Proteomic Profiling Identifies Afamin as a Potential Biomarker for Ovarian Cancer

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Abstract

Purpose: To discover and validate serum glycoprotein biomarkers in ovarian cancer using proteomic-based approaches.

Experimental Design: Serum samples from a "discovery set" of 20 patients with ovarian cancer or benign ovarian cysts or healthy volunteers were compared by fluorescence two-dimensional differential in-gel electrophoresis and parallel lectin-based two-dimensional profiling. Validation of a candidate biomarker was carried out with Western blotting and immunoassay ($n = 424$).

Results: Twenty-six proteins that changed significantly were identified by mass spectrometric sequencing. One of these, confirmed by Western blotting, was afamin, a vitamin E binding protein, with two isoforms decreasing in patients with ovarian cancer. Validation using cross-sectional samples from 303 individuals (healthy controls and patients with benign, borderline, or malignant ovarian conditions and other cancers) assayed by ELISA showed significantly decreased total afamin concentrations in patients with ovarian cancer compared with healthy controls ($P = 0.002$) and patients with benign disease ($P = 0.046$). However, the receiver operating characteristic areas for total afamin for the comparison of ovarian cancer with healthy controls or benign controls were only 0.67 and 0.60, respectively, with comparable figures for CA-125 being 0.92 and 0.88 although corresponding figures for a subgroup of samples analyzed by isoelectric focusing for afamin isoform 2 were 0.85 and 0.79. Analysis of a further 121 samples collected prospectively from 9 patients pretreatment through to relapse indicated complementarity of afamin with CA-125, including two cases in whom CA-125 was noninformative.

Conclusions: Afamin shows potential complementarity with CA-125 in longitudinal monitoring of patients with ovarian cancer, justifying prospective larger-scale investigation. Changes in specific isoforms may provide further information.

The most widely used marker for ovarian cancer is CA-125 (1). However, its poor sensitivity in detecting stage I disease and lack of specificity preclude its sole use in screening and limit its value in differential diagnosis of pelvic masses, particularly in premenopausal women. Although recommendations about the optimal interpretation or definition of changes in CA-125 levels vary, there is growing consensus that the main utility of CA-125 lies in the assessment of response to therapy and in the longitudinal monitoring and detection of disease recurrence, providing a median lead time of 3 to 4 months over clinical or radiological assessment. However, CA-125 alone is not informative in 10% to 20% of patients with advanced disease and further complementary markers are needed.

Several potential candidate markers are the subject of current evaluation, including OVX1, macrophage colony-stimulating factor, inhibin, kallikreins, tissue polypeptide-specific antigen, and lysophosphatidic acid (1, 2). The concept of using multiple markers to overcome heterogeneity and enhance sensitivity and specificity is also being explored using both specific proteins and pattern recognition analysis of mass spectrometric profiles (3–6). With technological improvements allowing more sensitive, reproducible, and higher-throughput profiling and identification of proteins and their posttranslational modifications, proteomics-based approaches are increasingly being used (7, 8) with several studies illustrating the potential for ovarian cancer (6, 9–12).

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Table 1. Characteristics of the patients (N = 303) providing samples used in the cross-sectional part of this study

Group	Median age (range), y	Number (n = 303)
Ovarian cancer—pretreatment [FIGO stage I/II (n = 15) and stage III/IV (n = 42)]	62.0 (19-87)	57
Serous (n = 26)		
Endometrioid (n = 9)		
Clear cell (n = 5)		
Mucinous (n = 4)		
Other (n = 13)		
Primary peritoneal carcinoma	67.5 (56-81)	10
Advanced ovarian cancer	61.0 (39-84)	38
At relapse (n = 9)		
On chemotherapy (n = 16)		
Palliative care (n = 13)		
Cancer metastatic to ovary (8 endometrial carcinoma or sarcomas and 1 melanoma)	63.0 (43-90)	9
Benign gynecologic conditions	46.0 (19-83)	60
Non-inflammatory (includes fibroids, cysts; n = 37)		
Inflammatory (includes endometriosis; n = 19)		
Mature teratoma (n = 4)		
Borderline ovarian tumors (serous and mucinous)	56.5 (20-81)	26
Healthy controls	51.0 (27-87)	39
Other cancers (42 endometrial, 9 breast, 6 renal, 5 cervix, 1 colorectal, 1 fallopian tube)	65.0 (30-81)	64

NOTE: With the exception of the patients with advanced ovarian cancer, all samples were obtained before surgery or other treatment.
Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.

Glycoproteins are pivotal in many processes involving cellular interactions such as tumor metastasis, with abundant evidence of altered glycosylation in cancer (13). Such multiple protein isoforms are readily seen with two-dimensional PAGE and when combined with downstream blotting with lectins (glycan-binding proteins with specific but often overlapping affinities for particular glycans), disease-related differences in glycoforms can be detected (14, 15). In ovarian cancer, immunohistochemical differences in lectin reactivity and altered glycosylation of serum proteins such as α_1-antitrypsin have been reported (16–20).

The aim of this study was, first, to discover novel serum markers for ovarian cancer using a small "discovery set" of serum samples with analysis by the low-throughput multiplexed approach of two-dimensional differential in-gel electrophoresis (DIGE; using minimal lysine labeling with Cy3 and Cy5 dyes for the simultaneous fluorescent analysis of two samples in one two-dimensional gel; ref. 21) in parallel with lectin-based profiling of two-dimensional PAGE blots; second, to validate potential markers using higher-throughput immunoassays on larger sample sets.

Patients and Methods

Extended details are provided in Supplementary data.

Patients and routine clinical chemistry measurements. Serum and EDTA plasma samples were obtained in Leeds (n = 271) and Oxford (n = 32) from healthy controls (nonpatient volunteers) and patients before treatment (except the advanced ovarian cancer group; Table 1) following ethical approval and informed consent and stored at -80°C. Serum concentrations of C-reactive protein (CRP) and total protein were analyzed using a Bayer Advia 1650 analyzer and CA-125 using a Bayer Centaur analyzer. For the longitudinal study, 121 samples from 9 patients with ovarian cancer before treatment and at intervals until relapse were collected at the Medical University of Innsbruck with CA-125 determined using a microparticle enzyme immunoassay and the Abbott IMx analyzer. Reference ranges were <10 mg/L, 64 to 83 g/L, and <35 units/mL for CRP, total protein, and CA-125 (both assays), respectively.

Initial discovery study using two-dimensional DIGE and two-dimensional PAGE lectin blotting. The initial discovery set consisted of serum samples from five patients with International Federation of Gynecology and Obstetrics stage III serous (two with serous and endometrioid) ovarian cancer before treatment, five patients with advanced relapsed serous ovarian cancer (one being subsequently reassessed as primary peritoneal carcinoma), five patients with benign serous cystadenomas, and five female healthy controls of similar age to the ovarian cancer patients; samples in the benign and control groups were pooled to form a control pool against which all samples were directly compared.

For two-dimensional DIGE, the method was essentially as previously described (21). Briefly, cancer samples were diluted to 5 mg protein/mL in DIGE labeling buffer and minimally labeled with lysine-labeling Cy3. The pooled control sample was labeled with Cy5 and coelectrophoresed with each of the 10 Cy3-labeled cancer serum samples using 24-cm 3-10NL immobilized pH gradient strips (GE Healthcare) with 75 μg of labeled serum proteins per dye per strip. Following separation on 10% acrylamide SDS-PAGE gels and imaging, gels were analyzed using DeCyder v.4.0 software (GE Healthcare). Spots were selected for subsequent analysis on the basis of an average alteration in standardized volume ratio between control and patient samples of ≥1.5-fold ($P \leq 0.05$, log-transformed t test). Using two dyes rather than three overcomes much of the dye labeling bias associated with the Cy2 dye and reduces the noise (22). In initial differential labeling studies using same-same comparisons, only 6 of 1,077 spots changed and accordingly reciprocal labeling was not carried out in line with our previous findings (23). With the very low variance associated with this analytic system, technical replicates were not used with the focus being on the biological replicates as recommended (24).

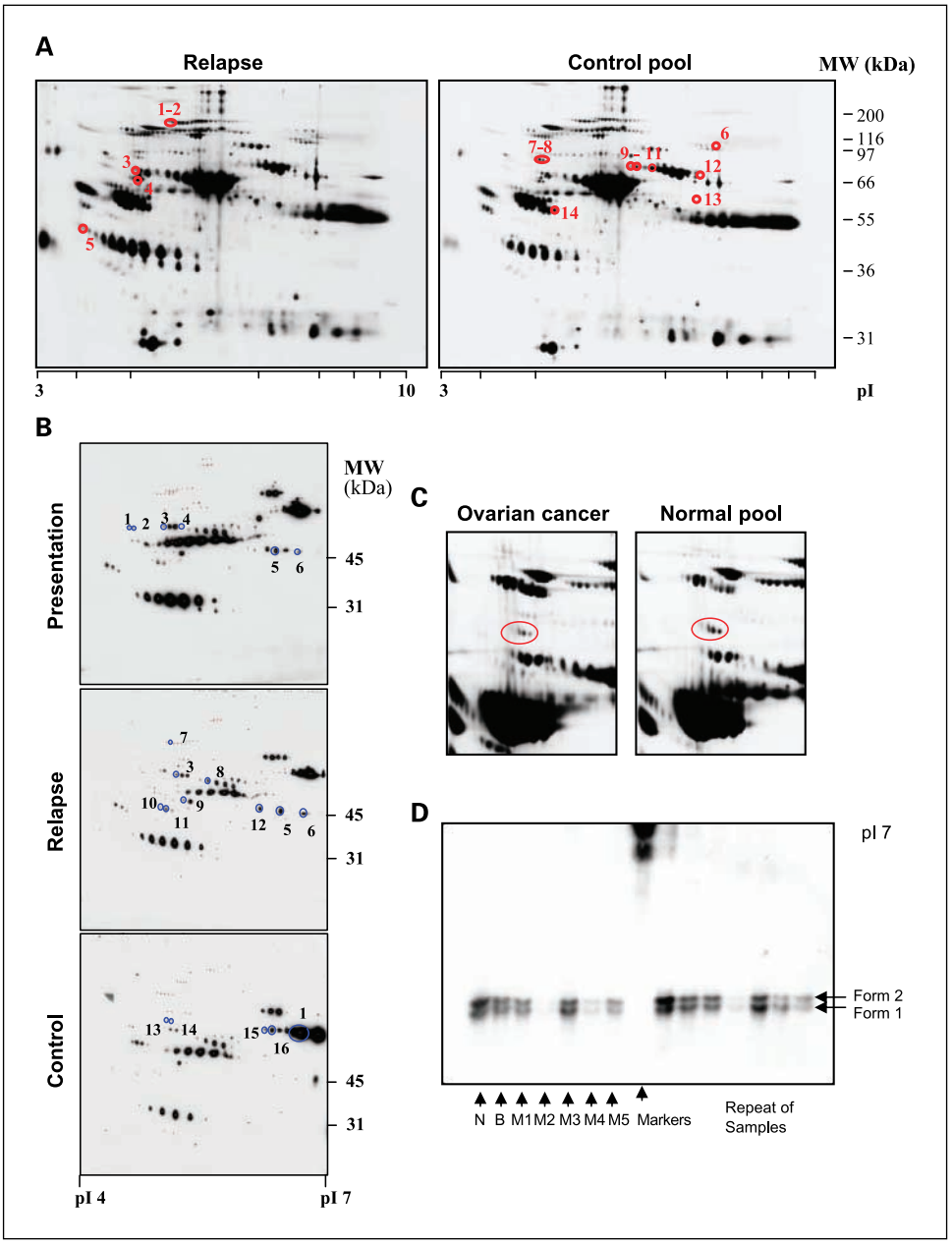

Fig. 1. Representative examples of gels using either the DIGE (*A*) or lectin blotting (*B*) approach to illustrate the differences seen in serum proteins between the groups with the different afamin isoforms illustrated by DIGE (*C*) and one-dimensional isoelectric focusing (*D*). *A,* for the DIGE screen, proteins up-regulated in cancer are shown in a sample from a relapsed patient with those down-regulated in cancer shown in the control pool. *B,* for the lectin screen, examples of lectin serum blots using samples from patients at presentation or during relapse and the control pool are shown with marked protein spots indicating those present at higher intensities in each group. In all cases, the ID numbers refer to those listed in Table 2. *C,* in the enlarged version of a region on the two-dimensional DIGE figure, several forms of the subsequently identified protein afamin are apparent (*circled*). The two most dominant basic forms (forms 1 and 2) correspond to spots 7 and 8 in *A* and spots 13 and 14 in *B. D,* these two forms can also be seen by one-dimensional isoelectric focusing and Western blotting with a specific antibody against afamin, with comparison of the bands being seen for serum samples from a normal healthy volunteer (*N*), a patient with benign serous cystadenoma (*B*), and five patients with stage III or IV serous ovarian cancer (*M1-M5*). The equivalent of 1/11 μL of serum was loaded in each case.

For lectin-based profiling, each of the 10 patient sera and two replicates of the control pool sample were subjected to isoelectric focusing on 18-cm pH 4-7 immobilized pH gradient strips with 300 μg protein/strip (25) followed by electrophoresis using 8% SDS-PAGE gels together with biotinylated molecular weight markers. After equilibration of the gel in Towbin's buffer for 30 min, proteins were transferred onto Hybond-C Super nitrocellulose membrane and blocked in 4% (w/v) bovine serum albumin in lectin buffer containing Tween 20 (LB-T; 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 0.05% v/v Tween 20, pH 7.6). Blots were then probed with elderberry bark lectin (50 ng/mL in LB-T/2% (w/v) bovine serum albumin) for 1 h, washed, and incubated in streptavidin-horseradish peroxidase [1/3,000 dilution in LB-T/2% (w/v) bovine serum albumin] for an hour. Blots were developed using SuperSignal West Pico chemiluminescent substrate (Pierce), exposed to Biomax MS film, and scanned images were analyzed using Melanie III (Genebio). Between-blot variation was corrected for by adjusting for the ratio of marker intensities between blots. Differentially expressed spots were defined as those showing ≥2-fold change in their normalized intensity in at least three of five of the samples in the group relative to the control pool.

Protein identification. Proteins for sequence analysis were prepared using preparatory gels containing 2-mg unlabeled protein stained with colloidal Coomassie blue. For the lectin blotting study, similar gels (1.5 mg) were used and specific spots identified by protein profile pattern comparison of the preparatory gel against the original blots and parallel aligned whole-protein silver-stained images. Tryptic digestion and mass spectrometry (MS) identification of proteins were done essentially as previously described (21) with extracts being analyzed using a Voyager-DE STR MALDI-TOF or 4700 TOF-TOF (Applied Biosystems) and database searching using Mascot (Matrix Science).

Analysis of afamin isoforms by isoelectric focusing and immunoblotting. Serum (n = 98 from the various groups in Table 1) was subjected to isoelectric focusing using isoelectric point 3-7 isoelectric

focusing gels (Novex) with the Novex Minicell system using an anode buffer of 7 mmol/L phosphoric acid and a cathode buffer of 40 mmol/L lysine. Each sample was diluted and 10 μL (equivalent to 1/11 μL of serum) were loaded per lane in duplicate. After focusing, gels were incubated in 50% Towbin's buffer/10% (v/v) methanol, transferred onto polyvinylidene difluoride (Immobilon P), and blocked with 10% (w/v) Marvel/TBS-T before being probed with monoclonal N13 anti-afamin antibody (26), which recognizes all forms of afamin, at 1.26 μg/mL in 1% Marvel/TBS-T. After development using Envision horseradish peroxidase–conjugated antimouse immunoglobulin (1/100) followed by SuperSignal West Pico substrate and Biomax MS film, scanned images were used for quantification of blot intensity using ImageQuant (GE Healthcare). Polyvinylidene difluoride membranes were stained with Coomassie blue to allow correction for protein loading per lane. Levels of the afamin isoforms were expressed semiquantitatively relative to an arbitrary control serum included on every gel.

Measurement of afamin in plasma samples. Total afamin levels in plasma and serum samples from the 302 patients and controls indicated in Table 1 and the 9 patients for whom longitudinal samples were available were assayed using a sandwich ELISA as previously described (26).

Statistical analyses. The SAS statistical software package (SAS Institute) was used in all analyses. Comparison of variables was carried out using ANOVA with the Wilcoxon rank-sum test being used to compare pairs of groups. For correlation analysis, Spearman rank correlation coefficients were calculated. Receiver operating characteristic (ROC) curves were produced using the results of the univariate analysis and confidence limits for the area under the curve (AUC) estimated by the method of Delong et al. (27). Logistic regression was also done to

Table 2. Summary of MS sequenced proteins showing potentially significant differences in serum concentration by two-dimensional DIGE or lectin blotting

Protein	Swiss-Prot accession no.	Study type and spot ID		Fold alteration (mean levels vs control)	MS analysis		
		DIGE	Lectin		MS or MS/MS	No. unique peptides	% Coverage
Complement factor H	P08603	1	N/A	↑1.6× all	MS	22	21
		2		↑1.5× all	MS and MS/MS	25 and 4	22 and 2
α_1-B-glycoprotein	P04217	3	3	DIGE ↑1.6× met; Lectin ↑3.1× local/6.3× met	MS and MS/MS	10 and 12	28 and 24
		N/A	4	↑3.6× local	MS	8	27
Complement C9	P02748	4	N/A	↑2.8× met/2.4× all	MS	19	32
Leucine-rich $z\alpha_2$-glycoprotein	P02750	5	N/A	↑2.7× met/2.2× all	MS	8	39
Plasminogen	P00747	6	N/A	↓2.2× local/2.0× met/2.1× all	MS and MS/MS	24 and 32	32 and 8
Afamin	P43652	7	13	DIGE ↓1.6× local, met and all; Lectin ↓ 5.2× local	MS and MS/MS	22 and 28	34 and 31
		8	14	DIGE ↓1.9× local/2.1× met/2.0× all; Lectin ↓77.3 local/6.8× met	MS and MS/MS	17 and 33	24 and 36
IgM C region	P01871	9	15	DIGE ↓2.2× met and all; Lectin ↓ 38.5× met	MS/MS	6	14
		10	16	DIGE ↓2.4× met/2.1 × all; Lectin ↓3.5× local/17.3× met	MS/MS	2	5
Transferrin	P02787	12	N/A	↓1.6× local/2.3× met/2.0× all	MS and MS/MS	22 and 8	42 and 10
		N/A	17	↓3.3× met	MS	23	33
Complement factor B	P00751	13	N/A	↓2.9 local, met and all	MS/MS	4	5
Vitamin D binding protein	P02774	14	N/A	↓5.2× all	MS	17	41
Vitronectin	P04004	N/A	1	↑7.6 local	MS/MS	7	10
		N/A	2	↑5.3 local	MS/MS	12	14
Apolipoprotein H	P02749	N/A	5	↑15.2 local/19.6 met	MS/MS	4	8
		N/A	6	↑30.3 local/33.2 met	MS/MS	6	12
		N/A	12	↑met (absent in control)	MS/MS	4	9
α_1-antitrypsin	P01009	N/A	7	↑11.6 met	MS	19	51
		N/A	10	↑8.7 met	MS	15	43
		N/A	11	↑2.9 met	MS	16	43
Hemopexin	P02790	N/A	8	↑6.0 met	MS	14	31
Antithrombin III	P01008	N/A	9	↑3.0 met	MS	9	30

NOTE: Summary of those proteins sequenced by MS that show potentially significant differences in serum concentration by two-dimensional DIGE or lectin blotting in patients with localized (n = 5) or metastatic ovarian cancer (n = 5) or both groups combined (n = 10), compared with a control pool (n = 10, normal healthy volunteers and benign gynecologic conditions) with the mean fold change shown in each significant comparison. The changes in lectin blotting are fold changes in densitometric readings of lectin blots and therefore do not reflect linear measurements of protein per se. The spot IDs relate to the gel examples provided in Fig. 1 for each of the DIGE and lectin blotting studies and, if listed on the same line, indicate the same protein in both studies. One additional protein spot (ID no. 11) on the DIGE gel is not listed here but contains components of proteins present in DIGE spots 9, 10, and 12.

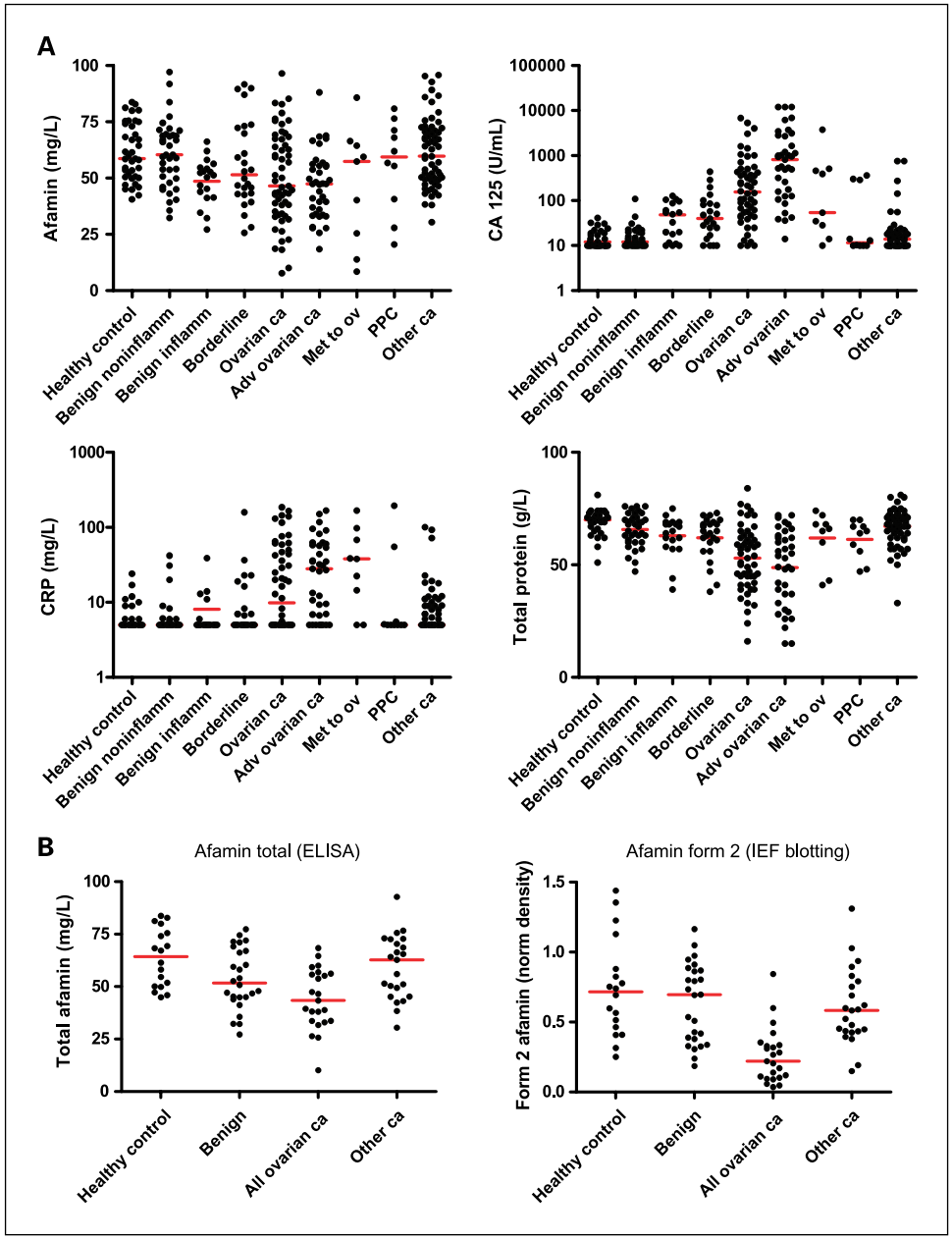

Fig. 2. *A,* dot plots showing total afamin, CA-125, and CRP as measured by ELISA and total protein concentrations in serum/plasma samples from healthy controls (n = 39), patients with benign gynecologic conditions (n = 60), borderline ovarian tumors (n = 26), ovarian cancer at presentation (n = 57), advanced ovarian cancer at relapse or during treatment (n = 38), other cancers metastatic to ovary (n = 9), primary peritoneal carcinomatosis (*PPC*; n = 10), and other cancers (n = 64; Table 1). The lines indicate the median levels with CA-125 and CRP being plotted on a logarithmic scale. *B,* dot plots showing total afamin concentrations measured by ELISA in a subgroup of samples from healthy controls (n = 18), benign gynecologic conditions (n = 26), ovarian cancer at presentation or during relapse and treatment (n = 23), and other cancers (n = 23) compared with the normalized densitometric quantitation of afamin isoform 2 in the same patients. Lines, median values.

find the best-fitting multivariate model for each of the comparison groups (CA-125 was transformed to the log scale in these analyses). The cutoff for afamin was defined as <45 mg/L (10th percentile in this study but 5th percentile from an Austrian study involving >400 healthy individuals).[10] No significant correlation was seen for afamin and age in the control group.

Results

Identification of proteins altered in ovarian cancer serum by two-dimensional DIGE. In total, 62 protein spots from an average of 1,134 spots were detected as changing significantly ($P < 0.05$ with most <0.01) in either the group of patients with ovarian cancer before treatment or the group with ovarian cancer following relapse compared with the control pool. Of these, 14 that changed significantly in all five patients in each group or in both groups combined compared with the control/benign pool were selected for sequencing (Fig. 1A; Table 2). Of the remaining proteins, several were tentatively identified as isoforms of α_1-acid glycoprotein, leucine-rich α_2-glycoprotein, haptoglobin, CRP, α_2-HS glycoprotein, and prothrombin by comparison with in-house reference gels and SWISS-2DPAGE.

Identification of proteins altered in ovarian cancer serum by lectin blotting. Using elderberry bark lectin blotting, a total of 50 lectin-reactive spots changed significantly between either or both cancer groups and the control pool from ~100 resolved per blot (versus up to 1,000 detected by silver staining in the same region). Of these, 17 that did not correspond to known changes already identified from reference maps were selected

[10] H. Dieplinger, unpublished data.

for sequencing and identified as isoforms of nine protein species (Table 2; Fig. 1B).

Validation of afamin changes and comparison with CA-125, CRP, and total protein. Of the five sequenced proteins changing in both approaches (Table 2), afamin, with two isoforms shown to be decreased in patients with ovarian cancer ($P < 0.001$), was selected for further validation. The initial confirmation of the two-dimensional gel results was carried out on a small number of samples using one-dimensional isoelectric focusing with Western blotting, which allows discrimination between the two main isoforms on the basis of isoelectric point and which showed several samples from patients with ovarian cancer to have much weaker or absent bands by Western blotting (Fig. 1C and D).

When total afamin was assayed by ELISA in the larger study, significant differences for afamin concentrations were apparent between the groups overall ($P < 0.001$; Fig. 2A; Table 3). Compared with the healthy control group, significantly lower afamin concentrations were found only in patients with ovarian cancer, either at presentation or with advanced disease at relapse or during treatment ($P = 0.002$ and $P < 0.001$ respectively). The benign group overall was just significantly higher than the ovarian cancer presentation group ($P = 0.046$) but the subgroup with non-inflammatory benign conditions had significantly higher afamin levels than patients with ovarian cancer at presentation or those with advanced disease undergoing treatment or at relapse ($P = 0.004$ and $P = 0.03$, respectively). Afamin concentrations were also significantly lower in the ovarian cancer group compared with the other cancers examined ($P < 0.002$). No significant differences in afamin concentration were apparent between stage I/II and stage III/IV ovarian cancers at presentation, with 6 of 14 stage I/II disease patients having levels <45 mg/L, or between serous and other histologic types.

To enable comparisons to be made consistently, estimates were made at a fixed specificity of 0.95 for CA-125, CRP,

Table 3. Summary statistical analyses with respect to circulating concentrations of afamin, CA-125, CRP, and total protein

(A) Descriptive and main group comparisons

Group	Median (5th-95th percentiles)			
	Afamin (mg/L)	CA-125 (IU/mL)	CRP (mg/L)	Total protein (g/L)
Healthy controls ($n = 39$)	58.7 (42.4-82.9)	12.0 (<10-32.0)	<5 (<5-17.0)	70.0 (58.0-74.0)
Benign gynecologic conditions ($n = 60$)	54.4 (33.5-80.6)	14.0 (<10-108.5)	<5 (<5-31.0)	67.0* (47.0-76.0)
Ovarian cancer—presentation ($n = 57$)	46.5* (18.2-83.4)	155.5 † (<10-4,022)	9.9* (<5-144.0)	54.5 † (29.0-76.0)
Ovarian cancer—advanced/relapse ($n = 38$)	47.5 † (25.7-68.9)	810.0 † (36.0->12,000)	28.0 † (<5-151.0)	53.0 † (15.0-72.0)

(B) Sensitivity values (95% CI) at a fixed specificity of 0.95 and ROC area (95% CI) for the study comparisons indicated

Comparison	Parameter	Afamin (mg/L)	CA-125 (units/mL)	CRP (mg/L)	Total protein (g/L)
Ovarian cancer (at presentation) vs normal controls	Cutoff point	44.72	33	19	57
	Sensitivity	0.47 (0.34-0.60)	0.85 (0.76-0.95)	0.44 (0.31-0.58)	0.56 (0.42-0.69)
	ROC area	0.67 (0.56-0.78)	0.92 (0.84-0.99)	0.69 (0.79)	0.85 (0.76-0.93)
Ovarian cancer (at presentation) vs benign	Cutoff point	34.23	107	21	50
	Sensitivity	0.25 (0.13-0.36)	0.59 (0.46-0.72)	0.39 (0.26-0.52)	0.44 (0.31-0.58)
	ROC area	0.60 (0.49-0.70)	0.88 (0.81-0.94)	0.69 (0.60-0.78)	0.76 (0.67-0.85)
Ovarian cancer (at presentation) vs other cancers	Cutoff point	40.8	148	25	53
	Sensitivity	0.35 (0.23-0.48)	0.52 (0.38-0.65)	0.37 (0.24-0.50)	0.48 (0.35-0.62)
	ROC area	0.66 (0.56-0.76)	0.84 (0.73-0.95)	0.73 (0.59-0.88)	0.86 (0.74-0.92)

(C) Spearman correlation coefficients of variables using all groups ($n = 287$ except for afamin vs age where $n = 303$)

	Age	CA-125	CRP	Total protein
CA-125	0.11 (NS)			
CRP	0.24 ‡	0.44 ‡		
Total protein	-0.12§	-0.33 ‡	-0.19‖	
Afamin	-0.05 (NS)	-0.24 ‡	-0.32 ‡	0.24 ‡

Abbreviation: NS, not significant ($P > 0.05$).
*$P \leq 0.01$, compared with the healthy controls.
†$P \leq 0.001$, compared with the healthy controls.
‡$P \leq 0.0001$.
§$P \leq 0.05$.
‖$P \leq 0.001$.

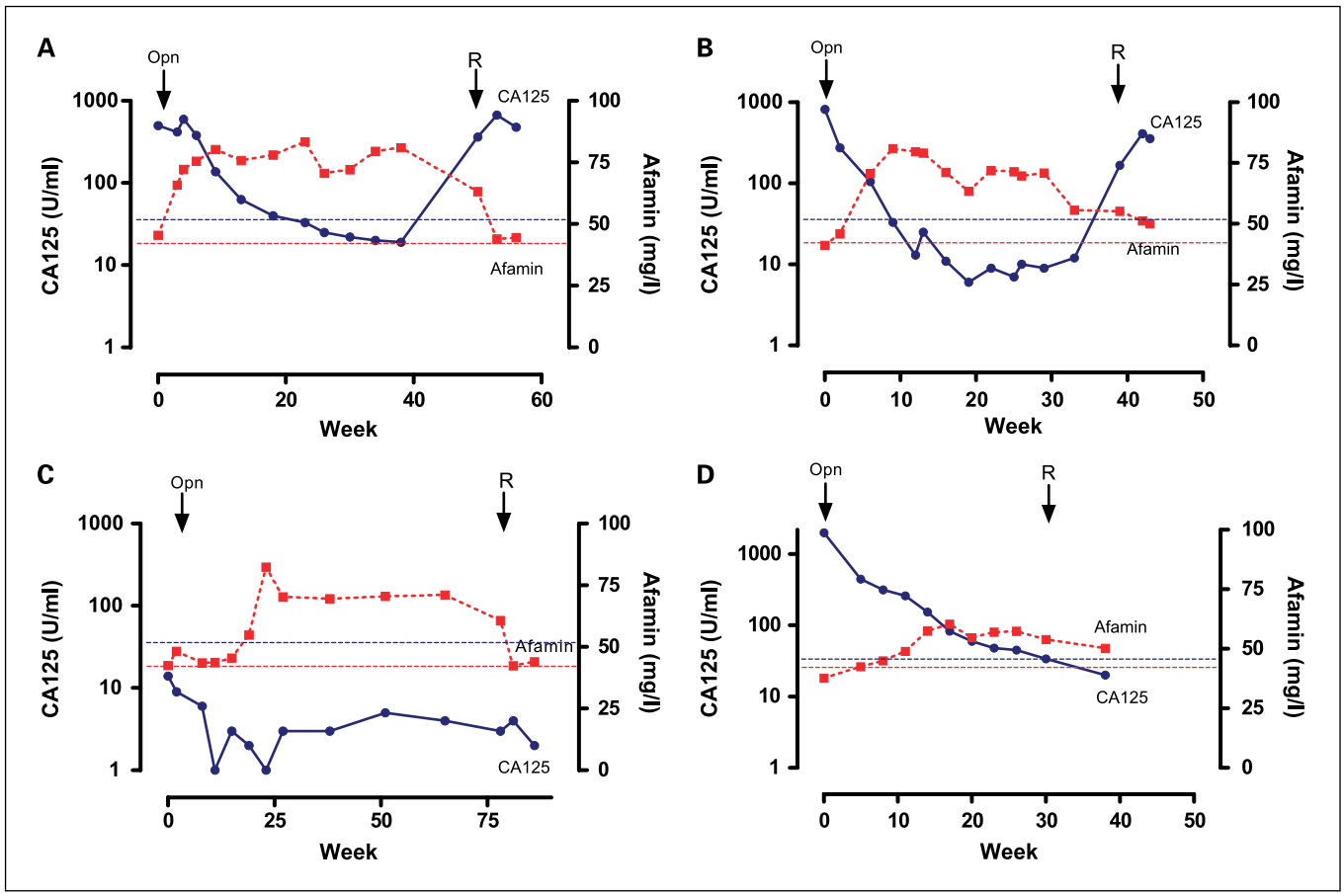

Fig. 3. Serum CA-125 (*blue line*; log scale) and plasma afamin (*red line*) concentrations measured longitudinally by ELISA in four patients with ovarian cancer from presentation until following relapse. Periods of chemotherapy and surgery are indicated together with the time of clinical diagnosis of relapse (*R*). Dotted lines, upper limit of normal reference range for CA-125 (*blue*) and the lower limit of normal reference range for afamin (*red*). All patients had stage IIIc serous papillary adenocarcinoma with the exceptions of patient C, who was stage IIIb.

total protein, and afamin. CA-125 was found to provide the best sensitivity [0.85; 95% confidence interval (95% CI), 0.76-0.95] in comparison of ovarian cancer versus normal controls, with lower sensitivity when the cancer group was compared with the benign group (0.59; 95% CI, 0.46-0.72) or the inflammatory benign conditions only (0.57; 95% CI, 0.44-0.71; Table 3B). The corresponding figures for afamin showed much poorer sensitivity for both the normal control comparison (0.46; 95% CI, 0.34-0.60) and benign conditions (0.25; 95% CI, 0.13-0.36). Of 11 benign samples in which CA-125 was abnormal, afamin was normal and, conversely, 2 of 13 benign samples in which afamin was abnormal had abnormal CA-125 values. However, examination of ROC curves for CA-125, comparing patients with ovarian cancer at presentation against those with benign conditions, showed an AUC of 0.88 (95% CI, 0.81-0.94) compared with 0.60 (CI 0.49-0.70) for afamin, and in multivariate models, afamin or CRP together with CA-125 did not seem to improve the discrimination over and above that for CA-125 alone. In the smaller subset examined for afamin isoform 2, it did seem that the discrimination between ovarian cancer and other cancers (but not benign conditions) was slightly improved with addition of isoform 2 to CA-125 in the model (sensitivity 0.57; 95% CI, 0.31-0.83; ROC area, 0.88; 95% CI, 0.75-1.00).

For all data, a moderate negative correlation between CRP and afamin (Table 3C; Supplementary Fig. S1) was found, which was stronger if the ovarian cancer groups (presentation and advanced disease) alone were considered (r = -0.54). However, 45% of 81 samples with afamin <45 mg/L had normal CRP values. CA-125 was also significantly correlated with CRP for both the total data sets (r = 0.44) and this was also seen for the data for the ovarian cancer patients alone (r = 0.47). Overall, there was a moderate positive correlation between circulating total protein and afamin levels, which was almost absent in the ovarian cancer group alone, indicating that the decrease in afamin was not just a reflection of the lower total protein concentrations seen in these patients. This was also supported following normalization of the data for total serum protein, when although the significant differences between the group medians disappeared, the distributions of values were very different between the ovarian cancer patients and the remaining groups, with ~25% of ovarian cancer patients still having lower afamin levels than normal but a similar number having higher values.

Analysis of afamin isoforms by one-dimensional isoelectric focusing and Western blotting. The initial one-dimensional isoelectric focusing validation work on a limited number of samples was extended to a total of 90 samples (Fig. 2B).

Whereas the total afamin levels measured by ELISA of this subset of samples clearly showed similar differences to those of the full groups, examination of the isoform 2 in particular indicated that this may provide more discrimination between the groups than total afamin by ELISA. In support of this, although the correlation between the ELISA results for total afamin and isoform 2 isoelectric focusing, for example as the most discriminant isoform, was significant ($P < 0.0001$), it was not absolute ($r = 0.63$). Similarly, the sensitivity figures at a fixed specificity of 0.95 for the ovarian cancer at presentation group versus healthy controls or benign controls were 0.64 (CI 0.39-0.90) and 0.29 (0.05-0.52), respectively. The corresponding ROC areas were 0.85 (0.72-0.99) and 0.79 (0.64-0.93), which were clearly much improved over total afamin and approached CA-125 (Table 3B).

Longitudinal measurements of afamin and CA-125. Figure 3 with four representative examples clearly shows the inverse relationship between CA-125 and afamin concentrations (patients A and B), which was seen in five of the six patients in whom CA-125 increased with relapse. Importantly, afamin levels at the time of relapse were within the reference range in many cases, emphasizing the importance of assessing individual baseline levels (i.e., following successful treatment) and monitoring changes with time. The potential complementarity of afamin with CA-125 was also seen in three patients in whom CA-125 was relatively uninformative, although the changes in afamin were modest. Specifically, in two of these patients shown here, in patient C, CA-125 remained in the reference range throughout although exhibiting a small change postoperatively, whereas afamin increased markedly postoperatively and declined at the time of relapse. In patient D, the decline of CA-125 following surgery was slow, potentially indicating residual disease, and declined still even at the time of relapse at week 30, whereas afamin increased postoperatively to a maximum by week 17 but then declined slowly but steadily by 18% through to week 38.

Discussion

This study has shown the potential of proteomic profiling in identifying potential biomarker candidates in serum using small discovery sets of selected samples of similar clinical stage with further subsequent validation of a novel candidate, afamin, on a larger independent sample set using a robust quantitative assay. Clear complementarity with CA-125 is apparent in longitudinal monitoring of patients and should be further validated in prospective studies in terms of potential use in patients in whom CA-125 is noninformative. For diagnostic discrimination, afamin alone is poor but the potential for the isoforms, in particular isoform 2, for complementing CA-125 or other markers should be explored further in a larger study with an independent test set as described for B7-H4 (5).

Very little information is currently available about optimal sample numbers for such proteomic comparisons in terms of the power of the experiment to detect changes. This is dependent not only on the degree of biological variation and the magnitude of change expected but also on the technical variation of the specific approach, which is also laboratory dependent, and the analysis software used. Based on an examination of the variance of technical replicates using Cy3/Cy5 comparisons, the calculated power of three replicates for detecting either a 1.5-fold or 2-fold change in protein was >0.9 in both cases (22), which corroborates findings of a high degree of technical reproducibility in the lab in which this study was done although the variation was also dependent on spot volume (23). With the focus on biological replicates and the avoidance of technical replicates in the same study (24), the biological variation is likely to be greater and, therefore, potentially more replicates are needed; however, if only proteins that change in relatively large magnitude are needed as is the case for markers, then the group sizes used here may be expected to be sufficient for the initial discovery phase, provided stringent validation is undertaken, and this seems to be the case for several proteins studied.

The parallel approaches have allowed discovery of proteins that change in amount and also in glycoform. The absolute magnitude of the changes cannot be compared between approaches due to the less quantitative nature of the blotting approach, their different sensitivities, and the protein-specific dependence of the lectin blotting results, potentially reflecting protein concentration, lectin accessibility/nature of the glycosylation, and site occupancy. The apparent change in afamin glycosylation requires a more quantitative and high-throughput assay for further investigation. Potential approaches include either immunoassay, which would necessitate the generation of a glycoform-specific antibody, which is in itself dependent on the nature of the specific form and potentially a tremendous challenge, or, alternatively, the development of an immuno-mass spectrometry–based assay for the detection of the specific posttranslationally modified peptides. There is precedent for analysis of specific glycoforms being of increased clinical use; characterization of the glycoforms of prostate-specific antigen and CA-125 shows that specific disease-associated differences may ultimately prove to be more informative (28, 29), shed glycans in ovarian cancer sera contain at least 15 unique glycans (30), and a novel glycosylated form of eosinophil-derived neurotoxin is elevated specifically in urine samples from patients with ovarian cancer (11).

Afamin (α-albumin, α_1T-glycoprotein) is the newest member of the albumin family comprising albumin, α-fetoprotein, and vitamin D binding protein. With a predicted mass of ~65 kDa and apparent molecular mass of 87 kDa due to N-glycosylation, similarity is greatest (~60%) with α-fetoprotein (31, 32). Further characterization showed a molecular mass of 74,400 Da by matrix-assisted laser desorption/ionization (33) with five N-linked sialylated biantennary complex type chains (26, 33). Now known to possess vitamin E binding activity (26, 34), afamin is present in plasma/serum, cerebrospinal fluid, and follicular fluid (26, 31) with at least three different forms in the 5.05 to 5.25 isoelectric point range, with isoform 2 described here being the more basic of these. Afamin mRNA expression is predominantly in liver and kidney (Unigene Hs168718) but lysates from ovarian cell lines have also been shown to contain afamin (35) although of a different form (68,981 kDa/isoelectric point 7.66 fraction) from that seen here and in other studies using serum/plasma. It is therefore difficult to interpret whether the changes in

circulating afamin represent primary or secondary changes. The findings in relation to CRP indicate that it is unlikely that the decrease is due to acting as a negative acute phase protein, however. Similarly, the lower concentrations did not occur solely as part of a generalized decrease in total protein concentration seen in many patients. Indeed, in some patients with very low total protein, an apparent increase in afamin was seen. Interestingly, decreased tissue levels of afamin have been reported in hepatocellular carcinoma although serum levels were not examined, with some evidence using transfectants that down-regulation of afamin may promote proliferation (36). We found no evidence of changes in the majority of serum samples from patients with endometrial, breast, or renal cancers examined. Given the nature of afamin as a vitamin E binding protein, the effect of potential confounding factors such as diet would also be explored in future studies.

Several proteins have previously been implicated as changing in ovarian cancer, in some cases as part of the acute phase response. These include antithrombin III, α_1-antitrypsin, and serotransferrin. Both vitamin D binding protein (Gc globulin) and α_1-B-glycoprotein are little studied but further investigation would need to address whether polymorphic variation in these proteins contributed to the changes seen and also the estrogen dependence of vitamin D binding protein synthesis (37, 38). There is currently no evidence for any estrogen-mediated regulation of afamin, with no age dependence of circulating concentrations being apparent in this study and ongoing analysis of premenopausal versus postmenopausal samples indicating minimal, if any, differences.[11] Vitronectin has been implicated in cancer metastasis and complement factor H and its C-truncated form, complement factor H–like protein, are thought to exert an immunoprotective effect based on inhibition of complement lysis and have previously been shown to be secreted from ovarian cancer cells (39). Complement factor H–like protein has utility in bladder cancer such as in the BTA stat test (40).

Clearly, further prospective studies of afamin in larger groups of patients of varying stages and grade are warranted to determine its clinical utility in detecting relapse or response to therapy and its complementarity with CA-125. With many of the changes occurring within the "reference range" for afamin, the need to establish individual profiles is apparent. This has also recently been shown for CA-125 where a progressive low-level increase even within the reference range is strongly predictive of relapse (41). Whether the baseline levels are informative in predicting risk of relapse as reported for CA-125 in patients achieving normalization before maintenance chemotherapy remains to be seen (42, 43). There are many challenges in the area of clinical proteomics (8), particularly in the development of prefractionation techniques, but these findings using a glycoprotein-based selection illustrate the potential of proteomics-based approaches in biomarker discovery.

[11] B. Dieplinger, unpublished data.

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