JOURNAL OF PROTEOMICS 96 (2014) 291–299

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Technical note

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

Jung-hyun Rho[a], *Judson R. Mead*[a], *W. Shea Wright*[b], *Dean E. Brenner*[c,e], *James W. Stave*[d], *Jeffrey C. Gildersleeve*[b], *Paul D. Lampe*[a,*]

[a]*Translational Research Program, Human Biology and Public Health Sciences Divisions, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, WA 98109, USA*
[b]*Chemical Biology Laboratory, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, MD 21702, USA*
[c]*Great Lakes New England (GLNE) Clinical Validation Center of EDRN, University of Michigan Medical Center, Ann Arbor, MI 48109, USA*
[d]*SDIX, LLC., Newark, DE 19702, USA*
[e]*VA Medical Center, Ann Arbor, MI 48105, USA*

ARTICLE INFO

Article history:
Received 31 July 2013
Accepted 19 October 2013
Available online 1 November 2013

Keywords:
Glycoproteins
Glycans
Sialyl Lewis A
Lewis X
Cancer biomarker
Antibody array

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.

Biological 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

* *Corresponding author at:* Fred Hutchinson Cancer Research Center, M5-C800, 1100 Fairview Ave. North, Box 19024, Seattle, WA 98109, USA. Tel.: +1 206 667 4123; fax: +1 206 667 2537.
E-mail address: plampe@fhcrc.org (P.D. Lampe).

1874-3919/$ – see front matter
http://dx.doi.org/10.1016/j.jprot.2013.10.030

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.

1. Introduction

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 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 29× higher reactivity to dimeric Lewis X, 12× for Lewis A-Lewis X, 6× for lacto-N-hexose (Gal-GlcNAc) and 4× 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.

1.1. *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 (10,800 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 contents 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