original article

Circulating biomarker tissue kallikrein-related peptidase KLK5 impacts ovarian cancer patients' survival

J. Dorn[1]*, V. Magdolen[1], A. Gkazepis[1], T. Gerte[1], A. Harlozinska[2], P. Sedlaczek[2], E. P. Diamandis[3,4,5], T. Schuster[6], N. Harbeck[1†], M. Kiechle[1] & M. Schmitt[1]

[1]Department of Obstetrics and Gynecology, Technical University of Munich, Munich, Germany; [2]Department of Clinical Immunology, Wrocław Medical University, Wrocław, Poland; [3]Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Canada; [4]Department of Clinical Biochemistry, University Health Network and Toronto Medical Laboratories, Toronto, Canada; [5]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada; [6]Institute for Medical Statistics and Epidemiology, Technical University of Munich, Munich, Germany

Background: Effective cancer biomarkers for early detection, prognosis, or therapy response prediction are urgently needed in ovarian cancer. Kallikrein-related peptidases, including KLK5, have been reported to play an important role in the course of the disease.

Patients and methods: KLK5 antigen content was determined by enzyme-linked immunosorbent assay in ovarian cancer patients' [FIGO (International Federation of Gynecology and Obstetrics) stages I–IV, $n = 52$] serum as well as ascitic fluid and compared with KLK5 content in serum of patients with benign ovarian tumors ($n = 45$).

Results: KLK5 antigen content was significantly elevated in the serum of ovarian cancer patients compared with the serum of patients with benign ovarian tumors. Forty-two of 52 ovarian cancer serum samples, 42 of 43 benign ovarian tumor serum samples, and all 41 ascitic fluid samples were KLK5 positive. Elevated KLK5 antigen in serum and ascitic fluid of ovarian cancer patients was a prognostic factor for progression-free survival.

Conclusions: Our data support the finding that ovarian cancer patients release significant amounts of KLK5 into serum and ascitic fluid but KLK5 antigen is low in serum of patients with benign ovarian tumors. Increased serum and ascitic fluid KLK5 levels are associated with poor patient outcome, thus underlining the importance of KLK5 as a biomarker for early detection as well as for disease management in ovarian cancer.

Key words: kallikrein-related peptidase 5, KLK5, ovarian cancer, prognosis, serum cancer biomarker

introduction

Ovarian cancer is the third most common neoplasm of the female reproductive tract but the leading cause of death from a gynecological malignancy [1]. Early detection could potentially decrease mortality, but early-stage tumors commonly show no obvious and often only unspecific signs or symptoms [2]. Numerous studies have focused on understanding the underlying tumor biology of ovarian cancer and to develop improved screening methods or to tailor more effective ways of treatment, based on the tumor's unique molecular signature [3]. In addition, by finding reliable prognostic and/or predictive factors, patients at high risk might be identified and directed to tailored therapeutic regimens [4, 5].

The tissue kallikrein family genes (kallikrein-related peptidases, KLK1–15) are clustered on chromosome 19q13.4. Evidence for involvement of the human tissue kallikrein serine protease family in ovarian cancer progression is emerging [6–8]. Several kallikreins are implicated to participate within a proteolytic cascade in neoplastic progression by promoting or inhibiting cancer cell proliferation, angiogenesis, invasion, and metastasis [9, 10].

KLK5, -6, -8, -10, and -11 seem to be useful diagnostic serum markers in ovarian cancer with additional value to that of the tumor marker CA 125 [11]. KLK5 is present in the serum of more than two-thirds of ovarian cancer patients of any stage but undetectable in healthy individuals [12]. When measured serially before and after the first cycle of chemotherapy in serum of ovarian cancer patients, rapid decreases of KLK5 levels appear to be associated with favorable response. Higher KLK5 concentration at baseline indicate worse outcome [13].

Correspondence to: Dr J. Dorn, Department of Obstetrics and Gynecology, Technische Universität München, Ismaninger Str. 21, D-81576 München, Germany.
Tel: +49-89-4140-2442; Fax: +49-89-4140-4811; E-mail: julia.dorn@lrz.tum.de

[†]Present address: Breast Center, Department of Obstetrics and Gynecology, University of Cologne, Cologne, Germany.

In tumor tissue of ovarian cancer patients, KLK4–8, -10, -11, -13, and -15 are differentially expressed at messenger RNA and protein expression levels [14]. KLK4, -5, -6, -7, and -10 have been reported as markers of more aggressive tumors with poor prognosis [15]. KLK5 is significantly elevated in ovarian cancer tissues compared with low-malignant-potential (LMP) tumors. High KLK5 tumor tissue levels are associated with advanced stage of the disease and significantly shorter progression-free survival (PFS) and overall survival [16, 17]. In contrast, KLK8, -11, and -13 seem to be favorable tumor biomarkers [10, 18]. Hence, among the human kallikrein-like peptidases, KLK5 may serve as a valuable poor prognostic biomarker in ovarian cancer.

The present study was conducted to evaluate the kallikrein-related peptidase KLK5 as new serum biomarker in ovarian cancer, which (i) may serve as a screening tool, e.g. within a multiparametric score, to identify those patients who need to be referred for further diagnostic work-up; (ii) may allow assessment of prognosis in patients with ovarian cancer, i.e. to identify those patients who may benefit from a therapy that is different from the standard regime; and (iii) may identify KLK5 as a potential new target for therapy.

The circulating cancer biomarker KLK5 is easy to assess by enzyme-linked immunosorbent assay (ELISA) in serum and ascitic fluid of ovarian cancer patients before or at the time of surgery. It therefore seems to be useful for ovarian cancer screening and as a candidate marker for assessment of prognosis, as well as for tailoring or targeting of therapy in ovarian cancer. In the present analysis, KLK5 antigen levels were determined by ELISA in sera of patients with benign ovarian tumors, and, for the first time ever, in matched sera and ascitic fluids of ovarian cancer patients, and the prognostic impact of KLK5 antigen content assessed.

materials and methods

patients

Fifty-two patients (median age: 53 years, range: 37–75 years) with histologically confirmed primary ovarian carcinoma FIGO (International Federation of Gynecology and Obstetrics) stages I–IV were enrolled in this study. Patients were treated at the 1st Gynecological Department of the Wrocław Medical University, Poland, according to international treatment guidelines for ovarian cancer patients, including primary cytoreductive surgery followed by platinum-containing chemotherapy. The KLK5 study was approved by the local ethics committee and was conducted cooperatively by the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität Muenchen, Munich, Germany; the Department of Clinical Immunology, Wrocław Medical University, Poland; and the Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Canada. Median follow-up of patients still alive at time of analysis was 22 (1–40) months. Detailed clinical patient data are shown in Table 1. Residual tumor mass was assessed using the now old clinical classification of ≤2 or >2 cm, which was standard at the time of surgery. Thus, in the present retrospective study, data referring to the current classification of optimal and suboptimal debulking (≤1 or >1 cm and/or the size of residual tumor in millimeter) cannot be supplied.

In addition to ovarian cancer patients' samples, a collective of sera obtained from 45 patients with benign ovarian neoplasms encompassing serous cysts ($n = 30$, 67%), mucinous adenoma ($n = 8$, 18%), and serous

Table 1. Ovarian cancer patients' clinical data ($n = 52$)

		n	%
Median age, years (range)		53.0 (37–75)	
Median follow-up, months (range)		22 (1–40)	
FIGO stage	I	8	15
	II	6	12
	III	23	44
	IV	15	29
FIGO stage (early versus late)	I/II	14	27
	III/IV	38	73
Nuclear grade	G1	9	17
	G2	17	33
	G3	26	50
Nuclear grade (low versus high)	G1/G2	26	50
	G3	26	50
Age (years)	≤60	32	62
	>60	19	36
	Missing	1	2
Histological subtype	Serous	26	50
	Endometrioid	11	21
	Undifferentiated	11	21
	Mucinous	3	6
	Clear cell	1	2
Residual tumor mass (cm)	≤2	20	38
	>2	31	60
	Missing	1	2
Deceased	No	36	69
	Yes	15	29
	Missing	1	2
Progress	No	20	38
	Yes	31	60
	Missing	1	2

FIGO, International Federation of Gynecology and Obstetrics.

cystadenoma ($n = 7$, 16%) was studied. Median age of these patients was 48 years (18–77 years).

Blood was drawn preoperatively and serum collected. Ascitic fluid was obtained during surgery by collection into a sterile bag after flushing the needle and bag with heparin. The ascitic fluid was cleared by centrifugation at 10 000 g for 30 min and fat and debris discarded. Ascitic fluids were stored at −80°C until further use.

KLK5 ELISA

Antigen content of KLK5 was quantified using a highly sensitive and specific noncompetitive in-house 'sandwich-type' immunoassay [12, 19]. In essence, at first, wells of a polystyrene microtiter plate are incubated with a capture antibody (monoclonal mouse anti-KLK5) solution. This capture antibody was generated by immunizing mice with recombinant KLK5 protein, and the polyclonal detection antibody by immunizing rabbits with the same KLK5 protein. Secondly, a KLK5-containing test solution is added to the wells and detected by addition of the polyclonal antibody to KLK5 followed by addition of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch, West Grove, PA). KLK5 content is visualized by addition of diflunisal phosphate (DFP). After removal of a phosphate ester of DFP by alkaline phosphatase, the resulting DF forms a complex with Tb^{3+}–EDTA, which upon excitation at 336 nm causes Tb^{3+} fluorescence at 624 nm. Fluorescence signal detection and data analysis were handled by the time-resolved CyberFluor 615

Immunoanalyzer (MDS Nordion, Kanata, Canada). All samples were analyzed in duplicates. Lower and upper detection limits of this sensitive assay are 0.1 and 25 ng/ml, respectively. No cross-reactivity of the KLK5 ELISA with any of the other 14 members of the kallikrein-like peptidase family was observed.

production of human KLK proteins

Recombinant pro-forms of KLK3–15 were designed and produced in-house in a similar fashion as described previously by Debela et al. [20]. Briefly, KLK complementary DNAs encompassing the full-length coding sequence were isolated from breast or ovarian cancer tissues, or from cancer cell lines, and fusion genes generated encoding the native pro-forms of the respective KLK plus an N-terminally located histidine tag. Subsequently, recombinant KLK proteins were produced in transformed *Escherichia coli* cells, enriched, and purified under denaturing/slightly reducing conditions [20].

western blot analysis

For western blots and immunohistochemistry, a commercially available immunopurified goat polyclonal antibody directed to KLK5, different from the ELISA antibody (AF1108, #1108-SE; R&D Systems, Minneapolis, MN), was used. The protein used for affinity purification of AF1108 is the mature form of recombinantly expressed KLK5, expressed in a murine myeloma NS20-derived cell line. AF1108 is not part of the KLK5 ELISA. To demonstrate specificity of AF1108, recombinant KLK proteins 3–15, plus tissue factor representing an irrelevant control protein, were subjected to electrophoresis in 12% sodium dodecyl sulfate polyacrylamide gels and then separated proteins transferred on to polyvinylidene fluoride membranes (PALL, Dreieich, Germany) for 3 h at 75 mA per membrane, using a semidry transfer device (Whatman Biometra, Göttingen, Germany). After transfer, membranes were blocked with 5% skim milk powder (#70166; Sigma-Aldrich, Munich, Germany) in phosphate-buffered saline (PBS; 0.1%)–Tween-20 (pH 7.4, 1 h, room temperature). Subsequently, blots were incubated overnight at 4°C with KLK5-directed goat polyclonal antibody AF1108 (0.4 μg/ml) followed by three washes, 10 min each, in PBS–Tween-20 at room temperature. Binding of the antibody to the target protein was visualized by incubation of the membrane with horseradish peroxidase-conjugated rabbit anti-goat IgG (#A5420; Sigma-Aldrich) diluted 10 000-fold in PBS–Tween-20 containing 5% skim milk powder, followed by chemiluminescence reaction (Amersham Biosciences, Little Chalfont, UK). For determination of the relative molecular mass of the various KLK proteins, the prestained Protein IV Marker set (PeqLab, Erlangen, Germany) was used.

immunohistochemical visualization of KLK5 in normal and diseased ovarian tissues

Formalin-fixed, paraffin-embedded ovarian tissue specimens were retrieved from the archives of the Institute of Pathology of the Technical University of Munich, Germany. Full-face sections (2 μm) were deparaffinized and hydrated by passing them through xylene twice (10 min) and a descending series of graded ethanol (5 min each step). Then, the sections were subjected to antigen retrieval by pressure cooking in 10 mM citrate buffer, pH 6.0. Endogenous peroxidase activity in the tissue sections was quenched by blocking solution #K5361 (room temperature, 5 min) containing 0.5% H_2O_2, detergents, and enzyme inhibitors (DAKO, Hamburg, Germany). Sections were incubated overnight at 4°C with antibody AF1108 directed to KLK5, diluted 1 : 100 (2 μg/ml) in antibody diluent #S2022 (DAKO). Binding of the antibody to the KLK5 target protein was visualized following the EnVision protocol employing a dextran polymer conjugated with horseradish peroxidase-labeled antibodies to goat IgG and the peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride chromogen (K5361; DAKO), which forms a brown product at the site of enzyme reaction.

Nuclei were counterstained with Mayer's acid hematoxylin for 10 s, rinsed under running tap water, transferred to distilled water, and mounted with mounting medium Histokitt (#1025-250; Assistent, Sondheim, Germany).

Immunohistochemical images were captured using the digital slide scanner Hamamatsu NanoZoomer RS (Hamamatsu Photonics, Munich, Germany), at ×200 magnification, and slightly processed with a brightness/contrast tool (Microsoft PowerPoint 2007).

statistical analysis

The established prognostic clinical factors such as age, FIGO stage, nuclear grade, and residual tumor mass were coded as binary variables (age: ≤60 versus >60 years; FIGO stage: I/II versus III/IV; residual tumor mass: ≤2 versus >2 cm). To meet the nonnormal distribution of KLK5, nonparametric tests were used for statistical analysis. Spearman's rank correlation was applied to assess bivariate relationships of different biomarkers. Comparison of biomarker expression between low- and high-risk patient groups was carried out by the Mann–Whitney U Test. Outcome variables were PFS and overall survival. Corresponding survival probabilities were estimated according to Kaplan–Meier method and plotted. Comparison between low- and high-risk groups was carried out using the log-rank test. Uni- and multivariate Cox proportional hazard regression analyses were carried out to investigate the statistically independent impact of clinical factors or KLK5 on survival; estimates of hazard ratios were provided with 95% confidence intervals. All statistical tests were carried out two-sided and a P value ≤0.05 was considered statistically significant. In general, the present investigation has to be considered exploratory in nature. Therefore, to retain maximum statistical power in the primary interesting analyses, no correction for multiple testing was carried out. Our results may serve to generate hypotheses, which can then be validated in prospective and adequately powered analyses.

results

The present retrospective investigation focuses on the clinical impact of cancer biomarker KLK5 in patients with primary ovarian cancer (n = 52), encompassing different tumor stages (FIGO I, II, III, and IV). Table 1 summarizes the clinical and histopathological patient characteristics. Higher FIGO stage III/IV is associated with poor clinical outcome compared with early stage of the disease FIGO I/II (P = 0.006 for PFS), demonstrating that our results are in agreement with those reported by others [21, 22].

In general, KLK5 protein is highly expressed in the skin, with lower concentrations in the breast, esophagus, and salivary gland. By immunohistochemistry, we analyzed the expression pattern of KLK5 in benign and malignant ovarian neoplastic tissues, applying a polyclonal antibody (AF1108) directed to amino acids 67–293 of human KLK5. In western blot analysis, this antibody does not cross-react with any of the KLK family members tested (Figure 1A). KLK5 protein is, if at all, expressed very weakly in an endometriosis cyst, a benign disorder of the ovary (57-year-old patient) (Figure 1B) but distinctly expressed in primary serous ovarian cancer tissue (59-year-old patient, FIGO stage IV, nuclear grade 3) (Figure 1C).

As analyzed by ELISA, KLK5 is released into the blood and ascitic fluid of ovarian cancer patients (Table 2) but is not detected in sera of normal female donors [23]. As depicted in Table 2 and Figure 2A, 48 of 52 ovarian cancer serum samples and 42 of 43 serum samples of patients presenting with benign ovarian tumor were positive for KLK5 employing a highly
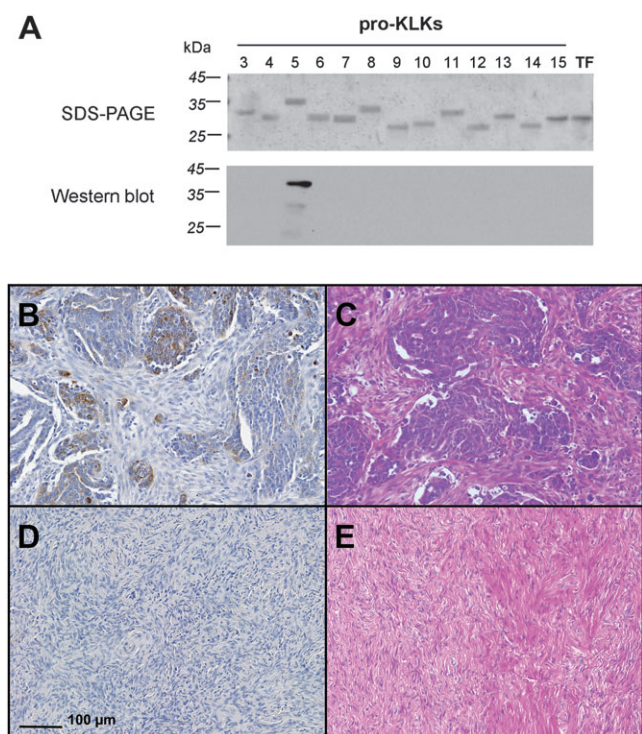
Figure 1. Immunohistochemical staining for presence of kallikrein-related peptidase 5 (KLK5) in benign and malignant ovarian tumor tissues. Specificity of the KLK5-directed goat polyclonal antibody was tested by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis. After separation by 12% SDS–PAGE (A), His-tagged recombinant pro-forms of KLK3–15 (1 µg each), plus an irrelevant protein (His-tagged tissue factor), were blotted to a polyvinylidene fluoride membrane and reacted with polyclonal goat AF1108 antibody directed to KLK5. KLK3, KLK4, and KLK6–15 did not react with the antibody, only KLK5, with an apparent molecular weight of 40 kDa. The additional faint bands seen in the KLK5 lane correspond to small amounts of degradation products of KLK5, which arise during purification of recombinant KLK5. Thus, no cross-reactivity of the AF1108 antibody with other KLKs except KLK5 was observed. KLK5 is most closely related to KLK4, -7, -8, -9, -11, -13, and -14 (amino acid sequence identity between 48% and 52%). The other members of the KLK family display an identity that is lower than KLK5 versus trypsin. To control the protein concentrations used, another gel electrophoresis with an identical loading was carried out and the proteins visualized by Coomassie blue staining. Representative tissues of (B, C) primary ovarian cancer, FIGO (International Federation of Gynecology and Obstetrics) stage IV, nuclear grade 3, and (D, E) benign ovarian disorder (endometriosis cyst) were stained with hematoxylin–eosin (C, E) and with antibody AF1108 to KLK5 (B, D), respectively. KLK5: brown color; nuclei were counterstained with hematoxylin (blue color). Images were scanned and digitized by the Hamamatsu NanoZoomer Digital Pathology virtual microscope (Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany) with a ×40 lens. Note: KLK5 protein expression is almost absent in the endometriosis cyst tissue but present in several tumor cells within the ovarian cancer tissue nests. Likewise, determined in tumor tissue extracts of the patients by KLK5-specific ELISA, antigen expression is low in the benign case but elevated in the tumor tissue of the ovarian cancer patient.

specific KLK5 ELISA test. Interestingly, only low amounts of KLK5 are present in the serum of patients with benign ovarian

disease (Figure 2A). All 41 ascitic fluid samples were KLK5 positive.

Levels of KLK5 and the established ovarian cancer tumor marker CA 125 did not correlate, neither when measured in serum nor when measured in ascitic fluid; CA 125 was a good discriminator between benign and malignant disease (data not shown). KLK5 content in serum was significantly, but weakly, correlated with KLK5 content in ascitic fluid of ovarian cancer patients ($r = 0.39$, $P = 0.012$, $n = 41$). There is a nonsignificant trend of the preoperative serum concentration of KLK5 toward lower values in patients with a small residual tumor mass after surgery (≤2 cm) than in those with tumor mass >2 cm and poor surgical outcome ($P = 0.22$) (Figure 2B).

To evaluate the impact of clinical factors and biomarkers on prognosis, uni- and multivariate Cox regression analyses were carried out, as shown in Table 3. Significant variables of this statistical model regarding PFS and overall survival in univariate analysis were FIGO stage (I/II versus III/IV), residual tumor mass after surgery (≤2 versus >2 cm), age, and KLK5 protein content (cut-off: median value) in serum and ascitic fluid. Figure 3 exemplifies the statistical significance of both serum ($P = 0.034$) and ascitic fluid ($P = 0.041$) KLK5 levels for PFS. The Kaplan–Meier curves are dichotomized using the median of the KLK5 concentration in serum and ascitic fluid.

In multivariate analysis for PFS (Table 3), however, only age was found to be a statistically significant predictor ($P = 0.044$). Regarding overall survival (Table 3), only FIGO stage was statistically significantly associated with an increased risk for death ($P = 0.043$).

discussion

Despite numerous efforts, there are still no established, effective circulating biomarkers for the early diagnosis of or risk assessment in ovarian cancer, neither for the early nor for the advanced stages of the disease [24]. Although the CA 125 serum biomarker is a valuable marker for monitoring the clinical course of ovarian cancer patients, its sensitivity is very low for early detection or prognosis of ovarian cancer [24, 25]. Furthermore, only little information on patient outcome in relation to preoperative CA 125 level has been reported [26]. Nonetheless, there is an urgent need to identify additional circulating biomarkers that could diagnose ovarian cancer at an early stage before it becomes clinically detectable, especially since ∼20% of ovarian cancers have little or no expression of CA 125 [27] and that owing to the absence of specific symptoms and lack of effective screening strategies, >70% of the women are diagnosed at late stage of disease (FIGO stage III or IV) [1, 24, 25, 27].

Recently, additional circulating serum cancer biomarkers have been evaluated alone and in combination with CA 125, which may have a potential for screening, diagnosis, prognosis, and monitoring of ovarian cancer patients' clinical outcome [24, 25]. In this respect, the most promising serum ovarian cancer biomarkers include human epididymis protein 4 (HE4), mesothelin, macrophage colony-stimulating factor, osteopontin, soluble epidermal growth factor receptor, and the tissue kallikrein-related peptidases [28], formerly known as tissue kallikreins [9].

Table 2. KLK5 antigen concentration in serum and ascites of ovarian cancer patients and in serum of patients with benign ovarian tumor

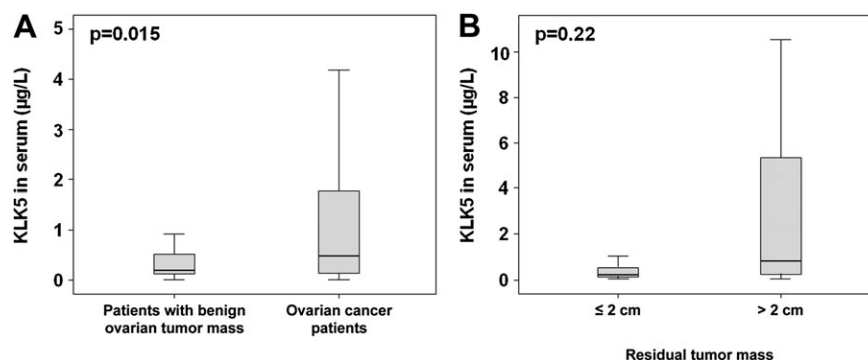|	KLK5 (µg/l) in serum of ovarian cancer patients	KLK5 (µg/l) in serum of patients with benign ovarian tumors	KLK5 (ng/mg protein) in ascitic fluid of ovarian cancer patients
n	52	43	41
Missing	0	2	11
Range	0.1–118.2	0.1–1.4	0.1–156.6
Interquartile range (25%–75% quartile)	0.13–1.81	0.12–0.54	0.10–1.37
Median	0.47	0.19	0.21
No. of samples below detection limit	4	1	0

KLK5, kallikrein-related peptidase 5.

Figure 2. Kallikrein-related peptidase 5 (KLK5) antigen content in serum. KLK5 is significantly elevated in serum of ovarian cancer patients ($n = 52$, with four samples below detection limit) compared with patients with benign ovarian tumor ($n = 43$, with one sample below detection limit) (A). Furthermore, KLK5 is elevated in serum of ovarian cancer patients with residual tumor tissue mass >2 cm ($n = 31$) compared with patients with residual tumor mass ≤2 cm ($n = 20$), although this is a nonsignificant trend only ($P = 0.22$) (B). Boxes represent the interquartile range (IQR) with median (horizontal line within the box) and whiskers represent the farthest data points that are within 1.5 times the IQR from the lower/upper edge of the box. Data points outside the range of whiskers are not displayed.

Table 3. Uni- and multivariate Cox regression analysis for progression-free and overall survival

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Progression-free survival						
FIGO I/II versus III/IV	5.55	1.65–18.66	0.006	–	–	n.s.
Residual tumor mass ≤2 versus >2 cm	4.56	1.76–11.77	0.002	–	–	n.s.
Age ≤60 versus >60 years	2.05	1.0–4.19	0.049	2.12	1.02–4.39	0.044
KLK5 in serum >0.47[a] versus ≤0.47	2.23	1.06–4.69	0.034	–	–	n.s.
KLK5 in ascitic fluid >0.21[a] versus ≤0.21	2.35	1.04–5.33	0.041	–	–	n.s.
Overall survival						
FIGO I/II versus III/IV	7.15	0.93–54.67	0.058	10.7	1.08–106.0	0.043
Age ≤60 versus >60 years	2.68	0.94–7.67	0.066	–	–	n.s.
Residual tumor mass ≤2 versus >2 cm	2.24	0.71–7.05	0.17	–	–	n.s.
KLK5 in serum >0.47[a] versus ≤0.47	1.71	0.61–4.81	0.31	–	–	n.s.
KLK5 in ascites >0.21[a] versus ≤0.21	1.67	0.54–5.12	0.37	–	–	n.s.

[a]Median cut-off value.

HR, hazard ratio; CI, confidence interval: FIGO, International Federation of Gynecology and Obstetrics; n.s., nonsignificant; KLK5, kallikrein-related peptidase 5.
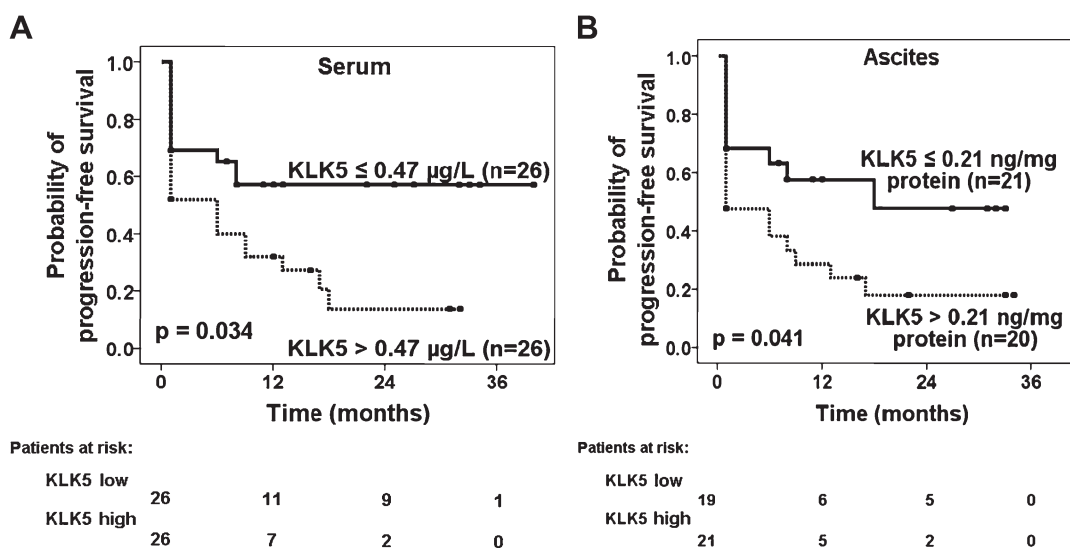
Figure 3. Probability of progression-free survival of ovarian cancer patients ($n = 52$) stratified by kallikrein-related peptidase 5 (KLK5) content in serum (A) or ascitic fluid (B). (A) Patients with higher KLK5 content in serum (dichotomized by the median, >0.47 µg/l) have a significantly worse prognosis than patients with a lower KLK5 content in serum (≤0.47 µg/l; $P = 0.034$). (B) The same can be observed for the KLK5 content in ascitic fluid: progression-free survival is worse in patients with high KLK5 levels in ascitic fluid (>0.21 ng/mg protein, the median) versus low KLK5 levels (≤0.21 ng/mg protein; $P = 0.041$).

Oikonomopoulou et al. [13] examined a panel of 10 serum biochemical parameters for their ability to predict response to chemotherapy, progression, and survival of ovarian cancer patients. Sera from ovarian cancer patients were analyzed for content of CA 125, B7-H4, regenerating protein IV, spondin-2, as well as KLK5, -6, -7, -8, -10, and -11. All markers examined, except KLK7 and regenerating protein IV, were powerful predictors of time to progression among chemotherapy responders. A rapid decrease in KLK5 serum levels after the first chemotherapy cycle was associated with a favorable response to chemotherapy. Likewise, Zheng et al. [29] examined a panel of 11 biochemical variables, including nine KLKs plus B7-H4 and CA 125, measured in cytosolic extracts of ovarian cancer tissues for their power to diagnose, give prognostic information, or predict chemotherapy response in ovarian cancer patients. In this study, KLK5 and KLK6 were positively associated with progression by univariate analysis; KLK10 and KLK11 both predicted time to progression in a multivariate statistical model.

The view that tumor-associated overexpression of kallikrein-related peptidases contributes to ovarian cancer progression is strongly supported by an experimental mouse cancer model study that showed that overexpression of KLK4, -5, -6, and -7 increases the malignant phenotype of ovarian cancer cells. Simultaneous expression of these KLKs resulted in a considerable increase of tumor burden compared with the vector control cell line [30].

We would like to mention, that, in addition to KLK5, we also have assessed the impact of other kallikrein-related peptidases such as KLK6, -7, -8, -10, and -11 for ovarian cancer prognosis. Since only KLK5 showed to be of statistical significance to assess PFS in this ovarian cancer cohort, we presented data for this promising cancer biomarker only.

KLK5 is a trypsin-like secreted serine protease of hormonally regulated tissues and is produced as an inactive preproenzyme

that is transformed during secretion into the proenzyme and then to the active enzyme by extracellular cleavage [31]. It is capable of digesting various extracellular matrix and plasma proteins; its proteolytic activity is inhibited by several protein inhibitors such as α_2-antiplasmin, antithrombin III, and α_2-macroglobulin as well as by Zn^{2+} and other divalent cations [20, 32]. Together with other kallikreins, KLK5 seems to participate in skin desquamation, a process that is similar to detachment and dissemination of migrating cells in the process of metastasis [31].

Using a highly specific and sensitive ELISA immunoassay, KLK5 was identified primarily in the skin, but also in breast, salivary glands, esophagus, and other tissues of normal individuals, as well as in serum and malignant ascites of ovarian cancer patients [17]. Yousef et al. [12] analyzed serum samples of healthy individuals as well as of patients with various malignancies including breast, ovarian, prostate, and colon cancers. Whereas KLK5 levels were below the detection limit in normal individuals and most patients with diverse malignancies, 67% of ovarian cancer and 49% of breast cancer patients were KLK5 positive, with serum levels up to 20 times higher than the detection limit. Besides that, high levels of KLK5 were detected in the ascitic fluid obtained from advanced ovarian cancer patients [12]. KLK5 expression in ovarian cancer tissues was also demonstrated at the gene and protein level [16, 17, 23, 33]. Thus, KLK5 appears to be a promising disease forecast marker for predicting the clinical course of ovarian cancer.

In this study, for the first time ever, serum and ascitic fluid from the same ovarian cancer patient cohort as well as serum of patients presenting with benign ovarian tumors have been examined for antigen content of KLK5. For the ovarian cancer patients, the prognostic impact of these antigen levels was analyzed. We confirm the findings of Yousef et al. [12], acquired in another patient collective, that ovarian cancer

patients release significant amounts of KLK5 into the blood stream. With respect to patients with benign ovarian tumor, we now, for the first time, report that KLK5 is also released into the blood of these patients but at a much lower rate. Accordingly, we found that KLK5 protein is not or only very weakly expressed in benign ovarian tumors but distinctly expressed in ovarian cancer tissue. These findings are in line with the results of Yousef et al. [12], who found higher amounts of KLK5 in malignant ovarian cytosols compared with normal or benign tumors. KLK5 in serum or ascitic fluid did not correlate with the established ovarian cancer serum biomarker CA 125, thus demonstrating that KLK5 provides independent information from CA 125 in ovarian cancer. In this study, the observational period was fairly short (22 months); yet, a considerable number of the patients had already progressed early on or after primary surgery. Despite the overall poor prognosis of this group of patients, the impact of the established clinical factors like FIGO stage, age, and residual tumor mass after primary surgery on PFS was confirmed.

In univariate analyses, higher KLK5 levels both in serum and in ascites were significant predictors of shorter PFS in ovarian cancer patients. This finding is in line with that by Kim et al. [17], who showed that KLK5 protein expressed in ovarian cancer tissue also significantly impacts survival of ovarian cancer patients. These data, taken as a whole, endorse present knowledge that KLK5—both in tumor tissue and in serum—is a valid and important biomarker in ovarian cancer patients. Using the additional information given by the KLK5 serum content regarding prognosis in patients who receive standard ovarian cancer therapy, this standard therapy regimen could, for instance, be adjusted by sparing patients with a very poor prognosis from potential toxic therapies if they are already in poor health status or by including these patients in trials exploring alternative treatment regimens or drugs if they are clinically fit enough.

Since KLK5 protein is not present in normal female donors and low in patients with benign ovarian tumors but increased in ovarian cancer patients, we propose that KLK5 could serve as a potential serum biomarker to identify patients afflicted with ovarian tumors, either in the benign or in the malignant state, who should be referred to further diagnostic work-up such as vaginal ultrasound. Our hypothesis-generating results may help to set up clinically driven validation studies in the near future.

acknowledgements

funding

disclosure

The authors declare no conflict of interest.

references

1. Cannistra SA. Cancer of the ovary. N Engl J Med 2004; 351: 2519–2529.

2. Nossov V, Amneus M, Su F et al. The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? Am J Obstet Gynecol 2008; 199: 215–223.

3. Crijns AP, Duiker EW, de Jong S et al. Molecular prognostic markers in ovarian cancer: toward patient-tailored therapy. Int J Gynecol Cancer 2006; 16 (Suppl 1): 152–165.

4. Yap TA, Carden CP, Kaye SB. Beyond chemotherapy: targeted therapies in ovarian cancer. Nat Rev Cancer 2009; 9: 167–181.

5. Olopade OI, Grushko TA, Nanda R et al. Advances in breast cancer: pathways to personalized medicine. Clin Cancer Res 2008; 14: 7988–7999.

6. Emami N, Diamandis EP. Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers. Clin Chem 2008; 54: 1600–1607.

7. Clements JA, Willemsen NM, Myers SA, Dong Y. The tissue kallikrein family of serine proteases: functional roles in human disease and potential as clinical biomarkers. Crit Rev Clin Lab Sci 2004; 41: 265–312.

8. Schmitt M, Magdolen V. Using kallikrein-related peptidases (KLK) as novel cancer biomarkers. Thromb Haemost 2009; 101: 222–224.

9. Sotiropoulou G, Pampalakis G, Diamandis EP. Functional role of human kallikrein-related peptidases. J Biol Chem 2009; 284: 32989–32994.

10. Borgono CA, Diamandis EP. The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer 2004; 4: 876–890.

11. Rosen DG, Wang L, Atkinson JN et al. Potential markers that complement expression of CA125 in epithelial ovarian cancer. Gynecol Oncol 2005; 99: 267–277.

12. Yousef GM, Polymeris ME, Grass L et al. Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer [erratum appears in Cancer Res 2003; 63 (17): 5647]. Cancer Res 2003; 63: 3958–3965.

13. Oikonomopoulou K, Li L, Zheng Y et al. Prediction of ovarian cancer prognosis and response to chemotherapy by a serum-based multiparametric biomarker panel. Br J Cancer 2008; 99: 1103–1113.

14. Yousef GM, Polymeris ME, Yacoub GM et al. Parallel overexpression of seven kallikrein genes in ovarian cancer. Cancer Res 2003; 63: 2223–2227.

15. Obiezu CV, Diamandis EP. Human tissue kallikrein gene family: applications in cancer. Cancer Lett 2005; 224: 1–22.

16. Diamandis EP, Borgono CA, Scorilas A et al. Immunofluorometric quantification of human kallikrein 5 expression in ovarian cancer cytosols and its association with unfavorable patient prognosis. Tumour Biol 2003; 24: 299–309.

17. 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: 643–650.

18. Kishi T, Grass L, Soosaipillai A et al. Human kallikrein 8, a novel biomarker for ovarian carcinoma. Cancer Res 2003; 63: 2771–2774.

19. Christopoulos TK, Diamandis EP. Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. Anal Chem 1992; 64: 342–346.

20. Debela M, Goettig P, Magdolen V et al. Structural basis of the zinc inhibition of human tissue kallikrein 5. J Mol Biol 2007; 373: 1017–1031.

21. Colombo N, Van Gorp T, Parma G et al. Ovarian cancer. Crit Rev Oncol Hematol 2006; 60: 159–179.

22. van der Burg ME. Advanced ovarian cancer. Curr Treat Options Oncol 2001; 2: 109–118.

23. Shaw JL, Diamandis EP. Distribution of 15 human kallikreins in tissues and biological fluids. Clin Chem 2007; 53: 1423–1432.

24. Gagnon A, Ye B. Discovery and application of protein biomarkers for ovarian cancer. Curr Opin Obstet Gynecol 2008; 20: 9–13.

25. Bast RC Jr, Badgwell D, Lu Z et al. New tumor markers: CA125 and beyond. Int J Gynecol Cancer 2005; 15 (Suppl 3): 274–281.

26. Høgdall E. Cancer antigen 125 and prognosis. Curr Opin Obstet Gynecol 2008; 20: 4–8.

27. Gadducci A, Cosio S, Tana R, Genazzani AR. Serum and tissue biomarkers as predictive and prognostic variables in epithelial ovarian cancer. Crit Rev Oncol Hematol 2009; 69: 12–27.

28. Jurisicova A, Jurisica I, Kislinger T. Advances in ovarian cancer proteomics: the quest for biomarkers and improved therapeutic interventions. Expert Rev Proteomics 2008; 5: 551–560.

29. Zheng Y, Katsaros D, Shan SJ et al. A multiparametric panel for ovarian cancer diagnosis, prognosis, and response to chemotherapy. Clin Cancer Res 2007; 13: 6984–6992.

30. Prezas P, Arlt MJ, Viktorov P et al. Overexpression of the human tissue kallikrein genes KLK4, 5, 6, and 7 increases the malignant phenotype of ovarian cancer cells. Biol Chem 2006; 387: 807–811.

31. Brattsand M, Egelrud T. Purification, molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. J Biol Chem 1999; 274: 30033–30040.

32. Debela M, Beaufort N, Magdolen V et al. Structures and specificity of the human kallikrein-related peptidases KLK 4, 5, 6, and 7. Biol Chem 2008; 389: 623–632.

33. Dorn J, Harbeck N, Kates R et al. Disease processes may be reflected by correlations among tissue kallikrein proteases but not with proteolytic factors uPA and PAI-1 in primary ovarian carcinoma. Biol Chem 2006; 387: 1121–1128.