MATERIALS AND METHODS

Study subjects

Serum samples were obtained from residents of one village in Miyazaki, Japan, who attended free, government-sponsored health examinations from November 1984 to November 2000. The prevalence of antibody to HCV (anti-HCV) in this village was 23% [1].

Serum samples

Sequential serum samples were evaluated from six anti-HCV positive individuals who developed liver cancer between 1984 and 1999. Each of the six liver cancer cases was matched to two anti-HCV-positive controls by gender, age, and year of study enrollment, except for case no. 6, who was only matched with one control (a total of six cases and 11 controls). All 63 serum samples (23 from cases and 40 from controls) were coded. Samples had undergone fewer than two freeze–thaw cycles and had otherwise been kept at −80 °C until use.

Reverse transcription-polymerase chain reaction

RNA was extracted from 0.25 mL of each serum sample and cDNA was synthesized. The 176 base pair (bp) HCV E2 fragment (containing the 81-nucleotide HVR1 and an additional 35 nucleotides upstream and 60 nucleotides downstream) and the 256-bp 5′-noncoding region (5′-NCR) fragment were amplified with gene-specific primers using nested polymerase chain reaction (PCR) (Table 1) [2].

To amplify the HVR1 fragment, the first round of PCR was performed with one sense primer [HCVHf1 (Table 1)] and a mixture of two antisense primers (HCVHb1 and HCVHb2); the second round of PCR was performed with one sense primer (HCVHf2) and a mixture of two antisense primers (HCVHb3 and HCVHb4). The amplification of the 5′-NCR fragment was carried out by conventional nested PCR using one sense (P-285) and one antisense primer (P-43) for the first round of PCR and one sense (P-276) and one antisense primer (P-50) for the second round of PCR [2]. The amplified HVR1 fragment was a 176-bp product from position +1366 to +1541 according to the numbering system of Choo *et al.* [3]. The amplified 5′-NCR fragment was a 256-bp product that extended from nucleotide position −276 to −21.

Cloning and sequencing of PCR products

Polymerase chain reaction products were purified and cloned. Plasmid DNA was then extracted and sequenced on an automated DNA sequencer (model P310; PE/ABI, Foster City, CA, USA) with the BigDye Terminator Cycle Sequence Ready Reaction Kit (PE/ABI) using a universal M13 reverse primer according to protocols provided by the manufacturer. For most cases and controls, 10 clones from the HVR1 and five clones from the 5′-NCR were studied. However, for cases no. 3 and 5, and controls no. 3 and 5, 50 clones of the HVR1 fragment from each serum sample were amplified and sequenced in order to determine the extent to which examining additional clones would increase the number of quasispecies detected.

Sequence analysis

The number of viral variants and the genetic diversity of the HCV quasispecies were assessed by examining viral sequences of the HVR1 and 5′-NCR genes. DNA sequence data were analysed with Edview software (PE/ABI).

Table 1 Primers for amplifying HVR1 and 5′-NCR of HCV

Name	Sequence	Positions
HVR1 – first PCR		
HCVHf1	5′-GCC ATA TAA CGG GTC ACC GCA TGG C-3′	1208 to 1232
HCVHb1	5′-CCC CAC GAC AAC AGG AC-3′	1811 to 1827
HCVHb2	5′-TCC CAC CAC CAC GGG GC-3′	1811 to 1827
HVR1 – second PCR		
HCVHf2	5′-ATG GTG GGG AAC TGG GCG AAG G-3′	1366 to 1387
HCVHb3	5′-ATG TGC CAA CTG CCG TTG GT-3′	1522 to 1541
HCVHb4	5′-AGG TGC CAA CTG CCG TTG GT-3′	1522 to 1541
5′-NCR – first PCR		
P-285	5′-ACT GTC TTC ACG CAG AAA GCG TCT AGC CAT-3′	−285 to −256
P-43	5′-CGA GAC CTC CCG GGG CAC TCG CAA GCA CCC-3′	−14 to −43
5′-NCR – second PCR		
P-276	5′-ACG CAG AAA GCG TCT AGC CAT GGC GTT AGT-3′	−276 to −247
P-50	5′-TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG-3′	−21 to −50

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Sequence alignment and phylogenetic analysis were performed using MacVector V6.5 software (Oxford Molecular Group, Madison, WI, USA) and the GCG Wisconsin Package software (GCG, Madison, WI, USA). Genetic distance, a pairwise comparison of evolutionary distance between aligned sequences, was determined using the GCG Wisconsin Package software (GCG). For each serum sample (from the six cases and 11 controls), the DNA sequences from each of 10 clones of the PCR products were compared with the other nine clones (a total of 100 comparisons for each serum sample). The numerical result produced by the software reflects the number of substitutions per 100 nucleotides as well as the nature of the substitutions.

RESULTS

The average genetic distance among 10 clones prepared from each serum sample was 18.3 among six cases (23 serum samples) and 6.7 among 11 controls (40 serum samples) ($P < 0.001$) (Fig. 1). Initially, 10 clones of HVR1 were sequenced from each serum sample. The number of quasispecies detected in each serum sample did not differ significantly between cases (average of 6.6 quasispecies) and controls (average of 5.1 quasispecies) when only 10 clones were sequenced from each sample (Fig. 2). In addition, no differences were seen in the number of quasispecies detected in early and late serum samples from the same individuals among both cases and controls when only 10 clones were sequenced (data not shown).

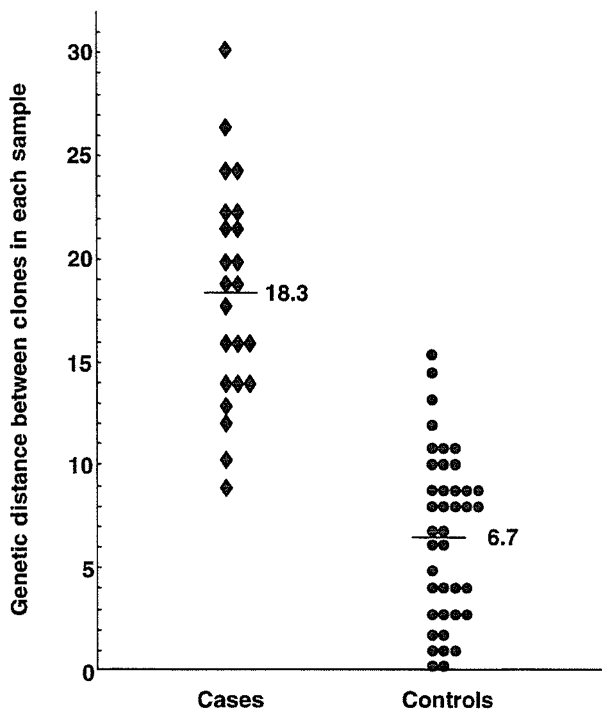

Fig. 1 Each mark represents the 'genetic distance' (see text for definition) between HCV RNA in the 10 clones from each of the serial serum samples from six liver cancer cases and 11 controls (63 serum samples total). Genetic distance, a pairwise comparison of evolutionary distance, was expressed as substitutions per 100 nucleotides. The average genetic distance within each serum specimen was 18.3 in cases and 6.7 in controls.

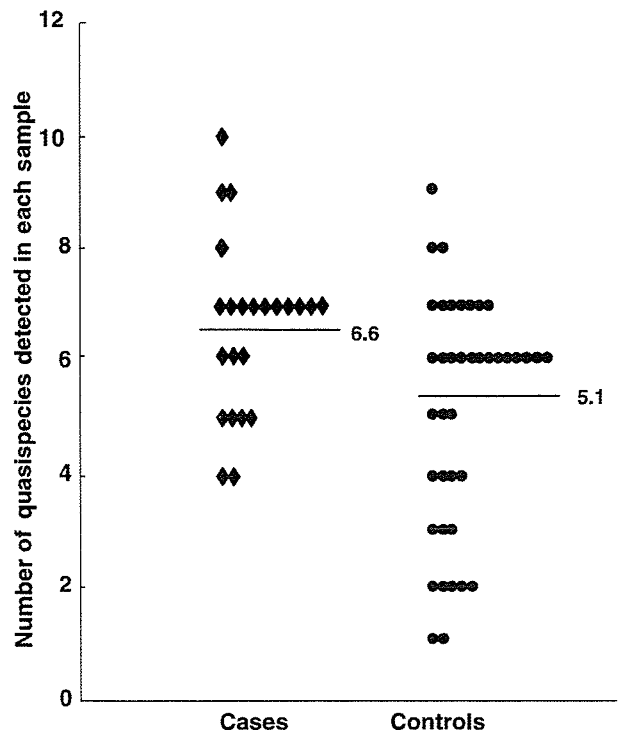

Fig. 2 Number of HCV quasispecies detected by sequencing 10 clones for each of 63 serum samples. There was no statistically significant difference between cases (average of 6.6 quasispecies) and controls (average of 5.1 quasispecies).

Forty additional clones each were analysed in sequential groups of 10 clones from cases no. 3 and 5 and their matched controls in samples obtained at the time of entry into the study, 1 year later, and 5 or 10 years later, for a total of 50 clones from each serum sample. After having sequenced 40 clones per sample, sequencing an additional 10 clones per sample detected only one or no additional quasispecies (Fig. 3).

During disease progression, the number of quasispecies detected by analysing 50 clones per sample in case no. 3 increased from 16 in 1984 to 22 in 1989, in control no. 3 from eight in 1984 to 15 in 1989, in case no. 5 from 18 in 1989 to 25 in 1994, and in control no. 5 increased from 6 in 1984 to 23 in 1994 (Fig. 3). The difference in the number of quasispecies between these cases and controls at the time of entry into the study (1984, average of 17 quasispecies in cases and seven in controls) was higher than 5–10 years later (1989 or 1994, average of 23.5 quasispecies in cases and 19 in controls).

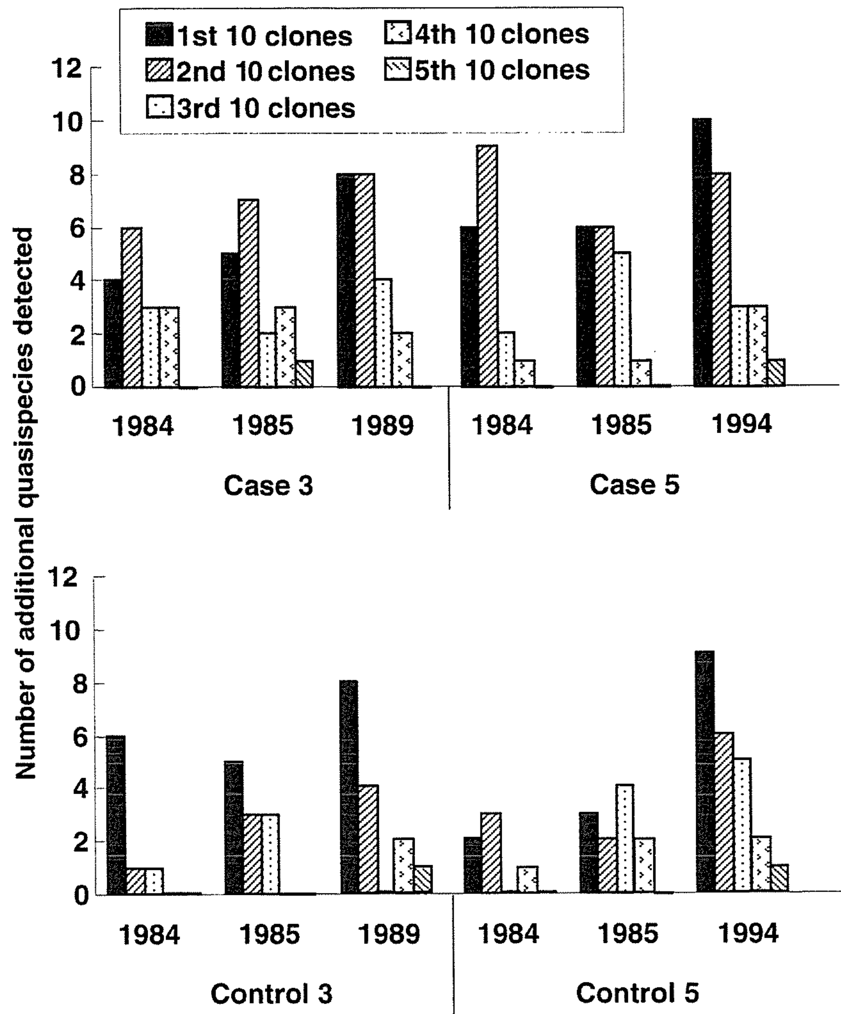

Fig. 3 Correlation between the number of clones sequenced and the number of *additional* quasispecies detected. Fifty clones were sequentially analysed in incremental groups of 10 clones each from cases no. 3 and 5 and controls no. 3 and 5, using serum samples obtained at entry to the study, 1 year later, and 5 or 10 years later. The bars indicate the number of *additional* quasispecies detected among those 10 clones (over and above those detected by testing prior groups of 10 clones each). The ability to detect additional quasispecies decreased as more clones were analysed. Nearly all detectable quasispecies were detected in the first 40 clones (i.e. one or no additional quasispecies were detected by testing 50 clones, compared with testing 40 clones).

DISCUSSION

The quasispecies nature of HCV is thought to play an important role in maintaining and modulating viral replication [4,5], and the increasing diversity of the quasispecies in one individual is associated with more advanced liver damage caused by HCV [6]. In the present study, the quasispecies diversity in those who subsequently developed hepatocellular carcinoma (HCC) was greater than that in controls (average genetic distance 18.3 in cases and 6.7 in controls).

In the present study, the number of quasispecies was seen to increase dramatically over 5–10 years in four patients and controls from whom 50 clones per sample were studied, reaching a level between 15 and 25 quasispecies per serum sample. However, overall sample-to-sample fluctuation was seen for each of the other seven patients and controls, from whom only 10 clones per sample were studied.

The present study shows the importance of sequencing a large number of clones in order to obtain the most accurate assessment of quasispecies diversity. Most published studies of HCV quasispecies have reported the results of sequencing only three to five clones per sample. However, Torres-Puente *et al.* [7] recently reported that no significant difference was observed in genetic variability tested in small numbers of samples (10 sequences) compared with large numbers (100 sequences). In contrast, the present study shows that sequencing a small number of clones per sample provides less information about quasispecies diversity than sequencing 20, 30, or 40 clones per sample. The data in this study also suggest that the maximum number of quasispecies in a serum sample usually can be detected by sequencing about 40 clones per sample.

Obtaining an accurate picture of the quasispecies diversity in the serum of individuals infected with HCV may lead to a better understanding of the pathogenesis. There may be differences in virulence among quasispecies; some of the quasispecies in an individual are not transmitted in at least some cases of maternal–fetal transmission [8], needlestick accidents [2], or experimental transmission to chimpanzees [9]. Theoretically, some quasispecies might bind to cellular receptors more readily or might escape the host immune

response more easily. The association of quasispecies diversity with the emergence of escape mutants [10], and the correlation of greater HCV quasispecies diversity with more severe liver damage [11], indicate that quasispecies diversity plays an important role in the course of HCV infection.

REFERENCES

1 Okayama A, Stuver SO, Tabor E *et al.* Incident hepatitis C virus infection in a community-based population in Japan. *J Viral Hepat* 2002; 9: 43–51.

2 Gao G, Buskell Z, Seeff L *et al.* Drift in the hypervariable region of the hepatitis C virus during 27 years in two patients. *J Med Virol* 2002; 68: 60–67.

3 Choo Q-L, Richman KH, Han JH *et al.* Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA* 1991; 88: 2451–2455.

4 Domingo E, Holland JJ. RNA virus mutations and fitness for survival. *Annu Rev Microbiol* 1997; 51: 151–178.

5 Korenaga M, Hino K, Katoh Y *et al.* A possible role of hypervariable region 1 quasispecies in escape of hepatitis C virus particles from neutralization. *J Viral Hepat* 2001; 8: 331–340.

6 Koizumi K, Enomoto N, Kurosaki M *et al.* Diversity of quasispecies in various disease stages of chronic hepatitis C virus infection and its significance in interferon treatment. *Hepatology* 1995; 22: 30–35.

7 Torres-Puente M, Bracho MA, Jimenez N, Garcia-Robles I, Moya M, Gonzalez-Candelas F. Sampling and repeatability in the evaluation of hepatitis C virus genetic variability. *J General Virol* 2003; 84: 2343–2350.

8 Weiner AJ, Thaler MM, Crawford K *et al.* A unique, predominant hepatitis C virus variant found in an infant born to a mother with multiple variants. *J Virol* 1993; 67: 4365–4368.

9 Sugitani M, Shikata T. Comparison of amino acid sequences in hypervariable region-1 of hepatitis C virus clones between human inocula and the infected chimpanzee sera. *Virus Res* 1998; 56: 177–182.

10 Farci P, Bukh J, Purcell RH. The quasispecies of hepatitis C virus and the host immune response. *Springer Semin Immunopathol* 1997; 19: 5–26.

11 Duffy M, Salemi M, Sheehy M *et al.* Comparative rates of nucleotide sequence variation in the hypervariable region of E1/E2 and the NS5b region of hepatitis C virus in patients with a spectrum of liver disease resulting from a common source of infection. *Virology* 2002; 301: 354–364.

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Usefulness of a new immuno-radiometric assay to detect hepatitis C core antigen in a community-based population

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SUMMARY. A new immuno-radiometric assay (IRMA) to detect hepatitis C virus (HCV) core antigen (HCVcAg) has been developed. The aim of the present study was to investigate the sensitivity and specificity of this IRMA to measure HCV antigenemia, based on the detection of HCV RNA as the gold standard, and to assess the utility of the IRMA in a community-based population. Anti-HCV positive residents in a hyperendemic area of HCV infection in Japan were studied. Serum levels of HCVcAg were measured using IRMA, and the presence of HCV RNA was determined by a qualitative reverse transcription-polymerase chain reaction (RT-PCR) assay. The sensitivity and the specificity of the IRMA were 96.4 and 100%, respectively. The sensitivity of the IRMA was similar between serological HCV group I (HCV genotypes 1a and 1b) (97.6%) and group II (HCV genotypes 2a and 2b) (94.0%). There was a strong correlation between serum HCVcAg level and HCV-RNA measured by a quantitative RT-PCR ($r = 0.832$, $P < 0.0001$). There also was a very strong correlation of HCVcAg level between IRMA measurements performed on serum and those performed on plasma ($r = 0.984$, $P < 0.0001$). In conclusion, this new IRMA is useful for the detection of HCV core antigen in a community-based population.

Keywords: community-based population, hepatitis C virus core antigen, immuno-radiometric assay, serological hepatitis C virus group.

INTRODUCTION

Persistent infection with hepatitis C virus (HCV), as well as hepatitis B virus (HBV), is a primary cause of chronic liver disease, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). In Town C of Miyazaki Prefecture in Japan, the average annual mortality from liver disease was estimated to be 102.5/100 000 between 1995 and 1997, which was substantially higher than the estimate of 37.6/100 000 for all of the prefecture, as reported by the prefectural public health service. In 1993, over 4000 residents of Town C were tested for antibody to HCV (anti-HCV) and HBV surface antigen (HBsAg) in conjunction with the local, government-sponsored general health examination conducted in the town. The prevalence of anti-HCV positivity was found to be 22.5% and that of HBsAg positivity, 1.1%. Thus, the elevated mortality from liver disease in Town C is considered to be due to the high prevalence of HCV infection in this population.

Measurement of anti-HCV is generally used to screen for HCV infection. However, not all anti-HCV positive people are persistently infected with the virus. Therefore, a simple, quick, and inexpensive method for measuring HCV viremia is required for population-based testing. Although the detection of HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR) represents the most sensitive method for determining persistent HCV infection, the assay is time-consuming, costly, and technically demanding. In contrast, enzyme immunoassays (EIAs) to detect HCV core antigen (HCVcAg) are simple and relatively inexpensive [1]. A number of reports have demonstrated the utility of measuring HCVcAg using EIAs [2–5]. Recently, a new immuno-radiometric assay (IRMA) to detect HCVcAg was developed.

The aim of the present study was to investigate the sensitivity and specificity of this new IRMA to measure HCV antigenemia, based on the detection of HCV RNA as the gold

Abbreviation: EIA, enzyme immunoassay; HCC, hepatocellular carcinoma; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction; US, ultrasonography.

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standard, and to assess the utility of the IRMA in a community-based population such as Town C.

Methods and subjects

Since 1994, 1083 Town C residents have been identified as being positive for anti-HCV and have been screened annually for the early detection of HCC by ultrasonography (US) examination. To elucidate the natural history of HCV infection and the risk factors for HCC, additional virologic, epidemiologic, and clinical data also have been collected at these US examinations, beginning in 2001. Blood samples for CBC, liver function test, tumour markers (alpha-fetoprotein and PIVKA-II), hepatic fibrosis markers (hyaluronic acid and type IV collagen) and HCV-associated markers (anti-HCV antibody titre, serological genotyping of HCV, virus load, presence of HCV RNA) are collected after taking an informed consent. In 2002, 931 subjects were invited to the annual US examination, of whom 674 (72%) attended and provided a blood sample.

Anti-HCV antibody

Anti-HCV antibody was measured by chemiluminescent enzyme immunoassay using a third generation HCV antibody kit (Lumipulse Ortho II; Ortho-Clinical Diagonostics K. K., Tokyo, Japan).

HCV antigen load

At the 2002 US screening, the new IRMA (Ortho HCV Ag IRMA Test; Ortho-Clinical Diagnostics K. K.) was used to detect HCVcAg. Testing using the IRMA was carried out as indicated by the manufacturer. Briefly, serum or standard sample was added to pretreatment solution in a plastic tube and incubated at 56–60 °C. After adding the reaction solution and one bead coated with two different monoclonal antibodies against HCVcAg to each tube, the mixture was incubated at room temperature. Two different horse-radish peroxidase-conjugated monoclonal antibodies against HCVc Ag was added to each tube. ^{125}I-conjugated polyclonal antibodies against peroxidase then were added, and the radioactivity of the sample and the standard was measured with a γ-scintillation counter. The concentration of HCVcAg was expressed as femto-mol/L (fmol/L). The range of the IRMA was from 20 to 20 000 fmol/L. When the titre of HCVcAg was less than 20 fmol/L, the sample was judged as negative. The plasma levels of HCVcAg also were measured, by the same IRMA method, for a subset of 40 subjects.

HCV RNA detection and quantification

The presence of HCV RNA in serum was determined by a qualitative RT-PCR assay kit (Amplicore® HCV; Nippon Roche, Tokyo, Japan). The detectable HCV-RNA by this assay kit was 10 copy/mL. The serum HCV RNA level was measured using a quantitative RT-PCR assay kit (Amplicore® GT HCV Monitor v2.0; Nippon Roche) on a random sample of 100 HCV RNA positive subjects. However, four of the subjects had an insufficient sample for measuring HCV RNA level. The concentration of HCV RNA was expressed as KIU/mL, and the range was from 0.5 to 850 KIU/mL. When the titres of HCV-RNA were less than 0.5 KIU/mL, the sample was judged as negative.

Serological HCV group

Serological HCV groups were determined by a serological genotyping assay (Immunocheck F-HCV Grouping; International Reagents Co., Kobe, Japan) [6]. When the serological group could not be clearly classified by this assay, HCV genotypes were determined by the RT-PCR method [7]. Genotypes 1a and 1b were defined as serological HCV group I, and genotypes 2a and 2b as group II.

RESULTS

A total of 613 (90.9%) of the 674 residents who attended the 2002 US screening and provided a blood sample were anti-HCV antibody positive and 61 were negative. Thirty-eight per cent of the subjects studied were men ($n = 233$), and the overall mean age was 69.3 years.

The results of the IRMA testing are summarized in Table 1. HCVcAg was detected by IRMA in 424 (69.2%) of the 613 anti-HCV positive subjects; HCV RNA was detected by RT-PCR in 440 (71.8%). Based on the HCV RNA status as the gold standard, the sensitivity of the IRMA was 96.4% (424/440), with a specificity of 100%. All 61 subjects who were anti-HCV negative were negative for both HCVcAg and HCV-RNA. Ninety-six randomly selected serum samples were tested for HCV-RNA level using a quantitative RT-PCR assay (Table 2). Neither HCVcAg by IRMA nor HCV-RNA by RT-PCR was detected in four samples. In three samples, HCV-RNA was detected only by RT-PCR. The HCV-RNA level of these samples was 1.3, 7.1 and 19 KIU/mL. There were no samples that were HCVcAg positive but negative for HCV-RNA (Tables 1 and 2). Figure 1 shows a strong correlation

Table 1 Comparison of the detection of HCV core antigen by immuno-radiometric assay with the detection of HCV-RNA by RT-PCR

	HCV-RNA by qualitative RT-PCR	
HCVcAg by IRMA	+	–
+	424	0
–	16	173

HCV, hepatitis C virus; RT-PCR, reverse transcription-polymerase chain reaction.

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Table 2 Comparison of the detection of HCV core antigen by IRMA and HCV-RNA by quantitative RT-PCR in HCV-RNA positive subjects

HCVcAg by IRMA	HCV-RNA by quantitative RT-PCR	
	+	–
+	89	0
–	3	4

HCV, hepatitis C virus; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction.

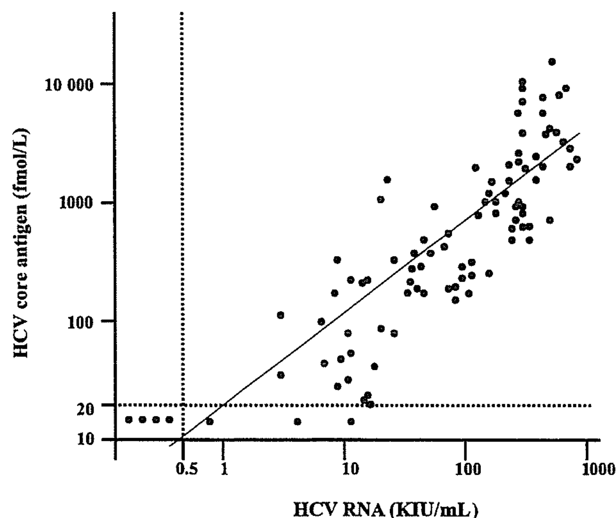

Fig. 1 Correlation between concentration of HCV RNA measured by AMPLICOR Monitor test and HCV core antigen measured by immuno-radiometric assay. The serum HCV RNA level was measured using a quantitative RT-PCR assay kit (Amplicore® GT HCV Monitor v2.0) on a random sample of ninety-six HCV RNA positive subjects. The levels of serum HCVcAg measured by IRMA were strongly correlated with HCV-RNA levels by RT-PCR (Pearson correlation coefficient, $r = 0.832$, $P < 0.0001$).

between serum HCVcAg level measured by IRMA and HCV-RNA level by RT-PCR ($r = 0.832$, $P < 0.0001$).

The HCV genotype group could be determined by serological genotyping or RT-PCR assay for 524 subjects who attended the 2002 screening (Table 3). The distribution of subjects by HCV genotype group was 356 (67.9%) of the 524 subjects in group I and 168 (32.1%) in group II. The sensitivity of the IRMA was similar in the two serological HCV genotype groups (97.6% in group I and 94.0% in group II) (Table 3). It is noteworthy that the average amount of HCVcAg detected by the IRMA was similar in the two-genotype groups (median of 3060 fmol/L in group I and median of 3350 fmol/L in group II).

The correlation between serum and plasma levels of HCVcAg detected by IRMA was analysed in a subset of the Town C residents who attended the 2002 US screening (Fig. 2). The levels of HCVcAg in plasma were strongly correlated with those in serum (Pearson correlation coefficient, $r = 0.984$, $P < 0.0001$).

Table 3 Comparison of the detection of HCV core antigen by IRMA with the detection of HCV RNA by RT-PCR, by serological HCV group

HCVcAg by IRMA	HCV-RNA by RT-PCR	
	+	–
Serological HCV group I		
+	282	0
–	7	67
Serological HCV group II		
+	141	0
–	9	18

HCV, hepatitis C virus; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction.

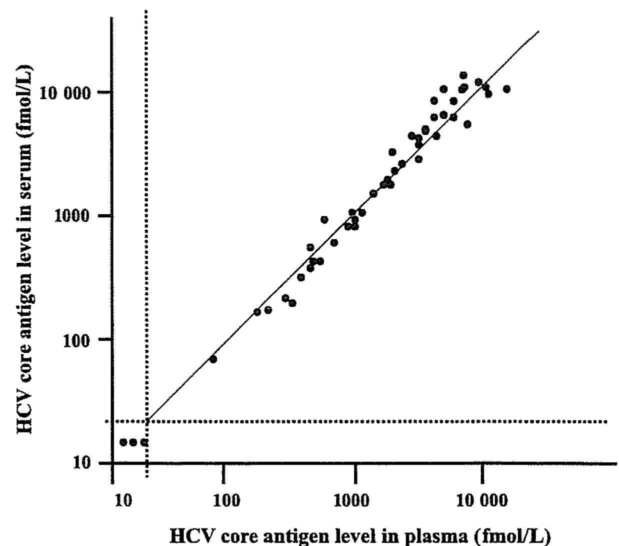

Fig. 2 Correlation between HCV core antigen levels in serum and in plasma measured by immuno-radiometric assay. The subjects were classified into six groups according to their serum HCVcAg levels (<20, 20–100, 100–1,000, 1000–10 000, 10 000–20 000, >20 000 fmol/L). Plasma samples from five or six subjects were randomly selected from each group for a total of 40 subjects and were measured by IRMA. The levels of HCVcAg in plasma were strongly correlated with those in serum (Pearson correlation coefficient, $r = 0.984$, $P < 0.0001$).

DISCUSSION

HCV core antigen was first detected in the circulation of HCV-infected hosts using EIA-based methods [1,8,9]. Many

reports have demonstrated the utility of EIA to detect HCVcAg for monitoring the effect of interferon therapy [1], for following liver transplantation patients with HCV recurrence [2], for quantitative evaluation of HCV viremia in anti-HCV positive patients [3], and as a marker of HCV viremia in the serological window-phase period [4]. However, the first versions of the EIA for HCVcAg had some limitations. They could not detect HCVcAg below a level of 20 KIU/mL of HCV RNA, so that their use was limited to the monitoring of late events during and after antiviral treatment [10]. In addition, the pretreatment process of the sample was somewhat complicated, with three steps required to expose the epitopes of HCVcAg bound by low-density lipoprotein or anti-HCV core antibody. The sensitivity of the EIA also was affected by mutations in the HCV core region [11]. Moreover, differences in the detectable levels of HCVcAg titres between the serological HCV genotype groups resulted in a lower sensitivity of the EIA among people infected with HCV genotype 2 [12].

In 1999, Aoyagi and colleagues developed a modified version of the EIA for HCVcAg [5]. In this version, the epitope of HCVcAg could be easily exposed, and binding by anti-HCV core antibody in the serum could be reduced by incubation with three types of detergents. Since the modified EIA required only one pretreatment step, it was simpler than the first generation versions of the assay. In addition, it had a 100-fold increase in sensitivity over the earlier versions. Tanaka *et al.* demonstrated that the second generation of the EIA for HCVcAg was useful for the diagnosis of acute and chronic hepatitis C and for predicting and monitoring the effect of interferon treatment [13]. More recently, a new IRMA-based test for detecting HCVcAg, which is a further modification of the Aoyagi *et al.* EIA method, was developed.

In the present study, we investigated the sensitivity and specificity of this IRMA, to detect HCV persistent infection based on the presence of HCV RNA as the gold standard. The sensitivity of the IRMA was 96.4%. In contrast, we tested for HCVcAg by a first generation assay at the 2001 US screening and found its sensitivity was 85.4% (data not shown). Of the randomly selected 96 samples, there was a strong correlation between serum HCVcAg level measured by the IRMA and HCV-RNA level by a commercial quantitative RT-PCR assay. This result is the same as that previously reported by Tanaka *et al.* [13]. Moreover, the IRMA for HCVcAg overcomes the problem of the effect of serological HCV genotype group on the level of HCVcAg detectable by EIA in serum [12]. In our population, the sensitivity of the first generation EIA was significantly lower in serological group II (HCV genotypes 2a and 2b) (74.7%) than in group I (HCV genotypes 1a and 1b) (90.5%) (data not shown). In striking contrast, the sensitivity of the IRMA was 94% or higher in both serological HCV genotype groups. It is not clear why the sensitivity of the IRMA is not affected by HCV genotype group. Both the EIA [1] and the IRMA [5] use high-affinity monoclonal antibodies that recognize amino acid sequences known to be relatively well conserved across the six HCV genotypes. However, small differences in these amino acid sequences between genotype groups I and II may exist, which render the monoclonal antibodies in the first generation EIA less sensitive to detect genotype group II core antigen. Moreover, the four different monoclonal antibodies used in the second generation IRMA may enhance the ability of this assay to detect HCVcAg in persons infected with HCV group II genotypes.

The presence of HCV RNA is considered the gold standard for determining HCV persistence, and RT-PCR is a very sensitive method to detect HCV RNA. However, the RT-PCR assay requires additional amplification procedures and specially-trained personnel and carries the risk of contamination. In addition, it has been reported that the RT-PCR method produces false negative results for heparinized samples [14] and may not detect the lower HCV RNA levels found for persons infected with HCV genotypes 2a or 2b [15]. There also is a progressive and significant loss of HCV RNA activity when the time from the formation of the clot until centrifugation is longer than 2 h from the collection of the blood specimen [16]. In contrast, EIA-based methods to detect HCVcAg offer several advantages over RT-PCR for measuring HCV persistence. First, EIAs are not influenced by the anticoagulants EDTA, heparin, or sodium citrate [5]. In the current study, there was a very strong correlation between HCVcAg levels in serum and those in plasma as measured by the new IRMA. Second since the HCVcAg detected by EIA is stable [5], extra precautions in processing and storing specimens also should not be necessary. Moreover, the IRMA appears to be sufficiently sensitive to identify persistent group II HCV infection. Finally, the cost of the IRMA kit is less than one-third that of the RT-PCR assay.

The majority of HCV carriers are asymptomatic, and some may display advanced liver disease, including liver cirrhosis and HCC, without the awareness that they are infected with HCV. Thus it is important that persons with persistent HCV infection can be identified. In the present study, we demonstrated the usefulness of a new IRMA for the detection of HCV antigenemia in the community-based Town C population in Japan. The assay is relatively simple and inexpensive and has a high sensitivity to detect HCVcAg in people infected with HCV genotypes 1 or 2. Thus, this new IRMA is an economically viable option for identifying individuals with chronic HCV infection when screening large numbers of people on a population level.

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REFERENCES

1 Tanaka T, Lau JYN, Mizokami M *et al.* Simple fluorescent enzyme immunoassay for detection and quantification of hepatitis C viremia. *J Hepatol* 1995; 23: 742–745.

2 Dickson RC, Mizokami M, Orito E, Qian KP, Lau JY. Quantification of serum HCV core antigen by a fluorescent enzyme immunoassay in liver transplant recipients with recurrent hepatitis C – clinical and virologic implications. *Transplantation* 1999; 68: 1512–1516.

3 Komatsu F, Takahashi K. Determination of serum hepatitis C (HCV) core protein using a novel approach for quantitative evaluation of HCV viremia in anti-HCV-positive patients. *Liver* 1999; 19: 375–380.

4 Widell A, Molnegren V, Pieksma F, Calmann M, Peterson J, Lee SR. Detection of hepatitis C core antigen in serum or plasma as a marker of hepatitis C viremia in the serological window-phase. *Transfusion* 2002; 12: 107–113.

5 Aoyagi K, Ohue C, Iida K *et al.* Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. *J Clin Microbiol* 1999; 37: 1802–1808.

6 Tsukiyama-Kohara K, Yamaguchi K, Maki N *et al.* Antigenicities of grouping I and II hepatitis C virus polypeptides – molecular basis of diagnosis. *Virology* 1993; 192: 430–437.

7 Ohno T, Mizokami M, Wu RR *et al.* New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35: 201–207.

8 Takahashi K, Okamoto H, Kishimoto S *et al.* Demonstration of a hepatitis C virus-specific antigen predicted from the putative core gene in the circulation of infected hosts. *J Gen Virol* 1992; 73: 667–672.

9 Kashiwakuma T, Hasegawa A, Kajita T *et al.* Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). *J Immunol Methods* 1996; 190: 79–89.

10 Bouvier-Alias M, Patel K, Dahari H *et al.* Clinical utility of total HCV core antigen quantification: a new indirect marker of HCV replication. *Hepatol* 2002; 36: 211–218.

11 Tokita H, Kaufman G, Matsubayashi M *et al.* Hepatitis C virus core mutations reduce the sensitivity of a fluorescence enzyme immunoassay. *J Clin Microbiol* 2000; 38: 3450–3452.

12 Orito E, Mizokami M, Tanaka T *et al.* Quantification of serum hepatitis C virus core protein level in patients chronically infected with different hepatitis C virus genotypes. *Gut* 1996; 39: 876–880.

13 Tanaka E, Ohue C, Aoyagi K *et al.* Evaluation of a new enzyme immunoassay for hepatitis C virus (HCV) core antigen with clinical sensitivity approximating that of genomic amplification of HCV RNA. *Hepatology* 2000; 32: 388–393.

14 Willems M, Moshage H, Nevens F, Fevery J, Yap SH. Plasma collected from heparinized blood is not suitable for HCV-RNA detection by conventional RT-PCR assay. *J Virol Methods* 1993; 42: 127–130.

15 Davis GL, Lau YYN, Urdea MS *et al.* Quantitative detection of hepatitis C virus RNA with a solid-phase signal amplification method: detection of optimal conditions for specimen collection and clinical application in interferon-treated patients. *Hepatology* 1994; 19: 1337–1341.

16 Hawkins A, Davidson F, Simmonds P. Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotype 1, 2, and 3 by Quantiplex HCV assay versions 1 and 2, Roche Monitor assay, and in-house limiting dilution methods. *J Clin Microbiol* 1997; 35: 187–192.

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The peroxisome proliferator-activated receptor-γ agonist, pioglitazone, inhibits fat accumulation and fibrosis in the livers of rats fed a choline-deficient, L-amino acid-defined diet

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Abstract

Administration of a choline-deficient, L-amino acid-defined (CDAA) diet to rats causes steatohepatitis, hepatic fibrosis, and hepatocellular carcinoma, a pathology similar to that observed in non-alcoholic steatohepatitis (NASH). The aim of this study was to evaluate if a peroxisome proliferator-activated receptor (PPAR)-γ agonist, pioglitazone (PGZ), could ameliorate CDAA diet-induced fatty liver and cirrhosis. Rats were fed a CDAA diet for 1 week and were given the CDAA diet for an additional week with or without PGZ (2-week model). Also, after administration of the CDAA diet for 12 weeks, rats were administered the CDAA diet for an additional 4 weeks with or without PGZ (16-week model). The CDAA diet, administered for either one or 12 weeks, induced fatty liver or cirrhosis with up-regulation of hepatic PPAR-γ expression, respectively. In the 2-week model, rats treated with PGZ for 1 week demonstrated significantly lower hepatic triglyceride content and serum levels of tumor necrosis factor-α. In the 16-week model, treatment for 4 weeks with PGZ ameliorated hepatic fibrosis with a decrease in the expression of procollagen, α-smooth muscle actin, and transforming growth factor-β1 in comparison to rats without PGZ. These results suggest that PPAR-γ agonist is a potential therapeutic modality to treat NASH.
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Keywords: CDAA; NASH; PPAR-γ agonist; Fatty liver; Hepatic fibrosis

1. Introduction

Non-alcoholic steatohepatitis (NASH) is caused by the accumulation of excess fat in the liver. This disease may progress to cirrhosis, occasionally causing hepatocellular carcinoma [1,2], NASH is also closely associated with type 2 diabetes mellitus and obesity; insulin resistance has been implicated as a possible factor in NASH development [3–5]. The mechanism of this disease is still obscure, however, in part due to the lack of an appropriate experimental model. The choline-deficient, L-amino acid-defined (CDAA) diet, a semi-synthetic diet containing no choline and limited methionine in the absence of any known carcinogens, may prove such a model. Administration of the CDAA diet to rats induces hepatocellular carcinoma (HCC) in conjunction with fatty liver, hepatocyte injury and regeneration, fibrosis, and cirrhosis [6,7]. This phenotype is similar to the development of human HCC with cirrhosis, especially that observed in NASH.

Peroxisome proliferator-activated receptor-γ (PPAR-γ), a member of the nuclear receptor superfamily, was originally defined as an essential factor in adipogenesis [8]. The PPAR subfamily consists of three isotypes, PPAR-α, PPAR-γ, and PPAR-β/δ; these three PPAR isotypes exhibit distinct

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patterns of tissue distribution [9]. PPAR-γ agonists are widely used in patients with insulin resistance and diabetes. PPAR-γ agonists have also been reported to inhibit cell proliferation and collagen expression in primary hepatic stellate cells (HSC) both in vitro and in vivo [10,11]. These results suggest a potential therapeutic value for PPAR-γ agonists in treating NASH. It is unclear, however, if the dosages of PPAR-γ agonist used for the treatment for patients with type 2 diabetes mellitus will be effective for NASH therapy. Even if effective for the early prevention of liver disease, it remains unknown whether PPAR-γ agonist will be effective if given after the disease has progressed to fatty liver or liver cirrhosis.

This study examined the effects of treating CDAA-fed rats with the PPAR-γ agonist, pioglitazone (PGZ; 1.0 mg/(kg day)), an agent widely used to treat patients with type 2 diabetes mellitus, after the development of fatty liver and liver cirrhosis. We have demonstrated that PGZ administration suppresses fat accumulation in the liver and hepatic fibrosis in these rats, suggesting that hepatic PPAR-γ may be a useful therapeutic target in humans suffering from NASH.

2. Materials and methods

2.1. Animals

Six-week-old male Fischer 344 rats were purchased from Kyushu Experimental Animal Supply (Kumamoto, Japan). The animals were maintained at constant room temperature (25 °C) and provided free access to water and food throughout the study. After a 1-week acclimation period on a standard diet, rats were switched to a CDAA diet (Dyets Inc., Bethlehem, PA) and were sacrificed at 1, 2, 12, and 16 weeks after beginning the CDAA diet. Blood was obtained by cardiac puncture. The liver was immediately excised and the wet weight of the liver was determined. Samples were either immediately subjected to histological analysis or frozen in liquid nitrogen and stored at −80 °C until analysis. All animal procedures were performed according to approved protocols in accordance with the ethical committee of University of Miyazaki.

2.2. Compounds and animal treatment

The PPAR-γ agonist PGZ was kindly provided by Takeda Pharmaceutical Co. (Tokyo, Japan). All rats were continuously fed a CDAA diet with or without PGZ until the end of the experiment. When indicated, PGZ was given by gavage once a day at a dose of 1 mg/(kg day), a dosage comparable to that used to treat humans. Rats were randomly divided into four groups. Groups 1 and 3, administered the CDAA diet for 2 or 16 weeks, respectively, served as control groups. One or 12 weeks after beginning administration of the CDAA diet, the experimental animals in groups 2 and 4 received PGZ for an additional one or 4 weeks with the CDAA diet, respectively. Thus, groups 1 and 2 were 2-week model, and group 3 and 4 were also 16-week model.

2.3. Serum markers

Serum levels of alanine aminotransferase (ALT), glucose, insulin, and hyaluronic acid were determined in the experiments. Serum levels of tumor necrosis factor (TNF)-α were measured using a rat cytokine ELISA kit (Biosource, Camarillo, CA).

2.4. Reverse transcription-polymerase chain reaction

Total RNA was extracted from liver tissue using the acid guanidinium thiocyanate:phenol:chloroform method. One microgram of RNA in a 20-μl mixture was reverse transcribed with Molony murine leukemia virus reverse transcriptase MMLV (TaKaRa, Tokyo, Japan) at 42 °C for 60 min in the presence of random hexamers. The following primers were used: 5′-ATCAGCTCTGTGGACCTCTCTGTG-3′ (sense) and 5′-AGCTTCAATCGGATGGTTCTTCGG-3′ (antisense) for rat PPAR-γ (product size 380 base pairs [bp]), 5′-AACAGGACTTCTTCGGAACCAC-3′ (sense) and 5′-CATTTGCACCACTTGTGGCTTC-3′ (antisense) for rat procollagen α2(I) (product size 422 bp) [12], and 5′-ACTCTACCCACGGCAAGTTCA-3′ (sense) and 5′-GGCAGTGATGGCATGGACT-3′ (antisense) for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (product size 483 bp). Five microliters of the reverse-transcribed mixture was amplified by polymerase chain reaction (PCR) in a 25-μl volume (TaKaRa). PCR reactions were initially denatured at 94 °C for 2 min and cycled at 94 °C for 30 s, 53 °C (PPAR-γ), 56 °C (type I procollagen α2), or 59 °C (GAPDH) for 30 s, and 72 °C for 30 s. Thirty-two cycles were performed to amplify PPAR-γ and procollagen α2(I), while 30 cycles were performed to amplify GAPDH using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA). Type I procollagen α2 PCR products were examined by 1.2% agarose gel electrophoresis and visualized with ethidium bromide. Densitometric analysis examined PCR product semi-quantities by measuring the absorbance with a Bio-1D apparatus (M & S Instruments Trading Inc., Tokyo, Japan). The magnitudes of gene expression were calculated as relative intensity against GAPDH mRNA levels. The mean relative intensity of type I procollagen α2 in group 3 was normalized to a value of 1.

2.5. Histopathological and immunohistochemical analysis

Liver samples were fixed in 10% formalin and embedded in paraffin. For histological examination, 5-μm slices were stained with hematoxylin and eosin (HE) or Azan dye. Immunohistochemical analysis of α-smooth muscle actin (ASMA) (Dako Japan, Kyoto, Japan) was performed with paraffin-embedded sections as previously described

[13]. Rabbit anti-rat IgG (Novocastra, Burlingame, CA) was applied to samples, followed by an avidin–biotin-peroxidase complex and chromatin 3′,3′-diaminobenzidine for detection of bound antibody.

2.6. Western blot analysis

Liver tissues were solubilized using T-PER tissue protein extraction reagent (Piece Biotechnology, IL). Sample proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidine difluoride filters. After blocking with 1% bovine serum albumin, filters were incubated with anti-PPAR-γ polyclonal antibody (Santa Cruz Biotechnology, CA) or anti-ASMA monoclonal antibody (Dako Japan). Bound antibody was detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham Biosciences, Buckinghamshire, UK), respectively. Proteins were then visualized with ECL Western blotting detection kit (Amersham).

2.7. Hepatic triglyceride content and TGF-β1 expression

Hepatic triglyceride content was determined as previously described [14]. Triglyceride content is expressed as mg/g of the wet liver. TGF-β1 expression in the liver was measured using a Quantikine assay kit (R & D systems, Minneapolis, MN). After the preparation of liver lysates, samples were equalized for protein concentration as previously described prior to measuring TGF-β1 levels [13]. TGF-β1 protein levels measured the active form, detected by the addition of HCl [15]. The resulting values are expressed as ng/g of the wet liver.

2.8. Statistical analysis

Results are presented as means ± S.D. Statistical parameters were ascertained using StatView J-4.5 software (Abacus Concepts, Inc., Berkeley, CA). Differences between means were assessed by the Mann–Whitney *U*-test. The significance level was set at $P < 0.05$.

3. Results

3.1. PPAR-γ expression in the liver during CDAA diet administration

We have previously reported that diffuse fatty liver was observed in the animals 1 week after beginning of the CDAA diet [16]. The development of cirrhosis was also recognized at 12 weeks [16]. As PPAR family members are implicated in adipogenesis, we examined PPAR-γ expression during stages of the CDAA diet in which fat accumulation and liver fibrosis were induced. Although no hepatic expression of PPAR-γ could be observed in normal rats, PPAR-γ expression was observed after one and 12 weeks of CDAA diet administration (Fig. 1). These findings indicate that increased expression of PPAR-γ was observed in conjunction with the development of fatty liver and liver cirrhosis. Thus, PPAR-γ is a potential therapeutic target for treatment of fatty liver and hepatic fibrosis in this animal model.

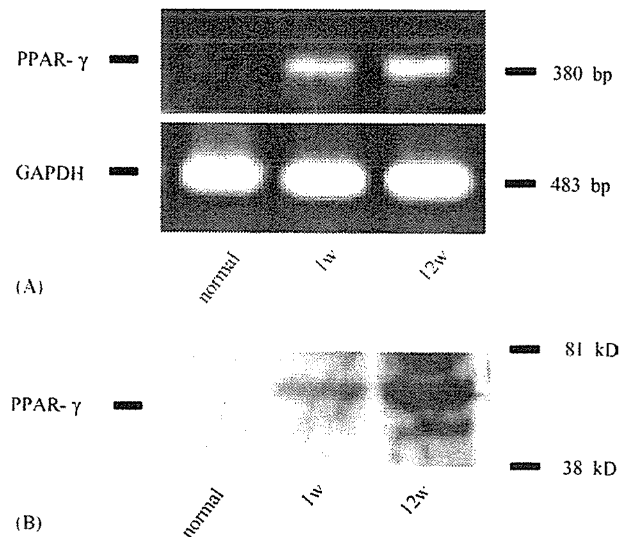


Fig. 1. Hepatic PPAR-γ expression in rats fed a CDAA diet. PPAR-γ expression in the livers of four rats at each time point was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. No hepatic expression of PPAR-γ mRNA was observed in normal rats. Administration of a CDAA diet induced the up-regulation of PPAR-γ expression in the liver at weeks 1 and 12. Representative results of RT-PCR (A) and Western blot analysis of PPAR-γ protein expression (B) are shown.

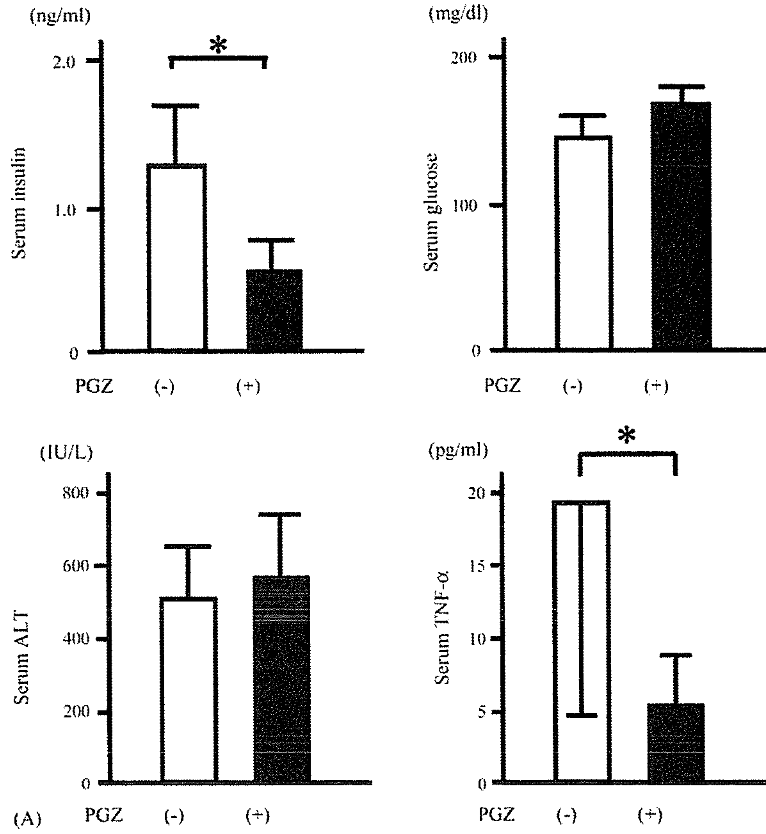
3.2. Serological and histological analysis during fatty liver development (2-week model)

We examined the effects of PGZ administration on various serum markers. Serum insulin levels were significantly reduced in the PGZ-treated rats (Fig. 2A), whereas serum levels of glucose and ALT were unaffected by this treatment. PGZ treatment also lowered serum levels of TNF-α. We examined the effect of PGZ treatment on fat accumulation in the liver. HE staining demonstrated that administration of the CDAA diet to animals for 2 weeks resulted in marked fat accumulation (Fig. 2B-a, group 1). Treatment with PGZ (group 2) slightly improved this fat accumulation (Fig. 2B-b), in comparison with the extent observed in group 1.

PGZ also significantly decreased triglyceride content in the liver (Fig. 2C), indicating that PGZ resolves the fatty changes in liver tissues induced by the CDAA diet.

3.3. Histological and biochemical analysis during development of liver cirrhosis (16-week model)

In group 3 animals, livers were enlarged, yellowish, and multinodular (Fig. 3A-a). Marked liver fibrosis was observed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Serum insulin
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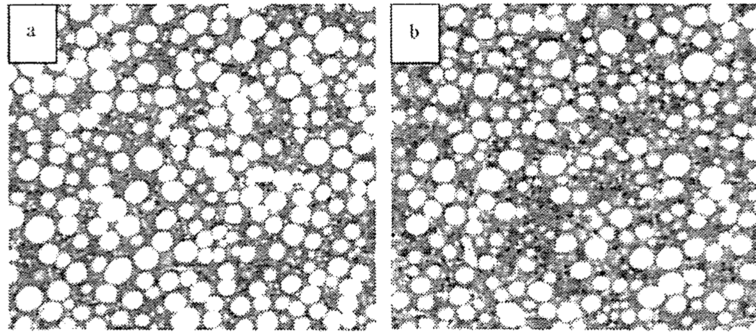
a
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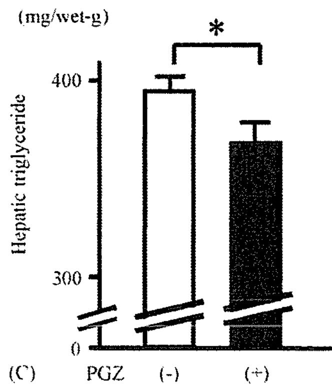
(mg/wet-g)
400
300
0
Hepatic triglyceride
*
(C) PGZ (-) (+)

after a 16-week administration of a CDAA diet (Fig. 3A–c). In group 4, a 4-week PGZ treatment during weeks 12–16 significantly decreased liver fibrosis (Fig. 3A–b and A–d). PGZ treatment also decreased type I procollagen α2 mRNA expression (Fig. 3B) and hepatic TGF-β1 concentration (Fig. 3C). Although serum levels of ALT and glucose were indistinguishable between groups 3 and 4, serum concentrations of insulin and hyaluronic acid were significantly reduced in comparison to control (non-PGZ-treated) rats given a CDAA diet (Fig. 3D). These results suggest that PGZ resolved hepatic fibrosis.

3.4. ASMA expression in the liver of rats fed a CDAA diet and the effects of PGZ treatment (16-week model)

We examined the effects of PGZ on hepatic stellate cell (HSC) activation during the development of fibrosis using immunohistochemical analysis of ASMA expression. Although the number of activated HSCs expressing ASMA increased during administration of the CDAA diet, PGZ treatment from weeks 12 to 16 dramatically reduced the number of ASMA-positive cells in the liver (Fig. 4A). In addition, the expression of ASMA protein decreased in the liver at week 16 (Fig. 4B).

These results suggest that treatment with PGZ ameliorates CDAA diet-induced liver fibrosis via suppression of HSC activation and proliferation.

4. Discussion

The pathological changes of the livers of rats fed the CDAA diet, characterized by diffuse fatty infiltration and inflammation followed by development of cirrhosis and HCC, are similar to those changes seen in patients with NASH. Additionally, increases in hepatic mRNA expression and serum level of TNF-α were observed in these rats compared with normal rats (data not shown). TNF-α is known to play an important role in the development of hepatic steatosis; patients with NASH exhibit significant increases in serum TNF-α [17]. Therefore, although the pathogenic mechanisms underlying NASH are not well understood, a rat animal model fed a CDAA diet is considered to be an appropriate model to investigate the pathogenesis of NASH and examine potential therapeutic agents.

In this study, we demonstrated that PPAR-γ expression was upregulated in the livers of rats fed a CDAA diet. The expression of PPAR-γ is closely associated with the development of fatty liver and hepatic steatosis [18–21]. Schadinger et al. [22] recently reported that PPAR-γ2 induces steatosis in hepatocytes via pathways regulating de novo lipid synthesis. Nakae et al. [6] have reported that oxidative DNA damage induced by the CDAA diet was significantly greater than that resulting from a choline-deficient diet. As oxidative signals induce and activate PPAR-γ [23], the induction of oxidative stress in the livers of rats fed the CDAA diet likely stimulates hepatocytes expression of PPAR-γ. In agreement with previous studies demonstrating a role for oxidative stress in human NASH [24,25], the expression of PPAR-γ may be associated with NASH development. Therefore, rats fed a CDAA diet are a suitable experimental model for NASH. In addition, PPAR-γ expression may be a potential therapeutic target for the treatment of human NASH.

As NASH is associated with insulin resistance, preliminary data using PPAR-γ agonists to improve this condition in patients with NASH have suggested a potential reversal of the adverse effects of NASH on the liver through selective modulation of PPAR-γ activity [26–28]. These reports demonstrated that hepatic fat content was decreased and hepatic fibrosis was significantly improved in patients with NASH treated with PPAR-γ agonists (PGZ or rogiglitazone) for 48 weeks. In addition, serum insulin levels, but not serum glucose levels, were reduced by PGZ administration in humans and rats [27,29]. The presence of insulin resistance in our experimental model appears to be negative; serum levels of glucose and insulin did not differ between rats fed a normal diet and those fed a CDAA diet for 1 week, respectively (data not shown). Serum insulin levels, however, were significantly reduced in PGZ-treated rats, despite unaltered serum glucose levels (Fig. 2A), indicating that insulin sensitivity is increased in these animals. These results also suggest that PGZ modulates PPAR-γ activity in human NASH patients in a manner similar to that seen in our experimental model.

While PPAR-γ agonists have improved ALT levels and steatosis in NASH patients [27–29], these agonists have also been reported to induce liver injury [30,31]. In this report, however, it was not observed any alterations in serum ALT levels which result was similar to other report [32]. Previously, we demonstrated an increased apoptosis of hepatocytes in rats fed a CDAA diet, which did not correlate with changes in serum ALT levels [16]. Hepatocyte apoptosis is also significantly increased in patients with NASH [33,34]. In addition, PPAR-γ agonists inhibit the production of monocyte inflammatory cytokine, which may contribute to tissue damage [35]. In this study, serum levels of TNF-α were significantly reduced by PGZ administration. As TNF-α contributes to the

Fig. 2. Serum markers, histopathological findings and hepatic triglyceride levels during fatty liver development after a 2-week administration of the CDAA diet. (A) Treatment with PGZ significantly reduced serum levels of insulin, while serum glucose and ALT levels were unaffected by treatment (with or without a 1-week treatment with PGZ; $n = 4$). Serum TNF-α expression was also significantly reduced by a 1-week treatment with PGZ ($n = 8$) compared with untreated CDAA-fed controls ($n = 8$) ($^{*}P < 0.05$). (B) Representative photomicrographs of liver from treated animals are shown. Two-week administration of the CDAA diet resulted in a marked fat accumulation (a). A 1-week administration of PGZ slightly reversed this fat accumulation (b) (HE staining, original magnification ×100). (C) Hepatic triglyceride levels were significantly reduced by a 1-week treatment with PGZ beginning at week 2 ($n = 4$) in comparison with untreated CDAA-fed controls ($n = 4$) ($^{*}P < 0.05$).

induction of hepatocyte apoptosis, PGZ treatment may reduce hepatocyte apoptosis without altering serum ALT levels especially in this model. Furthermore, the differences between rat and human, variations in the amount of specific cofactors, or the presence of different isoforms of PPAR-γ [23,36] may be due to different effect of PGZ on serum ALT levels.

PGZ administration reduced hepatic triglyceride levels in rats fed a CDAA diet. Although the mechanisms by which PPAR-γ agonists reduce hepatic triglyceride content have not been clarified, PPAR-γ regulates the expression of genes involved in adipocyte differentiation and lipid storage. PPAR-γ agonists reduced the total lipid content of the livers of ob/ob mice or alcohol administered rat [37]. PPAR-γ agonists up-regulate the expression of lipid-related genes, including fatty acid transporters and uncoupling protein (UCP)-2 [38], and down-regulate the expression of leptin. In addition, non-parenchymal cells, such as Kupffer cells, may be a potential pharmaceutical target of PGZ in the early stages of hepatocarcinogenesis in rats fed a CDAA diet [39]. Prostaglandin E2 derived from activated Kupffer cells results in triglyceride accumulation in the liver; PPAR-γ agonists suppress Kupffer cell activation [40,41]. Increases in the hepatic mRNA

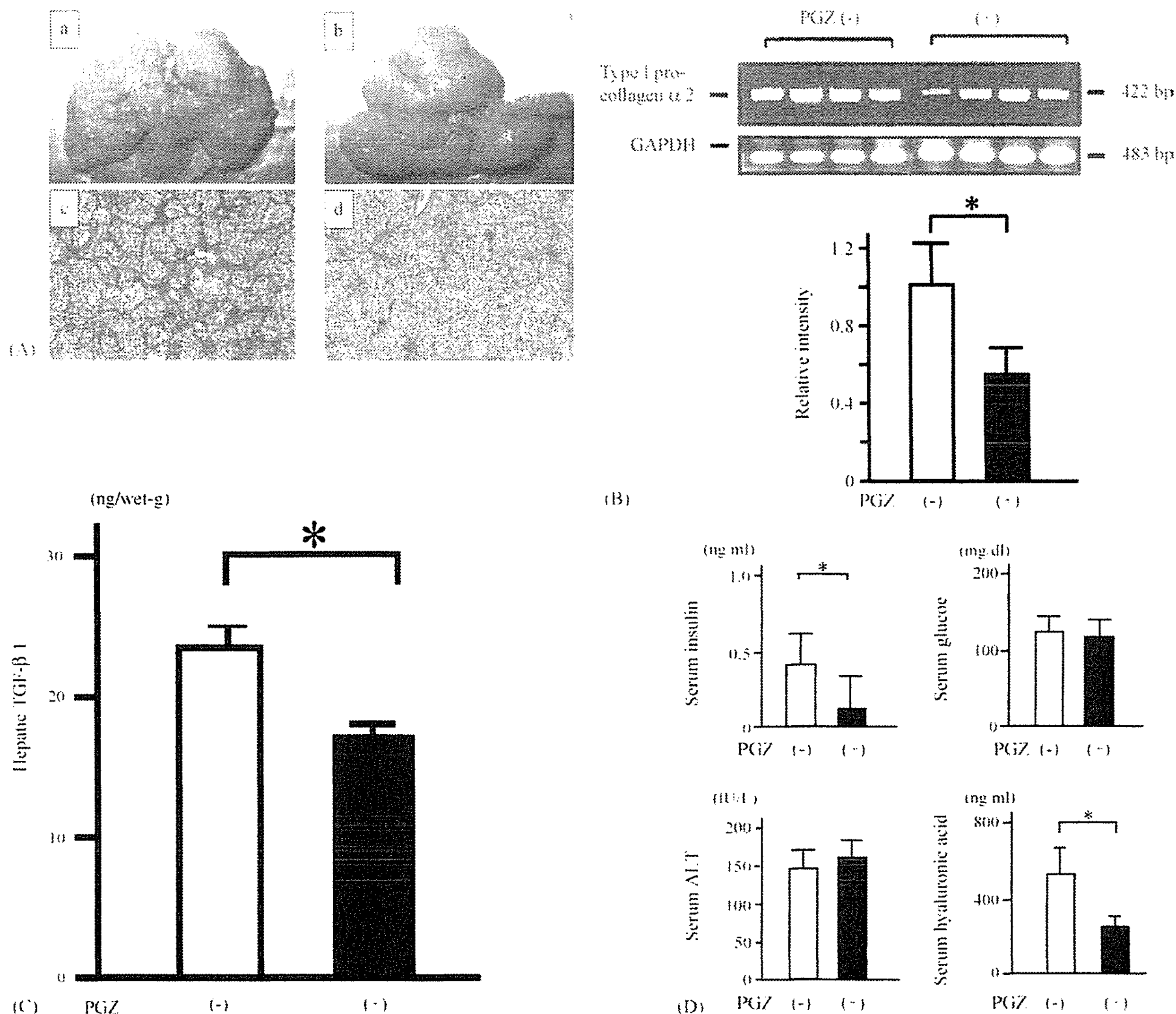

Fig. 3. Histopathological findings, serum markers, type I procollagen α2 mRNA and hepatic TGF-β1 protein levels during the development of liver cirrhosis. (A) Liver tissues were obtained from rats fed a CDAA diet for 16 weeks with (b and d) or without (a and c) PGZ treatment during the final 4 weeks. CDAA diet administration resulted in the development of multinodular liver (a) and liver fibrosis (c). PGZ treatment from weeks 12 to 16 decreased liver surface nodules (b) and fibrosis (d). (a and c) Representative macroscopic appearance of livers. (c and d) Representative photomicrographs of liver sections (Azan staining, original magnification ×40). (B and C) PGZ treatment during weeks 12–16 decreased type I procollagen α2 mRNA expression (B) and hepatic TGF-β1 protein content (C) in the liver at week 16 (with or without PGZ; $n = 4$) ($^*P < 0.05$). (D) PGZ treatment during weeks 12–16 also significantly decreased serum concentrations of insulin and hyaluronic acid at week 16 (with or without 4 weeks of PGZ treatment; $n = 4$) ($^*P < 0.05$). Neither serum glucose nor ALT levels differed between these two groups.

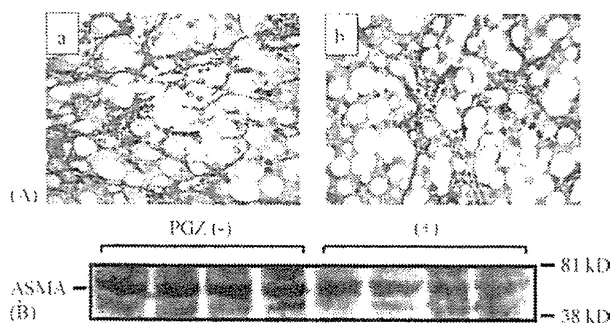

Fig. 4. The expression of α-smooth muscle actin (ASMA). (A and B) Immunohistochemical (A) and Western blot analysis (B) of ASMA examined the effects of PGZ treatment on fibrosis development. Liver tissues were obtained from rats fed a CDAA diet for 16 weeks with (b) or without (a) PGZ treatment during weeks 12–16 ($n = 4$). (A) Representative photomicrographs of liver sections are shown (original magnification ×100). PGZ treatment reduced the number of ASMA-positive cells (b). (B) PGZ treatment also decreased the overall expression of ASMA protein in the liver at week 16.

expression of TNF-α in CDAA treated-rats in comparison to the levels observed in normal rats (data not shown) might also be associated with Kupffer cell activation. As adipocyte differentiation, lipid storage, apoptosis and Kupffer cell activation may contribute to the development of fatty liver and hepatocyte injury and the ability to influence these processes with agonists appears to be a therapeutic approach.

Kawaguchi et al. [32] recently reported that PGZ improved hepatic steatosis and prevented liver fibrosis in rats fed a CDAA diet. As the PGZ was mixed into CDAA chow and was administered from the beginning of CDAA feeding, the daily dose of PGZ could not be evaluated. In this study, we administered 1.0 mg/(kg day) of PGZ, an identical dose as that used for the treatment for patients with type 2 diabetes mellitus (approximately 0.5–1.0 mg/(kg day)). Using a rat model of NASH, this study demonstrated that clinical doses of PGZ effectively reduced steatosis and liver fibrosis, hepatic levels of TGF-β1, and the expression of type I procollagen α2 mRNA. In addition, serum hyaluronic acid levels, which reflect the degree of hepatic fibrosis in patients with chronic liver disease such as NASH [17], were also reduced in this model following PGZ treatment. These data document that clinical use of PGZ for NASH is both practical and effective.

We have also demonstrated that PGZ induced an anti-fibrotic effect in a rat model of liver fibrosis induced by administration of the CDAA diet. Oral administration of PPAR-γ agonists has been reported to reduce extracellular matrix deposition and HSC activation in both toxic and cholestatic models of liver fibrosis in vitro and in vivo [11,42]. PPAR-γ agonists inhibit the proliferation of HSCs; increased levels of PPAR-γ inhibit TGF-β1-induced collagen synthesis [11]. TGF-β plays an important role in liver fibrogenesis and activates HSC autocrine mechanisms [43]. While the expression of TGF-β1 is stimulated during liver fibrogenesis in rats fed a CDAA diet [16], TGF-β1 expression was suppressed by PGZ treatment in this study. Furthermore, PGZ treatment decreased the expression of ASMA, as assessed by immunohistochemical analysis and Western blot analysis. Although PGZ administration did not significantly affect PPAR-γ expression in liver tissues at 16 weeks of CDAA diet (data not shown), a decrease in hepatic expression of TGF-β1 and ASMA is likely due to the inhibition of HSC proliferation and activation by PGZ.

In this study, we demonstrate that PPAR-γ expression is stimulated in the livers of rats fed a CDAA diet. Administration of a clinical dose of a PPAR-γ agonist, PGZ, effectively reduced fatty accumulation in the liver tissues and inhibited the development of cirrhosis. As rats fed a CDAA diet are an appropriate experimental model for the liver phenotype exemplified in NASH, PGZ may be a useful therapy for patients with NASH. Further investigation will be needed to clarify the role of PPAR-γ in NASH development and to evaluate the effect of other PPAR-γ agonists on hepatic steatosis and fibrosis.

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References

[1] Bugianesi E, Leone N, Vanni E, et al. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 2002;123:134–40.

[2] Shimada M, Hashimoto E, Taniai M, et al. Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. J Hepatol 2002;37:154–60.

[3] Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. Hepatology 1990;12:1106–10.

[4] Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. Hepatology 1999;30:1356–62.

[5] Marchesini G, Brizi M, Marsell-Labate AM, et al. Association of nonalcoholic fatty liver disease with insulin resistance. Am J Med 1999;107:450–5.

[6] Nakae D, Yoshiji H, Maruyama H, Kinugasa T, Denda A, Konishi Y. Production of both 8-hydroxydeoxyguanosine in liver DNA and γ-glutamyltransferase-positive hepatocellular lesions in rats given a cholin-deficient, L-amino acid-defined diet. Jpn J Cancer Res 1990;81:1081–4.

[7] Nakae D, Yoshiji H, Mizumoto Y, et al. High incidence of hepatocellular carcinomas induced by a cholin-deficient, L-amino acid-defined diet in rats. Cancer Res 1992;15:5042–5.

[8] Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 1994;79:1147–56.

[9] Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev 1999;20:649–50.

[10] Miyahara T, Scherum L, Rippe R, et al. Peroxisome proliferator-activated receptors and hepatic stellate cell activation. J Biol Chem 2000;275:35715–22.
[11] Galli A, Crabb DW, Ceni E, et al. Antidiabetic thiazolidinedions inhibit collagen synthesis and hepatic stellate cell activation in vivo and in vitro. Gastroenterology 2002;122:1924–40.
[12] Dooley S, Hamzavi J, Breitkopf K, et al. Smad 7 prevents activation of hepatic stellate cells and liver fibrosis in rats. Gastroenterology 2003;125:178–91.
[13] Yoshiji H, Kuriyama S, Yoshii J, et al. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. Hepatology 2001;34:745–50.
[14] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509.
[15] Danielpour D. Improved sandwich enzyme-linked immunosorbent assays for transforming growth factor beta 1. J Immunol Methods 1993;158:17–25.
[16] Onaga M, Ido A, Hasuike S, et al. Enhanced expression of growth factors and imbalance between hepatocyte proliferation and apoptosis in the livers of rats fed a choline-deficient, L-amino acid-defined diet. Hepatol Res 2004;28:94–101.
[17] Kugelmas M, Hill DB, Vivian B, Marsano L, McClain CJ. Cytokines and NASH: a pilot study of the effects of lifestyle modification and vitamin E. Hepatology 2003;38:413–9.
[18] Vidal-Puig A, Jimenez-Lian M, Lowell BB, et al. Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. J Clin Invest 1996;97:2553–61.
[19] Buramt CF, Sreena S, Hirano K, et al. Troglitazone action is independent of adipose tissue. J Clin Invest 1997;100: 2900–8.
[20] Chao L, Marcus-Samuels B, Mason MM, et al. Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. J Clin Invest 2000;106:1221–8.
[21] Yu S, Matsusue K, Kashireddy P, et al. Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor γ1 (PPARγ1) overexpression. J Biol Chem 2003;278:498–505.
[22] Schadinger SE, Bucher NLR, Schreiber BM, Farmer SR. Am J Physiol Endcrinol Metab 2005 [Epub ahead of print].
[23] Iwashima Y, Eto M, Horinouchi S, Sano H. Advanced glycation end product-induced peroxisome proliferator-activated-γ receptor gene expression in the cultured mesangial cells. Biochem Biophys Res Commun 1999;264:441–8.
[24] Lieber CS. CYP2E1: from ASH to NASH. Hepatol Res 2004;28:1–11.
[25] Weltman MD, Farrell GC, Hall P, Ingelman-Sundberg M, Liddle C. Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis. Hepatology 1998;27:128–33.
[26] Promrat K, Lutchman G, Uwaifo GI, et al. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. Hepatology 2004;39:188–96.
[27] Neuschwander-Tetri BA, Brunt EM, Wehmeier KR, Sponseller CA, Hampton K, Bacon BR. Interim results of a pilot study demonstrating the early effects of the PPAR-γ ligand rosiglitazone on insulin sensitivity, aminotransferases, hepatic steatosis and body weight in patients with non-alcoholic steatohepatitis. J Hepatol 2003;38:434–40.
[28] Neuschwander-Tetri BA, Brunt EM, Kehmeier KR, Oliver D, Bacon BR. Improved nonalcoholic steatohepatitis after 48 weeks of treatment with the PPAR-γ ligand rosiglitazone. Hepatology 2003;38:1008–17.
[29] Ikeda H, Taketomi Y, Sugiyama Y, et al. Effects of pioglitazone on glucose and lipid metabolism in normal and insulin resistant animals. Drug Res 1990;40:156–62.
[30] Forman LM, Simmons DA, Diamond RH. Hepatic failure in a patient taking rosiglitazone. Ann Intern Med 2000;132:118–21.
[31] May LD, Lefkowitch JH, Kram MT, Rubin DE. Mixed hepatocellular-cholestatic liver injury after pioglitazone. Ann Intern Med 2002;136:449–52.
[32] Kawaguchi K, Sakaida I, Tsuchiya M, Omori K, Takami T, Okita K. Pioglitazone prevents hepatic steatosis, fibrosis, and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. Biochem Biophys Res Commun 2004;315:187–95.
[33] Feldstein AE, Canbay A, Angulo P, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology 2003;205:437–43.
[34] Ribeiro PS, Cortez-Pinto H, Sola S, et al. Hepatocyte apoptosis, expression of death receptors, and activation of NF-kappaB in the liver of nonalcoholic and alcoholic steatohepatitis patients. Am J Gastroenterol 2004;99:708–17.
[35] Jiang C, Ting AT, Seed B. PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. Nature 1998;391:82–6.
[36] Camp HS, Li O, Wise SC, et al. Differential activation of peroxisome proliferator-activated receptor-γ by troglitazone and rosiglitazone. Diabetes 2000;49:539–47.
[37] Memon RA, Tecott LH, Nonogaki K, et al. Up-regulation of peroxisome proliferator-activated receptors (PPAR-alpha) and PPAR-gamma messenger ribonucleic acid expression in the liver in murine obesity: troglitazone induces expression of PPAR-gamma-responsive adipose tissue-specific genes in the liver of obese diabetic mice. Endocrinology 2000;141:4021–31.
[38] Spiegelman BM. PPAR-γ; adipogenic regulator and thiazolidinedione receptor. Diabetes 1998;47:507–14.
[39] Denda A, Kitayama W, Murata A, et al. Increased expression of cyclooxygenase-2 protein during rat hepatocarcinogenesis caused by a choline-deficient, L-amino acid-defined diet and chemopreventive efficacy of a specific inhibitor, nimesulide. Carcinogenesis 2002;23:245–56.
[40] Enomoto N, Ikejima K, Yamashina S, et al. Kupffer cell-derived prostaglandin E2 is involved in alcohol-induced fat accumulation in rat liver. Am J Physiol 2000;279:G100–6.
[41] Uchimura K, Nakamuta M, Enjoji M, et al. Activation of retinoic X receptor and peroxisome proliferator-activated receptor-γ inhibits nitric oxide and tumor necrosis factor-α production in rat Kupffer cells. Hepatology 2001;33:91–9.
[42] Kon K, Ikejima K, Hirose M, et al. Pioglitazone prevents early-phase hepatic fibrogenesis caused by carbon tetrachloride. Biochem Biophys Res Commun 2002;291:55–61.
[43] Tahashi Y, Matsuzaki K, Date M, et al. Differential regulation of TGF-beta signal in hepatic stellate cells between acute and chronic rat liver injury. Hepatology 2002;35:49–61.

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.