Cellular distribution and heterogeneity of PSA and PSMA expression in normal, hyperplasia and human prostate cancer

Awatef Ben Jemaa, Yosra Bouraoui, Sataa Sallami, Ahmed Banasr, Yassine Nouira, Ali Horchani, Ridha Oueslati

Unit of Immunology, Environmental Microbiology and Cancerology, Faculty of Sciences Bizerte, University of Carthage, Tunis, Tunisia. Faculty of Medicine of Tunis, University of Tunis El Manar, La Rabta Hospital, Department of Urology, Tunis, Tunisia

A. Ben Jemaa, Y. Bouraoui, S. Sallami, A. Banasr, Y. Nouira, A. Horchani, R. Oueslati

Distribution cellulaire et Hétérogénéité d'expression du PSA et PSMA dans la prostate normale, l'hyperplasie bénigne et le cancer de la prostate chez l'homme

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R É S U M É

Prérequis : Comme étant des cibles prometteuses pour le diagnostic, le pronostic et le traitement du cancer de la prostate (CaP) la distribution et le mode d'expression de l'antigène spécifique de la prostate (PSA) et l'antigène de membrane spécifique de la prostate (PSMA) dans les tumeurs prostatiques présentent un intérêt particulier.

Buts : Comparer la distribution cellulaire et l'hétérogénéité de l'expression du PSA et PSMA dans la prostate normale (PN), l'hyperplasie bénigne de la prostate (HBP) et le CaP primitif et analyser leur corrélation avec l'activité angiogénique en fonction du Score de Gleason (faible, moyen et élevé).

Méthodes: L'étude a été réalisée sur 6 PN, 44 HBP et 39 CaP. L'analyse immunohistochimique a été la méthode employée. Les anticorps monoclonaux 3E6 et ER-PR8 ont été utilisés pour évaluer l'expression du PSMA et PSA respectivement. L'évaluation de l'angiogenèse a été faite par l'immunomarqueur CD34.

Résultats: Dans notre étude, nous avons remarqué de différences dans la localisation intracellulaire du PSMA entre le tissu prostatique normal et pathologique. La localisation du PSMA a été détectée au niveau apical chez la quasi-totalité des patients avec CaP (28/39). En revanche, la majorité des échantillons NP et HBP (4/6 et 30/44, respectivement) ont montré une localisation cytoplasmique de PSMA dans les cellules épithéliales luminales. Contrairement au PSMA, le PSA a été préférentiellement localisé dans le compartiment cytoplasmique dans les trois types de prostate (PN, HBP et CaP). Une corrélation a été démontrée entre le grade histologique, l'expression du PSMA et l'activité angiogénique chez les patients cancéreux.

Conclusions: L'immunomarquage simultanée avec le PSA et PSMA dans les tissus prostatiques pourrait améliorer le taux de détection et d'identification des cancéreux présentant un haut risque de progression vers un phénotype métastatique. Nos résultats supportent la faisabilité mais aussi le potentiel de l'utilisation plutôt du PSMA que du PSA comme cible dans les approches thérapeutiques en particulier pour ceux atteints d'un adénocarcinome peu différencié.

M o t s - c l é s

PSA, PSMA, mode d'immunomarquage, score de Gleason, activité angiogénique

S U M M A R Y

Background: As promising targets for in vivo diagnostic, prognostic and therapeutic approaches, the distribution and staining pattern of prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) in tumors are of significant interest.

Aims: To compare the cellular distribution and heterogeneity of PSA and PSMA expression in normal prostate (NP), benign prostatic hyperplasia (BPH) and primary prostatic tumors and to analyze their relation with the angiogenic activity according to Gleason grade (low, medium and high) in primary PC.

Methods: The study was carried out in 6 NP, 44 BPH and 39 PC. Immunohistochemical analysis was performed. Monoclonal antibodies 3E6 and ER-PR8 were used to assess PSMA and PSA expression respectively. The evaluation of angiogenesis was made by CD34 immune marker.

Results: In our study we noticed differences in the intracellular localization of the PSMA immunostaining which seem to be related to the normal and pathological context. A significant number of primary tumors presented with apical pattern of PSMA (28/39); whereas a relevant part of NP samples and BPH samples showed cytoplasmic localization (4/6 and 30/44, respectively) in luminal epithelial cells. Compared to PSMA, PSA was preferentially localized in cytoplasmic compartment in all type of prostate. A direct correlation between histological grade, PSMA expression and angiogenic activity could be demonstrated in primary PC.

Conclusions: Simultaneous stains with PSA and PSMA in individual prostate tissue will greatly improve the detection rate and identify a high risk PC that could progress to metastatic phenotype. Our findings clearly support the feasibility but also direct the potential of PSMA-targeted in vivo therapeutic approaches in PC patients rather than PSA especially those with poorly differentiated adenocarcinoma.

K e y - w o r d s

PSA, PSMA, staining pattern, Gleason score, angiogenic activity

Prostate epithelial cells produce tissue differentiation markers, prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) even in the case of the derangement of the prostate gland, such as benign prostate hyperplasia (BPH) and prostate carcinoma (PC) (1, 2). The PSA is a 33 KDa glycoprotein and secreted into the seminal plasma (3). PSA belongs to the family of serine proteases and member of the tissue kallikrein family (4). This protease is a widely used serum marker for PC but has limited specificity for distinguishing early PC from BPH, because both pathologies release PSA into the serum (5). To improve the clinical value of PSA, the determination of the ratio free/total PSA in serum is the clinical usefulness of PSA testing in PC screening (6). The in vivo antibody binding of PSA gives a promising opportunity for targeted therapeutic applications in PC disease (7). Nevertheless, PSMA is a 100 kDa type II membrane protein with enzymatic functions, acting as a glutamate-preferring carboxypeptidase in human prostate tissue, which plays a role in folic acid utilization and metabolism (8, 9). An increase in expression of PSMA was reported in patients with hematogenous micrometastases of prostate carcinoma (10). In addition to the prostate, PSMA expression has also been reported in the newly formed vessels resembling tumor related angiogenesis in many types of carcinomas (2, 11). PSMA is alternatively spliced to produce PSMA' variant which is remains intracellular. Both variants are recognized by commercially available highly specific monoclonal antibodies, e.g. 7E11 or 3E6 which are applicable for immunohistochemistry (8, 12). The in vivo antibody binding of surface PSMA gives a promising opportunity for targeted imaging and therapeutic applications (13). Several next-generation anti-PSMA antibodies are now either fully human or humanized, thus making them even more likely to be diagnostically and therapeutically effective (8, 14).

The aim of our study was to examine and compare the cellular localization and variability of PSA and PSMA expression as revealed on tissue level by immunohistochemistry in normal, hyperplasia and primary prostate carcinomas. Furthermore, we analyzed the relationship between tissues PSA, PSMA and angiogenesis among clinical stage in primary PC. Information on target heterogeneity and cellular distribution should help to identify factors that may interfere with the clinical utilization of PSA and PSMA based approaches.

PATIENTS AND METHODS

Prostates were obtained from: (a) transurethral resections from 44 men (aged from 61 to 85 years) diagnosed clinically and histopathologically with BPH; (b) radical prostatectomy from 39 men (aged from 57 to 88 years); 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: mouse anti-human PSMA (3E6), mouse anti-human PSA (ER-PR8) and mouse anti-human CD34 (QBend10) (Dako, Glostrup, Denmark). CD34 antibody was used for analysis of angiogenic activity in the prostate tissues.

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 TM (3 m thick) were processed following the NovoLink 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 1/50 (PSMA) and 1/100 (PSA, CD34) in antibody diluents (Dako, Glostrup, Denmark) at room temperature for 30 minutes.

Afterwards, the sections were incubated with Post Primary Block for 30 minutes to block non-TM specific polymer binding. The sections were incubated with NovoLink 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 (O.D) 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 same results were obtained by two different observers. The number of sections examined was determined by successive approaches to obtain the minimum number required to reach the lowest SEM. The PSMA and PSA immunostains were evaluated according to the staining intensity: Negative (O.D = 0), Weak (O.D < 15), Moderate (O.D between 15-20) and Strong (O.D > 20).

The statistical significance between means of the different prostate group's samples was assessed by the one-way ANOVA test and $p<0.05$ was considered significant (GraphPad PRISMA 5.0 computer program).

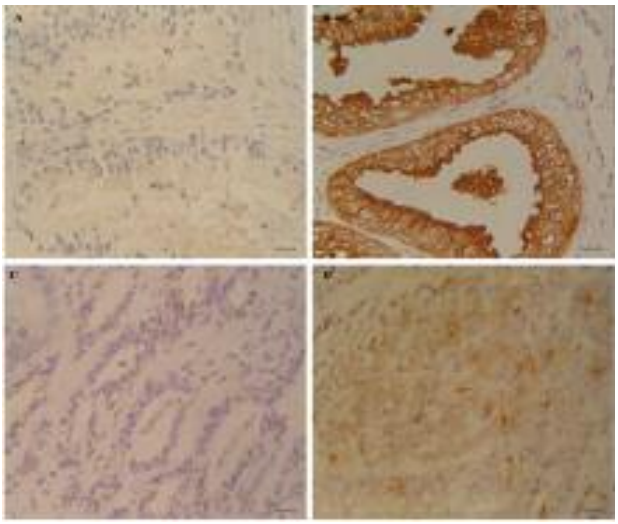
RESULTS

The cellular localization of the confirmed positive PSA and PSMA cases was denoted in Table 1 and Table 2 respectively. We found immunoexpression of PSA and PSMA in different compartment of prostate epithelial cells (Fig. 1 and Fig. 2). As in normal and in pathological prostate tissue, PSA was detected mainly in the cytoplasm of epithelial cells (Fig. 1 and TABLE 1).

Table 1 : Cellular localization of PSA in normal prostate (NP), benign prostatic hyperplasia (BPH) and prostate cancer (PC) tissues.

Positive PSA Staining	Cellular Localization of PSA		
	Apical	Membranous	Cytoplasmic
NP (5/6)	**0**	1	4
BPH (33/44)	**1**	2	30
PC (29/39)	**2**	4	23

Figure 1 : NP showing weak cytoplasmic staining for PSA (A) in epithelial cells. BPH showing strong cytoplasmic staining for PSA in prostatic epithelial cells (B). Low (C) and intermediate (D) PSA expression in cytoplasm of neoplastic acinar structures in prostatic carcinoma. Scale bars: 20μm.

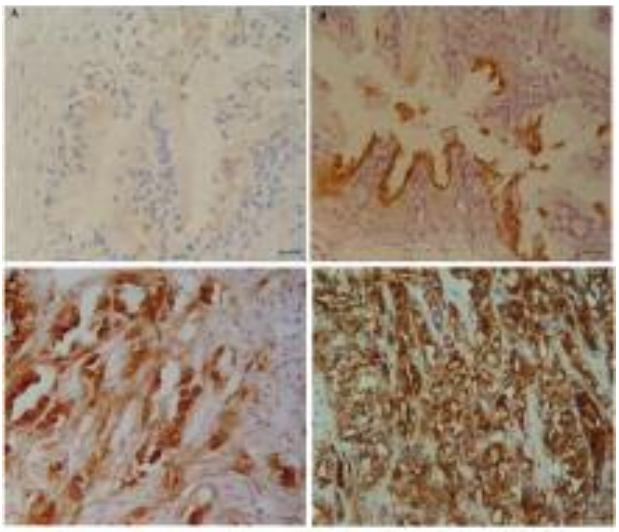


Like PSA, PSMA expression was seen exclusively at luminal cells of prostatic glandular structures in all prostate tissues (Fig. 2). Endotheliums of all prostatic samples were deprived of both PSMA and PSA immunoreactions (Fig. 1 and Fig. 2). Both normal (4/6) and benign epithelial (30/44) cells showed cytoplasmic localization of PSMA in a relevant part of prostate tissues (TABLE 2). Prostatic cancer glandular epithelium expressed PSMA mostly in an apical pattern (28/39) (Table 2), whereas scanty prostatic samples showed cytoplasmic localization of PSMA (3/39) (Table 2 and Fig. 2).

Table 2 : Cellular localization of PSMA in normal prostate (NP), benign prostatic hyperplasia (BPH) and prostate cancer (PC) tissues.

Positive PSA Staining	Cellular Localization of PSA		
	Apical	Membranous	Cytoplasmic
NP (5/6)	**0**	1	4
BPH (38/44)	**2**	6	30
PC (38/39)	**28**	7	3

Figure 2 : NP showing weak cytoplasmic staining for PSMA (A) in epithelial cells. BPH showing intermediate membranous staining for PSMA in prostatic epithelial cells (B). Strong and diffuse cytoplasmic PSMA expression in infiltrating malignant cells in prostatic carcinoma (C and D). Scale bars: 20μm.



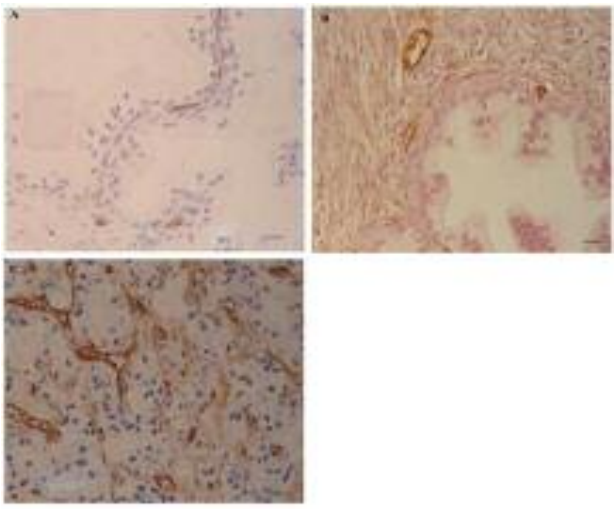
As shown in Figure 3, immunoreactivity to CD34 was found exclusively in the membranous endothelium of both normal and pathological prostate tissues. Capillary vessel network observed, especially near the basal membranes of acinar structures in NP (Fig. 3A) and in BPH (Fig. 3B). Prostatic carcinomas were characterized by a high density of intratumoral capillary vessel network (Fig. 3C).

The overall intensity (optical density) per unit surface area of the IHC staining was determined with an automatic image analyzer.

Table 3 : Summary of PSMA, PSA and CD34 expression according to the Gleason score of prostate cancer patients. Average of optical densities (O.D.) ± SEM (%) were evaluated only 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.

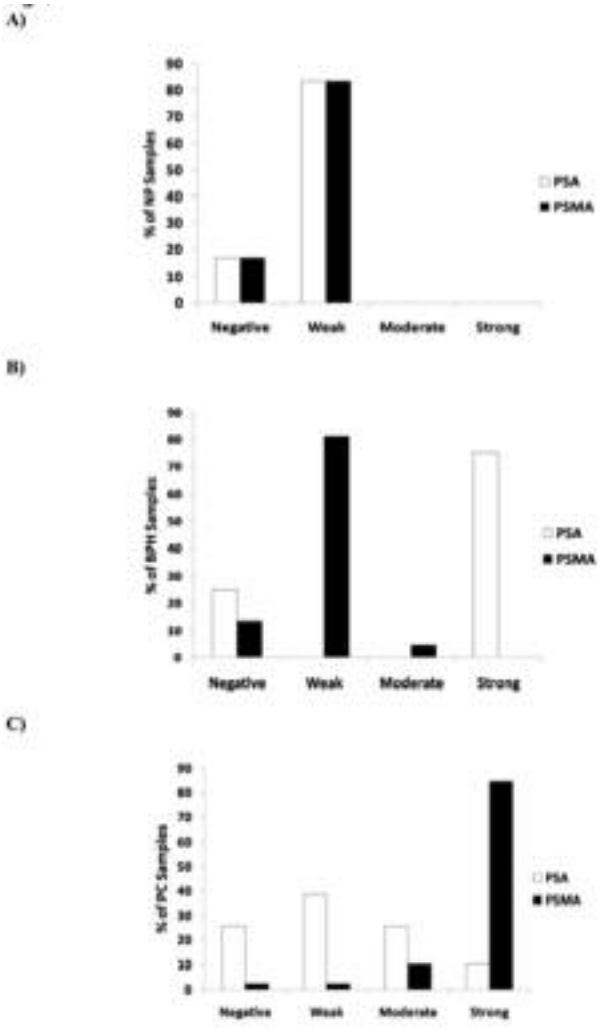
Gleason score	Number of patients	PSMA		PSA		CD34	
		(%)	O.D	(%)	O.D	(%)	O.D
3-6	13	12 (92%)	20.28±2.22a	9 (69%)	14.18±0.67a	13(100%)	7.88±0.13a
7	5	5 (100%)	32.47±1.41b	4 (80%)	20.49±0.21b	5 (100%)	10.08±0.18b
8-10	21	21 (100%)	47.24±0.16c	16 (76%)	15.57±1.19a	21 (100%)	15.77±0.14c

Figure 3 : Capillary vessel network observed, especially near the basal membranes of acinar structures in NP (A) and in BPH (B). Prostatic carcinoma with high density of intratumoral capillary vessel network (C). Scale bars: 20μm.



The PSA and PSMA immunostains were evaluated according to the staining intensity: Negative, Weak, Moderate and Strong. Results of PSA and PSMA immunoreactivity in normal and pathological human prostates tissues are summarized in Fig. 4. PSA and PSMA positivity were evident by immunohistochemistry in the great majority of the evaluated NP, BPH and prostate carcinomas (TABLE 1 and TABLE 2). Normal prostatic glandular epithelium expressed PSA and PSMA with weak intensity in 5/6 cases of NP (83.3%). Only a single case was completely negative for PSA and PSMA in normal prostate (16.6%). A negative PSMA staining was observed in 6/44 BPH samples (13.6%) and in 1/39 primary prostate cancer (2.5%) (Fig. 4). Of 44 BPH samples, 11 (25%) cases were negative for PSA, whereas 10/39 (25.6%) PC cases don't express this latter protein. The staining intensity for PSMA was observed as weak in 81.8% cases of BPH and in 2.5% of primary PC. In addition, moderate intensity for PSMA was found in 4.5% of BPH and in 10.2% of PC samples. Surprisingly, we don't found a strong intensity for PSMA in benign prostatic tissues, while most PC patients (84.6%) revealed a strong immunoreactivity for this protein. Inversely to PSMA, strong intensity for PSA was found in most of BPH tissues (75%). In PC patients, intensity for PSA was observed as weak in 38.4% cases, moderate in 25.6% cases and strong in 10.2% cases.

Figure 4 : Distribution of PSMA and PSA immunostaining intensities in normal prostate (NP) (A), benign prostatic hyperplasia (BPH) (B) and prostate cancer (PC) (C). The PSMA and PSA immunostains were evaluated according to the staining intensity: Negative (% O.D = 0), weak (% O.D < 15), Moderate (% O.D between 15-20) and Strong (% O.D > 20).



PSMA, PSA and CD34 related parameters were further evaluated in the context of the differentiation level by the use of the Gleason score (GS) of the primary tumors. Among PC patients, we found 13 cases with well differentiated tumors (GS between 3 and 6), 5 cases with GS 7 and 21 patients with poorly differentiated tumors (GS between 8 and 10). A tendency and statistical correlation of increased PSMA expression could be observed in regard to Gleason score in primary prostate cancer samples ($p<0.05$); whereas the highest PSA expression was found for PC patients with Gleason score 7 (20.49 ± 0.21) ($p<0.05$) (Table 3). As listed in TABLE 3, the intensity of PSMA expression was significantly increased in PC with Gleason score 8-10 compared to those with Gleason score 7 (47.24 ± 0.16 and 32.47 ± 1.41, respectively) ($p<0.05$). Similar to PSMA, the angiogenic activity increased with increased Gleason score: 7.88 ± 0.13 (GS 3-6), 10.08 ± 0.18 (GS 7) and 15.77 ± 0.14 (GS 8- 10) ($p<0.05$).

DISCUSSION

The transition from normal cells to prostatic benign hyperplasia and localized carcinoma are in part, thought to be the consequence of the deregulation of tissue differentiation markers such as PSA and PSMA (1,2). In the present study, the staining pattern of PSA was compared to that of PSMA in normal and pathologic (hyperplasia and cancer) prostate tissues. Here, we demonstrated that PSA was most expressed in the cytoplasm of both normal and pathologic glandular epithelia. However, PSA was preferentially expressed in BPH rather than normal and prostate adenocarcinoma. Although the PSMA was exclusively localized at luminal cells of prostatic glandular its staining pattern fluctuates between normal prostate, BPH and PC. Cell surface positivity for PSMA was observed in the relevant part of the evaluated PC samples, whereas cytoplasmic staining without evident membrane positivity observed in a scanty part of the evaluated tumor samples. Nevertheless, both normal and benign prostate epithelial cells showed cytoplasmic localization of PSMA in most of prostate tissues. In our study, we noticed that PSMA has been shown to be weakly expressed in both normal prostate and BPH; whereas it's strongly expressed in PC. Our data were concordant with the results of Mhawech-Fauceglia et al (15). According to their study, the strongest staining pattern of PSMA was apical or with membranous accentuation in luminal cells of vast majority of PC (15). Our findings suggest that in addition to their organ specificity (1, 2), the characteristic stain pattern of PSA and PSMA as revealed by immunohistochemistry in individual hyperplasia and prostate carcinomas can provide valuable information regarding the detection of PC. In fact, high PSA expression is likely reflective of BPH disease; whereas strong PSMA expression with apical pattern is likely indicative of PC disease. As targets for in vivo prognostic and therapeutic approaches (7, 14) our findings suggest that distribution and stain pattern of PSA and PSMA in primary tumors are of significant interest. The efficacy of these approaches highly depends on a homogenous and tumor cell-selective membrane expression
of these molecules. Any variation (negativity, heterogeneity of expression, lack of membrane localization) may significantly limit the access of the therapeutic agent to the target cells resulting in therapy failure (16). Previous studies described cytoplasmic PSMA as a splice variant (PSMA') which lost its ability to be integrated in the lipid bilayer as a transmembrane protein. The biological relevance of this variant is not yet known (17). We interpret, that cytoplasmic PSMA positivity presented in our cases represent the overexpression of the PSMA' splice variant in prostate cancer. This kind of overexpression may have a clinical impact as this PSMA splice variant will not be accessible for antibodies in vivo despite the immunohistochemical positivity. Therefore, cytoplasmic PSMA positivity should be considered equal to PSMA negativity in future immunohistochemistry based studies. Our present study confirmed the frequent expression and the target potential of PSMA rather than PSA in primary prostate carcinomas. In prostate carcinomas, it was previously showed that PSA was preferentially expressed in well-differentiated adenocarcinoma rather than in poorly differentiated adenocarcinoma (18, 19). Consistent with this finding, the present study showed that stratified according to Gleason score, PSA expression increases several fold in Gleason grade 7 compared to well and less differentiated adenocarcinoma. In contrast to PSA, in an earlier report by Perner et al, about 48% of the evaluated primary tumors showed PSMA overexpression, which was associated with a high Gleason score (8-10) indicating relatively less differentiated late stage prostate carcinomas (12). In line with this study, we also found higher expression of PSMA in high-grade versus low-grade cancers. Consistent with the correlation between PSMA expression and tumor stage, increased levels of PSMA are associated with androgen-independent PC (20). Unlike expression of PSA, which is downregulated after androgen ablation, PSMA expression is significantly increased in hormone-naive metastases as compared with localized prostate cancer cases (2, 12). Interestingly, we demonstrated a trend of increased PSMA expression concomitant with increased of angiogenic activity in the groups of primary prostatic carcinoma with high Gleason score compared to those with low or medium grade cancers. Nevertheless, according to pathologic stage we found that PSA immunoreactivity is inversely related to PSMA expression and angiogenic activity in primary prostatic carcinomas. Our data suggest that PSA and PSMA are regulated differentially in localized PC. In fact, PSA and PSMA may be causally involved in a reciprocal manner in the development of localized prostate cancer and its progression to metastatic disease. Since PSA decreased in expression with Gleason grade, angiogenic activity and also malignant transformation (21), it might be involved in the early steps of prostate cancer development. Further, PSA might have an anti-angiogenic and anti- metastatic role in PC, where low PSA expression was associated with a high angiogenesis and poorly differentiated adenocarcinoma (22,23). However, enhanced expression of PSMA could be closely associated with aggressiveness and severity of PC. The function

of PSMA in late prostate cancer is unknown, but its ability to remodel extracellular matrix by proteolytic cleavage might be important (24). As a metallopeptidase, PSMA has been documented experimentally to facilitate both malignant transformation as well as progression to a metastatic phenotype (24, 25).

Furthermore, these findings on PSA and PSMA staining patterns and their correlation with angiogenic activity among Gleason grade have important implications for disease-specific therapeutic options. Targeting PSMA seems to be more suitable than PSA in the therapeutic approach and diagnostic imaging with antibody radioconjugates. In fact, according to our results, PSMA seems to fulfill criteria of target for immunotherapy (2) rather than PSA such as: primarily restricted to the prostate, abundantly expressed as protein at all stages of disease and presented at the cell surface.

On the basis of the above results, there was a highly significant difference pattern among BPH and locally confined PC in the expression of both PSA and PSMA. Simultaneous stains with PSA and PSMA in individual prostate tissue will greatly improve the detection rate and identify a high risk PC that could progress to metastatic phenotype. As PC is a heterogeneous disease, significant attention should be give to PSA and PSMA expression as revealed by immunohistochemistry in individual prostate carcinomas which can provide information regarding indication and pitfalls of PSA or PSMA based anticancer treatment antibody therapy.

Competing Interests No competing interests
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