WNT5A gene and protein expression in endometrial cancer

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Abstract

Introduction. WNT5A (Wnt family member 5A) belongs to the WNT family of secreted signaling glycoproteins that play essential role in developmental, physiological and pathological processes. WNT5A was shown to take part in carcinogenesis process playing both oncogenic and suppressor functions in various types of human malignancies. This study aimed to assess the expression of the WNT5A gene at the mRNA and protein levels in the specimens derived from endometrial cancer (EC) or unchanged control endometrium. The associations between the WNT5A expression levels and clinicopathological characteristics and survival of EC patients were evaluated.

Materials and methods. Total RNA was isolated in order to assess the relative amounts of WNT5A mRNA by quantitative polymerase chain reaction (QPCR) in samples of unchanged endometrial control (n = 8) and tumor samples of EC patients (n = 28). Immunohistochemistry (IHC) was used to determine the presence of WNT5A protein in the sections of formalin-fixed, paraffin-embedded tissue specimens derived from unchanged endometrial controls (n = 6) and EC tumors (n = 19). Significance of differences in WNT5A expression levels between the studied groups of EC patients and correlations between the WNT5A and demographic data, pathological features, hematological parameters and overall survival of the patients were evaluated by statistical analysis.

Results. The level of WNT5A mRNA was decreased in EC in comparison to unchanged endometrium. WNT5A expression was associated with primary tumor invasion status exhibiting reduced level of transcripts in EC that involved organs beyond the uterus when compared to the uterus-confined cancers. WNT5A immunoreactivity was visualized in the cytoplasm and nuclei of EC cells as well as in the luminal and glandular epithelial cells of unchanged endometrium. WNT5A mRNA expression levels correlated negatively with cytoplasmic, and positively with nuclear immunoreactivity of the WNT5A protein in the EC cells. In addition, the relationships between blood leucocyte count (in particular granulocytes and lymphocytes) of patients with EC and their WNT5A mRNA and protein expression levels were established. A positive correlation between the nuclear immunoeexpression of WNT5A protein in the cancer cells in cell nuclei and mean platelet volume in blood was also found.

Conclusions. The results of the first study of WNT5A expression at the transcript and protein levels indicate that it could be considered as a potential marker of molecular changes that take place during EC development. (Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 2, 84–93)

Key word: WNT5A expression; endometrial cancer; platelets volume; QPCR; IHC
Introduction

Endometrial cancer (EC) is the most common gynecological neoplasm diagnosed in developed countries, the fourth most common cancer in women after breast, lung, and colorectal cancer. Its incidence is increasing [1]. Exposure to endogenous and exogenous estrogens associated with obesity, diabetes, early onset of menarche, nulliparity, late onset of menopause, older age (> 55 years) and use of tamoxifen, are considered as the main risk factors leading to EC development [2, 3]. Patients are often diagnosed when the disease is still limited to the uterus, what results in a good prognosis. Thus, the 5-year overall survival (OS) for EC varies from 74% to 91% in non-metastatic patients [4].

Major diagnostic and prognostic problems of EC assessment are often associated with histopathological evaluation. According to WHO classification, seven subtypes of EC can be distinguished [5]. The most common subtype of EC is an endometrial endometrioid adenocarcinoma (EEAC), classified as type I or estrogen-dependent cancer [6]. Endometrial serous adenocarcinoma (ESC) and clear cell endometrial carcinoma (ccEC), aggressive neoplasia carrying a poor prognosis, are estrogen-independent and are classified as Type II [6]. Several research teams have defined immunohistochemical and/or mutation profiles to aid in distinguishing EC subtypes [7–9]. However, classification of EC by histomorphological criteria has a limited reproducibility and is not accurate or precise enough to effectively triage patients into optimal treatment groups. Thus, there is a necessity to develop better diagnostic and prognostic markers that could improve the histopathological examination [10] and would provide more biologically informative data from endometrial tumors that could assist in planning the optimal course of treatment for the affected individuals.

The WNT family consists of 19 secreted glycoproteins which participate in organogenesis during the fetal life as well as in physiological and pathological states postnatally [11]. Intracellular signal transduction pathways regulated by WNT proteins result in cell proliferation, differentiation, migration, adhesion and change of cell polarity [11]. The conservatively preserved WNT5A (Wnt family member 5A) protein is crucial for prenatal development, and its absence causes intrauterine fetal death associated with developmental defects [12]. Alternative splicing results in the formation of WNT5A protein isoforms [13] which can fulfill both oncogenic and suppressor functions in the process of carcinogenesis [14]. Moreover, WNT5A influences the cancer stem cell population and tumor microenvironment. It is an important molecule for the regulation of cellular senescence, epithelial-mesenchymal transition (EMT), metastasis, tumor cell metabolism and chemotherapy resistance of cancer cells in various cancer types [15]. The expression of the WNT family members, including WNT5A, in EC was evaluated by Bui et al. [16]; however, they analyzed only a very small group of patients with EC. Therefore, the present study was designed and performed to analyze and compare the expression of the WNT5A gene in unchanged endometrial control and tumor samples of EC patients at both mRNA and protein levels by the use of quantitative PCR (QPCR) and immunohistochemistry (IHC), respectively. We correlated the obtained data with clinicopathological features of the patients as well as with the OS to assess whether the evaluation of WNT5A gene or protein expression can provide a new diagnostic and/or prognostic marker in EC.

Material and methods

Patients. After receiving institutional review board approval, patients were recruited at Division of Gynecology and Obstetrics and Gynecology Oncology Center at The Regional Specialist Hospital in Olsztyn, Faculty of Medicine, University of Warmia and Mazury in Olsztyn, Poland. All participants provided written informed consent for tissue and blood sample donation. The control group (n = 8) included women (age: 47 ± 7.63, mean ± SD; BMI 18.5–25, n = 4; BMI 25–30, n = 2; BMI > 30, n = 2), subjected to diagnostic curettage of the uterus and surgery, in which histopathological examination of EC and endometrial hyperplasia (hyperplasia simplex and complex, without atypia and with atypia) proved to be negative. The EC group (n = 28) included women (age: 66.95 ± 10.38, mean ± SD; BMI 18.5–25, n = 3; BMI 25–30, n = 7; BMI > 30, n = 18) with histologically diagnosed EC of the uterus, detected by diagnostic curettage of the uterine cavity and treated with surgery (hysterectomy, salpingo-oophorectomy, cytological peritoneal washing and brushing, and pelvic and aortic lymph node dissection). Diagnosis of uterine cancer and its status were established by means of clinical, laboratory and histopathological evaluation. None of the patients received any anticancer therapy before the surgery. Ongoing acute illnesses (i.e. infection, non-infectious inflammation, cardiovascular events) or disease that had occurred within the last 30 days, known active malignancy, long-term estrogen therapy, polycystic ovarian syndrome, obesity, diabetes mellitus and hypertension as well as drug or alcohol abuse, excluded patients from the study. After the surgery, data on the OS were collected for all patients.
Material collection and clinical parameters estimation. Blood samples were collected from the fasting patients before surgery. Blood morphology parameters and activated partial thromboplastin time (APTT) were measured using Cobas 6000 multianalyzer (Roche Diagnostics Ltd., Basel, Switzerland).

Tissue samples (5 × 5 × 5 mm) were taken from areas of endometrial tumors without blood-cysts, hemorrhage or necrosis which could disturb further molecular assays. Multiple sample blocks were taken from the same tumor-suspected area to compensate for eventual tumor heterogeneity. The samples were snap-frozen in liquid-nitrogen immediately after excision and stored at −80°C for mRNA isolation and further molecular studies. Tissue sections for routine diagnostic pathomorphological assessment and IHC were fixed in a 10% neutral-buffered formalin. After 12 h sections were dehydrated in a graded ethanol series (50–96%), cleared in xylene and embedded in paraffin for further processing.

Tumor phenotype classification. The criteria of TNM system following American Joint Committee on Cancer (AJCC) were adopted to establish primary tumor status [17]. Patients classified as T1a (n = 10) and T1b (n = 6), T2 (n = 7) and T3 (n = 5) were considered. Subsequently, the patients were grouped depending on: the depth of myometrium invasion as less or more than halfway (n = 12 and n = 16, respectively), uterine cervix infiltration as negative and positive (n = 14 and n = 14, respectively) and uterus-limited tumor growth or involvement of other organs by direct extension beyond the uterus (n = 23 and n = 5, respectively). Pathomorphological assessment was based on a routine light microscopy evaluation. Malignancy grade was assessed according to the International Federation of Gynecology and Obstetrics (FIGO) system [18], as low grade (G1; n = 2), moderate grade (G2; n = 19), and high grade (G3; n = 7). Grades 1 and 2 were grouped together (named further G2). The absence (n = 15) or presence (n = 13) of endometrial fibroids (myomas) were also recorded for each patient.

Total RNA isolation and Real time PCR. Endometrial tissues were homogenized using MagNA Lyser Instrument and MagNA Lyser Green Beads (Roche Molecular Systems, Inc., Pleasanton, CA, USA). Extraction of total RNA was conducted using mirVana Isolation Kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the protocol provided by manufacturers. The quality and quantity of isolated RNA was estimated with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Obtained RNA samples were stored at −80°C until further analysis. The reverse transcription was conducted according to the protocol of HighCapacity cDNA Reverse Transcription Kit (Applied Biosystem; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Quantification of WNT5A and peptidylprolyl isomerase A (PPIA) cDNA in collected samples (HS00998537_m1 and HS99999904_m1) was carried out using ABI 7500 Fast Real-Time PCR System and TaqMan® Fast Advanced Master Mix (all: Applied Biosystem; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions, and using the following conditions: polymerase activation for 20 sec at 95°C, followed by 40 cycles of denaturation at 95°C for 3 sec and annealing/extension at 60°C for 30 sec. All samples were quantified in duplicate. No template control reactions were performed for each QPCR run. Standard curves consisting of serial dilutions of the appropriate cDNA were used to control the efficiency of QPCR. Relative quantification of WNT5A expression was evaluated using the ddCt method [19]. Quantified gene had the Ct normalized against PPIA expression (dCt) as the reference [20].

Immunohistochemistry. Formalin-fixed, paraffin-embedded blocks of unchanged (control) endometrium obtained from 6 patients without EC, and fragments of tumor samples from 19 patients with EC, were cut into 5 μm-thick sections, soaked in xylene and rehydrated in a series of decreasing ethanol dilutions (96–50%). Antigen retrieval was carried out using Epitope Retrieval Solution (pH 6; Dako; Agilent Technologies Inc., Santa Clara, CA, USA), microwaving for 12 min at 97°C. Endogenous peroxide activity was blocked in a solution of 3% hydrogen peroxide in methanol for 10 min at room temperature (RT). Tissue sections were incubated with 2.5% normal horse serum (NHS; Vector Laboratories; Maravaí Life Sciences, Inc., Chicago, IL, USA) in buffer containing 0.1 M PBS and 3% bovine serum albumin (BSA; Sigma-Aldrich; Merck, Darmstadt, Germany) for 30 min at RT. Rabbit anti-human WNT5A (H-58) antibody (sc-30224; Santa Cruz Biotechnology Inc., Dallas, TX, USA), were diluted in NGS/PBS/BSA buffer (1:100). After overnight incubation at 4°C with primary antibody, slides were washed 3 times with PBS containing 0.1% Tween20 (Sigma-Aldrich; Merck, KGaA) and incubated for 30 min at RT with goat anti-rabbit antibody diluted according to the protocol provided by manufacturers (ImmPRESS Universal reagent Anti-Mouse/Rabbit Ig: Vector Laboratories; Maravaí Life Sciences, Inc.). Reaction was visualized after short incubation with 3,3’-diaminobenzidine (DAB; Liquid DAB + Substrate Chromogen System; Dako; Agilent Technologies Inc.). After counterstaining in Mayer’s hematoxylin (Sigma-Aldrich; Merck, KGaA), dehyration in series of alcohol dilutions (50–96%) and mounting in DPX medium (Sigma-Aldrich; Merck, KGaA) slides were died using XC-50 camera mounted on Olympus BX-41 (Olympus Corporation, Tokyo, Japan) light microscope by a pathologist who was blinded to the patients’ clinical data. Pictures were taken with Panoramic Digital Slide Scanner MIDI (3DHistech Ltd., Budapest, Hungary). Negative controls were obtained by incubation of sections without primary antibody.
The estimation of WNT5A staining intensity and heterogeneity. The classification of intensity and heterogeneity of the staining were conducted according to the IRS (immunoreactivity score) scale as described by Remmele and Stegner [21]. Briefly, the staining intensity was estimated as 1 (the weakest), 2 (moderate), or 3 (the most intense). Heterogeneity characterized the proportion of positively stained tumor cells was evaluated as 0 points, absence of cells with positive reaction; 1 point, 1–10%; 2 points, 1–50%; 3 points, 51–80%; 4 points, > 80% cells with positive reaction by estimation on screening wide areas within each tissue section. The obtained values of intensity and heterogeneity were multiplied and scored from 0 to 12 points. All cases with negative immunoreactivity were scored as 0.

Statistical analysis. All statistical analyses were conducted using GraphPad PRISM v. 6.0 software (GraphPad Software, Inc., San Diego, CA, USA). Comparisons of WNT5A mRNA and immunoreactivity levels in unchanged and endometrial tumor samples were conducted with Mann-Whitney tests. Statistical differences of mRNA levels between EC tissue groups in respect to: primary tumor status, tumor myometrial invasion, infiltration of the tumor on the cervix, invasion beyond the uterus, malignancy grade and presence of endometrial fibroids (myomas) were examined using Kruskal-Wallis and Mann-Whitney tests. All correlations of WNT5A gene and protein expression in both studied patient groups were calculated using a Spearman test. Survival curves were plotted according to the Kaplan-Meier method and the significance of differences in OS between groups of patients was evaluated by the log-rank test. Differences were considered to be statistically significant at p value less than 0.05.

Results

Characteristics of the patients
Demographic data and blood parameters of control and EC patients included in the study are presented in Table 1.

WNT5A expression at the mRNA level in EC
In both control and EC samples, the presence of WNT5A mRNA was observed. Samples with WNT5A expression ratio below 1.0 were considered as down-regulated and those with ratio above 1.0 us up-regulated. The WNT5A transcript level was decreased in 24/28 (85.72%) and was elevated in 4/28 (14.28%) patients with EC. The mean WNT5A gene expression was reduced in EC patients compared to control group (p < 0.0001; Fig. 1A).

WNT5A mRNA levels were significantly lower in the group of patients characterized by tumor invasion beyond the uterus when compared to the uterus-confined EC samples (p < 0.05; Fig. 1E). WNT5A expression also tended to correlate with the depth of myometrial invasion exhibiting lower transcript level in tumors that have grown deeper than halfway through the myometrium as compared to those which invaded less than the half of myometrium thickness, but this difference was not confirmed by the statistics (p = 0.06; Fig. 1C). There were no statistically significant associations between the WNT5A mRNA levels and primary tumor status (established as T1a, T1b, T2 and T3; Fig. 1B), infiltration of the tumor on the cervix (absent or present; Fig. 1D), malignancy grade (G2 and G3; Fig. 1F) and occurrence of uterine fibroids (absent or present; Fig. 1G).

The immunoreactivity of the WNT5A protein in control and EC tissue
Tissue samples of 6 control and 19 EC patients previously analyzed for their WNT5A mRNA expression levels were subjected to IHC in order to assess the immunorepression of WNT5A protein in the normal endometrium and EC cancer cells.

Representative microphotographs of IHC presenting the location of WNT5A protein in the control endometrium and tumor tissue of EC patients are

<p>| Table 1. Demographic data and blood parameters of the studied cohort of patients |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Unit</th>
<th>Control</th>
<th>Endometrial cancer</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47 ± 7.63</td>
<td>66.95 ± 10.38</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.88 ± 5.92</td>
<td>31.39 ± 5.47</td>
</tr>
<tr>
<td>RBC (10³/μl)</td>
<td>4.57 ± 0.50</td>
<td>4.77 ± 0.45</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>12.16 ± 1.89</td>
<td>13.86 ± 1.36</td>
</tr>
<tr>
<td>Ht (%)</td>
<td>38.28 ± 4.63</td>
<td>41.88 ± 3.17</td>
</tr>
<tr>
<td>WBC (10³/μl)</td>
<td>7.18 ± 1.60</td>
<td>7.36 ± 2.14</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>63.59 ± 7.10</td>
<td>60.32 ± 8.74</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>61.34 ± 8.08</td>
<td>57.95 ± 9.26</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>1.6 ± 0.73</td>
<td>1.94 ± 1.28</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.52 ± 0.26</td>
<td>0.43 ± 0.26</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>28.46 ± 6.07</td>
<td>30.46 ± 7.83</td>
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<tr>
<td>Monocytes (%)</td>
<td>5.79 ± 1.06</td>
<td>6.49 ± 1.81</td>
</tr>
<tr>
<td>Platelets (10³/μl)</td>
<td>296.4 ± 78.52</td>
<td>229.9 ± 60.26</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>10.5 ± 0.96</td>
<td>10.93 ± 0.78</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>26.47 ± 1.75</td>
<td>27.49 ± 3.61</td>
</tr>
</tbody>
</table>

The results are presented as means ± SD. Abbreviations: BMI — Body Mass Index; RBC — Red Blood Cells; Hb — Hemoglobin; Ht — Hematocrit; WBC — White Blood Cells; MPV — Mean Platelet Volume; APTT — Activated Partial Thromboplastin Time.
shown in Figure 2. The figures presenting negative control samples were not shown.

The histologically-unchanged endometrial fragments showed dominant, strong, homogenous positive WNT5A immunoreactivity in the cytoplasm and, sporadically, in the nuclei of epithelial cells, whereas in tumor tissue, WNT5A protein staining were mainly present in the nuclei of cancer cells. When control and EC tissue sections were compared, qualitative differences between the immunoreactivity in cytoplasmic and nuclear compartments were observed (Fig. 2). However, there were no quantitative alterations of
WNT5A expression in endometrial cancer

WNT5A immunoreactivity in cytoplasm and nuclear compartments between the unchanged endometrium and EC samples (p > 0.05; Fig. 3 A and B, respectively).

WNT5A protein immunoreactivity was measured on the basis of the IRS scale in the cytoplasmic and nuclear compartments of EC cells. It did not correlate with clinicopathological characteristics of the patients (data not showed).

**Correlations between WNT5A mRNA levels and WNT5A protein immunoreactivity in the cytoplasm and nuclei of tumor cells**

A negative correlation was observed between WNT5A mRNA expression and protein immunoreactivity in the cytoplasm of EC cells (Rho = -0.55; p < 0.05). In contrast, a positive correlation was observed between the WNT5A transcript levels in EC tumor samples and...
WNT5A immunoreactivity in the nuclei of cancer cells (Rho = 0.60; p < 0.01).

**Correlations between blood parameters and WNT5A mRNA expression levels and protein immunoreactivity in EC specimens**

In patients with EC, WNT5A gene expression correlated positively with the count of leukocytes (WBCs; p < 0.05) and the percentage of granulocytes (p < 0.05), and negatively with the percentage of lymphocytes in blood (p < 0.5; Table 2).

In patients with EC, we observed negative correlation between the cytoplasmic immunoreactivity of the WNT5A protein and percentage of granulocytes (p < 0.01) or neutrophils (p < 0.01) in blood when the protein in the cytoplasm of EC cells was concerned (Table 2). The percentage of lymphocytes in blood positively correlated with the immunoreactivity of WNT5A in the cytoplasm of EC cells (p < 0.01; Table 2). Mean platelet volume (MPV) was the only blood parameter which positively correlated with WNT5A protein immunoreactivity in the nuclei of EC cells (p < 0.05). No correlations with age, BMI as well as or other estimated blood parameters such as RBC, WBC and platelets counts, types of leukocytes, hemoglobin (Hb), hematocrit (Ht), MPV and APTT, were found when nuclear localization of WNT5A was considered in EC patients (Table 2).

**Correlations between WNT5A gene and protein expression and OS of patients with EC**

The evaluation of the WNT5A gene expression level as a prognostic factor in the studied group of the patients with EC was assessed after the follow up of patients (n = 28) and the median observation period was 59 months. During the observation period 25% (n = 7) patients died. We found a significant correlation between the tumor grade and OS of the patients (p < 0.001; Fig. 4A).

In order to investigate associations between OS and WNT5A expression EC specimens were divided into the respective cohorts. Based on the median WNT5A mRNA relative expression (RQ = 0.495) the patients were divided into two groups regarded as < 0.495 (n = 14) and > 0.495 (n = 14). Consequently, the patients were dichotomously divided on the basis of their median value for the cytoplasmic (IRS = 4) or nuclear (IRS = 6) WNT5A immunoreactivity score. We found that the expression level of WNT5A mRNA (Fig. 4B) as well as the intensity of cytoplasmic or nuclear WNT5A immunoreactivity (Fig. 4C and D, respectively) did not correlate with patients’ OS.

**Discussion**

There is an urgent need for intense research in the field of molecular biology of endometrial tumors in order to detect the molecular markers of carcinogenesis process. The present study is the first that used both QPCR and IHC techniques to establish WNT5A expression at the transcript and protein levels in the EC tissue. Our study demonstrated that WNT5A mRNA is down-regulated in the tumor samples as compared to normal endometrial tissue fragments. These findings support the study of Bui et al. [16] who investigated the expression of seven genes belonging to the WNT family in neoplastically-altered and normal endometrium by ribonuclease protection assay. Their results suggested reduced level of WNT5A expression in EC tumor tissue and human EC cell lines [16]. Unfortunately, the study of Bui et al. involved only four patients with EC and 11 control cases and its findings were neither supported by statistical analysis nor the correlations between WNT5A expression and clinico-pathological parameters [16]. Therefore, our research was carried out on a larger cohort of patients and is the first study which shows associations between the reduced WNT5A expression and increasing invasive properties of EC tumors.

In our cohort of the patients with EC the WNT5A gene expression was markedly reduced in the tumors that spread outside the uterus as compared to the organ-confined cases. Our results suggest that

<table>
<thead>
<tr>
<th>Blood Parameters</th>
<th>WNT5A mRNA</th>
<th>WNT5A Protein</th>
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<tbody>
<tr>
<td>Age</td>
<td>−0.22</td>
<td>0.18</td>
</tr>
<tr>
<td>BMI</td>
<td>0.31</td>
<td>−0.39</td>
</tr>
<tr>
<td>RBC</td>
<td>−0.01</td>
<td>−0.19</td>
</tr>
<tr>
<td>Hb</td>
<td>−0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>Ht</td>
<td>0.24</td>
<td>0.05</td>
</tr>
<tr>
<td>WBC</td>
<td>0.39*</td>
<td>−0.36</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>0.42*</td>
<td>−0.66**</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.32</td>
<td>−0.61**</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>−0.03</td>
<td>−0.04</td>
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<tr>
<td>Basophils</td>
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<td>0.12</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>−0.38*</td>
<td>0.67**</td>
</tr>
<tr>
<td>Monocytes</td>
<td>−0.09</td>
<td>0.22</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.10</td>
<td>0.006</td>
</tr>
<tr>
<td>MPV</td>
<td>0.30</td>
<td>−0.07</td>
</tr>
<tr>
<td>APTT</td>
<td>−0.09</td>
<td>−0.18</td>
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Abbreviations as in the legend to Table 1. Asterisks indicate statistical significance; *p < 0.5; **p < 0.01.
Figure 4. Analysis of the overall survival (OS) of patients with endometrial cancer (EC). Kaplan-Meier survival curves of EC patients relating to (A) the histological grade of EC tumor (n = 28), (B) levels of WNT5A mRNA (n = 28), (C) cytoplasmic (n = 19) and (D) nuclear (n = 19) immunoreactivity of the WNT5A protein. P-values of log-rank test are shown.

decreased WNT5A mRNA level could also correlate with the depth of invasion through myometrium. Although these findings could suggest tumor suppressor role of WNT5A in EC, we did not disclose any significant relationship between its expression levels and overall survival of the patients. Indeed, the precise status of the WNT5A gene as a factor that might have influenced cancer progression has not been established yet and the opposing observations in other human tumors imply that role of WNT5A protein could be tissue- and/or cancer-specific [14]. In the female reproductive system the decreased expression of WNT5A was documented in epithelial ovarian cancer and this reduction was attributed to the hypermethylation of the gene promoter [22]. In contrast to the latter study, Peng et al. found that WNT5A immunoreactivity was increased in the ovarian cancer cells as compared to benign tumors and normal ovarian tissue [23]. Furthermore, elevated level of WNT5A protein was associated with shorter overall and progression-free survival of the patients with ovarian cancer [23]. Also in patients with cervical cancer WNT5A expression was found to be either decreased [13] or increased [24]. Using QPCR and IHC techniques Lin et al. [24] showed that the expression of WNT5A mRNA was up-regulated in the primary cervical tumors and WNT5A immunoreactivity in cancer cells correlated with the occurrence of distant metastases and worse prognosis. These discrepancies may be partially explained by the findings of Bauer et al. [13] who demonstrated that the tumor-promoting or tumor-suppressing role of WNT5A in cervical cancer can be attributed to the differential expression of two WNT5A isoforms that have distinct effects on cancer-relevant signaling pathways.

In our study the immunoreactivity of the WNT5A protein was observed not only in the cytoplasm but also in the nuclei of EC cells and, to a lesser extent, in some epithelial cells of the control endometrial tissues. Interestingly, WNT5A transcription can result in the synthesis of two different isoforms, long (380 amino-acids; The Consensus CDS accession number: CCDS46850) and short (365 amino-acids; CCDS58835) [25] that could be characterized by distinct biological activity [13]. The results of in silico analysis of their amino-acid sequences using nuclear localization signals (NLS) mapper [26] recognized a single putative NLS within the N-terminus of long WNT5A isoform and lack of it in the short isoform. Basing on NLS prediction it could be assumed that localization of WNT5A immunoreactivity within the cell may differ depending on the type of expressed
isoform, with long isoform being directed both to the cytoplasm and nucleus and short isoform expressed exclusively in the cytoplasm [26]. Therefore, we suggest that the changes in the subcellular localization of WNT5A immunoreactivity are associated with the altered expression pattern of WNT5A isoforms as it was demonstrated in other human cancers such as cervical cancer, breast cancer and neuroblastoma [13]. The QPCR assay used in our study did not discriminate between the mRNA variants encoding for long and short WNT5A isoforms. In our immunohistochemical study, we could detect both WNT5A protein isoforms, since the applied primary antibody recognizes 23–80 aa of the long isoform as well as 15–72 aa of the short WNT5A isoform, respectively. Thus, further studies aiming to determine the expression of WNT5A isoforms in EC samples could clarify whether the possible role of this protein in EC pathogenesis is isoform-specific. Furthermore, our results showed that WNT5A mRNA correlated negatively with cytoplasmic and positively with nuclear WNT5A immunoreactivity levels in EC cells. We believe that the latter observation could be, at least partially, also elucidated by the analysis of WNT5A expression with respect to the expressed variants of transcript or isoforms.

Our study revealed that WNT5A mRNA and immunoreactivity levels correlated with preoperative counts of white blood cells and MPV. Since the correlations with granulocytes and lymphocytes count were found for the cytoplasmic but not nuclear immunoreactivity of WNT5A, its role as secretory molecule and modulator of immune processes can be considered [15]. Other authors have shown that biomarkers of inflammation such as elevated neutrophil to lymphocyte ratio or platelet to lymphocyte ratio are significant prognostic factors in EC and correlate with worse prognosis [27]. In our study the lower cytoplasmic immunoreexpression of WNT5A was positively associated with lymphocyte and negatively with neutrophils counts. This relationships could indicate the existence of direct or indirect links between the WNT5A expressed in endometrial tumor and inflammatory processes. Indeed, WNT5A protein have been considered tumor-derived immunomodulator that has been shown to influence chemotactic cell migration in ovarian cancer cell line SKOV-3 [28] and promote differentiation of regulatory T-cells by activation of dendritic cells in melanoma [29]. Interestingly, it was demonstrated that WNT5A potentiated thromboxane A2 mimetic-induced platelet aggregation and therefore the secretion of this protein by circulating cancer cells might promote metastatic progression suggesting that WNT5A can promote tumor progression by its interaction with the platelets [30]. It cannot be excluded that in EC, WNT5A has analogous functions to those reported in the aforementioned studies [28–30]. However, further experiments are needed to evaluate its importance in establishing of tumor microenvironment and blood-borne metastasis.

In conclusion, the investigations of the WNT5A expression in the endometrial cancer suggest that this signaling molecule could function in the development of this malignancy as tumor suppressor and/or modulator of immune response and could be considered as a potential marker in EC. However, the precise role of WNT5A in the pathogenesis of endometrial cancer must be elucidated in further experiments.

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References
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