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Androgen Receptor Mutations in Prostate Cancer[1]

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ABSTRACT

We analyzed the frequency and relevance of mutations in the coding region of the androgen receptor (AR) in genomic DNA extracted from 137 specimens of prostate cancer. The specimens were obtained from the primary tumors of patients affected by stage B disease [15 nonmicrodissected (group 1A) and 84 microdissected (group 1B)] and from the metastatic deposits of individuals with stage D1 disease [8 nonmicrodissected (group 2A) and 30 microdissected (group 2B)] who had not undergone androgen ablation therapy. The study was conducted by PCR-single strand conformational polymorphism (SSCP) analysis of exons 2–8 in the four groups and direct sequence analysis of exon 1 in group 1B. As positive and negative controls, we used genomic DNA extracted from genital skin fibroblasts of patients affected by various forms of androgen resistance with known mutations in the AR. To control for genetic instability, PCR-SSCP analysis of exon 2 of the human progesterone receptor was carried out on each specimen.

The overall number of mutations detected was 11 (8%). No mutations were detected in any of the 99 patients with stage B disease. Eleven mutations were detected in exons 2–8 in 8 of the 38 patients with stage D1 disease (all in group 2B). Simultaneous analysis of exon 2 of the progesterone receptor was carried out, and no SSCP changes were identified.

These data suggest that AR mutations are rare and presumably do not play a role in the initial phase of prostatic carcinogenesis. The presence of a significant number of AR mutations in metastatic disease indicates that mutations of this molecule may play a role in the most advanced phases of the natural history of this disease, either by facilitating growth or acquisition of the metastatic phenotype.

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy in men and the second leading cause of cancer death in men in the United States (1). The development, growth, and differentiation of the normal prostate is regulated by androgens, which exert their effects by interacting with the AR,[3] a member of a superfamily of ligand-activated nuclear transcription factors (2). Abnormal androgen biosynthesis or inactivating mutations of AR are associated with absent or rudimentary development of the prostate in 46,XY individuals (3). There is anecdotal evidence that prostate cancer does not develop in men castrated before puberty, and that androgen is required for the experimental induction of prostate cancer in animals. Therefore, a normal AR signaling pathway is a necessary requirement, not only for the development of a normal prostate but presumably also for the development of prostate cancer.

Radical prostatectomy is the main curative treatment for men with organ-confined disease. Most men with non-organ-confined disease will undergo palliation with radiation or androgen ablation. Androgen ablation successfully shrinks primary and metastatic lesions by inducing apoptosis of androgen-dependent prostate cancer cells (4) in up to 80% of the cases (5). Unfortunately, prostate cancer is a heterogeneous lesion and at the time of diagnosis may contain foci of both androgen-dependent and -independent cells (6). Androgen-independent cells escape apoptosis induced by androgen ablation (7) and by many cytotoxic drugs. They continue to proliferate and metastasize, despite profound changes in the surrounding hormonal milieu, and represent the most direct threat to patient survival. There is evidence that the AR is expressed in all stages of prostate cancer evolution, including prostatic intraepithelial neoplasia (8), primary (9, 10) and metastatic (11, 12) disease, both before and after androgen ablation therapy. Few prostate cancers are AR negative (13), and thus, even the androgen-independent tumors express the AR protein.

In the wake of the earlier identification of "outlaw estrogen receptors" in breast cancer (14), the possibility that point mutations in the AR may account for progression from androgen-dependent to androgen-independent growth has been a popular theory. Numerous investigators have used PCR-SSCP of DNA extracted from foci of prostate cancer to search for AR variants, both in clinically detectable disease and in latent prostate cancer.

A review of published data (Refs. 15–36; reviewed in Ref. 37) shows that there is still considerable controversy in the field. A total of 581 cases of clinically detectable prostate cancer have been analyzed at the molecular level for the presence of AR mutations. To date, a total of 47 mutations (frequency, 8%) causing an amino acid change or addition have been detected. Interestingly, 22 of these mutations (46% of total) were reported by three groups from a total of 59 patients (frequency of mutations in these 59 patients, 37%; Refs. 21, 26, and 34). The remaining 25 mutations were detected in 522 patients (frequency, 4.7%). Methodological variables and patient sampling may be responsible for these discrepancies in the literature. For instance, Tilley *et al.* (34) demonstrated that variables correlating with increased prevalence of AR mutations are the use of tumor-enriched DNA after microdissection of the sample and analysis of exon 1. Another potential methodological variable is the quality of DNA extracted from paraffin-embedded tissue, which according to some reports is not optimal and may account for an increased frequency of PCR infidelity (38).

In view of the controversies still existing in the field, we have studied the molecular structure of the AR in a large series of specimens derived from stage B prostate cancers and from metastatic deposits to pelvic lymph nodes (stage D1). We have determined the importance of using microdissected *versus* nonmicrodissected specimens, of extracting DNA from fresh *versus* archival tissue, and of analyzing exon 1 to the overall incidence of detection of receptor mutations. We report a significant increase in AR mutations in metastatic (stage D1) compared with localized (stage B) prostate cancers.

MATERIALS AND METHODS

Tissue. Specimens used in these studies were obtained from the tissue bank of one of the authors (M. I.) and from the tissue bank of the Baylor Specialized Programs of Research Excellence for prostate cancer. The protocol was approved by the Institutional Review Board of Baylor College of Medicine. We

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[3] The abbreviations used are: AR, androgen receptor; SSCP, single-stranded conformational polymorphism; PR, progesterone receptor.

analyzed DNA from 99 tumors of patients with stage B disease who underwent total prostatectomy between 1986 and 1998. In 15 of these patients, genomic DNA was extracted from frozen nonmicrodissected cancer specimens of ~10 mg (group 1A), which were isolated according to the procedure of Wheeler and Lebovitz (39) and contain at least 25% of cancer. The remaining 85 samples (group 1B) were snap frozen and extracted after ascertaining the presence of at least 50% neoplastic tissue by frozen section analysis, as described by Ittmann *et al.* (40). High molecular weight genomic DNA was extracted from the powdered samples of group 1A using 10 volumes of extraction buffer [10 mM Tris-Cl (pH 8.0), 0.1 M EDTA (pH 8.0), 10 μg/ml RNase, and 0.5% SDS] for 1 h at 37°C. Proteinase K at a final concentration of 100 μg/ml was then added and incubated at 50°C for 3 h. After three phenol extractions, the aqueous phase was dialyzed in 50 mM Tris (pH 8.0) at 4°C for 24 h. One μg of this highly purified material was used in each PCR reaction.

We also studied 38 paraffin-embedded pelvic lymph nodes resected from patients with stage D prostate cancer (group 2). Of these, 8 were not microdissected and contained 20–25% cancer (group 2A). Thirty were microdissected and contained 90–95% cancer (group 2B). To enrich for tumor-derived DNA, specimens of group 2B were cut in three sections of 4, 25, and 4 μm. After H&E staining, a pathologist identified the region containing 100% cancer in the two 4-μm sections. The 25-μm region located between the two adjacent sections containing 100% cancer was microdissected and then deparaffinized in xylene and ethanol and precipitated by microcentrifugation at 4°C. Genomic DNA was extracted through digestion in a 100-μl volume [10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, and 0.1 mg of proteinase K] at 65°C for 2 h, followed by denaturation of proteinase K at 95°C for 10 min. Depending on the final yield, 4–10 μl of genomic DNA were used in each PCR reaction.

PCR-SSCP. PCR amplification of exons 2–8 was performed using a MJ Research Thermal Cycler and the primers reported previously (41), with the exception of exon 4, which was amplified using two sets of overlapping primers to generate products within the acceptable range that maintains SSCP sensitivity. The primers used for exon 4 were the following: IV-S, TGATAAATTCAAGTCTCTCTTCCTT; IV-AS, ACACACTACACCTGGCTCAATGGCTT; IVB-S, CAGTGTCACACATTGAAGGCTATGAA; and IVB-AS, CACTAAATATGATCCCCCTTATCTC.

Eight sets of overlapping sense (S) and antisense (AS) primers were used to amplify exon 1. The coordinates of these oligonucleotides are derived from the sequence of Tilley *et al.* (2). Primer E1-VIIIAS is derived from the region of intron 1 adjacent to the 3′ of exon 1 (41). The eight sets of primers are: E1-IS, ^{139}TGGAAGATTCAGCCAAGCTCAAG^{161}; E1-IAS, ^{417}CTGCTGCTGCCTGGGGCTAGTCTC^{394}; E1-IIS, ^{280}AGGCACCCAGAGGCCGCGAGCGCAG^{304}; E1-IIAS, ^{579}GCTGGCGGCCACGGCGGCTCCAGG^{556}; E1-IIIS, ^{514}TCGGCCCTGGAGTGCCACCCCGAGA^{538}; E1-IIIAS, ^{912}CGCCTCCACACCCAGGCCCATGGA^{889}; E1-IVS, ^{612}GGACGAGGATGACTCAGCTGCCCCA^{636}; E1-IVAS, ^{1113}TAGCCCTTTGGTGTAACCTCCCTTG^{1089}; E1-VS, ^{803}CTCCCACTTCCTCCAAGGACAA^{824}; E1-VAS, ^{1264}AGCCAGTGGAAAGTTGTAGTAGTC^{1240}; E1-VIS, ^{1207}GCACTGGACGAGGCAGCTGCGTAC^{1230}; E1-VIAS, ^{1506}CCACACGGTCCATACAACTGGCC^{1483}; E1-VIIS, ^{1401}CGCGGGTGCAGCGGGACCCGGTTCT^{1425}; E1-VIIAAS, ^{1660}ACACATCAGGTGCGGTGAAGTCGCT^{1636}; E1-VIIIS, ^{1578}GGGAGCTGTAGCCCCCTACGGCTAC^{1602}; and E1-VIIIAS, CGAAAGCGACATTTCTGGAAGGAAA. Exon 2 of the human PR was also amplified and used as a control using oligonucleotides: PR-S, GCATTTCAAGGCCGGATTCAGAAGC; and PR-AS, AGGAGCCTACCTTCCATTGCCC (42).

Because the process of confirming the identification of point mutations used very stringent criteria and numerous PCR reactions (see below), there was insufficient genomic DNA available to analyze exon 1 of the *AR* gene in groups 1A, 2A, and 2B; however, we were able to analyze all eight exons in group 1B.

The PCR reaction involved denaturation at 100°C for 30 s, annealing and extension at 68°C for 90 s for 35 cycles using 2.5 units of Taqara (Pan Vera Corporation, Madison WI), and 1 μl of [^{32}P]dCTP per reaction. The size and integrity of the PCR product and the absence of contamination in the negative control sample (in which no DNA was added) were confirmed on 2% agarose gels. SSCP analysis was performed according to the published procedures (43–45). Briefly, 5 μl of PCR sample plus 20 μl of formamide loading dye (0.05% bromphenol blue, 0.05% xylene cyanol, 50 mM EDTA, and 20 mM NaOH in deionized formamide) were boiled for 10 min, snap-frozen in dry ice, and thawed. Six μl of the samples were electrophoresed on a 6% nondenaturing polyacrylamide gel at 400 V for 14–24 h, depending on the fragment generated. A nondenatured wild-type AR control was run in parallel with the other denatured samples to determine the mobility of the double-stranded DNA. As an internal control, each gel was run with a positive and a negative control (*i.e.*, a PCR product generated from the genomic DNA of patients with testicular feminization that were known to have a wild-type or mutated sequence in exons 2–8). This genomic DNA was used to optimize the SSCP conditions. For instance, in most cases a run of 12–14 h was sufficient to detect the abnormal SSCP migration of the positive control; however, in the case of exons 3 and 7, a run of 22–24 h was necessary. This genomic DNA was the generous gift of Dr. M. J. McPhaul (University of Texas Southwestern Medical Center, Dallas, TX). Each of the positive mutant AR controls used in these studies has been characterized and published (41, 46–53). After electrophoresis, gels were dried and exposed to X-ray films for autoradiography at −80°C.

DNA Sequencing. Stringent criteria were adopted to eliminate PCR artifacts. The presence of a variant SSCP shift was confirmed in three independent PCR reactions. These PCR products were divided into three aliquots, of which one was run in the SSCP gel and one in an agarose gel to verify the presence of the correct amplified product and the absence of background in the negative control. The third aliquot was TA-subcloned into the pCR2.1-TOPO plasmid (Invitrogen, Carlsbad, CA) and dideoxy-sequenced (54) in a denaturing polyacrylamide gel, using the Sequenase 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH). Each mutation was confirmed a minimum of three times from multiple clones (up to 20) obtained from at least three independent PCR amplifications. Sequencing was performed in both the sense and antisense orientations.

Exon 1 of group 1B was analyzed by direct sequencing of the PCR product using an automated sequencer (Perkin-Elmer Sequencer 310). Sequence analysis was performed multiple times (up to 10) from the product of at least two PCR amplifications. Each reaction mix consisted of 30–90 ng of the purified PCR product, 3.2 pmol of primer, and 4 μl of Big Dye Terminator dye mix (Perkin-Elmer Applied Biosystems, Foster City CA). PCR sequencing was performed at 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min for a total of 25 cycles. The resulting DNA was then precipitated in ethanol, dissolved in 12 μl of formamide, and analyzed on the automatic sequencer. Computer analysis, and careful reading of the sequencer print out were used to distinguish mutations. All nucleotides of exon 1 were sequenced except the polyQ and polyG repeats. AR amino acid numbering reported in this study is based on an assumed length of 919 amino acids to be consistent with the AR mutation web site.[4] To determine the sensitivity of our automated sequencing technique, we performed serial dilutions of mutant and wild-type genomic DNA [ranging from 1:1 to 1:1/10 (wild type:mutant)]. Each dilution was amplified and sequenced, and in each instance a mutation of the correct nucleotide was detected (not shown). These data indicate that direct sequencing identifies point mutations if they are present in 10% of the genomic DNA. Because previous pilot experiments in our laboratory have demonstrated that a mutation can correctly be identified by SSCP if it is present in 10–15% of the genomic DNA, we conclude that direct sequencing is at least as sensitive as SSCP in detecting point mutations.

RESULTS

Nonmicrodissected Stage B Prostate Cancers (Group 1A). SSCP analysis of 15 nonmicrodissected specimens isolated from patients with stage B disease showed the absence of AR variants in each of the seven exons (2–8) analyzed. Representative pictures showing SSCP analysis of exons 3 and 7 are shown in Fig. 1. In view of the absence of reproducible SSCP shifts, sequencing analysis was not performed with these samples, and it was assumed that no mutation was present. Reproducible SSCP shifts were obtained in genomic DNA extracted from patients with androgen resistance and proven mutations of AR (Fig. 1), thus demonstrating that the SSCP conditions were optimized to detect mutations. No SSCP shifts were detected in

[4] Internet address: http://www.mcgill.ca/androgendb/data.htm.

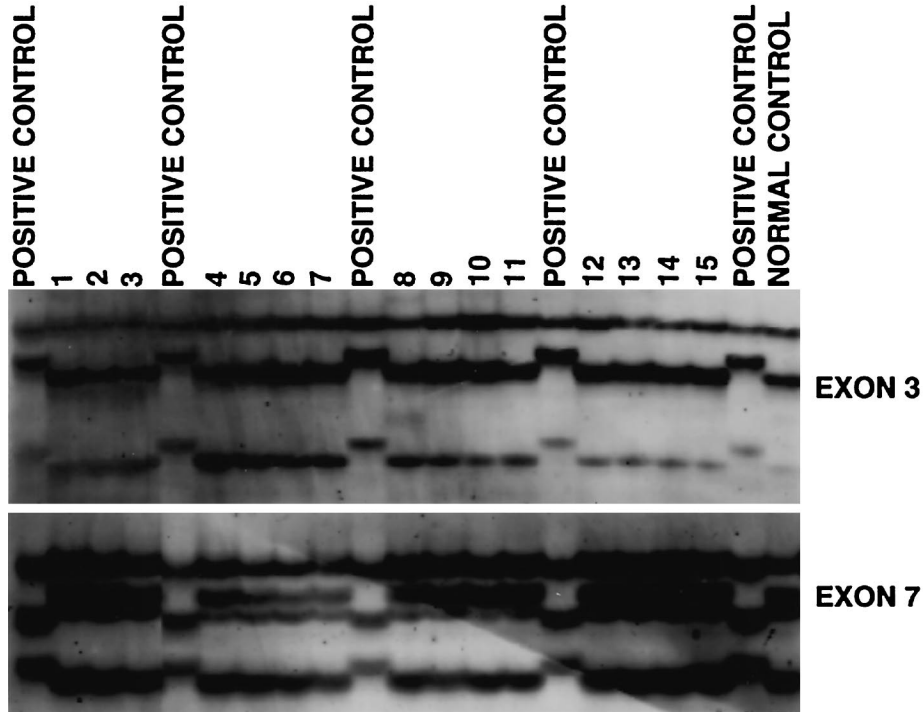

Fig. 1. SSCP analysis of exons 3 and 7 of the androgen receptor in 14 nonmicrodissected stage B prostate cancers (group 1A). Samples were run in a nondenaturing polyacrylamide gel, as explained in "Materials and Methods." Known mutants for exons 3 (48) and 7 (53) were loaded as a positive control in each reaction.

samples containing exon 2 of the human PR that were run in parallel (not shown).

Microdissected Stage B Prostate Cancer (Group 1B). Group 1B was microdissected to contain a minimum of 50% neoplastic tissue by frozen section analysis. Analysis of exons 2–8 in the 84 patients belonging to this group showed 14 SSCP variants. Sequence analysis (in each case, a minimum of 10 templates) did not show any mutation in 13 of these variants. In one case, we detected a 1-bp change corresponding to codon 580 (AAG-AAA) that did not change the amino acid residue. This was a germ-line polymorphism that was also detected in the surrounding normal tissue and confirmed the ability of our technique to detect single bp changes in the AR sequence. Direct sequencing of exon 1 did not identify any mutation of the coding sequence. No sequence abnormalities were detected in exon 2 of the PR that was run in parallel. The positive controls from exons 2–8 yielded SSCP shifts in the exon, where they had been identified previously, demonstrating that we were using optimized conditions for AR mutation analysis.

Metastatic Lesions (Groups 2A and 2B). SSCP analysis of specimens from groups 2A and 2B showed 19 reproducible shifts (in at least two separate PCR reactions) in the seven exons that were analyzed (four shifts in group 2A and 15 in group 2B). Sequence analysis revealed 11 mutations (all in group 2B), 10 missense and 1 nonsense mutations (Table 1). Each mutation was detected at least once in each of the three PCR amplifications performed for each exon during the SSCP screening (Table 1). Fig. 2 shows the SSCP pattern of the mutations detected in patients 3 and 20. Each of these mutations was a somatic event, because wild-type sequences were also detected. Four of these mutations were detected in patient 1 and involved exons 5 (A-748-T), 7 (S-865-P and Q-867-stop), and 8 (Q-919-R). Because each exon was amplified individually from a pool of DNA derived from many cells, it is impossible to know if these mutations were present in the same AR molecule or even the same cell. We consistently found two mutations in exon 7 of this patient. Because they were never detected in the same template, they most likely represent separate events that affected two individual AR molecules. The remaining seven mutations were detected in seven different patients and affected exons 2 (in four cases), 3, 5, and 7.

The remaining six SSCP shifts of group 2B and the four SSCP shifts of group 2A did not yield a reproducible base change when subcloned and sequenced. Despite extensive sequence analysis of these samples (in each case, at least 20 templates from three different PCR amplifications), we were unable to identify a base change that recurred more than once. In each instance, the majority were wild-type sequences, and a minority (in 25–35% of the templates) were unreproducible base changes (*i.e.*, base changes that were never found more than once in the 20 different templates that were sequenced). Because we were unable to identify these base changes more than once, they have not been taken into account. Because the phenomenon of SSCP shifts followed by identification of unreproducible base change was observed only in DNA obtained from paraffin-embedded tissue, it is possible that fixation may affect the quality of this DNA. This in turn may affect the fidelity of the PCR reaction, as described previously by Shiao *et al.* (38). Exon 2 of the PR did not show any SSCP abnormality.

Table 1 *Summary of the mutations identified in group 2B*

Tumor	Exon	Mutation	Amino acid change	No. of PCR-SSCP amplifications	No. of mutated templates[a]	No. of templates sequenced
1	5	GCC-ACC	A-748-T	3	8	12
1	7	TCC-CCC	S-865-P	3	8	11
1	7	CAG-TAG	Q-867-stop	3	4	16
1	8	CAG-CGG	Q-919-R	3	3	20
3	2	GCC-GTC	A-586-V	3	3	20
5	2	AAG-AGG	K-580-R	3	3	20
8	7	AGA-GGA	R-846-G	3	8	13
11	2	ACA-GCA	T-575-A	3	5	15
12	2	GCT-TCT	A-587-S	3	3	20
18	3	TGT-TAT	C-619-Y	3	6	18
20	5	GTC-GCC	V-757-A	3	5	17

[a] In every case, wild-type sequences were also identified in other templates, indicating that each mutation was a somatic event. Each of these mutations was identified in at least three separate PCR amplifications.

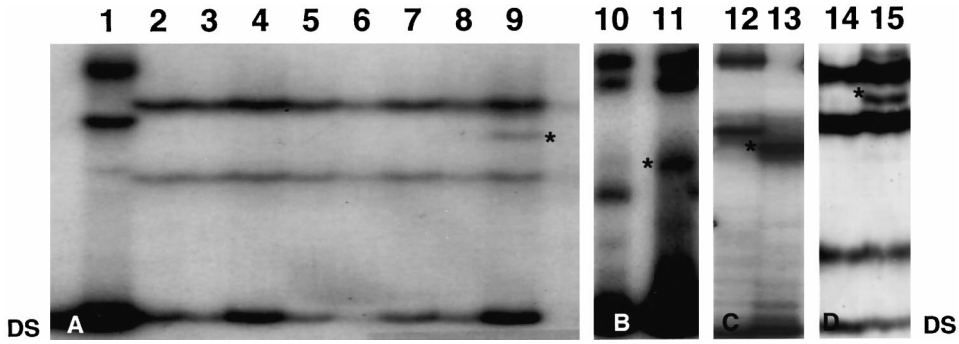

Fig. 2. SSCP analysis of exons 5 (*A*), 2 (*B*), 3 (*C*), and 8 (*D*) in patients with stage D prostate cancer. *A, Lane 9* ∗, SSCP pattern detected in exon 5 of patient 20. The sample run in *Lane 1* was an exon 5 mutant (W739R) described previously in a patient with androgen resistance (49). The sample run in *Lane 2* was a control with known wild-type exon 5. The samples run in *Lanes 3–8* were from six patients with prostate cancer and had an SSCP migration pattern undistinguishable from the wild-type. *B, Lane 11* ∗, abnormal SSCP pattern detected in exon 2 of patient 3, compared with the migration pattern of a wild-type exon 2 in *Lane 10*. *C, Lane 13* ∗, abnormal SSCP pattern detected in patient 18, compared with the migration pattern of a wild-type exon 3 in *Lane 12*. *D, Lane 15* ∗, abnormal SSCP pattern detected in patient 1 (exon 8), compared with the migration pattern of a wild-type exon 8 in *Lane 14*.

DISCUSSION

Summary of the Data

Groups 1A and 1B. Two types of samples from patients affected by stage B disease were studied. The analysis of group 1A (nonmicrodissected) was significant for the complete absence of AR mutations. Because positive controls with known mutant DNA from patients with androgen resistance syndromes were analyzed in each reaction and SSCP variants were identified in each of these specimens, we conclude that the absence of AR mutations in these samples was unlikely attributable to imperfect SSCP conditions. However, because we were concerned that this negative result was because of a failure to microdissect the tissue or to analyze exon 1, we obtained 84 prostate cancers specimens (group 1B) that had been microdissected to contain a minimum of 50% neoplastic tissue. Previous analysis of this material demonstrated loss of heterozygosity on 13q in ~30% of the samples (55). This result is in agreement with several studies by other groups and indicates that this DNA was indeed enriched with tumor DNA. Because a large amount of genomic DNA was available for group 1B, we were able expand our analysis to exon 1. Sequence analysis demonstrated conclusively that no amino acid changes were present in this group of patients, although a somatic polymorphism was detected.

On the basis of the studies performed with groups 1A and 1B, which represent almost 50% of all published stage B cases that have been analyzed for AR mutations, we conclude that AR mutations are quite rare in patients with clinically localized disease and usually do not play a role in the initial phases of prostatic carcinogenesis. These conclusions are largely based on the use of SSCP or direct sequencing as screening techniques to detect mutations, and both these procedures are sensitive if a mutation is present in at least 10–15% of the genomic DNA. Thus, it could be argued that techniques more sensitive could alter the apparent frequency of mutations detected. In agreement with our findings, previous studies of other authors have detected AR mutations only in 2.1% of patients with stage B disease (15–18, 22–24, 29, 32, 33, 35). Thus, we believe that AR mutations are exceptionally rare in this early phase of the natural history of prostate cancer.

Metastatic Disease. In view of the controversies existing in the field, we investigated patients with metastatic prostate cancer. We microdissected metastatic tissue obtained from patients who were unexpectedly found to have non-organ-confined disease on the frozen sections of the regional lymph nodes analyzed at the time of radical prostatectomy. Analysis of these cancers, all obtained before implementation of androgen ablation, demonstrated that AR mutations occur in metastatic prostate cancer in contrast to stage B disease. A total of 11 mutations was detected in this group of 38 patients. Because four of these 11 mutations were in the same patient, AR variants were present in 8 patients of 38 screened (*i.e.*, 21%). We were unable to analyze exon 1 in this group of patients, and a previous report (34) has identified a significant number of AR mutations in exon 1. Thus, the exact prevalence of AR mutations in stage D1 prostate cancer may be even higher than that established by our study.

Because no SSCP abnormalities were found in exon 2 of the PR, we conclude that the relatively high prevalence of mutations in stage D1 disease is not attributable to generalized genetic instability, and that specific mechanisms selecting these mutations may be involved. Because the patients described in this report were analyzed prior to initiation of hormonal therapy, our findings also support the hypothesis that cells with mutant AR can preexist androgen-ablative treatments. The presence of AR mutations in metastatic deposits obtained from patients with stage D1 disease confirms previous reports by Taplin *et al.* (26, 27) in stage D2 and suggests that AR mutations may play an active role in promoting the metastatic phenotype.

Characteristics of the Various Mutations Detected

A detailed analysis of the functional phenotype created by each mutation is beyond the scope of this report and will be reported in separate manuscripts. The mutations found in metastatic specimens, S-865-P, Q867-stop, Q-919-R, A-586-V, K-580-R, R-846-G, T-575-A, A-587-S, C-619-Y, and V-757-A, have never been described before. Additionally, these amino acid residues have never been affected by a mutation, according to the AR database web site.[4] Although mutation A-748-T has never been described before, two reports have identified mutations of codon 748. In one case (49), mutation A-748-D was detected in a patient with a Reifenstein phenotype, and the functional analysis showed an increased dissociation rate of [^{3}H]mibolerone from receptors containing that mutation. In the second case, A-748-V was detected in the prostate of a Japanese patient affected by latent prostate cancer (25). No functional analysis of this mutation was done. However, because residue 748 is in a highly conserved region of the hormone-binding domain, mutations of this codon were predicted to inactivate the AR protein.

Mutation Q-867-stop has never been described before. However, previous investigators have studied the transcriptional activity of AR molecules containing premature stop codons in regions surrounding Q-867. Because these mutants [W-796-stop (46) and S-853-stop (53)]

were both transcriptionally silent, we predict by analogy that also Q-867 is an AR mutation causing loss of transcriptional activity.

Role Played by the Different Methodological Variables

Differences in the methodologies of the various investigators may account for the discrepant results in the different series. Failure to use tumor-enriched DNA may account for the negative results that some authors have published in the past. In view of this, we expanded our analysis of patients with stage B disease to an additional 84 samples, after failing to detect any mutation in the initial nonmicrodissected 15 specimens. Despite this precaution, we were unable to detect any mutations in the stage B patients analyzed, regardless of enrichment with genomic DNA extracted from tumor. However this may simply reflect a feature of stage B prostate cancers where few AR mutations are present; tumor enrichment may very well be important to facilitate detection of mutations in more advanced cancers. This argument is confirmed by the identification of AR mutations in group 2B (microdissected) and not 2A (nonmicrodissected). It is likely that more sensitive techniques, such as laser microdissection, will improve our ability to detect AR mutations in specimens where nonmalignant tissue has carefully been removed.

An additional variable may be the analysis of exon 1. Tilley *et al.* (34) have identified the majority of their mutations in this segment of AR. Many investigators have neglected analysis of exon 1 for different reasons. It is difficult to obtain sufficient DNA to perform 16 PCR amplifications. In addition, many authors have been influenced by the molecular analysis of AR in patients with androgen resistance syndromes, where the majority of the mutations were identified in exons 2–8. We were unable to study the incidence of mutations in exon 1 of groups 1A, 2A, and 2B; however, no mutations were detected in exon 1 of group 1B. Again, this may simply reflect the low prevalence of AR mutations in prostate cancer at this early stage.

A final methodological variable that may have overinflated the number of mutations in other series is the use of DNA derived from paraffin-embedded tissue. As noted above, a large number of base changes were detected only in archival tissue, suggesting that the process of paraffin embedment has detrimental effects on the quality of genomic DNA. However, one could argue that the absence of SSCP variants in exon 2 of PR in paraffin-embedded tissue should rule out artifact as the cause of these irreproducible base changes. This issue has been investigated in a concomitant investigation,[5] where we have performed parallel sequence analysis of DNA coming from paraffin-embedded and frozen tissue without SSCP bandshifts. This analysis demonstrated that irreproducible base changes are uniquely present in DNA derived from paraffin-embedded tissue. Thus, our experience suggests that extreme care should be placed in interpreting data derived from genomic DNA extracted from paraffin-embedded tissue. Nonetheless, our stringent criterion for identification of mutations (*i.e.*, a high percentage of a single mutation by sequencing multiple independent PCR reactions) rules out the possibility that the difference we observed between stages B and D1 disease is a function of differences in the tissue processing.

Conclusions

An investigation into the role played by AR mutations on the natural history of prostate cancer was particularly needed in view of the controversial data reported in the literature. The data presented suggest that this molecule does not play a significant role in the initial phases of prostate cancer, other than signaling its normal functions. Interestingly, a relatively high number of mutations were detected in metastatic tissue, arguing for a contribution by variant AR molecules to the development of metastatic disease and/or androgen-independent growth. Although a careful functional analysis of these mutations will clarify the basic biology of their contribution to disease progression, ongoing studies are evaluating other aspects of AR mutations in prostate cancer, such as their prevalence in patients that have failed hormonal treatments, and in specimens obtained from the same patient before and after androgen-ablative treatments.

[5] D. J. Lamb, N. L. Weigel, and M. Marcelli, manuscript in preparation.

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