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Human Kallikrein 8, a Novel Biomarker for Ovarian Carcinoma[1]

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ABSTRACT

Human kallikrein 8 (hK8; neuropsin) is a serine protease and new member of the hK family. The aim of this study was to examine if hK8 may serve as a novel cancer biomarker. An hk8-ELISA, developed in-house, was used to study the distribution of hK8 in various biological fluids and tissue extracts from healthy individuals and ovarian cancer patients of different stages of the disease (International Federation of Obstetrics and Gynecology II–IV). For ovarian cancer patients, very high levels in ascites fluid were observed (≤1000 μg/liter; $n = 85$ samples). Elevated serum levels were seen in 24 of 40 (62%) of ovarian cancer patients. Higher ascites fluid hK8 concentration was associated with better ovarian cancer progression-free survival ($P = 0.02$). In both serum and ascites fluid, there is a significant correlation between hK8 and CA125 concentration ($r = 0.51$ and 0.58, respectively). The serum concentration of hK8 was an indicator of progression on regression on longitudinal monitoring of an ovarian cancer patient. These data suggest that hK8 protein is detectable in ovarian cancer tissue extracts, serum, and ascites fluid, indicating that it may serve as a new ovarian cancer marker.

INTRODUCTION

The human tissue kallikrein gene family, a subfamily of serine proteases, is now known to include 15 members (1, 2). All genes in this family localize to chromosome 19q13.4 and share significant similarities at both the DNA and amino acid level. The hK[3] family includes hK3/PSA, which is the most important biomarker for prostate cancer (3). Recently, three other members of this family, hK6/neurosin, hK10/normal epithelial cell-specific 1, and hK11/trypsin-like serine protease, have been shown to be potential biomarkers for ovarian and prostate cancer (4–6). In addition, recent reports also suggest that many other members of this family are associated with cancers of the breast, ovary, prostate, and testis, as well as with diverse diseases of the central nervous system, skin, etc. (reviewed in Ref. 7).

KLK8/neuropsin is a member of the hK family (1, 2) (*KLK8* is a gene, and hK8 is a protein, according to the official kallikrein gene nomenclature; Ref. 8). Originally, *KLK8* was cloned from a human skin cDNA library as a homologue of mouse neuropsin (9). The mouse homologue has highest expression in skin and brain, especially the hippocampus, and was assumed to be associated with neural plasticity, memory formation, and some forms of epilepsy (10–13). *KLK8* mRNA is increased in Alzheimer's disease hippocampus compared with controls, which suggests that *KLK8* may indeed have a relationship with neural plasticity in humans (14). *KLK8* transcripts in ovarian cancer tissues are expressed at higher levels than in controls (15). Two splice variants of *KLK8* have been detected in ovarian cancer (16). Because the *KLK8* gene is predicted to encode for a secreted serine protease, it is possible that the gene product, hK8, may have diagnostic value for cancer, similarly to hK3 (PSA; Ref. 3), hK6, hK10 (normal epithelial cell-specific 1), and hK11 (trypsin-like serine protease; Refs. 4–7). To date, there is no literature describing any relationship between hK8 protein expression and cancer. We here report for the first time elevation of hK8 protein in serum, ascites fluid, and tumor cytosol fractions of advanced stage ovarian cancer patients.

MATERIALS AND METHODS

hK8 Immunoassay Procedure. We developed a highly sensitive and specific immunoassay for hK8, based on polyclonal mouse and rabbit antibodies (17). The assay has a detection limit of 0.2 μg/liter, is precise (<10% coefficient of variation), and has no cross-reactivity with any other member of the kallikrein family (hK1-hK15). This assay was used to quantify hK8 by using recombinant hK8 as a standard, produced in yeast (17). All samples were measured in duplicate. Details about the generation and purification of recombinant hK8, polyclonal mouse and rabbit antibodies, development, and evaluation of this assay have been published elsewhere (17).

Cytosolic Fractions of Ovarian Cancer Tissues. Preparation of cytosol fractions from normal, benign, and primary cancer tissues at the Department of Obstetrics and Gynecology, Technical University of Munich, Germany, is described by Schmalfeldt *et al.* (18). Cytosol fractions were stored in liquid nitrogen until use. Ascites fluids were obtained from patients with advanced stage ovarian cancer stage International Federation of Obstetrics and Gynecology II ($n = 7$), III ($n = 20$), and IV ($n = 4$) by tapping the patients and collection of the fluid into a sterile bag by flushing the needle and bag with heparin. The ascites fluids were then centrifuged at 10,000 × *g* for 30 min to sediment cells and any debris. The supernatants were harvested, aliquoted, and stored at −80°C until use. An additional set of 54 ascites fluids from ovarian cancer patients with unknown stage was also used. The study to collect tissue and ascites fluid from ovarian cancer patients to assess the patients' risk profiles was approved by the Ethics Committee of the University Hospital (Klinikum rechts der Isar) of the Technical University of Munich. All patients received treatment according to consensus recommendations at that time.

Fractionation of Biological Fluids with Gel Filtration High-pressure Liquid Chromatography. To determine the molecular mass of the protein detected in the biological fluids and tissue extracts, ascites fluid and ovarian cancer serum were fractionated with gel filtration chromatography, as described elsewhere (19). The fractions were then collected and analyzed for hK8 by using the hK8-ELISA.

Statistical Analysis. Statistical analysis was performed with SAS software (SAS Institute, Cary, NC). All data were analyzed with nonparametric tests, and relationships between different variables were assessed by Spearman or Pearson correlation after logarithmic transformation of data. Survival probability was plotted according to Kaplan-Meier, and differences between curves were evaluated by the Log-rank test. A $P < 0.05$ was considered statistically significant.

RESULTS

Ascites Fluid of Ovarian Cancer Patients. We quantified hK8 in 31 ascites fluid samples obtained from patients with metastatic ovar-

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[3] The abbreviations used are: hK, human kallikrein; PSA, prostate-specific antigen.

ian carcinoma and known stage. All samples were positive for hK8 (Fig. 1). The following statistical parameters describe the findings: lowest value, 5 μg/liter; highest value, 487 μg/liter; mean $\pm$ SD, 129 $\pm$ 149 μg/liter; median, 62 μg/liter.

After correcting for total protein in these ascites samples, the hK8 concentration, expressed as nanograms of hK8 per milligram of total protein, was 3.51 $\pm$ 0.66 ng/mg (mean $\pm$ SD), the range was 0.34–12.9 ng/mg, and the median was 1.73 ng/mg. For these patients, we also had information on age, various clinicopathological variables, including International Federation of Obstetrics and Gynecology stage, and outcomes (progression-free and overall survival). We found no association between ascites fluid hK8 levels and patient age. However, there was an inverse association between ascites fluid hK8 and tumor stage (Fig. 1), as well as progression-free survival ($P = 0.022$), but not overall survival ($P = 0.45$). In a separate series of 54 ascites fluid samples with no clinical information, we quantified hK8 and CA125. The results are shown in Fig. 2. We found a significant correlation between ascites fluid hK8 and CA125 ($r = 0.58$, after log transformation of data).

We have also analyzed a total of 36 peritoneal dialysis fluids from patients with renal failure. Of these, 26 were noninfected, and 10 were infected by bacteria. The concentration of hK8 was 2.3 $\pm$ 2.1 μg/liter (mean $\pm$ SD) in the noninfected samples and 3.7 $\pm$ 1.5 μg/liter in the infected samples. These means were ~56 and 35 times lower than the mean seen in the ascites fluid from ovarian cancer patients ($P < 0.01$ by the Mann-Whitney test).

Ovarian Cancer Cytosolic Extracts. We analyzed hK8 protein in cytosol fractions of 20 ovarian cancer tissues, along with extracts from 10 normal ovarian tissues and 10 ovarian tissues with benign disease. The data were expressed as nanograms of hK8 protein per milligram of protein (Fig. 3). The highest levels of hK8 were <4.8 ng/mg protein in normal tissues, whereas only 3 samples (30%) from the benign disease group exceeded this level. However, 11 of 20 samples (55%) from the ovarian cancer tissues demonstrated higher levels of hK8, as compared with normal tissues. These data suggest that in ~55% of patients, hK8 protein is up-regulated in the cancerous tissue, in comparison with normal tissues.

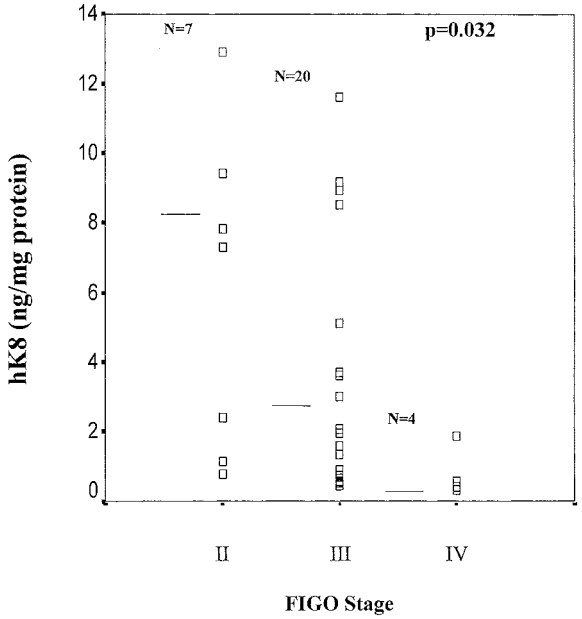

Fig. 1. Levels of hK8 (nanograms per milligram of protein) in ascites fluid of women with advanced ovarian cancer. Higher levels are seen in lower stage disease. The *P* was calculated by the Kruskal-Wallis test. *Horizontal lines*, median values; *N*, number of patients.

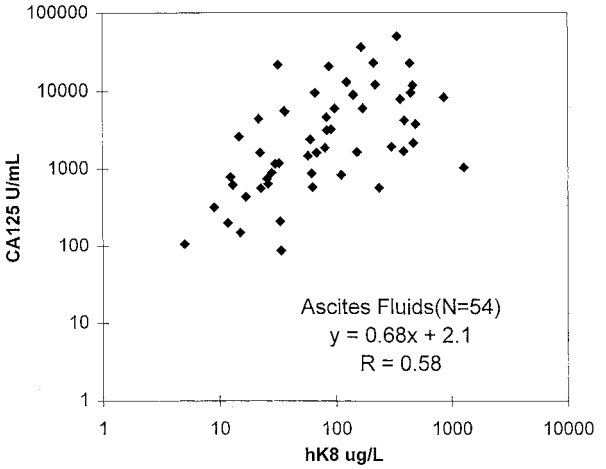

Fig. 2. Correlation between CA125 and hK8 concentrations in ascites fluids of ovarian cancer patients. The Pearson correlation coefficient [R] was calculated after logarithmic transformation of the data. For discussion, see text.

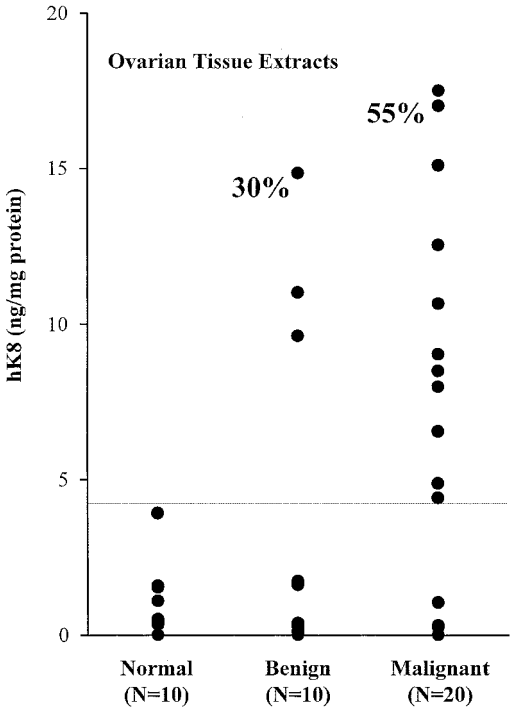

Fig. 3. Concentration of hK8 protein in tissue extracts from normal ovaries, ovaries with benign disease, and ovarian cancer tissues. *N*, the number of tissues extracted. The results are expressed as nanograms of hK8 per milligrams of total protein. The percentage of samples containing higher levels than normal tissue extracts is shown.

Serum of Cancer Patients. We have initially analyzed a total of 36 serum samples from patients with various malignancies, including prostate ($n = 6$), breast ($n = 6$), liver ($n = 6$), testicular ($n = 6$), colon ($n = 6$), and ovarian cancer ($n = 6$), along with 6 serum samples from healthy male and 10 serum samples from healthy female subjects. The highest level in normals was 5 μg/liter. In the cancer patients, we found 1 patient with breast cancer (8.2 μg/liter) and 1 patient with colon cancer (7.4 μg/liter) with elevated hK8 levels. However, in the ovarian cancer group, we found 4 patients with elevated levels (6.4–12.9 μg/liter). In view of this finding, we analyzed another series of 40 sera from normal women and 40 sera from ovarian cancer patients of various stages. The data are shown in Fig. 4. When a cutoff of 7.5 μg/liter is used for classification (97.5% specificity), the positivity

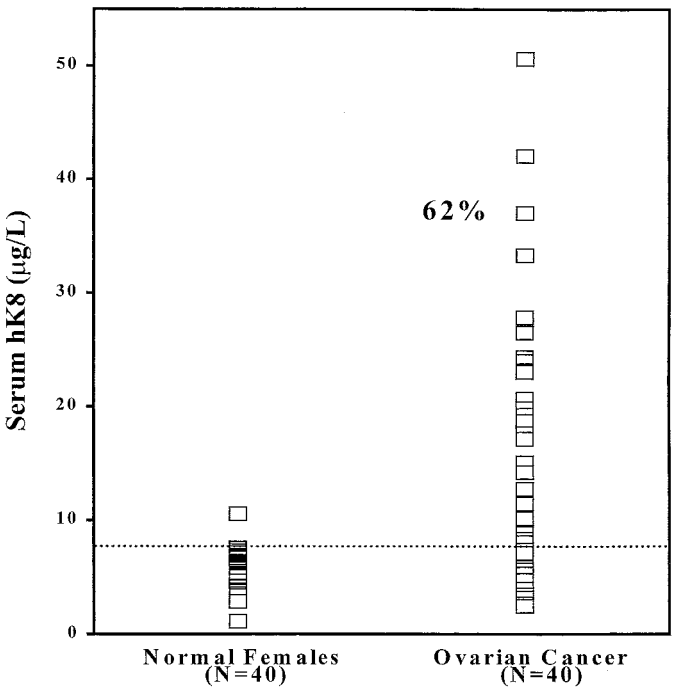

Fig. 4. Distribution of serum hK8 concentration in 40 normal females and 40 patients with ovarian cancer of various stages. When a cutoff of 7.5 μg/liter was selected (97.5% specificity; *horizontal line*), the sensitivity is 62%. For discussion, see text.

rate (sensitivity) of this test for ovarian cancer patients is ~62% (Fig. 4). When we plotted serum hK8 and CA125 levels, we found a significant correlation between these parameters (Fig. 5). We have further analyzed a series of serum samples obtained from 1 patient over a period of ~1 year. The levels of CA125 and hK8 correlated considerably, indicating that hK8 could also have value for disease monitoring.

High-performance Liquid Chromatography. Many enzymes circulate in serum as complexes with proteinase inhibitors. For some kallikreins, including PSA, the major circulating form is a complex with a proteinase inhibitor (*e.g.*, PSA is complexed with α-1 antichymotrypsin). To investigate if hK8 is circulating in various molecular forms, we have fractionated serum of an ovarian cancer patient with elevated hK8, with gel filtration chromatography, as described earlier (19) and analyzed all fractions with the hK8-ELISA. The hk8 immunoreactivity eluted as a single peak with a molecular mass of ~30 kDa, consistent with the molecular weight of free (uncomplexed) hK8.

DISCUSSION

The hK gene locus on chromosome 19q13.4 has now been well characterized (1, 2). This gene family includes 15 members, all encoding for secreted serine proteases. Among all members, PSA is the most valuable biomarker for prostate cancer (3).

Previously, a number of other kallikreins, including KLK4, KLK5, KLK6, KLK7, KLK8, KLK9, KLK10, KLK11, KLK14, and KLK15, have been associated with various forms of malignancy and especially cancers of the ovary, breast, prostate, and testis (reviewed in Refs. 1 and 2).

We have reported previously that at least three kallikreins, hK6, hK10, and hK11, constitute new serum biomarkers for ovarian carcinoma (4–6). We, and others, have also shown that at least nine kallikrein genes are differentially expressed in ovarian cancer, at the mRNA level (1, 2, 15, 16, 20, 21). We recently reported that the *KLK8* gene, at the mRNA level, is up-regulated in ovarian cancer and that its higher expression is associated with favorable outcome (16). However, no literature exists on levels of hK8 protein in either tissues or serum. We here demonstrate that hK8 protein expression is higher in cancerous tissues, in comparison with benign and normal tissues. Furthermore, we report for the first time very high levels of hK8 in ascites fluid of women with advanced ovarian cancer (stage II–IV) and that higher ascites fluid hK8 concentration is seen in patients who have lower stage disease. Similarly, progression-free survival in patients with higher levels of hK8 in ascites fluid is longer. We found a significant correlation between ascites fluid hK8 and CA125. Peritoneal fluids from noncancer patients do not contain much hK8, suggesting that this enzyme is likely secreted by tumor cells in ovarian cancer. These data suggest that hK8 concentration in ovarian cancer may be a marker of differentiation, with higher levels seen in more differentiated and less aggressive tumors. These proposals need verification with a larger series of specimens.

We also report here, for the first time, elevation of serum hK8 concentration in a proportion of patients with ovarian cancer. Our preliminary data indicate that patients with other cancers rarely have elevations of serum hK8. Further verification of these findings will require a larger series of patients with well-defined clinical data. However, it is interesting to note that the degree of hK8 overexpression at the ovarian cancer tissue level (55%) is similar to the percentage of patients with elevated serum levels (62%). These data prompt us to speculate that it's likely the subset of patients with elevated serum levels are those in whom hK8 is overexpressed in the cancerous tissues. This proposal should be examined in the future by analyzing a larger set of tissues and serum from the same patients. Furthermore, we provide preliminary evidence that hK8 may have some value for ovarian cancer patient monitoring (Fig. 6).

Comparison of the data presented here for hK8 with information published previously on elevated serum and tissue levels of kallikreins

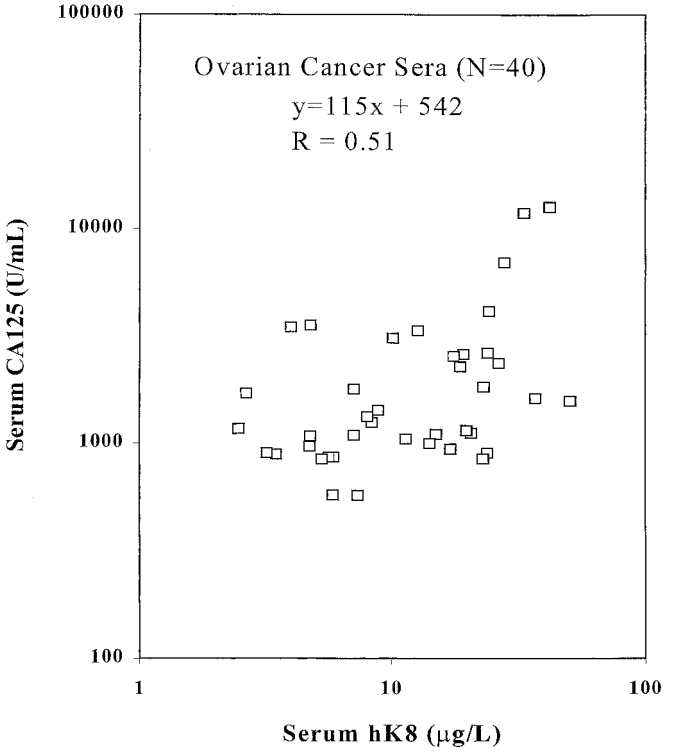

Fig. 5. Correlation between serum CA125 and hK8 concentration for 40 patients with ovarian cancer. The Pearson correlation coefficient was calculated after logarithmic transformation of the data. For discussion, see text.

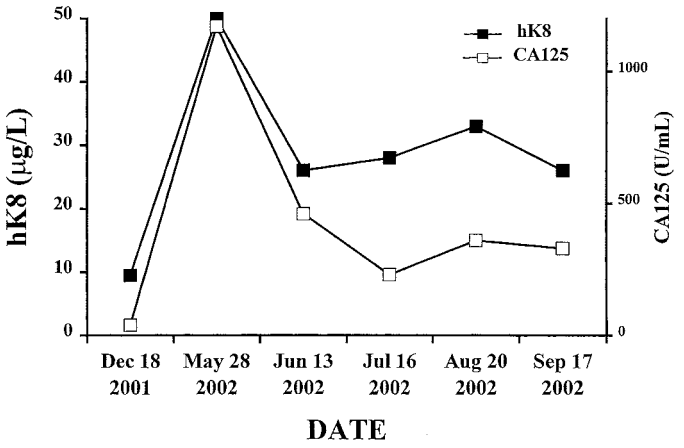

Fig. 6. Monitoring of an ovarian cancer patient with serum hK8 and serum CA125 analysis. Over the course of ~1 year, the fluctuations of the two markers were highly correlated. For discussion, see text.

hK6, hK10, and hK11 (4–6, 22) suggests that these four kallikreins, which are all elevated in ovarian cancer, may be part of an enzymatic cascade pathway involving multiple kallikreins (23).

It is now well accepted that a single cancer biomarker will likely not provide all of the necessary information for cancer diagnosis, monitoring, and prediction of therapeutic response. It seems reasonable that the classical ovarian cancer biomarker, CA125, should be tested in combination with the four kallikreins that have been reported to increase in ovarian cancer (hK6, hK8, hK10, and hK11), as well as other candidate biomarkers, to devise a diagnostic/prognostic panel for ovarian cancer (24, 25). In this respect, hK8 could be a member of this panel.

Many kallikreins interact with various circulating proteinase inhibitors (26, 27). We examined the molecular forms of hK8 in serum. Our assay detected a single peak of ~30 kDa, which corresponds to the free hK8 protein. It is thus likely that hK8 circulates in serum in its free form. Alternatively, it is possible that the fraction of hK8, which is bound to proteinase inhibitors (like α_2-macroglobulin), is not recognized by hk8-ELISA. More studies will be necessary to clarify these issues.

In conclusion, we here report, for the first time, hK8 overexpression in ovarian cancer tissues and high levels of hK8 in serum and ascites fluid of ovarian cancer patients. This kallikrein may be an additional diagnostic, prognostic, monitoring, and predictive marker of ovarian carcinoma.

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