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Human Kallikrein-Related Peptidase 12: Antibody Generation and Immunohistochemical Localization in Prostatic Tissues

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BACKGROUND. Human tissue kallikrein-related peptidases (genes, *KLK*s; proteins, KLKs) are a subgroup of serine proteases present in a variety of tissues and biological fluids. A number of human tissue KLKs are established or candidate serologic biomarkers for prostate cancer. Human kallikrein-related peptidase 12 (*KLK12*, KLK12), recently identified in our laboratory, is a novel member of the *KLK* gene family. Here, we report generation of antibodies against the full-length recombinant KLK12 (classical form) and the immunohistological localization of this KLK in normal and malignant prostate tissues.

METHODS. The mature form of *KLK12* cDNA was amplified using PCR and cloned into a plasmid vector for protein production in *E. coli*. Following identification by mass spectroscopy, recombinant KLK12 was purified and used as immunogen in rabbits. Anti- KLK12 antibody was used for immunostaining of paraffin-embedded sections of human prostate tissue. Immunoexpression of KLK12 in benign and malignant prostate tissue was evaluated using a prostate cancer tissue array.

RESULTS. Anti-KLK12 antibody showed a predominantly apical and membranous staining of the luminal cells of the normal prostate in contrast with the predominantly diffuse cytoplasmic staining observed in both prostatic intra-epithelial neoplasia and adenocarcinomas. This was occasionally associated with an intense granular supranuclear staining. More than 95% of the prostate cancers on the tissue microarray were KLK12 positive.

CONCLUSION. Higher levels of KLK12 in malignant prostatic glands, and the shift in subcellular localization of KLK12 in prostate cancer observed in this study point to the potential role of this kallikrein during prostate carcinogenesis. *Prostate 67: 1465–1474, 2007.*

KEY WORDS: **cancer biomarkers; KLK12; prostate cancer; protein expression; serine proteases**

INTRODUCTION

The human tissue kallikrein-related peptidase gene family is a group of 15 closely related genes, located on chromosome 19 (q13.4) in tandem, which encode for secreted serine proteases with various physiological functions [1,2]. Pancreatic/renal kallikrein (KLK1), human glandular kallikrein 2 (KLK2) and prostate-specific antigen (KLK3, PSA) are amongst the first kallikrein-related peptidases identified. In addition to these ''classical'' *KLK*s, in recent years, 12 additional *KLK* genes have been identified in the same locus [3,4].

Abbreviations: PSA, prostate-specific antigen; RT, reverse transcription; MS, mass spectrometry; IPTG, isopropyl β-D-thiogalactoside; Ab, antibody; AP, alkaline phosphatase; BCA, bicinchoninic acid; bp, base pair; ELISA, enzyme-linked immunosorbent assay; Ni-NTA, nickel-nitrilotriacetic acid; Kb, kilobase; TMA, tissue micro-array; PIN, prostatic intra-epithelial neoplasia.

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Human tissue *KLK* gene family members have attracted significant attention, mainly due to their association with various malignancies and their potential applicability as novel biomarkers [5]. KLK2, KLK3, and KLK11 are established or candidate serologic biomarkers in early diagnosis and monitoring of prostate cancer [6–11]. In addition to prostate cancer, many of the newly identified *KLKs* are differentially expressed in ovarian [12–18], breast [19–26], testicular [27–31], pancreatic [32], cervical [33,34], and colorectal [35] cancer. In recent years, the expression of some of the newly identified KLKs in normal human tissue has been examined by immunohistochemistry [36–38]. Using immunohistochemistry, the differential expression of KLK8 during various stages of ovarian cancer [39], up-regulation of KLK4 in prostate cancer [40], KLK6 in renal cell carcinoma [41] and KLK14 in breast cancer [26] has been documented. No rich natural source for many of the newly identified KLKs is currently available; consequently the production of recombinant tissue KLKs for antibody generation and functional studies in recent years is highly pursued.

Human kallikrein-related peptidase 12 (*KLK12*) was identified in our laboratory by using the positional candidate gene cloning approach. This gene was originally named as *KLK-L5* (kallikrein-like gene 5). With the construction of the detailed map of the human kallikrein gene locus [1,42] and adoption of a new nomenclature for human kallikreins [43], the name *KLK-L5* was replaced with *KLK12* gene symbol (GenBank Accession number AF135025). A revised nomenclature for human and animal kallikreins is now in place [44].

Similar to other kallikreins, KLK12 is a proteolytic enzyme with serine protease activity [45]. KLK12 mRNA is highly expressed in a variety of tissues including the prostate, and is up-regulated by steroid hormones in human prostate and breast cancer cell lines [46]. *KLK12* has four alternatively spliced forms [47]. Three of these splice variants occur in the protein coding region resulting in three different protein products. All splice forms of KLK12 are predicted to produce secreted proteins, however the ''classical'' form of KLK12 (GenBank Accession number NM_145894) is the only protein product that represents a typical kallikrein-like enzyme with expected serine protease activity. The classical form of KLK12 is synthesized as a preproenzyme which contains an N-terminal signal peptide of 17 amino acids and a cleavage site for activation between the 21 and 22 residues. The classical KLK12 in its mature form consists of 227 amino acids with a calculated molecular weight of 24.5 kDa.

In this study we report the production of the first antibodies against the full-length mature form of classical KLK12 and utilization of these antibodies in the immunohistochemical localization of this KLK in normal prostate tissue. Since in addition to KLK3 a number of recently identified KLKs (KLK2, KLK4, KLK11, and KLK15) appear to be promising prostatic biomarkers, anti-KLK12 antibodies generated here were applied to a prostate cancer tissue array, and the immunoexpression of KLK12 in benign and malignant prostate tissue was evaluated.

MATERIALS AND METHODS

Production and Purification of KLK12 in *E. Coli*

PCR amplification of the mature form of classical KLK12: Total human lung tissue RNA was reverse-transcribed to cDNA using the Superscript™ preamplification system (Gibco BRL, Rockville, MD) according to the manufacturer's recommendations. Two rounds of polymerase chain reaction (PCR) were conducted using P*fu* Turbo DNA polymerase (Stratagene Cloning Systems, La Jolla, CA) and a thermal cycler (Eppendorf gradient Mastercycler). Initially, the prepro-form of *KLK12* from start codon to stop codon was amplified (forward primer: 5′-CACCATGGGG-CTCAGCATCTT-3′; reverse primer: 5′- TCAGTTGT-TCCTCATG ATCATCCG-3′) using 39 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 1 min and extension at 72°C for 50 sec, and a final extension at 72°C for 10 min. PCR products were separated on a 1% agarose gel and the PCR band corresponding to the classical form of KLK12 (GenBank Accession number NM_145894) was excised and purified using Qiagen gel extraction kit (Qiagen, Inc., Valencia, CA) according to the vendor's instructions. The purified DNA product was diluted and used as template in a second round of PCR amplification to generate mature form of classical *KLK12*. The mature form of *KLK12* cDNA was generated using 30 PCR cycles and similar amplification conditions that were used for the generation of the prepro-form of KLK12, with the exception that the forward primer: 5′-CACCATTTTCAATG GCACT-GAGTG TGGG-3′ was used. The final PCR product was gel-purified and used for cloning.

pET100-TOPO cloning of KLK12: The PCR product was ligated into pET-100/D TOPO plasmid vector containing an N-terminal polyhistidine $(His)_6$ tag and was used to transform TOP10 *E. coli* cells according to vendor's instructions (Invitrogen, catalogue # K100-01). Positive clones were selected on ampicillin agar plates and plasmid DNA was isolated and used for DNA sequencing. The sequences (both directions) were subjected to homology search using the BLASTN algorithm.

Production of rKLK12: *E. coli* strain BL21(DE3) was transformed with pET100-*KLK12* and protein produc-

tion was induced using 0.5 mM IPTG. Samples were collected prior to IPTG addition and every hour thereafter for 4 hr. BL21 *E. coli* culture was then harvested by centrifugation and the cell pellet was kept frozen at −20°C until use. Whole cell lysates of cultured BL21 *E. coli* cells were subjected to SDS-PAGE and Western blotting. The identity of rKLK12 was confirmed by tandem mass spectrometry.

Purification of rKLK12 and mass spectrometry: Procedures for rKLK12 purification are essentially similar to procedures detailed for KLK9 purification in our previous study [48]. rKLK12 was initially isolated in inclusion bodies. Purified inclusion bodies were further subjected to metal affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Inc.) under denaturing conditions according to manufacturer's instructions. KLK12 was further purified using reversed-phase high performance liquid chromatography (HPLC) and the denaturing agent (8 M urea) was removed. The identity of the purified protein was confirmed by mass spectrometry. Protein samples were resolved by SDS-PAGE, the corresponding band was excised from the gel, digested by trypsin and analyzed by nanoelectrospray tandem mass spectroscopy (LTQ linear ion trap; Thermo Electron Corporation, Waltham, MA) as detailed elsewhere [49].

Production of Polyclonal Antibodies Against KLK12 and Cross-Reactivity Analysis

Purified rKLK12 was used as an immunogen for production of polyclonal antibodies in female New Zealand white rabbits. One hundred microgram of purified KLK12 was diluted 1:1 in complete Freund's adjuvant for the first subcutaneous injection and in incomplete Freund's adjuvant for subsequent injections. Test bleeds were drawn and the serum was tested for antibody titer and the presence of anti-KLK12 antibodies using an antibody capture assay: 96 well micro-titer plates, coated with 50 ng of rKLK12 per well were incubated with various dilutions of KLK12 rabbit antiserum. Following wash steps, bound KLK12 rabbit antiserum in each well was probed with AP-conjugated goat anti-rabbit IgG as the secondary antibody. The fluorescence signal was measured with a time-resolved fluorometer, as detailed elsewhere [49]. Sera obtained from pre-immune rabbits were used in parallel as negative controls. Specificity and potential cross-reactivity of KLK12 rabbit serum against other members of human tissue KLK family was assessed by Western blotting.

Electrophoresis and Western Blot Analysis

Electrophoresis and electroblotting were performed essentially as detailed elsewhere [48]. In brief, protein samples were subjected to SDS-PAGE using gradient 4–12% bis-tris polyacrylamide mini-gels. Gels were stained using Coomassie G-250 solution or processed further for Western blotting. Electroblotting of proteins onto nitrocellulose membranes was conducted at a constant voltage of 30 V for 1 hr. Membranes were blocked overnight in blocking solution (PBS + 5 % non-fat dry milk, w/v), washed in TBST (20 mM Tris–HCl, 140 mM NaCl, pH 7.5 plus 0.1% Tween-20, v/v), and then incubated for 2 hr with KLK12 rabbit antiserum diluted 6,000-fold in dilution buffer (TBST + 5% non-fat dry milk, w/v). The blots were then washed with TBST, and incubated for 1 hr with AP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:15,000 in dilution buffer. Nitrocellulose membranes were then washed in TBST, and AP activity was detected on an X-ray film. Pre-immune serum was used in parallel as negative control. For cross-reactivity analysis, 50 ng of purified KLK1-KLK15 were loaded per lane, electrophoresed and probed with KLK12 rabbit antiserum as outlined above.

Immunohistochemical Staining of Prostate Tissue Sections

Formalin-fixed paraffin embedded prostate tissue sections (4 μm thick) were dewaxed with xylene and hydrated through graded ethanol solutions. Immunohistochemistry was performed using the avidin–biotin peroxidase complex technique (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) according to vendor's instructions. In brief, the slides were placed in 3% hydrogen peroxide and then blocked in 1% normal goat serum. The sections were then incubated with rabbit-anti-KLK12 antiserum (1:500 dilution) for 1 hr. After washing, the slides were incubated with biotinylated goat anti-rabbit IgG for 30 min, washed and further incubated with avidin-peroxidase reagent for 30 min. Peroxidase in tissue sections was visualized as brown color using a DAB substrate solution. The sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted in Permount. Prostate tissue sections treated with no primary antibody (PBS alone) or pre-immune rabbit serum instead of the primary antibody were used as negative controls. Polyclonal antibodies against recombinant human tissue kallikrein 9 [48] and hepsin (our unpublished data) generated in house using the same protein expression system and antibody production, as well as monoclonal antibodies against PSA were also used as controls.

Construction of the Tissue Array

A total of 82 radical prostatectomy specimens were selected for the tissue microarray (TMA). Transurethral

resections of the prostate of 10 men with benign prostatic hyperplasia, negative for malignancy, were used as normal controls. The tissue microarray was constructed as described by Kononen et al. [50]. Six cylindrical tissue cores (0.6 mm in diameter) were taken from each of the control samples. Of the 80 radical prostatectomy specimens, a total of 255 tissue cores were collected in the TMA. Of each patient 2–10 core biopsies were inserted in the TMA depending on the number of different Gleason grade areas (range 2–4). The distribution of pathological stage was 83% pT2, 15% pT3 and 2% pT4, while 4%, 6%, 51%, 33%, and 6% of the cancers were Gleason score 4, 5, 6, 7, and 8 respectively.

Patients and Tissue Samples (Clinical and Pathological Data)

Conventional sections of three paraffin embedded radical prostatectomy specimens were used for titration of the antibody and for comparison of staining results within the same specimen on benign peripheral and transition zone prostatic glands, high grade PIN and prostate cancer. The prostatectomy slides were selected for simultaneous presence of adenocarcinoma and PIN within one slide.

Evaluation of Immunohistochemical Staining of KLKI2 Expression inTMA

For the various Gleason grades of adenocarcinoma, the immunostaining results were scored per tissue core by one investigator as negative, positive and the presence of granular staining was separately recorded. For benign glands, high grade PIN and the various Gleason grades of adenocarcinoma present in individual cores, the subcellular localization of the immunostaining was also recorded (membranous, diffuse non-granular cytoplasmic and granular staining). Staining was scored as membranous if any of the glandular areas (benign, PIN, carcinoma) showed a typical membranes pattern irrespective of the presence of cytoplasmic staining. Significant differences in staining pattern between the four groups were calculated using the Pearson Chi-square test with $P < 0.05$ indicating significance.

RESULTS

Production and Purification of rKLKI2

Two rounds of PCR, as detailed earlier, resulted in amplification of the classical, mature form of *KLK12* as a single band. DNA sequencing of the *KLK12*/pET100 construct confirmed the presence of the insert with no mutation, in-frame with the N-terminal $(His)_6$ tag. In Figure 1, production and purification of rKLK12 from transformed bacterial cells, as detected by SDS-PAGE (Coomassie stain), are shown. KLK12 was not produced in BL21 cells prior to the addition of IPTG (lane 1A) or in BL21 cells transformed with *KLK12* construct at the end of the experiment in the absence of IPTG (lane 2A). However, KLK12 was visible as a strong band in cell extracts from BL21 cells that were transformed with pET100-KLK12 construct 1 hr post-IPTG stimulation (lane 3A). At 3 (lane 4A) and 4 hr (lane 5A) post-IPTG stimulation, rKLK12 was detected as the most prominent protein in the whole cell bacterial extracts.

A high degree of rKLK12 enrichment was achieved by isolation of inclusion bodies (lane 7A). KLK12 fusion protein dissolved in 8 M urea was eluted from Ni-NTA beads by lowering the pH to 5.0 (lanes 8A–9A). The

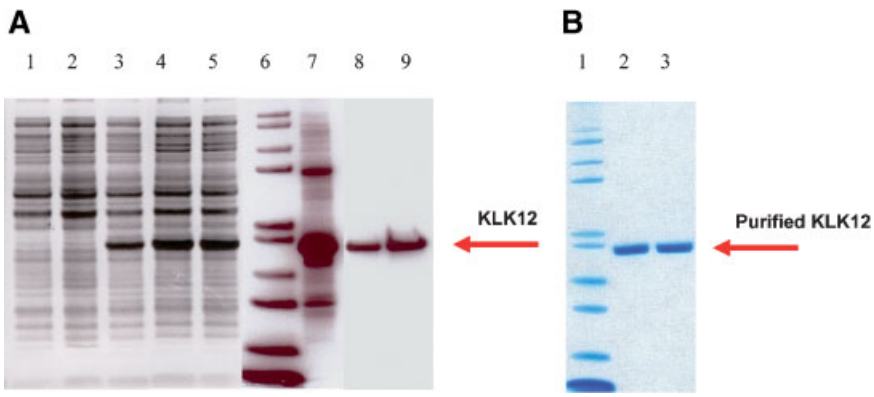

Fig. 1. Detection of KLKI2 fusion protein in bacterial cell pellets prior and after purification, as detected by SDS-PAGE (Coomassie stain). **Lane IA**, BL2I *E. coli* cell pellet prior to IPTG induction; **Lane 2A**, BL2I cell pellet (containing KLKI2 insert) in the absence of IPTG, at 3 hr; **Lane 3A**, BL2I cell pellet (containing KLKI2 insert) 1 hr post-IPTG stimulation; **Lane 4A**, BL2I cell pellet (containing KLKI2 insert) 3 hr post IPTG stimulation; **Lane 5A**, BL2I cell pellet (containing KLKI2 insert) 4 hr post IPTG stimulation; **Lane 6A**, "Mark I2" molecular mass marker; **Lane 7A**, Purified inclusion bodies; **Lane 8A–9A**, Purified KLKI2 after Ni-NTA metal affinity chromatography; **Lane IB,** "Mark I2" molecular mass marker; **Lanes 2B–3B**, Purified KLKI2 fractions following reversed phase HPLC.

eluted KLK12 was further purified using reversed-phase HPLC, which also facilitated removal of urea. The above procedures resulted in highly purified KLK12 (a single band) as seen in Figure 1 (lanes 2B–3B). A final yield of 6 mg of purified KLK12 per liter of BL21 culture could be routinely obtained. The identity of the purified recombinant fusion protein as KLK12 was confirmed by mass spectrometry. Recombinant KLK12 was enzymatically inactive, but suitable for polyclonal antiserum production.

Characterization of Anti-KLKI2 Rabbit Anti-Serum

Using the KLK12 rabbit antiserum, rKLK12 was readily detected by Western blotting as seen in Figure 2A. Whole cell extracts from BL21 *E. coli* cells without insert (lane 9) and BSA (lane 7) used as negative controls did not result in detection of any protein bands. KLK12 rabbit antiserum at a dilution of 1:6,000 could readily detect un-purified KLK12 in the whole cell lysate of transformed BL21 cells (lane 8), as well as purified KLK12 (lane 6). Parallel control Western blot (lanes 1–5) performed simultaneously using pre-immune rabbit antiserum (replacing the primary antibody) resulted in detection of no protein bands. KLK12 rabbit antiserum specificity was further tested by evaluating its possible cross-reactivity with all other 14 recombinant kallikrein proteins, available in-house. Western blot analysis (Fig. 2B) indicated that KLK12 was the only KLK detected when probed with KLK12 rabbit serum. Antibody titer and specificity of the KLK12 rabbit polyclonal was also tested using ELISA with coated rKLK12. KLK12 rabbit serum, at dilutions as high as 1,000,000-fold in dilution buffer (containing 6% BSA), produced a fluorescence signal which was at least 2-fold higher than the pre-immune rabbit control serum (data not shown).

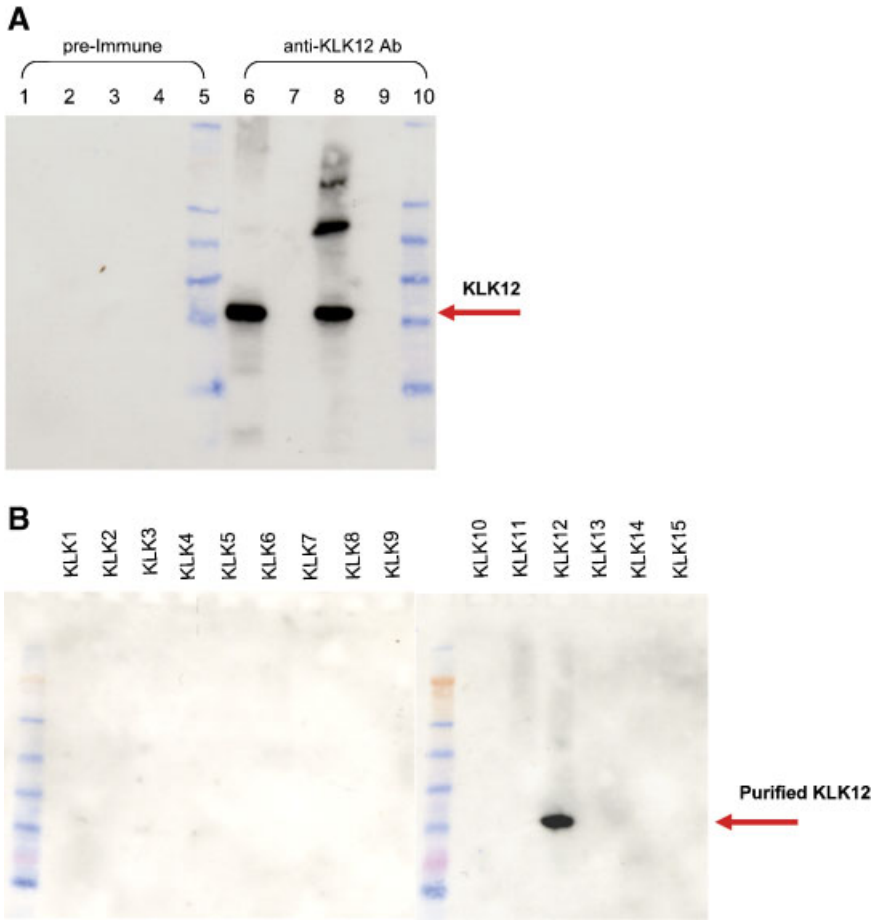

Fig. 2. **A**: KLKI2 fusion protein as detected by Western blotting using KLKI2 rabbit serum. **Lanes I – 4** are the same as lanes 6 -9 but probed with pre-immune rabbit serum; **Lanes 5 and I0,** "See blue plus2" molecular mass marker; **Lane 6,** purified KLKI2 fusion protein; **Lane 7,** BSA; **Lane 8,** KLKI2 in whole cell lysate of BL2I cells transformed with KLKI2 construct; **Lane 9,** Whole cell lysate of BL2I cells with no insert. **B**: Western blot to test for cross-reactivity of KLKI2 rabbit antiserum with all other members of *KLK* gene family. Fifty nanogram of each protein was loaded into wells of polyacrylamide gels, electrophoresed, and probed with KLKI2 rabbit antiserum. No cross reactivity with any of the tissue KLK family members was observed.

Immunostaining of Prostatic Tissues

Immunostaining of paraffin embedded sections of human prostate tissue with anti-KLK12 antibody resulted in a predominantly apical and typical membranous staining of the luminal cells of the normal prostate (Fig. 3B). Staining of the basal cells surrounding the prostatic glands was negative. Prostate fibromuscular stromal cells did not show a specific staining pattern, but peripheral nerves showed a faint cytoplasmic staining pattern. In contrast to anti-KLK12 antibody, when benign prostatic gland was stained with anti-KLK3 (PSA) antibody, the staining of the luminal cells was diffuse cytoplasmic, and generally showed no accentuation of the apical membranes (Fig. 3D). No staining was seen when sections were treated with the pre-immune rabbit serum as the primary antibody (Fig. 3A). Strikingly, both prostatic intra-epithelial neoplasia (PIN) and adenocarcinomas showed a moderate to intense diffuse cytoplasmic expression of KLK12 (Fig. 3C). Occasionally, an intense granular supranuclear staining in high grade PIN (Fig. 3E) and

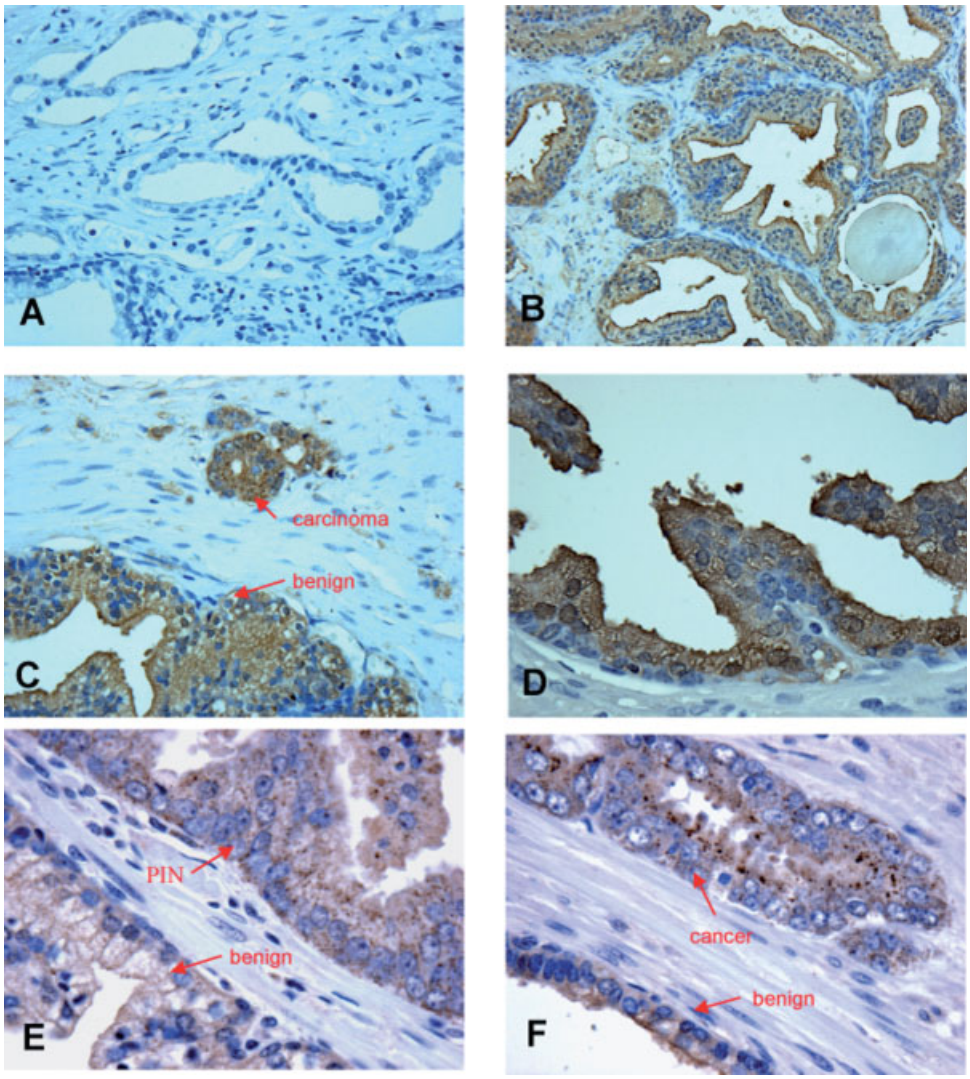

Fig. 3. Immunohistochemical localization of KLK12 in benign and malignant prostate tissue. **A**: Negative control: prostate tissue stained with pre-immune rabbit serum (100×). **B**: Overview showing benign glands with predominant apical staining of luminal, but not basal cells by anti-KLK12 antibody (100×). **C**: Higher magnification (200×) of benign gland and adenocarcinoma gland (upper gland). Benign glands show a membranous staining along the luminal surface and the adenocarcinoma glands have a diffuse cytoplasmic staining. **D**: Benign prostatic gland, stained with anti-hK3 (PSA) antibody. The staining of the luminal cells is diffuse cytoplasmic, showing no accentuation of the apical membranes (400×). **E**: Micrograph (400×), stained with anti-KLK12 antibody, revealing the presence of intensely stained granules in the PIN gland (upper gland), but not in the benign gland (lower middle gland). **F**: Micrograph (400×) of benign prostatic gland and carcinoma, showing the intense granular cytoplasmic staining of KLK12 in the carcinoma gland.

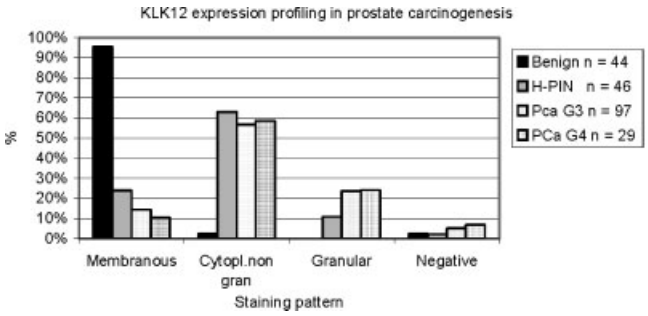

Fig. 4. The subcellular distribution of KLK12 in benign glands, PIN, Gleason grade 3 and grade 4 adenocarcinoma. A significant difference in membranous and granular staining pattern was found between benign glands and PIN and between benign glands and carcinoma ($P < 0.001$).

carcinoma glands (Fig. 3F, upper gland) was observed. This differential staining pattern in benign glands, PIN and adenocarcinoma was further confirmed by analysis of the TMA (Fig. 4). Virtually, all TMA cores with benign glands showed membranous staining, while the proportion of carcinomas, respectively PIN with membranous staining was reduced ($P < 0.001$, respectively $P = 0.01$), but the difference between PIN and carcinoma was not significant ($P = 0.1$). A significantly larger proportion of PIN as well as carcinomas showed granular staining for KLK12, when compared to benign glands ($P = 0.008$, respectively $P < 0.001$), while the difference between PIN and carcinomas was nearly significant ($P = 0.06$). Although in 10 prostate cancers heterogeneity in staining was observed, only three cases (seven TMA cores) were negative for hK12 expression (4%). Cytoplasmic granular staining was not observed in benign glands and in one of eight prostate cancers with Gleason pattern 2, while these supranuclear cytoplasmic speckles were present in 24% of the samples with Gleason pattern 3 and Gleason pattern 4. Cytoplasmic granular staining was not observed when anti-PSA, anti-KLK9, or anti-hepsin antibodies were used as controls (data not shown).

DISCUSSION

Among the 15 members of the human tissue kallikrein-related peptidase gene family, *KLK12* is the only kallikrein for which production of antibodies has not been reported. In order to obtain sufficient amounts of KLK12 for antibody generation we expressed the recombinant form of KLK12 using an *E. coli* protein expression system. Due to the presence of different KLK12 mRNA splice variants, which result in predicted proteins of varying lengths [47], we used RT-PCR using primers specifically designed to amplify and clone the mature form of ''classical'' KLK12 which represents a typical kallikrein-like enzyme with serine protease activity. The apparent molecular mass of 28 kDa observed for recombinant KLK12 fusion protein on SDS-PAGE was slightly higher than its predicted molecular mass of 24.5 kDa due to the presence of the 3 kDa N-terminal fusion tag. Following confirmation of protein identity using mass spectroscopy, highly purified KLK12, seen as a homogenous single band (Fig. 1), was used as antigen for antibody generation. KLK12 rabbit antibody did not react with any of the proteins in the whole cell lysate of control BL21 cells. The ''classical'' KLK12 has 48% amino acid sequence identity with KLK8, 46% identity with KLK10, and 38% identity with both PSA and KLK2 [46]; however, in our cross reactivity analysis, KLK12 rabbit antiserum had no cross-reactivity with any of these or other KLKs (Fig. 2B) indicating that our anti-KLK12 rabbit antibody is highly specific.

Since classical KLKs such as KLK3 and KLK2 have already proved to be amongst the best prostatic biomarkers, and due to recognition of a number of newly identified tissue kallikreins as emerging biomarkers of prostate cancer [5], anti-KLK12 antibodies generated here were used for immunostaining of paraffin-embedded sections of human normal and malignant prostate tissue. In contrast to PSA, KLK12 in normal peripheral and transition zone prostate tissue displayed a predominantly apical staining of the luminal cells, accentuated at the membrane. The apical immunostaining pattern of normal prostate tissue using anti-KLK12 antibody indicates that KLK12 displays its activity at the luminal surface, allowing interaction with the secretions within the prostatic glandular lumina.

The reason for the intense granular supranuclear staining of KLK12 observed in about 24% of prostate cancer samples having a Gleason grade 3/4, but mostly absent in well differentiated, low grade prostate cancers or normal prostatic tissue observed in this study is currently unknown. This granular staining was absent when anti-PSA, anti-KLK9, or anti-hepsin control antibodies were used (data not shown). Aberration in secretory pathways involved in the transport of KLK12 following its synthesis, or perhaps presence of a non-secreted splice variant of KLK12 may result in the cytoplasmic accumulation of this KLK in prostate cancer. Alternative splicing is prevalent within the KLK gene locus. The presence of at least 82 KLK gene transcript forms has recently been reported [47] and the number of identified transcripts is still rising. The four splice variants of KLK12 known to date are predicted to encode for secreted proteins. The polyclonal antibody generated here may detect all currently known splice variants of this kallikrein. However, the presence of yet unidentified splice variants of KLK12

lacking a signal peptide cannot be ruled out. Presence of splice variants resulting in altered cellular localization has also been reported for other serine proteases. Alternative splicing of the transmembrane serine protease hepsin results in an isoform which lacks the transmembrane domain and is thus found in the cytoplasm. When compared to the transmembrane isoform, the non-transmembrane hepsin isoform was reported to have a different expression pattern in a number of colon adenocarcinoma cell lines [51].

Immunohistochemical localization of KLK6 and KLK10 in diverse normal human tissues indicates that these KLKs are mainly expressed by glandular epithelia, the choroid plexus, peripheral nerves, and several neuroendocrine tissues [36,37]. Intense staining of KLK11 in the supranuclear cytoplasm of small intestine epithelial cells has been reported [52]. Expression of KLKs mainly by glandular epithelia, as well as presence of many KLKs tested so far in biological fluids, indicate that KLKs are secreted proteins [1,3]. However, immunohistological analysis of a recently identified isoform of KLK4 which lacks the signal peptide due to alternative splicing has revealed a nuclear localization of this isoform in prostate tissue [40]. An increasing number of reports indicate that the KLK transcriptome is altered during neoplastic progression. Various splice variants of KLK4, KLK5, KLK7, and KLK8 are overexpressed in ovarian cancer. KLK 13 splice variants are exclusively expressed in the normal testis, but are absent in the adjacent cancerous tissue [3,27]. An aberrant form of KLK12 (due to splice-site genetic polymorphism) and its association with primary gastric cancer has previously been described [45]. The presence of aberrant forms of KLK12 in prostate cancer merits further investigation.

In contrast to earlier reports indicating down-regulation of KLKs in breast, prostate and testicular tumors [reviewed in Borgono and Diamandis [3]], more recent immunohistological analyses of various KLKs indicate that contrary to PSA, many of the newly identified KLKs are up-regulated in cancer. Recent immunohistochemical analysis of KLK2 and KLK4 in prostate cancer [53], KLK14 in breast cancer [26], and KLK7 in squamous cervical cancer [33] indicate that the expression levels of these KLKs are increased in cancer. Higher levels of KLK12 in malignant prostatic glands observed in this study are interesting, since the increased levels were also detected in high grade PIN lesions. Since the latter lesions represent a putative precursor for prostate cancer this finding would suggest a possible role for KLK12 in prostate carcinogenesis. The recombinant KLK12 and antibodies generated against this KLK reported in this study may help elucidate the functional role of this peptidase in prostate carcinogenesis.

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