

Human Kallikrein 5: A Potential Novel Serum Biomarker for Breast and Ovarian Cancer¹

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ABSTRACT

The kallikrein family is a group of 15 serine protease genes clustered on chromosome 19q13.4. *Human kallikrein (hK) gene 5 (KLK5)* is a member of this family and encodes for a secreted serine protease (hK5). *KLK5* was shown to be differentially expressed at the mRNA level in breast and ovarian cancer. Until now, detection of hK5 protein in either biological fluids or tissues has not been described due to lack of suitable reagents and methods. The aim of this study was to develop immunological reagents and a sensitive and specific fluorometric immunoassay (ELISA) for hK5, to examine the presence of hK5 in human tissues and biological fluids, and to study the possible clinical utility of hK5 as a biomarker for endocrine-related malignancies. Recombinant hK5 protein was produced and purified using a *Pichia pastoris* yeast expression system. The protein was used as an immunogen to generate mouse and rabbit polyclonal anti-hK5 antibodies. A sandwich-type microplate immunoassay (ELISA) was developed using these antibodies, coupled with a time-resolved fluorometric detection technique. The ELISA assay was then used to measure hK5 in various biological fluids, tissue extracts, and serum samples from normal individuals and patients with various malignancies. The hK5 ELISA immunoassay has a lower detection limit of 0.1 µg/liter, is specific for hK5, and has no cross-reactivity with other homologous kallikreins. The dynamic range is 0.1–25 µg/liter, and within-run and between-run coefficients of variation within this range are <10%. hK5 is found in many tissues, with the highest expression levels seen in the skin, breast, salivary gland, and esophagus. hK5 is present at relatively high levels in milk of lactating women. Whereas the levels of hK5 are almost undetectable in serum of normal individuals (male and female) and patients with diverse malignancies, higher concentrations were found in a proportion of patients with ovarian (69%) and breast (49%) cancer. High levels were also detected in ascites fluid from metastatic ovarian cancer patients and in ovarian cancer tissue extracts. In conclusion, we report development of the first immunofluorometric assay for hK5 and describe the distribution of hK5 in biological fluids and tissue extracts. Our preliminary data indicate that hK5 is a potential biomarker in patients with ovarian and breast cancer.

INTRODUCTION

The hK³ gene family was, until recently, thought to consist of only three genes: (a) pancreatic/renal kallikrein (*KLK1*, encoding for hK1 protein); (b) human glandular kallikrein 2 (*KLK2*, encoding for hK2 protein); and (c) hK3 (*KLK3*, encoding for hK3 protein or PSA). This gene family has already contributed an excellent biomarker (PSA, hK3) that is currently used for diagnosis and monitoring of prostate

cancer and hK2 as another promising prostate cancer biomarker (1). More recently, new members of the hK gene family have been discovered. This gene family now includes 15 genes which are all encoding for serine proteases, showing significant homologies at both the DNA and the amino acid level and they are all localized in tandem on human chromosome 19q13.4. Recent developments on this gene family have been reviewed (2, 3).

The *hK gene 5 (KLK5)*, according to the official kallikrein gene nomenclature; Ref. 4), previously known as kallikrein-like gene 2 (*KLK-L2*; Ref. 5) or *human stratum corneum tryptic enzyme (HSCTE*; Ref. 6), is a newly identified member of the hK gene family that maps to chromosome 19q13.4, close to other kallikrein genes (7). By reverse transcription-PCR analysis, it has been reported that this gene is mainly expressed in the skin, testis, breast, and brain (5, 6). The protein sequence has the conserved catalytic triad of serine proteases (5). The hK5 protein is predicted to be a secreted serine protease, and the enzyme was found to have proteolytic activity (6). *KLK5* gene expression at the mRNA level was also found to be regulated by steroid hormones in the BT-474 breast cancer cell line (5). We have recently shown that *KLK5* is differentially expressed at the mRNA level in many endocrine-related malignancies, including ovarian (8), breast (9), and testicular cancer (10), and that it has the potential of being a new cancer biomarker. Here, we study the expression of hK5 at the protein level in various tissues and biological fluids and provide the first evidence that hK5 protein is elevated in serum of patients with ovarian and breast cancer and in the ascites fluid of patients with advanced ovarian cancer.

MATERIALS AND METHODS

Production and Purification of Recombinant hK5

Cloning of *KLK5* cDNA into a Yeast Expression System. Recombinant hK5 was produced using the *Pichia pastoris* yeast expression system (Invitrogen, Carlsbad, CA). Two primers were designed to amplify the *KLK5* cDNA sequence: (a) 5'-AGC-AGC-CGA-TCG-ATC-AAT-GGA-TCC-GAC-TG-3' (forward primer); and (b) 5'-CTG-AGT-CCT-GGG-ATG-TCT-AGA-GAG-TTG-GC-3' (reverse primer). Human breast cDNA was used as a template. PCR was carried out in a 50-µl reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 10 µM deoxynucleoside triphosphates, 30 µM primers, and 0.75 µl of Expand Long Template PCR polymerase mix (Roche Diagnostics, Mannheim, Germany), using an Eppendorf master cycler. The PCR conditions were 94°C for 2 min, followed by 94°C for 10 s, 54°C for 30 s, and 68°C for 1 min for 40 cycles and a final extension at 68°C for 7 min. The PCR product was then cloned into the yeast expression vector pPICZα using standard procedures (11). The sequence of the construct was confirmed with an automated DNA sequencer.

Production of hK5 in Yeast. The pPICZαC vector containing the mature *KLK5* cDNA sequence was introduced into the yeast strains X-33, KM71, and GS115. A stable clone was selected from the KM71 strain following the manufacturer's recommendations (Invitrogen). hK5 was produced by growing the stable yeast clone in a medium containing 10 g/liter yeast extract, 20 g/liter peptone, 100 mM potassium phosphate (pH 6.0), 13.4 g/liter yeast nitrogen base, and 40 mg/liter biotin in a 30°C shaking incubator (250 rpm). hK5 production was induced with 1% methanol/day over 6 days. The cells were

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³ The abbreviations used are: hK, human kallikrein; PSA, prostate-specific antigen; MES, 4-morpholineethanesulfonic acid; FIGO, International Federation of Gynecologists and Obstetricians; CGAP, Cancer Genome Anatomy Project; EST, expressed sequence tag; DHT, dihydrotestosterone; MS-MS, tandem mass spectrometry.

subsequently spun down, and the supernatant was collected. A hK5 rabbit polyclonal peptide antibody (produced in-house) was used to monitor hK5 production by Western blot analysis.

Purification of hK5 with Cation-Exchange and Reversed-Phase Chromatography. Because the hK5 protein is predicted to be a secreted protein, recombinant hK5 was purified from yeast culture supernatants by cation-exchange chromatography using CM-Sepharose fast flow (Pharmacia Biotech, Piscataway, NJ) and reversed-phase liquid chromatography using a C₄ column (0.45 × 5 cm; Vydac). The presence of hK5 in various fractions was identified by Western blotting using an anti-hK5 peptide antibody. In summary, the CM-Sepharose beads, previously activated with 1 M KCl, were equilibrated in 10 mM MES buffer (pH 6.5). The yeast culture supernatant was then diluted 4-fold with 10 mM MES buffer (pH 6.5), the pH was adjusted to 6.5, and the supernatant was applied to the column. After washing the column, hK5 was eluted with 300 mM KCl in 10 mM MES buffer (pH 6.5). Trifluoroacetic acid as ion-pairing agent was added into this eluate (final concentration, 10 ml/liter), which was then loaded on a C₄ column and equilibrated with 1 ml/liter trifluoroacetic acid in water. A linear gradient (1%/min) of acetonitrile from 15% to 50% in 1 ml/liter trifluoroacetic acid was then performed. The fraction containing hK5 was evaporated on a SpeedVac (Savant). The purified material was then separated by SDS-PAGE and stained with Coomassie Blue to assess its purity and its molecular mass. The protein concentration of the purified hK5 was determined by the bicinchoninic acid method, which uses BSA as calibrator (Pierce Chemical Co., Rockford, IL).

Characterization of hK5 by Mass Spectrometry. Purified hK5 was applied to polyacrylamide gels (~1 μg) and stained with Coomassie G-250 for visualization. The stained band was subsequently excised and destained with 300 ml/liter acetonitrile in 100 mM ammonium bicarbonate. The band was then reduced [10 mM DTT in 50 mM ammonium bicarbonate (pH 8.3)] and alkylated [50 mM iodoacetamide in 50 mM ammonium bicarbonate (pH 8.3)] before overnight trypsin digestion. Peptide fragments were then extracted with 50 ml/liter acetic acid, evaporated dry on a Savant concentrator, and reconstituted in 10 μl of a solution of methanol-water-acetic acid (500:495:5 by volume).

All nanoelectrospray mass spectrometry experiments were conducted on a Q-star (PE/Sciex, Concord, Ontario, Canada) hybrid quadrupole/time-of-flight instrument, for high resolution and online tandem mass spectrometry (MS-MS) experiments (12). Conventional mass spectra were obtained by operating the quadrupole in a radiofrequency-only mode while a pusher electrode was pulsed at a frequency of approximately 7 kHz to transfer all ions to the time-of-flight analyzer. MS-MS experiments on trypsin-digested peptides identified in survey scans were conducted using a nanoelectrospray source. Precursor ions were selected by the first quadrupole while a pusher electrode was pulsed at a frequency of approximately 7 kHz to transfer fragment ions formed in the radiofrequency-only quadrupole cell to the time-of-flight analyzer. Mass spectral resolution was typically 9,000–10,000. A scan duration of 1 and 2 s was set for conventional and MS-MS mass spectral acquisition, respectively. Collisional activation was performed using nitrogen collision gas with typically a 30-V offset between the DC voltage of the entrance quadrupole and the radiofrequency-only quadrupole cell. Data were acquired and processed using LC Tune and Biomultiview programs from PE/Sciex.

Production of Antibodies against hK5

The purified recombinant hK5 protein was used as an immunogen to immunize rabbits and mice. hK5 (100 μg) was injected s.c. into Balb/C female mice and New Zealand White female rabbits. The protein was diluted 1:1 in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for the subsequent injections. Injections were repeated six times at 3-week intervals. Blood was drawn from the animals and tested for antibody generation. To test for production of anti-hK5 polyclonal antibodies in mice and rabbits, we used the following immunoassay. Sheep antimouse or goat antirabbit IgG, respectively (Jackson ImmunoResearch, West Grove, PA), was immobilized on 96-well white ELISA plates. The mouse/rabbit serum was then applied to the plates at different dilutions ranging from 1:500 to 1:50,000. After incubation (1 h) and washing, biotinylated recombinant hK5 was added to each well (5–10 ng/well). Finally, after incubation (1 h) and washing, alkaline phosphatase-conjugated streptavidin was added, incubated for 15 min, and washed, and the alkaline phosphatase activity was detected with time-resolved fluorescence (for details, see below).

Immunofluorometric Assay (ELISA) for hK5

Standard Assay Procedure. A sandwich-type, noncompetitive immunoassay (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 mM Tris buffer (pH 7.80)] in each well. The plates were then washed six times with the washing buffer [9 g/liter NaCl and 0.5 g/liter Tween 20 in 10 mM Tris buffer (pH 7.4)]. Mouse anti-hK5 polyclonal antiserum was diluted 2,000-fold in a general diluent (60 g/liter BSA, 50 mM Tris (pH 7.80), and 0.5 g/liter sodium azide), and 100 μl were applied to each well. After a 2-h incubation, the plates were washed six times with washing buffer.

hK5 calibrators or samples were then pipetted into each well (50 μl/well) along with 50 μl of the general diluent and incubated for 1 h with shaking; the plates were then washed with washing buffer six times. Subsequently, 100 μl of rabbit anti-hK5 antiserum, diluted 500-fold in buffer A (containing the components of the general diluent plus 25 ml/liter normal mouse serum, 100 ml/liter normal goat serum, and 10 g/liter bovine IgG), were applied to each well and incubated for 1 h; plates were then washed as described earlier. Finally, 100 μl of alkaline phosphatase-conjugated goat antirabbit IgG, Fc fragment-specific antibody (Jackson ImmunoResearch), diluted 3,000-fold in buffer A, were added to each well and incubated for 45 min, and plates were washed as described above.

Diflunilal phosphate (100 μl of a 1 mM solution) in substrate buffer [0.1 M Tris (pH 9.1), 0.1 M NaCl, and 1 mM MgCl₂] was added to each well and incubated for 10 min. Developing solution (100 μl, containing 1 M Tris base, 0.4 M NaOH, 2 mM TbCl₃, and 3 mM EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was then 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 (13).

Determination of the Sensitivity of the hK5 Immunoassay. Recombinant hK5 was used to generate the calibration curve. hK5 calibrators were prepared by diluting the purified recombinant hK5 in the general diluent. These calibrators were then used to define the detection limit of the assay.

Determination of the Specificity of the hK5 Immunoassay. Biological fluids and recombinant hK5 were used to determine the specificity of the developed immunoassay. These samples were first measured with the standard assay procedure described above. The mouse and rabbit anti-hK5 antisera were then successively replaced with sera from the same animals, obtained before immunization (preimmune sera). The samples were measured again, and the fluorescence counts were compared with the counts obtained by the standard assay. The cross-reactivities of other homologous proteins were investigated using purified recombinant hK1-hK15 (available in-house), using 100–1,000-fold higher concentrations, all diluted in the general diluent and measured using the same assay. All other kallikreins were also calibrated by the bicinchoninic acid method using BSA as calibrators.

Determination of Assay Precision, Linearity, and Recovery. To assess the precision of the assay, hK5 calibrators and various clinical samples (two milks, two seminal plasmas, and three amniotic fluids) were analyzed either within-run (10 replicates) or between-run (10 replicates over 2 weeks). To assess the linearity of the assay, two milks, two seminal plasmas, two amniotic fluids, two sera with elevated hK5 values, one skin extract, and one ovarian cancer tissue extract were serially diluted with 60 g/liter BSA and reassayed. Recovery was assessed by adding recombinant hK5 to milk, ascites fluid from ovarian cancer patients, and serum samples from men and women.

Preparation of Human Tissue Extracts and Biological Fluids

The following human tissues (adult and fetal) were used for screening: esophagus; tonsil; skin; testis; kidney; salivary gland; breast; fallopian tube; adrenal gland; bone; brain; cerebellum; colon; endometrium; liver; lung; muscle; ovary; pancreas; pituitary gland; prostate; seminal vesicle; small intestine; spinal cord; spleen; stomach; thyroid; trachea; and ureter. Human tissue extracts were prepared as follows: frozen human tissues (0.2 g) were pulverized on dry ice to fine powders. Two ml of extraction buffer [50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 5 mM EDTA, and 1% NP40 surfactant] were 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 centrifuged at $14,000 \times g$ at 4°C for 30 min. The supernatants representing the tissue extracts were collected and stored at -80°C until use. The biological fluids (amniotic fluid, breast milk, cerebrospinal fluid, follicular fluid, serum, and seminal plasma) were leftovers of samples submitted for routine biochemical testing and stored at -80°C until use. Our procedures have been approved by the Institutional Review Board of Mount Sinai Hospital.

Preparation of cytosol fractions from normal tissues as well as from tissues of patients with benign ovarian disease or from tumor tissues of patients with primary ovarian cancer, at the Department of Obstetrics and Gynecology, Technical University of Munich (Munich, Germany), was described previously by Schmalfeldt *et al.* (14). Information on stage and grade was not available. Cytosol fractions were stored in liquid nitrogen until use. Ascites fluids were obtained from patients with ovarian cancer FIGO stage II ($n = 9$), III ($n = 18$), and IV ($n = 4$) by tapping the patients and collecting fluid into a sterile bag, after flushing the needle and bag with heparin. The ascites fluids were then centrifuged at $10,000 \times g$ for 30 min to sediment cells and any debris. The supernatants were harvested, aliquoted, and stored at -80°C until use. The study to collect tissue and ascites fluid from ovarian cancer patients to assess the patients' risk profiles was approved by the Ethics Committee of the University Hospital (Klinikum rechts der Isar) of the Technical University of Munich, and patients have signed the informed consent form to allow their tissues and ascites fluids to be used for scientific purposes. All patients received their treatment according to consensus recommendations at that time.

Recovery

Recombinant hK5 was added to a general diluent (control), male and female sera, and various biological fluids at different concentrations, and the spiked samples were measured. Recoveries were then calculated after subtraction of the endogenous concentrations.

Fractionation of Biological Fluids with Size-Exclusion High-Performance Liquid Chromatography

To determine the molecular mass of the hK5 protein detected in the biological fluids and tissue extracts, various samples were fractionated with gel filtration chromatography, as described elsewhere (15). The fractions were collected and analyzed for hK5 using the developed immunoassay. For comparison purposes, we also analyzed fractions for hK6 using a previously published assay (16).

Cancer Cell Lines and Hormonal Stimulation Experiments

The breast cancer cell lines MDA-MB-231, BT-474, T-47D, ZR-75, and MCF-7; the ovarian cancer cell line HTB-75 (Caov-3); and the prostate carcinoma cell lines LNCaP and PC-3 were purchased from American Type Culture Collection (Manassas, VA). The BG-1 ovarian cancer cell line was kindly provided by Dr. Henri Rochefort (Montpellier, France), and the PC-3 cell line stably transfected with androgen receptor [PC-3 (AR)₆] was kindly provided by Dr. Theodore Brown (Toronto, Ontario, Canada). Cells were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with glutamine (200 mM) and fetal bovine serum (10%) in plastic flasks to near confluence. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluence. Twenty-four h before the experiments, the culture medium was changed to medium containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media at a final concentration of 10^{-8} M (ethanol final concentration, 0.1%). Cells stimulated with ethanol were included as controls. The cells were grown for 7 days, and the tissue culture supernatants were collected for hK5 quantification by fluorometric ELISA.

In Silico Analysis of KLK5 Expression

Analysis of KLK5 mRNA expression in a variety of normal and cancer cell libraries obtained from different tissues was performed using the database of the CGAP of the National Cancer Institute. Quantitative KLK5 transcript levels were assessed by "Virtual Northern" analysis of the Serial Analysis of Gene Expression database (SAGEmap). Comparison between normal and cancerous cDNA libraries was done using the EST database of the CGAP

through the "Digital Differential Display" search engine and the SAGEmap database through the "Xprofiler" search engine.

RESULTS

Production and Purification of hK5 Recombinant Protein. The cDNA encoding for the active form of hK5 was cloned into a *P. pastoris* yeast expression system. Expression in yeast produced a protein migrating around 35 kDa. The protein was purified by ion-exchange and reverse-phase chromatography essentially as described previously for hK10 protein (17). The purified protein was then run on a SDS-PAGE gel and stained with Coomassie Blue, and the band was excised from the gel and hydrolyzed by trypsin digestion. No other impurities were identified after Coomassie Blue staining. The tryptic digests were subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry, and selected fragments were further sequenced by using tandem mass spectrometry, as described previously (17). This analysis confirmed that the recombinant protein produced in yeast is hK5. For example, the peptides LGHYSL-SPVYESGQQMFQ and DVRPINVSSHCPASGTK correspond to the amino acid sequences 118–135 and 168–184 of hK5 protein (GenBank accession number AF135028). Because the predicted molecular mass of nonglycosylated hK5 is approximately 32 kDa (5), we assume that the recombinant hK5 protein, like other kallikreins produced in *Pichia*, is glycosylated.

Recombinant hK5 protein was injected into mice and rabbits as described in "Materials and Methods" to generate polyclonal antibodies. High titers of specific antibodies were detected in serum of mice and rabbits after the third booster injection. These antibodies were used for the development of the hK5 immunofluorometric assay. We adopted a sandwich-type assay configuration (ELISA) in which the capture antibody was generated in mice, and the detection antibody was generated in rabbits. This assay configuration does not necessitate any prior antibody purification and was found previously to be highly specific and sensitive for other kallikreins, including hK6 (16), hK10 (17), and hK11 (18).

Sensitivity, Specificity, Linearity, and Precision of the hK5 Immunofluorometric Assay. The detection limit of the hK5 immunofluorometric assay, defined as the concentration of hK5 that can be distinguished from zero with 95% confidence, was $0.1 \mu\text{g/liter}$, and the dynamic range extends to $25 \mu\text{g/liter}$. We have further confirmed that the assay specifically measures hK5. When we successively replaced mouse and rabbit antisera with preimmune mouse and rabbit sera, the fluorescence signals of standards or hK5-positive samples were reduced to nearly zero (data not shown). Because hK5 is a member of the hK family, it shares significant amino acid homology with other kallikreins (2). The hK5 protein shows 50–51% identity with hK8, hK9, hK11, hK13, and hK14; 45–49% identity with hK6, hK7, hK12, and hK15; and 38–44% identity with hK1, hK2, hK3, hK4, and hK10. To demonstrate that there is no interference from these homologous proteins, the cross-reactivities of recombinant hK1, hK2, hK3, hK4, hK6, hK7, hK8, hK9, hK10, hK11, hK12, hK13, hK14, and hK15 were examined. All recombinant proteins produced no measurable readings, even at concentrations 1000-fold higher than that of hK5. These data demonstrate that this immunoassay can efficiently discriminate hK5 from other homologous proteins and that it measures hK5 with high specificity.

To assess the linearity of this assay, various clinical samples were diluted serially, and hK5 was re-measured. These samples included milks, seminal plasmas, and amniotic fluids. In all samples, we found a good dilution linearity with this assay, suggesting freedom from matrix effects (obtained values with diluted samples were within 10% of values obtained with undiluted samples). Within-run and between-

run precision was assessed with various hK5 calibrators and clinical samples over 2 weeks ($n = 10$). In all cases, the coefficients of variation were between 3% and 8% within the dynamic range of the assay (0.1–25 $\mu\text{g/liter}$).

hK5 Recovery from Biological Fluids. We tested the recovery of added recombinant hK5 in 6% BSA (as a control) and in milk, ascites fluid, and serum. Samples were incubated for 1 h at room temperature after spiking. Recoveries ranged from 90–100% in BSA, 80–90% in milk, 60–75% in ascites fluid, and 50–60% in male and female serum. About the same recovery was obtained when serum samples from males and females were spiked with native hK5 present from milk (data not shown). The recovery data in serum suggested that hK5 may be sequestered in serum by proteinase inhibitors, similarly to other kallikreins, including hK3 (PSA) and hK2 (Refs. 19–22; see also the text below).

Expression of hK5 in Human Tissues and Presence in Biological Fluids. The expression of hK5 in human tissues was investigated by analyzing tissue extracts with the developed hK5 immunoassay. Tissues were collected at autopsy. The data for adult and fetal tissues are graphically shown in Fig. 1. The highest hK5 levels in adult tissues were seen in skin, followed by breast, salivary gland, esophagus, cerebellum, seminal vesicle, hippocampus, spinal cord, axillary lymph node, pituitary gland, testis, and lung. The following tissues were either weakly positive or negative for hK5: bone; kidney; colon; liver; muscle; pancreas; prostate; spleen; thyroid; stomach; small intestine;

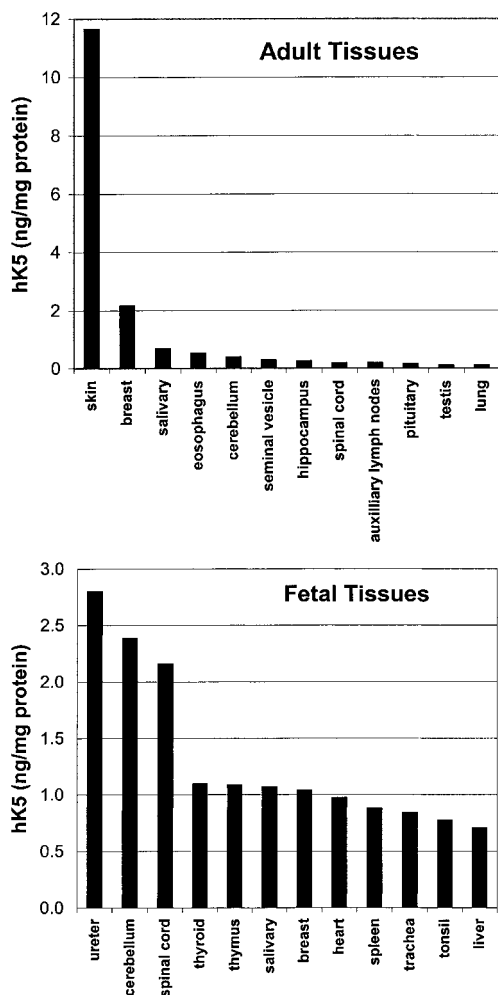


Fig. 1. hK5 content of cytosolic extracts of various adult and fetal human tissues. All hK5 concentrations were corrected for the amount of total protein.

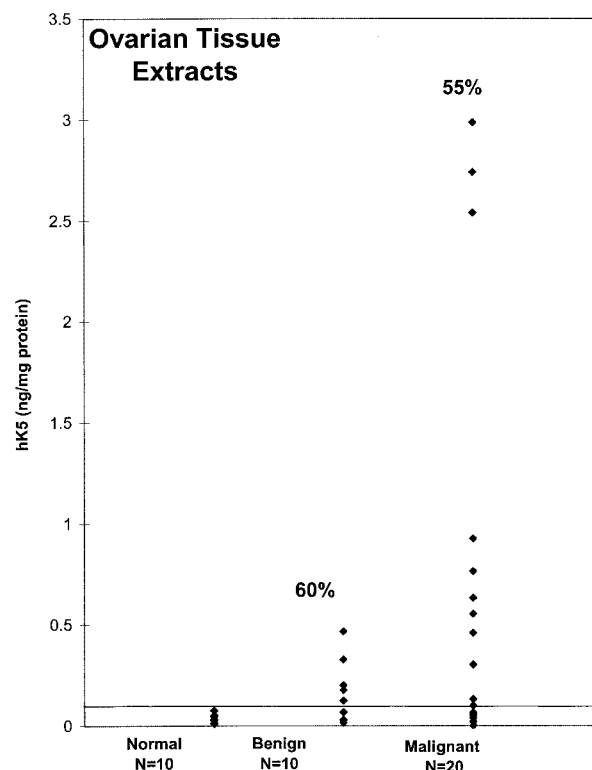


Fig. 2. hK5 protein content (ng/mg total protein) of cytosolic extracts of normal ovaries, benign ovarian tissue, and ovarian cancer. Note the higher levels of hK5 in about 55–60% of extracts from benign ovarian tissue and cancer. The highest levels are seen in cancer tissues. $N =$ number of tissues extracted. The horizontal line indicates 100th percentile for normal tissues.

trachea; endometrium; fallopian tube; ovary; ureter; mesentery lymph node; uterus; tonsil; frontal cortex; medulla; midbrain; occipital cortex; pons; and temporal lobe. In fetal tissues, the highest levels of hK5 were seen in the ureter, cerebellum, and spinal cord, and lower levels were seen in many other tissues (Fig. 1).

We also quantified hK5 protein in various biological fluids of human origin. The highest levels were seen in milk of lactating women. Among six samples, the range was 10–409 $\mu\text{g/liter}$, with a mean of 120 $\mu\text{g/liter}$ and a median of 61 $\mu\text{g/liter}$. The other tested fluids (6 samples/fluid type) had much lower amounts (up to 0.17 $\mu\text{g/liter}$ for cerebrospinal fluid, up to 0.9 $\mu\text{g/liter}$ for amniotic fluid, up to 0.4 $\mu\text{g/liter}$ for seminal plasma, and up to 0.35 $\mu\text{g/liter}$ for follicular fluid).

Ovarian Tissue Extracts. Preliminary data indicated that this gene, *KLK5*, like other kallikreins (23–26), may be up-regulated in ovarian cancer (8). To examine this further, we prepared cytosolic extracts from 10 normal ovarian tissues, 10 tissues from benign ovarian disease patients, and 20 tissues from ovarian cancer. These tissues were obtained at the time of surgery and before initiation of any other treatment. We then quantified hK5 protein with the developed immunoassay. After correction for the total protein content of each extract, the results are graphically presented in Fig. 2. Clearly, hK5 protein expression is higher in about 55–60% of patients with either benign ovarian disease or ovarian cancer. However, the levels of hK5 protein in the ovarian cancer tissue extracts is significantly higher than the levels in either normal or benign disease tissue extracts.

Ascites Fluid of Ovarian Cancer Patients. We quantified hK5 in 31 ascites fluid samples obtained from patients with metastatic ovarian carcinoma (stages II, III, and IV disease). All samples were positive for hK5, with values ranging from 1 to 300 $\mu\text{g/liter}$, with a

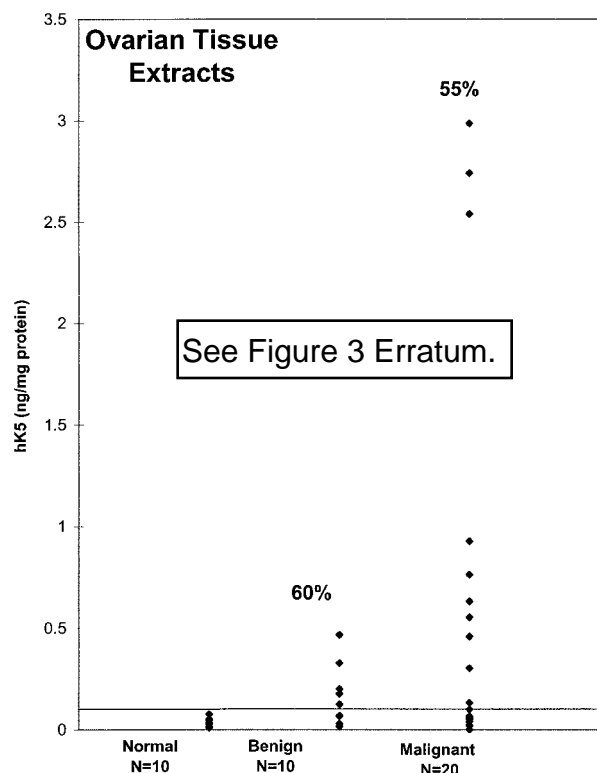
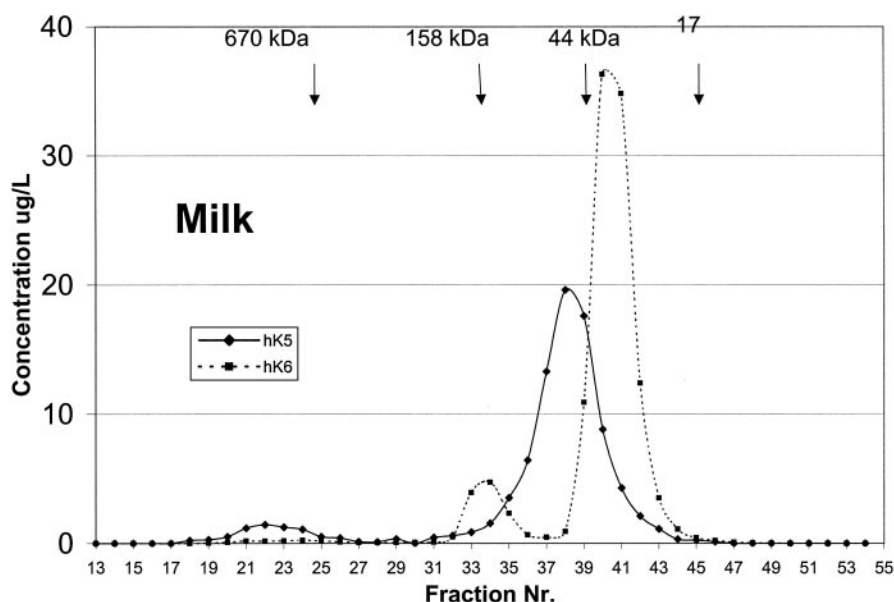


Fig. 3. Distribution of hK5 concentration in serum of patients diagnosed with various malignancies. Sera from normal female and male subjects were also included. High hK5 concentration (>100th percentile of normals) is found in a proportion of patients with ovarian and breast cancers. For more details on patient numbers and proportion of patients with elevated values, see Results section.

mean of $32 \mu\text{g/liter}$ and a median of $6.8 \mu\text{g/liter}$. After correcting for total protein in these ascites samples, the hK5 concentration (expressed as $\mu\text{g hK5/g total protein}$) was 0.094 ± 0.068 (mean \pm SD), the range was $0.01\text{--}0.30 \mu\text{g/g}$, and the median was $0.07 \mu\text{g/g}$. For these patients, we also had information on age, serum CA125, and FIGO stage. Statistical analysis has indicated that there were no significant correlations between ascites fluid hK5 concentration and either patient age, CA125 concentration, or FIGO stage ($P > 0.05$ by Fisher's exact test; data not shown).

See Figure 4 Erratum.

Fig. 4. Fractionation of three biological fluids (serum, ascites fluid from an ovarian cancer patient, and breast milk) by size-exclusion liquid chromatography. The elution profile of molecular mass standards is denoted by arrows. In serum, hK5 elutes as two immunoreactive peaks: one with an apparent molecular mass of 50 kDa (fractions 37–39); and one with an apparent molecular mass of approximately $150\text{--}180 \text{ kDa}$ (fractions 31–33). The elution profile of another kallikrein with a similar theoretical molecular mass, hK6, is also shown by dashed lines. This kallikrein elutes at a molecular mass of $\sim 35 \text{ kDa}$, corresponding to free hK6. In ascites fluid, the same comments apply as for serum. In breast milk, hK5 elutes mainly as a single immunoreactive peak. hK6 elutes as two distinct peaks, one at a molecular mass of $\sim 35 \text{ kDa}$ and another one at a molecular mass of $\sim 100 \text{ kDa}$.

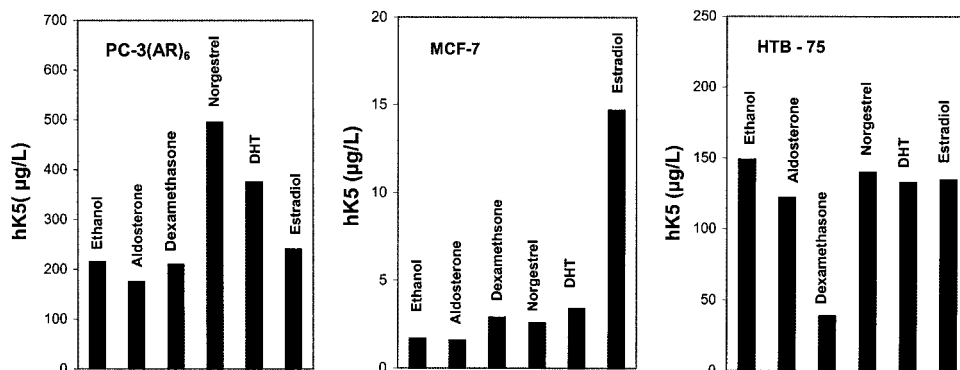


hK5 Is a Potential Serum Biomarker for Ovarian and Breast Cancer. A total of 263 serum samples were analyzed for hK5 by the developed ELISA assay. These samples were obtained from normal individuals [males ($N = 40$) and females ($N = 40$)] and patients diagnosed with various malignancies including prostate ($N = 21$), breast ($N = 39$), ovarian ($N = 69$), thyroid ($N = 10$), colon ($N = 25$), pancreatic ($N = 9$), and hepatocellular carcinomas ($N = 10$). The results are graphically presented in Fig. 3. A cutoff value of $0.1 \mu\text{g/liter}$ was chosen, which represents the detection limit of the assay and the 100th percentile of normals. We found none of the normals and only four (15%) patients with colon cancer with hK5 concentrations slightly above the cutoff value (up to $0.4 \mu\text{g/liter}$). However, 67% of patients with ovarian cancer and 49% of patients with breast cancer had elevated hK5 levels in serum. In many of these patients, the serum levels were more than 20 times higher than the cutoff value (Fig. 3).

Fractionation of Biological Fluids with Size-Exclusion High-Performance Liquid Chromatography. To determine the molecular mass of the protein detected in biological fluids, samples were fractionated on a gel filtration column. The presence of hK5 in the various fractions was then assessed with the developed immunoassay. For milk, when the hK5 concentration in fractions was plotted against the fraction number, a peak around fraction 38 (corresponding to a molecular mass of $\sim 50 \text{ kDa}$) was detected (Fig. 4). A small peak around the void volume of the column ($\sim 700 \text{ kDa}$) could also be seen. In ascites fluid from ovarian cancer patients and in serum, another smaller peak corresponding to a molecular mass of $\sim 160\text{--}180 \text{ kDa}$ was also detected. This likely represents hK5 bound to a proteinase inhibitor or another interacting protein. The higher-than-expected ($30\text{--}35 \text{ kDa}$) molecular mass of hK5 in serum, ascites fluid, and milk was verified by measuring another kallikrein, hK6, in the same samples (Fig. 4). It is possible that native hK5 is highly glycosylated or that it may interact with the gel filtration column, leading to delayed retention.

Production of hK5 by Cell Lines and Hormonal Regulation. We tested several cancer cell lines for constitutive hK5 expression and for hK5 expression after steroid hormone stimulation. The cell lines PC-3 (AR)₆, HTB-75 (Caov-3), and MCF-7 produced relatively large amounts of hK5, whereas the cell lines MDA-MB-231, BG-1, T-47D, BT-474, PC-3, LNCaP, and ZR-75 did not. Among the three cell

Fig. 5. Expression of hK5 protein by various cell lines. On the Y axis, we present hK5 concentration in tissue culture supernatants after incubation with the indicated steroids at a concentration of 10⁻⁸ M for 6 days. Ethanol is used as control. Note the up-regulation of hK5 expression by norgestrel and DHT in the PC-3 (AR₆) prostate cancer cell line, the up-regulation of hK5 expression by estradiol in the MCF-7 breast carcinoma cell line, and the down-regulation of hK5 expression by dexamethasone in the HTB-75 ovarian cancer cell line.



lines producing hK5, PC-3 (AR)₆ secreted the highest levels (tissue culture supernatant concentration of ~1300 µg/liter, compared with 147 µg/liter for HTB-75 cells and 1.5 µg/liter for MCF-7 cells). These data refer to the supernatants collected from cells without any hormonal stimulation. Upon stimulation by steroid hormones, hK5 concentration increased by about 2–3-fold after norgestrel and DHT treatment in the PC-3 (AR)₆ cell line. We further observed a 3.5-fold reduction of hK5 concentration upon stimulation by dexamethasone in the HTB-75 (Caov-3) cell line and an 8-fold increase upon stimulation by estradiol in the MCF-7 cell line (Fig. 5).

In Silico Analysis of *KLK5* Gene Expression. The expression pattern of *KLK5* mRNA was analyzed using various CGAP databases and analysis tools. Virtual Northern analysis of a *KLK5*-unique SAGETag (TCTCCTGGAC) against the SAGEmap database indicated that whereas no expression was identified in normal ovarian libraries, *KLK5* expression was detected in five ovarian cancer libraries from different sources. In addition, *KLK5* was detected in five normal mammary gland libraries, consistent with our protein expression results of Fig. 1. The results of screening of the EST database for *KLK5* ESTs are shown in Table 1. Of 27 positive adult clones, 9 clones were from the same ovarian adenocarcinoma cell line, 6 were from different other ovarian cancer libraries, 3 were from squamous cell carcinoma of the skin, 2 were from uterine cancer libraries, and 1 was from squamous cell carcinoma of the tongue. These data are suggestive of an association between *KLK5* expression and certain malignancies (ovarian, skin, uterine, and tongue malignancies). In addition, ESTs were isolated from testis, breast, and lung libraries, consistent with our protein expression results.

DISCUSSION

Many kallikreins have recently been shown to be differentially expressed in hormone-related malignancies (2, 3). Of particular interest is the link between kallikreins and ovarian cancer (2, 3, 8, 18, 23–29). We have recently shown that hK6 (zyme/protease M) is a potential new serum biomarker for ovarian cancer (25). hK10 is also elevated in serum of ovarian cancer patients and has a role in diagnosis and prognosis (26). More recently, hK11 levels were shown to be elevated in serum of 70% of women with ovarian cancer (18). In this study, we provide preliminary evidence indicating that hK5 is a potential serum biomarker for ovarian cancer. Our results are based on the finding of significant elevations of serum hK5 concentration in ovarian cancer patients (Fig. 3) and the identification of high levels of hK5 in ascites fluid from ovarian cancer patients and in ovarian cancer tissue extracts (Fig. 2). We found no significant correlation between ascites fluid hK5 concentration and either serum CA125 or FIGO stage, but this finding needs further verification because the number of patients in this series was relatively small. At the mRNA level, we have previously shown that *KLK5* is a marker of unfavorable prognosis in ovarian cancer (8). Additional studies will be necessary to establish the diagnostic and prognostic value of hK5, either alone or in combination with other biomarkers, in ovarian cancer patients.

We have also recently shown that *KLK5* mRNA can be used as a marker of unfavorable prognosis in breast cancer (9). Here, we provide evidence of serum hK5 protein elevation in a subset of patients with breast cancer (Fig. 3). It will be worthwhile to examine hK5 protein levels in serum and tissues and correlate these data with patient clinicopathological parameters. It has been reported previously

Table 1 *hK5* expression in normal and pathological conditions as estimated from EST analysis

Tissue	Library type	Matching clones	Library ID	Source
Ovary	Adenocarcinoma cell line	9	NIH_MGC_9	NIH ^a
Ovary	5 Pooled tumors	1	NCLCGAP.OV23	CGAP
Ovary	Adenocarcinoma	2	NYHLMGC.66	NIH
Ovary	Ovarian cancer	2	Soares ovary tumor NbHOT	LLNL ^b
Ovary	Fibrotheoma	1	NCLCGAP.ov18	CGAP
Skin	Squamous cell carcinoma	3	NCLCGAP.skn4	CGAP
Endometrium	Adenocarcinoma cell line	2	NIH_MGC.44	NIH
Tongue	Squamous cell carcinoma	1	NCLCGAP.HN3	CGAP
Testis	Normal testis	1	Soares.testis_NHT	LLNL
Stomach	Normal adult	1	ST0296	LICR ^c
Stomach	Normal adult	2	S17N258215	CRIBB ^d
Breast	Adult female	1	BT0590	LICR
Larynx	Normal larynx	1	NCLCGAP.Lar1	CGAP
Lung	Fetal lung	1	Soares.fetal_lung_NbHL19W	LLNL
Heart	Fetal heart	4	Soares.fetal_heart_NbHH19W	LLNL

^a NIH mammalian gene collection.

^b Lawrence Livermore National Laboratory.

^c Ludwig Institute for Cancer Research.

^d Korea Research Institute of Bioscience and Biotechnology.

that another kallikrein, *KLK10*, is down-regulated at the mRNA level in breast cancer (30–32) and that high protein levels in cytosols are associated with resistance to chemotherapy (33).

hK5 protein was previously purified from the human skin and shown to be activated by trypsin (6). However, hK5 was not previously detected in any other tissue or fluid due to the lack of specific reagents and sensitive analytical methods. We have therefore undertaken this study to develop such reagents and methods. Using the highly sensitive and specific hK5 ELISA assay, we detected hK5 in various tissue extracts but predominantly in the skin, breast, salivary glands, esophagus, and a few other tissues (Fig. 1). The tissue expression pattern found at the protein level correlates well with previous reports on mRNA expression, with the highest levels found in skin and breast (2, 3, 5, 6). We further identified relatively large amounts of hK5 in milk, suggesting secretion of this protein by epithelial cells, similarly to another two kallikreins, hK6 and hK10, as shown by immunohistochemistry (34, 35). The concentration of hK5 in serum of healthy men and women appears to be very low, close to the detection limit of our assay (0.1 $\mu\text{g/liter}$). The results of tissue expression and the presence of hK5 in biological fluids are further supported by our Virtual Northern analysis data and the summary of bioinformatic analysis of Table 1.

Many kallikreins circulate in biological fluids as complexes with proteinase inhibitors (19–22). The free as well as the bound forms of these enzymes are useful biomarkers for the differential diagnosis of cancer (1, 22). Here we report that in serum and ascites fluid, hK5 protein is present in two forms, one at a relatively lower molecular mass (around 50 kDa), and another one around 150–180 kDa. These data suggest that hK5 likely interacts with proteinase inhibitors or other interacting proteins in these two fluids. Further evidence for such speculation is provided by the lower-than-expected recovery of added recombinant hK5 in biological fluids. More studies will be necessary to characterize the various molecular forms of hK5 in biological fluids and identify its interacting proteins.

Like many other kallikreins (2), hK5 is expressed by prostate, breast, and ovarian cancer cell lines, and this expression is modulated by steroid hormones. As shown in Fig. 5, we found a significant up-regulation of hK5 protein expression by estradiol in supernatants of MCF-7 breast cancer cells and up-regulation of hK5 protein expression by norgestrel and DHT in PC-3 (AR₆) prostate cancer cells. Furthermore, a significant down-regulation of hK5 expression by dexamethasone in the HTP-75 cell line was observed (Fig. 5). It will be necessary to further characterize the promoter of this gene to explain the effect of steroid hormones on hK5 expression.

In addition to various experimental approaches, bioinformatic analysis of gene expression can now provide important information on the frequency of mRNA transcript abundance in various cDNA libraries. The data of Table 1 confirm the experimentally established expression of hK5 in ovary, skin, testis, breast, and other tissues. Furthermore, and in accordance with the data of Fig. 2 and Table 1, it is clear that hK5 protein is significantly overexpressed in ovarian cancer, in comparison with normal tissues. Table 1 further suggests that hK5 measurements may be useful in cancers of the skin and endometrium. These suggestions need experimental verification.

In conclusion, we here present the first evidence that hK5 concentration is elevated in the serum of patients with ovarian and breast cancer. These data, combined with previous reports of higher serum and tissue concentrations of multiple kallikreins in ovarian cancer, suggest that this gene family may represent an enzymatic cascade pathway that is activated in ovarian and other cancers (36, 37). It is possible that multiple members of this gene family could serve as valuable diagnostic and prognostic markers for ovarian and other cancers. Furthermore, the delineation of kallikrein biological activity

in ovarian cancer tissue may lead to therapies that target the enzymatic activity of these proteolytic enzymes.

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Correction

In the article by G. M. Yousef *et al.*, entitled “Human Kallikrein 5: A Potential Novel Serum Biomarker for Breast and Ovarian Cancer,” which appeared in the July 15, 2003 issue of *Cancer Research* (pp. 3958–3965), Figure 3 was printed incorrectly. Below is the correct figure and caption.

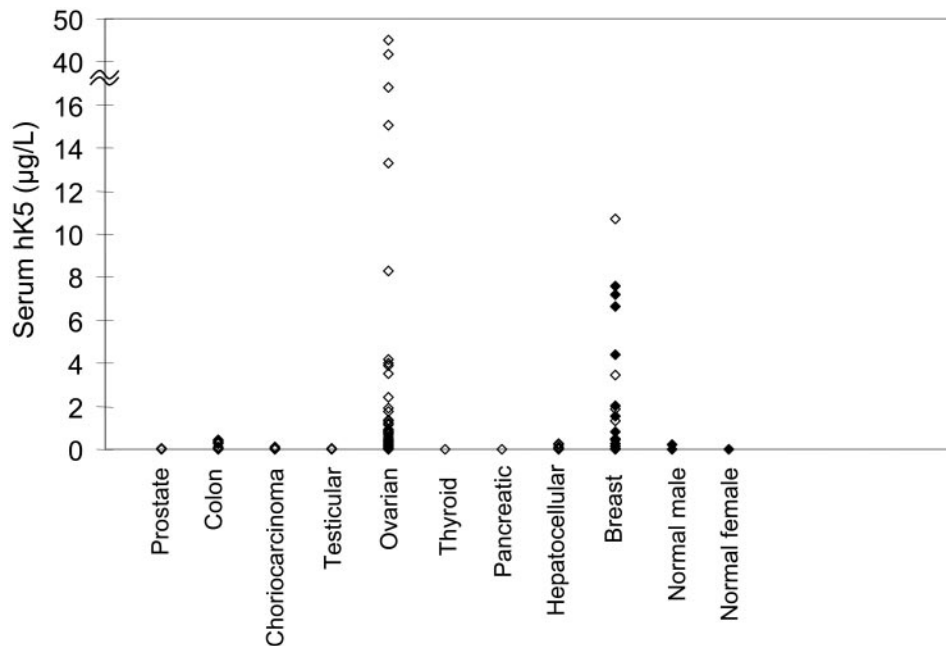


Fig. 3. Distribution of hK5 concentration in serum of patients diagnosed with various malignancies. Sera from normal female and male subjects were also included. High hK5 concentration ($>100^{\text{th}}$ percentile of normals) is found in a proportion of patients with ovarian and breast cancers. For more details on patient numbers and proportion of patients with elevated values, see text. Filled diamonds represent overlapping values of hK5.

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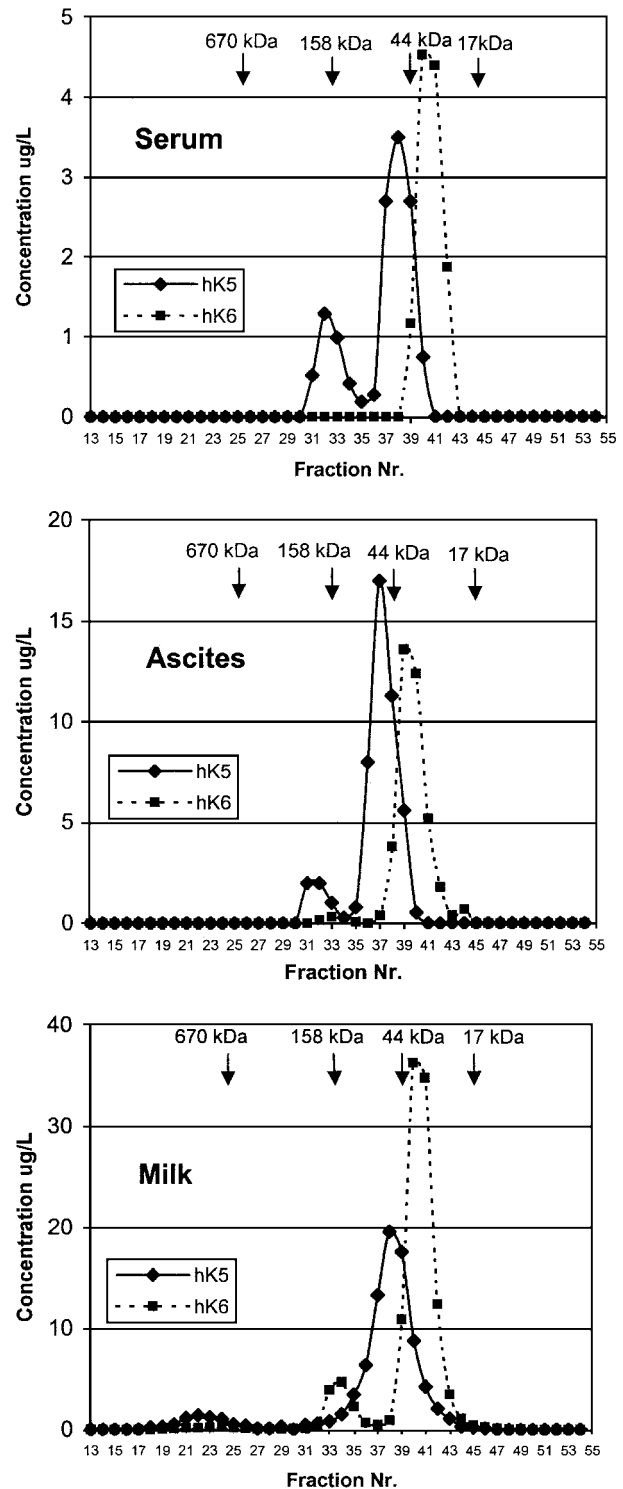


Fig. 4. Fractionation of three biological fluids (serum, ascites fluid from an ovarian cancer patient, and breast milk) by size-exclusion liquid chromatography. The elution profile of molecular mass standards is denoted by *arrows*. In serum, hK5 elutes as two immunoreactive peaks, one with an apparent molecular mass of 50 kDa (*fractions 37–39*) and one with an apparent molecular mass of approximately 150–180 kDa (*fractions 31–33*). The elution profile of another kallikrein with a similar theoretical molecular mass, hK6, is also shown by *dashed lines*. This kallikrein elutes at a molecular mass of ~35 kDa, corresponding to free hK6. In ascites fluid, the same comments apply as for serum. In breast milk, hK5 elutes mainly as a single immunoreactive peak. hK6 elutes as two distinct peaks, one at a molecular mass of ~35 kDa and another one at a molecular mass of ~100 kDa.