C.I., conidence interval.

*Child-Pugh class A includes patients without cirrhosis.

**Evaluated by pathologic examination of resected specimens.

TECHNICAL BRIEF

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

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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

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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.

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Figure 1. Efficient construction of WFA-positive rhM2BP as a calibrator for a quantitative lectin–antibody sandwich immunoassay. Cell glycomes of culture supernatants of CHO and HEK293 cells cultivated in RPMI or DMEM were analyzed using a 45-lectin microarray (A). The obtained signal intensity was normalized against the means of signals on the 45 lectins. The asterisk indicates the signal intensity of WFA. (B) Comparative analysis of glycan profiles among four different rhM2BP production lots. Respective glycan profiles of two purification samples per production lot were averaged and are represented as a bar graph with error bars. The reactivity to WFA is displayed separately in the *left* panel of (C). The interlot variability on the WFA reactivity was further estimated by standardizing the signal on WFA using SSA in the *right* panel of (C).