

# Polyclonal antibodies against kallikrein-related peptidase 4 (KLK4): immunohistochemical assessment of KLK4 expression in healthy tissues and prostate cancer

Lina Seiz<sup>1,a</sup>, Matthias Kotzsch<sup>2,a</sup>, Nicolai I. Grebenchtchikov<sup>3</sup>, Anneke J. Geurts-Moespot<sup>3</sup>, Susanne Fuessel<sup>4</sup>, Peter Goettig<sup>5</sup>, Apostolos Gkazepis<sup>1</sup>, Manfred P. Wirth<sup>4</sup>, Manfred Schmitt<sup>1</sup>, Arndt Lossnitzer<sup>2</sup>, Fred C.G.J. Sweep<sup>3</sup> and Viktor Magdolen<sup>1,\*</sup>

<sup>1</sup> Clinical Research Unit, Department of Obstetrics and Gynecology, Technical University of Munich, Ismaninger Str. 22, D-81675 Munich, Germany

<sup>2</sup> Institute of Pathology, Dresden University of Technology, Fetscherstr. 74, D-01307 Dresden, Germany

<sup>3</sup> Department of Laboratory Medicine, Radboud University Nijmegen Medical Centre, Geert Grooteplein 8, NL-6525 GA Nijmegen, The Netherlands

<sup>4</sup> Department of Urology, Dresden University of Technology, Fetscherstr. 74, D-01307 Dresden, Germany

<sup>5</sup> Structural Biology Group, University of Salzburg, Billrothstr. 11, A-5020 Salzburg, Austria

\* Corresponding author  
e-mail: viktor.magdolen@lrz.tum.de

## Abstract

KLK4 is a member of the human kallikrein-related peptidase family of (chymo)trypsin-like serine proteases. The aim of the present study was to generate polyclonal antibodies (pAb) directed against KLK4 for the analysis of KLK4 by immunohistochemistry in human tissues. Recombinantly expressed human mature KLK4 was used for immunization of chickens. pAb 617A is an affinity-purified monospecific pAb fraction reacting with a linear epitope within a flexible surface-exposed loop of KLK4. pAb 617C is the KLK-directed pAb fraction completely depleted from pAb 617A. In healthy adult tissues, KLK4 was immunodetected by both antibody fractions in kidney, liver, and prostate, but not in other organs such as colon and lung. To evaluate protein expression of KLK4 in prostate cancer, samples of tumor tissue plus corresponding tumor-free areas of 44 prostate cancer patients, represented on a tissue microarray, were investigated. Distinct KLK4 immunostaining was observed with both antibodies in cancerous glandular epithelial cells, but not in surrounding stromal cells. KLK4 expression was lower in stage pT3+4 than in pT1+2 tumors, which was highly significant when employing pAb 617A. Thus, our results indicate that KLK4, which is expressed in the healthy pros-

tate, is upregulated in early-stage but not late-stage prostate cancer.

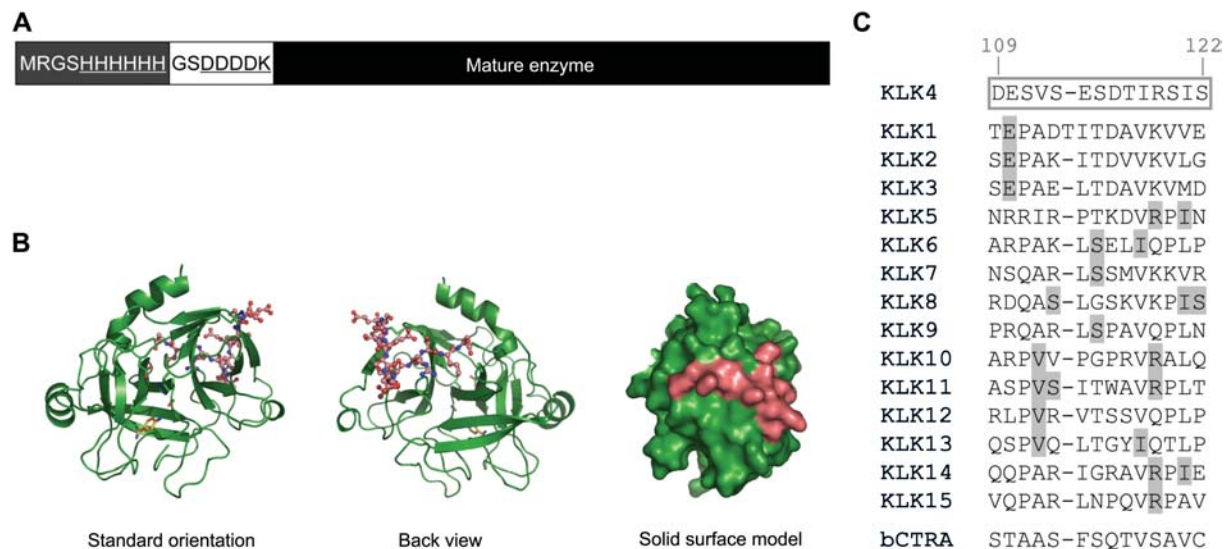
**Keywords:** immunohistochemistry; kallikrein-related peptidase 4; KLK4; prostate; prostate cancer.

## Introduction

The human tissue kallikrein gene (*KLK*) family codes for 15 highly conserved, secreted serine proteases with either trypsin or chymotrypsin-like substrate specificity. The kallikrein genes are localized on chromosome 19q13.3–4, where they represent the largest uninterrupted cluster of protease genes in the human genome. Many *KLK* genes, including *KLK4*, are transcriptionally regulated by steroid hormones (Nelson et al., 1999; Lai et al., 2009). Strikingly, specific members of the kallikrein family are known to be coexpressed in various normal and malignant tissues, thus raising the possibility that KLKs might participate in proteolytic cascades (Borgoño and Diamandis, 2004; Clements et al., 2004; Pampalakis and Sotiropoulou, 2007; Lundwall and Brattsand, 2008).

Initially, several kallikrein-related peptidases were the focus of attention as potential disease biomarkers, particularly in hormone-regulated malignancies. To date, PSA (prostate-specific antigen, KLK3) is the best-established clinical marker indicating the existence and also the progression of prostate cancer. However, owing to limitations such as false positive results in patients with benign prostatic hyperplasia and false negative results in the case of residual disease, additional markers are highly needed. One such candidate for improving diagnosis of prostate cancer or monitoring of residual disease could be represented by KLK4 (Day et al., 2002), also known as prostase, owing to its expression in the prostate and its presence in seminal plasma (Nelson et al., 1999; Obiezu et al., 2002, 2005). Indeed, under physiological conditions, KLK4, together with PSA/KLK3 and other prostatic KLKs, is part of a highly regulated proteolytic cascade, which upon ejaculation leads to activation of pro-KLK3 and subsequent semen liquefaction (Pampalakis and Sotiropoulou, 2007). Moreover, KLK4 was independently identified as enamel matrix serine protease 1 (EMSP1; Simmer et al., 1998) and found to play an essential role in enamel biomineralization by degrading matrix proteins prior to their removal from the maturing enamel during tooth formation (Simmer et al., 2009). Although KLK4 was originally described as a protease with prostate-restricted expression (Nelson et al., 1999), later studies suggested that it is

<sup>a</sup>These authors contributed equally to this work.



**Figure 1** Location of peptide KLK<sub>109–122</sub> used for selection of monospecific polyclonal antibodies directed against rec-KLK4.

(A) Structure of the immunogen rec-KLK4. The sequence encoding mature human KLK4 is preceded by an N-terminal 17 aa-extension harboring a (His)<sub>6</sub>-tag and an enterokinase cleavage site. (B) Location of the peptide KLK<sub>109–122</sub> within the structure of KLK4 (Debela et al., 2006a). Stereo ribbon plots are shown in the standard orientation (the substrate binding site from N- to C-terminus runs horizontally from left to right) as well as from the back of the protease. As seen by solid surface representations, the selected peptide (red) is located on the surface of the molecule. Figures were created with the software PyMOL (DeLano, 2002). (C) Multiple sequence alignment for human kallikrein-related peptidases and bovine chymotrypsin (bCTRA) encompassing the (non-conserved) sequence between positions 109 and 122 (according to the chymotrypsin numbering).

expressed in a wide array of healthy adult tissues (Shaw and Diamandis, 2007).

KLK4 displays trypsin-like activity (Debela et al., 2006a,b) and is able to convert pro-urokinase-type plasminogen activator (pro-uPA) into active uPA, which is known to be a key player in extracellular matrix remodeling, angiogenesis, wound healing, embryogenesis, tumor invasion, and metastasis (Takayama et al., 2001; Beaufort et al., 2006). In addition, KLK4 can modulate cell surface-associated proteolytic activity and alter the adhesive properties of tumor cells by cleaving the uPA receptor (uPAR) (Beaufort et al., 2006). Moreover, PAR-1 and PAR-2, members of the protease-activated receptor family of G-protein-coupled receptors, have been demonstrated to be activated by KLK4 (Mize et al., 2008; Ramsay et al., 2008). In the case of PAR-1, it has been proposed that KLK4, expressed by prostate cancer cells, activates PAR-1 in the surrounding stroma, which in turn releases interleukin-6 and other cytokines, which then stimulate proliferation of the cancer cells (Wang et al., 2010). PAR-2 has also been shown to be coexpressed with KLK4 in prostate cancer (Ramsay et al., 2008). Therefore, KLK4-mediated modulation of the tumor-associated uPA/uPAR system and cell signaling via PARs could contribute to angiogenesis, invasiveness or progression of (prostate) cancer. Evidence for overexpression of KLK4 mRNA has been shown for both prostate and ovarian cancer (Dong et al., 2001; Xi et al., 2004), being associated with progression and unfavorable prognostic outcome in ovarian cancer patients (Obiezu et al., 2001). Significantly higher KLK4 mRNA levels were also found in cancerous breast tissue compared with the normal breast (Mangé et al., 2008).

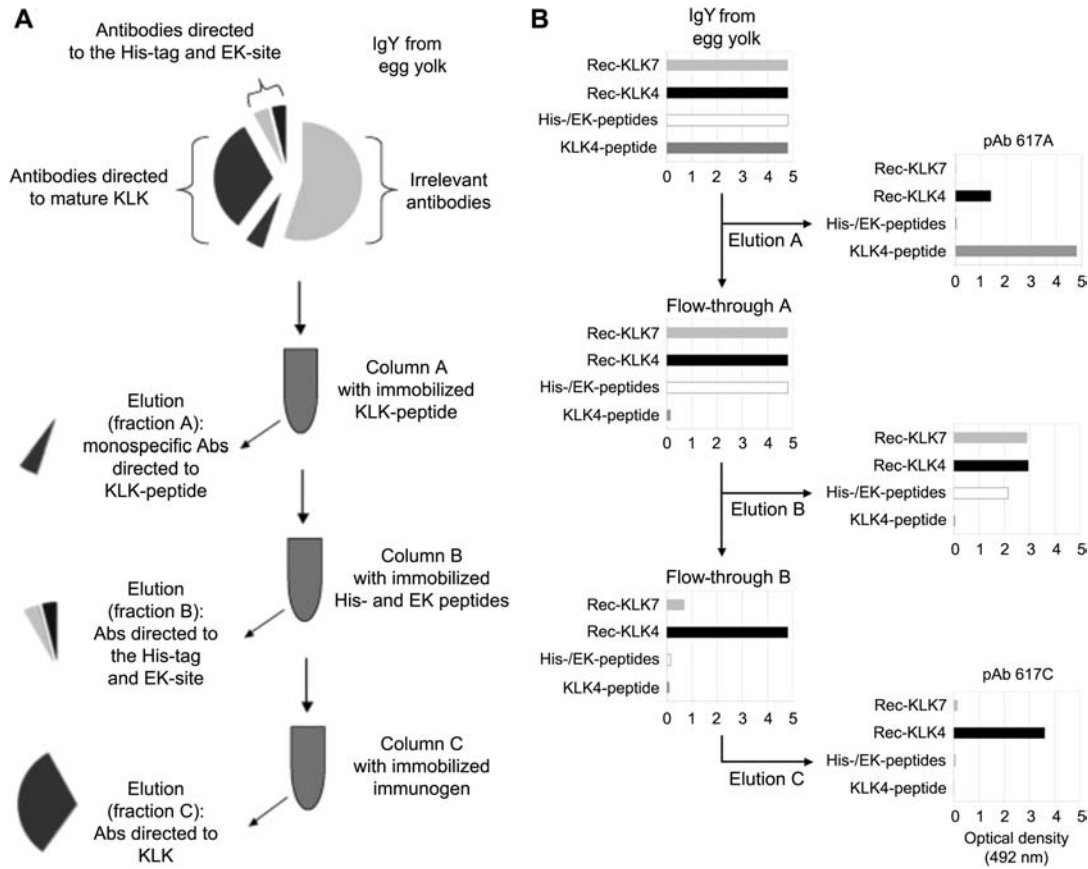
Nevertheless, the function of KLK4 in hormone-dependent human malignancies is still unclear and requires further investigation. Thus, the need for reliable tools to characterize KLK4 expression in human tissues incited us to generate specific antibodies directed to KLK4 to analyze KLK4 protein expression in healthy human organs as well as tumor tissue of prostate cancer patients.

## Results

### Generation of polyclonal antibodies directed to KLK4 in chickens

Purified and refolded recombinant (non-glycosylated) human KLK4 (rec-KLK4), carrying an N-terminal extension of 17 amino acids encompassing a histidine (His)<sub>6</sub>-tag and an enterokinase (EK) cleavage site (DDDDK↓) was used for immunization of chickens (Figure 1A). Antibodies from egg yolk were subsequently purified by affinity chromatography by three consecutive procedures: (a) against a unique peptide of KLK4 (Figure 1B, C) to select for monospecific polyclonal antibodies; (b) by a 'negative' purification step using columns with immobilized peptides covering the tag of the recombinant protein, and (c) against the immunogen (Figure 2).

For selection of the peptide epitope for purification of monospecific antibodies, we searched – based on the X-ray structure of KLK4 – for regions which are surface-exposed and not involved in secondary structures. Such surface-exposed flexible loops are known to be immunogenic, and



**Figure 2** Affinity purification of polyclonal antibodies 617A and 617C.

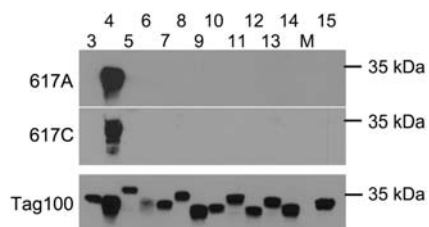
(A) Purification scheme. The IgY fraction from egg yolk of a chicken, immunized with rec-KLK4, is passed over column A with immobilized KLK<sub>109–122</sub>. The elution (fraction A: pAb 617A) contains monospecific, polyclonal antibodies directed against the linear epitope 109–122 of KLK4. The flow-through of column A is applied to column B with immobilized peptides GSHHHHHHGS (His-peptide) plus HHHGSDDDDK (EK-peptide). The elution (fraction B) contains antibodies directed to the N-terminal extension of rec-KLK4, i.e., the (His)<sub>6</sub>-tag and the EK-site. The flow-through of column B is passed over column C with immobilized rec-KLK4. The elution (fraction C: pAb 617C) contains polyclonal antibodies directed against rec-KLK4. (B) One-side ELISA. Elution A (pAb 617A) reacts with peptide KLK<sub>4109–122</sub> and with rec-KLK4; elution B reacts with rec-KLK4, rec-KLK7 (harboring the identical N-terminal, 17aa-extension), and with His- plus EK-peptides. Elution C (pAb 617C) reacts with rec-KLK4 but not with KLK<sub>4109–122</sub>, demonstrating that this fraction is completely depleted from antibodies directed against the epitope 109–122 of KLK4.

furthermore often represent linear (and not conformational) epitopes. In addition, we searched for regions, which are not highly conserved among the members of the KLK protease family. By this strategy, we selected the region encompassing amino acids 109–122 of KLK4 (according to the chymotrypsin numbering) (Figure 1B, C).

#### Purification and characterization of polyclonal antibodies directed to KLK4 in chickens

Three types of affinity chromatography columns were produced: in the first, the synthetic KLK4-derived peptide 109–122 (KLK<sub>4109–122</sub>) was coupled to the column material, for the second, peptides covering the (His)<sub>6</sub>-tag (GSHHHHHHGS) plus the EK-site (HHHGSDDDDK) were coupled, and the third column was modified by covalent addition of the immunogen, rec-KLK4 (Figure 2A). The IgY fraction extracted from egg yolks was applied onto the first

column, and the elution of this column contained monospecific polyclonal antibodies (pAbs) against KLK<sub>4109–122</sub> (fraction 617A). The reaction pattern of pAb 617A was analyzed by ‘one-side ELISA’ assays, in which the antigen, the KLK4-derived peptide, or control proteins/peptides were used for coating, demonstrating a specific, strong reaction with both the immunogen (rec-KLK4) as well as the peptide KLK<sub>4109–122</sub> (Figure 2B). The flow through was applied to the column linked to the (His)<sub>6</sub>-tag plus EK-site peptides, for negative selection of antibodies generated against the N-terminal, non-KLK related sequences of rec-KLK4. The flow through of the second column, which now was depleted from antibodies directed to the non-KLK related sequences of rec-KLK4, was applied to the third column, coupled with the immunogen. As seen in the ‘one-sided ELISA’ assay, antibodies eluted from the third column, fraction 617C, react distinctly with the immunogen (rec-KLK4), but not with peptide KLK<sub>4109–122</sub> (Figure 2B), demonstrating that pAb 617C



**Figure 3** Analysis of specificity of polyclonal antibodies 617A and 617C by Western blot analysis.

Recombinant pro-forms of KLK3–15 (~1 µg each) were subjected to 12% SDS-PAGE, blotted onto a PVDF membrane and then reacted with pAb 617A or pAb 617C. Pro-KLK3 and pro-KLK5–15 did not react with the antibodies, only pro-KLK4 did, with an apparent molecular mass of approximately 33 kDa. Thus, no cross-reactivity of the antibodies with other KLKs was observed. The transfer of the proteins was verified by reaction with an antibody directed against the C-terminally located Tag100 epitope present in all recombinant pro-KLK proteins.

is the antibody fraction completely depleted from pAb 617A. Thus, pAb 617A and pAb 617C are directed against different epitopes of KLK4.

In Western blot analyses, pAb 617A and pAb 617C were found to be specific for KLK4, as there was no crossreaction with either the KLK proteases rec-KLK2 (data not shown), rec-KLK3, and rec-KLK5–15 (Figure 3), or other His- and/or EK-tagged proteins (data not shown).

#### Immunohistochemical analyses of KLK4 expression in healthy adult human tissues employing the antibodies 617A and 617C

In immunohistochemical analyses of representative conventional tissue sections of prostate tissues, both antibody fractions 617A and 617C were found to be suitable for detection of KLK4 in human tissues. Using a tissue microarray containing a variety of normal adult tissues, KLK4 was immu-

**Table 1** Expression pattern of KLK4 in healthy human tissues using pAbs 617A and 617C.

	617A <sup>a</sup>	617C <sup>a</sup>
Kidney	++	++
Prostate gland	+	+
Uterus	-	-
Small intestine	-	-
Lung	-	-
Tonsil	-	-
Liver	++	++
Skeletal muscle	-	-
Cerebellum	-	-
Colon	-	-
Skin	-	-

<sup>a</sup>KLK4 expression: ++, high; +, moderate; -, negative.

nodetected with high frequency in liver hepatocytes and renal tubular, but not in glomerular cells. Staining intensity in the renal cortex was higher compared with renal medullary cells (Figure 4A, B). Other normal tissues such as colon, lung, skin or skeletal muscle were not immunoreactive for KLK4 with either of the antibodies (Figure 4C, D, Table 1). For both antibodies, competition experiments were carried out on normal prostate sections to confirm their specificity (data not shown).

#### Immunohistochemical analyses of KLK4 in prostate cancer patients by antibodies 617A and 617C and its association with clinicopathological parameters

To evaluate protein expression of KLK4 in prostate cancer by immunohistochemistry, conventional tissue sections of prostate cancer specimens as well as samples of tumor tissue (Tu) and the corresponding tumor-free (Tf) areas of 44 prostate cancer patients, represented on a tissue microarray, were investigated using antibodies 617A and 617C.

Distinct KLK4 immunostaining was observed with both antibodies in malignant glandular epithelial cells, but not in the basal layer of surrounding normal tissue or fibromuscular stromal cells. Typically, an intense granular cytoplasmic staining of cancer glands was observed. There was no nuclear staining with either of the antibodies. Furthermore, in corresponding tumor-free areas we found only a weak KLK4 staining in glandular luminal cells of non-malignant prostate tissue (Figure 5).

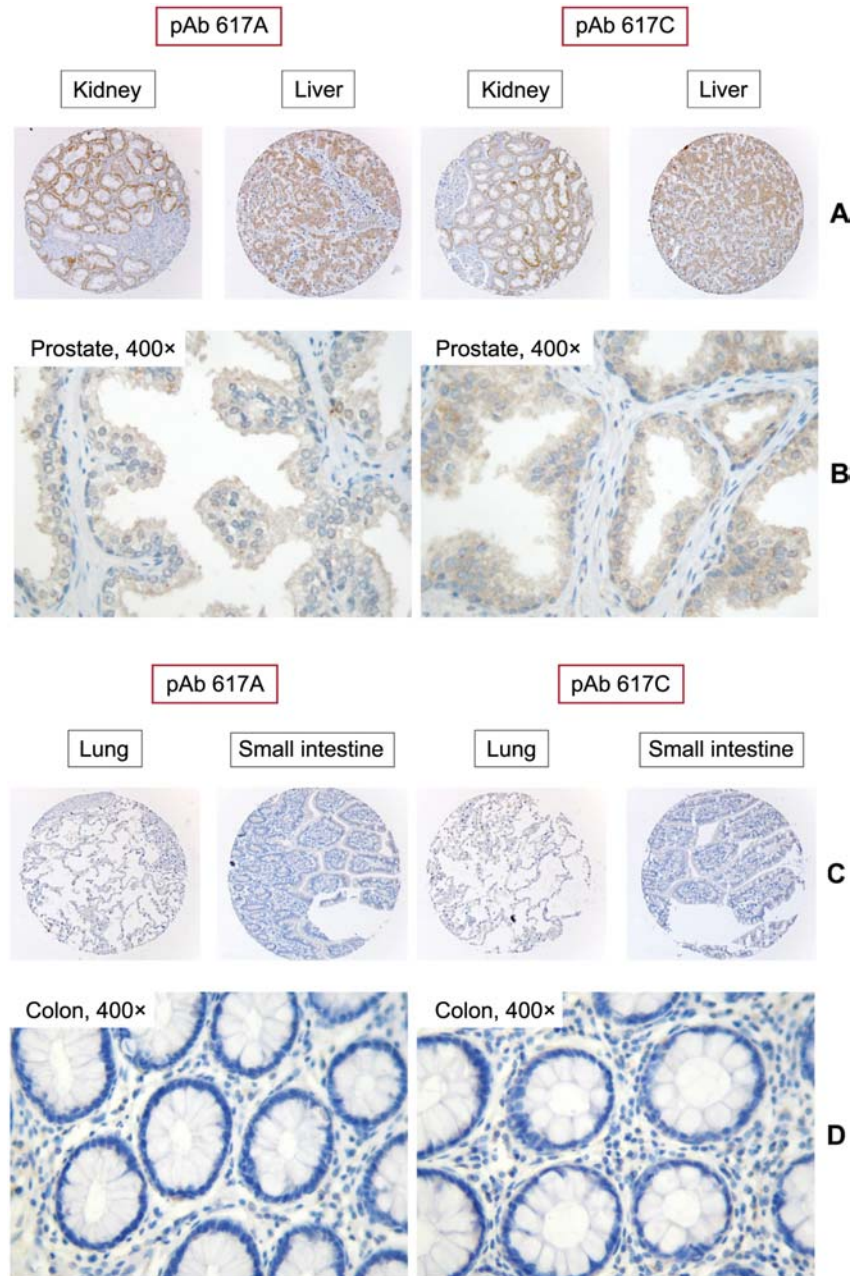
For estimation of KLK4 immunoreactivity, a semiquantitative score based on KLK4 staining intensity and the percentage of KLK4-positive cancer cells (KLK4-Tu) as well as

**Table 2** Association of KLK4-Tu score values of pAbs 617A and 617C with clinicopathological parameters of prostate cancer patients (n=44).

Clinicopathological parameters	No. of patients	KLK4-Tu 617A <sup>a</sup>	KLK4-Tu 617C <sup>a</sup>
Total	44	0.43	0.38
Nodal status <sup>b</sup>		<i>p</i> =0.683	<i>p</i> =0.120
No	37	0.48	0.45
Yes	5	0.20	0
Grading <sup>c</sup>		<i>p</i> =0.572	<i>p</i> =0.912
Grade I	8	0.34	0.34
Grade II	17	0.65	0.57
Grade III	9	0.31	0.22
Gleason score <sup>c</sup>		<i>p</i> =0.455	<i>p</i> =0.459
Low (2–6)	15	0.42	0.33
Intermediate (7)	17	0.56	0.57
High (8+9)	12	0.25	0.17
Tumor stage <sup>b</sup>		<i>p</i> =0.005	<i>p</i> =0.139
T1+2	25	0.68	0.54
T3+4	19	0.09	0.17

<sup>a</sup>Mean tissue score values; <sup>b</sup>Mann-Whitney test; <sup>c</sup>Kruskal-Wallis test.

The number of patients does not always sum up to 44 because of missing data.



**Figure 4** KLK4 immunoeexpression in healthy adult human tissues.

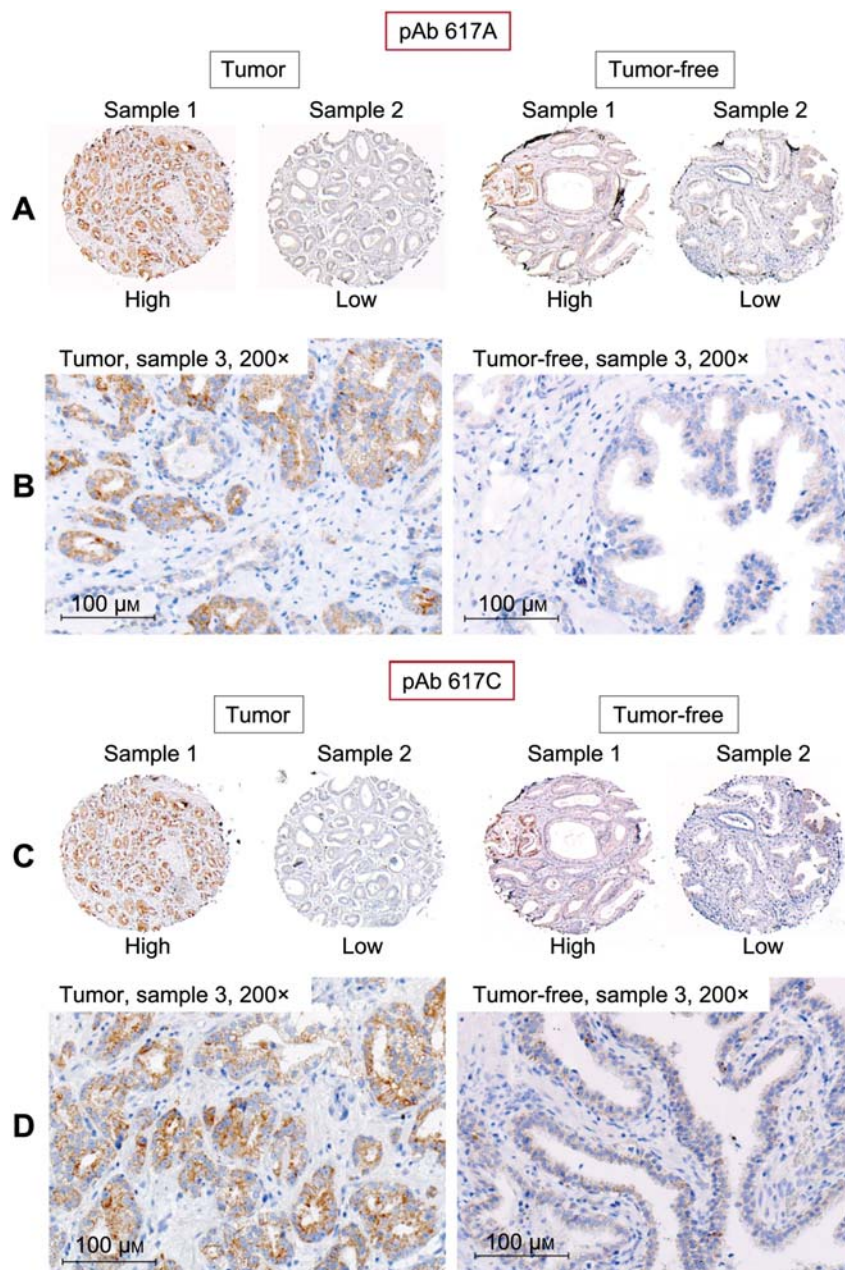
(A, B) KLK4 is expressed in healthy adult kidney, liver, and prostate tissue. (A) Core punches within the tissue microarray stained with pAb 617A or 617C and using the Vectastain ABC-kit (Vector Laboratories) (original magnification  $\times 40$ ). (B) Full-face tissue sections stained using the EnVision system (Dako) (original magnification  $\times 400$ ). (C, D) KLK4 is not expressed in healthy adult lung, small intestine and colon tissue. (C) Core punches from tissue microarray stained using the Vectastain ABC-kit (Vector Laboratories) (original magnification  $\times 40$ ). (D) Full-face tissue sections stained using the EnVision system (Dako) (original magnification  $\times 400$ ).

KLK4-positive prostate glandular cells in tumor-free areas (KLK4-Tf) was used.

A strong, highly significant correlation was observed between pAb 617A and pAb 617C, both with regard to KLK4 expression in tumor cells ( $r_s=0.81$ ,  $p<0.001$ ) and non-malignant cells in tumor-free areas ( $r_s=0.83$ ,  $p<0.001$ ). For KLK4-Tu versus KLK4-Tf values, there was, however, a weaker correlation for both pAb 617A and pAb 617C ( $r_s=0.63$ ,  $p<0.001$ , and  $r_s=0.33$ ,  $p=0.026$ , respectively).

Furthermore, a distinct elevated KLK4 expression was observed in tumor cells versus non-malignant cells in tumor-free areas: in the case of pAb 617A, the mean score values were 0.43 (range 0–5.25) for KLK4-Tu versus 0.17 (range 0–2.0) for KLK4-Tf; in the case of pAb 617C the mean values were 0.38 (range 0–6.0) for KLK4-Tu versus 0.01 (range 0–0.5) for KLK4-Tf.

Immunohistochemical expression of KLK4 was analyzed for potential associations with clinical parameters. The rela-



**Figure 5** KLK4 expression in tumor tissue and corresponding tumor-free areas of prostate cancer specimens. Full-face sections and tissue microarray sections were stained with pAb 617A (A, B) or pAb 617C (C, D) using the Vectastain ABC-kit (Vector Laboratories). (A, C) Representative core punches within the tissue microarray are shown presenting different tissue score values (original magnification  $\times 40$ ). (B, D) Representative areas within full-face tissue sections at higher magnification (original magnification  $\times 200$ ).

tionship between KLK4-Tu score values of antibodies 617A and 617C and relevant clinicopathological characteristics of prostate cancer patients is summarized in Table 2. Interestingly, a significant association was observed between KLK4-Tu score values of pAb 617A and tumor stage, which is a strong prognostic indicator of prostate cancer. KLK4 expression was significantly lower in patients with stage pT3 and pT4 tumors, i.e., non-organ confined disease, compared with patients with stage pT1 and pT2 tumors, i.e., organ confined disease (Table 2). There was no significant relationship between KLK4-Tu score values of pAb 617C and tumor

stage. Furthermore, there was no significant association between KLK4-Tu and KLK4-Tf score values and any of the other clinicopathological parameters including preoperative PSA serum levels (Table 2 and data not shown).

## Discussion

In past years, we have established a prokaryotic expression system allowing the recombinant expression and purification

of synthetic pro-enzyme forms of several members of the human kallikrein-related peptidase family. After refolding and removal of the non-natural propeptide by enterokinase, we were able to solve the crystal structure of mature enzymes and perform enzyme kinetic studies for a series of the KLK proteases, including KLK4 (Debela et al., 2006a,b, 2008). In the present study, we used the recombinant, refolded synthetic pro-form of KLK4 as the immunogen. Injection of folded (native) antigens mostly induces generation of antibodies directed against conformational epitopes, i.e., the binding sites of these antibodies are constituted by the three-dimensional structure of the antigen and can be composed of non-continuous peptide sequences. Such antibodies could be very useful for ELISA or FACS analyses, in which antigens in their native conformation are detected. However, for other applications, in which the antigens are partly denatured, e.g., in immunohistochemistry or Western blot analyses, it is more favorable to use antibodies directed against linear epitopes of the antigen. Therefore, often short peptide sequences derived from the target protein are employed to generate anti-peptide antibodies (Harvey et al., 2003). We reasoned that among the polyclonal antibodies raised against the (folded) immunogen rec-KLK4, a fraction will also be directed against such linear peptide epitopes. Thus, based on the X-ray structure of KLK4 (Debela et al., 2006a), we searched for short surface-exposed sequences that are not part of secondary structures ( $\alpha$ -helices or  $\beta$ -sheets). Such rather flexible loop structures are good candidates for the generation of antibodies directed against linear epitopes. Importantly, one of these regions, corresponding to amino acids 109–122 of KLK4 is not well conserved among the other members of the KLK protease family (Figure 1C), minimizing the risk of crossreaction with these otherwise highly homologous proteins. Using a synthetic KLK4<sub>109–122</sub>-peptide for affinity chromatography, we were able to isolate an antibody fraction, pAb 617A, which reacts with the KLK4<sub>109–122</sub> peptide and the full-length KLK4 immunogen, respectively. Even more importantly, the antibody fraction pAb 617C was found to be completely devoid of antibodies directed against KLK4<sub>109–122</sub>. Although present in the entire polyclonal serum, these two antibody fractions are clearly directed against different epitopes: pAb 617A against the sequence KLK4<sub>109–122</sub>, pAb 617C against epitopes residing in the other parts of the mature KLK4 protein.

The two antibody fractions were independently used in comparative immunohistochemical analyses of normal human tissues resulting in identical staining intensity and pattern in the corresponding tissue sections (Figure 4, Table 1). These findings strongly indicate that the two antibodies, directed to different epitopes, specifically detect KLK4 in various normal tissues and prostate cancer specimens. Specificity of the antibodies was also assessed by Western blot analyses concerning cross-reaction with other members of the KLK family. KLK4 exhibits a minimum of 37% (versus KLK10) and maximum of 52% (versus KLK5) identical amino acids at homologous positions within the KLK family (Lundwall and Brattsand, 2008). For the recombinant pro-forms of KLK2 to KLK15 no crossreaction was detected with both antibody fractions (Figure 3).

Incipiently, KLK4 had been detected in the normal and neoplastic prostate, only (Nelson et al., 1999). Owing to this apparently prostate-restricted expression pattern, it became generally known as prostatic. However, subsequent studies could not confirm KLK4 expression being restricted to the prostate. In independent studies on tooth formation, an enamel matrix serine protease 1 (EMSP1) was identified (Simmer et al., 1998) and shown to play an important role in the process of enamel maturation (Simmer et al., 2009). Human EMSP1 turned out to be identical to KLK4 (Simmer and Bartlett, 2004). KLK4 has also been detected in a large panel of healthy adult tissues including prostate, liver, breast, skin, and brain (Obiezu et al., 2005). In our immunohistochemical stainings with pAb 617A and pAb 617C we found high KLK4 immunoreactivity in liver hepatocytes and renal tubular cells as well as moderate staining intensity in the healthy prostate (Figure 4A,B, Table 1), which is in agreement with previously reported data (Shaw and Diamandis, 2007). With regard to the absence of staining in colon, lung, skin, and tonsil tissue (Figure 4C,D, Table 1), our results proved to be consistent with findings obtained from analyses of healthy adult tissue extracts by ELISA (Shaw and Diamandis, 2007).

In the present study, we further investigated KLK4 expression in prostate tumor tissue and the corresponding tumor-free areas of 44 prostate cancer patients. We observed a distinctly elevated KLK4 antigen expression in prostate cancer cells compared with nearby non-malignant prostate epithelial cells. Contrary to previous data reporting mainly nuclear KLK4 expression in prostate cancer, based on the proposed existence of a controversially discussed short *KLK4* transcript lacking the signal sequence (Simmer and Bartlett, 2004; Klock et al., 2007), we exclusively observed a cytoplasmic staining pattern in prostate cancer glands only, similarly to the staining of epithelial cells in healthy adult tissues (Figure 5). Nuclear immunostaining did not occur with any of our two antibodies, being in line with experimental evidence, that kallikrein-related peptidases, including KLK4, are produced in the cytoplasm and secreted into the extracellular matrix (Simmer et al., 1998; Obiezu et al., 2005; Shaw and Diamandis, 2007).

To evaluate the relevance of elevated KLK4 expression in prostate cancer patients we analyzed the relationship between KLK4-Tu tissue score values and clinicopathological patients' characteristics (Table 2). Strikingly, we found a statistically significant, inverse correlation between KLK4-Tu score values obtained with pAb 617A and tumor stage ( $p=0.005$ ). Prostate cancer specimens classified as stage pT3 or T4 tumors showed significantly lower KLK4 expression (mean score value 0.09) than stage pT1 or T2 tumors (mean score value 0.68). Thus, we conclude that KLK4 expression is closely linked to tumor stage, which is a strong prognostic factor in prostate cancer patients. A very similar phenomenon has been observed in a study where KLK4 mRNA levels were monitored in breast cancer patients. In parallel to our findings, increased KLK4 expression has been found in pT1 and pT2 tumors compared with normal breast tissues, whereas pT3 tumors displayed less elevated KLK4 expression levels (Mangé et al., 2008). Interestingly, another member of

the KLK family, KLK5, has also been shown to be inversely associated with tumor stage (as well as Gleason score) in prostate cancer patients (Yousef et al., 2002; Korbakis et al., 2009). Gene expression of both, *KLK4* and *KLK5*, seems to be regulated by androgens (Korbakis et al., 2009; Lai et al., 2009) and can thus be downregulated in hormone-refractory prostate cancer in comparison to androgen-sensitive tumors. Therefore, decrease in *KLK4* and *KLK5* expression could be associated with a more advanced, potentially already castration-resistant, disease. Future studies with an increased number of tissues could allow an analysis, if expression of these factors in prostate cancer is continuously and statistically significant decreasing from pT1 to pT4.

Although the expression of *KLK4* in normal and neoplastic prostate epithelial tissues has been extensively investigated, the role of *KLK4* in development and progression of hormone-regulated malignancies such as prostate cancer remains to be further elucidated. By generating highly specific antibodies directed to *KLK4*, we now provide a reliable tool for the fine analysis of the expression and functional role of *KLK4* in healthy and diseased human organs. Our results indicate that *KLK4* expression levels in prostate cancer patients carry important stage-related prognostic information. In this regard, authentic monitoring of *KLK4* expression is a crucial prerequisite for utilizing *KLK4* as a target for cancer diagnosis and therapy.

## Materials and methods

### Reagents

Peptides *KLK*<sub>109–122</sub> (for the sequence see Figure 1C), GSHHHH-HHGS and HHHGSDDDDK [corresponding to (His)<sub>6</sub>-tag and the EK cleavage site of rec-*KLK4*, respectively] were synthesized by the Service Center of the Max-Planck-Institute of Biochemistry, Martinsried, Germany.

### Recombinant kallikrein peptidases

Recombinant *KLK4* and *KLK7*, both harboring an N-terminal (His)<sub>6</sub>-tag followed by an EK-site, were expressed in *Escherichia coli* and purified as described previously (Debela et al., 2006a,b). In addition, expression plasmids encoding native pro-forms of *KLK* proteases (*KLK2* to *KLK15*) plus an N-terminally located histidine-tag and a C-terminal Tag100 epitope were generated and used for production of the respective recombinant pro-forms.

Expressed (non-glycosylated) proteins were purified via their histidine-tag by nickel-nitrilotriacetic acid agarose affinity chromatography (Qiagen, Hilden, Germany) under denaturing/slightly reducing conditions. Subsequently, refolding procedures were employed, using reduced and oxidized glutathione as redox reagents (Sigma, Deisenhofen, Germany) to promote protein renaturation.

### Generation of polyclonal antibodies directed against *KLK4* in chickens

Chickens were immunized intramuscularly (pectoral muscle) with 20 µg of rec-*KLK4* per injection following the protocol by McKiernan et al. (2008). Antibodies (IgY, avian analog of IgG) were isolated from egg yolk using a standard step precipitation procedure

utilizing increasing concentrations of polyethyleneglycol (PEG precipitation) as described previously (Grebenschikov et al., 1997). This procedure yielded approximately 95% pure IgY as analyzed by SDS-PAGE and subsequent Coomassie Blue staining.

### Affinity purification of polyclonal antibodies directed to *KLK4*

Monospecific chicken antibodies were purified from the IgY fraction using affinity chromatography employing three consecutive steps: (i) affinity column with covalently linked peptide *KLK4*<sub>109–122</sub>; (ii) affinity column with immobilized peptides covering the tag of the recombinant protein (GSHHHHHHGS and HHHGSDDDDK corresponding to (His)<sub>6</sub>-tag and the EK cleavage site of rec-*KLK4*, respectively); and (iii) affinity column, coupled with the immunogen, i.e., rec-*KLK4*.

The coupling of recombinant *KLK4* protein and peptides to the affinity columns was performed according to the standard BioRad procedure. After coupling, the gel slurry was packed into disposable plastic columns (Pierce, Rockford, IL, USA). For each particular column, approximately 2 ml of a 1:1 mixture of AffiGel<sup>®</sup>10 and AffiGel<sup>®</sup>15 (Bio-Rad Laboratories, Hercules, CA, USA) was used.

After filtration, at first, the antibody solution (~8 ml) was passed twice through the affinity column coupled with the *KLK4*<sub>109–122</sub>-peptide. The obtained flow through was then passed twice through the affinity column containing the mixture of immobilized poly-His and EK-peptides. Finally, the flow-through of that purification step was passed twice through the affinity column containing immobilized rec-*KLK4*. After washing with phosphate-buffered saline (PBS), bound antibodies of all three columns were eluted with 4 ml of 0.1 M glycine/HCl buffer, pH 2.4, followed by an immediate restoring of a neutral pH using appropriate amounts of a KOH solution. The purified antibodies were finally concentrated by an Amicon Ultra-15 centrifugal filter device (Millipore, Amsterdam, The Netherlands), diluted 1:1 with glycerol, and stored at -20°C until use.

### One-sided ELISA

Purified antibodies were characterized using microtiter plate-based 'one-sided ELISA' assays in which the antigen or irrelevant proteins were coated onto the wells of a microtiter plate.

Briefly, Nunc Maxisorp<sup>™</sup> flat-bottomed immuno plates (Life Technologies, Breda, The Netherlands) were coated overnight at 4°C with testing proteins (rec-*KLK4*, rec-*KLK7*) and peptides (*KLK4*<sub>109–122</sub>, His-tag- and EK-site-derived), respectively, diluted in coating buffer (15 mM Na<sub>2</sub>CO<sub>3</sub> and 35 mM NaHCO<sub>3</sub>, pH 9.6) at a concentration of 0.1 µg/ml. Afterwards, plates were washed twice (Tecan, Männedorf, Switzerland) and blocked with blocking buffer (1% bovine serum albumin, BSA, in PBS) for 2 h at 37°C followed by washing each of the wells four times with 300 µl washing buffer per well (0.1% Tween-20 in PBS). The last cited washing procedure was repeated after each single incubation step. Next, wells were incubated with the antibody containing samples appropriately diluted in dilution buffer (1% BSA in washing buffer) for 2 h at room temperature. Subsequently, incubation with horseradish peroxidase (HRP)-labeled rabbit anti-chicken IgY (Sigma) as detection antibody was performed for 2 h at room temperature. After incubation, substrate solution consisting of ortho-phenyldiamine (Dako, Glostrup, Denmark) in color-developing buffer (phosphate-citrate buffer with sodium perborate; Sigma) was added, and plates incubated in the dark for 30 min at room temperature. After color development, 100 µl of 1 M H<sub>2</sub>SO<sub>4</sub> per well was added to stop the



reaction. Optical density was determined at 492 nm using an automated ELISA reader (Lab Systems, Oy, Helsinki, Finland).

### Western blot analysis

To demonstrate specificity of pAb 617A and 617C, samples of recombinant pro-KLK3–15 were subjected to 12% SDS-PAGE, and separated proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (PALL, Dreieich, Germany) using a semi-dry transfer device. Subsequently, membranes were blocked with 5% skim milk powder in PBS-0.1% Tween-20 buffer (pH 7.4) for 1 h at room temperature. Then, blots were incubated overnight at 4°C with the respective antibodies, followed by three washes, 10 min each, in PBS-0.1% Tween-20 buffer at room temperature. Binding of the antibodies to the target protein was visualized by incubation of the membrane with HRP-conjugated goat anti-chicken IgY (Sigma) diluted 1:10 000 in PBS-0.1% Tween-20 buffer containing 5% skim milk powder, followed by chemiluminescence reaction using ECL (Amersham Biosciences, Little Chalfont, UK). For determination of the relative molecular mass of the various KLK proteins, the prestained Protein IV-Marker set (PepLab, Erlangen, Germany) was employed.

### Patients and tissues

In the present study, tissue samples of patients with primary adenocarcinoma of the prostate who underwent radical prostatectomy at the Department of Urology of the Dresden University Medical Center were used. Formalin-fixed, paraffin-embedded prostate tissue specimens as well as normal adult tissues were obtained from archival material of the Center's Institute of Pathology. The study adhered to national regulations on ethical issues and was approved by the local Ethical Committee.

Conventional sections from two prostate cancer samples were selected for titration of the antibodies and for comparison of staining results between tumor and corresponding tumor-free areas within the same specimen.

Matched pairs of malignant and non-malignant prostate carcinoma specimens were used for the generation of a prostate cancer tissue microarray. Patients' age at diagnosis ranged from 50 to 76 (median 64) years. In some cases, the tissue specimens were not adequately represented on the stained tissue microarray, were damaged during immunohistochemical procedures, or the cores were floated off from the slides for unknown reasons. Moreover, only cases for which the two tumor tissue cores and the tumor-free tissue core were evaluable were included in the study. Therefore, 44 prostate cancer patients were finally included in the statistical evaluation with regard to its association with clinical parameters. The histopathological examination of corresponding autologous specimens (Tu and Tf samples), which were simultaneously embedded in paraffin, was performed according to the Union Internationale Contre le Cancer (UICC) classification system. Tumors were graded according to the Gleason score and the World Health Organization grading system as modified by the German Prostate Cancer Study Group (Hulpap, 1998). Patient and tumor characteristics are summarized in Table 2.

### Tissue microarrays

The technique of tissue microarray production has been described and validated previously by others (Skacel et al., 2002). In brief, morphologically most representative regions of tumor and corresponding tumor-free areas of the same specimens were marked on the original hematoxylin/eosin-stained tissue section. Using these

sections for orientation, two (tumor area) or one (tumor-free area) tissue core punches of 0.6 mm in diameter were taken out from the selected area of each individual paraffin-embedded prostate cancer tissue (donor block), and were precisely mounted into a recipient paraffin block using a manual tissue microarray device (Beecham Instruments, Silver Springs, MD, USA). Sections of 4–5  $\mu$ m thickness of the resulting tissue microarray blocks were transferred to adhesive coated glass slides using the paraffin sectioning aid system (adhesive coated slides PSA-CS4x, adhesive tape, UV lamp; Instrumedics Inc., Hackensack, NJ, USA) to support the cohesion of 0.6 mm array elements. For the construction of the prostate cancer tissue microarray block, tumor specimens from 84 prostate cancer patients were available. Similarly, tissue microarrays containing tissue cores punches of various healthy adult human tissues (four cores for each type of tissue) were constructed.

### Immunohistochemistry

Sections of tissue microarrays as well as full-face sections were stained using pAbs 617A and 617C. The tissue sections were dewaxed, rehydrated, and treated for antigen retrieval by pressure cooking (15 min, 120°C, 0.1 M citrate buffer, pH 6.0). After several washes with Tris-buffered saline (TBS, pH 7.6), 0.3% H<sub>2</sub>O<sub>2</sub> in TBS was applied for 10 min at room temperature to block endogenous peroxidase activity. Normal goat serum diluted 1:100 in TBS was applied for 45 min at room temperature to block nonspecific antibody binding. Subsequently, primary antibodies (617A or 617C) were allowed to react overnight at 4°C followed by incubation with biotinylated goat anti-chicken IgY (Vector Laboratories, Burlingame, CA, USA) for 50 min at room temperature. After several washes, the Vectastain Elite ABC-reagent (Vector Laboratories) was applied for 50 min at room temperature and the washing steps were repeated. The peroxidase reaction was developed with 3,3'-diaminobenzidine (DAB, Dako, Hamburg, Germany) for 10 min at room temperature in the dark. Finally, counterstaining of sections was performed with hematoxylin. As a negative control, the primary antibody was omitted and replaced by PBS or by irrelevant antibodies.

In some cases (normal colon and prostate tissues, as indicated), a rabbit anti-chicken IgY was applied as the secondary antibody, and specific antibody binding was detected using the EnVision+ peroxidase polymer system along with DAB as the chromogen (Dako).

### Quantification of KLK4 immunostaining on tissue microarrays

For evaluation of KLK4 immunostaining intensity and location, a semiquantitative score based on staining intensity and percentage of positive cells was created. Staining intensity was classified on a scale of 0 to 3 (0 – no staining; 1 – weak staining; 2 – moderate staining; 3 – strong staining). The percentage of positively stained cells was scored by cell counts after examination of tissue cores on a scale of 1 to 3: 1 – staining of <10% of cells; 2 – 11–50%, and 3 >50%. Based on these scores, a final immunoreactivity score was created by multiplication of the intensity score values with the positivity score values thus obtaining a maximum score of 9. As from each tumor area two cores were evaluated, the mean score value of the two readings was used for all statistical calculations.

### Statistical analyses

The correlation between score values (Tu- and Tf-scores obtained with either pAb 617A or 617C) was analyzed using the Spearman rank correlation ( $r_s$ ). The relation of KLK4 protein expression values

with clinicopathological parameters was determined using non-parametric Mann-Whitney or Kruskal-Wallis tests. The statistical analyses were two-sided,  $p$ -values  $<0.05$  were considered to be statistically significant. Calculations were performed using the StatView 5.0 statistical package (SAS Institute, Cary, NC, USA).

## Acknowledgments

This study was supported in part by the Kommission Klinische Forschung der TU München. We thank Jörg Hofmann and Sabine Creutzburg for their excellent technical assistance, and Julia Dorn for helpful discussions.

## References

- Beaufort, N., Debela, M., Creutzburg, S., Kellermann, J., Bode, W., Schmitt, M., Pidard, D., and Magdolen, V. (2006). Interplay of human tissue kallikrein 4 (hK4) with the plasminogen activation system: hK4 regulates the structure and functions of the urokinase-type plasminogen activator receptor (uPAR). *Biol. Chem.* 387, 217–222.
- Borgoño, C.A. and Diamandis, E.P. (2004). The emerging roles of human tissue kallikreins in cancer. *Nat. Rev. Cancer* 4, 876–890.
- Clements, J.A., Hooper, J.D., Odorico, D.M., and Dong, Y. (2004). The tissue kallikrein gene cluster. In: *Handbook of Proteolytic Enzymes*, A.J. Barrett, N.D. Rawlings and J.F. Woessner, eds. (London, UK: Elsevier Academic Press), pp. 1569–1577.
- Day, C.H., Fanger, G.R., Retter, M.W., Hylander, B.L., Penetrante, R.B., Houghton, R.L., Zhang, X., McNeill, P.D., Filho, A.M., Nolasco, M., et al. (2002). Characterization of KLK4 expression and detection of KLK4-specific antibody in prostate cancer patient sera. *Oncogene* 21, 7114–7120.
- Debela, M., Magdolen, V., Grimminger, V., Sommerhoff, C., Messerschmidt, A., Huber, R., Friedrich, R., Bode, W., and Goettig, P. (2006a). Crystal structures of human tissue kallikrein 4: activity modulation by a specific zinc binding site. *J. Mol. Biol.* 362, 1094–1107.
- Debela, M., Magdolen, V., Schechter, N., Valachova, M., Lottspeich, F., Craik, C.S., Choe, Y., Bode, W., and Goettig, P. (2006b). Specificity profiling of seven human tissue kallikreins reveals individual subsite preferences. *J. Biol. Chem.* 281, 25678–25688.
- Debela, M., Beaufort, N., Magdolen, V., Schechter, N.M., Craik, C.S., Schmitt, M., Bode, W., and Goettig, P. (2008). Structures and specificity of the human kallikrein-related peptidases KLK 4, 5, 6, and 7. *Biol. Chem.* 389, 623–632.
- DeLano, W.L. (2002). The PyMOL Molecular Graphics System on World Wide Web <http://www.pymol.org>.
- Dong, Y., Kaushal, A., Bui, L., Chu, S., Fuller, P.J., Nicklin, J., Samaratunga, H., and Clements, J.A. (2001). Human kallikrein 4 (KLK4) is highly expressed in serous ovarian carcinomas. *Clin. Cancer Res.* 7, 2363–2371.
- Grebenschikov, N., Geurts-Moespot, A., De Witte, H., Heuvel, J., Leake, R., Sweep, F., and Benraad, T. (1997). A sensitive and robust assay for urokinase and tissue-type plasminogen activators (uPA and tPA) and their inhibitor type I (PAI-1) in breast tumor cytosols. *Int. J. Biol. Markers* 12, 6–14.
- Harvey, T.J., Dong, Y., Bui, L., Jarrott, R., Walsh, T., and Clements, J.A. (2003). Production and characterization of antipeptide kallikrein 4 antibodies. Use of computer modeling to design peptides specific to kallikrein 4. *Methods Mol. Med.* 81, 241–254.
- Helpap, B. (1998). Prognosefaktoren des Prostatakarzinoms. *Pathologie* 19, 42–52.
- Klokk, T.I., Kilander, A., Xi, Z., Waehre, H., Risberg, B., Danielsen, H.E., and Saatcioglu, F. (2007). Kallikrein 4 is a proliferative factor that is overexpressed in prostate cancer. *Cancer Res.* 67, 5221–5230.
- Korbakis, D., Gregorakis, A.K., and Scorilas, A. (2009). Quantitative analysis of human kallikrein 5 (KLK5) expression in prostate needle biopsies: an independent cancer biomarker. *Clin. Chem.* 55, 904–913.
- Lai, J., Myers, S.A., Lawrence, M.G., Odorico, D.M., and Clements, J.A. (2009). Direct progesterone receptor and indirect androgen receptor interactions with the kallikrein-related peptidase 4 gene promoter in breast and prostate cancer. *Mol. Cancer Res.* 7, 129–141.
- Lundwall, A. and Brattsand, M. (2008). Kallikrein-related peptidases. *Cell. Mol. Life Sci.* 65, 2019–2038.
- Mangé, A., Desmetz, C., Berthes, M.L., Maudelonde, T., and Solasol, J. (2008). Specific increase of human kallikrein 4 mRNA and protein levels in breast cancer stromal cells. *Biochem. Biophys. Res. Commun.* 375, 107–112.
- McKiernan, E., O'Brien, K., Grebentchikov, N., Geurts-Moespot, A., Sieuwerts, A.M., Martens, J.W., Magdolen, V., Evoy, D., McDermott, E., Crown, J., et al. (2008). Protein kinase C $\delta$  expression in breast cancer as measured by real-time PCR, Western blotting and ELISA. *Br. J. Cancer* 99, 1644–1650.
- Mize, G.J., Wang, W., and Takayama, T.K. (2008). Prostate-specific kallikreins-2 and -4 enhance the proliferation of DU-145 prostate cancer cells through protease-activated receptors-1 and -2. *Mol. Cancer Res.* 6, 1043–1051.
- Nelson, P.S., Gan, L., Ferguson, C., Moss, P., Gelinas, R., Hood, L., and Wang, K. (1999). Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. *Proc. Natl. Acad. Sci. USA* 96, 3114–3119.
- Obiezu, C.V., Scorilas, A., Katsaros, D., Massobrio, M., Yousef, G.M., Fracchioli, S., Rigault de la Longrais, I.A., Arisio, R., and Diamandis, E.P. (2001). Higher human kallikrein gene 4 (KLK4) expression indicates poor prognosis of ovarian cancer patients. *Clin. Cancer Res.* 7, 2380–2386.
- Obiezu, C.V., Soosaipillai, A., Jung, K., Stephan, C., Scorilas, A., Howarth, D.H., and Diamandis, E.P. (2002). Detection of human kallikrein 4 in healthy and cancerous prostatic tissues by immunofluorescence and immunohistochemistry. *Clin. Chem.* 48, 1232–1240.
- Obiezu, C.V., Shan, S.J., Soosaipillai, A., Luo, L.Y., Grass, L., Sotiropoulou, G., Petraki, C.D., Papanastasiou, P.A., Levesque, M.A., and Diamandis, E.P. (2005). Human kallikrein 4: quantitative study in tissues and evidence for its secretion into biological fluids. *Clin. Chem.* 51, 1432–1442.
- Pampalakis, G. and Sotiropoulou, G. (2007). Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer. *Biochim. Biophys. Acta* 1776, 22–31.
- Ramsay, A.J., Dong, Y., Hunt, M.L., Linn, M., Samaratunga, H., Clements, J.A., and Hooper, J.D. (2008). Kallikrein-related peptidase 4 (KLK4) initiates intracellular signaling via protease-activated receptors (PARs). KLK4 and PAR-2 are co-expressed during prostate cancer progression. *J. Biol. Chem.* 283, 12293–12304.
- Shaw, J.L.V. and Diamandis, E.P. (2007). Distribution of 15 human kallikreins in tissues and biological fluids. *Clin. Chem.* 53, 1423–1432.

- Simmer, J.P. and Bartlett, J.D. (2004). Kallikrein 4 is a secreted protein. *Cancer Res.* *64*, 8481–8483.
- Simmer, J.P., Fukae, M., Tanabe, T., Yamakoshi, Y., Uchida, T., Xue, J., Margolis, H.C., Shimizu, M., DeHart, B.C., Hu, C.C., et al. (1998). Purification, characterization, and cloning of enamel matrix serine proteinase 1. *J. Dent. Res.* *77*, 377–386.
- Simmer, J.P., Hu, Y., Lertlam, R., Yamakoshi, Y., and Hu, J.C. (2009). Hypomaturational enamel defects in Klk4 knockout/LacZ knockin mice. *J. Biol. Chem.* *284*, 19110–19121.
- Skacel, M., Skilton, B., Pettay, J.D., and Tubbs, R.R. (2002). Tissue microarrays: a powerful tool for high-throughput analysis of clinical specimens: a review of the method with validation data. *Appl. Immunohistochem. Mol. Morphol.* *10*, 1–6.
- Takayama, T.K., McMullen, B.A., Nelson, P.S., Matsumura, M., and Fujikawa, K. (2001). Characterization of hK4 (prostase), a prostate-specific serine protease: activation of the precursor of prostate-specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. *Biochemistry* *40*, 15341–15348.
- Wang, W., Mize, G.J., Zhang, X., and Takayama, T.K. (2010). Kallikrein-related peptidase-4 initiates tumor-stroma interactions in prostate cancer through protease-activated receptor-1. *Int. J. Cancer* *126*, 599–610.
- Xi, Z., Klock, T.I., Korkmaz, K., Kurys, P., Elbi, C., Risberg, B., Danielsen, H., Loda, M., and Saatcioglu, F. (2004). Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer. *Cancer Res.* *64*, 2365–2370.
- Yousef, G.M., Scorilas, A., Chang, A., Rendl, L., Diamandis, M., Jung, K., and Diamandis, E.L. (2002). Down-regulation of the human kallikrein gene 5 (KLK5) in prostate cancer tissues. *Prostate* *51*, 126–132.

Received October 15, 2009; accepted November 25, 2009