incidence. Subjects with an elevated sTNF-R2 level experienced a HCC risk that was ~6.5 times greater than that of subjects with a lower sTNF-R2 level following adjustment for all markers (OR, 6.4; 95% CI, 2.0-20.6), whereas an association with increased HCC risk was no longer observed for sICAM-1 and sCD30 (OR, 1.3; 95% CI, 0.6-3.1 and OR, 1.0; 95% CI, 0.4-2.6, respectively). These associations were not substantially different after multivariable adjustment for alcohol consumption and HCV serotype or when restricted to those HCC cases diagnosed >2 years after their first available prediagnostic sample (data not shown).

Discussion

In this prospective, community-based study, we found that an apparent up-regulated immune response, as characterized by the studied serum immune markers, was positively associated with an increased HCV-related HCC risk. Markers of both type 1 and type 2 immune responses were shown to be elevated in prediagnostic serum samples of HCC cases compared with HCV-infected controls. The increased levels of these serum immune markers may reflect an activated immune response that could predispose individuals to more severe liver disease, ultimately resulting in the development of HCC.

In its function as a receptor for the proinflammatory cytokine TNF-α, sTNF-R2 has been shown in many studies to be directly associated with HCV-related chronic hepatitis and histologic fibrosis (27, 28, 42). Itoh et al. (43) reported high correlations between sTNF-R2 and several liver disease markers (e.g., alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transpeptidase) as well as with Knodell's histologic activity index score in HCV chronically infected subjects. It has also been suggested that, at low concentrations, as observed during chronic HCV infection (13), TNF-α preferentially binds TNF-R2 over TNF-R1 (42). Furthermore, studies in murine models have shown that the binding of TNF-R2 initiates signals for the proliferation of thymocytes and cytotoxic T cells (44). Thus, the binding of TNF-R2 by TNF-α could contribute to a persistent low-level immune response that exacerbates ongoing liver injury.

ICAMs, which are readily expressed on the surface of hepatocytes, also seem to play a major role in HCV-associated chronic inflammation and persistent liver damage. Although sICAM-1 is secreted by various cell types, circulating levels have been suggested to parallel the level of liver inflammation (31, 45-47). For example, circulating levels of sICAM-1 have been reported to increase in patients who are progressing from chronic hepatitis to cirrhosis and HCC and are strongly correlated with indices of hepatic injury, including alanine aminotransferase (48, 49). In addition, Hamazaki et al. (50) observed a strong correlation between sICAM-1 serum level and tumor size in HCC patients. In the present prospective evaluation to examine the effects of prediagnostic levels of sICAM-1 on HCV-associated HCC risk, we found that subjects with elevated levels of prediagnostic sICAM-1 experienced a greater risk of HCC compared with individuals with lower levels. This finding is consistent with our hypothesis that an activated type 1 or cell-mediated immune response during chronic HCV infection increases the risk for developing HCC.

High levels of circulating sCD30 levels were also positively associated with increased HCC risk. Patients who fail to eliminate the virus and progress to chronic HCV infection have been found to have peripheral evidence of a strong type 2 immune response (25). The present findings agree with that of Gramenzi et al. (51) who recently reported that a predominant type 2 profile was associated with more severe liver disease. However, Gramenzi et al. also reported that a shift to a type 1 cytokine profile of peripheral blood mononuclear cells was associated with a more favorable clinical outcome, which is not consistent with the present findings. In fact, the current findings suggest that, in addition to an elevated sCD30 level, elevated type 1 immune markers may also contribute to a general dysregulation of the host immune status before HCC diagnosis, which ultimately predisposes the subject to increased immunopathogenesis of the liver.

It is of interest to note that simultaneous adjustment for all three immune markers revealed that only sTNF-R2 was significantly associated with increased incidence of HCC. That sTNF-R2 is independently associated with HCC after adjusting for sICAM-1 and sCD30 may reflect the significance of the immune response that is triggered by the binding of TNF-α to sTNF-R2 (44, 52). Because elevated levels of TNF-α are found in chronic HCV infection (26), it is possible that the cytotoxic T cells recruited by the TNF-α signaling system are more important in exacerbating ongoing liver injury. Given the correlation of sICAM-1 and sCD30 with sTNF-R2, as well as the stronger association of sTNF-R2 with HCC risk, it is not surprising that the association of sICAM-1 and sCD30 with HCC incidence became closer to the null with simultaneous adjustment for all three immune markers. Alternatively, the observed association between sTNF-R2 and HCC may be attributed to the bias resulting from imprecise measurement of correlated exposures (53). In the present study, sCD30 was measured with the greatest imprecision, whereas sTNF-R2 was measured with the smallest variability.

Unique to this study is the use of prediagnostic serum samples to measure the levels of sTNF-R2, sICAM-1, and sCD30. Therefore, the possibility that the tumor caused the elevation of circulating serum immune markers is unlikely. We also excluded HCC cases diagnosed within 2 years of the serum sample tested to minimize the possibility of reverse causation and found the associations with the immune markers to be unchanged. In addition, the community-based setting of the study provides a novel perspective in determining the natural history of HCV-induced HCC. The present findings show that prediagnostic serum levels of select immune biomarkers can be useful in predicting HCC incidence within a nonpatient population.

The present study has some limitations. Although information on alcohol consumption was obtained, the lack of quantitative data for all subjects may have resulted in residual confounding (i.e., 15 subjects were missing information on alcohol consumption). Nevertheless, because alcohol consumption is reportedly inversely associated with type 1 immune markers (54, 55), any residual confounding would be expected to result in an underestimation of the true relation between elevated type 1 markers and HCC incidence. In addition, although smoking was not adjusted for due to unavailable data, confounding by smoking was presumed to be minimal; Kuper et al. (56) found a significant dose-response, positive association between smoking and HCC risk only among HCV-negative subjects and concluded that smoking was less important as a risk factor for HCC among HCV-positive subjects. It is also important to consider that the immune marker data were obtained from the peripheral blood compartment, which may only partially reflect immune events occurring within the infected liver. However, Sobue et al. (14) reported a correlation in the helper T-cell type 1 and type 2 ratio between the peripheral blood and the liver and that the immune response of peripheral blood shifted toward a type 1 cytokine profile as liver damage progressed.

In summary, the observed association between elevated serum type 1 and type 2 immune markers and HCC risk supports the hypothesis that subjects with a dysregulated immune response experience greater hepatocyte damage, including hepatocarcinogenesis, as a result of HCV-induced immunopathogenesis. The association of sTNF-R2 and HCC risk after adjustment for sICAM-1 and sCD30 suggests a greater role for an activated type 1 response, although further

study is required. The present findings also show that prediagnostic serum levels of sTNF-R2, sICAM-1, and sCD30 can be useful in predicting HCC incidence within a community-based study population. This finding needs to be confirmed in other population studies.

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Interleukin-10 or tumor necrosis factor-α polymorphisms and the natural course of hepatitis C virus infection in a hyperendemic area of Japan

Kazunori Kusumoto [a], Hirofumi Uto [a], Katsuhiro Hayashi [a], Yuka Takahama [b], Hiroyuki Nakao [c], Robert Suruki [d], Sherri O. Stuver [d,e], Akio Ido [f], Hirohito Tsubouchi [f,g,*]

[a] *Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan*
[b] *Miyazaki Prefectual Industrial Support Foundation, 16500-2 Higashikaminaka, Sadowara, Miyazaki 880-0303, Japan*
[c] *Department of Public Health, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan*
[d] *Department of Epidemiology, Harvard School of Public Health, 677 Huntington Av., Boston, MA 02115, USA*
[e] *Department of Epidemiology, Boston University School of Public Health, 715 Albany Street, T324E, Boston, MA 02118, USA*
[f] *Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-850, Japan*
[g] *Department of Digestive and Life-style related Disease, Kagoshima University Graduate School of Medical and Dental Science, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan*

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Abstract

We investigated the effects of polymorphisms in interleukin (IL)-10 and tumor necrosis factor (TNF)-α on the natural course of hepatitis C virus (HCV) infection in a community-based population in Japan. A total of 460 anti-HCV antibody seropositive individuals were classified into two groups, those who were positive or negative for HCV RNA. In HCV RNA-positive individuals with at least four annual alanine aminotransferase (ALT) measurements taken between 1993 and 2003, 74 exhibited persistently normal ALT levels, while 211 had one or more elevated ALT level tests. We examined the relationships between polymorphisms in the genes encoding IL-10 (−1082, −819, −592) or TNF-α (−308, −238) and HCV clearance, ALT abnormalities, or serum level of type IV collagen 7S, a marker of hepatic fibrosis. These polymorphisms were equally distributed among the patient subgroups with differential HCV RNA clearances or ALT abnormalities. Serum levels of type IV collagen 7S, however, were significantly higher in individuals with an A at position −238 or −308 in the TNF-α gene promoter than in individuals lacking these polymorphisms. We conclude that, while the relationships between inherited variations in IL-10 or TNF-α expression are not associated with alterations in HCV clearance or ALT levels, TNF-α polymorphisms may be associated with hepatic fibrosis.

Keywords: Interleukin-10; Tumor necrosis factor-α; Hepatitis C; Gene polymorphism; Hepatic fibrosis

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of acute and chronic hepatitis. HCV infects an estimated 170 million people worldwide, most of whom fail to clear the virus and develop chronic hepatitis. Some of those with chronic hepatitis may progresses to cirrhosis or hepatocellular carcinoma (HCC) [1]. Chronic HCV infection develops in most (75–85%) infected persons; persistently elevated or fluctuating alanine aminotransferase (ALT) levels indicate active liver disease in 60–70% of chronically infected patients. In the remaining 30–40%, ALT levels remain normal [2–7]. Although immune responses are known to play an important role in both HCV clearance

* Corresponding author. Fax: +81 992 75 0588.
E-mail address: htsubo@m2.kufm.kagoshima-u.ac.jp (H. Tsubouchi).

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and ALT abnormalities [2], host genetic factors have not been elucidated.

Interleukin (IL)-10 and tumor necrosis factor (TNF)-α play key roles in the regulation of cellular immune responses in HCV infection [8–11]. Production of these cytokines is regulated at the transcriptional, posttranscriptional, and translational levels [12–15]. In patients chronically infected with HCV, the production of inappropriate amounts of cytokines, such as IL-10 and TNF-α were reported to be associated with HCV clearance, fibrogenesis, and even resistance to interferon therapy [16–18].

A number of reports indicate that single nucleotide polymorphisms (SNPs) in the promoter regions of the IL-10 and TNF-α genes influence the spontaneous clearance of HCV infection [19–21]. Conflicting studies, however, have reported no significant associations between some SNPs and HCV clearance [22–24]. Although this discrepancy may be due to ethnic differences in the populations examined, leading to a differential distribution of IL-10 promoter genotypes, the role of these polymorphisms in HCV infection remains unclear. In addition, the association between either IL-10 or TNF-α gene polymorphisms and ALT elevation or hepatic fibrosis in patients with chronic HCV infection has not been fully evaluated.

Since 1994, a cohort study has examined the natural history of HCV infection in adult residents of a community-based population in a HCV hyperendemic area in Japan [25]. The overall rate of anti-HCV antibody positivity was 22.5% in inhabitants more than 40 years of age. All of these individuals were Japanese; movement of residents in and out of the region has been rare. This area provided an appropriate setting to investigate the effects of genetic background on HCV infection, because stratification of the population and the resulting tendency to detect false-positive associations could be minimized. In this study, we evaluated the effects of functionally relevant cytokine polymorphisms in the promoters of the IL-10 (−1082G/A, −819C/T, and −592C/A) [14] and TNF-α (−308G/A and −238G/A) [26] genes on the course of HCV infection.

2. Materials and methods

2.1. Study population

A total of 460 anti-HCV-positive residents in Town C were evaluated in this study. The Town C HCV study is a cohort study examining the natural course of HCV infection in adult residents of a community in Miyazaki Prefecture, Japan. Residents that were identified as anti-HCV-positive at general health examinations were invited to participate in annual liver disease examinations. None of this study population had received interferon therapy or was positive for hepatitis B surface antigen. These individuals were classified into the following two groups. The persistent infection group was comprised of individuals positive for anti-HCV antibodies and HCV RNA; the viral clearance group included individuals who were positive for anti-HCV antibody that had no evidence positive for HCV RNA on at least two occasions. We divided individuals with persistent HCV infections who had at least four annual ALT measurements available during 1993 and 2003 into two groups. The persistent normal ALT group included individuals who had persistently normal ALT levels (<35 IU/L), while the abnormal ALT group was composed of individuals who had ALT levels fluctuating across 35 (IU/L) during the follow-up period or elevated ALT levels (≥35 IU/L) in all measurements. Individuals who were negative for anti-HCV antibodies in Town C were evaluated as controls. Informed consent was obtained from participants at the time of enrollment. This study was approved by the human subjects committees of the University of Miyazaki, Faculty of Medicine, Japan, the Harvard School of Public Health, and the Boston University School of Public Health.

2.2. Blood examination of hepatic fibrosis markers, anti-HCV antibodies, and HCV RNA levels

ALT was examined annually between 1993 and 2003. Hyaluronic acid (HA) and type IV collagen 7S (IVcol.7S), two markers of hepatic fibrosis in chronic HCV infection, were measured by a latex bead agglutination assay (LPIA-ACE HA, Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan; normal range: ≤50 ng/ml) or radioimmunoassay (Type IV collagen · 7S kit, Mitsubishi Kagaku Iatron, Inc.; normal range: ≤6.0 ng/ml), respectively. These markers were evaluated in 2003. Anti-HCV antibody titers were measured by chemiluminescence enzyme immunoassay using a third-generation kit (Lumipulse Ortho II, Ortho-Clinical Diagnostics K. K., Tokyo) at least once between 2001 and 2003. The presence of serum HCV RNA was determined by qualitative reverse transcription polymerase chain reaction (PCR) with a cut-off for a positive result of 10 copies/mL (Amplicore HCV, Nippon Roche, Tokyo). HCV core antigen levels were measured by radio-enzyme immunoassay (Ortho HCV Ag IRMA test; Ortho-Clinical Diagnostic, K. K., Tokyo, Japan).

2.3. DNA extraction

Ten microliters of whole blood was drawn into an EDTA vacutainer by venipuncture. Within 1 h, the buffy coat was separated from the blood by centrifugation at 3000 rpm for 10 min. Genomic DNA was extracted from the buffy coat using a MagExtractor System MFX-2000 (Toyobo, Osaka, Japan), according to the manufacturer's protocols.

2.4. Real-time PCR allelic discrimination assays

PCR with sequence-specific primers was used to define the IL-10 promotor SNPs at the −1082, −819, and −592 positions as described [14]. These sites have also been cited as positions −1117, −854, and −627, respectively [27]. We also evaluated TNF-α promoter SNPs at the −308 and −238 positions [28]. The positions of these polymorphic

variations have been previously published [8,29]. Real-time PCR allelic discrimination assays were designed using TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA). Assays perform genotyping of the G → A −1082 (dbSNP ID:rs1800896, TaqMan® SNP Genotyping Assays ID: C_1747360_10) and C → A −592 (dbSNP ID:rs1800872, TaqMan® SNP Genotyping Assays ID: C_1747363_10) SNPs in the IL-10 promoter region and the G → A −308 (dbSNP ID:rs1800629, TaqMan® SNP Genotyping Assays ID: C_7514879_10) and G → A −238 (dbSNP ID:rs361525, TaqMan® SNP Genotyping Assays ID: C_2215707_10) SNPs in the TNF-α promoter region were commercially available (Applied Biosystems). Genotyping of the C → T −819 (dbSNP ID:rs1800871) SNP in the IL-10 promoter region utilized the primers, IL-10–819F (AGTAGGGTGAGGAAACCAAATTCTC) and IL-10–819R (CATGACCCCTACCGTCTCTATTTT), and the probes, IL-10–819F (ACAGAGATGTTACATCAC, labeled with the dye VIC) and IL-10–819R (CACAGAGAT ATTACATCAC, labeled with the dye FAM), in a Custom TaqMan® Genomic Assay. Briefly, 3 ng DNA was mixed with Allelic Discrimination Assay Mix (900 nM each forward and reverse primer, 200 nM each reporter dye FAM or VIC-labeled probe) and TaqMan Universal PCR Master Mix (Applied Biosystems). PCRs were carried out in a total volume of 3 μl/well in 96-well PCR plates. The PCR conditions subjected samples to 50 °C for 2 min for contamination control with AmpErase uracil *N*-glycosylase and 95 °C for 10 min to activate the AmpliTaq Gold Enzyme, followed by 40 cycles of 92 °C for 15 s for denaturation, and 60 °C for 1 min for annealing and extension. Genotypes were assessed by the TaqMan allele-specific assay method using the ABI Prism® 7000 Sequence Detection System, according to the manufacturer's protocols (Applied Biosystems). All genotypes were scored using the allelic discrimination program of ABI software.

2.5. Statistical evaluation

Results obtained for genotypes were analyzed according to etiology and severity using Pearson's^2 contingency tables and Fisher's exact test, respectively. Logistic regression analysis examined the relationship between genotype and clinical characteristics, allowing for additional variables. The differences in mean values were assessed by Mann–Whitney *U* test. All statistical analyses were performed using SPSS version 11.01 statistical analysis software (SPSS Inc., Chicago, IL). All differences were considered to be significant at *P* values less than 0.05.

3. Results

3.1. The base line characteristics of the individuals in this study (Table 1)

Table 1 details the demographic and clinical features of all cases and controls. Evidence of viral clearance, defined as the absence of detectable HCV RNA, was observed in 114 (24.8%) of 460 residents positive for anti-HCV antibodies (Table 1A). Female gender was associated with persistently normal ALT levels: 77.0% of subjects with persistently normal ALT were female in comparison to 62.6% of patients with persistently abnormal ALT ($P < 0.001$) (Table 1B). Viral group 1 was significantly associated with abnormal ALT levels; 55.4% of subjects with persistently normal ALT levels were viral group 1 in comparison to 65.0% of subjects with abnormal ALT levels ($P < 0.05$) (Table 1B).

3.2. Differential distribution of IL-10 and TNF-α alleles or genotypes and HCV clearance (Table 2)

Four hundred and sixty and 454 residents were successfully genotyped for IL-10 and TNF-α, respectively. None of the IL-10 single polymorphisms at positions −1082, −819, or −592 were differentially distributed between the persistent infection and viral clearance groups. The IL-10 promoter haplotype frequencies were also not significantly different between these two groups. The two SNPs (−308 and −238) in the TNF-α promoter were also not differently distributed among these resident subgroups. The distribution of cytokine genotypes and allelic frequencies in our blood donor control group was similar to previously published data for other Japanese populations (data not shown) (see Table 2).

Table 1A
Clinical and virological characteristics in 460 residents positive for anti-HCV antibodies and in 63 residents negative for anti-HCV antibodies

	Persistent infection $n = 346$ (%)	Viral clearance $n = 114$ (%)	Healthy Control $n = 63$ (%)
Age[a]	63.4 ± 9.6	67.9 ± 11.3	63.4 ± 9.66
Gender			
Male	126 (36.4)	36 (31.6)	16 (25.4)
Female	220 (63.6)	78 (68.4)	47 (74.6)
Viral group		NE	NE
Group 1	218 (63.0)		
Group 2	112 (32.4)		
Undetermined	16 (4.6)		
HCV core Ag (fmol/L)[a]	4879 ± 4856	NE	NE
		*	
ALT (IU/L)[a]	47.9 ± 46.9	21.4 ± 11.5	24.9 ± 16.3
		*	
Platelet ($\times 10^4$/ml)[a]	19.2 ± 6.2	23.3 ± 5.78	24.1 ± 6.6

HCV, hepatitis C virus; Ag, antigen; ALT, alanine aminotransferase; Group 1, genotype la, 1b or serotype 1; Group 2, genotype 2a, 2b or serotype 2; NE, not examined; *$P < 0.001$.
[a] Values are means ± SD.

Table 1B
Clinical and virological characteristics in individuals with persistent HCV infection who had at least four annual ALT measurements available during 1993 and 2003

	Normal ALT $n = 74$ (%)	Abnormal ALT $n = 211$ (%)
Age[a]	71.0 ± 10.3	69.7 ± 9.13
Gender		‡
Male	17 (23.0)	79 (37.4)
Female	57 (77.0)	132 (62.6)
Viral group		†
Group 1	41 (55.4)	137 (65.0)
Group 2	31 (41.9)	64 (30.3)
Undetermined	2 (2.7)	10 (4.7)
HCV core Ag (fmol/L)[a]	5032 ± 4270	4661 ± 795
		*
ALT (IU/L)[a]	21.6 ± 7.6	54.2 ± 45.1
		*
Platelet ($\times 10^4$/ml)[a]	23.7 ± 5.4	18.0 ± 5.9

HCV, hepatitis C virus; Ag, antigen; ALT, alanine aminotransferase; Group 1, genotype 1a, 1b or serotype 1; Group 2, genotype 2a, 2b or serotype 2; ‡$P < 0.05$; †$P < 0.001$; *$P < 0.001$.
[a] Values are means ± SD.

3.3. Differential distribution of IL-10 and TNF-α alleles or genotypes and ALT abnormality (Table 3)

No single polymorphism in the IL-10 gene at positions −1082, −819, or −592 was differentially distributed between the persistently normal ALT and abnormal ALT groups. Differences in the IL-10 promoter haplotype frequencies were also not statistically significant between these two groups. The two SNPs (−308 and −238) in the TNF-α promoter were not differentially distributed among the resident subgroups (see Table 3).

3.4. Association of IL-10 and TNF-α gene alleles or genotypes and a hepatic fibrosis marker in chronic HCV infection (Fig. 1)

To analyze the effect of IL-10 and TNF-α gene promoter polymorphisms on hepatic fibrosis in individuals with persistent HCV infections who had at least four annual ALT measurements available during 1993 and 2003, we examined type IVcol.7S and HA levels in individuals separated on the basis of the presence or absence of these SNPs. Serum levels of HA did not differ significantly between individuals with and without these IL-10 or TNF-α SNPs. In contrast, serum levels of type IVcol.7S were significantly higher in individuals with GA at position −238 in the TNF-α promoter than in those with GG at this position ($P = 0.013$). Serum levels of IVcol.7S were also significantly higher in individuals with an A at either position −308

Table 2
Differential distribution of IL-10 genotype, haplotype or TNF-α genotype in 346 individuals with chronic HCV infection or in 114 individuals with spontaneous clearance of HCV infection

		Persistent infection	Viral clearance	OR	95% C.I.
IL-10 genotype		$n = 346$ (%)	$n = 114$ (%)		
IL-10 (−1082)	GG	0 (0)	0 (0)		
	GA	30 (8.7)	11 (9.6)	0.889	0.430–1.837
	AA	316 (91.3)	103 (90.4)	Ref.	
IL-10 (−819)	CC	30 (87)	9 (7.9)	0.793	0.355–1.770
	CT	160 (46.2)	46 (40.4)	0.76	0.488–1.185
	TT	156 (45.1)	59 (51.7)	Ref.	
IL-10 (−592)	CC	30 (8.7)	9 (7.9)	0.793	0.355–1.770
	CA	160 (46.2)	46 (40.4)	0.76	0.488–1.185
	AA	156 (45.1)	59 (51.7)	Ref.	
IL-10 haplotype	Phenotype				
GCC/GCC	High	0 (0)	0 (0)		
GCC/ACC	Intermediate	10 (2.9)	1 (0.1)	0.266	0.034–2.113
GCC/ATA	Intermediate	20 (5.8)	10 (8.8)	1.322	0.585–2.990
ACC/ACC	Low	20 (5.8)	8 (7.2)	1.058	0.442–2.532
ACC/ATA	Low	140 (40.5)	36 (31.6)	0.680	0.434–1.091
ATA/ATA	Low	156 (45.1)	59 (51.8)	Ref.	
TNF-α genotype		$n = 346$ (%)	$n = 108$ (%)		
TNF-α (−308)	GG	336 (97.1)	105 (97.2)	Ref.	
	GA	10 (2.9)	3 (2.8)	0.960	0.259–3.553
	AA	0 (0)	0 (0)		
TNF-α (−238)	GG	339 (98.0)	106 (98.1)	Ref.	
	GA	7 (2.0)	2 (1.9)	1.066	0.212–5.360
	AA	0 (0)	0 (0)		

Table 3
Differential distribution of IL-10 genotype, haplotype or TNF-α genotype in 74 individuals with persistently normal ALT or in 211 individuals with abnormal ALT

		Persistently normal ALT	Abnormal ALT	OR	95% C.I.
IL-10 genotype		$n = 74$ (%)	$n = 211$ (%)		
IL-10 (−1082)	GG	0 (0)	0 (0)		
	GA	9 (12.2)	18 (8.5)	0.674	0.288–1.573
	AA	65 (87.8)	193 (91.5)	Ref.	
IL-I0 (−819)	CC	8 (10.8)	17 (8.1)	1.226	0.489–3.076
	CT	28 (37.8)	95 (45.0)	0.768	0.437–1.349
	TT	38 (51.4)	99 (46.9)	Ref.	
IL-10 (−592)	CC	8 (10.8)	17 (8.1)	1.226	0.489–3.076
	CA	28 (37.8)	95 (45.0)	0.768	0.437–1.349
	AA	38 (51.4)	99 (46.9)	Ref.	
1L-10 haplotype	Phenotype				
GCC/GCC	High	0 (0)	0 (0)		
GCC/ACC	Intermediate	1 (1.4)	8 (3.8)	0.327	0.040–2.694
GCC/ATA	Intermediate	8 (10.8)	10 (4.7)	2.084	0.765–5.677
ACC/ACC	Low	7 (9.5)	9 (4.3)	2.026	0.705–5.826
ACC/ATA	Low	20 (27.0)	85 (40.3)	0.613	0.332–1.133
ATA/ATA	Low	38 (51.4)	99 (46.9)	Ref.	
TNF-α genotype					
TNF-α (−308)	GG	70 (94.6)	206 (97.6)	Ref.	
	GA	4 (5.4)	5 (2.4)	0.952	0.205–4.444
	AA	0 (0)	0 (0)		
TNF-α (−238)	GG	74 (100)	205 (97.2)		
	GA	0 (0)	6 (2.8)		
	AA	0 (0)	0 (0)		

or −238 in the TNF-α promoter than in patients without these SNPs ($P = 0.004$).

4. Discussion

Host immune factors are important in the outcome of HCV infection [30]. While multiple studies have reported the association of IL-10 and TNF-α gene promoter polymorphisms with the natural course of HCV infection [8,9,16–24,31–38], these results remain controversial as many of these studies have used relatively small subject numbers in regionally disparate areas. In contrast, our study included 460 HCV patients and 63 healthy controls, all from a single area hyperendemic for HCV in Japan [25]. Thus, this is a large study capable of addressing the impact of cytokine polymorphisms on the course of HCV infection in a statistically meaningful manner. Our results indicate that there is no significant association between polymorphisms in the IL-10 (−1082, −819, and −592) or TNF-α (−308, −238) genes or cytokine haplotype and either HCV clearance or severity of hepatitis.

IL-10, whose secretion is regulated by monocytes and lymphocytes [14,39], plays an important role in the pathogenesis of HCV infection [8–11]. Three larger studies examined the relationship between IL-10 polymorphisms and viral clearance; a study of 659 subjects revealed an association of the G/G genotype at position −1082 with persistent infection [38]. Two additional studies, examining 606 [24] and 259 [37] subjects, as well as this study, could not confirm this association. An effect of IL-10 haplotype on IL-10 production has also been reported [14,40]. Mangia et al. found that the IL-10 ATA haplotype was more frequent in patients exhibiting spontaneous HCV clearance than in those with persistent infection [21]. In our study, however, there was no association of IL-10 haplotype with spontaneous HCV clearance. Although the lower frequencies of the G/G genotype or GCC haplotype in Asian populations than those seen in Caucasians may be one reason for this discrepancy [41], we do not have any evidence to believe that IL-10 genotype is associated with spontaneous HCV clearance.

TNF-α, a multifunctional proinflammatory cytokine, is an important pathogenic mediator [31,42,43]. As there are striking differences in the ability to produce cytokines between individuals with genetic polymorphisms within cytokine regulatory regions [44], it is logical to suppose that polymorphisms in the TNF-α promoter region could affect the outcomes of HCV infection. We did not detect, however, any association of TNF-α (−308, −238) polymorphisms with HCV clearance, confirming several previous reports [21–23]. Evaluation of the combined TNF-α and IL-10 genotype revealed a significantly increased proportion of an "anti-inflammatory genotype" (low-TNF-α/high-IL-10 producers) in patients whose HCV infections resolved in comparison to patients with persistent HCV infection [19,20]. As none of the subjects in our study exhibited a high-IL-10 producer phenotype, we could not fully examine the anti-inflammatory genotype (low-TNF-α/high-IL-10 producers). In addition, these results may be influenced by ethnic variations.

In patients with HCV-related chronic hepatitis associated with persistently normal ALT levels, disease grade does not increase with time; progression to cirrhosis is slow or absent [45]. Therefore, we also evaluated the association of SNPs with the severity of hepatitis by evaluating sequential ALT measurements or markers of hepatic fibrosis. As assessed by sequential ALT measurements, our study suggested that IL-10 and TNF-α polymorphisms do not contribute to ALT abnormalities, which may be associated with the severity of hepatitis. These data were similar to previous reports indicating that IL-10 and TNF-α polymorphisms did not correlate with sequentially acquired ALT levels [21,22,31–33].

Analysis of TNF-α gene genotype (−308A and −238A) in 114 HCV-infected subjects was reported to confer an increased rate of fibrosis [46]. Two additional studies, however, examining 128 [9] and 153 [47] subjects, could not confirm this association. Serum levels of IVcol.7S typically accompany the progression of hepatic fibrosis [48]. In our study, serum levels of IVcol.7S were significantly higher in individuals of the GA genotype at position −238 in the TNF-α promoter than in those with the GG genotype. We also observed higher IVcol.7S levels in individuals with GA at position −308 than in individuals with the GG genotype, nevertheless this trend was not statistically significant ($P = 0.08$). In addition, serum levels of IVcol.7S in individuals with an A at either position −238 or −308 in the TNF-α gene promoter were significantly higher than those in individuals without those SNPs (Fig. 1). Increasing serum levels of IVcol.7S with more advanced hepatic fibrosis stage, has been reported [48]. These observations, in conjunction with our analysis of 283 individuals, suggest that TNF-α polymorphisms are associated with disease progression during persistent HCV infection.

Although serum levels of HA are also markers of hepatic fibrosis and persistently high ALT levels induced hepatic fibrosis, TNF-α SNPs were not associated with either of these parameters in spite of the association between TNF-α SNPs and IVcol.7S. The production of HA increases under various inflammatory conditions or aging [49]; thus, increases in the serum levels of HA may be partially responsible for these discrepancies. In addition, these contrasting findings may result from differing definitions of the cut-offs defining ALT normalcy or the time frame for defining persistence. Further studies using a larger sample population and additional analyses, including confirmation of severe hepatic fibrosis by liver biopsy, are needed to verify our results.

Our evaluation of the relationship between inherited variations in cytokine expression and the course of chronic hepatitis C indicated that neither IL-10 nor TNF-α gene promoter polymorphisms are associated with HCV clearance or ALT abnormalities in a large-scale population in a hyperendemic area of Japan. TNF-α gene promoter polymorphisms, however, were linked to disease progression in patients with persistent HCV infection.

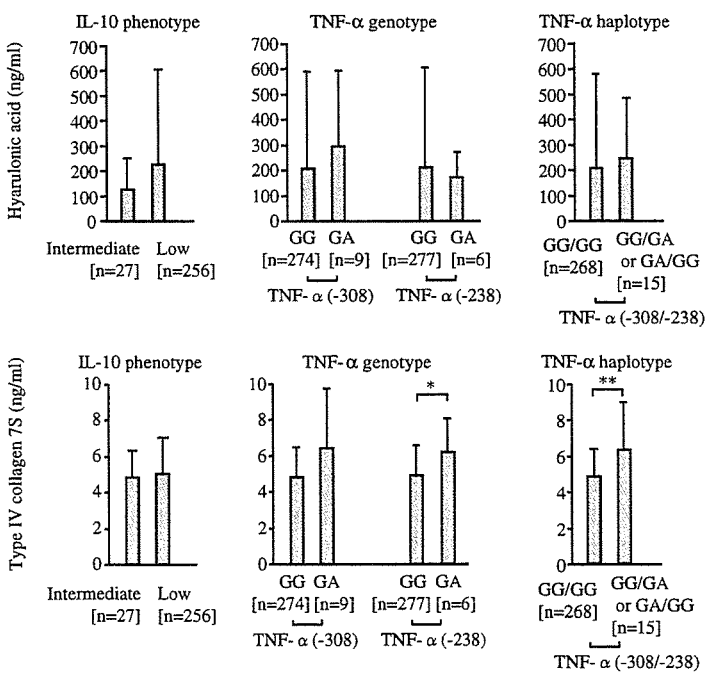

Fig. 1. Serum levels of type IV collagen 7S and hyaluronic acid in 283 individuals with persistent HCV infection who had at least four annual ALT measurements available during 1993 and 2003. Data are shown as means ± SD. Mann–Whitney U test was used for evaluating statistical significance. $^{*}P = 0.013$, $^{**}P = 0.004$.

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Alanine aminotransferase level as a predictor of hepatitis C virus-associated hepatocellular carcinoma incidence in a community-based population in Japan

Robert Suruki[1]*, Katsuhiro Hayashi[2], Kazunori Kusumoto[2], Hirofumi Uto[2], Akio Ido[2], Hirohito Tsubouchi[2] and Sherri O. Stuver[1,3]

[1]*Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA*
[2]*Department of Internal Medicine II, University of Miyazaki Faculty of Medicine, Miyazaki, Japan*
[3]*Department of Epidemiology, Boston University School of Public Health, Boston, MA, USA*

We evaluated the utility of alanine aminotransferase (ALT) measurements in predicting the incidence of hepatocellular carcinoma (HCC) in a cohort of 667 adults with chronic hepatitis C virus (HCV) infection from a community-based population in Japan, between 1994 and 2003. Cox proportional hazards regression analysis was used to describe the relationship between prediagnostic levels of ALT and the rate of HCC, after adjusting for age and gender; hazard ratios (HRs) and 95% confidence intervals (CIs) were obtained. Over an average of 8 years of follow-up, 52 HCC cases were identified. A significant association between a 20 IU/L difference in higher ALT level and subsequent HCC incidence was observed (HR = 1.2; 95% CI: 1.1, 1.3). An abnormal ALT level (≥35 IU/L) increased the HCC rate by 4-fold compared to a normal ALT level (HR = 4.1; 95% CI: 2.1, 8.0). Among 551 subjects with at least 4 repeated measurements of ALT, those with persistently abnormal ALT levels (n = 118) had a strong, significantly increased HCC rate compared to those with persistently normal ALT levels (n = 296) (HR = 23.2; 95% CI: 3.0, 178.5). This study demonstrates that elevated ALT levels, measured on an average of 8 years before HCC diagnosis, predict an increased rate of HCV-associated HCC in a community-based population and that utilizing serial measurements to identify persistent ALT abnormality may be useful in determining HCC risk.

Key words: alanine aminotransferase; chronic hepatitis C virus infection; hepatocellular carcinoma

Hepatitis C virus (HCV) infection has become a major public health concern, with ~170 million persons chronically infected worldwide.[1] Approximately 70% of those acutely infected will become persistently infected with HCV, leading to an increased risk of liver fibrosis, liver cirrhosis and development of hepatocellular carcinoma (HCC).[2–4] Furthermore, with an increasing global incidence of ~80% over the past 20–30 years, HCC has become the fifth most common cancer and ranks third with respect to cancer-related mortality.[5–7] In developed countries, including the United States and Japan, HCV infection has been identified as the main risk factor for HCC.[8–10]

Although the exact pathogenesis of HCV-associated HCC is unknown, chronic inflammation related to the immune response to HCV infection, with a resultant increased proliferation of hepatocytes, likely promotes carcinogenesis in the liver. Consequently, this process of persistent injury and regeneration creates a procarcinogenic environment in which frequent genetic mutations and/or instability are common. Recently, Tanaka *et al.* reported that an elevated serum alanine aminotransferase (ALT) level at blood donation was positively associated with subsequent HCC risk among HCV-infected donors in Japan.[7] ALT is an enzyme present in the liver, which is released into the blood stream with increasing liver tissue damage, and thus represents activated inflammatory necrosis of hepatocytes. Other studies in Japan have shown that sustained elevated ALT levels in HCV-infected liver disease patients lead to increased HCC risk and recurrence of HCC.[11,12]

Since most individuals infected with HCV remain asymptomatic, studies based on clinic patients may overestimate the true effect of ALT on HCC risk. In contrast, the community-based setting offers a unique advantage, in that it is mostly composed of infected individuals who are asymptomatic, minimizing the bias introduced by using patients who may have more severe liver disease. We conducted a prospective study to examine the utility of ALT measurements in predicting the incidence of HCC in chronically HCV-infected subjects in a community-based population in Japan.

Material and methods

Study Population

Subjects in this study were participants in a community-based cohort study conducted within the adult population of Town C in southwestern Miyazaki Prefecture, Japan; characteristics of the study population have been described previously.[13] Briefly, beginning in 1993, anti-HCV positive residents have been identified in conjunction with the annual government-sponsored general health examinations conducted in Town C. As of 1994, such residents have been invited to take part in a liver disease screening program to monitor HCC development. The screening program involves annual ultrasonographic liver disease examinations, which was augmented with a self-administered questionnaire and collection of blood sample since 2001. Serum markers of liver disease are measured in the blood samples provided (e.g. ALT). Additional measurements of ALT were available, beginning in 1993, for the study subjects who attended the government-sponsored general health examinations.

For the present analysis, the follow-up period started at the date (year) of first ALT measurement (baseline) and ended at the year of diagnosis of HCC, year of death, or December 31, 2003, whichever came first. There were no subjects lost to follow-up. Subjects without evidence of chronic HCV infection were not included in the present analysis. Chronic HCV carriers were defined as persons with at least 1 HCV RNA or HCV core antigen positive result, between 1995 and 2003. However, individuals with a positive RNA or core antigen result followed by 2 consecutive negative results were considered not to be chronic carriers.

A total of 52 incident HCC cases occurring between the years 1994 and 2003 were included in this analysis. Suspected liver cancer cases were identified at the liver disease screenings, and the diagnosis of HCC was subsequently confirmed by their primary physicians. For 40 cases of HCC, the diagnosis was determined on the basis of information collected via biopsy and/or imaging analysis using magnetic resonance imaging, computed tomography scan, angiography or ultrasonographic tomography. An additional 12 HCC cases were identified based on death certificate information, which was obtained from routine searches of vital statistics records that

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Hirohito Tsubouchi's current address is: Digestive Disease and Lifestyle related Disease Health Research Human and Environmental Science, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima, Japan.
***Correspondence to:** Harvard School of Public Health, 677 Huntington Ave., Kresge 826, Boston, MA 02115, USA. Fax: +617-432-4626.
E-mail: rsuruki@hsph.harvard.edu
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are collected and maintained by the Municipal Public Health Department; for these cases, the year of death was used as the year of diagnosis. For 4 HCC cases, HCV viremia status could not be determined; however, since all 4 cases were anti-HCV seropositive, HCV chronicity was assumed.

Laboratory assays

Anti-HCV antibodies were measured by a third-generation enzyme-linked immunosorbent assay (Lumipulse Orhto II; Ortho-Clinical Diagnostics, K.K., Tokyo, Japan). Specimens were tested for HCV RNA using a reverse-transcriptase polymerase chain reaction assay (Amplicore HCV, Nippon Roche, Tokyo, Japan). Between 1995 and 2001, HCV core antigen level was measured by a fluorescent enzyme immunoassay (Immunocheck F-HCV core antigen; Kokusai Shiyaku, Kobe, Japan); starting in 2002, an immunoradiometric assay replaced the fluorescent enzyme immunoassay to measure HCV core antigen (Ortho HCV Ag IRMA Test; Ortho-Clinical Diagnostic, K.K., Tokyo, Japan).

Statistical analyses

The incidence of HCC was evaluated in the 667 adult residents of Town C who were determined to be chronically infected with HCV. Cox proportional hazards regression was used to describe the relationship between prediagnostic levels of ALT, and the rate of HCC. Hazard ratios (HRs) and associated 95% confidence intervals (CIs) were computed, while adjusting for age group and gender. The first available ALT measurement was evaluated as both a continuous and a dichotomous (≥35 IU/L *vs.* <35 IU/L) variable. In addition, a time-dependent variable based on multiple ALT values obtained between 1993 and 2002 was also assessed as both a continuous and dichotomous (≥35 IU/L *vs.* <35 IU/L) factor. In Instances when ALT values were not available from consecutive years, the last observed value was carried forward until the next available measurement. ALT measurements obtained during the same calendar year as HCC diagnosis were excluded. For the subset of 551 subjects who had at least 4 ALT measurements, 3 mutually-exclusive groups were evaluated: (i) persistently abnormal ALT (all values ≥35 IU/L, $n = 118$), (ii) persistently normal ALT (all values <35 IU/L, $n = 137$), and (iii) fluctuating ALT ($n = 296$). We examined the association between ALT group and HCC incidence following the fourth ALT measurement. The SAS statistical program (v. 8.0) was used in all analyses, the p-values quoted are 2-sided, and the statistical significance was set at $p < 0.05$.

Results

Among the 667 subjects analyzed, 52 HCC cases occurred over an average of 7.9 years of follow-up, representing a total of 5,292 person-years. The study population had a mean age at baseline of 62.6 years (range: 34–90) and was comprised of 288 (43.2%) men (Table I). Men experienced a rate of HCC incidence that was 3-fold greater than that of women (HR = 3.0; 95% CI: 1.7, 5.4). There was a 20% increase in the HCC incidence rate associated with a 10-year increase in age (HR = 1.2; 95% CI: 0.9, 1.7), which was not statistically significant ($p = 0.16$). Subsequent Cox models were adjusted for gender and categories of age at first ALT measurement.

After adjusting for categories of age and gender, a 20 IU/L difference in baseline ALT was associated with a statistically significant 20% increase in the rate of HCC incidence (Table II). When baseline ALT was dichotomized, an abnormal ALT level (≥35 IU/L) increased the rate of HCC by 4 times compared to a normal ALT level (<35 IU/L). In comparison, when ALT was examined as a time-varying covariate based on measurements obtained between 1993 and 2002, the same effect for a 20 IU/L increased difference in ALT was observed, and the higher HCC rate associated with abnormal ALT (≥35 IU/L) was only slightly attenuated (Table II). When we examined the relationship by length of follow-up, we observed a similar 3- to 4- fold association between an abnormal baseline ALT level and HCC development for regardless of length of follow-up (Table III). A sensitivity analysis was performed to examine the validity of the assumption of chronic HCV infection in the 4 HCC cases from which viremia information was not available. Excluding these 4 HCC cases did not remarkably change the effect estimate of elevated ALT on the hazard of liver cancer (data not shown).

Within the sub-group of 551 subjects with at least 4 ALT measurements, 26 HCC cases occurred over an average of 5.3 years of follow-up. The characteristics of these subjects were the same as those of the total cohort studied (data not shown); of note, subjects with persistently abnormal ALT were more likely to be younger and to be men compared to subjects with persistently normal ALT ($p < 0.05$ and $p < 0.0001$, respectively). There was a strong significant age- and gender-adjusted increased rate of HCC associated with persistently abnormal ALT compared to those with persistently normal ALT (Table IV). Subjects with fluctuating ALT experienced a three-fold greater age- and gender-adjusted rate of HCC incidence compared to subjects with normal ALT, although the association was not statistically significant ($p = 0.3$) and very unstable as evidenced by the wide 95% CI.

TABLE I – BASELINE CHARACTERISTICS OF HCV CHRONICALLY INFECTED PARTICIPANTS

Characteristic	Total n (%)
Age (years)	
<50	78 (11.7)
50–59	124 (18.6)
60–69	305 (45.7)
≥70	160 (24.0)
Male	288 (43.2)
Mean ALT (SD)	45.5 (41.5)
ALT categories	
<35 IU/L	349 (52.3)
≥35 IU/L	318 (47.7)
Mean years of follow-up (SD)	7.9 (2.9)

$n = 667$; Town C HCV Study, 1994–2003; SD, standard deviation.

TABLE II – HAZARD RATIO ESTIMATES FOR ALT AS CATEGORIZED AND HCC INCIDENCE AMONG SUBJECTS WHO ARE HCV CHRONICALLY INFECTED

	HR[1]	95% CI
ALT level (20 IU/L increase)		
Baseline	1.2	(1.2–1.3)
Time-varying	1.2	(1.1–1.2)
Abnormal ALT (≥35 IU/L)[2]		
Baseline	4.0	(2.1–7.9)
Time-varying	3.7	(1.9–7.3)

ALT, alanine aminotransferases; HR, hazard ratio; CI, confidence interval.

Town C HCV Study, 1994–2003.

[1]Adjusted for age groups and gender.–[2]Reference group is normal ALT (<35 IU/L).

TABLE III – HAZARD RATIO ESTIMATES FOR ABNORMAL BASELINE ALT (≥35 IU/L), STRATIFIED BY LENGTH OF FOLLOW-UP

	HR[1]	95% CI	n (HCC)
Follow-up ≥1	4.2	(2.1–8.1)	667 (52)
Follow-up >1	3.7	(1.8–7.2)	647 (47)
Follow-up >2	3.1	(1.5–6.2)	617 (41)
Follow-up >3	3.4	(1.6–7.2)	598 (35)
Follow-up >4	4.5	(1.8–11.2)	576 (29)
Follow-up >5	4.3	(1.6–11.7)	552 (23)
Follow-up >6	3.1	(1.0–10.0)	519 (15)
Follow-up >7	2.5	(0.6–10.0)	483 (9)
Follow-up >8	3.2	(0.3–31.2)	423 (4)

HR, hazard ratio; CI, confidence interval; HCC, hepatocellular carcinoma.

[1]Adjusted for age groups and gender.

TABLE IV – HAZARD RATIO ESTIMATES FOR ALT CLASSIFICATION GROUPS AND HCC INCIDENCE AMONG SUBJECTS AMONG THE SUBSET OF SUBJECTS WITH AT LEAST 4 REPEATED MEASUREMENTS OF ALT IN THE TOWN C HCV STUDY, 1994–2003

	HR[1]	95% CI
Persistently normal[2]	1.0	
Fluctuating	2.9	(0.4–23.7)
Persistently abnormal	19.8	(2.6–152.6)

ALT, alanine aminotransferases; HR, hazard ratio; CI, confidence interval.
[1]Adjusted for age groups and gender.–[2]Reference group is normal ALT (<35 IU/L).

Discussion

In this large, prospective, community-based study, elevated ALT level predicted an increased rate of HCV-associated incident HCC during a mean follow-up of almost 8 years. The present findings are consistent with the hypothesis that the relative risk of HCC increases with severity of liver damage, as indexed by elevated ALT. Similar results were reported by Tanaka *et al.* for anti-HCV seropositive blood donors, aged between 16 and 64 years, in Japan.[7] In that study, the investigators found that, compared to subjects with an ALT of ≤29 Karumen Units, subjects with a higher ALT experienced a significantly greater risk of developing HCC over a mean follow-up of 8 years. An association of higher ALT level and HCC incidence was observed even within the normal range of values utilized in the study by Tanaka *et al.* (HR = 6.23; 95% CI: 2.7, 13.5). Although direct comparisons with the results presented by Tanaka *et al.* were not possible due to differences in the normal cut-off values used, a significantly increased rate of HCC associated with ALT levels between 20 and 34 IU/L compared to lower levels was not observed in the present study (HR = 1.5; 95% CI: 0.3, 7.0).

Chronic injury of hepatocytes in subjects infected with HCV is largely a result of both virus-specific and virus-nonspecific immune responses. This chronic inflammation contributes to the procarcinogenic environment by causing ongoing regeneration and proliferation of hepatocytes, which invariably increases genetic instability. However, for hepatocarcinogenesis to occur, these accumulating genetic alterations must lead to a malignant transformation. The resulting activated necrosis of hepatocytes can be measured by serum ALT. Of note, the predictive capacity of abnormal ALT increased almost 6-fold when serial measurements of ALT were used to identify subjects with consistently elevated ALT, relative to using one-time, baseline ALT measurement only.

It is known that ALT levels begin to decrease with greater liver injury, as the damaged hepatocytes become unable to produce ALT.[14] Thus, those subjects who developed HCC after a relatively shorter follow-up time might be misclassified as having normal ALT related to underlying liver damage. As a result, the estimate of the effect of elevated ALT on HCC incidence could have been underestimated in the present study. However, when the analysis was stratified by length of follow-up, an abnormal baseline ALT level was consistently associated with a 3- to 4-fold increased rate of HCC development regardless of length of follow-up. Therefore, it is unlikely that the estimate of association observed was attenuated as a result of more severe liver injury with concomitant lower ALT among the HCC cases.

There were several limitations to the present analysis. We were unable to adjust for interferon treatment, since this information was not available for all subjects. It is possible that the association of ALT with the rate of HCC development may differ by interferon treatment status. Several studies have shown that interferon treatment reduces HCC risk among subjects infected with HCV and, in some instances, independent of its effect on HCV RNA clearance.[15–17] Ikeda *et al.* reported that, regardless of HCV RNA clearance among subjects with persistently normal ALT, the rate of HCC development was significantly lower among patients treated with interferon therapy compared to untreated patients.[16] Thus, by ignoring treatment history, the estimate of the association between ALT levels and HCC incidence may have been overestimated in the present study, if study subjects with normal ALT were more likely to have been previously treated with interferon than subjects with abnormal ALT. However, among subjects in the present study with information on interferon treatment (n = 405), normal baseline ALT was associated with a lower likelihood of being ever treated with interferon compared to abnormal baseline ALT (data not shown). We also did not have information on history of heavy alcohol consumption at baseline, which is known to be associated with an increased risk for the development of HCC.[18,19] However, since alcohol does not appear to increase the risk for HCC through mechanisms other than ones involving liver injury, not adjusting for this risk factor is unlikely to have biased the observed effect estimates.

ALT levels are known to be associated with HCC[7,11,12]; however, the relationship has not been described prospectively in a community-based setting. The present study demonstrates that elevated ALT levels, measured on average 8 years before cancer diagnosis, were associated with an increased rate of HCC among subjects chronically infected with HCV. Furthermore, utilizing serial measurements to identify persistent ALT abnormality revealed a stronger association with HCC risk. The results of this study demonstrate the utility and effectiveness of clinically available data in HCV endemic regions to minimize HCC-associated morbidity and mortality.

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Spontaneous elimination of hepatitis C virus RNA in individuals with persistent infection in a hyperendemic area of Japan

Hirofumi Uto [a], Katsuhiro Hayashi [a], Kazunori Kusumoto [a], Satoru Hasuike [a], Kenji Nagata [a], Mayumi Kodama [a], Akio Ido [b], Michinori Kohara [c], Sherri O. Stuver [d,e], Hirohito Tsubouchi [b,f,*]

[a] *Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan*
[b] *Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-850, Japan*
[c] *Department of Microbiology and Cell Biology, The Tokyo Metropolitan Institute of Medical Science, 18-22 Honkomagome 3-chome, Bunkyo-ku, Tokyo 113-8613, Japan*
[d] *Department of Epidemiology, Boston University School of Public Health, 715 Albany Street, T324E, Boston, MA 02118, USA*
[e] *Department of Epidemiology, Harvard School of Public Health, 677 Huntington Av., Boston, MA 02115, USA*
[f] *Digestive Disease and Life-style related Disease, Health Research Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima, Kagoshima 890-8520, Japan*

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Abstract

The natural course of hepatitis C virus (HCV) carriers is not well understood. We examined the clinical characteristics of individuals exhibiting spontaneous elimination of HCV as part of a cohort study of residents of a HCV hyperendemic area in Japan. In individuals who were judged to have persistent HCV infection in 1995, 302 had at least 4 annual ALT measurements between 1993 and 2000, and had not been treated with IFN. They were tested for the presence of HCV RNA in 2001 and/or 2002 and HCV RNA could not be detected in 20 of the 302 individuals. In these 20 individuals, 7 were confirmed to have detectable HCV RNA and 13 were not until 2000. Thus, 2.4% (7/289) were judged to have spontaneously eliminated the HCV infection during that 6-year period. Although there were no differences in age, sex, ALT levels, or serologically defined HCV genotype between individuals with and without exhibiting spontaneous elimination, there was a significant relationship between the elimination of HCV RNA and a low level of HCVcAg (<20 pg/mL) ($P < 0.001$) upon testing in 1995. These results suggest that spontaneous elimination of HCV RNA following persistent infection is rare and appears to be related to viral load.

Keywords: HCV; HCV core antigen; ALT; Community-based population

1. Introduction

Hepatitis C virus (HCV) infection is one of the most common causes of acute or chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1,2]. Persistent viral infection is estimated to occur in approximately 40–60% of patients with symptomatic acute hepatitis C [3,4]. The clinical features and progression of HCV in these carriers, however, have not been fully characterized.

Although the spontaneous elimination of HCV is thought to be rare in individuals with persistent viral infection in comparison to those with acute disease, the reported frequencies have varied considerably [5–9]. Furthermore, spontaneous elimination during chronic infection has been reported to be related to several factors, such as age, sex, parturition, additional surgical procedure, or stages of HCC [6–10]. The occurrence of elimination and the associated predisposing factors in the general population, however, have not been examined sufficiently.

* Corresponding author. Tel.: +81 992 75 5326; fax: +81 992 75 0588.
E-mail address: htsubo@m2.kufm.kagoshima-u.ac.jp (H. Tsubouchi).

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In this study, we sought to elucidate the clinical and virological features of individuals with persistent HCV infection in a hyperendemic population of Japan. We also evaluated the frequency of spontaneous elimination of chronic HCV infection in this population.

2. Materials and methods

2.1. Study population

Town C is a small town in midwestern Miyazaki Prefecture, Japan, located in a rural area isolated by densely forested mountains. Farming is the principal occupation. A local government-sponsored general health examination program, begun in 1993, has been conducted annually for residents over 20 years of age. Collected blood samples were sent to a commercial laboratory in Miyazaki City for routine blood chemistry analyses. Additional blood samples were stored at or below −30 °C until testing. As part of a collaborative effort between the University of Miyazaki, Faculty of Medicine and the local government and public health service, an ultrasonography screening program began in 1994 to detect HCC in Town C residents who have been identified as positive for anti-HCV antibodies. A research study was initiated in 2001, in which additional virologic and epidemiologic data were collected in addition to the ultrasonography liver disease screenings.

2.2. Serologic studies and viral markers

Study individuals were comprised of Town C residents, who had received a government-sponsored general health examination between 1993 and 1995 that included testing for antibodies against HCV (anti-HCV) using an enzyme immunoassay (EIA) kit (Immunocheck F-HCV Ab, International Reagents Co., Kobe, Japan). This kit was a second-generation assay in which HCV-derived recombinant polypeptides c11 (a structural core protein) and c7 (a nonstructural (NS) protein covering NS3) were used. Anti-HCV titers were determined from the ratio of response intensity of the sample determined by EIA to that determined for a negative control [(intensity of sample − intensity of negative control)/(cut-off − intensity of negative control)]. Ratio values lower than 1.0 were considered to be negative for anti-HCV antibody. Since 2001, anti-HCV was measured in serum samples by a third generation chemiluminescent enzyme immunoassay (CLEIA), in accordance with the manufacturers' instructions (Ortho-Clinical Diagnostics, Raritan, NJ). This assay uses HCV-derived recombinant polypeptides c25 (a structural and a NS protein), c33c (a protein covering NS3) and NS5 (a protein covering NS5 region). Assay results (cut-off index) are calculated as a normalized signal relative to the cut-off value (signal/cut-off [S/C] ratio). The cut-off value was calculated using the formula; luminescence of standard HCV positive serum × 0.28. A sample with an S/C ratio of ≥1.00 was considered to test positive.

We also evaluated the results of biochemical tests measuring ALT [normal value (nl): <35 IU/L], aspartate aminotransferase (AST; nl: <40 IU/L), and γ-glutamyltranspeptidase (γ-GTP; nl: male <70 IU/L, female <30 IU/L) measured from 1993 to 2000.

Serum levels of HCV core antigen (HCVcAg) were tested in all individuals who were anti-HCV antibody-positive using a fluorescence enzyme immunoassay (FEIA) (Immunocheck F-HCVAg Core, International Reagents Co., Kobe) [11,12]. The use of two high-affinity monoclonal antibodies directed against amino acids 21–40 or 41–60 of the HCVcAg in the FEIA gave a lower limit of detection of 8 pg/mL HCVcAg. Using the detection of HCV RNA as the gold standard, we determined the sensitivity and specificity of the FEIA to be 84.5 and 99.4%, respectively [13].

In 1995, we assessed the presence of HCV RNA by qualitative reverse transcription polymerase chain reaction (RT-PCR) (Amplicore HCV, Nippon Roche, Tokyo, Japan) among those individuals whose HCVcAg levels were below the 8 pg/mL limit of detection of the FEIA. In 2001–2002, we also tested for the presence of HCV RNA in all available samples, both stored and newly acquired, by RT-PCR (Amplicore HCV v2.0, Nippon Roche).

Serologically defined genotype (serotype) of HCV was determined using a serological genotyping kit (Immunocheck F-HCV Grouping, International Reagents Co., Kobe).

2.3. Statistical analysis

All statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA). Gender, prevalence of HCV serotype, and frequency of HCV RNA elimination were compared using one-factor ANOVA, χ^2-test or Fisher's exact test, as appropriate. Additional parameters were compared using Scheffe's test or the Mann–Whitney U test, as appropriate. A p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Prevalence of anti-HCV positivity

Initial testing of Town C demonstrated an overall prevalence of anti-HCV antibody positivity of 20.6% (1151/5577), which gradually increased with age (Table 1). There were no differences in the prevalence of anti-HCV antibody positivity between males and females. Eight hundred thirty-six of the 1151 anti-HCV antibody-positive individuals were tested for HCVcAg levels in 1995, at least 6 months after the measurement of anti-HCV antibody titers. Five hundred twenty-eight (63.2%) of those tested were HCVcAg-positive by FEIA, but were not examined for HCV RNA by RT-PCR; 63 (7.5%) were HCVcAg-negative, but tested positive for HCV RNA

Table 1
The age-specific prevalence of confirmed anti-HCV positivity in 1993–1995 in Town C, Japan

Age (years)[a]	Male	Female	All
–29	2/119 (1.7%)	6/202 (3.2%)	8/321 (2.5%)
30–39	22/193 (11.4%)	29/442 (6.6%)	51/635 (8.0%)
40–49	52/403 (12.9%)	46/503 (9.1%)	98/906 (10.8%)
50–59	81/407 (19.9%)	130/607 (21.4%)	211/1014 (20.8%)
60–69	185/693 (26.7%)	277/919 (30.1%)	462/1612 (28.7%)
70–	122/385 (31.7%)	199/704 (28.3%)	321/1089 (29.5%)
Total	464/2200 (21.1%)	687/3377 (20.3%)	1151/5577 (20.6%)

[a] In 1995.

by RT-PCR in 1995. These 591 anti-HCV antibody-positive individuals who also tested positive for either HCVcAg or HCV RNA 6 months or more after the initial anti-HCV antibody testing were judged as having a persistent HCV infection.

3.2. Serum ALT and HCV core antigen in anti-HCV positive residents

Those individuals harboring persistent HCV infections were similar in both gender and age to previously infected individuals (Table 2). Although the mean titers of anti-HCV antibodies were greater in those individuals positive for HCVcAg in comparison to those negative for HCVcAg, but HCV RNA-positive, AST, ALT, and γ-GTP levels were similar between these two groups (Table 2). The frequency of HCV serotype I was significantly higher in the HCVcAg-positive individuals in comparison to the HCVcAg-negative, HCV RNA-positive individuals. The increased sensitivity of the HCVcAg FEIA in carriers infected with HCV serotype I may explain this observation [13]. The serum levels of both AST and ALT were significantly higher in individuals with persistent HCV infections than in those lacking any evidence of HCVcAg or HCV RNA. The serum levels of γ-GTP were significantly higher in HCVcAg-positive individuals than in those lacking detectable HCVcAg or HCV RNA. In chronically infected individuals, however, no correlation between HCVcAg concentrations and ALT levels could be observed (data not shown).

Of the 591 individuals diagnosed with persistent HCV infection in 1995, 511 had at least four available annual ALT measurements between 1993 and 2000. Sixty-three of these individuals reported having received interferon (IFN) treatment before 2002.

Of the 448 individuals who had not been treated with IFN, 162 (36.2%) had normal ALT levels in all tests (<35 IU/L) (Group N), while 286 (63.8%) had at least one abnormal ALT level (≥35 IU/L) during the examination period (Group A). There were no differences between these two groups in the HCVcAg levels determined in 1995 (data not shown).

3.3. Spontaneous elimination of HCV RNA

In 591 individuals who were judged as having a persistent HCV infection in 2000, serum samples in 2001 or 2002 were obtained from 302 individuals who had at least four available annual ALT measurements between 1993 and 2000 and had not been treated by IFN until 2002. These 302 serum samples were tested of HCV RNA using RT-PCR and a positive HCV RNA was detected in 282 of these 302 serum samples. Of the 20 individuals who were HCV RNA-negative, four individuals (case A, C, F and G in Fig. 1 and Table 3) were only positive for HCV RNA in 1995, 3 were confirmed positive for HCV RNA in any of the available stored samples until 2000 and 13 did not have detectable HCV RNA in any of the available stored samples spanning from 1995 to 2000. As a result, seven individuals (2.4%, 7/289) were determined to have spontaneously eliminated the HCV infection, based on RT-PCR (Fig. 1). For cases A, C, D, F, and G, there were insufficient serum quantities to re-test the 1995 samples. The titers of anti-HCV in the samples of cases F and G were very low in 1993 and became negative after 2001. The titers of anti-HCV in cases A–C also appeared to decrease over time (Fig. 1). In the seven individuals with apparent

Table 2
Characteristics of anti-HCV antibody-positive Town C residents, separated by HCV core antigen and HCV RNA status in 1995[a]

Characteristics	HCV core antigen ≥8 pg/mL (n = 528)	HCV core antigen (–) and HCV-RNA (+) (n = 63)	HCV core antigen (–) and HCV RNA (–) (n = 245)	p value*
Gender, male female	209/319	26/37	90/155	0.69
Age	65.2 ± 10.4	66.5 ± 10.3	64.0 ± 10.9	0.19
Anti-HCV titer	9.0 ± 1.3[b,c]	6.6 ± 2.0[d]	5.0 ± 2.5	<0.001
HCV Serotype (I/II)[e]	303/115	30/22	NT[f]	0.03
AST	48.4 ± 41.2 (493)[c]	52.1 ± 35.1 (60)[d]	27.2 ± 149(231)	<0.001
ALT	42.9 ± 40.4 (493)[c]	44.8 ± 36.8 (60)[d]	25.0 ± 16.5 (231)	<0.001
γ-GTP	38.6 ± 51.5(493)[c]	41.4 ± 25.4 (60)	25.4 ± 293 (231)	<0.001

[a] Data shown as the means ± S.D. (number of individuals examined).
[b] $p < 0.001$ vs. HCV core antigen (–) and HCV RNA (+) group, by Scheffe's test.
[c] $p < 0.001$ vs. HCV core antigen (–) and HCV RNA (–) group, by Scheffe's test.
[d] $p < 0.01$ vs. HCV core antigen (–) and HCV RNA (–) group, by Scheffe's test.
[e] Excluding individuals whose HCV serotype was undetermined.
[f] Not tested.
* Based on one-factor ANOVA, χ^2 test, or Fisher's exact test, as appropriate for the comparison across the groups.

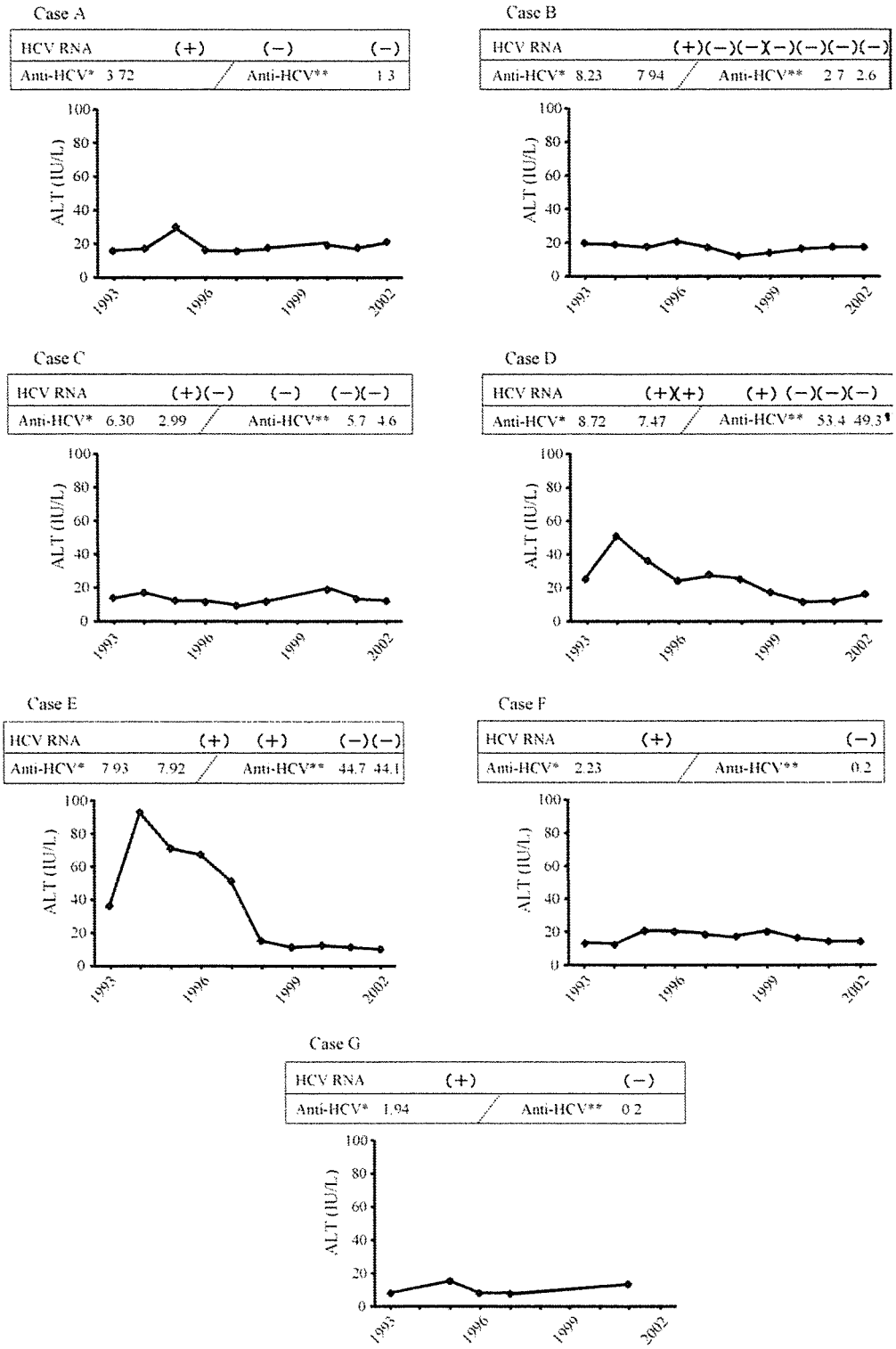

Fig. 1. Clinical course of individuals with spontaneous elimination of serum HCV RNA. Panels (A)–(G) represent the seven cases that were followed for 8 or 9 years. *Antibody against HCV (anti-HCV) were tested by a second generation enzyme immunoassay kit; titers higher than 1.0 were considered to be positive for anti-HCV antibody. **Since 2001, anti-HCV was evaluated by a third generation chemiluminescent enzyme immunoassay; samples with a signal/cut-off ratio of ≥1.0 were considered to be positive. ¶Data was from 2003.

Table 3
Characteristics of anti-HCV positive residents diagnosed with persistent HCV infection in 1995 whose serum HCV RNA were spontaneously eliminated between 1996 and 2002

Case	Age[a]/sex	HCV core antigen (pg/mL)	HCV serotype	ALT abnormality[b] (Group)	Platelets[c] ($\times 10^4/\mu L$)	Type IV collagen 7S[c] (ng/mL)
A	55/F	<8	ND[d]	N	25.5	4.7
B	60/F	33.4	I	N	25.0	3.9
C	64/F	<8	II	N	23.1	3.6
D	66/M	<8	II	A	25.3	3.9
E	68/M	28.2	II	A	17.1	3.3
F	73/M	<8	ND	N	20.6	4.5
G	76/M	<8	ND	N	20.7	3.8

[a] In 1995.
[b] Data were obtained between 1993 and 2000. N; persistently normal ALT levels, A; fluctuating or persistently abnormal ALT levels.
[c] Data were obtained in 2001, with the exception of case F for which data were obtained in 2002.
[d] Individuals whose HCV serotype was not determined.

Table 4
Comparison of demographic and virologic data of seven individuals whose serum HCV RNA were spontaneously eliminated to those of individuals remaining HCV RNA-positive

Characteristics	Elimination of HCV RNA ($n = 7$)	Remained positive for HCV RNA ($n = 282$)	p value*
Gender, male/female[a]	4/3	92/190	0.23
Age[a,b]	66.0 ± 7.2	65.3 ± 8.3	0.95
HCV core antigen (<20/>=20)[a]	5/2	33/249	<0.001
HCV Serotype (I/II)[a,c]	1/3	169/79	0.10
ALT[a,b]	28.9 ± 20.9	40.9 ± 38.7	0.16
ALT group (N/A)[d]	5/2	97/185	0.10

[a] Data were obtained in 1995.
[b] Data shown as the means ± S.D.
[c] Excluding individuals whose HCV serotype was undetermined or was not examined.
[d] N: persistently normal ALT levels, A: fluctuating or persistently abnormal ALT levels.
* Based on Mann–Whitney U test or Fisher's exact test as appropriate.

clearance of HCV RNA, five had HCVcAg levels below the assay's limit of detection; only HCV RNA could be detected by RT-PCR at initial testing in 1995 (Table 3). The incidence of low HCVcAg levels (below 20 pg/mL) [14] determined in 1995 was significantly higher in the individuals with spontaneous elimination of serum HCV RNA (5/7, 71.4%) than in those who remained HCV RNA-positive (33/282, 11.7%) ($P < 0.001$) (Table 4). Five of the seven individuals (71.4%) also had persistently normal ALT levels (Group N), while two had at least one abnormal ALT level (Group A) in the measurements taken between 1993 and 2000 (Fig. 1, Table 3). In comparison to the individuals who remained HCV RNA-positive, those spontaneously eliminating HCV RNA were more likely to be male (57 versus 33%; $p = 0.23$), be infected with HCV serotype II (75 versus 32%; $p = 0.10$), and exhibit persistently normal ALT levels (71 versus 39%; $p = 0.10$), although none of these trends were statistically significant (Table 4). In addition, platelet counts and type IV collagen 7S levels, which reflect the degree of liver fibrosis [15,16], were normal in all available measurements taken from these seven individuals in 2001 or 2002 (Table 3). The serum ALT levels were also normal during this period (Fig. 1).

4. Discussion

This study was performed in an area of Japan hyperendemic for HCV infection, where the prevalence of anti-HCV antibody positivity is 4- to 12-fold higher than that seen in the surrounding areas. The frequency of anti-HCV antibody positivity in this community is similar to that reported for other endemic areas of Japan [5]. In this population, as most residents displayed asymptomatic infections, with less than 5% reporting a history of acute hepatitis or jaundice (data not shown), the exact date of infection were typically unknown. Residents who tested positive for HCVcAg and/or HCV RNA more than 6 months after demonstrating anti-HCV seropositivity were considered to have a persistent HCV infection. The 70.5% prevalence of persistent infection in this population was similar to that previously reported [5,17]. The HCV serotype I was the most common seen in the study population, as reported for other Japanese populations [18,19], suggesting that Town C is similar to other HCV endemic areas of Japan.

In the seven individuals who eliminated HCV RNA, the results of five individuals (cases A–C, F and G) were positive for HCV RNA only once, two of whom (cases F and

G) also were negative for HCV RNA only once. These data do not necessarily exclude the possibility of false positive or false negative HCV RNA results; however, the titers of anti-HCV in all five individuals were lower when the individuals were HCV RNA negative than when they were HCV RNA positive. Although the anti-HCV assays used were different before and after 2001, data from the individuals in the cohort who remained positive for HCV RNA did not show a similar decrease in titer values between the two assays (data not shown). Thus, the results of the HCV RNA and anti-HCV antibody testing, albeit not definitive, would seem to support the spontaneous elimination of HCV RNA in these individuals.

We demonstrated that serum HCV RNA was spontaneously eliminated in 7 of 20 individuals who were HCV RNA-negative in 2001/2002. The remaining 13 individuals who were HCVcAg-positive in 1995 were found to be HCV RNA-negative upon re-testing of all the available stored samples taken between 1995 and 2000. Although these 13 individuals may have undergone spontaneous HCV elimination, we could not confirm the elimination of HCV RNA using RT-PCR as the gold standard. Although the reason underlying this inconsistency is unclear, HCV RNA may have degraded in those blood samples that were stored at −30 °C until testing. As a result, we have conservatively estimated the frequency of HCV elimination in persistent infection at 2.4% (7/282).

Although HCV RNA detection was not performed every year, the presence of HCV RNA was assessed more than 6 years after the initial testing for of HCVcAg or HCV RNA in 1995; thus, the elimination rate was estimated to be approximately 0.4%/year, which is similar to that reported by Watanabe et al. [5]. In contrast, some investigators have reported higher elimination rates than that seen in this study [6–9]. Hattori et al. [7] demonstrated that 14% of pregnant female patients with chronic HCV infections lost positivity for serum HCV RNA without treatment during the follow-up (duration of average follow-up; 5.8 years) of parturition. Fujisawa et al. [6] showed that 8.3% of children with chronic hepatitis C exhibit spontaneous clearance of serum HCV RNA during follow-up (duration of infection; 5.5 years). Fukuizumi et al. [8] estimated a natural disappearance rate of serum HCV RNA positivity at 2.8% per year. These differences may be related to differences in the immune systems of pregnant and non-pregnant women or children and adults. In addition, there may be differences in the initial serum HCV RNA levels, the rate of HCV mutation, the duration of infection, or the HLA allelic frequencies. Further investigations of the factors influencing spontaneous HCV clearance will be necessary to address these discrepancies, which are ongoing in this cohort study.

The spontaneous elimination of HCV RNA has been observed to occur more frequently in females and in individuals with persistently normal ALT levels [5,20]. These observations, however, included individuals who had recovered from acute HCV infection. Although these individuals were positive for anti-HCV antibodies, positive for HCV RNA were not shown before the infection was cleared. In this study, we did not observe a relationship between female gender and the spontaneous elimination of HCV in individuals with persistent infections, which correlated well with the results of Fukuizumi et al. [8]. These results indicate that there is no significant association between gender and spontaneous HCV elimination in individuals with persistent infections.

Yokosuka et al. reported that 6 (2%) of 310 patients with type C chronic liver disease became negative for HCV RNA detection over a 3-year period; all 6 patients exhibited liver cancer with chronic active hepatitis or liver cirrhosis [9]. Individuals became seronegative for HCV RNA in the terminal stages of liver cancer, prompting the hypothesis that a reduction in the amount of the virus occurs with the loss of a suitable environment in which the virus can replicate. In this study, none of the individuals displaying a spontaneous loss of HCV infection had any evidence of hepatocellular carcinoma or liver cirrhosis (data not shown). There were significant differences in the HCVcAg levels measured initially in 1995 between those who eliminated the virus and those supporting persisting infections. Thus, an initial low HCV viral load may be an important factor in the clearance of HCV infection [5–8].

Fukuizumi et al. reported that spontaneous elimination was associated with low ALT levels [8]. In our study, Town C residents exhibiting spontaneous elimination of HCV RNA also tended to have low ALT levels in 1995 and persistently normal ALT levels during 1995 and 2000. However, this tendency was not statistically significant and Fukuizumi et al. did not investigate the association between spontaneous elimination of HCV RNA and sequential ALT levels [8]. The exact mechanism that serum HCV RNA was spontaneously eliminated is unclear. However, HCV elimination in HCV carriers with normal or only mildly elevated ALT levels likely differs from that in individuals with acute infection, in whom cellular immune responses induce severe liver enzyme elevation and these acute responses are critical for the clearance of HCV. The role for HCV elimination in individual with persistent infection requires further investigation.

In summary, we have provided evidence for the spontaneous elimination of HCV RNA. This elimination occurred only rarely and was associated with low initial HCV viral loads, but was not associated with age, gender, or ALT levels. In addition, we could not observe an association between HCV viral load and ALT levels.

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