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Research article

PP1 catalytic isoforms are differentially expressed and regulated in human prostate cancer

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ABSTRACT

The Ser/Thr-protein phosphatase PP1 (PP1) is a positive regulator of the androgen receptor (AR), which suggests major roles for PP1 in prostate carcinogenesis. However, studies dedicated to the characterization of PP1 in PCa are currently scarce. Here we analyzed the expression and localization of the PP1 catalytic (PP1c) isoforms in formalin-fixed, paraffin-embedded prostate tissue samples, as well as in PCa cell lines. We also analyzed well-characterized PCa cohorts to determine their transcript levels, identify genetic alterations, and assess promoter methylation of PP1c-coding genes. We found that PP-1A was upregulated and relocalized towards the nucleus in PCa and that *PPP1CA* was frequently amplified in PCa, particularly in advanced stages. PP-1B was downregulated in PCa but upregulated in a subset of tumors with *AR* amplification. PP-1G transcript levels were found to be associated with Gleason score. PP1c-coding genes were rarely mutated in PCa and were not prone to regulation by promoter methylation. Protein phosphorylation, on the other hand, might be an important regulatory mechanism of PP1c isoforms' activity. Altogether, our results suggest differential expression, localization, and regulation of PP1c isoforms in PCa and support the need for investigating isoform-specific roles in prostate carcinogenesis in future studies.

1. Introduction

The Ser/Thr-protein phosphatase PP1 (PP1) regulates the activity of several tumor suppressor and oncogenic proteins, thereby determining the flow of key oncogenic signaling cascades [1]. It exists within cells as an oligomer composed by a catalytically active and highly efficient subunit (PP1c), which is coupled to at least 1 regulatory subunit (PP1r) to compensates for the lack of PP1c's substrate specificity.

PP1 functional multiplicity has been mostly attributed to PP1rs, which also exist as individual entities and have PP1-independent cellular functions [2]. However, increasing evidence suggests that, at the most basic level, PP1 diversity is also determined by differential expression and localization of PP1c isoforms [3–6].

In human cells, PP1c is encoded by 3 distinct genes—*PPP1CA* (11q13.2), *PPP1CB* (2p23.2), and *PPP1CC* (12q24.11)—giving rise to 3 highly homologous canonical isoforms: PP-1A, PP-1B, and PP-1G, respectively. Their central cores are conserved and only minor differences are registered at terminal regions [7]. Canonical PP1c isoforms are believed to be ubiquitously expressed and their expression have been demonstrated in a variety of cancers. However, they remain barely investigated in oncology even though an increasing number of studies suggest non-redundant and even antagonizing roles [8,9].

Abbreviations: AR, androgen receptor; CRPC, castration-resistant prostate cancer; ETS, erythroblast transformation-specific; GS, Gleason score; IHC, immunohistochemistry; IRS, immunoreactive score; NEPC, prostate neuroendocrine carcinoma; NPT, normal prostate tissue; PAP, prostatic acid phosphatase; PCa, prostate cancer; PIN, prostatic intraepithelial neoplasia; PP1, serine/threonine-protein phosphatase PP1; PP1c, PP1 catalytic subunit; PP1r, PP1 regulatory subunit; PSA, prostate-specific antigen; Ser, serine; Thr, threonine; Tyr, tyrosine.

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In PCa, PP1 enhances the stability and transcriptional activity of the androgen receptor (AR), a central player in all stages of prostate carcinogenesis and progression to metastatic castration-resistant PCa (CRPC)—the leading cause of mortality associated with the disease [10–14]. Besides the canonical AR isoform, PP1 also upregulates the splicing variant AR-V7, which lacks ligand-binding domain and, therefore, has been implicated in cancer cell survival during androgen deprivation therapy [14]. Despite the strong evidence suggesting major roles for PP1 in PCa, comprehensive studies devoted to the characterization of PP1c isoforms in PCa are still lacking.

In this study we aimed to investigate the expression of PP1c isoforms and eventual associations with clinicopathological parameters. Our results show that PP1c isoforms are differentially regulated in normal prostate and PCa, uncovering the need for future dedicated studies to understand their potentially non-redundant functions in prostate carcinogenesis. Moreover, we suggest that PP-1A and PP-1G worth further investigation as diagnostic and prognostic marker, respectively.

2. Materials and methods

2.1. Human samples

After approval by the institutional review board (Comissão de Ética para a Saúde do Instituto Português de Oncologia do Porto, CES-IPOFG_EPE 019/08), formalin-fixed, paraffin-embedded tissue samples (prostate tumor tissue, n = 12, and morphologically normal prostate tissue [NPT], n = 4) were obtained from the archive of the Department of Pathology of the Portuguese Oncology Institute of Porto, Portugal. NPT was collected from the peripheral zone of patients submitted to cystoprostatectomy due to bladder cancer. Tissue collection and histopathological evaluation was performed according to institutional guidelines as previously described [15]. Relevant clinicopathological data was collected retrospectively from medical records (Table 1).

2.2. Immunohistochemistry

Immunohistochemistry (IHC) studies were performed using the Novolink™ Polymer Detection System (Leica Biosystems, Wetzlar, Germany) as previously described [16]. Antigen retrieval was heat-induced in a microwave in the presence of 1 × sodium citrate buffer solution, pH 6.0, for anti-PP-1G antibody, or 1 × EDTA buffer solution, pH 8.0, for anti-PP-1A and anti-PP-1B antibodies, as determined by initial optimization tests. Tissue sections were incubated with the primary antibody (1:500) for 1h at room temperature (RT), in a humidified chamber.

Three slides were used from each patient included in the study (1 for each isoform) and the experiments were performed in parallel. Positive controls were selected from the analysis of the Human Protein Atlas database (https://www.proteinatlas.org/humanproteome/tissue, date of access: Feb 2019) [17]. Slides were analyzed by an experienced Uropathologist regarding the percentage of positive cells, the staining intensity of immunoreactive cells, and the cellular localization of the signal. Immunostaining results were expressed as immunoreactive score (IRS, range 0–6) that reflected the positivity score (0: no positive cells; 1: 1–50% positive cells; 2: 51%–90% positive cells; 3: more than 90% positive cells) plus the staining intensity score (0: no cell staining; 1: weak cell staining; 2: moderate cell staining; 3: strong cell staining). Protein localization was classified as present in the cytoplasm, nucleus, or both.

Table 1
Clinicopathological data of the PCa patients.

Characteristics	Study population (N = 12)
Age, years	64 (60–75)
Initial PSA, ng/mL	10.2 (4.8–17.5)
Pathological stage, n (%)	
pT2b	3 (25.0)
pT2c	2 (16.7)
pT3a	6 (50.0)
pT3b	1 (8.30)
Gleason score, n (%)	
6 (3 + 3)	5 (41.7)
7 (3 + 4)	5 (41.7)
8 (3 + 5)	2 (16.7)

Values are presented as median (range) unless otherwise indicated.

2.3. Cell culture

Human prostate epithelial cells (PNT2 cell line) and PCa cells (LNCaP [androgen-dependent] and PC3 [androgen-independent] cell lines) were grown in RPMI 1640 Medium with L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin solution (5000 U/mL). All media and supplements were from Gibco™ (by Thermo Fisher Scientific, Massachusetts, USA). Cell cultures were maintained at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. Cells were routinely checked for mycoplasma contamination and kept at low passage for the assays.

2.4. Antibodies

Rabbit polyclonal anti-PP-1A and anti-PP-1G antibodies were previously produced in-house [4]. Mouse monoclonal anti-PP-1B (sc-365678) and anti-tubulin beta chain (β-tubulin, sc-5274) antibodies were acquired from Santa Cruz Biotechnology (Texas, USA). Rabbit monoclonal anti-PP-1A phospho-Thr320 antibody (ab62334) was obtained from Abcam (Cambridge, UK). Secondary antibodies IRDye® 800CW goat anti-mouse and IRDye 680RD® goat anti-rabbit were acquired from LI-COR® Biosciences (Nebraska, USA). Goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody Alexa Fluor® 594 (A-11037) and goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody Alexa Fluor® Plus 488 (A32723) were from Invitrogen™ (by Thermo Fisher Scientific, Massachusetts, USA).

2.5. Western blot

Cells were lysed in 1 × RIPA lysis buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) (Merck™, Darmstadt, Germany) supplemented with 1 × cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and 1% Phosphatase Inhibitor Cocktail II (Alfa Aesar by Thermo Fisher Scientific, Massachusetts, USA). Whole-cell extracts were sonicated and centrifuged at 16,000×*g* for 20 min at 4 °C. Supernatants were collected and protein concentration was determined using the Pierce™ BCA Protein Assay (Thermo Scientific™ by Thermo Fisher Scientific, Massachusetts, USA), according to the manufacturer's instructions. Equal amounts of protein (30 μg) were diluted in 4 × Laemmli buffer, boiled for 5 min, and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gels were electrotransferred onto GE Healthcare Amersham™ Protran™ NC Nitrocellulose Membranes, 0.45 μm pore size (GE Healthcare, Illinois, USA). Membranes were blocked in 5% Blotto non-fat dry milk (Santa Cruz Biotechnology, Texas, USA) or 5% albumin bovine fraction V (BSA) for phospho-antibody, in tris-buffered saline (TBS), for 1 h at RT. Incubation with the primary antibodies occurred for 2 h at RT using the following dilutions: anti-PP-1A (1:5000), anti-PP-1B (1:1000), anti-PP-1G (1:5000), anti-PP-1A phospho-Thr320 (1:1000), and anti-β-tubulin (1:1000). After being washed in TBS with 0.1% Tween™ 20 (TBS-T) (Fisher BioReagents™ by Thermo Fisher Scientific, Massachusetts, USA), membranes were incubated with either IRDye® 800CW goat anti-mouse (1:10,000) or IRDye 680RD® goat anti-rabbit (1:20,000) for 1 h at RT. Membranes were scanned using the Odyssey Infrared Imaging

System (LI-COR® Biosciences, Nebraska, USA).

The expression levels of PP1 isoforms were represented as relative abundance to the loading control, β-tubulin.

2.6. Immunofluorescence

Cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.2% Triton™ X-100 (Fisher BioReagents™ by Thermo Fisher Scientific, Massachusetts, USA) in 1 × PBS for 15 min. Non-specific binding sites were blocked with 5% normal goat serum (Sigma-Aldrich®, Missouri, USA) and 1% BSA (NZYTech, Lisbon, Portugal) in 1 × PBS/0.2% Triton™ X-100 for 1 h. Cells were then incubated for 1 h with the primary antibodies—anti-PP-1A (1:250), anti-PP-1B (1:100), or anti-PP-1G (1:500), diluted in 1% BSA/1 × PBS/0.2% Triton™ X-100. After washing, cells were incubated with goat anti-Rabbit IgG, Alexa Fluor 594 (1:1000) or goat anti-Mouse IgG, Alexa Fluor Plus 488 (1:1000) for 1 h, protected from light. Nucleus were stained with Hoechst 33258 (Abcam, Cambridge, UK) and coverslips were mounted onto microscope slides using Mowiol mounting medium. All steps were performed at RT. Images were acquired in an Olympus IX81 motorized inverted microscope (Olympus®, Lisbon, Portugal).

2.7. Databases and data mining

2.7.1. Catalogue of somatic mutations in cancer (COSMIC)

The occurrence of somatic point mutations in PP1c-coding genes in PCa was investigated through COSMIC database, v90, release date Sep 5, 2019 (https://cancer.sanger.ac.uk/cosmic) [18]. COSMIC is the largest repository of somatic mutations occurring in human cancers and provides comprehensive data to explore their impact [18]. The search was performed using the Genome Reference Consortium Human Build 38 (GRCh38) genome version. *PPP1CA*, *PPP1CB* and *PPP1CC* were searched in all screen types, using the coordinate system 'Amino-acid', and the tissue filter specified to 'Prostate'.

2.7.2. cBioPortal for cancer genomics

Insights into PCa genomics were also obtained through comprehensive search in cBioPortal, v3.2.2, release date Feb 6, 2020 (https://www.cbioportal.org) [18–20]. This platform was developed at the Memorial Sloan Kettering Cancer Center to allow the integration and exploration of datasets from large-scale genomic studies. At the time of this study, cBioPortal integrated 21 PCa studies, accounting for a total of 6836 samples from 6550 patients. These included primary and metastatic samples from different cancer types, namely prostate adenocarcinoma, CRPC, and prostate neuroendocrine carcinoma (NEPC). We first queried cBioPortal for mutations and copy-number alterations in PP1c-coding genes in a global case set consisting of all samples analyzed in the 21 PCa studies. After determining the studies with relevant data for our study, we selected the most appropriated to use in each analysis considering the type of genomic and clinical data provided by each dataset (Suppl. Table 1). Since some samples were common to more than 1 dataset, we also took this into account when choosing the datasets to avoid data duplication.

2.7.3. UALCAN

Expression of PP-1A, PP-1B and PP-1G at transcript levels was analyzed using The Cancer Genome Atlas (TCGA) datasets—the benchmark of cancer genomics [21]—through the web resource UALCAN (http://ualcan.path.uab.edu/index.html) [22]. UALCAN is a user-friendly tool that allows to perform differential analyses from RNA-Seq Level-3 expression data. It includes 2 prostate datasets: prostate adenocarcinoma and metastatic PCa (MET500). By data mining these datasets, we compared the expression of PP1cs between primary prostate tumors and prostate normal tissues, as well as across tumors with different Gleason score (GS) and molecular signatures. We also analyzed promoter methylation levels, which were defined as beta value ranging from 0 (unmethylated) to 1 (fully methylated). Hypermethylation and hypomethylation were considered for beta values of 0.5–0.7 and 0.25–0.3, respectively.

2.8. Statistical analysis

Statistical significance of tissue- and cell-based assays was calculated using chi-square, Kruskal-Wallis, or Mann-Whitney tests, wherever appropriate. Statistical analysis was performed by using the IBM® SPSS® Statistics software, version 25.0 (New York, United States). Statistical analysis of genomic studies was performed via UALCAN database using the *t*-test to estimate the significance of difference in gene expression levels between groups [22]. The significance level was set to 0.05.

3. Results

3.1. Expression and localization of PP1c isoforms in NPT and PCa tissues

All samples were positively stained for the 3 isoforms (representative figures in Fig. 1a), albeit with varying intensities (Suppl. Table 2). The IRS calculated for each isoform in each specimen was comparable in NPT—100% of the samples scored 4 for PP-1A and PP-1G and 5 for PP-1B—, while variations were observed among PCa samples (Suppl. Table 2). Mean IRS showed that PP-1A, but not PP-1B and PP-1G, was overexpressed in PCa ($p = 0.0039$, Fig. 1b).

The localization of PP1c isoforms was consistent in all NPT samples. PP-1A and PP-1G were detected in the cytoplasm, while PP-1B was detected in both cytoplasm and nucleus (Fig. 1c). In contrast, their localization varied among PCa samples. Cytoplasmic and nuclear localization was observed in 67%, 75%, and 25% of cases for PP-1A, PP-1B, and PP-1G, respectively (Fig. 1c). These results suggest PP-1A re-localization towards the nucleus in PCa ($p = 0.021$; Fig. 1c). In the remaining cases, positive staining was restricted to the cytoplasm (Fig. 1c). Of mention, at least 1 isoform was present in the nucleus in each specimen (data not shown).

No significant associations were identified between protein expression/localization and patients' clinicopathological parameters, including pT stage (pT2: $n = 5$; pT3: $n = 7$) and GS (GS6: $n = 5$; GS7: $n = 5$; GS8:$n = 2$) for any PP1c isoform (Suppl. Fig 1).

3.2. Expression and localization of PP1c isoforms in androgen-dependent and castration-resistant cell lines

PP-1A was expressed at lower levels in LNCaP cells than in PC3 and PNT2 cells, which exhibited similar levels (Fig. 2a). This might suggest a more prominent role for PP-1A in androgen-dependent rather than in castration-resistant stages. The expression of PP-1G was increased in PCa cells, particularly PC3 cells, when compared to normal-like prostate cells (Fig. 2a). PP-1B was found in similar levels in all cell lines (Fig. 2a).

We also investigated PP1 phosphorylation using a phospho-specific antibody for the most widely recognized phospho-residue, Thr320. The results suggested that PP1c isoforms might be differentially regulated between androgen-dependent and castration-resistant cells (Fig. 2b).

Immunofluorescence analysis revealed differences in PP1c isoforms' cell distribution patterns (representative figures in Fig. 2c). In LNCaP cells, PP-1A and PP-1G were found to be particularly localized in the nucleus, while PP-1B was minimally localized or even totally absent from the nucleus (Fig. 2c). All isoforms were observed in the cytoplasm of both cell lines, albeit distinctive distribution patterns had been identified: PP-1A was more restricted to the perinuclear space than PP-1G, which was found widely dispersed within the cytoplasm; PP-1B showed cytoplasmic and membrane staining consistent with filament network and cell-cell adhesion configurations (Fig. 2c).

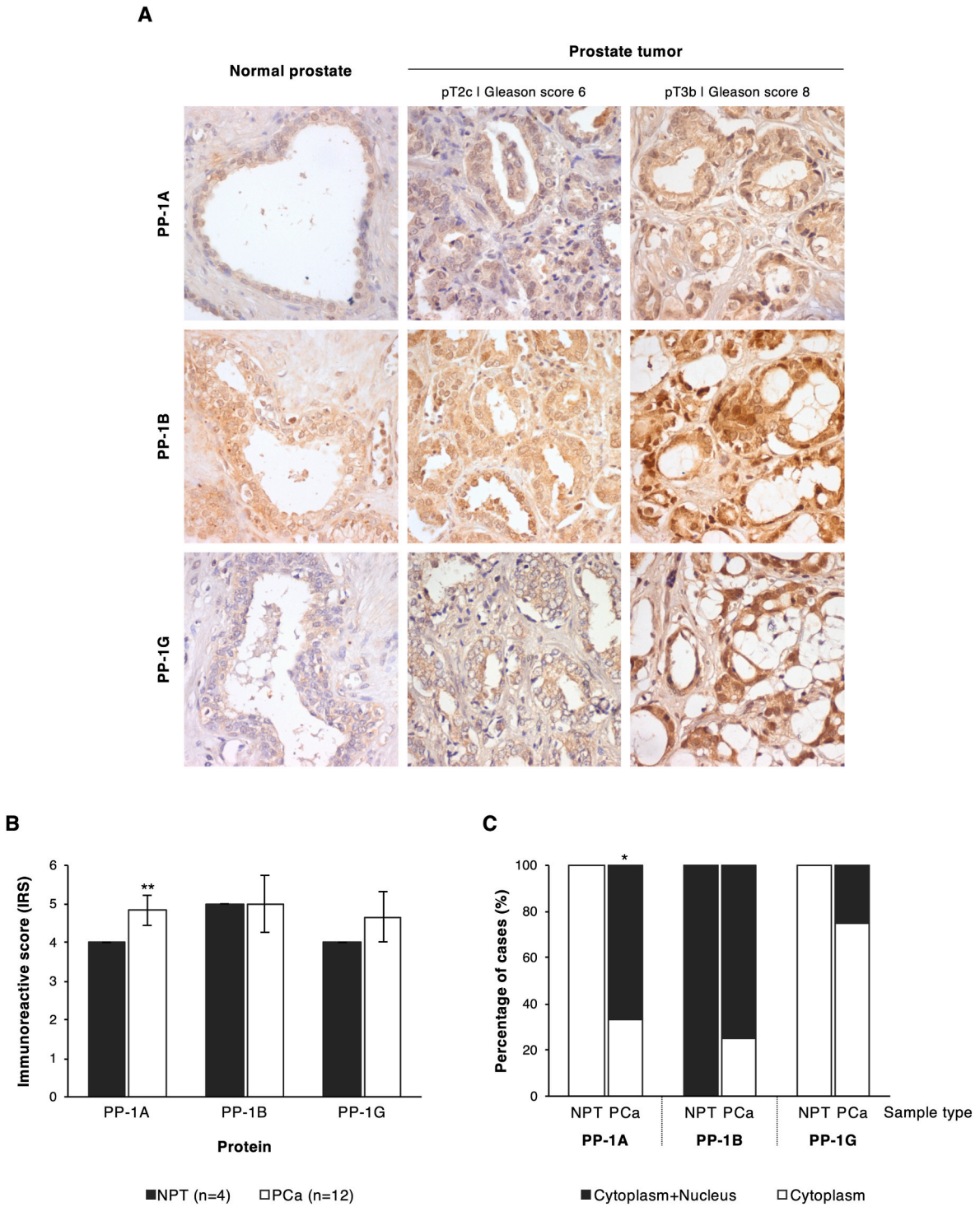

Fig. 1. Immunoexpression of PP1c isoforms in normal prostate (NPT) and prostate cancer (PCa) tissues. A – Representative images of the immunostaining of PP-1A, PP-1B and PP-1G (top to bottom) in NPT and PCa with increasing aggressivity (left to right) (magnification × 400). Nuclei were counterstained with hematoxylin solution. The images in each column corresponds to the same patient. B – Mean immunoreactive score of each isoform in NPT (n = 4) and PCa (n = 12). Results are expressed as mean ± SD. Statistical significance was calculated using the Mann-Whitney test. C – Localization of the isoforms in the cases assessed. Results are expressed as percentage of cases observed in cytoplasm or in cytoplasm and nucleus. Statistical significance was calculated using the Chi-square test. *p < 0.05, **p < 0.01.

3.3. Expression of PP1c transcripts in TCGA PCa cohorts (normal vs tumor)

PP-1A was upregulated (p = 1.96E-05; Fig. 3a) and PP-1B was downregulated (p = 0,009; Fig. 3b) in prostate primary tumors when compared to normal tissue. On the other hand, despite the similar levels observed for PP-1G in normal prostate and primary prostate tumors (Fig. 3c), it seems to be particularly overexpressed in tumors with higher GS (Fig. 3d). GS6 tumors exhibited significantly lower PP-1G levels than GS7 (p = 0.025), GS8 (p = 0.009), GS9 (p = 0.004), and GS10 tumors (p = 8.19E-04) (Fig. 3d). PP-1G was also significantly overexpressed in GS10 tumors when compared to GS7 tumors (p = 0.017) (Fig. 3d).

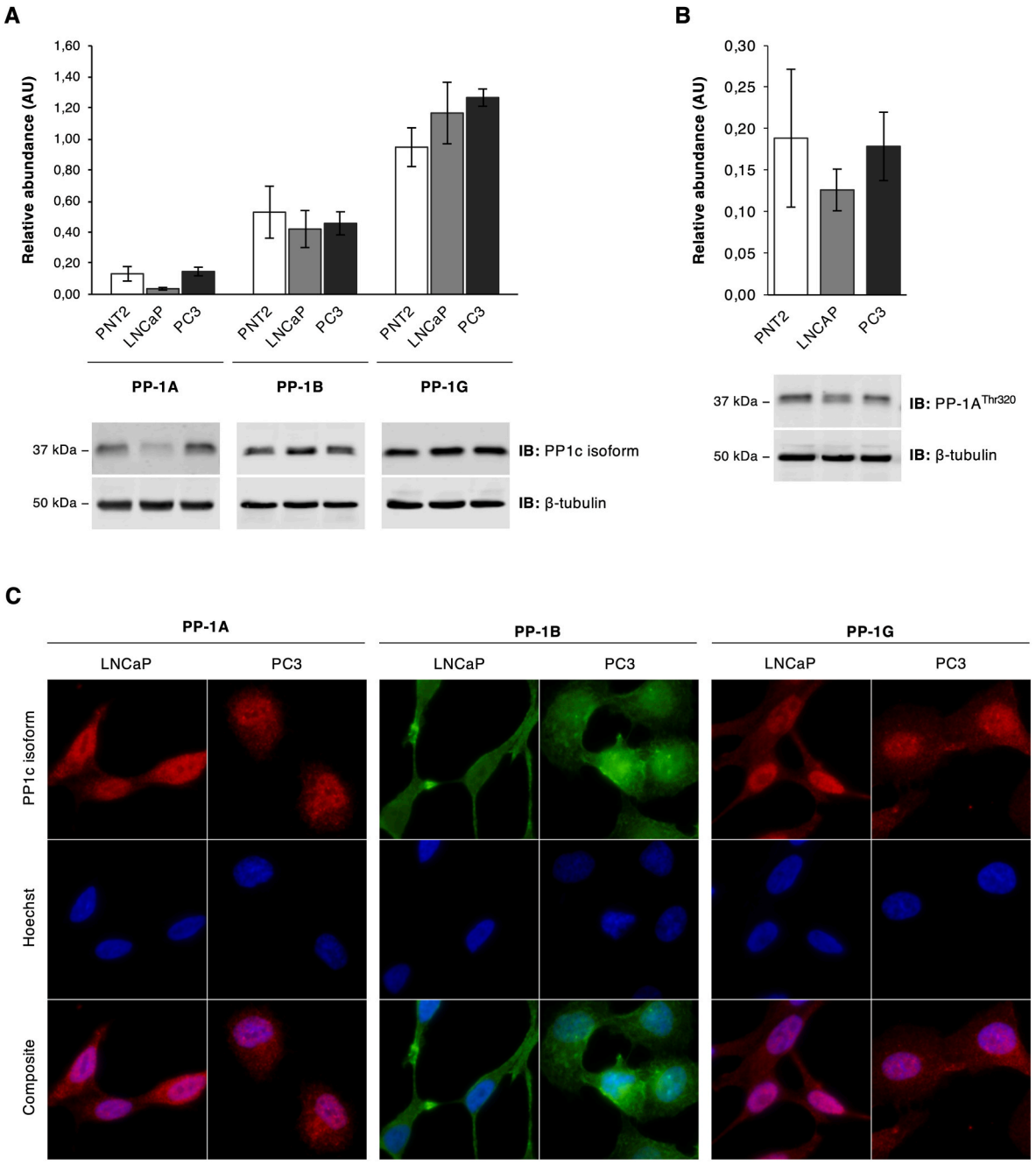

Fig. 2. Protein expression of PP1c isoforms in human PCa cell lines. Western blot analysis of PP-1A, PP-1B and PP-1G expression (A) and phosphorylation (B) in LNCaP (androgen-dependent) and PC3 (castration-resistant) cells using isoform-specific and phospho-specific antibodies, respectively. Immortalized normal prostate cells, PNT2, were used as positive control. Graphs depict the expression levels normalized to the loading control, β-tubulin, from triplicate experiments. Results are expressed as mean ± SD. C – Representative images of PP-1A, PP-1B and PP-1G (top to bottom) in LNCaP and PC3 cells. Nucleus were stained with Hoechst. Images were acquired in an Olympus IX81 motorized inverted microscope (× 100).

Neither PP-1A nor PP-1B expression were associated with GS (Suppl. Fig. 2a–b).

3.4. Expression of PP1c transcripts in PCa subtypes

PCa heterogeneity is well-recognized and a major obstacle for the establishment of successful management strategies. In recent years, great effort has been put into defining molecular alterations that allow the identification of PCa subtypes. Hence, we analyzed the expression of PP-1A, PP-1B, and PP-1G in association with known molecular signatures [23]. PP-1B was overexpressed in tumors with *AR* amplification from the metastatic PCa cohort MET500 PRAD dataset [24] ($p = 0.042$; Fig. 3e). No significant differences were observed for PP-1A and PP-1G (Suppl. Fig. 2c–d).

Additional associations were investigated in the TCGA prostate adenocarcinoma dataset. PP-1A mRNA expression was significantly higher in tumors with *SPOP* mutation ($p = 6.20E-04$) or *ETV1* fusion ($p = 0.017$) in comparison to those with *ERG* fusion (Fig. 3f). PP-1B mRNA expression was lower in tumors with *SPOP* mutation ($p = 0.007$) or *ETV4* fusion ($p = 0.046$), than in those with *ERG* fusion (Fig. 3g). Similar expression levels were found for PP-1G in the different PCa molecular subtypes (Suppl. Fig. 2e).

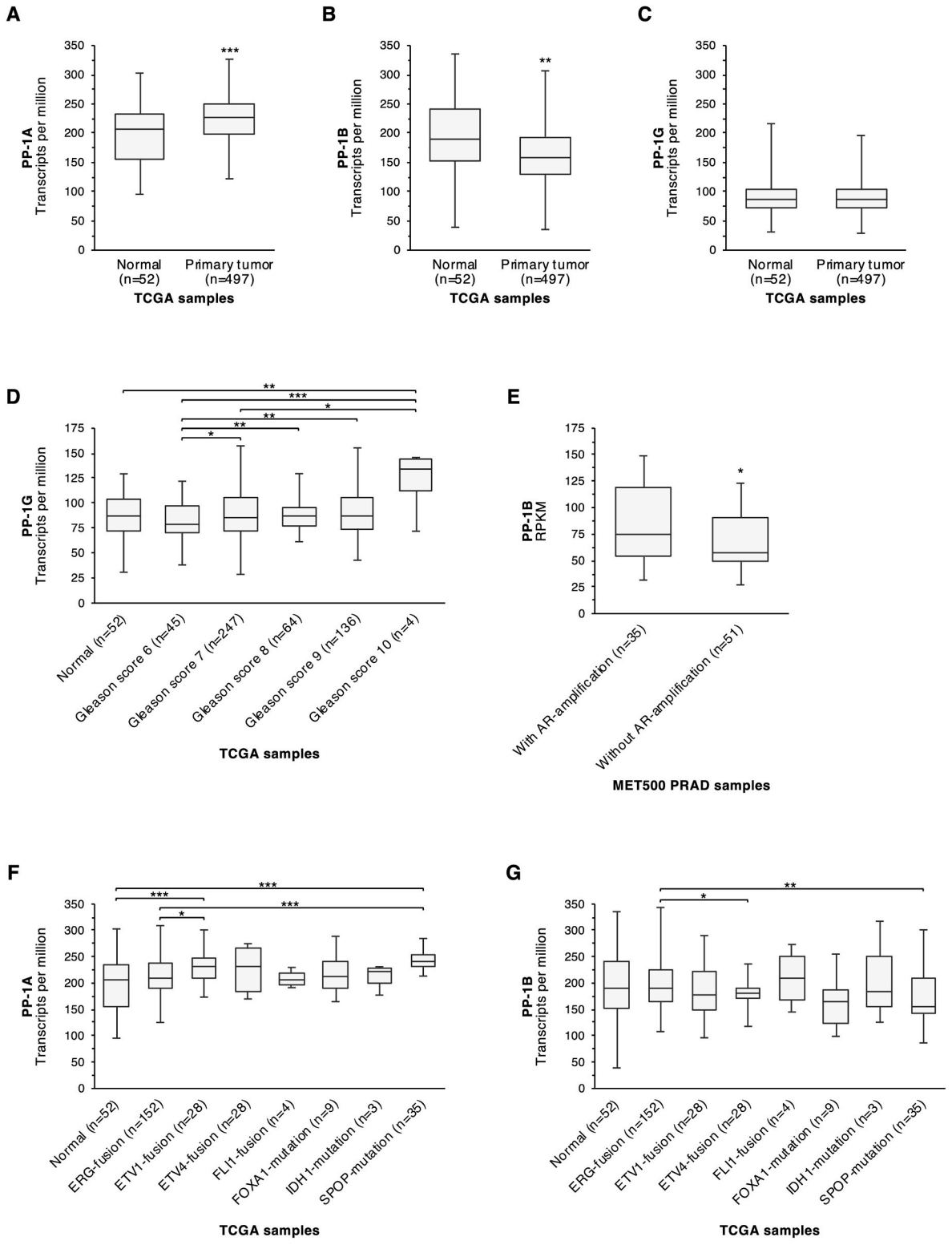

Fig. 3. Expression of PP1c transcripts in normal prostate and primary tumors from TCGA PCa cohorts. A–C – mRNA expression levels of PP-1A (A), PP-1B (B) and PP-1G (C) in normal prostate and primary tumors. D – mRNA expression levels of PP-1G in tumors with different GS. E – mRNA expression levels of PP-1B in tumors with or without androgen receptor (*AR*) amplification. Data is presented as reads per kilobase per million reads (RPKM). F–G – mRNA expression levels of PP-1A (F) and PP-1B (G) in tumors with distinct molecular signatures. Data was reproduced and analyzed through UALCAN. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

3.5. Point mutations in PP1c-coding genes in PCa

The occurrence of somatic mutations in PP1c-coding genes (ie, *PPP1CA*, *PPP1CB*, and *PPP1CCI*) was explored using COSMIC and cBioPortal databases. Point mutations were identified in prostate carcinomas, but not in hyperplasia, prostatic intraepithelial neoplasia (PIN) or adenomas (data not shown). The calculated mutation frequency was low for the 3 genes (Suppl. Table 3). Except for 1 primary tumor sample that exhibited an intronic substitution in both *PPP1CB* and *PPP1CC*, no sample was mutated in more than 1 gene simultaneously

(Suppl. Table 3). Also, only 5 samples displayed 2 distinct mutations in the same gene and only 6 mutations were observed in more than 1 sample (Suppl. Table 3).

Few of the identified mutations had known impact on the amino acid sequence of PP1c isoforms (Fig. 4a and Suppl. Table 3). Five missense mutations were identified in *PPP1CA*, with 3 affecting the catalytic core sequence and 2 the *C*-terminal region (Fig. 4a and Suppl. Table 3). Two of them, R221H and A299P, were associated with copy gain (Fig. 4a and Supp. Table 3). Two missense mutations were identified in *PPP1CC*: 1 at the catalytic core sequence that was associated with copy gain (F227S) and 1 at the *C*-terminal (K319R) (Fig. 4a and Supp. Table 3). A *PPP1CB* splicing site variant was detected in a metastasis sample (Fig. 4a and Supp. Table 3).

3.6. Amplification/deletion of PP1c-coding genes in PCa

The analysis of 8 non-redundant studies from cBioPortal database revealed that PP1c-coding genes were amplified in nearly 40% of CRPC and 30% of NEPC samples (Fig. 4b). Both amplification and deletion were detected in prostate adenocarcinoma samples, though deletions occurred in a lower frequency (8,8% and 0,62%, respectively) (Fig. 4b).

Except for NEPC in which the amplification frequency of *PPP1CB* was slightly higher, *PPP1CA* was the most frequently altered in all cancer subtypes, followed by *PPP1CC* (Table 2). On the other hand, *PPP1CC* registered the highest number of deletions in prostate adenocarcinoma samples (Table 2).

3.7. Promoter methylation of PP1c-coding genes in PCa

All PP1c-coding genes exhibited residual or hypomethylated levels in both normal prostate and primary tumors (Fig. 4c–e). When comparing the 2 conditions, promoter methylation of *PPP1CA* ($p = 0.003$) and *PPP1CB* ($p = 4.09E-06$) were significantly lower in primary tumors than in normal prostate (Fig. 4c–d). On the other hand, primary tumors presented significantly higher *PPP1CC's* promoter methylation than normal samples ($p = 1.63E-12$; Fig. 4e).

4. Discussion

This study provides the first comprehensive characterization of PP1c isoforms in PCa and underlines the importance of investigating isoform-specific roles in prostate carcinogenesis.

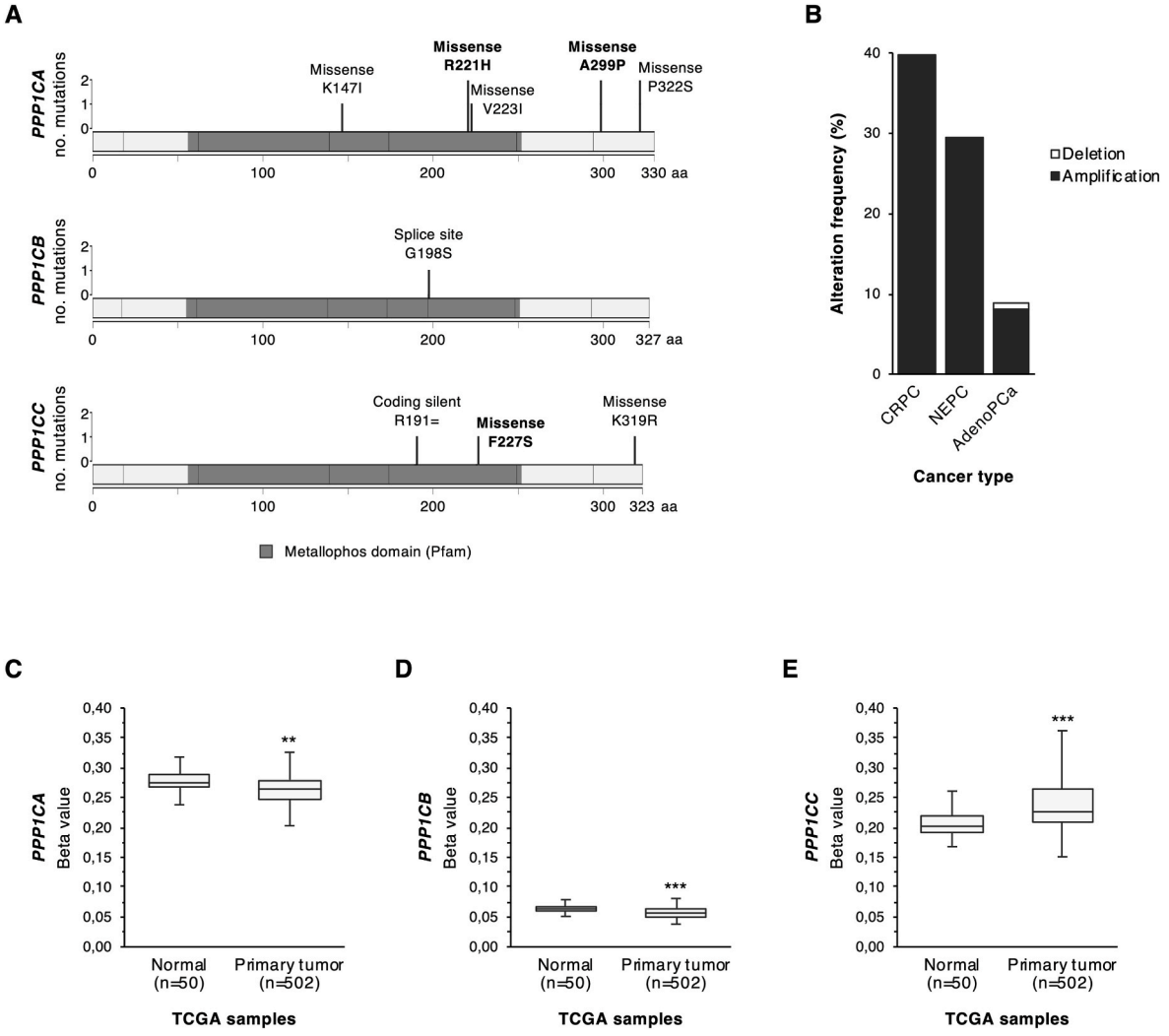

Fig. 4. Genetic alterations and promoter methylation of PP1c-coding genes in PCa. A – Mutation diagram of *PPP1CA*, *PPP1CB* and *PPP1CC* genes in PCa. Mutations in bold are associated with copy gain. Data retrieved from COSMIC database, v90 (https://cancer.sanger.ac.uk/cosmic, accessed on Jan 9, 2020) and cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on Jan 10, 2020). B – Alteration frequency of PP1c-coding genes in castration-resistant prostate cancer (CRPC; N = 63), prostate neuroendocrine carcinoma (NEPC; N = 44), and prostate adenocarcinoma (AdenoPCa; N = 1443). Data retrieved from cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on Jan 10, 2020). C–E – Promoter methylation levels of *PPP1CA* (C), *PPP1CB* (D) and *PPP1CC* (E) genes in normal prostate and primary tumors from TCGA PCa cohorts. Data was reproduced and analyzed through UALCAN. ***$p < 0.001$; **$p < 0.01$.

Table 2
Copy number alterations in *PPP1CA*, *PPP1CB* and *PPP1CC* genes in PCa.

Cancer type	*PPP1CA*		*PPP1CB*		*PPP1CC*	
	Amp	Del	Amp	Del	Amp	Del
Castration-resistant prostate cancer (CRPC) (N = 63)	19 (30.16)	0	8 (12.70)	0	13 (20.63)	0
Prostate neuroendocrine carcinoma (NEPC) (N = 44)	9 (20.45)	0	8 (18.18)	0	7 (15.91)	0
Prostate Adenocarcinoma (N = 1443)	75 (5.27)	1 (0.07)	29 (2.01)	2 (0.14)	36 (2.49)	7 (0.49)

Values are presented as number (%).
Abbreviations: Amp, amplification; Del, deletion. Data retrieved from cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on January 10, 2020). Datasets: NEPC (Multi-Institute 2016), Prostate (SU2C 2019), Prostate (MICH), Prostate (FHCRC, 2016), The MPC Project, Prostate (Eur Urol 2017), Prostate (TCGA PanCan 2018), Prostate (MSKCC 2010).

By combining IHC studies using isoform-specific antibodies with data mining of comprehensive TCGA prostate adenocarcinoma cohorts, we showed that PP-1A is overexpressed in PCa (Figs. 1b and 3a). We also show a shift towards its nuclear expression (Fig. 1c). Previous analyses using tissue microarrays reported higher expression of PP-1A in prostate tumors than in benign hyperplastic tissue [25]. The authors observed both cytoplasmic and nuclear localizations and identified a correlation between increased PP-1A cytoplasmic expression and higher GS [25]. In this study, we were not able to identify any association between PP-1A expression and/or localization and tumor grading (Suppl. Fig. 1), probably due to the small size of our cohort and the limited number of poorly differentiated tumors (GS8, n = 2) when compared to moderately differentiated tumors (GS6 and GS7, n = 10) (Suppl. Fig. 1). PP-1A overexpression has also been reported in other malignancies, including glioblastoma and bladder cancer tissues [26–28]. Consistent with our findings, these studies found that PP-1A was weakly expressed or absent from the nucleus of normal cells, but highly expressed in tumor cells [26–28]. Increased nuclear expression of PP-1A was particularly observed in mitotic cells and in more aggressive tumors [27,28].

In contrast to PP-1A, we found PP-1B mRNA expression to be lower in PCa than in normal tissue (Fig. 3b). Though in our study we did not observe a corresponding reduction in PP-1B protein levels (Fig. 1b), previous studies reported significantly lower levels in PCa than in benign prostatic hyperplasia [29]. In addition, PP-1B was the only isoform found in the nucleus of normal prostate cells, whereas 25% of the tumors showed nuclear exclusion of the protein (Fig. 1c). The redistribution of PP1c isoforms is not surprising as they are highly dynamic molecular entities and their presence have been described in several subcellular compartments [3,30]. Most likely, their localization depends on the cellular context (e.g. cell cycle stage) and their affinity for interacting proteins [31–33]. Depletion of PP-1B was shown to instigate massive nuclear abnormalities [34]. For instance, the disruption of the interaction between PP-1B and the protein phosphatase 1 regulatory subunit 12A, a key targeting subunit to myosin light chain and other substrates that specifically interacts with PP-1B and PP-1G, but not with PP-1A, could lead to nuclear lamina rupture [7,34]. This effect was shown to be particularly dramatic in cancer cell lines, which seem to be more sensitive to actomyosin-mediated nuclear dysmorphia [34].

PP-1G was the only isoform whose expression was associated with tumor differentiation (Fig. 3d). The association between PP-1G expression and tumor grading has been reported for other malignancies, as hepatocellular carcinomas and brain tumors [35,36]. These reports provided evidence for the use of PP-1G as a marker of poor prognosis in both hepatocellular cancer and glioma [35,36].

The ambiguity of the findings obtained when studying the role of PP1c isoforms in cancer somehow impairs the development of subsequent studies to address these divergences. One hypothesis is that such divergencies could result from differential expression among cancer subtypes. For instance, low levels of PP-1A protein expression were associated with estrogen receptor-negative breast tumors [37], while its overexpression was associated with poor overall survival and progression free survival in *TP53*-expressing glioblastomas [27,28].

PCa is widely recognized as a disease with substantial inter- and intra-heterogeneity, and great effort has been put into defining molecular features that aid tumor categorization. Here we showed that PP-1A and PP-1B were differentially expressed in tumors with *SPOP* mutations. *SPOP* mutations are the most commonly identified point mutations in primary PCa (up to 15% of the cases) and are considered driver lesions for the disease [38]. Moreover, they enhance cancer cell survival and resistance to docetaxel [39].

In PCa, *SPOP* mutations are mutually exclusive from rearrangements involving the erythroblast transformation-specific (ETS) family members, such as *ERG*, *ETV1*, and *ETV4* [38]. These and other members of the ETS family of oncogenic transcription factors are frequently fused with androgen-regulated genes [38]. In this study, we found increased PP-1A and PP-1B mRNA expression in tumors with *ETV1* and *ETV4* fusions, respectively, when compared to tumors with *ERG* fusion (Fig. 3f–g). Whether ETS gene rearrangements are mutually exclusive among themselves is still dubious [40], but they might support distinct oncogenic events in PCa. Interestingly, *ETV1* but not *ERG* promotes AR transcriptional activity and enhances autonomous testosterone production [41]. *ETV1* and *ETV4* seem to promote metastasis formation and have both specific and overlapping targets and functions [42].

Furthermore, PP-1B mRNA expression was higher in metastatic tumors with *AR* amplification than in those without *AR* amplification (Fig. 3e). *AR* amplification is common in metastatic PCa, but not in most clinically localized tumors, and is believed to contribute to therapeutic resistance [43]. Altogether, these results suggest a differential regulation of PP1c isoforms in PCa tissue and disclose a potential association between their expression and PCa molecular subtypes.

We also investigated differences in the expression and localization of PP1c proteins in androgen-dependent and castration-resistant PCa cells. The most evident alteration was the decrease in PP-1A levels observed in LNCaP cells comparing with those found for PC3 cells, which suggest a role for PP-1A in androgen-dependent stages. The similar PP-1A expression levels in PNT2 and PC3 cells does not indicate absence of functional role of PP1c in PC3 cells (Fig. 2a). In addition, the variation observed in Thr320 phospho-levels (Fig. 2b) also suggests that PP1c isoforms are differentially regulated by phosphorylation between the cell lines. Phosphorylation of Thr320 inhibits PP1c activity and is highly recognized as the mechanism that silences PP-1A during cell cycle progression [44–46]. However, Thr320 is conserved by all PP1c isoforms. Hence, our results suggest that overall PP1c activity might be increased in androgen-dependent cells, despite other mechanisms may also affect PP1c activity. These results would benefit from in-depth studies to identify and characterize PP1c phospho-forms in PCa. We also found that PP1c isoforms were widely distributed in both LNCaP and PC3 cells (Fig. 2c). Similar staining patterns were identified for PP-1A and PP-1G, but not for PP-1B. Altogether, these results suggest that PP1c isoforms may play distinct roles in androgen-dependent and castration-resistant PCa cells. In fact, all PP1c isoforms were found to interact with AR, but only the interaction with PP-1A has been investigated in further detail [10,13]. PP-1A enhances AR nuclear localization and transcriptional activity through dephosphorylation of Ser650 [10]. The interaction between PP-1A and AR is supported by a positive feedback loop in which AR acts as a PP1c regulator besides also being a substrate. AR targets PP-1A to chromatin, where it catalyzes the dephosphorylation of cyclin-dependent kinase 9. As a result, positive transcriptional elongation factor b is mobilized to induce AR transactivation [13]. In addition, PP-1A inhibits AR polyubiquitylation by dephosphorylating and inactivating proteins involved in AR

ubiquitylation and degradation [11]. PP-1B may also contribute to this effect through interaction and downregulation of the serine/threonine-protein kinase PAK 6, a cyclin-dependent kinase inhibitor 1-activated kinase that mediates AR degradation [47,48]. The effect of PP1c isoforms on AR signaling has also shown to be dependent on PP1rs, which direct the action of the catalytic subunits and might dictate different outcomes [49]. Henceforth, PP1c isoforms might play distinct roles, albeit cooperative at least in part, in regulating AR signaling in PCa.

We went further to analyze if genetic variations and changes in promoter methylation might explain, at least in part, the alterations observed in PP1c isoforms expression. We found that PP1c-coding genes are rarely mutated in PCa (Suppl. Table 3). This was not surprising since on the one hand the occurrence of point mutations in PCa is not frequent [38] and, on the other hand, PP1c-coding genes are believed to be highly resistant to mutation. However, *de novo* variants have been described in patients with relevant clinical phenotypes [50–52]. Moreover, PP1c-coding genes were found to be frequently amplified in PCa, particularly in advanced stages (Fig. 4b). Among the 3 genes, *PPP1CA* was the most frequently amplified (Table 2). *PPP1CA* is located on a frequently overrepresented chromosomal band [25]. Amplification of *PPP1CA* was recently described in both localized and metastatic castration-resistant prostate cancers (7% and 17% of the cases analyzed, respectively) [53]. The authors also reported a frequent co-occurrence with the amplification of G1/S-specific cyclin-D1 [53]. The amplification of *PPP1CA* might explain in part the overexpression of PP-1A in PCa, as previously suggested [25].

DNA methylation of PP1c genes' promoters have been described in other pathophysiological contexts [54,55]. Data on tumors is rather limited, but decreased mean methylation of *PPP1CA* and *PPP1CB*, as well as increased mean methylation of *PPP1CC* were reported in non-small cell lung cancer specimens [56]. In our study, we found that PP1c-coding genes are unmethylated or hypomethylated in normal prostate and primary prostate tumors (Fig. 4c–e). Therefore, promoter methylation does not seem to be a crucial regulatory mechanism of PP1c isoforms expression in PCa.

This study has some limitations. First, the cohort used in IHC studies is small and included a higher number of PCa specimens than normal prostate controls. This could lead to biased results. However, this is a preliminary study for the general characterization of PP1c isoforms in PCa and we aimed to include representative cases from different PCa stages. Also, we complemented our study cohort with a large, well-characterized, publicly available cohort which allowed us to further characterize the expression of PP1c isoforms in PCa. Second, the findings showed some inconsistencies between human tissues and human cell lines (eg, the increase in PP-1A expression observed in prostate tumor tissues was not reflected in PCa cells). However, it should be noted that we used PNT-2 cells as positive control for protein expression assays. This is an immortalized normal prostate epithelium cell line and, therefore, cells might have molecular alterations that are not found in primary prostate cells. Hence, to better understand the differential role and regulation of PP1c isoforms in prostate cells, primary prostate cells should be used in future studies. Moreover, we used PCa cell lines that mimic different stages in PCa progression: androgen-dependency and castration-resistance. Similar classification was not made for PCa tissues, which might also contribute to some inconsistencies (eg, PP-1B was found in both nucleus and cytoplasm in human PCa tissue, while in PCa cells it seems minimally expressed in the nucleus, particularly in LNCaP cells). Whether this and other findings are related to AR signaling remains to be further elucidated. To that end, it would be interesting to assess potential changes in LNCaP sublines developed after long-term androgen ablation.

5. Conclusion

Our results, together with findings from previous studies, suggest that: PP-1A might function as an oncoprotein in PCa, being involved in cell malignant transformation; PP-1B could have specific roles in the regulation of AR-mediated signaling; and PP-1G might be involved in PCa progression, given the association of its expression with GS. These and other hypotheses would be of interest to address in future studies. Moreover, studies in larger cohorts would be essential for determining the potential added-value of PP-1A and PP-1G in the diagnosis and prognosis of PCa.

Credit author statement

Conceptualization: JF and MF; Methodology: JF, VC, JL and IC. Formal analysis: JF and RH; Writing - original draft preparation: JF; Writing - review and editing: JL, BM, CJ and MF. Funding acquisition: MF and CJ. Supervision: MF and CJ.

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Ethics approval

This study was approved by the institutional review board (Comissão de Ética para a Saúde do Instituto Português de Oncologia do Porto, CES-IPOFG_EPE 019/08).

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2022.113282.

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