Expression and prognostic significance of kallikrein-related peptidase 8 protein levels in advanced ovarian cancer by using automated quantitative analysis

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Summary

Kallikrein-related peptidases, a subgroup of the serine protease enzyme family, are considered to be important prognostic biomarkers in cancer. In this study we sought to determine the prognostic value of kallikrein-related peptidase 8 (KLK8,hK8,KLK-8) in ovarian cancer using a novel method of compartmentalised in situ protein analysis. A tissue array composed of 150 advanced stage ovarian cancers, uniformly treated with surgical debulking followed by platinum-paclitaxel combination chemotherapy, was constructed. For the evaluation of kallikrein-related peptidase 8 protein expression, we used an immunofluorescence-based method of automated in situ quantitative protein analysis (AQUA). Mean follow-up time of the cohort was 34.35 months. One hundred twenty-six of 150 cases had sufficient tissue for AQUA analysis. There were significant correlations between tumour mask KLK8 protein expression levels and clinicopathological variables, including grade (p=0.0011), residual disease (p=0.0063) and clinical response to chemotherapy(p=0.0346). In univariate survival analysis there was a significant correlation between KLK8 tumour mask expression and five years progression-free survival, meanwhile it was not associated with five-year overall survival (p =0.0694). Specifically, low KLK8 expression correlated with better outcome (top vs. bottom quartile, p=0.0319). In multivariate survival analysis, adjusting for well-characterised prognostic variables, tumour KLK8 expression level retained its prognostic significance for progression-free survival (95%CI: 0.341–1.027, p=0.045). The possibility that KLK8 may be a suitable candidate as a diagnostic and prognostic marker warrants further investigation.

Keywords

Kallikrein-related peptidase 8, AQUA, advanced ovarian cancer, prognosis

Thromb Haemost 2009; 101: 541–546

Introduction

Ovarian cancer is the most fatal gynecologic cancer. The poor outcome may be related to the fact that approximately 75% of women with ovarian cancer are diagnosed with advanced disease (1).

The current management of patients with advanced disease (stages III and IV) involves optimal surgical debulking followed by chemotherapy. Although 75% of women initially respond to chemotherapy, emergence of resistance hinders successful treatment outcomes. Clinicopathologic parameters do not accurately classify patients in terms of prognosis (2). Several investigators are exploring potential biomarkers that will identify patients in need of more aggressive or experimental treatment.

Kallikrein-related peptidases are a subgroup of the serine protease enzyme family which containes 15 members (3). The kallikrein-related peptidases gene locus is localised on chromosome 19q13.4 (4). Kallikrein-related peptidases are expressed in several human tissues, such as breast, ovary, prostate and testis (5–7). It seems that multiple members of the kallikrein-related

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Received: January 26, 2008
Accepted after major revision: September 2, 2008

Prepublished online: January 15, 2009
doi:10.1160/TH08-01-0052

peptidases gene family are deregulated in ovarian cancer and are involved in several cancer related processes (8–11). The kallikrein-related peptidases may be able to activate each other or other molecules like cytokines and growth factors in a cascade of events leading to tumorigenesis (12). Their possible biologic mechanisms include their ability to degrade extracellular matrix, thus facilitating invasion and metastasis (13). It is possible that some of these proteases have applications as disease biomarkers. The most well-known member of the family is the KLK3/PSA, which is the most widely used biomarker for prostate cancer (14).

Here, we sought to determine whether kallikrein-related peptidase 8 (KLK8) protein level is associated with clinical outcome in a large cohort of uniformly treated patients with epithelial ovarian cancer using a novel in-situ quantitative method of protein expression (15).

Materials and methods

Patient population

Inclusion criteria were primary epithelial ovarian cancer patients (FIGO stages III and IV) who had undergone surgical resection in the Department of Gynecology of Alexandra University Hospital in Athens, Greece, between 1996 and 2003, and who were treated postoperatively with carboplatin and paclitaxel chemotherapy. In all cases an effort was made for optimal surgical cytoreduction and adequate staging, which included at least total abdominal hysterectomy (TAH) with bilateral salpingoophorectomy (BSO), inspection and palpation of all peritoneal surfaces and retroperitoneal area, biopsies of suspect lesions for metastases, infracolic omentectomy and peritoneal washings. Grading was performed by evaluation of tumor architecture, the amount of solid neoplastic areas, nucleus-cytoplasm ratio, and nuclear pleomorphism. The tumours were subdivided into three groups: well-differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3), according to these criteria. Informed consent was obtained from all of the patients .

Chemotherapy was instituted two to three weeks after surgery. All of the patients received platinum-paclitaxel chemotherapy. Gynecological examination, CA-125 assay, and radiological investigations, if necessary, were performed monthly for the clinical assessment of response, which was recorded according to WHO criteria(16). Follow-up examinations were performed every month.

Tissue microarray construction

A tissue microarray consisting of tumours from each patient in the cohort was constructed at the Yale University Tissue Microarray Facility. Following institutional review board approval the tissue microarray was constructed as previously described (17), including 150 cases. Tissue cores 0.6 mm in size were obtained from paraffin-embedded formalin-fixed tissue blocks from the Alexandra University Hospital Department of Pathology archives. Hematoxylin and Eosin stained slides from all blocks were first reviewed by a pathologist to select representative areas of invasive tumour to be cored. The cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD, USA). All of the tumours were represented with two-fold redundancy. Previous studies have demonstrated that the use of tissue microarrays containing one to two histospots provides a sufficiently representative sample for analysis by immunohistochemistry. The addition of a duplicate histospot, while not necessary, does provide marginally improved reliability (18). The tissue microarray was then cut to yield 5-µm sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ, USA) with UV cross-linking.

Quantitative immunohistochemistry

Tissue microarray slides were deparaffinised and stained as previously described (16). In brief, slides were deparrafinised with xylene followed by ethanol. Following rehydration in dH_20, antigen retrieval was accomplished by pressure cooking in 0.1 M citrate buffer (ph 6.0). Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 minutes. Non-specific antibody binding was then blocked with 0.3% bovine serum albumin (BSA) for 30 minutes at room temperature. Primary rabbit polyclonal antibody to kallikrein-related peptidase 8 (KLK8) was used at 1:40 dilution in 0.3% BSA/TBS. This antibody has been validated in previous studies using immunohistochemistry (IHC) , Western blotting and ELISA analysis of normal and neoplastic tissue (19–21).

Following these steps, slides were incubated with primary antibody at 4°C overnight. Subsequently, slides were incubated with goat anti-rabbit secondary antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp., Carpinteria, CA, USA) for one hour at room temperature. The tumour cells were identified by use of anticytokeratin antibody cocktail (mouse anti-pancytokeratin antibody z0622; DAKO Corp.) with subsequent goat anti-mouse antibody conjugated to Alexa546 fluourophore (A11035, Molecular Probes, Eugene, OR, USA). Target (KLK8) molecules were visualised with a fluorescent chromogen (Cy-5-tyramide; Perkin Elmer Corp., Wellesley, MA, USA). Cy-5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue autofluorescence. The array was 4', 6-diamidino-2-phenylindole (DAPI) counterstained to visualise nuclear compartment. Slides were mounted with a polyvinyl alcohol-containing aqueous mounting media with antifade reagent (n-propyl gallate, Acros Organics, Vernon Hills, IL, USA) (Fig. 1).

Automated image acquisition and analysis

Automated image acquisition and analysis using AQUA has previously been described (22). In brief, monochromatic, high-resolution (1024 x 1024 pixel; 0.5-µm) images were obtained of each histospot. We distinguished areas of tumour from stromal elements by creating a mask from the cytokeratin signal. DAPI signal was used to identify nuclei, and the cytokeratin signal was used to define cytoplasm. Overlapping pixels (to a 99% confidence interval) were excluded from both compartments. The KLK8 signal (AQUA score) was scored on a normalised scale of 1–255 expressed as pixel intensity divided by the target area. AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumour.

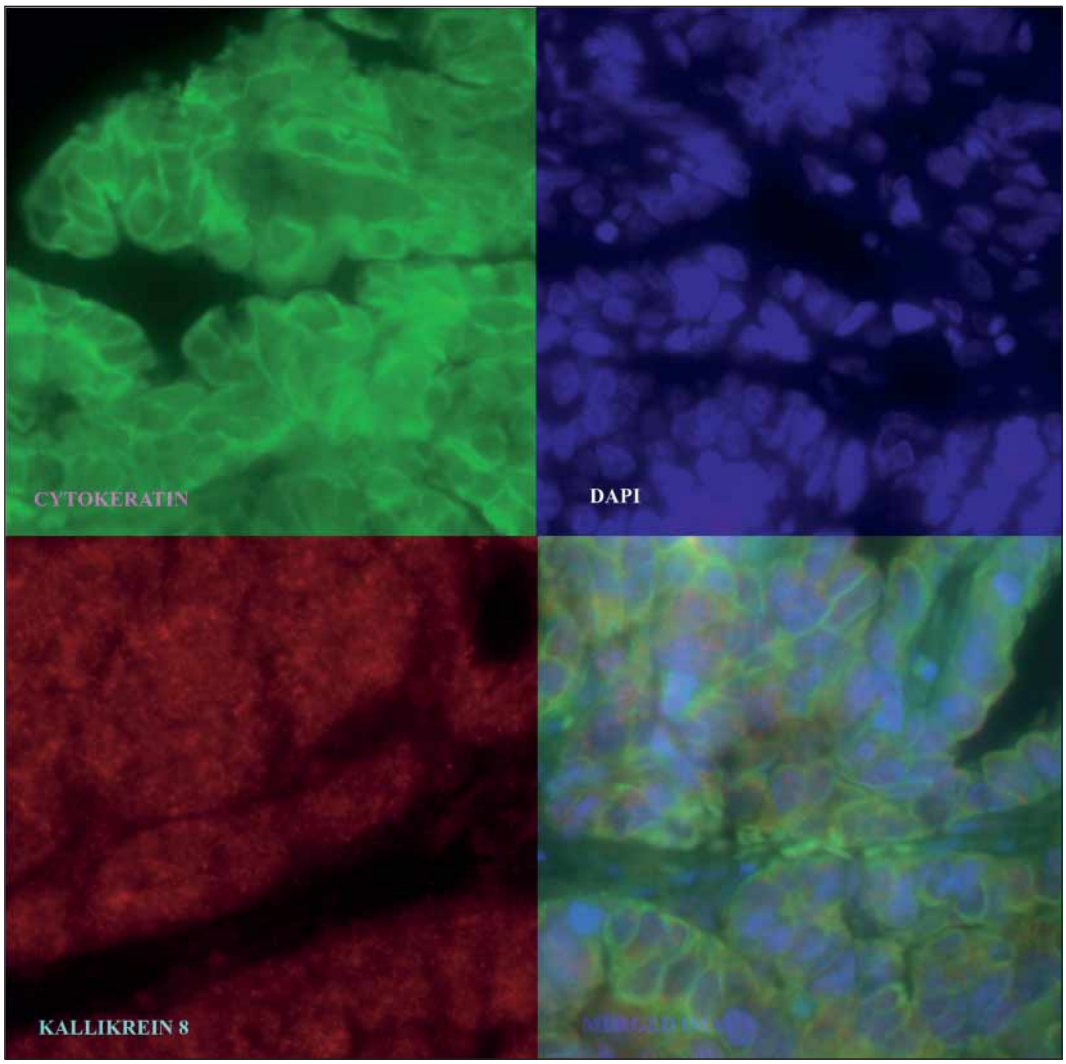

Figure 1: Protein expression of kallikrein-related peptidase 8 was determined using AQUA analysis based on immunofluorence. Digital images of each tumour spot were captured using Cy3 anticytokeratin antibody to generate a tumour mask. 4,6-Diamidino-2-phelynindole (DAPI) was used to visualise nuclei ,and Cy5 was used to visualise KLK8. The individual images are related to adjacent areas of the original tissue section and only the bottom right image is a three-colour merged image . The tumour tissue is an epithelial adenocarcinoma of poor differentiation and the patient has been classified as FIGO stage III.

Statistical analysis

Histospots containing <10% tumour as assessed by mask area (automated), were excluded from further analysis. AQUA scores represent expression of a target protein on a continuous scale from 1–255. Progression-free survival and overall survival were subsequently assessed as continuous variables for determining statistical significance . All survival analysis was performed at five-year cutoffs. Confidence intervals were assessed by multivariate Cox proportional hazards model. Overall survival was defined as the time from first day of chemotherapy to death from any cause. Progression-free survival was defined as the time from first day of chemotherapy to the first of either death from any cause or disease progression (assessed by CA-125 increase and/or imaging studies). Performance status was dichotomised into "0" versus all others, histologic type into serous versus all others and clinical response into complete response and partial response versus all others. Although several cutoff values of residual volume tumour have been proposed, it has been reported that progressive gradations of residual disease can affect ovarian cancer prognosis. Our patient population was divided into two groups according to the extent of residual disease at first surgery; less than or equal to 2 cm and greater than 2 cm. Comparisons of KLK8 expression with age, histology, clinical response , residual disease , FIGO stage, performance status and grade were made by ANOVA test. All calculations and analyses were performed with Statview version 5.0.1. (S.A.S. Institute, Cary, NC, USA).

Results

Clinical and pathological variable analysis

One hundred fifty patients were included in the study. Mean follow-up time (range) for the entire cohort was 34.35 months (range 1 to 91.7). Demographic and clinicopathological variables for the cohort are summarised in Table 1.

Quantitative immunohistochemistry for KLK8 protein expression

Of the 150 patients included in this study, 126 (84%) had sufficient tissue for analysis of KLK8 protein expression by AQUA. Tissues that were deemed insufficient had less than 10% tumour mask within the histospot, as represented on the tissue microarrays. Normalised AQUA scores were represented on a 1–255

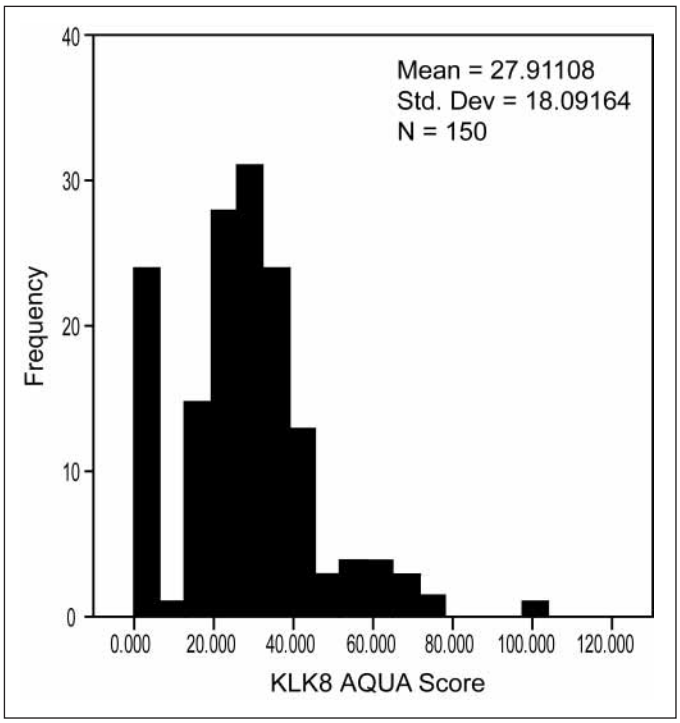

Figure 2: AQUA analysis showed a left skewed distribution for kallikrein-related peptidase 8 tumour expression.

Table 1: Demographic, clinical and pathologic data (KLK8).

Variable	n (with AQUA data)	Mean KLK8 AQUA score ± Std Dev	p
Age			
≤60	65	32.1 ± 14.4	0.40
>60	61	34.3 ± 14.7	
Differentiation			
Poor	75	29.8 ± 13.08	0.0011
Moderate	39	40.3 ± 16.4	
Well	12	32.4 ± 9.05	
Initial histology			
Serous	89	34.5 ± 15.05	0.13
All others	37	30.1 ± 12.9	
FIGO stage			0.75
II	7	32.07 ± 17.3	
III	92	33.8 ± 14.9	
IV	27	31.4 ± 12.7	
Residual disease (cm)	35	27.5 ± 11.3	0.0063
≤2	91	35.4 ± 15.1	
>2			
Clinical response to chemotherapy	72	35.5 ± 16.6	0.035
CR+PR	54	30.07 ± 10.5	
All others			
Performance status			
No impairment	87	33.7 ± 15.8	0.53
All others	39	32 ± 11.2	

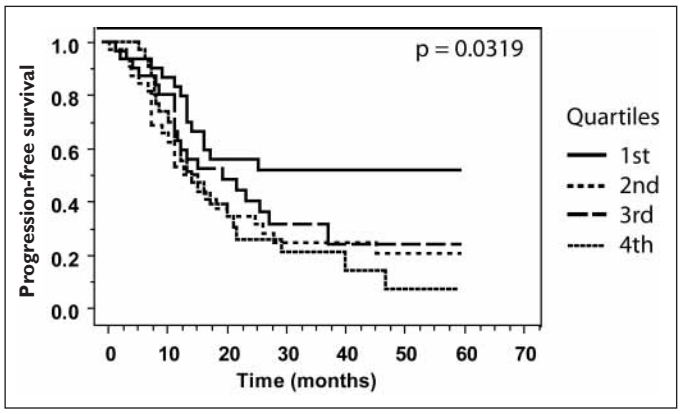

Figure 3: Dividing the cohort into quartiles based on KLK8, low KLK8 expression correlates with better outcome (p=0.0319, top vs. bottom quartile).

scale. KLK8 expression followed a skewed distribution as expected for a cancer tissue biomarker (Fig. 2).

Association of KLK8 expression and clinicopathological variables

There was significant association between tumour mask KLK8 protein expression and some clinicopathological variables, including differentiation, residual disease, clinical response to chemotherapy. To the contrary , there was no association between KLK8 expression and other clinicopathologic factors, such as age, histological type, FIGO stage and performance status (Table 1).

Univariate survival analysis

Tumour mask AQUA expression levels of KLK8 were examined for association with five-year overall survival (OS) and progression-free survival (PFS) as continuous variables. The KLK8 tumour mask expression was not associated with five-year overall survival (p=0.0694). There was a significant correlation between KLK8 tumour mask expression and five-year progression-free survival. To graphically represent the association of KLK8 with outcome, we divided the cohort into quartiles based on KLK8 expression. This graph demonstrates that low KLK8 expression correlates with better outcome (p=0.0319, top vs. bottom quartile) (Fig. 3).

Multivariate progression-free survival analysis

Using the Cox proportional hazards model, we performed multivariate analysis to assess the predictive value of tumour KLK8 expression. Tumour KLK8 expression by AQUA was analysed for five-year progression-free survival. We also included the following known prognostic variables in the regression model: age, FIGO stage, differentiation grade, residual disease, performance status, response to chemotherapy and histological type. Residual disease (95%CI: 0.165–0.752, p=0.007) along with FIGO stage (IV/all others) (95%CI: 1.364–4.037, p=0.002) and tumour KLK8 expression level (95%CI: 0.341–1.027, p=0.045) were significant predictor variables of progression-free survival. To the contrary, age, histological type, differentiation grade, per-

formance status and response to chemotherapy were not significant predictor variables of progression-free survival. Results of multivariate survival analyses are summarised in Table 2.

Table 2: Multivariate five-year progression-free survival analysis by Cox regression (KLK8).

Variable	Hazard ratio (95% confidence interval)	P
Progression-free survival		
Age	1.194 (0.738–1.933)	0.47
Histology (serous/all others)	1.232 (0.711–2.136)	0.46
FIGO stage(IV/all others)	2.347 (1.364–4.037)	0.002
Grade (poor/all others)	0.751 (0.450–1.254)	0.27
Clinical response to chemotherapy (CR + PR/all others)	0.860 (0.497–1.487)	0.59
Residual disease (≤2/>2cm)	0.352 (0.165–0.752)	0.007
KLK8 tumour mask	1.013 (0.341–1.027)	0.045

Discussion

Ovarian cancer remains the most lethal disease among all gynecological malignancies. Traditional clinical and pathological parameters do not accurately classify patients in relation to prognosis. The discovery of new biomarkers that are suitable for early disease diagnosis and prognosis may ultimately lead to improved patient management and outcomes. For that purpose, we sought to determine the prognostic value of quantitatively assessed KLK8 protein expression in ovarian cancer.

Experiments conducted in mice found that KLK8 (neuropsin) is highly expressed in the brain and plays an important role in embryonic cell differentiation and in neural plasticity processes (23, 24). Experiments in human specimens found that KLK8 is expressed in the brain, skin and ovaries (14, 25, 26) . The high expression in human brain tissues may indicate that KLK8 plays a role in development, plasticity and cancer progression (27, 28).

Previous studies have found that KLK8 exists in higher levels in ovarian cancer tissues compared to benign and normal ovarian tissue. In their studies, Borgono et al. (21) and Shigemasa et al. (29) found that human kallikrein 8 protein is a favourable prognostic marker in ovarian cancer. Magklara et al. (30) also found that higher KLK8 levels in the tumour was related to more favourable features, such as lower grade disease, smaller residual tumour and better progression- free and overall survival.

Our study is the first to examine KLK8 protein expression in ovarian carcinoma using a novel quantitative in-situ method of protein analysis (AQUA) in advanced-stage ovarian cancer. Our goal was to quantitatively assess expression of KLK8 on a cohort of ovarian cancer specimens in an objective, automated fashion and to evaluate the association between KLK8 expression and clinical outcome . This method allows measurements of protein expression within subcellular compartments that results in a number directly proportional to the number of molecules expressed per unit area. Thus, we avoid biases introduced from the arbitrary cutoff points used in conventional immunohistochemistry studies while at the same time preserving spatial and morphological information that techniques such as Western blotting lose.

The pathophysiological role of KLK8 has not been clearly defined. Sher et al (31) found that by using in situ degradation and cell adhesion assays in invasive lung cancer cell lines, protein products of *KLK8* splice variants degrade fibronectin and modify the extracellular microenvironment. The degradation of fibronectin by KLK8 suppresses integrin signaling and reduces cancer cell motility. The authors also showed that KLK8 suppresses tumour growth and invasion *in vitro* and *in vivo*. High KLK8 levels were associated with longer time to postoperative recurrence in early-stage non-small cell lung cancer patients. On the contrary, Rajapakse et al. (32) reported that KLK8 degrades extracellular matrix proteins as fibronectin and collagen type IV, via its own activity and through the activity of the plasmin by converting single-chain t PA to two chain t PA. The degradation of these proteins may be a crucial step for cancer cells to invade and metastasise, hence it could be speculated that KLK8 may be involved in the metastatic processes of ovarian cancer.

We demonstrated that KLK8 expression is an adverse predictor of progression-free survival time in our cohort of patients with advanced ovarian cancer as low KLK8 expression correlated with better outcome. Although our findings seem to be contradictory with the results of previous studies (25, 29, 30), it should be highlighted that our patients had advanced-stage disease. In the aforementioned studies, when the patients were divided into subgroups, KLK8 expression was a favourable prognostic marker in patients with early stages while its expression was lower or absent in advanced stages and higher grade disease.

In conclusion, Kallikrein-related peptidase 8 might play an adverse role in the prognosis in patients with advanced ovarian cancer. The possibility that KLK8 may be a suitable candidate as a valuable prognostic marker merits further investigation.

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