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Host Immune Status and Incidence of Hepatocellular Carcinoma among Subjects Infected with Hepatitis C Virus: A Nested Case-Control Study in Japan

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Abstract

A nested case-control study was conducted to examine the association between host immune status, as characterized by serum immune marker levels, and the development of hepatocellular carcinoma (HCC) up to 8 years later in persons with chronic hepatitis C virus (HCV) infection. Cases (n = 39) and matched controls (n = 117) were selected from participants of the Town C HCV Study in Japan between 1996 and 2004 and matched on age at first available sample (±1 year), gender, and length of follow-up. Separate analyses were done for each of three serum immune markers: soluble tumor necrosis factor-receptor II (sTNF-R2) and soluble intercellular adhesion molecule-1 (sICAM-1), as indicators of type 1, cell-mediated immune response, and soluble CD30 (sCD30), as an indicator of type 2, humoral immune response. The median concentrations of sTNF-R2, sICAM-1, and sCD30 among controls were 3,170 pg/mL, 305 ng/mL, and 3.0 units/mL, respectively, and were higher among cases (3,870 pg/mL, 372 ng/mL, and 3.3 units/mL, respectively). The risk of developing HCC among subjects with immune marker concentrations above the median levels of the controls was >2-fold greater than among subjects with lower concentrations for all three markers [sTNF-R2: odds ratio (OR), 6.9; 95% confidence interval (95% CI), 2.4-20.5; sICAM-1: OR, 2.0; 95% CI, 0.9-4.1; and sCD30: OR, 2.1; 95% CI, 1.0-4.7]. Simultaneous adjustment for all three markers revealed only sTNF-R2 to be associated with HCC risk (OR, 6.4; 95% CI, 2.0-20.6). Adjustment for alcohol consumption and HCV serotype did not materially alter these associations. Results from this prospective, community-based study suggest that a dysregulation in both type 1-related and type 2-related host immunity contributes to the development of HCV-associated HCC. (Cancer Epidemiol Biomarkers Prev 2006;15(12):2521-5)

Introduction

Hepatocellular carcinoma (HCC) is the predominant histologic subtype of primary liver cancer (1). Although relatively infrequent in developed countries, the incidence of HCC has increased in the United States and Japan over the past 20 to 30 years (1, 2). Such increases have been partially attributed to the emergence of the hepatitis C virus (HCV), an established risk factor for HCC (3, 4). Despite worldwide endemicity, the prevalence of HCV infection varies significantly by geographic region. For example, higher HCV infection prevalence rates have been reported in African and Asian countries, whereas prevalence rates among industrialized nations in North America, northern and western Europe, and Australia have generally been lower (5).

The great majority of HCV-infected individuals fail to eliminate the virus and progress onto chronic HCV infection (6-9). Explanations for this phenomenon include the presence of HCV quasispecies, the development of mutations in key areas of the viral genome, and the direct interference by the virus of the host immune response (10-12). A strong cell-mediated immune response is thought to lead to clearance of HCV, whereas an elevated humoral response or an only moderately increased cell-mediated response pattern has been reported in patients with chronic infection (7, 8, 13-16). The tension between the continued replication of the virus and a persistent attempt by a less than optimal immune response to eliminate HCV-infected cells within chronically infected persons is implicated in hepatocyte damage and, in some instances, progression to HCC. This continuous inflammation and hepatocyte regeneration in the setting of chronic hepatitis and progression to cirrhosis is thought to lead to an accumulation of chromosomal damage and possibly to initiate hepatic carcinogenesis (17).

The immune response to virus infection consists of two major components: the innate and adaptive response. The innate response is the first to respond to invading pathogens and involves natural killer cells, complement, cytokines, and apoptosis (18). Natural killer cells rely on antigen-independent mechanisms to inhibit viral replication (19, 20). In contrast, the adaptive response requires recognition of a specific viral epitope and is divided into two effector types: cell-mediated type 1 response and humoral type 2 response (18). The function of these two effector responses is tightly regulated within immunocompetent persons; however, dysregulation of type 1 and/or type 2 response can occur in cases of infectious, neoplastic, and inflammatory diseases (21).

The type 1/type 2 cytokine balance in sera, liver tissue, and culture supernatant of lymphocytes has been studied extensively in HCV-infected patients, but inconsistent results have failed to provide definitive information about the role of cytokines in HCV disease pathology (13-15, 22-29). Nevertheless, data from several studies suggest that a dysregulation of

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the host immune status may be important in the progression of HCV-related liver disease (14, 22-25). A shift to a type 1 cytokine profile in patients with chronic hepatitis C is correlated with liver disease activity and progression (14). Similarly, an elevation of soluble CD30 (sCD30), a marker of type 2 response, has also been reported to be correlated with liver disease progression and severity in HCV-infected patients (25).

Tumor necrosis factor (TNF)-α is a mediator of innate inflammation and cellular immune response produced primarily by activated monocytes and Kupffer cells and plays a role in initiating fibrogenesis through binding to specific cellular receptors [TNF-receptors (TNF-R); ref. 26]. After cellular stimulation, extracellular domains of these receptors can be proteolytically cleaved, resulting in two soluble forms: soluble TNF-R1 (sTNF-R1) and sTNF-R2. High concentration of sTNF-R2 has been observed for prolonged periods in the circulation of patients with various inflammatory diseases, including HCV infection, making sTNF-R2 an ideal serum biomarker to characterize the type 1 immune response (27-30). Activation of the immune response in chronic hepatitis has also been shown by means of using circulating levels of intercellular adhesion molecule (ICAM)-1 (31, 32). Soluble ICAM-1 (sICAM-1) is an important adhesion molecule that is thought to be involved in liver inflammation. sCD30 is a member of the TNF/nerve growth receptor family and is preferentially expressed and secreted by human CD4 T cells producing type 2 cytokines (33, 34). Elevated levels of sCD30 have been detected in patients with conditions attributed to type 2 cytokine immunity, such as systemic lupus erythematosus and Omenn's syndrome, as well as in patients with HCV-associated liver disease (25, 35, 36).

We undertook the present study to elucidate the role of host immune status in the incidence of HCV-associated HCC in a prospective community-based cohort of HCV-infected persons in Japan. Given their extremely short half-life and the potential effects of freeze/thaw cycles (37), direct measurement of cytokines is not feasible in a community-based study using archived frozen serum samples. Serum proteins that are less labile and documented to be correlated with type 1 and type 2 response, particularly with respect to HCV infection, represent a more feasible alternative (28-32, 35, 36). For these reasons, we selected sTNF-R2 and sICAM-1 as markers of a type 1 cytokine milieu and sCD30 as a surrogate marker of a type 2 cytokine environment. Using prediagnostic serum levels of these serologic immune markers, we hypothesized that host immune dysregulation suggesting an up-regulated type 1 (cell mediated) and/or type 2 (humoral) response against HCV in a community-based setting increases HCC incidence. The propensity of HCV to cause clinically inapparent disease underlines the importance of assessing the informativeness of these biomarkers in identifying chronically infected subjects who may be predisposed to develop HCC.

Materials and Methods

Study Population. Data collected as part of the Town C HCV Study were used for the present analysis (38). Briefly, this community-based cohort study is being conducted within the adult population of a community (Town C) in southwestern Miyazaki Prefecture, Japan. Beginning in 1994, anti-HCV-positive residents identified at annual government-sponsored general health examinations in Town C were invited to participate in a liver disease screening program to monitor the development of HCC. In 2001, as a collaborative effort between Harvard School of Public Health (Boston, MA) and University of Miyazaki Faculty of Medicine (Miyazaki, Japan), a research component was incorporated into the ongoing liver disease screenings, which augmented these annual ultrasonography-based liver examinations with a self-administered questionnaire and the collection of a blood sample. Frozen aliquots of the blood samples are retained and stored at −80°C at the University of Miyazaki Faculty of Medicine, with duplicate aliquots shipped to Harvard School of Public Health where they are also stored at −80°C. In addition, between 1995 and 2000, serum samples collected from identified anti-HCV-positive Town C residents, in conjunction with the government-sponsored general health examinations, also have been frozen and stored at −20°C at the University of Miyazaki Faculty of Medicine. This population-based study was approved by the Human Subjects Committees of Harvard School of Public Health and University of Miyazaki Faculty of Medicine.

A total of 70 incident HCC cases has been identified among the study population of HCV-seropositive Town C residents (n = 1,311; mean age, 62 years) between 1994 and 2004. Of these, 39 cases occurred between the years 1996 and 2004 and had a prediagnostic serum sample obtained at least 1 year before the HCC diagnosis, for measuring the selected immune markers, and had evidence of chronic HCV infection, defined as having at least one HCV RNA or HCV core antigen-positive result between 1995 and 2004. For 32 of the 39 cases, the diagnosis was determined based on information collected via biopsy and/or imaging analysis using magnetic resonance imaging, computed tomography scan, angiography, or ultrasonographic tomography. An additional seven HCC cases were identified by means of death certificate information; for these cases, the year of death was used as the year of diagnosis.

We used incidence density sampling to select controls from the set of subjects at risk at the time of diagnosis of each HCC case (39). Subjects with evidence of chronic HCV infection, at least 1 year of follow-up, and an available sample were eligible for inclusion in the risk set (n = 676; mean age, 64 years). Three controls were randomly selected from the risk set for comparison with the index HCC case. A risk set was defined by the gender, age (±1 year) at first available sample, and length of follow-up (equal or greater than case) of the case. A total of 117 controls was matched to the 39 cases. Due to the matching criteria, the number of potential subjects within a risk set was relatively small; thus, the controls were made up of 99 unique individuals and included 15 controls that were selected more than once.

Laboratory Methods. Specimens were tested for HCV RNA using a reverse transcription-PCR 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). HCV serotype was determined by an enzyme immunoassay (Immunocheck F-HCV Grouping, International Reagents Co., Kobe, Japan). When the serologic group could not be clearly classified by this assay, HCV genotypes were determined by the reverse transcription-PCR method (40). Genotypes 1a and 1b were defined as serologic HCV group 1 and genotypes 2a and 2b as group 2. The above serum testing was completed by a commercial laboratory in Japan.

Serum immune marker testing of archived baseline specimens was completed by the General Clinical Research Center Core Laboratory at Massachusetts Institute of Technology (Boston, MA). The samples were sent in randomly ordered batches, and laboratory personnel were blinded to the case-control status of the specimens. The levels of sTNF-R2 and sICAM-1 were measured by means of ELISA (Quantikine and Paramter, respectively, R&D Systems, Minneapolis, MN); these assays have an interassay variability that ranges from 6% to

Table 1. Baseline characteristics of cases and matched control subjects, including median and interquartile range of serum immune marker levels, in the Town C HCV Study, Japan

Characteristics	Cases (n = 39)	Controls (n = 117)
Gender, n (%)		
Men	25 (64.1)	75 (64.1)
Women	14 (35.9)	42 (35.9)
Age (y), mean (SD)	65.3 (7.5)	65.2 (7.5)
Length of follow-up (y), mean (SD)	4.4 (2.1)	4.4 (2.0)
Alcohol consumption, n (%)*		
None	21 (55.3)	53 (51.5)
Occasional	4 (10.5)	18 (17.5)
Daily, low (≤60 g alcohol per day)	10 (26.3)	26 (25.2)
Daily, high (>60 g alcohol per day)	3 (7.9)	6 (5.8)
HCV serotype, n (%)[†]		
1	29 (76.3)	78 (66.7)
2	9 (23.7)	39 (33.3)
sTNF-R2 (pg/mL)		
Median	3,870.0	3,170.0
Interquartile range	3,350-4,820	2,670-3,970
Minimum, maximum	2,330, 6,740	1,740, 7,150
sICAM-1 (ng/mL)		
Median	372	305
Interquartile range	287-449	228-367
Minimum, maximum	55, 891	58, 699
sCD30 (units/mL)		
Median	3.3	3.0
Interquartile range	2.67-4.89	2.17-3.87
Minimum, maximum	0.5, 10.0	0.5, 18.8

*One case and 14 controls missing alcohol information.
† One case missing serotype information.

10% according to the manufacturer. Levels of sCD30 were also determined by means of an ELISA (ZyQuick sCD30 ELISA kit, Zymed Laboratories, Inc., San Francisco, CA), with an inter-assay variability ranging from 9.4% to 17.5%.

Statistical Analysis. Cases and controls were compared by medians for continuous variables and by contingency tables for qualitative data. The association between biomarker levels and the risk of HCC was analyzed using conditional logistic regression, which accounts for the matching within the risk sets. With risk set sampling, the odds ratio (OR) derived from the conditional logistic regression analysis directly estimates the hazard ratio (39, 41). Because serum immune marker levels were skewed and no cutoff levels for an elevated value have previously been determined, the serologic biomarkers were modeled as dichotomous variables using the median value among the controls. We also evaluated alcohol consumption (none, occasional, or daily) at baseline and HCV serotype (serotype 1 versus serotype 2) as potential confounders in multivariable regression models. Alcohol consumption was determined based on responses to a questionnaire administered by the public health nurses at the first liver disease screening program examination attended by the resident. The "daily" drinkers were further categorized into high (>60 g alcohol per day) and low (≤60 g alcohol per day) groups. In instances where data from the public health nurses' questionnaire were not available, "never" drinkers could be identified using the study-related questionnaire obtained beginning in 2001 and were thus included in the "none" category (n = 7). To evaluate the potential effect of reverse causation (i.e., preclinical HCC causing the elevation of serum immune markers), the analyses also were restricted to HCC cases diagnosed >2 years after their first available prediagnostic sample. All P values are two tailed, and P values of <0.05 were considered to indicate statistical significance. All analyses were done with the use of Statistical Analysis System software version 8.2 (SAS Institute, Cary, NC).

Results

The baseline (i.e., at first available serum sample) characteristics for the 39 cases and 117 matched controls are shown in Table 1. The mean age of the study participants was 65.2 years (SD, ±7.4) and 64.1% were men. Cases and controls were comparable with respect to age, gender, and length of follow-up by the matched design. There also was no difference in alcohol consumption between cases and controls; however, cases were more likely to be infected with HCV serotype 1 than were controls, although the difference was not statistically significant.

With respect to the type 1 immune markers, the median concentration of sTNF-R2 and sICAM-1 was statistically significantly higher among HCC cases than among controls (Table 1). The risk of developing HCC among subjects with type 1 immune marker levels above the median value of the controls was approximately 6- and 2-fold greater than among subjects with lower levels for sTNF-R2 and sICAM-1, respectively (Table 2). These observations suggest that HCC cases experience an elevated type 1 immune response before the development of HCC. Levels of sTNF-R2 and sICAM-1 were positively correlated with one another; the age- and gender-adjusted partial correlation coefficient among controls was 0.44 (P < 0.0001). Multivariable regression analysis revealed that adjusting for alcohol consumption and HCV serotype did not materially change these associations (data not shown).

About the type 2 serum immune marker among HCC cases, the median level of sCD30 was marginally significantly greater compared with controls (Table 1). An increased sCD30 level was also positively associated with a 2-fold greater HCC risk compared with subjects with levels below the median value of the controls in the conditional logistic regression analysis (Table 2). sCD30 levels were significantly correlated with the type 1 immune markers; the age- and gender-adjusted partial correlation coefficients among controls for sCD30 with sTNF-R2 and sICAM-1 were 0.64 (P < 0.0001) and 0.40 (P < 0.0001), respectively. Again, adjustment for alcohol consumption and HCV serotype did not notably alter the observed association for sCD30 (data not shown).

There were seven HCC cases that were diagnosed within 2 years of their first available prediagnostic sample. To evaluate the potential effect of reverse causation, we removed these seven cases from the analysis and found that the observed associations remained unchanged: $OR_{sTNF\text{-}R2}$, 6.0 [95% confidence interval (95% CI), 2.0-17.9]; $OR_{sICAM\text{-}1}$, 2.2 (95% CI, 1.0-5.0); and OR_{sCD30}, 2.3 (95% CI, 1.0-5.5).

Evaluation of the independent effect of each serum immune marker after adjusting for the other two markers showed that only sTNF-R2 was significantly associated with HCC

Table 2. OR estimates for the association of greater than median level of serum immune markers with HCC among HCV-infected subjects in the Town C HCV Study

	Cases/controls	OR* (95% CI)
sTNF-R2		
≤3,170 pg/mL[†]	6/60	1.0
>3,170 pg/mL	33/57	6.9 (2.4-20.5)
sICAM-1		
≤305 ng/mL[†]	13/59	1.0
>305 ng/mL	26/58	2.0 (0.9-4.1)
sCD30		
≤3.0 units/mL[†]	13/59	1.0
>3.0 units/mL	26/58	2.1 (1.0-4.7)

*Conditional logistic regression, matched on age, gender, and minimum follow-up.
† Median value of controls.

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

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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.
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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

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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 (×10^4/ml)[a] *	23.7 ± 5.4	18.0 ± 5.9

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; ‡$P < 0.05$; †$P < 0.001$; *$P < 0.001$.
[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-10 (−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.	
IL-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.

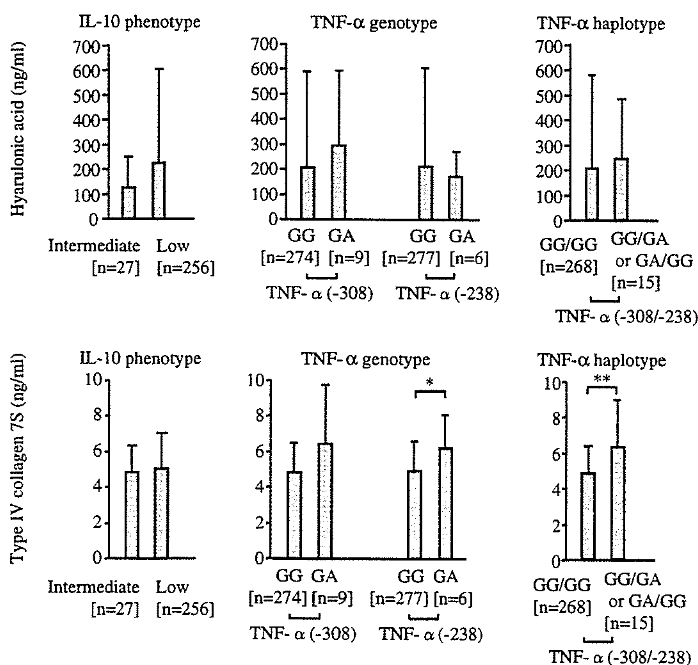

Fig. 1. Serum levels of type IV collagen 7S and hyaluronic acid in 283 individuals with persistent HCV infection who had at least four annual ALT measurements available during 1993 and 2003. Data are shown as means ± SD. Mann–Whitney U test was used for evaluating statistical significance. $^{*}P = 0.013$,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

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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

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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.
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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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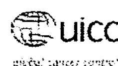
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are collected and maintained by the Municipal Public Health Department; for these cases, the year of death was used as the year of diagnosis. For 4 HCC cases, HCV viremia status could not be determined; however, since all 4 cases were anti-HCV seropositive, 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

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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.
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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.

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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