Multiplex Analysis of Serum Biomarkers in Ovarian Cancer Patients Using Bio-Plex® Suspension Array System

V. Gupta, C. Reyes, W. Geng, J. Fedynyshyn and W. Tan

Life Science Group
2000 Alfred Nobel Drive
Hercules, CA 94547 USA

Abstract

Ovarian cancer is the fifth leading cause of cancer deaths among North American women. CA-125 tumor antigen has been the standard for monitoring response of ovarian cancer patients to therapy. However, the use of this marker for detecting ovarian cancer has been limited by both false positive and false negative results. The analysis of novel cancer biomarkers in combination with this established marker as a composite profiling tool is expected to further benefit early detection, screening, and prediction of the disease. In this study, we profiled the levels of 37 potential biomarkers. These are circulating proteins in sera collected from ovarian cancer patients in different stages. The results showed that eight markers, angiopoietin-2, G-CSF, IL-6, IL-10, procalcitonin, ferritin, haptoglobin, and CRP were highly elevated in ovarian cancer samples as compared to age-matched healthy controls. In contrast, the levels of leptin, PECAM-1, PAI-1, and visfatin were found to be reduced in the cancer patients.

Introduction

In the United States alone, approximately 23,000 new cases of ovarian cancer are diagnosed each year, with a mortality count of close to 15,000 per year (www.ovariancancer.org). Contributing to the poor prognosis is the lack of symptoms in the early stages of the disease. As a result, more than 75% of the diagnoses are made in stages III and IV, in which the survival rate is less than 30% (Schink 1999). Despite great attention to the use of CA-125 as a reliable biomarker for detecting ovarian cancer, many attempts are falling short of demonstrating its clinical utility as an early diagnostic marker, especially in the high-risk group of women. In many documented cases, the level of CA-125 was found to be elevated above reference levels in only 50% of clinically detectable early stage disease (Posadas 2004). Ultimately, combining new biomarkers with CA-125 as a composite of markers would improve the diagnostic value of CA-125 for early detection of the disease.

In this study, we evaluated the levels of 37 serum proteins in sera collected from ovarian cancer patients in stages I–III. These markers were initially chosen to profile their clinical relevance to ovarian carcinoma. Targets with a high frequency of citations (Table 1) reflect the significant association of these markers to ovarian cancer. These 37 markers covered cytokines, angiogenic factors, acute phase proteins, hormones, and other serum proteins. The levels of these markers were also measured in healthy subjects to establish baseline.

Table 1. Citation matches for serum proteins relevant to early detection of ovarian cancer.

Source	VEGF	G-CSF	TNF-α	Insulin	IL-6	tPA*	IL-8	PECAM-1 (CD31)	PAI-1	Ferritin	Haptoglobin	CRP	Leptin
PubMed citation	515	427	423	391	206	150	107	73	72	72	48	37	20
Google hits (08/28/08)	225,000	39,300	110,000	185,000	124,000	66,700	69,600	23,900	146,000	58,000	26,200	56,300	99,500
ScienceDirect	76	36	72	59	59	18	42	8	18	2	6	12	2

* tPA, tissue plasminogen activator.

Methods

The 37 serum markers are available commercially in five separate Bio-Plex suspension array panels: Two Bio-Plex Pro™ human acute phase panels, Bio-Plex Pro™ human angiogenesis, and human diabetes panels, and the Bio-Plex® Precision Pro™ human cytokine panel. Analysis was carried out on a Bio-Plex suspension array system (Figure 1), which permits the simultaneous measurement of multiple serum proteins in a single well in 3 hr, using as little as 12.5 µl of serum or 50 µl of tissue culture supernatant.

Fig. 1. Bio-Plex suspension array system. The complete system includes an array reader, microplate platform and high-throughput fluidics (HTF) system, in addition to the standard assay modules and reagent and diluent kits.

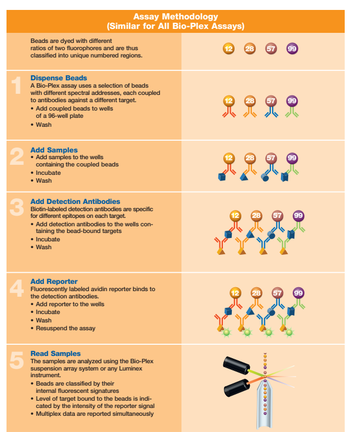

Fig. 2. General assay methodology.

The magnetic bead-based assay and its platform integrate a series of color-coded beads, each of which is coupled to a unique antibody specific for a biochemical marker. Each magnetic particle is dyed with two fluorophores. Each of the classification dyes emits at a distinct wavelength with its absorption maximum at 635 nm. The reporter is a third fluorophore, phycoerythrin (PE), which absorbs maximally at 532 nm and emits at a third distinct wavelength. Phycoerythrin was chosen for its high molar extinction coefficient, quantum yield, resistance to photobleaching, minimal self-quenching, and excellent stability. The detector consists of a flow cell designed such that the magnetic particles flow in single file (laminar flow) through a region illuminated by two lasers. The particles emit light at 3 wavelengths, two from the classification dyes and one from the reporter dye. These capture antibody-coupled beads serve as solid phase for the capture of the desired serum or plasma proteins using a standard sandwich-based detection format. For preliminary screening, the levels of these markers were measured from sera collected from 12 ovarian cancer patients and their matching controls (ILSbio, LLC.) The disease samples were diagnosed with stages I–III and grades 1–3 of ovarian cancer. The average age of the diseased subjects was 54 years, with a range of 43–75. Control subjects were age matched to the disease subjects. All serum samples were diluted 1 in 4 with the appropriate diluents prior to assay. For some targets, the serum samples were subjected to higher dilution in order to bring the measurement to within assay range. Hemolyzed samples were excluded from the study. A general assay workflow is described in Figure 2.

Results

Assay Specifications

The 37 serum proteins were grouped into five individual assay panels (Table 2). The design, validation, and verification of these multiplex assay panels followed a standard workflow that addressed intra- and inter-assay %CV, standard and sample recovery, limit of detection (LOD), as well as assay range.

Table 2: Precision and accuracy of assays.

	Human Angiogenesis 9-Plex	Human Diabetes 12-Plex	Human Acute Phase 5-Plex	Human Acute Phase 4-Plex	Human Cytokine 10-Plex
	Angiopoietin-2	C-peptide	Procalcitonin	A2M	IL-1β
	Follistatin	Ghrelin	Ferritin	Haptoglobin	IL-2
	HGF	GIP	Fibrinogen	CRP	IL-4
	IL-8	GLP-1	tPA	SAP	IL-5
	G-CSF	Glucagon	SAA		IL-6
	PDGF-BB	IL-6			IL-7
	VEGF	Insulin			IL-10
	Leptin	Leptin			IL-12 (p70)
	PECAM-1	PAI-1			IFN-γ
	Resistin				TNF-α
	TNF-α				
	Visfatin				
Precision (%CV)					
Intra-assay	≤15%	≤20%	≤15%	≤15%	≤10%
Inter-assay	≤25%	≤30%	≤25%	≤25%	≤15%
Accuracy (%Recovery)	70–130%	70–130%	70–130%	70–130%	80–120%

The assay range was determined from five independent analyses to obtain mean lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ). Within this range, the performance of each assay was defined by intra-assay replicate precision and acceptable recovery of the standard points as well as spiked samples. Table 3 lists the specifications for assay range, LOD, and dilutions required to bring measurements to within assay range.

Table 3. Assay range, limit of detection, and sample dilution of different targets.

Target	Assay Range, pg/ml	LOD, pg/ml	Sample Dilution
IL-1β	0.2–556	0.08	4
IL-2	4.5–4,570	0.41	4
IL-4	0.2–2,611	0.09	4
IL-5	5–4,355	1.37	4
IL-6	5–18,618	0.34	4
IL-13	1–11,850	0.17	4
IL-10	1.7–3,994	0.13	4
IL-12 (p70)	0.3–2,406	0.24	4
IFN-α	0.7–1,814	0.27	4
IL-8	1–7,475	0.2	4
PDGF-BB	3–24,423	1.3	4
VEGF	1–16,906	0.8	4
C-peptide	371–5,400	44	4
GIP	17–13,000	1.6	4
GLP-1	188–27,000	6.5	4
Glucagon	137–18,000	13	4
PAI-1	37–18,000	3.7	4
Resistin	3–1,100	0.3	4
TNF-α	0.9–3,200	0.2	4
HGF	20–20,201	7.2	4
G-CSF	11–25,665	2.3	4
Follistatin	25–25,584	3.6	4
PECAM-1	45–32,691	5.8	4
Leptin	8–36,256	3.9	4
Ghrelin	35–50,000	2.2	4
Insulin	250–32,000	40	4
Angiopoietin-2	59–59,784	10.1	4
Visfatin	427–500,000	137	4
Ferritin	3–50,000	1.3	100
Fibrinogen	5,000–813,000	2,800	100
Procalcitonin	14–10,000	11.3	100
SAA	1,000–700,000	1,100	100
tPA	28–5,000	6.15	100
A2M	500–1,875,000	250	10,000
CRP	10–50,000	4	10,000
SAP	100–250,000	63	10,000
Haptoglobin	100–500,000	70	10,000

In the initial screen, the levels of 37 serum proteins were compared between 12 ovarian cancer patients and 12 age-matched healthy women. Age matching was to ensure comparable immune response in both groups. The difference in expression between the two groups is summarized in Table 4.

Table 4. Marker presentation between women with ovarian cancer and healthy women.

		Mean		Median		Range	
Target	Units	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
Angiopoietin-2	pg/ml	434	731	375	786	143–901	285–1,294
Follistatin	pg/ml	634	1,121	476	656	321–1,343	422–2,931
G-CSF	pg/ml	106	169	95	183	37–149	79–252
HGF	pg/ml	1,143	1,334	1,122	1,302	777–1,695	673–1,835
IL-β	pg/ml	185	52	69	27	9–820	12–142
Leptin	pg/ml	17,425	1,803	11,154	1,562	3,489–39,905	926–3,200
PDGF	pg/ml	4,824	3,747	4,104	2,729	2,003–10,621	1,541–8,121
PECAM-1	pg/ml	9,826	5,208	7,856	4,342	3,889–19,579	3,148–11,697
VEGF	pg/ml	70	81	55	62	31–131	34–181
C-peptide	pg/ml	1,970	1,480	1,113	1,194	657–5,510	533–4,179
Ghrelin	pg/ml	643	115	213	115	0–1,581	0–130
GIP	pg/ml	113	61	115	47	9–229	0–163
GLP-1	pg/ml	236	336	156	325	97–699	167–575
Glucagon	pg/ml	105	134	125	135	0–222	0–351
Insulin	pg/ml	403	96	22	0	0–1,742	0–1,058
PAI-1	pg/ml	28,900	8,930	15,395	7,334	8,194–62,801	5,223–33,731
Resistin	pg/ml	2,963	2,016	2,750	1,713	2,093–4,923	704-5,925
TNF-α	pg/ml	9	10	6	10	4–21	5-21
Visfatin	pg/ml	11,211	2,536	6,063	2,519	749–34,807	331–5,222
IL-1β	pg/ml	2.8	0.8	0.74	0.1	0.02–11.3	0–7.7
IL-2	pg/ml	2.0	0.1	0.00	0.0	0–23.9	0–1.0
IL-4	pg/ml	0.4	0.1	0.04	0.0	0–3.4	0–0.4
IL-5	pg/ml	1.1	0.2	0.00	0.0	0–8.7	0–2.6
IL-6	pg/ml	11.8	309.1	0.00	200	0–117.5	0–1,393
IL-10	pg/ml	3.2	94.6	2.55	14.2	1.1–9.66	3.8–372
IL-12 (p70)	pg/ml	3.9	0.0	0.00	0.0	0–28.5	0–0.3
IL-13	pg/ml	0.2	0.1	0.00	0.0	0–1.4	0–0.2
IFN-γ	pg/ml	1.3	0.2	0.00	0.0	0–9.98	0–2.5
Procalcitonin	pg/ml	1.4	22	1.3	15.8	634–2,418	807–2,346
Ferritin	ng/ml	18.4	226	18.9	114	6.5–30.6	2.4–733
tPA	ng/ml	0.8	11	0.0	0.0	2.4–7.4	5.9–82
A2M	µg/ml	1,491	1,262	1,370	1,186	944–2,488	573–1,936
Haptoglobin	µg/ml	1,337	4,006	654	2,701	308–6,060	298–12,778
CRP	µg/ml	2	95	1	64.3	0–4	3–383
SAP	µg/ml	30	34	29	32.7	26–40	15–46
Fibrinogen	µg/ml	1.8	43	1.8	16.1	0.9–3.3	0.9–262
SAA	µg/ml	3.1	502	2.1	143	1.2–7.5	0.4–1,698

Marker Profiles

Of the 37 serum proteins studied, serum concentration of 8 markers (IL-6, IL-10, procalcitonin, ferritin, haptoglobin, CRP, angiopoietin-2, and G-CSF) showed significant elevation in the disease group as compared to the healthy controls ($P < 0.003$ to $P < 0.03$). In contrast, 4 serum markers (letpin, PAI-1, PECAM-1, and visfatin) showed significant decrease in the diseased group ($P < 0.01$ to $P < 0.04$). The levels of fibrinogen, SAA, GLP-1, and follistatin showed marked (though not statistically significant) increase in the disease group. The serum profiles of these markers are shown in Figure 3. Similar findings on IL-6 and IL-10 were reported by Yurkovetsky et al. 2007 and Lambeck et al. 2007. The findings on leptin and PAI-1 were also reported by Mor et al. 2005 and Ho et al. 1999, respectively.

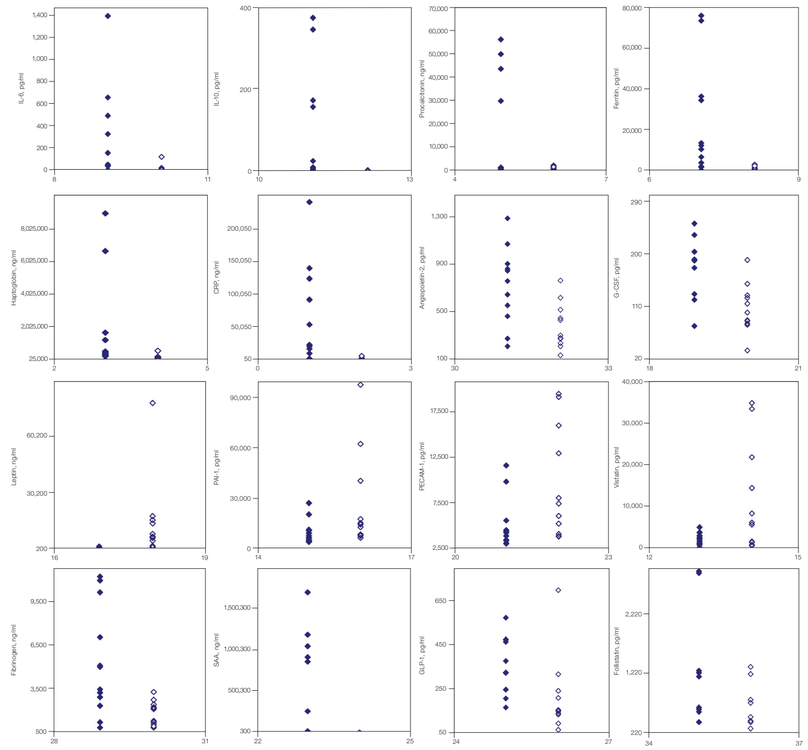

Fig. 3. Circulating levels of 16 serum markers in ovarian cancer patients and healthy controls. Sera were collected from 12 individuals with stages I–III ovarian cancer and 12 age-matched healthy women. Solid and open diamonds denote cancer and control groups respectively. ◆, sera from cancer patients, ◇, sera from control subjects.

Table 5 summarizes the statistical significance of the detected marker levels evaluated using standard Student's *t*-test. *P* values of less than 0.05 (two-tailed) were considered statistically significant. A larger sample size plus broader disease stages and grades would be required to further characterize the potential association of these markers to early detection of the disease.

Table 5. Statistical difference in serum levels between ovarian cancer and control groups. *P* values highlighted in green indicate levels that have statistically significant difference relative to the control group ($P < 0.05$). *P* values highlighted in blue indicate insignificant difference.

G-CSF	Leptin	CRP	Procalcitonin	Angiopoietin-2	Ferritin	PECAM-1	IL-6	PAI-1	Visfatin	Haptoglobin	IL-10	Resistin	SAA	Fibrinogen	GIP	Follistatin	IL-8	IL-1β
0.0028	0.0091	0.0098	0.0102		0.0158	0.0219	0.0251	0.0261	0.0335	0.0339	0.0395	0.0407	0.0657	0.0699	0.0796	0.0856	0.0983	0.1198	0.1369

GLP-1	Insulin	IL-12 (p70)	HGF	tPA	A2M	IL-4	IFN-γ	PDGF-BB	IL-5	IL-2	Ghrelin	C-Peptide	Glucagon	SAP	TNF-α	IL-13	VEGF
0.1413	0.1641	0.1752	0.1846	0.2008	0.2061	0.2415	0.2530	0.2838	0.3094	0.3675	0.3772	0.4016	0.4025	0.4297	0.4540	0.4731	0.5459

Conclusion

In this study, targets from five Bio-Plex assay panels were used to evaluate serum proteins that have potential association with ovarian cancer. The levels of eight biomarkers, G-CSF, CRP, procalcitonin, angiopoietin-2, ferritin, IL-6, IL-10, and haptoglobin were significantly higher in patients with ovarian cancer relative to the control group. In contrast, the levels of four biomarkers, leptin, PECAM-1, PAI-1, and visfatin, were found to be lower in the disease group. Further evaluation with larger sample size plus broader characterization on histology, stage, and grade will be useful in identifying these targets as potential candidate markers for detecting ovarian carcinoma. In combination with CA-125, these protein markers, specifically G-CSF, CRP, procalcitonin, angiopoietin-2, ferritin, IL-6, IL-10, and haptoglobin, are also likely to become important composite markers for future generations of ovarian cancer screening algorithms. The multiplex immunoassay platform is capable of measuring the levels of multiple targets in a single well of a 96-well microplate in less than 3 hr, using as little as 12.5 µl of serum, plasma, and other matrices. This significantly reduces the time and cost spent on preliminary screening of serum samples for biomarker profiling.

References

Ho CH et al. (1999). Diagnostic and prognostic values of plasma levels of fibrinolytic markers in ovarian cancer. Gynecol Oncol 75, 397–400.

Khoo US et al. (1999). Somatic mutations in the BRCA1 gene in chinese sporadic breast and ovarian cancer. Oncogene 18, 4,643–4,646.

Lambeck AJA et al. (2007). Serum cytokine profiling as a diagnostic and prognostic tool in ovarian cancer: A potential role for Interleukin 7. Clin Cancer Res 13, 2,385–2,391.

Mor G et al. (2005). Serum protein markers for early detection of ovarian cancer. Proc Natl Acad Sci U S A 102, 7,677–7,682.

Posadas EM et al. (2004). Proteomics and ovarian cancer: Implications for diagnosis and treatment: A critical review of the recent literature. Curr Opin Oncol 16, 478–484.

Schink JC (1999). Current initial therapy of stage III and IV ovarian cancer: Challenges for managed care. Semin Oncol 26, 2–7.

Yurkovetsky Z et al. (2007). Development of multimarker panel for early detection of endometrial cancer. High diagnostic power of prolactin. Gynecol Oncol 107, 58–65.