# Clinicopathological characteristics of androgen-dependent advanced prostate cancer patients with $\alpha_2$-macroglobulin deficiency

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**Abstract.** α2-macroglobulin (α2M) is thought to be involved in cancer metastasis and inflammatory reaction through its functions as a proteinase inhibitor and carrier protein for interleukin-6 (IL-6). We previously reported that advanced prostate cancer (PCa) patients with multiple distant bone metastases had markedly decreased serum α2M levels (<20 mg/dl) and no detection of α2M by immunoelectrophoresis (defined as α2M deficiency). We also showed a relationship between serum α2M levels and acute inflammatory biomarkers in PCa patients with or without α2M deficiency. In this study, we analyzed in detail the clinicopathological characteristics and pathogenesis of α2M deficiency in androgen-dependent advanced PCa patients. In this study, 15 PCa patients were diagnosed at the Kitasato University Hospital. α2M levels were determined by laser-nephelometry and immunoelectrophoresis, and PSA levels were determined by enzyme immunoassay. IL-6 levels were measured by a specific luminescence sandwich-type enzyme-linked immunosorbent assay, and CRP levels were determined by latex nephelometry. Immunohistochemical staining for PSA in PCa specimens was also performed. The binding assay for purified α2M and PSA was analyzed by western blotting. α2M deficiency was specific for advanced PCa patients with multiple distant bone metastases. PSA was markedly detected in sera and prostate specimens of advanced PCa patients with α2M deficiency, and there was a negative correlation between serum α2M and PSA levels during the course of clinical treatment. Acute inflammatory biomarkers such as IL-6 and CRP were within reference range in α2M-deficient patients. The binding assay showed that PSA easily bound to α2M, which was detected as an approximately 800-kDa complex by western blotting. Further, genetic analysis of a α2M-deficient patient showed no mutations in the α2M gene. These results suggested that α2M deficiency develops from catabolism of α2M in androgen-dependent advanced PCa patients, and serum α2M level may be an indicator of PCa disease progression in addition to PSA level.

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*Key words:* α2-macroglobulin, prostate-specific antigen, interleukin-6, C-reactive protein, androgen-dependent advanced prostate cancer

## Introduction

α2-macroglobulin (α2M) is a 720-kDa glycoprotein mainly produced by kupper cells of the liver and is the most abundant proteinase inhibitor in blood. Serum α2M levels generally increase in various disorders, but it has been shown to decrease in some pathological conditions such as disseminated intravascular coagulation (DIC), hematologic malignancy, advanced prostate cancer (PCa) and inherited α2M deficiency (1-4). α2M has an important biological function as a carrier protein for interleukin-6 (IL-6) and transforming growth factor (5,6), as well as an inhibitor of the activity of various proteases, and it is also involved in coagulation, fibrinolytic activity, inflammatory reaction and cancer metastasis *in vivo*.

Prostate-specific antigen (PSA) is a 32-kDa glycoprotein secreted by glandular epithelial cells of the prostate gland. This enzyme is a serine protease with properties similar to the kallikrein-type enzymes and serves to liquefy seminal coagulum (7). PSA is present as a free molecule or as a complex with α1-antichymotrypsin (ACT) or α2M in the blood, and it has been shown to be a specific marker for diagnosis, prognosis and monitoring of therapeutic effects in PCa patients (8-10). We previously reported that the relative proportion of free and complexed forms of PSA is different in patients with localized PCa and benign prostate hypertrophy (BPH) (11).

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 (12-14). IL-6 is a 23-kDa multi-functional cytokine that regulates the production of CRP and SAA in liver cells, and plays an important role in the inflammatory reac-

tion *in vivo* (15,16). CRP is a 120-kDa sensitive inflammatory biomarker and most widely used in routine clinical examination.

It is thought that α2M is involved in inflammatory reaction through its function as a carrier protein of IL-6 (5). 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. We 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) (1,2). We also showed a relationship between serum levels of α2M and acute inflammatory biomarkers in PCa patients with or without α2M deficiency (17). In this study, we analyzed in detail the clinicopathological characteristics and pathogenesis of α2M deficiency in androgen-dependent advanced PCa patients. Although this study includes only a limited number of PCa patients, it is the first report on the investigation of the clinicopathological characteristics of androgen-dependent advanced PCa patients with α2M deficiency.

## Materials and methods

*Fifteen untreated adult men, who were diagnosed with prostatic disease at the Kitasato University Hospital, participated in this study*. The 15 patients had PCa at stage M1b (mean age 71.8 years, range 62-85 years), and the pathological diagnosis was adenocarcinoma in all cases. α2M deficiency in the advanced PCa patients was defined as serum α2M levels markedly decreased to <20 mg/dl and lack of a precipitation line denoting α2M by immunoelectrophoresis using anti-whole human serum and antiserum to α2M in routine clinical examination. Histology was confirmed by six-sextant biopsy and/or transurethral resection in all cases. PCa was staged clinically according to the TNM classification (18). 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. We analyzed the changes in serum levels of α2M, PSA and CRP after transurethral resection of the prostate (TURP) and administration of bicaltamide, an anti-androgen reagent. We obtained informed consent from all subjects for this study.

*Measurement of α2M, PSA, IL-6 and CRP levels in serum*. α2M levels were determined by laser-nephelometry (Behring-Nephelometer Analyzer, Behring Diagnostics, Sommerville, NJ, USA). PSA levels were measured by enzyme immunoassay (Tosoh, AIA-600, Tokyo, Japan). Measurement of IL-6 levels was performed by a specific luminescence sandwich-type enzyme-linked immunosorbent assay (ELISA), using a previously described method with modifications (19). 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. CRP levels were measured by latex nephelometry using the LX-M (Eiken Chemical Co., Tokyo, Japan).

*Immunoelectrophoresis*. Immunoelectrophoresis was performed with anti-whole human serum (Fujirebio, Tokyo, Japan) and antiserum to α2M (Dako, Carpinteria, CA, USA).

*Bone scintigraphy*. 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.

*Immunohistochemical staining for PSA*. Sections of prostate cancer specimens were fixed in 10% formalin, embedded in paraffin and used for immunohistochemical analysis. Rabbit anti-human PSA antibody (Dako, Glostrup, Denmark) was diluted 3000-fold and used according to the labeled streptavidin-biotinylated antibody (LSAB) method. Paraffin sections (3 $\mu$m) were treated with organic solvents to remove the paraffin, washed with water and then soaked with methanol containing 0.3% hydrogen peroxide for 20 min to inactivate endogenous peroxidase. The sections were washed with water, incubated in phosphate-buffered saline (PBS) (0.01 M, pH 7.4) overnight at 4˚C, and reacted with the primary antibody. The sections were then washed with PBS and incubated with biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted 200-fold for 20 min, followed by treatment with streptavidin biotinylated-horseradish peroxidase complex (Amersham/GE Healthcare, Piscataway, NJ, USA) diluted 1/100 for 30 min at room temperature. Subsequently, the sections were incubated with diaminobenzidine (DAB) solution to visualize the staining, and the nuclei were stained with methyl green. PSA expression in the specimens was classified as very weak (positive cells were detected in <5% of the tumor mass), weak (positive cells were detected in 5-50% of the tumor mass), or strong expression (positive cells were detected in >50% of the tumor mass).

*Binding assay and western blotting*. Binding capability between purified α2M (Protogen AG, Laufelfingen, Switzerland) and PSA (Chemicon/Millipore, Billerica, MA, USA) was analyzed by western blotting using a 5-15% SDS-polyacrylamide gel and staining with anti-PSA sera (Dako, USA), according to the Laemmli method (20). α2M and PSA of 1:1 molar concentrations were mixed and incubated for 20 min at 37˚C before gel electrophoresis.

*Analysis of α2M gene*. DNA was extracted from a formalin-fixed liver specimen obtained from one of the PCa patients with α2M deficiency with DNA from healthy volunteers as controls. The α2M gene was analyzed in these liver DNA extracts using a polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) assay, as described in our previous report, to detect exon 24 (21).

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

*Ethical approval*. This 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.

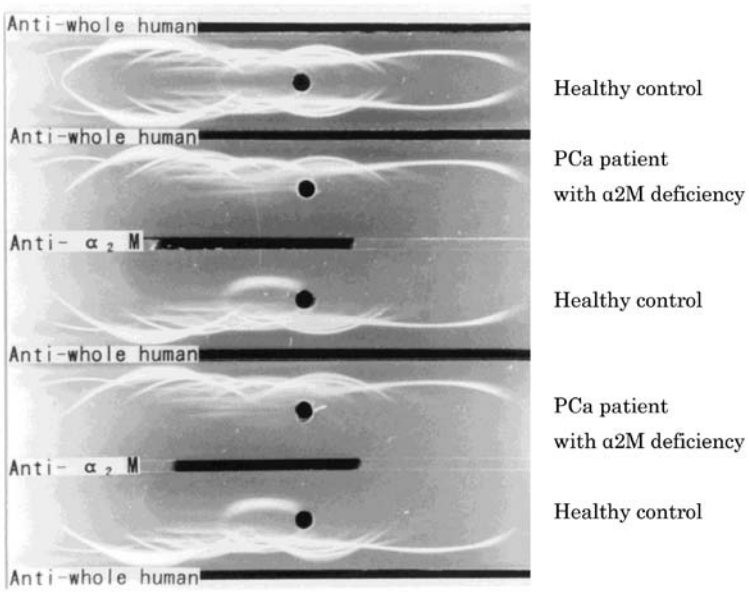


Figure 1. Immunoelectrophoretic pattern in serum of androgen-dependent advanced PCa patients with α2M deficiency.

Table I. Immunological findings of androgen-dependent advanced prostate cancer (PCa) patients with α2M deficiency.

| | Reference range | Median value (range) |
|---|---|---|
| α2M (mg/dl) | 100-250 | 10 (6-19) |
| PSA (ng/ml) | <2.0 | 6,800 (1,530-14,746) |
| IL-6 (pg/ml) | <10 | 1.95 (0.5-5.8) |
| CRP (μg/dl) | <300 | 170 (49-313) |

α2M, α2-macroglobulin; PSA, prostate-specific antigen; IL-6, interleukin-6; CRP, C-reactive protein.

## Results

*Immunological findings in serum of androgen-dependent advanced PCa patients with α2M deficiency.* Since our previous study indicated a relationship between α2M and inflammatory markers in PCa patients with or without α2M deficiency, we further analyzed the clinicopathological characteristics and pathogenesis of α2M deficiency in this study to understand the underlying mechanisms. Table I shows the concentrations (median value, range) of α2M, PSA, IL-6 and CRP in the serum of PCa patients with α2M deficiency. PCa patients with α2M deficiency had multiple distant bone metastases in all cases. Serum PSA levels were markedly increased in these patients while serum IL-6 and CRP levels were within reference range.

*Immunoelectrophoretic pattern in serum of androgen-dependent advanced PCa patients with α2M deficiency.* There was no precipitation line depicting α2M in the immunoelectrophoresis assay using anti-whole human serum and antiserum to α2M in PCa patients with α2M deficiency (Fig. 1).

*Skeletal scintigraphy of androgen-dependent advanced PCa patients with α2M deficiency.* All 15 patients enrolled in this study were diagnosed with M1b, thus skeletal scintigraphy was performed to confirm this. Anterior view before treatment showed an abnormal uptake of $^{99m}$Tc-MDP in skull, scapula, thoracic vertebra and hip bone (Fig. 2).

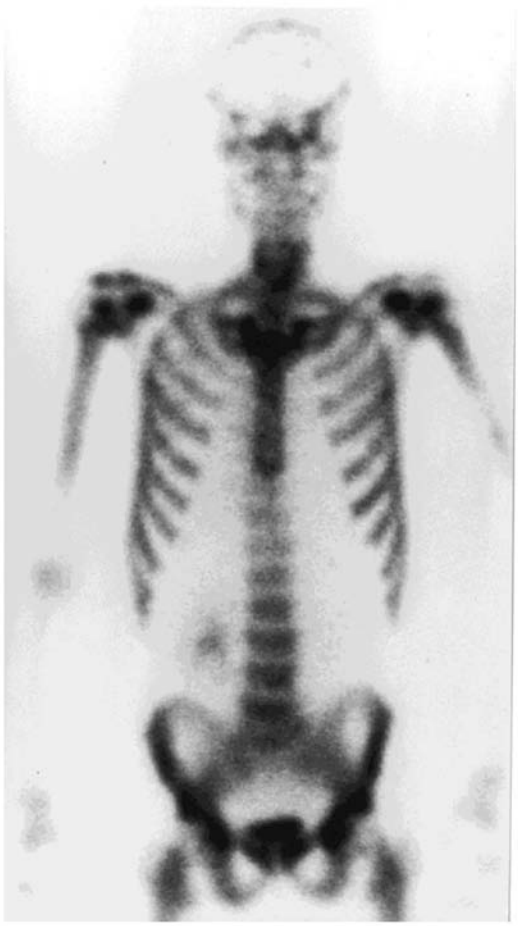

Figure 2. Skeletal scintigraphy of androgen-dependent advanced PCa patients with α2M deficiency.

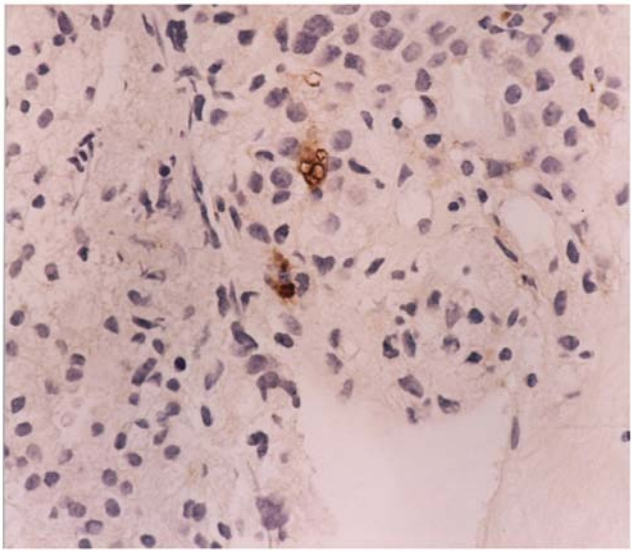

Figure 3. Immunohistochemical staining for PSA in the prostate specimens of advanced PCa patients without α2M deficiency (x200). PSA was weakly expressed in epithelial cells of the PCa specimens.

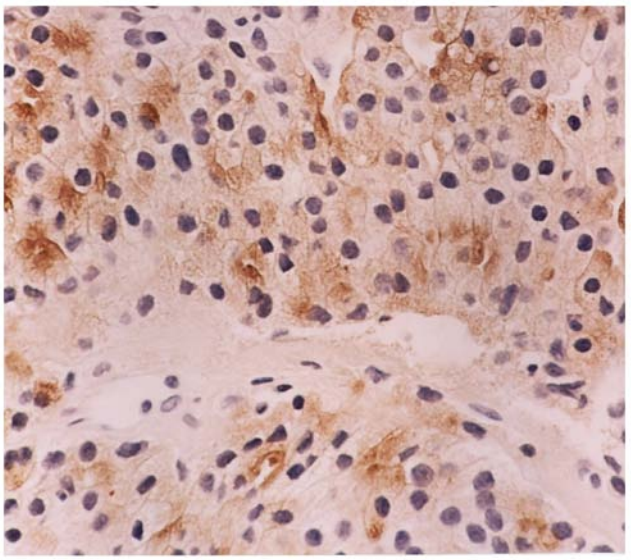

Figure 4. Immunohistochemical staining for PSA in the prostate specimens of advanced PCa patients with α2M deficiency (x200). PSA was expressed very strongly in epithelial cells of the PCa specimens.

*Immunohistochemical staining for PSA*. Immunohistochemical analysis of the prostate specimens was performed to evaluate the expression of PSA in PCa patients with or without α2M deficiency. PSA was weakly expressed in epithelial cells of the PCa specimens from patients without α2M deficiency (Fig. 3), while this protein was expressed very strongly in epithelial cells of the PCa cases with α2M deficiency (Fig. 4).

*Protein interaction between α2M and PSA*. To verify the presence and size of the α2M-PSA complex, purified α2M and PSA were mixed, and the complex was identified by western blotting as a band of ~800 kDa. Free PSA bands in the region of 30-40 kDa were also observed (Fig. 5).

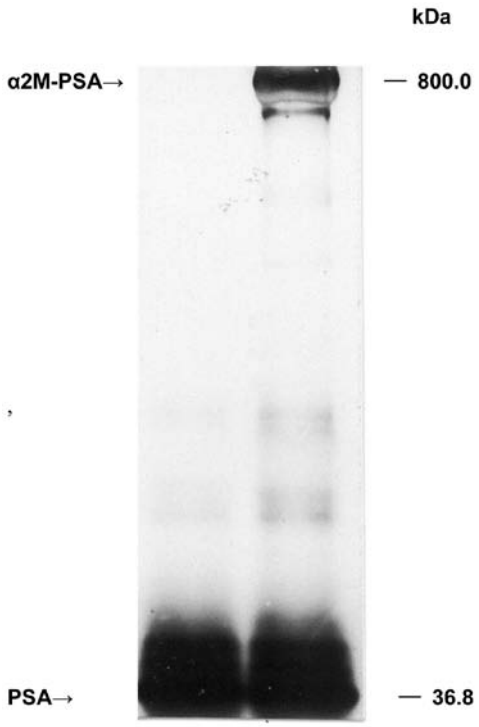


Figure 5. Binding interaction and complex formation between α2M and PSA as detected by western blotting.

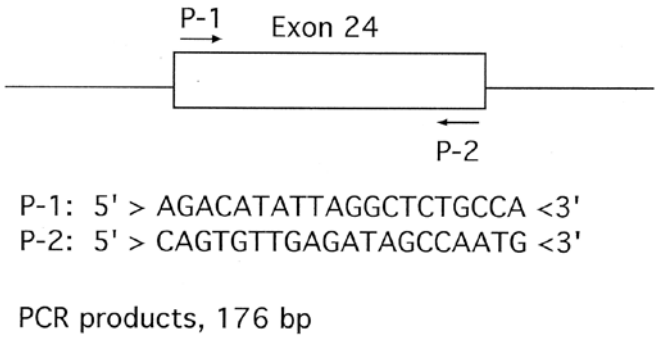


Figure 6. Genetic analysis of the α2M gene.

*Genetic analysis of the α2M gene*. We performed PCR analysis of exon 24 of the α2M gene to determine if the PCa cases harbored any mutations in this gene. Exon 24 contains a thiolester site, which is essential for the function of α2M as a proteinase inhibitor. The site was amplified with two primers by PCR (Fig. 6). The 176-bp amplification product of exon 24 from a PCa patient with α2M deficiency was identical to those derived from control samples (Fig. 7).

*Clinical course of an androgen-dependent advanced PCa patient with α2M deficiency*. To determine the relationship between PSA and α2M levels with respect to the clinical course and treatment, we followed one PCa patient with α2M deficiency. Serum PSA level was abnormally high while serum α2M level was extremely low in the patient upon admission to the hospital. Clinical improvement was accompanied by a decrease in serum PSA level, while conversely, serum α2M level increased after

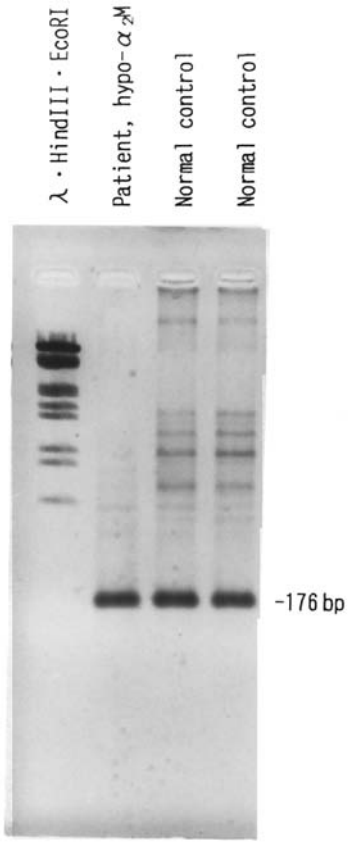


Figure 7. PCR of exon 24 in the α2M gene.

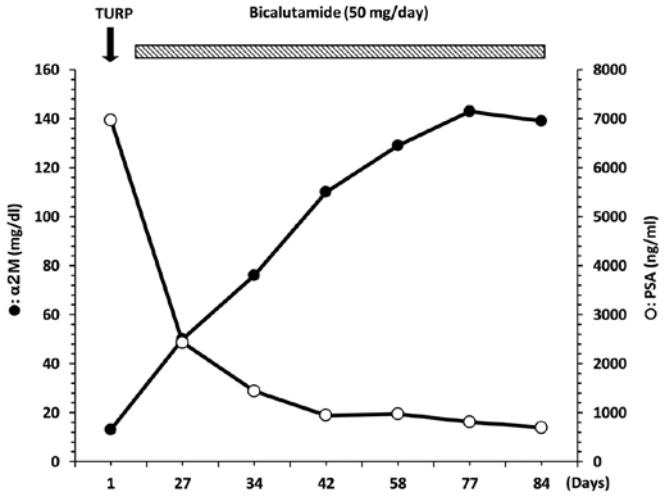


Figure 8. Clinical course of an androgen-dependent advanced PCa patient with α2M deficiency.

TURP and administration of bicaltamide, an anti-androgen reagent. Moreover, serum α2M and PSA levels changed inversely during the course of clinical treatment (Fig. 8).

*Change in serum CRP level in an androgen-dependent advanced PCa patient with α2M deficiency*. We further investigated the relationship between CRP and α2M levels with respect to the clinical course and treatment. Serum CRP level was within the

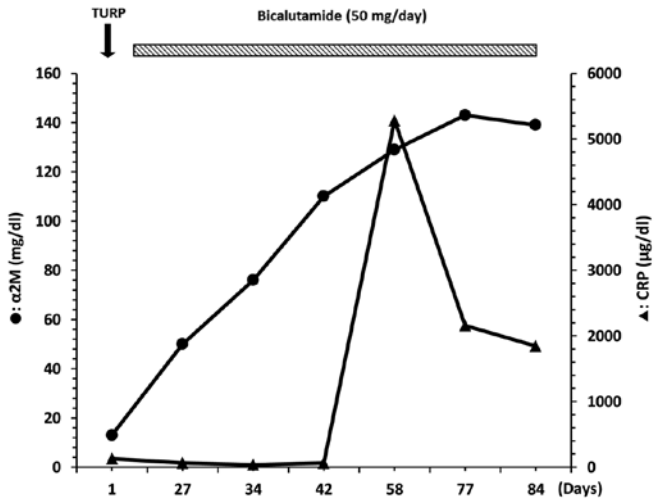


Figure 9. Change in serum CRP level in an androgen-dependent advanced PCa patient with α2M deficiency.

reference range to approximately the 50th day of treatment, but thereafter, it increased to an extremely high level after serum α2M level improved to within the reference range (Fig. 9).

## Discussion

The α2M protein is involved in fibrinolytic activity, cancer metastasis and inflammatory reaction *in vivo* through its functions as a protease inhibitor and carrier protein for IL-6 (5). We previously reported that the quantitative imbalance between proteases, such as PSA and matrix metalloproteinase-2, and their inhibitors, including α2M and α2-plasmin inhibitor, is a causative factor in invasion and metastasis of PCa (22). Furthermore, we showed a relationship between serum levels of α2M and acute inflammatory biomarkers such as IL-6 and CRP in PCa patients with or without α2M deficiency (17). We also 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) (1,2). In this study, we are the first to analyze and report in detail the clinicopathological characteristics and pathogenesis of α2M deficiency in androgen-dependent advanced PCa patients.

All cases of α2M deficiency were advanced PCa patients with multiple distant bone metastases, but some cases were not complicated by DIC. α2M deficiency in these 15 cases was verified as shown by lack of a precipitation line depicting α2M in the immunoelectrophoretic assay using anti-whole human serum and antiserum to α2M, and serum α2M levels were <20 mg/dl. On the other hand, PSA was expressed very strongly in the epithelial cells of the PCa specimens with α2M deficiency as compared to without α2M deficiency, which correlated with the notable increase in serum PSA levels. It is thought that PSA is expressed in epithelial cells of the prostate gland and secreted into the blood. It is also reported that PSA is detected in blood either as a free molecule or as a complex with α2M and ACT (8-11). In this study, a binding assay was performed whereby pure α2M and PSA were combined *in vitro*, and the binding interaction was detected by western blotting

in order to confirm the complex formation between α2M and PSA. The bands representing the α2M-PSA complex and free PSA were observed by immunoblotting. It is thought that the α2M-PSA complex retains weak enzymatic activity, but residual protease activity is rapidly eliminated from the blood by cells of the reticuloendothelial system, such as macrophages and kupper cells of the liver (23,24).

PCa treatment consists of anti-androgen therapy, irradiation and finally prostatectomy. Anti-androgen therapy is at first generally very effective for androgen-dependent advanced PCa, yielding survival benefits for most patients (25-27). In the present study, clinical improvement was accompanied by a decrease in serum PSA levels, and serum α2M and PSA levels changed inversely during the course of clinical treatment using TURP and anti-androgen therapy. Therefore, serum α2M levels appear to be affected by serum PSA levels. Based on these results, it was speculated that α2M deficiency in advanced PCa patients was caused by elimination of the large amount of α2M-PSA complex.

In order to determine if α2M deficiency was due to mutational changes in the α2M gene, genetic analysis was performed and we found that the 176-bp amplification product of exon 24 from a PCa patient with α2M deficiency was identical to that derived from normal control subjects, indicating that the α2M gene (exon 24) of the patient had no mutations. These results suggested that α2M deficiency developed from catabolism of α2M rather than loss of α2M through mutation in androgen-dependent advanced PCa patients.

The α2M protein is involved in inflammatory reaction through its function as a carrier protein of IL-6 which promotes the production of other acute inflammatory biomarkers such as CRP and SAA in liver cells (15,16). We previously reported that serum levels of α2M-dependent acute inflammatory biomarkers such as CRP, SAA and IL-6 were decreased in PCa patients with α2M deficiency (17). In this study, serum IL-6 levels were within reference range in PCa patients with α2M deficiency. Furthermore, serum CRP levels were also within reference range, but only until day 50 of treatment, following which the marker increased dramatically to extremely high levels while serum α2M level improved to within reference range during the clinical course. This suggests that the production of acute inflammatory biomarkers such as IL-6 and CRP is affected by α2M levels in serum.

In conclusion, the present study demonstrated that α2M deficiency is specific for advanced PCa patients with multiple distant bone metastases. PSA was markedly detected in sera and prostate specimens of advanced PCa patients with α2M deficiency. PSA easily bound to α2M *in vitro*, as detected by western blotting. Furthermore, there was a negative correlation between serum α2M and PSA levels during the course of clinical treatment. Based on these results, it is speculated that α2M deficiency in advanced PCa patients is caused by elimination of the large amount of α2M-PSA complex in the blood, and not by mutational changes in the α2M gene since no mutations were found in an α2M-deficient patient. These results suggested that α2M deficiency developed from catabolism of α2M in advanced PCa patients, and serum α2M levels may be an indicator of PCa disease progression in addition to PSA levels.

**Acknowledgements**

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