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Serum expression level of cytokine and chemokine correlates with progression of human ovarian cancer

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Summary

Objective: This work was designed to determine the relationship between serum expression level of cytokines and chemokines and progression of human ovarian cancer, and to evaluate the utility and diagnostic value of target markers as risk indicators. *Materials and Methods:* A set of candidate cytokines and chemokines (GM-CSF, IFN-γ, GRO, IL-1β, IL-2, IL-6, IL-8, MCP-1, TNF-α, VEGF, EGF, RANTES, CCL21/6Ckine, and SDF-1/CXCL12) were measured using Luminex liquid chip technique in healthy women (n=75) and in women with ovarian cancer (n=77). *Results:* EGF, IL-6, MCP-1, 6Ckine, RANTES, and IL-10 were significantly overexpressed in the tumor group compared to those in normal controls, while IL-2 was reduced. The combined markers (EGF, MCP-1, 6Ckine, IL-6, and TNF-α) achieved 91.1% sensitivity, 65.8% specificity, and 83.3% area under the ROC curve (AUC) in distinguishing serous ovarian cancer from health controls. *Conclusion:* This study suggested that serum expression level of cytokines and chemokines correlate with progression of human ovarian cancer. The association of EGF, MCP-1, 6Ckine, IL-6, and TNF-α may contribute to increase diagnosis rate of malignant ovarian tumors

Key words: Cytokine; Chemokine; Serum; Ovarian cancer detection.

Introduction

Ovarian cancer is the ninth most common female cancer worldwide and the fifth leading cause of cancer-related deaths in women [1]. The aggressive behavior attributes to the lack of specific symptomatology and the absence of appropriate risk indicator. Human epithelial cancers contain a significant number of host leukocytes, mainly macrophages and lymphocytes, and express a complex network of cytokines and chemokines [2]. It is now being reported that many of these cytokines and chemokines are also associated with ovarian cancer. Earlier studies indicated higher cytokines and cytokine receptors in human EOC cell lines and solid cancers [3-5]. Yigit *et al.* measured the cytokine production profiles and found both IL-6 and IL-8 overexpressed in EOC ascites [6].

Cytokines and chemokines are secreted by both the epithelial cells of cancer and the stromal components, such as fibroblasts, mesenchymal stem cells, inflammatory infiltrating cells or endothelial cells. They are critical regulators of immune responses, inflammatory reactions, apoptosis, cell proliferation, and formation of new blood vessels [7]. Via their receptors they play important roles in both physiological and pathological processes of inflammation. Chemokines are involved in the physiological process of ovulation in the normal ovary. Therefore it raises the possibility that cytokines and chemokines are involved in the inflammatory process and mediate immune responses that may favor or inhibit tumor progression [8, 9]. Another reason for ovarian cancer is retrograde menstruation via the fallopian tube, where the flow of endometrial fluid contain inflammatory molecules such as CXCL8 (IL-8), tumor necrosis factor alpha (TNF-a) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [10].

Recently, some studies reported the possible diagnostic and prognostic use of cytokine levels in newly diagnosed malignant tumor. Cytokines or their receptors may also represent a target for specific anticancer therapy at the molecular level.

The present authors hypothesized that serum cytokines/chemokines would be elevated in patients with ovarian cancer compared to a similar control group of age and sex matched women. Hence the identification of serum biomarkers could possibly be used clinically to distinguish people at increased risk of developing ovarian malignant disease. Therefore, the aim of the present study was to investigate the expression of target markers in ovarian cancer patients compared to normal controls, to determine the relationship between serum expression level of cytokines and chemokines, and progression of human ovarian cancer, and finally to evaluated the utility and diagnostic value of target markers as risk indicators in distinguishing malignant from health controls. In this study, the authors limited the analysis to these factors to maintain statistical power, to limit ovarian cancer only to serous

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Table 1. — *Clinicopathologic features of serous ovarian adenocarcinoma patients.*

Variable	Clinicopathologic parameter	Samples
Case		152
Normal control		75
Ovarian cancer (serous)	51.12 ± 13.02	77
FIGO* Stage	I	11
	II	4
	III	57
	IV	5
Grade of differentiation	Low-grade	59
	Mid-grade	14
	High-grade	4

*International Federation of Gynecology and Obstetrics.

Table 2. — *Serum concentrations of candidate markers in serous ovarian adenocarcinoma.*

Variable	Ovarian cancer (pg/ml)	Health controls (pg/ml)	*p*-value
EGF	153.28 (98.56−229.58)	85.61 (42.13−136.35)	0.000
GM-CSF	1.53 (0.87−2.92)	1.26 (0.62−3.32)	0.727
GRO	712.29 (516.44−1023.24)	790.88 (701.92−915.45)	0.379
IFN-γ	1.22 (0.60−2.84)	1.06 (0.60−2.35)	0.597
IL-2	0.44 (0.15−0.83)	0.54 (0.34−1.45)	0.045
IL-6	3.84 (1.40−9.05)	1.20 (0.70-1.90)	0.000
IL-8	54.62 (27.71−103.08)	44.91 (27.06−73.60)	0.241
MCP-1	886.60 (604.66−1342.50)	611.69 (438.46−782.90)	0.001
TGF-α	7.99 (4.76−14.14)	8.70 (7.27−12.38)	0.192
TNF-α	12.88 (8.31−19. 61)	11.15 (7.59−15.37)	0.116
VEGF	230.89 (93.22−395.01)	191.81 (97.85−298.72)	0.247
6Ckine	142.93 (103.04−192.81)	88.74 (60.48−129.27)	0.000
SDF1-a+β	1895.32 (1210.97−2487.78)	2238.49 (1096.03−2869.73)	0.221

Variable are expressed as median (25–75th), analyzed by Mann-Whitney U-test. $p < 0.05$ was considered to be statistically significant.

ovarian adenocarcinoma, to minimize false positive results, and to further elucidate mechanisms that may link inflammatory reaction with factors that promote initiation of ovarian tumors.

Materials and Methods

Patients and specimen collection

The authors obtained 152 serum samples between November 2012 and August 2013 at Chinese PLA General Hospital, Beijing, China. These included 75 normal serum from healthy volunteers without history of malignancy and having similar age (median = 45.5, range = 20–89 years old). In 77 of serous ovarian adenocarcinoma patients, the aged ranged from 16 to 82 years. Tumor grading and staging were established according to International Federation of Gynecology and Obstetrics (FIGO) criteria. Four were grade I, 14 were grade II, and 59 were grade III tumors. Eleven cases were staged as FIGO I, four cases as FIGO II, 57 cases as FIGO III, and five cases as FIGO IV (Table 1). Specimens were aliquoted and stored at -80°C until analysis. All patients and health controls provided written informed consents and this study was approved by the Committee on Ethics of the Chinese PLA General Hospital.

The authors evaluated serum levels of the following 16 candidate markers selected from cytokines and chemokines correlated with human ovarian cancer according to the previous literature. This analysis, tested by Luminex liquid chip technology, therefore included epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), granulocyte macrophage colony-stimulating factor (GM-CSF), transforming growth factor α (TGF-α), tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), growth-regulated oncogene (GRO), monocyte chemotactic protein-1(MCP-1), chemokine(C-Cmotif)ligand21 (CCL21/6Ckine), stromal cell derived factor 1 (SDF-1α+β/CXCL12) and regulated upon activation normal T-cell expressed and secreted (RANTES). All analytes were measured with human cytokine/chemokine kits according to the manufacturer's instructions.

Standard curve (ranging from 3.2 to 10,000 pg/ml) was made by five-fold dilutions of the working standards in the provided buffer. SDF-1α+β ranging from 97.7 to 10,000 pg/ml and CCL21/6Ckine ranging from 19.5 to 10,000 pg/ml. Standards, controls 1 and 2, and patient serum samples were pipetted at 25 µl per well in duplicate. About 25 µl of serum was added to the standards and controls, and 25 µl of provided assay buffer was added to the serum samples. After adding 25 µl of the bead mixture, the microplate was incubated overnight at 4°C. Wells were vacuumed and washed twice with wash buffer. Twenty-five µl detection antibodies were added, and the microplate was incubated for 30 minutes in the dark on a microtiter shaker. Twenty-five µl streptavidin-phytoerythrin were added per well and incubated for 30 minutes. The microplate was washed twice. Sheath fluid was added to each well, and samples were read. Data were analyzed using a five-parametric-curve fitting.

There was no or negligible cross-reactivity between the antibody and any of the other analytes in reaction system. Assay sensitivities minimum detectable concentrations (minDC pg/ml) were calculated by immunoassay analysis software (EGF: 2.7 pg/ml, VEGF: 5.8 pg/ml, GM-CSF: 9.5 pg/ml, TGF-α: 0.4 pg/ml, TNF-α: 0.1 pg/ml, IFN-γ: 0.1 pg/ml, IL-1β: 0.4 pg/ml, IL-2: 0.3 pg/ml, IL-6: 0.3 pg/ml, IL-8: 0.2 pg/ml, IL-10: 0.3 pg/ml, GRO: 10.1 pg/ml, MCP-1: 0.9 pg/ml, CCL21/6Ckine: 18.9 pg/ml, SDF-1α+β/CXCL12: 83.5 pg/ml, and RANTES: 1.0 pg/ml).

Statistical analysis

The calculations were performed with SPSS 19.0 statistical software. Results were presented as medians and range 25th/75th percentiles. The Mann-Whitney test was used to test statistical significance. The analysis of variables beyond the detection range was initially performed using the Chi-square test. The diagnostic value of over-expressed cytokine and chemokine was considered using receiver operating characteristic (ROC) curves that were constructed by plotting sensitivity versus 1-specificity and the areas under the curve (AUC) were calculated. The bootstrap method was used to calculate the confidence intervals for AUC. *P*-value < 0.05 was considered to be statistically significant.

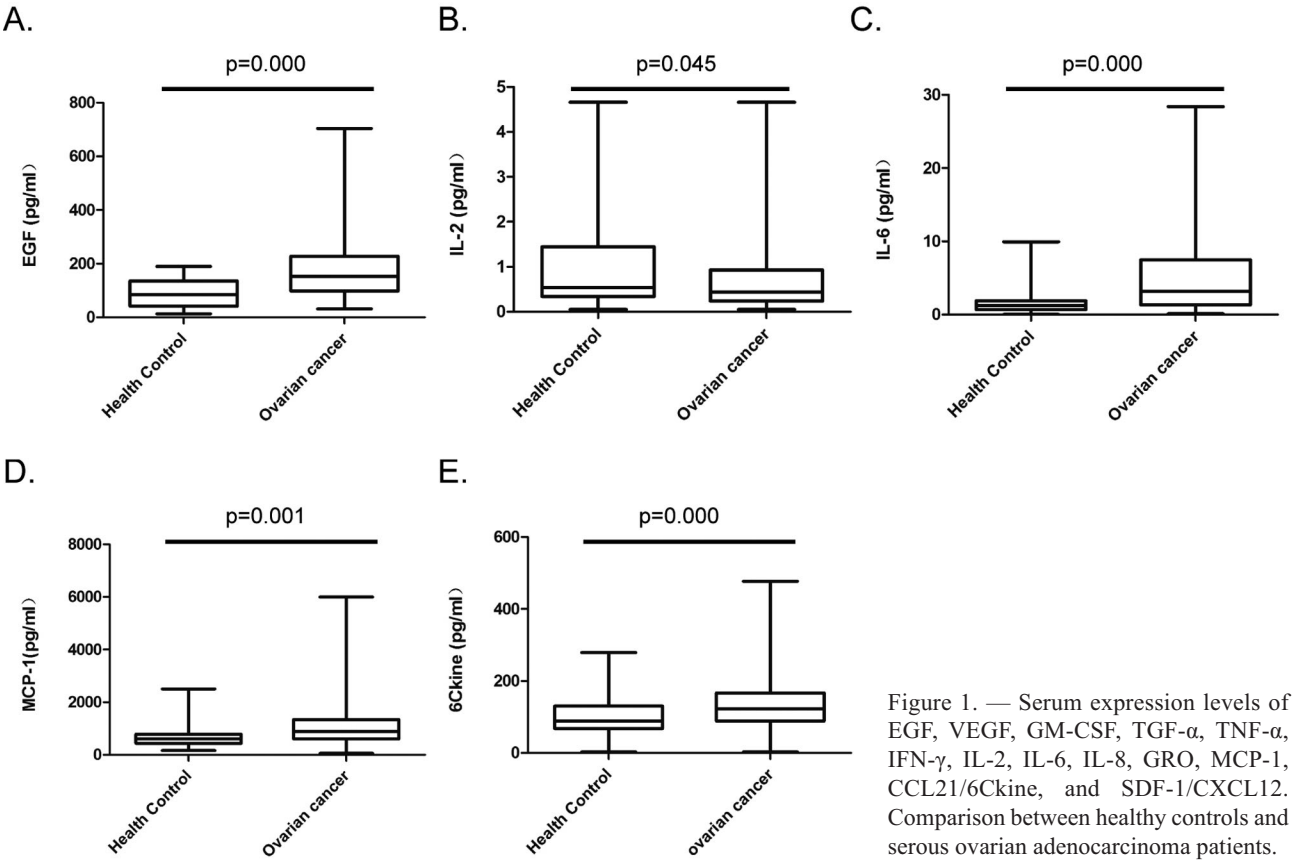

Figure 1. — Serum expression levels of EGF, VEGF, GM-CSF, TGF-α, TNF-α, IFN-γ, IL-2, IL-6, IL-8, GRO, MCP-1, CCL21/6Ckine, and SDF-1/CXCL12. Comparison between healthy controls and serous ovarian adenocarcinoma patients.

Results

Presence of circulating EGF, VEGF, GM-CSF, TGF-α, TNF-α, IFN-γ, IL-2, IL-6, IL-8, GRO, MCP-1, CCL21/6Ckine, and SDF-1/CXCL12

Serum samples were obtained and analyzed concurrently and the values with their range are shown in Table 2. The median concentrations of EGF, IL-6, MCP-1, and CCL21/6Ckine were significantly higher in serum from ovarian cancer patients compared to healthy volunteers ($p < 0.001$), while serum levels of IL-2 were reduced. In addition to the above five cytokines, three out of the remaining cytokines on the array had a large proportion of results that were beyond the limits of detection for the assays.

High concentrations of EGF in serum were observed in patients with a median of 153 pg/ml (98.56−229.58). The highest median concentration (703.88 pg/ml) was observed in the group with advanced stage disease. The lowest median concentration (31.76 pg/ml) was found in the group with early stage disease, high-grade of differentiation (Figure 1A)

The proinflammatory IL-6 levels between health controls and patients was statistically significant ($p < 0.05$). The highest median IL-6 concentration (28.38 pg/ml) was observed in patients with poorly differentiated serous cystadenocarcinoma. The median concentration was 1.1 pg/ml in patients in early stage (n=15), while 4.34 pg/ml in those in late stage (n=61) (Figure 1C).

The highest median MCP-1 concentration (5996.37 pg/ml) was observed in patients with low-grade differentiated ovarian serous adenocarcinoma. It appears that the relative expression of MCP-1 was increased in serum of higher malignancy patients. The median concentration of MCP-1 ranged from 780.73 pg/ml to 938.53 pg/ml according to its stage status (Figure 1D).

The difference in 6Ckine levels between diseases compared to controls was statistically significant ($p < 0.05$). The median concentration was 140.44 pg/ml in patients with early stage which was much higher than those in volunteers. The highest median concentration (476.62 pg/ml) was observed in patients with poorly differentiated and late stage tumor (Figure 1E).

Second major finding was that serum levels of IL-2 differed among the three groups, the median concentration of IL-2 (0.36 pg/ml) was observed in patients with early stage (I, n=11). In patients with Stage (III, IV), the median concentration was 0.54 pg/ml (Figure 1B). Although by no means definitive, this observation suggests an interaction between systemic and serum levels of IL-2, possibly reflecting systemic production and consequent renal clearance.

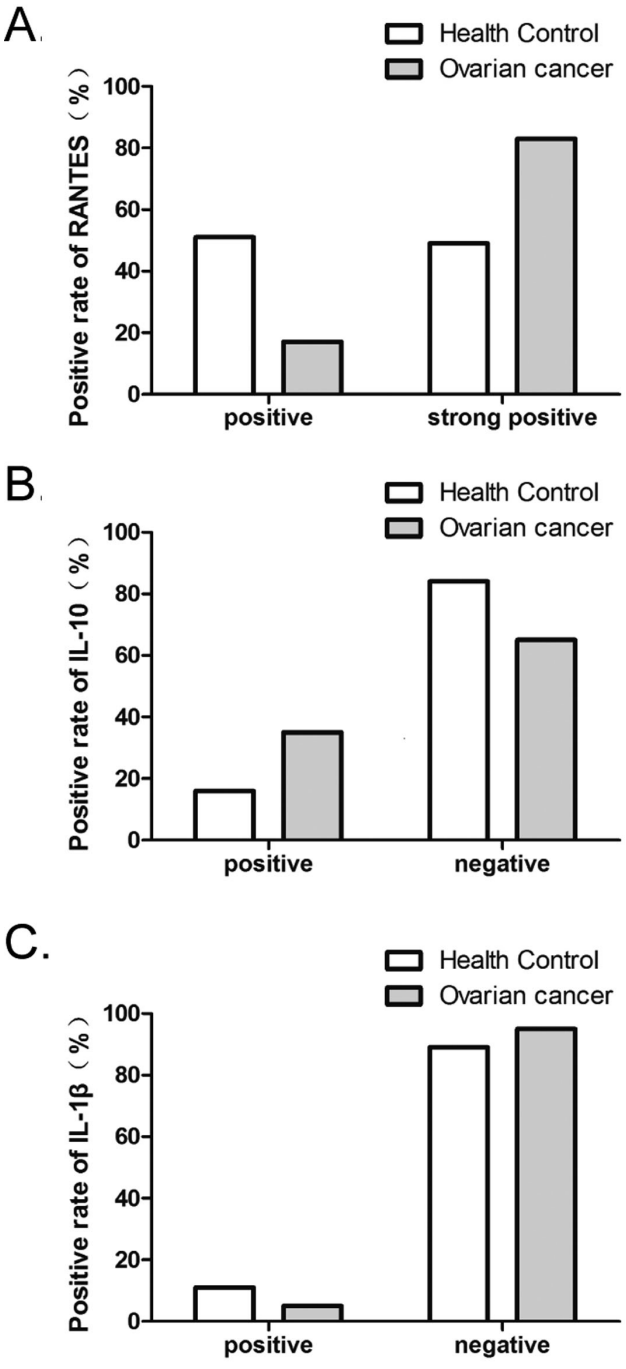

Figure 2. — Positive rate of IL-1β, IL-10, and RANTES in serum-comparison between healthy controls and serous ovarian adenocarcinoma.

Frequency distribution of circulating RANTES, IL-10, and IL-1β

RANTES was out of the highest detectable concentrations levels in 83.1% of the serum samples (64/77), compare with 50.7% (38/75) out of range in health controls. Positive ratio contrasted by using Chi-Square test (x^2 = 34.26, p = 0.000) (Figure 2A). IL-10 and IL-1β were below detectable levels of analyte on the contrary. IL-10 was detectable in 27 out of 77 patients (35.1%) and in 12 out of 75 cases with health controls (16.0%) (x^2 = 43.35, p = 0.000) (Figure 2B). Detectable levels of IL-1β were observed in just four out of 77 patients (5.2%) and in eight out of 75 cases with health controls (10.7%) (x^2 = 108.47, p = 0.000) (Figure 2C). Frequency distribution is reported in Table 3.

Table 3. — *Frequency distribution of candidate markers out of reading range.*

		Ovarian Cancer N(COL%)	Health Controls N(COL%)	*p*-value
IL-10#	Negative	50 (64.9)	63 (84.0)	0.000**
	Positive	27 (35.1)	12 (16.0)	
IL-1β#	Negative	73 (94.8)	67 (89.3)	0.000**
	Positive	4 (5.2)	8 (10.7)	
RANTES#	Strong positive	64 (83.1)	38 (50.7)	0.000**
	Positive	13 (16.9)	37 (49.3)	

#Candidate markers out of reading range, compared constituent ratio using Chi-Square test. $P < 0.05$ was considered to be statistcally significant. **$p < 0.01$.

Table 4. — *Comparison of the sensitivities for ovarian cancer detection.*

Biomarker	AUC	CI	Sensitivity	Specificity	*p*-value
EGF	0.764	0.658−0.862	0.468	0.923	0.000
MCP-1	0.697	0.575−0.805	0.727	0.667	0.003
6Ckine	0.746	0.642−0.857	0.820	0.615	0.000
IL-6	0.738	0.629−0.845	0.643	0.789	0.000
TNF-α	0.673	0.556−0.791	0.532	0.718	0.007
Overall	0.833	0.746−0.921	0.911	0.658	0.000

Presence of candidate markers in women with different stages and grade of the tumor

The authors also attempted to evaluate the level of the panel of target markers expression status within the different stages of the tumor. A comparison of the serum concentrations of all the candidate biological indicators, the serum levels of EGF, VEGF, GM-CSF, TGF-α, TNF-α, IFN-γ, IL-2, IL-6, IL-8, GRO, MCP-1, CCL21/6Ckine, and SDF-1/CXCL12 of the cases were significantly higher than the controls for the four stages. However, the serum levels of these cytokines and chemokines in the cases were not significantly different among the four stages, respectively. Moreover, there was no apparent and remarkable difference in the expression levels of these indicators between high and low grade disease.

The diagnostic performance of candidate markers in discriminating malignant from health controls

The diagnostic performance of candidate markers in

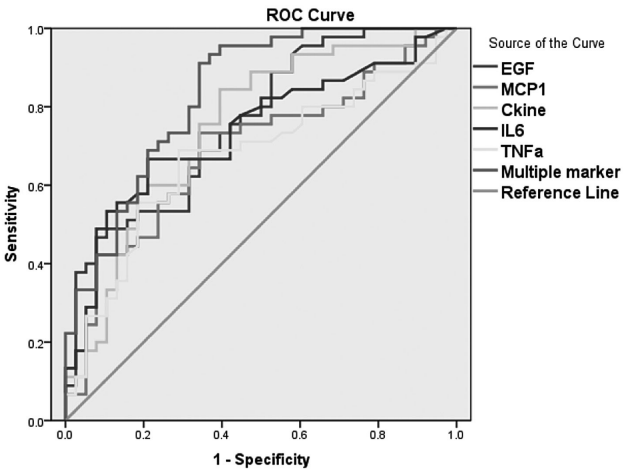

Figure 3. — The prognostic value of cytokine and angiogenesis chemokine profiles as biomarkers for discriminating serous ovarian adenocarcinoma from health controls evaluated by areas under the ROC curves.

discriminating malignant from health controls was verified using ROC analysis. The resultant accuracy (ROC area) values for EGF, MCP-1, 6Ckine, IL-6, TNF-α, and their corresponding ROC curves are shown in Figure 3. The results in Table 4 show that combined markers achieved 91.1% sensitivity, 65.8% specificity, and attained 83.3% area under the ROC curve (AUC) for distinguishing serous ovarian adenocarcinoma from health controls.

Discussion

Multiplex chemokines are involved in all steps of tumor development, including tumor cell proliferation and apoptosis, tumor angiogenesis, invasion of peripheral tissues, and specific homing to metastatic sites. Cytokines mainly originate from a number of sources including the tumor itself, the surrounding stroma, or systemic tissues involved in the response. On the contrary, the inflammation-mediated release of cytokines, chemokines, and growth factors can also promote migration and proliferation of leukocytes, epithelial, and endothelial cells [11]. In the present study, the cytokine production profiles in EOC serum were measured by Luminex liquid chip technology.

The authors found median concentrations of EGF, IL-6, MCP-1, and 6Ckine to be significantly elevated in serum of women with ovarian cancer, compared to health controls. This study showed similar results with the data obtained by previous studies [11-13]. Regarding IL-2, similar to Giuntoli *et al.*, a reduction in serum of ovarian cancer patients was observed [14].

In high-grade ovarian cancer cultures, it has been shown that EGF induces cell invasion by activating an epithelial-mesenchymal transition (EMT) [15]. In borderline tumors, immunohistochemical studies have shown that EGF and EGFR are expressed, but there is no difference in EGFR staining intensity among benign, borderline, and malignant ovarian tumors [16]

IL-6 is a pleiotropic inflammatory cytokine, first discovered as a B-cell growth factor. In vitro and in vivo studies have indicated that IL-6 may provide autocrine and paracrine growth stimulation in EOC cells [17]. It has been involved in the autocrine growth of ovarian cancer cells, most likely by increasing their capacity to secrete matrix metalloproteinase (MMP)-9 [18], The activation of the IL-6 complex activates Janus kinases (JAK), signal transducers, and activators of transcription (STATs), which regulate cell proliferation and apoptosis [19].

In this study, markedly elevated levels of serum CCL21/6Ckine were demonstrated in ovarian cancer group. CCL21/6Ckine act as CXCR3 chemokine ligands that have anti-angiogenic properties through direct interaction with the endothelium [20]. Recent findings, however, suggest that the tumor cells themselves generate a gradient of CCL21/6Ckine, which creates a continuous cycle of T or NK cells recruitment [21]. A synergistic anti-tumor effect has been observed when CCL21/6Ckine was combined with IL-2 [22]. CCL21 also co-stimulates expansion of naive T-cells and increases secretion of Th1 cytokines, TNF-α and IFN-γ [23].

The present data showed that most serum RANTES levels were out of the highest detectable range in ovarian cancer patients. However, the higher levels of RANTES than highest detectable range were observed in 64 out of all 77 patients. The serum RANTES concentration determined by ELISA was significantly elevated in the ovarian cancer patients as control values correlating with the stage of disease and the extent of residual tumor mass [24]. The chemokine CCL5/RANTES could chemo-attract monocytes into ovarian cancer and contribute to the disease in this manner. The importance of RANTES would probably not only induce monocyte migration. Another view assumed that high RANTES concentrations in the serum of ovarian cancer patients would “break” the chemotactic gradient or induce functional desensitization of RANTES receptors [25]. Tumor microenvironmental RANTES secreted by tumor cells and tumor-derived fibroblasts could induce tumor cell gene expression of various MMPs, especially MMP9, which degrade the extracellular matrix and favour tumor invasion [26, 27].

In another analyses, it seemed that the relative expression level of VEGF, IL-8, and TNF-α were increased in serum of ovarian malignancy diseases. However, there were no significant differences by statistical analysis. Some previous research related to these factors has been done. VEGF overexpression has been demonstrated in ovarian cancer and has shown to be a poor prognostic factor [28]. VEGF evaluated in human primary ovarian tumor cells that were exposed to the hypoxic tumor microenvironment [29].

IL-8 is necessary for stimulation of angiogenesis and tumor cell proliferation, therefore playing an important role in tumor growth and metastasis formation [30]. VEGF act as potent angiogenic factors, as well as less potent but possibly relevant factors such as IL-8 and other ELR-positive CXC chemokines [31]. IL-8 plays a role as key mediator that induces NF-κB activation and VEGF upregulation [32].

SDF-1, also named CXCL12, exists in two alternative splicing variants, α and β, is a chemokine of CXC subfamily originally characterized as a pre-B-cell stimulatory factor cloned from bone marrow cell supernatants. Recent data suggest that SDF-1α induces DNA synthesis in ovarian cancer cells in vitro through ERK1/2 activation [33], stimulates ovarian cancer cell growth through the EGF receptor transactivation [34], induces tumor cell migration and invasion, increases integrin expression, induces MMP1 mRNA expression, induces TNF expression, stimulates angiogenesis through its sole receptor CXCR4, attracts and modulates dendritic cells (DCs) into tumor, and presence of Treg cells prevent immune cell migration to the immunosuppressive tumor site [35].

In the present present study, the serum levels of EGF, MCP-1, 6Ckine, IL-6, and TNF-α in patients were significantly higher than controls, which indicated that EGF, MCP-1, 6Ckine, IL-6, and TNF-α may be involved in disease.

The cytokine and chemokine network in human tumors is complex and its role is only partially understood. In fact, accumulating evidence suggests that cytokine and chemokine may contribute directly to the diagnostic of tumor diseases. IL-8 can be used as a non-invasive prognostic marker for mammary gland cancer [36]. VEGF expression was an independent prognostic factor [37]. RANTES could be used as a prognostic indicator in both breast and cervical cancers [38]. Since single predictor is not a specific marker, it may be necessary to combine with other cancer biomarkers to increase its predictive value. The association of EGF, MCP-1, 6Ckine, IL-6, and TNF-α have a sensitivity of 91.1% and a specificity of 65.8% in predicting ovarian cancer, and ROC curve analysis using five combined markers yielded an AUC of 83.3%. These data support the view that the panel of indicators (EGF, MCP-1, 6Ckine, IL-6, and TNF-α) may contribute to increase diagnosis rate of malignant ovarian tumors. These markers may identify the risk of ovarian cancer if strong association is confirmed by future longitudinal prospective studies.

In this study, the authors assessed serum cytokines and chemokines by using the Luminex technology. According to reports in the literature, the results of this detection method were stable [39]. Luminex technology showed sufficient sensitivity and temporal reproducibility in serum. This study had some limitations. Serum may not be representative of in-vivo conditions because proteases are released from platelets together with other blood cells and activated during blood clotting [40].

In summary, the knowledge gained from multi-analytical determination of cytokines and chemokine could allow better diagnosis and management of malignant tumor of ovary. In this regard, utilizing multiplex markers out of cytokines and chemokines may increase the sensitivity for the detection of ovarian cancer while maintaining the specificity. Serum expression profiling of the cytokines and chemokine present in ovarian cancer represents an effective method to explore for screening in symptomatic women.

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