

Frequent Detection of Codon 877 Mutation in the Androgen Receptor Gene in Advanced Prostate Cancers¹

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Abstract

Prostatic tissue specimens derived from transurethral resections of patients with metastatic prostate cancer were analyzed for genetic alterations in the hormone-binding domain of the androgen receptor (AR) gene. Direct sequencing of the polymerase chain reaction-derived DNAs of 6 of 24 specimens revealed a codon 877 mutation (ACT → GCT, Thr → Ala) in the hormone-binding domain of the AR gene. This same AR mutation has been reported previously in a metastatic prostate cancer cell line, LNCaP, where this mutation confers upon the AR an altered ligand-binding specificity which is stimulated by estrogens, progestagens, and antiandrogens. It is possible that analogous to an activated/altered growth factor receptor oncogene, codon 877 mutant AR with altered ligand binding may provide a selective growth advantage in the genesis of a subset of advanced prostate cancer. Although estrogens are used infrequently, antiandrogens are used increasingly in hormonal therapy for patients with advanced prostate cancer. The stimulatory effect of these therapeutic agents on the codon 877 mutant AR further suggests that this frequently observed AR mutation may contribute to the treatment refractory disease.

Introduction

Prostate cancer is the most frequently diagnosed solid tumor and the second leading cause of cancer death in men in the United States (1). Although potentially curable in its early stages, metastatic prostate cancer is incurable despite temporary remissions commonly achieved with hormonal therapy. Historically, 40–50% of patients with this disease present with locally advanced or metastatic disease (2). The cornerstone of therapy in patients with metastatic disease is androgen ablation, commonly referred to as “hormonal therapy,” since Huggins and Hodges’ seminal discovery in 1941 (3). Androgen ablation can be achieved by orchiectomy, by the administration of estrogens, or more recently by one of the luteinizing hormone-releasing hormone agonists. Recent clinical trials have demonstrated the efficacy of combining an antiandrogen to orchiectomy or a luteinizing hormone-releasing hormone to block the remaining androgens produced by the adrenal glands (4). Although approximately 80% of patients initially respond to hormonal ablation, the vast majority of patients eventually relapse (5), presumably due to neoplastic clones of cells which become refractory to this therapy.

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Androgen ablation therapy in prostate cancer involves the inhibition or blockade of androgen signaling pathway(s) via AR³ in prostatic epithelial cells. It has been postulated that alterations of the AR may play an important role in prostate cancer development. Earlier studies using radioligand binding showed that lower levels of the nuclear AR correlated with a poor response to hormonal therapy (6). However, the major concern regarding these observations was the accuracy of the assays using homogenized specimens containing both normal and tumor cells (6). Recent studies using AR immunostaining have shown the presence of AR in prostate cancer specimens resistant to hormonal therapy (7–10), and more careful analysis has revealed a correlation between heterogeneous AR staining within specimens and a poor response to hormonal therapy (11). Although the analysis of AR protein may approximate the presence or absence of AR in prostate cancer specimens, these methods are not able to address alterations of AR functions resulting from mutations in the transcription activation region, DNA binding, or HBD of the AR gene (12). There have been only a few reports describing infrequent AR mutations in prostate cancer to date. A well characterized cell line, LNCaP, derived from a lymph node of a patient being treated with estrogen and orchiectomy for metastatic prostate cancer, is known to harbor a mt AR with a point mutation in the HBD (13, 14). The possibility of cell culture artifact for this AR mutation has not been excluded. The same LNCaP AR mutation has been described either as a codon 877 (ACT → GCT) of a 919-codon AR complementary DNA (13) or as a codon 868 (ACT → GCT) in a 910-codon AR complementary DNA (14). A recent study has analyzed 26 early stage B prostate cancers and reported codon 730 AR mutation in one specimen (15). Recent studies of the AR gene in advanced prostate cancers have also revealed mutations in codons 701 and 877 in 1 of 8 specimens (16), in codons 340 and 798 in 2 of 10 specimens (17), and in codon 715 in 1 of 7 specimens (18). It is interesting that most of the reported AR mutations in prostate cancers were present in the HBD. It is critically apparent that alteration of AR in advanced prostate cancer needed to be evaluated further. Toward this goal, we have analyzed specimens of TUR of prostate from 24 patients with advanced disease for alterations in the HBD of AR.

We have found the frequent presence (6 of 24) of the codon 877 mutation (ACT → GCT) in prostate cancer specimens studied in our laboratory. The detailed analysis of the complete HBD of the AR in two specimens positive for the codon 877 mutation and one specimen negative for this mutation did not reveal any other mutation. This is the first report demonstrating the frequent mutation of codon 877 in advanced prostate cancers, and the implications of our findings are discussed.

³The abbreviations used are: AR, androgen receptor; HBD, hormone-binding domain; mt, mutant; TUR, transurethral resection; PCR, polymerase chain reaction; wt, wild-type.

Materials and Methods

Tumor Specimens and Cell Lines. Clinical pathological data on all patients were obtained from the tumor registry review at Walter Reed Army Medical Center (Washington, DC). A total of 24 patients with advanced prostate cancer were selected for this study. The prostate cancer cell lines LNCaP, DU 145, and PC-3 were obtained from American Type Culture Collection (Rockville, MD).

Archival paraffin-embedded channel TUR specimens of these selected patients were analyzed by hematoxylin and eosin staining of 4- μ m-thick sections for the presence of tumor cells. Regions of the tumor tissue sections containing a high fraction of the neoplastic cells (20–100%) were identified by one author (I. A. S.).

DNA Extraction. Corresponding tumor regions from hematoxylin and eosin slides were marked on the tissue embedded in paraffin blocks and were microdissected. DNA was then extracted following the method of Moul *et al.* (19) where the tissue was deparaffinized by xylene and DNA was extracted by proteinase K treatment followed by phenol extraction and ethanol precipitation.

PCR/DNA Sequencing. The DNAs were amplified by PCR for the five exons of HBD of the *AR* gene using the primers described by Lubahn *et al.* (20), except for the upstream primer of exons D and G. The primers used for exons D and G are 5'-GTAGTTGCATTGTGTGTTTTTGACC-3' and 5'-GTCAAGTCTGTGGTCAGAAAACCTGG-3', respectively. The PCR of genomic DNAs were essentially performed as described earlier (21). The specific PCR reaction conditions were: 95°C for 30 s; 60°C for 45 s, and 72°C for 60 s for 40 cycles followed by 72°C for 5 min for 1 cycle.

To generate the template for DNA sequencing, a second PCR reaction comprising an aliquot of the first PCR product and the primers set including one biotin-labeled primer was performed. Additionally, to increase the specificity of the PCR reaction, the second PCR was always performed with at least one primer nested to the primers used in the first PCR reaction. The primers used for the second PCR were: Exon D, B-5'-CCACTGAGGAGACAAC-CCAGAAGC-3', 5'-GATCCCCCTTATCTCATGCTCCC-3'; Exon E, B-5'-CAACCCGTCAGTACCCAGACTGACC-3', 5'-ACCAACCAGGTCTGGC-CAAGCTGC-3'; Exon F, B-5'-CTCTGGGCTTATTGGTAAACTTCC-3', 5'-TGGTCTCTCTGAATCTCTGTGC-3'; Exon G, B-5'-TGTCTAATGCTC-CTTCGTGGGC-3', 5'-CTCTATCAGGCTGTTCTCCCTGAT-3'; Exon H, B-5'-GAGCCACCTCTTGTCAACCCTG-3', 5'-GGAGTAGTGCAGAG-TTATAACAGGC-3'. The primers with the prefix B refer to biotinylated primers. The second PCR reaction conditions were similar to the first PCR except that the amplification was only for 30 cycles. The 5'-biotinylated single stranded DNA was purified using streptavidin magnetic beads (Dynal, NY) and DNAs were sequenced with complementary sequencing primers using Sequenase version 2.0 (United States Biochemicals).

Results

For archival paraffin-embedded prostate TUR specimens of 24 patients used in this study, the patients had a known history of advanced prostate cancer and presented with the symptoms of bladder outlet obstruction despite treatment with hormonal and/or radiation therapy (Table 1). The DNA sequence of 3 of the first 11 specimens analyzed revealed the presence of a point mutation in codon 877 (ACT → GCT) of exon H of the *AR* gene (Fig. 1). Additionally, sequencing of both strands revealed the same mutation without any other alteration of exon H. Since we detected the same mutation previously reported for LNCaP cell line and our analysis used PCR, we wanted to rule out any accidental contamination of prostate cancer specimens with LNCaP DNA from our laboratory, as well as the possibility of cross-contamination between specimens. For this purpose, we repeated the entire procedure starting from the same genomic DNA or a new DNA preparation, and we reproducibly detected the codon 877 mutation in the same specimens. The only variation between three such repeated experiments was the varying intensities of a residual wt band in the codon 877 in DNA sequencing gels (Fig. 1). The detection of the wt band is most likely due to the extraction of DNA from normal cells present in the tumor specimens, and the

Table 1 Androgen receptor gene mutation and stage of prostate cancer specimens

| Patient | Stage at TUR | Codon 877 <i>AR</i> mutation |
|----------------|----------------|------------------------------|
| 1 | D ₁ | – |
| 2 | C | – |
| 3 | D ₁ | – |
| 4 | D ₁ | – |
| 5 | D ₂ | + |
| 6 | C | – |
| 7 | C | – |
| 8 | D ₀ | – |
| 9 | D ₁ | – |
| 10 | C | + |
| 11 | D ₂ | – |
| 12 | D ₂ | – |
| 13 | D ₁ | – |
| 14 | D ₂ | – |
| 15 | D ₂ | – |
| 16 | D ₂ | + |
| 17 | D ₂ | – |
| 18 | D ₁ | + |
| 19 | D ₂ | – |
| 20 | D ₁ | + |
| 21 | D ₁ | – |
| 22 | D ₂ | – |
| 23 | D ₀ | + |
| 24 | D ₂ | – |
| LNCaP | Cell line | + |
| DU 145 | Cell line | – |
| PC 3 | Cell line | – |
| Human placenta | Control | – |

variability in detection of the wt band is most probably due to variations in yields of the PCR product used for DNA sequencing. We also ruled out any cross-contamination between specimens from an independent study from our laboratory, showing that the same DNA specimens used for *AR* mutation study exhibited different mutations of the *p53* gene. A mock DNA extraction, along with tumor tissue DNA extraction or negative controls used in PCR reaction, also did not reveal any detectable product when compared to the reactions containing DNA. Furthermore, a human placental DNA, DNAs from two prostate cancer cell lines (DU 145 and PC-3), and the prostate cancer specimens exhibiting wt sequence for the codon 877 of *AR* reproducibly displayed wt codon 877 sequence (Table 1). Thus, on the basis of these results we feel confident that the codon 877 mutation in prostate cancer specimens reported here truly represent *in vivo* mutation. We extended the analysis of this mutation to an additional 13 specimens, and these specimens were processed for DNA extraction in a separate laboratory. Three more specimens from this group exhibited the same codon 877 mutation. Altogether, we identified 6 of 24 patients with an identical mutation of the *AR* gene (Fig. 1; Table 1). We next analyzed selected specimens for the rest of the HBD (12) encompassing part of exon D and complete exons of E, F, and G by DNA sequencing. Two specimens positive for codon 877 mutation did not reveal any other mutation in the remainder of the HBD. Furthermore, one specimen negative for codon 877 mutation and human placental DNA revealed wt *AR* sequence in the entire HBD. Thus, these results further suggest the *in vivo* selection of the codon 877 mutation in the HBD (Fig. 2) of the *AR* in prostate cancer specimens analyzed here.

Discussion

Although infrequent *AR* mutations in prostate cancers have been described recently (15–18), it is striking that we have frequently detected the codon 877 (ACT → GCT) mutation in a number of advanced prostate cancer specimens (6 of 24 patients). The same *AR* mutation has been described earlier in a metastatic prostate cancer cell line, LNCaP, but the *in vivo* origin of this *AR* mutation has not been completely established. Our studies, along with the recent report of Suzuki *et al.* (16), describing the same codon 877 *AR* mutation in

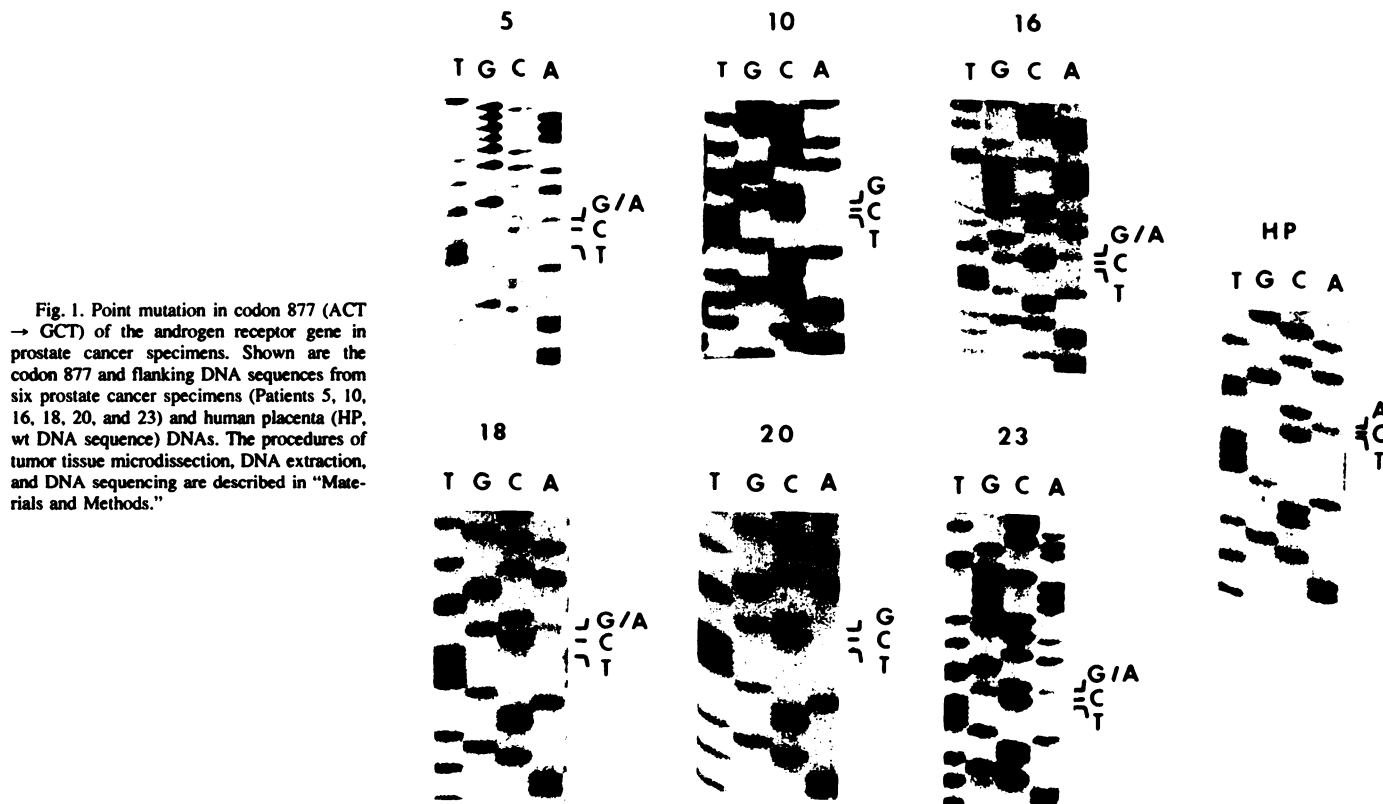


Fig. 1. Point mutation in codon 877 (ACT → GCT) of the androgen receptor gene in prostate cancer specimens. Shown are the codon 877 and flanking DNA sequences from six prostate cancer specimens (Patients 5, 10, 16, 18, 20, and 23) and human placenta (HP, wt DNA sequence) DNAs. The procedures of tumor tissue microdissection, DNA extraction, and DNA sequencing are described in "Materials and Methods."

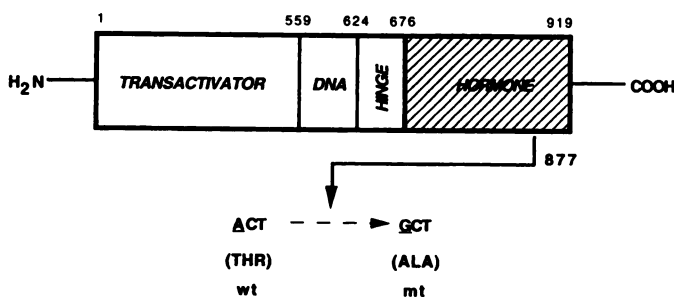


Fig. 2. Schematic representation of androgen receptor codon 877 mutation detected in prostate cancer specimens. Androgen receptor gene mutation and the corresponding amino acid change detected in the hormone-binding domain (■) of the androgen receptor protein. Adapted from the paper of Janne *et al.* (12), with permission.

metastatic specimens of one patient, provide strong support for the *in vivo* significance of this AR mutation. More importantly, the results reported here provide the first evidence for a mutational "hot spot" in the AR gene in a subset of prostate cancer.

Our results differ from other studies (15–18) in the frequent detection of this codon 877 mutation and are best explained by the larger number of specimens, the quality of the specimens, and the method by which tumor cell populations were selected and processed. In our study, hematoxylin and eosin staining was used to denote the most abundant concentration of tumor cells. The concentration of phenotypically similar tumor cells within the same specimen was quantitated, and the region containing tumor cells was microdissected to ensure that the inevitable inclusion of normal cells was kept to a minimum. Since the AR gene is present on chromosome X and there is only one X allele present in males, the presence of both the wt and mt codon 877 sequence in some specimens (Fig. 1) is most likely due to the presence of normal cells in tumor specimens. The DNA from the specimens containing 90–100% of tumor cells

(analyzed by I. A. S.) exhibited only the mt band in the codon 877, further supporting the presence of normal cells in those tumor specimens exhibiting both wt and mt codon 877 sequence.

Mutations in the DNA-binding domain and HBD are described in androgen resistance syndromes (22). Recent studies have also shown mutations of AR in the DNA-binding domain in male breast cancer (23). Mutational analysis of the AR gene *in vitro* has shown that deletions of HBD can constitutively activate the receptor (24). Thus, mutations of a functionally important region in the AR gene may also abrogate AR signaling pathways. It is conceivable that these mutations of the AR gene contribute to the aggressive and hormone-refractory phenotype of advanced prostate cancer. Some of the implications of our observation can be extrapolated from studies on the functional characteristics of the mutant AR with a known codon 877 point mutation that is present in the LNCaP prostate cancer cell line (14). LNCaP cells were derived from a metastatic lesion of a patient with treatment-resistant prostate cancer and have been shown to have altered receptor specificity in binding to progestagens, estrogens, and antiandrogens (14). It is interesting to note that antiandrogens have an unexpected stimulatory effect on the growth of LNCaP cells. All the specimens analyzed here were also from patients with advanced prostate cancers. Of six specimens positive for the codon 877 mutation, a majority of them represented stage D disease (C, 1; D₀, 1; D₁, 2; D₂, 2). On the basis of the altered ligand-binding specificity of AR due to codon 877 mutation, it is tempting to suggest that analogous to an activated/alternated growth factor receptor oncogene (25), the mt AR may provide a selective growth advantage in a subset of advanced prostate cancer. In contrast to the expected inhibitory effect of the agents used in hormonal therapy, their stimulatory effect on the codon 877 mt AR (14) may provide one of the mechanisms involved in the treatment refractory prostate cancers. Therefore, follow-up of these interesting observations have the potential to unravel the molecular mechanisms involved in the genesis of treatment-resistant advanced prostate cancers.

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Note Added in Proof

Recently we have obtained additional experimental evidence which further rules out any accidental contamination of the LNCaP DNA in prostate biopsy specimens. PCR based analysis of the polymorphic CAG repeat region of exon A revealed distinct band patterns for three biopsy DNA specimens exhibiting codon 877 AR mutation in comparison to LNCaP DNA. These results provide additional support for an *in vivo* origin of the codon 877 mutation reported here.

References

- Boring, C. C., Squires, T. S., and Tong, T. Cancer statistics; 1994. *CA Cancer J. Clin.*, 43: 7-26, 1994.
- Veterans Administrative Cooperative Urological Research Group. Carcinoma of the prostate: treatment comparisons. *J. Urol.*, 98: 516-522, 1967.
- Huggins, C., and Hodges, C. V. Studies on prostatic cancer, effects of castration, of estrogens and of androgen injection on serum phosphatase in metastatic carcinoma of the prostate. *Cancer Res.*, 1: 293-297, 1941.
- Crawford, E. D., Eisenberger, M. A., McLeod, D. G., Spaulding, J.-T., Benson, R., Dorr, F. A., Blumenstein, B. A., Davis, M. A., and Goodman, P. J. A controlled trial of leuprolide with and without flutamide in prostatic carcinoma. *N. Engl. J. Med.*, 321: 419-424, 1989.
- Blackard, C. E., Byar, D. P., and Jordan, W. P., Jr., for the Veterans Administration Cooperative Urological Research Group. Orchiectomy for advanced carcinoma: a re-evaluation. *Urology*, 1: 533-560, 1973.
- Barrack, E. R., and Tindall, D. J. A critical evaluation of the use of androgen receptor assays to predict the androgen responsiveness of prostatic cancer. *Prog. Clin. Biol. Res.*, 239: 155-187, 1987.
- Sadi, M. V., Walsh, P. C., and Barrack, E. R. Immunohistochemical study of androgen receptors in metastatic prostate cancer. *Cancer (Phila.)*, 67: 3057-3064, 1991.
- van Der Kwast, T. H., Schalken, J., Ruizeveld De Winter, J. A., van Vroonhoven, C. C. J., Mulder, E., Boersma, W., and Trapman, J. Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int. J. Cancer*, 48: 189-193, 1991.
- Brolin, J., Lowhagen, T., and Skoog, L. Immunocytochemical detection of the androgen receptor in five needle aspirates from benign and malignant human prostate. *Cytopathology*, 3: 351-357, 1992.
- Miyamoto, K. K., McSherry, S. A., Dent, G. A., Sar, M., Wilson, E., French, F. S., Sharief, Y., and Mohler, J. L. Immunohistochemistry of the androgen receptor in human benign and malignant prostate tissue. *J. Urol.*, 149: 1015-1019, 1993.
- Sadi, M., and Barrack, E. R. Image analysis of the androgen receptor immunostaining in metastatic prostate cancer. Heterogeneity as a predictor of response to hormonal therapy. *Cancer (Phila.)*, 71: 2574-2580, 1993.
- Janne, O. A., Palvimo, J. J., Kallio, P., and Mehto, M. Androgen receptor and mechanism of androgen action. *Ann. Med.*, 25: 83-89, 1993.
- Harris, S. E., Rong, Z., Harris, M. A., and Lubahn, D. D. Androgen receptor in human prostate carcinoma LNCaP/ADEP cells contains a mutation which alters the specificity of the steroid-dependent transcriptional activation region. *Endocrinology*, 126 (Suppl.): 93, 1990.
- Veldscholte, J., Ris-Stalpers, C., Kuiper, G. G. J. M., Jenster, G., Berrevoets, C., Claassen, E., van Rooij, H. C. J., Trapman, J., Brinkman, A. O., and Mulder, E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.*, 173: 534-540, 1990.
- Newmark, J. R., Hardy, D. O., Tonb, D. C., Carter, B. S., Epstein, J. I., Isaacs, W. B., Brown, T. R., and Barrack, E. R. Androgen receptor gene mutations in human prostate cancer. *Proc. Natl. Acad. Sci. USA*, 89: 6319-6323, 1992.
- Suzuki, H., Sato, N., Watabe, Y., Masai, M., Seino, S., and Shimazaki, J. Androgen receptor gene mutations in human prostate cancer. *J. Steroid Biochem. Mol. Biol.*, 46: 759-765, 1993.
- Castagnaro, M., Yandell, D. W., Dockhorn-Dworhiczak, B., Wolfe, H. J., and Poremba, C. Human androgen receptor gene mutations and *p53* gene analysis in advanced prostate cancer. *Verh. Dtsch. Ges. Pathol.*, 77: 119-123, 1993.
- Cullig, Z., Hobisch, A., Cronauer, M. V., Cuto, A. C. B., Hittmair, A., Radmayr, C., Eberle, J., Bartsch, G., and Klocker, H. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. *Mol. Endocrinol.*, 7: 1541-1550, 1993.
- Moul, J. W., Friedrichs, P. A., Lance, R. S., Theune, S. M., and Chang, E. H. Infrequent *ras* oncogene mutations in human prostate cancer. *Prostate*, 20: 327-328, 1992.
- Lubahn, D. B., Brown, T. R., Simental, J. A., Higgs, H. N., Migeon, C. J., Wilson, E. M., and French, F. S. Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. *Proc. Natl. Acad. Sci. USA*, 86: 9534-9538, 1989.
- Srivastava, S., Zou, Z., Pirollo, K., Blattner, W., and Chang, E. H. Germ-line transmission of a mutated *p53* gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature (Lond.)*, 348: 747-749, 1990.
- Sultan, C., Lumbroso, S., Poujol, N., Belon, C., Boudon, C., and Lobaccaro, J. M. Mutations of androgen receptor gene in androgen insensitivity syndromes. *J. Steroid Biochem. Mol. Biol.*, 46: 519-530, 1993.
- Wooster, R., Mangion, J., Eeles, R., Smith, S., Dowsett, M., Averill, D., Barrett-Lee, P., Easton, D. F., Ponder, B. A. J., and Stratton, M. R. A germ line mutation in the androgen receptor gene in two brothers with breast cancer and Reifenstein syndrome. *Nat. Genet.*, 2: 132-134, 1992.
- Simental, J. A., Sar, M., Lane, M. V., French, F. S., and Wilson, E. M. Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J. Biol. Chem.*, 266: 510-518, 1991.
- Aaronson, S. A. Growth factors and cancer. *Science (Washington DC)*, 254: 1146-1153, 1991.

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