High-throughput Multidimensional Mass Spectrometry Analysis for the Detection of Early Stage Epithelial Ovarian Cancer: A Serum Test for Ovarian Cancer

David A. Fishman[1], Zhiqi Hao[2], Colleen K. Van Pelt[2], Wesley S. Wiggins[3], Partha Seshaiah[3], Timothy A. Coleman[3], and Ben A. Hitt[3]

[1]Obstetrics and Gynecology, Northwestern University, Chicago, IL, [2]Advion BioSciences, Inc., Ithaca, NY, [3]Correlogic Systems, Inc., Bethesda, MD

Overview

Purpose

- To develop a high-throughput procedure for serum sample preparation and analysis using chip-based, automated nanoelectrospray ionization (nanoESI; NanoMate® 100, Advion BioSciences, Inc.) mass spectrometry (MS).
- To develop a model for the early detection of epithelial ovarian cancer from nanoESI mass spectra using Proteome Quest® software (Correlogic Systems, Inc.).

Methods

- 586 samples consisting of 291 epithelial ovarian cancer (EOC) cases and 295 high risk (HR) controls were obtained from the National Ovarian Cancer Early Detection Program.
- Serum was diluted 1:250 in 50/50 acetonitrile/water containing 0.2% formic acid.
- Insoluble material was removed by centrifugation.
- Samples were automatically infused and analyzed using the NanoMate 100 and an Applied BioSystems/MDS Sciex API QSTAR® Pulsar i.
- Models were built using Proteome Quest software which identifies *seromic*™ patterns that differentiate two biological states. (Serome: serum proteome-metabolome)

Results

- Preliminary modeling results demonstrated 71 samples were inappropriate for the study; further examination identified these as:
  (1) No Evidence of Disease
  (2) Post Surgery
  (3) Non-epithelial Ovarian Cancer
- Remaining files (220 EOC cases, 295 HR controls) were used for modeling.
- Modeling to discriminate between the two states demonstrated 92% sensitivity and 93% specificity in testing and 97% sensitivity and 94% specificity in validation.

Introduction

Ovarian cancer kills more women than all other gynecologic malignancies combined primarily due to our inability to detect early stage disease. Of the three main types of ovarian tumors, epithelial tumors are the most common. Our goal is to develop an effective high-throughput serum-based test that achieves early detection of epithelial ovarian cancer.

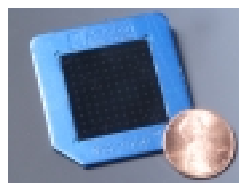
The multifactor nature of cancer and other diseases suggests that single biomarkers may not be accurate predictors of disease. Alternatively, the pattern formed by a combination of several biomarkers could result in both early detection and more accurate diagnosis. To identify such patterns, it is necessary to use high-throughput serum profiling combined with powerful bioinformatics tools for data processing, analysis, and pattern recognition. Proteomics approaches employing SELDI-TOF MS combined with bioinformatics tools have been used to identify biomarker patterns for ovarian, prostate, bladder, and breast cancers (1-3).

In this study, sera obtained after IRB consent from 295 healthy high-risk individuals and 291 women with stage I-IV epithelial ovarian cancer were evaluated. Proteome Quest modeling yielded a model with 92% sensitivity and 93% specificity on testing data and 97% sensitivity and 94% specificity on validation data. Additionally, on a blinded examination of the entire sample set, the modeling process correctly identified 71 specimens which were neither normal nor epithelial ovarian cancer.

Methods

The ESI Chip™

- A 10 x 10 microarray of nanoelectrospray nozzles on a 2.25 mm pitch.
- A separate nozzle is used for each sample, eliminating the possibility of cross-contamination.



ESI Chip

The NanoMate 100

- Fully-automated nanoelectrospray system.
- Optimally positions the ESI chip in relation to the MS orifice.
- Aspirates samples from a 96-well plate using a separate pipette tip for each sample.

NanoMate 100

Sample Preparation

1) Dilute serum samples 1:250 in 50/50 acetonitrile/water containing 0.2% formic acid.
2) Remove insoluble material by centrifugation at 13,000*g* for 15 minutes at 4°C.
3) Transfer samples to 96-well plate. Samples are ready for automated nanoESI/MS infusion analysis.

Representative Plate Map

Day 1/ Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	RC1	RC1	RC2	RC2	EOC+	EOC-	O406263	C427506	O62	C424119	O326	C423853
B	O400011	C427466	O106	C425323	O351	C420695	O414380	C430086	O123	C427474	O336	C426007
C	O408919	C427278	O121	C429659	O406975	C427134	O177	C430054	O40908	C424099	O48	C419907
D	O42	C420927	O402658	C427294	O132	C424043	O98	C429926	O411404	C427270	O19	C422759
E	O6	C419787	O49346	C427014	O101	C421707	O412108	C427888	O133	C424334	O403423	C423877
F	O35	C430325	O141	C420351	O411964	C427713	O50	C423893	O411791	C423885	O409884	C430014
G	O403308	C423949	O147	C423507	O413387	C429902	O421535	C427086	O70	C427350	O64	C424166
H	O424992	C420123	O49427	C427896	O140	C424182	RC1	RC1	RC2	RC2	EOC+	EOC-

This is a schematic of a representative plate containing 84 experimental samples consisting of EOC cases (O) and HR controls (C), process controls (RC1, RC2), EOC positive controls (EOC+), and EOC negative controls (EOC-).

Instrumentation

- All data were collected using an Advion NanoMate100 and an Applied Biosystems/MDS Sciex API QSTAR Pulsar i.
- Nanoelectrospray was generated by applying 0.6 psi of spray pressure and 1.6 kV of spray voltage on the conductive tip. Five µL of the diluted, centrifuged sample was aspirated and delivered at an estimated flowrate of 100 nanoliters per minute into the inlet of the ESI chip.
- The QSTAR was operated in positive TOF-MS mode with a 2-second scan rate. Data for each sample were acquired for 1 minute from m/z 300 to m/z 3000 in the MCA 'On' mode with an intensity threshold of 0.

Model Building Using Proteome Quest

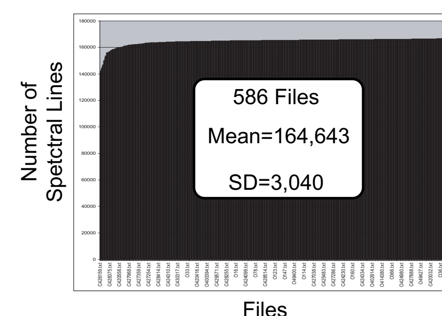
- A randomly selected set of samples was used as a training set. The model identifies features that separate disease/biological states.
- The remaining samples were used in testing and validation.
- Spectra were randomly asigned into three groups for modeling, as follows:

	HR Control	EOC
Training	103	94
Testing	82	65
Validation	110	61
TOTAL	295	220

- Each sample in testing and validation is given a score based on an N-dimensional match to the feature set identified in the model.

Results

Data Consistency



As a preliminary assessment of data consistency, sample spectra line numbers were compared. This graph shows the number of data lines acquired for each of the 586 EOC and HR control samples. The data are sorted from smallest to largest by line number. The mean number of lines per file was 164,643, with a standard deviation of 3,040. Data consistency was high as judged by the low standard deviation of 1.8% relative to the mean.

Model Building

Model development involves training, testing, and validation sets. In the present study, cases are defined as pre-treatment epithelial ovarian cancer, and controls as high risk without disease. Preliminary modeling utilizing 586 spectra identified a set of 71 samples that were not appropriate for inclusion in the study. These 71 samples were excluded as inappropriate because they were identified as:

(1) No Evidence of Disease

(2) Post Surgery

(3) Non-epithelial Ovarian Cancer

The remaining 515 mass spectra (220 EOC cases, 295 HR controls) were randomly assigned to training, testing, and validation sets. Once this random assignment was made, spectra were only employed for the assigned function, i.e., training spectra were only used for training, testing spectra were only used for testing, and validation spectra were only used for final validation.

Model Description

Node	Count	State	StateSum	Error	m/z 762.0388	787.1278	869.1978	883.2139	890.6626	1054.8270	1108.8980	1151.3774
0	133	0	41	41	0.0030	0.0038	0.1418	0.1806	0.2454	0.2774	0.5413	1.0000
1	43	1	33	10	0.0009	0.0185	0.1642	0.2092	0.3684	0.3504	0.5896	1.0000
2	2	1	2	0	0.0000	0.0214	0.2164	0.4410	0.5897	0.6401	0.8065	1.0000
3	13	1	13	0	0.0012	0.0091	0.1771	0.2265	0.6367	0.3526	0.5672	1.0000
4	2	1	1	1	0.0081	0.0000	0.0872	0.1768	1.0000	0.1746	0.2736	0.4683
5	4	1	4	0	0.0108	0.0038	0.2111	0.2781	0.9792	0.3749	0.5573	0.8753

Using 515 spectra (220 EOC, 295 HR controls), a model was built that separated the two states. The model contained eight features with m/z values of 762, 787, 869, 883, 890, 1054, 1108 and 1151. Six nodes were formed: Node 0 describing HR controls (no disease) and Nodes 1-5 describing disease. For each node, the normalized relative values for the selected features are shown.

Model Results

Testing Results

Score	Control	Cancer	Total
0.308271	76	5	81
0.5	1	8	9
0.767442	4	31	35
1	1	21	22
Total	82	65	147

Sensitivity: 92%
Specificity: 93%

Score ≥0.5	Cancer	Score <0.5	Control

Validation Results

Score	Control	Cancer	Total
0.308271	103	2	105
0.5	0	6	6
0.767442	6	32	38
1	1	21	22
Total	110	61	171

Sensitivity: 97%
Specificity: 94%

Score ≥0.5	Cancer	Score <0.5	Control

Using a cut-off score of 0.5, the model demonstrated a sensitivity of 92% and a specificity of 93% in testing with 147 samples. Using a validation set of 171 samples, the model demonstrated a sensitivity of 97% and a specificity of 94%.

Modeling Results by Stage

In Study	Training	Stage	Testing	Validation	Totals
66	32	Stage I	12/14	19/20	31/34
38	13	Stage II	11/14	10/11	21/25
87	35	Stage III	29/29	23/23	52/52
19	11	Stage IV	5/5	3/3	8/8
10	3	Unknown	3/3	4/4	7/7
220	**94**	**Totals**	**60/65**	**59/61**	**119*/126**

*PPV=TP/(TP+FP)
=119/132
=90%

This table shows the distribution of the 220 disease files across the entire study, indicating the number of samples for each cancer stage that were used. Ninety-four samples representing all four of the different stages of epithelial ovarian cancer were used to train the model as shown. Sixty-five cancer samples were used in testing and 61 samples were used in validation. Most notably, the model correctly identified all (60 out of 60) Stage III and Stage IV cancers. For the early stage cancers (I & II), this model was able to correctly identify 52 out of 59 in testing and validation.

This model reported 132 samples in total as disease, of which 119 were true disease samples. The Positive Predictive Value [True Positives / (True Positives + False Positives)], based on this study, was calculated to be 90%.

Conclusions

- Using this semi-automated high-throughput method, we were able to process and generate spectral data at an average rate of 96 samples approximately every two hours. Data consistency was high as judged by sample spectra line number.
- Spectral data from 586 samples were used to generate a series of preliminary models from which we identified a subset of 71 cases that did not meet the inclusion criteria.
- The remaining 515 samples were used to train, test, and validate a model that describes disease state differences between epithelial ovarian cancer and high risk controls. This model demonstrated sensitivity of 92% and specificity of 93% in testing for 147 samples and sensitivity of 97% and specificity of 94% in validation for 171 samples.
- This process and platform appear to be applicable to other disease states, and in view of its rate of throughput and data quality, we will continue its development toward clinical applications.

References:

1.) Petricoin III, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., Mills, G. B., Simone, C., Fishman, D. A., Kohn, E. C., Liotta, L. A. Use of Proteomic Patterns in Serum to Identify Ovarian Cancer. *Lancet* **2002**, 359, 572-577.

2.) Issaq, H. J., Conrads, T. P., Prieto, D. A., Tirumalai, R., Veenstra, T. D. SELDI-TOF MS for Diagnostic Proteomics. *Anal. Chem.* **April 2003**, 149-155.

3.) Issaq, H. J., Veenstra, T. D., Conrads, T. P., Felschow, D. The SELDI-TOF MS Approach to Proteomics: Protein Profiling and Biomarker Identification. *Biochem. Biophys. Res. Commun.* **2002**, 292, 587-592.

Acknowledgments:

Samples were provided by NCI-EDRN Grant UO1CA85133.

Katherine Dunsmore, Advion BioSciences, Inc.