Factors Predicting HBsAg Seroclearance and Alanine Transaminase Elevation in HBeAg-Negative Hepatitis B Virus-Infected Patients withPersistently Normal Liver Function

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Abstract

Background
A certain proportion of hepatitis B virus (HBV)-infected patients with persistently normal alanine transaminase (ALT) levels have significant fibrosis. Using liver stiffness measurements (Fibroscan®) and laboratory data, including serum ALT, quantitative HBsAg (qHBsAg), and HBV DNA, we attempted to predict the natural histories of these patients.

Methods
Non-cirrhotic HBeAg-negative chronic hepatitis B patients with persistently normal ALT were followed up prospectively with the end points of HBsAg seroclearance and ALT elevation above the upper limit of normal. The factors that were predictive of the end points were identified.

Results
A total of 235 patients with an average age of 48.1 +/- 10.7 years were followed up for 7 years. Eight patients (3.4%) lost HBsAg, and 15 patients (6.4%) experienced ALT elevation. The overall cumulative HBsAg seroclearances were 0.4%, 1.3% and 2.3% at years 1, 3 and 5, respectively. Regarding HBsAg seroclearance, the qHBsAg (< 30 IU/ml) cutoff resulted in a hazard ratio (HR) of 19.6 with a 95% confidence interval (CI) of 2.2–166.7 (P = 0.008). The baseline ALT level (odd ratio (OR) 1.075, 95% CI 1.020–1.132, P = 0.006) and a qHBsAg above 1000 IU/ml (3.7, 1.1–12.4, P = 0.032) were associated with ALT elevation. Limited to men, the baseline liver stiffness (1.6, 1.0–2.5, P = 0.031) and a qHBsAg above 1000 IU/ml (10.4, 2.1–52.4, P = 0.004) were factors that were independently associated with ALT elevation.
Conclusion
A low qHBsAg level predicted HBsAg clearance. Baseline ALT and a qHBsAg above 1000 IU/ml were independent predictive factors for ALT elevation. Among the men, the independent predictive factors for ALT elevation were qHBsAg and liver stiffness.

Introduction
Most of the treatment guidelines suggest that chronic hepatitis B (CHB) patients with persistently normal alanine aminotransferase (AL) levels should not undergo antiviral therapy with the exceptions of liver cirrhosis patients and patients with liver biopsies that exhibit significant fibrosis. Previous studies have demonstrated that high serum HBV DNA levels increase the risks of liver cirrhosis and/or hepatocellular carcinoma (HCC). A certain proportion of patients with normal ALT levels have significant inflammation or fibrosis [1,2]. Most of the treatment guidelines suggest that chronic hepatitis B (CHB) patients with persistently normal alanine aminotransferase (ALT) levels should not undergo antiviral therapy with the exceptions of liver cirrhosis patients and patients with liver biopsies that exhibit significant fibrosis. Previous studies have demonstrated that high serum HBV DNA levels increase the risks of liver cirrhosis and/or hepatocellular carcinoma (HCC). A certain proportion of patients with normal ALT levels have significant inflammation or fibrosis [1,2]. One report found that among the patients with ages older than 40 years and ALT levels in the high normal range, up to 47% of patients with persistently normal ALT and an HBV DNA value > 10,000 copies/ml exhibit significant fibrosis and inflammation on liver biopsy. In contrast, the other two studies showed that most of the patients in the immune tolerant phase of chronic HBV infection exhibited no or minimal fibrosis despite high HBV DNA levels [3,4]. These data indicate that duration of infection and age are important for assessing the severity of liver injuries in patients with high HBV DNA but normal ALT levels. In recent years, the quantification of HBsAg has become an important factor in the evaluation of viral activity. Brunetto et al. [5] first studied the relationship between the serum HBsAg levels and the clinical stages of HBeAg-negative HBV carriers with genotype D infections. They found that a combination of single measurement HBV DNA (≤ 2000 IU/ml) and quantification of HBsAg (≤ 1000 IU/ml) could identify inactive carriers with 94.3% diagnostic accuracy [5]. Tseng et al. [6] reported that risks of HBeAg-negative hepatitis, hepatitis flares, and cirrhosis were low in patients with qHBsAg (≤ 1000 IU/ml) [6]. Lower levels of both quantitative HBsAg and HBV DNA might represent an inactive HBV infection [7,8]. Hepatic fibrosis indicates accumulated liver damage and is also a prognostic factor for chronic hepatitis B disease. A non-invasive method, such as transient elastography, makes the measurement of fibrosis easy and available. In this study, we used the baseline ALT and HBV DNA levels, the qHBsAg levels and Fibroscan® results to predict the natural courses of HBeAg negative hepatitis B virus-infected patients with persistently normal ALT.

Patients and Methods
Study populations
All the HBsAg tests from 1999 to 2010 were searched in our computerized database of central laboratories. HBeAg-negative patients were enrolled. Those aged over 20 with persistent normal ALT for more than 1 year before entering the study and positive HBsAg and negative
A-HCV statuses during a 2-year period and who were subjected to regular follow-up by four hepatologists were enrolled in this prospective study. Each patient was followed at 6-month intervals over 2 years before entering the study to ensure that the liver enzymes were within the normal limits. HBV–related test results were identified for 130,306 patients, and positive HBsAg positivity was noted in 24,182 patients. A total of 984 person-times from the 33,768 person-times satisfied the conditions. Two hundred ninety-three patients signed the forms and joined the study. Fifty-eight patients were excluded due to HBeAg positivity, liver cirrhosis and loss to follow-up. A total of 235 HBeAg-negative patients were enrolled in this study. Our Institutional Review Board approved the study.

Follow-up

These 235 patients were followed from November 2007 to November 2014. The exclusion criteria included HBeAg positivity, liver cirrhosis based on ultrasound, co-infection with HCV or HIV, hemodialysis, chemotherapy and transplantation. Pregnant and breastfeeding women were also excluded. At the initial visit, the following laboratory data were collected: hemoglobin level, platelet count, aspartate transaminase (AST), ALT, alpha fetoprotein (AFP), total bilirubin, quantitative HBsAg (qHBsAg), hepatitis B virus DNA (HBV DNA), Fibroscan results, lipid profile and body mass index (BMI). The patients were followed up on a 6- or 12-month basis.

Laboratory methods

SeroLOGY study. The following parameters were measured: HBsAg (MEIA, Abbott, North Chicago, IL, or Elecsys HBsAgII, Roche Diagnostics GmbH, Mannheim Germany), Roche Diagnostics GmbH (Mannheim, Germany), quantitative HBsAg level (HBs Ag quantitative kit (Abbott, North Chicago), AST (ULN: 37 U/L with GOT-L-J2 reagent, WAKO, Japan), ALT (ULN: 40 U/L; by GPT-L-J2 reagent, WAKO, Japan.), CBC (with a Sysmex XE-5000 Hematology Analyzer KOB, Koba, Japan), and AFP (with an ARCHITECT AFP assay; Abbott Ireland). The serum HBV DNA was quantified using TaqMan HBV analyte-specific reagent (ASR; Roche Molecular Systems, Inc., Branchburg, New Jersey, USA), which has a detection limit of 70 copies/ml.

Liver stiffness measurements. A one-dimensional transient elastography technique applied with the Fibroscan® (Echosens, Paris, France) system was used for the liver stiffness measurements. During the examinations, the patients were lying on their backs with their right arms raised and tucked behind their head. A staff member placed the probe against the skin (in an intercostal space) and located a suitable portion of the liver of at least 6 cm in thickness that was free of large vascular structures. The acceptable range of the measurement depth was between 2.5 cm and 6.5 cm. Ten successful measurements were required for reliable results [9].

Statistical analysis. All data were recorded and analyzed using the paired t-test, chi-square test, Cox regression analysis, the Kaplan–Meier survival curve with the log-rank test and binary logistic regression for the analyses. The results are expressed as the means ± the standard deviations (SDs). A ROC analysis was performed to define the most suitable quantitative HBsAg value based on Yuden’s index (max [sensitivity +specificity-1]). The statistical procedures were performed with the SPSS statistical software version 17. P values < 0.05 were considered significant.

Ethics. Every patient agreed and signed the informed consent. The local institutional review board in Chang Gung Memorial Hospital approved the study.
Results

A total of 235 HBeAg-negative patients including 131 men and 104 women were enrolled. The endpoints were HBsAg seroclearance and ALT elevation (i.e., at least one episode of >40 u/l (the upper limit of normal, ULN) during the follow-up period. Eight patients (3.4%) exhibited HBsAg seroclearance, 15 patients (6.4%) experienced ALT elevation, and 212 patients (90.2%) did not exhibit HBsAg seroclearance or ALT elevation (Fig 1).

HBsAg seroclearance

Among the 8 patients who exhibited HBsAg seroclearance, the HBsAg data at the entry point were lost; thus, we used the data from the remaining 5 patients for the statistical analysis (Table 1). The most important factor for predicting HBsAg seroclearance was the qHBsAg. The median age of the HBsAg seroclearance patients was 51.6 years +/- 7.5 years. The cutoff point was identified via a ROC analysis. The area under the curve was 0.835. The best cutoff points were 28.9 IU/ml (in this study, we set the cutoff at 30 IU/ml, which yielded a sensitivity and specificity of 80% and 84%, respectively) and 97.5 IU/ml (in this study, we set the cutoff at 100 IU/ml, which yielded a sensitivity and specificity of 80% and 76%, respectively). The overall cumulative proportions of HBsAg seroclearance patients were 0.4%, 1.3% and 2.3 at years 1, 3 and 5, respectively (Fig 2A). Using a qHBsAg cutoff point of 30 IU/ml, the HBsAg...
seroclearance rates were 5%, 7.5% and 10.3% at years 1, 3 and 5, respectively (Fig 2B). Using an HBsAg cutoff point of 100 IU/ml, the HBsAg seroclearance rates were 3.4%, 5.2% and 7.1% at years 1, 3 and 5, respectively (Fig 2C). The hazard ratio (HR) for the 30 IU/ml qHBsAg cut-off was 19.6 with a 95% confidence interval (CI) of 2.2–166.7 (P = 0.008, Table 2).

Elevation of ALT above the ULN

Of the 235 HBeAg-negative patients, 15 patients (6.4%) experienced ALT elevations. These patients were predominantly male (male:female, 12:3, P = 0.051) and had higher baseline ALT levels (32.2 +/-12.2 vs. 23.9+/-8.8 u/l, P = 0.001), higher qHBsAg values (3.1+/-0.7 vs 2.4+/-1.1 log10 IU/ml, P = 0.003), and greater liver stiffness levels (5.1 +/-1.2 kPa vs 4.4 +/-1.3 kPa, P = 0.037, Table 3). Univariate analyses indicated that the factors that predicted ALT elevation were the baseline ALT level, liver stiffness, a HBV DNA >2000 IU/ml and the qHBsAg value. The multivariate analysis revealed that the baseline liver stiffness (OR 1.6, 95% CI 1.0–2.5, P = 0.031) and a qHBsAg above

Table 1. Basic characteristics of the HBsAg seroclearance and non-seroclearance patients.

| Group                        | HBsAg Seroclearance | HBsAg Non-Seroclearance | P value* |
|------------------------------|---------------------|-------------------------|----------|
| Patient Number               | 5                   | 227                     |          |
| Age (years)                  | 51.6±7.5            | 47.9±10.8               | 0.445    |
| Sex (Male:Female)            | 4:01                | 102:125                 | 0.267    |
| Genotype (B, C, unknown)     | 3:02:00             | 168:47:12               | 0.533    |
| DM (%)                       | 0 (0%)              | 8 (3.5%)                | 0.589    |
| Hypertension (%)             | 0(0%)               | 28(12.3%)               | 0.29     |
| BMI (kg/m2) (no. 5–225)      | 23.5±3              | 23.3±3.2                | 0.933    |
| Total cholesterol (mg/dl)    | 184.7±15.5          | 189.8±33.6              | 0.463    |
| Triglyceride (mg/dl)         | 146.7±119.3         | 96.6±58.4               | 0.764    |
| HDL (mg/dl)                  | 50.7±11.3           | 60.0±14.2               | 0.197    |
| LDL (mg/dl)                  | 104.7±14.2          | 110.2±28.8              | 0.703    |
| Baseline ALT (u/l)           | 22.4±6              | 24.5±9.2                | 0.614    |
| ALT (> 30 u/l)               | 1(20%)              | 59(26%)                 | 0.762    |
| Total Bilirubin (mg/dl)      | 1.0±0.3             | 0.8±0.2                 | 0.250    |
| Platelets (10^3/μL)          | 197±19              | 220±49                  | 0.301    |
| Hemoglobin (Hb; g/dl)        | 15.1±1.4            | 14.2±1.5                | 0.183    |
| AFP (α-fetal protein; ng/ml) | 6.5±7.6             | 3.1±1.6                 | 0.376    |
| HBV DNA levels (log10 IU/ml) | 2.3±1.5             | 3.0±1.2                 | 0.158    |
| HBV DNA > 2000 IU/ml        | 2 (40%)             | 105 (46.3%)             | 0.781    |
| HBV DNA > 20000 IU/ml       | 0(0%)               | 38(16.7%)               | 0.317    |
| qHBsAg (log10 IU/ml)         | 0.3±1.4             | 2.5±1.0                 | 0.005†   |
| qHBsAg > 30 IU/ml (%)        | 1 (20%)             | 191 (84.1% %)           | <0.001†  |
| qHBsAg > 100 IU/ml (%)       | 1 (20%)             | 173 (76.2% %)           | 0.004†   |
| Stiffness (kPa)              | 5.1±2.8             | 4.5±1.2                 | 0.301    |

*: Based on chi-square and independent samples t-tests.
†: P value < 0.05.
Fig 2. Relationship between quantitative HBsAg (qHBsAg) and the rate of hepatitis B surface antigen (HBsAg) seroclearance in the natural courses of the HBeAg-negative patients. Overall Cumulative HBsAg seroclearance (A), HBsAg seroclearance with a qHBsAg cutoff value of 30 IU/ml (B), and HBsAg seroclearance with a qHBsAg cutoff value of 100 IU/ml (C). The patients were stratified based on the baseline qHBsAg status (B, C).

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Discussion

HBsAg seroclearance is a landmark in the treatment of chronic HBV infection because hepatitis B is mainly immune-mediated, and the elimination of HBsAg is considered to be an indicator of the effective treatment of HBV infection. A lower HBsAg level may indicate better host immune control of HBV duplication and a decreased level of intrahepatic covalently closed circular DNA [10]. The annual HBsAg seroclearance rate of hepatitis B patients is between 0.5% and 2.26% per year depending on enrollment in treatment [11–13]. In a study conducted in Taiwan regarding spontaneous HBsAg seroconversion, the average follow-up period of hepatitis B patients was 7.4 years, and 18 patients of 3,000 cleared HBsAg with an annual rate of 0.6%. At 1 year after HBeAg seroconversion, low serum levels of HBsAg predicted the elimination of HBsAg in patients with genotype B or C infections [14]. A Korean study demonstrated that the overall annual HBsAg seroclearance rate was 1.8%, but the annual HBsAg seroclearance rate of the older-aged group was higher, i.e., the annual seroclearance rate was 2.69% among patients aged over 60, whereas the annual seroclearance rate was 1.91% among the patients aged between 40 and 59. The cumulative probabilities of HBsAg seroclearance were 1.2%, 3.8% and 8.7% for patients who were followed for 1, 3 and 5 years, respectively [15]. The annual HBsAg seroclearance rate in our study was 0.48%, i.e., 8 patients exhibited HBsAg seroclearance in the 1645 person-years study, and the rate was 0.3%, i.e., 5 patients exhibited HBsAg seroclearance in the 1624 person-years study. The overall cumulative HBsAg seroclearance rates were 0.4%, 1.3% and 2.3% for the patients who were followed up for 1, 3 and 5 years,

### Table 2. Univariate and Multivariate Analyses of the Factors Associated With HBsAg Seroclearance.

| Variables                  | Comparison                  | Univariate OR (95% CI) | P value | Multivariate OR (95% CI) | P value |
|----------------------------|------------------------------|------------------------|---------|--------------------------|---------|
| Age (years)                | Increase per one year       | 1.031 (0.952–1.118)    | 0.453   |                          |         |
| Sex (Male:Female)          | Female vs. Male             | 3.236 (0.361–28.571)   | 0.294   |                          |         |
| DM (%)                     | +/-                          | 0.047 (0.0–52261727)   | 0.774   |                          |         |
| Hypertension (%)           | +/-                          | 0.041 (0.0–4866)       | 0.592   |                          |         |
| BMI (kg/m2) (no. 5:225)    | Increase per kg/m2          | 1.015 (0.773–1.331)    | 0.916   |                          |         |
| Total cholesterol (mg/dL)  | Decrease per one mg/dl      | 1.005 (0.974–1.036)    | 0.769   |                          |         |
| Triglyceride (mg/dL)       | Increase per one mg/dl      | 1.007 (0.998–1.015)    | 0.119   |                          |         |
| HDL (mg/dl)                | Decrease per one mg/dl      | 1.057 (0.968–1.152)    | 0.210   |                          |         |
| LDL (mg/dl)                | Decrease per one mg/dl      | 1.007 (0.969–1.046)    | 0.704   |                          |         |
| Baseline ALT (u/l)         | Decrease per one u/l        | 1.027 (0.926–1.140)    | 0.607   |                          |         |
| ALT (> 30 u/l)             | Less than 30 u/l            | 1.404 (0.156–12.5)     | 0.762   |                          |         |
| Total Bilirubin (mg/dl)    | Increase per one mg/dl      | 5.376 (0.782–36.954)   | 0.087   |                          |         |
| Platelets (10^3/μl)        | Decrease per one 10^3/μL    | 1.122 (0.908–1.386)    | 0.284   |                          |         |
| Hemoglobin (g/dl)          | Increase per one g/dl       | 1.555 (0.804–3.009)    | 0.19    |                          |         |
| HBV DNA levels (log10 IU/ml)| Decrease per 1 log 10 IU/ml | 1.739(0.805–3.759)     | 0.159   |                          |         |
| HBV DNA > 2000 IU/ml      | Less than 2000 IU/ml        | 1.305(0.218–7.812)     | 0.77    |                          |         |
| qHBsAg (log10 IU/ML)       | Decrease per 1 log 10 IU/ML | 2.257 (1.199–4.255)    | 0.012†  |                          |         |
| qHBsAg > 30 IU/ml          | Less than 30 IU/ml          | 19.607 (2.207–166.666) | 0.008†  | 19.607 (2.207–166.666)   | 0.008†  |
| qHBsAg > 100 IU/ml         | Less than 100 IU/ml         | 3.472 (1.161–10.387)   | 0.024†  |                          |         |
| Stiffness (kPa)            | Increase per one kPa        | 1.347 (0.770–2.357)    | 0.296   |                          |         |

†: P value < 0.05.

1000 IU/ml (OR 10.4, 95% CI 2.1–52.4, P value 0.004) were independent risk factors for ALT elevation (Table 5).
respectively. In the present study, the HBsAg seroclearance rate was lower than that in the aforementioned Korean study. In the Korean study, the patients were all genotype C, and approximately 20% of them were treated with an antiviral agent or interferon; however, in our study, the patients were all treatment-naïve, and genotype B was predominant.

Given that there is no evidence of liver cirrhosis or HCV/HDV super infection and the subject’s age is <50 years at the time of the HBsAg loss, there is a minimal risk of HCC development [16]. The REVEAL-HBV study demonstrated that HBsAg seroclearance is significantly associated with increased age, a low serum HBV DNA level (100000 vs ≥ 100000 copies/mL) and a high body mass index (30 vs 30 kg/m2) [17]. In the natural course of chronic HBV infection, a lower HBV DNA level has been reported to be important in determining the subsequent elimination of HBsAg, and the HBV DNA levels in the sera of 95.8% of patients have been found to be undetectable before the elimination of HBsAg [18]. The HBsAg level was a better indicator than the HBV DNA level regarding the prediction of HBsAg elimination in patients who were followed for 6 years. Comparison of the patients with HBsAg levels ≥ 1000 IU/ml with those with HBsAg levels that were 100–999 and < 100 IU/ml revealed that the hazard ratios were 4.4 (95% CI, 1.1–17) and 24.3 (8.7–67.5), respectively, which indicated that the HBsAg loss rate of the latter group was higher. [14]. In a longitudinal study of 117 patients performed in Hong Kong, during the natural course of chronic hepatitis B, a HBsAg

| Group                        | ALT Elevation | Non-Elevation | P value* |
|------------------------------|---------------|---------------|---------|
| Patient Number               | 15            | 220           |         |
| Age (years)                  | 46.5±9.2      | 48.1±10.8     | 0.571   |
| Sex (Male:Female)            | 12:03         | 119:101       | 0.051   |
| Genotype (B, C, unknown)     | 11:03:01      | 162:47:11     | 0.957   |
| DM (%)                       | 0 (0%)        | 8 (3.6%)      | 0.452   |
| Hypertension (%)             | 4(26.7%)      | 24(10.9%)     | 0.068   |
| BMI (kg/m2)                  | 24.5±3.6      | 23.3±3.1      | 0.171   |
| BMI (kg/m2) (23 ≥)           | 11(73.3%)     | 111(50.9%)    | 0.171   |
| Total cholesterol (mg/dl)    | 191.8±26.4    | 189.4±34      | 0.792   |
| Triglyceride (mg/dl)         | 89.9±35.8     | 97.5±60.9     | 0.634   |
| HDL (mg/dl)                  | 58.2±12.5     | 59.8±14.3     | 0.674   |
| LDL (mg/dl)                  | 115.5±25.8    | 109.9±28.8    | 0.463   |
| Baseline ALT (u/l)           | 32.2±12.2     | 23.96±5.8     | 0.001†  |
| Baseline ALT (> 30 u/l)      | 7(46.7%)      | 54(24.5%)     | 0.059   |
| Total Bilirubin (T-Bil; mg/dl)| 0.8±0.3       | 0.8±0.3       | 0.828   |
| Platelets (10^3/μL)          | 221±54        | 219±48        | 0.852   |
| AFP (α-fetal protein; ng/ml) | 3.2±1.1       | 3.1±2.0       | 0.882   |
| HBV DNA levels (log10 IU/ml) | 3.3±1.5       | 3.0±1.2       | 0.357   |
| HBV DNA > 2000 IU/ml         | 8(53.3%)      | 99(45%)       | 0.531   |
| HBV DNA > 20000 IU/ml        | 6(49%)        | 32(14.5%)     | 0.010†  |
| HBs Ag titer (log10 IU/ml)   | 3.1±0.7       | 2.4±1.1       | 0.003†  |
| qHBsAg > 1000 IU/ml (%)      | 11 (73.3%)    | 85(38.6%)     | 0.008†  |
| Stiffness (kPa)              | 5.1±1.2       | 4.4±1.3       | 0.037†  |
| Stiffness (>5 kPa) (%)       | 6(40%)        | 42(19.4%)     | 0.056   |
| HBsAg Seroclearance during follow-up (%) | 0 (0%) | 8(3.6%) | 0.452 |

*: Based on chi-square and independent samples t-tests.
†: P value < 0.05.

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| Variables                  | Comparison          | Univariate OR (95% CI) | P value | Multivariate OR (95% CI) | P value |
|---------------------------|---------------------|------------------------|---------|--------------------------|---------|
| Age (years)               | Decrease per one year | 1.014 (0.965–1.066) | 0.57    |                          |         |
| Sex (Male:Female)         | Male vs. Female     | 3.389 (0.931–1.234)   | 0.064   |                          |         |
| BMI (kg/m2)               | Increase 1 kg/m2    | 1.116 (0.953–1.307)   | 0.173   |                          |         |
| BMI (kg/m2) (23 >)        | Above 23 kg/m2      | 2.651(0.819–8.582)    | 0.104   |                          |         |
| Total cholesterol (mg/dL) | Increase 1 mg/dl    | 1.002(0.987–1.018)    | 0.791   |                          |         |
| Triglyceride (mg/dL)      | Increase 1 mg/dl    | 0.997(0.987–1.008)    | 0.633   |                          |         |
| HDL (mg/dL)               | Increase 1 mg/dl    | 0.992(0.955–1.030)    | 0.672   |                          |         |
| LDL (mg/dL)               | Increase 1 mg/dl    | 1.006(0.990–1.023)    | 0.461   |                          |         |
| Baseline ALT (u/l)        | Increase 1 μl       | 1.084(1.030–1.140)    | 0.002†  | 1.075 (1.020–1.132)      | 0.006†  |
| Baseline ALT (> 30 u/l)   | Above 30 u/l        | 2.690(0.932–7.762)    | 0.067   |                          |         |
| Total Bilirubin (T-Bil; mg/dL) | Increase per one mg/dl | 1.209(0.221–6.605)    | 0.827   |                          |         |
| Platelets (10^3/μL)       | Increase per one 10^3/μL | 1.010(0.909–1.122)    | 0.851   |                          |         |
| AFP (α-fetal protein; ng/ml) | Increase per one ng/ml | 1.019(0.797–1.303)    | 0.881   |                          |         |
| DNA (log 10 IU/ml)        | Increase per 1 log 10 IU/ml | 1.228(0.793–1.901)    | 0.357   |                          |         |
| HBV DNA > 2000 IU/ml     | Above 2000 IU/ml    | 1.397(0.489–3.986)    | 0.532   |                          |         |
| HBV DNA > 20000 IU/ml    | DNA > 20000 IU/ml   | 3.917 (1.305–11.753) | 0.015†  |                          |         |
| qHBsAg (log 10 IU/ml)     | Increase per 1 log 10 IU/ml | 2.248(1.089–4.643)    | 0.029†  |                          |         |
| qHBsAg > 1000 IU/ml      | qHBsAg > 1000 IU/ml | 4.368(1.347–14.157)   | 0.014†  | 3.718 (1.119–12.359)     | 0.032†  |
| Stiffness (kPa)           | Increase per one kPa | 1.431 (1.012–1.982)   | 0.043†  |                          |         |
| Stiffness (>5 kPa)        | Above > 5 kPa       | 2.778(0.937–8.233)    | 0.065   |                          |         |

†: P value < 0.05.

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| Variables                  | Comparison          | Univariate OR (95% CI) | P value | Multivariate OR (95% CI) | P value |
|---------------------------|---------------------|------------------------|---------|--------------------------|---------|
| Age (years)               | Decrease per one year | 1.019 (0.964–1.077) | 0.497   |                          |         |
| BMI (kg/m2)               | Increase 1 kg/m2    | 1.032 (0.857–1.242)   | 0.738   |                          |         |
| BMI (kg/m2) (23 >)        | Above 23 kg/m2      | 1.26(0.359–4.424)     | 0.718   |                          |         |
| Total cholesterol (mg/dl) | Increase 1 mg/dl    | 1.001(0.985–1.018)    | 0.884   |                          |         |
| Triglyceride (mg/dl)      | Increase 1 mg/dl    | 0.985(0.967–1.004)    | 0.122   |                          |         |
| HDL (mg/dl)               | Increase 1 mg/dl    | 1.022(0.979–1.067)    | 0.322   |                          |         |
| LDL (mg/dl)               | Increase 1 mg/dl    | 1.004(0.986–1.022)    | 0.693   |                          |         |
| Baseline ALT (U/L)        | Increase per one U/l | 1.069(1.002–1.142)    | 0.044†  |                          |         |
| Baseline ALT (> 30 u/l)   | Above 30 u/l        | 1.310(0.391–4.381)    | 0.662   |                          |         |
| Total Bilirubin (T-Bil; mg/dl) | Decrease per one mg/dl | 1.364(0.198–9.345)    | 0.752   |                          |         |
| Platelets (10^3/μL)       | Increase per one 10^3/μL | 1.020(0.894–1.175)    | 0.726   |                          |         |
| AFP (α-fetal protein; ng/ml) | Decrease per one ng/ml | 1.018(0.768–1.347)    | 0.902   |                          |         |
| DNA (log 10 IU/ml)        | Increase per 1 log 10 IU/ml | 1.509(0.853–2.667)    | 0.157   |                          |         |
| HBV DNA > 2000 IU/ml     | Above 2000 IU/ml    | 1.867(0.560–6.221)    | 0.309   |                          |         |
| HBV DNA > 20000 IU/ml    | DNA > 200000 IU/ml  | 4.952(1.392–17.615)   | 0.013†  |                          |         |
| qHBsAg (log 10 IU/ml)     | Increase per 1 log 10 IU/ml | 2.971(1.163–7.590)    | 0.023†  |                          |         |
| qHBsAg > 1000 IU/ml      | qHBsAg > 1000 IU/ml | 10.256(2.143–49.087) | 0.004†  | 10.447(2.081–52.441)     | 0.004†  |
| Stiffness (kPa)           | Increase per one kPa | 1.556(1.066–2.272)    | 0.022†  | 1.606(1.043–2.473)       | 0.031†  |
| Stiffness (>5 kPa)        | > 5 kPa             | 4.008(1.146–14.023)   | 0.03†   |                          |         |

†: P value < 0.05.

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reduction > 1 log10 IU/ml was associated with greater viral suppression [19]. Our study revealed that the baseline HBV DNA level had nothing to do with HBsAg seroclearance, and only the qHBsAg was associated with HBsAg seroclearance; at the cutoff of qHBsAg 30 IU/ml, the HR was 19.230, and 95% CI was 2.2–166.7. There was no significant relationship between genotype and HBsAg seroclearance in our study. This study suggested the existence of an uncoupled immune effect on HBsAg production and viral duplication. However, a low qHBsAg level might suggest a more complete immune clearance than a low HBV DNA level.

At present, there is little data available for predicting spontaneous ALT elevation above the ULN in asymptomatic chronic HBV-infected patients with persistently normal ALT. In a recent study performed in India with patients with HBeAg-negative chronic hepatitis B who were yet asymptomatic and had normal ALT levels, the factors that were found to be predictive of the occurrence of spontaneous ALT flares were the male gender, the presence of precore mutants and an age equal to or above 30 years old at baseline. ALT flares were defined as elevations in the serum ALT level to above 2 times the ULN in combination with an HBV DNA level $\geq$ 105 copies/ml or a 100-fold rise in the HBV DNA from the previous baseline level. The rate of ALT flares was approximately 4.3% per year. The examined patients were predominantly genotypes D and A [20]. In a prospective study performed in Canada, ALT elevation was defined as a change from a normal ALT (ALT $\leq$ 40 IU/ml) to an elevated ALT (ALT $>$ 40 IU/ml). This study followed 37 HBeAg-negative patients with normal ALT levels at baseline for a median of 3 years (0.67–4 years), and their baseline HBV DNA levels were found to be highly predictive of future ALT elevations above the ULN [21]. In contrast, a study of chronic hepatitis B among Asian American patients found that the ALT levels were normal (genotype B or C), and there were no strong associations of ALT flares with any of the assessed clinical factors, which included an age $\geq$ 50, gender, ALT at biopsy $\geq$ 1/2 ULN, fibrosis stage $>$ 1, inflammation grade $>$ 1, HBV DNA $\geq$ 100000 IU/ml or $\geq$ 20000 IU/ml, HBeAg positivity, HBV PC mutation, HBV BCP mutation, all combinations of PC and BCP mutations, and genotypes B or C. However, this study of Asian Americans did not test qHBsAg [22]. In our study, the annual rate of spontaneous ALT elevation was 0.9%, and the qHBsAg played a more important role in predicting ALT elevations. According to the medical care guide of the Taiwan National Health Insurance Administration, in the patient group with ALT elevations above the ULN, 7 of 15 patients (46.7%) would receive nucleoside/nucleotide analogues.

Arena et al. demonstrated that there is a strong association between liver stiffness and serum aminotransferase level [23]. In CHB patients with ALT flares, liver stiffness increases and returns to normal levels after 6 months [24]. A recent meta-analysis demonstrated that the FibroTest has good accuracy in terms of the identification of HBV-associated significant fibrosis and cirrhosis [25]. Regarding the prediction of ALT elevations above the ULN, the patients were further divided into male and female groups because different etiologies may be present in the different genders. However, there were only 3 females, so it was not possible to statistically analyze the females, and we only analyzed the male patients in the subgroup analysis. Baseline liver stiffness was correlated with future ALT elevation in our cohort. Across all patients, liver stiffness was only significant in the univariate analysis. However, in the subgroup analysis of the male patients, liver stiffness was significant in both univariate and multivariate analyses. We also analyzed the fibrosis-4 score (FIB-4) and AST to platelet ratio index (APRI). However, there was no significant relationship between these scores and ALT elevation.

qHBsAg provides not only a very useful means to identify the HBV-related cause of liver damage in HBsAg carriers but also it is the most important predictor of clinically relevant outcomes associated with HBV-induced liver disease. In a prospective study performed in France, reactivation of hepatitis among HBeAg-negative asymptomatic patients could be predicted by a combination of HBsAg $>$ 1000 IU/ml and HBV-DNA $>$ 200 IU/ml (92% sensitivity and
96% negative predictive value) [26]. Tseng TC et al. showed that HBsAg, ALT and age could be used as predictors for hepatocellular carcinoma in HBeAg negative patients with HBV DNA < 2000 IU/ml. This study revealed higher risk of HCC in patients with HBsAg ≥ 1000 IU/ml compared with those < 1000 IU/ml (adjusted hazard ratio 13.7, 95% confidence interval: 4.8–39.3) [27]. Another prospective study from Korea also demonstrated that combined HBsAg and HBV DNA (> 850 IU/ml and 850 IU/ml, respectively) could predict the reactivation of HBV with 84.6% diagnostic accuracy [28]. One recent study showed that combined HBV DNA with qHBsAg or liver stiffness measurement could correctly identify inactive carrier at a single time point [29]. Our study emphasizes the influence of single baseline ALT, qHBsAg and liver stiffness measurement on natural history of asymptomatic hepatitis B infected patients. However, our study has some limitations. This study conducted only on a small sample size of population with relatively short duration of follow-up. We used the same level of ALT normal cutoff value (40 IU/ml) for both genders (2016 American Association for the Study of Liver Disease Guideline: normal ALT levels are < 30 IU/ml for males and < 10 IU/ml for female) because we use the same normal ALT cutoff value in real-world clinical practice in Taiwan.

In conclusion, our study demonstrated that a single baseline qHBsAg is an important factor for future ALT elevations above the ULN and qHBsAg seroclearance. Very few studies have used baseline liver stiffness to predict future ALT elevations. Currently, no simple and readily available markers are able to accurately predict biochemical elevations in HBV-infected patients. Using the qHBsAg, liver stiffness and HBV DNA, we have demonstrated a new perspective for predicting the natural course of HBeAg-negative patients with persistently normal liver function.

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References

1. Lai M, Hyatt BJ, Nasser I, Curry M, Afdhal NH. The clinical significance of persistently normal ALT in chronic hepatitis B infection. J Hepatol 2007; 47:760–7. doi: 10.1016/j.jpedit.2007.07.022 PMID: 17928090

2. Kumar M, Sarin SK, Hissar S, Pande C, Sakhua P, Sharma BC, et al. Virologic and histologic features of chronic hepatitis virus-infected asymptomatic patients with persistently normal ALT. J Gastroenterol 2008; 134:1376–84.

3. Andreani T, Serfaty L, Mohand D, Dernaika S, Wendum D, Chazouilleres O, et al. Chronic hepatitis B virus carriers in the immunotolerant phase of infection: histologic findings and outcome. J Gastroenterol Hepatol 2007; 5:636–41. doi: 10.1016/j.jgh.2007.01.005 PMID: 17428739

4. Hui CK, Leung N, Yuen ST, Zhang HY, Leung KW, Lu L et al. Natural history and disease progression in Chinese chronic hepatitis B patients in immune-tolerant phase. J Hepatol 2007; 46:395–401.

5. Brunetto MR, Oliveri F, Colombatto P, Moriconi F, Ciccossi P, Coco B, et al. Hepatitis B surface antigen serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. J Gastroenterol 2010; 139:483–90.

6. Tseng TC, Liu CJ, Yang HC, Su TH, Wang CC, Chen CL, et al. Serum Hepatitis B Surface Antigen Levels Help Predict Disease Progression in Patients with Low Hepatitis B Virus Loads. J Hepatol 2013; 57:441–50.

7. Chen CH, Lee CM, Wang JH, Tung HD, Hung CH, Lu SN. Correlation of quantitative assay of hepatitis B surface antigen and HBV DNA levels in asymptomatic hepatitis B virus carriers. Eur J Gastroenterol Hepatol. 2004; 16:1213–8. PMID: 15489584

8. Kuo YH, Chang KC, Wang JH, Tsai PS, Hung SF, Hung CH, et al. Changing serum levels of quantitative Hepatitis B surface antigen and hepatitis B virus DNA in hepatitis B virus surface antigen carrier. Kaohsiung J Med Sci. 2015; 31:102–7. doi: 10.1016/j.kjms.2014.11.002 PMID: 25645989

9. Wang JH, Changchien CS, Hung CH, Eng HL, Tung WC, Kee KM, et al. FibroScan and ultrasonography in the prediction of hepatic fibrosis in patients with chronic viral hepatitis. J Gastroenterol 2009; 44:439–46. doi: 10.1007/s00535-009-0017-y PMID: 19308312

10. Chan HL, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, et al. Serum hepatitis B surface antigen quantification can reflect hepatitis B virus in the liver and predict treatment response. Clin Gastroenterol Hepatol 2007; 5:1462–68. doi: 10.1016/j.cgh.2007.09.005 PMID: 18054753

11. Chu CM, Liaw YF. HBsAg seroclearance in asymptomatic carriers of high endemic areas: appreciably high rates during a longterm follow-up. J Hepatol 2007; 45:1187–92.

12. McMahon BJ, Holck P, Bulkow L, SnowballM. Serologic and clinical outcomes of 1536 Alaska natives chronically infected with hepatitis B virus. Ann Intern Med 2001; 135:759–68. PMID: 11694101

13. Liu J, Yang HI, Lee MH, Lu SN, Jen CL, Wang LY, et al. Incidencend determinants of spontaneous hepatitis B surface antigen seroclearance: a community-based follow-up study. J Gastroenterol 2010; 139:474–82.

14. Tseng TC, Liu CJ, Su TH, Wang CC, Chen CL, Chen PJ, et al. Serum hepatitis B surface antigen levels predict surface antigen loss in hepatitis B e antigen seroconverters. J Gastroenterol 2011; 141:517–25.

15. Kwak Ms, Cho EJ, Jang ES, Lee JH, Yu SJ, Kim YJ, et al. Predictors of HBsAg seroclearance in HBsAg negative chronic hepatitis B patients. Digestion 2011; 84 (suppl 1):23–28.

16. Chen YC, Sheen IS, Chu CM, Liaw YF. Prognosis following spontaneous HBsAg seroclearance in chronic hepatitis B patients with or without concurrent infection. J Gastroenterol 2002; 123:1084–89.

17. Chen CJ, Yang HI. Natural history of chronic hepatitis B REVEALed. J Gastroenterol Hepatol 2011; 26:628–38. doi: 10.1111/j.1440-1746.2011.06695.x PMID: 21323729

18. Liu J, Yang HI, Lee MH, Lu SN, Jen CL, Wang LY, et al. Incidence and determinants of spontaneous hepatitis B surface antigen seroclearance: a community-based follow-up study. J Gastroenterol 2010; 139:474–82.

19. Chan HL, Wong VW, Wong GL, Tse CH, Chan HY, Sung JJ. A longitudinal study on the natural history of serum hepatitis B surface antigen changes in chronic hepatitis B. J Hepatol 2010; 52:1223–41.
20. Kumar M, Chauhan R, Gupta N, Hisar S, Sakhuja P, Sarin SK. Spontaneous increases in alanine aminotransferase levels in asymptomatic chronic hepatitis B virus-infected patients. J Gastroenterol 2009; 136: 1272–80.

21. Feld JJ, Ayers M, El-Ashry D, Mazziulli T, Tellier R, Heathcote EJ. Hepatitis B virus DNA prediction rules for hepatitis B e antigen-negative chronic hepatitis B. J Hepatol 2007; 46:1057–70.

22. Nguyen K, Pan C, Xia V, Hu J, Hu KQ. Clinical course of chronic hepatitis B presented with normal ALT in Asian American patients. J Viral Hepatol 2015; 22: 809–16.

23. Arena U, Vizzutti F, Corti G, Ambu S, Stasi C, Bresci S et al. Acute viral hepatitis increases liver stiffness values measured by transient elastography. J Hepatol 2008; 47: 380–4.

24. Fung J, Lai CL But D, Hsu A, Seto WK, Cheng C, et al. Reduction of liver stiffness following resolution of acute flares of chronic hepatitis B. Hepatol Int 2010; 4:716–22. doi: 10.1007/s12072-010-9189-z PMID: 21286342

25. Xu Xue-Ying, Kong Hong, Song Rui-Xiang, Zhai Yu-Han, Wu Xiao-Fei, Ai Wen-Si, et al. The Effectiveness of Noninvasive Biomarkers to Predict Hepatitis B-Related Significant Fibrosis and Cirrhosis: A Systematic Review and Meta-Analysis of Diagnostic Test Accuracy. PLoS One 2014; 9: e100182. doi: 10.1371/journal.pone.0100182 PMID: 24964038

26. Martinot-Peignoux M, Lapalus M, Lacroix C, Netto-Cardoso AC, Boyer N, Ripault MP, et al. Prediction of disease reactivation in asymptomatic hepatitis B e antigen-negative chronic hepatitis B patients using baseline serum measurements of HBsAg and HBV-DNA. J Clin Virol 2013; 58:401–7. doi: 10.1016/j.jcv.2013.08.010 PMID: 24004660

27. Tseng TC, Liu CJ, Yang HC, Su TH, Wang CC, Chen CL, et al. High levels of hepatitis B surface antigen increase risk of hepatocellular carcinoma in patients with low HBV load. J Gastroenterol 2012; 142:1140–9.

28. Park H, Lee JM, Seo JH, Kim HS, Ahn SH, Kim DY, et al. Chon CY. Predictive value of HBsAg quantification for determining the clinical course of genotype CHBeAg-negative carriers. Liver Int 2012; 32:796–802. doi: 10.1111/j.1478-3231.2011.02693.x PMID: 22128792

29. Maimone S, Caccamo G, Squadrino G, Alibrandi A, Saffioti F, Spinella R, et al. A Combination of Different Diagnostic Tools Allows Identification of Inactive Hepatitis B Virus Carriers at a Single Time Point Evaluation. Liver Int 2016 Sep 8.