Table 1. Prevalence of HBV genotypes in patients with acute hepatitis B during four chronological periods

Period	Genotype A	Genotype B	Genotype C	Others
1994-1998	11[a]	3	18	0
(n=32)	(34.4%)	(9.3%)	(56.3%)	
1994-1998	14[b]	4	20	0
(n=38)	(36.8%)	(10.5%)	(52.7%)	
1994-1998	28[c]	6	19	1
(n=54)	(51.9%)	(11.1%)	(35.1%)	(1.9%)
1994-1998	60 [a, b, c]	13	16	2
(n=91)	(65.9%)	(14.3%)	(17.6%)	(2.2%)
Total	113	26	73	3
(n=215)	(52.5%)	(12.0%)	(34.0%)	(1.5%)

[a]p=.0032

[b]p=.0014

[c]p=.02

Table 2. Baseline Characteristics and the Duration of HBsAg in Patients with Acute Hepatitis B with Different HBV Genotypes

Features	HBV Genotypes					
	A	**B**	**C**	**D**	**E**	**F**
	(n = 113)	(n = 26)	(n = 73)	(n = 1)	(n = 1)	(n = 1)
Age (years)	30.8 ± 9.5	32.3 ± 9.5	33.3 ± 10.9	27	26	58
Male	106 (93.8%)[a]	21 (80.7%)[b]	29 (39.7%)[a, b]	0	0	1 (100%)
Transmission routes Identified	102 (90.2%)	21 (80.8%)	53 (72.6%)	1 (100%)	1 (100%)	1 (100%)
Heterosexual	70 (68.6%)	19 (90.4%)	47 (88.7%)	1 (100%)	1 (100%)	1 (100%)
MSM	32 (31.4%)[c, d]	1 (4.8%)[c]	6 (11.3%)[d]	0	0	0
ALT (IU/L)	2,126 ± 938[e*]	2,394 ± 820	2,857 ± 1,668[e]	4,180	1,175	1,533
Bilirubin (mg/mL)	7.1 ± 6.4[f*]	4.8 ± 3.3[f, g]	9.0 ± 7.5[g]	6.8	3.9	3.5
HBV DNA (log copies/mL)	6.3 ± 1.7[h*]	5.5 ± 2.3	4.9 ± 1.5[h]	5.2	7.4	4.8
HBeAg	95/121 (77.3%)[i*]	24/28 (88.5%)	37/58 (65.5%)[i]	1/1 (100%)	1/1 (100%)	1/1 (100%)
Anti-HIV	7/72 (9.7%)	0/7 (0%)	0/23 (0%)	Not tested	0/1 (0%)	Not tested
Duration of HBsAg* Group (months)						
I (< 3)	35 (42.2%)	16 (64.0%)	31 (64.6%)	0	1	1
II (3–6)	34 (41.0%)	8 (32.0%)	11 (22.9%)	1	0	0
III (> 6 –12)	9 (10.8%)	0	6 (12.5%)	0	0	0
IV (> 12)	5 (6.0%)	1 (4.0%)	0	0	0	0

Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus type-1; MSM, men having sex with men

[a]$P < .001.$

[b]$P < .001.$

[c]$P = .017.$

[d]$P = .002.$

[e]$P = .002.$

[f]$P = .003.$

[g]$P < .001.$

[h]$P < .001.$

[i]$P = .036.$

[*]Data from anti-HIV-positive patients are excluded.

Legends to the Figures

Fig. 1. Evolutionary relationships of 86 HBV/A taxa, including 20 from the present cases. The evolutionary history, inferred using the Neighbor-Joining method, shows that all the 20 samples had similar nucleotide sequences close to previously reported genotype A2 sequences from Western countries.

Fig. 2. Levels of HBsAg in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B of genotype A *, $P < .05$; **, $P < .01$; and ***, $P < .001$.

Fig. 3. Levels of HBV DNA in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B of genotype A. *, $P < .05$; **, $P < .01$; and ***, $P < .001$.
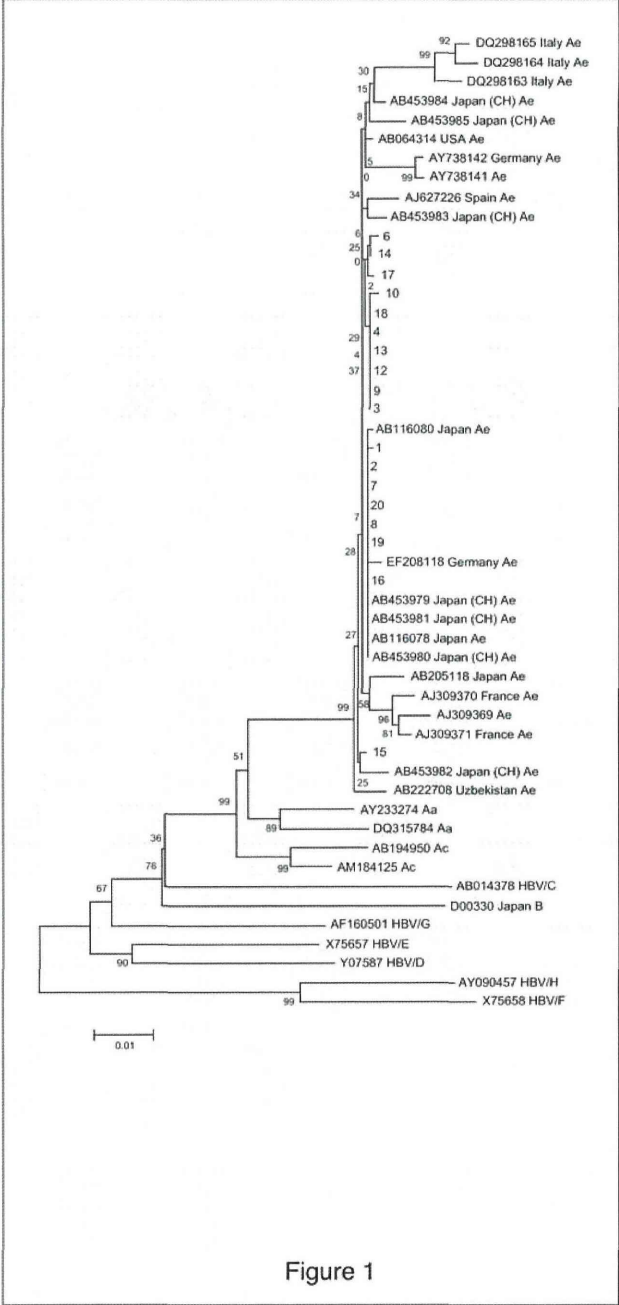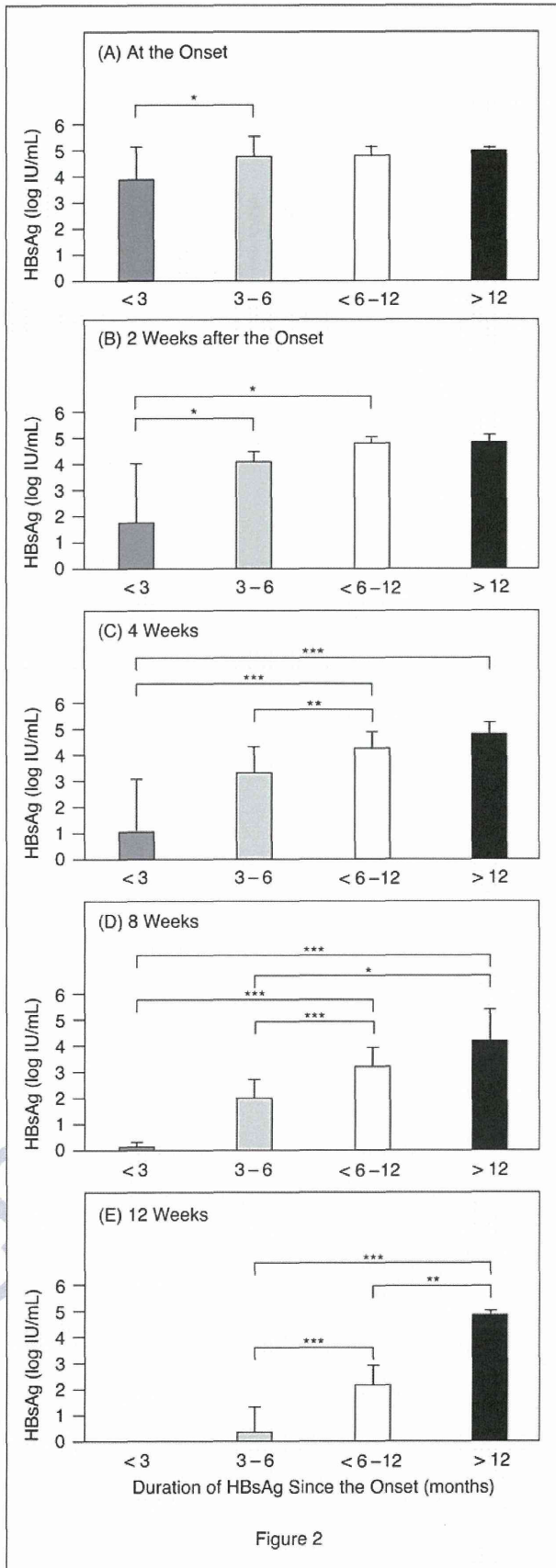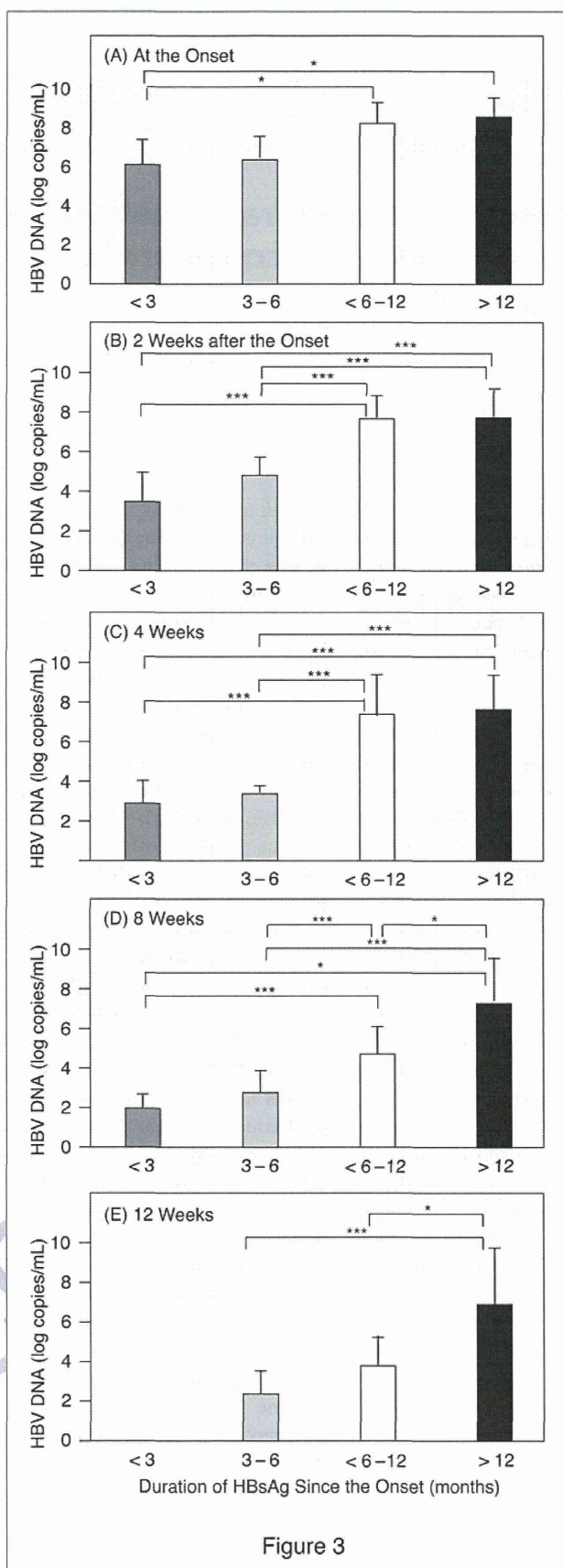
Figure 1

Figure 2

Figure 3

COMMUNICATION

Multilectin-assisted fractionation for improved single-dot tissue glycome profiling in clinical glycoproteomics†

Binbin Tan,[ab] Atsushi Matsuda,[a] Yan Zhang,[b] Atsushi Kuno[a] and Hisashi Narimatsu*[a]

To survey the glycome shift in cancer, single-dot tissue glycome profiling was improved by incorporating a lectin-assisted fractionation. The differential analysis of tissue specimens from colorectal cancer patients (*n* = 45) revealed that unfucosylated/α2,6-sialylated glycoproteins significantly increased in patients with poor prognoses. The detailed annotation will be an indispensable supplement for cancer-related glyco-biomarker discovery.

The glycome, the repertoire of glycans produced by individual biological systems (*e.g.*, cells, tissues, and organisms), has been widely reported to modulate numerous physiological and pathological states.[1,2] As important posttranslational modifications of proteins, glycans profoundly affect a wide range of biological processes such as apoptosis,[3] angiogenesis,[4] and microbial recognition.[5] In particular, the importance of the glycome has been highlighted by its role in immune responses to various disease states including cancer, which makes the glycome a promising new source for biomarker discovery.[2,6,7] Currently, mining of the glycome for cancer-associated biomarkers represents a new paradigm for cancer diagnosis and prognosis. However, the inherent diversity, complexity and heterogeneity of glycan structures make glycome analysis particularly challenging.[8]

For decades, capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) have been widely used for glycome analysis. CE is a rapid and reliable analytical tool for determining carbohydrate structures, but it is limited by the need for synthetic standards for every glycan analyzed.[8,9] HPLC is regularly used for glycan separation in most glycome analytical laboratories with excellent reproducibility.[10] Like CE, the utility of HPLC is limited to glycome analysis because of the requirement for chemical or enzymatic

liberation of glycans from glycoconjugates. MS has become an indispensable technology in the field of glycomics because of its powerful capacity for determining the structural details of glycans. Recently, new analytical technologies have been developed to optimize the MS-based glycome analysis platform.[11,12] However, the requirement for expensive equipment and professional personnel hinders the broad application of these techniques. Recently, lectin microarrays have emerged as an increasingly important technology for glycome investigation.[13] Compared with the technologies described above, lectin microarrays have been recognized as a simple, rapid, highly sensitive, and high-throughput platform that obtains the glycome profile (both *N*- and *O*-glycans) of diverse complex biological samples without releasing the glycan moieties from glycoconjugates.

Comprehensive elucidation of the glycome from biological samples is a significant challenge because of the complexity and microheterogeneity of glycan structures, which considerably hinder glycans from becoming potential informative biomarkers for understanding both normal biological functions and pathological processes.[14,15] Therefore, effective sample fractionation or enrichment is necessary for more in-depth investigation of glycomes. So far, lectin affinity chromatography (especially multilectin affinity chromatography) has been widely employed by researchers to isolate diverse glycan motifs in the extensive mining of glycomes for cancer biomarker discovery.[16,17] The series of lectins used in the chromatography are usually determined with an emphasis on comprehensiveness. This means a lack of systematic determination based on the differential glycome analysis using clinical specimens (*e.g.*, tissue and serum) reduces the efficiency of biomarker discovery.

Recently, with advances in analytical technologies, tissue glycome investigation is gaining momentum in biomarker discovery.[12,18,19] In fact, compared with serum glycome, tissue glycome is a more direct and authentic reflection of the disease state within the corresponding organ. It is now generally recognized that reliable cancer-specific biomarkers should be produced by the cancer cells themselves and are usually present

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in serum at only low levels, especially at the early stage of cancer.[20] For this reason, it is necessary to develop a novel method to explore tissue glycomes in detail. To date, there have been hardly any related reports focused on the development of a strategy for in-depth tissue glycome analysis. Therefore, we developed an ultrasensitive method for glycan analysis targeting small regions (i.e., single-dot tissue on tissue arrays) on formalin-fixed paraffin-embedded (FFPE) tissue sections by means of lectin microarrays.[21,22] Herein, we report an improved strategy integrating tissue arrays, lectin microarrays, and lectin-assisted fractionation techniques, optimized for in-depth tissue glycome investigation, which would be feasible for elucidation of subtle changes during pathological processes. Furthermore, our strategy provided a new idea for designing a sequential combination of lectins for multilectin affinity chromatography in the early phase of biomarker discovery (Fig. 1).

To prove our concept, we investigated the tissue glycome of colorectal cancer (CRC) with the aim of finding reliable CRC-related glyco-biomarkers for clinical application, i.e., prediction of CRC prognosis. To identify the glycan that specifically predicts CRC prognosis as 10 year survival after surgery, we first compared the glycome profiles of single-dot tissues in CRC patients with good ($n = 34$) and poor ($n = 11$) prognoses (Table S1, ESI†) based on FFPE colon tissue arrays and lectin microarrays (First LA in Fig. 1, experimental procedure described in the ESI†). The LecChip microarray (GlycoTechnica, Sapporo, Japan) consists of 45 lectins with a broad range of specificities, covering almost all of the important N- and O-linked glycans. Their carbohydrate specificities are listed in Table S2 (ESI†). Based on unbiased statistical analysis with all of the normalized data (Table S3, ESI†), we found that the signals of AAL and ABA on the LecChip significantly differentiated the two groups of CRC patients ($p < 0.05$). Specifically, the AAL signal was stronger in patients with good prognosis, and the ABA signal was much weaker in patients
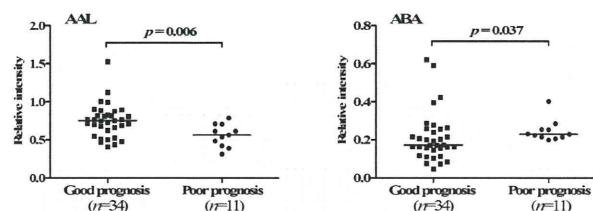

Fig. 2 Selected probe lectins for discrimination of CRC patients with good and poor prognoses.

with good prognosis than in those with poor prognosis (Fig. 2). AAL has specificity for fucosylated glycans,[23] which is known to increase in cancer-associated glycan alterations.[24,25] ABA has specificity for Gal-exposed O-linked glycans (i.e., Core1) and GlcNAc-exposed (i.e., agalactosylated) N-linked glycans,[26] and is reported to have a potent antineoplastic effect on malignant colon cells.[27,28] Considering the statistical results and the biological significance of AAL and ABA, we selected these two lectins as the probe lectins for further studies.

To further confirm the contribution of selected probe lectins (AAL and ABA) toward CRC prognosis, and for more effective enrichment of potential glyco-biomarker candidates, we combined the lectin microarray-based tissue glycome profiling analysis with lectin-assisted fractionation. For the lectin affinity capturing, 11 patients with good prognosis were selected from the original 34 cases with age ($p = 0.92$) and sex ($p = 0.14$) matched to the 11 original patients with poor prognoses. The clinical information including follow-up months for the selected subjects is provided in Table S4 (ESI†).

As shown in Fig. 3A, each Cy3-labeled glycoprotein sample extracted from single-dot tissues was divided into three aliquots (30 µL each) for AAL-affinity capturing, ABA-affinity capturing, and "Input". In our strategy, we collected the supernatants after AAL- and ABA-affinity capturing (i.e., the pass fractions: AAL(−)
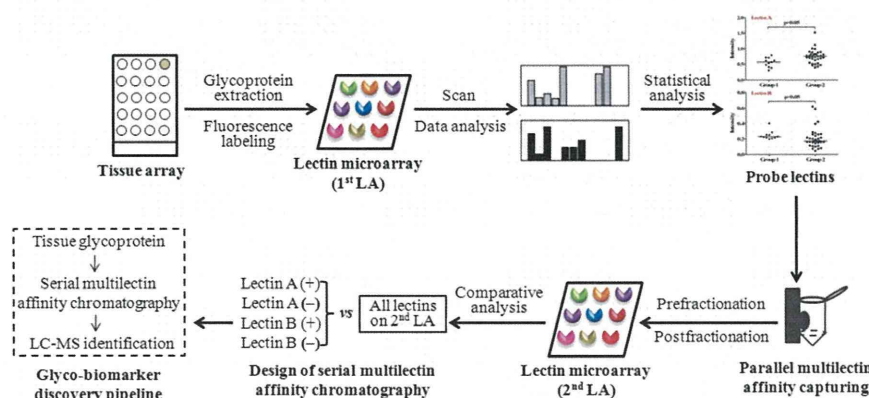
Fig. 1 Schematic diagram for glyco-biomarker discovery based on lectin microarrays and lectin-assisted fractionation. (1) Comparative analysis of tissue glycome profiling between two groups of study subjects based on FFPE tissue arrays and lectin microarrays (1st LA). (2) Statistical analysis for screening the probe lectins to distinguish different groups (e.g., lectins A and B). (3) Fractionation of tissue glycoproteins by affinity capturing using probe lectins in parallel, and then comparative analysis of glycan profiling between the pre- and post-fractionations based on lectin microarrays (2nd LA). (4) Selection of the most accurate biomarker candidates and optimization of the serial multilectin affinity chromatography for glyco-biomarker identification based on LC-MS in further studies.

A

B

Fig. 3 Mining of potential glyco-biomarkers for CRC prognosis. (A) Scheme for lectin microarray-based tissue glycome profiling analysis with AAL- and ABA-assisted fractionation. (B) A Venn diagram visualizing the results obtained by incorporating the lectin microarrays and lectin-assisted fractionation.

and ABA(−)). Each pass fraction was subjected to lectin microarray analysis (second LA in Fig. 1) instead of the elution fraction in order to minimize the experimental bias. Then, we defined "Input(−) minus AAL(−)" as the AAL captured fraction (*i.e.*, AAL(+)), and "Input(−) minus ABA(−)" as the ABA captured fraction (*i.e.*, ABA(+)). Based on the results of the LecChip analysis with lectin-assisted fractionation (AAL(+), AAL(−), ABA(+), ABA(−)), it was found that the signals of ABA in AAL(−) fractions showed the most significant differences between CRC patients with good and poor prognoses. Furthermore, the signals of α2,6-sialic acid binders such as *Sambucus nigra agglutinin* (SNA) and *Sambucus sieboldiana agglutinin* (SSA) both in AAL(−) and ABA(+) fractions were significantly increased in patients with poor prognosis, whereas both SNA and SSA signals in "Input(−)" showed no significant differences between the two groups of patients (Table 1). It has been reported that the activity of β-galactoside α2,6-sialyltransferase (ST6Gal.I) and SNA reactivity increase in human colon cancer tissues.[29] Taking the specificities of three lectins into account, sialylated mucins, which are well known as potential CRC markers,[30–32] might be rationally enriched in these fractions. This suggested that AAL(−)/ABA(+)/SNA(+) and AAL(−)/ABA(+)/SSA(+) fractions

contain a series of glycoproteins, which should be accurate glyco-biomarker candidates for CRC prognosis (Fig. 3B). Considering the potential bias caused by different pathological stages in CRC patients, 5 patients at stage III with good prognoses were selected from 11 patients with age ($p = 0.83$) and sex ($p = 1.0$) matched to 5 patients at stage III with poor prognoses for further analysis. The clinical information for the selected subjects is provided in Table S6 (ESI†). The signals of ABA in AAL(−) fractions also showed the largest differences between the two groups, despite the significance becoming weaker ($p = 0.058$) because of the limited sample size (5 *vs.* 5). Similarly, the signals of SNA and SSA in AAL(−) and ABA(+) fractions also increased in patients with poor prognosis (Table S7, ESI†). These results suggested the potential clinical value of our findings for CRC prognosis while comparing the patient groups at the same pathological stage. All results proved that it is necessary to fractionate glycoproteins for in-depth glycome analysis, and our method was successful in solving the technical problem. We propose an optimized scheme of serial multilectin affinity chromatography for MS-based glycoproteomics, which would contribute towards effectively specifying the target range needed to find novel and clinically promising biomarkers for

Table 1 Significant lectins in AAL and ABA affinity fractionations between CRC patients with good and poor prognoses

Lectin	Input(−)			AAL(+)			AAL(−)			ABA(+)			ABA(−)		
	Good	Poor	p	Good	Poor	p	Good	Poor	p	Good	Poor	p	Good	Poor	p
AAL	23 800	18 000	0.28	23 800	18 000	0.28	0	0	1.00	9200	9000	0.97	14 500	9700	0.12
ABA	6300	9800	0.07	6300	7900	0.49	0	2500	**0.01**	6300	9800	0.07	0	0	1.00
SNA	19 000	20 200	0.22	14 800	13 400	0.87	0	8800	**0.04**	4800	8500	**0.05**	12 100	11 300	0.82
SSA	19 400	20 500	0.21	17 000	14 800	0.87	0	8200	**0.04**	5100	8600	**0.03**	13 500	12 900	0.79

Values are the means of absolute signal intensities. *p*-values in bold indicate statistical significance by the Mann–Whitney *U* test. Good prognosis ($n = 11$), poor prognosis ($n = 11$).

CRC prognosis (as shown in Fig. S1, ESI†). We will conduct further validation of lectin binding and subsequent identification of the glyco-biomarker candidates in a larger clinical sample pool in a subsequent study.

Conclusions

As a proof of concept, this study confirmed the effectiveness and feasibility of our strategy on lectin microarray-based tissue glycome profiling for clinical applications of glyco-biomarkers. The significant advantages of our strategy include: (1) an ideal combination of an ultrasensitive lectin microarray and a FFPE tissue array with comprehensive clinical information that showed potential advantage in cancer-specific biomarker discovery, (2) simple manipulation and rigorous selection of probe lectins based on statistical analysis to ensure the reliable results, (3) a lectin microarray integrated with lectin-assisted fractionation that can provide more detailed and useful information for logical optimization of serial multilectin affinity chromatography before MS analysis, which would be feasible for glycoproteomics-based biomarker discovery, and (4) a universal platform in the uppermost stream of the pipeline of glyco-biomarker discovery, which can be useful for exploring clinically promising biomarkers in further studies.

Abbreviations

CE	Capillary electrophoresis
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
LA	Lectin microarray
FFPE	Formalin-fixed paraffin-embedded
CRC	Colorectal cancer
AAL	*Aleuria aurantia* lectin
ABA	*Agaricus bisporus* agglutinin
SNA	*Sambucus nigra* agglutinin
SSA	*Sambucus sieboldiana* agglutinin

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