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ORIGINAL ARTICLE

Prostate Cancer

Identification of speckle-type POZ protein somatic mutations in African American prostate cancer

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The speckle-type POZ protein (SPOP) is a tumor suppressor in prostate cancer (PCa). SPOP somatic mutations have been reported in up to 15% of PCa of those of European descent. However, the genetic roles of SPOP in African American (AA)-PCa are currently unknown. We sequenced the SPOP gene to identify somatic mutations in 49 AA prostate tumors and identified three missense mutations (p.Y87C, p.F102S, and p.G111E) in five AA prostate tumors (10%) and one synonymous variant (p.I106I) in one tumor. Intriguingly, all of mutations and variants clustered in exon six, and all of the mutations altered conserved amino acids. Moreover, two mutations (p.F102S and p.G111E) have only been identified in AA-PCa to date. Quantitative real-time polymerase chain reaction analysis showed a lower level of SPOP expression in tumors carrying SPOP mutations than their matched normal prostate tissues. In addition, SPOP mutations and novel variants were detected in 5 of 27 aggressive PCa and one of 22 less aggressive PCa ($P < 0.05$). Further studies with increased sample size are needed to validate the clinicopathological significance of these SPOP mutations in AA-PCa.

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INTRODUCTION

Prostate cancer (PCa) is the most prevalent malignancy and second most lethal cancer in men in the United States. The incidence and mortality rates of PCa in African American (AA) men are the highest among all ethnic groups.[1] However, the genetic basis of this disease and the PCa disparities among the AA population remain largely unknown.

A recent study showed that speckle-type POZ protein (SPOP) is the most frequently mutated gene in PCa.[2] Barbieri *et al.*[2] performed exome sequencing on 112 prostate tumors predominantly of European descent and identified SPOP mutations at a frequency of 6%–15% across localized and advance prostate tumors. Other studies reported SPOP mutation frequencies of 4.4% (2 of 45), 3.4% (2 of 58), and 28.6% (2 of 7).[3–5] SPOP is a CULLIN3-dependent ubiquitin ligase responsible for the ubiquitination and proteolysis of steroid receptor coactivator-3 (SRC-3/AIB1), an oncogene overexpressed in many human cancers including PCa.[6–8] SPOP functions as a tumor suppressor in prostate cells by promoting the ubiquitination and proteasomal degradation of the SRC-3 protein and suppressing androgen receptor transcriptional activity.[9] The tumor suppressor effect of SPOP is abrogated by PCa-associated SPOP mutations.[9] Moreover, transfection of PCa cells with the most common SPOP mutant (the F133V variant) or SPOP small interfering ribonucleic acid showed increased tumor invasion.[2] Tumors with SPOP mutations were positively associated with deletion of *CHD1*, *FOXO3,* and *PRDM,* suggesting that loss of one or more of these tumor suppressor genes may cooperate with SPOP mutations to promote tumorigenesis.[2]

We hypothesized that SPOP mutations may contribute to the aggressiveness of PCa in AA men. In this study, we sequenced the entire coding region of SPOP to identify somatic mutations and determined its expression and potential association with PCa aggressiveness in an AA-PCa cohort collected in the Southern Louisiana area.

MATERIALS AND METHODS

Prostate tissue specimens

Forty-nine anonymous, snap-frozen AA prostate tumors, their matched adjacent normal prostate tissues, and clinicopathological information were obtained from the Biospecimen Core of the Louisiana Cancer Research Consortium (LCRC). The clinical characteristics of this study's PCa cohort are summarized in **Table 1**. Specimens were collected at the Louisiana State University Health Sciences Center (LSUHSC) or Tulane University Medical Center between 2009 and 2013. Histological classification including Gleason score and tumor cell content were evaluated by two pathologists. This study was approved by the Institutional Review Board of LSUHSC.

Deoxyribonucleic acid extraction and speckle-type POZ protein mutation screening

Tumor tissues were macrodissected as previously described to enrich for tumor deoxyribonucleic acid (DNA).[10] Genomic DNA from tumors

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and matched normal tissues were extracted from frozen tissues using the DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA) according to the manufacturers' protocol. Exon-specific products were polymerase chain reaction (PCR) amplified using exon-specific primers (**Table 2**) in a 15 μl PCR reaction containing genomic DNA (3.5 ng), 1× AmpliTaq Gold PCR buffer, 2.0 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} of each dNTP, 0.4 μmol l^{-1} sense and 0.4 μmol l^{-1} antisense primer, and 0.5 units of AmpliTaq Gold DNA polymerase. PCR amplification was performed with a DNA Engine Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR amplicons were purified using exonuclease I (USB/Affymetrix, Santa Clara, CA, USA) and shrimp alkaline phosphatase (USB/Affymetrix), and submitted to direct Sanger sequencing using the forward and reverse PCR primers. Primer sets for the amplification and sequencing of exons 6 and 7 have previously been described.[2] Mutations were confirmed by bi-directional sequencing of a second independent amplified PCR product from the original genomic DNA template. All sequencing was performed at the LCRC Genomics Facility (New Orleans, LA, USA).

Real-time-polymerase chain reaction and restriction enzyme digest analysis of speckle-type POZ protein for structural variation determination

Total ribonucleic acid (RNA) was extracted from frozen PCa tissue using TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturers' protocol. RNA was treated with deoxyribonuclease, and complementary DNA (cDNA) was generated with the SuperScript III First-Strand synthesis system (Invitrogen). The full-length SPOP cDNA was amplified using primers designed to amplify a product of approximately 1.25 kb that covers the entire coding region of SPOP: 5'-ACAGAAATCTTTGCCCCCTGAC-3' (forward) and 5'-GGTGGTCAGTGGCAGCAACAG-3' (reverse). The real-time PCR (RT-PCR) products were digested with *PvuII* (Promega, Madison, WI, USA) and analyzed on a 2% Tris-borate-EDTA agarose gel.

Real-time quantitative polymerase chain reaction analysis of speckle-type POZ protein expression

Quantitative real-time polymerase chain reaction was performed using the cDNA templates synthesized from RNA samples and a SPOP TaqMan probe purchased from Applied Biosystems (SPOP ID: HS00737433; Foster City, CA, USA). Briefly, 1 μl of cDNA was mixed in a total volume of 5 μl with 2× TaqMan Universal Master Mix (Applied Biosystems), 0.25 μl SPOP primer/probe mix, and 1.25 μl of water. The thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s followed by 60°C for 1 min. Data were collected and analyzed with a 7900 HT RT-PCR instrument (Applied Biosystems). SPOP gene expression levels were normalized to a calibrator (pool of tumor cDNAs) and the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The comparative cycle threshold method ($2^{-\Delta\Delta Ct}$) was used to analyze relative gene expression differences between paired-set samples of normal tissue and AA-PCa tumor tissue.

Statistical analysis

Statistical analysis of the messenger RNA (mRNA) expression results was performed using Wilcoxon's signed rank test. Clinical variables, such as age and Gleason score, were compared using the chi-squared test. Statistical analyses were performed using GraphPad Prism6 (GraphPad Software, Inc., La Jolla, CA, USA), and $P < 0.05$ was considered as statistically significant.

RESULTS

We screened for somatic variants in exons six and seven of the SPOP gene in 49 AA prostate tumor tissues because all of the SPOP mutations previously reported were within these two exons.[2] Three somatic SPOP missense mutations were identified in exon six in five tumor tissues (10%), and one somatic variant was identified in one tumor sample (**Figure 1** and **Table 3**). The c.A260G (p.Y87C) and c.T305C (p.F102S) mutations were recurrent, being identified in two different tumor samples, whereas the c.G332A (p.G111E) mutation and c.C318T synonymous variant (p.I106I) were detected once. Because none of the corresponding blood DNA samples showed evidence of mutations, it was concluded that the observed mutations arose somatically. No variants were detected in exon seven. We also screened for somatic variants in the remaining SPOP exons (1–5 and 8–12) and detected no variants.

Table 1: Clinical characteristics of the African American prostate cancer cohort

Clinic characteristics	*Number of cases*	*Values*
Age (year)		
Mean (range)	49	59.5 (50–72)
Gleason score		
Gleason<7	9	5.9 (5–6)
Gleason=7	31	7.0 (7–7)
Gleason>7	9	8.4 (8–9)
PSA level (ng ml^{-1})		
Median (range)	42	13.84 (0.4–170.4)

PSA: prostate-specific antigen

Table 2: Primers used for the PCR amplification and sequencing of SPOP exons

Exons	*Forward primer (5'→3')*	*Reverse primer (5'→3')*	*PCR size (bp)*
Exon 1	CGCATGCGCACTAGGAAAGA	CCCCACTCCGACAGGACAACC	369
Exon 2	CAGTAGGTTTCGGCCATTGGG	CCAACATGGCAAAACCTCAACT	242
Exon 3	GGATACAGTTAGTGGTGTTTAG	CCTTAAGGACACTGGGCTATGCG	254
Exon 4	GGACCTCAGTGGTAAGGAGCC	GGCAGTTATGTTCCAGAGAAAG	387
Exon 5	CTGCTATCTTCCTATCAGGATC	CAAAAGTCCTGGCCTTCATGG	350
Exon 6–7*	TTCTATGGGGCCTGCATTT	CTCCACTTGGGGCTTTTTCT	628
Exon 6**	TTTTCTATCTGTTTTGGACAGG	CAAAGCCACAACTTGTCAGTG	248
Exon 7**	TTTGCGAGTAACCCCAAAG	CTCATCAGATCTGGGAACTGC	450
Exon 8	CTGAATGTACTACAGCAGTTAGG	GGTGATTATGCAATCACTTG	346
Exon 9	GAGGTAGAGACCTTTCCTATAGC	GTGGGAAATCATCTGACTCTAG	241
Exon 10	CAAAGTTCTCTCCTCACTAGAGC	CCACAATGCAACATAGAATCCTG	309
Exon 11	CATGGTCTGTGCAGTTCTATTC	GCTACTTGCCTGCTTTACCCAC	363
Exon 12–1	CTTGCATTGACACCCTTTATACC	CAGACAGGTGTCTCCGAAGTAC	572
Exon 12–2	CTCTGTTGTGAGGGGAAGAG	CCACATCAGAACACTCATCTTC	585
Exon 12–3	CAAGCATCAGAAGAGCTCTCTTG	CCCTGGAGGAAGAGGGGCTAGTC	492
Exon 12–4	CCCAGCCCAGATTGCTCAGC	GAACATTCTTGTGAGCTCCAG	615

*Primer set used to amplify exons 6 and 7, **Sequencing primers for exons 6 or 7. PCR: polymerase chain reaction; SPOP: speckle-type POZ protein

Table 3: Summary of SPOP mutations identified in African American prostate tumors

Sample ID	*Age*	*PSA*	*Gleason score*	*Nucleotide change*	*Amino acid change*
AA-10	53	NR	7	c. 260A>G	p.Y87C
AA-39	57	5.2	7	c. 260A>G	p.Y87C
AA-29	60	4	7	c. 305T>C	p.F102S
AA-37	62	4.6	5	c. 305T>C	p.F102S
AA-50	56	0.4	7	c. 332G>A	p.G111E
AA-36	58	11.1	8	c. 318C>T	p.I101I

NR: not recorded; PSA: prostate specific antigen; SPOP: speckle-type POZ protein

To determine the molecular impact of the SPOP mutations on SPOP expression, we performed RT-quantitative PCR (RT-qPCR) analysis of the six AA prostate tumors carrying SPOP mutations or the novel coding variant. As shown in **Figure 2a**, SPOP mRNA expression was significantly reduced in all six of the AA-PCa tumors carrying SPOP mutations or the variant compared with their matched normal tissue ($P = 0.0313$), supporting the notion that SPOP functions as a tumor suppressor gene.

To determine the potential association between SPOP mutations and the aggressiveness of AA-PCa, we classified the 49 tumors into two groups: aggressive and less aggressive. Tumors from patients age 60 years and less with a Gleason score 7 or greater were classified as aggressive PCa, and those from patients age 60 and less with a Gleason score <7 or over age 60 regardless of Gleason score were classified as less aggressive PCa. We found that five of 27 aggressive tumors harbored SPOP mutations or the novel variant, while only one of 22 less aggressive tumors harbored a mutation ($P < 0.05$; **Table 3**).

DISCUSSION

This is the first study to identify SPOP somatic mutations in AA prostate tumors. We performed Sanger sequencing on DNA isolated from enriched tumor cells from snap-frozen prostate tissues and demonstrated that 10% (5/49) of localized AA prostate tumors harbored SPOP somatic mutations. The frequency of mutations identified in our AA prostate tumor cohort is comparable to the frequency of the 6%–15% reported for European prostate tumor cohorts.[2] Until date, more than 30 SPOP somatic mutations have been reported in cohorts of prostate tumors; 20 (65%) of these mutations were identified in exon seven, and 11 (35%) were found in exon six (**Figure 3**).[2–4] Interestingly, two missense mutations (p.G111E and p.F102S) and the synonymous variant (p.I106I) have not been reported in other prostate tumors cohorts; thus, they represent novel somatic mutations in our AA-PCa tumors. Although the 102 codon mutations were detected in AA and European prostate tumors, the p.F102S mutation identified in the AA tumors is different from the p.F102C mutation detected in European tumors. Recently, it was demonstrated that mutations within exon six, for example p.Y87C and p.F102C, attenuated the binding of SCR-3 to SPOP and increased androgen receptor transcriptional activity. We speculate that several novel mutations identified in our AA-PCa cohort, for example p.G111E and p.F102, will have similar functional effects on SCR-3 binding and androgen receptor activity.

The different landscape of SPOP mutations in exons six and seven between AA and European tumors led us to consider the possibility that additional mutations may exist in other SPOP coding regions in AA tumors. Therefore, we amplified and sequenced the remaining exons (1–5 and 8–12) in all 49 tumor tissues. However, no additional somatic mutations or coding variants were identified. To exclude the possibility of insertion or deletion events in the SPOP gene, which may result in exon skipping, we amplified the entire SPOP mRNA from 39 tumors for which mRNA was available and performed *PvuII* digestion. We did not detect deletions/insertions or exon skipping (data not shown), which further strengthens our conclusion that our AA prostate tumor cohort only harbors SPOP mutations in exon six.

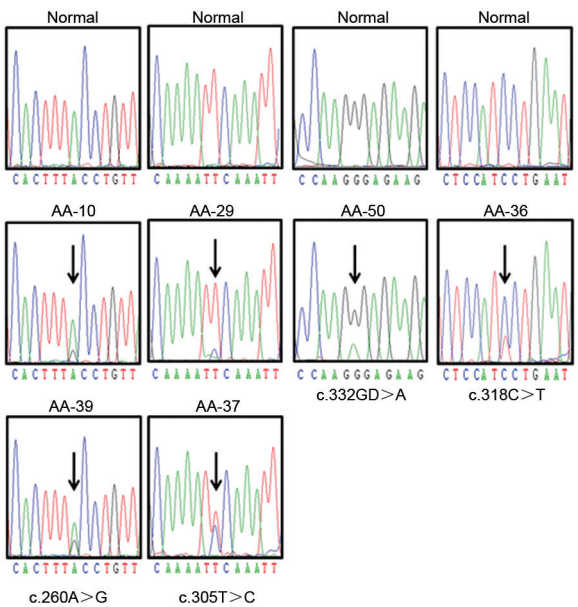

Figure 1: Chromatograms from African American prostate tumors showing speckle-type POZ protein (SPOP) variants. Deoxyribonucleic acid chromatogram sequences (sense direction) of SPOP exon six, demonstrating the heterozygous mutated alleles compared with the normal homozygous allele. Mutations are indicated by an arrow and were confirmed by the bi-directional sequencing of two independent polymerase chain reaction products from the original genomic template.

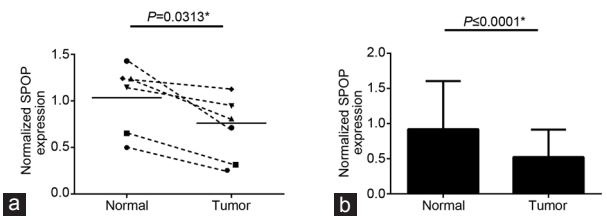

Figure 2: Expression of speckle-type POZ protein (SPOP) messenger ribonucleic acid (mRNA) in normal and prostate cancer tumor tissues from African American patients. (**a**) Expression of SPOP mRNA in the six tumors in which mutations were identified. Matched normal and tumor samples are represented by the same symbol and connected by a line. Horizontal bars represented the median. (**b**) Expression of SPOP mRNA in the 25 normal-tumor matched samples used in this study. Expression of SPOP mRNA was determined by real-time-polymerase chain reaction as outlined in Materials and Methods and normalized to glyceraldehyde-3-phosphate dehydrogenase expression. The bar represents the mean value of two independent experiments.

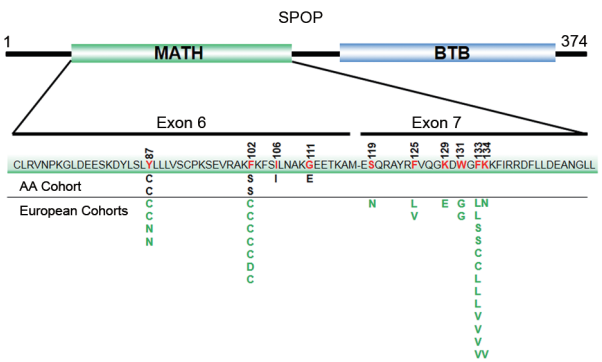

Figure 3: Speckle-type POZ protein (SPOP) somatic mutations identified in African American (AA) or European prostate tumors. Schematic representation of the SPOP protein showing the positions of individual somatic mutations identified among AA prostate cancer tumors relative to the functional domains and in comparison with mutations identified in European cohorts.[2] Mutated amino acids are shown in red.

All AA prostate tumors harboring SPOP mutations or the novel variant showed relatively low SPOP expression. However, RT-qPCR analysis of SPOP mRNA from 19 AA prostate tumors lacking SPOP mutations that had matched normal tissue mRNA available also demonstrated a reduction in the SPOP expression level compared with matched normal tissues ($P = 0.0024$; data not shown). Therefore, as shown in **Figure 2b**, the overall expression of SPOP in all 25 of the AA prostate tumors was reduced ($P \leq 0.0001$), suggesting that other regulatory mechanisms such as epigenetic regulation might be involved in the regulation of SPOP mRNA expression in addition to the SPOP mutations.

We defined aggressive PCa in our cohort based on an age of onset at 60 with a Gleason score of 7 or greater. Based on this criterion, SPOP mutations were associated with the aggressive AA-PCa samples in this study. However, the relatively young age of our cohort (median age: 59.5, range: 50–72) and relatively small number of tumors analyzed limit the power and significance of this conclusion. Larger sample sets of aggressive and less aggressive AA prostate tumors are needed to validate this potentially important finding.

CONCLUSIONS

We have identified two novel missense SPOP somatic mutations in AA prostate tumors and demonstrated reduced SPOP expression in AA prostate tumors. We hope that these interesting findings from this initial and limited case analysis will stimulate studies in larger cohorts of AA prostate tumors to validate these mutations and determine the molecular and pathological significance of these novel and unidentified AA-PCa SPOP mutations in PCa aggressiveness and PCa disparity in the AA population.

AUTHOR CONTRIBUTIONS

EB, CQ, SM, JZ, and AT performed the experiments and analyzed data. JC provided the PCa tissues for this study. EB and WL wrote and revised the manuscript. JE, JW, and WL provided information and supervised the study. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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