# Ultrasensitive Assay for Hepatitis B Core-Related Antigen Predicts Hepatocellular Carcinoma Incidences During Entecavir

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**Serum hepatitis B core-related antigen (HBcrAg) and surface antigen (HBsAg) are surrogate markers of intrahepatic covalently closed circular DNA. The measurement range of the current HBcrAg assay is relatively narrow. Thus, we examined the potential of HBcrAg and HBsAg measured by ultrasensitive assays for predicting hepatocellular carcinoma (HCC) development in patients with chronic hepatitis B treated with entecavir (ETV). We conducted a retrospective cohort study of 180 patients who received ETV for >1 year. All patients had hepatitis B e-antigen negativity at baseline. Serum HBcrAg and HBsAg levels at baseline and year 1 were measured in all patients by ultrasensitive assays using immunoassay for total antigen including complex by pretreatment (iTACT) technology. During the median follow-up of 11.0 years, 22 patients developed HCC (11.8/1,000 person-years). Baseline HBsAg levels were not associated with HCC development during ETV treatment. However, high HBcrAg levels at baseline and at year 1 were significantly associated with HCC development (log-rank test; $P < 0.001$). In 110 patients (61.1%) with ≥4.0 log U/mL at baseline (high HBcrAg cohort), HBcrAg declined to ≤2.9 log U/mL at year 1 in 25 patients (22.7%). The adjusted hazard ratio for HCC incidence was significantly lower in patients with HBcrAg ≤2.9 log U/mL at year 1 than in those in the high HBcrAg cohort. *Conclusion:* Measurement of HBcrAg by ultrasensitive assay has better potential for predicting HCC during antiviral treatment than the current HBcrAg assay. (*Hepatology Communications* 2022;6:36-49).**

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More than 257 million people worldwide are persistently infected with hepatitis B virus (HBV), with 887,000 chronic hepatitis B (CHB) carriers progressing to hepatocellular carcinoma (HCC), resulting in death.[1] Treatment of patients with CHB with nucleos(t)ide analogs (NAs) reduces their HBV DNA levels and decreases the risk of HCC.[2-5] However, NA treatment does not completely eliminate the risk of HCC; some patients still develop HCC despite long-term NA treatment.

Treatment response to NA and follow-up in untreated patients with CHB are generally monitored by measuring serum HBV DNA, hepatitis B e antigen (HBeAg) and antibody levels, and hepatitis B surface antigen (HBsAg) levels. Additionally, many studies have recently focused on hepatitis B core-related antigen (HBcrAg), which

*Abbreviations: ALP, alkaline phosphatase; CHB, chronic hepatitis B; CI, confidence interval; ETV, entecavir; GGT, gamma glutamyltransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio; IQR, interquartile range; iTACT, immunoassay for total antigen including complex by pretreatment; NA, nucleos(t)ide analog; ROC, receiver operating characteristic.*

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is a new serum biomarker of HBV.[6] Serum HBcrAg and HBsAg are surrogate markers of intrahepatic covalently closed circular DNA (cccDNA).[7-9] Some studies have shown that HBcrAg is a useful marker for predicting HCC in untreated and NA-treated patients with CHB.[10-13] However, changes in HBsAg and HBcrAg titers have not been well characterized in patients with CHB. In up to 40% of CHB carriers with HBsAg seroclearance according to older generation HBsAg assays, HBV antigens could be detected using Lumipulse HBsAg-HQ and/or HBcrAg kits.[14] The lower limits of quantification in commonly used commercial HBsAg assays are 0.05-0.005 IU/mL. The lower cutoff of the current HBcrAg assay is 3.0 log U/mL. This low sensitivity of the current HBcrAg assay is one of its current limitations.[15] Therefore, HBcrAg status <3.0 log U/mL should be evaluated.

Regarding HBsAg, a new ultrasensitive assay using immunoassay for total antigen including complex by pretreatment (iTACT) technology has recently been published.[16] The lower cut-off value of this iTACT-HBsAg assay is 0.0005 IU/mL, which is 10-100 times more sensitive than current HBsAg assays. Moreover, iTACT-HBcrAg quantitative reagent, which has been modified by a complete serum treatment process, has recently been developed.[17] This iTACT-HBcrAg assay is approximately 8 times more sensitive than the current HBcrAg assay. It has been reported that iTACT-HBsAg and iTACT-HBcrAg have the potential to predict HCC in patients who achieve HBsAg seroclearance using current assays.[17] Therefore, residual low levels of viral antigen might be associated with HCC development in patients who achieve sufficient viral suppression by potent NA treatment.

In this study, we aimed to determine whether the assessment of baseline and on-treatment serum HBcrAg and HBcrAg levels by ultrasensitive assays were predictive of HCC development in patients with CHB who received long-term entecavir (ETV) treatment.

# Patients and Methods

## STUDY POPULATION

We conducted a retrospective cohort study of patients who received ETV for more than 1 year at our institute. These patients had chronic HBV infection and had a confirmed HBsAg-positive status for at least 6 months with no prior history of HCC. The treatment criteria were based on Japanese Society of Hepatology (JSH) guidelines for the management of HBV infection.[18] A total of 407 consecutive patients with baseline HBeAg negativity began to receive ETV 0.5 mg/day before December 2013. Of the 407 patients, 227 were excluded due to follow-up period of <1 year, HCC development within 1 year of ETV treatment initiation, written informed consent for this study not obtained during the study enrollment period, or nonstorage of adequate serum samples. None of the patients had coinfection with hepatitis C or human immunodeficiency virus. The remaining 180 patients were included in the analysis (Supporting Fig. S1A). Written informed consent for this study and serum sample storage were obtained from each patient. The study protocol complied with the ethical guidelines of the Declaration of Helsinki and the ethical guidelines

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for medical and health research involving human subjects of the Ministry of Health, Labor, and Welfare in Japan. The study was approved by the Toranomon Hospital Ethics Committee (ID 1392).

## STUDY DESIGN AND CLINICAL DATA COLLECTION

The baseline date (day 0) was defined as the date of ETV initiation (Supporting Fig. S1B). We collected data on baseline characteristics, cirrhosis status, biochemistry, and viral markers. Serum HBcrAg and HBsAg levels at baseline and year 1 were measured in all patients by ultrasensitive assays using iTACT technology (Fujirebio Inc., Tokyo, Japan) from their stored serum samples as described in the following sections. Patients were followed up until a confirmed diagnosis of HCC at 1 year after the start of observation (primary outcome) or until the last follow-up (before December 2020) (Fig. 1B). All patients were followed at 1- to 3-month intervals, during which biochemical markers, serum HBV viral markers, blood counts, tumor markers, and HCC status were monitored. All patients also underwent ultrasonography, helical dynamic computed tomography, or magnetic resonance imaging at intervals of 3-6 months for those with cirrhosis or 6-12 months for those without cirrhosis. Cirrhosis was determined by laparoscopy, liver biopsy, imaging modalities, or portal hypertension. The diagnosis of HCC was predominantly based on imaging findings, including dynamic computed tomography, magnetic resonance imaging, and/or digital subtraction angiography.

## iTACT-HBsAg ASSAY

The iTACT-HBsAg assay used in this study (sensitivity, 0.0005 IU/mL) was an improved reagent compared to that reported.[16] The sensitivity of iTACT-HBsAg is approximately 100 times higher than that of conventional HBsAg assays. This assay was fully automated with a Lumipulse Presto II (Fujirebio Inc.) automated chemiluminescent enzyme immunoassay system. We added 90 μL of acidic solution to 50 μL of the sample, which was then stirred and incubated at 37°C for 6.5 minutes. Fifty microliters of antibody-coated bound particle solution was dispensed, stirred, and incubated at 37°C for 8 minutes. After the complexes of antigen–antibody-coated particles were mixed, they were attracted to a magnet and the magnetic field vector (B) and/or force vector (F) were separated. The complex was then washed 3 times with a Lumipulse Presto washing buffer (Fujirebio Inc.) solution. Next, 50 μL of an alkaline phosphatase (ALP)-labeled antibody solution was dispensed, stirred, and incubated for 8 minutes at 37°C. After the complex was washed again, a chemiluminescent substrate was added and incubated for 4 minutes at 37°C. Finally, the amount of luminescence was measured at 463 nm.

## iTACT-HBcrAg ASSAY

The iTACT-HBcrAg assay used in this study (sensitivity of 2.1 log U/mL) was developed based on the conventional reagent Lumipulse HBcrAg (Fujirebio Inc.). The iTACT-HBcrAg assay has approximately 8 times higher sensitivity than the Lumipulse assay. The iTACT-HBcrAg achieves higher sensitivity than the Lumipulse HBcrAg by changing the measurement system and optimizing sample pretreatment conditions and reagent composition conditions. The measurement principle and antibodies used for both methods are similar, and the correlation of iTACT-HBcrAg with Lumipulse HBcrAg for positive samples was good (data not shown). In this study, HBcrAg was measured using only iTACT-HBcrAg. Two types of cut-off values were set, the highly sensitive iTACT cut-off value (2.1 log U/mL) and the current cut-off value (3.0 log U/mL). The antibody-bound particles and ALP-labeled antibodies in the iTACT-HBcrAg assay reagent are also used as materials for the Lumipulse HBcrAg. The iTACT HBcrAg assay was performed with a Lumipulse Presto II after manual pretreatment of the samples. We mixed 150 μL of specimen with 300 μL of pretreatment solution containing detergent mixture; this was incubated for 5 minutes at 80°C with agitation. Then, 100 μL of the pretreated samples was incubated with 50 μL of the on-board pretreatment solution for 6.5 minutes at 37°C, and 50 μL of antibody-coated particle solution was dispensed, stirred, and incubated at 37°C for 8 minutes. After the antigen–antibody-coated particle complex was washed 3 times with the Lumipulse Presto washing buffer, 50 μL of an ALP-labeled antibody solution was dispensed, stirred, and incubated for 8 minutes at 37°C. After the complex was washed again, a chemiluminescent substrate was added and incubated for 4 minutes at 37°C. Finally, the amount of luminescence was measured at 463 nm.

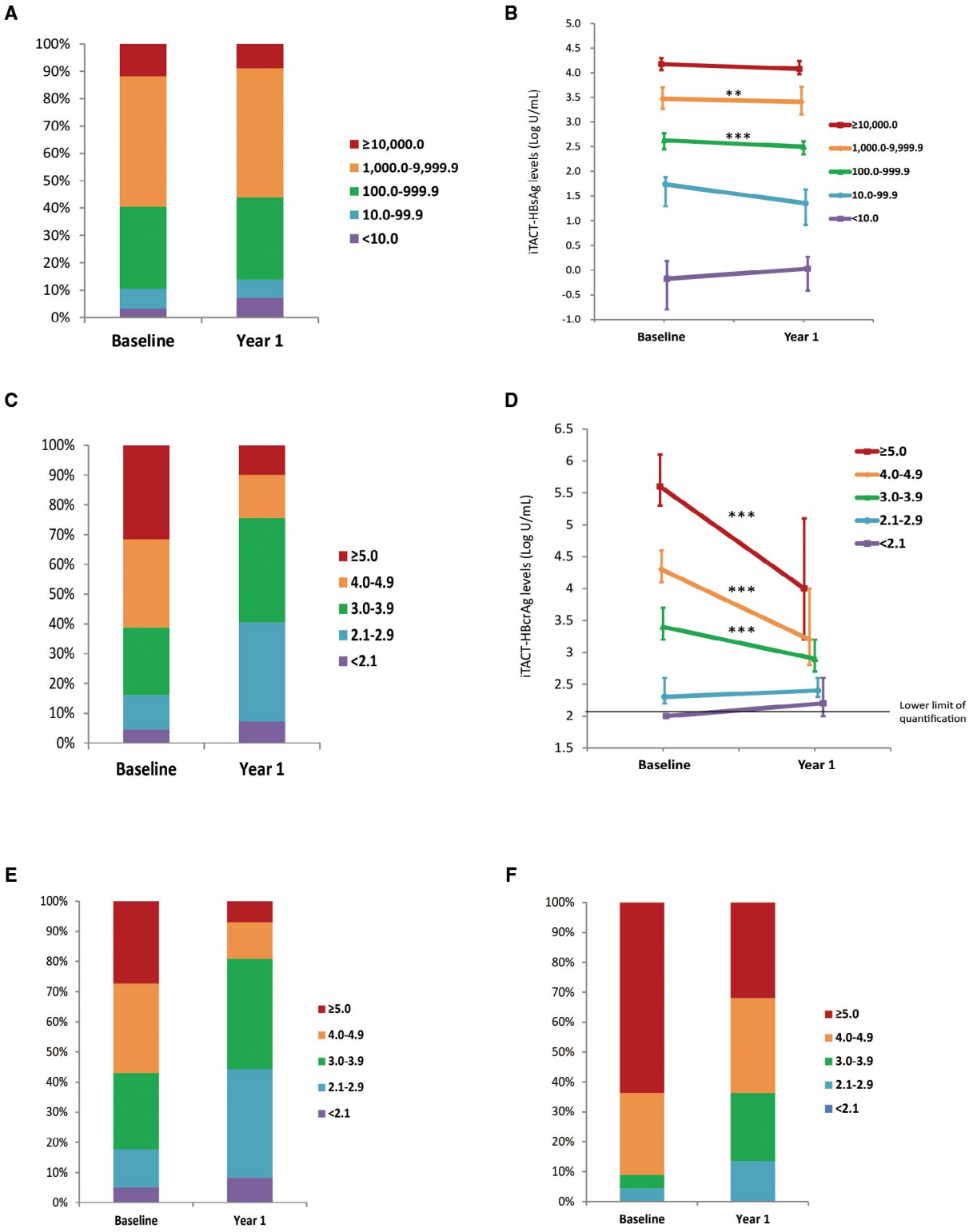



**FIG. 1.** Changes in iTACT-HBsAg and iTACT-HBcrAg from baseline to year 1. (A) Distribution of iTACT-HBsAg at baseline and year 1 in the entire cohort. (B) Kinetics of iTACT-HBsAg from baseline to year 1 by baseline iTACT-HBsAg levels. Data show median (25th to 75th percentile). (C) Distribution of iTACT-HBcrAg at baseline and year 1 in the entire cohort. (D) Kinetics of iTACT-HBcrAg from baseline to year 1 by baseline iTACT-HBsAg levels. Data show median (25th to 75th percentile). (E) Distribution of iTACT-HBcrAg at baseline and year 1 in patients who did not develop HCC (n = 158). (F) Distribution of iTACT-HBcrAg at baseline and year 1 in patients who developed HCC (n = 22). $^{**}P < 0.01$; $^{***}P < 0.001$.

## OTHER HBV MARKERS

HBV DNA was quantified using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of >2.6-7.6 log copies/mL, or the COBAS TaqMan HBV Test (version 2.0; Roche Diagnostics), which has a dynamic range of >2.1-9.0 log copies/mL. HBV DNA levels were converted from log copies/mL to log IU/mL according to the manufacturer and JSH recommendations. HBeAg status was determined using a commercially available enzyme immunoassay (EIA) kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV genotypes were also serologically determined using a commercial kit (HBV Genotype EIA; Institute of Immunology) that detected the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the eight major genotypes (A-H).

## STATISTICAL ANALYSIS

Categorical data were compared using the chi-squared or Fisher's exact test. Continuous variables with a non-normal distribution were analyzed with the Mann-Whitney U test, while those with a normal distribution were analyzed with Student *t* test. Patients with the following events were censored: loss to follow-up, discontinuation of NA, or death before HCC development. Time-dependent receiver operating characteristic (ROC) curves were used to predict the incidence of HCC until year 5 or year 10 using Kaplan-Meier estimates. DeLong's test was used to compare two ROC curves. The optimal cut-off values were determined using the Youden index (sensitivity plus specificity minus one). We calculated the metrics for predicting HCC incidence, including sensitivity, specificity, positive predictive value, negative predictive value, and accuracy when the optimal cut-off values were applied. The cumulative HCC rates were analyzed using the Kaplan-Meier method, and differences in the resulting curves were evaluated using log-rank tests. Cox regression analyses were used to assess variables that were significantly associated with the development of HCC. A multivariate Cox proportional-hazards regression was used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for HCC development after controlling for potential predictors of HCC. The multivariate models included variables that exhibited significant associations ($P < 0.05$) with HCC incidence in the univariate analysis. Because the number of events was relatively small in this study, we also analyzed the multivariate models with the inclusion of two variables that were associated with HCC in the univariate analysis in order to avoid overfitting the model. Significance level was defined as $P < 0.05$ for all two-tailed tests. Data analyses were performed using SPSS software (version 25.0; IBM Corp., Armonk, NY) and R software (version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org).

# Results

## PATIENT CHARACTERISTICS

Baseline characteristics and demographics of the patients are shown in Table 1. During a median follow-up of 11.0 years, 22 patients developed HCC (11.8/1,000 person-years). There were significant differences in some baseline characteristics between HCC and non-HCC cases (Table 1). A total of 171 patients (95%) achieved virologic response at year 1, which was defined as a value below the lower limit of quantification (<1.3 log IU/mL). No patients experienced virologic breakthrough during ETV treatment in the entire study cohort.

## iTACT-HBcrAg AND iTACT-HBsAg LEVELS

The median baseline iTACT-HBcrAg and iTACT-HBsAg levels were 4.2 log U/mL (interquartile range [IQR], 3.3-5.1) and 1,752.62 IU/mL (IQR, 417.84-4,876.15), respectively (Table 1). Twenty-one patients (11.7%) were categorized into ≥10,000.0 IU/mL, 86 patients (47.8%) into 1,000.0-9,999.9, 54 patients (30.0%) into 100.0-999.9, 13 patients (7.2%) into 10.0-99.9, and 6 patients (3.3%) into <10.0 of baseline iTACT-HBsAg (Table 1; Fig. 1A). There were no differences in baseline iTACT-HBsAg levels between the HCC and non-HCC cases (Table 1; Fig. 1A). Few changes in iTACT-HBsAg were observed from baseline to year 1 (Fig. 1A,B).

Fifty-seven patients (31.7%) were categorized into ≥5.0 log U/mL, 53 patients (29.4%) into 4.0-4.9, 41 patients (22.8%) into 3.0-3.9, 21 patients (11.7%) into

**TABLE 1. BASELINE CHARACTERISTICS**

| Baseline characteristics | All (N = 180) | Non-HCC (n = 158) | HCC (n = 22) | *P* Value |
|---|---|---|---|---|
| Age (years) | 51 ± 9.90 | 51 ± 9.86 | 53 ± 10.12 | 0.220 |
| Sex (male) | 111 (61.7%) | 96 (60.4%) | 15 (71.4%) | 0.350 |
| Preexisting cirrhosis | 48 (26.7%) | 35 (22.2%) | 13 (59.1%) | <0.001 |
| HBV genotype (A:B:C:D:unclassified/missing) | 8:51:114:1:6 | 5:47:99:1:4 | 3:4:15:0:0 | 0.171 |
| HBV DNA (log IU/mL) | 4.8 (3.5-5.6) | 4.8 (3.2-5.5) | 5.4 (4.4-6.3) | 0.033 |
| iTACT-HBsAg (IU/mL) | 1,752.62 (417.84-4,876.15) | 1,545.84 (369.05-4,749.25) | 2,795.30 (1,041.99-5,305.13) | 0.199 |
| ≥10,000.0 | 21 (11.7%) | 19 (12.0%) | 2 (9.1%) | |
| 1,000.0-9,999.9 | 86 (47.8%) | 71 (44.9%) | 15 (68.2%) | |
| 100.0-999.9 | 54 (30.0%) | 50 (31.6%) | 4 (18.2%) | |
| 10.0-99.9 | 13 (7.2%) | 12 (7.6%) | 1 (4.5%) | |
| <10.0 | 6 (3.3%) | 6 (3.8%) | 0 (0%) | |
| iTACT-HBcrAg (log U/mL) | 4.2 (3.3-5.1) | 4.1 (3.2-5.0) | 5.4 (4.9-5.7) | <0.001 |
| ≥5.0 | 57 (31.7%) | 43 (27.2%) | 14 (63.6%) | |
| 4.0-4.9 | 53 (29.4%) | 47 (29.7%) | 6 (27.3%) | |
| 3.0-3.9 | 41 (22.8%) | 40 (25.3%) | 1 (4.5%) | |
| 2.1-2.9 | 21 (11.7%) | 20 (12.7%) | 1 (4.5%) | |
| <2.1 (not detected) | 8 (4.4%) | 8 (5.1%) | 0 (0%) | |
| AST (IU/L) | 42 (29-69) | 42 (29-68) | 43 (33-71) | 0.573 |
| ALT (IU/L) | 51 (31-98) | 50 (32-108) | 55 (31-86) | 0.846 |
| GGT (IU/L) | 30 (19-65) | 30 (19-61) | 40 (21-90) | 0.107 |
| Serum albumin (g/L) | 3.8 (3.5-4.0) | 3.9 (3.8-4.1) | 3.8 (3.5-4.0) | 0.009 |
| Platelet ($10^5$/$mm^3$) | 17.0 ± 5.48 | 17.5 ± 5.31 | 13.6 ± 5.57 | 0.001 |
| AFP (ng/dL) | 4 (3-6) | 4 (2-5) | 7 (4-22) | <0.001 |

All values are expressed as mean ± SD, median (IQR, twenty-fifth to seventy-fifth percentile), number (percentage of total), or number.
Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

2.1-2.9, and 8 patients (4.4%) into <2.1 (not detected) of baseline iTACT-HBcrAg (Table 1; Fig. 1C). iTACT-HBcrAg levels significantly declined in patients with ≥3.0 log U/mL of baseline iTACT-HBcrAg (Fig. 1D). In 110 patients with baseline high iTACT-HBcrAg (≥4.0 log U/mL), HBcrAg declined to ≤2.9 a log U/mL at year 1 in 25 patients (22.7%) and to 3.0-3.9 log U/mL in 41 patients (37.3%). HBcrAg declined slower in patients who developed HCC than in those who did not (Fig. 1E,F).

## PREDICTIVE CAPABILITIES OF HCC

The time-dependent areas under the ROC curves of baseline iTACT-HBcrAg for discriminating the 5- and 10-year incidence of HCC were 0.702 (95% CI, 0.594-0.811) and 0.700 (95% CI, 0.588-0.813), respectively; those of baseline iTACT-HBsAg were 0.551 (95% CI, 0.414-0.688) and 0.551 (95% CI, 0.431-0.672), respectively; and those of baseline HBV DNA were 0.660 (95% CI, 0.522-0.798) and 0.618 (95% CI, 0.486-0.750), respectively (Fig. 2A,C). The AUROCs of on-treatment HBcrAg at 1 year for the 5- and 10-year incidence of HCC were 0.664 (95% CI, 0.536-0.791) and 0.742 (95% CI, 0.622-0.862), respectively; those of iTACT-HBsAg at year 1 were 0.555 (95% CI, 0.427-0.683) and 0.571 (95% CI, 0.455-0.688), respectively (Fig. 2B,D). Baseline and on-treatment iTACT-HBcrAg had a better predictive capability for the 10-year incidence of HCC than iTACT-HBsAg ($P < 0.05$ for both).

Next, we calculated the optimal cut-off values of baseline and on-treatment iTACT-HBcrAg for predicting the 5- and 10-year incidence of HCC using the ROC curves as described above. The optimal cut-off values of baseline iTACT-HBcrAg for predicting the 5- and 10-year incidence of HCC were 4.4 log U/mL and 4.7, and those of iTACT-HBcrAg at year 1 were 2.8 and 4.0, respectively (Supporting

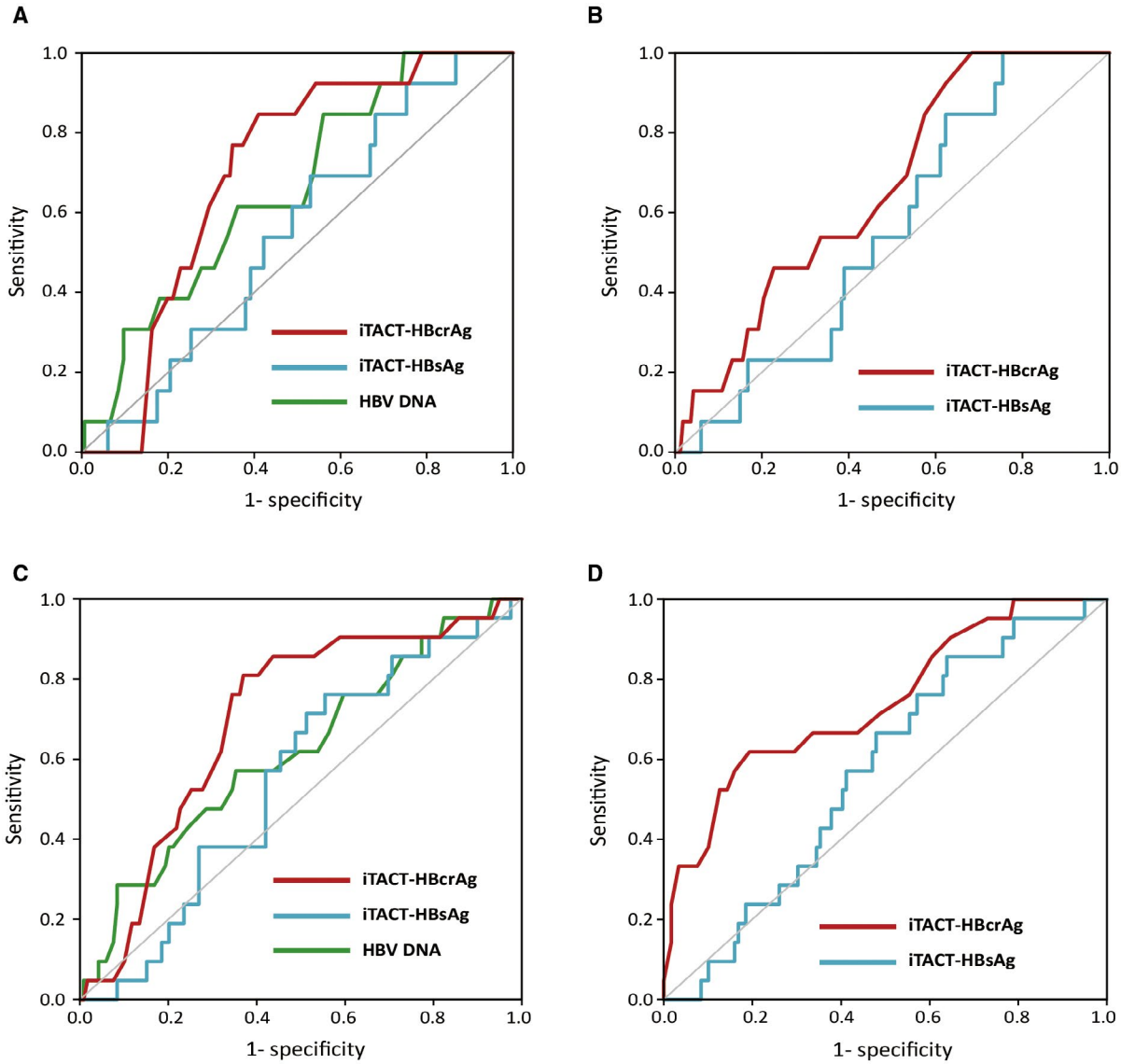
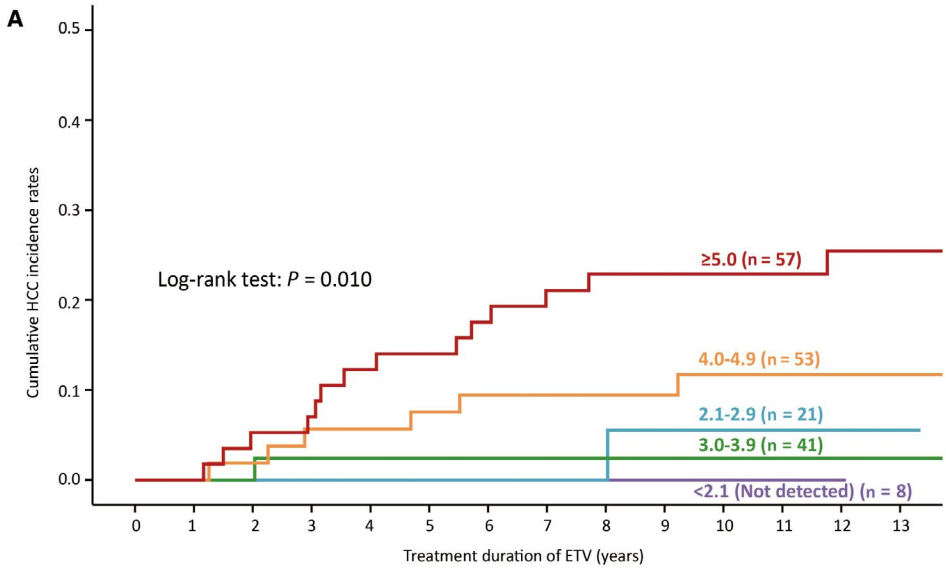



**FIG. 2.** ROC curves for predicting HCC incidence. (A) ROC curves of baseline values for predicting HCC incidence in 5 years. (B) ROC curves of baseline values for predicting HCC incidence in 10 years. (C) ROC curves of values at year 1 for predicting HCC incidence in 5 years. (D) ROC curves of values at year 1 for predicting HCC incidence in 10 years.

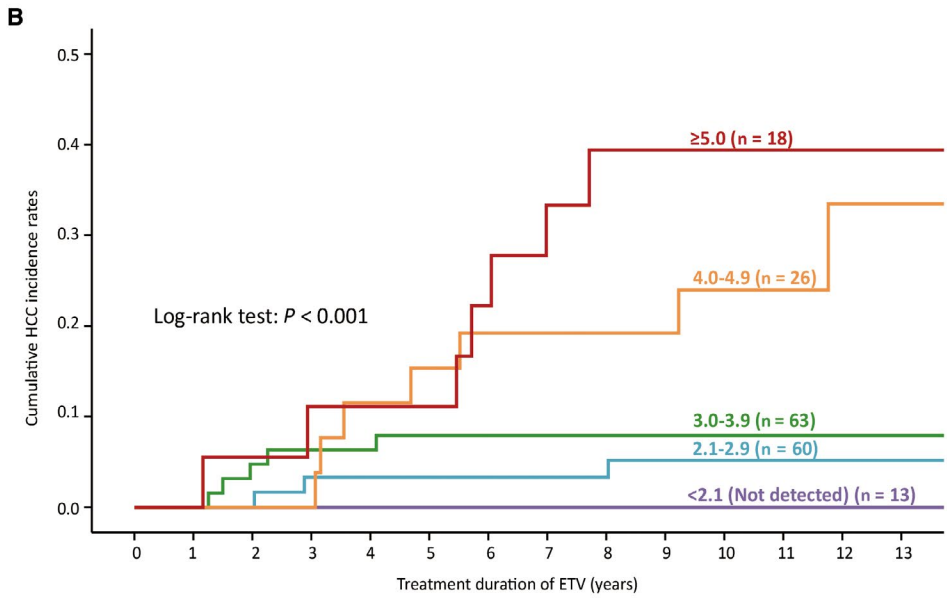
Table S1). The metrics of the optimal cut-off values in iTACT-HBcrAg for predicting HCC incidence are shown in Supporting Table S1. The accuracy of iTACT-HBcrAg at year 1 (cutoff, 4.0 log U/mL) for predicting 10-year incidence of HCC was 0.779. This was better than that of the baseline cutoff.

## FACTORS ASSOCIATED WITH HCC

Patients with high iTACT-HBcrAg levels at baseline or year 1 were likely to develop HCC in a level-dependent manner (Fig. 3A,B). Cumulative incidences of HCC by iTACT-HBcrAg levels with or without cirrhosis are shown in Supporting Fig. S2. Nine patients without cirrhosis and 13 patients with cirrhosis developed HCC. Patients without cirrhosis with high iTACT-HBcrAg levels at baseline or year 1 were likely to develop HCC in a level-dependent manner as well as overall results shown in Fig. 3A,B (Supporting Fig. S2A,C). In particular, significant differences were observed using log-rank tests stratified by iTACT-HBcrAg levels at year 1 (Supporting Fig. S2C).




| No. at risk | Baseline | Year 1 | Year 2 | Year 3 | Year 4 | Year 5 | Year 6 | Year 7 | Year 8 | Year 9 | Year 10 | Year 11 | Year 12 | Year 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ≥5.0 | 57 | 57 | 54 | 53 | 50 | 49 | 47 | 45 | 42 | 40 | 38 | 35 | 24 | 16 |
| 4.0-4.9 | 53 | 53 | 52 | 50 | 50 | 49 | 48 | 48 | 44 | 41 | 38 | 28 | 14 | 6 |
| 3.0-3.9 | 41 | 41 | 41 | 40 | 40 | 40 | 40 | 39 | 38 | 36 | 31 | 21 | 15 | 9 |
| 2.1-2.9 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 18 | 12 | 9 | 5 | 2 | 1 |
| <2.1 (Not detected) | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 5 | 5 | 3 | 1 | 1 | 0 |




| No. at risk | Year 1 | Year 2 | Year 3 | Year 4 | Year 5 | Year 6 | Year 7 | Year 8 | Year 9 | Year 10 | Year 11 | Year 12 | Year 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ≥5.0 | 18 | 17 | 16 | 16 | 16 | 14 | 12 | 10 | 9 | 8 | 7 | 4 | 2 |
| 4.0-4.9 | 26 | 26 | 26 | 23 | 22 | 21 | 21 | 19 | 17 | 15 | 13 | 6 | 5 |
| 3.0-3.9 | 63 | 60 | 59 | 59 | 58 | 58 | 58 | 53 | 52 | 49 | 37 | 23 | 11 |
| 2.1-2.9 | 60 | 60 | 58 | 58 | 58 | 58 | 57 | 52 | 44 | 37 | 26 | 17 | 12 |
| <2.1 (Not detected) | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 12 | 10 | 7 | 6 | 1 |

**FIG. 3.** Cumulative HCC incidence rates using Kaplan-Meier curves. (A) HCC incidence rates by baseline iTACT-HBcrAg levels. (B) HCC incidence rates by iTACT-HBcrAg levels at year 1.

**TABLE 2. FACTORS ASSOCIATED WITH HCC INCIDENCE DURING ETV TREATMENT USING UNIVARIATE AND MULTIVARIATE COX REGRESSION**

| Models | HR (95% CI) | *P* Value |
|---|---|---|
| Unadjusted | | |
| Baseline HBV DNA (per 1 log IU/mL increase) | 1.40 (1.03-1.90) | 0.034 |
| Baseline iTACT-HBcrAg (per 1 log U/mL increase) | 1.74 (1.25-2.43) | 0.001 |
| On-treatment iTACT-HBcrAg at year 1 (per 1 log U/mL increase) | 2.30 (1.56-3.37) | <0.001 |
| Adjusted for baseline iTACT-HBcrAg | | |
| On-treatment iTACT-HBcrAg at year 1 (per 1 log U/mL increase) | 1.96 (1.19-3.22) | 0.008 |
| Adjusted for baseline HBV DNA | | |
| On-treatment iTACT-HBcrAg at year 1 (per 1 log U/mL increase) | 2.17 (1.46-3.24) | <0.001 |
| Adjusted for cirrhosis | | |
| On-treatment iTACT-HBcrAg at year 1 (per 1 log U/mL increase) | 1.93 (1.25-2.97) | 0.003 |
| Adjusted for platelet | | |
| On-treatment iTACT-HBcrAg at year 1 (per 1 log U/mL increase) | 2.05 (1.38-3.06) | <0.001 |
| Adjusted for GGT | | |
| On-treatment iTACT-HBcrAg at year 1 (per 1 log U/mL increase) | 2.22 (1.51-3.25) | <0.001 |
| Adjusted for albumin | | |
| On-treatment iTACT-HBcrAg at year 1 (per 1 log U/mL increase) | 2.15 (1.47-3.14) | <0.001 |
| Adjusted for all variables associated with HCC in the univariate analysis* | | |
| On-treatment iTACT-HBcrAg at year 1 (per 1 log U/mL increase) | 1.70 (1.09-2.66) | 0.020 |

*Adjusted for HBV DNA, cirrhosis, GGT, albumin, and platelets.

In patients with cirrhosis, a similar trend was observed, but there were no significant differences because of a smaller sample size. Baseline factors associated with HCC incidence, as identified in the univariate analysis, are shown in Supporting Table S2. Baseline iTACT-HBcrAg, HBV DNA, and on-treatment HBcrAg at year 1 were associated with HCC in the univariate analysis (Table 2). We then used multivariate Cox regression analysis to estimate the risk of developing HCC after adjusting for multiple baseline variables and HBV markers identified as significant in the univariate analysis. Multivariate analysis showed that on-treatment iTACT-HBcrAg at year 1 was significantly associated with HCC after adjustment for baseline HBcrAg, HBV DNA, preexisting cirrhosis, gamma-glutamyltransferase (GGT), albumin, and platelet count (Table 2). In addition, on-treatment iTACT-HBcrAg was associated with HCC with adjustment for multiple baseline characteristics identified as significant in the univariate analysis, including preexisting cirrhosis, GGT, albumin, platelet count, and HBV DNA (Table 2).

We also conducted multivariate analysis as a subanalysis using the two optimal cut-off values of on-treatment iTACT-HBcrAg as calculated above (2.8 log U/mL and 4.0) (Supporting Tables S3 and S4). In particular, the results using 4.0 as the cutoff were similar to the main analysis (Supporting Table S4).

## REDUCTION IN iTACT-HBcrAg AFFECTS HCC INCIDENCE

To analyze the impact of a reduction in iTACT-HBcrAg on HCC development, we stratified the entire cohort into three groups as follows: the High→High group comprised patients with a persistently high HBcrAg level (≥4.0 log U/mL); the High→Low group comprised those with a high baseline (≥4.0 log U/mL) and low on-treatment HBcrAg level (<4.0 log U/mL); and the Low→Low group comprised those with persistently low HBcrAg levels (<4.0 log U/mL). The HCC incidence rates were significantly higher in the High→High group than in the other two groups ($P < 0.001$; Fig. 4A). We also analyzed these data using a Cox regression model after adjusting for multiple baseline variables and including the above three groups. We found that patients in the High→Low and Low→Low groups were less likely to develop HCC compared to those in the High→High group (HR [High→Low], 0.32; 95% CI, 0.11-0.92; $P = 0.034$; HR [Low→Low], 0.17; 95% CI, 0.03-0.89; $P = 0.036$) (Tables 3 and 4).

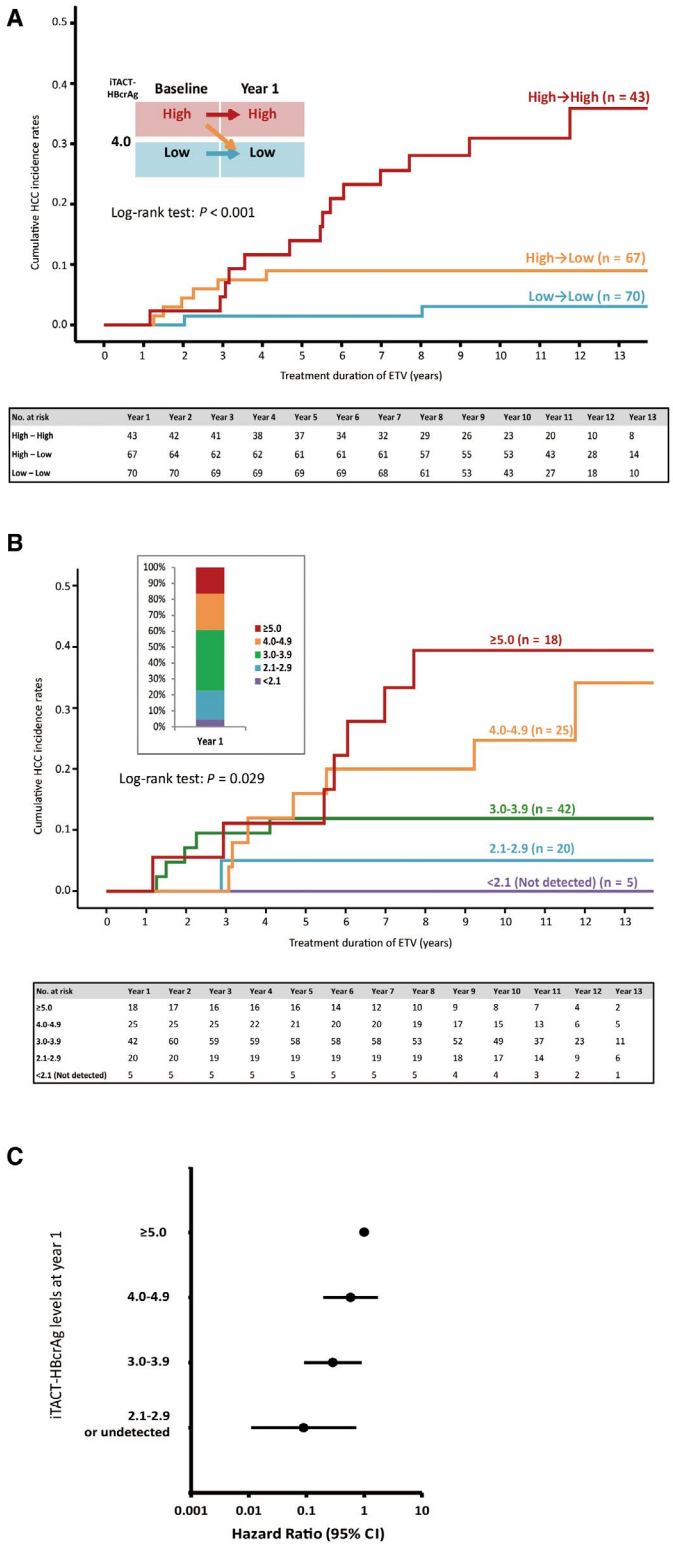



**FIG. 4.** HCC incidence rates and iTACT-HBcrAg levels. (A) Cumulative HCC incidence rates using Kaplan-Meier curves for three subgroups stratified by the reduction in iTACT-HBcrAg from baseline to year 1. (B) Distribution of iTACT-HBcrAg at year 1 in patients with high baseline HBcrAg levels (≥4.0 log U/mL) and cumulative HCC incidence rates using Kaplan-Meier curves by iTACT-HBcrAg levels at year 1 in patients with high baseline HBcrAg. (C) HRs (95% CIs) of HCC by iTACT-HBcrAg levels at year 1 using univariate Cox regression in patients with high baseline HBcrAg when the reference was set at HBcrAg ≥5.0 log U/mL.

**TABLE 3. HAZARD RATIO FOR HCC INCIDENCE IN THE THREE SUBGROUPS STRATIFIED BY REDUCTION IN iTACT-HBcrAg FROM BASELINE TO YEAR 1 USING UNIVARIATE AND MULTIVARIATE COX REGRESSION**

| Models | Categories | HR (95% CI) | *P* Value |
|---|---|---|---|
| Unadjusted | High→High | 1 (reference) | — |
| | High→Low | 0.25 (0.10-0.66) | 0.005 |
| | Low→Low | 0.08 (0.02-0.35) | 0.001 |
| Adjusted for cirrhosis | High→High | 1 (reference) | — |
| | High→Low | 0.37 (0.13-1.06) | 0.065 |
| | Low→Low | 0.12 (0.02-0.54) | 0.006 |
| Adjusted for platelet | High→High | 1 (reference) | — |
| | High→Low | 0.33 (0.12-0.86) | 0.024 |
| | Low→Low | 0.11 (0.02-0.47) | 0.003 |
| Adjusted for GGT | High→High | 1 (reference) | — |
| | High→Low | 0.26 (0.10-0.66) | 0.005 |
| | Low→Low | 0.11 (0.02-0.37) | 0.001 |
| Adjusted for albumin | High→High | 1 (reference) | — |
| | High→Low | 0.24 (0.09-0.62) | 0.003 |
| | Low→Low | 0.08 (0.02-0.36) | 0.001 |
| Adjusted for all variables associated with HCC in the univariate analysis* | High→High | 1 (reference) | — |
| | High→Low | 0.32 (0.11-0.92) | 0.034 |
| | Low→Low | 0.17 (0.03-0.89) | 0.036 |

*Adjusted for HBV DNA, cirrhosis, GGT, albumin, and platelets.

**TABLE 4. HRS OF iTACT-HBcrAg AT YEAR 1 FOR HCC INCIDENCE USING UNIVARIATE AND MULTIVARIATE COX REGRESSION IN PATIENTS WITH HIGH BASELINE HBCRAG LEVELS (≥4.0 LOG U/ML)**

| Models | HR (95% CI) | *P* Value |
|---|---|---|
| Unadjusted (per 1 log U/mL increase) | 1.96 (1.26-3.07) | 0.003 |
| Adjusted for platelet | 1.72 (1.07-2.74) | 0.024 |
| Adjusted for cirrhosis | 1.69 (1.02-2.77) | 0.041 |
| Adjusted for GGT | 1.90 (1.22-2.95) | 0.004 |

Finally, we evaluated whether a reduction in iTACT-HBcrAg had an impact on HCC incidence in 110 patients with baseline high HBcrAg (≥4.0 log U/mL) who had a high risk of HCC. In these 110 patients, iTACT-HBcrAg changed to <2.1 at year 1 in 5 (4.5%), 2.1-2.9 in 20 (18.2%), 3.0-3.9 in 42 (38.2%), 4.0-4.9 in 25 (22.7%), and ≥5.0 log U/mL in 18 (16.4%) patients (Fig. 4B). Low iTACT-HBcrAg in patients was associated with a lower cumulative HCC incidence rate ($P$ = 0.029; Fig. 4B). The univariate hazard ratios of HCC were 0.01 (95% CI, 0.01-0.71) in patients with HBcrAg ≤2.9 log U/mL at year 1, 0.28 (95% CI, 0.09-0.89) with HBcrAg 3.0-3.9, and 0.68 (95% CI, 0.24-1.94) with HBcrAg 4.0-4.9, when the reference was set at HBcrAg ≥5.0 log U/mL at year 1 (Fig. 4C). Multivariate Cox regression showed that iTACT-HBcrAg levels at year 1 were significantly associated with HCC incidence with adjustments for baseline variables, including preexisting cirrhosis, GGT, albumin, and platelet count, in 110 patients with baseline high HBcrAg (≥4.0 log U/mL) (HR, 1.64; 95% CI, 1.01-2.66; $P$ = 0.046) (Tables 3 and 4).

# Discussion

Our study showed that iTACT-HBcrAg had a better potential for predicting HCC in patients who were HBeAg negative during ETV treatment than the current HBcrAg assay. In particular, iTACT-HBcrAg levels at year 1 after starting ETV exhibited a stronger association with the development of HCC than with the baseline HBcrAg level. Although cirrhotic status was a common risk factor of HCC, similar results were observed even in patients without cirrhosis (Supporting Fig. S2C). A lower on-treatment iTACT-HBcrAg was associated with a lower risk of HCC. Patients with undetected on-treatment iTACT-HBcrAg were unlikely to develop HCC during ETV treatment. Although this finding was similar to our previous work, there was a concern

about the lower sensitivity of the current HBcrAg assay.[15] In this study, the new iTACT-HBcrAg assay could overcome this concern and stratify the risk of HCC during NA treatment with greater ability. On the other hand, baseline iTACT-HBsAg, which is a new and ultrasensitive assay, was not associated with HCC development.

HBcAg, HBeAg, and 22-kDa precore protein (p22cr) antigens are encapsulated in HBV complete virions (Dane particles) and HBV incomplete particles (hollow particles) in the bloodstream. The advantage of the HBcrAg assay is that these three types of HBV core proteins can simultaneously be measured by denaturing the various antigens form immune complexes with endogenous antibodies, which are underestimated in other assays using the sample treatment solution.[19,20] Results from one study showed there was a good correlation between HBV cccDNA and HBcrAg levels in any group of untreated patients with CHB, with or without HBeAg, and HBcrAg is thought to reflect the viral load of HBV in liver tissue.[21] HBcrAg also reflects the transcriptional activity of HBV.[9] Therefore, the longitudinal kinetics of HBcrAg can show the antiviral effects of NAs independent of HBV DNA during NA treatment.[12, 20] The kinetics of HBcrAg during NA treatment vary widely among individuals. In this study, three types of HBcrAg kinetics were observed, as shown in Fig. 4A. There were more patients for whom HBcrAg at year 1 could be quantified with the iTACT cutoff (2.1 log U/mL) than with the current cutoff (3.0 log U/mL) (92.8% vs. 59.5%) (Fig. 1C). Most patients with low levels of iTACT-HBcrAg (<3.0 log U/mL) had quantifiable iTACT-HBcrAg. HCC risk was reported to become higher in a level-dependent manner of the conventional HBcrAg.[11] Therefore, we stratified iTACT-HBcrAg levels by per 1.0 log in order to evaluate the HCC risk of patients with each iTACT-HBcrAg range. Consequently, we could stratify the risk of HCC during ETV treatment in an HBcrAg level-dependent manner in the present study compared to our previous study. The iTACT-HBcrAg achieved higher sensitivity than the current HBcrAg assay by changing the measurement system and optimizing sample pretreatment conditions and reagent composition conditions. The supersensitization of HBcrAg will be more useful for evaluating the antiviral effects and HCC risk than the current assay. The results of this study require validation by future studies.

We could quantify the low levels of HBcrAg using iTACT assay even under ETV or other NA treatment. Actually, only 7.2% of patients had undetectable iTACT-HBcrAg at year 1 (Fig. 1C). A recent report also showed that 97.5% of patients treated with NA had detectable iTACT-HBcrAg (≥2.1 log U/mL) at their last visit.[22] Quantifying HBcrAg levels in most patients is the strength of iTACT-HBcrAg measurement for prediction of HCC development in patients who received ETV or other NA. According to time-dependent ROC curve analysis in this study, the two optimal cut-off values of iTACT-HBcrAg at year 1 were 2.8 log U/mL for the 5-year incidence of HCC and 4.0 for the 10-year incidence, respectively (Supporting Table S1). Therefore, HBcrAg of 4.0 log U/mL was still a good cutoff for predicting HCC incidence, as shown for the untreated cohort.[11] However, we might be able to stratify the detailed HCC risks using the iTACT-HBcrAg assay if future studies with a larger population and more time points could be conducted.

The novel finding of this study was that the risk of HCC decreased if on-treatment iTACT-HBcrAg levels decreased (Fig. 4). This finding was not fully observed by the current HBcrAg assay in a previous study.[12] Monitoring on-treatment iTACT-HBcrAg can be more useful for predicting HCC development during NA treatment than the current HBcrAg assay. No patients with undetected iTACT-HBcrAg developed HCC in this study, and such patients may have a very low risk of HCC. However, the number of patients with undetected iTACT-HBcrAg was small. It will be important to evaluate in the future whether the prevalence of patients with undetected iTACT-HBcrAg increases over 1 year after NA treatment.

Although iTACT-HBcrAg was associated with HCC development during ETV treatment, iTACT-HBsAg was not associated with HCC. This result was similar to that of our previous report in patients who were HBeAg negative. The new feature of the principle of iTACT-HBsAg measurement is the inactivation of patient-oriented hepatitis B surface antibody by acid pretreatment; this is in contrast to the current HBsAg assay (Lumipulse HBsAg-HQ by Fujirebio).[16] There was a strong correlation between the levels of iTACT-HBsAg and the current HBsAg assay in the relatively high HBsAg zone.[16] More than half of the patients in this study had iTACT-HBsAg levels of 1,000 IU/mL or more. This may explain why iTACT-HBsAg was

not associated with HCC. The sensitivity of iTACT-HBsAg is about 100 times higher than that of conventional HBsAg assays and 10 times higher than that of the Lumipulse HBsAg-HQ assay. Recently, it was reported that residual low HBsAg by the iTACT-HBsAg assay might predict HCC development even if HBsAg seroclearance was achieved according to a conventional assay.[17] The ultrasensitive assay of HBsAg will be helpful for monitoring HBsAg seroclearance and HBV reactivation other than on-treatment monitoring.

Some limitations to this study should be noted. First, this was a retrospective cohort study conducted at a single institution, and iTACT-HBcrAg was not measured continuously over a long period. Therefore, larger studies with larger sample sizes and more time points are needed in the future to confirm the findings which were observed in this study. Second, the study population and number of events were relatively small. This is because we only had a short period to obtain written informed consent from study participants. Third, a virologic evaluation of liver tissues was not conducted. There are no studies on the correlation between iTACT assays and HBV in liver tissues; this needs to be evaluated in future studies. Fourth, iTACT-HBcrAg and iTACT-HBsAg assays could be measured only during research. It is necessary to widely evaluate and validate this iTACT assay under various conditions in future research and real-life settings. Finally, age and sex, which are well-known risk factors of HCC, were not associated with HCC. Regarding sex, our previous study also showed that sex was not associated with HCC in patients who were HBeAg negative.[12] There were no differences in patients' characteristics and HBV markers between sexes in the present study, and this issue needs further evaluation. Regarding age, our previous study showed that age was associated with HCC even in patients who were HBeAg negative.[12] The number of events and population were lower in the present study than the previous study, and this might be attributed to the lower sample size. In addition, the multivariate analysis adjusted for age or sex was similar to the main analysis (data not shown).

In conclusion, the present study indicated that the measurement of HBcrAg by an ultrasensitive assay has better potential for predicting HCC in patients who are HBeAg negative during ETV treatment. HBcrAg levels were quantified below the cutoff of the current HBcrAg assay in some patients. The risk of HCC decreased in a level-dependent manner with regard to on-treatment HBcrAg. The measurement of HBcrAg by the ultrasensitive assay will be helpful for managing patients with HBV infection.

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# Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1819/suppinfo.