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RESEARCH ARTICLE

Assessment of Expression of Homeobox A5 in Endometrial Cancer on the mRNA and Protein Level

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Abstract: ***Background*:** Endometrial cancer is one of the most common gynecological cancer in the developed countries and occurs mainly in postmenopausal women. Angiogenesis is important for cancer formation as it provides nutrients for growing tumor mass. Most tumors do not show detectable Homeobox A5 (HOXA5 level), suggesting its potential role as a cancer suppressor. It was demonstrated that HOXA5 is involved in the progression of various types of cancer and the loss of its expression correlates with higher pathological grade and poorer outcome.

***Objective*:** The aim of the study was to evaluate HOXA5 expression at transcriptome and protein levels.

***Materials and Methods*:** The study enrolled 45 women diagnosed with endometrial cancer and 15 without neoplastic changes. The histopathological examination allowed us to divide cancer tissue samples according to the degree of histological differentiation: G1, 17; G2, 15; G3, 13. The expression of the HOXA5 protein was determined by immunohistochemistry. Microarray and RT-qPCR techniques were used to assess *HOXA5* expression at the mRNA level.

***Results*:** The reaction to the HOXA5 protein was only visible in glandular cells in G1 endometrial cancer and was lower compared to the control. In grades 2 and 3, reactions were noted at the limit of the method's sensitivity. In addition, reduced *HOXA5* expression was observed at the transcriptome level.

***Conclusion*:** HOXA5 may become a potential complementary molecular marker, allowing early detection of neoplastic changes in the endometrium. It also seems that detection of HOXA5 at the mRNA and protein levels may be helpful in improving the accuracy of diagnosis and planning effective oncological therapy.

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1. INTRODUCTION

Endometrial cancer is one of the most common gynecological cancer in the developed countries and occurs mainly in postmenopausal women [1]. It can be divided into three grades [G1-G3] according to the degree of histological differentiation, as recommended by the Federation of Gynecology and Obstetrics (FIGO). G1 demonstrates ≤5% solid growth pattern, G2 shows between 6 and 50% solid growth pattern, while in G3 >50% solid growth pattern is observed [2].

The homeobox family is a highly conserved group of morphoregulatory genes that, in a coordinated manner, activate or suppress the expression of many genes. It participates in embryonic development, cell differentiation [3, 4], the occurrence of malignant tumors [5], as well as controls angi-

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ogenesis in the complex tumor microenvironment. Homeobox A5 (HOXA5), belonging to the HOXA protein family, participates in the development and progression of tumor [6, 7]. In contrast to *HOX3*, the expression of *HOXA5* gene is observed in inactive vessels, whereas it disappears in the vessels associated with the tumor [8]. Forced expression of HoxA5 in activated, cultured vascular endothelial cells leads to a decrease in the proangiogenic receptor (VEGFR2) level with the simultaneous increase in the expression of antiangiogenic factor thrombospondin-2 [9, 10]. In addition, in cultured vascular endothelial cells, HoxA5 reduces migration and endothelial permeability by stabilizing the adherens junctions [11]. It was demonstrated that HOXA5 expression is significantly downregulated in esophageal squamous cell carcinoma and correlates with a clinical-stage (TNM), tumor size, and lymph node metastases [12]. Furthermore, the loss of HOXA5 resulted in the limitation of p53 expression in human breast tumors [13]. Reduction of HOXA5 expression was reported in 7 out of 10 cases of breast cancer in women, indicating a correlation of its expression with disease progression. It is also worth paying attention to the role of HOXA5 as an indicator of precancerous changes in the ovaries, contributing to the ovarian epithelial inclusion cysts. These observations suggest that the gene encoding HOXA5 acts as a suppressor gene. Reduced HOXA5 expression in biopsies of colorectal tumors correlates with elevated levels of nuclear β-catenin, and therefore HOXA5 has an important role in signal transduction of the WNT/β-catenin pathway. The critical role of HOXA5 in the said cascade is supported by the fact that HOXA5 expression is observed in developing lungs [13, 14]. All these findings indicate that HOXA5 may have a high therapeutic potential, reducing tumor angiogenesis and stabilizing hyperpermeable tumor vascularization.

The aim of the study was to evaluate HOXA5 expression at transcriptome and protein levels using immunohistochemistry, microarray and RT-qPCR techniques.

2. MATERIALS AND METHODS

The material for the study included endometrial tissue samples collected from patients who had undergone a hysterectomy. The study group consisted of 45 women diagnosed with endometrioid endometrial cancer, while 15 women without neoplastic changes constituted a control group. The groups of patients were characterized by age, height, weight and BM, which were described as mean ± standard deviation: (C = 54.53 ± 10.41 years, 1.62 m ± 0.07 m, 73.79kg ± 19.22kg, 28.21 ± 7,91 - overweight; G1 = 66.64 ± 6.07 years, 1.61 m ± 0.04 m, 74.38kg ± 14.32kg, 28.77 ± 5.73 - overweight; G2 = 67.4 ± 11.89 years, 1.57 m ± 0.05 m, 84.77kg ± 25.63kg, 34.63 ± 11.63 - class I obesity; G3 = 63.38 ± 8.16 years, 1.58 m ± 0.06 m, 83.15 kg ± 10.16kg, 33.87 ± 4.87 - class I obesity). The criteria for exclusion from the study group included non-endometrioid endometrial cancer, coexisting cervical cancer, adenocarcinoma with squamous elements, diagnosed endometriosis or adenomyosis, extreme obesity [BMI > 40] and the use of hormone therapy in 24 months prior to surgery. The histopathological examination allowed to divide the study group according to the degree of histological differentiation: well-differentiated (G1; <5% solid cancer) 17 patients, moderately differentiated (G2; 6-50% solid cancer) 15 patients and poorly differentiated (G3; >50% solid cancer) 13 patients. Approval for the study was obtained from the Bioethical Committee of the Medical University of Silesia, no. KNW/0022/KB/237/16.

The paraffin blocks were provided by the Laboratory of Pathomorphology of Beskid Center of Oncology in Bielsko-Biała. Rabbit anti-HOXA5 polyclonal antibody (Novus Biological) was used to perform immunohistochemical staining of the prepared slides. Antigens were retrieved by incubating slides in citrate buffer (10 mM, pH 6.0) at 95°C for 30 min in a water bath and then cooling for 30 min. The next step included blocking the endogenous peroxidase activity with 0.3% (v/v) hydrogen peroxide and 0.1% NaN_3 in PBS for 10 min. To block non-specific antibody binding sites, 1% BSA solution in PBS was used for 30 min at room temperature. The solution was then removed and the primary antibody was applied to the slides. The incubation was carried out for 20h at 4°C. Biotinylated secondary antibodies were applied to the slides, followed by avidin-biotinylated peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories). Diaminobenzidine (DAB) was used to visualize the bound antibodies, according to the manufacturer's instructions. The slides were then stained with Gill's hematoxylin, dehydrated, and cover-slipped. Negative controls consisted of slides in which the primary antibody was replaced with rabbit IgG at the same concentration as the primary antibody. Nikon Eclipse E200 light microscope with Nikon DS-Fi1 digital camera was used to examine the prepared slides and take pictures under 200x magnification. In total, 15 photos were taken for each paraffin block (3 slides, 5 photos for each of them). In each positively stained cell, the intensity of staining was measured as the optical density of the reaction product. To calculate the average optical density, the image analysis program NISAR (Nikon) was used.

The expression profile of *HOXA5* was also evaluated at the mRNA level using microarray technique (HG-U133A; Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. The procedures were explained in detail in our previous paper [15]. In addition, the real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was used to validate the results of *HOXA5* expression obtained by the microarray technique. It was carried out using SensiFAST SYBR No-ROX One-Step Kit (Bioline, London, UK) and Opticon^{TM} DNA Engine Sequence Detector (MJ Research Inc., Watertown, MA, USA) as recommended by the manufacturer. *18S rRNA* was used as an endogenous control. The reaction was carried out using the following primer pairs: *HOXA5* (forward: 5' TGACTAGTGACTCTGTGATG 3', reverse: 5' CACAGTTTGCTTAAAACAGC 3'), *18S rRNA* (forward: 5' CGGACAGGATTGACAGATTGA 3', reverse: 5' GCCAGAGTCTCGTTCGTTAT 3'). The RT-qPCR thermal profile included: reverse transcription (45ºC for 10 min), polymerase activation (95ºC for 2 min), 40 cycles including denaturation (95ºC for 5 s), annealing (60ºC for 10 s) and elongation (72ºC for 5 s).

Statistical analysis was carried out using the Statistica 13.0 PL software (StatSoft, Cracow, Poland). The normality of the data distribution was confirmed with the Shapiro-Wilk test. Then, the one-way ANOVA was conducted. The values obtained using immunohistochemistry are presented as mean ± standard deviation.

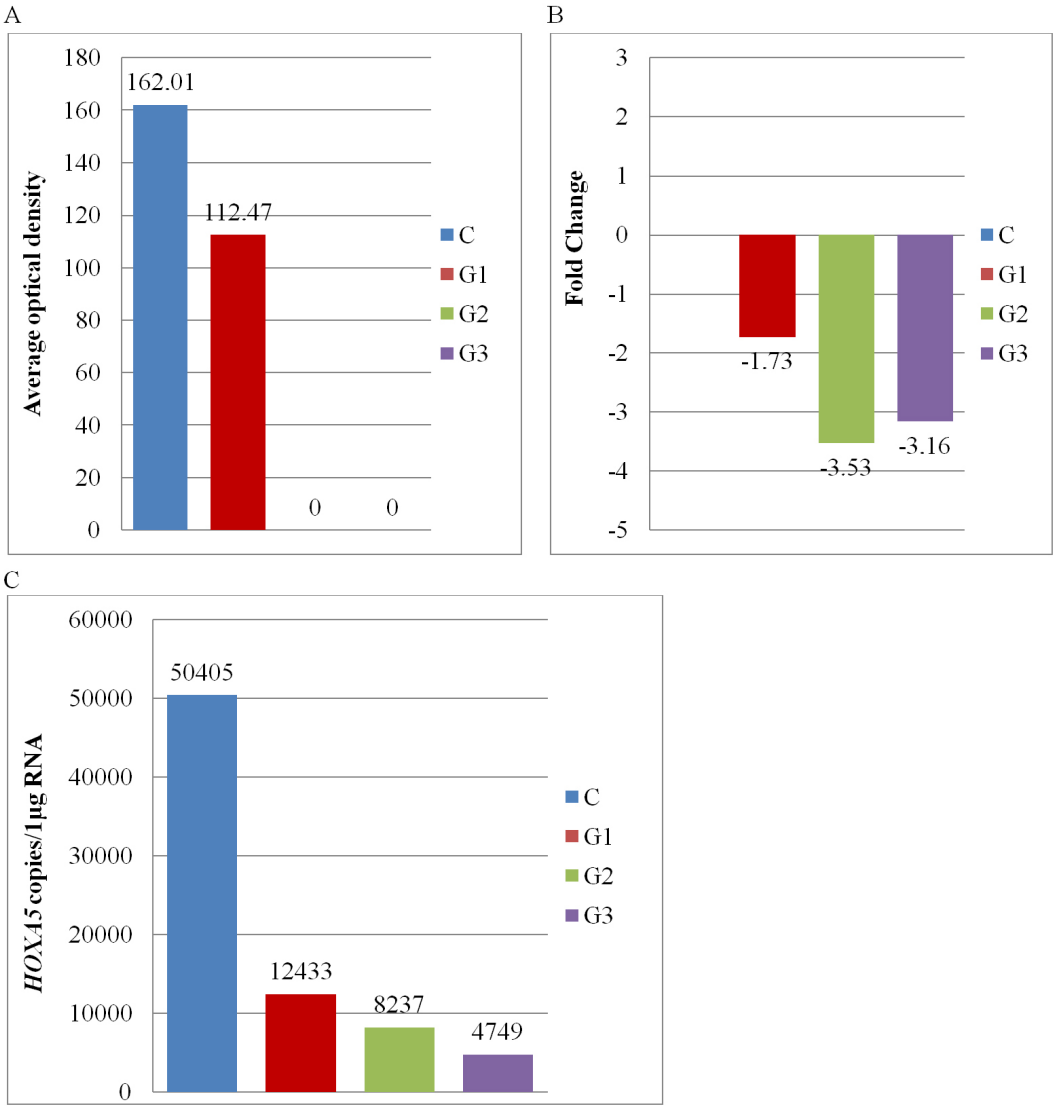

Fig. (1). The results of HOXA5 expression at protein (**A**) and mRNA levels (**B-C**). C, control; G1-G3, grades of endometrial cancer.

The microarray data was analyzed using GeneSpring 13.0 software (Agilent Technologies, Inc., Santa Clara, CA, USA) and DNA Microarray Integromics analysis platform PL-Grid Infrastructure (http://www.plgrid.pl/en).

3. RESULTS

Table **1** and Fig. (**1 A-C**) show the results of HOXA5 expression at both mRNA and protein levels. HOXA5 protein was well expressed in the control samples. The reaction to this protein was only visible in glandular cells (Fig. **2**). It was found that HOXA5 protein expression appeared only in G1 endometrial cancer and was lower by about 31% compared to the control. In the remaining grades, G2 and G3, reactions at the limit of the method's sensitivity were observed. Taking into account the microarray data, it can be observed that regardless of endometrial cancer grade, *HOXA5* level was reduced compared to control. The obtained results showed that as the cancer grade increases, *HOXA5* transcriptional activity decreases (from -1.73 in G1 to -3.16 in G3). The last part of the molecular analysis was RT-qPCR to validate the *HOXA5* microarray profile. The results confirmed the direction of changes in the *HOXA5* level determined during microarray analysis ($p<0.05$). However, the differences in the *HOXA5* expression pattern observed between G2 and G3 cancer in RTqPCR appear to be higher compared to microarray analysis (Table **1**, Fig. **1**). Fig. (**3**) shows the melting curve for *HOXA5*, which confirms the specificity of RTqPCR.

4. DISCUSSION

Angiogenesis is a complex, multistep process not only important for embryonic development, wound healing, but also cancer formation as it provides nutrients for growing tumor mass [11]. Cancer can activate angiogenesis through multiple signaling pathways and mediators [16]; therefore it is important to carry out studies to identify new molecular markers and therapeutic targets.

Table 1. Expression of HOXA5 at the protein and mRNA levels.

Group	Protein		mRNA			
			Microarrays		RT-qPCR [*HOXA5* Copies/1µg RNA]	
	M	SD	FC	Up/Down	M	SD
C	162.01	13.16	-	-	50405	2369
G1	112.47*	9.84	-1.73	down	12433*	385
G2	-	-	-3.53	down	8237*	720
G3	-	-	-3.16	down	4749*	535

M, mean; SD, standard deviation; FC, fold change; C, control; G, grade of endometrial cancer. *$p<0.05$ *vs*. C group.

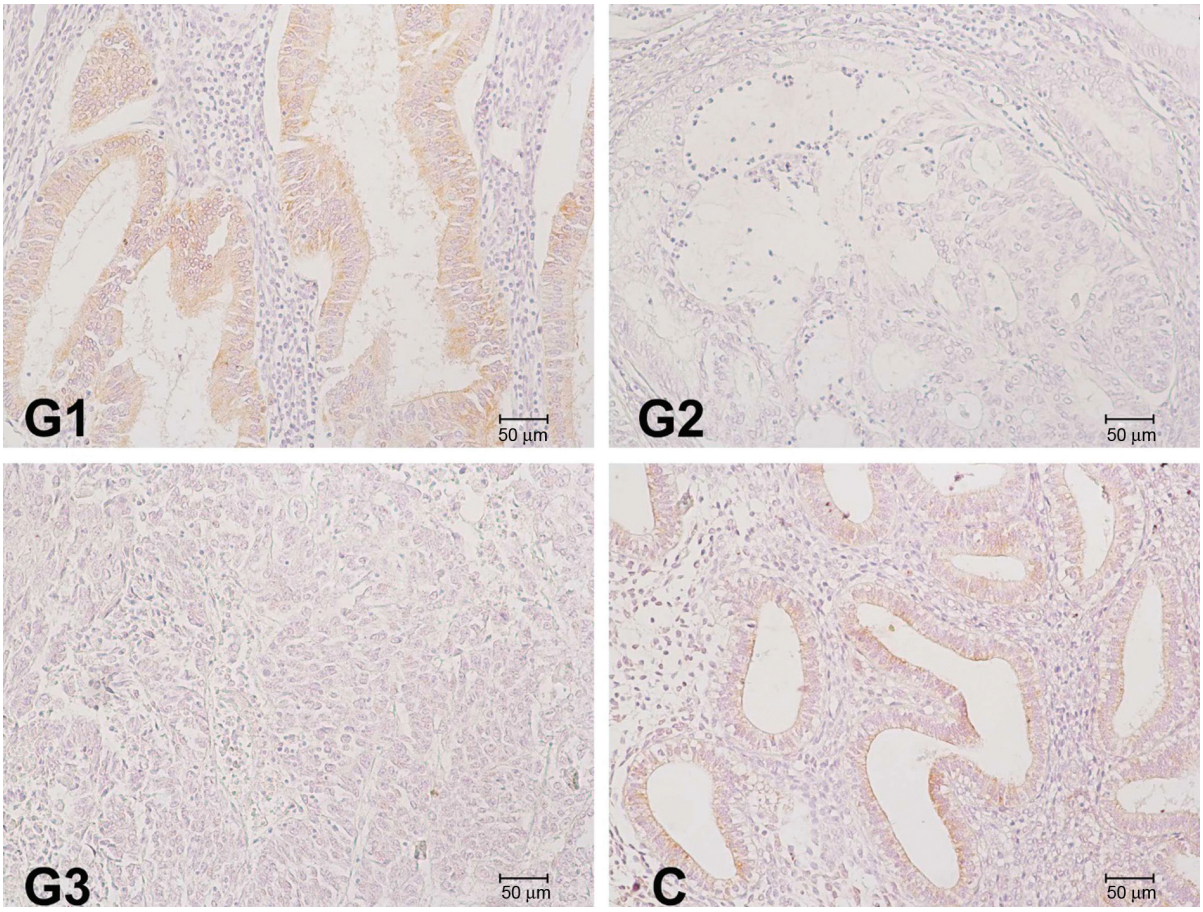

Fig. (2). Immunohistochemical localization of HOXA5 protein in different grades of endometrial cancer and control. C, control; G1-G3, grades of endometrial cancer. Positive reaction - brown color. 200x magnification.

Most tumors do not show detectable HOXA5 levels, suggesting its potential role as a cancer suppressor. It was demonstrated that HOXA5 is involved in the progression of various types of cancer and the loss of its expression correlates with higher pathological grade and poorer outcomes [17]. A decreased level of HOXA5 was found in breast, colorectal, prostate cancer, as well as in gastric cancer cells [18-21]. In our study, HOXA5 expression was determined at both mRNA and protein levels, which gave the opportunity to determine changes in its level at two levels of genetic information flow. In addition, a similar expression pattern of *HOXA5* results from two independent techniques realistically reducing the risk of false-positive and false-negative results. We observed a statistically lower level of HOXA5 protein in G1 endometrial cancer than in the control group. In turn, HOXA5 expression was not found in biopsies of G2 and G3 endometrial cancer. The relatively high inhibition of HOXA5 expression seems to indicate that as the cancer grade increases, the cells present different metabolism, protein expression and triggered signal cascades [22]. It is there-

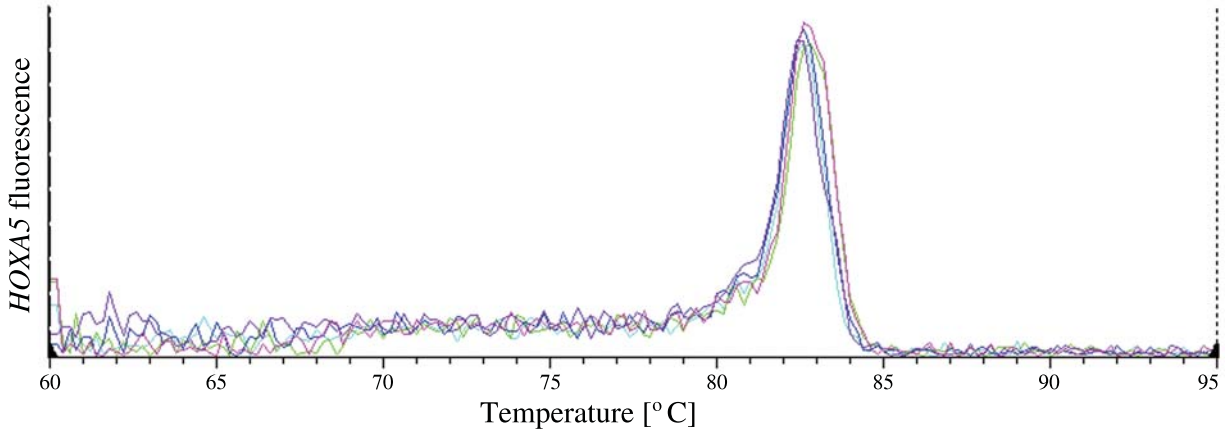

Fig. (3). *HOXA5* melting curve that confirms the specificity of RT qPCR. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

fore important to evaluate variances in HOXA5 expression in different grades of endometrial cancer. In addition, it should be remembered that the process of neoplastic transformation is extremely dynamic and varies to some extent in different types of cancer [23]. The study of Ordóñez-Morán *et al.* using IHC staining in colorectal cancer showed a low level of HOXA5 in stem cells and high expression in differentiated villi, suggesting that HOXA5 is strongly associated with regulation of metastatic potential. They also indicated that HOXA5 could be a promising target for a new modern therapeutic strategy [24]. It is possible that the expression of HOXA5 observed in our analysis may suggest that as the cancer grade increases, the pool of stem cells forming the tumor microenvironment increases and/or the cells forming the tumor mass and its microenvironment express genes and proteins characteristic of the stem cells. It would confirm an increase in metastatic potential with a decrease in the degree of endometrial cancer differentiation. Therefore, our observations may be a premise to continue research on the significance of stem cells in endometrial cancer.

It should be noted that epigenetic mechanisms regulating gene expression and signaling pathways play an important role in carcinogenesis [25, 26]. Watson *et al.* suggested that one of the likely reasons for the reduction of *HOXA5* expression at the mRNA level could be hypermethylation of the *HOXA5* gene promoter sequence. In addition, the simultaneous excessive methylation of the *HOX* genes, *p16* and *MGMT* leads to an increasing number of aberrant cells at the stage of tumor stage [27]. In turn, Li *et al.* analyzed changes in the methylation profile of *HOXA2, HOXA5, HOXA6* in colorectal cancer. The highest methylation level was observed for *HOXA2* and *HOXA5* in the first, non-invasive stage of this cancer. They observed that the expression of three *HOXA* isoforms was downregulated [28]. Methylation has been described as an extremely important epigenetic modification during the early stages of tumorigenesis, which probably determines the metastasis potential of cancer cells [29]. Our observations are in line with the findings of Wang *et al.* who showed a decrease in HOXA5 expression using RT-qPCR and Western blot. They also pointed out that HOXA5 may be a factor inhibiting the development of cervical cancer, which is most likely associated with the function of HOXA5 as a regulator of the AKT/p27 pathway [30].

Taking into account the role of HOXA5 as a cancer suppressor [17] and our results obtained using microarrays, RT-qPCR and IHC staining, it appears that G2 and G3 cancer cells have a higher proliferate potential compared to G1 samples. In studies on invasive breast cancer, it was reported that HOXA5 is expressed in physiological vessels, but not in angiogenic vessels associated with cancer [31]. Cuevas *et al.* demonstrated that HOXA5 expression in endothelial cells significantly reduced angiogenesis during cancer progression and inhibited transition into the dysplastic phenotype, accompanied by an increase in thrombospondin-2 level and a decrease in VEGFA expression [10]. In order to limit the progression of cancer, an antiangiogenic approach focused on the restoration of HOXA5 expression may be proposed.

In this study, HOXA5 protein expression has not been observed in G2 and G3 endometrial cancer samples, which may be due to increasing the role of post-transcriptional regulation. One of the widely described mechanisms involved in gene expression changes is microRNA molecules (miRNAs). Through sequence-specific silencing, they affect processes such as cell differentiation and regulation of signal cascades [32]. Their importance is emphasized especially in the context of the neoplastic transformation [33, 34] and molecular marker systems [35]. Liu *et al.* reported that miR-196a suppressed the expression of HOXA5 in non-small cell lung cancer at both mRNA and protein levels, leading to the promotion of cell proliferation, migration and invasion [36]. Therefore, it seems likely that with the advancement of endometrial cancer, the activity of miRNAs increases, which results in the lack of HOXA5 expression. Finding the HOXA5 protein only in G1 endometrial cancer highlights two important aspects. First, it will allow early detection of neoplastic changes of the endometrium, increasing the possibility of using HOXA5 as a complementary molecular marker. Secondly, a statistically significant difference in the HOXA5 protein level between grade 1 samples and control may indicate that miRNAs potentially regulating HOXA5 expression at an early stage of endometrial cancer development are silenced [37].

It should be noted that the analysis carried out in this study allows a comprehensive assessment of changes in HOXA5 expression in endometrial cancer biopsies. The

strengths of our research include the use of modern and complex molecular biology methods. In addition, the inclusion and exclusion criteria are very strict, which allowed to create a homogeneous group and obtain reliable results. Changes in the expression profile of *HOXA5* determined using microarrays were confirmed by RT-qPCR. Admittedly, it may seem that the size of individual groups [C, G1-G3] is relatively small, but it is associated with study eligibility criteria. The molecular analysis carried out does not involve additional burden for patients, as tissue samples are taken as a standard for histopathological examination [38]. The extension of the analysis to recognize changes in the expression of selected factors in the context of endometrial cancer will allow the classification of obtained biopsies to the appropriate cancer grade [G1-G3]. This is due to the fact that molecular changes are ahead of phenotypic changes [39].

CONCLUSION

In summary, the present study at the protein and mRNA level indicates an important role of HOXA5 expression in endometrial cancer. Our results and observations of other researchers have suggested that HOXA5 expression is lowered regardless of the cancer type. Epigenetic mechanisms, *i.e*. DNA methylation and miRNAs, seem to play a key role in HOXA5 expression regulation. Furthermore, it can be assumed that the expression pattern of HOXA5 in endometrial cancer samples suggests that as the cancer grade increases, the percentage of stem cells forming the tumor mass and microenvironment and/or cells that express stem cell markers also increases. Therefore, a thorough analysis is necessary.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval of the Bioethical Committee of the Medical University of Silesia, no. KNW/0022/KB/237/16 has been obtained for this study.

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

CONSENT FOR PUBLICATION

Informed consent was obtained from all of the patients recruited.

AVAILABILITY OF DATA AND MATERIALS

The data used to support the findings of this study are included within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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All authors were responsible for the concept and design of the study, collection and collation of data, analysis and interpretation of data, writing of the article, reviewing, and final reviewing of this article and graphics performance. We thank Wojciech Peszek for comments that greatly improved the manuscript.

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