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ScienceDirect
Clinical Biochemistry 40 (2007) 104–110
CLINICAL BIOCHEMISTRY

Development of an immunofluorometric assay for human kallikrein 15 (KLK15) and identification of KLK15 in tissues and biological fluids

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Received 6 June 2006; received in revised form 19 July 2006; accepted 27 July 2006
Available online 25 August 2006

Abstract

Background: Human kallikrein 15 (KLK15) may have some utility as a prostate, ovarian, and breast cancer biomarker, based on previous studies, which examined mRNA levels of *KLK15*. The aim of this study was to develop analytical technology for human kallikrein 15, including recombinant protein, specific antibodies, and a sensitive and specific ELISA immunoassay. The assay was then used to examine levels of KLK15 in tissues and biological fluids.

Methods: We produced human, recombinant pro-KLK15 in HEK 293 cells. Recombinant KLK15 was purified with various chromatographic steps and used to immunize rabbits and mice for production of KLK15 polyclonal antibodies. We used these antibodies to develop a highly sensitive and specific KLK15 immunoassay and to study KLK15 expression in various tissues and biological fluids.

Results: Large amounts of pure, recombinant KLK15 have been produced and characterized. KLK15 mouse and rabbit polyclonal antibodies have been employed for development of a KLK15 immunoassay. This assay has a lower detection limit of 0.05 μg/L, and no cross-reactivity with any of the other fourteen kallikreins. Using this assay, KLK15 was detected in prostate, colon, and thyroid tissues, as well as in breast milk and seminal plasma.

Conclusions: The KLK15 reagents developed here will allow for analysis of KLK15 protein expression levels in tissues and biological fluids, both normal and cancerous. This will expand upon previously characterized tissue *KLK15* mRNA expression studies which suggested that KLK15 might be useful as a biomarker for breast, ovarian, and prostate cancer. KLK15 is another serine protease that is produced in prostate and other tissues and is secreted in seminal plasma and other fluids. Its physiological function needs to be further elucidated.

Keywords: Kallikrein; KLK15; Immunoassay; Tissue expression; Serine proteases

Introduction

The human tissue kallikrein family, located on chromosome 19q13.4, consists of 15 genes, all predicted or shown to encode secreted serine proteases [1,2]. Kallikrein 15 (*KLK15*, *prostinogen*, *ACO protease*) is the most recently cloned member of the kallikrein gene family and maps between the two classical kallikreins, *KLK1* and *KLK3*, on the kallikrein locus [3]. mRNA studies indicate that *KLK15* is highly expressed in the thyroid, salivary, and adrenal glands, prostate, and colon ([1], and our unpublished observations). *KLK15* has also been found to be up-regulated by steroid hormones in the prostate cancer cell line LNCaP [3].

The function of the protein encoded by *KLK15* (KLK15) is currently unknown. However, KLK15 is predicted to be a secreted protein [1]. Preliminary functional studies indicate that KLK15 is a trypsin-like serine protease, preferring to cleave after arginine and/or lysine [4,5]. KLK15 has also been shown to cleave and activate pro-PSA (KLK3) into active PSA [3], indicating that perhaps KLK15 may be involved in an enzymatic cascade within the prostate [6].

Many kallikreins have been found to be useful cancer biomarkers (reviewed in [7,8]). KLK3 (PSA) and KLK2 have

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doi:10.1016/j.clinbiochem.2006.07.011

proven useful in diagnosing and monitoring prostate cancer patients [9,10]. mRNA studies indicate that *KLK15* may also have some utility as a cancer biomarker. *KLK15* has been shown to be up-regulated in cancerous versus non-cancerous prostate tissues, as well as in more aggressive prostate tumors, at the mRNA level [3,11], indicating that it may be useful for distinguishing between more or less aggressive forms of prostate cancer. *KLK15* may also serve as an unfavorable marker for ovarian cancer as it was found to be up-regulated in cancerous versus benign ovarian tumors [12]. *KLK15* was found to be a predictor of reduced progression-free and overall survival for ovarian cancer [12]. For breast cancer, however, mRNA studies suggest that *KLK15* may serve as a predictor of longer progression-free and overall survival [13].

Thus far, the clinical utility of *KLK15* as a cancer biomarker has been studied only at the mRNA level, and KLK15 protein levels in tissues and biological fluids have not been examined. This is due to the lack of reagents for monitoring KLK15 protein levels. The physiological function of KLK15 is currently unknown, and PSA is the only proposed KLK15 substrate [4]. To further elucidate this protein's role as a biomarker and to study its physiological role, well-characterized, recombinant protein is required. Recombinant KLK15 can also be used for production of specific monoclonal and polyclonal antibodies, for development of a KLK15 immunoassay, for enzyme kinetic studies and KLK15 substrate analysis.

We have expressed and characterized recombinant KLK15 using a mammalian expression system and used this recombinant protein to produce KLK15 polyclonal antibodies. Using our rabbit polyclonal antibody in combination with a KLK15 monoclonal antibody (obtained as a pre-release reagent from R&D Systems Inc.), we have developed a novel KLK15 "sandwich-type" immunoassay. We used this assay to analyze KLK15 expression in various human tissues and biological fluids. KLK15 is found primarily in prostate, colon, and thyroid tissues. KLK15 is also found in seminal plasma, confirming that it is a secreted protein.

Materials and methods

Cloning of KLK15 into a mammalian expression vector

KLK15 mRNA was obtained from LNCaP prostate cancer cells (purchased from ATCC), by Trizol (Invitrogen) extraction, as per the manufacturer's instructions. *KLK15* mRNA was reversed transcribed into first strand cDNA using superscript first strand synthesis (Invitrogen). *KLK15* cDNA (NM_017509) was amplified by PCR using the forward primer 5′-cacccaggatggtgacaagttg-3′ and reverse primer 5′-gtcacttcctcttcatggtttccc-3′. PCR was performed in a 25 μL reaction mixture containing 15 ng cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 100 ng of primers and 2.5 U of *pfu* turbo DNA polymerase (Stratagene). The PCR conditions were 94°C for 2 min, followed by 94°C for 1 min, 66°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR product was subsequently cloned into the pcDNA 3.1-v5-HIS-Topo vector (Invitrogen), using the manufacturer's recommended method. The correct sequence of the above construct was confirmed by sequencing.

Production of KLK15 in human embryonic kidney (HEK 293) cells

HEK 293 cells were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS). The *KLK15*-pcDNA3.1 construct was introduced into the HEK 293 cells using Fugene 6 transfection agent (Roche), as per the manufacturer's recommendations. pcDNA3.1 is neomycin (G418) resistant, and 48 h following transfection, 30 μg/mL G418 was added to the medium, as a positive selection agent. Massive cell death occurred within 2 weeks of G418 addition. Viable clones were visualized under the light microscope, marked, and picked via pipetting. Stable clones were picked and grown to confluency in DMEM containing 10% FBS and 15 μg/mL G418. KLK15 expression was monitored by Western blotting, using a rabbit polyclonal antibody developed in our laboratory against recombinant KLK15 protein produced in *E. coli*. The clone which produced the highest levels of KLK15 (clone C4) was characterized further. Once confluent in serum containing medium, C4 cells were grown in serum free CD CHO (BD Biosciences) medium containing 15 μg/mL G418, for 10 days, after which the cells were pelleted by centrifugation and the supernatant was retained for purification.

KLK15 purification from HEK 293 cells using cation exchange and reversed phase chromatography

Recombinant KLK15 was purified from HEK 293 cell culture supernatant using two stages of chromatography. Firstly, cation exchange chromatography was performed using an SP sepharose fast flow column (5 mL; GE Healthcare) and, secondly, reversed phase chromatography was performed using a C_4 column (Vydac). Briefly, SP sepharose beads previously activated with 1 M NaCl were equilibrated with 50 mM sodium acetate (pH 5.2). C4 cell supernatant was pumped through the SP sepharose column at a flow rate of 1.0 mL/min to allow for protein binding to the beads. The beads were then washed with 50 mM sodium acetate (pH 5.2). KLK15 was eluted using a step gradient starting with a linear gradient from 0 to 200 mM NaCl elution over 20 min followed by constant 200 mM NaCl over 20 min. This step was followed by a second linear gradient from 200 mM to 1 M NaCl over 40 min. KLK15 was eluted around 400 mM NaCl. Trifluoroacetic acid, as an ion-pairing agent, was added to this eluate (final concentration 10 mL/L), which was then loaded on a C_4 column equilibrated with 1 mL/L trifluoroacetic acid in water. A step gradient increasing from 28 to 40% in 1% increments over 80 min of acetonitrile in 1 mL/L trifluoroacetic acid was then performed. The fraction containing KLK15 was concentrated by evaporation of the acetonitrile. The purified material was