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

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 antiandrogen 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.

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 mm³ 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 antiandrogen ablation, mice were surgically castrated after the xenograft volume reached 150 mm³. In the bicalutamide + castration group, bicalutamide administration was started when xenograft volume was over 150 mm³ 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
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-ACCTTCAGATGACCTC-3' and 5'-CAGCTGACAGATGATCTC-5'. 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 tetrahydrochloride. All sections were counterstained with hematoxylin.

Sequencing analysis. Genomic DNA was extracted using a QiAamp 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 × 10⁶ 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. 1A). He was treated with maximal androgen blockade (combination of luteinizing hormone–releasing hormone analogues 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 chemoeostimulatory therapy.

blockade therapy, serum PSA levels increased as liver metastasis progressed (Fig. 1B 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 mm³ 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. 2A) and Western blotting (Fig. 2B) and PSA production by KUCaP using an immunohistochemical assay (Fig. 2C). In addition, serum PSA level in mice bearing KUCaP xenografts was linearly correlated with xenograft volume (Fig. 2D).

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. 3A). 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. 3B). 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.

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 androgen 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.
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 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.

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.

Acknowledgments

Received 3/10/2005; revised 8/17/2005; accepted 9/1/2005.

Grant support: Grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Isao Kanatani, Tomoko Matsushita and Chie Hagihara for their valuable technical assistance.
References


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, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/21/9611

Cited articles
This article cites 20 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/21/9611.full.html#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
/content/65/21/9611.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.