Human Kallikrein 14: A New Potential Biomarker for Ovarian and Breast Cancer

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ABSTRACT

Human kallikrein gene 14 (KLK14) is a recently discovered member of the tissue kallikrein family of secreted serine proteases, which includes hK3/prostate-specific antigen, the best cancer biomarker to date. Given that KLK14 is hormonally regulated, differentially expressed in endocrine-related cancers, and a prognostic marker for breast and ovarian cancer at the mRNA level, we hypothesize that its encoded protein, hK14, like hK3/prostate-specific antigen, may constitute a new biomarker for endocrine-related malignancies. The objective of this study was to generate immunological reagents for hK14, to develop an ELISA and immunohistochemical techniques to study its expression in normal and cancerous tissues and biological fluids. Recombinant hK14 was produced in Pichia pastoris, purified by affinity chromatography, and injected into mice and rabbits for polyclonal antibody generation. Using the mouse and rabbit antisera, a sandwich-type immunofluorometric ELISA and immunohistochemical methodologies were developed for hK14. The ELISA was sensitive (detection limit of 0.1 μg/liter), specific for hK14, linear from 0 to 20 μg/liter with between-run and within-run coefficients of variation of <10%. hK14 was quantified in human tissue extracts and biological fluids. Highest levels were observed in the breast, skin, prostate, seminal plasma, and amniotic fluid, with almost undetectable levels in normal serum. hK14 concentration was higher in 40% of ovarian cancer tissues compared with normal ovarian tissues. Serum hK14 levels were elevated in a proportion of patients with ovarian (65%) and breast (40%) cancers. Immunohistochemical analyses indicated strong cytoplasmic staining of hK14 by the epithelial cells of normal and malignant skin, ovary, breast, and testis. In conclusion, we report the first ELISA and immunohistochemical assays for hK14 and describe its distribution in tissues and biological fluids. Our preliminary data indicate that hK14 is a potential biomarker for breast and ovarian cancers.

INTRODUCTION

Human tissue kallikreins are secreted serine proteases encoded by 15 structurally similar, hormonally regulated genes that colocalize to chromosome 19q13.4 in a 300-kB region (1, 2). (Please note that kallikrein gene and protein symbols are “KLK” and “hK,” respectively, as described in the official nomenclature; Ref. 3). All of the kallikreins studied to date are differentially expressed at the mRNA level, we hypothesize that its encoded protein, hK14, like hK3/prostate-specific antigen, may constitute a new biomarker for endocrine-related malignancies. Therefore, the purpose of the present study was to produce recombinant hK14 and generate specific polyclonal antibodies in mice and rabbits to develop ELISA and immunohistochemical methodologies. This allowed us to examine hK14 levels in normal and cancerous human tissues and biological fluids, and to determine its clinical utility as a biomarker for hormone-dependent malignancies.

MATERIALS AND METHODS

Cloning, Production, Purification, and Characterization of Recombinant hK14m•His

Cloning of KLK14 into P. pastoris Expression Vector pPICZαA. KLK14 cDNA encoding the 227 amino acids of the mature form of the hK14 protein (corresponding to amino acids 25–251 of GenBank accession no. AAK48524; Ref. 14) was PCR amplified from vector construct pPICZαA-KLK14 (10 ng), produced previously by cloning amplified mature KLK14 cDNA into expression vector pPICZαA of the Easyselect P. pastoris yeast expression system (Invitrogen, Carlsbad, CA). The reaction was performed in a 50-μl reaction mixture containing Pfu DNA polymerase buffer (200 mM Tris-HCl (pH 8.8), 20 mM MgSO4, 100 mM KCl, 100 mM (NH4)2SO4, 1% Triton X-100, and 1 mg/ml nuclease-free BSA), 2 mM MgCl2, 200 μM deoxynucleoside triphosphates, 100 ng of primer FP14-His (5’ GAA GCT GAA TTC ATA ATT GGT GG 3’) and RP14-His (5’ TTT GGT CTA GAG CTT TGT CCC), and 0.5 μl (1.25 units) of PfuTurbo DNA polymerase (Stratagene, La Jolla, CA), on an Eppendorf master cycler. The PCR cycling conditions were 95°C for 1 min, followed by 95°C for 30 s, 56°C for 1 min, 72°C for 1 min for 40 cycles, and a final extension at 72°C for 7 min. After PCR, amplified KLK14 was

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visualized with ethidium bromide on 2% agarose gels, extracted, digested with EcoRI/XhoI, and ligated into expression vector pPICZaA (Invitrogen) at corresponding restriction enzyme sites using standard techniques. Because the 5’ end of KKL14 insert was cloned in-frame with the yeast α-factor secretion signal and the 3′ end in-frame with COOH-terminal c-myc epitope and poly-histidine (His$_x$) tags, the construct was denoted pPICZaA-KKL14myc-His and recombinant protein, hK14myc-His. The KKL14 sequence within the construct was confirmed with an automated DNA sequencer using vector-specific primers in both directions.

**Protein Production.** Pmel1-linearized pPICZaA-KKL14myc-His, as well as empty pPICZaA (negative control), were transformed into chemically competent *P. pastoris* strain X-33 after which they integrated into the yeast genome by homologous recombination. Transformed X-33 cells were then plated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 m sorbitol, and 2% agar) plates containing Zeocin, a selective reagent. A stable yeast transformant was selected as per the manufacturer’s recommendations, inoculated in buffered minimal glycerol-complex (BMGY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 0.5 M sodium nitrate, and 1% glycerol), and overnight incubated at 30°C on a plate agitator at 250 rpm, diluted to $A_{600} = 1.0$ in BMMY (same as BMGY except that 1% glycerol is replaced with 0.5% methanol) and incubated under the same conditions as above for 6 days with a daily supplement of 1% methanol. The supernatant was collected by centrifugation at 4000 × g for 20 min.

**Protein Purification.** Recombinant hK14myc-His was purified from the yeast culture supernatant by immobilized metal affinity chromatography using a Ni$^{2+}$-nitrilotriacetic acid column (Qiagen, Valencia, CA). Briefly, the yeast culture supernatant was diluted four times in equilibration buffer [50 mM Na$_2$HPO$_4$, 300 mM NaCl, and 10 mM imidazole (pH 8.0)] and loaded onto a column containing Ni$^{2+}$-nitrilotriacetic acid resin equilibrated previously with the same buffer. The column was then washed twice with 5 volumes of equilibration buffer and the adsorbed hK14myc-His eluted with a 20, 100, 250, 500, and 1000 mM imidazole step gradient. All of the fractions were analyzed as described below, and those containing hK14myc-His were pooled and concentrated by ultrafiltration with an Amicon YM10 membrane (Millipore Corporation, Bedford, MA). The total protein concentration was subsequently determined using the Bradford bicinchoninic acid method with BSA as a standard (Pierce Chemical Co., Rockford, IL).

**Detection of hK14myc-His.** To monitor recombinant hK14myc-His production and purification, samples were subjected to SDS PAGE using the NuPAGE Bis-Tris electrophoresis system and 4–12% gradient polyacrylamide gels at 200 V for 30 min (Invitrogen). Proteins were visualized with a Coomassie G-250 staining solution, SimplyBlue SafeStain (Invitrogen), according to the manufacturer’s instructions. For Western blot analysis, proteins were transferred to a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) at 30 V for 1 h, after separation by SDS-PAGE. The membrane was blocked with Tris-buffered saline-Tween [0.1 mol/liter Tris-HCl buffer (pH 7.5) containing 0.15 mol/liter NaCl and 0.1% Tween 20] supplemented with 5% nonfat dry milk overnight at 4°C. Subsequently, the membrane was probed with a mouse anti-His (COOH-terminal) monoclonal antibody (Invitrogen; diluted 1:5000 in Tris-buffered saline-Tween) for 1 h at room temperature. After washing the membrane three times for 15 min with Tris-buffered saline-Tween, it was treated with horseshad peroxidase-conjugated goat antimouse antibody (1:20,000 in Tris-buffered saline-Tween; Amersham Biosciences) for 1 h at room temperature. Finally, the membrane was washed again as above, and fluorescence was detected on X-Ray film using the SuperSignal West Pico chemiluminescent substrate (Pierce Chemical Co.).

**Mass Spectrometry.** The identity of hK14myc-His was confirmed by tandem mass spectrometry, as described previously in detail for recombinant hKL10(19).

**NH$_2$-Terminal Sequencing.** NH$_2$-terminal sequence analysis was performed to identify the amino acids at the NH$_2$-terminal end of recombinant hK14myc-His. Recombinant hK14myc-His (20 μg/lane) was first separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences), immersed previously in 100% methanol, at 30 V for 1 h. After the transfer, the membrane was removed and rinsed with deionized water three times for 5 min before staining. Coomassie blue R-250 (0.1% solution in 40% methanol) was used subsequently to stain the membrane (5 min) followed by destaining (5 min) in a 50% methanol solution. The membrane was then thoroughly washed with deionized water and air-dried. hK14myc-His was subjected to automated NH$_2$-terminal Edman degradation consisting of 5 cycles of Edman chemistry on a Forton/Beckman Gas-phase Microsequencer, followed by phenylthiohydantoin analysis on an high-performance liquid chromatography. The amino acid sequence was determined by an Applied Biosystems model 477A amino acid analyzer.

**Glycosylation Status.** hK14myc-His was incubated with Peptide/N-glycosidase F (New England Biolabs, Beverly, MA), an amidase of 36 KDa which cleaves N-glycan chains from glycoproteins (between the innermost N-acetyl-glucosamine and Asn). Briefly, 15 μg of purified hK14 was denatured in 2 μl of denaturing buffer (5% SDS and 10% β-mercaptoethanol) at 100°C for 5 min and immediately transferred to ice for an additional 5 min. One-tenth the volume of both G7 buffer (0.5 M sodium phosphate (pH 7.5)) and 10% NP40 were then added, followed by 1 μl of Peptide/N-glycosidase F. The reaction was incubated at 37°C for 2 h.

Two identical polyacrylamide gels containing 10 μg/lane of purified hK14myc-His, purified deglycosylated hK14myc-His, as well as horseradish peroxidase (a glycoprotein of ~40 KDa; positive control) and soybean trypsin inhibitor (an unglycosylated protein of ~21.5 KDa; negative control) were subjected to SDS-PAGE. One gel was stained with SimplyBlue SafeStain (Invitrogen) a Coomassie G-250 staining solution, and the other using the GelCode Glycoprotein staining kit (Pierce Chemical Co.). The latter allows for detection of glycoprotein sugar moieties on polyacrylamide gels. This gel was treated with periodic acid, which oxidizes the glycols present in glycoproteins to aldehydes, followed by immersion in the GelCode Glycoprotein Stain, containing acidic fuchsin sulfite, the active agent.

**Production of Antibodies against hK14myc-His.** Purified recombinant hK14myc-His (~100 μg) was used as an immunogen and injected s.c. into Balb/C female mice and New Zealand White female rabbits for polyclonal antibody production. The protein was diluted 1:1 in complete Freund’s adjuvant for the first injection and in incomplete Freund’s adjuvant for subsequent injections. Injections were repeated three times for mice and six times for rabbits at 3-week intervals. Blood was drawn from the animals and tested for antibody generation every 2 weeks. To test for production of anti-hK14 polyclonal antibodies in mice and rabbits, the following immunosassay was used: sheep antimiast or goat antirabbit IgG (Jackson ImmunoResearch, West Grove, PA) was immobilized on 96-well opaque polystyrene plates. The mouse/rabbit serum was then applied to the plates at different dilutions ranging from 1:250 to 1:100,000. After incubation (1 h) and washing, biotinylated recombinant hK14myc-His was then added to each well (50 ng/well). Finally, after incubation (1 h) and washing, alkaline phosphatase-conjugated streptavidin was added, incubated (15 min), and washed. Diflunal phosphate (100 μl of a 1 mmol/liter solution) in substrate buffer [0.1 mol/liter Tris (pH 9.1), 0.1 mol/liter NaCl, and 1 mmol/liter MgCl$_2$] was added to each well and incubated for 10 min. Developing solution (100 μl containing 1 mol/liter Tris base, 0.4 mol/liter NaOH, 2 mol/liter TdC$_5$, and 3 mmol/liter EDTA) was added to each well and incubated for 1 min. The fluorescence was measured with a time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, Ontario, Canada). The calibration and data reduction were performed automatically, as described elsewhere (20, 21).

**ELISA for hK14**

**Standard Assay Procedure.** A sandwich-type polyclonal (mouse/rabbit) ELISA was developed as follows: white polystyrene microtiter plates were coated with sheep antimiast IgG, Fc fragment-specific antibody (Jackson ImmunoResearch) by overnight incubation of 100 μl of coating antibody solution [containing 500 ng of antibody diluted in 50 mmol/liter Tris buffer (pH 7.80)] in each well. The plates were then washed three times with washing buffer [9 g/liter NaCl and 0.5g/liter Tween 20 in 10 mmol/liter Tris buffer (pH 7.40)]. Mouse anti-hK14 polyclonal antisem was diluted 2000-fold in a general diluent [60 g/liter BSA, 50 mmol/liter Tris (pH 7.8), and 0.5 g/liter sodium azide], and 100 μl was applied to each well. After a 1-h incubation, the plates were washed six times with washing buffer.

hK14 calibrators or samples were then pipetted into each well (100 μl/well, diluted 1:1 in general diluent), incubated for 1 h with shaking, and then washed six times. Subsequently, 100 μl of rabbit anti-hK14 antisem diluted 2000-fold in buffer A (containing the components of the general diluent plus 25 μl/liter normal mouse serum, 100 ml/liter normal goat serum, and 10 g/liter bovine IgG) was applied to each well and incubated for 1 h; plates were then washed as described above. Finally, 100 μl/well of alkaline phosphatase-conjugated goat
antirabbit IgG, Fc fragment-specific (Jackson ImmunoResearch), diluted 1000-fold in buffer A were added to each well, incubated for 30 min, and washed as above. Difluorophosphate in substrate buffer was added to each well and incubated for 10 min, followed by developing solution pipetted for 1 min. The fluorescence was measured with the CytoFluor 615 Immunolizer.

**Determination of Sensitivity, Specificity, and Linearity.** Recombinant hK14myc-His was used to generate the calibration curve. hK14myc-His calibrators were prepared by diluting purified recombinant hK14myc-His in the general diluent. These calibrators were then used to define the detection limit of the assay.

**Specificity.** Recombinant hK14myc-His, a biological fluid (seminal plasma), and a tissue extract (breast cancer cytosol), containing high hK14 levels, were used to determine the specificity of the developed immunoadassay. These samples were first measured by the standard assay procedure described above. The mouse and rabbit anti-hK14 anti-sera were then successively replaced with sera from the same animals obtained before immunization (preimmune sera). The samples were remeasured, and fluorescence counts were compared with those obtained by the standard assay. For further confirmation of the specificity of this assay, recombinant hK14myc-His (1 μg, 100 ng, and 20 ng) was subjected to Western blot analysis using mouse and rabbit polyclonal preimmune and immune antisera (all diluted 1:2000), separately, as primary antibodies.

In addition, the cross-reactivities of other homologous proteins were investigated using purified recombinant hK2-hK15 (produced in-house), all at a concentration of 1000 μg/liter. Furthermore, hK14 (without c-myc and His epitopes; produced using similar techniques as hK14myc-His) was also measured.

**Linearity.** To determine the linearity of the hK14 immunoadassay, seminal plasma, and breast cancer cytosol samples with high hK14 levels were serially diluted in the general diluent, and the amount of hK14 was measured using the standard assay procedure.

**Preparation of Human Tissue Cytosolic Extracts and Biological Fluids**

The presence of hK14 in normal human tissues (i.e., esophagus, tonsil, skin, testis, kidney, salivary gland, breast, fallopian tube, adenral, bone, colon, endometrium, liver, lung, muscle, ovary, pancreas, pituitary, prostate, seminal vesicle, small intestine, spinal cord, spleen, stomach, thyroid, trachea, and ureter), areas of the human brain (i.e., frontal cortex, cerebellum, hippocampus, medulla, midbrain, occipital cortex, pons, and temporal lobe), and cancerous breast and ovarian tissues was determined using the hK14 immunoadassay. 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 mmol/liter Tris (pH 8.0), 150 mmol/liter NaCl, 5 mmol/liter EDTA, 10 g/liter NP40 surfactant, 1 mmol/liter phenylmethylsulfonyl fluoride, 1 g/liter aprotinin, and 1 g/liter leupeptin) was added to the tissue powders, and the mixture was incubated on ice for 30 min with repeated shaking and vortex-mixing every 10 min. Mixtures were then centrifuged at 14,000 × g at 4°C for 30 min. The supernatants were then collected. The levels of hK14 in ovarian and breast cancer cytosols were also determined. The biological fluids (seminal plasma, amniotic fluid, breast milk, cerebrospinal fluid, follicular fluid, and serum, and ascites fluid from women with advanced ovarian cancer) screened were residual samples submitted for routine biochemical testing. All of the tissue cytosolic extracts and biological fluids were stored at −80°C until use. The Institutional Review Board of Mount Sinai Hospital has approved these procedures.

**Recovery**

Recombinant hK14myc-His was added to the general diluent (control), normal serum (male and female), seminal plasma, amniotic fluid, and breast cancer cytosols at different concentrations (5 and 10 μg/liter), and measured with the hK14 immunoadassay. Recoveries were then calculated after subtraction of the endogenous concentrations.

**Hormonal Stimulation Experiments**

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI medium (Invitrogen) supplemented with glutamine (200 mmol/liter) and fetal bovine serum (10%), in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. Twenty-four h before the hormonal stimulation experiments, the culture medium was changed into a medium containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added to the culture medium, at a final concentration of 10−8 mol/liter. Steroids tested were aldosterone (mineralocorticoid), deoxymethasone (glucocorticoid), norgestrel (androgenic progestin), dihydrotestosterone (androgen), and estradiol (estrogen). Unstimulated cells and cells stimulated with 100% ethanol were included as controls. The cells were grown for 7 days, and the cell culture supernatant was collected for hK14 measurement using the developed ELISA. These experiments were repeated twice.

**Immunohistochemistry**

Immunohistochemical staining was performed according to a streptavidin-biotin-peroxidase protocol using the DAKO LSABKit Peroxidase (DAKO, Glostrup, Denmark) as described previously in detail for hK6 (22), hK10 (23), and hK13 (24). The hK14-specific rabbit polyclonal antibody, raised against full-length recombinant hK14myc-His protein produced in yeast, was used as the primary antibody.

**RESULTS**

Recombinant hK14myc-His was produced in the *P. pastoris* expression system, as a fusion protein consisting of the enzymatically active form of hK14 and COOH-terminal c-myc and His tags, with a predicted molecular mass of ~28 kDa. As detected by Western blot analysis, the protein was expressed and secreted into the culture medium of a highly expressing X-33 *P. pastoris* clone in two forms, indicated by two distinct bands of ~28 and 25 kDa, with the former being the predominant species (Fig. 1A, Lanes 3–5). Furthermore, the protein appears to degrade after 4 days of methanol induction, as shown in Fig. 1 by the lower molecular mass bands (~21.5 kDa) in *Lanes 4–5*. Recombinant hK14myc-His was detected in the culture supernatant after 1 d of methanol induction (data not shown) with highest levels after 6 days. hK14myc-His was not observed in the supernatant of cells before induction (Fig. 1A, Lane 2) or in the induced yeast cells transformed with the pPICZαA vector only (Fig. 1A, Lane 6).

Recombinant hK14myc-His was purified by immobilized metal affinity chromatography after 6 days of methanol induction from the culture supernatant with 1 mg of purified hK14myc-His per 250 ml of culture supernatant obtained, on average. As shown in Fig. 1B, purified hK14myc-His is visualized as two bands of 28 and 25 kDa on a Coomassie blue stained SDS-PAGE gel. These bands were excised, digested with trypsin, and sequenced by tandem mass spectrometry. The m/z values of the tryptic peptides extracted from the nanoelectrospray mass spectrum (data not shown) allowed for the calculation of their molecular masses. Confirmation of the sequence assignment was achieved using tandem mass spectrometry on selected precursor ions. The partial sequences of these tryptic peptides were identified from their molecular masses. Confirmation of the sequence assignment was achieved using tandem mass spectrometry. For example, partial sequenced tryptic peptides, SSQPWQAAALLGPR and AVRPIEVQCASPGTSCR, matched precisely with hK14 amino acid sequences 34–47 and 126–144, respectively (GenBank accession no. AAK48524).

The NH2-terminal sequence of the 28 kDa form of purified hK14myc-His was Glu-Ala-Glu-Phe-Ile-Ile. The first two amino acids identified, Glu-Ala, correspond to the last two amino acids of the yeast secretion α-factor. Although two potential cleavage sites exist.
for the removal of the yeast secretion α-factor, in this case, the dipeptidyl aminopeptidase involved in the maturation of α-factor (Ste 13 gene product) cleaved at the NH₂-terminal side of Glu, resulting in only a partial removal of the α-factor. The other cleavage site is located at the COOH-terminal end of Ala, and if used, the Glu-Ala dipeptide, would not have been incorporated at the NH₂ terminus of hK14myc-His. The next two amino acids, Glu-Phe, represent the amino acids of the EcoRI restriction enzyme site, which were used to clone KLK14. The last two, Ile-Ile, match those of mature hK14, residues 25 and 26 in the protein sequence.

Given that two forms of hK14myc-His were produced and that hK14 possesses a potential glycosylation site (Asn-Ile-Ser) in its primary sequence recognized by P. pastoris, we originally hypothesized that the higher molecular mass species of 28 kDa was glycosylated. To included as positive and negative controls, respectively. On treatment glycoprotein stain (Fig. 2).

Horseradish peroxidase, a glycoprotein, amide gels, one stained by Coomassie-blue and the other with a PNGase-F, was separated by SDS-PAGE on two identical polyacrylamide gels, Lane 1, recombinant hK14myc-His purified by immobilized metal affinity chromatography from the yeast culture supernatant. Elution fractions were concentrated 20 times (1.5 mg/ml as determined by the bicinchoninic acid total protein assay). M, molecular weight marker.

Characteristics of the hK14 ELISA

Configuration. The generation of polyclonal antibodies against recombinant hK14myc-His was accomplished by injecting the recombinant protein into mice and rabbits using standard techniques (29). The mouse and rabbit antisera demonstrated increasing hK14 immunoreactivity (higher titers) up to and including the third booster injection. No immunoreactivity significantly higher than background was noted when preimmune rabbit or mouse serum was substituted for the respective immune serum. Thus, the mouse and rabbit antibodies obtained after the third booster injection were used to develop the hK14 immunofluorometric assay. A “sandwich-type” polyclonal immunoassay configuration, in which the capture antibody was generated in mice and the detection antibody in rabbits, was adopted. A secondary goat antirabbit polyclonal antibody, labeled with alkaline phosphatase, was also used, and the activity of alkaline phosphatase was measured by time-resolved fluorometry (20). This assay configuration does not necessitate any prior antibody purification and was found previously to be highly specific and sensitive for other kallikreins, including hK4 (30), hK5 (8), hK6 (31), hK8 (32), hK10 (19), hK11 (12), and hK13 (33).

Sensitivity. A typical calibration curve for the hK14 ELISA is shown in Fig. 3. Purified recombinant hK14myc-His, diluted in 60 g/liter BSA to 0.1, 0.5, 1, 5, and 20 µg/liter were used as calibrators. Over this range, the assay showed a strong, linear relationship. The detection limit, defined as the concentration of hK14 that can be distinguished from zero with 95% confidence (mean ± 2 SD of zero calibrator), was 0.1 µg/liter.

Specificity. The specificity of the hK14 ELISA was confirmed by performing several experiments. First, immunoassay and Western blots were performed using either preimmune or immune mouse and rabbit sera. When mouse and rabbit antisera was replaced with preimmune mouse and rabbit sera in the immunoassay, fluorescence signals pertaining to recombinant hK14myc-His (20 µg/liter) and hK14-positive samples (semenal plasma and breast tissue extract; ≈350,000 arbitrary units) were reduced to background signals (≈16,000 arbitrary units; Fig. 4). This experiment demonstrates that the fluorescence counts generated with the hK14 immunoassay represent the specific binding of mouse and rabbit anti-hK14 polyclonal antibodies be glycosylated in vivo, similar to other native kallikreins including hK1 (25), hK2 (26, 27), and hK3 (28).

Fig. 1. Expression and purification of recombinant hK14myc-His. A, detection of recombinant hK14myc-His by Western blot analysis using anti-His antibody. Lane 1, molecular weight markers; Lane 2, culture supernatant from a yeast clone transformed with pPICZαA vector containing KLK14 cDNA before methanol induction (Day 0); Lanes 3–5 correspond to 2, 4, and 6 days of induction with 1% methanol. Lane 6, supernatant from an X-33 strain transformed with empty pPICZαA (negative control) after 6 days of methanol induction (1%). B, the proteins were separated by SDS-PAGE and stained with Coomassie blue. Lane 1, recombinant hK14myc-His purified by immobilized metal affinity chromatography from the yeast culture supernatant. Elution fractions were concentrated 20 times (1.5 mg/ml as determined by the bicinchoninic acid total protein assay). M, molecular weight marker.

Fig. 2. SDS-PAGE of purified hK14myc-His before and after treatment with PNGase-F. In both A and B, Lane 1 corresponds to purified hK14myc-His; Lane 2, purified hK14myc-His incubated with PNGase-F (38 kDa); Lane 3, horseradish peroxidase (40 kDa), positive control; and Lane 4, soybean trypsin inhibitor (21.5 kDa), negative control. (Note that in Lane 2 of A the top band represents PNGase-F). In A, the gel was stained with Coomassie blue, and there is no noticeable shift in the band representing hK14myc-His in Lane 2, as would be expected after deglycosylation. In B, the gel was stained with acidic fuchsin sulfite, a glycoprotein stain. Only the glycoprotein horseradish peroxidase (positive control) in Lane 3 is stained, additionally confirming that recombinant hK14myc-His is not glycosylated. M, molecular weight marker.
to hK14. Second, Western blot analysis of purified recombinant hK14<sub>myc-His</sub> using rabbit preimmune and immune sera produced bands corresponding to hK14<sub>myc-His</sub> (1 μg and 100 ng) only when the membrane was probed with immune rabbit sera (Fig. 5). No bands representing hK14<sub>myc-His</sub> were observed when the membranes were incubated with either preimmune or immune mouse sera (data not shown). Third, the cross-reactivities of the polyclonal mouse and rabbit antibodies were assessed. Because hK14 is a member of the human kallikrein family, it shares significant amino acid similarity with other kallikreins, in particular to hK6 showing 48% amino acid identity. Thus, the cross-reactivities of recombinant hK2-hK15 were examined. All of the recombinant proteins produced readings comparable with background signals, even at concentrations 1000-fold higher than hK14. Furthermore, no cross-reactivity was observed when other His-tagged proteins (e.g., recombinant hK5<sub>myc-His</sub>) were examined. In addition, recombinant hK14 (without c-myc/His epitopes) was tested, and the established assay was able to detect hK14 with or without the c-myc/His epitopes equally well. In effect, these data confirm that the immunoassay measures hK14 with high specificity, efficiently discriminates hK14 from other similar proteins, and does not detect the polyhistidine tag.

**Linearity and Precision.** To assess the linearity of this assay, various samples (serum, seminal plasma, and breast cancer cytosol) were serially diluted, and hK14 was remeasured (data not shown). Good dilution linearity was observed with this assay, suggesting freedom from matrix effects. Within- and between-run precision was assessed with various hK14 calibrators and clinical samples. In all of the cases, the imprecision of the assay (coefficient of variation) was <10%.

**Identification of hK14 in Human Tissue Extracts and Biological Fluids**

**Distribution of hK14 in Human Tissue Cytosolic Extracts.** The levels of hK14 in various adult male and female tissues were quantified using the developed immunoassay. The data are presented graphically in Fig. 6. The amount of hK14 in these extracts was corrected for the total protein content and expressed as ng of hK14 per g of total protein. Highest hK14 levels were observed in the breast followed by skin, prostate, midbrain, and axillary lymph nodes. Lower levels were seen in the lung, stomach, and testis. No immunoreactivity was detected in the other tissues examined (Please see “Materials and Methods”).

**Immunohistochemical Localization of hK14.** hK14 was immunohistochemically localized in the glandular epithelial cells derived from a variety of nonmalignant and malignant tissues (Fig. 7). Strong immunostaining was generally observed in the cytoplasm of these epithelial cells, whereas stroma was typically negative.

**hK14 in Biological Fluids.** The concentration of hK14 in various biological fluids was quantified, as shown in Table 1. Highest levels of this kallikrein were seen in seminal plasma, followed by amniotic fluid and follicular fluid. Lower levels were obtained in male serum samples, whereas female serum, cerebrospinal fluid, ascites fluid, and breast milk were all negative for hK14 (concentrations below the detection limit of 0.1 μg/liter).

**Recovery of hK14 from Biological Fluids.** The recovery of hK14 in various biological fluids was incomplete and ranged from 24–60% in male serum, 18–35% in female serum, 30–64% in seminal plasma, 35–49% in amniotic fluid, and 40–56% in breast cancer cytosols.

**Hormonal Regulation of hK14.** To study the hormonal regulation pattern of hK14, breast cancer cell line BT-474 was cultured, stimulated with various steroids at 10<sup>−8</sup> mol/liter final concentration, and tissue culture supernatants were analyzed after 7 days of incubation with the hK14 immunoassay. As illustrated in Fig. 8, the steroid that produced the most significant increase (38-fold) in hK14 concentration as compared with baseline hK14 levels (alcohol stimulation) was estradiol. Dihydrotestosterone caused a 4-fold increase in hK14 lev-
HUMAN KALLIKREIN 14 IN CANCER PATIENTS

**DISCUSSION**

Extensive research throughout the past few decades has focused on the identification of tumor markers to aid in cancer screening, diagnosis, monitoring, prognosis, and ultimately, to increase patient survival. Traditional and emerging tumor markers, either causally involved in carcinogenesis or incidental byproducts of malignant transformation, range from oncogenes, suppressor genes, cytokines, angiogenic factors, carbohydrate antigens, and proteases, to cell-free nucleic acids, autoantibodies, adhesion proteins, and circulating cancer cells (34). Proteases, in particular, have received a great deal of attention for their fundamental roles in tumor progression and metastasis (35, 36). Human tissue kallikreins are among proteases of the serine class, which have been implicated in carcinogenesis (1, 2). This family of enzymes includes established (hK3/PSA) and prospective (hK2, hK5, hK6, hK8, hK10, and hK11) serological cancer biomarkers, as well as many potential prognostic/predictive indicators (5). Among the latter is **hK14**, a hormonally regulated kallikrein gene, differentially expressed in several hormone-dependent cancers and a prognostic indicator in breast and ovarian carcinomas (14, 16, 18). Given the above, we speculate that this kallikrein, at the protein level, will also possess clinical utility as a cancer biomarker. Yet, until now, specific reagents and tools necessary to qualitatively and quantitatively examine hK14 were not available.

In the present study, we produced recombinant hK14<sup>myc-His</sup> in the *P. pastoris* expression system, and purified and administered this protein as an immunogen to mice and rabbits for polyclonal antibody generation. We used these antibodies to: (a) develop a highly sensitive and specific ELISA suitable for hK14 quantification in biological fluids and tissue extracts; and (b) perform immunohistochemical studies.

Using our ELISA, we measured hK14 in several biological fluids with the highest levels in seminal plasma and amniotic fluid (Table 1). This observation confirms that, in vivo, hK14 is a secreted protein. In contrast, the concentration of hK14 in the serum of healthy men and women, and in follicular and ascites fluids was extremely low, close to the detection limit of the immunoassay (0.1 μg/liter). The recovery of recombinant hK14<sup>myc-His</sup> from biological fluids was also incomplete (ranging from 18% to 64%), a common finding among other kallikreins, including, hK3, hK6, hK8, hK10, hK11, and hK13 (12, 19, 31–33, 37). One possible explanation for this phenomenon is that hK14 forms complexes with protease inhibitors, rendering them undetectable by immunoassay, and resulting in an underestimation of...
hK14 concentration (38). In fact, it has been well documented that other kallikreins, including hK2, hK3, and hK6, are sequestered (mainly in serum) by circulating protease inhibitors including α2-macroglobulin, protein C inhibitor, α1-antichymotrypsin, α2-antiplasmin, α1-antitrypsin, antithrombin, and protease inhibitor 6, forming complexes that are often not easily quantified (39–46). Because the free, as well as the bound forms of these kallikreins are useful biomarkers for the differential diagnosis of cancer (43, 47), additional studies will be necessary to characterize the various molecular forms of hK14 in biological fluids, identify the interacting proteins, and determine their clinical value.

The tissue expression pattern of hK14 was determined by analyzing a panel of adult human tissue extracts with our ELISA. The protein was detectable in a few tissues, specifically the breast, skin, prostate, midbrain, axillary lymph nodes, lung, stomach, and testis. Furthermore, as is the case for hK2, hK3, hK6, hK7, hK9, hK10, hK11, and hK13 (12, 19, 22–24, 48–50), hK14 was immunohistochemically localized in the cytoplasm of glandular epithelial cells from various tissues, both healthy and cancerous (Fig. 7), likely within the Golgi apparatus or secretory vesicles, additionally implying that this protein is secreted. These findings correlate well with each other and our previous report on KLK14 mRNA expression by reverse transcription-PCR, indicating that KLK14 is transcribed in the brain, breast, prostate, and testis (14).

One discrepancy was found, however, between the expression of human kallikrein 14 mRNA and protein levels in central nervous system (CNS) tissues. We have reported previously that KLK14 mRNA levels are highest in CNS tissues (i.e., brain, cerebellum, and spinal cord; Ref. 14), in contrast to a paper by Hooper et al. (15) indicating restricted KLK14 expression in the prostate, spleen, and skeletal muscle, and to our findings that suggest that the hK14 protein is not detected in any CNS tissue, with the exception of the midbrain, in which relatively low levels were observed (Fig. 6). Furthermore, hK14 was undetectable in cerebrospinal fluid (Table 1). These data suggest that human kallikrein 14, at the protein level, is not significantly expressed in the CNS. The difference between KLK14 and hK14 levels in the CNS may be attributed to: (a) post-translational regulation of the KLK14 gene; (b) efficient KLK14 transcription but rapid degradation of KLK14 mRNA (due to mRNA instability and short half-life); or (c) efficient translation of hK14 but rapid degradation shortly after synthesis.

Most, if not all, members of the kallikrein gene family are regulated by steroid hormones in the prostate, breast, and ovarian cancer cell lines studied (1, 2). Certain genes are predominately up-regulated by androgens and androgenic progestins (e.g., KLK2, KLK3, KLK4, KLK13, and KLK15), whereas others are primarily responsive to estrogens (e.g., KLK5, KLK6, KLK7, KLK9, KLK10, and KLK11; Ref. 2). Promoter/enhancer regions have only been characterized for KLK1, KLK2, and KLK3. With respect to the KLK14 gene, sequence analysis of its promoter region revealed the presence of a putative androgen response element and preliminary hormonal regulation studies indicate that KLK14 mRNA levels are predominately up-regulated by androgens in the breast (including BT-474 cells) and ovarian cancer cell lines tested (16). However, the immunofluorometric quantification of hK14 levels in the supernatant of the androgen and
estrogen receptor-positive breast cancer cell line BT-474, after stimulation with various steroid hormones, indicated that the \textit{KLK14} gene is significantly up-regulated by estrogens, followed by androgens (dihydrotestosterone) and androgenic progestins (norgestrel). Although these results suggest that \textit{KLK14} is both androgen and estrogen responsive in the BT-474 cancer cell line, it is unclear whether androgens or estrogens are the primary up-regulating steroid hormones, due to the conflicting mRNA and protein data.

Despite this inconsistency, these data are still in accord with the tissue expression pattern of hK14, which shows relatively high levels of hK14 in the breast (an estrogen-regulated tissue) and the prostate (an androgen-regulated tissue) from which it is likely secreted (15), to form a constituent of seminal plasma, where it is also found at high levels. It will be necessary to additionally characterize the promoter/enhancer regions of the \textit{KLK14} gene to explain the effect of steroid hormones on hK14 expression.

It has been reported previously that the \textit{KLK14} gene is differentially expressed, and has prognostic value in ovarian and breast cancer (14, 16, 18). Because no literature exists on hK14 protein expression in either the tissues or serum of ovarian and breast cancer patients, we used our ELISA to quantify hK14 in normal, benign, and cancerous ovarian tissue extracts, and in serum from normal individuals and patients with ovarian and breast cancer. We found elevated hK14 levels in ovarian cancer tissue extracts (Fig. 10) and in the serum of a subset of ovarian and breast cancer patients, compared with normal (Table 2; Fig. 9). It is likely that hK14 elevation in ovarian cancer tissues may account for its elevation in the serum of ovarian cancer patients. Accordingly, the group of patients with elevated serum levels may also be those in whom tissue hK14 levels are overexpressed. On the basis of these collective findings, we consider that hK14 is a potential ovarian and breast cancer biomarker, in addition to its prognostic value at the mRNA level. These proposals warrant further investigation with a larger patient series, and well-defined clinical and pathological data.

In addition to hK14, other kallikrein proteins, including hK5 (8), hK6 (9, 51), hK8 (10), hK10 (11), hK11 (12), and hK13 (33) are also elevated in the serum and/or tissues of ovarian cancer patients. Serum hK5 is also higher in a subgroup of breast cancer patients (8), whereas higher levels hK3/PSA and hK10 are associated with a poor response to tamoxifen therapy (52, 53). Given that these kallikreins are coexpressed and likely coordinately regulated, it is not unreasonable to speculate that they may form an enzymatic cascade pathway involved in ovarian and breast carcinogenesis by, as yet, unknown mechanisms (13). hK14 may also be included in a panel with other ovarian and breast cancer biomarkers.

\begin{table}[h]
\centering
\caption{Concentration of hK14 in sera of patients with various malignancies}
\begin{tabular}{llll}
\hline
Malignancy & Number of serum samples & Patients (%) with hK14 serum values $> \text{normal}^{a}$ & \\
\hline
Ovarian cancer & 20 & 13 (65\%) & \\
Breast cancer & 20 & 8 (40\%) & \\
Prostate cancer & 31 & 5 (16\%) & \\
Colon cancer & 10 & 2 (20\%) & \\
Testicular cancer & 10 & 1 (10\%) & \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Concentration of hK14 in various biological fluids}
\begin{tabular}{llllll}
\hline
Biological fluid & Number of samples tested & hK14 concentration (\textmu{g/liter}) & \\
 & & Range & Mean (SD) & Median & Positivity (\%) & \\
\hline
Seminal plasma & 30 & 0.6–23.6 & 10.8 (6.0) & 10.7 & 100 & \\
Amniotic fluid & 46 & 0.5–19.8 & 4.6 (4.7) & 2.6 & 100 & \\
Follicular fluid & 4 & 0–0.8 & 0.2 (0.4) & 0 & 25 & \\
Ascites fluid & 51 & 0–0.77 & 0.063 (0.16) & 0 & 18 & \\
Serum &  &  &  &  &  & \\
\begin{tabular}{l}
Female \end{tabular} & 27 & 0–0.16 & 0.02 (0.05) & 0 & 11 & \\
\begin{tabular}{l}
Male \end{tabular} & 28 & 0 & 0 (0) & 0 & 0 & \\
Cerebrospinal fluid & 6 & 0 & 0 & 0 & 0 & \\
Breast milk & 5 & 0 & 0 & 0 & 0 & \\
\hline
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Amniotic fluid & 46 & 0.5–19.8 & 4.6 (4.7) & 2.6 & 100 & \\
Follicular fluid & 4 & 0–0.8 & 0.2 (0.4) & 0 & 25 & \\
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\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig8}
\caption{Hormonal regulation of hK14 in the breast cancer cell line BT-474. hK14 is mainly up-regulated by estradiol, followed by dihydrotestosterone (DHT) and norgestrel.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9}
\caption{Concentration of hK14 in extracts from normal, benign, and cancerous ovarian tissues. n = number of tissues extracted. The percentage of samples containing higher hK14 levels compared with highest normal tissue extract is shown.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig10}
\caption{Serum hK14 (\textmu{g/liter}) levels in patients with ovarian and breast cancer compared with normal females. Serum hK14 is elevated in 65% of women with ovarian cancer and 40% with breast cancer when using a cutoff value equal to the lower detection limit (0.1 \textmu{g/liter}) of the immunoassay (indicated by \textsuperscript{*}).}
\end{figure}
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breast cancer biomarkers, including other kallikreins, to improve the diagnostic/prognostic potential for these lethal malignancies.

In conclusion, this is the first report to describe the development of specific reagents (recombinant hK14 and polyclonal antibodies) and methodologies (ELISA and immunohistochemistry) for the quantitative and qualitative study of human kallikrein 14 at the protein level. We provide initial insight into the tissue expression pattern, hormonal regulation, and potential clinical utility of hK14, and present preliminary data suggesting that this kallikrein may be clinically useful as a biomarker for breast and ovarian cancers. Our reagents and technologies will allow for additional detailed basic and clinical investigations to additionally elucidate the role of hK14 in human physiology and as a cancer biomarker.

REFERENCES


