PSMA-PSA clones drived by full Akt phosphorylation (T308+,S473+) recapitulate molecular features of human prostate cancer

Les clones PSMA-PSA contrôlés par la phosphorylation totale d'Akt (T308+,S473+) récapitulent les caractéristiques moléculaires du cancer de la prostate humain

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RÉSUMÉ

Prérequis : PSMA+,PSA+) et (PSMA+,PSA-) sont les deux clones identifiés comme étant les plus exprimés au cours de la progression du cancer de la prostate (CaP). Toutefois, les signatures moléculaires associées à ces clones et leur impact sur la progression tumorale prostatique ne sont pas encore élucidés.

But : Etant une voie majeure impliquée dans les événements conduisant à la transformation maligne de la prostate, nous avons étudié l'impact de l'activation totale de l'Akt (T308+, S473+), en relation avec les taux du PSA sérique, l'expression tissulaire du PSMA et l'activité angiogénique, sur l'émergence des clones prostatiques cancéreux (PSMA+,PSA+) et (PSMA+,PSA-).

Méthodes: L'étude a été réalisée sur 6 prostates normales (PN), 25 hyperplasies bénignes de la prostate (HBP) et 23 CaP. L'analyse immunohistochimique a été la méthode employée dans l'immunomarquage du PSMA, PSA, pAkt(T308), pAkt(S473) et CD34 dans les tissus prostatiques. L'évaluation de l'angiogenèse a été faite par l'immunomarqueur CD34. Les concentrations du PSA sériques sont mesurées par l'automate Immulite.

Résultats: Dans notre étude, nous avons montré qu'au sein du profil pAkt (T308+,S473+), les cancéreux avec un clone (PSMA+,PSA+) sont caractérisés par un taux élevé de PSA sérique, une forte expression du PSMA et une activité angiogénique intense par rapport à ceux avec un clone (PSMA+,PSA-). Dans le profil pAkt (T308+,S473+), bien qu'ayant le même clone (PSMA+,PSA+), les caractéristiques moléculaires des patients HBP sont totalement différentes des cancéreux. En effet, parmi les patients présentant une activation maximale d'Akt, les cancéreux avec un clone (PSMA+,PSA+) ont un taux élevé de PSA sérique, du PSMA tissulaire et une forte activité angiogénique par rapport aux bénins possédant le même clone.

Conclusion: Nos résultats soulignent le rôle potentiel de l'activation totale d'Akt (T308+,S473+) dans l'expansion de plusieurs clones PSMA-PSA de la prostate capables de conduire l'initiation du CaP humain ainsi que la progression vers un phénotype métastatique. Identifier les patients selon les clones PSMA-PSA pourrait récapituler les caractéristiques histologiques et moléculaires du CaP humain et former une nouvelle approche pour contrôler les métastases prostatiques.

Mots-clés
pAkt, PSMA, PSA, clones, métastases, cancer de la prostate.

SUMMARY

Background: (PSMA+,PSA+) and (PSMA+,PSA-) are the two most individual clones that we have previously identified during prostate cancer (PC) progression. However, molecular signatures associated with these distinct PSMA-PSA prostate clones and their specific correlation with disease outcome is yet to be defined.

Aim: Since Akt is a major pathway involved in the critical activating events that leads to malignant form of the disease, we studied the involvement of full Akt activation (T308+,S473+) connected with serum PSA levels, tissue PSMA expression and angiogenic activity on the emergence of (PSMA+,PSA+) and (PSMA+,PSA-) PC clones.

Methods: The study was carried out in 6 normal prostate, 25 benign prostate hyperplasia (BPH) and 23 (PC). Immunohistochemical analysis was performed to study the expression of PSMA, PSA, pAkt(T308), pAkt(S473) and CD34 in prostate tissues. The evaluation of angiogenesis was made by CD34 immune marker. Serum levels of PSA were assayed by Immulite autoanalyser.

Results: The most relevant result showed that, among PC patients with pAkt (T308+,S473+) profile, patients that exhibit the (PSMA+,PSA+) clone have higher serum PSA levels, tissue PSMA expression and angiogenic activity than those with (PSMA+,PSA-) clone. Although have the same (PSMA+,PSA+) prostate clone, BPH patients have distinct molecular-biological features compared to PC patients among pAkt (T308+,S473+) profile. In fact, among patients with maximal Akt activation, the (PSMA+,PSA+) PC clone is characterized by higher serum PSA levels, tissue PSMA production and intensive angiogenic activity than (PSMA+,PSA+) BPH clone.

Conclusion: These findings emphasize the potential role of the full Akt activation (T308+,S473+) in expansion of several PSMA-PSA prostate clones capable of driving both human PC initiation as well as progression to a metastatic phenotype. Pinpoint patients according to PSMA-PSA clones could recapitulate the histological and molecular features of human PC and may offer a novel approach for controlling metastasis.

Key-words
pAkt, PSMA, PSA, clones, metastases, prostate cancer.

Tumors that arise from prostate tissue exhibit heterogeneity which presents a serious challenge for clinical prostate cancer management (1,2). Although prostate cancer is a disease of older men, studies of prostate specimens from healthy men suggest that prostate cancer initiation may have already taken place at a relatively early age (3,4). These findings have been proposed the existence of multiple neoplastic transformation events, many of which may give rise to latent prostate cancer that, does not progress to clinically detectable disease. Alternatively, a proportion will give rise to aggressive potential metastatic forms of the disease (3-5). Subtypes of prostate cancer can reflect the emergence of distinct individual clones during prostate cancer progression (2,6). Based on prostate specific membrane antigen (PSMA) and prostate specific antigen (PSA) profiles, the two most individual clones that we have previously identified during prostate cancer progression are : (PSMA+,PSA+) and (PSMA+,PSA-) (7). The PSMA is a type II transmembrane protein endowed with enzymatic activities and rapid internalization (8,9). PSMA causes prostate cancer cells to behave more aggressively, making them more likely to move and invade healthy tissue surrounding the tumor (10,11). PSA is used extensively as a serum biomarker to screen for prostate cancer, to detect recurrence following local therapies, and to follow response to systemic therapies for metastatic disease. Nevertheless, as a functional product of normal prostate epithelial tissue, serum PSA levels will reflect changes due to inflammation, trauma or benign proliferation (12,13). The molecular mechanism leading to the emergence of several clones of prostate cancer has been poorly defined. Several intracellular transduction pathways are involved in the critical activating events that lead to clinical prostate disease even more metastatic prostate cancer (14). Akt is a major pathway activated constitutively in metastatic human prostate cancer relating to the stimulation of cell migration and invasion (15,16). Akt activation involves the phosphorylation of two residues: threonine 308 (T308) in the activation loop by PDK1 (phosphoinositoldependent kinase 1) and serine 473 (S473) in the C-terminal hydrophobic motif by PDK2. A number of kinases have been suggested to function as the so-called PDK2 including mammalian target of rapamycin complex 2 (mTORC2) and integrin-linked kinase (ILK) (17,18). Phosphorylation of Akt at S473 has been extensively studied in prostate tumor samples as a correlate for Akt activity, yet the phosphorylation profiling of Akt (T308/S473) in the process of clonal prostate cancer evolution is not assessed. For this purpose, we studied at first the relevance of the phosphorylation profiling of Akt (T308/S473) in the evolution of (PSMA+,PSA+) and (PSMA+,PSA-) prostate clones from normal, benign prostate hyperplasia to prostate cancer. Furthermore, we restricted our investigation on the involvement of full Akt activation (T308+,S473+), connected with serum PSA levels, tissue PSMA and angiogenic activity, on the emergence of (PSMA+,PSA+) and (PSMA+,PSA-) prostate cancer clones.

METHODS

Prostates were obtained from: (a) transurethral resections from 25 men (aged from 61 to 85 years) diagnosed clinically and histopathologically with benign prostate hyperplasia (BPH); (b) radical prostatectomy from 23 men (aged from 57 to 88 years) diagnosed with prostate cancer (PC) ; and (c) histologically normal prostates (NP) obtained at autopsy (8-10 hours after death) from 6 men (aged from 21 to 40 years) without histories or reproductive, endocrine or related diseases. All pathological, clinical and personal data were anonymized and separated from any personal identifiers. All the procedures followed were examined and approved by the Hospital of La Rabta of Tunis and the Hospital of Charles Nicolle of Tunis (Tunisia).

Antibodies

The primary antibodies used were rabbit anti-human pAkt (T308), rabbit anti-human pAkt (S473) (Bioworld Technology, USA), mouse anti-human PSMA (3E6), mouse anti-human PSA (ER-PR8), and mouse anti-human CD34 (QBend10) (Dako, Glostrup, Denmark). The CD34 antibody was used for analysis of angiogenic activity in the prostate tissues.

Immunochemical procedure specificity was checked using negative controls. For negative controls, tissues of each type (NP, BPH, and PC) were incubated with blocking peptides (Santa Cruz Biotechnology, CA, USA) at the same immunoglobulin concentration used for each antibody.

Hematoxylin and eosin staining

For hematoxylin and eosin staining, tissues were fixed for 24 hours at room temperature in 0.1 M phosphate-buffered 10% formaldehyde, dehydrated and embedded in paraffin. Sections (3 µm thick) were processed to deparaffinization, hydrated through graded alcohols and washed in de-ionized water. Slides were then immersed in hematoxylin for 5 min and washed twice in distilled water for 15 s. After immersing in eosin for 2 min sections were dipped in 50% ethanol for 30 s, washed in distilled water for 15 s then immersed in 95% ethanol for 30 s, washed again in distilled water for 15 s and then dehydrated in 2 changes of 100% ethanol for 1 min each. Slides were then immersed in 3 changes of xylene by immersion for 1 min each. Stained tissues were visualized with a microscope equipped with digital camera.

Immunohistochemistry (IHC):

For immunohistochemistry analysis, tissues were fixed for 24 hours at room temperature in 0.1 M phosphate-buffered 10% formaldehyde, dehydrated and embedded in paraffin. Sections (3 µm thick) were processed following the NovoLinkTM Polymer Detection Systems (Novocastra Laboratories Ltd, Newcastle, UK) method. Following deparaffinization, sections were hydrated through graded alcohols and washed in de-ionized water. To retrieve the antigen, the sections were incubated with 0.1 M citrate buffer (pH 6) for 20 minutes in a 98°C water bath. Slides were allowed to cool for another 20 min, followed by washing in de-ionized water. Endogenous peroxidase activity was quenched by incubation with Peroxidase Block for 5 minutes. Each incubation step was carried out at room temperature and was followed by two sequential washes (5 min each) in TBS. Sections were incubated with Protein Block for 5 minutes to prevent non-specific binding of the first antibody. Thereafter, the primary antibodies were applied at a dilution of 1/100 (pAkt (T308), pAkt (S473), PSA and CD34) and of 1/50 PSMA in antibody diluents (Dako, Glostrup, Denmark) at room temperature for 60 minutes. Afterwards, the sections were incubated with Post Primary Block for 30 minutes to block non-specific polymer binding. The sections were incubated with NovoLinkTM Polymer for 30 minutes followed by incubations with 3, 3'-

diaminobenzidine (DAB) working solution for 5 minutes to develop peroxidase activity. Slides were counterstained with hematoxylin and mounted. Immunochemical procedure specificity was checked using negative controls. Prostatic tissues of each type were incubated with blocking peptides (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in place of primary antibody.

Evaluation and interpretation of IHC staining:

A comparative quantification histological of immunolabeling among the different types of prostates was performed for each of the three antibodies. Of each prostate, six histological sections were selected at random. In each section, the staining intensity (optical density) per unit surface area was measured with an automatic image analyzer (Motic Images Advanced version 3.2, Motic China Group Co., China) in 5 light microscopic fields per section, using the X40 objective. Delimitation of surface areas was carried out manually using the mouse of the image analyzer. For each positively immunostained section, one negative control section (the following in a series of consecutive sections) was also used, and the optic density of this control section was taken away from that of the stained section. From the average values obtained (by the automatic image analyzer) for each prostate, the means ± SEM for each prostatic type (normal prostate, BPH and PC) were calculated. The number of sections examined was determined by successive approaches to obtain the minimum number required to reach the lowest SEM. The statistical significance between means of the different prostate group's samples was assessed by the Fisher exact and the one-way ANOVA test. For all statistical tests, $p<0.05$ was considered significant (GraphPad PRISMA 5.0 computer program).

PSA immunoassays:

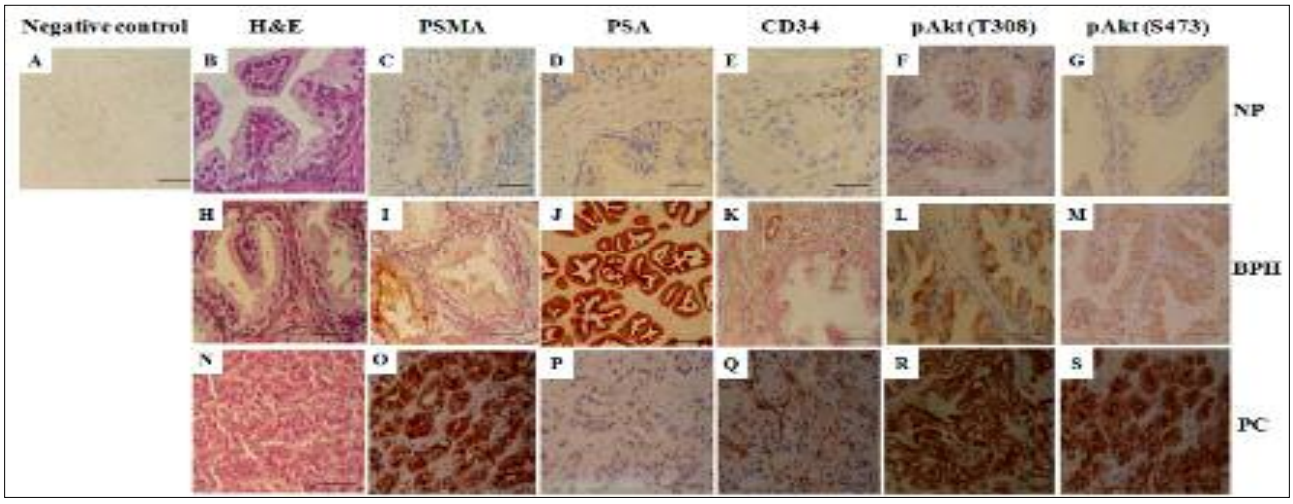
PSA DPC Immulite assays (gifts of Diagnostics Products Corporation, Los Angeles, CA) were used to determine serum PSA levels from these patients before the transurethral resection or the radical prostatectomy. These assays were performed according to the manufacturer's instructions. It's an immunoassay type sandwich, double site and in solid phase, using the monoclonal and polyclonal antibodies anti-PSA. The reaction was developed with an enhanced chemiluminescence. The normal PSA level by DPC was 4ng/mL.

RESULTS

Immunohistochemical localizations of tissue PSMA, PSA, CD34, pAkt (T308) and pAkt (S473) in normal, hyperplastic and carcinomatous prostatic tissue

To determine the expression of tissue PSMA, PSA, CD34, pAkt (T308) and pAkt (S473) in prostate tissue, we used immunohistochemistry analysis. No immunoreactivity was observed in the negative controls incubated with blocking peptides (Figure. 1A). PSMA, PSA, pAkt (T308) and pAkt (S473) were exclusively detected at luminal cells of prostatic glandular structures in all prostate tissues. We found PSMA, PSA, pAkt (T308) and pAkt (S473) in different pattern of prostatic epithelial cells (Figure. 1). PSMA was mainly expressed in the cytoplasm in normal epithelial cells; whereas in pathological prostate tissues (BPH and PC) PSMA was detected both in the cytoplasm and membrane of luminal cells (Figure. 1 C, I, O). As in normal and pathological prostate samples, PSA was mainly detected in the cytoplasm of epithelial cells (Figure. 1 D, J, P). In NP, BPH and PC samples, immunoexpressions for pAkt (T308) and pAkt (S473) were detected mainly in both membrane and cytoplasm of epithelial cells (Figure. 1). As shown in Figure. 1, immunoreactivity to CD34 was found exclusively in the endothelium of normal and pathological (both benign and malignant) prostate tissue.

Figure 1 : Expression of PSMA, PSA, CD34, pAkt (T308) and pAkt (S473) in human normal (NP), benign (BPH) and prostate cancer (PC) tissues. Histologic specimens of normal (B), benign (H) and cancer (N) human prostate tissues stained with hematoxylin and eosin (H&E). A. Representative of negative control. NP showing weak cytoplasmic staining for PSMA (C), PSA (D), pAkt (T308) (F) and pAkt (S473) (G) in epithelial cells. BPH showing membranous staining for PSMA (I) and both membranous and cytoplasmic staining for PSA (J), pAkt (T308) (L) and pAkt (S473) (M) in prostatic epithelial cells. CD34 was found at low level in membranous and cytoplasmic endothelial cells in NP (E) and BPH (K). Strong and diffuse PSMA (O), pAkt (T308) (R) and pAkt (S473) (S) expression in the membrane and the cytoplasm of neoplastic acinar structures in prostatic carcinoma. PSA (P) showed weak cytoplasmic immunoreactions of epithelial cells in PC. Prostatic carcinoma with high density of intratumoral capillary vessel network (Q). Scale bars: A-I, K-S, 20 μm; J, 30 μm.



Distribution of tissue PSMA, PSA, CD34, pAkt (T308) and pAkt (S473) among prostate specific antigen sera levels in normal and prostatic pathologies

In a further approach, we examined the expression of tissue PSMA, PSA, CD34, pAkt (T308) and pAkt (S473) in NP, BPH and PC samples among sera PSA levels depending on cut-off of PSA 4ng/mL: 0-4ng/mL, 4-20ng/mL and PSA>20ng/mL (TABLE 1). As listed in the TABLE 1, the mean of PSMA expression was significantly increased in benign prostate glands compared with normal prostate tissue (17.62 ± 3.38 and 3.7 ± 0.91, respectively). The highest level of PSMA expression was found in primary prostate cancer (27.54 ± 2.29) which significantly differed from benign ($p < 0.0001$) and normal prostatic tissue ($p < 0.0001$) (TABLE 1). According to sera PSA levels, immunorectivity for tissue PSMA appeared in all BPH and PC patients with sera PSA levels >20ng/mL. Interestingly, positivity of tissue PSMA was highest in PC compared to BPH patients for sera PSA groups between 0-4 ng/mL and those between 4–20 ng/mL (TABLE 1).

When tissue PSA is evaluated by means immunohistochemistry, immunoreactions for tissue PSA was found the highest in hyperplastic epithelial cells. The intensity of immunoreactions to tissue PSA decreased from BPH samples to prostate adenocarcinoma (30.93 ± 5.37 and 11.18 ± 2.72, respectively) ($p < 0.0001$) (TABLE 1). As shown in the TABLE 1, PSA staining was present in 50% of BPH patients with sera PSA levels between 0-4 ng/mL and PSA >20 ng/mL. For BPH patients with sera PSA levels between 4-20 ng/mL, tissue PSA staining was detected in 77.7% of this later PSA group's. However, the distribution of tissue PSA seems to be different in PC patients. Indeed, no immunoreactions to tissue PSA were observed in PC patients with sera PSA levels between 0-4 ng/mL; whereas its positivity gradually increases from PC patients with sera PSA levels between 4-20 ng/mL to the PC PSA group's > 20 ng/mL (TABLE 1).

When we looked at the vasculature in our samples, we found that immunoreactions to CD34 were detected in all groups of NP, BPH and PC patients. Measuring the optical density of CD34 immunostaining, we observed that there is a significant difference in vasculature density between normal, hyperplasia and tumors in our collection (TABLE 1). Interestingly, the degree of vascularization was higher in PC patients who mostly have sera PSA levels >20 ng/mL than BPH patients who mostly have sera PSA levels between 0-4 ng/mL or between 4-20 ng/mL. Indeed, CD34 staining was found more abundant in PC specimens (14.29 ± 0.99), compared with NP and BPH ($p <0.0001$). Vessel density was higher in BPH compared to NP samples (8.21 ± 1.94 and 2.34 ± 0.82, respectively) ($p < 0.0001$) (TABLE 1).

Regarding pAkt (T308) and pAkt (S473) staining, the highest optical densities were found in cancer epithelial cells (33.79 ± 2.42 and 35.28 ± 2.33, respectively) (TABLE 1). As listed in the TABLE 1, mean pAkt (T308) expression was significantly increased in benign prostate glands compared with normal prostate tissue (19.56 ± 3.16 and 7.6 ± 0.4, respectively) ($p<0.0001$). Similar to pAkt (T308), immunostaining intensity to pAkt (S473) was higher in BPH than in NP samples (17.71 ± 2.13 and 6.44 ± 0.17, respectively) ($p<0.0001$) (TABLE 1).

Comparison of pAkt (T308) and pAkt (S473) staining among sera PSA levels revealed differences between three prostate groups. BPH patients with sera PSA levels between 0-4ng/mL presented a same percentage (30%) of both pAkt (T308) and pAkt (S473) expression. However, pAkt (T308) was preferentially more expressed than pAkt (S473) in BPH patients with sera PSA levels between 4-20ng/mL and >20ng/mL (55.5% and 33.3%, 50% and 16.6%, respectively) (TABLE 1). Contrary to BPH patients, the three sera PSA groups of PC patients have shown more than 50% of patients with positive immunoreactions to either pAkt (T308) or pAkt (S473). Indeed, immunoreactions to pAkt (T308) or pAkt (S473) were observed in all PC patients with sera PSA levels between 0-4ng/mL. Moreover, for patients with sera PSA levels

Table 1 : Percentage of patients showing positive immunohistochemical reactions to PSMA, PSA, CD34, pAkt (T308) and pAkt (S473) according to groups: normal prostate (NP), benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC). BPH and PC groups were subdivided according to PSA levels: 0-4, 4-20 and >20 ng/ml. Values were expressed as mean ± SEM. Average optical densities were only evaluated in patients showing positive immunoreactions. Values denoted by different superscripts are significantly different from each other. Those values sharing the same superscript are not statistically different from each other. Statistical analysis refers to each antibody separately. Significance was determined at p<0. 05.

PSA levels (ng/ml)	PSMA (%)	O.D.	PSA (%)	O.D.	CD34 (%)	O.D.	pAkt T308 (%)	O.D.	pAkt S473 (%)	O.D.
NP (6)										
<4 (6)	5 (83.3%)	3.7 ± 0.91[a]	5 (83.3%)	3.07 ± 0.56[a]	6 (100%)	2.34 ± 0.82[a]	2 (33.3%)	7.6 ± 0.4[a]	2 (33.3%)	6.44 ± 0.17[a]
BPH (25)	20 (80%)	17.62 ± 3.38[b]	15 (60%)	30.93 ± 5.37[b]	25 (100%)	8.21 ± 1.94[b]	11 (44%)	19.56 ±3.16[b]	7 (28%)	17.71 ± 2.13[b]
0-4 (10)	7 (70%)		5 (50%)		10 (100%)		3 (30%)		3 (30%)	
4-20 (9)	7 (77.7%)		7 (77.7%)		9 (100%)		5 (55.5%)		3 (33.3%)	
>20 (6)	6 (100%)		3 (50%)		6 (100%)		3 (50%)		1(16.6%)	
PC (23)	22 (95.6%)	27.54 ± 2.29[c]	17 (73.9%)	11.18 ± 2.72[c]	23 (100%)	14.29 ± 0.99[c]	18 (78.2%)	33.79 ± 2.42[c]	17 (73.9%)	35.28 ± 2.33[c]
0-4 (2)	2 (100%)		0 (0%)		2 (100%)		2 (100%)		2 (100%)	
4-20 (7)	6 (85.7%)		5 (71.4%)		7 (100%)		5 (71.4%)		4 (57.1%)	
>20 (14)	14 (100%)		12 (85.7%)		14 (100%)		11 (78.5%)		11 (78.5%)	

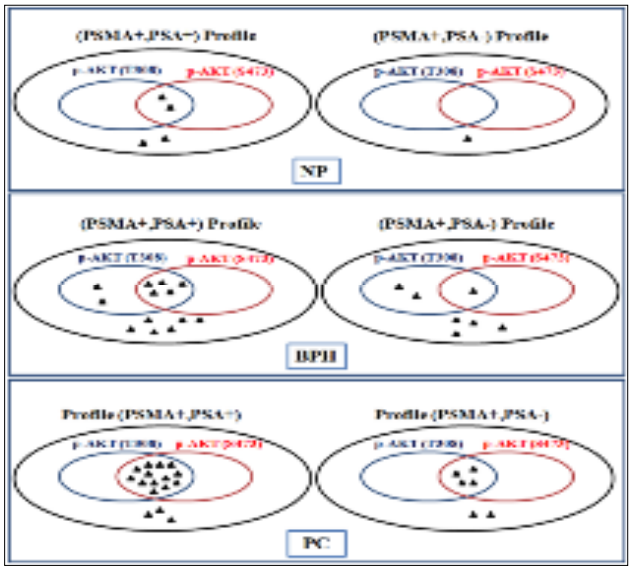
between 4-20ng/mL, the positivity for either pAkt (T308) and pAkt (S473) increased from BPH to PC patients (55.5% and 71.4%, 33.3% and 57.1%, respectively) (TABLE 1). In another hand, in patients with sera PSA levels >20ng/mL the positivity for either pAkt (T308) or pAkt (S473) increased significantly from BPH to PC patients (50% and 78.5%, 16.6% and 78.5%, respectively) (TABLE 1).

Phosphorylation profiling of Akt (T308/S473) connected with PSMA-PSA prostate tumor clonal progression

As we have previously reported, (PSMA+,PSA+) and (PSMA+,PSA-) are the two most abundant PSMA-PSA prostate subgroups in normal, benign prostatic hyperplasia and prostate cancer tissue (7). According to Gleason score (GS), patients from (PSMA+,PSA+) subgroup have a high grade prostate cancer (GS ≥ 8). However, most of patients from (PSMA+,PSA-) subgroup have an intermediate gleason grade prostate cancer (GS = 6).

To highlight the effect of phosphorylation profiling of Akt (T308/S473) on PSMA-PSA prostate tumor clonal progression we examined at first the distribution of distinct phospho-Akt profiles among (PSMA+,PSA+) and (PSMA+,PSA-) prostate subgroups according to NP, BPH and PC specimens. As shown in Figure. 2, immunoreactions to pAKT (T308+,S473+) gradually increased from NP, BPH to PC samples either in (PSMA+,PSA+) or (PSMA+,PSA-) prostate subgroups. Interestingly, pAKT (T308+,S473+) profile was absent in (PSMA+,PSA-) subgroup from NP tissue; whereas this latter profile was more immunoexpressed in (PSMA+,PSA+) compared to (PSMA+,PSA-) prostate subgroups either in normal, benign hyperplasia or prostate carcinomas samples. For the remaining pAkt (T308,S473) profiles, we found that pAkt (T308-,S473+) profile was not detected in all prostate groups; whereas pAkt (T308+,S473-) profile was only immunoexpressed in BPH patients from (PSMA+,PSA+) and (PSMA+,PSA-) subgroups (Figure. 2).

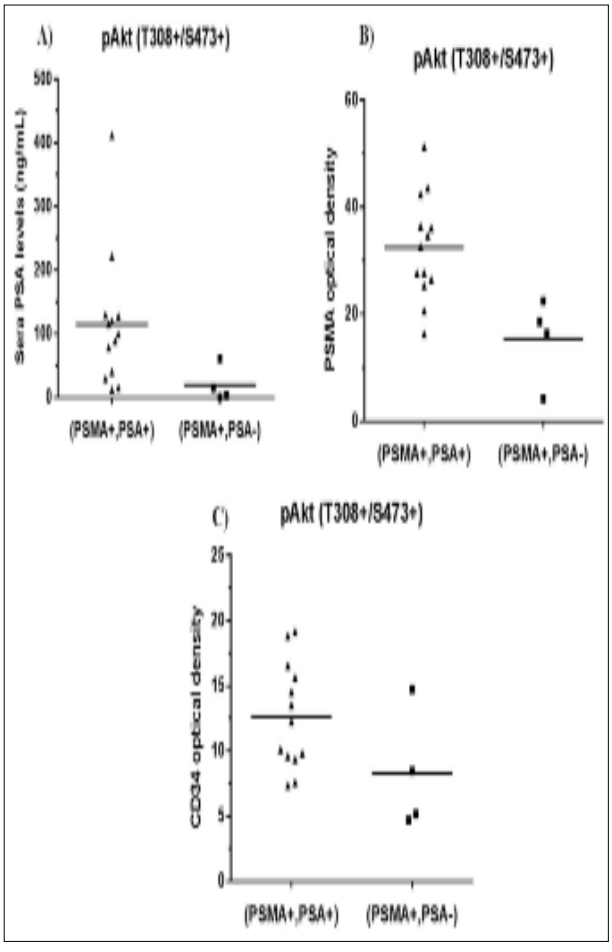
Figure 2 : Distribution of phosphorylation profiling of Akt (T308/S473) in (PSMA+,PSA+) and (PSMA+,PSA-) subgroups according to normal prostate (NP) (A), benign prostatic hyperplasia (BPH) (B) and prostatic carcinoma (PC) (C) specimens.



For the last pAkt (T308-,S473-) profile, we showed that this latter profile was detected in all prostate tissue; whereas its slightly more detected in (PSMA+,PSA+) compared to (PSMA+,PSA-) prostate subgroups in NP, BPH and PC samples (Figure. 2). Comparison of the relevance of pAKT (T308+,S473+) and pAkt (T308-,S473-) in each PSMA-PSA prostate subgroups showed that pAkt (T308-,S473-) profile was more detected than pAKT (T308+,S473+) profile in both (PSMA+,PSA+) and (PSMA+,PSA-) subgroups from BPH patients. Contrary to BPH patients, pAKT (T308+,S473+) profile was more immunoexpressed than pAkt (T308-,S473-) profile in both (PSMA+,PSA+) and (PSMA+,PSA-) subgroups from PC patients (Figure. 2).

In a further approach, we examined the expression of distinct prostate cancer indicator parameters among the most immunoexpressed pAkt (T308+,S473+) profile in both (PSMA+,PSA+) and (PSMA+,PSA-) subgroups from PC patients. As shown in Figure. 3A, the mean sera PSA levels was higher in (PSMA+,PSA+) subgroup compared to those (PSMA+,PSA-) from PC patients (116.5 ng/mL and 20.5 ng/mL, respectively).

Figure 3 : Comparison of sera PSA levels (A), PSMA (B) and CD34 (C) expression between (PSMA+,PSA+) and (PSMA+,PSA-) subgroups from prostate cancer (PC) samples with pAkt (T308+/S473+) profile. Average optical densities were evaluated only in patients showing immunopositivity

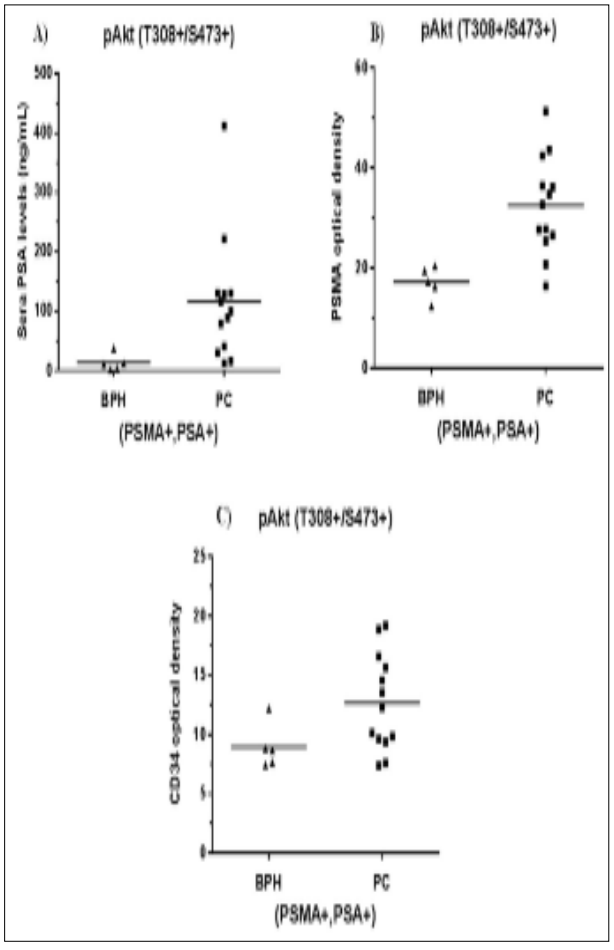


Immunostaining intensity to tissue PSMA was also higher in (PSMA+,PSA+) subgroup compared to those (PSMA+,PSA-) from PC patients with pAkt (T308+,S473+) profile (mean PSMA optical density was 32.46 and 15.43, respectively) (Figure. 3B).

As displayed in Figure. 3C, (PSMA+,PSA+) subgroup was associated with high intratumoral angiogenesis activity compared to (PSMA+,PSA-) from PC patients with pAkt (T308+,S473+) profile (mean CD34 optical density was 12.64 and 8.26, respectively).

Finally, to more elucidate the effect of phosphorylation profiling of Akt (T308/S473) on PSMA-PSA prostate tumor clonal progression we examined the crosstalk between the full Akt activation (T308+,S473+) and several PC parameters in the most immunoexpressed PSMA-PSA prostate tumor clone (PSMA+,PSA+) in BPH and PC patients. As shown in Figure. 4, sera PSA levels, tissue PSMA expression and also angiogenic activity assessed by CD34 antibody increased significantly in (PSMA+,PSA+) prostate clone with pAkt (T308+,S473+) profile from BPH to PC patients (15.6 ng/mL and 116.5 ± ng/mL , 16.14 and 32.46, 8.09 and 12.64, respectively).

Figure 4 : Repartition of sera PSA levels (A), PSMA (B) and CD34 (C) expression among (PSMA+,PSA+) subgroup from benign prostatic hyperplasia (BPH) and prostate cancer (PC) samples with pAkt (T308+/S473+) profile. Average optical densities were evaluated only in patients showing immunopositivity.



DISCUSSION

Our findings emphasize the potential role of the phosphorylation profiling of Akt (T308,S473) in expansion of several PSMA-PSA prostate clones capable of driving both human prostate cancer initiation as well as progression to a metastatic phenotype. But, even more importantly, we postulate that pinpoint patients according to PSMA-PSA phenotype could recapitulate the histological and molecular features of human prostate cancer.

Although most prostate cancers are slow-growing and indolent, a proportion is aggressive, developing metastasis and resistance to androgen deprivation treatment (5,19). Since the existence of cells that will give rise to indolent or to potential metastatic forms of the disease from the early stages of the formation of the primary prostate tumor (20), identification of molecular signatures of cancer initiation is a prerequisite. Akt is shown to be crucial in prostate tumor growth as well as in the multistep processes of invasion and metastasis, including migration and angiogenesis (15,16). Several studies aimed at evaluating the involvement of Akt phosphorylation for patient prostate cancer invasion and metastasis have focused on S473 rather than T308 phosphorylation, probably in large part because phosphoS473 antibodies are relatively effective, particularly in immunohistochemical analysis. As a consequence, few studies have analyzed Akt phosphorylation on T308 (15,16). Given that PSA is an extracellular protease that has been documented experimentally to facilitate both malignant transformation as well as progression to a metastatic phenotype (21) we analyzed at first distinctive Akt phosphorylation at T308 and S473 among sera PSA levels according the group 0-4, 4-20 and >20 ng/mL. Our results demonstrated that among sera PSA levels, according the cut-off, the expression of pAkt (T308) and pAkt (S473) were different in BPH and PC samples. In PC patients, pAkt (T308) and pAkt (S473) were higher expressed in the group of PSA levels between 4-20ng/mL and >20 ng/mL compared to BPH patients. The highest tissue PSA was found in BPH patients who mostly have a serum PSA levels <20 ng/mL compared to PC patients who mostly have a serum PSA levels >20 ng/mL. Moreover, in PC there was an association between the high phosphorylation levels of Akt either at T308 and S473 residues and elevated PSA serum levels: >20 ng/mL. These findings suggest that Akt activation could be implicated indirectly to leakage PSA in the circulation as well increased serum PSA levels in PC patients. Our results are consistent with the literature that showed a correlation between Akt activation and serum PSA levels in PC patients (22,23). Remarkably the highest levels of tissue PSMA and angiogenic activity were found in primary PC who mostly have sera PSA levels >20 ng/mL. Since the increase in pAkt and tissue PSMA have been associated with bad prognosis in prostate cancer (23), the evaluation of the cross-talk between Akt activation, tissue PSMA and angiogenic activity among sera PSA levels might be important to assess malignancy. However, sera PSA levels cannot distinguish between the life-threatening and the relatively harmless forms of the disease (24). Combined with the evidence of the phenotypic heterogeneity of metastatic prostate cancer, it is conceivable that clinical PC initiates from a different pathogenic program (6,25). Therefore, profiling analyses of prostate specimens may strictly define molecular signatures associated with distinct cancer

subtypes that specifically correlate with disease outcome. Profiling analyses of normal, benign hyperplasia and prostate cancer specimens allowed us to identify two most individuals prostate clones: (PSMA+,PSA+) and (PSMA+,PSA-) (7). In order to assess the molecular signatures of each PSMA-PSA clone in NP, BPH and PC, as starting point we compared the phosphorylation profiling of Akt (T308/S473) between (PSMA+,PSA+) and (PSMA+,PSA-) prostate clones. According to our study, the preponderance of each pAkt (T308,S473) profile is largely depends to the cellular context. Interestingly, we showed that full Akt activation gradually increased from NP, BPH to PC samples in the two PSMA-PSA clones. The expression of this latter pAkt profile was lower than pAkt (T308-,S473) profile in both (PSMA+,PSA+) and (PSMA+,PSA-) subgroups from BPH patients. However, full Akt activation was more immunoexpressed than pAkt (T308-,S473-) profile in both (PSMA+,PSA+) and (PSMA+,PSA-) subgroups from PC patients. For the remaining pAkt (T308,S473) profiles, we found that pAkt (T308-,S473+) profile was not detected in all prostate groups; whereas pAkt (T308+,S473-) profile was only immunoexpressed in BPH patients from (PSMA+,PSA+) and (PSMA+,PSA-) subgroups. Apparition of several pAkt (T308,S473) profiles in NP, BPH and PC samples may be explain by the fact that Akt activity is controlled by the phosphorylation and dephosophorylation cycles (26). At the membrane, PDK1 phosphorylates Akt on T308; whereas Akt-S473 phosphorylation is mediated by PDK2 (17,18). In contrast, PP2A (protein phosphatase 2A) negatively regulates Akt activity by inducing Akt T308 dephosphorylation. Furthermore, PHLPP (PH domain leucine-rich repeat protein phosphatase) suppresses Akt activity by dephosphorylating Akt at S473 (26). Although T308 phosphorylation is necessary and sufficient for Akt activation, maximal activation requires additional phosphorylation at S473. T308 phopshoryaltion of Akt directly was shown to control Akt activity. While S473 phopshorylation does not directly control Akt activity, it may facilitate T308 phosphorylation (18,26). Based on our results, we suggest a more extensive role of full Akt phosphorylation in malignant prostate compared to NP and BPH tissue. Therefore, our results seem to indicate that maximal Akt activation, as phosphorylated at T308 and S473, might enhance the development of malignant prostate phenotype. We have also demonstrated that pAkt (T308+,S473+) profile was more immunoexpressed in (PSMA+,PSA+) compared to (PSMA+,PSA-) clone either in normal, benign prostate hyperplasia and prostate carcinomas samples. Accordingly, full Akt phosphorylation is might be implicated in the emergence of PSMA-PSA clones which presumably characterized by different pathogenic program leading to PC disease. In support of this view, in a human prostate tissue transformation model, it was recently reported that cells expressing the oncogene myristoylated Akt can initiate heterogeneous tumors (27). In order to better clarify these observations, we have analyzed distinct indicators PC parameters such as sera PSA levels, tissue PSMA expression and angiogenic activity in (PSMA+,PSA+) and (PSMA+,PSA-) prostate cancer subgroups with pAkt (T308+,S473+) profile. Surprisingly, the highest immunoexpression of each above PC parameters has been detected when PSMA and PSA are coexpressed in PC patients with maximal Akt activation. Increased sera PSA levels are observed in primary localized prostate cancer, but are commonly found at high levels in androgen-independent human prostate cancers with sites of bony and soft tissue metastases (28). Even as they lose the morphologic characteristics of the normal prostate gland and become increasingly less differentiated PC cells continues to express PSMA in high levels especially in hormone refractory aggressive metastatic prostate cancer cells (11,29). As stated, these cells continue to express androgen receptor (AR) that is functional in the low levels or absence of its normal ligand (androgen) (30). However, it is not known whether PSMA and PSA remain under AR control in androgen-independent disease or becomes regulated by other transcriptional pathways (13,31). Accordance to our results, we suggest that PSMA and PSA are coexpressed in primary prostate cancer and synergize with full Akt phosphorylation (T308+,S473) to undergo androgen-independent metastatic prostate cancer phenotype. The data presented in our study support this concept and showed clearly that PC patients from (PSMA+,PSA+) subgroup are poorly differentiated adenocarcinoma; whereas most of PC patients from (PSMA+,PSA-) subgroup are low grade (GS = 6). Indeed, patients with high Gleason score are more likely to develop malignant subtype of prostate carcinomas compared to those with low Gleason score (32).

Based on their different molecular phenotypes, (PSMA+,PSA+) and (PSMA+,PSA-) variants of prostate cancer might be arising from distinct cells of origin. Prostate adenocarcinoma can be serially propagated by cells with a luminal phenotype; whereas it have been recently reported that human prostate tumors may set aside a subset of basal cells within the tumor to ensure continuous production of malignant luminal-like cancer cells (27). The Akt and AR signaling pathways have recently been shown to regulate each other through complex reciprocal feedback mechanisms (33). Accordingly, the cooperative effects of Akt and AR in basal or luminal cells might recapitulate the histological and molecular features of human prostate cancer, with expansion of luminal cells expressing or not PSMA and PSA.

Notably, coexpression of PSMA and PSA are commonly found in normal, primary PC cells and androgen-dependent metastatic PC cells (like LNCaP prostate cancer cell line derived from lymph node metastasis) (13,34). In the present study, PSMA and PSA are mostly coexpressed in normal, BPH and PC samples. However, the feature of the (PSMA+,PSA+) clone is not the same in BPH compared to PC patients with pAkt (T308+,S473+) profile. In fact, in patients with maximal Akt activation, the (PSMA+,PSA+) clone in PC samples is characterized by higher production of sera PSA levels, tissue PSMA and intensive angiogenic activity than (PSMA+,PSA+) clone in BPH samples. These findings suggest that dual activation of Akt at T308 and S473 is responsible of PSMA and PSA production in cellular context-specific manner.

While PSMA and PSA coexpression is maintained in androgen-dependent metastatic LNCaP cell line, the problem of the loss of their expression upon cancer progression from androgen-dependent to androgen-independent stage like in case of two metastatic cell lines PC-3 (derived from bone metastasis) and Du-145 (derived from brain metastasis) remains open (13,34). Despite the phenotypic heterogeneity of metastatic prostate cancer (25), molecular and cytogenetic analyses showed that multiple metastases in the same patient are clonally related (35), indicating that advanced prostate cancer is monoclonal (35,36). These observations lead us to conclude

that, in the same patient, malignant luminal cancer cells may switches from (PSMA+/-,PSA+/-) to a final (PSMA-,PSA-) phenotype during their serially propagation to the bone or to the brain. This switch seems to implicate several mechanisms involved in phosphorylation profiling of Akt at T308 and S473. In support of this concept, evidence indicates that, while mammalian target of rapamycin complex 2 (mTORC2) acts as the PDK2 in LNCaP (PSMA+,PSA+) cells, integrin-linked kinase (ILK) plays a major role in facilitating S473-Akt phosphorylation in PC-3 (PSMA-,PSA-) cells (37). Surprisingly, the angiogenic factor basic fibroblast growth factor (bFGF) involved in Akt activation (38) has been shown to restore the expression of PSMA in markedly dedifferentiated androgen-independent metastatic prostate cancer PC-3 and Du 145 cells (34). The expression levels of pAkt have been proposed as a useful indicator of the severity of the disease in prostate cancer. Its low expression in normal prostate epithelial cells increased several fold in high-grade prostate cancers, in metastatic and in androgen-insensitive prostate carcinoma (16,39). Accordingly, the progressive emergence of (PSMA-,PSA-) clones in response to phosphorylation of Akt at T308 and S473 during prostate tumor progression may represent one mechanism by which prostate cancer cells escape hormonal control.

CONCLUSION

The present results may have important implications in understanding tumorigenic hierarchies in human prostate cancer clones and phenotypic heterogeneities of metastatic prostate cancer, which may prove useful for properly targeted therapy of metastatic disease in advanced prostate cancer.

Competing interests

The authors declare that they have no competing interests.

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