Promoter hypermethylation of the tumor-suppressor genes *RASSF1A*, *GSTP1* and *CDH1* in endometrial cancer

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Abstract. Endometrial cancer is a common gynecological malignancy with a good prognosis in early stages of the disease. The CpG island in the promoter region of tumor-suppressor genes are frequently methylated in various types of human cancers. In the present study, we examined the methylation status of the *GSTP1*, *CDH1* and *RASSF1A* genes in endometrioid endometrial cancer (EEC), endometrial complex hyperplasia (EHP) and healthy endometrium with the aim to identify correlations between promoter hypermethylation, disease risk and clinicopathological parameters. A nested two-stage methylation-specific PCR (MSP) was performed to analyze the promoter CpG methylation status of *GSTP1*, *CDH1* and *RASSF1A* genes in the population studied. A total of 92 subjects were initially included in the study of which 41 EEC, 19 EHP and 20 controls were processed for final analyses. A significant difference was found between the study groups and the presence of promoter CpG hypermethylation status in the *GSTP1* ($P<0.05$) and *RASSF1A* ($P<0.0001$) genes. *RASSF1A*, *GSTP1* and *CDH1* gene promoter methylation was present in 85.4, 68.3 and 31.4% of EEC samples when compared to that in the controls with 30.0, 35.0 and 20.0%, respectively. CpG methylation of all three investigated tumor-suppressor genes was found in 12.2% of EEC patients, in 4.2% of EHP patients and in 3.7% of the controls, respectively. Positive findings for the promoter methylation of two investigated genes were found in 48.7% of EEC patients, 26.0% of EHP patients and in 18.5% of the controls. With regard to histopathological variables and CpG methylation, we found significant correlations between the *RASSF1A* and *GSTP1* genes and higher tumor grade, deeper myometrial invasion and positive metastatic involvement of pelvic lymph nodes. No associations were noted between promoter hypermethylation of the *CDH1* gene and biological features of the endometrial cancer cases. The results indicate that aberrant CpG methylation of the promoter region in the *GSTP1* and *RASSF1A* tumor-suppressor genes is an important event in carcinogenesis of endometrial cancer and may have an impact on tumor aggressiveness. Finally, the present study suggests that epigenetic alterations may be of diagnostic value for the better clinical management of premalignant endometrial lesions.

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Introduction

Endometrial carcinoma is the most common malignancy of the female reproductive tract and is the fourth most common cancer among women in Europe (1) and the second most common cancer among women in Slovakia (2). Contrary to its high incidence, the mortality rate is the lowest of all gynecological malignancies (19.5-23.7/100,000) indicating a good prognostic outcome when detected in early stages (1).

Endometrioid carcinoma of the endometrium (EEC), also known as type I, is the most common histological type of the disease and accounts for ~80% of cases of endometrial carcinoma (3). This type of the disease is associated with an endocrine milieu of estrogen predominance and develops from endometrial hyperplasia. The majority of cases are presented in early stages, are usually well differentiated, are associated with a favorable prognosis (4) compared to type II carcinomas (e.g. papillary serous and clear cell) and are sensitive to endocrine treatment (5). The transition from normal endometrium to a malignant tumor is thought to involve a stepwise accumulation of alterations in cellular mechanisms leading to dysfunctional cell growth (6). Type I and type II carcinoma also present different molecular pathways in evolution.

The risk factors for EEC include obesity, anovulatory states, early onset of menarche, late menopause, nulliparity and exogene exposure of estrogene therapy (hyperestrogenic status), that promote the development of endometrial hyperplasia (mainly complex) with or without atypia which has been proposed as a possible precursor lesion of EEC (7,8) with progression rates into carcinoma of up to 40% (9).

Endometrial carcinogenesis is a complex process requiring the acquisition of genetic abnormalities in oncogenes, tumor-suppressor genes and genes involved in DNA repair. Among such genes, *GSTP1*, *CDH1* (*E-cadherin*) and *RASSF1A* are

included. The *GSTP1* gene is localized on chromosome 11q13 and encodes production of glutathion-S-transferases and plays a role in processes such as cell metabolism, response to stress stimuli, cell proliferation, apoptosis, carcinogenesis, response to chemotherapy, interaction with cellular proteins and cellular detoxification (10). The *CDH1 (E-cadherin*) gene is localized on chromosome 16q21 and belongs to the calcium-dependent cell adhesion molecule family. Predominantly, it is found in epithelial cells where it is responsible for intercellular adhesive junctions. The loss of *CHD1* expression leads to the loss of tissue homogeneity and predisposes to early invasion and metastatic spread of malignant cells. Thus, its downregulation is associated with poor prognosis in many epithelial tumor types (11). The *RASSF1A* gene is localized on chromosome 3p21.3 and encodes production of Ras-superfamily GTP-ases. The Ras superfamily comprises many guanine nucleotide-binding proteins (G proteins) that are essential to intracellular signal transduction. These proteins act biologically as molecular switches, which, cycling between OFF and ON states, play a fundamental role in cell biological processes, e.g. regulation of cell proliferation, differentiation, motility and apoptosis (12,13).

Previously, mainly genetic mutations (*PTEN*, *p53* and *KRAS* oncogenes) have been the scope of molecular studies; yet, recently it has become clear that epigenetic alterations (e.g. methylation, histone deacetylation or miRNA expression) may underline the molecular biology of endometrial lesions (10,14). These changes are defined as heritable alterations in gene expression without alteration of the nucleotide sequence (15) and are the most common molecular alterations in human neoplasias (16). Carcinogens may act by altering the normal epigenetic control of gene activity in specialized cells, and thereby produce aberrant heritable phenotypes (17). These phenotypes can be utilized not only at the level of diagnosis, but also in early prevention (18). Moreover, epigenetic changes are dynamic and modifiable upon treatment with pharmacological agents; thus, potential targets to halt carcinogenesis for example by using histone deacetylase inhibitors (HDACIs) and/or DNA methyltransferase inhibitors (DNMTIs) (19,20) have been studied.

While epigenetics refers to broad changes in several types of malignancies, including gynecological (21-23), we focused on the role of DNA methylation in relation to endometrial carcinogenesis. We aimed to investigate the aberrant methylation of CpG islands within the promoter regions of three tumor-suppressor genes, *GSTP1*, *CDH1* and *RASSF1A*, in endometrioid endometrial carcinomas and endometrial complex hyperplasias in order to define the frequency of the epigenetic alterations in comparison to healthy controls and to determine the possible impact on the disease histological pattern.

Materials and methods

Patient population. This was a prospective study enrolling initially a total of 92 subjects referred to the Department of Obstetrics and Gynecology, Jessenius Faculty of Medicine, Comenius University, Bratislava, Slovak Republic for surgical treatment due to uterine pathology. All participants were of Caucasian race and residents of the geographic area of Slovakia. After initial consultation, all subjects signed an informed consent and subsequently underwent biological sample collection during surgery (hysteroscopy, hysterectomy, uterine curettage) or by a pathologist during procurement of a frozen section in case of known endometrial malignancy. The retrieved tissue samples, sized 3-5 x 5 x 3-5 mm, and the obtained tissue samples were immediately placed in plastic tubes with mRNA stable solution and stored frozen at -20˚C for later epigenetic analysis. Exclusion criteria were the history of previous endometrial surgery, history of a previous gynecological malignancy, synchronous malignancy and cases with an endometrial malignancy other than endometrioid adenocarcinoma (e.g. serous or clear cell type). For healthy controls, we used histologically negative endometrial samples from paraffin-embedded tissue blocks from cases operated on for benign uterine fibroids. Of all the enrolled subjects, only samples with retrieved sufficient mRNA and later DNA were included in the final analyses: endometroid adenocarcinoma (EEC, n=41/41), endometrial complex hyperplasia with/without atypia (EHP, n=19/24), and cases with healthy endometrium (controls, n=20/27). The stratification into the observed groups (EEC and EHP) was based retrospectively according to the histopathological report. The Regional Ethics Committee of the Jessenius Faculty of Medicine (registered under IRB00005636 at the Office for Human Research Protection, U.S. Department of Health and Human Services) approved the study protocol (codes IRB 169/2011). The study was carried out in accordance with the Declaration of Helsinki for experiments involving humans.

Histopathological analysis. Histological samples were fixed in 10% formol solution, and assessments were performed using 4- to 5-μm hematoxylin and eosin-stained sections of formalin-fixed, paraffin-embedded tumors. Typing was evaluated according to the WHO Classification of Tumours (24), and histological grading and staging was carried out according to the revised pTNM FIGO 2009 classification (25-27).

DNA isolation from formalin-fixed paraffin-embedded tissue. Formalin-fixed paraffin-embedded tissue was deparaffinized by organic solvent-xylene and a series of alcohol solutions, and removed of their water and air-dried at room temperature. Dry tissue was suspended in 180 μl of lysis buffer and digested by proteinase K for 3 days at 56˚C, following genomic DNA extraction using the DNeasy blood and tissue kit (Qiagen, Heidelberg, Germany) according to the manufacturer's instructions.

Genomic DNA isolation from fresh endometrial tissue. Fresh endometrial tissue was cut into 0.3-cm thick pieces, and each piece was sampled into a 1.5-ml tube containing RNAlater® protect reagent (Qiagen). Tissue was stored at 4˚C for one day until DNA extraction was performed. Protected tissue was centrifuged 1 min at 14,000 rpm at 4˚C. RNAlater was removed, and one stainless steel bead 5 mm in diameter (stainless steel beads, 5 mm; Qiagen), 300 μl of RLT buffer (Qiagen) and β-mercaptoethanol and 1 μl Reagent DX (Qiagen) were added into each tube. The tissue was homogenized in TissueLyser LT (Qiagen) at 50 Hz for 2 min until the tissue was completely disturbed. Homogenized tissue was incubated at 56˚C for 12 h with addition of 60 μl proteinase K into each sample. DNA

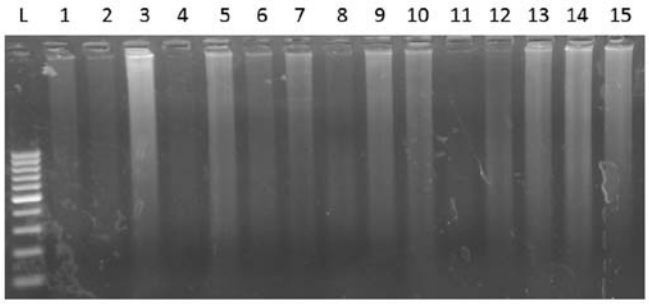

Figure 1. Quality and concentration of the DNA extracted from the paraffin-embedded tissue as determined by 1.5% agarose electrophoresis. In lanes 4 and 11 poor quality of DNA is noted and these samples were not suitable for further analysis.

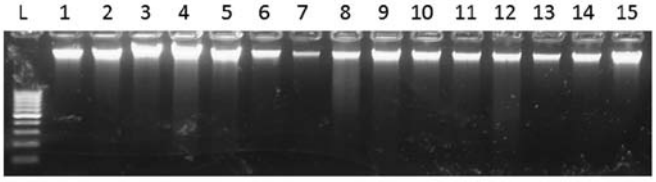

Figure 2. Quality of DNA extracted from the endometrial tissue as displayed on 1.5% agarose electrophoresis stained with ethidium bromide. The band intensity depends on the DNA concentration. In lanes 7 and 13 poor quality of DNA is noted and these samples were not suitable for further analysis.

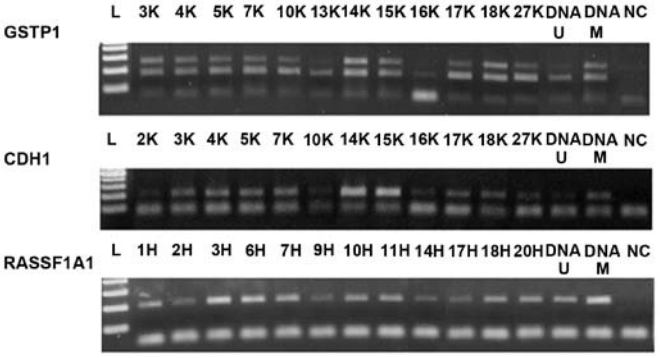

Figure 3. PCR products of the first step of nested-MSP with flanking primers for *GSTP1*, *CDH1* and *RASSF1A1*. Positive and negative controls are included. L, 100-bp DNA ladder; DNA U, positive control for umnethylated DNA; DNA M, positive control for methylated DNA; NC, negative control.

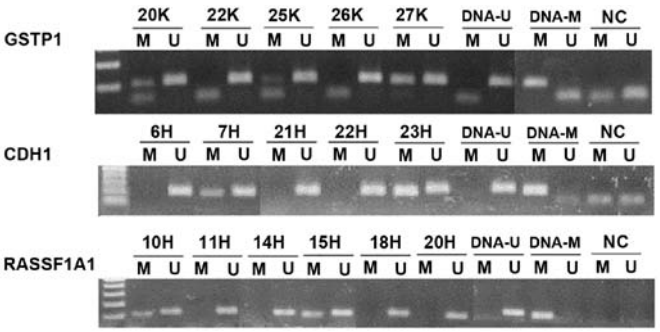

Figure 4. Representative results of the second step of nested-MSP. In *GSTP1*, samples 20K, 25K, 27K were methylated, while 22K and 26K were unmethylated in the promoter region; in *CDH1*, samples 7H and 23H were methylated, while 6H, 21H and 22H were unmethylated in the *CDH1* promoter region. On the last *RASSF1A1* segment, samples 10H and 15H were methylated, while 11H, 14H, 18H and 20H were unmethylated in the *RASSF1A1* promoter region. DNA-U, positive control for umnethylated DNA; DNA-M, positive control for methylated DNA; NC, negative control.

was isolated from the lysed tissue using the DNeasy blood and tissue kit according to the manufacturer's instructions.

DNA quality control. DNA was eluted in 60 μl of elution buffer in both procedures, and its quality was confirmed by electrophoretic separation on a 1.5% agarose gel stained by ethidium bromide (Figs. 1 and 2). DNA concentration was estimated by UV spectrophotometry at a wavelength of 260 nm.

Bisulfite conversion. Bisulfite conversion of DNA takes advantage of the bisulfite-mediated chemical conversion of unmethylated cytosine residues into uracil. Methylated cytosine residues remain unchanged. DNA methylated and unmethylated genomic regions after bisulfite conversion can be distinguished by sequence-specific PCR primers (28). Aliquots of 1 μg of each DNA sample were subjected to bisulfite treatment using the EpiTect bisulfite modification kit (Qiagen) following the manufacturer's protocol. Briefly, 1 μg of DNA was mixed with 85 μl of bisulfite mix and 35 μl of DNA protect buffer in a total volume of 140 μl. Bisulfite conversion was performed using the following thermal cycling program: denaturation at 95˚C for 5 min, incubation at 60˚C for 25 min, denaturation at 95˚C for 5 min, incubation at 60˚C for 85 min, denaturation at 95˚C for 5 min and incubation at 60˚C for 175 min. After cycling, converted DNA was purified using an automatic preparation in QIAcube (Qiagen). Converted DNA was subsequently eluted in 20 μl of elution buffer and stored at -20˚C. Unmethylated and methylated DNA was included in every bisulfite treatment as a control sample.

Methylation-specific nested PCR (MSP). Nested PCR is a two-step PCR; in the first step (Fig. 3), PCR initially amplifies bisulfite-modified DNA with the use of flanking PCR primers. In the second step (Fig. 4), the amplified external product is used as the template for the methylation-specific PCR assay using internal methylated and unmethylated primers. The modified DNA was subject to methylation-specific nested PCR (N-MSP) to investigate the methylation status of the promoter region of the *GSTP1*, *CDH1* and *RASSF1A* genes. Primer sequences, annealing temperatures and the product lengths are listed in Table I.

The first step of PCR was carried out in a total volume of 25 μl per reaction, containing 1 U of FastStart Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA), 2.5 μl of 10X PCR buffer, 2.5 mmol/l $MgCl_2$, 1.0 mmol/L dNTPs (dNTP Mix; Applied Biosystems, Foster City, CA, USA) and 10 pmol/l of each external primer. The first step was run in a thermal cycler using the following conditions: initial denaturation at 95˚C for 5 min; then 35 cycles at 95˚C for 30 sec, 56˚C (*GSTP1* and *RASSF1A*) or 60˚C (*CDH1*) for 30 sec and 72˚C for 30 sec, and a final extension at 72˚C for 5 min. The first step PCR product (5 μl) was mixed with 2 μl of 6X DNA loading dye (Fermentas, Germany) and analyzed on 1.75% agarose gel electrophoresis and visualized by ethidium bromide staining.

Table I. Primer sequences of external and specific internal primers, annealing temperatures, sizes of the PCR products and studies where the primers were published.

Gene	Primer type	Sequence	Annealing temp. (˚C)	Size (bp)	Study (ref.)
External primers					
GSTP1	Forward	5'-GGGATTTTAGGGYGTTTTTTTG-3'	56	159	(29)
	Reverse	5'-ACCTCCRAACCTTATAAAAATAATCCC-3'			
CDH1	Forward	5'-GTGTTTTYGGGGTTTATTTGGTTGT-3'	60	186	(29)
	Reverse	5'-TACRACTCCAAAAACCCATAACTAACC-3'			
RASSF1A	Forward	5'-TTGAGTTGYGGGAGTTGGTATT-3'	56	210	(30)
	Reverse	5'-CCCAAATAAATCRCCACAAAAAT-3'			
Internal methylated					
GSTP1	Forward	5'-TTCGGGGTGTAGCGGTCGTC-3'	62	91	(29)
	Reverse	5'-GCCCCAATACTAAATCACGACG-3'			
CDH1	Forward	5'-TGTAGTTACGTATTTATTTTTAGTGGCGTC-3'	62	112	(29)
	Reverse	5'-CGAATACGATCGAATCGAACCG-3'			
RASSF1A	Forward	5'-GTGTTAACGCGTTGCGTATC-3'	58	94	(31)
	Reverse	5'-AACCCCGCGAACTAAAAACGA-3'			
Internal unmethylated					
GSTP1	Forward	5'-GATGTTTGGGGTGTAGTGGTTGTT-3'	59	97	(29)
	Reverse	5'-CCACCCCAATACTAAATCACAACA-3'			
CDH1	Forward	5'-TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT-3'	61	120	(29)
	Reverse	5'-ACACCAAATACAATCAAATCAAACCAAA-3'			
RASSF1A	Forward	5'-TTTGGTTGGAGTGTGTTAATGTG-3'	60	108	(31)
	Reverse	5'-CAAACCCCACAAACTAAAAACAA-3'			

Table II. Demographics and clinical features of the studied groups.

Features	EEC (n=41)	EHP (n=24)	Controls (n=27)	P-value
Mean age (years)	63.1	52.8	48.7	0.0001
Onset of menarche (years)	13.5	13.3	12.9	NS
Parity	2.3	2.0	2.5	NS
Smoking (%)	60.3	39.7	44.9	NS
History of OC (%)	14.6	12.5	11.1	NS
History of HRT (%)	4.8	4.1	3.7	NS

EEC, endometrioid endometrial cancer; EHP, endometrial complex hyperplasia; OC, oral contraceptives; HRT, hormone replacement therapy; NS, not significant.

First step PCR products were diluted 500-fold, and 2 μl was added to a second PCR in a 25 μl volume, with primers specific for methylated or unmethylated alleles. The second step PCR comprised 30 cycles for all genes at 95˚C for 30 sec, annealing at 62˚C (*GSTP1* and *CDH1*-methylated), 61˚C (*CDH1*-unmethylated), 60˚C (*RASSF1A1*-unmethylated), 59˚C (*GSTP1*-unmethylated) or 58˚C (*RASSF1A1*-methylated) for 30 sec and extension at 72˚C for 30 sec. Five microliters and 2 μl of 6X DNA loading dye were loaded onto 1.75% agarose gel and visualized by ethidium bromide staining (Fig. 4).

Statistical analysis. We used descriptive statistics expressed as means ± standard deviation (±SD) or as a number (percentage) to provide a summary of the data for continuous and categorical variables, respectively. The homogeneity of the studied groups was assessed using the Student's t-test. For statistical analysis between the CpG methylation and histological findings, the Chi-square (χ^2) test was used based on Pearson's distribution. The correlations between tumor-suppressor gene promoter methylation and histopathological variables were assessed using Pearson's correlation coefficient. The trendlines were

Table III. Presence of CpG promoter hypermethylation in tumor-suppressor genes according to histopathology.

CpG methylation positivity	EEC (n=41)	EHP (n=19)	Controls (n=20)	P-value
GSTP1 methylated	68.3	52.6	35.0	
GSTP1 unmethylated	31.7	47.4	65.0	<0.05
CDH1 methylated	31.4	21.1	20.0	
CDH1 unmethylated	68.6	78.9	80.0	NS
RASSF1A methylated	85.4	36.8	30.0	
RASSF1A unmethylated	14.6	63.2	70.0	<0.0001

Data are represented as percentages. EEC, endometrioid endometrial cancer; EHP, endometrial complex hyperplasia.

Table IV. Analysis of previously published data concerning methylation profiles in endometrioid endometrial cancer (EEC).

	Cases with CpG island promoter methylation in EEC		
	RASSF1A	*GSTP1*	*CDH1*
Pallarés *et al* (34)	74.0	-	-
Pijnenborg *et al* (41)	85.0	-	-
Seeber *et al* (40)	79.0	15.0	-
Arafa *et al* (38)	74.0	-	-
Liao *et al* (39)	61.5	-	-
Kang *et al* (36)	81.0	-	42.9
Nieminen *et al* (57)	54.0	-	15.0
Di Domenico *et al* (14)	-	-	61.5
Banno *et al* (48)	-	-	14.0
Saito *et al* (47)	-	-	37.8
Moreno-Bueno *et al* (46)	-	-	21.2
Chan *et al* (55)	-	30.9	-
Fiolka *et al* (present study)	85.4	68.3	31.4

Table V. Associations between biological features of EEC and promoter CpG methylation of tumor-suppressor genes.

CpG methylation positivity	*GSTP1*	*CDH1*	*RASSF1A*
Tumor grade	r=0.3875	r=0.2301	r=0.5199
	p=0.0123	p=0.1708	p=0.0005
Myometrial invasion	r=0.4325	r=0.1849	r=0.3727
	p=0.0047	p=0.2732	p=0.0164
Metastatic pelvic lymph nodes	r=0.3044	r=0.1589	r=0.3211
	p=0.0530	p=0.3477	p=0.0407

r, Pearson's correlation coefficient.

achieved by applying linear regression model analysis. The statistical level of significance was set to P≤0.05. All statistical calculations were performed by MedCalc 11.1 (MedCalc Software Inc., Mariakerke, Belgium) software for Windows.

Results

A total of 92 subjects was initially included in the study of which 41 EEC, 19 EHP and 20 controls were processed for final analyses due to sufficient DNA extraction and quality. The mean age of the EEC patients was 63.1 (±9.3) years, the mean age of the EHP patients was 52.8 (±9.2) years and 48.7 (±11.1) years for the controls (P<0.0001). There was no significant difference in onset of menarche, parity, history of oral contraceptives and hormonal replacement therapy and smoking among the patients and controls, thus, proving homogeneity of the studied cohort samples (Table II).

A significant difference was found between the studied groups and the presence of the promoter CpG hypermethylated status of the G*STP1* (P<0.05) and *RASSF1A* (P<0.0001) genes. Promoter methylation of the *RASSF1A*, *GSTP1* and *CDH1* genes was present in 85.4, 68.3 and 31.4% of EEC samples when compared to the controls with 30.0, 35.0 and 20.0%, respectively (Table III) and was in line with previously published studies (Table IV). The CpG methylation status of all three investigated tumor-suppressor genes was found in 12.2% of EEC patients, in 4.2% of EHP patients and in 3.7% of the controls, respectively. The positive findings for promoter methylation in two investigated genes were revealed in 48.7% of EEC patients, 26.0% of EHP and in 18.5% controls.

In regards to the histopathological variables and CpG methylation in the promoter region of the tumor-suppressor genes we found significant correlations between the *RASSF1A* and *GSTP1* genes and higher tumor grade, myometrial invasion and positive metastatic involvement of pelvic lymph nodes (Table V). No associations were noted between promoter hypermethylation of the *CDH1* gene and the biological features of endometrial cancer; however, a trend of higher tumor grade, deeper myometrial invasion and metastatic spread to pelvic lymph nodes was observed (see regression lines of positive correlations) (Fig. 5).

Discussion

There is no effective screening or diagnostic tool for the prevention of endometrial cancer; thus, its incidence is rising when compared to other gynecological malignancies, e.g.

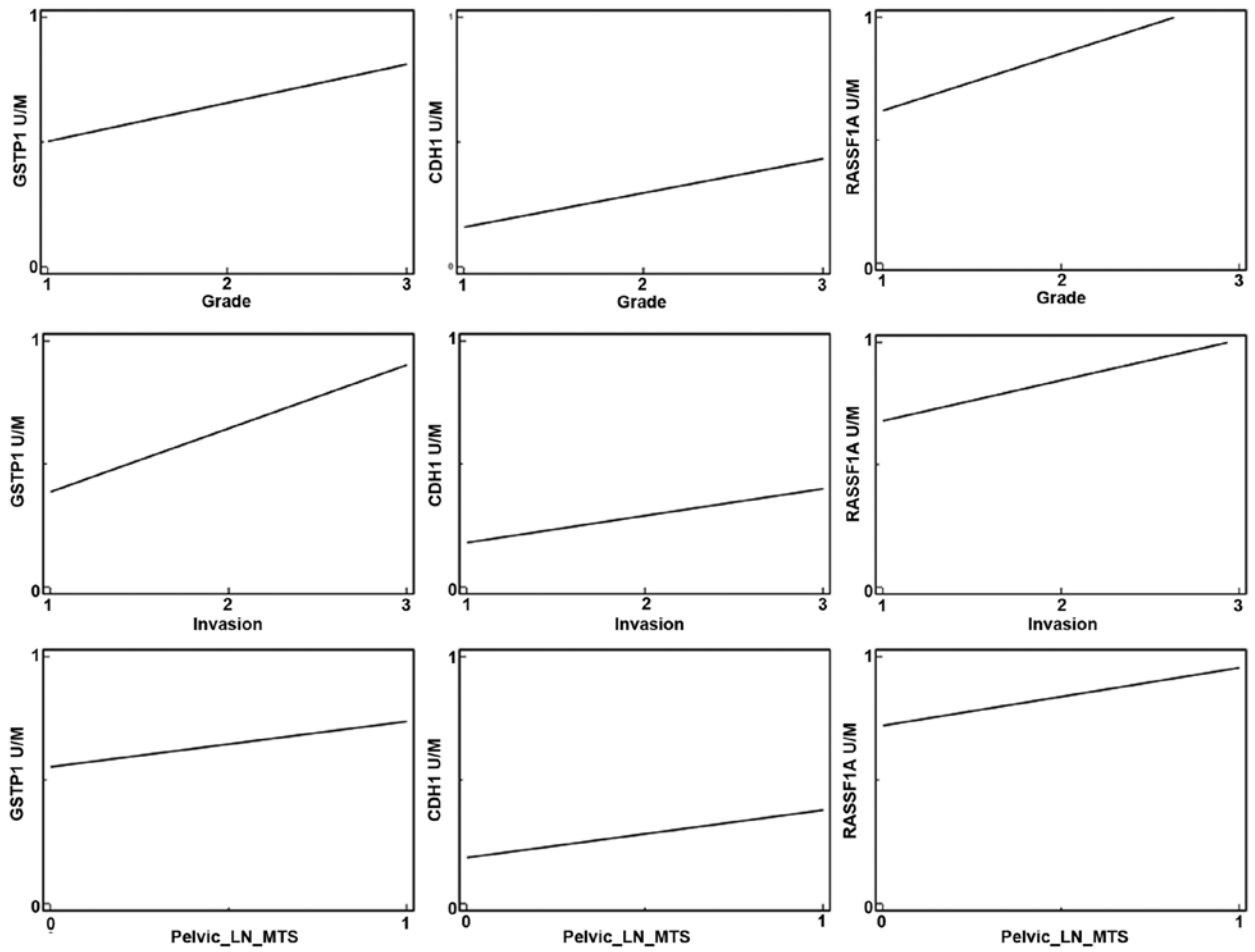

Figure 5. Correlations between the biological features of EEC and CpG hypermethylation of the analyzed genes obtained from the linear regression model. U, unmethylated status; M, methylated status; 0, negative pathological pattern; 1, positive pathological pattern.

cervical cancer. In contrast, common risk and predisposing epidemiological and histopathological factors associated with the development of uterine carcinomas have been identified. Based on these factors, we are able to select the women at a higher risk for disease origin and offer them increased attention. Moreover, scientists are still in search for new screening methods toward the aim of detecting premalignant at risk lesions or early stages of the disease. In the present study, we took advantage of the techniques of epigenetics and proteomics with their high sensitivity and specificity in the detection of human malignancies and their new therapeutic approaches (32,33). The most common epigenetic alterations analyzed in human cancers are CpG promoter methylation, histone deacetylation and miRNA expression. Epigenetic inactivation is defined as a change imposed onto the functionality of a gene that does not involve alteration of its coding sequence. Transcriptional silencing by hypermethylation of CpG islands in the promoter regions of tumor-suppressor genes has become recognized as a common phenomenon in carcinogenesis, including endometrial cancer (34).

As endometrial carcinogenesis is a multistep process involving precursor lesions, the aim of the present study was to analyze the methylation frequency of three tumor-suppressor genes (*RASSF1A*, *CDH1*/*E-cadherin* and *GSTP1*) in endometrioid endometrial carcinomas, complex endometrial hyperplasias and in healthy endometrium with an aim to find the potential value for early cancer diagnosis and the association with the clinicopathological pattern of the disease. The results indicated that the pattern of gene promoter methylation was associated with the biological aggressiveness of the carcinoma and that the frequency of methylated genes progressively increased with the type of histological features from normal endometrium to endometrial hyperplasia and endometrioid carcinomas. Thus, the panel of examined genes as well as the frequency of the methylation of these genes may be useful to distinguish between EHP and EEC, and low vs. high-grade carcinomas in daily pathology practice. Moreover, it can be useful for oncologists for assessment of the prognosis of EEC.

In the present study, a significant difference was found between the studied groups and the presence of promoter CpG hypermethylation status in the *RASSF1A* and *GSTP1* genes. Promoter methylation of the *RASSF1A*, *GSTP1* and *CDH1* genes was present in 85.4, 68.3 and 31.4% of EEC samples when compared to the controls with 30.0, 35.0 and 20.0%, respectively. The CpG methylation in all three investigated tumor-suppressor genes was noted in 12.2% of EEC patients, in 4.2% of EHP patients and in 3.7% of the controls, respectively. Positive findings for promoter methylation in two investigated genes was noted in 48.7% of EEC patients, 26.0% of EHP patients and in 18.5% of the controls.

The first report of *RASSF1A* methylation in association with endometrial cancer was published in 2004 by Fiegl *et al* (35) in a sample of 15 patients aimed for detection of endometrial cancer using epigenetic markers. Later, several studies focused on this epigenetic event in endometrial carcinomas as well as in hyperplasias (34,36-40).

Similar to our study, Seeber *et al* (40) analyzed the promoter hypermethylation of the *RASSF1A* and *GSTP1* genes in endometrial carcinomas and detected 79 and 15% methylation positivity for the observed genes and a significantly higher cumulative methylation index of tumor-suppressor genes in EC type I compared to type II. Similarly, *RASSF1A* was shown to be frequently (74%) methylated in EEC also in another study where it was a common finding in advanced-stage disease (34). The high frequency of methylation of the *RASSF1A* gene in EEC and atypic EHPs was revealed also by Arafa *et al* (38) where a methylated promoter occurred in 74 and 50% of subjects, respectively. No significant results were obtained for the other genes (*P16*, *MGMT* and *GSTP1*). Notably, 36% of histologically normal endometrial tissues adjacent to EEC showed *RASSF1A* gene methylation, indicating that CpG promoter region methylation is a markedly heterogeneous process, even in the absence of morphological or other molecular alterations which may point to the active cancerization process in the surrounding endometrium region. The 70 and 50% CpG promoter methylation positivity in the *RASSF1A* gene in EEC and EHP was detected also by Pijnenborg *et al* (41). Collectively, these studies confirmed a high frequency (33% up to 85%) of CpG promoter methylation of the *RASSF1A* gene in endometrial carcinomas. Moreover, this epigenetic alteration showed different frequencies according to the type of disease, with a higher incidence in endometrioid compared to serous or clear cell carcinomas (39,40).

Furthermore, our association analysis demonstrated that hypermethylation of CpG islands was correlated with clinicopathological parameters (tumor grade, myometrial invasion and nodal involvement). There were significant correlations between the *RASSF1A* and *GSTP1* genes and higher tumor grade, deeper myometrial invasion and positive metastatic involvement of pelvic lymph nodes. Similar results were found by Liao *et al* (39) who detected higher *RASSF1A* hypermethylation in type I endometrioid EC compared to type II carcinomas, advanced stage and myometrial invasion. The advanced stage of the disease (FIGO stage III, IV), metastatic lymph node involvement, and high grade (G3) were more frequent in patients with *RASSF1A* hypermethylation than in those without as revealed in a study by Jo *et al* (42). Thus, this epigenetic event has the potential to be used as a molecular marker for cancer diagnosis and prognosis. Based on these findings, there is increased importance of CpG promoter methylation of the *RASSF1A* gene for clinicians due to its potential association with survival outcome. There is research where this epigenetic event was reported to be associated with poor survival showing higher incidence of recurrences (77.8%) and lower disease-free survival (DFS) (97.0%) at 5 years for methylated and unmethylated patients (42). However, in another study this association was controversial (41). Nevertheless, the positive prognostic outcome is augmented by the findings that EC cells with *RASSF1A* promoter hypermethylation treated with 5-aza-2-deoxycytidine demonstrated reexpression and demethylation of the promoter region of *RASSF1A*. This suggests that aberrant hypermethylation of this gene is directly responsible for transcriptional inactivation of its expression in EC cell lines (43,44) and its restriction may improve the survival in EC patients. Moreover, *RASSF1A* hypermethylation was found to be significantly associated with microsatellite instability in endometrial carcinomas and loss of heterozygosity in cervical cancers; thus, we could block the increasing rate of genetic abnormalities in uterine carcinogenesis (43,45).

A few studies have evaluated the promoter methylation of *CDH1*/*E-cadherin*, a possible tumor-suppressor gene, in endometrial cancer (14,46-49). In the present study, we detected CpG promoter methylation of this gene in 31.4% of EEC samples, 21.1% of EHP cases and in 20.0% of cases with healthy endometrium. No associations were found between promoter hypermethylation of the *CDH1* gene and biological features of endometrial cancer, and there was no difference across histologic types of the disease (EEC vs. EHP vs. healthy control endometrium). A higher methylation rate of the *CDH1* gene (42.9% in EEC) was detected by Kang *et al* (36) who also described the high frequency of this event in cervical squamous cell carcinoma (80.6%). The association between *CDH1* promoter hypermethylation and endometrial cancer was also analyzed by Yi *et al* (49) who found that the hypermethylation of the *CDH1* promoter, which caused low expression of E-cadherin in endometrial cancer, was associated with not only clinicopathological progression of endometrial cancer but also with the overall 5-year clinical survival rate. The findings provide a potential therapeutic and prognostic target molecule for patients with endometrial cancer. Furthermore, it has been suggested that epigenetic change in E-cadherin expression could allow dissociation of individual cells from the primary tumor mass to facilitate invasion or metastasis (50). The positive associations between *E-cadherin* promoter methylation, higher tumor grade, myometrial invasion and involvement of pelvic lymph nodes revealed by Saito *et al* (47), suggest a possible role of *CDH1* in endometrial cancer progression. However, additional studies did not confirm this and found that *CDH1* promoter hypermethylation, noted in 21.2% of endometrial carcinomas, was not associated with clinicopathological or immunohistochemical variables (46,51). Moreover, controversial findings of this epigenetic event in endometrial carcinogenesis were presented by Pijnenborg *et al* (37) who did not find *CDH1* gene promoter methylation in the tested endometrial tumors, although the absence of E-cadherin expression was detected and found to be associated with the development of distant metastases. Although the role of *CDH1* promoter methylation in endometrial carcinogenesis must be analyzed in further studies, similar to hypermethylation of the *RASSF1A* gene, *CHD1* hypermethylation can be modified by DNA methyltransferases. It was shown that CpG methylation in the promoter region of the *E-cadherin* gene and induction of *E-cadherin* after treatment with the DNA methyltransferase inhibitor 5-azacytidine in human cancer cell lines lacked E-cadherin expression (52). In other words, the positive demethylation effect of DNMTIs and HDACIs on *CDH1* promoter methylation was observed and resulted in the suppression of growth of endometrial cancer cells (20,53).

CDH1 suppressor gene-mediated invasion in human cancer is silenced by an epigenic mechanism (54).

The frequency of CpG methylation in the promoter of the *GSTP1* gene in endometrial cancer was reported by Chan *et al* (55) who found a frequency of 30.9% and Seeber *et al* (40) who found a 15% rate for this epigenetic alteration. In the present study, we detected a frequency of 68.3 and 52.6% of CpG methylation in EEC and EHP samples with significant correlations to tumor aggressiveness. *GSTP1* promoter methylation and linkage to tumor biologic patterns was the scope of the study of Chan *et al* (55), who revealed that the extent of myometrial invasion was significantly correlated with both the methylation status and the protein expression of the *GSTP1* gene. Moreover, they postulated that hypermethylation of the G*STP1* gene promoter region may act as a dynamic regulation mechanism contributing to reduced *GSTP1* expression, which is associated with the myometrial invasion potential of endometrial carcinoma.

As evidenced from our study and the above mentioned studies, epigenetic molecular changes are commonly present in EEC and its precursor lesions, and these changes in endometrial tissue can be detectable several years before endometrial carcinoma in genetically predisposed individuals. Additionally, the recent data confirm that the methylation profile of the peritumoral endometrium is different from the altered molecular background of benign endometrial polyps and hyperplasias. Therefore, these findings suggest that the methylation of tumor-suppressor genes may clearly distinguish between benign and malignant lesions (14) which can be utilized in wide diagnostics or disease prevention. For example, *RASSF1A* promoter methylation in cervical cell smears can predict the presence of endometrial cancer with a 63.0% sensitivity and 96.3% specificity (56). The clinical importance of epigenetic abnormalities is also heightened by its power to distinguish the biologic risk of a lesion as it has been proven that abnormal DNA mismatch repair and methylation classify normal endometrium and simple hyperplasia into one category and complex hyperplasia without atypia, complex hyperplasia with atypia, and endometrial carcinoma into another, suggesting that, contrary to a traditional view, complex hyperplasia without atypia and complex hyperplasia with atypia are equally important as precursor lesions of endometrial carcinoma (57). Generally, hypermethylation of tumor-suppressor genes can be used in the early disease detection and prediction of the risk of malignant conversion.

In conclusion, promoter methylation of common tumor-suppressor genes is a frequent epigenetic event in EEC and EHP indicating their active and flexible role via control of gene expression in early carcinogenesis. Furthermore, the high methylation incidence of the *RASSP1A* and *GSTP1* genes in high-grade and advanced-stage carcinomas emphasizes their prognostic value in EEC which collectively represents a clinical tool for the proper management of the disease.

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