Human Kallikrein 11: A New Biomarker of Prostate and Ovarian Carcinoma

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ABSTRACT

Human kallikrein 11 (hK11) is a putative serine protease of the human kallikrein gene family. Currently, no methods are available for measuring hK11 in biological fluids and tissues. Our aim was to develop immunological reagents and assays for measuring hK11 and examine if the concentration of this kallikrein is altered in disease states. We produced recombinant hK11 protein in a baculovirus system and used it to develop monoclonal and polyclonal antibodies against hK11. We then developed an immunofluorometric procedure for measuring hK11 in biological fluids and tissue extracts with high sensitivity and specificity. We further quantified hK11 in various biological fluids and in serum of patients with various cancers. The hK11 immunofluorometric assay is highly sensitive (detection limit, 0.1 ng/ml) and specific (no detectable cross-reactivity for other homologous kallikreins). We established the tissue expression pattern of hK11 at the protein level and found the highest levels in the prostate, followed by stomach, trachea, skin, and colon. We have immunohistochemically localized hK11 in epithelial cells of various organs. We further detected hK11 in amniotic fluid, milk of lactating women, cerebrospinal fluid, follicular fluid, and breast cancer cytosols. However, highest levels were seen in prostatic tissue extracts and seminal plasma. hK11 in seminal plasma and prostatic extracts is present at ~300-fold lower levels than prostate-specific antigen and at approximately the same levels as hK2. hK11 expression in breast cancer cell lines is up-regulated by estradiol. Elevated serum levels of hK11 were found in 70% of women with ovarian cancer and in 60% of men with prostate cancer. This is the first reported immunological assay for hK11. Analysis of this biomarker in serum may aid in the diagnosis and monitoring of ovarian and prostatic carcinoma.

INTRODUCTION

PSA2 is the best described cancer marker and is currently used widely for diagnosis and monitoring of prostatic carcinoma (1). PSA is a member of the hK family of serine proteases and has chymotrypsin-like enzymatic activity. Another member of the hK family, glandular hK2 is a new potential prostatic biomarker (2). Recently, the hK gene family has been expanded to include 15 members that share significant similarities at both the DNA and amino acid level (3, 4). All members of the hK gene family localize on chromosome 19q13.4 and encode for secreted serine proteases. Recently, it has been reported that two members of this family, hK6 and hK10, are potential biomarkers for diagnosis and monitoring of ovarian cancer (5, 6). In addition, many other members of the same family have been found to be overexpressed or underexpressed in ovarian and other cancers (7–16).

The gene encoding hK11 was first cloned by Yoshida et al. (17) and was named trypsin-like serine protease. With the newly established kallikrein gene nomenclature, this gene is now known as KLK11 (the protein is designated as hK11; Ref. 18). Although it has been pre...
positive clones were expanded sequentially in 24-well plates and 6-well plates. Supernatants were further characterized by performing IgG isotyping, and clones were subjected to limiting dilutions. The clones were then expanded in flasks to generate large amounts of supernatants in serum-free medium. The monoclonal antibodies were purified from the supernatants by using protein G-affinity chromatography.

**Immunofluorometric Assay for hK11.** In the standard assay procedure, one purified anti-hK11 monoclonal antibody diluted in coating buffer (containing 50 mM Tris, pH 7.80) was dispensed into a 96-well white polystyrene microtiter plate (100 μl/500 ng/well) and incubated overnight at room temperature. The plates were then washed three times with washing buffer (containing 9 g/l NaCl and 0.5 g/l Tween 20 in 10 mM Tris-buffer, pH 7.8). One hundred μl of hK11 calibrators (recombinant hK11 in 60 g/l BSA) or samples were applied into each well along with 50 μl of assay buffer. The assay buffer was a 50 mM Tris-buffer (pH 8.0), containing per liter 60 g of BSA, 0.5 mol of KCl, 0.5 g of Tween 20, 10 g of bovine immunoglobulins, 100 mM of goat serum, and 25 μl of mouse serum. The plates were incubated for 2 h on an orbital shaker to allow for hK11 molecules to bind to the plates. The plates were then washed six times. Subsequently, the plates were incubated for 1 h with 100 μl/well of a rabbit anti-hK11 polyclonal antisera (unpurified), diluted 2000-fold in assay buffer. The plates were then washed six times with washing buffer. One hundred μl of alkaline phosphatase-conjugated goat antirabbit antibody (Jackson Immuno Research), diluted 3000-fold in assay buffer, were added to each well, incubated for 30 min, and washed six times, as described above. Finally, 100 μl of 1 mM of dinitrophenyl phosphate, diluted in substrate buffer [0.1 M Tris (pH 9.1), 0.1 M NaCl, and 1 mM MgCl₂] were added into each well for 10 min. One hundred μl of developing solution (1 mM Tris-base, 0.4 mM sodium hydroxide, 2 mM TbCl₃, and 3 mM EDTA) were pipetted into each well for 1 min. The fluorescence was measured with a time-resolved fluorometer on the CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, Ontario, Canada), as described previously (22). The calibration and data reduction was performed automatically.

**Determination of Assay Characteristics.** The assay characteristics were determined essentially as described elsewhere (20).

**Human Tissue Cytosolic Extracts and Biological Fluids.** Human tissue cytosolic extracts were prepared as follows. Various frozen human tissues (0.2 g) were pulverized on dry ice to fine powders. Extraction buffer [1 ml, containing 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN₃, and 1 μg/ml leupeptin] was added to the tissue powders, and the mixture was incubated on ice for 30 min with repeated shaking and mixing every 10 min. Mixtures were then centrifuged at 14,000 rpm at 4°C for 30 min. The supernatants (cytosolic extracts) were then collected. The biological fluids tested were leftovers of clinical samples submitted for routine biochemical testing at Mount Sinai Hospital. All tissue cytosolic extracts and biological fluids were stored at −80°C until use.

**Recovery.** Recombinant hK11 was added to human serum samples at different concentrations and measured with the developed hK11 immunoassay. Recoveries were then calculated after subtraction of the endogenous concentrations.

**Fractionation of Biological Fluids with Size-Exclusion high-performance liquid chromatography.** Biological fluids were fractionated on a silica-based gel filtration column essentially as described elsewhere (20). The fractions were collected and analyzed for hK11 with the developed immunoassay.

**Immunohistochemistry.** A mouse monoclonal antibody was raised against hK11 full-size recombinant protein produced in yeast cells, as described above. Immunohistochemical staining for hK11 was performed according to a standard immunoperoxidase method. Briefly, paraaffin-embedded tissue sections (4 μm) were fixed and dewaxed. Endogenous peroxidase activity was blocked with 3% aqueous hydrogen peroxide for 15 min. Sections were then treated with 0.4% pepsin at pH 2.0 for 5 min at 4°C and blocked with 20% protein block (Signet Labs) for 10 min. The primary antibody was then added at 1:3000 dilution for 1 h at room temperature. After washing, biotinylated anti-mouse antibody (Signet Labs) was added, diluted 4-fold in antibody dilution buffer (Dako). After incubation and washing, streptavidin-tagged horseradish peroxidase was added for 30 min at room temperature. After washing, detection was achieved with amino ethyl carbazol for 5–10 min. The slides were then counterstained with hematoxylin and then mounted with coverslips.

**Hormonal Regulation of hK11.** To study the hormonal regulation of hK11, various cell lines were cultured and then stimulated with different steroids, essentially as described elsewhere (23). All steroid hormones were used at the final concentration of 10⁻⁸ M. Tissue culture supernatants were collected after 7 days and used for analysis of hK11; PSA was used as a control. PSA analysis was performed with the method described elsewhere (24).

**RESULTS**

We were able to produce recombinant hK11 protein in baculovirus, bacterial, and yeast expression systems (data not shown). The protein was purified using successive cycles of ion-exchange, affinity, and reverse-phase liquid chromatography, as described previously (20). The identity of the protein was verified by nanoelectrospray mass spectrometry (20). The concentration of the highly purified protein (>99% by SDS-PAGE) was established by the bicinchoninic acid total protein method, with BSA as a standard.

We raised monoclonal mouse antibodies and polyclonal rabbit antibodies using standard techniques (21). One monoclonal antibody was used for coating microtiter plates (capture antibody), and the polyclonal rabbit antibody was used for detection in an ELISA-type sandwich assay. A secondary goat antirabbit polyclonal antibody, labeled with alkaline phosphatase, was also used, and the activity of alkaline phosphatase was detected by time-resolved fluorometry, as described previously (22). We have carefully optimized this assay in terms of amounts of reagents used and incubation times to obtain the lowest possible detection limits. The optimal conditions are described in “Materials and Methods.”

A typical calibration curve for this assay is shown in Fig. 1. The detection limit, defined as the concentration of analyte that can be distinguished from zero with 95% confidence, is 0.1 μg/l, and the dynamic range extends to 50 μg/l. Within-run and day-to-day precision studies (10 runs over 2 weeks) yielded coefficients of variation <10% within the measurement range. Recovery of added recombinant hK11 to serum averaged 50% (Table 1). We have further evaluated the cross-reactivity of this assay against other homologous kallikreins. We found no detectable cross-reactivity from hK2, hK4, hK6, hK10, and hK13 when these recombinant proteins (produced in-house) were tested at levels up to 1000 μg/l. For PSA (hK3), we detected some cross-reactivity when the PSA used was purified from seminal plasma. At 1,000 and 10,000 μg/l of seminal plasma PSA, the hK11 equivalent concentrations were 0.23 and 3.2 μg/l, respectively.

![Fluorescence vs hK11 (μg/L)](image)

**Fig. 1.** Calibration curve of the newly developed hK11 immunofluorometric assay. The assay has a dynamic range of 0.1 μg/l (lowest detection limit) to 50 μg/l. The fluorescence of the zero standard was subtracted from all other measurements.
yielding an average cross-reactivity of 0.028%. However, when we tested the cross-reactivity with recombinant PSA, produced in bacteria, the cross-reactivity was much less (0.0003%). We thus concluded that the higher cross-reactivity found with seminal plasma PSA was likely attributable to hK11 contamination in this preparation. Further evidence that PSA does not cross-react with the hK11 immunoassay is provided below.

To study the distribution of hK11 protein in various human tissues, we have prepared cytosolic extracts, as described in “Materials and Methods” and quantified hK11 by the developed immunoassay. All values were corrected for the total protein content of the extracts. The data are shown in Fig. 2. Highest levels of hK11 were found in the prostate, followed by stomach, trachea, skin, and colon. Lower levels were found in pituitary, testis, lung, small intestine, and seminal vesicles. To study the cellular distribution of hK11, we have immunohistochemically localized it in paraffin-embedded tissue in intestinal epithelium (Fig. 3, A and B). Staining was restricted to the cytoplasm of epithelial cells.

We have further tested for the presence of hK11 in various biological fluids. The data are shown in Table 2. We detected hK11 in all fluids tested, with highest levels seen in amniotic fluid and milk of lactating women. The presence of hK11 in these fluids provides further evidence that hK11 is a secreted protein.

We have further quantified hK11 in seminal plasma, given the fact that this serine protease is present at highest levels in the prostate gland (Fig. 2). We have also quantified hK11 in five different prostatic tissue extracts, along with PSA (hK3), to establish their relative abundances. These data are shown in Table 3. hK11 concentration in seminal plasma is, on average, >100-fold higher than in any other biological fluid shown in Table 2. In the prostatic tissue extracts, the levels of hK11 are ~250 times lower than PSA levels, and there is no apparent correlation between PSA and hK11 levels. In seminal plasma, the levels of hK11 are also ~300 times lower than PSA. The lack of correlation between PSA and hK11 concentration in seminal plasma (Table 3) further suggests that our assay is not affected by the relatively huge amounts of PSA in this fluid. Another prostatic kallikrein, hK2, is present in seminal plasma at levels 300 times lower than PSA and at levels roughly equivalent to those of hK3. We have previously established tissue culture systems for studying the hormonal regulation of hK3 and hK2 (23). We used the cell lines LNCaP (prostatic carcinoma), PC-3 (AR; a prostatic carcinoma cell line stably transfected with androgen receptor), MCF-7 (breast carcinoma), MFM-223 (breast carcinoma), ZR-75 (breast carcinoma), BT-474 (breast carcinoma), T-47D (breast carcinoma), BT20 (breast carcinoma), and BG-1 (ovarian carcinoma). From all of these cell lines, only two (MCF-7 and BT-474) were able to produce and secrete detectable hK11 upon hormonal stimulation. As can be seen from Fig. 4, hK11 protein production is highly stimulated, mainly by estradiol in both cell lines, and to a lesser degree by other steroid hormones. In contrast, PSA (hK3) is highly up-regulated by dihydrotestosterone and the progesterin norgestrel in BT-474 cells. In accordance with previous data, we did not find any production of PSA in the MCF-7 cell line (23). These data strongly suggest that the KLK11 gene is up-regulated mainly by estrogens in these cell lines.

We have quantified hK11 in the serum of 40 apparently healthy females (ages 25 to 60 years) and 32 males (ages 30 to 65 years). Males have approximately three times higher levels, apparently because of the presence of the prostate. The medians of males (0.32 µg/l) and females (0.11 µg/l) differ significantly (P < 0.01 by Mann-Whitney test). For further analyses, we considered the upper reference range for normal persons as 0.25 µg/l for women and 0.50 µg/l for men (95th percentile).

We then analyzed a total of 114 serum samples from patients with various malignancies to examine whether hK11 is elevated in any of them. The data are presented in Table 4. Highest percentages of hK11 elevations were seen in patients with ovarian and prostate cancers. These data should be considered as preliminary, because the types of malignancies tested are limited and the number of patients in each group is small. The immunohistochemical localization of hK11 in an ovarian cancer tissue is shown in Fig. 3, C and D.

To examine the molecular forms of hK11 in seminal plasma and serum, as determined by our assay, we separated, on a gel filtration column, one seminal plasma and one serum sample with high hK11. The data are shown in Fig. 5. hK11 in seminal plasma elutes as a single peak corresponding to a molecular weight of M, 30,000 (free hK11). In serum, in addition to the major M, 30,000 form, there is a small peak (<10%) corresponding to a molecular mass of ~100 kDa. This may represent hK11 bound to serum proteinase inhibitors, as has been shown for PSA (26, 27).

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### Table 1 Recovery of added recombinant hK11 to human serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initially present</th>
<th>Added</th>
<th>Recovered</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 g/l bovine serum albumin (control)</td>
<td>0</td>
<td>4.9</td>
<td>4.9</td>
<td>100</td>
</tr>
<tr>
<td>Male serum #1</td>
<td>0.27</td>
<td>4.9</td>
<td>2.2</td>
<td>46</td>
</tr>
<tr>
<td>Male serum #2</td>
<td>0.17</td>
<td>4.9</td>
<td>2.7</td>
<td>55</td>
</tr>
<tr>
<td>Female serum #1</td>
<td>0.24</td>
<td>4.9</td>
<td>2.4</td>
<td>48</td>
</tr>
<tr>
<td>Female serum #2</td>
<td>0.23</td>
<td>4.9</td>
<td>2.4</td>
<td>48</td>
</tr>
</tbody>
</table>

**Average**

Average 50 µg/l bovine serum albumin (control) 4.9 4.9 100

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**Fig. 2.** hK11 content of cytosolic extracts of various human tissues. All hK11 concentrations were corrected for the amount of total protein. For details and discussion, see text.
DISCUSSION

Among all known cancer biomarkers, PSA is the most valuable, because of its tissue specificity. Elevated serum PSA levels are seen in patients with prostatic carcinoma, and the test is widely used for diagnosis and monitoring of this disease. PSA is a member of the human tissue kallikrein gene family, and it is a secreted serine protease of 30-kDa molecular mass (1–4). Another member of this family, hK2, is an emerging biomarker for prostatic carcinoma (2). More recently, two other members of the kallikrein gene family, hK6 and hK10, have been proposed as new biomarkers for ovarian carcinoma (5, 6). Although there are now 15 known kallikrein genes, many family members have not been studied in detail (3, 4). We have reviewed recently the potential utility of kallikreins as cancer biomarkers (28). The success of other kallikreins as cancer biomarkers has prompted us to speculate that hK11 may also represent a novel biomarker. However, no methods are currently available for hK11 quantification in biological fluids or tissues.

We have produced recombinant hK11 protein in baculovirus, yeast, and bacterial expression systems. These proteins were highly purified by chromatography and used as immunogens to produce monoclonal and polyclonal antibodies. We used these antibodies to develop a highly sensitive immunoassay that is suitable for hK11 quantification in biological fluids or tissues.

We have produced recombinant hK11 protein in baculovirus, yeast, and bacterial expression systems. These proteins were highly purified by chromatography and used as immunogens to produce monoclonal and polyclonal antibodies. We used these antibodies to develop a highly sensitive immunoassay that is suitable for hK11 quantification in biological fluids or tissues. Similar to a few other kallikreins (hK2, hK3, hK4, hK6, and hK10; Refs. 3, 4), hK11 is highly expressed in the prostate gland and to a lower degree in a number of other tissues (Fig. 2). Many biological fluids, including amniotic fluid and milk of lactating women, contain considerable amounts of hK11 (Table 2). However, highest levels are sheen in seminal plasma (Table 3).

hK11 is secreted by epithelial cells and has been immunolocalized in the supranuclear compartment, likely representing the Golgi apparatus of these cells (Fig. 3). Similar to many other members of this family, hK11 is up-regulated by steroid hormones, especially estradiol in two breast cancer cell lines (Fig. 4). Other members of this family

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Sample number</th>
<th>hK11, µg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk of lactating women</td>
<td>1 2 3 4 5 6</td>
<td>0.8 40 1.6 9.4 0.7 8.1</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>1 2 3 4 5 6</td>
<td>5.2 45 2.0 7.8 10.5 11.3</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>1 2 3 4 5 6</td>
<td>2.9 3.0 1.5 1.6 1.3 1.7</td>
</tr>
<tr>
<td>Follicular fluid</td>
<td>1 2 3 4 5 6</td>
<td>0.5 0.7 0.7 7.3 0.6 2.2</td>
</tr>
<tr>
<td>Breast cancer cytosol</td>
<td>1 2 3 4 5 6</td>
<td>0.16 0.13 &lt;0.1 0.31 0.1 0.8</td>
</tr>
</tbody>
</table>

Table 2 Concentration of hK11 in biological fluids

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Sample number</th>
<th>PSA, µg/l</th>
<th>hK11, µg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostatic tissue extracts</td>
<td>1 2 3 4 5 6</td>
<td>69.000 11.000 12.000 57.000 192.000</td>
<td>766 192 146 204 428</td>
</tr>
<tr>
<td>Seminal plasma samples</td>
<td>1 2 3 4 5 6</td>
<td>3.5 × 10^6 1.1 × 10^6 0.3 × 10^6 0.9 × 10^6 0.6 × 10^6</td>
<td>2.8 × 10^3 1.7 × 10^3 1.4 × 10^3 3.0 × 10^3 3.4 × 10^3</td>
</tr>
</tbody>
</table>

Table 3 Concentrations of PSA (hK3) and hK11 in five prostatic tissue extracts and five seminal plasma samples
(e.g., PSA, hK2, and hK4) are up-regulated by androgens (3, 4, 23), whereas other kallikreins are also up-regulated by estrogens (3, 4, 29).

Our assay detects mainly the free form of hK11 in biological fluids (Fig. 5). Our data with serum suggest that hK11 may also be present in a complexed form with proteinase inhibitors, such as other kallikreins (26, 27). However, we do not know whether the traces of bound hK11 (Fig. 5A) represent the true concentration of this analyte or whether our assay does not efficiently measure the complex form of this serine protease. More studies will be necessary to delineate this issue. The lower recovery in serum (~50%; Table 1) further suggests that hK11 may bind to proteinase inhibitors that inhibit or partially inhibit its immunological activity in our assay, upon entrance into the circulation.

Although hK11 is present in the prostate, its concentration is substantially lower than PSA (Table 3). However, it appears that hK11 and hK2 concentrations are approximately equal in both prostatic tissue extracts and seminal plasma (25). It will be interesting to examine the biological role of this kallikrein in prostatic tissue and its secretions. It has been shown previously that PSA can cleave semenogelins and facilitate semen liquifaction (30). Additionally, hK2 can activate the pro-form of PSA (31–33). More recently, hK15, another kallikrein expressed in the prostate (12), was shown to activate the pro-form of PSA more efficiently than hK2 (34). It will be interesting to examine whether hK11 also participates in this cascade of activation reactions among kallikreins expressed in the prostate.

The availability of recombinant hK11 protein will facilitate further studies into the understanding of the physiological functions of this serine protease in the prostate and other tissues.

Our preliminary investigations indicate that serum hK11 concentration is elevated in the majority of patients with ovarian and prostate cancer (Table 4). Because the malignancies and number of serum samples tested were limited, these data should be considered preliminary. We were able to immunohistochemically localize hK11 protein in ovarian cancer tissue (Fig. 3). Positivity was predominantly seen in the cytoplasm of tumor cells. It will be worthwhile to examine whether this biomarker has utility in the early diagnosis of ovarian cancer, in combination with other markers and in the differential diagnosis between prostate cancer and benign prostatic hyperplasia. These possible applications are of great clinical importance.

In conclusion, we present, to our knowledge, the first method for hK11 quantification and provide information regarding its tissue expression, hormonal regulation, and possible diagnostic value as an ovarian and prostatic cancer biomarker. The availability of this technology will allow more detailed investigations in the future.

**REFERENCES**
