Urinary proteomic profiles of prostate cancer with different risk of progression and correlation with histopathological features

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ABSTRACT

Prostate cancer (PCa) is the most common tumor in men with extremely variable outcome, varying from latent or indolent form to very aggressive behavior. High grade tumors, expansions exceeding the prostatic capsule into the surrounding soft tissues and spreading through lymph vascular channels, represent the most consistent unfavorable prognostic factors. However, accuracy in the prediction of the disease progression is sometimes difficult. Along with new molecular diagnostic techniques and more accurate histopathological approaches, proteomic studies challenge to identify potential biomarkers predictive of PCa progression. In our study we analyzed the urinary proteomes of 42 patients affected by PCa through two-dimensional electrophoresis associated with mass spectrometry. Proteomic profiles were correlated to histopathological features including pTNM stage and tumor differentiation in order to provide new promising markers able to define more accurately the PCa aggressiveness and driving new therapeutic approaches.

1. Introduction

Prostate cancer (PCa) is the most common male cancer in industrialized countries with a frequency increasing with patient age [1]. Many PCas show unspecific clinical presentation and are diagnosed in advanced stage. Metastatic PCa exhibits aggressive clinical behavior and, at time, hormon-resistance or chemo-resistance. The definition of localized or advanced tumor has now been replaced by the concept of Risk (R) of disease progression based on 5 classes: R very low, R low, R intermediate, R high and R very high. This classification is based on disease characteristics such as stage, grading and prostate specific antigen (PSA) levels [2-5]. Important known unfavorable histological prognostic features, such as high grade cancer (Gleason grade ≥ 4/ Prognostic Grade Group System ≥4) [6-8], vascular lymphatic invasion, extraprostatic extension and advanced stage p disease [9], are not prognostic reliable predictors as opposed to periodic serum PSA level assay, which is now considered the gold standard for PCa monitoring. Although new histopathological diagnostic approaches are emerging, such as early detection of neoplasia [10-13], there are still a few studies on the validation of proteomic markers.

The aim of the study is to identify by proteomics, specifically employing two-dimensional electrophoresis (2-DE) associated with mass spectrometry (MS), potential prognostic biomarkers of PCa in urine, a biological sample readily available with non-invasive procedure. In this study, the urinary proteomes of patients diagnosed with PCa were analyzed and studied by 2-DE coupled to MS. Moreover, obtained results were further validated by the immunochemistry technique Western Blot (WB).

2. Materials and methods

2.1. Patients enrolled

Forty-two patients affected by PCa and candidates for radical prostatectomy were selected for this preliminary study. The selection was made at the Department of Urology of the University Hospital of Modena, after authorization by the local Ethics Committee [Code n°57/08]. Screening was carried out among patients with the following characteristics: age between 60 and 80 years, absence of systemic diseases and negative history of relevant events in the 6 months prior to selection.

2.2. Histological examination

Radical prostatectomies, including prostate gland, deferens and seminal vessels, were en bloc examined in the Pathological Laboratory of University of Modena. The prostates were measured and inked on external surface the sliced transversally from the apex to base at 0,5 cm thick, and put in larger tissue holder [14,15]. Then, standard, 3-micron thick routine sections were obtained and stained with haematoxylin and eosin. Tumors were graded using the ISUP modification on Gleason grading on prostatic cancer [16] and classified by the 8th edition TNM staging system, 2017 [17]. For all cases other histopathological conditions were recognized, such as type of inflammation, pre-malignant conditions (*e.g.* prostatic intraepithelial neoplasia and adenosis) and unfavorable histological aspects (*e.g.* lymphovascular invasion (LVI)). Due to their limited number, patients with LVI (n = 5) were not enrolled in the study, while the remaining 37 patients were divided into three groups according to disease progression risk: PCa group with low risk (PCa_LR); PCa group with intermediate risk (PCa_IR) and PCa group with high risk (PCa_HR). Patients' histological characteristics are highlighted in Table 1. Slides representative for the different tumor tissues included in the study are shown in Fig. 1.

2.3. Urine samples preparation

Urine morning samples (10 mL) were centrifuged at 800 $\times g$ at 4 °C for 10 min and then aliquoted and stored at -80 °C until proteomic analysis that was performed on urinary pools prepared with patients' urine of each group. Pools (4 mL) were concentrated/desalted using Amicon filters, 3 kDa molecular wheight (MW) cut-off (Amicon Ultra-4, Millipore); concentrated protein pools were quantified by Bradford's spectrophotometric method [18] using Multiskan FC microplate reader (Thermo Scientific).

2.4. Two-dimensional electrophoresis

Urinary pools (7 μg of proteins) were solubilized with solubilization buffer composed of 6 M urea, 2 M thiourea, 4% CHAPS, 25 mM dithiothreitol and 0.2% ampholytes pH 3–10. The successive two-dimensional electrophoresis (2-DE) was performed following the protocol described in Bergamini et al. [19] using 7 cm Immobilized pH Gradient strips (IPG strips), wide pH range 3–10 (Bio-Rad) and mini 8–16% polyacrylamide gradient gel (separation in second dimension). Gels were subsequently stained with a Silver Nitrate protocol, as reported in detail in Bellei et al. [20]. Finally, the stained gel images were acquired with a calibrated densitometer (GS800, Bio-Rad) and then analyzed with the "PDQuest 2-D Analysis Software" (version 7.3.1, Bio-Rad) that identified the differential expression of the protein spots according to their staining intensity, reported as optical density (OD), and spot area (mm^2). Spots with fold-change in expression >1.5 were considered differently expressed.

2.5. Protein identification by mass spectrometry

Before mass spectrometry (MS) analysis, protein spots were

Table 1
Patients' subdivision in accordance with disease progression risk.

Group	GS/GG	TNM parameters		
		T	N	M
PCa_LR (n = 16)	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2c	N0	MX
	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2a	NX	MX
	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2c	N0	MX
	G6(3 + 3)/GG1	pT2c	NX	MX
	G6(3 + 3)/GG1	pT2a	NX	MX
	G6(3 + 3)/GG1	pT2c	N0	MX
	G6(3 + 3)/GG1	pT2c	N0	MX
	G6(3 + 3)/GG1	pT1a	NX	MX
PCa_IR (n = 14)	G7(3 + 4)/GG2	pT2a	NX	MX
	G7(3 + 4)/GG2	pT2a	NX	MX
	G7(3 + 4)/GG2	pT2a	NX	MX
	G7(3 + 4)/GG2	pT2c	N0	MX
	G7(3 + 4)/GG2	pT2c	N0	MX
	G7(3 + 4)/GG2	pT2c	N0	MX
	G7(3 + 4)/GG2	pT2c	N0	MX
	G7(4 + 3)/GG3	pT2c	NX	MX
	G7(4 + 3)/GG3	pT2c	NX	MX
	G7(4 + 3)/GG3	pT2c	NX	MX
	G7(4 + 3)/GG3	pT2c	NX	MX
	G7(4 + 3)/GG3	pT2c	NX	MX
	G7(4 + 3)/GG3	pT2c	NX	MX
	G7(4 + 3)/GG3	pT2c	NX	MX
PCa_HR (n = 7)	G7(3 + 4)/GG2	pT3a	NX	MX
	G7(3 + 4)/GG2	pT3a	NX	MX
	G7(3 + 4)/GG2	pT3a	NX	MX
	G7(4 + 3)/GG3	pT3a	N0	MX
	G7(4 + 3)/GG3	pT3b	N0	MX
	G8(4 + 4)/GG4	pT3a	N0	MX
	G8(5 + 3)/GG4	pT3a	N0	MX

Abbreviations: GS = Gleason Score; GG = ISUP Grade Group; PCa_LR = PCa with low risk of progression; PCa_IR = PCa with intermediate risk of progression; PCa_HR = PCa with high risk of progression.

manually removed from the gel and subjected to "in-gel trypsin digestion" as previously fully described [20]. The peptides obtained were extracted with trifluoroacetic acid at 1%/50% ACN, dried by vacuum centrifuge (Savant Speed-Vac), resuspended in 10 μL of formic acid ACN/0.1% and subjected to MS analysis. MS analysis was performed by a Nano LC-CHIP-MS system consisting of a Nano HPLC/Chip microfluidic device (Agilent Technologies Inc.) associated with a 6520 Accurate-Mass Quadrupole-Time-of-Flight Liquid Chromatography/Mass Spectrometry (Agilent Technologies Inc.), as previously described in detail [20]. The MS results obtained were processed using online available programs (Search engine: "MASCOT MS/MS Ion search"; Database: Swiss-Prot.).

2.6. Western Blot analysis

The Western Blot immunochemistry technique was performed to validate Serotransferrin (TRFE), Ganglioside GM2A (SAP3) and Prostatic Acid Phosphatase (PPAP) identifications. An aliquot of each urinary pool was subjected to one-dimensional electrophoresis under denaturing conditions, according to the Laemmli procedure [21]. Protein separation was performed on gel precast Bolt ™ 12% polyacrylamide Bis-Tris Plus (Life Technologies), in electrophoretic chambers Mini Gel Tank (Life Technologies) containing Running Buffer MES SDS 1× (Life Technologies). The separated proteins were then transferred onto a polyvinylidene fluoride membrane (porosity 0.2 μm,
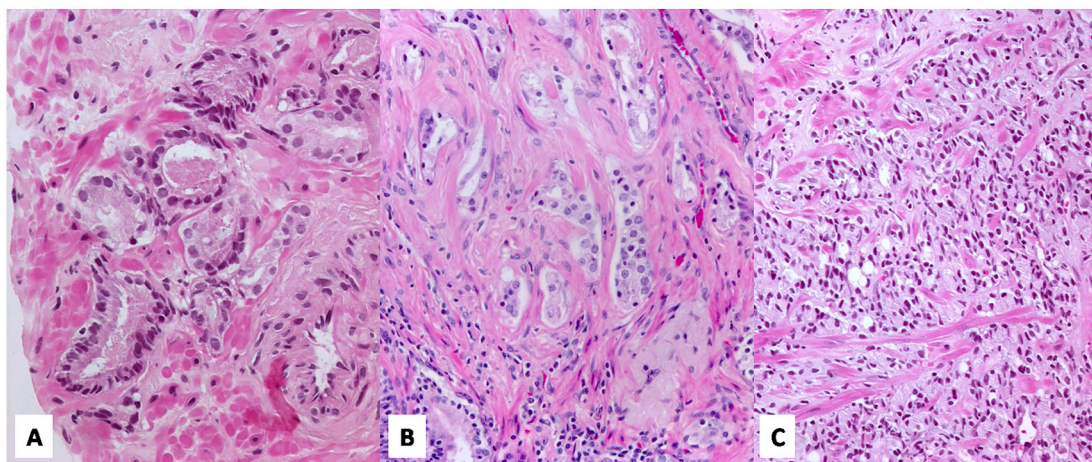
Fig. 1. Histological slides of the prostatic carcinomas included in the study: G6 (3 + 3) Gleason Score tumor; G7 (4 + 3) Gleason Score tumor; G8 (4 + 4) Gleason Score tumor.

Life Technologies) by electroblotting in cold transfer buffer (100 V for 1 h), blocked with 5% non-fat milk and then incubated overnight at 4 °C in slight continuous stirring with primary antibodies (all Abcam): anti TRFE (rabbit polyclonal, 1:500), anti PPAP (rabbit monoclonal, 1:1000), anti SAP3 (rabbit polyclonal, 1:100). Subsequently, membranes were incubated (1 h in continuous agitation) with secondary antibody: Anti-Rabbit IgG VHH Single Domain Antibody (1:6250, Abcam). After membrane incubation (5 min in the dark) with enhancer chemiluminescent solution (ECL, WesternSure TM PREMIUM Chemiluminescent substrate), C-DiGit® Blot Scanner (LI-COR Bioscences) was used to detect protein signals whereas Image Studio™ Lite software was used for signal acquisition and quantification.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by Student's *t*-test (two-tail) considering a p-value ≤0.05 and ≤0.01 as statistically significant.

3. Results

The results of our study can be summarized as follow.

3.1. Patient's clinical data

No significant differences in age and PSA values were identified between the considered groups: patients' mean age and PSA values (± SD) were 67.06 ± 6.34 and 5.27 ± 1.69 ng/mL respectively in PCa_LR group; 67.85 ± 4.81 and 5.91 ± 1.48 ng/mL inPCa_IR group; 66.83 ± 5.27 and 5.12 ± 0.81 ng/mL in PCa_LR group.

3.2. Two-dimensional electrophoresis and proteins identification by MS analysis

All groups were subjected to 2-DE analysis. The analysis of protein maps, using PDQuest software, showed 1 spot expressed only in PCa_LR and PCa_IR, 1 spot expressed only in PCa_HR, and spots present in all groups. The subsequent analysis of the spots permitted the identification of the proteins listed in Table 2 and highlighted in Fig. 2; among those found in all groups, six were differently expressed (spot with a fold change of OD *mm2 ≥ 1.5) (Table 3): Serotransferrin (TRFE), Zinc-alpha-2-glycoprotein (ZA2G), Keratin, type I cytoskeletal 10 (K1C10), Prostate-Specific Antigen (KLK3, also known as PSA), Ganglioside GM2 activator (SAP3) and Ubiquitin-40S ribosomal protein S27a (RS27A).

Table 2
Proteins identified by ESI-Q ToF LC/MS.

Proteins	Entry name	AC no.	Score	Mass (kDa)	Seq. sig.	Cov. (%)
Beta-2-microglobulin[a]	B2MG	P61769	47	13,820	2	21
Prostatic acid phosphatase[b]	PPAP	P15309	25	44,880	3	17
Serotransferrin[c]	TRFE	P02787	799	79,294	20	45
Zinc-alpha-2-glycoprotein[c]	ZA2G	P02531	271	34,465	10	44
Alpha-1-microglobulin[c]	AMBP	P02760	154	39,886	8	36
Keratin, type I cytoskeletal 10[c]	K1C10	P13645	85	59,020	9	21
Prostate-specificantigen[c]	KLK3	P07288	43	29,293	3	20
Prostaglandin-H2 D-isomerase[c]	PTGDS	P41222	284	21,243	9	41
Basement membrane-specific heparan sulfate proteoglycan core protein[c]	PGBM	P98160	599	479,253	11	2
Ganglioside GM2 activator[c]	SAP3	P17900	123	21,281	7	32
Transthyretin[c]	TTHY	P02766	127	15,991	11	66
Ubiquitin-40S ribosomal protein S27a[c]	RS27A	P62979	42	18,296	3	24
Protein S100-A8[c]	S10A8	P05109	26	10,885	1	29

Entry name: protein entry name from UniProt Knowledge database; AC no: primary accession number from Uniprot database; score: highest scores obtained with MASCOT search engine; Mass (kDa): theoretical protein MW; seq. sig.: total number of peptides matching the identified proteins; cov %: % of amino acids sequenced for each detected protein.
[a] Protein identified in PCa_LR and PCa_IR.
[b] Protein identified in PCa_HR.
[c] Protein identified in all group examined.

3.3. Western Blot results

TRFE, SAP3 and PPAP identifications were validated by WB immunochemistry method (Fig. 3). TRFE signals were detected at 77 kDa in all groups: signal significantly higher in PCa_HR than PCa_LR (p = 0.009) and PCa_IR (p = 0.007), and in PCA_IR respect to PCa_LR (p = 0.03); 21 kDa signal, corresponding to SAP3 and similarly detected in all groups, was more intense in PCa_IR respect to PCa_LR (p = 0.04) and PCa_HR (p = 0.002), with a statistical significance; finally the PPAP signal, at 45
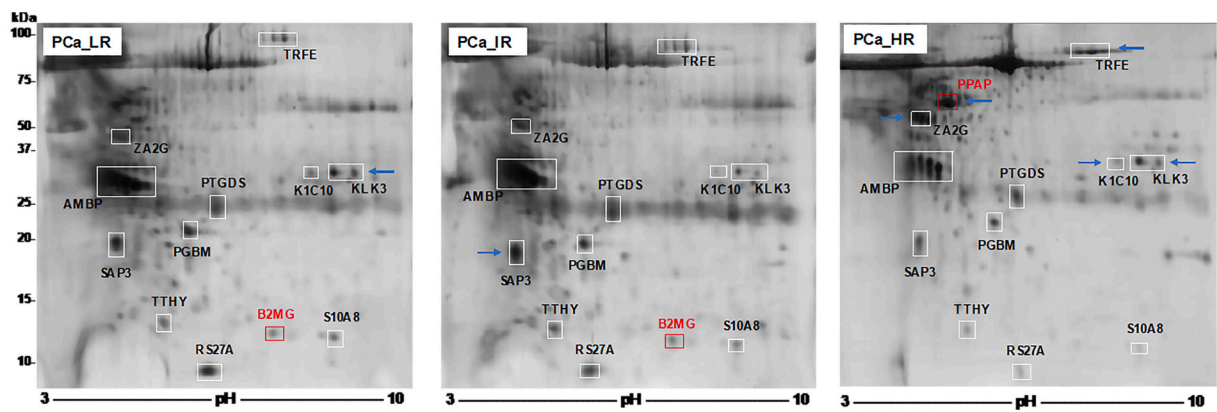
Fig. 2. Urinary protein profile of the PCas with low, intermediate and high progression risk and MS analysis. Representative protein maps obtained by 2-DE analysis from each group: PCa_LR, PCa_IR and PCa_HR. In the figure are reported: marker MW expressed in kDa; strip's pH range used for first-dimension separation; protein identified through MS analysis is indicated by Entry name corresponding to those indicated in Table 2. The proteins common in all three maps are indicated in white rectangles, while those detected only in some maps are indicated in red rectangles; the arrows indicate the spots differently expressed between the groups examined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Proteins differently expressed in PCa_IR, PCa_IRandPCa_HR.

Protein	Protein spots intensity (O.D.)			Expression	Fold
Name	PCa_LR	PCa_IR	PCa_HR	Protein change	Change
TRFE	86,81	89,45	557,53	↑ HR *vs* LR	+6.42
				↑ HR *vs* IR	+5.61
ZA2G	120,70	95,77	314,31	↑ HR *vs* LR	+2.60
				↑ HR *vs* IR	+3.28
K1C10	23,40	15,71	30,67	↑ HR *vs* IR	+1.95
KLK3	72,79	38,32	123,16	↑ HR *vs* LR	+1.58
				↑ HR *vs* IR	+3.18
				↑ LR *vs* IR	+2.01
SAP3	93,52	120,63	69,71	↑ IR *vs* HR	+1.73
RS27A	61,22	27,23	16,71	↓ HR *vs* LR	−3.66
				↓ HR *vs* IR	−2.25
				↓ IR *vs* LR	−2.24

Abbreviations used: O.D. = optical density; PCa_LR = PCa with low progression risk; PCa-IR = PCa with intermediate progression risk; PCa_HR = PCa with high progression risk; TRFE = Serotransferrin; ZA2G = Zinc-alpha-2-glycoprotein, K1C10 = Keratin, type I cytoskeletal 10, KLK3 = Prostate-specific antigen; SAP3 = Ganglioside GM2 activator (SAP3); RS27A = Ubiquitin-40S ribosomal protein S27A; ↑ increase expression protein; ↓ decrease expression protein. Spot with a fold change of O.D. *mm2 ≥ 1.5 were considered expressed differently.

kDa, was detected only in PCa_HR. Results obtained by WB analysis, executed in duplicate, confirmed 2-DE/MS data.

4. Discussion

The 2-DE/MS analysis allowed characterizing the urinary protein profile of PCa. Notably, we have identified 13 proteins: B2MG, detected only in PCa_LR and PCa_IR; PPAP only in PCa_HR; and other 11 proteins, TRFE, ZA2G, AMBP, K1C10, KLK3, PTGDS, PGBM, SAP3, TTHY, RS27A, S10A8, detected in all groups (Fig. 2 and Table 2). According to the literature data, a remarkable number of these proteins, such as B2MG, PPAP, TRFE, ZA2G, K1C10, SAP3 and RS27A, are considered indicators of disease progression. High serum levels of B2MG protein are predictive of an unfavorable prognosis associated with disease progression in patients with haematological tumors (including myeloma, lymphomas and leukemia) [22-25], colorectal and prostate cancer [26,27].

In our study, the B2MG protein was identified only in PCa_LR and PCa_IR, but not in PCa_HR; the protein could therefore be considered an index of aggressiveness of tumors morphologically classified as low or medium risk.

PPAP was identified only in PCa_RH and this result has been

confirmed by WB analysis (Fig. 2). PPAP has been defined as a progression risk factor for PCa [28,29] and has been observed highly expressed in PCa bone metastases [30]. In our study, PPAP detection is correlated with unfavorable histological factors including an advanced pathological stage, extraprostatic tumor extension, angioinvasion and extraprostatic nerve infiltration. Based on these results, PPAP could become a useful urinary marker for a clinical staging of prostate cancer.

TRFE protein resulted more expressed in PCa_HR respect to PCa-LR and PCa_IR and moreover WB analysis showed a statistically significant increase in PCA_IR compared to those in PCa_LR (Fig. 2).These results correlate with its role as an unfavorable prognostic factor: TRFE is an iron transport protein that increases in tumors because actively proliferating tumor cells need more iron [31,32]. Its detection in prostate cancer patients' urine could improve their stratification in risk classes, becoming an important index for the choice of therapy to be applied.

Literature data attribute different roles to ZA2G, such as the function as a biomarker for the early diagnosis of PCa [33] and PCa progression index being related to cancer cell proliferation and their attitude to metastatize [34,35]. We found ZA2G more expressed in PCa characterized by a high mitotic rate and a high Gleason/GG degree of differentiation (PCa_HR) than PCa_LR and PCa_IR, confirming its role as a marker of disease progression.

K1C10 was more expressed in PCa_HR *vs* PCa-IR: protein expression is closely related to the aggressiveness of the tumor and its prognostic significance has been reported in different epithelial types of tumors [36-38].

SAP3 protein was found increased in PCa_IR but not inPCa_HR supporting its potential significance in tumor progression. This result was confirmed by WB analysis. Clinical relevance of high plasma levels of SAP3 has been investigated in neuroblastoma, melanoma, lymphoma, glioma and lung cancer and breast cancer [39], reporting significative correlation with poor prognosis and short disease-free survival. However there are no previous studies correlating urine SAP3 protein level and PCa prognosis.

RS27A was found significantly less expressed in PCa_HR than PCa_LRand in PCa_IR respect to PCa_LR. RS27A belongs to the ubiquitous protein-protein complex responsible for protein degradation and involved in various biological processes including cell cycle regulation, apoptosis andgenotoxic stress-responses [40]. The down-regulation of this protein could be considered an index of PCa aggressiveness.

Finally, KLK3 (PSA) was found significantly more expressed in PCa_LR respect to PCa_IR and PCa_HR. This result seems to confirm the literature data, according to which PSA may not segregate clinically
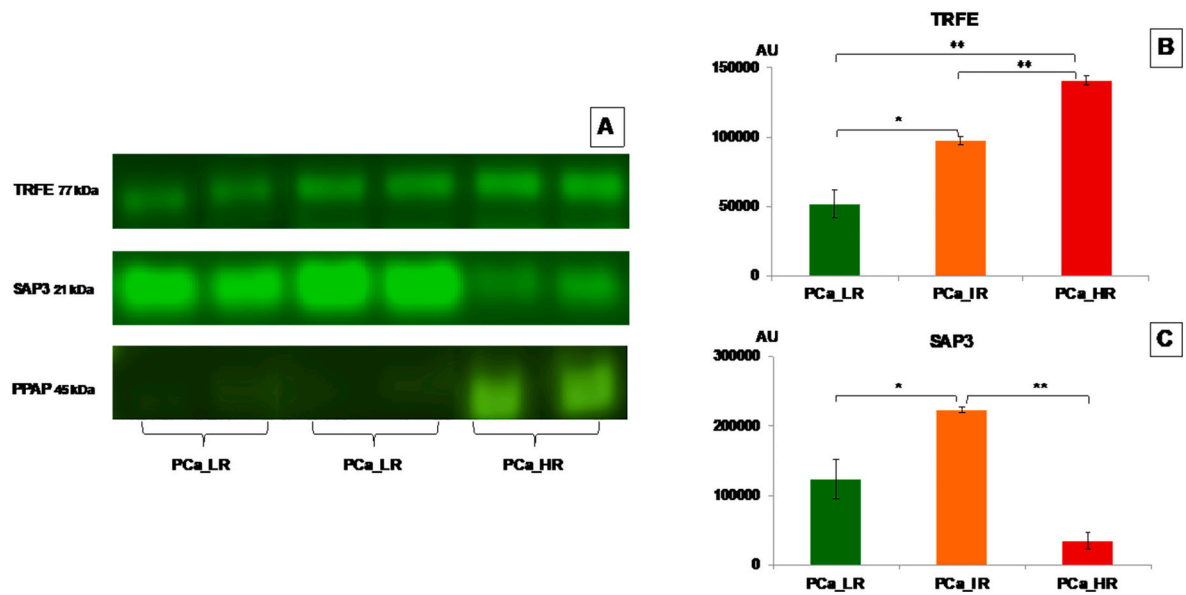
Fig. 3. Serotransferrin (TRFE), Ganglioside GM2 activator (SAP3) and Prostatic acid phosphatase (PPAP) validation by WB analysis. A, representative WB images that show the MW (kDa) of the bands in which the signal proteins were detected. B and C reported, respectively, graphical representation of TRFE and SAP3 signal intensity (expressed in arbitrary units) detected in the three groups considered; in B, TFRE intensity statistically higher in PCa_HR respect to PCa-LR and PCa_IR (**$p < 0.01$ in both cases), and in PCa_IRvsPCa_LR (*$p < 0.05$); in C, SAP3 intensity statistically higher in PCa_IR respect to PCa-LR (*$p < 0.05$) and PCa_HR (**$p < 0.01$).

important tumors from the low-risk ones [41].

In conclusion, despite the small number of cases examined, we have shown that the urinary proteomic profile of aggressive PCa differs from that of less aggressive tumors [42]. B2MG, PPAP, TRFE, ZA2G, K1C10, SAP3 and RS27A, are potential prognostic biomarkers of PCa. In association with traditional histological features they may provide further valuable indications on the progression of the disease. These novel insights might prompt the most appropriate treatment hence resulting into an increase in PCa survival.

Declaration of competing interest

Acknowledgments

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