Identification of candidate biomarkers using the Experion™ automated electrophoresis system in serum samples from ovarian cancer patients

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Abstract. Ovarian cancer is the most common cause of disease-related death in women globally. Detection of ovarian cancer using new biomarkers is necessary for early diagnosis. To date, there have been no obvious biomarkers for ovarian cancer detection in the incipient stage. In this study, we discovered potential diagnostic serological biomarkers for ovarian cancer using the Experion™ automated electrophoresis system. Sera from 14 healthy women and 84 ovarian cancer patients at stages I- IV were applied to the Experion to compare the protein expression levels. To examine the protein expression pattern of Experion data, proteins in the samples were resolved using 10 and 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by silver staining. The candidate biomarkers elevated in ovarian cancer were purified and determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. α-2-macroglobulin (173.7 kDa), ceruloplasmin (147 kDa), inter-α-trypsin inhibitor family heavy chain-related protein (126 kDa), C-1 inhibitor (115.2 kDa) and hemoglobin α/β (14.4 kDa were overexpressed in the ovarian cancer sera. This study documents a novel way to measure ovarian cancer or cancer-related proteins for biomarkers using the Experion assay system, which should be easily adaptable for high-throughput diagnosis to establish databases of ovarian cancer for clinical applications.

Introduction

Ovarian cancer is a significant cause of death in females globally (1). This cancer is hard to detect at an early stage because of the non-specific symptoms and misdiagnosis as other disease. The high mortality associated ovarian cancer is due to delayed diagnosis after metastasis to other organs (2). Therefore, it is important to find new biomarkers for detecting and monitoring of ovarian cancer at early stage (3).

Early stage detection of ovarian cancer has a survival rate of over 90% (4). Presently, the cancer antigen CA-125 is increased in more than 80% of patients with fairly advanced ovarian cancer (5-7). The level of CA-125 has become established as a useful biomarker for the assessment of ovarian cancer (8-10). The serum level of CA-125 and ultrasonography are used to standardize diagnosis for advanced ovarian cancer determination. Tumor serum markers could provide reliable and reproducible information for evaluation of disease. It has been reported that CA-125 is the most useful target for prognosis of ovarian cancer (11-14). However, CA-125 monitoring at an early stage of ovarian cancer is difficult (15). CA-125 lacks sensitivity and specificity for screening of ovarian cancer. The combined use of haptoglobin-α (Hp-α) and CA-125 has 91% of sensitivity and 96% of specificity in the serum of ovarian cancer patients (16). Therefore, many studies have sought to find biomarkers that can overcome the deficiencies of CA-125 (17). Biomarkers capable of early detection would improve ovarian cancer patient survival rate.

More recently, biological tools including microarrays and proteomics have been explored in identifying new biomarkers for ovarian cancer. In this study, a combined approach based on the Experion automated electrophoresis system (Bio-Rad, Hercules, CA) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to identify highly sensitive and specific serum biomarkers in patient serum from 14 healthy women and 84 ovarian cancer patients at stages I-IV. The Experion system is able to quantify protein expression levels through high throughput screening. These candidate markers were identified by MALDI-TOF-MS. The distinctive polypeptides were identified as α-2-macroglobulin (173.7 kDa), ceruloplasmin (147 kDa), inter-α-trypsin inhibitor family heavy chain-related protein (IHRP; 126 kDa), C-1 inhibitor (115.2 kDa and hemoglobin α/β (14.4 kDa).

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Table I. Clinical characteristics and ages of the patients.

	Diagnostic groups and FIGO stages				Age	Histologic subtypes		
	a	b	c	n	Mean ± SD (median)	Epithelial ovarian tumor	Stromal cell	Other
Control				10	39±5 (40)			
Stage I	11	1	7	19	45±13 (49)	11	3	5
Stage II	2	2	6	10	46±13 (52)	8		2
Stage III		1	40	41	57±13 (62)	37		4
Stage IV				14	54±11 (56)	8		6

FIGO, International Federation of Gynecology and Obstetrics.

Table II. Protein peaks identified by the Experion system.

Healthy women		Ovarian cancer patients				
MW (kDa)	Con (μg/μl)	Stage I Con (μg/μl)	Stage II Con (μg/μl)	Stage III Con (μg/μl)	Stage IV Con (μg/μl)	Average (stages I-IV) Con (μg/μl)
9.9	13.2	9.4	6.3	6.3	6.1	7.0
12.5	1.2	5.3	0.0	0.0	0.0	1.3
14.4	**2.7**	29.7	38.8	18.6	62.8	**37.5**
17.1	**5.4**	2.1	3.2	1.1	2.2	**2.2**
19.1	11.6	12.4	10.2	12.2	17.6	13.1
24.1	88.2	75.7	56.1	65.3	70.7	67.0
28.8	253.3	225.4	258.2	228.1	290.6	250.6
36.0	125.9	99.8	104.4	112.1	135.4	112.9
38.4	0.0	31.9	124.4	23.7	40.2	55.1
41.4	15.7	15.7	0.0	0.0	0.0	3.9
47.8	0.0	1.0	3.6	6.5	2.3	3.4
54.3	21.9	19.3	16.9	18.5	16.4	17.8
56.5	0.0	0.0	35.2	9.8	9.4	13.6
64.4	2,160.4	2,093.9	2,419.2	1,862.6	2,295.0	2,167.7
78.6	62.6	99.8	0.0	33.0	0.0	33.2
81.9	53.7	57.8	134.0	65.9	57.0	78.7
90.4	208.1	189.0	180.3	123.4	161.6	163.6
94.6	0.0	0.0	203.7	38.7	40.3	70.7
110	**54.5**	28.2	36.9	24.5	26.2	**29.0**
115.2	**0.0**	40.2	54.4	52.0	63.7	**52.6**
126	**1.4**	7.5	9.5	13.3	12.4	**10.7**
147	**1.1**	2.3	4.5	6.7	7.9	**5.4**
176.3	**6.0**	11.2	9.0	14.6	15.3	**12.5**

Con, concentration.

Materials and methods

Ovarian cancer patients and specimens. Our analysis included 10 healthy women and 84 ovarian cancer patients. The average age of the ovarian cancer patients was 46 years (Table I). The stages of tumors from the ovarian cancer patients were assigned according to the guidelines provided the International Federation of Gynecology and Obstetrics. Each serum sample was provided by Kang Nam St. Mary's Hospital of Catholic Medical School according to the procedures approved by the Institutional Review Board of the Catholic University of Korea (IRB no. KCM07MI020).

Experion™ system of automated electrophoresis. The Experion automated electrophoresis system (Bio-Rad) integrates protein quantitation into a single process in which protein separation,

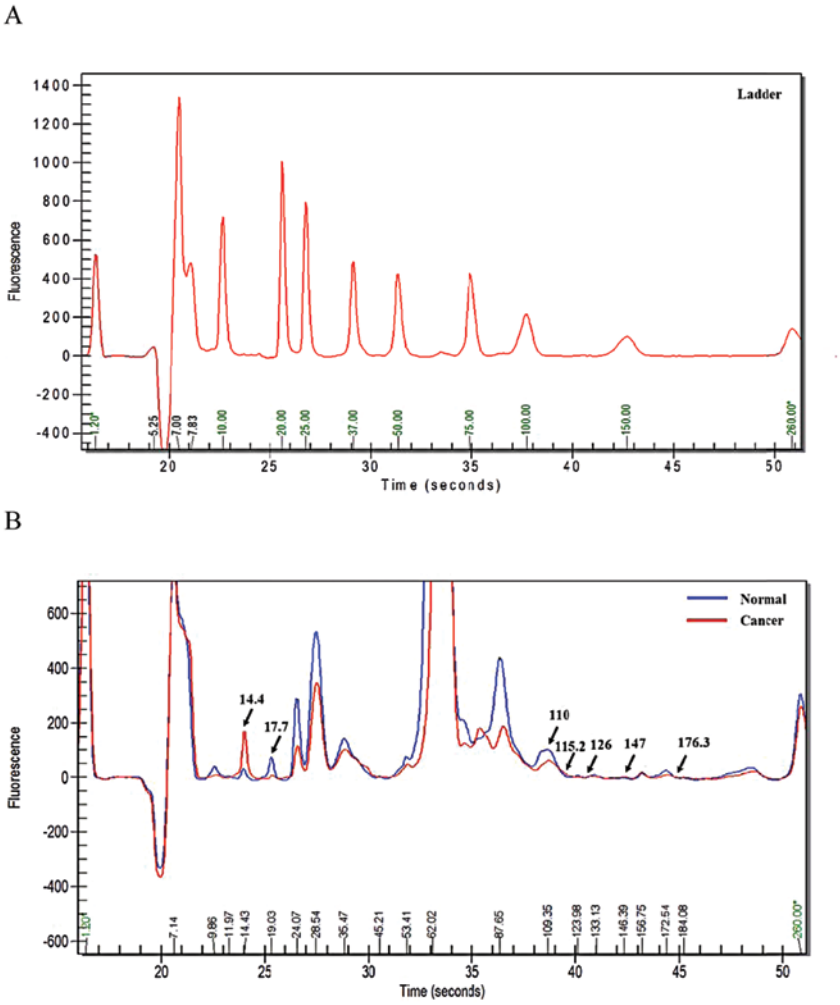

Figure 1. Spectra pattern differences between normal and ovarian cancer serum in Experion analysis. The purified protein fractions were monitored by Experion system. (A) Spectra of standard molecular marker. (B) Serum protein from ovarian cancer patients and normal healthy controls.

staining, band detection and quantitation are automatically executed (18). All procedures followed the manufacturer's protocol.

In-gel digestion with trypsin and extraction of peptides. The procedure for in-gel digestion of protein spots from silver stained gels were done as previously described (19). Pieces of stained gel were washed in 25 mM ammonium bicarbonate buffer (pH 7.8) containing 50% (v/v) acetonitrile (ACN) for 1 h at room temperature. The gels were dehydrated by speed vacuum for 10 min and then rehydrated in trypsin solution (Promega, Madison, WI) at 37˚C overnight. The tryptic peptides were incubated with 5 μl of 0.5% trifluoroacetic acid (TFA) containing 50% (v/v) ACN for 40 min with mild sonication. The eluted peptides were enriched up to 1 μl volume using vacuum centrifugation (20). To perform mass spectrometric analysis, each peptide solution was applied to a desalting column (GE loader tip; Eppendorf, Hamburg, Germany) (21,22). Eluted samples from desalting column were dropped onto a MALDI plate (96x2; Applied Biosystems, Foster City, CA) for analysis as described below.

Analysis of peptides using MALDI-TOF MS for identification of proteins. MALDI-TOF mass spectrometry was performed using a Voyager-DE STR mass spectrometer (Applied Biosystems) in the reflectron positive ion mode (19). The proteins were matched by peptide mass fingerprinting searching against the Swiss-Prot and NCBI databases, using the search program MS-Fit (http://prospector.ucsf.edu).

Results

Quantification of protein expression in ovarian cancer sera. To ascertain the protein expression patterns in the ovarian cancer patient sera, we used an Experion™ Pro260Chip and analyzed the distinction between ovarian cancer patient sera and sera from normal females. The protein quantification profile of the serum samples revealed higher protein concentrations in the ovarian cancer sera than normal sera for proteins migrating with an electrophoretic mobility of 14.4, 115.2, 126, 147 and 176.3 kDa (Table II). On the other hand, normal control samples were higher than ovarian cancer serum in 17.1- and 110-kDa proteins. These proteins all displayed concentration differences exceeding 1.5-fold between normal and ovarian cancer serum. Although the spectra profiles of the serum of ovarian cancer and normal were comparable, seven different peak patterns were expressed (Fig. 1). These results enabled the detection of potential biomarkers of ovarian cancer. To

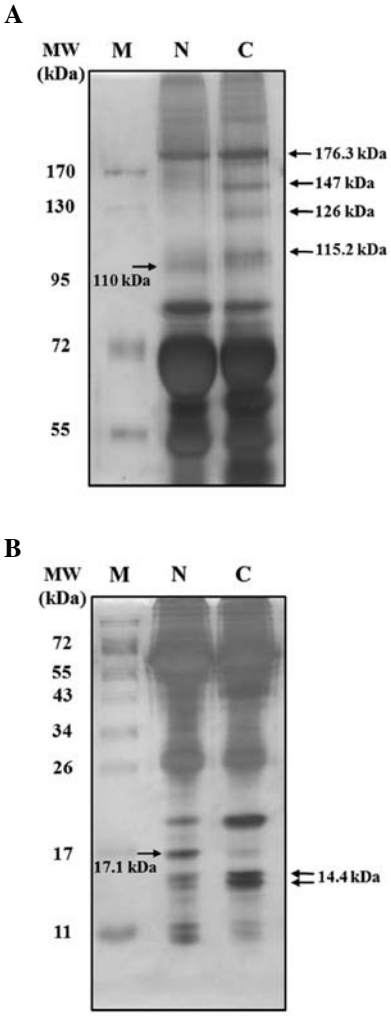

Figure 2. Protein expression level of normal and ovarian cancer patient serum in silver stained gel. (A) Proteins (176.3, 147, 126, 115.2 and 110 kDa) were marked in 10% SDS-PAGE gel. (B) Proteins (17.1 and 14.4 kDa) were marked in 15% SDS-PAGE gel.

confirm the elevated protein expression level in ovarian cancer serum, the serum samples were examined using 10 and 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining. The silver stained 10% gel images show that proteins of 115.2, 126, 147 and 176.3 kDa were elevated in ovarian cancer sera, whereas a 110-kDa species was increased in normal sera (Fig. 2A). In 15% gels, a 14.4- kDa species was increased and a 17.1-kDa protein was decreased in ovarian cancer sera (Fig. 2B).

Purification and identification of biomarker candidates. To further characterize the candidate biomarkers, the fractions eluted from the gel were analyzed by MALDI-TOF-MS, which confirmed the purification of the polypeptide peaks (Fig. 3). Analysis focused on regions showing reproducible differences in increased intensity between sera of ovarian cancer patients and healthy individuals. The differential expression of the protein content between the two groups was determined using MALDI-TOF-MS analysis. Table III lists the identified proteins, theoretical pI value, molecular weight, Z score and number of peptides used for identification and protein coverage.

Seven proteins were determined to be α-2-macroglobulin (176.3 kDa), ceruloplasmin (147 kDa), IHRP (126 kDa), C-1 inhibitor (115.2 kDa), P130 (110kDa), transthyretin (TTR; 17.1 kDa) and hemoglobin β/hemoglobin α (14.4 kDa) (Table III). Expression of P130 (110 kDa) and TTR (17.1 kDa) were lower in serum from ovarian cancer patients than in healthy women, whereas expression of α-2-macroglobulin (176.3 kDa), ceruloplasmin (147 kDa), IHRP (126 kDa), C-1 inhibitor (115.2 kDa) and hemoglobin β/hemoglobin α (14.4 kDa) were increased.

Discussion

In this study, we used the Experion protein quantification system to detect biomarkers for diagnosis in ovarian cancer patient. The Experion automated electrophoresis system easily enables protein quantitation and performs high-throughput screening for detecting candidate biomarkers in ovarian cancer. The present findings are hopeful, given that ovarian cancer is one of the detrimental causes of death in females in the world (1), yet no apparent clinical prognoses or characteristics have been demonstrated at the initial stage of ovarian cancer.

We detected hemoglobin β chain, hemoglobin α chain together with α-2-macroglobulin, ceruloplasmin, IHRP, C-1

Table III. Proteins identified by peptide mass fingerprinting using MALDI-TOF-MS.

Experion Data (kDa) up		MALDI-TOF-MS result				
		Identified protein	MW	pI	Est'd Z (95%≥1.65)	Coverage (%)
176.3	C	α-2-macroglobulin	164.72	6.0	2.37	21
147	C	Ceruloplasmin	120.87	5.4	2.24	22
126	C	IHRP	103.60	6.5	1.57	18
115.2	C	C-1 inhibitor	32.75	8.8	2.30	24
110	N	P130	129.78	7.4	0.89	8
17.1	N	TTR	12.83	5.3	1.42	67
14.4	C	Hemoglobin β	15.98	6.8	2.00	45
		Hemoglobin α	10.69	7.1	1.34	40

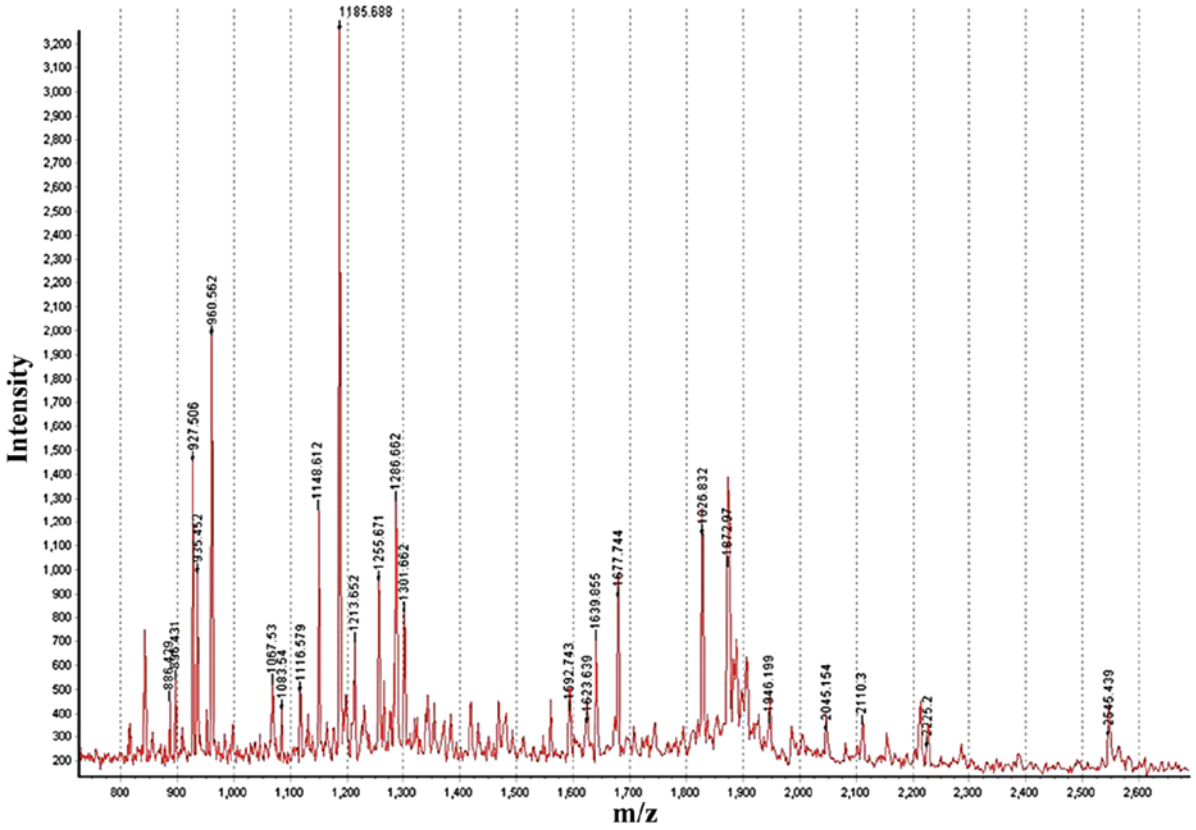

Figure 3. MALDI-TOF MS analysis of serum proteins.

inhibitor, P130 and TTR. Of these proteins, P130 and TTR were decreased in cancer serum samples. Our analysis included 10 healthy individuals and 84 ovarian cancer patients.

α-2-macroglobulin (A2M) is a protease inhibitor in mammals (23). It is reported that A2M is secreted in serum of women with inflammatory and neoplastic ovarian lesions. A2M has also been semi-quantitatively identified in ovarian cancer-related proteins (24).

Ceruloplasmin is a member of a family of copper transport metalloproteins. It has important roles in iron metabolism and antioxidant defense (25). Ceruloplasmin blocks the copper ion-activated production of toxic oxygen compounds and protects cells from oxidative stress (26). Ceruloplasmin protein is expressed in pancreatic, nasopharyngeal and germline ovarian cancers (27). Ceruloplasmin promoter activation is specifically and efficiently enhanced in ovarian cancer (28).

IHRP is an acute phase protein and glycoprotein in mammals, which is cleaved to different length fragments (29). IHRP inhibits polymerization through binding to actin and protects cells from phagocytosis. IHRP concentrations are elevated in patient serum of inflammatory disease. So, IHRP has been implicated as an anti-inflammatory protein (30).

C-1 inhibitor is a protease inhibitor that regulates vascular permeability and suppression of inflammation (31). C-1 inhibitor is an acute phase protein that inhibits complement system protease. Also, C-1 inhibitor is proposed to play a role in inhibition of alternative complement activation and inflammation.

Hemoglobin is an iron-containing oxygen transporter in red blood cells. Hemoglobin is overexpressed in ovarian cancer (16,32). Previously, our group also reported that the potential value of the hemoglobin-α and -β subunits as serum biomarkers for the early diagnosis and prognosis of ovarian cancer (33).

In contrast, our results show that P130 and TTR were decreased in ovarian cancer patient serum. Commonly, it has been reported that these protein expressions were decreased in cancer patient serum.

P130 (also known as pRb2), is a member of a family of retinoblastoma proteins. Proteins of the pRB family regulate transcription and progression of the cell cycle (34). P130 may operate as a tumor suppressor in small-cell lung carcinoma (35). The decline of P130 causes tumorigenesis in mouse model of human lung adenocarcinoma (36).

TTR is a carrier of the thyroid hormone thyroxine and retinol in serum and cerebrospinal fluid. TTR is reduced in ovarian cancer as well as cervical and endometrial carcinomas (37,38).

The present study demonstrates the potential of the Experion quantitation method that covers high sensitivity and specificity biomarker discovery in ovarian cancer. Our group has previously found that hemoglobin β/α and ceruloplasmin are increased in ovarian cancer serum using the Experion assay system (39). Hemoglobin β/α has been identified as an ovarian cancer biomarker by using the surface enhanced laser desorption/ionization time-of-flight mass spectrometry mass method (33). Thus, hemoglobin β/α must be regarded as a strong candidate ovarian cancer biomarker. α-2-macroglobulin, IHRP, C-1 inhibitor, P130 and TTR were all also presently altered in expression (overexpressed or decreased) in ovarian cancer. Further studies are needed to determine their relevance as

ovarian cancer biomarkers. In addition, C-1 inhibitor and IHRP were increased in stage I ovarian cancer serum compared with normal serum (Table II), implicating the two proteins as strong candidate biomarkers for the early detection of ovarian cancer.

Our study shows that the Experion system is able to identify new biomarkers selectively and correctly. Also, the Experion system could be applied to find other disease biomarkers.

In conclusion, α-2-macroglobulin, ceruloplasmin, IHRP, C-1 inhibitor, P130, TTR and hemoglobin β/α were identified in ovarian cancer using the Experion assay system. The findings provide evidence for the use of these proteins as new potential biomarkers for ovarian cancer diagnosis. Identification of potential biomarkers provides opportunities to develop non-invasive diagnosis and further improves the understanding of ovarian cancer development.

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