Novel Mutations of Androgen Receptor: A Possible Mechanism of Bicalutamide Withdrawal Syndrome

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ABSTRACT

Most prostate cancers (PCs) become resistant to combined androgen blockade therapy with surgical or medical castration and antiandrogens after several years. Some of these refractory PCs regress after discontinuation of antiandrogen administration [antiandrogen withdrawal syndrome (AWS)]. Although the molecular mechanisms of the AWS are not fully understood because of the lack of suitable experimental models, one hypothesis of the mechanism is mutation of androgen receptor (AR). However, bicalutamide, which has become the most prevalent pure antiandrogen, does not work as an agonist for any mutant AR detected thus far in PC. To elucidate the mechanisms of the AWS, we established and characterized novel LNCaP cell sublines, LNCaP-cxDs, which were generated *in vitro* by culturing androgen-dependent LNCaP-FGC human PC cells in androgen-depleted medium with bicalutamide to mimic the combined androgen blockade therapy. LNCaP-FGC cells did not grow at first, but they started to grow after 6–13 weeks of culture. Bicalutamide stimulated LNCaP-cxD cell growth and increased prostate-specific antigen secretion from LNCaP-cxD cells both *in vitro* and *in vivo*. Sequencing of AR transcripts revealed that the AR in LNCaP-cxD cells harbors a novel mutation in codon 741, TGG (tryptophan) to TGT (cysteine; W741C), or in codon 741, TGG to TTG (leucine; W741L), in the ligand-binding domain. Transactivation assays showed that bicalutamide worked as an agonist for both W741C and W741L mutant ARs. Importantly, another antiandrogen, hydroxyflutamide, worked as an antagonist for these mutant ARs. In summary, we demonstrate for the first time that within only 6–13 weeks of *in vitro* exposure to bicalutamide, LNCaP-FGC cells, whose growth had initially been suppressed, came to use bicalutamide as an AR agonist via W741 AR mutation to survive. Our data strongly support the hypothesis that AR mutation is one possible mechanism of the AWS and suggest that flutamide might be effective as a second-line therapy for refractory PC previously treated with bicalutamide.

INTRODUCTION

Because most cases of PC[2] are initially dependent on androgen for growth, androgen suppression by castration or luteinizing hormone-releasing hormone analogues can successfully control the growth of PC. Usually, the patient is additionally given an AR antagonist to achieve a complete androgen blockade, because the adrenal glands still produce considerable amounts of adrenal androgens. However, essentially all of the patients who show initial favorable responses to this combined androgen blockade eventually become refractory after several years (1). Paradoxically, a decline in serum PSA levels and a regression of tumors occur after discontinuation of antiandrogen administration in some of these patients with recurrent PCs (2, 3). This phenomenon is termed the AWS. The molecular mechanisms of the

AWS are still unclear because of the lack of suitable experimental models. One possible mechanism responsible for the AWS is mutation of AR because hydroxyflutamide, the active metabolite of an antiandrogen flutamide, works as an agonist for some mutant ARs (4–6). However, to our knowledge, bicalutamide, which has become the most prevalent pure antiandrogen, does not act as an agonist for any mutant AR detected thus far in PC, although bicalutamide also induces the bicalutamide withdrawal syndrome (2, 3). Nor have we verified that the AR gene in PC cells is altered by antiandrogens. Therefore, we could not confirm that AR mutation is the cause of the AWS. In this study, we established novel LNCaP cell sublines by *in vitro* long-term culture of LNCaP-FGC cells in the presence of bicalutamide. The characterization of these cell lines directly reveals that PC cells can alter their AR gene in such a way as to use bicalutamide as an AR agonist to survive.

MATERIALS AND METHODS

Cell Culture. The human PC cell line LNCaP-FGC (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) was maintained in RPMI 1640 with 10% FBS (Trace Scientific Ltd., Melbourne, Australia). After LNCaP-FGC cells started growing in phenol red-free RPMI 1640 with 10% DCC-FBS in the presence and absence of bicalutamide (purchased as Casodex tablets and extracted in our company), the cell lines were designated as LNCaP-cxD (LNCaP-cxD2, -cxD11, and -cxD12) and LNCaP-hr, respectively. LNCaP-cxD2 and LNCaP-cxD12 were maintained in phenol red-free RPMI 1640 with 10% DCC-FBS in the presence of 1 μM bicalutamide, and LNCaP-cxD11 was maintained in phenol red-free RPMI 1640 with 10% DCC-FBS in the presence of 0.1 μM bicalutamide. LNCaP-hr was maintained in phenol red-free RPMI 1640 with 10% DCC-FBS. DCC-FBS was prepared by incubating 500 ml of FBS with 25 ml of DCC solution (5% charcoal activated; Sigma, St Louis, MO) and 0.05% T-70 dextran (Pharmacia, Uppsala, Sweden) in Dulbecco's PBS (Wako, Osaka, Japan) at 45°C for 30 min, followed by collection of the supernatant after centrifugation at $1800 \times g$ for 30 min. All cell lines were maintained at 37°C in 5% CO_2 atmosphere.

Cell Growth and PSA Assays. In time course studies, cells were seeded into 24-well plates at 4×10^4 cells/well in phenol red-free RPMI 1640 containing 10% DCC-FBS with or without bicalutamide. After 1–7 days, cells were counted with a particle counter (Beckman Coulter, Tokyo, Japan), and the PSA levels in the conditioned media were determined with an enzyme immunoassay kit (Dainippon Pharmaceutical Co., Ltd.). In dose-response studies, cells were seeded into 24-well plates at 4×10^4 cells/well in phenol red-free RPMI 1640 containing 10% DCC-FBS, and bicalutamide (0.01–30 μM) was added on the next day. Cells were counted 3 days after treatment with bicalutamide, and the PSA levels in the conditioned media were also determined.

Animal Experiments. The protocols of animal experiments were approved by the Takeda Experimental Animal Use and Care Committee in accordance with NIH standards. Five-week-old male BALB/c athymic nude mice were purchased from Charles River Japan (Kanagawa, Japan). LNCaP-cxD2 or LNCaP-hr cells [5×10^6 cells/100 μl Matrigel (BD Biosciences, Bedford, MA)] were injected s.c. into one flank of each castrated mouse. Three days before tumor injection, the treatment with either vehicle (20% benzyl benzoate in corn oil) or bicalutamide (2.1, 21, or 210 mg/kg/week, s.c., twice a week) was started. The tumor size was measured with a caliper and expressed in mm³ using the formula $0.5 \times a \times b^2$, where a is the largest diameter, and b is largest

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diameter perpendicular to *a*. At the end of experiments, blood samples were obtained to measure the serum PSA levels.

Sequencing of cDNA after Reverse Transcription-PCR. Total RNA was isolated from LNCaP cell lines, and cDNA was synthesized using avian myeloblastosis virus reverse transcriptase XL (Takara Shuzo Co., Ltd., Shiga, Japan). The entire AR protein-coding region was amplified by PCR with seven sets of oligonucleotide primers [5′-GCCTGTTGAACTCTTCTGAG-3′ and 5′-TGTCCTTGGAGGAAGTGGGAG-3′, 5′-AAGCCCATCGTAGAGGCC-CCA-3′ and 5′-GGAAAGTTGTAGTAGTCGCGACTCTG-3′, 5′-GCACT-GGACGAGGCAGCTGCGTAC-3′ and 5′-CCACCACACGGTCCATACA-ACT-3′, 5′-CGCATCAAGCTGGAGAACCCGCTG-3′ and 5′-CAGGTG-CGGTGAAGTCGCTTTCCT-3′, 5′-ACAGCCGAAGAAGGCCAGTTGT-3′ and 5′-ACACACTACACCTGGCTCAAT-3′, 5′-CAGTGTCACACATTGA-AGGCT-3′ and 5′-CTTGCACAGAGATGATCTCTGC-3′, and 5′-GTCCTG-GAAGCCATTGAGCCA-3′ and 5′-CAGGCAGAAGACATCTGAAAG-3′), some of which were synthesized in reference to previously published sequences (7). Both sense and antisense DNA strands of the PCR products were directly sequenced with the ABI PRISM Big Dye Terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan). The mutation in codon 741 (TGG to TGT and TGG to TTG) was confirmed in two independent PCR-amplified DNA fragments. Furthermore, independently, PCR amplifications of the entire AR protein-coding region were performed with cDNA from LNCaP-cxD2 cells using two sets of primers (5′-GATGCCGCGGGCCACCATGGA-AGTGCAGTTAGGGCTGGGAAG-3′ and 5′-GATGGGTACCACACATC-AGGTGCGGTGAAG-3′, 5′-GATGGGTACCCTGGCGGCATGGTG-AGCAGAG-3′ and 5′-GATGCCGCGGGCGGCCGCCTGGGTGTGGAA-ATAGATGGGCTTGAC-3′). The PCR products were cloned, and three clones each were sequenced. The mutation in codon 741 (TGG to TGT) was found in two of the three clones. In contrast, the mutation in codon 741 was not detected in any of the 27 cloned PCR-amplified AR transcripts from LNCaP-FGC cells.

Site-directed Mutagenesis. The AR coding region amplified by PCR from transcripts of LNCaP-cxD2 cells was inserted into pcDNA3.1. The single mutants W741C, W741L, and T877A and the wild-type AR expression plasmids were created with three primer sets (5′-AGAGAGCTGGCATCAGT-TCACTTTTGACCTGCTAATCAAG-3′ and 5′-CTTGATTAGCAGGTCAA-AAGTGAACTGAGTCAGCTCTCT-3′, 5′-CTGTCATTCAGTACTCCTTG-ATGGGGCTCATGGTGTTTG-3′ and 5′-CAAACACCATGAGCCCCATC-AAGGAGTACTGAATGACAG-3′, and 5′-CTGTCATTCAGTACTCCTGG-ATGGGGCTCATGGTGTTTG-3′ and 5′-CAAACACCATGAGCCCCATC-CAGGAGTACTGAATGACAG-3′) by site-directed oligonucleotide mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA. These constructs were confirmed by DNA sequencing.

Transactivation Assays. After COS-7 cells were cotransfected with pcDNA3.1-AR and pGL3-PSA-luc vector, which consists of two tandem PSA promoter and the luciferase gene, by using SuperFect transfection reagent (Qiagen, Hilden, Germany), these cells were trypsinized and seeded equally into 96-well plates at 1×10^4 cells/well in phenol red-free DMEM with 10% DCC-FBS (2 ng of pcDNA3.1-AR and 60 ng of pGL3-PSA-luc vector/well). Drugs were added 3 h after transfection. After a 24-h incubation at 37°C in a 5% CO_2 atmosphere, luciferase activity in the cell lysate was measured using the Steady-Glo Luciferase Assay System (Promega, Madison, WI) and the Wallac 1420 ARVO SX multilabel counter (EG&G Wallac, Turku, Finland).

Statistical Analysis. Differences between the control group and the drug-treated groups were analyzed by unpaired Student's *t* test (Fig. 1) or Dunnett's test (Figs. 2 and 3). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

We established and characterized a novel LNCaP PC cell subline, LNCaP-cxD2, which was generated *in vitro* by long-term culture of LNCaP-FGC cells in androgen-depleted medium with 1 μM bicalutamide to mimic the clinical situation in which PC patients receive medical or surgical castration in combination with antiandrogen therapy. LNCaP-FGC cells did not grow at first but started to grow after approximately 6 weeks of culture. Independently, we established two other cell lines by the same method with 0.1 and 1 μM bicalutamide and designated them LNCaP-cxD11 and LNCaP-cxD12, respectively.

In each case, it took less than 13 weeks for LNCaP-FGC cells to start growing. To our knowledge, this is the first study that shows the establishment of bicalutamide-resistant cell lines by incubation of PC cells with bicalutamide. We also established another cell line, LNCaP-hr, which was generated *in vitro* by culturing LNCaP-FGC cells in androgen-depleted medium without bicalutamide to mimic the clinical situation in which PC patients receive castration monotherapy. In this case, LNCaP-FGC cells started to grow after less than 4 weeks of culture. The steady-state levels of the AR determined by immunoblot analysis in LNCaP-cxD2, LNCaP-cxD11, LNCaP-cxD12, and LNCaP-hr cells are 0.93-, 1.3-, 0.97-, and 2.0-fold as high as that in parental LNCaP-FGC cells (data not shown). The steady-state PSA levels in LNCaP-cxD2, LNCaP-cxD11, LNCaP-cxD12, and LNCaP-hr cells are 0.99-, 1.4-, 2.3-, and 0.65-fold as high as that in parental LNCaP-FGC cells (data not shown).

In cell growth studies, withdrawal of bicalutamide from culture media suppressed LNCaP-cxD2 cell proliferation (Fig. 1*A*) and reduced secretion of PSA, a marker of PC (1), in LNCaP-cxD2 cells (Fig. 1*B*). Because these findings are consistent with clinically observed AWS (2, 3), LNCaP-cxD2 cells might be an appropriate model for studying the mechanisms of AWS. Bicalutamide stimulated LNCaP-cxD2 cell proliferation in a biphasic manner, with a peak at 1 μM (Fig. 2*A*). Because androgen stimulated LNCaP-FGC cell proliferation also in a biphasic manner (Ref. 8; data not shown), our data suggest that bicalutamide might become an AR agonist in LNCaP-cxD2 cells. Consistently, bicalutamide increased PSA secretion, a marker of androgen action, in a dose-dependent manner in LNCaP-cxD2 cells (Fig. 2*B*), as androgen increased PSA secretion in a dose-dependent manner in LNCaP-FGC cells (data not shown). The growth curve of LNCaP-cxD12 was quite similar to that of LNCaP-cxD2 in that the peak was at 1 μM bicalutamide (Fig. 2, *A* and *C*), where both of these two cell lines were maintained. The growth curve of LNCaP-cxD11 was also bell-shaped (Fig. 2*C*). Interestingly, the growth-promoting concentrations in LNCaP-cxD11 cells, which were maintained with 0.1 μM bicalutamide, were lower than those in LNCaP-cxD2 and LNCaP-cxD12 (Fig. 2, *A* and *C*), suggesting that LNCaP-FGC cells could elaborately adapt to the culture condition, the bicalutamide concentration in the medium. PSA secretion was increased in a dose-dependent manner by bicalutamide in both LNCaP-cxD11 and LNCaP-cxD12 cells (Fig. 2*D*). These findings suggest that bicalutamide might also become an AR agonist in both LNCaP-cxD11 and LNCaP-cxD12 cells. In contrast to LNCaP-cxD cells, bicalutamide inhibited proliferation (Fig. 1*A* and Fig. 2*A*) and reduced PSA secretion (Fig. 1*B* and Fig. 2*B*) in LNCaP-FGC and LNCaP-hr cells. Bicalutamide also reduced androgen-induced PSA secretion (data not
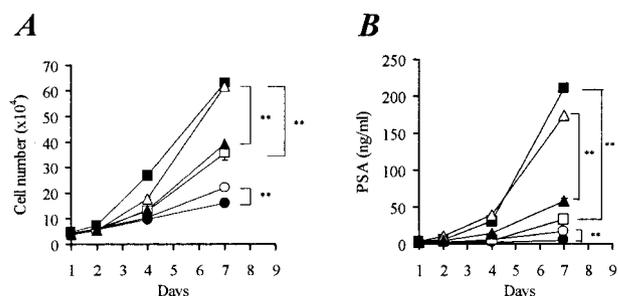

Fig. 1. Bicalutamide withdrawal phenomena in LNCaP-cxD2 cells. LNCaP-FGC (● and ○), LNCaP-cxD2 (■ and □), and LNCaP-hr (▲ and △) cells were plated in 24-well plates (4×10^4 cells/well) in 1 ml of RPMI 1640 with 10% DCC-FBS in the presence (●, ■, and ▲) or absence (○, □, and △) of 1 μM bicalutamide. After 1, 2, 4, and 7 days, cells were counted, and the culture medium was collected for measurement of PSA levels. *A*, growth; *B*, PSA secretion. Data are expressed as the mean of quadruplicate determinations. *Bars*, ±SE. ∗∗, $P < 0.01$ on day 7.
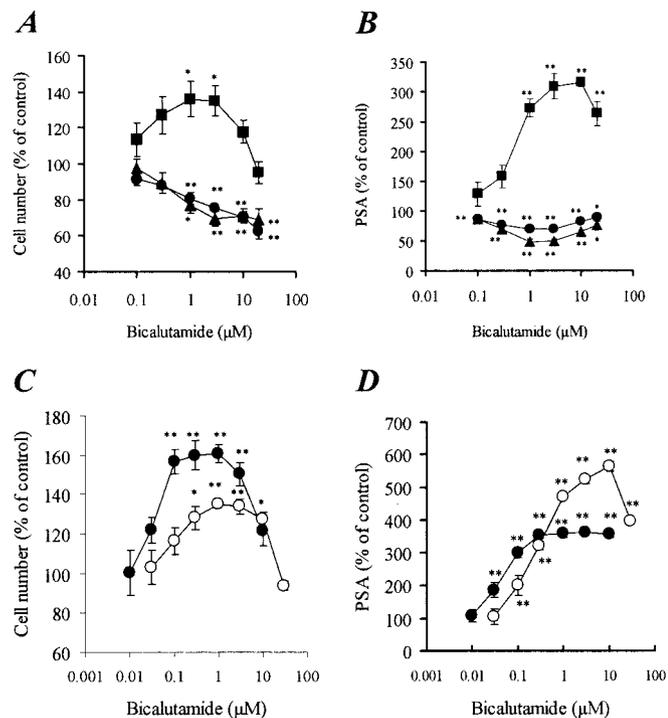
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Fig. 2. Bicalutamide promotes growth and PSA secretion in LNCaP-cxD cell lines. *A* and *B*, LNCaP-FGC (●), LNCaP-cxD2 (■), and LNCaP-hr (▲) cells were plated in a 24-well plate (4×10^4 cells/well) in 1 ml of RPMI 1640 with 10% DCC-FBS. The next day, bicalutamide (0.1, 0.3, 1, 3, 10, or 20 μM) was added. After 3 days, cells were counted, and the culture medium was collected for measurement of PSA levels. *C* and *D*, LNCaP-cxD11 (●) and LNCaP-cxD12 (○) cells were plated in a 24-well plate (4×10^4 cells/well) in 1 ml of RPMI 1640 with 10% DCC-FBS. The next day, bicalutamide (0.01, 0.03, 0.1, 0.3, 1, 3, 10, or 30 μM) was added. After 3 days, cells were counted, and the culture medium was collected for measurement of PSA levels. *A* and *C*, growth; *B* and *D*, PSA secretion. Data are expressed as the mean of triplicate determinations. *Bars*, ±SE. *, $P < 0.05$; **, $P < 0.01$ *versus* vehicle control.

shown), demonstrating that bicalutamide remains an antagonist in LNCaP-FGC and LNCaP-hr cells. Our results suggest that LNCaP-FGC cells could survive even under the growth-suppressive pressure of bicalutamide by changing their AR functions in such a way as to use bicalutamide as an agonist and also by adjusting the intensity of AR signals to their optimum. Our data also suggest that the switch of bicalutamide from antagonist to agonist might require the presence of bicalutamide.

To determine whether bicalutamide also promotes tumor growth *in vivo*, we established castrated nude mouse models of LNCaP-cxD2 and LNCaP-hr tumor xenografts. Bicalutamide significantly accelerated LNCaP-cxD2 tumor growth (Fig. 3*A*). Furthermore, blood PSA levels in mice bearing LNCaP-cxD2 tumors were significantly increased by bicalutamide (Fig. 3*B*). In contrast, bicalutamide significantly inhibited LNCaP-hr tumor growth (Fig. 3*C*). Consistently, blood PSA levels in mice bearing LNCaP-hr tumors were significantly decreased by bicalutamide (Fig. 3*D*). Our results demonstrate that bicalutamide promotes LNCaP-cxD2 tumor growth *in vivo* at the doses lower than or equal to those at which bicalutamide inhibited LNCaP-hr tumor growth.

To study the molecular mechanism for the change of AR functions in LNCaP-FGC cells, we sequenced entire AR transcripts in LNCaP-FGC, LNCaP-hr, and LNCaP-cxD2 cells. Consistent with previous findings (9, 10), we detected only the T877A mutation in LNCaP-FGC and LNCaP-hr cells (Fig. 4). In contrast, we detected a novel mutation in codon 741, TGG (tryptophan) to TGT (cysteine; W741C), in the ligand-binding domain in addition to the T877A mutation in LNCaP-cxD2 cells (Fig. 4). All nucleotide sequences of the AR

transcripts except those in codon 741 were the same among these three cell lines. Furthermore, we sequenced the ligand-binding domain of the AR transcripts in LNCaP-cxD11 and LNCaP-cxD12 cells. We found exactly the same W741C mutation in LNCaP-cxD12 cells (Fig. 4). Surprisingly, we detected another point mutation in codon 741, TGG to TTG (leucine; W741L), in addition to the T877A mutation in LNCaP-cxD11 cells (Fig. 4). Our data suggest that the switch of bicalutamide from antagonist to agonist in LNCaP-cxD cell lines might depend on an additional AR mutation in codon 741 and that the emergence of the mutation might require the existence of bicalutamide.

To clarify the role of the AR mutation in codon 741 on conversion of bicalutamide to an agonist, we assessed the transcriptional responses to bicalutamide of the wild-type; the single mutants W741C, W741L, and T877A; and the double mutants W741C/T877A and W741L/T877A ARs in transactivation assays. Interestingly, the single
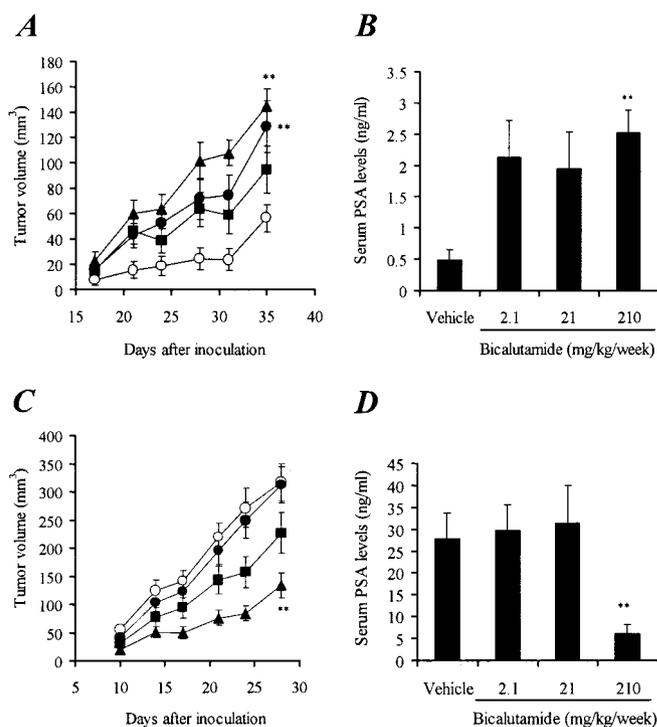

Fig. 3. Bicalutamide promotes LNCaP-cxD2 tumor growth and elevates serum PSA levels. Castrated nude mice bearing LNCaP-cxD2 (*A* and *B*) or LNCaP-hr (*C* and *D*) tumors were treated with 2.1, 21, and 210 mg bicalutamide/kg/week. *A* and *C*, tumor growth. ○, vehicle; ●, 2.1 mg bicalutamide/kg/week; ■, 21 mg bicalutamide/kg/week; ▲, 210 mg bicalutamide/kg/week. *B* and *D*, serum PSA levels in nude mice bearing LNCaP-cxD2 tumors 35 days after inoculation (*B*) or in nude mice bearing LNCaP-hr tumors 28 days after inoculation (*D*). Data represent the mean. *Bars*, ±SE. $n = 16-17$. **, $P < 0.01$ *versus* vehicle control on day 35 (*A* and *B*) or day 28 (*C* and *D*).

Ligand-binding domain

AR cDNA	exon 1–4	exon 5	exon 6, 7	exon 8
	AR type	Codon 741		Codon 877
	Wild	TGG (Trp)		ACT (Thr)
Cell line				
LNCaP-FGC	T877A	TGG (Trp)		GCT (Ala)
LNCaP-hr	T877A	TGG (Trp)		GCT (Ala)
LNCaP-cxD2	W741C&T877A	TGT (Cys)		GCT (Ala)
LNCaP-cxD11	W741L&T877A*	TTG (Leu)		GCT (Ala)
LNCaP-cxD12	W741C&T877A*	TGT (Cys)		GCT (Ala)

Fig. 4. A single nucleotide change in codon 741 of AR in LNCaP-cxD cell lines. *, only cDNA sequence in the ligand-binding domain was analyzed. The G→T point mutation is shown in *bold*.
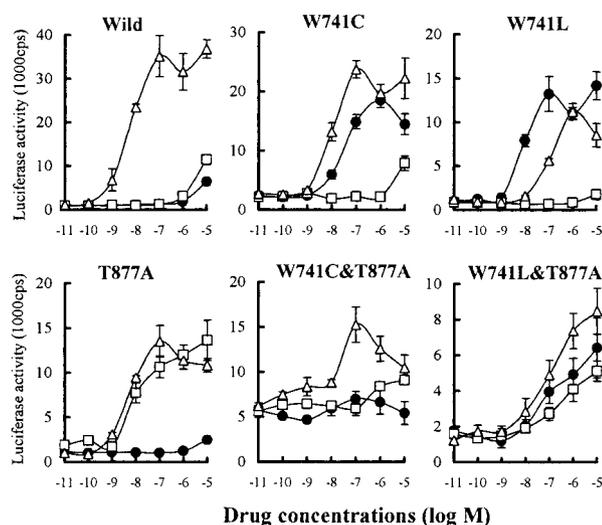
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Fig. 5. The W741C and W741L mutant ARs are activated by bicalutamide. COS-7 cells were cotransfected with wild-type or mutant AR expression vectors and the pGL3-PSA-luc vector and treated with drugs (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M). Luciferase activity was assayed 24 h after drug treatment. ●, bicalutamide; □, hydroxyflutamide; △, DHT. Basal luciferase activities of the wild-type, W741C, W741L, T877A, W741C/T877A, and W741L/T877A ARs were 911 ± 49, 2453 ± 144, 973 ± 81, 1049 ± 68, 6952 ± 580, and 1618 ± 172 counts per second (cps), respectively. Data are expressed as the mean of quadruplicate determinations. Bars, ±SE.

mutants W741C and W741L and the double mutant W741L/T877A ARs had a substantial transactivation response to bicalutamide (Fig. 5). In contrast, the wild-type and the T877A mutant ARs responded only slightly to bicalutamide (Fig. 5). Our data demonstrate the critical role of the AR mutation in codon 741 on the switch of bicalutamide to an agonist. The sensitivity of the W741C and W741L mutant ARs is similar (Fig. 5), which suggests that the elaborate adaptation of LNCaP-FGC cells to the culture condition of bicalutamide levels (Fig. 2, *C* and *D*) might not depend on the type of AR mutation but on other mechanisms. Surprisingly, the W741C/T877A double mutant AR showed constitutively active characteristics in this assay and did not become activated further by bicalutamide (Fig. 5), which is not consistent with the results in cell growth assays using LNCaP-cxD2 or LNCaP-cxD12 cells (Fig. 2). Further study is required to clarify this discrepancy. It might depend on the difference of the intracellular environments.

Mutations in the AR in PCs have been well-documented (11–13). Several mutant ARs such as T877A, T877S, H874Y, V715M, and L701H/T877A have a broadened spectrum of ligand responsiveness, and hydroxyflutamide works as an agonist for these mutants (4–6). However, to our knowledge, bicalutamide does not work as an agonist for any mutant AR detected thus far in PC. Therefore, this is the first study showing that bicalutamide works as an agonist for mutant ARs in PC cells. Furthermore, in this study, we demonstrate for the first time *in vitro* that bicalutamide can cause the antiandrogen withdrawal phenomenon via AR mutation in PC cells. Our data strongly support the hypothesis that AR mutation is one possible mechanism of the AWS, although mechanisms other than AR mutation may be also involved in the AWS (10).

Because it took only 6–13 weeks for bicalutamide to switch from an antagonist to an agonist by the W741C mutation in AR, strong mutation pressure from the presence of bicalutamide should be directed toward the emergence of a mutation in codon 741 in the AR gene. It has been reported that T877 is a hot spot of the AR mutation selected by treatment with flutamide in PC patients (14), and the W741C AR mutation has recently been discovered in recurrent PC samples from patients treated with combined androgen blockade

therapy using bicalutamide (15). Therefore, W741 may be a hot spot of the mutation selected by treatment with bicalutamide.

Currently, the mechanism of the quick emergence of the mutant AR in PC cells is unclear. To our knowledge, any single AR mutation other than T877A has not been documented thus far in any cell line derived from LNCaP; however, we cannot completely exclude the possibility that LNCaP-FGC originally harbored cells with the double mutant W741C/T877A or W741L/T877A ARs. Another possibility is that mutation occurred spontaneously and randomly at an extremely high rate, and cells that accidentally obtained the W741C or W741L mutation were selected. Recently, resistance to the Abl tyrosine kinase inhibitor STI-571, which is known as a molecular targeted drug, has been reported to develop as a result of the T315I mutation in the target protein, Abl tyrosine kinase, in chronic myeloid leukemia (16). What is common in both bicalutamide and STI-571 is that although cancer cells have alternatives to depend on for growth-promoting signals other than the drug-targeted protein, they stick to the drug-targeted signal. The different point is that in this study, PC cells not only became unresponsive to the antagonistic activity of bicalutamide but also came to use bicalutamide as an agonist, whereas the T315I mutant Abl tyrosine kinase is only unresponsive to STI-571 (16). There might be some unknown mechanisms that PC could actively make the AR gene mutated to survive against antiandrogens. Although LNCaP cells, which are derived from a metastatic lesion of PC and exist with an abnormal karyotype (17), may be unusually susceptible to selective pressure from antiandrogens, elucidation of the molecular mechanisms for the quick emergence of the mutant AR with this simple *in vitro* culture system using LNCaP-FGC cells might help us to create drugs without drug resistance or drugs that prevent drug resistance in the future.

It has been reported that bicalutamide is effective as a second-line therapy for some PC that has been treated with a combined androgen blockade therapy using flutamide and contains the T877A mutant AR (14). This observation is consistent with the findings that hydroxyflutamide worked as an agonist and bicalutamide worked as an antagonist for the T877A mutant AR in transactivation assays (Ref. 4; Fig. 5). The single mutant W741C and W741L ARs responded only slightly to hydroxyflutamide (Fig. 5), or rather hydroxyflutamide blocked the action of DHT for these mutant ARs (Fig. 6). Bicalutamide could not block the action of DHT for these mutant ARs (Fig. 6). Our data suggest that after PC progresses during bicalutamide administration, flutamide might be effective as a second-line therapy.
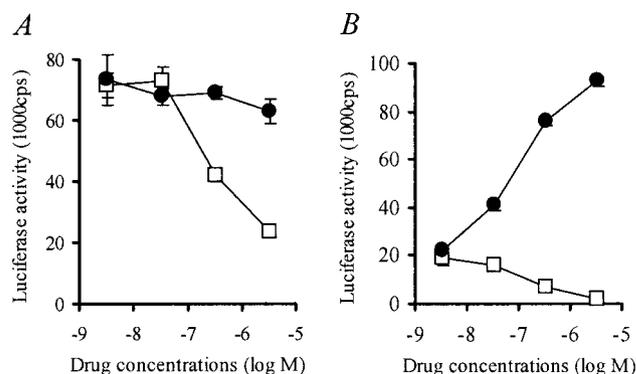

Fig. 6. Hydroxyflutamide works as an antagonist for the W741C and W741L mutant ARs. COS-7 cells were cotransfected with the W741C (*A*) or W741L (*B*) mutant AR expression vectors and the pGL3-PSA-luc vector and treated with the indicated concentrations of bicalutamide or hydroxyflutamide in the presence of 40 nM (*A*) or 200 nM (*B*) DHT. Luciferase activity was assayed 24 h after drug treatment. ●, bicalutamide; □, hydroxyflutamide. Luciferase activities of the W741C and W741L ARs without antiandrogens in the presence of DHT were $69,728 \pm 1,914$ and $24,384 \pm 2,052$ cps, respectively. Data are expressed as the mean of triplicate determinations. Bars, ±SE.

In this study, we demonstrated *in vitro* that PC cells actively alter their AR gene to convert antiandrogen into agonist. If we can predict with this *in vitro* assay system which types of AR mutation emerge clinically when one antiandrogen is used for a long time, and if we can design other antiandrogens effective for the emergent mutant AR, then we could continue to control PC growth by serial antiandrogen treatment. Alternatively, mixtures of several antiandrogens with different chemical structures might be effective because there is an extremely low possibility that AR will be altered in such a way as to become activated by all of the different antiandrogens simultaneously.

ACKNOWLEDGMENTS

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