American Journal of Obstetrics and Gynecology (2006) **194**, 1296–302

ELSEVIER

American Journal of Obstetrics & Gynecology

www.ajog.org

Overexpression of kallikrein 10 (hK10) in uterine serous papillary carcinomas

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Received for publication June 3, 2005; revised July 27, 2005; accepted October 21, 2005

KEY WORDS
Uterine serous papillary carcinoma
Kallikrein 10
Biomarkers
Serine proteases
Tumor markers

Objective: Kallikrein 10 is a secreted serine protease recently implicated in the growth and invasion of several human tumors. The goal of this study was to investigate the expression and secretion levels in vitro and in vivo of kallikrein 10 in uterine serous papillary carcinoma, a highly aggressive variant of endometrial tumor.

Study design: Human kallikrein 10 gene expression levels were evaluated in 11 snap-frozen uterine serous papillary carcinoma biopsies and 6 normal endometrial cell biopsies by real-time polymerase chain reaction. Secretion of kallikrein 10 protein by 10 primary tumor cultures including 3 uterine serous papillary carcinomas, 2 endometrioid carcinomas, and 5 ovarian serous papillary tumors was measured using a sensitive ELISA. Finally, kallikrein 10 concentration in 75 serum and plasma samples from 22 healthy women, 20 women with benign diseases, 21 women with endometrioid carcinomas, and 12 uterine serous papillary carcinoma patients was studied.

Results: Kallikrein 10 gene expression levels were significantly higher in uterine serous papillary carcinoma when compared with normal endometrial cell biopsies (mean copy number by real time polymerase chain reaction = 743 versus 1.4; uterine serous papillary carcinoma versus endometrioid carcinoma: $P < .02$). In vitro kallikrein 10 secretion was detected in all primary uterine serous papillary carcinoma cell lines tested (mean = 2.7 μg/L), and the secretion levels were not significantly different to those found in primary ovarian serous papillary tumor cultures (mean 4.2 μg/L). In contrast, no kallikrein 10 secretion was detectable in primary endometrioid carcinomas. Kallikrein 10 serum and plasma concentrations (μg/L; mean ± SEM) among normal

Supported in part by grants from the Angelo Nocivelli and the Camillo Golgi Foundation, Brescia, Italy, and a grant from the Italian Institute of Health (to A. S.).

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doi:10.1016/j.ajog.2005.10.794

healthy females (0.6 ± 0.04), patients with benign diseases (0.6 ± 0.06), and patients with endometrioid carcinomas (0.7 ± 0.06) were not significantly different. In contrast, serum and plasma kallikrein 10 values in uterine serous papillary carcinoma patients (1.2 ± 0.1) were significantly higher than those in the non-cancer group ($P = .002$), benign group ($P = .002$), and endometrioid carcinoma patients ($P = .005$).

Conclusion: Kallikrein 10 is highly expressed in uterine serous papillary carcinoma, and it is released in the plasma and serum of uterine serous papillary carcinoma patients. Kallikrein 10 may represent a novel biomarker for uterine serous papillary carcinoma.

Endometrial cancer is the most prevalent cancer of the female genital tract in North America. On the basis of clinical and histological variables, 2 main types of endometrial cancer have been described. Type I tumors are usually well differentiated and endometrioid in histology and account for the majority of cases, whereas type II endometrial cancers are poorly differentiated tumors, often with serous papillary (USPC) or clear cell histology.[1] Although most endometrioid tumors (ECs) are 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, USPCs and clear cells are poorly differentiated, biologically aggressive tumors, which, although they represent only 10% to 15% of all endometrial carcinomas, are responsible for about 50% of all relapses.[2-4] The discovery of novel diagnostic and therapeutic markers against these aggressive subsets of endometrial cancers remains a high priority.

In an effort to identify genes up-regulated in USPCs that might serve as potential tumor markers for this disease, we recently performed transcriptional profiling utilizing ribonucleic acid (RNA) isolated from highly purified primary USPCs and samples taken from normal endometrial cells (NECs).[5] When relative levels of gene expression were compared between USPCs and NECs, the gene encoding for human kallikrein 10 (hK10, also known as normal epithelial cell-specific 1) was consistently found as 1 of the most highly up-regulated genes in USPCs.[5]

Human K10, originally identified by Liu et al,[6] is a secreted enzyme belonging to a large family of kallikrein proteases that encode for trypsin-like or chymotrypsin-like serine proteases (reviewed by Diamandis and Yousef[7]). Human K10 was originally postulated to be a tumor suppressor gene because it was found abundantly expressed in a normal breast cell line, 76N-MEC, but down-regulated in the radiation-transformed equivalent line, N76-R30.[6] Similarly, hK10 is down-regulated in prostate cancer cell lines,[8] and transfection of hK10 cDNA into a highly aggressive hK10-negative breast cancer cell line dramatically reduced its tumorigenic phenotype.[8] Importantly, hK10 has recently been found at high levels in the circulation of a subset of ovarian cancer patients characterized by high resistance to chemotherapy and short survival.[9-12] This discovery has highlighted the potential of hK10 as a novel biomarker in ovarian cancer. This is the first study to investigate hK10 gene expression and protein secretion in endometrial cancer in vitro as well as in vivo.

Material 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 (F.I.G.O) operative staging system. Only specimens with more than 75% tumor content were used for all experiments. Fresh tumor biopsies from gynecologic tumors including 11 USPCs, 2 ECs, and 5 serous papillary ovarian carcinomas (OSPC) derived from patients harboring advanced stage disease (III-IV) were obtained at the time of surgery through the Gynecologic Oncology Division and the Pathology Department, University of Arkansas for Medical Sciences, under approval of the institutional review board. Patient characteristics from which tumor biopsies were obtained are described in Table I.

Total abdominal hysterectomy and bilateral lymph node dissection were performed in all USPC patients. Six normal endometrial control cell samples (NECs) were obtained from biopsies of benign hysterectomy specimens from women of similar age. Some primary tumor biopsies (ie, 3 USPCs, 2 ECs, and 5 OSPCs) were also established as short-term cultures following previously reported standard tissue culture techniques.[5] Briefly, tumor tissues obtained from cancer patients were mechanically minced and enzymatically dissociated with 0.14% collagenase type I (Sigma, St. Louis, MO) and 0.01% DNAse (Sigma, 2000 KU/mg) in RPMI 1640 media, as described previously by Santin et al.[5] After 1 to 2 hours' 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 medium (Sigma) containing 10% fetal bovine serum (FBS) (Gemini, Woodland, CA). The final pellet was then placed in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% FBS, 200 μg/ml penicillin,

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

	NEC n = 6	EC n = 2	USPC n = 11	OSPC n = 10
Age (mean ± SD)	49 ± 8	59 ± 4	64 ± 9	61 ± 10
Stage				
I	—	—	1	—
II	—	—	1	—
III	—	2	5	3
IV	—	—	4	2
Grading				
G1	—	—	—	—
G2	—	—	—	—
G3	—	2	11	5

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 performed 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 previously described.[5] Only primary cultures that had at least 90% viability and contained more than 99% epithelial cells were used for hK10 quantification by enzyme-linked immunosorbent assay (ELISA) as described below.

RNA isolation

RNA isolation from all primary snap-frozen samples including 11 USPCs as well as 6 normal endometrial cell controls was performed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To verify integrity, 4 μg of RNA from each sample was run in 1% agarose gel using 18S+28S ribosomal RNA (Sigma) as positive control. RNA extracted from the OVCAR-3 serous papillary ovarian cancer cell line, previously reported to express hK10,[10] was used as a positive control.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (q-RT-PCR) was performed with an ABI Prism 7000 sequence analyzer (Applied Biosystems, Foster City, CA) using the manufacturer's recommended protocol to evaluate expression of hK10 gene in samples from all snap frozen biopsies. Each reaction was run in triplicate. The comparative threshold cycle method (Applied Biosystems) was used to determine gene expression in each sample relative to the value observed in the nonmalignant endometrial epithelial cells, using glyceraldehyde-3-phosphate dehydrogenase (assay-on-demand Hs99999905_m1) RNA as an internal control. Briefly, 5 μg of total RNA from each sample was reverse transcribed using SuperScript III first-strand cDNA synthesis (Invitrogen). Ten microliters of reverse-transcribed RNA samples (from 500 μl of total volume) were amplified by using the TaqMan universal polymerase chain reaction (PCR) master mix (Applied Biosystems) to produce PCR products specific for hK10. The hK10 primers were obtained from Applied Biosystems as assay on demand products (assay identification: Hs 00173611_m1).

Analysis of hK10 secretion

An important issue is whether hK10 gene expression differences in tumor tissues result in meaningful differences in protein expression. To validate hK10 data obtained by RT-PCR on snap-frozen USPC and NEC samples at the protein level as well as to compare hK10 secretion among different gynecologic malignancies, supernatants obtained from 10 primary gynecologic tumors including 3 USPCs, 2 ECs, and 5 primary OSPCs were evaluated by ELISA. Briefly, tumor supernatants tested for hK10 secretion were collected by primary tumor cell lines seeded at a density of 1×10^5 cells/mL in tissue culture Petri dishes (Corning) in RPMI 1640 media, supplemented with 10% FBS. After 48 hours' incubation at 37°C, supernatants were aspirated, rendered cell free by centrifugation at 1500 rpm for 10 minutes, and stored at –20°C before being analyzed for hK10 by a sandwich ELISA.

hK10 concentration was quantified in the serum and plasma of 22 apparently healthy women, 20 women with benign diseases, 21 women with histologically proven primary ECs, and 12 women with histologically proven primary USPCs by ELISA as previously described by Luo et al.[9] This assay incorporates 2 hK10-specific antibodies in a sequential 2-site immunometric format with time-resolved fluorescence detection.[9] The assay has a detection limit of 0.05 μg/L and a dynamic range up to 20 μg/L. Variability was less than 10% within the measurement range. Briefly, white polystyrene microtiter plates were coated with anti-hK10 monoclonal antibody.[9] Five hundred micrograms per 100 μL of coating antibody solution (50 mM Tris buffer, 0.05% sodium azide [pH 7.8]) was added to each well and incubated overnight at room temperature.

The plates were washed 3 times with washing buffer (5 mM Tris buffer, 150 mM NaCl, 0.05% Tween 20 [pH 7.8]). Fifty microliters of hK10 calibrators or samples and 50 μl assay buffer (50 mM 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 M 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 6 times with washing buffer, after which 100 μL of biotinylated detection antibody solution (25-50 ng anti-hK10 in assay buffer) were added to each well and incubated for 1 hour at room temperature with shaking. The plates were then washed 6 times with washing buffer.

Subsequently, 5 ng/well of alkaline phosphatase-conjugated streptavidin solution (Jackson ImmunoResearch) in 6% bovine serum albumin buffer (in 50 mM 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 6 times with washing buffer, and then 100 μL of substrate buffer (0.1 mol/L Tris buffer [pH 9.1]) containing 1 mmol/L diflunisal phosphate, 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$ were added to each well and incubated for 10 minutes with shaking at room temperature. One hundred microliters of developing solution (1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, 3 mmol/L EDTA) were added to each well and incubated for 1 minute with shaking at room temperature. The fluorescence was measured with a time-resolved fluorometer (Cyberfluor 615 Immunoanalyzer, MDS Nordion, Toronto, Ontario, Canada).

Patient characteristics from which plasma and serum samples were obtained are described in Table II. Serum and plasma samples were analyzed in duplicate with inclusion of 3 quality-control samples in every run. Variability between serum and plasma samples was less than 0.3 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.

Statistical analysis

The analyses of differences between USPCs and NECs in the q-RT-PCR expression data and between hK10 serum and plasma concentrations among supernatants obtained from gynecologic tumor cultures with different histologies were performed with the Student *t* test at an alpha of 0.05.

Results

hK10 expression in snap-frozen endometrial cancer by q-RT-PCR

USPCs are rare tumors that may present in either pure forms or admixed with endometrioid or clear cell tumor cells (ie, mixed USPCs).[4] 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 hK10 expression by RT-PCR from 11 primary USPCs with single-type differentiation (ie, pure USPCs). A comparison of the q-RT-PCR data for hK10 in USPCs and NECs is shown in Figure 1. Significant expression differences between USPCs and NECs were readily apparent (Figure 1, **A** and **B**). All USPC samples (11 of 11 is 100%, mean copy number ± SEM 743 ± 214, range from 49 to 2557) were found positive for hK10 expression by RT-PCR (Figure 1B). In contrast, only low levels of hK10 gene expression were found in the NEC control cultures tested (mean copy number ± SEM 1.4 ± 0.2, range from 1 to 1.6) (Figure 1, **B** USPCs versus NECs: $P < .02$).

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

Variable	Healthy female n = 22	Benign diseases n = 20	EC n = 21	USPC n = 12
Age (mean ± SD)	43.6 ± 12.9	50.2 ± 14.6	60.8 ± 9.2	63.5 ± 9.5
Stage				
I	—	—	17	1
II	—	—	3	1
III	—	—	1	6
IV	—	—	—	4
Grading				
G1	—	—	13	—
G2	—	—	6	—
G3	—	—	2	12

hK10 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 (eg, monocytes, dendritic cells, lymphocytes). Primary short-term tumor cultures, minimizing the risk of a selection bias inherent in any long-term in vitro growth, may provide an opportunity to study differential hK10 secretion between highly enriched populations of tumor-derived epithelial cells. Cell-free supernatants from 10 freshly isolated gynecologic malignancies including 3 USPCs, 2 high-grade ECs, and 5 high-grade OSPCs were collected and analyzed for the levels of hK10 expression by ELISA.

Because prolonged passages in vitro are known to alter the physiology and phenotype of primary tumor cells, we performed all our experiments with highly purified fresh tumor cells grown for less than 10 passages in vitro. Growth control medium was always analyzed at the same time. In this regard, RPMI 1640 media containing 10% FBS had no detectable endogenous levels of hK10 immunoreactivity by ELISA (data not shown). As shown in Figure 2, all primary USPC tumor cell lines tested secreted significant amounts of

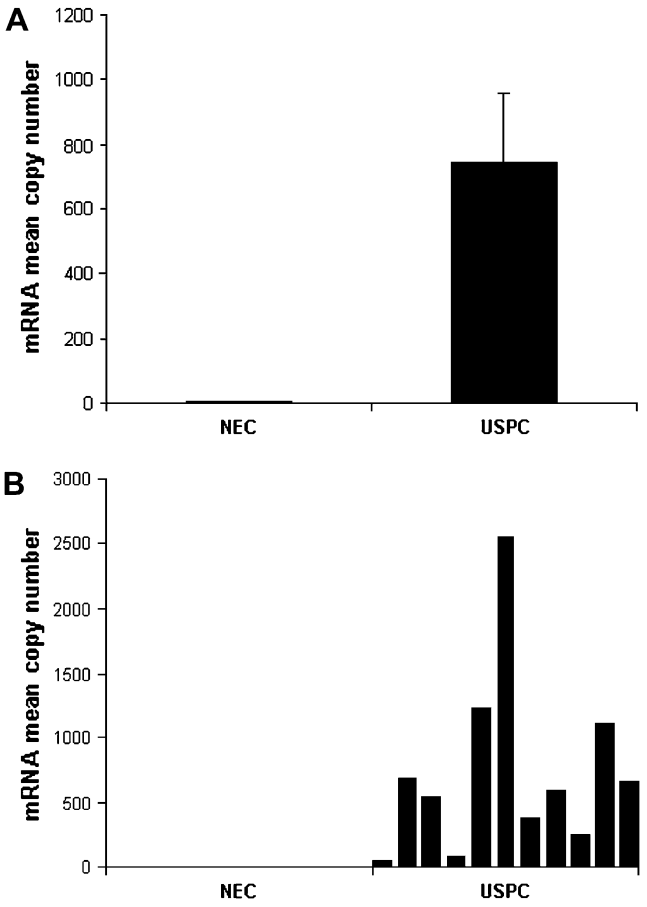

Figure 1 hK10 messenger RNA copy number by quantitative RT-PCR in 17 snap-frozen endometrial biopsies. **A,** hK10 mRNA mean copy number ± SEM in 6 NEC control samples and 11 USPCs. **B,** hK10 messenger RNA copy number in individual NEC controls and USPC samples.

hK10 (mean 2.7 μg/L, range between 1.8 and 3.4 μg/L per 10^5 cells per 48 hours). These levels were not significantly different when compared with those detected in 3 of 5 primary serous ovarian tumor cultures used as positive controls (mean 4.2 μg/L, range between 0.0 and 15.8 μg/L per 10^5 cells per 48 hours) (Figure 2). In contrast, hK10 secretion was negligible in the 2 primary short-term EC cultures derived from G3 tumors (Figure 2, USPCs versus OSPCs: not significant; USPCs versus ECs: $P < .02$).

Plasma and serum hK10 concentration in endometrial cancer and noncancer patients

To investigate whether hK10 is detectable in the plasma and serum of patients harboring endometrial cancer, plasma and serum samples from 21 EC patients and 12 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. The

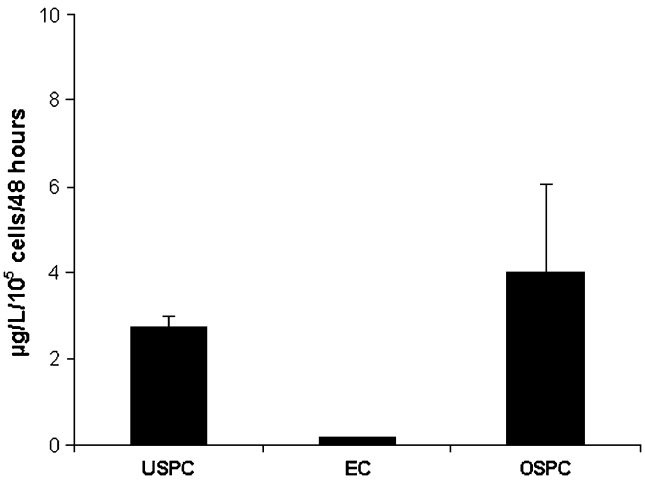

Figure 2 hK10 levels by ELISA in the supernatants from 10 freshly isolated gynecologic malignancies including 3 USPCs, 2 ECs and 5 high-grade OSPCs.

Table III Serum and plasma hK10 in noncancer (healthy), benign disease, and EC and USPC patients

Variable	Mean ± SEM	Range
Noncancer (n = 22)	0.6 ± 0.04	0.3 to 1.2
Benign disease (n = 20)	0.6 ± 0.06	0.2 to 1.3
EC (n = 21)	0.7 ± 0.06	0.2 to 1.3
USPC (n = 12)	1.2 ± 0.1*	0.6 to 2.1

* *P* value: noncancer cells versus USPC = 0.002; benign versus USPC = 0.002; EC versus USPC = 0.005.

hK10 serum and plasma levels (in micrograms per liter) from 22 healthy female controls, (mean ± SEM 0.6 ± 0.04, range from 0.3 to 1.2), 20 patients with benign gynecologic diseases (mean ± SEM 0.6 ± 0.06, range from 0.2 to 1.3), and 21 patients with ECs (mean ± SEM 0.7 ± 0.06, range from 0.2 to 1.3) were not statistically significantly different (Table III). In contrast, serum and plasma hK10 values in USPC patients (mean ± SEM 1.2 ± 0.1, range from 0.6 to 2.1) were significantly higher than those in the noncancer ($P = .002$), benign group ($P = .002$), and EC patients ($P = .005$).

Comment

Kallikreins belong to a family of serine proteases endowed with well-characterized roles in diverse cellular activities including blood coagulation, wound healing, digestion, and immune responses as well as tumor invasion and metastasis. Secreted serine proteases such as prostate-specific antigen and kallikrein 2 have already found important clinical applications as prostate cancer biomarkers.[7] This report represents the first evaluation

of hK10, a member of the expanded human kallikrein gene family,[7] as a novel biomarker in USPCs. We report a high level of expression of the hK10 gene in USPCs. The hK10 gene expression was strikingly higher in USPCs when compared with NECs by RT-PCR, with a mean copy number of hK10 gene messenger RNA in USPCs 531 times higher when compared with NECs. Consistent with these findings in-snap frozen tissue, highly purified primary USPC cultures were found to secrete high levels of hK10 by ELISA. In contrast, hK10 was not detected in either of the 2 EC cell lines tested in this study. When USPC secretion of hK10 was compared with that of primary serous ovarian cancer cell lines in vitro, no significant differences were found. The high levels of hK10 secreted by 3 of 5 primary ovarian tumors tested in our assay are consistent with recent reports showing high levels of hK10 expression in tumor tissue and serum of ovarian cancer patients.[11,12]

In these studies, ovarian cancer patients showing high concentrations of hK10 in ovarian tumor cytosol or with high preoperative serum levels of hK10 had significantly worse prognosis than patients with low preoperative hK10.[11,12] The hK10 overexpression in these previously published studies[11,12] was found to correlate with intrinsic resistance to adjuvant chemotherapy. Our results showing high levels of hK10 secretion by USPCs, a variant of uterine carcinoma notorious for its high resistance to chemotherapy and its aggressive biologic behavior, are consistent with these findings. Taken all together, these data further support the view that high expression of hK10 in ovarian serous papillary carcinoma and USPCs may identify gynecologic malignancies characterized by an aggressive biologic behavior and resistance to chemotherapy.

Next we evaluated hK10 levels in the plasma and serum of endometrial cancer patients. We found high levels of hK10 in USPC patients but not in patients harboring ECs or benign gynecologic disease, when compared with the levels found in healthy women. These in vivo data agree 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 vivo as well as in vitro. In agreement with previous reports,[11,12] we found no elevation of hK10 in the serum and plasma of patients harboring benign gynecologic disease or in healthy females.

The use of hK10 as a novel biomarker for the early diagnosis, monitoring, and prediction of response to treatment in USPCs might greatly contribute to the improvement of clinical management and outcomes of these patients. Although CA 125 may be elevated in metastatic endometrial tumors with endometrioid histology,[13,14] this marker appears to have limited utility in monitoring the effects of adjuvant therapy or in the prediction of tumor recurrence in USPC patients.[15] These data suggest that kallikrein 10 may represent a promising novel biomarker for early detection of recurrent USPC disease and/or the monitoring USPC response to adjuvant therapy.

In this single-institution pilot work, the majority of USPC patients were found to harbor advanced disease. This point is noteworthy because, although most of our patients were considered to have clinical stage I disease and up-graded only at the time of comprehensive surgical staging laparotomy, it remains possible that the elevated levels of hK10 may reflect a bias related to advanced stage. Larger studies including more USPC patients harboring surgically confirmed early-stage disease will be necessary to exclude this possibility. Nevertheless, of the 2 patients included in this work who had surgically confirmed early stage disease, 1 was found to have elevated levels hK10, suggesting no advanced stage bias. It is important to point that out because of the propensity of USPCs to rapidly manifest extrauterine disease; the USPC series reported here is representative of the advanced disease commonly found in comprehensively surgically staged USPC patients.[2,16]

Multiple kallikrein gene family members, including the enzymes hK4, hK5, hK6, hK7, hK8, and hK9 have recently been shown to have prognostic significance in ovarian cancer.[7,17] Because USPC is histologically similar to high-grade serous ovarian carcinoma, it seems very likely that, analogous to ovarian cancer, multiple members of the human kallikrein gene family may be also dysregulated in USPC. Consistent with this view, our group has recently reported the high expression of hK6 in USPC patients,[5] further supporting the potential use of multiple kallikreins as biomarkers for not only ovarian carcinoma but also USPC.[18] Because serine proteases are involved in cancer progression, they may be suitable candidates not only for diagnostic and prognostic purposes but also as novel therapeutic targets.

Consistent with this view, we have recently reported the definition of an immunogenic region within the stratum corneum chymotryptic enzyme (SCCE/kallikrein 7/hK7), which incorporates multiple CD8+ cytotoxic T lymphocyte epitopes as well as CD4+ T helper epitopes.[19,20] This discovery has highlighted the potential use of serine proteases as attractive target antigens for the immunotherapy of human cancer patients refractory to standard treatment modalities.[19,20] On the basis of these findings, the antigenic potential of other kallikreins highly differentially overexpressed in human tumors is currently being investigated in our laboratory.

In conclusion, we report here the first evidence that hK10 is highly expressed in USPC and that high concentrations of hK10 are present in the plasma and serum of USPC patients. Our results strongly support the hypothesis that hK10 may represent a novel biomarker for this highly aggressive variant of endometrial cancer.

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