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 (hK15, hK15, protein) [2]. mRNA studies suggest that hK15 is overexpressed in ovarian cancer tissues, and in more aggressive forms of prostate cancer [3]. These preliminary experimental findings implicate hK15 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 necessary. 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 mammalian expression systems. hK15 cDNA obtained from P3C prostate cancer cells was amplified by PCR and cloned into the pPIC3 expression vector downstream of the AOX1 gene, making hK15 expression inducible by methanol. KM71 P. pastoris cells were transformed with the hK15-pPIC3 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, by 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.

hK15 cDNA was also cloned into pDONR201-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 of 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 specific antibodies that developed against recombinant 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.

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 evidence for 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.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acids (hK15 proform)</th>
</tr>
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<tbody>
<tr>
<td>LLEGEECAPSSPSVALWTER</td>
<td>22-42</td>
</tr>
<tr>
<td>FGNCAGSKLPHLLSAHGRSR</td>
<td>45-68</td>
</tr>
<tr>
<td>VLLQGQHLK</td>
<td>70-78</td>
</tr>
<tr>
<td>LGSEHHRL</td>
<td>72-79</td>
</tr>
<tr>
<td>RDGPEGLR</td>
<td>80-87</td>
</tr>
<tr>
<td>DGPEGLR</td>
<td>81-87</td>
</tr>
<tr>
<td>LNPQVRPAVLPTR</td>
<td>118-130</td>
</tr>
<tr>
<td>FNCGASLISPHWVLSAAHCQSR</td>
<td>45-66</td>
</tr>
<tr>
<td>LGEHNLRK</td>
<td>72-79</td>
</tr>
<tr>
<td>LNPQVRPAVLPTR</td>
<td>118-130</td>
</tr>
<tr>
<td>CPHPSGACWGSVGLSNPEPTAGCS</td>
<td>151-158</td>
</tr>
<tr>
<td>LTLMNCACAGESR</td>
<td>188-200</td>
</tr>
<tr>
<td>VCHYLHWRU</td>
<td>242-250</td>
</tr>
</tbody>
</table>

Glycosylation Analysis

38 kDa is larger than the expected molecular weight of hK15, 30 kDa. We hypothesized this was due to glycosylation of hK15 and assessed 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.

Production of hK15 Antibodies

Purified recombinant hK15 was used as an immunogen in 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 1: Protein hK15 positive 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 2: 10 ug of purified hK15 was treated with PNGaseF according to the manufacturer’s protocol (A). The left panel shows western blotting with an hK15 polyclonal antibody. The right panel shows mass spectrometry analysis of the purified hK15.

Identification of hK15 from biological samples was performed 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.

Recombinant hK15 was purified from P. pastoris culture supernatant using the same purification steps as used for mammalian hK15. The purified hK15 was confirmed to recognize various forms of hK15, and the results are shown in figure 3.

Figure 3: 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 for detection. This immunoassay recognized recombinant hK15 produced by yeast, mammalian, and E. coli expression systems. Following the optimization of our assay, we used 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).

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 used an hK15 mouse monoclonal antibody (obtained as a prerelease reagent from R&D Systems, Inc) for capture, and our rabbit polyclonal for detection. This immunoassay recognized recombinant hK15 produced by yeast, mammalian, and E. coli expression systems. Following the optimization of our assay, we used 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).

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.

REFERENCES: