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 and/or protein level in endocrine-related malignancies including cancers of the ovary, breast, prostate, and testis, and several are implicated in carcinogenesis (2). It is conceivable that this gene family may harbor many potential cancer biomarkers (4, 5). Among all of the kallikreins, hK3 or prostate-specific antigen (PSA) is a valuable biomarker for screening, diagnosis, and monitoring of prostate cancer (6). Recent reports indicate that many other kallikreins, including hK2, hK5, hK6, hK8, hK10, and hK11 are prospective serological screening and/or diagnostic biomarkers for several cancer types including ovarian, breast, and prostate (7–12), and at least 13 of the 15 kallikrein family members possess prognostic/predictive value in certain malignancies, primarily ovarian cancer (13).

Human kallikrein gene 14 (*KLK14*, formerly known as *KLK-L6*), identified recently by the positional candidate cloning approach, is a steroid hormone-regulated serine protease gene and a novel member of the human kallikrein gene family (14–16). Whereas *KLK14* mRNA was only detected in three tissues by Northern blot analysis (a ~1.5kb splice variant predominantly expressed in the prostate, with lower levels in the spleen, and a ~1.9kb transcript found exclusively in skeletal muscle; Ref. 15), a reverse transcription-PCR study indicated that *KLK14* is transcribed predominantly in central nervous system tissues, as well as in endocrine-regulated tissues, including the breast, prostate, and testis (14). *In situ* hybridization additionally demonstrated that *KLK14* is localized within the epithelial cells of the benign and malignant prostate gland (15).

Preliminary evidence indicates that *KLK14* is implicated in hormone-dependent malignancies and has clinical utility. For instance, *KLK14* is differentially expressed, at the mRNA level, in breast, ovarian, prostate, and testicular cancer tissues, and two breast cancer cell lines (14). Subsequent quantitative reverse transcription-PCR studies demonstrated that *KLK14* is significantly up-regulated in cancerous *versus* normal prostate tissues, primarily in more aggressive forms of the disease (17), and that its expression in breast and ovarian tumor tissues has independent prognostic value (16, 18). Given the above, we postulate that the secreted serine protease encoded by the *KLK14* gene, denoted hK14, has clinical value as a biomarker for cancer. Thus far, quantitative and qualitative studies on hK14 have not been feasible due to a lack of suitable reagents and techniques. 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 hK14^myc-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 pPICZα A-*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 MgSO$_4$, 100 mM KCl, 100 mM (NH$_4$)$_2$SO$_4$, 1% Triton X-100, and 1 mg/ml nuclease-free BSA], 2 mM MgCl$_2$, 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 GTT 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

visualized with ethidium bromide on 2% agarose gels, extracted, digested with *Eco*RI/*Xba*I, and ligated into expression vector pPICZαA (Invitrogen) at corresponding restriction enzyme sites using standard techniques. Because the 5′ end of *KLK14* 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)$_6$ tags, the construct was denoted pPICZαA-*KLK14*$^{myc-His}$ and recombinant protein, hK14$^{myc-His}$. The *KLK14* sequence within the construct was confirmed with an automated DNA sequencer using vector-specific primers in both directions.

Protein Production. *Pme*I-linearized pPICZαA-*KLK14*$^{myc-His}$, as well as empty pPICZαA (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 (pH 6.0), 1.34% yeast nitrogen base, 40 mg/liter biotin, and 1% glycerol] overnight 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 hK14$^{myc-His}$ was purified from the yeast culture supernatant by immobilized metal affinity chromatography using a Ni^{2+}-nitriloacetic 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+}-nitriloacetic acid resin equilibrated previously with the same buffer. The column was then washed twice with 5 volumes of equilibration buffer and the adsorbed hK14$^{myc-His}$ 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 hK14$^{myc-His}$ 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 hK14$^{myc-His}$. To monitor recombinant hK14$^{myc-His}$ 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 onto 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 horseradish 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 hK14$^{myc-His}$ was confirmed by tandem mass spectrometry, as described previously in detail for recombinant hK10 (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 hK14$^{myc-His}$. Recombinant hK14$^{myc-His}$ (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. hK14$^{myc-His}$ was sub-

jected to automated NH$_2$-terminal Edman degradation consisting of 5 cycles of Edman chemistry on a Porton/Beckman Gas-phase Microsequencer, followed by phenylthiohydantoin analysis on an high-performance liquid chromatography column.

Glycosylation Status. hK14$^{myc-His}$ 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*-acetylglucosamine 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 hK14$^{myc-His}$, purified deglycosylated hK14$^{myc-His}$, 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 hK14$^{myc-His}$. Purified recombinant hK14$^{myc-His}$ (~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 development. 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 immunoassay was used: sheep antimouse 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 hK14$^{myc-His}$ 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. Diflunisal 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 mmol/liter TbCl$_3$, 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 antimouse 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 antiserum 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 antiserum diluted 2000-fold in buffer A (containing the components of the general diluent plus 25 ml/l normal mouse serum, 100 ml/l normal goat serum, and 10 g/l 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. Diflunisal phosphate 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 Cyberfluor 615 Immunoanalyzer.

Determination of Sensitivity, Specificity, and Linearity. Recombinant hK14$^{myc-His}$ was used to generate the calibration curve. hK14$^{myc-His}$ calibrators were prepared by diluting purified recombinant hK14$^{myc-His}$ in the general diluent. These calibrators were then used to define the detection limit of the assay.

Specificity. Recombinant hK14$^{myc-His}$, 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 immunoassay. 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 hK14$^{myc-His}$ (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 hK14$^{myc-His}$) was also measured.

Linearity. To determine the linearity of the hK14 immunoassay, serum, 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, adrenal, 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 immunoassay. 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, 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 hK14$^{myc-His}$ 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 immunoassay. 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 nmol/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), dexamethasone (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 hK14$^{myc-His}$ protein produced in yeast, was used as the primary antibody.

RESULTS

Recombinant hK14$^{myc-His}$

Recombinant hK14$^{myc-His}$ 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. 1*A, 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 hK14$^{myc-His}$ was detected in the culture supernatant after 1 d of methanol induction (data not shown) with highest levels after 6 days. hK14$^{myc-His}$ was not observed in the supernatant of cells before induction (Fig. 1*A, Lane 2*) or in the induced yeast cells transformed with the pPICZαA vector only (Fig. 1*A, Lane 6*).

Recombinant hK14$^{myc-His}$ was purified by immobilized metal affinity chromatography after 6 days of methanol induction from the culture supernatant with 1 mg of purified hK14$^{myc-His}$ per 250 ml of culture supernatant obtained, on average. As shown in Fig. 1*B,* purified hK14$^{myc-His}$ 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 on 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 the *m/z* spacing between adjacent fragments ions. For example, partially sequenced tryptic peptides, SSQPWQAALLAGPR and AVRPIEVTQACASPGTSCR, matched precisely with hK14 amino acid sequences 34–47 and 126–144, respectively (GenBank accession no. AAK48524).

The NH$_2$-terminal sequence of the 28 kDa form of purified hK14$^{myc-His}$ 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
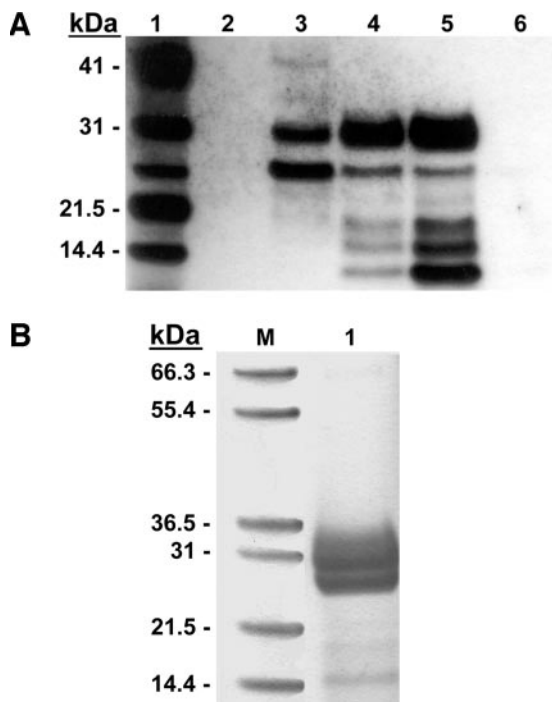
Fig. 1. Expression and purification of recombinant hK14[myc-His]. *A,* detection of recombinant hK14[myc-His] by Western blot analysis using anti-His antibody. *Lane 1,* molecular weight markers; *Lane 2,* culture supernatant from a yeast clone transformed with pPICZαA vector containing *KLK14* cDNA 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 hK14[myc-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.

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 NH$_2$-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 NH$_2$ terminus of hK14[myc-His]. The next two amino acids, Glu-Phe, represent the amino acids of the *Eco*RI 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 hK14[myc-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 determine this, purified hK14[myc-His], before and after incubation with PNGase-F, was separated by SDS-PAGE on two identical polyacrylamide gels, one stained by Coomassie-blue and the other with a glycoprotein stain (Fig. 2). Horseradish peroxidase, a glycoprotein, and soybean trypsin inhibitor, a nonglycosylated protein, were also included as positive and negative controls, respectively. On treatment with PNGase-F, the molecular weight of hK14[myc-His] did not change (Fig. 2*A*). Furthermore, only the positive glycoprotein control was visualized on the gel stained for glycoproteins with acidic fuschin sulfite (Fig. 2*B*). Collectively, the results indicate that hK14[myc-His] is not glycosylated. Because both versions of recombinant hK14[myc-His] are unglycosylated, it is most probable that the 25 kDa version of hK14[myc-His] is a degraded species that arose via autocleavage or through cleavage by other proteases present in the yeast culture. These results, however, do not rule out the possibility that native hK14 may

be glycosylated *in vivo,* similar to other native kallikreins including hK1 (25), hK2 (26, 27), and hK3 (28).

Characteristics of the hK14 ELISA

Configuration. The generation of polyclonal antibodies against recombinant hK14[myc-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 hK14[myc-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 hK14[myc-His] (20 μg/liter) and hK14-positive samples (seminal 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
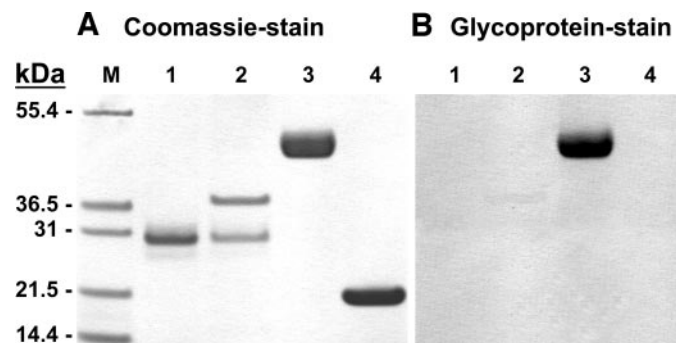

Fig. 2. SDS-PAGE of purified hK14[myc-His] before and after treatment with PNGase-F. In both *A* and *B, Lane 1* corresponds to purified hK14[myc-His]; *Lane 2,* purified hK14[myc-His] incubated with PNGase-F (38 kDa); *Lane 3,* horseradish peroxidase (40 kDa), positive control; and *Lane 4,* soybean 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 hK14[myc-His] in *Lane 2,* as would be expected after deglycosylation. In *B,* the gel was stained with acidic acidic fuchsin sulfite, a glycoprotein stain. Only the glycoprotein horseradish peroxidase (positive control) in *Lane 3* is stained, additionally confirming that recombinant hK14[myc-His] is not glycosylated. *M,* molecular weight marker.
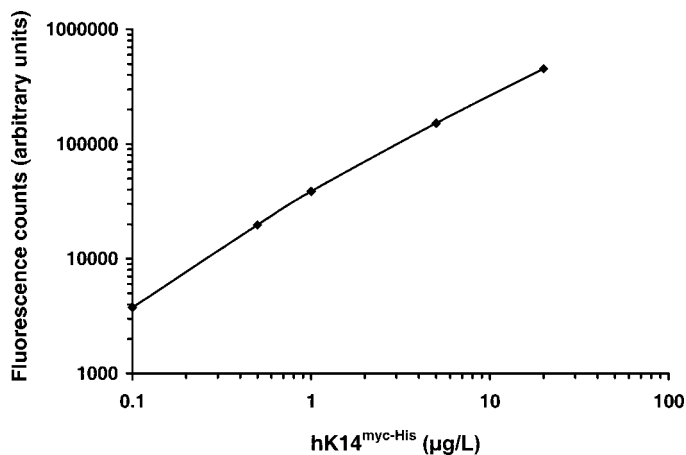
Fig. 3. A typical calibration curve for the hK14 ELISA. Background fluorescence (zero calibrator) has been subtracted from all measurements. The dynamic range of this assay is 0.1–20 μg/liter.
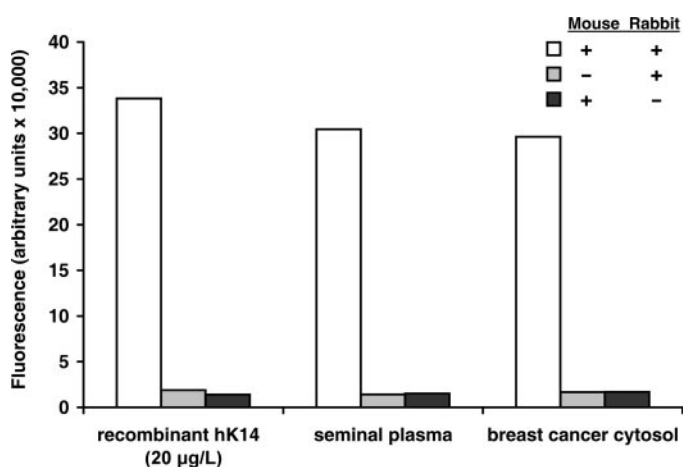

Fig. 4. Bar diagram showing the specificity of the hK14 immunoassay. The immunoreactivity of preimmune and immune mouse and rabbit sera was assessed by performing the immunoassay with mouse and rabbit immune sera (□), replacing mouse serum with preimmune mouse serum (▨) and replacing rabbit serum with preimmune rabbit serum (■). Note the significant decrease in fluorescence (immunoreactivity) when immune hK14-sera are replaced with preimmune sera.

to hK14. Second, Western blot analysis of purified recombinant hK14$^{myc-His}$ using rabbit preimmune and immune sera produced bands corresponding to hK14$^{myc-His}$ (1 μg and 100 ng) only when the membrane was probed with immune rabbit sera (Fig. 5). No bands representing hK14$^{myc-His}$ 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$^{myc-His}$) 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^{-8} 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-
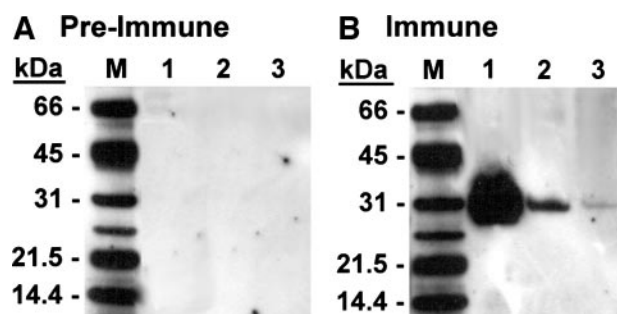

Fig. 5. Specificity of rabbit anti-hK14$^{myc-His}$ polyclonal antibodies. Purified recombinant hK14$^{myc-His}$ (1000, 100, and 20 ng/lane, in *Lanes 1, 2,* and *3,* respectively) was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with preimmune (*A*) and immune (*B*) rabbit sera (1:2000). Note the absence of bands in *A* indicating the absence of hK14-specific antibodies, as anticipated. In *B,* hK14 specific bands are visualized. Band intensity diminishes with decreasing amounts of hK14$^{myc-His}$. *M,* molecular weight marker.
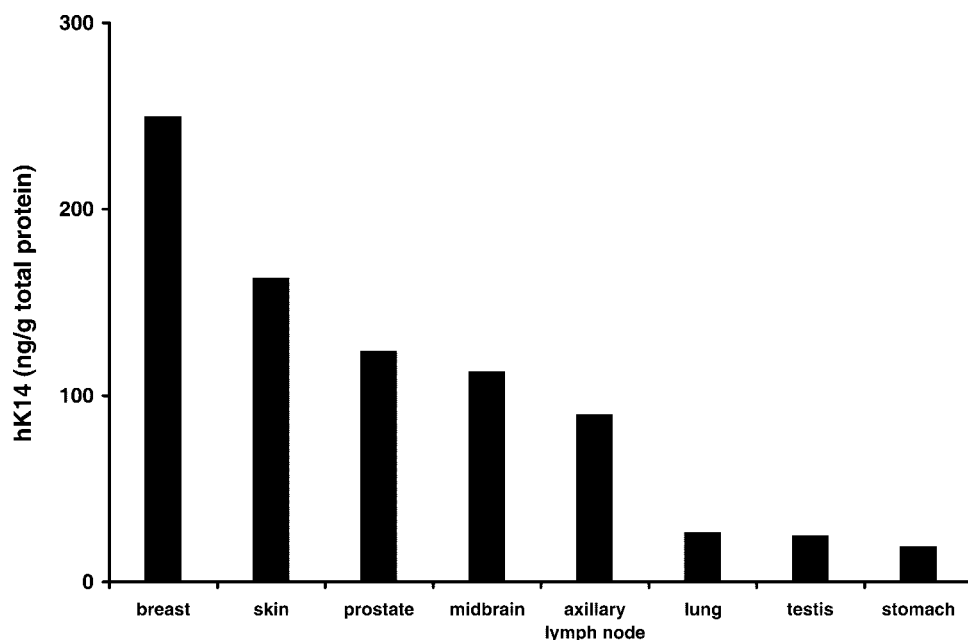
Fig. 6. Concentration of hK14 in various adult human tissues.

els, whereas norgestrel produced a 2.8-fold increase. These data suggest that *KLK14* gene expression is mainly up-regulated by estrogens in the BT-474 breast cancer cell line.

hK14 Expression in Normal, Benign, and Cancerous Ovarian Cytosols. The levels of hK14 in 20 ovarian cancer tissue extracts, along with 10 normal ovarian tissues and 10 from patients with benign disease, were quantified using the hK14 immunoassay. The hK14 values were corrected in terms of total protein and expressed as ng of hK14 per mg total protein. The results are displayed in Fig. 9. The levels of hK14 in normal ovarian tissue extracts did not exceed 32.9 ng/g. Although 5 of 10 extracts from the benign disease group surpassed this level, 4 of these values (with the exception of 1 extract measuring 338 ng/g) remained below 81.3 ng/g. Interestingly, 8 of 20 (40%) ovarian cancer tissue extracts contained even higher levels of hK14, all greater than 143 ng/g, in comparison with that of normal and benign tissues (with that one exception). These data imply that in ovarian cancer patients, hK14 is generally overexpressed, in contrast to those with normal or benign disease.

hK14 in the Serum of Cancer Patients. hK14 levels were analyzed in a total of 91 serum samples derived from patients with various malignancies, including ovarian ($n = 20$), breast ($n = 20$), prostate ($n = 31$), testicular ($n = 10$), and colon ($n = 10$) cancers, along with 27 and 28 serum samples from healthy normal male and female subjects, respectively. hK14 was not detected in the serum of normal females, and reached a high level of 0.16 μg/liter in healthy male sera examined (Table 1). Among patients with cancer (Table 2), 13 (65%) women with ovarian cancer and 8 (40%) with breast cancer demonstrated elevated levels of hK14, 0.12–1.58 μg/liter and 0.12–0.3 μg/liter, respectively (Fig. 10). Five individuals (16%) with prostate cancer displayed elevated hK14 levels (0.23–0.62 μg/liter), whereas only 2 (20%) patients with colon cancer (0.18 and 0.26 μg/liter) and 1 (10%) with testicular carcinoma (0.49 μg/liter) possessed eminent hK14 concentrations.

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 sur-

vival. 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 *KLK14*, 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, the specific reagents and tools necessary to qualitatively and quantitatively examine hK14 were not available.

In the present study, we produced recombinant hK14[myc-His] 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[myc-His] 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
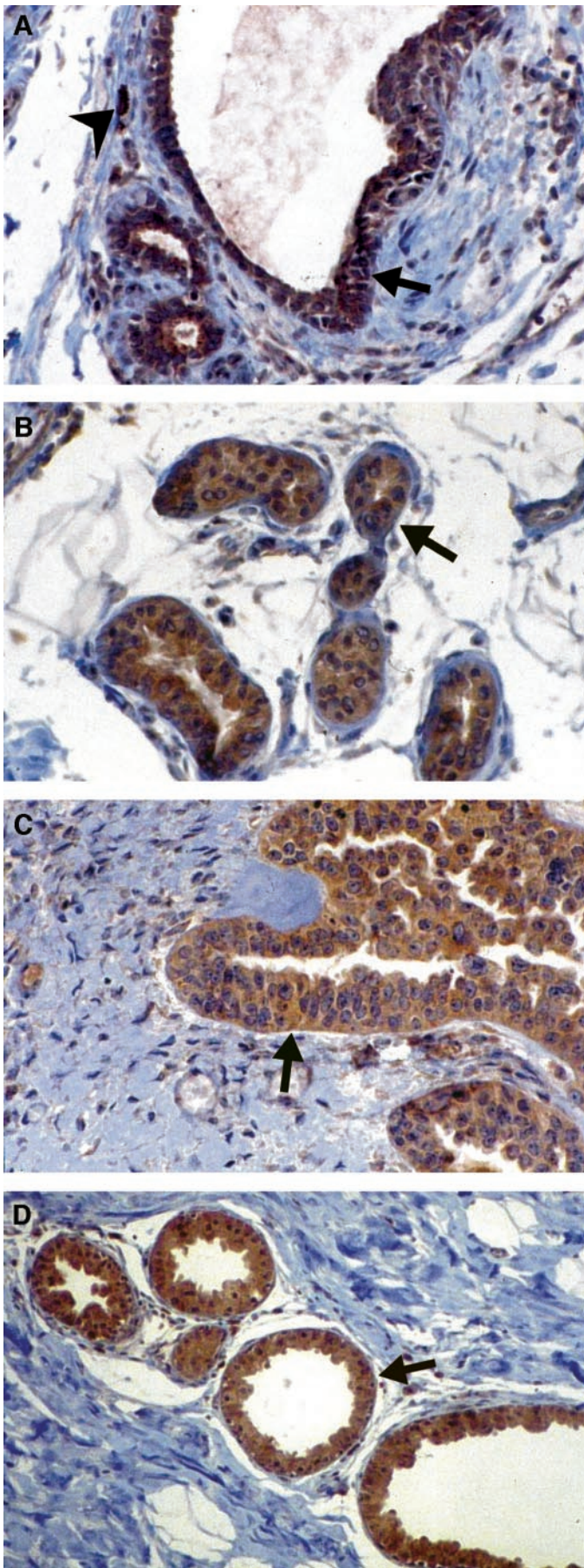
hK14 concentration (38). In fact, it has been well documented that other kallikreins, including hK2, hK3, and hK6, are sequestered (mainly in serum) by circulating protease inhibitors including α_2-macroglobulin, protein C inhibitor, α_1-antichymotrypsin α_2-antiplasmin, α_1-antitrypsin, antithrombin, 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

Fig. 7. Immunohistochemical localization of hK14 in normal (*A* and *B*) and malignant (*C* and *D*) tissues with the polyclonal hK14 rabbit antibody (dilution, 1:400). *A,* cytoplasmic immunoexpression in ductal columnar and myoepithelial cells of the breast (magnification ×200). *B,* strong immunoexpression by the epithelium of the eccrine glands of the skin (magnification ×200). *C,* staining by a moderately differentiated cystadenocarcinoma of the ovary (magnification ×200). *D,* strong cytoplasmic immunoexpression by epithelium with apocrine metaplasia of the breast (magnification ×100).

Table 1 *Concentration of hK14 in various biological fluids*

| Biological fluid	Number of samples tested	hK14 concentration (μg/liter)			Positivity (%)
		Range	Mean (SD)	Median	
Seminal plasma	36	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[a]	51	0–0.77	0.063 (0.16)	0	18
Serum					
Male	27	0–0.16	0.02 (0.05)	0	11
Female	28	0	0 (0)	0	0
Cerebrospinal fluid	6	0	0	0	0
Breast milk	5	0	0	0	0

[a] From ovarian cancer patients.
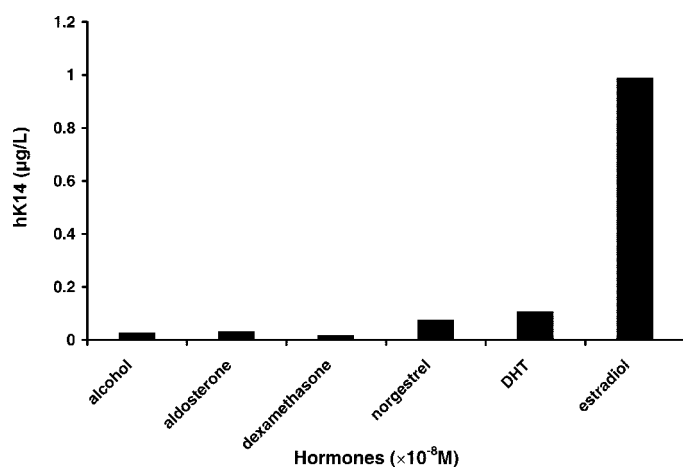
Fig. 8. Hormonal regulation of hK14 in the breast cancer cell line BT-474. hK14 is mainly up-regulated by estradiol, followed by dihydrotestosterone (*DHT*) and norgestrel.
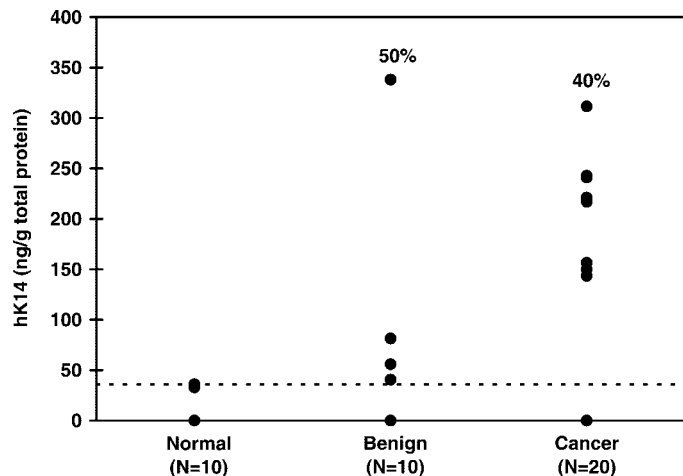

Fig. 9. Concentration of hK14 in extracts from normal, benign, and cancerous ovarian tissues. *n* = number of tissues extracted. The percentage of samples containing higher hK14 levels compared with highest normal tissue extract is shown.

estrogen receptor-positive breast cancer cell line BT-474, after stimulation with various steroid hormones, indicated that the *KLK14* gene is significantly up-regulated by estrogens, followed by androgens (dihydrotestosterone) and androgenic progestins (norgestrel). Although these results suggest that *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 *KLK14* gene to explain the effect of steroid hormones on hK14 expression.

It has been reported previously that the *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

Table 2 *Concentration of hK14 in sera of patients with various malignancies*

Malignancy	Number of serum samples	Patients (%) with hK14 serum values > normal[a]
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%)

[a] Normal female = 0 μg/liter; normal male ≤ 0.16 μg/liter.
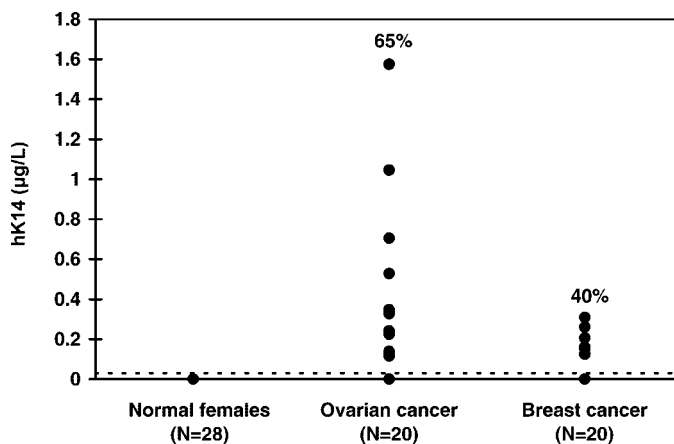

Fig. 10. Serum hK14 (μg/liter) levels in patients with ovarian and breast cancer compared with normal females. Serum hK14 is elevated in 65% of women with ovarian cancer and 40% with breast cancer when using a cutoff value equal to the lower detection limit (0.1 μg/liter) of the immunoassay (indicated by ⋯).

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.

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