Hepatitis C Virus Infection and Risk of Hepatocellular Carcinoma Among Japanese: Possible Role of Type 1b (II) Infection

Keitaro Tanaka, Hideyuki Ikematsu, Tomio Hirohata, Seizaburo Kashiwagi*

Background: Although hepatitis C virus (HCV) infection is recognized as an important risk factor for hepatocellular carcinoma (HCC), the strength of this association has been inconsistent. In addition, the role of specific HCV genotypes in HCC progression has not yet been determined. Purpose: We conducted a case-control study to estimate the relative risk (RR) of HCC in relation to HCV infection among residents of the Fukuoka Prefecture, where HCC risk is among the highest in Japan, and to examine whether the risk differs according to HCV genotypes and/or HCV RNA titers. Methods: Stored serum samples obtained from 91 patients with HCC and 410 healthy control subjects, who had been frequency matched to the patients with regard to sex and age, were tested for antibodies to HCV by use of second-generation immunoradiometric and immunoblot assays. The presence of serum HCV RNA and of specific HCV genotypes was determined by use of polymerase chain reaction-based assays, and HCV RNA titers were measured by use of a branched DNA assay. Results: Antibodies to HCV were detected in 71 patients (78.0%) and in 30 control subjects (7.3%), of whom 57 patients and 25 control subjects had serum HCV RNA. One patient was positive for HCV RNA but not for antibodies to HCV. The second age-adjusted RR of HCC among individuals positive for antibodies to HCV was estimated to be 53.7 (95% confidence interval [CI] = 27.1-106.2). Antibodies to HCV were much more prevalent among patients negative for serum hepatitis B surface antigen (HBsAg) (69 of 72, 95.8%) than among HBsAg-positive patients (two of 19, 10.5%); the RR increased to 339.6 (95% CI = 96.5-1195.8) in the separate analysis of HBsAg-negative subjects. The most frequent genotype among HCV RNA-positive subjects was type 1b (also called type II) (found in 49 [86.0%] of 57 patients and in 15 [60.0%] of 25 control subjects); individuals with type 1b infection experienced a significantly elevated risk (RR = 3.8; 95% CI = 1.0-13.9) compared with the risk observed for individuals with type 2a (also called type III) infection. No statistically significant association between HCV RNA titers and HCC was evident. Conclusions: HCV infection, particularly type 1b infection, plays an important role in the development of HCC among the study population. We estimated that approximately 78% (95% CI = 69%-86%) of the HCCs that occur in this high-risk area are attributable to HCV infection, if we assume that the patients in this study were representative population samples. Implications: Further studies are needed to clarify potential risk factors, including specific HCV genotypes, for progression to HCC among HCV carriers. [J Natl Cancer Inst 1996;88:742-6]

Rapidly accumulated evidence from both epidemiologic and laboratory investigations has suggested that hepatitis C virus (HCV) infection plays a crucial role in the etiology of hepatocellular carcinoma (HCC) (1). However, the strength of this linkage as reflected by the relative risk (RR) has varied widely from study to study, partly because of problems inherent in both the sensitivity and the specificity of different assays for detecting antibodies to HCV when these assays are applied to stored sera (2,3). In addition, the contribution of HCV to the occurrence of HCC appears to vary substantially between geographic regions, being generally low in areas endemic for hepatitis B virus (HBV) infection and considerably high in Japan and some parts of southern Europe (1,4).

A great deal of attention also has been paid to the role of HCV genotypes in the development of chronic liver disease and HCC (5-7) and in different responses to interferon therapy (8,9). Although it is known that most patients with HCV-related chronic liver disease or HCC have type 1b (also called type II) infection (5,6,10,11), few controlled studies (7) have been conducted that have taken into account the background prevalence of different HCV genotypes. The amount of HCV RNA in the serum that possibly reflects viral replication in liver cells may also be associated with HCC risk.

We previously performed a case-control study of HCC in Fukuoka Prefecture, where HCC risk is among the highest in Japan (12,13); on the basis of first-generation immunologic assays, we found an extremely elevated risk among subjects positive for antibodies to HCV (RR adjusted for demographic factors = 52.3), and attributable risk calculations suggested that HCV infection could account for more than half of the HCCs occurring in this area (12).

In the present study utilizing stored sera from the same case-control series, we used second-generation immunologic assays to evaluate the risk of HCC. We sought additional supporting evidence by using a polymerase chain reaction (PCR)-based assay to detect HCV genomes. We examined whether the risk differs according to specific HCV genotypes and/or HCV RNA titers.

Subjects and Methods

Study Subjects

The study subjects and methods have been fully described elsewhere (12,13). In brief, our study involved 91 case patients with HCC (73 males and 18 females). These patients had been admitted to Kyushu University Hospital between December 11, 1985, and June 30, 1989, and their sera were stored and made available for testing for antibodies to HCV and HCV RNA status. The patients met the following selection criteria: (a) initially diagnosed as having HCC within 1 year before identification for participation in this study, (b) aged 40-69 years, (c) residing in Fukuoka or Saga Prefecture (neighboring Fukuoka Prefecture), and (d) of Japanese nationality. The median ages were 59.0 years for the
male patients and 56.5 years for the female patients. Of the 91 patients, 89 were residents of Fukuoka Prefecture, whereas only two resided in Saga Prefecture. The disease in 30 patients was histologically confirmed; in the remaining 61 patients, it was diagnosed by angiography, ultrasonography, and/or computed tomography as well as by ascertainment of elevated α-fetoprotein levels. Seventy-six patients (83.5%) had pre-existing liver cirrhosis, and nine patients (9.9%) had some form of chronic hepatitis.

The control subjects consisted of 410 persons (291 males and 119 females) aged 40-69 years who resided in Fukuoka City and who underwent health examinations at a public health center located near Kyushu University Hospital between January 1, 1986, and July 31, 1989. They were selected so that their distribution by sex and age would be as similar as possible to that of the patients. Those who had definite chronic liver diseases such as chronic hepatitis and liver cirrhosis or those from whom blood specimens could not be obtained were excluded. The median ages were 57.0 years for the male control subjects and 56.0 years for the female control subjects.

Despite the above selection procedure, the control subjects had a higher proportion of females than did the patients (P = .07 by chi-squared test), and the male patients were significantly older than the male control subjects (P<.05 by Wilcoxon test). Thus, whenever possible, sex and age were considered in the estimation of RRs for relevant factors because of their possible confounding effects. Although the distribution of occupational history and duration of education (i.e., socioeconomic indicators that can be possible confounders) also differed slightly but significantly between the patients and control subjects, the adjustment for these factors had little effect on the RR estimates.

Serologic Tests

Information on the status of serum hepatitis B surface antigen (HBsAg), determined by a reverse passive hemagglutination method (Aussell; Abbott Laboratories, Chicago, IL), was obtained from the medical records of the patients. For the control subjects, sera were tested for HBsAg by the reverse passive hemagglutination method. Until tested for antibodies to HCV, sera from 91 patients and from 410 control subjects were kept frozen at -70 °C. A second-generation immunoradiometric assay (IRMA II; Ortho Diagnostic Systems Inc., Raritan, NJ) was used to detect antibodies to HCV. This assay detects antibodies to the C22-3 (core protein), C200 (NS3 and NS4 proteins), and C100-3 (NS3 and NS4 proteins) (1). The sera that were positive in IRMA II were further confirmed for positivity with a second-generation recombinant immunoblot assay (RIBA II; Chiron Therapeutics, Emeryville, CA). RIBA II separately detects antibodies to the C22-3 (core protein), C33c (NS3 protein), C100-3 (NS3 and NS4 proteins), and 5-1-1 (NS4 protein) (1). Following the manufacturer’s instructions, we considered samples positive for two or more of the four antigens as being “reactive,” those positive for only one antigen as being “indeterminate,” and those negative for any antigen as being “nonreactive”; in our examinations, no samples were positive for the control band of superoxide dismutase.

Detection, Genotyping, and Titration of HCV RNA

The presence or absence of serum HCV RNA was determined for all 91 patients. For 45 control subjects who were positive in IRMA II, and for 20 control subjects who were randomly selected from 365 IRMA II-negative control subjects, RNA was extracted from 0.1 mL of frozen serum and analyzed for HCV RNA by nested PCR with primers derived from the 5' untranslated region, as described by Okamoto et al. (14). Each specimen was examined at least in duplicate, and only repeatedly positive results were considered as being positive. The results in the above experimental series were further confirmed by use of a commercially available assay (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). Our dilution study suggested that the Amplicor kit could detect 20-60 HCV RNA genome equivalents/0.1 mL [genotype II by the classification system of Okamoto et al. (10)] or 1 by the classification system of Simmonds et al. (15)]. Throughout these examinations, we avoided false-positive results by strictly adhering to the techniques described by Kwoh and Higuchi (16). For HCV RNA-positive specimens, we determined HCV genotypes by amplifying a putative core-region sequence with genotype-specific primers, as described by Okamoto et al. (10). By this method, four genotypes [I, II, III, and IV, which correspond to 1a, 1b, 2a, and 2b, respectively, by the classification system of Simmonds et al. (15)] could be identified. For convenience, we followed the nomenclature proposed by Simmonds et al. (17).

The sera that tested positive for HCV RNA were further subjected to a branched DNA assay (Quantiplex HCV RNA; Chiron Therapeutics) for titration of the amount of HCV RNA. According to information supplied by the manufacturer, the detection limit of the assay is approximately 0.5 genome megaequivalent (MEq)/mL.

Statistical Analysis

Using the SAS/PC statistical package (SAS Institute Inc., Cary, NC) (18,19), we estimated the RR, as approximated by the odds ratio, along with its 95% confidence interval (CI); to do so, the data were modeled by use of unconditional logistic regression analysis to control for possible confounders including sex and age category (40-49 years, 50-59 years, and 60-69 years). All reported P values are two-tailed.

Results

Seventy-three (80.2%) of 91 patients and 45 (11.0%) of 410 control subjects were found to be positive by IRMA II. Of 73 IRMA II-positive patients, 71 (97.3%) were found to be reactive by RIBA II, whereas 30 (66.7%) of 45 IRMA II-positive control subjects were reactive. Table 1 shows the prevalence of antibodies to HCV among the patients and control subjects by results from both IRMA II and RIBA II as well as by sex. Among males, 59 (80.8%) of 73 patients were positive by both IRMA II and RIBA II, whereas 25 (8.6%) of 291 control subjects were positive. Among females, the corresponding prevalence was 66.7% for the patients and 4.2% for the control subjects.

Table 2 presents the detection rate of serum HCV RNA according to the combined results from IRMA II and RIBA II. Among IRMA II-negative subjects (consisting of 18 patients and 20 randomly selected control subjects), only one patient was found to have serum HCV RNA. None of the two patients and 15 control subjects who were positive by IRMA II but either nonreactive or indeterminate by RIBA II had detectable serum HCV RNA. Of the 71 patients and

Table 1. Prevalence of antibodies to HCV among study subjects by results from IRMA II and RIBA II assays and by sex*

<table>
<thead>
<tr>
<th></th>
<th>IRMA II</th>
<th>RIBA II</th>
<th></th>
<th>No. of males (%)</th>
<th></th>
<th>No. of females (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCC patients</td>
<td>Control subjects</td>
<td>HCC patients</td>
<td>Control subjects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>12 (16.4)</td>
<td>256 (88.0)</td>
<td>6 (33.3)</td>
<td>109 (91.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>0 (0.0)</td>
<td>2 (0.7)</td>
<td>0 (0.0)</td>
<td>1 (0.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>2 (2.7)</td>
<td>8 (2.7)</td>
<td>0 (0.0)</td>
<td>4 (3.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>59 (80.8)</td>
<td>25 (8.6)</td>
<td>12 (66.7)</td>
<td>5 (4.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>73 (100)</td>
<td>291 (100)</td>
<td>18 (100)</td>
<td>119 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*HCV = hepatitis C virus; IRMA II = second-generation immunoradiometric assay; RIBA II = second-generation recombinant immunoblot assay; HCC = hepatocellular carcinoma.
30 control subjects who tested positive by both IRMA II and RIBA II, 57 patients (80.3%) and 25 control subjects (83.3%) had serum HCV RNA. Because of these findings, thereafter only IRMA II-positive and RIBA II-reactive results were considered as reflecting positivity to antibodies to HCV.

In Table 3, the distribution of subjects positive for antibodies to HCV and for HCV RNA is displayed according to HCV genotypes. The most prevalent genotype was type 1b in both the patients (86.0%) and the control subjects (60.0%), followed by type 2a (10.5% of the patients and 28.0% of the control subjects) and type 2b (none of the patients and 4.0% of the control subjects); no type 1a was detected. Mixed genotypes were observed for one patient (strongly positive for type 2a and weakly positive for type 2b) and for one control subject (strongly positive for type 2a and weakly positive for type 1b). For one patient and one control subject, HCV genotypes could not be determined despite the repeated detection of HCV RNA. One patient negative for antibodies to HCV but positive for HCV RNA had a type 2a genotype. The sex- and age-adjusted RR for patients positive for type 1b in comparison with those positive for type 2a was estimated to be 3.8 (95% CI = 1.0-13.9; \( P = 0.04 \)), and this estimate decreased slightly to 3.4 (95% CI = 1.0-12.0; \( P = 0.06 \)) when we included in the analysis one patient with type 2a infection who was negative for antibodies to HCV.

Table 2. Detection rate of HCV RNA according to antibodies to HCV status*

<table>
<thead>
<tr>
<th>IRMA II</th>
<th>RIBA II</th>
<th>HCC patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>Not tested</td>
<td>1/18 (5.6)</td>
<td>0/20 (0.0)</td>
</tr>
<tr>
<td>+</td>
<td>Nonreactive</td>
<td>0/0 (0.0)</td>
<td>0/3 (0.0)</td>
</tr>
<tr>
<td>+</td>
<td>Indeterminate</td>
<td>0/2 (0.0)</td>
<td>0/12 (0.0)</td>
</tr>
<tr>
<td>+</td>
<td>Reactive</td>
<td>57/11 (80.3)</td>
<td>25/30 (83.3)</td>
</tr>
</tbody>
</table>

*HCV = hepatitis C virus; IRMA II = second-generation immunoradiometric assay; RIBA II = second-generation recombinant immunoblot assay; HCC = hepatocellular carcinoma.

Table 3. Distribution of subjects with antibodies to HCV and HCV RNA in the serum and relative risks by HCV genotypes and by HCV RNA titers*

<table>
<thead>
<tr>
<th>HCV genotypes</th>
<th>No. of HCC patients (%)</th>
<th>No. of control subjects (%)</th>
<th>Relative risk†</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>49 (86.0)</td>
<td>15 (60.0)</td>
<td>3.8</td>
<td>1.0-13.9</td>
</tr>
<tr>
<td>2a</td>
<td>6 (10.5)</td>
<td>7 (28.0)</td>
<td>1.0</td>
<td>Reference</td>
</tr>
<tr>
<td>2b</td>
<td>0 (0.0)</td>
<td>1 (4.0)</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>2a + 1b</td>
<td>0 (0.0)</td>
<td>1 (4.0)</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>2a + 2b</td>
<td>1 (1.8)</td>
<td>0 (0.0)</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>ND</td>
<td>1 (1.8)</td>
<td>1 (4.0)</td>
<td>1.2</td>
<td>0.1-22.9</td>
</tr>
<tr>
<td>Total</td>
<td>57 (100)</td>
<td>25 (100)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HCV RNA titers</th>
<th>No. of HCC patients (%)</th>
<th>No. of control subjects (%)</th>
<th>Relative risk†</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.5</td>
<td></td>
<td>17 (29.8)</td>
<td>6 (24.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5-1.9</td>
<td>17 (29.8)</td>
<td>6 (24.0)</td>
<td>1.0</td>
<td>0.3-4.0</td>
</tr>
<tr>
<td>2.0-9.9</td>
<td>18 (31.6)</td>
<td>7 (28.0)</td>
<td>1.1</td>
<td>0.3-4.4</td>
</tr>
<tr>
<td>≥10.0</td>
<td>5 (8.8)</td>
<td>6 (24.0)</td>
<td>0.3</td>
<td>0.1-1.3</td>
</tr>
<tr>
<td>Total</td>
<td>57 (100)</td>
<td>25 (100)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*HCV = hepatitis C virus; HCC = hepatocellular carcinoma.
†Adjusted for sex and age category whenever possible.
§ND = not determined. HCV genotypes could not be determined for these subjects.
†One patient negative for antibodies to HCV and positive for HCV RNA showed type 2a. When this patient was included in the analysis, the relative risk (and 95% confidence interval) for type 1b versus type 2a became 3.4 (1.0-12.0).

Discussion

In our previous analysis (12) that was based on first-generation enzyme immunoassay and recombinant immunoblot assay, we found approximately 51% of the patients and 3% of the control subjects to be positive for antibodies to HCV. In the present study that was based on second-generation assays, we found that the prevalence of positive antibodies to HCV increased to approximately 78% for the patients and 7% for the control subjects. More than 80% of the subjects positive for antibodies to HCV had serum titers (>10 MEq/mL) was somewhat lower among the patients (8.8%) than among the control subjects (24.0%), but positive for HCV RNA had a reading of 0.4 MEq/mL.

The sex- and age-adjusted RRs of HCC were estimated to be 53.7 (95% CI = 27.1-106.2) for positive antibodies to HCV and 16.0 (95% CI = 6.4-39.7) for positive HBsAg. Table 3 shows the distribution of the patients and control subjects and the RRs according to the status of both HBsAg and antibodies to HCV. The prevalence of positive antibodies to HCV was much higher among HBsAg-negative patients (69 of 72 patients, 95.8%) than among HBsAg-positive patients (two of 19 patients, 10.5%) (P for difference <.001 by Fisher's exact test). Among HBsAg-negative subjects, those positive for antibodies to HCV showed the sex- and age-adjusted RR of 339.6 (95% CI = 96.5-1195.8). In contrast, among subjects negative for antibodies to HCV, those positive for HBsAg had a sex- and age-adjusted RR of 293.7 (95% CI = 68.7-1255.6). Only two patients and no control subjects had duplicate infections, leading to an infinite RR with uncertain precision.
HCV RNA. This result indicated that the above prevalences were not substantially distorted by false-positives, as is frequently observed in sera stored for long periods (2). Although PCR used for detection of HCV RNA has problems with regard to both sensitivity and specificity (20), we confirmed the PCR results by repeated tests on each specimen and by using a standardized commercial kit; the performance of this kit had been validated earlier.

In accord with our previous findings (12), in the present analysis we again demonstrated an extremely elevated risk of HCC related to HCV infection. The sex- and age-adjusted RR for subjects positive for antibodies to HCV reached 53.7, and this estimate increased to 339.6 when we examined HBsAg-negative subjects separately. This change in RR was due to the strong negative relationship between the status of HBsAg and antibodies to HCV among the patients (Table 4). Accordingly, a similar increase in RR was observed associated with chronic HBV infection; while the sex- and age-adjusted RR for positive HBsAg was estimated to be 16.0, which is close to the estimates obtained from most of the other analytic epidemiologic studies [reviewed in (27)], the analysis of subjects negative for antibodies to HCV led to an RR estimate of 293.7. Each viral infection appears to be separately associated with most HCCs; thus, our study could not address the possible interactive role of HBV and HCV infections in the development of HCC (22,23).

Several case–control studies (23,24-29) based on second-generation assays reported RRs ranging from 1.1 to 27—RRs that are substantially lower than our estimates. The reason for this discrepancy is largely unknown. However, in some studies, contamination of false-positive or indeterminate results may have biased the RR estimates toward unity. Another possibility may be related to the relative importance of HBV and HCV infections in the etiology of HCC among the Japanese, compared with other risk factors such as alcohol consumption and cigarette smoking (13,30-33). In our study population, both infections may represent distinct essential determinants, without any other comparable determinants that possibly include aflatoxin exposure (34), whereas alcohol consumption and cigarette smoking may act as contributory, but not always necessary, factors. If so, the occurrence of HCC without either viral infection would be extremely low, which, in turn, could lead to extremely elevated RRs for each infection such as our estimates.

Our findings and interpretations may not be generalizable to other settings in Japan. For example, Ohkoshi et al. (35) indicated that, in a town in Japan where both HBV and HCV infections are endemic, HCV-infected individuals did not seem to develop progressive liver diseases. However, this result may be related to the retrospective nature of their study. In addition, these investigators did observe abnormal liver enzyme levels (mostly fluctuating) in approximately 80% of HCV carriers and ultrasonographic findings suggestive of cirrhosis in about 12% of these carriers. Epidemiologic studies among other Japanese populations are needed to confirm our results.

Our key finding is that individuals with type 1b HCV infection were about three to four times more likely to develop HCC compared with those with type 2a infection. Ichimura et al. (7) reported that type 1b infection was significantly more prevalent among patients with chronic ac-
measurement may fluctuate in the course of disease progression.

In conclusion, our results revealed an extremely important role for HCV infection, particularly type 1b infection, in the etiology of HCC among the study population. If one assumes that the exposure prevalences among the patients in this study were representative of those among all HCC patients in Fukuoka, attributable risk calculations (38) suggest that HCV and HBV infections may account for approximately 78% (95% CI = 69%-86%) and 21% (95% CI = 12%-29%), respectively, of the HCCs occurring among residents of this region of Japan. Thus, strategies directed against HCV infection will be central to the prevention of HCC in this high-risk area. Further studies are required to clarify potential risk factors, including specific HCV genotypes, that affect the development of HCC among patients.

References


Notes

Supported in part by grants 0670264 and 07707256 from the Ministry of Education, Science and Culture, Japan; and by the Fukuoka Cancer Society.

We thank Yoshitaka Etoh of Rintec Inc. for his technical assistance. We also thank the staff members of the following institutions for assisting in blood and data collection: First and Third Departments of Internal Medicine and Second Department of Surgery, Kyushu University; A School of Medicine, and Hakata Public Health Center.

Manuscript received October 3, 1995; revised March 5, 1996; accepted March 11, 1996.