Microvascular Research 107 (2016) 91–96

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

The associations between serum VEGF, bFGF and endoglin levels with microvessel density and expression of proangiogenic factors in malignant and benign ovarian tumors

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ARTICLE INFO

Article history:
Received 17 February 2016
Revised 27 May 2016
Accepted 12 June 2016
Available online 14 June 2016

Keywords:
Angiogenesis
Ovarian cancer
Ovarian tumor
VEGF
bFGF
Endoglin
Microvessel density

ABSTRACT

Aim of the study: To investigate whether serum levels of VEGF, bFGF and endoglin correlate with tumor VEGF and bFGF expression or microvessel density (MVD) in ovarian cancer.
Patients and methods: Forty five patients with epithelial ovarian cancers (EOCs) and 38 patients with benign ovarian tumors (BOTs) were included into the study. Serum levels of VEGF, bFGF and endoglin were assessed using ELISA. The expression of VEGF and bFGF in tumor samples were evaluated using ELISA of supernatants obtained from tumor homogenization. MVD was analyzed using immunohistochemistry with antibodies against CD31, CD34 and CD105.
Results: Serum VEGF levels were significantly higher in EOCs than in BOTs (436.6 pg/ml [19.67–2860] vs 295.5 pg/ml [123–539], $P = 0.025$). Serum endoglin levels were lowered in the group EOCs when compared to BOTs (33,720 g/ml [12,220–73,940] vs 42,390 pg/ml [19,380–56,910], $P = 0.015$). There were no differences in bFGF levels between studied groups. EOCs have significantly higher CD105 MVD (25 vessels/mm2 [0–57] vs 6 vessels/mm2 [0–70], $P < 0.001$) and tumor VEGF (405.9 pg/mg protein [0–3000] vs 2.225 [0–634.7], $P < 0.001$) expression than BOTs, while, bFGF expression was higher in BOTs than in EOCs (2076 pg/mg protein [668.1–8718] vs 847.3 pg/mg protein [188.9–8333], $P = 0.003$). In patients with EOCs we have observed negative correlation between serum VEGF concentration and its tissue expression (r Spearman $= -0.571$, $P = 0.0261$), and serum VEGF concentration correlated positively with CD34-MVD (r Spearman $= 0.545$, $P = 0.0289$). In a multiple regression analysis we have observed only the negative correlation between serum VEGF and CD105-MVD ($r = -0.5288$, $P = 0.0427$).
Conclusions: Serum VEGF is a useful marker for prediction of ovarian cancer MVD and tumor VEGF expression.

1. Introduction

Development and incorporation of bevacizumab, a humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF), have started the new era of the management of epithelial ovarian cancer (EOC), fallopian tube cancer (FTC) and primary peritoneal cancer (PPC) patient. Current data indicate bevacizumab to prolong overall survival during primary treatment of high-risk, non-optimally debulked EOC, FTC and PPC patients (Perren et al., 2011). Patients with recurrent malignancies, both platinum-resistant and platinum-sensitive, may also benefit from bevacizumab administration (Pujade-Lauraine et al., 2014; Aghajanian et al., 2012). Although not confirmed in third phase clinical trials, bevacizumab seems to be effective in palliative support for heavily pretreated patients (Cannistra et al., 2007; Burger et al., 2007) (Garcia et al., 2008; Han et al., 2010).

Unfortunately not all EOC, FTC and PPC patients received overall survival prolongation with bevacizumab treatment. Only thanks to careful evaluation of study subgroups we could find patients who may have profit from the treatment (Perren et al., 2011; Burger et al., n.d..). The more we know about tumor angiogenesis and microenvironment the more individualized management we can offer. We know that several features of tumor neovascularization may indicate patients' prognosis. The studies focus mainly on evaluation of tumor microvessel density (MVD) and expression of proangiogenic factors. Although not all studies

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http://dx.doi.org/10.1016/j.mvr.2016.06.002
0026-2862/

are concordant, high tumor microvessel density (MVD) is regarded as a marker of poor prognosis (Rubatt et al., 2009). There is even data showing MVD is useful for prediction of response to antiangiogenic therapy in ovarian cancer patients (Han et al., 2010). Increased tumor expression of several proangiogenic factors, like vascular-endothelial growth factor (VEGF) suggests shortened survival in ovarian cancer (Han et al., 2010; Yu et al., 2013; Smerdel et al., 2009). On the other hand, lowered bFGF expression may indicate high grade epithelial ovarian cancers (Szubert et al., 2014).

Examination of tumor sample is expensive, time-consuming and subjective. While the evaluation of patient serum is quick, easy to obtain, safe and the results could be standardized. In several studies, including various human neoplasms (e.g. breast cancer, osteosarcoma, gastrointestinal stromal tumor), serum VEGF levels could accurately predict tissue VEGF expression and MVD (Iovino et al., n.d.; Chen et al., 2012; Wang et al., 2009a). However, there is lack of comprehensive studies concerning this issue in ovarian cancer. Thus, the main aim of this study was to assess whether serum levels of selected proangiogenic factors may predict their tissue expression and MVD.

2. Materials and methods

2.1. Study group

The study group included 83 patients treated due to ovarian tumor in the Division of Gynecological Surgery, Poznan University of Medical Sciences, Poland, during the years 2007–2012. Tumors removed during surgery were examined histopathologically and classified according to WHO criteria. After histological examination the study group was divided into 2 subgroups: 1) epithelial ovarian cancers (n = 45) and 2) benign ovarian tumors (n = 38). The histological types of the malignant ovarian tumors included in the study were as follows: 20 serous, 4 mucinous, 4 endometrioid, 4 clear cell adenocarcinomas, 9 undifferentiated carcinomas, 3 metastatic ovarian tumors and one ovarian carcinosarcoma. The subgroup of benign ovarian tumors included the following: 6 serous and 5 mucinous cystadenomas, 8 endometrioid cysts, 11 adult teratomas, 3 fibrothecomas, one struma ovari, one Brenner tumor and 3 tubo-ovarian abscesses. Malignant tumors were classified into 3 histological grades: 10, 8, and 27 tumors were classified as grades 1, 2, and 3, respectively. The clinical stage of the disease was specified using the criteria of the International Federation of Gynecology and Obstetrics (FIGO). Malignant tumors were classified according to the FIGO stage of the disease as follows: 11 stage I patients, 7 stage II patients, 20 stage III patients, and 7 stage IV patients.

We obtained tissue samples during surgery. The materials were divided into 2 parts. The first part was fixed in buffered formalin, and the second part was frozen just after collection and stored at −82 °C.

This study received local Ethics Committee approval.

2.2. Serum levels of VEGF, bFGF and endoglin (CD105)

Prior to operation, patients' sera were collected for determination of VEGF, bFGF and endoglin concentration. Blood samples were collected within 5 days before surgical treatment. Blood samples were centrifuged, and the sera were stored at −82 °C. VEGF, bFGF and endoglin serum levels were analyzed by EIA assays (catalog no. DY293B, DY233 and DY1097 respectively, R&D Systems Minneapolis, MN, USA).

2.3. Expression of VEGF and bFGF in tumor samples

The tissue expression of VEGF and bFGF was assessed by ELISA in supernatants obtained from tumor homogenization. The homogenates were obtained from freshly frozen tissue samples. The analyzed tissue specimens were homogenized mechanically in a buffer containing 150 mM NaCl (Sigma Aldrich, USA), 5 mM EDTA (Sigma Aldrich, USA), 50 mM Tris-HCl, pH 7.4 (Sigma Aldrich, USA), 1% Triton X-100 (Sigma Aldrich, USA), and a protease inhibitor cocktail (Sigma Aldrich, USA, Catalog number S8820). The homogenates were centrifuged for 15 min in Eppendorf tubes at 10,000 rpm. The supernatants were used for ELISA to measure the concentration of VEGF and bFGF (catalog no. DY293B and DY233 respectively, R&D Systems Minneapolis, MN, USA). The expression of VEGF and bFGF is reported as the tissue protein content (pg/mg total protein). Protein concentrations were assessed according to Lowry's method. No repeated freeze-thaw cycles were performed before ELISA analysis in any case. Before analysis, we have performed micro-dissection to exclude connective tissue and large vessels from the specimen. The samples were not matched for the analysis and until statistical analysis all the members of the team involved in ELISA analysis were blinded against studied groups. The analyzes were performed in duplicates for each series and following coefficients of variation (cv) were calculated for intra-assay variability: VEGF = 13% and bFGF = 6.98%.

2.4. Immunohistochemistry and MVD assessment

Formalin-fixed tissue samples were used to evaluate MVD. Immunohistochemistry was used for endothelium labeling with antibodies against CD105 monoclonal mouse anti-human, clone MM0049; Novus Bilogicals, Product No. NB110–93,509; dilutation 1:500, CD31 (monoclonal mouse anti-human, clone C70A; Dako, Product No. M0823; dilutation 1:200), and CD34 (monoclonal mouse anti-human, clone QBEND-10; Dako, Product No. M7165; dilutation 1:200). A modified protocol proposed by Rubatt et al. (Han et al., 2010) was used for the assessment of MVD. Briefly, one observer screened the whole sample by light microscopy at 40× magnification to identify the 3 largest microvessel clusters ("hot spots"). Only hot spots located near neoplastic cells were analyzed. Subsequently, microvessels were counted in each of the selected hot spots at 400× magnification. Only microvessels with lumen were considered. Large vessels and vessels with muscular walls were not counted. The median number of microvessels from 3 hot spots was used for the final analysis. MVD assessment was conducted by researcher experienced in angiogenesis studies. Examples of immunohistochemistry photograms are presented in Fig. 1 (Fig 1.)

Figs. 1 A, B, C present immunohistochemistry photograms of ovarian mucinous cystadenoma with vessels stained with anti-CD34, -CD31 and -CD105 antibodies respectively (×50 magnification). Please note the solely stained vessels with anti-CD105 antibodies of the same tumor presented at photogram D (×200 magnification). The photograms E, F and G present immunohistochemical staining of CD34, CD31 and CD105 of serous high-grade adenocarcinoma of the ovary under (×50 magnification). The photogram H presents the same tumor stained with anti-CD105 antibodies under magnification of ×200. Please note higher microvessel density when compared with benign ovarian tumor (photogram D).

2.5. Statistical analysis

Statistical analysis was performed with WEKA 3.6, R3.1.1 and MedCalc 15.11.4. The Mann-Whitney test was used for the comparison of serum levels of VEGF, bFGF and endoglin, tissue VEGF and bFGF expression, and MVD between the group of malignant and benign ovarian tumors. We have performed Spearman's rank correlations and least squares multiple regression analysis to find relationship between serum levels of proangiogenic factors (VEGF, bFGF and endoglin) with their tissue expression (VEGF and bFGF) and MVD.

3. Results

We have found significantly elevated serum VEGF levels in the group of malignant ovarian tumors when compared to benign ovarian tumors. On the other hand, serum endoglin levels were lowered in the group of epithelial ovarian cancer when compared to benign ovarian tumors.