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Original Article

Identification of a novel germline missense mutation of the androgen receptor in African American men with familial prostate cancer

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Abstract

Race, family history and age are the unequivocally accepted risk factors for prostate cancer (PCa). Androgen receptor (AR)-dependent signaling is an important element in prostate carcinogenesis and its progression to metastatic disease. We examined the possibility of genomic changes in the *AR* in association with familial PCa in African Americans who have a higher incidence and mortality rate and a clinically more aggressive disease presentation than Caucasians. Genomic DNAs of 60 patients from 30 high-risk African American and Caucasian families participating in the Louisiana State University Health Sciences Center genetic linkage study of PCa were studied. Exon-specific polymerase-chain reaction, bi-directional automated sequencing and restriction enzyme genotyping were used to analyze for mutations in the coding region of the *AR* gene. We identified a germline *AR* (A1675T) (T559S) substitution mutation in the DNA-binding domain in three PCa-affected members of an African-American family with a history of early-onset disease. The present study describes the first *AR* germline mutation in an African-American family with a history of familial PCa. The *AR* (T559S) mutation may contribute to the disease by altering AR DNA-binding affinity and/or its response to androgens, non-androgenic steroids or anti-androgens. Additional studies will be required to define the frequency and contribution of the *AR* (A1675T) allele to early-onset and/or familial PCa in African Americans.

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1 Introduction

Prostate cancer (PCa) aggregation in families has led many investigators to believe that it has a strong hereditary component. Linkage analyses in families with hereditary PCa have identified several possible

susceptibility or genetic predisposition loci suspected to harbor gene mutations conferring an increased PCa risk. These include highly penetrant susceptibility genes such as *HPC1*, *HPC2* and *CAPB* [1–4]. In addition, mutations in the *BRCA1* and *BRCA2* genes and polymorphic variants of candidate genes such as the 5α-reductase and vitamin D receptor have been suggested to influence the risk for PCa [5–9].

The androgen receptor gene (*AR*) has also been suggested as a PCa susceptibility gene. Alterations in the *AR* gene can have profound effects on AR expression, the activity of its target genes, and its responsiveness to androgen, non-androgenic steroids and anti-androgens during the natural history of PCa. Somatic *AR* mutations are rarely found in untreated localized PCa (< 2%), but are detected at a higher frequency after androgen ablation therapy and in hormone-refractory or metastatic tumors [10, 11]. In contrast, germline *AR* mutations are rarely found. So far, the R726L germline *AR* mutation has been reported to be the only mutation contributing to 2% of both sporadic and familial PCa in the Finnish population [12, 13].

We evaluated the possibility of genomic changes in the *AR* in African Americans and Caucasian families with a history of familial PCa, which is defined as having equal to or more than three patients with PCa. We screened the *AR* coding region in 60 PCa cases from 15 African American and 15 Caucasian families. In an African American family, we identified a novel germline *AR* missense mutation in three siblings with early-onset PCa, referring to the X-linked transmission pattern.

2 Materials and methods

2.1 Study subjects

We screened the genomic DNAs of two PCa patients from each of the 15 African American and 15 Caucasian families with at least three PCa-affected members. Recruitment of these families began in March 2000 as an effort to initiate a genetic linkage study of PCa at Louisiana State University Health Sciences Center, New Orleans [14]. In each family, a reliable and informative contact person (proband) was communicated to discuss the nature of the study with both affected and non-affected family members. Family history was obtained and a report on any type of cancer was documented. A family pedigree was prepared from information provided by the proband and verified by communicating with other relatives. Written informed consent was obtained from all patients and their family members. All study-related protocols were approved by the Institutional Review Board. Medical records were reviewed to confirm the primary site of cancer.

2.2 Analysis of AR sequence in familial PCa cases

Whole blood samples were used to extract genomic DNA using a Puregene DNA isolation kit (Gentra Systems Inc., Minneapolis, MN, USA). Polymerase chain reaction (PCR) amplification was performed by using the previously well-characterized exon-specific primer sequences covering *AR* gene intron/exon boundaries [15].

For exon 2 (the site of *AR* mutation), we used 5′-GCCTGCAGGTTAATGCTGAAGACC-3′ (sense; position 99133–99156) and 5′-CCTAAGTTATTTGATA GGGCCTTGCC-3′ (antisense; position 99511–99486, Accession No. NC_000023). In all, 50 ng of genomic DNA was amplified by 35 cycles of PCR in 50 μL containing 0.2 μmol L^{-1} of each primer, 0.2 mmol L^{-1} of dNTPs, 1.5 mmol L^{-1} of $MgCl_2$ and 2.5 units of GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR conditions were 95ºC for 5 min, followed by 95ºC for 45 s, 55ºC for 45 s and 72ºC for 1 min, with a 10-min extension at 72 ºC after the last cycle. The correct band size was verified by running a 1.2% agarose gel. PCR products were gel-purified by using a Qiagen PCR-cleaning kit (Qiagen, Inc., Valencia, CA, USA). Sequencing was performed in both directions and repeated independently to ensure the accuracy of the data. The reported sequence was examined by Chromas LITE software (version 2.0) and compared with *AR* gene in the NCBI gene database (Accession No. NM_000044). After detecting the *AR* (A1675T) mutation in two PCa-affected brothers, we examined available genomic DNAs from five other siblings from this specific African American family.

2.3 Establishment of BsbI restriction enzyme assay for AR (A1675T) genotyping

We developed a rapid enzymatic assay to detect *AR* (A1675T) genotype using an amplified PCR product and a *Bsb*I restriction enzyme to digest the constitutive *Bsb*I sites in the normal A-allele. The PCR condition included a hot start (95ºC/10 min) and 35 cycles of 95ºC for 45 s, 56ºC for 45 s and 72ºC for 40 s, followed by a final extension at 72ºC for 10 min. The primers sense, 5′-AAGACCTGAGACTTCACTTGC-3′, and

AR (A1675T) antisense, 5′-AAGACCTTGCAGCTTCCACAT-3′, were used to amplify the DNA fragment containing the allele of interest at the AR-1675 position. Gel-purified PCR products were cleaned and, before digestion with the enzyme, the accuracy of the PCR products was confirmed by sequencing. *Bsb*I restriction enzyme digestion was performed in a 15 μL volume including 1.5 μL of 10 × NEB Buffer 2, 1.5 μL of 10 × BSA solution, 1 μL of *Bsb*I (5U, New England Biolabs, Ipswich, MA, USA) and 11 μL of amplified PCR product. Digestion products were incubated at 37ºC for 3 h and loaded in a 2.5% agarose gel. *Bsb*I enzyme sites are 5′…GAAGAC$(N)_2$…3′ and 3′…CTTCTG$(N)_6$…5′.

2.4 Determination of African ancestry

The African ancestry of the family members with *AR* (A1675T) mutation was confirmed by using the *Sty*I restriction enzyme and PCR for FY-null genomic marker, a population-specific allele for Africans as described by Tournamille *et al.* [16] and Parra *et al.* [17].

2.5 Analysis of polymorphic AR CAG and GGC repeats

Several studies have suggested an association between the polymorphic $(CAG)_n$/$(GGC/N)_n$ repeat length of *AR* and PCa risk [18]. To evaluate the possibility of a linkage disequilibrium between the CAG/GGC repeat length and *AR* (A1675T) allele, we genotyped the study subjects by PCR and sequencing using the A1/A2 and A7/A8 primers, as described by Lubahn *et al*. [15]. PCR was performed under the following conditions: an initial denaturation step at 95ºC for 5 min for CAG repeat and 95ºC for 10 min for GGC repeat (as a hot-start), followed by 35 cycles of denaturation at 95ºC for 45 s, annealing at 55ºC (for CAG) or 60ºC (for GGC) for 45 s, extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min.

2.6 Determination of PSA-ARE1 (–158 G/A) genotype

PSA-ARE1 (–158 G/A) single-nucleotide polymorphism (SNP; rs266882) alone or in association with the polymorphic CAG repeat of *AR* has been the subject of numerous studies to identify its association with PCa risk or susceptibility [19–22]. To analyze the possibility of a linkage disequilibrium between *PSA*-G/A SNP and the *AR* (A1675T) allele, we genotyped all our study subjects by sequencing the PCR product and using the *Nhe*I restriction enzyme digestion, as described by Binnie *et al*. [23].

2.7 Statistical methods

Marker allele frequencies from sibship data were estimated using MENDEL v9.0 (UCLA, Los Angeles, CA, USA) [24]. Linkage analyses for *PSA* SNP were carried out using the reconstruction-combination transmission disequilibrium test (RC-TDT). Linkage analyses for *AR*-(A1675T) allele were performed using the X-linked reconstruction combination-transmission/disequilibrium test (XLRC-TDT) [25]. Reconstruction combination tests were used because parental genotypes were not available. The *AR* gene is on the X-chromosome and PCa is limited to males; thus, the usage of XLRC-TDT procedure is justified. Comparisons of average CAG and GGC repeats by race and *AR* status were performed using the MIXED procedure of SAS v9.1 (SAS Institute Inc., Cary, NC, USA) to account for the sibship membership to nuclear families; *P*-values were adjusted for multiple testing where necessary to maintain a family-wise significance level of 0.05.

3 Results

3.1 Identification of AR (A1675T) (T559S) mutation in a high-risk African American family

Sequencing studies conducted in 15 African American and 15 Caucasian families with a history of familial PCa identified a single base substitution (ACC to TCC) of the *AR* gene in two PCa-affected members in an African American family. This single base substitution led to a missense mutation changing the amino acid, threonine, to serine at codon 559 of the DNA-binding domain (exon 2) of *AR*. A normal A-allele, a mutant T-allele and a normal female with heterozygous AT alleles are shown in of Figure 1. This family had nine PCa cases with five cases confirmed by medical records and four by description by relatives (Figure 2). Genomic DNAs were available from family members 1, 3, 12, 13, 14, 15 and 16. Of these, members 1, 3, 13 and 16 showed a mutant T-allele, member 12 showed a normal A-allele, and female members 14 and 15 were heterozygous at AT-alleles with no history of cancer (Figure 1C). Although family member 16 showed the mutant T-allele and was reported by family members to have severe urological problems, no medical confirmation of urological malignancy exists because of refusal of medical attention. The alignment of amino acid sequences of the human AR before and after the *AR* (A1675T)

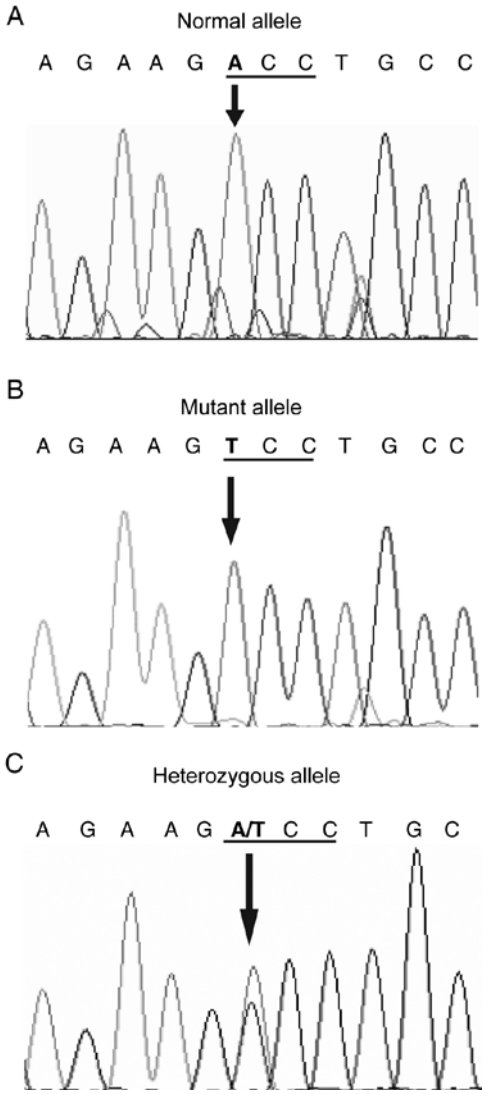

Figure 1. The *AR* (A1675T) (T559S) mutation. PCR amplification of genomic DNA with exon-specific primers and bi-directional sequence analysis revealed the presence of a single base-substitution mutation, which resulted in threonine to serine amino-acid change in the early region of DNA-binding domain of *AR*. (A): A normal male with A-allele; (B): An African-American patient with familial PCa and a mutant T-allele; (C): His sister with normal phenotype and heterozygous AT alleles.

(T559S) position showed that the mutant amino acid is located in the N-terminal portion of the DNA-binding domain, which is highly conserved and showed 100% homology with mouse, rat and monkey. Our analysis of genomic DNA from 150 normal unrelated individuals (75 African Americans and 75 Caucasians) excluded the possibility for *AR* (A1675T) as a polymorphic variant.

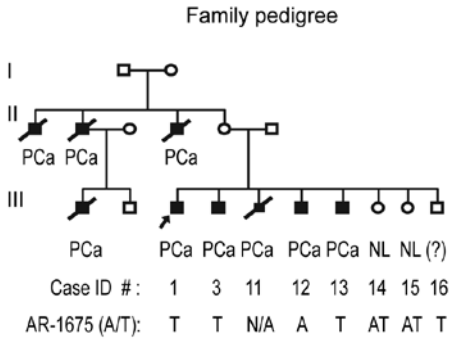

Figure 2. The patients' family tree. The family members with the *AR* (A1675T) (T559S) are indicated, as are those who presented with heterozygous AT-alleles (female). Affected siblings of the proband (contact person), except family member 12, showed a mutant T-allele and both females had heterozygous AT-alleles. Family member 11 had confirmed medical diagnosis of PCa and died of the disease before being recruited into the study. Family member 16, a mutation carrier, had severe urological problems; no medical confirmation of urological malignancy exists because of refusal of medical attention. Squares indicate male family members; circles, female family members; solid symbols, affected family members; slash, deceased. The proband is indicated by an arrow.

Overall, the *AR* (A1675T) (T559S) might present as a PCa-predisposing germline mutation and/or a novel SNP on the X-chromosome in familial PCa.

3.2 AR (A1675T) genotype identification by BsbI restriction digestion

As shown in Figure 3A, digestion with *Bsb*I identified three possible genotypes: a mutant T-allele with a 206-bp band, a heterozygous AT-allele showing 70-, 136- and 206-bp bands and existing only in normal females (carrier of the mutant T-allele), and a normal A-allele with two bands of 70 and 136 bp.

3.3 Determination of African ancestry

The African ancestry of the PCa-affected family members with *AR* (A1675T) mutation was confirmed by *Sty*I restriction enzyme digestion and by PCR for FY-null genomic marker, a population-specific allele for Africans [16, 17]. As shown in Figure 3B, all members in this specific family showed a similar restriction banding pattern.

3.4 Correlation with demographics and clinical data

The clinical features of the *AR* (A1675T) mutation-

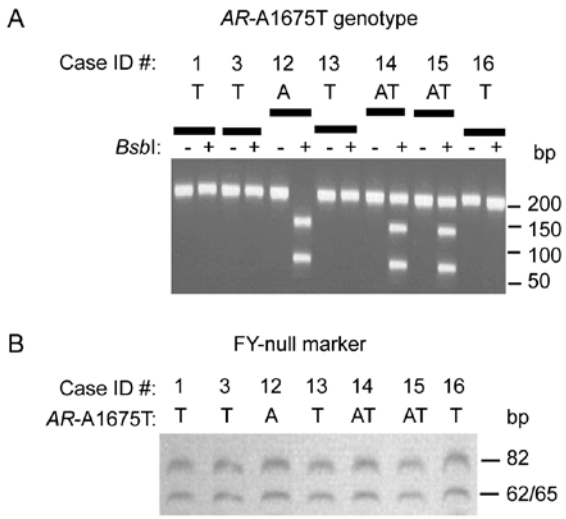

Figure 3. (A): Identification of *AR* (A1675T) genotype by *Bsb*I restriction enzyme digestion. Genomic DNAs were amplified and digested with 5 U of *Bsb*I to destroy a constitutive *Bsb*I site in *AR* containing the normal A-allele. Digested PCR products were loaded on a 2.5% agarose gel, visualized and photographed. Using *Bsb*I digestion, the mutant allele in family members 1, 3, 13 and 16 leads to a single 206-bp band, whereas the PCa-affected member 12 with normal A-allele shows two bands (70 and 136 bp). Family members 13 and 14, two normal females with heterozygous AT-alleles (one mutant and one wild type), produce three bands (70, 136 and 206 bp). (B): Determination of African ancestry by restriction analysis of the PCR-amplified FY-null locus in a PCa-affected family with *AR* (A1675T) mutation. African ancestry was analyzed by *Sty*I-restriction analysis of the PCR-amplified FY-null locus as a population-specific allele in all available members of the PCa-affected family with *AR* (A1675T) mutation.

positive PCa patients are shown in Table 1. The average age at PCa diagnosis was 57.6 years, which is considered early-onset. Serum-PSA level at PCa diagnosis was also low (3.5–7.4 ng mL^{-1}). The Gleason score for patients with the mutant T-allele was also at the intermediate level.

3.5 Distribution of AR (A1675T) genotype and polymorphic AR-CAG/GGC repeat and PSA-ARE1 (G/A) sites

The polymorphic CAG and GGC repeats of *AR* gene alone or in combination with *PSA*-ARE1 (G/A) SNP have been suggested as the contributing factors for PCa susceptibility or risk [18–22]. We tested the CAG/GGC repeat length in all our cases and in particular in the members of the family presented with the *AR* (A1675T) mutation. With the exception of family member 12 with CAG_{20} and GGC_{20}, all other members of this family showed the CAG_{23} and GGC_{16} repeat length (Table 1). This difference might be a reflection of the inheritance of different alleles from the maternal X-chromosome containing the normal A-allele.

The number of CAG repeats varied from 15 to 27 in African Americans, with 23 repeats being the most frequent (n = 9), and from 18 to 28 in Caucasians, with 21, 23 and 26 repeats being the three frequent alleles (n = 5). The number of CAG repeats was 21.3 ± 0.5 (mean ± SE) in African Americans and 23.2 ± 0.6 (mean ± SE) in Caucasians, which was significantly different (P = 0.01). The number of GGC repeats varied from 14 to 25 in African American families, with

Table 1. Demographics and allelic distribution of common polymorphic sites in the African American family with germline *AR* (A1675T) mutation[1].

Case ID # [2]	1	3	11[5]	12	13	14	15	16
PCa [3]	+	+	+	+	+	Normal female	Normal female	? [4]
AR (A1675T)	T	T	N/A	A	T	TA	TA	T
CAG_n	23	23	N/A	20	23	23	23	23
GGC_n	16	16	N/A	20	16	16	16	16
ARE1-PSA	AG	AG	N/A	AG	AG	AG	AG	AG
Age[6] (years)	58	62	56	51	57	60	57	55
PSA (ng mL^{-1})	6.2	3.5	N/A	7.4	4.3	N/A	N/A	N/A
Gleason score	6	7	N/A	9	7	N/A	N/A	N/A

Abbreviations: PCa, prostate cancer; N/A, denotes not available; ARE1, Androgen-response element 1; PSA, Prostate-specific antigen, [1]This family was the only one out of 15 high-risk African American families identified with germline *AR* (A1675T) mutation; [2]Case ID numbers are based on family pedigree; [3]PCa diagnosis was confirmed by medical records; [4]This family member carries mutation and has reported urological problems, but no additional medical information was available; [5]Family member 11 had confirmed medical diagnosis of PCa and died of the disease before being recruited for the study; [6]Age at which PCa was diagnosed.

23 repeats being the most frequent (n = 9), and from 17 to 26 in Caucasian families, with 23 repeats being the most frequent (n = 15). The mean number of GGC repeats was 20.7 ± 0.5 in African Americans, which was significantly different from that in Caucasians 23.3 ± 0.5 ($P < 0.001$). All members of the family showed the AG genotype for *PSA*-ARE1 (–158 G/A) SNP (Table 1). In our data, no association existed between the PSA-ARE1 (G/A) polymorphism and CAG (≤ 22) and GGC (≤ 16) repeats or *AR* (A1675T) alleles, or between the *AR* (A1675T) mutation and the GGC repeat length in African Americans (Table 2). However, a linkage may exist ($P = 0.06$) between the *AR* (A1675T) mutation and the CAG repeat length (≤ 22 *vs.* ≥ 23). Considering that the investigation was performed on a random set of African American families with at least three PCa-affected members, it is estimated that the mutant T-allele may exist with a prevalence of 16.2% in the previous generation and in 4.2% of the current generation among nuclear families of African Americans, with at least two sibs being affected with PCa.

Table 2. *AR* CAG and GGC and PSA (G/A) frequency distribution and association with *AR* (A1675T) mutation in African Americans and Caucasian Americans with familial prostate cancers.

Characteristics	African American	Caucasians
No. of high-risk families	15	15
No. of PCa-affected males[1]	37	30
PSA allele frequency[2]		
A	0.484	0.548
G	0.516	0.452
AR (A1675T) allele frequency[3]		
A	0.958	N/A
T	0.042	N/A
PSA genotype linkage[4] with:		
AR (A1675T) mutation status	1.0	N/A
CAG repeats (≤ 22)	0.857	1.0
GGC repeats (≤ 16)	1.0	1.0
AR mutation linkage[5] with:		
CAG repeats (≤ 22)	1.0	N/A
GGC repeats (≤ 16)	0.20	N/A

Abbreviations: PCa, prostate cancer; AR, androgen receptor; PSA, prostate-specific antigen; N/A, not applicable.
[1]PCa-affected males are brothers within a nuclear family; [2]Estimated with Mendel v9.0, autosomal; [3]Estimated with Mendel v9.0, X-linked; [4]Exact *P*-value using Reconstruction-combination transmission disequilibrium test procedure assuming full sibships; [5]Exact *P*-values using X-linked reconstruction combination-transmission/disequilibrium Test procedure assuming full sibships.

4 Discussion

African American men present with a higher incidence and mortality rate and more aggressive PCa at a younger age than other ethnic groups [26]. A family history of PCa significantly increases their risk [27]. *AR* genetic aberrations in the form of mutation, amplification or polymorphisms have been considered major contributors in pathophysiology of PCa and its progression to advanced stages. AR protein expression is estimated to be 22% higher in the benign prostate and 81% higher in the malignant prostate of African Americans compared with Caucasians [28].

In this study, we report the first germline *AR* (A1675T) (T559S) mutation in a high-risk nuclear family of African Americans with nine PCa-affected cases. This mutation may present as an interesting PCa-susceptibility allele in African Americans with familial inheritance. The localization of the T559S mutation in the DNA-binding domain makes it a likely candidate to affect the AR-binding affinity to its target genes. However, substitution of an uncharged polar amino acid (threonine) for another uncharged polar amino acid (serine) might not affect the AR surface charge, expression or its interactions with coactivators.

The existing reports on *AR* germline mutations in PCa are limited to Caucasian patients. The R726L mutation was reported in Finnish patients with sporadic or familial PCa [12, 13]. Additional reports include those on two unrelated PCa patients with G2T and C214A mutations within the 5′-UTR (non-coding) region of the *AR* [29]. One final report showed the AR-Q798E mutation in both the PCa tissue and the genomic DNA of a patient [30].

In vitro biofunctional assays have shown an inverse relationship between polymorphic CAG and GGC repeats and AR activity status. Based on these data, it has been widely hypothesized that the CAG/GGC repeat length inversely correlates with PCa risk or early-onset disease [18, 20, 31]. In our study, the difference between mean CAG and GGC repeat in African Americans and Caucasian was significant ($P < 0.05$). This might be due to racial difference or a characteristic of familial PCa. The absence of linkage disequilibrium between the X-linked *AR* (A1675T) mutation and GGC repeat might indicate the lack of their significance or contribution at least in familial PCa in African Americans. It is noteworthy that a recent comprehensive analysis on a large nested case-control study in the Physicians' Health Study, together

with a meta-analysis of the published studies, did not find any significant association between *PSA*-ARE1 SNP and PCa risk [32]. In contrast to GGC repeats, there may be a linkage between the *AR* (A1675T) mutation and CAG repeat length. Owing to the relatively small number of families investigated and the detection of *AR* (A1675T) mutation in only one out of 15 African American families, our results should be interpreted with caution.

Analysis of the European genomic contribution of 10 different populations of African descent in the United States revealed that the level of European admixture, mainly French, is the highest in New Orleans, which is the source of our study population [17]. In future large-scale studies, determination of African Ancestry and admixture population might be necessary to map this specific *AR* germline mutation to a founder PCa allele of a specific African or European descent.

Additional large-scale population studies are currently being conducted to determine the frequency of *AR* (A1675T) germline mutation in both sporadic and familial PCa and to elucidate its biological significance and potential as a predisposing genetic marker or a modifier variant in African Americans versus Caucasians.

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