent factor for the development of HCC.[19,21] The previous study confirmed that conclusion.

Increasing patient age at the time of diagnosis of chronic liver disease was shown to be associated with increased histologic severity and cirrhosis in many previous studies. Multivariate analysis in those studies confirmed increasing patient age at the time of diagnosis to be an independent factor for progression from chronic hepatitis to cirrhosis[22] and the incidence of HCC.[23] However, aging was associated with histologic severity and induced carcinogenesis, and the authors did not provide clear data regarding a relation between aging and HCC development by multivariate Cox regression analyses. The results of the current study demonstrated that age at diagnosis was an independent factor for HCC development by multivariate Cox regression analyses in a cohort study. In the current study, the risk of developing HCC in the group of patients age ≥ 56 years at the time of study entry was increased 7.84-fold compared with the group of patients age < 56 years. Age was found to be the most important risk factor for the development of HCC.

The most striking finding of the current study was that the majority of patients with posttransfusion chronic HCV developed HCC after the age of 60 years regardless of when they acquired the HCV infection. Of 52 HCC patients, 92% were age > 60 years at the time of diagnosis. The mean age of patients at the time of HCC diagnosis was 65 years (standard deviation of 4.8 years; range, 58–79 years). Thirty-eight of the 52 HCC patients (73%) were in their 60s and only 4 patients were age < 60 years at the time of diagnosis (Fig. 2). Some previous studies indicated that patient age at the time of diagnosis of HCC among chronically HCV-infected patients ranged from 63–68 years.[24] Shiratori et al. reported that 70% of HCC patients who were HCV carriers (n = 48) were age > 60 years at the time of HCC diagnosis.[25] Shimauchi et al. reported that among 648 HCC patients who were HCV antibody-positive, 24% were age < 60 years, and that heavy drinking and the presence of HBV coinfection were found to be related independently to the development of HCV antibody-positive HCC at a younger age.[26] However, these reports were not prospective studies and included patients who were infected with HCV through various routes. Because the history of posttransfusion hepatitis is long, to our knowledge there are only limited numbers of reports regarding when the patients develop HCC. Kiyosawa et al. found in their prospective study that the mean interval between the blood transfusion (the presumed source of HCV infection) and the diagnosis of HCC was approximately 29 years in the 21 patients in their study.[5] In the current study, the mean age of patients at the time of HCC diagnosis was 64.4 years (standard deviation of 5.8 years; range, 53–74 years) and only 4 patients were age < 60 years. The current study data and those of Kiyosawas et al.[5] indicate that it is rare to find HCC in a patient with posttransfusion hepatitis C who is age < 60 years.

Figure 3 shows the relation between the period from blood transfusion to the diagnosis of HCC and the age of the patient at the time of blood transfusion. There was a significantly negative correlation between the period from blood transfusion to the diagnosis of HCC and the age of the patient at blood transfusion (correlation coefficient = 0.702; $P < 0.0001$; Y = 61.1–