Discovery of altered protein profiles in epithelial ovarian carcinogenesis by SELDI mass spectrometry

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Summary

Objective: Identification of proteomic alterations in epithelial ovarian tumorigenesis may facilitate the understanding of progression of this disease. *Methods:* Specific protein peak patterns were identified in 20 microdissected epithelial ovarian tumors (13 epithelial ovarian cancers (EOCs) and 7 low malignant potential (LMP) tumors), as well as in the matched normal cells. Protein profiles were generated by surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) from all the different types of cells. *Results:* Among seven protein peaks from EOC cells, six were significantly increased while one was decreased compared with normal cells, and three peaks from LMP cells were markedly increased while one was decreased compared with normal cells. *Conclusions:* The combination of SELDI and laser capture microdissection (LCM) is effective in finding the key molecules in ovarian tumorigenesis. Further identification of these protein peaks is important and these malignant protein signatures lend themselves to identification of populations at high-risk for EOC and for monitoring response to EOC chemopreventive agents.

Key words: Epithelial ovarian cancer; Surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS); Laser capture microdissection (LCM).

Introduction

Epithelial ovarian cancer (EOC) remains a major cause of gynecologic cancer mortality in women, with an estimated 15,310 deaths in 2006 in the United States [1]. Given our knowledge about the steep survival gradient relative to the stage at which then disease is diagnosed, it is reasonable to suggest that early detection remains the most promising approach to improve the long-term survival of ovarian cancer patients. The key molecular events in the pathogenesis of EOC are not well defined or understood. It is becoming increasingly clear that because of the inherent molecular heterogeneity and multifocal nature of EOC [2], additional improvement in early detection, diagnosis, and prognosis will likely require the measurement of a panel of biomarkers. These cancers are embedded in a heterogeneous tissue. This has also blocked the study of molecular mechanisms in cancerous pathways, but now the advent of LCM can isolated separate pure cell populations [3]. There are many techniques in the research of molecular mechanisms in ovarian carcinogenesis, including patterns of single nucleotide polymorphisms, DNA methylation or changes in mRNA/protein expression [4-6]. It has been demonstrated, however, that there is often no predictive correlation between mRNA abundance and the quantity of the corresponding functional protein present within a cell. Hence, since proteins represent the preponderance of the biologically active molecules responsible for most cellular functions, it is believed that the direct measurement of protein expression can more accurately indicate cellular dysfunction underlying the development of disease.

The proteome is the full complement of proteins that regulate the physiological and pathophysiological phenotype of a cell. Because proteins initiate all cell functions and pathways, identifying differentially expressed proteins between normal and pathological states can lead to a better understanding of the cellular mechanisms involved in cancer. The identification of changes in protein expression and modifications that occur in the early stages of a developing cancer could lead to the discovery of protein biomarkers and novel strategies for the improvement of early detection, diagnosis, and therapy of cancer. SELDI-TOF-MS can provide a rapid protein expression profile from a variety of biological samples. This technology has been used effectively in several cancers for analyzing protein expression [7-9]. The advantage of the SELDI is the ability to simultaneously detect multiple protein changes with a high degree of sensitivity in a rapid high throughput process. The precision makes it possible to delineate very small proteins and peptides.

The first study that utilized SELDI-TOF-MS in cancer research was for the detection of ovarian cancer utilizing serum samples [10, 11]. In this report we have used SELDI-TOF-MS to take comparative analyses of proteins in LMP, EOC and each matched normal cells procured by LCM. The objectives of our research were to discover potential biomarkers that could be used to differentiate malignant from the nonmalignant cell populations, and to understand the key molecular events in the pathogenesis of ovarian cancers.

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Materials and Methods

Patients and specimens

We investigated 20 cases of specimens from patients who were surgically treated at the Women's Hospital School of Medicine, Zhejiang University from 2003-2006. Specimens included 13 EOCs and seven LMPs and the matched normal ovarian tissues in the same patients. None of the patients had received chemotherapy or radiotherapy before surgical resection. All cases were reevaluated and classified according to the classification recently accepted by the World Health Organization (WHO). The degree of histological grade was also classified according to WHO criteria. After surgical removal, all tissues were immediately frozen in liquid nitrogen and stored at -70°C until analysis. Ethical approval was obtained before this study was undertaken.

Laser capture microdissection

Frozen samples were embedded in OCT medium, cut in a cryostat at 5 μm thickness, and mounted on membrane-based slides. LCM was performed on the ovarian tissue specimens to enrich cell populations (Figure 1). To assure visual discrimination of specific cell populations procured by LCM, slides were fixed in 70% alcohol for 30 sec and stained with hematoxylin, and one section from each of the frozen samples was stained with H&E, and examined by a pathologist. Frozen sections were dehydrated for 5 sec in 70, 90, and 100% ethanol with a final 5 min dehydration step in xylene. Air-dried sections were laser capture microdissected by a Leica LCM system (Germany). The objective cells were identified and targeted through a microscope, and a 15 μm laser beam pulse activated the film on a CapSure LCM Cap (Arcturus Engineering). In each case, ~$1x10^4$ cells were captured. LCM cells were pooled from multiple caps, which were stored on dry ice until dissection was complete. On the basis of careful review of the histological sections, each microdissection was estimated to contain 95% of desired cells. The time of microdissection was less than 30 min for each slide to avoid proteolysis.

Cell lysates

Microdissected cells for SELDI studies were lysed in 6 μl lysis buffer (7 mol/l urea, 4% CHAPS, 2 mol/l Thiourea, 50 mmol/l DTT and 0.2% protease inhibitor mixture) directly on the LCM cap and incubated for 20 min at 4°C, and then centrifuged briefly to remove cellular debris. The lysates were frozen immediately and stored at -70°C.

SELDI analysis

Ciphergen SELDI-TOF-MS (PBS-II plus) and CM10 ProteinChip (weak cation exchanger) were purchased from Ciphergen Biosystems. Sinapinic acid (SPA) was purchased from Fluka (USA). All other reagents were purchased from Sigma (USA).The samples were thawed in ice and 20 μl of sodium acetate (50 mM, pH4) was added and was further agitated on a platform shaker at 4°C for 2 min. CM10 chips were activated by adding 200 μl of sodium acetate and agitated for 5 min twice. Diluted samples (20 μl) were applied to each spot of the bioprocessor (Ciphergen Biosystems) that contains the ProteinChip arrays. The bioprocessor was then sealed and agitated on a platform shaker for 60 min at 4°C. The excess of serum mixtures was discarded. The chips were then washed three times with 200 μl of sodium acetate and another two times with deionized water. Finally, the chips were removed from the bioprocessor and air-dried. Prior to the SELDI-TOF-MS analysis, 1 μl of a saturated solution of SPA in 0.5 ml/l CAN and 5 ml/l trifluoroacetic acid was applied onto each chip twice and the chips were again air-dried. Chips were detected by the PBS-II plus mass spectrometer reader. Data were obtained by averaging of 140 laser shots with an intensity of 200, a detector sensitivity of 8, a high mass of 100,000 Da and an optimized range of 2000-20,000 Da. Mass accuracy was calibrated by the all-in-one peptide molecular mass standard (Ciphergen Biosystems).

Experiment data analysis by ZUCIPDAS

The total experimental data was handled by the Zhejiang University Cancer Institute ProteinChip Data Analysis System (ZUCIPDAS, www.zlzx.net) which was designed by Yu Jiekai including preprocessed and model construction data. Firstly, the original data were handled by using an undedicated discrete wavelet transform method and denoising the signals. Secondly, according to three labeled peaks (M/Z 4096, 6637 and 1 3764Da) which appeared in all the selected spectra, we adjusted the intensity scale. The spectra were subjected to baseline corrections by aligning with a monotone local minimum curve and mass calibration. The proteomic peaks were detected and quantified by an algorithm that takes the maximal height of every denoised, baseline-corrected, and calibrated mass spectrum into account. Thirdly, we filtered out the peaks with signal-to-noise (S/N) of more than three. Finally, to match peaks across spectra, we pooled the detected peaks if the relative difference in their mass sizes was no more than 0.3%. The minimal percentage of each peak, appearing in all the spectra, is specified to ten. The matched peak across spectra is defined as a peak cluster. If a spectrum does not have a peak within a given cluster, the maximal height within the cluster will be assigned to its peak value. The identified peak clusters was normalized together.

The preprocessed data were used to establish models. In this experiment, we used a nonlinear SVM classifier with a radial based function kernel, and with the parameter gamma of 0.6, being the cost of the constrain violation of 19 to discriminate the different groups. The diagnostic model was evaluated and validated by leaving one cross validation. The principle of validation is that the approach takes out one sample each time as the test set and keeps the remaining samples as the training set, and then the test is repeated until each sample has been taken once as a test sample. Each peak in experiment data was estimated by the p value of the Wilcoxon T-test. The top ten peaks with the smallest p value were selected for further analysis. Combinations with the highest accuracy in distinguishing different groups of data were selected as potential biomarkers. The SVM model with the highest Youden's index was selected as the model for detecting esophageal carcinoma and precancerous lesions.

Results

Sample harvest and SELDI array

The pure populations of ovarian malignant epithelial, low malignant potential epithelial cells and organ-matched normal or benign epithelial cells from seven LMP and 13 EOC specimens were selectively microdissected. In two of the EOC specimens, benign, low malignant potential and malignant cell types were identified and harvested. An average of 5,000 cells was microdissected in duplicate for each cell type. Afterwards filtrat-

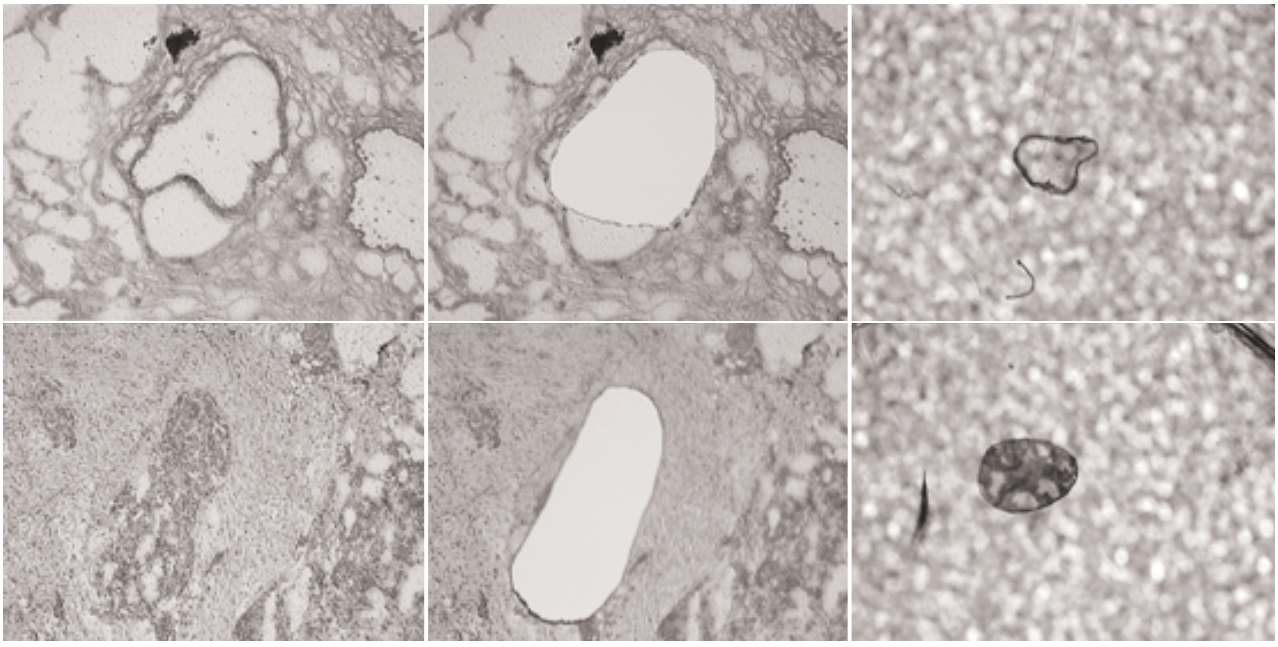

Figure 1. — Laser capture microdissected (LCM) representative sections from LMP and EOC. (A) epithelial cells in LMP. (B) LCM removed the epithelial cells. (C) Cap containing only selected epithelial cells. (D) Epithelial cells in EOC. (E) LCM removed the epithelial cells. (F) Cap containing only selected epithelial cells.

ing noise by Ciphergen ProteinChip Software 3.1, 200 peaks were detected. The peaks were 2 kDa to 30 kDa. Peaks with m/z < 2 kDa were mainly ion noise from the matrix and therefore excluded.

Protein profiles of malignant and the matched normal cells in the same patients from LCM

We found that intensities for seven peaks detected in the mass spectra were significantly different between tumor cells and normal or benign cells. These peaks were m/z of 11659 Da, 4697 Da, 10143 Da, 5077 Da, 10182 Da, 9970 Da and 10107 Da. Among them, six peaks from tumor cells showed marked increases when compared with normal or benign cells, but one peak (4697 Da) was overexpressed in normal or benign cells than in tumor cell lysates ($p < 0.05$). Figure 2 and Table 1 give the descriptive statistics of the seven peaks.

Table 1. — *Seven peaks (m/z) were significantly different between cancer and normal or benign cells (Da, mean ± SD).*

Peak	Cancer	Normal or benign	p value
11659Da	674.44 ± 635.59	206.59 ± 395.06	0.0065688018
4697Da	382.79 ± 109.45	422.93 ± 422.88	0.0089127029
10143Da	1311.78 ± 973.24	453.42 ± 576.53	0.0159413125
5077Da	586.56 ± 313.05	369.52 ± 203.67	0.0274450994
10182Da	599.57 ± 646.83	289.10 ± 498.77	0.0312522389
9970Da	536.45 ± 414.62	225.91 ± 202.23	0.0355038906
10107Da	2315.86 ± 812.91	850.72 ± 535.75	0.0455002639

Protein profiles of LMP and the matched normal or benign ovarian cells in the same patients from LCM

The peaks with m/z of 4951 Da, 4481 Da, 10852 Da, 4433 Da were significantly different between LMP and normal or benign ovarian cells ($p < 0.05$). Among them, three peaks from LMP cells were overexpressed when compared with normal or benign cells, but one peak (4951 Da) was decreased ($p < 0.05$). Figure 2 and Table 2 give the descriptive statistics of the four peaks.

Table 2. — *Four peaks were significantly different between LMP and normal or benign ovarian cells (Da, mean ± SD).*

Peak	LMP	Normal or benign	p value
4951Da	3024.26 ± 808.28	5690.49 ± 618.73	0.0131642013
4481Da	618.05 ± 375.06	224.39 ± 211.45	0.0323777399
10852Da	328.76 ± 351.67	22.72 ± 30.92	0.04066462695
4433Da	3122.67 ± 769.00	1252.13 ± 270.91	0.0422811156

Comparison of protein profiles of malignant and LMP cells

In our samples, two of the EOC specimens held the benign, LMP and malignant cell types. We found that the peaks at 10181Da and 10852Da were elevated both in malignant and LMP cells compared with normal or benign cells, and that the peaks were more overexpressed in malignant cells than in LMP cells, as shown in Figure 2 (C-F).

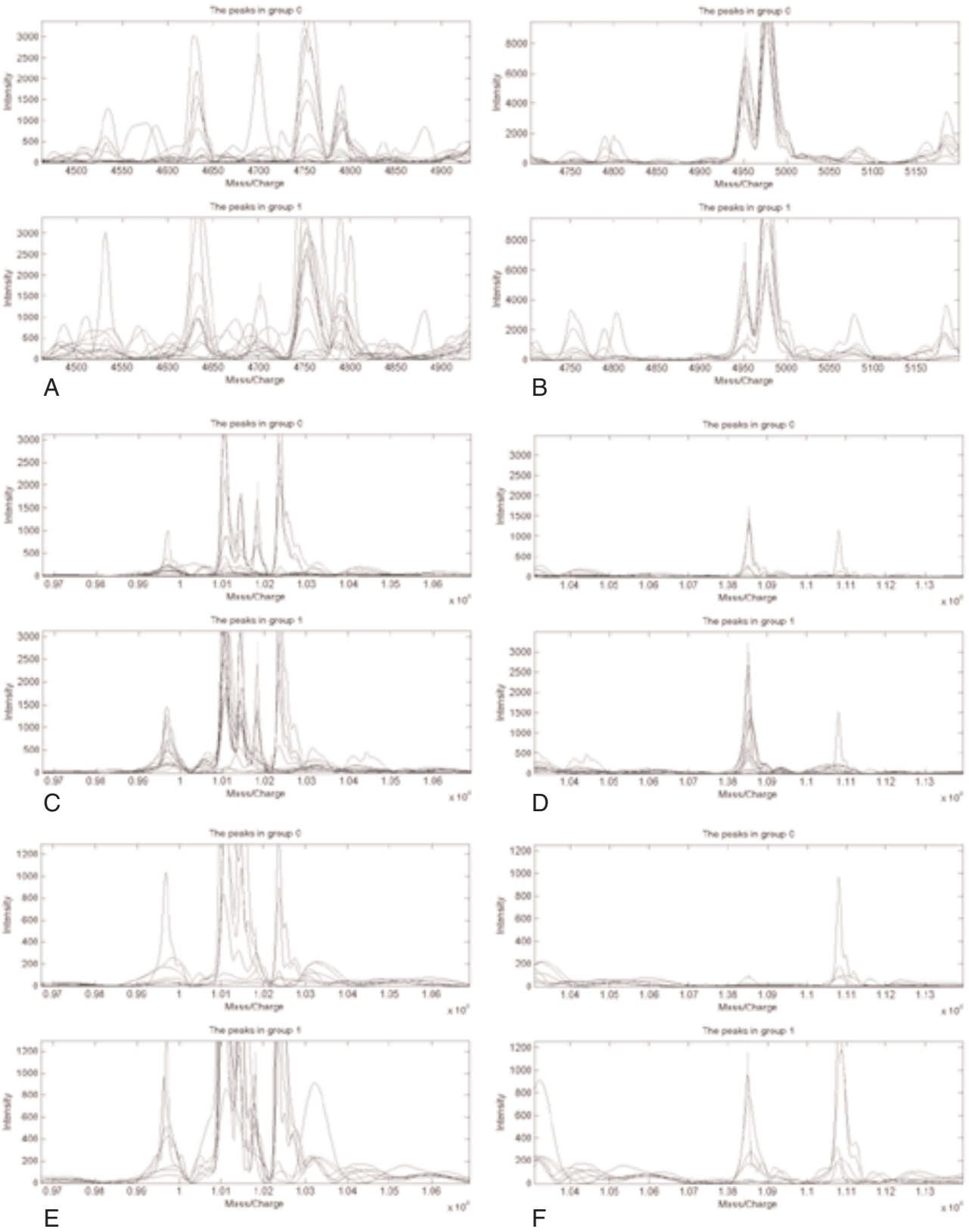

Figure 2. — Representative examples of SELDI-MS spectra on EOC, LMP and matched normal or benign cells. (Group 0) normal or benign cells. (Group 1) tumor cells. (A.C.D) EOC. (B.E.F) LMP. (A) m/z,4697Da. (B) m/z,4951Da. (C.E) m/z,10181Da. (D.F) m/z 10852Da.

Comparison of protein profiles of different histological type of EOCs

We did not find that different histological types of EOC cells shared any of the same peaks.

Discussion

EOC is a highly heterogeneous group of cancers. It is the fourth most common cause of death from cancer among women in the United States [1]. The 5-year survival rate is less than 40% [12] because of the presentation of the majority of cases at an advanced stage, and the etiology and precursor lesions of EOC are poorly understood. The development of EOC is a multistep process encompassing multiple events involving oncogenic and tumor suppressor gene products. These events can occur pre- or post-translationally and will be reflected in differential changes in a lot of proteins [13]. Analyzing the proteomic changes that occur in EOC progression is very difficult because of the biological heterogeneity of it. To compare protein expression in EOC, sufficient numbers of these cells are needed. One solution is microdissection of pure cell populations. SELDI has provided an approach for the sensitive and direct analysis of proteins in complex biological samples. The principle of this technique is very simple. A few microliters of an interesting sample are deposited on the chromatographic surface. The protein chip arrays are incubated and then washed with an appropriate buffer. The proteins of interest are captured on the chromatographic surface by adsorption, partition, electrostatic interaction or affinity chromatography depending on their properties, and analyzed by TOF-MS. The result is a mass spectrum comprised of the mass-to-charge (m/z) values and intensities of the bound proteins/peptides [14]. A unique strength of SELDI is its ability to analyze proteins from a variety of crude sample types, with minimal sample consumption. The number of publications describing the use of this technology has increased significantly in the past few years. In particular, the technology has been applied extensively in cancer research. Previous studies have demonstrated the successful application of this technology in the serum of EOC patients [10]. In this study, we first combined SELDI with LCM to get the protein profiles from microdissected epithelial ovarian tumors. Our findings indicate specific protein peaks that show a marked change in expression associated with EOC or LMP. The patterns of increases and decreases in protein levels were observed when protein profiles of tumor cells were compared with normal or benign cells. Seven peaks detected in the mass spectra were significantly different between tumor cells and normal or benign cells. Among them, six peaks from tumor cells showed marked increases when compared with normal or benign cells, but one peak (4697 Da) was more overexpressed in normal or benign cells than in tumor cell lysates. The four peaks were significantly different between LMP and normal or benign ovarian cells. Among them, three peaks from LMP cells were overexpressed when compared with normal or benign cells, but one peak (4951Da) was decreased. Interestingly, some of these peaks appeared in serum from some of the same patients in this study (data not shown). This approach could also lead to a several new possibilities in EOC detection including evaluation of ''malignant'' peptide expression patterns in tissue and serum samples on high throughput platforms, serving as screening tools for populations at high risk for EOC or LMP, and for monitoring response to EOC chemopreventive agents, therefore identity of the peaks and the examined serum samples will need to be done to confirm these results.

EOC appears to arise via one of at least two pathways; spontaneously and aggressively, with no precursor lesion (Type 2), or by slower development from an inclusion cyst to a benign adenoma or cystadenoma of LMP, through to metastatic adenocarcinoma (Type 1) [15]. Previous studies have shown a cytogenetic link between LMP and low-grade EOC strengthening its role as a precursor lesion [16, 17]. Therefore, the identification of proteins specifically associated with LMP would have a tremendous impact as markers for the early detection of EOC. In our study, two of the EOC specimens held the benign, LMP and malignant cell types. We found that the peaks at 10181Da and 10852Da were elevated both in malignant and LMP cells compared with normal or benign cells, and the peaks were more overexpressed in malignant cells than in LMP cells. EOC and LMP cells exhibited similar SELDI protein profiles underscoring the phenotypic similarity of these two disease states.

A recent profiling study compared serous, mucinous, endometrioid and clear cell EOC with normal OSE brushings, as well as the fallopian tube, endometrium and colon [18]. When compared with normal OSE, the changes in gene expression noted in serous EOC correlated with those found in the fallopian tube, but not in other normal tissues. Similarly, differences between mucinous EOC and normal OSE correlated with those in normal colon, and differences between both endometrioid and clear cell cancers and OSE correlated with those in normal endometrium. In this research, we did not find different histological types of EOC cells sharing any of the same peaks. All these studies identify the specific molecular alterations and pathways that lead to the different EOC histotypes, as well as identifying important histotype-specific biomarkers.

Conclusion

The application of molecular biology techniques to tumor biology has produced information about the cellular processes that regulate proliferation, differentiation, and apoptosis in normal cells, and disrupting these normal functions in cancer initiation and progression. It is clear that a new approach is needed to accurately distinguish between normal cells, early precancerous lesions, and malignant diseases. A variety of mass spectrometry-based platforms are currently available for providing information on both protein patterns and protein

identity. In our research, differential SELDI protein profiles were observed for cell lysates prepared from microdissected LMP, EOC and normal cells. Additional studies are under way to identify and characterize these potential peptide/protein biomarkers. Once identified, characterization of their function and biological role in ovarian tumorigenesis may lead to their potential use as diagnostic and prognostic biomarkers as well as conceivable therapeutic targets.

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