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Original Research Article

Profiling of selected angiogenesis-related genes in serous ovarian cancer patients

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ABSTRACT

Purpose: Since angiogenesis plays an important role in the pathogenesis of ovarian cancer the aim of the study was to compare the expression of the most relevant angiogenesis-related genes in serous ovarian cancer samples. Genes were divided into 5 subgroups according to their angiogenic potential: growth factors and their receptors; cytokines/chemokines; adhesion molecules and other matrix related proteins; transcriptions factors and signaling molecules; morphogenic factors, and angiogenesis inhibitors.
Materials/methods: Twenty-nine patients were involved in the study: 20 with serous ovarian cancer and 9 healthy controls. All neoplasms were confirmed by histopathological examination. Healthy ovarian control samples were obtained from women diagnosed with fibroids and had previously scheduled operations. Real-time PCR gene arrays were used to examine the expression of 84 human angiogenesis-related genes and expression of selected proteins was assessed with ELISA.
Results: Significantly higher expressions of 46 genes were found in the ovarian cancer group compared to the healthy control group. By the use of ELISA we confirmed the expression of three proteins i.e.: angiopoietin-2, angiopoietin-like protein 3, and angiopoietin receptor 2. Only angiopoietin-2 and angiopoietin receptor 2 showed significant differences between ovarian cancer and healthy controls.
Conclusions: Changes in the expression of selected genes associated with angiogenesis may add new information to the pathogenesis of ovarian cancer. Although the angiopoietin-2 signaling pathway may play an important role in neovascularization in ovarian cancer, the role of angiopoietin-like protein 3 is yet to be established.

1. Introduction

Ovarian cancer is the leading cause of mortality among female patients with reproductive tract neoplasms. It is the fifth leading cause of cancer-related deaths in female patients in developed countries [1].

Angiogenesis plays a crucial role in the spreading of cancer cells in all types of neoplasms and can be regulated by different natural or pharmacological stimulators and inhibitors. There are two different mechanisms of angiogenesis. The first one is creating a new blood vessel by splitting an existing blood vessel into two separate capillaries. The second one is sprouting a new blood vessel from pre-existing capillaries. Angiogenic growth factors activate receptors on endothelial cells present in pre-existing blood vessels. Then activated endothelial cells release proteases that destroy the basement membrane, allowing endothelial cells to proliferate into the surrounding matrix. Tumors induce angiogenesis by secreting various growth factors. VEGF (Vascular Endothelial Growth Factor) has been demonstrated to be a major factor involved in angiogenesis by causing a signaling cascade in endothelial cells. Binding to VEGF receptor-2 (VEGFR-2) starts a tyrosine kinase signaling cascade that stimulates the production of factors which stimulate vessel permeability (nitric oxide), cell proliferation (basic Fibroblast Growth Factor – bFGF; angiopoietins – Ang), as well as cell migration (metalloproteinases – MMPs; Intercellular Adhesion Molecules – ICAMs) and finally differentiation into mature

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vessels. Angiopoietin pathways interacting with Tie2 receptors in endothelial cells can mediate a neovascularization process in tumor tissue due to a VEGF-dependent mechanism [2–6].

Many angiogenesis-related genes and proteins have already been studied in gynecological malignancies [3,4,7]. Our study focused on the most prominent 84 factors with potential angiogenic and antiangiogenic activity as divided into 5 subgroups based on their possible influence on angiogenesis. This study is a continuation of previously published research on profiling selected angiogenesis-related genes in non-malignant gynecological lesions [8,9].

2. Material and methods

2.1. Patients

Twenty-nine patients were involved in the study. Patients with ovarian cancer underwent a hysterectomy in the Department of Gynecology and Gynecological Oncology at the Medical University of Białystok, Poland and Department of Gynecological Oncology in Gdansk, Poland. Only patients with histopathologically confirmed serous ovarian cancer were included in the study. Healthy ovarian control samples were obtained from pre- and postmenopausal women who had previously scheduled laparotomies due to fibroids. The study protocol was approved by the Local Ethics Committee of the Medical University of Bialystok (R-I-002/324/2009, R-I-002/324/2013). Informed signed consents were obtained from all patients.

2.2. RNA isolation and quality control

Extraction of total RNA from tissue samples was performed using a Macherley-Nagel (Duren, Germany) isolation kit (NucleoSpin RNA/Protein, Macherley-Nagel) according to the manufacturer's protocols. RNA concentration and quality were determined using a NanoDrop 2000c spectrophotometer (Kisker, Steinfurt, Germany) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Perlan, Poland). Only samples with RIN (RNA Integrity Number) higher than 7 were further processed [9].

2.3. PCR array

The cDNA (100 ng/μl) for each RNA sample (3 μl) was obtained using a Super Array RT2 First Strand kit (SABiosciences Corporation, Prospekta, Poland) according to the manufacturer's protocol. The PCR array was designed to study the profiles of 84 human angiogenesis-related genes in quadruplicates (list available at: www.sabiosciences.com/rt_pcr_product/HTML/PAHS-024Z.html). PCR array experiments were performed with an ABI 7900HT instrument (Life Technologies, Applied Biosystems, Poland). Conditions for amplification were as follows: 1 cycle of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C [9].

Gene expression values were calculated based on the ΔΔCt method where one sample was designated as the calibrator against which all other samples were analyzed. For the calibrator sample, i.e. reference RNA from a normal ovary (Life Technologies, Ambion, Poland), the equation was: relative quantity = $2 - 1$ which was 1; therefore, every other sample was expressed relative to this value.

2.4. ELISA

We performed enzyme-linked immunosorbent assays (ELISA) for three proteins encoded by overexpressed genes i.e.: angiopoietin-2 (Ang2; detection range 46.9–3000 pg/mL, R&D Systems), angiopoietin-like protein 3 (Angl3; detection range 0.156–10 ng/mL, R&D Systems), and angiopoietin receptor 2 (Tie2; detection range 0.156–10 ng/mL, R&D Systems). All measurements were made in duplicate and averaged. The methods used were those described in the protocols accompanying the ELISA kits.

2.5. Statistical analysis

The Mann–Whitney–Wilcoxon test was used to compare the expression of genes for the two groups for quantitative data due to the non-normal distribution of the tested variables. Calculations were performed in Microsoft Excel spreadsheets and STATISTICA (StatSoft, Inc. Ver. 7.1. statistical package and data analysis software). The Mann–Whitney–Wilcoxon test was also used to compare differences in the expression of the previously mentioned three proteins in the two groups. p-Values (Two-Sided $\Pr > |Z|$) of the two-sided Mann–Whitney–Wilcoxon test was evaluated using The NPAR1WAY Procedure in the SAS System.

3. Results

Patients' clinical characteristics are presented in Table 1. Among all of the studied 84 genes (Table 2) we found significantly higher expressions of 46 in all five groups: in the growth factors and their receptors group: EGF, TYMP, FGFR3, FIGF, TGFA, TGFB1, TGFBR1, MDK; in the cytokines/chemokines group: IL1B, IL6, IL8, CXCL1, CXCL3, CCL11, CCL2, IFNB1, TNF; in the adhesion molecules and other matrix related proteins group: MMP9, ANPEP, CDH5, PLAU, ITGAV, PLG, ITGB3, THBS2; in the transcription factors and signaling molecules group: AKT1, JAG1, TEK, HAND2, ENG, FLT1, HIF1A, NOTCH4, PTGS1, STAB1, SPHK;, and in the morphogenic factors and angiogenesis inhibitors group: ANGPT2, ANGPTL3, BAI1, EFNA1, EFNA3, EPHB4, PF4, NRP1, NRP2, S1PR1.

Detailed values are presented in Table 2. Interestingly we found no differences between ovarian cancer and healthy patients in the expression of genes such as VEGF-A and VEGF-C which are often associated with tumor neovascularization in other neoplasms. We showed that expression of proteins angiopoietin-2 (Ang2) and angiopoietin receptor 2 (Tie2) had significant differences between ovarian cancer and healthy controls (Table 3).

4. Discussion

We evaluated the expression of 84 most prominent genes with potential angiogenic and antiangiogenic activity as divided into 5 subgroups depending on their possible influence on angiogenesis. ELISA confirmed the expression of three proteins encoded by the most homogeneous overexpressed genes, i.e.: angiopoietin-like protein 3, angiopoietin-2, and angiopoietin receptor 2. Statistically significant differences between ovarian cancer and healthy controls were found only for proteins Ang2 and Tie2.

Table 1
Patients characteristics.

Characteristics	Number	%
Ovarian cancer patients	20	100
Mean age (range)	65.8 (49–80)	
Histopatologic type of cancer	17 adenocarcinoma serosum	85
	3 cystadenocarcinoma serosum	15
FIGO stage of disease	3 IC	15
	3 IIC	15
	1 IIIA	5
	12 IIIC	60
	1 IV	5
Grade	3 Low grade tumors	15
	17 High grade tumors	85
Control group	9	100
Types of tissues	9 normal ovaries	100
Mean age (range)	49.3 (43–58)	