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Original article

SPOP and MMR/MSI alterations in prostate cancer: relationship with PD-L1, TILs and AR expression

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Summary

Objective. Despite the promising introduction of anti-PD-L1 therapy for advanced stage of prostate cancer (PCa), recent studies have demonstrated limited success, suggesting the need to improve patient selection.

Methods. We retrospectively selected 153 PCa patients. We performed SPOP mutational analysis and evaluated PD-L1 expression, MMR/MSI status, TIL (as CD4/CD8 ratio), and the mRNA expression of AR and CD274. Using SPOP interfering-RNA in two PCa cell lines (LNCaP, PC3) and western-blot analysis, we examined the role of SPOP silencing on CD274 expression.

Results. Functionally altered SPOP mutations (14 out of 153 samples, 9.15%) and MMR/MSI status (3.3%) were associated with higher PD-L1 expression (both $p < 0.0001$), lower TIL ($p < 0.0001$ and $p = 0.0004$), and higher Gleason scores (both $p < 0.05$). SPOP-mutated patients exhibited significantly higher CD274, and AR mRNA expression compared to those without mutations ($p = 0.0006$ and $p = 0.0148$). Reducing SPOP expression in cancer cell lines resulted in a significant upregulation of PD-L1 expression.

Conclusions. Our analysis identifies SPOP mutations and MMR/MSI status as cofactors in high PD-L1 expression and CD8/TIL presence in PCa, representing potential markers for selecting patients who are more likely to respond immunotherapy or to combined treatment.

Key words: SPOP, MMR/MSI, Prostate cancer, Immunotherapy

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Introduction

Prostate cancer (PCa) remains a significant global health burden, ranking as the fifth most common cause of cancer-related death among males. While traditional treatment approaches, including surgery, radiation therapy, and androgen deprivation therapy, have demonstrated efficacy in localized and locally-advanced disease, the prognosis for patients with metastatic disease remains poor despite various treatments, including chemotherapy [1,2].

The advent of immune checkpoint inhibitors (ICIs) has transformed the

cancer treatment landscape, offering durable responses and improved survival outcomes in several tumour types. Unfortunately, the efficacy of ICIs in PCa has been modest, particularly in monotherapy. Nevertheless, in-depth analysis of clinical trials evaluating the efficacy of immunotherapeutic agents in metastatic or locally-advanced prostate cancer have shown that a small subgroup of patients exibited satisfactory objective responses to these drugs [1,2]. The reasons for these differing responses in this subset have not been thoroughly investigated, and the patient's enrolment did not consider tumour molecular characteristics. When assessed, only PD-L1 was evaluated with a low positivity threshold (1%) and often with different immunostaining scores [1,2]. In this way, a further analysis correlating the patient's cancer-immune system interaction with the molecular tumour characteristics could be useful in identifying possible predictive biomarkers that can accurately select PCa patients who are most likely to respond to ICI therapy [2].

SPOP (Speckle-type POZ protein) is an adaptor protein of cullin-based E3 ubiquitin ligase that plays a crucial role in the degradation of various proteins involved in cell growth, differentiation, and apoptosis. Inactivating mutations in the SPOP gene have been identified in approximately 6-15% of PCas, leading to the accumulation of its target proteins, such as the androgen receptor (AR) [3] or steroid receptor coactivator 3 (SRC3) [4]. promoting tumorigenesis [5]. Several studies have indicated that SPOP alterations play a pivotal role in genome stability via multiple pathways affecting impaired DNA damage repair (DDR) or the TOPO2A gene[5], and in immune-escape through increased PD-L1 expression and decreased numbers of cancer related CD8+/tumour-infiltrating lymphocytes (TILs) [6].

Microsatellite instability (MSI) results from defects in the DNA mismatch repair (dMMR) system, which normally corrects errors that occur during DNA replication. MSI or dMMR is associated with an increased number of mutations within the tumour and, consequently, in the production of neoantigens that can be recognised by the immune system. MSI status is frequently related to higher expression of PD-L1 and may help to identify patients who are more susceptible to ICIs in several tumours [7-9].

In this retrospective study, we aimed to investigate the relationship between SPOP mutations and MMR/MSI status with AR and PD-L1 expression in a cohort of PCa patients. We hypothesised that these molecular alterations may serve as predictive markers for PD-L1 expression and potentially guide ICI or combined therapies in PCa.

Materials and methods

Patients' features

A retrospective observational multicentre study was conducted at the Unit of Pathology and Urology of Fondazione Policlinico Universitario "Agostino Gemelli" – IRCCS, Catholic University of Sacred Heart, Rome, and at the Unit of Pathology and Urology of the University of Messina. The clinical and pathological records of 153 patients diagnosed with PCa after transrectal biopsy between January 2020 and January 2022 were reviewed. Two expert genitourinary pathologists (MM and FP) confirmed the diagnoses. Patients were eligible for inclusion in the study if they had: (i) pathologically confirmed prostate acinar adenocarcinoma, and (ii) availability of tumour tissue samples. Patients were excluded for: (i) inadequate tumour tissue, (ii) prior radiochemotherapy or other treatments, (iii) presence of other tumours, or (iv) incomplete clinical and follow-up data. All patient data were collected anonymously, and the study was conducted in accordance with the Declaration of Helsinki. All patients signed an informed consent form approved by the local Ethics Committee (number CE 2019-3668, Fondazione Policlinico Universitario "Agostino Gemelli" – IRCCS, Catholic University of Sacred Heart, Rome). This study followed REMARK criteria to identify new biomarkers [10].

SPOP mutational analysis

Genomic DNA from tumours was extracted from paraffin-embedded (FFPE) tissues using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Tissue samples contained at least 70% of the tumour. PCR was performed in reactions containing genomic DNA (100 ng), 0.2 µmol/L of primers, and 2x PCRBIO HS Taq Mix (PCR Biosystems Inc., Wayne, Pennsylvania, USA). SPOP exon 5, 6, 7, 8, and 9 amplifications were performed on a C1000 Touch Thermal Cycler (BioRad, Hercules, CA, USA). Primer sequences are reported in Supplementary Table SI. The fragments were separated by electrophoresis on 2% agarose gels containing ethidium bromide and visualised by UV illumination. PCR products were treated with EXOSap (UBS, Sial, Rome, Italy), following the manufacturer's protocol, and directly sequenced using a BigDye Terminator kit v3.1 (Applied Biosystem, Foster City, CA, USA) with forward and reverse primers in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) [11].

Immunohistochemistry for CD4, CD8, and PD-L1, and staining evaluation score

Briefly, 4-µm thick sections were obtained from formalin-fixed paraffin-embedded (FFPE) blocks. For antigen retrieval, deparaffinised and rehydrated sections were treated with citric acid buffer (pH 6.0) for three cycles of 5 minutes each in a 750 W microwave oven, followed by inhibition of endogenous peroxidase with 3% H_2O_2 for 10 minutes at room temperature. The sections were then incubated with a mouse anti-CD4 monoclonal antibody (Clone 4B12; Dako, Carpinteria, CA, USA) and a mouse anti-CD8 monoclonal antibody (Clone 1A5; Ventana Inc., Tucson, AZ). The primary antibodies were visualised using the avidin–biotin–peroxidase complex method (UltraTek HRP Anti-polyvalent; ScyTek, Logan, Utah, USA), according to the manufacturer's instructions. 3,3'-Diaminobenzidine tetrahydrochloride was used as the enzyme substrate to observe the specific antibody localisation, and Mayer's haematoxylin was used as a nuclear counterstain.[11] Human lymph node or tonsil tissues (for CD4, CD8, and PD-L1) were used as positive controls. Negative controls were obtained by replacing the primary antibody with a non-immune immunoglobulin of the same isotype or phosphate-buffered saline solution (PBS, pH 7.4). The results of immunohistochemical (IHC) reactions were independently evaluated by two pathologists (VF and FP) who were blinded to the clinicopathological data. Variations in the expression value within a range of 5% were re-evaluated on a consensus basis using a double-headed microscope [12].

The CD4+/CD8+ T cell tissue ratio was calculated by dividing the CD4+ T cell mean value by the CD8+ T cell for each specimen. IHC-stained samples were examined under a light microscope (Olympus BX-51). The TILs were counted in 10 different areas at 400× magnification for each case. Only TILs within the borders of prostate cancer were evaluated (within 0.5 mm of the tumour). The immune cell count was collected for each of 10 fields and averaged to calculate the mean number for one computerised 400× microscopic field (0.1590 mm2/field). Quantification of CD4+ and CD8+ T cells was performed microscopically and expressed as the average value of 10 high-power fields (HPFs) for each lineage. The immunohistochemical cut-off of 0.2, as the average of the values obtained, discriminates between samples with high or low CD4/CD8 ratios [12].

PD-L1 expression was evaluated using an anti-PD-L1 (Clone SP263) rabbit monoclonal antibody on the Ventana BenchMark platform (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions. Tumour cell PD-L1 expression was evaluated using the CPS score, expressed as the number of PD-L1 positive tumour cells, lymphocytes, and macrophages divided by the total number of viable tumour cells, multiplied by 100. Any perceptible and convincing partial or complete linear membranous staining of viable tumour cells that was distinct from cytoplasmic staining was considered positive PD-L1 staining and was included in the scoring. Likewise, any membranous and/or cytoplasmic staining of mononuclear inflammatory cells within tumour nests and/or adjacent supporting stroma was counted as positive PD-L1 staining and was included in the CPS numerator. Neutrophils, eosinophils, plasma cells, and inflammatory cells associated with in-situ components, benign structures, or ulcers were excluded from the CPS [13]. At least 100 viable tumour cells must have been present on the PD-L1-stained slide to be considered adequate for evaluation. Tumour cells must show partial or complete membrane staining to be counted as "stained," whereas immune cells are counted if any staining appears. An immunohistochemical cut-off of CPS≥1 was used to discriminate samples with high or low PD-L1 expression.

The appropriate cut-off for the CD4/CD8 ratio and PD-L1 was determined using ROC curve analysis (data not shown).

The agreement index (Cohen's K) between the two pathologists was very good for the different antibodies, ranging between k = 0.82 and k = 0.87.

Assessment of MMR/MSI status

MMR status was assessed on FFPE tumour tissue samples using antibodies against MLH1, MSH2, MSH6, and PMS2. Briefly, FFPE sections (4 µm thick) were mounted on positively charged glass slides. For antigen retrieval, deparaffinised and rehydrated sections were treated with citric acid buffer (pH 6.0), two cycles of 3 minutes each at 500 W, followed by inhibition of endogenous peroxidase with 3% H_2O_2 for 5 minutes. Then, sections were incubated for 1 hour at room temperature with mouse monoclonal anti-MLH-1, anti-MSH2, and anti-MSH6 (clone M1; clone G219-1129; clone 44; Roche, Monza, Italy) and mouse monoclonal anti-human PMS2 (clone 2G5; Novus Biologicals, Milan, Italy). The primary antibodies were visualised using the avidin–biotin–peroxidase complex. Samples were stained multiple times, and results were highly reproducible. Staining patterns of MMR proteins were evaluated using normal epithelial, stromal, or inflammatory cells, or lymphoid follicles as internal controls. Deficiency of any products of these four MMR proteins was classified as MMR-deficient (MMRd), while proficient MMR was determined if all MMR proteins were expressed.

MSI analysis was also carried out on all samples. DNA from tumour tissues was analysed with the EasyPGX ready MSI (Diatech Pharmacogenetics, Jesi,

Italy) following the manufacturer's protocol. MSS was defined if no instability at any of the loci was detected, MSI-low (MSI-L) was defined if instability at a single locus was detected, and MSI-high (MSI-H) was defined if two or more loci demonstrated instability. Only patients with MSI-high were classified as positive for microsatellite instability. The concordance rate between MMR and MSI status evaluation was 98.9%. Discordant cases were resolved by repeating the IHC (independently evaluated by two pathologists, MM and FP, and then re-evaluated on a consensus basis using a double-headed microscope) and MSI analysis on a different FFPE tissue block.

Real-time expression of AR and CD274

Total RNA was isolated from paraffin-embedded tissues using the RNeasy Kit (Qiagen, Milan, Italy) following the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the Im-Prom™ Reverse Transcription System (Promega, Milan, Italy) and random hexamers. Briefly, quantitative PCR of biological samples was performed in 20 µl total volume with 2 µl RT products, 10 µl of the 2× KAPA SYBR FAST Universal qPCR Kit, and 250 nM of each primer. All reactions were performed in triplicate. The specificity of each PCR product was validated by melting curve analysis at the end of the PCR cycles. The relative gene expression of CD274 and AR was calculated using the 2-DDCt method, and the expression data were normalised to endogenous β-actin. The sequences of the primers are reported in Supplementary Table SI.[14]

Cell lines, cell cultures, and transfections

LNCaP and PC3 cell lines from ATCC were cultured in RPMI supplemented with 10% FBS, l-Glutamine, non-essential amino acids and penicillin/streptomycin. Cells were transfected with 1, 10, and 20 nM of each SPOP interfering RNA (iSPOP1, iSPOP2, and iSPOP3), purchased from TriFECTa® RNAi Kit (IDT, IA, USA) following the manufacturer's protocol.[14]

Western-blot analysis for SPOP and CD274

Briefly, cell samples were incubated in hypotonic lysis buffer (10 mM Hepes, pH 7.5, 10 mM KCl, 3 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) without detergent to swell the cells. The plasma membrane of the swollen cells was then lysed by adding a non-ionic, non-denaturing detergent (NP-40, 0.5%), and the nuclei were pelleted. LNCaP and PC3 cells were extracted in 50 mM HEPES (H3375 Sigma-Aldrich, Rome, Italy), 1% Triton X-100 (T8787 Sigma-Aldrich), 100 mM NaCl (S7653 Sigma-Aldrich), 10 mM $MgCl_2$ (M8266 Sigma-Aldrich), 10% glycerol (G5516 Sigma-Aldrich), 0.5 mM dithiothreitol (DTT) (D9779 Sigma-Aldrich), 10 mM β-glycerophosphate (G9422 Sigma-Aldrich), 0.1 mM sodium orthovanadate (450243 Sigma-Aldrich), and protease and phosphatase inhibitor cocktail (PPC1010 Sigma-Aldrich) to obtain whole protein extract. Protein extracts were then denatured in sample buffer (Tris-HCl [pH 6.8], 10% SDS, 36% glycerine, 5% 2-mercaptoethanol, 0.03% bromophenol blue), boiled for 5 minutes, and then separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Gels were blotted with transfer buffer (30 mM Tris, 240 mM glycine, 20% methanol) directly onto pure nitrocellulose membrane (Bio-Rad, Milan, Italy) at 330 mA for 1 hour. Blots were probed with anti-SPOP (rabbit polyclonal antibody, 1:500 dilution, Thermo Fisher Scientific, Milan, Italy), CD274 (PD-L1, mouse monoclonal antibody, 1:500 dilution, Thermo Fisher Scientific), and β-tubulin (mouse monoclonal antibody, clone 2 28 33, 1:1000 dilution, Thermo Fisher Scientific) in TBST with gentle shaking. After membrane incubation with goat anti-mouse HRP-conjugated antiserum (1:1000; BD Biosciences, Milan, Italy) or goat anti-rabbit HRP-conjugated antiserum (1:1000; BD Biosciences) in TBST for 1 hour at room temperature with gentle shaking, blots were covered with enhancing chemiluminescence solution (GE Healthcare, United Kingdom) for 1 minute. The bands were subjected to densitometric analysis using the ChemiDoc Imaging Systems (Bio-Rad) or ImageJ software (NIH), after normalisation with the intensity of the reference gene [14].

Statistical Analysis

The objective of this analysis was to explore the correlation between clinical and biological parameters, SPOP mutations, MMR/MSI status, TILs (reported as CD4/CD8 tissue ratio), PD-L1 expression, and AR expression in a cohort of patients with PCa.

Statistical analysis was performed using GraphPad Prism 6 software (Graph Pad Software, San Diego, CA) and MedCalc version 19 (MedCalc Software, Mariakerke, Belgium) [12].

A statistical comparison of continuous variables was performed using the Mann-Whitney U-test (t-test), as appropriate. Categorical variables were compared using Chi-square statistics and Fisher's exact test (data is reported as a Forest plot). We calculated the inter-rater agreement (Kappa) using MedCalc software to evaluate the agreement between the two pathologists regarding immunohistochemistry. The area under the receiver operating characteristics (ROC) curve was calculated and tested for significance using the z-test. Statistical significance was set at $p < 0.05$.

Results

Table I. Patient characteristics.

	n = 153
Age, mean (±SD)	73 (8.4)
PSA at diagnosis *Mean (±SD)* *Median (Min, Max)*	 26 (9.4) 13.3 (2.25; 400)
T Stage at diagnosis, n (%) *1/2* *3/4*	 80 (52.3) 73 (47.7)
N stage at diagnosis, n (%) *0* *1*	 112 (73.2) 41 (26.8)
M stage at diagnosis, n (%) *0* *1*	 109 (71.2) 44 (28.8)
Gleason score *> 7* *≤ 7*	 40 (26.1) 113 (73.9)
SPOP, n (%) *mutated* *wildtype*	 14 (9.1) 139 (90.1)
MSI/MMR, n (%) *positive* *negative*	 5 (3.3) 148 (96.7)
PD-L1 expression, CPS (%) *< 1* *≥ 1*	 132 (86.3) 21 (13.7)
CD4+/CD8+ ratio (%) *≥ 0.2* *< 0.2*	 120 (78.4) 33 (21.6)

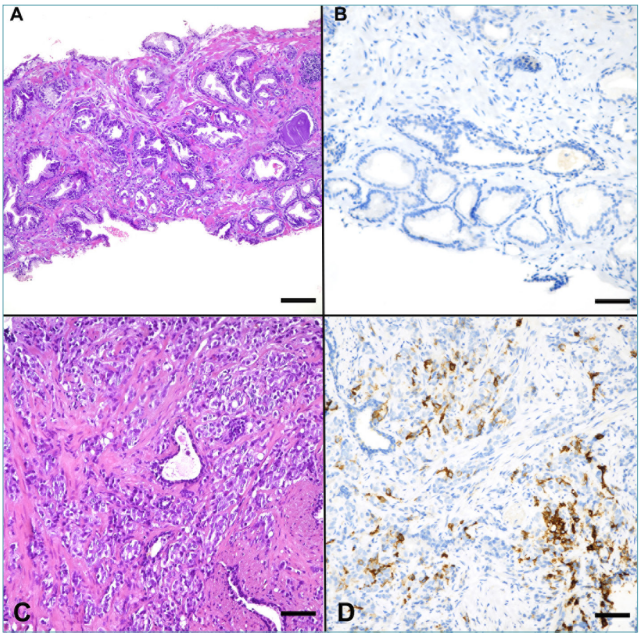

Figure 1. Immunohistochemical analysis of PD-L1 protein expression. The figure shows two representative cases of PCa (panel A and C, respectively, E&E, 100X magnification) analyzed with SP263 clone (panel B and D, respectively; 200x magnification), having higher PD-L1 expression (CPS > 1) in panel D and negative PD-L1 expression (CPS < 1) in panel B.

Clinical and molecular cohort characteristics

Table I shows the main clinicopathological and molecular characteristics of the 153 patients included in this study.

At the time of diagnosis, the mean age was 73 years, and the mean PSA serum level was 26 ng/ml. Seventy-three out of 153 (47.7%) patients had a T3/4 stage, while 41 (26.8%) patients had lymph node involvement and 44 (28.8%) had a metastatic stage. Forty out of 153 patients (26.1%) were diagnosed with a Gleason score greater than 7 (Gleason group 4-5). We identified a SPOP mutation in 14 patients (9.15%). F133 was the most frequently mutated SPOP residue, occurring in 8 out of 14 patients (57.1%; 4 patients with F133L mutation, 3 patients with F133S mutation, and 1 patient with F133V mutation), while 5 patients had a mutation of the F102 residue (35.7%; 3 patients with F102C mutation and 2 with F102V mutation), and 1 patient (7.1%) had a W131G mutation (Supplementary Figure 1). Five patients out of 153 (3.3%) exhibited MMR defect (3 with a loss of expression of MSH2/MSH6 and 2 with a loss of MLH1/PMS2 proteins), resulting in MSI-high. The concordance rate between MMR and MSI status evaluation was 98.9% (151 out of the 153 cases). Only two cases showed discordance between MMR status and MSI analysis. In detail, one case had loss of PMS2 protein expression, and the other one showed mild and very focal MSH6 immunostaining. These two cases, classified as MMRd, showed an MMS analysis. The IHC and MSI analysis was repeated on different FFPE tissue blocks, and MMR status, in accordance with MSI analysis, resulted proficient. Moreover, 21 patients (13.7%) had high PD-L1 expression with a CPS level greater than 1, while lower than 1 or negative PD-L1 expression was observed in 132 patients (86.3%; Fig. 1). The TILs, evaluated as a CD4/CD8 ratio, were below the 0.2 cut-off in 33 (21.6%) patients and above the 0.2 cut-off in 120 (78.4%) patients.

Correlation between clinical, biological, and molecular parameters

The principal correlations between clinicopathological and molecular parameters are reported in Figure 2.

We found a significant association between SPOP mutations and higher Gleason scores ($p < 0.0001$; OR 13.91, 95% CI from 3.639 to 53.16), higher PSA levels (cut-off 10 ng/ml; $p < 0.0001$; OR 0.06, 95% CI from 0.008 to 0.516), absence of metastatic status

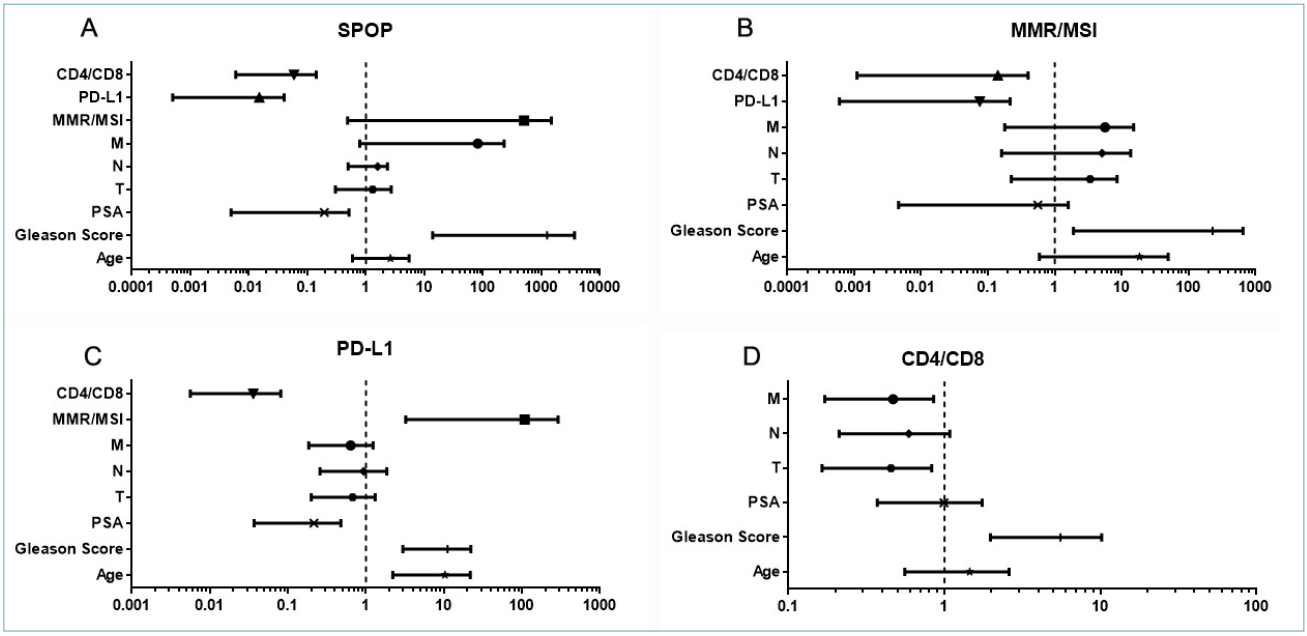

Figure 2. The figure shows the Forest plots regarding the correlation of SPOP expression (panel A), MMR/MSI status (panel B), PD-L1 expression (panel C) and TILs, as CD4/CD8 ratio (panel D), with the main molecular and clinic-pathological parameters.

(p = 0.011; OR 13.51, 95% CI from 0.788 to 231.8), higher PD-L1 expression (p < 0.0001; OR 0.005, 95% CI from 0.0005 to 0.0406), and lower CD4/CD8 ratios (p < 0.0001; OR 0.03, 95% CI from 0.006 to 0.142). No significant correlation was found between SPOP mutation and age, T stage, N stage, or MMR/MSI status (Fig. 2, panel A).

MMR/MSI status showed a significant association with higher Gleason scores (p = 0.001; OR 35.17 95% CI from 1.896 to 652.2), higher PD-L1 expression (p < 0.0001; OR 0.011, 95% CI from 0.0006 to 0.214), and lower CD4/CD8 ratios (p = 0.0004; OR 0.21, 95% CI from 0.0011 to 0.400). No significant correlation was found between MMR/MSI status and age, T stage, N stage, M stage, or PSA serum level (Fig. 2, panel B).

Higher PD-L1 expression (CPS ≥1) was significantly correlated with higher Gleason scores (p < 0.0001; OR 8.15, 95% CI from 2.988 to 22.25), age under 70 (p = 0.0003; OR 6.97, 95% CI from 2.218 to 21.90), higher PSA levels (cut-off 10 ng/ml; p = 0.0007; OR 0.135 95% CI from 0.038 to 0.48), and lower CD4/CD8 ratios (p < 0.0001; OR 0.021, 95% CI from 0.006 to 0.0812). No significant correlation was found between PD-L1 expression and T stage, N stage, or M stage (Fig. 2, panel C).

Moreover, we found a significant correlation between lower CD4/CD8 ratios and higher Gleason scores (p = 0.0005; OR 4.48, 95% CI from 1.973 to 10.18), metastatic status (p = 0.0284; OR 0.38, 95% CI from 0.171 to 0.853), and T stage (p = 0.018; OR 0.37, 95% CI from 0.168 to 0.830). No significant correlation was found between CD4/CD8 ratio and age, PSA serum level, T stage, or N stage (Fig. 2, panel D).

Correlation between SPOP mutation and higher CD274 and AR expression

We compared the AR and PD-L1 (CD274) mRNA expression in two patient groups: one with SPOP mutations (14 cases) and another group without SPOP mutations (28 cases). We found that the relative expressions of AR and CD274 were significantly higher (by approximately 2.1 and 2.6 times, respectively) in patients with SPOP mutations compared to those without alterations (p = 0.0006, Figure 3 panel A, for CD274; p = 0.0148, Fig. 3 panel B, for AR).

Silencing of the SPOP gene resulted in higher CD274 expression in two prostate cancer cell lines

We examined the ability of specific SPOP siRNAs to reduce the CD274 protein level in two PCa cell lines, LNCaP and PC3. We selected these two PCa cell lines as they are known to have SPOP wild-type [15]. We transfected LNCaP and PC3 cells with three different SPOP-specific siRNAs. The western blot analysis showed that the different SPOP-specific siRNAs had

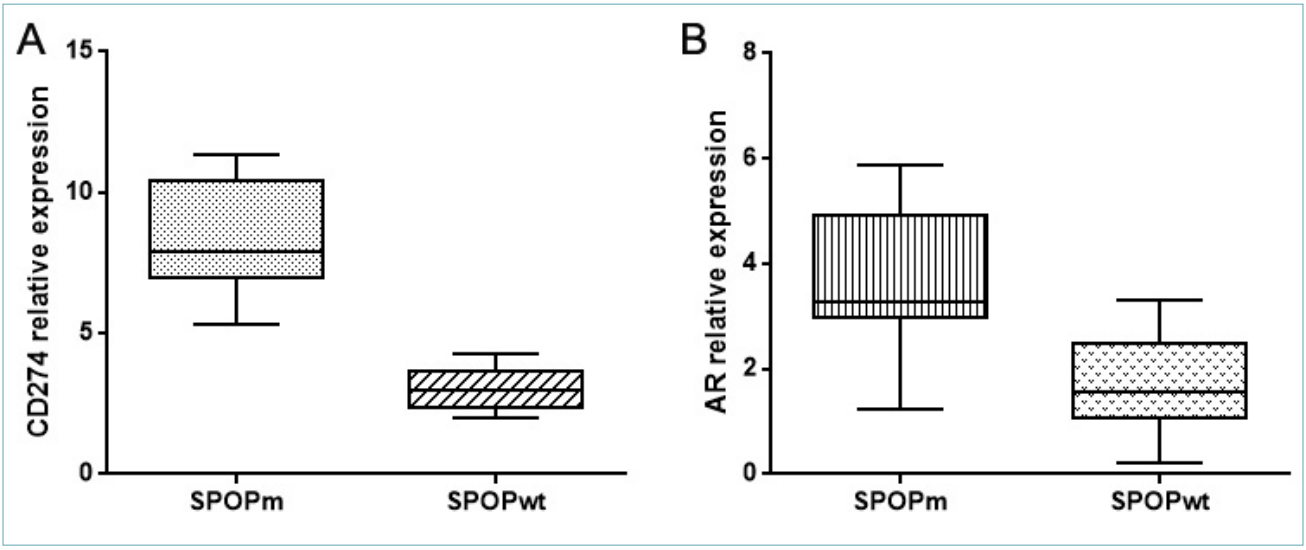

Figure 3. The figure shows the significant high expression of CD274 and AR in two PCas subgroups with SPOP mutations in comparison to those without mutations (p = 0.0006 and p = 0.0148, respectively).

varying levels of efficiency in downregulating the expression of the SPOP protein (Fig. 4, panel A and B). The greatest interference in SPOP protein expression was achieved by transfection with siRNA-3 (86.4% in LNCaP cell line and 66.7% in PC3 cell line; Fig. 4, panel A, B, and C) and, to a lesser extent, with siRNA-1 (75.1% in LNCaP cell line and 63.3% in PC3 cell line; Fig. 4, panel A, B, and C). The cells transfected with siRNA-2 exhibited no significant reduction in SPOP protein levels (data not shown). Alongside the reduction of SPOP expression, we conversely found a significant upregulation of CD274 expression of 1.6 times (in LNCaP cell line) and 1.2 times (in PC3 cancer cell line) using SPOP siRNA-1, and of 4.2 times (in LNCaP cell line) and 1.8 times (in PC3 cancer cell line) using SPOP siRNA-3 (Fig. 4, panel C).

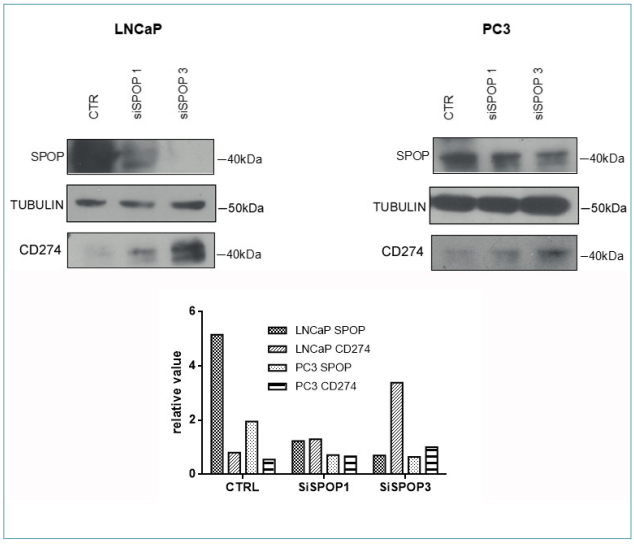

Figure 4. The figure shows the significant down-regulation of SPOP protein in two PCa cell lines (LNCaP cell line, in panel A and PC3 cell line, in panel B) after transfection with two specific siRNA (siSPOP1 and siSPOP3) and the contemporarily up-regulation of CD274 (PD-L1) in the same cell lines. Panel C shows the graphic representation of the relative quantization of SPOP and CD274 proteins in the panel A and B.

Discussion

This study investigated the relationship between SPOP mutations, microsatellite instability, and the expression of AR, PD-L1, TILs (as CD4/CD8 ratio), and the main clinical features in a cohort of prostate cancer patients.

As previously reported in other studies, SPOP acts as a tumour suppressor gene in many tumour types, including PCa. Silencing mutations of SPOP lead to the inactivation of this gene, promoting the stabilisation of SPOP targets and determining the malfunction of various key regulatory pathways involved in the tumourigenesis of this cancer [5,6,16,17]. In line with the literature, we found SPOP mutations, all in the MATH domain, in 9.15% of our patients. Moreover, patients with SPOP

mutations exhibited significantly higher PD-L1 expressions, lower CD4/CD8 ratios (both $p < 0.0001$), and high levels of AR mRNA expression ($p = 0.0148$). As described, the inactivating SPOP mutations result in reduced function of this E3 ubiquitin ligase adaptor protein, leading to decreased degradation of multiple protein substrates, including the androgen receptor, steroid receptor coactivator, and PD-L1 [6,17,18]. We also confirmed this molecular mechanism in an in-vitro experiment, where, using two specific SPOP siRNAs in two SPOP-wild-type prostate cancer cell lines (LNCaP and PC3), we demonstrated a significant increase in the expression of the CD274 protein (PD-L1) concurrent with SPOP downregulation. Unlike Cavalcante L. et al., who reported that patients with SPOP mutations had a tumour microenvironment characterised by a low presence of CD8+ cells with no significant increase in PD-L1 compared to non-mutated cases [19], we found that mutated patients had a tumour-related infiltrate rich in CD8+ cells, the main effectors of the antitumour immune response, and, in agreement with others [6], significantly high PD-L1 levels. This apparent discordance can be explained by comparing the different techniques used for evaluating the tumour immune infiltrate: tissue-specific cell RNA analysis versus the peritumoral evaluation of CD4 and CD8 lymphocytes in our work. Furthermore, we used the clone antibody SP263 and the CPS score for evaluating PD-L1 expression, in comparison to the SP142 clone and TPS used in the report by Cavalcante L. et al. [19].

SPOP mutations are involved in genome instability via multiple pathways affecting the DNA damage repair system (DDR). This could, in turn, lead to an increase in PD-L1 expression, as frequently demonstrated in several tumours [20], and a greater antitumour inflammatory response (increased presence of neoantigens), which would promote CD8+ cell recruitment [21] and, through a greater microenvironment secretion of interferon gamma, PD-L1 upregulation [12,22].

In agreement with the literature, our cohort showed that patients with SPOP mutations had a significant association with high pretreatment serum PSA levels, high Gleason scores, and a non-metastatic disease status [23,24].

Likewise, we also found a significant association between microsatellite instability and high levels of PD-L1 expression and lower CD4/CD8 values ($p < 0.0001$ and $p = 0.0004$, respectively). Consistent with previous reports, we found a prevalence of 3.3% of MMR defects in our cohort.[7] The association between PD-L1 expression and MMR/MSI status is debated and not consistent across different clinical studies; this discrepancy largely depends on the different methodologies used to evaluate the state of microsatellite instability (MMR or MSI) and the various types of antibodies, platforms, scores, and cut-offs employed to quantify PD-L1 expression [7,9,25]. In our cohort, although the number of cases was limited, there is a significant association between MMR/MSI and PD-L1 expression, suggesting that there may be a pathogenetic mechanism in this type of cancer similar to that described in other tumour types.

The association between MMR/MSI status and a greater infiltrate of peritumoral CD8+ cytotoxic lymphocytes is also debated; however, in line with what was reported by some authors, our data seem to support this hypothesis [25].

From a clinical perspective, and in accordance with literature data, we also found a significant association between MMR/MSI and high Gleason scores [24,25].

Although ICI therapy has improved survival and response outcomes with a favourable safety profile in many advanced malignancies, its efficacy has been modest in patients with prostate cancer [26]. Nevertheless, the good response to these drugs in some patients with metastatic PCa (especially those with dMMR/MSI-high), the debated and “heterogeneous” use of predictive signatures regarding PD-L1 evaluation or dMMR/MSI-high in PCa monotherapy trials, and the recent clinical studies highlighting the significant response of advanced PCa to ICIs in combination with other treatments, have raised the need for a more in-depth study of genetic alterations, immunological mechanisms, and the microenvironment in this type of cancer. This would specifically identify immunotherapy biomarkers, even in combination, which can better select patients who are most likely to respond to ICI treatment [26]. In this scenario, SPOP mutations and MMR/MSI status may influence the tumour immune microenvironment (TILs) and PD-L1 expression in PCa, suggesting potential correlations between these molecular alterations and the response to immune checkpoint inhibition (ICI) therapy, especially in combination treatment with anti-androgen receptor therapy, given the effect of SPOP mutations on increased AR expression in this tumour. In a clinical and diagnostic scenario, it could be considered beneficial to introduce immunotherapy, in association or not with other drugs, in a subset of PCa patients with advanced or metastatic prostate cancer, for whom there are no other therapeutic options. In these patients, it could be useful to perform a mutational analysis of the most frequently mutated exons of the SPOP gene and MMR/MSI analysis, to identify who could best respond to immunotherapy. If, through further prospective studies with a larger case series, this were to prove valid, the tests could also be extended to other groups of patients with early stages of PCa. The main

limitations of our study are its retrospective design and the relatively small, albeit homogeneous, cohort of patients, necessitating confirmation of our data in other independent studies, including prospective trials with combined therapy, to validate our parameters as a prognostic tool.

Conclusion

In conclusion, this study provides preliminary evidence suggesting that SPOP mutations and MMR/MSI status may serve as potential biomarkers for PD-L1 expression and ICI therapy response, including combined treatment protocols (for example, with anti-androgen receptor therapy) in PCa. Further research is necessary to confirm these findings and develop more personalised treatment strategies for PCa patients.

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Conflicts of interest statement

The authors declare no conflict of interest.

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Authors' contributions

V.F., E.G., F.P. and M.M. were the principal authors and the main contributors in writing the manuscript. A.T. and V.F. collected the clinical data. A.O., E.G., E.Gu., G.R. and V.Z. analysed and interpreted the patient data and reviewed the literature. M.M., V.F., F.P. and I.V.Z. performed the immunohistochemistry analysis. S.C. and T.C. performed the mutational analysis. E.G. performed the real-time analysis. E.Gu. and S.D. performed the cell lines transfection and western-blot analysis. M.M., V.F., A.T., C.R. and G.F. read and corrected the manuscript. G.F. and V.F. corrected the English language errors. All authors read and approved the final manuscript.

Ethical consideration

This study was approved by the Institutional Ethics Committee of Fondazione Policlinico Universitario "Agostino Gemelli", IRCCS, Roma (approval number CE 2019-3668).

The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki. Written informed consent was obtained from each participant/patient for study participation and data publication. The report does not present identifying images or other personal or clinical details of participants that compromise anonymity.

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