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α1,2-Fucosylated and β-*N*-acetylgalactosaminylated prostate-specific antigen as an efficient marker of prostatic cancer

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A prostate-specific antigen (PSA) is widely used as a diagnostic marker for prostate cancer (PC) because of its high specificity. However, elevated serum PSA does not occur only in PC but also in benign prostatic hyperplasia (BPH). Since the structural changes of *N*-glycans during carcinogenesis are common phenomena, we investigated whether PC-specific *N*-glycans are linked to PSA. We first analyzed the carbohydrate structures of PSA derived from seminal fluid, serum of BPH and PC patients, and PC cell line, namely, LNCaP using eight lectin-immobilized columns and then with enzyme-linked immunosorbent assay (ELISA). The fraction of serum PSA from PC patients bound to both Fucα1-2Gal and βGalNAc binding *Trichosanthes japonica* agglutinin-II (TJA-II) column, while that from BPH patients did not exhibit this binding ability, thereby implying that there is elevated expression of α1,2-fucosylation and β-*N*-acetylgalactosaminylation of PSA during carcinogenesis. We then performed a real-time polymerase chain reaction (PCR) and confirmed that these structural changes correlated with the elevated expression of fucosyltransferase I (FUT1) and β-*N*-acetylgalactosaminyltransferase 4(B4GALNT4). Second, we measured TJA-II-bound PSA contents and the binding ratios of TJA-II column chromatography in serum PSA samples from 40 patients of both PC and BPH. The results indicated that both TJA-II-bound PSA content and TJA-II binding ratios (%) could be used to discriminate between PC and BPH with more than 95% probability, and TJA-II-bound PSA can be regarded as a potential marker of PC.

Keywords: glycosyltransferase/lectin/prostate cancer/prostate-specific antigen

Introduction

Prostate cancer (PC) is the most prevalent form of cancer in men in developed countries. In the past decade, there has been a remarkable increase in the incidence of PC throughout the world, with the mortality rates increasing in many countries with considerable geographic variation. The increased incidence of PC is related to the widespread use of serum prostate-specific antigen (PSA)-based screening of asymptomatic men. PSA is a serine protease encoded by the *KLK3* gene (Lundwall and Lilja 1987), which contains 237 amino acids and a single *N*-linked glycan at Asn45 (Bélanger et al. 1995). PSA produced by the prostate is primarily excreted in the semen but a small proportion enters the blood. Diagnosis of PC through PSA detection is based on the fact that cancerous prostate tissues release 30 times more PSA into circulation than normal prostate tissues (Stamey et al. 1987), and the serum PSA levels are proportional to the increasing palpable stages of prostate cancer as determined by digital rectal examination (DRE). However, the elevated serum PSA concentration occurs not only in PC but also in benign prostatic hyperplasia (BPH). The serum PSA is found either in free form or bound form with plasma proteins. The major binding proteins are α1-antichymotrypsin (Christensson et al. 1990) and α2-macroglobulin, both of which are extracellular protease inhibitors abundantly present in the serum. Measuring free PSA (F) and total PSA (T) in serum and determining F/T ratio are found to improve the diagnosis of prostate cancer (Lilja et al. 1991; Stenman et al. 1991). Recent trends in PC diagnosis (e.g., lower serum PSA cutoff (Catalona et al. 1997), F/T ratio (Stenman et al. 1991; Catalona et al. 1995), complexed PSA (Brawer et al. 1998), pro PSA, and BPSA (benign prostate-specific antigen) (Okihara and Babaian 2001)) have led to an increase in the number of men treated for presumed PC. However, these new methods of PSA diagnosis still cannot discriminate between PC and BPH, and there is a clear need to develop novel PC-specific molecular markers.

It is well known that the structure of carbohydrates produced by cells changes during carcinogenesis (Yamashita and Kobata 1996a, 1996b); in keeping with this fact, numerous tumor-specific carbohydrate markers, such as CA19-9, have been used for clinical purposes. The structures of carbohydrates on PSAs derived from human seminal fluid, serum and tissues of BPH and PC patients, and PC cell lines have been analyzed and reported by many researchers. The main structures of seminal PSA glycans were found to be sialylated biantennary complex-type oligosaccharides (Bélanger et al. 1995; Sumi et al. 1999; Prakash and Robbins 2000; Okada et al. 2001; Peracaula et al. 2003; Ohyama et al. 2004; Tajiri et al. 2008). PSAs from PC tissues and a PC cell line, namely, LNCaP, were reported to contain complex-type oligosaccharides with higher antennas than those from BPH tissues and seminal fluid (Sumi et al. 1999; Prakash and Robbins 2000). Ohyama et al. (2004) performed lectin column chromatography and determined the amount of free PSA; they found that the amount of *Maackia amurensis* lectin (MAL)-bound fraction of free PSA, which contains Siaα2-3Gal residues, in the serum of PC patients was higher than that in the serum of BPH patients. However, the clinical application of MAL is limited as the target glycans of MAL are linked to

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α1-antichymotrypsin (Laine et al. 1991) and α2-macroglobulin (Bezouška et al. 1985); therefore, it is necessary to identify new components with the ability to bind free PSA, which is lowered in the serum of PC patients. The structure of peripheral carbohydrates of serum PSA has not yet been elucidated because the amount of serum PSA is too low; however, studies on PSA derived from LNCaP cells suggest that these PSAs contain fucosylated and *N*-acetylgalactosaminylated residues (Peracaula et al. 2003). In this study, we analyzed the structures of peripheral carbohydrates of PSA from seminal fluid, serum of BPH and PC patients, and LNCaP cells using β-*N*-acetylgalactosamine (β-GalNAc)- and (Fucα1-2Gal)-specific lectin column chromatography and enzyme-linked immunosorbent assay (ELISA). The results of our study suggest that PSA-containing cancer-specific glycans are efficient diagnostic markers of PC and can be used to discriminate between PC and BPH.

Results

Comparative studies of the sugar chain structures of PSAs derived from the serum of PC and BPH patients, LNCaP cells, and seminal fluid of normal subjects using various lectin columns

Several papers have elaborated the carbohydrate structures of PSA derived from PC and BPH tissues, seminal fluid, and LNCaP cells (Sumi et al. 1999; Prakash and Robbins 2000; Okada et al. 2001; Peracaula et al. 2003; Ohyama et al. 2004; Tajiri et al. 2008). However, the information on serum PSA is limited because of its low concentration. Since PSA has only 1 *N*-linked glycan at the Asn45 of the mature peptide (Bélanger et al. 1995), the carbohydrate structures can be analyzed quantitatively using lectin column chromatography (Yamashita and Kobata 1996a). To determine the structures of *N*-linked glycans of serum PSA, we compared the lectin-binding abilities of free PSA derived from the serum of BPH and PC patients, seminal fluid of normal subjects, and LNCaP cells by performing lectin column chromatography, as summarized in Table I. The serum samples were fractionated in eight different lectin columns and the amount of free PSAs in each eluate was measured using the free PSA analyzing kit. To check whether the respective lectin column chromatography interacted with glycans of PSA specifically, the recovery was calculated after eluting with the corresponding haptenic sugars and 0.1 N acetic acid for DSA (Table I). Except MAL-Sepharose column chromatography, the recovery of PSA from the remaining seven lectin columns was constantly over 95%, suggesting that the binding abilities for these seven lectin column chromatographies used in this study reflect the corresponding carbohydrate structures of PSA quantitatively. First, the *N*-glycan backbone structures were compared using UDA, LCA, and DSA: UDA recognizes high-mannose-type glycans (unpublished data); LCA recognizes the fucosylated tri-mannosyl core of complex-type *N*-glycans (Kornfeld et al. 1981); and DSA recognizes tri- and tetra-antenna *N*-glycans (Yamashita et al. 1987). About 10% of the PSAs in the eluates bound to the UDA-agarose column and over 60% bound to the LCA column, suggesting that the PSAs mainly contained complex-type glycans. Furthermore, a large amount of seminal PSA and serum PSA of BPH patients passed through the DSA-Sepharose column, while substantial amount of serum PSA from PC patients and PSA from LNCaP cells was bound and eluted with 1% GlcNAc oligomer and sequential 0.1 N acetic acid, thereby indicating that the amount of tri- or tetra-antennary complex-type *N*-glycans increased during the development of PC. The recovery of DSA columns using 1% GlcNAc oligomer was between 41% and 78%. It was because 2,4-branched triantennary glycans linked to PSA were eluted by 1% GlcNAc oligomer, and 2,6-branched ones were not eluted by 1% GlcNAc oligomer, but 0.1N acetic acid same as those of serum transferrin purified from hepatocellular carcinoma (Yamashita et al. 1989). Second, the structures of nonreducing terminal sides were compared using the following lectins: MAL, which specifically recognizes Siaα2-3Galβ1-4GlcNAc residues (Wang and Cummings 1988); TJA-I, which recognizes the Siaα2-6Galβ1-4GlcNAc residue (Yamashita et al. 1992a); TJA-II, which recognizes both Fucα1-2Gal and β-GalNAc residues but not Siaα2-6GalNAcβ residues (Yamashita et al. 1992b); UEA-I, which recognizes the Fucα1-2Galβ1-4GlcNAc residue (Hindsgaul et al. 1982); and WFA, which recognizes the nonsubstituted terminal β-GalNAc residue but not Siaα2-6GalNAcβ residue (Ohkura et al. 2002). The serum PSA of BPH patients was bound to the TJA-I-Sepharose column and subsequently eluted with 0.1 M lactose, whereas most of the serum PSA of BPH patients passed through the TJA-II-, UEA-I-, WFA-, and MAL-Sepharose columns (Table I, Figure 1A), indicating that serum PSA of BPH patients contains exclusively Siaα2-6Galβ1-4GlcNAc residues and it does not contain Siaα2-3Galβ1-4GlcNAc, Fucα1-2Galβ1-4GlcNAc or nonsubstituted terminal β-GalNAc residues. The TJA-II-binding ability (%) of serum PSA of BPH patients did not change after

Table I. Lectin-binding abilities (%) of free PSAs from BPH serum, PC serum, PC cell line, LNCaP, and human seminal fluid

Lectin	Recognition sugar	BPH serum PSA[a]	PC serum PSA[b]	LNCaP cell PSA	Seminal PSA	Haptenic sugar
UDA	High-mannose-type glycans	8.2%	8.8%	16%	7.3%	1% GlcNAc oligomer
LCA	Fucosylated tri-mannosyl core	60.6%	65%	60%	72%	0.1M Methyl-O-Man
DSA	Tri- or tetra-antenna glycans	5.3% (20%)[c]	27% (57%)[c]	6.0% (58%)[c]	5.0% (20%)[c]	1% GlcNAc oligomer
MAL	Siaα2→3Galβ1→4GlcNAc	2.5%[d]	10.8%[d]	6.2%[d]	3.9%[d]	0.4 M lactose
TJA-I	Siaα2→6Galβ1→4GlcNAc	65%	45%	26%	54%	0.1 M lactose
TJA-II	Fucα1→2Galβ1→4(3)GlcNAc and GalNAcβ1→	2.0(2.0%)[e]	16% (59%)[e]	57% (65%)[e]	2% (8.4%)[e]	10 mM lactose
UEA-I	Fucα1→2Galβ1→4GlcNAc	<1%	5%	8.3%	<1%	50 mM fucose
WFA	GalNAcβ1→	<1%	11%	54%	<1%	10 mM GalNAc

[a]BPH serum was from a BPH patient (total PSA, 16.9 ng/mL; F/T, 16); [b]PC serum was from a PC patient (total PSA, 3597 ng/mL; F/T, 3.9); [c]Percent ratios in parentheses indicate DSA binding abilities (%) by eluting with 0.1 N acetic acid; [d]The recovery from MAL columns by 0.4 N lactose was 45% of BPH serum PSA, 40% of PC serum PSA, 43% of LNCaP cell PSA, and 78% of seminal PSA and sequential elution with 0.1 N acetic acid or 1% ethylenediamine could not recover any PSA; [e]Percent ratios in parentheses indicate TJA-II binding abilities (%) after *Arthrobactor* sialidase digestion.

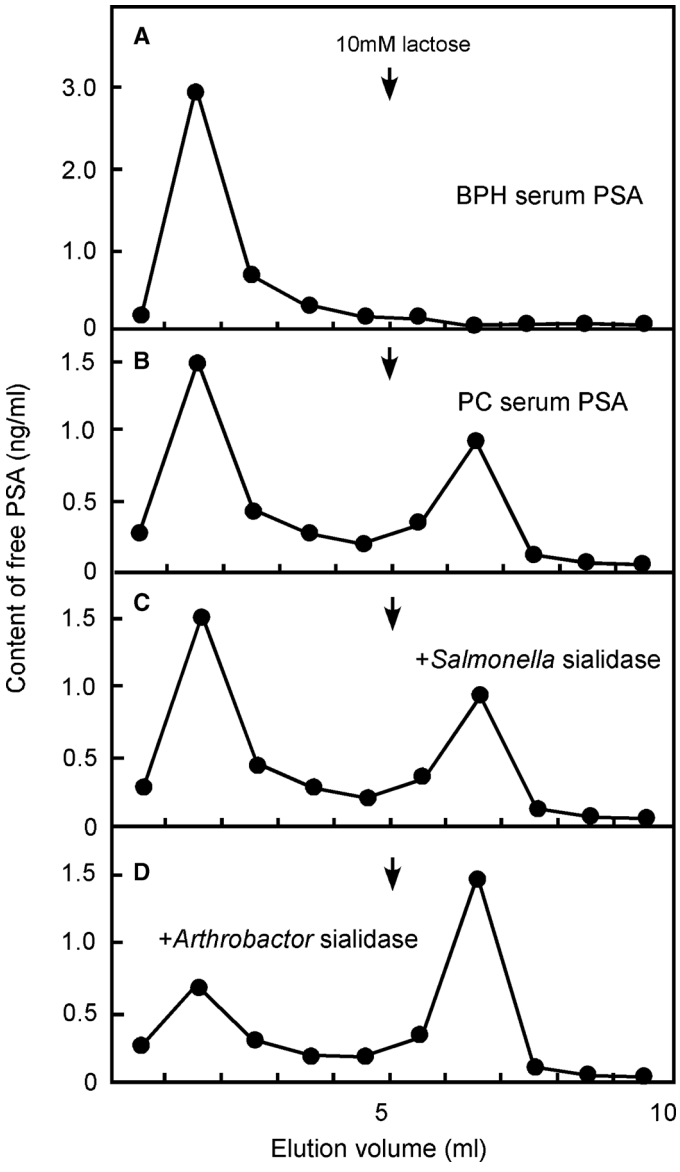

Fig. 1. TJA-II-Sepharose column chromatography of PSAs derived from the serum of BPH patients (**A**) and serum of PC patients (**B**)–(**D**). Serum samples (5 μL for PC patients, 50 μL for BPH patients) were diluted with PBS containing 0.1% BSA (PBS 0.1% BSA), applied to TJA-II-Sepharose columns (3 mg TJA-II/mL gel bed volume), and fractionated by using PBS-0.1% BSA. In the next step, PBS containing 0.1% BSA and 10 mM lactose was used as the buffer and the samples were fractionated (**A**), (**B**). *Salmonella* sialidase-treated serum of PC patients (**C**) and *Arthrobacter* sialidase-treated serum of PC patients (**D**) were also fractionated by a similar procedure. The contents of free PSA were measured using a free PSA analyzing kit. Arrows indicate the position where buffers were switched. PSAs were assayed by ELISA, as described in *Materials and methods*.

Siaα2-3(6)Gal(GalNAc) hydrolyzing *Arthrobacter* sialidase treatment (Uchida et al. 1974), suggesting that the serum PSA of BPH patients does not contain any Siaα2-6GalNAcβ- residues.

In contrast, free PSA from the serum of PC patients bound to TJA-II-, UEA-I-, WFA-, and MAL columns and was subsequently eluted with 10 mM lactose, 50 mM fucose, 10 mM *N*-acetylgalactosamine, and 0.4 M lactose, respectively, indicating that the amount of Fucα1-2Galβ1-4GlcNAc, GalNAcβ1-4GlcNAc, and Siaα2-3Galβ1-4GlcNAc residues increased during carcinogenesis. Increase of MAL-binding ability of the serum PSA of PC patients was previously reported by Ohyama et al. (2004); however, the recovery of these PSAs from the MAL-Sepharose column was too low and the assessment of the quantity of PSAs containing Siaα2-3Gal residues was difficult. *Arthrobacter* sialidase-treated PSA could not be recovered from the MAL column, suggesting that PSA might interact non-specifically with MAL via peptide. Since TJA-II-binding ability of serum PSA from PC patients did not change after Siaα2-3Gal-hydrolyzing *Salmonella* sialidase treatment (Ohkura et al. 2002) (Figure 1C, Table I) and increased from 16% to 59% after Siaα2-3(6)Gal(GalNAc) hydrolyzing *Arthrobactor* sialidase digestion (Figure 1D, Table I), the Siaα2-6GalNAcβ- residues also appear to be increased during carcinogenesis. Mass-spectrometric data of previously reported studies have suggested the existence of the HexNAc-HexNAc sequence (Okada et al. 2001) and real-time PCR analyses performed in this study have revealed increased amounts of GalNAcβ1-4GlcNAc (LacdiNAc)-specific B4GALNT4 (Gotoh et al. 2004) (Figure 7 and Table II); these findings indicate that β-GalNAc residues might be linked to β-GlcNAc at the C-4 position.

Table II. Quantitative real-time RT-PCR analysis of glycosyltransferases in normal prostate tissues and three prostate cancer cell lines. The relative expression levels of FUT1, FUT2, B4GALNT2, B4GALNT3, and B4GALNT4 genes were normalized to the expression levels of the GAPDH gene. All samples were amplified in triplicate

	Normal1	Normal2	LNCaP	PC3	DU145
FUT1	0.71	0.61	9.3	1.4	3.3
FUT2	<0.5	<0.5	0.72	1	1.3
B4GALNT2	<0.005	<0.05	0.01	<0.005	0.01
B4GALNT3	<0.5	<0.5	1	<0.5	2.9
B4GALNT4	0.4	0.81	15	3.9	4
ST6GAL1	0.3	1.0	1.8	0.26	0.22
ST6GAL2	<0.02	<0.02	<0.02	0.04	<0.02

Tajiri et al. (2008) reported that the structures of the sugar chains of free PSA were the same as those of PSA bound to α1-antichyrmotrypsion, which were released by chemical treatment. The distinct structural features of the *N*-glycans of serum PSA from BPH and PC patients, seminal PSA from normal subjects, and PSA from LNCaP cells as determined by lectin column chromatography are summarized in Figure 2. The serum PSA from BPH patients mainly contained α2-6 sialylated bi-antenna complex-type glycans (Figure 2) and the serum PSA from PC patients and PSA from LNCaP cells contained bi-, tri-, and tetra-antennary complex-type glycans expressing Fucα1-2Galβ1-4GlcNAc, Siaα2-6Galβ1-4GlcNAc, ±Siaα2-6GalNAcβ1-4GlcNAc, and Siaα2-3Galβ1-4GlcNAc residues (Figure 2).

TJA-II-Sepharose column chromatography of serum PSA from PC and BPH patients

PSA circulates in the blood either in the free form or bound form with plasma proteins, mainly, α1-antichymotrypsin and α2-macroglobulin, both of which are extracellular protease inhibitors present in large amounts in the serum. According to the previous reports, the *N*-glycans of α1-antichymotrypsin and α2-macroglobulin are bi- and tri-antennary complex-type glycans and do not contain Fucα1-2Galβ1-4GlcNAc and

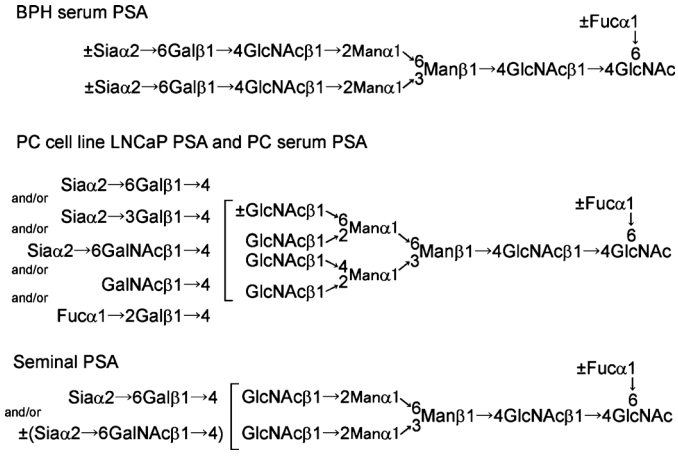

Fig. 2. Predicted structures of the main *N*-linked glycans of PSAs derived from serum of BPH and PC patients, PC cell line LNCaP, and seminal fluid.

±Siaα2-6-β-GalNAc residues (Bezouška et al. 1985; Laine et al. 1991). The preliminary experiments of our study indicated that there was no difference in the ratios of TJA-II-bound PSA to unbound PSA between free PSA and total PSA. Accordingly, to detect PC serum PSA-specific glycans, both type 2H and lacdiNAc recognizing TJA-II-Sepharose column was used, because TJA-II fundamentally recognizes a β-galactosyl residue and the binding strength increases on substitution of the hydroxyl group at the C-2 position with a fucosyl or actamido group (Yamashita et al. 1992b). Furthermore, to confirm whether type 2H and lacdiNAc containing PSA is quantitatively analyzed with TJA-II-Sepharose column chromatography, PSA secreted from LNCaP cells was sequentially analyzed using TJA-II-, UEA-I-, and WFA-Sepharose column chromatographies (Figure 3) and surface plasmon resonance assay as shown in Figure 4. 84% of PSA from LNCaP cells passed through the UDA column (Figure 3A, a). The UDA passed-through fraction was applied to TJA-II column, and 57% of PSA bound to the TJA-II column (Figure 3B, b). 8.3% of TJA-II(+) PSA bound to the UEA-I column (Figure 3C, d) and the remaining part of TJA-II(+)UEA-I(-) PSA (Figure 3C, c) exclusively bound to WFA column, suggesting that 49% of PSA had only lacdiNAc residues (Figure 3D, e). A part of TJA-II(+)UEA-I(+) PSA also bound to the WFA column, suggesting that both type 2H and lacdiNAc residues are linked to the different branches of 5% PSA (Figure 3E, g) and that the remaining 3.3% PSA had only type 2H residues (Figure 3E, f). Next, to provide more rigid data showing that PSA itself bound to TJA-II, surface plasmon resonance analysis was performed. After PSA from LNCaP cells was immobilized via an anti-PSA monoclonal antibody, TJA-II bound to PSA dose dependently and was released by the addition of 10 mM lactose (Figure 4). Because of heterogeneity of PSA glycans, the K_D value was calculated as about 10^{-7}M using BI-Aevaluation software. These results confirmed that PSA from LNCaP cells bind quantitatively to TJA-II via the carbohydrate residues. On the basis of these results, we fractionated the serum samples from PC and BPH patients by TJA-II-Sepharose column chromatography and measured the amount of total PSAs in each eluate, as shown in Figure 5.

The amount of total PSA from PC and BPH patients is partially overlapped, as reported in previous studies (Figure 5A).

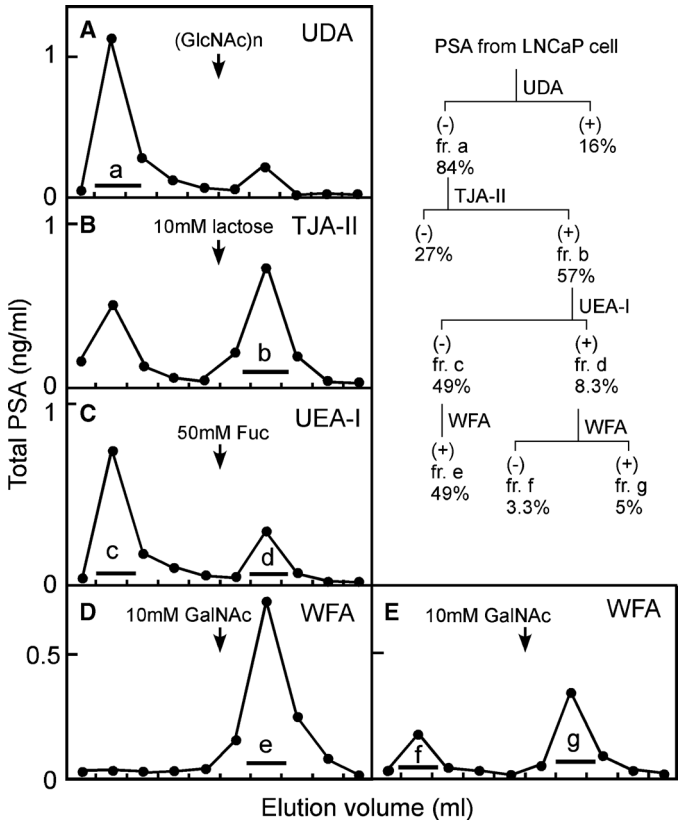

Fig. 3. Sequential lectin column chromatography of PSA from LNCaP cells. Cell culture media were applied to a UDA column and fractionated same as Table I (**A**). Passed-through fraction a was applied to a TJA-II column and bound fraction b was collected (**B**). Fraction b was applied to a UEA-I column, and passed-through fraction c and bound fraction d were collected (**C**). Fractions c and d were applied to WFA columns and fractions e, f, and g were collected (**D**), (**E**). The contents of total PSA were measured using total PSA analyzing kit. Arrows indicate the position where buffers containing haptenic sugars were switched.

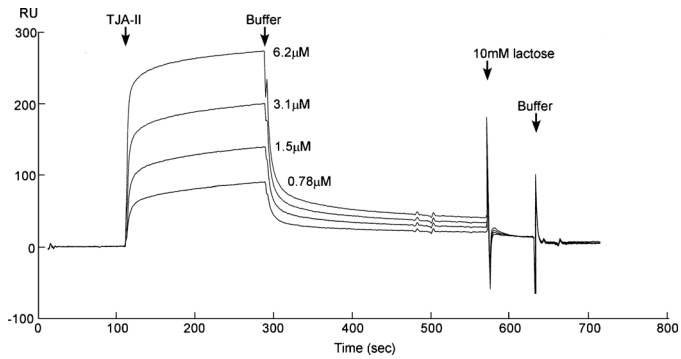

Fig. 4. Surface plasmon resonance analysis of TJA-II binding to PSA from LNCaP cells. An anti-PSA monoclonal antibody was immobilized on a CM5 sensor chip by the amine-coupling method. Culture media of LNCaP cells were introduced onto the chip and PSA was captured (1750 RU). Various concentrations of TJA-II were introduced onto the PSA-captured surface for 180 s at a flow rate of 20 μL/min. The relative response was determined by subtracting the blank values on the non-immobilized surface from the values on the PSA-captured surface.

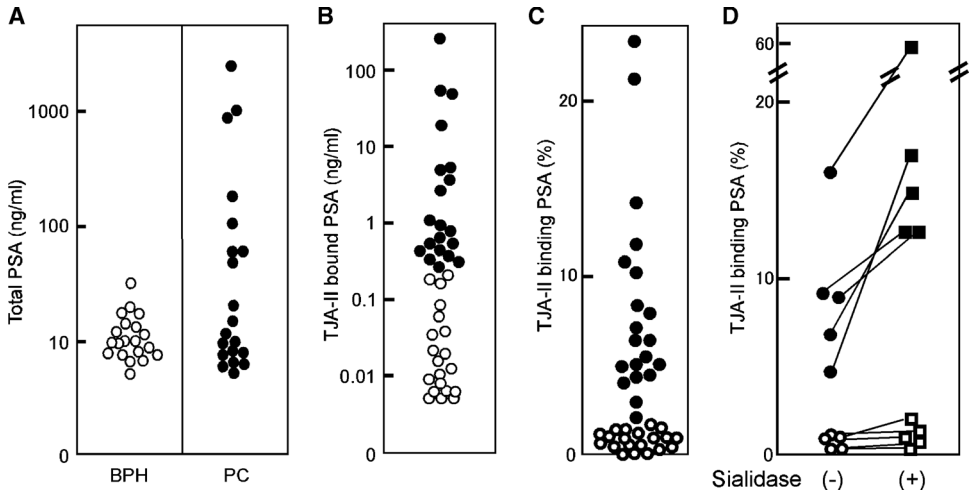

Fig. 5. TJA-II binding ratios (%) and TJA-II-bound PSA concentrations from the sera of PC and BPH patients. (**A**) Total PSA concentrations in sera from 20 BPH patients (○) and 20 PC patients (●). Serum samples were also used in (**B**) and (**C**). (**B**) Total PSA concentrations of TJA-II bound fractions. (**C**) TJA-II binding ratios (%). (**D**) TJA-II binding ratios (%) of *Arthrobactor* sialidase-treated serum from five sera from BPH patients (○) and sialidase-treated sera from BPH patients (□), and five sera from PC patients (●) and sialidase-treated sera from PC patients (■).

However, the amount of TJA-II-bound total PSA in the serum of PC patients was significantly more than that in the serum of BPH patients ($P < 0.05$) (Figure 5B). As determined by TJA-II-Sepharose column chromatography, the mean ± SD (%) of the ratio of TJA-II bound PSA to total PSA of PC and BPH patients were 8.3 ± 5.6% and 1.0 ± 0.55%, respectively, and the difference was statistically significant (Figure 5C). These results suggested that the amount of α1,2-linked fucose and β-GalNAc residues in the total PSA derived from the serum of PC patients was higher than that in the serum of BPH patients. Furthermore, in order to elucidate whether Siaα2-6GalNAcβ1-4GlcNAc is constantly present in the serum PSA of BPH patients, five serum samples of both BPH and PC patients were treated with *Arthrobactor* sialidase and subsequently subjected to TJA-II column chromatography. TJA-II binding ratios (%) of the serum samples of BPH and PC patients were determined to be 1.5 ± 0.78% and 23 ± 20%, respectively (Figure 5D). These results indicate that TJA-II-binding analysis of sialidase-treated serum PSAs of BPH and PC patients is more effective for discriminating between PC and BPH. In addition, the relationship between TJA-II binding% ratio and clinical stage/Gleason score was investigated to understand the relationship between the% ratio of TJA-II binding PSA and the malignancy in prostate cancer. As summarized in Figure 6, TJA-II binding PSA seemed to show some reciprocal relationship to clinical stage and Gleason score, suggesting that the expression of TJA-II binding serum PSA is useful to diagnose the early stages of PC and discriminate BPH and PC.

Measurement of the glycosyltransferase transcript levels by real-time PCR

To identify the glycosyltransferases responsible for increased expression of Fucα1-2Galβ1-4GlcNAc, GalNAcβ1-4GlcNAc, and Siaα2-6GalNAcβ1-4GlcNAc residues, the expression levels of sialyltransferases, fucosyltransferases, and *N*-acetylgalactosaminyltransferases were analyzed using real-time PCR. The candidate enzymes responsible for Fucα1-2Gal formation are fucosyltransferases I (FUT1) (Larsen et al. 1990) and II (FUT2) (Kelly et al. 1995), for GalNAcβ1-4GlcNAc formation are *N*-acetylgalactosaminyltransferases III (B4GALNT3) (Sato et al. 2003) and IV (B4GALNT4) (Gotoh et al. 2004), and for GalNAcβ1-4Gal formation it is *N*-acetylgalactosaminyltransferase II (B4GALNT2) (Montiel et al. 2003). Sialyltransferases, namely, ST6GAL1 and ST6GAL2 (Takashima et al. 2002), are responsible for the decrease of Siaα2-6GalNAc residues and the exposure of GalNAcβ- residues. Because the tissue samples obtained from PC patients were very limited, we first compared the FUT1, FUT2, B4GALNT2, B4GALNT3, B4GALNT4, ST6GAL1, and ST6GAL2 cDNA levels between the normal tissues and

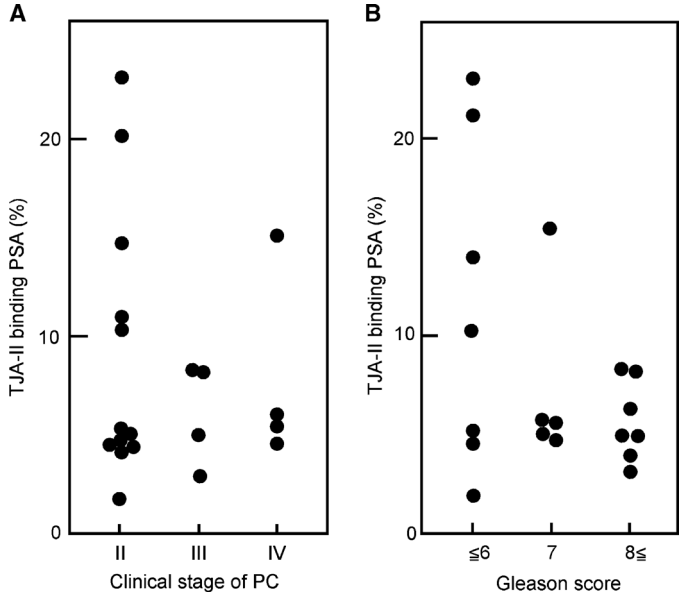

Fig. 6. Distribution of serum TJA-II binding ratios (%) by clinical stage (**A**) and Gleason score (**B**). TJA-II binding ratios (%) of PSA from 20 PC patients as same as Figure 5C were shown in relation to clinical stages (**A**) or Gleason score (**B**).

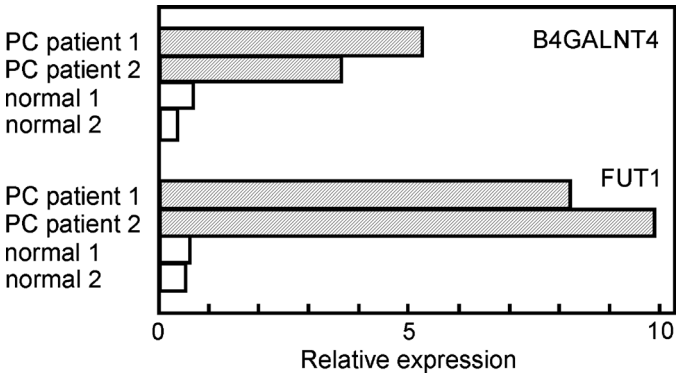

Fig. 7. The relative expression levels of *B4GALNT4* and *FUT1*. The total RNAs from two normal and two PC patients tissues were isolated, reverse transcribed, and amplified by real-time PCR. The mRNA levels of *B4GALNT4* and *FUT1* genes were estimated by analyzing the kinetics of cDNA amplification of the PCR reactions and were normalized to that of GAPDH.

the following PC cell lines: LNCaP, PC3, and DU145. The expression of *FUT1* and *B4GALNT4* was significantly higher in cancer cell lines than that in normal tissues, as shown in Table II. No significant change was observed in the levels of ST6GAL1 mRNA. However, the expression of *FUT2*, *B4GALNT3*, and *ST6GAL2* in normal tissues was below the threshold limit of detection using *GAPDH* gene expression as the standard. These results suggested that the increased levels of Fucα1-2Galβ1-4GlcNAc, GalNAcβ1-4GlcNAc, and Siaα2-6GalNAcβ1-4GlcNAc residues were due to increased levels of *FUT1* and *B4GALNT4*, not but that of *ST6GAL1* itself. Next, we compared the *FUT1* and *B4GALNT4* gene expression in two normal tissues with that in PC tissues from two patients. The *FUT1* and *B4GALNT4* gene expression appeared to increase during carcinogenesis (Figure 7). These results indicated that FUT1 and B4GALNT4 are responsible for the increase of Fucα1-2Galβ1-4GlcNAc, GalNAcβ1-4GlcNAc, and Siaα2-6GalNAcβ1-4GlcNAc residues on PSA molecules.

Discussion

It is well known that the structures of carbohydrates on the cell surface change during carcinogenesis (Yamashita and Kobata 1996a, 1996b). The analysis of the carbohydrate structures is difficult because of low levels of glycoprotein as tumor markers in the serum, such as PSA; however, it has been proposed that glycoproteins containing tumor-specific carbohydrates exist. Lectin column chromatography is a powerful tool to elucidate both core structures and peripheral structures of carbohydrates linked to at least several pg per mL of glycoproteins that are detectable by ELISA. First, we evaluated the carbohydrate structures of PSAs derived from seminal fluid of normal subjects, serum of PC and BPH patients, and a PC cell line, namely, LNCaP using several kinds of lectin columns. Previous studies have already demonstrated that seminal PSA is mainly composed of sialylated biantennary complex-type sugar chains and that a large amount of highly branched sugar chains are found on PSA derived from PC tissues, LNCaP cells, and some serum samples from PC patients containing >10,000-fold higher contents of PSA (Sumi et al. 1999; Prakash and Robbins 2000; Okada et al. 2001; Peracaula et al. 2003; Ohyama et al. 2004; Tajiri et al. 2008). Using a DSA column, we showed that 25% of free PSA from BPH patients, 25% of seminal PSA, 84% of free PSA from PC patients, and 64% of PSA from LNCaP cells had higher branched glycans than biantennary glycans. The increase in the amount of highly branched glycans is commonly observed during carcinogenesis (Yamashita and Kobata 1996a); therefore, PSA, which contains highly branched glycans, can be considered as a potential candidate marker of PC. We achieved less than 50% recovery from DSA-Sepharose columns by hapten sugars in this study, and serum-PSA-binding proteins α1-antichymotrypsin and α2-macroglobulin are known to have bi- or tri-antennary glycans (Bezouška et al. 1985; Laine et al. 1991); therefore, this technique appears to have limited applications in the clinical field. Similar to the results of Ohyama et al. (2004), our findings also indicate an increase in the MAL-bound PSA fraction (Table I). However, the recovery from MAL was too low to calculate the binding ratios and could not be improved by changing the elution conditions. Furthermore, the content of free PSA, which decreased to <20% of total PSA in the sera of PC patients, should be detected followed by MAL-Sepharose column chromatography. On the other hand, the TJA-II-bound fraction of PSA in the serum PSA from PC patients and that from LNCaP cells were markedly higher than that of seminal PSA and serum PSA from BPH patients because the recovery from TJA-II was constantly >95% and total PSA could be detected. Hence, we used TJA-II column chromatography to further study the clinical application of PSA.

The results of this study clearly suggested that the TJA-II-binding ability of serum PSA can be used to discriminate between PC and BPH (Figure 5). TJA-II specifically binds to both Fucα1-2Gal and GalNAcβ- residues (Yamashita et al. 1992b); therefore, the level of PSA containing Fucα1-2Gal and GalNAcβ- residues was higher in the sera of PC patients than in the sera of BPH patients. The serum-free PSA from PC patients bound not only to the TJA-II column but also to the UEA-I column, which was specific for Fucα1-2Galβ1-4GlcNAc residue, and the WFA column, which was specific for GalNAcβ-residues, as shown in Table I. On the other hand, seminal PSA did not bind to TJA-II-, UEA-I-, and WFA-Sepharose columns. Accordingly, the levels of both Fucα1-2Gal and nonsubstituted GalNAcβ- residues were found to increase during carcinogenesis. It has been previously reported that the Fucα1-2Gal residue in PSA from LNCaP cells is linked to 15% of the total *N*-glycan (Peracaula et al. 2003); however, we are the first to report the presence of Fucα1-2Galβ1-4GlcNAc residues on the serum PSA of PC patients and the increase of Fucα1-2Gal residues during the development of PC. We have previously reported that the expression of Fucα1-2Galβ1-4GlcNAc residues on alkaline phosphatases in the sera of choriocarcinoma patients (Fukushima et al. 1998) and that of Fucα1-2Galβ1-4(±Fucα1-3)GlcNAc residues (type 2H and Le^y antigen) on the carcinoembryonic antigen in the tissues of adenocarinoma patients were increased (Fukushima et al. 1995). Kannagi et al. (1986) also reported that abnormally Le^y antigen levels were observed only in patients with hepatoma and not in patients with nonmalignant liver diseases, including liver cirrhosis. However, the physiological significance of the presence of Fucα1-2Gal residues on cancer cells remains unknown.

We first demonstrated the presence of the nonreducing terminals containing GalNAcβ- residues in the serum PSA from PC

patients. Previous reports have suggested that HexNAc-HexNAc residues are linked to seminal PSA (Okada et al. 2001) and PSA from LNCaP cells (Peracaula et al. 2003) using mass spectrometry and β-hexosaminidase digestion in combination with high-performance liquid chromatography (HPLC); however, these methods did not clarify the presence of GalNAcβ- residues. Determination of the nonreducing terminal of GalNAcβ- residues was based on the binding specificities of these residues to TJA-II and WFA-Sepharose columns. We further found that the expression of *B4GALNT4* was higher in PC tissues than in normal prostate tissues. B4GALNT4 has been implicated in the synthesis of LacdiNAc residues (Gotoh et al. 2004); thus, GalNAcβ-residues recognized with TJA-II- and WFA-Sepharose columns could be a part of LacdiNAc residues and not Sd or Cad antigens. LacdiNAc residues have not been commonly found on human tissues, except in the case of human glycoprotein hormones. Since the detection of LacdiNAc residues from cell lines derived from solid tumors has been reported previously (Jaques et al. 1996; Do et al. 1997), LacdiNAc residues might be one of the cancer-specific carbohydrate markers. However, lack of direct methods of detection of LacdiNAc residues complicates the confirmation of these findings.

HexNAc-HexNAc residues are found in seminal PSA (Okada et al. 2001); however, in our experiments, seminal PSA did not bind to TJA-II or WFA columns (Table I). Since Siaα2-6GalNAcβ1-4GlcNAc residues did not bind to TJA-II and WFA columns (Ohkura et al. 2002), whether sialidase-treated seminal PSA could bind to the TJA-II column was examined. We found that 8.4% of seminal PSA bound to the TJA-II column (Table I), suggesting that seminal PSA contained Siaα2-6GalNAcβ1-4GlcNAc residues and not GalNAcβ1-4GlcNAc residues. The serum PSA from PC patients also contained substantial amounts of Siaα2-6GalNAcβ1-4GlcNAc residues as indicated by the high binding ratios of TJA-II after *Arthrobacter* sialidase digestion (Table I, Figures 1D and 5D).

Finally, despite advance understanding of PSA-based screening methods, its optimal clinical performance remains unknown. A significant number of cases can remain undiagnosed if a static trigger point of 4 ng/mL is used for early detection; on the other hand, a low PSA cutoff value can increase the sensitivity of detection of PC, but it simultaneously decreases the diagnostic specificity by increasing the biopsy rate. These results clearly indicate that there is an urgent need for the development of novel biomarkers to improve the specificity of early detection of clinically significant PC. Our data suggested that the detection of TJA-II bound PSA could be a promising tool for diagnosis of prostate cancer, which can clearly discriminate between BPH and PC.

Material and methods

Materials

PSA purified from human seminal fluid was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). *Salmonella typhimurium* LT2 α-sialidase (Ohkura et al. 2002) and *Arthrobacter ureafaciens* sialidase (Uchida et al. 1974) were obtained from Takara Biochemicals (Kyoto, Japan) and Nakalai Tesque (Tokyo, Japan), respectively. *Ulex europaeus* agglutinin (UEA)-I-agarose (gel concentration, 2.7 mg/mL) was purchased from J-Oil Mills, Inc. (Tokyo, Japan). *Trichosanthes japonica* agglutinin (TJA)-I (Yamashita et al. 1992a) and TJA-II (Yamashita et al. 1992b), MAL (Wang and Cummings 1988), and *Datura stramonium* agglutinin (DSA) (Yamashita et al. 1987) were purified and bound to cyanogen bromide (CNBr)-activated Sepharose 4B (gel concentration, 3 mg/mL) (Cuatrecasas and Anfinsen 1971). CNBr-activated Sepharose 4B was purchased from GE Healthcare (England, UK). *Wisteria floribunda* agglutinin (WFA)-agarose (gel concentration, 3 mg/mL), *Lens Culinaris* agglutinin (LCA)-agarose (gel concentration, 4–5 mg/mL), and *Urtica dioica* agglutinin (UDA)-agarose (gel concentration, 1.5–3 mg/mL) were purchased from EY Laboratories Inc. (San Mateo. CA).

PC cell lines

Human PC cell lines, namely, LNCaP (RCB2144) and DU145 (RCB2143), were provided by RIKEN Bioresource Center through the National Bio-Resource Project of the MEXT, Japan. Human PC cell line, PC-3 (JCRB9110), was obtained from the Health Science Research Resources Bank, Japan. Cells were cultured in an RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS).

Patient characteristics

Serum samples were obtained from 20 randomly selected patients who were newly diagnosed with PC at various clinical stages and 20 patients with BPH. Clinical staging was decided on the basis of the results of DREs and bone scintigraphy. Computed tomography (CT) and/or magnetic resonance imaging (MRI) were also used to determine the T stage of the cancer. The clinical T stages of the cancer ranged from T1cN0M0 to T4N1M1b as per the definitions of the 2002 American Joint Committee on Cancer. Histopathologic findings of the prostate needle biopsy specimens confirmed the diagnosis for all PC patients (Matsumoto et al. 2005; Satoh et al. 2005) and the findings of systemic 22-core transperineal ultrasound-guided template prostate biopsy specimens confirmed the diagnosis for all BPH patients (Satoh et al. 2005). The final pathological diagnosis (including determination of the Gleason score) was made by a single pathologist in Kitasato University Hospital.

Human serum and tissue samples

We obtained 40 prostate tissue specimens from patients who had undergone systemic 22-core transperineal ultrasound-guided template prostate biopsy at the Kitasato University Hospital. We removed two samples from the same site on the prostate based on template information. If the pathological findings of the first sample were positive for PC, the second sample was also defined as a PC. However, if all systemic 22-cores were negative for cancer, the second sample was defined as BPH. Few tissue samples were placed in a 1.5 mL tube, which contained a 10:0.5 (v/v) ratio of RNAlater solution (Ambion Inc., Japan) to prostate tissue. Each eligible patient signed a study-specific informed consent form, and this study was approved by the Institutional Review Board at our hospital (approval number C08-467).

Lectin column chromatography and detection of PSA

Eight separate columns containing 1 mL of TJA-I-Sepharose, TJA-II-Sepharose, DSA-Sepharose, MAL-Sepharose, UDA-agarose, UEA-I-agarose, LCA-agarose, and WFA-agarose were

equilibrated with phosphate-buffered saline (PBS) (containing 0.02% NaN_3) and 0.1% bovine serum albumin (BSA). Next, 1–100 μL serum containing 0.4–1 ng PSA, cell culture media, or seminal PSA was diluted with the equilibrated buffer up to 300 μL; applied to the column; and incubated at 4°C for 15 min. The following solutions were used for elution: 5 mL of 0.1% BSA-PBS at 4°C, 5 mL of 0.1% BSA-PBS containing lactose (10 mM for TJA-II, 0.1 M for TJA-I, and 0.4 M for MAL columns), 10 mM *N*-acetylgalactosamine (WFA column), 50 mM fucose (UEA-I column), 0.1 M α-methylmannoside (LCA column), and 1% *N*-acetylglucosamine oligomers (DSA and UDA columns). For the precolumn, pass-through, and bound fractions, total PSA and free PSA were measured by the Hybritech® PSA test kit and Hybritech® free PSA kit (Beckman Coulter, Inc.), respectively. The relative amount of the lectin-bound fraction was calculated against the precolumn amount and the amount recovered from columns. The samples were diluted with 0.1% BSA-PBS and digested using 50 mU of *Arthrobacter* or *Salmonella* sialidases at 37°C for 1 h before application to TJA-II-Sepharose columns.

Surface plasmon resonance analysis of TJA-II binding to PSA from LNCaP cells

The affinity of TJA-II to PSA from LNCaP cells was measured by the SPR assay using the BIAcore 2000 instrument. An anti-PSA monoclonal antibody (Mikuri Immunological Laboratories Co., Ltd, Kyoto, Japan) was immobilized on a CM5 sensor chip by the amine-coupling method. The coupling density was 5500 RU. The culture medium of LNCaP cells was diluted in the HPS-EP buffer, and injected onto the sensor chip at 5 μL/min. The captured PSA from LNCaP cells was 1750 RU. Then, various concentrations of TJA-II were introduced onto the PSA-captured surface. The sensor surface was regenerated by 10 mM lactose.

Measurement of mRNA expression levels by the real-time polymerase chain reaction

The real-time polymerase chain reaction (PCR) was used to measure the mRNA expression of glycosyltransferases in cells and prostate tissues. The total RNA from RNAlater-stored tissues and cells was prepared using ISOGEN (Nippon Gene Co., Ltd, Tokyo, Japan) and subsequently extracted using chloroform and isopropyl alcohol. The extracted RNA was ethanol precipitated, resuspended in DEPC-treated distilled water, and reverse transcribed using Superscript III (Invitrogen) with oligo(dT). Real-time PCR was performed using Power SYBR® Green PCR master mix (Life Technologies, Japan) and gene-specific primers in a thermal cycler Dice® real-time system (TP800, Takara, Japan). The primers used for amplification were as follows: FUT1, 5′-AACGCCTCCTCTTCCTGTC-3′ and 5′-TGGGGTAGACAGTCCAGGTG-3′ (GenBank accession no. NM_000148); FUT2, 5′-CCTCAACATCAAAGGCACTG-3′ and 5′-GGCCTATTGCATTGATCGTC-3′ (GenBank accession no. NM_000511); B4GALNT2, 5′-GATTTTTCCAACCCCT GGAT-3′ and 5′-GAAGTTGACCACGCCACTG-3′ (GenBank accession no. NM_153446); B4GALNT3, 5′-AGGTCACGC GAGTCTTCTTG-3′ and 5′-ACAATGCGCTGTAGCTGGTA-3′ (GenBank accession no. NM_173593); B4GALNT4, 5′-ACTGGGAGCTCCTGGACA-3′ and 5′-TGGTGATAGAAA TTCCGCAGT-3′ (GenBank accession no. NM_178537); ST6GAL1, 5′-TCAGCGGGATCTCTGAAGTC-3′ and 5′-AAACCTCAGGACTGCGTCA-3′ (GenBank accession no. NM_003033); ST6GAL2, 5′-TCCTTGGGCGAGGAAATAG-3′ and 5′-CCCAACATCTTTCTCATAACCAC-3′ (GenBank accession no. NM_006927); GRPDH, 5′-ATCCACATCGC TCAGACAC-3′, and 5′-GCCCAATACGACCAAATCC-3′ (GenBank accession no. NM_002046). The genes encoding glycosyltransferases and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by real-time PCR; the PCR program included 40 cycles of 95°C for 10 s and 60°C for 40 s. Each primer pair produced a single and sharp peak, thereby indicating that the primers amplified only one specific PCR product. No primer dimers were observed. All samples were amplified in triplicate.

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Abbreviations

BPH, benign prostatic hyperplasia; BSA, bovine serum albumin; DSA, *Datura stramonium* agglutinin; FCS, fetal calf serum; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GlcNAc, *N*-acetylglucosamine; LCA, *Lens Culinaris* agglutinin; MAL, *Maackia amurensis* lectin; Man, mannose; PBS, phosphate-buffered saline; PC, prostate cancer; PCR, polymerase chain reaction; PSA, prostate-specific antigen; Sia, sialic acid; TJA, *Trichosanthes japonica* agglutinin; UDA, *Urtica dioica* agglutinin; UEA, *Ulex europaeus* agglutinin; WFA, *Wisteria floribunda* agglutinin.

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