in patients with resolved HBV infection,[1,4,5] but a standard man agement according to the risk of HBV reactivation has not been established as yet. We hope that the additional information can help readers regarding the optimal interval and sensitivity of the HBV DNA monitoring assay. In addition, if the reappearance of HBsAg and viral mutations related to viral replication are good predictive markers for severe hepatitis resulting from HBV reactivation, we can recommend that antiviral treatment should be started immediately for those patients with HBV reactivation.

SHIGERU KUSUMOTO, M.D., PH.D.[1]
YASUHITO TANAKA, M.D., PH.D.[2]
MASASHI MIZOKAMI, M.D., PH.D.[3]
RYUZO UEDA, M.D., PH.D.[4]
[1]Department of Medical Oncology and Immunology
Nagoya City University Graduate School of Medical Sciences
Nagoya, Japan
[2]Department of Virology and Liver Unit
Nagoya City University Graduate School of Medical Sciences
Nagoya, Japan
[3]The Research Center for Hepatitis and Immunology
National Center for Global Health and Medicine
Ichikawa, Japan
[4]Department of Tumor Immunology
Aichi Medical University School of Medicine
Aichi, Japan

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Reply:

We thank Dr. Kusumoto and his colleagues for their comments on our study.[1] Management of chemotherapy induced hepatitis B virus (HBV) reactivation in lymphoma patients with resolved HBV infection is a challenging issue in endemic areas because 50% 60% of the general population in those areas have resolved HBV infec tion, and the clinical courses of HBV reactivation in this patient population vary greatly. Dr. Kusumoto pointed out several impor tant points that warrant further investigation.

With increasing sensitivity of the HBV DNA tests, chemotherapy induced HBV reactivation can be detected in about 20% of lymphoma patients who receive rituximab/CHOP chemo therapy and can be detected earlier (median, 11.8 weeks earlier than the less sensitive assay).[1,2] However, in our study, most of the additional HBV reactivations detected by the more sensitive assay were asymptomatic. Only 1 patient had a hepatitis flare, which resolved spontaneously. It is difficult to analyze whether earlier use of antiviral therapy by a more sensitive assay can further reduce the number of HBV related hepatitis flares, given the small number of patients. Therefore, the clinical benefit of HBV reactivation detected by the more sensitive assay for pre emptive antivirals remains to be established.

Reappearance of hepatitis B surface antigen (HBsAg), which can be measured conveniently in the clinic by chemiluminescent immunoassay, such as the Abbott Architect assay (Abbott Labora tories, Abbott Park, IL), was found, in our study, to be signifi cantly associated with HBV related hepatitis flares. The sensitivity of HBsAg tests currently used in the clinic can be as low as 0.05 IU/mL. Most of the patients were found to be $HBsAg^{+}$ at the time of hepatitis flare. Therefore an assay for HBsAg during the chemotherapy course is strongly advocated, in addition to HBV DNA. Whether HBsAg assay is useful, and more cost effective for managing HBV reactivations in lymphoma patients, warrants a prospective study. Finally, we agree with Dr. Kusumoto that persistence of HBsAg positivity for more than 6 months may negatively influence long term outcome, and long term follow up of patients with reappearance of HBsAg is ongoing.

Dr. Kusumoto pointed out the potential importance of viral factors, including the HBV replication levels and the presence of precore/basal core promoter mutations, in the development of severe hepatitis flare. Because the majority of the patients did not have detectable HBV DNA at baseline, we could not study the viral factors before therapy. For those patients with HBV reactivations, we are currently characterizing the viral genotypes and variants. However, because the patient number is still limited, studies of larger sample size and more comprehensive evaluation of the viral and host factors that may contribute to HBV reactivation are definitely needed.

Dr. Huang and his colleagues recently reported on a random ized trial of prophylactic antiviral therapy in lymphoma patients with resolved HBV infection.[2] Although the short term efficacy in preventing HBV viral reactivation was clearly demonstrated, future studies are warranted to evaluate the optimal duration of antiviral therapy, the high risk patient population for whom prophylactic antiviral therapy is most indicated, and the effect of prophylactic antiviral therapy on long term outcome. In the endemic area, the management strategies have to take additional factors, especially cost effectiveness, into account.

CHIUN HSU, M.D., PH.D.[1,4]
PEI JER CHEN, M.D., PH.D.[2,3]
ANN LII CHENG, M.D., PH.D.[1,4]
Departments of [1]Oncology and [2]Internal Medicine
National Taiwan University Hospital
Taipei, Taiwan

Chapter 23

Differential Glycan Analysis of an Endogenous Glycoprotein: Toward Clinical Implementation—From Sample Pretreatment to Data Standardization

Atsushi Kuno, Atsushi Matsuda, Sachiko Unno, Binbin Tan, Jun Hirabayashi, and Hisashi Narimatsu

Abstract

There are huge numbers of clinical specimens being stored that contain potential diagnostic marker molecules buried by the coexistence of high-abundance proteins. To utilize such valuable stocks efficiently, we must develop appropriate techniques to verify the molecules. Glycoproteins with disease-related glycosylation changes are a group of useful molecules that have long been recognized, but their application is not fully implemented. The technology for comparative analysis of such glycoproteins in biological specimens has tended to be left behind, which often leads to loss of useful information without it being recognized. In this chapter, we feature antibody-assisted lectin profiling employing antibody-overlay lectin microarray, the most suitable technology for comparative glycoanalysis of a trace amount of glycoproteins contained in biological specimens. We believe that sharing this detailed protocol will accelerate the glycoproteomics-based discovery of glyco-biomarkers that has attracted recent attention; simultaneously, it will increase the value of clinical specimens as a gold mine of information that has yet to be exploited.

Key words Glycoprotein, Glycan analysis, Lectin microarray, Clinical specimen, Biomarker

1 Introduction

It is evident that almost all secreted proteins are glycosylated via the glycosynthetic pathway in the endoplasmic reticulum and the Golgi apparatus. Because this glycosylation is characteristic of the extent of cell differentiation and the state of the cell, i.e., the origin of the tissue, its developmental stage, and the presence of malignancy, blood glycoproteins consist of a mixture of heterogeneous molecules derived from many origins [1, 2]. Thus, glycoproteins that are present in serum and that exhibit cancer-associated changes in glycosylation (glyco-alteration) have potential as biomarkers (glyco-biomarkers) for cancer diagnosis. Owing to the rapid advances in glycomics/glycoproteomics technologies, numerous glycoproteins

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have now been identified as candidate glyco-biomarkers. These glyco-biomarkers have been attracting a great deal of attention in the "discovery phase" [3, 4], and are expected to move toward clinical implementation by a process similar to that followed for alpha-fetoprotein (AFP). In the early 1990s, increased fucosylation of complex-type *N*-glycans was detected in some glycoproteins from hepatocellular carcinoma (HCC) patients [5]. More than 30 % of all AFP glycoforms were found to react to a fucose-binding lectin, *Lens culinaris* agglutinin (LCA). This fraction, designated as AFP-L3, was subsequently approved by the US FDA in 2005 as the first glycoprotein biomarker. Such a scenario is the ideal for identification of subsequent candidate molecules; however, it requires a systematic verification procedure for selection of the most appropriate candidate from hundreds identified in the preceding discovery phase. The lack of such a system has been a major obstacle in the mass spectrometry-based development of glyco-biomarkers.

Lectin microarray is a twenty-first century technology for glycan analysis of proteins [6]. Although the early developments of the methodology focused on high-sensitivity analysis of the microheterogeneity of glycan structures on a target glycoprotein [7–9], the majority of its applications seem to have shifted to the comparative glycome analysis of crude samples, e.g., cultured cells [10–13], bacteria [14, 15], and viruses [16]. We note that direct labeling is mostly used as the detection principle, in which more than 100 ng of the glycoprotein is usually needed prior to Cy3 labeling to assure highly practical and reproducible analysis (although the analysis itself can be satisfactorily performed with even 1 ng of the analyte sample). This is clearly a serious disadvantage for the analysis of less available endogenous glycoproteins, e.g., those contained in clinical samples. To overcome this problem, antibody-overlay lectin microarray was developed as an alternative approach to detecting specific interactions between a target glycoprotein (analyte) and multiple lectins immobilized on a microarray. This is achieved with the aid of antibodies raised against the "core protein" moiety [17]. In addition, the only pretreatment or prior processing required with the use of a specific antibody (either polyclonal or monoclonal) is immunoprecipitation. This extreme simplicity allows us to analyze sub-picomole (nanogram) amounts of a target glycoprotein preparation by means of lectin microarray, with a much greater throughput. In fact, this technical modification of the lectin microarray was made specifically for glyco-biomarker verification [18], and enables high-throughput glycan analysis of over a hundred clinical samples targeting a particular candidate glycoprotein [19, 20]. However, despite the attention it has attracted, the use of this verification method has not been sufficiently popularized in the field of glyco-biomarker development. This can be demonstrated by the fact that there are few studies concerning glyco-biomarkers except those published by a small

Table 1
Examples of focused glycan profiling by antibody-overlay lectin microarray analysis

Target glycoprotein	Clinical specimen		Detection method	Reference
Tissue				
Podoplanin	FFPE testis tissue from seminoma patient	3.6 mm^3	ALP	Kuno et al. [17]
PSA, MME	Frozen OCT-embedded prostate tissue from prostate cancer	200 μg total protein	ALP	Li et al. [21]
Serum				
Fetuin-A	Sera of patients with pancreatic cancer	100 μL	DL	Kuwamoto et al. [22]
AGP	Sera of chronic hepatitis C patients	0.5 μL	ALP	Kuno et al. [19]
Mac-2 binding protein	Sera of chronic hepatitis C patients and healthy volunteers	2.0 μL	ALP	Kuno et al. [20]
CPN2, CSF 1R, SPARCL1, ICOSLG, PIGR,	Sera of HCC patients and healthy volunteers	10 μL	ALP	Kaji et al. [23]
Other body fluid				
Transferrin	Cerebrospinal fluid		ALP	Futakawa et al. [24]
L1CAM	Bile of patients with CC and hepatolithiasis	100 μL	ALP	Matsuda et al. [25]

ALP anitbody-assisted lectin profiling method, *DL* direct labeling method

number of groups focusing on glyco-biomarker development (*see* Table 1). In this chapter, we would like to introduce the simple and versatile methodology of lectin microarray by example, with detailed protocols including sample pretreatment for two endogenous glycoproteins, Mac-2 binding protein (M2BP) and MUC1.

2 Materials

Prepare all solutions using ultrapure water (e.g., Milli-Q water). Prepare and store all reagents at room temperature (RT) unless otherwise indicated.

2.1 M2BP Immunoprecipitation Components

1. Phosphate buffered saline (PBS).
2. Tris buffered saline (TBS): 10 mM Tris–HCl, 150 mM NaCl, pH 7.6.

3. TBS containing 1.0 % Triton X-100 (TBSTx).
4. Magnetic beads (SA-MB): Dynabeads® MyOne Streptavidin T1 (Life Technologies, Carlsbad, CA).
5. Antibody solution (bio-Ab): biotinylated goat anti-human M2BP polyclonal antibody (R&D Systems, Inc., Minneapolis, MN) in PBS (50 ng/μL).
6. (Optional) Biotinylation reagent: biotin labeling kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan).
7. (Optional) Affinity column for antibody purification: HiTrap Protein G HP, 1 mL (GE Healthcare UK Ltd., Little Chalfont, UK).
8. Elution buffer (EB): TBS containing 0.2 % SDS.
9. Low-retention tubes: Eppendorf® Protein LoBind Microcentrifuge Tubes, 1.5 mL (Eppendorf Co., Hamburg, Germany).
10. Low-retention tips: epT.I.P.S. LoRetention, 2–200 μL (Eppendorf Co.).
11. Magnet stand (MgS): DynaMag-2 (Life Technologies).
12. Reaction mixer: Thermomixer Comfort (Eppendorf Co.).
13. Multipurpose spin down mixer: Bug Crasher GM-01 (Taitec Co., Nishikata, Japan).
14. Heat block: Dry Thermo Unit (Taitec Co.).

2.2 M2BP Western Blot Components

1. Electrophoresis gel: 5–20 % Gradient gel (DRC, Tokyo, Japan).
2. PVDF membrane: Immun-Blot® PVDF Membrane for protein blotting (0.2 μm) (Bio-Rad Laboratories, Hercules, CA).
3. Blotter: Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories).
4. Blocking solution (BS): 4.0 % Block Ace powder (DS Pharma Biomedical Co., Ltd., Osaka, Japan) in Milli-Q water.
5. TBS containing 0.05 % Tween-20 (TBST).
6. Alkaline phosphatase–streptavidin (Jackson ImmunoResearch Laboratories Inc., Philadelphia, PA).
7. WB substrate: Western Blue® stabilized substrate for alkaline phosphatase (Promega, Madison, WI).

2.3 M2BP Antibody-Overlay Lectin Microarray Components

1. Lectin microarray slides: LecChip™ (GlycoTechnica Ltd., Yokohama, Japan, *see* Fig. 1).
2. Array scanner: GlycoStation™ Reader 1200 (GlycoTechnica Ltd.).
3. Imaging software: Array Pro Analyzer v. 4.5 (Media Cybernetics, Inc., Bethesda, MD) or GlycoStation ToolsPro Suite v. 2.0 (GlycoTechnica Ltd.).

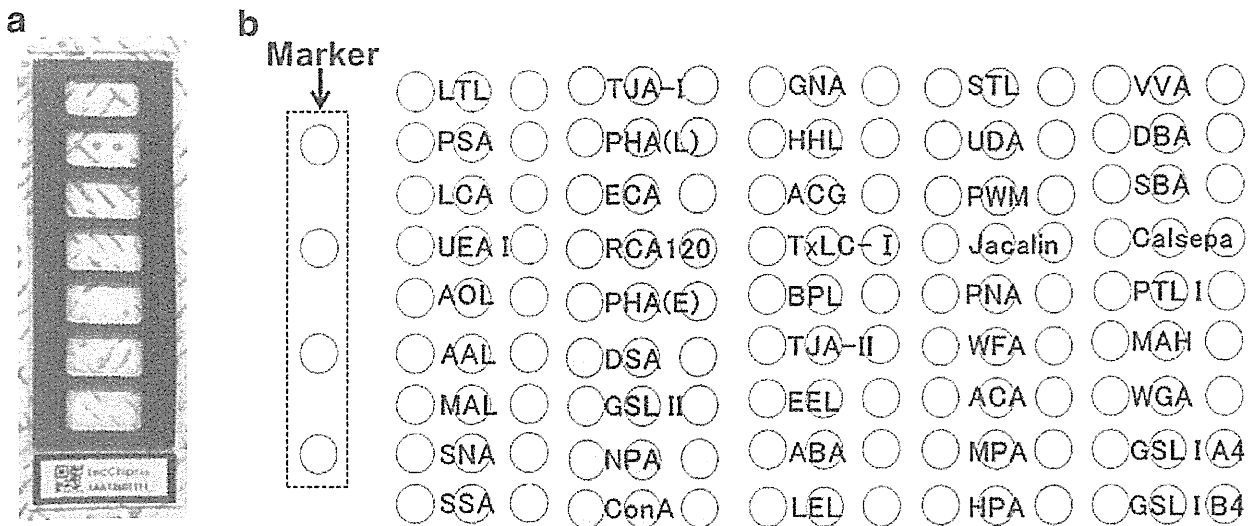

Fig. 1 Overview of LecChip instrument (**a**) and array format (**b**)

4. Chip incubator: to keep mixing the reaction solution on the chip under constant conditions at 20 °C with high humidity, set Bio shaker SHM-311 (SCINICS) in the incubator MIR-153 (SANYO Electric Co., Ltd., Moriguchi, Japan).
5. Humidified incubation chamber: incubation chamber, dark orange (Cosmo Bio Co., Ltd., Tokyo, Japan).
6. Probing buffer (PB): TBS containing 1 % (v/v) Triton X-100, 1.0 mM $CaCl_2$, and 1.0 mM $MnCl_2$.
7. Chip cleaner wipes: Kimtex white 115 mm × 230 mm (Nippon Paper Crecia Co., Ltd., Tokyo, Japan).
8. PBS containing 1.0 % Triton X-100 (PBSTx).
9. Antibody solution (bio-Ab): biotinylated goat anti-human M2BP polyclonal antibody in PBS (50 ng/μL).
10. Blocking reagent (BR): IgG from human serum (about 10 mg/mL, Sigma-Aldrich Co., St. Louis, MO).
11. Cy3-labeled streptavidin solution (Cy3-SA): Cy3-streptavidin (GE Healthcare) in PBS (50 ng/μL).

2.4 MUC1 Cell Culture Components

1. Media: RPMI1640 medium containing 5 % fetal bovine serum (FBS), serum-free RPMI1640 medium (Life Technologies).
2. Antibiotics: 100 units/mL penicillin, 100 mg/mL streptomycin (Life Technologies).
3. 150 cm^2 cell culture flasks (Becton Dickinson Co., Franklin Lakes, NJ).
4. CO_2 incubator (Thermo Fisher Scientific Inc., Fremont, CA).
5. Centrifugal filters: Amicon Ultra 10 kDa (Merck, Darmstadt, Germany).

2.5 MUC1 Immunohistochemistry Components

1. Xylene.
2. 100 % EtOH.
3. 95 % (v/v) EtOH.
4. 90 % (v/v) EtOH.
5. 80 % (v/v) EtOH.
6. 70 % (v/v) EtOH.
7. PBS.
8. Biotin labeling kit-NH_2 (Dojindo Laboratories).
9. 10 mM sodium citrate buffer (pH 6.0).
10. Autoclave (Tomy Seiko Co., Ltd., Tokyo, Japan).
11. Methanol containing 0.3 % H_2O_2.
12. VECTASTAIN ABC Kit (Vector Laboratories, Ltd., Burlingame, CA).
13. 2 % normal horse serum.
14. ImmPACT™ DAB Peroxidase Substrate (Vector Laboratories, Ltd).
15. Vector hematoxylin (Vector Laboratories, Ltd.).
16. VECTASHIELD mounting medium (Vector Laboratories, Ltd.).
17. Incubation chamber: a "Chip incubator" for lectin microarray analysis is recommended (*see* Subheading 2.3).
18. 1 % (w/v) eosin solution (Wako Pure Chemical Industries, Ltd.).
19. 0.2 % HCl–EtOH solution.

2.6 Dissection of Tissue Fragments and Protein Extractic Components

1. Surgical formalin-fixed paraffin-embedded tissue (FFPT) sections (5 μm thickness).
2. Disposable scalpels No. 12 (As One Corp., Osaka, Japan).
3. Low-retention tubes: Eppendorf® Protein LoBind microcentrifuge tubes, 1.5 mL (Eppendorf Co.).
4. 10 mM sodium citrate buffer (pH 6.0).
5. Coprecipitant: 50 % slurry of Avicel PH-101 (Sigma-Aldrich, Co.) in PBS.
6. PBS containing 0.5 % NP-40.
7. PBS.
8. Centrifuge (Tomy Seiko Co., Ltd.).
9. Sonicator (Tamagawa Seiki Co., Ltd., Iida, Japan).
10. Microscopy: Olympus CKX41 (Olympus Co., Tokyo, Japan).

2.7 MUC1 Immunoprecipitation Components

1. Anti-sialyl-MUC1 antibody (MY.1E12).
2. SA-MB (*see* Subheading 2.1, **item 4**).
3. DynaMag™-2 magnetic particle concentrator (Life Technologies).
4. PBS.
5. PBSTx.
6. PBS containing 0.2 % SDS.
7. Reaction mixer: Thermomixer Comfort (Eppendorf Co.).

2.8 MUC1 Antibody-Overlay Lectin Microarray Components

1. Lectin microarray slides.
2. GlycoStation™ Reader 1200 (GlycoTechnica Ltd.).
3. PBSTx.
4. Blocking reagent (BR): IgG from human serum (about 10 mg/mL, Sigma-Aldrich Co.).
5. Cy3-labeled streptavidin solution (Cy3-SA): Cy3-streptavidin (GE Healthcare) in PBS (50 ng/μL).
6. Imaging software: Array Pro Analyzer v. 4.5 (Media Cybernetics, Inc.) or GlycoStation ToolsPro Suite v. 2.0 (GlycoTechnica Ltd.).
7. Chip incubator.

3 Methods

Lectin microarray enables high-sensitivity glycan analysis of glycoproteins in clinical specimens. In this situation, quite a small amount of the analyte glycoproteins will be handled during the sample preparation and lectin microarray analysis. Therefore, low-retention tips and tubes should be used to prevent unintended loss of proteins. In addition, all buffer solutions used here contain surfactants such as SDS and Triton X-100 (*see* **Note 1**). Handle all reagents and perform all processes at RT unless otherwise specified.

3.1 Differential Glycan Analysis of M2BP from Culture Supernatants

M2BP is ubiquitously expressed in several kinds of cancer cells at different expression levels with various glycosylation modifications (A. Kuno, unpublished observation). In this protocol, we determined the conditions for the sample preparation and lectin microarray analysis based on the experimental results for six hepatocellular carcinoma cell lines (Huh7, HepG2, HAK1A, HAK1B, KYN-1, and KYN-2), which were cultivated in serum-free culture media.

3.1.1 Pretreatment of Magnetic Beads

1. Put the required amount of 10 mg/mL SA-MB (e.g., [number of samples + one for reserve] × 10 μL) in a microtube (*see* **Note 2**).
2. Place the tube on an MgS until the solution becomes clear.

3. After discarding the supernatant and removing bubbles (if any), take the tube from the MgS and add TBSTx (5× the volume of SA-MB).
4. After mixing thoroughly and flash-spinning (F-S) the SA-MB solution with a Bug Crasher, place the tube on the MgS until the solution becomes clear.
5. Repeat the washing process twice.
6. After discarding the supernatant, resuspend the SA-MB with TBSTx (1/2 volume of the beads).
7. Store the twofold-concentrated SA-MB (2× SA-MB) at 4 °C until use for immunoprecipitation.

3.1.2 Immunoprecipitation of M2BP

1. Put the culture supernatant containing 1 μg of the total protein, previously determined by the Bradford protein assay kit, into a 1.5 mL microtube.
2. Adjust the reaction solution to 93 μL with TBSTx.
3. Add 2 μL of bio-Ab and incubate at 4 °C for 30 min with gentle mixing with the reaction mixer.
4. F-S and add 5 μL of the pretreated 2× SA-MB, and then further incubate for 30 min.
5. F-S and keep the tube on the MgS until the reaction solution becomes clear.
6. Separate the supernatant and keep it as the "pass-through fraction (TF)."
7. Take the tube from the MgS and add TBSTx (200 μL).
8. Resuspend the SA-MB conjugated with Ab and M2BP with thorough mixing using the Bug Crasher.
9. F-S and keep the tube on the MgS until the reaction solution becomes clear.
10. Repeat the washing process twice.
11. After discarding the supernatant, resuspend the SA-MB in EB (10 μL) with gentle tapping.
12. Heat-treat at 70 °C for 5 min and then immediately place on ice (1 min).
13. Next keep at RT for 5 min, F-S, and add 10 μL of TBSTx.
14. F-S and keep the tube on the MgS until the reaction solution becomes clear.
15. Separate the supernatant and keep it as the "elution fraction (EF)."
16. Store both the TF and EF at −30 °C until used for subsequent quantitative and qualitative analysis.

3.1.3 Quantitation and Characterization of Enriched M2BP by Western Blot Analysis

1. Electrophorese half the volume of EF (10 μL) on a 5–20 % gradient gel under the following conditions: current 20 mA/gel, running time 40–50 min (until the front line migrates about 4 cm).
2. Transfer the electrophoresed proteins to a PVDF membrane by blotting.
3. Wash the membrane twice with TBS for 5 min.
4. Immerse the membrane in TBS at 37 °C for 30 min.
5. Wash the membrane three times with TBST for 5 min.
6. Immerse the membrane in 0.15 ng/μL bio-Ab diluted in TBST at 37 °C for 50 min.
7. Wash the membrane three times with TBST for 5 min.
8. Immerse the membrane in the alkaline phosphatase–streptavidin solution diluted 10,000-fold with TBST at 37 °C for 40 min.
9. Wash the membrane three times with TBST for 5 min.
10. Place the membrane in TBS and incubate for 5 min.
11. Immerse the membrane in the WB substrate.
12. Stop the chromogenic reaction by placing the membrane in Milli-Q water (*see* the expression pattern for each cell line in Fig. 2a).

3.1.4 Antibody-Overlay Lectin Microarray

1. Bring the LecChip, which is prepacked in a lightproof zipper bag, from the freezer (−20 °C) and leave on a bench without opening until it reaches RT.
2. Open the package and wash each well on the chip three times with 100 μL of PB.
3. Place the chip on a chip cleaner to remove excess solution (*see* **Note 3**).
4. Add PB (100 μL) into each well and store the chip in a humidified incubation chamber at 4 °C.
5. Remove the PB completely, and add 52 μL of PBSTx and 8 μL of EF to each well.
6. Incubate the chip in the chip incubator overnight.
7. Add 2 μL/well of BR and incubate the chip for 30 min.
8. Wash each well three times with 60 μL of PBSTx, and add 56 μL of PBSTx and 2 μL of BR.
9. Mix the solution gently, add 2 μL of bio-Ab, and incubate the chip for 1 h.
10. Remove the antibody solution completely and wash each well three times with 60 μL of PBSTx.
11. Add 56 μL of PBSTx and 4 μL of Cy3-SA and incubate the chip for 30 min.

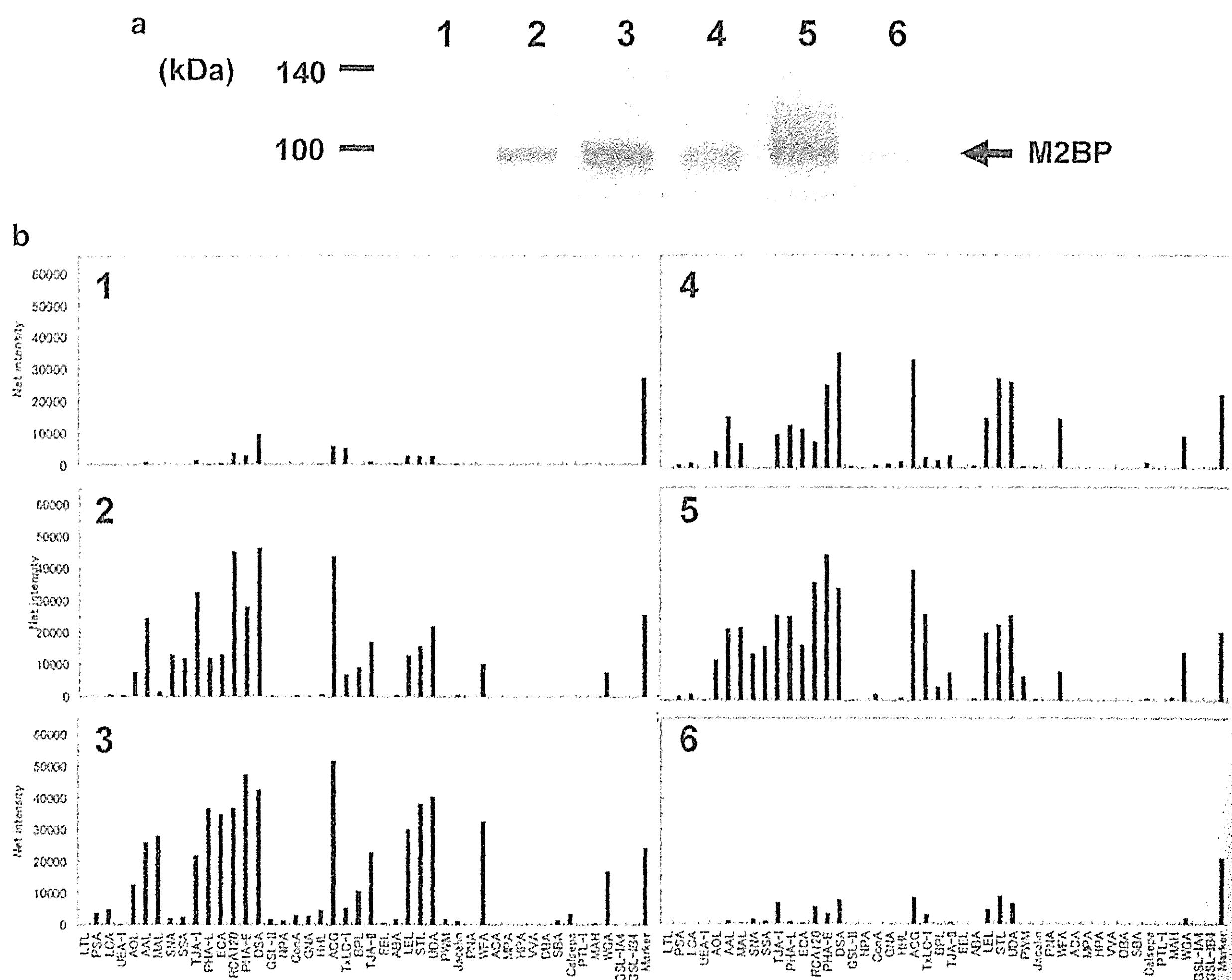

Fig. 2 Quantitation and characterization of endogenous human M2BP from culture supernatants of six hepatocellular carcinoma cell lines (*1*, Huh7; *2*, HepG2; *3*, HAK1A, *4*, HAK1B; *5*, KYN-1; *6*, KYN-2) by western blot (**a**) and lectin microarray (**b**)

12. Remove the solution completely and wash each well three times with 60 μL of PBSTx.
13. Wipe the sides and back of the chip with the chip cleaner while keeping the buffer in each well of the chip.
14. Scan the chip with the scanner (*see* **Note 4**).
15. Save the resulting fluorescent images as TIFF files and convert them to signal counts with the imaging software.
16. Calculate the net intensity value for each spot by subtracting the background value from the mean of the signal intensity values of three spots.
17. Characterize or compare the obtained glycan profiles using MS Excel (*see* the signal pattern obtained for each cell line in Fig. 2b).

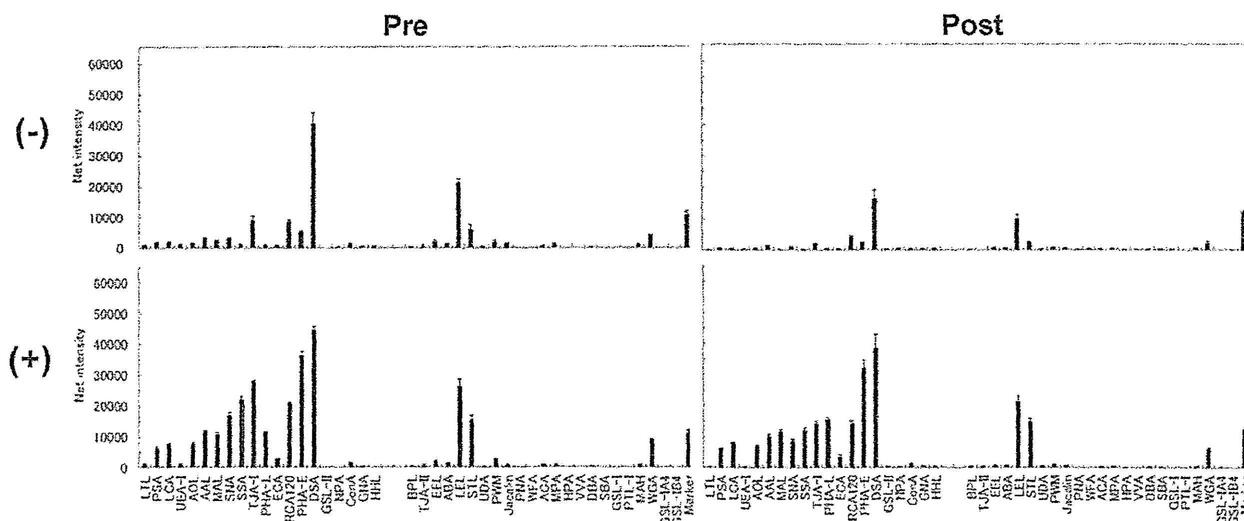

Fig. 3 Effect of the contaminant glycoproteins in the detection antibody solution prior to biotinylation on glycan profiles of human M2BP. Glycan profiles were obtained in the presence (+) or absence (–) of serum M2BP by overlaying the detection antibody (pre: commercial anti-human M2BP antibody was used without purification; post: the same antibody was used after repurification)

3.1.5 (Optional) Antibody Biotinylation

If a biotinylated antibody is not commercially available, free antibodies are routinely labeled with a biotin labeling kit-NH_2. The biotinylated antibody should be characterized by both western blot and lectin microarray before its use in immunoprecipitation and the differential glycan analysis.

3.1.6 (Optional) Antibody Purification

If a background signal for the negative control (without M2BP) is obtained on some lectins, repurification of the detection antibody using a protein G HP column may be helpful to reduce signal noise (*see* Fig. 3).

3.2 Differential Glycan Analysis of Human Serum M2BP

3.2.1 Serum Pretreatment

1. Dilute 10 μL of serum with PBS (88 μL) and then add 10 % SDS solution (2 μL) in a tube.
2. Heat-treat the tube at 95 °C for 20 min and put on ice for 1 min.
3. Mix gently and F-S with the Bug Crasher.
4. Store at −80 °C until use for immunoprecipitation (*see* **Note 5**).

3.2.2 Immuno-precipitation of Serum M2BP

1. Perform this using 20 μL of the heat-treated serum solution.
2. Refer to the protocol for culture supernatants (*see* Subheading 3.1, **step 2**).

3.2.3 Antibody-Overlay Lectin Microarray

1. Perform this using 8 μL of the EF.
2. Refer to the protocol for culture supernatants (*see* Subheading 3.1, **step 4**).

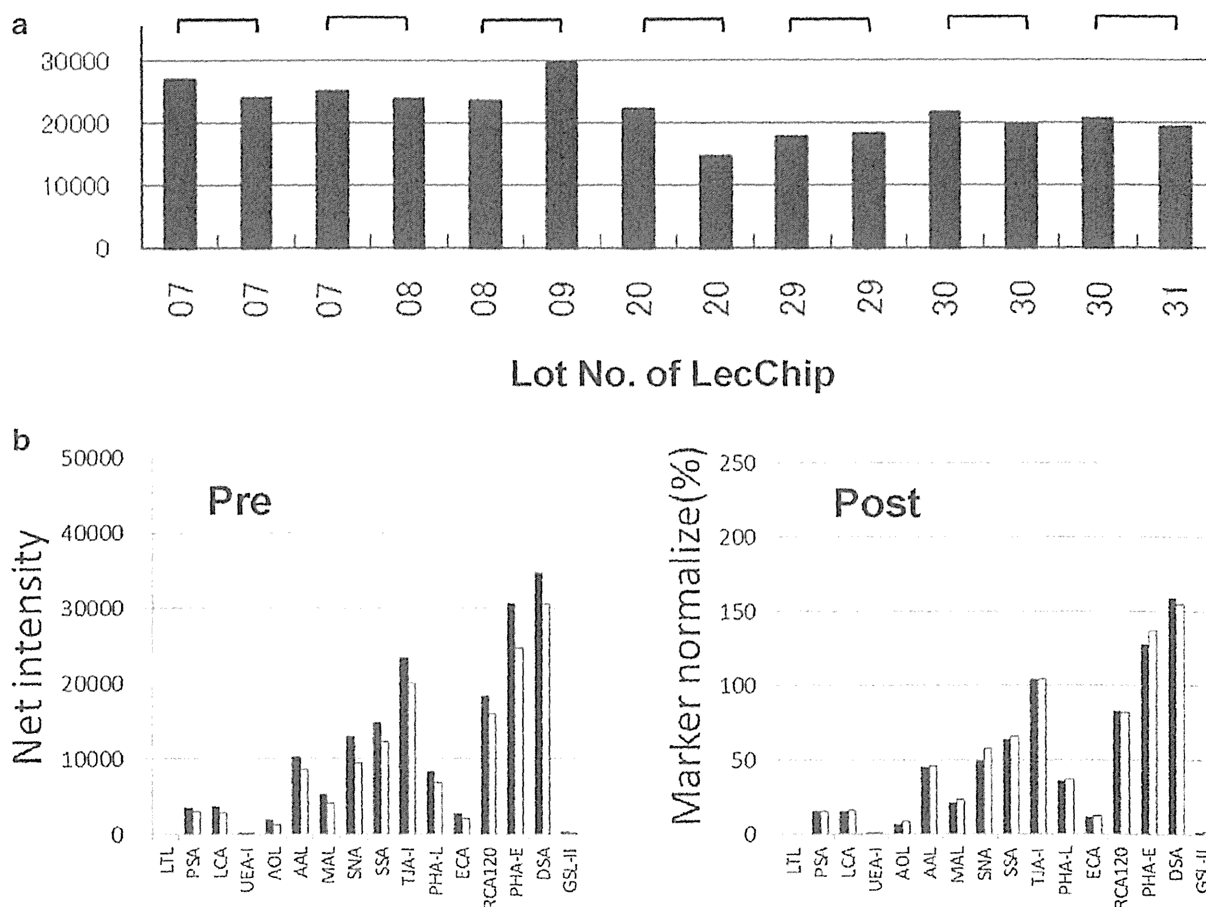

Fig. 4 Importance of an internal standard for data acquisition. (**a**) Effect of the time after powering up the system on inter- or intraday variation of the array signal intensity. When we performed a large-scale validation (about 500 patient sera) of serum M2BP using the lectin microarray, over 70 slides were used with some product lot variation. The figure shows the signal intensity of the marker in the first well of the last chip in each antibody-overlay lot. The *brackets* indicate the measurements made in the morning and afternoon of the same day. The results clearly indicate that the time from powering up until use affects the signals, as does lot variation. (**b**) Signal intensity of the positive controls (IP elution fraction from type-A serum) before and after standardization. The same IP elution fraction was added to the first well of the chips from different lots and scanned on different days

3.2.4 (Optional) Data Standardization

The relative intensity of the lectin-positive samples can be determined from the ratio of their fluorescent intensity to that of the internal standard spots designated as "markers" (*see* Fig. 1). Also *see* **Note 6** for statistical analysis using the array data and the results in Fig. 4.

The following three protocols are optimized for the differential glycan analysis of sialylated MUC1 derived from cell culture supernatants, tissue sections, and body fluids. Quantification of proteins in these types of samples is quite difficult. Although the glycan profiling of sialyl-MUC1 described in this paragraph is not a direct quantitative measurement of the alterations, the signals obtained are dose dependent and enable us to analyze the glycosylation alteration in a quantitative manner using statistics.

3.3 Differential Glycan Analysis Targeting Sialyl-MUC1 from Culture Supernatants of Biliary Tract Cancer Cell Lines

3.3.1 Preparation of Culture Supernatants

1. Prepare RPMI1640 medium supplemented with 5 % FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin for cell culture.
2. Culture 100,000 cells/cell line in 40 mL of the culture medium in a 150 cm^2 flask in 5 % CO_2 at 37 °C.
3. After cells are confluent, wash cells five times with serum-free RPMI1640 medium to completely remove FBS.
4. Culture cells in serum-free RPMI1640 medium for 2 days.
5. Collect the supernatant and filter it through a 0.22 μm filter.
6. Concentrate the supernatant tenfold by ultrafiltration.

3.3.2 Immunoprecipitation of Sialyl-MUC1 with Monoclonal Antibody, MY.1E12

1. Purify MY.1E12 from the culture supernatant of hybridoma cells (*see* **Note 7**) and label using a biotin labeling kit-NH_2.
2. React biotin-labeled MY.1E12 (500 ng) with SA-MB in 20 μL of PBSTx in a reaction mixer set at 4 °C and 1,400 rpm for 1 h.
3. Wash the beads conjugated with biotin-labeled MY.1E12 three times with 200 μL of PBSTx.
4. Dilute 20 μL of the culture supernatant to 40 μL with PBSTx and add to the beads.
5. Incubate the mixture overnight at 4 °C for the reaction to occur.
6. Wash the beads three times with 200 μL of PBSTx and add 10 μL of PBS containing 0.2 % SDS to the beads.
7. Elute the bound material by heat denaturing at 95 °C for 10 min.
8. Collect the supernatant for complete depletion of the contaminating biotinylated MY.1E12 antibody.
9. Add 40 μL of SA-MB to the collected solution.
10. Incubate for 1 h at 4 °C.
11. Collect the supernatant as the immunoprecipitated sample.

3.3.3 Antibody-Overlay Lectin Microarray Analysis Targeting Sialyl-MUC1

1. Dilute the immunoprecipitated sialyl-MUC1 solutions (2.5–20 μL) obtained from KMC cells to 60 μL with PBSTx and apply to the lectin microarray slides (*see* **Note 8**).
2. Place the slides in a humidified incubation chamber and incubate at 20 °C overnight.
3. After incubation, add 2 μL/well of BR and incubate at 20 °C for 30 min.
4. Wash each slide three times with 60 μL of PBSTx, add 100 ng of Cy3-SA in PBSTx and incubate at 20 °C for 25 min.
5. Wash the slide three times with 60 μL of PBSTx and scan with the GlycoStation™ Reader (*see* **Note 9**).
6. Analyze the data with the Array Pro Analyzer.

The dilution curves of sialyl-MUC1 obtained from KMC cell culture supernatants confirm the detection of lectin signals for sialyl-MUC1 in the linear response range using 5 μL supernatants (*see* Fig. 5b).

3.3.4 Differential Glycan Profiling of Sialyl-MUC1 from Biliary Tract Cancer Cell Lines

1. Apply 5 μL of immunoprecipitated sialyl-MUC1 solutions obtained from 20 μL of eight biliary tract cancer cell lines to the lectin microarray slides for antibody-overlay lectin microarray.

Glycan profiling data are obtained in four cell lines as sialyl-MUC1 positive (KMC, TGBC-TKB-1, TGBC-TKB-44, and SZchA-1) (*see* Fig. 5c) and not obtained in four cell lines as sialyl-MUC1 negative (KMCH, TGBC-TKB-2, SkchA-1, and MZchA-2).

3.4 Antibody-Overlay Lectin Microarray Analysis of Sialyl-MUC1 Derived from Surgical Cholangiocarcinoma FFPT Sections

3.4.1 Immunohistochemistry of Surgical Tissue Sections with MY.1E12

1. Deparaffinize the FFPT sections by soaking twice in xylene for 10 min and then soak twice in 100 % EtOH for 10 min.
2. Soak the sections in 95, 90, 80, and 70 % EtOH for 10 min each and wash with PBS.
3. Immerse the sections in 10 mM sodium citric acid buffer (pH 6.0) and autoclave at 110 °C for 10 min for antigen retrieval.
4. Wash the sections with PBS and incubate with MeOH containing 0.3 % H_2O_2 for 15 min to block the endogenous peroxidase.
5. Wash the sections twice with PBS and incubate with PBS containing 2 % normal horse serum at RT for 15 min to block nonspecific binding.
6. Remove the excess blocking solution (*see* **Note 10**) and incubate the sections with the primary antibody solution (biotin-labeled MY.1E12, 0.5 μg/mL in PBS containing 2 % normal horse serum) in a humidified incubation chamber at RT for 1 h.
7. Wash the sections twice with PBS for 3 min and incubate with the secondary reagents (VECTASTAIN ABC reagents) at RT for 30 min in the humidified incubation chamber.
8. Wash the sections three times with PBS and stain with ImmPACT DAB reagents.
9. Wash the sections under tap water flow and incubate with hematoxylin for 20 s to counterstain.
10. Wash the section under warm tap water flow for 10 min and mount with VECTASHIELD mounting medium. A typical immunohistologic image is shown in Fig. 6a.

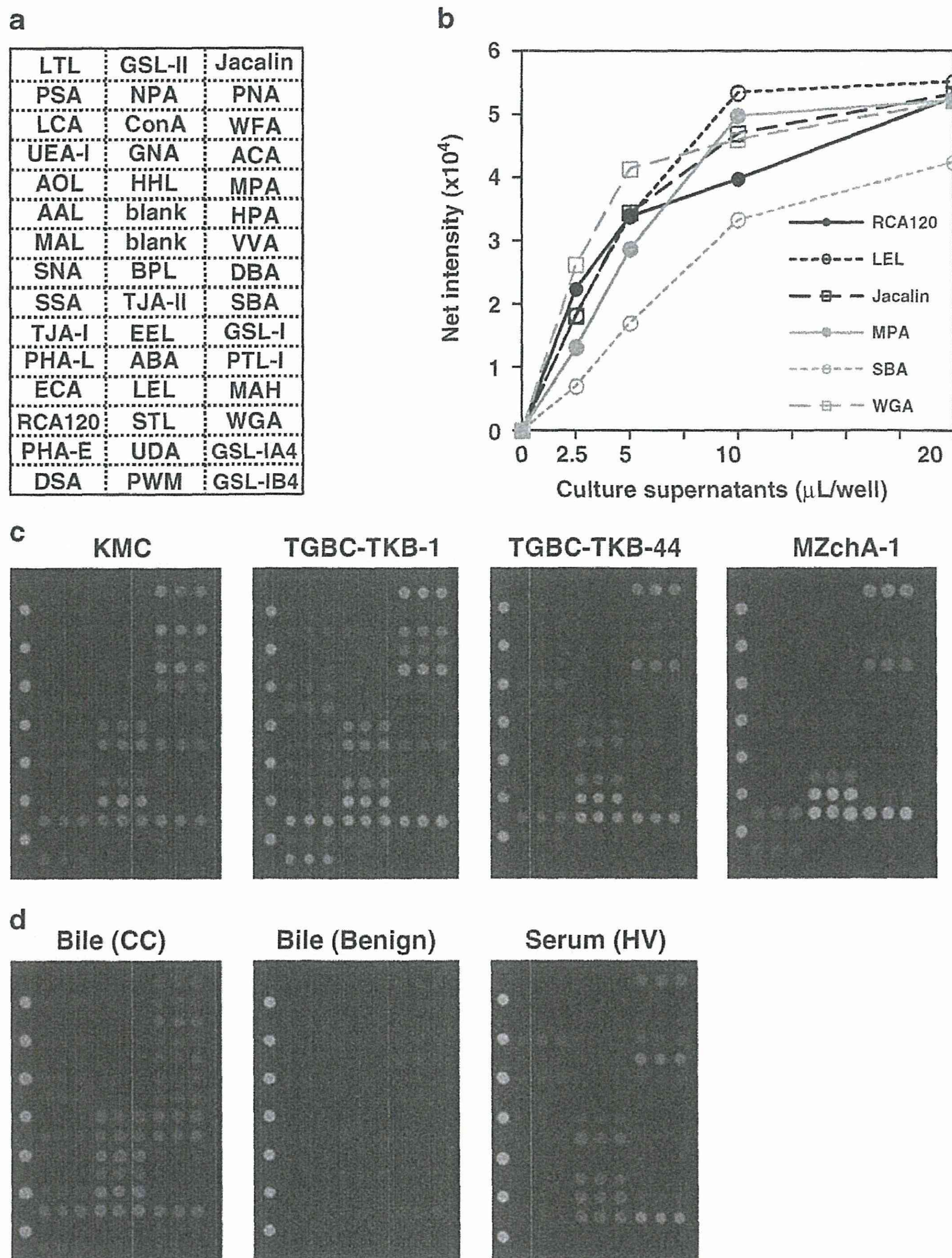

Fig. 5 Antibody-overlay lectin microarray analysis of sialyl-MUC1 obtained from culture supernatants, bile, and serum. (**a**) Array format of in-house lectin array. (**b**) Dose dependency of the antibody-overlay lectin microarray in cell culture supernatants of KMC. KMC cell culture supernatants were applied to the lectin microarray at various volumes (2.5–20 μL/well) to determine the appropriate amount required for a reliable analysis. (**c**) Glycan profiles of the sialyl-MUC1-producing biliary tract cancer cell lines. Immunoprecipitation of sialyl-MUC1 was performed using the same volume of samples. (**d**) Typical profiles of human body fluid specimens (bile and serum). Bile (CC), pooled bile from CC patients; Bile (Benign), pooled bile from patients with hepatolithiasis; serum (HV), serum from a healthy volunteer without any hepatic disease

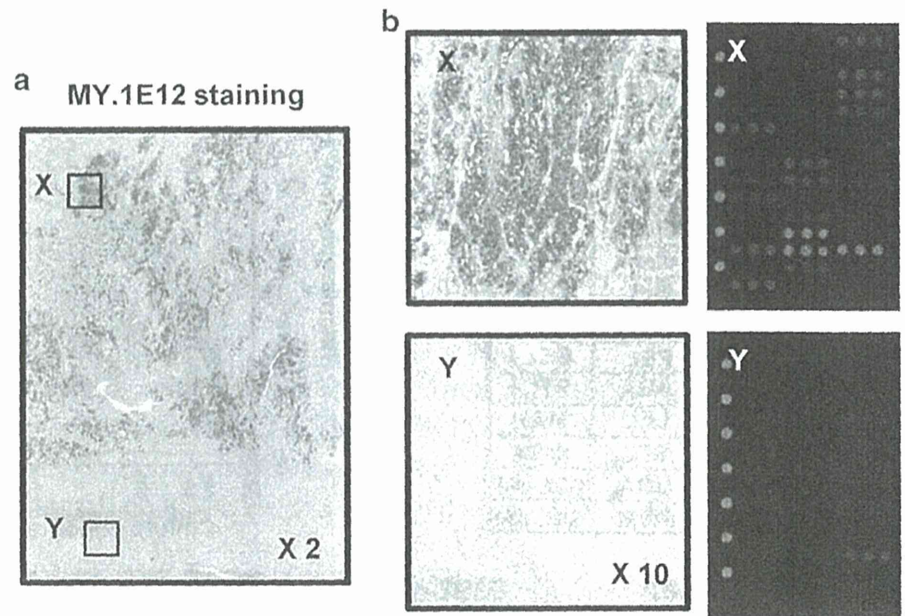

Fig. 6 Antibody-overlay lectin microarray analysis of tissue sections. An antibody-overlay lectin microarray was performed on the glycoproteins extracted from tissue sections. (**a**) Immunohistochemistry with MY.1E12. The lesion stained with MY.1E12 (*X*: MY.1E12-positive area) and unstained lesion (*Y*: MY.1E12-negative area). (**b**) Immunohistochemistry with MY.1E12 and scanned images of antibody-overlay lectin microarray in the MY.1E12-positive and -negative areas

11. For hematoxylin and eosin staining, soak the deparaffinized tissue sections in hematoxylin and incubate at RT for 20 s.
12. Wash the sections under flowing tap water and soak in eosin at RT for 5 min.
13. Wash the sections with tap water (ten passes) and mount with VECTASHIELD mounting medium.

3.4.2 Dissection and Protein Extraction from Tissue Sections

This step is performed as described in previous studies [26, 27].

1. Deparaffinize the FFPT sections as described above.
2. Wash the sections with PBS and visualize under a microscope after hematoxylin staining.
3. Wash the sections with PBS and scratch the tissue fragments from the sections off the slides with a scalpel under a microscope (*see* **Note 11**).
4. Typical staining for the comparative analysis between MY.1E12-positive and -negative tissue areas is shown in Fig. 6a, b.
5. Collect the fragments into a 1.5 mL microtube containing 200 μL of 10 mM sodium citrate buffer acid.
6. Add 4 μL of a cellulose solution (50 % slurry of Avicel) to each suspension as a coprecipitant and incubate at 95 °C for 1 h for antigen retrieval.

7. Centrifuge the tube at 20,000×*g* at 4 °C for 5 min, remove the supernatant and add 200 μL of PBS to the pellet.
8. Centrifuge the tube at 20,000×*g* at 4 °C for 5 min, remove the supernatant and add 20 μL of PBS containing 0.5 % NP-40 to the tube for solubilization.
9. Sonicate the solution gently three times for 10 s and leave the tube on ice for 1 h.
10. Centrifuge the tube at 20,000×*g* at 4 °C for 5 min. The supernatants are used as the protein extraction samples.

3.4.3 Immunoprecipitation of Sialyl-MUC1 from Tissue Lysates with Monoclonal Antibody, MY.1E12

1. Perform this procedure using 20 μL of the protein extraction samples.
2. Refer to the protocol for immunoprecipitation using culture supernatants (*see* Subheading 3.3, **step 2**).
3. The resulting supernatants are designated as the immunoprecipitated samples from tissue lysates.

3.4.4 Glycan Profiling of Sialyl-MUC1 by Antibody-Overlay Lectin Microarray

1. Perform this procedure using the immunoprecipitated samples.
2. Refer to the protocol for immunoprecipitation using culture supernatants (*see* Subheading 3.3, **step 3**).
3. As the amount of the target protein obtained is quite small, the total volume of EF (20 μL) should be applied to each well.

Typical scan data from lectin microarray for the comparative analysis between MY.1E12-positive (X) and -negative (Y) tissue areas in Fig. 6b show specific detection of the glycan profile of sialyl-MUC1.

3.5 Differential Glycan Analysis of Sialyl-MUC1 Derived from Bile and Serum of Normal Controls and Patients with Cholangiocarcinoma and Benign Diseases

1. Immunoprecipitation of sialyl-MUC1 from bile and serum with monoclonal antibody, MY.1E12:
   Based on our previous studies, the outcome will be best with 20 μL of bile or serum specimens. Refer to the protocol for immunoprecipitation using culture supernatants (*see* Subheading 3.3, **step 2**). The resulting supernatants are designated as immunoprecipitated samples from bile and serum.
2. Glycan profiling of sialyl-MUC1 with antibody-overlay lectin microarray:
   Perform it using the immunoprecipitated samples. Refer to the protocol for antibody-overlay lectin microarray using culture supernatants (*see* Subheading 3.3, **step 3**). Dilute 5 μL of immunoprecipitated sialyl-MUC1 with PBSTx to 60 μL and apply to the lectin microarray slides (*see* **Note 12**).

Typical scan data of bile or serum sialyl-MUC1s are shown in Fig. 5d. Unlike the results in serum, although some of the bile samples from benign disease patients show a high level of sialyl-MUC1,

biliary sialyl-MUC1 increases significantly in CC compared with benign bile duct disease [28]. The results for the bile and serum samples represent quantitative alterations and qualitative alterations, respectively.

4 Notes

1. The procedures from pretreatment to lectin array analysis involve very small amounts of target proteins. Use of buffer solutions containing minimal levels of surfactants is effective to reduce physical adsorption into tubes and tips, which results in stable recovery of the target proteins. Particularly when handling heat-denatured samples, it is necessary to keep surfactants in buffers at a certain level to avoid aggregation of proteins.
2. For pretreatment of the SA-MB, when five times the required volume of the beads exceeds 1.5 mL, use a 2.0 mL low-retention tube. When more beads are necessary, divide the beads into multiple tubes, wash, and follow the procedure until twofold-concentrated beads are obtained. Combine the obtained 2× SA-MB before storing. When a Bug Crasher is not available, mix the solutions thoroughly by tapping and inverting the tube and then F-S.
3. Handle the LecChip on a sheet of Kimtex throughout the washing process. As shown in Fig. 7, after removing the solutions from the wells, place the LecChip facedown to absorb excess solution from the wells. Turn the chip face up and immediately add the solution of the following step to prevent wells from drying. A handheld electric pipettor that can dispense a desired amount of solution is useful for efficient processing.

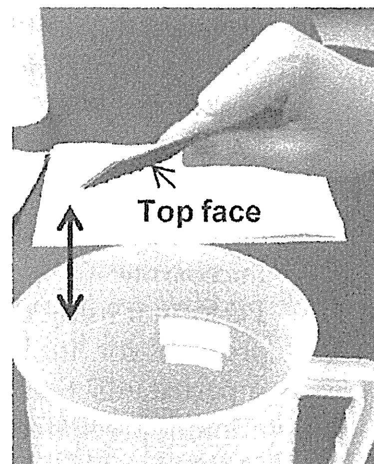

Fig. 7 Efficient procedure for removing solutions from the chip without contamination. Turn the chip facedown and shake three times to remove the excess solution

4. Check the scanned images for undesired stain, etc., on the bottom or sides of LecChips. If any is present, remove it with a Kimwipe soaked with ultrapure water, then dry off the area with a dry Kimwipe. If the stain remains, use 70 % ethanol, followed by ultrapure water, then dry off with a Kimwipe. Do not wipe roughly as the rubber is only lightly covering the chip.
5. When handling serum from HBV- or HCV-infected patients, we inactivate the virus by heat treatment in a P2 room prior to the experiment. For complete inactivation, we dilute the serum tenfold with PBS containing 0.2 % SDS, then heat at 95 °C for 20 min. This treatment may affect the yield of the target protein in the following immunoprecipitation depending on the type of antibodies used.
6. When handling a large number of samples, be aware of the following:
   (a) Prepare the required amounts of biotinylated antibodies, TBSTx, and magnetic beads for the immunoprecipitation of "number of samples + one reserve" prior to the experiment.
   (b) Wash the magnetic beads prior to every use. Do not store the beads after washing or use stored washed beads.
   (c) The number of samples used for one immunoprecipitation should be no more than 22, as handling many samples leads to time gaps within the batch because of washing procedures, etc. The whole process is relatively short, so perform several batches of immunoprecipitation in one day when handling more than 22 samples.
   (d) Use a total of 22 samples including the negative (TBSTx instead of serum) and positive (commercial serum derived from a healthy volunteer) control for each immunoprecipitation.
   (e) Note that signal intensities obtained can vary depending on the scanner conditions (e.g., time from turning on the lamp until use) and even when scanning the same chip at the same gain setting (Fig. 4a). Therefore, data standardization is necessary when comparing a large number of samples (>50) (Fig. 4b).

In addition, as ten LecChip are manufactured and supplied as one lot, inter-lot variation of chips is not negligible when handling more than 70 samples. Therefore, we use aliquots of the same immunoprecipitation EF as the positive control in one well per lot to confirm there is no large deviation within the lot. In our previous studies performed in accordance with this protocol, the positive control was prepared by a single immunoprecipitation of the required amount of the immunoprecipitation EF of a commercial type-A serum. Antibody-overlay lectin microarray was conducted on up to eight chips per day allowing one well per chip for the positive control.