disruption of mammary epithelial architecture. *J. Cell Biol.* **2004**, *165* (2), 263–73.
(37) Cheung, K. J.; Libbrecht, L.; Tilleman, K.; Deforce, D.; Colle, I.; Van Vlierberghe, H. Galectin-3-binding protein: a serological and histological assessment in accordance with hepatitis C-related liver fibrosis. *Eur. J. Gastroenterol. Hepatol.* **2010**, *22* (9), 1066–73.
(38) 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* (6), 1606–13.
(39) Alexopoulou, A.; Papatheodoridis, G. V. Current progress in the treatment of chronic hepatitis C. *World J. Gastroenterol.* **2012**, *18* (42), 6060–9.
(40) Shoeb, D.; Dearden, J.; Weatherall, A.; Bargery, C.; Moreea, S.; Alam, S.; White, E.; Vila, X.; Freshwater, D.; Ryder, S.; Mills, P. R.; Alexander, G. J.; Forton, D.; Foster, G. R. Extended duration therapy with pegylated interferon and ribavirin for patients with genotype 3 hepatitis C and advanced fibrosis: final results from the STEPS trial. *J. Hepatol.* **2013**, DOI: 10.1016/j.jhep.2013.11.011.

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Multilectin-assisted fractionation for improved single-dot tissue glycome profiling in clinical glycoproteomics†

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To survey the glycome shift in cancer, single-dot tissue glycome profiling was improved by incorporating a lectin-assisted fractionation. The differential analysis of tissue specimens from colorectal cancer patients (*n* = 45) revealed that unfucosylated/α2,6-sialylated glycoproteins significantly increased in patients with poor prognoses. The detailed annotation will be an indispensable supplement for cancer-related glyco-biomarker discovery.

The glycome, the repertoire of glycans produced by individual biological systems (*e.g.*, cells, tissues, and organisms), has been widely reported to modulate numerous physiological and pathological states.[1,2] As important posttranslational modifications of proteins, glycans profoundly affect a wide range of biological processes such as apoptosis,[3] angiogenesis,[4] and microbial recognition.[5] In particular, the importance of the glycome has been highlighted by its role in immune responses to various disease states including cancer, which makes the glycome a promising new source for biomarker discovery.[2,6,7] Currently, mining of the glycome for cancer-associated biomarkers represents a new paradigm for cancer diagnosis and prognosis. However, the inherent diversity, complexity and heterogeneity of glycan structures make glycome analysis particularly challenging.[8]

For decades, capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) have been widely used for glycome analysis. CE is a rapid and reliable analytical tool for determining carbohydrate structures, but it is limited by the need for synthetic standards for every glycan analyzed.[8,9] HPLC is regularly used for glycan separation in most glycome analytical laboratories with excellent reproducibility.[10] Like CE, the utility of HPLC is limited to glycome analysis because of the requirement for chemical or enzymatic liberation of glycans from glycoconjugates. MS has become an indispensable technology in the field of glycomics because of its powerful capacity for determining the structural details of glycans. Recently, new analytical technologies have been developed to optimize the MS-based glycome analysis platform.[11,12] However, the requirement for expensive equipment and professional personnel hinders the broad application of these techniques. Recently, lectin microarrays have emerged as an increasingly important technology for glycome investigation.[13] Compared with the technologies described above, lectin microarrays have been recognized as a simple, rapid, highly sensitive, and high-throughput platform that obtains the glycome profile (both *N*- and *O*-glycans) of diverse complex biological samples without releasing the glycan moieties from glycoconjugates.

Comprehensive elucidation of the glycome from biological samples is a significant challenge because of the complexity and microheterogeneity of glycan structures, which considerably hinder glycans from becoming potential informative biomarkers for understanding both normal biological functions and pathological processes.[14,15] Therefore, effective sample fractionation or enrichment is necessary for more in-depth investigation of glycomes. So far, lectin affinity chromatography (especially multilectin affinity chromatography) has been widely employed by researchers to isolate diverse glycan motifs in the extensive mining of glycomes for cancer biomarker discovery.[16,17] The series of lectins used in the chromatography are usually determined with an emphasis on comprehensiveness. This means a lack of systematic determination based on the differential glycome analysis using clinical specimens (*e.g.*, tissue and serum) reduces the efficiency of biomarker discovery.

Recently, with advances in analytical technologies, tissue glycome investigation is gaining momentum in biomarker discovery.[12,18,19] In fact, compared with serum glycome, tissue glycome is a more direct and authentic reflection of the disease state within the corresponding organ. It is now generally recognized that reliable cancer-specific biomarkers should be produced by the cancer cells themselves and are usually present

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in serum at only low levels, especially at the early stage of cancer.[20] For this reason, it is necessary to develop a novel method to explore tissue glycomes in detail. To date, there have been hardly any related reports focused on the development of a strategy for in-depth tissue glycome analysis. Therefore, we developed an ultrasensitive method for glycan analysis targeting small regions (*i.e.*, single-dot tissue on tissue arrays) on formalin-fixed paraffin-embedded (FFPE) tissue sections by means of lectin microarrays.[21,22] Herein, we report an improved strategy integrating tissue arrays, lectin microarrays, and lectin-assisted fractionation techniques, optimized for in-depth tissue glycome investigation, which would be feasible for elucidation of subtle changes during pathological processes. Furthermore, our strategy provided a new idea for designing a sequential combination of lectins for multilectin affinity chromatography in the early phase of biomarker discovery (Fig. 1).

To prove our concept, we investigated the tissue glycome of colorectal cancer (CRC) with the aim of finding reliable CRC-related glyco-biomarkers for clinical application, *i.e.*, prediction of CRC prognosis. To identify the glycan that specifically predicts CRC prognosis as 10 year survival after surgery, we first compared the glycome profiles of single-dot tissues in CRC patients with good (n = 34) and poor (n = 11) prognoses (Table S1, ESI†) based on FFPE colon tissue arrays and lectin microarrays (First LA in Fig. 1, experimental procedure described in the ESI†). The LecChip microarray (GlycoTechnica, Sapporo, Japan) consists of 45 lectins with a broad range of specificities, covering almost all of the important *N*- and *O*-linked glycans. Their carbohydrate specificities are listed in Table S2 (ESI†). Based on unbiased statistical analysis with all of the normalized data (Table S3, ESI†), we found that the signals of AAL and ABA on the LecChip significantly differentiated the two groups of CRC patients (p < 0.05). Specifically, the AAL signal was stronger in patients with good prognosis, and the ABA signal was much weaker in patients

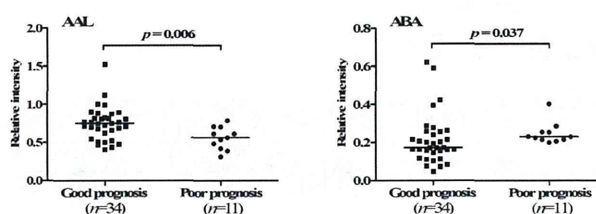

Fig. 2 Selected probe lectins for discrimination of CRC patients with good and poor prognoses.

with good prognosis than in those with poor prognosis (Fig. 2). AAL has specificity for fucosylated glycans,[23] which is known to increase in cancer-associated glycan alterations.[24,25] ABA has specificity for Gal-exposed *O*-linked glycans (*i.e.*, Core1) and GlcNAc-exposed (*i.e.*, agalactosylated) *N*-linked glycans,[26] and is reported to have a potent antineoplastic effect on malignant colon cells.[27,28] Considering the statistical results and the biological significance of AAL and ABA, we selected these two lectins as the probe lectins for further studies.

To further confirm the contribution of selected probe lectins (AAL and ABA) toward CRC prognosis, and for more effective enrichment of potential glyco-biomarker candidates, we combined the lectin microarray-based tissue glycome profiling analysis with lectin-assisted fractionation. For the lectin affinity capturing, 11 patients with good prognosis were selected from the original 34 cases with age (p = 0.92) and sex (p = 0.14) matched to the 11 original patients with poor prognoses. The clinical information including follow-up months for the selected subjects is provided in Table S4 (ESI†).

As shown in Fig. 3A, each Cy3-labeled glycoprotein sample extracted from single-dot tissues was divided into three aliquots (30 μL each) for AAL-affinity capturing, ABA-affinity capturing, and "Input". In our strategy, we collected the supernatants after AAL- and ABA-affinity capturing (*i.e.*, the pass fractions: AAL(−)

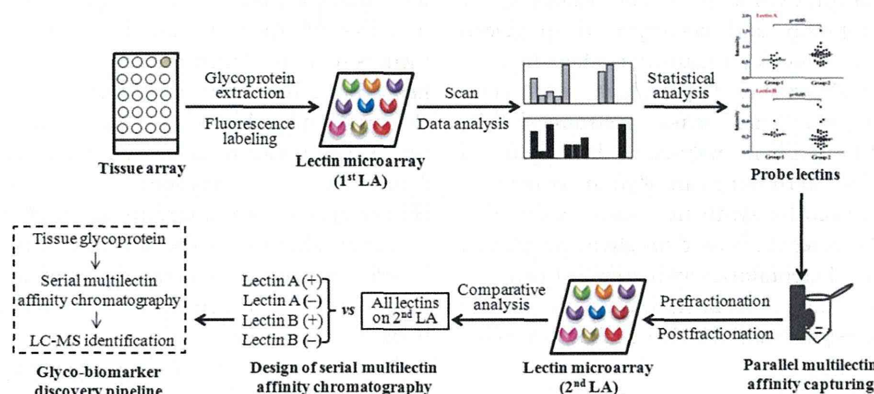

Fig. 1 Schematic diagram for glyco-biomarker discovery based on lectin microarrays and lectin-assisted fractionation. (1) Comparative analysis of tissue glycome profiling between two groups of study subjects based on FFPE tissue arrays and lectin microarrays (1st LA). (2) Statistical analysis for screening the probe lectins to distinguish different groups (*e.g.*, lectins A and B). (3) Fractionation of tissue glycoproteins by affinity capturing using probe lectins in parallel, and then comparative analysis of glycan profiling between the pre- and post-fractionations based on lectin microarrays (2nd LA). (4) Selection of the most accurate biomarker candidates and optimization of the serial multilectin affinity chromatography for glyco-biomarker identification based on LC-MS in further studies.

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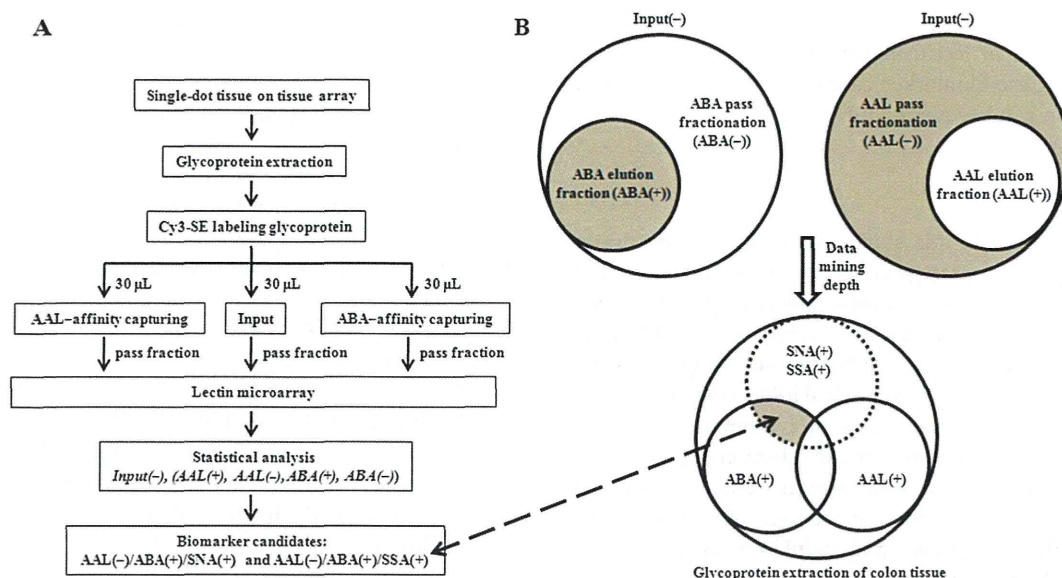

Fig. 3 Mining of potential glyco-biomarkers for CRC prognosis. (A) Scheme for lectin microarray-based tissue glycome profiling analysis with AAL- and ABA-assisted fractionation. (B) A Venn diagram visualizing the results obtained by incorporating the lectin microarrays and lectin-assisted fractionation.

and ABA(−)). Each pass fraction was subjected to lectin microarray analysis (second LA in Fig. 1) instead of the elution fraction in order to minimize the experimental bias. Then, we defined "Input(−) minus AAL(−)" as the AAL captured fraction (*i.e.*, AAL(+)), and "Input(−) minus ABA(−)" as the ABA captured fraction (*i.e.*, ABA(+)). Based on the results of the LecChip analysis with lectin-assisted fractionation (AAL(+), AAL(−), ABA(+), ABA(−)), it was found that the signals of ABA in AAL(−) fractions showed the most significant differences between CRC patients with good and poor prognoses. Furthermore, the signals of α2,6-sialic acid binders such as *Sambucus nigra agglutinin* (SNA) and *Sambucus sieboldiana agglutinin* (SSA) both in AAL(−) and ABA(+) fractions were significantly increased in patients with poor prognosis, whereas both SNA and SSA signals in "Input(−)" showed no significant differences between the two groups of patients (Table 1). It has been reported that the activity of β-galactoside α2,6-sialyltransferase (ST6Gal.I) and SNA reactivity increase in human colon cancer tissues.[29] Taking the specificities of three lectins into account, sialylated mucins, which are well known as potential CRC markers,[30–32] might be rationally enriched in these fractions. This suggested that AAL(−)/ABA(+)/SNA(+) and AAL(−)/ABA(+)/SSA(+) fractions contain a series of glycoproteins, which should be accurate glyco-biomarker candidates for CRC prognosis (Fig. 3B). Considering the potential bias caused by different pathological stages in CRC patients, 5 patients at stage III with good prognoses were selected from 11 patients with age (p = 0.83) and sex (p = 1.0) matched to 5 patients at stage III with poor prognoses for further analysis. The clinical information for the selected subjects is provided in Table S6 (ESI†). The signals of ABA in AAL(−) fractions also showed the largest differences between the two groups, despite the significance becoming weaker (p = 0.058) because of the limited sample size (5 *vs.* 5). Similarly, the signals of SNA and SSA in AAL(−) and ABA(+) fractions also increased in patients with poor prognosis (Table S7, ESI†). These results suggested the potential clinical value of our findings for CRC prognosis while comparing the patient groups at the same pathological stage. All results proved that it is necessary to fractionate glycoproteins for in-depth glycome analysis, and our method was successful in solving the technical problem. We propose an optimized scheme of serial multilectin affinity chromatography for MS-based glycoproteomics, which would contribute towards effectively specifying the target range needed to find novel and clinically promising biomarkers for

Table 1 Significant lectins in AAL and ABA affinity fractionations between CRC patients with good and poor prognoses

	Input(−)			AAL(+)			AAL(−)			ABA(+)			ABA(−)		
Lectin	Good	Poor	p	Good	Poor	p	Good	Poor	p	Good	Poor	p	Good	Poor	p
AAL	23 800	18 000	0.28	23 800	18 000	0.28	0	0	1.00	9200	9000	0.97	14 500	9700	0.12
ABA	6300	9800	0.07	6300	7900	0.49	0	2500	**0.01**	6300	9800	0.07	0	0	1.00
SNA	19 000	20 200	0.22	14 800	13 400	0.87	0	8800	**0.04**	4800	8500	**0.05**	12 100	11 300	0.82
SSA	19 400	20 500	0.21	17 000	14 800	0.87	0	8200	**0.04**	5100	8600	**0.03**	13 500	12 900	0.79

Values are the means of absolute signal intensities. p-values in bold indicate statistical significance by the Mann–Whitney U test. Good prognosis (n = 11), poor prognosis (n = 11).

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CRC prognosis (as shown in Fig. S1, ESI†). We will conduct further validation of lectin binding and subsequent identification of the glyco-biomarker candidates in a larger clinical sample pool in a subsequent study.

Conclusions

As a proof of concept, this study confirmed the effectiveness and feasibility of our strategy on lectin microarray-based tissue glycome profiling for clinical applications of glyco-biomarkers. The significant advantages of our strategy include: (1) an ideal combination of an ultrasensitive lectin microarray and a FFPE tissue array with comprehensive clinical information that showed potential advantage in cancer-specific biomarker discovery, (2) simple manipulation and rigorous selection of probe lectins based on statistical analysis to ensure the reliable results, (3) a lectin microarray integrated with lectin-assisted fractionation that can provide more detailed and useful information for logical optimization of serial multilectin affinity chromatography before MS analysis, which would be feasible for glycoproteomics-based biomarker discovery, and (4) a universal platform in the uppermost stream of the pipeline of glyco-biomarker discovery, which can be useful for exploring clinically promising biomarkers in further studies.

Abbreviations

CE	Capillary electrophoresis
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
LA	Lectin microarray
FFPE	Formalin-fixed paraffin-embedded
CRC	Colorectal cancer
AAL	*Aleuria aurantia* lectin
ABA	*Agaricus bisporus* agglutinin
SNA	*Sambucus nigra* agglutinin
SSA	*Sambucus sieboldiana* agglutinin

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References

1 J. Hirabayashi, Y. Arata and K.-i. Kasai, *Proteomics*, 2001, **1**, 295–303.

2 H. J. An, S. R. Kronewitter, M. L. A. de Leoz and C. B. Lebrilla, *Curr. Opin. Chem. Biol.*, 2009, **13**, 601–607.

3 E. Rapoport and J. Le Pendu, *Glycobiology*, 1999, **9**, 1337–1345.

4 T. Saito, E. Miyoshi, K. Sasai, N. Nakano, H. Eguchi, K. Honke and N. Taniguchi, *J. Biol. Chem.*, 2002, **277**, 17002–17008.

5 Y. van Kooyk and G. A. Rabinovich, *Nat. Immunol.*, 2008, **9**, 593–601.

6 R. T. Kam and T. W. Poon, *Clin. Proteomics*, 2008, **4**, 67–79.

7 S. Hua, C. Lebrilla and H. J. An, *Bioanalysis*, 2011, **3**, 2573–2585.

8 J. F. Rakus and L. K. Mahal, *Annu. Rev. Anal. Chem.*, 2011, **4**, 367–392.

9 Y. Mechref and M. V. Novotny, *Mass Spectrom. Rev.*, 2009, **28**, 207–222.

10 L. Royle, M. P. Campbell, C. M. Radcliffe, D. M. White, D. J. Harvey, J. L. Abrahams, Y.-G. Kim, G. W. Henry, N. A. Shadick, M. E. Weinblatt, D. M. Lee, P. M. Rudd and R. A. Dwek, *Anal. Biochem.*, 2008, **376**, 1–12.

11 S. Yang, Y. Li, P. Shah and H. Zhang, *Anal. Chem.*, 2013, **85**, 5555–5561.

12 S. J. Yang, S. T. Eshghi, H. Chiu, D. L. DeVoe and H. Zhang, *Anal. Chem.*, 2013, **85**, 10117–10125.

13 J. Hirabayashi, A. Kuno and H. Tateno, *Electrophoresis*, 2011, **32**, 1118–1128.

14 R. A. Dwek, *Chem. Rev.*, 1996, **96**, 683–720.

15 D. Aldredge, H. J. An, N. Tang, K. Waddell and C. B. Lebrilla, *J. Proteome Res.*, 2012, **11**, 1958–1968.

16 Z. Yang, L. E. Harris, D. E. Palmer-Toy and W. S. Hancock, *Clin. Chem.*, 2006, **52**, 1897–1905.

17 M. Madera, Y. Mechref, I. Klouckova and M. V. Novotny, *J. Proteome Res.*, 2006, **5**, 2348–2363.

18 Z. Dai, J. Fan, Y. Liu, J. Zhou, D. Bai, C. Tan, K. Guo, Y. Zhang, Y. Zhao and P. Yang, *Electrophoresis*, 2007, **28**, 4382–4391.

19 A. Matsuda, A. Kuno, H. Matsuzaki, T. Kawamoto, T. Shikanai, Y. Nakanuma, M. Yamamoto, N. Ohkohchi, Y. Ikehara, J. Shoda, J. Hirabayashi and H. Narimatsu, *J. Proteomics*, 2013, **85**, 1–11.

20 H. Narimatsu, H. Sawaki, A. Kuno, H. Kaji, H. Ito and Y. Ikehara, *FEBS J.*, 2010, **277**, 95–105.

21 A. Matsuda, A. Kuno, H. Ishida, T. Kawamoto, J.-i. Shoda and J. Hirabayashi, *Biochem. Biophys. Res. Commun.*, 2008, **370**, 259–263.

22 A. Kuno, A. Matsuda, Y. Ikehara, H. Narimatsu and J. Hirabayashi, in *Methods in Enzymology*, ed. F. Minoru, Academic Press, 2010, vol. 478, pp. 165–179.

23 K. Matsumura, K. Higashida, H. Ishida, Y. Hata, K. Yamamoto, M. Shigeta, Y. Mizuno-Horikawa, X. Wang, E. Miyoshi, J. Gu and N. Taniguchi, *J. Biol. Chem.*, 2007, **282**, 15700–15708.

24 E. Miyoshi, K. Moriwaki, N. Terao, C.-C. Tan, M. Terao, T. Nakagawa, H. Matsumoto, S. Shinzaki and Y. Kamada, *Biomolecules*, 2012, **2**, 34–45.

25 Y. Takeda, S. Shinzaki, K. Okudo, K. Moriwaki, K. Murata and E. Miyoshi, *Cancer*, 2012, **118**, 3036–3043.

26 S. Nakamura-Tsuruta, J. Kominami, A. Kuno and J. Hirabayashi, *Biochem. Biophys. Res. Commun.*, 2006, **347**, 215–220.

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27 L. Yu, D. G. Fernig, J. A. Smith, J. D. Milton and J. M. Rhodes, *Cancer Res.*, 1993, **53**, 4627–4632.
28 L.-G. Yu, D. G. Fernig, M. R. H. White, D. G. Spiller, P. Appleton, R. C. Evans, I. Grierson, J. A. Smith, H. Davies, O. V. Gerasimenko, O. H. Petersen, J. D. Milton and J. M. Rhodes, *J. Biol. Chem.*, 1999, **274**, 4890–4899.
29 F. Dall'Olio, M. Chiricolo, C. Ceccarelli, F. Minni, D. Marrano and D. Santini, *Int. J. Cancer*, 2000, **88**, 58–65.
30 S. H. Itzkowitz, E. J. Bloom, W. A. Kokal, G. Modin, S.-I. Hakomori and Y. S. Kim, *Cancer*, 1990, **66**, 1960–1966.
31 S. Nakamori, M. Kameyama, S. Imaoka, H. Furukawa, O. Ishikawa, Y. Sasaki, T. Kabuto, T. Iwanaga, Y. Matsushita and T. Irimura, *Cancer Res.*, 1993, **53**, 3632–3637.
32 R. S. Bresalier, S. B. Ho, H. L. Schoeppner, Y. S. Kim, M. H. Sleisenger, P. Brodt and J. C. Byrd, *Gastroenterology*, 1996, **110**, 1354–1367.

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HEPATOLOGY

Prediction of liver stiffness hepatocellular carcinoma in chronic hepatitis C patients on interferon-based anti-viral therapy

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chronic hepatitis C, hepatocellular carcinoma, liver stiffness, risk factor.

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Disclosure: The authors declare no conflict of interest.

Abstract

Background and Aim: The purpose of this study was to evaluate the usefulness of liver stiffness measurement (LSM) for assessing the risk of hepatocellular carcinoma (HCC) in chronic hepatitis C (CHC) patients receiving interferon (IFN) therapy.

Methods: One hundred fifty-one CHC patients who underwent LSM and received IFN therapy were included in the estimation cohort, and 56 were included in the validation study. The cumulative HCC incidences were evaluated using Kaplan–Meier plot analysis and the log-rank test. Multivariate Cox proportional hazard analyses were used to estimate the hazard ratios (HRs) of variables for HCC.

Results: In the estimation cohort, 9 of 151 patients developed HCC during the median follow-up time of 722 days. Multivariate analysis identified three independent risk factors for HCC: LSM (≥ 14.0 kPa, HR 5.58, $P = 0.020$), platelet count ($< 14.1 \times 10^4/\mu L$, HR 5.59, $P = 0.034$), and non-sustained virological response (HR 8.28, $P = 0.049$). The cumulative incidence of HCC development at 3 years was 59.6%, 8.2%, and 0.0% in patients with all three risk factors, one to two risk factors, and none of these risk factors, respectively. The incidence of HCC was significantly different between these groups ($P < 0.001$). In the validation cohort, HCC incidence was also significantly different with respect to these risk factors ($P = 0.037$).

Conclusion: LSM, platelet count, and IFN-therapeutic effect could be used to successfully stratify the risk of HCC in patients receiving IFN therapy and demonstrate the usefulness of LSM before IFN therapy for the management of CHC patients.

Introduction

Persistent hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease leading to the development of HCC, the fifth most common cancer, and the third most common cause of cancer-related death worldwide.[1] HCV is responsible for 27–75% of the HCC cases in Europe and the United States and > 80% of the HCC cases in Japan.[2,3] In fact, HCV-positive patients have a 20-fold higher risk of developing HCC than HCV-negative patients,[4] indicating a significant carcinogenic role for persistent HCV infection. Because of this connection, many chronic hepatitis C (CHC) patients are treated with interferon (IFN)-based antiviral therapy because it not only eradicates HCV but also reduces the rate of HCC development. IFN therapy is most effective at decreasing the risk of developing HCC in patients that achieve a sustained virological response (SVR);[5–7] however, the risk of HCC development persists after IFN therapy even in patients who do achieve SVR.[8] HCC might develop immediately after IFN therapy in some cases, or during long-term IFN therapy in others.[9,10] Because assessing the risk of developing HCC is clinically important in the management of CHC patients, it is necessary to establish predictors for HCC development in patients who receive IFN therapy.

Some factors reported to predict the risk of HCC development after IFN therapy are older age, male gender, and severe fibrosis,[11,12] with advanced fibrosis and cirrhosis significantly correlating with the risk of HCC development.[13] To date, liver biopsy has been the gold standard for assessing the severity of liver fibrosis and cirrhosis,[14] although sampling errors and intraobserver and interobserver variability can lead to understaging.[15,16] In addition, it is difficult to perform liver biopsy for all patients because of its invasiveness and rare but potentially life-threatening complications.[14] As a result, liver stiffness measurement (LSM), a type of transient elastography, has become a reliable alternative for assessing hepatic fibrosis and cirrhosis mainly in patients with CHC.[17,18] LSM is non-invasive, reproducible, can be expressed numerically as continuous values, and has a wide dynamic range in the evaluation of hepatic fibrosis. These advantages over liver biopsy

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suggest the clinical usefulness of LSM for predicting HCC development. Here, we evaluated factors that affect the occurrence of HCC in CHC patients receiving IFN therapy, with a special focus on the predictive value of LSM.

Methods

Patients. Between October 2007 and April 2011, a total of 207 consecutive CHC patients who underwent a successful LSM and then received IFN-based antiviral therapy at the Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, Shizuoka, Japan, were retrospectively enrolled in this study. CHC diagnosis was based on serum HCV-RNA positivity. Exclusion criteria were as follows: (i) hepatitis B surface antigen positivity; (ii) other causes of liver disease of mixed etiologies, including autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease; (iii) evidence of hepatocellular carcinoma (HCC) on ultrasonography or computed tomography; (iv) previous history of liver transplantation; and (v) treatment for HCC. This study was approved by the Ethics Committee of Juntendo University Shizuoka Hospital in accordance with the Helsinki Declaration, and all patients provided written informed consent.

Of these 207 patients, 151 underwent ultrasonography-guided percutaneous liver biopsy within a week before treatment initiation. Liver biopsy specimens were embedded in paraffin and stained with hematoxylin-eosin, Azan-Mallory, and reticulin silver impregnation. The specimens were evaluated by an experienced pathologist who was blinded to the patients' clinical data. Histological evaluation was based on the METAVIR criteria.[19] Hepatic fibrosis was defined as follows: F0, no fibrosis; F1, periportal fibrous expansion; F2, portal fibrous widening with bridging fibrosis; F3, bridging fibrosis with lobular distortion; and F4, liver cirrhosis. On the basis of the degree of lymphocyte infiltration and hepatocyte necrosis, inflammation was scored from A0 to A3, with higher scores indicating more severe inflammation. The 151 patients who underwent liver biopsy were enrolled into the estimation group for the identification of risk factors for HCC development, and the remaining 56 patients who did not undergo liver biopsy were enrolled into a group for the validation of these identified risk factors.

All laboratory tests were performed for each patient just before initiation of IFN therapy. Blood cell counts, serum alanine transaminase, gamma-glutamyl transpeptidase, hemoglobin A1c, total bilirubin, albumin, prothrombin time, and alpha-fetoprotein (AFP) were measured using commercially available assays. The HCV genotype was determined using polymerase chain reaction with the HCV Genotype Primer Kit (Institute of Immunology Co., Ltd., Tokyo, Japan) and classified as genotype 1, genotype 2, or other, according to Simmonds' classification system. Serum HCV viral load was determined using quantitative reverse transcription polymerase chain reaction using the COBAS TaqMan HCV Test (Roche Diagnostics, Branchburg, NJ, USA).

Treatment protocol. The treatment protocol for CHC patients consisted of 1.5 μg/kg of pegylated IFN-α-2b or 180 μg of pegylated IFN-α-2a once a week, combined with ribavirin at an oral dose of 600–1000 mg/day. Duration of the treatment was 48–72 weeks for those with HCV genotype 1 and a serum HCV viral load > 5 log IU/mL. For all other patients, treatment lasted for 24 weeks. SVR was defined as undetectable serum HCV-RNA at 24 weeks after the end of treatment.

Measurement of liver stiffness. Measurement of liver stiffness by transient elastography was performed using FibroScan (Echosens, Paris, France) within a week before treatment initiation. Technical details of the examination and procedure have been reported previously.[17] Ten validated measurements were made on each patient, and results were expressed in kilopascals (kPa). Only procedures with 10 validated measurements and a success rate of at least 60% were considered reliable, and the median value was considered representative of the liver elastic modulus.

Patient follow-up and HCC diagnosis. Serum AFP was measured every month, and ultrasonography or computed tomography were performed at least every 3–6 months for HCC surveillance during and after treatment, with a minimum follow-up duration of 6 months after the initiation of IFN therapy. HCC was diagnosed by histological examination and/or triphasic computerized tomography, in which hyperattenuation in the arterial phase with washout in the late phase is pathognomonic for HCC.[20] The status of patients enrolled in this study was confirmed as of March 2012.

Statistical analyses. All analyses were conducted using IBM SPSS version 19 (IBM SPSS, Chicago, IL, USA), and P values less than 0.05 were considered statistically significant. Continuous variables and categorical variables were summarized as median (range) and percentage, respectively. Mann–Whitney U and chi-square tests were used when appropriate. The strength of the association between LSM and the histological fibrosis stage was estimated using the Spearman's rank correlation coefficient. Cumulative incidences of HCC development were estimated by Kaplan–Meier analysis and compared using the log-rank test. Cox logistic regression analysis was used for multivariate analysis to identify factors that were independently associated with HCC development. The cut-off value of each factor for predicting the development of HCC was determined using receiver operator characteristics analysis.

Results

Patient characteristics. A total of 229 patients received LSM followed by IFN-based antiviral therapy at Juntendo Shizuoka Hospital during the study period. Twenty-two patients (9.6%) were excluded because of LSM failure and/or an invalid LSM. Of the remaining 207 patients, 151 underwent liver biopsy prior to IFN therapy and together formed the risk factor-estimation cohort. The clinical, anthropometric, and laboratory data of the estimation cohort are summarized in Table 1. The 151 patients (83 male and 68 female) had a median age of 62 years (range 22–82 years) and a median LSM of 8.8 kPa (range 2.8–45.7 kPa). There was a significant positive association between LSM and histological fibrosis stage ($r = 0.59$, $P < 0.001$). The prevalence of genotype

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Table 1 Baseline characteristics of the estimation cohort

Variables	All	HCC development (+)	HCC development (–)	*P*-value
Number of patients	151	9	142	
Age (years)	62 (22–82)	67 (60–82)	61 (22–80)	0.010†
Male (%)	55	55.6	54.9	1.000‡
BMI (kg/m²)	23.5 (18.1–36.8)	23.8 (23.3–25.7)	23.4 (18.1–36.8)	0.217†
Habitual drinker (%)	10.6	11.1	10.6	1.000‡
Fibrosis stage (F0–2/F3–4)	115/36	5/4	110/32	0.048‡
Inflammatory grade (A0–1/A2–3)	33/118	0/9	33/109	0.101‡
LSM (kPa)	8.8 (2.8–45.7)	14.8 (9.8–45.7)	8.7 (2.8–34.8)	0.002†
Observation period (days)	722 (189–1378)	688 (189–1217)	733 (190–1378)	0.467†
Genotype 1 (%)	56.3	100	53.5	0.065‡
HCV-RNA (log IU/mL)	6.4 (0.0–7.7)	6.5 (2.9–7.2)	6.3 (0.0–7.7)	0.168†
Albumin (g/dL)	4.1 (3.4–4.8)	4.1 (3.5–4.6)	4.1 (3.4–4.8)	0.390†
ALT (IU/L)	59 (10–410)	75 (27–181)	57 (10–410)	0.467†
Total bilirubin (mg/dL)	0.7 (0.3–1.8)	0.8 (0.5–1.3)	0.7 (0.3–1.8)	0.070†
γGTP (IU/L)	44 (4–517)	75 (31–129)	41 (4–517)	0.120†
Hemoglobin A1c (%)	5.1 (3.7–8.2)	5.1 (3.7–6.1)	5.1 (4.2–8.2)	0.561†
Ferritin (ng/mL)	134 (8–2096)	215 (8–1026)	134 (9–2096)	0.675†
White blood cell count (× 10³/μL)	4.9 (2.0–10.3)	4.3 (3.0–7.3)	4.9 (2.0–10.3)	0.496†
Hemoglobin (g/dL)	13.8 (8.9–17.5)	13.3 (9.9–17.5)	13.8 (8.9–17.1)	0.376†
Platelet count (× 10⁴/μL)	16.3 (5.2–37.0)	9.6 (5.2–19.4)	16.5 (5.8–37.0)	0.004†
Prothrombin time (%)	100 (70–157)	93 (79–120)	102 (70–157)	0.185†
AFP (ng/mL)	6 (1–306)	14 (4–109)	6 (1–306)	0.004†
SVR rate (%)	55	11.1	57.7	0.011‡

Scale data are shown as median (range). *P* values are for comparisons between patients with and without HCC development.
†Mann–Whitney *U* test.
‡Chi-square test.
γGTP, γ-glutamyl transpeptidase; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; BMI, body mass index; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LSM, liver stiffness measurement; SVR, sustained virological response.

1 HCV infection was 56.3%. Following IFN-based antiviral therapy, SVR was obtained in 83 of the 151 patients (55%). During the median follow-up period of 722 days (range 189–1378 days), nine patients (6.0%) developed HCC. The cumulative incidence of HCC estimated using the Kaplan–Meier method was 1.3%, 4.5%, and 9.0% at 1, 2, and 3 years, respectively (Fig. 1). Compared with patients who had not developed HCC, HCC patients were of advanced age and had a high LSM, a high fibrosis stage, a low platelet count, and a low SVR rate (Table 1).

Risk analyses. Univariate analysis revealed that age ($P = 0.029$), LSM ($P = 0.005$), platelet count ($P = 0.002$), AFP ($P = 0.003$), and non-SVR ($P = 0.011$) were associated with HCC development (Table 2). Multivariate Cox logistic regression analysis identified three independent risk factors: LSM ≥ 14.0 kPa (hazard ratio [HR] 5.58, 95% confidence interval [CI] 1.32–23.64, $P = 0.02$), non-SVR (HR 8.28, 95% CI 1.01–68.05, $P = 0.049$), and platelet count < 14.1 × 10⁴/μL (HR 5.59, 95% CI 1.14–27.53, $P = 0.034$), Table 3. The 1-, 2-, and 3-year cumulative incidence rates of HCC development in patients with LSM < 14.0 kPa were 0.8%, 2.3%, and 4.6%, respectively, whereas those with LSM ≥ 14.0 kPa were 3.2%, 12.0%, and 22.2%, respectively ($P = 0.005$) (Fig. 2a). The cumulative incidence rates of HCC development in patients with SVR were 0.0%, 2.0%, and 2.0%, respectively, whereas those without SVR were 3.0%, 7.4%, and 17.1%, respectively ($P = 0.011$) (Fig. 2b). The cumulative incidence rates of HCC development in patients with a platelet count ≥ 14.1 × 10⁴/μL were 0.0%, 0.0%, and 4.2%, respectively, whereas those with a platelet count < 14.1 × 10⁴/μL were 4.0%, 13.4%, and 19.1%, respectively ($P = 0.002$) (Fig. 2c).

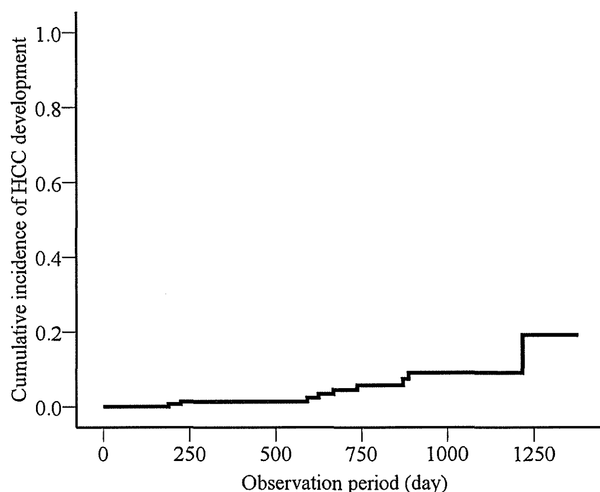

Figure 1 Incidence of hepatocellular carcinoma (HCC) in 151 patients with chronic hepatitis C receiving interferon-based anti-viral therapy estimated using the Kaplan–Meier method.

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Table 2 Univariate analysis of factors associated with hepatocellular carcinoma development

Variables	n	Cumulative incidence of HCC (%)		P-value
		1 year	3 years	
Age (years)				
< 60	63	0.0	0.0	0.029
≥ 60	88	2.3	13.6	
Sex				
Female	68	1.5	12.1	0.910
Male	83	1.2	6.7	
BMI† (kg/m^2)				
< 23.8	50	0.0	5.3	0.250
≥ 23.8	42	2.4	6.0	
Habitual drinker				
No	135	0.8	9.6	0.905
Yes	16	6.2	6.2	
Fibrosis stage				
F0–2	115	0.9	6.7	0.228
F3–4	36	2.9	15.0	
LSM (kPa)				
< 14	119	0.8	4.6	0.005
≥ 14	32	3.2	22.2	
ALT (IU/L)				
< 55	71	0.0	4.9	0.123
≥ 55	80	2.5	12.9	
γGTP† (IU/L)				
< 55	83	0.0	5.2	0.057
≥ 55	67	3.0	13.5	
Hemoglobin A1c† (%)				
< 5.5	109	0.9	6.8	0.219
≥ 5.5	25	0.0	18.8	
Ferritin† (ng/mL)				
< 210	74	1.4	10.0	0.175
≥ 210	43	2.3	16.3	
Platelet count (× 10^4/μL)				
≥ 14.1	101	0.0	4.2	0.002
< 14.1	50	4.0	19.1	
AFP† (ng/mL)				
< 10	95	0.0	5.6	0.003
≥ 10	38	4.9	22.3	
SVR				
Yes	83	0.0	2.0	0.011
No	68	3.0	17.1	

†Data not available for all patients.
AFP, alpha-fetoprotein; ALT, alanine aminotransferase; BMI, body mass index; γGTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; LSM, liver stiffness measurement; SVR, sustained virological response.

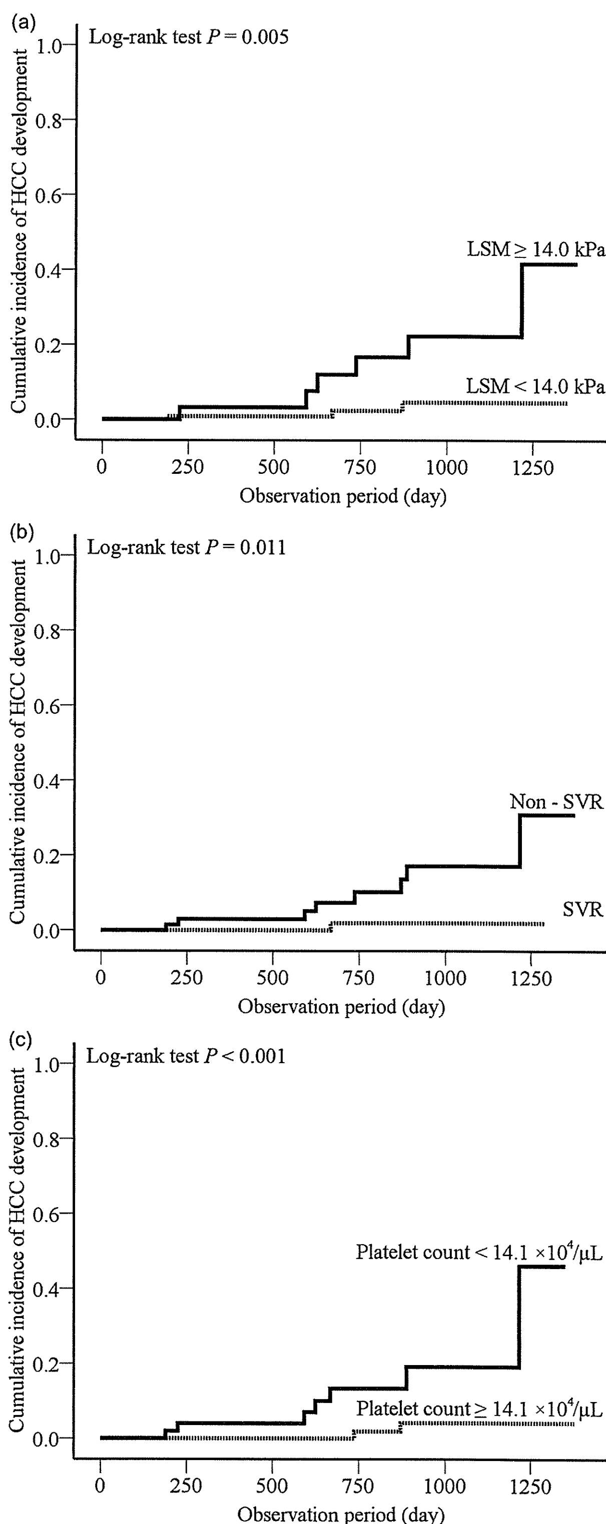

Figure 2 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development. Patients were stratified according to liver stiffness measurement (LSM) (a), sustained virological response (SVR) (b), and platelet count (c).

Number of risk factors and HCC development. The number of risk factors varied between patients: 12 patients (7.9%) had all three risk factors, 32 patients (21.2%) had two, 50 patients (33.1%) had one, and 57 patients (37.7%) had none of these risk factors (Fig. 3). Patients without these risk factors did not develop HCC during the study period. In patients with 1 or 2 risk factors, the cumulative incidence rates at 1, 2, and 3 years were 1.2%, 3.1%, and 8.2%, respectively, whereas patients with all three risk

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Table 3 Multivariate analysis of factors associated with hepatocellular carcinoma development

Variable		Hazard ratio (95% CI)	*P*-value
LSM (kPa)	< 14.0	1.00	0.020
	≥ 14.0	5.58 (1.32–23.64)	
SVR	SVR	1.00	0.049
	Non-SVR	8.28 (1.01–68.05)	
Platelet count (× 10^4/μL)	> 14.1	1.00	0.034
	≤ 14.1	5.59 (1.14–27.53)	

CI, confidence interval; LSM, liver stiffness measurement; SVR, sustained virological response.

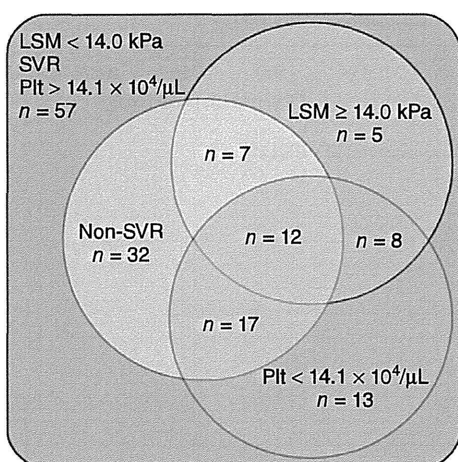

Figure 3 Patient distribution at each risk factor. LSM, liver stiffness measurement; Plt, platelet count; SVR, sustained virological response.

factors had significantly higher cumulative incidence rates (9.1%, 39.4%, and 59.6% at 1, 2, and 3 years, respectively; log-rank test, $P < 0.001$) (Fig. 4).

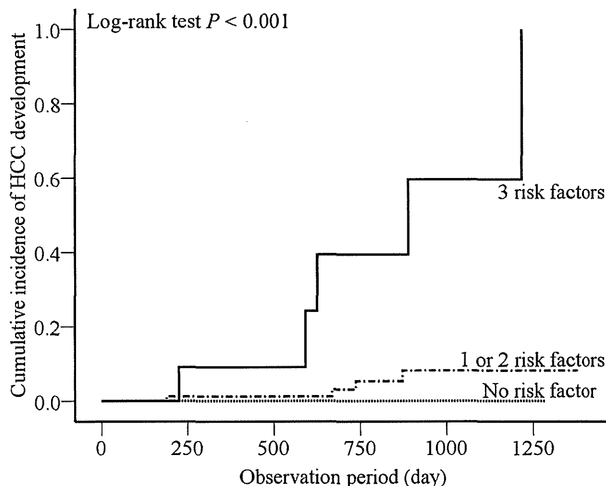

Figure 4 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development. Patients were stratified according to the number of risk factors.

The relationship between the number of risk factors and HCC development in the validation cohort. Fifty-six patients who received IFN therapy without liver biopsy were enrolled into the validation group for analysis of these three risk factors. The 56 patients (33 male and 23 female) had a median age of 65 years (range 35–79 years) and a median LSM of 8.0 kPa (range 2.6–32.0 kPa). There were no significant differences in clinical, anthropometric, and laboratory findings between the validation and estimation cohorts (data not shown). In the validation cohort, seven patients (12.5%) had all three risk factors, 25 patients (44.6%) had one or two risk factors, and 24 patients (42.9%) had none of these risk factors. Patients without these risk factors did not develop HCC during the study period. In patients with one or two risk factors, and patients with all three risk factors, the cumulative incidence rates at 3 years were 12.7% and 28.6%, respectively. There was also a significant difference in the cumulative incidences of HCC development according to the number of risk factors ($P = 0.037$, Fig. 5).

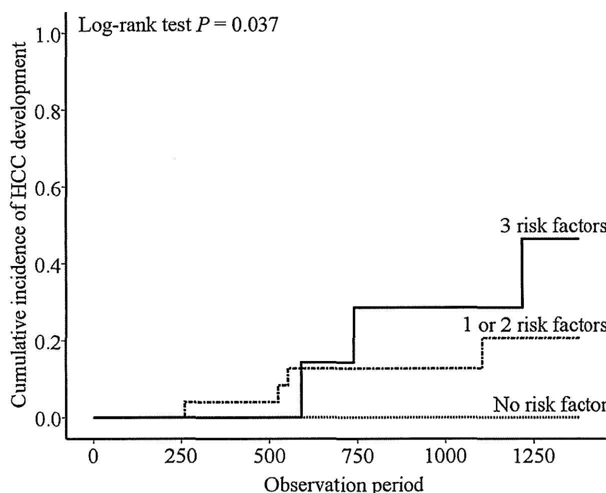

Figure 5 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development in the validation cohort. Patients were stratified according to the number of risk factors they had.

Discussion

Patients with liver cirrhosis or pre-existing severe hepatic fibrosis have a higher risk of developing HCC,[2] even after IFN-based therapy with SVR.[9,10] Clinical diagnosis of liver cirrhosis can be easily made in cases showing stigmata of end-stage liver disease, such as ascites, jaundice, variceal bleeding, and hepatic encephalopathy; however, diagnosis becomes difficult if the liver shows compensation, and normal or near-normal laboratory findings. Liver biopsy has been considered the only diagnostic method for the assessment of early compensated cirrhosis, although

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several studies have pointed out sampling variability as a potential limitation of biopsy to diagnose cirrhosis.[21,22] Given the importance of assessing the HCC risk factors in managing CHC patients, we evaluated factors that affect the occurrence of HCC in CHC patients receiving IFN therapy, with a special focus on the predictive value of LSM as an alternative to liver biopsy.

Our data identified three risk factors for developing HCC after IFN therapy. Consistent with previous reports,[5–7] we found that failure to achieve SVR was a significant predictor of HCC development among patients receiving IFN therapy. Although it is possible that IFN therapy itself reduces the risk of HCC,[6,7] non-SVR patients had an approximately eightfold higher risk of developing HCC than SVR patients. In addition, we identified both high LSM and low platelet count as significant predictors of HCC development independently of non-SVR. The LSM threshold ≥ 14.0 kPa identified here as a risk factor for HCC is in agreement with previously reported cut-off values for liver cirrhosis,[15,16] further supporting the idea that pre-existing liver cirrhosis increases the risk of HCC development. Similar to LSM, the platelet count reflects the severity of CHC[21] and is used to estimate the degree of fibrosis.[23–25] Previous reports have also shown low platelet counts to represent a risk of HCC.[23,24] Our cohort showed that LSM was sometimes high even in patients without a low platelet count, whereas other patients had a low platelet count without LSM elevation. Such patients are nevertheless at risk of HCC, suggesting that LSM and platelet count indicate advanced fibrosis or compensated cirrhosis in a complementary manner.

In agreement with a previous report, our findings indicate that LSM could be used to stratify the risk of HCC development in CHC patients.[26] Moreover, combination of LSM with platelet count and the IFN-therapeutic effect could be used to stratify the risk of HCC in patients receiving IFN therapy. Patients without all three risk factors had a very low risk of HCC development, and patients with 1 or 2 risk factors had a moderate risk. Conversely, patients with all three risks had an extremely high risk. In clinical practice, frequency of HCC surveillance should be decided based on HCC risk. Indeed, each of these three factors has previously been shown to be associated with the risk of developing HCC. However, here, we have proposed a new, non-invasive risk assessment based on the combination of LSM and two other factors. In the present study, we did not identify advanced histological fibrosis stage F3–4 as a risk factor for HCC likely because of liver biopsy sampling variability because patients were not excluded based on the length of liver biopsy samples, an important factor affecting variability in histological assessment of liver fibrosis.[15] Taken together, these findings suggest that LSM would be more useful than liver biopsy for diagnosis of patients with liver cirrhosis who are at high risk of HCC, especially those with compensated cirrhosis.

Our data indicate patients with all of the three risk factors require the most intensive HCC surveillance; however, this study does have a few limitations. One drawback is that LSM failure and unreliable results occur in some patients. In our cohort, 9.0% of patients who received LSM did not yield reliable results. Because subcutaneous fat attenuates the transmission of share waves and the ultrasonic signals into the liver used to determine LSM, obesity is the principal reason for LSM failure.[27] In addition, it is likely that obesity itself is associated with an increased risk of HCC.[28] As a result, our findings might not reflect the risk of HCC in obese patients. Another recent report demonstrated that a new FibroScan XL probe, designated for use in obese patients, could reduce LSM failure and facilitate reliable results.[29] A study using this new probe will more accurately evaluate the predictive value of LSM for the risk of HCC development.

In conclusion, our findings indicate that LSM, platelet count, and IFN-therapeutic effect could be used to successfully stratify the risk for HCC development in patients receiving IFN-based antiviral therapy and demonstrate the usefulness of LSM before IFN therapy for the management of CHC patients.

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References

1 El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557–76.
2 Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127** (Suppl.): S35–50.
3 Kiyosawa K, Umemura T, Ichijo T *et al.* Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; **127** (Suppl.): S17–26.
4 Sun CA, Wu DM, Lin CC *et al.* Incidence and cofactors of hepatitis C virus-related hepatocellular carcinoma: a prospective study of 12,008 men in Taiwan. *Am. J. Epidemiol.* 2003; **157**: 674–82.
5 Cammà C, Giunta M, Andreone P, Craxi A. Interferon and prevention of hepatocellular carcinoma in viral cirrhosis: an evidence-based approach. *J. Hepatol.* 2001; **34**: 593–602.
6 Papatheodoridis GV, Papadimitropoulos VC, Hadziyannis SJ. Effect of interferon therapy on the development of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis: a meta-analysis. *Aliment. Pharmacol. Ther.* 2001; **15**: 689–98.
7 Shiratori Y, Ito Y, Yokosuka O *et al.* Antiviral therapy for cirrhotic hepatitis C: association with reduced hepatocellular carcinoma development and improved survival. *Ann. Intern. Med.* 2005; **142**: 105–14.
8 Morgan TR, Ghany MG, Kim HY *et al.* Outcome of sustained virological responders with histologically advanced chronic hepatitis C. *Hepatology* 2010; **52**: 833–44.
9 Kanda T, Imazeki F, Mikami S *et al.* Occurrence of hepatocellular carcinoma was not a rare event during and immediately after antiviral treatment in Japanese HCV-positive patients. *Oncology* 2011; **80**: 366–72.
10 Lok AS, Everhart JE, Wright EC *et al.* Maintenance peginterferon therapy and other factors associated with hepatocellular carcinoma in patients with advanced hepatitis C. *Gastroenterology* 2011; **140**: 840–9.
11 Makiyama A, Itoh Y, Kasahara A *et al.* Characteristics of patients with chronic hepatitis C who develop hepatocellular carcinoma after a sustained response to interferon therapy. *Cancer* 2004; **101**: 1616–22.
12 Ikeda M, Fujiyama S, Tanaka M *et al.* Risk factors for development of hepatocellular carcinoma in patients with chronic hepatitis C after sustained response to interferon. *J. Gastroenterol.* 2005; **40**: 148–56.
13 Yoshida H, Shiratori Y, Moriyama M *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis

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C in Japan. IHIT study group. Inhibition of hepatocarcinogenesis by interferon therapy. *Ann. Intern. Med.* 1999; **131**: 174–81.

14 Bravo AA, Sheth SG, Chopra S. Liver biopsy. *N. Engl. J. Med.* 2001; **344**: 495–500.

15 Bedossa P, Dargère D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology* 2003; **38**: 1449–57.

16 Rousselet MC, Michalak S, Dupré F *et al.* Sources of variability in histological scoring of chronic viral hepatitis. *Hepatology* 2005; **41**: 257–64.

17 Ziol M, Handra-Luca A, Kettaneh A *et al.* Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48–54.

18 Castéra L, Vergniol J, Foucher J *et al.* Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343–50.

19 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513–20.

20 Bruix J, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208–36.

21 Abdi W, Millan JC, Mezey E. Sampling variability on percutaneous liver biopsy. *Arch. Intern. Med.* 1979; **139**: 667–9.

22 Maharaj B, Maharaj RJ, Leary WP *et al.* Sampling variability and its influence on the diagnostic yield of percutaneous needle biopsy of the liver. *Lancet* 1986; **1**: 523–5.

23 Matsumura H, Moriyama M, Goto I, Tanaka N, Okubo H, Arakawa Y. Natural course of progression of liver fibrosis in Japanese patients with chronic liver disease type C—a study of 527 patients at one establishment. *J. Viral Hepat.* 2000; **7**: 268–75.

24 Pohl A, Behling C, Oliver D, Kilani M, Monson P, Hassanein T. Serum aminotransferase levels and platelet counts as predictors of degree of fibrosis in chronic hepatitis C virus infection. *Am. J. Gastroenterol.* 2001; **96**: 3142–6.

25 Degos F, Christidis C, Ganne-Carrie N *et al.* Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; **47**: 131–6.

26 Masuzaki R, Tateishi R, Yoshida H *et al.* Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. *Hepatology* 2009; **49**: 1954–61.

27 Castéra L, Foucher J, Bernard PH *et al.* Pitfalls of liver stiffness measurement: a 5-year prospective study of 13,369 examinations. *Hepatology* 2010; **51**: 828–35.

28 Polesel J, Zucchetto A, Montella M *et al.* The impact of obesity and diabetes mellitus on the risk of hepatocellular carcinoma. *Ann. Oncol.* 2009; **20**: 353–7.

29 Myers RP, Pomier-Layrargues G, Kirsch R *et al.* Feasibility and diagnostic performance of the FibroScan XL probe for liver stiffness measurement in overweight and obese patients. *Hepatology* 2012; **55**: 199–208.

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Accepted Article

TECHNICAL BRIEF

Reconstruction of a robust glycodiagnostic agent supported by multiple lectin-assisted glycan profiling

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Accepted Article

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Abbreviations: CH, chronic hepatitis; **CHO**, Chinese hamster ovary; **COI**, cutoff index; **HEK293**, human embryonic kidney 293; **hM2BP**, human Mac-2 binding protein; **HV**, healthy volunteer; **M2BPGi**, Mac-2 binding protein glycosylation isomer; **mAb**, monoclonal antibody; **rhM2BP**, recombinant human Mac-2 binding protein; **WFA**, *Wisteria floribunda* agglutinin; **WFA^{+}-hM2BP**, *Wisteria floribunda* agglutinin-positive human Mac-2 binding protein

Keywords: antibody / glycoprotein / lectin / liver fibrosis / sandwich immunoassay

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2500 words

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Accepted Article

Statement of clinical relevance

"Glycodiagnosis" is a new paradigm of clinical diagnosis based on quantitation of glyco-alteration of cells and their secreted molecules as a biomarker that reflects disease progression well. To quantitate such proteins bearing disease-specific changes in glycosylation, a lectin–antibody sandwich immunoassay has been proposed for developing glycodiagnosis. This study mostly focused on the construction of a robust calibrator and a standardized parameter (the cutoff index) involved in a glycodiagnostic agent, FastLec-Hepa. This agent has been developed for use in the assessment of disease severity and in evaluating the efficacy of therapy; for instance, monitoring of the degree of liver fibrosis along with antiviral therapy in cases of viral hepatitis. This unique technical approach supported by multiple lectin-assisted glycan profiling is applicable to the development pipeline for a wide variety of glycodiagnostic tools. We believe our approach is capable of revolutionizing the use of glycodiagnosis in clinical medicine and provides a framework for the development of a new generation of biomarker assays.

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Accepted Article

Abstract

Purpose: *Wisteria floribunda* agglutinin-positive human Mac-2 binding protein (WFA$^+$-hM2BP) was recently validated as a liver fibrosis glycobiomarker with a fully automated lectin-antibody sandwich immunoassay. In this study, we supplied recombinant WFA$^+$-hM2BP as the standard glycoprotein and the overlaid antibody to enhance the robustness of WFA$^+$-hM2BP quantification.

Experimental design: The optimum conditions for producing recombinant WFA$^+$-hM2BP were selected by cell glycome analysis based on a lectin microarray. Interlot variability of recombinant WFA$^+$-hM2BP was determined using an antibody-overlay lectin microarray. Screening of anti-M2BP monoclonal antibody was completed by incorporating a WFA-antibody sandwich ELISA and an antibody-overlay lectin microarray.

Results: The lectin microarray analysis revealed that human embryonic kidney 293 (HEK293) cells efficiently and stably produced WFA$^+$-hM2BP in DMEM containing 10% FCS without any lot variation in the M2BP glycosylation level. A spiking experiment with recombinant WFA$^+$-hM2BP was mostly effective for antibody screening. The reconstituted sandwich immunoassay was useful for the continuous quantification and cutoff index (COI) expression of serum

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WFA^{+}-hM2BP.

Conclusions and clinical relevance: The multiple use of lectin-assisted glycan profiling enabled us to construct a reliable sandwich assay kit for monitoring liver fibrosis in patients with viral hepatitis. This will assist in the development pipeline for other glycodiagnostic agents.

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The close relationship of alterations in glycosylation (glyco-alterations) with the biological phenomena or diseases has long been reported in many studies. The annotation of such glyco-alterations on specific glycoproteins has been challenging for technical reasons and few outputs feasible for clinical use have been proposed [1]. A few excellent examples of diagnostic systems, referred to as "glycodiagnostic agents", have been developed based on highly practical and promising markers such as the ratio of core fucosylation in α-fetoprotein (AFP-L3%) [2]. In the past decade, biomarker development has been pipelined along with the assistance of recent revolutionary progress in proteomic technologies [3] and the pathway for applying glycoprotein biomarkers has been established successively from the discovery phases to verification (see Supporting Information Fig. S1) [4]. Furthermore, various unique systems assisted by advanced technologies such as "lab-on-a-chip" have been proposed for the detection of glycoprotein biomarkers [5, 6]. However, a complete form of the "glycodiagnostic agent" has not yet been developed.

To develop reliable systems toward clinical implementation and therapeutic benefits, glycoprotein biomarkers should be quantified by a sandwich immunoassay based on an anti-glyco-epitope reagent [7], in which lectins are

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employed widely as the reagents. The sandwich immunoassay can be classified unambiguously based on the immobilized substance on the plate: either as an antibody raised against the protein moiety or as a lectin probing the glyco-epitope [8]. No matter which system is selected, a complex problem of detection noise will arise from unfavorable direct interactions between the lectin and antibody, as well as the abundant serum proteins such as albumin and IgG. However, there is no versatile method to resolve this, so researchers have been addressing these problems specifically with regard to the detection of each target molecule [8–12]. In addition, a diagnostic system has to be developed along with the establishment of a recombinant glycoprotein as a robust calibrator and the cutoff index (COI) for quality control before commercialization for practical use.

Here, we introduce our efficient approach to these important tasks based on glycan profiling including a lectin array analysis (Supporting Information Fig. S1) [13], which was employed in the development of our novel glycan-based sandwich immunoassay system for quantifying fibrosis, FastLec-Hepa [8].

FastLec-Hepa automatically detects the *Wisteria floribunda* agglutinin (WFA)-positive human Mac-2 binding protein (WFA^{+}-hM2BP), which has been confirmed as a marker glycoprotein having fibrosis-related glyco-alteration. In

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