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KLK15 is a prognostic marker for progression-free survival in patients with radical prostatectomy

Anja Rabien[1], Florian R. Fritzsche[2,3], Monika Jung[1], Angelika Tölle[1], Eleftherios P. Diamandis[4], Kurt Miller[1], Klaus Jung[1,5], Glen Kristiansen[2,3†] and Carsten Stephan[1,3†]

[1] Department of Urology, Charité - Universitätsmedizin Berlin, Campus Charité Mitte, Berlin, Germany
[2] Institute of Pathology, Charité - Universitätsmedizin Berlin, Campus Charité Mitte, Berlin, Germany
[3] Institute of Pathology, Universitätsspital Zürich, Zürich, Switzerland
[4] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada
[5] Berlin Institute for Urologic Research, Berlin, Germany

In search of biomarkers for prostate cancer, we evaluated the expression of the human kallikrein-related peptidase KLK15 in samples of prostatic adenocarcinomas from radical prostatectomies. Twenty-five pairs of cancerous and adjacent normal prostatic tissue were selected by laser capture microdissection. The tissue was used for quantification of *KLK15* mRNA by reverse-transcriptase polymerase chain reaction. Immunohistochemical expression of the KLK15 protein in 193 samples of prostatic adenocarcinoma was analysed in relation to clinicopathological parameters of the patients and disease progression. Expression of KLK15 correlated with the pathological tumour stage and Gleason score of the cases, both at mRNA and at protein level. While mRNA expression in the tumour was elevated, the protein level of KLK15 was reduced compared with adjacent normal tissue and to prostatic intraepithelial neoplasia. Univariate Kaplan-Meier analysis showed a significant association of dichotomised KLK15 levels with disease progression defined by prostate-specific antigen relapse ($p = 0.001$). Multivariate analysis according to the Cox proportional hazards regression model identified dichotomised KLK15 expression, corrected for the patient parameters age, preoperative prostate-specific antigen level, pathological tumour stage, Gleason score and surgical margin status, as an independent prognostic factor for poor outcome (inclusion model, hazard ratio 1.802, 95% confidence interval 1.037–3.132, $p = 0.037$). We suggest KLK15 as a new independent tumour marker for patients at risk for disease progression after radical prostatectomy.

With 15 members up to now, the human kallikrein-related peptidases (KLKs, formerly "kallikreins"[1] are the largest group of human serin proteases, localised on chromosome 19q13.4. Kallikreins act as secreted proteins in proteolytic cascade pathways to activate each other, process growth factor binding proteins and cleave components of the extracellular matrix.[2,3] Naturally designed to regulate cell growth, migration and angiogenesis, KLKs contribute to invasive and metastatic processes in carcinogenesis.[4] Several kallikreins are found in the prostate and in the prostate fluid, in which they regulate semen liquefaction at ejaculation.[3] Characteristic patterns for prostate cancer were already found for some KLKs, *e.g.*, for the commonly used prostate-specific antigen (PSA, KLK3).[4–10]

Although PSA is in use as serum biomarker for detection of prostate adenocarcinoma and as indicator of the biochemical relapse after radical prostatectomy, its limitations still dissatisfy regarding sensitivity and specificity.[10] Although improving, the latter is true as well for different forms or combinations of PSA with other parameters.[10] Tissue-associated biomarkers, which could help to specify diagnosis, prognosis and therapeutic approaches for prostate cancer, did not reach the level of common clinical use up to now, but there already is an example of recent advances in diagnostics. Weaknesses of single biomarkers are compensated in combining immunohistochemical staining of basal cells (p63) and of alpha-methylacyl-CoA racemase (AMACR) with a third and fourth staining of golgi phosphoprotein 2 (GOLPH2)

Key words: KLK15, prostate cancer, prognostic marker, RT-PCR, immunohistochemistry

Abbreviations: ALAS1: aminolevulinate synthase 1; CI: confidence interval; EAU: European Association of Urology; HPRT1: hypoxanthine phosphoribosyl transferase 1; K-ALPHA-1: K-alpha-1 tubulin; KLK: kallikrein-related peptidase; PIN: prostatic intraepithelial neoplasia; PSA: prostate-specific antigen; RT-PCR: reverse-transcriptase polymerase chain reaction

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Correspondence to: Anja Rabien, Department of Urology, Research Division, Charité - Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany, Tel.: +49 30 450515035, Fax: +49 30 450515904, E-mail: anja.rabien@charite.de

and fatty acid synthase (FASN) to correctly diagnose 99% of prostate carcinoma at the Institute for Clinical Pathology, Universitätsspital Zürich.[11] The 3 tissue markers PSA, prostate-specific membrane antigen (PSMA) and the androgen receptor are used to assess the metastatic state of prostate carcinoma patients.[11] Nevertheless, new biomarkers are sought, which could considerably improve the informational content of the material from biopsies or prostatectomies, perhaps avoiding annoying checkups. Discriminative tissue markers for cases which will undergo recurrence after radical prostatectomy or which will define survival could avoid overtreatment. Therefore, in particular, prognosis of prostate adenocarcinoma still needs an update. Since our initial investigations on the youngest member of the human kallikrein-related peptidases, KLK15, suggested a suitability as biomarker for prostate cancer,[12,13] our retrospective study focused on this target in a wider approach.

In time with our first description of the KLK15 gene,[13] the human kallikrein-related peptidase was found as "prostin" supposed to cleave pro-PSA.[14] KLK15 has an assumed trypsin-like activity[15] and is up-regulated by steroid hormones.[13,16] It is expressed among others in seminal plasma and in the prostate, where abundant amounts of *KLK15* mRNA were opposite to rather low concentrations of the protein, detected by an immunofluorometric assay.[13,17,18] Real-time reverse transcriptase polymerase chain reaction (RT-PCR) experiments proposed *KLK15* as an independent favourable prognostic marker for progression-free and overall survival of breast cancer patients[16] and, by contrast, to be an independent unfavourable marker for ovarian cancer.[19] Ovarian carcinomas had elevated levels of *KLK15* compared with benign ovarian tissues.[19] Analyses of *KLK15* mRNA in prostate cancer described an increase compared to normal prostatic tissue as well, associating *KLK15* with more aggressive tumours.[12,13]

In view of these promising results, we chose a comprehensive immunohistochemical approach to the KLK15 protein in prostate cancer, which is closer to practice. Expression of KLK15 should be localised and correlated to clinicopathological parameters including postoperative PSA levels. Additional quantification of *KLK15* mRNA in laser microdissected material should more exactly define the transcriptional expression levels in prostatic adenocarcinoma.

Material and Methods

Patients

One hundred ninety-three patients with prostatic adenocarcinoma who underwent radical prostatectomy at the Department of Urology, Charité University Hospital between 1989 and 2001 were included in the immunohistochemical study with permission of the local ethics committee. Tumour stages were determined according to L'Union Internationale Contre le Cancer[20] and tumour grades according to Gleason. Gleason scores were centrally reviewed for our retrospective study.

Table 1. Clinicopathological characteristics of prostate cancer patients[1]

Patient characteristics	
Age (years)[2]	61 (46–73)
Preoperative PSA level (ng/ml)[2,3]	9.4 (0.5–150)
Follow-up time (months)[2]	60 (10–188)
PSA defined recurrence	
Yes – no. (%)	63 (32.6)
No – no. (%)	130 (67.4)
Time (months)[2]	27 (2–144)
Tumour characteristics	**n (%)**
pT status	
pT2	108 (56.0)
pT3	80 (41.5)
pT4	5 (2.6)
Gleason score	
3	7 (3.6%)
4	13 (6.7%)
5	29 (15.0%)
6	30 (15.5%)
7	64 (33.2%)
8	33 (17.1%)
9	16 (8.3%)
10	1 (0.5%)
Surgical margin status	
R0	109 (56.5%)
R1	82 (42.5%)
Rx	2 (1.0%)

[1]193 patients evaluated for immunohistochemical expression of KLK15. [2]Data are presented as median and range (in parentheses). [3]Interquartile range (6–15) ng/ml; 15 values were missing.

Clinicopathological patient parameters are listed in Table 1 with follow-up time lasting from the date of surgery to the recent determination of PSA level. A PSA recurrence, which was considered to indicate progression of prostate cancer, was defined as a persistent increase of PSA to $\geq$0.2 ng/ml following the recommendations of the Prostate Cancer Guidelines Update Panel.[21]

For mRNA analysis, 25 patients with radical prostatectomies of the years 2003 and 2004 were included in the study under the conditions as mentioned earlier. The median age was 62 (range: 46–70 years), the median preoperative PSA level was 7.1 ng/ml (range: 1.8–35.1 ng/ml) and the median follow-up time was 51 months (range: 4–81 months) with 5 (20%) cases of PSA defined recurrence. Median time to recurrence was 28 months (range: 2–45 months). The prostate adenocarcinomas of the 25 patients were histologically characterised as follows: pN0, pM0; 1x pT2a, 1x pT2b, 15x pT2c, 6x pT3a, 2x pT3b; Gleason score: 2x Gleason 5, 6x Gleason

6, 10x Gleason 7, 5x Gleason 8, 2x Gleason 9; surgical margin status: 18x R0, 5x R1, 2x Rx.

All cases were selected according to tissue availability and were not stratified in any way.

Tissue collection and laser capture microdissection

Fresh surgical specimens of tissue from radical prostatectomies were snap-frozen in liquid nitrogen and stored at −80°C until further processing. Tissue cryosections of 7 μm were mounted on polyethylene terephtalate coated slides (Micro Dissect GmbH, Mittenaar, Germany), stained with cresyl violet and desiccated. Glands of prostate cancer and adjacent normal tissue, specified by a genitourinary pathologist, were dissected with a laser capture microdissection system (Leica AS LMD, Leica, Wetzlar, Germany), collected in 70 μl RNA lysis/binding buffer of the RNeasy Micro Kit (Qiagen, Hilden, Germany) per tube, including 1% beta-mercaptoethanol, and stored at −80°C.

Isolation of RNA and quantitative RT-PCR

Four tubes of about 5,000–10,000 cells in total were pooled for isolation of RNA with the RNeasy Micro Kit (Qiagen) pursuant to the manufacturer's instructions. Concentration of RNA was determined by measurements in the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). Reverse transcription of 12–50 ng total RNA was performed with the Sensiscript Reverse Transcriptase Kit (Qiagen) according to the manufacturer's protocol. The real-time RT-PCR was performed on the LightCycler Instrument 1.5 (Roche Applied Science, Mannheim, Germany). The PCR assay for KLK15 (accession no. NM_017509.2) was designed using the ProbeFinder software (Roche) by combination of the gene-specific forward primer 5′-CTT CCT GCT GGC ATC CAC-3′ and the reverse primer, 5′-AGT GGG GTG CAC ACT CGT-3′ with the hydrolysis probe #18 from the Universal ProbeLibrary (cat.no. 04686918001). The amplicon size amounted to 71 bp. The PCR was run with the ready-to-use LightCycler TaqMan Master (Roche), 1 μl cDNA, 0.1 μM of the probe and 0.2 μM of each primer in a total volume of 10 μl. The TaqMan Master contained a FastStart Taq DNA Polymerase for hot start PCR. The PCR conditions were: pre-incubation step of 10 min at 95°C; 45 cycles of denaturation for 10 sec at 95°C, annealing for 30 sec at 60°C and elongation for 5 sec at 72°C with a temperature transition rate of 20°C/sec. Each PCR run included a non-template control, a cDNA pool with known *KLK15* mRNA yield used as standard for quantification, and a cDNA sample as run-to-run precision control. A standard curve for calibration of amplification rates was generated by serial dilutions of an undiluted cDNA pool and resulted in a scale of arbitrary units of gene expression. All paired samples were measured in one run. The inter-run variation of the quantities was 6.17%, corresponding to a crossing point value variation of 0.31%. The PCR efficiency for *KLK15* was 1.85. For normalisation of different starting RNA yields, KLK15 gene expression was related to the geometric mean of 3 suitable reference genes for prostate tissue, hypoxanthine phosphoribosyl transferase 1 (HPRT1, PCR efficiency of 2.00), aminolevulinate synthase 1 (ALAS1, PCR efficiency of 1.99) and K-alpha-1 tubulin (K-ALPHA-1, PCR efficiency of 1.94), which were quantified according to Ohl *et al.*[22] The ratio of KLK15 gene expression to the geometric mean of the expression of the 3 reference genes was given as arbitrary units.

Generation of polyclonal rabbit antibody to KLK15 and Western Blotting

Purified NSO-derived recombinant human KLK15, isoform 1, amino acids 17–256, accession no. Q9112R5 (R&D Systems, Minneapolis, MN, USA) was subcutaneously injected into New Zealand White rabbits (Charles River Laboratories, Montréal, QC, Canada) to obtain polyclonal antibodies. After the first protein injection with Freund's adjuvant, the protein was diluted in incomplete Freund's adjuvant for further injections. Injections were repeated 6 times at 3-week intervals. Every 2 weeks, serum samples of the rabbits were tested for antibody generation. The serum could be used without any further purification.

To depict the protein pattern recognised by our KLK15 antibody (below: "in-house KLK15 antibody"), we used Western blotting as described before.[23] Briefly, tissue of prostate adenocarcinoma and of adjacent normal tissue was lysed in a buffer of 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.25% (v/v) Triton X-100, pH 7.5 including protease inhibitors. Thirty micrograms of total protein each were separated on a 12.5% sodium dodecylsulfate polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). After blocking with a solution of 2% ECL Advance blocking agent (GE Healthcare, München, Germany) in Tris-buffered saline/0.1% Tween-20, the membrane was incubated with our in-house KLK15 antibody, diluted 1:20,000 in this solution overnight at 4°C. For competition experiments, the antibody was pre-incubated with about 10-fold excess of the recombinant human KLK15 (R&D Systems Inc., Minneapolis, MN, USA), calculated per estimated weight of rabbit serum immunoglobuline. This peptide had been used to generate the in-house KLK15 antibody. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2,500; DakoCytomation, Hamburg, Germany) served as secondary antibody. Bands were detected by enhanced chemiluminescence (GE Healthcare) in a Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA, USA) and quantified using the Quantity One 1-D Analysis Software Version 4.3.0 (Bio-Rad Laboratories).

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections of 2–3 μm were immunostained as described.[24] In brief, antigens of deparaffinised and re-hydrated sections were retrieved by pressure cooking in 0.01 M citrate buffer for 5 min. After blocking, slides were incubated for 1 hr with a 1:1,000 solution of

our in-house KLK15 antibody, which was generated as described above. Antibody detection was performed with a labelled streptavidin biotin kit using alkaline phosphatase (LSAB2 System-AP, DakoCytomation) and Fast-Red (Sigma-Aldrich, Munich, Germany) staining according to the manufacturer's instructions. Nuclei were briefly counterstained with Mayer's acidic hemalum solution (Hollborn, Leipzig, Germany). The slides were aquaeously mounted. Constant quality during staining procedures was guaranteed with positive and negative controls. Because of the heterogeneous character of prostate cancer which will create a bias in estimating the percentage of stained cells of a phenotype, scoring was kept simple, classifying all epithelial cells of the prostate glands with average scores for each phenotype: Staining intensities of prostate adenocarcinomas, prostatic intraepithelial neoplasia (PIN) and adjacent normal glands ranging from negative (0) over weak (1) and moderate (2) to strong (3) were evaluated by 2 genitourinary pathologists and an experienced scientist in a joint session at a multi-headed microscope. Questionable cases were discussed until consensus was reached. The observers were blinded for patient outcome.

Statistical analysis

Calculations were performed with GraphPad Prism 4.03 (GraphPad Software, San Diego, USA) and SPSS for Windows 12.0 (SPSS Inc., Chicago, IL, USA). The Student's *t* test was used for paired RNA data, while association of KLK15 with Gleason scores was tested by chi-square according to Pearson. Differences in mean expression of the KLK15 protein in 3 types of tissue were analysed with the Tukey's multiple comparison test (one-way analysis of variance). Associations between KLK15 protein expression and clinicopathological data were investigated with Fisher's exact test and with Spearman's bivariate correlation. Prognostic significance was determined in Kaplan-Meier analysis (log rank test) and in Cox proportional hazards regression analysis (likelihood ratio test) related to PSA relapse-free survival. Cox analyses were done with the models of inclusion and forward as well as backward stepwise selection. Bootstrap analyses with 1,000 and 2,000 cycles were calculated, to validate the sample size for Cox regression. Significance was defined as $p < 0.05$; all *p*s were two-sided.

Results

Expression of KLK15 mRNA

Pairs of adjacent normal and cancerous prostatic glands from 25 patients were selected by laser capture microdissection and analysed by real-time RT-PCR. Normalisation of *KLK15* expression to the best fitting reference genes for prostate cancer, HPRT1, ALAS1 and K-ALPHA-1[22] gave the average ratios for normal and tumour tissue analysed with the Student's *t* test of paired data (Fig. 1). With 17 increases and 8 reductions, increased levels of *KLK15* in malignant prostate tissue were significant ($p = 0.045$). While the majority of cases with an increase (82.4%) had a Gleason score ≥ 7, all cases with a decrease had been characterised as Gleason ≤ 7 (Pearson's chi-square test with trisection of Gleason 3–6, 7, 8–10; $p = 0.035$). All cases with PSA defined recurrence (5 out of 25 cases) were found to have an increase in *KLK15*.

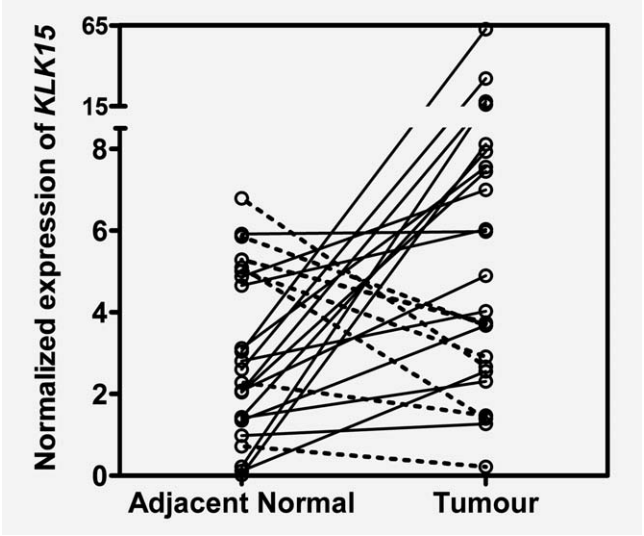

Figure 1. Analysis of *KLK15* mRNA. Average expression ratio of *KLK15* normalised to the reference genes hypoxanthine phosphoribosyl transferase 1, aminolevulinate synthase 1, and K-alpha-1 tubulin in 25 pairs of laser microdissected cancerous and adjacent normal tissue of prostates. Expression in tumour cells is significantly higher than in the normal matches ($p = 0.045$, paired *t* test). Pairs with an increase in tumour tissue (solid line) are distinguished from those with a decrease (dotted line) compared with adjacent normal tissue.

Comparison to clinicopathological parameters, such as age of the patient, preoperative PSA level, tumour stage, tumour grade and surgical margin status were also done with mean-normalised data of *KLK15* expression. It correlated with the pT status (Spearman's rank correlation coefficient r_s 0.419, $p = 0.037$) and with the tumour grading according to Gleason (r_s 0.528, $p = 0.007$) in prostatic adenocarcinomas. Higher *KLK15* expression above the median of 4.02 was significantly associated to higher Gleason scores of 7–10 (Fisher's exact test, $p = 0.030$). As the number of cases was limited, due to laser microdissection of prostate glands, association to PSA relapse-free time after radical prostatectomy could only show a trend without significance. Nevertheless, discrimination of *KLK15* levels in tumour tissue according to median suggested an association of higher *KLK15* expression with shorter progression free survival, the latter defined by PSA relapse ($p = 0.105$, Supporting Information Fig. 1).

Immunohistochemical analysis of KLK15 expression

The clinicopathological characteristics of the 193 cases of prostate cancer used for immunohistochemical KLK15 analysis are given in Table 1. Expression of KLK15 was mostly

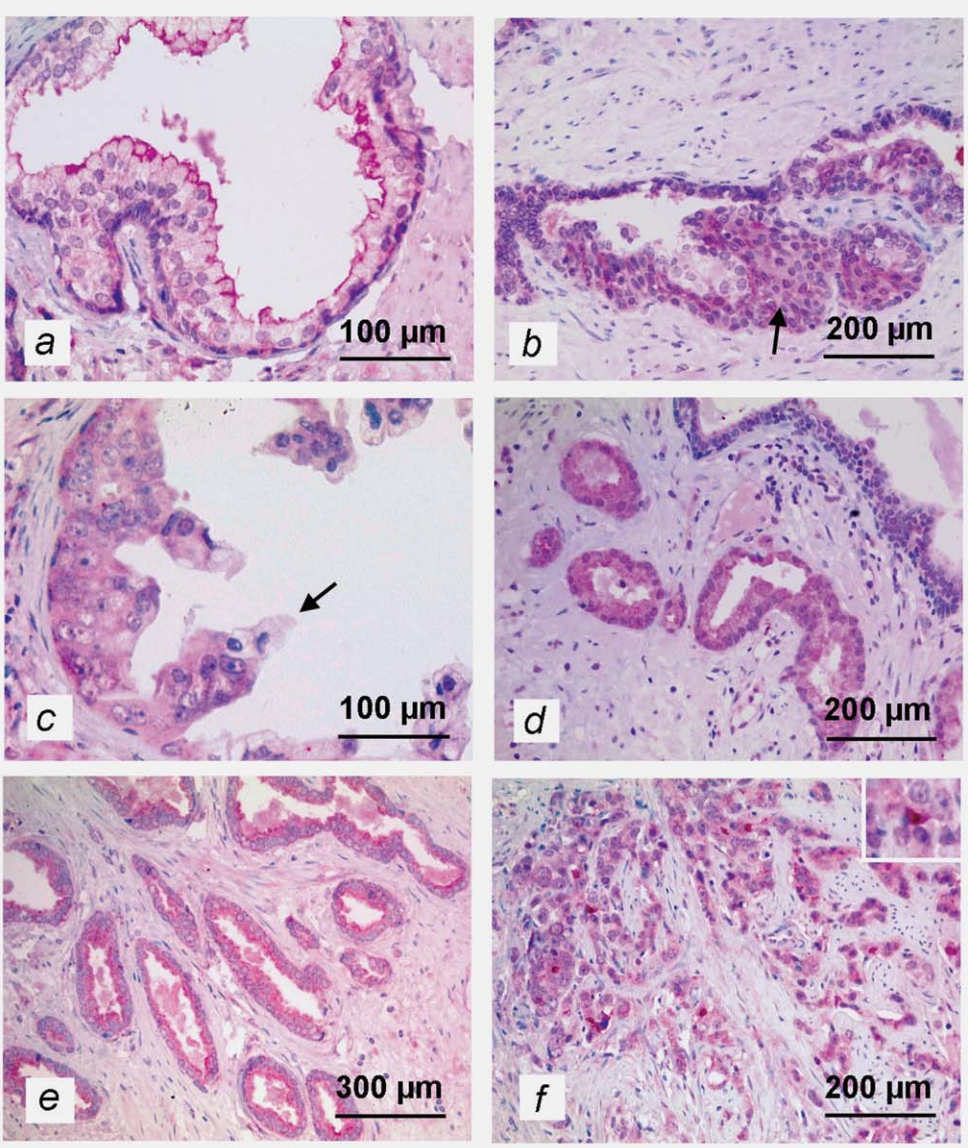

Figure 2. Immunostaining of KLK15 in the tumour-bearing prostate. (*a*) Adjacent normal prostatic tissue with a weak cytoplasmic, in this case predominantly membraneously accentuated KLK15 immunoreactivity. Also, a minimal staining of basal cells can be observed. (*b*) Adjacent normal prostatic tissue with basal cell hyperplasia (arrow). Note strong KLK15 expression in hyperplastic basal cells. (*c*) Moderate KLK15 expression in prostatic intraepithelial neoplasia, note lack of immunoreactivity in single non-neoplastic cells (arrow). (*d*) Strong KLK15 expression in a moderately differentiated (Gleason 3 + 3) adenocarcinoma of the prostate, in the right upper corner a normal gland displaying markedly reduced KLK15 levels. (*e*) Gleason 3 + 3 prostate adenocarcinoma with strong KLK15 expression. Minimal expression of KLK15 is seen in muscular fibrils of the stroma. (*f*) Gleason 4 + 3 prostate adenocarcinoma with strong KLK15 expression. Single cells are found with very high levels of KLK15 within the whole cell (Insert). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

restricted to the cytoplasm of prostatic glandular cells, often apically accentuated in the secretory cells, whereas a slight stromal immunostaining concentrated on muscular fibrils (Fig. 2). Characteristic staining patterns according to the neoplastic status of the tissue could not be recognised. While atrophic cells were barely stained, basal cells showed a heterogeneous pattern with partly strong expression of KLK15, *e.g.*, in basal cell hyperplasia (Fig. 2*b*). While staining intensities for adjacent normal tissue and PIN were splitted equally between low expression (0 and 1) and high expression (2 and 3) of KLK15, low KLK15 expression prevailed in tumour tissue with about 70% (Table 2). Non-parametric tests of bi-variate correlations revealed a highly significant positive correlation of KLK15 expression in cancerous prostatic glands, in adjacent normal tissue, and in PIN with each other (r_s between 0.607 and 0.908, $p < 0.001$). Specificity of our in-house KLK15 antibody, which was already used before in Western blots,[17] was demonstrated by competition experiments using

Table 2. KLK15 staining in prostate tissue of 193 patients

	Adjacent normal		PIN[1]		Tumour	
KLK15 Staining score[2]	Cases (%)	%[3]	Cases (%)	%[3]	Cases (%)	%[3]
0	13 (6.7)		14 (7.3)		29 (15.0)	
		51.8		50.8		70.5
1	87 (45.1)		84 (43.5)		107 (55.4)	
2	76 (39.4)		79 (40.9)		46 (23.8)	
		48.2		49.2		29.5
3	17 (8.8)		16 (8.3)		11 (5.7)	
KLK15 mean value	1.5		1.5		1.2	
KLK15 median	1.0		1.0		1.0	

[1]Prostate intraepithelial neoplasia. [2]Average staining score for each phenotype of a section. [3]KLK15 staining levels 0 and 1 were defined as low, staining levels 2 and 3 were defined as high expression according to median.

immunohistochemical staining (Supporting Information Fig. 2) as well as Western blotting (Supporting Information Fig. 3).

Association of KLK15 expression to clinicopathological parameters

Comparative studies on expression of KLK15 and the clinicopathological parameters, such as age of the patient, preoperative PSA level, tumour stage, tumour grade and surgical margin status showed a positive correlation of KLK15 expression in tumour tissue with the pT status (r_s 0.163, $p = 0.024$) and with the Gleason score (r_s 0.282, $p < 0.001$). Expression of KLK15 in PIN correlated positively with the Gleason score as well (r_s 0.177, $p = 0.014$). Dichotomised data were used to analyse the association of KLK15 expression in tissue of prostate cancer ($n = 193$) with clinicopathological parameters. As described earlier (Table 2), KLK15 staining levels 0 and 1 were defined as low, staining levels 2 and 3 were defined as high expression in the tumour (mean value 1.2; median 1.0 for 193 patients). According to the Fisher's exact test, cancerous KLK15 expression was significantly associated with the Gleason score (Gleason 3–6 *versus* Gleason 7–10, $p = 0.010$). Associations of KLK15 with the age of the patient or the preoperative PSA level (dichotomised according to median), with the pT stage (pT2 *versus* pT3–4) or the surgical margin status (R0 *versus* R1) were statistically not significant. The chi-square test according to Pearson grouped 79 cases of Gleason 3–6 to 81% cases with low KLK15 levels *versus* 19% cases with high KLK15 levels, 64 cases of Gleason 7 to 72% low and 28% high expression of KLK15 and 50 cases of Gleason 8–10 to 52% low and 48% high KLK15 expression ($p = 0.002$). Positive association of cancerous KLK15 expression with the Gleason score was compatible with a comparable analysis of KLK15 difference between tumour and adjacent normal tissue, which was just used to create data similar to the RNA analysis. While the majority of cases with a decrease in tumour tissue (81.2%) had a Gleason score ≤ 7, 65% of the cases with an increase or with similar expression levels had been characterised as Gleason ≥ 7 (Pearson's chi-square test with trisection of Gleason 3–6, 7, 8–10; $p = 0.047$). Further analysis of KLK15 differences between tumour and adjacent normal tissue was avoided, since 117 cases (60.6%) were equally assessed in both types of tissue.

Relationship between KLK15 expression and PSA relapse-free time after radical prostatectomy

The prostate carcinoma cases were analysed univariately and multivariately concerning clinicopathological parameters and expression of KLK15. About one third of the patients had PSA defined recurrence (Table 1) as expected for prostate cancer cases.[25] The follow-up time was >2 years in 190 cases, >5 years in 91 cases and >10 years in 16 cases. Univariate Kaplan-Meier analyses were performed with dichotomised variables (according to median as shown in Table 3) and the time parameter of PSA relapse-free time indicating progression-free survival. Highly significant discrimination with higher preoperative PSA levels, higher tumour stages and grades, and with positive surgical margin status going along with shortened PSA relapse-free survival and with increased 5-year PSA relapse rates reflected representative tumour cases (data not shown). Higher KLK15 expression in prostate carcinoma showed significant disadvantages as well. Progression-free time was significantly shorter with higher expression of KLK15, displaying an elevated 5-year relapse rate of (53.1 $\pm$ 7.3)% *versus* (23.9 $\pm$ 4.1)% for low expression of KLK15. Kaplan-Meier survival curves are shown in Figure 3. Univariate Cox proportional hazards regression analyses of dichotomised clinicopathological parameters and cancerous KLK15 levels gave similar results (Table 3, univariate analysis). The parameter patient age was not significant. Calculation with all 6 variables in the multivariate inclusion analysis showed significance of preoperative PSA, Gleason sum and the KLK15 level, with a 1.8-fold higher risk of PSA relapse for prostatectomised patients with high expression of KLK15 in the tumour (Table 3). Stepwise elimination procedures retained these 3 variables and resulted in little changes of the hazard ratio, with forward and backward likelihood ratio

Table 3. Cox proportional hazards regression analysis of clinicopathological parameters and expression of KLK15[1]

		Univariate analysis		Multivariate inclusion analysis		Stepwise selection[2]	
Variable	Stratification	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age[3]	≤61/> 61	0.732 (0.445–1.204)	0.219	0.827 (0.481–1.422)	0.492	–	–
Preoperative PSA[3,4]	≤9.4/> 9.4 ng/ml	2.595 (1.467–4.591)	0.001	1.990 (1.100–3.601)	0.023	2.159 (1.207–3.859)	0.009
Tumour stage	pT2/pT3–4	2.027 (1.224–3.359)	0.006	1.251 (0.688–2.275)	0.463	–	–
Tumour grade	Gleason 3–6/7–10	3.150 (1.731–5.732)	<0.001	2.483 (1.211–5.094)	0.013	2.846 (1.434–5.649)	0.003
Surgical margin status[5]	R0/R1	1.683 (1.013–2.797)	0.044	1.223 (0.686–2.180)	0.495	–	–
Expression of KLK15[6]	low/high	2.229 (1.353–3.673)	0.002	1.802 (1.037–3.132)	0.037	1.819 (1.053–3.141)	0.032

[1]Analysis in relation to the risk of PSA relapse as indicator of prostate cancer progression for 193 patients (multivariate analyses: 177 patients). [2]Forward and backward stepwise elimination procedures including all six variables gave the same result. [3]Dichotomised according to median. [4]15 preoperative PSA values were missing. [5]2 cases were Rx. [6]Staining levels 0 and 1 are defined as low, staining levels 2 and 3 are defined as high expression in the tumour. HR: Hazard ratio; CI: confidence interval.

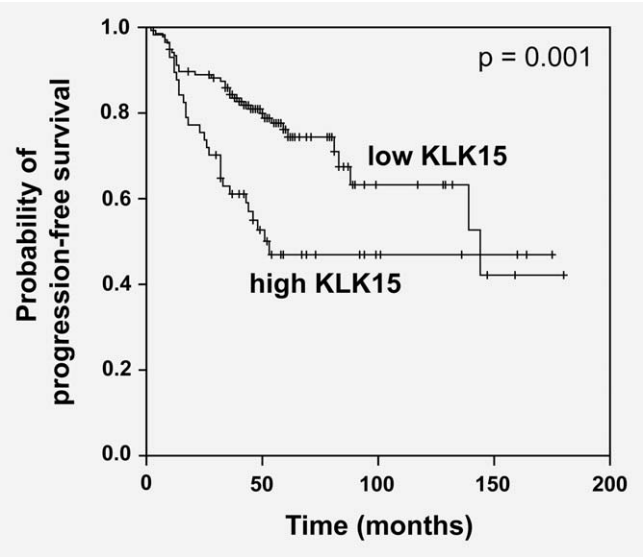

Figure 3. Kaplan-Meier analysis of KLK15 expression in prostate cancer, related to PSA relapse-free time indicating progression-free survival. The 193 cases were divided into subgroups with low expression of KLK15 (136 cases, 35 events) and with high expression of KLK15 (57 cases, 28 events) in prostatic adenocarcinomas. Staining levels 0 and 1 are defined as low; staining levels 2 and 3 are defined as high expression in the tumour. Censored cases are marked (+). High levels of KLK15 in prostate cancer significantly went along with shorter progression-free survival.

equalling under the conditions of entry up to $p = 0.05$ and elimination ≥0.10 (Table 3, right). Thus, KLK15 expression in prostate cancer was found to be an independent prognostic factor adjusted for patient age, preoperative PSA, tumour stage, Gleason sum and surgical margin as possible confounders.

Discussion

The human kallikrein-related peptidase KLK15 has been barely characterised up to now. This retrospective study on KLK15 in prostatic adenocarcinoma could not only refine data for the transcriptional level, but also provides the first analysis of the KLK15 protein in cancer. As urgently recommended,[26] we followed the "Reporting Recommendations for Tumor Marker Prognostic Studies"[27] in our KLK15 study (Supporting Information Table 1).

Our positive correlation of *KLK15* mRNA to tumor aggressiveness conforms to the literature,[12,13] but additionally giving exact discrimination between the different neoplastic stages of prostatic glandular cells without contamination by other cell types. The low sample number is a potential bias of *KLK15* data in comparison to clinicopathological parameters and in particular for survival analyses. Nevertheless, our results for *KLK15* mRNA are well in line with our immunohistochemical data and, moreover, they suggest that with progressing malignancy hitherto unknown processes cause a change to (partly considerable) increases in KLK15 in the tumour. Two other studies on laser microdissected material from radical prostatectomy identified increased androgen receptor mRNA and decreased PSA/HK3 mRNA to be associated with disease progression, defined by biochemical failure.[28,29]

Cytoplasmic and often luminal concentrated localisation, as observed for the KLK15 protein, is described for several other human kallikrein-related peptidases.[7,30,31] Unlike our KLK14 staining, which was not significantly different in prostatic tumour and adjacent normal tissue,[7] immunohistochemical studies of Petraki *et al.*[30] showed lower expression of KLK6, KLK10 and KLK13 in prostate cancer compared with non-malignant prostates. We also observed less KLK15 staining in the tumour, but we avoided an inter-patient bias by comparing within one tissue sample, a method that, on the other hand, bears the risk of a tumoural influence on the

adjacent "benign" tissue. Reduction of KLK15 is surprising, as cancerous KLK15 is significantly related to a worse prognosis for the patients, expected to at least reach tumour-surrounding levels of the normal glandular cells. The lower staining level could be due to a shortened half life of KLK15 in the tumour or to a higher turnover, yielding more of the secreted form, which was not our topic here. Additional studies on serum samples are needed to explore the relevance of secreted KLK15. A specific immunofluorometric assay that can be used for serum tests was developed recently.[17]

Despite rather low levels of the protein detected by the immunofluorometric assay in prostatic tissue,[17] our immunohistochemical analysis defines a considerable part of prostatic glands with high KLK15 expression. This is the advantage of exact tissue determination using immunohistochemistry for protein analysis – or laser microdissected material for mRNA analysis. However, immunohistochemical studies are cheaper and closer to practical use than routine tests for mRNA expression, so that our study mainly focused on immunohistochemistry. Specificity of the antibody used for immunostaining is a weak point of this method, which we tried to assess by competition experiments. Competition revealed a residual risk of cross reactivity that should not be excluded, but most of the members of the KLK family show a smaller apparent molecular weight than the 37 kDa we determined for KLK15 in prostate tissue. This molecular weight is in line with the literature describing a 38 kDa band for KLK15 produced in human embryonic kidney cells HEK293.[17] Bootstrapping confirmed the validity of our sample size for immunohistochemistry, and the tumour parameters proved it to be representative. The follow-up time, however, should have been longer to gather a reliable recurrence rate, since 10 years are considered to be sufficient.[32] The use of PSA relapse as an endpoint surrogate for patient survival is another limiting bias of our study, but it is generally accepted according to the EAU guidelines on prostate cancer.[33] In particular, for patients after radical prostatectomy, a marked increase of PSA over time was shown to correlate with a higher rate of prostate cancer-specific mortality,[34] indicating the utility of PSA follow-ups. Several studies on tissue biomarkers for patients after radical prostatectomy used biochemical failure as an indicator of disease progression and found the cell cycle inhibitors $p27^{Kip1}$ and p53, the transcriptional repressor EZH2 (enhancer of zeste homolog 2), the proliferation marker Ki-67, the anti-apoptotic protein Bcl-2, the structural protein caveolin-1 and the microvessel density to be of predictive value (for review see Ref. 35). Other potential candidates remained controversial.[35] Our recent immunohistochemical studies identified a regulator of mRNAs, the ELAV-like protein HuR,[36] the A disintegrin and metalloprotease ADAM9,[23] the histone deacetylase HDAC2[37] and KLK14[7] as independent prognostic markers, but unlike *KLK15*, data for *KLK14* mRNA did not show any significance.[7] In the field of epigenetics, promoter methylation of the pituitary homeobox 2 (PITX2) gene, which encodes a transcription factor, was found to be a strong prognostic marker for patients after radical prostatectomy.[38] However, there is no marker that is able to replace PSA recurrence up to now, although predictive accuracy could be amended using nomograms.[32,35] Our findings of significant KLK15 association with disease progression, indicated by PSA relapse, disclose the probable prognostic potential of this human kallikrein-related peptidase. It should, however, be validated by external, prospective studies and is restricted to patients after radical prostatectomy so far. As an assumed activator of PSA and plasmin, KLK15 is supposed to cause degradation of the extracellular matrix, contributing to tumour growth, metastasis and invasion.[3] Further studies will be informative not only of prognostic approaches, but also of the therapeutic potential of KLK15.

In conclusion, our study proposes KLK15 to become a prognostic marker for patients after radical prostatectomy. As independent prognostic factor for disease recurrence, KLK15 is suggested to improve postoperative care, supplementing PSA time courses, but larger studies should further define the role of KLK15 to validate its use for clinical implementation.

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