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Research Article

APOL2: A New Candidate Gene Associated with Hereditary Prostate Cancer

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Abstract

Background: Family history is a well-recognized risk factor for prostate cancer (PCa), but germline variants in *BRCA1/2*, and other DNA or mismatch repair genes explain less than half of hereditary cases. The identification of rare, highly penetrant PCa genes has been extremely challenging, and the role of polygenic risk adds to the complexity of this research. Methods: Integrated molecular genetic analysis of a family with clinical criteria for hereditary PCa: targeted *BRCA1/2* testing, multigene panel testing and whole-exome sequencing (WES). Bioinformatic and in silico analyses of WES data identified gene variants for protein studies and prevalence analysis in healthy controls. A functional viability study of the top candidate was performed to confirm its relevance in PCa. Results: no pathogenic variant either in targeted or commercial multigene testing was observed, but analysis of WES data identified ten variants of interest. After segregation studies and review of their functions, *APOL2* and *PELP1* genes were selected for protein expression studies. These suggested a higher *APOL2* specificity for prostate tissue compared to *PELP1*; also, the *APOL2* variant was not present in male controls, while the *PELP1* variant was observed in 3% of these. Functional studies disclosed a decreased viability in APOL2-silenced PCa cell lines. Conclusions: The *APOL2* gene is a good candidate for further studies in hereditary PCa.

Keywords: Familial prostate cancer, *APOL2*, susceptibility, mutation

Introduction

Prostate cancer (PCa), one of the leading causes of cancer-related deaths in men [1], is also one of the most heritable cancers [2]. This disease has been associated with several hereditary cancer syndromes and more than 100 common variants [3,4], but germline mutations in PCa patients were mostly found in *BRCA2* (5%), *ATM* (2%), *CHEK2* (2%), *BRCA1*(1%), *RAD51D* (0,4%), *PALB2* (0,4%), *ATR* (0,3%) and *NBN,PMS2*,-*GEN1,MSH2,MSH6,RAD51C*, *MRE11A*, *BRIP1*, *HOXB13* or *FAM175A* [5]. Most of these genes are directly or indirectly associated with DNA-repair and homologous recombination. If a pathogenic germline variant is identified in those genes, it allows for cascade testing in family relatives and precision management regarding the prognosis and treatment of PCa patients. Indeed, a high frequency of *BRCA2*, *CHECK2* and *ATM* [5, 6] variants were observed more frequently in men with advanced PCa [5] than in men with localized disease [7], and targeted treatment with PARP inhibitors are more effective in PCa patients with germline variants in those genes [8].

BRCA2 mutations have been associated with a 2- to 6-fold increase in the risk for prostate cancer [9-11]. Current guidelines recommend germline *BRCA1/2* testing for PCa patients with familial history and patients with high-risk or metastatic disease [12]. Since PCa is included in the Hereditary Breast Ovarian Cancer (HBOC) syndrome, some authors defend changing its designation to King Syndrome to include *BRCA1/2* testing in the routine management of PCa patients [13, 14]. Men with Lynch syndrome (germline mutations in *MLH1, MSH2, MSH6, PMS2*, or *EPCAM*) have a 2- to 5.8- fold increase in risk for prostate cancer [15]. However, there are no current NCCN guidelines regarding any specific prostate cancer screening recommendations for men with this syndrome [12].

In the absence of a pathogenic germline variant, criteria for classifying a PCa family as hereditary include 1) three or more first-degree relatives with PCa, or 2) three successive generations of PCa, or 3) two relatives with PCa diagnosed at age ≤55 years [16]. Inherited cancer assessment and local resources may

decide on *BRCA1/2* or MMR targeted screening based on family history of other cancers, or upfront or sequential multigene testing. Nevertheless, even with increasing access to testing panels, including already known PCa genes, a significant number of these families will not identify a conclusive germline variant. In the era of precision medicine, these patients and their families represent an unmet need in routine clinical practice.

In this study, we searched for new PCa genes through an integrated clinical and molecular analysis of PCa and non-PCa patients belonging to a family with clinical criteria for hereditary PCa.

Material and Methods

Institutional approval

This study was approved by the Ethics Committee of Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG, UIC/829). PCa and control patients gave written informed consent to participate in the study.

Family pedigree, biological samples and sequential DNA testing

The pedigree of the family of interest for this study is shown in Figure 1. This family has African-Portuguese ancestry. Initially peripheral leukocyte DNA was obtained from PCa patients (III.1, III.2, III.3 and III.5) and targeted *BRCA1/2* testing was performed by CSCE (Conformational Sensitive Capillary Electrophoresis)]. In 2018, when III.5 was diagnosed with a second neoplasia (ductal biliary cancer), this patient consented on multigene testing (*BRCA* Hereditary Cancer MASTR Plus panel from Multiplicom, Niel, Belgium). This panel was run in a MiSeq NGS platform from Illumina.

Control DNA samples of 100 healthy males (average age: 62 years old) were requested from Biobanco-iMM, Lisbon Academic Medical Center, Lisbon, Portugal.

Whole exome sequencing (WES)

DNA was extracted from leukocytes as previously described [17]. Genomic DNA from 3 representative members of this family was analyzed through WES (patients III.1, III.3 and III.4, Figure 1).Whole exome sequencing was performed in the Erasmus Medical Center (Rotterdam, The Netherlands), using the SureSelect Human All Exon V4 capture kit (Agilent, Technologies, Santa Clara, California, USA). The captured exonic sequences were sequenced in a HiSeq2500 (Illumina Inc., San Diego CA, USA), with a v2 rapid flowcell for PE 100bp with indices. The reads were aligned against the human reference genome version GRCh38 using the BWA-backtrack software. Aligned reads were converted (SAM to BAM), and between 94% and 99% of reads were successfully mapped to the Human Genome. Duplicates were removed with Picard MarkedDuplicates and subsequenctly the variants were called using GATKv2 tool and annotated with Annovar.

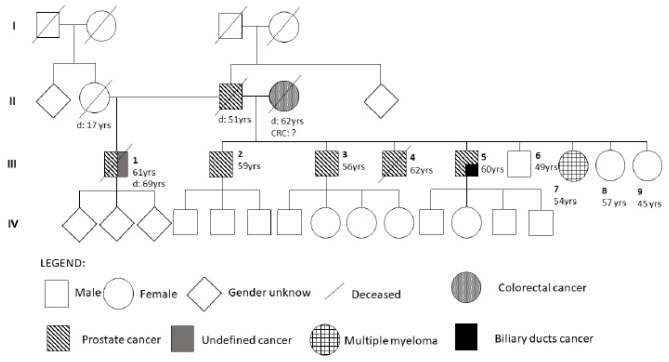

Figure 1. Prostate cancer family pedigree. Six first-degree patients affected with prostate cancer are represented in this family with African-Portuguese ancestry.

Bioinformatic analysis

The bioinformatic analysis of the sequencing data was initially performed by Bioinf2Bio (Porto, Portugal). Briefly, fastq files were converted in SAM and then in BAM files, to enable easy visualization in the Integrative Genomics Viewer. IGV is a tool for interactive exploration of integrated genomic datasets, and to perform sequencing data analysis. The details of subsequent analyses were previously described [17]. Selection of potential pathogenic variants was performed as shown in Figure 2.

Genetic variants' validation

DNA from patients III-1, III-2, III-3, III-5, III-7, III-8, and III-9 were used for validation of variants' selected after bioinformatic analysis (Figure 1 and Supplementary Table 1) as previously described in [17]. Primer sequences and PCR conditions

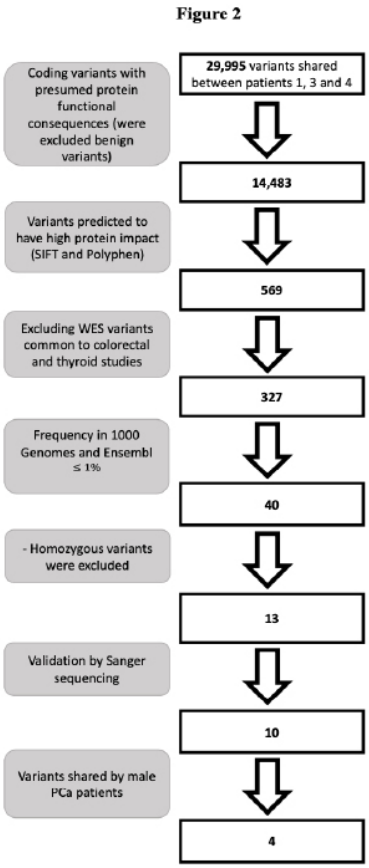

Figure 2. WES data analysis workflow. Only variants with potential protein consequences were selected. Exclusion criteria related to allele frequency higher than 1% (in the global, European and African populations) and homozygous variants. Only variants validated by Sanger sequencing and segregated with PCa patients were included for further analyses.

Table 1: Primer sequences and melting temperature (Tm). For each gene variant specific primers were designed.

Gene	Primer Forward	Primer Reverse	Tm (ºC)
ACIN1	GTACTCATGCCAACCCTCGT	cacccgggattctctcatac	60
ANKRD53	CTTCCACCCCTCTGTGGAT	CTCCAGGAAGCTGCTGAAGT	60
APOL2	atgagctgctgggaagttgt	ggacatgggggtagatcaca	60
KCNJ18	AGTTCCTGCTGCCCAGTG	TCTCTGACCCCCGTCTGTAG	60
KRT3	CTTTGCAGGTGGCTATGGAG	GGCTGCAGGAGACTCTGGT	60
OR10A7	GCCATAGGCTCTTGGATGTC	ACAGGAGTGTGGAGGCAAGT	60
PELP1	TCAACAGCAGTGATGAAGAGG	ccaaactccaggtcttccac	60
MUC6	GCCAACAGGTACCATTCCTC	TGCGTGTACTAGTGGGGTTG	60

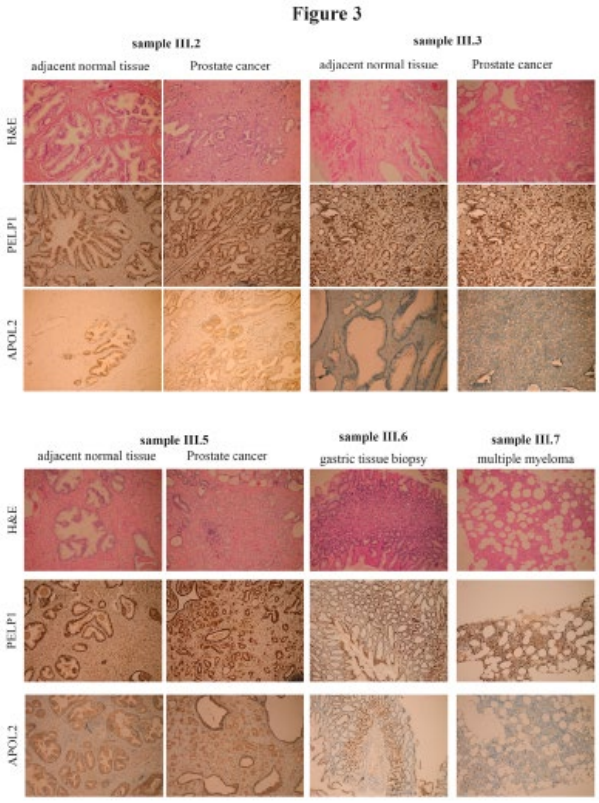

Figure 3. Immunostaining of PELP1 and APOL2 in pathology specimens. Prostate tumor samples (including adjacent normal tissue) from III.2, III.3 and III.5 and begnin gastritis (III.6) as well as multiple myeloma (III.7) specimens were analyzed.

melting are indicated in Table 1.

Immunohistochemistry analysis

Protein expression of the genes of interest was analysed through staining of prostate cancer FFPE samples from patients III.2, III.3 and III.5. A benign gastric biopsy from family member III.6 (Figure 1) and a bone marrow sample from a sibling with multiple myeloma (III.7 Figure 1) were also tested. Tissues sections were stained on a Ventana Benchmark Ultra using CC1 standard antigen retrieval. The primary antibody against APOL2 and PELP1 were used at a final dilution of 1:500 (Anti-APOL2 antibody (Invitrogen LTI A5-36425) and Anti-PELP1 (STJ24959). Positive controls (normal prostate tissue) were tested using the same antibodies. OptiView (Roche) was used as detection system. All samples were reviewed centrally by an expert pathologist.

Cell culture

Prostate cancer cell lines, PC3 and LNCaP were kindly supplied by Professor Carmen Jerónimo, from Instituto Português de Oncologia do Porto FG, EPE (IPO-Porto). Cells were cultured in RPMI medium with Hepes supplemented with 1% L-glutamine 1% antibiotic-antimycotic (all from Gibco®, Life Technologies, Paisley, UK), and 10% (v/v) fetal bovine serum (FBS) (Merck Millipore, Berlin, Germany). The cell lines were free of mycoplasma by the universal mycoplasma detection kit (ATCC® 30-1012K™, Manassas, USA).

siRNA transfection and cell viability

APOL2 siRNA smart pool (D-017407-01, Dharmacon, CO, USA) and siRNA control non-targeting (D-001210-02-05, Dharmacon) were individually transfected into PC3 and LNCaP cells with DharmaFECT 1 transfection reagent (T-2001-02 Dharmacon, CO, USA) following the manufacturer's instructions. Cells were seeded in 6-well plates at an initial concentration of 6×104 PC3 cells/well and 9×104 LNCaP cells/well. After 48 hours the cells were harvested for cell viability assay by trypan blue (Gibco®, Life Technologies, Paisley, UK) exclusion assay. The viable cells were counted in a hemocytometer (0.100 mm, Neubauer Improved, Erlangen, Germany).

RNA extraction, cDNA synthesis, and gene expression analyses

RNA was extracted from PCa cell lines after 48 hours of siRNA treatment using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and quantified by UV spectrophotometry (NanoDrop ND-1000). cDNA was synthesized from 1 µg of total RNA, using random primer p(dN)6 (Roche Diagnostics Corporation, Indianapolis, IN, USA) and SuperScript II reverse transcriptase (Thermos scientific, CA, USA).

APOL2 quantitative RT-PCR assays were performed with forward primer CACGCGCAGACCTTCGTT and reverse primer CCATGGAGGGCGGATTG. PCR amplifications were performed using 10 µM of each primer and Power SYBR Green PCR Master Mix (Applied Biosystems, CA USA), according to the manufacturer's protocol. Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) expression was used as endogenous control, with forward primer TGACACTGGCAAAACAATGCA and reverse primer GGTCCTTTTCACCAGCAAGCT.

Statistical analysis

All experiments were performed in at least three independent assays. The results are expressed as the mean + standard deviation. T-test was performed to assess statistical differences using GraphPad Prism software (version 5.0). p values <0.05 were considered statistically significant.

Results

Sequential DNA testing

No pathogenic *BRCA1/2* variants were identified in III.1, III.2, III.3 and III.5. Patient III.5 also consented on multigene testing after a second cancer diagnosis, but again no pathogenic variant was identified. Due to its likely autosomal dominant pattern of transmission, with no genetic cause identified, this family was selected for WES.

Whole exome sequencing and segregation analysis

WES was performed on DNA from III.1, III.3 and III.5. A total of 29,995 variants were shared by these patients (Figure 2), but several were excluded considering criteria such as the predicted high impact in protein function and prediction tools (SIFT and Polyphen). Only rare (<1%), heterozygous variants shared by all patients with prostate cancer were selected for further analyses. Consequently, we obtained 13 rare, heterozygous and potentially functional variants (Figure 2). After validation by Sanger sequencing (Table 2) and segregation (Table 3), variants in 3 genes (*KCNJ18, MUC6* and *ZNF17* genes) were excluded. Segregation analysis for the resulting 10 variants, including all available DNAs from family members was performed (Supplementary Table 1). Four variants in 4 different genes (*ACIN1, APOL2, PELP1* and *CIB1*), were shared by the 4 affected male PCa patients: These variants led to a frameshift in 3 cases: *ACIN1, PELP1* and *APOL2* and to splice acceptor disruption (Table 3) in *CIB1*. Segregation studies revealed that gene variants in detected *ACIN1* and *CIB1* were shared by all siblings (males and females with or without any cancer history), suggesting that they could be benign polymorphisms. *APOL2* and *PELP1* were then selected for further studies.

Immunochemistry of tumor specimens

APOL2 and *PELP1* protein expression was evaluated by immunostaining of cancer and normal adjacent prostate tissue of patients III.2, III.3 and III.5. Samples of benign gastric tissue (III.6) and multiple myeloma (III.7) from family relatives were also analyzed (Figure 3).

APOL2, was clearly observed in the cytoplasm of prostate cells (similar between cancer and non-cancer cells), in contrast with other tissues (Figure 3). Indeed, although cytoplasmic staining in gastric epithelial cells, bone marrow megakaryocytes and endothelial cells could not be excluded, this was clearly different from prostate staining (III.6 and III.7, Figure 3).

As for *PELP1*, its expression was observed both in the nucleus and cytoplasm of both normal and neoplastic prostate, as well as in the other tissues tested (Figure 3).

Studies of PELP1 and APOL2 variants in healthy male controls

Although immunostaining suggested *APOL2* as a more prostate-specific gene, the role of *PELP1* in this family PCa could not be excluded. Although the frequency of *PELP1* and *APOL2* gene variants is very low (< 1%) as reported in 1000 Genomes and Ensemble databases, we tested 100 healthy male controls for the variants of interest. As shown in Table 4, while the *APOL2* variant was not found in any of the controls the variant in the *PELP1* was found in 3% of those. Taken together these data, *APOL2* was selected as the best candidate for functional studies.

Figure 4

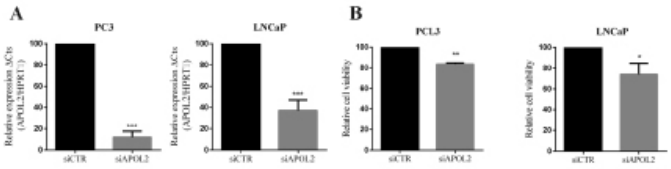

Figure 4. APOL2 silencing reduces prostate cancer cell lines viability. APOL2 mRNA expression (A) and cell viability (B) in prostate cell lines (PC3 and LNCaP) 48 hours after siRNA transfection (A) or APOL2 silencing (B).

Functional studies

Satisfactory levels of *APOL2* silencing after 48h of siRNA transfection, were obtained (> 60%) (Figure 4A) and we observed a significant reduction of cell viability (20% and 30% in PC3 and LNCaP, respectively) (Figure 4B), in both cell lines.

Discussion

In this study, whole-exome sequencing analysis of PCa patients belonging to a family with criteria for hereditary PCa was performed, since no pathogenic variant in known PCa genes had been previously identified, either by targeted *BRCA1/2* or multigene testing. After bioinformatic and segregation analysis, four variants of interest were observed in *ACIN1, APOL2, PELP1*, and *CIB1* genes. Further expression and functional studies identified *APOL2* as the best candidate to explain the family phenotype.

Like many others in clinical practice, the family selected for this study fulfills criteria for hereditary PCa. However, commercially available tests did not disclose a genetic cause for the familial phenotype. Since several relatives consented to genet-

Table 2. Whole exome sequencing variants selected for further studies

Chr	Pos GRCh38	Ref	Alteration	Gene
14	23079574	AGAACGT-GAACGTGA	AGAACGT-GAACGT GAACGTGA	*ACIN1*
2	70984816	TGCCCAAGC-CCA	TGCCCA	*ANKRD53*
22	36239444	GG-GATCTTCCTCTG	GG	*APOL2*
17	21703981	C	T	*KCNJ18*
12	52795629	ACCAAAGCCAC-CAG CCCCTCCAAAG-CCA CCAGCCCCTC-CAAA GCCACCAGC	ACCAAAGC-CACCAG CCCCTC CAAAGCCAC-CAGC	*KRT3*
12	55221522	TCC	TC	*OR10A7*
17	4672141	ATCCTCCTC	ATCCTC	*PELP1*
1	3186163	C	T	*PRDM16*
13	24692541	G	A	*ATP12A*
2	74492217	C	G	*TTC31*
3	75738469	T	C	*ZNF717*
15	90231215	TAGAGAG	TAG	*CIB1*
11	1017183	G	T	*MUC6*

Table 3. Segregation analysis. Validation of variants by Sanger sequencing previously obtained in WES analysis.

		samples						
		brothers	**sisters**					
Genes	**Consequence**	**III-1**	**III-2**	**III-3**	**III-5**	**III-7**	**III-8**	**III-9**
ACIN1	frameshift	**present**	**present**	**present**	**present**	**present**	**present**	**present**
ANKRD53	3_prime_UTR	**present**	absent	**present**	**present**	**present**	**present**	**present**
APOL2	frameshift	**present**	**present**	**present**	**present**	**present**	absent	**present**
KCNJ18	stop_gained	absent	absent	absent	absent	absent	absent	absent
KRT3	frameshift	**present**	absent	**present**	**present**	absent	absent	absent
OR10A7	frameshift	**present**	absent	**present**	**present**	absent	absent	absent
PELP1	frameshift	**present**	**present**	**present**	**present**	**present**	**present**	absent
PRDM16	missense	**present**	absent	**present**	**present**	absent	absent	absent
ATP12A	missense	present	absent	**present**	**present**	absent	absent	absent
TTC31	missense	present	absent	**present**	**present**	absent	absent	absent
ZNF717	missense	absent	absent	absent	absent	absent	absent	absent
CIB1	splice_acceptor	**present**	**present**	**present**	**present**	**present**	**present**	**present**
MUC6	missense	absent	absent	absent	absent	absent	absent	absent

Table 4. Frequency in healthy donors

	Frequency %	Age (average)
APOL2	0 % (0/100)	62 years
PELP1	3 % (3/100)	62 years

ic and segregation studies, and DNA and pathology specimens, were available, searching for new PCa genes through WES was decided.

Bioinformatics' analysis and stringent criteria to navigate the vast amount of WES data, allowed first the identification of variants in four genes (*ACIN1, APOL2, PELP1* and *CIB1*), selected either because they were predicted to be pathogenic based on in-silico prediction tools, or because of their previously described function [18-21]. Segregation analysis further narrowed the candidates to *APOL2* and *PELP1*, since the variants in *ACIN1* (coding a caspase-3-activated protein required for apoptotic chromatin condensation [21-23]) and *CIB1* (coding a calcium and integrin-binding protein 1 involved in cell survival, proliferation, migration, adhesion, and apoptosis [24]), genes were shared by all siblings. Additionally, *CIB1* upregulation frequently correlates with oncogenic mutations of *KRAS* (27), and these are infrequent in PCa (24, 25).

APOL2 and *PELP1* genes have an essential function in tissue homeostasis [18, 19], and our immunochemistry data revealed the maintenance of protein expression for both genes. These observations suggest that both frameshift variants (one in each gene) not affect protein expression, or the wild-type allele may have compensated for the frameshift variant identified. However, we observed a higher affinity of *APOL2* for prostate tissue, since contrasting with *PELP1, APOL2* expression was lower in all other tissues tested. Besides this higher affinity for prostate tissue, the *PELP1* variant was observed in healthy male controls, selecting *APOL2* as the top PCa candidate gene.

Is there a possible monogenic effect, for *APOL2*, that could explain the phenotype observed in the family included in this study? This gene encodes Apolipoprotein L2, a protein found to be upregulated in the brains of African-Americans schizophrenic patients [25], with functions mostly unknown [19]. *APOL1* and *APOL6* have been described as novel pro-death BH3-only proteins [19] that are also capable of regulating autophagy. *APOL2* has also been associated with autophagy-mediated by Bcl-2 [19] and described as an anti-apoptotic protein in the human bronchial epithelium stimulated by the cytotoxic effects of IFN-γ [26]. *APOL2* includes 6 exons, but most transcripts described include only 5 exons [27]. Importantly this gene was found to be highly expressed in the lung, pancreas, prostate, spleen, liver, and placenta [27]. We demonstrated that *APOL2* silencing led to decreased viability in PCa cell lines.

Our study is the first to describe the association of *APOL2* with PCa, but it is remarkable that its location on chromosome 22q.12, matches with one of the two recently identified PCa susceptibility loci [28, 29]. The functional studies we performed also add to the possible role of *APOL2* in the modulation of prostate cancer cell survival, adding to its recognized functions of autophagy and apoptosis (32, 39).

Our data cannot exclude the *PELP1* gene as a PCa gene of interest, involved either in monogenic or polygenic cancer risk. This gene encodes a protein expressed in both in the nucleus and cytoplasm (28) that is a co-regulator of several transcription factors and a substrate of several kinases (31). It also functions as a coactivator of the estrogen receptor (ER) being upregulated in breast cancer (31), and is involved in the androgen receptor complex [30], which has been proposed as a putative targeted therapy in PCa [30]. Neither *PELP1*, whose variant identified in this study may be a polymorphism, neither *ACIN1* nor *CIB1*, can be excluded as PCa genes of interest for future studies.

Conclusions

In this study, and to our knowledge, the *APOL2* gene is associated, for the first time, with PCa risk. Further to our research, its location on chromosome 22q.12, a recently identified PCa susceptibility locus [28, 29] reinforces APOL2 as a new PCa candidate gene.

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Conflicts of Interest

The authors declare no conflict of interest

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