IMMUNOHISTOCHEMICAL LOCALIZATION AND EXPRESSION PROFILING OF HUMAN TISSUE KALLIKREIN 12 IN HUMAN PROSTATE CANCER TISSUES

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ABSTRACT

Human tissue kallikreins (genes, KLKs; proteins, hKs) are a subgroup of serine proteases present in a variety of tissues and biological fluids. A number of human tissue kallikreins are established or candidate serologic biomarkers for prostate cancer. Human tissue kallikrein 12 (KLK12, hK12), recently identified in our laboratory, is a novel member of the kallikrein gene family. KLK12 mRNA is expressed in a variety of human tissues including the prostate gland. Here, we report the generation of the first antibodies against the full-length recombinant hK12 (classical form) and immunohistological localization of this kallikrein in normal and malignant prostate tissue. Mature form of KLK12 cDNA was amplified using PCR and cloned into pET/100 TOPO plasmid vector for protein production in E. coli. Recombinant hK12 was purified and used as immunogen in rabbits. After confirmation of antibody specificity using antibody capture assay and Western blotting, anti-hK12 antibody was used for immunostaining of paraffin embedded sections of human prostate tissue.

Anti-hK12 antibody showed a predominantly apical staining of the luminal cells of the normal prostate in contrast with the diffuse cytoplasmic staining observed using anti-hK3 (PSA) polyclonal antibodies. No staining was seen when primary antibody was omitted or replaced with pre-immune serum. Strikingly, both prostatic intra-epithelial neoplasia and adenocarcinomas showed a diffuse cytoplasmic expression of hK12, occasionally associated with an intense granular supranuclear staining. Analysis of a prostate cancer tissue microarray containing about 500 cores of 83 prostatectomy specimens revealed that more than 90% of prostate cancers were hK12 positive. The observed shift in subcellular localization of hK12 in prostate cancer may point to its potential role during prostate carcinogenesis.

INTRODUCTION

- Human tissue kallikreins (KLK) are a subgroup of serine proteases with diverse physiological function present in a variety of tissues and biological fluids.
- For many years the human tissue kallikrein gene family was considered to be composed of only three genes: the pancreatic/renal kallikrein (KLK1), the human glandular kallikrein 2 (KLK2), and prostate specific antigen (KLK3).
- These three genes are now considered as the classical human tissue kallikrein. In the recent years twelve additional genes with significant homology with the previously known kallikreins have been identified by different investigators.
- All 15 human tissue kallikrein genes are tandemly located in a 300 kb region on chromosome 19q13.3-19q13.4 and are composed of five coding exons of similar or identical size.
- The most well known member of this group of enzymes KLK3, also known as Prostate-Specific Antigen (PSA), is currently used in the diagnosis and monitoring of prostate cancer.
- In 1987, Stamey et al. showed that serum concentrations of PSA increase in patients with prostate cancer, and the elevated levels correlate with tumor volume and clinical stage of disease.
- Since then numerous studies have confirmed the value of serum PSA determinations in the diagnostic and monitoring of prostate cancer patients. Recent reports indicate that KLK2 may soon find applicability as an additional prostatic and breast cancer biomarker.
- In the recent years, the value of the newly identified members of the human kallikrein gene family as potential biomarkers of cancer is being intensely investigated.
- In recent years the expression of some of the newly identified tissue kallikreins in normal human tissue has been examined by immunohistochemistry.
- In addition, using immunohistochemistry the differential expression of hK8 during various stages of ovarian cancer, up-regulation of hK4 in prostate cancer, hK6 in renal cell carcinoma, and hK14 in breast cancer has recently been documented.
- Kallikrein 12 (*KLK12*), identified in our laboratory, is one of the newly characterized members of the kallikrein gene family.
- Similar to other kallikreins, *KLK12* gene product (hK12) is predicted to be a secreted serine protease. hK12 is synthesized as a prepro-enzyme and is then processed into the mature form with a predicted molecular weight of 24.5 KDa.
- In this study recombinant hK12 and anti-hK12 antibodies were generated. Anti-hK12 antibodies were used to perform immunohistochemistry for this kallikrein for the first time, and the cellular localization of hK12 in normal prostate tissue was identified.
- Since in addition to hK3 a number of recently identified kallikreins (hK2, hK4, hK11, and hK15) have proven to be promising prostatic biomarkers, anti-hK12 antibodies generated here were further applied to a prostate cancer tissue array, and the immunoexpression of hK12 in benign and malignant prostate tissue was evaluated.

METHODOLOGY:

- Total lung tissue mRNA was reverse-transcribed to cDNA. PCR was conducted using the proof-reading enzyme *Pfu* and oligonucleotide primers specific to classical human *KLK12*.
- The amplified cDNA was cloned into pET/100 TOPO plasmid vector. Plasmid DNA containing the pET-*KLK12* construct was used to transform the *E.coli* strain BL21(DE3) cells. Protein production was detected using SDS-PAGE and western blotting.
- rhK12 was purified using Ni-NTA metal affinity chromatography followed by RP-HPLC. Purified r*KLK12* was used as antigen to generate polyclonal antibodies. Antibody specificity was confirmed using antibody capture assay and Western blotting.
- Anti-hK12 antibody was used for immunostaining of formalin-fixed paraffin embedded prostate tissue sections (4 µm thick) using the avidine-biotin peroxidase complex technique. Rabbit-anti-hK12 antiserum (1:500 dilution) and biotinylated goat anti-rabbit IgG were used as the primary and secondary antibody.
- Peroxidase in tissue sections was visualized as brown color using DAB substrate solution. The sections were counterstained with hematoxylin. Prostate tissue sections treated with no primary antibody, or pre-immune rabbit serum were used as negative controls.

RESULTS:

Fig. 1 Purification of rhK12 fusion protein using Ni-NTA chromatography as detected by SDS-PAGE.
Lane 1) Bacterial pellet prior to purification; Lane 2) Soluble impurities removed; Lane 3) purified inclusion bodies; Lane 4-6) wash steps; Lane 7) purified rhK12, first elute pH 3.8; Lane 8) second elute pH 3.8; Lane 9) third elute pH 3.8; Lane 10) MW marker

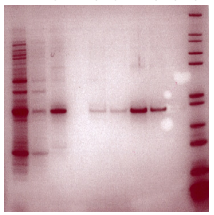


Fig. 2 Purification of rhK12 fusion protein using reversed-phase HPLC as detected by SDS - PAGE (Coomassie stain)
Lane 1) Molecular marker; Lanes 2-10) HPLC fractions

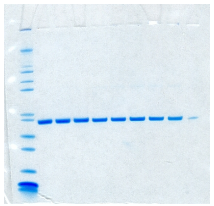


Fig. 3 hK12 fusion protein as detected by Western blotting using hK12 rabbit serum. Lane 1-4) Same as lanes 6-9 but probed with non-immune rabbit serum; Lane 5 and 10) molecular mass marker; Lane 6) BSA; Lane 7) purified hK12 fusion protein; Lane 8) hK12 in whole cell lysate of BL21 cells transformed with hK12 construct; Lane 9) Whole cell lysate of BL21 cells with no insert.

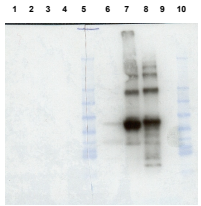


Fig. 4 Immunohistochemical localization of hK12 in benign and malignant prostate tissue; Negative control: prostate tissue stained with non-Immune rabbit serum.

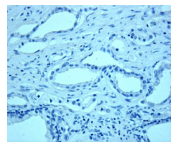


Fig. 5 Immunohistochemical localization of hK12 in benign prostate tissue; Benign glands stained with anti-hK12 rabbit serum showing membranous staining of luminal, but not basal cells. Note predominant apical staining.

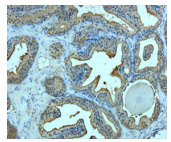


Fig. 6 hK12 in benign gland and adenocarcinoma gland (upper glands). The adenocarcinoma glands have a predominant cytoplasmic staining.

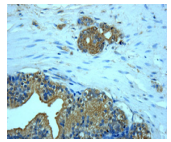
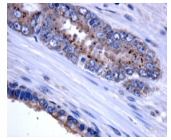


Fig. 7 hK12 in benign gland (lower glandular cells) and speckles in carcinoma gland (upper gland). Cytoplasmic granular staining was not observed in benign glands or prostate cancers with a low Gleason pattern, however these cytoplasmic speckles were present in 32.5 % of the samples with Gleason pattern 3, and 29% of the samples with Gleason pattern 4.



DISCUSSION:

- Among the fifteen members of the human tissue *KLK* gene family, *KLK12* is the only kallikrein for which the production of antibodies against its native or recombinant form has not been reported.
- Similar to many of the newly identified tissue kallikreins, no rich natural source from which these proteins could be isolated is currently available. In order to obtain sufficient amounts of hK12 for antibody generation we expressed the recombinant form of hK12 using an *E. coli* protein expression system.
- Due to the presence of different *KLK12* mRNA splice variants which result in proteins of various length, we used RT-PCR followed by two rounds of PCR using primers specifically designed to amplify and clone the mature form of "classical" *KLK12* (GenBank Accession number NM_145894) which represents the hK12 as a typical kallikrein-like enzyme with serine protease activity.
- The apparent molecular mass 28 kDa observed for recombinant hK12 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 hK12 was used as antigen for antibody generation. hK12 rabbit antibody did not react with any of the proteins in the whole cell lysate of control BL21 cells. The "classical" hK12 has 48% amino acid sequence identity and 57% overall similarity with hK8, 46% identity with hK10, and 38% identity with both PSA and hK2, however, in our cross reactivity analysis, hK12 rabbit antiserum had no cross-reactivity with any of these or any other tissue kallikreins.
- Since classical kallikreins such as hK3 and hK2 have already proved to be amongst the best prostatic biomarkers, anti-hK12 antibodies generated here were used for immunostaining of paraffin-embedded sections of human prostate tissue.
- hK12 in normal prostate tissue mainly displayed a cytoplasmic staining. With the exception of hK4 which is a predominantly nuclear protein, many of the kallikreins tested so far generally localize in the cytoplasm. Expression of human tissue kallikreins mainly by glandular epithelia, as well as presence of all tissue kallikreins tested so far in biological fluids indicate that kallikreins are secreted proteins. Immunostaining pattern of normal prostate tissue using anti-hK12 antibody indicate that, similar to other kallikreins, hK12 is a secreted protein.
- To-date, the expression of kallikrein 12 (at the protein or mRNA level) in prostate cancer has not been investigated. Since in addition to hK3 a number of recently identified kallikreins (hK2, hK4, hK11, and hK15) have proven to be promising prostatic biomarkers, anti-hK12 antibodies generated here were further applied to a prostate cancer tissue array, and the immunoexpression of hK12 in benign and malignant prostate tissue was evaluated.
- Intense granular supranuclear staining of hK12 in about 30% of prostate cancer samples having a Gleason grade ¾ was observed, this intense staining was totally absent in low grade cancers or normal prostatic tissue.
- The reason for this expression pattern is currently unknown. In high grade prostate tumors, aberration in secretary pathways involved in the transport of hK12 following its synthesis, or perhaps presence of a non-secreted splice variant of hK12 may result in the cytoplasmic accumulation of this kallikrein in prostate cancer. All four splice variants of hK12 known to date however, are predicted to encode for secreted protein.

CONCLUSION:

The production of the recombinant hK12 and anti-hK12 antibodies reported in this study provide the research tools required to characterize the structure and function of this kallikrein. Utilization of hK12 specific antibodies may be valuable in prostate cancer diagnosis.

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