A Multiparametric Panel for Ovarian Cancer Diagnosis, Prognosis, and Response to Chemotherapy

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Abstract

Purpose: Our goal was to examine a panel of 11 biochemical variables, measured in cytosolic extracts of ovarian tissues (normal, benign, and malignant) by quantitative ELISAs for their ability to diagnose, prognose, and predict response to chemotherapy of ovarian cancer patients.

Experimental Design: Eleven proteins were measured (9 kallikreins, B7-H4, and CA125) in cytosolic extracts of 259 ovarian tumor tissues, 50 tissues from benign conditions, 35 normal tissues, and 44 tissues from nonovarian tumors that metastasized to the ovary. Odds ratios and hazard ratios and their 95% confidence interval were calculated. Time-dependent receiver operating characteristic curves for censored survival data were used to evaluate the performance of the biomarkers. Resampling was used to validate the performance.

Results: Most biomarkers effectively separated cancer from noncancer groups. A composite marker provided an area under the curve of 0.97 (95% confidence interval, 0.95-0.99) for discriminating normal and cancer groups. Univariately, hK5 and hK6 were positively associated with progression. After adjusting for clinical variables in multivariate analysis, both hK10 and hK11 significantly predicted time to progression. Increasing levels of hK13 were associated with chemotherapy response, and the predictive power of hK13 to chemotherapy response was improved by a panel of five biomarkers.

Conclusions: The evidence shows that a group of kallikreins and multiparametric combinations with other biomarkers and clinical variables can significantly assist with ovarian cancer classification, prognosis, and response to platinum-based chemotherapy. In particular, we developed a multiparametric strategy for predicting ovarian cancer response to chemotherapy, comprising several biomarkers and clinical features.

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Epithelial ovarian cancer is the fourth leading cause of cancer-related deaths and the most lethal gynecologic malignancy among women in the United States (1). Early-stage (stage I or II) ovarian cancer has excellent prognosis if treated, but late ovarian cancer (stage III-IV), which is found in ~70% of all patients, is associated with poor survival (10-30%; ref. 2). Overall, survival rates for this cancer have not changed over the past 2 decades despite the availability of new cytotoxic treatments (3). Hence, improvement of long-term survival in patients with ovarian cancer is dependent on early detection. New technological advances, including microarrays and proteomics, promise to identify molecular signatures of early disease and novel ways for early diagnosis, classification, and prognosis (4). However, until effective screening strategies become available, the optimal management of ovarian cancer patients will depend partially on biochemical and clinical prognostic and predictive factors.

Prognostic indicators improve the accuracy of predicting patient outcomes. On the other hand, predictive indicators help institute more individualized treatments because they guide the physician on the likelihood of response to specific therapeutic

agents. The traditional clinicopathologic variables of prognosis in ovarian cancer, such as stage, grade, tumor size, residual tumor after surgery, age, and presence or absence of ascites, although highly useful, still have limitations in predicting the outcome of individual patients due to disease heterogeneity. Therefore, there is a need to discover and validate biomarkers that provide independent prognostic and predictive information so that treatment strategies can be tailored to individual patients. A large number of new biomarkers with prognostic and predictive potential in ovarian cancer have been discovered (5–7). A 115-gene prognostic signature known as "Ovarian Cancer Prognostic Profile," which seems to have independent prognostic value, was recently identified by microarray analysis (6, 7).

Despite many efforts to discover discrete novel prognostic, predictive, and diagnostic biomarkers for many cancer types, it is now believed that multiparametric analysis of many different markers offers several advantages (8). In this respect, the group of serine proteases, known as human tissue kallikreins, is highly suited for such multiparametric analysis. Already, many members of this family have been shown in previous studies to have independent prognostic, predictive, and diagnostic value. These findings have been recently reviewed (9, 10). Another protein, known as B7-H4, has recently been shown to have value as a serologic diagnostic marker for ovarian cancer (11). Highly sensitive and specific ELISAs have now been developed for multiple kallikreins and B7-H4 (10, 11).

The aim of the present study was to analyze by quantitative ELISA methodologies nine members of the human tissue kallikrein family, B7-H4, and the traditional ovarian cancer biomarker, CA125, in a large collection of ovarian carcinoma tissue cytosolic extracts and correlate, at univariate and multivariate levels, and with various statistical methods, their ability to separate cancer from noncancer patients, their combined prognostic value on patient survival, and their predictive value on response to chemotherapy.

Materials and Methods

Ovarian cancer patients and specimens. Two hundred and fifty-nine patients with primary epithelial ovarian cancer were included in this study, ranging in age from 20 to 85 years, with a median of 57 years. Patients were monitored for survival and disease progression. Among these 259 patients, 126 experienced at least one disease recurrence and death occurred in 117 patients. A total of 149 experienced either disease progression or death, with a median progression-free survival time of 30 months. The median follow-up time of patients alive was 50 months (range, 1-150 months). Among 240 patients with known response to chemotherapy, 19 (8%) experienced progression or had no change, 41 (17%) had partial responses, and 180 (75%) had complete responses.

After surgery, all patients were treated with platinum-based chemotherapy. The first-line chemotherapy regimens included cisplatin (for 56% of patients), carboplatin (30%), cyclophosphamide (41%), doxorubicin (7%), epirubicin (12%), paclitaxel (16%), and methotrexate (1%). To assess response to chemotherapy, we defined complete response as a resolution of all evidence of disease for at least 1 month; partial response was defined as a decrease (for at least 1 month) of at least half in the diameters of all measurable lesions without the development of new lesions; stable disease was defined as a decrease of <25% in the diameters of all measurable lesions; and progressive disease was defined as an increase of at least 25%.

Histologic examination, done during intrasurgery frozen section analysis, allowed representative portions of each tumor, containing >80% tumor cells, to be selected for storage until analysis. Clinical and pathologic information documented at the time of surgery included disease stage, tumor grade, histotype, and debulking success. The staging of tumors was in accordance with the International Federation of Gynecologists and Obstetricians criteria (12), grading was established according to Day et al. (13), and the classification of histotypes was based on both the WHO and International Federation of Gynecologists and Obstetricians recommendations (14).

Patients with disease of clinical stages I to IV and tumor grades 1 to 3 were represented in this study. Of the 250 tumors with known histologic type, 110 (44%) were of the serous papillary histotype, 84 (34%) represented other epithelial histotypes, and 56 (22%) were undifferentiated.

Included in this study were also 50 tissues obtained at surgery from patients with benign gynecologic conditions (including endometriosis, mucinous cystadenomas, dermoid cysts, ovarian benign teratomas, and corpus luteum), 44 tissues from patients with non-ovarian primary tumors that metastasized to the ovary (from the gastrointestinal tract, endometrium, uterus, or breast), and 35 tissues from patients with ovaries without any pathologies (normal ovarian tissues). Age distributions of the four groups were similar; median ages for patients with primary ovarian cancer, benign diseases, metastatic cancer, and normal ovarian tissues were 57, 50, 55, and 50, respectively.

Investigations were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983, and were approved by the Institutional Review Boards of Mount Sinai Hospital and the University of Turin (all clinical samples came from the latter institution).

Preparation of cytosolic extracts. All specimens were snap frozen in liquid nitrogen immediately after surgery and stored at -80°C until extraction. Frozen tissues (20-100 mg) were pulverized on dry ice to a fine powder and added to 10 volumes of extraction buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 g/L NP40 surfactant, 1 mmol/L phenylmethylsulfonyl fluoride, 1 g/L aprotinin, 1 g/L leupeptin]. The resulting suspensions were incubated on ice for 30 min with repeated shaking and vortexing every 10 min. The mixtures were then centrifuged at 14,000 × *g* at 4°C for 30 min and the supernatant (cytosolic extract) was collected and stored at -80°C until further analysis. Protein concentration of the extracts was determined using the bicinchoninic acid method with bovine serum albumin as standard (Pierce Chemical Co.).

Measurement of biomarkers in ovarian cytosolic extracts. The concentration of the examined biomarkers in cytosolic extracts was measured by using highly sensitive and specific noncompetitive "sandwich-type" ELISAs, developed either at Mount Sinai Hospital (nine kallikreins) or at diaDexus (B7-H4). Most of these assays have been evaluated and published elsewhere (10, 11). In short, all assays are based on mouse monoclonal antibody capture and detection antibodies, except hK4, hK11, and hK14 (mouse monoclonal capture; rabbit polyclonal for detection).

The concentration of the classic ovarian cancer biomarker CA125 in tumor cytosols was measured using the Immulite 2000 automated assay (Diagnostic Products Corp.).

The concentration of all analytes was expressed as pg of analyte per mg of total protein or unit/mg of total protein (for CA125) to account for the amount of tissue extracted.

Data analysis and statistics. The relationships between biomarkers with patient and tumor characteristics were examined with Kruskal-Wallis test, a nonparametric method for examining differences among multiple groups. Spearman's rank correlation coefficient was used to assess the correlations among biomarkers. The primary outcome for survival analyses was the progression-free survival, defined as the time from diagnosis to ovarian cancer recurrence or death from any cause. Patients alive and not meeting any events, as defined by these end points, were censored at the time the last vital status was ascertained.

Kaplan-Meier curves were used to present the survival probabilities as a function of time among groups of patients, defined by the tertile of the marker values, and log-rank tests were used to examine the overall difference among the curves. Cox regression model was applied to evaluate the hazard ratios (HR) of biomarkers on progression-free survival. Clinical variables, including age, stage, grade, debulking, and histologic type, were adjusted in multivariate Cox proportional hazards models. Logistic regression was done to calculate the odds ratio (OR) that defines the relation between biomarkers and response to therapy, where the outcome is response (partial response or complete response) versus no response (no change or progression). Both HR and OR were calculated on log-transformed biomarkers and represented with their 95% confidence interval (95% CI) and two-sided *P* values.

To further evaluate the diagnostic or prognostic usefulness of the markers based on dichotomous classification, we considered receiver operating characteristic (ROC) curve analysis. A cutoff point was used to define a positive or negative marker result. For markers measured on continuous scales, a ROC curve is a plot of the true-positive fraction versus the false-positive fraction, evaluated for all possible cutoff point values. For binary outcome (i.e., response to chemotherapy), the ROC curve quantifies the discriminatory ability of a marker for separating those who responded from those who did not. For time to progression analysis, where the disease outcome is not concurrent with the test and the accuracy is a function of time, time-dependent ROC techniques (15) for censored survival times were considered. We compared the true-positive fraction, P (marker > cutoff point|death within t year), and false-positive fraction, P (marker > cutoff point|survived beyond t year), across all possible cutoff points, and for t equal to 1 and 5 years, respectively. For each ROC curve, we calculated the area under the curve (AUC), which ranges from 0.5 (for a noninformative marker) to 1 (for a perfect marker) and corresponds to the probability that a randomly selected patient who dies within t years has a higher marker value than a randomly selected patient who survived. Bootstrap method was used to calculate the confidence intervals for AUC.

The ROC analysis was first conducted on individual markers and then in combination to explore the potential that a marker panel can lead to improved performance. We considered an algorithm that renders a single composite score using the linear predictor fitted from a binary regression model. This algorithm has been justified to be optimal under the linearity assumption (16, 17) in the sense that ROC curve is maximized (i.e., best sensitivity) at every threshold value. In particular, a weighted logistic regression that is appropriate for censored failure time data was used (18) for deriving the prognostic index. A stepwise regression procedure was used to select markers in the panel, sometimes along with clinical variables.

Because an independent validation series was not available for this study, the predictive accuracy of the composite scores was evaluated based on resampling of the original data. Specifically, we randomly split the data into a training set and a validation set. The training set included two thirds of the observations, and the validation set included one third of the observations. Using the training set, we first did model selection from which the final selected model gave rise to the linear combination rule. We then calculated two ROC curves for the linear score: one using data from the training set and the other from the validation set. The vertical differences between the two ROC curves gave the overestimation of the sensitivities at given specificities. The whole procedure was repeated 200 times, and these differences were averaged to yield an estimate of the expected overestimation. We present both the original ROC curves and the ROC curves that are corrected for overestimation.

All analyses were done using Statistical Analysis System 9.1 (SAS Institute) and S-Plus 7.0 software (Insightful Corp.).

Results

Distribution of biomarkers in ovarian tissues. Ovarian tissue extracts from four groups of patients were used: healthy women

Table 1. Associations of biomarkers with clinical features

	n	CA125 (median*)	hK5 (median)	hK6 (median)	hK7 (median)	hK8 (median)	hK14 (median)	B7-H4 (median)
Age (y)								
≤55	113	1,354	0.84	2.43	3.19	0.43	0.04	758
>55	134	1,281	0.79	2.98	2.24	0.64	0.05	1,101
P †		0.89	0.74	0.54	0.28	0.43	0.60	0.19
Debulking								
OD	140	843	0.29	1.73	1.52	0.54	0.03	650
SD	103	1,738	1.90	4.19	4.47	0.63	0.10	1,414
P		0.07	<0.001	0.0002	<0.001	0.24	0.0010	0.001
Grade								
G_1	58	1,324	0.18	1.59	1.42	0.38	0.03	419
G_2	45	1,470	0.35	1.75	1.47	0.63	0.04	623
G_3	139	1,353	1.93	3.50	4.34	0.58	0.07	2,200
P		0.95	<0.001	0.04	<0.001	0.72	0.08	<0.001
Stage								
I	65	422	0.12	0.32	0.67	0.11	0.02	500
II	20	2,860	0.32	3.33	1.66	0.56	0.03	973
III	143	1,470	1.82	3.84	3.89	0.68	0.08	1,229
IV	18	1,166	1.87	3.94	4.06	0.42	0.05	2,121
P		0.07	<0.001	<0.001	<0.001	0.02	0.002	0.003
Histology								
Serous	110	1,805	1.54	4.40	3.86	0.71	0.08	1,269
Epi	84	623	0.18	1.32	1.21	0.23	0.02	839
Undiff	56	584	0.80	1.67	2.36	0.39	0.04	628
P		0.0004	<0.001	<0.001	<0.001	0.002	0.05	0.30

Abbreviations: OD, optimal debulking; SD, suboptimal debulking; Epi, nonserous epithelial; Undiff, undifferentiated.
*Values are in unit/mg of total protein for CA125 and pg/mg of total protein for all other variables.
† *P* values are from global nonparametric Kruskal-Wallis test for testing the association between a marker and a clinical variable.

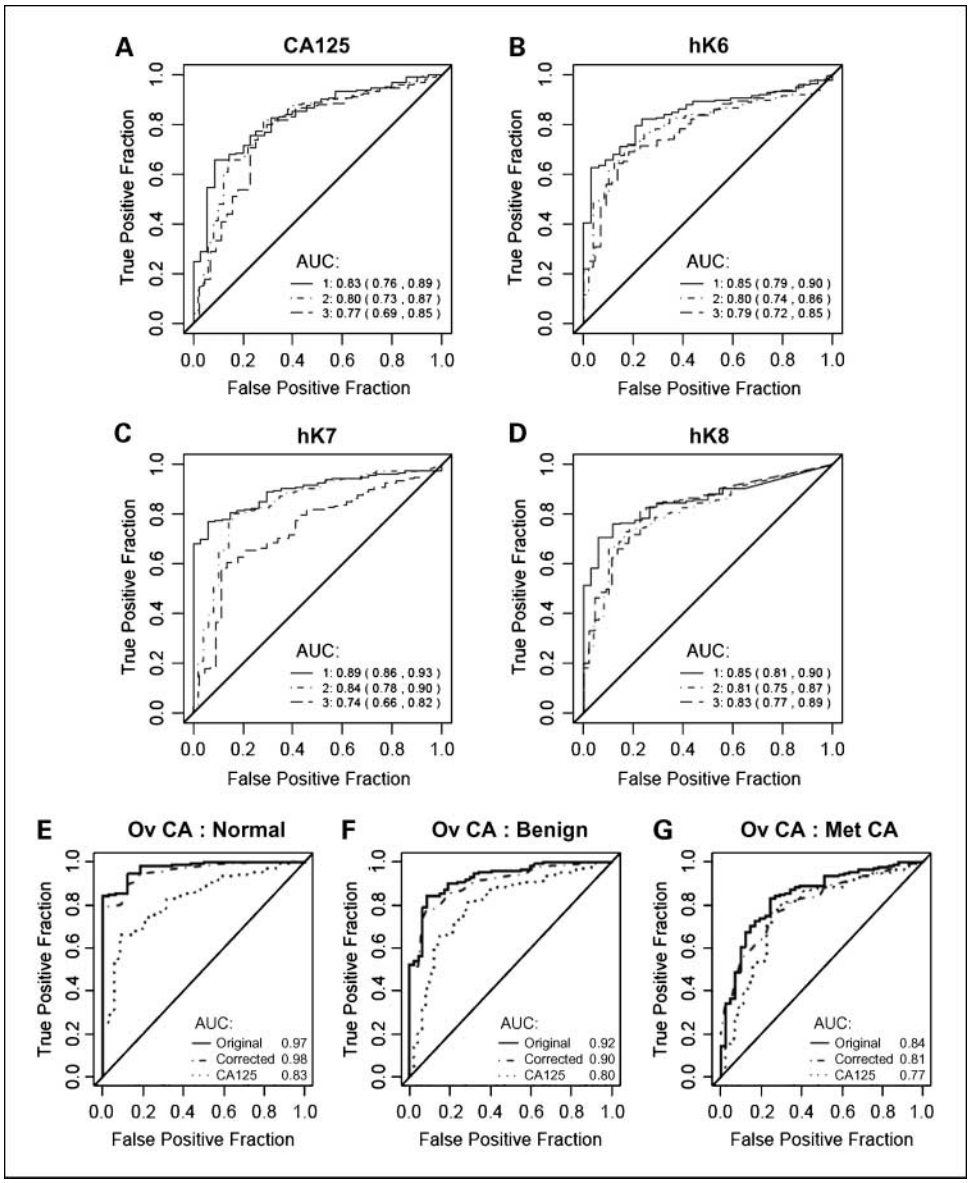

Fig. 1. *A* to *D,* ROC curves for CA125, hK6, hK7, and hK8. Each panel presents ROC curves of an individual marker for distinguishing primary ovarian tumors from normal ovarian tissue (*solid line; 1*), benign (*dotted line; 2*), and other primary cancers that metastasized to ovary (*dashed line; 3*). *E* to *G,* ROC curves for the combined marker, without (*Original*) and with correction for overfitting (*Corrected*), and for CA125 alone (*CA125*). The correction for overfitting was done by the cross-validation procedure described in Materials and Methods. The combined model for ovarian cancer versus the other three groups is further described in Table 1. *Ov CA,* ovarian cancer; *Met CA,* primary nonovarian cancer metastatic to ovary.

(n = 35), benign gynecologic diseases (n = 50), primary tumors from other organs that metastasized to the ovary (n = 44), and primary ovarian cancer (n = 259). Most of the kallikreins (except hK4) have very good discriminatory capacity in distinguishing between ovarian cancer patients and women with either no pathology (normal) or benign conditions (all P values of pairwise Wilcoxon Rank sum test < 0.01). Their diagnostic/discriminatory performances were comparable or superior to that of CA125. Many of these markers (e.g., hK6, hK7, and hK8) showed good accuracy in separating primary ovarian cancer from other primary cancers that metastasized to ovary, indicating that they may represent relatively specific markers for this disease.

Table 1 presents distributions of individual markers, stratified by clinical features. Only markers that were significantly associated with at least one clinical variable are included in the table. Higher values of CA125 and hK8 are significantly associated with serous histology types (P = 0.0004 and 0.002, respectively), whereas hK5, hK6, and hK7 are associated with all clinical variables, including debulking success, grade, stage, and histology type (all $P < 0.05$).

B7-H4 levels are associated with debulking success, grade, and stage but not histology type. Median values are comparable between the two age groups across all markers.

Figure 1A to D presents the ROC curves for CA125 and three selected kallikreins. The descriptive analyses of the distributions of all individual biomarkers, their relation to disease stage, and their individual ability to discriminate between the four groups of patients are given as Supplementary Data (Supplementary Figs. S1-S11). We also developed a "combined" marker using logistic regression with stepwise selection. Figure 1E to G summarizes these data. The combined marker was far superior to CA125 or any other individual marker in separating ovarian cancer from the other three groups after adjusting for overfitting. In Supplementary Table S1, we summarize the AUC for all individual markers and the combined marker for distinguishing ovarian cancer from all other groups. An AUC of 0.97 (highest value) was achieved with the combined marker.

Table 2. Univariate and multivariate Cox regression analysis for progression-free survival

Clinical variables	*n* (%)	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Age (y)					
≤55	113 (46)	1.00	0.11	1.00	0.18
>55	134 (54)	1.31 (0.94-1.82)		1.27 (0.9-1.78)	
Stage					
I	65 (26)	1.00	<0.001	1.00	0.0013
II	20 (8)	1.01 (0.33-3.07)		0.99 (0.32-3.01)	
III	143 (58)	6.43 (3.67-11.26)		3.08 (1.59-5.97)	
IV	18 (7)	15.21 (7.34-31.51)		4.79 (2.02-11.35)	
Grade					
G_1	58 (24)	1.00	<0.001	1.00	0.41
G_2	45 (19)	3.28 (1.72-6.24)		1.6 (0.77-3.31)	
G_3	139 (57)	5.07 (2.89-8.89)		1.56 (0.79-3.11)	
Histology					
Serous	110 (44)	1.00	<0.001	1.00	0.96
Epi	84 (34)	0.46 (0.31-0.67)		0.94 (0.61-1.46)	
Undiff	56 (22)	0.63 (0.41-09.6)		1.00 (0.63-1.58)	
Debulking					
OD	140 (58)	1.00	<0.001	1.00	<0.001
SD	103 (42)	6.27 (4.37-8.99)		2.85 (1.84-4.42)	
Markers				**Adjusting for clinical variables**	
CA125	259	1.06 (0.89-1.25)	0.53	0.82 (0.65-1.04)	0.10
hK4	259	1.07 (0.9-1.27)	0.45	1.15 (0.96-1.39)	0.13
hK5	259	1.39 (1.15-1.67)	0.0005	0.86 (0.69-1.06)	0.16
hK6	259	1.39 (1.15-1.67)	0.0005	1.02 (0.8-1.29)	0.89
hK7	259	1.23 (0.97-1.55)	0.08	0.76 (0.58-1.01)	0.06
hK8	259	1.12 (0.96-1.3)	0.15	0.84 (0.69-1.03)	0.09
hK10	259	1.03 (0.87-1.2)	0.75	0.78 (0.64-0.95)	0.01
hK11	259	0.87 (0.71-1.06)	0.16	0.73 (0.57-0.94)	0.01
hK13	259	0.94 (0.81-1.09)	0.43	0.87 (0.73-1.05)	0.14
hK14	259	1.11 (0.98-1.25)	0.12	0.91 (0.79-1.05)	0.20
B7-H4	259	1.22 (1-1.49)	0.05	0.82 (0.64-1.05)	0.11

Association of biomarkers with clinicopathologic factors. Significant correlations were observed between most of the biomarkers, particularly among CA125 and the kallikreins, including hK5 to hK14, for which Spearman's correlations range from 0.28 to 0.83 (all $P < 0.05$). The correlation between all tested biomarkers is given in Supplementary Data (Supplementary Fig. S12), along with a hierarchical clustering analysis (Supplementary Fig. S13), showing closest correlation between hK6 and hK8 ($r_s = 0.83$).

Associations of biomarkers with progression-free survival. We investigated whether each of the biomarkers by itself is predictive of ovarian cancer progression and survival. The results of the Cox univariate and multivariate analyses are summarized in Table 2. With univariate Cox regression models, all clinical factors correlated significantly with progression-free survival (all $P < 0.0001$). Among the 11 biomarkers, hK5 and hK6 are predictive of progression, both with HR of 1.39 (95% CI, 1.15-1.67). This is consistent with the results of the nonparametric method of Kaplan-Meier for each marker. Indeed, when we divided patients into three groups by the tertile values of the individual marker, the progression-free rates are statistically different among the three groups for hK5 and hK6 (Fig. 2). After adjusting for all the clinical variables in multivariate analysis, both hK10 (HR, 0.78; 95% CI, 0.64-0.95) and hK11 (HR, 0.73; 95% CI, 0.57-0.94) significantly predicted time to progression at 0.05 level. Interestingly, when we added response to chemotherapy in the multivariate regression model along with the other clinical variables, CA125, hK7, hK10, and hK11 became independent predictors of ovarian cancer progression, with HR of 0.74 (95% CI, 0.59-0.94), 0.71 (95% CI, 0.54-0.95), 0.77 (95% CI, 0.63-0.94), and 0.73 (95% CI, 0.56-0.93), respectively.

To assess whether the markers have good capacity in discriminating between subjects who progress before a given time t and those who survive beyond t, the time-dependent ROC method was used to evaluate the prognostic accuracy of the biomarkers. We constructed ROC curves at 1 and 5 years to investigate whether they have the accuracy to separate short-term survivors (failed by 1 year versus alive beyond 1 year) and long-term survivors (failed by 5 years versus alive beyond 5 years), respectively. Although univariately the predictive accuracy of each individual marker is quite low, the method was useful in identifying a panel of markers that offers improved prognostic accuracy. We used a weighted logistic regression for survival data and aimed to find a linear combination of markers with which the area under the time-dependent ROC curve is maximized at each t. For 1-year follow-up, hK6, hK8, hK11, and hK13 were selected in the marker panel; for 5-year follow-up, hK6, hK7, hK11, hK14, and B7-H4 were selected. Different markers were included, as they may represent different biological or clinical capacities when predicting long-term versus short-term survival. In Fig. 3A to D, we display the resulting ROC curves and their 95% CIs. The AUC for the ROC curve at $t = 1$ year, for example, is 0.76

(95% CI, 0.70-0.85), suggesting that the marker panel can be fairly accurate at predicting progression at 1 year; the performance at 5 years is similar (AUC, 0.76; 95% CI, 0.69-0.82). Because we used the same data to select the marker panel, derive linear score, and construct ROC curves to evaluate the marker performance, future experiments should validate the performance of this marker panel in an independent data set. A cross-validation method, described in Materials and Methods, was used to correct for potential overfitting. Indeed, the AUC of corrected ROC curves dropped slightly to 0.70 after this correction.

The clinical variables are, in fact, very predictive for progression as shown in Table 2. We sought to evaluate whether a marker panel could provide incremental value in prognostic accuracy beyond these clinical variables. Indeed, a model with both clinical variables and several biomarkers seems to provide improved prognostic accuracy for both 1- and 5-year outcomes (Fig. 3A and B). For example, for a 1-year outcome, a predictive model that includes both biomarkers and clinical variables (stage, debulking, and response to chemotherapy) had an AUC of 0.90 (95% CI, 0.86-0.96) compared with a model with only clinical variables, including stage, debulking, and response to chemotherapy (AUC, 0.74; 95% CI, 0.60-0.86). When we corrected for potential overfitting, combining markers with clinical variables yielded an AUC of 0.80 (95% CI, 0.86-0.94), suggesting that the panel of biomarkers can provide incremental value over a model that includes only clinical variables. Similar conclusions can be drawn for the 5-year outcomes (Fig. 3C and D).

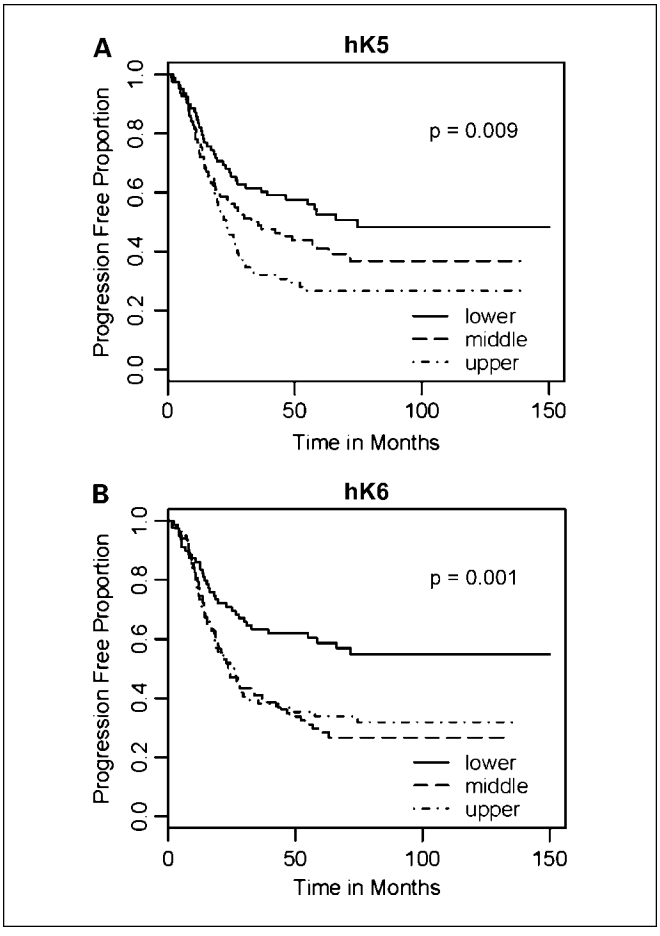

Fig. 2. Progression-free survival curves for hK5 and hK6 among three groups defined by tertiles of marker levels. *P* values were calculated by log-rank tests.

Associations of biomarkers with response to chemotherapy. We found that, among the 11 considered biomarkers, hK13 levels significantly predicted response to chemotherapy. Higher levels of hK13 were significantly associated with better clinical response, with a *P* value of 0.006 from nonparametric Kruskal-Wallis test among groups of patients with no change, progression, partial response, and complete response. This finding was further confirmed by the logistic regression models presented in Table 3. In univariate logistic regression analyses, the OR of hK13 for response to chemotherapy is 1.73 (95% CI, 1.13-2.65; $P = 0.01$). In a multivariate model adjusting for clinical variables, increasing levels of hK13 are again associated with responses, with an OR of 2.32 (95% CI, 1.3-4.14; $P = 0.005$). Furthermore, the clinical value of the marker was confirmed by ROC analysis. hK13 seems to have discriminatory capacity for classifying patients into responders (partial or complete response) and nonresponders (progressed or no change), with an AUC of 0.70 (95% CI, 0.58-0.82). Combining several of the markers improved the classification capacity: a panel of markers, including hK6, hK8, and hK13, yielded an AUC of 0.75 (95% CI, 0.63-0.85; Fig. 3E and F). Further, combination of these markers with clinical variables (stage and debulking) increased the discriminatory capacity for classifying patients into responders and nonresponders with an AUC of 0.91 (95% CI, 0.87-0.97). The clinical variables alone gave an AUC of 0.70 (95% CI, 0.54-0.86).

Discussion

Using traditional methods, as well as microarray- and proteomic-based expression profiling, several tumor-associated prognostic biomarkers with biological relevance in ovarian tumorigenesis or tumor progression have been discovered. Several classes of proteolytic enzymes (such as serine, cysteine, and metalloproteinases) have emerged as important prognostic indicators in ovarian cancer (19). Among these enzymes, many members of the human tissue kallikrein family of secreted serine proteases have shown potential as diagnostic, prognostic, and predictive indicators in ovarian cancer (10).

Previous work has focused on the individual value of each tissue kallikrein in ovarian cancer diagnostics. For example, the following kallikreins (transcripts or proteins) are overexpressed in cancerous tissues, in comparison with normal or benign ovarian tissues, and to correlate with higher stage and grade and poor survival (in general, unfavorable prognosis) in ovarian cancer: KLK4 (20–23), KLK5 (10, 24–28), KLK6 (29–39), KLK7 (5, 27, 35, 40–42), KLK10 (26, 31, 35–39, 43–48), KLK11 (26, 49), and KLK15 (50). On the other hand, another group of kallikreins was also found to be overexpressed in ovarian cancer but this overexpression correlated with lower stage and grade and increased patient survival (favorable prognosis) as follows: KLK8 (26, 35, 51), KLK9 (52), KLK11 (35, 36), KLK13 (26, 53), and KLK14 (40).

Among the nonkallikrein markers tested, B7-H4 has previously been examined as a candidate serum and tissue biomarker for ovarian cancer. In multivariate logistic regression analyses, B7-H4 was additive to CA125, especially in the detection of

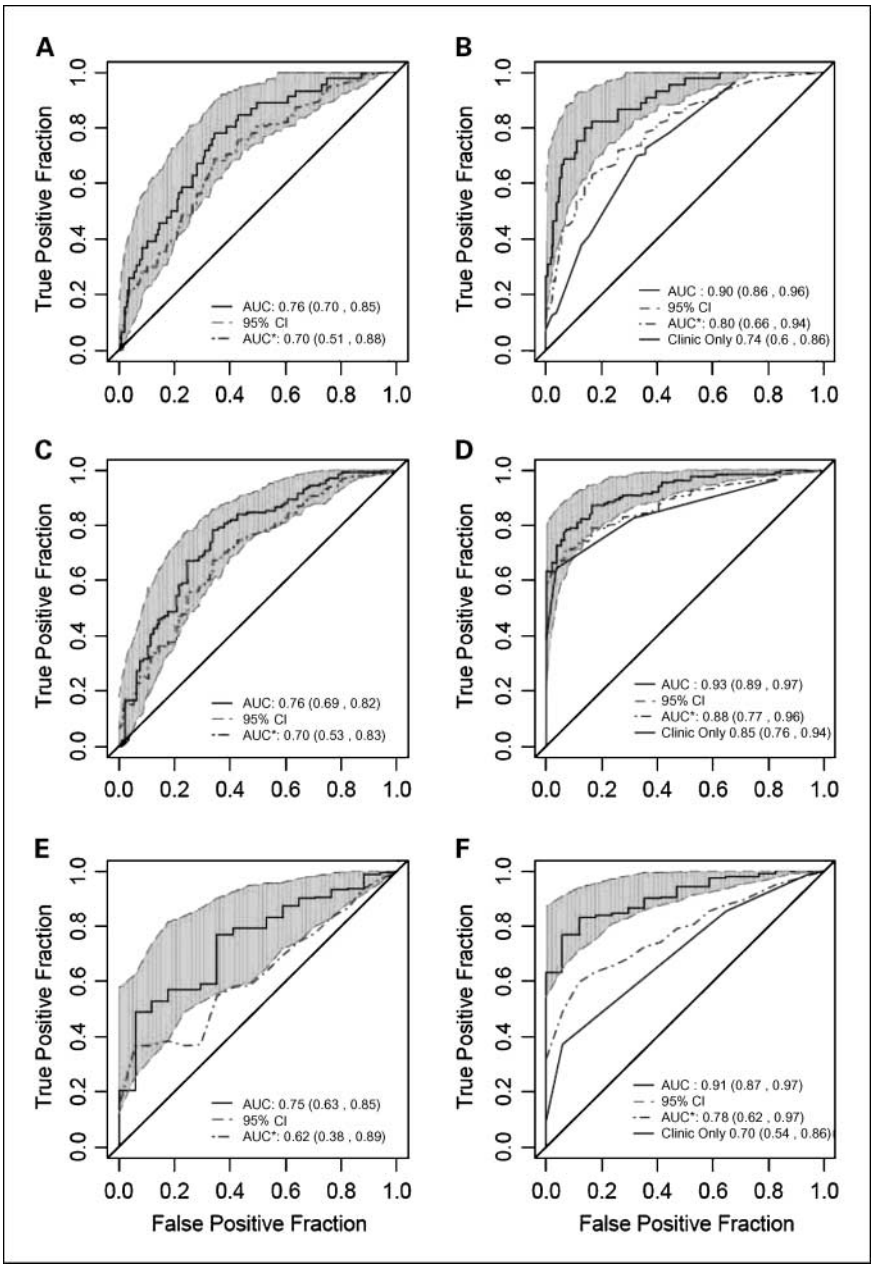

Fig. 3. ROC curves for 1-year (*A* and *B*) and 5-year (*C* and *D*) progression-free survival using groups of biomarkers (combined marker), without and with clinical markers. Presented in each of the left panel is the original ROC curve with its 95% CI (*shaded area*) and the ROC curve calculated using cross-validation to correct for overfitting (*AUC**). Combined marker for 1 y: hK6, hK8, hK11, and hK13. Combined marker for 5 y: hK6, hK7, hK11, hK14, and B7-H4. Presented in each of the right panel is the original ROC curve for the combined marker and clinical variables, such as stage, debulking, and response to chemotherapy, along with its 95% CI (*shaded area*), the ROC curve calculated using cross-validation to correct for overfitting (*AUC**), and the ROC curve calculated using only the clinical variables (*Clinic Only*). *E* and *F*, ROC curves for response to chemotherapy. In each panel, the original ROC curve with its 95% CI (*shaded area*) and the ROC curve corrected for overfitting (*AUC**) are presented. Left, ROC curves using a group of biomarkers (hK6, hK8, and hK13); right, ROC curves using a group of biomarkers (CA125, hK8, and hK13) and clinical variables, including stage and debulking, as well as a ROC curve based on only clinical variables (*Clinic Only*). CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

early-stage cancer (11). Additionally, immunohistochemical studies showed overexpression and membranous staining in serous ovarian cancer (54). B7-H4 has potential in complementing CA125 for diagnosis (11). Others reported overexpression of B7-H4 in ovarian tumors (54). We have also recently confirmed overexpression of this antigen in ovarian tumor cytosols and its adverse prognosis in ovarian cancer patients.[7] A recent immunohistochemical study examined the expression of 10 potential serum markers of epithelial ovarian cancer, in comparison with normal ovaries and other normal tissues. Among the most consistently overexpressed biomarkers were hK10 and hK6 (33). Another recent study (55) assessed KLK levels in effusions and confirmed the overexpression of KLK6, KLK7, KLK8, and KLK10 in ovarian cancer ascites and their discriminating potential between malignant and benign effusions.

In this study, we attempted to evaluate the possible diagnostic, prognostic, and predictive value of 11 biochemical variables, including 9 tissue kallikreins, in a relatively large collection of primary ovarian cancer cytosolic extracts and in extracts from normal ovaries, ovaries with benign gynecologic diseases, as well as primary tumors from other organs that metastasized to the ovary. Our data confirm previous findings that almost all kallikreins and B7-H4 are highly overexpressed in primary ovarian cancer tissues, in comparison with normal, benign, or nonovarian tumors metastatic to the ovary (Supplementary Figs. S1-S11). Many of the tested biomarkers (including hK5, hK6, hK7, hK8, hK11, hK13, and B7-H4) were superior to CA125 in discriminating ovarian cancer from the other three groups (Supplementary Table S1). A

[7] Unpublished data.

multiparametric panel (combined marker) had an AUC of 0.97 for separating primary ovarian cancer from normal tissues and very high capacity for discriminating between ovarian cancer and either benign conditions (AUC, 0.92) or nonovarian tumors metastatic to the ovary (AUC, 0.84). These data support the view that kallikrein and B7-H4 overexpression is associated with the genotype and phenotype of ovarian cancer cells. These proteins, as previously suggested (9–11), may have value as serologic biomarkers for this disease.

We have further shown strong associations of hK5, hK6, hK7, hK8, hK14, and B7-H4 with clinical features such as debulking success, grade, stage, and histologic type (Table 1). Many of these biomarkers correlate with each other (weakly to strongly) as shown by Spearman's correlation and hierarchical clustering (Supplementary Figs. S12 and S13).

As expected, clinical variables such as stage, grade, histology, and debulking correlate strongly with progression-free survival at univariate analysis. The strongest biochemical variables associated with adverse progression-free survival were the kallikreins hK5 and hK6. These data were also confirmed with Kaplan-Meier survival analysis (Fig. 2) and they are in accord with previous studies (9–11). When we combined a group of kallikreins (hK6, hK8, hK11, and hK13), we were able to predict patient progression at 1 year with good accuracy (AUC, 0.76). Another panel of biomarkers consisting of hK6, hK7, hK11, hK14, and B7-H4 was able to effectively predict progression at 5 years (AUC, 0.76; Fig. 3). Furthermore, we identified a model consisting of clinical variables and several biomarkers to provide improved prognostic accuracy of progression-free survival at 1 and 5 years (with an AUC of 0.90 and 0.93, respectively) over a model consisting of only clinical variables (Fig. 3). Further studies to confirm the incremental value of the combined model over a model with only clinical variables would be important.

hK13 was found here to have favorable prognostic value, in accordance to our previous report (53), and significant predictive value for response to chemotherapy (a new finding). In multivariate analysis, the predictive value of hK13 for response to chemotherapy was stronger than any of the other biochemical markers or clinical variables, including stage, grade, histology, and debulking success (Table 3). These data suggest that hK13 is a new, powerful, and independent biochemical variable of response to chemotherapy and may have clinical value for selecting patients who are likely to respond or fail chemotherapy treatments. The predictive value of hK13 was further augmented with a panel of biomarkers, including hK6, hK8, and hK13, with an AUC of 0.75 (Fig. 3). The predictive power of this biochemical marker panel was further enhanced by including clinical variables such as stage and debulking to increase the AUC to 0.91 (Fig. 3).

In the emerging era of individualized therapy (56), cancer patients are stratified by biochemical, molecular, genomic, or proteomic technologies according to their likelihood of responding to specific treatments. This approach holds great

Table 3. Univariate and multivariate logistic regression analysis for response to chemotherapy

Clinical variables	*n* (%)	Univariate analysis		Multivariate analysis	
		OR (95% CI)	*P*	OR (95% CI)	*P*
Age (y)					
≤55	113 (46)	1.00	0.15	1.00	0.20
>55	134 (54)	0.45 (0.16-1.31)		0.45 (0.13-1.54)	
Stage					
I	65 (26)	1.00	0.003	1.00	0.10
II	20 (8)	0.31 (0.02-5.14)		0.33 (0.02-6.32)	
III	143 (58)	0.19 (0.02-1.48)		1.64 (0.1-27.04)	
IV	18 (7)	0.03 (0-0.29)		0.36 (0.02-7.32)	
Grade					
G_1	58 (24)	1.00	0.05	1.00	0.70
G_2	45 (19)	3.28 (1.72-6.24)		1.83 (0.87-3.85)	
G_3	139 (57)	5.07 (2.89-8.89)		1.58 (0.77-3.24)	
Histology					
Serous	110 (44)	1.00	0.53	1.00	0.43
Epi	84 (34)	1.27 (0.40-4.04)		0.6 (0.14-2.50)	
Undiff	56 (22)	0.63 (0.21-1.93)		0.41 (0.11-1.60)	
Debulking					
OD	140 (58)	1.00	0.0006	1.00	0.04
SD	103 (42)	0.07 (0.02-0.33)		0.08 (0.01-0.84)	
Markers				**Adjusting for clinical variables**	
CA125	259	0.98 (0.60-1.60)	0.93	0.8 (0.38-1.69)	0.57
hK4	259	1.16 (0.68-1.96)	0.59	1 (0.54-1.87)	0.99
hK5	259	0.95 (0.56-1.6)	0.85	1.21 (0.6-2.46)	0.59
hK6	259	0.98 (0.60-1.61)	0.95	1.28 (0.64-2.56)	0.49
hK7	259	0.92 (0.50-1.71)	0.80	1.07 (0.46-2.48)	0.88
hK8	259	1.27 (0.89-1.79)	0.18	1.51 (0.89-2.56)	0.12
hK10	259	1.4 (0.95-2.07)	0.09	1.39 (0.80-2.41)	0.24
hK11	259	1.49 (0.85-2.61)	0.17	1.3 (0.62-2.72)	0.49
hK13	259	1.73 (1.13-2.65)	0.01	2.32 (1.30-4.14)	0.005
hK14	259	1.06 (0.75-1.50)	0.74	1.07 (0.67-1.71)	0.78
B7-H4	259	1.28 (0.71-2.33)	0.41	2 (0.87-4.59)	0.10

promise for improving clinical outcomes by administering tailored drugs to those who are likely to respond, while sparing others from harmful side effects, or directing them to alternative therapeutic options. Examples of successful drugs for individualized treatments include tamoxifen, Herceptin, Gleevec, Tarceva, and Avastin (56). These drugs are administered only after assessing a predictive marker of therapeutic response. The value of these predictive biomarkers for improving clinical outcomes is unquestionable. We here reveal for the first time that hK13 concentration in tumor cell extracts has important predictive value for response to chemotherapy of ovarian cancer patients. This prediction can be further augmented by including additional kallikrein and nonkallikrein biomarkers as well as clinical variables. The AUC in ROC analysis for predicting response to chemotherapy was 0.91. It will be necessary to further validate these findings with an independent set of samples. We hypothesize that additional biochemical markers, assessed in ovarian tumor cytosols, may further increase the predictive value of this algorithm to a point that may allow implementation into routine clinical practice.

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