Retrospective cohort study (April 2000 – March 2023)

Screening of non-HBV HCC patients

+

Inclusion Criteria
Patients with complete clinical information and preserved serum for hHBsAg+ testing

Analysis

- Prognosis impact of pHBV and hHBsAg+ in non-HBV HCC
- Overall survival (OS)

*non-HBV HCC, non- hepatitis-B virus related hepatocellular carcinoma; pHBV, previous HBV infection; hHBsAg+, high sensitivity hepatitis B surface antigen; AFP, alpha fetoprotein

Non-HBV HCC (N=401)

With pHBV (n=288)

Group 1

Without pHBV (n=113)

Group 2

pHBV without hHBsAg+ (n=254)

Group 3

pHBV with hHBsAg+ (n=34)

More advanced HCC stage

High levels of serum AFP

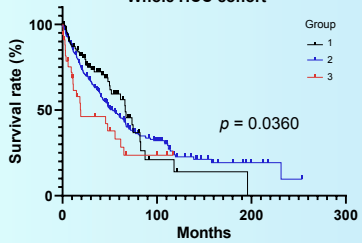
High vascular invasion

Short OS

Results

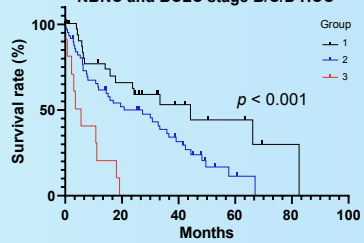
Comparison of OS in three groups

Whole HCC cohort



Group	Previous HBV infection	High sensitivity HBsAg	Median survival
1	(−)	(−)	66.4 (50.2 – 82.1)
2	(+)	(−)	56.6 (43.4 – 67.2)
3	(+)	(+)	19.3 (10.0 – 61.5)

NBNC and BCLC stage B/C/D HCC



Group	Numbers at risk					Median survival
1	37	18	8	4	1	44.5 (16.2 – NA)
2	57	26	13	2	0	20.9 (11.9 – 36.2)
3	11	0	0	0	0	5.9 (0.9 – 11.4)

- In the whole cohort, multivariant Cox regression analysis revealed that the median OS for patients with pHBV and hHBsAg+ was significantly associated with shorter OS (HR, 2.954; 95% CI, 1.394 – 6.264; $p = 0.005$)
- In patients with NBNC etiology HCC and BCLC stage B/C/D, multivariant cox regression analysis revealed that median OS for patients with pHBV and hHBsAg+ was significantly associated with shorter OS (HR, 7.43; 95% CI, 2.95 – 17.83; $p < 0.001$)
- Presence of hHBsAg+ and pHBV infection is strongly and independently connected to poor prognosis for patients with non-HBV HCC

Positivity of High-Sensitivity HBsAg Test, not Previous HBV Infection, Indicates Poor Prognosis in Patients with Non-HBV-Related HCC

Word count (excluding summary): 2803 (main text)

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Ethics approval and informed consent

The ethics committee of Hokkaido University Hospital confirmed that the study protocol (IRB number: 019-0400) conformed to the ethical guidelines of the Declaration of Helsinki. Patients who provided written informed consent or did not decline to participate were included in the study. The ethics committee specifically approved the inclusion of patients who did not decline

to participate in the study instead of obtaining written informed consent from the included patients.

Data availability statement All data generated or analysed during this study are included in this article. Further inquiries can be directed to the corresponding author.

Funding

This study was supported in part by grants from the Japan Agency for Medical Research and Development (AMED; Grant Nos. JP24fk0210126, JP24fk0310501, JP24fk0210121, JP24fk0210112, JP24fk0210142, JP24fk0210111, JP24fk0310524, JP24fk0210123, JP24fk0210157, JP24fk0310518, JP24fk0210103, JP24fk0210104, JP24fk0210113, and JP24fk0210143).

Disclosure and declarations

Professor Naoya Sakamoto received lecture fees from Bristol Myers Squibb and Pharmaceutical K.K, grants and endowments from MSD K.K and Chugai Pharmaceutical Co., Ltd, and a research grant from Gilead Sciences, Inc. and AbbVie Inc. Dr. Goki Suda received

research grants from Bristol Myers Squibb, MSD K.K, and Gilead Sciences. The other authors have no conflicts of interest to disclose.

Author contributions

Yasuura and Suda designed the study. Yasuura, Suda and Ohara performed the statistical analyses. Yasuura, Ohara, and Suda wrote and revised the manuscript. Yasuura, Suda, Ohara, Meno, Sho, Kohya, Sasaki, Yoda, Yoshida, Fu, Yang, Hosoda, Maehara, Ohnishi, Baba, Kitagataya, Kawagishi, Nakai, Saitou, Natsuizaka, and Ogawa collected the data. Taketomi, Fukuhara, Sugiyama, and Sakamoto provided hepatological advice and edited the manuscript. Sakamoto revised the manuscript for important intellectual content.

Abstract

Background and Aims: The prognostic impact of previous-HBV-infection (pHBV) in non-HBV-related hepatocellular carcinoma (non-HBV-related-HCC) and the prevalence, characteristics, and significance of recently developed high-sensitivity HBs antigen positivity (hHBsAg+) in these patients remain unclear. We aimed to close these gaps.

Methods: We retrospectively screened patients with newly diagnosed non-HBV-related-HCC (standard HBsAg-test negative) at Hokkaido University. Patients with complete clinical information and preserved serum for hHBsAg+ were included. We evaluated the prevalence, characteristics, and prognostic impact of pHBV and hHBsAg+ in non-HBV-related-HCC.

Results: Four hundred one non-HBV-related-HCC patients were included (288 with pHBV/113 without pHBV). In non-HBV-related-HCC, pHBV did not affect overall survival (OS). Among non-HBV-related-HCC patients with pHBV, 11.8% (34/288) were hHBsAg+ and had more advanced stages of HCC, higher AFP levels, higher vascular invasion rates, and significantly shorter OS than others (OS: 19.3 vs 61.4 months, $p = 0.012$). Comparison of OS among non-HBV-related-HCC patients without pHBV (Group 1), those with pHBV and without hHBsAg+ (Group 2), and those with pHBV and hHBsAg+ (Group 3) revealed significantly shorter OS in Group 3 (19.3, 56.6, and 66.4 months in Groups 1, 2, and 3, respectively; $p = 0.036$). Multivariate Cox regression indicated that compared with Group 1,

only Group 3 was significantly and independently associated with shorter OS (HR: 2.044, p = 0.011). Subgroup analysis revealed that this association was particularly evident in non-HBV-related-HCC patients with non-B-non-C aetiology and advanced HCC.

Conclusions: In non-HBV-related-HCC patients, hHBsAg+, not pHBV, is significantly and independently associated with poor prognosis.

Keywords: non-HBV-related HCC, HBV, high-sensitivity HBs antigen test, prognosis, biomarker

Introduction

Hepatocellular carcinoma (HCC) ranks among the most fatal cancers,[1] and there is an ongoing rise in the number of HCC-diagnosed cases.[2] Thus, identifying optimal therapeutic options and clarifying prognostic factors and their underlying mechanisms is clinically important. The aetiology of HCC is varied, including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, metabolic dysfunction-associated steatotic liver disease (MASLD), and alcohol use[3]. Importantly, the aetiology of HCC is associated with molecular subclasses,[3] with HBV-related HCC being classified into the proliferation class, characterised by more aggressive tumours, poor cell differentiation, and an active or exhausted immune response compared with HCV or MASLD-related HCC.[3] Moreover, in recently developed anti-HCC therapies, it has been reported that the aetiology of HCC might affect the therapeutic response and prognosis.[4, 5]

HBV infection affects more than 296 million individuals globally, leading to nearly 800,000 fatalities each year.[6] Additionally, it is anticipated that the number of patients with previous HBV infection (pHBV) is several times higher than that of patients with current HBV infections.

121 It has been reported that pHBV can be a risk factor for hepatocarcinogenesis;[7] however, this
122 remains controversial.[8-10] Additionally, how pHBV affects the biological characteristics of non-
123 HBV-related HCC has not been well clarified. Recently, it has been reported that HBV
124 integration is not rare in HCC patients with pHBV and observed in driver genes in some cases.[11]
125 Moreover, HCC with more HBV-DNA integration into the host genome is associated with poor
126 prognosis.[12] Thus, in HCC patients with pHBV, some may have significant HBV-DNA
127 integration, which might affect the biological characteristics of HCC and prognosis.
128
129 Recently, a high-sensitivity Hepatitis B surface antigen (HBs) antigen test has been developed.
130 Using this high-sensitivity HBs antigen test,[13] very low titres of serum HBs antigen can be
131 detected, which were previously undetectable. In addition, it has been reported that in some
132 patients with low HBV replication, integrated HBV DNA mainly produces HBsAg
133 independently of viral replication.[14] Thus, in non-HBV related HCC patients with pHBV, the
134 positivity of high-sensitivity HBs-antigen (hHBsAg+) might reflect HBV-DNA integration and
135 could affect the characteristics of HCC and prognosis. However, the prevalence and
136 significance of hHBsAg+ in non-HBV HCC patients with pHBV have not been fully clarified.
137 Therefore, in the present study, we sought to address these gaps in knowledge.
138

Materials and methods

Patients and study design

In this retrospective study, we screened patients with initially diagnosed HCC[15] without current HBV infection at Hokkaido University and related hospitals between April 2000 and March 2023. We included patients with newly diagnosed non-HBV related HCC (negative for HBs antigen using the Architect HBsAg-QT assay, Abbott Laboratory, Tokyo, Japan; a quantification range of 0.05–250 IU/mL) if they had comprehensive clinical information and preserved serum at the time of HCC diagnosis. If information on HBV infection status was lacking, and the patients had preserved serum, we evaluated the status using the preserved serum. Patients with HCC were excluded if they had current HBV infection (HBs antigen positive by the standard HBs antigen test of Architect HBsAg-QT assay and/or HBV-DNA positive), lacked proper clinical information, or did not have preserved serum for analysis using the high-sensitivity HBs antigen assay (Lumipulse HBsAg-HQ (Fujirebio Inc., Tokyo, Japan; quantification range of 0.005–150 IU/mL).

We collected baseline laboratory data, including details regarding tumour markers, patient gender, age, HCC aetiology, disease stage (classified by the Barcelona Clinic Liver Cancer [BCLC] system), liver function (based on the Child-Pugh classification), treatment protocols,

HCV infection, and overall survival (OS). We evaluated the prevalence and characteristics of hHBsAg+ in HCC patients with pHBV. We analysed the prognostic impact of pHBV or hHBsAg+ on prognosis in the entire cohort, consisting of non-HBV-related HCC patients with and without pHBV. Additionally, we conducted subgroup analysis in non-HBV-related HCC patients stratified by HCC aetiology and/or BCLC stage.

This study strictly followed the ethical guidelines outlined in the Declaration of Helsinki. The study protocol received approval from the ethics committee of Hokkaido University Hospital (approval number: 019-0400). Participation in the study was contingent upon patients providing written informed consent or not expressing refusal to participate. The ethics committee granted explicit permission for the inclusion of certain patients under these conditions, even in cases where written informed consent was not obtained.

Evaluation of HBV markers

We defined pHBV as HBsAg negative, HBVDNA negative, and anti-HBs positive or anti-HBc positive. We analysed the levels of serum HBV markers, including the standard HBs antigen, anti-HBc antibody, and anti-HBs antibody, in all included patients using previously reported methods.[16-21] If possible, we also collected data on HBV-DNA or analysed HBV-DNA levels

using preserved serum. We quantified standard HBsAg titres utilising a chemiluminescence-based immunoassay system, the Architect HBsAg-QT assay (Abbott Laboratory, Tokyo, Japan), which has a quantification range of 0.05–250 IU/mL. Additionally, HBV-DNA levels were determined via a real-time TaqMan polymerase chain reaction assay, specifically the Cobas AmpliPrep/Cobas TaqMan HBV test (version 2.0, Roche Diagnostics, Basel, Switzerland), offering a quantification range of 1.3–8.3 log IU/mL. The detection and quantification of serum anti-HBc antibodies were conducted using Architect Anti-HBc chemiluminescence immunoassays (Abbott). Serum anti-HBs were assessed using chemiluminescence immunoassays (Architect anti-HBs assay, with a lower limit of detection of 10 mIU/mL [Abbott]). Specifically, high-sensitivity HB antigen quantification was performed using Lumipulse HBsAg-HQ (Fujirebio Inc., Tokyo, Japan), with a quantification range of 0.005–150 IU/mL.[22]

Statistical analysis

For the analysis of categorical data, we utilised the chi-squared test or Fisher's exact test as appropriate. Continuous variables were evaluated using either the Student's t-test or the Mann-Whitney U test, depending on the data distribution. Survival curves were constructed using the Kaplan-Meier method, and differences between these curves were assessed using the log-rank

test. Multivariate Cox regression analysis was conducted to clarify the factors associated with prognosis, including the positivity of high-sensitive HBsAg, in the entire non-HBV related HCC patient cohort and subgroups. A p-value <0.05 was considered statistically significant. All statistical evaluations were performed using the Prism 9.41 (GraphPad Software, La Jolla, California) and EZR software (Saitama Medical Center at Jichi Medical University, Saitama, Japan).

Results

Prevalence and characteristics of hHBsAg+ in non-HBV-related HCC patients with pHBV

We screened a total of 553 non-HBV-related HCC patients and included 401 initially diagnosed non-HBV-related HCC patients (Figure 1). A total of 147, 2, and 3 patients were excluded due to recurrence of HCC, positive HBV-DNA, and insufficient clinical data and/or preserved serum, respectively (Figure 1). Of the 401 included patients, 288 had pHBV and 113 did not. Among the 288 patients with pHBV, 34 (11.8%) were hHBsAg+. Comparisons between non-HBV related HCC patients with or without hHBsAg+ revealed that HCC patients with hHBsAg+ had significantly more advanced BCLC stages ($p = 0.002$), higher AST levels ($p = 0.025$), higher AFP levels ($p = 0.03$), and a higher rate of vascular invasion ($p < 0.001$; Table

1). Additionally, we analysed the relationship between hHBsAg+ and the status of HBs and HBc antibodies (Table S1). Among the three groups of patients—those positive for anti-HBs only, for anti-HBc only, and for both anti-HBs and anti-HBc—the rate of hHBsAg+ was similar (p=0.996). All 110 patients without pHBV were negative for hHBsAg+ (Table S1).

Effect of pHBV or hHBsAg+ on prognosis in patients with initially diagnosed non-HBV-related HCC

We evaluated the impact of the presence of pHBV or hHBsAg+ on the prognosis of patients with non-HBV-related HCC in the entire cohort (Table S2). As shown in Figure 2A, the comparison of overall survival (OS) between non-HBV-related HCC patients with or without pHBV revealed similar median OS between the two groups. However, as shown in Figure 2B, non-HBV-related HCC patients with hHBsAg+ had significantly shorter median OS compared to those without (19.3 months [95% CI: 10.0–61.5 months] vs. 61.4 months [95% CI: 49.7–68.1 months], p = 0.012). Subsequently, we compared the median OS among three groups: non-HBV-related HCC patients with pHBV and hHBsAg+, those with pHBV and without hHBsAg+, and those without pHBV (Table S3). As shown in Figure 3A, median OS was significantly different among the three groups, with the shortest OS in patients with pHBV and hHBsAg+ (19.3 months [95% CI: 10.0–61.5 months] vs. 56.6 months [95% CI: 43.4–67.2 months] vs. 66.4 months [95% CI: 50.2–82.1 months], p = 0.036).

In multivariate Cox regression analysis regarding factors associated with OS, BCLC stage, Child-Pugh grade, platelet counts, AST, and AFP were significantly associated with median OS (Table 2). Additionally, although the median OS for patients with pHBV and without hHBsAg+ was similar to the reference (HR: 1.351; 95% CI: 0.927–1.969; p = 0.118), the median OS for patients with pHBV and hHBsAg+ was significantly and independently shorter than that of the reference group (HR: 2.044; 95% CI: 1.181–3.538; p = 0.011; Table 2).

Effect of pHBV or hHBsAg+ on prognosis in subgroups of patients with non-HBV-related HCC

Non-B Non-C (NBNC) aetiology HCC

We conducted a subgroup analysis in patients with NBNC aetiology of HCC (Table S4). As shown in Figure 3B, comparison of the median OS among patients without pHBV, those with pHBV and without hHBsAg+, and those with pHBV and hHBsAg+ revealed a significantly shorter median OS in patients with pHBV and hHBsAg+ (11.4 months [95% CI: 3.0–19.3 months] vs. 44.6 months [95% CI: 33.6–60.3 months] vs. 66.4 months [95% CI: 48.1–82.8 months], p = 0.035). In multivariate Cox regression analysis, BCLC stage, Child-Pugh grade, platelet counts, AST, and AFP were significantly associated with the median OS (Table 2).

Additionally, the median OS for patients with pHBV and hHBsAg+ was significantly shorter than that of the reference group (HR: 2.954; 95% CI: 1.394–6.264; p = 0.005) (Table 2).

HCV-related HCC

In subgroup analysis of HCV-related HCC patients (Table S5), Kaplan-Meier analysis and multivariate Cox regression analysis revealed that hHBsAg+ did not affect the prognosis of patients with HCV-related HCC (Figures 3C and S1, Table S6).

BCLC stage 0/A

We conducted a subgroup analysis in patients with BCLC stage 0/A of non-HBV-related HCC. Baseline patient characteristics are shown in Table S7. As shown in Figure 4A, comparison of median OS among non-HBV-related HCC patients without pHBV, those with pHBV and without hHBsAg+, and those with pHBV and hHBsAg+ revealed a similar median OS among the groups. Multivariate Cox regression analysis showed that hHBsAg+ was not associated with OS (HR: 1.061; 95% CI: 0.383–2.942; p = 0.909; Table S8). In addition, progression-free survival (PFS) was also similar among non-HBV-related HCC patients without pHBV, those with pHBV and without hHBsAg+, and those with pHBV and hHBsAg+ (Figure S2A).

BCLC stage B/C/D

Next, we conducted a subgroup analysis in patients with BCLC stage B/C/D of non-HBV-related HCC. Baseline patient characteristics are shown in Table S9. As shown in Figure 4B, comparison of the median OS among non-HBV-related HCC patients with pHBV and hHBsAg+, those with pHBV and without hHBsAg+, and those without pHBV revealed a significantly shorter median OS in patients with pHBV and hHBsAg+ (11.1 months [95% CI: 2.9–19.3 months] vs. 21.5 months [95% CI: 15.2–33.1 months] vs. 44.5 months [95% CI: 23.8-not reached], $p = 0.013$). Multivariate Cox regression analysis revealed that hHBsAg+ was significantly associated with shorter OS (HR: 4.460; 95% CI: 2.144–9.277; $p < 0.001$; Table 2).

NBNC HCC with BCLC stage 0/A

We conducted a subgroup analysis in patients with NBNC HCC and BCLC stage 0/A (Table S10). In these patients, hHBsAg+ did not affect prognosis (Figure 4C, Table S11). Similarly, in these patients, hHBsAg+ did not affect PFS (Figure S2B).

NBNC HCC with BCLC stage B/C/D

Next, we conducted a subgroup analysis in patients with NBNC HCC and BCLC stage B/C/D (Table S12). As shown in Figure 4D, comparison of the median OS among HCC patients with

pHBV and hHBsAg+, those with pHBV and without hHBsAg+, and those without pHBV revealed a significantly shorter median OS in patients with pHBV and hHBsAg+ (5.9 months [95% CI: 0.9–11.4 months] vs. 20.9 months [95% CI: 11.9–36.2 months] vs. 44.5 months [95% CI: 16.2-not reached], $p < 0.001$). Multivariate Cox regression analysis revealed that the median OS in patients with pHBV and hHBsAg+ was significantly shorter than that of the reference group (HR: 7.426; 95% CI: 2.946–17.830; $p < 0.001$; Table 2).

Changes in hHBsAg+ after curative treatment of HCC

Finally, we analysed changes in high-sensitivity HBs antigen levels after curative treatment of HCC. We obtained preserved serum from ten non-HBV-related HCC patients with positive high-sensitivity HBs antigen who underwent curative HCC treatment (resection of HCC or RFA). Of these, 80% (8/10) became negative for high-sensitivity HBs antigen after curative HCC treatment (Table 3).

Discussion

The influence of previous HBV infection (pHBV) on the biological characteristics of non-HBV-related HCC and its prognostic implications remains inadequately elucidated. The present study revealed that the presence of pHBV did not affect the prognosis of patients with initially

diagnosed non-HBV-related HCC. Among the non-HBV-related HCC patients with pHBV, 11.8% (34/288) were hHBsAg+, and these patients had a significantly shorter OS compared to other non-HBV-related HCC patients (median OS: 19.3 months [95% CI: 10-61.5 months] vs. 61.4 months [95% CI: 49.7-68.1 months], p = 0.012). Patients with pHBV and hHBsAg+ exhibited higher serum AFP levels and a higher rate of vascular invasion, which are typical phenotypes of HBV-related HCC[2]. Multivariate Cox regression analysis revealed that the median OS in non-HBV-related HCC patients with pHBV and hHBsAg+ was significantly and independently associated with shorter OS (HR: 2.044; 95% CI: 1.181–3.538; p = 0.011, Table 2). Notably, subgroup analysis revealed that this association was particularly evident in non-HBV-related HCC patients with NBNC aetiology and BCLC stage B/C/D (HR: 7.43; 95% CI: 2.95-17.83, p < 0.001).

The reason for why hHBsAg+ causes poor prognosis has not fully been clarified, but several hypotheses exist. Recent efforts integrating genomic, epigenomic, histological, and immunological insights have led to the development of a molecular and immune-based classification system for HCC.[2, 23] These classes are linked to distinct genomic alterations, histopathological markers, the aetiology of HCC, and clinical prognosis.[2] Approximately half of HCC cases are classified as the Proliferation class, which is particularly prevalent among

319 HBV-related HCC and associated with poorer prognosis.[2] This class is characterised by higher
320 AFP levels, higher rates of vascular invasion, and poor prognosis.[2] Similarly, non-HBV-related
321 HCC patients with pHBV and hHBsAg+ showed higher AFP levels, higher vascular invasion
322 rates, and poorer prognosis compared to other non-HBV related HCC patients. Thus, non-HBV-
323 related HCC with pHBV and hHBsAg+ might have characteristics similar to those of HBV-
324 related HCC. Recent studies have shown that in low HBV replication conditions, integrated
325 HBV DNA mainly produces HBsAg, independently of viral replication.[14] Therefore, hHBsAg+
326 in non-HBV-related HCC patients might be a surrogate marker of significant HBV-DNA
327 integration into the host genome, characteristic of HBV-related HCC.[2] This has important
328 clinical implications because recent studies have shown that the aetiology of HCC might affect
329 the outcome of systemic therapy.[4, 24] Especially, recent reports showed that non-viral HCC,
330 particularly NASH-related HCC, may show reduced sensitivity to immune checkpoint
331 inhibitors (ICIs).[4] Even in cases of NASH-related HCC, those with hHBsAg+ may exhibit
332 characteristics of HBV-related HCC, potentially enhancing the efficacy of ICIs. However,
333 further analysis, including analysis of HBV integration into the HCC genome, is required to
334 validate this hypothesis.
335

In HCV-related HCC patients, hHBsAg+ did not affect prognosis. Thus, in cases of HCV-related HCC, the impact of HCV infection might be significant, potentially limiting the prognostic influence of the high-sensitivity HBs antigen.

In patients with BCLC stage 0/A non-HBV-related HCC, hHBsAg+ did not affect their prognosis and PFS. The reason for this is not entirely clear. The sample size and observational period might affect the results as early-stage HCC could be expected to have longer survival than advanced HCC. Another hypothesis is that curative treatments such as radiofrequency ablation (RFA) or surgery are feasible for HCC classified as BCLC stage 0/A. Therefore, even in non-HBV-related HCC with numerous HBV-DNA integrations, which might enhance the cancer's malignant potential, the prognosis may not be adversely affected due to the possibility of curative intervention. In fact, in patients with hHBsAg+ who underwent curative treatment of HCC and had preserved serum after treatment, most became negative for high-sensitivity HBs antigen (80%, 8/10). These findings might suggest that the high-sensitivity HBs antigen likely originates predominantly from the HCC itself and not from non-cancerous background liver tissue. However, further analysis, including HBV-DNA integration into the HCC genome, should be required to validate this hypothesis.

354 The reason for why the presence of anti-HBs antibodies did not influence hHBsAg+ has not
355 been fully understood. However, it is possible that the Lumipulse HBsAg-HQ (Fujirebio Inc.,
356 Tokyo, Japan) assay, which involves pretreatment of samples with a solution containing
357 surfactant to disrupt HBV particles, leads to dissociation of HBsAg from HBsAg–anti-HBs
358 complexes and linearisation of epitopes,[25, 26] which may play a role. Nevertheless, further
359 investigation is required to confirm this.
360
361 Moreover, to further investigate the persistence of hHBsAg+, we conducted an additional
362 analysis. As shown in Table S13, we analysed serum samples from three patients whose serum
363 had been preserved prior to the onset of hHBsAg+ in this study A total of 67% (2/3) of patients
364 had persistent hHBsAg positivity over time before intervention for HCC. This persistence of
365 hHBsAg positivity was similarly observed in a previous report on HBV reactivation during
366 immunosuppressive therapy.[27]
367
368 Furthermore, there are unresolved issues regarding the impact of hHBsAg+ on clinical practice
369 for patients with pHBV. In chronic HBV-infected patients in which the HBs antigen becomes
370 negative, a decrease in the occurrence of HCC has been reported.[28] However, the proportion of
371 cases in which hHBsAg becomes positive for patients with pHBV and without HCC, and how

hHBsAg+ relates to hepatocarcinogenesis in such cases, requires further investigation. In addition, the immune response against HBV, including T follicular helper cells, is import to seroclearance of the HBs antigen.[29] Thus, among patients with pHBV, the immune status may differ between those with and without hHBsAg+, potentially affecting the prognosis. However, further analysis is required.

This research had limitations. It was a retrospective study, and we could not conduct pathological and genetic analyses. Therefore, a prospective large-scale study, including pathological and genetic analyses, is required to validate our results.

Conclusion

In summary, in patients with non-HBV-related HCC, hHBsAg+ is significantly and independently associated with poor prognosis. This association is particularly evident in HCC patients with NBNC aetiology and advanced HCC. These findings have potential utility in the clinical management of and therapeutic strategies for patients with non-HBV-related HCC.

Acknowledgements

The authors would like to thank all patients and their families.

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Figure legends

Fig 1. Flowchart of the study

Abbreviations: HCC, hepatocellular carcinoma; HBsAg, Hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus (HCV) positive; HCV-RNA positive + post sustained viral response by anti-HCV therapy

Fig 2. Comparison of the overall survival (OS) of (A) HCC patients with or without pHBV, and (B) HCC patients with or without positivity of high-sensitivity HBs antigen

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen

Fig 3. Comparison of overall survival (OS) in the entire cohort and a subgroup stratified based on HCC aetiology

A. Comparison of OS among HCC patients according to three groups: group 1 - without pHBV, group 2 - with pHBV and without hHBsAg+, and group 3 - with pHBV and hHBsAg+.

B. Comparison of OS among NBNC aetiology HCC patients according to groups 1–3.

C. Comparison of OS among HCV-related HCC patients according to groups 1–3.

Abbreviations: pHBV, previous HBV infection; hHBsAg+, positivity of high-sensitivity HBs antigen test; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen

Fig 4. Comparison of overall survival (OS) in subgroups stratified by BCLC stage and HCC aetiology

A. Comparison of OS among BCLC stage 0/A HCC patients according to three groups: group 1 - without pHBV, group 2 - with pHBV and without hHBsAg+, and group 3 - with pHBV and hHBsAg+.

B. Comparison of OS among BCLC stage B/C/D HCC patients according to groups 1–3.

C. Comparison of OS among NBNC and BCLC stage 0/A HCC patients according to groups 1–3.

D. Comparison of OS among NBNC and BCLC stage B/C/D HCC patients according to groups 1–3.

Abbreviations: pHBV, previous HBV infection; hHBsAg+, positivity of high-sensitivity HBs antigen test; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen

1 **Tables**

2 **Table 1.** Comparison of the baseline characteristics of patients with non-HBV HCC and

3 those with high-sensitivity HBsAg (-) or (+)

Variables	High-sensitivity HBsAg (-) (n=367)	High-sensitivity HBsAg (+) (n=34)	P-values
Sex			0.074
Male	257 (70.0%)	29 (85.3%)	
Female	110 (30.0%)	5 (14.7%)	
Age (years)	73 (23–95)	70.5 (51–89)	0.146
HCV status			0.785
non-infection	208 (56.7%)	18 (52.9%)	
post SVR	30 (8.2%)	2 (5.9%)	
HCV-RNA+	128 (34.9%)	14 (41.2%)	
Not Available	1 (0.3%)	0 (0%)	
BCLC stage			0.002
0	94 (25.8%)	4 (11.8%)	
A	137 (37.5%)	11 (32.4%)	
B	79 (21.6%)	5 (14.7%)	
C	46 (12.6%)	14 (41.2%)	
D	9 (2.5%)	0 (0%)	
Not Available	2 (0.5%)	0 (0%)	
Vascular invasion			<0.001
(-)	329	23	
(+)	33	11	
Child-Pugh grade			0.123
A	267 (74.0%)	21 (61.8%)	
B	82 (22.7%)	10 (29.4%)	
C	12 (3.3%)	3 (8.8%)	
Not Available	6 (1.6%)	0 (0%)	
Follow-up period, months	36.7 (0–253.9)	13.1 (0.6–116.9)	0.009
Platelet counts ($\times 10^4/\mu L$)	14.0 (2.9–40.0)	15.15 (2.7–61.0)	0.432
AST (IU/L)	47 (11–886)	63.5 (20–213)	0.025
ALT (IU/L)	34.5 (3–936)	41 (16–105)	0.636

γ-GT (IU/L)	72 (10–1035)	84 (10–1555)	0.916
FIB-4 index	4.1 (0.6–37.3)	5.1 (1.2–13.5)	0.349
AFP (ng/mL)	12.8 (1.0–740761)	21.1 (1.4–74579)	0.03

Data are presented as numbers, percentages of patients, or median (range) values.

AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; BCLC, Barcelona Clinic Liver Cancer; FIB-4 index, fibrosis-4 index; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; SVR, sustained virological response; γ-GT, γ-glutamyl transferase.

Table 2. Multivariate cox regression analysis of factors associated with overall survival

Variables	Whole cohort		NBNC cohort		BCLC stage B/C/D		NBNC and BCLC stage B/C/D	
	HR (95% CI)	P-values	HR (95% CI)	P-values	HR (95% CI)	P-values	HR (95% CI)	P-values
Sex, Male vs. Female	1.054 (0.758–1.467)	0.754	1.040 (0.645–1.677)	0.873	0.804 (0.463–1.396)	0.439	0.894 (0.452–1.767)	0.747
Age (years)	0.996 (0.981–1.010)	0.520	1.015 (0.993–1.038)	0.192	0.999 (0.977–1.020)	0.888	1.015 (0.986–1.046)	0.317
Without pHBV	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
pHBV without hHBsAg+	1.351 (0.927–1.969)	0.118	1.344 (0.840–2.150)	0.217	2.264 (1.311–3.911)	0.003	1.999 (1.051–3.803)	0.035
pHBV with hHBsAg+	2.044 (1.181–3.538)	0.011	2.954 (1.394–6.264)	0.005	4.460 (2.144–9.277)	<0.001	7.426 (2.946–17.830)	<0.001
HCV-RNA (-) vs. (+)	1.277 (0.910–1.794)	0.158	(–)	(–)	1.118 (0.633–1.975)	0.701	(–)	(–)
BCLC stage 0 /A vs. B/C /D	3.208 (2.357–4.365)	<0.001	4.198 (2.693–6.542)	<0.001	(–)	(–)	(–)	(–)
Child-Pugh grade A vs. B or C	2.325 (1.680–3.216)	<0.001	2.573 (1.696–3.903)	<0.001	3.635 (2.275–5.807)	<0.001	3.427 (1.984–5.919)	<0.001
Platelet counts ($\times 10^4/\mu L$)	1.039 (1.018–1.059)	<0.001	1.038 (1.013–1.063)	0.002	1.064 (1.034–1.094)	<0.001	1.060 (1.024–1.097)	0.001
AST (IU/L)	1.004 (1.002–1.006)	<0.001	1.004 (1.001–1.007)	0.004	1.004 (1.001–1.006)	<0.001	1.004 (1.001–1.006)	0.004
ALT (IU/L)	0.997 (0.993–1.001)	0.152	0.995 (0.989–1.002)	0.174	0.996 (0.990–1.001)	0.135	0.995 (0.986–1.003)	0.215

γ-GT (×10^2 IU/L)	1.108 (0.990–1.241)	0.075	1.073 (0.939–1.227)	0.299	1.141 (0.991–1.314)	0.067	1.095 (0.922–1.301)	0.990
AFP (×10^3 ng/mL)	1.004 (1.001–1.006)	0.015	1.004 (1.001–1.007)	0.016	1.003 (0.9998–1.006)	0.063	1.004 (1.000–1.007)	0.028

AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; BCLC, Barcelona Clinic Liver Cancer; CI, confidence interval; HR, hazard ratio; HBsAg, hepatitis B surface antigen, HCV, hepatitis C virus; hHBsAg+, positivity of High-sensitivity hepatitis B surface antigen Test; pHBV, previous HBV infection; γ-GT, γ-glutamyl transferase.

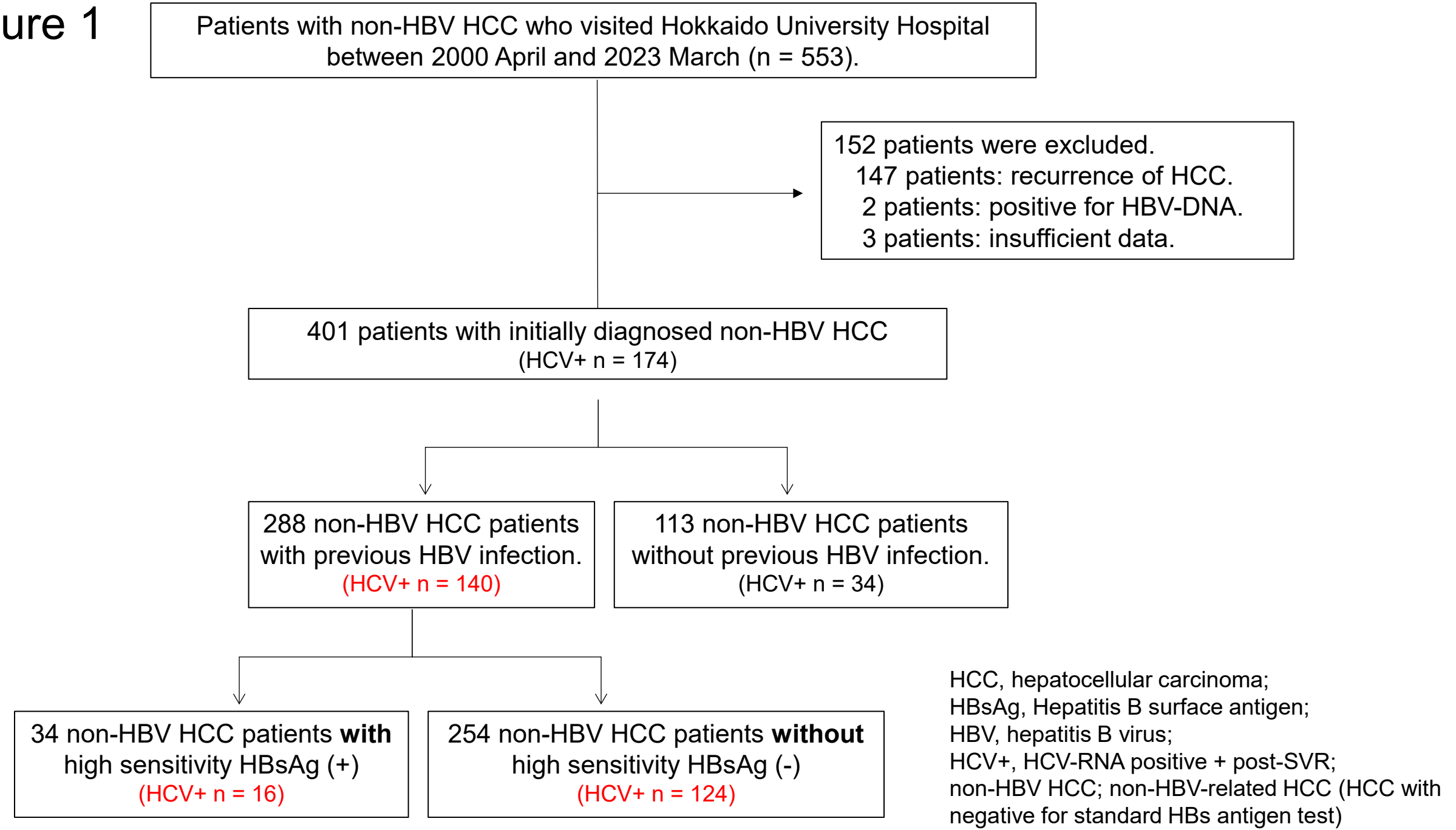
Table 3. Changes in high-sensitivity HBsAg after curative treatment of HCC in non-HBV-related HCC patients positive for high-sensitivity HBsAg

High-sensitivity HBsAg after curative treatment	n
Negative	**8 (80%)**
Positive	**2 (20%)**

HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen.

34

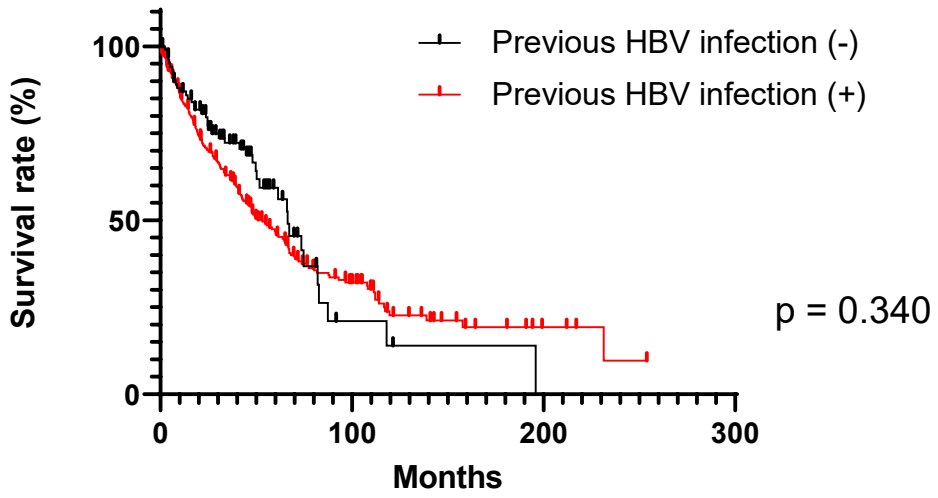
Figure 1



HCC, hepatocellular carcinoma;
HBsAg, Hepatitis B surface antigen;
HBV, hepatitis B virus;
HCV+, HCV-RNA positive + post-SVR;
non-HBV HCC; non-HBV-related HCC (HCC with negative for standard HBs antigen test)

Figure 2

A

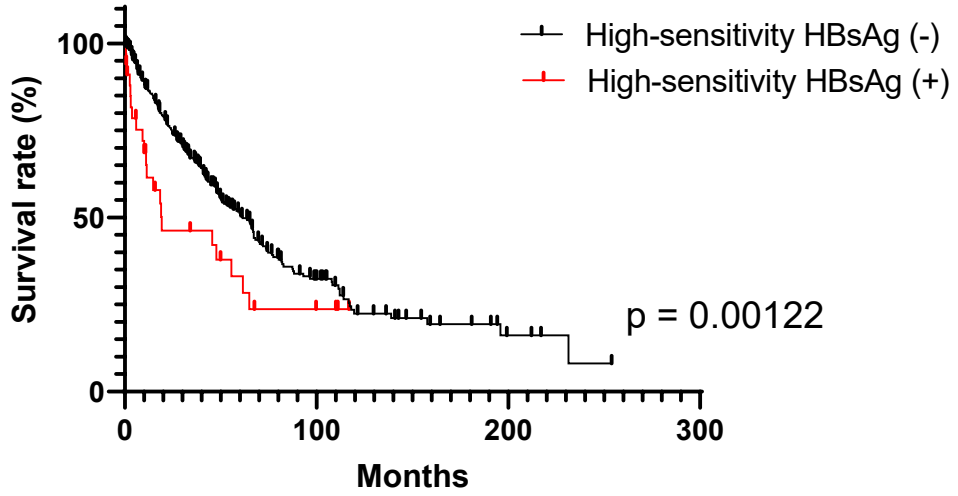


Number at risk

Previous HBV infection (-)	113	27	3	1	0	0
Previous HBV infection (+)	288	99	40	12	4	1

	n	Median survival	P value
Previous HBV infection (-)	113	66.4 (50.2 - 82.1)	0.34
Previous HBV infection (+)	288	53.2 (42.7 - 65.1)	

B



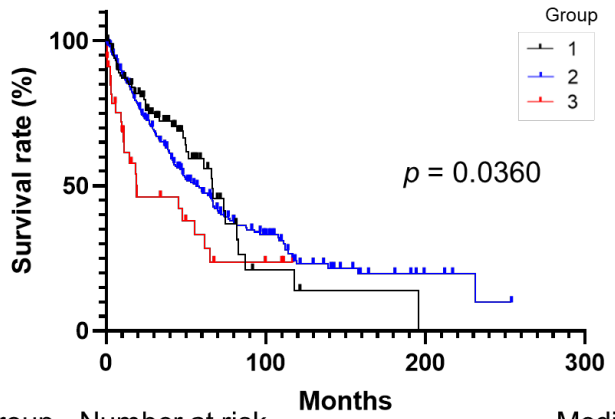
Number at risk

High-sensitivity HBsAg (-)	367	118	40	13	4	1
High-sensitivity HBsAg (+)	34	8	3	0	0	0

	n	Median survival	P value
High-sensitivity HBsAg (-)	367	61.4 (49.7 - 68.1)	0.0122
High-sensitivity HBsAg (+)	34	19.3 (10.0 - 61.5)	

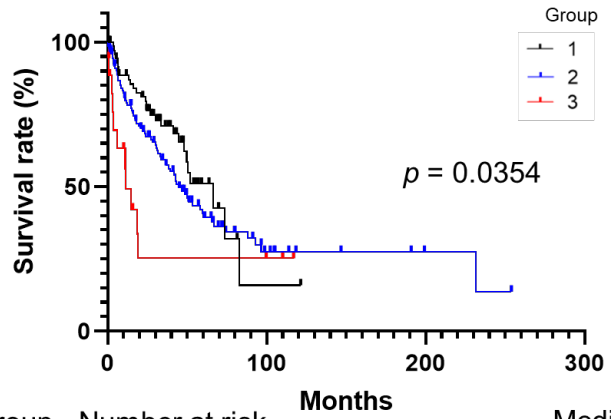
Figure 3

A



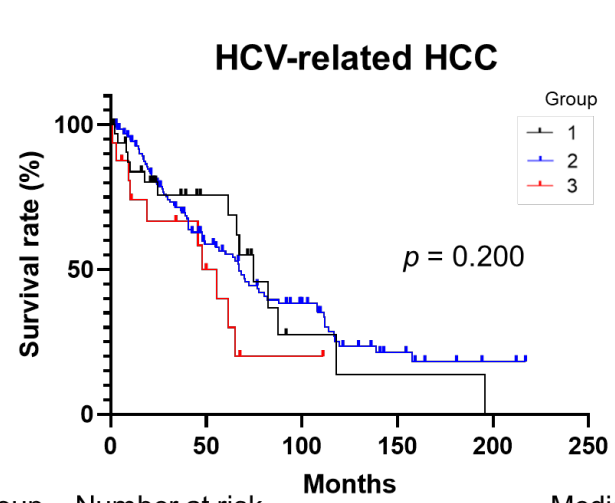
Group	Number at risk						Median survival
1	113	27	3	1	0	0	66.4 (50.2 - 82.1)
2	254	91	37	12	4	1	56.6 (43.4 - 67.2)
3	34	8	3	0	0	0	19.3 (10.0 - 61.5)

B



Group	Number at risk						Median survival
1	79	16	1	0	0	0	66.4 (48.1 - 82.8)
2	129	37	10	4	2	1	44.6 (33.6 - 60.3)
3	18	3	2	0	0	0	11.4 (3.0 - 19.3)

C



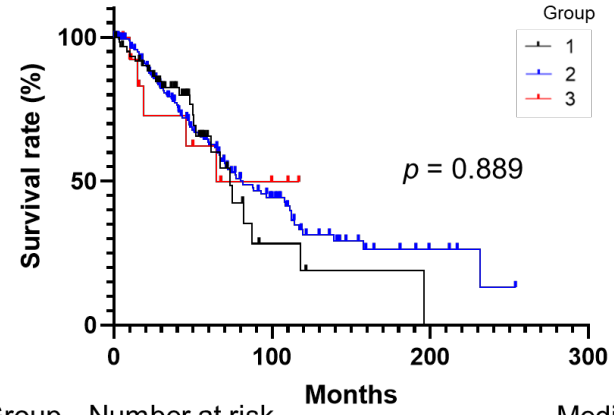
Group	Number at risk					Median survival
1	34	11	2	1	0	74.7 (61.4 - 118.0)
2	124	54	27	8	2	67.2 (47.9 - 81.8)
3	16	5	1	0	0	47.7 (10.0 - 64.9)

	Previous HBV infection	High sensitivity HBsAg
1	(−)	(−)
2	(+)	(−)
3	(+)	(+)

Figure 4

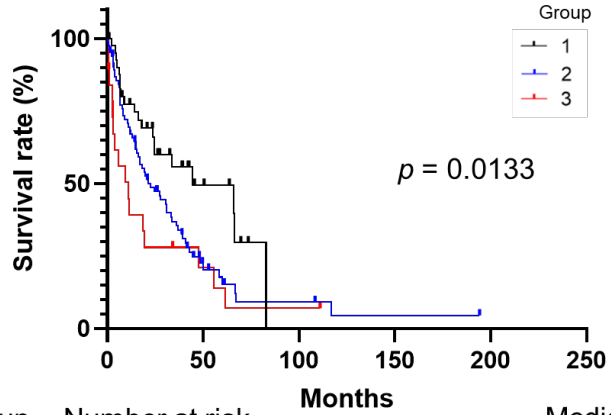
	Previous HBV infection	High sensitivity HBsAg
1	(−)	(−)
2	(+)	(−)
3	(+)	(+)

A



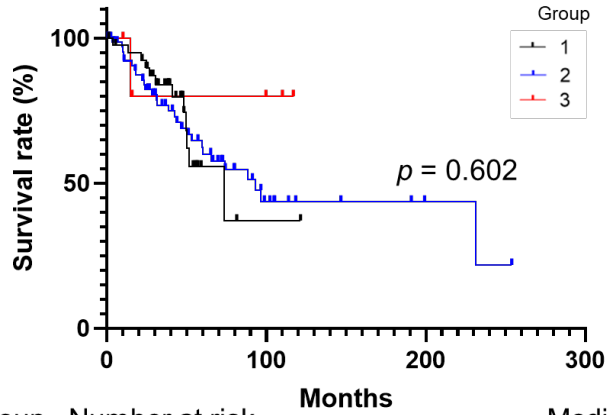
Group	Number at risk						Median survival
1	62	20	3	1	0	0	73.5 (51.8 - 87.4)
2	169	82	34	11	4	1	80.3 (66.8 - 111.4)
3	15	5	2	0	0	0	64.9 (14.8 - NA)

B



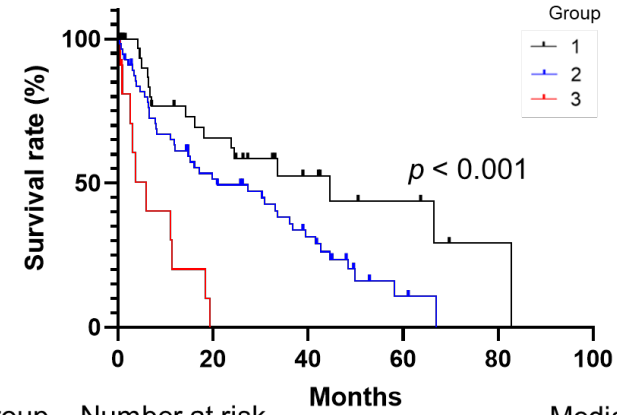
Group	Number at risk					Median survival
1	49	7	0	0	0	44.5 (23.8 - NA)
2	85	9	3	1	0	21.5 (15.2 – 33.1)
3	19	3	1	0	0	11.1 (2.9 – 19.3)

C



Group	Number at risk						Median survival
1	41	11	1	0	0	0	73.5 (49.7 - NA)
2	72	33	10	4	2	1	93.1 (59.7 - NA)
3	7	3	2	0	0	0	NA (114.8 - NA)

D

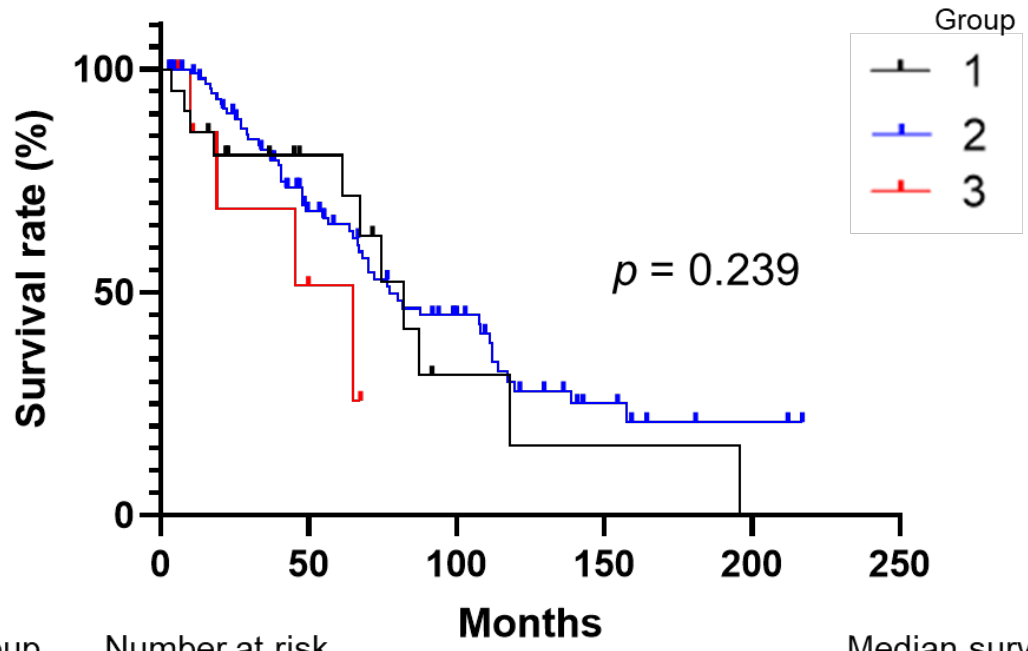


Group	Number at risk					Median survival
1	37	18	8	4	1	44.5 (16.2 - NA)
2	57	26	13	2	0	20.9 (11.9 - 36.2)
3	11	0	0	0	0	5.9 (0.9 - 11.4)

Supplementary Figure S1

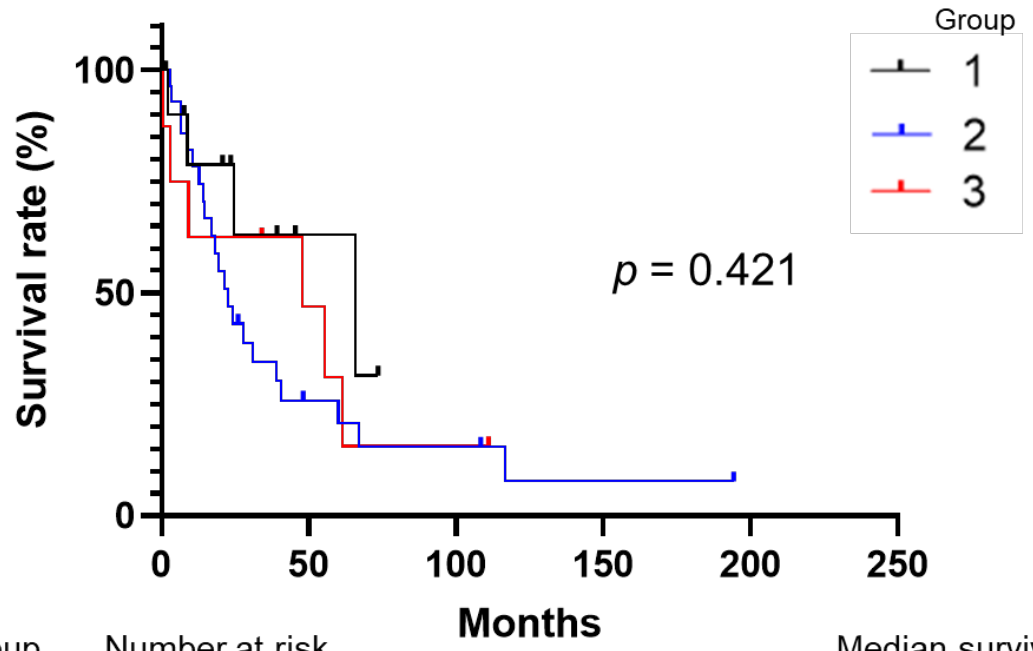
	Previous HBV infection	High sensitivity HBsAg
1	(−)	(−)
2	(+)	(−)
3	(+)	(+)

A



Group	Number at risk					Median survival
1	21	9	2	1	0	82.1 (61.4 – 118.0)
2	96	49	24	7	2	77.4 (66.8 – 112.0)
3	8	2	0	0	0	64.9 (10.0 - NA)

B

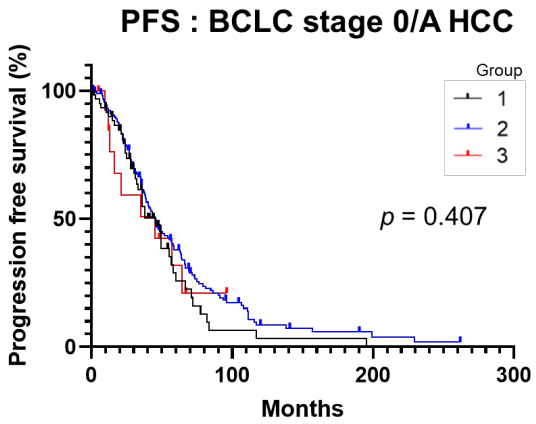


Group	Number at risk					Median survival
1	12	2	0	0	0	66.1 (2.2 – NA)
2	28	5	3	1	0	22.6 (14.4 – 38.9)
3	8	3	1	0	0	37.7 (0.7 – 61.5)

Supplementary Figure S2

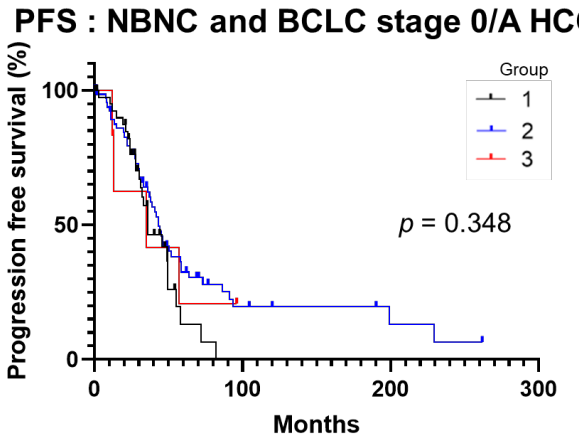
	Previous HBV infection	High sensitivity HBsAg
1	(−)	(−)
2	(+)	(−)
3	(+)	(+)

A



Group	Number at risk						Median PFS
1	62	13	2	1	0	0	45.8 (32.0 - 55.0)
2	169	59	17	5	2	1	45.4 (38.5 - 55.0)
3	15	4	0	0	0	0	45.3 (13.0 - 64.2)

B



Group	Number at risk						Median PFS
1	41	5	0	0	0	0	36.0 (30.4 - 49.6)
2	72	22	6	4	2	1	43.0 (36.0 - 58.0)
3	7	2	0	0	0	0	35.0 (12.0 - NA)