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MUTATION OF THE ANDROGEN-RECEPTOR GENE IN METASTATIC ANDROGEN-INDEPENDENT PROSTATE CANCER

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Abstract Background. Metastatic prostate cancer is a leading cause of cancer-related death in men. The rate of response to androgen ablation is high, but most patients relapse as a result of the outgrowth of androgen-independent tumor cells. The androgen receptor, which binds testosterone and stimulates the transcription of androgen-responsive genes, regulates the growth of prostate cells. We analyzed the androgen-receptor genes from samples of metastatic androgen-independent prostate cancers to determine whether mutations in the gene have a role in androgen independence.

Methods. Complementary DNA was synthesized from metastatic prostate cancers in 10 patients with androgen-independent prostate cancer, and the expression of the androgen-receptor gene was estimated by amplification with the polymerase chain reaction. Exons B through H of the gene were cloned, and mutations were identified by DNA sequencing. The functional effects of the mutations were assessed in cells transfected with mutant genes.

PROSTATE cancer is the most commonly diagnosed malignant condition and the second leading cause of cancer-related death in American men.¹ In its early stage the disease is sometimes curable by radical prostatectomy or radiation therapy. However, metastases are common at presentation and they ultimately afflict many patients who were treated with curative intent when they had early-stage disease. The only effective treatment for metastatic prostate cancer is reduction of testosterone and 5 α -dihydrotestosterone concentrations (androgen ablation), by either orchiectomy or the administration of an agonist of luteinizing hormone-releasing hormone (LHRH). The rate of response to androgen ablation can be as high as 80 percent, but the duration of response is only 12 to 18 months.² The ef-

Results. All androgen-independent tumors expressed high levels of androgen-receptor gene transcripts, relative to the levels expressed by an androgen-independent prostate-cancer cell line (LNCaP). Point mutations in the androgen-receptor gene were identified in metastatic cells from 5 of the 10 patients examined. One mutation was in the same codon as the mutation found previously in the androgen-independent prostate-cancer cell line. The mutations were not detected in the primary tumors from two of the patients. Functional studies of two of the mutant androgen receptors demonstrated that they could be activated by progesterone and estrogen.

Conclusions. Most metastatic androgen-independent prostate cancers express high levels of androgen-receptor gene transcripts. Mutations in androgen-receptor genes are not uncommon and may provide a selective growth advantage after androgen ablation. (N Engl J Med 1995; 332:1393-8.)

fect of androgen ablation may be augmented by flutamide, an androgen antagonist that acts on the androgen receptor to block the effects of androgens derived from the adrenal gland.³

The mechanisms by which tumor cells escape androgen ablation and become independent of the need for androgen are not understood. Salvage therapy with cytotoxic chemotherapy has generally not been effective in patients with androgen-independent prostate cancer.⁴ Secondary hormonal treatments with estrogens, progesterones, or glucocorticoids can induce short-term partial responses in a minority of patients.⁴ Interestingly, a rate of improvement of 30 to 40 percent after flutamide withdrawal has been reported in patients with androgen-independent prostate cancer who received both flutamide and testicular androgen ablation.⁵

Androgens are required for the development of both the normal prostate and prostate cancer.⁶ Androgens act through the androgen receptor, which belongs to the steroid-receptor superfamily of ligand-dependent transcription factors.^{7,8} This superfamily includes receptors for thyroid hormone, retinoic acid, estrogen, progesterone, glucocorticoids, and other steroid hor-

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mones. The structure of the androgen receptor is prototypical, with an N-terminal transactivating domain (exon A) and a C-terminal hormone-binding domain (exons D through H) (Fig. 1). Between these areas lies the DNA-binding domain (exons B and C), with its two zinc fingers that bind to specific DNA sequences, termed androgen-responsive elements. Once bound to androgen, the androgen receptor regulates the expression of androgen-responsive genes by binding to their androgen-responsive elements and by interacting with other transcription factors. A notable gene regulated by androgen in prostate cells encodes the prostate-specific antigen.⁹

Androgen ablation causes dramatic regression of prostate cancer and normal prostate tissue in most patients and in animal models of prostate cancer.¹⁰ At the cellular level, androgen ablation induces programmed cell death, or apoptosis,¹¹ which appears to be mediated directly or indirectly by unligated androgen receptors. It seems, therefore, that androgen-independent prostate-cancer cells can escape the apoptosis induced by androgen ablation. A mechanism for this escape could be by mutations in the androgen-receptor gene that allow the receptors to stimulate the growth of prostate cells in the absence of androgen. Mutations in the androgen-receptor gene have been shown to cause the androgen-insensitivity syndrome¹²⁻¹⁴ and the spinal and bulbar muscular atrophy syndrome,¹⁵ but they have been detected only rarely in prostate cancer. In a large number of primary prostate cancers analyzed by several groups, only a single mutant androgen-receptor gene was identified.¹⁶⁻²⁰

The androgen-receptor genes of metastatic androgen-independent prostate cancers have not been examined extensively, owing partly to difficulty in obtaining the appropriate tissue. Nevertheless, recent reports suggest that mutations in the androgen-receptor gene can occur in androgen-independent prostate cancer.^{18,19,21} In this report we show that in 5 of 10 patients with metastatic androgen-independent prostate cancer, the androgen-receptor genes had point mutations, all in the hormone-binding domain. One mutation was in the same codon as the previously described mutation in the prostate-cancer cell line (LNCaP).²² Functional studies showed that two of the mutant androgen receptors could be activated by progesterone and estrogen. These results suggest that mutant androgen-receptor genes are a selective advantage in metastatic androgen-

independent prostate cancer, perhaps because they remain active after androgen ablation.

METHODS

Tissue Procurement and Synthesis of Complementary DNA

Prostate-tumor tissue was obtained from patients with metastatic prostate cancer in relapse after androgen ablation by either orchiectomy or the administration of an LHRH agonist. When possible, bone marrow biopsies of posterior iliac-crest metastases were done at sites that correlated with abnormalities identified on bone scans. Other bone marrow samples were obtained at sites of pathologic fractures or from other metastatic sites. All samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis. RNA was extracted from 4 to 10 frozen sections measuring $10\ \mu\text{m}$, and complementary DNA (cDNA) was synthesized according to standard methods, as described previously.²³ Additional frozen sections were analyzed microscopically to confirm the presence of prostate cancer in the samples.

Quantitation of cDNA and Expression of Androgen Receptors

To estimate the amount of cDNA in each sample, amplifications with the polymerase chain reaction (PCR) were carried out with primers for the ubiquitously expressed protein beta₂-microglobulin.²⁴ Serial dilutions of cDNA were amplified by PCR with a beta₂-microglobulin sense primer spanning exons 1 and 2 (ATCCAGCGTACTCCAAAGATTCAG) and an antisense primer in exon 4 (AAATTGAAAGTTAAGTATGCACGC). Each $20\text{-}\mu\text{l}$ reaction mixture contained cDNA, 50 ng of each primer, 100 μg of bovine serum albumin per milliliter, 0.2 mM deoxynucleoside triphosphates, 1.5 mM magnesium chloride, 50 mM potassium chloride, 10 mM TRIS (pH 9.0 at 25°C), and 1 U of *Taq* DNA polymerase. PCR amplifications were done for 25 to 30 cycles at 94°C for 20 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, followed by a 7-minute extension at 72°C . The PCR products were separated on agarose gels, underwent Southern blotting with a beta₂-microglobulin probe (CACGTCATCCAGCAGA, the sense primer in exon 2), and were quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, Calif.).

Approximately equal amounts of cDNA, on the basis of the expression of beta₂-microglobulin, were then amplified by PCR with androgen-receptor primers 3 and 5, located in exons A and C, respectively (Fig. 1), and primers for beta₂-microglobulin. The PCR reactions were carried out for 24 cycles exactly as described above, except for the inclusion of 50 ng of each androgen-receptor primer. This number of cycles was determined in preliminary experiments to precede the plateau phase of the reaction. The products were analyzed on agarose gels and then hybridized with internal oligonucleotide probes specific for beta₂-microglobulin (see above) and androgen receptor (primer 4).

PCR Amplification, Cloning, and Sequencing of the Androgen Receptor

The androgen-receptor gene was amplified by PCR with primers 1 and 7, located at the 3' end of exon A and in the 3' untranslated region, respectively (Fig. 1). A seminested PCR was then carried out

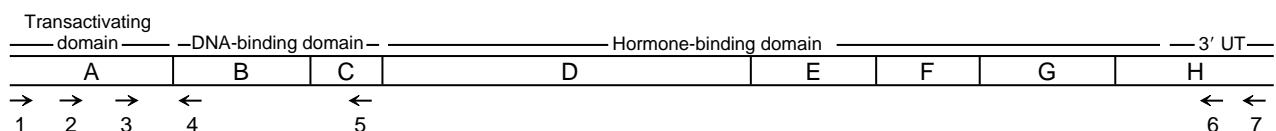


Figure 1. Structure of the Androgen Receptor and Primers Used for PCR Amplification.

Exons A through H and the locations of the transactivating, DNA-binding, and hormone-binding domains are shown. The positions and orientations of the primers used are also shown. The sequences of the primers are as follows: 1, CTACGGCTACTCGGC-CCCCTCA; 2, CGACTTCACCGCACCTGATGTGT; 3, GCATGGTGAGCAGAGTGCCCTATC; 4, AAACATGGTCCCTGGCAGTCTCCA; 5, TCCCAGAGTCATCCCTGCTTCAT; 6, TAACAGGCAGAAGACATCTGAAAG; and 7, ACAGACTGTACATCAATAGAGGAAATTC.

UT denotes untranslated.

with an internal primer in exon A (primer 2). The PCR products were then digested with *KpnI* and *PstI* (which cut the sample just 3' of primer 2 in exon A and 5' of primer 7 in exon H) and cloned into the *PstI* and *KpnI* sites of pBluescript. Bacterial colonies containing the androgen receptor were identified by hybridization, and multiple isolates from each patient were selected. Plasmids were purified and subjected to double-stranded sequencing with Sequenase and a series of internal sequencing primers, as described by the manufacturer (United States Biochemicals, Cleveland). Between 10 and 14 plasmids from each patient were sequenced. Additional sequencing of plasmids and direct sequencing of PCR products were carried out on a DNA sequencer (model 373A, Applied Biosystems, Foster City, Calif.) with *Taq* DNA polymerase and fluorescent dideoxynucleotides (Applied Biosystems). Base changes were determined to be mutations, rather than *Taq* polymerase errors, on the basis of their identification in multiple plasmids and their isolation after a second independent PCR amplification and analysis.

Functional Analysis of Mutant Androgen Receptors

Mutations in the androgen-receptor genes were cloned into the androgen-receptor expression vector pSVAR₀ (kindly provided by Dr. Albert Brinkmann, Erasmus University, Rotterdam, the Netherlands).²⁵ The reporter plasmid MMTVpA3LUC was derived from pHHLUC²⁶ and contained the luciferase gene under the control of an androgen-responsive element in the mouse-mammary-tumor-virus long terminal repeat (kindly provided by Dr. Richard Pestell, Northwestern University Medical School, Chicago). All experiments also included a pSV- β -galactosidase plasmid to control for the efficiency of transfection (Promega, Madison, Wis.). Transient transfections into CV-1 cells were carried out in triplicate with calcium phosphate (Mammalian Cell Transfection Kit, Specialty Media, Lavallete, N.J.) as described by the manufacturer. Approximately 12 hours after the transfections were initiated, the cells were washed and placed in medium containing steroid hormone-free fetal-calf serum for 4 hours. Hormones were then added, and the cells were incubated for another 24 hours. The cells were then lysed, and luciferase and β -galactosidase activity were measured.

RESULTS

Expression of Androgen Receptors in Bone Marrow Metastases from Patients with Androgen-Independent Prostate Cancer

Patients with advanced androgen-independent prostate cancer generally have widespread metastatic disease in the bone marrow. Previous studies showed that the level of expression of androgen receptors by normal lymphoid and myeloid cells was quite low or absent.²⁷ We therefore used a semiquantitative reverse-transcriptase PCR method to measure androgen-receptor transcripts in bone marrow in the presence or absence of prostate cancer.

Figure 2 shows that bone marrow samples from four patients with androgen-independent prostate cancer who were treated by androgen ablation had readily detectable levels of androgen-receptor gene transcripts (lanes 1, 3, 4, and 5). The levels in these marrow samples were similar to the level expressed by LNCaP (lane 7). In contrast, expression of the androgen-receptor gene was not detectable in samples from patients who were in complete remission after androgen ablation (Fig. 2, lane 2, and data not shown). Since none of these samples contained pure populations of tumor cells (Fig. 2), the average level of androgen-receptor transcripts in the tumor cells was high relative to that in LNCaP. Figure 2 also shows the level of androgen-receptor gene transcripts in a biopsy specimen obtained

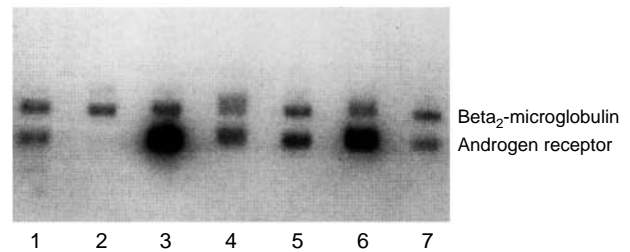


Figure 2. Results of Semiquantitative Reverse-Transcriptase PCR Amplification of the Androgen Receptor.

Samples were amplified simultaneously to measure the amount of beta₂-microglobulin and androgen-receptor cDNA, subjected to Southern blotting, and hybridized with internal oligonucleotide probes for beta₂-microglobulin and androgen receptor. Lanes 1, 3, 4, and 5 show bone marrow specimens from patients with advanced androgen-independent prostate cancer (the sample in lane 5 was completely replaced by tumor, whereas the other biopsy specimens had scattered foci of tumor); lane 2, a bone marrow specimen from a patient with prostate cancer in complete remission after androgen ablation; lane 6, cells obtained during a channel transurethral prostatectomy in a patient with androgen-independent prostate cancer; and lane 7, LNCaP cells.

during a channel transurethral prostatectomy in a patient with androgen-independent prostate cancer (lane 6). This sample contained a large proportion of tumor cells and showed a correspondingly high level of androgen-receptor gene transcripts.

These results confirmed that metastatic androgen-independent prostate cancers contain high levels of androgen-receptor gene transcripts, whereas cells in normal bone marrow have relatively little. On the basis of these observations, we proceeded to analyze the androgen-receptor genes expressed in bone marrow and other metastatic sites in 10 patients with androgen-independent prostate cancer.

Identification of Androgen-Receptor Mutations

Tumor cells from 10 patients with metastatic androgen-independent prostate cancer (Table 1) were ob-

Table 1. Clinical Characteristics of 10 Patients with Prostate Cancer.

PATIENT NO.	INITIAL STAGE	GLEASON SCORE	LOCAL TREATMENT	TIME TO METASTASIS	HORMONAL TREATMENT	TIME TO ANDROGEN-INDEPENDENT CANCER
				mo		mo
1	B	Unknown	Radiation	36	Leuprolide Flutamide	14
2	B	Unknown	Radiation	180	Leuprolide Flutamide	8
3	C	8	Surgery	7	Orchiectomy	1
4	D2	8	None	—	Leuprolide Flutamide	4
5	D2	Unknown	None	—	Orchiectomy	32
6	D2	Unknown	None	—	Orchiectomy	14
7	B	9	Radiation	19	Orchiectomy	14
8	B	Unknown	Radiation	72	Orchiectomy	48
9	C	9	Surgery Radiation	36	Leuprolide Flutamide	18
10	D2	Unknown	None	—	Orchiectomy	34

tained from bone marrow (Patients 1, 2, 3, 4, 6, 8, 9, and 10), pleural fluid (Patient 5), or a skin nodule (Patient 7). The DNA-binding and hormone-binding domains of the androgen-receptor gene were amplified by PCR and cloned. Multiple isolates (10 to 14 from each patient) were sequenced. Androgen-receptor gene mutations were identified in 5 of the 10 patients (Table 2). Each of these mutations was confirmed by independent PCR amplifications.

Four of the bone marrow samples (Patients 1, 2, 3, and 4) contained androgen-receptor genes with single point mutations in the hormone-binding domain. The mutation in Patient 1 was in the same codon as the mutation reported previously in LNCaP, but resulted in a different amino acid change (threonine to serine, rather than threonine to alanine).²² Both mutant and wild-type androgen-receptor genes were identified in Patients 1, 3, and 4. The wild-type genes could have been derived from normal cells in the biopsy specimens or from tumor cells with wild-type androgen-receptor genes. In contrast, every androgen-receptor gene isolated from Patient 2 had a mutation in codon 874, raising the possibility that this variation was a polymorphism and not a somatic mutation related to the malignant cell itself. To distinguish between these two possibilities, genomic DNA was isolated from peripheral-blood mononuclear cells and amplified with exon H-specific primers. The germ-line androgen-receptor gene in this material was wild type. Thus, the codon 874 variant in Patient 2 was a somatic mutation (data not shown).

Paraffin blocks containing primary-tumor specimens obtained during a prostatectomy (Patient 3) and a core biopsy of the prostate (Patient 4) were available. Areas containing tumor were microdissected, and the DNA was extracted and amplified by PCR with exon-specific primers. The androgen-receptor genes in both cases were wild type, whereas these genes were mutated in the metastatic tumors.

The cells from Patient 5, which were derived from a malignant pleural effusion, had an androgen-receptor gene with four mutations located in exons D, E, and H (Table 2). Every isolate from two separate PCR

Table 2. Mutations in the Androgen-Receptor Gene Identified in Five Patients with Androgen-Independent Prostate Cancer.

PATIENT No.	CODON*	EXON	TYPE OF MUTATION	FREQUENCY (%)†
1	877	H	ACT (Thr)→AGT (Ser)	86
2	874	H	CAT (His)→TAT (Tyr)	100
3	902	H	CAA (Gln)→CGA (Arg)	37
4	721	D	GCC (Ala)→ACC (Thr)	20
5‡	647	D	AGC (Ser)→AAC (Asn)	100
	724	E	GGC (Gly)→GAC (Asp)	
	880	H	CTG (Leu)→CAG (Gln)	
	896	H	GCA (Ala)→ACA (Thr)	

*Numbering of the codons is based on the results of Lubahn et al.²⁸

†The frequency is based on the fraction of isolates in the initial cloning process that contained the mutation.

‡One androgen receptor with four mutations was isolated.

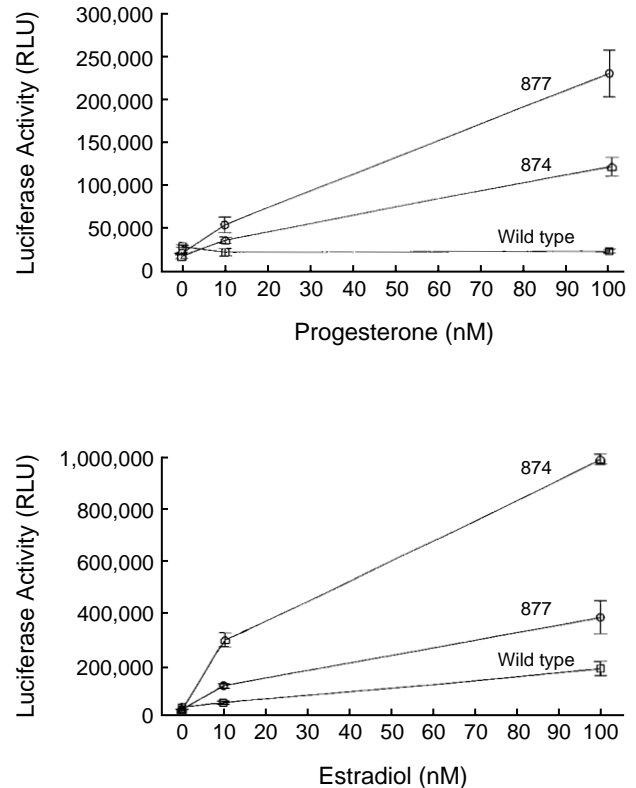


Figure 3. Functional Analysis of Androgen-Receptor Mutations. Wild-type or mutant androgen receptors were transiently expressed in CV-1 cells with a luciferase reporter gene and various concentrations of estradiol or progesterone. The luciferase activity (expressed in relative light units [RLU]) was normalized for β -galactosidase activity to yield a standardized RLU. The maximal responses to androgen (5α -dihydrotestosterone) for each androgen receptor averaged approximately 2,200,000 RLU for the wild-type androgen receptor, 1,500,000 RLU for the codon 874 androgen receptor, and 800,000 RLU for the codon 877 androgen receptor in three experiments. All determinations were done in triplicate, and the mean (\pm SE) results of a representative experiment are shown.

amplifications contained these four mutations, which were also evident when the amplified PCR products were sequenced directly (data not shown). This patient's prostate cancer was unusual clinically in that it presented in an inguinal lymph node and the subsequent malignant pleural effusion, which contained cells weakly positive for prostate-specific antigen, resolved with flutamide treatment. Analysis of DNA from peripheral-blood mononuclear cells with primers specific for exons E and H demonstrated that the mutations in these exons were not polymorphisms (data not shown).

Functional Analysis of Mutant Androgen Receptors

The mutant androgen-receptor genes from Patients 1 and 2, which contained single point mutations and appeared to be expressed by the majority of tumor cells, were studied functionally. The cloned genes from these two tumors were transfected into CV-1 cells, and their ability to respond to progesterone and estradiol was as-

sessed. The wild-type and mutant androgen receptors were inactive in the absence of androgen but could be stimulated by androgen (Fig. 3). As has been shown previously,²² the wild-type androgen receptor was specific for androgens and was only weakly stimulated by estrogen or progesterone (Fig. 3). In contrast, the mutant androgen receptors from both patients were stimulated by estrogen and progesterone (Fig. 3).

DISCUSSION

We showed that semiquantitative amplification with reverse transcription PCR can detect transcripts of the androgen-receptor gene in metastatic androgen-independent prostate-cancer cells. The level of androgen-receptor gene expression in these samples was at least comparable to the amount expressed by LNCaP. This finding is consistent with results of immunohistochemical analysis of the expression of androgen-receptor genes in patients with advanced androgen-independent prostate cancer.^{20,29} They indicate that down-regulation of the gene cannot be a frequent mechanism of escape from programmed cell death triggered by androgen ablation. Instead, they suggest that tumor-cell growth after androgen ablation requires the expression of a functional androgen-receptor gene.

Sequence analyses revealed point mutations in the hormone-binding domain of the androgen-receptor gene in 5 of 10 patients with androgen-independent prostate cancer. Mutations in the androgen-receptor gene were not found in the two archival specimens of primary tumor examined, a result consistent with previous studies showing that these mutations are extremely rare in primary prostate cancer.¹⁶⁻²⁰ This finding indicates that androgen ablation selects for tumor cells with certain mutations of the androgen-receptor gene, presumably because these mutant androgen receptors do not require the usual levels of androgen to stimulate cell growth. Functional studies showing that these mutations alter the hormone specificity of the androgen receptor further support this conclusion.

The mutations in codons 874 and 877 studied in this report both generated androgen receptors that could be stimulated by estrogen and progesterone. The LNCaP mutation described previously in codon 877 (threonine to alanine) generated an androgen receptor that could also be activated by estrogen, progesterone, and flutamide.²² A mutant androgen receptor recently isolated from a patient with androgen-independent prostate cancer (with a substitution of methionine for valine at codon 715) could similarly be stimulated by progesterone.¹⁸ These observations, together with previous mutagenesis studies of codon 877,³⁰ indicate that a number of mutations in the hormone-binding domain can alter the specificity of the androgen receptor. In vitro studies indicate that the androgen receptor can also be constitutively activated by deletions in the hormone-binding domain,³¹ although such deletions have not yet been identified in vivo. Point mutations and deletions in the estrogen receptor have been found in breast cancer.^{32,33}

Clinical data indicate that many androgen-inde-

pendent prostate cancers are sensitive to secondary hormonal treatments, including flutamide withdrawal.^{4,5} These observations also suggest that functionally altered androgen receptors may be present in a substantial proportion of patients with androgen-independent prostate cancer. Nonetheless, there are other possible mechanisms of androgen independence,^{34,35} the importance of which remains to be established. It is most likely that androgen-independent prostate cancers are heterogeneous, even in individual patients. The relatively short-lived response to androgen ablation indicates that a large number of cells must be androgen-independent before androgen ablation as a result of independent mutations in the androgen-receptor gene and alterations in other proteins. Our results suggest that mutant androgen-receptor genes in androgen-independent prostate cancer could be useful targets of new drugs for the treatment of prostate cancer.

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