RESEARCH ARTICLE

Insulin-Like Growth Factors and Their Binding Proteins in Tumors and Ascites of Ovarian Cancer Patients: Association With Response To Neoadjuvant Chemotherapy

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Abstract

Purpose: Tumor cell growth and sensitivity to chemotherapy depend on many factors, among which insulin-like growth factors (IGFs) may play important roles. The aim of the present study was to evaluate the levels of insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) in primary tumors and ascites as predictors of response to neoadjuvant chemotherapy in ovarian cancer (OC) patients. **Materials and Methods:** Tumor tissue samples and ascitic fluid were obtained from 59 patients with advanced OC. The levels of IGF-I, IGF-II, IGFBP-3, IGFBP-4 and PAPP-A were determined using ELISA kits. Taking into account the data on expression of these IGF-related proteins and outcome, logistic regression was performed to identify predictors of response to neoajuvant chemotherapy. **Results:** Human ovarian tumors expressed IGFs, IGFBP-3, IGFBP-4 and PAPP-A and these proteins were also present in ascites fluid and associated with its volume. IGFs and IGFBPs in ascites and soluble PAPP-A might play a key role in ovarian cancer progression . However, levels of proteins of the IGF system in tumors were not significant predictors of objective clinical response (oCR). Univariate analysis showed that the level of IGF-I in ascites was the only independent predictor for oCR. **Conclusion:** The level of IGF-I in ascites was shown to be an independent predictor of objective clinical response to chemotherapy for OC patients treated with neoadjuvant chemotherapy and debulking surgery.

Keywords: Insulin-like growth factors- insulin-like growth factor binding proteins- ovarian cancer- ascites

Introduction

Tumor cell growth and sensitivity of tumor cells to chemotherapy depend on many factors, among which insulin-like growth factors (IGFs) play an important role. The IGF system includes IGF-I, IGF-II, their receptors (IGF-IR and IGF-II/mannose-6-phosphate receptor) and six IGF binding proteins (IGFBPs) (Firth and Baxter, 2002; Samani et al., 2007; Yunusova et al., 2016). Many proteases are involved in the regulation of IGFBPs. Among them, pregnancy-associated plasma protein (PAPP-A) is a metalloprotease involved in the hydrolysis of IGFBP-4 and IGFBP-5 (Yunusova et al., 2013; Thomsen et al., 2015). Upon binding of IGFs to IGF-IR, many signaling pathways can be activated, leading to stimulation of cell proliferation, motility and inhibition of apoptosis (Yunusova et al., 2015).

Ovarian cancer metastasis to the greater omentum and ascitic fluid accumulation in the peritoneal cavity indicate the disease progression. Malignant ascites constitute a tumor microenvironment promoting migration, survival and enhanced invasive activity of tumor cells due to significant amounts of growth factors, cytokines and fibronectin in ascites of ovarian cancer patients (Sodek et al., 2012; Latifi et al., 2012). The levels of IGFs and IGFBPs in ascites were similar or lower than those in serum. However, PAPP-A proteolytic activity was significantly higher in malignant ascites than in serum. Ascites was more potent than serum in activating the IGF-IR receptor in vitro (KIRA assay) (Thomsen et al., 2015).

The ability to perform optimal cytoreduction is currently regarded as the most important prognostic factor for patients with advanced ovarian cancer (Morimoto et al., 2016). In the absence of conditions for optimal primary cytoreductive surgery in patients with advanced ovarian cancer, neoadjuvant chemotherapy (NACT) may be administered. When compared with primary cytoreductive surgery, NACT is associated with a greater percentage of optimal cytoreduction, less blood loss and better quality of life (Weinberg et al., 2010). Chemoresistance in ovarian cancer cells may be associated with the overexpression of proteins of the IGF system or components of the IGF-mediated signaling pathway. Overexpression of IGF-IR and phosphatidylinositol 3-kinase were associated with platinum resistance of ovarian cancer cells (Eckstein

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et al., 2009). The ovarian cancer cell lines, resistant to taxanes, had a high level of IGF-II, and the decrease in the level of IGF-II restored the cells' sensitivity to taxanes (Huang et al., 2010). L.Lu et al. (2006) showed no association between the IGF-II and IGFBP-3 mRNA expression in tumors and response to platinum-based chemotherapy (Lu et al., 2006). It has recently been demonstrated that ascites is a platform for translational research in ovarian cancer. The accessibility of ascitic fluid and its cellular components make it an excellent source of tumor cells for the investigation of prognostic and predictive biomarkers, as well as for molecular profiling analysis (Sodek et al., 2012; Latifi et al., 2012). The elevated level of IGFBP-3 before chemotherapy and the high level of IGF-II after chemotherapy in ascites of ovarian cancer patients were shown to correlate with low overall survival, and the level of IGF-II after chemotherapy was an independent prognostic factor in Cox multivariate analysis (Slipicevic et al., 2009). Thus, no strong association between the response to chemotherapy using the ovarian cancer cell lines and the response to chemotherapy in human ovarian cancer was found (translational studies). The aim of this study was to evaluate protein levels of the IGF system in the primary tumor and ascites of ovarian cancer patients and to identify the predictors of response to NACT.

Materials and Methods

Patients. The study was approved by the Local Ethics Committee of the Cancer Research Institute of Tomsk National Research Medical Center. All patients provided informed written consent before being included in this study. Tissue samples of primary tumors and ascites were obtained from 59 patients with IIIC-IV stages of ovarian cancer (FIGO, 2013). Patients with stage IB and IC ovarian cancer constituted a comparison group. All patients with early ovarian cancer underwent radical surgery. Between 2012 and 2015, the patients were treated at the Department of Gynecology of the Cancer Research Institute of Tomsk National Research Medical Center. They were Caucasian and resided in the Western Siberia region. All ovarian cancer patients underwent diagnostic laparoscopy for morphological verification and surgical staging as well as for sampling of tumor tissue and ascites. Histological sections were prepared from paraffin blocks according to the standard method with the staining with hematoxylin-eosin. In our study, all ovarian cancers were characterized as high-grade serous adenocarcinomas. R. V. Verhaak et al. (2013) in their multicenter study reported that up to 80% of all malignant epithelial ovarian tumors were high-grade serous adenocarcinomas (Verhaak et al. (2013). All patients were tested for the presence of BRCA1 mutations insC 5382 by allele-specific PCR in real time. Patients included in this study were BRCA1-negative. The volume of ascites was measured clinically and by ultrasound examination using the LOGIQ S6 machine. In accordance with the recommendations of T. Feidenberg et al. (2014), the patients were divided into 3 subgroups: patients having low-volume (<200 mL), moderate-volume (200-1,000 mL) and high-volume ascites (> 1,000 mL)

(Feidenberg et al., 2014). All 59 patients with advanced ovarian cancer met the recommendations for ASCO and SGO Release Clinical Practice Guideline on Neoadjuvant Therapy for Newly Diagnosed Advanced Ovarian Cancer (2016). The NACT regimen was administered as follows: carboplatin plus paclitaxel, 175mg/ m², carboplatin AUC 6 on day 1, every 21 days, 3-4 courses. Clinical response to treatment was determined after 3-4 cycles by RECIST criteria (2009), according to which the result was regarded as an objective clinical response (oCR) in patients with partial or complete response, while patients who had a stable disease or disease progression were as non- responders. Pathological complete response was defined as no residual tumor in abdominal cavity and lymph nodes. The Gynaecologic Oncology Group (GOG) currently defines optimal cytoreduction as leaving residual disease less than 1 cm in maximum tumor diameter.

Serum CA125 and HE4 assay. CA-125 and HE4 were used for monitoring epithelial ovarian cancer (Wang et al., 2013, Steffensen et al., 2016; Feng et al., 2016), therefore, we measured CA-125 and HE4 serum levels before NACT and analyzed with oCR. We used ELISA kits developed by Fujirebio Diagnostic, Inc (Sweden).

Ascites supernatants and cleared tissue homogenate preparation. Tumor samples (100 mg) were homogenized and then resuspended in 300 μL of 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM ATP, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, and 100 mM NaCl. The homogenate was centrifuged at 100,009g for 60 min at 4 oC as previously described (Spirina et al., 2012). For preparation of supernatant aliquot of ascites (about 10 ml) was centrifuged at 4°C and 900 g for 20 minutes. The supernatants of the ascites were collected, frozen and stored at -200C prior to the studies. For the preparation of clarified homogenates, tumor tissue (approximately 100 mg) was homogenized in liquid nitrogen, then resuspended in 300 300 μL of 50 mM tris-HCl-buffer (pH 7.5). The homogenate was centrifuged for 60 min at 10000 g and 4C.

ELISA. The levels of IGF-I, IGF-II, IGFBP-3, IGFBP-4 and PAPP-A were evaluated in the clarified tumor homogenates and ascites supernatants using the ELISA kits (R&D Systems, Mediagnost, Abcam) in Anthos 2020 microplate reader. The protein concentration in homogenates and supernatants was determined by Lowry.

Flow cytometry. Preparing cell suspension. Samples of ovarian cancer tissues were disintegrated on Becton Dickinson (BD) Medimashine System using BD "Cell Wash" buffer. Cell suspension was filtrated through a 50 μm Syringe Filcons nylon filter. This procedure is optimal for cell suspensions from tissues due to the absence of their contamination. Number of cells was counted in BD Trucount tubes (BD, USA) by flow cytometry. Flow cytometry. Extracellular staining was performed with CD221 (IGR-IR) phycoerithrin (PE) antibody (BD). For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm kit (BD, USA) (standard protocol). Aliquots of cell suspensions were incubated with conjugated antibodies at 200 C for 30 minutes. We used anti-cytokeratin 18 fluorescein isothiocyanate as a primary antibody (FITC) (BD, Santa Cruze). Results were

Table 1. IGFs, IGFBPs and PAPP-A Level (ng/mg of Protein) in Primary Tumor and Ascites in Patients with Early and Advanced Ovarian Cancer, Me (25-75%)

Sample type	Parameters				
	IGF-I	IGF-II	IGFBP-3	IGFBP-4	PAPP-A
Primary tumor (IB,C Stage), n=15	0.1 (0.1-0.4)	3.4 (2.0-3.6)	17.9 (16.2-24.4)	6.0 (3.6-12.4)	0.4 (0.01-0.4)
Primary tumor (IIIC-IV Stage), n=59	0.1 (0.060-0.305)	5.6 (3.28-8.49)	5.9 (0.90-8.10)	3.6 (0.6-5.6)	0.3 (0.09-0.32)
Ascites (IIIC-IV Stage), n=53	2.5 (0.9-2.7) $P_{2,3}<0.05$	115.0 (89.0-183.0) $P_{1,2}<0.05$ $P_{2,3}<0.05$	190.0 (140.0-266.0) $P_{1,2}<0.05$ $P_{2,3}<0.05$	62.3 (51.4-77.8) $P_{2,3}<0.05$	0.2 (0.05-0.42)

Note, 1,2 – changes between protein levels in tumor samples in early and advanced ovarian cancer; 2,3 – changes between protein levels in primary tumor and ascites

analyzed using the FACS Diva 6.1. software. Percentage of cells specifically stained for IGF-IR was analyzed (%).

Statistical analyses. Statistical analysis was performed using Statistica 10.0 software. All data were expressed as medians with interquartile ranges or as means with standard errors. To evaluate the difference, either Mann-Whitney or Kruskal-Wallis test was used. Correlation analysis on data was carried out with Spearman Rank Correlation test. P-values < 0.05 were considered statistically significant. Univariate logistic regression analysis was performed to predict oCR.

Results

The IGFs and IGFBPs levels were 10 times higher in ascites than in tumor tissue. There was no significant difference in the concentration of PAPP-A between primary tumor and ascites (Table 1). The level of IGF-II was significantly higher and the level of IGFBP-3 was significantly lower in patients with advanced ovarian cancer than in patients with early ovarian cancer (p<0.05). However, the expression of tyrosine kinase receptor IGF-IR was higher in early than in advanced cancer samples (91.6% (89.0-96.0) versus 79.6% (68.0-90.0) of cytokeratin-18 positive tumor cells, p <0.05). Multiple correlations were revealed between IGFs and IGFBPs levels in ascites and between IGFs and IGFBPs in tumors (Table 2). There was no correlation between IGF-IR expression and IGFs/IGFBPs levels in tumors.

The levels of IGFs, IGFBPs and PAPP-A were then investigated in ascitic fluid in relation with the ascites volume (Table 3). Patients with high-volume ascites had decreased IGF-II level. The level of PAPP-A metalloproteinase was significantly higher in patients with moderate- and high-volume ascites compared to that observed in patients with low-volume ascites (p <0.05).

Taking into account the data on the expression of the IGF- related proteins associated with ovarian cancer chemoresistance to identify predictors of response to NACT, the logistic regression analysis was performed. A total of 40 patients had achieved oCR, and 19 patient were non-responders. Only 4 patients (6.7%) had pathological complete response. Thirty patients (51%) had optimal and twenty-nine (49%) had suboptimal cytoreduction. We analyzed not only clinical characteristics (patients' age, FIGO stage and ascites volume) but also the levels of CA125 and HE4 as well as the levels of proteins of the IGF system in tumor tissue and ascites before NACT. The association between these parameters and oCR is shown in Table 4. The levels of proteins of the IGF system in tumor were not significant predictors of oCR. The univariate analysis showed that the level of IGF-I in ascites was the only independent predictor for cOR (Table 4).

Discussion

In our study, the high levels of IGFs and IGFBPs in a cell-free compartment of ascites were mainly

Table 2. Spearman Rank Order Correlations between Concentrations of IGF Related Peptides in Tumors and Ascites

	IGF-Itumor	IGF-IItumor	IGFBP-3tumor	IGFBP-4tumor	PAPP-Atumor	IGF-Iascites	IGF-IIascites	IGFBP-3ascites
IGF-Itumor			0.4	0.4		0.7	-0.4	0.6
IGF-IItumor			-0.5					
IGFBP-3tumor	0.4	-0.5		0.4		0.6	-0.5	
IGFBP-4tumor	0.4		0.4		0.3	0.7	-0.7	0.6
PAPP-Atumor				0.3		0.7		0.8
IGF-Iascites	0.7		0.6	0.7	0.7			0.8
IGF-IIascites	-0.4		-0.5	-0.7				
IGFBP-3ascites	0.6		0.5	0.5	0.8	0.8		
IGFBP-4ascites					0.4			
PAPP-A ascites	-0.5							

Notes, Only P-values below 0.05 are listed; All measurements were performed in tumor and ascites; Each correlation is based on 50 paired observations

Table 3. IGFs, IGFBPs and PAPP-A Level (ng/mg of Protein) in Ascites in Patients with Advanced Ovarian Cancer in Ascites Depending on Its Volume, Me (25-75%)

Ascites volume (mL)	Parameters				
	IGF-I	IGF-II	IGFBP-3	IGFBP-4	PAPP-A
Low volume (less 200 mL), n=10	1.9 (1.7-2.3)	193.0 (55.0-378.0)	96.9 (88.2-166.0)	5.9 (3.6-12.4)	0.04 (0-0.12)
Moderate volume, n=10	3.9 (0.5-9.5)	165.0 (84.0-281.0)	268.0 (100.0-404.0)	3.6 (0.6-5.6)	0.4 (0.13-0.5)
High volume, n=33	3.0 (1.3-5.0)	67.3 (28.7-124) **$P_{1,3}<0.05$** **$P_{2,3}<0.05$**	210.0 (140.0-266.0)	62.3 (51.4-77.8)	0.3 (0.14-0.5) **$P_{1,3}<0.05$** **$P_{1,2}<0.05$**

Note, 1,2 , changes between protein levels in low- and moderate-volume ascites samples; 1,3 , changes between protein levels in low- and high-volume ascites samples; 2,3, changes between protein levels in moderate- and high-volume ascites samples

associated with peritoneal ovarian cancer metastasis. Most authors reported that the elevated levels of total protein and growth factors in ascites were related with their increased secretion of both primary tumor cells and tumor/non-tumor cells floating in ascites (Sodek et al., 2012; Latifi et al., 2012). High levels of growth factors in ascites were explained by the increased peritoneal capillary permeability due to excessive production of vascular endothelial growth factor by these cells, thereby promoting the release of growth factors from

Table 4. Univariate Analysis to Predict Objective Clinical Response

Clinicopathological characteristics	Univariate analysis		
	Odds ratio	95%CI	P value
Age (years):			
≤50, n=24	1		
>50, n=35	1.1	0.4-3.3	0.86
FIGO stage:			
IIIC, n=47	1		
IV, n=12	1.1	0.3-4.2	0.92
Ascites (ml):			
≤1000, n=20	1		
>1000, n=33	0.5	0.1-1.7	0.19
CA125 level before NACT (IU/ml)			
<500, n=27	1		
≥ 500, n=30	0.7	0.2-2.0	0.50
HE4 level before NACT (pmol)			
<500, n=29	1		
≥500, n=30	0.5	0.2-1.7	0.16
Level of proteins of IGF-system in ascites before NACT (ng/mg of protein)			
IGF-I			
≥2.48, n=26	1		
<2.48, n=27	3.7	1.0-6.7	**0.048**
IGF-II			
<115, n=26	1		
≥115, n=27	1.6	0.4-4.8	0.48
IGFBP-3			
<190, n=26	1		
≥190, n=27	1.9	0.5-5.0	0.32
IGFBP-4			
<97.0, n=27	1		
≥97.0, n=26	0.9	0.4-2.4	0.57
PAPP-A			
Absent, n=23	1		
Present, n=30	0.6	0.3-1.9	0.44

the capillaries into the peritoneal cavity (Staldmann et al., 2005). Our data, indicating the multiple relationships between the levels of IGFs in ascites and the levels of IGFs and IGFBPs in tumor, confirmed this hypothesis. We revealed that the IGF-II level was 10 times higher than the IGF-I level in both tumor tissue and tumor ascites. A similar trend in malignant ascites was previously shown (Thomsen et al., 2015). The difference in the IGF-II/ IGF-I ratio in our study and in the study of Thomsen J. et al. may be due to different study design. Our findings in measuring the IGF-II/ IGF-I ratio were not completely consistent with those reported by Thomsen J. et al. (2015). In their study, the concentration of IGF- related peptides in ascites was expressed as micrograms per liter of ascitic fluid. In our study, as the protein concentration in ascites varied from 10 to 26 mg per mL, the concentrations of IGFs, IGFBPs and PAPP-A in ascites were calculated as the amount of protein in ascites.

We revealed the relationship between the levels of IGF-II and PAPP-A and the volume of ascites. The high level of PAPP-A metalloproteinase in ascites was associated with the high-volume ascites. Bold H.B. et al. (2011) showed that the enhanced cellular proliferation, increased vascularization and cellular invasion into Matrigel occurred in ovarian cancer cell line with a high PAPP-A expression compared to that with a low PAPP-A expression. We previously found higher expression of PAPP-A in peritoneal metastases of ovarian cancer as compared to that in the primary tumor (Yunusova et al., 2015). Although the PAPP-A levels in tumors and ascites were not associated with oCR in advanced ovarian cancer, further studies are needed to evaluate cellular and extracellular forms of PAPP-A in order to clarify the role of this protease in the ovarian cancer metastasis, chemoresistance and progression.

Data on different molecular profiles of high-grade serous carcinomas with low- (200 mL) and high- (more than 1,000 mL) volume ascites showed that differences were mainly related to the expression of genes and proteins associated with regulation of immune response. For high-grade serous ovarian cancer, the presence of large-volume ascites was associated with low rates of optimal cytoreduction and overall survival. Better surgical outcome and longer overall survival were observed in patients with low-volume ascites (less 200 mL) (Feidenberg et al., 2014). In our study, the ascites volume was not associated with response to NACT. There was an insignificant tendency towards a decrease in the rate of oCR in patients with high-volume ascites (Table 4). Published reports on the prognostic significance of ascites volume in ovarian cancer are often contradictory. In the study of Angioli R. et al (2013), ascites volume of less than 500 mL combined with preoperative serum HE4 level of less than 262 pmol/l were able to target candidates with the potential for optimal cytoreduction (all histotypes of advanced ovarian cancer). Feigenberg T. et al. (2014), demonstrated better surgical outcome and prolonged overall survival for the low-volume ascites group compared to the high-volume ascites group. However, the prognostic value of the ascites volume appeared to be limited by the existence of a large group of patients with moderate volume of ascites.

Until now, there are only a few clinical (volume of ascitic fluid, FDG-PET metabolic response), laboratory (CA125 and HE4 levels) and molecular (survivin expression, IGF-II and IGFBP-3 levels in ascites) parameters that can be used as predictive or prognostic markers in ovarian cancer patients treated with NACT and debulking surgery. Among them, there are no strong predictors of oCR (Avril et al., 2005; Slipcevic et al., 2009; Angioli et al., 2013; Gasowska-Bodnar et al., 2014; Morimoto et al., 2016). Our results were consistent with several recent reports demonstrating a 4-10-fold increase in the concentration of IGF-II in ascites as compared to that of IGF-I, thus indicating that IGF-II can contribute significantly to IGF-IR activation. On the other hand, IGF-II only has an IGF-IR cross-reactivity of 12% relative to IGF-I. Hence, it is reasonable to assume that the majority of the signal obtained by the bioassay originates from IGF-I (Chen et al., 2003; Thomsen et al., 2015). Further investigations for evaluating the function and bioavailability of IGF-I in ascites are need to clarify the role of IGF-I as a biomarker in ovarian cancer.

Conclusion. The IGFs and IGFBPs levels were 10 times higher in ascites than in tumor tissue. There was no significant difference in the concentration of PAPP-A between primary tumor and ascites. We were the first to show the relationship between the levels of IGF-II and PAPP-A and the volume of ascites. The univariate analysis showed that the level of total IGF-I in ascites was an independent predictor of oCR to chemotherapy for OC patients treated with neoadjuvant chemotherapy and debulking surgery.

Acknowledgments

Abbreviations
IGF-I and IGF-II - insulin-like growth factors, IGFBPs - IGF binding proteins, IGF-IR - insulin-like growth factor receptor I type, PAPP-A - pregnancy-associated plasma protein A, cOR – objective clinical response.

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