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Clinical relevance of kallikrein-related peptidase 6 (KLK6) and 8 (KLK8) mRNA expression in advanced serous ovarian cancer

Abstract: Most members of the kallikrein-related peptidase family have been demonstrated to be dysregulated in ovarian cancer and modulate tumor growth, migration, invasion, and resistance to chemotherapy. In the present study, we assessed the mRNA expression levels of KLK6 and KLK8 by quantitative PCR in 100 patients with advanced serous ovarian cancer FIGO stage III/IV. A pronounced correlation between KLK6 and KLK8 mRNA expression ($r_s = 0.636$, $p < 0.001$) was observed, indicating coordinate expression of both peptidases. No significant associations of clinical parameters with KLK6, KLK8, and a combined score KLK6 + KLK8 were found. In univariate Cox regression analysis, elevated mRNA levels of KLK6 were significantly linked with shortened overall survival (OS) (hazard ratio [HR] = 2.07, $p = 0.007$). While KLK8 values were not associated with patients' outcome, high KLK6 + KLK8 values were significantly associated with shorter progression-free survival (HR = 1.82, $p = 0.047$) and showed a trend towards significance in the case of OS (HR = 1.82, $p = 0.053$). Strikingly, in multivariable analysis, elevated KLK6 mRNA values, apart from residual tumor mass, remained an independent predictive marker for poor OS (HR = 2.33, $p = 0.005$). As KLK6 mRNA and protein levels correlate, KLK6 may represent an attractive therapeutic target for potent and specific inhibitors of its enzymatic activity.

Keywords: kallikrein-related peptidase; KLK6; KLK8; ovarian cancer; quantitative PCR.

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Introduction

Ovarian cancer represents the fifth most common cause of cancer death among women and the most lethal gynecological neoplasm (Prat, 2012). Annually, approximately 6.1 per 100 000 women are newly diagnosed with ovarian cancer (Ferlay et al., 2015). Owing to its intraperitoneal location, the biological behavior of most epithelial tumors, and lack of early symptoms, more than 70% of patients are diagnosed in late stages of the disease [International Federation of Gynecology and Obstetrics (FIGO) classifications: stage III–IV] in which the 5-year survival rate is around 30% (Kipps et al., 2013; Prat, 2014). The most important clinical factor, and the only one to be influenced by the surgeon so far, is residual tumor mass after standard debulking operation (Dorn et al., 2011). Thus, valid biomarkers to predict prognosis and/or response to chemotherapy are urgently needed.

The family of the human kallikrein-related peptidases (KLK) serves as a promising pool of biomarkers for ovarian cancer (among other diseases). Its 15 members (KLK1-15) constitute a subgroup of the serine protease

enzyme family and are encoded within a gene cluster localized on chromosome 19q13.3-4. Nearly all members of the KLK family are dysregulated in ovarian cancer and associated with prognosis. In fact, there is growing evidence that KLKs play an important role in cancer biogenesis including tumor growth, migration, invasion, and chemoresistance, thus making them potential targets for anti-cancer therapeutic agents (Dorn et al., 2014; Kryza et al., 2016).

In human tissues, KLKs are often co-expressed and functionally linked to each other. For example, in the skin, KLKs 5, 6, 7, 8, and 14 are involved in corneocyte desquamation and/or regulation of inflammatory reactions or modulation of the lipid-permeability barrier. Prostatic KLKs, i.e. KLK3 in concert with KLK2 and KLK4, are responsible for semen liquefaction. In the central nervous system (CNS), 12 of the 15 members of the KLK family, including KLK6 (neurosin) and KLK8 (neuropsin), are expressed under physiological conditions (Prassas et al., 2015).

KLK6 seems to be the most abundant serine protease in the CNS and participates in numerous physiological and pathological processes. Together with KLK1 and KLK8, KLK6 seems to play an important role in brain injury (Scarisbrick, 2012). KLK6 levels are reduced in cerebrospinal fluid, serum, and plaques in Alzheimer's disease, which can be employed to differentiate the disease from vascular dementia and pseudodementia patients (Bayani and Diamandis, 2012). KLK6 serum levels are significantly increased in multiple sclerosis (MS) and correlate with poor prognosis of MS patients (Bayani and Diamandis, 2012). In Parkinson's disease, KLK6 was implicated in cleavage of α-synuclein preventing its polymerization in Lewy bodies (Tatebe et al., 2010). KLK6 expression is dysregulated in different cancers such as pancreatic (Ruckert et al., 2008), breast (Wang et al., 2008), colon (Vakrakou et al., 2014), and gastric cancer (Kolin et al., 2014). In ovarian cancer, KLK6 overexpression has been reported to be associated with poor prognosis, advanced clinical disease, shorter disease-free and overall survival, and chemotherapy resistance (Diamandis et al., 2003; Kountourakis et al., 2008; White et al., 2009; Loessner et al., 2012; Seiz et al., 2012).

KLK8 is moderately expressed in neurons of the hippocampus, playing essential roles in synaptic plasticity as basis of learning and memory formation, while it displays elevated expression levels following cerebral injuries and in the hippocampus of Alzheimer patients (Shimizu-Okabe et al., 2001; Yoshida, 2003). KLK8 is overexpressed in the tumor tissue of cervix (Cané et al., 2004) and colon cancer (Yousef et al., 2004). Furthermore, its overexpression has been reported to have an – albeit

controversial – impact on prognosis in lung cancer (Sher et al., 2006; Planque et al., 2010). In ovarian cancer, Kishi et al. (2003) reported high expression of KLK8 in serum, ascitic fluid, and tumor tissues obtained from primary ovarian cancer patients, being correlated with the protein expression of tumor-associated CA-125. High KLK8 protein expression was found to be associated with a favorable prognosis, lower nuclear grade, earlier clinical stage and extended overall and disease-free survival of ovarian cancer patients (Shigemasa et al., 2004; Borgono et al., 2006). On the contrary, Kountourakis et al. (2009) reported that low KLK8 protein expression was associated with better outcome in ovarian cancer. To date, there are no data describing the relationship between KLK8 mRNA expression and survival in patients with advanced serous ovarian cancer.

As KLK6 has been described to be a valuable biomarker in ovarian cancer, and is often co-expressed with KLK8 in other organs such as the CNS, in the present study, we evaluated the predictive value of KLK6 and KLK8 mRNA levels as well as their combined expression score for progression-free (PFS) and overall (OS) survival in a cohort of patients with advanced high-grade serous ovarian cancer FIGO stage III/IV. Expression levels of KLK6 and KLK8 were determined by qPCR and the association of KLK mRNA levels with clinical parameters and with survival of the patients was analyzed.

Results

KLK6 and KLK8 mRNA expression in tumor tissue of advanced ovarian cancer patients and relation to patient and tumor characteristics

KLK6 and KLK8 mRNA levels were determined by qPCR in tumor tissues of a homogenous patient cohort (n = 100) encompassing patients with serous ovarian cancer FIGO stage III/IV, only. The relative KLK6 and KLK8 qPCR mRNA levels, normalized to the expression levels of the housekeeping gene HPRT, ranged from 0.38 to 1067.49 (median: 119.42) and from 0.02 to 86.42 (median: 10.75), respectively. As evident by Mann-Whitney test analysis ($p < 0.001$) and by nonparametric Spearman rank correlation analysis ($r_s = 0.636$, $p < 0.001$), a highly significant, pronounced positive correlation between KLK6 and KLK8 mRNA expression is observed (Figure 1A). Based on the observed co-expression of KLK6 and KLK8,
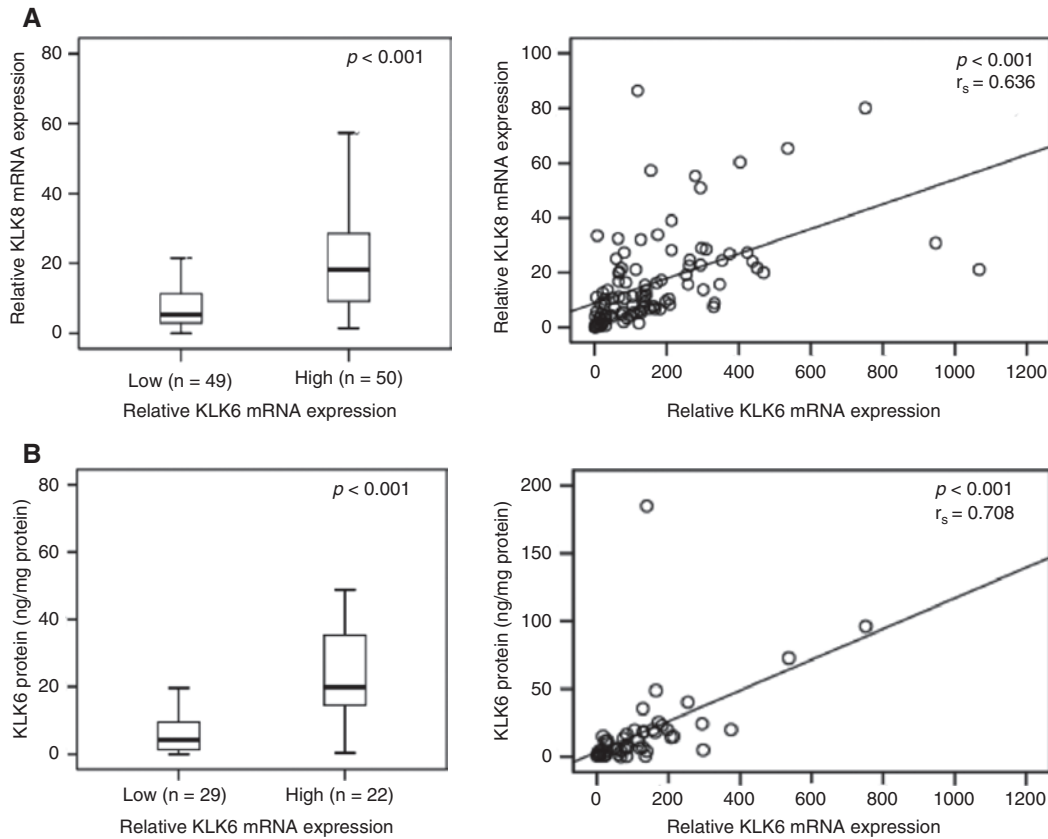
Figure 1: Correlations of KLK6 mRNA expression with KLK6 protein levels and KLK8 mRNA expression levels in tumor tissues of advanced ovarian cancer patients (FIGO III/IV).

(A) KLK6 mRNA expression is significantly correlated with KLK8 mRNA levels in tumor tissue. KLK6 and KLK8 mRNA levels were determined by qPCR with normalization to HPRT mRNA levels. In the left panel, KLK6 mRNA levels were dichotomized into low and high by the median and analyzed for association with KLK8 mRNA values (Mann-Whitney test; $p < 0.001$). In the right panel, KLK6 mRNA and KLK8 mRNA levels were analyzed as linear factors (Spearman correlation; $r_s = 0.636$, $p < 0.001$). (B) KLK6 mRNA expression is significantly correlated with its protein levels (determined by ELISA) in tumor tissue. In the left panel, KLK6 mRNA levels were dichotomized into low and high by the median and analyzed for association with KLK6 antigen levels (Mann-Whitney test; $p < 0.001$). In the right panel, KLK6 mRNA and antigen levels were analyzed as linear factors (Spearman correlation; $r_s = 0.708$, $p < 0.001$).

we further categorized the expression levels of both factors in a KLK6 + KLK8 low-expressing group (both KLK6 and KLK8 mRNA levels below the median) versus a high-expressing group (KLK6 and/or KLK8 mRNA values above the median) for statistical analysis (see below). In case of KLK6, protein expression values determined by ELISA in tumor tissue extracts were available for 51 patients (Dorn et al., 2015). As depicted in Figure 1B, a strong positive correlation between KLK6 mRNA and KLK6 protein levels is found using both Mann-Whitney test ($p < 0.001$) and Spearman rank correlation ($r_s = 0.708$, $p < 0.001$) analyses.

Table 1 depicts the association between dichotomized KLK6 and KLK8 mRNA expression levels (low/high) and the combined KLK6 and KLK8 values (KLK6 + KLK8) in relation to established clinical parameters in ovarian cancer such as age, pre-operative ascites fluid volume,

and post-operative residual tumor mass. The mRNA levels of KLK6, KLK8, and KLK6 + KLK8 do not differ significantly between tumors in relation to these clinical parameters, apart from a trend towards significance ($p = 0.062$) for low KLK6 + KLK8 levels in optimally debulked patients versus patients displaying a residual tumor mass.

Association of KLK6, KLK8, and KLK6 + KLK8 mRNA values with progression-free (PFS) and overall (OS) survival: univariate analysis

The strength of association between traditional clinical parameters, and KLK6, KLK8, and KLK6 + KLK8 mRNA expression levels with patients' 5-year PFS and OS by univariate analysis is summarized in Table 2. Patients with residual tumor mass after debulking surgery and

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

Clinical parameters	N[a]	KLK6[b] low/high	KLK8[b] low/high	KLK6+KLK8[c] low/high
Total no. of patients	100	49/50	50/50	34/65
Age		$p = 0.919$	$p = 0.841$	$p = 0.942$
≤ 60 years	49	24/25	25/24	17/32
> 60 years	51	25/25	25/26	17/33
Residual tumor mass		$p = 0.230$	$p = 0.359$	$p = 0.062$
0 mm	45	25/20	25/20	20/25
> 0 mm	54	23/30	25/29	14/39
Ascitic fluid volume		$p = 0.875$	$p = 0.551$	$p = 0.264$
≤ 500 ml	46	25/21	26/20	20/26
> 500 ml	38	20/18	19/19	12/26

[a]Number of patients.
[b]χ^2 Test (cut-off point = median).
[c]High KLK6+KLK8: KLK6 and/or KLK8 above median; low KLK6+KLK8: KLK6 and KLK8 both below median.

Table 2: Univariate Cox regression analysis of clinical outcome in advanced ovarian cancer patients (FIGO III/IV) with respect to clinical parameters and tumor biological factors.

Clinical parameters		OS			PFS	
	N[a]	HR (95% CI)[b]	p	N[a]	HR (95% CI)[b]	p
Age						
≤ 60 years	43	1		41	1	
> 60 years	48	1.66 (0.98–2.82)	0.061	44	1.39 (0.82–2.35)	0.228
Residual tumor mass						
0 mm	41	1		40	1	
> 0 mm	49	4.63 (2.53–8.48)	<0.001	44	3.31 (1.89–5.78)	<0.001
Ascitic fluid volume						
≤ 500 ml	41	1		39	1	
> 500 ml	35	2.17 (1.22–3.85)	0.008	31	2.27 (1.27–4.05)	0.006
KLK6 mRNA(med)[c]						
Low	44	1		43	1	
High	46	1.78 (1.04–3.03)	0.035	41	1.29 (0.76–2.18)	0.343
KLK6 mRNA(61st)[d]						
Low	54	1		53	1	
High	36	2.07 (1.22–3.50)	0.007	31	1.15 (0.67–1.97)	0.608
KLK8 mRNA[c]						
Low	44	1		41	1	
High	47	1.07 (0.64–1.80)	0.802	44	1.42 (0.84–2.40)	0.194
KLK6+KLK8 mRNA[c]						
Low	29	1		28	1	
High	61	1.82 (0.99–3.33)	0.053	56	1.82 (1.01–3.30)	0.047

[a]Number of patients.
[b]HR: hazard ratio (CI: confidence interval) of univariate Cox regression analysis.
[c]Dichotomized into high and low levels by the median.
[d]Dichotomized into high and low levels by the 61st percentile.

those patients with high pre-operative amounts of ascitic fluid have a significantly shorter OS (HR = 4.63, 95% CI = 2.53–8.48, $p < 0.001$ and HR = 2.17, 95% CI = 1.22–3.85, $p = 0.008$, respectively) and PFS (HR = 3.31, 95% CI = 1.89–5.78, $p < 0.001$ and HR = 2.27; 95% CI = 1.27–4.05, $p = 0.006$, respectively). KLK6 mRNA levels dichotomized by the median represent a significant predictive

factor for OS (HR = 1.78, 95% CI = 1.04–3.03, $p = 0.035$) indicating an about two-fold increased probability of death in the KLK6 high-expressing group. Optimization of the KLK6 cut-off value (61st percentile) does slightly increase the predictive power for OS (HR = 2.07, 95% CI = 1.22–3.50, $p = 0.007$), whereas the association remains not significant concerning PFS. KLK8 mRNA

expression is neither associated with OS nor PFS, yet, high KLK6 + KLK8 values show a trend towards significance in case of OS (HR = 1.82, 95% CI = 0.99–3.33, $p = 0.053$) and are significantly associated with shorter PFS (HR = 1.82, 95% CI = 1.01–3.30, $p = 0.047$). These findings were also confirmed by Kaplan-Meier estimation: the association of KLK6, KLK8, and KLK6 + KLK8 mRNA levels with OS and PFS is visualized by the respective survival curves (Figure 2). Here, high KLK6 + KLK8 mRNA levels are significantly associated with both shorter OS and PFS ($p = 0.048$ and $p = 0.042$, respectively).

Association of KLK6, KLK8, and KLK6 + KLK8 mRNA values with progression-free (PFS) and overall (OS) survival: multivariable analysis

The independent relationship of KLK6, KLK8, and KLK6 + KLK8 with OS and PFS was studied by multivariable Cox hazard regression analysis, including the factors age, ascites fluid volume, and presence of residual tumor mass (base model). In this base model, residual tumor mass is the only clinical parameter representing a predictive marker for OS and PFS (HR = 3.56, 95% CI = 1.63–7.78,
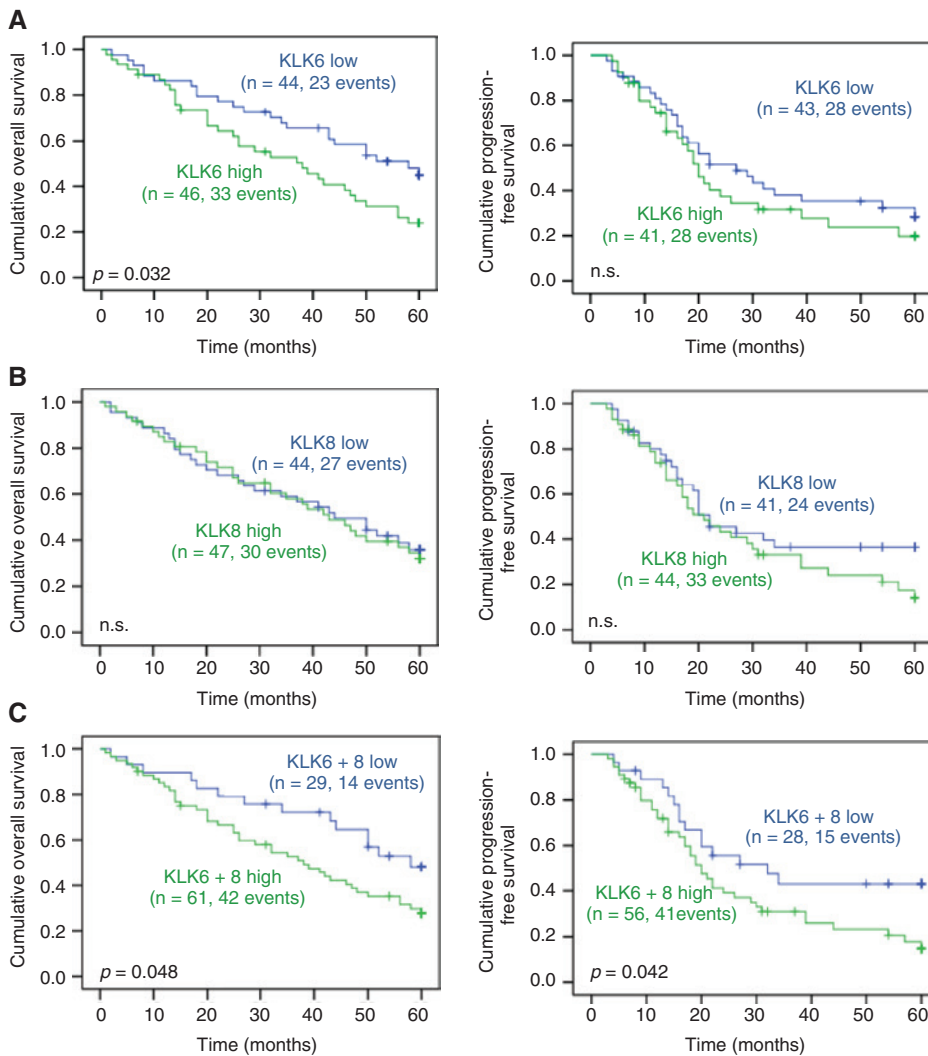

Figure 2: Probability of overall survival (OS) and progression-free survival (PFS) of patients with advanced ovarian cancer (FIGO III/IV) as stratified by KLK6, KLK8 and KLK6 + KLK8 mRNA expression levels, respectively, in primary tumor tissues.
(A) Patients with low KLK6 mRNA expression show significantly better OS (Kaplan-Meier analysis, $p = 0.032$) (left panel) than the group of patients with high KLK6 mRNA expression, but not for progression-free survival (right panel). (B) KLK8 mRNA levels have no impact on both OS (left panel) and PFS (right panel). (C) Patients with high KLK6 and/or high KLK8 expression (KLK6+8 high) display significantly shorter OS (Kaplan-Meier analysis, $p = 0.048$) (left panel) and PFS ($p = 0.042$) (right panel), compared to patients with low mRNA expression levels of both KLK6 and KLK8 (KLK6+8 low). In all of the analyses, KLK6 and KLK8 mRNA levels were dichotomized into low and high by the median.

$p = 0.001$ and HR = 2.30, 95% CI = 1.06–4.96, $p = 0.034$, respectively), while the pre-operative ascitic fluid volume loses its predictive significance for both OS and PFS when subjected to multivariable analysis (Table 3). Among the tumor biological factors (added separately to the base model), only KLK6 mRNA levels (dichotomized by either the median or the 61st percentile) significantly contribute to the base model for OS (KLK6 [median]: HR = 1.85, 95% CI = 1.03–3.31, $p = 0.039$; KLK6 [61st percentile]: HR = 2.33, 95% CI = 1.29–4.19, $p = 0.005$). KLK8 mRNA shows a trend towards significance for PFS (HR = 1.66, 95% CI = 0.92–3.02, $p = 0.094$), while the combined KLK6 + KLK8 mRNA values lose their predictive value in the multivariate model for both OS and PFS.

Discussion

In ovarian cancer, co-expression of KLK6 and KLK8 has been observed (Yousef et al., 2003b; Zheng et al., 2007; Bayani et al., 2008; Avgeris et al., 2012) implicating that both proteases may contribute to tumor invasion and metastasis through cleavage of extracellular matrix components such as laminin, collagens, fibronectin, or vitronectin (Ghosh et al., 2004; Pampalakis and Sotiropoulou, 2007). In the current study, we did not only find co-expression of KLK6 and KLK8 in advanced ovarian cancer (FIGO III/IV), but there was clear evidence as well for coordinated regulation of expression of both factors on the mRNA level ($r_s = 0.636$; $p < 0.001$). Other KLKs such as KLK13 or KLK14 display only a weak correlation to either KLK6 or KLK8 or each other in the same ovarian cancer cohort (with r_s-values around 0.4 or lower; N. Ahmed and L. Dettmar, personal communication). In addition, on the protein level (Zheng et al., 2007), KLK6 expression was found to be strongly correlated with KLK8 expression in tumor tissue of ovarian cancer patients ($r_s = 0.829$). The coordinated regulation of gene expression may, therefore, point towards an involvement of both proteases in shared cascades/pathways.

Table 3: Multivariable Cox regression analysis of clinical outcome in advanced ovarian cancer patients (FIGO III/IV) with respect to clinical parameters and tumor biological factors.

Clinical parameters		OS			PFS	
	N[a]	HR (95% CI)[b]	p	N[a]	HR (95% CI)[b]	p
Age						
≤60 years	37	1		35	1	
>60 years	38	1.55 (0.86–2.82)	0.148	34	1.06 (0.57–1.94)	0.860
Residual tumor mass						
0 mm	36	1		35	1	
>0 mm	39	3.56 (1.63–7.78)	0.001	34	2.30 (1.06–4.96)	0.034
Ascitic fluid volume						
≤500 ml	41	1		39	1	
>500 ml	34	0.97 (0.48–1.98)	0.941	30	1.38 (0.67–2.84)	0.382
KLK6 mRNA(med)[c]						
Low	40	1		39	1	
High	35	1.85 (1.03–3.31)	0.039	30	1.21 (0.66–2.21)	0.539
KLK6 mRNA (61st)[d]						
Low	49	1		48	1	
High	26	2.33 (1.29–4.19)	0.005	21	1.21 (0.65–2.27)	0.541
KLK8 mRNA[c]						
Low	40	1		37	1	
High	35	0.91 (0.50–1.65)	0.756	32	1.66 (0.92–3.02)	0.094
KLK6 + KLK8 mRNA[c]						
Low	28	1		27	1	
High	47	1.40 (0.73–2.68)	0.317	42	1.68 (0.89–3.20)	0.113

[a]Number of patients.
[b]HR: hazard ratio (CI: confidence interval) of multivariable Cox regression analysis. Biological markers were separately added to the base model of clinical parameters: age, residual tumor mass, and ascitic fluid volume.
[c]Dichotomized into high and low levels by the median.
[d]Dichotomized into high and low levels by the 61st percentile.

In fact, KLK6 and KLK8 are co-expressed in several human organs and tissues, a fact that militates in favor of common pathways of activation, upregulation and/or interrelated participation in numerous physiological and pathological activities. In CNS, KLK6 and KLK8 are expressed abundantly, regulating neural plasticity, development and demyelination. They contribute synergistically to degeneration of the spinal cord after injury, as highly reactive oligodendrocytes express both proteases (Yoshida, 2003; Yousef et al., 2003a; Terayama et al., 2004). Interestingly, Kishibe et al. (2007) showed that both KLK6 and KLK8 are upregulated in a parallel manner in skin epidermis upon treatment with 12-O-tetra-decanoyl-phorbol acetate (TPA), which induces epidermal hyperplasia similar to psoriatic lesions. Similarly, KLK6 and KLK8 are highly abundant in intercellular spaces of the stratum spinosum and stratum granulosum of the skin and it was proposed that they work together in a cascade-like pathway during wound healing, whereby KLK8 directly or indirectly upregulates and activates KLK6 contributing to keratinocytes migration and proliferation in early stages of wound healing (Kishibe et al., 2007, 2012). Subsequently, KLK6 will activate the protease activated receptor 2 (PAR2) which is believed to be responsible for further keratinocyte differentiation in the late stages of wound healing (Kishibe et al., 2007, 2012). Moreover, both KLKs have been implicated in pathological skin disorders such as psoriasis: here, high KLK6 and KLK8 protein levels were found in synovial fluid, skin lesions, and serum of psoriasis patients. In particular, elevated KLK8 levels were associated with disease severity (Eissa et al., 2013).

In the present study, KLK6 was found to be a strong predictor of OS (but not PFS), i.e. patients with high expression of KLK6 displayed an about two-fold increased risk of death. By multivariable analysis, KLK6 retained its statistical significance as an independent predictive biomarker. Likewise, several other independent studies previously reported that KLK6 overexpression is associated with unfavorable patients' outcome in colon, pancreas, lung, and also ovarian cancer (Diamandis et al., 2003; Kountourakis et al., 2008; Ruckert et al., 2008; Nathalie et al., 2009; White et al., 2009; Seiz et al., 2012). Notably, KLK6 protein in serum and ascitic fluids of ovarian cancer patients – in contrast to KLK6 from cerebrospinal fluid of healthy individuals – was characteristically associated with a modification of the single N-glycosylation site by α2-6-linked sialic acid which may enable the development of tumor-specific antibodies to detect and/or target KLK6 (Kuzmanov et al., 2009).

Interestingly, in an immunohistochemical study, overexpression of KLK6 protein in ovarian cancer stromal cells was found to be significantly associated with unfavorable prognosis. This fact, together with the role of KLK6 in cleavage of ECM proteins, elucidate the contribution of KLK6 to tumor invasiveness and metastasis (Ghosh et al., 2004; Prezas et al., 2006; Seiz et al., 2012). Moreover, KLK6 in concert with KLKs 4, 5, and 7 provokes transforming growth factor (TGF-β) signaling that may be the cause for taxane drug resistance in ovarian cancer (Loessner et al., 2012). Thus, KLK6 expression may be linked to chemoresistence and this may be the reason for the shortened overall survival of the patients, which all had received adjuvant chemotherapy following surgery. Furthermore, in various *in vitro* cell culture model systems, KLK6 was shown to support cancer cell proliferation, to inhibit apoptosis, and to increase tumor cell invasiveness and migration through laminin and Matrigel matrices in gastric, colon, non-small cell lung, and pancreatic cancer cells by a wide variety of mechanisms, such as increase of ADAM10 expression, inhibition of E-cadherin expression, or PAR2-mediated activation of the epidermal growth factor receptor (EGFR) which in turn contributes to c-Myc expression regulation and interaction with angiogenic factors (Klucky et al., 2007; Henkhaus et al., 2008; Ruckert et al., 2008; Nathalie et al., 2009; Kim et al., 2011; Kryza et al., 2016).

KLK8 is upregulated in ovarian cancer compared to normal ovaries or ovaries with benign disease (Underwood et al., 1999; Kishi et al., 2003; Yousef et al., 2003b; Hibbs et al., 2004) and has previously been proposed as a favorable prognostic marker. Kishi et al. (2003), Shigemasa et al. (2004), and Borgono et al. (2006) demonstrated that KLK8 protein overexpression either detected by ELISA or immunohistochemistry in ovarian cancer was significantly associated with good prognosis. Magklara et al. (2001) determined KLK8 mRNA expression levels by qPCR and found a favorable prognostic impact of high mRNA expression as well. It is, however, important to note that all the studies mentioned above included also early stage patients (FIGO I and/or II) and other histologic subtypes in addition to high-grade serous carcinoma, and demonstrated that KLK8 expression is higher in early-stage and lower-grade tumors as compared to advanced-stage and high-grade tumors.

Among reports that analyzed the predictive impact of KLK8 expression in ovarian cancer, the study by Kountourakis et al. (2009) was the only one using a patient cohort similar to our study considering only advanced ovarian cancer patients FIGO stage III/IV and 69% high-grade serous subtype. Applying an automated quantitative analysis technique (AQUA), which evaluates tumor protein molecule expression per unit areas, high KLK8

expression was found to be associated with poor outcome (Kountourakis et al., 2009). Thus, elevated KLK8 may mediate some adverse effects in late stages of ovarian cancer. This would be in line with findings from our current study and others showing that expression levels of KLK6 and KLK8 are strongly and positively correlated (Zheng et al., 2007) and that KLK6 is associated with an unfavorable outcome (Diamandis et al., 2003; Kountourakis et al., 2008; White et al., 2009; Seiz et al., 2012). Although, in the present study, KLK8 alone showed no statistically significant impact on both OS and PFS, and, thus, may not represent a strong stand-alone biomarker in ovarian cancer, patients displaying both low KLK6 and low KLK8 intratumoral mRNA expression had a significantly longer OS and PFS compared to patients displaying high KLK6 and/or KLK8 levels (log-rank analysis). It should be stressed again that KLK6 levels alone were statistically significantly associated with OS, but not with PFS. Thus, KLK8, in combination with KLK6, may aid to identify patients with better outcome, especially concerning PFS. Interestingly, KLK6 and KLK8 are members of a multiparametric panel of KLKs (KLK's 6, 8, 11, and 13), which has been demonstrated to predict patient progression at 1 year with good accuracy (Zheng et al., 2007).

In conclusion, our current study is the first to quantify KLK6 and KLK8 mRNA expression levels in a homogenous patient cohort of advanced serous ovarian cancer FIGO III/IV. Both mRNAs were found to be widely expressed and the mRNA expression levels were not associated with clinical factors. KLK6 was identified as a statistically independent unfavorable predictive factor for OS, while KLK8 mRNA levels were neither associated with OS nor with PFS. Nevertheless, the use of KLK8 in combination with KLK6 allowed for the identification of a patient group with better outcome (low KLK6 + low KLK8) for both OS and PFS. All in all, together with findings of other studies, KLK6 may be to be considered as validated predictive biomarker in ovarian cancer. As elevated levels of this proteolytic enzyme are associated with poor patient outcome, KLK6 represents an attractive target for tumor therapy, e.g. *via* the development of potent and specific inhibitors of its enzymatic activity.

Materials and methods

Patients

One hundred patients afflicted with advanced serous ovarian cancer FIGO stage III/IV, treated between 1990 and 2012 at the

Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universität München, Munich, Germany, were enrolled in this study. The study was approved by the local Ethics Committee and written informed consent was obtained from all patients. Median patients' age at time of surgery was 61 years (range 23–88 years). All patients initially underwent standard stage-related primary radical debulking surgery. Forty-five patients (45%) were optimally debulked with complete removal of all 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 any neoadjuvant therapy before primary surgery. Median time of follow-up was 41 months for overall survival (OS; range 1–262 months after primary tumor resection) and 20 months for PFS (range 3–262 months). Clinical and histomorphological parameters documented at the time of surgery included histologic subtype, absence or presence of residual tumor mass (0 mm [no visible or palpable tumor left after surgery] vs. > 0 mm [any residual tumor left after surgery]) and ascitic fluid volume (estimated preoperatively by vaginal ultrasound). During the analyzed follow-up time of 5 years, 57 of the 85 patients with available data for PFS had relapsed, and 57 of the 91 patients with available data for OS had died.

RNA isolation from cell lines

For preliminary establishment of the quantitative polymerase chain reaction (qPCR) assays for both KLK6 and KLK8, human ovarian cancer OV-MZ-6 cells stably transfected with pRcRSV-derived expression plasmids encoding the complete coding region (pre-pro-proteins) of either KLK6 or KLK8 (OV-KLK6 and OV-KLK8), were employed (Prezas et al., 2006). Total RNA was isolated from these cell lines using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

RNA isolation from tumor tissue

Deep-frozen tumor tissue samples of ovarian cancer patients stored in liquid nitrogen were selected from the tissue storage facility of the Tumor Bank of the Medical Faculty of the Technical University of Munich, Germany. Tissue samples were sliced to obtain 10–20 µg of still-frozen tumor tissue material, which was immediately dissolved in 600 µl of RLT plus buffer (Qiagen) containing 6 µl 2-mercaptoethanol. Simultaneous purification of total DNA and RNA was performed on the automated QIAcube sample preparation machine (Qiagen) using the All Prep DNA/RNA Universal kit for RNA and DNA extraction from tissues (Qiagen) following the manufacturer's instructions.

In brief, DNA was first purified by use of a DNA spin column, eluted, and stored at –20°C. The flow through of this purification step, containing the RNA, was supplemented with 150 µl chloroform to optimize the RNA purification for samples with higher content of fatty tissues. Samples were thoroughly vortexed and centrifuged (4°C for 3 min, $20\,000 \times g$) to separate the phases. Then, the aqueous phase was carefully transferred to a 2 ml collection tube and an optimized RNA clean-up run on the QIAcube was performed together with a proteinase K digestion step and ethanol supplementation for

optimized binding of total RNA to the RNeasy Mini spin column. A DNase I digestion step ensured high-yields of DNA-free RNA, which was eluted in 50 µl RNase-free H_2O.

RNA concentration and purity was assessed using the Nano Drop 2000c spectrophotometer and the Nano Drop 2000/2000c software (Thermo Fisher Scientific, Wilmington, USA). Quality of RNA was assessed by determination of absorbance ratios at 260/280 nm and 260/230 nm according to the manufacturer's instructions. RNA samples were stored at −80°C until use.

Reverse transcription and cDNA synthesis

Reverse transcription of the isolated RNA (input: 1000 and 500 ng per reaction for RNA of cell lines and tumor tissues, respectively) was conducted using the cloned AMV first strand cDNA Synthesis Kit (Invitrogen, Darmstadt, Germany) following the manufacturer's instructions and final elution of samples in RNase-free H_2O, resulting in a final cDNA concentration of 10 and 5 ng/µl for cell lines and clinical samples, respectively. cDNA samples were stored at −20°C until further use.

Real-time polymerase chain reaction

To account for sample heterogeneity, different extraction/conversion efficiencies, and mastermix variations, the efficiency of KLK6 and KLK8 assays was validated by standard dilution series (Bustin and Nolan, 2013). A 2-fold dilution series of RNA from both cell lines, OV-KLK6 and OV-KLK8, respectively, with five dilution steps (DNA0-DNA4; range 30–1.875 ng), was used to establish a standard dilution curve for each KLK6, KLK8, and the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). The logarithm of each known concentration in the dilution series (x-axis) was plotted against the cycle of threshold (Ct) value for the respective sample (y-axis). Linear regression analysis resulted in an R^2 coefficient and efficiency (E) was calculated from the linear slope by using the following calculation formula: $E = 10exp − (1/slope)$. One hundred percent efficiency corresponds to an E-value of 2. A ΔE between KLK6 or KLK8 and HPRT1 was calculated for estimation of Δ-efficiency-related error margins. Gene specific primers were designed with the Universal Probe Library Assay Design Center software (https://lifescience.roche.com/shop/products/universal-probelibrary-system-assay-design). The following primers (Metabion, Martinsried, Germany) and hydrolysis probes from the Universal Probe Library (Roche, Penzberg, Germany) were used:

KLK6 (numbers for the location of the primers are according to the NCBI entry NM_002774): KLK6-forward (261–280), TGGTGCT-GAGTCTGATTGCT; KLK6-reverse (302–320), CGCCATGCACCAACT-TATT (reaction concentration: 400 nм each); amplicon size: 60 bp. The assay detects KLK6 mRNA variants A and B, both encoding the identical, full length KLK6 protein. The other known variants C, D, and E, encoding a truncated version of KLK6 are not detected.

KLK8 (NM_007196): KLK8-forward (782–799), CAGCAAA-GGGGCTGACAC; KLK8-reverse (869–886), GACCTCCCACAGGGGTCT (reaction concentration: 400 nм each); KLK8-probe, 5′-FAM-TGCC-CTGG (reaction concentration: 200 nм); amplicon size: 105 bp. The assay detects the two major KLK8 mRNA transcript variants 1 and 2,

encoding full length KLK8, plus variants 3, 5, and 6. mRNA variant 4 (potentially encoding a peptide of only 32 amino acids) is not detected.

HPRT1 (NM_000194): HPRT1-forward (218–241), TGACCTT-GATTTATTTTGCATACC; HPRT1-reverse (300–319), CGAGCAAGAC-GTTCAGTCCT (reaction concentration: 400 nм each); HPRT1-probe, 5′-FAM-GCTGAGGA (reaction concentration: 200 nм); amplicon size: 102 bp.

For KLK6, the assay was optimized to use a SYBR-Green based real-time PCR assay using Brilliant III SYBR Green Mastermix (Agilent Technologies, Darmstadt, Germany), while for KLK8 and HPRT1, Taqman-based technology (FAM-labeled Universal Probe Library Taqman probes) with Brilliant III QPCR Master Mix with low ROX (Agilent Technologies, Darmstadt) was used. Ten µl of the respective 2× QPCR mastermix were supplemented with primer, probes and cDNA/controls and adjusted with water to an end volume of 20 µl.

All reactions were performed in triplicates (input: 15 ng/well and 30 ng/well for clinical samples and cell lines, respectively) in 96-well plates. The following cycling program was used: 95°C, 3 min (polymerase activation step), followed by 40 cycles at 95°C, 15 sec (denaturation) plus 60°C, 1 min (annealing/elongation) using the MxPro Software version 4.10. The instrument was set to detect and report fluorescence at each cycle during the 60°C annealing/extension step. Cycle threshold values (Ct) were determined automatically for each marker separately by the MxPro software (version 4.10; standard evaluation settings) by the course of the fluorescent signal readout during the PCR cycle program and including adaptive baseline correction and amplification-based threshold value (in the area of 5–60% total fluorescence level, averaged over technical replicates). For specificity and sensitivity, two negative controls comprising a no-template control (RNA-free water) and total RNA (30 ng; no reverse transcriptase reaction) of ovarian cancer samples were included in duplicates in each run to test for false-positive results. Additional control runs were performed on a set of patient samples in independent repetitions over all sample batches to assess assay and sample robustness in the study. The different expression levels were normalized to human HPRT and a calibrator (OV-KLK6, OV-KLK8 for KLK6 and KLK8, respectively), which was included in each run to allow normalization of samples. Relative target gene expression was calculated using $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct$ sample − ΔCt calibrator and $\Delta Ct = Ct_{target} − Ct_{HPRT}$ (Pfaffl, 2012). Relative error propagation (EP) was calculated for each ΔCt analysis step by the following formula:

$$EP(\Delta Ct) = SQRRoot(((STDEV_{marker})^2 + (STDEV_{HPRT})^2)/2)$$

and

$$EP(\Delta\Delta Ct) = SQRRoot(((STDEV\Delta Ct_{sample})^2 + (STDEV\Delta Ct_{calibrator})^2)/2).$$

Absolute error was calculated by the following formula: ln $2^*EP(\Delta\Delta Ct)^* 2^{-\Delta\Delta Ct}$ (sample). STDEV stands for standard deviation; SQRRoot for square root; ln for natural logarithm.

Due to possible detection limitation and variances of sample qualities and qPCR efficiencies, the following quality criteria were applied to exclude unassertive results. Samples were excluded from the study if (1) the Ct value for HPRT was > 35, (2) the 2 exp−ΔΔCt error progression % was > 30% even after repetition, and (3) the % STDEV of the 2 exp−ΔΔCt for 2 valid runs was higher than 47.1%.

Quantification of KLK6 antigen concentrations in ovarian cancer tissue extracts

Preparation of extracts from ovarian cancer tissues and determination of KLK6 antigen concentrations in these tumor extracts by a noncommercial in-house ELISA test format were previously described (Dorn et al., 2015).

Statistical analyses

All statistical analyses were performed with the SPSS statistical analysis software (version 20.0; SPSS Inc., Chicago, IL, USA). The association of KLK6 and KLK8 mRNA expression levels with clinical characteristics of the patients was evaluated using the χ^2 test. Correlations between continuous variables of tumor biological markers (KLK6 mRNA, KLK8 mRNA, and KLK6 protein) were calculated using the Mann-Whitney U test and Spearman rank correlation (r_s). Associations of tumor biological factors and clinical parameters with patient's survival were analyzed 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 established clinical parameters in ovarian cancer such as age, presence of residual tumor mass, and ascitic fluid volume. For survival analyses, OS and PFS of ovarian cancer patients were used as follow-up end points. Survival curves were plotted according to Kaplan-Meier, using the log-rank model to test for differences. p-Values ≤ 0.05 were considered statistically significant.

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