N-glycosylation status of ß-haptoglobin in sera of patients with prostate cancer vs. benign prostate diseases

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Abstract. N-glycosylation status of purified ß-haptoglobin separated from sera of patients with prostate cancer was studied in comparison to that of sera from patients with benign prostate diseases, or normal subjects. Two different approaches, as summarized below, one based on binding of lectins and antibodies to ß-haptoglobin, the other on mass spectrometry of released N-linked glycans from ß-haptoglobin, were performed. Some of the results were useful for distinction of prostate cancer vs. benign prostate diseases. i) Binding of *Phaseolus vulgaris*-L lectin (PHA-L), defining the GlcNAcß6Manα6Man side chain present in tri- or tetra-antennary N-linked glycans, to ß-haptoglobin was higher for cases of prostate cancer and high-grade prostate intraepithelial neoplasia than for benign diseases. Binding of *Aleuria aurantia* lectin (AAL) defining Fucα3-, α4-, or α6-GlcNAc, or monoclonal antibody directed to sialyl-Le^x, to ß-haptoglobin was also higher for some of the cancer cases than for benign diseases. Many other lectins and antibodies showed no binding to ß-haptoglobin, or showed no significant difference between cancer vs. benign diseases. ii) Mass spectrometric analysis of N-linked glycans of ß-haptoglobin released by Peptide N-glycosidase-F showed enhanced expression of monosialyl tri-antennary structures in prostate cancer cases. Thus, binding of PHA-L to affinity-purified ß-haptoglobin from sera of patients could lead to development of useful tools for differential diagnosis of prostate cancer vs. benign prostate diseases.

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Abbreviations: AAL, *Aleuria aurantia* lectin; ASAP, atypical small acinar proliferation; BSA, bovine serum albumin; CaP, prostate cancer; ELISA, enzyme-linked immunosorbent assay; HGPIN, high-grade prostatic intraepithelial neoplasia; HRP, horseradish peroxidase; LTL, *Lotus tetragonolobus* lectin; PHA-L, *Phaseolus vulgaris*-L lectin; PSA, prostate-specific antigen; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNA, *Sambucus nigra* lectin; TBS, Tris-buffered saline

Key words: haptoglobin, prostate cancer, lectin blotting, N-linked glycan, mass spectrometry

Introduction

Specific glycosyl epitopes defined by monoclonal antibodies (mAbs), such as sialyl-Le^a, sialyl-Le^x, sialyl-Tn, Tn, Gb3, globo-H, and GD3, are expressed preferentially in some types of human cancer. These epitopes have been used successfully for diagnosis (reviewed in ref. 1) and to some extent for therapy of human cancers (2-5); (reviewed in ref. 6 and 7). On the other hand, various tumors express higher levels of tri- or tetra-antennary structures as compared to bi-antennary structures (8,9), due to enhanced synthesis of the GlcNAcß6Manα6Man side chain, presumably by enhanced GlcNAc transferase-V expression (10,11). These changes of N-linked glycan structure in tumors may have diagnostic and prognostic value (9,12).

Haptoglobin, the hemoglobin-binding protein, is one of the major serum components. It consists of S-S linked α and ß subunits, with genetically defined polymorphism characteristic of the individual, and associated with diseases (13). Elevated levels of haptoglobins with different N-glycosylation status have been associated with cancer, inflammation and other diseases (reviewed in ref. 14). An increase of fucosylated haptoglobin (probed by *Lotus tetragonolobus* lectin) was found in sera of patients with various types of cancer (15).

Recently, the N-glycosylation status of haptoglobin from sera of patients with cancer has been studied in detail (16-18). The N-glycosylation sites of haptoglobin were found

exclusively in the ß-subunit and the level of N-glycosylation was enhanced at Asn 207 and 211 as compared to Asn 241 or Asn 184 of the ß-subunit isolated from prostate cancer, as compared to benign prostate disease (16). Haptoglobin isolated from sera of patients with pancreatic cancer was found to show enhanced reactivity with *Aleuria aurantia* lectin (AAL), suggesting the presence of Fucα6GlcNAc linked to Asn (17). However, the fucosylation site of haptoglobin in prostate cancer may be different from that in pancreatic cancer, since extensive mass spectrometry of released N-linked glycans from prostate cancer cases indicated that the fucosylation site was at the peripheral GlcNAc through α3 or α4 fucosylation at Asn 207 and 211, rather than α6 fucosylation at the core GlcNAc (16). Similarly, site-specific expression of sialyl-Le^x in the tri-antennary N-linked glycan at the Asn 211 site of haptoglobin in pancreatic cancer was clearly demonstrated recently (18).

The present study addressed a basic question, whether tri- or tetra-antennary N-linked glycan levels are increased relative to the bi-antennary glycan level in purified ß-chain of haptoglobin from sera of patients with prostate cancer vs. benign prostate diseases. For this purpose, blottability of purified ß-haptoglobin by *Phaseolus vulgaris*-L lectin (PHA-L) as compared to various other lectins and antibodies, was studied initially. Binding of PHA-L, but not of other lectins, was correlated with various cases of prostate cancer as opposed to benign prostate diseases. The structural basis of enhanced N-linked glycans associated with cancer was studied by mass spectrometry. Results indicated that enhanced expression of monosialylated, but not multi-sialylated, structures is closely associated with cancer cases.

Materials and methods

Serum samples. Serum samples of 21 patients with 'prostate cancer' (CaP, n=6, mean age 72 years), 'high-grade prostate intraepithelial neoplasia' (HGPIN, n=5, mean age 68), 'benign' (n=5, mean age 61), 'inflammatory prostate disease' (n=2, mean age 60), 'prostatitis' (n=2, mean age 54) and 'atypical small acinar proliferation (ASAP)' (n=1, age 63) were obtained from Seattle Urological Associates, Swedish Medical Center, Seattle, WA, USA. Because all patients had an enlarged prostate on digital rectal exam and/or rising or elevated PSA, they were subjected to prostate biopsies. Cores 9-21 (the number varied depending on the case) were taken in each biopsy section and categorization of patients as benign disease or prostate cancer was based on results from biopsy examination by a pathologist. The project was approved by the Ethics Committees of the participating hospitals and Pacific Northwest Research Institute. Serum samples of healthy volunteers (n=2, mean age 53) and combined samples from other healthy volunteers (n=7, mean age 49), were obtained in our laboratory. We did not examine PSA value, or perform rectal exam or prostate biopsy, for healthy volunteers, since all volunteers were young, healthy persons and many were female.

Antibodies and lectins. mAb (mouse IgM) directed to sialyl-Le^x (SNH3) (19) was established and prepared in our lab. mAb (mouse IgG1) directed to sialyl-Le^a (CA-19-9) (20), obtained from ATCC, was grown in our lab. Rabbit anti-human haptoglobin antibody was from Dako (Carpinteria, CA). Goat anti-rabbit IgG-horseradish peroxidase (HRP) was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse IgM-HRP and goat anti-mouse IgG-HRP were from Southern Biotech (Birmingham, AL). Lectins *Phaseolus vulgaris*-L-HRP (to Galß4GlcNAcß6Manα6Man side chain of tri- or tetra-antennary N-linked glycan), *Sambucus nigra*-HRP (to NeuAcα6- or -α3Gal/GalNAc), *Erythrina cristagalli*-HRP (defining Galß4GlcNAc), *Maackia amurensis*-HRP (to NeuAcα3Gal), *Wistaris floribunda*-HRP (to GalNAca6Gal) were from EY Laboratories (San Mateo, CA). Biotinylated *Aleuria aurantia* lectin (to Fucα3-, -α4-, or -α6GlcNAc) and biotinylated *Lotus tetragonolobus* lectin (to Fucα2Galß4 [Fucα3]GlcNAcß3Gal (H/Le^y)) were from Vector Laboratories (Burlingame, CA).

Preparation of hemoglobin. Hemoglobin was purified from human blood as described previously (21), with some modification. Briefly, blood cells were separated from blood by centrifugation at 3,000 rpm for 15 min and washed 5x with 3 volumes of PBS by similar centrifugation. Erythrocytes were lysed by addition of pure water (2 volumes) and centrifuged at 5,000 rpm for 30 min at 4˚C with angle rotor (Sorvall SA-600, Dupont, Newtown, CT). The lysate separated was added with ammonium sulfate until saturation (0.767 g/ml), stirred, stood in ice 30 min and centrifuged at 5,500 rpm for 40 min at 4˚C. The supernatant was dialyzed against 20 mM sodium phosphate, pH 8.0, overnight at 4˚C (molecular weight cutoff 12,000-14,000). Dialyzed solution of hemoglobin was concentrated through Centricon Plus 70 (NMWL 10,000, Millipore) by centrifugation at 3,000 rpm for 10 min (swing rotor). The concentration of hemoglobin as a total protein was determined by Micro BCA™ Protein assay kit (Pierce Biotechnology, Rockford, IL) based on standard curve using BSA (Sigma) in the concentrated solution when diluted 1: 10,000 by volume (132 mg/ml).

Preparation of Sepharose-4B column conjugated with hemoglobin. Conjugation of hemoglobin to Sepharose-4B was performed as described previously (21). Briefly, 5 g cyanogen bromide (CNBr) conjugated to Sepharose-4B (Sigma) was activated by suspending in 1 mM HCl (30 ml) at room temp for 30-60 min and filtered by gravity through glass filter, applying vacuum briefly at the end. The gel was washed twice with 20 ml 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0, transferred to a beaker and suspended in 25 ml of the same buffer. Small portions of concentrated hemoglobin stock solution (132 mg/ml, as above) were dropped into gel suspension under stirring, one by one, in total 50 mg, stirring continued 1 h at room temperature, filtered through glass filter without vacuum, washed with 200 ml 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0, transferred to a beaker, added with 25 ml 1 mM HCl for 2 h and filtered. Finally, the gel was washed with 200 ml 20 mM PBS with 0.15 M NaCl, pH 7.2, and the suspension was kept at 4˚C until use, or kept as column (e.g., 1.3x16 cm; volume ~14 ml). Hemoglobin or its conjugate to Sepharose is commercially available (Sigma).

Separation of haptoglobin from sera of patients by affinity chromatography on hemoglobin-Sepharose-4B column.

Venous blood was drawn into special plastic tubes (BD Vacutainer®, BD Diagnostics, Franklin Lake, NJ) containing clot activator, and centrifuged at 3,000 rpm for 10 min. Serum, separated as clear supernatant in 5 ml aliquots, was stored at -80˚C, or subjected to hemoglobin-Sepharose-4B column as follows: i) Serum placed on the column was drawn, 2 ml PBS was added slowly along the wall of the column, further washed with the same PBS buffer as above, flow rate 2 ml/min, fraction size 5 ml and washed continuously for fractions 1-12. ii) Eluted with 0.15 M NaCl, pH 11.0, flow rate 2 ml/min, fraction size 5 ml, took fractions 13-26. iii) Eluted with 5 M urea/0.15 M NaCl, pH 11.0, flow rate 2 ml/min, fraction size 5 ml, took fractions 27-36. Measured absorbance (λ=280 nm) using 100 μl fractions. Pooled this eluent, dialyzed in 0.1% acetic acid (MW cutoff 3,500) for 2-3 days and lyophilized.

Separation of haptoglobin ß-chain by gel filtration. Haptoglobin ß-chain was separated from crude hetero-tetramer haptoglobin, and cross-linked with inter and intra S-S bridges $(\alpha ß)_2$, which were cleaved as described below (21). Briefly, S-S linkage of lyophilized haptoglobin fraction from affinity chromatography on hemoglobin-Sepharose-4B column as above was reduced with 150 mM dithioerythritol, alkylated with 300 mM acrylamide, and subjected to Superdex 200 10/30 GL column chromatography (10x300 mm, GE Healthcare, Uppsala, Sweden), following filtration with Millipore Ultra-free-MC (0.45 μm). The flow rate and fraction size were 0.4 ml/min and 0.4 ml/tube, respectively. The fractions containing pure ß-chain, confirmed by silver staining, were pooled, dialyzed in distilled water and lyophilized. The protein quantity of ß-chain was determined by Micro BCA™ Protein assay kit based on standard curve using human haptoglobin (Sigma) and material was subjected to Western blot analysis, lectin blot, ELISA assay and MALDI-TOF mass spectrometry.

SDS-PAGE, lectin blot and Western blot analysis. Aliquots of 200 ng ß-haptoglobin based on Micro BCA™ Protein assay were mixed with SDS-PAGE sample buffer containing 5% 2-mercaptoethanol (2-ME) and boiled for 5 min. Proteins were separated on 10% polyacrylamide gel, transferred to PVDF membrane (Millipore Corp., Billerica, MA) and blocked with 3% BSA in TBS (140 mM NaCl, 10 mM Tris-HCl, pH 8.0) for 1 h at room temp. The BSA-blocked PVDF membrane was then blotted with HRP-labeled or biotinylated lectins, followed by re-blotting with anti-haptoglobin antibodies as follows: i) For HRP-labeled lectin, the membrane was washed 5x with TBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.1 mM $MnCl_2$ [TBS(+)] and incubated overnight at 4˚C with 20 μg/ml HRP-labeled lectin in TBS(+). The membrane was then washed gently 5x with TBS(+) and incubated with Supersignal West Pico Chemiluminescence substrate kit (Pierce). ii) For blotting with biotinylated lectin (e.g., biotinylated-AAL or -LTL), the membrane was incubated with the lectin overnight, then incubated for 30 min at room temp with Vectatastain ABC reagent (Vectastain Elite ABC kit, Vector Labs, Burlingame, CA), which was freshly prepared 30 min before use. iii) For the loading control of ß-haptoglobin, the same blot as in i) or ii) above was reblotted with anti-haptoglobin antibody as follows: The membrane was incubated with Stripping buffer (2% SDS, 62.6 mM Tris-HCl, pH 6.7, 0.78% 2-ME) for 20 min at 60˚C. After rinsing 5x (5 min each) with TBS/0.05% Tween-20 [T-TBS], the PVDF membrane was blocked with 5% non-fat dry milk in T-TBS for 2 h, washed and incubated overnight with primary anti-human haptoglobin rabbit antibody (Dako). HRP-coupled goat anti-rabbit IgG was used as secondary antibody. Supersignal West Pico Chemiluminescence substrate kit was used to enhance the bands as above. The intensity of bands blotted by lectins and anti-haptoglobin was determined by densitometric analysis using Scion imaging program (www.scioncorp.com/).

The relative intensity of band blotted by lectin between samples per same intensity of ß-haptoglobin blotted by anti-haptoglobin was calculated and termed 'Blotting index'.

Enzyme-linked immunosorbent assay (ELISA) of ß-haptoglobin. Since sensitivity of Western blot analysis with anti-carbohydrate antibodies was not satisfactory and results were often ambiguous, antibody binding to purified ß-haptoglobin was studied by ELISA procedure. ELISA plates (Pro-Bind™, #353915, Becton-Dickinson Labware, Franklin, NJ) were coated with 2 μg purified ß-haptoglobin (50 μl of 40 μg/ml haptoglobin solution on each well), and incubated overnight at 4˚C. After aspiration and washing 3x with 100 μl of 10 mM PBS/0.14 M NaCl, pH 7.0, plates were blocked with 200 μl of 3% BSA, washed 3x with 200 μl TBS (10 mM Tris-HCl/0.14 mM NaCl, pH 8.0) and incubated for 1 h at room temp with anti-sialyl-Le^x IgM mAb SNH3 or anti-sialyl-Le^a IgG1 mAb CA19-9. Each well was aspirated, washed 3x with 200 μl TBS and incubated with 50 μl HRP-labeled secondary antibodies for 1 h at room temp. For quantity control of ß-haptoglobin, the same amount of purified ß-haptoglobin from each case was coated on the same plate and the procedure described above was followed. After blocking with 3% BSA, the well was washed and incubated with 100 μl anti-haptoglobin (2 μg/ml) for 1 h at room temperature. Each well was aspirated, washed 3x with 200 μl TBS and incubated with 100 μl anti-rabbit IgG-HRP secondary antibody for 1 h at room temperature. Level of sialyl-Le^x or sialyl-Le^a in the same amount of purified ß-haptoglobin was determined by absorbance (λ=405, λ=630) using 0.5 mg/ml ABTs [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate in 50 mM citrate buffer (pH 4.0)/0.03% H_2O_2].

Analysis of N-linked glycans by MALDI-TOF mass spectrometry. Purified ß-haptoglobin preparations from prostate cancer (n=5) and benign prostate disease (n=5) were each reduced for 1 h at 37˚C in 50 mM Tris-HCl buffer (pH 8.5) containing a 4-fold excess of dithiothreitol and carboxymethylated with a 2-fold molar excess of iodoacetic acid for 1 h at room temperature in the dark. Following dialysis at 4˚C for 72 h against 4x4.5 liters of cold 50 mM ammonium bicarbonate, pH 7.5 and lyophilization, the samples were digested with sequencing-grade trypsin (Promega) (1 μg in 50 mM ammonium bicarbonate, pH 8.5, for 18 h at 37˚C). The reactions were stopped by adding a few drops of acetic acid to the solution. The samples were lyophilized, dissolved in 150 μl (5% (v/v) acetic acid and purified using a SepPak cartridge C_{18} (Waters Corp). The purified glycopeptides were

digested with PNGase-F (Roche Applied Science) in 50 mM ammonium bicarbonate (pH 8.5) containing 10 units of enzyme at 37˚C over 18 h. The sample was lyophilized and the released N-glycans were purified using a SepPak cartridge C_{18} (Waters Corp). Permethylation and sample clean-up were performed using the sodium hydroxide protocol, as described previously (22).

MALDI-TOF MS and MS/MS data for the permethylated samples were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. For MS/MS experiments, the collision energy was set to 1 kV and argon was used as collision gas. Samples were dissolved in 10 μl methanol and mixed at a 1:1 ratio (v/v) with 2,5-dihydro-benzoic acid as matrix.

The obtained MS data were subjected to semi-quantitative comparisons. The data were processed using Data Explorer 4.9 Software (Applied Biosystems). Each spectrum was baseline corrected (default settings) and noise filtered (with correction factor of 0.7). Customized peak de-isotoping was carried out before the MS data were converted to ASCII format. Peak lists were generated by manual peak picking using GlycoWorkbench (23) and data were analyzed using Microsoft Excel 2003.

Results

Clinical status of patients. Clinical diagnosis of prostate disease was established by prostate biopsy of all 21 cases. For prostate biopsy, there were 9 cores taken from 1 case (Case #6), 10 cores from 1 case (Case #4), 11 cores from 1 case (Case #12), 12 cores from 4 cases (Case #1, 8, 11, 21), 13 cores from 1 case (Case #2), 14 cores from 5 cases (Case #3, 9, 10, 13, 18), 15 cores from 3 cases (Case #16, 19, 20), 16 cores from 3 cases (Case #7, 14, 15), 19 cores from 1 case (Case #5) and 21 cores from 1 case (Case #17), as shown in Table IA. The degree of differentiation in prostate carcinoma was expressed as the Gleason score (24) diagnosed by a pathologist. Dates of birth of patients, sample collection date, stages and types of cancer, types of inflammation (acute or chronic), are summarized in Table IA. Of these, 5 cases (Case #4, 7, 10, 19, 21) were identified as high-grade prostate intraepithelial neoplasia (HGPIN), 6 cases (Case #6, 9, 11, 14, 15, 16) as prostate cancer (CaP), 5 cases (Case #3, 5, 8, 12, 18) as benign disease, 2 cases (Case #1, 20) as inflammatory prostate disease, 2 cases (Case #13, 17) as prostatitis, and 1 case (Case # 2) as atypical small acina proliferation (ASAP). Patchy chromic inflammation (Case #1), prostatitis (Case #13, 17) and benign (Case #3, 5, 8, 12, 18, 20) were categorized as benign disease. ASAP (Case #2) and HGPIN (Case #4, 7, 10, 19, 21) were categorized as neither cancer nor benign disease. Surgical operation was not yet performed in any of the cases. Local radioactive 'seed' therapy (brachytherapy, often with Strontium 90 plaque) was applied, or prepared for application, in some cases. Changes in PSA levels before and after treatment, and other follow-up information, are shown in Table IB.

Separation and purity of ß-haptoglobin. ß-haptoglobin was purified from sera of 21 patients by affinity chromatography on hemoglobin-Sepharose-4B column followed gel filtration chromatography on Superdex 200 10/30 GL column, as described in Materials and methods. Typical examples of affinity chromatography and gel filtration pattern are as shown previously (16). The elution profile of ß-haptoglobin on gel filtration chromatography and silver staining pattern of each fraction showing absorbance at 280 nm, are shown in Fig. 1. The chromatogram showed two peaks between fraction (Fr.) number 26 and 40 (Fig. 1A). The silver staining following 10% SDS-PAGE showed that ß- and α2-haptoglobin were eluted from Fr. 26 to Fr. 35 and from Fr. 34 to Fr. 37, respectively (Fig. 1B). Because Fr. 34 and 35 contained both ß- and α2-haptoglobin, we pooled the fractions from Fr. 26-33 containing pure ß-haptoglobin (without contaminant such as α2-haptoglobin), dialyzed, lyophilized, dissolved in distilled water and used for Western blot analysis, lectin blot, ELISA and N-linked glycan analysis.

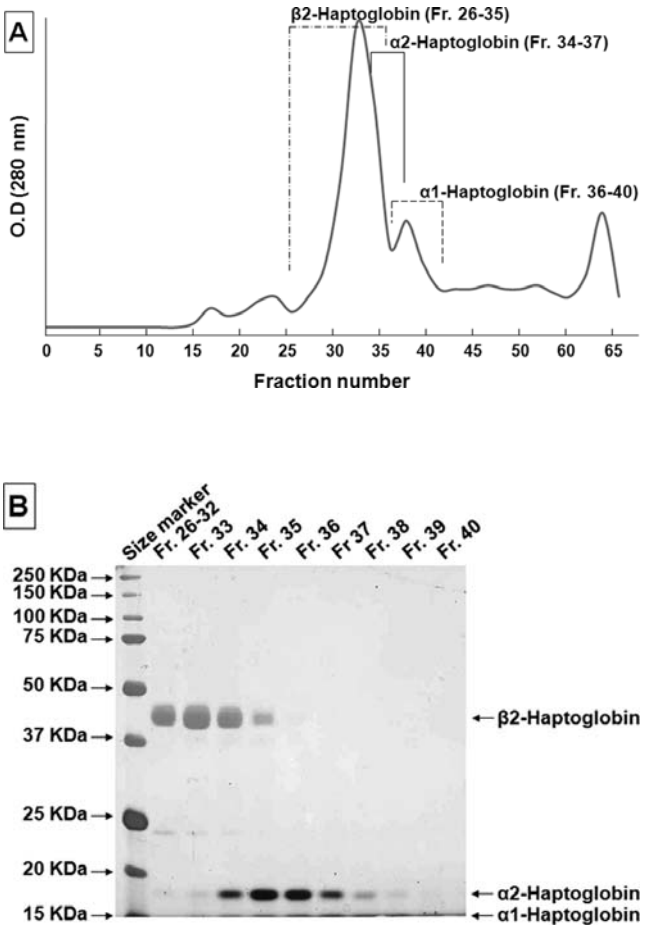

Figure 1. Gel filtration chromatography and silver staining. (A) Gel filtration chromatography. The lyophilized powder from hemoglobin-bound fraction was subjected to gel filtration chromatography using Superdex 200 10/30 GL column, following reduction and alkylation as described in Materials and methods. Elution profiles of haptoglobin chains were presented as chromatograms based on silver staining of each fraction, as shown in (B). (B) Silver staining. Ten μl of each fraction from gel filtration chromatography as in (A) was subjected to SDS-PAGE as described in Materials and methods, followed by silver staining (SilverSNAP Stain Kit II, Pierce, Rockford, IL) according to the manufacturer's instruction. Fractions containing pure ß-haptoglobin without contaminant were pooled, dialyzed, lyophilized and used for further experiments shown in Figs. 2, 3 and 4. Size marker, protein size marker; Fr. 26-32, pooled fraction numbers 26-32. Fr. 33 to 40, fraction number 33 to 40. Locations of α2- and ß-haptoglobin are indicated.

Table I. Clinical data.

A, Biopsy-based diagnosis and brief follow-up information of each case.

Case #	Date of birth	Date of sample collection	Biopsy		Follow-up information
			Cores taken	Diagnosis	
1	08/23/1948	02/16/2007	12	Patchy chronic inflammation	No further treatment/therapies
2	03/07/1944	02/16/2007	13	Atypical small acinar proliferation (ASAP)	No further treatment/therapies
3	02/12/1952	02/21/2007	14	Benign	No follow-up
4	07/10/1938	03/01/2007	10	High-grade prostate intraepithelial neoplasia (HGPIN)	No further treatment/therapies
5	04/14/1941	03/01/2007	19	Benign	No follow-up
6	01/10/1928	03/02/2007	9	Prostate cancer (CaP), Gleason score 4+4=8	Casodex & finasteride therapy. PSA declining, patient to follow-up in 6 months
7	04/18/1940	03/08/2007	16	High-grade prostate intraepithelial neoplasia (HGPIN)	Consistent PSA, no further treatment. Evaluate every 6 months
8	01/20/1943	04/05/2007	12	Benign	No follow-up
9	01/07/1939	04/09/2007	14	Multi focal HGPIN and single focus of moderately differentiated adenocarcinoma, Gleason score 3+3=6	Has elected for robotic assisted laparoscopy. Radical prostatectomy with colleague in different location
10	08/19/1931	04/09/2007	14	High-grade prostate intraepithelial neoplasia (HGPIN) and foci of atypia, highly suspicious for adenocarcinoma	DRE revealed enlarged prostate w/out evidence of nodules or induration. Reassess in 6 months
11	07/17/1934	04/13/2007	12	Prostate cancer (CaP), Gleason score 4+3 =7	see Table IB
12	11/21/1947	04/13/2007	11	Benign (suspected malignancy)	see Table 1B
13	02/05/1950	06/05/2007	14	Prostatitis	Rx quinolone; no further treatment/ therapies since
14	06/17/1951	06/05/2007	16	Prostate cancer (CaP), Gleason score 3+3=6	see Table IB
15	07/14/1924	06/08/2007	16	Prostate cancer (CaP), Gleason score 3+4=7	6/19/07 CaP discussion with MD; 9/11/07 steriotactic radiotherapy. Asked pt to return for follow-up in 3 months. No visit since
16	02/28/1934	06/14/2007	15	Prostate cancer (CaP), Gleason score 3+3=6	Bx 6/14/07, 7/6/07 CaP discussion with MD; referred to outside physician/ surgeon
17	03/28/1956	06/15/2007	21	Prostatitis	Rx quinolone; no further treatment/ therapies since
18	11/18/1942	06/15/2007	14	Benign	No follow-up
19	09/13/1942	06/15/2007	15	High-grade prostate intraepithelial neoplasia (HGPIN)	see Table 1B

Table IA. Continued.

Case #	Date of birth	Date of sample collection	Biopsy: Cores taken	Biopsy: Diagnosis	Follow-up information
20	02/02/1946	06/15/2007	15	Benign w/focal acute inflammation	Discussion re increasing PSA risks with MD, if continue to rise will req additional biopsy. Due in 12 months
21	03/03/1941	06/18/2007	12	High-grade prostate intraepithelial neoplasia (HGPIN)	see Table 1B

B, Cancer cases receiving specific treatment (cases #11, 14, 19, 21), a benign case (#12) displaying high variation of PHA-L blotting index and their follow-up information.

Case #	Sample collection	Follow-up information and PSA values (ng/ml)
11	4/13/2007	Biopsy 04/13/07 (biopsy used for study), diagnosis = prostate cancer; prostate cancer discussion 05/14/07 with physician; 08/22/07 brachytherapy; no further treatment/ therapies since. PSA: 4.4 on 03/02; 4.4 on 06/15; 3.79 on 09/12; 1.50 on 09.27.
12	4/13/2007	Biopsy 04/13/07 (biopsy used for study), diagnosis = benign prostate hypertrophy; patient to follow-up with PSA in 6 months; no further treatments/therapies since. PSA: 3.67 on 02/12; 3.9 on 03/09.
14	6/5/2007	Biopsy 06/05/07, diagnosis = prostate cancer (biopsy used for study); 06/20/07 prostate cancer discussion with physician; patient has not had treatment for prostate cancer as of 10/12/07, but likely to undergo brachytherapy in October sometime; no further treatment/ therapies since. PSA: 6.2 on 04/04; 7.9 on 05/10.
19	6/15/2007	Biopsy 11/03/06, diagnosis = high-grade prostatic intraepithelial neoplasia (HGPIN) (biopsy used for study); repeat biopsy on 07/20/07 with focal HGPIN again; patient to follow-up with PSA in 6 months; no further treatment/therapies since. PSA: 2.2 on 05/04; 3.0 on 08/14.
21	6/18/2007	Biopsy 06/18/07, diagnosis = HGPIN (biopsy used for study); patient to follow-up with PSA in 6 months; no further treatments/therapies since. PSA: 2.41 on 04/16; 2.93 on 05/21.

Blotting data of purified ß-haptoglobin with PHA-L and other lectins. Binding of PHA-L lectin to affinity-purified ß-haptoglobin, based on Western blot analysis pattern of 200 ng ß-haptoglobin, was higher for prostate cancer cases than for benign prostate diseases or normal subjects. The analysis was repeated 6 times for 200 ng samples from 21 cases. Relative densitometric intensity of PHA-L lectin binding pattern, divided by intensity of 200 ng ß-haptoglobin band stained by its antibody, from each case, is shown in Fig. 2A. Relative densitometric intensity of ß-haptoglobin from pooled normal sera (N) and that of commercial ß-haptoglobin (C), are also shown in Fig. 2A. Variation of the 6 determinations represented in each column is indicated by vertical bar.

Relative densitometric intensity of PHA-L binding vs. anti-ß-haptoglobin antibody binding was termed 'blotting index'. Out of 6 prostate cancer cases, the index was >0.6 in 4 cases, 0.4-0.6 in 1 cases, and 0.2-0.4 in 1 cases; there was no case with index <0.2. Out of 5 HGPIN cases, the index was >0.6 in 3 cases, 0.4-0.6 in 1 cases, and 0.2-0.4 in 1 case; there was no case with index <0.2. The index of one case with ASAP was 0.2-0.4. In contrast, out of 9 cases of benign prostate diseases, there was no case with index >0.6; index was 0.4-0.6 in 2 cases; 0.2-0.4 in 5 cases; and <0.2 in 2 cases. Commercial haptoglobin and all normal subjects showed index 0.2-0.4 and <0.2, respectively (Table II).

Reactivity of ß-haptoglobin with *Aleuria aurantia* lectin (AAL) (Fig. 2B) or with *Sambucus nigra* lectin (SNA) (Fig. 2C) was associated with some malignant cases, but the degree of this association was less than that for PHA-L (Fig. 2A). Various other lectins used (see Materials and methods) did not show clear binding to 200 ng ß-haptoglobin of any case (data not shown).

Reactivity of mAbs directed to sialyl-Le^x or to sialyl-Le^a with ß-haptoglobin. Previous mass spectrometric analysis of N-linked glycans released from haptoglobin ß-chain indicated the presence of Fucα3- or -4 linked to penultimate GlcNAc of NeuAcα3Galß4- or -3GlcNAc (16). Therefore, reactivity of ß-haptoglobin with mAb SNH3 (anti-sialyl-Le^x) or with mAb CA-19-9 (anti-sialyl-Le^a) was determined by ELISA, rather

Table II. Blotting index of prostate cancer, high-grade prostate intraepithelial neoplasia, atypical small acinar proliferation, benign cases and normal cases.

	Blotting index			
	<0.2	0.2-0.4	0.4-0.6	>0.6
Prostate cancer (CaP)				
Case 6 (CaP, 4+4=8)				+
Case 9 (multifocal HGPIN & adenocarcinoma, 3+3=6)				+
Case 11 (CaP, 4+3=7)			+	
Case 14 (CaP, 3+3=6)		+		
Case 15 (CaP, 3+4=7)				+
Case 16 (CaP, 3+3=6)				+
High-grade prostate intraepithelial neoplasia (HGPIN)				
Case 4 (HGPIN)				+
Case 7 (HGPIN)				+
Case 10 (HGPIN & foci of atypia)				+
Case 19 (HGPIN)		+		
Case 21 (HGPIN)			+	
Atypical small acinar proliferation (ASAP)				
Case 2 (ASAP)		+		
Benign				
Case 1 (patchy chronic inflam.)	+			
Case 3 (benign)		+		
Case 5 (benign)			+	
Case 8 (benign)			+	
Case 12 (benign)			+	
Case 13 (prostatitis)	+			
Case 17 (prostatitis)		+		
Case 18 (benign)		+		
Case 20 (benign)	+			
Normal subject	+			
Commercial haptoglobin		+		

Positive cases for CaP	(>0.6, 4/6)		67%
	(>0.4, 5/6)		83%
Positive cases for HGPIN	(>0.6, 3/5)		60%
	(>0.4, 4/5)		80%
Positive cases for ASAP	(>0.4, 0/1)		0%
	(0.2-0.4, 1/1)		100%
Positive cases for benign	(≥0.6, 0/9)	+	0%
	(0.4-0.6, 3/9)		
	(0.2-0.4, 3/9)	-	100%
	(<0.2, 3/9)		

than Western blot analysis, since reactivity of these anti-carbohydrate mAbs is often undetectable by Western blot analysis. Results are shown in Fig. 3A and B. There was no clear association with cancer vs. benign diseases, except that #11 (CaP) and #19 (HGPIN) showed high binding to anti-SLe^x. These cases, however, showed moderate binding (index 0.2-0.6) to PHA-L. This finding suggests that binary binding pattern with anti-SLe^x and with PHA-L has some advantage (see Discussion).

Mass spectrometric profiling of ß-haptoglobin N-linked glycans. N-linked glycans released by PNGase-F from samples 1-10 were analyzed by MALDI-TOF mass spectrometry as described in M&M. Data are shown in Fig. 4. The benign samples (1, 2, 3, 5, 8) are in Fig. 4A and the cancer samples (4, 6, 7, 9, 10) in Fig. 4B. Assignments for each of the molecular ions are given in Fig. 4C. The spectra are broadly similar, with biantennary glycans being the most abundant (m/z 2431, 2605, 2792 and 2966), together with lesser

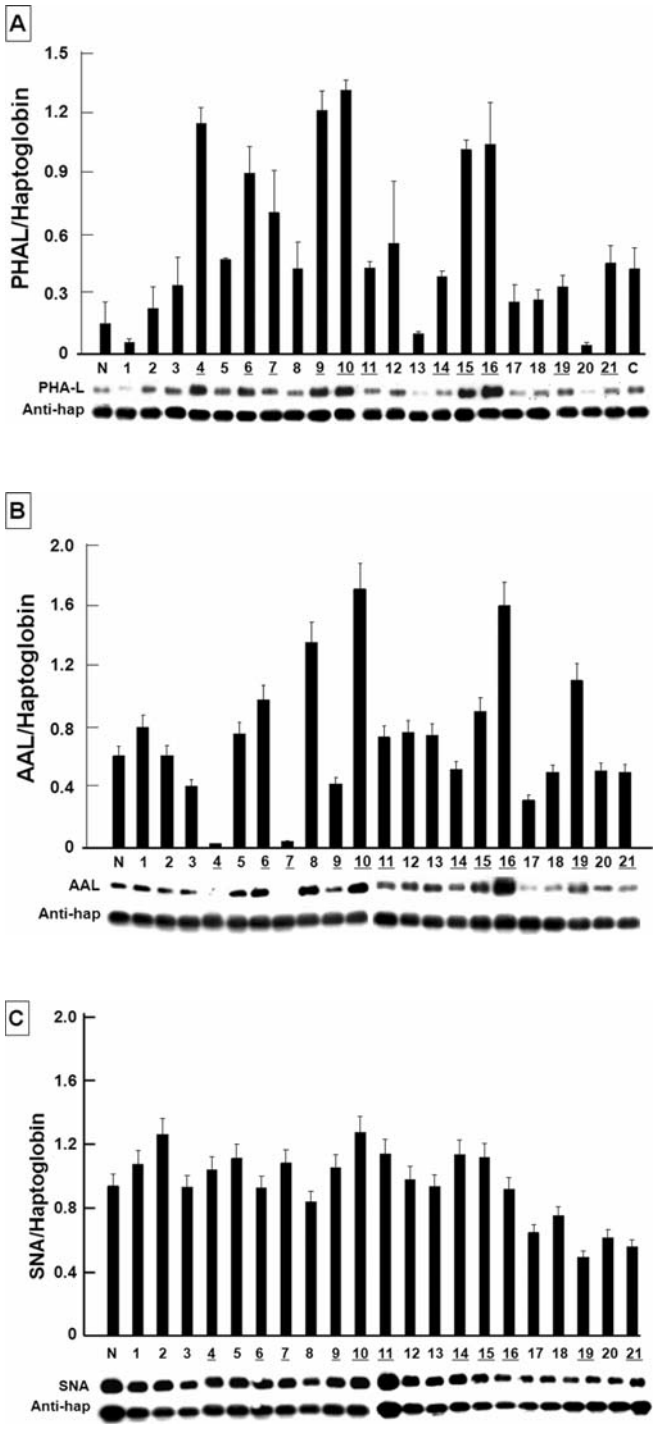

Figure 2. Lectin binding activity of purified ß-haptoglobin, by Western blot analysis with densitometric Scion image. Approximately 200 ng of purified ß-haptoglobin as shown in Fig. 1, determined by Micro BCA™ protein assay kit, from each of 21 cases as explained in Tables IA and B (abscissa; cancer cases indicated by underline), was subjected to lectin blot using HRP-labeled or biotinylated lectin, and the same blot was re-blotted with anti-ß-haptoglobin as loading control, as described in Materials and methods. ß-haptoglobins from pooled sera from 9 normal volunteers ('N'), or commercial source ('C'), were analyzed simultaneously under the same conditions. Densitometric comparison of each band was performed by Scion image program. The ratio of density by lectin to density by anti-haptoglobin antibody was expressed as 'blotting index' (ordinate). Mean ± SD is presented in each panel. (A) Blotting index of ß-haptoglobin given by HRP-labeled *Phaseolus vulgaris*-L lectin (PHA-L)/anti-haptoglobin antibody. Analysis was repeated 6 times. (B) Blotting index of ß-haptoglobin given by biotinylated *Aleuria aurantia* lectin (AAL)/anti-haptoglobin antibody. Analysis was repeated 3 times. (C) Blotting index of ß-haptoglobin given by HRP-labeled *Sambucus nigra* lectin (SNA)/anti-haptoglobin antibody. Analysis was repeated 3 times.

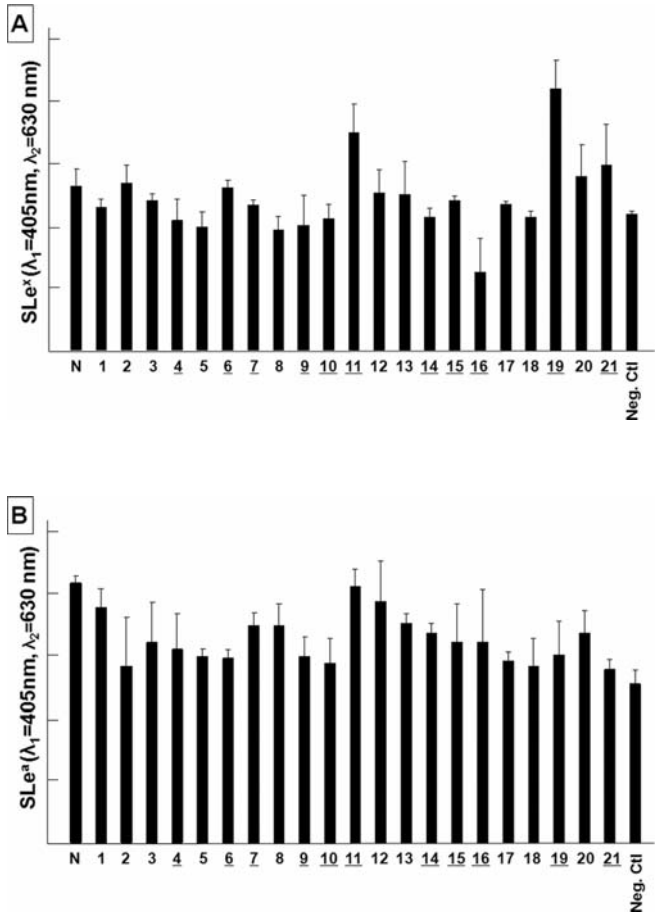

Figure 3. Enzyme-linked immunosorbent assay (ELISA) of ß-haptoglobin with mAb directed to defined tumor-associated glycosyl epitope. Purified ß-haptoglobin (2 μg) from serum of each of 21 cases as in Tables IA and B (abscissa; underlined numbers as in Fig. 2), or from pooled sera from 9 normal volunteers ('N'), was placed on the same ELISA plate at the same time, and subjected to ELISA procedure as described in Materials and methods. Assay was repeated 3 times and mean ± SD is presented. Neg. Ctl: As negative control, coating haptoglobin was omitted and distilled water was added to well instead of haptoglobin. (A) ELISA with IgM mAb SNH3 directed to sialyl-Le^x. (B) ELISA with IgG1 mAb CA-19-9 directed to sialyl-Le^a.

amounts of tri- (m/z 2880, 3054, 3242, 3416, 3603, 3777 and 3951) and tetra- (m/z 3330, 3691, 3865, 4052 and 4226) antennary glycans. All the glycans are sialylated. In addition, some carry a fucose on one or two antennae (m/z 2605, 2966, 3054, 3416, 3777, 3865, 3951, 4226), in accordance with previous studies (16). Semi-quantitative comparisons of the abundances of each of the molecular ions in the benign and cancer spectra were carried out as described in Materials and methods (Table III). This analysis indicated that levels of fucosylation were similar in all samples. However, there were clear differences between benign and cancer samples with respect to the amount of sialylation. This was significantly higher in the benign samples than in the cancer samples, as exemplified by the ratios of the disialyl:monosialyl bi-antennary glycans (m/z 2792:m/z 2431) which, with one exception (sample 3), were between 0.6 and 0.9 for benign samples, and between 0.06 and 0.4 for cancer samples. Similarly, with one exception (sample 7), monosialyl tri-antennary glycans (m/z 2880) were more abundant in the cancer samples, whereas disialylated triantennary glycans

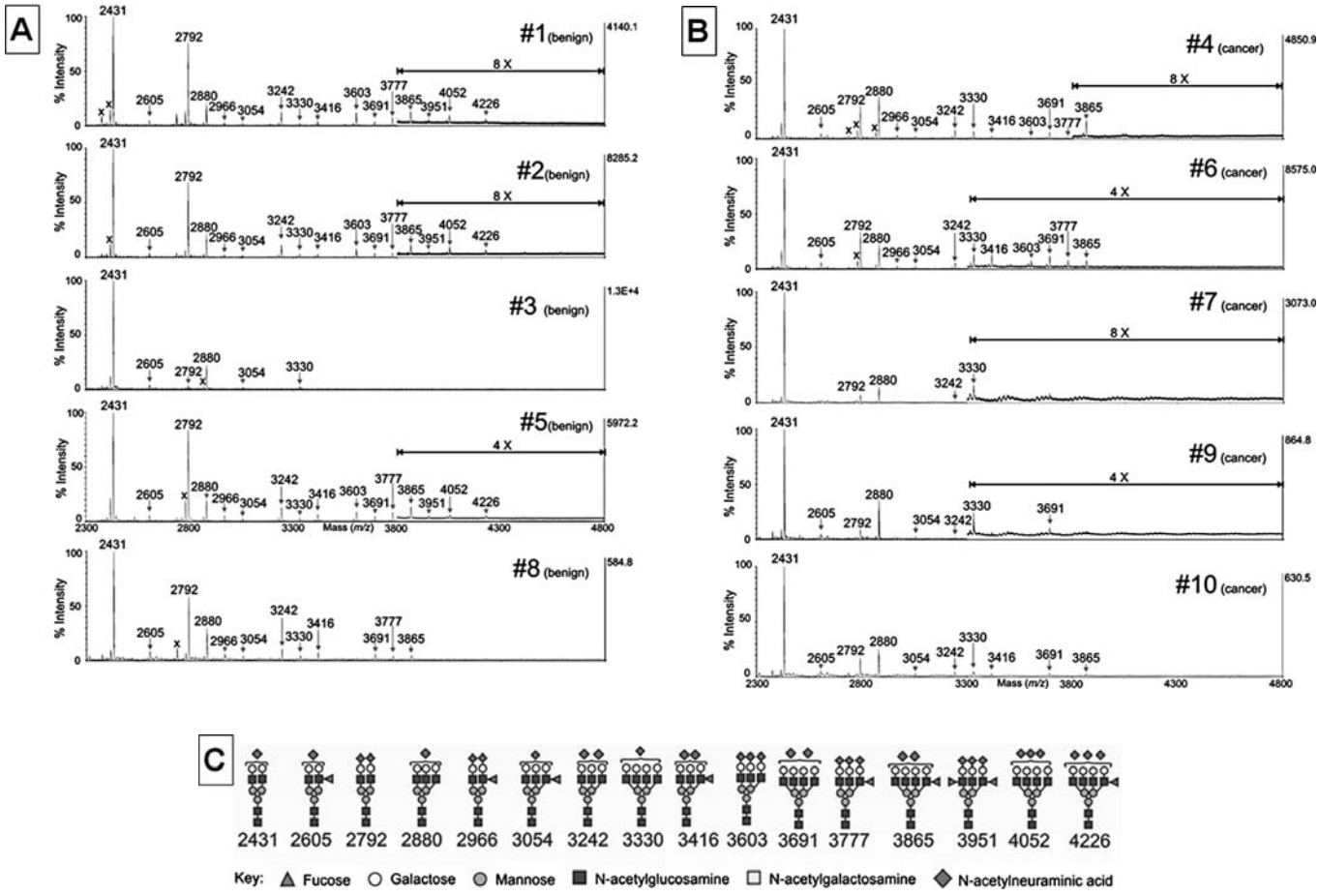

Figure 4. MALDI-TOF mass spectra and structures of 16 N-linked glycans from ß-haptoglobin. (A) MALDI-TOF mass spectra of N-linked glycans released from purified ß-haptoglobin from benign cases in Table IA. (B) MALDI-TOF mass spectra of N-linked glycans released from purified ß-haptoglobin from cancer cases in Table IA. (C) Structures, each assigned by mass number, of 16 N-linked glycans from ß-haptoglobin released by PNGase-F treatment, followed by MALDI-TOF mass spectrometry, as described in Materials and methods.

(m/z 3242), again with the single exception of sample 3, were more abundant in the benign samples (see Table III). Thus, the mass spectrometric data suggest that cases expressing high PHA-L binding may be correlated with high expression of monosialyl tri-antennary structures.

Discussion

Haptoglobin is an 'acute-phase protein' (25) that displays a rapid increase in response to inflammation, injury, wound healing, cancer and many other pathobiological processes, similarly to fibrinogen, ceruloplasmin, α-1 acid glycoprotein and C-reactive protein (CRP) (15,26). The increase of haptoglobin with aberrant glycosylation, probed by various lectins, has been a target of cancer diagnosis for many years (14-17). An increased level of haptoglobin with enhanced expression of Fucα6GlcNAc linked to Asn, defined by AAL binding, was claimed to be associated with pancreatic cancer (17). The glycosyl epitope was identified recently as a tri-antennary N-linked glycan with the sialyl-Le^x epitope present at Asn 211 site of ß-haptoglobin (18).

Aberrant glycosylation in prostate cancer was initially studied based on enhanced expression of the lacto-series type 2 chain epitope, defined by mAb 1B2, Le^y, defined by mAb AH6, or sialyl-Le^x, defined by mAb SNH3, in many cases of invasive prostate cancer (27). Much later, the 'RM2 antigen' expressed in renal cell carcinoma was identified as GalNAcß1-4 disialyl-Lc4 (28). The same RM2 antigen was later found to be highly expressed in prostate cancer tissue and was associated in a prostate cancer cell line with a ~40 kDa band on Western blot analysis, which was assumed to be haptoglobin (29,30).

Results of the present study, as summarized in Table II, clearly indicate higher blottability with PHA-L lectin for ß-haptoglobin from patients with prostate cancer (CaP) or high-grade prostate intraepithelial neoplasia (HGPIN; a precursor to development of invasive prostatic adenocarcinoma), compared to patients with benign prostate diseases. Higher PHA-L binding due to enhanced expression of GlcNAc transferase-V in tumors is caused by the direct effect of the Ets transcription factor, which is up-regulated by activation of oncogenes Src, Ras and Raf, leading to enhanced signaling causing cell proliferation (10); (reviewed in ref. 31). Enhanced GlcNAc transferase-V may cause activation and stabilization of matriptase (32) and the transferase *per se* causes tumor-associated angiogenesis (33), leading to enhanced metastasis.

Increased PSA level in serum cannot be considered a conclusive biomarker of prostate cancer, since PSA also

Table III. Structure and distribution pattern of N-linked glycans in cancer vs. benign cases.

		Benign case #					Cancer case #				
Structure	m/z	#1	#2	#3	#5	#8	#4	#6	#7	#9	#10
Bi-antennary	2431	100	100	100	100	100	100	100	100	100	100
	2605	6	4	5	6	9	5	6	0	5	6
	2792	65	80	6	92	58	31	41	7	6	15
	2966	4	3	0	6	5	4	3	0	0	0
Tri-antennary	2880	25	27	23	23	34	43	26	16	37	29
	3054	4	2	2	4	4	2	3	0	3	3
	3242	11	15	0	14	11	10	8	2	3	5
	3416	4	5	0	8	9	2	4	0	0	3
	3603	9	13	0	10	0	1	3	0	0	0
	3777	7	7	0	10	5	1	4	0	0	0
	3951	0	0	0	0	0	0	0	0	0	0
Tetra-antennary	3300	4	5	3	4	5	8	4	2	6	5
	3691	4	6	0	6	6	8	5	0	2	2
	3865	2	2	0	3	6	2	3	0	0	1
	4052	1	2	0	1	0	0	0	0	0	0
	4226	0	1	0	1	0	0	0	0	0	0

increases in benign prostate hyperplasia and prostatitis (34-36). Early-stage prostate cancer with PSA value 4-10 ng/ml cannot be distinguished from normal subjects. Furthermore, highly malignant prostate cancer often displays a low PSA value (34-36). The PSA value may reflect the size of the prostate gland, rather than the degree of active proliferation of prostate cells.

Structural analysis of N-linked glycans released by PNGase-F from ß-haptoglobin of cases #1-10 indicates the presence of 16 structures as shown in Fig. 4C. The abundance of monosialyl tri-antennary structure (m/z 2880), which is higher in the cancer samples, correlates with the PHA-L blotting index of ß-haptoglobin from prostate cancer, which is higher than that from benign prostate disease. A similar sialylation trend was observed for the biantennary glycans. Thus, a higher expression of monosialyl bi-antennary structure (m/z 2431) compared with its disialylated counterpart (m/z 2792) was observed in cancer cases than in benign prostate disease (Table III), although this structure does not react with PHA-L.

These monosialyl structures in ß-haptoglobin may be associated with specific Asn glycosylation sites, since recent studies indicate that α1-3 fucosylation to form sialyl-Le^x is located at Asn 211 of ß-haptoglobin from sera of pancreatic cancer patients (18). A possibility is opened that monosialyl N-linked glycans, regardless of bi-, tri-, or tetra-antennary structure, could be clustered at Asn 207 and 211 sites, since our previous studies indicate that N-linked glycans are more clustered at these sites, as compared to Asn 241 or 184 sites (16). Such clustered glycans at defined glycosylation sites may be more accessible to lectins and antibodies, because of the preferred conformational structure of haptoglobin.

Aberrant glycosylation of haptoglobin, associated with prostate cancer, was efficiently probed by lectins or antibodies, particularly PHA-L, AAL, SNA, and anti-sialyl-Le^x antibody. However, higher association of monosialyl N-linked glycans with cancer, as compared to multi-sialyl N-linked glycans, regardless of bi-, tri-, or tetra-antennary structure, was only detectable through mass spectrometric analysis. None of the lectins or antibodies could distinguish monosialyl vs. multi-sialyl N-linked glycans. Therefore, a combination of two approaches is needed in the discovery phase of putative biomarker identification: one involving binding assays of lectins or antibodies to intact ß-haptoglobin and the other systematic mass spectrometry of released oligosaccharides from ß-haptoglobin.

Mass spectrometry has been used successfully to solve many problems of structure and function of glycoconjugates (37-40). However, such approach is currently impractical for clinical diagnosis because of the limitations of existing technologies and the high operating costs of mass spectrometric instruments. Furthermore, reliable and reproducible mass spectrometric technologies for high-throughput glycomics are still under development. On the other hand, the utilization of lectin or antibody blotting of purified or semi-purified serum glycoprotein components, as described here, may lead to simpler and practical procedures for clinical diagnosis or prognosis.

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