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Androgen receptor gene mutations in human prostate cancer

(polymerase chain reaction/denaturing gradient gel electrophoresis/somatic mutation)

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ABSTRACT **We screened human prostate cancer tissues for the presence of somatic mutations in the hormone binding domain of the androgen receptor (AR) gene. Exons E–H were amplified from genomic DNA using the polymerase chain reaction and analyzed by denaturing gradient gel electrophoresis (DGGE), which separates DNA fragments that differ by only a single base. We detected a mutation in exon E of the hormone binding domain in 1 of 26 specimens of untreated organ-confined stage B prostate cancer. The mutation was not detectable in peripheral blood lymphocyte DNA. Lymphocyte DNA (wild-type AR) migrated in DGGE as a single band. The tumor DNA migrated in DGGE as four bands, consistent with the presence of cells with mutant AR plus cells with wild-type AR and indicating that the tumor contained a somatic mutation. To our knowledge, a somatic AR gene mutation has not been reported previously. Sequencing revealed a G → A substitution in codon 730, changing valine to methionine. Codon 730 is in a region highly conserved among all steroid receptors. The abundance of the mutated fragment (about 50% of the DNA in the specimen) indicates its presence in cells with a growth advantage. A somatic mutation could be detected by DGGE if it represented at least 10% of the sample. Failure to detect mutations in other specimens analyzed may be due to this limit of sensitivity, the presence of mutations in other parts of the AR, or a low frequency of mutations in early stage disease.**

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer deaths in men in the U.S. (1). Recognition that androgen is required for the development of prostate cancer (2) and its growth (3) has been the basis for continuing interest in the role of the androgen receptor (AR) in prostate cancer (1, 4–6). The AR is a member of the superfamily of genes that code for the steroid and thyroid hormone receptor family of ligand-dependent nuclear transcription factors, all of which have an N-terminal domain that affects transcription efficiency, a central DNA binding domain that binds to a target gene hormone response element and thereby determines target gene specificity, and a C-terminal hormone binding domain (HBD) (7–9). Studies on the role of AR in prostate cancer have focused on measurements of AR based on ligand binding or immunohistochemistry (4–6). However, it is now clear that ligand binding and immunoreactivity are not adequate indicators of a functional AR, since mutant ARs can be generated that bind steroid but are nonfunctional or that do not bind steroid but are nevertheless constitutively active (10–12). Deletion of all or part of the N-terminal domain or of the DNA binding domain renders the AR transcriptionally inactive despite its ability to still bind androgen with high affinity (11, 12). Most remarkably, deletion of the HBD renders the AR unable to bind steroid but constitutively transcriptionally active even in the absence of hormone (10–12). Thus, knowledge of the integrity of the AR gene or gene product may be a more accurate index of potential AR function in prostate cancer than immunoreactivity or ligand binding.

Mutated ARs occur naturally as a result of AR gene mutations (13–22). AR gene mutations were first described in complete androgen insensitivity syndrome (CAIS), an X chromosome-linked inherited disorder that causes XY genotypic males to develop as phenotypic females because of defective AR (13–18). AR gene mutations in CAIS have been found in the N-terminal domain (17, 18), the DNA binding domain (17), or the HBD (13–17). Each of these mutations inactivates AR function, even though some of the mutant AR proteins produced still bind androgen (15–17).

Not all naturally occurring AR gene mutations inactivate AR function, as first documented in a human prostate cancer cell line (21, 22). The LNCaP human prostate cancer cell line is androgen independent (23, 24), but growth *in vitro* can be stimulated by androgen, estrogen, progesterone, or antiandrogen (21, 22). These cells contain AR but no estrogen receptors or progesterone receptors; however, the AR gene contains a point mutation in the HBD that converts a threonine to alanine (21, 22). The position of this mutation was reported by Harris *et al.* (21) to be codon 877 of a 919 codon AR cDNA (7, 14, 21) and by Veldscholte *et al.* (22) to be codon 868 of a 910 codon AR cDNA (12, 22); these are equivalent positions. Remarkably, this mutant AR has altered steroid specificity compared to wild-type AR; mutant AR binds and is transcriptionally activated by androgen, estrogen, progesterone, or antiandrogen (21, 22). Although the possibility has not been ruled out that this AR gene mutation occurred during establishment of the LNCaP cell line *in vitro*, the mutation might well have accounted for growth of the androgen-independent lymph node metastasis from which the LNCaP cells were aspirated after estrogen therapy (23). Clearly, if other tumors contained mutations like that in LNCaP, they would appear AR-rich but would progress on androgen ablative therapies that include estrogen or antiandrogen.

Intrigued by the exciting possibility that human prostate cancer might contain AR gene mutations that alter AR function or inactivate it and thereby affect the cancer cell phenotype, we initiated a study to screen for mutations in the HBD of the AR gene in human prostate cancer tissue specimens.

MATERIALS AND METHODS

Human Prostate Tissues and Cell Lines. Tissue was obtained from 26 men undergoing radical prostatectomy for clinical stage B prostate cancer. Based on hematoxylin/eosin staining of frozen sections of the tumor nodule, the blocks

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Abbreviations: AR, androgen receptor; HBD, hormone binding domain; DGGE, denaturing gradient gel electrophoresis; CAIS, complete androgen insensitivity syndrome.

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were trimmed to remove nonmalignant tissue and enrich the proportion of tumor in the section (25). Normal peripheral prostate tissue or peripheral blood also was obtained from prostatectomy patients. Prostate cancer was obtained also from 2 patients with stage D2 metastatic disease who had failed hormonal therapy and who were undergoing transurethral resection of the prostate to relieve bladder outlet obstruction by their tumor. Four established human prostate cancer cell lines (LNCaP, DU145, TSU, PC-3) and one transplantable xenograft (PC-82) (24) were also studied.

Amplification of Genomic DNA by the Polymerase Chain Reaction (PCR). Genomic DNA was isolated (25). AR exons E–H plus their intron/exon borders were amplified from genomic DNA by PCR using oligonucleotide primers designed to permit detection of splice site and coding sequence mutations (14) and modified to include a 40-nucleotide GC tail at the 5′ end of each 5′ primer (15). These GC tails become amplified during PCR and create a 5′ GC clamp that resists strand separation in denaturing gradient gel electrophoresis (DGGE) and increases the resolution of mutations in DGGE (26). PCR conditions were as described (15). PCR products of the expected size and relative amount were confirmed by electrophoresis in a 3% nondenaturing agarose gel and ethidium bromide staining.

DGGE. Although analysis of PCR fragment size by agarose gel electrophoresis can be sufficient to diagnose the presence of sizeable insertions or deletions, additional analysis is required to detect point mutations or insertions/deletions too small to cause a noticeable change in mobility in agarose. When a DNA fragment enters a concentration of denaturant that causes melting, its mobility is slowed. Because the melting temperature (t_m) of DNA is sequence dependent, mutant fragments have a different t_m and therefore melt at a different concentration of denaturant (26). PCR-amplified fragments were electrophoresed in a 6.5% polyacrylamide gel with a 35–75% gradient of denaturant (100% denaturant is 40% formamide and 7 M urea) in a recirculating Tris/acetate/EDTA buffer bath at 60°C for 1300 V·hr (CBS Scientific, Del Mar, CA) (26). Gels were stained with ethidium bromide.

DNA Sequencing. DNA was amplified using primers without a GC tail (14, 15) and direct sequencing of PCR products was carried out using the dideoxynucleotide chain-termination method (15, 27). The sense and antisense strands were sequenced.

RESULTS

Detection of AR Gene Mutations by DGGE of PCR-Amplified DNA Fragments. Exons E–H of the HBD of the AR gene (Fig. 1 *Upper*) could be amplified from genomic DNA of all specimens, indicating the absence of genomic exon deletions. Amplified DNA fragments were then analyzed by DGGE.

Fig. 2 illustrates our ability to detect by DGGE the presence of the point mutation in exon H of LNCaP cells (21, 22), which migrates faster (i.e., melts at a higher concentration of denaturant) than exon H of wild-type AR. All other specimens appeared to have a wild-type exon H.

The AR gene is on the X chromosome (7, 17), so normal cells in an XY male have only one copy of the AR gene. If all cells have the same AR gene sequence, PCR-amplified DNA fragments migrate as a single band in DGGE. LNCaP cells contain three X chromosomes (23), but exon H migrated as a single band in DGGE (Fig. 2), indicating the presence of only mutant DNA. Thus, the AR mutation in LNCaP must have occurred before the development of aneuploidy.

Stage B Prostate Cancer Contains a Somatic Mutation in Exon E of the AR Gene. When PCR-amplified exon E from human prostate cancer tissue specimens and cell lines was screened by DGGE, we detected a somatic mutation in a

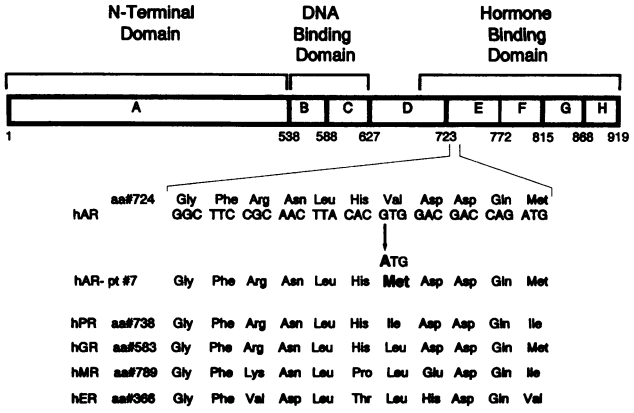

FIG. 1. (*Upper*) Schematic of human AR structure. Amino acid positions of each exon are noted below the bar (14). (*Lower*) Wild-type AR sequence is compared with that in prostate cancer patient no. 7 and in equivalent regions of the human progesterone receptor (hPR), glucocorticoid receptor (hGR), mineralocorticoid receptor (hMR), and estrogen receptor (hER). Wild-type sequences are from ref. 14. The AR gene mutation at codon 730 in patient no. 7 is in a region of the HBD highly conserved among steroid receptors.

patient (no. 7) with organ-confined, untreated stage B prostate cancer (Fig. 3, lane 5). The mutation was not detectable in peripheral blood lymphocyte DNA (Fig. 3, lane 4). Lymphocyte DNA (wild-type AR) migrated in DGGE as a single band because there is only one allele per cell and all cells have the same allele. The tumor DNA migrated in DGGE as four bands, which represent mutant DNA from cells with a mutant AR gene, wild-type DNA from cells in the same specimen that contain wild-type AR, and two heteroduplex forms. Each heteroduplex, which consists of one wild-type strand and one mutant strand, is destabilized by the presence of a mismatch and therefore melts at a lower concentration of denaturant than either the wild-type homoduplex or mutant homoduplex (26). The presence of more than one allele in the same specimen was evidence that the tumor contained a somatic mutation. The presence of a mutation in patient no. 7 exon E was confirmed by independent amplifications of additional aliquots of genomic DNA and DGGE analysis of these PCR products.

Patient no. 7 had clinical stage B prostate cancer (Gleason score 8) and elevated serum prostate specific antigen (PSA) (21.4 ng/ml) but no evidence of metastasis. His postoperative PSA was normal (<0.3 ng/ml), and 3 years later he remains disease free.

AR Gene Mutation in Stage B Prostate Cancer Codes for Amino Acid Change in a Conserved Region of the HBD. Because of redundancy in the genetic code, some DNA mutations are silent, causing no change in amino acid coding. It was important, therefore, to determine whether the somatic mutation we detected in the patient no. 7 prostate

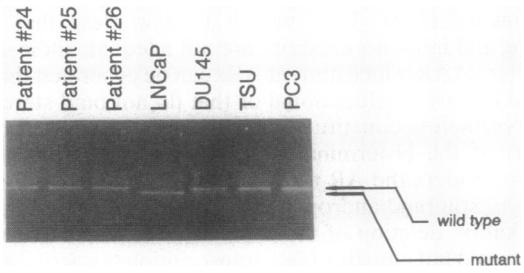

FIG. 2. DGGE of PCR-amplified exon H in three stage B prostate cancers and four cell lines. LNCaP exon H contains a point mutation and has a mobility different from other specimens that migrate at the position of wild-type exon H.

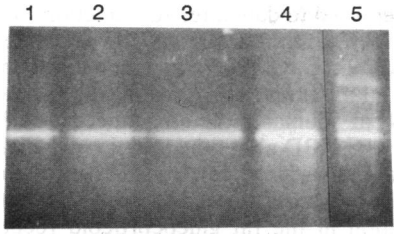

FIG. 3. DGGE of exon E. Lanes 1–3, 3 prostate cancers that contain wild-type exon E; lane 4, exon E of peripheral lymphocytes from patient no. 7 migrates as a single band at the position of wild-type exon E; lane 5, stage B prostate cancer tissue from patient no. 7 contains a somatic AR gene mutation in exon E; note the four bands.

cancer caused a change in the amino acid coding sequence or changed the splice site sequence. Exon E was amplified from another aliquot of genomic DNA, and the PCR product was sequenced directly (15, 27). The DNA sequence of exon E from normal peripheral blood lymphocytes of the same patient (Fig. 4) was identical to that of wild-type AR (14).

In contrast, the sequencing gel of the tumor exon E revealed the presence of two bases, G and A, at the first nucleotide position of codon 730 (using the numbering in ref. 14) (Fig. 4). This is consistent with the presence of two DNA fragments that have a different sequence, again diagnostic of the presence of a somatic mutation. The wild-type AR codon 730 is GTG, which codes for valine; the presence of A at the first position changes the sequence to ATG, which codes for methionine (Fig. 4). Sequencing of the opposite DNA strand confirmed these results. All other nucleotides were identical to wild type, indicating that tumor exon E differed from normal by only a single base substitution. These data attest to the sensitivity of detecting a DNA sequence alteration by DGGE.

Fig. 1 illustrates the position of this mutation in the coding sequence of the AR and shows the surrounding sequence in the human AR and in the equivalent sequences of other members of the steroid receptor family, including the progesterone receptor, glucocorticoid receptor, mineralocorticoid receptor, and estrogen receptor (7). AR codon 730 is located in a region of the HBD that is highly conserved among members of the steroid receptor family, but the amino acid at codon 730 is unique to the AR.

Sequencing of the mutation in patient no. 7 stage B prostate cancer revealed that it disrupted a *Pml* I restriction site. Exon E of wild-type AR contains only one *Pml* I site (14); therefore *Pml* I digestion of wild-type PCR product [327 base pairs (bp)] should yield two bands, 109 bp and 218 bp, but *Pml* I should not digest the mutant 327-bp fragment. *Pml* I digestion of the exon E PCR product from normal peripheral lymphocyte DNA of patient no. 7 indeed yielded two bands as expected (Fig. 5, lane 4). *Pml* I digestion of the prostate cancer exon E yielded three bands: a 327-bp band that resisted digestion

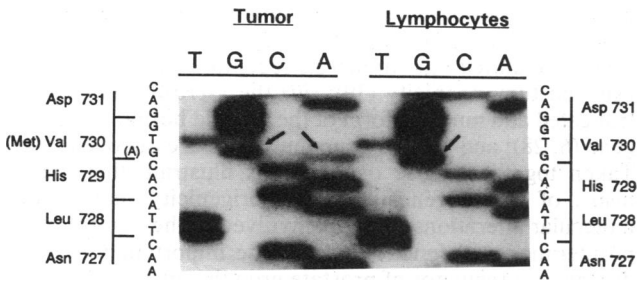

FIG. 4. DNA sequencing gel of exon E in patient no. 7 tumor and normal lymphocytes. The tumor shows a G and A in codon 730 where lymphocytes contain only A at the position noted by the arrow.

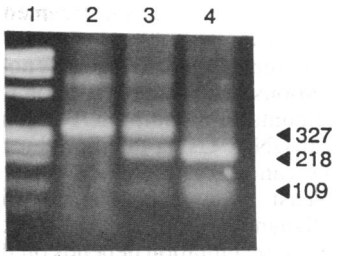

FIG. 5. *Pml* I digestion of exon E PCR product. Lane 1, molecular weight markers; lane 2, no *Pml* I; lane 3, patient no. 7 tumor plus *Pml* I; lane 4, patient no. 7 normal lymphocytes plus *Pml* I. Arrowheads indicate size in bp.

and represents the mutant fragment and two bands of 109 bp and 218 bp that resulted from digestion of wild-type DNA. The presence of three bands confirms the presence of a somatic mutation, and the relative amount of the 327-bp fragment reflects the relative amount of mutant allele in the specimen.

Sensitivity of Detecting Somatic Mutations by DGGE. We wondered whether our inability to detect mutations in exons E–H of the other 25 stage B cancer specimens might be due to the presence of an amount of mutant DNA below the limit of sensitivity of our screening method. We therefore designed an experiment to determine how much mutant DNA must be present to be detected by DGGE. We amplified exon G from wild-type AR and from a mutant AR (exon G mutant described in ref. 15) and mixed them in various proportions. By DGGE, a single band was present in mixtures containing only wild-type DNA or only mutant DNA, and these bands had different mobilities (Fig. 6). Mixtures of wild type and mutant gave rise to multiple bands in DGGE, as expected, and these could be seen when 10% of the sample contained a mutation (Fig. 6). Because of this limit of sensitivity, we cannot rule out the possibility that other prostate cancer specimens we screened may also contain mutations in exons E–H; if present, they are in <10% of the cells in the specimen. If mutations were in fact present, and in cells with a growth advantage, we might detect them only at later stages of tumor progression.

DISCUSSION

Receptor mutations reported to date were discovered in specimens previously characterized biochemically as having defective or abnormal receptors (13–22). In our study, we were able to screen for the presence of mutations in large numbers of prostate cancer specimens having unknown AR properties by analyzing the mobility of PCR-amplified DNA

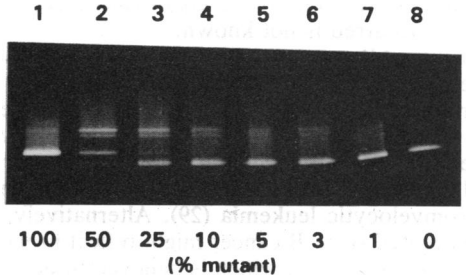

FIG. 6. Sensitivity of detecting a mutation in a mixture of wild type and mutant by DGGE. Numbers below each lane represent the percentage of sample that is mutant. Mutant (lane 1) and wild-type (lane 8) exon G have a different mobility. Mutant homoduplex and heteroduplex forms are readily seen when 10% of the sample is mutant (lane 4).

fragments in DGGE. Only DNA fragments that have a mobility different from that of wild type are sequenced.

Our ability to detect mutations depends not only on the use of optimal conditions of DGGE but also on the proportion of the sample that contains mutant DNA and on the sensitivity of detecting that DNA. Using ethidium bromide staining of DGGE gels, we can detect the presence of a mutation if it represents at least 10% of the sample. Since all tumors contain nonmalignant supporting stroma and blood vessels, detection of a somatic mutation depends on the proportion of tumor and nontumor cells in the specimen and on the proportion of tumor cells with the mutation. Detection of multiple bands by DGGE, though diagnostic of a somatic mutation, does not indicate whether the wild-type allele is present in malignant cells that lack the mutation, in nonmalignant cells, or in both.

With this approach we identified an AR gene mutation in a specimen of stage B prostate cancer, which we diagnosed based on the presence of multiple bands in DGGE. The mutation was not detectable in peripheral blood lymphocyte DNA. Therefore, the tumor contained a somatic mutation. To our knowledge, a somatic AR gene mutation has not been reported previously. AR gene mutations in CAIS, by contrast, are germ-line mutations, present in all cells in the body. If the mutation in the tumor had simply been an inherited mutation or polymorphism, all cells would have had the same allele, and the tumor and peripheral lymphocytes would have yielded a single band in DGGE with the same mobility, but a mobility different from wild type.

Screening the HBD of the AR gene revealed a mutation in only 1 of 26 stage B prostate cancer specimens. Possible reasons for failure to detect mutations in the HBD of the other specimens analyzed are (*i*) the presence of a mutation in <10% of the cells, the limit of sensitivity of the method we used; (*ii*) the presence of a mutation in other parts of the AR (the HBD represents only 21% of the coding sequence of the AR); and/or (*iii*) a low frequency of mutations in early stage disease. If mutations reflect tumor progression, the mutation frequency may be higher in late stage disease.

It was clear from the intensity of the multiple bands detected by DGGE and by *Pml* I digestion that the mutant allele in prostate cancer patient no. 7 was present in a substantial percentage of the specimen (see Figs. 3 and 5). We estimated it to be about 50%. The presence of a substantial wild-type band suggests the presence of a substantial proportion of cells with wild-type AR, at least some of which are likely to be nonmalignant cells. Others may be malignant cells that lack the mutation. We infer that the mutation occurred in cells with a growth advantage. If the original cell with the somatic mutation had not had a growth advantage, it would have remained an insignificant percentage of the tumor, becoming diluted by other cells with a growth advantage, and it would not have been detectable by DGGE. Whether the AR gene mutation itself conferred a growth advantage on the cell in which it occurred is not known.

Finding an AR gene mutation in a surgical specimen of untreated, organ-confined stage B disease raises the question whether mutated AR might have oncogenic potential, as appears to be the case for the v-*erbA* oncogene, which is a truncated mutant of the thyroid hormone receptor gene (9, 28), and the translocated chimeric retinoic acid receptor α in acute promyelocytic leukemia (29). Alternatively, mutated AR in untreated stage B cancer might reflect progression to androgen independence, which is believed to occur prior to androgen ablation (30).

The 100% conservation of amino acid sequence of the HBD and DNA binding domain of the AR in evolutionarily distant species (human, mouse, rat) (18) suggests that conservation of wild-type function is dependent on conservation of wild-type sequence. Indeed, all single amino acid mutations in the AR HBD reported to date alter AR function, but in different ways. In CAIS, all AR mutants are unable to activate transcription at physiological androgen concentrations, though not all mutations abrogate androgen binding (15–18). In the LNCaP human prostate cancer cell line, a point mutation in the HBD alters the steroid specificity of the AR (i.e., increases its affinity for nonandrogenic steroids) but it is still an active transcription factor (21, 22). Interestingly, a point mutation in the rat glucocorticoid receptor HBD at cysteine 656 produces a "super" receptor that has a 9-fold higher affinity and specificity for glucocorticoid and that is more potent and efficacious as a transcription activator *in vivo* than wild-type receptor (31). Creation of a super AR, by analogy, might allow continued prostate cancer growth in an androgen-poor environment.

It is likely, therefore, that the amino acid change at AR codon 730 also alters ligand binding and/or function, but how it is affected remains to be determined. In addition to its role in ligand binding, the HBD also contains a nuclear localization signal (11, 12), a dimerization domain (11, 12, 28, 29), hsp90 interaction domain(s) (7, 32), and transcription activation domains (9, 10–12, 15–17). Therefore, mutations in the HBD may affect more than just ligand binding. HBD mutants that bind ligand can have altered transcriptional activity (15, 16), and mutant receptors unable to bind ligand may still be able to affect transcription, either by ligand-independent activation (33) or by interaction with other transcription factors (28, 29, 34).

The mutation at AR codon 730 is in a conserved region of the HBD involved in binding to ligand and to hsp90 (7, 32). It has been suggested that unliganded steroid receptors (full-length wild type) are transcriptionally inactive because hsp90 binding to the HBD prevents receptor binding to target genes and that HBD deletion mutants are constitutively active because hsp90 cannot bind. Deletion of this conserved region in the glucocorticoid receptor abrogates binding to hsp90 and creates a constitutively active receptor (32), but the effect of point mutations in this region has not been investigated.

In addition to finding AR gene mutations in human prostate cancer tissue (this study), an established prostate cancer cell line (21, 22), CAIS (13–18), hypospadias (20), and X chromosome-linked spinal and bulbar muscular atrophy (19), naturally occurring mutations also have been found recently in other members of the steroid receptor superfamily: estrogen receptors in human breast cancer (34), vitamin D receptors in hereditary rickets (35), retinoid receptors in acute promyelocytic leukemia (29), glucocorticoid receptors in glucocorticoid-resistant cells (36), and thyroid hormone receptors in thyroid hormone resistance syndrome (37). Notably, an estrogen receptor mRNA variant has been found in breast cancer that lacks part of the HBD (34); remarkably, it yields a mutant receptor that is constitutively active in the absence of estrogen (34), providing a potential mechanism for estrogen-independent breast cancer growth. Mutations in other important signal transducing molecules confer oncogenic potential—for example, (*i*) mutated plasma membrane-associated GTP-binding proteins $G_{s\alpha}$ and $G_{i\alpha}$ are oncogenes (38); (*ii*) mutated α_{1B}-adrenergic receptors enhance mitogenesis and tumorigenicity (39); (*iii*) the v-*erbA* oncogene is a truncated and mutated form of the thyroid hormone receptor gene (6, 28); and (*iv*) *ras* gene mutations are oncogenic (40). Taken together, these observations illustrate that mechanisms of carcinogenesis and tumorigenicity may involve molecular alterations of genes involved in the control of cell proliferation and function. Given the important role of androgen as a regulator of prostate growth and function (1, 3), the requirement of androgen for prostate carcinogenesis (2), and the persistent expression of AR in androgen-independent prostate cancer (6, 21, 22), which raises the possibility of

continued AR activity even in the absence of androgen, it is tempting to hypothesize that mutations in the AR gene might be involved in the development or progression of prostate cancer. Our discovery of a somatic AR gene mutation in early stage prostate cancer, even prior to androgen ablation, warrants further investigation of this hypothesis.

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