# Focused Differential Glycan Analysis with the Platform Antibody-assisted Lectin Profiling for Glycan-related Biomarker Verification*[S]

Atsushi Kuno‡, Yukinari Kato‡, Atsushi Matsuda, Mika Kato Kaneko, Hiromi Ito, Koh Amano, Yasunori Chiba, Hisashi Narimatsu, and Jun Hirabayashi§

**Protein glycosylation is a critical subject attracting increasing attention in the field of proteomics as it is expected to play a key role in the investigation of histological and diagnostic biomarkers. In this context, an enormous number of glycoproteins have now been nominated as disease-related biomarkers. However, there is no appropriate strategy in the current proteome platform to qualify such marker candidate molecules, which relates their specific expression to particular diseases. Here, we present a new practical system for focused differential glycan analysis in terms of antibody-assisted lectin profiling (ALP). In the developed procedure, (i) a target protein is enriched from clinic samples (*e.g.* tissue extracts, cell supernatants, or sera) by immunoprecipitation with a specific antibody recognizing a core protein moiety; (ii) the target glycoprotein is quantified by immunoblotting using the same antibody used in (i); and (iii) glycosylation difference is analyzed by means of antibody-overlay lectin microarray, an application technique of an emerging glycan profiling microarray. As model glycoproteins having either *N*-linked or *O*-linked glycans, prostate-specific antigen or podoplanin, respectively, were subjected to systematic ALP analysis. As a result, specific signals corresponding to the target glycoprotein glycans were obtained at a sub-picomole level with the aid of specific antibodies, whereby disease-specific or tissue-specific glycosylation changes could be observed in a rapid, reproducible, and high-throughput manner. Thus, the established system should provide a powerful pipeline in support of ongoing efforts in glyco-biomarker discovery.** ***Molecular & Cellular Proteomics 8:99–108, 2009.***

Glycan synthesis in individual cells is regulated by harmonized expression of more than a hundred glycosyltransferases. Importantly, detectable dynamics occurring on each cell surface during diverse biological events, *e.g.* differentiation, proliferation, and signal induction, indicate drastic changes in the glyco-machinery (1). During the last few decades, there have been enormous advances in the findings of glycosylation alterations related to oncogenesis. Cell surface sialylation and $\beta$1–6 branching of *N*-linked glycans are strongly correlated with metastatic potential of cancer cells (2, 3). Metastatic ability is also reflected by dramatic alteration of core structures of *O*-glycans (4). These observations suggest that novel tumor-specific glycoproteins accompanying substantial structural changes in glycan moieties will become reliable biomarkers with higher specificity than those established previously, *e.g.* CA19-9 (5), carcino-embryonic antigen family (6), $\alpha$-fetoprotein (AFP)[1] (7), and prostate-specific antigen (PSA) (8).

Recent advances in technologies such as mass spectrometry (MS), microarray, and laser microdissection have strongly advanced the proteomics-based biomarker discovery phase targeting both cultured cells and tissue specimens (9–14). This has resulted in the emergence of an extensive range of biomarker "candidates", many of which are glycoproteins. However, these candidate molecules need to be subsequently subjected to a verification step prior to a much large scale validation phase (*e.g.* treating with >1,000 incidences). Antibody microarray has taken the place of ELISA as a more versatile and high-throughput technique, and has enabled multiplexed quantitative analysis for over a hundred proteins with sufficient sensitivity (15). As an application of MS technology, multiple-reaction monitoring-MS has also been developed (16–19). However, taking into consideration the critical glycosylation changes occurring on diverse glycoproteins, differential analysis of respective glycans is necessary in parallel with quantitative protein analysis. In this context, Chen *et al.* (20) have recently developed a lectin-overlay antibody microarray with the intention of discovering glycoprotein biomarkers. However, the proposed strategy requires repeated

From the Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Central 2, 1-1-1, Umezono, Ibaraki 305-8568, Japan

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[1] The abbreviations used are: AFP, $\alpha$-fetoprotein; ALP, antibody-assisted lectin profiling; PSA, prostate-specific antigen; $\alpha$PSA, mouse anti-PSA monoclonal antibody; spPSA, seminal plasma PSA; CHO, Chinese hamster ovary; hpIgG, human polyclonal IgG; LEC, lymphatic endothelial cell; MS, mass spectrometry; TBS, Tris-buffered saline; Pod, podoplanin; Sia, sialic acid; hTf, human serum transferrin; Tf, transferrin; PBS, phosphate-buffered saline; ST, sialylated T; diST, disialylated T antigen; PBSTx, 1% Triton X-100 in PBS; AR, androgen receptor; GalNAc, *N*-acetylgalactosamine; Tn, GalNAc$\alpha$-Ser/Thr.

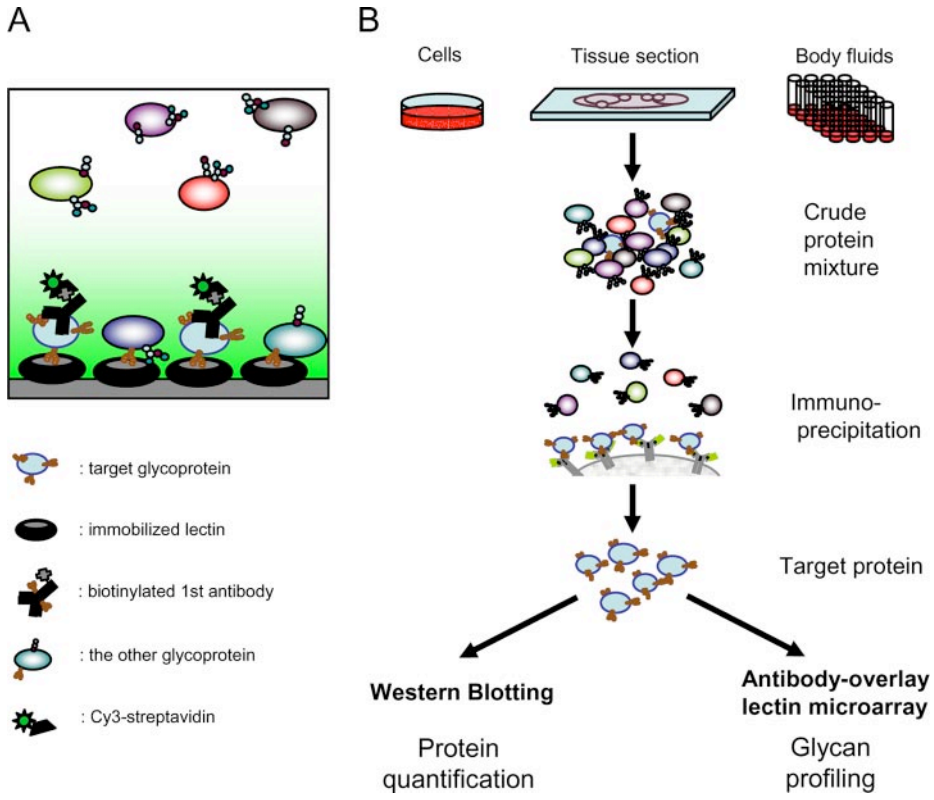



FIG. 1. *A*, alternative approach to fluorescent detection of lectin-glycan interaction. To observe lectin-glycan interactions in terms of fluorescent signals in the lectin microarray, there are two approaches to fluorescence labeling of glycoconjugates, *i.e.* direct and indirect methods. In the former direct labeling method, as it means, appropriate fluorescent reagents (*e.g.* Cy3-SE) are used to label target glyco-conjugates directly prior to a binding reaction. This method enables profiling of a mixture of glycoproteins from whole cell lysate and tissue extract. Therefore, this approach aiming at total glycan profiling is suited for glycomics-based analysis. In the latter case (indirect labeling), a target glycoprotein is detected with the aid of both biotinylated specific antibody, raised against the core protein of the target molecule, and fluorescently labeled streptavidin. *B*, schematic overview of focused differential glycan profiling using antibody-assisted lectin microarray. Over 50% of proteins expressed in cell are glycosylated. A variety of glycoproteins exist in abundance, with different proportions in crude protein samples such as serum and lysates from cells and tissue sections. These would interfere with the lectin-analyte interactions. To profile the glycan structure of an extremely small amount of a target glycoprotein with the aid of antibody-overlay lectin microarray, (i) the protein is enriched from such crude samples; (ii) glycoprotein thus enriched is quantified by Western blotting; and (iii) the residual protein is subjected to antibody-overlay lectin microarray. This sequential procedure is defined as a novel and feasible glyco-biomarker discovery pipeline.

assays with more than 30 lectins to search for critical differences in glycan structures. In the present study, an alternative approach, *i.e.* "antibody-overlay lectin microarray" (21), was taken, which is based on the lectin microarray platform originally developed in our laboratory (22–24). In this strategy, a specific glycan profile of a target glycoprotein is acquired with the aid of a specific antibody raised against the core protein moiety, removing the need to covalently label each glycoprotein with a fluorescent reagent (Refs. 22–29 and Fig. 1*A*). Once a target glycoprotein ($<$100 ng) is enriched from a trace amount of crude samples by a microliter-scale immunoprecipitation procedure with the biotinylated antibody used for the above detection, a precise glycan profile of the target protein is obtained in a highly reproducible and high-throughput manner (see Fig. 1*B*). The system is unique in its ability to distinguish critical differences in both core and terminal structures occurring on *N*- and *O*-glycans attached to the proteins. The combined procedure, consisting of antibody-enrichment, quantification and overlay detection of lectin microarray, is designated antibody-assisted lectin profiling (ALP). The system provides a feasible and powerful advance toward "focused differential glycoproteomics".

## EXPERIMENTAL PROCEDURES

*Reagents, Cell Lines, and Tissue Specimen*—Reagents were purchased from Sigma, Wako Chemicals (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan) unless otherwise noted. Seminal plasma PSA and human serum transferrin (hTf) were obtained from R&D Systems, Inc. (Minneapolis, MN) and Athens Research & Technology (Athens, GA), respectively. Biotinylated mouse anti-PSA monoclonal antibody (bio-$\alpha$PSA) and rabbit anti-hTf polyclonal antibody (bio-$\alpha$hTf) were obtained from R&D Systems, Inc. and Abcam, Inc. (Cambridge, MA), respectively. Chinese hamster ovary (CHO) and prostate cancer cell lines (LNCaP and PCa2b) were obtained from the American Type Culture Collection (ATCC). Fifteen glioblastoma cell lines (LN18, LN215, LN229, LNZ308, LN319, LN340, LN428, LN464, U87, U178,

U251, U373, A1207, SF763, and T98G) were donated by Dr. Webster K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA). Two prostate cancer cell lines (PC3, DU145) were obtained from Tohoku University. Lymphatic endothelial cells (LECs) were purchased from AnioBio (Del Mar, CA). FLAG-tagged hPod-transfected CHO cell line was prepared as described previously (30). These cell lines were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air in RPMI 1640 medium (for CHO, PC3, and DU145) or Dulbecco's modified Eagle's medium (for glioblastoma cell lines, LNCaP and PCa2b) supplemented with 1 or 10% heat-inactivated fetal bovine serum (Sigma), 2 mM L-glutamine (Invitrogen), and antibiotics (100 $\mu$g/ml of kanamycin for CHO, 100 IU/ml of penicillin, and 100 $\mu$g/ml of streptomycin for PC3, DU145, LNCaP, and PCa2b). For a pilot experiment of lectin microarray analysis using tissue sections, glioblastoma cell line LN319 was transplanted into BALB/c nude mice. The grown glioblastoma-like tumor was dissected at 5 $\mu$m for immunohistochemical and lectin microarray analyses. Seminoma specimen was purchased from Cybrdi, Inc. (Frederick, MD).

*Large-scale Purification of hPods*—hPod/CHO and hPod/LN319 were purified as described by Kato *et al.* (31). Briefly, cultured cells ($1 \times 10^{10}$ cells/preparation) were lysed with PBS containing 0.25% Triton X-100. The lysate from CHO was applied on anti-FLAG antibody-conjugated-agarose gel (M2; Sigma). After washing the gel, the captured hPod/CHO was eluted with 100 $\mu$g/ml FLAG peptide (Sigma). To purify hPod/LN319, the cell lysate was applied on NZ-1 antibody-immobilized resin. After washing the gel, the captured hPod/CHO was eluted with hpp38–51 peptide (EGGVAMPGAEDDVV) (31).

*Preparation of Recombinant hPods by a Yeast Genetic Engineering System*—A set of recombinant hPods with defined *O*-glycan structures were prepared as described by Amano *et al.* (32). Briefly, recombinant glutathione *S*-transferase and $His_6$-tagged hPods with Tn and Core1 structures were expressed in TN-1 and T-1 cells, respectively. The hPods purified by affinity chromatography were digested with Precision protease. After removing glutathione *S*-transferase tag fragments, both recombinant hPods were subsequently reacted with appropriate glycosyltransferases to build up STn and Core3 structures on the hPod having Tn and Core2, sialylated T (ST) and disialylated T antigen (diST) structures on the hPod having T (Core1), respectively.

*Immunohistochemical Analysis*—Tissue sections on glass slides were deparaffinized and rehydrated. Testicular tumor specimens were incubated first with NZ-1 for Pod (a rat monoclonal antibody; 1 $\mu$g/ml) at room temperature for 1 h, then with biotin-conjugated secondary anti-rat IgG antibody (Dako, Glostrup, Denmark) for 1 h, and finally with peroxidase-conjugated biotin-streptavidin complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) for 1 h. Color was developed using 3,3′-diaminobenzene tetrahydrochloride tablet sets (Dako) for 3 min. In the case of PSA, prostate cancer or benign prostate hyperplasia specimens were incubated with bio-$\alpha$PSA at room temperature for 1 h.

*Immunoprecipitation*—For analysis of endogenous hPod, each cell line was solubilized with lysis buffer (1% Triton X-100 in PBS (PBSTx)). Immunohistochemically hPod-positive cell populations from tissue sections were excised (about 3.5 $mm^3$) and solubilized with ProteoSOL Tissue Extraction System (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The hPods in these lysates were immunoprecipitated using 10 $\mu$g of NZ-1 antibody immobilized-Sepharose. After washing the resin with 1 ml of PBS, captured hPods were released with 20 $\mu$l of SDS-PAGE sample buffer excluding bromphenol blue, reducing agent and glycerol by heat denaturation. For PSA analysis, PSA was immunoprecipitated from culture supernatant concentrate (20 times) of each cell line using 500 ng of biotinylated anti-PSA monoclonal antibody pre-conjugated to 10 $\mu$l of streptavidin-immobilized magnetic beads, Dynabeads MyOne™ Streptavidin T1 (DYNAL Biotech ASA, Oslo, Norway) conjugate. The beads thus capturing PSA were washed three times with 200 $\mu$l of PBSTx, and then the bound PSA was eluted with 10 $\mu$l of the elution buffer (TBS containing 0.2% SDS) by heat denaturation. After dilution by an equal volume of 1% Triton X-100 in TBS, the contaminant antibody was completely depleted by addition of 20 $\mu$l of the streptavidin-immobilized magnetic beads.

*Western blot Analysis*—The purified hPods were electrophoresed under reducing conditions on 10–20% polyacrylamide gels. The separated proteins were transferred to a polyvinylidene difluoride membrane. After blocking with 4% skim milk in PBS, the membrane was incubated with NZ-1 antibody, then with peroxidase-conjugated anti-rat antibody (1/1000 diluted; GE Healthcare) and developed for 1 min with ECL reagents (GE Healthcare) using Kodak X-Omat AR film. In the case of PSA, the membrane was incubated with biotinylated anti-PSA monoclonal antibody, then with alkaline phosphatase-conjugated streptavidin (1/5000 diluted with TBST; ProZyme, Inc., San Leandoro, CA). The treated membrane was reacted with Western Blue® stabilized substrate for alkaline phosphatase (Promega).

*Antibody-overlay Lectin Microarray*—Antibody-overlay lectin microarray was basically performed as described previously (22–24). Briefly, the purified proteins were diluted to 60 $\mu$l with PBSTx or TBSTx and then applied to the lectin array containing triplicate spots of 43 lectin (see supplemental Fig. S1) into each of 8-divided incubation baths on the epoxy-coated glass slide from Schott AG (Mainz, Germany). After incubation at 20 °C for 12 h, 20 $\mu$g of human serum polyclonal IgG was added to the glass slide followed by 30-min incubation. The reaction solution was discarded, and the glass slide was washed three times with PBSTx. 60 $\mu$l of biotinylated antibody solution in PBSTx was applied to the array and then incubated at 20 °C for 1 h. After washing three times with PBSTx, 60 $\mu$l of Cy3-labeled streptavidin (GE Healthcare) solution in PBSTx was added to the array and then incubated at 20 °C for 30 min. The glass slide was rinsed with PBSTx and scanned by an evanescent-field fluorescence scanner, GlycoStation™ (Moritex, Co., Tokyo, Japan). All data were analyzed with the Array-Pro analyzer version 4.5 (Media Cybernetics, Inc.). The net intensity value for each spot was calculated by subtracting background value from signal intensity values of three spots.

*Glycosidase Treatment*—To remove Sia residues, hPods were incubated with Sialidase A™ from *Arthrobacter ureafaciens* (ProZyme, Inc.) or $\alpha$-2,3-Neuraminidase from *Macrobdella decora* expressed in *Escherichia coli* (ProZyme, Inc.) at 37 °C for 2 h. hPods were also digested with both Sialidase A™ and *O*-Glycanase™ from *Streptococcus pneumoniae* expressed in *E. coli* (ProZyme, Inc.) at 37 °C for 12 h to remove Core1-related *O*-glycans completely.

*Platelet Aggregation Assay by WBA Carna*—Platelet aggregating activity was measured by WBA Carna (IMI, Saitama, Japan) as described previously (31). Heparinized mouse whole blood was drawn from BALB/c mice. Two hundred microliters each of mouse whole blood samples and NZ-1 or control rat IgG in four reaction tubes was stirred at 1,000 rpm at 37 °C, and pre-incubated for 2 min, followed by the addition of 12 $\mu$l each of cells ($2 \times 10^7$ cells/ml). One to five minutes later, whole blood samples were sucked to detect aggregation pressure at a rate of 200 $\mu$l/6.4 s using a syringe containing screen microsieves made of nickel, 3.7 mm in diameter, with 300 openings of $20 \times 20$ $\mu m^2$ in a 1-mm diameter area. The final platelet aggregation pressure of each reaction tube was determined at the pressure rate (%) of a pressure sensor connected to the syringe.

## RESULTS

*Fabrication of Antibody-overlay Lectin Microarray*—To achieve extremely high throughput and feasibility, the antibody-overlay lectin microarray is considered to be an ideal

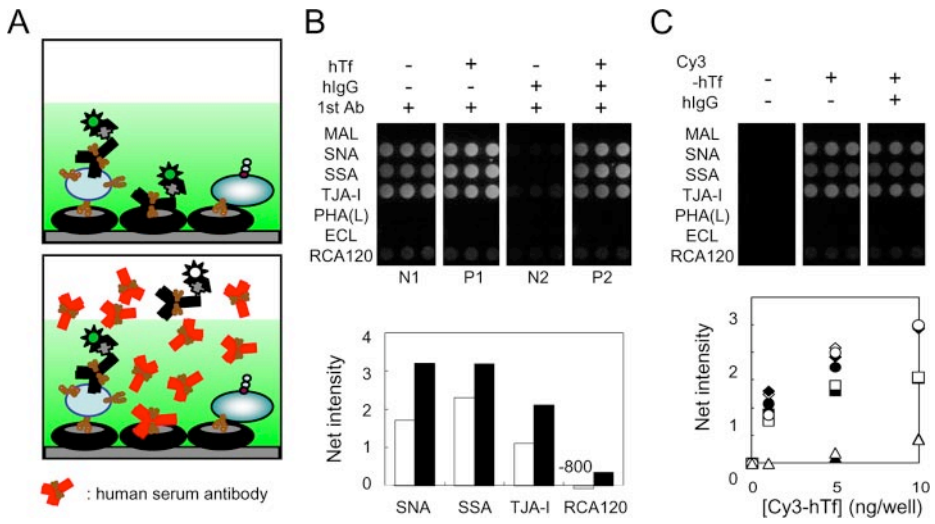



FIG. 2. **Optimization of experimental conditions for antibody-overlay lectin microarray.** *A*, a concept for noise reduction without loss of specific signals for an analyte. Glycans connected to a detection antibody potentially interact with lectins immobilized on the glass slide (*top*). To avoid this, the analyte-bound microarray is blocked with hpIgG prior to incubation with a detection antibody (*bottom*). *B*, effect of the blocking reagent on the background noise reduction because of detection antibody. A scan image of antibody-overlay lectin microarray for hTf (*top*). Positive signal intensities (*P*; in the presence of hTf) are subtracted by negative one (*N*; in the absence of hTf). The resultant intensities with or without blocking treatment are indicated as *closed* or *open bars*, respectively (*bottom*). *C*, lectin microarray analysis of Cy3-labeled hTf in the presence (*black*) or absence (*white*) of hpIgG. Cy3-labeled hTf-bound lectin microarray was incubated with an excess amount of hpIgG for 1 h followed by scanning (*top*). The obtained signals on SNA (●), SSA (■), TJA-I (◆), and RCA120 (▲) are elevated in a dose-dependent manner (*bottom*). No substantial signal because of the blocker hpIgG was observed upon binding of Cy3-labeled hTf.

approach to achieve differential glycan analysis targeting a particular glycoprotein, by using a lectin microarray infrastructure (21). From a practical viewpoint, there is a critical issue to be overcome when using antibodies for glycoprotein detection, since the antibody glycans potentially interact with lectins immobilized on the glass slide (Fig. 2*A*, *top*). To examine this possibility, we first carried out a pilot experiment of the antibody-overlay lectin microarray using hTf, which mainly contained sialylated biantennary, complex-type *N*-glycans at two *N*-glycosylation sites (22), as a model glycoprotein. Lectin microarray comprising 43 lectins (supplemental Fig. S1) was incubated first with or without 1 ng of hTf and then with 20 ng of biotinylated rabbit anti-hTf polyclonal antibody followed by Cy3-streptavidin detection as described under "Experimental Procedures". Signals were observed both in the presence and absence of hTf on extensive spots of $\alpha$2–6 Sia-binders, SSA, SNA, and TJA-I, as well as a $\beta$-Gal-binder, RCA120 (Fig. 2*B*). This indicates a typical glycan profile to serum antibody, *i.e.* partially $\alpha$2–6 sialylated biantennary complex type *N*-glycan. In fact, positive signal intensities (*P*) (in the presence of hTf) were almost comparable with those of negative ones (*N*) (in the absence of hTf), indicating the derived signals were not reliable. To suppress such undesirable background noise, attributed to the detection antibody, while preserving specific signals of hTf, effective blocking of the analyte (hTf)-bound microarray was attempted using an excess amount of non-labeled "human" polyclonal IgG (hpIgG) prior to detection by anti-hTf antibody (Fig. 2*A*, *bottom*). The blocker, hpIgG, having substantially the same glycoforms as those of the detection antibody ("rabbit" polyclonal antibody in this case), but lacking the epitope (biotin) for fluorescence detection, is expected to work for masking the residual binding sites on the array. Technical consideration was also taken to minimize the blocking time (30 min) to suppress the analyte exchange between the pre-captured analyte (hTf) and an excess amount of the blocker hpIgGs. However, no detectable signal reduction was observed even after 2 hours of incubation with the blocker hpIgG, as examined by direct probing of the array with Cy3-labeled hTf (Fig. 2*C*). As a result of blocking with 20 $\mu$g of hpIgG, the background signals totally disappeared in the absence of hTf, whereas intense (*i.e.* specific) signals were observed, *e.g.* on RCA120, in the presence of the analyte (Fig. 2*B*). The obtained signal pattern was closely correlated with the reported glycan structures of hTf (21).

*Focused Differential Glycan Profiling of an N-glycosylated Protein, PSA*—To profile a relatively small amount ($<$100 ng) of a target glycoprotein with the aid of antibody-overlay lectin microarray, the target should be enriched in advance from crude samples. Here, a crucial point is how ALP is designed to realize an effective procedure for the verification of glyco-biomarker candidates. In our development concept, a single antibody plays the following three roles; (i) prior enrichment by immunoprecipitation; (ii) quantification by Western blotting; and (iii) glycan profiling by antibody-overlay lectin microarray (Fig. 1*B*). Based on these criteria, we attempted differential glycan profiling of prostate-specific antigen (PSA), which is known to be specifically expressed in AR-positive prostate cancer cells, and has a

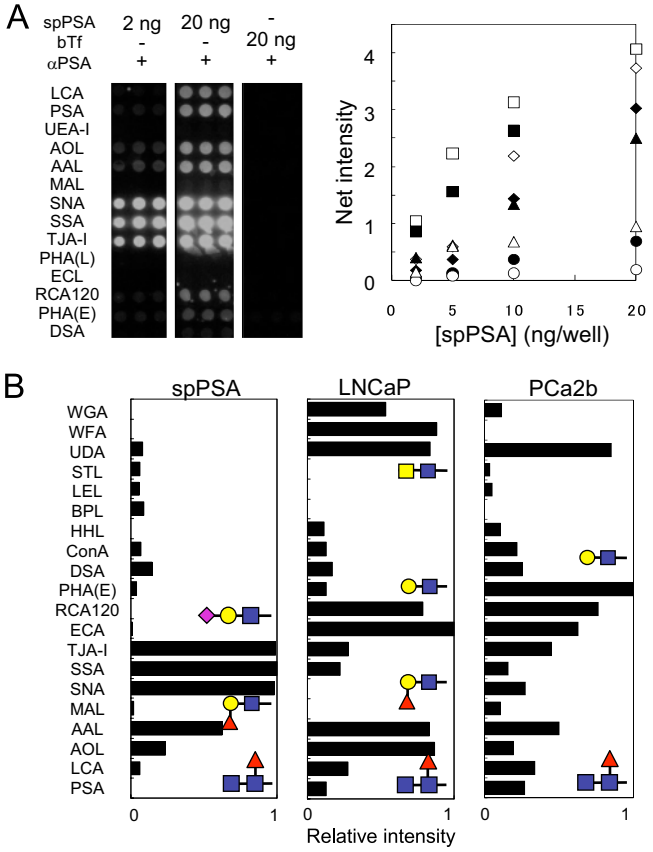



FIG. 3. **Focused differential glycan profiling of PSA.** *A*, dose-dependent fluorescent signals of the spPSA for 14 lectins (*left*). No signal was obtained for bovine serum transferrin (bTf) as a negative control. The binding isotherms of interaction of eight positive lectins with spPSA (*right*) are shown except for SNA, SSA, and TJA-I; AAL (□), AOL (■), LCA (◇), *Pisum sativum* agglutinin (◆), PHA(E) (△), RCA120 (▲), PHA(L) (○), and MAL (●), respectively. *B*, differential glycan profiling among PSAs from different origin. The microarray was incubated with PSAs enriched from seminal plasma and culture supernatants of LNCaP and PCa2b cells. Relative intensities of 20 lectins (*i.e.* maximal fluorescent intensity defined as 1 on each lectin array) are indicated as *horizontal bars*. Typical glycan epitopes (structural units) relevant to the present analysis are also indicated by the Consortium for Functional Glycomics format.

single *N*-glycosylation site (33, 34). Firstly, various concentrations (2∼20 ng/60 μl) of normal seminal plasma PSA (spPSA) were applied to the lectin microarray followed by hpIgG blocking as described, and detection with 50 ng of biotinylated mouse anti-PSA monoclonal antibody (bio-αPSA). As shown in Fig. 3*A*, specific signals were obtained in a dose-dependent manner, whereas essentially no signal was obtained for bovine serum transferrin as a negative control. The resultant glycan profile of spPSA provides the following information: (i) modification of biantennary *N*-glycan with Core α1–6 fucosylation as evident in signals on LCA, *Pisum sativum* agglutinin, AOL, and AAL; (ii) heavy α2–6 sialylation associated with strong signals on SNA, SSA, and TJA-I; and (iii) partial α2–3 sialylation attributed to a weak signal on MAL. All data are consistent with a recent analysis by high performance liquid chromatography and MS (34).

Glycan analysis was then performed with the optimized procedure toward PSA enriched from the culture supernatant of a prostate cancer cell line, LNCaP. Following 20-fold concentration of the supernatant by ultrafiltration, PSA secreted from LNCaP (designated hereafter PSA/LNCaP) was immunoprecipitated from 20 μl of the concentrate with 500 ng of bio-αPSA, which had previously been conjugated with 10 μl of streptavidin-coated magnetic beads. After extensive washing, the captured PSA was released by heat treatment in the presence of 0.2% SDS, and the denatured bio-αPSA co-eluted with PSA was selectively recaptured with 20 μl of the streptavidin-coated beads. Thus enriched PSA was quantified by Western blotting to yield about 20 ng (1 ng/μl). Subsequently, the residual fraction of enriched PSA (5 ng) was subjected to antibody-overlay lectin microarray using the same bio-αPSA. Intense signals were observed on some lectin spots. No substantial signal was obtained by direct probing (*i.e.* without enrichment) the microarray even with 20-fold concentrated crude culture supernatant (supplemental Fig. S2). Although a residual amount of bio-αPSA (*i.e.* 10 ng or 2% of the input antibody estimated by Western analysis) actually contaminated the analyte, it did not cause substantial background noise nor interference with the specific signals. PSA/LNCaP showed notable differences compared with spPSA described above (Fig. 3*B* and supplemental Fig. S2). (i) Drastic reduction in sialylation signals on both α2–6 Sia-binders, SNA, SSA, and TJA-I and an α2–3 Sia-binder, MAL. (ii) Complementary increment of terminal Lac/LacdiNAc structures associated with signals on RCA120, ECA, WGA and WFA. (iii) A higher terminal fucose content represented by Lewis antigens (Fucα1–3Gal or Fucα1–4Gal) and H-antigen (Fucα1–2Gal) evident on AAL and AOL signals. These structural features correlate well with results in previous reports (33, 34). Thus, the developed system allows differential glycan profiling of a target glycoprotein even in a small quantity contained in a cultured supernatant.

To extend this approach, three more human prostate cancer cell lines; AR-positive MDA PCa2b, AR-negative PC3 and DU145, were analyzed. Twenty-microliter of each concentrated culture supernatant was used for enrichment as described. Western blot quantification revealed that PCa2b gave ∼3 times lower amount (*i.e.* ∼0.33 ng PSA/μl of elution fraction) of the analyte (PSA) compared with LNCaP. Thus, 15 μl of the elution fraction of PSA/PCa2b (5 ng) was applied to the lectin microarray (supplemental Fig. S3). It was found that the signal pattern of PSA/PCa2b was clearly different from those of spPSA and PSA/LNCaP (Fig. 3*B*). Although there has been no report on glycan structures of PSA/PCa2b, notable features of PSA/PCa2b can be documented as regards comparison with psPSA: (i) drastic decrease in α2–6 sialylation as observed in PSA/LNCaP; (ii) corresponding increment of ter-

minal α2–3 sialylation and LacNAc structures evident by signals on an α2–3 Sia-binder, MAL, and LacNAc-binders, RCA120 and ECA, respectively; and (iii) higher frequency of core-fucosylation on biantennary complex type *N*-glycans revealed by signals on LCA and *Pisum sativum* agglutinin. These signals also imply an increase in high-mannose structures together with other mannose-binders, UDA, HHL and ConA. On the other hand, when compared with the previous cell line, LNCaP; (i) drastic reduction in signals on WGA as well as WFA (both having affinity to *N*-acetyl group); and in contrast (ii) dramatic increase in PHA(E) signal suggesting the presence of bisecting GlcNAc are depicted. Throughout the analysis, α2–6 de-sialylation of *N*-glycan is a consistently evident feature shared by the AR-positive prostate cancer cell lines (LNCaP and PCa2b). However, their profiles did not necessarily show particular similarity to the one reported recently for serum PSA derived from prostate cancer (PC) patients, except for α2–3 Sia increment. It should be mentioned, however, that serum PSA level is abnormally high (>10 μg/ml serum) in these patients (35). From a clinical viewpoint, it is important to differentiate PSA glycoforms of malignant prostate cancer from benign prostate hyperplasia. With the ultrasensitivity and preciseness of the developed system, it will be possible to achieve differential profiling using patient sera containing even a small amount (<100 ng/ml serum) of PSA.

*Focused Differential Glycan Profiling of a Mucin-like Glycoprotein, Podoplanin*—Glycosylation change in mucin has also been a potential target for a glyco-biomarker in the context of tumor progression and metastatic potential, particularly because it represents a major component of epithelia, the major origin of tumorigenesis. Human platelet-aggregating factor podoplanin (hPod) was chosen as a model mucin-like glycoprotein from both practical and biological points of view. hPod is expressed in many tumor cells, such as testicular germ cell tumors, brain tumors, several squamous cell carcinomas, and malignant mesothelioma (31, 36–39), while it is also known as a lymphatic endothelium marker (40). Recent structural analysis of glioblastoma-derived hPod demonstrated that it mainly bears fully sialylated Core1, *i.e.* diST (30, 31, 41). More recently, we produced recombinant hPods by a yeast genetic engineering system, which have homogenous *O*-glycan structures (32). In this study, we prepared a set of recombinant hPods with defined *O*-glycan structures, *i.e.* Tn, T (Core1), Core2, Core3, sialylated Tn (STn), ST, or diST. As a result of antibody-overlay lectin microarray analysis with the aid of an established monoclonal antibody, NZ-1 (31), the seven recombinant glycoproteins (each 10 ng) gave totally different signal patterns from one another, whereas Core1 and its structural derivatives were in part contaminated with Tn. The results of ten lectins showing critical difference are summarized in Fig. 4, where a unique representation format allows us to follow structural changes easily (for details, see legend to Fig. 4). For instance, 6-OH modification of the reducing terminal GalNAc is rejected by a Core1/GalNAc-binder, Jacalin

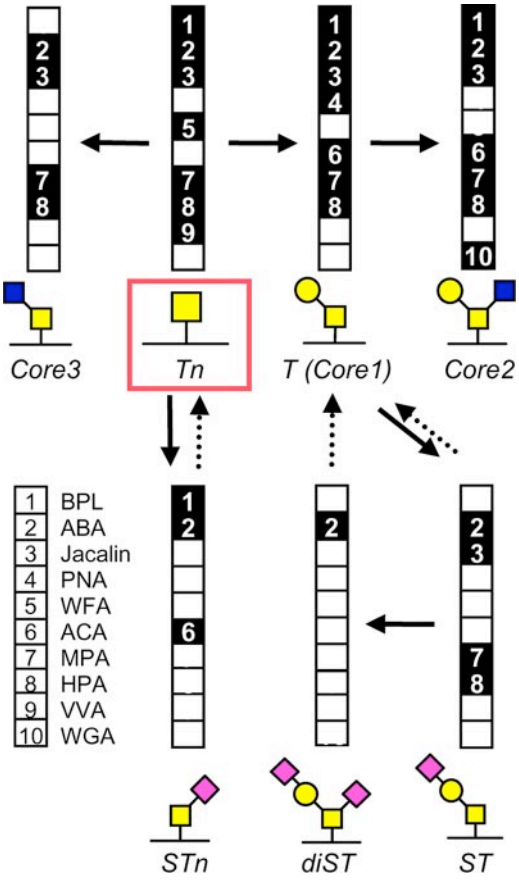



FIG. 4. **Focused differential glycan profiling of podoplanins (Pods).** Relation of signal patterns of ten lectins with alterations of *O*-glycan structure in hPod. hPod with a specific *O*-glycan, *i.e.* either Tn or T (Core1), was over-expressed in yeast as described previously (32). Both recombinant hPods were subsequently reacted with appropriate glycosyltransferases (each step was indicated by an arrow) to build up Core3 and STn structures on the hPod having Tn, or Core2, ST, and diST structures on the hPod having T (Core1), respectively. Glycan changes by Sialidase A™ treatment were noted by dashed arrows. *O*-glycan structures are shown by the Consortium for Functional Glycomics format. Each of the engineered hPods was analyzed by antibody-overlay lectin microarray with biotinylated NZ-1 antibody. The binding signals of the seven analytes observed on selected ten lectin spots are shown as a patterning diagram with ten-divided columns: positive and negative interactions are indicated as *black* and *white boxes*, respectively. For instance, Jacalin (column 3) cannot bind to STn and diST, of which positions are colored in white. Thus, Jacalin rejects 6-OH modification of reducing terminal GalNAc residue. In other words, Jacalin works as a "zone-discriminator" regarding 6-OH modification, showing no binding to modified structures. On the other hands, PNA (column 4) works as an "epitope detector", indicating the presence of Core 1 (T). ABA can react with all of the *O*-glycan structures relevant to this study. Core3 and ST structures, which show identical pattern diagrams to each other, can be clearly differentiated after Sialidase A™ treatment, which specifically works on ST.

(42), while it is accepted by another Core1-binder, ACA. It is known that difference in sialylation frequency affects the platelet-aggregating activity of hPod (31) (also see Fig. 5*A*). This fact is easily traced with the combination of five lectins,

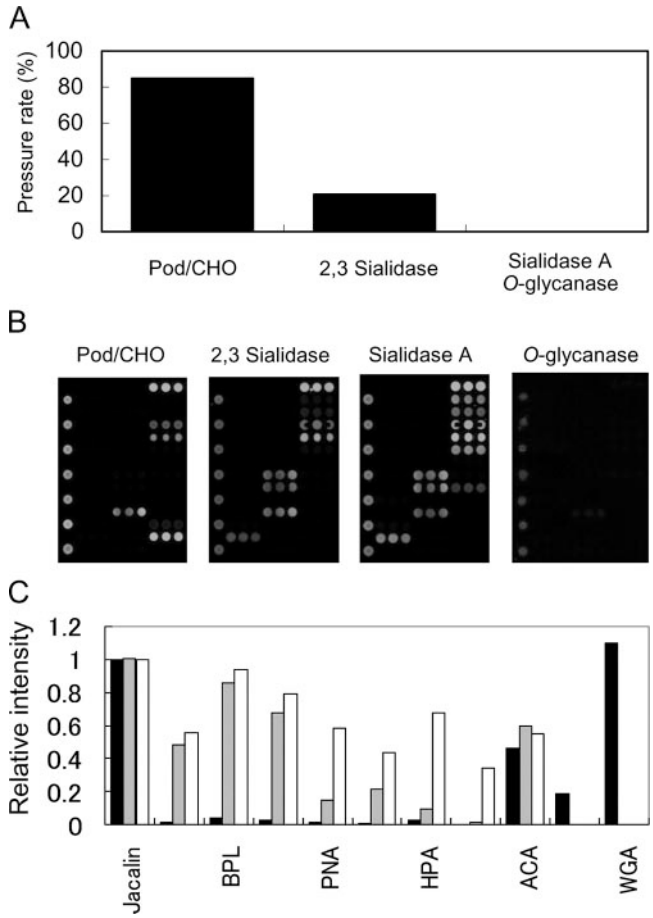



FIG. 5. **Effect of sialylation of hPod on its platelet aggregating activity.** A sialylated mucin-like glycoprotein hPod/CHO was purified as described previously (30), then reacted with Sialidase A™ or α2,3-Neuraminidase to obtain desilalylated hPods with different degree of sialylation. The platelet aggregating activity of each hPod was evaluated as described under "Experimental Procedures" (*A*). The obtained hPods, together with hPod/CHO deglycosylated by *O*-glycanase, were qualified by antibody-overlay lectin microarray as described under "Experimental Procedures" (*B*). Relative intensities of eleven lectins with hPod/CHO derivatives determined from the ratio to the fluorescent intensity on Jacalin are indicated with *black* (intact), *gray* (with α2,3-Neuraminidase), and *white bars* (with Sialidase A™), respectively.

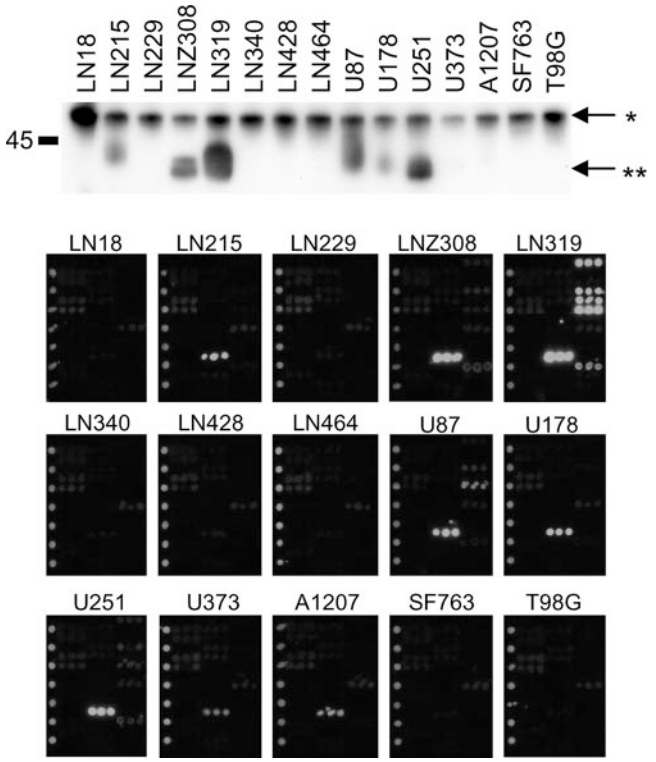
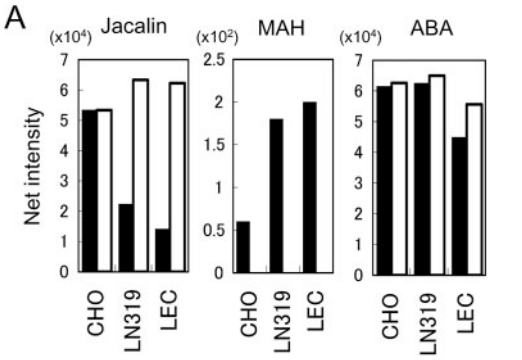



FIG. 6. **Differential analysis of fifteen kinds of glioblastoma cells.** Every cell line was cultured, harvested and lysed with PBS containing 0.25% Triton X-100. Each lysate was incubated with NZ-1 antibody immobilized resin and captured hPods were eluted in SDS-PAGE sample buffer with heating denature. The obtained hPods were quantified by Western blotting (*top*), and subjected to antibody-overlay lectin microarray (*bottom*). *Asterisks* (* and **) denote the bands corresponding to heavy chain of Nz-1 antibody and hPod, respectively. Evidently the signal intensity of each cell line was correlated well with the expression level of hPod revealed by Western analysis.

*i.e.* Jacalin, BPL, PNA, MAH and WGA, where their fluorescence intensities are normalized relative to Jacalin (also see Fig. 5, *B* and *C*).

To prove the ultra-sensitivity of ALP from a more practical viewpoint, we extended the approach to LEC, because glycosylation features of hPod/LEC have never been characterized because of its low natural abundance. hPod/LEC was immunoprecipitated from 1-dish cultured cells (~5 × $10^6$) with 10 μl of NZ-1-Sepharose, and the obtained glycan profile was compared with those of both recombinant hPod over-expressed in CHO cells (hPod/CHO) and endogenous hPod from human glioblastoma LN319 cell line (hPod/LN319), the latter being the most over-expressing cell line of glioblastoma (see Refs. 30, 31 and Fig. 6). Notably, glycan profile of the hPod/CHO thus enriched differed from that of hPod/CHO obtained by a large scale preparation described above. This is probably because of the difference in elution conditions: *i.e.* in the former procedure, the bound antigen was eluted by heat denaturation in the presence of SDS, while in the latter, it was eluted more mildly by adding a competitive peptide having affinity to immobilized Nz-1 antibody. This may affect the binding pattern on less stable lectins, *e.g.* WGA. Nevertheless, we confirmed that the resultant signal patterns of desialylated forms of the three hPod preparations were closely similar to one another (supplemental Fig. S4*A*). However, when untreated hPods were compared, a glycan profile of hPod/LEC considerably differed from hPod/CHO, while it closely resembled that of hPod/LN319 (supplemental Fig. S4*A*). The observed differences are interpreted as higher frequency of α2–6 sialylation at the reducing terminal GalNAc, which is evident from both increment of the MAH signals and complementary decrement of the Jacalin signals (Figs. 4 and 7).




B

| | Median (SD) | | *P*-value |
|---|---|---|---|
| *n*=4 | CHO | LEC | |
| Jacalin/ABA | 0.61 (0.029) | 0.17 (0.016) | <0.001 |
| MAH/ABA | 0.032 (0.012) | 0.076 (0.010) | 0.0017 |

FIG. 7. **Differential glycan profiling among hPod/CHO, hPod/LN319, and hPod/LEC.** The three hPods were analyzed as their sialylated and desialylated forms (for details, see Fig. S4). Net intensities of three representative lectins (Jacalin, MAH, and ABA) with sialylated and desialylated forms are indicated with *close* (sialidase minus) and *open bars* (sialidase plus), respectively (*A*). Regarding hPod/CHO and hPod/LEC, differences in the signal ratios of the relevant lectins, *i.e.* Jacalin/ABA and MAH/ABA, were found to be statistically significant based on the Student's *t* test ($p < 0.01$) in the microarray analyses ($n = 4$) (*B*).

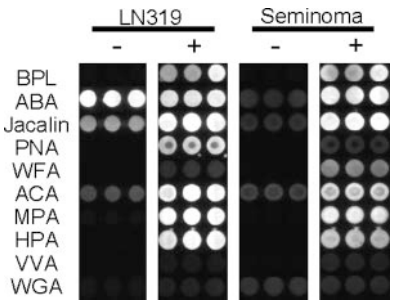



FIG. 8. **Challenge to glycan profiling of hPod enriched from a tissue lysate.** Podoplanin-positive area on the counterpart sections was selectively dissected (excised volume estimated to be ~3.6 $mm^3$). hPod was enriched with NZ-1-immobilized-Sepharose and was analyzed by antibody-overlay lectin microarray. For comparison, hPod enriched from tumor section of LN319 cell-transplanted mice was also analyzed as a positive control. Desilalylated hPods were also subjected to antibody-overlay lectin microarray.

As mentioned above, expression of endogenous hPod has been found histologically in an extensive range of tumors. A comprehensive glycan analysis of hPod from various types of tumor cells should give a direct answer to whether or not the sialylated *O*-glycans are actually involved in platelet aggregating-activity of hPod, which might lead to enhanced metastatic potential. It was therefore decided to apply the developed ALP system to hPod extracted from a relevant tissue section. For this purpose, hPod-positive seminoma cells (~3.6 $mm^3$) were scratched from a deparaffinized and formalin-fixed testicular tissue section followed by extraction of membrane proteins. After immunoprecipitation as described, a 1/10 aliquot of the enriched protein was subjected to antibody-overlay lectin microarray to obtain a unique signal pattern to hPod derived from seminoma (supplemental Fig. S5). Although significant signals were observed on ABA and Jacalin in the desialylated form of hPod/seminoma as well as xenograft LN319, intact hPod showed strong signals on ACA and WGA. Since signals on both ABA and Jacalin were greatly reduced, it strongly implies a high degree of 6-OH modification of the reducing terminal GalNAc residue of Core1, *e.g.* diST (Fig. 8). Notably, the WGA signal remained even after desialylation (Figs. 4 and 8). By comparison with the patterning diagrams obtained for the engineered hPods (Fig. 4), where WGA signal was specifically observed in Core2-hPod probe, the Core2 structure is the most likely glycoform expressed on hPod/seminoma. Furthermore, the binding signals on terminal GalNAc binders, WFA and VVA (see *pattern* diagrams of STn and Tn in Fig. 4), rather than that on a Core1-binder, PNA, were specifically observed in desialylated hPod/seminoma. These results strongly suggest that hPod/seminoma expresses STn structure, *i.e.* the 6-OH sialylated form of an immature Core1 (Tn), as well as diST and Core2. This structural diversity of hPod/seminoma is considered to represent the heterogeneous nature of the tissue specimens. It should be emphasized that such information has never been obtained by conventional procedures requiring a much larger amount of pure samples. The developed system ALP therefore constitutes a great practical advance toward the realization of ultra-sensitive, highly precise glycan profiling of marker candidate glycoproteins.

## DISCUSSION

Glycosylation alteration occurring on glycoproteins has often been discussed in relation to oncogenesis and tumor progression and provides potential targets for serological protein biomarker development. Recent advances in glycoproteomics produced a number of glycoproteins, which have been nominated as disease-related biomarker candidates (14, 43–47). As a standard procedure employed in concurrent proteomics-based biomarker development, the candidates are first quantified with the samples used in the candidate identification phase, then verified via a hundred clinical samples before the last validation phase (10). In general, serum concentrations of diagnostic glycoproteins are too low to detect their glycosylation changes in an unambiguous manner. In the case of hepatocellular carcinoma (HCC), the presence of >35% AFP-L3 fraction, *i.e.* LCA-reactive AFP, among the total AFP glycoforms, has been established as a diagnostic and prognostic marker with 100% specificity (7). Nevertheless, for this diagnosis, total AFP values in patient serum are in the range 10–200 ng/ml (48). Other glycosylation alterations include a serological prostate cancer marker, PSA, for which a gray zone

concentration to distinguish prostate cancer from benign prostate hyperplasia resides between 4 and 10 ng/ml in serum. A reliable technology, from sample preparation to analysis, enabling a nanogram-scale analysis is urgently required for these low-abundance target molecules. To improve the present diagnostic approach, ALP is considered to be the best candidate as a verification system. Its feasibility depends on dual focusing of analytes in crude glycoprotein solution with antibody, *i.e.* nanogram-scale immunoprecipitation and subsequent antibody-overlay detection, which has proven to be an ultrasensitive system for "focused differential glycan analysis".

Technical points to be refined in the above profiling system are sensitivity and noise reduction because of the antibodies used. In fact, previous investigators attempting a similar approach used up to a microgram order of glycoproteins, which severely restricted an analytical range to relatively abundant target proteins (21). Our recently improved lectin microarray system assures ultra-high sensitive analysis, *i.e.* a nanogram order of glycoproteins (24). The problem of background noise was solved by an extremely simple masking procedure; *i.e.* using non-labeled heterospecies IgG. Successful analysis of endogenous glycoproteins, PSA and Pod, representing *N*- and *O*-glycoproteins, was demonstrated using a small amount of sample comparable with Western blotting analysis. This significant improvement will help to make the developed ALP system widespread over the relevant fields including molecular, cellular, and tissue biologies, as well as clinical sciences.

In conclusion, a highly feasible system, ALP, has been proposed as the best-focused differential glycan profiling technology. With this system, a glyco-biomarker discovery pipeline targeting proteomics-based biomarker discovery as described by Rifai *et al.* (10) will be rapidly established. Protocols for nanogram-scale verification have been constructed for over twenty kinds of glycoprotein marker candidates toward the forthcoming validation phase[2], where in turn an immune-based sandwich assay should work better, *i.e.* lectin-overlay antibody microarray (20). For realization of this, selection of appropriate lectin probes is critical to differentiate disease-specific glycan alterations. Both microarray technologies are necessary and should be combined to achieve rapid glyco-biomarker development in the near future.

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[2] A. Kuno, H. Narimatsu, and J. Hirabayashi, unpublished results.

[S] The on-line version of this article (available at http://www.mcp.org) contains Supplemental Figs. S1–S5. All lectin abbreviations are given in Supplemental Table 1 and Supplemental Fig. S1.

‡ Both authors contributed equally to this study.

§ To whom correspondence should be addressed: Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), AIST Tsukuba Central 2, 1-1-1 Umezono, Ibaraki 305-8568, Japan. Ph.: 81-29-861-3124; Fax: 81-29-861-3125. E-mail: jun-hirabayashi@aist.go.jp.

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