Pathologic predictive factors for late recurrence of hepatocellular carcinoma in chronic hepatitis

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Pathologic predictive factors for late recurrence of hepatocellular carcinoma in chronic hepatitis

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<TABLE OF CONTENTS>

ABSTRACT ·····1

I. INTRODUCTION ·····3
II. MATERIALS AND METHODS ·····5
1. Case selection and clinical information ·····5
2. Evaluation of the histopathologic parameters of the background liver related to late recurrence of HCC ·····6
3. Evaluation of immunohistochemical markers of the background liver related to late recurrence of HCC ·····9
A. Patients and samples for reverse phase protein array (RPPA) experiments ·····9
B. Proteomic data from the RPPA experiments ·····9
C. Immunohistochemical analyses ·····10
D. Statistical analyses ·····11
III. RESULTS ·····13
1. Pathologic features of the background liver in the training and validation cohorts ·····13
2. Protein markers associated with late recurrence of HCC ·····15
3. Examination of the pathologic features and expression of immunohistochemical makers related to late recurrence of HCC ···17
4. Development and validation of a predictive model for late recurrence of HCC ·····23
5. Analyses of the HBV hepatitis patient subgroup ·····38
IV. DISCUSSION ·····46
V. CONCLUSION ·····55

REFERENCES ·····56

ABSTRACT (IN KOREAN) ·····62

LIST OF FIGURES

Figure 1. Study design ……5
Figure 2. Histopathologic features of the non-tumor liver parenchyma ……8
Figure 3. Expression of proteins associated with late recurrence of HCC ……16
Figure 4. Expressions of the immunohistochemical markers ……17
Figure 5. Kaplan–Meier curve of late recurrence-free survival according to the combinations of the immunohistochemical marker proteins; pSTAT3, pEKR1/2, and SYK in the training cohort ……25
Figure 6. Nomogram of the predictive model for 3- and 5-year probability of late recurrence of HCC ……29
Figure 7. Calibration plots for the predictive ability of the nomogram 31
Figure 8. Discrimination of the nomogram with time–dependent AUC (iAUC) comparison ……35

LIST OF TABLES

Table 1. Activity grading and fibrosis staging system ·····7
Table 2. Information on primary antibodies·····11
Table 3. Baseline characteristics of the training and validation cohorts 14
Table 4. Characteristics of training cohort (n = 402) without and with late recurrence of HCC ·····18
Table 5. Characteristics of the validation cohort (n = 243) without and with late recurrence of HCC ·····21
Table 6. Univariate Cox proportional hazards regression analysis for late recurrence of HCC in the training cohort ·····23
Table 7. Selection of variables using multiple Cox proportional hazards regression analyses ·····28
Table 8. Score on each point of the nomogram ·····30
Table 9. Comparison among the predictive abilities of the three models ·····33
Table 10. Comparison of the performance of the predictive models with immunohistochemical markers ·····37
Table 11. Characteristics of the HBV hepatitis patient subgroup of the training cohort (n = 335) without and with late recurrence of HCC ···39
Table 12. Characteristics of the HBV hepatitis patient subgroup of the validation cohort (n = 188) without and with late recurrence of HCC 41
Table 13. Univariate Cox proportional hazards regression analysis for late recurrence of HCC in the HBV hepatitis patient subgroup of the training cohort (n = 335) ·····43
Table 14. Multiple Cox proportional hazards regression analyses for late recurrence of HCC in the HBV hepatitis patient subgroup of the training cohort (n = 335) ·····45

ABSTRACT

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Background: Hepatocellular carcinoma (HCC) is associated with poor outcomes with a cumulative 5-year recurrence rate of >70% even after curative resection. Late recurrence (occurring >2 years after resection) is regarded as de novo HCC that typically develops from chronic liver disease via a multistep process. Recent reports have shown that the molecular genetic profile of the background liver rather than that of HCC itself predicts late HCC recurrence. In this study, we aimed to develop and validate a predictive model based on the histopathologic features and immunohistochemical marker expression that can prove useful in clinical practice.

Material and methods: Patients who had undergone curative resection for HCC were included to the training (n = 402) and validation (n = 243) cohorts, independently. Histopathologic features including lobular and porto-periportal inflammatory activity, fibrosis stage, and small or large liver cell changes were evaluated. To evaluate changes in the protein expression of genes related to late recurrence, 95 non-tumor liver tissue samples from HCC patients were screened using reverse phase microarray analysis. The expression of phosphorylated

signal transducer and activator of transcription 3 (pSTAT3), phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2), plasminogen activator inhibitor-1 (PAI-1), and spleen tyrosine kinase (SYK) was assessed using immunohistochemical staining. A predictive model was constructed using independent parameters selected using multiple Cox proportional hazards regression analysis. The same analysis was performed for the validation cohort to verify the reliability of the model for predicting late recurrence.

Results: In the training cohort, late recurrence of HCC was observed in 74 (18%) patients with a median follow-up period of 82 months. In HCC patients, late recurrence was correlated with cirrhosis (fibrosis stage 4); small and large liver cell changes; and upregulation of pSTAT3, pERK1/2, and PAI-1 levels ($p < 0.05$ for all). Cirrhosis (odds ratio [OR] = 2.0; 95% confidence interval [CI]: 1.2–3.2); moderate or severe lobular activity (OR = 21.4; 95% CI: 4.4–104.2); and expression of one or more of pSTAT3, pERK1/2, and SYK (OR = 6.0; 95% CI: 2.0–17.5) as detected with immunohistochemical staining were independently associated with late recurrence of HCC according to multivariate Cox regression analysis ($p < 0.05$ for all). A nomogram based on these variables was created to predict late recurrence of HCC, and Harrell's C index was 0.701 (95% CI: 0.64–0.75). In the validation cohort, 47 patients (19%) showed late recurrence with a median follow-up period of 56 months, and the Harrell's C index was 0.719 (95% CI: 0.64–0.79) for predicting late recurrence of HCC, demonstrating the high reliability of the predictive model.

Conclusion: Our predictive model that was based on cirrhosis (fibrosis stage 4); moderate or severe lobular activity; and expression of one or more of pSTAT3, pERK1/2, and SYK in the non-tumor tissue of the background liver is useful for predicting late recurrence of HCC following curative resection.

Key words: chronic hepatitis, hepatocellular carcinoma, late recurrence, lobular inflammation, fibrosis, cirrhosis, pSTAT3, pERK/12, SYK

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I. INTRODUCTION

Hepatocellular carcinoma (HCC) generally develops through a multistep process in the background liver with chronic hepatitis with various etiologies such as chronic inflammation, hepatocyte injury, regeneration, and fibrosis[1]. The cumulative 5-year recurrence rate of HCC after curative resection is as high as 70%[2,3], mainly owing to intrahepatic micro-metastasis from primary HCC or multi-centric occurrence from a background of inflamed liver parenchyma as de novo secondary HCC[4]. Early recurrence of HCC within 2 years of curative resection is termed intrahepatic metastasis and is associated with microvascular invasion, poor tumor differentiation, and microsatellite nodules of HCC[5]. In contrast, late recurrence (>2 years after curative resection) is considered to be related to chronic hepatitis of the background liver as a field effect[6].

Although several pathologic studies have predicted HCC recurrence, these mainly focused on the histopathologic and molecular features of the tumor itself that are related to and predict early recurrence[7-11]. Several studies on late recurrence of HCC have used clinical data, such as serum levels of alpha-fetoprotein, hepatitis B virus (HBV) antigen, HBV DNA, or aspartate

aminotransferase/platelet ratio index, age, and remaining liver function, to predict outcome[7,12-16]. With these factors, the status of the remnant liver tissue after curative resection of HCC has been consistently considered an important risk factor for late recurrence[6,15,16]. The annual incidence of late recurrence of HCC is 25%–35%, whereas the incidence of new cases is 1%–7%[6]; therefore, the risk of developing secondary HCC is increased in patients with a history of HCC. Moreover, advances in early detection and treatment of HCC have increased patient survival period, increasing the risk of developing late recurrence. Therefore, the prediction of late recurrence and provision of individual treatment to prevent late recurrence of HCC is crucial.

The risk of multiple occurrence or late recurrence of HCC has been reportedly predicted based on the expression profiles of specific genes in non-tumor liver tissues after surgical resection[8,17]. Such gene signatures are associated with inflammatory and oxidative stress response and growth signaling. The molecular signatures of hepatic injury and regeneration (HIR) of the background (non-tumor) liver were recently reported to be closely associated with late recurrence and poor prognosis[8,17,18] based on gene profiling using cDNA and RNA from the non-tumor liver tissue. However, these genetic signatures are too complex to be applicable in daily clinical practice.

In the present study, we investigated the histopathologic parameters and immunohistochemical molecular marker expressions in the background liver that were associated with late recurrence of HCC after curative resection in two independent cohorts. A model for predicting late recurrence of HCC based on the resected HCC specimens was developed and validated; this model can be useful in clinical practice.

II. MATERIALS AND METHODS

1. Case selection and clinical information

The patients included in this retrospective study were assigned to two independent cohorts from different institutes: a training cohort to establish a predictive model and a validation cohort to confirm its reliability. The training cohort included consecutive patients who underwent curative resection for HCC at Severance Hospital from March 2006 to February 2011. Of these, 402 were eligible for evaluation of the non-tumor liver tissue. The median follow-up period was 82 months (range: 1–135 months). The validation cohort comprised 243 consecutive patients who underwent curative resection for HCC at Seoul National University Bundang Hospital from January 2003 to February 2013. The median follow-up period was 56 months (range: 1–155 months) (Figure 1). Clinical data regarding age, sex, etiology of chronic liver disease, and time to recurrence were retrospectively reviewed from the electronic medical records. Early and late recurrences were defined as recurrence within or after 2 years, respectively, of curative resection for HCC[8].

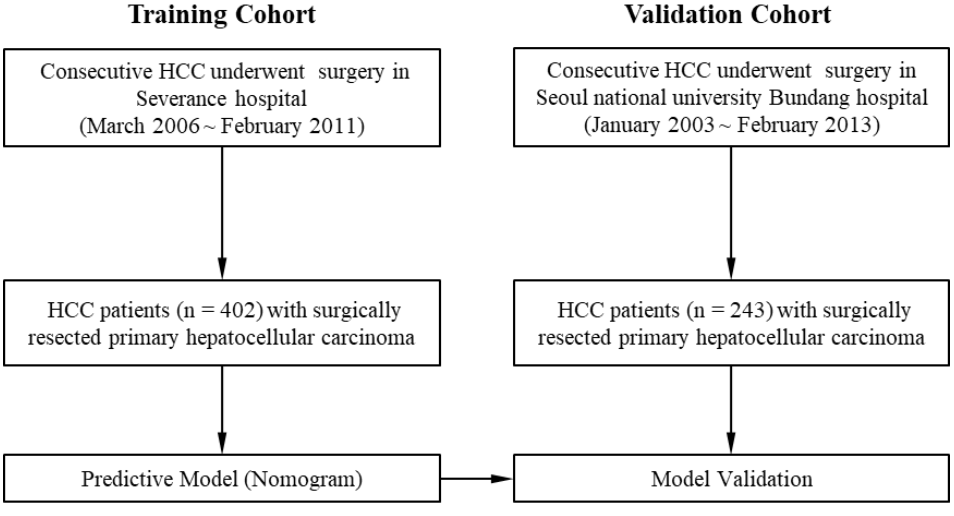

Figure 1. Study design. A total of 402 non-tumor liver tissue specimens in the

training cohort were evaluated and used to generate a predictive model that was validated using data from an independent validation cohort.

This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (2017-3562-001), and Seoul National University Bundang Hospital (B-1804-460-305), and the need for patient consent was waived.

2. Evaluation of the histopathologic parameters of the background liver related to late recurrence of HCC

For histopathologic evaluation of the background liver, representative paraffin-embedded sections of the non-tumor liver parenchyma were examined using hematoxylin and eosin and Masson's trichrome staining. Lobular and porto-periportal inflammatory activity and fibrosis stage of chronic hepatitis were assessed as per the criteria proposed by the Korean Study Group for the Pathology of Digestive Disease [19,20] (Table 1 and Figure 2A–C). For the stage 4 fibrosis (cirrhosis), it was divided into three subcategories, 4a, 4b, and 4c, according to septal thickness[21,22]. Small and large liver cell changes (SLCCs and LLCCs, respectively) were also evaluated as per previously established criteria[23-25]. LLCCs were defined as hepatocytes with both nuclear and cellular enlargement, preserved nucleus/cytoplasm ratio, nuclear pleomorphism, frequent nuclear hyperchromasia, and multinucleation[23]. SLCCs were defined as hepatocytes with reduced cell volume, increased nucleus/cytoplasm ratio, mild nuclear pleomorphism and hyperchromasia, and cytoplasmic basophilia that gave the impression of nuclear crowding[24] (Figure 2D). The proportion of liver cell changes in the whole section was estimated; a positive result was defined as the presence of at least 5% of each type of liver cell change.

Table 1. Activity grading and fibrosis staging system

Features	Score	Definition
Lobular activity	Grade 0 (no)	No necrosis
	Grade 1 (minimal)	Sinusoidal lymphocytosis ± one or fewer necrotic area per 10× objective field
	Grade 2 (mild)	2 –5 necrotic areas per 10× objective field
	Grade 3 (moderate)	6 –10 necrotic areas per 10× objective field
	Grade 4 (severe)	≥10 necrotic areas per 10× objective field or confluent necrosis (zone 3)
Porto-periportal activity	Grade 0 (no)	<Mild portal inflammation
	Grade 1 (minimal)	>Mild portal inflammation ± focal piecemeal necrosis in a few portal tracts
	Grade 2 (mild)	Piecemeal necrosis, focal in some or most portal tracts or <50% in a few portal tracts
	Grade 3 (moderate)	Piecemeal necrosis, <50% in most portal tracts or >50% in a few or some portal tracts
	Grade 4 (severe)	Piecemeal necrosis, >50% in most portal tracts/septal surfaces or bridging necrosis
Fibrosis	Stage 0 (no)	Normal connective tissue
	Stage 1 (portal)	Fibrous portal expansion
	Stage 2 (periportal)	Periportal fibrosis with short septa extending into lobules or rare porto-portal septa (intact architecture)
	Stage 3 (septal)	Fibrous septa reaching adjacent portal tracts and terminal hepatic venule (architectural distortion but no obvious cirrhosis)
	Stage 4 (cirrhosis)	Diffuse nodular formation
	Stage 4a	Most septa are thin (one broad septum allowed)
	Stage 4b	At least two broad septa, but no very broad septa
	Stage 4c	At least one very broad septum or more than half of minute nodules (micronodular cirrhosis)

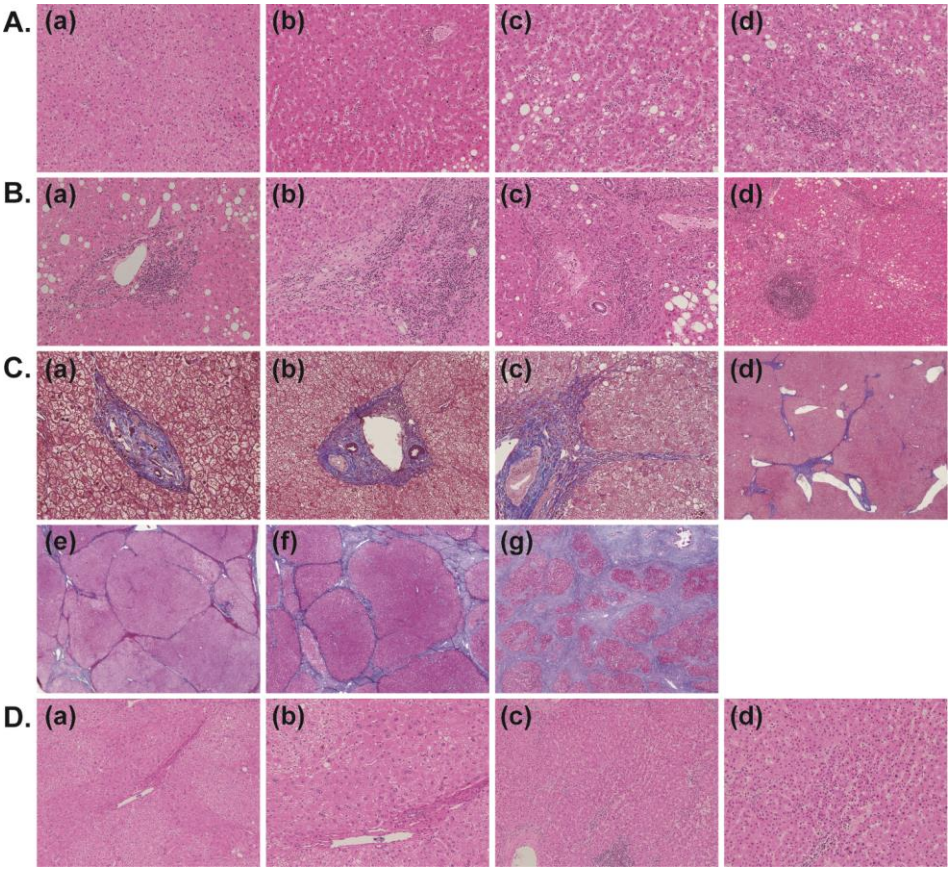

Figure 2. Histopathologic features of the non-tumor liver parenchyma. A. Lobular activity: (a) grade 0 (hematoxylin and eosin [HE], 200×), (b) grade 1 (HE, 200×), (c) grade 2 (HE, 200×), (d) grade 3 (HE, 200×). B. Porto-periportal activity: (a) grade 1 (HE, 200×), (b) grade 2 (HE, 200×), (c) grade 3 (HE, 200×), (d) grade 4, bridging necrosis (HE, 100×). C. Fibrosis: (a) stage 0 (Masson's trichrome [TRC], 200×), (b) portal fibrosis (TRC, 200×), (c) periportal fibrosis (TRC, 100×), (d) septal fibrosis (TRC, 40×), (e) cirrhosis 4a, (TRC, 40×), (f) cirrhosis 4b, (TRC, 40×), (g) cirrhosis 4c, (TRC, 40×). D. Liver cell changes (LCCs): (a) Large LCC (LLCC; HE, 100×), (b) LLCC, (HE, 200×), (c) small LCC (SLCC; HE, 100×), (d) SLCC (HE, 200×).

3. Evaluation of immunohistochemical markers of the background liver related to late recurrence of HCC

A. Patients and samples for reverse phase protein array (RPPA) experiments

Protein expression profiling was performed with an RPPA platform using archived non-tumor liver tissue samples of 95 HCC patients (MD Anderson Cancer Center cohort) who underwent hepatectomy as primary treatment. Samples were frozen in liquid nitrogen and stored at −80°C until protein extraction. The study protocol was approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center.

B. Proteomic data from the RPPA experiments

RPPA experiments were performed using 95 surgically resected, frozen, non-tumor surrounding tissue samples as previously described[26-30]. Briefly, protein was extracted from the tissue using RPPA lysis buffer composed of 1% Triton X-100, 50 nmol/L HEPES (pH 7.4), 150 nmol/L NaCl, 1.5 nmol/L $MgCl_2$, 1 mmol/L EGTA, 100 nmol/L NaF, 10 nmol/L Na pyrophosphate, 10% glycerol, 1 nmol/L phenylmethylsulfonyl fluoride, 1 nmol/L Na_3VO_4, and 10 mg/mL aprotinin. Lysis buffer was used to lyse the frozen tumors by homogenization using a Precellys homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). The protein concentration in the lysates was adjusted to 1 μg/μL, as determined with the bicinchoninic acid assay, and the samples were boiled in 1% sodium dodecyl sulfate. Serial 5-fold dilutions were prepared with lysis buffer. An Aushon Biosystems 2470 arrayer (Burlington, MA, USA) was used to print 1056 samples onto nitrocellulose-coated slides (Grace Bio-Labs, Bend, OR, USA), which were then probed with 172 validated primary antibodies followed by corresponding secondary antibodies (goat anti-rabbit or -mouse or rabbit anti-goat IgG). Signals were visualized with

diaminobenzidene colorimetric reaction using a DakoCytomation catalyzed signal amplification system (Dako, Glostrup, Denmark). Slides were scanned with a CanoScan 9000F (Canon, Tokyo, Japan). Spot intensity was analyzed and quantified using Arraypro (http://www.mediacy.com/index.aspx?page=ArrayPro) (Level 1 data). SuperCurveGUI software[31] (available at http://bioinformatics.mdanderson.org/Software/supercurve/) was used to estimate the half-maximal effective concentration of proteins in each dilution series (in log2 scale). The RPPA data were processed and normalized as previously described[26-30]. All samples were processed and printed as a single batch. Final antibody selection was dictated by the availability of high-quality antibodies that consistently passed a strict validation process[32]. These antibodies were assessed for specificity, quantifiability, and sensitivity (dynamic range) using protein extracts from cultured cells or tumor tissue. The probability of HIR was determined, as previously reported[18], based on the mRNA expression data of the same tissue samples.

C. Immunohistochemical analyses

Representative paraffin-embedded sections of the non-tumor liver parenchyma were used for tissue microarray construction and immunohistochemical analyses. All the hematoxylin and eosin-stained slides were reviewed, and the representative areas were carefully selected and marked on individual paraffin blocks; 2.0-mm tissue cores were collected from each tumor specimen.

Details of the antibodies used are summarized in Table 2. Immunohistochemical staining was performed using the Ventana BenchMark XT automated immunostainer and reagents from Ventana Medical Systems (Tucson, AZ, USA). The slides were dried at 60℃ for 1 h

and deparaffinized using EZ Prep at 75℃ for 4 min. Cell conditioning was performed using CC1 solution at 100℃ for 64 min. Signals were detected using the OptiView DAB IHC Detection kit (Ventana Medical Systems). Counter staining was performed with hematoxylin I for 4 min at room temperature. Positive expression was defined as ≥1% staining.

Table 2. Information on primary antibodies

Antibody	Source	Clone	Dilution	Antigen retrieval
pSTAT3	Cell signaling technology, Danvers, MA, USA	D3A7	1:300	Microwave, Tris-EDTA (pH 8.0)
p-ERK1/2 (T202/Y204)	Cell signaling technology, Danvers, MA, USA	D13.14.4E	1:100	Automated immunostainer
SYK	Cell signaling technology, Danvers, MA, USA	D2Z1E	1:50	Automated immunostainer
PAI-1	Abcam, Cambridge, UK	1D5	1:100	Microwave, Citrate (pH 6.0)

pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase; PAI-1, plasminogen activator inhibitor-1

D. Statistical analyses

Descriptive statistics of the baseline characteristics of the training and validation cohorts are expressed as mean ± standard deviation values for continuous variables and as frequencies (percentages) for categorical variables. Data were analyzed using the t-test and Mann–Whitney U-test for continuous variables and χ^2 test or Fisher's exact test for the categorical variables. Univariate Cox proportional hazards regression analyses were performed to identify significant risk factors among histopathologic parameters and immunohistochemical markers. To confirm the significance of differences in late recurrence-free survival of HCC between the immunohistochemical marker combinations (all negative or one, two, or

three with positive expression), survival curves were generated using the Kaplan–Meier product-limit method, and late recurrence-free survival was compared across immunohistochemical marker combinations using the log-rank test.

The following three potential predictive models were considered: histopathologic parameters only (Model 1), histopathologic parameters + individual immunohistochemical markers (Model 2), and histopathologic parameters + immunohistochemical marker combinations (Model 3). Final risk factors from the histopathologic parameters were selected using a stepwise variable selection method, and multiple Cox proportional hazards regression analyses were performed for each of the three models.

A nomogram was used to provide a visual explanation of the survival outcome predicted by the model. A point system was used for scoring, based on the weighted (relative) importance of individual risk factors; individual probabilities for 3- and 5-year recurrence-free survival of HCC patients were calculated. The nomogram validation comprised two parts—calibration and discrimination.

Calibration was performed by comparing the means of the nomogram-predicted late recurrence with those of the actual late recurrence distributions as determined using the Kaplan–Meier estimates. It was assessed by stratifying the patients into different groups according to predictive probability, and a bootstrap method was used to reduce bias. If the predictive model is well calibrated, the calibration plot should indicate an agreement between the predicted and observed probability of late recurrence HCC with a smooth line.

The predictive accuracy of the final model was assessed by discrimination using the training and validation cohorts. Discrimination was evaluated by calculating the concordance index (Harrell's C index[33]) and measured as the time-dependent incremental area under the receiver operating

characteristic curve [Heagerty's integrated AUC (iAUC)[16]] for the following three models: histopathologic parameters only (Model 1), histopathologic parameters + individual immunohistochemical markers (Model 2), and histopathologic parameters + immunohistochemical marker combination (Model 3). Differences and 95% confidence intervals (CIs) between the outcome and model were calculated using a bootstrapping method: values ranged from 0.5 (no discrimination) to 1.0 (perfect discrimination).

We also computed the net reclassification improvement (NRI) and the integrated discrimination improvement (IDI) at 3 and 5 years to assess performance improvements in the predictive model with immunohistochemical markers[34]. The same comparison was conducted for the validation cohort.

Two-sided *p* values of <0.05 and excluding 0 in the 95% CI were considered to indicate statistical significance. Statistical analyses were performed using SPSS v.23.0 (SPSS Inc., Chicago, IL, USA), SAS v.9.2 (SAS Institute, Cary, NC, USA), and R v.3.2.5 (survival, rms, and risksetROC packages) (R Foundation for Statistical Computing, Vienna, Austria).

III. RESULTS

1. Pathologic features of the background liver in the training and validation cohorts

In the training cohort (n = 402), early recurrence (n = 132, 33%) and late recurrence (n = 74, 18%) patients were included during the 82-month follow-up period. In the validation cohort (n = 243), early recurrence (n = 75, 30%) and late recurrence (n = 47, 19%) patients were included during the 56-month follow-up period. Histopathologic features examined were lobular

or porto-periportal activity, fibrosis stage, and proportion of LLCCs and SLCCs (Table 3).

Table 3. Baseline characteristics of the training and validation cohorts

	Training Cohort (Severance hospital) (n = 402)	**Validation Cohort (SNUBH) (n = 243)**	**p-value**
Clinical characteristics			
Age (year, mean ± SD)	55.21 ± 10.48	56.83 ± 10.56	0.070
Sex (Male/Female)	330(82%)/72(18%)	189(78%)/54(22%)	0.181
Etiology (HBV/HCV/Alcohol/Unknown)	335(83%)/16(4%)/17(4%)/34(8%)	188(77%)/14(6%)/16(7%)/25(10%)	0.273
Histopathologic features			
Grading			
Lobular activity (no/minimal/mild/moderate/severe)	18(4%)/70(18%)/310(77%)/4(1%)/0(0%)	10(4%)/53(22%)/178(73%)/2(1%)/0(0%)	0.587
Periportal activity (no/minimal/mild/moderate/severe)	18(5%)/82(20%)/260(65%)/41(10%)/1(0%)	13(5%)/70(29%)/142(58%)/17(7%)/1(0%)	0.108
Staging			
Fibrosis (0/1/2/3/4A/4B/4C)	5(1%)/11(3%)/62(15%)/116(29%)/52(13%)/129(32%)/27(7%)	4(2%)/14(6%)/39(16%)/62(26%)/40(16%)/74(30%)/10(4%)	0.266
Cirrhosis (present/absent)	194(48%)/208(52%)	119(49%)/124(51%)	0.861

Liver cell change			
Liver cell change (LLC) (No/Large LCC only/Small LCC only/ Both LCC)	117(29%)/164(41%)/3(1%)/118(29%)	69(28%)/95(39%)/6(3%)/73(30%)	0.336
Large LCC (%, mean ± SD)	10.08 ± 11.23	8.35 ± 9.12	0.168
Small LCC (%, mean ± SD)	2.95 ± 5.71	2.75 ± 4.63	0.620
Follow-up data			
Recurrence (yes/no)	196(49%)/206(51%)	121(50%)/122(50%)	0.798
Early recurrence (<2 years)/Late recurrence (≥2years)	132(33%)/74(18%)	75(30%)/47(19%)	0.866
Death (yes/no)	85(21%)/317(79%)	36(15%)/207(85%)	**0.046***
Follow-up period (months) (Median [range])	82 (1-135)	56 (1-155)	**<0.001***

2. Protein markers associated with late recurrence of HCC

We generated proteomic data from 95 non-tumor liver tissue samples using RPPA. In a previous study, we established the genomic HIR probability that is significantly associated with de novo late recurrence[18]. To identify protein markers associated with late recurrence of HCC, we examined proteins whose expression or phosphorylation state was significantly correlated with HIR probability ($p < 0.05$); thus, we identified the following eight proteins: spleen tyrosine kinase (SYK), neurofibromin 2 (NF2), pSTAT3 (Y705), plasminogen

activator inhibitor (PAI)-1, RAB25, phosphorylated mitogen-activated protein kinase (pMAPK) (T202), phosphorylated estrogen receptor α (pERα) (S118), and 14-3-3ε (Figure 3). Among these, the levels of SYK, NF2, pSTAT3 (Y705), PAI, and RAB25 were positively correlated, whereas those of PI3K-p110α, pMAPK (T202), pERα (S118), and 14-3-3ε were negatively correlated, with HIR gene signature.

Among the eight proteins that showed significant correlation with known HIR probabilities, pSTAT3, pERK1/2, SYK, and PAI-1, were suitable for immunohistochemistry. The nuclear expression of pSTAT3 and pERK1/2 and the cytoplasmic expression of SYK and PAI-1 were evaluated (Figure 4).

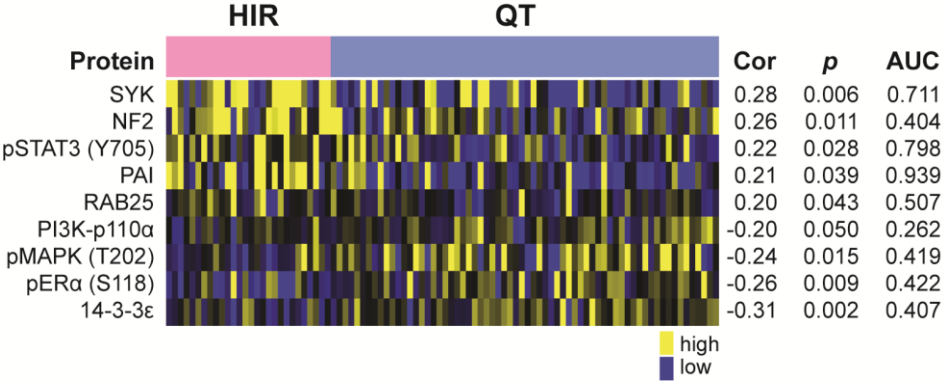

Figure 3. Expression of proteins associated with late recurrence of HCC. Proteins with expressions significantly correlated with hepatic injury and regeneration (HIR) probability were identified as potential markers of late recurrence. Of 202 proteins, only eight were significantly associated ($p < 0.05$) with the probability of HIR subtype associated with late recurrence of HCC. QT, quiescent; Cor, Pearson correlation; *p*, p-value; AUC; area under the receiver operating characteristic curve for late recurrence of HCC; SYK, spleen tyrosine kinase; NF2, neurofibromin 2; pSTAT3, phosphorylated signal transducer and activator of transcription 3; PAI-1, plasminogen activator inhibitor-1; PI3K-p110α, phosphatidylinositol-4,5-biphosphate3-kinase; pMAPK, phosphorylated mitogen-activated protein kinase, pERα, phosphorylated

estrogen receptor α.

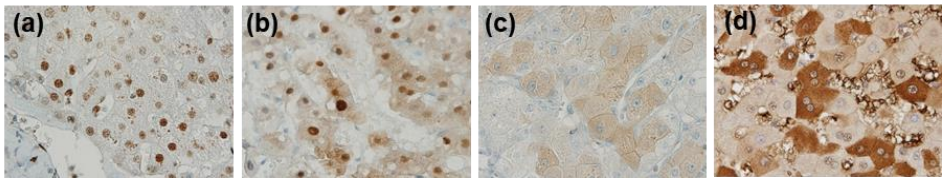

Figure 4. Expressions of the immunohistochemical markers. Hepatocytes in chronic liver disease expressing (a) pSTAT3, (b) pERK1/2, (c) SYK, and (d) PAI-1 (200×).

3. Examination of the pathologic features and expression of immunohistochemical markers related to late recurrence of HCC

In the training cohort, HCC patients with late recurrence showed a higher frequency of cirrhosis (fibrosis stage 4) (p = 0.012) and more extensive LLCCs and SLCCs (p = 0.006 and 0.013, respectively) in the background liver (Table 4). pSTAT3, pERK1/2, SYK, and PAI-1 were selected as markers related to late recurrence of HCC for immunohistochemical analysis because suitable antibodies were available for these proteins that were applicable to formalin-fixed, paraffin-embedded tissue. pSTAT3, pERK1/2, and PAI-1 protein levels were higher in HCC patients with than in those without late recurrence ($p < 0.05$ for all) (Table 4).

Table 4. Characteristics of training cohort (n = 402) without and with late recurrence of HCC (after 2 years)

	No late recurrence (n=328)	Late recurrence (after 2 years) (n = 74)	p-value
Clinical characteristics			
Age (year, mean ± SD)	55.31 ± 10.64	54.78 ± 9.74	0.543
Sex (male/female)	268 (82%)/60 (18%)	62 (84%)/12 (16%)	0.674
Etiology (HBV/HCV/Alcohol/Unknown)	272 (83%)/10 (3%)/16 (5%)/30 (9%)	63 (85%)/6 (8%)/1 (2%)/4 (5%)	0.082
Histopathologic features			
Grading			
Lobular activity (no/minimal/mild/moderate/severe)	14 (4%)/60 (18%)/252 (77%)/2 (1%)/0 (0%)	4 (5%)/10 (14%)/58 (78%)/2 (3%)/0 (0%)	0.303
Periportal or septal activity (no/minimal/mild/moderate/severe)	14 (4%)/69 (21%)/215 (66%)/29 (9%)/1 (0%)	4 (5%)/13 (18%)/45 (61%)/12 (16%)/0 (0%)	0.381
Staging			
Cirrhosis	160 (49%)	48 (65%)	**0.012***
Fibrosis (0/1/2/3/4a/4b/4c)	5 (1%)/10 (3%)/52 (16%)/101 (31%)/41 (12%)/97 (30%)/22 (7%)	0 (0%)/1 (1%)/10 (14%)/15 (20%)/11 (15%)/32 (43%)/5 (7%)	0.233
Liver cell change			
Liver cell change (LLC)	100 (30%)/138 (42%)/2 (1%)/88 (27%)	17 (23%)/26 (35%)/1 (1%)/30 (41%)	0.102

(No/Large LCC only/ Small LCC only/ Both LCC)			
Large LCC (%, mean ± SD)	9.33 ± 10.82	13.39 ± 12.43	**0.006***
Small LCC (%, mean ± SD)	2.62 ± 5.29	4.39 ± 7.14	**0.013***
Immunohistochemical marker expression			
pSTAT3 (positive/negative)	47 (14%)/281 (86%)	18 (24%)/56 (76%)	**0.035***
pERK1/2 (positive/negative)	90 (27%)/238 (73%)	35 (47%)/39 (52%)	**0.001***
SYK (positive/negative)	94 (29%)/234 (71%)	25 (34%)/49 (66%)	0.383
PAI-1 (positive/negative)	181 (55%)/147 (45%)	53 (72%)/21 (28%)	**0.010***

pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase; PAI-1, plasminogen activator inhibitor-1.

In the validation cohort, HCC patients with late recurrence showed a higher frequency of moderate to severe lobular activity, cirrhosis (fibrosis stage 4), LLCCs, and SLCCs than those without ($p < 0.05$ for all). In addition, the rates of pSTAT3 and pERK1/2 positivity were higher in HCC patients with late recurrence than in those without ($p = 0.045$ and 0.040, respectively), although SYK expressions of the two groups were not significantly different ($p = 0.294$) (Table 5).

Table 5. Characteristics of the validation cohort (n = 243) without and with late recurrence of HCC (after 2 years)

	No late recurrence (n=196)	Late recurrence (after 2 years) (n = 47)	p-value
Clinical characteristics			
Age (year, mean ± SD)	56.42 ± 10.49	58.57 ± 10.77	0.266
Sex (male/female)	150 (77%)/46 (23%)	39 (83%)/8 (17%)	0.340
Etiology (HBV/HCV/Alcohol/Unknown)	153 (78%)/13 (7%)/12 (6%)/18 (9%)	35 (75%)/1 (2%)/4 (8%)/7 (15%)	0.403
Histopathologic features			
Grading			
Lobular activity (no/minimal/mild/moderate/severe)	9 (5%)/32 (16%)/155 (79%)/0 (0%)/0 (0%)	1 (2%)/21 (45%)/23 (49%)/2 (4%)/0 (0%)	**<0.001***
Periportal or septal activity (no/minimal/mild/moderate/severe)	10 (5%)/59 (30%)/116 (59%)/10 (5%)/1 (1%)	3 (6%)/11 (24%)/26 (55%)/7 (15%)/0 (0%)	0.182
Staging			
Cirrhosis	92 (47%)	32 (68%)	**0.009***
Fibrosis (0/1/2/3/4a/4b/4c)	4 (2%)/13 (7%)/37 (19%)/50 (25%)/27 (14%)/56 (29%)/9 (4%)	0 (0%)/1 (2%)/2 (4%)/12 (26%)/13 (28%)/18 (38%)/1 (2%)	**0.035***
Liver cell change			
Liver cell change (LLC)	57 (29%)/82 (42%)/5 (3%)/52 (26%)	12 (25%)/13 (28%)/1 (2%)/21 (45%)	0.098

(No/Large LCC only/ Small LCC only/ Both LCC)			
Large LCC (%, mean ± SD)	10.33 ± 9.40	14.68 ± 12.57	**0.038***
Small LCC (%, mean ± SD)	3.65± 6.72	5.72 ± 7.94	**0.041***
Immunohistochemical marker expression			
pSTAT3 (positive/negative)	54 (28%)/142 (72%)	20 (43%)/27 (57%)	**0.045***
pERK1/2 (positive/negative)	57 (29%)/139 (71%)	21 (45%)/26 (55%)	**0.040***
SYK (positive/negative)	33 (17%)/163 (83%)	11 (23%)/36 (77%)	0.294

pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase

4. Development and validation of a predictive model for late recurrence of HCC

A univariate Cox proportional hazards regression analysis was used to identify the significant risk factors for late recurrence of HCC among the histopathologic parameters and expressed immunohistochemical markers. (Table 6). HCV, lobular activity, periportal activity, cirrhosis, liver cell change, and pSTAT3, pERK1/2, SYK immunohistochemical expression were significantly associated with increasing later recurrence of HCC.

Table 6. Univariate Cox proportional hazards regression analysis for late recurrence of HCC in the training cohort (n = 402)

		Univariate analysis	
Parameters		Hazard ratio (95% CI)	p-value
Clinical characteristics			
Age (ref ≤ 60 years)	≥ 60	0.8 (0.5–1.3)	0.362
Sex (ref = male)	Female	0.7 (0.4–1.4)	0.315
Etiology (ref = HBV)	HCV	2.9 (0.3–6.9)	**0.012***
	Alcohol	0.5 (0.1–3.2)	0.436
	Unknown	0.6 (0.2–1.6)	0.291
Histopathologic features			
Grading			
Lobular activity (ref = no, minimal, mild)	Moderate, severe	32.7 (6.9–154.4)	**<0.001***
Periportal or septal activity (ref = no, minimal, mild)	Moderate, severe	2.0 (1.1–3.7)	**0.030***
Staging			
Cirrhosis (ref = fibrosis stage < 4)	≥ stage 4	2.3 (1.4–3.6)	**0.001***
Liver cell change			
Liver cell change (ref = no)	LLCC only	1.01 (0.5–1.9)	0.973

	SLCC only	2.4 (0.3–17.7)	0.407
	LLCC and SLCC	1.9 (1.0–3.4)	**0.040***
Immunohistochemical marker expression			
pSTAT3 (ref = negative)	Positive	2.3 (1.4–3.9)	**0.002***
pERK1/2 (ref = negative)	Positive	2.3 (1.5–3.7)	**<0.001***
SYK (ref = negative)	Positive	1.8 (1.1–2.9)	**0.021***
PAI-1(ref = negative)	Positive	1.6 (0.9–2.6)	0.081
3 Immunohistochemical marker combination (pSTAT3 or pERK1/2 or SYK) (ref = all negative)	1 positive	2.1 (1.2–3.7)	**0.009***
	2 positive	3.5 (1.9–6.5)	**<0.001***
	3 positive	5.2 (1.8–15.2)	**0.024***

CI, confidence intervals; pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase; PAI-1, plasminogen activator inhibitor-1; LLCC, large liver cell change; SLCC, small liver cell change.

The various combinations of pSTAT3, pERK1/2, and SYK (all negative or one, two, or three with positive expression) had different associations with late recurrence-free survival in HCC patients using the log-rank test ($p < 0.05$ for all) (Figure 5).

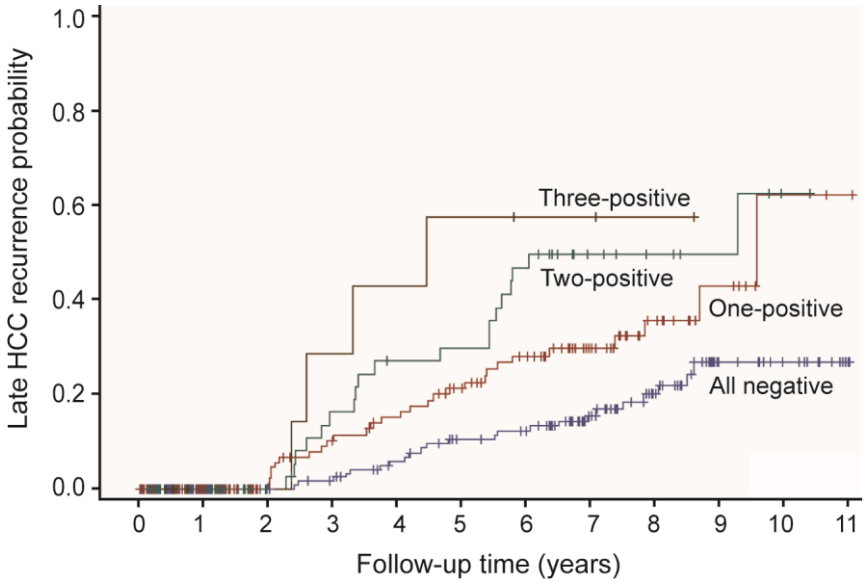

Figure 5. Kaplan–Meier curve of late recurrence-free survival according to the combinations of the immunohistochemical marker proteins; pSTAT3, pEKR1/2, and SYK in the training cohort. All negative (0, blue line); one positive (1, red line); two positive (2, green line); and three positive (3, brown line). $p = 0.005$*, 0 vs 1; $p < 0.001$*, 0 vs 2; $p < 0.001$*, 0 vs 3, $p = 0.373$, 1 vs 2; $p = 0.636$, 1 vs 3; $p = 0.003$*, 2 vs 3.

Cirrhosis and lobular activity were retained as histopathologic parameters, whereas pSTAT3, pERK1/2, and SYK were retained as immunohistochemical markers in the multiple Cox proportional hazards regression model using a stepwise variable selection method (Table 7). To design a nomogram for predicting late recurrence of HCC, two selected histopathologic parameters (cirrhosis and lobular activity) and immunohistochemical markers (pSTAT3, pERK1/2, and SYK) were used to calculate regression coefficients, and the following three models were tested: Model 1, histopathologic parameters only; Model 2, histopathologic parameters and single application of pSTAT3, pERK1/2, or SYK; and Model 3, histopathologic parameters and combined

application of pSTAT3, pERK1/2, and SYK (Table 7).

The risk factors were proportionally assigned as points on a scale of 0–100 in the nomogram based on their regression coefficient for 3- and 5-year recurrence-free survival (Table 8). The 3- and 5-year survival rates predicting the probability of late recurrence of HCC were estimated by comparing the total points calculated with the sum of the points for each risk factor (Figure 6).

The equation $S(t, X) = [S_0(t)]^{\exp(LP)}$ was generated to evaluate the probability of late recurrence of HCC based on the results of the multivariate Cox proportional hazards regression analysis of the histopathologic parameters and marker combinations (pSTAT3, pERK1/2, and SYK) (Model 3). The letters in the equation represent the following: S = score; t = time (3- or 5-year); X = three variables associated with late recurrence (cirrhosis, lobular activity, and immunohistochemical marker combination); $S_0(t)$ = constant value for 3- or 5-year prediction [S_0 (3) = 0.9375271; S_0 (5) = 0.09803303]; LP, linear predictor = $\sum_{i=1}^{p} \beta_i \times (x_i - \bar{x})$; LP = 0.6822 × (a − 0.5174129) + 3.0680 × (b − 0.009950249) + 0.6847 × (c1 − 0.3557214) + 1.0628 × (c2 − 0.1691542) + 1.7885 × (c3 − 0.02487562); a = cirrhosis (0, no cirrhosis; 1, cirrhosis); b = lobular activity (0, no or minimal or mild; 1, moderate or severe); c1 = one positive marker (0, none positive; 1, one positive); c2 = two positive markers (0, none or one positive; 1, two positive); and c3 = three positive markers (0, none or two positive; 1, three positive).

The nomogram comprised eight rows representing the following. The first row (Points) was the point assignment for each variable. The following three rows were assigned to each of the three variables (cirrhosis, lobular activity, and marker combination), and a point value according to each variable was obtained by plotting a vertical line between each patient's variable value and the Points line. Total points (row 5) were calculated by summing all the assigned points for the three variables. The predictive probability of 3- and

5-year risk of late recurrence of HCC can be obtained by drawing a vertical line between the total points and the 3- or 5-year survival probability (rows 6 and 7) (Figure 6).

Table 7. Selection of variables using multiple Cox proportional hazards regression analyses

Parameters	[Model 1] Histologic parameter only		[Model 2] Histologic parameter + individual immunohistochemical marker		[Model 3] Histologic parameter + immunohistochemical marker combination	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Histopathologic parameters						
Cirrhosis (ref = no fibrosis)	2.2 (1.3–3.5)	**0.0015***	2.0 (1.2–3.2)	**0.006***	2.0 (1.2–3.2)	**0.006***
Lobular activity (ref = no, minimal, mild) Moderate or severe	23.2 (4.9–110.6)	**<0.0001***	24.7(5.1–119.3)	**<0.001***	21.4 (4.4–104.2)	**<0.001***
Immunohistochemical markers						
pERK1/2 (ref = no expressed)			2.0(1.3–3.2)	**0.003***		
SYK (ref = no expressed)			1.7(1.0–2.7)	**0.044***		
Marker combination (pSTAT3 + pERK1/2 + SYK)						
1 (Positive1 + Negative2)					2.0 (1.1–3.5)	**0.016***
2 (Positive2 + Negative1)					2.9 (1.6–5.4)	**<0.001***
3 (Positive + Positive + Positive)					6.0 (2.0–17.5)	**0.001***

HR, hazard ratio; CI, confidence intervals; pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase

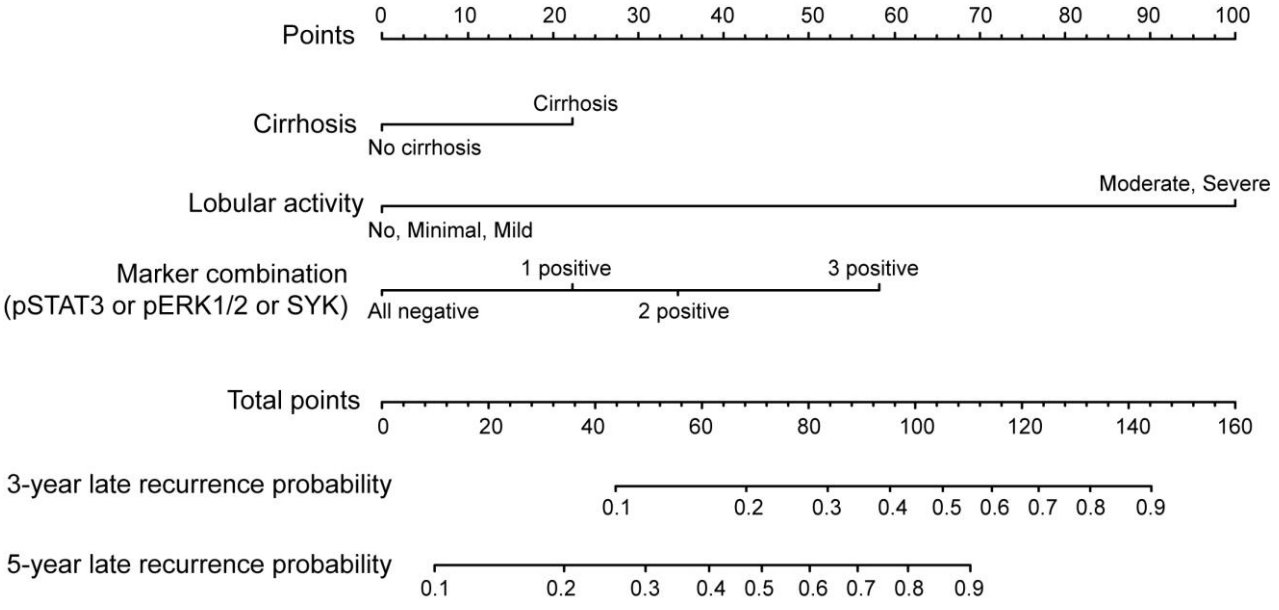

Figure 6. Nomogram of the predictive model for 3- and 5-year probability of late recurrence of HCC. The first row ("Points") is the point assignment for each variable. The following three rows are assigned to each variable (cirrhosis, lobular activity, and marker combination); a point value according to each variable can be obtained by drawing a vertical line between each patient's variable value and the Points line. Total points (row 5) can be calculated by summing all the assigned points for the three variables. The ability to predict late recurrence of HCC can be determined by drawing a vertical line between "Total points" (row 5) and "3-year late recurrence probability" or "5-year late recurrence probability" (rows 6 and 7).

Table 8. Score on each point on the nomogram

Variables		**Points**
Cirrhosis		
	Fibrosis 0–3	0
	Cirrhosis (fibrosis > 4)	22
Lobular activity		
	No, minimal, or mild (grade 0–2)	0
	Moderate or severe (grade 3–4)	100
Marker combination		
(pSTAT3 or pERK1/2 or SYK)	All negative	0
	One positive	22
	Two positive	35
	Three positive	58

pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase

The calibration plot of the model showed the mean bootstrap-predicted and actual probabilities of late recurrence of HCC at 3 and 5 years, represented by a parallel line close to the reference (diagonal) line in the training cohort. The plots of the training and validation cohorts presented an optimal agreement between the nomogram prediction and the actual observation for the 3- and 5-year recurrences (Figure 7).

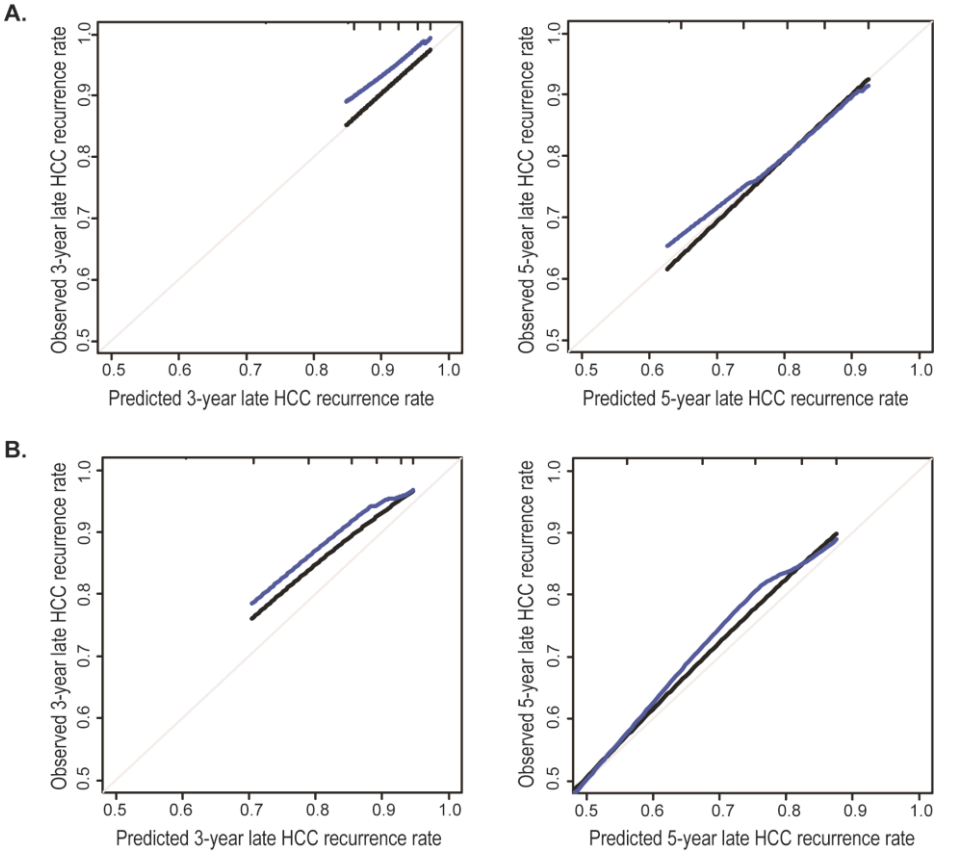

Figure 7. Calibration plots for the predictive ability of the nomogram. (A) Calibration plot for Model 3 in the training cohort reliably predicted 3- and 5-year recurrence of HCC, as revealed by its parallel position close to the diagonal (ideal) line. (B) A similar parallel line close to the reference line was observed for the validation cohort. Gray, ideal line; black, observed line; blue, optimism corrected line.

After designing the nomogram, the predictive accuracy of the final model was assessed using data from the training and validation cohorts. Model 3 showed the highest predictive ability with the training cohort dataset (Harrell's C index = 0.701, 95% CI = 0.642–0.758 and Heagerty's iAUC = 0.675, 95% CI = 0.615–0.731), followed by Model 2 (Harrell's C index = 0.653, 95% CI =

0.592–0.714 and Heagerty's iAUC = 0.630, 95% CI = 0.570–0.692) and then by Model 1 (Harrell's C index = 0.619, 95% CI = 0.557–0.679 and Heagerty's iAUC = 0.598, 95% CI = 0.538–0.662). The differences in iAUC between Models 3 and 1 as well as between Models 2 and 1 were significant (difference = 0.077, 95% CI = 0.029–0.131, p = 0.004 and difference = 0.032, 95% CI = 0.012–0.060, p = 0.014, respectively). The difference between Models 3 and 2 was not statistically significant (difference = 0.046, 95% CI= −0.011–0.102, p = 0.100). The best predictive ability for the dataset of the validation cohort was exhibited by Model 3 (Harrell's C index = 0.719, 95% CI = 0.644–0.790 and Heagerty's iAUC = 0.707, 95% CI = 0.637–0.773), followed by Model 2 (Harrell's C index = 0.672, 95% CI = 0.598–0.739 and Heagerty's iAUC = 0.663, 95% CI = 0.590–0.737) and then by Model 1 (Harrell's C index = 0.656, 95% CI = 0.587–0.717 and Heagerty's iAUC = 0.640, 95% CI = 0.570–0.707). The iAUC for Model 3 was significantly higher than that for Model 1 (difference = 0.067, 95% CI = 0.024–0.122, p = 0.021). Thus, the predictive ability based on Heagerty's incident/Dynamic AUC (3 year) and Heagerty's incident/Dynamic AUC (5 years) was similar to these results (Table 9 and Figure 8).

Table 9. Comparison among the predictive abilities of the three models

	Harrell's C index (95% CI)	**Heagerty's iAUC** (95% CI)	**Heagerty's incident/Dynamic AUC (3 years)** (95% CI)	**Heagerty's incident/Dynamic AUC (5 years)** (95% CI)
Training cohort				
[Model 1] Histologic parameter only	0.619 (0.557–0.679)	0.598 (0.538–0.662)	0.594 (0.536–0.653)	0.594 (0.535–0.655)
[Model 2] Histologic parameter + individual immunohistochemical marker	0.653 (0.592–0.714)	0.630 (0.57–0.692)	0.624 (0.567–0.684)	0.623 (0.566–0.683)
[Model 3] Histologic parameter + immunohistochemical marker combination	0.701 (0.642–0.758)	0.675 (0.615–0.731)	0.676 (0.613–0.735)	0.671 (0.612–0.727)
p-value (1) vs. (2)	**0.015***	**0.014***	**0.032***	**0.026***
p-value (1) vs. (3)	**0.006***	**0.004***	**0.003***	**0.003***
p-value (2) vs. (3)	0.093	0.100	0.083	0.075
Validation cohort				
[Model 1]	0.656 (0.587–0.717)	0.640 (0.57–0.707)	0.641 (0.572–0.705)	0.640 (0.567–0.718)

Histologic parameter only				
[Model 2]				
Histologic parameter	0.672 (0.598–0.739)	0.663 (0.59–0.737)	0.663 (0.593–0.732)	0.656 (0.583–0.735)
+ individual immunohistochemical marker				
[Model 3]				
Histologic parameter	0.719 (0.644–0.790)	0.707 (0.637–0.773)	0.718 (0.643–0.783)	0.682 (0.610–0.760)
+ immunohistochemical marker combination				
p-value (1) vs. (2)	0.219	**0.029***	0.067	0.110
p-value (1) vs. (3)	**0.030***	**0.021***	**0.016***	0.162
p-value (2) vs. (3)	0.130	0.156	0.096	0.402

iAUC, integrated area under the curve; CI, confidence intervals.

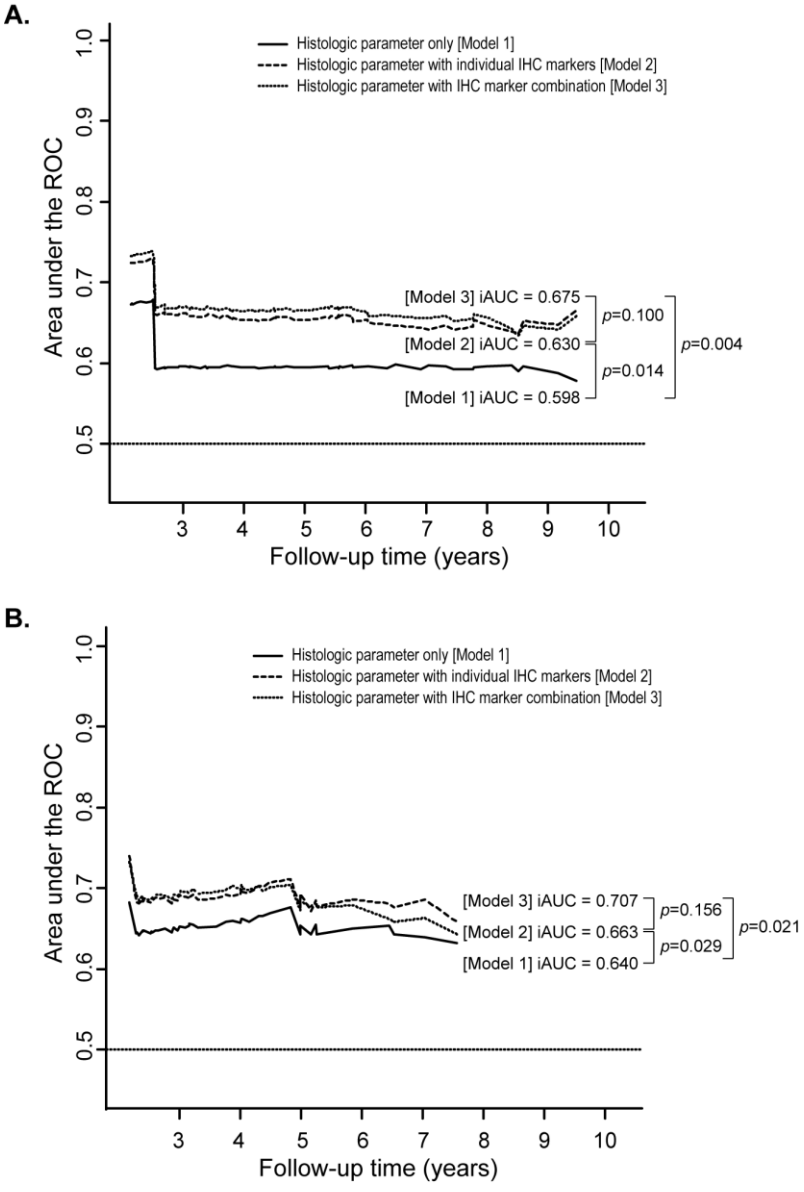

Figure 8. Discrimination of the nomogram with time-dependent AUC (iAUC) comparison. (A) Discrimination of the nomogram in the training cohort. The highest predictive value was observed for Model 3, followed by Model 1 and Model 2. (B) The predictive values of the validation cohort were similar.

Furthermore, to estimate the incremental value of the immunohistochemical markers to predict late recurrence of HCC, we calculated the NRI and IDI of the multivariate Cox regression models using histologic parameters that incorporated the immunohistochemical markers (Model 2) or marker combinations (Model 3) and those that did not (Model 1). The addition of individual immunohistochemical markers (Model 2) yielded significant positive value of NRI for 3- and 5-year late recurrences (NRI = 0.447, 95% CI = 0.130–0.574, p = 0.010 and NRI = 0.265, 95% CI = 0.073–0.397, p = 0.010, respectively). Adding the immunohistochemical marker combination (Model 3) also yielded significantly positive value of NRI for 3- and 5-year late recurrences (NRI = 0.403, 95% CI = 0.065–0.537, p = 0.024 and NRI = 0.249, 95% CI = 0.054–0.387, p = 0.012, respectively). IDI exhibited significant positive value in both Model 2 and Model 3 compared with that in Model 1 for the 5-year late recurrence (0.039, 95% CI = 0.007–0.106, p = 0.002 and 0.048, 95% CI = 0.013–0.145, p = 0.002, respectively); IDI was also positive for the 3-year late recurrence but was not significant for both models. In the validation cohort, the IDI of Model 3 for the 5-year late recurrence had significant positive value (0.060, 95% CI = 0.010–0.173, p = 0.022). NRI and IDI were positive; however, they were not significant for the 3- and 5-year late recurrences in Model 2 (Table 10).

Table 10. Comparison of the performance of the predictive models with immunohistochemical markers

	3-year				5-year			
	NRI (95% CI)	p-value	**IDI** (95% CI)	p-value	**NRI** (95% CI)	p-value	**IDI** (95% CI)	p-value
Training cohort								
[Model 1]	Ref		Ref		Ref		Ref	
Histologic parameter only								
[Model 2]	0.447	**0.010***	0.022	0.060	0.265	**0.010***	0.039	**0.002***
Histologic parameter + individual immunohistochemical marker	(0.130–0.574)		(−0.002–0.070)		(0.073–0.397)		(0.007–0.106)	
[Model 3]	0.403	**0.024***	0.027	0.082	0.249	**0.012***	0.048	**0.002***
Histologic parameter + immunohistochemical marker combination	(0.065–0.537)		(−0.008–0.145)		(0.054–0.387)		(0.013–0.145)	
Validation cohort								
[Model 1]	Ref		Ref		Ref		Ref	
Histologic parameter only								
[Model 2]	0.149	0.254	0.016	0.240	0.239	0.124	0.031	0.090
Histologic parameter + individual immunohistochemical marker	(−0.118–0.392)		(−0.008–0.083)		(−0.142–0.447)		(−0.004–0.127)	
[Model 3]	0.088	0.290	0.009	0.354	0.285	0.056	0.060	**0.022***
Histologic parameter + immunohistochemical marker combination	(−0.129–0.362)		(−0.018–0.131)		(−0.005–0.466)		(0.010–0.173)	

NRI, net reclassification improvement; IDI, integrated discrimination improvement; CI, confidence intervals.

5. Analyses of the HBV hepatitis patient subgroup

We attempted to compare the valuable late recurrence predictive parameters according to the etiologies; however, the number of patients with late recurrence, except for those with HBV hepatitis, was too small (HCV, 6 of 16 cases; alcohol, 1 of 17 cases; unknown, 4 of 34 cases) to perform statistically valid Cox proportional hazards regression analyses with subgroups. We performed the analysis with only HBV hepatitis patients as a subgroup, and the results were similar to those of the complete data. The HBV hepatitis subgroup of the training cohort comprised 335 patients; among them, 63 patients with late recurrence had greater LLCC and high expression of pERK1/2 and PAI-1 ($p < 0.05$ for all, Table 11). In the validation cohort, among the 188 HBV hepatitis patients, 35 patients with late recurrence had a more advanced lobular activity and fibrosis as well as higher expression of pERK1/2 ($p < 0.05$ for all, Table 12). Univariate Cox proportional hazards regression analyses were performed to identify the significant risk factors for late recurrence of HCC in HBV hepatitis subgroup of the training cohort, lobular activity, cirrhosis, and immunohistochemical markers of pSTAT3, pERK1/2, and SYK were significantly associated with increasing the risk of late HCC recurrence ($p < 0.05$ for all, Table 13). The histologic parameters (lobular activity and cirrhosis) and individual immunohistochemical markers (pSTAT3, pEKR1/2, and SYK) or immunohistochemical marker combinations (pSTAT3, pERK1/2, and SYK) were subjected to multiple Cox proportional hazard regression analyses. As similar with Model 2 and Model 3 of total dataset, histologic parameters and immunohistochemical markers of pERK1/2 and SYK or those and immunohistochemical marker combinations were selected as predictive markers for late recurrence of HCC (Table 14).

Table 11. Characteristics of the HBV hepatitis patient subgroup of the training cohort (n = 335) without and with late recurrence of HCC (after 2 years)

	No late recurrence (n = 272)	**Late recurrence (after 2 years) (n = 63)**	**p-value**
Clinical characteristics			
Age (year, mean ± SD)	53.55 ± 9.72	52.62 ± 8.54	0.412
Sex (male/female)	219 (80%)/53 (20%)	54 (86%)/9 (14%)	0.338
Histopathologic features			
Grading			
Lobular activity (no/minimal/mild/moderate/severe)	0 (0%)/54 (20%)/217 (80%)/1 (0%)/0 (0%)	0 (0%)/10 (16%)/51 (81%)/2 (3%)/0 (0%)	0.085
Periportal or septal activity (no/minimal/mild/moderate/severe)	0 (0%)/60 (22%)/193 (71%)/18 (7%)/1 (0%)	0 (0%)/13 (21%)/44 (70%)/6 (10%)/0 (0%)	0.828
Staging			
Cirrhosis	146 (54%)	42 (67%)	0.061
Fibrosis (0/1/2/3/4a/4b/4c)	1 (0%)/5 (2%)/33 (12%)/87 (32%) /38 (14%)/89 (33%)/19 (7%)	0 (0%)/0 (0%)/7 (11%)/14 (22%) /10 (16%)/27 (43%)/5 (8%)	0.575
Liver cell change			
Liver cell change (LCC) (No/Large LCC only/Small LCC	68 (25%)/123 (45%)/1 (0%)/80 (30%)	14 (22%)/22 (35%)/1 (2%)/26 (41%)	0.175

only/Both LCC)			
Large LCC (%, mean ± SD)	10.20 ± 11.25	14.10 ± 12.92	**0.042***
Small LCC (%, mean ± SD)	2.90 ± 5.54	4.48 ± 7.30	0.052
Immunohistochemical marker expression			
pSTAT3 (positive/negative)	41 (15%)/231 (85%)	14 (22%)/49 (78%)	0.168
pERK1/2 (positive/negative)	72 (27%)/200 (73%)	29 (46%)/34 (54%)	**0.002***
SYK (positive/negative)	74 (27%)/198 (73%)	22 (35%)/41 (65%)	0.222
PAI-1 (positive/negative)	150 (55%)/122 (45%)	46 (73%)/17 (27%)	**0.009***

pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase; PAI-1, plasminogen activator inhibitor-1.

Table 12. Characteristics of the HBV hepatitis patient subgroup of the validation cohort (n = 188) without and with late recurrence of HCC (after 2 years)

	No late recurrence (n = 153)	**Late recurrence (after 2 years) (n = 35)**	**p-value**
Clinical characteristics			
Age (year, mean ± SD)	54.09 ± 10.23	55.51 ± 9.53	0.379
Sex (male/female)	110 (72%)/43 (28%)	28 (80%)/7 (20%)	0.328
Histopathologic features			
Grading			
Lobular activity (no/minimal/mild/moderate/severe)	5 (3%)/25 (16%)/123 (80%)/0 (0%)/0 (0%)	0 (0%)/18 (51%)/15 (43%)/2 (6%)/0 (0%)	**<0.001***
Periportal or septal activity (no/minimal/mild/moderate/severe)	7 (4%)/52 (34%)/85 (56%)/8 (5%)/1 (1%)	0 (0%)/9 (26%)/20 (57%)/6 (17%)/0 (0%)	0.096
Staging			
Cirrhosis	71 (46%)	25 (71%)	**0.008***
Fibrosis (0/1/2/3/4a/4b/4c)	2 (1%)/12 (8%)/29 (19%)/39 (26%) /22 (14%)/43 (28%)/6 (4%)	0 (0%)/0 (0%)/0 (0%)/10 (29%)/11 (31%)/14 (40%)/0 (0%)	**0.008***
Liver cell change			
Liver cell change (LCC) (No/Large LCC only/Small LCC	43 (28%)/61 (40%)/3 (2%)/46 (30%)	9 (26%)/9 (26%)/1 (2%)/16 (46%)	0.282

only/Both LCC)			
Large LCC (%, mean ± SD)	11.08 ± 9.77	15.71 ± 13.46	0.082
Small LCC (%, mean ± SD)	4.09 ± 7.18	6.26 ± 8.44	0.097
Immunohistochemical marker expression			
pSTAT3 (positive/negative)	43 (28%)/110 (72%)	20 (57%)/15 (43%)	0.088
pERK1/2 (positive/negative)	40 (26%)/113 (74%)	17 (49%)/18 (51%)	**0.009***
SYK (positive/negative)	22 (14%)/131 (86%)	9 (26%)/26 (74%)	0.103

pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase

Table 13. Univariate Cox proportional hazards regression analysis for late recurrence of HCC in the HBV hepatitis patient subgroup of the training cohort (n = 335)

Parameters		Univariate analysis	
		Hazard ratio (95% CI)	p-value
Clinical characteristics			
Age (ref ≤ 60)	≥60	0.9 (0.4–1.9)	0.738
Sex (ref = male)	Female	0.6 (0.3–1.2)	0.156
Histopathologic features			
Grading			
Lobular activity (ref = no, minimal, mild)	Moderate, severe	27.0 (5.7–127.8)	**<0.001***
Periportal or septal activity (ref = no, minimal, mild)	Moderate, severe	1.5 (0.6–3.4)	0.389
Staging			
Cirrhosis (ref = fibrosis < 4)	≥4	2.1 (1.2–3.5)	**0.007***
Liver cell change			
Liver cell change (ref = no)	LLCC only	0.8 (0.4–1.6)	0.517
	SLCC only	2.0 (0.3–15.0)	0.514
	LLCC and SLCC	1.6 (0.9–3.0)	0.175
Immunohistochemical marker expression			

pSTAT3 (ref = negative)	Positive	2.0 (1.1–3.6)	**0.025***
pERK1/2 (ref = negative)	Positive	0.6 (0.5–0.9)	**0.001***
SYK (ref = negative)	Positive	1.8 (1.1–3.1)	**0.023***
PAI-1 (ref = negative)	Positive	1.7 (0.9–2.9)	0.075
3 Immunohistochemical marker combination			
(pSTAT3 or pERK1/2 or SYK) (ref = all negative)			
	1 positive	2.1 (1.2–3.8)	**0.012***
	2 positive	3.1 (1.6–6.2)	**0.001***
	3 positive	4.9 (1.7–14.4)	**0.004***

CI, confidence intervals; pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase; PAI-1, plasminogen activator inhibitor-1.

Table 14. Multiple Cox proportional hazards regression analyses for late recurrence of HCC in the HBV hepatitis patient subgroup of the training cohort (n = 335)

	[**Model 1**] Histologic parameter only		[**Model 2**] Histologic parameter + individual immunohistochemical marker		**[Model 3]** Histologic parameter + immunohistochemical marker combination	
Parameters	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Histopathologic parameters						
Cirrhosis (ref = no fibrosis)	2.0 (1.2–3.4)	**0.012***	1.8 (1.1–3.1)	**0.029***	1.8 (1.1–3.1)	**0.027***
Lobular activity (ref = no, minimal, mild) Moderate or severe	20.5 (4.3–97.96)	**<0.0001***	22.3 (4.6–107.8)	**<0.001***	20.3 (4.1–99.6)	**<0.001***
Immunohistochemical markers						
pERK1/2 (ref = not expressed)			1.9 (1.2–3.2)	**0.010***		
SYK (ref = not expressed)			1.7 (1.0–3.0)	**0.036***		
Marker combination (pSTAT3 + pERK1/2 + SYK)						
1 (Positive1 + Negative2)					2.0 (1.1–3.7)	**0.019***
2 (Positive2 + Negative1)					2.5 (1.2–5.1)	**0.010***
3 (Positive + Positive + Positive)					5.7 (1.9–16.9)	**0.002***

HR, Hazard ratio; CI, confidence intervals; pSTAT3, phosphorylated signal transducer and activator of transcription 3; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; SYK, spleen tyrosine kinase

IV. DISCUSSION

Chronic hepatitis due to various etiologies, including viral infection and alcohol, results in hepatocyte injuries and necrosis. These injuries and cell death induce perpetual regeneration and proliferation of the hepatocytes and activation of the stellate cells, promoting liver fibrosis[1]. Persistent liver fibrosis may result in cirrhosis, with accumulation of genetic instabilities of the hepatocytes, eventually leading to HCC. Hepatocarcinogenesis is a multistep process manifested by the subsequent development of hyperplastic nodules, dysplastic nodule, early HCC, and finally progressive HCC[1,35-37].

HCC has poor prognosis, with a cumulative 5-year recurrence rate of >70% following curative resection[33]. Recurrence rates and prognoses for most cancer types are determined by the degree of cancer malignancy. However, HCC demonstrates a different recurrence pattern in that the recurrence rate decreases following curative resection but remains unaltered or increases after few years[6]. Hepatocarcinogenesis of the background liver due to chronic hepatitis is considered a major cause of postoperative recurrence. In early recurrences, the aggressiveness of the primary liver cancer; defined by microvascular invasion, capsular invasion, tumor differentiation, and serum tumor marker levels have been considered risk factors. In contrast, the known risk factors of late recurrences are chronic inflammation from viral infection, cirrhosis, and multi-centric occurrence[4,6,16,38,39]. The cut-off between these two types of recurrences is 2 years, the intercept value of 2 linear regression lines obtained by evaluating the data regarding disease-free survival[4].

It is crucial to study late recurrences of HCC because they account for 25%–35% of all HCC recurrences[6,38,40]. Moreover, approximately 1%–7% of all patients with chronic hepatitis develop HCC; however, this rate of de novo HCC development is several times higher for patients with previous HCC[6,41]. Thus, proper and timely management of chronic hepatitis is essential. Moreover, advances in HCC imaging allow early detection and timely surgical treatment,

improving early HCC recurrence-free survival[42]. As the survival rate increases, the rate of late recurrence increases; therefore, it is clinically important to establish a predictive model to accurately predict the late recurrence of HCC and provide individual treatment.

Studies have shown that late recurrence may be predicted using clinical characteristics, including serum alpha-fetoprotein, HBV antigen, HBV DNA, serum aspartate aminotransferase/platelet ratio index, age, and indocyanine green retention rate at 15 min that provide an assessment of liver function[6,12,14,15]. Some predictive models for HCC recurrence have been introduced using these clinical characteristics[7,13,43]; however, to our knowledge, no predictive model composed of histologic parameters or molecular markers has been established thus far.

To identify the predictive factors of late recurrence of HCC, pathologic parameters of the training cohort were evaluated. The univariate Cox hazards regression analyses revealed that histopathologic features that increased the risk of late recurrence were lobular activity, periportal activity, cirrhosis, and liver cell changes. These histopathologic features were analyzed using multivariate proportional hazards regression analyses; the results showed that lobular activity and cirrhosis were independent prognostic factors of late recurrence of HCC.

Background liver fibrosis and inflammation are two important risk factors of late recurrence of HCC[4,6,15,16]. Several studies have demonstrated that the risk of developing HCC increases with the progression of liver fibrosis in chronic hepatitis patients with or without a history of HCC[20,44,45]. Thus, cirrhosis may help predict late recurrence of HCC, supporting the results of our study. Stage 4 fibrosis (cirrhosis) is divided into the following three subcategories: 4a, 4b, and 4c, according to septal thickness. Further, studies have shown that the risk of developing HCC increases with increase in septal thickness[21,22]. However, in our study, univariate Cox hazards regression analyses did not reveal any

difference among the three subcategories. This may be attributable to the fact that the sample of cirrhosis patients was insufficient for regression analysis with subcategorization for late recurrence.

Several studies have shown that lobular activity may be considered a risk factor for late recurrence of HCC in chronic hepatitis patients who are at a high risk of HCC[6,15,16,20]. The pathogenic role of inflammation in hepatocarcinogenesis is explained as interaction between activated inflammatory cells; such as activated type II macrophages, which were modulated by inflammation microenvironment with increased interleukin (IL) 6 or transforming growth factor-β excretion and liver stem cells, which were activated by signaling pathway regarding Wnt/β catenin and STAT3/NF-κB[46]. These studies support our findings regarding the predictive power of lobular activity. Lobular activity, rather than porto-periportal activity, was reported as a risk factor for late recurrence of HCC[6,20]. It was suggested that persistent lobular necroinflammation destroys the lobular structure; further, it accelerates regeneration and accumulation of the genetic alteration of hepatocytes. Therefore, chronic hepatitis with severe lobular activity could be an independent predictive factor for the development of de novo HCC, supporting our decision to retain lobular activity as a predictive factor in the prediction model.

To identify the protein markers related to the late recurrence of HCC, RPPA was performed on the non-tumor liver tissue. RPPA is a very precise and sensitive technology that can perform quantitative measurements of hundreds of signaling proteins in several samples[47]. This array involves the advantage of enabling simultaneous quantification and phosphorylation of proteins in multiple samples. We identified eight proteins that showed significant correlation with late recurrence of HCC in patients with HIR signatures on their non-tumor liver tissue. The AUC was used to check the degree to which the expression of these proteins can predict the late recurrence of HCC. SYK,

pSTAT3, and PAI had values of >0.7; therefore, they were selected for analysis. pMAPK (pERK1/2), known to be involved in the carcinogenesis of several organs, was also included in the analysis. Immunohistochemical staining of these four IHC markers showed that in the training cohort, univariate Cox hazards regression analysis of these markers on their non-tumor liver tissue showed that pSTAT3, pERK1/2, and SYK significantly increased the risk of late recurrence.

Most of these markers are known to exert a carcinogenesis effect through their roles in signaling pathways, fibrosis, and inflammation. However, they have not been reported as risk factors of late recurrence of HCC. Based on studies related to hepatocarcinogenesis, we expected these markers to predict late recurrence owing to the fact that they share the molecular process of hepatocarcinogenesis.

In a previous study, STAT3 was identified as the central gene within the HIR signatures through gene network analysis; it was also reported that its expression is increased in patients with late recurrence of HCC[18]. STAT3 becomes phosphorylated when activated owing to various stresses via the intracellular and extracellular pathways and moves to the nucleus; further, it upregulates several genes associated with cell proliferation, survival, tumor invasion, and metastasis. It also induces the expression of cytokines, such as IL-6 and chemokines. These effects activate STAT3, forming a loop, inducing inflammation, and promoting cancer development[48-53]. In addition, as per a previous study, in patients with HBV hepatitis, the activation of STAT3 inhibits apoptosis, increases HBV replication and acute phase responses, thereby promoting progression to HCC[54]. Thus, STAT3 was considered a key protein marker for predicting the progression of hepatocytic alteration in the background of chronic hepatitis and predicting the late recurrence of HCC.

SYK is known to modulate liver inflammation, cell death, and steatosis in HCV hepatitis or alcoholic liver diseases[55]. Further, SYK is involved in increased inflammation of the background liver tissue that leads to the

progression to carcinoma by an increase in the level of cytokines through the toll-like receptor or immunoreceptor tyrosine-based activation motif[56,57]. Recently, an increased number of SYKs from stellate cells and interactions with increased SYKs from the hepatocytes following chronic liver injury have been shown to increase fibrosis, eventually leading to the development of HCC[58]. Therefore, SYK was also regarded as an important predictive factor associated with the inflammatory process through interaction with the stellate cells for predicting the late recurrence of HCC in patients with chronic hepatitis.

Upregulated pMAPK (pEKR1/2) through the AKT/mTOR and Ras/MAPK pathways or other pathways, regulates various cell functions, such as proliferation, survival, and differentiation[59,60]. The stress-activated MAPK cascade plays an important role in hepatocarcinogenesis from chronic hepatitis, and the HBx of HBV affects this pathway by continuing cell proliferation and survival[61,62]. Upregulated pERK1/2 indicated consistent increase in cell growth signals; thus, it was expected to help predict late recurrence of HCC.

PAI-1 is the main inhibitor of plasminogen activators; therefore, it inhibits fibrosis. Moreover, although only few studies have assessed its role in the liver, elevated levels of PAI-1 is associated with the progression of inflammation and fibrosis in steatosis and alcoholic liver disease[63,64]. It is also known to affect liver fibrosis and carcinogenesis by inducing extracellular matrix accumulation when expressed through TGF-b/Smad2/3 signaling[65]. Although PAI-1 was not identified as an independent predictive factor, it is expected to play a role in HCC development because the progression of fibrosis is an important part of hepatocarcinogenesis.

Univariate Cox hazards regression analyses showed that the three immunohistochemical markers—pSTAT3, pERK1/2, and SYK—significantly increase the risk of late recurrence of HCC. Our results were consistent with previous reports; the selected markers reflecting the inflammatory process would increase the risk of late recurrence of HCC and could be candidate

predictive markers.

In contrast, excluding the correlation that may exist among these three markers, pSTAT3, pERK1/2, and SYK were simply combined to achieve a complementary effect for multivariate analyses. Kaplan–Meier curve and log-rank test were used to rule out whether the IHC marker combination increased the risk of late recurrence of HCC. A combination of these three markers (all negative or one, two, or three with positive expressions) showed increasing significance in predicting a higher risk of late recurrence when the presence of positive expression increased. Several previous studies in other organs have shown that a combined assessment of molecular markers improves prognostic power and accurate predictive ability of outcomes compared with individual markers owing to the association with different pathways for cancer progression[66-68]. Thus, a combination of immunohistochemical markers was included in the analyses for selecting the final predictive model and was expected to improve predictive ability.

Multiple Cox hazards regression analysis was performed to evaluate whether the markers of each model could independently predict late recurrence of HCC with the highest predictive accuracy. When each IHC marker was applied to the two selected histologic parameters cirrhosis and lobular activity, (Model 2) only pERK1/2 and SYK significantly predicted late recurrence. In contrast, when the IHC marker combination was applied (Model 3), it significantly predicted late recurrence. The Harrell's C index and Heagerty's iAUC that were analyzed for discrimination assessment were the highest for Model 3. The Harrell's C index with its respective CI provides a more comprehensive measure of discrimination, obtained using the bootstrap method[42], and C index indicates a higher probability for the predicted risk for an event than for a non-event[14]. The Heagerty's iAUC is a weighted average of the AUC across the follow-up period and is a measure of the overall predictive accuracy of the survival model[16,35,36]. AUC is a more commonly used discrimination assessment method; however, it

is known to have lower sensitivity for comparing the prediction ability of the models to that of the baseline model. Thus, Harrell's C index and Heagerty's iAUC were more appropriate validation analyses for discrimination in our predictive model with follow-up time, and those showed superior predictive ability when the IHC marker combination was added to the histopathologic parameters as a base model (differences between Model 1 and Model 3 of Harrell's C index = 0.082, $p = 0.006$).

For further analyses, NRI and IDI were assessed to determine the improvement in predictive ability for late recurrence of HCC, with Model 1 (histologic parameter only) as the baseline model for the application of IHC markers (Model 2 and Model 3)[35,44]. These analyses showed that the addition of a new biomarker to a baseline model can accurately stratify individuals into clinically significant higher and lower risk categories[40]. It is a more important and sensitively discriminating method in clinical application as a predictive model compared with AUC. NRI is the net increase or decrease in the risk categories among event cases minus that among non-event cases[44]. IDI is the difference between the discrimination slopes of the models with and without a new marker[22]. The NRI of Model 2 and Model 3 for 3-year late HCC recurrence was >0.4 (0.447 and 0.403, respectively) with intermediate predictive ability, and the IDI of Model 2 and Model 3 for 5-year late HCC recurrence was >0 (0.039 and 0.048, respectively), representing good discrimination. Therefore, the application of IHC markers on the baseline model could improve the predicted probability. Although the NRI of Model 2 was higher than that of Model 3, the C index, iAUC, and IDI were higher in Model 3. Therefore, it was expected that a combination of IHC markers could improve the prognostic predictive ability.

Therefore, Model 3 (histologic parameters and IHC marker combination) was selected as the final prediction model. The regression coefficient of each variable was calculated, and a score was assigned for each point based on the

weighted importance. A nomogram was used to provide visual explanation for the survival outcome predicted by the model. The nomogram was designed to visually demonstrate the rate of 3- and 5-year late recurrence following curative resection. Calibration plots of Model 3 for 3- and 5-year late recurrence showed optimal agreement between the prediction and actual observation with fit and line close to the diagonal ideal line. Thus, we confirmed that the nomogram based on Model 3 had good performance with accurate and reliable predictive ability.

The validation and training cohorts did not differ significantly in terms of age, sex, cause of hepatitis, and rate of late recurrence. The histopathologic features of the two groups were not significantly different for all four parameters. Thus, the validation cohort was comparable and was used to verify the reliability of Model 3. The predictive ability with calibration plot, Harrell's C index, and Heagerty's iAUC were tested using the prediction model, showed good agreement with fit and close plot to ideal line and revealed high predictive ability with high Harrell's C index and Heagerty's iAUC (0.719 and 0.707, respectively). The results of NRI and IDI assessments showed positive values; however, the improvement in discrimination was not statistically significant, except in IDI for 5-year late recurrence of HCC (IDI = 0.060, $p = 0.022$).

The Harrell's C index and Heagerty's iAUC were higher in the validation cohort than in the training cohort, while the NRI and IDI were lower, but not significantly lower, in the validation cohort than in the training cohort. Generally, the C index or iAUC of the validation cohort is lower than that of the training cohort because the model is fit with the training cohort. However, in rare situations, increased predictive ability was observed in the validation cohort. Variables constituting the model would be more fit in the validation cohort because the cohorts did not exhibit any significant differences, except in the follow-up period and survival rates. In contrast, decreased NRI and IDI could be due to the relatively lower number of late recurrences in the validation cohort

to evaluate the prediction ability of developed model. The direction of positive value was concordant between the two cohorts; however, variance would be relatively increased in the validation cohort, so the discrimination of the model might do not statistically significant. Moreover, the follow-up period and survival rates were different in the two cohorts. The disease progression status at the time of curative surgery and relatively shorter follow-up time could be the reason for the lower predictive ability of the model in the validation cohort. Therefore, further evaluation of the predictive model is warranted on a larger validation cohort with a longer follow-up period to obtain more accurate results.

To our knowledge, this is the first nomogram based on histologic parameters and immunohistochemical protein markers that predicted late recurrence of HCC in patients who have undergone curative resection for HCC with a chronic hepatitis background. We identified that three immunohistochemical markers (pSTAT3, pERK1/2, and SYK), histopathologic features, cirrhosis, and lobular activity can independently predict the risk of late recurrence of HCC related to HIR. Further, we developed and validated prediction models based on these parameters. The established model showed a high degree of agreement and predictive ability, and thus, it may be useful for predicting the risk of late recurrence of HCC. It would be helpful for clinicians in the prediction of late recurrence of HCC and the establishment of treatment and follow-up plans.

The predominant etiology of the two cohorts was HBV hepatitis. We attempted to compare the predictive parameters as per the etiologies by dividing them into subgroups. However, the number of patients with late recurrences owing to other etiologies, HCV, alcohol, and unknown etiologies was too small, and the statistical power was too low to allow valid Cox regression analysis. The analysis showed that the HBV subgroup had similar predictive factors as the total cohort. Further research with a larger sample of subjects with other etiologies is required for evaluating the predictive factors of late recurrence of HCC due to each etiology.

V. CONCLUSION

In conclusion, we found that cirrhosis, higher lobular activity, and overexpression of a HIR-related marker combination (pSTAT3, pERK1/2, and SYK) in non-tumor liver parenchyma are independent predictors of late recurrence or de novo HCC following surgical resection. In patients with chronic liver disease, the possibility of late recurrence should be considered because it actively influences the prognosis. Our predictive model is expected to provide important information that can aid clinical decisions regarding the treatment for hepatitis or fibrosis.

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ABSTRACT (IN KOREAN)

만성 간염에서의 간세포암종의 2년 후 재발에 대한 병리학적 예측

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남 지 해

배경: 간세포암종은 치료적 절제술 후에도 5년 재발율이 70%를 넘어 좋지 않은 예후를 보이며, 치료적 절제술 후 2년 후에 발생하는 2년 후 재발은 대부분 만성 간염에서 다단계 과정을 통해 새롭게 발생하는 간세포암종으로 생각된다. 최근 간세포암종의 2년 후 재발에는 간세포암종 자체 보다는 만성간염의 기저 간질환 (비종양 간조직)의 분자유전학적 프로파일이 관련된다고 보고되었다. 이 연구에서는 비종양 간조직의 조직병리학적 특징과 면역조직화학염색 마커에 기초한 2년 후 재발에 대한 예측 모델을 개발하고 이의 유용성을 입증하고자 하였다.

재료 및 방법: 간세포암종으로 치료적 절제술을 받은 환자를 대상으로 모델 수립군 (402명)과 모델 검증군 (243명)을 독립적으로 구축하였다. 조직병리학적 소견은 간소엽 및 문맥역/문맥주변부 간염활성, 섬유화, 작은 간세포 변화 및 큰

간세포 변화의 정도를 평가하였다. 간세포암종의 2년 후 재발과 연관된 유전자의 단백질 발현변화를 발굴하기 위하여 간세포암종 환자 (95명)의 비종양 간조직을 이용하여 역상 마이크로어레이 분석을 수행하였으며, 이 중 phosphorylated signal transducer and activator of transcription (pSTAT)3, phosphorylated extracellular signal-regulated kinase (pERK)1/2, plasminogen activator inhibitor (PAI)-1, and spleen tyrosine kinase (SYK)에 대한 면역조직화학 염색을 수행하였다. 모델 수립군에 대해 다변량 로지스틱 분석을 시행하여, 독립적으로 예측 가능한 인자를 이용하여 예측모델을 수립하였다. 이 예측모델을 통해 모델 확인군에서도 2년 후 재발을 잘 예측할 수 있는지 검증하였다.

결과: 모델 수립군에서 간세포암종의 2년 후 재발은 74 (18%) 명의 환자에서 관찰되었으며, 평균 추적기간은 82개월이었다. 간세포암종의 2년 후 재발은 간경화 (4기 섬유화), 작은 간세포 및 큰 간세포 변화, pSTAT3, pERK1/2, 및 PAI-1의 발현 증가와 유의한 관련성을 보였다 (모두 $p < 0.05$). 다변량 로지스틱 분석 결과 간경화 (odds ratio [OR] = 2.0, 95 % confidence interval [CI]: 1.2–3.2), 중등도 이상의 간소엽 간염활성 (OR = 21.4, 95 % CI: 4.4–104.2), pSTAT3, pERK1/2 및 SYK 발현 (OR = 6.0, 95 % CI: 2.0-17.5)이 간세포암종의 2년 후 재발과 독립적으로 연관되었다 (모두 P < 0.05). 이러한 변수를 기반으로 간세포암종의 2년 후 재발을 예측하기 위한 nomogram을 수립하였고, Harrell's C index가 0.701 (95% CI: 0.64–0.75)이었다. 모델검증군에서

간세포암종의 2년 후 재발은 47 (19%) 명의 환자에서 발생되었고, 평균 추적기간은 56개월이었다. 모델확인군에서 간세포암종의 2년 후 재발은 중등도 이상의 간소엽 간염활성, 간경화, 작은 간세포 및 큰 간세포 변화, pSTAT3와 pEKR1/2의 발현 증가와 연관성을 보였다 (모두 $p < 0.05$). 모델확인군에서도 다변량 로지스틱 회귀 분석을 통해 간경화 (OR = 3.0, 95 % CI: 1.5-5.8), 중등도 이상의 간소엽 간염활성 (OR = 14.2, 95 % CI: 3.0–67.3), pSTAT3, pERK1/2 및 SYK (OR = 2.6, 95 % CI: 1.2–5.3)의 발현이 독립적으로 연관이 있는 것을 확인하였다 (모두 $p < 0.05$). 간세포암종의 2년 후 재발을 예측하기 위한 nomogram을 적용하였을 때 Harrell's C index가 0.719 (CI: 0.64–0.79)로 모델확인군에서도 높은 예측력을 보이는 것을 검증하였다.

결론: 간세포암종의 2년 후 재발을 예측하기 위하여 간세포암종의 비종양 간조직에서 간경화 (4기 섬유화), 중등도 이상의 간소엽 간염활성 및 pSTAT3, pERK1/2 및 SYK 중 하나 이상의 발현에 기초한 예측모델을 수립하였으며, 간세포암종의 2년 후 재발 예측에 유용함을 검증하였다.

핵심되는 말: 만성 간염, 간세포암종, 2년 후 재발, 간소엽 간염활성, 섬유화, 간경화, STAT3, pERK1/2, SYK