DNA methylation of selected tumor suppressor genes in endometrial hyperplasia

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Aims. To investigate DNA methylation of specific gene promoters in endometrial hyperplasia compared to normal endometrial tissue.
Materials and Methods. To search for epigenetic events, methylation-specific multiplex ligation-dependent probe amplification was employed to compare the methylation status of 64 tissue samples with atypical endometrial hyperplasia, 60 tissue samples with endometrial hyperplasia without atypia, and 40 control tissue samples with normal endometrium.
Results. Differences in DNA methylation among the groups were found in *PTEN*, *CDH13*, and *MSH6* promoters (*PTEN*: atypical hyperplasia 32%, benign hyperplasia 6.8%, normal endometrium 10%; *P*=0.004; *CDH13*: atypical hyperplasia, 50%; benign hyperplasia, 43%; normal endometrium 8.1%; *P*=0.003; *MSH6* atypical hyperplasia 84%, benign hyperplasia, 62%; normal endometrium, 52%; *P*=0.008.) Higher rates of *CDH13* promoter methylation were identified in the groups with both forms of endometrial hyperplasia when compared to the control group (atypical hyperplasia, *P*=0.003, benign hyperplasia, *P*=0.0002). A higher rate of DNA methylation of the *PTEN* and *MSH6* promoters was observed in samples with atypical endometrial hyperplasia than in samples with benign endometrial hyperplasia (*PTEN*: *P*=0.02; *MSH6*: *P*=0.01) and samples with normal endometrial tissue (*PTEN*, *P*=0.04; *MSH6*, *P*=0.006).
Conclusion. DNA methylation of *CDH13*, *PTEN*, and *MSH6* appear to be involved in the development of endometrial hyperplasia.

Key words: methylation, *CDH13, PTEN, MSH6*, endometrial hyperplasia, epigenetics

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INTRODUCTION

Endometrial hyperplasia is characterized by the excessive proliferation of endometrial cells. Endometrial hyperplasia is usually caused by high levels of free estrogen combined with insufficient levels of progesterone-like hormones, which usually oppose the proliferative effects of estrogen on the endometrium. Endometrial hyperplasia represents a physiological response of endometrial tissue to the growth-promoting actions of estrogen. The predisposing factors include obesity, polycystic ovary syndrome, estrogen-producing tumors, late menopause, and unopposed estrogen use.

Endometrial hyperplasia is classified into two categories by the World Health Organization: endometrial hyperplasia without atypia (synonym: benign endometrial hyperplasia) and endometrial atypical hyperplasia/endometrioid intraepithelial neoplasia (EIN) (ref.[1]). The gland-forming cells of endometrial hyperplasia may undergo progressive changes that result in endometrioid type of endometrial cancer (EC), which is one of the most common cancers of the female genital tract[2]. Endometrial hyperplasia usually occurs after menopause, when ovulation stops and progesterone is no longer produced, as well as during perimenopause when women experience irregular ovulation[3]. The most common symptoms of endometrial hyperplasia are abnormal uterine bleeding, including menorrhagia, intermenstrual bleeding, postmenopausal bleeding, and irregular bleeding on hormone replacement therapy or tamoxifen[4-6].

About 25–40% of patients with atypical hyperplasia subsequently progress to endometrial cancer; endometrial cancer has also been found to coexist in 13–43% of patients with atypical hyperplasia[7]. The risk of progression of benign hyperplasia to endometrial cancer is less than 5% in over 20 years[8]. Therefore, atypical hyperplasia is considered a precancerous lesion, whereas the risk of developing invasive carcinoma in patients with benign hyperplasia is very low. This means that benign hyperplasia and atypical hyperplasia require different treatment approaches, with atypical hyperplasia requiring hysterectomy, while benign hyperplasia can be treated with hormonal treatment, such as progestin therapy, in some cases[9]. This makes the differentiation between benign hy-

perplasia and atypical hyperplasia very important to avoid undertreatment or overtreatment of patients.

Changes in DNA methylation of tumor suppressor genes have been shown to be an early step in the carcinogenesis of endometrial tissue[10]. However, information on whether there are differences in DNA methylation of tumor suppressor genes between atypical endometrial hyperplasia and benign endometrial hyperplasia are limited.

To fill this knowledge gap, a retrospective study was carried out to identify DNA methylation changes in tumor suppressor genes among subsets of endometrial samples with atypical hyperplasia, benign hyperplasia, and normal histopathological findings. In this study, a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) probe set was used to analyze DNA methylation changes in the promoter regions of 25 selected tumor suppressor genes.

MATERIALS AND METHODS

In this cross-sectional study, formalin-fixed and paraffin-embedded samples of benign endometrial hyperplasia and atypical endometrial hyperplasia, and samples of normal endometrial tissue in a total of 164 samples were obtained from women treated at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic. All women were treated from 2007 to 2014, and all were Caucasian. Samples of normal endometrium were obtained from patients who were treated surgically for a nonmalignant diagnosis, mostly after surgical treatment of uterine prolapse or uterine leiomyomas. Paraffin blocks were retrieved from the archive of the Fingerland Department of Pathology, University Hospital Hradec Kralove. All the slides were reviewed by a professor of pathology with subspecialization in gynecological pathology (JL). DNA was extracted from formalin-fixed paraffin-embedded samples using a Qiagen DNA extraction kit (Hilden, Germany). The study was approved by the Ethics Committee, of the Faculty Hospital Hradec Kralove and the institutional review board committee (r.n. 20120-4 S21P).

Benign endometrial hyperplasia

Benign endometrial hyperplasia was defined as irregularity and cystic expansion of glands (simple) or crowding and budding of glands (complex) without significant cytologic atypia of the glandular epithelium[1].

Atypical endometrial hyperplasia

Atypical endometrial hyperplasia was defined as simple or complex architectural changes of endometrial glands, with atypical changes of glandular epithelium, including cell stratification, tufting, loss of nuclear polarity, enlarged nuclei, and an increase in mitotic activity. However, stromal invasion characterized by loss of intervening stroma, presence of desmoplastic stromal reaction or very complex (e.g., villoglandular, papillary) architecture was absent, as it already indicates progression into endometrioid endometrial carcinoma[1].

Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA)

The MS-MLPA probe set ME002-B1 (MRC-Holland, Amsterdam, The Netherlands), which was used in this study, could simultaneously check for aberrant methylation in 25 tumor suppressor genes. Probe sequences, gene loci, and chromosomal locations are available at http://www.mlpa.com. Individual genes were evaluated using two probes that recognized different Hha1 restriction sites in their respective regions. The procedure was performed according to the manufacturer's instructions with minor modifications. Briefly, DNA (100 ng) was dissolved in 5 μL of TE buffer (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0), denatured, and subsequently cooled down to 25 °C. After the probe mix was added, the probes were allowed to hybridize (overnight at 60 °C). Subsequently, the samples were divided into two groups: in one half, the samples were directly ligated, while in the other half, ligation was combined with the HhaI digestion enzyme. This digestion resulted in ligation of methylated sequences only. PCR was performed on all samples using a standard thermal cycler (GeneAmp 9700, Applied Biosystems, Foster City, CA, USA), with 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min with a final extension of 20 min at 72 °C. Aliquots of 0.6 μL of the PCR reaction were combined with a 0.2 μL LIZ-labeled internal size standard (Applied Biosystems), and 9.0 μL of deionized formamide. After denaturation, the fragments were separated and quantified by electrophoresis on an ABI 3130 capillary sequencer and analyzed using GeneMapper4.0 (both Applied Biosystems). Peak identification and values corresponding to the peak size in base pairs (bp) and peak areas were used for further data processing. The methylation dosage ratio was obtained using the following equation: Dm = (Px/Pctrl)Dig/(Px/Pctrl)Undig where Dm is the methylation dosage ratio, Px is the peak area of a given probe, Pctrl is the sum of the peak areas of all control probes, Dig is the HhaI-digested sample, and Undig is the undigested sample. Dm can vary between 0 and 1.0 (corresponding to 0–100% of methylated DNA). Based on previous experiments, we considered a promoter to show methylation if the methylation dosage ratio was ≥0.15, which corresponds to 15% of methylated DNA (ref.[11]). CpG universal methylated and unmethylated DNA (Zymoresearch, Irvine, CA, USA) was used as a control in every run.

Statistical analysis

Demographic and clinical characteristics were compared using a nonparametric Mann-Whitney *U*-test for continuous variables and are presented as median values (range). Categorical variables were compared using Fisher's exact test or chi-square test, as appropriate, and are presented as numbers (%). Spearman's partial correlation was used to adjust the results for all potential confounders (age, BMI, diabetes mellitus, and hypertension). Differences were considered statistically significant at $P<0.05$. Gene methylation results were controlled for multiple comparisons, and differences were considered

significant at $P<0.002$. All *P*-values were obtained from two-sided tests, and all statistical analyses were performed using GraphPad Prism 6 for Mac OS X (GraphPad Software, San Diego, CA, USA) or the SPSS version 19.0 statistical package for Mac OS X (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographic and clinical characteristics of the study population

In total, 167 endometrial samples were included in the study. Three samples were excluded because of human errors during storage. Therefore, the remaining samples from 164 women were included in the analyses: 64 samples of atypical hyperplasia, 60 samples of benign hyperplasia, and 40 samples of normal endometrial tissue as the control group. Six samples failed to be analyzed, all of which were atypical hyperplasia samples. The demographic and clinical characteristics of women with respect to the presence of benign hyperplasia and atypical hyperplasia are presented in Table 1. All variables that were significant in the univariate analysis (female age, body mass index [BMI], diabetes mellitus [DM], and hypertension disorders [HT]) were considered as potential confounders.

Presence of gene methylation among women: with normal endometrial tissue, with benign hyperplasia and with atypical hyperplasia

Differences in DNA methylation among the subgroups were found in the *TP53*, *PTEN*, *CDH13*, *MSH6*, and *THBS1* promoters in a crude analysis (*TP53* $P=0.049$, *PTEN* $P<0.0001$, *CDH13* $P<0.0001$, *MSH6* $P=0.002$, *THBS1* $P=0.01$). After adjustment for multiple comparisons, differences in DNA methylation remained significant only for the *PTEN*, *CDH13*, and *MSH6* promoters (Table 2). These differences remained significant after adjusting for potential confounders (*PTEN*, $P=0.004$, *CDH13* $P=0.003$, *MSH6* $P=0.008$).

The samples with atypical hyperplasia had higher rates of methylation of *PTEN* and *MSH6* promoters than those with benign hyperplasia (*PTEN* $P=0.02$, adj. $P=0.02$; *MSH6* $P=0.05$, adj. $P=0.01$) and the samples with normal endometrial tissue (*PTEN*, $P=0.001$ adj. $P=0.04$; *MSH6* $P=0.02$, adj. $P=0.006$) in crude analyses and even after adjustments for potential confounders. No differences in the methylation rates of *PTEN* and *MSH6* were found between the samples with normal endometrial tissue and those with benign hyperplasia (*PTEN*, $P=0.76$; *MSH6*, $P=0.54$). The samples with atypical hyperplasia and benign hyperplasia had higher rates of methylation of the *CDH13* gene than the samples with normal endometrial tissue (atypical hyperplasia: $P<0.0001$, adj. $P=0.003$; benign hyperplasia: $P=0.0002$, adj. $P<0.0001$). No difference in the rate of methylation of the *CDH13* gene was found between women with benign hyperplasia and atypical hyperplasia ($P=0.76$).

DISCUSSION

Carcinogenesis is generally driven by progressive genetic alterations that are dependent on a wide range of internal and external factors. DNA methylation is a stable epigenetic marker that is frequently altered in tumors. Given its stability in biological specimens, it has become an attractive biomarker for disease[12]. This study showed significant aberrant changes in the DNA methylation of three tumor suppressor genes, namely *PTEN*, *MSH6*, and *CDH13*.

PTEN

Phosphatase and tensin homolog (*PTEN*) is a protein that, in humans, is encoded by the *PTEN* gene. *PTEN* is a major tumor suppressor gene (*TSG*) that is commonly inactivated in various cancers and has been implicated in many aspects of malignant phenotypes, such as proliferation, transformation, invasion, and metastasis[13-16]. During carcinogenesis, mutations and deletions of *PTEN* lead to silencing of its enzymatic activity, which in turn leads to increased cell proliferation and reduced cell death. Frequent genetic inactivation of *PTEN* has been observed in endometrial cancer, prostate cancer, and glioblastoma. Reduced *PTEN* expression is found in many other tumor

Table 1. Demographical and clinical findings of women with atypical endometrial hyperplasia, with benign endometrial hyperplasia and with normal endometrial findings.

	Control group n = 40	Benign hyperplasia n = 60	Hyperplasia with atypia n = 58	*P*
Age [years, median (range)]	65 (50–83)	50 (25–78)	62 (38–84)	**<0.0001**
BMI [kg/m^2, median (range)]	27.3 (19.8–37.3)	28 (18.9–45.0)	32.5 (21.1–51.9)	**<0.0001**
Hypertension [number (%)]	12	20 (35%)	42 (72%)	**<0.0001**
Diabetes [number (%)]	2 (5%)	3 (5.2%)	14 (24%)	**0.002**
Breast cancer [number (%)]	4 (10%)	2 (3.5%)	1 (1.7%)	0.14
Smoking [number (%)]	2 (5%)	6 (10.5%)	4 (7%)	0.58

Continuous data are presented as median (range), categorical data presented as n (%).
Significant *P*-values are shown in bold.

Table 2. Frequency of DNA methylation of the selected genes among the subsets of the women with atypical endometrial hyperplasia, with benign endometrial hyperplasia and with normal endometrial findings.

Gene	Control group	Benign hyperplasia	Hyperplasia with atypia	*P*
	n = 40	n = 60	n =58	
BRCA1 [number (%)]	0 (0%)	0 (0%)	0 (0%)	-
BRCA2 [number (%)]	0 (0%)	0 (0%)	0 (0%)	-
ATM [number (%)]	0 (0%)	0 (0%)	0 (0%)	-
TP53 [number (%)]	1 (3%)	10 (17%)	11 (19%)	**0.049**
PTEN [number (%)]	4 (10%)	4 (7%)	19 (33%)	**<0.0001***
MGMT [number (%)]	0 (0%)	2 (3%)	3 (5%)	0.36
PAX5 [number (%)]	0 (0%)	2 (3%)	4 (7%)	0.21
CDH13 [number (%)]	3 (8%)	25 (43%)	29 (50%)	**<0.0001***
TP73 [number (%)]	0 (0%)	0 (0%)	0 (0%)	-
WT1 [number (%)]	0 (0%)	3 (5%)	9 (16%)	0.12
VLH1 [number (%)]	0 (0%)	0 (0%)	0 (0%)	-
GSTP1 [number (%)]	0 (0%)	0 (0%)	0 (0%)	-
CHFR [number (%)]	0 (0%)	1 (2%)	1 (2%)	0.71
EST1 [number (%)]	0 (0%)	0 (0%)	0 (0%)	-
RP1 [number (%)]	0 (0%)	0 (0%)	0 (0%)	-
MSH6 [number (%)]	21 (52%)	36 (62%)	49 (84%)	**0.002***
MGMT [number (%)]	7 (18%)	4 (7%)	7 (12%)	0.27
THBS1 [number (%)]	0 (0%)	2 (3%)	8 (14%)	**0.01**
CADM1 [number (%)]	0 (0%)	1 (2%)	1 (2%)	0.71
STK11 [number (%)]	0 (0%)	0 (0%)	0 (0%)	–
PYCARD [number (%)]	1 (3%)	1 (2%)	1 (2%)	0.95
PAX6 [number (%)]	0 (%)	0 (0%)	0 (0%)	–
CDKN2A [number (%)]	0 (0%)	0 (0%)	0 (0%)	–
GATA5 [number (%)]	0 (0%)	1 (2%)	5 (9%)	0.053
RARB [number (%)]	0 (0%)	0 (0%)	0 (0%)	–
CD44 [number (%)]	0 (0%)	3 (5%)	2 (3%)	0.36
RB1 [number (%)]	0 (0%)	0 (0%)	0 (0%)	–

Categorical data are presented as n (%).
Significant *P*-values are shown in bold.
Significant *P*-values after correction for multiple comparisons are indicated with *.
– – can not be calculated because either one group was not found for a given gene methylation.

types, such as lung and breast cancers. Furthermore, *PTEN* mutations also cause a variety of inherited predispositions to cancer[17-21]. The 2017 European Society of Gynecological Oncology guidelines recommend the use of immunohistochemistry for *PTEN* to improve the differential diagnosis of endometrial pathologies. *PTEN*-positive glands are seen in high numbers in benign hyperplasia, whereas complete loss of *PTEN* protein expression is most commonly found in endometrial carcinoma and atypical hyperplasia[22]. In a systematic review of the loss of *PTEN* expression as a diagnostic marker of endometrial precancer, the authors stated that several studies in the literature showed a highly variable degree of association between the loss of *PTEN* expression and atypical hyperplasia, making it difficult to analyze its diagnostic accuracy[23]. Another study suggested the usefulness of *PTEN* expression in endometrial hyperplasia as an early warning for heightened cancer risk[24]. This correlates with the results of the present study which showed significantly higher methylation of *PTEN* gene in the group with atypical hyperplasia (33%) compared to the group with benign hyperplasia (7%) and the control group with normal endometrial tissue (10%).

MSH6

The *MSH6* gene belongs to a set of genes known as the mismatch repair (*MMR*) genes. *MSH6* provides instructions for making a protein and is essential for DNA repair. Another crucial role of *MSH6* is fixing errors incurred during DNA replication in preparation for cell division. The *MSH6* protein attaches to the *MSH2* protein (produced from the *MSH2* gene) to form a protein complex. This complex can identify locations on DNA with mistakes that occurred during DNA replication, which are then repaired after marking by the *MLH-PMS2* protein complex. Hypermethylation of the promoter *MSH6*, seems to be a frequent early event in breast cancer and prostate cancer[11]; however, information and knowledge regarding *MSH6* and its mutations in connection with endometrial hyperplasia are very limited.

In recent literature, a few studies have addressed or described the relationship between the *MSH6* gene and endometrial hyperplasia. In one study, the authors showed that the loss of expression of *MSH6* was higher in endometrial hyperplasia with coexisting endometrial cancer or in endometrial hyperplasia with subsequent progression to endometrial cancer[25]. In the present study, *MSH6* methylation was frequent in each group; however, there was an increasing number of *MSH6* gene hypermethylation from the control group to the group with atypical hyperplasia: control group (52%), benign hyperplasia (62%), atypical hyperplasia (84%).

CDH13

CDH13 (H-cadherin) belongs to the cadherin gene superfamily. Cadherin proteins are a class of type-1 transmembrane proteins. They act as negative regulators of axon growth during neural differentiation, protect vascular endothelial cells from apoptosis due to oxidative stress, and are associated with resistance to atherosclerosis. As transmembrane proteins, cadherins play an important role in cell adhesion by forming adherent junctions to bind cells within tissues[26]. Control of cellular adhesion and motility is one of the crucial mechanisms responsible for tumor initiation and progression[27]. DNA methylation of cadherins has been described in several cancer types, including bladder cancer[28], prostate cancer[29], ovarian cancer[30], and endometrioid carcinoma of the endometrium.

In the present study, statistically significant higher methylation of *CDH13* ($P<0.0001$) was found in samples with atypical hyperplasia (50%) and benign endometrial hyperplasia (43%) when compared to the control group with normal endometrial tissue (8%). This is consistent with earlier findings, which showed that *CDH13* promoter was not only frequently methylated in endometrial cancer samples (81%), but also in benign hyperplasia (50%) and in atypical hyperplasia samples (52%) (ref.[31]).

CONCLUSION

The current study has some strengths: a relatively large homogeneous cohort of women (Caucasian women from the eastern part of the Czech Republic) were used in this study; all samples were reviewed by a highly experienced pathologist with expertise in gynecological oncology. However, a major limitation of this study is that only a selected panel of tumor suppressor genes was assessed. However, this panel was developed and used in our previous study on endometrial cancer[31].

The findings of this study suggest that DNA methylation of *PTEN* and *MSH6* can help in separating precancerous atypical endometrial hyperplasia from benign endometrial hyperplasia. In contrast, DNA methylation of *CDH13* suggests an important role early in the process of excessive proliferation of endometrial tissue that leads to endometrial hyperplasia.

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