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Clinical value of protein expression of kallikrein-related peptidase 7 (KLK7) in ovarian cancer

Abstract: Expression of the kallikrein-related peptidase 7 (KLK7) is dysregulated in ovarian cancer. We assessed KLK7 expression by ELISA and quantitative immunohistochemistry and analyzed its association with clinicopathological parameters and patients' outcome. KLK7 antigen concentrations were determined in tumor tissue extracts of 98 ovarian cancer patients by ELISA. For analysis of KLK7 immunoexpression in ovarian cancer tissue microarrays, a manual quantitative scoring system as well as a software tool for quantitative high-throughput automated image analysis was used. In immunohistochemical analyses, expression levels of KLK7 were not associated with patients' outcome. However, in multivariate analyses, KLK7 antigen levels in tumor tissue extracts were significantly associated with both overall and progression-free survival: ovarian cancer patients with high KLK7 levels had a significantly, 2-fold lower risk of death [hazard ratio (HR)=0.51, 95% confidence interval (CI)=0.29–0.90, p=0.019] or relapse [HR=0.47, 95% CI=0.25–0.91, p=0.024), as compared with patients who displayed low KLK7 levels. Our results indicate that – in contrast to earlier findings – high KLK7 antigen levels in tumor tissue extracts may be associated with a better prognosis of ovarian cancer patients.

Keywords: ELISA; immunohistochemistry; kallikrein-related peptidases; prognosis; tumor tissue.

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Introduction

Numerous studies have focused on improved understanding of the underlying tumor biology in ovarian cancer and biomarkers associated with this fatal disease (Cannistra, 2004; Yoshida et al., 2009; Swisher et al., 2012). However, for both early and advanced ovarian cancer, clinical, histomorphological, and tumor biological biomarkers useful for diagnosis, prognosis, and/or therapy response prediction are in short supply (Rosen et al., 2005). Cancer biomarkers that would reliably predict the risk of an ovarian cancer patient to experience untimely disease recurrence followed by early death or response to preoperative, adjuvant, or palliative therapy are urgently needed (Bast et al., 2005). In this respect, tumor-associated proteases such as several of the members of the serine protease-type kallikrein-related peptidase family of genes (KLK1–15), located on chromosome 19q13.3-4, may function as tumor biomarkers in ovarian cancer (Yousef and Diamandis, 2009; Seiz et al., 2012).

Only a few other established tumor biological markers do serve as cancer biomarkers for this kind of disease, the most important being the serum marker CA125 encoded by the MUC16 gene (Whitehouse and Solomon, 2003). Serum CA125 half-life was reported by various authors to be an independent prognostic factor for the survival of patients with advanced epithelial ovarian cancer and treated with chemotherapy. Uzunoglu et al. (2013), however, are favoring the use of CA125 antigen determined at different times during the chemotherapy cycles to assess response to chemotherapy and to predict clinical outcome of the ovarian cancer patients. Besides that, the serum-based OVA1 test (consisting of CA125, transferrin,

β_2-microglobulin, apolipoprotein A1, and prealbumin) as well as the ROMA score (Risk of Malignancy Algorithm, consisting of human epididymis protein HE4, CA125, and menopausal status) can help to evaluate the likelihood that a woman's ovarian tumor mass is malignant or benign and then support the clinician's decision whether to transfer the patient to a gynecological oncologist for surgery (Yip et al., 2011). Yet, although any of these factors additionally to CA125 are considered valuable biomarkers for ovarian cancer detection or disease recurrence monitoring, none of these single markers was regarded as suitable for prediction of ovarian cancer prognosis (Høgdall et al., 2010; Clarke et al., 2011; Kim et al., 2012; Macuks et al., 2012).

Recent studies suggest that several members of the KLK family, in conjunction with other histo-/biomarkers, can be used to predict surgical success (Dorn et al., 2007); others, including KLK7, may be assessed to predict response to chemotherapy or overall and progression-free survival (OS and PFS, respectively) (Kishi et al., 2003; Kyriakopoulou et al., 2003; Shan et al., 2006; Oikonomopoulou et al., 2008; Psyrri et al., 2008; Dong et al., 2010; Dorn et al., 2011a,b).

KLK7, initially described as human stratum corneum chymotryptic enzyme, was first identified in the human skin (Hansson et al., 1994) and is supposed to be involved in the process of skin desquamation (Caubet et al., 2004). In addition to skin, in normal tissue, KLK7 is predominantly produced by the esophagus, kidney, lung, stomach, cervix uteri, ovary, and the vagina (Shaw and Diamandis, 2007) and is upregulated in tumor tissues of patients afflicted with cancer of the cervix uteri (Termini et al., 2010), colon (Talieri et al., 2009), pancreas (Avgeris et al., 2010), and ovary (Dorn et al., 2006, 2007; Shan et al., 2006; Psyrri et al., 2008) and downregulated in prostate cancer (Xuan et al., 2008), breast cancer (Holzscheiter et al., 2006; Mangé et al., 2008), and kidney cancer (Gabril et al., 2010).

KLK7 expression in normal and cancerous tissues has been determined at the mRNA level by microarray analysis (Hibbs et al., 2004) and quantitative PCR (Kyriakopoulou et al., 2003; Holzscheiter et al., 2006) and at the protein level by immunoenzymometric assays (Dorn et al., 2006; Shan et al., 2006) and immunofluorescence techniques (Psyrri et al., 2008).

In the present study, we aimed at determining KLK7 protein expression in ovarian cancer tissues applying two different quantitative approaches, ELISA and quantitative immunohistochemistry (IHC), using well-characterized antibodies to KLK7. KLK7 immunoreactivity was estimated manually and by an automated image analysis software (Conway et al., 2008). We compared these findings with clinical and histomorphological parameters and related them to OS and PFS of the ovarian cancer patients.

Results

Characterization of the polyclonal antibody AF2624 directed to KLK7

In previous studies from our groups on different sets of ovarian cancer patients, KLK7 antigen levels were determined in ovarian cancer tissue extracts using a highly sensitive ELISA test format. There, it was shown that KLK7 is highly overexpressed in malignant ovarian tumors compared with healthy or benign ovarian tissues (Dorn et al., 2006, 2007; Paliouras and Diamandis, 2006; Shan et al., 2006). The antibodies used in the KLK7-ELISA did not prove to be of use in staining of formalin-fixed, paraffin-embedded tissue (data not shown). Therefore, we screened other antibodies directed to KLK7 for the potential use in IHC and selected the highly specific, affinity-purified polyclonal goat IgG antibody (pAb) AF2624 (R&D Systems, Wiesbaden, Germany).

To validate the antibody, we analyzed the specificity of pAb AF2624 using Western blot analysis (Figure 1). Purified human KLK1 as well as recombinant human proforms of KLK2-15 (~1 µg each) were subjected to 12% SDS-PAGE, blotted onto a PVDF membrane, then reacted with pAb AF2624, and subsequently visualized by reaction with an HRP-labeled antibody to goat IgG. Pro-KLK7 was the single antigen to show reactivity, thus indicating the specificity of the selected antibody. The proper transfer of

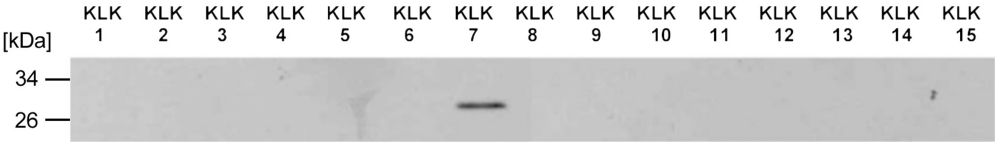

Figure 1 Characterization of polyclonal antibody AF2624 directed to pro-KLK7.
Analysis of antigen-binding specificity of pAb AF2624 directed to recombinant KLK7 by Western blot analysis. Only pro-KLK7, with an apparent molecular mass of approximately 26 kDa, but no other recombinant (pro-)KLKs, displayed immunoreactivity.

KLK proteins onto the membrane was verified by reaction with an antibody directed against the C-terminally located Tag100 epitope present in pro-KLK3-15 (Seiz et al., 2012, data not shown).

To further characterize the KLK7-directed antibody, we used different tissues with a known KLK7 expression level for IHC. In previous studies, KLK7 immunoexpression has been demonstrated in various tissues with highest staining intensity in the skin. Using pAb AF2624, and in line with previous findings, we in fact observed high immunoexpression in the stratum corneum of the skin, moderate expression in normal breast tissue, and low expression in skeletal muscle and benign ovarian tumor tissue (Figure 2).

Comparison of KLK7 levels by Western blotting, IHC, and ELISA

To further compare the different techniques of Western blot analysis, ELISA, and IHC for KLK7 expression, we randomly selected tissue specimens from nine ovarian cancer patients with either low (n=5) or high (n=4) KLK7 antigen levels as assessed by ELISA and analyzed these samples by Western blot analysis as well. Here, we observed reactivity of pAb AF2624 with a protein doublet, with an apparent molecular weight of about 32 and 30 kDa, respectively (Figure 3A). These two bands may well

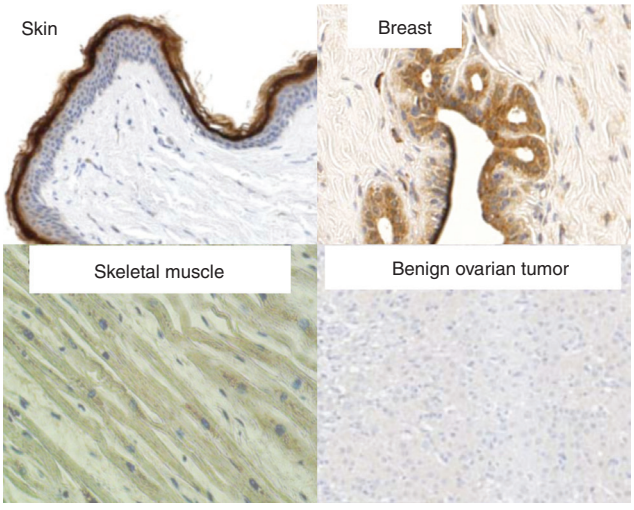

Figure 2 Immunohistochemical staining of KLK7 in normal and benign tissues.
Strong KLK7 expression is observed in the outer layer (stratum corneum) of the skin, moderate staining in normal breast tissue, low staining in skeletal muscle, and no KLK7 immunoexpression in cystoma, a benign tumor of the ovary.

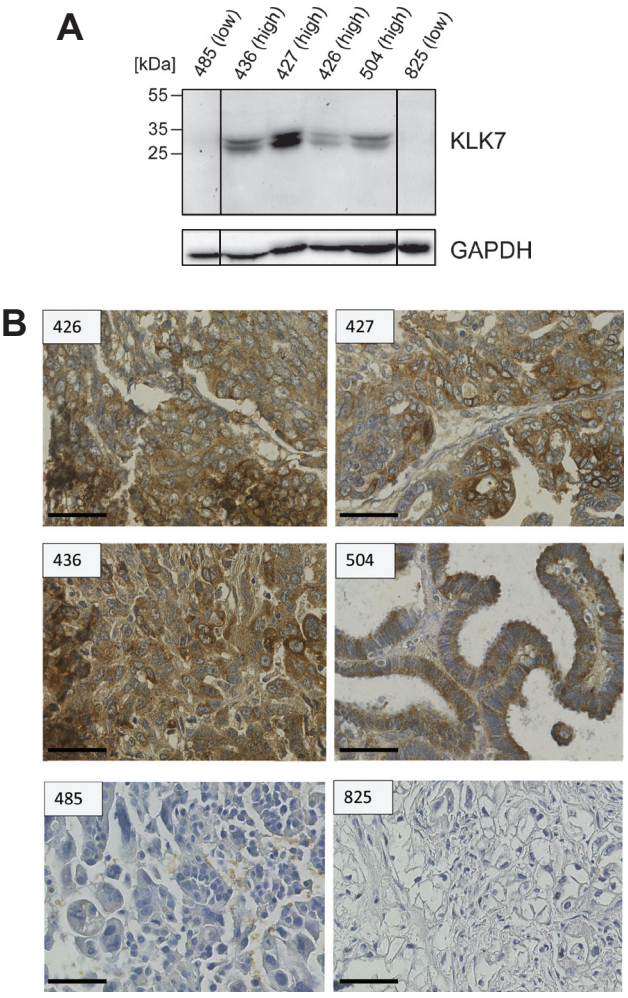

Figure 3 Analysis of the protein expression pattern of KLK7 in ovarian cancer by Western blot analysis and IHC.
(A) Ovarian cancer detergent-released tissue extracts assessed for KLK7 antigen expression by Western blot analysis. Samples showing high or low antigen levels by KLK7-ELISA were analyzed by Western blot analysis. The ELISA values (ng KLK7/mg total protein) of the samples were the following: 504, 20.32; 426, 9.01; 427, 22.29; 436, 16.89; 485, 0.21; 825, 0.37; 433, 0.86. The antigen levels in the tumor extracts of 503 and 416 were below the lower detection limit of the KLK7-ELISA (0.2–10 ng/ml). pAb AF2624 showed reactivity with a protein doublet, with an apparent molecular weight of 32 and 30 kDa, probably corresponding to pro-KLK7 and activated KLK7, respectively. The intensity of the two bands corresponds well with the classification high and low KLK7 by ELISA. For the estimation of the loading of the gel and protein transfer to the membrane, the blots were additionally reacted with an antibody directed to GAPDH (lower panel).
(B) Paraffin-embedded, formalin-fixed ovarian cancer tissues preselected by ELISA (high vs. low KLK7 protein expression) as assessed by IHC. Note that high KLK7 antigen content (504, 426, 427, and 436) matches with pronounced cytoplasmic staining of tumor cells and absence of stromal cell staining; low ELISA KLK7 antigen content (485, 503, 825, 416, and 433) is compatible with a faint cytoplasmic staining of tumor cells and absence of stromal cell staining (scale bar: 50 μm).

correspond to pro-KLK7 and activated KLK7, respectively, as active KLK is generated by clipping off the short N-terminal pro-peptide of the KLK7 zymogen (corresponding to seven amino acids). The intensity of the two bands corresponds well with the classification high and low KLK7 levels as assessed by ELISA (for ELISA values see Figure 3).

Then, tissue microarray sections of the selected cases were stained with pAb AF2624 using the Dako EnVision method (Dako, Hamburg, Germany). Again, in these cases, pAb AF2624 reactivity in immunohistochemical staining showed to be high and low, respectively, concordant with the KLK7 antigen concentration determined by ELISA (Figure 3B).

Relationship between KLK7 expression levels in ovarian cancer tissue

We then aimed at comparing the well-established KLK7-ELISA format with IHC using the sensitive and specific pAb AF2624 in 98 ovarian cancer patients. In ovarian cancer tissue, we observed prominent staining of tumor cells but less staining of stromal cells. Because both stromal and tumor cells express KLK7 in ovarian cancer tissue, we aimed at analyzing the expression pattern individually in these cell types. There are several examples in the literature demonstrating a prognostic impact of the expression pattern of a given biomarker either present in stromal or tumor cells (see, e.g., Kotzsch et al., 2010; Seiz et al., 2012). Therefore, for the evaluation of observer-assisted analysis of KLK7 immunostaining, a quantitative score based on staining intensity and percentage of positively stained cells was designed (see Materials and methods) for tumor cell (KLK7-RT) and stromal cell (KLK7-RN) KLK7 expression. The mean KLK7-RT and KLK7-RN scores values of two or three cores from each tumor sample and the summary score of both score values (KLK7-RT+RN) were used for statistical analyses. In addition, another method for analysis of total KLK7 expression, namely automated image analysis of KLK7 immunostaining utilizing positive pixel algorithms (see Materials and methods section) was performed. Again, after analyzing two or three cores from each tumor sample, the mean score value (KLK7-AV) of the readings was used for statistical calculation.

A distinctly elevated KLK7 expression was observed in tumor cells compared with stromal cells in tumor tissue: the mean score value of KLK7-RT was higher (5.5, range 0–30) than that of KLK7-RN (1.9, range 0–10). The frequency of score values greater than zero was much higher for tumor cells (71 vs. 27 negative) compared with stromal cells (24 vs. 74 negative). The overall KLK7-RT+RN and KLK7-AV score values ranged from 0 to 40 (mean 7.4) and from 63 to 5201 (mean 1126), respectively. The mean KLK7-ELISA (KLK7-E) value was 5.5 ng/mg protein (range 0–45.9).

We found a moderate but statistically significant correlation between KLK7-RT and KLK7-RN values (r_s=0.46, p<0.001). A strong, significant correlation was observed among KLK7-RT, KLK7-RN, and KLK7-RT+RN score values (KLK7-RT/KLK7-RT+RN, r_s=0.89, p<0.001; KLK7-RN/KLK7-RT+RN, r_s=0.70, p<0.001). Furthermore, KLK7-AV score values were significantly correlated with KLK7-RT (r_s=0.74, p<0.001), with KLK7-RN (r_s=0.54, p<0.001) and with the overall KLK7-RT+RN score values (r_s=0.75, p<0.001).

Next, we evaluated the relationship between KLK7 immunoexpression and KLK7 antigen levels in tumor tissue extracts by ELISA (KLK7-E). The KLK7-E concentration was only weakly (but significantly) correlated with KLK7-RT, KLK7-RN, KLK7-RT+RN, and KLK7-AV immunoscore values (r_s=0.39, p<0.001; r_s=0.30, p<0.01; r_s=0.30, p<0.001; r_s=0.27, p<0.01). When we grouped the KLK7 immunoexpression in tumor cells (KLK-RT) into three categories (by the 33rd and 66th percentiles) and analyzed their relation to KLK7-ELISA, applying box plot analyses, significant differences of KLK7-ELISA values between the groups were observed (p=0.001, Kruskal-Wallis test; Figure 4). Because there may exist an *a priori* ordering of the three groups analyzed, additionally, the Jonkheere-Terpstra test was used to test for associations. In fact, a highly significant positive association was found between KLK7 values and KLK-RT groups (p<0.001).

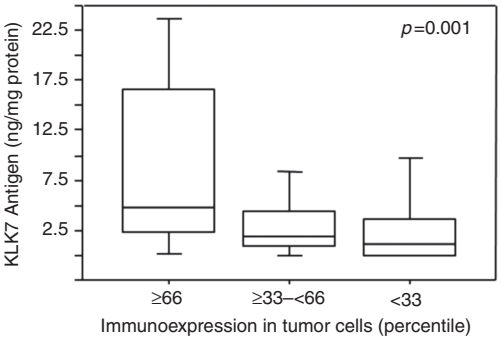

Figure 4 Association of KLK7 immunoexpression and antigen levels in tumor tissue.
Box plot analyses of the association of KLK7 immunoexpression in tumor cells (KLK7-RT) and KLK7-ELISA after grouping the KLK7-RT values by the 33rd and 66th percentiles into the category high, medium, and low. Significant differences of KLK7-ELISA values between the ≥66 group and the two other groups were observed (p=0.001, Kruskal-Wallis test; groups ≥66 vs. ≥33–<66, p=0.012; ≥66 vs. <33, p<0.001; ≥33–<66 vs. <33, p=0.252, Mann-Whitney U-test).

Association of KLK7 expression with clinicopathological parameters

As justified by the results depicted in Figure 4, for all statistical analyses, the 66th percentile of the immunoscore values was applied as cutoff to classify immunohistochemical KLK7 expression as high (>66%) or low (0–66%): KLK7-RT, cutoff=5 (KLK7-RT high with immunoscore >5.01: 30 cases, KLK7-RT low: 68 cases); KLK7-RN, cutoff=0 (KLK7-RN high with immunoscore >0: 24 cases, KLK7-RN low: 74 cases); KLK7-RT+RN, cutoff=6 (KLK7-RT+RN high with immunoscore >6.01: 33 cases, KLK7-RT low: 65 cases); KLK7-AV, cutoff=1100 (KLK7-AV high with immunoscore >1101: 33 cases, KLK7-AV low: 65 cases). Likewise, the KLK7-E concentrations were divided into groups with high or low antigen levels by the 66th percentile (4.0 ng/mg protein; KLK7-E high with values >4.01: 33 cases, KLK7-E low: 65 cases).

KLK7 values stratified into low and high expression groups by the 66th percentile were used for assessing the association of KLK7 with clinical and histomorphological parameters. The relationship between KLK7 immunoscore values and KLK7-E antigen levels – and relevant clinicopathological parameters of ovarian cancer patients – is summarized in Table 1. High levels of KLK7-RT, KLK7-RT+RN, and KLK7-AV are related to the presence of residual tumor mass (p=0.033, p=0.010, and p=0.018, respectively). Furthermore, a significant association was observed between high KLK7-RT+RN score values and high ascitic fluid volume (p=0.004). In addition, high KLK7-AV values are associated with FIGO stage III/IV (p=0.034), high KLK7-E concentrations with response to chemotherapy (p=0.034), whereas high KLK7-RN values were strongly related to low nuclear grade G1+2 (p=0.004) (Table 1).

Association of clinicopathological parameters and KLK7 expression with patient's survival

The association of relevant clinicopathological parameters and KLK7 protein expression levels with OS and PFS is presented in Tables 2 and 3. All clinicopathological variables included in the Cox model, such as FIGO stage I/II vs. III/IV nuclear grade G1/2 vs. G3/4, presence of residual tumor mass and ascitic fluid volume, but not age, were univariate predictors for OS in the ovarian cancer

Table 1 Association between clinical/histomorphological characteristics of patients and tumor biological factors (n=98).

Clinicopathological parameters	No. of patients	KLK7-RT[a,b] low/high	KLK7-RN[a,b] low/high	KLK7-RT+RN[a,b] low/high	KLK7-AV[a,b] low/high	KLK7-ELISA[a] low/high
Total no. of patients	98	68/30	74/24	65/33	65/33	65/33
Age		NS	NS	NS	NS	NS
≤60 years	60					
>60 years	38					
FIGO stage		NS	NS	NS	p=**0.034**	NS
I+II	21				18/3	
III+IV	77				47/30	
Nuclear grade		NS	p=**0.004**	NS	NS	NS
G1+G2	37		22/15			
G3+G4	61		52/9			
Residual tumor mass		p=**0.033**	NS	p=**0.010**	p=**0.018**	NS
0 mm	52	40/12		41/11	40/12	
>0 mm	41	23/18		22/19	22/19	
Ascitic fluid volume		NS	NS	p=**0.004**	NS	NS
≤500 ml	64			49/15		
>500 ml	32			15/17		
Response to CTX		*p=0.052*	NS	NS	NS	p=**0.034**
No	13	12/1				12/1
Yes	63	41/22				39/24

Bold values are significant results (p<0.005); values in italic/not bold are have missed significance.

[a]χ^2-test (cutoff point >66th percentile).

[b]KLK7 immunoreactivity scores (KLK7-RT: in tumor cells; KLK7-RN: in stromal cells; KLK7-RT+RN: addition of KLK7-RT and KLK7-RN score values; KLK7-AV.: immunoscore by automated image analysis).

Table 2 Univariate Cox regression analysis to determine disease survival in patients with ovarian cancer (n=98).

Clinicopathological parameters	No. of patients	OS		PFS	
		HR (95% CI)[a]	*p*-Value	HR (95% CI)[a]	*p*-Value
Total no. of patients	98				
Age					
≤60 years	60	1			
>60 years	38	1.49 (0.91–2.42)	NS	1.26 (0.73–2.19)	NS
FIGO stage					
I+II	21	1		1	
III+IV	77	4.89 (2.10–11.4)	<**0.001**	7.98 (2.48–25.7)	<**0.001**
Nuclear grade					
G1+G2	37	1		1	
G3+G4	61	1.88 (1.10–3.18)	**0.020**	1.64 (0.91–2.96)	NS
Residual tumor mass					
0 mm	52	1		1	
>0 mm	41	5.69 (3.28–9.87)	<**0.001**	5.30 (2.91–9.63)	<**0.001**
Ascitic fluid volume					
≤500 ml	64	1		1	
>500 ml	32	3.35 (2.03–5.52)	<**0.001**	2.85 (1.61–5.05)	<**0.001**
KLK7-RT[b,c]					
Low	68	1		1	
High	30	1.36 (0.82–2.27)	NS	1.53 (0.87–2.69)	NS
KLK7-RN[c]					
Low	74	1		1	
High	24	1.02 (0.59–1.77)	NS	0.83 (0.42–1.61)	NS
KLK7-RT+RN[b,c]					
Low	65	1		1	
High	33	1.50 (0.91–2.46)	NS	1.41 (0.80–2.48)	NS
KLK7-AV[b,c]					
Low	65	1		1	
High	33	1.53 (0.92–2.53)	*0.098*	1.39 (0.78–2.47)	NS
KLK7-ELISA[b]					
Low	65	1		1	
High	33	0.71 (0.42–1.20)	NS	0.66 (0.36–1.20)	NS

[a]HR (95% CI) of univariate Cox regression analysis.

[b]Dichotomized in high and low levels by the 66th percentile (low: 0th–66th, high: >66th percentile).

[c]KLK7 immunoreactivity scores (KLK7-RT: in tumor cells; KLK7-RN: in stromal cells; KLK7-RT+RN: addition of KLK7-RT and KLK7-RN score values; KLK7-AV: immunoscore by automated image analysis).

cohort (Table 2). Likewise, in univariate analysis for PFS, all of the clinicopathological parameters, except age and nuclear grade, reached statistical significance (Table 2). Conversely, the expression level of KLK7 in tumor cells or stromal cells as detected by IHC, the combined overall score KLK7-RT+RN, and the KLK7-E values were not associated with patients' outcome in univariate Cox regression analysis (Table 2).

Strikingly, in multivariate analysis, KLK7-E values, in addition to FIGO stage and residual tumor mass, were significantly associated with survival (Table 3). Ovarian cancer patients with high KLK7-E levels had a significantly, 2-fold lower risk of death or relapse with a hazard ratio (HR) of 0.51 (95% CI=0.29–0.90, p=0.019) and 0.47 (95% CI=0.25–0.91, p=0.024), respectively, as compared with patients who displayed low KLK7-E levels (Table 3).

In addition, we performed statistical analyses in the subgroup of patients with advanced ovarian cancer (FIGO stage III/IV; n=77). Here, the clinical variables age (only for OS), presence of residual tumor mass, and high ascitic fluid volume were univariate predictors for both OS and PFS. Furthermore, we found a significant association between low KLK7-E antigen levels in tumor tissue extracts and an increased risk of death for FIGO III/IV patients in univariate Cox regression analysis (Table 4). On the contrary, KLK7 immunoscore values were not significantly related with OS and PFS in FIGO III/IV ovarian cancer patients.

Table 3 Multivariate Cox regression analysis to determine the association of tumor biological factors with disease survival in patients with ovarian cancer (n=91).

Clinicopathological parameters	No. of patients	OS		PFS	
		HR (95% CI)[a]	*p*-Value	HR (95% CI)[a]	*p*-Value
Total no. of patients	91				
Age					
≤60 years	57	1		1	
>60 years	34	1.37 (0.81–2.30)	NS	1.16 (0.64–2.10)	NS
FIGO stage					
I+II	18	1		1	
III+IV	73	2.57 (0.95–6.99)	*0.064*	3.80 (1.10–13.2)	**0.035**
Nuclear grade					
G1+G2	34	1		1	
G3+G4	57	1.20 (0.69–2.08)	NS	0.92 (0.50–1.70)	NS
Residual tumor mass					
0 mm	51	1		1	
>0 mm	40	3.59 (1.75–7.35)	<**0.001**	3.43 (1.65–7.12)	**0.001**
Ascitic fluid volume					
≤500 ml	63	1		1	
>500 ml	28	1.14 (0.60–2.15)	NS	1.15 (0.57–2.33)	NS
KLK7-RT[b,c]					
Low	62	1		1	
High	29	1.12 (0.65–1.93)	NS	1.21 (0.67–2.18)	NS
KLK7-RN[c]					
Low	71	1		1	
High	20	0.62 (0.32–1.21)	NS	0.58 (0.28–1.21)	NS
KLK7-RT+RN[b,c]					
Low	62	1		1	
High	29	0.97 (0.56–1.68)	NS	1.02 (0.55–1.88)	NS
KLK7-AV[b,c]					
Low	61	1		1	
High	30	1.37 (0.76–2.47)	NS	1.20 (0.63–2.26)	NS
KLK7-ELISA[b]					
Low	60	1		1	
High	31	0.51 (0.29–0.90)	**0.019**	0.47 (0.25–0.91)	**0.024**

[a]HR (95% CI) of multivariate Cox regression analysis. Biological markers were separately added to the base model of clinical parameters: age, FIGO stage, nuclear grade, residual tumor mass, and ascitic fluid volume.

[b]Dichotomized in high and low levels by the 66th percentile (low: 0th–66th, high: >66th percentile).

[c]KLK7 immunoreactivity scores (KLK7-RT: in tumor cells; KLK7-RN: in stromal cells; KLK7-RT+RN: addition of KLK7-RT and KLK7-RN score values; KLK7-AV: immunoscore by automated image analysis).

In multivariate Cox analysis for advanced ovarian cancer patients, the presence of residual tumor mass remained a strong, statistically significant parameter for both OS and PFS (Table 4). As already shown in the whole patient cohort, in multivariate analysis, high KLK7-E values were strongly associated with a longer OS (HR=0.40, 95% CI=0.22–0.73, p=0.003) and PFS (HR=0.45, 95% CI=0.23–0.87, p=0.018). These findings were confirmed by Kaplan-Meier estimation; the association of KLK7-E levels with OS and PFS is visualized by the respective survival curves (Figure 5). All other analyzed clinical, histomorphological, and biological parameters as well as biological factors were not significantly related with OS and PFS in FIGO stage III/IV ovarian cancer patients (data not shown).

Discussion

This study aimed at examining KLK7 expression in ovarian cancer tissue and analyzing its association with the course of the disease. KLK7 has been described to be overexpressed in ovarian cancer compared with healthy ovarian tissue and benign ovarian tumors and to be associated with more advanced and aggressive disease and

Table 4 Univariate and multivariate Cox regression analysis to determine the association of tumor biological factors with disease survival in FIGO III/IV ovarian cancer patients (n=77 and n=73, respectively).

Clinicopathological parameters	No. of patients	OS		PFS	
		HR (95% CI)[a]	*p*-Value	HR (95% CI)[a]	*p*-Value
Univariate analysis					
Total no. of patients	77				
Age					
≤60 years	48	1		1	
>60 years	29	1.82 (1.08–3.04)	**0.023**	1.47 (0.83–2.62)	NS
Residual tumor mass					
0 mm	33	1		1	
>0 mm	41	4.30 (2.39–7.73)	<**0.001**	3.60 (1.93–6.69)	<**0.001**
Ascitic fluid volume					
≤500 ml	46	1		1	
>500 ml	30	3.06 (1.81–5.19)	<**0.001**	2.62 (1.45–4.73)	**0.001**
KLK7-ELISA[b]					
Low	51	1		1	
High	26	0.55 (0.31–0.95)	**0.032**	0.56 (0.30–1.05)	*0.071*
Multivariate analysis					
Total no. of patients	73				
Residual tumor mass					
0 mm	33	1		1	
>0 mm	40	3.49 (1.68–7.22)	<**0.001**	3.31 (1.59–6.90)	**0.001**
KLK7-ELISA[b]					
Low	47	1		1	
High	26	0.40 (0.22–0.73)	**0.0030**	0.45 (0.23–0.87)	**0.018**

[a]HR (95% CI) of multivariate Cox regression analysis. Biological markers were separately added to the base model of clinical parameters in the FIGO III/IV subgroup: age, nuclear grade, residual tumor mass, and ascitic fluid volume.

[b]Dichotomized in high and low levels by the 66th percentile (low: 0th–66th, high: >66th percentile).

poor outcome (Dong et al., 2003, 2010; Kyriakopoulou et al., 2003; Bignotti et al., 2006; Shan et al., 2006; Zheng et al., 2007; Psyrri et al., 2008; Girgis et al., 2012). Most findings, so far, have been acquainted by analyses of the KLK7 mRNA expression level or, on the protein level, by ELISA.

We first analyzed the performance of the commercially available polyclonal antibody AF2624 to KLK7 by

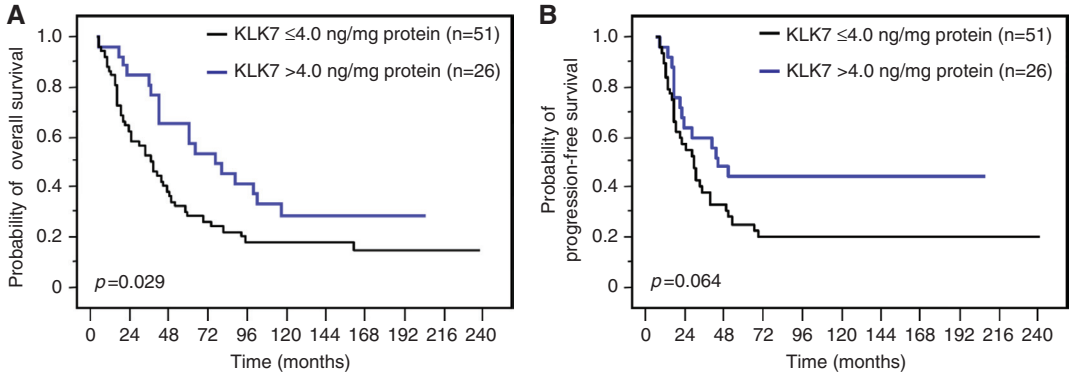

Figure 5 Probability of OS and PFS of advanced ovarian cancer patients stratified by antigen levels in tumor tissue.
Advanced stage ovarian cancer patients (FIGO III/IV), with elevated KLK7 levels (>4.0 ng/mg protein) in tumor tissue, as assessed by ELISA, show significantly better OS (Kaplan-Meier analysis, p=0.029) (A) and a trend for better PFS (p=0.064) (B) than the group of patients with low KLK7 levels (≤4.0 ng/mg protein).

Western blot analyses and, through this, excluded cross-reactivity with the other 14 serine proteases of the human KLK family. Next, we used antibody AF2624 in IHC to evaluate KLK7 protein expression in various types of tissues. Overall, KLK7 immunoreactivity has been described to be cytoplasmatic, not tissue-specific, but with characteristic expression patterns depending on the origin of the tissue. Glandular epithelia constitute the main sites of KLK immunoexpression; positive immunoreactivity in their secretions suggests that KLKs are secreted proteins (Petraki et al., 2006).

KLK7 is known to be primarily expressed in the skin, suitable to its supposed physiological role in desquamation and keratinization processes, specifically in the stratum corneum, but also in endocrine sweat and apocrine glands (Petraki et al., 2006). This is in line with our findings. For breast tissue, moderate staining has been described by Petraki et al. (2006), findings we could reproduce using pAb AF2624 as well. Mesenchymal tissue and benign ovarian tumors have been reported to show no or only weak staining for KLK7 (Dong et al., 2003). In line with these reports, we observed weak KLK7 protein expression when staining cystoma of the ovary or skeletal muscle specimens.

The major aims of our study were to compare the technique of observer-assisted, manual analysis with automated quantification of KLK7 immunostaining and to analyze the similarities and differences in results acquired by ELISA and Western blot. When applying these techniques to a chosen set of ovarian cancer specimens with low and high KLK7 levels as determined by ELISA in tumor tissue extracts, Western blot analyses and IHC staining intensity were found to be concordant with the ELISA results. However, when analyzing KLK7 antigen levels quantified in tumor tissue extracts by ELISA compared with KLK7 tissue immunoreactivity in all cases of the cohort (n=98) using Spearman correlation, association between KLK7-ELISA levels and KLK7 immunoscore values is only moderate indicating that the different technical approaches can be compared, but with limitations.

The moderate association between KLK7-ELISA and KLK7-IHC values is not unique for this serine protease. In fact, such a weak correlation has been demonstrated for the serine protease urokinase-type plasminogen activator (uPA) as well (Lang et al., 2013). One reason is that, for IHC, only a thin section of tumor tissue is investigated, whereas for the ELISA test, proteins are extracted from relatively large pieces of tumor tissue and then the protein of interest is determined in this tissue extract. A second reason could be the use of different KLK7 antibodies in ELISA and in IHC because the antibodies applied in the ELISA test were not suitable for IHC staining.

Another aim of the study was to determine the clinical impact of KLK7 expression levels to predict the course of the ovarian cancer disease. KLK7, among other KLKs, has been reported to be involved in ovarian tumor progression and to predict OS and PFS as well as surgical outcome and response to chemotherapy (Kyriakopoulou et al., 2003; Borgono and Diamandis, 2004; Shan et al., 2006; Dorn et al., 2007; Oikonomopoulou et al., 2008; Psyrri et al., 2008; Dong et al., 2010). In our study, in Cox univariate and multivariate analysis, neither KLK7 IHC scores by observer-assisted analysis nor by automated quantification, showed statistically significant prognostic impact. Thus, KLK7 IHC results (regardless of how they are assessed) are not useful for predicting the clinical outcome of ovarian cancer patients. This finding is in line with reports regarding assessment of the clinical impact of the protease uPA and its inhibitor PAI-1: regarding various types of cancer, quantitation of uPA and/or PAI-1 is performed by ELISA and not by IHC (Schmitt et al., 2008).

Strikingly, however, at the multivariate level, KLK7-ELISA (KLK7-E) values, in addition to the established prognostic clinical factors FIGO stage and residual tumor mass, were significantly associated with both OS and PFS. One possible explanation for the significance of KLK7 expression at the multivariate but not the univariate level are differences in the number of patients included in the analyses, as some are lost for the multivariate analysis due to missing values (in our cohort, 98 patients for univariate analysis, 91 for multivariate analysis; in the group of FIGO III/IV patients: 77 vs. 73). Apart of this, the prognostic effect of KLK7 could be modulated by the other variables included in the multivariate analysis. Ovarian cancer patients with high KLK7-E levels had a significantly 2-fold lower risk of death or relapse, as compared with patients who displayed low KLK7-E levels. This is in contrast to earlier findings, where high KLK7 levels were associated with more advanced disease, later stage, and shorter PFS as estimated by ELISA (Shan et al., 2006), quantitative PCR (Kyriakopoulou et al., 2003), and immunofluorescence analysis (Psyrri et al., 2008).

However, contradictory findings have already been reported for KLK7 in breast cancer: Holzscheiter et al. (2006) analyzed the expression of full-length KLK7 mRNA encoding for the active protease in breast cancer tissue and found high levels of KLK7 to be a favorable prognostic marker (for disease-free survival; as a

continuous marker as well as using an optimized cutoff). In contrast to this, Talieri et al. (2004) reported KLK7 mRNA levels (full-length and variant) to be significantly lower in breast cancer patients of low stage (I/II) and with positive progesterone receptors and that patients with KLK7-positive tumors had a shorter disease-free and OS compared with patients with KLK7-negative tumors, as assessed by semiquantitative RT-PCR. A possible explanation for this discrepancy might be the fact that in the cohort analyzed by Talieri et al. (2004), most of the patients were subjected to adjuvant therapy, whereas the patients in the study of Holzscheiter et al. (2006) might disclose more the natural course of the cancer disease by being least influenced by postoperative systemic treatments.

Our ovarian cancer cohort has been uniformly treated by adjuvant platinum-containing polychemotherapy, except in those cases where a polychemotherapy was not indicated (FIGO I) or not suitable due to the patient's general condition. In contrast to this, only 30% of the patients included in the study by Kyriakopoulou et al. (2003) were treated with carboplatin and only 16% with paclitaxel. Thus, a therapy effect analogous to the one hypothesized by Holzscheiter et al. (2006) is possible. Furthermore, in the patient cohort analyzed by Shan et al. (2006), more patients were of earlier stage (30% FIGO I/II, as compared with 20% FIGO I/II in our cohort), which translates into a decease rate of 50% in the cohort of Shan et al. (2006) compared with 67% in our patient group. Nevertheless, the pathophysiological function of most of the KLKs including KLK7 in ovarian cancer is not clear and needs further investigation using patient samples from multiple institutions to be able to explain contradictory findings in different patient cohorts.

In summary, we report on the evaluation of a commercially available antibody against KLK7 and its utility for IHC. The selected antibody AF2624 shows good concordance with previous results when staining normal and benign tissues. The two IHC scoring methods applied for the estimation of KLK7 tissue expression (observer-assisted and automated scoring by image analysis) prove to be comparable. Nevertheless, according to our results, scoring the KLK7 immunoreactivity in formalin-fixed paraffin-embedded ovarian cancer tissue is not a measure to predict the clinical outcome of the ovarian cancer patients. KLK7 levels as assessed by ELISA were, however, found to be significantly associated with ovarian cancer patient's outcome in the present study. Yet, contrary to earlier findings (Kyriakopoulou et al., 2003; Dorn et al., 2006, 2007; Shan et al., 2006), in our cohort, high KLK7 levels in tumor tissue extracts are associated with prolonged OS and PFS. This might be explained by differences in the patient cohorts (tumor stage) as well as therapy effects.

Materials and methods

Patients

A total of 98 patients afflicted with ovarian cancer FIGO stage I–IV (International Federation of Gynaecology and Obstetrics) between 1990 and 1999 were enrolled in a retrospective study conducted at the Department of Obstetrics and Gynaecology, Klinikum rechts der Isar, Technische Universität München, Munich, Germany. Standard surgical procedures were performed, including pelvic and paraaortic lymphadenectomy and – if indicated – partial resection of the small and large intestine, peritonectomies, and upper abdominal surgery. In younger patients (<35 years) with tumor FIGO stage I, less radical surgery was performed to preserve patient fertility. All of the patients gave written informed consent for the use of tissue material for scientific and teaching purposes. The study was approved by the local ethics committee. Following surgery, all patients received adjuvant treatment according to consensus recommendations at that time, including platinum-based chemotherapy. None of the patients received neoadjuvant therapy before surgery.

The median age of the patients at surgery was 57 years (range 20–85 years). The median follow-up time was 57 and 32.5 months for OS and PFS, respectively (range 1–244 months after primary tumor resection for both). Clinical and histomorphological factors documented at the time of surgery included FIGO stage, nuclear grade, presence of residual tumor mass (defined as largest intra-abdominal tumor diameter left at the end of the operation), and ascitic fluid volume (estimated preoperatively by vaginal ultrasound) (see Table 1). Of the 98 ovarian tumors, 74 were of serous papillary histotype, 4 endometrioid, 10 undifferentiated, 8 mucinous, and 2 clear cell types. During the follow-up period of the study, 52 (53.1%) of the patients relapsed and 66 (67.3%) died.

Preparation of tumor tissue extracts and KLK7-ELISA

Tissue extracts from primary tumors of ovarian cancer patients were prepared as described previously (Dorn et al., 2006). In brief, fresh tissue samples were collected during surgery, classified by a pathologist, and stored in liquid nitrogen. Deep-frozen specimens of 200–500 mg wet weight were pulverized and resuspended in Tris-buffered saline (TBS; 0.02 M Tris-HCl, 0.125 M sodium chloride, pH 8.5), containing 1% w/v Triton X-100 (all: Sigma, Munich, Germany). After extraction and ultracentrifugation, the supernatant was collected, aliquoted, and stored in liquid nitrogen until further analysis. KLK7 antigen concentrations were determined in tissue extracts using a noncommercial in-house KLK7-ELISA format. The detection limit of this KLK7-ELISA is 0.2 to 20 ng/ml (Paliouras and Diamandis, 2006). KLK7 antigen levels are expressed as nanograms per milligram of total protein. The protein content in tumor tissue extracts was determined using the BCA method (Pierce, Rockford, IL, USA).

Western blot analysis

Samples were subjected to 12% SDS-PAGE, and the separated proteins transferred onto PVDF membranes (Pall, Dreieich, Germany) using a semidry transfer device. Membranes were incubated for 60 min at room temperature in PBS-T (phosphate-buffered saline, pH 7.4, containing 0.1% Tween 20) plus 5% w/v skim milk powder (Fluka, Munich, Germany). Subsequently, the blots were incubated overnight with polyclonal goat antibody AF2624 (R&D Systems, Abingdon, UK) directed to purified NS0-derived recombinant human KLK7 (aa 23–252), diluted in the same buffer (final concentration: 0.2 μg/ml). After three washes, 10 min each, in PBS-T at room temperature, the binding of the antibodies to the target protein was visualized by incubation of the membrane with horseradish peroxidase-conjugated rabbit anti-goat IgG (#A5420; Sigma, Munich, Germany) diluted 10,000-fold in PBS-T plus 5% skim milk powder, followed by chemiluminescence reaction (Amersham Biosciences, Little Chalfont, UK). For apparent molecular mass determination, the Page-Ruler Plus prestained protein ladder was used as a standard (range 10–250 kDa; Fermentas Life Sciences, St. Leon-Rot, Germany).

Tissue preparation, and microarray construction

Tissue samples were obtained during surgery, inspected by a pathologist from the Institute of Pathology of the Technische Universität München, immediately fixated in neutral-buffered formalin, and then embedded in paraffin. The technique of tissue microarray production has been described in detail elsewhere (Seiz et al., 2012). In brief, morphologically, most representative areas of the tissue were marked on the original hematoxylin/eosin-stained tissue section. Using these sections for orientation, three cylindrical core tissue punches of 1 mm in diameter were taken from the selected area of each individual paraffin-embedded ovarian cancer tissue block (donor block) and precisely mounted into a paraffin block (recipient block) using a manual tissue microarray device (Beecham Instruments, Silver Springs, MD, USA). Sections of 2 μm thickness of the resulting tissue microarray blocks were transferred to surface-coated glass slides and deparaffinized. For the construction of the tissue microarray block, fixed tumor specimens from 98 ovarian cancer patients were available.

Immunohistochemistry

Tissue sections were dewaxed, rehydrated, and treated for antigen retrieval by pressure cooking (4 min, 120°C, 0.1 M citrate buffer, pH 6.0). After several washes with TBS (pH 7.6), endogenous peroxidase activity was quenched by incubating the tissue sections at room temperature for 5 min with endogenous enzyme block solution (#K5361; Dako, Hamburg, Germany). After washing with TBS, sections were incubated at 4°C overnight with the human KLK7-directed primary polyclonal antibody AF2624, diluted to a final concentration of 0.4 μg/ml in antibody diluent (Dako). After washing with TBS, mouse-anti-goat IgG (Jackson Immunoresearch, Baltimore, MD, USA) diluted in antibody diluent was applied to all tissue slides (30 min, room temperature). Following another washing step with TBS, sections were reacted with a dextran polymer conjugated with horseradish peroxidase and secondary antibodies with anti-rabbit and anti-mouse specificity (30 min, room temperature; EnVision; Dako). After an additional washing step, the chromogenic reaction was carried out (10 min, room temperature) using the peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB+; Dako), which forms a brown product at the site of enzyme reaction. Finally, counterstaining of the sections was performed with Meier's hematoxylin. As a negative control, the primary antibody was omitted and replaced by TBS or by irrelevant antibodies.

Quantification of KLK7 immunostaining

For the evaluation of observer-assisted analysis of KLK7 immunostaining intensity and tissue location, a quantitative score based on staining intensity and percentage of positive cells was created by inspecting at least two tissue cores from each case. KLK7 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 1–10, with grade 1: staining of ≤10% of cells; grade 10, with >90% of positive cells. Based on these scores, a final immunoreactivity score was created by multiplying the intensity score values with the cell positivity score values for each tumor cells (KLK7-RT) and stromal cells (KLK7-RN). In addition, an overall score was created by adding up the KLK7-RT and KLK7-RN score values (KLK7-RT+RN).

The Hamamatsu Nanozoomer HT system (Hamamatsu Photonics, Herrsching, Germany) was used to capture digital images. For automated image analysis of KLK7 immunostaining in ovarian cancer tumor tissue specimens, the software product OpTMA (SlidePath, Dublin, Ireland) was used. OpTMA facilitates high-throughput automated image analysis utilizing positive pixel algorithms. The results are presented as quantitative data. The software fully automates the dearraying process of TMAs and then automatically associates tissue spots with intensity data. From each tumor sample, at least two cores were evaluated, and the mean score values (KLK7-AV) of the readings were used for statistical calculations.

Statistics

The levels of significance 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 66th percentile) with clinical and histomorphological parameters was evaluated using the χ^2-test. For survival analyses, the OS and PFS of ovarian cancer patients were used as follow-up end points. The association of KLK7 antigen values and of clinicopathological factors with OS and PFS was analyzed using the Cox univariate and multivariate proportional hazards regression models and expressed as HR and its 95% confidence interval (95% CI). The multivariate Cox regression model was adjusted for known clinical prognostic factors in ovarian cancer patients: age, FIGO stage, nuclear grade, presence of residual tumor mass, and ascitic fluid volume. In subgroup analysis of FIGO III/IV patients, the clinical factors age, nuclear grade, presence of residual tumor mass, and ascitic fluid volume were used. Survival curves were generated by Kaplan-Meier analysis 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.

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