MAJOR ARTICLE

High Levels of Hepatitis B Virus After the Onset of Disease Lead to Chronic Infection in Patients With Acute Hepatitis B

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Background. Some patients with acute hepatitis B virus (HBV) infection develop chronic infection. However, the method for identifying these patients has not been established.

Methods. We followed 215 Japanese patients with acute HBV infection until the clearance of hepatitis B surface antigen (HBsAg) or the development of chronic infection. Levels of HBsAg and HBV DNA were serially monitored from the onset.

Results. Of the 215 patients, 113 (52.5%) possessed HBV genotype A, 26 (12.0%) genotype B, and 73 (34.0%) genotype C. Twenty-one of the 215 (9.8%) developed chronic infection, with the persistence of HBsAg for >6 months. The rate of chronicity of genotype A, B, and C was 12.4%, 3.8%, and 8.2%. Of the 21 patients, only 6 (2.8%) patients, including 5 with genotype A, failed to clear HBsAg within 12 months. Levels of HBsAg at 12 weeks and HBV DNA at 4 weeks were useful for distinguishing the patients who became chronic from those who did not ($P < .001$ and $P < .001$, respectively). Likewise, the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks were useful for discriminating between the patients who lost HBsAg within 12 months and those who did not ($P < .01$ and $P < .05$, respectively).

Conclusions. In acute HBV infection, clearance of HBV may happen between 6 and 12 months from the onset. Only those who fail to clear HBV within 12 months from the onset may develop chronic infection.

Keywords. hepatitis B virus antigen; hepatitis B virus; genotype.

The clinical outcome of acute hepatitis B is self-limited in the majority of immunocompetent adults. However, some patients run a prolonged or even chronic course, or are complicated by acute liver failure. Several factors are implicated in different clinical courses.

Hepatitis B virus (HBV) genotypes and subtypes are known to influence the clinical outcome of acute hepatitis B. For instance, HBV subgenotype B1 is associated with fulminant hepatic failure in acute hepatitis B [1]. On the other hand, genotype A is associated with chronic sequelae [2–5]. Furthermore, patients with subgenotype C2 are more likely to develop chronic infection than those with subgenotype B2 [6]. These characteristics may reflect viral kinetics in acute HBV infection that would differ among HBV infections with distinct genotypes/subgenotypes, but little is known about them.

Quantitation of hepatitis B surface antigen (HBsAg), in addition to HBV DNA, has been introduced to analysis of viral kinetics in patients with chronic hepatitis B in recent years. HBsAg levels are also useful for estimating

Received 14 February 2013; accepted 9 May 2013; electronically published 23 May 2013.

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Clinical Infectious Diseases 2013;57(7):935–42

DOI: 10.1093/cid/cit348

viral loads and predicting the response to antiviral treatments [7–9], and for determining the natural history of chronic hepatitis B [10, 11]. Therefore, HBsAg and HBV DNA would be instrumental in foretelling the outcome of acute hepatitis B. However, the clinical utility of these markers in patients with acute hepatitis B is largely unknown.

Therefore, the aim of the present study was to examine differences in viral kinetics among patients with acute hepatitis B, who were infected with HBV of different genotypes, and evaluate the usefulness of quantifying HBsAg and HBV DNA for predicting the clinical outcome.

PATIENTS AND METHODS

Patients

This was a retrospective study of patients who were diagnosed with acute hepatitis B in our institutions during 1994 through 2010. Criteria for the diagnosis of acute hepatitis B were (1) acute onset of liver injury without a previous history of liver dysfunction; (2) detection of HBsAg in the serum; (3) immunoglobulin M (IgM) antibody to HBV core (anti-HBc) in high titers (detectable in serum samples diluted 10-fold) [3]; (4) absence of a past or family history of chronic HBV infection; and (5) exclusion of coinfection with hepatitis A virus, hepatitis C virus, or other hepatotropic viruses by serologic testing. Among the 232 patients who met these criteria, 215 patients (159 men and 56 women with a mean age of 31.8 ± 10.0 years) whose serum samples were available for virologic analyses were included in the study. No patient developed liver failure.

No patient received antiviral treatment. Of the 215 patients, 159 (74.0%) patients could be regularly followed up until the confirmation of clinical outcomes. Based on the duration of HBsAg (defined as the interval between the onset [defined by the first visit] and the last visit with detectable HBsAg), we classified the 159 patients into the following 4 groups (the duration of HBsAg is indicated in parentheses): group 1 (<3 months); group 2 (3–6 months); group 3 (>6–12 months); and group 4 (>12 months). Changes in virologic parameters were analyzed in relation with clinical characteristics. The study was approved by the ethics committees of our institutions, and written informed consent was obtained from each patient.

Quantification of Serologic Markers for HBV Infection and HBV DNA

HBsAg had been measured quantitatively by chemiluminescent enzyme-linked immunosorbent assay (ELISA; Sysmex JAPAN Co, Ltd, Kobe, Japan) every 2–4 weeks, until the clinical outcome was known. It has a dynamic range of 0.03–2, 500 IU/mL. Serum samples scaling out from this range were diluted so as to contain them within it. Antibody to hepatitis B s antigen (anti-HBs), hepatitis B e antigen (HBeAg), and IgM anti- HBc were determined by ELISA (Abbott JAPAN Co, Ltd, Tokyo, Japan). Levels of HBV DNA were determined using the COBAS TaqMan HBV v.2.0 kit (Roche Diagnostics, Basel, Switzerland), which has a dynamic range over 2.1–9.0 log copies/mL.

HBV Genotyping

The HBV genotype was determined by a genotype-specific probe assay (Smitest HBV genotyping Kit, Genome Science, Fukushima, Japan) as previously reported [12].

Molecular Evolutionary Analyses

HBV genotype A started to prevail in Japan merely several years ago, suggesting that it was imported to Japan only recently [3, 13]. Therefore, genomic sequences of HBV genotype A (HBV/A), recovered from sera of patients with acute HBV infection, would be closely related to one another and with those reported from abroad. To evaluate this possibility, 20 HBV/A samples were selected randomly and sequenced by the method reported previously [14].

The number of nucleotide substitutions per site was estimated by the 6-parameter method [15], and a phylogenetic tree was constructed by the neighbor-joining method [16] based on the numbers of substitutions. To confirm the credibility of phylogenetic analyses, bootstrap resampling tests were carried out 1000 times [17].

Statistical Analyses

Categorical variables were compared by χ^2 test or Fisher exact test, and continuous variables by the Mann-Whitney U test. $P < .05$ was considered statistically significant. Receiver operating characteristic (ROC) analysis was performed to compute the area under the ROC curves for viral markers to determine cutoff points for predicting the outcome.

RESULTS

Distribution of HBV Genotypes in Patients With Acute Hepatitis B

HBV genotypes were determined in 215 of the 232 (93%) patients with acute hepatitis B. Of the 215 patients, genotype A was detected in 113 (52%), B in 26 (12%), C in 73 (33%), D in 1 (1%), E in 1 (1%), and F in 1 (1%). The distribution of genotypes was compared among 4 periods during 1994 through 2010 (Table 1). The proportion of patients with genotype A gradually increased to 65.9% in 2007–2010; it was higher than those in the earlier periods (34.4% in 1994–1998 [$P = .002$], 36.8% in 1999–2002 [$P = .002$], and 51.9% in 2003–2006 [$P = .093$]).

Phylogenetic Relationship Among HBV Strains of Genotype A

We randomly selected 11 HBV/A strains sampled in 2007–2010 and 9 of those in 2001–2006, and constructed a molecular evolutionary tree (Figure 1). All 20 samples had similar nucleotide sequences with a concordance of 99%. They were close to previously

Table 1. Prevalence of Hepatitis B Virus Genotypes in Patients With Acute Hepatitis B During 4 Chronologic Periods

Period	Genotype A	Genotype B	Genotype C	Others
1994–1998 (n = 32)	11[a] (34.4%)	3 (9.3%)	18 (56.3%)	0
1994–1998 (n = 38)	14[b] (36.8%)	4 (10.5%)	20 (52.7%)	0
1994–1998 (n = 54)	28[c] (51.9%)	6 (11.1%)	19 (35.1%)	1 (1.9%)
1994–1998 (n = 91)	60[a,b,c] (65.9%)	13 (14.3%)	16 (17.6%)	2 (2.2%)
Total (N = 215)	113 (52.5%)	26 (12.0%)	73 (34.0%)	3 (1.5%)

[a] $P = .0032$.

[b] $P = .0014$.

[c] $P = .02$.

reported genotype A2 sequences from Western countries. The results support the possibility that HBV/A was imported to Japan only recently and has been spreading throughout the country.

Clinical Features Among Patients Infected With HBV of Different Genotypes

Clinical features of patients with acute hepatitis B of different genotypes are compared in Table 2. The mean age was no different among patients infected with HBV of different genotypes. The proportion of men was higher in genotype A or B than C infection (93.8% or 80.7% vs 39.7% [A vs C, $P < .001$; B vs C, $P < .001$]).

The maximum alanine aminotransferase (ALT) level was lower in patients with genotype A than in those with genotype C (2126 ± 938 vs 2857 ± 1668 IU/L, $P = .002$). The maximum bilirubin level was higher in patients with genotype A (7.1 ± 6.4 mg/dL) or C (9.0 ± 7.5 mg/dL) than in those with genotype B (4.8 ± 3.3 mg/dL) (A vs B, $P = .003$; B vs C, $P < .001$). Regarding viral markers, the peak HBV DNA level was higher in patients with genotype A than in those with genotype C (6.3 ± 1.7 vs 4.9 ± 1.5 log copies/mL, $P < .001$). HBeAg was detected in 95 of the 121 (77.3%) patients with genotype A, 24 of the 28 (88.5%) with genotype B, and 37 of the 58 (65.5%) with genotype C (A vs C, $P = .036$). Men who have sex with men were more frequently represented among patients with genotype A than B or C (31.4% vs 4.8% or 11.3% [A vs B, $P = .017$; A vs C, $P = .002$]). Antibody to human immunodeficiency virus (anti-HIV) was examined in 72 of the 113 (63.7%) patients with genotype A, 7 of the 26 (26.9%) with genotype B, 58 of the 73 (79.5%) with genotype C, and 1 with genotype E. Anti-HIV was detected in 7 of the 72 (9.7%) patients with genotype A, and the other 96 patients tested for anti-HIV showed negative results. All of the 7 patients with anti-HIV cleared HBsAg from the serum within 6 months without antiviral treatment.

Among the 215 patients whose HBV genotypes were determined, 159 could be followed until the confirmation of clinical outcomes. The distribution of HBsAg-positive period is compared among patients with different genotypes. Group 1 (HBsAg persisting for <3 months) comprised 84 patients; group 2 (3–6 months) comprised 54 patients; group 3 (>6–12 months) comprised 15 patients; and group 4 (>12 months) comprised 6 patients. HBsAg remained >6 months in 21 of the 215 (9.8%) patients, including 14 of the 113 (12.4%) with genotype A, 1 of the 26 (3.8%) with genotype B, and 6 of the 73 (8.2%) with genotype C. Among the 21 patients, 15 (71.4%) cleared HBsAg within 12 months from the onset, and were classified into group 3. The remaining 6 (5 with genotype A and 1 with genotype B) who failed to clear HBsAg within 12 months were classified into group 4. All of the 6 were negative for anti-HIV. The proportion of group 4 was 6.0% in the patients with genotype A, 4.0% in those with genotype B, and 0% in those with genotype C.

The mean duration of HBsAg was 13.9 ± 8.7 weeks in patients with genotype A, 7.1 ± 5.3 weeks in those with genotype B, and 9.6 ± 7.6 weeks in those with genotype C, presuming the duration of HBsAg in group 4 at 12 months. The duration was longer in patients with genotype A than in those with B or C (A vs B, $P < .001$; A vs C, $P = .04$).

Prediction of the Outcome by the Duration of HBsAg

Table 2 shows that the duration of HBsAg among patients with genotype A varied to a higher extent than that among those with other genotypes. Therefore, we determined HBsAg and HBV DNA levels serially, and evaluated them for the ability to predict the outcome of acute hepatitis B in patients with genotype A.

Serial changes in HBsAg levels are shown in Supplementary Figure 1*A*. HBsAg levels declined more slowly in group 2 than group 1, as well as in group 3 than group 2. In group 4, HBsAg reelevated at 12 weeks after the onset. Figure 2 compares HBsAg levels among groups 1–4 at different intervals from the onset. HBsAg at 8 weeks from the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBsAg at 12 weeks from the onset was helpful for discriminating among groups 2, 3, and 4.

Prediction of the Outcome by HBV DNA

We also studied serial changes of HBV DNA in patients with genotype A, and examined if they also were useful for predicting the clinical outcome of acute hepatitis B. Supplementary Figure 1*B* shows serial changes in HBV DNA levels in patients in 4 groups. Although the reelevation of HBV DNA was not observed, the decline of HBV DNA was quite slow in group 4. Figure 3 compares HBV DNA levels among groups 1–4 at different intervals from the onset. HBV DNA at 4 weeks from

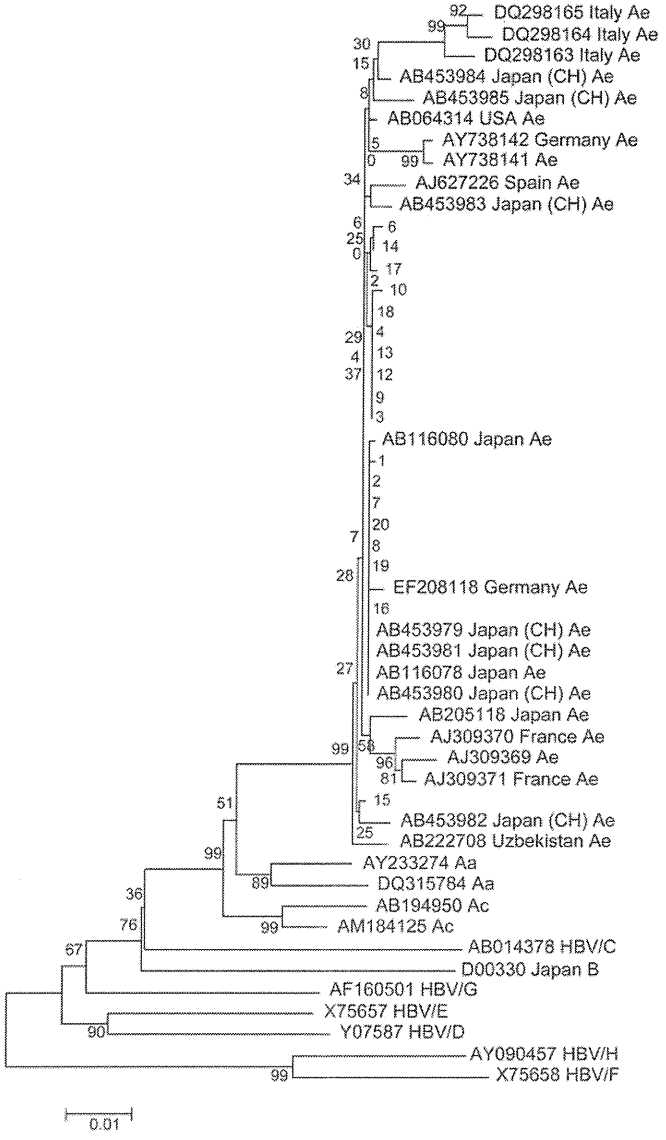

Figure 1. Evolutionary relationships of 86 hepatitis B virus genotype A taxa, including 20 from the present cases. The evolutionary history, inferred using the neighbor-joining method, shows that all 20 samples had similar nucleotide sequences close to previously reported genotype A2 sequences from Western countries.

the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBV DNA levels at 8 weeks from the onset were useful for discriminating between group 4 and group 3, as well as for distinguishing group 3 or 4 from group 1 or 2.

Levels of HBsAg and HBV DNA for Predicting Persistent Infection

As the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks from the onset were useful for distinguishing group 4 from the other groups, we evaluated the appropriate levels for predicting persistent infection in patients with genotype A. When we set the cutoff value of HBsAg at 1000 IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (98.1%). Likewise, when we set the cutoff value of HBV DNA at 10^6 log IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (96.4%). Therefore, HBsAg at 12 weeks >1000 IU/mL or HBV DNA at 8 weeks >10^6 log copies/mL is useful for predicting persistent infection.

DISCUSSION

In Japan, as shown in Table 1, the dominant HBV in acute hepatitis has been shifting from genotype C to A [3, 5, 14, 18]. The fact that nucleotide sequences of HBV/A isolates from patients

Table 2. Baseline Characteristics and the Duration of Hepatitis B Surface Antigen in Patients With Acute Hepatitis B Virus With Different Hepatitis B Virus Genotypes

	HBV Genotypes					
Features	A (n = 113)	B (n = 26)	C (n = 73)	D (n = 1)	E (n = 1)	F (n = 1)
Age, y	30.8 ± 9.5	32.3 ± 9.5	33.3 ± 10.9	27	26	58
Male	106 (93.8%)[a]	21 (80.7%)[b]	29 (39.7%)[a,b]	0	0	1 (100%)
Transmission routes Identified	102 (90.2%)	21 (80.8%)	53 (72.6%)	1 (100%)	1 (100%)	1 (100%)
Heterosexual	70 (68.6%)	19 (90.4%)	47 (88.7%)	1 (100%)	1 (100%)	1 (100%)
MSM	32 (31.4%)[c,d]	1 (4.8%)[c]	6 (11.3%)[d]	0	0	0
ALT, IU/L	2126 ± 938[e,*]	2394 ± 820	2857 ± 1668[e]	4180	1175	1533
Bilirubin, mg/dL	7.1 ± 6.4[f*]	4.8 ± 3.3[f,g]	9.0 ± 7.5[g]	6.8	3.9	3.5
HBV DNA, log copies/mL	6.3 ± 1.7[h,*]	5.5 ± 2.3	4.9 ± 1.5[h]	5.2	7.4	4.8
HBeAg	95/121 (77.3%)[i,*]	24/28 (88.5%)	37/58 (65.5%)[i]	1/1 (100%)	1/1 (100%)	1/1 (100%)
Anti-HIV	7/72 (9.7%)	0/7 (0%)	0/23 (0%)	Not tested	0/1 (0%)	Not tested
Duration of HBsAg*						
Group (mo)						
1 (<3)	35 (42.2%)	16 (64.0%)	31 (64.6%)	0	1	1
2 (3–6)	34 (41.0%)	8 (32.0%)	11 (22.9%)	1	0	0
3 (>6–12)	9 (10.8%)	0	6 (12.5%)	0	0	0
4 (>12)	5 (6.0%)	1 (4.0%)	0	0	0	0

Abbreviations: ALT, alanine aminotransferase; anti-HIV, antibody to human immunodeficiency virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MSM, men who have sex with men.

[a] $P < .001$.

[b] $P < .001$.

[c] $P = .017$.

[d] $P = .002$.

[e] $P = .002$.

[f] $P = .003$.

[g] $P < .001$.

[h] $P < .001$.

[i] $P = .036$.

* Data from anti-HIV-positive patients are excluded.

with acute hepatitis B in this study were very close to one another suggests that most HBV/A strains were imported recently and have spread rapidly, which may be attributed to the features of HBV/A in transmission routes and viral kinetics. We have reported that patients with genotype A tend to have multiple sexual partners [5]. Consequently, chances of secondary transmission of HBV/A would be higher than those of other genotypes, which may increase the number of patients who contract HBV/A infections. On the other hand, HBsAg persisted longer in patients with genotype A than B or C, which is consistent with the in vivo experiment using chimera mice carrying human hepatocytes showing that proliferation of HBV starts later and lasts longer in genotype A than in B or C infection [19].

Our results have shown that 6% of the patients with genotype A develop persistent infection. Because liver cirrhosis or hepatocellular carcinoma can develop in a substantial population of HBV carriers [20, 21], it is important to distinguish the patients in whom HBV infection becomes chronic, and follow them carefully. Although polymorphisms in host genes may be useful for identifying patients who are prone to develop chronic HBV infection [22], simple surrogate markers for the outcome have not been reported. Our data indicate that it would be difficult to predict the clinical outcome based on serum levels of viral markers at the first visit alone. This is understandable, because the dose of infecting virus, as well as the interval between infection and the first visit, can vary widely. Hence, we set out to analyze changes in serum levels of viral markers.

As seen in Figure 2, HBsAg levels at 12 weeks from the onset were most useful for discriminating among groups 2, 3, and 4 in the genotype A infection. Therefore, the outcome of acute hepatitis B may be predictable at this time point. Of note is the reelevation of HBsAg observed in group IV (Supplementary Figure 1*A*). Reelevation of viral markers suggests prolonged viral proliferation in the liver, and may be useful to identify the patients who may develop chronic infection.

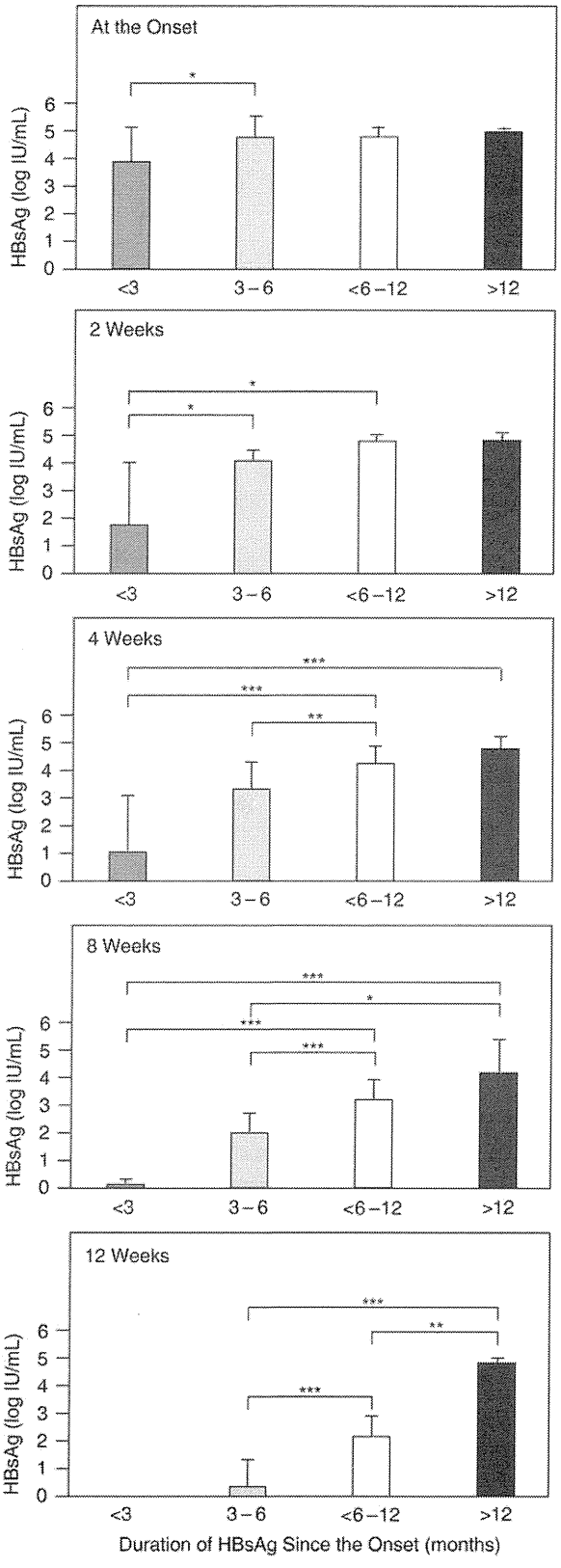

Figure 2. Levels of hepatitis B surface antigen in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviation: HBsAg, hepatitis B surface antigen.

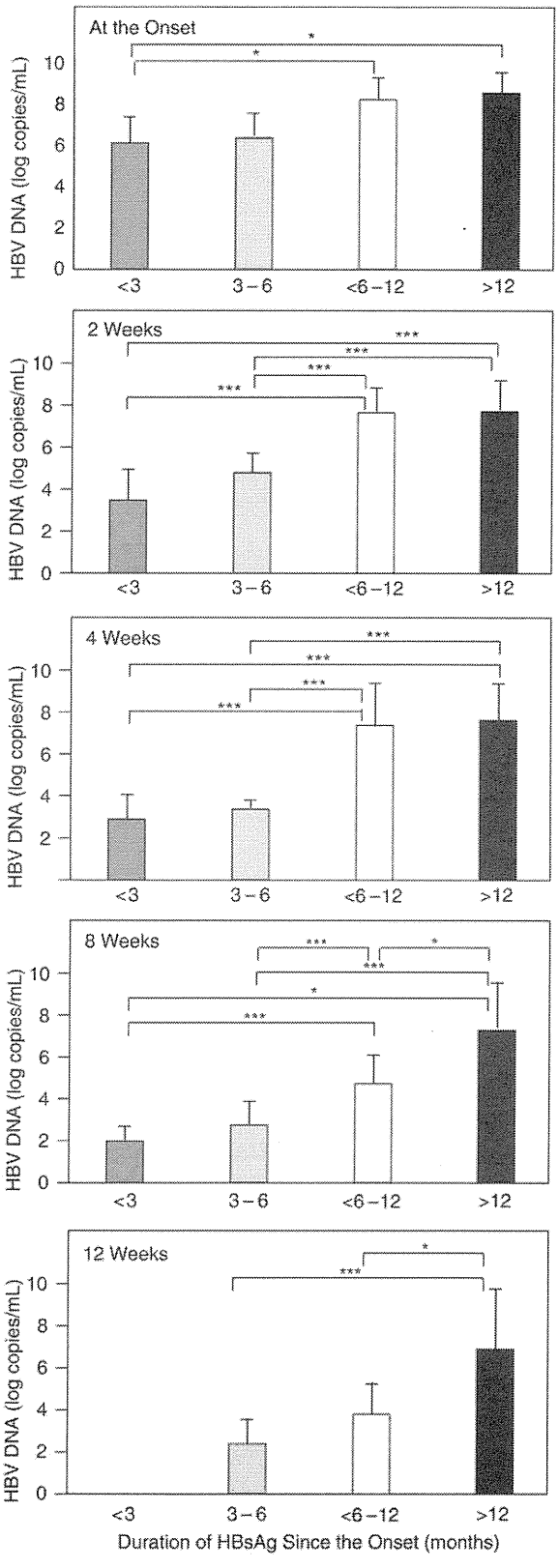

Figure 3. Levels of hepatitis B virus DNA in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A. *$P < .05$; **$P < .01$; ***$P < .001$. Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

As shown in Figure 3, HBV DNA levels at 4 weeks from the onset can discriminate groups 1/2 from groups 3/4. Furthermore, HBV DNA levels at 8 weeks from the onset can distinguish group 4 from group 1, 2, or 3. Therefore, the combination of HBV DNA levels at weeks 4 and 8 would be useful for predicting the outcome. For the prediction of a chronic outcome, HBV DNA level at 8 weeks from the onset is a useful surrogate marker of the outcome as well as HBsAg level at 12 weeks. There were differences in viral kinetics among groups 1, 2, 3, and 4.

Our present study showed that 15 of the 215 patients (7.0%) cleared HBsAg from >6 to 12 months after the onset. Sixty percent of the 15 patients had HBV/A. Although these patients met the criteria of chronic infection, they finally cleared HBsAg from the sera. Therefore, we would like to propose that transition to chronic infection in acute hepatitis B be judged at 12 months from onset in patients with genotype A; further studies in larger cohorts are necessary. One reason for our proposal is the indication of antiviral treatment. Antiviral treatment in patients with acute hepatitis B is not indicated because previous studies failed to show the efficacy of antiviral treatments in the patients with acute hepatitis B [23, 24]. However, if patients who actually develop chronic infection can be identified and treated by antiviral treatment, the number of those who develop secondary infection may be markedly reduced. Evaluation of the efficacy of antiviral treatments by prospective studies, based on surrogate markers for the outcome, should be conducted as the next step. HBeAg, which was reported to be useful as a surrogate marker for chronicity, should also be assessed as a surrogate marker [25, 26].

Our study has some limitations. First, the lack of data in early stages made it difficult to study viral kinetics precisely. Second, viral kinetics in the infection with each HBV genotype were obtained from a restricted number of patients, not large enough to establish the usefulness of changes in viral markers in earlier stages of HBV infection. Third, anti-HIV was not checked in all patients due to the lack of informed consent. Fourth, HBsAg and HBV DNA were not determined 24 weeks after onset when discrimination between groups 3 and 4 may be possible more easily. Fifth, the maximum levels of ALT and bilirubin may be affected by the time of blood test. Validation studies in larger cohorts are necessary to evaluate the feasibility of our hypotheses.

In conclusion, we have shown that viral kinetics and the clinical outcome are different among patients with acute hepatitis B who are infected with HBV of distinct genotypes. HBsAg levels at 12 weeks and HBV DNA at 8 weeks after the onset would be useful to predict the clinical outcome of patients with acute hepatitis B.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Financial support. This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Author Manuscript Published OnlineFirst on September 17, 2013; DOI: 10.1158/1055-9965.EPI-13-0363
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Cancer Epidemiology, Biomarkers & Prevention

Plasma Levels of Adiponectin and Primary Liver Cancer Risk in Middle-aged Japanese Adults with Hepatitis Virus Infection: A Nested Case-control Study

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Cancer Epidemiol Biomarkers Prev Published OnlineFirst September 17, 2013.

Author Manuscript Published OnlineFirst on September 17, 2013; DOI: 10.1158/1055-9965.EPI-13-0363
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Plasma Levels of Adiponectin and Primary Liver Cancer Risk in Middle-aged Japanese Adults with Hepatitis Virus Infection: A Nested Case-control Study

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Running title: plasma adiponectin levels and primary liver cancer risk

Keywords: adiponectin, primary liver cancer, nested case-control study, hepatitis virus infection, Japan

Grant Support

This study was supported by National Cancer Center Research and Development Fund (M. Inoue, N. Sawada, S. Sasazuki, M. Iwasaki, T. Shimazu, T. Yamaji, and S. Tsugane), A Grant-in-Aid for Cancer Research (M. Inoue and S. Tsugane), Research on Hepatitis (M. Inoue), and by the Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour and Welfare, Japan (S. Tsugane).

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest to disclose.

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Word count: 3,192 words in text excluding abstract and 246 words in abstract
3 Tables and 1 figure

Abstract

Background: Excess body weight is an independent risk factor for primary liver cancer, and the role of adiponectin in the pathogenesis of obesity-related malignancies is a focus of research interest. Few prospective studies have examined the association between circulating adiponectin and liver cancer risk, so we investigated this association in a nested case-control study of a population-based prospective cohort in Japan.

Methods: From 18,628 target participants aged 40 to 69 years who returned the baseline questionnaire and provided blood samples, we selected those with either hepatitis B virus or hepatitis C virus infection at baseline (n=1,544). Among these, 90 were newly diagnosed with primary liver cancer from 1993 through 2006, and matched to 177 controls. The odds ratios of liver cancer development based on plasma levels of adiponectin were estimated with a conditional logistic regression model.

Results: Median values of total and high-molecular-weight adiponectin tended to be higher in the patients with liver cancer, and plasma levels of adiponectin were positively associated with liver cancer risk. Body mass index- and diabetes-adjusted odds ratios for the highest tertile of total and high-molecular-weight adiponectin levels versus the lowest were 3.30 (95% confidence interval=1.45-7.53, p trend<0.01) and 3.41 (1.50-7.73, p trend<0.01), respectively. There was no effect modification by body mass index and diabetes.

Conclusions: Higher plasma adiponectin levels were associated with an increased risk of primary liver cancer in middle-aged Japanese adults with hepatitis virus infection.

Impact: Circulating adiponectin levels may be a risk marker for primary liver cancer.

Introduction

Accumulating epidemiological evidence suggests that excess body weight is an independent risk factor for primary liver cancer (1, 2). Even in Japan, where the prevalence of overweight and obesity is lower than that in Western countries (3) and the main cause of liver cancer is chronic hepatitis C virus (HCV) infection (4), overweight or obesity appear to increase the risk of liver cancer (5). We have also reported that high body mass index (BMI) is associated with an increased risk of primary liver cancer, irrespective of hepatitis virus infection (6). The causal association between excess body weight and liver cancer development is probably connected with insulin resistance (7, 8). However, the underlying mechanism by which excess body weight promotes liver cancer development is still not fully understood.

The role of adiponectin in the pathogenesis of obesity-related malignancies is a focus of research interest. Adiponectin is a physiologically active polypeptide secreted by adipose tissues, and circulating levels are inversely associated with obesity (9). Earlier experimental studies suggested that adiponectin played a protective role in carcinogenesis via insulin sensitization, antiproliferation, antiinflammation, and angiogenesis regulation (10), and data supported epidemiologic evidence that adiponectin levels were inversely associated with the risk of obesity-related malignancies such as breast, colorectal, endometrium, and prostate cancers (10). These results also suggested that elevated levels of adiponectin would be associated with a reduced risk of primary liver cancer linked with obesity, and that hyperadiponectinemia might suppress liver tumorigenesis (11). Indeed, experimental studies

indicated that adiponectin treatment increased apoptosis of hepatocelluar carcinoma (HCC), the most common form of primary liver cancer, and inhibited its proliferation (12, 13). However, it has been pointed out that hyperadiponectinemia reflects the progression of liver disease leading to the development of liver cancer, because the liver is the main organ of adiponectin metabolism (9, 11). A recent hospital-based cohort study showed that high serum levels of adiponectin were positively associated with the development of HCC in patients with chronic hepatitis C (14). Since there have been few prospective studies (14-16), further examination of the potential association between adiponectin and liver cancer risk is warranted.

The aim of the present study was to investigate the association between plasma adiponectin levels and primary liver cancer risk in middle-aged adults with hepatitis virus infection. We used a nested case-control design based on data from a large-scale population-based prospective cohort study in Japan.

Materials and Methods

Study population

The Japan Public Health Center-based Prospective Study (JPHC Study) is an ongoing cohort study of cancer, cardiovascular disease and other lifestyle-related diseases. The first cohort (Cohort I) started in 1990 and the second cohort (Cohort II) in 1993. In Cohort I, the study population included all registered Japanese residents aged 40 to 59 years of 5 public

health center areas, and in Cohort II included all residents aged 40 to 69 years of 6 other areas; the study design is described elsewhere (17). We investigated the hepatitis B surface antigen (HBsAg) and anti-HCV antibody (anti-HCV) measurements from the Cohort II data. Our study was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan.

In Cohort II (1993-1994), 56,542 participants (response rate: 82%) answered a baseline questionnaire in socio-demographic characteristics, medical history, smoking and drinking habits, diet and so on. Of the questionnaire respondents, 37% voluntarily provided 10 mL of blood in health checkups during the baseline survey (1993-1995). The blood samples were divided into plasma and buffy layers and preserved at −80°C until analysis. Study participants were informed of the objectives and methods of the study in writing, and those who responded to the questionnaire and donated blood were regarded as having given informed consent to participate. Of these, we selected only those who had no history of cancer at baseline and had provided data on basic characteristics, leaving us with a total of 18,628 participants (6,401 men, 12,227 women).

Follow-up

Participants were followed up from the date of blood collection until December 31, 2006. Residence status and survival were confirmed annually through residential registers in the respective public health center areas. During the follow-up period, 0.3% (n=49) of participants were lost to follow-up.

Incident cases of primary liver cancer were identified by active patient notification from major hospitals in the study area and data linkage with population-based cancer registries. Death certificates were used as a supplementary information source. Cases were coded using the International Classification of Diseases for Oncology, Third Edition (code: C22.0) (18). In our cancer registry system, the proportion of cases for which information was available from death certificates only was 4.7%.

Selection of cases and controls

From the 18,628 participants, we selected those with either HBV or HCV infection at baseline (n=1,544). Plasma samples were screened for HBsAg by reversed passive hemagglutination with a commercial kit (Institute of Immunology Co., Ltd., Tokyo, Japan) and for anti-HCV with a third-generation immunoassay (Lumipulse II Ortho HCV, Ortho-Clinical Diagnosis K.K., Tokyo, Japan) (19). In this study, positivity for HBsAg was regarded as indicating HBV infection, and positivity for anti-HCV as indicating HCV infection (20). Up to the end of the study period after blood collection, we identified 91 new cases of primary liver cancer among the 1,544 participants. For each patient newly diagnosed with liver cancer, we selected 2 controls at random from among the participants with no history of liver cancer when the diagnosis was made. Controls were matched to each patient in term of age (within 5 years), sex, public health center area, fasting status at blood collection, baseline menopausal status (for women), and hepatitis virus infection status (HBV/HCV). No appropriate matched controls were found for 1 patient, and only 1 matched

control for each of 3 other patients, so a total of 90 patients and 177 controls were included in the present analysis.

Laboratory assay for adiponectin

Circulating adiponectin levels have been reported to be stable over time and to have high reliability (21-23), so this biological marker is likely to be useful in epidemiological studies. From the blood samples collected at baseline, plasma levels of total adiponectin and high-molecular-weight (HMW) adiponectin, which is considered to be the active form of the hormone (10) were analyzed with a Human Adiponectin ELISA Kit for Total and Multimers (Sekisui Medical, Co. Ltd, Tokyo, Japan) by the enzyme-linked immunosorbent assay method. Cases and matched controls were assayed in the same batch. The minimum detection level was 0.39 μg/ml for both total and HMW adiponctin. For the assays, intra-assay and intra-assay coefficients of variation from the company's quality control samples were ≤3.9% and ≤5.7% for total adiponectin, and ≤3.3% and ≤6.2% for HMW adiponectin, respectively. All samples were analyzed at a single laboratory (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) by technicians blinded to case-control status.

Statistical analysis

All analyses were performed with STATA version 11 (STATA Corporation, College Station, Texas, USA). All p values reported are two sided, and significance level was set at

$p<0.05$.

Comparisons of the baseline characteristics between the cases and controls were made by the chi-square test or Mann-Whitney test, as appropriate. Because adiponectin levels are reportedly low in obese people and in patients with type 2 diabetes (9, 24), we confirmed whether the relation between plasma levels of total/HMW adiponectin and BMI/diabetes in the controls did not contradict existing knowledge. Spearman's rank correlation coefficients were calculated for adiponectin levels and BMI. Comparative adiponectin levels between the participants with diabetes and those without were evaluated by the Mann-Whitney test. In this study, diabetes was defined as a self-reported history of diabetes, and/or anti-diabetic medication use, and/or blood glucose ≥5.55 mmol/l (100 mg/dl) fasting or ≥7.77 mmol/l (140 mg/dl) non-fasting (20).

For total and HMW adiponectin, participants were classified into sex-specific tertiles according to the frequency of distribution among the controls. Using a conditional logistic regression model, we calculated odds ratios (ORs) and 95% confidence intervals (CIs) of primary liver cancer for plasma levels of total and HMW adiponectin. Dummy variables were created for the categories of plasma adiponectin levels, and the lowest category was used as the reference category. To investigate whether adiponectin was associated with liver cancer through the pathway for insulin resistance (24), the ORs were adjusted for BMI (<18.5, 18.5-21.9, 22.0-24.9, ≥25.0 kg/m^2) and diabetes (yes or no). We also adjusted for the following variables previously associated with liver cancer risk (20, 25, 26): alcohol consumption (never, past, <150, 150 to <450, ≥450 g/week ethanol); coffee consumption

(almost never, 1 time/week to <1 cup/day, ≥1 cup/day); and serum alanine aminotransferase (ALT) levels (<30, 30-69, ≥70 IU/L). Because adjustment for smoking status (a suspected risk factor for liver cancer) and vegetable and fish intake (factors previously associated with liver cancer risk (27, 28)) produced ORs that were almost identical to those without adjustment, results for these variables are not presented in this paper. Linear trends for ORs were tested using the exposure categories as ordinal variables. An earlier study demonstrated that high adiponectin levels might be a proxy marker increasing the likelihood of subsequent liver cancer development (14). Therefore, we performed additional analyses after excluding 17 cases of liver cancer diagnosed in the first 3 years after blood collection because of checking for temporality. In addition, subgroup analyses were performed for 71 cases of liver cancer death where cancer development was likely to have been observed in the early follow-up period, and after limiting analysis to or excluding 53 cases of liver cancer diagnosed in the first 7 years after blood collection.

Additionally, we investigated whether the association between adiponectin levels and primary liver cancer risk was modified by sex, hepatitis virus infection status (HBV/HCV), BMI (<25.0, ≥25.0 kg/m^2), and diabetes (yes or no). For these stratified analyses, participants were divided into 2 groups based on median plasma levels of total and HMW adiponectin in the controls. We used unconditional logistic regression for BMI and diabetes, and included matching factors in the multivariable models. Statistical interaction between adiponectin levels and stratified variables was tested with a likelihood ratio test.

Results

Table 1 shows the baseline characteristics of the case and control groups. The prevalence of overweight, diabetes, and high ALT levels was tended to be higher and that of daily coffee consumption was lower in the case group than in the control group. Participants in the control group tended to be light (<150 g/week ethanol) to moderate (150 to <450 g/week ethanol) drinkers. The median values of total adiponectin were statistically higher among the cases than the controls (6.16 vs. 5.11 μg/ml, p=0.03) and those of HMW adiponectin were marginally higher in the case group (2.66 vs. 2.06 μg/ml, p=0.06) (Figure 1). In the control group, Spearman's rank correlation coefficients of BMI with total and HMW adiponectin were −0.40 (p<0.01) and −0.25 (p<0.01), respectively. Total and HMW adiponectin levels were lower among the participants with diabetes than those without (median: 3.45 vs. 5.34 μg/ml, p=0.02 for total adiponectin; 1.26 vs. 2.25 μg/ml, p=0.04 for HMW adiponectin).

Table 2 presents the conditional logistic regression results for the association between total and HMW plasma adiponectin levels and the risk of primary liver cancer. After adjustment for BMI and diabetes, we found a statistically significant positive association between plasma adiponectin and liver cancer risk (p for trend<0.01 for both total and HMW adiponectin). The ORs for the highest tertile of total and HMW adiponectin levels versus the lowest tertile were 3.30 (95% CI=1.45-7.53) and 3.41 (1.50-7.73), respectively. The positive association remained significant for total adiponectin (p for trend=0.04) and marginal for