Risk of Hepatocellular Carcinoma and Secondary Structure of Hepatitis C Virus (HCV) NS3 Protein Amino-Terminus, in Patients Infected with HCV Subtype 1b

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We conducted a retrospective study of 65 patients with chronic hepatitis C, to determine whether the secondary structure of the amino-terminal 120 residues of the hepatitis C virus (HCV) NS3 protein is associated with an increased risk of development of hepatocellular carcinoma (HCC). The cumulative incidence of HCC was highest among patients infected with group B HCV-1b, wherein the risk of HCC significantly increased compared with that among patients infected with group A (hazard ratio, 4.95 [95% CI, 1.43–17.11]) after adjustment for age and histological stage. This HCV-1b grouping may be a useful marker for detecting the risk of development of HCC.

Hepatocellular carcinoma (HCC) is a common malignancy, and the mortality associated with it is increasing in Japan [1]. The 3 major categories of risk factors that appear to influence the incidence of HCC are host, viral, and environmental factors. Epidemiologic and clinical studies have shown that chronic hepatitis C virus (HCV) infection is a major cause of HCC [2].

At present, HCV is classified into at least 6 genotypes and >60 subtypes [3, 4]. HCV subtype 1b (HCV-1b) is most common in Asian countries, including Japan [5]. Patients infected with HCV-1b have a greater risk of progression to HCC than do those infected with other subtypes [6]. However, it remains unclear whether all HCV strains are associated with HCC to an equal degree.

We have been interested in the possible involvement of the HCV NS3 region in hepatocarcinogenesis. It has been reported that an amino-terminal portion of NS3 (aa 1027–1295 and aa 1008–1246) has the potential to transform NIH 3T3 and rat fibroblast cells [7, 8]. Moreover, studies have reported that an amino-terminal portion of NS3 (aa 1027–1459) renders NIH 3T3 cells more resistant to DNA damage–induced apoptosis [9, 10], which is thought to be a prerequisite for the malignant transformation of cells, and that NS3 interacts differentially, in a sequence-dependent manner, with the p53 tumor suppressor [11].

Recently, we reported that HCV-1b strains can be classified into different groups based on the secondary structure of an amino-terminal portion of the NS3 protein and that group B strains are more prevalent among patients with HCC [12]. These results suggest the possibility that HCV-1b strains of group B cause HCC more frequently than do group A strains.

Using a retrospective cohort study design, we precisely assessed the possible association between the HCV-1b NS3-protein group and the risk of development of HCC.

Patients and methods. This retrospective cohort study enrolled outpatients infected with HCV-1b, who were referred from general medical or hepatology clinics to the Yamagata University Hospital, to further investigate their liver status. They all had been diagnosed histologically as having chronic hepatitis or cirrhosis. Patients with alcohol-related liver injury or autoimmune hepatitis or who were positive for hepatitis B surface antigen were excluded. Furthermore, patients who were followed up for <12 months were excluded, to rule out the possibility that the cancer was present at the start of the study. Twenty patients were excluded because samples available for group analysis were inadequate. We consequently examined 65 patients. Observation began in October 1981 and ended in December 2005. The baseline condition was considered to be the initial histologic diagnosis, and the end points were considered to be either (1) development of HCC or (2) last ultrasound (US) or computed tomogram (CT) without a diagnosis of HCC. At baseline, none of the patients had a diagnosis of HCC, on the basis of screening tests using US, CT,
Table 1. Baseline characteristics of patients infected with group A, B, and C strains of HCV.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group A (n = 18)</th>
<th>Group B (n = 44)</th>
<th>Group C (n = 3)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;55 years</td>
<td>11 (61)</td>
<td>18 (41)</td>
<td>1 (33)</td>
<td>.37</td>
</tr>
<tr>
<td>&gt;55 years</td>
<td>7 (39)</td>
<td>26 (59)</td>
<td>2 (67)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13 (72)</td>
<td>21 (48)</td>
<td>2 (67)</td>
<td>.20</td>
</tr>
<tr>
<td>Female</td>
<td>5 (28)</td>
<td>23 (52)</td>
<td>1 (33)</td>
<td></td>
</tr>
<tr>
<td>Stage of liver fibrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1 or F2</td>
<td>10 (56)</td>
<td>22 (50)</td>
<td>3 (100)</td>
<td>.30</td>
</tr>
<tr>
<td>F3 or F4</td>
<td>8 (44)</td>
<td>22 (50)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Grade of inflammatory activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1 or A2</td>
<td>13 (72)</td>
<td>28 (64)</td>
<td>3 (100)</td>
<td>.52</td>
</tr>
<tr>
<td>A3</td>
<td>5 (28)</td>
<td>16 (36)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 IU/L</td>
<td>9 (50)</td>
<td>27 (61)</td>
<td>0 (0)</td>
<td>.11</td>
</tr>
<tr>
<td>&gt;100 IU/L</td>
<td>9 (50)</td>
<td>17 (39)</td>
<td>3 (100)</td>
<td></td>
</tr>
<tr>
<td>Time since diagnosis of chronic liver injury, years</td>
<td>1.3 (0.2–9.3)</td>
<td>0.7 (0.3–8.5)</td>
<td>1.5 (0.5–1.5)</td>
<td>.51</td>
</tr>
<tr>
<td>Time since blood transfusion, years</td>
<td>28.6 (5.7–35.6)</td>
<td>26.9 (5.5–36.0)</td>
<td>...</td>
<td>.67</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of subjects, unless otherwise indicated.

* By Fisher’s exact test.

b By Kruskal-Wallis test.

c Based on data from those patients who had a history of blood transfusion, including 10 infected with group A strains and 17 infected with group B strains.

d By Mann-Whitney test.

or serum ?-fetoprotein (AFP) level. None of the patients without HCC died during the follow-up period. All of the patients underwent screening examination for HCC, by either US or CT, at least once every 6 months, as well as blood testing, including testing for AFP, every 1 or 2 months. None of the personnel involved in the decision to screen and in selection of participants in the study were aware of the group status. There was no evident difference in screening frequency among the groups. The cirrhotic patients at baseline were all in a state of Child class A, which means that the clinically evaluated severity of liver disease was mild. The HCV genotype was determined by reverse-transcription polymerase chain reaction (RT-PCR) using genotype-specific primers [13].

On the basis of the secondary structure of NS3 protein, the HCV-1b isolates from all patients were classified further—into group A, group B, and an indeterminate group, group C, as reported elsewhere [12]. After October 2001, stored frozen samples collected during the follow-up period were used to determine the group. To identify whether the group shifted to another group during the infection period, the group of 8 patients was examined by use of paired serum samples from each of them.

Histological findings were scored to determine the stage of liver fibrosis and the grade of inflammatory activity, according to the classification system of Desmet et al. [14], by institutional pathologists who were blinded to the subjects’ respective subgroup classifications. The stage of fibrosis was assessed as ranging from stage F0 (no fibrosis) to F4 (cirrhosis), and the grade of inflammatory activity was scored from grade A0 (minimal) to grade A3 (severe).

Written informed consent was obtained from all the participants. The study was approved by the Ethics Committee of Yamagata University.

The characteristics of the groups of patients were compared by use of Fisher’s exact test, Mann-Whitney test, or Kruskal-Wallis test. Cumulative incidence curves were estimated by use of the Kaplan-Meier method; differences between the groups were assessed by use of the log-rank test. The risk of development of HCC was evaluated by use of the hazard ratio (HR) and its 95% confidence interval (CI), which were estimated by
use of the Cox proportional hazard model. The following 5 variables at baseline and the average HCV RNA level during the follow-up period were analyzed to assess potential confounding for liver carcinogenesis: age (≥55 years or >55 years), sex (male or female), stage of liver fibrosis (F1/F2 or F3/F4), grade of inflammatory activity (A1/A2 or A3), alanine aminotransferase level (≤100 IU/L or >100 IU/L), and HCV RNA level (low or high). The serum HCV RNA level was designated as a high viral load when it was either >10⁶ equivalents/mL, on the basis of branched DNA probe assay, or >10⁵ copies/mL, on the basis of combined RT-PCR assay (Amplicor-HCV monitor assay). Also, patients who received interferon therapy, which is effective against HCV infection and is known to reduce the risk of development of HCC in patients with chronic hepatitis C, were compared with those who had not received it [15].

It was difficult to clarify the time when some of the patients in the present study became infected with HCV. Therefore, we evaluated 2 factors as a proxy of the infection period before enrollment: (1) time since diagnosis of chronic liver injury and (2) time since blood transfusion in 27 patients who had a history of transfusion. The proportional hazard assumption was checked for all covariates, and no relevant violations were found. We used SAS statistical software (version 8.2; SAS Institute, Inc.) for the analyses. P < .05 was considered to be statistically significant.

Results. Of the 65 HCV-1b isolates analyzed, 18 were classified as group A, 44 as group B, and 3 as group C. Baseline characteristics of the patients infected with group A, B, and C strains are summarized in Table 1. For the factors listed in the table, there were no significant differences between the groups. The HCV RNA level also did not differ significantly between the 3 groups (P = .158): the proportions of patients with a high viral load were 67%, 86%, and 100% in those infected with group A, B, and C strains, respectively. The median follow-up period was 12.8 years (range, 1.0–19.5 years). The subgroups with group A, B, and C strains, respectively, were compared with those who had not received it [15].

Figure 1 depicts the cumulative incidence of HCC (determined by the Kaplan-Meier method) in the 3 groups. The 10-year cumulative incidences for patients infected with group A strains and for those infected with group B strains were 0.16 (95% CI, 0.00–0.37) and 0.43 (95% CI, 0.28–0.58), respectively; the 15-year rates for these 2 groups were 0.16 (95% CI, 0.00–0.37) and 0.72 (95% CI, 0.54–0.90), respectively. Cumulative incidence differed significantly between patients infected with group A strains and those infected with group B strains (P < .01, by log-rank test).

For estimation of risk of development of HCC, we focused on group A strains and group B strains, because the number of group C strains was too small to allow accurate evaluation of this association. The crude HR for development of HCC among patients infected with group B strains (when the crude HR among patients infected with group A strains was considered to be the reference) was 4.92 (95% CI, 1.48–16.32), and group B strains were significantly associated with HCC after adjustment for each potential confounding factor: HR, 5.15 (95% CI, 1.52–17.38); HR, 5.79 (95% CI, 1.70–19.77); HR, 5.10 (95% CI, 1.53–17.04); HR, 4.05 (95% CI, 1.21–13.56); and HR, 5.77 (95% CI, 1.68–19.83) for age, sex, grade of inflammatory activity, stage of fibrosis, and HCV RNA level, respectively. On the basis of the results of 31 cases of development of HCC, we included 2 important factors—namely, age and...
histologic stage—in the multivariate model; after adjustment for these 2 factors, the risk was significantly greater among the patients infected with group B strains (HR, 4.95 [95% CI, 1.43–17.11]).

Discussion. The findings of the present study suggest that the secondary structure of the amino-terminal 120 residues of the HCV NS3 protein may be independently associated with the risk of development of HCC. The mechanism underlying the relationship between hepatocarcinogenesis and sequence diversity remains unclear. It has been speculated that the difference in the secondary structure of NS3 causally associates with HCC, which reflects a conformational difference that might consequently affect interaction with the p53 tumor-suppressor gene [11]. However, there is another possibility—that the NS3-protein group B strains exert oncogenic function via a mechanism independent of interaction with p53. Moreover, we cannot exclude the possibility that the other biological functions of NS3, such as serine protease activity and antiapoptotic capacity, vary between different groups. In a previous study, which included 35 (54%) of the patients in the present study, we analyzed the amino acid sequences of the HCV isolate from patients with or without HCC and, among the majority of isolates from patients with HCC, did not find, at the primary-structure level, any particular residue that might be contributing to HCC [12]. Further experimental studies are necessary to elucidate these issues.

Long-term chronic inflammation is considered to be related to carcinogenesis. Unfortunately, it was difficult, in some patients in the present study, both to determine when they became infected with HCV and to evaluate the true duration of their infection. We chose, as the baseline, the time when liver biopsy was performed, because histologic characteristics are strong factors for development of HCC [15]. Although the presumed infection period before enrollment was not significantly different between the groups, it should be noted that there might have been unmeasured confounding, because of the limitation posed by the present study's lack of data with respect to true disease-inception status.

In conclusion, the present study shows that the long-term cumulative incidence of HCC in patients infected with HCV group B is significantly different from that in patients infected with HCV group B—and that, after adjustment for age and stage of fibrosis, the latter patients have a markedly increased HR. These findings suggest that HCV-1b grouping, based on the secondary structure of an amino-terminal portion of the NS3 protein, is a potential marker for a high risk of development of HCC in patients with chronic hepatitis C. It is important to further test and replicate the present study's results, in other populations and in a larger number of subjects who have a defined time point of infection, such as blood-transfusion recipients.

Acknowledgment

We thank L. Shao for helpful comments.

References