further confirmation by longer and larger studies is required for the use of finasteride as a treatment option for patients with PSA recurrence after radical prostatectomy.

Eventually, the treatment of PSA recurrence by suspected distant metatstasis involves hormonal manipulation, but at present the type and timing of such treatment are still based on physician and patient preference.

CONCLUSIONS

A significant proportion of patients who undergo a radical prostatectomy for localized prostate cancer develop PSA recurrence. In terms of the treatment for such PSA recurrence, some patients may be good candidates for local radiotherapy, whereas others may be indicated to undergo hormonal manipulation rather than radiotherapy. Although the pathological findings and post-operative serum PSA parameters may be useful for predicting the pattern of recurrence, it is still quite difficult to identify the most appropriate candidates for each type of treatment. The optimal type and timing of hormonal manipulation have yet to be elucidated. Further prospective randomized trials are thus still necessary to reach a consensus regarding the ideal treatment protocols for PSA recurrence after radical prostatectomy.

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Antiandrogen Bicalutamide Promotes Tumor Growth in a Novel Androgen-Dependent Prostate Cancer Xenograft Model Derived from a Bicalutamide-Treated Patient

Toru Yoshida, Hidefumi Kinoshita, Takehiko Segawa, Eijiro Nakamura, Takahiro Inoue, Yousuke Shimizu, Toshiyuki Kamoto, and Osamu Ogawa

Department of Urology, Kyoto University Graduate School of Medicine, Kyoto, Japan

Abstract

Androgen ablation therapies are effective in controlling prostate cancer. Although most cancers relapse and progress despite androgen ablation, some patients experience antiandrogen withdrawal syndrome, in which those treated with antiandrogen show clinical improvement when antiandrogen is discontinued. Although the androgen receptor (AR) is suggested to play an important role in prostate cancer progression even after the androgen ablation, limited tissue availability for molecular studies and small numbers of human prostate cancer cell lines have restricted prostate cancer research. Here, we describe KUCaP, a novel serially transplantable human prostate cancer xenograft model. We established KUCaP from liver metastatic tissue of a patient treated with antiandrogen bicalutamide. KUCaP expressed the AR with a point mutation at amino acid 741 (tryptophan to cysteine; W741C) in the ligand-binding domain. This mutation was also present in cancerous tissue used for generation of KUCaP. Although the growth of KUCaP in male mice was androgen dependent, bicalutamide aberrantly promoted the growth and prostate-specific antigen production of KUCaP. For the first time, we show the agonistic effect of bicalutamide to a xenograft with clinically induced AR mutation. This bicalutamide-responsive mutant AR will serve in the development of new therapies for androgen ablation–resistant prostate cancers. (Cancer Res 2005; 65(21): 9611-6)

Introduction

Most prostate cancers are initially dependent on androgens for their growth. Androgen ablation therapies are effective in controlling prostate cancer. However, virtually all prostate cancers relapse and progress after the initial response to androgen ablation therapy. Recent studies show that the androgen receptor (AR) signaling continues to influence prostate cancer growth even after androgen ablation (1). Increased bioavailability of the ligands in the prostate, increased expression of AR in prostate cancer, structural changes of AR by mutations, changes of AR coregulators levels, and ligand-independent activation of AR are the possible mechanisms for the growth of prostate cancer in this stage. Some investigators propose the term "androgen ablation–resistant" cancer instead of the term "androgen-independent" cancer, because prostate cancers may never encounter a completely "androgen-independent" environment despite androgen ablation (2). Through the course of progression to androgen ablation–resistant prostate cancer, the so-called "antiandrogen withdrawal syndrome" has been observed in 30% to 50% of patients (3). This phenomenon is characterized by the observation that patients show clinical improvement when therapeutic antiandrogen is discontinued, suggesting aberrant effects of antiandrogens on prostate cancer cells. Mutant ARs, such as T877A or H874Y, were reported to be activated by antiandrogen flutamide, which is considered to be a potential mechanism for antiandrogen withdrawal syndrome.

There are few *in vivo* models of prostate cancer that reflect various stages of the disease (4, 5). Here, we report the development and characterization of a novel androgen-dependent prostate cancer xenograft, KUCaP, which was established from a patient treated with antiandrogen bicalutamide. KUCaP had W741C mutation in its *AR* gene and our experimental results revealed that bicalutamide promoted the growth of KUCaP. Sequencing analysis suggested that this mutant AR was developed in the liver metastatic tissue during the treatment. This article shows for the first time the agonistic effect of bicalutamide to a xenograft with clinically induced AR mutation.

Materials and Methods

Patient and tissue samples. Clinical materials used for the establishment of KUCaP were obtained at autopsy from a 64-year-old Japanese male patient who died of androgen ablation–resistant prostate cancer after obtaining informed consent according to protocols approved by the Institutional Review Board at Kyoto University Hospital.

Animals. All experiments involving laboratory animals were done in accordance with the Guideline for Animal Experiments of Kyoto University and approved by Animal Research Committee at Kyoto University Graduate School of Medicine. C.B-17/IcrCrj severe combined immune-deficient (SCID) mice (Charles River Japan, Yokohama, Japan) were used.

Generation and serial transplantation of KUCaP. Liver metastatic tissue was harvested and immediately submerged in ice-cold RPMI 1640 supplemented with 10% FCS, minced into 20 to 30 mm^3 tumor bits, and transplanted into 5-week-old male mice. Tumor bits were transplanted s.c. with 50 μL Matrigel (Becton Dickinson, Bedford, MA) injected around the implant. At sacrifice, the xenograft was harvested and was serially transplanted into male mice as minced tumor pieces.

Hormonal manipulation on mice bearing KUCaP. To determine the response of KUCaP to androgen ablation, mice were surgically castrated after the xenograft volume reached 150 mm^3. In the bicalutamide + castration group, bicalutamide administration was started when xenograft volume was over 150 mm^3 and castration was done 7 days later. Bicalutamide (dissolved in benzyl benzoate and corn oil, 150 mg/kg/wk, administered thrice a week) or vehicle was injected s.c. during the experimental periods. Tumor volumes were measured with a caliper using the formula, $a \times b^2 \times 0.52$, where a is the largest diameter and

Requests for reprints: Osamu Ogawa, Department of Urology, Kyoto University Graduate School of Medicine, 54 Shogoinkawahara-cho, Sakyo-ku, 606-8507 Kyoto, Japan. Phone: 81-75-751-3325; Fax: 81-75-761-3441; E-mail: ogawao@kuhp.kyoto-u.ac.jp.
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b is the largest diameter perpendicular to a. Sera samples were obtained when animals were sacrificed and stored at −80°C until prostate-specific antigen (PSA) determination by Tandem-R assay (Hybritech, San Diego, CA).

Reverse transcription-PCR, Western blotting, and immunohistochemistry. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized with a First Strand cDNA synthesis kit (Amersham Biosciences, Buckinghamshire, United Kingdom) and amplified by PCR. Primer sequences for *AR* were as follows: 5′-GATGA-ACTTCGAATGAACTAC-3′ and 5′-CACTTGCACAGAGATGATCTC-3′. PCR conditions were as follows: 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 45 seconds, followed by a final extension step at 72°C for 5 minutes. For Western blotting, 40 μg of whole cell extracts were separated on a 10% polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore Co., Bedford, MA). The blot was blocked and then incubated with antihuman AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 16 hours at 4°C. The bands were detected with a horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences) and ECL plus detection system (Amersham Biosciences). Standard H&E staining was done on formalin-fixed and paraffin-embedded sections. Immunohistochemical assays were done using rabbit polyclonal antibody to human PSA (DakoCytomation, Glostrup, Denmark). Reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochroride. All sections were counterstained with hematoxylin.

Sequencing analysis. Genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany). All exons of the *AR* gene were PCR amplified. Individual exons or overlapping fragments (exons 1 and 4) were amplified as previously described (6). PCR amplicons were purified using a PCR purification kit (Qiagen) and then sequenced directly with the same primers used for the initial PCR using the ABI Prism 310 genetic analyzer system (Applied Biosystems, Warrington, United Kingdom).

Transactivation assays. Human mutant AR expression vectors, pCMV-AR-W741C and pCMV-AR-T877A, were constructed from pCMV-AR (7) by site-directed mutagenesis or subcloning of PCR products. PC-3 human prostate cancer cells were seeded into 24-well plates at 1.5×10^5 per well in phenol red–free RPMI 1640 supplemented with charcoal/dextran–treated FCS and were transiently cotransfected with 50 ng of human AR expression vector (wild-type, T877A, or W741C), 500 ng of p5.3PSAp-Luc, and 5 ng of pTK-RL using LipofectAMINE 2000 reagent (Invitrogen). Luciferase activity of the cell lysate in the indicated concentration of dihydrotestosterone, hydroxyflutamide, or bicalutamide was measured (8) using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) with a luminometer (MicroLumat Plus LB96V, Berthold Technologies, Bad Wildbad, Germany).

Results and Discussion

We established KUCaP, a novel serially transplantable human prostate cancer xenograft, by transplantation of liver metastatic tissue into male SCID mice. Tissue samples were obtained from a 64-year-old Japanese male patient who was 27 months postpresented with advanced prostate cancer (Fig. 1*A*). He was treated with maximal androgen blockade (combination of luteinizing hormone–releasing hormone analogue and antiandrogen) therapy using bicalutamide. After initial response to maximal androgen

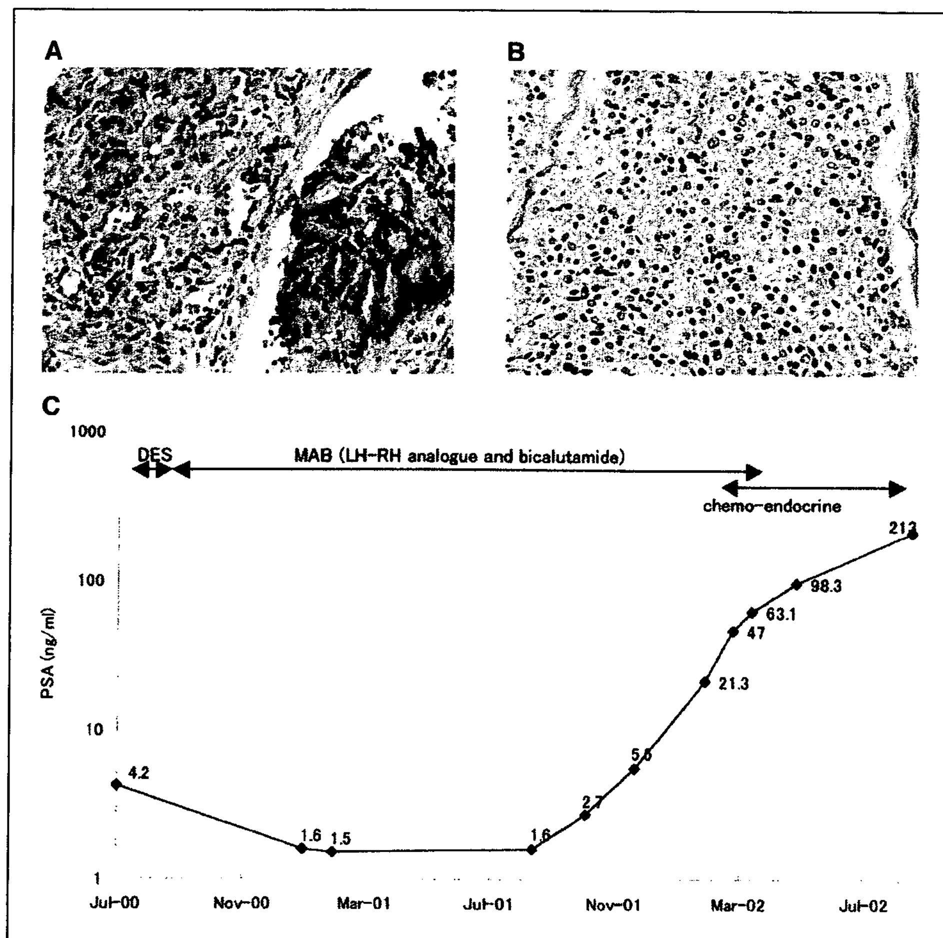

Figure 1. Clinical course of the patient. *A,* prostate needle biopsy specimen showed prostatic adenocarcinoma of Gleason score 4 + 5. H&E, ×400. *B,* liver tumor tissue obtained at autopsy showed metastatic adenocarcinoma. H&E, ×400. *C,* summary of clinical course of the patient. Initial treatment with diethylstilbestrol (*DES*) was followed by maximal androgen blockade (*MAB*) therapy using bicalutamide. After the progression with reelevation of serum PSA level, bicalutamide was discontinued and the patient received chemoendocrine therapy. The patient died of cancer in October 2002. *LH-RH,* luteinizing hormone–releasing hormone.

blockade therapy, serum PSA levels increased as liver metastasis progressed (Fig. 1*B* and *C*). There was no apparent progression in bone lesion during this period.

Tumor tissues obtained from prostate and liver metastases were transplanted s.c. into male SCID mice. One tumor piece from liver metastasis showed initial growth 6 months post-implantation and was designated as xenograft tumor KUCaP. KUCaP was maintained by serial passages in male mice as minced tumor pieces. The volume of KUCaP reached 100 mm^3 4 to 8 weeks posttransplantation. The tumor take rate of KUCaP was over 80%.

Androgen dependency and PSA expression are very important clinical features of human prostate cancer. AR is a key molecule that mediates the biological effects of androgens on prostate cancer cells (9). We confirmed AR expression by KUCaP using reverse transcription-PCR (RT-PCR; Fig. 2*A*) and Western blotting (Fig. 2*B*) and PSA production by KUCaP using an immunohistochemical assay (Fig. 2*C*). In addition, serum PSA level in mice bearing KUCaP xenografts was linearly correlated with xenograft volume (Fig. 2*D*).

We sequenced all *AR* exons from KUCaP genomic DNA. Sequencing results showed a missense substitution of TGG (tryptophan) to TGT (cysteine) at codon 741 (W741C) in the ligand-binding domain. W741C mutation was confirmed in KUCaP mRNA by sequencing RT-PCR products. DNA sequencing analysis of clinical materials from the patient revealed that only wild-type AR was present in prostatic tumors and in lymph node metastatic tissues. However, wild-type and W741C mutant AR coexisted in liver metastatic tissues used for the establishment of KUCaP (Fig. 3*A*). These results clearly indicated that W741C mutation in KUCaP AR was not developed after transplantation into mice but in liver metastatic tissues during the clinical course of the patient.

Normally, AR is specifically activated by testosterone and dihydrotestosterone (10). Mutations in ligand-binding domain often widen this stringent specificity and, as a result, prostate cancer cells with mutant AR can proliferate and avoid apoptosis by using other circulating steroid hormones or sometimes antiandrogens as substitute for androgens when androgen levels are low (11–13). It is reported that AR mutations are uncommon in primary prostate cancer without androgen ablation therapy (14). However, the frequency of mutations of AR is significantly increased in tumors after maximal androgen blockade therapy, suggesting strong selection pressure from antiandrogen on *AR* gene mutation (15, 16).

Recently, Hara et al. (17) established novel LNCaP cell sublines that had W741C mutation in addition to T877A, which parental LNCaP cells possess in AR, by exposing LNCaP cells to bicalutamide. Bohl et al. (18) reported the X-ray crystal structure of bicalutamide bound to codon 741 mutant AR and offered suggestion of structural modifications of this bicalutamide-mutant AR complex. Taplin et al. (15) and Haapala et al. (19) reported the discovery of W741C mutant AR in patients treated with bicalutamide. To investigate the role of W741C mutant AR in bicalutamide treatment *in vitro*, we transfected PC-3 prostate cancer cells, which does not express AR, with wild-type or mutant ARs (W741C or T877A) and assessed the transcriptional response to dihydrotestosterone and antiandrogens. *In vitro* transactivation assay showed that W741C mutant AR was aberrantly activated by bicalutamide, whereas wild-type and T877A mutant ARs were not stimulated by bicalutamide (Fig. 3*B*). In addition, when LNCaP cells, which possess T877A mutant AR, were transiently transfected with W741C mutant AR in steroid hormone-depleted medium, the administration of bicalutamide increased PSA concentration in the medium (data not shown). These results suggested that bicalutamide worked as an agonist for W741C mutant AR *in vitro*.

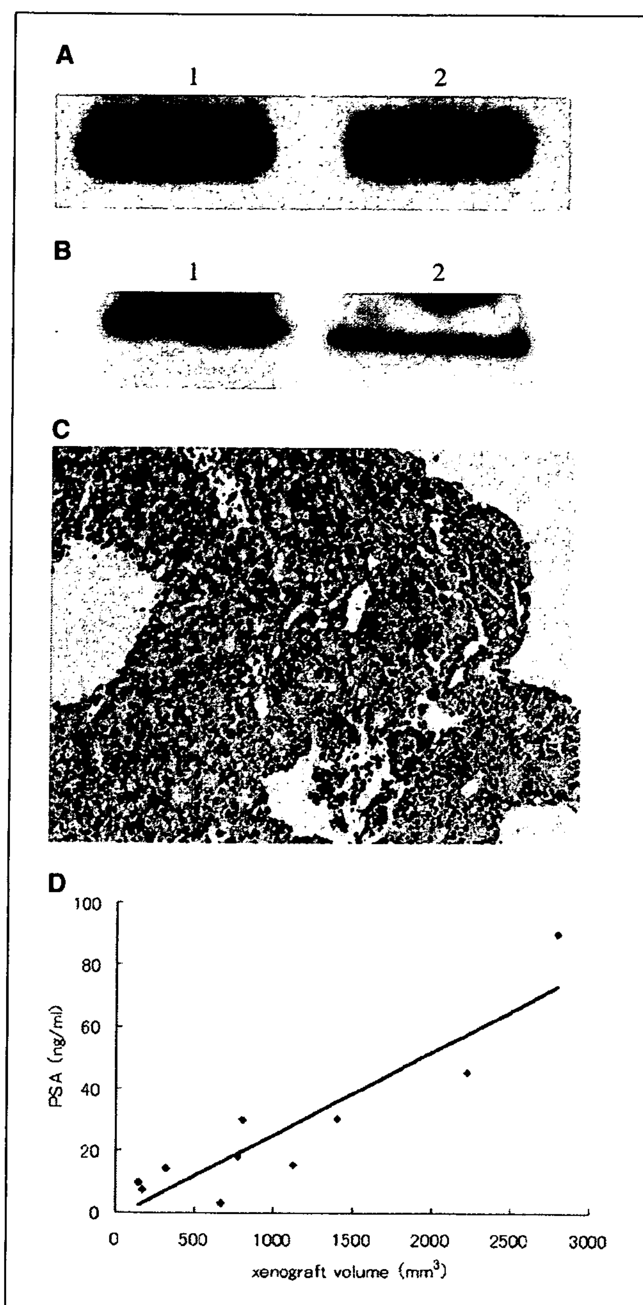

Figure 2. AR and PSA expression of KUCaP. *A,* AR expression was confirmed by RT-PCR assay. *Lane1,* LNCaP (AR- and PSA-positive prostate cancer cell line); *lane2,* KUCaP. *B,* AR expression was confirmed by Western blotting. *Lane 1,* LNCaP; *lane 2,* KUCaP. *C,* immunohistochemistry assay for human PSA. ×200. *D,* serum PSA levels of mice bearing KUCaP xenograft were measured by Tandem-R assay. Serum PSA levels were linearly correlated to xenograft volume.

Next, we investigated KUCaP growth in response to androgen or bicalutamide *in vivo*. Immediately after the castration of mice

bearing KUCaP, the volume of the xenograft began to decrease (Fig. 4A), indicating that the growth of KUCaP was androgen dependent. We investigated whether KUCaP growth would be promoted by bicalutamide as suggested by transactivation assay using W741C mutant AR. Administration of bicalutamide or vehicle to mice bearing KUCaP was started 1 week before the castration and continued for 3 more weeks until the sacrifice. Although KUCaP treated with vehicle regressed after castration, KUCaP treated with bicalutamide continued to grow even after castration (Fig. 4B). In castrated mice treated with vehicle, PSA level was <0.2 ng/mL in all animals. However, serum PSA level in castrated mice treated with bicalutamide did not decrease—93.5 ± 30.7 ng/mL (mean ± SD)—but is even higher than PSA levels in mice without hormonal manipulation (Fig. 4C). These results suggested that antiandrogen bicalutamide had an agonistic activity to W741C mutant AR *in vivo*. Bicalutamide-treated KUCaP in castrated mice had very similar histology to KUCaP in male mice without hormonal manipulation, suggesting that bicalutamide functioned as a substitution of testicular androgen even after castration in KUCaP (Fig. 4D-F).

Mutant ARs are implicated in antiandrogen withdrawal syndrome (3). Nonsteroidal antiandrogens, such as bicalutamide or flutamide, bind to AR and prevent androgen-induced conformational change of AR. They are used in androgen ablation therapy in combination with medical or surgical castration. In antiandrogen withdrawal syndrome, clinical characteristics of a patient improve when therapeutic antiandrogen is withdrawn, suggesting aberrant agonistic activity of antiandrogens to AR. Antiandrogen withdrawal syndrome can be observed in 30% to 50% of patients who manifest disease progression during maximal

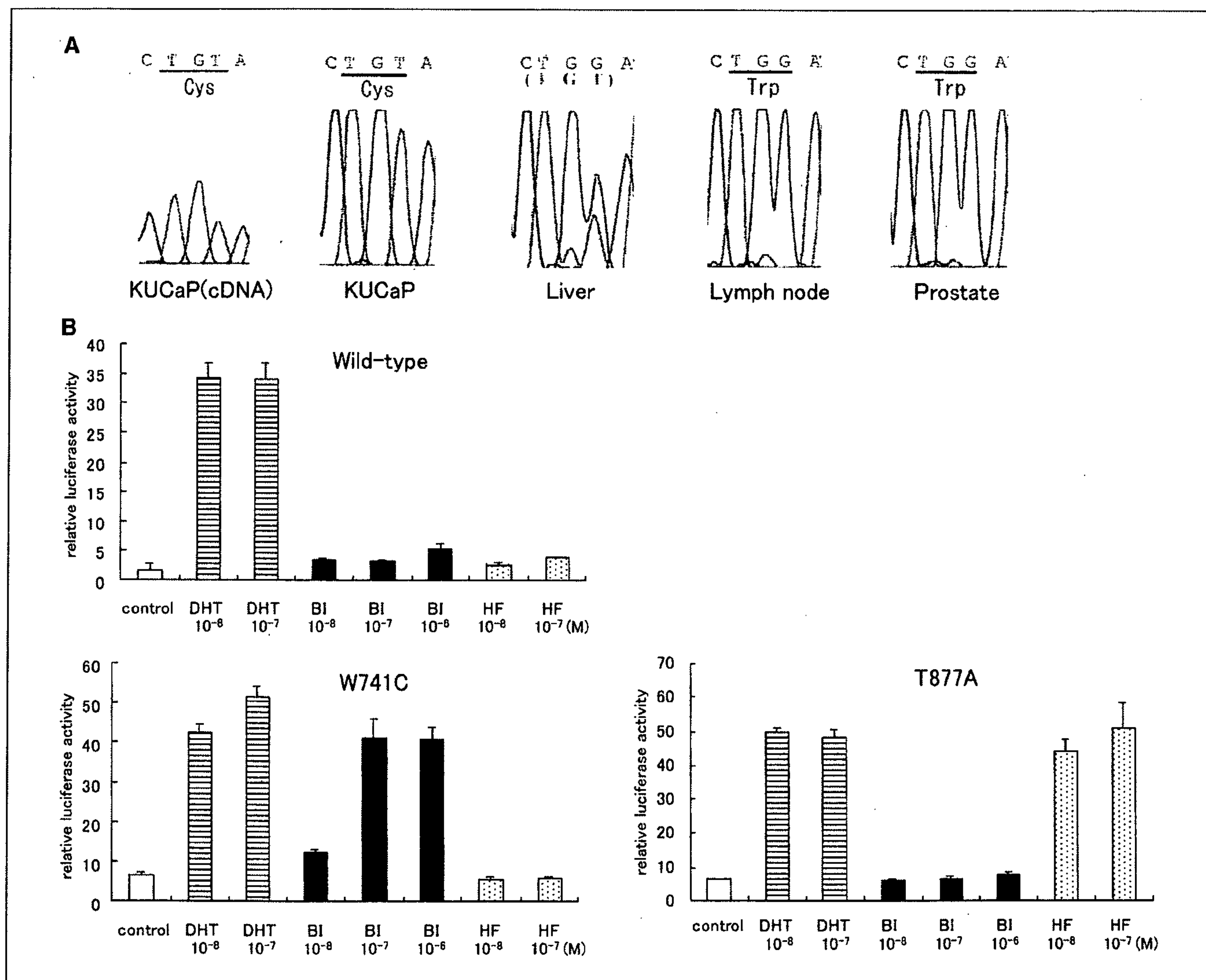

Figure 3. Bicalutamide works as an agonist for W741C mutant AR of KUCaP *in vitro*. *A*, W741C mutant AR developed in the liver metastasis and was maintained in KUCaP xenograft. *Left* to *right*, W741C in KUCaP cDNA, W741C in KUCaP genome, mixture of W741C and wild-type in patient's liver metastasis, wild-type in patient's lymph node metastasis, and wild-type in patient's prostate cancer. *B*, *in vitro* agonistic activity of bicalutamide to W741C mutant AR. PC-3 prostate cancer cells transfected with human AR expression vectors (wild-type, T877A, or W741C) were treated with dihydrotestosterone (*DHT*), bicalutamide (*BI*), or hydroxyflutamide (*HF*) at the indicated concentration. Luciferase activity was measured 48 hours after drug treatment. Human wild-type AR was specifically activated by dihydrotestosterone. W741C mutant AR was activated by dihydrotestosterone and bicalutamide, not by hydroxyflutamide. T877A mutant AR was activated by dihydrotestosterone and hydroxyflutamide, not by bicalutamide. Values are presented as the ratio of PSA-Luc versus *Renilla* Luc activity. *Columns*, mean of quadruplicated experiments; *bars*, SD.

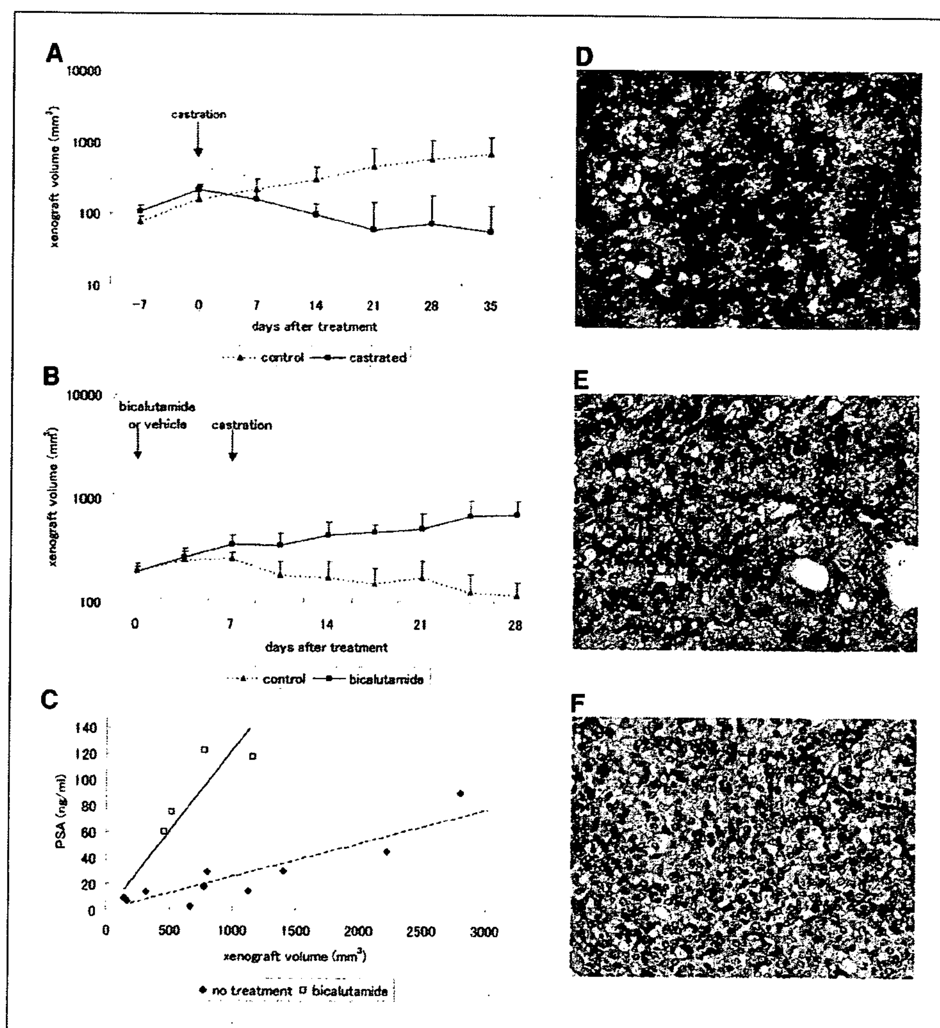

Figure 4. Effect of androgen ablation or bicalutamide administration on KUCaP. *A,* growth of KUCaP xenograft in intact male SCID mice (control, $n = 11$) and growth regression of KUCaP xenograft after surgical castration (castrated, $n = 4$). Mice were castrated after the xenograft volume reached 150 mm^3. Immediately after castration, xenograft growth was repressed and xenograft volume decreased. *B,* mice bearing KUCaP were treated with bicalutamide ($n = 6$) or vehicle ($n = 5$) and castrated on day 7. Growth of KUCaP was promoted by bicalutamide even after castration of mice. *Points,* mean; *bars,* SD. *C,* serum PSA levels at 3 weeks after castration in bicalutamide-treated mice were rather higher compared with male mice without hormonal manipulation. *D,* histology of KUCaP of a mouse treated with bicalutamide and castration. *E,* histology of KUCaP of a mouse without hormonal manipulation. *F,* histology of KUCaP of a mouse treated with vehicle and castration. H&E, ×200.

androgen blockade therapy. *In vitro* studies have suggested several potential mechanisms involved in this phenomenon. They include *AR* gene mutations, *AR* gene amplifications, and AR coregulatory protein alterations (3). Several mutant ARs, such as T877A and H874Y, are reported to be activated by flutamide *in vitro*. However, Shao et al. (20) found that H874Y mutant AR in CWR22 xenograft did not significantly affect *in vivo* steroid specificity and concluded that an *in vivo* model is essential for determining the effect of an AR mutation.

We have shown that KUCaP derived from clinical materials with W741C mutant AR maintained the mutation in the xenografts and that the response of KUCaP to bicalutamide *in vivo* was in concordance with the results of transactivation assays using W741C mutant AR. We believe that it is very important that W741C mutant AR, which showed aberrant response to bicalutamide, was not generated in the laboratory but in a patient treated with bicalutamide, the most widely used pure nonsteroidal antiandrogen. The fact that serum PSA values in this patient increased as liver metastasis progressed during bicalutamide treatment was compatible with our experimental results with KUCaP. The patient did not show antiandrogen withdrawal syndrome clinically. The finding that tumor cells other than liver metastatic cells did not have W741C mutant AR might be one of the reasons. However, the velocity of PSA elevation after bicalutamide discontinuation was lowered (Fig. 1*C*), suggesting partial effect of bicalutamide withdrawal.

In conclusion, we have established a novel androgen-dependent prostate cancer xenograft model KUCaP that may account for prostate cancer progression during bicalutamide treatment. KUCaP shows the possibility that clinically induced mutations of AR may actually play an important role in antiandrogen withdrawal syndrome or androgen ablation–resistant prostate cancer progression. The recognition of the presence of bicalutamide-responsive mutant AR in clinical settings will be important in the development of new forms of therapy for the treatment of patient suffering from androgen ablation–resistant prostate cancer.

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Prevention of Cancer Cachexia by a Novel Nuclear Factor κB Inhibitor in Prostate Cancer

Kenji Kuroda,[1] Yutaka Horiguchi,[1] Jun Nakashima,[1] Eiji Kikuchi,[1] Kent Kanao,[1] Akira Miyajima,[1] Takashi Ohigashi,[1] Kazuo Umezawa,[2] and Masaru Murai[1]

Abstract **Purpose:** To investigate the association between serum interleukin-6 (IL-6) and cachexia in patients with prostate cancer and the inhibitory effect of a new nuclear factor κB (NF-κB) inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), on IL-6 production and cachexia in an animal model of hormone-refractory prostate cancer.

Experimental Design: The association between serum IL-6 levels and variables of cachexia was evaluated in 98 patients with prostate cancer. The inhibitory effects of DHMEQ on IL-6 secretion and cachexia were investigated in *in vitro* and *in vivo* studies using JCA-1 cells derived from human prostate cancer.

Results: Serum IL-6 levels were significantly elevated and cachexia developed in JCA-1 tumor-bearing mice as well as in prostate cancer patients with progressive disease. IL-6 secretion was significantly inhibited in JCA-1 cells exposed to DHMEQ. Intraperitoneal administration of DHMEQ (8 mg/kg) to tumor-bearing mice produced a significant amelioration of the reduction in body weight, epididymal fat weight, gastrocnemius muscle weight, hematocrit, and serum levels of triglyceride and albumin when compared with administration of DMSO or no treatment. DHMEQ caused a significant decrease of serum IL-6 level in JCA-1 tumor-bearing mice (all $P < 0.05$).

Conclusions: These results suggested an association between serum IL-6 and cachexia in patients with prostate cancer and in JCA-1 tumor-bearing mice and that a new NF-κB inhibitor, DHMEQ, could prevent the development of cachexia in JCA-1 tumor-bearing mice presumably through the inhibition of IL-6 secretion. DHMEQ seems to show promise as a novel and unique anticachectic agent in hormone-refractory prostate cancer.

Cancer cachexia, which features the loss of muscle and fatty tissue as well as anorexia, asthenia, and anemia (1, 2), makes therapeutic intervention difficult (3) and is an important cause of death in cancer patients (4). Although little is known about the detailed mechanisms of cachexia, recent studies have revealed that inappropriate production and release of cytokines such as interleukin-6 (IL-6) is involved in the induction of cachexia (5-8). Progressive prostate cancer is often associated with anorexia, weight loss, and accelerated malnutrition that lead to cachexia, even if metastases are confined to the bones. It has been reported that human prostate cancer cells produce IL-6 and that the serum level of IL-6 is elevated in patients with prostate cancer (9, 10). However, few studies have investigated the association between the serum IL-6 level and cachexia in patients with prostate cancer. Production of IL-6 is regulated by several transcription factors, among which nuclear factor κB (NF-κB) is one of the pivotal regulators of cytokine-inducible gene expression (11). Schwarz et al. (12) have suggested that suppression of NF-κB may result in the amelioration of cachexia in a mouse tumor model. It is also well known that NF-κB shows often constitutive activation in hormone-refractory prostate cancer cells (13, 14). As far as we know, no investigators have explored a treatment strategy for cachexia based on the regulation of NF-κB by administration of a compound synthesized from a natural product. Recently, we have investigated the effectiveness of a new NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), which is a 5-dehydroxymethyl derivative of epoxyquinomicin C that shows anti-NF-κB activity in cultured human leukemia Jurkat cells and inhibits type II collagen-induced rheumatoid arthritis in mice (15). The present study was undertaken to evaluate the association between IL-6 and cachexia in patients with prostate cancer, as well as the inhibitory effect of DHMEQ on IL-6 production and cachexia in an animal model of hormone-refractory prostate cancer.

Authors' Affiliations: [1]Department of Urology, Keio University, School of Medicine, Tokyo, Japan and [2]Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan

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Requests for reprints: Jun Nakashima, Department of Urology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: 81-3-5363-3825; Fax: 81-3-3225-1985; E-mail: njun@sc.itc.keio.ac.jp.

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Materials and Methods

Patients. The association between serum IL-6 and cachexia in patients with prostate cancer was evaluated in this retrospective study. Ethics approval was obtained from our institutional ethics committee. Ninety-eight archival serum samples from patients with histologically

confirmed prostate cancer were examined. There were 55 patients with untreated disease, 23 patients in remission after endocrine therapy, and 20 patients with relapse. The definitions of remission and tumor progression were previously reported (8). Remission included any of the following: (*a*) reduction or disappearance of tumor masses; (*b*) a decrease in the number, size, or intensity of lesions on successive bone scans; or (*c*) a significant decrease of serum prostate-specific antigen. In addition, there had to be no new lesions and no deterioration of symptoms or performance status. Any of the following events was considered evidence of tumor progression: (*a*) the appearance of any new metastasis; (*b*) an increase in the number, size, or intensity of lesions on successive bone scans; or (*c*) significant cancer-related deterioration of symptoms or performance status. Classification of the patients with prostate cancer was done in accordance with Modified Jewett Staging System (16). Serum levels of IL-6 were measured using the Quantikine enzyme-linked immunoadsorbent assay kit (R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. Laboratory tests included analysis of serum albumin and hematocrit. Performance status was assessed in accordance with the Eastern Cooperative Oncology Group scale, in which 0 indicated that the patient had no symptoms; 1, the patient had symptoms but was ambulatory; 2, the patient was bedridden less than half the day; 3, the patient was bedridden half the day or longer; and 4, the patient was chronically bedridden and required assistance with activities of daily living. Body mass index (BMI) was calculated by the following formula: weight (kg) / height^2 (m^2).

Cell line. JCA-1 cells derived from human prostate cancer (17) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin (Life Technologies, Inc., Grand Island, NY), and 100 IU/mL penicillin (Life Technologies).

Chemicals. DHMEQ was synthesized in our laboratory (15). We also referred to the article by Suzuki et al. (18) in which the molecular structure was shown. It was dissolved in DMSO to prepare a 10 mg/mL solution and was subsequently diluted in culture medium to a final DMSO concentration of <0.1%.

In vitro ***interleukin-6 assay.*** JCA-1 cells (1×10^5) were seeded in a total volume of 1 mL of medium in each well of 24-well tissue culture plates and allowed to grow overnight. Then cells were treated with 1.0 or 1.5 μg/mL of DHMEQ, whereas other cells treated with the same concentrations of DMSO served as controls. After 48 hours of incubation, the supernatant of each well was collected and stored at −80°C until assay, and the number of viable cells was determined by trypan blue dye exclusion. The IL-6 concentration was measured using an enzyme immunoassay specific for human IL-6 (R&D Systems QuantiGlo Human IL-6 Immunoassay kit) according to the instructions of the manufacturer.

Animal model. All procedures involving animals and their care in this study were approved by the animal care committee of our institution in accordance with institutional and Japanese government guidelines for animal experiments. Male Balb/C-nu/nu mice were obtained from Sankyo Lab Service Corp. (Tokyo, Japan). The mice were housed at a constant temperature and humidity and received a standard diet and water. JCA-1 cells (1×10^7) were inoculated s.c. into the right flank of each mouse. When the tumors reached ~ 10 mm in diameter, mice were randomly assigned to three groups. DHMEQ (8 mg/kg) was administered i.p. in a volume of 0.2 mL once daily for 25 days to group 2 (n = 12). This group was labeled Tumor (+), DHMEQ. To clarify the effect of the vehicle, the same dose of DMSO given to group 2 was injected in another group of mice (group 3, n = 16), and this group was labeled Tumor (+), DMSO, and another group of tumor-bearing mice was observed without any treatment [group 4, n = 11; this group was labeled Tumor (+), No drug]. As a healthy control, age-matched mice were observed without any treatment [group 1, n = 14; this group was labeled Tumor (−), No drug]. During the treatment period, mice were carefully monitored and body weight was measured every other day. At the time of sacrifice, the tumor, gastrocnemius muscle, and epididymal fat were dissected and weighed. Blood samples were collected into nonheparinized tubes, and serum was separated within 1 hour of sacrifice. The serum samples were stored at −80°C and thawed just before testing. Serum IL-6 activity was determined using an enzyme immunoassay specific for human IL-6 (R&D Systems QuantiGlo Human IL-6 Immunoassay kit) according to the instructions of the manufacturer. At the same time, the hematocrit and the serum levels of triglycerides and albumin were also measured in each mouse.

Statistical analysis. All values are expressed as the mean ± SE. Variables for different groups were compared using Student's t test or ANOVA; $P < 0.05$ was considered statistically significant.

Results

The serum IL-6 level of 20 patients with relapse was significantly higher than that of 55 untreated patients or that of 23 patients in remission (Table 1). Serum levels of prostate-specific antigen were significantly higher in patients with relapse than in untreated patients or patients with remission. Serum albumin levels, hematocrit, and BMI were significantly lower in patients with relapse than in untreated patients or patients in remission (Table 1). The performance status was also significantly worse in patients with relapse when compared with untreated patients or patients in remission (Table 1). The serum albumin level and hematocrit were significantly lower (all $P < 0.05$) in patients with serum IL-6 level ≥ 7 pg/mL than in patients with serum IL-6 level < 7 pg/mL (data not shown). BMI was significantly lower in patients with serum IL-6 level ≥ 7 pg/mL (19.38 ± 0.41 kg/m^2) than in patients with serum IL-6 level < 7 pg/mL (22.94 ± 0.28 kg/m^2; $P < 0.0001$; Fig. 1).

The IL-6 level in the culture medium of JCA-1 cells treated with DHMEQ at 1.0 or 1.5 μg/mL for 48 hours was 4.03 ± 0.35 and 2.98 ± 0.17 pg/mL/10^5 cells, respectively, being significantly lower than when cells were treated with DMSO alone (10.54 ± 3.73 pg/mL/10^5 cells; P = 0.031 and P = 0.015, respectively; Fig. 2).

When mice had developed tumors ~ 10 mm in diameter after inoculation of JCA-1 cells, treatment was initiated and the day when treatment was started was designated as day 0. At the end of experiments, the mean weight of tumors of DHMEQ-treated mice was 3.02 ± 0.54 g, which was smaller than that of DMSO-treated mice (4.16 ± 0.81 g) and untreated mice (4.15 ± 1.10 g), but the differences were not statistically significant. The body weight, epididymal fat weight, gastrocnemius muscle weight, hematocrit, and serum levels of triglyceride and albumin were significantly lower in untreated JCA-1 tumor-bearing mice (group 4) than in healthy control mice without tumors (group 1), and serum IL-6 levels were significantly elevated in group 4 mice at the time of sacrifice (Figs. 3 and 4; Table 2). Although the body weight of untreated tumor-bearing mice and tumor-bearing mice treated with DMSO decreased in a time-dependent manner, the weight of JCA-1 tumor-bearing mice treated with DHMEQ did not decline significantly (Fig. 3). On day 26, body weight (28.24 ± 1.44 g), epididymal fat weight (197.11 ± 31.67 mg), and gastrocnemius muscle weight (499.27 ± 30.26 mg) were significantly greater in DHMEQ-treated mice (group 2) than in mice treated with DMSO alone (group 3; 24.09 ± 1.30 g, 117.12 ± 19.10 mg, and 306.28 ± 24.46 mg; P = 0.018, P = 0.044, and $P < 0.001$, respectively) or untreated mice (group 4; 21.46 ± 1.08 g, 43.48 ± 2.97 mg, and 261.13 ± 14.54 mg; P = 0.002, P = 0.001, and $P < 0.001$, respectively; Table 2).

In addition, the hematocrit (41.89 ± 1.54%) and the serum triglyceride level (60.63 ± 7.36 mg/dL) were significantly higher in group 2 mice than in group 3 mice (36.71 ± 1.81% and

Table 1. Disease status and variables

	No. patients	Serum levels of IL-6 (pg/mL)		Rate of serum IL-6 levels of ≥7 pg/mL (%)	Prostate-specific antigen (ng/mL)	Albumin (g/dL)	Hematocrit (%)	BMI (kg/m²)	Performance status
		Range	Mean ± SE		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Untreated patients									
Total	55	0.69-111.06	5.21 ± 2.06	10.9 (6/55)	171.09 ± 58.73	3.58 ± 0.06	36.26 ± 0.70	22.74 ± 0.31	0.75 ± 0.14
Stage A	3	0.86-1.6	1.20 ± 0.22	0 (0/3)	2.27 ± 0.71	3.57 ± 0.27	41.73 ± 3.35	20.97 ± 1.54	0
Stage B	15	0.69-5.65	2.45 ± 0.40	0 (0/15)	42.52 ± 19.18	3.57 ± 0.09	38.27 ± 1.26	22.21 ± 0.46	0.33 ± 0.13
Stage C	11	1.07-7.45	2.16 ± 0.45	9.1 (1/11)	50.94 ± 14.90	3.76 ± 0.06	38.16 ± 1.07	23.18 ± 0.65	0.09 ± 0.09
Stage D	26	1.08-111.06	8.55 ± 4.30	19.2 (5/26)	315.57 ± 118.37	3.52 ± 0.11	33.67 ± 0.94	23.05 ± 0.50	1.35 ± 0.22
Patients with remission as a result of endocrine therapy									
	23	0.26-4.77	2.45 ± 0.26	0 (0/23)	79.34 ± 53.48	3.78 ± 0.09	36.60 ± 1.03	23.12 ± 0.59	0.48 ± 0.16
Patients with relapsed bone metastatic disease after endocrine therapy									
	20	4.46-135.53	41.58 ± 8.07	85.0 (17/20)	14.35 ± 1210.34	2.78 ± 0.08	31.00 ± 1.37	18.99 ± 0.32	2.83 ± 0.19

NOTE: The untreated patients were separated into subgroups of stage A to D in accordance with Modified Jewett Staging System. Performance status was assessed in accordance with the Eastern Cooperative Oncology Group scale.

36.50 ± 6.16 mg/dL; $P = 0.018$ and $P = 0.020$, respectively) or group 4 mice (34.70 ± 1.80% and 32.00 ± 2.91 mg/dL; $P = 0.005$ and $P = 0.007$, respectively). The serum level of albumin was significantly higher in group 2 (2.04 ± 0.07 g/dL) than in group 4 (1.77 ± 0.08 g/dL; $P = 0.019$; Table 2). The serum IL-6 level of group 2 (238.83 ± 72.59 pg/mL) was significantly lower than that of group 3 (1,009.51 ± 316.35 pg/mL; $P = 0.030$) or group 4 (1,312.09 ± 368.66 pg/mL; $P = 0.006$; Fig. 4).

Discussion

It has been reported that elevation of the serum levels of IL-6 is strongly associated with cachexia in patients with various types of advanced cancer. Serum cytokine levels are increased in prostate cancer patients who have weight loss when compared with those who show no weight loss (8, 19). In the present study, prostate cancer patients with relapse showed more severe cachexia and had higher serum IL-6 levels than untreated patients or patients in remission. In addition, the prostate cancer patients with higher serum IL-6 levels were more cachectic than those with lower IL-6 levels. Wallenius et al. investigated the effect of IL-6 on the physique in mice lacking the gene encoding IL-6 ($Il6^{-/-}$ mice) and found that they developed mature-onset obesity that was partly reversed by IL-6 replacement. Taken together, these results suggest a strong relationship between the serum level of IL-6 and weight loss (20). In the present study, DHMEQ produced a significant decrease in the IL-6 level and a significant improvement in the body weight of tumor-bearing mice.

Alexandrakis et al. (21) showed that IL-6 was significantly higher and hemoglobin was significantly lower in patients with multiple myeloma than in the controls, and a significant decrease in hemoglobin concentration and hematocrit was also found in patients with higher serum IL-6 levels. Ishiko et al.

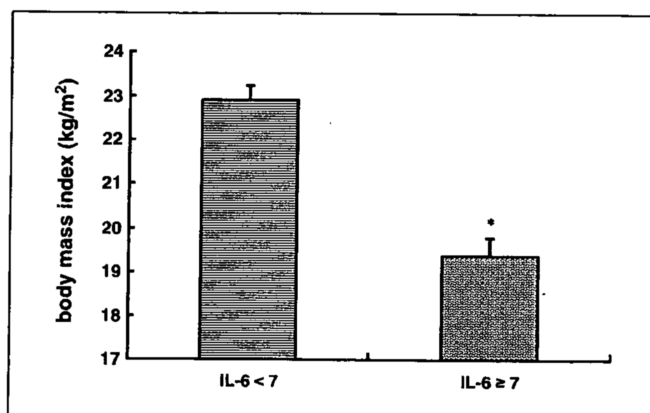

Fig. 1. Relationship between IL-6 and BMI. BMI was significantly lower in patients with serum IL-6 level ≥ 7 pg/mL (19.38 ± 0.41 kg/m²) than in patients with serum IL-6 level < 7 pg/mL (22.94 ± 0.28 kg/m²; $P < 0.0001$). *, significantly different from the mean value of patients with serum IL-6 level < 7 pg/mL.

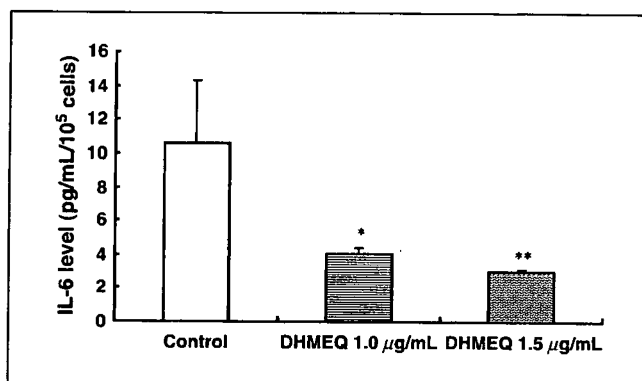

Fig. 2. Effect of DHMEQ on IL-6 secretion by JCA-1 cells. At concentrations of 1.0 and 1.5 μg/mL DHMEQ significantly inhibited IL-6 secretion by JCA-1 cells. *Columns,* mean value of samples; *bars,* SE. *, significantly different from control (DMSO alone; $P = 0.031$). **, significantly different from control (DMSO alone; $P = 0.015$).

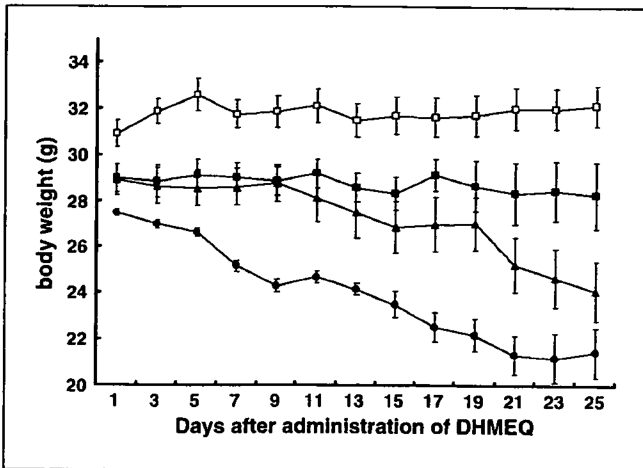

Fig. 3. Effect of DHMEQ on body weight. JCA-1 cells (1×10^7) were inoculated s.c. into the right flank of each mouse. When palpable tumors had developed (largest diameter >10 mm), the mice were randomly assigned to three groups. Then DHMEQ (8 mg/kg) was administered i.p. once daily for 25 days to one group of mice [■, Tumor (+), DHMEQ; $n = 12$], DMSO (the vehicle for DHMEQ) was given to another group [▲, Tumor (+), DMSO; $n = 16$], and no drug was given to another group [●, Tumor (+), No drug; $n = 11$]. The group Tumor (−), No drug were healthy control mice with no tumors and no treatment (□; $n = 14$). *Points,* mean body weight of mice per group; *bars,* SE. Body weight of group Tumor (+), DMSO was statistically significantly lower at day 23 ($P = 0.032$) and day 25 ($P = 0.018$) than that of group Tumor (+), DHMEQ, and body weight of group Tumor (+), No drug was statistically significantly lower at each day than that of group Tumor (+), DHMEQ (all $P < 0.05$).

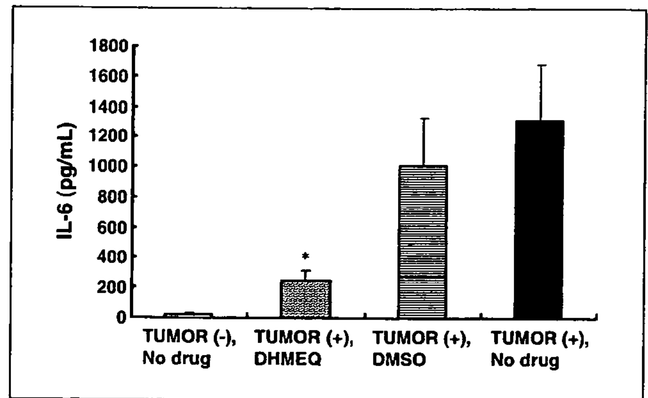

Fig. 4. Effect of DHMEQ on serum IL-6 level. Serum IL-6 levels were significantly lower in Tumor (+), DHMEQ mice than in Tumor (+), DMSO mice or Tumor (+), No drug mice. *Columns,* mean IL-6 levels of samples; *bars,* SE. *, significantly different from Tumor (+), DMSO ($P = 0.030$) and Tumor (+), No drug ($P = 0.006$).

(22) showed that there is severe anemia in cancer-bearing rabbits, whereas the mean hemoglobin value of normal rabbits was much higher. In our study, the serum IL-6 level was significantly higher and hematocrit was significantly lower in JCA-1 tumor-bearing mice than in healthy control mice, and DHMEQ significantly prevented the development of anemia and the increase of serum IL-6.

Soda et al. (23) transplanted mice with clone 20 (a subclone of murine colon adenocarcinoma), causing profound weight loss by 15 days after inoculation, and showed that body fat was lost preferentially along with a decrease in the plasma triglyceride level. Fat constitutes 90% of the fuel reserve in adults and depletion of fat is commonly seen in cancer patients with cachexia, whereas Strassmann and Kambayashi (24) reported that IL-6 may decrease carcass lipids. Path et al. investigated the effects of IL-6 on adipocyte functions. They found that chronic incubation of adipocytes with 1 nmol/L IL-6 during differentiation reduced glycero-3-phosphate dehydrogenase activity, a marker of adipocyte differentiation, and triglyceride synthesis to 67 ± 9% of the basal level ($P < 0.05$; ref. 25). Naoe et al. (26) showed that a significant decrease in the circulating levels of triglyceride was found in the saline-treated tumor-bearing mice compared with the saline-treated normal mice. In the present study, DHMEQ significantly inhibited the decrease of epididymal fat weight and serum triglycerides, presumably through the suppression of IL-6 production.

Oka et al. reported that serum albumin levels were significantly lower in patients with esophageal squamous cell carcinoma who had high serum levels of IL-6 when compared with patients who had lower IL-6 levels. They suggested that IL-6, which is produced by tumor cells, may be related to various disease variables in patients with esophageal squamous cell carcinoma, as well to the nutritional status (27). Tisdale (28) also reported that IL-6 may play a role in muscle wasting in certain animal tumors, possibly via both lysosomal (cathepsin) and nonlysosomal (proteasome) pathways. We showed that JCA-1 tumor-bearing mice showed a significant decrease in gastrocnemius muscle weight and serum albumin, and changes were significantly prevented by DHMEQ.

The regulation of many cytokine genes, including IL-6, is relatively simple, and the transcriptional factor NF-κB has been reported to up-regulate various cytokines (29-31). Several approaches to inhibit the activation of NF-κB have been investigated (32). A recombinant adenovirus vector expressing

Table 2. Effects of DHMEQ on epididymal fat weight, gastrocnemius muscle weight, hematocrit, and serum levels of triglyceride and albumin

	Tumor (−), No drug	Tumor (+), DHMEQ	Tumor (+), DMSO	Tumor (+), No drug
Epididymal fat weight (mg)	314.40 ± 40.23	197.11 ± 31.67*	117.12 ± 19.10	43.48 ± 2.97
Gastrocnemius muscle weight (mg)	558.66 ± 19.62	499.27 ± 30.26*	306.28 ± 24.46	261.13 ± 14.54
Hematocrit (%)	42.14 ± 1.07	41.89 ± 1.54*	36.71 ± 1.81	34.70 ± 1.80
Triglyceride (mg/dL)	80.82 ± 13.77	60.63 ± 7.36*	36.50 ± 6.16	32.00 ± 2.91
Albumin (g/dL)	2.45 ± 0.09	2.04 ± 0.07†	1.95 ± 0.08	1.77 ± 0.08

*Significantly different from the group of Tumor (+), DMSO and Tumor (+), No drug.
†Significantly different from the group of Tumor (+), No drug (all $P < 0.05$).

the stable form (IκBα) induces apoptosis of cancer cells showing constitutive NF-κB activity *in vitro* (33). Kawamura et al. developed synthetic double-stranded oligodeoxynucleotides for use as "decoy" *cis* elements that block the binding of nuclear factors to the promoter regions of target genes. They injected decoy oligodeoxynucleotide targeting NF-κB directly into adenocarcinoma colon 26 tumors in mice to examine whether or not cachexia was alleviated by inhibiting the action of cytokines, and their results suggested that cytokines regulated by NF-κB may play a pivotal role in the induction of cachexia in the colon 26 model (34). However, the clinical feasibility of gene therapy for inhibiting NF-κB is limited by the need for intratumoral delivery of a vector that expresses the NF-κB inhibitor, and few studies have assessed the usefulness of this strategy with *in vivo* models. In contrast, we assessed a novel agent for inhibiting the activity of NF-κB. There have been no previous reports about the therapeutic effect of an agent synthesized from a natural product on cachexia mediated through the regulation of cytokines by NF-κB.

We have previously reported that DHMEQ produces a significant decrease in NF-κB activity in JCA-1 cells with constitutive NF-κB activation (35). Because of these encouraging *in vitro* findings, we investigated the effect of DHMEQ on cachexia induced by JCA-1 tumor secreting IL-6. We found that DHMEQ significantly inhibited IL-6 production and significantly prevented the development of cachexia in a JCA-1 tumor model.

In conclusion, we showed a significant association between IL-6 and cachexia in patients with progressive prostate cancer, as well as in JCA-1 tumor–bearing mice, and we showed that DHMEQ inhibits NF-κB and thus prevents the development of cachexia induced by prostate cancer in an animal model. Prevention of the complex syndrome of cachexia will improve the quality of life for cancer patients. The new NF-κB inhibitor, DHMEQ, seems to be a promising novel anti-cachectic agent for the treatment of hormone-resistant prostate cancer.

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Pretreatment Serum Testosterone Level as a Predictive Factor of Pathological Stage in Localized Prostate Cancer Patients Treated with Radical Prostatectomy

Takashi Imamoto[a,*], Hiroyoshi Suzuki[a], Satoshi Fukasawa[a], Masaki Shimbo[a], Masahiko Inahara[a], Akira Komiya[a], Takeshi Ueda[a], Taizo Shiraishi[b], Tomohiko Ichikawa[a]

[a]*Department of Urology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan*
[b]*Department of Pathology, Faculty of Medicine, Mie University, Mie 514-8507, Japan*

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Abstract

Objective: Pretreatment serum level of testosterone (T) is a potential prognostic factor for prostate cancer. The present study was conducted to evaluate the clinical significance of pretreatment serum T level in patients with clinically localized prostate cancer.

Materials and Methods: The subjects were 82 clinically localized prostate cancer patients treated with radical prostatectomy, whose pretreatment T levels were recorded. We investigated clinical and pathological factors such as pretreatment serum T level, age, pretreatment PSA or pathological Gleason score concerning the association with pathological stage and biochemical recurrence.

Results: The mean pretreatment T level was significantly lower in patients with non-organ-confined prostate cancer (pT3–T4, N1; 3.44 ± 1.19 ng/ml) than in patients with organ-confined cancer (pT2; 4.33 ± 1.42 ng/ml) ($p = 0.0078$). Multivariate analysis demonstrated that pathological Gleason score, pretreatment serum T level and pretreatment PSA were significant predictors of extraprostatic disease. When the patients were divided into high and low T level groups according to the median value, pretreatment T levels were not significantly associated with PSA recurrence rates ($p = 0.7973$).

Conclusions: A lower pretreatment T level appears to be predictive of extraprostatic disease in patients with localized prostate cancer.

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Keywords: Prostate cancer; Testosterone; Radical prostatectomy; Pathological stage; Prognosis

1. Introduction

A number of clinical, endocrinological and pathological prognostic factors for prostate cancer have been reported [1–16]. Knowledge of these factors will likely contribute to determining the most appropriate therapy for individual patients. Several studies have identified relationships of pretreatment serum level of testosterone (T) with clinical stage of prostate cancer and patient survival, suggesting that pretreatment serum T level has potential as a prognostic factor for prostate cancer [1–10].

There are epidemiological and genetic differences in prostate cancers between patients in Japan and the US. *p53* gene mutational analysis, which often provides information about etiological factors, has revealed clear differences in *p53* gene mutational spectra between Japanese and Western cases [17]. The usefulness of T level as a prognostic factor in Japanese prostate cancer patients has yet to be determined. In the present study of 82 Japanese localized prostate cancer patients who underwent radical prostatectomy as an initial treatment, we investigated the predictive

* Corresponding author. Tel. +81 43 226 2134; Fax: +81 43 226 2136.
E-mail address: t-imamo@pg7.so-net.ne.jp (T. Imamoto).

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value of pretreatment T level for pathological staging of clinically localized prostate cancer. In addition, we examined possible relationships between pretreatment serum T level and several clinical factors.

2. Materials and methods

The subjects were 82 patients (mean age, 66.6 ± 5.3 years; range, 50–77 years) with prostate cancer (ICD7 code 177) that was treated with radical prostatectomy at Chiba University Hospital between April 1996 and March 2004, whose pretreatment T levels were recorded. Patients who received neo-adjuvant treatment before surgery were excluded from the study.

Blood samples were taken in the morning. Serum T level was measured by radioimmunoassay using the DPC total testosterone kit (Nippon DPC Corp., Tokyo, Japan). Serum prostate-specific antigen (PSA) values were determined using the Tandem R kit (Hybritech Inc., San Diego, CA, USA). Staging was based on the 1997 TNM classification.

All subjects underwent retropubic radical prostatectomy as the first-line treatment. Pathology of prostate cancer was evaluated by whole mount step sectioning. Organ-confined disease (pT2) was defined as cancer that had no capsular penetration, seminal vesicle involvement, or pelvic lymph node involvement. Non-organ-confined cancer (pT3–T4, N1; extraprostatic disease) was defined as cancer with capsular penetration or involvement of seminal vesicles or pelvic lymph nodes.

Biochemical recurrence of prostate cancer was defined as two consecutive increases of serum PSA by greater than 0.4 ng/ml. Biochemical-recurrence-free survival rate was calculated using the Kaplan–Meier method. Statistical significance was examined using Student's *t*-test, one-way analysis of variance (one-way ANOVA), and the log-rank test. Multivariate analysis was performed using a Cox regression model. A *p*-value below 0.05 was considered to indicate significance.

3. Results

Overall pretreatment data for age and serum levels of total T and PSA are shown in Table 1. Table 2 shows surgical factors for all 82 patients. Pathological stages were as follows: pT2a, 36 patients (43.9%); pT2b, 22 patients (26.8%); pT3a, 14 patients (17.1%); pT3b, 9 patients (11.0%); pT4, 1 patient.

Table 3 shows nonparametric univariate analysis of the relationship of pretreatment T to clinical and pathological factors. The mean pretreatment T level was significantly lower in patients with non-organ-confined prostate cancer (pT3–T4, N1; 3.44 ± 1.19 ng/ml) than in patients with organ-confined disease (pT2, 4.33 ± 1.42 ng/ml) ($p = 0.0078$) (Fig. 1). In contrast, pretreatment T levels were not significantly associated with age, pretreatment PSA or pathological Gleason score. Serum T level did not significantly correlate with age, although previous studies with large populations of healthy men have shown that serum T levels decline with age [18,19].

Univariate analysis of the relationship of selected factors to extraprostatic disease (pT2 versus pT3–T4, N1) demonstrated that pretreatment serum levels of PSA and pathological Gleason score were the significant predictors of pathological stage ($p = 0.0006$ and 0.0035, respectively). Table 4 shows a multivariate analysis of

Table 1
Overall pretreatment characteristics of 82 patients

Variable	No. patients (%)
Total testosterone (ng/ml)	
<2.0	4 (4.9)
2.0–2.9	18 (22.0)
3.0–3.9	22 (26.8)
4.0–4.9	18 (22.0)
>4.9	20 (24.4)
Mean/median	4.06/3.87
Age (years)	
<60	9 (11.0)
60–64	23 (28.0)
65–69	20 (24.4)
70–74	25 (30.5)
>74	5 (6.1)
Mean/median	66.6/67
PSA (ng/ml)	
0–4	1 (1.2)
4.1–10	42 (51.2)
10.1–20	28 (34.1)
>20	10 (12.2)
Unknown	1 (1.2)
Mean/median	12.3/9.1

Table 2
Surgical factors in 82 patients

Variable	No. patients (%)
Treatment modality	
Primary radical prostatectomy only	78 (95.1)
Radical prostatectomy + adjuvant	4 (4.9)
Pathological Gleason score	
≤4	4 (4.9)
5–6	37 (45.1)
7	34 (41.5)
8–10	7 (8.5)
Pathological stage	
pT2a	36 (43.9)
pT2b	22 (26.8)
pT3a	14 (17.1)
pT3b	9 (11.0)
pT4	1 (1.2)
Seminal vesicle status	
Positive	4 (4.9)
Negative	78 (95.1)
Lymph node status	
Positive	4 (4.9)
Negative	78 (95.1)

Table 3
Nonparametric univariate analysis of relationship of pretreatment testosterone with clinical and pathological factors

Variable	Testosterone (ng/ml)			p-value
	Mean	Median	Range	
Age (years)				0.947
<67 (median)	4.068	3.89	1.71–9.43	
≥67	4.047	3.82	1.46–6.64	
PSA (ng/ml)				0.453
0–10	4.183	4.21	1.46–6.64	
>10	3.944	3.63	1.61–9.43	
Pathological Gleason score				0.114
≤6	3.810	3.63	1.61–6.64	
7–10	4.304	4.19	1.46–9.43	
Pathological stage				0.008
pT2	4.328	4.20	1.46–5.85	
pT3–T4 or N1	3.438	3.50	1.61–9.43	

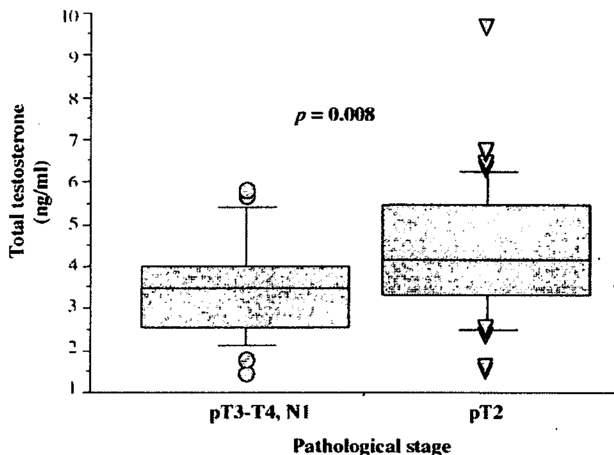

Fig. 1. Nonparametric univariate analysis of relationship between pretreatment T and pathological stage.

statistically significant predictors of extraprostatic disease (pT2 versus pT3–T4, N1) according to the Cox regression model. The pretreatment T level was an independent significant predictor of extraprostatic disease ($p = 0.0034$). Pathological Gleason score and pretreatment serum levels of PSA were also independently significant ($p = 0.0024$ and 0.0081, respectively).

Four patients who had pT4 or pN1 disease received adjuvant hormonal treatment immediately after radical prostatectomy. Biochemical recurrence was investigated using the remaining 78 patients who did not receive adjuvant therapy immediately after surgery. The mean follow-up for the patients was 20.0 months, and PSA recurrence was detected in 9 patients (11.5%) during the follow-up. Table 5 shows univariate analysis of the relationship of selected factors to biochemical recurrence using the Kaplan–Meier method. When the patients were divided into high and low T level groups according to the median value (3.87 ng/ml), pretreatment T levels were not significantly associated with PSA recurrence rates ($p = 0.7973$). Pretreatment PSA and pathological stage were also insignificant. Even in analysis confined to pT2 or pT3 patients, pretreatment T levels were not significantly associated with biochemical recurrence rates (not shown). In contrast, age and pathological Gleason score were significant predictors of PSA recurrence ($p = 0.0369$ and 0.0485, respectively).

Table 5
Univariate analysis of relationship of selected factors to biochemical recurrence

Factor	No. patients	% 3-Yr. Biochemical-recurrence-free survival rate	p-value
Age (years)			0.0369
<67 (median)	35	60.4	
≥67	43	90.6	
PSA (ng/ml)			0.9829
0–10	42	77.9	
>10	35	72.3	
Pathological stage			0.1006
pT2	57	81.6	
pT3	21	63.2	
Total testosterone (ng/ml)			0.7973
<3.87 (median)	37	76.1	
≥3.87	41	77.1	

4. Discussion

Prostate cancer is one of the most common cancers among Western populations, and its incidence is increasing in Asia. Prostatic development and growth depend on androgenic stimulation. In 1941, Huggins

Table 4
Multivariate analysis of significant predictors of extraprostatic disease (pT2 versus pT3–T4, N1) according to the Cox regression model

Variable	Relative hazards ratio	95% confidence interval	p-value
PSA (ng/ml) (≤10/>10)	0.191	0.056–0.650	0.0081
Pathological Gleason score (2–6/7–10)	0.125	0.033–0.478	0.0024
Total testosterone (ng/ml)	2.167	1.291–3.637	0.0034

et al. published the first report a relationship between serum T and prostate cancer [20]. Currently, the association of serum T with prostate cancer is incompletely understood. Adlercreutz et al. were the first to demonstrate that the mean serum T level was significantly higher in patients with a good response to endocrine therapy than in those with a poor response [1]. Other reports suggest that prognosis can be predicted from serum T level, performance status, serum alkaline phosphatase level, and the extent of disease grade on bone scan in stage D2 patients [2–10]. Evaluation of clinically localized prostate cancer has shown no correlation of serum total, free or percent free T levels with known clinicopathological features such as PSA, Gleason score or extraprostatic extension [21].

The finding of extraprostatic disease in radical prostatectomy specimens is an adverse prognostic factor; such tumors are significantly more likely to progress than organ-confined cancers [22]. In addition, extraprostatic disease is associated with a greater risk of a positive surgical margin, which further decreases the likelihood of long-term cancer control. Consequently, it is important for prognosis to determine whether a patient has extraprostatic disease. The present study is the first to analyze T data of Japanese men with clinically localized prostate cancer. We found that the mean pretreatment T level was significantly lower in patients with non-organ-confined prostate cancer (pT3–T4, N1) than in patients with organ-confined cancer (pT2). Multivariate analysis demonstrated that pathological Gleason score, pretreatment serum T level and pretreatment PSA were independent significant predictors of extraprostatic disease. Monda et al. reported that serum total T values are not useful in predicting the pathological stage in patients with clinically localized prostate cancer [23]. In 2000, Hoffman et al. performed an investigation similar to the present study, and obtained results suggesting that low serum free T is a marker of more aggressive disease [24], but they noted no significant differences based on total T. That finding is not surprising, because free T is considered the more biologically active form, and the discrepancy may also be due to their relatively small cohorts of patients. Massengill et al. found that total T was a predictor of extraprostatic disease in univariate and multivariate analyses of localized prostate cancer patients [25]. Thus, the available evidence suggests that pretreatment total T level is useful in the management of localized prostate cancer.

The mechanism and of the relationship between serum T and prognosis of prostate cancer remains to be elucidated, and attempts have been made to define the influence of the prostate on serum hormone levels. Miller et al. reported that serum levels of gonadotropins and total and free T were significantly elevated after radical prostatectomy in 63 patients with prostate cancer [21]. Their results suggest that one or more factors from a normal or malignant prostate can generate negative feedback in the hypothalamus-pituitary axis. Inhibin is considered a candidate for such an inhibitory factor. In animal and cell culture models, inhibin produced in the testes and prostate can inhibit the production and secretion of pituitary gonadotropins [26,27]. Ghosh et al. noted that levels of T and enzymes involved in T production increased in rats after prostatectomy [28]. Studies of inhibin have been limited to animal prostates; consequently, the role of inhibin in human prostate tissue has not been well defined. Most recently, Zhang et al. investigated both serum total and free T levels, before and after radical prostatectomy in 79 patients [29]. They found that serum total and free T levels were significantly elevated after surgery, and that patients with high-grade prostate cancer had significantly lower levels of both total and free T than patients with moderate-grade prostate cancer.

Similarly, Schatzl et al. reported that patients with high Gleason score prostate cancer had lower serum testosterone levels and their gonadotropins were lower in parallel [30]. These findings suggest that the factor or factors produced in the prostate originates from prostate cancer cells rather than normal prostate gland cells.

It is generally recognized that pretreatment PSA, Gleason score and pathological stage are significant predictors of biochemical recurrence in clinically localized prostate cancer patients treated with radical prostatectomy. In the present study, age and pathological Gleason score were significant predictors of PSA recurrence. In contrast, pretreatment T levels, pretreatment PSA and pathological stage were not significantly associated with PSA recurrence rates. Even in analysis confined to pT2 and pT3 patients, pretreatment T levels were not significantly associated with biochemical recurrence rates. Although the reason of this contradiction is unclear, the limitations of the present study include its retrospective nature and small population size. Further study with large clinical trials is needed to investigate the relation of these prognostic values with biochemical recurrence.

5. Conclusion

In the present study, total T was an independent significant predictor of extraprostatic disease in uni-

variate and multivariate analyses. A lower pretreatment T level appears to be predictive of non-organ-confined disease in patients with localized prostate cancer. However, pretreatment serum T level was not associated with biochemical recurrence after radical prostatectomy.

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Multigene Methylation Analysis for Detection and Staging of Prostate Cancer

Hideki Enokida,[1,3] Hiroaki Shiina,[2] Shinji Urakami,[1,2] Mikio Igawa,[2] Tatsuya Ogishima,[1] Long-Cheng Li,[1] Motoshi Kawahara,[3] Masayuki Nakagawa,[3] Christopher J. Kane,[1] Peter R. Carroll,[1] and Rajvir Dahiya[1]

Abstract **Purpose:** Aberrant gene promoter methylation profiles have been well-studied in human prostate cancer. Therefore, we rationalize that multigene methylation analysis could be useful as a diagnostic biomarker. We hypothesize that a new method of multigene methylation analysis could be a good diagnostic and staging biomarker for prostate cancer.

Experimental Design: To test our hypothesis, prostate cancer samples (170) and benign prostatic hyperplasia samples (69) were examined by methylation-specific PCR for three genes: adenomatous polyposis coli (*APC*), glutathione S-transferase pi (*GSTP1*), and multidrug resistance 1 (*MDR1*). The methylation status of representative samples was confirmed by bisulfite DNA sequencing analysis. We further investigated whether methylation score (M score) can be used as a diagnostic and staging biomarker for prostate cancer. The M score of each sample was calculated as the sum of the corresponding log hazard ratio coefficients derived from multivariate logistic regression analysis of methylation status of various genes for benign prostatic hyperplasia and prostate cancer. The optimal sensitivity and specificity of the M score for diagnosis and for staging of prostate cancer was determined by receiver-operator characteristic (ROC) curve analysis. A pairwise comparison was employed to test for significance using the area under the ROC curve analysis. For each clinicopathologic finding, the association with prostate-specific antigen (PSA) failure-free probability was determined using Kaplan-Meier curves and a log-rank test was used to determine significance. The relationship between M score and clinicopathologic findings was analyzed by either the Mann-Whitney *U* test, Kruskal-Wallis test, or the Spearman rank correlation test.

Results: The frequency of positive methylation-specific PCR bands for *APC, GSTP1*, and *MDR1* genes in prostate cancer samples was 64.1%, 54.0%, and 55.3%, respectively. In benign prostatic hyperplasia samples, it was 8.7%, 5.8%, and 11.6%, respectively. There was a significant correlation of M score with high pT category ($P < 0.001$), high Gleason sum ($P < 0.001$), high preoperative PSA ($P = 0.027$), and advanced pathologic features. For all patients, the M score had a sensitivity of 75.9% and a specificity of 84.1% as a diagnostic biomarker using a cutoff value of 1.0. In patients with low or borderline PSA levels (<10.0 ng/mL), the M score was significantly higher in prostate cancers than in benign prostatic hyperplasias (2.635 ± 0.200 and 0.357 ± 0.121, respectively). ROC curve analysis revealed that the M score had a sensitivity of 65.4% and a specificity of 94.2% when 1.0 was used as a cutoff value. For all patients, M score can distinguish organ-confined ($\leq pT_2$) from locally advanced cancer ($\geq pT_3$) with a sensitivity of 72.1% and a specificity of 67.8%. Moreover, considering patients with PSA levels of <10 ng/mL, the M score has a sensitivity of 67.1% and a specificity of 85.7%. The ROC curve analysis showed a significant difference between M score and PSA ($P = 0.010$).

Conclusions: This is the first report demonstrating that M score is a new method for multigene methylation analysis that can serve as a good diagnostic and staging biomarker for prostate cancer.

The discovery and widespread use of serum prostate-specific antigen (PSA) has had a tremendous impact on all aspects of management of prostate carcinoma (1-3). PSA is the most sensitive diagnostic biomarker for prostate cancer detection to date. However, its low specificity has led to unnecessary biopsy for verifying the presence of prostate cancer (2, 3). Aberrant methylation profiles of gene promoters have been well-investigated in prostate cancer (4-6). Recent studies have shown that the methylation status of glutathione S-transferase pi (*GSTP1*) or adenomatous polyposis coli (*APC*) could individually distinguish prostate cancer from benign prostatic hyperplasia (4-6). Some investigators have shown that *GSTP1* hypermethylation can be easily detected in body fluids such as

Authors' Affiliations: [1]Department of Urology, Veterans Affairs Medical Center and University of California, San Francisco, California; [2]Department of Urology and Biochemistry, Shimane University, Izumo; and [3]Department of Urology, Kagoshima University, Kagoshima, Japan

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Requests for reprints: Rajvir Dahiya, Urology Research Center, Department of Urology, Veterans Affairs Medical Center, University of California, San Francisco, CA 94121. Phone: 415-750-6964; Fax: 415-750-6639; E-mail: rdahiya@urol.ucsf.edu.

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