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Ovarian cancer specific kallikrein profile in effusions

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Abstract

Objective. Kallikreins belong to the serine protease family and their roles as cancer associated markers have been proposed. However, a comprehensive and parallel analysis of different secreted kallikreins in ovarian cancer has not been performed. This study was undertaken to profile the secreted kallikreins in cancer effusion supernatants.

Methods. We applied ELISA to measure the protein levels of nine kallikreins (4–8, 10, 11, 13, and 14) in a total of 221 effusion supernatants obtained from ovarian cancer, benign non-neoplastic diseases and a variety of other neoplastic diseases.

Results. Our results demonstrated that ovarian cancer effusions contained higher levels of all kallikreins analyzed except kallikrein 4, as compared to benign effusions ($p<0.0005$) and other cancer types ($p<0.03$). Unsupervised principal component analyses demonstrated a unique cluster of ovarian cancer samples which were distinct from benign effusions and other cancer groups based on measurements of secreted kallikreins 5–8, 10, 11, 13 and 14. Supervised combinations of the eight kallikreins achieved areas under ROC curve of 0.994 and 0.961 in separating ovarian cancer from benign effusion groups and other cancer groups, respectively. Among kallikreins, kallikreins 6, 7, 8, and 10 showed the highest statistical power in distinguishing ovarian cancer from benign controls and other cancer groups and these kallikreins could diagnose false negative cases based on cytology.

Conclusions. The above findings indicate that kallikreins 6, 7, 8 and 10 are the four most specific secreted kallikreins in ovarian cancer. These kallikreins may have clinical implications in the differential diagnosis of ovarian carcinoma from benign diseases and other cancer types.

Keywords: Ovarain cancer; Ascites; Kallikrein

Introduction

Human tissue kallikreins are serine proteases encoded by 15 tandemly located genes on the chromosome 19q13.4 [1,2]. Kallikreins participate in a variety of physiological processes from regulation of blood pressure and electrolyte balance to tissue remodeling, prohormone processing, neural plasticity and skin desquamation. In human cancer tissues, several kallikreins have been found overexpressed in major types of carcinomas [3]. For example, kallikreins 4, 5, 6, 7, 9, 10, 13 and 14 have been reported to be biomarkers associated with ovarian, breast, prostate and testicular cancer [4–6]. Recent studies further suggest that kallikreins are directly involved in the development of cancer by participating in extracellular matrix degradation, cellular invasion and metastasis [3,7–9]. All kallikreins are secreted proteins and can be detected in body fluids, suggesting a role of kallikreins in cancer diagnosis [4,10–12]. In fact, kallikrein 3, also known as prostate specific antigen (PSA), is currently the most useful tumor marker for prostate cancer screening, diagnosis, prognosis and monitoring. In addition to

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kallikrein 3, several kallikreins are also detected in body fluids. For example, kallikreins 5, 6, 8, 10, 11 and 14 have been proposed as serum markers for prognosis and monitoring in ovarian carcinomas and kallikreins 3, 5 and 14 are serum markers for diagnosis and prognosis in breast carcinomas [4].

Although kallikreins have been well studied in human cancer [3], previous reports have focused on a single kallikrein in a specific cancer type and parallel analysis of different secreted kallikreins in the same set of clinical samples has not been performed. It is essentially not well-known which secreted kallikreins predominate in various types of human cancers. This question appears critical, not only because of biological interest but also for clinical applications of kallikreins as cancer diagnostic markers. In this study, we applied ELISA and comprehensively measured the protein concentrations of nine kallikreins (4–8, 10, 11, 13, and 14) in effusion supernatants from a total of 221 patients. The reasons to focus on analyzing effusion samples are as follows: First, it is thought that effusions are ideal samples to study secreted proteins as they are directly released from tumor cells into the effusion fluid. Therefore, the tumor-secreted proteins are enriched in the supernatants and measurement of kallikreins in effusion samples may facilitate the differential diagnosis of malignant versus benign diseases and among different malignant diseases. Second, cancer effusions are commonly found in cancer patients at late stages and with recurrent diseases; therefore, a better understanding of the pathogenesis of effusions may be important in the development of novel strategies to control effusion formation in patients. The most common neoplastic diseases that generate effusions are those developed from ovarian, breast, lung, liver and gastrointestinal carcinomas. Our results demonstrated a unique profile of secreted kallikreins in ovarian cancer effusions, in which kallikreins 6, 7, 8 and 10 were the four kallikreins showing the highest statistical power in separating ovarian cancer from benign effusions and other types of major cancers. This restricted cancer-type specific secretion of kallikreins may have biological implications and suggests that these kallikrein markers are ideal for ovarian cancer diagnosis.

Materials and methods

Samples

The effusion samples included a total of 221 anonymous effusions (78 ascites samples and 143 pleural effusions) from 195 patients which were collected at the Innsbruck University Hospital in Austria. All specimens used were approved by the local institutional review boards. The samples were collected in a consecutive fashion regardless of clinical and cytological diagnoses. The clinical diagnosis of the samples was listed in Table 1. The diagnoses of benign effusions were listed in Table 2. The diagnoses of effusions were based on clinical diagnosis and final pathology reports. All samples were collected from individual patients, except 25 patients who had two effusions and 1 patient with three effusions, which were collected from the same patients at least 1 month between the collections. The recurrent effusion samples were excluded in the primary analysis. All samples were analyzed in a blinded fashion without prior knowledge of the specimen identity. Samples were generally centrifuged within 30 min after specimen acquisition and the supernatants were collected in 1.5 microfuge tubes and were frozen shortly in −80 °C. For ELISA, the effusion supernatant was thawed on ice and centrifuged again at 10,000 rpm and 200 μl of the supernatant was collected for kallikrein assays within 30 min after complete thawing.

Table 1
Patient profile included in this study

Diagnosis	Case number
Non-neoplastic diseases	71
Neoplastic disease	
Ovarian cancer	23
Lung cancer	37
Breast cancer	26
Digestive organ cancer	23
Hepatocellular cancer	7
Hematological cancer	10
Sarcoma	4
Cervical cancer	3
Miscellaneous cancers	17
Total	221

Kallikrein measurements

For all kallikrein measurements in effusions, we used ELISA-type immunofluorometric procedures developed in-house. Most of these methods have been described and validated in previous publications. In Table 3, we provide information on these methods along with appropriate references [6,12–14]. We have tested the cross-reactivity of these ELISA assays against all other kallikreins and we found no cross-reactions in all cases. The precision of all assays within the dynamic range cited in Table 3 was <10%. These assays were standardized using recombinant proteins produced in yeast or mammalian expression systems, as previously described [6,12–14].

Statistical analysis

The results of the kallikrein levels were plotted according to disease diagnosis and *t*-test was used to analyze the difference between groups for each kallikrein. Receiver operating characteristics (ROC) curves were constructed for kallikrein levels in supernatants of effusion samples as a diagnostic marker by plotting sensitivity versus 1-specificity. Area under the curve (AUC) was calculated to demonstrate the performance of each kallikrein or kallikreins in combination in the differential diagnosis among different experimental groups. Unsupervised principal component analysis was used to cluster ovarian cancer, non-ovarian cancer and benign effusion groups. In addition, supervised UMSA component analysis [15,16] was used analyze and visualize the separation between the groups. Linear regression was used to assess the correlation in the effusion levels of different kallikreins.

Results

The concentrations of nine kallikreins including kallikreins 4–8, 10, 11, 13 and 14 in the effusion supernatants were determined using ELISA previously developed and validated [6,12–14]. The levels of each kallikrein in effusion samples (excluding the recurrent specimens from the same patients) were shown in Fig. 1 and Table 4. As compared to benign effusions, the majority of ovarian cancer effusions showed higher levels of all kallikreins except kallikrein 4. The kallikrein concentrations in non-ovarian cancers were generally low, except for a few cancer cases from breast, lung and digestive organs with high kallikrein levels, notably of kallikreins 5–8 and 10. The levels of kallikreins 5–8, 10, 11 and 13 were significantly higher in ovarian cancer than in benign effusions

Table 2
Diagnosis of benign effusions

Diagnosis	Patient number
Cardiomyopathy	26
Liver cirrhosis	21
Pneumonia/tuberculosis	15
Others	
Pulmonary embolus	2
Meig's syndrome	1
Gorham disease	1
Congenital chylothorax	1
Arthritis	1
Renal failure	3
Total	71

($p \leq 0.002$) and non-ovarian cancer groups ($p \leq 0.05$). ROC curve analysis was then used to assess the performance of individual kallikreins in distinguishing ovarian cancers from benign effusions and different types of non-ovarian cancer (Fig. 2). We found that kallikreins 6, 10, 8 and 7 were the top four kallikreins that showed the highest area under the ROC curve (AUC) in separating ovarian cancer effusions from benign effusions with AUC of 0.992, 0.962, 0.953 and 0.870, respectively. Similarly, kallikreins 8, 10, 6 and 7 also showed the highest AUC to differentiate ovarian cancer from non-ovarian cancer effusions with AUC of 0.926, 0.900, 0.894 and 0.840, respectively. When the non-ovarian cancers were classified into different cancer types, kallikreins 6, 8 and 10 remained the top three kallikreins to distinguish ovarian carcinomas versus breast (AUC > 0.890), hepatocellular (AUC > 0.920), gastrointestinal (AUC > 0.830), hematopoietic neoplasms (AUC > 0.920) and non-small cell lung carcinomas (AUC > 0.870). The concentrations of kallikreins 6, 7, 8 and 10 in ovarian cancer effusions were highly correlated to each other (Pearson correlation, $r > 0.62$), especially between kallikreins 6 and 7 with a correlation coefficiency of 0.854. There was no statistical correlation between kallikrein levels and patients' age (Pearson correlation, $r < 0.2$) and sites (abdominal or pleural) of effusions ($p > 0.05$) in the ovarian cancer group. Twenty-five cancer patients had recurrent effusions. Although we did not include those recurrent effusions in the above analysis, we compared the levels of kallikreins 6, 7, 8 and 10 between the primary and recurrent effusions and found that there was no statistically significant difference of kallikrein levels between primary and recurrent effusions. The p values of paired t-test for kallikrein 6, 7, 8 and 10 were 0.846, 0.589, 0.394 and 0.163, respectively.

Unsupervised principal component analysis was used to evaluate the performance by combining different kallikreins in the diagnosis of ovarian cancer. All but kallikrein 4 were included in the analysis because kallikrein 4 was the only one that did not have statistical power ($p > 0.90$) in distinguishing ovarian cancer from benign effusions and other cancer types based on AUC. As shown in Fig. 3A, all ovarian cancer samples clustered close to each other and distantly from the benign effusion group. Similarly, the ovarian cancer group was also separated from the majority of non-ovarian cancer groups although there are a few overlapping cases. However, the non-ovarian cancer samples significantly overlapped with benign effusions, and there was a lack of the separation between non-ovarian cancer and benign effusions. In order to better present the relationship among ovarian cancer, non-ovarian cancer and benign effusion groups, we analyzed two of the groups at one time using a three-dimensional view which is based on a supervised component analysis by combining the levels of kallikrein 5–8, 11, 13 and 14. As shown in Fig. 3B, we were able to demonstrate the high performance of secreted kallikreins in separating ovarian cancer and benign effusion groups, and ovarian cancer and other cancer groups with AUC of 0.994 and 0.961, respectively. Similar to the unsupervised principal component analysis, there was only a limited power of kallikrein in distinguishing non-ovarian cancers and benign effusions, with an AUC of 0.694.

The cytology reports were available from 20 ovarian cancer effusion cases which were used to compare the cytology diagnosis to the kallikrein levels. Using the cutoffs determined by the kallikrein concentrations that yielded the highest sensitivity at 100% specificity (based on the ROC curve analysis) to define cancer cases, the sensitivity of kallikreins 6, 8 and 10 ELISA was 100%, 95% and 95%, respectively, which was higher than 75% in routine cytology examination. Therefore, kallikrein 6 ELISA assay was able to diagnose five additional cases of ovarian carcinoma which were not diagnosed by routine cytology.

Discussion

Currently, there are very few secreted cancer biomarkers that have a major clinical impact, with the exception of prostate-specific antigen (kallikrein 3) and CA125. One of the major difficulties in applying cancer-associated markers for cancer detection, diagnosis and monitoring is the fact that most of the current cancer biomarkers are neither cancer specific nor tumor-type specific; their levels in body fluids are elevated in malignant diseases as well as in benign conditions. Therefore, most candidate cancer biomarkers lack such high sensitivity and specificity that are required for clinical applications. With the discovery of the complete kallikrein gene family (1, 2) and the establishment of methods to quantify different kallikreins (Table 3), previous studies have demonstrated promising sensitivity and specificity for applying individual kallikreins

Table 3
hK-specific ELISA assays used in the present study

Kallikrein	Coating/detection Ab [a]	Dynamic range to (ng/l)	Detection limit (ng/l)	Reference
hK4	mono/poly	20,000	100	[14]
hK5	mono/mono	25,000	100	[6]
hK6	mono/mono	50,000	100	[12]
hK7	mono/mono	20,000	200	[15]
hK8	mono/mono	20,000	200	[16]
hK10	mono/mono	20,000	50	[17]
hK11	mono/mono	50,000	100	[18]
hK13	mono/mono	20,000	50	[19]
hK14	mono/poly	20,000	100	[20]

[a] Mono, monoclonal mouse antibody; poly, polyclonal rabbit antibody.

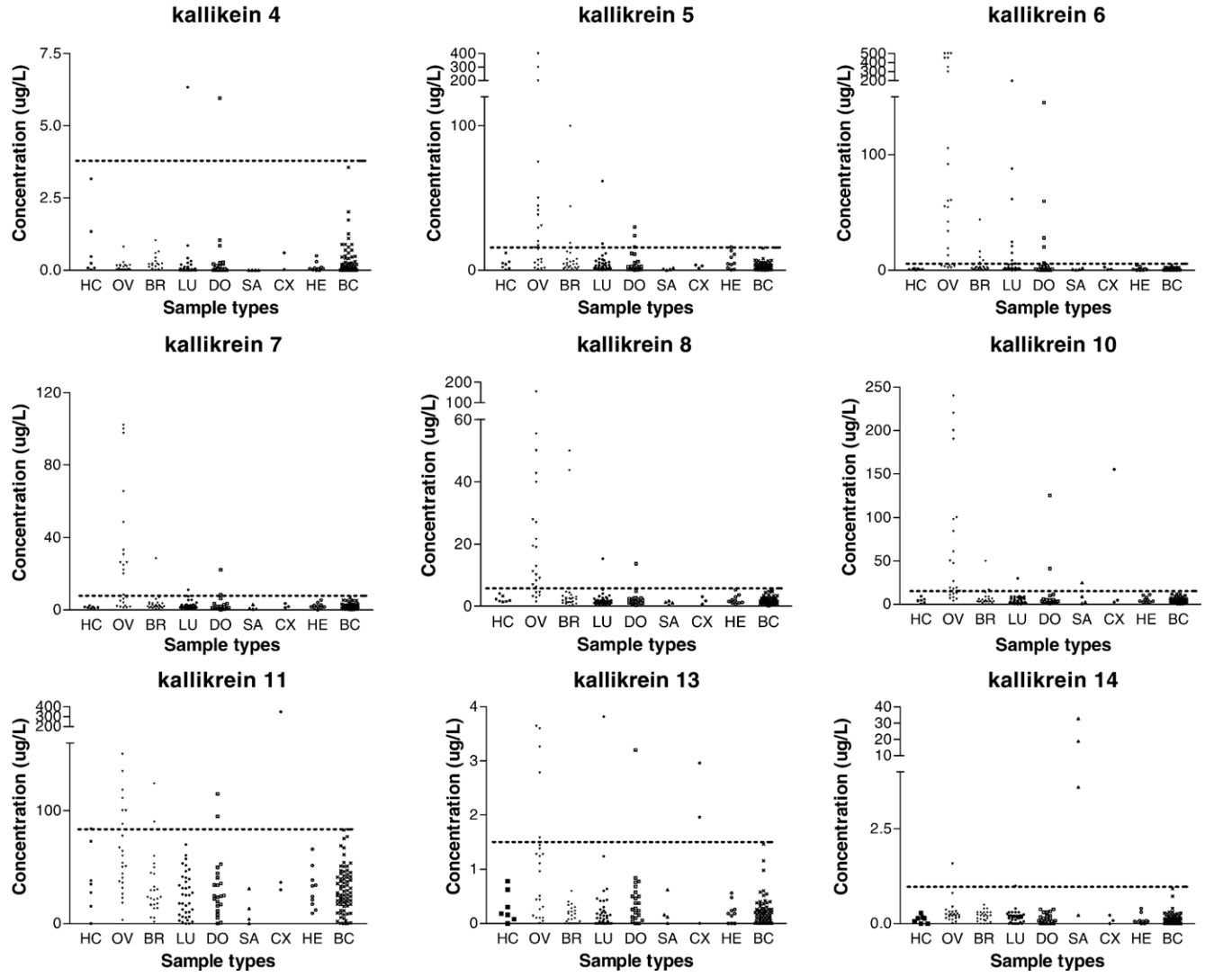

Fig. 1. Scatter plots of individual kallikrein levels in effusion supernatants from different cancer types and benign effusions. Each symbol represents an individual effusion sample. The dash line in each panel indicates the highest kallikrein concentration in the benign effusion group. HC: hepatocellular carcinoma, OV: ovarian cancer; BR: breast carcinoma; LU: non-small cell lung carcinoma; DO: carcinoma from digestive organs; SA: sarcoma; CX: cervical carcinoma; HE: hematopoietic neoplasm; BC: benign effusion.

in distinguishing cancer from its corresponding benign tissue [2,6], suggesting that kallikrein family members are potential cancer biomarkers. With parallel analysis of the secreted tissue kallikreins 4–8, 10, 11, 13 and 14 on the same set of clinical effusion specimens, the results from current study provided compelling evidence that specific kallikreins appear to be ovarian cancer-specific as they are present at high levels in ovarian cancer effusions but rarely in non-ovarian cancer effusions and benign effusions. Furthermore, combination of these kallikreins using ROC curve analysis gave a remarkably high sensitivity and specificity for the diagnosis of ovarian cancer in effusion samples.

In contrast to other kallikreins analyzed in this study, kallikrein 4 is the only one in which its expression level fails to distinguish ovarian cancers from either non-ovarian cancers or benign effusions. The concentration of kallikrein 4 in ovarian cancer effusions was generally very low, a finding in contrast to other reports showing expression of kallikrein 4 in ovarian cancer tissues [17–19]. The discrepancy is likely due to that the previous studies analyzed the gene expression levels in tumor cells by RT-PCR and immunoblotting while the current study measured the secreted level of kallikrein 4 by ELISA, suggesting that kallikrein 4 can be expressed in ovarian cancer cells but not secreted to the extracellular environment. In contrast, we demonstrated that the secreted levels of kallikreins 5–8, 10, 11, 13 and 14 in effusions were significantly higher in ovarian cancer than in benign effusions and other non-ovarian cancer groups, a finding consistent with previous reports showing overexpression of kallikreins 5–8, 10, 11 and 14 in ovarian carcinoma tissues [6] and tumor cells in ascites [14]. In this study, we further tested if a combination of a selected panel of biomarkers was more effective than the measurement of a single tumor marker in cancer diagnosis [20–22]. A linear combination of all different kallikreins (except kallikrein 4) achieved an AUC of 0.994 which was higher than the AUC of any single kallikrein in the diagnosis of ovarian versus benign

Table 4
The mean levels of kallikrein in samples analyzed

Kallikrein	OVCA	Non-OVCA	Benign
Kallikrein 4	0.128 (0.190)[a]	0.354 (0.882)	0.283 (0.595)
Kallikrein 5	62.2 (104)	6.60 (12.4)	2.73 (2.51)
Kallikrein 6	144 (188)	10.0 (29.0)	0.942 (0.778)
Kallikrein 7	27.6 (33.1)	2.78 (3.71)	2.00 (1.43)
Kallikrein 8	23.2 (32.9)	3.19 (6.53)	1.88 (1.05)
Kallikrein 10	57.0 (66.4)	10.1 (21.8)	3.95 (2.82)
Kallikrein 11	63.9 (40.6)	38.1 (44.7)	29.9 (19.5)
Kallikrein 13	1.00 (1.05)	0.378 (0.611)	0.214 (0.271)
Kallikrein 14	0.301 (0.330)	0.520 (3.23)	0.121 (0.154)

OVCA: ovarian cancer effusions.
Non-OVCA: effusions from non-ovarian cancer.
Benign: benign effusions.
[a] Mean and one standard deviation in parenthesis.

effusions, but the increase in AUC was only very mild as compared to kallikrein 6 (AUC = 0.992). This is probably because the performance of kallikrein 6 in distinguishing ovarian versus benign effusions is excellent and other kallikrein has minimal value in increasing the diagnostic power. Besides, measurement of kallikrein levels is also useful in differential diagnosis of ovarian versus other non-ovarian cancers as the AUC is as high as 0.961. The lack of separation power for kallikreins to distinguish non-ovarian cancers and benign effusions is likely due to the fact that the secreted levels of all kallikreins in non-ovarian cancer group are generally low and are similar to the levels in the benign effusion group.

The mechanisms of increased levels of kallikreins 6, 8 and 10 in ovarian cancer body fluids remain to be determined. Although many amplicons in chromosome 19 such as cyclin E, AKT-2 and Notch-3 loci are known in ovarian cancer, the DNA copy number at 19q13.4, where the kallikrein genes are located, is rarely increased [23]. Therefore, elevated kallikrein expression in ovarian cancer is probably a result of promoter activation by the estrogen receptor signaling pathway [24]. Alternatively, it is also likely that high kallikrein levels in ovarian cancer effusions are a result of enhanced secretion efficiency, increased cellular necrosis and/or decreased kallikrein degradation in ovarian cancer effusions. As kallikreins have been shown to promote tumor progression by participating in extracellular matrix degradation and release of bioactive peptides that may regulate tissue remodeling [3,7–9], the high concentrations of kallikreins 6, 8 and 10 in ovarian cancer effusions imply that ovarian cancers utilize these kallikreins to transform the tissue microenvironment of peritoneal walls and facilitate tumor implantation and dissemination by the floating tumor cells in effusions. It is also likely that kallikreins secreted by the tumor cells in the effusion may directly contribute to the formation of effusion because kallikreins may enhance the permeability and/or destroy the angiolymphatic vessels on the peritoneal wall. Further studies are required to address the etiologic roles of kallikreins in the formation of effusions in ovarian cancer and determine if ovarian cancer-specific kallikreins can be used in target-based therapy and in prevention of effusions.

Effusions either in abdominal or pleural cavities are common clinical presentations in patients who suffer from a variety of medical conditions including inflammatory disorders, infectious diseases, renal, liver and cardiac diseases as well as malignant neoplasms [25–28]. Cytological examination is routinely performed to distinguish malignant from benign diseases. Although the sensitivity and specificity of cytology

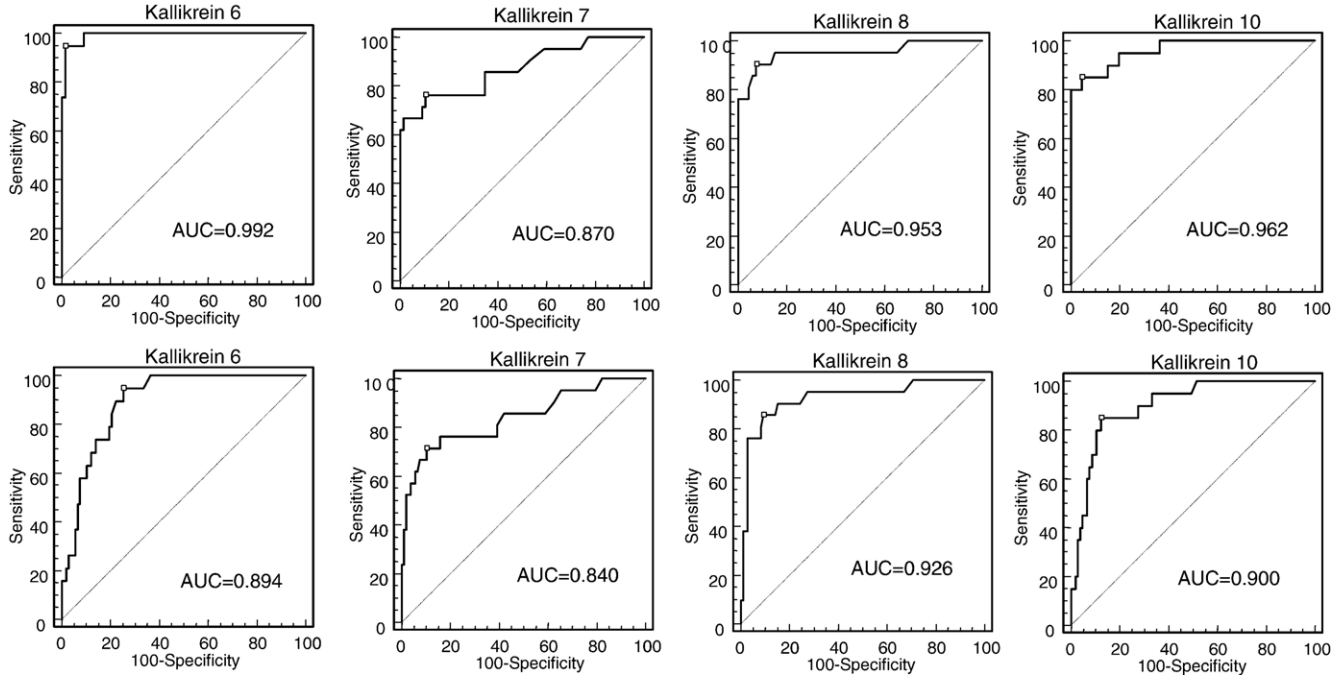

Fig. 2. ROC curve analysis of kallikreins 6, 7, 8 and 10 in distinguishing ovarian cancer cases versus benign effusions (upper panels) and in distinguishing ovarian cancer and non-ovarian cancer cases (bottom panels). The area under the ROC curve (AUC) is given in each panel.

when combined with immunocytochemistry are generally high in the diagnosis of malignant effusions [29,30], they may not be satisfactory in many cases and the differential diagnosis among different cancer types may be difficult [31]. Thus, the measurement of kallikreins could have diagnostic utility in assisting cytology diagnosis of ovarian cancer effusion samples. It should be noted that a high kallikrein concentration in effusions detected in cytology negative cases may be via an indirect route as the solid ovarian tumor in the peritoneal wall, omentum or ovaries secrete kallikrein into circulation then into effusion. Because secreted kallikreins 6, 7, 8 and 10 were specific to ovarian cancer, the kallikrein ELISA should be useful in diagnosing ovarian cancer in patients who are clinically suspicious for this neoplastic disease (in ovarian cancer patients who develop ascites after debulking surgery and chemotherapy). However, its value in differential diagnosis among different types of cancer is limited. Nevertheless, kallikreins 6, 7, 8 and 10 can be combined with other cancer type specific markers for such purpose.

Although a near perfect performance of the combined ELISA assays (AUC>0.99) was obtained from this study, it should be noted that further multi-institutional studies should be performed to validate our results in a larger series of cases and controls. Thus, it is conceivable that a combination of the selected kallikreins and other markers can further increase the sensitivity and specificity of kallikrein-based diagnostics in clinical applications. Secreted tumor markers other than kallikreins have been reported in malignant effusions of ovarian cancer patients [32]. For example, lysophosphatidic acid (LPA) was first identified in ascites of ovarian cancer patients and this molecule has been demonstrated to play a biological role in ovarian cancer cell growth [33]. Urokinase plasminogen activator (uPA), a critical component of the metastatic cascade, is also found at high concentrations in ovarian cancer ascites, and the levels of uPA correlate inversely with prognosis. LPA induced a consistent increase in uPA promoter activity and mRNA levels, suggesting that increased uPA production is, at least in part, transcriptional [34]. It has also been reported that ovarian and breast ascites specimens contained much higher concentrations of secretory HLA-G than the benign ascites specimens [35].

In summary, we profiled secreted kallikreins in several common types of human cancer and demonstrated that among those tumor types examined, ovarian cancer was the main tumor type that secreted kallikreins. We also reported that kallikreins 6, 7, 8 and 10 were the major kallikreins primarily associated with ovarian cancer effusions. Our results suggest that measurement of kallikreins in effusions can be a useful adjunct approach to diagnose ovarian cancer in effusion samples together with imaging techniques. Future studies are required to address the clinical potential in applying kallikrein profiles to detect early ovarian cancer in serum samples.

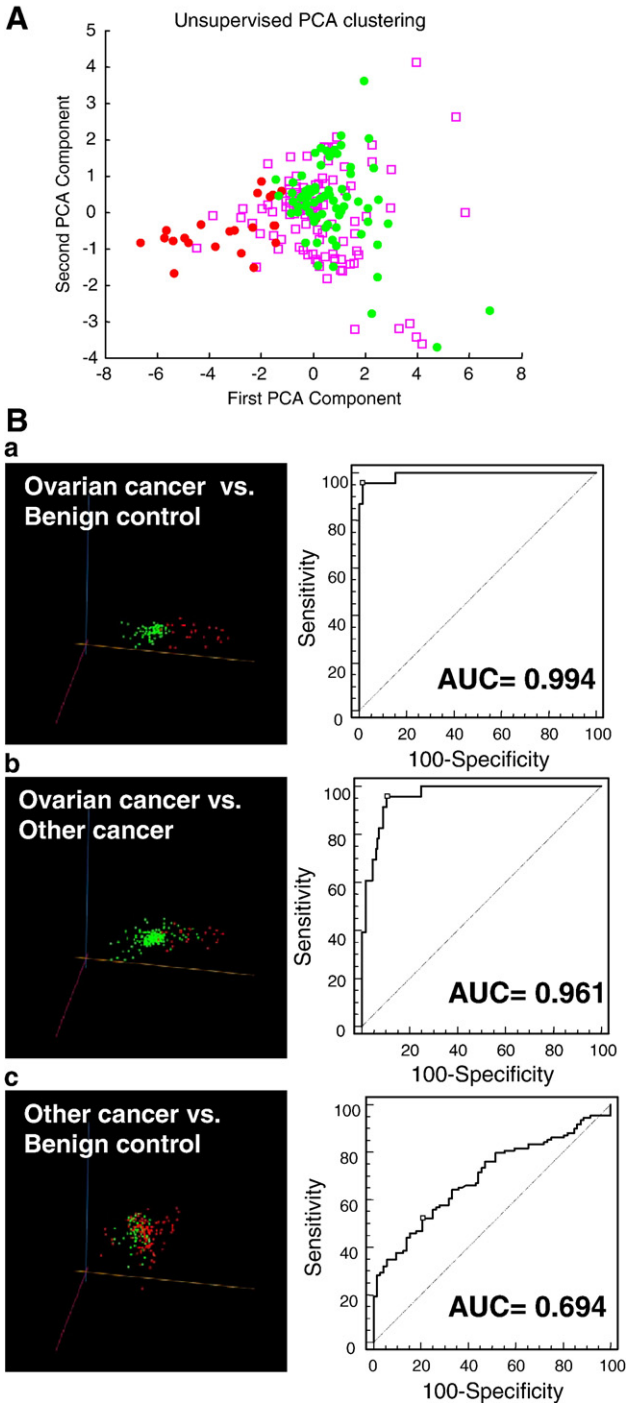

Fig. 3. A: Principal component analysis (PCA) showing clusters of samples. Ovarian cancer group (red filled circles) is distinct from benign effusions (green filled circles). Similarly, ovarian cancer group can also be separated from the majority of non-ovarian cancer group (open pink squares) although there are a few overlapping cases. B: Three-dimensional clustering using the UMSA component analysis, a supervised component analysis method, in ovarian cancer, non-ovarian cancer and benign effusion groups. The first axes correspond to direction along which samples have the best separation. ROC analysis in each figure is performed using data projection on the first axes of the corresponding figure. It is a linear combination of the levels of kallikreins 5–8, 10, 11, 13 and 14. The upper panel: Comparison of ovarian cancer group (red dots) versus benign effusion group (green dots). The middle panel: Comparison of ovarian cancer group (red dots) versus non-ovarian cancer group (green dots). The bottom panel: Comparison of non-ovarian cancer group (red dots) versus benign effusion group (green dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

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