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Expression analysis and study of the *KLK15* mRNA splice variants in prostate cancer and benign prostatic hyperplasia

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Prostate cancer is the most commonly diagnosed malignancy in male populations in the Western world. The *KLK15* gene, the newest member of the kallikrein family, is expressed in the prostate gland. The purpose of this study is the expression analysis and the clinical evaluation of the *KLK15* mRNA spliced variants in prostate cancer (CaP) and benign prostatic hyperplasia (BPH) patients. Total RNA was isolated from 104 CaP and BPH tissue specimens. After testing the quality of the RNA, cDNA was produced by reverse transcription, and PCR was performed for the amplification of the *KLK15* mRNA transcripts. *GAPDH* and *HPRT* genes were used as endogenous controls. Our data revealed that mRNA spliced variants of *KLK15* were differentially expressed in prostate tissue specimens. Analysis of data showed a statistically significant ($P < 0.001$) increase in the frequency of overexpression of *KLK15* transcripts encoding for both the active isoform and for the isoform 3 in CaP compared to BPH samples. Furthermore, *KLK15* transcripts were found to be highly expressed in more aggressive tumors ($P = 0.017$). These results suggest that *KLK15* expression analysis could be employed as a valuable tool for the discrimination between BPH and CaP tissue specimens and as an unfavorable prognostic marker for prostate cancer. (*Cancer Sci* 2010; 101: 693–699)

Prostate cancer (CaP) is the most common non-skin male malignancy, accounting for almost 33% of all cancer incidents. Also, it is the second-most common cause of cancer-related deaths in most Western countries.[1,2] Although many risk factors, such as alterations in androgen biosynthesis and metabolism, and diet and chronic inflammation, have been associated with the disease, the etiology of prostate cancer remains uncertain.[1,3,4] The most common form of the disease is sporadic prostate cancer, which is expected to be developed by non-random somatic genome alterations such as loss or gain of chromosomal regions (6q, 7q, 8p), abnormal DNA methylation, mutations of oncogenes (*EGFR*, *MYC*) and tumor suppressor genes (*NXK3.1*, *GSTP1*), and alterations regarding the androgen receptor (AR).[2,5–7] AR is an intracellular ligand-dependent transcription factor, which plays an essential role in the survival and growth of prostate cells, as well as in the initiation and the progression of prostate tumors. As a result, the disease treatment aims at the regulation and manipulation of the AR metabolic pathway. However, AR function evolves as the cell changes toward a clinical androgen-depletion independent state.[8,9]

Recently published data show that benign prostatic hyperplasia (BPH) occurs to about 8% of men aged from 31 to 40 years, to one-quarter of men in their 50s, to one-third of men in their 60s, to about half of all men aged 80 years or older and increases to about 90% by the ninth decade of life.[10]

Since the discovery of prostate-specific antigen (PSA) also known as, kallikrein (KLK) 3 routine PSA testing has become the mainstay of prostate cancer detection.[11] However, PSA lacks diagnostic specificity, given that PSA levels can be increased in non-malignant conditions. Consequently, a variety of molecular biomarkers, including other kallikrein members, could play a potential role in the clinical diagnosis of prostate cancer.[11–15]

The human tissue kallikreins (KLKs) comprise a subgroup of secreted serine proteases that represent the largest contiguous cluster of protease genes in the human genome and are located in the chromosome locus 19q13.4.[16,17] The process of alternative splicing seems to be a rather usual event within the kallikrein family; approximately 70 alternatively spliced variants of kallikrein genes have been indentified up to date.[16–20]

Kallikreins are widely expressed in diverse tissues and are implicated in a variety of diverse normal physiologic functions, from the regulation of blood pressure to skin desquamation.[16,21–23] It is essential for kallikreins to be regulated at various levels, including transcription, translation, and post-translation.[16,24,25]

It is widely accepted that *KLK*s are involved in various neoplastic conditions and they are proposed to serve as new biomarkers for diagnosis, prognosis, and monitoring of cancer.[26] *KLK4*, *KLK11*, *KLK14*, and *KLK15* have been shown in particular to be highly expressed at the mRNA level in prostate cancer.[26–32]

The *KLK15* gene is the newest member of the kallikrein family. It maps to the human kallikrein locus, between *KLK1* and *KLK3* genes, and bares structural resemblance to the *KLK3* (PSA) gene. *KLK15* is formed of five coding exons and four intervening sequences.[33] Alternative splicing is a common event in the kallikrein locus and *KLK15* is not an exception. Therefore, apart from the classical isoform, three more splice variants have been identified. However, only the classical mRNA isoform encodes for an active protein; the other splice variants are thought to encode for truncated proteins.[18,33]

KLK15 is primarily expressed in the thyroid gland. Other organs where *KLK15* mRNA expression has also been detected are the prostate gland, salivary glands, colon, testis, and kidney. The expression of *KLK15* is up-regulated, at the mRNA level, by steroid hormones in prostate cancer cell lines. Nevertheless, tissue expression levels are not affected by preoperative antiandrogen treatment given to patients.[33]

During 2007, KLK15 protein (hK15) expression levels were analyzed in various human tissues, and biological fluids.[34] KLK15 is most abundant in the breast and fetal skin. In addition, KLK15 protein presence was also examined in human biological fluids and was found mainly in seminal plasma, breast milk, and

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the cervicovaginal fluid. The physiologic role of hK15 remains uncertain, as it is only known to activate proPSA.[33]

The expression of *KLK15* at the mRNA level has been shown to be significantly higher in various types of cancerous tissues compared to either their noncancerous counterparts. *KLK15* has been found to be overexpressed in prostate and ovarian cancer and it is regarded as an unfavorable prognostic marker for these types of cancer.[28,35] On the contrary, *KLK15* could constitute a favorable prognostic marker for breast cancer, given that higher expression of *KLK15* was present in node-negative patients and *KLK15* expression was found to be a significant predictor of progression-free survival.[36]

Materials and Methods

Prostate tissues. Prostate tissue specimens were obtained from 52 patients with BPH, and 52 patients with CaP, collected at Laiko General Hospital, Athens, Greece, from June 2005 to July 2007. The age of patients diagnosed with BPH ranged from 49 to 83 years (median, 70 years), whereas the age of those suffering from CaP varied from 52 to 76 years (median, 65 years). Patients had not received any hormonal therapy prior to surgery.

The tissue portions from the BPH patients were received from the adenomatectomy specimen, removed either in a transurethral or open prostatectomy method. Immediately after the removal of the prostate gland by radical prostatectomy in CaP patients, a tissue sample of about 200 mg was sectioned from the peripheral zone of the prostate gland guided by the preoperative features of the biopsy, which namely are the location of the tumor and the number and percentage of the cores with established malignancy, as well as by the macroscopic findings of the tissue specimen. The sample was then separated into two mirror-image sections, one of which was always evaluated by the same pathologist in order to confirm, or not, the presence of malignancy. The other section of the sample was rapidly frozen in liquid nitrogen and stored at −80°C until the analysis. From each frozen prostate cancer sample section, only the cores with confirmed malignancy were used for total RNA extraction.

Total PSA (tPSA) serum concentrations were precisely measured for all patients before operation. Data including the demographic features, PSA values, digital rectal examination findings, clinical stage of the disease, parameters of the biopsy of the prostate gland, parameters of the pathology report, and medical history of all patients was thoroughly recorded (Table 1).

Our study was performed with respect to the ethical standards of the 1975 Declaration of Helsinki, as revised in 1983. Institutional approval for the use of the prostate samples was obtained from the ethical committee of Laiko General Hospital.

Table 1. Distribution of patients on the basis of the examined clinicopathological factors

Variables	Mean ± SE
Benign prostatic hyperplasia (BPH) (*n* = 52)	
PSA (ng/mL)	4.91 ± 0.79
Age (years)	70.27 ± 1.07
Prostate cancer (CaP) (*n* = 52)	
PSA (ng/mL)	9.55 ± 0.89
Age (years)	64.8 ± 0.8
Patient stage	No. of patients (%)
pT1	0 (0)
pT2	33 (63.5)
pT3	16 (30.8)
pT4	1 (1.9)
x	2 (3.8)
Tumor size	
<1 cm, non-invasive	10 (19.2)
≤1 cm, invasive	10 (19.2)
>1 cm, invasive	28 (53.8)
x	4 (7.7)
Gleason score	
≤6	16 (30.8)
7	28 (53.8)
>7	5 (9.6)
x	3 (5.8)

PSA, prostate-specific antigen.

Total RNA extraction, RNA quality evaluation, and cDNA synthesis. Prostate tissue frozen specimens of 40–90 mg, obtained from the BPH patients' sample, and only the cores with confirmed malignancy from the CaP tissue samples, were used for total RNA extraction and cDNA synthesis. Noncancerous portions of prostate cancer patients' samples were excluded from the analysis. Consequently, the mRNA molecules that were reversed-transcribed into cDNA were obtained from either well-defined benign portions from BPH or malignant cores from CaP patients. After pulverization with BioPulverizer (Biospec, Bartlesville, OK, USA) on dry ice, total RNA was isolated from the resulting specimens' powder using TRI-Reagent (Ambion, Austin, TX, USA). All RNA samples were preserved with RNA-Storage solution (Ambion) and stored at −80°C. Total RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm. RNA integrity was evaluated by agarose gel electrophoresis. One μg of total RNA from each tissue sample was reverse-transcribed into first-strand cDNA in a 20-μL reaction, using M-MuLV Reverse Transcriptase RNase H^- (Finnzymes Oy, Espoo, Finland).

Table 2. Primers used for RT-PCR

Method	Gene	Sequence	Tm (°C)	Product size (bp)
RT-PCR	*GAPDH*	(F) CCACATCGCTCAGACACCAT	59.4	240
		(R) TGACAAGCTTCCCGTTCTCA	59.6	
	HPRT	(F) TGGAAAGGGTGTTTATTCCTCAT	60.5	151
		(R) ATGTAATCCAGCAGGTCAGCAA	60.6	
	KLK15 (classical mRNA isoform)	(F) CACGTCTCGGGTCATTCCAC	61.4	229
		(R) TGGGAGACTCACTTGTGACCG	61.2	
	KLK15 (splice variants)	(F) CATCATGTTGCTGCGCCTAGT	61.7	391 (classical isoform)
		(R) CAGGCTTGGTGGTGTTGTCA	60.1	273 (splice variant 1)
				254 (splice variant 2)
				136 (splice variant 3)

All nucleotide sequences are given in the 5′ → 3′ direction. Tm was calculated using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). F, forward primer; R, reverse primer. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *HPRT*, hypoxanthine-guanine phosphoribosyltransferase; *KLK15*, kallikrein 15.

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Polymerase chain reaction (PCR). Gene specific primers (Table 2) were designed for *GAPDH* (glyceroldehyde 3-phosphate dehydrogenase, housekeeping gene), *HPRT* (hypoxanthine-guanine phosphoribosyltransferase, housekeeping gene), *KLK15* classical mRNA isoform, and *KLK15* splice variants based on cDNA sequences from the NCBI Sequence database (GenBank accession nos. NM_002046 for *GAPDH*, NM_000194.2 for *HPRT*, NM_017509 for the *KLK15* classical isoform, NM_023006, NM_138463, NM_138464 for the *KLK15* splice variants), using the Primer Express software (Applied Biosystems, Foster City, CA, USA). The use of *HPRT* as a second endogenous reference gene and the determination of its expression levels, in a significant number of randomly selected CaP and BPH specimens, attributes to the examination of the *GAPDH* transcription impact on the *KLK15* expression analysis.[37]

PCR was performed for the *KLK15* classical mRNA isoform using 2.5 μL of cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM dNTPs, 1 μM primers, and 1.25 units of GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) in a Labnet thermocycler (Labnet International, Berkshire, UK). The optimum cycling conditions were 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR reaction for the *KLK15* splice variants required alternatively 1.5 μL cDNA, 1.5 mM $MgCl_2$, and an annealing temperature of 59°C. Eventually, PCR amplification for the housekeeping genes *GAPDH* and *HPRT* was performed using 1.2 μL of cDNA, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM primers, 1.25 units of GoTaq Flexi DNA polymerase (Promega), and 5× reaction buffer (Promega), with optimum cycling conditions: 95°C for 2 min, followed by 36 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. For negative control, in each PCR reaction, a sample without cDNA template was used. Equal amounts (15 μL) of the *GAPDH, HPRT*, and the *KLK15* transcript PCR products (Table 2) were electrophoresed on 1.5% agarose gel electrophoresis, visualized using ethidium bromide staining, and photographed under ultraviolent light. The images were analyzed in order to evaluate the levels of gene expression with the NIH Image program developed at the US National Institutes of Health. For assessment of the levels of expression, arbitrary units were used derived from the fraction: *KLK15* mRNA transcripts expression levels/*GAPDH* expression levels. Specimens which failed to express *KLK15* at detectable levels were attributed gene expression equal to half of the arbitrary units of the sample with the lowest expression levels (half of the detection limit).

Statistical analysis. Due to the fact that the distribution of *KLK15* expression levels in both the BPH and CaP patients was not Gaussian, analysis of differences between these parameters, in the two groups, was performed with the nonparametric Mann–Whitney and Kruskal–Wallis tests. Correlations between different variables were assessed by Spearman correlation coefficient. Receiver–operator curves (ROC) were constructed for *KLK15* expression levels and total PSA, by plotting sensitivity *versus* (1-specificity), and the areas under the ROC curves (AUC) were analyzed by Hanley and McNeil method. The ability of the variables to predict presence of prostate cancer was studied using univariate and multivariate unconditional logistic regression analysis.

Results

Expression analysis of *KLK15* splice variants in prostate tissue specimens. Several studies have proposed the use of *GAPDH* and *HPRT* housekeeping genes as endogenous reference controls for gene expression analysis in prostate tissue specimens.[37] In the current study, analysis of the *KLK15* expression between the CaP and BPH patients was performed, employing *GAPDH* as an endogenous control. The use of *HPRT* as a second reference endogenous control for the normalization of *KLK15* expression levels did not affect the *KLK15* expression analysis demonstrated using the *GAPDH* housekeeping gene.

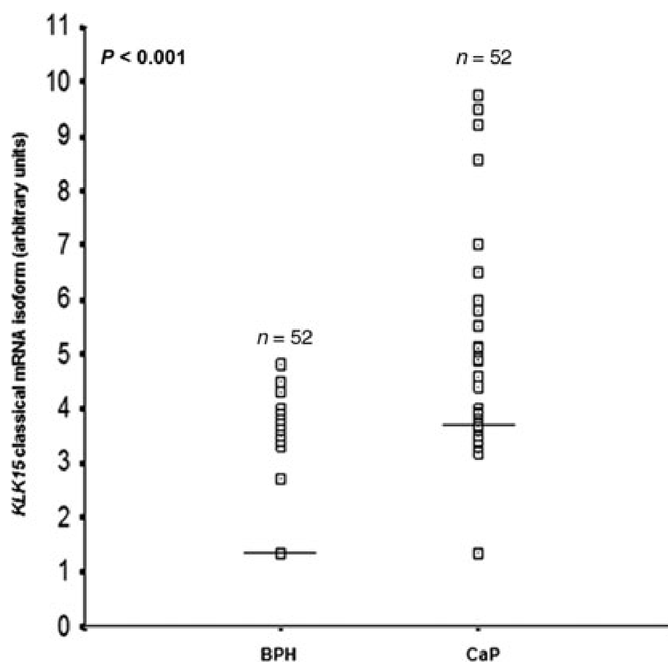

Fig. 2. Distribution of kallikrein 15 (*KLK15*) classical mRNA levels in benign prostatic hyperplasia (BPH) and prostate cancer (CaP) patients. The bold lanes represent the median value (50th percentile) for each patient cohort. (*P*-value was calculated by the Mann–Whitney test.)

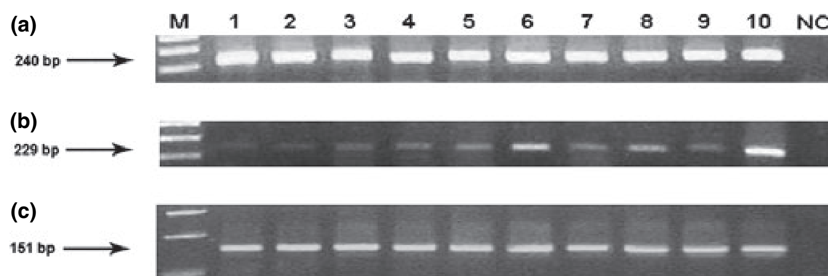

Fig. 1. Expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (a), kallikrein 15 (*KLK15*) splice variants (b), and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) (c) in benign prostatic hyperplasia (BPH) and prostate cancer (CaP) samples. 1, 3, 5, 7, 9: BPH samples; 2, 4, 6, 8, 10: CaP samples; M: molecular weight marker; NC: negative control.

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This observation underlines that *KLK15* expression analysis is independent of the chosen endogenous reference gene.

The expression of the *KLK15* classical mRNA isoform was examined in human prostate tissue specimens, both cancerous (n = 52) and hyperplastic (n = 52). The classical mRNA isoform of *KLK15* was expressed in both CaP and BPH samples (Fig. 1), though its levels of expression were found statistically significantly (P < 0.001) higher in the CaP samples compared to BPH samples (Fig. 2). More precisely, the expression levels of the *KLK15* classical mRNA isoform in the BPH samples (n = 52) ranged from 1.35 to 4.80 arbitrary units (mean ± SE, 2.09 ± 0.16; median, 1.35) (Table 3), while in the CaP samples (n = 52) the corresponding levels ranged from 1.35 to 9.76 arbitrary units (mean ± SE, 3.79 ± 0.31; median, 3.75) (Table 4). These results, depicted in Figure 2, indicate a statistically significant (P < 0.001) increase in *KLK15* classical mRNA isoform expression levels in the CaP samples in comparison with the BPH ones. It was also observed that gene expression in the cancerous samples was higher compared to the benign ones in the 50th (median), 75th, and 90th percentiles.

The expression of the *KLK15* alternative spliced variants was also examined in the same human prostate cancer tissue specimens (n = 52), as well as in benign prostate hyperplasia samples (n = 52). Splice variants 1, 2, and 3 were expressed in both CaP and BPH samples (Fig. 3). In particular, the expression levels of the *KLK15* splice variants 1 and 2 in the BPH samples (n = 52) varied from 1.10 to 10.60 arbitrary units (mean ± SE, 3.62 ± 0.40; median, 3.30) (Table 3), while in the CaP samples (n = 52) the corresponding levels ranged from 1.10 to 12.20 arbitrary units (mean ± SE, 3.99 ± 0.40; median, 4.00) (Table 4). These results indicate a slight, not statistically significant (P = 0.52), increase in the levels of expression of the *KLK15* splice variants 1 and 2. As far as the splice variant 3 of the *KLK15* gene is concerned, its expression levels fluctuated from 0.90 to 7.10 arbitrary units in the BPH samples (n = 52) (mean ± SE, 2.31 ± 0.28; median, 0.90) (Table 3), while in the CaP samples (n = 52) the respective levels ranged from 0.90 to 8.90 arbitrary units (mean ± SE, 3.07 ± 0.32; median, 2.90) (Table 4). This increase in the *KLK15* splice variant 3 levels of expression in the CaP in relation to the BPH samples was rated with a P-value of 0.085.

Assessment of *KLK15* mRNA expression in the discrimination of CaP from BPH. ROC curve and logistic regression analysis were used to evaluate the differential expression of both the *KLK15* classical isoform and the splice variants of the gene, as a tool in the discrimination of CaP from BPH. ROC analysis

Table 3. Descriptive statistics of continuous variables in the studied BPH patients

Variables	Mean ± SE (n = 52)	Range	Percentiles (median)				
			10	25	50	75	90
KLK15 classical mRNA isoform†	2.09 ± 0.16	1.35–4.80	1.35	1.35	1.35	3.40	3.97
KLK15 splice variants 1 and 2†	3.62 ± 0.40	1.10–10.60	1.10	1.10	3.30	5.55	8.56
KLK15 splice variant 3†	2.31 ± 0.28	0.90–7.10	0.90	0.90	0.90	4.07	5.81
PSA (ng/mL)	4.91 ± 0.79	0.40–25.55	1.05	1.64	3.82	5.68	11.39
Age (years)	70.27 ± 1.07	49.0–83.0	61.5	66.2	70.0	75.0	80.0

†*KLK15* mRNA transcript expression levels/*GAPDH* mRNA expression levels in arbitrary units. BPH, benign prostatic hyperplasia; *KLK15*, kallikrein 15; PSA, prostate-specific antigen.

Table 4. Descriptive statistics of continuous variables in the studied CaP patients

Variables	Mean ± SE (n = 52)	Range	Percentiles (median)				
			10	25	50	75	90
KLK15 classical mRNA isoform†	3.79 ± 0.31	1.35–9.76	1.35	1.35	3.75	4.89	6.85
KLK15 splice variants 1 and 2†	3.99 ± 0.40	1.10–12.2	1.10	1.10	4.00	5.45	8.00
KLK15 splice variant 3†	3.07 ± 0.32	0.90–8.90	0.90	0.90	2.90	4.45	6.07
PSA (ng/mL)	9.55 ± 0.89	2.56–41.80	3.78	5.28	8.50	11.80	16.06
Age (years)	64.8 ± 0.8	52.0–76.0	56.1	61.0	65.0	69.4	73.0

†*KLK15* mRNA transcript expression levels/*GAPDH* mRNA expression levels in arbitrary units. CaP, prostate cancer; *KLK15*, kallikrein 15; PSA, prostate-specific antigen.

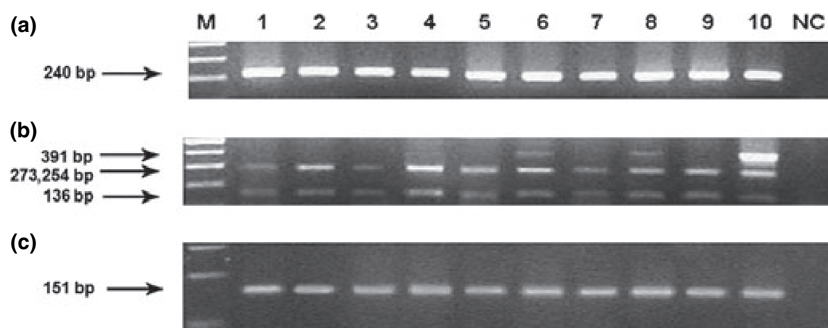

Fig. 3. Expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (a), kallikrein 15 (*KLK15*) splice variants (b), and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) (c) in benign prostatic hyperplasia (BPH) and prostate cancer (CaP) samples. 1, 3, 5, 7, 9: BPH samples; 2, 4, 6, 8, 10: CaP samples; M: molecular weight marker; NC: negative control.

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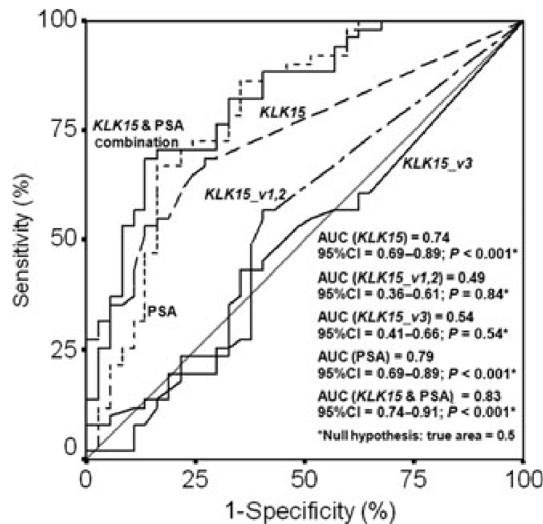

Fig. 4. Receiver–operator curve (ROC) analysis for kallikrein 15 (*KLK15*) expression and the serum total prostate-specific antigen (tPSA) concentration, constructed by plotting sensitivity *versus* (1-specificity).

(Fig. 4) for *KLK15* classical mRNA isoform expression (AUC = 0.74, 95%CI = 0.69–0.89, $P < 0.001$) and tPSA serum concentration (AUC = 0.79, 95%CI = 0.69–0.89, $P < 0.001$) unraveled their value in the differential diagnosis between patients suffering from CaP and BPH. To further investigate the discriminatory value of the *KLK15* expression in relation to tPSA serum concentration, another logistic regression model (function combination [FC] = 0.553**KLK15* + 0.178*PSA−2.389) was developed and adjusted only for these two variables. We calculated log likelihood scores for this multivariate logistic regression model, which incorporated both of the variables, for each patient. The increased AUC (AUC = 0.83, 95%CI = 0.74−0.91; $P < 0.001$) revealed the improved discrimination of the malignant from the benign prostate tissue origin. The corresponding ROC analysis for the splice variants 1 and 2 (AUC = 0.49, 95%CI = 0.36−0.61, $P = 0.84$) and splice variant 3 (AUC = 0.54, 95%CI = 0.41−0.66, $P = 0.54$) did not show any statistically significant discriminative value between CaP and BPH samples.

Univariate logistic regression analysis models (Table 5) were also developed to assess the differential expression of the *KLK15* classical mRNA isoform and the alternative spliced *KLK15* variants between CaP and BPH patients. *KLK15* classical isoform mRNA levels (crude odds ratio = 1.79, 95%CI = 1.34−2.39, $P < 0{,}001$) were statistically significantly elevated in CaP patients and this observation can be used to distinguish them from those suffering from BPH. As it was expected, the same could be concluded for tPSA (crude odds ratio = 1.24, 95%CI = 1.09−1.41, $P = 0{,}001$). In addition, patients with high *KLK15* splice variant 3 mRNA expression (crude odds ratio = 1.17, 95%CI = 0.97−1.41, $P = 0.085$) possessed a higher risk of suffering from prostate cancer. On the contrary *KLK15* splice variants 1 and 2 (crude odds ratio = 1.04, 95%CI = 0.91−1.19, $P = 0.52$) could not be used for the differential diagnosis between CaP and BPH. Finally, the multivariate logistic models that were developed (Table 5) revealed the statistically significant differential diagnostic value of *KLK15* classical mRNA isoform expression (crude odds ratio = 1.96, 95%CI = 1.31−2.95, $P = 0.001$) in discerning between CaP and BPH.

Association of *KLK15* mRNA expression levels with clinicopathological variables in prostate cancer patients. The median of *KLK15* mRNA transcripts expression levels/*GAPDH* mRNA expression levels was used to investigate the possible relationship between *KLK15* expression and various clinical data derived from patients with prostate cancer (Table 6). As far as the patients' TNM stage is concerned, the expression of *KLK15* splice variants 1 and 2 was found to be higher (median, 4.50 arbitrary units) to a statistically significant degree ($P = 0.035$) in patients with advanced TNM stage (pT3/pT4) compared to early stage (pT1/pT2) patients (median, 3.00 arbitrary units). However, a safe conclusion could be reached for neither the classical isoform, nor the splice variant 3. Furthermore, Gleason score was found to be positively correlated ($P = 0.017$) with high *KLK15* classical mRNA isoform expression as patients with Gleason score ≤6 were characterized by lower levels of *KLK15* classical isoform expression (median, 2.27 arbitrary units) compared to those with Gleason score equal to 7 (median, 3.90 arbitrary units) or to those with Gleason score >7 (median, 4.60 arbitrary units). The same conclusions could be drawn for splice variants 1 and 2 ($P = 0.042$) which were expressed in higher levels in patients with elevated Gleason score (median, 7.30 arbitrary units for Gleason score >7; median, 3.90 for Gleason score equal to 7; median, 2.30 for Gleason score ≤6). However, *KLK15* splice variant 3 did not display any relationship with Gleason score. Finally, the expression levels of the *KLK15* classical isoform were significantly elevated ($P = 0.007$) in patients with invasive, large tumors (median value for invasive tumors >1 cm = 3.85 arbitrary units; median value for invasive tumors ≤1 cm = 3.90) in comparison with patients possessing non-invasive small tumors ≤1 cm (median value, 1.35 arbitrary units). As far as the splice variants of the *KLK15* gene are concerned, no statistically significant correlation was observed between their levels of expression and the size and/or invasion of the tumor.

Correlations between continuous variables of the study in CaP and BPH patients. Correlations between different variables were assessed by Spearman correlation coefficient. This analysis showed that there was a strong positive correlation ($r = 0.688$) between the levels of expression of the *KLK15* splice variants 1 and 2 and the splice variant 3 in the BPH samples ($P < 0.001$), while no other correlation was observed to be statistically signif-

Table 5. Logistic regression analysis of BPH and CaP patients for predicting the presence of prostate cancer

Covariant	Univariate analysis			Multivariate analysis†		
	Crude odds ratio	95% CI	*P*-values‡	Crude odds ratio	95% CI	*P*-values‡
KLK15 classical mRNA isoform	1.79	1.34–2.39	<0.001	1.96	1.31–2.95	0.001
PSA	1.24	1.09–1.41	0.001	1.21	1.04–1.39	0.012
Age	0.88	0.82–0.95	0.001	0.86	0.79–0.94	0.001
KLK15 splice variants 1 and 2	1.04	0.91–1.19	0.52	–	–	–
KLK15 splice variant 3	1.17	0.97–1.41	0.085	–	–	–

†Logistic regression models were adjusted for *KLK15*, PSA, and patients' age. ‡Test for trend. BPH, benign prostatic hyperplasia; CaP, prostate cancer; CI, confidence interval; *KLK15*, kallikrein 15; PSA, prostate-specific antigen.

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Table 6. Relationships between KLK15 expression status, patient stage, tumor size, and Gleason score

Variable	No. of patients	*KLK15* classical mRNA isoform†	*KLK15* splice variants 1 and 2†	*KLK15* splice variant 3†
Patient stage				
pT1/pT2	33	3.67	3.00	2.80
pT3/pT4	17	3.80	4.50	2.80
x	2	(P = 0.52‡)	(P = 0.035‡)	(P = 0.51‡)
Tumor size				
≤1 cm, non-invasive	10	1.35	3.65	0.90
≤1 cm, invasive	10	3.90	1.10	4.05
>1 cm, invasive	28	3.85	4.30	2.90
x	4	(P = 0.007§)	(P = 0.42§)	(P = 0.48§)
Gleason score				
≤6	16	2.27	2.30	0.90
7	28	3.90	3.90	2.90
>7	5	4.60	7.30	3.00
x	3	(P = 0.017§)	(P = 0.042§)	(P = 0.39§)

†Median of *KLK15* mRNA transcript expression levels/*GAPDH* mRNA expression levels in arbitrary units. ‡Calculated by Mann–Whitney test. §Calculated by Kruskal–Wallis test. *KLK15*, kallikrein 15; x, unknown.

icant. Contrariwise, CaP specimens revealed various relationships among the variables studied. First, a positive association was noticed between the expression levels of the *KLK15* splice variants 1 and 2 and splice variant 3 (r = 0.618, P < 0.001) and the classical mRNA isoform as well (r = 0.344, P = 0.012). Furthermore, the classical mRNA isoform expression was proved to be positively correlated with the levels of tPSA (r = 0.274, P = 0.052) and the patients' age (r = 0.273, P = 0.052). The tPSA serum concentration was also associated with both the expression levels of *KLK15* splice variants 1 and 2 (r = 0.269, P = 0.056) and patients' age (r = 0.318, P = 0.023). Finally, due to the fact that the correlation between tPSA and *KLK15* classical isoform, as well as between tPSA and *KLK15* splice variants, was found to be borderline statistically significant, we also examined these relations, employing Pearson correlation analysis, which further confirmed the statistical importance (P < 0.05) of the aforementioned results.

Discussion

Proteases are implicated in various physiological and pathological conditions in the human body, including tumor progression and tumor invasion.[7,25] Human tissue kallikreins represent an enormous cluster of 15 genes in the human genome, encoding for secreted serine proteases, which possess a unique strategic role in a wide range of regulatory functions of the human organism. However, they have been repeatedly accused of participating in a number of abnormal conditions; mainly in tumor progression and metastasis, as they are differentially expressed in many types of cancer.[16,26,27]

Prostate cancer remains the second-most common cause attributed to cancer deaths in Western populations.[1] Moreover, prostate cancer is very frequently diagnosed (one in six males), mainly using the routine PSA (kallikrein 3) testing. However, PSA lacks diagnostic specificity.[11] Consequently, many molecular biomarkers, including other kallikrein members, are suspected to play a potential role in the diagnosis of prostate cancer.[14] It is widely known and accepted that there is a strong correlation between cancer incidence and alterations in the expression of certain genes. More precisely, in prostate cancer, many kallikrein genes, including *KLK15,* have been found to be highly expressed at the mRNA level.[26] *KLK15* maps between *KLK1* and *KLK3* genes and displays significant structural similarity to the *KLK3* (PSA) gene. It is expressed in a variety of human tissues and its expression is regulated by steroid hormones.[33] Alternative splicing of the gene produces, 3 additional splice variants that differ from the classical mRNA isoform. *KLK15*, like many other *KLK* genes, has been shown to be associated with various clinicopathological characteristics of certain malignancies. *KLK15* mRNA levels have been previously depicted to be elevated in cancerous parts of the prostate gland and this overexpression is associated with advanced tumor stage. *KLK15* is also expressed at higher levels in ovarian cancer tissue specimens compared with benign ovarian tissues and could also constitute an independent marker of unfavorable prognosis in ovarian cancer.[28,35] Contrariwise, in breast cancer, *KLK15*, similarly to *KLK3*, is rated as an independent marker of favorable prognosis.[36]

Until the present study, there has been no report of an independent expression analysis, accompanied with a corresponding clinical evaluation, for the classical *KLK15* mRNA isoform, the only one which encodes for an active hk15 protein. It is thought that the physiological role of hK15 is to regulate PSA activity by activating proPSA.[33] Additionally, the expression pattern of the alternatively spliced *KLK15* mRNA and its clinical utility are investigated independently for the first time in this study in the same tissue specimens used for the analysis of the classical *KLK15* mRNA isoform. The *KLK15* alternatively spliced variants are thought to encode for truncated proteins and may possess a regulatory role.

In the present study, it is reported that the classical mRNA isoform and the alternatively spliced variants of the gene are expressed in both CaP and BPH samples (Figs 1,3). For the first time, it is pinpointed that the classical mRNA isoform of the *KLK15* gene is expressed at a higher level in the CaP samples (Tables 3,4, Fig. 2), in comparison with the BPH samples (P < 0.001). Same conclusions can be drawn for the splice variant 3 as well (Tables 3,4), but the observed differences in the expression did not seem to have any statistically significant discriminatory value (P = 0.085). These results are in accordance with other published data that suggest overexpression of the *KLK15* gene in cancerous prostate tissues, when they are compared to their non-malignant pairs.[28,33]

Taking a step forward, we investigated the value of this *KLK15* expression pattern in the case of discrimination between CaP and BPH tissue specimens. ROC (Fig. 4) and logistic regression analysis (Table 5) revealed that the classical mRNA isoform of the *KLK15* gene is differentially expressed at a statistically significant (P < 0.001) level and can be used independently as a valuable tool for differential diagnosis between CaP and BPH tissue samples. Moreover, the combination of the *KLK15* classical mRNA isoform expression levels and tPSA serum concentrations was shown to generate an enhanced tool for the efficient discrimination between BPH and CaP samples (P < 0.001). On the other hand, none of the expression profiles of any of the splice variants seemed to shed any light on this purpose, at least not in a statistically significant manner.

KLK15 expression was also scanned for any potential prognostic value which could arise from any relationships with the clinicopathological data of the patients examined (Table 6). As a result, the expression of *KLK15* splice variants 1 and 2 were found to be increased in a statistically significant degree (P = 0.035) in patients with advanced stage (pT3/pT4) compared to early stage (pT1/pT2) patients. Besides, the Gleason score was found to be positively correlated (P = 0.017) with high *KLK15* classical mRNA isoform expression, as patients with Gleason score ≤6 were characterized by lower levels of *KLK15* classical isoform expression compared to those with Gleason score ≥7. The results concerning the splice variants 1

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and 2 ($P = 0.042$), which were expressed in higher levels in patients with elevated Gleason score, led us to the same conclusion. In addition, the expression levels of *KLK15* classical isoform were significantly elevated ($P = 0.007$) in patients with invasive, large tumors in relation to patients possessing non-invasive small tumors. Consequently, these results verified those already published that propose *KLK15* as an unfavorable prognostic marker for prostate cancer, because patients with more aggressive types of cancer would present higher *KLK15* mRNA levels than those with less developed and less aggressive malignancy.[28] Our data has enriched this field by suggesting specific transcripts that can be used to monitor the progress of prostate cancer.

A strong positive correlation ($P < 0.001$) between the *KLK15* splice variants 1 and 2 and the splice variant 3 levels of expression was unveiled in the BPH samples, using the Spearman correlation coefficient (r_s). The relationship mentioned above was also observed in the CaP specimens ($r = 0.618$, $P < 0.001$), where those variants were found to correlate with the classical mRNA isoform ($r = 0.344$, $P = 0.012$). Furthermore, tPSA was shown to be positively associated with the classical mRNA isoform expression levels ($r = 0.274$, $P = 0.052$) and with the expression levels of *KLK15* splice variants 1 and 2 ($r = 0.269$, $P = 0.056$). The correlations between tPSA and *KLK15* expression were confirmed using Pearson correlation analysis ($P < 0.05$).

In conclusion, our data propose the kallikrein gene 15 (*KLK15*) as a potential biomarker for the discrimination between CaP and benign BPH tissue specimens. Moreover, *KLK15* could constitute an unfavorable marker in the prognosis of the disease and in the determination of suitable therapeutic treatment. Finally, attention has been drawn to the fact that it is wiser to choose a specific transcript expression analysis (either that of the classical *KLK15* mRNA isoform or one of the alternative spliced ones) in the miscellaneous fields of differential diagnosis and prognosis of prostate cancer.

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Disclosure Statement

The authors have no conflict of interest.

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