Development of a Novel Clinical Diagnostic Method for the Detection of Glycosylation Changes in Liver Fibrosis

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Diagnosis and treatment of a patient with chronic hepatitis or hepatic cirrhosis require assessment of the progression of liver fibrosis using simplified and highly accurate diagnostic approaches. While liver biopsy is considered the gold standard for diagnosis, it is a highly invasive procedure that it is not easily implementable. Also, conventional serum tests have issues with diagnostic accuracy. Thus, a simplified blood test is desirable as recommended in "A 7-year strategy plan for Hepatitis research" by the Ministry of Health, Labour and Welfare. Since 2009, when Sysmex participated in the New Energy and Industrial Technology Development Organization, hereinafter "NEDO" project, we engaged in the development of a practical assay kit HISCL-M2BPGi reagent (Sysmex Corporation, Kobe, Japan) together with the National Institute of Advanced Industrial Science and Technology (AIST). The kit provides an available measurement result in a minimum of 17 minutes after blood sampling. This kit enables us to assess the progression of liver fibrosis using a simplified and highly accurate measurement.

Key Words M2BPGi (Mac-2 Binding Protein Glycosylation Isomer), Liver Fibrosis, Wisteria Floribunda Agglutinin, Glycosylation Maker

INTRODUCTION

Worldwide research of liver fibrosis biomarkers has long been implemented. However, most markers have just focused on the extent of expression in conjunction with individual differences and variable living habits, and have not captured minimal structural changes of the tissue after many years, such as liver fibrosis. Therefore, the National Institute of Advanced Industrial Science and Technology (AIST) has been researching these biomarkers by focusing on the structural change of glycosylation and the carrier protein, allowing the capturing of quantitative changes associated with liver fibrosis. After implementing a basic study, M2BPGi: Mac-2 binding protein glycosylation isomer, Wisteria floribunda agglutinin (WFA) was identified as a lectin that is most correlated with progression of liver fibrosis. From the beginning, binding forces between glycosylation and lectin were an issue, so it was considered that measurement by an automated analyzer would be difficult. However, Sysmex improved the stability for long-term storage using liquid reagents and reactivity. As a result, Sysmex has achieved a measurement test for determination of the glycosylation isomer by using lectin and an antibody and which can be completed within a short time (17 minutes). Regulatory approval of this *in vitro* diagnostic was obtained in December 2013. Currently, this test assay kit is used and applied to a number of patient samples at several medical institutions, and there are accumulating reports of its utility. Here, we will show the usefulness of this glycosylation marker and also technical issues in implementing measurement of glycosylation structure, including an explanation of the demand for a liver fibrosis test, and also the background of development of the M2BPGi test assay its current clinical applicability.

Chronic hepatitis and liver fibrosis test

In Japan, chronic hepatitis is mainly caused by infection by the hepatitis B virus (HBV) or hepatitis C virus (HCV). The number of HBV and HCV infections in Japan is estimated to be 1.1 to 1.4 million and 1.9 to 2.3 million, respectively. Continuous HBV and HCV infection results in chronic inflammation and the disruption of hepatic cells (chronic hepatitis) in the liver over a period of 20 to 25 years, resulting in hepatocellular fibrosis, and progressing to liver cirrhosis and finally transformation into liver cancer. It is important to assess the rate of disease progression in the process of diagnosis and treatment of chronic hepatitis and liver cirrhosis. [1)]

To evaluate the rate of progression of liver fibrosis, histopathological testing (i.e., biopsy) is often used. As the tissue sample is removed from the liver with a needle, there is a risk of bleeding to the patient, so performing biopsies requires patients to stay at hospital due to physical burden, with patients' social and economic activities thereby strictly limited over this period. Furthermore, there are other risks, such as sampling errors, questions regarding the anatomical location to remove the hepatic tissue, and existing hospital-to-hospital differences in testing. [2)] In addition to liver biopsy, clinical findings, the number of blood platelets, blood enzymes, and biomarkers (e.g., type IV collagen and hyaluronic acid), and diagnostic imaging are used as

diagnostic indices. Unfortunately, these do not provide satisfactory clinical performance and, thus, a new noninvasive testing method is an unmet medical need. These pathological situations indicate that reducing physical burden and provision of noninvasive evaluation methods to assess liver fibrosis are required. These are also covered as goals in the "A 7-year strategy plan for Hepatitis research" released in 2008.

What is a glycosylation marker?

Glycosylation is the number of simple sugar binding-like chain rings and glycoprotein alternations found on the surface of various proteins *in vivo*. H. Narimatsu of AIST likened glycosylation to "clothes being dressed by the cell and protein." Features and analogies are as follows [3]. 1) According to cell maturation, the glycosylation structure (clothes) is changed. 2) There is tissue specificity in the glycosylation structure. 3) Individual glycosylation structures exist, such as with blood types. 4) Each glycosylation type has a unique glycosylation structure. That is to say, by identifying which type of glycosylation is occurring on the protein and how it transforms, disease-specific glycosylation markers may be clinically useful markers.

Measurement principle

As mentioned above, although it is clear that glycosylation and the change of the glycosylation structure possess useful information, there are still few reports of its clinical application as a simple and rapid test due to issues regarding analysis and measurement, which remain to be solved. As one of the main causes, the force of lectin binding to glycosylation is weak, so that constructing an enzyme-linked immunosorbent (ELISA) method is generally difficult. To resolve this issue, A. Kuno attempted to find a biomarker with a liver fibrosis-specific glycosylation structural change to meet the 3 following specificities from among the lectin chromatography, IGOT, and glycoproteomics (such as lectin array): 1. Exists in a large number of glycoproteins in the blood serum; 2. Several types of glycoalterations per molecule are present; 3. Other contaminating glycoproteins binding to disease-specific probe lectin (WFA) would not exist in blood serum and the Signal/Noise ratio should be preferable. Following this approach, the WFA (probe) and M2BPGi (candidate molecule) were selected [4].

Lectin (WFA) used in this test kit was discovered by Kurokawa et al., and the use of this lectin for the alpha-GalNAc-Ser/Thr-bearing glycoprotein action of α/β-N-acetylgalactosamine binding to galactose was reported by Piller [5,6]. The target core protein, M2BP, is a secretory glycoprotein known as ligand of Mac-2 and is a macrophage-associated lectin. The molecular structure consists of 585 amino acid residues, and there are 7 glycosylation N-binding forms in the primary sequence, all of which undergo glycoalteration. Furthermore, since it exists *in vivo* as ring structure of dimer-octamer, it is considered to have strong affinity to lectin (as much as glycosylation per molecule, enabling greater capability of binding to WFA [7]. To utilize this feature, Sysmex developed a method of densely immobilizing WFA on the surface of magnetic particles with this test kit. This method enables us to capture M2BPGi that has reactivity to WFA using extremely small amounts of blood serum (10μL). By later using our developed alkaline phosphatase conjugated anti-M2BP antibody which never prevents binding of WFA and glycosylation, and CDP-Star® we successfully developed the first *in vitro* diagnostic using chemiluminescence and rapidity (17 minutes) to assess progression of liver fibrosis in the world.

In addition, by transfecting human M2BP expression vectors in HEK293 cells, which are generally used for the expression of recombination protein, M2BPGi with reactivity to WFA is obtained. This standard control was developed to be the basis for collaboration with ASIT.

This test kit is used in its automated immunoassay

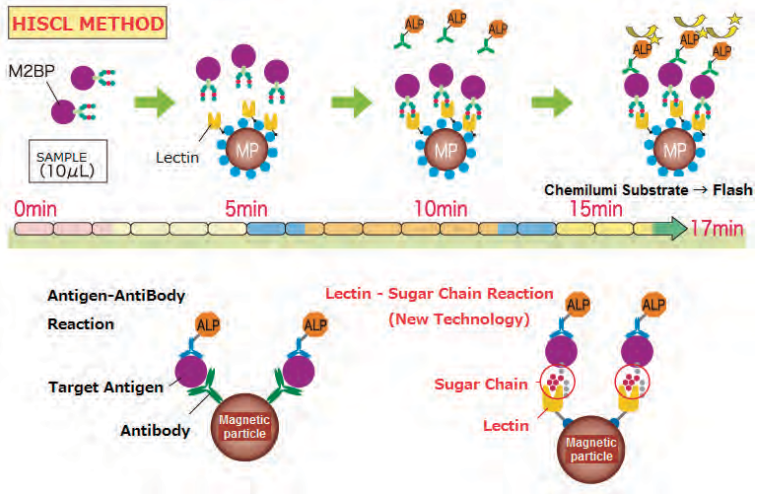

Fig. 1 *Lectin - Glycosylation reaction method*

systems, referred to as the HISCL Series of Sysmex (***Fig. 1***).

METHODS AND RESULTS

1. WFA-antibody sandwich immunoassay for M2BPGi by HISCL

All assays used an automated immunoassay system HISCL-5000 (Sysmex Corporation, Kobe, Japan).
The fibrosis-specific form of glycosylated M2BP (M2BPGi) was measured using a sandwich immunoassay approach. M2BPGi was captured by WFA immobilized on magnetic beads, and the bound product was assayed with an anti-human M2BP monoclonal antibody linked to alkaline phosphatase (ALP-αM2BPmAb). Two reagent packs (M2BP-WFA detection pack and a chemiluminescence substrate pack) were loaded in the HISCL. The detection pack comprised 3 reagents: a reaction buffer solution (R1), a WFA-coated magnetic bead solution (R2), and an ALP-αM2BPmAb solution (R3). The chemiluminescence substrate reagent pack contained a buffer solution (R4) and a CDP-Star® substrate solution (R5). Typically, serum (10μL) was diluted to 60μL with R1 and then mixed with R2 (30μL). After the binding reaction, R3 (100μL) was added to the reaction solution. The resultant conjugates were magnetically separated from unbound components, and mixed well with R4 (50μL) and R5 (100μL) before reading of the fluorescence. The chemiluminescent intensity was acquired within a period of 17 minutes in the operation described above. The reaction chamber was kept at 42°C throughout.

2. Reproducibility

To determine reproducibility on HISCL, 10 repeat measurements of samples (L and H) were run.
The within-run reproducibility of the results of these samples in terms of coefficient of variation (CV) was 5.0% and 2.6% (***Table 1***).

3. Minimum detection limit

Samples having 1.22 cut off index (C.O.I.) and 1.36 (C.O.I.) of M2BPGi were serially diluted with HISCL diluent buffer.
To determine the minimum measured concentration not falling within the range of the mean count +2 standard deviations (SD) of the 0 concentration control (Blank), 5

Table 1 *Reproducibility*

	Specimen-1		Specimen-2	
	Counts	C.O.I.	Counts	C.O.I.
n	10	10	10	10
mean	112,652	0.08	26,416,899	21.68
SD	2,568	0.004	672,463	0.55
CV(%)	2.3%	5.0%	2.5%	2.6%
Min	106,306	0.08	25,282,900	20.75
Max	115,529	0.09	27,380,802	22.48

Table 2 *Limit Of Detection*

Specimen-3

Dilution ratio		Blank	20 times	10 times	4 times	2times	1.3times	Undiluted
Chemiluminescent intensty（Counts）	N=1	1464	83521	143742	339124	679686	1008789	1414415
	N=2	1863	83584	138087	343425	664811	1020573	1394639
	N=3	2080	80054	144236	335463	685916	1026554	1448181
	N=4	1828	84874	150417	327176	671048	1053708	1443207
	N=5	1724	84733	138859	330339	672739	993574	1482622
Average		1,792	83,353	143,068	335,105	674,840	1,020,640	1,436,613
SD		224	1,948	4,959	6,540	8,148	22,369	33,722
CV(%)		12.5%	2.3%	3.5%	2.0%	1.2%	2.2%	2.3%
C.O.I.		0.00	0.06	0.12	0.28	0.57	0.86	1.22
Ave.+2SD		2,241	87,250	152,985	348,185	691,135	1,065,378	1,504,057
Ave.-2SD		1,343	79,457	133,151	322,025	658,545	975,902	1,369,169

Specimen-4

Dilution ratio		Blank	20 times	10 times	4 times	2times	1.3times	Undiluted
Chemiluminescent intensty（Counts）	N=1	1,464	85,156	154,219	394,511	810,982	1,181,591	1,619,297
	N=2	1,863	82,548	159,649	369,170	822,505	1,229,165	1,482,512
	N=3	2,080	85,681	152,214	385,780	802,001	1,232,270	1,663,469
	N=4	1,828	83,936	161,830	394,528	800,356	1,192,584	1,650,309
	N=5	1,724	84,016	159,662	387,400	789,357	1,165,829	1,585,720
Average		1,792	84,267	157,515	386,278	805,040	1,200,288	1,600,261
SD		224	1,216	4,085	10,369	12,424	29,381	72,351
CV(%)		12.5%	1.4%	2.6%	2.7%	1.5%	2.4%	4.5%
C.O.I.		0.00	0.07	0.13	0.32	0.68	1.02	1.36
Ave.+2SD		2,241	86,700	165,685	407,015	829,889	1,259,051	1,744,963
Ave.-2SD		1,343	81,835	149,345	365,541	780,191	1,141,525	1,455,559

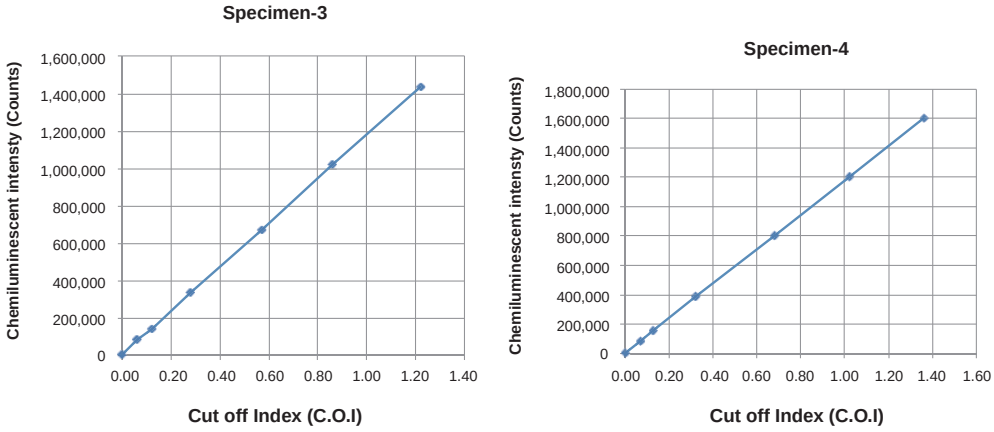

Fig. 2 *Minimum detection limit*

repeat measurements were made with each diluted sample. The minimum measured concentration samples were 0.06 (C.O.I.) and 0.07 (C.O.I.)
(***Table 2, Fig. 2***).

DISCUSSION

The minimum detection level of HISCL M2BPGi in our experiment was almost 0.1 (C.O.I.). This result suggests the possibility of quantitative monitoring of M2BPGi at levels as low as < 1.0 (C.O.I.), which can contribute to diagnosis and treatment.

CONCLUSION

Clinical performance

M2BP is even expressed in the blood serum of healthy subjects. In 2009, although there was a report of the expression of liver fibrosis and M2BP by Cheung et al., there was no satisfactory report of it meeting clinical performance for diagnosing each fibrosis stage of the disease [8]. Accordingly, "quantitative change" of the protein is not only treated as an index, but also the change of glycosylation structure associated with the disease state on the surface of the glycoprotein. In other words, it can become a more useful marker by concomitantly measuring the “qualitative change” compared to the use of a conventional marker.
By adding 2.5 SD to the average value of the luminescence intensity obtained from 800 healthy blood samples, luminescence intensity criteria are defined as following: cut off index (C.O.I) = 1.00, C.O.I < 1.00 is negative (nonchronic hepatitis group); 1.00 ≤ C.O.I < 3.00 is positive (1+) (chronic hepatitis group: F0-F3); C.O.I ≥ 3.00 is positive (2+) (liver cirrhosis group: F4). Because of conducting clinical assessment, the measurement value of the Mac-2 binding protein glycosylation isomer (M2BPGi) has been indicated as a preferable correlation with liver biopsy, which is considered the gold standard. In all stages of disease, a useful advantage was shown, and clinical performance with the same or more than the conventional serum marker was also demonstrated [9]. In addition, clinical assessment of M2BPGi is currently implemented in several medical institutes and interesting clinical data that cannot be seen in liver biopsy or by other serum tests are accumulating. For example, according to the report by Yamazaki et al., in which the use of data stratification by categorizing “M2BPGi value (C.O.I.)” as either less than 1.00, over 1.00 and less than 4.00, over 4.00, or other than fibrosis stage, it was found that accumulating data for liver cancer rates at 5 and 10 years indicated differing occurrence rates, which are not discrete variables, such as the F0 to F4 stages obtained with liver biopsy. Moreover, with a variable sequence showing the state of liver, it is expected that M2BPGi may be indicated as a risk marker for liver cancer [10]. According to a science study outcome report by the Department of Health, Labour and Welfare, (Representative: H. Narimatsu), the measurement result indicated preferable correlation with that of liver stiffness measurement using ultrasound waves and elastography. In the case of extensively progressed liver stiffness, M2BPGi provided a better diagnosis compared to liver stiffness measurement. In assessing fibrosis progression of nonalcoholic fatty liver disease, although the cutoff value showed lower tendency than hepatitis virus caused by HCV, it is reported as a useful marker as it can distinguish between stages F2 and F3. Through ongoing accumulating clinical results, we are trying to discover other clinical utilities for this assay beyond fibrosis progression.

Prospective view

As previously described, glycosylation involves qualitative changes on the surface of proteins in disease-specific glycosylation markers. There exist many clinical applications that have not been realized with conventional markers targeting quantitative changes of the protein. Japan is a leading country in glycosylation research, and AIST is the center of enthusiastic research

and development conducted from the base technology to the eventual realization of a diagnostic method. Therefore, there already exist some new disease specific-glycosylation marker candidates, such as M2BPGi. Also Sysmex accelerates strong industry-government-academia relationships by applying its expertise in rapid measurement using glycosylation structural change brought from M2BPGi using lectin, thereby offering a new “high-value test.” This is one way that may lead to improvement in the quality of life of patients, and in the progress of medical technology and treatment. Also, development of glycosylation disease-specific markers should be an important clinical development priority, which could bring a paradigm shift in the clinical testing market.

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