Levels of acute inflammatory biomarkers in advanced prostate cancer patients with α_2-macroglobulin deficiency

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Abstract. C-reactive protein (CRP), serum amyloid A (SAA), interleukin-6 (IL-6), α1-antitrypsin (α1AT), α1-acid glycoprotein (α1AG) and ceruloplasmin (CP) are acute inflammatory biomarkers that increase in various conditions including infection, inflammation, malignancy and tissue disturbance. In contrast, α_2-macroglobulin (α2M) is involved in inflammation through its function as a carrier protein of IL-6. We had previously reported on advanced prostate cancer (PCa) patients with multiple distant bone metastases in whom serum α2M levels were markedly decreased (α2M deficiency). However, the relationship between serum levels of α2M and acute inflammatory biomarkers in PCa patients with or without α2M deficiency has not been demonstrated. In the present study, we examined serum levels of CRP, SAA, IL-6, α1AT, α1AG and CP in PCa patients with or without α2M deficiency to establish clinical significance and changes in these biomarkers during PCa disease progression. We found that upon addition of recombinant IL-6 (rIL-6) to serum from PCa patients with α2M deficiency, since a function of α2M is to bind and stabilize IL-6, the α2M-IL-6 complex and free endogenous IL-6 were not detectable. Serum levels of the α2M-independent markers, α1AT, α1AG and CP, in all PCa patients regardless of α2M deficiency were significantly higher than in healthy controls, but those of the α2M-dependent molecules, CRP, SAA and IL-6, were not increased in PCa patients with α2M deficiency. Therefore, quantitation of both α2M-dependent (CRP, SAA and IL-6) and α2M-independent (α1AT, α1AG and CP) acute inflammatory biomarkers in advanced PCa patients may be an auxiliary indicator, together with prostate-specific antigen (PSA), to monitor PCa disease progression.

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Introduction

α_2-macroglobulin (α2M) is the most abundant proteinase inhibitor in the blood and inhibits the activity of various proteases through direct interaction. Moreover, a major biological function of α2M is as a carrier protein for interleukin-6 (IL-6) or growth hormone (1,2). Therefore, α2M is involved in coagulation, fibrinolytic activity and inflammatory reaction *in vivo*. Serum α2M levels generally increase in various disorders, such as nephrotic syndrome, inflammatory diseases and malignancy. On the other hand, it is also known that serum α2M levels decrease in very few disorders such as disseminated intravascular coagulation (DIC) and hematologic malignancy. We had previously reported on advanced prostate cancer (PCa) with multiple distant bone metastases, in whom serum α2M levels were markedly decreased to <20 mg/dl (α2M deficiency), while serum prostate-specific antigen (PSA) levels were greatly increased (3,4). These cases were not complicated by DIC.

IL-6, C-reactive protein (CRP) and serum amyloid A (SAA) are widely used as acute inflammatory biomarkers in various conditions such as infection, inflammation, malignancy and tissue disturbance (5-7). IL-6 is a multi-functional cytokine that regulates the production of CRP and SAA in liver cells in various conditions (8,9). CRP is most widely used as a sensitive inflammatory biomarker in routine clinical examination. SAA exists as a high density lipoprotein (HDL)-complex with a molecular weight of 200-400 kDa, which is generally increased in patients with viral infection or in corticosteroid-treated patients in contrast to CRP (6,10).

α1-antitrypsin (α1AT) is a plasma glycoprotein, which inhibits the activity of proteases, such as trypsin and elastase, derived from macrophage or pancreas cells. α1-acid glycoprotein (α1AG) is also a plasma glycoprotein that inhibits the activity of progesterone. Ceruloplasmin (CP) is a copper-binding protein and is involved in metabolism of copper or iron.

It is thought that α2M is involved in inflammatory reaction through its function as a carrier protein of IL-6 (1). Furthermore, it has been suggested that the concentration of α2M affects the levels of IL-6, CRP and SAA produced by liver cells through its regulation of IL-6. However, the relationship between serum levels of α2M and acute inflammatory biomarkers in PCa

patients with or without α2M deficiency has not been demonstrated. Therefore, we quantified serum levels of CRP, SAA, IL-6, α1AT, α1AG and CP in PCa patients with or without α2M deficiency to establish the clinical significance and changes of these acute inflammatory biomarkers during PCa disease progression. Although this study includes only a limited number of PCa patients, it is the first report to investigate the clinical significance and changes of acute inflammatory biomarkers in PCa patients with regard to α2M deficiency.

Materials and methods

Subjects. Forty-three untreated adult men participated in this study, of whom 10 were healthy controls (mean age 62.8 years, range 52-70) and 33 were diagnosed with prostatic disease at the Kitasato University Hospital. The thirty-three patients had PCa at stage M1b (mean age 67.8 years, range 55-79) without inflammatory disorders. α2M deficiency was defined as serum α2M levels <20 mg/dl and there was no precipitation line of α2M on immunoelectrophoresis with anti-whole human serum and antiserum to α2M in routine examination. These patients also had markedly increased serum PSA levels and multiple distant bone metastases. The histological diagnoses in the patients were confirmed by six-sextant biopsy and/or transurethral resection in all cases. PCa was staged clinically following the TNM classification (11). 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. The M1 stage is defined by the presence of distant metastasis and M1b by bone metastasis. Serum samples were obtained from these patients and stored at -80˚C until use.

Ethical approval. The study was conducted in accordance with the Declaration of Helsinki. This study had no influence on the management of patients, and informed consent was obtained from all subjects.

Acute inflammatory biomarkers. Measurement of α2M, α1AT, α1AG and CP levels in serum was performed by laser nephelometry using a Behring Nephelometer Analyzer (Behring Diagnostics, Westwood, MA). CRP and SAA levels in serum were measured by latex nephelometry using the LX-M (Eiken Chemical Co., Japan). IL-6 levels in serum were measured by a specific luminescence sandwich-type enzyme-linked immunosorbent assay (ELISA), using a previously described method with modifications (12). The assay employed 2 monoclonal anti-human IL-6 antibodies (clones IG61 and IG67; Toray, Tokyo, Japan) and alkaline phosphatase with the chemiluminescent substrate Lumi-phos (Lumigen Inc., Detroit, MI) and was sensitive to 0.5 pg/ml.

Bone scintigraphy. Bone metastasis in PCa patients was diagnosed by bone scintigraphy using ^{99m}Tc-labeled methylene diphosphonate (^{99m}Tc-MDP).

Western blotting. Binding assays for purified α2M (Protogen AG, Laufelfingen, Switzerland) and recombinant IL-6 (rIL-6) (Genzyme, Cambridge, MA) were analyzed by Western blotting with 5-15% SDS-polyacrylamide gel electrophoresis and staining with anti-IL-6 sera (Dako, Glostrup, Denmark) according to the Laemmli method (13). α2M and IL-6 of 1:1 molar concentrations were mixed and incubated for 20 min at 37˚C.

High performance liquid chromatography. We analyzed the IL-6 elution profiles of a mixture of purified α2M (50 μg) and rIL-6 (2 μg) or serum (10 μl) from patients with α2M deficiency mixed with rIL-6 (2 μg) by high performance liquid chromatography (HPLC). Fractionation of serum samples was performed by HPLC using a TSKG-3000 SWXL (Tosoh, Tokyo, Japan) column, and IL-6 level in each fraction was measured. Flow rate was 1 ml/min and fractions of 0.5 ml volume were collected. The column was calibrated using 20 mM phosphate buffer (pH 7.5).

Statistical analysis. The Wilcoxon and the Mann-Whitney U-tests were used for statistical analyses, and $p<0.05$ was considered statistically significant.

Results

Concentrations of α2M, CRP, SAA, IL-6, α1AT, α1AG and CP in serum. Table I shows the concentrations (mean ± SD) of CRP, SAA, IL-6, α1AT, α1AG and CP in the sera of healthy controls and PCa patients with or without α2M deficiency. α2M deficiency was defined as serum α2M levels <20 mg/dl. The serum levels of CRP (Fig. 1), SAA (Fig. 2) and IL-6 (Fig. 3) in PCa patients without α2M deficiency were significantly higher than in healthy controls and PCa patients with α2M deficiency (all $p<0.01$). On the other hand, serum levels of α1AT (Fig. 4), α1AG (Fig. 5) and CP (Fig. 6) in PCa patients with or without α2M deficiency were significantly higher than in healthy controls ($p<0.05$). However, there was no significant difference between PCa patients with and without α2M deficiency.

Western blotting for α2M and IL-6 complex. To verify the detection and size of the α2M-IL-6 complex, purified α2M and rIL-6 were mixed, and the complex was identified using Western blotting as a band of ~800 kDa (Fig. 7).

IL-6 elusion profiles of mixture of purified α2M and rIL-6, and serum from patients with α2M deficiency mixed with rIL-6. Fig. 8 shows the IL-6 elution profiles of a mixture of purified α2M and rIL-6 (dots) and of serum from patients with α2M deficiency mixed with rIL-6 (triangles) by HPLC. Three peaks were identified from the mixture of purified α2M and rIL-6: the first peak at 800 kDa corresponding to the α2M-IL6 complex; a second peak at ~50 kDa corresponding to a polymer of IL-6; and a third peak at ~20 kDa representing free IL-6. When the serum from PCa patients with α2M deficiency was mixed with rIL-6 the first and third peaks corresponding to α2M-IL-6 complex and free IL-6, respectively, were undetectable.

Discussion

CRP, SAA, IL-6, α1AT, α1AG and CP are known as acute inflammatory biomarkers that increase in various conditions such as infection, inflammation, malignancy and tissue

Table I. Concentration (mean ± SD) of α_2-macroglobulin (α2M), C-reactive protein (CRP), serum amyloid A (SAA), interleukin-6 (IL-6), α1 anti-chymotripsin (α1AT), α1 acid glycoprotein (α1AG) and ceruloplasmin (CP) in sera of healthy controls and advanced prostate cancer (PCa) patients with or without α2M deficiency.

		Advanced PCa patients	
	Healthy controls (n=10)	without α2M deficiency (n=23)	with α2M deficiency (n=10)
α2M (mg/dl)	151.7±25.30	252.45±125.07[a]	12.75±5.60[b]
CRP (μg/dl)	109.58±89.66	1534.6±1482.8[a]	173±90.3[b]
SAA (μg/ml)	9.96±4.16	54.7±44.5[a]	2.15±1.68[b]
IL-6 (pg/ml)	7.58±6.0	46.50±40.65[a]	3.38±2.61[b]
α1AT (mg/dl)	122±28.0	293.76±68.51[c]	305.71±71.30[c]
α1AG (mg/dl)	67.5±25.5	102±39.36[c]	100.8±38.90[c]
CP (mg/dl)	29.0±8.0	37.74±10.93[c]	37.6±10.89[c]

[a]p<0.01, healthy controls vs. PCa patients without α2M deficiency; [b]p<0.01, PCa patients without α2M deficiency vs. PCa patients with α2M deficiency; [c]p<0.05, healthy controls vs. PCa patients with or without α2M deficiency.

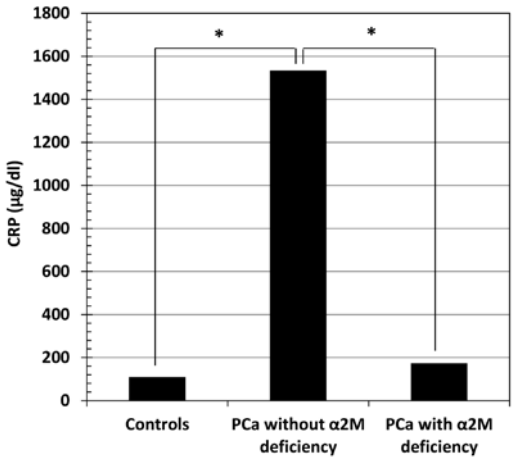

Figure 1. Serum CRP levels in healthy controls and advanced PCa patients with or without α2M deficiency. Serum CRP levels were significantly increased in PCa patients without α2M deficiency as compared with healthy controls and PCa patients with α2M deficiency. PCa, prostate cancer; *p<0.01.

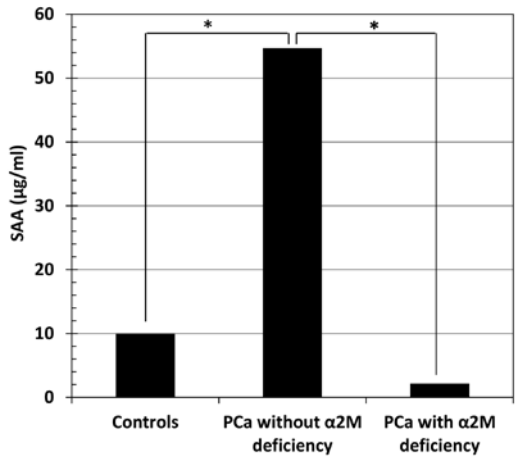

Figure 2. Serum SAA levels in healthy controls and advanced PCa patients with or without α2M deficiency. Serum SAA levels were significantly increased in PCa patients without α2M deficiency as compared with healthy controls and PCa patients with α2M deficiency. PCa, prostate cancer; *p<0.01.

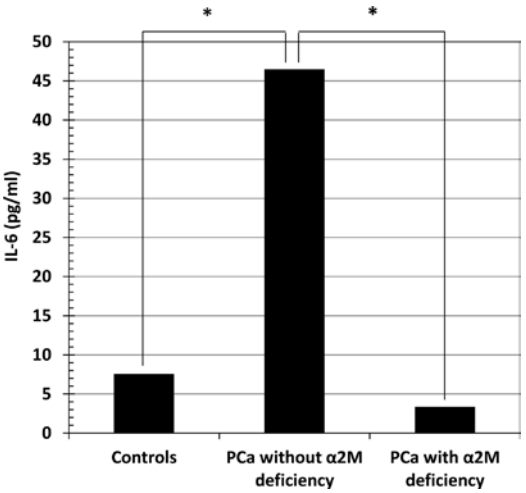

Figure 3. Serum IL-6 levels in healthy controls and advanced PCa patients with or without α2M deficiency. Serum IL-6 levels were significantly increased in PCa patients without α2M deficiency as compared with healthy controls and PCa patients with α2M deficiency. PCa, prostate cancer; *p<0.01.

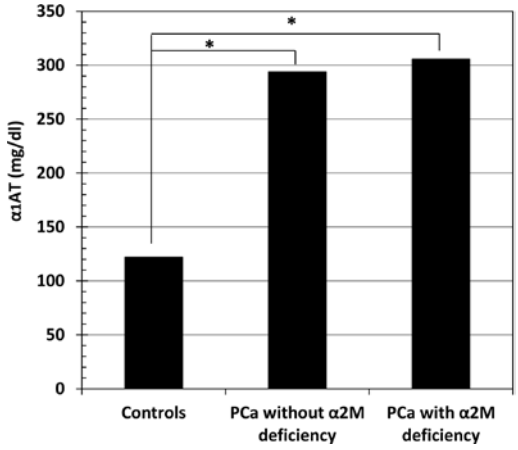

Figure 4. Serum α1AT levels in healthy controls and advanced PCa patients with or without α2M deficiency. Serum α1AT levels were significantly increased in PCa patients with or without α2M deficiency as compared with healthy controls. PCa, prostate cancer; *p<0.05.

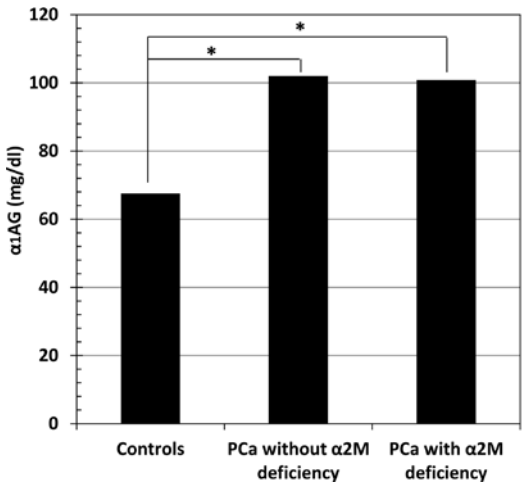

Figure 5. Serum α1AG levels in healthy controls and advanced PCa patients with or without α2M deficiency. Serum α1AG levels were significantly increased in PCa patients with or without α2M deficiency as compared with healthy controls. PCa, prostate cancer; *p<0.05.

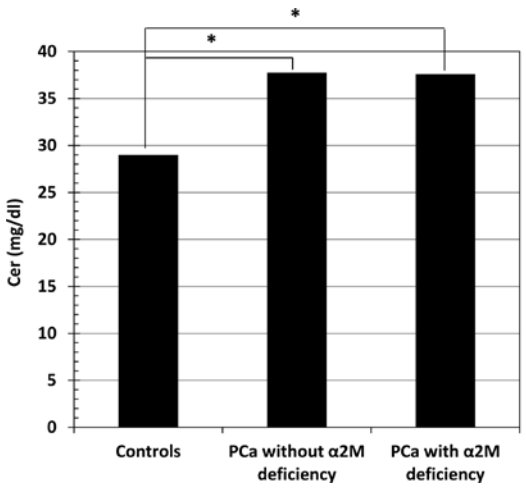

Figure 6. Serum CP levels in healthy controls and advanced PCa patients with or without α2M deficiency. Serum CP levels were significantly increased in PCa patients with or without α2M deficiency as compared with healthy controls. PCa, prostate cancer; *p<0.05.

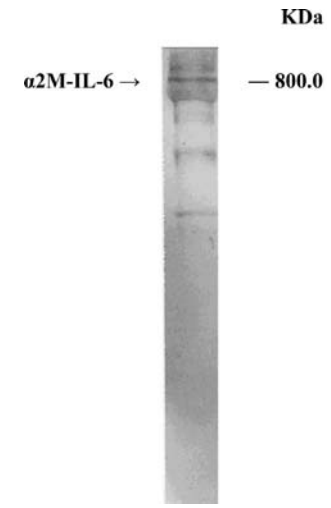

Figure 7. Western blotting for α2M and IL-6 complex. The band at ~800 kDa corresponds to the complex between purified α2M and recombinant IL-6.

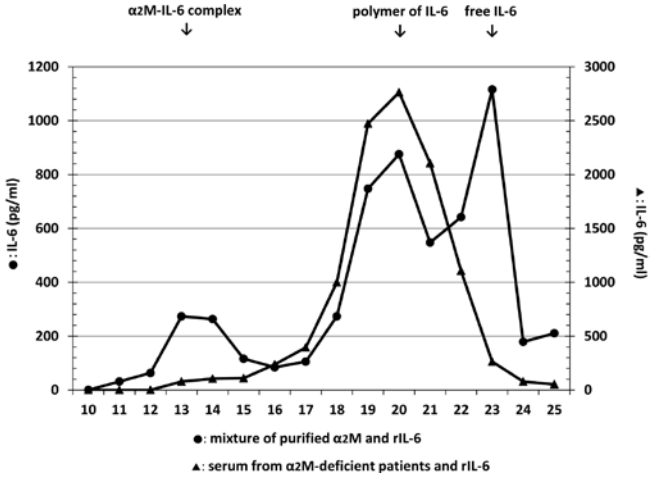

Figure 8. IL-6 elusion profiles of a mixture of α2M and recombinant IL-6 (rIL-6), and serum from patients with α2M deficiency mixed with rIL-6. Using HPLC, the mixture of purified α2M and rIL-6 generated three peaks: the first peak at 800 kDa corresponding to a α2M-IL6 complex; a second peak at ~50 kDa corresponding to a polymer of IL-6; and a third peak at ~20 kDa representing free IL-6.

disturbance (5-7,14). On the other hand, α2M is involved in the inflammatory reaction through its function as a carrier protein of IL-6 which promotes the production of acute inflammatory biomarkers in liver cells (1). Therefore, it has been suggested that the concentration of α2M affects the levels of IL-6, CRP and SAA produced by the liver cells through its regulation of IL-6. We had previously reported on advanced PCa patients with multiple distant bone metastases in whom serum α2M levels were markedly decreased to <20 mg/dl (α2M deficiency) (3,4). However, the relationship between serum levels of α2M and acute inflammatory biomarkers in PCa patients with or without α2M deficiency has not been demonstrated. In this study, we examined serum levels of CRP, SAA, IL-6, α1AT, α1AG and CP in PCa patients with or without α2M deficiency to establish clinical significance of these biomarkers during PCa disease progression. This is the first report that analyzed the changes of acute inflammatory biomarkers in PCa patients with or without α2M deficiency.

CRP is a plasma protein produced in liver cells by cytokine stimulation, mainly IL-6, but also interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) secreted from macrophages (8,9). Serum CRP levels are increased in various conditions including infection, inflammation and tissue disturbance such as malignancy or myocardial infarction, but rarely in viral infection, multiple myeloma and non-active systemic lupus erythematosus (6,15,16). It has been reported that CRP and SAA may be important prognostic markers for long-term survival in breast cancer patients (17). Fujikawa *et al* showed that elevated serum CRP levels are associated with tumor progression and poor prognosis of esophageal cancer (7). In recent years, the determination of high sensitivity CRP (hs-CRP) is possible due to the wide use of low concentration range measurements in

routine clinical examination. It has been reported that hs-CRP reflects the degree of localized vascular inflammation and is a useful prognostic marker of cardiovascular events (18,19).

SAA is a plasma protein produced in liver cells by cytokine stimulation mainly IL-1β, as well as IL-6 and TNF-α, and it exists as a high density lipoprotein (HDL)-complex with a molecular weight of 200-400 kDa. SAA is generally increased in patients with viral infection and corticosteroid-treated patients, a characteristic that differs from CRP (6,10). It has been hypothesized that the degree of change in serum SAA levels is larger as compared to CRP in various conditions such as infection, inflammation, malignancy and tissue disturbance, and IL1-β stimulation of SAA production is hard to suppress by corticosteroid (10). Cocco *et al* showed that SAA may be a novel biomarker for endometrial cancer to monitor disease recurrence and response to therapy (20). It is also reported that extremely elevated plasma levels of CRP and SAA are high risk factors for development of gastric cancer (21).

IL-6 is the most sensitive inflammatory cytokine which promotes the production of both CRP and SAA in liver cells, similar to IL-1β and TNF-α. It has been shown that serum IL-6 and CRP levels may be useful in the differentiation between diagnosis of pancreatic cancer and chronic pancreatitis (22). Kim *et al* reported that preoperative serum IL-6 and CRP levels may be markers of tumor invasion, lymph node metastasis and TNM stage (23). In the present study, CRP, SAA and IL-6 levels in serum were significantly increased in PCa patients without α2M deficiency as compared with healthy controls and PCa patients with α2M deficiency.

To further study the relationship between IL-6 and α2M, we demonstrated and confirmed here that IL-6 can easily complex with α2M *in vitro* as detected by immunoblotting. Furthermore, using HPLC, we showed that the IL-6 elution profiles of a mixture of purified α2M and rIL-6 generated three peaks corresponding to the α2M-IL-6 complex, polymer of IL-6 and free IL-6. In contrast, a mixture of serum from α2M-deficient PCa patients and rIL-6 did not demonstrate the peaks representing the α2M-IL-6 complex and free IL-6. These results suggest that a function of α2M is to bind and stabilize IL-6 by forming a complex. Based on these findings, IL-6 may be unstable in PCa patients with α2M deficiency, leading to decreased production of CRP and SAA in liver cells. This is supported by the evidence that the serum levels of α2M-dependent acute inflammatory biomarkers such as CRP, SAA and IL-6 were indeed decreased in PCa patients with α2M deficiency.

Some other acute inflammatory biomarkers including α1AT, α1AG and CP are also known to increase in various conditions. It has been reported that α1AT may be a significant factor in the differential diagnosis of serous effusion in patients with malignant disease (24). Yildrim *et al* reported that α1AG is a useful prognostic factor in addition to performance status in patients with primary lung cancer (25). It has been also shown that CP may be a reliable marker for prostate cancer where is not accompanied by elevation of serum PSA (26). Further, Kasprzyk *et al* have reported that α1AT, α1AG and CP are useful prognostic factors in the risk assessment of non-small lung cancer recurrence after surgical management (27). In the present study, serum levels of α1AT, α1AG and CP in PCa patients with and without α2M deficiency were significantly higher than in healthy controls. Moreover, there was no significant difference in levels of these three factors between PCa patients with and without α2M deficiency. These results suggest that α1AT, α1AG and CP are acute inflammatory biomarkers which are independent of α2M function as a carrier protein of IL-6.

In conclusion, the present study demonstrated that α2M can stabilize IL-6 by forming a complex, and in a mixture of serum from α2M-deficient PCa patients and rIL-6, the α2M-IL-6 complex and free IL-6 could not be detected. Furthermore, serum levels of CRP, SAA and IL-6, which are dependent on α2M function, were not increased in PCa patients with α2M deficiency. Therefore, measurement of the acute inflammatory biomarkers (CRP, SAA and IL-6) that depend on α2M in combination with those independent of α2M function (α1AT, α1AG and CP) in PCa patients may be an auxiliary indicator, together with PSA, to monitor PCa disease progression.

Acknowledgements

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