S3 Table. Counts of NS3 amino acid at position 80 binned by genotype in reported sequences retrieved from the Los Alamos HCV sequence database.
(XLSX)

S4 Table. Counts of NS3 amino acid at position 80 binned by sampled country in reported sequences retrieved from the Los Alamos HCV sequence database.
(XLSX)

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Author Contributions

Conceived and designed the experiments: MO HY. Performed the experiments: MO HY TT. Analyzed the data: MO HY TT HO WS. Contributed reagents/materials/analysis tools: HG SO. Wrote the paper: MO HY HO WS KM SK KK.

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Original Article

Potential associations between perihepatic lymph node enlargement and liver fibrosis, hepatocellular injury or hepatocarcinogenesis in chronic hepatitis B virus infection

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Aim: Although perihepatic lymph node enlargement (PLNE) is frequently observed in chronic liver disease, little is known about PLNE in chronic hepatitis B virus (HBV) infection. We aimed to evaluate this issue.

Methods: We originally enrolled a consecutive 502 patients with chronic HBV infection. Among them, 288 patients without history of interferon-based or nucleoside analog treatment and hepatocellular carcinoma (HCC) were primarily analyzed.

Results: PLNE was detected in 27 of 288 (9.4%) patients, which was fewer than that in chronic hepatitis C patients but more than that in subjects undertaking a general health examination as previously reported. The presence of PLNE was significantly associated with a higher probability of having an aspartate aminotransferase (AST) platelet ratio index of more than 1.5 (11.1% vs 1.5%, $P = 0.01$), a higher AST level (38.0 vs 26.8 U/L, $P = 0.001$), a higher alanine aminotransferase level (50.1 vs 28.0 U/L, $P < 0.0001$), and a lower platelet count (18.6 vs 20.6 $\times 10^4/\mu$L, $P = 0.048$) after adjustment for sex and age. However, in our original sample ($n = 502$), PLNE was observed in 1.4% of the patients with HCC and/or its history whereas 9.2% of the patients without HCC, and the proportion was significantly lower in patients with HCC and/or its history ($P = 0.03$).

Conclusion: PLNE was associated with liver fibrosis and hepatocellular injury, but was negatively associated with HCC in chronic HBV infection.

Key words: fibrosis, hepatitis B, immune response, inflammatory activity, perihepatic lymph node enlargement

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is an important cause of chronic liver disease globally, with an estimated 350 million carriers worldwide.[1] Chronic HBV infection is a major risk factor for several liver diseases, such as chronic hepatitis B (CHB), liver cirrhosis and hepatocellular carcinoma (HCC),[2] which is the fifth most common cancer worldwide.[3] HBV infection itself is non-cytopathic, and it is the immune response to the viral antigens that are thought to be responsible for the necroinflammatory process involved in chronic infection, cirrhosis and HCC.[4]

Perihepatic lymph node enlargement (PLNE) is frequently observed in patients with chronic liver disease,[5] especially in those with hepatitis C virus (HCV) infection.[6,7] Although some studies have reported that PLNE was associated with inflammatory activity, stage of liver fibrosis or hepatitis viral load,[7–12] such associations were inconsistent among other studies,[6–8,10–15] suggesting that the clinical significance of PLNE in HCV infection has not been fully established yet. We have recently reported that PLNE is negatively associated with the response to interferon (IFN)-based treatment or development of HCC in patients with chronic hepatitis C (CHC).[16,17] It is

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reported that PLNE is a relatively common finding in patients with primary biliary cirrhosis among other liver diseases.[15]

On the other hand, the reports regarding PLNE in patients with chronic HBV infection have been scarce. The purpose of the present study was to evaluate the clinical significance of PLNE in chronic HBV infection.

METHODS

Patients and screening for PLNE

WE ENROLLED 502 consecutive patients with chronic HBV infection who underwent ultrasonography (US) between November 2012 and April 2013 at the Department of Clinical Laboratory, the University of Tokyo Hospital. Patients with chronic HBV infection were defined as those positive for hepatitis B surface antigen for at least 6 months. Patients who were positive for HCV RNA and had a history of other hepatobiliary disease were excluded. All patients took laboratory blood tests at the time they underwent US. Aspartate aminotransferase (AST) platelet ratio index (APRI) was used to assess liver fibrosis, and APRI of more than 1.5 was classified as bridging fibrosis or cirrhosis (F stage 3–4).[18] The criteria to identify PLNE were previously described; PLNE was defined as a lymph node at the perihepatic area measuring 1 cm or more in the longest axis.[17] In the analysis to examine the association between PLNE and clinical findings, we excluded the patients receiving IFN or nucleoside analog treatment, or having HCC or a history of HCC from the primary analysis.

The present study was carried out in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Research Ethics Committees of the authors' institutions.

Study end-points

We examined the association between PLNE and clinical findings such as liver fibrosis, hepatocellular injury or the presence of HCC in patients with chronic HBV infection (the primary end-point of this study). We previously reported the prevalence of PLNE in the patients with CHC[17] or the patients underwent general health examinations (general population).[19] Using the results of our previous studies, we then compared the prevalence of PLNE in the patients with chronic HBV infection to patients with CHC or general population (the secondary end-point).

Statistical analysis

Continuous variables were presented as the mean ± standard deviation (SD), while categorical variables were expressed as frequencies (%). Categorical data were analyzed using the χ^2-test or Fisher's exact test, and stepwise logistic regression model analyses were used to adjust the contribution of PLNE by other covariates such as sex or age. For continuous data, the univariate associations were evaluated using Student's *t*-test. Stepwise regression model analyses were used to adjust the contribution of PLNE by other covariates such as sex or age. The Cochran–Armitage trend test was used for assessing increasing or decreasing trends in binomial proportions. All statistical analyses were two-sided, and the threshold of the reported *P*-values for significance was accepted as less than 0.05. All statistical analyses were performed using R statistic software version 2.15.2 (http://www.r-project.org).

RESULTS

Patient characteristics and association between PLNE and clinical findings

TO EXAMINE THE association between PLNE and clinical findings, patients receiving IFN or nucleoside analog treatment, and those with HCC or past history of HCC were excluded from the primary analysis, because antiviral treatment or HCC could directly influence PLNE. As a result, the data of 288 among 502 patients were primarily analyzed. Characteristics of these patients are shown in Table 1. Overall, 51.0% (147) were male, and the mean age was 53.72 years. PLNE were detected in 27 of 288 (9.4%) patients, and the mean length of the longest axis was 1.6 cm (range, 1.0–3.2).

Table 2 shows the relationships between PLNE and various clinical findings. The presence of PLNE was significantly associated with a higher APRI value of more than 1.5 ($P = 0.01$), a higher serum AST level ($P = 0.001$), a higher serum alanine aminotransferase (ALT) level ($P < 0.0001$), and a lower platelet count ($P = 0.048$) after adjustment for sex and age, suggesting that PLNE may be observed in patients with more liver fibrosis and more hepatocellular injury. We also compared the correlation between PLNE and clinically diagnosed liver cirrhosis. Diagnosis of cirrhosis is based on the presence of clinical and laboratory features of portal hypertension (the presence of esophageal varices and/or collateral circulation at endoscopy and ultrasound) and/or liver stiffness measurement value more than

Table 1 Patient characteristics ($n = 288$)

Parameter	Values
Mean age (years)	53.72 ± 14.74
Sex	
Male	147 (51.0%)
Female	141 (49.0%)
PLNE	
Present	27 (9.4%)
Absent	261 (90.6%)
Perihepatic lymph node diameter†	1.6 (1.0–3.2)‡
APRI score	
>1.5	7 (2.4%)
≤1.5	281 (97.6%)
Mean HBV DNA (log^{10} copies/mL)	3.97 ± 2.13
Mean AST (U/L)	27.83 ± 17.37
Mean ALT (U/L)	30.09 ± 30.68
Mean TB (mg/dL)	0.93 ± 0.57
Mean albumin (g/dL)	4.20 ± 0.34
Mean platelet count ($\times 10^4/\mu L$)	20.43 ± 6.01
Mean γ-GT (U/L)	39.26 ± 108.55
Mean PT-INR	0.95 ± 0.14

†In PLNE positive patients.
‡Median (range).
Continuous variables are represented as the mean ± standard deviation and categorical variables were as number and frequencies (%).
γ-GT, γ-glutamyltransferase; ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; HBV, hepatitis B virus; PLNE, perihepatic lymph node enlargement; PT-INR, prothrombin time international normalized ratio; TB, total bilirubin.

16.9 kPa which was reported to be the optimal diagnostic accuracy.[20] Of the patients, 18.5% (5/27) with PLNE and 2.7% (7/261) without PLNE were clinically diagnosed with liver cirrhosis. The prevalence of the patients with clinically diagnosed liver cirrhosis was significantly higher in the patients with PLNE ($P = 0.0009$) after adjustment for sex and age (data not shown). In addition, there was a trend that serum HBV DNA level was higher in patients with PLNE than in those without, although not statistically significant (Table 2).

We then compared the prevalence of PLNE among asymptomatic carrier (APRI, ≤1.5; ALT, ≤30), patients with chronic hepatitis (APRI, ≤1.5; ALT, >30) and patients with cirrhosis (APRI, >1.5). PLNE was detected in 8.3% (17/206) of asymptomatic carriers, 9.3% (7/75) of patients with chronic hepatitis and 42.9% (3/7) of patients with cirrhosis (Fig. 1). The progression of liver disease was significantly associated with higher prevalence of PLNE ($P = 0.03$), as demonstrated by the Cochran–Armitage trend test. We also examined the association between hepatitis B e (HBe) status and the prevalence of PLNE. PLNE was detected in 18.8% (6/32) of the patients positive for HBe antigen (HBeAg) and 8.3% (21/251) of the patients negative for HBeAg. Prevalence of PLNE was higher in the patients with positive for HBeAg, however, the difference did not reach statistical significance ($P = 0.11$).

Comparison of the frequency of PLNE in patients with HCC and/or its history and those without HCC

Because our current findings suggest the associations between PLNE and liver fibrosis, hepatocellular injury or serum HBV DNA level in patients with chronic HBV infection, we wondered whether PLNE might be observed more frequently in those with HCC and/or its past history than in those without. On the other hand, PLNE was reportedly a negative risk for hepatocarcinogenesis in CHC patients. Thus, these results prompted us to examine how PLNE could be associated with HCC in patients with chronic HBV infection.

To address this question, we compared the frequency of PLNE in patients with HCC and/or its history and in those without in our original sample ($n = 502$). Table 3 shows the patient characteristics and the associations between prevalence of HCC and clinical findings. PLNE was detected in 1.4% (1/69) of the patients with HCC and/or its history and 9.2% (40/433) of the patients without HCC, where the patients receiving IFN or nucleoside analog treatment were also included in the analysis. As shown in Figure 2 and Table 3, the frequency of PLNE was significantly lower in patients with HCC and/or its history than in those without ($P = 0.03$). Then, we tested PLNE and the following variables on multivariate analysis: age, sex, APRI score, ALT, total bilirubin, albumin and γ-glutamyltransferase. As a result, the association between PLNE and lower probability of HCC was noted, although not statistically significant ($P = 0.057$). In this multivariate analysis, higher prevalence of HCC was significantly associated with older age ($P = 0.01$), male sex ($P = 0.009$), higher APRI score ($P = 0.0002$) and lower ALT level ($P = 0.0006$) (data not shown).

Comparison of the frequency of PLNE with liver diseases of other etiology

Perihepatic lymph node enlargements were detected in 27 of 288 (9.4%) of the patients without treatment for

Table 2 Associations between PLNE and clinical findings ($n = 288$)

Variable	n (proportion)/mean (SD)		P	
	PLNE positive group ($n = 27$)	PLNE negative group ($n = 261$)	P-value	Adjusted P-value‡
APRI >1.5†	3 (11.1%)	4 (1.5%)	0.02	0.01§
HBV DNA (log^{10} copies/mL)	4.70 (2.97)	3.89 (2.01)	0.2	0.13¶
AST (U/L)	38.0 (33.5)	26.8 (14.5)	0.09	0.001¶
ALT (U/L)	50.1 (70.1)	28.0 (22.4)	0.11	<0.0001¶
TB (mg/dL)	0.93 (0.44)	0.93 (0.58)	0.98	-
Albumin (g/dL)	4.14 (0.29)	4.20 (0.34)	0.33	-
Platelet count ($\times 10^4/\mu L$)	18.6 (6.96)	20.6 (5.87)	0.12	0.048¶
γ-GT (U/L)	44.6 (84.1)	38.7 (110.9)	0.74	-
PT-INR	0.99 (0.09)	0.95 (0.14)	0.11	-
AFP (ng/mL)	3.22	4.68	0.15	-

†Odds ratio (95% confidence interval) for PLNE positive group was 7.85 (1.59–38.76).
‡Adjusted for sex and age at enrollment (independent variables). The dependent variables of each P-value are the items in the leftmost fields of corresponding rows (the proportion of having APRI >1.5, AST, ALT, TB and so on).
§P-value by stepwise logistic regression analysis.
¶P-value by stepwise regression analysis.
γ-GT, γ-glutamyltransferase; AFP, α-fetoprotein; ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; HBV, hepatitis B virus; PLNE, perihepatic lymph node enlargement; PT-INR, prothrombin time international normalized ratio; SD, standard deviation; TB, total bilirubin.

HBV or history of HCC and in 41 of 502 (8.2%) of the whole patients, and the proportion of PLNE was not significantly different between these two patient groups ($P = 0.65$, χ^2-test). In addition, in patients receiving IFN or nucleoside analog treatment and/or having HCC or its past history, PLNE was found in 7.0% patients. Thus, PLNE may be observed in as much as 10% of patients with chronic HBV infections. Then, we compared the frequency of PLNE in subjects with different backgrounds; in our previous studies, PLNE was detected in 20.0% (169/846) of patients with CHC[17] and in 1.6% (69/4234) of subjects who underwent a general health examination.[19] As shown in Figure 3, the frequency of PLNE in patients with chronic HBV infection was significantly higher than that in subjects undertaking a general health examination ($P < 0.0001$) but lower than that in CHC patients ($P < 0.0001$).

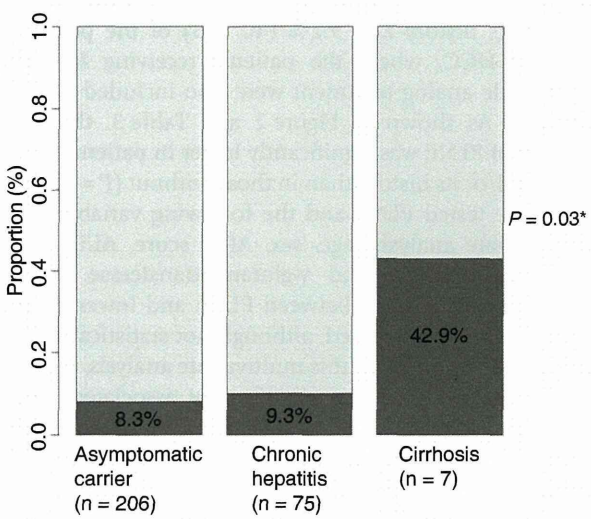

Figure 1 Bar plot of the proportion of perihepatic lymph node enlargement (PLNE) positive patients in asymptomatic carriers, patients with chronic hepatitis and patients with cirrhosis. *P-value by the Cochran–Armitage trend test. □, PLNE negative; ■, PLNE positive.

DISCUSSION

ALTHOUGH PLNE IS one of the common findings in chronic liver disease,[8–12,16,17] it has yet remained unclear how frequent PLNE would be observed or what would be the clinical significance of PLNE in patients with chronic HBV infection. In the current study, in patients without HBV treatment or HCC, PLNE was significantly associated with a higher probability of having an APRI of more than 1.5, a higher serum AST level and a higher ALT serum level. Also, a significantly increasing trend of PLNE prevalence across asymptomatic carriers, patients with chronic hepatitis and patients with

Table 3 Patient characteristics according to prevalence of HCC in the original cohort ($n = 502$)

Variable	n (proportion)/mean (SD)		P
	HCC positive group ($n = 69$)	HCC negative group ($n = 433$)	
PLNE positive	1 (1.4%)	40 (9.2%)	0.03
APRI >1.5	8 (11.6%)	14 (3.2%)	0.005
HBV DNA (log^{10} copies/mL)	1.19 (1.67)	3.00 (2.36)	<0.0001
AST (U/L)	30.4 (17.8)	28.3 (27.3)	0.40
ALT (U/L)	27.2 (19.1)	29.5 (38.1)	0.42
TB (mg/dL)	1.02 (0.45)	0.95 (0.56)	0.29
Albumin (g/dL)	4.08 (0.41)	4.20 (0.39)	0.03
Platelet count ($\times 10^4/\mu L$)	14.0 (5.41)	19.7 (6.10)	<0.0001
γ-GT (U/L)	48.8 (50.2)	40.9 (98.9)	0.31
PT-INR	1.00 (0.12)	0.98 (0.25)	0.60

γ-GT, γ-glutamyltransferase; ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PLNE, perihepatic lymph node enlargement; PT-INR, prothrombin time international normalized ratio; SD, standard deviation; TB, total bilirubin.

cirrhosis was observed, suggesting that PLNE may be associated with progress of liver fibrosis and severer hepatocellular damage in patients with chronic HBV infection. Furthermore, the frequency of PLNE in patients with chronic HBV infection was significantly lower than that in CHC patients. To the best of our knowledge, this is the first study showing the clinical significance of PLNE in chronic HBV infection.

It is well known that liver fibrosis and hepatocellular injury are associated with HCC occurrence generally in chronic liver disease.[21] Furthermore, recent evidence has revealed that serum HBV DNA level is a risk for hepatocarcinogenesis in chronic HBV infection.[22] In the current study, PLNE was associated with liver fibrosis, hepatocellular injury and serum HBV DNA level, however, PLNE was less frequently observed in patients with HCC and/or its history than in those without HCC in our original sample ($n = 502$). Although this paradoxical result is interesting, its mechanism remains to be clarified. Of note, we previously reported that PLNE was a negative risk for HCC occurrence in CHC patients.[17] Thus, the result in the present study may imply the similar association between PLNE and the development of HCC in patients with chronic liver disease in general. One probable explanation for this paradoxical result is that the presence of PLNE may reflect a stronger immune response to hepatitis virus, which could exert also an antitumor immune response.

On multivariate analysis regarding HCC prevalence and clinical parameters, lower ALT levels were significantly associated with higher prevalence of HCC. This

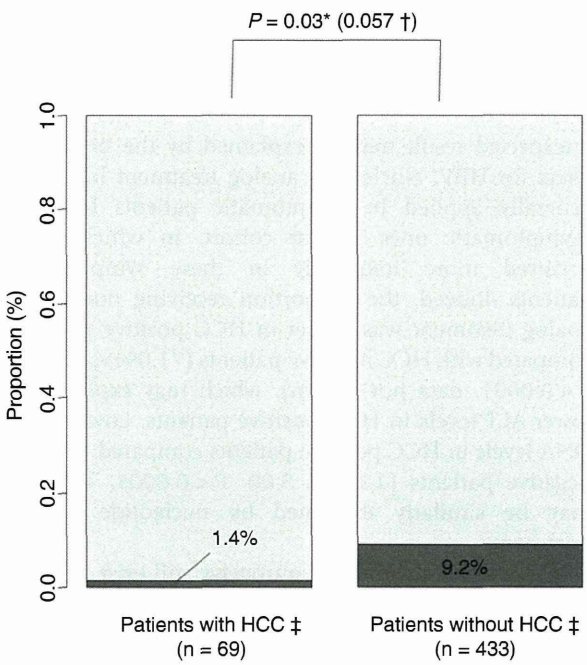

Figure 2 Bar plot of the proportion of perihepatic lymph node enlargement (PLNE) positive patients with and without hepatocellular carcinoma (HCC). **P*-value by Fisher's exact test. †*P*-value by logistic regression test (adjustment for age, sex, aspartate aminotransferase-to-platelet ratio index score, alanine aminotransferase, total bilirubin, albumin and γ-glutamyltransferase). ‡Patients receiving interferon or nucleoside analog treatment were also included. □, PLNE negative; ■, PLNE positive.

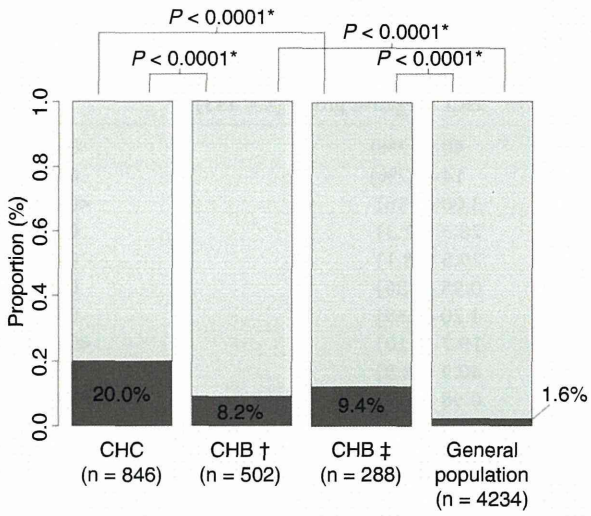

Figure 3 Bar plot of the proportion of perihepatic lymph node enlargement (PLNE) positive patients in chronic hepatitis B (CHB), chronic hepatitis C (CHC) and general health examination. **P*-value by the χ^2-test. †Total patients; ‡Patients without history of hepatocellular carcinoma or treatment for CHB. □, PLNE negative; ■, PLNE positive.

unexpected result may be explained by the treatment effect for HBV. Nucleotide analog treatment has been generally applied in symptomatic patients but not asymptomatic ones in our cohort, in which HCC occurred more frequently in these symptomatic patients. Indeed, the proportion receiving nucleotide analog treatment was higher in HCC positive patients compared with HCC negative patients (71.0% vs 33.4%, $P < 0.0001$, data not shown), which may explain the lower ALT levels in HCC positive patients. Lower HBV DNA levels in HCC positive patients compared to HCC negative patients (1.19 vs 3.00, $P < 0.0001$, Table 3) may be similarly explained by nucleotide analog treatment.

It should be noted that controversy still exists regarding whether PLNE could be associated with hepatocellular damage or liver fibrosis in patients with chronic HCV infection.[6–9,11,13,14] These results raise a possibility that the clinical significance of PLNE may be distinct regarding the association with hepatitis activity between chronic HBV infection and chronic HCV infection. The distinct PLNE frequency between chronic HBV infection and chronic HCV infection observed in the current study may be in line with this concept. Hyperplasia of regional lymph nodes are generally considered to reflect inflammatory responses in the adjacent organs. Especially in chronic HCV infection, PLNE are thought to reflect the immunological response of the host.[11] Indeed, HCV-specific IFN-γ production and proliferative response of T cells were found commonly in perihepatic lymph nodes,[23] suggesting that PLNE indicates an active host immune response in chronic hepatitis C. The humoral immune response plays an essential role in HBV and HCV infection.[24] Almost 40% of patients infected with HCV were reported to develop at least one extrahepatic manifestation during the course of the disease.[25] CHC patients were shown to be associated with mixed cryoglobulinemia, chronic thyroiditis, Sjögren's syndrome or membranoproliferative glomerulonephritis.[26–31] On another front, extrahepatic manifestations of hepatitis B were reported to be present in 1–10% of HBV-infected patients, which is lower than that of HCV-infected patients, including serum sickness-like syndrome, acute necrotizing vasculitis (polyarteritis nodosa), membranous glomerulonephritis or popular acrodermatitis of childhood (Gianotti–Crosti syndrome).[32,33] Thus, there may be an apparent incompatibility between patients with chronic HBV and HCV infection in terms of the component of immune system. The result of the present study may support this concept.

In patients not receiving IFN or nucleoside analog treatment, and those without having HCC or its past history for comparison with other background ($n = 288$) to avoid an influence of antiviral treatment or HCC occurrence, the frequency of PLNE was 9.4%. Although these patients appear to be biased toward a less severely ill patient population, the frequency of PLNE in these patients was not significantly different from that in the original 502 patients (8.2%). Of note, the frequency of PLNE in CHB patients was much lower than that in chronic hepatitis C patients, as previously reported. As suggested earlier, the different components of the immune system in patients with chronic HBV and HCV infections could explain this difference.

This study is limited by the absence of some important clinical details such as information about the histological findings of fibrosis and inflammation. Although the APRI is a useful index for the prediction of fibrosis, the limitation of this score has been reported in previous studies.[34,35] However, we also showed that the prevalence of the patients with clinically diagnosed liver cirrhosis was also significantly higher in the patients with PLNE ($P = 0.0009$), which may minimize this limitation. Another limitation to consider is the cross-sectional design of the present study, which does not allow causal inferences and limits any assumptions about the duration of the existence of any of the criteria,