Associations of IgG N-Linked Oligosaccharide Chains and Proteases in Sera of Prostate Cancer Patients With and Without α 2-Macroglobulin Deficiency

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We previously reported on a number of cases of metastatic prostate cancer (PCa) in which serum α 2-macroglobulin (α 2M) levels were markedly decreased to less than 20 mg/dl (α 2M deficiency). All PCa patients with α 2M deficiency had multiple bone metastases. Proteases in ten PCa patients with and without α 2M deficiency were studied and compared against ten healthy controls in order to elucidate the relationships between changes in sugar chain structure and neoplasia. We assessed the relationship between ratios of Fr4 to Fr1 and Fr2 (Fr4/Fr1+Fr2 ratios) of oligosaccharide chains, and ratios of free prostate-specific antigen (PSA) to total PSA (*F/T* ratios), and serum levels of matrix-metalloproteinase-2 (MMP-2) in PCa progression. Measurement of serum α 2M concentration was performed by laser

nephelometry. Serum PSA and MMP-2 levels were determined by enzyme immunoassay and free PSA by radioimmunoassay. N-linked oligosaccharides of human serum immunoglobulin G were analyzed using fluorophore-associated carbohydrate electrophoresis. In those PCa patients with α 2M deficiency: (a) serum α 2M and *F/T* ratios were lower ($P < 0.05$) and (b) Fr4/Fr1+Fr2 ratios and serum MMP-2 levels were higher when compared with those PCa patients without α 2M deficiency. There was a significant correlation between Fr4/Fr1+Fr2 ratios and *F/T* ratios or serum MMP-2 levels in PCa with α 2M deficiency ($P < 0.05$). Therefore, these markers may serve as an auxiliary serum tumor marker for monitoring of the bone metastases or progression of disease in PCa. *J. Clin. Lab. Anal.* 23:125–131, 2009. © 2009 Wiley-Liss, Inc.

Key words: α 2-macroglobulin; prostate-specific; antigen; matrix-metalloproteinase-2; N-linked oligosaccharides

INTRODUCTION

Serum α 2-macroglobulin (α 2M) is a 720 kDa glycoprotein that inhibits many protease activities and is involved in coagulation, fibrinolysis, and inflammatory reactions in vivo. Serum α 2M levels show slight increases in disorders such as nephrotic syndrome or malignancy. On the other hand, decreases of α 2M levels are known only in very few disorders, such as disseminated intravascular coagulation (DIC) or hematologic malignancies. We previously showed in a small number of cases of prostate cancer (PCa) with bone metastases that serum α 2M levels were markedly decreased to less than 20 mg/dl (α 2M deficiency), whereas serum prostate-specific antigen (PSA) levels

were remarkably increased (1). These cases were not complicated by DIC.

PSA is a 33 kDa glycoprotein secreted by prostatic glandular epithelial cells. We previously reported that

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PSA was strongly expressed in PCa cells by immunohistochemical analyses of metastatic PCa specimens (2). PSA is a serine protease similar to the enzyme kallikrein and serves to liquefy seminal coagulum (3,4). PSA is present as a free molecule and complexes with α 1-antichymotrypsin (ACT) or α 2M in the blood (5,6). PSA is widely used as a specific marker in the diagnosis, prognosis, and therapeutic monitoring of the PCa patients (7–9). It is recognized that the relative proportions of free and PSA complexes differ between PCa and benign prostate hypertrophy (BPH), and that the ratio of free PSA to total PSA (*F/T* ratio) is useful for differential diagnosis of localized PCa and BPH (10,11).

Matrix-metalloproteinases (MMPs), or cancer cell-derived proteases, play an important role in the invasion and metastasis of cancer cells through the degradation of the extracellular matrix, similar to urokinase-type plasminogen activator (uPA) (12,13). Of the different MMPs, matrix-metalloproteinase-2 (MMP-2) (gelatinase A) and MMP-9 (gelatinase B) have been shown to degrade type IV collagen, which is a major component of cellular basement membranes, and to participate in the invasion and metastasis of cancer cells (14).

On the other hand, oligosaccharide chains bind to glycoproteins and help to maintain the three-dimensional structure of proteins. They also play an important role in the physiological activity of the proteins. Changes in oligosaccharide structure affect the molecular structure of immunoglobulin G (IgG) and the ability of Fc receptors to bind to the macrophage surface (15,16). Serum IgG oligosaccharide chains lacking galactose (Gla) (agalactosyl IgG oligosaccharides) were frequently found in sera of rheumatoid arthritis (RA) patients by high-performance liquid chromatography (17). It is thought that agalactosyl IgG oligosaccharides activate the complement system via mannose-binding protein and participate in the pathogenesis of RA (18). Fucosylated oligosaccharides were also found in sera of nonsmall cell lung cancer patients (19). We previously showed that agalactosyl IgG oligosaccharides increased significantly during the progression of cancer using fluorophore-associated carbohydrate electrophoresis (FACE) analysis (20).

However, there are no reports on the relationships between changes in sugar chain structure and proteases associated with neoplastic development, such as in PCa patients with and without α 2M deficiency. Therefore, in this study, serum IgG *N*-linked oligosaccharide chain structures in PCa patients were studied and the relationship between Fr4/Fr1+Fr2 ratios of oligosaccharide chains and *F/T* ratios or serum MMP-2 levels in PCa patients with and without α 2M deficiency was evaluated.

MATERIALS AND METHODS

Patient Population

There were 20 untreated subjects who participated in this study. Ten healthy adult males were enrolled in the control group (mean age 62.4 years, range 54–70 years). Ten prostate diseases diagnosed as PCa were composed of the following: stages T1–T2—five cases and M1b—five cases. The mean age was 68.2 years and ranged from 54 to 78 years. The patients were enrolled from the Kitasato University Hospital and cooperating hospitals. PCa patients with α 2M deficiency had multiple bone metastases and serum α 2M levels were markedly decreased to less than 20 mg/dl. None of the cases had DIC, which was screened by measurement of fibrinogen, fibrin/fibrinogen degradation products (FDP) and D-dimer. Histology was confirmed by six-sextant biopsy and/or transurethral resection in all cases. PCa was clinically staged following the TNM classification (21). Briefly, stage T1 is defined as tumor not clinically recognizable and identifiable only by histological examination of prostatic tissue. Stage T2 tumors are palpable but confined within the prostate. Stage T3 tumors are palpable and extend through the prostatic capsule with unilateral or bilateral extension. M1 stage is defined by the presence of distant metastasis and M1b by bone metastases. Bone metastasis in PCa patients was diagnosed by bone scintigraphy using ^{99m}Tc -labeled methylene diphosphonate (^{99m}Tc -MDP). All PCa patients with stage M1b showed multiple sites of accumulation of the isotope. The serum samples were stored at -80°C until use. Informed consent was obtained from all participants in this study.

The Determination of α 2M, PSA, and MMP-2 Levels in Serum

Measurement of α 2M concentration was performed by laser nephelometry (Behring nephelometer analyzer, Behring). PSA levels were determined by enzyme immunoassay (AIA-600, TOSOH, Tokyo, Japan) and free PSA by radioimmunoassay (WALLAC-1460, Pharmacia, Tokyo, Japan). Bone metastasis in PCa patients was diagnosed by bone scintigraphy using ^{99m}Tc -labeled methylene diphosphonate. All PCa patients with stage M1b showed multiple sites of isotope accumulation. Serum MMP-2 levels were determined by measuring pro-MMP-2 using a one-step sandwich enzyme immunoassay for MMP-2 (Fuji Chemical Industries, Toyama, Japan) according to the manufacturer's instructions.

Purification of Serum IgG

Serum (300–500 μl) was diluted four-fold with 0.01 M phosphate buffer (pH 7.0), and applied to a Protein G column (Pharmacia Biotech Inc., Uppsala, Sweden). After washing the column with 5 ml of 0.01 M phosphate buffer,

protein was eluted with 3 ml of 0.1 M glycine-HCl buffer (pH 3.0) and 0.5 ml of 1 M Tris-HCl (pH 9.0) buffer. The protein was dialyzed against distilled water for 48 hr using a dialysis membrane (Sanko Junyaku Inc., Tokyo, Japan) and lyophilized. The purity of the IgG was confirmed by immunoelectrophoresis using anti-human whole serum antibody and anti-human serum IgG antibody.

Release of *N*-Linked Oligosaccharide Chains From Serum IgG

Purified IgG (250 µg) was dissolved in 25 µl of distilled water, and 25 µl of 0.1 M phosphate buffer (pH 7.4), 1 µl of 5% SDS, and 1.5 µl of 1.44 M 2-mercaptoethanol were added. The mixture was heated at 100°C for 5 min and then treated with 2.5 ml of 7.5% Nonidet P-40 and 2 µl of recombinant peptide *N*-glycosidase F (PNGase F, EC 3.5.1.52, Seikagaku Kogyo Inc., Tokyo, Japan) at 37°C for 2 hr. Subsequently, anhydrous ethanol (171 µl) was added and the mixture was cooled for 10 min. After centrifugation of the mixture at 15,000 rpm for 5 min at 4°C, the supernatant containing the released oligosaccharides was evaporated to dryness and recovered.

Fluorescence Labeling of *N*-Linked Oligosaccharide Chains From Serum IgG

Five microliters of 0.15 M 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) in 15% acetic acid and 5 µl of 1.0 M sodium cyanoborohydride in 1.0 M dimethyl sulfoxide were added to the oligosaccharides in the residue and the mixture was incubated at 37°C for 16 hr.

Electrophoresis and Imaging Analysis

The ANTS-labeled oligosaccharides were separated by electrophoresis (SDS-PAGE) on a FACE-*N*-linked oligosaccharide gel (Glyko Inc., Novato, CA) at a constant current of 15 mA for 90 min. After the termination of electrophoresis, the gel was imaged with a FACE IMAGER scanner (Glyko Inc.), and the fluorescent fraction (Fr) patterns were analyzed in five fractions by FACE Imaging Software version 2.47 (Glyko Inc.). An oligo ladder standard (Glyko Inc.) containing ANTS-labeled glucose polymers composed of 1–20 glucose residues was applied to the gel as the marker. The determination of each Fr band was calculated by comparing with a standard degree of polymerization (DP) of G4, composed of four glucose residues, and is shown as a percentage (%) in Table 3.

Sugar Chain Structures of the Respective Fractions

Healthy controls were analyzed by using exoglycosidase and the sugar chain structures were categorized

into the following five fractions (17): Fr1, IgG oligosaccharides with two Gla (digalactosyl IgG oligosaccharides); Fr2, with one Gla (monogalactosyl IgG oligosaccharides); Fr3 with one sialic acid (Sia) (monosialyl oligosaccharides); Fr4 without Gla (agalactosyl IgG oligosaccharides); and Fr5 with two Sia (disialyl oligosaccharides). The pattern of serum IgG *N*-linked oligosaccharide chains by SDS-PAGE was illustrated in a previous report (20).

Statistical Analysis

The gel images were extracted from the FACE Imaging Software into Adobe PhotoShop version 5.5 after converting them to PICT files. The Wilcoxon test and Mann-Whitney *U*-test were used for statistical analysis and $P < 0.05$ was considered as statistically significant.

RESULTS

The Concentrations (Median Values, Range) of α 2M, PSA, and Free PSA/Total PSA (*F/T*) Ratio

Table 1 gives the concentrations of α 2M, PSA, and *F/T* ratios in the sera of healthy controls and in PCa patients with and without α 2M deficiency. In PCa patients with α 2M deficiency and who had multiple bone metastases, serum PSA levels were significantly higher compared with healthy controls and PCa patients without α 2M deficiency ($P < 0.05$). In addition, serum α 2M levels and *F/T* ratios in PCa patients with α 2M deficiency were significantly lower than those in healthy controls and in PCa patients without α 2M deficiency ($P < 0.05$).

The Concentrations (Median Values, Range) of MMP-2

Table 2 gives the concentrations of MMP-2 in the sera of healthy controls and in PCa patients with and without α 2M deficiency. In PCa patients with α 2M deficiency, serum MMP-2 levels were significantly higher than those in healthy controls and PCa without α 2M deficiency ($P < 0.05$).

Serum IgG *N*-Linked Oligosaccharide Chains (% of Total; Median Values, Range) and Fr4/Fr1 + Fr2 (Ratio)

Table 3 gives the percentage of each fraction of serum IgG *N*-linked oligosaccharide chains in healthy controls and in PCa patients with and without α 2M deficiency. In healthy controls, the most abundant fraction according to the DP value was Fr2 (37.5%), followed by Fr1 (22.85%), Fr3 (22.75%), Fr5 (8%), and finally Fr4

TABLE 1. Concentrations (Median Values, Range) of α 2-Macroglobulin (α 2M), Prostate-Specific Antigen (PSA), and Free PSA/Total PSA (F/T) Ratios in Sera of Healthy Controls and Prostate Cancer (PCa) Patients With or Without α 2M Deficiency

	Healthy controls	PCa with α 2M deficiency	PCa without α 2M deficiency	<i>P</i> -value of healthy controls vs. PCa with α 2M deficiency	<i>P</i> -value of healthy controls vs. PCa without α 2M deficiency	<i>P</i> -value of PCa with α 2M deficiency vs. PCa without α 2M deficiency
α 2M (mg/dl)	146.5 (114–196)	9 (6–19)	204 (159–366)	<0.05	NS	<0.05
PSA (ng/ml)	2.15 (1.1–2.8)	4,210 (1,300–7,680)	11.5 (4.3–51)	<0.05	NS	<0.05
F/T (ratio)	8.75 (4–37.2)	15.7 (12–28)	15.7 (12–28)	<0.05	NS	<0.05

NS, no statistical significance.

TABLE 2. Concentrations (Median Values, Range) of Matrix-Metalloproteinase-2 (MMP-2) in Sera of Healthy Controls and Prostate Cancer (PCa) Patients With or Without α 2M Deficiency

	Healthy controls	PCa with α 2M deficiency	PCa without α 2M deficiency	<i>P</i> -value of healthy controls vs. PCa with α 2M deficiency	<i>P</i> -value of healthy controls vs. PCa without α 2M deficiency	<i>P</i> -value of PCa with α 2M deficiency vs. PCa without α 2M deficiency
MMP-2 (ng/ml)	649 (614–782)	924 (635–1,030)	742 (686–831)	<0.05	NS	<0.05

NS, no statistical significance.

TABLE 3. Serum IgG N-Linked Oligosaccharide Chains (% of Total; Median Values, Range) and Fr4/Fr1+Fr2 (Ratios) in Healthy Controls and Prostate Cancer (PCa) Patients With or Without α 2M Deficiency

	Healthy controls	PCa with α 2M deficiency	PCa without α 2M deficiency	<i>P</i> -value of healthy controls vs. PCa with α 2M deficiency	<i>P</i> -value of healthy controls vs. PCa without α 2M deficiency	<i>P</i> -value of PCa with α 2M deficiency vs. PCa without α 2M deficiency
Fr1 (%)	22.85 (18.5–29.6)	14 (8–16.5)	19.3 (16.8–21.9)	<0.05	NS	<0.05
Fr2 (%)	37.5 (22.5–44.1)	32.6 (30.5–35.1)	36 (33.1–41.5)	<0.05	NS	<0.05
Fr3 (%)	22.75 (17.6–25.9)	16.8 (10.4–22.1)	17.9 (12.4–21.8)	NS	NS	NS
Fr4 (%)	4.61 (4.2–16.2)	22 (17.8–35.5)	17.1 (12.6–23.2)	<0.05	NS	NS
Fr5 (%)	8 (6.1–8.6)	13.6 (7–15.5)	9.6 (7.4–14.3)	<0.05	NS	NS
Fr4/Fr1 + Fr2 (ratio)	0.23 (0.07–0.31)	0.48 (0.35–0.87)	0.31 (0.23–0.42)	<0.05	NS	NS

NS, no statistical significance; IgG, immunoglobulin G.

(4.61%). Serum IgG oligosaccharide chains of PCa patients were separated into five fractions as in healthy controls. Fr1 and Fr2 showed a significant decrease in PCa patients with α 2M deficiency when compared with both healthy controls and PCa without α 2M deficiency ($P<0.05$). Although Fr3 decreased in PCa patients compared with healthy controls, the values were not significant. Fr4 and Fr5 both increased in PCa patients

over healthy controls, and those increases in PCa patients with α 2M deficiency compared with healthy controls were significant ($P<0.05$). We calculated the ratio of Fr4 without Glc to Fr1 and Fr2 containing Glc (Fr4/Fr1+Fr2 ratio). The Fr4/Fr1+Fr2 ratios increased in PCa patients compared with healthy controls and was significantly higher in PCa with α 2M deficiency ($P<0.05$).

Correlation of Free PSA/Total PSA (F/T) Ratio and $Fr4/Fr1+Fr2$ Ratio

The relationship between the F/T ratios and the $Fr4/Fr1+Fr2$ ratios in PCa patients was examined (Fig. 1). There was a significant correlation between the F/T ratios and the $Fr4/Fr1+Fr2$ ratios in PCa patients with $\alpha 2M$ deficiency ($r = 0.65$, $P < 0.05$). On the other hand, there was no significant correlation in PCa patients without $\alpha 2M$ deficiency ($r = 0.09$).

Correlation of MMP-2 and $Fr4/Fr1+Fr2$ Ratio

The relationship between the concentrations of serum MMP-2 and the $Fr4/Fr1+Fr2$ ratios in PCa patients was also studied (Fig. 2). There was a significant correlation between the serum MMP-2 levels and the $Fr4/Fr1+Fr2$ ratios in PCa patients with $\alpha 2M$

Fig. 1. Correlation of free PSA/total PSA (F/T) ratio and $Fr4/Fr1+Fr2$ ratio in prostate cancer (PCa) patients with or without $\alpha 2M$ deficiency. PSA, prostate-specific antigen; $\alpha 2M$, $\alpha 2$ -macroglobulin.

Fig. 2. Correlation of matrix-metalloproteinase-2 (MMP-2) and $Fr4/Fr1+Fr2$ ratio in prostate cancer (PCa) patients with or without $\alpha 2M$ deficiency. $\alpha 2M$, $\alpha 2$ -macroglobulin.

deficiency ($r = 0.53$, $P < 0.05$). On the other hand, there was no significant correlation in PCa patients without $\alpha 2M$ deficiency ($r = 0.20$).

DISCUSSION

$\alpha 2M$, the most abundant protease inhibitor in blood, inhibits many protease activities related to fibrinolytic action and inflammatory reactions. $\alpha 2M$ has also been reported to inhibit the activity of proteases such as MMPs or uPA, and thus may inhibit the ability of cancers to infiltrate and metastasize (12,13). The $\alpha 2M$ -protease complex retains a weak enzyme activity, but the residual protease activity is rapidly eliminated from blood by the reticuloendothelial system (22,23). On the other hand, PSA is a serine protease secreted by prostate epithelial cells. PSA is present as a free molecule and can form a complex with ACT or $\alpha 2M$ in blood (5,6). PSA is useful as a specific tumor marker for PCa, with the F/T ratio showing particular usefulness in the differential diagnosis of localized PCa and BPH (10,11). We, as well as others, reported that the F/T ratio is useful to differentiate PCa from BPH in patients with intermediate levels of PSA (4–10 ng/ml) (24). In this study, serum $\alpha 2M$ levels tended to decrease markedly (< 20 mg/dl) in cases where serum PSA levels showed striking increases ($> 1,300$ mg/dl). All cases of PCa patients with $\alpha 2M$ deficiency had advanced cancers having multiple bone metastases and high levels of serum PSA. The decrease in serum $\alpha 2M$ levels may be owing to the reticuloendothelial system rapidly removing many $\alpha 2M$ -PSA complexes from the blood in PCa patients with extremely high levels of serum PSA. PSA levels in serum were significantly higher and F/T ratios were significantly lower in PCa patients with $\alpha 2M$ deficiency than in those of healthy controls and in PCa patients without $\alpha 2M$ deficiency. The F/T ratios of PCa patients are considered useful for monitoring of the bone metastases or progression in PCa.

Of the different MMPs, MMP-2 as well as MMP-9 is thought to be involved in the invasion and metastasis of cancer cells (14). These are important proteases that degrade type IV collagen, a major component of the cellular basement membrane. It has been reported that expressions of MMP-2 and MMP-9 were significantly higher in PCa tissue than in BPH tissue (25). This information may help in predicting the progression of PCa. Hoikkala et al. demonstrated the increased expressions of MMP-2 and MMP-9 in lung cancer tissues, which turned out to have high prognostic value in lung carcinoma (26). These reports are consistent with the idea that MMP-2 and MMP-9 are involved in the invasion and metastasis of cancer. We also showed previously that serum MMP-2 levels may be an auxiliary

indicator to serum PSA for monitoring of the PCa progression (2). MMP-2 levels in serum were significantly higher in PCa patients with $\alpha 2M$ deficiency than in those of healthy controls and PCa patients without $\alpha 2M$ deficiency. Therefore, MMP-2 may be useful for monitoring of the bone metastases or progression in PCa.

The sugar chain structure of IgG is formed after the addition and/or repair of sugar chains by glycosyltransferase. In plasma cells, this process is initiated in the endoplasmic reticulum and transmitted to the Golgi body. Glc is linked to IgG sugar chains by the action of galactosyltransferase (Gal-T) (27). Although the exact mechanism responsible for altered glycosylation remains to be discovered, it is thought that Gal-T activity in plasma cells is down-regulated during the process of carcinogenesis or cancer progression. Thus, tumor progression can cause a significant increase of agalactosyl IgG oligosaccharides (Fr4). The serum IgG N-linked oligosaccharide chains from PCa patients could be separated into the same five fractions as healthy controls. Fr1 and Fr2 that have Glc were decreased, whereas Fr4 without Glc was increased in PCa patients compared with healthy controls. The Fr4/Fr1+Fr2 ratios were increased in PCa patients with $\alpha 2M$ deficiency compared with healthy controls and PCa without $\alpha 2M$ deficiency, and were significantly higher in PCa patients with $\alpha 2M$ deficiency.

In conclusion, the Fr4/Fr1+Fr2 ratios were significantly increased in PCa patients with $\alpha 2M$ deficiency, and there was a significant correlation between the Fr4/Fr1+Fr2 ratios and *F/T* ratios or serum MMP-2 levels in PCa patients with $\alpha 2M$ deficiency. Therefore, these markers may serve as auxiliary serum tumor markers for monitoring of the bone metastases or progression in PCa.

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