2000; Tanaka et al., 2001]. The extent to which the TTV sequence has been preserved varies by the genomic region; the coding region is variable, whereas the noncoding region is well conserved. Hence, the genomic areas that are selected for designing primers for polymerase chain reaction (PCR) amplification of TTV DNA, considerably influence the rate of detection of TTV DNA by PCR [Takahashi et al., 1998; Itoh et al., 1999]. Namely, PCR using primers based on the sequence of a coding region (N22 PCR) can detect essentially TTV of genotypes 1–6 in group 1, but PCR using primers based on the sequence of a noncoding region (UTR PCR) can detect nearly all genotypes/genetic groups [Okamoto et al., 1998b, 2000b]. Accumulating lines of evidence indicate that TTV is acquired in early childhood [Davidson et al., 1999; Gerner et al., 2000], and that it is prevalent in adults, who are co-infected frequently with TTVs of distinct genotypes/genetic groups [Ball et al., 1999; Okamoto et al., 1999b; Niel et al., 2000].

The role of TTV in hepatic diseases has not yet been defined, and remains controversial [Charlton et al., 1998; Tanaka et al., 1998; Gimenez-Barcons et al., 1999; Ikeda et al., 1999; Matsumoto et al., 1999; Tagger et al., 1999; Zein et al., 1999; Trimoulet et al., 2000; Yoshida et al., 2000; Moriyama et al., 2001; Tokita et al., 2001c]. However, it was reported recently that the TTV viral load may reflect the immune status of patients infected with human immunodeficiency virus type 1 (HIV), and serve as independent predictors of survival [Christensen et al., 2000; Shibayama et al., 2001]. Therefore, the aims of the present study were to investigate the prevalence and titer of TTV DNA, and to examine the relationship between the extent of TTV viremia and the complications of cirrhosis and hepatocellular carcinoma (HCC) among 237 patients with hepatitis C virus (HCV)-related chronic liver disease.

## MATERIALS AND METHODS

### Patients

This study included 237 patients with HCV-related chronic liver disease [115 males and 122 females; age, $63 \pm 10$ years (mean ± standard deviation, SD; range 28–91 years), 147 patients without cirrhosis, and 90 patients with cirrhosis, who were seen in the Department of Gastroenterology at National Tokyo Hospital between July and December 2000]. Among 42 patients with HCC, 12 patients had accompanying HCC at the first coming to our department and followed for $21 \pm 22$ months (range, 0–60 months). Among 32 cirrhotic patients who had HCC, 23 patients developed HCC $57 \pm 35$ months (range, 2–120 months) after being followed for cirrhosis. The diagnosis of chronic HCV infection was based on continuous positivity for second-generation antibodies to HCV (Dainabot, Tokyo, Japan) and positivity for HCV RNA (Amplicor HCV kit: Nippon Roche, Tokyo, Japan) in the serum for more than 6 months.

The diagnoses of noncirrhotic chronic hepatitis and cirrhosis were based on abnormalities of the liver function tests and liver imaging (ultrasonography/computed tomography) and/or liver histological examination at the time of TTV determination. The diagnosis of HCC was made using liver imaging (ultrasonography, computed tomography, or magnetic resonance imaging) and/or angiography and/or tumor biopsy. The following patients were excluded: patients who had received interferon therapy within 1 year before TTV DNA measurement; patients who had a history of blood transfusion for the treatment of liver disease; patients with ongoing hepatitis B virus (HBV) infection who were positive for hepatitis B surface antigen and/or had high-titer antibodies against hepatitis B core antigen; patients with autoimmune liver disease; and patients with a known daily alcohol intake of >80 g. No patient had antibodies to HIV (Dainabot). This study was approved by the Ethics Committee at the National Tokyo Hospital, and informed consent was obtained from each patient.

### Measurements of Various Parameters

The serum hyaluronan level was determined by the sandwich enzyme-binding protein assay reported by Chichibu et al. [1989]. The serum type IV collagen level was determined by the enzyme immunoassay reported by Obata et al. [1989]. The spleen size was measured by ultrasonography using the method of Matsutani et al. [1991]. Briefly, on the section that contained the splenic hilum and the largest area of spleen, the distance between the hiler indentation and anterior cranial end (A cm) and length of the line perpendicular to it (B cm) were measured. The product of $A \times B$ was used as spleen size ($cm^2$).

### Quantitation of HCV RNA and Determination of HCV Genotypes

Quantitation of HCV RNA was carried out by a commercially available kit (Amplicor HCV Monitor assay, version 2.0; Nippon Roche). HCV genotypes of 1a, 1b, 2a, 2b, and 3a were determined by the method described previously [Okamoto et al., 1993] with a slight modification. In brief, the method included 2 stages: amplification of a C region of the HCV genome by PCR using universal primer pairs in the first stage; and selective amplification of the first stage PCR products using a nested pair of a genotype-specific antisense primer and a universal sense primer in the second stage. The original genotype 1b-specific antisense primer (No. 133) was replaced by another primer, No. 492 [Holland et al., 1996]. The product from the second stage PCR was electrophoresed in agarose gel, and HCV genotypes were identifiable by the length of the amplified sequence because the genotype-specific primers used in the second stage were chosen to amplify a different size sequence for each genotype (49 base pairs [bp] for genotype 1a, 144 bp for 1b, 174 bp for 2a, 123 bp for 2b, and 88 bp for 3a).

### Detection and Quantitation of TTV DNA

From 100 μl of the serum sample, nucleic acids were extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany), and dissolved in 50 μl of nuclease-free distilled water. The extracted nucleic acids corresponding to 10 μl of the serum served as the template for the detection of TTV DNA by PCR.

Two different PCR methods for the detection of TTV DNA (N22 PCR and UTR PCR) were used [Okamoto et al., 1998b, 2000b]. N22 PCR can detect primarily TTV of genotypes 1–6 classifiable in group 1, whereas UTR PCR can detect essentially all TTV genotypes in groups 1–5 [Muljono et al., 2001; Shibayama et al., 2001; Peng et al., 2002]. N22 PCR was performed using Perkin-Elmer AmpliTaq DNA Polymerase (Roche Molecular Systems, Inc., Branchburg, NJ) and semi-nested primers, NG059 and NG063 in the first round and NG061 and NG063 in the second round, which were derived from the N22 region [Nishizawa et al., 1997] in the center of open reading frame 1 (ORF1) of TTV genotypes 1 and 2 [Okamoto et al., 1998a], by the method described elsewhere [Okamoto et al., 1998b]. The amplification product of the first-round PCR was 286 bp, and that of the second-round PCR was 271 bp.

UTR PCR was carried out in the presence of Perkin-Elmer AmpliTaq Gold (Roche Molecular Systems) and nested primers by the method described previously [Okamoto et al., 2000b], with slight modifications. Briefly, primers NG472 (sense: 5′-GCG TCC CGW GGG CGG GTG CCG-3′ [W = A or T]) and NG352 (antisense: 5′-GAG CCT TGC CCA TRG CCC GGC CAG-3′ [R = A or G]) were used for the first-round PCR, and primers NG473 (sense: 5′-CGG GTG CCG DAG GTG AGT TTA CAC-3′ [D = G, A or T]) and NG351 (antisense: 5′-CCC ATR GCC CGG CCA GTC CCG AGC-3′) were used for the second-round PCR, which were derived from the same well-conserved area in the UTR of the TTV genome as in the original method [Okamoto et al., 2000b]. The size of the amplification product of the first-round PCR was 91 bp, and that of the second-round PCR was 71 bp.

TTV DNA was quantitated by real-time detection PCR using 5 μl of the nucleic acid solution as a template, primers NG473-NG352, a doubly labeled probe [NG369-P: 5′-(Fam)-AGT CAA GGG GCA ATT CGG GCT CGG GA-(Tamra)-3′], and the LightCycler-Fast-Start DNA Master Hybridization Probes kit (Roche Diagnostics GmbH). This method is based on real-time glass capillary thermocycling and fluorescence resonance energy transfer. PCR amplification was started with an initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 10 s and annealing-extension at 62°C for 30 s. All reactions were carried out in a LightCycler Apparatus (Roche Diagnostics GmbH). The quantification limit of the system was 3 to 5 copies per test capillary (20 μl of reaction mixture). Intra- and inter-assay reproducibility was determined by testing on 5 different days 10 independent DNA extractions of two reference TTV positive sera, the %CV (coefficient of variance based on the value of crossing point on the LightCycler) being 0.7 and 1.8, respectively. Overall variation was less than 0.53 log. Assay specificity was confirmed by sequencing the amplicons of randomly selected samples. All samples were tested in duplicate from independent extractions and mean values are shown in Results.

The genomic DNA of TTV-like mini virus (TLMV), which has a smaller genome (2.8–3.0 kb) [Takahashi et al., 2000] than TTV, was considered to be not amplifiable by this real-time PCR method, since the sequences of primers NG473 and NG352 and a probe NG369-P used differed by 33–67% (2–4 of the 3′-terminal 6 nucleotides), 54–63% (13-15 of the entire 24 nucleotides), and 12–23% (3–6 of the entire 26 nucleotides), respectively, from those of the corresponding areas of the 12 TLMV isolates with the known full-length sequence, which were available from the DDBJ/GenBank/EMBL databases as of October 19, 2001.

### Statistical Analysis

Statistical analysis was carried out using the Mann-Whitney U-test for comparison of continuous variables between two groups, and the Fisher's exact test or $\chi^2$-test for comparison of proportions between two groups. The multivariate logistic regression model was used to assess the independency of high TTV viremia as a risk factor for HCC. Differences were considered to be statistically significant at $P < 0.05$. Data are presented as mean ± SD.

## RESULTS

### Comparison of Various Features Between TTV DNA-Positive and-Negative Patients

Two hundred nineteen (92%) of the 237 patients with HCV-related chronic liver disease were positive for TTV DNA detectable by UTR PCR. When the background features of the TTV DNA-positive and-negative groups were compared for the 18 variables listed in Table I, only past history of interferon treatment (37% vs. 67%, $P < 0.05$) was significantly different between the two groups.

Among all 237 patients studied, 72 (30%) were positive for TTV DNA detectable by N22 PCR: patients who were negative for TTV DNA detectable by UTR PCR were also negative for TTV DNA detectable by N22 PCR. The features listed in Table I were also compared between the N22 PCR-positive group (n = 72) and the N22 PCR-negative group (n = 165). Past history of blood transfusion was observed significantly more frequently in the N22 PCR-positive group than in the-negative group (71% vs. 55%, $P < 0.05$). Conversely, past history of interferon treatment was significantly less frequently observed in the N22 PCR-positive group than in the -negative group (29% vs. 44%, $P < 0.05$). However, other features, namely, the complication of cirrhosis or HCC, HCV RNA titer, and platelet count, did not differ

TABLE I. Comparison of Various Features Between the UTR PCR-Positive and -Negative Groups Among the 237 Patients With HCV-Related Chronic Liver Disease

| Feature[a] | UTR PCR-positive (n = 219) | UTR PCR-negative (n = 18) | *P* value* |
|---|---|---|---|
| Age (year) | 64 ± 10 | 58 ± 14 | NS (0.09)[b] |
| Sex (male/female) | 109/110 | 6/12 | NS (0.22)[c] |
| Past history of blood transfusion (+) | 132 (60%) | 10 (56%) | NS (0.80)[c] |
| Duration after blood transfusion (year) | 36 ± 9 (n = 132) | 34 ± 13 (n = 10) | NS (0.97)[b] |
| Past history of interferon treatment (+) | 82 (37%) | 12 (67%) | <0.05[c] |
| Liver cirrhosis (+) | 82 (37%) | 8 (44%) | NS (0.62)[c] |
| Hepatocellular carcinoma (+) | 40 (18%) | 2 (11%) | NS (0.75)[c] |
| HCV RNA titer (K IU/ml) | 681.1 ± 502.9 | 779.9 ± 420.5 | NS (0.25)[b] |
| High HCV load ≥100 K IU/ml | 193 (88%) | 17 (94%) | NS (0.70)[c] |
| HCV genotype (1b/2a/2b) | 172/29/18 | 15/2/1 | NS (0.88)[d] |
| AST (IU/l) | 64 ± 43 | 68 ± 42 | NS (0.45)[b] |
| ALT (IU/l) | 66 ± 52 | 80 ± 45 | NS (0.28)[b] |
| γ-globulin (g/dl) | 1.8 ± 0.6 | 1.7 ± 0.5 | NS (0.37)[b] |
| Hyaluronan (ng/ml) | 242.7 ± 324.2 | 246.6 ± 262.6 | NS (0.79)[b] |
| Type IV collagen (ng/ml) | 187.0 ± 106.3 | 172.0 ± 72.5 | NS (0.77)[b] |
| Platelet count ($\times 10^4$/μl) | 12.9 ± 5.6 | 12.9 ± 5.6 | NS (0.97)[b] |
| AFP (ng/ml) | 610.9 ± 6255.3 | 105.0 ± 312.4 | NS (0.87)[b] |
| Spleen size ($cm^2$) | 18.4 ± 10.8 | 19.1 ± 9.9 | NS (0.65)[b] |

*NS, not significant.
[a]Abbreviations (normal range) are: AST, aspartate aminotransferase (9–31 IU/l); ALT, alanine aminotransferase (4–34 IU/l); AFP, α-fetoprotein (<10 ng/ml); and normal ranges are: γ-globulin, 0.8–2.0 g/dl; hyaluronan, <50.0 ng/ml; type IV collagen, <137.0 ng/ml; platelet count, 15–30 $\times 10^4$/μl; spleen size, <20 $cm^2$.
[b]Mann-Whitney U-test.
[c]Fisher's exact test.
[d]$\chi^2$-test.

significantly between the N22 PCR-positive and N22 PCR-negative groups. Of note, among the 147 non-cirrhotic patients, the platelet count was significantly lower in the N22 PCR-positive group (n = 46) than in the-negative group (n = 101) (13.9 ± 4.3 vs. 16.4 ± 4.9 × $10^4$/μl, $P < 0.005$), corroborating a previous report [Tokita et al., 2001c].

### Comparison of Various Features of the 237 Patients According to the TTV Viremia Level

Among the 219 patients with HCV-related chronic liver disease who were positive for TTV DNA detectable by UTR PCR, the TTV viremia ranged from $1.3 \times 10^2$ to $2.1 \times 10^6$ copies/ml (median, $1.6 \times 10^4$ copies/ml; mean ± SD, $6.2 \times 10^4 \pm 1.8 \times 10^5$ copies/ml), and 132 (56% of the total) patients had a high TTV viremia of $\geq 10^4$ copies/ml. Various features were compared between the patients with a high TTV viral load (n = 132) and those with low TTV viral load or who were negative for TTV DNA (n = 105) (Table II). When the N22 PCR status was compared between the two groups, the N22 PCR-positive rate was significantly higher in the High TTV Viral Load group (45% vs. 11%, $P < 0.0001$). As indicated in Table II, high TTV viral load was significantly associated with several features including older age ($P < 0.05$), more frequent past history of blood transfusion ($P < 0.001$), less frequent past history of interferon treatment ($P < 0.005$), complication of cirrhosis ($P < 0.05$) and HCC ($P < 0.0005$), lower HCV RNA titer ($P < 0.05$), less frequent HCV RNA titer of ≥ 100 K IU/ml ($P < 0.05$) or lower platelet count ($P < 0.01$).

### Comparison of Various Features Between the Patients Who Did or Did Not Have HCC Among All 237 Patients or the 90 Cirrhotic Patients

Among the 237 patients with HCV-related chronic liver disease, 42 (18%) had accompanying HCC. Table III compares various features of the patients who did (n = 42) or did not have HCC (n = 195). The positive rate of TTV DNA detectable by N22 PCR or UTR PCR did not differ significantly between the two groups. However, high TTV viremia was significantly more frequent among the patients with HCC than among those without HCC (81% vs. 50%, $P < 0.0005$). The TTV viral load was significantly higher in the patients with HCC than in those without HCC ($1.3 \times 10^5 \pm 3.8 \times 10^5$ vs. $4.6 \times 10^4 \pm 9.6 \times 10^4$ copies/ml, $P < 0.0005$). Furthermore, HCC was significantly associated with higher age ($P < 0.0001$), longer duration after blood transfusion ($P < 0.05$), complication of cirrhosis ($P < 0.0001$), lower HCV RNA titer ($P < 0.01$), lower frequency of high HCV viremia ($P < 0.05$), higher levels of AST ($P < 0.001$), γ-globulin ($P < 0.001$), hyaluronan ($P < 0.0001$), Type IV collagen ($P < 0.0001$) and α-fetoprotein ($P < 0.0001$), lower platelet count ($P < 0.0001$), and larger spleen size ($P < 0.0001$).

Similarly, various features were compared between the 90 cirrhotic patients who did (n = 32) or did not (n = 58) have HCC. The occurrence of HCC among the cirrhotic patients was significantly associated with high TTV viremia ($P < 0.05$), and higher levels of hyaluronan ($P < 0.05$), Type IV collagen ($P < 0.05$) and α-fetoprotein ($P < 0.0001$), but not with past history of blood transfusion, duration after blood transfusion, and higher age.

TABLE II. Comparison of Various Features of the Patients With HCV-Related Chronic Liver Disease According to the Viremia Level of TTV Detectable by UTR PCR

| Feature[a] | UTR PCR titer | | P value* |
|---|---|---|---|
| | $\geq 10^4$ copies/ml (n = 132) | $<10^4$ copies/ml (n = 105) | |
| Age (year) | 65 ± 9 | 61 ± 12 | <0.05[b] |
| Sex (male/female) | 69/63 | 46/59 | NS (0.24)[c] |
| Past history of blood transfusion (+) | 92 (70%) | 50 (48%) | <0.001[c] |
| Duration after blood transfusion (year) | 37 ± 10 (n = 92) | 34 ± 10 (n = 50) | NS (0.14)[b] |
| Past history of interferon treatment (+) | 40 (30%) | 54 (51%) | <0.005[c] |
| Liver cirrhosis (+) | 60 (45%) | 30 (29%) | <0.05[c] |
| Hepatocellular carcinoma (+) | 34 (26%) | 8 (8%) | <0.0005[c] |
| N22 PCR-positive | 60 (45%) | 12 (11%) | <0.0001[c] |
| HCV RNA titer (K IU/ml) | 619.3 ± 474.3 | 775.7 ± 513.3 | <0.05[b] |
| High HCV load ≥ 100 K IU/ml | 111 (84%) | 99 (94%) | <0.05[c] |
| HCV genotype (1b/2a/2b) | 103/17/12 | 84/14/7 | NS (0.79)[d] |
| AST (IU/l) | 66 ± 43 | 63 ± 41 | NS (0.54)[b] |
| ALT (IU/l) | 65 ± 50 | 70 ± 53 | NS (0.66)[b] |
| γ-globulin (g/dl) | 1.8 ± 0.5 | 1.8 ± 0.6 | NS (0.24)[b] |
| Hyaluronan (ng/ml) | 256.7 ± 353.1 | 225.7 ± 271.9 | NS (0.19)[b] |
| Type IV collagen (ng/ml) | 194.0 ± 116.2 | 175.6 ± 85.9 | NS (0.14)[b] |
| Platelet count ($\times 10^4/\mu l$) | 12.0 ± 5.3 | 14.0 ± 5.7 | <0.01[b] |
| AFP (ng/ml) | 974.0 ± 8045.9 | 67.7 ± 269.5 | NS (0.10)[b] |
| Spleen size ($cm^2$) | 19.3 ± 11.9 | 17.4 ± 8.9 | NS (0.17)[b] |

*NS, not significant.
[a]See Table I for abbreviations (normal range).
[b]Mann-Whitney U-test.
[c]Fisher's exact test.
[d]$\chi^2$-test.

To examine whether high TTV viremia is a significant risk factor for the occurrence of HCC, multivariate logistic regression analysis was carried out with other variables (complication of cirrhosis, age ≥65 years, past history of interferon treatment, and high HCV viremia). High TTV viremia $\geq 10^4$ copies/ml was found to be a significant risk factor for the occurrence of HCC ($P < 0.05$) among patients with HCV-related chronic liver disease (Table IV). Furthermore, high TTV viral load ($\geq 10^4$ copies/ml) and high level of hyaluronan

TABLE III. Univariate Analysis of Various Features for the Occurrence of HCC Among Patients With HCV-Related Chronic Liver Disease

| Feature[a] | Patients with HCV-related chronic liver disease | | P value* |
|---|---|---|---|
| | With HCC (n = 42) | Without HCC (n = 195) | |
| Age (year) | 70 ± 6 | 62 ± 11 | <0.0001[b] |
| Sex (male/female) | 22/20 | 93/102 | NS (0.61)[c] |
| Past history of blood transfusion (+) | 27 (64%) | 115 (59%) | NS (0.60)[c] |
| Duration after blood transfusion (year) | 40 ± 7 (n = 27) | 35 ± 10 (n = 115) | <0.05[b] |
| Past history of interferon treatment (+) | 12 (29%) | 82 (42%) | NS (0.12)[c] |
| Liver cirrhosis (+) | 32 (76%) | 58 (30%) | <0.0001[c] |
| N22 PCR-positive | 13 (31%) | 59 (30%) | NS (>0.99)[c] |
| UTR PCR-positive | 40 (95%) | 179 (92%) | NS (0.75)[c] |
| UTR PCR titer $\geq 10^4$ copies/ml | 34 (81%) | 98 (50%) | <0.0005[c] |
| UTR PCR titer (copies/ml)[e] | $1.3 \times 10^5 \pm 3.8 \times 10^5$ (n = 40) | $4.6 \times 10^4 \pm 9.6 \times 10^4$ (n = 179) | <0.0005[b] |
| HCV RNA titer (K IU/ml) | 518.1 ± 435.4 | 725.3 ± 502.8 | <0.01[b] |
| High HCV load ≥ 100 K IU/ml | 33 (79%) | 177 (91%) | <0.05[c] |
| HCV genotype (1b/2a/2b) | 34/3/5 | 153/28/14 | NS (0.31)[d] |
| AST (IU/l) | 85 ± 57 | 60 ± 37 | <0.001[b] |
| ALT (IU/l) | 73 ± 67 | 66 ± 48 | NS (0.60)[b] |
| γ-globulin (g/dl) | 2.1 ± 0.6 | 1.8 ± 0.5 | <0.001[b] |
| Hyaluronan (ng/ml) | 480.4 ± 514.9 | 191.8 ± 230.8 | <0.0001[b] |
| Type IV collagen (ng/ml) | 265.7 ± 161.0 | 168.6 ± 77.6 | <0.0001[b] |
| Platelet count ($\times 10^4/\mu l$) | 9.4 ± 4.8 | 13.6 ± 5.4 | <0.0001[b] |
| AFP (ng/ml) | 3144.9 ± 14140.3 | 18.4 ± 46.0 | <0.0001[b] |
| Spleen size ($cm^2$) | 25.3 ± 14.5 | 17.0 ± 9.1 | <0.0001[b] |

*NS, not significant.
[a]See Table I for abbreviations (normal range).
[b]Mann-Whitney U-test.
[c]Fisher's exact test.
[d]$\chi^2$-test.
[e]Titers were compared among the patients who were positive for TTV DNA by UTR PCR.

TABLE IV. Multivariate Logistic Regression Analysis of Various Parameters for the Occurrence of HCC Among Patients With HCV-Related Chronic Liver Disease or Liver Cirrhosis

| Factor | Category | Odds ratio (95% CI)[a] | P value* |
|---|---|---|---|
| Among patients with HCV-related chronic liver disease | | | |
| UTR PCR titer | $<10^4$ copies/ml | 1 | |
| | $\geq 10^4$ copies/ml | 3.1 (1.3–7.6) | <0.05 |
| Liver cirrhosis | No | 1 | |
| | Yes | 5.4 (2.4–12.2) | <0.0001 |
| Age | <65 years | 1 | |
| | ≥65 years | 3.1 (1.2–7.9) | <0.05 |
| Past history of interferon treatment | No | 1 | |
| | Yes | 1.0 (0.4–2.3) | NS (0.98) |
| HCV RNA titer | ≥100 K IU/ml | 1 | |
| | <100 K IU/ml | 2.3 (0.8–6.4) | NS (0.12) |
| Among patients with HCV-related liver cirrhosis | | | |
| UTR PCR titer | $<10^4$ copies/ml | 1 | |
| | $\geq 10^4$ copies/ml | 4.0 (1.3–12.7) | <0.05 |
| Hyaluronan | <300 ng/ml | 1 | |
| | ≥300 ng/ml | 3.6 (1.3–9.7) | <0.05 |
| HCV RNA titer | ≥100 K IU/ml | 1 | |
| | <100 K IU/ml | 1.6 (0.4–6.6) | NS (0.49) |

*NS, not significant.
[a]95% CI, 95% confidence interval.

(≥300 ng/ml) were presumed to be independent risk factors for the complication of HCC among the HCV-related cirrhotic patients ($P < 0.05$, $P < 0.05$; respectively).

## DISCUSSION

The development of HCC is a major problem in HCV infection, and persistent HCV infection is known to be an important risk factor for HCC. The mortality rate of HCC is estimated to be higher than 23 deaths per $10^5$ persons per year in Japan, where HCV presently accounts for 76% of HCC cases [Lemon et al., 2000]. Once cirrhosis is established, the risk of HCC is approximately 1 to 4% per year [Colombo et al., 1991; Tsukuma et al., 1993]. However, HCC can occur without cirrhosis [De Mitri et al., 1995], as was also shown in the present study; 10 patients (24%) were noncirrhotic among the 42 HCV-related HCC patients studied. Hence, many risk factors for HCV-related HCC have been reported [Colombo, 1999]. Patients with concurrent HBV or HIV infection or heavy alcohol intake, which are known to be risk factors for HCV-related HCC [Colombo, 1999], were excluded from the present study. Some of our subjects may have had occult HBV infection. However, an essential role of occult HBV infection in hepatocarcinogenesis in patients with chronic hepatitis C remains debated [Cacciola et al., 1999; Shintani et al., 2000].

The present study suggested that high viremia of concurrent TTV is an independent risk factor for the complication of HCC among patients with HCV-related chronic liver disease. Namely, in addition to cirrhosis and age, which are well-known risk factors for HCC [Tsukuma et al., 1993; Colombo, 1999], high TTV viremia was estimated to be another independent risk factor for the complication of HCC among the 237 studied patients with HCV-related chronic liver disease in a multivariate logistic regression model. The levels of hyaluronan and type IV collagen are utilized as sensitive markers for liver fibrosis [Engstrom-Laurent et al., 1985; Ueno et al., 1993; Murawaki et al., 1996], and these two markers as well as high TTV viral load were significantly associated with the complication of HCC among the 90 HCV-related cirrhotic patients ($P < 0.05$; $P < 0.05$; $P < 0.05$, respectively, in univariate analysis). On multivariate analysis, high TTV viremia was also presumed to be a risk factor for HCC, independent from a high hyaluronan level (≥300 ng/ml), among the 90 HCV-related cirrhotic patients. Therefore, it seems likely that a high viral load of co-infecting TTV is a significant, independent risk factor for the complication of HCC among patients with HCV-related chronic liver disease.

The underlying reason for the independent association of high TTV viremia with the complication of HCC among the patients with HCV-related chronic liver disease, remains unknown. However, there are two possible explanations. One explanation is that TTV infection may influence the progression of chronic liver disease in concert with concurrent HCV and may be associated with the development of HCC. Zein et al. [1999] reported that TTV infection was more prevalent among patients with advanced HCV-associated liver disease (decompensated cirrhosis and HCC) than in those with stable disease (chronic hepatitis and compensated cirrhosis). It was suggested recently that infection of certain TTV genotypes may interfere with the improvement of liver function following the start of abstinence, in patients with alcoholic liver disease

[Tokita et al., 2001a], and influence the necrosis and inflammation of hepatocytes and liver fibrosis in patients with nonalcoholic fatty liver disease [Tokita et al., 2001b]. Furthermore, Moriyama et al. [2001] reported that the score of irregular regeneration of hepatocytes in TTV-infected cirrhotic patients with chronic hepatitis C was higher than that in the nonviremic patients, suggesting that TTV infection may influence the development of HCV-related HCC. This is also supported by the previous report of Shibata et al. [1998] that severe irregular regeneration of hepatocytes was the strongest risk factor for the development of HCC in patients with chronic hepatitis C and liver cirrhosis. These findings support that TTV plays a role in the development of cirrhosis and subsequent complications. However, it is important to consider an alternative explanation of the obtained results. It has been reported that the TTV viral load is inversely correlated with the CD4 cell count among patients infected with HIV, and that it may reflect the immune status of these immunocompromised hosts [Christensen et al., 2000; Shibayama et al., 2001]. A possible relationship between the prevalence of elevated TTV viral load and the level of immunocompetence among TTV-infected patients on maintenance hemodialysis or with diabetes mellitus has been suggested, and arguments for the increased replication of TTV in hosts with immunological disorders and for the existence of immune control of TTV viral load have been provided by Touinssi et al. [2001]. Therefore, it is likely that an impaired immune system or suppression of the immune system is involved in elevated TTV viremia in HCC patients.

Besides the association of high TTV viremia with the complication of HCC in patients with HCV-related chronic liver disease, several other findings that should be discussed were obtained in the present study. High TTV viremia was associated with low HCV RNA titer, as previously described for the two groups of chronic hepatitis C patients with or without TTV genotype 1 [Nishizawa et al., 2000]. Such a reciprocal inverse relationship has also been observed between replications of HBV and HCV [Pontisso et al., 1993]. The presence of TTV DNA detectable by either N22 PCR or UTR PCR was associated with lower prevalence of past history of interferon treatment (Table I). These results corroborate the previous finding that TTV is sensitive to interferon treatment [Akahane et al., 1999; Chayama et al., 1999]. In this regard, the question as to whether the results obtained in the present study are biased by the effect of past interferon therapy in 94 patients, may be raised. However, even when the analyses in Tables II and III were performed among the remaining 143 patients who had no history of interferon treatment, high TTV viremia was significantly associated with the complication of cirrhosis ($P < 0.01$) or HCC ($P < 0.05$), and conversely, the occurrence of HCC was significantly associated with high TTV load ($P < 0.05$), in which all of the differences were assessed by Fisher's exact test.

In conclusion, high TTV viremia was significantly associated with the occurrence of HCC in patients with HCV-related chronic liver disease in the present study. However, it is not clear whether high TTV viremia plays a role in the development of cirrhosis and subsequent complications; whether it is a cofactor in the progression of liver disease; or whether it is a serological marker reflecting the host's immune status. Prospective studies need to be conducted to elucidate whether high TTV viral load is associated with the development of HCC and whether it has clinical significance in predicting the outcome of patients with HCV-related chronic liver disease. In this context, future studies on the role of high TTV viral load in patients with hepatic or extrahepatic disease of unknown etiology are warranted.

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## REFERENCES

Akahane Y, Sakamoto M, Miyazaki Y, Okada S, Inoue T, Ukita M, Okamoto H, Miyakawa Y, Mayumi M. 1999. Effect of interferon on a nonenveloped DNA virus (TT virus) associated with acute and chronic hepatitis of unknown etiology. J Med Virol 58:196–200.

Ball JK, Curran R, Berridge S, Grabowska AM, Jameson CL, Thomson BJ, Irving WL, Sharp PM. 1999. TT virus sequence heterogeneity in vivo: evidence for co-infection with multiple genetic types. J Gen Virol 80:1759–1768.

Cacciola I, Pollicino T, Squadrito G, Cerenzia G, Orlando ME, Raimondo G. 1999. Occult hepatitis B virus infection in patients with chronic hepatitis C liver disease. N Engl J Med 341:22–26.

Charlton M, Adjei P, Poterucha J, Zein N, Moore B, Therneau T, Krom R, Wiesner R. 1998. TT-virus infection in North American blood donors, patients with fulminant hepatic failure and cryptogenic cirrhosis. Hepatology 28:839–842.

Chayama K, Kobayashi M, Tsubota A, Kobayashi M, Arase Y, Suzuki Y, Saitoh S, Murashima N, Ikeda K, Okamoto K, Hashimoto M, Matsuda M, Koike H, Kobayashi M, Kumada H. 1999. Susceptibility of TT virus to interferon therapy. J Gen Virol 80:631–634.

Chichibu K, Matsuura T, Shichijo S, Yokoyama MM. 1989. Assay of serum hyaluronic acid in clinical application. Clin Chim Acta 181:317–324.

Christensen JK, Eugen-Olsen J, Sorensen M, Ullum H, Gjedde SB, Pedersen BK, Nielsen JO, Krogsgaard K. 2000. Prevalence and prognostic significance of infection with TT virus in patients infected with human immunodeficiency virus. J Infect Dis 181:1796–1799.

Colombo M. 1999. Natural history and pathogenesis of hepatitis C virus related hepatocellular carcinoma. J Hepatol 31:25–30.

Colombo M, De Franchis R, Del Ninno E, Sangiovanni A, De Fazio C, Tommasini M, Donato MF, Piva A, Di Carlo V, Dioguardi N. 1991. Hepatocellular carcinoma in Italian patients with cirrhosis. N Engl J Med 325:675–680.

Davidson F, MacDonald D, Mokili JL, Prescott LE, Graham S, Simmonds P. 1999. Early acquisition of TT virus (TTV) in an area endemic for TTV infection. J Infect Dis 179:1070–1076.

De Mitri MS, Poussin K, Baccarini P, Pontisso P, D'Errico A, Simon N, Grigioni W, Alberti A, Beaugrand M, Pisi E, Brechot C, Patrizia P. 1995. HCV-associated liver cancer without cirrhosis. Lancet 345:413–415.

Engstrom-Laurent A, Loof L, Nyberg A, Schroder T. 1985. Increased serum levels of hyaluronate in liver disease. Hepatology 5:638–642.

Gerner P, Oettinger R, Gerner W, Falbrede J, Wirth S. 2000. Mother-to-infant transmission of TT virus: prevalence, extent and mechanism of vertical transmission. Pediatr Infect Dis J 19: 1074–1077.

Gimenez-Barcons M, Forns X, Ampurdanes S, Guilera M, Soler M, Soguero C, Sanchez-Fueyo A, Mas A, Bruix J, Sanchez-Tapias JM, Rodes J, Saiz JC. 1999. Infection with a novel human DNA virus (TTV) has no pathogenic significance in patients with liver diseases. J Hepatol 30:1028–1034.

Heller F, Zachoval R, Koelzer A, Nitschko H, Froesner GG. 2001. Isolate KAV: a new genotype of the TT-virus family. Biochem Biophys Res Commun 289:937–941.

Holland PV, Barrera JM, Ercilla MG, Yoshida CFT, Wang Y, De Olim GAB, Betlach B, Kuramoto K, Okamoto H. 1996. Genotyping hepatitis C virus isolates from Spain, Brazil, China, and Macau by a simplified PCR method. J Clin Microbiol 34:2372–2378.

Ikeda H, Takasu M, Inoue K, Okamoto H, Miyakawa Y, Mayumi M. 1999. Infection with an unenveloped DNA virus (TTV) in patients with acute or chronic liver disease of unknown etiology and in those positive for hepatitis C virus RNA. J Hepatol 30:205–212.

Itoh K, Takahashi M, Ukita M, Nishizawa T, Okamoto H. 1999. Influence of primers on the detection of TT virus DNA by polymerase chain reaction. J Infect Dis 180:1750–1751.

Itoh Y, Takahashi M, Fukuda M, Shibayama T, Ishikawa T, Tsuda F, Tanaka T, Nishizawa T, Okamoto H. 2000. Visualization of TT virus particles recovered from the sera and feces of infected humans. Biochem Biophys Res Commun 279:718–724.

Lemon SM, Layden TJ, Seeff L, Suzuki H, Nishioka K, Mishiro S, Johnson L. 2000. The 20th United States-Japan joint hepatitis panel meeting. Hepatology 31:800–806.

Matsumoto A, Yeo AE, Shih JW, Tanaka E, Kiyosawa K, Alter HJ. 1999. Transfusion- associated TT virus infection and its relationship to liver disease. Hepatology 30:283–288.

Matsutani S, Kimura K, Ohto M, Okuda K. 1991. Ultrasonography in the diagnosis of portal hypertension. In: Okuda K, Benhamou JP, editors. Portal hypertension clinical and physiological aspects. Tokyo: Springer-Verlag. p 197–206.

Miyata H, Tsunoda H, Kazi A, Yamada A, Khan MA, Murakami J, Kamahora T, Shiraki K, Hino S. 1999. Identification of a novel GC-rich 113-nucleotide region to complete the circular, single-stranded DNA genome of TT virus, the first human circovirus. J Virol 73:3582–3586.

Moriyama M, Matsumura H, Shimizu T, Shioda A, Kaneko M, Miyazawa K, Miyata H, Tanaka N, Uchida T, Arakawa Y. 2001. Histopathologic impact of TT virus infection on the liver of type C chronic hepatitis and liver cirrhosis in Japan. J Med Virol 64:74–81.

Muljono DH, Nishizawa T, Tsuda F, Takahashi M, Okamoto H. 2001. Molecular epidemiology of TT virus (TTV) and characterization of two novel TTV genotypes in Indonesia. Arch Virol 146:1249–1266.

Murawaki Y, Ikuta Y, Koda M, Yamada S, Kawasaki H. 1996. Comparison of serum 7S fragment of type IV collagen and serum central triple-helix of type IV collagen for assessment of liver fibrosis in patients with chronic viral liver disease. J Hepatol 24:148–154.

Mushahwar IK. 2000. Recently discovered blood-borne viruses: Are they hepatitis viruses or merely endosymbionts? J Med Virol 62:399–404.

Mushahwar IK, Erker JC, Muerhoff AS, Leary TP, Simons JN, Birkenmeyer LG, Chalmers ML, Pilot-Matias TJ, Dexai SM. 1999. Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. Proc Natl Acad Sci USA 96:3177–3182.

Niel C, Saback FL, Lampe E. 2000. Coinfection with multiple TT virus strains belonging to different genotypes is a common event in healthy Brazilian adults. J Clin Microbiol 38:1926–1930.

Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. 1997. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. Biochem Biophys Res Commun 241:92–97.

Nishizawa Y, Tanaka E, Orii K, Rokuhara A, Ichijo T, Yoshizawa K, Kiyosawa K. 2000. Clinical impact of genotype 1 TT virus infection in patients with chronic hepatitis C and response of TT virus to α-interferon. J Gastroenterol Hepatol 15:1292–1297.

Obata K, Iwata K, Ichida T, Inoue K, Matsumoto E, Muragaki Y, Ooshima A. 1989. One step sandwich enzyme immunoassay for human type IV collagen using monoclonal antibodies. Clin Chim Acta 181:293–304.

Okamoto H, Tokita H, Sakamoto M, Horikita M, Kojima M, Iizuka H, Mishiro S. 1993. Characterization of the genomic sequence of type V (or 3a) hepatitis C virus isolates and PCR primers for specific detection. J Gen Virol 74:2385–2390.

Okamoto H, Nishizawa T, Kato N, Ukita M, Ikeda H, Iizuka H, Miyakawa Y, Mayumi M. 1998a. Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown etiology. Hepatol Res 10:1–16.

Okamoto H, Akahane Y, Ukita M, Fukuda M, Tsuda F, Miyakawa Y, Mayumi M. 1998b. Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A-G hepatitis. J Med Virol 56:128–132.

Okamoto H, Nishizawa T, Ukita M, Takahashi M, Fukuda M, Iizuka H, Miyakawa Y, Mayumi M. 1999a. The entire nucleotide sequence of a TT virus isolate from the United States (TUS01): comparison with reported isolates and phylogenetic analysis. Virology 259:437–448.

Okamoto H, Takahashi M, Nishizawa T, Ukita M, Fukuda M, Tsuda F, Miyakawa Y, Mayumi M. 1999b. Marked genomic heterogeneity and frequent mixed infection of TT virus demonstrated by PCR with primers from coding and noncoding regions. Virology 259:428–436.

Okamoto H, Ukita M, Nishizawa T, Kishimoto J, Hoshi Y, Mizuo H, Tanaka T, Miyakawa Y, Mayumi M. 2000a. Circular double-stranded forms of TT virus DNA in the liver. J Virol 74:5161–5167.

Okamoto H, Takahashi M, Kato N, Fukuda M, Tawara A, Fukuda S, Tanaka T, Miyakawa Y, Mayumi M. 2000b. Sequestration of TT virus of restricted genotypes in peripheral blood mononuclear cells. J Virol 74:10236–10239.

Peng YH, Nishizawa T, Takahashi M, Ishikawa T, Yoshikawa A, Okamoto H. 2002. Analysis of the entire genomes of thirteen TT virus variants classifiable into the fourth and fifth genetic groups, isolated from viremic infants. Arch Virol 147:21–41.

Pontisso P, Ruvoletto MG, Fattovich G, Chemello L, Gallorini A, Ruol A, Alberti A. 1993. Clinical and virological profiles in patients with multiple hepatitis virus infections. Gastroenterology 105: 1529–1533.

Rodriguez-Inigo E, Casqueiro M, Bartolome J, Oritiz-Movilla N, Lopez-Alcorocho JM, Herrero M, Manzarbeitia F, Oliva H, Carreno V. 2000. Detection of TT virus DNA in liver biopsies by in situ hybridization. Am J Pathol 156:1227–1234.

Shibata M, Morizane T, Uchida T, Yamagami T, Onozuka Y, Nakano M, Mitamura K, Ueno Y. 1998. Irregular regeneration of hepatocytes and risk of hepatocellular carcinoma in chronic hepatitis and cirrhosis with hepatitis-C-virus infection. Lancet 351:1773–1777.

Shibayama T, Masuda G, Ajisawa A, Takahashi M, Nishizawa T, Tsuda F, Okamoto H. 2001. Inverse relationship between the titre of TT virus DNA and the CD4 cell count in patients infected with HIV. AIDS 15:563–570.

Shintani Y, Yotsuyanagi H, Moriya K, Fujie H, Tsutsumi T, Takayama T, Makuuchi M, Kimura S, Koike K. 2000. The significance of hepatitis B virus DNA detected in hepatocellular carcinoma of patients with hepatitis C. Cancer 88:2478–2486.

Tagger A, Donato F, Ribero ML, Binelli G, Gelatti U, Portera G, Albertini A, Fasola M, Chiesa R, Nardi G, the Brescia HCC study. 1999. A case-control study on a novel DNA virus (TT virus) infection and hepatocellular carcinoma. Hepatology 30:294–299.

Takahashi K, Hoshino H, Ohta Y, Yoshida N, Mishiro S. 1998. Very high prevalence of TT virus (TTV) infection in general population of Japan revealed by a new set of PCR primers. Hepatol Res 12:233–239.

Takahashi K, Iwasa Y, Hijikata M, Mishiro S. 2000. Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Arch Virol 145:979–993.

Tanaka H, Okamoto H, Luengrojanakul P, Chainuvati T, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. 1998. Infection with an unenveloped DNA virus (TTV) associated with posttransfusion non-A to G hepatitis in hepatitis patients and healthy blood donors in Thailand. J Med Virol 56:234–238.

Tanaka Y, Primi D, Wang RY, Umemura T, Yeo AE, Mizokami M, Alter HJ, Shih JW. 2001. Genomic and molecular evolutionary analysis of a newly identified infectious agent (SEN virus) and its relationship to the TT virus family. J Infect Dis 183:359–367.

Todd D, McNulty MS, Mankertz A, Lukert PD, Randles JW, Dale JL. 2000. Family *Circoviridae*. In: Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB, editors. Virus

taxonomy. Classification and nomenclature of viruses, Seventh Report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press. p 299–303.

Tokita H, Murai S, Kamitsukasa H, Yagura M, Harada H, Tawara A, Takahashi M, Okamoto H. 2001a. Influence of TT virus on the clinical course of alcoholic liver disease. Hepatol Res 19:180–193.

Tokita H, Murai S, Kamitsukasa H, Yagura M, Harada H, Hebisawa A, Takahashi M, Okamoto H. 2001b. Influence of TT virus on the histopathological features of nonalcoholic fatty liver disease. Hepatol Res 19:197–211.

Tokita H, Murai S, Kamitsukasa H, Yagura M, Harada H, Takahashi M, Okamoto H. 2001c. Influence of TT virus infection on the thrombocytopenia of patients with chronic liver disease. Hepatol Res 20:288–300.

Touinssi M, Gallian P, Biagini P, Attoui H, Vialettes B, Berland Y, Tamalet C, Dhiver C, Ravaux I, De Micco P, De Lamballerie X. 2001. TT virus infection: prevalence of elevated viraemia and arguments for the immune control of viral load. J Clin Virol 21:135–141.

Trimoulet P, De Ledinghen V, Ekouevi D, Bernard PH, Merel P, Chene G, Couzigou P, Fleury H. 2000. TT virus infection during chronic hepatitis C. Am J Gastroenterol 95:1765–1769.

Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H, Kawashima T. 1993. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 328: 1797–1801.

Ueno T, Inuzuka S, Torimura T, Tamaki S, Koh H, Kin M, Minetoma T, Kimura Y, Ohira H, Sata M, Yoshida H, Tanikawa K. 1993. Serum hyaluronate reflects hepatic sinusoidal capillarization. Gastroenterology 105:475–481.

Yoshida H, Kato N, Shiratori Y, Lan KH, Ono-Nita SK, Feng Z, Shiina S, Omata M. 2000. Poor association of TT virus viremia with hepatocellular carcinoma. Liver 20:247–252.

Zein NN, Arslan M, Li H, Charlton MR, Gross JB Jr, Poterucha JJ, Therneau TM, Kolbert CP, Persing DH. 1999. Clinical significance of TT virus infection in patients with chronic hepatitis C. Am J Gastroenterol 94:3020–3027.

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# Influence of TNF Gene Polymorphism and HLA-DRB1 Haplotype in Japanese Patients With Chronic Liver Disease Caused by HCV

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**OBJECTIVE:** To clarify the host genomic role in chronic liver disease associated with hepatitis C virus (HCV), we investigated the relationship between the severity of hepatitis and the polymorphisms of the tumor necrosis factor (TNF) gene or the human leukocyte antigen (HLA)-DRB1 haplotypes.

**METHODS:** We analyzed 40 healthy subjects, 50 patients with chronic inactive hepatitis caused by HCV with mean serum ALT concentrations under 40 IU/ml (group A), and 50 patients with chronic active liver disease caused by HCV and mean ALT concentrations over 50 IU/ml (group B).

**RESULTS:** There were no significant differences in the frequencies of TNF promoter gene variants at positions −238 and −308 between the groups. Regarding polymorphisms at the TNF-β NcoI site, the frequency of B1B1 homozygotes in group A was significantly increased, compared with the healthy subjects and those in group B (controls 7.5%, group A 34%, group B 10%). Regarding the analysis of HLA-DRB1, DRB1*0901 was significantly more frequent in group A than in group B (group A 19%, group B 5%). TNF B1B1 homozygotes were associated with HLA-DRB1*0901 and *1302, and negatively associated with DRB1*0405. Combination analysis revealed that HCV was inactive in the majority of patients who were both DRB1*0901 and B1B1 homozygotes.

**CONCLUSION:** Our data suggest that TNF gene polymorphisms and HLA-DRB1 haplotype may influence the activity of HCV in chronic liver disease. (Am J Gastroenterol 2003;98:160–166. © 2003 by Am. Coll. of Gastroenterology)

## INTRODUCTION

Although chronic hepatitis C is an infectious disease, mechanisms other than a direct cytotoxic effect on hepatocytes by the virus may be involved in the development of liver damage because the severity of chronic hepatitis C is not always related to virus-associated factors, such as the serum concentration of viral particles or viral type (1, 2). These findings suggest that host-associated factors are involved in the development of chronic hepatitis C.

Several lines of evidence suggest the importance of tumor necrosis factor (TNF)-α in hepatitis C infection. Patients with acute and chronic hepatitis C have elevated plasma concentrations of TNF-α (3, 4). Liver-infiltrating cytotoxic T lymphocytes have been shown to secrete TNF-α and interferon-γ (5, 6). In addition, hepatitis C virus (HCV) induces TNF-α production in human hepatocytes (7). A recent study found that nonresponsiveness to interferon-α treatment is related to high pretreatment TNF-α concentrations (8).

Variations in the production rates of cytokines between individuals have been observed (9, 10). Some of these interindividual differences in cytokine production may be related to polymorphisms in the cytokine genes themselves or genes, which regulate cytokine gene transcription. The genes of TNF-α and TNF-β, the latter also known as lymphotoxin-α, are located in the HLA class III region in the 6p21.3 band of the short arm of chromosome 6.

Recent studies have described extensive polymorphisms within the TNF promoter region at −238 and −308 and at the NcoI site in the TNF-β gene, and currently there is considerable interest in the relationships between these TNF gene polymorphisms and the susceptibility/resistance to both autoimmune and infectious diseases (10–12). *In vitro* studies have shown that certain polymorphic alleles may be associated with higher or lower levels of TNF-α production (13). Regarding the relationship between TNF promoter gene polymorphisms and HCV infection, Hohler *et al.* and Yee *et al.* reported that TNF gene promoter −238 or −308 polymorphisms were associated with chronic liver disease caused by HCV (14, 15). However, they did not correlate this relationship with hepatitis activity, such as serum ALT concentration, nor with the HLA-DRB1 haplotypes, which have been reported to be associated with the extent of hepatitis (16, 17). In this study, we investigated whether TNF polymorphisms influence the extent of hepatitis in those infected with hepatitis C, especially comparing those with inactive and active chronic hepatitis. We also investi-

**Table 1.** Clinical and Virological Features of HCV-Infected Patients

| | Group A (Mean ALT <40 U/L) | | Group B (Mean ALT >50 U/L) | | Controls |
|---|---|---|---|---|---|
| Number | 50 | | 50 | | 40 |
| Age (yr) | 50.4 ± 14.2 | | 55.1 ± 11.2 | | 51.3 ± 18.7 |
| Gender (M/F) | 16/34 | | 35/15 | | 25/15 |
| Pathology | CPH | 4 (8) | CAH | 16 (32) | |
| | CAH | 1 (2) | LC | 8 (16) | |
| | Biopsy (−) | 45 (90) | Varices (+) | 8 (16) | |
| | | | Biopsy (−) | 18 (36) | |
| LC | 0 (0) | | 16 (32) | | |
| HCV genotype | 1a | 1 (2) | 1a | 0 (0) | |
| | 1b | 33 (66) | 1b | 32 (64) | |
| | 2a | 6 (12) | 2a | 6 (12) | |
| | 2b | 6 (12) | 2b | 4 (8) | |
| | 2a + 1b | 2 (4) | 2a + 1b | 1 (2) | |
| | Unknown | 2 (4) | Unknown | 7 (14) | |
| HCV RNA titer (mEq/ml) | 12.12 ± 11.58 | | 11.27 ± 14.52 | | |
| Follow-up duration (yr) | 5.07 ± 2.64 | | 6.23 ± 4.12 | | |

Numbers in parentheses indicate percentages. CAH = chronic active hepatitis; CPH = chronic persistent hepatitis; LC = liver cirrhosis.

gated the relationship between HLA-DRB1 haplotypes and TNF gene polymorphisms.

## MATERIALS AND METHODS

### *Patients*

We examined 100 patients with chronic liver disease and 40 healthy controls. HCV-infected individuals were positive for both anti-HCV antibody and serum HCV RNA. All patients were negative for HBs antigen, had no history of alcohol abuse, and were negative for autoimmune hepatitis by the international criteria. Serum data including ALT concentration were obtained for over 2 yr every 1 or 2 months in all patients. Mean ALT concentration was calculated by averaging all measurements for each individual. Based upon the mean ALT concentrations, the patients with chronic liver disease were divided into two groups. Group A patients had a mean ALT concentration under 40 UI/ml, whereas that of group B patients was over 50 IU/ml. Histological studies were performed on liver biopsy specimens from five patients in group A and 24 patients in group B. Sixteen patients of group B had liver cirrhosis, which was diagnosed from histological examination or the presence of portal hypertension, such as esophageal varices and splenomegaly. The detailed clinical and virological data from the patients are shown in Table 1. None of group A patients had been receiving interferon therapy or stronger neominophagen C therapy. All patients and healthy controls gave informed consent before entry into the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by our institution's genomic research committee.

### *Analysis of TNF Gene Polymorphism*

Genomic DNAs were obtained from peripheral blood leukocytes by standard phenol-chloroform extraction (18). Screening for TNF polymorphisms was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism. PCR was carried out using Taq polymerase (TOYOBO, Tokyo, Japan) at 1.25 $\mu$l reaction, dNTP (TOYOBO) at 200 $\mu$M each and PCR primers at 1 $\mu$M final concentration. Restriction digests were performed on unpurified PCR products, adding the specific restriction buffer to achieve optimal reaction conditions.

A single-base polymorphism at TNF-$\alpha$ promoter position −308 was screened using flanking PCR primers (19), the forward primer incorporating a base mismatch to create an NcoI recognition site (primers: forward 5′ AGG CAA TAG GTT TTG AGG GCC AT 3′, reverse 5′ TCC TCC CTG CTC CGA TTC CG 3′), using PCR conditions as previously described (20). After digestion with NcoI (TOYOBO), the PCR products were fractionated on a 3% agarose gel (Aga-

**Table 2.** Distribution of TNF-$\alpha$ Promoter Variants at Position −238 in Chronic Liver Disease Patients Infected With HCV and Healthy Controls

| TNF-$\alpha$ 238 | G/G | G/A | A/A | G:A Allele |
|---|---|---|---|---|
| Control (n = 40) | 37 (92.5) | 3 (7.5) | 0 (0) | 77:3 |
| Group A (n = 50) | 47 (94) | 3 (6) | 0 (0) | 97:3 |
| Group B (n = 50) | 48 (96) | 2 (4) | 0 (0) | 98:2 |
| [LC (n = 16) | 16 (100) | 0 (0) | 0 (0) | 32:0] |

Numbers in parentheses indicate percentages. LC = liver cirrhosis.

**Table 3.** Distribution of TNF-α Promoter Variants at Position −308 in Chronic Liver Disease Patients Infected With HCV and Healthy Controls

| TNF-α 308 | G/G | G/A | A/A | G:A Allele |
|---|---|---|---|---|
| Control (n = 40) | 38 (95) | 2 (5) | 0 (0) | 78:2 |
| Group A (n = 50) | 47 (94) | 3 (6) | 0 (0) | 97:3 |
| Group B (n = 50) | 45 (90) | 5 (10) | 0 (0) | 95:5 |
| [LC (n = 16) | 13 (81.2) | 3 (18.8) | 0 (0) | 29:3] |

Numbers in parentheses indicate percentages. LC = liver cirrhosis.

rose-1000, Gibco BRL, Rockville, MD) and stained with ethidium bromide (product size after digestion: allele G = 87 and 20 base-pair [bp], allele A = 107 bp).

A single-base polymorphism at TNF-α promoter position −238 was screened using flanking PCR primers (21). The reverse primer was incorporated in a base mismatch to create an Eco47I recognition site (primers: forward 5′ GAA GCC CCT CCC AGT TCT AGT TC 3′, reverse 5′ CAC TCC CCA TCC CTG GTC 3′), using PCR conditions as previously described (21). After digestion with Eco47I (TOYOBO), the PCR products were fractionated on a 3% agarose gel (Agarose-1000) and stained with ethidium bromide (product size after digestion: allele G = 77, 63, 49, and 21 bp, allele A = 77, 70, and 63 bp).

A single-base polymorphism in the first intron of the TNF-β gene was screened using PCR amplification of flanking regions. A 782-fragment of genomic DNA containing the polymorphic NcoI restriction site was amplified (primers: forward 5′ CCG TGC TTC GTG CTT TGG ACT A 3′, reverse 5′ AGA GGG GTG GAT GCT TGG GTT C 3′), using PCR conditions as previously described (19). After digestion with NcoI, the PCR products were fractionated on a 2% agarose gel (Agarose-ME, Iwai Kagaku, Tokyo, Japan) and stained with ethidium bromide (product size after digestion: allele B1 = 586 and 196 bp, allele B2 = 782 bp).

### *Analysis of HLA-DRB1 Gene Polymorphism*

Analysis of HLA-DRB1 haplotypes was performed using the PCR-restriction fragment length polymorphism method with some modification, as previously described (22, 23). In brief, genomic DNA was obtained as described in the TNF polymorphism section. PCR amplification of the sample DNA was carried out using DRB1 group-specific primers, as previously described (22, 23). Next, the amplified products were cleaved with restriction enzymes and subjected to electrophoresis on a 10% polyacrylamide gel. The restriction fragments were detected by staining with ethidium bromide and observed under ultraviolet light. The HLA genotypes were determined on the basis of the restriction fragment length polymorphism patterns of the digested fragments.

### *Statistical Analysis*

Statistical analysis presented in Tables 2–7 was performed by $\chi^2$ and Fisher's exact test. Multivariate analyses were performed with SAS System 8e (SAS Institute, Cary, NC). To clarify the association between severity of hepatitis and polymorphisms of TNF gene or HLA-DRB1 haplotypes, ORs and 95% CIs were estimated by multivariate unconditional logistic regression model. The confounding factors included in the multivariate model were age and gender. The influence of profile, interaction, and multicollinearity was examined by regression diagnostics.

## RESULTS

### *TNF Gene Polymorphism*

Detailed clinical and virological data of the patients are shown in Table 1. HCV genotype, HCV RNA titer and follow-up duration were not significantly different between group A and B patients. The percentage of women in group A was higher than that in group B. Based upon histological examination of liver biopsy specimens from five patients in group A, four had chronic persistent hepatitis, and one had chronic active hepatitis. Histological examination revealed that 16 patients in group B had chronic active hepatitis, and eight had liver cirrhosis.

Tables 2–4 show the frequencies of the three TNF gene variants analyzed. There was no difference between the groups in the frequency of each of the polymorphisms at position −238 in the TNF promoter. Regarding position

**Table 4.** Distribution of TNF-β NcoI Site Polymorphism in Chronic Liver Disease Patients Infected With HCV and Controls

| TNF-β NcoI Site | B1B1 | B1B2 | B2B2 | B1:B2 Allele |
|---|---|---|---|---|
| Control (n = 40) | 3 (7.5) | 21 (52.5) | 16 (40) | 27:53 |
| Group A (n = 50) | 17 (34)* | 20 (40) | 13 (26) | 54:46† |
| Group B (n = 50) | 5 (10) | 25 (50) | 20 (40) | 35:65 |
| [LC (n = 16) | 0 (0) | 8 (50) | 8 (50) | 8:24] |

Numbers in parentheses indicate percentages. LC = liver cirrhosis.
* Group A *vs* controls, $p = 0.0027$ ($\chi^2$ test); $p = 0.0042$ (Fisher's exact test).
Group A *vs* group B, $p = 0.0038$ ($\chi^2$ test); $p = 0.0070$ (Fisher's exact test).
† Group A *vs* controls, $p = 0.0067$ ($\chi^2$ test); $p = 0.0071$ (Fisher's exact test).
Group A *vs* group B, $p = 0.0069$ ($\chi^2$ test); $p = 0.0012$ (Fisher's exact test).

**Table 5.** Frequencies of HLA-DRB1 Haplotypes in Chronic Liver Disease Patients Infected With HCV

| HLA-DRB1 Allele | Group A (n = 100 Alleles) | Group B (n = 100 Alleles) | Uninfected Normal† Subjects (n = 1832 Alleles) |
|---|---|---|---|
| 0101 | 1 (1) | 3 (3) | (5) |
| 0401 | 0 (0) | 1 (1) | |
| 0403 | 1 (1) | 7 (7) | (3) |
| 0405 | 15 (15) | 21 (21) | (16) |
| 0410 | 3 (3) | 4 (4) | |
| 0802 | 0 (0) | 4 (4) | (5) |
| 0803 | 10 (10) | 7 (7) | (8) |
| 0901* | 19 (19) | 5 (5) | (12) |
| 1101 | 4 (4) | 2 (2) | (3) |
| 1301 | 3 (3) | 0 (0) | |
| 1302 | 16 (16) | 12 (12) | (5) |
| 1401 | 5 (5) | 9 (9) | (4) |
| 1403 | 1 (1) | 1 (1) | |
| 1405 | 1 (1) | 1 (1) | |
| 1406 | 1 (1) | 0 (0) | |
| 1501 | 9 (9) | 5 (5) | (6) |
| 1502 | 10 (10) | 12 (12) | (9) |
| 1602 | 0 (0) | 1 (1) | |

Numbers in parentheses indicate percentages.
* Group A *vs* group B, $p = 0.0023$ ($\chi^2$ test); $p = 0.0039$ (Fisher's exact test).
† Result of study by Kuzushita *et al.* (16).

−308, the frequency of the 308A allele in group B was slightly higher than that in the healthy controls, but the difference was not statistically significant (frequency of −308 A/G: controls 5%, group A 6%, group B 10%). Regarding the TNF-β NcoI site, the incidence of TNF B1B1 homozygotes was significantly higher in group A than in group B and the healthy controls (controls 7.5%, group A 34%, group B 10%). In addition, the allelic frequency of TNF B1 allele was higher in group A than that in group B and in the healthy controls (controls 34%, group A 54%, group B 35%). These data suggest that the TNF-β gene genotype might influence the disease activity of chronic liver disease caused by HCV.

### *HLA-DRB1 Haplotype*

Table 5 shows the frequency of the HLA-DRB1 haplotypes in groups A and B. The frequency of the DRB1*0901 allele was significantly higher in group A than in group B. DRB1*1302 and DRB1*1501 were also increased in group A, although the increases were not significant. DRB1*0405 and DRB1*0403 were increased in group B, although they were not significantly higher.

### *Relationship Between TNF-β Gene Genotype and HLA-DRB1 Haplotype*

The association between the HLA-DRB1 haplotype and TNF-β gene genotype is shown in Table 6. The DRB1*0901 and DRB1*1302 haplotypes were associated with TNF B1B1 homozygotes, whereas DRB1*0405 was associated with TNF B2B2 homozygotes and negatively associated with TNF B1B1 homozygotes. There was no association between any other DRB1 haplotype and the TNF-β gene genotype.

The combination analysis is shown in Table 7. Of the 11 patients who were both DRB1*0901 and B1B1 homozygotes, nine (82%) belonged to the inactive group (group A). Although two other patients belonged to the active group (group B), they did not have liver cirrhosis, and their platelet count remained within normal limits.

In contrast, of the 17 patients who were both DRB1*0405 and B2B2 homozygotes, 12 patients (71%) belonged to the active group (group B).

### *Multivariate Analysis*

Table 8 shows the results of the multivariate analysis. Accordingly, HLA-DRB1*0901 and TNF-β gene genotype

**Table 6.** Linkage Dysequilibrium Between the HLA-DRB1 Haplotype and the TNF-β Gene Genotype in Chronic Liver Patients Infected With HCV

| | TNF-β Genotype | | | |
|---|---|---|---|---|
| DRB1 | B1B1 | B1B2 or B2B2 | Number | *p* |
| 0901+ | 11 | 12 | 100 | 0.0007* |
| 0901− | 11 | 66 | | 0.0015† |
| 1302+ | 12 | 15 | 100 | 0.001* |
| 1302− | 10 | 63 | | 0.0021† |
| 0405+ | 1 | 32 | 100 | 0.0013* |
| 0405− | 21 | 46 | | 0.0008† |
| | TNF-β Genotype | | | |
| DRB1 | B2B2 | B1B2 or B1B1 | Number | *p* |
| 0405+ | 17 | 16 | 100 | 0.0057* |
| 0405− | 16 | 51 | | 0.0073† |

* $\chi^2$ test.
† Fisher's exact test.

**Table 7.** Influence of the Combination of TNF-β Gene Genotype and HLA-DRB1 Haplotype

| TNF-β Gene | DRB1 | Group A | Group B | Comparison | *p* |
|---|---|---|---|---|---|
| B1B1 | *0901 | | | | |
| + | + | 9 | 2 | ++*vs*−− | 0.0048*<br>0.0072† |
| + | − | 8 | 3 | +−*vs*−− | 0.0235*<br>0.0437† |
| − | + | 9 | 3 | −+*vs*−− | 0.0127* |
| − | − | 24 | 42 | | 0.0233† |
| B2B2 | *0405 | | | | |
| + | + | 5 | 12 | ++*vs*−− | 0.0499*<br>0.0912† |
| + | − | 8 | 8 | +−*vs*−− | ns |
| − | + | 8 | 8 | −+*vs*−− | ns |
| − | − | 29 | 22 | | |

* $\chi^2$ test.
† Fisher's exact test.

proved to be significant independent factors. Age and gender also exhibited significant differences between group A and group B.

## DISCUSSION

In previous studies, Hohler *et al.* reported that the frequency of the TNF 238A allele was significantly higher in hepatitis C-infected patients compared with controls (14). Yee *et al.* reported that the TNF promoter variant 308A was more common in HCV-associated liver cirrhosis patients (15). However, Hohler *et al.* did not investigate the relationship between the extent of chronic liver disease and TNF gene polymorphisms, nor did they examine the frequency of the TNF-β gene genotypes (14). Yee *et al.* did not investigate the relationship between TNF gene polymorphisms and HLA-DRB1 haplotypes, nor the possible gene factors in those with chronic inactive hepatitis (15). We first investigated the relationship between the extent of chronic liver disease caused by HCV and TNF gene polymorphisms, with special attention directed to those with inactive hepatitis. We also analyzed the relationship between TNF gene polymorphisms and HLA-DRB1 haplotypes. The difference between our results and those of Hohler *et al.* (14) and Yee *et al.* (15) might be related to racial differences. Negoro *et al.* reported that in Japanese patients with Crohn's disease, TNF gene polymorphisms at −308 and −238 are not associated with disease location (24). However, Louis *et al.* reported that in Crohn's disease in whites, the −308 TNF gene polymorphism may exert a slight influence on the behavior of the disease (25). Therefore, in the Japanese population, the −238 and −308 TNF gene polymorphisms might not be very important for HCV-associated hepatitis.

We used the mean serum ALT concentrations as an indicator of activity of liver disease. One reason for this choice was that TNF is more associated with inflammation than fibrosis. Second, liver fibrosis is progressive over many years in those with chronic active hepatitis. Therefore, it would be difficult to compare the genetic backgrounds with the grade of fibrosis or the histological findings at a single

**Table 8.** Multivariate Analysis

| | | | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|---|---|
| | Group A (n = 50) | Group B (n = 50) | Crude OR | (95% CI) | *p* | Adjusted OR | (95% CI) | *p* |
| Age | | | | | | | | |
| Per 10-yr decrease | 50.4 ± 14.2 | 55.1 ± 11.2 | 0.75 | (0.54–1.03) | 0.073 | 0.58 | (0.38–0.87) | 0.009 |
| Gender | | | | | | | | |
| Male | 16 (32) | 35 (70) | 1.00 | | | 1.00 | | |
| Female | 34 (68) | 15 (30) | 0.20 | (0.90–0.47) | <0.001 | 0.16 | (0.06–0.43) | <0.001 |
| HLA-DRB1 | | | | | | | | |
| Other | 32 (64) | 45 (90) | 1.00 | | | 1.00 | | |
| *0901 | 18 (36) | 5 (10) | 0.20 | (0.07–0.59) | 0.004 | 0.23 | (0.07–0.79) | 0.020 |
| TNF-β genotype | | | | | | | | |
| 1/2 or 2/2 | 33 (66) | 45 (90) | 1.00 | | | 1.00 | | |
| 1/1 | 17 (34) | 5 (10) | 0.22 | (0.07–0.64) | 0.006 | 0.28 | (0.08–0.99) | 0.048 |
| TNF-α 308 | | | | | | | | |
| G/A | 3 (6) | 5 (10) | 1.00 | | | 1.00 | | |
| G/G | 47 (94) | 45 (90) | 0.57 | (0.13–2.55) | ns | 1.76 | (0.31–10.15) | ns |

Numbers in parentheses indicate percentages. Ranges in parentheses indicate 95% CIs.

time point. Persico *et al.* reported that in patients with HCV-related chronic hepatitis associated with persistently normal serum ALT concentrations, the grade of disease activity does not increase over the years, and the progression to cirrhosis is slow or absent (26). Therefore, it was reasonable to analyze the differences in genetic factors between chronic HCV patients with persistently low ALT concentrations and those with persistently high ALT concentrations and who had been observed over several years. Regarding the observation time of ALT concentration, the argument was valid. However, Kuzushita *et al.* defined HCV carriers as those who persistently showed normal ALT values for more than 2 yr during periodic biochemical examinations (once every 1 or 2 months) (16). In another report, they defined HCV carriers as those whose ALT levels were within normal levels for 8–153 months (mean 25.7 months) (27). Our observation duration of group A was a minimum of 2 yr, and the mean duration was 5.07 yr (60.8 months). We consider that our observation period was sufficient when compared with other studies.

Stuber *et al.* reported that in sepsis patients with TNF-$\beta$ genotype B2B2, serum concentration of TNF-$\alpha$ was higher than that in sepsis patients with B1B1 or B1B2 (28). Pociot *et al.* reported that *in vitro* stimulated mononuclear cells from individuals who were B1B1 produced lower concentrations of TNF-$\alpha$ than individuals who were B1B2 heterozygotes or B2B2 homozygotes (10). These data are consistent with our findings that the TNF-$\beta$ gene genotype may play a role in hepatitis associated with chronic HCV infection. We performed some preliminary studies on the serum concentrations of TNF-$\alpha$, but did not find any significant differences among the patients (serum levels of TNF-$\alpha$ [pg/ml], 0.76 $\pm$ 1.65 [B1B1, n = 18]; 2.23 $\pm$ 7.90 [B1B2, n = 31]; 2.04 $\pm$ 6.73 [B2B2, n = 23]). It is possible that in cases of chronic disease, a single determination of these concentrations in the serum may not detect differences in TNF-$\alpha$ production.

Gender and age differences also need to be considered. Sixty-eight percent of group A patients were women. In contrast, only 30% of group B patients were women. Kuzushita *et al.* reported that a higher percentage of HCV patients with low hepatitis activity are women (16). However, we believe that gender had little effect on our results. The frequency of B1B1 homozygotes in the male group A was 37.5%, almost the same as that of the entire group A patients (34%). The frequency of B1B1 homozygotes in the female group B was 13%, almost the same as that of the entire group B (10%). In addition, multivariate analysis proved that HLA-DRB1*0901 and TNF-$\beta$ gene genotype are independent significant factors to affect HCV activity.

Regarding the HLA-DRB1 analysis, Kuzushita *et al.* reported that DRB1*1302 was associated with a low activity of chronic HCV infection, whereas DRB1*0405 was negatively associated with a low activity (16). Aikawa *et al.* reported that DRB1*0901 was more frequent in noncirrhotic HCV patients than in patients with cirrhosis (17). Our results were similar. In our study, linkage analysis showed that B1B1 homozygotes were associated with DRB1*0901 and *1302, and negatively associated with DRB1*0405. Data from Germany and Denmark suggested that there is positive linkage between TNF B1 and DRB1*0301, *1302, and positive linkage between TNF B2 and DRB1*1501, *0405 (10, 29). As there was no patient with DRB1*0301 in our study, we did not find a significant association between TNF B1 and DRB1*0301. The association between TNF B1B1 homozygotes and DRB1*1302 is shown in Table 6. As a new finding, we found a significant association between TNF B1B1 homozygotes and DRB1*0901 in Japanese patients. In our study, TNF B2 was not associated with DRB1*1501. DRB1*0405 was also significantly associated with TNF B2B2 homozygotes.

The combination of the TNF-$\beta$ gene polymorphism and DRB1 haplotype was also investigated. HCV was inactive in the majority of patients with DRB1*0901 and homozygous for B1. In contrast, HCV was active in the majority of patients with DRB1*0405 and homozygous for B2. Therefore, the combination of TNF-$\beta$ gene genotype and the DRB1 haplotype might yield prognostic information on chronic HCV infection. In the future, genomic analyses might be useful for predicting the activity of hepatitis C infection.

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## REFERENCES

1. Yamada M, Kakumu S, Yoshioka K, et al. Hepatitis C virus genotypes are not responsible for development of serious liver disease. Dig Dis Sci 1994;39:234–9.
2. McGuinness PH, Bishop GA, Painter DM, et al. Intra-hepatic hepatitis C RNA levels do not correlate with degree of liver injury in patients with chronic hepatitis. Hepatology 1996;23: 676–87.
3. Tilg H, Wilmer A, Vogel W, et al. Serum levels of cytokines in chronic liver diseases. Gastroenterology 1992;103:264–73.
4. Torre D, Zeroli C, Giola M, et al. Serum levels of interleukin-1$\alpha$, interleukin-1$\beta$, interleukin-6, and tumor necrosis factor in patients with acute viral hepatitis. Clin Infect Dis 1994;18: 194–8.
5. Lohr HF, Schlaak JF, Gerken G, et al. Phenotypical analysis and cytokine release of liver-infiltrating and peripheral blood T lymphocytes from patients with chronic hepatitis of different etiology. Liver 1994;14:161–6.
6. Koziel MJ, Dudley D, Afdhal N, et al. HLA class-I-restricted cytotoxic T lymphocytes specific for hepatitis C virus. Identification of multiple epitopes and characterization of patterns of cytokine release. J Clin Invest 1995;96:2311–21.
7. Gonzalez-Amaro R, Garcia-Mozon C, Garcia-Buey L, et al. Induction of tumor necrosis factor-a production by human hepatocytes in chronic viral hepatitis. J Exp Med 1994;179: 841–8.
8. Larrea E, Garcia N, Qian C, et al. Tumor necrosis factor alpha

gene expression and the response to interferon in chronic hepatitis C. Hepatology 1996;23:210–7.
9. Wilson A, Giovine FD, Duff G. Genetics of TNFα in autoimmune, infectious and neoplastic diseases. J Inflamm 1995; 45:1–12.
10. Pociot F, Briant L, Jongeneel CV, et al. Association of tumor necrosis factor (TNF) and class II major histocompatibility alleles with the secretion of TNF alpha and TNF beta by human mononuclear cells; a possible link to insulin dependent diabetes mellitus. Eur J Immunol 1993;23:224–31.
11. McGuire W, Hill AVS, Allsopp CE, et al. Variation in the TNF-α promoter region associated with susceptibility to cerebral malaria. Nature 1994;371:508–11.
12. McGuire W, Knight JC, Hill AVS, et al. Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. J Infect Dis 1999;179: 287–90.
13. Wilson AG, Symons JA, McDowell TL, et al. Effect of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci USA 1997;94:3195–9.
14. Hohler T, Kruger A, Gerkan G, et al. Tumor necrosis factor alpha promoter polymorphsim at position −238 associated with chronic active hepatitis. J Med Virol 1998;54:173–7.
15. Yee LJ, Tang J, Herrera J, et al. Tumor necrosis factor gene polymorphisms in patients with cirrhosis from chronic hepatitis C virus infection. Gene Immun 2000;1:386–90.
16. Kuzushita N, Hayashi N, Moribe T, et al. Influence of HLA haplotypes on clinical courses of individuals infected with hepatitis C virus. Hepatology 1998;27:240–4.
17. Aikawa T, Kojima M, Onishi H, et al. HLA DRB1 and DQB1 alleles and haplotypes influencing the progression of hepatic C. J Med Virol 1996;49:274–8.
18. Kaneshige T, Takagi K, Nakamura S, et al. Genomic analysis using fingernail DNA. Nucl Acids Res 1992;20:5489–90.
19. Bernal W, Donaldson P, Underhill J, et al. Tumor necrosis factor genomic polymorphism and outcome of acetaminophen (paracentamol)-induced acute liver failure. J Hepatol 1998;29: 53–9.
20. Wilson AG, di Giovine FS, Blakemore AIF, et al. Single base polymorphism in the human tumor necrosis factor alpha gene detectable by NcoI restriction of PCR product. Hum Mol Genet 1992;1:353.
21. Gordon MA, Oppenheim E, Camp NJ, et al. Primary biliary cirrhosis shows association with genetic polymorphism of tumor necrosis factor alpha promoter region. J Hepatol 1999; 31:242–7.
22. Kaneshige T, Hashimoto M, Matsumoto Y, et al. Serologic and nucleotide sequencing analyses of a novel DR52-associated DRB1 allele with the DR' NJ25' specificity, designated DRB1*1307. Hum Immunol 1994;41:151–9.
23. Moribe T, Kaneshige T, Hirakata M, et al. Identification of DRB1*0405 variant (DRB1*04052) using the PCR-RFLP method. Tissue Antigens 1996;47:450–3.
24. Negoro K, Kinouchi Y, Hiwatashi N, et al. Crohn's disease is associated with novel polymorphisms in the 5'-flanking region of the tumor necrosis factor gene. Gastroenterology 1999;117: 1062–8.
25. Louis E, Peeters D, Frachimont L, et al. Tumor necrosis factor (TNF) gene polymorphism in Crohn's disease (CD) influence on disease behaviour?. Clin Exp Immunol 2000;119:64–8.
26. Persico M, Persico E, Supzzo R, et al. Natural history of hepatitis C virus carriers with persistently normal aminotransferase levels. Gastroenterology 2000;118:760–4.
27. Kuzushita N, Hayashi N, Katayama K, et al. Increased frequency of HLA DR13 in hepatitis C virus carriers with persistently normal ALT levels. J Med Virol 1996;48:1–7.
28. Stuber F, Petersen M, Bokelmann F, et al. A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-α concentrations and outcome of patients with severe sepsis. Crit Care Med 1996;24:381–4.
29. Bettinotti MP, Hartung K, Deicher H, et al. Polymorphism of the tumor necrosis factor beta gene in systemic lupus erythematosus: TNFB-MHC haplotypes. Immunogenetics 1993;37: 449–54.

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# Processing of Hepatitis C Virus Core Protein Is Regulated by Its C-Terminal Sequence

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Polyprotein processing of plus-strand RNA viruses is important in the regulation of gene production and replication. The core protein of hepatitis C virus (HCV), constructing the viral particle, is processed from its precursor polyprotein and observed as two forms, p23 and p21. Production of p21 by cleavage at the C-terminus of p23 is considered crucial to viral assembly and replication. In this study, this processing step was compared between clones isolated from two patients with fulminant hepatitis and from five patients with chronic hepatitis by an in vitro translation assay and cell transfection assay. The p21 core protein was predominant from the clone isolated from one of the fulminant hepatitis patient (p21 core protein production was 65.98%), while p23 was abundant with clones from five chronic hepatitis patients (p21 core protein production was 7.11 ± 1.62%) and clone from another fulminant hepatitis patient (p21 core protein production was 13.36%). Investigations with chimeric and mutation-introduced constructs revealed that four amino acid residues in the C-terminus of the core region are responsible for this difference. The data suggest that core protein processing is regulated by C-terminus mutations. ***J. Med. Virol. 69:357–366, 2003.***


**KEY WORDS:** HCV; fulminant hepatitis; p23; signal peptide peptidase; endoplasmic reticulum

## INTRODUCTION

Polyprotein processing of plus-strand RNA viruses is important in the regulation of gene production [Hellen et al., 1989; Palmenberg, 1990; Lohmann et al., 1996]. The efficiency of mature viral protein production from a precursor polyprotein can be modulated both by substrates and by proteases [Ypma-Wong et al., 1988; Amberg et al., 1994; Lemm et al., 1994]. Particular mutations in substrate amino acid sequences at the cleavage site of the viral precursor protein may alter the efficiency of cleavage and change viral replication and virulence [Lee et al., 2000].

Hepatitis C virus (HCV), one of the plus-strand RNA viruses, is a primary cause of posttransfusion and sporadic acute hepatitis [Choo et al., 1989; Kuo et al., 1989]. Infection with HCV leads to chronic liver diseases, including cirrhosis and hepatocellular carcinoma, because most patients fail to clear the virus and persistent infection follow [Kiyosawa et al., 1990; Saito et al., 1990; Poynard et al., 2000]. HCV belongs to the flaviviruses, and consists of ~9,600 nucleotides (nt) encoding a single open reading frame (ORF) of ~3,000 amino acid (aa) residues [Kato et al., 1990; Choo et al., 1991; Takamizawa et al., 1991; Reed and Rice, 2000]. [The nucleotide sequences in this article will appear in the DDBJ/EMBL/GenBank with the following accession numbers: AB047639 (JFH-1), AB077951 (JFH-2), and AB047640-AB047644 (JCH-1–JCH-5).] From this polyprotein, cleavage by host signal peptidases produces three structural proteins: core, envelope (E) 1, and E2 proteins. Of the structural proteins, the core protein seems to be a key molecule for both viral replication and pathogenesis because it forms the viral particle and also regulates multiple functions in host cells, such as

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apoptosis, transcription, signal transduction, immune presentation, and cell transformation [Shirai et al., 1994; Kita et al., 1995; Ray et al., 1995, Ray et al., 1996a,b; Honda et al., 2000; Kato et al., 2000; McLauchlan, 2000]. During virus assembly, the core protein undergoes two consecutive membrane-dependent cleavages, and it appears as two forms, p23 and p21, in both in vitro translation assays and cell culture systems [Liu et al., 1997]. The p21 core protein is cleaved from the endoplasmic reticulum (ER)-bound p23 core protein or the longer precursor polyprotein by the host signal peptide peptidase, and this protease was recently identified [Hussy et al., 1996; McLauchlan et al., 2002; Weihofen et al., 2002]. The p21 core protein was observed predominantly in patient serum containing native viral particles [Yasui et al., 1998]. Thus, the p21 core protein is the mature and stable form that accumulates in the cell and eventually constitutes the viral capsid. Although the production of p21 core protein is considered critical in viral assembly, the mechanisms of this processing and the association to viral characteristics remain unclear.

Recently, we reported a case of fulminant hepatitis in which HCV RNA was detected in serum and the entire genome of HCV was recovered from this patient's serum [Kato et al., 2001]. This isolated clone, JFH-1, demonstrated several substitutions in the core region from other clones isolated from patients with chronic hepatitis. In the present study, the differences in p21 production depending on the amino acid sequence of the C-terminus of the core region were examined among clones isolated from patients with fulminant or chronic hepatitis.

## MATERIALS AND METHODS

### HCV Clones From Hepatitis Patients

As reported previously, clone JFH-1 was isolated from the patients with fulminant hepatitis (32-year-old man), and clone JCH-1 to JCH-5 were isolated from the patients with chronic hepatitis (59-year-old woman, 47-year-old man, 49-year-old man, 59-year-old woman, and 39-year-old man, respectively). All viral markers of the other hepatitis virus were negative. Further detailed information of these patients were also described in previous report [Kato et al., 2001]. HCV cDNA was also cloned from another fulminant hepatitis patient, a 62-year-old man who had a history of coronary artery bypass operation without blood transfusion (clone JFH-2). One year after the operation, he was admitted for acute auditory disorder and received a course of β-methasone therapy. After withdrawal of β-methasone, he developed fulminant hepatitis, as diagnosed by acute liver failure associated with stage II encephalopathy and low prothrombin time. HCV RNA was detected by reverse transcription-polymerase chain reaction (RT-PCR) in his serum during the acute phase. All viral markers of the other hepatitis viruses were negative. All these clones were clustered into the genotype 2a and were found to contain substitutions in the core region. Consensus sequence of HCV clones in each patient were determined by sequencing of five clones and adapted to this study.

### Constructs of HCV cDNA

Expression vectors encoding the HCV core-E1 region (pJFH-1/CE1 and pJCH-1/CE1) were constructed with two clones (pJFH-1/CE1 and pJCH-1/CE1) isolated from previously reported patients with fulminant hepatitis (clone JFH-1) and chronic hepatitis (clone JCH-1) (Fig. 1). HCV cDNA fragments were recovered by PCR with DNA polymerase (Platinum Pfx, GIBCO-BRL, Rockville, MD). PCR primers were adapted to the 20-mer sequences of the 5′ untranslated region (nt 335–354) and the E1 region (nt 1341–1360), and *Eco*RI and *Xba*I restriction enzyme sites were added to the 5′ end of the oligomers. Expression vectors were constructed with these digested fragments inserted into the vector pEF1/Myc-His B (Invitrogen, Carlsbad, CA). These constructs possess T7 promoter sequences upstream of the Kozak sequences and the ATG initiator codon of the HCV core region.

Expression vectors encoding the HCV core region (pJFH-1, pJFH-2/core, and pJCH-1–pJCH-5/core) were constructed with clones JFH-1, JFH-2, and JCH-1–JCH-5 (Figs. 1–4). PCR primers were adapted to the 20-mer sequence of the 5′ untranslated region (nt 335–354) and the 3′ end of core region (nt 894–913), and *Eco*RI and *Xba*I restriction enzyme sites were added to the 5′ end of the oligomers. After digestion with *Eco*RI and *Xba*I, these fragments were inserted into the expression vectors.

Six chimeric constructs (pC/F 60, pC/F 90, pC/F 160, pF/C 60, pF/C 90, and pF/C 160) were generated from expression vectors pJFH-1/core and pJCH-1/core (Figs. 4 and 5). After each fragment of JFH-1 and JCH-1 was amplified by PCR with overlapped primers, chimeric fragments were obtained by the fusion PCR method with appropriate primers containing *Eco*RI and *Xba*I restriction enzyme sites in the 5′ end of the oligomers. After digestion with *Eco*RI and *Xba*I, these fragments were inserted into the expression vectors. The construct pC/F 60 contains a sequence of clone JCH-1 from aa 1–60 and a sequence of clone JFH-1 from aa 61–191. The constructs pC/F 90 and pC/F 160 have a clone JCH-1 sequence, aa 1–90 and aa 1–160, respectively, and a clone JFH-1 sequence in the remaining region. Likewise, pF/C 60, pF/C 90, and pF/C 160 have clone JFH-1 sequences aa 1–60, aa 1–90, and aa 1–160, respectively, and a clone JCH-1 sequence in the remaining region.

Mutation-introduced constructs containing one to three amino acid mutations in the core region were generated from expression vectors pJFH-1/core and pJCH-1/core by PCR amplification with mutation containing primers. Four amino acid residues of clone JFH-1 in the C-terminus of the core region, tyrosine at aa 164 ($^{164}$Y), phenylalanine at aa172 ($^{172}$F), proline at aa 173 ($^{173}$P), and valine at aa 187 ($^{187}$V), were mutated singly or in combinations with the residues of clone JCH-1,

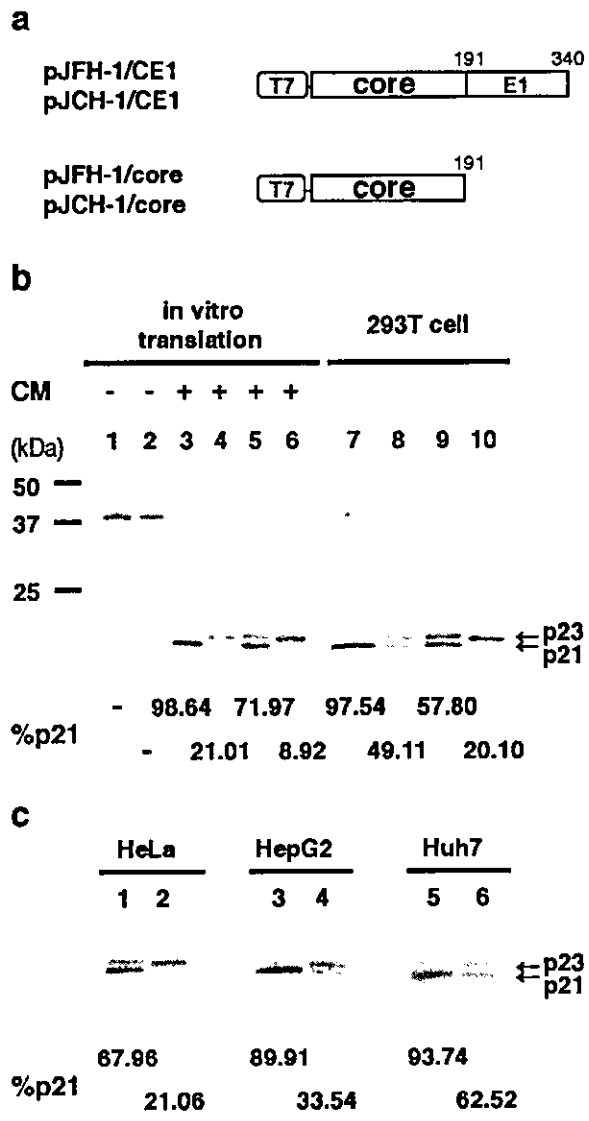


Fig. 1. Hepatitis C virus (HCV) core protein production in the in vitro translation assay and the cell culture system. **a:** HCV expression vectors were constructed with cDNA of the core and E1 regions (pJFH-1/CE1 and pJCH-1/CE1) or core regions (pJFH-1/core and pJCH-1/core) under the control of the T7 promoter. Clone JFH-1 was isolated from a patient with fulminant hepatitis, and clone JCH-1 was isolated from a patient with chronic hepatitis. **b:** HCV core proteins were expressed in the in vitro translation assay without (**lanes 1 and 2**) and with (**lanes 3–6**) canine pancreatic microsomal membrane (CM) or cultured 293T cells (**lanes 7–10**). HCV core proteins were detected using constructs pJFH-1/CE1 (**lanes 1, 3, 7**), pJCH-1/CE1 (**lanes 2, 4, 8**), pJFH-1/core (**lanes 5 and 9**), and pJCH-1/core (**lanes 6 and 10**). Arrows on the right indicate the two forms of HCV core protein, p23 and p21. The sizes of the protein molecular markers are indicated on the left. The ratios of p21 in the detected core proteins are indicated as %p21 at the bottom. **c:** HCV core proteins were expressed in HeLa, HepG2, and Huh7 cells as indicated. The pJFH-1/core (**lanes 1, 3, 5**) and pJCH-1/core (**lanes 2, 4, 6**) were transfected and HCV core proteins were detected in cell lysates. The ratios of p21 in the detected core proteins are indicated as %p21 at the bottom.

phenylalanine at aa 164 ($^{164}$F), cysteine at aa 172 ($^{172}$C), serine at aa 173 ($^{173}$S), and threonine at aa 187 ($^{187}$T), respectively, and vice versa. These constructs were named with the mutated amino acid residues (Figs. 4 and 6–8). All constructs were verified by sequencing with Big Dye Terminator Mix and an automated DNA sequencer model 310 (PE Biosystems, Foster City, CA).

### In Vitro Transcription and Translation Assays

With expression vectors as templates, in vitro transcription and translation assays were undertaken using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instruction. The reaction mixtures were incubated at 30°C for 30 min. The reaction was terminated by adding an equal volume of 2× sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 0.15% bromophenol blue, 20% glycerol, and 10% 2-mercaptoethanol. The mixture was heated at 95°C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

### Cell Culture System

The 293T cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn bovine serum under 5% $CO_2$ conditions. HeLa and HuH7 cells were grown in DMEM containing 10% fetal bovine serum. HepG2 cells were grown in modified Eagle's medium containing 10% fetal bovine serum. Cells were seeded into 6-well plates with $2 \times 10^5$ cells per well and cultured overnight. The cultured cells were then infected with the T7 recombinant vaccinia virus (vTF7-3, provided from Dr. Bernard Moss) and subjected to DNA transfection with transfection reagent (FuGENE 6, Roche Diagnostics, Mannheim, Germany). At 4 hr after transfection, the cells were harvested. The cell pellet was dissolved with RIPA buffer containing 1% SDS, 0.5% Nonidet P-40 (NP-40), 0.15 M NaCl, 10 mM Tris (pH 7.4), and 1 mM EDTA, then sonicated for 30 sec × 3, and subjected to SDS-PAGE.

### Western Blot Analysis of HCV Core Protein

The protein samples were separated on a 12% polyacrylamide gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA) with a semidry blotting apparatus (Biocraft, Tokyo, Japan). Transferred proteins were incubated with blocking buffer containing 5% nonfat dry milk (Snow brand, Sapporo, Japan) in phosphate-buffered saline (PBS). Monoclonal anti-HCV core antibody C7-50A (1 μg/ml) and horseradish peroxidase (HRP)-labeled sheep anti-mouse IgG (1:2,000 dilution) (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used to detect HCV core protein [Moradpour et al., 1996]. The signals were detected with a chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech). Intensities of the resulting bands were quantified by optical density using a model GS-700 imaging densitometer and molecular

analyst software (Bio-Rad, Hercules, CA). The ratio of p21 core protein to total core protein was calculated and represented as %p21 [%p21 = p21 core protein/(p21 core protein + p23 core protein)].

## RESULTS

The expression vectors encoding the HCV core-E1 region with two clones (pJFH-1/CE1 and pJCH-1/CE1) isolated from patients with fulminant hepatitis (clone JFH-1) and chronic hepatitis (clone JCH-1) were subjected to in vitro transcription and translation assays with and without canine pancreatic microsomal membrane (CM) [Kato et al., 2001]. When core proteins were produced in this assay without CM, only precursor protein was observed (Fig. 1b, lanes 1 and 2). This result was compatible with previous data indicating that HCV core protein cleavage from the precursor depends on ER proteases [Hijikata et al., 1991; Hussy et al., 1996; Yasui et al., 1998]. With CM, p21 core protein production was more than that of p23 when pJFH-1/CE1 was used as a template (Fig. 1b, lane 3, %p21 = 98.64%). In contrast, with CM, p21 core protein production was less than that of p23 core protein when pJCH-1/CE1 was used as a template (Fig. 1b, lane 4, %p21 = 21.01%). Similar results were obtained in the cell culture system using 293T cell, %p21 = 97.54% with pJFH-1/CE1 and %p21 = 49.11% with pJCH-1/CE1 (Fig. 1b, lanes 7 and 8). Thus, the differences in p21 and p23 core protein production between these HCV clones were shown in both in vitro assay and cell transfection assay. To determine whether the difference in p21 production is due to the amino acid sequence of the core region or that following the E1 region, core proteins were expressed in vitro assay and cell transfection assay using the expression vectors encoding the HCV core region of each clone (pJFH-1/core and pJCH-1/core). In the in vitro translation assay, %p21 with pJFH-1/core was 71.97% and %p21 with pJCH-1/core was 8.92% (Fig. 1b, lanes 5 and 6). This in vitro assay was repeated 7 times to confirm the reproducibility and mean ± SE were 72.15 ± 1.71 for JFH-1 and 7.51 ± 0.469 for JCH-1 ($P < 0.0001$ by student's $t$-test). In the 293T cell, %p21 with pJFH-1/core was 57.80% and %p21 with pJCH-1/core was 20.10% (Fig. 1b, lanes 9 and 10). This cell transfection experiment was also repeated 6 times and mean ± SE 68.97 ± 7.63 for JFH-1 and 29.59 ± 7.24 for JCH-1 ($P < 0.0005$ by Student's $t$-test). The predominance of p21 core protein production with the JFH-1 clone was confirmed from both core constructs and CE1 constructs, although p21 core protein production from core constructs was less than that from CE1 constructs.

To determine the p21 core protein production in different cell types, we transfected pJFH-1/core and pJCH-1/core in HeLa, HepG2, and Huh7 cells. %p21 was 67.96% with pJFH-1/core, and was 21.06% with pJCH-1/core in HeLa cell, 89.91% and 33.54% in HepG2 cell, 96.35% and 74.28% in Huh7 cell, respectively (Fig. 1c). Compared with the non-liver-derived cell lines, 293T and HeLa cells, p21 core protein was produced preferentially in the liver-derived cell lines, HepG2 and Huh7. This result may indicate that intracellular processing depends on the relative abundance of signal peptidase, which could vary from cell type to cell type, such as non-liver- and liver-derived cell lines.

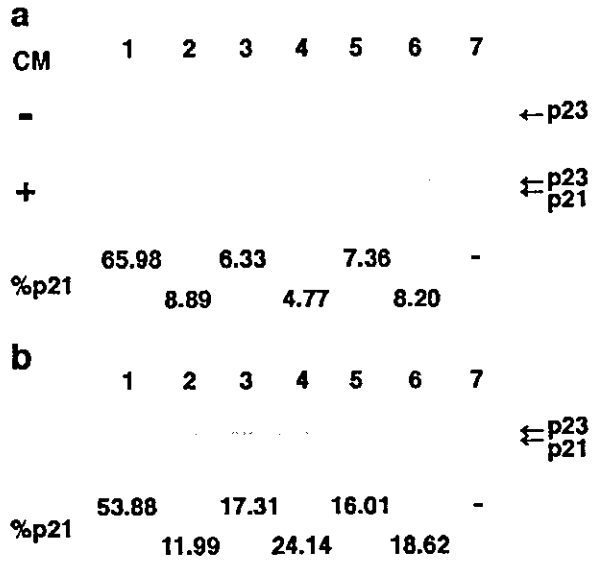


Fig. 2. Hepatitis C virus (HCV) core protein production using different HCV clones from patients with chronic hepatitis. HCV core proteins were detected using core-region constructs of clone JFH-1 (**lane 1**) and clones JCH-1–JCH-5 isolated from five patients with chronic hepatitis (**lanes 2–6**), and parental empty vector as a negative control (**lane 7**). **a:** These constructs were expressed in the in vitro transcription and translation assays without canine pancreatic microsomal membrane (CM) (–) and with CM (+) (upper and lower panel, respectively). **b:** The same constructs were transfected to 293T cells and HCV core protein expression in cell lysates was detected. The ratios of p21 in the detected core proteins are indicated as %p21 at the bottom.

To clarify further p21 core protein production in the other HCV clones, p21 core protein production from the construct pJFH-1/core was compared with those from constructs of HCV clones isolated from patients with chronic hepatitis (pJCH-1/core-pJCH-5/core). In the in vitro translation assay with CM, the average of %p21 with pJCH-1/core-pJCH-5/core was 7.11 ± 1.62, while %p21 with pJFH-1/core was 65.98% (Fig. 2a), in 293T cell, the average %p21 was 17.61 ± 4.41 with

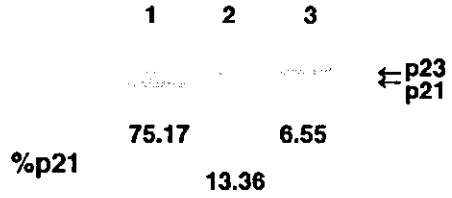


Fig. 3. Hepatitis C virus (HCV) core protein production using HCV clones from another patients with fulminant hepatitis (clone JFH-2). HCV core proteins were expressed in the in vitro translation assay with canine pancreatic microsomal membrane using core region constructs, pJFH-1/core (**lane 1**), pJFH-2/core (**lane 2**), and pJCH-1/core (**lane 3**). The ratios of p21 in the detected core proteins are indicated as %p21 at the bottom.

pJCH-1/core-pJCH-5/core and %p21 was 53.88% with pJFH-1/core (Fig. 2b). These results suggest that p21 core protein productions with clones isolated from patients with chronic hepatitis are less than that with clone JFH-1.

To determine whether this alteration of core protein processing founds in JFH-1 clone is common in all other HCV clones causing fulminant hepatitis, HCV cDNA was cloned from another fulminant hepatitis patient (clone JFH-2). Core region cDNA of JFH-2 was expressed in vitro and examined the p21 and p23 production as other clones. In the in vitro translation assay with CM, the %p21 of pJFH-2/core was 13.36%, a similar level to that of pJCH-1/core-pJCH-5/core (Fig. 3). These results suggested that the predominance of p21 core protein production was not always related to HCV clones causing fulminant hepatitis.

To determine the principal region affecting this processing step, chimeric constructs between pJFH-1/core and pJCH-1/core were used. Six chimeric constructs, pC/F 60, pC/F 90, pC/F 160, pF/C 60, pF/C 90, and pF/C 160, were subjected to the in vitro transcription and translation assays. The production of p21 was predominant with the pJFH-1/core construct (Fig. 5, lane 1,

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          1                                                  60 ←┐
JFH-1     MSTNPKPQRKTKRNTNRRPEDVKFPGGGQIVGGVYLLPRRGPRLGVRTTRKTSERSQPRG
JFH-2     ..........N.......Q.............................AA..........
JCH-1     ..................Q.............................A...A.......
JCH-2     ...T..............Q.............................A...........
JCH-3     .........Q.....S...Q..R........................A...........
JCH-4     ..................Q.............................A...........
JCH-5     ..................Q.............................A...........
          *** ***** * *** *** ** ************************  ** ********

          61                              90 ←┐
JFH-1     RRQPIPKDRRSTGKAWGKPGRPWPLYGNEGLGWAGWLLSPRGSRPSWGPTDPRHRSRNVG
JFH-2     ..........P...S.....Y.....................Q............R......
JCH-1     .......H......S.....Y..............................N.........
JCH-2     ..............S.....Y..............................N.........
JCH-3     ..............S..R..Y..............................N.........
JCH-4     ..............S.....Y..............................N.........
JCH-5     ..............S.....Y.....................................
          ******* ** *** ** ** ******************** ******** *** ******

                                                    164   172 173
          121                                  160 ←┐  ▽     ▽▽
JFH-1     KVIDTLTCGFADLMGYIPVVGAPLSGAARAVAHGVRVLEDGVNYATGNLPGFPFSIFLLA
JFH-2     ........................G.V...L.....A..............C........
JCH-1     ..........L..V.........V.S.L.............F.......CS.......
JCH-2     ........................G.V...L.............F.......CS.......
JCH-3     ........................G.V...L.....................CS.......
JCH-4     .........L..............G.V...L..........C.........CS.......
JCH-5     .....I..................G.V...L.............F.......CS.......
          ***** *** *** ** ******* * * * ***** **** * *******  *******

               187
          181   ▽   191
JFH-1     LLSCITVPVSA
JFH-2     ......T....
JCH-1     ......T....
JCH-2     ......T....
JCH-3     ..........
JCH-4     ..........
JCH-5     ......I....
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Fig. 4. Alignment of deduced amino acid sequences among clones JFH-1, JFH-2, and JCH-1–JCH-5 in the core region. Identical amino acids are indicated by dots. Arrows indicate the changing point of chimeric clones (Fig. 5). Open triangles indicate the mutated amino acid residues in mutation-introduced constructs (Figs. 6–8).