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Discovery of sialyl Lewis A and Lewis X modified protein cancer biomarkers using high density antibody arrays

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Abstract

We report on a high-dimensional method to globally profile glycoproteins that are modified with sialyl Lewis A or Lewis X glycans. Specifically, glycoproteins in serum or plasma are fractionated on a high-density antibody microarray (i.e., each are localized to their specific antibody spot) and are specifically detected via fluorescently labeled anti-sialyl Lewis A or anti- Lewis X antibodies with quantification in a microarray scanner. Non-glycosylated proteins or glycoproteins with other glycan motifs do not interfere with this assay. The whole process is very rapid and applicable for high-throughput screening without the need for purification of glycoproteins from the samples. Using these methods, sialyl Lewis A or Lewis X moieties were found to be expressed on many previously unreported secreted or membrane associated proteins. Furthermore, the combination of sialyl Lewis A or Lewis X content with protein level increased the ability of certain glycoproteins to distinguish 30 patients with stage III and IV colon cancer from 60 control samples. Thus, this highly sensitive method is capable of discovering novel specific glycan modifications on proteins, many of which will likely be useful for disease detection and monitoring.

Keywords

glycoproteins; glycans; sialyl Lewis A; Lewis X; cancer biomarker; antibody array

Most current clinical cancer biomarkers are specific for glycoproteins (e.g., CA125, CA15-3, PSA and CEA for ovarian, breast, prostate and colon cancer, respectively) or carbohydrate structures (e.g., CA19-9 for pancreas cancer). Approximately 50% of all proteins are estimated to be glycosylated [1] and glycan abundance and their micro- and macro-heterogeneity can be changed in a disease-specific manner [2]. This change in

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carbohydrate structure can have independent diagnostic value as well as supplemental benefit to known markers for better specificity and sensitivity [3–5]. Previously most glycoprotein screening studies have relied on immunoprecipitation or lectin affinity capture of whole glycoproteins and mass spectrometry identification of the de-glycosylated protein portion [6–8]. In a few cases, protein classes such as the mucins for pancreas cancer have been probed with lectins in an array format containing up to a few hundred antibodies [9–11]. A recent study used 58 different antibodies to a variety of serum proteins including mucins, matrix proteins, adhesion proteins, and cytokines on an array to capture potential CA19-9 antigen carrying proteins from sera of pancreas cancer patients [12]. In this report, glycoproteins in blood or tissue samples are specifically captured by over 3000 antibodies on an array, and the glycan moieties on proteins are detected by two different fluorescently-labeled anti-carbohydrate-specific antibodies. This approach allows us to discern how widespread a specific carbohydrate modification was across a significant portion of the plasma proteome.

We selected sialyl Lewis A and sialyl Lewis X for our prototypical glycoproteomic analyses. Sialyl Lewis A is the antigen for serological biomarker CA19-9 [13] which has been used for diagnosis and follow-up of gastrointestinal (GI) cancers [14, 15]. Reported CA19-9 carrying glycoproteins include MUC1, MUC5AC, MUC16, apolipoproteins and kininogen [7]. CA19-9 detection on MUC5AC or MUC16 showed improved sensitivity over the standard CA19-9 alone assay for pancreas cancer [12]. The anti-sialyl Lewis-A antibody used in this study (clone SLE121) has been reported to be highly specific for sialyl Lewis A in a monomeric form or as a part of elongated carbohydrate structures at non-reducing ends [25, 26]. Sialyl Lewis X is another cancer specific carbohydrate markers sometimes used for cancer staging, prognosis [16] and progression [17]. Reported sialyl-Lewis X carrying proteins include alpha 1-acid glycoprotein [18], CD66 [19] and MUC7 [20]. The specificity of the anti-sialyl Lewis X antibody (clone 258-12767) we used for this study had not been tested so we examined it using carbohydrate microarray profiling on over 200 glycan ligands and several abundant serum proteins. The antibody displayed binding affinity to its sialyl Lewis-X antigen but showed 29x higher reactivity to dimeric Lewis X, 12x for Lewis A-Lewis X, 6x for lacto-N-hexose (Gal-GlcNAc) and 4x for Lewis A (see supplemental figure). Since its specificity is not limited to the sialylated form, we refer to the antibody as Lewis X specific but caution that any glycoproteins identified with this antibody would need to be further validated.

Detection of sialyl Lewis A and Lewis X on affinity captured proteins by high density antibody array

Each array contained approximately 3600 human-protein specific antibodies to ~3000 different proteins printed in triplicate (10800 total spots) on N-hydroxysuccinimide (NHS)-ester reactive 3-D thin film surface slides (Nexterion H slide, Schott) as previously described [21, 22]. Microarray slides were blocked with 0.3% (v/v) ethanolamine in 50 mM sodium borate pH 8, washed, dried and incubated with sample. To detect levels of proteins in the plasma samples, we depleted albumin and IgG and 200 μg of the remaining protein from either the case or control sample was labeled with Cy5 and analyzed as previously described [21, 23]. To detect sialyl Lewis A or Lewis X carrying proteins, 10 μl of undepleted human plasma was diluted 1:8 in 0.05% Tween 20 in PBS, pipetted onto the slide at the microarray/coverslip (mSeries Lifter Slips, 22×25×1 mm, Thermo Scientific) junction and incubated for 60 min. Then, the slides were washed two times with 0.5% Tween 20 in PBS. Bound sialyl Lewis A or Lewis X carrying proteins were simultaneously detected after incubation with Cy3-anti-sialyl Lewis A and Cy3- or Cy5-anti-Lewis X monoclonal antibodies (US Biological; diluted to 5 μg/ml in 0.05% Tween 20 in PBS) for 60 min. The arrays were washed twice each with PBS/0.5% Tween 20, PBS and water

followed by drying by centrifugation. Finally, the slides were scanned on a GenePix 4200A microarray scanner (Axon Instruments) to produce green (Cy3) and red (Cy5) images. Spot intensities of the scanned array images are obtained using Genepix Pro 6.0 image analysis software. As a control to determine background levels of signal, the arrays were incubated with just Cy3- and Cy5-labled anti-sialyl Lewis A and Lewis X specific antibodies (no plasma added), and the resulting signals were used for background subtraction. Our choice of sialyl Lewis A and Lewis X structures, which are rarely expressed on antibodies, helped keep the background low even without chemical derivatization of the arrayed antibodies (normally required for lectin based analyses) [24].

Over half of the arrayed antibodies were directed to secreted and transmembrane proteins which are usually glycosylated. We analyzed the protein, sialyl Lewis A and Lewis X content of a colon cancer and an undiseased control plasma sample to test the specificity and utility of this glycoprotemic array in profiling glycan carrying proteins (the completely de-identified plasma samples used were collected prior to colonoscopy under an Institutional Review Board approved protocol and diagnosis was confirmed by colonoscopy/pathology review). As expected, antibodies to MUC1 and MUC5AC bound protein with sialyl Lewis A moieties (spot intensities 1619 and 539 after background subtraction, respectively), and MUC1 was detected with the Lewis X antibody (spot intensity 970 after background subtraction). In addition to the mucin proteins, a significant number of other proteins with, to our knowledge previously unreported, sialyl Lewis A and Lewis X modifications were detected as potential glycoprotein biomarkers. If we define a protein as positive by requiring at least 500 intensity value (a very clear spot) after background subtraction and sample/background signal ratio greater than two at all 3 spots of the triplicate, we obtain a total of 42 and 112 sialyl Lewis A and Lewis X modified proteins, respectively. Of these, 29 antibodies bound proteins with both glycan modifications. We suggest that the presence of both modifications on a protein might even be a better indication than either modification singularly or the protein alone and this technology can detect these proteins. Fig. 1 shows magnified proteomic (lower two rows) and glycan (upper two rows) signals from different array regions emphasizing three examples of sialyl Lewis A or Lewis X carrying proteins that showed much higher levels for glycan than proteomic changes in a case and control sample. Examining the protein signal, CD44, CTSL2 and PDGFA showed 1.6-, 1.1- and 1.6-fold changes, respectively, between the cancer and control samples (Fig. 1B). In contrast, their glycoproteomic changes detected by anti-sialyl Lewis A were more striking. CD44, CTSL2 and PDGFA proteins showed 7.4-, 2.7- and 3.5-fold increases, respectively (Fig. 1A). Fig. 1C–D shows DCD, HBEGF and VWF have 1.8-, 2.2- and 1.2-fold changes in protein levels, respectively (Fig. 1D), but Lewis X changes of 4.1-, 6.0- and 7.5-fold, respectively (Fig. 1C). By taking a ratio of the change in sialyl Lewis and protein levels, all 6 of these proteins show more cancer-specific glycosylation changes in the colon cancer sample. This result confirms that being able to detect the specific glycan content of proteins could increase their utility as biomarkers.

Since sialyl Lewis A and X are both fucosylated tetrasaccharides that only differ in the position of the fucose linkages (Fig. 2A), we had to test for antibody specificity and cross-reactivity. Thus, the colon cancer plasma sample was incubated on our antibody array and sialyl Lewis A and Lewis X on the captured proteins were simultaneously detected with scanning parameters set to yield 1:1 total spot intensity count ratio between the two wavelengths. The overlaid array image displayed a mixture of green, red and yellow spots suggesting the two antibodies detected their carbohydrate antigens independently (Fig. 2B). For example, FGFBP1 predominantly expressed sialyl Lewis A (spot intensity 3605) without detectable Lewis X and appeared as a green spot in the overlaid image (Fig. 2C). FBLN2 was detected with predominately Lewis X expression and appeared as a red spot in the overlay (spot intensity 34346). Some proteins expressed both glycans as exemplified by

the yellow NPY spot in the overlay (Fig. 2D, sialyl Lewis A spot intensity; 5160 and Lewis X intensity; 19476). To confirm that the two dyes do not indirectly bind to the carbohydrate structures and cause spectral bias, a plasma sample from the cancer patient used in Fig. 1 was incubated on array and Lewis X expression on the captured proteins was simultaneously detected with equal amounts of anti-Lewis X antibody each labeled with either Cy3 or Cy5, respectively. The resulting overlay image of the two dyes showed that virtually all the detected spots appeared yellow (not shown). When spots greater than 3000 intensity were examined, a plot of log2 transformed Cy5/Cy3 intensity ratios for each of the spots showed an average ratio of 1.01 ± 0.04 (mean ± std deviation), clearly verifying there is no spectral bias from Cy dyes using our approach.

Next, we assessed the reproducibility of the glycoproteomic array by calculating coefficient of variation after incubation with plasma from the colon cancer patient. Triplicate spots within the same array proved highly reproducible for both sialyl Lewis A and Lewis X expression with 94% and 93% of the spots, respectively, showing coefficients of variation (CV) of less than 10%. When three independent arrays were incubated with the same plasma sample, 60% and 71% of the spots showed less than 10% CV for sialyl Lewis A and Lewis X, respectively and over 94% showed CVs of less than 20% for detection of both glycans. These levels of reproducibility are certainly acceptable for discovery purposes.

Fig. 2C and F show the top 30 proteins with the highest sialyl Lewis A or Lewis X glycan content. Some proteins carry one carbohydrate structure dominantly over another, and 4 (C2, CD59, F5 and SPARC) had higher levels of both glycan structures. Proteins detected mainly with Sialyl Lewis A modifications include A2M, ADAM9, AFP, B3GNT5, CD44, CSF3, CTBS, CTSL2, EFNB3, EPHA3, FGFBP1, IGFBP5, IL18RAP, INHBC, MSLN, MUC1, NPY, PDGFA, PROM1, RAF1, S100A7, TFF3, TGFB2, TNFRSF10A, WISP1 and WNT9B. Proteins with dominant Lewis X expression include C4B/C4A, CD27, CD97, COL18A1, COL1A1, COL4A3, CTSD, DCD, DEFA1, EFNA5, FBLN2, FCGR2A, IL10, IL2RA, KRAS, LAMB1, LIFR, NPY, PDIA3, SERPINE1, SHBG, SPARC, TFRC, TGFA, VWF, WISP2 and WNT7B.

To determine whether microarray-discovered glycan modifications of the proteins could be validated via alternative methods, we immunoprecipitated proteins from a colon cancer patient plasma and detected the presence of their putative glycans by anti-sialyl Lewis A and Lewis X antibodies via Western blotting. We chose to examine C2 protein, which gave ~3500 and ~9500 spot intensities in sialyl Lewis A and Lewis X profiling microarrays, and PODXL which did not show any sialyl Lewis A and Lewis X carrier by microarray (as a negative control) because both proteins gave reasonable bands at the expected molecular size in Western immunoblot. As expected, immunoprecipitated C2 protein showed bands at 83 kDa both via immunoblot and when blotting with anti-sialyl Lewis A and anti-Lewis X antibodies (Fig. 2G). Immunoprecipitated POXDL protein gave a nice band via immunoblot but no signal from the anti-glycan antibodies (Fig. 2H). Negative controls with an additional antibody raised in the same host as the others and a no antibody control also showed no anti-glycan, C2 or PODXL signal (Fig. 2G & H).

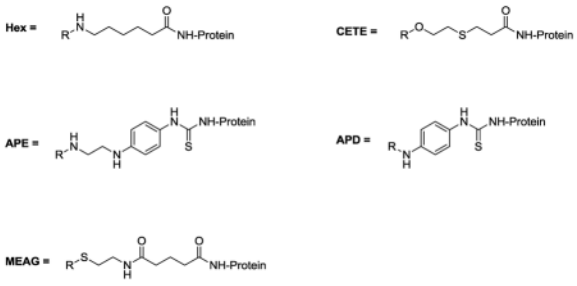
Given the high dimensionality of this assay, biomarker candidates would need to be tested in multiple sample sets prior to drawing any conclusions about their performance. However, we have performed a small study with plasmas from 30 late stage CRC patients (6 IIIa, 10 IIIb, 5 IIIc and 9 IV stages) and 60 healthy controls that were collected by the Great Lakes and New England Clinical Validation Center funded by the National Cancer Institute, Early Detection Research Network. Of the 30 patients, 20 were male with a median age of 59.5±10.6 years old and 10 were female at 62.0±13.3 years old. The analyses showed that there are glycoproteins that show statistically different protein, sialyl Lewis A and/or Lewis

X content when the plasma of late stage colorectal cancer (CRC) patients was compared to the controls (Table).

The table presents the top 29 glycoproteins that showed increased expression of either sialyl Lewis A or Lewis X in the cancer samples with p-values of ≤ 0.005. The q-value takes into account a correction for multiple comparison testing, and the coefficient is $\log_2$ of the intensity ratio between case and control samples. Of the 29 proteins with elevated glycan markers, only 5 were in our two lists of 30 most abundant glycoproteins shown in 2E and F indicating that most of the viable biomarkers might be from lower abundance glycoproteins. Of the 29, 15 showed decreases in protein level in cases, 6 of which were significant at p-value <0.05. The other 14 proteins showing increased protein included 8 sialyl Lewis A-modified proteins (CD44, CD46, CEACAM1, IL1B, INHBC, NOTCH4, PZP and TIMP2) and 11 Lewis X-modified proteins (B3GNT5, CD44, CST6, HSP90AB1, HSPG2, IL6, NOTCH2, PECAM1, PZP, TIMP2 and VWF). However, among them, only five proteins (B3GNT5, HSP90AB1, HSPG2, INHBC and NOTCH4) showed protein abundance changes with statistical significance at p<0.05. This demonstrates that our glycomic microarray could be used for biomarker discovery as a stand-alone-tool, but combinations of proteomic and glycomic levels of a protein might yield even better biomarker candidates. In the Table, we illustrate this point by examining the AUC for B3GNT5, CD44, HSPG2, IL6, INHBC, NOTCH4 and VWF each individually with sialyl Lewis A and/or Lewis X and then combined with protein levels. For example, VWF protein showed an AUC of 0.606 that increased to 0.900 when combined with Lewis X, and B3GNTS protein had an AUC of 0.796 that increased to 0.914 when combined with both sialyl Lewis A and Lewis X. Although this data is preliminary and any potential markers would have to be confirmed in multiple validation sample sets, it shows that colon cancer patients carry specific sialyl-Lewis A and/or Lewis X modified proteins and this array technique is capable of detecting them.

It should be noted that Lewis antigen production is controlled genetically and Lewis-negative individuals (10% in the Caucasian population [27]) do not produce sialylated Lewis antigens even when a large tumor is present [28]. The concentration of the marker is influenced by the patient's secretor status (FUT2 gene) and Lewis genotype (FUT3 gene) [29]. Despite this limitation, sialyl Lewis A (CA19-9) is the primary biomarker used for surveillance of gastrointestinal cancers. Identification of the specific protein carriers of CA19-9 in pancreas and colon cancer samples will allow us to test whether determination of the concentration and extent of modification of specific sialylated proteins will show improved biomarker sensitivity and specificity over the class as a whole as suggested previously for some mucins [9]. This method could also be applied to other interesting cancer-associated glycans to study including T (Thomsen-Friedenreich, Galβ1-3α-Ser/Thr), Tn (GalNAcα-Ser/Thr) and sialyl Tn (sialylα2-6GalNAcα-Ser/Thr) for O-glycan antigens [30] and, increased β1-6 branching and sialylation for N-glycan antigens [2].

In conclusion, we describe a method that allows rapid screening for the levels of modified carbohydrate in potential cancer modified glycoproteins with high dimensionality and throughput. This method confirmed known glycoproteins to contain sialyl Lewis A (CA19-9) and Lewis X moieties and identified many additional ones that could be potential cancer biomarkers.



Linkers

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Carbohydrates	
3′SLacNAc	Sialylα2-3Galβ1-4GlcNAc – BSA
3′Sia-3-FL	Siaα2-3Galβ1-4(Fucα1-3)Glc -BSA
3′-sulpho-LeA	3-SO3-Galβ1-3[Fucα1-4)GlcNAc-BSA
3′-sulpho-LeX	3-SO3-Galβ1-4[Fucα1-3)GlcNAc-BSA
6′Slac	Sialylα2-6Galβ1-4Glc-APD-HSA
6′-sulpho-LeA	6-SO3-Galβ1-3[Fucα1-4)GlcNAc-BSA
6′-sulpho-LeX	6-SO3-Galβ1-4[Fucα1-3)GlcNAc-BSA
2′FucLac	Fucα1-2Galβ1-4Glc-BSA (BG-H5)
Adi - 04	GalNAcα1-3Galβ-BSA (4/BSA)
Adi - 17	GalNAcα1-3Galβ-BSA (17/BSA)
A-LeB hexa	GalNAcα1-3(Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-BSA
alphaGal	Galα1-3Galβ1-4GlcNAc-BSA
alphaGal-6-deoxy	Galα1-3Galβ1-4(6deoxy-GlcNAc)-HSA (alphaGal)
Ara5	Araα1-5Araα1-5Araα1-5Araα1-5Araα1-BSA
Bdi	Galα1-3Gal – BSA
BG-A	GalNAcα1-3(Fucα1-2)Galβ- -BSA
BG-A1	GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA
BG-B (Dextra)	Galα1-3(Fucα1-2)Galβ-BSA from Dextra
BG-B (EMD)	Galα1-3(Fucα1-2)Galβ-BSA from EMD

BG-H1	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ APD-HSA
BG-H2	Fucα1-2Galβ1-4GlcNAcβ-HSA
Cellobiose	Glcβ1-4Glcβ-BSA
Cellotriose	Glcβ1-4Glcβ1-4Glcβ-BSA
Chito 3	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ-BSA (8/BSA)
Chito 3 - 20	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ-BSA (20/BSA)
DSLNT	Siaα2-3Galβ1-3(Siaα2-6)GlcNAcβ1-3Galβ1-BSA
Forssman Di - 04	GalNAcα1-3GalNAcβ1-BSA (4/BSA)
Forssman Di - 21	GalNAcα1-3GalNAcβ1-BSA (21/BSA)
Forssman Di - 31	GalNAcα1-3GalNAcβ1-BSA (31/BSA)
Fuc-a - 22	Fuc-α - BSA (22/BSA)
Fuc-a - 04	Fuc-α - BSA (4/BSA)
Fuc-b - 04	Fuc-β - BSA (4/BSA)
Fuc-b - 22	Fuc-β - BSA (22/BSA)
Fuc, Sia-LNnH-APD-HSA	Galβ1-4[Fucα1-3]GlcNAcβ1-6[Neu5Acα2-6Galβ1-4GlcNAcβ1-3]Galβ1-APD-HSA
G2M4	Manβ1-4(Galα1-6)Manβ1-4(Galα1-6)Manβ1-4Manβ1-BSA
GA1 - 06	Galβ1-3GalNAcβ1-4Galβ1-BSA (GA1tri or asialo-GM1; 6/BSA)
GA1 - 20	Galβ1-3GalNAcβ1-4Galβ1-BSA (GA1tri or asialo-GM1; 20/BSA)
GA1di	Galβ1-3GalNAcβ – HSA
GA2di - 04	GalNAcβ1-4Galβ - BSA (GA2di or asialo-GM2; 4/BSA)
GA2di - 16	GalNAcβ1-4Galβ - BSA (GA2di or asialo-GM2; 16/BSA)
GA2di - 37	GalNAcβ1-4Galβ - BSA (GA2di or asialo-GM2; 37/BSA)
GA2di - accurate	GalNAcβ1-4Galβ - BSA (from accurate chemicals)
Gal3	Galα1-3Galβ1-4Galα-BSA (Gal3)
Gal-a	Gal-α - BSA
Gala1-2Gal	Galα1-2Gal-BSA
Gala1-4Galb	Galα1-4Galβ-CETE-BSA
Galα3-type1	Galα1-3Galβ1-3GlcNAc-BSA
Gal-b	Gal-β - BSA
Galb1-6Man-a	Galβ1-6Man-α - BSA
Galilli	Galα1-3Galβ1-4Glc-BSA
GalNAc-a - 04	GalNAc-α - BSA (4/BSA)
GalNAc-a - 22	GalNAc-α - BSA (22/BSA)
GalNAca1-6Galb - 04	GalNAcα1-6Galβ-BSA (4/BSA)
GalNAca1-6Galb - 22	GalNAcα1-6Galβ-BSA (22/BSA)

GalNAc-b	GalNAc-β - BSA
Gb4	GalNAcβ1-3Galα1-4Galβ1-BSA
Glc-a	Glc-α - BSA
Glca1-6Glca1-4Glca1-4Glcb	Glcα1-6Glcα1-4Glcα1-4Glcβ-CETE-BSA
Glc-b	Glc-β - BSA
GlcNAca1-4Galb	GlcNAcα1-4Galβ-BSA (20/BSA)
GlcNAca1-4Galb - 03	GlcNAcα1-4Galβ-BSA (3/BSA)
GlcNAc-b	GlcNAc-β - BSA
GlcNAc-Man3	Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ-BSA
GlcNAc-Man5	Manα1-6(Manα1-3)Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ-BSA
GM1	Galβ1-3GalNAcβ1-4(Siaα2-3)Galβ-4(Glc)-HSA
GM3	Sialylα2-3Galβ1-4Glc-APD-HSA
Hep-N-acetylated	fully N-acetylated heparin polysaccharide-BSA
Hep-5000	heparin polysaccharide-BSA (MW ~5000)
Hya8	$(GlcNAcβ1\text{-}4GlcAβ1\text{-}3)_4β1$-BSA
Hya9	$(GlcAβ1\text{-}3GlcNAcβ1\text{-}4)_4β1$-3GlcAβ1-BSA
Hybrid-M5N4B	GlcNAcβ1-2Manα1-3[Manα1-3(Manα1-6)Manα1-6] (GlcNAcβ1-4)Manβ1-4GlcNAcβ1-BSA
iLNO	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6 (Galβ1-3GlcNAcβ1-3)Galβ1-BSA
Isomaltose	Glcα1-6Glcβ-BSA
LacNAc	Galβ1-4GlcNAc – BSA
LacNAc (trimeric)	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-APE-HSA
LacNAc-Man5	Manα1-6(Manα1-3)Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ-BSA
Lactose	Galβ1-4Glcβ – BSA
LeA	Galβ1-3[Fuca1-4)GlcNAcβ1-3Galβ1-4Glcβ- BSA
LeA-LeX	Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-APD-HSA
LeB	Fucα1-2Galβ1-3[Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ-BSA
LeC	Galβ1-3GlcNAcβ – BSA
LeX (dimeric)	Galβ1-4[Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-APE-BSA
LeX (monomeric)	Galβ1-4[Fucα1-3)GlcNAc-APD-HSA
LeY	Fucα1-2Galβ1-4[Fucα1-3)GlcNAc – HSA
LNH - 13	Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-BSA (13/BSA)
LNnH - 11	Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-BSA (11/BSA)
LNnT - 14	Galβ1-4GlcNAcβ1-3Galβ1-BSA (14/BSA)
LNnT - 04	Galβ1-4GlcNAcβ1-3Galβ1-BSA (4/BSA)

LNT - 05	Galβ1-3GlcNAcβ1-3Galβ-BSA (5/BSA)
LNT - 20	Galβ1-3GlcNAcβ1-3Galβ-BSA (20/BSA)
LSTa	Siaα2-3Galβ1-3GlcNAcβ1-3Galβ1-BSA
LSTb	Galβ1-3(Siaα2-6)GlcNAcβ1-3Galβ1-BSA
LSTc	Siaα2-6Galβ1-3GlcNAcβ1-3Galβ1-BSA
Maltopentaose	Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα-BSA
Maltose	Glcα1-4Glcβ-BSA
Man3	Manα1-6(Manα1-3)Manβ1-4GlcNAc -BSA
Man5	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAc-BSA
Man6 - II	Manα1-2Manα1-3Manα1-6(Manα1-2Manα1-3)Manβ1-BSA
Man6 - I	Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-BSA
Man7D1	Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc-BSA
Man7D3	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAc-BSA
Man8D1D3	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc-BSA
Man9	Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAc-BSA
Man-a	Man-α - BSA
Mana1-6Man-a	Manα1-6Man-α - BSA
Manb4	Manβ1-4Manβ1-4Manβ1-4Manβ1-BSA
ManT	Manα1-6[Manα1-3]Manβ -BSA
MFLNH I	Galβ1-4GlcNAcβ1-6 (Fucα1-2Galβ1-3GlcNAcβ1-3)Galβ1-BSA
MFLNH III	Galβ1-4(Fucα1-3)GlcNAcβ1-6 (Galβ1-3GlcNAcβ1-3)Galβ1-BSA
MSMFLNH I	Siaa2-6Galβ1-4GlcNAcβ1-6 (Fuca1-2Galβ1-3GlcNAcβ1-3)Galβ1-BSA
MSMFLnNH	Galβ1-4(Fucα1-3)GlcNAcβ1-6 (Siaα2-6Galβ1-4GlcNAcβ1-3)Galβ1-BSA
NA2	Galβ1-4GlcNAcβ1-2Manα1-6[Galb1-4GlcNAcb1-2Manα1-3]Manβ1-4GlcNAc -BSA
NA3	Galβ1-4GlcNAcβ1-2Manα1-6[Galb1-4GlcNAcb1-2(Galb1-4GlcNAcb1-2)Manα1-3]Manβ1-4GlcNAc -BSA
NA4	Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-6)Manα1-6[Galb1-4GlcNAcb1-2(Galb1-4GlcNAcb1- 4)Manα1-3]Manβ1-4GlcNAc -BSA
NGA2	GlcNAcβ1-2Manα1-6(GlcNAcb1-2Manα1-3)Manβ1-4GlcNAc -BSA
NGA2B	GlcNAcβ1-2Manα1-6(GlcNAcb1-2Manα1-3)(GlcNAcb1-4)Manβ1-4GlcNAc -BSA
NGA3	GlcNAcβ1-2Manα1-6[GlcNAcb1-2(GlcNAcb1-4)Manα1-3]Manβ1-4GlcNAc -BSA

NGA3B	GlcNAcβ1-2Manα1-6[GlcNAcb1-2(GlcNAcb1-4)Manα1-3](GlcNAcβ1-4)Manβ1-4GlcNAc-BSA
NGA4	GlcNAcβ1-2(GlcNAcβ1-6)Manα1-6[GlcNAcb1-2(GlcNAcb1-4)Manα1-3]Manβ1-4GlcNAc-BSA
NGA4(B)2	GlcNAcβ1-2(GlcNAcβ1-4)(GlcNAcβ1-6)Manα1-6[GlcNAcb1-2Manα1-3](GlcNAcβ1-4)Manβ1- 4GlcNAc -BSA
NGA5B	GlcNAcβ1-2(GlcNAcβ1-4)(GlcNAcβ1-6)Manα1-6[GlcNAcb1-2(GlcNAcb1-4)Manα1-3](GlcNAcβ1-4)Manβ1-4GlcNAc -BSA
P1	Galα1-4Galβ1-4GlcNAc-BSA
Pk or Gb3	Galα1-4Galβ1-4Glc-HSA
pLNH	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-BSA (21/BSA)
pLNH - 07	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-BSA (7/BSA)
Rha-a	Rha-α – BSA
Rha-b	Rha-β - BSA
Sialyl LeA	Siaα2-3Galβ1-3[Fucα1-4)GlcNAcβ1-3Galβ1-APD-HSA
Sialyl LeX	Sialylα2-3Galβ1-4[Fucα1-3)GlcNAc – BSA
TFiLNO(1-2,1-2,1-3)	Fuca1-2Galb1-3GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-6(Fuca1-2Galb1-3GlcNAcb1-3)Galb- BSA
Sia-LNnT	Siaα2-3Galβ1-4GlcNAcβ1-3Galβ1-APD-HSA
Sia-LNF V	Fucα1-2Galβ1-3[Neu5Acα2-6]GlcNAcβ1-3Galβ1-APD-HSA
X3Glc3	Xylα1-6Glcβ1-4(Xylα1-6)Glcβ1-4(Xylα1-6)Glcβ1-BSA
Xylβ4	Xylβ1-4Xylβ1-4Xylβ1-4Xylβ1-BSA

Glycoproteins

AGE30	Advanced glycation endproducts- Glucose + BSA on day 30 (AGE30)
AGE60	Advanced glycation endproducts- Glucose + BSA on day 60 (AGE60)
AGE90	Advanced glycation endproducts- Glucose + BSA on day 90 (AGE90)
Alpha-1-acid glycoprotein	alpha1 Acid Glycoprotein
Alpha-fetoprotein	alpha fetoprotein
BSM	Bovine submaxillary mucin (STn, STF, S-GlcNAcβ1-3, ~20% of Sia is acetylated at 7,8, or 9)
BSM (asialo)	Asialo-Bovine submaxillary mucin (aBSM, Tn, TF, GlcNAcβ1-3GalNAc)
BSM (deacetylated)	Deacetylated-Bovine submaxillary mucin
BSM (ox)	periodate oxidized bovine submaxillary mucin

CEA	carcinoembryonic antigen
FABP	Fatty Acid Binding Protein
fetuin	fetuin (Sia2-3LacNAc, Sia2-6LacNAc, SiaLeC, STF)
fetuin (asialo)	asialofetuin (Galb1-4GlcNAc, Galb1-3GlcNAc, Galb1-3GalNAc)
Fetuin (ox)	periodate oxidized fetuin
glycophorin (asialo)	asialo-glycophorin A
Glycophorin A	Glycophorin A
hsp90	Heat Shock Protein 90
KLH	Keyhole limpet hemocyanin
KLH (oxidized)	periodate oxidized Keyhole limpet hemocyanin
OSM	Ovine submaxillary mucin (94% STn, 4% TF, 2% Fuca1-2Galβ1-3GalNAc)
OSM (asialo)	asialo-Ovine submaxillary mucin
OSM (asialo, enzym)	enzyme treated asialo-OSM (almost all Tn)
OSM (enzym)	enzyme treated Ovine submaxillary mucin (almost all STn)
OSM (ox)	periodate oxidized ovine submaxillary mucin
ovalbumin	ovalbumin (56% Man5+Man6)
Ovalbumin (ox)	periodate oxidized ovalbumin
PSA	Prostate Specific Antigen; human seminal fluid
Tgl	Thyroglobulin

Peptides

Ac-A-Tn(Thr)-S-G - 05	Ac-Ala-(GalNAcα)Thr-Ser-Gly-Hex (5/BSA)
Ac-A-Tn(Thr)-S-G - 08	Ac-Ala-(GalNAcα)Thr-Ser-Gly-Hex (8/BSA)
Ac-A-Tn(Thr)-S-G - 23	Ac-Ala-(GalNAcα)Thr-Ser-Gly-Hex (23/ BSA)
Ac-G-V-Tn(Thr)-S-A-G - 04	Ac-Gly-Val-(GalNAcα)Thr-Ser-Ala-Gly-Hex (muc1) (4/BSA)
Ac-G-V-Tn(Thr)-S-A-G - 21	Ac-Gly-Val-(GalNAcα)Thr-Ser-Ala-Gly-Hex (muc1) (21/BSA)
Ac-P-Tn(Thr)-T-G - 05	Ac-Pro-(GalNAcα)Thr-Thr-Gly-Hex (muc2) (5/BSA)
Ac-P-Tn(Thr)-T-G - 08	Ac-Pro-(GalNAcα)Thr-Thr-Gly-Hex (muc2) (8/BSA)
Ac-P-Tn(Thr)-T-G - 22	Ac-Pro-(GalNAcα)Thr-Thr-Gly-Hex (muc2) (22/BSA)
Ac-S-S-S-G	Ac-Ser-Ser-Ser-Gly-BSA (24/BSA)

Ac-S-TF(Ser)-S-G - 04	AcSer-(Galβ1-3GalNAcα)Ser-Ser-Gly-Hex-BSA (4/BSA)
Ac-S-TF(Ser)-S-G - 16	AcSer-(Galβ1-3GalNAcα)Ser-Ser-Gly-Hex-BSA (16/BSA)
Ac-S-TF(Ser)-S-G - 28	AcSer-(Galβ1-3GalNAcα)Ser-Ser-Gly-Hex-BSA (28/BSA)
Ac-S-Thr-S-A-G - 18	Ac-Ser-Thr-Ser-Gly-Hex -BSA
Ac-S-Tn(Ser)-S-G - 04	AcSer-(GalNAcα)Ser-Ser-Gly-Hex-BSA (4/BSA)
Ac-S-Tn(Ser)-S-G - 22	AcSer-(GalNAcα)Ser-Ser-Gly-Hex-BSA (22/BSA)
Ac-S-Tn(Ser)-S-G - 33	AcSer-(GalNAcα)Ser-Ser-Gly-Hex-BSA (33/BSA)
Ac-S-Tn(Thr)-A-G - 04	Ac-Ser-(GalNAcα)Thr-Ala-Gly-Hex (4/BSA)
Ac-S-Tn(Thr)-A-G - 08	Ac-Ser-(GalNAcα)Thr-Ala-Gly-Hex (8/BSA)
Ac-S-Tn(Thr)-A-G - 22	Ac-Ser-(GalNAcα)Thr-Ala-Gly-Hex (22/BSA)
Ac-S-Tn(Thr)-G-G - 03	Ac-Ser-(GalNAcα)Thr-Gly-Gly-Hex (3/BSA)
Ac-S-Tn(Thr)-G-G - 07	Ac-Ser-(GalNAcα)Thr-Gly-Gly-Hex (7/BSA)
Ac-S-Tn(Thr)-G-G - 19	Ac-Ser-(GalNAcα)Thr-Gly-Gly-Hex (19/BSA)
Ac-S-Tn(Thr)-S-G - 04	AcSer-(GalNAcα)Thr-Ser-Gly-Hex-BSA (4/BSA)
Ac-S-Tn(Thr)-S-G - 24	AcSer-(GalNAcα)Thr-Ser-Gly-Hex-BSA (24/BSA)
Ac-S-Tn(Thr)-S-G HSA -04	AcSer-(GalNAcα)Thr-Ser-Gly-Hex-HSA (4/HSA)
Ac-S-Tn(Thr)-S-G HSA -23	AcSer-(GalNAcα)Thr-Ser-Gly-Hex-HSA (23/HSA)
Ac-S-Tn(Thr)-Tn(Thr)-G - 05	Ac-Ser-(GalNAcα)Thr-(GalNAcα)Thr-Gly-Hex (muc2) (5/BSA)
Ac-S-Tn(Thr)-Tn(Thr)-G - 09	Ac-Ser-(GalNAcα)Thr-(GalNAcα)Thr-Gly-Hex (muc2) (9/BSA)
Ac-S-Tn(Thr)-Tn(Thr)-G - 22	Ac-Ser-(GalNAcα)Thr-(GalNAcα)Thr-Gly-Hex (muc2) (22/BSA)
Ac-S-Tn(Thr)-V-G - 04	Ac-Ser-(GalNAcα)Thr-Val-Gly-Hex (4/BSA)
Ac-S-Tn(Thr)-V-G - 22	Ac-Ser-(GalNAcα)Thr-Val-Gly-Hex (22/BSA
Ac-TF(Ser)-G - 04	Ac(Galβ1-3GalNAcα)Ser-Gly-Hex-BSA (4/BSA)
Ac-TF(Ser)-G - 24	Ac(Galβ1-3GalNAcα)Ser-Gly-Hex-BSA (24/BSA)

Ac-Tn(Ser)-Tn(Ser)-Tn(Ser)-G-03	Ac(GalNAcα)Ser-(GalNAcα)Ser-(GalNAcα)Ser-Gly-Hex-BSA (3/BSA)
Ac-Tn(Ser)-Tn(Ser)-Tn(Ser)-G-16	Ac(GalNAcα)Ser-(GalNAcα)Ser-(GalNAcα)Ser-Gly-Hex-BSA (16/BSA)
Ac-Tn(Ser)-Tn(Ser)-Tn(Ser)-G-27	Ac(GalNAcα)Ser-(GalNAcα)Ser-(GalNAcα)Ser-Gly-Hex-BSA (27/BSA)
Ac-Tn(Thr)-G - 21	Ac(GalNAcα)Thr-Gly-Hex-BSA (21/BSA)
Ac-T-Tn(Thr)-P-G - 04	Ac-Thr-(GalNAcα)Thr-Pro-Gly-Hex (4/BSA)
Ac-T-Tn(Thr)-P-G - 08	Ac-Thr-(GalNAcα)Thr-Pro-Gly-Hex (8/BSA)
Ac-T-Tn(Thr)-P-G - 21	Ac-Thr-(GalNAcα)Thr-Pro-Gly-Hex (21/BSA)
Ac-Tn(Thr)-Tn(Thr)-Tn(Thr)-G -05	Ac-(GalNAcα)Thr-(GalNAcα)Thr-(GalNAcα)Thr-Gly-Hex (muc2) (5/BSA)
Ac-Tn(Thr)-Tn(Thr)-Tn(Thr)-G -08	Ac-(GalNAcα)Thr-(GalNAcα)Thr-(GalNAcα)Thr-Gly-Hex (muc2) (8/BSA)
Ac-Tn(Thr)-Tn(Thr)-Tn(Thr)-G -20	Ac-(GalNAcα)Thr-(GalNAcα)Thr-(GalNAcα)Thr-Gly-Hex (muc2) (20/BSA)
Ac-V-Tn(Thr)-S-G - 04	Ac-Val-(GalNAcα)Thr-Ser-Gly-Hex (4/BSA)
Ac-V-Tn(Thr)-S-G - 08	Ac-Val-(GalNAcα)Thr-Ser-Gly-Hex (8/BSA)
Ac-V-Tn(Thr)-S-G - 19	Ac-Val-(GalNAcα)Thr-Ser-Gly-Hex (19/BSA)
GTSSASTF(Thr)GHATPLPTD	G-T-S-S-A-S-TF(Thr)-G-H-A-T-P-L-P-T-D-BSA
GTSSATF(Ser)TF(Thr)GHATPLPTD	G-T-S-S-A-TF(Ser)-TF(Thr)-G-H-A-T-P-L-P-T-D-BSA
GTSSASTGHATPLPTD	G-T-S-S-A-S-T-G-H-A-T-P-L-P-T-D-BSA
GTSSATF(Ser)TGHATPLPTD	G-T-S-S-A-TF(Ser)-T-G-H-A-T-P-L-P-T-D-BSA
GTSSASTGHATF(Thr)PLPTD	G-T-S-S-A-S-T-G-H-A-TF(Thr)-P-L-P-T-D-BSA

Controls

BSA	Bovine serum albumin
Cy3	Cy3-BSA (20μg/mL + BSA, 125μg/mL total)
Cy5	Cy5-BSA (30μg/mL+ BSA, 125μg/mL total)
HSA	Human serum albumin (isolated from serum)
HSA (recomb)	human serum albumin (recombinant)
PEG-linker	HO-(CH2)2-NH-Gly-CO-PEG7-NH-(CO)Hept-SH-Mal-Cychex-CO-BSA

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Significance

In this paper, we show that we can detect cancer-specific glycan modifications on thousands of proteins using a high-density antibody array paired with a glycan specific antibody to probe the bound glycoproteins. To our knowledge, our array is by far the largest and densest that has ever been used for global profiling of specific glycan modification on proteins. Analysis of colon cancer patient plasma for sialyl Lewis A and Lewis X modifications revealed previously unknown protein carriers of these modifications and significant increases in these specific glycans on some proteins in people with cancer versus healthy controls, suggesting this method could be used to discover novel biomarkers.

Highlights

- We use high density antibody arrays to detect cancer specific glycans on proteins.
- Sialyl Lewis A and Lewis X carrying proteins were profiled from human plasma.
- 35 proteins showed overexpression of Lewis antigens in colon cancer.

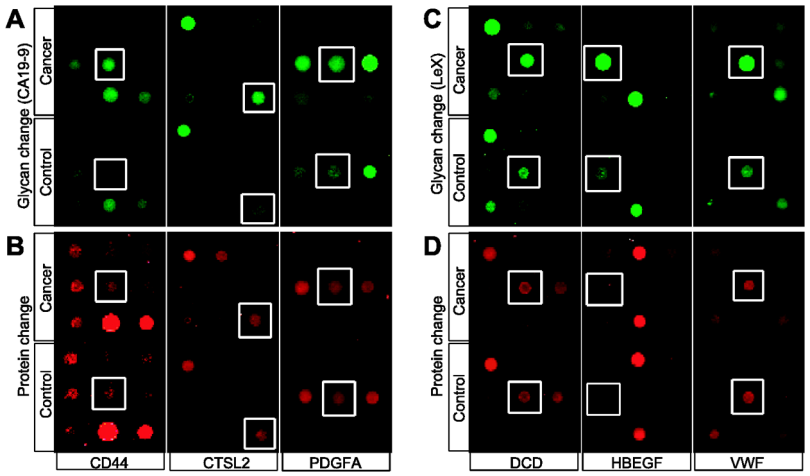

Figure 1. Protein and sialyl Lewis A and Lewis X expression in control and colon cancer plasma samples

Specific regions of the array are shown that include CD44, CTSL2, PDGFA, DCD, HBEGF and VWF proteins (boxed spots). (A, C) Glycan expression on proteins was profiled on the array with Cy3 labeled anti-sialyl Lewis A or Lewis X antibodies (shown as green). (B, D) The level of Cy-5 arrayed proteins for each sample at each spot is shown in red. Glycoproteins in the solid-outlined boxed regions show 1.1–2.2 fold change in colon cancer, but their sialyl Lewis A or Lewis X expression shows 2.7–7.5 fold higher levels in this specific cancer case than the healthy control.

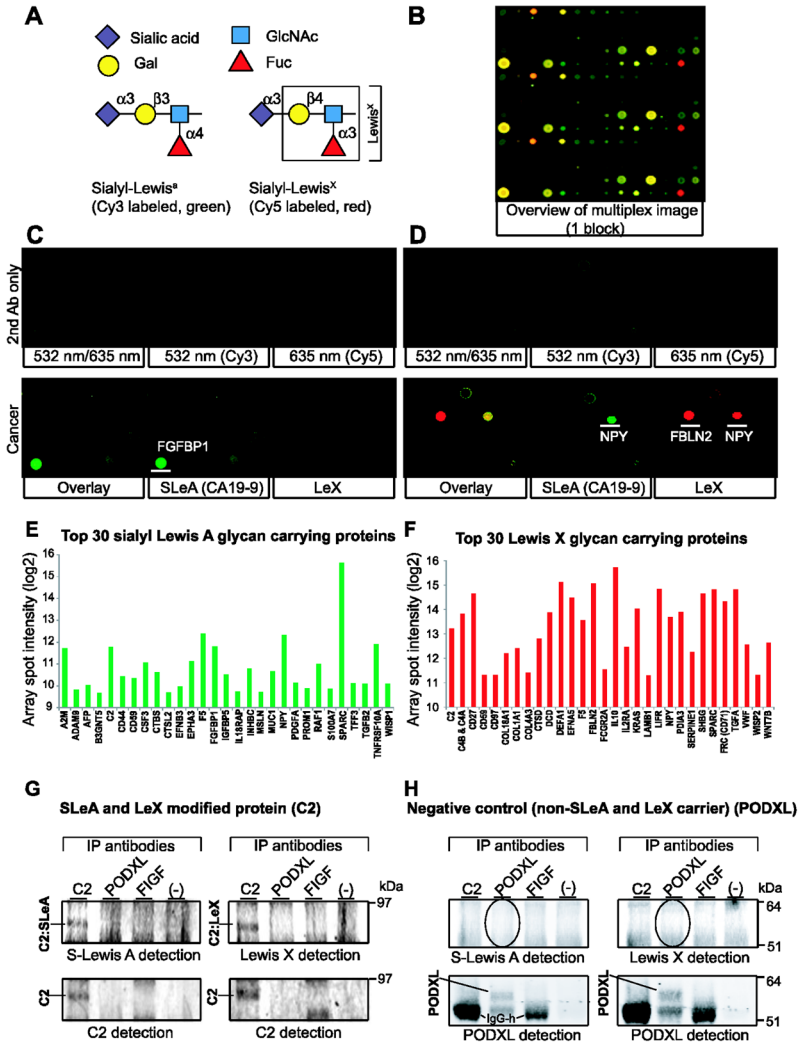

Figure 2. Performance of anti-sialyl Lewis A and Lewis X antibodies in multiplex array assays and detection of the higher abundance glycoproteins

(A) Sialyl Lewis A and X antigens are assembled from the same four monosaccharides and differ only in the position of their terminal fucose branches. The Lewis X portion of the structure is shown in the box. (B–D) A multiplex assay distinctively detected proteins expressing predominantly sialyl Lewis A (green spot), Lewis X (red spot) or both (yellow spots). (E,F) Plasma (10 μl) from a colon cancer patient was incubated on antibody array. Sialyl Lewis A and Lewis X expression on the captured proteins were measured and the 30 proteins with the highest glycan expression are presented after background subtraction. (G) Immunoprecipitated protein C2 (83 kDa) from a colon cancer patient plasma was detectable by anti-sialyl Lewis A and anti-Lewis X antibodies. As controls, proteins immunoprecipitated with other antibodies (PODXL and FIGF; generated in the same host and by the same manufacturer) and without any antibody (protein A resin only) were subjected to the same Western detection. No bands were detectable at the C2 protein size range. (H) PODXL protein (58 kDa) which showed no sign of sialyl-Lewis A and Lewis X modification by our microarray was not detectable by the same anti-sialyl Lewis A and anti-Lewis X antibodies. The similar control experiments as above were performed.

Table

Top 29 glycoproteins showing statistical significance for differences in 30 patients with late-stage colorectal cancers compared to 60 healthy controls.

	Sialyl Lewis A comparison					Lewis X comparison					Protein comparison					Combination markers					
																Protein+SLeA		Protein+LeX		Protein +SLeA+ LeX	
Gene name	Coef	p-value	q-value	AUC	* Sen	Coef	p-value	q-value	AUC	* Sen	Coef	p-value	q-value	AUC	* Sen	AUC	* Sen	AUC	* Sen	AUC	* Sen
ANXA2	0.328	0.003	0.165	0.730	0.433	0.447	<0.001	0.036	0.829	0.633	−0.410	0.035	0.055	0.724	0.482	n/d	n/d	n/d	n/d	n/d	n/d
B3GNT5	n /s	n/ s	n/ s	n /s	n/s	0.807	0.001	0.047	0.843	0.455	0.807	<0.001	0.008	0.796	0.467	0.811	0.500	0.895	0.727	0.914	0.773
CD44 (Ab 1)	0.525	0.003	0.165	0.755	0.310	1.083	<0.001	0.036	0.862	0.552	0.165	0.197	0.146	0.639	0.100	0.767	0.414	0.868	0.517	0.874	0.517
CD44 (Ab 2)	0.490	0.004	0.172	0.725	0.357	n/s	n/s	n/s	n/s	n/s	0.370	0.103	0.101	0.615	0.207	0.792	0.481	0.734	0.370	0.791	0.519
CD46	0.364	0.004	0.172	0.749	0.429	0.418	0.001	0.036	0.778	0.464	−0.447	0.001	0.008	0.787	0.273	n/d	n/d	n/d	n/d	n/d	n/d
CEACAM1	0.613	0.005	0.178	0.732	0.333	0.685	0.004	0.071	0.800	0.476	−0.612	0.021	0.044	0.714	0.333	n/d	n/d	n/d	n/d	n/d	n/d
CR3	n /s	n/ s	n/ s	n /s	n/s	0.311	0.003	0.062	0.762	0.444	−1.051	0.006	0.023	0.805	0.436	n/d	n/d	n/d	n/d	n/d	n/d
CSE	n /s	n/ s	n/ s	n /s	n/s	0.801	0.003	0.057	0.712	0.414	0.253	0.313	0.195	0.575	0.115	n/d	n/d	n/d	n/d	n/d	n/d
CTSE	n/s	n/s	n/s	n/s	n/s	0.502	<0.001	0.036	0.840	0.607	−0.567	0.018	0.038	0.685	0.203	n/d	n/d	n/d	n/d	n/d	n/d
DCD	n/s	n/s	n/s	n/s	n/s	0.561	0.003	0.060	0.740	0.241	−0.666	0.033	0.055	0.723	0.405	n/d	n/d	n/d	n/d	n/d	n/d
HSP90AB1	n/s	n/s	n/s	n/s	n/s	0.472	0.001	0.039	0.778	0.483	0.637	0.003	0.016	0.757	0.233	n/d	n/d	n/d	n/d	n/d	n/d
HSPC2	n/s	n/s	n/s	n/s	n/s	0.719	0.002	0.052	0.771	0.379	0.644	0.012	0.031	0.755	0.214	0.822	0.393	0.862	0.500	0.862	0.536
IL1B	0.630	0.001	0.085	0.698	0.241	0.829	<0.001	0.036	0.816	0.414	−0.376	0.113	0.107	0.619	0.143	n/d	n/d	n/d	n/d	n/d	n/d
IL6	n/s	n/s	n/s	n/s	n/s	0.676	0.002	0.057	0.760	0.379	0.457	0.072	0.081	0.658	0.172	0.685	0.250	0.774	0.464	0.773	0.464
INHBC	0.482	0.004	0.174	0.757	0.233	n/s	n/s	n/s	n/s	n/s	0.600	0.008	0.026	0.714	0.267	0.844	0.467	0.787	0.300	0.844	0.533
MASCO	n/s	n/s	n/s	n/s	n/s	0.454	<0.001	0.036	0.796	0.483	−0.324	0.158	0.129	0.617	0.293	n/d	n/d	n/d	n/d	n/d	n/d
MUC1	0.620	0.002	0.155	0.744	0.345	0.620	<0.001	0.036	0.844	0.621	−0.332	0.203	0.149	0.608	0.226	n/d	n/d	n/d	n/d	n/d	n/d
NOTCH2	n/s	n/s	n/s	n/s	n/s	0.618	0.003	0.062	0.734	0.367	0.098	0.697	0.319	0.547	0.000	n/d	n/d	n/d	n/d	n/d	n/d
NOTCH4	0.538	0.005	0.178	0.744	0.259	n/s	n/s	n/s	n/s	n/s	0.453	0.034	0.055	0.688	0.103	0.809	0.385	0.804	0.385	0.809	0.346
OAS1	n/s	n/s	n/s	n/s	n/s	0.507	0.004	0.071	0.705	0.433	−0.156	0.410	0.229	0.561	0.255	n/d	n/d	n/d	n/d	n/d	n/d
PECAM1	n/s	n/s	n/s	n/s	n/s	0.693	<0.001	0.036	0.844	0.500	0.290	0.473	0.249	0.602	0.200	n/d	n/d	n/d	n/d	n/d	n/d
PTPRC	n/s	n/s	n/s	n/s	n/s	0.460	0.001	0.045	0.773	0.407	-0.370	0.109	0.104	0.637	0.241	n/d	n/d	n/d	n/d	n/d	n/d
PZP	0.802	0.004	0.172	0.732	0.333	0.642	0.003	0.063	0.707	0.333	0.056	0.823	0.351	0.472	0.103	n/d	n/d	n/d	n/d	n/d	n/d
TIMP2	0.553	<0.001	0.074	0.810	0.517	0.676	<0.001	0.036	0.829	0.552	0.266	0.225	0.158	0.430	0.000	n/d	n/d	n/d	n/d	n/d	n/d
TLR2	n/s	n/s	n/s	n/s	n/s	0.313	0.004	0.064	0.772	0.483	−0.353	0.083	0.090	0.668	0.214	n/d	n/d	n/d	n/d	n/d	n/d
TNFSF9	0.615	<0.001	0.074	0.823	0.607	0.627	<0.001	0.036	0.806	0.571	−0.221	0.248	0.169	0.595	0.193	n/d	n/d	n/d	n/d	n/d	n/d

	Sialyl Lewis A comparison					Lewis X comparison					Protein comparison					Combination markers					
																Protein+SLeA		Protein+LeX		Protein +SLeA+ LeX	
VIL2	n/s	n/s	n/s	n/s	n/s	0.622	<0.001	0.036	0.840	0.586	−0.096	0.744	0.331	0.499	0.160	n/d	n/d	n/d	n/d	n/d	n/d
VWF	n/s	n/s	n/s	n/s	n/s	0.588	<0.001	0.036	0.855	0.741	0.298	0.168	0.133	0.606	0.214	0.787	0.360	0.900	0.720	0.884	0.680
WNT7B	n/s	n/s	n/s	n/s	n/s	0.640	<0.001	0.036	0.790	0.429	−0.113	0.620	0.297	0.515	0.175	n/d	n/d	n/d	n/d	n/d	n/d

*Sensitivity at 90% specificity, n/s: not signficant, n/d: not determined.