

Disease processes may be reflected by correlations among tissue kallikrein proteases but not with proteolytic factors uPA and PAI-1 in primary ovarian carcinoma

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Abstract

In epithelial ovarian cancer, the high mortality rate is usually ascribed to late diagnosis, since these tumors commonly lack early-warning symptoms, but tumor-associated biomarkers useful for prognosis or therapy response prediction are in short supply. However, members of the tissue kallikrein serine protease family, the serine protease uPA and its inhibitor PAI-1, are associated with tumor progression of ovarian cancer. Therefore, we used ELISA to determine uPA, PAI-1, and tissue kallikreins hK5–8, 10, 11, and 13 in extracts of 142 primary tumor tissue specimens from ovarian cancer patients and studied the strength of association between protein expression levels of these tumor tissue-associated factors. uPA, PAI-1, hK5, and hK8 were related to FIGO stage; hK5 expression was higher in FIGO III/IV than in FIGO I/II patient tissues. PAI-1 and hK5 differed significantly according to nuclear grading; expression of hK5 was higher in G3 than in G1/2 tumors. Associations between uPA, PAI-1, and the tissue kallikreins were weak. There were strong pairwise correlations within the cluster of tissue kallikreins hK5, 6, 7, 8, 10, and 11, but their bivariate distributions depended on nuclear grading. These results support the notion that several tissue kallikreins are co-expressed in ovarian cancer patients, substantiating the existence of a steroid hormone-driven tissue kallikrein cascade in this disease.

Keywords: ovarian cancer; PAI-1; plasminogen activator; plasminogen activator inhibitor type-1; proteolytic factors; tissue kallikreins; uPA; urokinase.

Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy. The high mortality rate is usually ascribed to late diagnosis, since epithelial ovarian tumors commonly

Regarding a recommendation for future nomenclature of kallikrein gene-derived proteases, see the article 'A comprehensive nomenclature for serine proteases with homology to tissue kallikreins' by Lundwall et al., *Biol. Chem.* 387 (2006), pp. 637–641.

lack early warning symptoms (Cannistra, 2004). Ovarian carcinomas are quite heterogeneous, and the molecular pathways underlying their progression are still unidentified, precluding the development of individualized treatment strategies. In early and advanced ovarian cancer, clinical, histomorphological, and tumor biological markers useful for diagnosis, prognosis, and/or therapy response prediction are in short supply (Rosen et al., 2005). Staging (FIGO I–IV) of the disease at the time of diagnosis according to the guidelines of the International Federation of Gynecology and Obstetrics represents the major prognostic factor in ovarian cancer. Other established prognostic markers are nuclear grade, patient age, presence or absence of ascitic fluid, and residual tumor mass after primary surgery. Only a few tumor biological markers may serve as diagnostic markers, the most important being CA125 encoded by the *MUC16* gene (Whitehouse and Solomon, 2003). Therefore, tumor biomarkers that reliably predict the risk of ovarian cancer patients experiencing untimely disease recurrence, early death, or response to preoperative, adjuvant or palliative therapy are urgently needed, both for the early (FIGO I/II) and advanced stages (FIGO III/IV) of the disease (Johann et al., 2004; Bast et al., 2005; Garner, 2005).

In patients afflicted with ovarian cancer, two serine protease-dependent biological systems are of prime tumor biological and clinical interest: the plasminogen activation system and the tissue kallikrein system. With regard to the plasminogen activation system, the serine protease uPA (urokinase-type plasminogen activator), its receptor CD87, and the uPA inhibitor PAI-1 have emerged as markers of poor prognosis in patients with advanced ovarian cancer. Elevation of these proteolytic factors in tumor tissues of ovarian cancer patients indicates an enhanced risk of disease recurrence. Thus, shorter survival of these patients compared to ovarian cancer patients with low levels of these proteolytic factors in tumor tissue is observed (Gleeson et al., 1996; van der Burg et al., 1996; Kuhn et al., 1999; Borgfeldt et al., 2001; Konecny et al., 2001). Remarkably, the finding that both uPA and PAI-1 are indicators of poor prognosis in patients with cancer of the ovary or other organs is in contrast to the known, classical role of the inhibitor PAI-1 in blocking uPA enzymatic action. This surprising feature may be explained by the additional multifunctional roles of uPA and PAI-1 in cell adherence, cell motility, cell signaling, and cell proliferation (Reuning et al., 2003; Durand et al., 2004).

Of particular interest is the recent discovery that several of the members of the serine protease-type tissue kallikrein family of genes (*KLK1–15*), located on chromosome 19q13.4, may serve as diagnostic, prognostic, and/or predictive tumor biomarkers in cancer patients. Tissue kallikreins are expressed in tumor tissues of patients with hormonally regulated malignancies, such as

in the prostate, testis, breast, and ovary (Borgono and Diamandis, 2004). It is noteworthy that certain tissue kallikreins activate the single-chain pro-enzyme form of uPA (pro-uPA) to generate proteolytically active two-chain HMW-uPA, suggesting an interfacing role of the tissue kallikrein system with the plasminogen activation system (Frenette et al., 1997; List et al., 2000; Takayama et al., 2001).

In ovarian cancer patients, six of the 15 tissue kallikreins (hK4, 5, 6, 7, 10, 15) are markers of poor prognosis, whereas higher levels of five other tissue kallikreins (hK8, 9, 11, 13, 14) in ovarian cancer patients are associated with favorable prognosis (Yousef et al., 2003, 2005; Borgono and Diamandis, 2004; Borgono et al., 2004; Obiezu and Diamandis, 2005). In addition, tissue kallikreins 4, 6, and 10 are highly expressed in serous epithelial ovarian tumors, whereas increased expression of tissue kallikreins 5, 11, and 13 is more frequently found in non-serous tumors. Only seven tissue kallikreins have been determined by ELISA (hK5, 6, 8, 10, 11, 13, 14), eight by RT-PCR (*KLK4–9, 14, 15*), and one by immunohistochemistry (hK4). Although previous findings support the notion that particular tissue kallikreins may be directly involved in ovarian cancer progression and metastasis, no direct comparison of the protein expression levels of tissue kallikreins in one set of tumor tissue extracts from a prospective clinical study has been available in the scientific literature up to now.

Proteolytic processes in apoptosis, matrix remodeling, and blood coagulation are often organized in cascades, in which several proteases activate each other in consecutive order (Schenone et al., 2004; Shi, 2004; Frederick et al., 2005; Skrzydlewska et al., 2005). This may also be true for the tissue kallikrein family and interfacing members of the plasminogen activation system (Yousef and Diamandis, 2002; Clements et al., 2004). Hence, the existence of correlations is of interest, and, in particular, different ovarian cancer disease stages (e.g., early and advanced) could be associated with changing joint distributions of protein expression levels of tissue kallikreins hK5, 6, 7, 8, 10, 11, and 13, or of plasminogen activation system components uPA and PAI-1 in tumor tissue extracts. We demonstrate that pairwise correlations of protein expression levels tend to be strong within the subset of tissue kallikreins hK5, 6, 7, 8, 10, and 11, but are weak between this cluster and uPA/PAI-1 and hK13. Moreover, we present evidence for the hypothesized relationship between disease stage (in particular, nuclear grading) and the strength of correlations among the tissue kallikreins hK5, 6, 7, 8, 10, and 11.

Results

Antigen levels of nine proteolytic factors (uPA, PAI-1, tissue kallikreins hK5, 6, 7, 8, 10, 11, 13) in detergent-released extracts of primary tumor tissues of ovarian cancer patients FIGO I–IV, stratified by FIGO stage

A total of 142 tumor tissue samples encompassing 35 early-stage ovarian cancer patients (FIGO I n=25; FIGO

II n=10) and 107 advanced-stage patients (FIGO III n=78; FIGO IV n=28) were enrolled in a prospective study conducted at the Department of Obstetrics and Gynecology, Technical University of Munich, Germany, between 1985 and 1999. Nuclear grade was distributed as follows: G1, n=14; G2, n=41; G3, n=86; and one unknown. Detergent-released extracts of primary tumor tissue specimens were assessed by ELISA for protein levels of the serine protease uPA and its inhibitor PAI-1. Likewise, protein levels of the serine proteases tissue kallikreins hK5, 6, 7, 8, 10, 11, 13 were determined in these tumor tissue extracts. Levels were expressed as ng of analyte per mg of total protein in the tissue extract.

In all of the tissue extracts examined, uPA antigen was detected using a commercial uPA ELISA. The distribution of uPA antigen levels (Table 1) was positively skewed, ranging from 0.02 to 24.0 ng/mg protein (median 0.9, mean 1.7, SD 2.6). The commercial PAI-1 ELISA failed to detect PAI-1 antigen in only one patient. Similarly to uPA, the distribution of PAI-1 antigen levels (Table 1) was positively skewed, ranging from 0 to 389.8 ng/mg protein (median 14.7, mean 32.8, SD 54.7). The median level of PAI-1 was thus approximately 16-fold that of uPA by weight in tumor tissue extracts (uPA $M_r \approx 49\,000$; PAI-1 $M_r \approx 50\,000$).

In view of these skewed distributions, it was of interest to investigate a possible association between high levels and FIGO stage. There was no significant difference between patients with early (FIGO I/II) vs. advanced (FIGO III/IV) stages of the disease with respect to *central tendency* of uPA, according to the Mann-Whitney test (Table 2). However, a borderline significant difference in central tendency ($p=0.075$) was observed for PAI-1. More subtle associations between these factors and FIGO stage are discussed below.

Protein levels of tissue kallikreins hK5, 6, 7, 8, 10, 11, 13 were also determined in these tissue extracts using in-house ELISAs (Paliouras and Diamandis, 2006). Ovarian cancer patients often express hK5, 6, 7, 8, 10, 11, 13 protein in their primary cancer tissue (Table 1), reaching antigen levels between 155 (hK11) and 478 (hK8) ng/mg of tissue protein extracted, except for hK13, with a low absolute level of expression (maximum 18 ng/mg protein). However, in contrast to uPA and PAI-1, one or more of the tissue kallikreins could not be positively detected in a considerable number of patients, although the in-house ELISA test formats are of high sensitivity and specificity (Borgono et al., 2004; Yousef et al., 2005; Paliouras and Diamandis, 2006). Remarkably, 40.8% (hK5), 4.9% (hK6), 21.8% (hK7), 13.4% (hK8), 14.8% (hK11), 23.2% (hK11) and 62.0% (hK13) of the tumor tissue specimens were not positive for the antigens examined. Note that the detection rate of <50% in the case of hK13 implies that the median (but of course not the mean) is zero (Table 1).

As for uPA and PAI-1, all tissue kallikrein levels had skewed distributions. Testing for a possible difference between patients with early (FIGO I/II) and advanced (FIGO III/IV) stage with respect to the tissue kallikreins, there was no significant difference in central tendency for hK6, 7, 8, 10, 11, or 13. However, for hK5 we observed a statistically significant difference ($p=0.007$) between

Table 1 Distribution of tissue kallikreins hK5–8, 10, 11 and 13, uPA, and PAI-1 in tumor tissue extracts of ovarian cancer patients (FIGO I–IV).

	hK5	hK6	hK7	hK8	hK10	hK11	hK13	uPA	PAI-1
Number of samples	142	141	142	142	141	141	141	141	141
n=0 ^a	58	7	31	19	21	33	88	0 ^e	1
n>0 ^b	84	134	111	123	120	108	53	141	140
%=0 ^c	40.8	4.9	21.8	13.4	14.8	23.2	62.0	0	0.7
%>0 ^d	59.2	95.1	78.2	86.6	85.2	76.8	38.0	100	99.3
Concentration (ng/mg protein)									
Minimum	0	0	0	0	0	0	0	0.02	0.0
Maximum	219.6	432.6	283.6	477.8	233.6	155.4	18.4	24.0	389.8
Median	0.4	9.4	2.1	9.5	3.9	1.5	0.0	0.9	14.7
Mean	3.1	20.5	7.8	28.9	10.7	8.3	0.6	1.7	32.8
SD	18.6	44.2	26.0	58.7	22.4	21.4	2.2	2.6	54.7

^aNumber of values below the detection limit.

^bNumber of values above the detection limit.

^cPercentage of values below the detection limit.

^dPercentage of values above the detection limit.

^eLower detection limits of the assays are as follows: hK5, 0.1 ng/ml; hK6, 0.5 ng/ml; hK7, 0.2 ng/ml; hK8, 0.2 ng/ml; hK10, 0.05 ng/ml; hK11, 0.1 ng/ml; hK13, 0.05 ng/ml. Note that the tissue kallikrein antigen, uPA and PAI-1 values are expressed as ng of analyte per mg of extracted tissue protein. Values below the lower detection limit of the assays are coded as zero.

FIGO I/II and FIGO III/IV patients, corresponding to an elevated median level of 0.5 ng hK5 in FIGO III/IV compared to a median of 0 ng in FIGO I/II. Again, a median of zero means that in this FIGO subgroup more than half the patients had hK5 levels below the limit of detection.

Defining quartiles of all proteolytic factors and classifying FIGO status by FIGO I/II vs. FIGO III/IV, we found that uPA, PAI-1, hK5, and hK8 had significant relationships to FIGO status according to χ^2 tests. Qualitatively describing these relationships, the lower FIGO group is underrepresented in the highest (fourth) quartile of uPA compared to the third quartile ($p=0.022$). For PAI-1, the lower FIGO group is over-represented in the lowest (first) quartile of PAI-1 and under-represented in the third quartile ($p=0.022$). For hK5, the lower group is over-represented in the first quartile and over-represented in the

fourth ($p=0.008$). For hK8, the lower group is over-represented in the first and fourth quartiles and under-represented in the third. For hK11, the lower group is over-represented in the third quartile and under-represented in the fourth.

The differing significance of associations according to Mann-Whitney and χ^2 tests does not represent a contradiction, but rather complementary information, since the Mann-Whitney test summarized above describes the central tendency, whereas the comparison of quartiles as reflected in the χ^2 test reflects more finely resolved features of the statistical distributions.

Antigen levels determined by ELISA of nine proteolytic factors (uPA, PAI-1, tissue kallikreins hK5, 6, 7, 8, 10, 11, 13) in extracts of primary tumor tissues of ovarian cancer patients FIGO I–IV, stratified by nuclear grade

Less than half of the 142 tumor specimens were of nuclear grade G1 (highly differentiated) or grade G2 (medium cellular differentiation) (G1, n=14, 9.9%; G2, n=41, 29.9%); the majority were undifferentiated (G3, n=86, 61.0%) and nuclear grade was unknown for one patient (FIGO II).

In view of the skewed distributions of tissue antigens, it was also of interest to investigate their possible association with nuclear grading (Table 2). There was no significant difference for the antigens among G1, G2 and G3 with respect to the central tendency, except for hK5 ($p=0.004$; Kruskal-Wallis test). Using quartiles of the proteolytic factor distributions as above to study more detailed putative relationships with nuclear grading (differentiation), we found that the distributions of PAI-1 ($p=0.012$) and again hK5 ($p=0.023$) were significantly related to nuclear grading. Qualitatively speaking, the fourth (highest) quartile of PAI-1 was over-represented in poorly differentiated (high grade) tumors, as was the

Table 2 Level of significance of differences between FIGO I/II vs. FIGO III/IV or between nuclear grade G1/G2/G3 regarding the antigen levels of individual proteolytic factors (uPA, PAI-1, hK5, 6, 7, 8, 10, 11, 13) expressed in primary tumor tissue specimens.

Proteolytic factor analyzed	Level of significance between factor present in tumor tissue extracts	
	FIGO I/II vs. FIGO III/IV patients (Mann-Whitney test)	G1 vs. G2 vs. G3 patients (Kruskal-Wallis test)
uPA	0.206	0.340
PAI-1	0.075	0.106
hK5	0.007	0.004
hK6	0.270	0.740
hK7	0.784	0.558
hK8	0.753	0.602
hK10	0.286	0.662
hK11	0.535	0.159
hK13	0.150	0.810

fourth (highest) quartile of hK5. This association between the high quartile of hK5 and poor differentiation could account for the significant relationship with respect to central tendency observed in the Kruskal-Wallis test reported above.

Assessment of correlation between proteolytic factors uPA, PAI-1, and tissue kallikreins hK5, 6, 7, 8, 10, 11, 13

Non-parametric (Spearman) correlations of protein expression of the nine proteolytic factors (uPA, PAI-1, and tissue kallikreins hK5, 6, 7, 8, 10, 11, 13) determined in extracts of ovarian cancer tissues by ELISA are summarized in Table 3. Associations between uPA or PAI-1 and the tissue kallikreins were weak, though in some cases significant, as were those between hK13 and the other tissue kallikreins.

In contrast, there were significant, and (with only a few exceptions) strong correlations up to approximately R=0.78 between all pairs within the cluster of tissue kallikreins hK5, 6, 7, 8, 10, and 11. The bivariate distributions among these factors often depended significantly on nuclear grading: generally, the tightest correlations occurred in low-grade (G1) patients, weakening with poorer differentiation. As a typical example, the combination of hK8 vs. hK11 is displayed as a scatter diagram in Figure 1. Here, it is evident that there is a much tighter relation (less scatter) between these tissue kallikreins in patients with highly differentiated tumors of grade G1 (R=0.9) compared to tumor specimens classified as G2 (R=0.465) or G3 (R=0.660). Even though the numbers are small for G1 (n=14), Spearman correlations are very strong for four additional combinations: hK6 vs. hK7 (G1 0.94; G2 0.749; G3 0.731), hK6 vs. hK8 (G1 0.94; G2

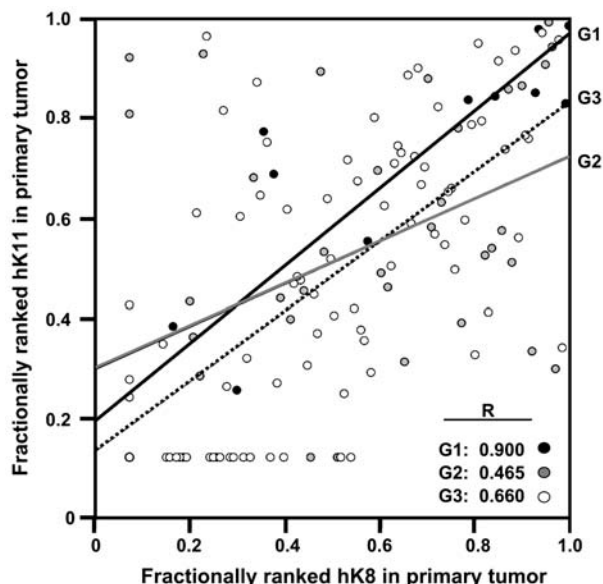


Figure 1 Correlation (Spearman) for hK8 vs. hK11 of fractionally ranked antigen values determined by ELISA in extracts obtained from primary tumor tissues of ovarian cancer patients (FIGO I-IV). The correlation factor R is depicted for patients with G1, G2, and G3 tumors separately.

0.85; G3 0.689), hK6 vs. hK11 (G1 0.8; G2 0.523; G3 0.595), and hK7 vs. hK8 (G1 0.87; G2 0.738; G3 0.796), all with R values exceeding 0.8.

It is also worth mentioning that nine of the G1 tumor specimens were from patients staged FIGO I (n=8) or FIGO II (n=1); only five cases of G1 were FIGO III (n=4) or FIGO IV (n=1).

Table 3 Correlation (R) between uPA, PAI-1, and tissue kallikreins hK5, 6, 7, 8, 10, 11, 13 with level of significance (p) determined in tumor tissue extracts of ovarian cancer patients FIGO I-IV.

		uPA	PAI-1	hK5	hK6	hK7	hK8	hK10	hK11
PAI-1	R	0.262							
	p	0.002							
	n	141							
hK5	R	0.086	-0.015						
	p	0.312	0.856						
	n	141	141						
hK6	R	0.181	-0.148	0.560					
	p	0.031	0.079	< 0.001					
	n	141	141	141					
hK7	R	0.229	-0.122	0.606	0.760				
	p	0.006	0.149	< 0.001	< 0.001				
	n	141	141	142	141				
hK8	R	0.139	-0.236	0.507	0.772	0.779			
	p	0.101	0.005	< 0.001	< 0.001	< 0.001			
	n	141	141	142	141	142			
hK10	R	0.176	-0.126	0.410	0.662	0.701	0.613		
	p	0.037	0.138	<0.001	< 0.001	< 0.001	< 0.001		
	n	141	141	141	141	141	141		
hK11	R	0.086	-0.273	0.323	0.594	0.635	0.627	0.682	
	p	0.310	0.001	<0.001	< 0.001	< 0.001	< 0.001	< 0.001	
	n	141	141	141	141	141	141	141	
hK13	R	-0.103	-0.060	0.152	0.218	0.171	0.160	0.248	0.270
	p	0.224	0.483	0.073	0.010	0.042	0.058	0.003	0.001
	n	140	140	141	140	141	141	140	140

Normal font: no or a low level of correlation; bold font: moderate to strong correlation.

Discussion

Tissue remodeling, involving the degradation of structural and extracellular matrix proteins – thereby mediating cell migration in embryogenesis, pregnancy, wound healing, and angiogenesis – is a key physiological function involving members of the plasminogen activation system such as uPA and its inhibitor PAI-1 (Schmitt et al., 1997; Reuning et al., 1998; Myohanen and Vaheri, 2004; Castellino and Ploplis, 2005). Tissue kallikreins, another type of degradative enzymes, display additional, diverse physiological functions, from the regulation of blood pressure and electrolyte balance to tissue remodeling, prohormone processing, neural plasticity, and skin desquamation (Borgono and Diamandis, 2004; Borgono et al., 2004). Recent evidence suggests that tissue kallikreins may be involved in cascade reactions, and that cross-talk may exist with proteases of similar or different catalytic types, such as the plasminogen activation system (Frenette et al., 1997; List et al., 2000; Takayama et al. 2001).

Dysregulated expression of tissue kallikreins, uPA, and PAI-1 is also a feature of malignancy (Schmitt et al., 1997; Borgono and Diamandis, 2004). In ovarian cancer patients, uPA and its inhibitor PAI-1 have emerged as markers of poor prognosis, indicating an elevated risk of early disease recurrence (metastasis) and poorer survival compared to patients with low levels of these proteolytic factors in tumor tissue (Gleeson et al., 1996; van der Burg et al., 1996; Kuhn et al., 1999; Borgfeldt et al., 2001). Several of the tissue kallikreins (hK4–8, 10, 11, and 13–15) are overexpressed in ovarian carcinoma tissues and are often associated with patient prognosis (Yousef et al., 2003; Borgono and Diamandis, 2004; Borgono et al., 2004; Obiezu and Diamandis, 2005). *KLK4*, *KLK5*/hK5, *KLK6*/hK6, *KLK7*, hK10, and *KLK15* mRNA or proteins are markers of poor prognosis in ovarian cancer; elevated levels are correlated with more aggressive forms of ovarian cancer and decreased disease-free and overall survival. *KLK8*, *KLK9*, hK11, hK13, and *KLK14*, however, are markers of favorable prognosis (Borgono et al., 2004).

Several reports have previously indicated an association between dysregulated tissue kallikrein expression and ovarian cancer progression and the potential use of tissue kallikreins as diagnostic/prognostic biomarkers for this type of cancer. However, little was previously known about the joint distributional characteristics of tissue kallikrein levels or about their correlations with the proteolytic factors uPA and PAI-1. Determination of these characteristics requires measurement and direct comparison of all these factors in one sample of tumor specimens, as carried out here.

We observed that uPA and PAI-1 are present in tissues from almost all of the ovarian cancer patients analyzed, independent of tumor stage or cellular differentiation, indicating concerted expression of these two proteolytic factors. We also noted that several of the ovarian cancer patients assessed did not express detectable tissue kallikrein protein at all, although the detection limit of the assays is in the low-nanogram range. This finding is especially remarkable for hK5, hK7, hK11, and hK13, with high proportions (41%, 22%, 23%, and 62%,

respectively) of patients negative for these tissue kallikreins, indicating non-concerted tissue kallikrein protein expression in some patients, but not in all.

An association of the central tendency (median) of the distributions of the nine proteolytic factors with FIGO stage was evident only in the case of hK5; the median for hK5 was lower in FIGO I/II than in FIGO III/IV patients. In addition, some of the quartiles for uPA, PAI-1, and hK8 depended on FIGO status. Similarly, only hK5 showed an association of the median with poorer grading (differentiation). The highest quartiles for both PAI-1 and hK5 were over-represented in G3 tumors.

The moderate to strong mutual correlations found within the cluster hK5, hK6, hK7, hK8, hK10, and hK11 (particularly among the trio hK6, hK7, hK8) for the collective as a whole would support the hypothesis of concerted expression of proteolytic factors within this cluster. The bivariate distributions among these factors depended significantly on nuclear grading, weakening with poorer differentiation. Particularly noteworthy were the tight correlations observed in the subgroup of G1 patients, particularly for the combinations of hK6 vs. hK7, hK6 vs. hK8, hK6 vs. hK11, hK7 vs. hK8, and hK8 vs. hK11, with $R > 0.8$.

On the other hand, for uPA, PAI-1, and hK13, no considerable correlations with any of the other proteolytic factors were observed, even considering the highly differentiated subgroup. From a tumor biological view, it might be considered that patients with highly differentiated tumors G1 were at an early stage of cellular dedifferentiation. Bearing in mind that approximately two-thirds of these patients were in early-stage FIGO I/II, we speculate that the particularly tight correlations among hK6, 7, 8, and 11 in this group of low-risk patients might hint at molecular processes with a common cause in the early phase of ovarian cancer progression. As tumors become less differentiated, it would generally be expected that additional processes would be unleashed, thereby adding additional scatter to these relationships, consistent with observations for the collective as a whole.

It should be pointed out that the tissue kallikreins are subject to regulation of gene expression and protein function, as all of the human tissue kallikrein genes are under steroid hormone regulation in endocrine-related tissues (Borgono and Diamandis, 2004; Borgono et al., 2004). In addition, while measuring tumor-associated proteolytic factors in tumor tissue extracts, possible post-translational modifications or proteolytic activation/degradation of the tissue kallikrein proteins expressed should be considered. In this case, certain putative molecular formations of the tissue kallikreins, including tissue kallikrein-inhibitor complexes, may escape detection by the antibodies used in the ELISA test kits, because these antibodies have been generated against recombinant tissue kallikreins.

In previous investigations, cut-off values have been estimated for particular tissue kallikreins, uPA, and PAI-1, to allow assessment of the univariate clinical impact of individual proteolytic factors (Kuhn et al., 1999; Diamandis et al., 2000, 2003; Luo et al., 2001; Luo et al., 2004; Magklara et al., 2001; Yousef et al., 2001; Borgono

et al., 2003; Kishi et al., 2003; Scorilas et al., 2004). Bearing in mind that multiple factors are involved, cut-off values for individual factors do not provide an adequate basis for clinical decision support, since classification of low- vs. high-risk groups according to a cut-off for one such factor will not necessarily be consistent with the classification according to a different factor.

The present work suggests that the role of these proteolytic factors as markers for processes associated with tumor progression could be reflected in their collective behavior. Hence, optimal use of these factors for the definition of prognostic risk groups and support of clinical decisions will require multivariate analyses and weighted risk assessment, taking into account not just single tissue kallikreins, but rather a panel of tissue kallikreins, uPA and PAI-1, as well as all available clinical and histomorphological parameters.

Multivariate scoring models are useful for combining multiple tumor biomarkers to achieve diagnostic, prognostic, or predictive accuracy greater than that of single tumor biomarkers alone. In view of the biological interrelationships among the proteolytic factors studied here, a particularly promising approach for discriminating and scoring model development could be to utilize advanced multivariate statistical methods (Kates et al., 2003) such as neural networks or decision tree analysis (Burke et al., 1997; De Laurentiis et al., 1999; Zhang et al., 1999; Harbeck et al., 2000; Jerez-Aragones et al., 2003) capable of modeling non-linear interactions. Appropriate statistical modeling techniques could further improve the clinical utility of tissue kallikreins, possibly in combination with other suitable molecules, as novel and powerful biomarkers for surgical success, disease progression, or response to therapy, ultimately providing considerable benefit to ovarian cancer patients.

Materials and methods

Patients

A total of 142 patients afflicted with ovarian cancer stage FIGO I–IV (Fédération Internationale de Gynécologie et d'Obstétrique) in 1985–1999 were enrolled in a prospective study conducted at the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technical University of Munich, Germany. Standard surgical procedures were performed, including partial resection of the small and large intestine, diaphragmatic peritoneum, peritonectomies and upper abdominal surgery, as well as pelvic and para-aortic lymphadenectomy if indicated (Kuhn et al., 1994, 1999; Schmalfeldt et al., 1995). In younger patients (<35 years) with tumor stage FIGO I, less radical surgery was performed to preserve patient fertility (Schmalfeldt et al., 1995). The study to collect tissue from ovarian cancer patients to assess the proteolytic factor profiles was approved by the Ethics Committee of the University Hospital Klinikum rechts der Isar of the Technical University of Munich. Following surgery, all patients received adjuvant treatment according to consensus recommendations at that time. None of the patients received any neoadjuvant therapy before surgery.

Laboratory methods

Tissue samples from primary ovarian cancer patients were collected during surgery, classified by a pathologist, and stored in

liquid nitrogen until use. Deep-frozen specimens of 200–500 mg wet weight were pulverized using a Micro-Dismembrator II bead mill apparatus (Sartorius, Göttingen, Germany) and immediately suspended in 2 ml of Tris-buffered saline (TBS; 0.02 M Tris-HCl, pH 8.5, 125 mM sodium chloride), 1% (w/v) Triton X-100 (Sigma, Munich, Germany). Extraction was conducted at 4°C for 12 h followed by ultracentrifugation at 100 000 *g* for 45 min to separate cell debris. The supernatant was collected, aliquoted, and stored in liquid nitrogen until further use.

uPA, PAI-1, and hK5, 6, 7, 8, 9, 11, 13 antigen concentrations were determined in the supernatant using commercially available ELISA kits (Imubind uPA #894, Imubind PAI-1 #821; American Diagnostica, Stamford, CT, USA) (Kuhn et al., 1999), and non-commercial in-house ELISA test formats for the respective tissue kallikreins (Paliouras and Diamandis, 2006). For this, capture antibodies were generated by immunizing mice with recombinant human tissue kallikrein proteins (monoclonal antibodies for hK5, hK11, hK13, polyclonal antibodies for hK6, hK7, hK8, hK10). Polyclonal detection antibodies were obtained by immunizing rabbits with these tissue kallikreins. Detection limits were as follows: hK5, 0.1–50 ng/ml; hK6, 0.5–200 ng/ml; hK7, 0.2–10 ng/ml; hK8, 0.2–20 ng/ml; hK10, 0.05–10 ng/ml; hK11, 0.1–50 ng/ml; and hK13, 0.05–20 ng/ml (Paliouras and Diamandis, 2006). In these ELISA formats, no cross-reactivity with any other member of the human tissue kallikrein family was detected. uPA, PAI-1, and the tissue kallikrein antigen values were expressed as ng/mg of protein, which was determined in the tissue extracts by the Pierce BCA method (Kuhn et al., 1999).

Statistics

Distributions were characterized in terms of minimum, maximum, median, mean, SD, and number of values detected. Antigen determinations were performed in duplicate. Since the univariate distributions of all proteolytic factors departed considerably from a normal distribution, fractional ranks were constructed for all these factors. Analyte measurements below the limits of sensitivity were coded as zero; note however, that since all statistical tests were non-parametric, coding these zero values as one-half of the detection limit would have led to the same results. For analytes with many zero values, the lowest fractional rank is obviously somewhat greater than zero, because the rank in case of ties is defined at the midpoint. Of interest are the joint distributions of proteolytic factors uPA, PAI-1, and the tissue kallikreins hK5, 6, 7, 8, 10, 11, 13 in the population as a whole, and particularly in subgroups defined by nuclear grades G1, G2, and G3 and FIGO classification I, II, III, and IV. All correlations are Spearman (rank) correlations with respect to the original analytes. Missing values were excluded pairwise. The level of significance was set to $p < 0.05$. We described correlations $R \geq 0.5$ as strong. Associations between FIGO status and central tendency (average rank) of proteolytic factors were studied by grouping factors in FIGO stage I/II or stage III/IV and then performing the Mann-Whitney U-test. Associations between nuclear grade (in the three classes G1, G2, and G3) and central tendency of proteolytic factor distributions were studied by performing the Kruskal-Wallis test. More detailed relationships (i.e., those not affecting central tendency) were studied by defining quartiles of all proteolytic factors and performing χ^2 tests.

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