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Profiling of selected angiogenesis-related genes in proliferative eutopic endometrium of women with endometriosis

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ABSTRACT

Objective: To compare the expression level of the most relevant angiogenesis-related genes in the eutopic endometrium of women with and without endometriosis.
Study design: 32 regularly menstruating patients (18 with endometriosis and 14 controls) underwent surgery in the proliferative phase of the cycle. Eutopic endometrium was collected by the use of aspirating biopsy prior to laparoscopy. Only patients with advanced (stage III and IV) histopathologically confirmed ovarian endometriosis were studied. Real-time PCR gene arrays were applied to examine the expression of 84 human angiogenesis-connected genes. Western-blot and enzyme-linked immunosorbent assays (ELISA) were used to confirm the expression of selected proteins.
Results: We found significantly higher levels of *AKT1* ($p = 0.003$), *TYMP* ($p = 0.02$), *JAG1* ($p = 0.007$), *LAMA5* ($p = 0.005$) and *TIMP-1* ($p = 0.03$) in eutopic endometrium of patients with endometriosis as compared with controls. By the use of Western blot we found clearly positive expression of AKT1 whereas ELISA assays confirmed expression of AKT1, TYMP, JAG1, LAMA5 and TIMP1.
Conclusion: Changes in the expression of selected genes might lead to or be a consequence of an early defect in the physiological activity of proliferative endometrium ultimately resulting in its overgrowth outside the uterine cavity.

1. Introduction

Endometriosis is defined as the presence of endometrial tissue, consisting of both glandular epithelium and stroma, outside the uterine cavity, and affects about 10% of women of reproductive age. In women undergoing laparoscopic surgery, however, to investigate the cause of their infertility or pelvic pain, the rates are about 30% and 50% respectively [1].

The most widely accepted theory for the development of endometriosis is the implantation theory of Sampson [2]. He proposed that endometrial tissue is retrogradely shed through the Fallopian tubes into the peritoneal cavity during menstruation, where it attaches and proliferates at ectopic sites. Retrograde menstruation is a common phenomenon occurring in more than 90% of women, but only 10% of those women develop endometriosis [3]. Therefore, further conditions must be in place to support excessive growth of eutopic endometrium outside the uterine cavity. It is becoming increasingly evident that the primary defect in endometriosis may be located in the eutopic endometrium [4].

One determining factor might be abnormal angiogenic potential of eutopic endometrium which could facilitate its overgrowth outside the uterine cavity [5]. While quite a number of angiogenesis-related genes and proteins have already been studied in eutopic and ectopic endometrium [4,6,7] in our study we concentrate on the 84 most prominent factors with potential angiogenic and antiangiogenic activities, divided into five groups depending on possible function in the pathogenesis of angiogenesis (Fig. 1). Since macro- and micro-array technologies have become widely used tools for analyzing the expression of a plethora of genes in a single experiment, the utility of various DNA microarray technologies in endometriosis has rapidly and tremendously evolved [8]. In our study we used a commonly accepted gene expression macroarray focused on potential angiogenesis-related genes [9,10]

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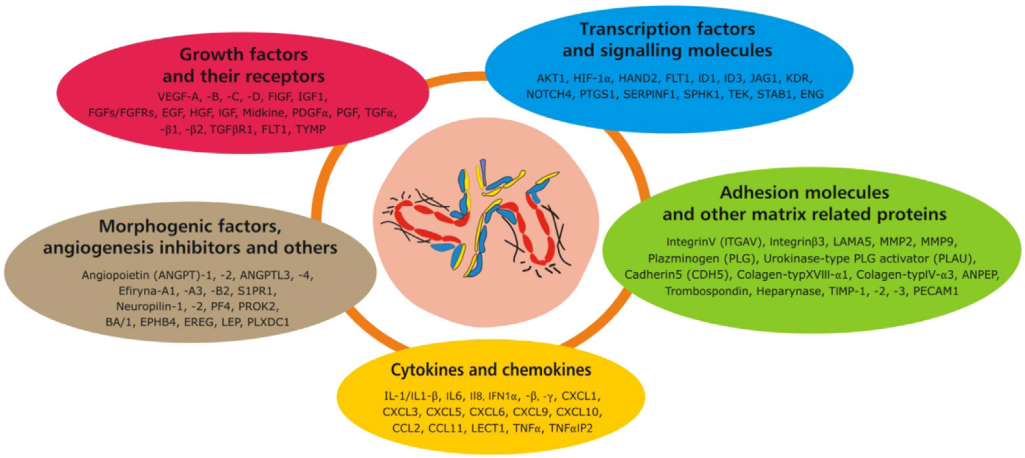

Fig. 1. Studied angiogenesis-related genes are presented in five major groups according to their function.

2. Material and methods

A total of 32 patients were recruited in the study. Endometrial samples were obtained from regularly menstruating premenopausal women aged 20–35 years, undergoing diagnostic or surgical laparoscopic surgery for non-malignant ovarian lesions. Patients with ovarian endometriosis, stages from III to IV, were diagnosed by laparoscopic findings according to the revised AFS classification of endometriosis [11] and each case was confirmed by histopathology.

Patients with autoimmune disease, pelvic inflammatory disease, adenomyosis, myomas, uterine adhesion and anomalies, dysfunctional uterine bleeding and those who took non-steroidal anti-inflammatory drugs, GnRH agonists and steroids for the past 3 months were excluded. Endometrial samples were collected by Pipelle suction in the operating room before the laparoscopic procedure and endometriosis samples were taken during laparoscopy. Endometrial tissue samples were classified by histological dating according to the method of Noyes et al. [12] and only patients in the proliferative phase (days 8–12) of the cycle were included in the study.

Among patients with endometriosis we were able to successfully collect endometrium-matched ovarian endometriosis samples with appropriate RNA quality (RNA integrity number (RIN) of at least 7) in 14 cases and we therefore chose only good quality samples for further analysis to make the subgroup sufficiently homogeneous for subsequent statistical analysis. The study protocol was approved by the Local Ethical Committee of Medical University of Bialystok, Poland, and informed consent was obtained from each patient.

2.1. RNA isolation and quality control

The extraction of RNA from the tissue samples was carried out using a Macherley-Nagel (Duren, Germany) isolation kit (NucleoSpin RNA/Protein, Macherley-Nagel) according to the manufacturers' protocols.

RNA concentration and quality were determined using the NanoDrop spectrophotometer (Kisker, Steinfurt, Germany) and the Agilent Bioanalyzer 2100 (Agilent Technologies, Perlan, Poland). Samples chosen for further analysis showed minimum signs of degradation as judged by the RIN, which was above 9 for all eutopic endometrium samples and above 7 for ovarian endometriosis.

2.2. RT2 Profiler PCR array

The cDNA (100 ng/μl) for each RNA sample (3 μl) was obtained using the Super Array RT2 First Strand kit (SABiosciences Corporation, Prospekta, Poland) according to the manufacturer's instructions. The PCR array was designed to study the profile 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 on 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.

Gene expression values were calculated based on the $\Delta\Delta C_t$ method, where one sample was designated the calibrator, through which all other samples were analyzed [13]. For the calibrator sample, i.e. reference RNA from normal endometrium (Life Technologies, Ambion, Poland), the equation is relative quantity = 2^{-0}, which is 1; therefore, every other sample is expressed relative to this.

2.3. Protein isolation and Western blot

The protein concentration in the supernatant was determined by the Bradford method using Bio-Rad Protein assay reagent (Biorad, Poland). Proteins were subsequently resolved by polyacrylamide gel electrophoresis with premade 4–20% polyacrylamide gels (Biorad, Poland) according to the manufacturer's instructions. Proteins in gels were transferred to PVDF (polivinyldifluoride) membranes (Whatman, Poland) in transfer buffer (Tris, glycine, 20% methanol) by the use of Mini Trans-Blot system (Biorad, Poland).

Expression of proteins was done by classical Western-blot analysis. In the first place membranes were incubated in blocking buffer (Sigma, Poland) for 1 h in order to prevent non-specific binding. Secondly, membranes were incubated at room temperature with following primary antibodies: mouse monoclonal anti-TYMP (sc-47702, Santa Cruz, Poland) in 1:100 dilution; mouse monoclonal anti-TIMP1 (from two manufacturers, i.e. sc-80365, Santa Cruz, Poland, and GTX108254, GeneTex, Poland) tested in the full range of concentrations with two types of antibodies; mouse monoclonal anti-AKT1 (sc-81434, Santa Cruz, Poland) in 1:200 dilution; mouse monoclonal anti-Jagged1 in the final 2 μg/ml concentration (from two manufacturers, i.e. MAB1277, R&D systems and ab7771, GeneTex, Poland); mouse monoclonal anti-Laminin5 tested in the full range of concentrations with two types of antibodies (from two manufacturers, i.e. MAB2144, R&D systems, Poland and ab55415, Abcam, Poland). After 5× washes with TBS–Tween (buffer Tris–HCl, 0.15% Tween-20), membranes were incubated with secondary antibody, goat anti-mouse HRP-conjugated (Santa Cruz, Poland) and analyzed with an enhanced chemiluminescence system (GE, Amersham, Poland). A tubulin