Human Kallikrein 6: A New Potential Serum Biomarker for Uterine Serous Papillary Cancer

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Abstract

Purpose: The discovery of novel biomarkers might greatly contribute to improve clinical management and outcomes in uterine serous papillary carcinoma (USPC), a highly aggressive variant of endometrial cancer.

Experimental Design: Human kallikrein 6 (*hK6*) gene expression levels were evaluated in 29 snap-frozen endometrial biopsies, including 13 USPC, 13 endometrioid carcinomas, and 3 normal endometrial cells by real-time PCR. Secretion of hK6 protein by 14 tumor cultures, including 3 USPC, 3 endometrioid carcinoma, 5 ovarian serous papillary carcinoma, and 3 cervical cancers, was measured using a sensitive ELISA. Finally, hK6 concentration in 79 serum and plasma samples from 22 healthy women, 20 women with benign diseases, 20 women with endometrioid carcinoma, and 17 USPC patients was studied.

Results: *hK6* gene expression levels were significantly higher in USPC when compared with endometrioid carcinoma (mean copy number by real-time PCR, 1,927 versus 239, USPC versus endometrioid carcinoma; $P < 0.01$). *In vitro* hK6 secretion was detected in all primary USPC cell lines tested (mean, 11.5 μg/L) and the secretion levels were similar to those found in primary ovarian serous papillary carcinoma cultures (mean, 9.6 μg/L). In contrast, no hK6 secretion was detectable in primary endometrioid carcinoma and cervical cancer cultures. hK6 serum and plasma concentrations (mean ± SE) among normal healthy females (2.7 ± 0.2 μg/L), patients with benign diseases (2.4 ± 0.2 μg/L), and patients with endometrioid carcinoma (2.6 ± 0.2 μg/L) were not significantly different. In contrast, serum and plasma hK6 values in USPC patients (6.1 ± 1.1) were significantly higher than those in the noncancer group ($P = 0.006$), benign group ($P = 0.003$), and endometrioid carcinoma patients ($P = 0.005$).

Conclusions: hK6 is highly expressed in USPC and is released in the plasma and serum of USPC patients. hK6 may represent a novel biomarker for USPC for monitoring early disease recurrence and response to therapy.

Uterine cancer is the most prevalent gynecologic tumor in women, with an estimated 40,100 cases and 6,800 deaths in the United States in 2003 (1). Based on clinical and histopathologic variables, two major subtypes of endometrial carcinoma, namely, uterine endometrioid tumors and uterine serous papillary carcinomas (USPC), have been described (2, 3). Although endometrioid carcinomas account for the majority (i.e., ~80%) of cases, these tumors are usually associated with a history of hyperestrogenism as the main risk factor, are histologically well or moderately differentiated, and typically have a favorable prognosis with appropriate therapy. In contrast, USPCs are poorly differentiated, biologically aggressive tumors, which although account for only ~10% of all endometrial carcinomas are responsible for ~50% of all relapses (4–12). The discovery of novel diagnostic and therapeutic markers against this aggressive subset of endometrial cancers remains a high priority.

With the goal of identifying genes with a differential pattern of expression between USPC and ovarian serous papillary carcinoma (OSPC) and to use this knowledge for the development of novel diagnostic and therapeutic markers against this disease, our group has recently used high-throughput technologies, such as high-density oligonucleotide arrays, to analyze USPC genetic fingerprints (13). Among the several candidate target genes identified, the gene encoding for human kallikrein 6 (*hK6*, also known as zyme/protease M/neurosin) was consistently found as one of the most highly

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up-regulated genes in USPC. The organization of the kallikrein locus, a gene family now consisting of 15 members that encode for trypsin-like or chymotrypsin-like serine proteases, has been elucidated recently (14). Serine proteases have well-characterized roles in diverse cellular activities, including blood coagulation, wound healing, digestion, and immune responses as well as tumor invasion and metastasis (reviewed in ref. 15). Importantly, secreted serine proteases, such as prostate-specific antigen, kallikrein 2, and other kallikreins, have already found important clinical applications as prostate cancer biomarkers (16). Of interest, hK6 has recently been found at high levels in the circulation of a subset of ovarian cancer patients harboring biologically aggressive tumors (16–18). This discovery has highlighted the potential of hK6 as a novel biomarker in ovarian cancer that may have value for disease diagnosis and prognosis. This report represents the first investigation examining hK6 expression and secretion in human endometrial carcinoma.

Patients and Methods

Primary tumors. Snap-frozen tumor biopsies and tumor samples were derived from primary specimens staged according to the International Federation of Gynecology and Obstetrics operative staging system. Only specimens with >75% tumor content were used for all experiments. Fresh tumor biopsies from gynecologic tumors, including 13 endometrioid carcinoma, 13 USPC, 5 OSPC derived from patients harboring advanced stage disease (III-IV), and 3 cervical carcinomas from stage IB to IIA patients, were obtained at the time of surgery through the Gynecologic Oncology Division and the Pathology Department, University of Arkansas for Medical Sciences (Little Rock, AR), under approval of the institutional review board. Patient characteristics from which tumor biopsies were obtained are described in Table 1. Total abdominal hysterectomy and bilateral lymph node dissection were done in all endometrial cancer patients. Normal endometrial control (NEC) cell samples were obtained from biopsies of benign hysterectomy specimens obtained from women of similar age. Some primary tumor biopsies (i.e., three USPC, three endometrioid carcinoma, five OSPC, and three cervical cancer) were also established as short-term cultures following previously reported standard tissue culture techniques (13, 19, 20). Briefly, tumor tissues obtained from cancer patients were mechanically minced and enzymatically dissociated with 0.14% collagenase type I (Sigma, St. Louis, MO) in RPMI 1640 as described previously by Santin et al. (13, 19, 20), respectively. After 1- to 2-hour incubation with enzyme on a magnetic stirring apparatus at 37°C in an atmosphere of 5% CO_2, the resulting suspension was collected by centrifugation at 100 × *g* for 5 to 10 minutes and washed twice with RPMI 1640 (Sigma) containing 10% fetal bovine serum (Gemini, Woodland, CA). The final pellet was then placed in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 200 units/mL penicillin, and 200 μg/mL streptomycin in tissue culture flasks or Petri dishes (Corning, Acton, MA). Tumor cells were then allowed to attach and proliferate. RNA extraction was done at a tumor cell confluence of 50% to 80% after a minimum of 2 to a maximum of 10 passages *in vitro*. The epithelial nature and the purity of tumor cultures were verified by immunohistochemical staining and flow cytometric analysis with antibodies against cytokeratin and vimentin as described previously (13, 19, 20). Only primary cultures that had at least 90% viability and contained >99% epithelial cells were used for hK6 quantification by ELISA as described below.

RNA isolation and cDNA synthesis. RNA isolation from all primary snap-frozen samples, including 26 primary endometrial cancers (i.e., 13 USPC and 13 endometrioid carcinoma) and 3 normal endometrial cell controls, was done using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To verify integrity, RNA (4 μg) from each sample was run in 1% agarose gel using 18S + 28S rRNA (Sigma) as positive control. First-strand cDNA was synthesized using 5 μg total RNA, 1× reverse transcription-PCR (RT-PCR) buffer, 5 mmol/L $MgCl_2$, 1 mmol/L deoxynucleotide triphosphates, 2.5 μmol/L random hexamers, 1 unit/μL RNase inhibitor, and 2.5 units/μL MulLV reverse transcriptase (GeneAmp RNA PCR kit, Applied Biosystems, Foster City, CA) in a total volume of 20 μL. RNA extracted from the CaOV3 serous papillary ovarian cancer cell line, reported previously to express hK6 (17), was used as a positive control.

Quantitative real-time reverse transcription-PCR. Quantitative real-time RT-PCR was done with an ABI Prism 7000 sequence analyzer using the manufacturer's recommended protocol (Applied Biosystems) to evaluate expression of *hK6* gene in samples from all snap-frozen biopsies. Each reaction was run in triplicate. The comparative threshold cycle method was used for the calculation of amplification fold as specified by the manufacturer. Briefly, total RNA (5 μg) from each sample was reverse transcribed using SuperScript III First-Strand cDNA synthesis (Invitrogen). Reverse-transcribed RNA samples (10 μL; from 500 μL of total volume) were amplified by using the Taqman Universal PCR Master Mix (Applied Biosystems) to produce PCR products specific for hK6. Primers specific for 18S rRNA and empirically determined ratios of 18S competimers (Applied Biosystems) were used to control for the amount of cDNA generated from each sample. hK6 primers were obtained from Applied Biosystems as Assay-on-Demand products.

Table 1. Characteristics of the patients from which primary tumor biopsies were obtained

	NEC (*n* = 3)	Endometrioid carcinoma (*n* = 13)	USPC (*n* = 13)	OSPC (*n* = 5)	Cervical cancer (*n* = 3)
Age (mean ± SD)	51 ± 7	58 ± 15	62 ± 3	60 ± 11	40 ± 6
Stage					
I	—	10	1	—	3
II	—	2	1	—	—
III	—	1	7	3	—
IV	—	—	4	2	—
Grading					
G1	—	8	—	—	—
G2	—	3	—	1	2
G3	—	2	13	4	1

Analysis of hK6 secretion. An important issue is whether *hK6* gene expression differences in tumor tissues result in meaningful differences in protein expression. To validate hK6 data obtained by RT-PCR on snap-frozen USPC and endometrioid carcinoma samples at the protein level as well as to compare hK6 secretion among different gynecologic malignancies, supernatants obtained from 14 primary gynecologic tumors, including 3 endometrioid carcinoma, 3 USPC, 5 primary OSPC, and 3 primary cervical cancer, were evaluated by ELISA. Briefly, tumor supernatants tested for hK6 secretion were collected by primary tumor cell lines seeded at a density of 1×10^5 cells/mL in tissue culture Petri dishes in RPMI 1640 supplemented with 10% fetal bovine serum (i.e., USPC, endometrioid carcinoma, and ovarian cancer cell lines) or serum-free keratinocyte medium (i.e., cervical cancer cell lines). After 48-hour incubation at 37°C, supernatants were aspirated, rendered cell-free by centrifugation at $1,500 \times g$ for 10 minutes, and stored at −20°C before being analyzed for hK6 by a "sandwich" ELISA.

hK6 concentration was quantified in the serum and plasma of 22 apparently healthy women, 20 women with benign diseases, 20 women with histologically proven primary endometrioid carcinoma, and 17 women with histologically proven primary USPC by ELISA as described previously by Diamandis et al. (21). This assay incorporates two hK6-specific mouse monoclonal antibodies, one for coating (clone 27-4) and the other for detection (clone E24) in a sequential two-site immunometric format with time-resolved fluorescence detection (21). The assay has a detection limit of 0.05 μg/L and a dynamic range up to 20 μg/L. Variability was <10% with in the measurement range. Briefly, white polystyrene microtiter plates were coated with anti-hK6 (27-4) monoclonal antibody (21). Coating antibody solution [500 μg/100 μL; 50 mmol/L Tris buffer, 0.05% sodium azide (pH 7.8)] was added to each well and incubated overnight at room temperature. The plates were washed thrice with the washing buffer [5 mmol/L Tris buffer, 150 mmol/L NaCl, 0.05% Tween 20 (pH 7.8)]. hK6 calibrators or samples (50 μL) and assay buffer [50 μL; 50 mmol/L Tris, 6% bovine serum albumin, 0.01% goat IgG, 0.005% mouse IgG (Fortron Bio Science, Inc., Morrisville, NC), 0.1% bovine IgG, 0.5 mol/L KCl, 0.05% sodium azide (pH 7.8)] were added to respective wells and incubated for 2 hours with shaking at room temperature. The plates were washed six times with the washing buffer, after which biotinylated detection antibody solution (100 μL; 25-50 ng anti-hK6 (E24) monoclonal antibody in assay buffer) was added to each well and incubated for 1 hour at room temperature with shaking. The plates were then washed six times with the washing buffer. Subsequently, alkaline phosphatase–conjugated streptavidin solution (5 ng/well; Jackson ImmunoResearch, Baltimore, PA) in 6% bovine serum albumin buffer [in 50 mmol/L Tris, 0.05% sodium azide (pH 7.8)] was added to each well and incubated for 15 minutes with shaking at room temperature. The plates were washed six times with the washing buffer, and substrate buffer [100 μL; 0.1 mol/L Tris buffer (pH 9.1)] containing 1 mmol/L diflunisal phosphate, 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$ was added to each well and incubated for 10 minutes with shaking at room temperature. Developing solution (100 μL; 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, 3 mmol/L EDTA) was added to each well and incubated for 1 minute with shaking at room temperature. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 immunoanalyzer (MDS Nordion, Toronto, Ontario, Canada).

Patient characteristics from which plasma and serum samples were obtained are described in Table 2. Serum and plasma samples were analyzed in duplicate with inclusion of three quality-control samples in every run. Variability between serum and plasma samples was <0.5 μg/L. Of the benign lesions, 12 were classified as endometriosis, 3 as mucinous cystadenomas, 2 as ovarian dermoid cysts, 1 as ovarian benign teratoma, 1 as corpus luteum, and 1 as serous cystadenoma. Serum and plasma samples from all patients were collected before surgery and stored at −80°C until analysis.

Table 2. Characteristics of the patients from which serum and plasma samples were obtained

Variable	Healthy females (n = 22)	Benign diseases (n = 20)	Endometrioid cancer (n = 20)	USPC (n = 17)
Age (mean ± SD)	43 ± 12	50 ± 14	62 ± 10	65 ± 8
Stage				
I	—	—	16	1
II	—	—	3	1
III	—	—	1	10
IV	—	—	—	5
Grading				
G1	—	—	12	—
G2	—	—	6	—
G3	—	—	2	17

Statistical analysis. Differences among USPC, endometrioid carcinoma, and NEC in the quantitative RT-PCR expression data were tested using the Wilcoxon rank-sum test at $\alpha = 0.05$. The analyses of differences between hK6 serum and plasma concentrations among the different groups of patients (i.e., healthy controls, benign gynecologic diseases, endometrioid carcinoma, and USPC) and among supernatants obtained from gynecologic tumor cultures with different histologies were done with the Student's t test.

Results

hK6 expression in snap-frozen endometrial cancer by quantitative real-time PCR. USPCs are rare tumors that may present in either pure forms or admixed with endometrioid or clear cell tumor cells (i.e., mixed USPC; ref. 11). To minimize the risk of contamination of USPC RNA with that of normal cells or tumor cells with different histology, we extracted RNA to be evaluated for hK6 expression by RT-PCR from 26 primary endometrial cancers with single-type differentiation (i.e., 13 USPC and 13 endometrioid carcinoma). A comparison of the quantitative RT-PCR data for hK6 in USPC and endometrioid carcinoma using NEC as controls is shown in Fig. 1. Significant expression differences between USPC and endometrioid carcinoma were readily apparent (Fig. 1A and B). All USPC samples [13 of 13 (100%); mean ± SE copy number, 1,927 ± 551; range, 111-6,295] and the majority of endometrioid carcinoma samples [10 of 13 (77%); mean ± SE copy number, 239 ± 86; range, 1.2-1,184] were found positive for hK6 expression by RT-PCR (Fig. 1B). However, only 1 of 13 (8%) of the endometrioid carcinoma had a mRNA copy number >350 (Fig. 1B). In contrast, 9 of 13 (69%) USPCs were found to highly express the *hK6* gene with mRNA copy number >350 (Fig. 1B; USPC versus endometrioid carcinoma: $P < 0.01$). Low levels of *hK6* gene expression were found in the NEC control cultures tested (mean ± SE copy number, 1.2 ± 0.2; range, 1-1.5; Fig. 1B).

hK6 secretion by primary gynecologic cancer cell cultures by ELISA. Snap-frozen biopsies may contain significant numbers of contaminant stromal cells as well as a variety of host-derived immune cells (e.g., monocytes, dendritic cells, and lymphocytes). Primary short-term tumor cultures, minimizing the risk of a selection bias inherent in any long-term *in vitro* growth,

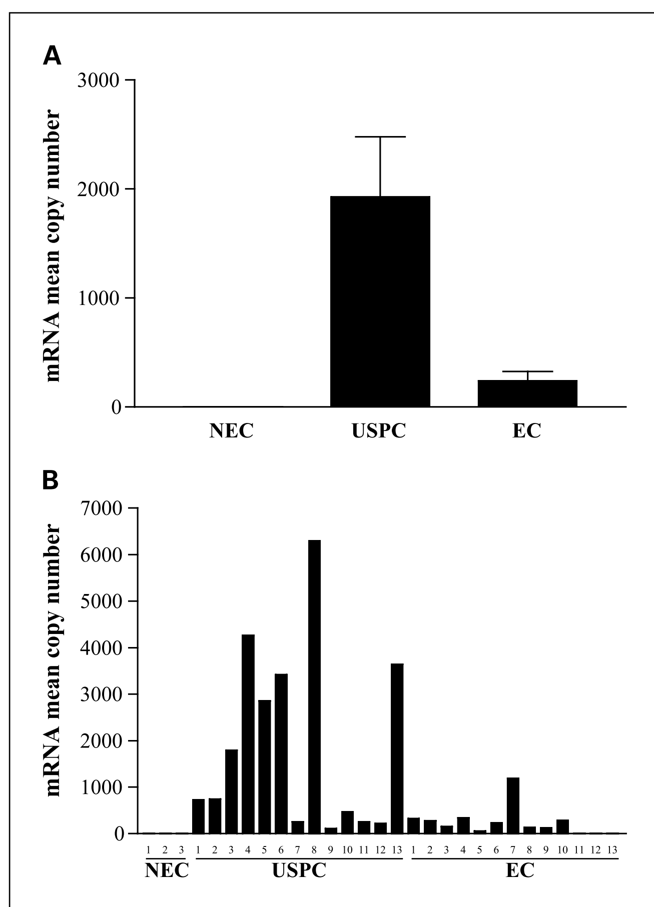

Fig. 1. hK6 mRNA copy number by quantitative RT-PCR in 29 snap-frozen endometrial biopsies. *A,* hK6 mRNA mean ± SE copy number in 3 NEC, 13 endometrioid carcinomas (*EC*), and 13 USPC. *B,* hK6 mRNA copy number in individual NEC controls, endometrioid carcinoma, and USPC samples. For discussion, see text.

may provide an opportunity to study differential hK6 secretion between highly enriched populations of tumor-derived epithelial cells. Cell-free supernatants from 14 freshly isolated gynecologic malignancies, including 3 USPC, 3 endometrioid carcinoma, 5 high-grade OSPC, and 3 squamous cervical carcinoma, were collected and analyzed for the levels of hK6 expression by ELISA. Because prolonged passages *in vitro* are known to alter the physiology and phenotype of primary tumor cells, we did all our experiments with highly purified fresh tumor cells grown for <10 passages *in vitro*. Growth control medium was always analyzed at the same time. In this regard, KSFM and RPMI 1640 containing 10% fetal bovine serum had no detectable endogenous levels of hK6 immunoreactivity by ELISA (data not shown). As shown in Fig. 2, all primary USPC tumor cell lines tested secreted large amounts of hK6 (mean, 11.5 μg/L; range, 0.4-26.6 μg/L/10^5 cells/48 hours). These levels were not significantly different when compared with those detected in three of five primary OSPC cultures used as controls (mean, 9.6 μg/L; range, 0.0-26.5 μg/L/10^5 cells/48 hours; Fig. 2). In contrast, no hK6 secretion was detected in the three primary short-term endometrioid carcinoma cultures derived from G2 tumors (1) and G3 tumors (2) and from the three primary short-term cervical cancer cultures tested (Fig. 2).

Plasma and serum hK6 concentration in endometrial cancer and noncancer patients. To investigate whether hK6 is detectable in the plasma and serum of patients harboring endometrial cancer, plasma and serum samples from 20 endometrioid carcinoma patients and 17 USPC patients were evaluated by ELISA. In addition, plasma and serum samples obtained from 22 healthy female controls and 20 patients diagnosed with benign gynecologic diseases were analyzed at the same time. hK6 serum and plasma levels (in μg/L) from 22 healthy female controls (mean ± SE, 2.7 ± 0.2; range, 1.4-4.4), 20 patients with benign gynecologic diseases (mean ± SE, 2.4 ± 0.2; range, 1.4-5.0), and 20 patients with endometrioid carcinoma (mean ± SE, 2.6 ± 0.2; range, 1.4-4.9) were not statistically significantly different (Table 3). In contrast, serum and plasma hK6 values in USPC patients (mean ± SE, 6.1 ± 1.1; range, 1.9-15.6) were significantly higher than those in the noncancer (P = 0.006), benign group (P = 0.003), and endometrioid carcinoma patients (P = 0.005). The distribution of hK6 concentration in the four groups of patients is presented in Fig. 3.

Discussion

The discovery of novel biomarkers for the early diagnosis, monitoring, and prediction of response to treatment in USPC, the most aggressive variant of endometrial cancer, might greatly contribute to the improvement of clinical management and outcomes of these patients. Unfortunately, no accepted and/or specific serum tumor markers have as yet been identified for this disease. In this regard, although it has been reported previously that the elevation of CA 125 level may be associated with an increase in the incidence of metastatic disease in endometrial tumors with endometrioid histology (22, 23), this marker seems to have limited utility in monitoring the effects of adjuvant therapy or in the prediction of tumor recurrence in USPC patients (24). This report represents the first evaluation of hK6, a member of the expanded human kallikrein gene family (14 – 16), as a novel biomarker in USPC.

In this study, we have quantified hK6 expression by RT-PCR in 26 snap-frozen endometrioid carcinoma and USPC specimens. In addition, we have studied hK6 protein secretion in 14 primary gynecologic malignancies, including endometrioid

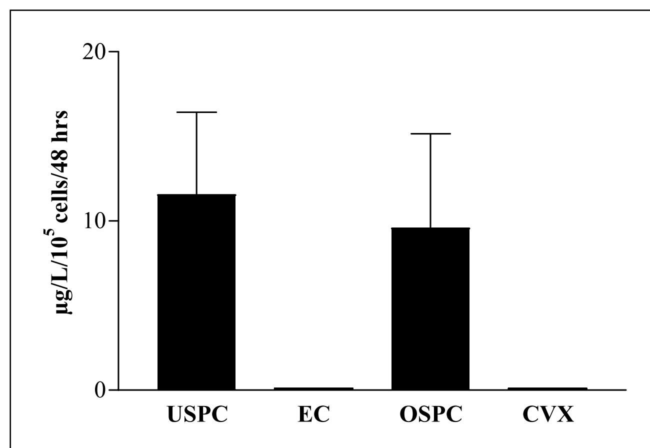

Fig. 2. hK6 levels by ELISA in the supernatants from 14 freshly isolated gynecologic malignancies, including 3 USPC, 3 endometrioid carcinoma, 5 high-grade OSPC, and 3 squamous cervical carcinomas (*CVX*).

Table 3. Serum and plasma hK6 in noncancer (healthy), benign disease, endometrioid carcinoma, and USPC patients

Patient group	Mean ± SE (μg/L)	Range (μg/L)
Noncancer (*n* = 22)	2.7 ± 0.2	1.4-4.4
Benign disease (*n* = 20)	2.4 ± 0.2	1.4-5.0
Endometrioid carcinoma (*n* = 20)	2.6 ± 0.2	1.4-4.9
USPC (*n* = 17)	6.1 ± 1.1*	1.9-15.6

*P = 0.006 for noncancer cells versus USCP, 0.003 for benign versus USCP, and 0.005 for endometrioid carcinoma versus USCP.

carcinoma, USPC, OSPC, and cervical cancer tumor cultures. We have confirmed the purity of the tumor cells in our short-term cultures by differential counts of Giemsa-stained cytospin slides as well as by cytokeratin expression using immunohistochemical techniques (data not shown). Our fresh tumor samples contained >99% tumor cells. Finally, we have studied hK6 levels in 79 serum and plasma samples derived from healthy donors, patients harboring benign gynecologic tumors, and endometrioid carcinoma and USPC patients.

We report for the first time a high level of expression of the *hK6* gene in USPC. Indeed, *hK6* gene expression was significantly higher in USPC when compared with NEC and endometrioid carcinoma by RT-PCR. Mean copy number of *hK6* gene mRNA in USPC was 8.1 times higher when compared with endometrioid carcinoma cells. Consistent with these findings, highly purified primary USPC cultures were found to secrete high levels of hK6 by ELISA. In contrast, hK6 was not detected in any of the three endometrioid carcinoma and three cervical cancer cell lines tested in this study. Our data highlight for the first time a major difference between type I (i.e., endometrioid carcinoma) and type II (i.e., USPC) endometrial cancer in the expression and secretion of hK6. Of interest, when USPC secretion of hK6 was compared with that of primary OSPC cell lines *in vitro*, no significant differences were found. The high levels of hK6 secreted by three of five primary ovarian tumors tested in our assay are consistent with recent reports showing high levels of hK6 expression in tumor tissue and serum of ovarian cancer patients (16, 18, 25). Of interest, in these recent studies, ovarian cancer patients with preoperative serum levels of hK6 above 4.4 μg/L had significantly worse prognosis than patients with low preoperative hK6 (18). Furthermore, hK6 overexpression was found to correlate with intrinsic resistance to adjuvant chemotherapy (18). Taken together, these data and our own results suggest that high expression of hK6 in gynecologic serous tumors, regardless of ovarian or uterine origin, may identify malignancies characterized by an aggressive biological behavior and resistance to chemotherapy. Consistent with this view, with the single exception of one patient diagnosed with early stage disease, all USPC patients evaluated in this study, despite aggressive adjuvant treatment (i.e., whole pelvis radiation and/or chemotherapy), either developed progressive disease during chemotherapy or recurred within 1 year after treatment was completed.

When hK6 levels were quantified in the plasma and serum of endometrial cancer patients, we found elevated levels in USPC patients but not in patients harboring endometrioid carcinoma or benign gynecologic disease when compared with the levels found in healthy women. Furthermore, in the limited number of USPC patients where sequential serum samples were available (i.e., five patients), a decrease in hK6 levels was observed postoperatively (data not shown). These *in vivo* data accord with our *in vitro* results with highly purified USPC short-term primary cultures and suggest that this serine protease is secreted by biologically aggressive USPC cells *in vitro* as well as *in vivo*. In agreement with previous reports (18), we found no elevation of hK6 in the serum and plasma of patients harboring benign gynecologic disease or in healthy females. We conclude that kallikrein 6 is a very promising novel biomarker for early detection of recurrent USPC disease and for the monitoring USPC response to adjuvant therapy.

It is worth noting that in our series of USPC patients, all of whom were surgically staged, the majority were found to harbor advanced disease. Thus, although most of our patients were considered to have clinical stage I disease and upgraded only at the time of comprehensive surgical staging laparotomy, it is possible that the elevated levels of hK6 may reflect a bias related to advanced stage. Although larger studies including more USPC patients harboring surgically confirmed early-stage disease will be necessary to exclude this possibility, it is worth noting that of the two patients included in this work who had surgically confirmed early-stage disease, one was found to have elevated levels of hK6. Furthermore, it is important to point out that because of the propensity of USPC to rapidly manifest extrauterine disease (i.e., positive lymph node metastases or spreading to the abdominal cavity), the USPC series reported here is likely most representative of the advanced stage disease commonly found in surgically staged USPC patients. In agreement with our data, Goff et al. found 75% of patients with clinical stage I/II USPC to have extracorporeal disease when comprehensively staged (6). Similarly, Bristow et al. showed 74.4% of USPC patients to have advanced stage disease

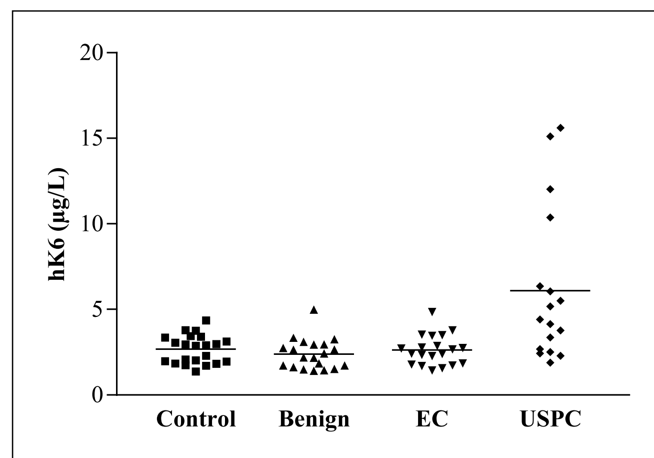

Fig. 3. hK6 levels by ELISA in serum and plasma from 22 healthy controls, 20 patients with benign diseases, 20 endometrioid carcinoma, and 17 USPC patients. Horizontal lines, mean values. Noncancer patients versus USPC, P = 0.006; benign versus USPC, P = 0.003; endometrioid carcinoma versus USPC, P = 0.005 by Student's t test.

following ovarian cancer–type surgical staging (26). These findings were also corroborated in studies by O'Hanlan et al. (27) and Geisler et al. (28).

Several other human kallikrein gene family members, including the enzymes hK4, hK5, hK7, hK8, hK9, and hK10, have recently been shown to have prognostic significance in ovarian cancer (14, 15, 29–37). Furthermore, serine proteases not belonging to the kallikrein family have also been shown to have prognostic significance in ovarian cancer, including trypsin, prostasin, hepsin, and testisin (38–40). Because USPC is histologically similar to high-grade OSPC, it seems very likely that, in analogy to ovarian cancer, multiple members of the human kallikrein gene family may be also dysregulated in USPC. It is thus possible that other members of this family will emerge as potential USPC biomarkers. If these proteases are involved in cancer progression, they may be suitable candidates as therapeutic targets (41). These possibilities will be investigated in future experiments.

In conclusion, we report here the first evidence that hK6 is highly expressed in USPC and that high concentrations of hK6 are present in the plasma and serum of USPC patients. Our results strongly support the hypothesis that hK6 may represent a novel biomarker for this highly aggressive variant of endometrial cancer. The current availability of a highly sensitive and specific immunofluorometric assay for measuring hK6 protein concentration in serum and biological fluids (21) will facilitate further studies to establish the clinical usefulness of the circulating levels of hK6 for the management of patients with USPC.

References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics. CA Cancer J Clin 2003; 53:5–26.
2. Bohkman JV. Two pathogenetic types of endometrial carcinoma. Gynecol Oncol 1983;15:10–7.
3. Rose PG. Endometrial carcinoma. N Engl J Med 1996;335:640–9.
4. Sherman ME, Bitterman P, Rosenshein NB, et al. Uterine serous carcinoma. A morphologically diverse neoplasm with unifying clinicopathological features. Am J Surg Pathol 1992;16:600–10.
5. Carcangiu ML, Chambers JT. Uterine papillary serous carcinoma: a study on 108 cases with emphasis on prognostic significance of associated endometrioid carcinoma, absence of invasion, and concomitant ovarian cancer. Gynecol Oncol 1992;47:298–305.
6. Goff BA, Kato D, Schmidt RA, et al. Uterine papillary serous carcinoma: pattern of metastatic spread. Gynecol Oncol 1994;54:264–8.
7. Carcangiu ML, Chambers JT. Early pathologic stage clear cell carcinoma and uterine papillary serous carcinoma of the endometrium, comparison of clinicopathological features and survival. Int J Gynecol Pathol 1995;14:30–8.
8. Levenback C, Burke TW, Silva E, et al. Uterine papillary serous carcinoma (USPC) treated with cisplatin, doxorubicin, and cyclophosphamide (PAC). Gynecol Oncol 1992;46:317–21.
9. Nicklin JL, Copeland LJ. Endometrial papillary serous carcinoma: pattern of spread and treatment. Clin Obstet Gynecol 1996;39:686–95.
10. Trope C, Kristensen GB, Abeler VM. Clear-cell and papillary serous cancer: treatment options. Best Pract Res Clin Obstet Gynaecol 2001;15: 433–46.
11. Hendrickson M, Ross J, Eifel P, Martinez A, Kempson R. Uterine papillary serous carcinoma: a highly malignant form of endometrial adenocarcinoma. Am J Surg Pathol 1982;6:93–108.
12. Chan JK, Loizzi V, Youssef M, et al. Significance of comprehensive surgical staging in noninvasive papillary serous carcinoma of the endometrium. Gynecol Oncol 2003;90:181–5.
13. Santin AD, Zhan F, Bellone S, et al. Discrimination between uterine serous papillary carcinomas and ovarian serous papillary tumors by gene expression profiling. Br J Cancer 2004;90:1814–24.
14. Yousef GM, Diamandis EP. The new human tissue kallikrein gene family: structure, function and association to disease. Endocr Rev 2001;21:184–204.
15. Borgono CA, Michael IP, Diamandis EP. Human tissue kallikreins: physiological roles and applications in cancer. Mol Cancer Res 2004;2:257–80.
16. Diamandis EP, Yousef GM. Human tissue kallikreins: a family of new cancer biomarkers. Clin Chem 2002; 48:1198–205.
17. Tanimoto H, Underwood LJ, Shigemasa K, Parmley TH, O'Brien TJ. Increased expression of protease M in ovarian tumors. Tumor Biol 2001;22:11–8.
18. Diamandis EP, Scorilas A, Fracchioli S, et al. Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. J Clin Oncol 2003;21:1035–43.
19. Santin AD, Hermonat PL, Ravaggi A, et al. Induction of human papillomavirus-specific CD4(+) and CD8(+) lymphocytes by E7-pulsed autologous dendritic cells in patients with human papillomavirus type 16- and 18-positive cervical cancer. J Virol 1999;73:5402–10.
20. Santin AD, Bellone S, Ravaggi A, et al. Induction of tumour-specific CD8(+) cytotoxic T lymphocytes by tumour lysate-pulsed autologous dendritic cells in patients with uterine serous papillary cancer. Br J Cancer 2002;86:151–7.
21. Diamandis EP, Yousef GM, Soosaipillai A, Grass L, Porter A, Little S, Sotiropoulou G. Immunofluorometric assay of human kallikrein 6 (zyme/protease M/neurosin) and preliminary clinical applications. Clin Biochem 2000;33:369–75.
22. Soper JT, Berchuck A, Olt GJ, Soisson AP, Clarke-Pearson DL, Bast RC Jr. Preoperative evaluation of serum CA 125, TAG 72, and CA 15-3 in patients with endometrial carcinoma. Am J Obstet Gynecol 1990; 163:1204–9.
23. Dotters DJ. Preoperative CA 125 in endometrial cancer: is it useful? Am J Obstet Gynecol 2000;182: 1328–34.
24. Price FV, Chambers SK, Carcangiu ML, Kohorn EI, Schwartz PE, Chambers JT. CA125 may not reflect disease status in patients with uterine serous carcinoma. Cancer 1998;82:1720–5.
25. Hoffman BR, Katsaros D, Scorilas A, et al. Immunofluorometric quantitation and histochemical localisation of kallikrein 6 protein in ovarian cancer tissue: a new independent unfavourable prognostic biomarker. Br J Cancer 2002;87:763–71.
26. Bristow RE, Asrari F, Trimble EL, Montz FJ. Extended surgical staging for uterine papillary serous carcinoma: survival outcome of locoregional (Stage I-III) disease. Gynecol Oncol 2001;81:279–86.
27. O'Hanlan KA, Levine PA, Harbatkin D, et al. Virulence of papillary endometrial carcinoma. Gynecol Oncol 1990;37:112–9.
28. Geisler JP, Geisler HE, Melton ME, Wiemann MC. What staging surgery should be performed on patients with uterine papillary serous carcinoma? Gynecol Oncol 1999;74:465–7.
29. Obiezu CV, Scorilas A, Katsaros D, et al. Higher human kallikrein gene 4 (KLK4) expression indicates poor prognosis of ovarian cancer patients. Clin Cancer Res 2001;7:2380–6.
30. Kim H, Scorilas A, Katsaros D, et al. Human kallikrein gene 5 (KLK5) expression is an indicator of poor prognosis in ovarian cancer. Br J Cancer 2001; 84:643–50.
31. Magklara A, Scorilas A, Katsaros D, et al. The human KLK8 (neuropsin/ovasin) gene: identification of two novel splice variants and its prognostic value in ovarian cancer. Clin Cancer Res 2001;7:806–11.
32. Luo LY, Katsaros D, Scorilas A, et al. Prognostic value of human kallikrein 10 expression in epithelial ovarian carcinoma. Clin Cancer Res 2001;7: 2372–9.
33. Luo L-Y, Bunting P, Scorilas A, Diamandis EP. Human kallikrein 10: a novel tumor marker for ovarian carcinoma? Clin Chim Acta 2001;306:111–8.
34. Dong Y, Kaushal A, Bui L, et al. Human kallikrein 4 (KLK4) is highly expressed in serous ovarian carcinomas. Clin Cancer Res 2001;7:2363–71.
35. Tanimoto H, Underwood LJ, Shigemasa K, et al. The stratum corneum chymotryptic enzyme that mediates shedding and desquamation of skin cells is highly overexpressed in ovarian tumor cells. Cancer 1999; 86:2074–82.
36. Underwood LJ, Tanimoto H, Wang Y, et al. Cloning of tumor-associated differentially expressed gene-14, a novel serine protease overexpressed by ovarian carcinoma. Cancer Res 1999;59:4435–9.
37. Yousef GM, Kyriakopoulou LG, Scorilas A, et al. Quantitative expression of the human kallikrein gene 9 (KLK9) in ovarian cancer: a new independent and favorable prognostic marker. Cancer Res 2001;161: 7811–8.
38. Shigemasa K, Underwood LJ, Beard J, et al. Overexpression of testisin, a serine protease expressed by testicular germ cells, in epithelial ovarian tumor cells. J Soc Gynecol Investig 2000;7:358–62.
39. Tanimoto H, Yan Y, Clarke J, et al. Hepsin, a cell surface serine protease identified in hepatoma cells, is overexpressed in ovarian cancer. Cancer Res 1997;57: 2884–7.
40. Hirahara F, Miyagi E, Nagashima Y, et al. Differential expression of trypsin in human ovarian carcinomas and low-malignant-potential tumors. Gynecol Oncol 1998;68:162–5.
41. Cannon MJ, O'Brien TJ, Underwood LJ, Crew MD, Bondurant KL, Santin AD. Novel target antigens for dendritic cell-based immunotherapy against ovarian cancer. Expert Rev Anticancer Ther 2002;2: 97–105.