Methylation analysis of tumor suppressor genes in endometroid carcinoma of endometrium using MS-MLPA

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Background. Epigenetic changes are considered to be a frequent event during tumor development. Hypermethylation of promoter CpG islands represents an alternative mechanism for inactivation of tumor suppressor genes, DNA repair genes, cell cycle regulators and transcription factors. The aim of this study was to investigate promoter methylation of specific genes in endometrial cancer by comparison with normal endometrial tissue.

Materials and Methods. We used MS-MLPA (Methylation-specific Multiplex ligation-dependent probe amplification) to compare the methylation status of 59 tissue samples of endometroid type of endometrial carcinoma with 20 control samples of non-neoplastic endometrium.

Results. Using 15% cut-off for methylation, we observed significantly higher methylation in the *CDH13* gene in endometrial cancer group. We observed significantly higher methylation in both *WT1* and *GATA5* genes in IB stage of endometroid carcinoma. We also observed significantly higher methylation in *GATA5* gene in the group of poorly differentiated endometroid carcinoma.

Conclusion. The findings suggest the importance of hypermethylation of *CDH13*, *WT1* and *GATA5* genes in endometrial carcinogenesis and could have implications for future diagnostic and therapeutic strategies of endometrial cancer based on epigenetic changes.

Key words: MS-MLPA, DNA methylation, endometrial cancer, *CDH13*, *WT1*, *GATA5*, epigenetics

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INTRODUCTION

Endometrial cancer is one of the three most common cancers in females in well developed countries. The vast majority of cases are diagnosed after the menopause, with the highest incidence around the seventh decade of life. The risk factors for the disease include obesity, hypertension, diabetes mellitus, late menopause and unopposed estrogen use¹. For all stages, the overall 5-year survival is around 80%. Two types of endometrial carcinoma are distinguished with respect to molecular genetic changes, biologic behaviour and prognosis: type I- endometroid and type II- non-endometroid carcinoma².

Aberrant methylation of normally unmethylated CpG islands, located in the 5' promoter region of genes, has been associated with transcriptional inactivation of several genes in human cancer, and can serve as an alternative to mutational inactivation³. Molecular events associated with tumor methylation hold promises for cancer risk assessment, diagnostic purpose and prognosis⁴. Moreover, epigenetic alterations are potentially reversible effects, which could be used for new therapeutic strategies in the future. Several methylation markers have been identified in endometrial cancer: *hMLH1*, *HOXA10*, *HOXA11*,

THBS2, *CDH13*, *HSPA2*, *RASSF1A*, *SOCS2*, *PER1*, *RARB2*, *GSTP1*, *SFN* (14-3-3 sigma), *SESN3* and *TITF1* (ref.⁵⁻⁸).

A number of methods have been developed for detection of methylation alterations in tumors, such as MSP (Methylation-specific PCR), MS-MLPA (Methylation-specific Multiplex ligation-dependent probe amplification), MS-HRM (Methylation-sensitive High resolution melting), DNA sequencing, microarrays and others⁹. Among these, MS-MLPA represents a rather novel cost-effective and time-efficient method and furthermore is an ideal technique to use in FFPE (formalin-fixed, paraffin-embedded) samples¹⁰. It permits simultaneous identification of epigenetic alterations in a predefined set of up to 25 genes. The present study applies a MS-MLPA analysis in endometrial cancer.

MATERIALS AND METHODS

Formalin-fixed and paraffin-embedded samples from both endometroid carcinoma of endometrium and normal endometrial tissue were obtained from 79 women (59 patients with endometrial cancer, 20 patients with normal endometrium) treated at the Department of Obstetrics

Table 1. Genes in the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) KIT ME002 Tumor suppressor-2 (MRC Holland).

Gene	Name	Probes	Chromosomal location
BRCA1	Breast cancer 1	03296-L01269	17q21.3
BRCA2	Breast cancer 2	02285-L01776	13q13.1
ATM	Ataxia telangiectasia mutated	03023-L02413	11q23
TP53	Tumor protein p53	02374-L02530	17p13.1
PTEN	Phosphatase and tensin homolog	03808-L02169	10q23.3
MGMTa	O-6-methylguanine-DNA methyltransferase	05670-L05146	10q26.3
PAX5	Paired box gene 5	03750-L03210	9p13
CDH13	Cadherin 13, H-cadherin	02257-L01742	16q23.3
TP73	Tumor protein p73	01684-L01264	1p36.3
WT1	Wilms tumor 1	02755-L02204	11p13
VHL	von Hippel-Lindau tumor suppressor	03818-L03850	3p25.3
GSTP1	Glutathione S-transferase pi 1	02747-L02174	11q13
CHFR	Checkpoint with forkhead and ring finger domains	02737-L02164	12q24.3
ESR1	Estrogen receptor 1	02746-L02173	6q25.1
RB1a	Retinoblastoma 1	02734-L02161	13q14.2
MSH6	MutS homolog 6	01250-L00798	2p16.3
MGMTb	O-6-methylguanine-DNA methyltransferase	13716-L15582	10q26.3
THBS1	Thrombospondin 1	01678-L17140	15q15
CADM1	Cell adhesion molecule 1	03816-L17141	11q23
STK1	Serine/threonine protein kinase	06783-L17143	19q13.3
PYCARD	PYD and CARD domain containing	02252-L01737	16p11.2
PAX6	Paired box gene 6	03749-L03209	11p13
CDKN2A	Cyclin-dependent kinase inhibitor 2A	01530-L00955	9p21.3
GATA5	GATA-binding protein 5	03752-L06199	20q13.3
RARB	Retinoic acid receptor, beta	04046-L02172	3p24.2
CD44	CD44 molecule (Indian blood group)	04500-L02761	11p12
RB1b	Retinoblastoma 1	04502-L02199	13q14.2

Fig. 1. Comparison of methylation frequencies (cut-off value 15%) of the 25 analyzed genes in endometrial cancer and control samples. There is significantly higher methylation in *CDH13* gene ($P < 0.001$) in endometrial carcinoma group compared with control group.

Fig. 2. Methylation of specific genes according to tumor stage. There is significantly higher methylation in *WT1* ($P=0.002$) and *GATA5* ($P=0.05$) genes in stage IB of endometrial carcinoma samples compared with stage IA samples.

Fig. 3. Methylation of specific genes according to tumor grade. There is significantly higher methylation in *GATA5* gene ($P=0.05$) in poorly differentiated carcinoma compared with grade 1 and grade 2 tumor samples.

and Gynecology, University Hospital Hradec Kralove, Czech Republic. The samples of normal endometrium were obtained from patients surgically treated for non-malignant diagnosis. The paraffin blocks were retrieved from the archive of the Fingerland Department of Pathology, University Hospital Hradec Kralove. All slides were reviewed by an experienced pathologist (J.L.). The tumors were classified according to the current WHO classification of tumors of the female reproductive system¹¹. The following clinicopathological data was recorded: patient's age, tumor stage and tumor grade. The study was approved by the Ethics Committee of University Hospital Hradec Kralove.

DNA was extracted from formalin-fixed, paraffin embedded samples using a Qiagen DNA extraction kit.

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

The present study used the MS-MLPA probe set ME002-B1 (MRC-Holland, Amsterdam, The Netherlands), which can simultaneously check for aberrant methylation in 25 tumor suppressor genes (Table 1). Probe sequences, gene loci and chromosome locations can be found at <http://www.mlpa.com>. Individual genes were evaluated by two probes, which recognized different HhaI restriction sites in their regions. The experimental

procedure was carried out according to the manufacturer's instructions, with minor modifications.

In short, DNA (100 ng) was dissolved up to 5 µL TE-buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0) denatured and subsequently cooled down to 25 °C. After adding the probe mix, the probes were allowed to hybridize (overnight at 60 °C). Subsequently, the samples were divided into two: in one half, the samples were directly ligated, while for the other half ligation was combined with the HhaI digestion enzyme. This digestion resulted in ligation of the methylated sequences only. PCR was performed on all the 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 0.2 µL LIZ-labeled internal size standard (Applied Biosystems), and 9.0 µL deionized formamide. After denaturation, 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 peak size in base pairs (bp), and peak areas were used for further data processing. Methylation dosage ratio was obtained by the following calculation: $Dm = (P_x/P_{ctrl})Dig/(P_x/P_{ctrl})Undig$, where Dm is the methylation dosage ratio, P_x is the peak area of a given probe, P_{ctrl} is the sum of the peak areas of all control probes, Dig stands for HhaI digested sample and Undig for 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.¹²). CpG universal methylated and unmethylated DNA (Chemicon International, Temecula, CA) were used in every run as controls.

Statistical analysis

Proportions were compared by two-tailed Fisher's exact test. Associations with P -value < 0.05 were considered to be significant.

RESULTS

In the present study we analyzed 79 samples of endometrial tissue (59 samples of endometroid carcinoma and 20 samples of normal endometrium). The median age of patients at the time of diagnosis was 65 years (range 44-84 years) in the carcinoma group and 60 years (range 50-79 years) in the control group.

We used the MS-MLPA probe set ME002 (MRC-Holland, Amsterdam, The Netherlands) to analyze samples of endometrium. Using 15% cut-off for methylation we observed statistically-significant higher methylation in *CDH13* gene ($P < 0.001$) and higher methylation in *WT1* gene ($P = 0.057$) in endometrial cancer patients compared to control group. For *MSH6* gene we observed high methylation (about 40%) in both endometrial can-

cer and control samples. For genes *BRCA1*, *BRCA2*, *ATM*, *TP53*, *PTEN*, *TP73*, *VHL*, *RBI*, *THBS1*, *STK11* and *RARB*, the methylation rate did not exceed the 15% threshold; the remaining genes also showed relevant differences in methylation between endometrial carcinoma and control samples (Fig. 1).

The methylation results from the endometrial cancer specimens were compared with clinicopathological characteristics, including tumor grade and tumor stage (pTNM). Both *WT1* ($P = 0.002$) and *GATA5* ($P = 0.05$) genes showed significantly higher methylation in stage IB compared with stage IA of endometrial cancer samples (Fig. 2). Methylation in *GATA5* gene ($P = 0.05$) was significantly higher in grade 3 of endometrial cancer samples compared with the group of grade 1 and grade 2 tumors (Fig. 3).

DISCUSSION

Endometrial carcinoma is the most common malignant tumor of the female genital system in developed countries. The biological features of endometrial cancer are determined by the underlying molecular alterations of tumor cells, including epigenetic inactivation of tumor suppressor genes as well as mutations and deletions. It is now clear that de novo promoter methylation is common mechanism for inactivation of tumor suppressor genes³. The promoter methylation status has been reported in several human neoplasms. The purpose of this study was to investigate promoter methylation in the set of common tumor suppressor genes in 59 endometrial cancer and 20 control samples. We used MS-MLPA and a threshold of 15% methylation was applied based on previous study¹².

We observed significantly higher methylation in *CDH13* gene and higher methylation in *WT1* and *CD44* genes in endometrial cancer compared with non-neoplastic samples indicating that promoter methylation of these tumor suppressor genes may play an important role in endometrial carcinogenesis. These findings could have implications for future diagnostic and therapeutic strategies of endometrial cancer based on epigenetic changes.

MSH6 was previously shown to be frequently methylated in breast cancer and also in normal breast tissue¹². In the present study, *MSH6* methylation was very frequent in both endometrial cancer and normal endometrial tissue (Fig. 1).

The gene *CDH13* (*H-cadherin*) encodes a member of the cadherin superfamily. The protein acts as a negative regulator of axon growth during neural differentiation, protects vascular endothelial cells from apoptosis due to oxidative stress and is associated with resistance to atherosclerosis. The gene is hypermethylated in many types of human cancer including ovarian and endometrial carcinomas^{13,14}. In the study using MS-MLPA probe mix ME001, targeting different CpG islands within promoter region of the *CDH13* gene, 93% of samples were methylated¹⁴. In our study, we observed almost 80% of methylated carcinoma samples. Methylation of *CDH1* (*E-cadherin*), another member of cadherin superfamily,

is also important event in endometrial carcinogenesis¹⁵. Aberrant methylation in promoter region of *CDH1* gene is associated with poor differentiation and myometrial invasion in endometrial carcinomas suggesting its possible role in tumor progression¹⁶. However, no association between *CDH1* hypermethylation and clinicopathological or immunohistological characteristics of endometrial cancer was found in other studies^{17,18}.

CD44 is a transmembrane receptor protein that belongs to the family of adhesion molecules and has a critical role in extracellular matrix adhesion and is implicated in a series of cellular events, such as lymphocyte homing, leukocyte activation, lymphopoiesis, embryogenesis, and wound healing¹⁹. With regard to *CD44* and its variants, several studies have investigated its expressions in endometrial pathologies, including adenocarcinomas²⁰⁻²⁵. In our study we observed higher methylation in *CD44* gene, but with no statistical significance.

According to tumor stage and grade we observed significantly higher methylation of *WT1* ($P=0.002$) and *GATA5* ($P=0.05$) genes in stage IB of endometrial carcinoma (Fig. 2) and significantly higher methylation of *GATA5* gene ($P=0.05$) in grade 3 of endometrial carcinoma (Fig. 3). These findings suggest that hypermethylation in *WT1* and *GATA5* genes could play an important role in tumor myometrial invasion and its aggressive behavior.

The Wilms' tumor gene *WT1* is overexpressed in various kinds of solid tumors. However, it remains unclear whether *WT1* plays a pathophysiological role in endometrial cancer^{26,27}. The *GATA* family of transcription factors plays essential role in cell growth and differentiation during embryogenesis and early development²⁸. *GATA5* have been implicated as important regulators in the normal development and differentiation of mesoderm- and endoderm-derived tissues, including lung, liver, gonad and pancreas²⁹. Loss of *GATA4* and *GATA5* expression second to promoter hypermethylation has been identified in primary ovarian, lung and gastrointestinal cancer³⁰⁻³³. Our present study is the first study to demonstrate methylation of *GATA5* in endometrial cancer.

There is an emerging evidence that epigenetic regulation of gene expression is at least as important to carcinogenesis as genetic disruption and more studies are needed to characterize the aberrant DNA methylation profile of endometrial carcinoma.

In conclusion, our study showed that there is significantly higher methylation in *CDH13* gene in the endometrial cancer group compared with samples of non-neoplastic endometrium. We also observed significantly higher methylation in *WT1* and *GATA5* genes in stage IB compared with stage IA of endometrial cancer samples. According to tumor grade, there was significantly higher methylation in *GATA5* gene in grade 3 of endometrial cancer samples compared with the group of grade 1 and grade 2 samples. The findings suggest the importance of hypermethylation of these genes in endometrial carcinogenesis and could have implications for future diagnostic and therapeutic strategies of endometrial cancer based on epigenetic changes.

ABBREVIATIONS

MS-MLPA, Methylation-specific Multiplex ligation-dependent probe amplification; MSP, Methylation specific PCR; PCR, Polymerase chain reaction; DNA, Deoxyribonucleic acid; MS-HRM, Methylation-sensitive High resolution melting; FFPE, Formalin-fixed, paraffin-embedded; WHO, World Health Organization; EDTA, Ethylenediaminetetraacetic acid; pTNM, pathologic TNM - T (tumor), N (nodes), M (metastasis).

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CONFLICT OF INTEREST STATEMENT

Author's conflicts of interest disclosure: *None declared.*

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