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.

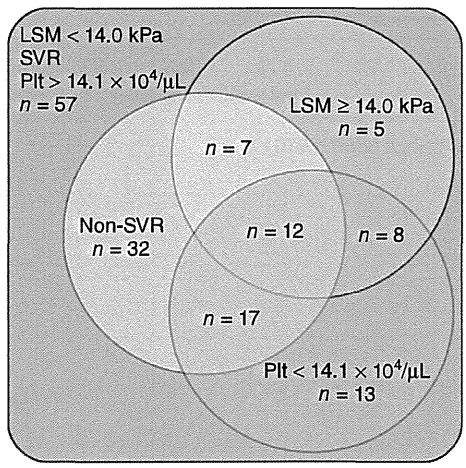

Figure 3 Patient distribution at each risk factor. LSM, liver stiffness measurement; Plt, platelet count; SVR, sustained virological response.

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).

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).

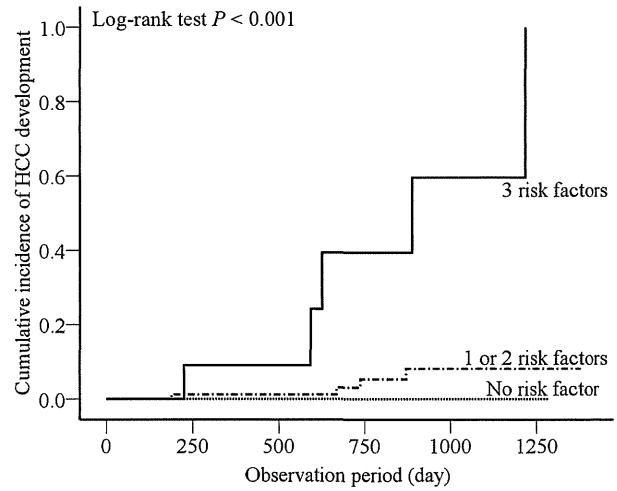

Figure 4 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development. Patients were stratified according to the number of risk factors.

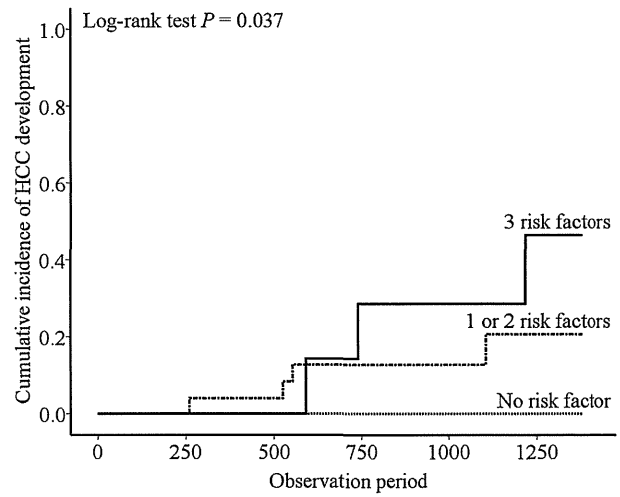

Figure 5 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development in the validation cohort. Patients were stratified according to the number of risk factors they had.

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

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.

Acknowledgment

This study was supported by a Health Labor Sciences Research Grant, Research on Measures for Intractable Diseases, from the Ministry of Health, Labor, and Welfare of Japan.

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TECHNICAL BRIEF

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

Atsushi Kuno[1#], Takashi Sato[1#], Hiroko Shimazaki[1], Sachiko Unno[1], Kozue Saitou[1], Katsue Kiyohara[1], Maki Sogabe[1], Chikayuki Tsuruno[2], Youichi Takahama[2], Yuzuru Ikehara[1], Hisashi Narimatsu[1*]

[1]Research Center for Medical Glycoscience (RCMG), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki, 305-8568, Japan.

[2]Immunology & Chemistry Product Engineering, ICH Business Unit, Sysmex Corporation, 4-4-4 Takatsukadai, Nishi-ku, Kobe, Hyogo 651-2271, Japan

#These authors contributed equally to this study.

Received: February 06, 2013; Revised: February 06, 2013; Accepted: February 25, 2013

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi:10.1002/prca.201300010

***Contact Information:** Hisashi Narimatsu, Ph.D., M.D.
Research Center for Medical Glycoscience (RCMG), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan.
Tel: +81-29-861-3200
E-mail: h.narimatsu@aist.go.jp

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

Total number of words (including references as well as figure and table legends).
2500 words

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.

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

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.

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

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

other words, it serves as a M2BP glycosylation isomer (M2BPGi) for estimating the progression level of fibrosis. We validated the correlation of FastLec-Hepa counts with the degree of fibrosis using samples from 209 patients with chronic hepatitis (CH) at two locations and documented its possible clinical utility for evaluating therapy by quantifying the degree of disease severity. In particular, the count-dependent monitoring for long-term follow-up (up to 3 years) of severely affected patients with any antiviral treatments was considered to be effective in the prevention of liver-related morbidity and mortality such as hepatic decompensation and hepatocellular carcinoma. To achieve the continuous monitoring based on the "on-site measurement", the assay must be standardized and the obtained counts should be normalized with an appropriate COI value. These might also achieve standardization in measuring disease severity and planning therapies. From these viewpoints, we decided that our validated FastLec-Hepa assay could be reconstructed effectively (see the final step of the developmental pipeline shown in Supporting Information Fig. S1) by creating a reliable calibrator.

In principle, as FastLec-Hepa monitors the level of fibrosis-related hM2BPGi, it requires an assay calibrator with dual epitopes: a glycan that is recognized by

WFA and an hM2BP peptide that reacts to an anti-hM2BP monoclonal antibody (mAb). Recombinant proteins for use as assay calibrators are generally produced in bacterial or yeast expression systems because of their high productivity and cost-effectiveness. However, these were not suitable for our purpose as the recombinant proteins produced from bacterial cells had no glycans and those from yeast cells contained only high mannose-type glycans, neither recognized by WFA. In our system, a mammalian cell line exhibiting WFA positivity was needed to produce the recombinant hM2BP (rhM2BP) as the assay calibrator. It was important to select a suitable host cell line for expressing rhM2BP because WFA positivity for hM2BP would depend on the glycosynthetic machinery of each cell line. To select host cells with prominent WFA reactivity, we first conducted a lectin microarray analysis on culture supernatants from two mammalian cell lines (Supporting Information Fig. S2), Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK293) cells, both of which are popular for producing recombinant proteins. Both cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in DMEM and RPMI-1460 medium (Gibco, Grand Island, NY) without FCS for 48 h. In the lectin microarray analysis under the direct-labeling method with Cy3, the culture supernatant of the

HEK293 cells exhibited higher WFA signals than that of the CHO cells (Fig. 1A). This result indicated that HEK293 cells had the potential to produce glycoproteins with higher WFA reactivity. Regarding the culture conditions, the lectin microarray also showed that DMEM was more suitable for a high WFA response than RPMI-1460 (Fig. 1A). Therefore, we finally established a stable cell line using HEK293 cells as a host.

To confirm the WFA positivity of rhM2BP expressed by HEK293 cells and to determine the interlot variability of production simultaneously, rhM2BP was subsequently expressed in HEK293 cells four times on different days and purified twice from each culture medium (Supporting Information Fig. S3 and Methods). A comparative glycan profiling of rhM2BP with signal intensities of the lectin microarray suggested WFA-positivity for rhM2BP without remarkable difference among the eight purification variants (Fig. 1B). Focusing on the WFA signals showing slight differences in intensity (CV = 15.7%, in the *left* panel of Fig. 1C), which might be attributed to variability in protein concentration, we further qualified the reactivity of each sample against WFA on the lectin microarray using data processing as described previously [12]. In this case, it would be appeared as a quantitative difference in rhM2BP in the level of a terminal Gal/GalNAc

marker of *N*-glycan, one of the WFA ligands [8]. The rate of produced asialo form shows an almost negative correlation with the ratio of sialylation. Therefore, data processing using the 6′-sialyllactosamine-binding lectin from *Sambucus sieboldiana* (SSA) as a normalizer enabled the precise qualification of WFA reactivity. As we expected, the result suggested that there was no qualitative difference among the production lots of rhM2BP in terms of the amount of the WFA ligand (CV = 5.6%, see the *right* panel of Fig 1C). Collectively, we constructed the protocol for production of rhM2BP as the assay calibrator.

We next examined the construction of an anti-rhM2BP mAb optimized for the sandwich immunoassay. Mice were immunized with rhM2BP and 13 productive mAb clones were selected based on their productivity and reactivity against rhM2BP. The mAb produced from each clone was purified (Supporting Information Fig. S4) and then biotinylated. Biotinylated mAb (25 ng per assay) was overlaid on the lectin microarray that had been incubated with rhM2BP overnight. The suitability of each mAb as the detecting antibody was assessed from the signal-to-noise ratio in the lectin–antibody sandwich immunodetection system (Supporting Information Figs S5 and S6). As a result, all mAb clones except for No. 2, with a high signal-to-noise ratio, were selected at this step. After

absence of potential interaction of HRP with WFA was confirmed (Supporting Information Fig. S7), the reactivity of each mAb clone against WFA-binding rhM2BP was further qualified in the HRP-assisted sandwich ELISA using a microtiter plate (Fig. 2A and Supporting Information Methods). Two clones (Nos. 11 and 28) were selected as the most feasible detection antibody along with a conventional accelerated stability test (Fig. 2B). In the following spiking experiment with rhM2BP (see Supporting Information Methods), we finally concluded that clone No. 28 was the best mAb with resistance against inactivation of the antigen–antibody reaction by serum components (Fig. 2C).

We prepared our diagnostic agent for the direct measurement of serum WFA^{+}-hM2BP with this antibody. To examine for variations, we performed triplicate measurements for rhM2BP on different days and different production lots. The intraday variations in measurements indicated high reproducibility of the assay (CV = 1.3, 0.7 and 2.3%) using the fully automatic immunoanalyzer HISCL-2000i (Sysmex Co., Hyogo, Japan), whereas the overall difference among the lots was not minimized (CV = 10.4%). Therefore, we reduced the interday and lot variations by indexing the measured values with the value of an exact amount of rhM2BP as a calibrator in the following procedure.

1. The mean +2.5 SD value of the measured values of 800 samples arbitrarily selected from the data set of sera from 1000 healthy volunteers (HV) that we recently reported [8] was assigned as a COI of 1.0 (Fig. 3).
2. An rhM2BP solution was prepared at a concentration to yield this COI value. This solution was designated as the master calibration solution for future production.
3. This calibrator used as a positive control and the buffer as a negative control was measured at least three times for each run of the HISCL-2000i.
4. The measured values (WFA^+-hM2BP) were indexed with the obtained values using the following equation:

   $COI = ([WFA^+\text{-hM2BP}]_{sample} - [WFA^+\text{-hM2BP}]_{NC}) / ([WFA^+\text{-hM2BP}]_{PC} - [WFA^+\text{-hM2BP}]_{NC})$

   $[WFA^+\text{-hM2BP}]_{sample}$, WFA^+-hM2BP count of serum sample (PC, positive control; NC, negative control).

As a result, the mean was 377710, the SD 272230, and the mean +2.5 SD value 1058285. Reliability was then validated using the remaining samples, where the number of samples having COI >1 was 10 out of 200 (5.0% of the total).

Subsequently, we evaluated the reconstructed system with the data set from our previous study [8]. A total of 232 samples from 3 different sites had been measured without dilution (direct measurement) and after 10-fold dilution, of which 117 HV samples were measured on different days with the kits of different production lots and 114 CH samples on the same day with the same production lot (1 CH sample was excluded as the direct measurement exceeded the LOQ). In the resulting 2D plots, both slopes were approximated at 0.96 with high correlation coefficients (R^2 = 0.98 and 0.99). For clinical use, the COI would help in understanding disease severity. The significance of the COI is currently under validation using over 10,000 samples from 15 different sites. The validation will not only support reliability of the glycodiagnostic agent, but also provide advantageous information on basic science, enabling the focused glycoproteomics with well-defined target cases and controls to clarify the structure of the disease-related glyco-alterations on M2BP. Further experiments for glycoproteomics and molecular pathology of M2BP will elucidate the mechanism of M2BP with such glyco-alterations secreted into serum.

In conclusion, we have reconstructed our sandwich assay system as a robust diagnostic agent thanks to the development of a robust calibrator. This was

achieved using the evidence-based technology of lectin-assisted glycan profiling focusing on cell-to-cell and protein-to-protein glyco-alterations. Our development pipeline is highly efficient and applicable to a wide variety of glycodiagnostic agents and might accelerate the incorporation of glycan-based technologies into the clinical arena.

We thank A. Togayachi, H. Kaji, A. Takahashi (AIST) and Y. Hamaguchi, T. Kagawa, S. Nagai, S. Matsubara, M. Terao (Sysmex Co.) for technical assistance, critical suggestions on assay development, or critically reading our manuscript. This work was supported in part by a grant from New Energy and Industrial Technology Development Organization of Japan.

The authors have declared no conflict of interest.

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