(Applied Biosystems) was used as an endogenous control. TaqMan gene expression assays were performed in duplicate in 20-μL reactions in Taqman Array 96-well plates using the StepOnePlus Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer's instructions. Standard curves were generated from threefold serial dilutions of cDNA, and the copy numbers of target genes were calculated according to the standard curves.

Immunohistochemistry Analysis

Formalin-fixed, paraffin-embedded sections of 69 surgical specimens were used for c-MET protein analysis. In the absence of a commercial antibody specific for L1-MET, a rabbit monoclonal antibody for c-MET (Cell Signaling Technology, Danvers, MA, USA) was used to evaluate total c-MET expression in HCC tumor tissues. Immunohistochemistry was performed following standard protocols, using a secondary horseradish peroxidase-tagged antibody labeled with anti-rabbit/mouse polymers (DAKO A/S, Glostup, Denmark).[13] HCC case with the highest c-MET mRNA expression was taken as a positive control. Phosphate buffered saline (PBS), instead of the primary antibody, was used for negative control. Staining was independently evaluated by two trained pathologists. c-MET expression in each tumor was assessed using a four-point scoring system as previously described, in which 0 = no staining observed in tumor cells, 1+ = weak staining in <10 % of cells, 2+ = weak staining in at least 10 % of cells or intense staining in 30 % or fewer tumor cells, and 3+ = intense staining in >30 % of tumor cells.[14] Scores of 0 and 1 were classified as c-MET low, and scores of 2+ and 3+ were classified as c-MET high.

Statistical Analysis

All statistical analyses were performed with statistical software SPSS version 21.0, (SPSS, Chicago, IL). Experimental results are expressed as the mean ± standard deviation. The chi-square test or Student's *t* test was used to compare values between the two groups. Overall survival (OS) and disease-free survival (DFS) curves were generated using the Kaplan–Meier method, and the differences were compared using log-rank test. Univariate and multivariate analysis were performed based on the Cox proportional hazard regression model to compute a hazard ratio (HR) according to LINE-1 methylation status. Correlation between *L1-MET* gene expression and LINE-1 methylation levels were calculated by Pearson correlation coefficients or chi-square test. For all statistical analyses, $p < 0.05$ was considered significant.

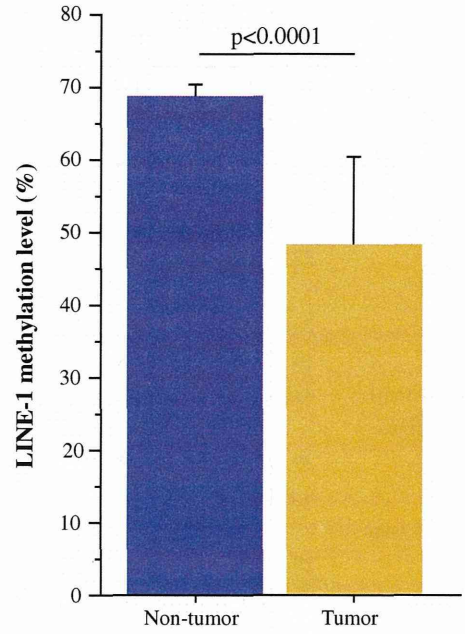

FIG. 1 Comparison of LINE-1 methylation between tumors and nontumors

RESULTS

LINE-1 is Hypomethylated in the HCC Tumor Tissues

LINE-1 methylation levels were evaluated by pyrosequencing analysis in 75 HCC tumors and their adjacent non-tumor tissues. Representative pyrograms for LINE-1 methylation in tumor and nontumor tissues are shown in Supplementary Fig. 1. LINE-1 was significantly hypomethylated in tumor tissues compared with nontumor tissues (48.3 ± 12.2 % vs. 68.2 ± 2.0 %, $p < 0.0001$, Fig. 1). Patients were divided into two groups, hypomethylation ($n = 38$) or hypermethylation ($n = 37$), according to the median LINE-1 methylation level (48.09 %) in the tumor tissue as cutoff value. We next compared the association of LINE-1 methylation levels with clinicopathological factors (Table 1). Besides sex, LINE-1 methylation was not significantly associated with other clinicopathological factors.

Association of LINE-1 Methylation Levels with HCC Patient Outcome

Patients with LINE-1 hypomethylation had significantly shorter DFS (median 22.1 months, log-rank $p < 0.05$) and OS rates (median 52.2 months, log-rank $p < 0.01$) compared with hypermethylation patients (Fig. 2). The 5-year survival rates were 44 and 94 % for LINE-1 hypo- and hypermethylation patients, respectively. Moreover, 5-year DFS in LINE-1 hypomethylation patients was 24 % compared with 57 % in hypermethylation patients. Multivariate

TABLE 1 Association of LINE-1 methylation with clinicopathological factors

Clinicopathological factors	LINE-1 hypermethylation ($n = 37$) (%)	LINE-1 hypomethylation ($n = 38$) (%)	p value
Age (yr)			0.41
<65	14 (37.8)	11 (28.9)	
≥65	23 (62.2)	27 (71.1)	
Sex			0.01
Male	23 (62.2)	33 (86.8)	
Female	14 (37.8)	5 (13.2)	
Size of tumor (cm)	4.8 ± 3.9	4.7 ± 3.1	0.94
Number of tumors			0.15
Single	29 (78.4)	24 (63.2)	
≥2	8 (21.6)	14 (36.8)	
Portal vein invasion			0.63
(−)	29 (78.4)	28 (73.7)	
(+)	8 (21.6)	10 (26.3)	
Hepatic vein invasion			0.41
(−)	35 (94.6)	34 (89.5)	
(+)	2 (5.4)	4 (10.5)	
Stage			0.12
I, II	26 (70.3)	20 (52.6)	
III, IV	11 (29.7)	18 (47.4)	
Intrahepatic metastasis			0.09
(−)	33 (89.2)	28 (73.7)	
(+)	4 (10.8)	10 (26.3)	
Differentiation			0.55
Well	7 (18.9)	5 (13.2)	
Moderate	24 (64.9)	29 (76.3)	
Poor	6 (16.2)	4 (10.5)	
HBs antigen			0.15
(−)	26 (70.3)	32 (84.2)	
(+)	11 (29.7)	6 (15.8)	
HCV antibody			0.12
(−)	25 (67.6)	19 (50)	
(+)	12 (32.4)	19 (50)	
Alcohol (g/day)			0.17
<60	36 (97.3)	34 (89.5)	
≥60	1 (2.7)	4 (10.5)	
Child-Pugh class			0.98
A	35 (94.6)	36 (94.7)	
B	2 (5.4)	2 (5.3)	
ICGR15			0.88
<15 %	24 (64.9)	24 (63.2)	
≥15 %	13 (35.1)	14 (36.8)	
MELD score			0.25
<9	35 (94.6)	33 (86.8)	
≥9	2 (5.4)	5 (13.2)	
ASA physical status			0.1
1, 2	36 (97.3)	33 (86.8)	
3	1 (2.7)	5 (13.2)	
Serum AFP (ng/ml)			0.08
<20	20 (55.6)	13 (35.1)	
≥20	16 (44.4)	24 (64.9)	
Serum PIVKAII (mAU/ml)			0.51
<40	11 (30.6)	9 (23.7)	
≥40	25 (69.4)	29 (76.3)	
Liver cirrhosis			0.95
NL	5 (13.5)	4 (11.1)	
LF/CH	22 (59.5)	22 (61.1)	
LC	10 (27.0)	10 (27.8)	

Abbreviations: *ICGR15* indocyanine green retention rate at 15 min, *MELD score* Model for end-stage liver disease score, *ASA Physical Status* American Society of Anesthesiologists Physical Status, *AFP* alpha-fetoprotein, *PIVKAII* protein induced by vitamin K absence or antagonist II, *NL* normal liver, *LF* liver fibrosis, *CH* chronic hepatitis, *LC* liver cirrhosis

analysis (Table 2) revealed that LINE-1 hypomethylation was an independent prognostic factor in DFS (HR 2.34; 95 % CI 1.02–5.38) and OS (HR 6.1; 95 % CI 1.18–31.45).

LINE-1 Methylation is Correlated with Expression of the c-MET Oncogene

Previous studies have shown that *c-MET* is inserted with LINE-1 (*L1-MET*), which is significantly associated with poor prognosis in bladder cancer and colorectal cancer metastasis.[12,15] Based on these data, we evaluated *L1-MET* expression in 53 paired HCC specimens by RT-PCR. *L1-MET* expression was higher in tumor tissues compared with nontumor tissues ($p < 0.01$, Fig. 3a). *L1-MET* expression was inversely correlated with LINE-1 methylation in all tumor and nontumor tissues ($R = -0.4$, $p < 0.001$; Fig. 3b). There was also inversely correlation between LINE-1 methylation and *L1-MET* expression only in tumor tissues ($R = -0.34$, $p = 0.01$; Supplementary Fig. 2a). *L1-MET* expression was significantly correlated with *c-MET* expression in HCC tumor tissues ($R = 0.58$, $p < 0.0001$, Supplementary Fig. 2b). We also examined c-MET protein expression in 69 HCC patients using immunohistochemistry (Supplementary Fig. 2c). Twenty-eight of 35 cases (80 %) exhibited higher c-MET expression in the hypomethylation group, whereas only 19 of 34 cases (55.9 %) exhibited high c-MET expression in the hypermethylation group ($p = 0.032$, chi-square test; Fig. 3c).

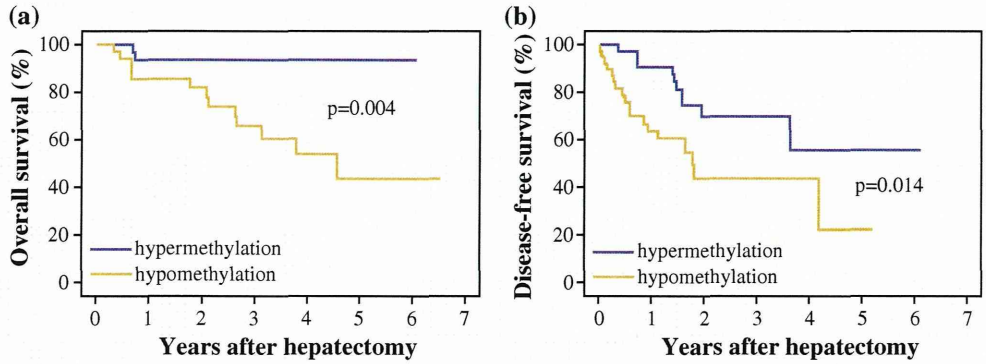

FIG. 2 Kaplan–Meier curves for overall survival (**a**) and disease-free survival (**b**) according to LINE-1 methylation levels in HCC

TABLE 2 Multivariate analysis of clinicopathological factors associated with overall survival and disease-free survival rates

	Overall survival			Disease-free survival		
	Univariate *p* value	Multivariate HR (95 % CI)	*p* value	Univariate *p* value	Multivariate HR (95 % CI)	*p* value
Age (<65 vs. ≥65)	0.168			0.366		
Sex (male vs. female)	0.935			0.103		
Size of tumor (<3.5 vs. ≥3.5 cm)	0.014	1.34–62.1	0.024	0.408		
Number of tumor (single vs. ≥2)	0.057			0.016	0.40–3.34	0.79
Portal vein invasion (+ vs. −)	0.356			0.957		
Hepatic vein invasion (+ vs. −)	0.002	0.12–2.28	0.385	0.001	1.42–19.86	0.013
Stage (I, II vs. III, IV)	0.032	1.70–175.89	0.016	0.155		
Intrahepatic metastasis (+ vs. −)	<0.001	2.15–95.96	0.006	0.004	0.4–4.82	0.603
Differentiation (well, mod vs. poor)	0.868			0.669		
ICG15 (<15 vs. ≥15 %)	0.363			0.004	1.78–10.36	0.001
Serum AFP (<20 vs. ≥20 ng/ml)	0.017	1.32–29.41	0.021	0.252		
PIVKAII (<40 vs. ≥40 mAU/ml)	0.280			0.121		
Liver cirrhosis (NL, LF/CH vs. LC)	0.123			0.213		
MELD score (<9 vs. ≥9)	0.458			0.66		
ASA physical status (1, 2 vs. 3)	0.471			0.299		
LINE-1 (hyper- vs. hypomethylation)	0.004	1.18–31.45	0.031	0.014	1.02–5.38	0.045

Cox proportional hazards regression model

Abbreviations: *ICGR15* indocyanine green retention rate at 15 min, *AFP* alpha-fetoprotein, *MELD score* Model for End-stage Liver Disease score, *ASA Physical Status* American Society of Anesthesiologists Physical Status, *PIVKAII* protein induced by vitamin K absence or antagonist II, *NL* normal liver, *LF* liver fibrosis, *CH* chronic hepatitis, *LC* liver cirrhosis

DISCUSSION

LINE-1 hypomethylation has previously been associated with poor prognosis in several types of cancer.[9,10] However, the role of LINE-1 hypomethylation in the pathogenesis of HCC still requires further clarification. In the current study, we demonstrate that LINE-1 is significantly hypomethylated in HCC tumor tissues and is significantly associated with poor OS and DFS. Moreover, LINE-1 hypomethylation led to activation of the *c-MET* oncogene, which may be involved in the progression of HCC. Taken together, these results indicate that LINE-1 hypomethylation may serve as a prognostic marker in HCC patients.

The biological function of LINE-1 remains incompletely understood. However, several hypotheses to explain LINE-1 involvement in tumorigenesis and cancer progression have been proposed. Environmental factors may initially change LINE-1 promoter methylation status, activating LINE-1 transcription; de novo LINE-1 retrotransposition into an oncogenic region may result in tumorigenesis; and once the tumor is established, the canonical LINE-1 promoter may become increasingly hypomethylated, potentially activating

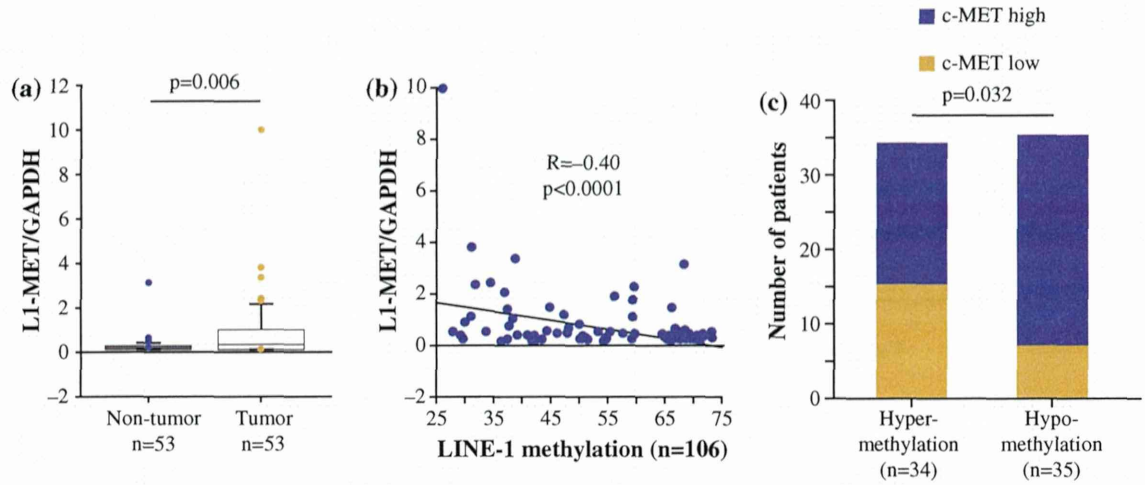

FIG. 3 *L1-MET* expression in HCC tissues and correlation with LINE-1 methylation levels. **a** Comparison of *L1-MET* expression between tumor and nontumor tissues ($p < 0.05$). **b** Correlation of *L1-MET* expression and LINE-1 methylation levels in all tumor and nontumors ($n = 106$, $R = -0.4$, $p < 0.001$). **c** Comparison of c-MET expression between LINE-1 hyper- and hypomethylation tumor tissues by immunohistochemistry ($p = 0.032$)

the antisense promoter and nearby genes.[16] LINE-1 ORF-1 protein overexpression has previously been shown to promote HepG2 cell proliferation and reduce the cytotoxicity of chemotherapy drugs.[17] Moreover, de novo LINE-1 retrotransposition suppresses tumor suppressor MCC, and activates ST18 in HCC.[18]

In our study, we evaluated the methylation level of three CpG sites in the LINE-1 promoter area (position 318–331, accession no. X58075) and found that the average methylation level in tumor tissues was 48.3 ± 12.2 %. Consistent with previous studies of cancer, global DNA was hypomethylated in HCC cancer tissues.[9,10] Clinicopathologically, we did not observe any correlation between LINE-1 methylation and tumor factors, such as tumor stage or size. In contrast, a previous study examining several single CpG sites in the LINE-1 promoter found that LINE-1 hypomethylation at the single CpG site was correlated with advanced tumor stage, larger tumor size, and tumor differentiation.[19] Because one CpG site in the previous study matches one of our target CpG sites analyzed in this study, these discrepant results may be due to the background of liver disease (HBV-related HCC vs. mixed cases) and/or the size of patients. Therefore, further studies using cohorts with larger size and different background liver diseases are required.

In this study, LINE-1 hypomethylation was associated with shorter OS (median 52.2 months) and DFS (median 22.1 months) in HCC patients. Our findings in HCC patients are in agreement with previous reports in other cancer patients, in which LINE-1 hypomethylation may serve as a prognostic marker.[19,20] Our results also indicate that LINE-1 hypomethylation is an independent prognostic factor for OS (HR 6.1; 95 % CI 1.18–31.45) and DFS (HR 2.34; 95 % CI 1.02–5.38). These results indicate that LINE-1 methylation may be a useful surrogate marker to evaluate patient outcome. However, the prognostic significance of LINE-1 methylation still requires further validation in a prospective and large-scale clinical study.

c-MET, a high-affinity receptor for hepatocyte growth factor (HGF), plays a critical role in cancer growth, invasion, and metastasis.[21] HCC patients with an active HGF/c-MET signaling pathway have a significantly worse prognosis, and in these patients, targeting c-MET represents a form of personalized treatment.[22–24] *L1-MET* is a fusion transcript, involving the *c-MET* gene and intronic LINE-1, located between the second and third exon of *c-MET*. The 5′UTR of the LINE-1 element harbors an antisense promoter that may drive *c-MET* transcription.[25] Hypomethylation of the specific LINE-1 promoter was also shown to induce expression of an alternate transcript of the *c-MET* oncogene in bladder tumors and colorectal cancer metastasis.[12,15] In the present study, we demonstrate that *L1-MET* expression is significantly upregulated in HCC tumor tissues and the *L1-MET* expression levels showed relative weak but significant negative correlation with LINE-1 methylation levels. Moreover, *L1-MET* expression was significantly correlated with *c-MET* expression and c-MET protein was significantly highly expressed in the LINE-1 hypomethylation tumor tissues. These results indicate that hypomethylated LINE-1 may affect HCC progression via activation of oncogenic c-MET expression. However, it is necessary to further investigate if there is any functional interference between *L1-MET* and c-MET in HCC. In addition, more than 1,500 genes contain insertions of full-length LINE-1. Therefore, the role of other candidate genes in carcinogenesis and cancer progression remains to be evaluated.

In summary, the present study suggests that hypomethylation of LINE-1 in HCC is significantly associated with poor prognosis, indicating that LINE-1 may be used as a biomarker to predict clinical outcome in HCC patients.

CONFLICT OF INTEREST We declare that no conflict of interest exist.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011;61:69–90.
2. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer.* 2006;6:674–687.
3. Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell.* 2012;150:12–27.
4. Weisenberger DJ. Characterizing DNA methylation alterations from The Cancer Genome Atlas. *J Clin Invest.* 2014;124:17–23.
5. Esteller M. Epigenetics in cancer. *N Engl J Med.* 2008;358:1148–1159.
6. Suzuki K, Suzuki I, Leodolter A, et al. Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. *Cancer Cell.* 2006;9:199–207.
7. Rodriguez J, Frigola J, Vendrell E, et al. Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Res.* 2006;66:8462–9468.
8. Cordaux R, Batzer MA. The impact of retrotransposons on human genome evolution. *Nat Rev Genet.* 2009;10:691–703.
9. Shigaki H, Baba Y, Watanabe M, et al. LINE-1 hypomethylation in gastric cancer, detected by bisulfite pyrosequencing, is associated with poor prognosis. *Gastric Cancer.* 2013;16:480–487.
10. Iwagami S, Baba Y, Watanabe M, et al. LINE-1 hypomethylation is associated with a poor prognosis among patients with curatively resected esophageal squamous cell carcinoma. *Ann Surg.* 2013;257:449–455.
11. Sigalotti L, Fratta E, Bidoli E, et al. Methylation levels of the "long interspersed nucleotide element-1" repetitive sequences predict survival of melanoma patients. *J Transl Med.* 2011;9:78.
12. Hur K, Cejas P, Feliu J, et al. Hypomethylation of long interspersed nuclear element-1 (LINE-1) leads to activation of proto-oncogenes in human colorectal cancer metastasis. *Gut.* 2014;63:635–646.
13. Iwahashi S, Utsunomiya T, Shimada M, et al. High expression of cancer stem cell markers in cholangiolocellular carcinoma. *Surg Today.* 2013;43:654–660.
14. Kondo S, Ojima H, Tsuda H, et al. Clinical impact of c-Met expression and its gene amplification in hepatocellular carcinoma. *Int J Clin Oncol.* 2013;18:207–213.
15. Wolff EM, Byun HM, Han HF, et al. Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. *PLoS Genet.* 2010;6:e1000917.
16. Carreira PE, Richardson SR, Faulkner GJ. L1 retrotransposons, cancer stem cells and oncogenesis. *FEBS J.* 2013; 281(1): 63–73.
17. Feng F, Lu YY, Zhang F, et al. Long interspersed nuclear element ORF-1 protein promotes proliferation and resistance to chemotherapy in hepatocellular carcinoma. *World J Gastroenterol.* 2013;19:1068–1078.
18. Shukla R, Upton KR, Munoz-Lopez M, et al. Endogenous retrotransposition activates oncogenic pathways in hepatocellular carcinoma. *Cell.* 2013;153:101–111.
19. Gao XD, Qu JH, Chang XJ, et al. Hypomethylation of long interspersed nuclear element-1 promoter is associated with poor outcomes for curative resected hepatocellular carcinoma. *Liver Int.* (2014); 34(1):136–146.
20. Benard A, van de Velde CJ, Lessard L, et al. Epigenetic status of LINE-1 predicts clinical outcome in early-stage rectal cancer. *Br J Cancer.* 2013;109:3073–3083.
21. Ma PC, Maulik G, Christensen J, Salgia R. c-Met: structure, functions and potential for therapeutic inhibition. *Cancer Metastasis Rev.* 2003;22:309–325.
22. Kaposi-Novak P, Lee JS, Gomez-Quiroz L, Coulouarn C, Factor VM, Thorgeirsson SS. Met-regulated expression signature defines a subset of human hepatocellular carcinomas with poor prognosis and aggressive phenotype. *J Clin Invest.* 2006;116:1582–1595.
23. Ueki T, Fujimoto J, Suzuki T, Yamamoto H, Okamoto E. Expression of hepatocyte growth factor and its receptor c-met proto-oncogene in hepatocellular carcinoma. *Hepatology.* 1997; 25:862–866.
24. You H, Ding W, Dang H, Jiang Y, Rountree CB. c-Met represents a potential therapeutic target for personalized treatment in hepatocellular carcinoma. *Hepatology.* 2011;54:879–889.
25. Weber B, Kimhi S, Howard G, Eden A, Lyko F. Demethylation of a LINE-1 antisense promoter in the cMet locus impairs Met signalling through induction of illegitimate transcription. *Oncogene.* 2010;29:5775–5784.

A Comparison of the Surgical Outcomes Among Patients With HBV-positive, HCV-positive, and Non-B Non-C Hepatocellular Carcinoma

A Nationwide Study of 11,950 Patients

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Objective: To compare the prognostic factors and outcomes after hepatic resection among patients with hepatitis B virus (HBV)-positive, hepatitis C virus (HCV)-positive, and negative for hepatitis B surface antigen and hepatitis C antibody, so-called "NBNC"-hepatocellular carcinoma (HCC) using the data from a nationwide survey.

Background: The incidence of NBNC-HCC is rapidly increasing in Japan.

Methods: A total of 11,950 patients with HBV-HCC (n = 2194), HCV-HCC (n = 7018), or NBNC-HCC (n = 2738) who underwent a curative hepatic resection were enrolled in this study. The clinicopathological features were compared among the groups. The significant prognostic variables determined by univariate analysis were subjected to a multivariate analysis using a Cox proportional hazard regression model.

Results: Liver function in the HCV-HCC group was significantly worse than that in the HBV-HCC and NBNC-HCC groups. The NBNC-HCC group had significantly more advanced HCC than the HCV-HCC group. The 5-year overall survival rates after hepatectomy in the HBV-HCC, HCV-HCC, and NBNC-HCC groups were 65%, 59%, and 68%, respectively. The 5-year recurrence-free survival (RFS) rates in these 3 groups were 41%, 31%, and 47%, respectively. Stratifying the RFS rates according to the TNM stage showed that the NBNC-HCC group had a significantly better prognosis than the HBV-HCC group in stages II, III, and IVA, and a significantly better prognosis than the HCV-HCC group in stages I and II. Multivariate analysis revealed a significantly better RFS rate in the NBNC-HCC group.

Conclusions: The findings of this nationwide survey indicated that patients with NBNC-HCC had a significantly lower risk of HCC recurrence than those with HBV-HCC and HCV-HCC.

Keywords: hepatic resection, nationwide study, non-B non-C hepatocellular carcinoma, prognosis

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The most prominent etiological factors associated with hepatocellular carcinoma (HCC) worldwide include chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), and chronic alcohol consumption. Although HCV-related HCC is responsible for the greatest proportion of HCC patients in Japan,[1,2] the proportion of HCC cases negative for hepatitis B surface antigen (HBsAg) and hepatitis C antibody (HCVAb), so-called "NBNC-HCC," is rapidly increasing.[3–5]

Many studies have compared the clinicopathological characteristics and prognosis after hepatic resection among patients with different background liver diseases, most of which compared HBV-HCC and HCV-HCC, and the results have been controversial.[6–8] Both HBV and HCV infections are hepatotrophic viral infections; however, there are significant differences between the molecular carcinogenic mechanisms of these 2 viruses.[9] In addition, several recent studies have compared the surgical outcomes between patients with NBNC-HCC and those with viral-related HCC (HBV-HCC and/or HCV-HCC), with inconsistent results, possibly owing to differences in demographic characteristics and tumor factors, and an insufficient number of patients in the cohorts.[10–21]

The exact background or molecular mechanisms underlying the sharp increase in the incidence of NBNC-HCC remain unclear; however, nonalcoholic steatohepatitis (NASH) and metabolic syndrome are suggested as important risk factors.[5,22] Differences in pathogenic mechanisms during hepatocarcinogenesis associated with the underlying chronic liver damage may explain the differences in the clinicopathological features and surgical outcomes observed in HCC patients who underwent hepatic resection. Characterizing the HCC based on the aspects of the background chronic liver damage may provide important insight into novel strategies for the prevention and treatment of the progression and recurrence of HCC.

The Liver Cancer Study Group of Japan (LCSGJ) has prospectively collected data on patients with HCC in Japan using biannual nationwide surveys since 1965. First, physicians in the participating institutions complete a questionnaire and check the accuracy of the data. Second, the nationwide survey committee checks the data, and with unusual data, the participating institution is requested to confirm the data to ensure its accuracy. Recently, this study group examined the

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prognostic factors and outcomes associated with 3 types of treatments in 4741 patients with NBNC-HCC and showed that "hepatic resection" offers a significant prognostic advantage over radiofrequency ablation and transcatheter arterial chemoembolization.[23] Therefore, this study compared the clinicopathological characteristics and prognosis after hepatic resection between the patients with NBNC-HCC and those with viral-related HCC based on data from the same nationwide follow-up survey conducted by the LCSGJ.

PATIENTS AND METHODS

A total of 62,321 patients with primary liver cancer were prospectively registered biannually from January 2000 to December 2005 by the LCSGJ, as reported previously.[23] This included 18,309 patients who underwent hepatic resection as the initial treatment, and 15,390 patients of these were histologically diagnosed with HCC in the resected specimens at each institution. Patients without data for hepatitis viral infection status of HBsAg and HCVAb were excluded. In addition, 309 patients positive for both HBsAg and HCVAb were excluded. Additional exclusions included extrahepatic metastasis, Child-Pugh C, and a noncurative hepatectomy. The study also excluded the 230 patients lacking outcome data. Finally, 11,950 HCC patients were included in this cohort study and were classified according to hepatitis virus infection status into HBV-HCC group (n = 2194, 18.4%), HCV-HCC group (n = 7018, 58.7%), and NBNC-HCC group (n = 2738, 22.9%).

Patients were prospectively followed up at each institution. Most patients were traditionally observed according to a protocol similar to the Japanese guidelines,[24] which recommended ultrasonography and tumor marker level measurement every 3 or 4 months, and enhanced computed tomography or magnetic resonance imaging every 6 or 12 months. The final prognosis of the registered patients was followed until confirmation of death at every survey. Although this study protocol was not submitted to the institutional review board of each institution participating in the nationwide survey, the collection and registration of the data for the patients with HCC were performed with the approval of each institution.

Statistical Analysis

The clinical characteristics of the 3 groups were compared by either the χ^2 test or the Kruskal-Wallis test. The overall and recurrence-free survival (RFS) rates after hepatectomy were calculated by the Kaplan-Meier method and were then compared by the log-rank test. Among the 11,950 patients included in this study, data on HCC recurrence were lacking in 674 patients. Therefore, the RFS rates were calculated for the remaining 11,276 patients. Nineteen clinical variables, including the hepatitis virus infection status, were evaluated by univariate analysis using the log-rank test to determine the risk factors for tumor recurrence after hepatic resection. The RFS rates were stratified according to the Child-Pugh classification and the TNM stage as defined by the LCSGJ (Table 1).[25] The RFS curves were compared among the different histological findings of the resected nontumor liver tissues.

Continuous variables were divided into 2 groups according to the median value. Significant variables with a value of $P < 0.05$ by univariate analysis were subjected to a multivariate analysis using a Cox proportional hazards regression model with a backward elimination method.[26] Significance tests were 2-tailed, and a value of $P < 0.05$ was considered statistically significant. Bonferroni correction was applied when the RFS rates were compared between the HBV-HCC, HCV-HCC, and NBNC-HCC groups (significance threshold, $P = 0.05$ divided by the number of groups: $P = 0.017$). All statistical analyses were performed using the Statistical Analysis System software program, version 9.1.3 (SAS Inc, Cary, NC).

TABLE 1. TNM Stage by the Liver Cancer Study Group of Japan

	T Category	N Category	M Category
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
Stage IVA	T4	N0	M0
	T1, T2, T3, T4	N1	M0
Stage IVB	T1, T2, T3, T4	N0, N1	M1

T category is determined on the basis of the "number," "size," and "vascular and/or bile duct invasion" by the tumor. N category: N0 indicates absence of lymph node metastasis, and N1 indicates presence of lymph node metastasis. M category: M0 indicates absence of distant metastasis, and M1 indicates presence of distant metastasis.

RESULTS

Clinical Characteristics of the Study Participants

The clinical characteristics in the 3 groups are summarized in Table 2. All of the 21 variables were significantly different among the groups. In particular, the age of the patients in the HBV-HCC group was significantly younger than that in the other 2 groups. The percentage of female patients in the HCV-HCC group was higher than that in the other 2 groups. For patients in the NBNC-HCC group, the percentage of habitual alcohol drinkers was significantly higher than that in the other 2 groups. The results of liver function tests in the HCV-HCC group were significantly worse. The incidence of a normal liver in the NBNC-HCC group (22%) was markedly higher than that in the HBV-HCC group (4%) and HCV-HCC group (2%). Conversely, the NBNC-HCC group had significantly more advanced HCC based on the tumor factors, including the tumor size and des-gamma-carboxy prothrombin. In contrast, the HCV-HCC group had the smallest tumor size and the lowest percentage of portal venous invasion (Table 2).

Prognosis of the Patients With HCC After Hepatic Resection

The prognosis after hepatic resection is summarized in Figure 1. The 5-year overall survival rates in the HBV-HCC, HCV-HCC, and NBNC-HCC groups were 65%, 59%, and 68%, respectively. The 5-year RFS rates were 41%, 31%, and 47%, respectively. Although the overall survival rate in the NBNC-HCC group was significantly better than that in the HBV-HCC and HCV-HCC groups, differences among the groups were relatively small (Fig. 1A), whereas differences in the RFS rates were more distinctive (Fig. 1B). Therefore, this study focused on the comparisons of the RFS rates among the 3 groups.

The RFS rate in the NBNC-HCC group was significantly better than that in the HBV-HCC and HCV-HCC groups. The RFS rate in the HBV-HCC group was comparable to that in the HCV-HCC group (Fig. 1B). After stratification according to the Child-Pugh class, the RFS rate in the NBNC-HCC group was significantly better than that in the HBV-HCC and HCV-HCC groups in Child-Pugh A patients (Fig. 2A). No significant differences were detected among the 3 groups in the Child-Pugh B patients, possibly due to the small number of patients classified as Child-Pugh B (Fig. 2B). When the RFS rates were stratified according to the TNM staging system (Fig. 3), the NBNC-HCC group had a significantly better prognosis than the HBV-HCC group in the stage II, III, and IVA patients, and a significantly better prognosis than the HCV-HCC group in the stage I and II patients. When the trends of the RFS curves were compared among the different histological findings of the resected nontumor liver tissues (Fig. 4), the curves in the HBV-HCC and NBNC-HCC groups

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TABLE 2. Clinical Characteristics in the 11,950 Patients With HCC Who Underwent Hepatic Resection

Variables	HBV (n = 2194)	HCV (n = 7018)	NBNC (n = 2738)	*P*
Age, yr	57 (40, 73)	67 (54, 78)	67 (51, 80)	<0.001
Sex				<0.001
Male	1796 (82%)	5225 (75%)	2253 (82%)	
Female	398 (18%)	1793 (26%)	485 (18%)	
Alcohol				<0.001
None	1550 (71%)	4752 (68%)	1535 (56%)	
Positive*	305 (14%)	1223 (17%)	845 (31%)	
Serum albumin, g/dL	4.0 (3.2, 4.7)	3.9 (3.0, 4.5)	4.0 (3.2, 4.7)	<0.001
Serum total bilirubin, mg/dL	0.9 (0.4, 1.5)	0.8 (0.4, 1.5)	0.9 (0.4, 1.5)	<0.001
ICG R15, %	13 (4, 28)	18 (6, 36)	14 (4, 32)	<0.001
Prothrombin activity, %	86 (62, 108)	86 (63, 108)	89 (66, 114)	<0.001
Esophageal varices				<0.001
None	1604 (73%)	4876 (70%)	2124 (78%)	
Positive	291 (13%)	1167 (17%)	250 (9%)	
Child-Pugh class				<0.001
A	1941 (89%)	5920 (84%)	2457 (90%)	
B	159 (7%)	760 (11%)	153 (6%)	
Alpha-fetoprotein, ng/mL	5467 (15, 35,000)	1647 (15, 4445)	3060 (15, 11,800)	<0.001
DCP, mAU/mL†	1602 (40, 10,000)	1111 (40, 10,000)	2194 (40, 10,000)	<0.001
Tumor number				<0.001
1	1624 (74%)	5106 (73%)	2126 (78%)	
2	312 (14%)	1091 (16%)	319 (12%)	
> 3	201 (9%)	662 (10%)	217 (8%)	
Tumor size, mm	4.8 (1.5, 12)	4.0 (1.4, 9)	5.8 (1.6, 14)	<0.001
Tumor differentiation‡				<0.001
Well	321 (15%)	1545 (22%)	598 (22%)	
Moderately	1417 (65%)	4319 (62%)	1708 (62%)	
Poorly	290 (13%)	710 (10%)	278 (10%)	
Portal venous invasion‡				<0.001
Negative	1384 (63%)	5108 (73%)	1826 (67%)	
Positive	683 (31%)	1542 (22%)	783 (29%)	
Hepatic venous invasion‡				<0.001
Negative	1749 (80%)	5938 (85%)	2232 (82%)	
Positive	283 (13%)	588 (8%)	337 (13%)	
IM‡				<0.001
Negative	1541 (70%)	5231 (75%)	2081 (76%)	
Positive	451 (21%)	1159 (17%)	783 (17%)	
Nontumor tissue‡				<0.001
Normal	76 (4%)	118 (2%)	611 (22%)	
Liver fibrosis	971 (44%)	3153 (45%)	1218 (45%)	
Liver cirrhosis	992 (45%)	3269 (47%)	703 (26%)	
TNM stage§				<0.001
I	301 (14%)	1047 (15%)	228 (8%)	
II	989 (45%)	3256 (48%)	1395 (51%)	
III	493 (23%)	1449 (21%)	578 (21%)	
IVA	138 (7%)	236 (3%)	136 (5%)	
Cause of death				<0.001
HCC related	305 (69%)	842 (56%)	239 (53%)	
Liver related	55 (13%)	252 (17%)	58 (13%)	
Operation related	8 (2%)	53 (3%)	12 (3%)	
Others	32 (7%)	236 (16%)	99 (22%)	
Median follow-up period, yr	1.6 (0.1, 5.1)	1.7 (0.1, 5.1)	1.5 (0.1, 5.1)	<0.001

Data are shown as the median (5 percentile, 95 percentile) unless specified.
*Eighty-six gram of alcohol daily for more than 10 yrs.
†Questionnaire sheet requested the actual value when it was between 40 and 10,000 mAU/mL.
‡Findings of histological examination.
§By the Liver Cancer Study Group of Japan.
Three groups (HBV, HCV, NBNC) were classified as described in "Patients and Methods" section.
DCP indicates des-gamma-carboxy prothrombin; ICGR15, indocyanine green retention rate at 15 min.

reached a plateau at 2 years after hepatic resection, but the RFS curve in the HCV-HCC group continued to decrease over time, even when patients had a normal liver. However, when patients had a cirrhotic liver, the curves in the HBV-HCC and NBNC-HCC groups decreased over time similar to the pattern observed in the HCV-HCC group.

Prognostic Factors Related to the RFS Rates After Hepatic Resection

Nineteen clinicopathological variables were screened as risk factors for HCC recurrence using a univariate analysis (Table 3). Age, sex, and habitual alcohol intake were not selected as prognostic

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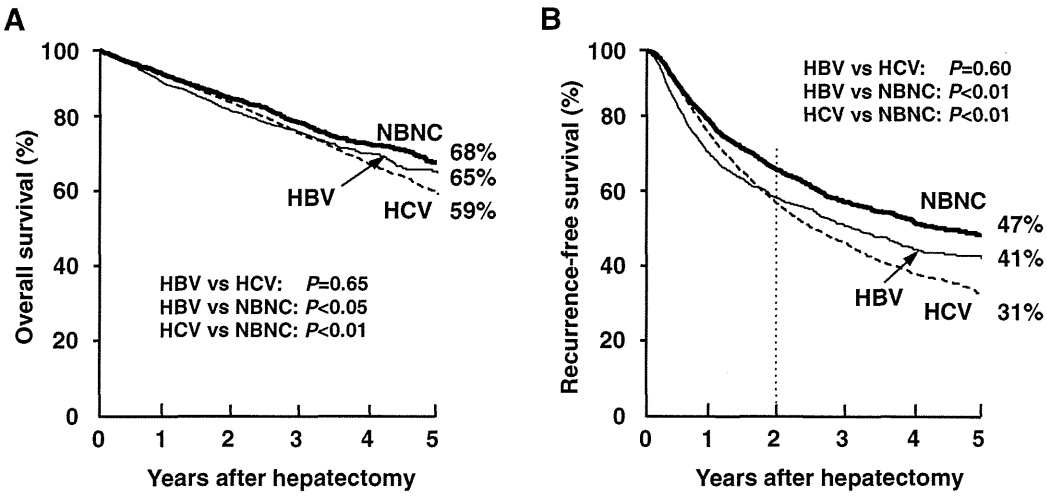

FIGURE 1. Comparisons of the survival outcomes after hepatic resection among the HBV-HCC, HCV-HCC, and NBNC-HCC groups. The overall survival rates (A) and RFS rates (B). The vertical dashed line indicates 2 years after hepatectomy. All comparisons were made using the log-rank test with Bonferroni correction.

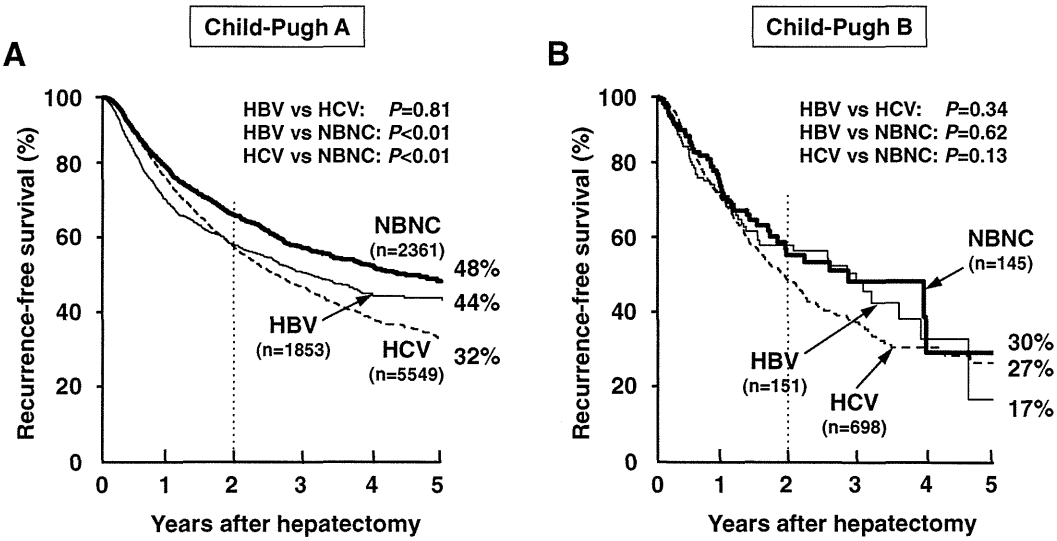

FIGURE 2. Comparisons of the RFS rates among the HBV-HCC, HCV-HCC, and NBNC-HCC groups. The RFS rates were stratified by Child-Pugh class into Child-Pugh A (A) and Child-Pugh B (B). The vertical dashed line indicates 2 years after hepatectomy. All comparisons were made by the log-rank test with Bonferroni correction.

factors, whereas the remaining 16 variables, including the hepatitis virus infection status, were significant risk factors for tumor recurrence after hepatic resection.

The initial multivariate model identified 11 variables as independent prognostic factors (Supplemental Table 1, available at http://links.lww.com/SLA/A587). Consequently, the final multivariate model identified 12 variables, which included the 11 variables selected by the initial multivariate model and the indocyanine green retention rate at 15 minutes, as independent prognostic factors (Table 4). The multivariate analysis revealed that the NBNC-HCC group had a significantly better RFS rate than the HBV-HCC group [hazard ratio (HR) = 1.21; 95% confidence interval (CI): 1.06–1.38, $P = 0.006$] or the HCV-HCC group (HR = 1.26; 95% CI: 1.13–1.41, $P < 0.001$). On the contrary, the HBV-HCC group had a comparable RFS rate with the HCV-HCC group (HR = 0.96; 95% CI: 0.86–1.07, $P = 0.43$).

DISCUSSION

Differences in the etiologies and pathogenic mechanisms of hepatocarcinogenesis may reflect the unique clinical characteristics and prognosis in patients with HCC.[5,27–29] Therefore, this study first compared the clinicopathological backgrounds among patients with HBV-HCC, HCV-HCC, and NBNC-HCC who underwent a curative hepatic resection.

It is well known that the age at occurrence of HCC is higher in HCV-HCC than in HBV-HCC patients.[6–8,10–21] This study also showed that NBNC-HCC occurs at a significantly higher age (nearly 10 years older) than HBV-HCC. It is noteworthy from the viewpoint of surveillance for HCC patients that NBNC-HCC (average, 67 years; range, 51–80 years) occurred at a wider range of ages than HCV-HCC (average, 67; range, 54–78 years). Indeed, a similar tendency has been observed in previous reports.[12,14,18,21] Several studies have shown that the percentage of female patients with HBV-HCC was lower

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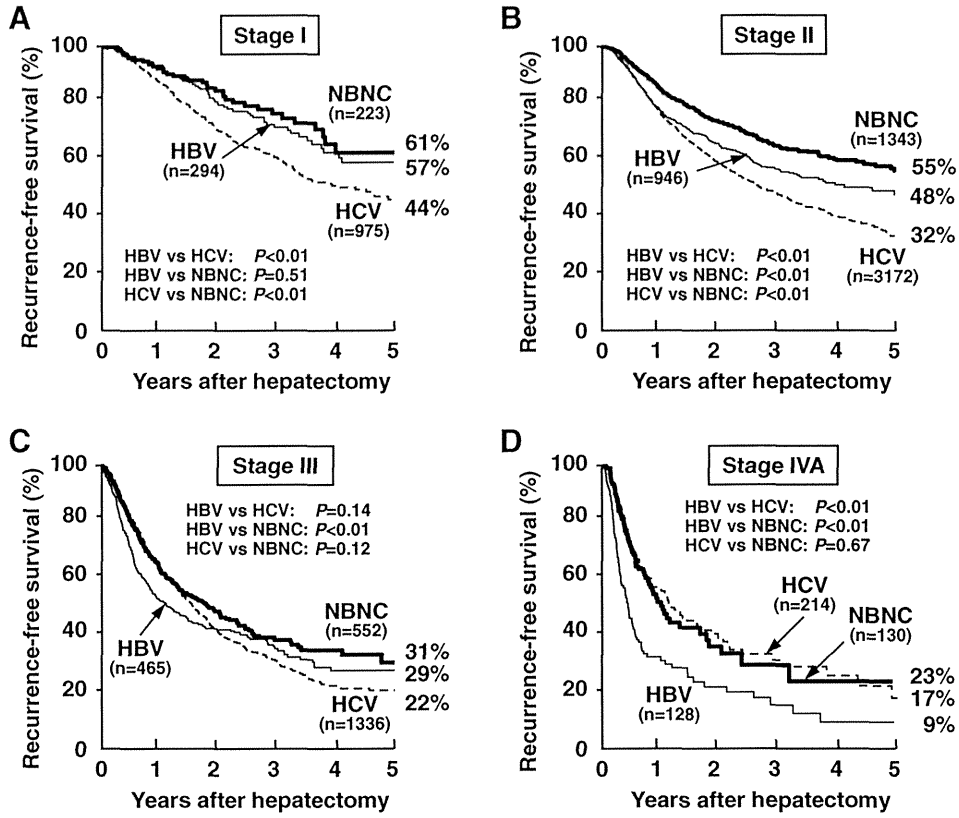

FIGURE 3. Comparisons of the RFS rates among the HBV-HCC, HCV-HCC, and NBNC-HCC groups. The RFS rates were stratified by the TNM stage[21] into stage I (A), stage II (B), stage III (C), and stage IVA (D). All comparisons were made by the log-rank test with Bonferroni correction.

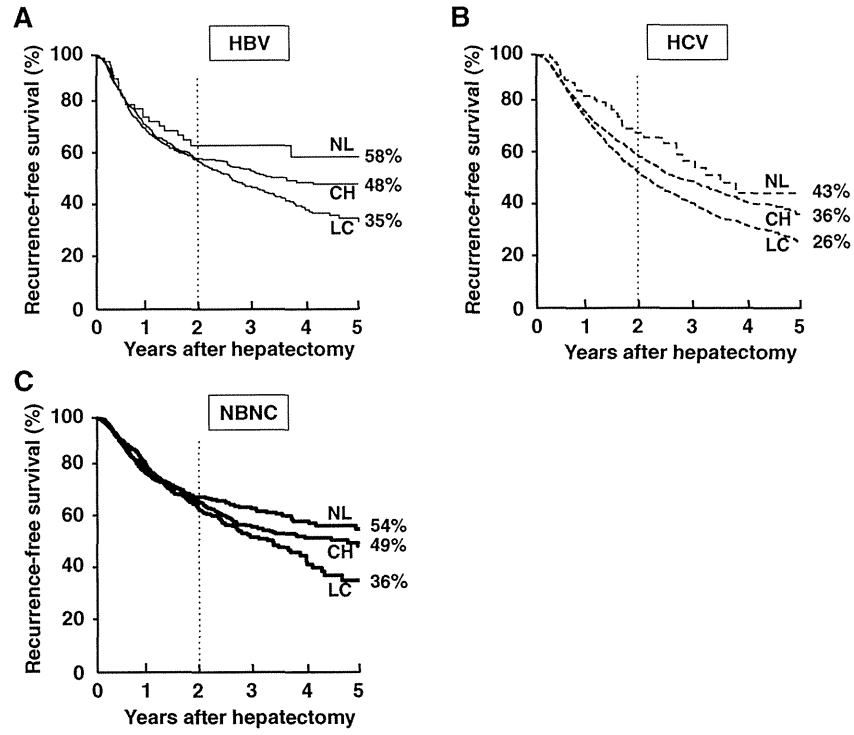

FIGURE 4. Comparisons of the RFS rates among the different histological findings of the resected nontumor liver tissues, including the NL (normal liver), CH (chronic hepatitis), and LC (liver cirrhosis) groups. The RFS rates were compared between patients with HBV-HCC (A), HCV-HCC (B), and NBNC-HCC (C). The vertical dashed line indicates 2 years after hepatectomy.

than that in patients with HCV-HCC[30,31] This study also indicated that the percentage of female patients in the NBNC-HCC group was significantly lower than that in the HCV-HCC group. Because more than 95% of the patients with habitual alcohol consumption in both groups were male (data not shown), which is consistent with previous reports from Japan,[32] the higher positive percent of habitual alcohol consumption in the NBNC-HCC group may at least partially reflect the lower percentage of female patients in the NBNC-HCC group. However, the precise reasons for the sex differences among the groups remain to be determined.

Histological examination revealed that the incidence of a normal liver in the NBNC-HCC group (22%) was markedly higher than

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TABLE 3. Risk Factors of Tumor Recurrence Determined by the Univariate Analysis in the Patients With HCC Who Underwent Hepatic Resection

Variables	No. Patient	DFS (%) 1-yr	3-yr	5-yr	*P*
All	11,276	75	49	37	
Age, yr					
<69	6533	75	50	38	Reference
≥69	4722	75	47	35	0.221
Sex					
Male	8722	75	49	36	Reference
Female	2554	75	50	40	0.269
Alcohol					
None	7389	74	49	37	Reference
Positive*	2245	76	49	37	0.499
Serum albumin, g/dL					
<3.9	4837	72	43	32	Reference
≥3.9	6097	77	53	41	<0.001
Serum total bilirubin, mg/dL					
<0.8	5553	75	51	39	Reference
≥0.8	5473	74	47	34	<0.01
ICGR15 (%)					
<14	4851	76	54	43	Reference
≥14	5530	73	44	31	<0.001
Prothrombin activity (%)					
<87	5097	73	46	35	Reference
≥87	5336	76	52	40	<0.001
Child-Pugh class					
A	9765	75	50	38	Reference
B	994	71	41	27	<0.001
Alpha-fetoprotein, ng/mL					
<15	4896	83	58	44	Reference
≥15	5886	68	42	31	<0.001
DCP, mAU/mL					
<148	5668	81	54	41	Reference
≥148	4413	66	42	31	<0.001
Tumor number					
1	8393	79	54	42	Reference
>2	2606	62	32	20	<0.001
Tumor size, mm					
<40	6288	81	55	41	Reference
≥40	4699	66	41	32	<0.001
Grade of differentiation†					
Well	2333	83	56	42	Reference
Moderately	7038	73	47	36	<0.001
Poorly	1215	63	43	32	<0.001
Portal venous invasion†					
Negative	7888	80	53	41	Reference
Positive	2811	60	39	27	<0.001
Hepatic venous invasion†					
Negative	9382	77	51	39	Reference
Positive	1132	57	34	25	<0.001
IM†					
Negative	8399	80	55	41	Reference
Positive	1942	54	27	18	<0.001
Nontumor tissue†					
Normal	777	77	62	53	Reference
Liver fibrosis	5098	75	52	41	<0.001
Liver cirrhosis	4621	73	44	29	<0.001
TNM stage‡					
I	1683	89	64	50	Reference
II	5834	80	54	40	<0.001
III	2724	62	35	26	<0.001
IVA	812	48	26	20	<0.001
Hepatitis virus infection status					
NBNC	2631	79	57	47	Reference
HBV	2094	70	50	41	<0.001
HCV	6551	75	46	31	<0.001

*Eighty-six gram of alcohol daily for more than 10 yrs.
†Findings of histological examination.
‡By the Liver Cancer Study Group of Japan.
DCP indicates des-gamma-carboxy prothrombin; ICGR15, indocyanine green retention rate at 15 min.

TABLE 4. Independent Risk Factors of Tumor Recurrence Determined by the Cox Proportional Hazard Regression Analysis With the Backward Elimination Method (Multivariate Final Model)

Variables	Patient No.	HR (95% CI)	*P*
Serum albumin, g/dL			
<3.9	3028	Reference	—
≥3.9	3833	0.84 (0.77–0.91)	<0.001
ICGR15, %			
<14	3189	Reference	—
≥14	3672	1.10 (1.01–1.20)	0.023
Alpha-fetoprotein, ng/mL			
<15	3121	Reference	—
≥15	3740	1.30 (1.19–1.41)	<0.001
DCP, mAU/mL			
<148	3851	Reference	—
≥148	3010	1.27 (1.16–1.39)	<0.001
Tumor size, mm			
<40	3905	Reference	—
≥40	2956	1.25 (1.14–1.38)	<0.001
Tumor number			
1	5216	Reference	—
>2	1645	1.26 (1.12–1.40)	<0.001
Portal venous invasion*			
Negative	5009	Reference	—
Positive	1852	1.26 (1.15–1.38)	<0.001
Hepatic venous invasion*			
Negative	6101	Reference	
Positive	760	1.25 (1.11–1.41)	<0.001
IM*			
Negative	5529	Reference	
Positive	1332	1.53 (1.37–1.70)	<0.001
Nontumor tissue*			
Normal	453	Reference	
Liver fibrosis	3394	1.09 (0.91–1.32)	0.347
Liver cirrhosis	3014	1.41 (1.16–1.72)	<0.001
TNM stage†			
I	1010	Reference	—
II	3671	1.31 (1.14–1.52)	<0.001
III	1682	1.63 (1.37–1.94)	<0.001
IVA	498	1.80 (1.44–2.24)	<0.001
Hepatitis virus infection status			
NBNC	1554	Reference	—
HBV	1263	1.21 (1.06–1.38)	0.006
HCV	4044	1.26 (1.13–1.41)	<0.001

*Findings of histological examination.
†By the Liver Cancer Study Group of Japan.
DCP indicates des-gamma-carboxy prothrombin; ICGR15, indocyanine green retention rate at 15 min.

that in the virus-related HCC cases (2%–4%). NBNC-HCC can occur with no features of chronic liver damage, and it is often associated with NASH and metabolic syndrome.[5,33,34] Because fewer patients in the NBNC-HCC group received regular follow-up for the liver disease, they were generally diagnosed at a more advanced stage of HCC.[35,36] However, in the HBV-HCC group, microscopic intrahepatic metastasis was more frequently observed compared with the NBNC-HCC group. Conversely, the HCV-HCC group had significantly less advanced tumors compared with the other 2 groups. Accordingly, HCC-related death predominated in the HBV-HCC group, whereas the liver-related death rate was higher in the HCV-HCC group. In the NBNC-HCC group, causes of death other than liver-related and HCC-related death ("Others" in the Table 2) were relatively more common (22%). To our knowledge, no previous studies have compared the cause of death after hepatectomy among patients with the different hepatitis virus infection status.[10–21] Metabolic

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syndrome-related deaths and other organ cancer-related deaths might also have been more common in the NBNC-HCC group, although the questionnaire sheet used in this survey did not include these questions.

Several studies have examined prognosis after hepatic resection for patients with NBNC-HCC.[10–21] Some showed that long-term surgical outcomes in patients with NBNC-HCC were comparable to those of patients with hepatitis virus-related HCC.[10,19,20] Cescon et al reported that NBNC-HCC patients had significantly better RFS rates than HBV-HCC patients.[15] Several others showed that NBNC-HCC patients had significantly better survival rates than HCV-HCC patients, but the rates were comparable with those of HBV-HCC patients.[11,14,17,18] Most studies enrolled 100 or fewer patients with NBNC-HCC, with inconclusive results. This study clearly showed that the RFS rate in the NBNC-HCC group (n = 2738) was significantly better than that in the HBV-HCC and HCV-HCC groups.

The RFS curves of the NBNC-HCC group and the HBV-HCC group were distinctly different within 2 years after hepatectomy, whereas these curves were almost parallel thereafter. There are 2 types of HCC recurrence; one is "early recurrence" mainly because of intrahepatic metastasis (IM) and the other is "late recurrence" because of multicentric (MC) hepatocarcinogenesis.[37] The results of this study suggest that the risk of IM recurrence in the NBNC-HCC group was lower than that in the HBV-HCC group, whereas the risk of MC recurrence was comparable between the 2 groups. These results may explain previous findings that NBNC-HCC patients had significantly better RFS rates than HBV-HCC patients when restricting analysis to those with liver cirrhosis.[13] In this study, the RFS curves of the NBNC-HCC and HCV-HCC groups gradually broadened over time. These findings may indicate that the risk of MC recurrence in the NBNC-HCC group was lower than that in the HCV-HCC group, whereas the risk of IM recurrence was comparable between the 2 groups. This study also revealed that although the difference in the RFS rates between the HBV-HCC and HCV-HCC groups was not statistically significant,[6,8] the patterns of recurrence (shapes of the RFS curves) between the 2 groups were substantially different (Fig. 1B).

The study stratified the patients on the basis of the TNM stage. The NBNC-HCC group showed a significantly better prognosis than the HBV-HCC group in the more advanced tumor-stages. In contrast, the NBNC-HCC group had a significantly better prognosis than the HCV-HCC group in the earlier tumor stages. The differences in the RFS rates between the 2 groups gradually broadened over time. It seems that MC recurrence in the HCV-HCC group was more frequent than that in the NBNC-HCC group during the early tumor stages, similar to previous findings that patients with HCV-HCC had a significantly poorer prognosis than those with NBNC-HCC, when the tumor size was less than 3 cm.[11]

It is important to note that differences in the overall survival rates among the 3 groups were smaller compared with those for the RFS rates as described in the previous reports.[12,13] The precise reasons for such discordant results remain unknown. However, difference in the patterns of HCC recurrence, which also reflect the therapeutic efficacy for recurrent HCC, may explain this finding.[12] Specifically, although the HCV-HCC group had a higher risk of recurrence, this group tended to develop MC recurrence rather than IM recurrence. Because MC recurrence is associated with a greater opportunity to receive potentially curative local therapies, such as hepatectomy and radiofrequency ablation, the overall survival rate of the HCV-HCC group may have been improved. In fact, it has been shown that the overall survival rate after MC recurrence was significantly better than that after IM recurrence.[38,39] Alternatively, metabolic syndrome-related death and other organ cancer-related death ("Others" in the Table 2) may account for the relatively worse overall survival in the NBNC-HCC group despite the lower risk of HCC recurrence in this group.

Twelve independent risk factors for tumor recurrence, including the hepatitis virus infection status, were identified using a Cox proportional hazards regression analysis. One previous study examined more than 100 patients with NBNC-HCC (n = 129) using a multivariate analysis to clarify the prognostic significance of the hepatitis virus infection status. However, the authors found no prognostic impact of the hepatitis virus infection status on the RFS rate after hepatectomy.[19] To our knowledge, this is the first study to demonstrate a significant prognostic advantage after hepatic resection in the NBNC-HCC group compared with the HBV-HCC and HCV-HCC groups using a multivariate analysis. Recently, Li et al[20] demonstrated that female sex was identified an independent risk factor for RFS rates in patients with NBNC-HCC. However, there were no significant sex differences in terms of the RFS rates in this study. In addition, sex was not selected as a prognostic factor in a previous nationwide study of NBNC-HCC patients.[23]

One limitation of this study was the relatively short follow-up period. However, the study did observe that the specific RFS curves reflected both early recurrence and late recurrence among the 3 examined groups. In addition, because the current study was not prospectively randomized, the treatment policies were not uniform, and the effectiveness of the treatment might not be comparable among the different institutions. No information regarding the possible etiologies of NBNC-HCC, such as occult HBV infection, NASH, diabetes mellitus, or metabolic syndrome (body mass index, etc) was available because it was not included on the questionnaire sheet used in this survey. The LCSGJ may have to consider the inclusion of such information in future questionnaire surveys.

CONCLUSIONS

This large nationwide study indicated that patients with NBNC-HCC had a significantly better overall survival rate after hepatectomy than those with HBV-HCC and HCV-HCC, although the differences in the overall survival rate among the 3 groups were relatively small. Much clearer differences were observed in the RFS rates among the groups. Patients with NBNC-HCC had a significantly lower risk of HCC recurrence after hepatectomy than those with HBV-HCC and HCV-HCC, possibly owing to the fact that NBNC-HCC may be a less aggressive tumor (lower risk of IM recurrence) than HBV-HCC, although NBNC-HCC patients and HBV-HCC patients have an almost identical risk of MC recurrence. In addition, NBNC-HCC may occur less frequently (lower risk of MC recurrence) than HCV-HCC, although NBNC-HCC patients and HCV-HCC patients have a comparable risk of IM recurrence. The findings of this study suggest that it is necessary to define uniform treatment strategies for HCC patients, based not only on the tumor stage or liver functional reserve but also on hepatitis viral infection status.

REFERENCES

1. Ikai I, Arii S, Okazaki M, et al. Report of the 17th nationwide follow-up survey of primary liver cancer in Japan. *Hepatol Res.* 2007;37:676–691.
2. Namiki I, Nishiguchi S, Hino K, et al. Management of hepatitis C: report of the consensus meeting at the 45th Annual Meeting of the Japan Society of Hepatology (2009). *Hepatol Res.* 2010;40:347–368.
3. Nishikawa H, Osaki Y. Non-B non-C hepatocellular carcinoma (review). *Int J Oncol.* 2013;43:1333–1342.
4. Tanaka H, Imai Y, Hiramatsu N, et al. Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003. *Ann Intern Med.* 2008;148:820–826.
5. Utsunomiya T, Shimada M. Molecular characteristics of non-cancerous liver tissue in non-B non-C hepatocellular carcinoma. *Hepatol Res.* 2011;41:711–721.

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6. Takenaka K, Yamamoto K, Taketomi A, et al. A comparison of the surgical results in patients with hepatitis B versus hepatitis C-related hepatocellular carcinoma. *Hepatology*. 1995;22:20–24.
7. Sasaki Y, Yamada T, Tanaka H, et al. Risk of recurrence in a long-term follow-up after surgery in 417 patients with hepatitis B- or hepatitis C-related hepatocellular carcinoma. *Ann Surg*. 2006;244:771–780.
8. Kao WY, Su CW, Chau GY, et al. A comparison of prognosis between patients with hepatitis B and C virus-related hepatocellular carcinoma undergoing resection surgery. *World J Surg*. 2011;35:858–867.
9. Honda M, Yamashita T, Ueda T, et al. Different signaling pathways in the livers of patients with chronic hepatitis B or chronic hepatitis C. *Hepatology*. 2006;44:1122–1138.
10. Pawlik TM, Poon RT, Abdalla EK, et al. Hepatitis serology predicts tumor and liver-disease characteristics but not prognosis after resection of hepatocellular carcinoma. *J Gastrointest Surg*. 2004;8:794–804.
11. Li Q, Li H, Qin Y, et al. Comparison of surgical outcomes for small hepatocellular carcinoma in patients with hepatitis B versus hepatitis C: a Chinese experience. *J Gastroenterol Hepatol*. 2007;22:1936–1941.
12. Tanaka K, Shimada H, Matsuo K, et al. Clinical characteristics and surgical outcome in hepatocellular carcinoma without hepatitis B virus surface antigen or hepatitis C virus antibody. *Ann Surg Oncol*. 2007;14:1170–1181.
13. Nanashima A, Abo T, Sumida Y, et al. Clinicopathological characteristics of patients with hepatocellular carcinoma after hepatectomy: relationship with status of viral hepatitis. *J Surg Oncol*. 2007;96:487–492.
14. Kondo K, Chijiiwa K, Funagayama M, et al. Differences in long-term outcome and prognostic factors according to viral status in patients with hepatocellular carcinoma treated by surgery. *J Gastrointest Surg*. 2008;12:468–476.
15. Cescon M, Cucchetti A, Grazi GL, et al. Role of hepatitis B virus infection in the prognosis after hepatectomy for hepatocellular carcinoma in patients with cirrhosis: a Western dual-center experience. *Arch Surg*. 2009;144:906–913.
16. Zhou Y, Si X, Wu L, et al. Influence of viral hepatitis status on prognosis in patients undergoing hepatic resection for hepatocellular carcinoma: a meta-analysis of observational studies. *World J Surg Oncol*. 2011;9:108.
17. Kaneda K, Kubo S, Tanaka H, et al. Features and outcome after liver resection for non-B non-C hepatocellular carcinoma. *Hepatogastroenterology*. 2012;59:1889–1892.
18. Kaibori M, Ishizaki M, Matsui K, et al. Clinicopathologic characteristics of patients with non-B non-C hepatitis virus hepatocellular carcinoma after hepatectomy. *Am J Surg*. 2012;204:300–307.
19. Nishikawa H, Arimoto A, Wakasa T, et al. Comparison of clinical characteristics and survival after surgery in patients with non-B and non-C hepatocellular carcinoma and hepatitis virus-related hepatocellular carcinoma. *J Cancer*. 2013;4:502–513.
20. Li T, Qin LX, Gong X, et al. Hepatitis B virus surface antigen-negative and hepatitis C virus antibody-negative hepatocellular carcinoma: clinical characteristics, outcome, and risk factors for early and late intrahepatic recurrence after resection. *Cancer*. 2013;119:126–135.
21. Kudo A, Tanaka S, Ban D, et al. Alcohol consumption and recurrence of non-B or non-C hepatocellular carcinoma after hepatectomy: a propensity score analysis. *J Gastroenterol*. 2014;49:1352–1361.
22. Welzel TM, Graubard BI, Zeuzem S, et al. Metabolic syndrome increases the risk of primary liver cancer in the United States: a study in the SEER-Medicare database. *Hepatology*. 2011;54:463–471.
23. Utsunomiya T, Shimada M, Kudo M, et al. Nationwide study of 4741 patients with non-B non-C hepatocellular carcinoma with special reference to the therapeutic impact. *Ann Surg*. 2014;259:336–345.
24. Makuuchi M, Kokudo N, Arii S, et al. Development of evidence-based clinical guidelines for the diagnosis and treatment of hepatocellular carcinoma in Japan. *Hepatol Res*. 2008;38:37–51.
25. The Liver Cancer Study Group of Japan. *The General Rules for the Clinical and Pathological Study of Primary Liver Cancer*. 2nd English ed. Tokyo, Japan: Kanehara & Co, Ltd; 2003.
26. Schneider HJ, Wallaschofski H, Völzke H, et al. Incremental effects of endocrine and metabolic biomarkers and abdominal obesity on cardiovascular mortality prediction. *PLoS One*. 2012;7:e33084.
27. Schlaeger C, Longerich T, Schiller C, et al. Etiology-dependent molecular mechanisms in human hepatocarcinogenesis. *Hepatology*. 2008;47:511–520.
28. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology*. 2012;142:1264–1273.
29. Arzumanyan A, Reis HM, Feitelson MA. Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma. *Nat Rev Cancer*. 2013;13:123–135.
30. Shiratori Y, Shiina S, Imamura M, et al. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C- viral infection in Japan. *Hepatology*. 1995;22:1027–1033.
31. Yang WJ, Chang CJ, Yeh SH, et al. Hepatitis B virus X protein enhances the transcriptional activity of the androgen receptor through c-Src and glycogen synthase kinase-3beta kinase pathways. *Hepatology*. 2009;49:1515–1524.
32. Tokushige K, Hashimoto E, Horie Y, et al. Hepatocellular carcinoma in Japanese patients with nonalcoholic fatty liver disease, alcoholic liver disease, and chronic liver disease of unknown etiology: report of the nationwide survey. *J Gastroenterol*. 2011;46:1230–1237.
33. Paradis V, Zalinski S, Chelbi E, et al. Hepatocellular carcinomas in patients with metabolic syndrome often develop without significant liver fibrosis: a pathological analysis. *Hepatology*. 2009;49:851–859.
34. Shrager B, Jibara G, Schwartz M, et al. Resection of hepatocellular carcinoma without cirrhosis. *Ann Surg*. 2012;255:1135–1143.
35. Abe H, Yoshizawa K, Kitahara T, et al. Etiology of non-B non-C hepatocellular carcinoma in the eastern district of Tokyo. *J Gastroenterol*. 2008;43:967–974.
36. Takuma Y, Nouso K, Makino Y, et al. Outcomes after curative treatment for cryptogenic cirrhosis-associated hepatocellular carcinoma satisfying the Milan criteria. *J Gastroenterol Hepatol*. 2011; 26:1417–1424.
37. Utsunomiya T, Shimada M, Imura S, et al. Molecular signatures of noncancerous liver tissue can predict the risk for late recurrence of hepatocellular carcinoma. *J Gastroenterol*. 2010;45:146–152.
38. Huang ZY, Liang BY, Xiong M, et al. Long-term outcomes of repeat hepatic resection in patients with recurrent hepatocellular carcinoma and analysis of recurrent types and their prognosis: a single-center experience in China. *Ann Surg Oncol*. 2012;19:2515–2525.
39. Wang B, Xia CY, Lau WY, et al. Determination of clonal origin of recurrent hepatocellular carcinoma for personalized therapy and outcomes evaluation: a new strategy for hepatic surgery. *J Am Coll Surg*. 2013;217:1054–1062.

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Elevated Serum Levels of *Wisteria floribunda* Agglutinin-Positive Human Mac-2 Binding Protein Predict the Development of Hepatocellular Carcinoma in Hepatitis C Patients

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The *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was recently shown to be a liver fibrosis glycobiomarker with a unique fibrosis-related glycoalteration. We evaluated the ability of WFA^+-M2BP to predict the development of hepatocellular carcinoma (HCC) in patients who were infected with the hepatitis C virus (HCV). A total of 707 patients who had been admitted to our hospital with chronic HCV infection without other potential risk factors were evaluated to determine the ability of WFA^+-M2BP to predict the development of HCC; factors evaluated included age, sex, viral load, genotypes, fibrosis stage, aspartate and alanine aminotransferase levels, bilirubin, albumin, platelet count, alpha-fetoprotein (AFP), WFA^+-M2BP, and the response to interferon (IFN) therapy. Serum WFA^+-M2BP levels were significantly increased according to the progression of liver fibrosis stage ($P<0.001$). In each distinctive stage of fibrosis (F0-F1, F2, F3, and F4), the risk of development of HCC was increased according to the elevation of WFA^+-M2BP. Multivariate analysis identified age >57 years, F4, AFP >20 ng/mL, WFA^+-M2BP $\geq$4, and WFA^+-M2BP 1-4 as well as the response to IFN (no therapy vs. sustained virological response) as independent risk factors for the development of HCC. The time-dependent areas under the receiver operating characteristic curve demonstrated that the WFA^+-M2BP assay predicted the development of HCC with higher diagnostic accuracy than AFP. *Conclusion:* WFA^+-M2BP can be applied as a useful surrogate marker for the risk of HCC development, in addition to liver biopsy. (HEPATOLOGY 2014;60:1563-1570)

The annual incidence of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV)-related cirrhosis ranges from 1% to 7%.[1,2] Therefore, reliable methods for the early identification of liver fibrosis progression and compensated liver cirrhosis are an essential part of an efficient surveillance program for the detection of HCC.[3]

Until recently, liver biopsy was considered the gold standard for assessing the severity of liver fibrosis and cirrhosis.[4,5] Although liver biopsy is generally accepted

Abbreviations: Ab, antibody; AFP, alpha-fetoprotein; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; AUROC, area under the ROC; CT, computed tomography; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MRI, magnetic resonance imaging; Peg-IFN, pegylated IFN; RBV, ribavirin; ROC, receiver operating characteristic; RT-PCR, reverse-transcriptase polymerase chain reaction; SVR, sustained virological response; US, ultrasound; WFA^+-M2BP, Wisteria floribunda *agglutinin-positive human Mac-2-binding protein.*

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to be a safe procedure, it can cause discomfort and carries a small risk of life-threatening complications.[6,7] Recently, an assay for *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was reported as a novel, noninvasive, and rapid bedside method to assess liver fibrosis.[8] M2BP has been shown to have multibranching and sialylated N-glycans. WFA is considered to recognize the GalNAc residue of N-glycans and O-glycans or the clustered LacNAc (Gal-GlcNAc) structure. Currently, we are analyzing the glycan structures of WFA^+-M2BP in detail using mass spectrometry–based technology.[9] Glycans can reflect the differentiation stage of cells, but not necessarily the level of cellular damage, and therefore they can be very effective markers for chronic disease. In the case of hepatitis, glycans are considered to reflect the progression of fibrosis more specifically than viral load. Several reports have identified M2BP as a potential marker of fibrosis progression in proteome study.[10-13] Kuno et al. were the first to report that a rapid, simple glycan-based immunoassay for WFA^+-M2BP can quantify fibrosis.[8,14]

On the other hand, we reported that alpha-fetoprotein (AFP) is a noninvasive predictive marker for the development of HCC in patients infected with HCV, which can be used to complement the information of fibrosis stage.[15]

In this report, we evaluated the utility of WFA^+-M2BP to predict the development of HCC in patients who were infected with HCV.

Patients and Methods

Patients. Between January 1992 and December 2003, 832 patients were determined to be positive for both anti-HCV by a second- or third-generation enzyme-linked immunoadsorbent assay and HCV RNA by polymerase chain reaction (PCR). They underwent liver biopsy guided by ultrasonography at the National Hospital Organization, Nagasaki Medical Center (Ōmura, Japan). Among them, 125 (15.0%) patients were excluded from enrollment in this retrospective analysis for the following reasons: (1) positivity for hepatitis B surface antigen (n = 12); (2) a heavy habitual drinking habit defined by an average daily consumption of >100 g of ethanol (n = 26); (3) autoimmune hepatitis (AIH), primary biliary cirrhosis, or idiopathic portal hypertension (n = 8); (4) positive antinuclear antibody (Ab; defined as titer >320×) without the diagnosis of AIH (n = 8); or (5) a short follow-up period <180 days (n = 71). The remaining 707 patients were analyzed retrospectively for the incidence of HCC.

For all patients in our cohort, a blood sample was taken on the day of the liver biopsy at our hospital. All samples were preceded to separate serum and stored at −20°C. At the time of blood withdrawal, all patients underwent liver biopsy. Their medical histories had been recorded, along with the results of routine tests for blood cell counts, liver biochemical parameters, and markers for HCV infection at the time of ultrasound (US)-guided liver biopsy and at regular intervals thereafter. Complete blood cell counts and biochemical tests were performed using automated procedures in the clinical pathological laboratories of our hospital.

Staging of Hepatic Fibrosis. Liver biopsies were taken by fine-needle aspiration (16G or 18G sonopsy) guided by US. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. They were evaluated for the stage of hepatic fibrosis by a pathologist according to the criteria of Desmet et al.[16]

Measurement of WFA^+-M2BP. WFA^+-M2BP quantification was measured based on a lectin-Ab sandwich immunoassay using the fully automatic immunoanalyzer, HISCL-2000i (Sysmex Co., Hyogo, Japan).[8] The measured values of WFA^+-M2BP conjugated to WFA were indexed with the obtained values using the following equation:

$$\text{Cutoff index (COI)} = \left(\left[\text{WFA}^+\text{-M2BP}\right]_{\text{sample}} - \left[\text{WFA}^+\text{-M2BP}\right]_{\text{NC}}\right) / \left(\left[\text{WFA}^+\text{-M2BP}\right]_{\text{PC}} - \left[\text{WFA}^+\text{-M2BP}\right]_{\text{NC}}\right)$$

where $[WFA^+\text{-M2BP}]_{sample}$ is the WFA^+-M2BP count of serum sample, PC is positive control, and NC is negative control. The positive control was supplied as

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a calibration solution preliminarily standardized to yield a COI value of 1.0.[14]

HCV RNA, HCV Core Antigen, and HCV Genotypes. HCV RNA was determined by reverse-transcriptase (RT)-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). HCV core antigen was determined using the Lumispot Eiken HCV antigen assay (Eiken Chemicals, Tokyo, Japan). HCV core antigen levels were classified into low and high with a cutoff at 1,000 fmol/mL.[17] Genotypes of HCV were determined by RT-PCR with genotype-specific primers (HCV RNA core genotype; Roche Diagnostics, Tokyo, Japan).[18]

Interferon Therapy. During the observation period, 373 of the 707 (52.8%) patients received interferon (IFN) monotherapy, pegylated (Peg)-IFN monotherapy, or combination therapy with IFN plus ribavirin (RBV) or Peg-IFN plus RBV. Sustained virological response (SVR) was defined as the absence of detectable HCV RNA at the end of 6 months or more of treatment, whereas patients who failed to meet these criteria were judged as having non-SVR. There was no relapse of viremia after 6 months among the SVR patients.

Diagnosis of HCC. Patients were followed up by hematological and biochemical tests at an interval of 1-12 months. Diagnostic imaging by US, computed tomography (CT), and magnetic resonance imaging (MRI) were performed in most patients. HCC was diagnosed by typical vascular patterns on CT, MRI, and angiography or by fine-needle biopsy of space-occupying lesions detected in the liver.

Ethical Considerations. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the *a priori* approval by the institution's human research committee.

Statistical Analysis. Continuous variables (platelet counts, albumin, total bilirubin, aspartate aminotransferase [AST], alanine aminotransferase [ALT], AFP, HCV core antigen, and WFA^+-M2BP) were dichotomized with respect to the median value or clinically meaningful values in the multivariate analysis. To estimate the cumulative risk of developing HCC, Kaplan-Meier's method and the log-rank test were used. Cox's proportional hazards regression analysis was performed to evaluate risk factors for HCC. Regression analysis was performed to calculate Spearman's rank-correlation coefficient. Kruskal-Wallis' analysis of variance (ANOVA), followed by the Games-Howel's posthoc test, was used to assess whether there were any significant differences in terms of fibrosis stages (F0-F1, F2, F3, and F4). The diagnostic performances of WFA^+-M2BP and AFP for censored development of HCC were assessed by using time-dependent receiver operating characteristic (ROC) curves by examining the area under the ROC (AUROC).[19] Inclusion of variables was assessed using a step-wise selection method. Cochran-Armitage's test for trend was used in the categorical data analysis to assess for the presence of an association between a variable with two categories and a variable with more than three categories. A P value of 0.05 was considered statistically significant. Data analysis was performed with SPSS statistical software (version 22.0; (SPSS, Inc., Chicago, IL) and JMP 10 (SAS Institute Inc., Cary, NC).

Table 1. Demographic, Clinical, and Virological Characteristics of the 707 Patients Persistently Infected With HCV

Age, years	57.0 (19-79)
Male, N (%)	351 (49.6)
Observation period, years	8.2 ± 4.4*
IFN therapy	373 (52.8%)
Habitual alcohol intake	135 (19.1%)
Pathological findings	
Fibrosis (N) 0-1/2/3/4	274/193/120/120
Activity (N) 0-1/2/3	199/365/143
Platelet count, $\times 10^4/mm^3$	15.6 (3.0-39.1)
Albumin, g/dL	4.2 (2.7-5.3)
Bilirubin, mg/dL	0.7 (0.1-2.5)
AST, IU/mL	53 (11-422)
ALT, IU/mL	82 (1-1,057)
AFP, ng/mL	6 (0.7-510)
HCV core antigen ≥1,000 fmol/L (%)	539 (76.2)
HCV genotype, N (%) 1b	510 (72.1)
2a/2b	195 (27.6)
Unknown	2 (0.3)
WFA^+-M2BP	1.9 (0.2-19.2)

Values are the medians with ranges in parentheses.
*Results are expressed as the mean ± standard deviation.

Results

Characteristics at Enrollment. The baseline characteristics of the 707 patients at enrollment are summarized in Table 1. Median age was 57.0 years; 120 (17.0%) patients were diagnosed histologically with liver cirrhosis (fibrosis stage F4) and the remaining 587 had chronic hepatitis (fibrosis stage F0, F1, F2, or F3). The median value of AFP was 6 ng/mL. The median value of WFA^+-M2BP was 1.9 (range, 0.2-19.2). The average follow-up period was 8.2 years.

WFA^+-M2BP Value and Fibrosis Stage. The average values (mean ± 1 standard error) for each fibrosis stage were 1.3 ± 0.1 in F0-F1 (n = 274), 2.2 ± 0.1 in F2 (n = 193), 3.3 ± 0.2 in F3 (n = 120),

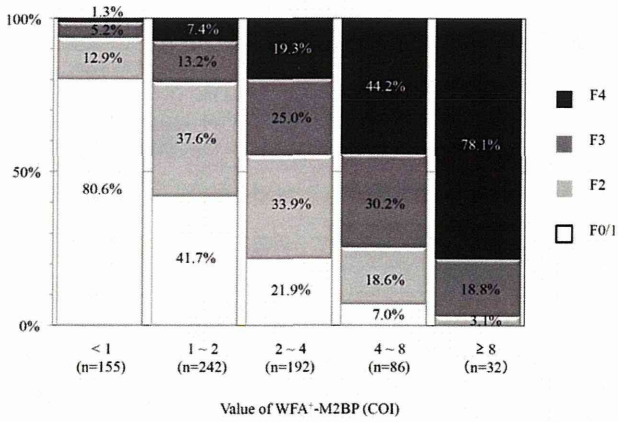

Fig. 1. Proportions of patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage. The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test), whereas that with F4 was increased ($P < 0.0001$; Cochran-Armitage's trend test).

and 5.2 ± 0.3 in F4 (n = 120). The degree of fibrosis was positively correlated with the median value of WFA$^+$-M2BP ($P < 0.001$) by a nonparametric method (Kruskal-Wallis' one-way ANOVA). Games-Howel's test confirmed that the WFA$^+$-M2BP value increased significantly with increasing stage of liver fibrosis: $P < 0.0001$ (F0-F1, compared with F2, F3, and F4); $P < 0.0001$ (F2, compared with F3 and F4); and $P < 0.0001$ (F3, compared with F4).

We estimated the diagnostic accuracy of WFA$^+$-M2BP for detecting stage F3-F4 disease. The AUROC in the prediction of ≥F3 was 0.815 (range, 0.782-0.842). The desired specificity level of 95% was achieved for a 4.0 threshold, and the sensitivity was 40.0%.

We analyzed the proportions of the patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage (Fig. 1). The proportion of patients with F1 was 125 cases (80.7%) in WFA$^+$-M2BP <1 (n = 155), 101 cases (41.7%) in WFA$^+$-M2BP ≤1 and <2 (n = 242), 42 cases (21.9%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 6 cases (7.0%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 0 cases (0.0%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test). Conversely, the proportion of patients with F4 was 2 cases (1.3%) in WFA$^+$-M2BP <1 (n = 155), 18 cases (7.4%) in WFA$^+$-M2BP ≤1, and <2 (n = 242), 37 cases (19.3%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 38 cases (44.2%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 25 cases (78.1%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F4 was increased with increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test).

Table 2. Step-wise Multiple Linear Regression Model to Identify Significant Independent Factors Affecting Serum WFA$^+$-M2BP Level

Final Fitted Model	Adjusted R^2	Standardized Coefficient β	P Value
Fibrosis stage		0.258	<0.001
AFP		0.187	<0.001
Albumin		−0.202	<0.001
AST (1: <53 IU/L; ≥2: 53 IU/L)		0.186	<0.001
Platelet	0.501	−0.147	<0.001
Sex (1: male; 2: female)		0.111	<0.001
HCV core antigen		−0.098	<0.001
Total bilirubin		0.091	0.001
Age		0.071	0.014

Relationship Between the WFA$^+$-M2BP Value and Baseline Biochemical Markers. To determine whether the WFA$^+$-M2BP value was associated with fibrosis stage, age, gender, platelet count, albumin, bilirubin, AST, ALT, AFP, HCV core antigen, HCV genotype, or histological grading, a step-wise multiple linear regression analysis was performed. Our results showed that independent variables, except for ALT, genotype, and histological grading, remained in the final equation (Table 2), suggesting that fibrosis stage was most closely associated with serum WFA$^+$-M2BP value (coefficient β, 0.258; $P < 0.001$).

Risk Factors for HCC. Cox's regression analysis was performed on several variables, including age, sex, alcohol consumption, IFN therapy during the observation period, biochemical and virological parameters, and serum WFA$^+$-M2BP level. The following factors were identified as posing an increased risk for HCC by the univariate analysis: age; response to IFN therapy (no therapy vs. SVR; $P < 0.001$); fibrosis stage (F3 and F4 vs. F0-F1; $P < 0.001$); platelet count (<15 × 10^4/mm^3 vs. ≥15 × 10^4/mm^3; $P < 0.001$); albumin (<4.2 vs. ≥4.2 g/mL; $P < 0.001$); AST (<53 vs. ≥53 IU/mL; $P < 0.001$), ALT (<82 vs. ≥82 IU/mL; $P = 0.035$), and AFP levels (≥20 and 6-20 vs. <6 ng/mL; $P < 0.001$); HCV genotype (1b vs. non-1b; $P = 0.025$); and serum WFA$^+$-M2BP level (≥4 and 1-4 vs. <1; $P < 0.001$). Multivariate analysis was performed on these factors (Table 3) and the following were identified as independent risk factors: fibrosis stage (F4); AFP (≥20 ng/mL); age (≥57 years); response to IFN therapy (no therapy vs. SVR); and WFA$^+$-M2BP (1-4 and ≥4).

Development of HCC. During the follow-up period, HCC developed in 110 (15.6%) patients. Of

Table 3. Factors Associated With Risk for HCC*

Features		HR (95% CI)	P Value
Fibrosis	F0-F1	1	
	F2	0.883 (0.411-1.897)	0.749
	F3	1.347 (0.624-2.906)	0.448
	F4	3.133 (1.536-6.390)	0.002
AFP	<6 ng/mL	1	
	6-20 ng/mL	1.710 (0.963-3.038)	0.067
	≥20 ng/mL	3.417 (1.807-6.460)	<0.001
Age	<57 years	1	
	≥57 years	2.039 (1.278-3.252)	0.003
IFN therapy	No therapy	1	
	Non-SVR	0.729 (0.467-1.137)	0.163
	SVR	0.089 (0.027-0.288)	<0.001
WFA^+-M2BP	<1	1	
	1-4	5.155 (1.180 – 22.500)	0.029
	≥4	8.318 (1.784 – 38.791)	0.007

Abbreviations: HR, hazard ratio; CI, confidence interval.
*Determined by multivariate analysis.

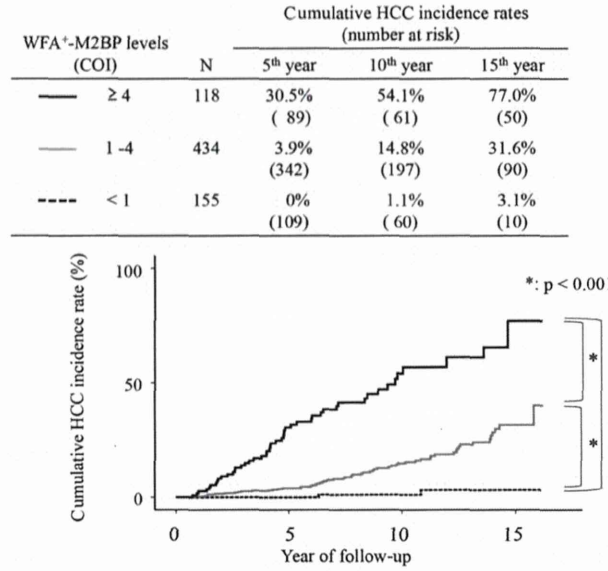

Fig. 2. Cumulative incidence of HCC according to WFA^+-M2BP level. Cumulative incidences of HCC according to the WFA^+-M2BP level were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA^+-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

the 110 patients with HCC, 58 (52.7%) were diagnosed with the disease by histological examination of biopsy-obtained or resected liver specimens. Of these 58 patients, 24 (41.3%) had hypovascular HCC.

Figure 2 shows the relation between Kaplan-Meier's estimates of the cumulative risk of HCC and the different WFA^+-M2BP levels at entry. The 10-year cumulative risk of HCC was 1.1% in the patients with WFA^+-M2BP <1 at entry, 14.8% among the patients with WFA^+-M2BP 1-4, and 54.1% in patients with WFA^+-M2BP >4. The incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

Figure 3 shows the relation between the cumulative incidence of HCC and WFA^+-M2BP levels, stratified by the fibrosis stage. In patients with fibrosis stage F0-F1, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of <1 ($P < 0.01$) and between those with WFA^+-M2BP levels of ≥4 and those with levels of <1 ($P < 0.01$). In patients with fibrosis stage F2-F3, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of ≤1 and those with levels of >4 ($P < 0.01$) and between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.001$). In patients with fibrosis stage F4, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.05$). As with WFA^+-M2BP levels, incidence rates increased with fibrosis stage, and the change in incidence was significant for each fibrosis stage.

Predictive Accuracy of Cumulative Incidence of HCC Compared With WFA^+-M2BP and AFP. AUROC analyses for prediction of the development of HCC at 1, 2, 3, 5, 7, and 10 years (range) were 0.762 (0.553-0.971), 0.792 (0.669-0.915), 0.832 (0.751-0.914), 0.858 (0.805-0.911), 0.821 (0.767-0.876), and 0.800 (0.745-0.855) in WFA^+-M2BP and 0.791 (0.684-0.898), 0.790 (0.723-0.857), 0.772 (0.693-0.850), 0.800 (0.741-0.858), 0.796 (0.745-0.848), and 0.821 (0.773-0.868) in AFP, respectively. The WFA^+-M2BP assay was superior to AFP for predicting the development of HCC at 3, 5, and 7 years.

Discussion

Liver biopsy has long been considered the gold standard for assessment of hepatic fibrosis,[20-23] and the Metavir[24] and Desmet et al.[16] staging systems are most commonly used. A higher degree of liver fibrosis is known to be the strongest risk factor for hepatocarcinogenesis in hepatitis C patients.[1,20] However, it also has its limitations for the staging of fibrosis because of the heterogeneous distribution of fibrosis in the liver,[25] and liver biopsy is an invasive procedure with

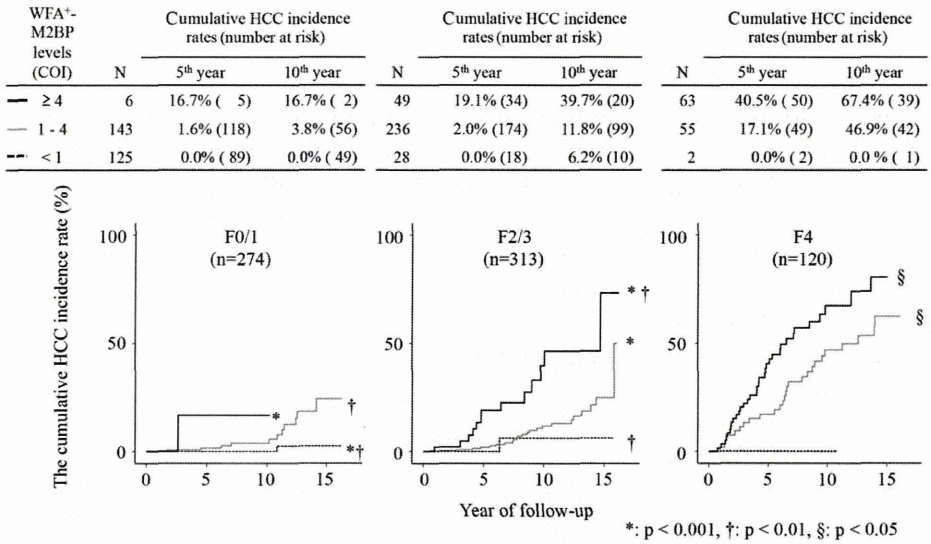

Fig. 3. Cumulative incidence of HCC according to WFA$^+$-M2BP levels, stratified by the fibrosis stage. Cumulative incidences of HCC, according to the WFA$^+$-M2BP level, stratified by the fibrosis stage were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA$^+$-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rates increased in accord with WFA$^+$-M2BP level.

associated morbidity (pain, bleeding, or hemobilia).[26] For these reasons, patients are often reluctant to undergo this invasive procedure and instead choose one of several noninvasive methods available for assessing the degree of liver fibrosis.

Nevertheless, in the past, no significant progress was made in the development of noninvasive biomarkers to guide clinical usage. WFA$^+$-M2BP was recently validated as a liver fibrosis glycobiomarker with a fully automated immunoassay.[8] In the present study, we assessed the performance of the WFA$^+$-M2BP assay in comparison with liver fibrosis stage and several serum markers, and, based on the results, we estimated whether WFA$^+$-M2BP is a useful predictor of the development of HCC as well as liver biopsy stage.

The first main finding of our study was that there was a significant correlation between the WFA$^+$-M2BP value and the fibrosis stage (Fig. 1). Moreover, step-wise multiple linear regression analysis showed that liver fibrosis stage was most closely associated with serum WFA$^+$-M2BP level. In addition, the degree of necroinflammation had no apparent effect on the WFA$^+$-M2BP value. Based on these results, we proposed a clinical management algorithm using a WFA$^+$-M2BP assay to predict the fibrosis stage. This approach could be used reliably for the first-line pretherapeutic evaluation of fibrosis in HCV-infected patients. On the other hand, the most widely used noninvasive techniques have recently shifted to physical measurements, such as FibroScan,[27-30] acoustic radiation force impulse, and real-time strain elastography. FibroScan has the advantages of being rapid and technically simple; however, operator skill affects its diagnostic success rate. Also, stiffness measurements can be difficult to obtain in obese patients and impossible in patients who have ascites. This is regarded as a limitation of transient elastography.[27,28] Therefore, we suggest that FibroScan, in conjunction with an assay of serum fibrosis biomarkers, would improve the diagnostic accuracy.

The second main finding of our study was the significant association between the WFA$^+$-M2BP level and the risk of HCC development in hepatitis C patients (Figs. 2 and 3). The diagnostic performance of WFA$^+$-M2BP, based on the AUROC values, was superior to that of AFP for predicting the development of HCC at 3, 5, and 7 years. The WFA$^+$-M2BP value can be used as a noninvasive predictor of HCC development and can be considered a surrogate marker for liver fibrosis. Various risk factors have been reported for HCC development among patients with HCV, including older age,[1] male sex,[1] heavy alcohol consumption,[31] obesity,[32] cirrhosis,[1,33] lower platelet count,[34-36] high serum AFP level,[15,36-44] low serum albumin level,[31] and high serum ALT and AST level.[45-47] Our results were consistent with these findings. Among them, liver fibrosis stage was the strongest prognostic indicator of chronic hepatitis. However, liver biopsy has several disadvantages. In our study, we have shown that the WFA$^+$-M2BP value is also a significant risk factor of HCC development independent of these factors. However, even though WFA$^+$-M2BP can be considered a surrogate marker for liver fibrosis, a distinct advantage of WFA$^+$-M2BP over liver biopsy is its wider dynamic range for the evaluation of liver cirrhosis. In the Metavir and Desmet et al. scoring systems, cirrhosis is represented by a single category (F4). However, the degree of fibrosis may vary widely

among patients in this category, and the risk of HCC may not be uniform. In our study, the risk of HCC development increased with increasing WFA^+-M2BP level as well as with increasing fibrotic stage. According to the elevation of WFA^+-M2BP value, the risk of development of HCC was increased (Fig. 3). In other words, each fibrosis stage can be further stratified with clinical relevance based on the WFA^+-M2BP level.

In our study, multivariate analysis identified fibrosis stage, high AFP level, older age, SVR to IFN therapy (no therapy vs. SVR), and high WFA^+-M2BP value as independent predictors of HCC development. The stratified WFA^+-M2BP value was independently associated with HCC development. These results indicate that the correlation between high WFA^+-M2BP and HCC development remains significant, even if HCC develops from a noncirrhotic background. Tateyama et al.[15] reported that AFP was a noninvasive predictive marker for the development of HCC in this same cohort; furthermore, not only high AFP levels ($\geq$20 ng/mL), but also slightly elevated AFP levels of between 6 and 20 ng/mL could indicate substantial risks for the development of HCC, complementing the fibrosis stage. Our present study was redesigned by the addition of one parameter (WFA^+-M2BP). Multivariate analysis did not identify slightly elevated AFP levels (6-20 ng/mL) as an independent risk factor, but did identify both stratified WFA^+-M2BP levels (1-4 and $\geq$4) as independent risk factors. Also, the time-dependent AUROC analysis suggested that WFA^+-M2BP is superior to AFP as a predictor for the development of HCC. These results mean that the WFA^+-M2BP level is the most reliable noninvasive predictive marker for the development of HCC in patients infected with HCV.

One of the limitations of the present study is that this cohort of 707 patients was analyzed retrospectively. There is thus need of a future study to prospectively analyze the efficacy of WFA^+-M2BP as a predictor of HCC development.

Another limitation is that the hepatocarcinogenesis of the patients who underwent IFN therapy was not evaluated. In this study, among the patients who achieved SVR (n = 139), 3 cases developed HCC during the follow-up period. The WFA^+-M2BP titers were 6.4, 5.6, and 1.5, respectively, in the 3 patients. All 3 cases obtained titers higher than 1, and 2 cases obtained titers higher than 4. This result suggests that patients with a high WFA^+-M2BP value should be monitored for the development of HCC even after achieving SVR. However, future assessments of the WFA^+-M2BP values at IFN administration and at posttreatment will be needed to verify this recommendation.

In conclusion, this study revealed an association between WFA^+-M2BP and the risk of HCC development in chronic hepatitis C patients. The results suggested that the WFA^+-M2BP assay should not be limited to use as a surrogate for liver biopsy, but rather could be applied as dynamic indicator of the risk of HCC development.

References

1. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med 1999; 131:174-181.
2. Liang TJ, Heller T. Pathogenesis of hepatitis C-associated hepatocellular carcinoma. Gastroenterology 2004;127(Suppl.):S62-S71.
3. Bolondi L, Sofia S, Siringo S, Gaiani S, Casali A, Zironi G, et al. Surveillance programme of cirrhotic patients for early diagnosis and treatment of hepatocellular carcinoma: a cost effectiveness analysis. Gut 2001;48:251-259.
4. Saadeh S, Cammell G, Carey WD, Younossi Z, Barnes D, Easley K. The role of liver biopsy in chronic hepatitis C. HEPATOLOGY 2001;33: 196-200.
5. Gebo KA, Herlong HF, Torbenson MS, Jenckes MW, Chander G, Ghanem KG, et al. Role of liver biopsy in management of chronic hepatitis C: a systematic review. HEPATOLOGY 2002;36:S161-S172.
6. Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). HEPATOLOGY 2000;32:477-481.
7. Dienstag JL. The role of liver biopsy in chronic hepatitis C. HEPATOLOGY 2002;36(Suppl.):S152-S160.
8. Kuno A, Ikehara Y, Tanaka Y, Ito K, Matsuda A, Sekiya S, et al. A serum "sweet-doughnut" 272 protein facilitates fibrosis evaluation and therapy assessment in patients 273 with viral hepatitis. Sci. Rep 2013; 3:1065.
9. Narimatsu Y, Kuno A, Ito H, Kaji H, Kaneko S, Usui J, et al. IgA nephropathy caused by unusual polymerization of IgA1 with aberrant N-glycosylation in a patient with monoclonal immunoglobulin deposition disease. PloS One 2014;9:e91079.
10. Sasaki T, Brakebusch C, Engel J, Timpl R. Mac-2 binding protein is a cell-adhesive protein of the extracellular matrix which self-assembles into ring-like structures and binds beta1 integrins, collagens and fibronectin. EMBO J 1998;17:1606-1613.
11. Iacovazzi PA, Trisolini A, Barletta D, Elba S, Manghisi OG, Correale M. Serum 90K/MAC-2BP glycoprotein in patients with liver cirrhosis and hepatocellular carcinoma: a comparison with alpha-fetoprotein. Clin Chem Lab Med 2005;39:961-965.
12. Artini M, Natoli C, Tinari N, Costantzo A, Marinelli R, Balsano C, et al. Elevated serum levels of 90K/MAC-2 BP predict unresponsiveness to alpha-interferon therapy in chronic HCV hepatitis patients. J Hepatol 1996;25:212-217.
13. Cheung KJ, Tilleman K, Deforce D, Colle I, Van Vlierberghe H. The HCV serum proteome: a search for fibrosis protein markers. J Viral Hepat 2009;16:418-429.
14. Kuno A, Sato T, Shimazaki H, Unno S, Saitou K, Kiyohara K, et al. Reconstruction of a robust glycodiagnostic agent supported by multiple lectin-assisted glycan profiling. Proteomics Clin Appl 2013;7:642-647.
15. Tateyama M, Yatsuhashi H, Taura N, Motoyoshi Y, Nagaoka S, Yanagi K, et al. Alpha-fetoprotein above normal levels as a risk factor for the