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Detection of angiogenic markers in endometriosis and endometrial carcinoma of the uterus

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Abstract Endometriosis is an estrogen-dependent disease characterized by the ectopic appearance of endometrial tissue. Pro and anti-angiogenic markers appear to be major factors influencing the development of inflammatory endometriosis. Vascular endothelial factor (VEGF) and placental growth factor (PlGF) belong to the group of pro-angiogenic factors. The class of anti-angiogenic markers includes e.g. soluble FMS-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng). The aim of the present study was to detect changes in the expression of selected proteins for and anti-angiogenic factors in the blood, studying to changes the expression of miR-17-5up levels and molecular changes on the DNA and chromatin level of the women with diseases of the female reproductive system.

Key words endometriosis, endometrial carcinoma, microRNA, angiogenic markers

1. INTRODUCTION

Oncological diseases of the female reproductive system are currently among the most common diseases. These are genetic diseases caused by uncontrolled cell proliferation - malignant tissue transformation. There are changes in the organization and sequence of the genome. Mutations of important regulatory genes are also present.

In various types of cancer, some cells of the body begin to divide without stopping and are transported by the blood or lymphatic system to the surrounding tissues where they create a new tumor secluded from the original tumor. The prevalence of cancer has a long-term growing nature. The causes of gynecological tumors are often times dependent on many factors, such as the individual's genetic predisposition.

Currently, the selection of the appropriate treatment type is based on the type, stage, and size of the tumor. The patient's general health and age also play an important role. Only 17 to 20% of women in Slovakia undergo regular gynecological examinations per year. Recently, there has been a significant increase in gynecological malignancies (Pleško et al., 2012).

Most oncological diseases are diagnosed only in advanced stages of the disease, so today's research focuses on identifying new biochemical and molecular markers that are needed for early diagnosis of cancer and non-cancer.

2. ENDOMETRIOSIS AND ENDOMETRIAL CANCER

Endometriosis is also a common gynecological inflammatory disease. It is an estrogen-dependent benign disease. It is characterized by the implantation of endometrial tissue in the pelvic area, which is sensitive to estrogen and significantly reduces the quality of life of women (Bulleti, 2010). Most often endometriosis affects the ovaries, fallopian tubes or tissues around the uterus. Typical symptoms of this inflammatory disease include recurrent pelvic pain, pain during the menstrual period, painful intercourse, urgent and sometimes painful emptying, and physical pain present during exercise, standing or walking. The inflammatory process that is caused by endometriosis can affect the fertility of women. Among the common causes of infertility are large pelvic bruising or peritubal adhesion, impairment of the fallopian tubes, or reduced oocyte release from the ovaries (Schenken RS., 1984). In women with endometriosis, there is an increased volume of peritoneal fluid with a high concentration of activated macrophages, prostaglandins, IL-1, TNF, and proteases. These changes are likely to have adverse effects on oocyte, sperm, embryo or oviduct function. The failure of fimbriae to capture eggs is the responsibility of an inhibitor of egg capture in the peritoneal fluid (Suginami H., 1988). Endometriosis is an inherited disease affected by environmental and genetic factors.

Although it is a benign disease that does not endanger women's lives, it is increasingly reducing the quality of life.

The most common female malignancies are uterine cancer. In the uterus, malignant proliferation occurs, which is endometrial cancer and less common uterine sarcoma. Endometrial cancer is a malignant tumor of the lining of the uterine cavity (endometrium) which results from abnormal growth of cells capable of attacking and spreading to other parts of the body. The incidence of this disease is increasing with age. Most women suffering from the disease are menopausal, with approximately 5% of patients under 40 years of age. Other risk factors include long-term estrogen therapy or obesity. Endometrioid carcinoma is one of the most common types of endometrial cancer and accounts for more than 80% of all cancers. The most common symptoms include abnormal uterine bleeding, vaginal discharge, haematuria, anemia, thrombocytosis, elevated blood glucose or abdominal pain (Walker S., 2013). Tumors originate from atypical hyperplasia, grow exophytically into the uterine cavity or infiltrate the uterine wall. Metastasis occurs later, when the lymph nodes of the pelvis are infected, but also distant organs.

3. ANGIOGENIC MARKERS

Angiogenesis contributes to the development of the vascular system during endometriosis but it also contributes to the development of the vascular system. In the developing embryo, it plays an important role in creating a functional circulatory system. Angiogenic markers and their receptors are involved in the regulation of placental vascular development (Park, Shim, Cha, 2015). The most studied are the placental growth factor (PlGF) and vascular endothelial growth factor (VEGF). Their antagonists include soluble endoglin (sEng) and soluble FMS-like tyrosine kinase (sFLT-1). The placental growth factor in humans is encoded by the PlGF gene (Maglione D., 1993) and located on chromosome 14q24. It is an important pro-angiogenic factor (Binder N., 2016). The placental trophoblast is the major source of PlGF during pregnancy, which plays an important role in the growth and differentiation of trophoblasts. It is expressed in many other tissues, including villous trophoblasts (Khalil A. et al., 2008). High expression is observed during pregnancy in the placenta as well as in the peritoneal fluid in patients with endometriosis (Binder N., 2016). Vascular Endothelial Growth Factor VEGF (vascular permeability factor VPF) is a signaling protein produced by cells, the role of which is to stimulate blood vessel formation. It plays an important role in the process of vasculogenesis as well as angiogenesis. The primary site of action is the vascular endothelium. It is involved in the stabilization of endothelial cells in blood vessels - in kidney, brain and liver. The receptors for this protein are on glomerular endothelial cells. Endoglin, known as CD105, is a TGF-β growth factor receptor. Its expression is upregulated in endothelial cells (Fonsatti E., 2001). It regulates cell proliferation, adhesion, migration, and differentiation (Wong SH., 2000). This glycoprotein acts as a tumor suppressor, inducing inflammation and the release of angiogenic factors from inflammatory cells in vivo (Hata A. et al., 1998).

Vascularization is required for tumor growth and metastasis, formation of new vessels. In the absence of blood, the tumor cell undergoes apoptosis (cell death) / necrosis. The main objective of endoglin therapeutic therapy in cancer is to target highly proliferating endothelial cells. This would inhibit metastasis and shrink tumors.

4. MICRO RNA

MicroRNA is a small non-coding RNA molecule, 19-25 nucleotides long single-stranded RNA. It occurs in most eukaryotes, including humans. MicroRNAs are involved in the regulation of gene expression, controlling a large number of physiological and pathological processes in the body, including malignant transformation. It is produced in the cytoplasm or nucleus from a long primary transcript (pri-miRNA) and hairpin precursor structure (pre-miRNA) by ribonucleases.

MicroRNA has an important function in the post-transcriptional regulation of gene expression of multiple oncogenes and tumor suppressors. These short, non-coding RNA molecules exhibit, in addition to specific tumor tissue expression profiles, other properties that make them an ideal tumor biomarker. These are high stability, easy detection, wide dynamic range and correlation with known clinical-pathological characteristics (Chan et al., 2011).

Circulating microRNAs in the diagnosis of various tumor types demonstrate good analytical properties (Brase et al., 2010). It proves very stable in body fluids, remains stable despite exposure to harsh conditions - high temperature, extreme pH, long-term storage (Li et al., 2015).

5. MATERIAL AND METHODS

The biological material of the experimental group in the study consisted of peripheral blood and urine of patients examined at the Gynecology and Obstetrics Clinic of UNLP in Košice. Patients in the experimental group (n = 15) were diagnosed with the frozen pelvis (FP), endometriosis, sacrouterine ligament (ESUL), and carcinoma of the endometrium of the uterus. The mean age of the patients was 41 years. The control group (n = 5) consisted of NTS UNLP blood donors.

Biological material from patients were collected in the form of whole blood collected by a closed collection system Vacutainer with the addition of K_3EDTA. Analysis of angiogenic marker expression was performed using an immuno-enzymatic ELISA method. A cell-based lysis kit was used to isolate miRNA from urine samples. The next step involved RNA precipitation, degradation, specific purification and elution of small miRNAs.

Specific RT reverse primers specific for the analysed miRNA (miR-17-5up) were used to reverse transcribe miRNA into cDNA. Analysis of specific miRNA expression levels was performed by Real-Time PCR using a Rotor-Gene Quadruplex Qiagene Cycler and TaqMan Gene Expression Assays. This laboratory technique monitors the amplification of the targeted DNA molecule during PCR. Subsequently, fluorescence radiation was detected by the detector during each cycle and its intensity correlated with the amount of amplicon in the reaction mixture. AFM was used to diagnose molecular changes in DNA and chromatin in patients with the frozen pelvis and endometrial carcinoma.

6. RESULTS

6.1 MicroRNA analysis – qRT-PCR

Changes in the expression of specific miR-17-5up levels were analysed in patients suffering from endometriosis sacrouterine ligament and frozen pelvis. From the measured values with respecting Ct and $2^{-\Delta\Delta CT}$ levels, it can be concluded that the target miR-17-5up is up-regulated. Significant changes in expression

levels were detected (Figure 1). The expression level of miR-17-5up in the experimental group in patients with endometriosis sacrouterine ligament was increased by 93% compared to the control group. The expression level of mir-17-5up in the experimental group in patients with frozen pelvis was increased by 712% compared to the control group.

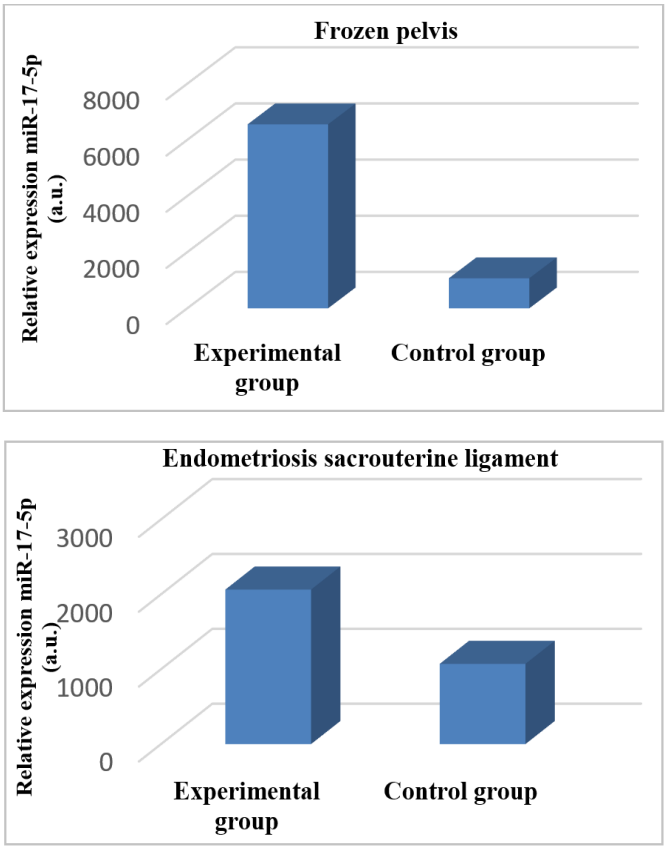

Figure 1. Change in miR-17-5up expression levels in patients with endometriosis sacrouterine ligament and frozen pelvis compared to control group

6.2 Analysis angiogenic markers – method ELISA

Expression of endoglin and FLT-1 anti-angiogenic marker proteins was detected using an ELISA method. Endoglin is a glycoprotein that functions as a tumor suppressor that induces inflammation and release of angiogenic factors from inflammatory cells. Elevated endoglin was observed in patients with endometriosis (inflammatory disease) compared to patients with endometrial cancer. Increased anti-angiogenic marker FLT-1 was observed in patients with endometrial cancer.

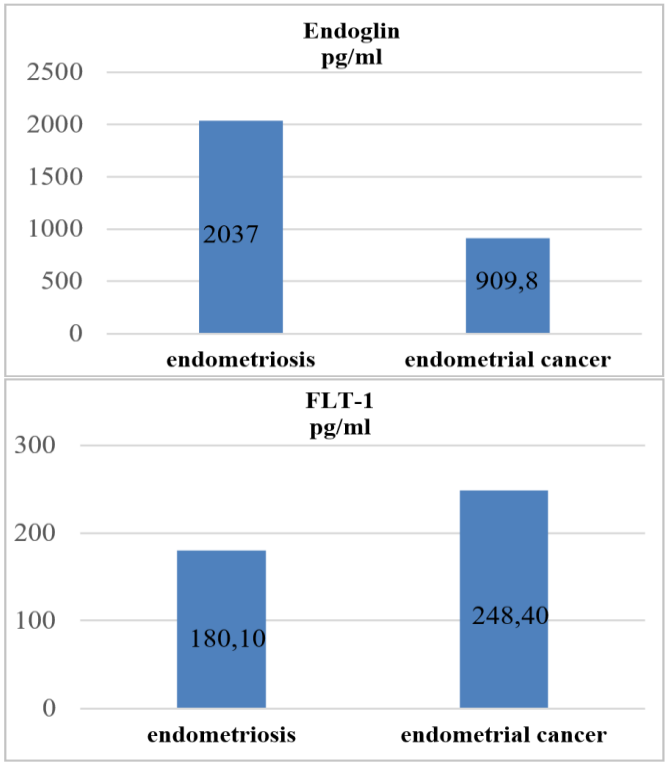

Figure 2. *Expression of endoglin and FLT-1 anti-angiogenic marker proteins in patients with various types of endometriosis and endometrial carcinoma*

Vascular endothelial growth factor VEGF plays an important role in stimulating blood vessel formation. It is one of the main inducers in the process of vasculogenesis as well as angiogenesis. Increased expression of pro-angiogenic VEGF marker proteins was observed in patients with endometrial cancer.

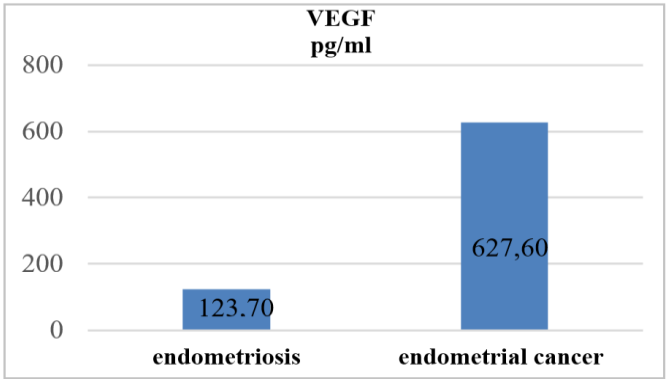

Figure 3. *Expression of proteins of the pro-angiogenic VEGF marker in patients with different types of endometriosis and endometrial cancer*

6.3 DNA and chromatin analysis – method AFM

Atomic Force Microscopy AFM is a technique that is used to display three-dimensional surface properties of samples. It is used to investigate the dispersion and aggregation of materials such as size, shape, sorption and structure.

The AFM method was used to compare the structure of ss and dsDNA, the size of individual nucleosomes, and the amplitude of the RMS spectrum, which expresses the roughness of the nuclear

material in the sample. AFM was used to diagnose molecular changes in DNA and chromatin in patients with the frozen pelvis and endometrial cancer compared to the control group. The results show that the width of ss, dsDNA, and nucleosome width was highest in endometrial cancer compared to the control group. Isolated single-strand and double-strand DNA systems in patients and control blood samples showed significant differences in nucleosome alignment and concentration and interconnections. In the DNA analysis of tissue isolated from a patient diagnosed with endometroid adenocarcinoma of corpus uteri, the ssDNA width showed a significant difference from the control sample (73 ± 5% higher, p <0.001), but the ssDNA height was at the control level. The width of the dsDNA showed similar values to that of the dsDNA, the maximum width of the dsDNA being approximately 77.8 ± 4% higher compared to the control (p <0.001). The isolated DNA showed an increased nucleosome width (of 73.4 ± 5% compared to the control sample (p <0.001).) The measured values showed that the width of ssDNA and dsDNA had a significant increase in endometrial cancer compared to controls group. controls. In opposite the height of ssDNA and dsDNA expressed maximal value in the sample with endometriosis. (Urdzík P. et al., 2019).

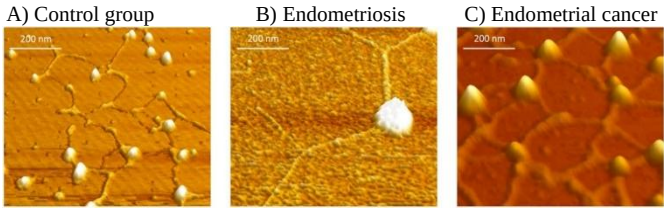

Figure 4. Results of ss and dsDNA and nucleosomes in patients with endometriosis and endometrial cancer compared to the control group

The second part was focused on the structure and compactness of chromatin (fig. 5). In endometriosis and endometrial cancer, the nucleosome width was lower compared to the control (33.3 ± 5%, p <0.01 and 23.8 ± 4%, p <0.05). The height of nucleosomes in the frozen pelvis sample showed the lowest value (Urdzík P. et al., 2019).

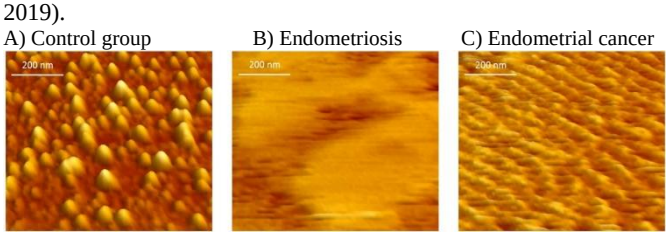

Figure 5. Results of isolated chromatin in patients with endometriosis and endometrial cancer compared to the control group

7. CONCLUSION

Endometriosis and endometrial cancer are among the most common female diseases that have an increasing incidence at present. The study of new biomarkers and laboratory procedures is important because cancer treatment has a higher success rate in the early stages. MicroRNA functions as an oncogene or tumor suppressor and the expression of these small molecules in tissue is tumor-specific. In addition, that miRNA have specific expression profiles in tumor tissue, have too other important properties that make them a interesting as a tumor biomarkers. Study of specific markers at the molecular level could assist in the development of new diagnostic procedures that would be useful not only in early diagnosis but also in monitoring the treatment of patients suffering from endometriosis and endometrial cancer.

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