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Multi-sequential surface plasmon resonance analysis of haptoglobin–lectin complex in sera of patients with malignant and benign prostate diseases

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ABSTRACT

Screening for prostate cancer remains unsatisfactory. Recent studies have examined the cancer diagnostic/prognostic values of various acute phase proteins, such as haptoglobin. We describe here a novel method of surface plasmon resonance (SPR) based on multi-sequential analysis with SNA-1, AAL, and PHA-L_4 lectin, to estimate the glycosylation status of haptoglobin in sera of patients with prostate cancer ($n = 15$), benign prostate disease (BPD) including benign prostatic hypertrophy ($n = 20$), and normal subjects ($n = 11$). The SPR-based analysis involves the use of anti-haptoglobin as ligand and dilution of the analyte to 1400-fold and filtration, followed by detection of the sugar chain by lectin solution. The normalized RU of lectin to haptoglobin represents the binding amount of lectin divided by that of haptoglobin. The normalized RU by SNA-1 of the prostate cancer group was significantly higher than those of the control and BPD group. SNA-1 detected NeuAcα2,6 in a biantennary sugar chain, whose content was the highest among the major glycoproteins in serum. Serum samples diluted about 7000-fold were subjected to microanalysis at 10 ng/μl and 10 μl/min for 4 min. The combination of SNA-1 and haptoglobin by SPR multi-sequential analysis offered the most accurate diagnosis of prostate cancer without any modification of serum glycoproteins.

The long-term outcome of cancer therapy depends on early diagnosis and the response to therapy. An important aspect of cancer management includes careful monitoring of cancer biomarkers in physiological fluids [1,2]. Measurement of the serum level of prostate-specific antigen (PSA)[1] has revolutionized the detection of prostate cancer [3–6]. Prostate cancer is the second most common cause of cancer death among men aged 60 to 79 years and the third most common among men aged over 80 [7]. However, high values of PSA are also found in some patients with benign prostatic hyperplasia (BPH). Specifically, the serum level of PSA in healthy men is ⩽4.0 ng/ml, and in some patients with highly suspicious prostate cancer, it could be within the "gray zone" (4–10 ng/ml) [8–10]. While a small proportion of PSA in the serum is in a free (F) form, the majority is complexed (C) with α1-antichymotrypsin (ACT) in prostate cancer. Serum total PSA and the F/T ratio are sometimes measured together in screening for prostate cancer especially in patients with serum PSA levels at the gray zone [11]. Evidence also suggests that measurement of the C-PSA is more accurate than the currently used total PSA [12].

The use of multiple markers for the diagnosis of early-stage carcinogenesis is important and should enhance screening for cancer [13]. Several studies have examined the diagnostic/prognostic values of various acute phase proteins, such as serum α1-antitrypsin (ATT), α1-acid glycoprotein (AGP), C-reactive protein (CRP), haptoglobin, and amyloid A proteins, in both inflammatory diseases and cancer [14,15]. In this regard, several studies have assessed the utility of haptoglobin in the diagnosis of hepatocellular carcinoma (HCC) [16], lung adenocarcinoma [17], ovarian cancer [18], pancreatic cancer [19], colon cancer [20], and breast cancer [21].

Changes in glycosylation occur in malignancy, immunodeficiency states, and autoimmune diseases. Naturally occurring glycans and neoexpressing glycans tend to be either under- or overexpressed in malignant tissues. Haptoglobin is a complementary marker to CA125 in ovarian cancer and induces chain glycosylation of both acute phase proteins and IgG [22]. Haptoglobin is fucosylated in pancreatic cancer [23] and HCC [24]. Site-specific analysis of *N*-glycan on haptoglobin has been applied recently in patients with pancreatic cancer [25] and a marked increase in serum levels of triantennary *N*-glycans containing Lewis X-type fucose at the Asn 211 site of haptoglobin was reported. The

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[1] *Abbreviations used:* BPD, benign prostate disease; BSA, bovine serum albumin; HCC, hepatocellular carcinoma; HRP, horseradish peroxidase; PSA, prostate-specific antigen; RU, resonance unit, SPR, surface plasmon resonance.

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α1-3/α1-4/α1-6 fucosylation of haptoglobin was also detected by lectin blotting with *Aleuriaaurantia* lectin (AAL).

The RM2 antigen (β1,4-GalNAc-disialyl-Lc_4) is a new marker of prostate cancer. Saito et al. [26] examined 75 patients with prostate adenocarcinoma and found weak to no expression of RM2 in 20 and moderate to high expression in 55 cases. In a previous report, we also studied the level and glycosylation status of haptoglobin in sera of patients with prostate cancer, and compared these levels to those in patients with BPD and normal subjects. Sialylated biantennary glycans were dominant in all three groups and triantennary, N-linked fucosylated glycans were predominantly located on N207 and N211 of haptoglobinin patients with prostate cancer. The expected presence of disialylated antennary with GalNAcβ4(NeuAcα3)Galβ3(NeuAcα6)GalNAcβGal, or its analog, despite cross-reactivity of prostate cancer haptoglobin with monoclonal antibody RM2, was not observed, and also, small levels of mono- and disialyl core type 1 O-linked structures were identified in prostate cancer [27]. Furthermore, RM2 immunoreactivity correlated significantly with high serum haptoglobin levels [28]. In this regard, serum haptoglobin β-chain can be identified by monoclonal antibody RM2, a novel serum marker of prostate cancer [28].

The current techniques employed to detect interactions between antigens and antibodies such as enzyme linked immunosorbent assay (ELISA), conventional methods used for the detection of cancer markers or acute phase proteins, usually require labeling with enzymes, radioactive isotopes, or fluorescein, and are thus complex and time-consuming procedures.

The above methods are also not a suitable platform for analysis of successive interactions that can simultaneously measure both the carrier protein level and the attached glycoform level [29,30]. Since the surface plasmon resonance (SPR) technique provides label-free and real-time detection, it has attracted attention as a biosensor [31,32]. Biosensors based on SPR are extensively used to monitor molecular interactions based on their outstanding sensitivity, reliability, reproducibility as well as capability of monitoring multiple successive interactions [33–38].

Here we report the development of a novel SPR multisequential analysis for the detection of haptoglobin and glycosylated haptoglobin using various lectins (SNA-1, AAL, and PHA-L_4). Subsequently, we used the calculated resonance unit (RU) ratio of the sugar/haptoglobin to compare the serum levels of haptoglobin and modified sugar chains among patients with prostate cancer and BPD and healthy subjects.

Material and methods

Materials

PHA-L_4 (*Phaseolus vulgaris*) and its horseradish peroxidase (HRP)-derivative, and AAL (*Aleuria aurantia*) and its biotin-derivative were purchased From J-Oil Mills (Tokyo, Japan). SNA-1 (*Sambucus nigra)* and MAA (*Maackia amurensis*) and the corresponding HRP-derivative and HRP-coupled AAA (*Angullla anguilla*) were from EY Laboratories (San Mateo, CA). Streptavidin-HRP conjugate was obtained from Invitrogen (Carlsbad, CA) (Table 1).Polyclonal rabbit anti-human haptoglobin was purchased from Dako cytomation (Glostrup, Denmark). Monoclonal mouse anti-sialyl-Lewis a was from Wako Pure Chemical Industries (Osaka, Japan). Anti-rabbit IgG-HRP and anti-mouse IgG-HRP were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Haptoglobin from pooled human plasma was purchased from Sigma–Aldrich (St. Louis, MO).

Collection of serum samples

Blood samples (5 ml each) were obtained from 15 patients with prostate cancer, 20 patients with BPD, and 11 healthy men at the Department of Urology, Juntendo University Hospital, Tokyo, Japan. The study protocol was approved by the Human Ethics Review Committee of Juntendo University Hospital and School of Medicine and a signed consent form was obtained from each subject. The collected samples were centrifuged at 3000 rpm for 15 min and the supernatant was divided into 0.3 ml aliquots and preserved at −80 °C until use. Serum protein concentration was measured by a BCA protein assay reagent kit (Thermo Fisher Scientific Inc., Rockford, IL) using a calibration curve constructed with bovine serum albumin (BSA) as a standard protein.

Measurement of serum PSA level and assessment of pathological state

Serum total PSA was measured by an AxSYM analyzer (Abbott Laboratories, Abbott Park, IL). The diagnoses of prostate cancer and BPD were based on histopathological examination of prostate tissue, and the grade of prostate malignancy was expressed by the Gleason score. Measurement of serum PSA level and histopathological examination were conducted at the Department of Clinical Laboratory and Department of Diagnostic Pathology, Juntendo University Hospital, respectively.

Surface plasmon resonance

SPR analysis was performed using a BIAcore2000 (GE Healthcare UK, Amersham Place, UK). Anti-haptoglobin polyclonal antibody was immobilized on the sensor chip C1 by the amine coupling method using the protocol supplied by the manufacturer. Prior to immobilization, the instrument with the docked sensor chip was washed. Briefly, a washing buffer consisting of 0.1 M glycine/NaOH, pH 12.0, and 0.3% Triton X-100 was injected three times for 2 min at a flow rate of 30 μl/min, using HBS-P buffer (10 mM Hepes, pH 7.4, 150 mM NaCl and 0.005% surfactant P20) as running buffer. Subsequently, the surface of the sensor was activated with a mixture of NHS (*N*-hydroxysuccinimide) and EDC (*N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide) and coupled with 200 μg/ml of anti-haptoglobin in 10 mM acetate buffer, pH 5.5, at a flow rate of 5 μl/min. Each step was performed over 7 min. The remaining residues were deactivated with 1 M ethanolamine hydrochloride, pH 8.5, for 12 min. This procedure immobilized about 1200 RU. Out of four flow cells, the first (Fc1) immobilized no ligand and was used as the blank cell.

Sequential analysis of haptoglobin binding and lectin

The protein concentration in serum samples was diluted to 1 mg/ml with running HBS-P (+) buffer (containing 0.9 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.1 mM $MnCl_2$, and 0.2 mg/ml BSA), and then filtered through 0.45 μm ultrafree-MC centrifugal filter devices (Millipore, Billerica, MA). The filtrate was diluted again to 50 ng/μl using the running buffer. Sequential binding analysis was performed and in the first step, the amount of haptoglobin in the diluted sera was measured with the sensor chip for 4 min at a flow rate of 10 μl/min (Fig. 1). In the second step, 100 μg/ml of lectin was injected sequentially for 4 min. The sensor chip was regenerated by repeating the injection 10 times for 1 min with a mixture containing 10 mM Gly/HCl, pH 1.7, 1 M NaCl, and 0.1% Tween 20. Each measurement was performed in duplicate. The binding RU of haptoglobin and sugar chain was measured, and the serum concentration of haptoglobin was determined using a calibration curve of multi standard V-SH2 (Nittobo Medical, Tokyo, Japan). The results were evaluated by BIA evaluation software 4.1. Lectin binding represented the net RU of lectin for 4 min (Fig. 1d) divided by the net RU of haptoglobin after 2 min from the end of haptoglobin injection (Fig. 1b). The value of normalized lectin binding was compared among patients with prostate cancer, BPD, and healthy