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Human tissue kallikrein 7, a novel biomarker for advanced ovarian carcinoma using a novel *in situ* quantitative method of protein expression

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Background: Kallikreins, a subgroup of the serine protease enzyme family, are considered important prognostic biomarkers in cancer. Here, we sought to determine the prognostic value of kallikrein 7 (hk7) in ovarian cancer using a novel method of compartmentalized *in situ* protein analysis.
Patients and methods: A tissue array composed of 150 advanced-stage ovarian cancers, uniformly treated with surgical debulking followed by platinum–paclitaxel (Taxol) combination chemotherapy, was constructed. For evaluation of kallikrein 7 protein expression, we used an immunofluorescence-based method of automated *in situ* quantitative measurement of protein analysis (AQUA).
Results: Mean follow-up time of the cohort was 34.35 months. One hundred and twenty eight of 150 cases had sufficient tissue for AQUA. In univariate survival analysis, low tumor hk7 expression was associated with better outcome for overall survival (OS) and disease-free survival in 3 years (P values 0.032 and 0.037, respectively). In multivariate survival analysis, adjusting for well-characterized prognostic variables, low tumor hk7 expression level was the most significant predictor variable for OS (95% confidence interval 0.125–0.729, $P = 0.007$).
Conclusions: High tumor hk7 protein expression is associated with inferior patient outcome in ovarian cancer. hk7 may represent a promising prognostic factor in ovarian cancer.
Key words: advanced ovarian cancer, AQUA, biomarker, hk7, prognosis

introduction

Ovarian cancer kills more women than all the other gynecological malignancies combined. If diagnosed at an early stage (I or II), patients with ovarian cancer have a 5-year survival rate of 85% [1]. However, over two-thirds of patients present with stage III or IV disease and have a 5-year survival of only 20% [1].

The current management of patients with advanced disease (stages III and IV) involves optimal surgical debulking followed by chemotherapy. The current standard chemotherapeutic approach for ovarian cancer patients includes taxane- and platinum-based regimens. Traditional clinicopathological factors have limited prognostic value and considerable interest lies in identifying molecular prognostic indicators in order to guide treatment decisions.

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CA 125 is the only well-validated ovarian cancer tumor marker. It has clinical value for disease monitoring and assessment of response to treatment [2]. However, its diagnostic or prognostic value is still under investigation. Several molecules have emerged as potential biomarkers for ovarian cancer. The family of human kallikreins includes members with potential prognostic value in ovarian cancer.

Kallikreins are a subgroup of the serine protease enzyme family which contains 15 members [3]. The human kallikrein gene locus is localized on chromosome 19q13.4 [4].The most well-known member of the family is the hk3/prostrate-specific antigen, which is the most widely used biomarker for prostate cancer. Kallikreins are expressed in several human tissues, mainly the hormone-producing or hormone-dependent ones such as breast, ovary, prostate and testis. In cancer cell lines, all kallikreins are under sex steroid hormone regulation [5]. It seems that multiple members of the human kallikrein gene family are disregulated in ovarian cancer [6]. The involvement of serine proteases in cascade pathways important in cancer progression such as coagulation, fibrinolysis and apoptosis is well documented and there are hypothetical

models implicating multiple kallikrein overexpression during progression of ovarian cancer into a more aggressive phenotype [7]. A possible biologic mechanism is their ability to degrade extracellular matrix, thus facilitating invasion and metastasis [8]. Therefore, some of these proteases may have applications as disease biomarkers and therapeutic targets.

Here, we sought to determine whether human tissue kallikrein 7 (hk7) 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.

patients and methods

patient population

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

Chemotherapy was instituted 2–3 weeks after surgery. All patients received platinum–paclitaxel chemotherapy. Gynecological examination, CA 125 assay and radiological investigations, if necessary, were carried out monthly for the clinical assessment of response. CT scans and X-rays were carried out every three cycles for the clinical assessment of response, which was recorded according to World Health Organization [9] criteria. Informed consent was obtained from all the patients.

tissue microarray construction

A tissue microarray consisting of tumors 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 [10], 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 tumor to be cored. The cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors 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. Addition of a duplicate histospot, while not necessary, does provide marginally improved reliability [10]. 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) with UV cross-linking.

quantitative immunohistochemistry

Tissue microarray slides were deparaffinized and stained as previously described [11]. In brief, slides were deparaffinized with xylene followed by ethanol. Following rehydration in dH_20, antigen retrieval was accomplished by pressure cooking in 0.1 mol/l citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 min. Nonspecific antibody binding was then blocked with 0.3% bovine serum albumin (BSA) for 30 min at room temperature. Primary rabbit polyclonal antibody to human kallikrein 7 (hk7) was used at 1 : 100 dilution in 0.3% BSA/tris-buffered saline. This antibody has been validated in previous studies using immunohistochemistry, RT–PCR and northern blot analysis of normal and neoplastic tissue [12, 13]. 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) for 1 h at room temperature. Tumor cells were identified by use of anti-cytokeratin antibody cocktail (mouse anti-pancytokeratin antibody z0622; DAKO Corp.) with subsequent goat anti-mouse antibody conjugated to Alexa546 fluorophore (A11035, Molecular Probes, Eugene, OR). We added 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Target (kallikrein 7) molecules were visualized with a fluorescent chromogen (Cy-5-tyramide; Perkin Elmer Corp., Wellesley, MA). Cy-5 binds to the hk7 detected by DAKO envision. Cy-5 (red) was used because its emission peak is well outside the green–orange spectrum of tissue autofluorescence. Slides were mounted with a polyvinyl alcohol-containing aqueous mounting media with antifade reagent (*n*-propyl gallate, Acros Organics, Vernon Hills, IL) (Figure 1).

automated image acquisition and analysis

Automated image acquisition and analysis using automated *in situ* quantitative measurement of protein analysis (AQUA) has been described previously [14]. In brief, monochromatic, high-resolution (1024 × 1024 pixel; 0.5-μm) images were obtained of each histospot. We distinguished areas of tumor 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 (CI)] were excluded from both compartments. The hk7 signal (AQUA score) was scored on a normalized 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 tumor.

statistical analysis

Histospots containing <10% tumor 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 to 255. It is often useful to categorize continuous variable in order to stratify patients into high versus low categories. Several methods exist to determine a cut point, including biological determination, splitting at the median and determination of the cut point which maximizes effect difference between groups. If the latter method (the so-called 'optimal *P* value' approach) is used, a dramatic inflation of type I error rates can result. A recently developed program, X-tile, allows determination of an optimal cut point while correcting for the use of minimum *P* value statistics [15]. As the AQUA technology is new, there are no established cut points available for quantitative hk7 expression. Therefore, for categorization of hk7 expression levels, the X-tile program was used to generate an optimal cut point. This approach has been successfully applied to AQUA data analysis. Two methods of statistical correction for the use of minimal *P* value approach were utilized. First, the X-tile program output includes calculation of a Monte Carlo *P* value for the optimal cut point generated. Cut points that yield Monte Carlo *P* values <0.05 are considered robust and unlikely to represent type I error. Secondly, the Miller–Siegmund (MS) minimal *P* value correction referenced by Altman et al. [16] was utilized.

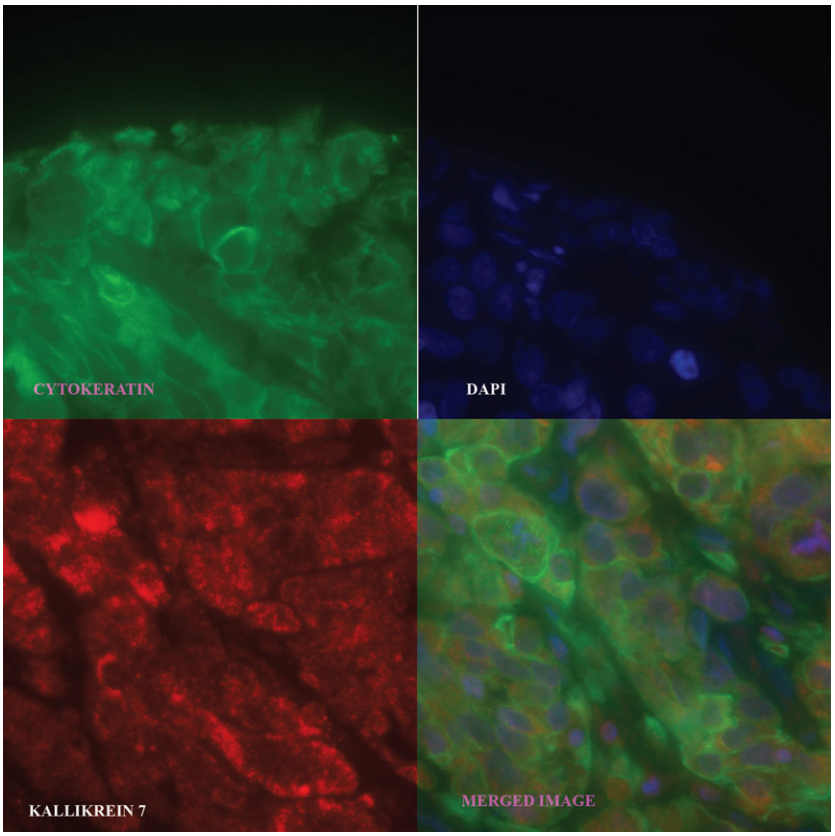

Figure 1. Protein expression of human kallikrein 7 was determined using automated *in situ* quantitative measurement of protein analysis on the basis of immunofluorescence. Digital images of each tumor spot were captured using Cy-3 anti-cytokeratin antibody to generate a tumor mask. 4′,6-Diamidino-2-phelynindole (DAPI) was used to visualize nuclei and Cy-5 was used to visualize hk7. A three-color merged image for each tumor is also shown.

This approach is accepted in the statistical literature, but relatively unknown in the medical/biological research community. Briefly, when making multiple comparisons to find the minimum *P* value using the log-rank test, the false high rate (i.e. the percentage of times a marker that has no true prognostic value will be found to have a $P < 0.05$) can approach 40%. Altman's statistical adjustment generates a minimum *P* value corrected to yield a true false-high rate of 5%. The corrected *P* value (P_{corr}) is calculated as follows: P_{corr} = phi(zeta)[zeta − (1/zeta)]log[e][(1-epsilon)^2/epsilon^2] + 4phi(zeta)/zeta (Where phi indicates the probability density function. P_{min} is the minimum p-value generated by evaluating multiple cut points. Zeta is the $(1-P_{min}/2)$-quantile of the standard normal distribution. Epsilon denotes the proportion of values excluded from consideration as an optimal cut point).* Our calculations were carried out using an epsilon of 0.10. Progression-free survival (PFS) and overall survival (OS) were subsequently assessed by Kaplan–Meier analysis with log rank for determining statistical significance, and only *P*-corrected was reported. This approach has been successfully applied to AQUA data analysis. All survival analysis was carried out at 3-year cut-offs. CIs were assessed by univariate and multivariate Cox proportional hazards model. OS was defined as time from first day of chemotherapy to death from any cause. PFS was defined as 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 dichotomized into '0' versus all others, histologic type into serous versus all others and clinical response into complete response (CR) versus all others. Although several cut-off values of residual volume tumor have been proposed, it has been reported that gradual 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, ≤2 and >2 cm. Comparisons of hk7 expression with age, FIGO stage, grade, performance status, histology, clinical response and residual disease were made by Mantel–Haenszel chi-square test. All calculations and analyses were carried out with Statview software (version 5.0.1.; SAS Institute Inc., Cary, NC).

results

clinical and pathological variable analysis

One hundred and fifty patients were included in the study. Mean follow-up time (range) for the entire cohort was 34.35 months (1–91.7). The median follow-up time was 29 months for OS and 16 months for disease-free survival (DFS). There were 117 (77.5%) FIGO stage III and 33 (22.5%) stage IV. One hundred and three (61%) patients had tumors of serous histology. Initial histologic grade was 14 well differentiated (9%), 49 moderately differentiated (33%) and 87 poorly differentiated (58%). Following initial surgical debulking, residual disease by size was distributed as follows: 38 (25%) with ≤2 cm and 112 (75%) with >2 cm. For clinical response to initial therapy, CR was recorded in 56 (37.3%) patients and partial response or stable disease/no response in 94 (62.7%) patients. Demographic and clinicopathological variables for the cohort are summarized in Table 1.

quantitative immunohistochemistry for hk7 protein expression and generation of optimal cut point by X-tile analysis

Of the 150 patients included in this study, 128 (85.3%) had sufficient tissue for analysis of hk7 protein expression by AQUA. Tissues deemed insufficient had <10% tumor mask within the histospot, as represented on the tissue microarrays. Normalized AQUA scores were represented on a 1–255 scale. Hk7 expression followed a skewed distribution as expected for a cancer tissue biomarker (Figure 2). When analyzed as a continuous variable, higher levels of Hk7 were associated with worse 3-year DFS and OS in a Cox univariate analysis ($P = 0.012$ and $P = 0.022$, respectively). We then used the X-tile program to identify subsets of tumors which exhibited markedly worse outcome. X-tile identified an optimal cut point for tumor hk7 levels at 54.2 AQUA units, with an MS minimal P value correction of 0.032 as determined by X-tile. MS P values <0.05 indicate robust and valid cut-point selection. Patients with tumor hk7 protein expression ≤54.2 were classified as hk7 low expressers ($n = 118$), and patients with tumor hk7 protein expression >54.2 were classified as hk7 high expressers ($n = 10$). This cut point corresponds with the tail of high expressers in the skewed hk7 distribution shown in Figure 2.

Table 1. Demographic, clinical and pathologic data

Variable	*n*	*n* (with AQUA data)	Hk7 low expressors	hk7 high expressors	*P*
Age (years)					
≤60	72	66	62	4	0.446
>60	78	62	56	6	
Differentiation					
Poor	86	75	70	5	0.307
Moderate	49	41	36	5	
Well	14	12	12	0	
Initial histology					
Serous	103	91	82	9	0.169
All others	47	37	36	1	
FIGO stage					
III	117	101	93	8	0.717
IV	33	27	25	2	
Residual disease (cm)					
≤2	38	35	34	1	0.2
>2	112	93	84	7	
Clinical response to chemotherapy					
Complete response	56	48	44	4	0.864
All others	94	80	74	6	
Performance status					
No impairment	103	88	83	5	0.182
All others	47	40	35	5	

AQUA, automated *in situ* quantitative measurement of protein analysis; FIGO, International Federation of Gynecology and Obstetrics.

association of hk7 expression and clinicopathological variables

There was no association between hk7 protein expression and clinicopathological variables including age, differentiation, histological type, histological grade, FIGO stage, residual disease, clinical response to chemotherapy and performance status (Table 1).

univariate survival analysis

Tumor hk7 levels were examined for association with 3-year OS and PFS using Kaplan–Meier survival analysis with log rank for determining significance. There were 128 patients, and 46 events for OS and 72 events for DFS were observed. As use of an optimized cut point can result in increased type I error, the MS correction method was applied to all Kaplan–Meier analyses. Kaplan–Meier survival curves generated for tumor hk7 protein expression, high versus low expression, are given in Figures 3 and 4. Low tumor hk7 protein expression was associated with improved outcome for OS and DFS, respectively (3-year OS: $P_{corr} = 0.032$; 3-year DFS: $P_{corr} = 0.037$) (Table 2).

multivariable survival analysis

Using the Cox proportional hazards model, we carried out multivariable analysis to assess the predictive value of hk7 expression. hk7 expression by AQUA was analyzed for OS and PFS. We also included the following known prognostic variables in the regression model: age, FIGO stage, differentiation grade, residual disease, response to chemotherapy and initial histology. Low hk7 level (95% CI 0.125–0.729, $P = 0.007$) along with performance status (95% CI 0.178–0.862, $P = 0.019$) were significant predictor variables of OS. FIGO stage (95% CI 1.665–4.859, $P = 0.0002$) along with residual disease (95% CI 0.083–0.456, $P = 0.0001$) were

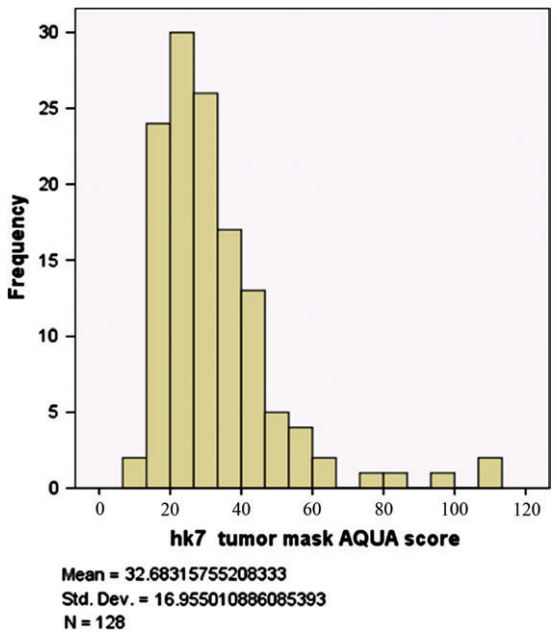

Figure 2. Hk7 expression followed a skewed distribution.

significant predictor variables of DFS. Results of multivariable survival analysis are summarized in Tables 3 and 4.

discussion

Ovarian cancer remains the most lethal disease among all gynecological malignancies. Traditional clinical–pathological parameters do not accurately classify patients in relation to prognosis. Hk7 is secreted like the other kallikreins, has a signal peptide and could potentially be used as a tumor marker similar to CA 125. In the present study, we sought to determine the prognostic value of quantitatively assessed hk7 protein expression in ovarian cancer.

The human kallikrein gene 7 (also known as the human stratum corneum chymotryptic enzyme) has been reported to be expressed at high levels in ovarian cancer [13, 17]. Hk7 is primarily expressed in the skin and specifically the stratum corneum [18]. The mechanism by which hk7 is expressed in ovarian cancer is unclear. In the skin, hk7 promotes epithelial cell desquamation and shedding via degradation of intercellular component of the matrix [19]. It is conceivable that hk7

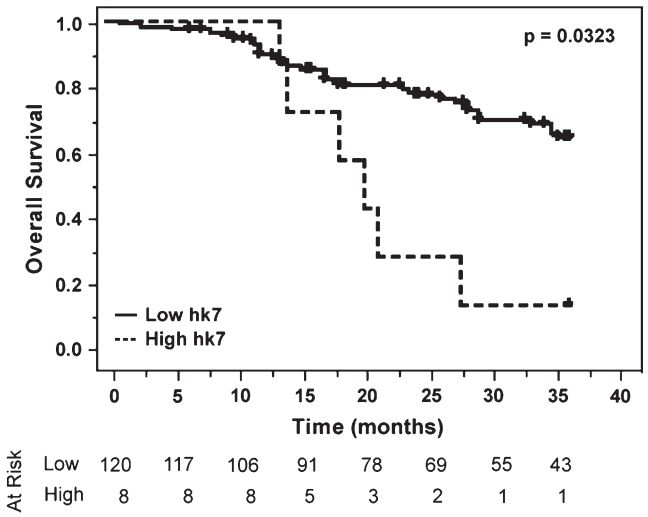

Figure 3. Kaplan–Meier survival analysis for overall survival (OS) by human kallikrein 7 expression levels as determined by automated *in situ* quantitative measurement of protein analysis. Patients with low hk7 expression had improved 3-year OS (P = Miller–Siegmund P values). The relative risk (RR) with 95% confidence interval for the hk7 high expressors = 3.57 (1.48–8.62). OS (number of patients): high = 8, low = 120. OS events: high = 6, low = 40. Censor times are denoted with ticks.

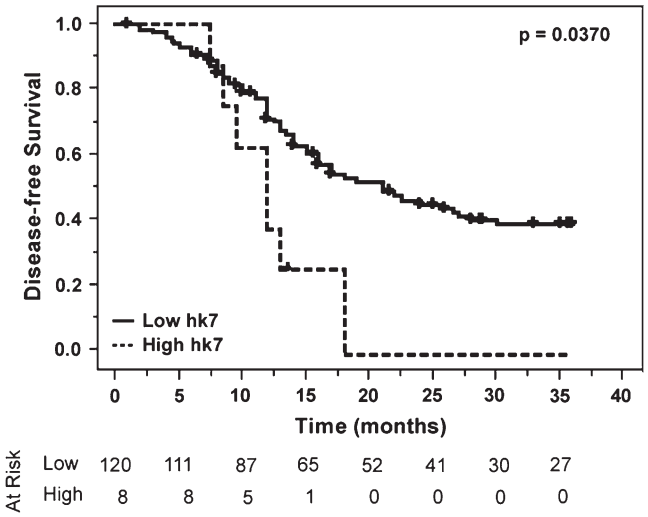

Figure 4. Kaplan–Meier survival analysis for disease-free survival (DFS) by human kallikrein 7 expression levels as determined by automated *in situ* quantitative measurement of protein analysis. Patients with low hk7 expression had improved 3-year DFS (P = Miller–Siegmund P values). The relative risk (RR) with 95% confidence interval for the hk7 high expressors = 2.74 (1.23–6.10). DFS (number of patients): high = 8, low = 120. DFS events: high = 7, low = 65. Censor times are denoted with ticks.

Table 2. Univariate 3-year survival analysis (Kaplan–Meier log rank)

Hk7 expression class	Median survival (months)	% Cumulative survival (95% confidence interval)	P_{corr}
Overall survival			
hk7 tumor mask low	19	65.5 (55.7–75.4)	0.032
hk7 tumor mask high	17	13.5 (0–37.9)	
Disease-free survival			
hk7 tumor mask low	16	39.1 (29.6–48.6)	0.037
hk7 tumor mask high	12	0	

P_{corr}, P values using the Miller–Siegmund method.

Table 3. Multivariate 3-year overall survival analysis by Cox regression

Variable	Hazard ratio (95% confidence interval)	P
Histology		
Serous	0.838 (0.403–1.74)	0.635
All others	1	
FIGO stage		
IV	1.927 (0.926–4.012)	0.079
III	1	
Grade		
Poor	0.829 (0.427–1.608)	0.578
All others	1	
Clinical response to chemotherapy		
Complete response	0.592 (0.3–1.171)	0.131
All others	1	
Residual disease (cm)		
≤2	0.719 (0.254–2.039)	0.535
>2	1	
Performance status		
No impairment	0.392 (0.178–0.862)	0.019
All others	1	
Hk7		
Tumor mask low	0.302 (0.125–0.729)	0.007
Tumor mask high	1	

FIGO, International Federation of Gynecology and Obstetrics.

Table 4. Multivariate 3-year disease-free survival analysis by Cox regression

Variable	Hazard ratio (95% confidence interval)	*P*
Histology		
Serous	0.946 (0.526–1.703)	0.853
All others	1	
FIGO stage		
IV	2.844 (1.665–4.859)	0.0001
III	1	
Grade		
Poor	0.774 (0.473–1.265)	0.306
All others	1	
Clinical response to chemotherapy		
Complete response	0.68 (0.417–1.107)	0.12
All others	1	
Residual disease (cm)		
≤2	0.195 (0.083–0.456)	0.0002
>2	1	
Performance status		
No impairment	1.089 (0.646–1.837)	0.748
All others	1	
Hk7		
Tumor mask low	0.522 (0.23–1.186)	0.12
Tumor mask high	1	

FIGO, International Federation of Gynecology and Obstetrics.

overexpression in ovarian cancer cells promotes peritoneal shedding and ascites formation. Hk7 expression is regulated by steroid hormones [20, 21]. Progesterone receptors (PRs) are frequently expressed in early stages of ovarian cancer and their presence is associated with favorable prognosis because progesterone promotes differentiation and apoptosis [22]. Poorly differentiated ovarian cancers are predominantly PR negative, whereas they have significantly higher hk7 messenger RNA (mRNA) levels.

Quantitative assessment of hk7 mRNA levels in 125 ovarian tumors of different disease stages and tumor grades demonstrated that high hk7 mRNA levels were associated with grade 3 tumors and suboptimal debulking [17]. Hk7 mRNA expression status in ovarian tissue was an independent predictor for PFS and OS in patients with early-stage disease and in optimally debulked patients with advanced disease. Higher hk7 expression in ovarian cancer tissue was associated with poorer prognosis of ovarian cancer patients, especially those with lower grade disease and those who had been optimally debulked [17]. Shan et al. [23] in studies done with enzyme-linked immunosorbent assay (ELISA) found that hk7 is associated with other unfavorable characteristics of ovarian cancer. Similarly, Prezas et al. [12] found that overexpression of the human tissue kallikrein genes hk 4, 5, 6 and 7 potentiates the malignant phenotype of ovarian cancer cells.

To our knowledge, our study is the first to examine hk7 protein expression in ovarian carcinoma using automated quantitative protein analysis. Our goal was to quantitatively assess expression of hk7 on a cohort of ovarian cancer specimens in an objective, automated fashion and to evaluate the association between hk7 expression and clinical outcome. We utilized a novel quantitative *in situ* method of protein analysis. 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 cut-off points used in conventional immunohistochemistry studies while at the same time preserving spatial and morphological information that techniques such as western blotting and ELISA lose. Both the optimal cut-point (X-tile) analysis and the skewed distribution of hk7 expression (Figure 2) demonstrate that tumors expressing abnormally high hk7 levels represent a distinct subset of tumors with markedly poor outcome. We demonstrated hk7 expression to be a robust predictor of DFS and OS times in our cohort of patients with advanced ovarian cancer. In multivariable analysis, hk7 protein expression status retained its prognostic significance for OS.

Our results indicate that human kallikrein 7 plays an adverse role in prognosis in patients with ovarian cancer. The possibilities that hk7 may be a suitable candidate as disease biomarker and prognostic factor merit further investigation.

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