Am J Cancer Res 2016;6(1):61-70
www.ajcr.us /ISSN:2156-6976/ajcr0018206

*Original Article*

# Assessment of kallikrein-related peptidase 5 (KLK5) protein expression in tumor tissue of advanced ovarian cancer patients by immunohistochemistry and ELISA: correlation with clinical outcome

Julia Dorn[1], Alexandra Yassouridis[1], Axel Walch[2], Eleftherios P Diamandis[3,4], Manfred Schmitt[1], Marion Kiechle[1], Ping Wang[1], Enken Drecoll[5], Barbara Schmalfeldt[1], Daniela Loessner[6], Matthias Kotzsch[7], Viktor Magdolen[1]

*[1]Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität München, Munich, Germany; [2]Institute of Pathology, Helmholtz Zentrum München, Neuherberg, Germany; [3]Department of Clinical Biochemistry, Mount Sinai Hospital and University Health Network, USA; [4]Division of Clinical Biochemistry, Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario, Canada; [5]Institute of Pathology, Technische Universität München, Munich, Germany; [6]Cancer Program, Faculty of Health, Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Australia; [7]Institute of Pathology, Technische Universität Dresden, Dresden, Germany*

Received October 20, 2015; Accepted October 30, 2015; Epub December 15, 2015; Published January 1, 2016

**Abstract:** Members of the human kallikrein-related peptidase (KLK) family, including KLK5, have been reported to play an important role in ovarian cancer progression. In the present study, we assessed KLK5 protein expression in ovarian cancer tissues by immunohistochemistry (IHC) and ELISA, and analyzed its association with clinicopathologic parameters and disease outcome in 95 patients with advanced ovarian cancer FIGO stage III/IV. KLK5 immunoexpression was evaluated in ovarian cancer tissue microarrays by IHC using a manual semiquantitative scoring system. KLK5 antigen levels were determined in ovarian cancer tumour tissue extracts by ELISA. KLK5 protein is expressed in ovarian cancer tissue by stromal and tumor cells. Mean KLK5 immunoscore values in tumor cells (KLK5-Tc; 5.7, range 0 to 12) were higher compared to stromal cells (KLK5-Sc; 1.2, range 0 to 9) but the correlation between KLK5-Tc and KLK5-Sc was rather low ($r_s$ = 0.34, $P < 0.05$). No significant associations of clinicopathological parameters with KLK5-Tc, KLK5-Sc, the combined overall score KLK5-Tc+Sc, or ELISA (KLK5-E) expression values were determined, except for KLK5-E protein expression with advanced age and high nuclear grade (G3). In univariate Cox regression analysis, elevated expression levels of KLK5-Sc are significantly linked with both prolonged overall survival (OS) (hazard ratio [HR] = 0.6, $P = 0.046$) and progression-free survival (PFS) (HR = 0.54, $P = 0.032$) of advanced ovarian cancer patients. KLK5-Tc and KLK5-Tc+Sc scores as well as the KLK5-E values were not associated with patients' outcome. In multivariable analysis, KLK5-Sc expression was found to be statistically significant for PFS. Patients with elevated KLK5-Sc had a two-fold lower risk of disease recurrence (HR = 0.53, $P = 0.037$) as compared to patients with low KLK5-Sc. For KLK5-Sc and OS, a trend towards statistical significance was observed (HR = 0.62, $P = 0.077$). These results indicate that KLK5 overexpression by stromal cells (KLK5-Sc) may be a positive modulator lowering aggressiveness of ovarian cancer.

**Keywords:** ELISA, immunohistochemistry, kallikrein-related peptidase, KLK5, ovarian cancer, outcome, tumor tissue

## Introduction

Ovarian cancer is the leading gynecologic malignancy in women resulting in death in the western world. Early ovarian cancer usually presents no obvious symptoms, therefore, the poor prognosis of this disorder is often the result of late diagnosis associated with untimely disease recurrence [1]. Currently, there is no suitable screening test for women at average risk of ovarian cancer which has been recognized to be helpful in the early detection of ovar-

**Table 1.** Association between clinical and histomorphological characteristics of advanced ovarian cancer patients FIGO III/IV and tumor biological factors

| Clinicopathological parameters | No. of patients | KLK5-Tc | KLK5-Sc | KLK5-Tc+Sc | KLK5-E |
|---|---|---|---|---|---|
| | | low/high[a] | low/high[b] | low/high[b] | low/high |
| Total | 95 | 58/34 | 58/32 | 48/42 | 62/33 |
| Age | | *P* = 0.886 | *P* = 0.418 | *P* = 0.931 | *P* = 0.009 |
| ≤ 60 years | 56 | 35/20 | 33/21 | 29/25 | 22/34 |
| > 60 years | 39 | 23/14 | 25/11 | 19/17 | 26/13 |
| Nuclear grade | | *P* = 0.712 | *P* = 0.791 | *P* = 0.924 | *P* = 0.015 |
| G1 + G2 | 27 | 15/10 | 16/8 | 13/11 | 19/8 |
| G3 | 68 | 43/24 | 42/24 | 35/31 | 29/39 |
| Residual tumor mass | | *P* = 0.477 | *P* = 0.411 | *P* = 0.489 | *P* = 0.833 |
| 0 cm | 39 | 27/12 | 22/15 | 22/15 | 20/19 |
| > 0 cm | 53 | 31/19 | 34/16 | 26/24 | 26/27 |
| Ascitic fluid volume | | *P* = 0.127 | *P* = 0.514 | *P* = 0.232 | *P* = 0.928 |
| < 500 ml | 53 | 37/16 | 31/20 | 30/21 | 27/26 |
| ≥ 500 ml | 40 | 20/17 | 25/12 | 17/20 | 20/20 |

$Chi^2$ test (cut-off point: median); No. of cases [a]n = 92; [b]n = 90.

ian cancer; at best, the combination of a thorough pelvic exam, transvaginal ultrasound, and testing for the blood tumor marker CA125 is offered to women who are considered high-risk ovarian cancer patients, or to women who have persistent, unexplained symptoms [1]. Thus, until now, no sufficiently accurate screening tests or biomarkers are available to tailor cancer therapy to the individual patient.

In this respect, it is worth to mention that several of the fifteen kallikrein-related peptidases (KLK) family members have been reported to contribute to ovarian cancer progression and metastasis (reviewed in [2, 3]). While e.g. KLK4, KLK6, and KLK15 are associated with poor clinical outcome of ovarian cancer patients, KLK9 and KLK14 are linked with a favorable course of the disease. For some KLKs (KLK7, 8, 10, 11, and KLK13) the clinical relevance is not clear yet. Previous findings made obvious that-either at the protein or gene level-elevated KLK5 concentrations present in ovarian cancer tumor tissue are associated with advanced disease stage as well as shorter disease-free and overall survival ([4-7]).

Like the other KLKs, KLK5 is a secreted serine protease. In healthy individuals, it is mainly present in the skin (often co-expressed with KLK7), but is also found in the breast, cervix, esophagus, heart, salivary gland, testis, thyroid, and vagina [8]. In the skin, KLK5 is involved in desquamation, probably acting as a physiological activator of KLK7 [9]. Interestingly, in advanced serous ovarian cancer tissue, a concordant higher expression of both KLK5 and KLK7 was observed when compared to normal or benign ovarian tissue [10].

In the present study, we assessed KLK5 protein expression in tumor tissues by two immunoenzymometric assays, immunohistochemistry (IHC) and ELISA, in a cohort of advanced ovarian cancer patients, employing well-characterized antibodies to KLK5. Localization and expression levels of KLK5 in formalin-fixed paraffin-embedded sections were determined separately for tumor cells and stromal cells. The findings were correlated with clinical and histomorphological parameters but also with clinical outcome of the ovarian cancer patients.

## Patients, material and methods

### *Patients*

Ninety-five patients afflicted with advanced ovarian cancer (FIGO stage III/IV), treated between 1990 and 1999, were enrolled in the present retrospective study conducted at the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität München, Germany. The study was approved by the local Ethics Committee. Median patients' age at time of surgery was 57 years (range 20-85 years). All patients initially underwent the standard stage-related primary radical

debulking surgery. Thirty-nine patients (41.1%) were optimally debulked with complete removal of macroscopically visible tumor manifestations. Following surgery, all of the patients received adjuvant treatment according to consensus recommendations at that time, including platinum-based chemotherapy. None of the patients received neoadjuvant therapy before primary surgery. Median time of follow-up was 40 and 23 months for overall survival (OS) and progression-free survival (PFS), respectively (range 1 to 244 months after primary tumor resection, for both). Clinical and histomorphological factors documented at the time of surgery included nuclear grade, presence of residual tumor mass (defined as largest intra-abdominal tumor diameter left at the end of surgery) and ascitic fluid volume (estimated preoperatively by vaginal ultrasound) (**Table 1**). During follow-up, 65 (68%) of the patients relapsed; 77 (81%) died.

*Tissue preparation, microarray construction, and immunohistochemistry*

Formalin-fixed, paraffin-embedded ovarian tissue specimens were retrieved from the archives of the Institute of Pathology of the Technische Universität München, Munich, Germany. Production of the tissue microarrays has been described previously [11, 12]. Immunohistochemical detection of KLK5 was performed as described [4]. In short, dewaxed and rehydrated tissue microarray sections were treated for antigen retrieval by pressure cooking (4 min, 120°C, 0.1 M citrate buffer, pH 6.0). After quenching endogenous peroxidase activity with a commercial blocking solution (#K5361, DAKO, Hamburg, Germany; 5 min, room temperature), the sections were reacted overnight at 4°C with goat polyclonal antibody AF1108 directed to KLK5 (#1108-SE; R&D Systems, Minneapolis, MN; 2 µg/ml) in the presence of antibody diluent #S2022 (DAKO). AF1108 was demonstrated to not cross-react with any of the other KLKs [4]. Interaction of antibody AF1108 with the KLK5 target protein was visualized by use of the EnVision protocol (DAKO). Nuclei were counterstained with Mayer's acid hematoxylin.

*Quantification of KLK5 immunostaining*

For evaluation of observer-assisted analysis of KLK5 tissue localization and immunostaining intensity, a quantitative score based on staining intensity and percentage of positive cells was applied. KLK5 staining intensity was classified on a scale of 0 to 3 (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining). The percentage of positively stained cells was scored by cell count on a scale of 0 to 4 (0: 0%; 1: 1-10%; 2: 11-50%; 3: 51-80%; 4: > 80%) [13]. A final immunoreactivity score was created by multiplication of the intensity score values with the cell positivity score values, separately for the tumor cells (KLK5-Tc) and the stromal cells (KLK5-Sc), respectively. In addition, an overall score was created by summing up the KLK5-Tc with the KLK5-Sc score values (KLK5-Tc+Sc).

*Preparation of tumor tissue extracts and quantification of KLK5 antigen levels by ELISA*

Tissue extracts from fresh-frozen primary tumor tissues of ovarian cancer patients were prepared as described previously [14]. The antigen content of KLK5 in tumor tissue extracts (expressed as ng per mg of protein) was quantified following the protocol of Dorn and co-workers [4] using a highly sensitive and specific in-house sandwich-type enzyme immunoassay (ELISA) [15]. Lower and upper detection limits of this assay are 0.1 and 25 ng/ml, respectively. No cross-reactivity of the KLK5 ELISA with any of the other member of the KLK family was observed. Protein content in tumor tissue extracts was determined by use of the BCA method (Pierce, Rockford, IL, USA).

*Statistical analyses*

The strengths of the associations between continuous variables of tumor biological markers were calculated using Spearman rank correlation ($r_s$). The relationship of biological marker expression levels (grouped according to the median) with clinical and histomorphological parameters was evaluated using the $Chi^2$-test. For survival analyses, overall survival (OS) and progression-free survival (PFS) of ovarian cancer patients were used as follow-up end points. Association of KLK5 and the clinical and histomorphological factors with OS and PFS was calculated by Cox univariate and multivariable proportional hazards regression models and expressed as hazard ratio (HR) and its 95% confidence interval (95% CI). The multivariable Cox regression model was adjusted for estab-

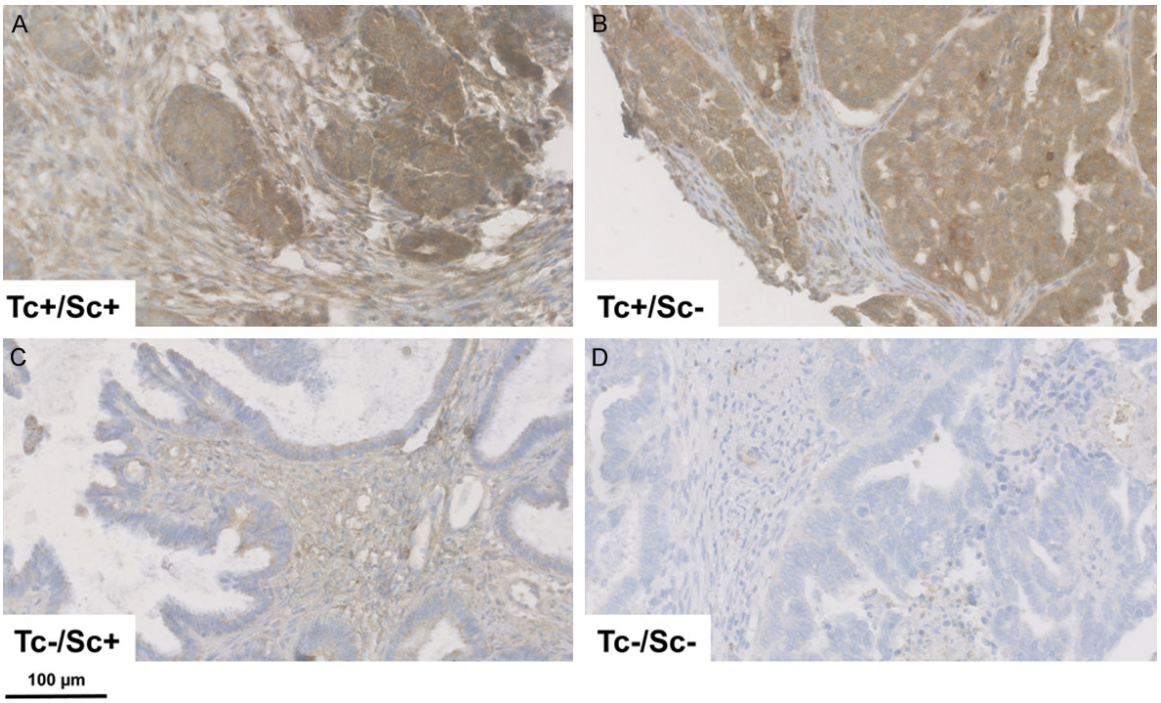


**Figure 1.** KLK5 immunoexpression in tumor tissue of ovarian cancer specimens. Tissue sections were stained with the goat polyclonal antibody AF1108 directed to KLK5 using the EnVision system (DAKO). Micrographs (A-D) illustrate representative core punches corresponding to high (+) or low (-) KLK5 immunoexpression in tumor cells (Tc) and stromal cells (Sc), respectively.

lished ovarian cancer factors such as age, nuclear grade, presence of residual tumor mass, and ascitic fluid volume. Survival curves were plotted according to Kaplan-Meier, using log-rank tests to test for differences. All calculations were performed using the StatView 5.0 statistical package (SAS Institute, Cary, NC, USA). *P*-values ≤ 0.05 were considered statistically significant.

## Results

### *Relationship between KLK5 expression levels in ovarian cancer tissue*

In the present retrospective study, we analyzed the clinical impact of KLK5 expression in tumor tissue by IHC and by ELISA, respectively, in patients with primary ovarian cancer (n = 95), encompassing advanced tumor stages (FIGO III/IV). IHC was performed on formalin-fixed, paraffin-embedded tumor tissue microarrays employing a KLK5-specific antibody, AF1108, following a previously established staining protocol [4]. Both stromal and tumor cells express KLK5 in ovarian cancer tissue (**Figure 1**), therefore, we individually evaluated the expression pattern in these cell types applying a score based on staining intensity and percentage of positively stained cells [13]. KLK5 expression was higher in tumor cells (Tc) compared to stromal cells (Sc): the mean score value of KLK5-Tc was 5.7 (range 0 to 12), that of KLK5-Sc 1.2 (range 0 to 9). The frequency of score values greater than zero was much higher for tumor cells (85, *versus* 7 negative) compared to stromal cells (32, *versus* 58 negative). The antigen content of KLK5 was quantified in tumor extracts of fresh-frozen tissue by ELISA. The mean KLK5-ELISA (KLK5-E) value was 2.3 ng/mg protein (range 0 to 30.9 ng/mg).

Although statistically significant, the correlation between KLK5-Tc and KLK5-Sc was rather low ($r_s$ = 0.34, $P < 0.05$). No statistically significant correlation was observed between KLK5-ELISA values and KLK5-Tc or KLK5-Tc+Sc values; for the correlation of KLK5-E with KLK5-Sc, only a very weak correlation was found ($r_s$ = 0.22, $P < 0.05$).

In the skin, KLK5 is coexpressed with KLK7 and very likely acts as the activator of KLK7 in desquamation. In a recent study, we analyzed the

**Table 2.** Univariate Cox regression analysis of clinical outcome in advanced ovarian cancer patients FIGO III/IV with respect to clinicopathological parameters and KLK5 tumor tissue expression

| Factor | No. of cases | Overall survival | | Progression-free survival | |
|---|---|---|---|---|---|
| | | HR (95% CI)[a] | *P* | HR (95% CI)[a] | *P* |
| Total number | 95 | | | | |
| Age | | | | | |
| ≤ 60 years | 56 | 1 | | | |
| > 60 years | 39 | 2.01 (1.27-3.18) | 0.003 | 1.76 (1.07-2.89) | 0.026 |
| Nuclear grade | | | | | |
| G1 + G2 | 27 | 1 | | 1 | |
| G3 | 68 | 1.59 (0.95-2.66) | 0.077 | 1.22 (0.71-2.10) | 0.475 |
| Residual tumor mass | | | | | |
| 0 mm | 39 | 1 | | 1 | |
| > 0 mm | 53 | 4.09 (2.44-6.85) | < 0.001 | 3.40 (1.98-5.84) | < 0.001 |
| Ascitic fluid volume | | | | | |
| < 500 ml | 53 | 1 | | 1 | |
| ≥ 500 ml | 40 | 3.12 (1.96-4.99) | < 0.001 | 2.59 (1.55-4.31) | 0.003 |
| KLK5-Tc[b] | | | | | |
| Low | 58 | 1 | | 1 | |
| High | 34 | 0.98 (0.61-1.57) | 0.932 | 1.01 (0.61-1.69) | 0.956 |
| KLK5-Sc[b] | | | | | |
| Low | 58 | 1 | | 1 | |
| High | 32 | 0.60 (0.32-0.99) | 0.046 | 0.54 (0.31-0.95) | 0.032 |
| KLK5-Tc+Sc[b] | | | | | |
| Low | 48 | 1 | | 1 | |
| High | 42 | 0.86 (0.54-1.36) | 0.508 | 0.86 (0.52-1.43) | 0.560 |
| KLK5-E[b] | | | | | |
| Low | 48 | 1 | | 1 | |
| High | 47 | 0.89 (0.56-1.39) | 0.599 | 0.89 (0.55-1.46) | 0.653 |

[a]HR: hazard ratio (95% confidence interval) of univariate Cox regression analysis. [b]Dichotomized into high and low levels by median.

clinical value of KLK7 protein expression in ovarian cancer in an overlapping patient cohort [12] and, thus, were able to examine whether there is coordinate expression of both proteases in ovarian cancer tissue as well. In fact, the KLK5 and KLK7 antigen levels determined by ELISA show a moderate correlation (KLK5-E vs. KLK7-E: $r_s = 0.55$, $P < 0.001$). By IHC, KLK5-Sc values were found to reasonably correlate with KLK7-Sc as well (KLK5-Sc vs. KLK7-Sc: $r_s = 0.42$, $P < 0.001$), whereas no correlation between KLK5-Tc and KLK7-Tc values and only a weak correlation between KLK5-Tc+Sc and KLK7-Tc+Sc ($r_s = 0.24$, $P < 0.05$) was seen.

**Table 1** summarizes the association between clinical and histopathological patient characteristics and KLK5 expression, either assessed by IHC or by ELISA. KLK5-E antigen levels were found to be significantly elevated in patients with advanced age and high nuclear grade (G3) versus low nuclear grade (G1/G2). Otherwise, no significant associations were observed for KLK5-Tc, KLK5-Sc, KLK5-Tc+Sc, or KLK5-E with nuclear grade, residual tumor or ascitic fluid volume.

*Association of clinical and histomorphological parameters and KLK5 expression with patients' survival*

Association of relevant clinicopathological parameters and KLK5 protein expression levels with OS and PFS, respectively, is presented in **Tables 2** and **3**. The clinicopathological variables age, residual tumor mass, and ascitic fluid volume are univariate predictors for both OS and PFS in the ovarian cancer cohort, nucle-

**Table 3.** Multivariable Cox regression analysis of clinical outcome in advanced ovarian cancer patients FIGO III/IV with respect to clinicopathological parameters and KLK5 tumor tissue expression

| Factor | No. of cases | Overall survival | | Progression-free survival | |
|---|---|---|---|---|---|
| | | HR (95% CI)[a] | *P* | HR (95% CI)[a] | *P* |
| Total number | 90 | | | | |
| Nuclear grade | | | | | |
| G1 + G2 | 26 | 1 | | 1 | |
| G3 | 64 | 1.23 (0.72-2.10) | 0.457 | 0.87 (0.49-1.56) | 0.644 |
| Residual tumor mass | | | | | |
| 0 mm | 39 | 1 | | 1 | |
| > 0 mm | 51 | 3.29 (1.69-6.41) | < 0.001 | 3.09 (1.57-6.07) | 0.001 |
| Ascitic fluid volume | | | | | |
| < 500 ml | 53 | 1 | | 1 | |
| ≥ 500 ml | 37 | 1.30 (0.71-2.39) | 0.393 | 1.25 (0.66-2.37) | 0.485 |
| KLK5-Tc[b] | | | | | |
| Low | 57 | 1 | | 1 | |
| High | 30 | 0.98 (0.58-1.63) | 0.929 | 0.90 (0.52-1.54) | 0.699 |
| KLK5-Sc[b] | | | | | |
| Low | 54 | 1 | | 1 | |
| High | 31 | 0.62 (0.37-1.05) | 0.077 | 0.53 (0.29-0.96) | 0.037 |
| KLK5-Tc+Sc[b] | | | | | |
| Low | 47 | 1 | | 1 | |
| High | 38 | 0.80 (0.49-1.31) | 0.376 | 0.78 (0.46-1.32) | 0.358 |
| KLK5-E[b] | | | | | |
| Low | 45 | 1 | | 1 | |
| High | 45 | 0.96 (0.59-1.58) | 0.879 | 1.03 (0.61-1.75) | 0.915 |

[a]HR: hazard ratio (95% confidence interval) of multivariable Cox regression analysis. Biological markers were added separately to the base model of clinical parameters: nuclear grade, residual tumor mass, and ascitic fluid volume. [b]Dichotomized into high and low levels by median.

ar grade G3 tumors compared to G1/2 tumors show a trend for poor patient survival (**Table 2**).

Strikingly, elevated expression levels of KLK5 in stromal cells, as detected by IHC, are significantly linked with both longer OS (HR = 0.6, 95% CI = 0.32-0.99, *P* = 0.046) and PFS (HR = 0.54, 95% CI = 0.31-0.95, *P* = 0.032). These findings were confirmed by Kaplan-Meier estimation: the association of KLK5-Sc levels with OS and PFS is visualized by the respective survival curves (**Figure 2**). By univariate Cox regression analysis, the tumor cell-derived score, KLK5-Tc, the combined overall score, KLK5-Sc+Tc, as well as the KLK5-E values were not associated with patients' outcome (**Table 2**). Since dichotomization of the tumor and stromal cell scores by the median leads to group sizes close to tertials (KLK5-Tc: median = 6.0, low: n = 58; high: n = 34; KLK5-Sc: median = 0, low: n = 58; high: n = 32), we grouped the ELISA values by tertials as well (KLK5-E: low = 62; high = 33) and performed univariate Cox regression analysis. Still, no association with patients' outcome was observed.

In multivariable analysis, residual tumor mass but not the ascitic fluid volume remained significantly associated with both OS and PFS (**Table 3**). Additionally, KLK5-Sc expression was found to be statistically significant for PFS: ovarian cancer patients with elevated KLK5-Sc levels had an about two-fold lower risk of disease recurrence with an HR of 0.53 (95% CI = 0.29-0.96, *P* = 0.037) as compared to patients with low KLK5-Sc levels (**Table 3**). Concerning OS, a trend towards statistical significance was observed for KLK5-Sc (HR: 0.62 (95% CI = 0.37-1.05, *P* = 0.077).

## Discussion

In previous studies, KLK5, a member of the kallikrein-related peptidase family, was pro-

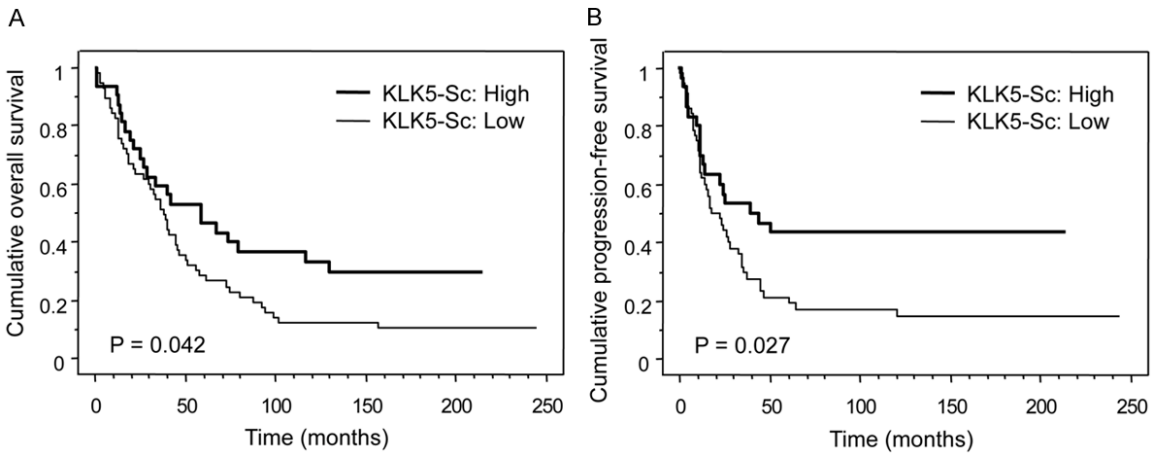


**Figure 2.** Probability of overall survival and progression-free survival of advanced ovarian cancer patients FIGO III/IV stratified by stromal cell-associated KLK5 expression levels in primary tumor tissues. Patients with positive KLK5 immunostaining of stromal cells in the tumor tissue (KLK5-Sc, n = 32) show significantly better overall survival (Kaplan-Meier analysis, *P* = 0.042) (A) and progression-free survival (*P* = 0.027) (B) than the group of patients with no detectable KLK5-Sc staining (n = 58).

posed as a valuable biomarker to predict poor clinical outcome of ovarian cancer patients. In the majority of ovarian cancer patients, KLK5 is present in their blood serum, in contrast to the blood serum of healthy women [4]. KLK5 may contribute additive value over CA125 as diagnostic biomarker in ovarian cancer [16]. Also, the initial KLK5 serum level and its decline during chemotherapy is an indicator for the response of ovarian cancer patients to cancer therapy [17, 18].

In ovarian cancer tumor tissue, higher KLK5 levels were found to be associated with an invasive (as compared to tumors with low-malignant potential) and more aggressive cancer phenotype connected with poor patient outcome, at both the mRNA and the protein level [5, 6]. The clinical observation, that KLK5 may be associated with the malignant phenotype of ovarian cancer has been further supported by experimental tumor models in mice [19, 20]: overexpression of KLK5 in tumor cells, among other KLKs, resulted in an increase of tumor load and intra-peritoneal spread as compared to the control group.

In the present retrospective study, we analyzed a cohort of 95 patients FIGO stage III/IV suffering from advanced primary ovarian cancer, treated with platinum-based chemotherapy. KLK5 protein expression was determined by IHC on a collection of primary tumor tissue microarrays, and by ELISA by assessing corresponding tumor tissue extracts. The impact of KLK5 protein expression on clinical outcome was evaluated by univariate and multivariable analysis. In IHC, we observed that both stromal and tumor cells express KLK5 in ovarian cancer tissue, with less negative cases and higher KLK5 levels in tumor cells. KLK5 expression in tumor cells correlated only weakly with that in stromal cells and not with KLK5 antigen levels as assessed by ELISA.

Recently, we determined KLK7 protein expression levels in an overlapping cohort of ovarian cancer patients by IHC and ELISA [12]. Concordant with the results for KLK5, KLK7 immunoscores in tumor cells showed less negative cases and higher KLK7 expression levels than KLK7 expressed in stromal cells. For both KLK5 and KLK7, we observed a weak but statistically significant correlation between their immunoscores determined in tumor cells and stromal cells, respectively. In contrast to KLK7, however, there was no significant correlation of KLK5 expression determined by IHC with KLK5 levels determined by ELISA, apart from a weak, but significant, correlation between KLK5-E and KLK5-Sc values.

In the healthy skin, both KLK5 and KLK7 are highly expressed and may be part of a proteolytic cascade [9]. Interestingly, in ovarian cancer, KLK5 and KLK7 are overexpressed as compared to normal or benign ovarian tissue [10]. Using the previous data for KLK7 [12] and the

data for KLK5 (from the present study), we examined whether KLK5 and KLK7 are co-expressed in ovarian tumor tissue. By IHC, a significant, moderate positive correlation was found regarding stromal cell expression and the combined score, but not for tumor cells. KLK5 and KLK7 antigen determined by ELISA showed a significant medium strong correlation.

Not surprisingly, the traditional clinical parameters age, residual tumor mass, and ascitic fluid volume are correlated with clinical outcome in this cohort of FIGO III/IV ovarian cancer patients. IHC expression levels of KLK5-Tc and KLK5-Tc+Sc as well as KLK5-E levels were not related to patients' outcome. Strikingly, however, high KLK5-Sc expression revealed KLK5 as a favorable biomarker for PFS and OS. Yet, this finding is not in line with earlier studies where KLK5 expression was shown to be associated with poor clinical outcome [5, 6]. It should, however, be pointed out that these studies used other methods for the assessment of KLK5 expression, either RT-PCR or ELISA. Furthermore, these studies included also early FIGO stage I/II patients in addition to advanced cancer stages. It, thus, is certainly of interest to explore the clinical relevance of stromal cell-associated KLK5 expression in early FIGO stage I/II patients in future studies as well.

Interestingly, in the earlier mentioned study of KLK7 expression in ovarian cancer tissue, we also found elevated KLK7 protein levels in tumor tissue extracts to be associated with longer PFS and OS [12]. In other types of cancers, such as breast cancer and oral squamous cell carcinoma [21, 22], concurrent downregulation of KLK5 and KLK7 have been reported indicating that reduction of the expression levels of these KLKs support tumorigenesis in these cancer types. In fact, a recent experimental study suggested that KLK5 may suppress breast cancer by inhibiting epithelial-to-mesenchymal transition and by modulating the mevalonate pathway [23].

The patient cohort analyzed in the present study encompasses advanced ovarian cancer stages (FIGO III/IV) only, characterized by very poor clinical outcome owing to the relatively unsatisfying operation results (optimally debulking rate of 41%) leading to high disease recurrence levels and short OS. It is tempting to speculate that KLK5 may play different roles in stromal and tumor cells due to differing substrate repertoires in the vicinity of the respective cell type. Therefore, high expression of KLK5 in stromal cells but not in tumor cells may lead to a less aggressive tumor phenotype and/or better response to chemotherapy.

Previously, we have examined tumor tissue localization and protein expression levels of another member of the KLK family, KLK6, in ovarian cancer by IHC [11]. Similar to the present study, we observed that stromal cell but not tumor cell-associated expression, is related to patients' outcome. In case of KLK6, elevated tissue expression levels were associated with reduced PFS and OS of ovarian cancer patients [11]. Thus, the results obtained with KLK5 and KLK6 point to an important contribution of tumor tissue-associated stromal cell proteases to tumorigenicity.

In conclusion, in this study, we examined KLK5 protein expression in the clinically important ovarian cancer subgroup stage FIGO III/IV, using IHC (tissue microarrays) and ELISA (tumor tissue extracts). Our results indicate that low KLK5 expression in stromal cells is related to an unfavorable clinical outcome of patients with advanced ovarian cancer by predicting shorter PFS and OS due to minor therapy response. Those patients could benefit from alternative therapy approaches and/or should be monitored more closely after primary therapy for recurrent disease.

## Acknowledgements

We thank D. Hellmann for technical assistance, A. Gkazepis for his help in the initial stages of the project, and R. Napieralski for helpful discussions. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (DFG) to J.D. and by a mobility grant (Personalized Medicine) from the German Academic Exchange Service (DAAD) to M.S., A.W., D.L., and V.M.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Viktor Magdolen, Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität München,

Ismaninger Strasse 22, 81675 Munich, Germany. Tel: +49-89-4140-2493; Fax: +49-89-4140-7410; E-mail: viktor.magdolen@tum.de

## References


[1] Kaygusuz I, Kosus A and Kafali H. Overview of gynecologic cancers. In: Noninvasive molecular markers in gynecologic cancers. In: Barh D and Gunduz M, editors. Boca Raton, FL, USA: CRC Press; 2015. pp. 3-42.

[2] Dorn J, Bayani J, Yousef GM, Yang F, Magdolen V, Kiechle M, Diamandis EP and Schmitt M. Clinical utility of kallikrein-related peptidases (KLK) in urogenital malignancies. Thromb Haemost 2013; 110: 408-22.

[3] Dorn J, Beaufort N, Schmitt M, Diamandis EP, Goettig P and Magdolen V. Function and clinical relevance of kallikrein-related peptidases and other serine proteases in gynecological cancers. Crit Rev Clin Lab Sci 2014; 51: 63-84.

[4] Dorn J, Magdolen V, Gkazepis A, Gerte T, Harlozinska A, Sedlaczek P, Diamandis EP, Schuster T, Harbeck N, Kiechle M and Schmitt M. Circulating biomarker tissue kallikrein-related peptidase KLK5 impacts ovarian cancer patients' survival. Ann Oncol 2011; 22: 1783-90.

[5] Diamandis EP, Borgono CA, Scorilas A, Yousef GM, Harbeck N, Dorn J, Schmalfeldt B and Schmitt M. Immunofluorometric quantification of human kallikrein 5 expression in ovarian cancer cytosols and its association with unfavorable patient prognosis. Tumour Biol 2003; 24: 299-309.

[6] Kim H, Scorilas A, Katsaros D, Yousef GM, Massobrio M and Fracchioli S. Human kallikrein gene 5 (KLK5) expression is an indicator of poor prognosis in ovarian cancer. Br J Cancer 2001; 5: 643-50.

[7] Dorn J, Bronger H, Kates R, Slotta-Huspenina J, Schmalfeldt B, Kiechle M, Diamandis EP, Soosaipillai A, Schmitt M and Harbeck N. OVSCORE-a validated score to identify ovarian cancer patients not suitable for primary surgery. Oncol Lett 2015; 1: 418-24.

[8] Goettig P and Magdolen V. Kallikrein-related peptidase 5. In: Handbook of proteolytic enzymes. In: Rawlings ND and Salvesen GS, editors. Oxford, Great Britain: Academic Press; 2013. pp. 2772-8.

[9] Brattsand M. Kallikrein-related peptidases and inhibitors of the skin. In: Kallikrein-related peptidases. Volume 1: Characterization, regulation, and interactions within the protease web. In: Magdolen V, Sommerhoff CP, Fritz H, Schmitt M, editors. Berlin: De Gruyter; 2012. pp. 329-349.

[10] Dong Y, Kaushal A, Brattsand M, Nicklin J and Clements JA. Differential splicing of KLK5 and KLK7 in epithelial ovarian cancer produces novel variants with potential as cancer biomarkers. Clin Cancer Res 2003; 9: 1710-20.

[11] Seiz L, Dorn J, Kotzsch M, Walch A, Grebenchtchikov NI, Gkazepis A, Schmalfeldt B, Kiechle M, Bayani J, Diamandis EP, Langer R, Sweep FC, Schmitt M and Magdolen V. Stromal cell-associated expression of kallikrein-related peptidase 6 (KLK6) indicates poor prognosis of ovarian cancer patients. Biol Chem 2012; 393: 391-401.

[12] Dorn J, Gkazepis A, Kotzsch M, Kremer M, Propping C, Mayer K, Mengele K, Diamandis EP, Kiechle M, Magdolen V and Schmitt M. Clinical value of protein expression of kallikrein-related peptidase 7 (KLK7) in ovarian cancer. Biol Chem 2014; 395: 95-107.

[13] Biesterfeld S, Veuskens U, Schmitz FJ, Amo-Takyi B and Böcking A. Interobserver reproducibility of immunocytochemical estrogen- and progesterone receptor status assessment in breast cancer. Anticancer Res 1996; 16: 2497-500.

[14] Dorn J, Harbeck N, Kates R, Magdolen V, Grass L, Soosaipillai A, Schmalfeldt B, Diamandis EP and Schmitt M. 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: 1121-8.

[15] Yousef GM, Polymeris ME, Grass L, Soosaipillai A, Chan PC, Scorilas A, Borgoño C, Harbeck N, Schmalfeldt B, Dorn J, Schmitt M and Diamandis EP. Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. Cancer Res 2003; 63: 3958-65.

[16] Rosen DG, Wang L, Atkinson JN, Yu Y, Lu KH, Diamandis EP, Hellstrom I, Mok SC, Liu J and Bast RC Jr. Potential markers that complement expression of CA125 in epithelial ovarian cancer. Gynecol Oncol 2005; 99: 267-77.

[17] Oikonomopoulou K, Li L, Zheng Y, Simon I, Wolfert RL, Valik D, Nekulova M, Simickova M, Frgala T and Diamandis EP. Prediction of ovarian cancer prognosis and response to chemotherapy by a serum-based multiparametric biomarker panel. Br J Cancer 2008; 99: 1103-13.

[18] Zheng Y, Katsaros D, Shan SJ, de la Longrais IR, Porpiglia M, Scorilas A, Kim NW, Wolfert RL, Simon I, Li L, Feng Z and Diamandis EP. A multiparametric panel for ovarian cancer diagnosis, prognosis, and response to chemotherapy. Clin Cancer Res 2007; 13: 6984-92.

[19] Prezas P, Arlt MJ, Viktorov P, Soosaipillai A, Holzscheiter L, Schmitt M, Talieri M, Diamandis EP, Krüger A and Magdolen V. Overexpression

of the human tissue kallikrein genes KLK4, 5, 6, and 7 increases the malignant phenotype of ovarian cancer cells. Biol Chem 2006; 387: 807-11.

[20] Loessner D, Rizzi SC, Stok KS, Fuehrmann T, Hollier B, Magdolen V, Hutmacher DW and Clements JA. A bioengineered 3D ovarian cancer model for the assessment of peptidase-mediated enhancement of spheroid growth and intraperitoneal spread. Biomaterials 2013; 34: 7389-400.

[21] Leusink FK, van Diest PJ, Frank MH, Broekhuizen R, Braunius W, van Hooff SR, Willems SM and Koole R. The co-expression of kallikrein 5 and kallikrein 7 associates with poor survival in non-HPV oral squamous-cell carcinoma. Pathobiology 2015; 82: 58-67.

[22] Li X, Liu J, Wang Y, Zhang L, Ning L and Feng Y. Parallel underexpression of kallikrein 5 and kallikrein 7 mRNA in breast malignancies. Cancer Sci 2009; 100: 601-7.

[23] Pampalakis G, Obasuyi O, Papadodima O, Chatziioannou A, Zoumpourlis V and Sotiropoulou G. The KLK5 protease suppresses breast cancer by repressing the mevalonate pathway. Oncotarget 2014; 5: 2390-403.