Mechanisms of disease

Use of proteomic patterns in serum to identify ovarian cancer

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Summary

Background New technologies for the detection of early-stage ovarian cancer are urgently needed. Pathological changes within an organ might be reflected in proteomic patterns in serum. We developed a bioinformatics tool and used it to identify proteomic patterns in serum that distinguish neoplastic from non-neoplastic disease within the ovary.

Methods Proteomic spectra were generated by mass spectroscopy (surface-enhanced laser desorption and ionisation). A preliminary "training" set of spectra derived from analysis of serum from 50 unaffected women and 50 patients with ovarian cancer were analysed by an iterative searching algorithm that identified a proteomic pattern that completely discriminated cancer from non-cancer. The discovered pattern was then used to classify an independent set of 116 masked serum samples: 50 from women with ovarian cancer, and 66 from unaffected women or those with non-malignant disorders.

Findings The algorithm identified a cluster pattern that, in the training set, completely segregated cancer from non-cancer. The discriminatory pattern correctly identified all 50 ovarian cancer cases in the masked set, including all 18 stage I cases. Of the 66 cases of non-malignant disease, 63 were recognised as not cancer. This result yielded a sensitivity of 100% (95% CI 93–100), specificity of 95% (87–99), and positive predictive value of 94% (84–99).

Interpretation These findings justify a prospective population-based assessment of proteomic pattern technology as a screening tool for all stages of ovarian cancer in high-risk and general populations.

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Introduction

Application of new technologies for detection of ovarian cancer could have an important effect on public health,[1] but to achieve this goal, specific and sensitive molecular markers are essential.[1–5] This need is especially urgent in women who have a high risk of ovarian cancer due to family or personal history of cancer, and for women with a genetic predisposition to cancer due to abnormalities in predisposition genes such as *BRCA1* and *BRCA2*. There are no effective screening options for this population.

Ovarian cancer presents at a late clinical stage in more than 80% of patients,[1] and is associated with a 5-year survival of 35% in this population. By contrast, the 5-year survival for patients with stage I ovarian cancer exceeds 90%, and most patients are cured of their disease by surgery alone.[1–6] Therefore, increasing the number of women diagnosed with stage I disease should have a direct effect on the mortality and economics of this cancer without the need to change surgical or chemotherapeutic approaches.

Cancer antigen 125 (CA125) is the most widely used biomarker for ovarian cancer.[1–6] Although concentrations of CA125 are abnormal in about 80% of patients with advanced-stage disease, they are increased in only 50–60% of patients with stage I ovarian cancer.[1–6] CA125 has a positive predictive value of less than 10% as a single marker, but the addition of ultrasound screening to CA125 measurement has improved the positive predictive value to about 20%.[6]

Low-molecular-weight serum protein profiling might reflect the pathological state of organs and aid in the early detection of cancer. Matrix-assisted laser desorption and ionisation time-of-flight (MALDI-TOF) and surface-enhanced laser desorption and ionisation time-of-flight (SELDI-TOF) mass spectroscopy can profile proteins in this range.[6–9] These profiles can contain thousands of data points, necessitating sophisticated analytical tools. Bioinformatics has been used to study physiological outcomes and cluster gene microarrays,[10–13] but to uncover changes in complex mass spectrum patterns of serum proteins, higher order analysis is required. We aimed to link SELDI-TOF spectral analysis with a high-order analytical approach using samples from women with a known diagnosis to define an optimum discriminatory PROTEOMIC PATTERN. We then aimed to use this pattern to predict the identity of masked samples from unaffected women, women with early-stage and late-stage ovarian cancer, and women with benign disorders.

Participants and methods

Study population

100 control samples (50 for the preliminary analysis and 50 for the masked analysis) were provided from the National Ovarian Cancer Early Detection Program (NOCEDP) clinic at Northwestern University

GLOSSARY

CLUSTER ANALYSIS

A means of plotting and analysing the protein patterns as clusters or groupings that are similar or not similar. This report uses a set of known "training" samples to segregate the data into two types of clusters: those containing samples from affected patients, and clusters containing samples from unaffected patients. After training, the pattern of an unknown sample is diagnostically classified by its similarity to the diseased or unaffected clusters found in the training set.

GENETIC ALGORITHM

A computer simulation of Darwinian evolution. The outcome is "survival" of the fittest subset of data in a sample. The genetic algorithm starts by randomly selecting many proteomic patterns within the training data for analysis. Each chosen pattern is tested to see how well it can discriminate affected from unaffected in the training set, by cluster analysis. Successful proteomic patterns are kept and recombined ("mated"), whereas unsuccessful patterns are discarded. Ultimately, a best pattern emerges after many successive iterations by the algorithm. This pattern, which best segregates the training sets, is used to classify diagnostically unknown samples.

MALDI-TOF and SELDI-TOF

Methods for profiling a population of proteins in a sample according to the size and net electrical charge of the individual proteins. The readout is a spectrum of peaks. The position of an individual protein in the spectrum corresponds to its "time of flight" because the small proteins fly faster and the large proteins fly more slowly. SELDI—a refinement of MALDI—preselects the proteins in the sample by allowing them to bind to the treated surface of a metal bar, which is coated with a specific chemical that binds a subset of the proteins within the serum sample.

PROTEOMIC PATTERN

The discriminating pattern formed by a small key subset of proteins or peptides buried among the entire repertoire of thousands of proteins represented in the sample spectrum. The pattern is defined by the peak amplitude values only at key mass/charge (*M/Z*) positions along the spectrum horizontal axis. For the discrimination of ovarian cancer, the pattern is formed by the combination of spectral amplitudes at five precise *M/Z* values.

Hospital (Chicago, IL, USA). 17 other control samples from anonymous women unaffected by cancer were provided by the Simone Protective Cancer Institute (Lawrenceville, NJ, USA). These 17 women had endometriosis (seven), uterine fibroids (three), sinusitis (four), rheumatoid arthritis (two), and ulcerative colitis (one) and were included in the masked validation set. Cases from the NOCEDP were self-referred under at least one of the following eligibility criteria: at least one affected first-degree relative; familial breast or ovarian cancer syndrome; positivity for *BRCA1* or *BRCA2* mutations; or personal history of breast cancer. *BRCA1/2* status was not made available to this analysis under the conditions of anonymisation. The high-risk population was chosen because availability of a viable management option is particularly important for women who are at increased risk of development of ovarian cancer.

All women received a yearly three-dimensional colour doppler flow ultrasound examination and measurement of CA125 concentration.[6] Cases were defined as unaffected if they had had a minimum of 5 yearly follow-up examinations without diagnosis of ovarian cancer. Cases with ovarian cancer were eligible if they had had a serum sample banked before pathological staging by a gynaecological oncologist.

Simple ovarian cysts were detected by ultrasonography in 38% of the unaffected women (table 1). All major epithelial subtypes of ovarian cancer were represented, and six of the cancer samples were from women with stage I disease, which mirrors the distribution of stage I ovarian cancer in the community. Reported oral contraceptive use and parity was not different between the groups. The median age in the healthy symptom-free control population was 49 years (range 21–75) in the preliminary set and 48 years (25–73) in the masked validation set. These ages were not substantially different from those for the cancer patients in the preliminary set (median 58 years [range 29–82]) and in the masked validation set (59 [30–80]), including only those with stage I cancers (57 [35–75]). On the basis of the age distribution, premenopausal and postmenopausal women were equally represented in both groups, thus menopausal status should not have been a discriminator in the detection algorithm.

	Preliminary set (n=100)	Masked set (n=116)
Unaffected women		
No evidence of ovarian cysts	37	24
Benign ovarian cysts <2·5 cm	11	19
Benign ovarian cysts >2·5 cm	2	6
Benign gynaecological disease*	0	10
Non-gynaecological inflammatory disorder*	0	7
Women with ovarian cancer		
Stage I	6	18
Stage II, III, IV	44	32

*Individuals recruited from general population. All others were from a high-risk clinic.

Table 1: **Disease status of preliminary and masked sets**

Serum samples were obtained before examination, diagnosis, or treatment and were immediately frozen in liquid nitrogen. Once at the laboratory, samples were thawed, separated into 10 μL portions, and refrozen. The pathological diagnosis was concealed from the operators before all analyses. Samples were obtained via protocols approved by the Institutional Review Board and reviewed by the National Institutes of Health Office of Human Subjects Research.

Proteomic analysis

Serum samples were thawed, added to a C16 hydrophobic interaction protein chip, and analysed on the Protein Biology System 2 SELDI-TOF mass spectrometer (Ciphergen Biosystems, Freemont, CA, USA).[9] Mass resolution (defined as m/Δm) is routinely achieved below 400. Mass accuracy is assessed daily through the use of angiotensin peptide calibrations. We achieve a mass accuracy of 0·1% with this system. Peptides and proteins below the 20 000 mass/charge (*M/Z*) range were ionised with α-cyano-4-hydroxy-cinnamic acid as a matrix, which is most effective for the detection of proteins and peptides in this mass range. The chips were analysed manually under the following settings: laser intensity 240, detector sensitivity 10, mass focus 6000, position 50, molecular mass range 0–20 000 Da, and a 50-shot average per sample. Data were collected without filters and were later used for analyses. Positives and controls were run concurrently, intermingled on the same chip and on multiple chips; the operators were unaware of which was which. None of the samples in the preliminary set were subsequently used in the masked validation set.

Serum from an unaffected woman and from a male control were individually applied to a single bait surface region on 100 separate C16 chips and on all eight bait surfaces of 12 separate chips for between-run and within-run analysis to determine reproducibility.

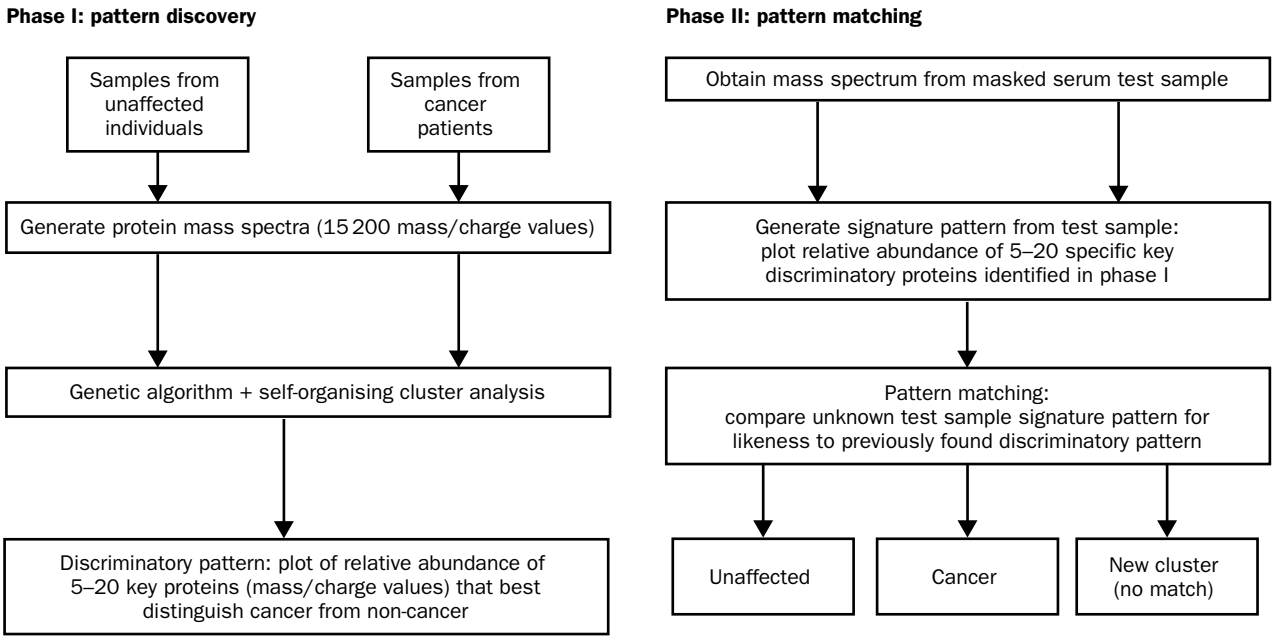

Figure 1: **Phases of N-dimensional proteomic analysis**

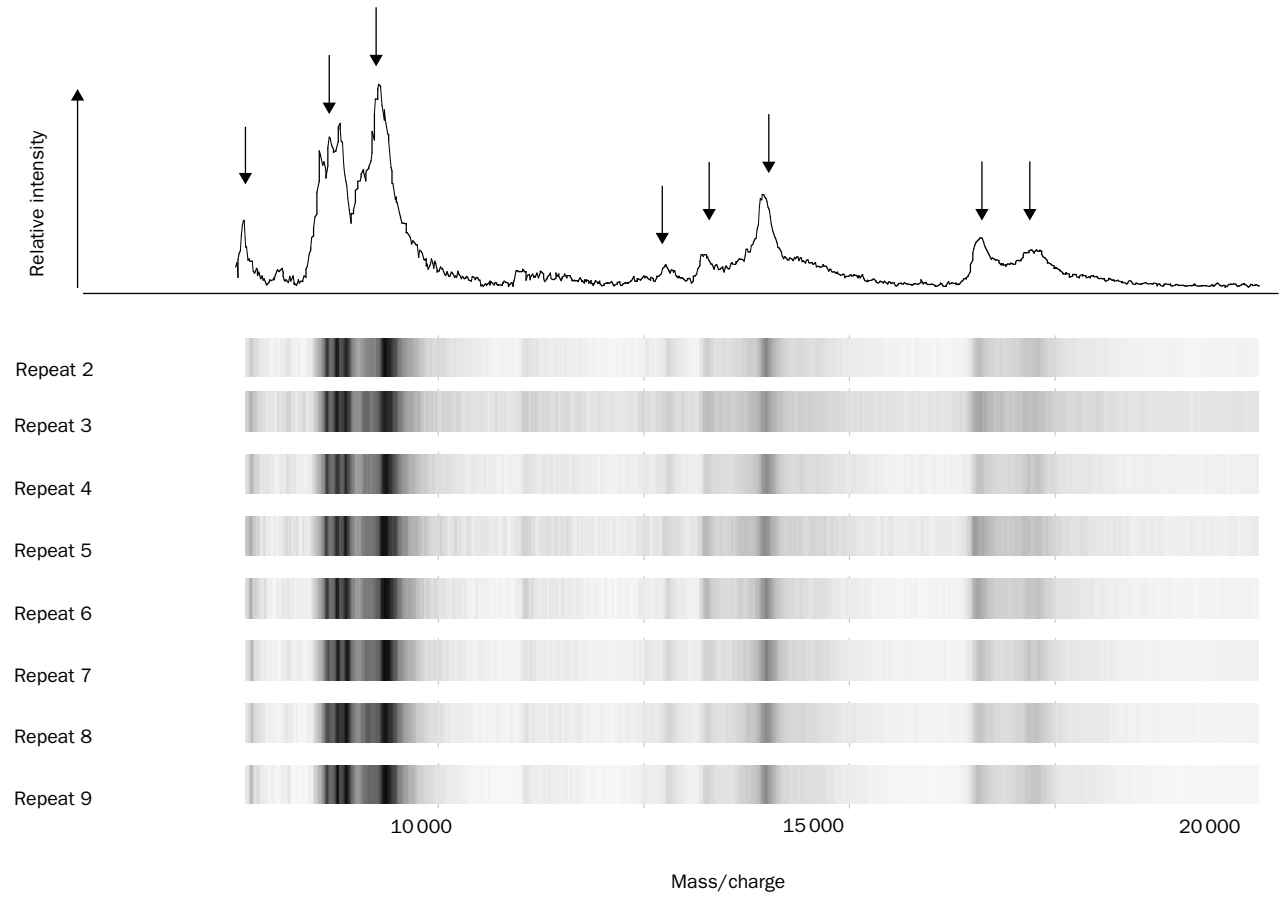

Figure 2: **Example of between-chip reproducibility of mass spectra**
Serum from an unaffected female control was individually applied to a single bait surface region on 100 separate C18 chips and analysed by SELDI-TOF. Nine randomly obtained spectra from the 100 used in the analysis are shown. The eight proteins with the highest consistent amplitudes (arrows), were used as a surrogate for reproducibility by calculation of the coefficient of variance of the normalised peak amplitudes for each of the eight.

Analytical procedure

We developed an analytical tool that combines elements from GENETIC ALGORITHMS first described by Holland[14] and CLUSTER ANALYSIS methods from Kohonen.[15,16] Genetic algorithms function in a manner similar to natural selection. The input data for analysis are ASCII files of proteomic spectra generated by SELDI-TOF. Each spectrum is composed of 15 200 *M/Z* values on the x axis, with a corresponding amplitude on the y axis. The output of the algorithm is the most fit subset of amplitudes at defined *M/Z* values that best segregates the preliminary data. Analysis was divided into two phases: a preliminary phase with knowns, and a testing phase with masked serum samples.

In phase I (figure 1), mass spectra from the two preliminary sets—ie, the 50 patients with biopsy-proven cancer and the 50 unaffected patients and controls—were compared. The algorithm identified a small subset of key values along the spectrum x axis using an iterative searching process. This subset was judged as important because the pattern of amplitudes at these *M/Z* values completely segregated the serum from patients with ovarian cancer from the unaffected populations.

The process starts with hundreds of random choices of small sets of exact *M/Z* values selected along the x axis in the SELDI-TOF mass spectra. Each candidate subset contains five to 20 of the 15 200 potential x-axis values that define the spectra. The fitness test consists of plotting the pattern formed by the combined y-axis amplitudes of the candidate set of key *M/Z* values in N-dimensional space, where N is the number of *M/Z* values in the test set. The pattern formed by the relative amplitude of the spectrum data for this set of chosen values is rated for its ability to distinguish the two preliminary populations. The *M/Z* values within the highest rated sets are reshuffled to form new subset candidates and the resultant y-axis-defined amplitudes are rated iteratively until the set that fully discriminates the preliminary set emerges.

The masked test spectra were analysed in phase II (figure 1). Only the key subset of the *M/Z* values identified in phase I was used for classification of the unknown samples. The pattern formed by the relative amplitudes of the key *M/Z* values in each unknown was matched to the optimum pattern defined in phase I. Each unknown was classified into three possible categories: cancer, unaffected, or new cluster. New cluster meant that the point in N-space of the unknown was outside the defined likeness boundaries of the cancer and unaffected clusters. The bioinformatics software developed and described herein is Proteome Quest beta version 1.0 (Correlogic Systems Inc, Bethesda, MD, USA).

A detailed description of the methods, presentation of the raw spectra (n=216), and analytical results can be found at http://clinicalproteomics.steem.com (accessed Jan 23, 2002).

Statistical analysis

50 cases had 96% power at the α=0·05 level to reject an 80% sensitivity or specificity in favour of a true value of 95%, using an exact test for single proportions, with cut-off points for rejection based on the cumulative binomial distribution.

The Cochran-Armitage trend test[17,18] and the Jonckheere test for trend[19] were used to test the significance of the classification of cancer versus new cluster versus unaffected, according to whether the true

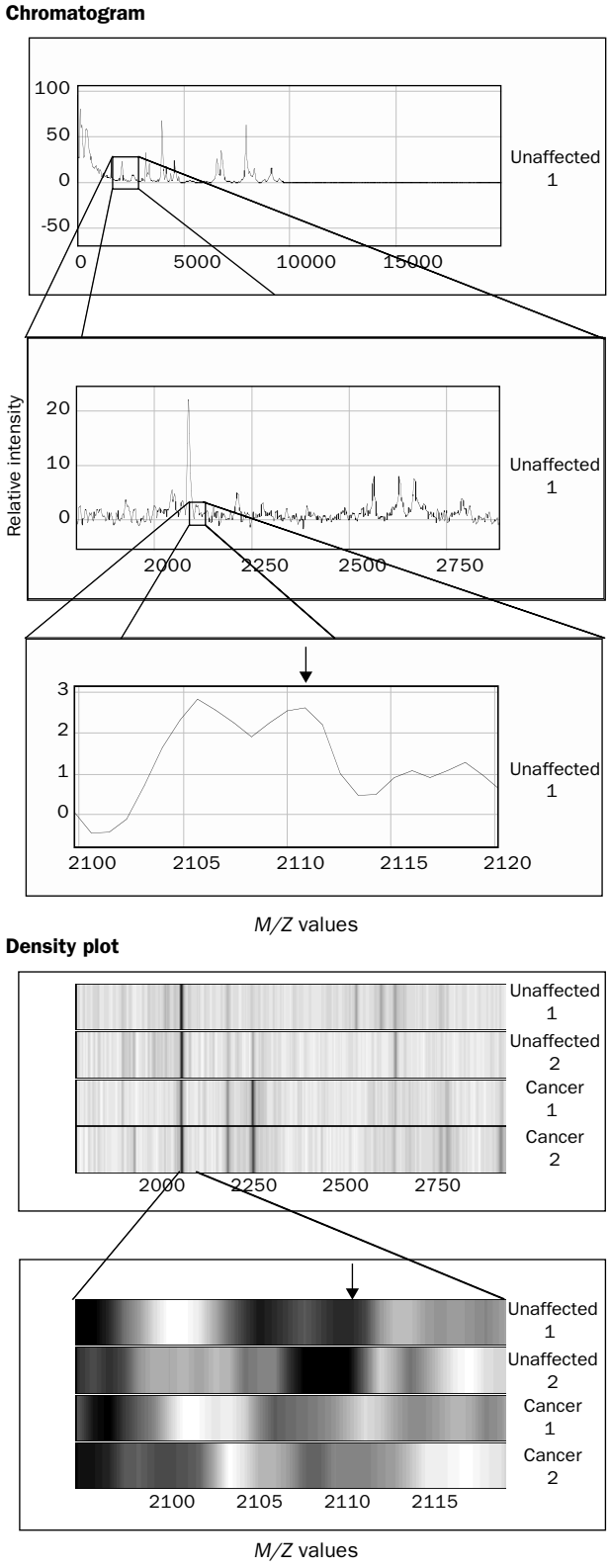

Figure 3: **Derivation of optimum discriminatory biomarker sets derived from mass spectroscopy protein mass/charge intensities**

A representative SELDI-TOF mass spectrum is shown with the molecular weight calculation (*M/Z* values) along the x-axis and relative intensity along the y-axis. The spectrum is represented as a mass chromatogram (top) or a density plot (bottom) of the same data. An increasing magnification of the boxed region of the spectrum is shown to reveal complexity of the spectrum. The protein peak at *M/Z* 2111 identified by the algorithm as belonging to the optimum discriminatory pattern is indicated by the arrow, and is distinguishable above background. The coefficient of variance for this peak with this serum sample from ten independent analyses and ten separate chips was 13%.

	Classification by proteomic pattern		
	Cancer	Unaffected	New cluster
Unaffected women			
No evidence of ovarian cysts	2/24	22/24	0/24
Benign ovarian cysts <2·5 cm	1/19	18/19	0/19
Benign ovarian cysts >2·5 cm	0/6	6/6	0/6
Benign gynaecological disease	0/10	1/10	9/10
Non-gynaecological inflammatory disorder	0/7	0/7	7/7
Women with ovarian cancer			
Stage I	18/18	0/18	0/18
Stage II, III, IV	32/32	0/32	0/32

Table 2: **Classification of serum samples from masked validation set by proteomic pattern**

disease state was presented in two levels or multiple ordered categories. All p values were two-tailed.

Role of the funding source

The funding source had no role in the design, conduct, analysis, or writing up of the study.

Results

Reproducibility and precision

An example of nine independently obtained spectra from the between-run analysis of the serum from the unaffected woman used to determine reproducibility of the mass spectra is shown in figure 2. The coefficient of variance (CV) for eight selected *M*/*Z* peaks with the highest amplitude was less than 10%. There was little variation with day-to-day sampling and instrumentation or chip variations. We calculated that mass spectrum patterns remained consistent (CV <10%) if serum samples were not frozen and thawed more than twice, and once thawed, kept at 4°C for less than 24 h.

The ability of the higher order bioinformatics tool to classify the same spectral data reliably was tested by importing independently generated serum spectra from two individuals: one unaffected and one with stage III ovarian cancer. The algorithm reliably identified 100% of the profiles in the course of 100 independent applications to the C16 chip surfaces (data not shown).

Detection of ovarian cancer

Examples of SELDI-TOF spectra from four patients in the preliminary set (two healthy and two with cancer) are shown in figure 3. The optimum discriminatory pattern in N-space for ovarian cancer was defined by the amplitudes at the key *M*/*Z* values 534, 989, 2111, 2251, and 2465. An example peak from the key subset (*M*/*Z*=2111) can be seen in the magnified view of the spectrum in figure 3, and is lower in the cancer sample than the unaffected sample. This peak is clearly distinguishable from the background noise. Analysis of spectra from the masked validation set (table 2), correctly classified 63 of 66 (95%) of the controls as not cancer, including correct classification of all 17 benign disease controls taken from the general population. 22 of 24 (92% [95% CI 73–99]) of the true normals were correctly classified, and all 50 cancer samples, including all 18 stage I cancers, were correctly classified as malignant.

This result yielded 100% sensitivity (95% CI 93–100) and 95% specificity (87–99). The positive predictive value for this sample set was 94% (84–99), compared with 35% for CA125 for the same samples. There was a strong statistical association between true disease state, whether categorised into two or three ordered groups, and classified state (p<0·001 for all such assessments). The pattern of key *M*/*Z* values segregating ovarian cancer was unable to classify serum samples from 266 men with benign and malignant prostate disease (data not shown).

Discussion

Complex serum proteomic patterns might reflect the underlying pathological state of an organ such as the ovary. This hypothesis is supported by the results of our masked analysis (table 2). Non-cancer control samples representing benign disease, gynaecological disorders, and inflammatory conditions were derived from patients in a high-risk clinic and from the general population (table 1). 63 of 66 samples were accurately classified as non-cancer, including all those from the general population. All ovarian cancers were correctly classified and distinguished from all non-malignant disorders, as were all stage I cancers confined to the ovary.

The cancer sets were derived from a population potentially enriched for ovarian cancer. The high-risk population was chosen as a control set because: (1) early diagnosis is a viable management option for women who are at an increased risk of development of ovarian cancer; (2) this is the population for whom a screening programme would first be used; and (3) serum samples, ultrasonography, and clinical follow-up information could be obtained for 5 years. This population allowed us to test the specificity of our method for classifying benign symptomless disorders such as ovarian cysts—a source of potential false positives—and to verify the 5-year disease-free status.

Proteomic pattern analysis does not merely involve recording and identifying a multiplexed list of biomarkers. The diagnostic pattern is coalesced as a point in N-dimensional space defined by the combination of the relative amplitudes at the subset of key *M*/*Z* spectrum values. The point in space for the unknown patient is compared for its proximity to clusters in N-dimensional space corresponding to the location of individuals in the preliminary set—an established pattern-recognition principle.[20] The preliminary "training" population for comparison becomes enriched as the system learns over time from the accumulated cases, and the outcome, in theory, will become more and more accurate.

The origin and full identity of the discriminating proteins or peptides is under investigation. They exist in the low-molecular-weight serum proteome, which is largely unknown at present. These proteins or peptides could be derived from the host organ, the cancer, or constitute metabolic fragments. The proteins or peptides are hydrophobic and of low molecular mass because of the specific ionisation and chip surface conditions used.

The mass spectrum data streams used for analysis are unfiltered, and therefore contain electronic noise, chemical noise due to contaminants and the ionisation matrix used, as well as real protein signatures. Intrinsic sample-to-sample, patient-to-patient, and day-to-day variability unrelated to the disease category will also influence the overall spectral signatures. Accordingly, we used the power of a genetic algorithm, which by definition applies a fitness test to pinpoint the candidate pattern that best segregates the training set. Amplitudes in the spectra that comprise noise will be successfully weeded from the population because they will not survive the fitness test. The use of a large training set ensures that the most fit pattern will ultimately have to transcend the background variables and noise to distinguish between the training cohorts.

The positive-predictive value for the masked validation set was 94%. This result can be directly compared to a value of 34% for CA125, which was measured in the same masked set. The value for CA125 probably reflects the young age of this group and the high frequency of benign pelvic disease—circumstances in which false positive CA125 results are much higher.[1–5] A positive-predictive value of 94% for the proteomic analysis method might be acceptable for high-risk population screening. However, in clinical practice, where the incidence of ovarian cancer in the population likely to be screened is low (about 1 in 2500), the positive-predictive value must be almost 100% to avoid generating a high number of false positives. The efficacy of screening with CA125 is increased when combined with ultrasonography or when measured over time. Similar approaches might improve the positive predictive value of proteomic analysis.

After proper validation, serum proteomic pattern analysis might ultimately be applied in medical screening clinics as a supplement to diagnostic work-up and assessment. A negative value, if the sensitivity remains at 100% on further trials, could be used for reassurance, whereas a positive value might be sufficient to warrant further investigation.

An important future goal is confirmation of sensitivity and specificity for the prospective detection of stage I ovarian cancer in trials of high-risk and low-risk women. Designing the trial to assess the efficacy of the approach as a stand-alone or combined with current screening options will be important. Such trials are underway at the National Cancer Institute.

Generation of the mass spectra requires only a small serum sample that could be obtained by fingerprick, and results are obtained in less than 30 min. Cost-effective, high-throughput screening is feasible. The concept and tool are flexible, and can be applied to any biological state and to data derived from future mass spectrometry platforms with higher resolution, sensitivity, and mass accuracy than the platform used herein. Samples can be applied to mass spectroscopy chips in the local laboratory and then transported to a central laboratory that houses the analytical software. Moreover, transportation of the raw spectra via the internet to a central site that incorporates an ever-expanding training set is feasible. By this approach, the pattern itself, independent of the identity of the proteins or peptides, is the discriminator, and might represent a new diagnostic paradigm.

Contributors

E Petricoin and L Liotta conceived the study, participated in modelling procedures and analysis, and wrote the report. B Hitt and P Levine conceived and developed the key software components and modelling procedures concerning biological states used in the study and assisted in the preparation of the paper. E Kohn, D Fishman, C Simone, and G Mills designed and wrote the participants section, provided serum sets used in the study, and assisted in the preparation of the paper. A Ardekani generated mass spectra and was responsible for archiving of serum samples. V Fusaro participated in the generation, analysis, and presentation of SELDI-TOF data. S Steinberg selected the statistical methods and did the data analysis.

Conflict of interest statement

B Hitt is the Chief Scientific Officer and P Levine is the President of Correlogic Systems Inc—the manufacturer of the bioinformatics software used in this study.

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