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High Expression of KLK14 in Prostatic Adenocarcinoma Is Associated with Elevated Risk of Prostate-Specific Antigen Relapse

Anja Rabien[a] Florian Fritzsche[b] Monika Jung[a] Eleftherios P. Diamandis[c] Stefan A. Loening[a] Manfred Dietel[b] Klaus Jung[a] Carsten Stephan[a] Glen Kristiansen[b]

[a]Department of Urology, and [b]Institute of Pathology, Charité-Universitätsmedizin Berlin, CCM, Berlin, Germany; [c]Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ont., Canada

Key Words
Kallikreins · Prostate cancer · Real-time reverse transcription polymerase chain reaction · Immunohistochemistry · KLK14

Abstract
Objective: Reliable prognostic tools for prostate cancer are still needed and KLK14, a young member of the growing family of human kallikrein-related peptidases, has been estimated to become a new significant marker. It is the aim of this study to analyze the clinical value of immunohistochemical expression of KLK14 in prostate cancer tissue samples. ***Methods:*** Protein expression of KLK14 was assessed immunohistochemically in 186 tissue samples from radical prostatectomies. Areas of normal prostatic tissue, of prostatic epithelial neoplasia and of prostatic adenocarcinoma were checked in relation to clinicopathological parameters of the patients. Expression of *KLK14* mRNA was quantified in 25 matches of normal and cancerous prostatic tissue, collected by laser capture microdissection. Real-time reverse transcriptase polymerase chain reaction analysis was used to supplement the immunohistochemical data. ***Results:*** Expression of the KLK14 protein correlated with the pathological tumor status in prostate cancer and was associated with disease progression defined by prostate-specific antigen relapse in univariate Kaplan-Meier analysis. The multivariate Cox proportional hazards regression model proved KLK14 to be an independent prognostic factor in prostate cancer. ***Conclusion:*** In conclusion, we consider KLK14 to be a suitable prognostic marker for the detection of cases at risk of disease progression after radical prostatectomy.

Introduction

Adenocarcinoma of the prostate is the most common type of cancer with 218,890 new cases and 27,050 deaths in the USA predicted for the year 2007 [1]. Since exact diagnostic and prognostic tools are lacking, recent efforts have been concentrated on finding new biomarkers for prostate cancer, which can support and complement the existing methods [2]. The family of the 15 human kallikrein-related peptidases (KLKs), formerly 'kallikreins' [3], includes, in addition to the generally used prostate-specific antigen (PSA, KLK3), numerous potential tumor

C.S. and G.K. contributed equally to this study.

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Anja Rabien, PhD
Department of Urology, Research Division
Charité-Universitätsmedizin Berlin, CCM, Charitéplatz 1
DE–10117 Berlin (Germany)
Tel. +49 30 450 515 035, Fax +49 30 450 515 904, E-Mail anja.rabien@charite.de

markers expressed in the prostate that have been investigated for diagnostic and prognostic purposes for prostatic adenocarcinoma [4–7]. As KLKs are secreted proteins involved in cell growth, migration and angiogenesis as well as in invasiveness and metastasis [4], these analytes offer the promising possibility to be used as serum markers. One of these interesting peptidases is KLK14.

The serine protease KLK14 was discovered 6 years ago [8, 9] and is supposed to cleave proteins from the extracellular matrix, e.g., collagens and laminin [5, 10]. It acts in response to steroid hormones [11, 12] with an assumed trypsin-like activity based on in vitro studies [10]. Proteolytic activity of the protease can be limited by autofragmentation, by the serpin class of endogenous inhibitors and by the inorganic ion zinc, while citrate has stimulatory effects [5]. Zinc is abundantly found in the prostate where KLK14 is most highly expressed [8, 11], apart from high levels of KLK14 in breast tissue [11, 13] and in the skin [11, 14, 15]. Although KLK14 can release angiostatic factors with tumor-suppressive functions [5], tumor-promoting actions probably prevail. The definite result is suggested to depend on cancer type and the microenvironment of the tumor [5].

While *KLK14* mRNA levels have been described to be reduced in breast cancer cell lines, in cancerous tissues of the breast, ovary, testis and the prostate compared with their normal counterparts [9, 12], other quantitative studies report an increase in breast, ovarian and prostate cancer [13, 16, 17]. Elevated protein expression of KLK14 in mammary and ovarian carcinoma was detected by ELISA (enzyme-linked immunosorbent assay) or immunohistochemically, whereas the latter is linked to higher aggressiveness [11, 13]. Increased levels of KLK14 were also found in the serum of patients with breast, ovarian and prostate tumors [5, 11]. Two studies indicated the prognostic value of higher *KLK14* expression for an unfavorable outcome in patients with breast cancer or carcinomas of the prostate [16, 18]. It was even of independent prognostic importance with reference to progression-free and overall survival in breast cancer (unfavorable prognosis) [18] and in ovarian carcinoma (favorable prognosis) [12].

In summary, there are conflicting data for the prostate cancer-specific regulation of *KLK14* mRNA, determined semiquantitatively or quantitatively in nonmicrodissected tissue samples [9, 16]. Corresponding analyses at the protein level are lacking, except for the first interesting hint to higher KLK14 expression in serum of patients with prostate cancer compared with healthy men [5]. Therefore, exact morphological determination and subsequent data concerning the prognostic significance, especially of KLK14 protein expression in prostate cancer, are needed. We aimed to (1) perform a large immunohistochemical expression study of KLK14 in tissue samples after radical prostatectomy, (2) complement immunohistochemical data with expression data of *KLK14* mRNA, (3) correlate KLK14 immunostaining with clinicopathological data, and (4) assess the diagnostic or prognostic significance of the KLK14 expression results with regard to the PSA relapse as surrogate marker for prostate cancer death.

Materials and Methods

Patients

One hundred and eighty-six patients with prostatic adenocarcinoma who had undergone radical prostatectomy at the Department of Urology, Charité University Hospital, between 1990 and 2001 were included in the immunohistochemical study, with permission of the local ethics committee. Tumor stages were determined according to L'Union Internationale contre le Cancer [19] and tumor grades according to Gleason. Gleason scores were centrally reviewed for our study. Clinicopathological parameters of the cohort 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 in PSA to $\geq$0.2 ng/ml following the recommendations of the Prostate Cancer Guidelines Update Panel [20]. Since 1995, at our department, total PSA was generally determined by Immulite PSA assays (Diagnostic Products Corp., Los Angeles, Calif., USA) with a lower limit of detection of 0.019 ng/ml [21], so that at least 92.5% of the follow-ups were uniformly measured.

For mRNA analysis, 25 patients with radical prostatectomies of the years 2003 and 2004 were included in the study. The median age was 62 years (range 46–70) and the median preoperative PSA level was 7.1 ng/ml (range 1.8–35.1). The prostate adenocarcinomas of the 25 patients were histologically characterized as follows: pN0, pM0; pT2a (n = 1), pT2b (n = 1), pT2c (n = 15), pT3a (n = 6), pT3b (n = 2); Gleason score: Gleason 5 (n = 2), Gleason 6 (n = 6), Gleason 7 (n = 10), Gleason 8 (n = 5), Gleason 9 (n = 2); surgical margin status: R0 (n = 18), R1 (n = 5), Rx (n = 2).

All cases were selected according to tissue availability and were not stratified in any way. None of the patients received antiandrogen pretreatment before surgery.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections of 2–3 μm were immunostained as described [22]. Slides were incubated for 1 h with a 1:500 solution of our polyclonal rabbit anti-human KLK14 antibody generated and validated in-house [13]. Constant quality during staining procedures was guaranteed with positive and negative controls. Staining intensities of prostate adenocarcinomas, prostatic epithelial 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 multiheaded microscope. Questionable cases were discussed until consensus was reached. The observers were blinded to patient outcome.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

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 terephthalate-coated slides (Micro Dissect GmbH, Mittenaar, Germany), stained with cresyl violet and desiccated. Glands of normal and cancerous prostatic 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% β-mercaptoethanol, and stored at –80°C.

Four tubes of about 5,000–10,000 cells in total were pooled for isolation of RNA with the RNeasy Micro Kit (Qiagen). Concentration of RNA was determined by measurements in the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, Del., USA). Because of the time-consuming dissection procedure, especially for the inhomogeneous morphology of prostate cancer tissue, total RNA yields of 12–50 ng were transcribed and later normalized to the geometric mean of 3 housekeeping genes [23]. Complementary DNA was synthesized with the Sensiscript Reverse Transcriptase Kit (Qiagen). *KLK14* mRNA was amplified with the QuantiTect SYBR Green PCR master mix (Qiagen) in reverse transcriptase polymerase chain reaction (RT-PCR) using a LightCycler (Roche Diagnostics, Mannheim, Germany) and the following gene-specific primers: forward 5′-AGT GGG TCA TCA CTG CTG CT-3′, reverse 5′-GAC CTC AAT GGG CCT GAC T-3′ (TIB MolBiol, Berlin, Germany). The PCR was run with 2 μl cDNA and 0.5 μM of each primer in a total volume of 20 μl as follows: preincubation step of 15 min at 95°C, 45 cycles of denaturation for 15 s at 94°C, annealing for 25 s at 61°C, and elongation for 20 s at 72°C with a temperature transition rate of 20°C per second. *KLK14* was quantified with a standard of diluted DU-145 cDNAs and exhibited a PCR efficiency of 1.98. The melting temperature analysis showed a single peak at 88.7°C. *KLK14* was related to the best fitting housekeepers for prostate cancer, hypoxanthine phosphoribosyltransferase 1 (PCR efficiency of 2.00), aminolevulinate synthase 1 (PCR efficiency of 1.99), and K-α_1 tubulin (PCR efficiency of 1.94) which were quantified according to Ohl et al. [24]. Matched samples were analyzed in duplicates in the same analysis to avoid between-run variations.

Statistical Analysis

Calculations were performed with GraphPad Prism 4.03 (GraphPad Software, San Diego, Calif., USA) and SPSS for Windows 12.0 (SPSS Inc., Chicago, Ill., USA). The Wilcoxon signed rank test was used for paired RNA data, and the mean expression of the protein was analyzed with Tukey's multiple comparison test (one way analysis of variance). Associations between protein expression of KLK14 and clinicopathological data were investigated with the χ^2 test, using linear-by-linear association, and with bivariate correlation according to Spearman. Prognostic significance was determined by SPSS Kaplan-Meier analysis and Cox proportional hazards regression analysis, related to PSA relapse-free survival. Significance was defined as $p < 0.05$.

Table 1. Clinicopathological characteristics of the 186 prostate cancer patients evaluated for immunohistochemical expression of KLK14

Patient characteristics	
Age, years	63 [47–74]
Preoperative PSA[1], μg/l	8.8 [0.56–56.3]
Follow-up	
Number of patients	146
Follow-up time, months	59 [10–180]
PSA-defined recurrence	
Yes	36 (24.7)
No	110 (75.3)
Time, months	30.5 [2–88]
Tumor characteristics	
pT status	
pT2	110 (59.1)
pT3	73 (39.2)
pT4	3 (1.6)
Gleason score	
3	8 (4.3)
4	15 (8.1)
5	27 (14.5)
6	30 (16.1)
7	61 (32.8)
8	27 (14.5)
9	17 (9.1)
10	1 (0.5)
Surgical margin status	
R0	109 (58.6)
R1	74 (39.8)
Rx	3 (1.6)

Data are presented as medians, with ranges in brackets, or as number of cases, with percentages in parentheses.

[1] Internal values of the clinic only.

Results

Localization and Expression Levels of KLK14

One hundred and eighty-six cases of prostate cancer used for immunohistochemical analysis of KLK14 are characterized clinicopathologically in table 1. KLK14 was expressed in the cytoplasm of prostatic glandular cells, often apically accentuated in the secretory cells, whereas stromal cells did not show any KLK14 immunostaining (fig. 1). Staining of KLK14 was divided into negative expression (0), weak expression (1) and high expression (2–3). Strong immunostaining (3) appeared rarely, 3 cases for tumor tissue, 1 case for PIN, and no case for adjacent normal tissue, so that they were pooled with moderate cases (2). Fifty-three cases (28.5%) of the tumor tissues were negative, 88 cases (47.3%) were weakly positive,

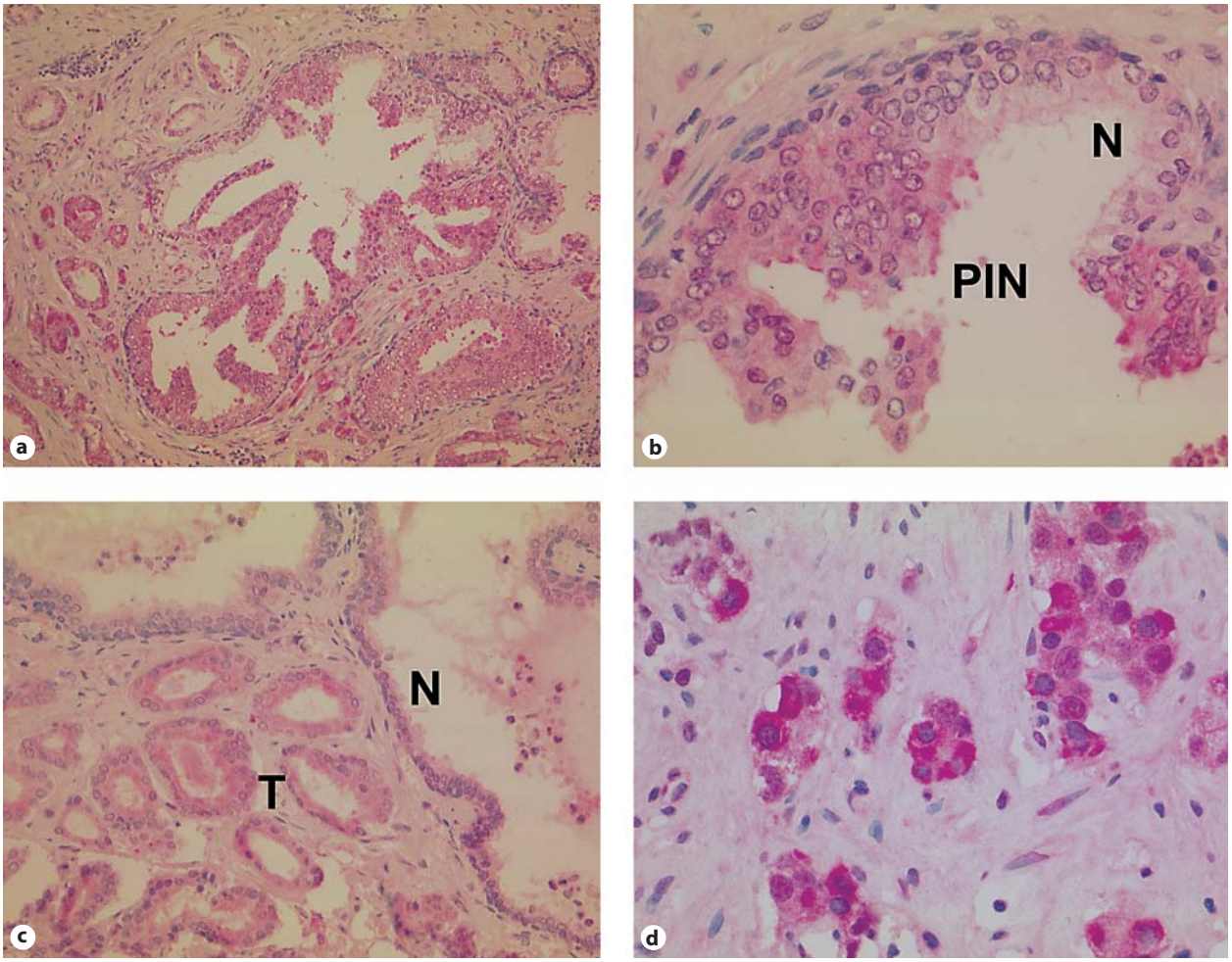

Fig. 1. Immunostaining of KLK14 in the prostate. **a** KLK14 is expressed in the cytoplasm of prostatic glandular cells. Magnification ×100. **b** In detail, an example of apical luminal accumulation in PIN is shown compared with adjacent normal tissue (N). Magnification ×400. **c** In many cases, KLK14 was equally expressed at different stages of neoplasia. T = Tumor tissue; N = adjacent normal tissue. Magnification ×200. **d** A Gleason 5 prostatic adenocarcinoma with high levels of KLK14. Magnification ×400.

and 45 cases (24.2%) were highly positive for KLK14 staining. Thirty-three cases (17.7%) of the normal matches did not show any expression of KLK14, 102 cases (54.8%) showed weak expression, and 51 cases (27.4%) showed high expression of KLK14. KLK14 expression in PIN was negative in 18.8% (n = 35) of the cases, weak in 50.5% (n = 94) of the cases, and high expression in PIN was detected in 30.6% (n = 57) of the cases. Mean staining levels of 0.97 ± 0.057 for the tumors, 1.08 ± 0.049 for normal prostatic glands and 1.12 ± 0.053 for PIN were not significantly different from each other. Nonparametric tests of bivariate correlations revealed a highly significant positive correlation of KLK14 expression in cancerous prostatic glands, in adjacent normal tissue, and in PIN with each other (Spearman's rank correlation coefficient r_s between 0.535 and 0.830, $p < 0.001$).

RT-PCR analysis of *KLK14* mRNA from laser-microdissected material was performed using cryosections from another 25 patients. Normalization of *KLK14* expression to the best fitting housekeepers for prostate cancer, hypoxanthine phosphoribosyltransferase 1, aminolevulinate synthase 1 and K-α_1 tubulin [24] gave the average ratios for normal and tumor tissue analyzed with the Wilcoxon signed rank test of paired data (fig. 2). There were no significant differences between pairs of malignant and nonmalignant tissue with a wide spread of 12 increases, 12

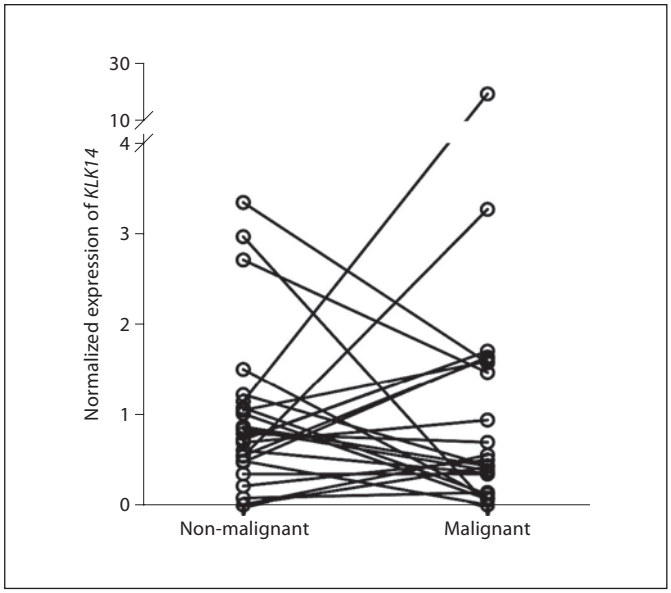

Fig. 2. Analysis of *KLK14* messenger RNA. Average expression ratio of *KLK14* normalized to the housekeeping genes hypoxanthine phosphoribosyl transferase 1, aminolevulinate synthase 1 and K-α_1 tubulin in 25 pairs of laser-microdissected normal and cancerous tissue of prostates. Expression of *KLK14* in malignant samples does not differ from nonmalignant matches (p = 0.456, paired t test).

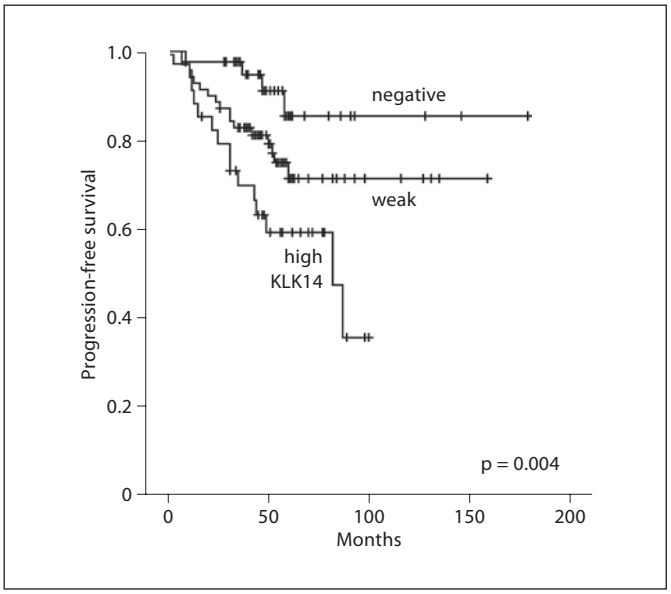

Fig. 3. Kaplan-Meier analysis of KLK14 expression in prostate cancer, related to PSA relapse-free time indicating progression-free survival. Curves are shown for 146 cases with follow-up. The cohort was divided into groups with negative, weak and high expression of KLK14 in prostatic adenocarcinomas. Censored cases are marked (+). Higher levels of KLK14 in prostate cancer significantly went along with shorter progression-free survival.

reductions and 1 equal value. Correlations with clinicopathological parameters were not detected either.

Association of KLK14 Expression with Clinicopathological Parameters

Comparative studies on the expression of KLK14 and clinicopathological parameters like the age of the patient, preoperative PSA level, tumor stage, tumor grade and surgical margin status showed a positive correlation of KLK14 expression in tumor tissue only with the pT status ($r_s = 0.169$, $p = 0.021$). Expression of KLK14 in PIN correlated positively with the pT status ($r_s = 0.247$, $p = 0.001$) and with tumor grading according to Gleason ($r_s = 0.189$, $p = 0.010$). According to the χ^2 test with linear-by-linear association, cancerous KLK14 expression was significantly associated with tumor staging ($p = 0.021$; table 2), which was also true for the expression of KLK14 in PIN ($p = 0.001$, data not shown).

Relationship between KLK14 Expression and PSA Relapse-Free Time after Radical Prostatectomy

Data of 146 patients with follow-up, about a quarter of them with PSA-defined recurrence (table 1), as expected for prostate cancer cases [25], were analyzed univariately and multivariately concerning clinicopathological parameters and expression of KLK14. The follow-up time was >2 years in 144 cases, >5 years in 66 cases and >10 years in 9 cases. Univariate Kaplan-Meier analyses were performed with the time parameter of PSA relapse-free time indicating progression-free survival. Highly significant discrimination with higher preoperative PSA levels, higher tumor stages and grades, and with positive surgical margin status along with shortened PSA relapse-free survival and with increased 5-year PSA relapse rates reflected the representative tumor cohort (data not shown). Higher KLK14 expression in prostate carcinoma showed significant disadvantages as well. Analysis of the 146 cases resulted in an increase in the 5-year relapse rate from 14.6 ± 7.2% for negative cases over 25.1 ± 5.6% for weak expression to 40.8 ± 8.9% for high expression of KLK14. Higher levels of KLK14 were associated with shortened progression-free times ($p < 0.004$). Corresponding Kaplan-Meier survival curves are shown in figure 3. Univariate Cox proportional hazards regression analyses of clinicopathological parameters and cancerous KLK14 levels gave similar results (table 3). With the multivariate

Table 2. χ^2 test: association of KLK14 expression in prostate cancer with clinicopathological parameters[1]

Characteristics	Total	Expression of KLK14[2]			p value[3]
		negative	weak	high	
All cases	186	53 (28.5)	88 (47.3)	45 (24.2)	
Age					0.949
≤63 years	109	31 (28.4)	52 (47.7)	26 (23.9)	
>63 years	77	22 (28.6)	36 (46.8)	19 (24.7)	
Preoperative PSA[4]					0.416
≤8.8 ng/ml	73	20 (27.4)	39 (53.4)	14 (19.2)	
>8.8 ng/ml	72	19 (26.4)	33 (45.8)	20 (27.8)	
pT stage					0.021
pT2	110	37 (33.6)	52 (47.3)	21 (19.1)	
pT3–4	76	16 (21.1)	36 (47.4)	24 (31.6)	
Histological grade					0.123
Gleason score 3–6	80	25 (31.3)	41 (51.3)	14 (17.5)	
Gleason score 7–10	106	28 (26.4)	47 (44.3)	31 (29.2)	
Surgical margin status[5]					0.581
R0	109	32 (29.4)	53 (48.6)	24 (22.0)	
R1	74	20 (27.0)	35 (47.3)	19 (25.7)	

Figures in parentheses are percentages.

[1] Dichotomized clinicopathological parameters, age of the patient and preoperative PSA level according to the median.

[2] Staining level 0 is defined as negative and 1 as weak, staining levels 2 and 3 are defined as high expression of KLK14 in the tumor.

[3] Linear-by-linear association

[4] One hundred and forty-five cases were available.

[5] Three cases were Rx.

Table 3. Cox proportional hazards regression analysis of clinicopathological parameters and expression of KLK14 in relation to the risk of PSA relapse as indicator of prostate cancer progression[1]

Variable	Relative risk (95% CI)	p value
Univariate analysis		
Age (≤63 years/>63 years)	1.013 (0.518–1.982)	0.971
Preoperative PSA[2] (≤8.8 ng/ml/>8.8 ng/ml)	3.088 (1.433–6.653)	0.004
Tumor stage (pT2/pT3–4)	2.343 (1.211–4.535)	0.011
Tumor grade (Gleason 3–6/Gleason 7–10)	4.066 (1.840–8.984)	0.001
Surgical margin status[3] (R0/R1)	1.630 (1.826–3.219)	0.159
Expression of KLK14[4] (negative/weak/high)	2.189 (1.351–3.545)	0.001
Multivariate analysis		
Preoperative PSA	2.174 (0.991–4.770)	0.053
Tumor stage	1.408 (0.676–2.930)	0.361
Tumor grade (Gleason)	2.998 (1.227–7.324)	0.016
Expression of KLK14	1.668 (1.003–2.775)	0.049

[1] Dichotomized data of clinicopathological parameters, age of the patient and preoperative PSA level according to the median. One hundred and forty-six cases with follow-up were available.

[2] Eight preoperative PSA values were missing.

[3] Two cases were Rx.

[4] Staining level 0 is defined as negative and 1 as weak, staining levels 2 and 3 are defined as high expression of KLK14 in the tumor.

analysis, including all significant variables shown in the univariate analysis, KLK14 expression in prostate carcinoma turned out to be an independent factor (table 3).

Discussion

In recent times, exploring the use of the new human KLK14 has been a promising research field which our present retrospective study on prostatic adenocarcinoma could considerably enhance. This is the first comprehensive immunohistochemical analysis of KLK14 expression in prostate cancer. KLK14 was observed in the cytoplasm of epithelial glandular cells as described previously in a study on breast cancer [13] as well as in a recent experimental review on several cancer types and KLKs, including prostate tissue [26]. Although immunostaining of KLK14 in the prostate was not specified, the authors noticed similar patterns of expression in different tissues shown for example in the testis [26]. We agree with their findings of KLK luminal apical accumulation in PIN [26] but we also detected this phenomenon in prostatic adenocarcinoma and in adjacent normal tissue, which has not been particularly emphasized in the literature [26]. KLK14 concentration to the luminal side could be explained by KLK14 being a secreted protein that is accumulated in the secretory cells of the prostate gland [27].

In contrast to increased KLK14 expression in breast and ovarian cancer [11, 13], KLK14 immunostaining revealed nearly equal amounts of the protein in prostatic adenocarcinomas, in matching normal tissue and in PIN and did not indicate any diagnostic benefit. These immunohistochemical data results are in line with our mRNA data. Our exact determination of *KLK14* mRNA provided no significant differences between the expression of *KLK14* in malignant and nonmalignant tissue. We refined conflicting data of cancer-specific down- and upregulation of *KLK14* mRNA [9, 16], using tissue selection by laser capture microdissection and a tailor-made set of housekeeping genes for prostate cancer. The size of the cohort with 25 patients should have been large enough to recognize potential differences so that further studies on mRNA levels of the peptidase in prostatic adenocarcinoma were not indicated.

The most important finding of our study is that immunostaining of KLK14 was able to distinguish patients with a higher risk of disease recurrence from lower-risk patients. The association of elevated KLK14 levels with higher aggressiveness or unfavorable prognosis corresponds to previous findings in breast carcinoma [13, 18], which further underscores that KLK14 adds to tumor progression. We could emphasize the prognostic value of KLK14 for patients after radical prostatectomy, since KLK14 was independent from significant clinicopathological parameters.

One limitation of our study is the use of PSA relapse as endpoint surrogate, but it is generally accepted according to the EAU Guidelines on Prostate Cancer [28]. In particular for patients after radical prostatectomy, a marked increase in PSA over time was shown to correlate with a higher rate of prostate cancer-specific mortality [29], indicating the utility of PSA follow-ups. It is left for a prospective study to analyze, if people with high prostatic tissue levels or even high serum levels of KLK14 are at higher risk of prostate cancer initiation or disease progression. This might be expected, since serum of patients with prostate cancer showed higher expression of KLK14 compared with serum of healthy males [5]. These data would eliminate the bias of varying follow-up times of our retrospective study. In order to develop a suitable prognostic system for patients after radical prostatectomy, KLK14 and PSA could be measured in time courses, as PSA velocity and PSA doubling time have been suggested to be the best markers for disease recurrence up to now [30]. However, in the present study, we renounced using those approaches, since PSA values need to be measured in equal intervals, and that precondition was not always fulfilled [31]. Because KLKs cooperate in proteolytic cascades [4], not only KLK14 but also further members of the family are expected to improve prognostic possibilities.

We conclude that KLK14 has the prognostic potential to recognize prostate cancer patients at higher risk of disease recurrence after radical prostatectomy. As an independent factor, KLK14 is expected to supplement postoperative PSA values for a more precise prognosis. Further studies of KLK14 in serum are indicated to approach clinical implementation.

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