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Immunohistochemical localization and analysis of kallikrein-related peptidase 7 and 11 expression in paired cancer and benign foci in prostate cancer patients

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Kallikrein-related peptidases 7 and 11 (KLK7/KLK11) share a high degree of structural similarity with PSA (KLK3) and other KLKs. The aim of this study was to evaluate differences in KLK7/ KLK11 expression in paired cancer/benign prostate foci and to determine possible associations with clinicopathological parameters.

Seventy archived paraffin-embedded tissue samples obtained from radical prostatectomy were stained for KLK7, KLK11, PSA and PSMA and expression was evaluated semiquantitatively.

The results showed statistically significant differences for all studied proteins between BPH and CaP foci. Both KLK7 (P=0.026) and KLK11 (P<0.001) expressions were decreased in prostate cancer cells compared to normal/benign prostate cells. Positive correlations were found for both KLK7 (Rs=0.74, P<0.001) and KLK11 (Rs=0.35, P=0.003) between CaP and BPH. We found a statistically significant upregulation of KLK11 in advanced cases compared to localized ones (P=0.026).

For the first time, we report lower expression of KLK11 in CaP compared to BPH and slight upregulation of KLK11 in advanced tumors compared to localized ones. Our observations support the diagnostic potential of KLK7/KLK11 for early prostate cancers but further studies on larger cohorts are needed in order to validate the clinical value of these biomarkers and clarify their biological role in prostate development and tumorigenesis.

Key words: kallikrein-related peptidases, KLK7, KLK11, prostate-specific antigen, prostate-specific membrane antigen, immunohistochemical analysis.

Prostate cancer (CaP) is the second most frequently diagnosed cancer and the third most common cause of cancer-related deaths in men [1]. Determination of the prostate specific antigen (PSA) also known as kallikrein-related peptidase 3 (KLK3) [2] has improved the detection and awareness of this malignancy. However, nonmalignant prostatic diseases, especially benign prostatic hyperplasia (BPH), acute prostatitis and prostate manipulations also cause serum PSA elevation, resulting in high false-positive rates of detection. This complicates the diagnosis of prostate cancer using PSA measurement alone [3]. Efforts have thus been made to increase the diagnostic specificity of PSA using concepts such as free to total PSA ratio (fPSA/tPSA), PSA velocity, PSA density and age specific PSA ranges [4]. Recently, the more accurate *phi* test (prostate health index) has emerged in clinical practice which is a combination of serum tPSA, % free PSA and p2PSA ([-2] proPSA isoform). *Phi* significantly increases the predictive value and specificity for CaP detection as well as reduces the number of negative biopsies within the tPSA range of the 2.5-10 ng/ml, i.e. "grey zone" [5].

Kallikrein-related peptidases (*KLK*-genes, KLK-proteins) represent a subgroup of the serine protease family consisting of 15 members located at q13.4 of chromosome 19. *KLK*s are tandemly arranged from centromere (*KLK*1, *KLK*15, *KLK*3, *KLK*2) to telomere (*KLK*4-*KLK*14) and are mainly under steroid hormone regulation [6, 7, 8]. The majority of kallikrein-related peptidases do not exhibit tissue specificity and are co-expressed in various organs such as CNS, skin, breast and almost all salivary glands, as proven in RT-PCR studies [8]. Both centromeric and telomeric groups of *KLK*s are highly expressed in the prostate suggesting their potential as novel diagnostic/prognostic markers [9].

Human kallikrein-related peptidase 7 (KLK7) was first found in the human stratum corneum where it plays a role

in desquamation processes. KLK7 is differentially expressed in ovarian and breast cancer and has been proposed as an unfavorable prognostic marker for these malignancies [10, 11]. A single study on KLK7 expression in CaP has shown its down-regulation at both mRNA (semiquantitative PCR) and protein level (western blot analysis, immunohistochemistry (IHC)) with a significant negative association to Gleason grade [12].

Human kallikrein-related peptidase 11 (KLK11), which was originally isolated from the human hippocampus, is under steroid hormone regulation and has three isoforms derived from alternative splicing: isoform 1 (brain-type), isoform 2 (prostate-type) and isoform 3 [13, 14]. The prostate-type variant is expressed only in the prostate while the brain-type can be detected in both brain and prostate [13]. High expression of *KLK11* mRNA and protein has been demonstrated in many normal tissues, including prostate, stomach, trachea, colon, brain, skin and salivary gland [6, 15]. KLK11 is a well-studied biomarker for ovarian cancer yielding high serum levels in 70% of cancer patients [15, 16]. Recent studies have shown that KLK11 serum levels are down-regulated and better discriminate CaP from BPH than total PSA, especially KLK11:total PSA ratio in combination with % free PSA (90% sensitivity, 51.5% specificity) (17). Overall, KLK11 seems to be a favorable prognostic marker for prostate cancer and could be used for discriminating BPH from CaP.

Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein playing a role in folic acid utilization and metabolism [18]. PSMA is highly expressed in normal prostate tissue [19] and it is negatively regulated by androgens (20). The expression increases in hPIN and is further enhanced in advanced, poorly-differentiated tumors, especially in androgen-independent prostate cancer [19, 20, 21].

The aim of this study was to analyze IHC expression of KLK7/KLK11, in parallel with well-known markers PSA and PSMA, in non-cancerous (normal/BPH) and cancerous foci and to determine any association with clinicopathological parameters.

Materials and methods

Patient and tissue specimen selection. Seventy cases of prostate cancer were identified as clinically localized (T1-T2, n=39) or locally advanced (T3-4, N0-1, M0, n=31). The study included patients who had not received neoadjuvant treatment but who had undergone radical prostatectomy (RP) as initial treatment following positive biopsy results. The study was approved by the ethics committee of the Medical Faculty of Palacky University. Clinical (TNM, clinical stage, serum total PSA) and pathologic (pT, Gleason score and grade, pN) characteristics were defined according to the WHO classification (Table 1). Paired CaP and BPH foci were identified in each patient and tissue sections from these were then used for further IHC analysis.

Table 1. Clinical and pathologic characteristics of patients

		Localized tumors (n)*	Advanced tumors (n)*
Age	51-59	15	8
	60-69	26	15
	70-73	1	5
Gleason Score	<7	21	8
	≥ 7	18	23
Clinical Stage	II	39	0
	III	0	25
	IV	0	6
Serum PSA	<4 ng/ml	8	3
	<8 ng/ml	14	10
	≥8 ng/ml	17	18

** Localized cases defined as pT1-pT2, advanced pT3-pT4*

Immunohistochemistry. IHC was performed according to our routine protocol. 70 archived paraffin-embedded tissues were dewaxed and rehydrated with xylene and ethanol. After immersion in citrate buffer (pH 6), the sections underwent pressure cooking pretreatment for 5 minutes for optimal antigen retrieval. The slides were immunostained for KLK7 and KLK11 by rabbit polyclonal primary antibodies (Abs) provided by Prof. Diamandis Lab "Mount Sinai" hospital, Toronto, Canada (dilution 1:200). PSA (clone ER-PR8, Dako, Denmark, dilution 1:25) and PSMA (clone YPSMA-1, Abcam, UK, dilution 1:1000) were stained by mouse monoclonal Abs and the secondary antibodies were applied for 60 minutes at room temperature. Specific binding was detected using the peroxidase/DAB based detection kit EnVision, Dako. Staining intensity, positive cell fraction, staining heterogeneity and cellular localization were determined for all four genes in both BPH and CaP foci for each patient. Positivity was classified according to the percentage of all positively stained tumor cells and the intensity of the IHC staining was categorized 0 to 2 as: 0 no staining, 0,5-very low, 1-moderate, 1,5-high and 2 strong positive staining. Staining intensity was then divided into a 3 grade scoring system, indicating 0-0,5 as no staining, 1-low, 1.5-moderate and 2 high staining, respectively. Histoscores were also derived for further statistical analysis (staining intensity x percentage of cells). Intracellular localization of the staining was determined and patterns of apical surface, cell membrane or cytoplasmic staining were documented.

Statistical analysis. The software Statistica 8.0 version for windows (Statsoft, CZ) was used for statistical analysis. As the distribution of variables was non Gaussian, the analysis of differences in protein expression between CaP and BPH was done using the nonparametric Wilcoxon Signed Ranks Test. Kruskal–Wallis one-way analysis of variance by ranks was used to test the equality of population means between low and high grade CaP (Gleason score <7 vs. ≥ 7), in organ-confined and advanced prostate cancers (pT2 vs. ≥ pT3, pT4)

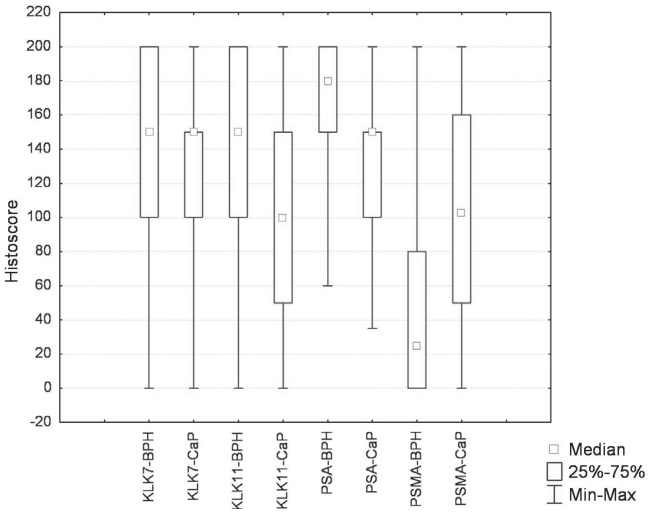

Figure 1. Differences in expression of selected proteins between BPH and CaP.

and in different clinical stages (II, III, IV). The correlation between different variables was assessed by the Spearman Rank correlation coefficient. $P<0.05$ was chosen as the level of significance.

Results

Staining was always cytoplasmic for both KLK7 and KLK11. Noteworthy was the positive staining of tumor neovasculature and muscle cells with both anti- KLK7 and anti- KLK11 Abs in some cases. Prostatic secretions were always strongly positive for KLK7/11. We also observed positive staining of urothelium and seminal vesicle epithelium for KLK7. The majority of CaP foci showed decreased expression of both KLK7 and KLK11 against BPH per section per patient ($P=0.026$ and $P=0.001$, respectively) (Figure 1), though expression heterogeneity was quite common, especially for KLK7 (Figure 2). Positive correlations were found for both KLK7 (Rs=0.74) and KLK11 (Rs=0.35) between CaP and BPH foci (Table 2). Moreover, a statistically significant correlation was found between KLK7 and KLK11 (Rs=0.242, $P=0.043$) for the BPH foci. This correlation did not seem to arise in the CaP foci. In addition, there was no significant correlation or significant differences in the staining intensity or percentage of cells stained between a Gleason score of <7 and ≥7 in prostate carcinoma, between pT2 and pT3-pT4 tumors and serum PSA values. However, we found a slight difference in KLK11 expression between advanced and localized cases with up-regulation in advanced ones ($P=0.049$).

We observed positive staining of both non-cancerous and cancerous foci with anti-PSA Abs which was cytoplasmic with apical accentuation. Prostatic secretions were strongly positive in almost all cases. Staining intensity was greater in BPH than prostate carcinoma ($P<0.001$) with a trend to further decrease in less differentiated cancers ($P=0.077$, Figure 3). PSA expression did not correlate significantly with other proteins or clinicopathological data.

We also detected high PSMA expression at the luminal-apical surface of glandular structures and in the neovasculature associated with cancer. Strong positive staining was also seen in some muscle cells and prostatic secretions. Cancer cells showed intense, significantly higher expression than normal/BPH glands (Figures 2 and 3), though cells lacking PSMA staining could also be identified close to the most intensively stained tumor cells indicating staining heterogeneity of tumor cells.

Table 2. Spearman's correlation coefficient (Rs) for biomarker expressions in BPH and CaP

		BPH				CaP			
		KLK7	KLK11	PSA	PSMA	KLK11	KLK7	PSA	PSMA
KLK7-BPH	Rs	1.000	**.242**	-.224	.125	.094	**.738**	-.082	.107
	p	.	**.043**	.062	.304	.437	**.000**	.502	.378
KLK11-BPH	Rs		1.000	.039	-.117	**.348**	.202	-.126	-.080
	p		.	.751	.335	**.003**	.093	.300	.512
PSA-BPH	Rs			1.000	-.040	.068	-.140	.130	.118
	p			.	.740	.579	.247	.282	.331
PSMA-BPH	Rs				1.000	.071	.168	.036	**.240**
	p				.	.562	.165	.764	**.046**
KLK11-CaP	Rs					1.000	.155	-.107	-.020
	p					.	.200	.377	.872
KLK7-CaP	Rs						1.000	-.226	.144
	p						.	.060	.235
PSA-CaP	Rs							1.000	-.024
	p							.	.842
PSMA-CaP	Rs								1.000
	p								.

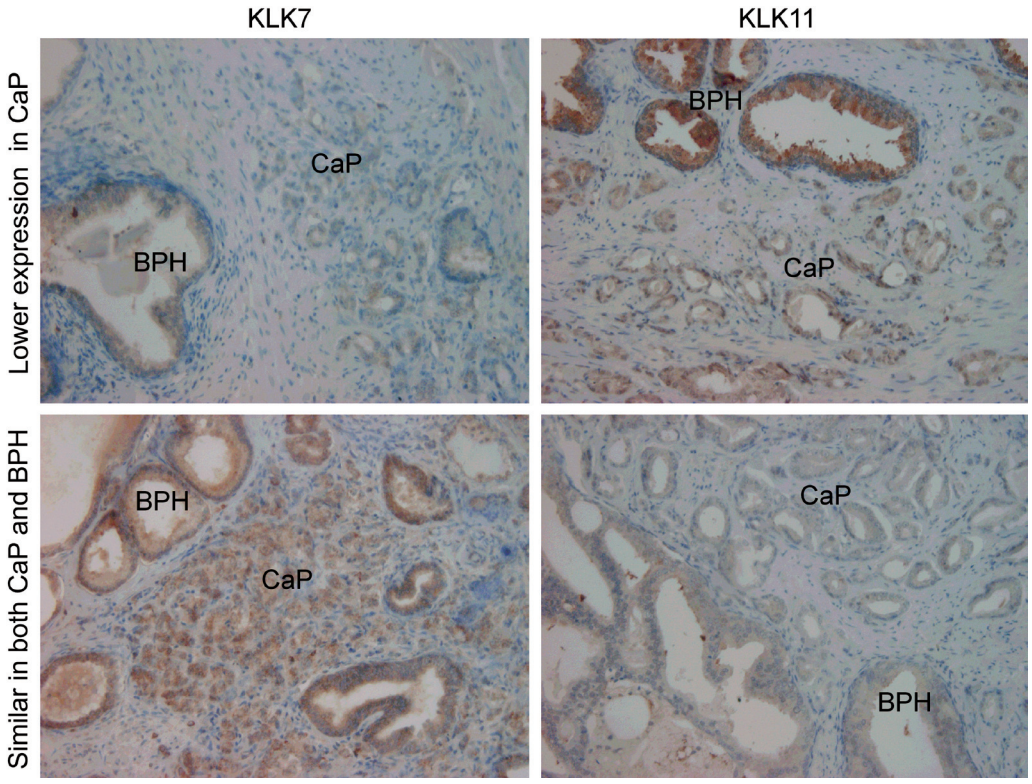

Figure 2. Heterogeneity of KLK7/11 expression in BPH and CaP.

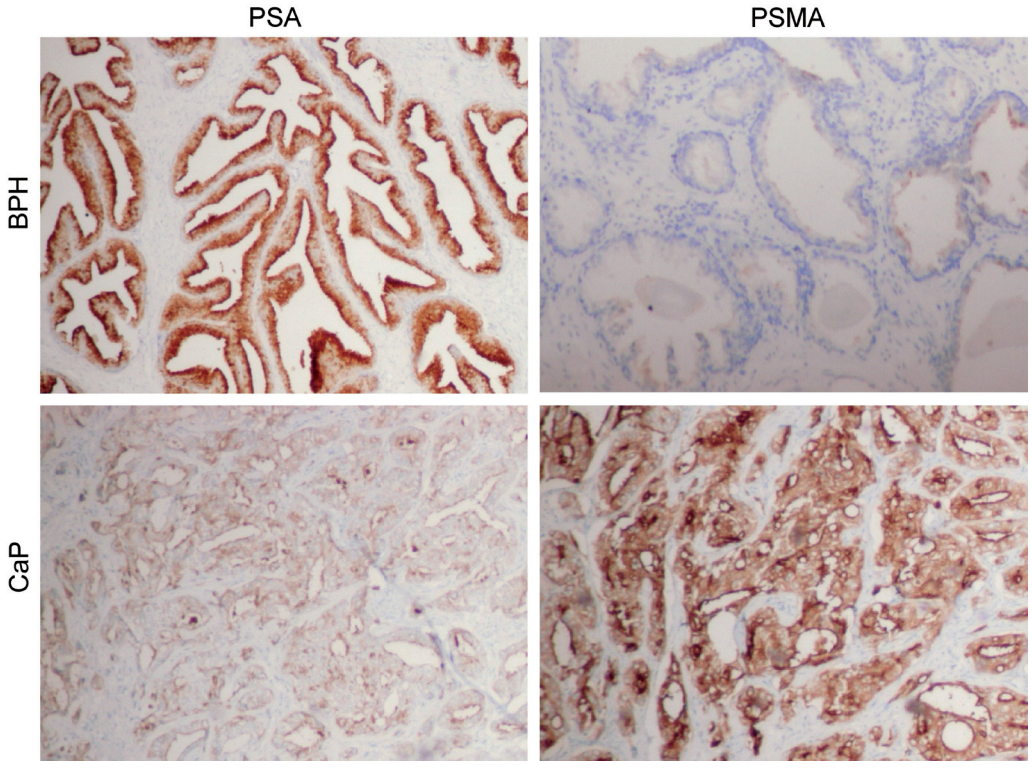

Figure 3. Expression of PSA and PSMA in BPH and CaP.

We also found a trend to increased PSMA expression in less differentiated cancer cells in relation to Gleason score (<7 vs. ≥ 7; P=0.114) and pT stage (pT2 vs. pT3-pT4; P=0.083).

Discussion

Our study focused on evaluation of KLK7 and KLK11 tissue expression along with PSA and PSMA. So far, KLK7 expression in prostate cancer has only been evaluated in a single study along with ALP (antileukoprotease) as a specific inhibitor of KLK7 (12). Both KLK7 and ALP have been shown to be down-regulated at mRNA and protein level, assessed by semi-quantitative PCR, western blot analysis and IHC. Significant negative association was found between Gleason grade and both KLK7 and ALP expression. In concordance with this study we confirmed down-regulation of KLK7 in malignant prostate epithelial cells compared to benign/normal prostate epithelial cells. We also observed positive staining of tumor neovasculature, some muscle cells, urothelium and seminal vesicle epithelium.

For the first time, we report down-regulation of KLK11 protein at tissue level. This is consistent with the serum levels reported by Nakamura et al (17). These authors evaluated different patients while we confirmed KLK11 down-regulation in the paired tissues of BPH and prostate carcinoma in the same patients. Expression differences for CaP and BPH foci were more significant for KLK11 than for KLK7. In other words, more heterogeneity was found for KLK7 than for KLK11. This could be related to their different regulation. Interestingly, *KLK11* mRNA expression in prostate cancer does not correspond with its protein levels. Such discordance has also been reported for other genes, for example in breast cancer, indicating complex regulation of protein expression (22). *KLK11* mRNA expression has been detected in both normal and cancerous prostate tissue but with significant up-regulation in cancer (14). Up-regulation of prostate-type *KLK11* was significantly associated with earlier stage, lower Gleason score and lower tumor grade but expression was again lowered with tumor progression (advanced stage, less-differentiated CaP) as confirmed by qRT-PCR analysis (23, 24). Also noteworthy was the inverse association between serum total PSA and *KLK11* mRNA overexpression, i.e. serum total PSA concentration was found to be lower in patients with overexpression of *KLK11* (25). No correlation or significant differences in relation to Gleason score, tumor grade, pT stage or serum PSA values were found. We observed slight up-regulation of KLK11 in advanced cases compared to localized cancers. However, this needs further validation by larger studies.

We confirmed decreased PSA expression in CaP compared to BPH. The trend to decreased PSA expression was also found in less differentiated cancers which is also in agreement with other IHC studies (26, 27). PSMA expression in cancer was the reverse of its PSA expression, i.e. PSMA expression was up-regulated in CaP vs BPH, in concordance with other IHC studies (19, 21). No statistically significant correlation with either Gleason score, tumor stage or PSA/PSMA expression was found. However, we detected high PSMA expression in the cancer-associated neovasculature, in some muscle cells and in prostatic secretions as reported in other studies [18, 19].

In conclusion, we report lower IHC expression of KLK11 in CaP tissue compared to normal/benign prostate tissue and also slight up-regulation of KLK11 in advanced tumors compared to localized ones. In concordance with the literature, we confirm both higher expression of PSMA, lower expression of KLK7 and PSA in CaP compared to BPH. Our observations support the diagnostic potential of KLK7/KLK11 for early prostate cancers but further studies on larger cohorts are needed in order to validate the clinical value of these biomarkers and clarify their biological role in prostate development and tumorigenesis.

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