Development of an Immunoassay for Human Kallikrein 15, a Potential Prostate and Ovarian Cancer Biomarker

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ABSTRACT AND INTRODUCTION

The kallikreins are a cluster of 15 genes on chromosome 19q13.4, encoding a subgroup of secreted serine proteases [1]. Many kallikreins are differentially expressed in hormone-regulated malignancies, implicating these proteins as potential cancer biomarkers [1]. The most recently cloned member of this gene family is kallikrein 15 (*KLK15*, gene, hK15, protein) [2]. mRNA studies suggest that *KLK15* is overexpressed in ovarian cancer tissues, and in more aggressive forms of prostate cancer [2-4]. These preliminary experiments implicate *KLK15* as a potential biomarker for ovarian and prostate cancer, however no studies have examined hK15 expression at the protein level.

To determine the clinical utility of hK15 as a cancer biomarker, a sensitive and specific hK15 serum assay is essential. Development of this assay requires large amounts of pure, recombinant hK15 for antibody production and assay standardization.

We have produced recombinant hK15 using *P. pastoris* and mammalian expression systems. *KLK15* cDNA obtained from PC3 prostate cancer cells was amplified by PCR and cloned into the pPIC9 expression vector downstream of the AOX1 gene, making *KLK15* expression inducible by methanol. KM71 *P. pastoris* cells were transformed with the *KLK15*-pPIC9 expression construct, creating a stable hK15-expressing clone. hK15 was secreted into, and purified from yeast culture supernatant using two stages of chromatography; cation exchange chromatography, using SP sepharose beads (GE Healthcare), and reversed phase chromatography using a C4 column (Vydac). Purified hK15 was identified by western blotting with an hK15 polyclonal antibody and was of the expected molecular mass of approximately 33 kDa. The presence of hK15 was also confirmed by tandem mass spectrometry.

KLK15 cDNA was also cloned into pcDNA3.1-V5-His and transfected into human embryonic kidney (HEK) 293 cells, from which a stable cell line was created. hK15 was secreted into the cell culture supernatant, and purified as described above. Purified hK15 was identified by western blotting and tandem mass spectrometry.

We used recombinant hK15 to immunize BALB/c mice and New Zealand white rabbits. Injections were repeated six times at 3-week intervals, following which, blood was drawn from the animals and tested for antibody generation. Western blotting and ELISA assays (plates coated with purified hK15) confirmed that the polyclonal antibodies generated recognized hK15 produced from yeast, mammalian, and *E. coli* expression systems.

Mouse and rabbit polyclonal antibodies were used to develop a sandwich type hK15 immunoassay. Our antibodies raised against mammalian hK15 produced were found to be optimal, and will be the focus from this point. A mouse monoclonal antibody (obtained as a prerelease reagent from R&D Systems, Inc.) was used for capture, and our rabbit polyclonal for detection. This immunoassay recognized recombinant hK15 produced by yeast, mammalian, and *E. coli* cells, and was used to detect hK15 in various tissue cytosols and biological fluids. hK15 was detected primarily in the adrenal gland, salivary gland, seminal plasma and breast milk.

AIM

We hypothesize that hK15 has clinical utility as a cancer biomarker. Examining this hypothesis requires the development of a sensitive and specific hK15 serum assay, for which we must produce large amounts of pure, recombinant hK15.

OBJECTIVES:

1) To produce large amounts of pure, recombinant hK15.
2) Use recombinant hK15 as an immunogen to produce monoclonal and polyclonal antibodies
3) Develop a sensitive and specific immunoassay for hK15
4) Examine hK15 levels in tissues and biological fluids
5) Perform clinical studies to determine hK15's utility as a biomarker

RESULTS AND DISCUSSION

Production of Recombinant hK15

hK15 positive purification fractions were identified by western blotting, and SDS-PAGE analysis (figure 1). Purified mammalian hK15 resolves as a diffuse band of approximately 38 kDa.

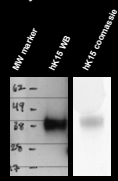

Figure 1: Pooled, hK15 positive fractions were resolved by SDS-PAGE. The left panel shows western blotting with an hK15 polyclonal antibody. The right panel shows coomassie staining of the 38 kDa hK15 band.

Identification of hK15

The presence of hK15 was confirmed by tandem mass spectrometry and N-terminal sequencing. The table below shows the 10 peptides identified by mass spectrometry. These peptides provide coverage of the full-length proform of hK15. N-terminal sequencing identified the first five amino acids of the hK15 proform (DGDLL), confirming the recombinant hK15 produced by HEK 293 cells is the expected proform.

Peptide	Amino Acids (hK15 proform)
LLEGDECAPHSQPWQVALYER	22-42
FNCGASLISPHWVLSAAHCQSR	45-66
VRLGEHNLR	70-78
LGEHNLRK	72-79
RDGPEQLR	80-87
DGPEQLR	81-87
LNPQVRPAVLPTR	118-130
CPHPGEACVVSGWGLVSHNEPGTAGSPR	131-158
LTNTMVCAGAEGR	188-200
VCHYLEWIR	242-250

Glycosylation Analysis

38 kDa is larger than the expected molecular weight of hK15, 30 kDa. We hypothesized this was due to glycosylation of hK15. hK15 contains two potential glycosylation sites at amino acids 171 and 232 We treated pure, recombinant hK15 with PNGaseF, an enzyme which removes N-glycan groups, and found that treatment with this enzyme caused hK15 to shift in size from 38 kDa to 30 kDa. Results of PNGaseF treatment are shown in figure 2.

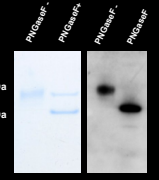

Figure 2: 10 ug of purified hK15 was treated with PNGaseF, according to the manufactuers directions. The left panel shows coomassie staining of hK15 before treatment and after treatment. The 37.5 kDa band seen after treatment is that of PNGaseF itself. The right panel shows a western blot of the identical samples. hK15 shifts in size after treatment, from 38 kDa to 30 kDa.

Production of hK15 Antibodies

Pure recombinant, mammalian hK15 was used to immunize mice and rabbits. Our antibodies were tested for their ability to recognize various forms of hK15, and the results are shown in figure 3.

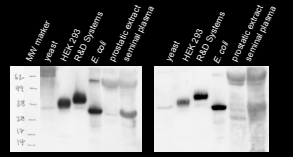

Figure 3: Various forms of recombinant hK15 as well as a prostatic extract, and seminal plasma were resolved by SDS-PAGE and blotted with our hK15 rabbit polyclonal antibody raised against mammalian hK15 (left panel), and hK15 mouse polyclonal antibody raised against hK15 mammalian protein (right panel).

Development of hK15 mono-poly Assay

Various pairs of antibodies were used to determine the optimal combination for sensitive and specific detection of hK15. We use an hK15 mouse monoclonal antibody (obtained as a prerelease reagent from R&D Systems, Inc) for capture, and our rabbit polyclonal antibody (raised against mammalian hK15) for detection. The detection limit of our assay is 0.05 ug/L hK15 (figure 4A). We have used our assay to examine hK15 expression levels in various tissues and biological fluids. Preliminary results indicate hK15 expression primarily in the salivary, thyroid, and adrenal glands figure 4B), as well as in seminal plasma (figure 4C).

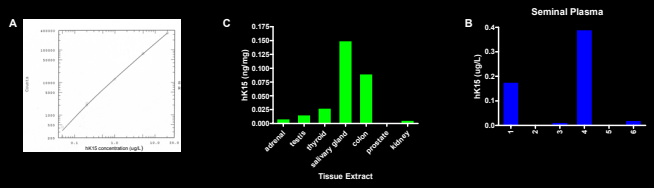

Figure 4: A) typical calibration curve for the hK15 mono-poly assay. B) hK15 expression in tissue extracts. C) hK15 expression in six seminal plasma samples.

METHODOLOGY:

Constructs

KLK15 cDNA was cloned into the mammalian expression vector, pcDNA3.1-V5-His. HEK293 cells were transfected using Fugene 6, and a stable cell line created.

Protein Expression

Once confluent in serum containing medium, hK15 expressing HEK 293 cells were grown in serum free CD CHO (BD Biosciences) medium containing 15 ug/mL G418, for 10 days, after which the cells were pelleted by centrifugation and the supernatant was retained for purification.

Protein Purification

Recombinant hK15 was purified from HEK 293 cell culture supernatant using two stages of chromatography. Firstly, cation exchange chromatography was performed using SP sepharose fast flow columns (5 mL; GE Healthcare) and secondly, reversed phase chromatography was performed using a C_4 column (Vydac). Briefly, SP sepharose beads previously activated with 1M NaCl were equilibrated with 50mM sodium acetate (pH 5.2). 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). hK15 was eluted using a step gradient starting with a linear gradient from 0-200 mM NaCl over 20 minutes, followed by constant 200 mM NaCl over 20 minutes. This step was followed by a second linear gradient from 200mM-1M NaCl over 40 minutes. hK15 was eluted in 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_2 column equilibrated with 1mL/L trifluoroacetic acid in water. A linear gradient (1.2% per min) of acetonitrile from 10 to 60% in 1mL/L trifluoroacetic acid was then performed. The fraction containing hK15 was concentrated be evaporation of the acetonitrile. The purified material was separated by SDS-PAGE and stained with coomassie blue to assess its purity and molecular mass. Bands stained with coomassie blue from this purified sample were subjected to mass spectrometry analysis to confirm their identity as hK15, as described previously [5].

Glycosylation Analysis

10 ug of purified hK15 was treated with PNGaseF, as per the manufactuer's recommendations (New England Biolabs).

Production of polyclonal antibodies

Purified, recombinant hK15 was used as an immunogen to immunize rabbits. hK15 (100 ug) was injected subcutaneously into New Zealand white rabbits. The protein was diluted 1:1 in complete Freund's adjuvant for the first injection, and in incomplete Freund's adjuvant for the subsequent injections. Injections were repeated six times at 3-week intervals. Blood was drawn from the animals and tested for antibody generation.

hK15 mono-poly Assay

White, polystyrene microtiter plates were coated with 250ng/well (diluted in 50 mM Tris, pH7.8) 252820 hK15 mouse monoclonal (obtained by a pre-release agreement with R&D Systems) and incubated overnight at room temperature. Plates were washed twice before standards and samples, diluted in 6% BSA were applied and incubated at room temperature for 2 hours, with continuous shaking. Plates were then washed six times, following which hK15 rabbit polyclonal antibody, diluted 1:3000 in assay buffer was added and incubated for 1 hour at room temperature with shaking. 100uL/well of alkaline phosphatase-conjugated goat anti-rabbit IgG, Fc fragment-specific (Jackson ImmunoResearch), diluted 1:5000 in 6% BSA was added to each well and incubated for 45 min. The plates were then washed as above. Diflunisal phosphate [100 uL of a 1 mmol/L solution in substrate buffer (0.1 mol/L Tris pH 9.1), 0.1 mol/L NaCl and 1 mmol/L MgCl2] was added to each well and incubated for 10 min. Developing solution (100 uL, containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L TbCl3, and 3 mol/L EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion). The calibration and data reduction were performed automatically, as described in detail elsewhere [6].

CONCLUSION:

We have developed a sensitive and specific hK15 immunoassay, which recognizes hK15 produced from various sources. Our assay is a tool, used for detection and quantification of hK15 levels in tissues and biological fluids. This will be necessary for determining hK15's potential as a cancer biomarker.

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