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TITLE: Determinants of hepatocellular carcinoma in cirrhotic patients treated with nucleos(t)ide analogues for chronic hepatitis B

Short title: Determinants of HBV-HCC under NUC

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Keywords: hepatitis B virus; liver cirrhosis; antiviral therapy; diabetes mellitus; risk stratification

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Objectives: We aimed to identify determinants of hepatocellular carcinoma (HCC) in cirrhotic patients who received nucleos(t)ide analogues for chronic hepatitis B (CHB).

Patients and methods: This retrospective-prospective study screened all patients (N=1,630) who received antiviral therapy for CHB between 1 September, 2007 and 31 March, 2013 at the E-Da Hospital, and enrolled 210 consecutive cirrhotic patients with pretreatment viral DNA >2,000 IU/mL. Those who developed HCC within 3 months of treatment were excluded. All participants were observed until occurrence of HCC, death, or 1 January 2014. The incidence and determinants of HCC were estimated using competing risk analyses adjusted for mortality.

Results: Thirty-five (16.7%) patients developed HCC during a median follow-up of 25.2 months (interquartile range, 16.3-37.3 months), with a cumulative incidence of 24.1% (95% confidence interval [CI], 16.3-32.0%) at 5 years. Multivariate–adjusted analyses identified age >55 years (adjusted hazard ratio [HR], 2.19; 95% CI, 1.03-4.66), male gender (adjusted HR, 3.07; 95% CI, 1.05-9.02), MELD score >12 points (adjusted HR, 2.16; 95% CI, 1.10-4.23), and diabetes mellitus (DM; adjusted HR, 3.49; 95% CI, 1.54-7.91) as independent risk factors after adjusting for multiple covariates including anti-diabetes medication. A scoring formula that used information of age, gender, MELD score, DM, and anti-diabetes regimen significantly discriminated patients at high or low risk of HCC, with sensitivity and specificity of

82.9% and 62.3%, respectively.

Conclusions: Age, gender, hepatic dysfunction, DM, and medication for DM are baseline factors that stratify the risk of HCC in cirrhotic patients who receive nucleos(t)ide analogues for CHB.

INTRODUCTION

Hepatitis B virus (HBV) infection is the leading etiology of liver-related morbidity and mortality, globally accounting for more than 50% of hepatocellular carcinomas (HCCs).[1, 2] Transcriptional and translational activity of the virus drives hepatocellular carcinogenesis in the natural history of chronic hepatitis B (CHB).[3, 4] Through inhibition of the viral polymerase, antiviral therapy using nucleos(t)ide analogue (NUC) potently suppresses HBV replication.[5] It can effectively ameliorate hepatitis, attenuate liver fibrosis, and delay disease progression.[6] Even overt cirrhosis may regress after long-term NUC therapy.[7, 8] Furthermore, a growing body of data has indicated that NUC treatment is associated with reduced occurrence and recurrence of HBV-related HCC.[9, 10]

Antiviral therapy may decrease but nevertheless does not eliminate the risk of HCC.[11] Some patients, especially those with existing cirrhosis, still develop HCC despite taking NUCs. The outcome determinants have not been elucidated in patients under antiviral treatment, and risk stratification in treated patients cannot rely on knowledge learned from untreated cohorts. This study aimed to investigate the chronological pattern and pretreatment risk factors of HCC in a CHB cohort with cirrhosis under continuous NUC therapy.

PATIENTS AND METHODS

Study design and patient population

This was a retrospective-prospective cohort study conducted in a teaching hospital in Taiwan (E-Da Hospital, Kaohsiung, Taiwan). The institutional review board of the hospital approved this study (protocol identification: EMRP-102-010). Through a computerized database, we first identified all CHB patients who received NUC between 1 September, 2007 and 31 March, 2013, and then manually reviewed their medical records to determine eligibility. The inclusion criteria were a positive serology of HBsAg or a documented history of HBV infection for 6 months or more, antiviral treatment with NUCs, presence of cirrhosis, and serum HBV DNA greater than 2,000 IU/mL. Cirrhosis was either histopathologically or clinically diagnosed. Clinical diagnosis was based principally on the sonographic evaluation of liver surface, parenchyma, vascular structure, and splenomegaly.[12] In the absence of histological proof, reimbursement of NUCs for the indication of CHB-related cirrhosis required presence of splenomegay or esophagogastric varices in addition to sonographic diagnosis.[9] Those who met any of the following criteria were excluded: superimposed infection with hepatitis C virus or human immunodeficiency virus, any malignant disease, organ transplantation, prior exposure to NUC or interferon, and occurrence of HCC within 3 months of therapy.

Antiviral treatment with NUC and surveillance for HCC

Enrolled patients received lamivudine 100 mg, entecavir 0.5 mg, telbivudine 600 mg, or tenofovir 300 mg once daily. Adefovir was not used in the first line but restricted in the rescue setting, per the regulation of the Taiwan National Health Insurance. For those who acquired on-treatment virological breakthrough, adefovir at a daily dose of 10mg was added. Occasionally, dosage might vary according to individual conditions such as renal impairment. All patients were followed up at an interval no longer than 3 months. All received HCC surveillance by means of ultrasonography and serum alpha-fetoprotein every 3 months in general.[13] HCC was diagnosed according to international guidelines.[2] Non-invasive diagnosis must fulfil characteristic features on dynamic images. Patients were observed from the initiation of NUC therapy until occurrence of HCC, death, loss to follow-up, or 1 January, 2014.

Assessment of clinical parameters and laboratory measurement

We manually reviewed and recorded clinical and laboratory data from the computerized database, including the behaviour of alcohol consumption with regard to the duration of drinking, types of beverage, and average amount per day. In principle, alcoholism was defined if the consumption exceeded 40g in men and 20g in women on a daily basis for 5 years.[14] Accuracy of the collected information was

audited by the principle investigator (YCH), who also ascertained the outcome of each enrolled subject. Serology of HBV was assayed by immunoassays (ABBOTT GmbH& Co., Wiesbaden, Germany). The serum level of HBsAg was semi-quantified with the upper bound of 250 IU/mL, per the manufacturer's protocol. Viral DNA was measured by the branched DNA assay (VERSANT® 440 Molecular System., Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) before 1 May, 2010, and afterward by the real-time PCR method (Roche COBAS® TaqMan® 48; Roche Diagnostics, Basel, Switzerland). The detection range was 357 to 17,857,100 IU/mL for the former assay and 6 to 110,000,000 IU/mL for the latter. Viral load was logarithmically transformed for expression, and values above the measurable range were recorded at one log above the upper bound. Virological breakthrough was defined if HBV DNA resurged to more than 10-fold from nadir; signature mutations for resistance were then sought. The Model for End stage Liver Disease (MELD) score,[15] the Aspartate aminotransferase to Platelet Ratio Index (APRI),[16] and the Risk Estimation for Hepatocellular Carcinoma in Chronic Hepatitis B (REACH-B) score were computed according to the original formulas.[17]

Data Analysis

Continuous variables were expressed with the median and interquartile range (IQR), and categorical variables with proportions. Death occurring prior to HCC was

considered as a competing risk event. The modified Kaplan-Meier method and the Gray's method were used to calculate the cumulative incidence of HCC.[18] Independent factors associated with HCC were analyzed by the modified Cox proportional hazard model that was adjusted for competing risks and multiple covariates.[19] The hazard ratio (HR) along with its 95% confidence interval (CI) was reported. Data was managed and analyzed by the commercially available software (Stata, version 9.1; Stata Corp, College Station, TX, USA). The competing risk analyses were performed using the R software with the "cmprsk_2.1-4" package. A p value <0.05 defined statistical significance.

RESULTS

Baseline characteristics of the study population

After screening a total of 1,630 consecutive patients (Figure 1), we finally enrolled 210 patients into analysis (Table 1). Thirty of the 49 diabetic patients had been using metformin. Drug resistance was detected in 2 patients taking entecavir (1.1% among all entecavir users). Cirrhosis was clinically diagnosed in most patients whereas histological proof was available in 24 participants (11.4%). Those who were clinically diagnosed appeared to be more severe on ultrasonography and endoscopy (Table 2).

HCC occurrence under continuous NUC therapy

Thirty five (16.7%) patients developed HCC during a median follow-up of 25.2 months (IQR, 16.3-37.3 months), with a cumulative incidence of 24.1% (95% CI, 16.3-32.0%) at 5 years (Figure 2). The vast majority of HCCs (n=34) occurred within 3 years of therapy.

Among 102 patients who had viral DNA data after one year, 86 patients (84.3%) found virus undetectable in serum. Except for 2 patients who were later confirmed to have drug resistance, all of the patients with detectable HBV DNA had viral load lower than 300 IU/mL (median 34 IU/mL, range 7- 248 IU/mL).

Univariate and multivariate-adjusted factors predictive of HCC under NUC

In the univariate Cox regression analyses (Table 3), age, gender, diabetes mellitus (DM), ascites, MELD and REACH-B scores were associated with HCC. In the multivariate-adjusted analysis including adjustment for anti-diabetes drugs in diabetic patients, older age, male gender, higher MELD score, and DM were independent risk factors.

Age >55 years, male gender, MELD score >12 points, and DM significantly discriminate the risk of HCC (Figure 3). Interestingly, the incidence of HCC was significantly lower in diabetic patients who took metformin than those who used other drugs (Supplementary Figure 1).

Risk score to predict the occurrence of HCC

These uncovered risk factors were weighted according to their regression coefficients in the Cox model (Table 4). The simplified calculation using integers was as accurate as that based on the original formula in predicting HCC (Figure 4A). Information of anti-diabetes medication significantly improved performance of the predictive model (Supplementary Figure 2). A risk score of 5 points or more significantly discriminate patients at high risk of HCC (Figure 4B), with sensitivity and specificity of 82.9% and 62.3%, respectively.

DISCUSSION

This study revealed that despite potent antiviral treatment, HCC still occurred frequently in cirrhotic patients with highly viremic CHB. Age, gender, hepatic dysfunction, and DM were independent risk factors unraveled in the multivariate-adjusted analysis. In addition, information of anti-diabetes medication was associated with improvement of risk stratification in diabetic patients. Our findings not only demonstrate that HCC surveillance remains essential in CHB patients under antiviral treatment, but also uncover those who require particular attention. Furthermore, this research underscores the unmet need for therapies beyond viral suppression to further attenuate risks in patients with advanced CHB.

Characterized by liver cirrhosis, male predominance, advanced age, and high serum level of HBV DNA, this cohort consists of patients at extremely high risk of HCC.[20, 21] Although emerging data indicates that cirrhosis may regress in NUC users, apparently it takes time, usually requiring 5 years or more.[7, 8] Importantly, we did not find markers of viral activity, i.e. concentration of viral DNA or serology of HBeAg, could stratify the risk, in contrast to previous studies of untreated CHB. Therefore, our findings exemplify the importance of different models for distinct scenarios. In view of the widespread use of NUCs for CHB, there is an urgent need for more knowledge to better understand the risk stratification in patients under treatment. Of note, our study focused on pretreatment factors that determined later development of HCC, but did not address their dynamic changes. Some parameters such as alpha-fetoprotein may change during treatment in association with occurrence of HCC.[22, 23]

Because age and hepatic dysfunction indicate chronicity and severity of accumulated hepatic damage, our data suggest that hepatocarcinogenesis in long-standing HBV infection cannot be sufficiently abolished by viral inhibition, at least not within 3 years of therapy. Longer observation is warranted to further elucidate the pattern and predictors of HCC occurring alongside the NUC treatment. Besides, the sexual dimorphism in HCC probably results from mechanisms beyond viral carcinogenesis,[24, 25] and therefore it may require a targeted therapy to attenuate

the risk conferred by male gender.

A number of studies have shown the association between DM and HCC,[26, 27] although the exact mechanism is incompletely understood.[28] Moreover, a recent research reported that diabetic patients were less likely to have cirrhosis regress after NUC therapy.[8] Our data further indicate that DM is becoming a major outcome determinant of CHB in the era of antiviral therapy. We also found an inverse association between metformin use and HCC risk, in line with existent literature.[29, 30] Because metformin is a first-line agent that diabetic patients usually start with, its use may identify those with early or mild DM and therefore result in the association. Whether there is anti-tumor efficacy associated with metformin is certainly interesting,[31] but beyond the scope of the present research. Regardless, our data supports that information of anti-diabetes medication is valuable for assessing the risk of HCC in diabetic patients.

Our study has the following strengths. First, stringent criteria for clinical diagnosis ascertained the presence of cirrhosis and enabled application of our findings to a clear patient group. Second, insomuch as virological data after one-year treatment attested potent viral inhibition, therapeutic efficacy was unlikely to confound the analysis. Furthermore, the competing risk analysis has accounted for influence of mortality on estimating the incidence of HCC.[32] Finally, all patients were followed up

at an interval shorter than 3 months, allowing timely detection of HCC.

The following limitations are noted. First, it requires external validation to extrapolate our conclusion to patients without cirrhosis. Second, we were unable to explore some potentially important factors including HBV genotype, familial predisposition, exposure to aflatoxin, and co-infection with hepatitis D virus (HDV). Incorporation of family history into analysis could introduce recall bias, especially when most participants were older than 50 years. Checkup of HDV is regrettably not a routine practice receiving reimbursement in Taiwan where the prevalence is low.[33, 34] Nevertheless, previous landmark studies from Asia did not find HDV was a significant determinant of HCC.[17, 20, 21] Finally, this single-center study from a referral hospital could not rule out the possibility of selection bias.

In summary, cirrhotic patients with HBV viremia still have a high risk of HCC despite treatment with NUCs, at least in the first 3 years of therapy. A clinically convenient model based on routinely available parameters that comprise age>55 years, male gender, MELD score>12 points, DM and medication for DM can stratify the risk.

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TRANSPARENCY DECLARATIONS

Yao-Chun Hsu reports having received lecture fees from GlaxoSmithKline, Novartis, Bristol-Myers Squibb, and the Harvester Trading Co. (the authorized distributor of tenofovir in Taiwan). Jaw-Town Lin reports having received research support in another study from the Gilead Sciences. There are no other conflicts of interest to declare. All other authors had nothing to declare.

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Table 1. Baseline characteristics of the study cohort

Characteristics	All (*n* = 210)	No HCC (*n* = 175)	HCC (*n* = 35)	*P*
Age, years	52.8 [46.0, 60.3]	52.0 [45.1, 59.9]	57.1 [50.8, 62.0]	0.01
Male gender, *n* (%)	154 (73.3%)	123 (70.3%)	31 (88.6%)	0.04
Body mass index, kg/m^2	25.6 [23.0, 28.2]	25.7 [23.0, 28.2]	24.0 [23.0, 26.4]	0.15
HBeAg positive, *n* (%)	46 (21.9%)	38 [21.7%]	8 [22.9%]	0.83
HBV DNA, log IU/ml	5.52 [4.22, 6.40]	5.44 [4.26, 6.31]	5.85 [4.21, 6.77]	0.49
HBsAg >100 IU/ml, *n* (%)	190 (90.5%)	159 (90.9%)	31 (88.6%)	0.75
AST, IU/L	66 [47, 98]	60 [43, 92]	86 [65, 127]	0.0003
ALT, IU/L	54 [42, 87]	53 [41, 83]	64 [48, 112]	0.07
Alpha-fetoprotein, ng/ml	7.77 [4.86, 15.84]	7.2 [4.7, 13.1]	14.3 [7.5, 26.6]	0.003
Bilirubin, mg/dL	1.26 [0.92, 1.77]	1.25 [0.91, 1.74]	1.38 [0.98, 2.34]	0.73
INR	1.12 [1.04, 1.22]	1.12 [1.03, 1.19]	1.16 [1.09, 1.28]	0.03
Creatinine, mg/dL	1.1 [1.0, 1.2]	1.1 [0.9, 1.2]	1.2 [1, 1.3]	0.004
Platelet, 10^3/μL	110 [75, 144]	111 [76, 145]	106 [68, 137]	0.7
Hemoglobin, g/dL	13.2 [11.3, 14.7]	13. 5[11.7, 14.7]	12.3 [10.9, 14.7]	0.23
Leucocyte, /μL	5230 [4260, 6810]	5230 [4260, 6630]	5200 [4290, 6820]	0.79
Diabetes mellitus, *n* (%)	49 (23.3%)	36 (20.6%)	13 (37.1%)	0.05
Hypertension, *n* (%)	31 (14.8%)	23 (13.1%)	8 (22.9%)	0.19
Dyslipidemia, *n* (%)	14 (6.7%)	13 (7.4%)	1 (2.9%)	0.47
Alcoholism, *n* (%)	28 (13.3%)	23 (13.1%)	5 (14.3%)	0.79
Splenomegaly, n (%)	157 (74.8%)	135 (77.1%)	22 (62.9%)	0.09
Ascites, *n* (%)	46 (21.9%]	35 (20.0%)	11 (31.4%)	0.18
Varices*, n/N (%)	63/122 (51.6%)	50/100 (50.0%)	13/22 (59.1%)	0.49
MELD score	10.17 [7.38, 12.38]	9.98 [7.33, 11.91]	11.46 [8.70, 14.97]	0.007
APRI	1.94 [1.03, 2.94]	1.70 [0.99, 2.86]	2.36 [1.60, 4.93]	0.01
REACH-B	11.5 [10,13]	11 [10, 13]	13 [11, 14]	0.005
Antiviral agent				0.04
Entecavir, *n* (%)	169 (80.5%)	137 (78.3%)	32 (91.4%)	
Tenofovir, *n* (%)	25 (11.9%)	25 (14.3%)	0	
Telbivudine, *n* (%)	11 (5.2%)	9 (5.1%)	2 (5.7%)	
Lamivudine, *n* (%)	5 (2.4%)	4 (2.3%)	1 (2.9%)	

Data are expressed as median [interquartile range) or number (percentage). *Only 122 patients had upper endoscopy at baseline; ALT, alanine aminotransferase; APRI, AST to platelet ratio index; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B s antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; INR, international normalized ratio; MELD, model for end-stage liver disease; REACH-B, risk estimation for hepatocellular carcinoma in chronic hepatitis B.

Table 2. Clinical evaluation of liver cirrhosis in patients with and without liver biopsy

	With liver histology (n=24)	Without liver histology (n=186)	P
Sonographic features			
Sonographic scores*	8 [7.5, 9]	9 [8, 10]	0.04
Splenomegaly, n (%)	13 (54.2%)	144 (77.4%)	0.02
Ascites, n (%)	2 (8.3%)	44 (23.7%)	0.12
Endoscopic findings#			
Esophageal varices, n (%)	3 (21.4%)	58 (53.7%)	0.04
Gastric varices, n (%)	1 (7.1%)	18 (16.7%)	0.69
Any varices, n (%)	4 (28.6%)	59 (54.6%)	0.09

Notes. *The sonographic scores comprised evaluation of liver surface, parenchyma, vascular structure, and splenomegaly, with a minimum of 4 and maximum of 11 points. # Endoscopy was performed in 122 patients at baseline.

Table 3. Univariate and multivariate Cox regression analyses for the risk factors of hepatocellular carcinoma

	Univariate analysis			Multivariate analysis		
Variable	Crude HR	95 % CI	*P*	Adjusted HR	95% CI	*P*
Age, per year	1.04	1.01~1.07	0.01			
Age >55 years	2.16	1.09~4.29	0.03	2.19	1.03-4.66	0.04
Male gender	3.05	1.08~8.64	0.04	3.07	1.05-9.02	0.04
Body mass index, kg/m^2	0.92	0.84~1.01	0.07			
HBsAg >100 IU/ml	0.54	0.19~1.55	0.25			
HBeAg positive	1.28	0.58~2.82	0.55			
HBV DNA, per log IU/ml	1.06	0.85~1.31	0.63			
AST, per 10U/L	1.0	0.99~1.01	0.90			
ALT, per 10U/L	1.0	0.98~1.01	0.76			
Alpha-fetoprotein, ng/ml	1.0	1.0~1.0	0.95			
Bilirubin, per mg/dL	1.0	0.90~1.10	0.93			
INR, per unit	2.26	0.72~7.10	0.16			
Creatinine, per mg/dL	1.14	0.93~1.41	0.20			
Platelet, per 10^3 cells/μL	1.0	0.99~1.01	0.73			
Hemoglobin, per g/Dl	0.92	0.80~1.05	0.21			
Leucocyte, per 10^3 cells/μL	1.06	0.94~1.20	0.32			
Diabetes mellitus*	2.13	1.07~4.23	0.03	3.49	1.54-7.91	0.003
Hypertension	1.63	0.74~3.60	0.22			
Dyslipidemia	0.34	0.05~2.50	0.29			
Alcoholism	1.16	0.45~3.00	0.76			
Splenomegaly	0.63	0.32~1.25	0.19			
Ascites	2.11	1.03~4.32	0.04			
MELD score, per point	1.06	1.01~1.12	0.02			
MELD >12 points	2.69	1.38~5.23	0.004	2.16	1.10-4.23	0.03
APRI, per point	1.01	0.95~1.06	0.83			
REACH-B, per point	1.23	1.04~1.45	0.02			
Antiviral therapy						
Entecavir	1					
Tenofovir	※					
Telbivudine	1.18	0.28~4.99	0.82			
Lamivudine	1.33	0.18~9.73	0.78			

*adjusted for use of metformin in the multivariate analysis, ※ not calculable due to no HCC in tenofovir users; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hazard ratio;

INR, international normalized ratio; MELD, model for end-stage liver disease; REACH-B, risk estimation for hepatocellular carcinoma in chronic hepatitis B.

Table 4. β coefficient in the Cox proportional hazard model and the corresponding risk scores to predict development of hepatocellular carcinoma

	β coefficient	95% CI	Score
Age			
> 55 years	0.78	0.08~1.48	2
Gender			
Male	1.11	0.05~2.17	3
MELD score			
> 12 points	0.76	0.07~1.45	2
Diabetes mellitus			
Diabetics using metformin	-0.55	-1.76~0.66	0
Diabetics without metformin	1.31	0.53~2.10	3

Figure 1

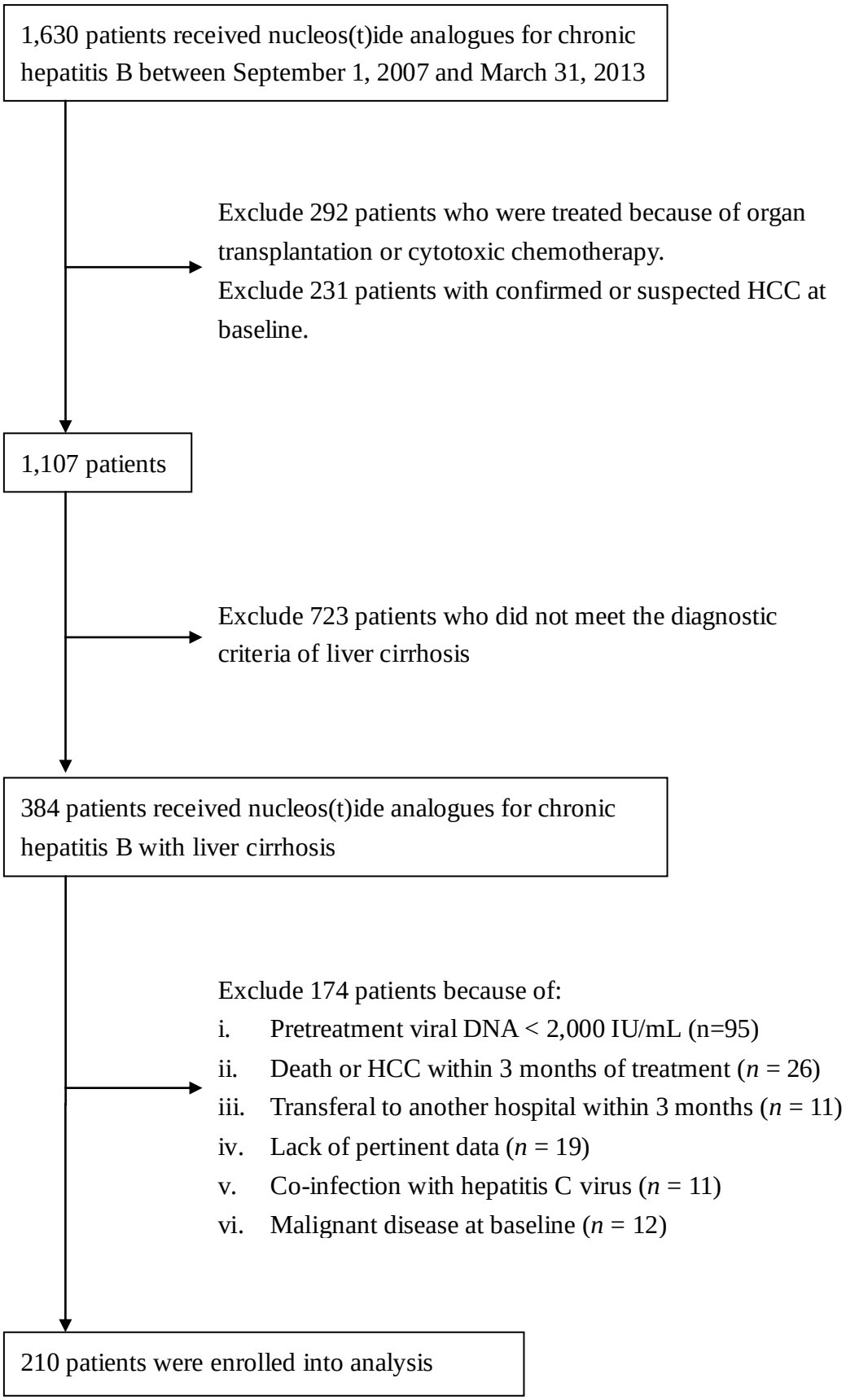

1,630 patients received nucleos(t)ide analogues for chronic hepatitis B between September 1, 2007 and March 31, 2013
Exclude 292 patients who were treated because of organ transplantation or cytotoxic chemotherapy.
Exclude 231 patients with confirmed or suspected HCC at baseline.
1,107 patients
Exclude 723 patients who did not meet the diagnostic criteria of liver cirrhosis
384 patients received nucleos(t)ide analogues for chronic hepatitis B with liver cirrhosis
Exclude 174 patients because of:
i. Pretreatment viral DNA < 2,000 IU/mL (n=95)
ii. Death or HCC within 3 months of treatment (n = 26)
iii. Transferal to another hospital within 3 months (n = 11)
iv. Lack of pertinent data (n = 19)
v. Co-infection with hepatitis C virus (n = 11)
vi. Malignant disease at baseline (n = 12)
210 patients were enrolled into analysis

Figure 2

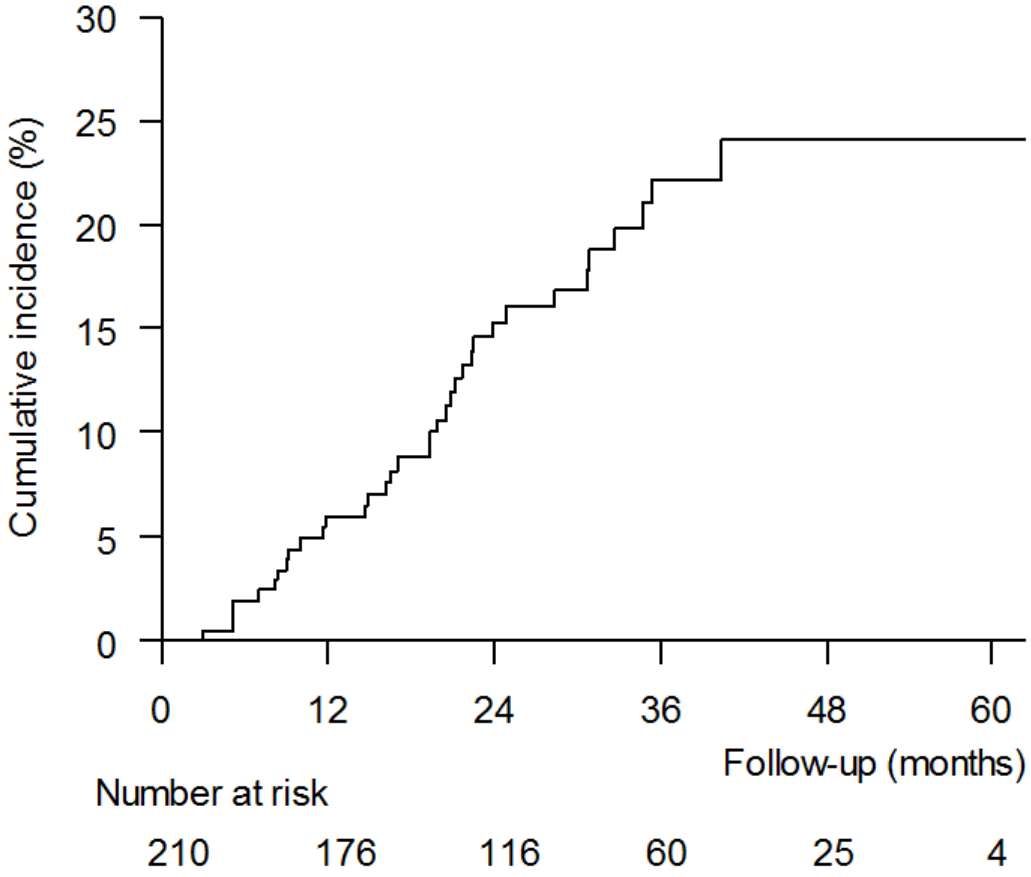

30
25
20
15
10
5
0
Cumulative incidence (%)
0
12
24
36
48
60
Follow-up (months)
Number at risk
210
176
116
60
25
4

Figure 3

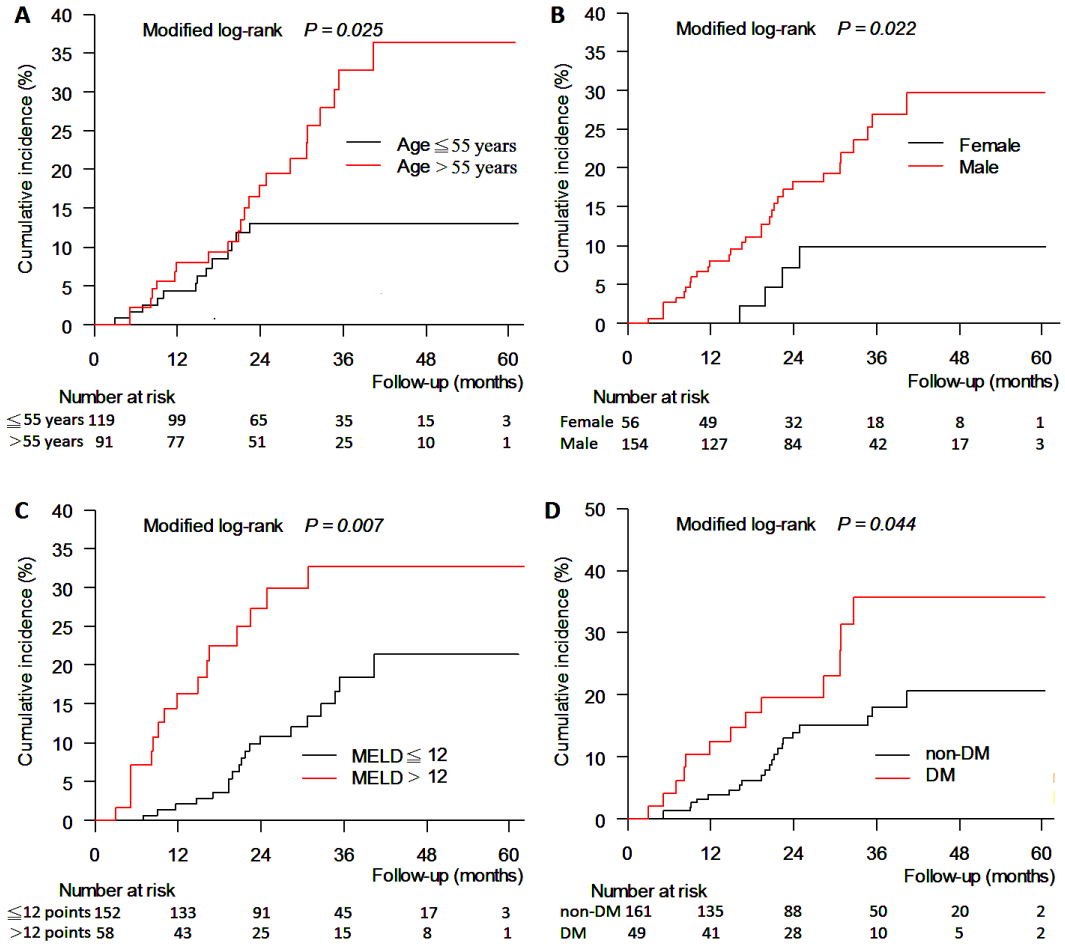


Figure 4

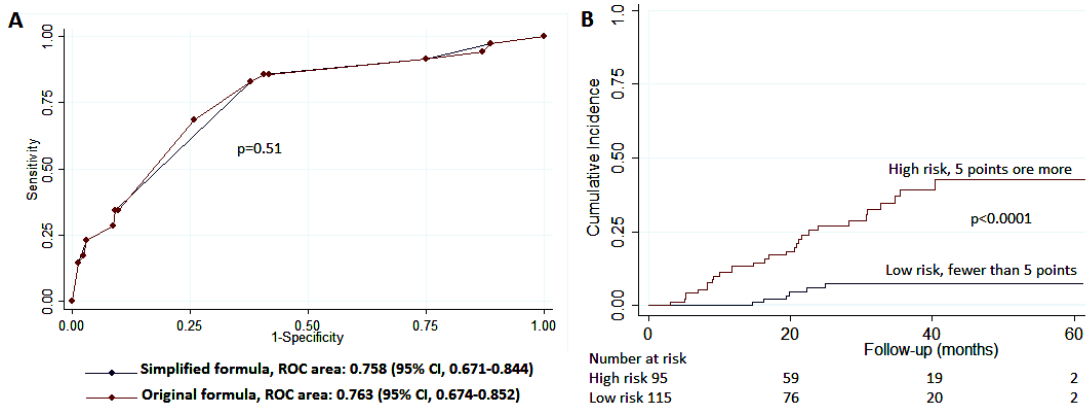


FIGURE LEGENDS:

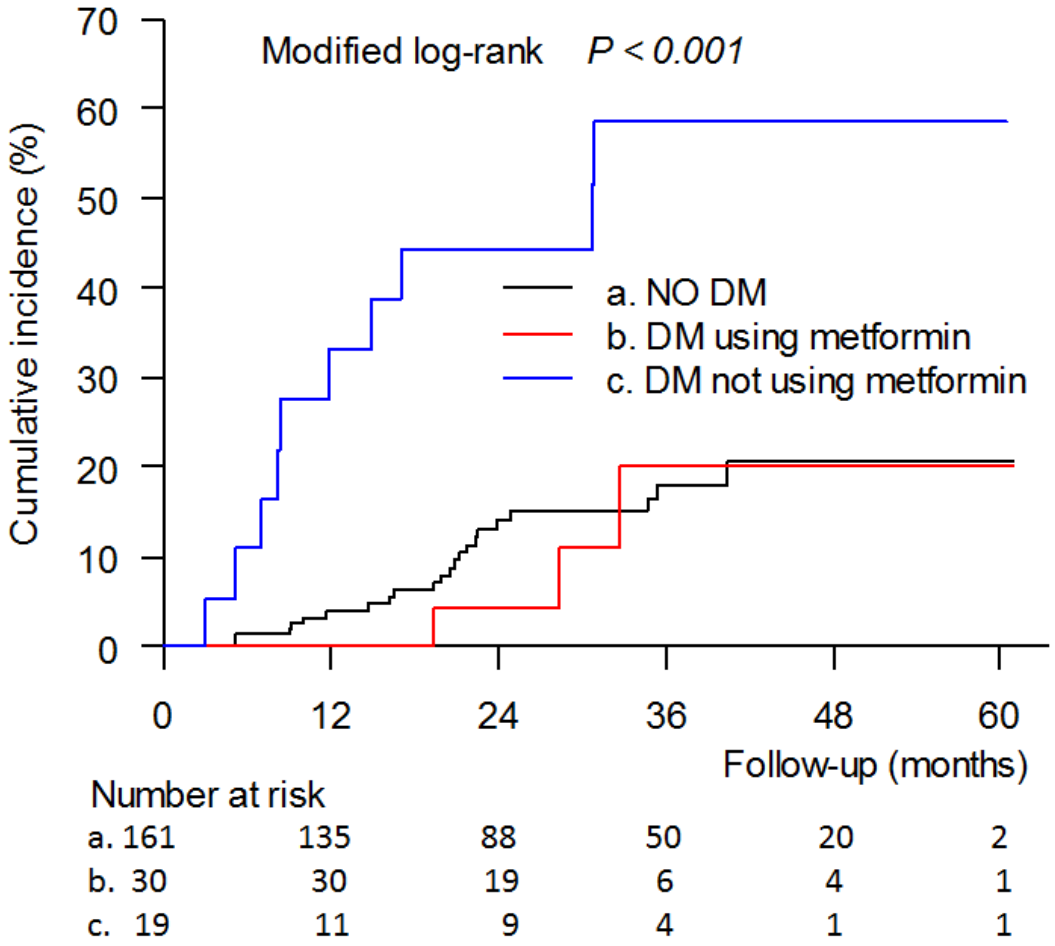
Figure 1: Flow chart of the enrollment process. HCC, hepatocellular carcinoma.

Figure 2: Incidence of hepatocellular carcinoma in cirrhotic patients under nucleos(t)ide analogues for chronic hepatitis B.

Figure 3: Incidence of hepatocelluar carcinoma stratified by risk factors at baseline. (A) stratified by age > or ≦55 years; (B) stratified by gendr; (C) stratified by MELD score > or ≦12 points; (D) stratified by DM; DM, diabetes mellitus; HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease.

Figure 4: Performance of the risk scores based on the baseline risk factors. (A) the receiver operating characteristic curves of the predictive formula to predict HCC; (B) a risk score of 5 points or more identifies patients at high risk of HCC. HCC, hepatocellular carcinoma.

Supplementary Figure 1



Supplementary Figure 2