Hypermethylation of selected genes in endometrial carcinogenesis

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Abstract

OBJECTIVES: Endometrial cancer is one of the most common malignancies in women. The prevention has failed so far to develop an effective screening program and its incidence is rising in proportion to the incidence of cervical cancer. In recent years the investigation of malignancy genomics (genetic and epigenetic changes) has become the main focus of scientists because of its high sensitivity and specificity.

MATERIAL AND METHODS: We conducted a prospective longitudinal study at the Dpt. of Gynaecology and Obstetrics of the Jessenius Faculty of Medicine in Martin from 2010 to 2012, in collaboration with the Institute of Pathology of the University Hospital in Martin. We analysed paraffin blocks of endometrial tissue from 123 women with endometrial cancer, hyperplasia and normal endometrial findings. By the use of bisulphidic modification technique and nested methylation-specific PCR (MSP), we analysed the methylation patterns of three genes: GSTP1, E-cad, RASSF1.

RESULTS: We found a statistically significant increase of methylation of the RASSF1 gene in endometrial cancer compared to simplex hyperplasia and intact endometrial tissue ($p<0.001$). GSTP1 and E-cad did not show any relevant methylation pattern in various endometrial lesions.

CONCLUSION: According to the results of our study, RASSF1 gene methylation could serve as a prognostic factor of endometrial carcinogenesis and could help to predict the behaviour of endometrial hyperplasia.

Abbreviations:

DNA	- deoxyribonucleic acid
EC	- endometrial cancer
EH	- endometrial hyperplasia
GSTP	- glutathione-S-transferase
K-ras	- Kirsten retrovirus-associated DNA sequences
hMLH	- human mutL homolog
hMSH	- human mutS homolog
IUD	- intrauterine
PTEN	- phosphatase and tensin homolog
RASSF1A	- ras-association domain family 1
RT PCR	- real time polymerase chain reaction
WHO	- World Health Organisation

ORIGINAL ARTICLE

INTRODUCTION

Endometrial cancer is one of the most common malignancies in women. The incidence is about 142 000 cases worldwide per year (Amant *et al.* 2005). In Slovakia 700 new cases are diagnosed each year and 200 women die of this disease (Health Yearbook 2002).

Scientists have failed so far to develop an effective screening program and so its incidence is rising in proportion to the incidence of cervical cancer. Several risk factors associated with the development of endometrial cancer have recently been identified revealing the pathomechanism of the malignant process. Based on data from epidemiological studies and observations, it is now possible to sort out high-risk women who may be at increased risk of developing endometrial cancer.

In recent years genomic research (genetic and epigenetic changes) has become a main focus of scientists because of its high sensitivity and specificity. Epigenetic modifications include DNA hyper- and hypomethylation and histone deacetylation. The detection of such changes may help us to understand the evolution of diseases before their clinical manifestation. The presence of DNA methylation can be determined not only in the affected tissue, but also in various body fluids.

The potential use of DNA methylation thus lies in the early diagnosis of malignant diseases, predicting their prognosis and response to treatment (demethylation agents and inhibitors of deacetylases). Some of the most important genes that are often hypermethylated in endometrial cancer are: GSTP1, RASSF1A, KAI1, alpha inhibitor and DAB21P (Egger *et al.* 2004; Li *et al.* 2005).

MATERIAL AND METHODS

We conducted a prospective longitudinal study at the Department of Gynaecology and Obstetrics of the Jessenius Faculty of Medicine in Martin from 2010 to 2012, in collaboration with the Institute of Pathology of the University Hospital in Martin. We analysed paraffin blocks of endometrial tissue from 122 women with endometrial cancer, hyperplasia and normal endometrial findings. These women all underwent surgical treatment during the study period. The study was approved by the ethics committee and from each patient informed consent was obtained. The exclusion criteria contained another co-incidence of histologically confirmed malignancy.

The patients were divided according to the result of histological examination into 4 groups:

1. patients with histologically confirmed endometrial cancer
2. patients with histologically proven simplex endometrial hyperplasia
3. patients with histologically proven atypical endometrial hyperplasia
4. patients with normal endometrial tissue

In each group we evaluated the following demographic parameters: age at the time of sampling, age at menarche period, parity and smoking history.

Samples of endometrial lesions and normal endometrium were fixed in 10% formalin solution for 18–24 hours, drained in descending order of alcohols and xylene and embedded in paraffin blocks. Tissue samples were cut into 4–5 µm thick sections and placed in tubes. Basic histological evaluation (typing and grading) of carcinomas was conducted on specimens stained HE. Tumour size in carcinomatous tissue was determined according to the criteria of TNM classification (UICC, 2002); the type of cancer was carried out according to WHO criteria (2003).

The tissue obtained from paraffin blocks was cut into 2 ml tubes. The paraffin was dissolved in an xylene solution followed by incubation of paraffin sections in alcohol. Genomic DNA was isolated from paraffin blocks using a commercially available kit QIAamp DNA Blood and easy yew kit (QIAGEN, Germany) according to the manufacturer's protocol. It is important that the tissue sample intended for the isolation of test DNA includes at least 50% of the tumour tissue. In the present case, with more normal tissue, the analysis results are not reliable. DNA concentration was determined using a spectrophotometer with a wavelength of 260 nm. For the bisulphidic modification we used 1–16 µl of DNA (max. 20 µl). Due to the degradation of DNA and low concentrations, some samples were not suitable for further processing.

Conversion of unmethylated cytosines to uracil of the DNA from the tissue samples was performed using a kit (EpiTect bisulfite, QIAGEN, Germany) according to the manufacturer's protocol. After the conversion PCR tubes were quickly centrifuged and transferred into 2 ml tubes and continuing refinement of the QIA cube then led to a pure product with bisulphidic DNA modification unmethylated cytosines to uracil. Nested PCR was carried out in 1 ml of DNA modified with sodium bisulfite in 25 ml of reaction mixture consisting of: 2.5 mmol/L MgCl, 2, 10 pmol/L of each forward and reverse primer, 1.0 mmol/L dNTPs, 2.5 mmol/L 10 × PCR buffer (ABgene®, United Kingdom). The first step PCR was carried out in the program, with an initial denaturation at 95 °C, 5 min. and subsequent 35 cycles with 30 second intervals and at an annealing temperature of 56 °C. For the second step we used an identical PCR program except for the annealing temperature. The annealing temperature was determined by the formula:

$$Tm = [4 (C + G) + 2 (A + T)] - 4$$

PCR reaction conditions had to be optimized to be as efficient as possible. To control contamination of PCR, i.e. presence of unwanted DNA in any of the components of the PCR reaction (e.g. contaminated buffer, dNTP-human, primers, water or sterile pipette reagents for the preparation of mastermix), we used a negative control containing all reagents except DNA. In

the first step of PCR we used external primers (Table 1) binding to the methylated and unmethylated DNA. The resulting products were diluted at a ratio of 1:500. 1 µl DNA was used in round 2 of PCR, where internal primers were used pertaining to specific methylation. The resulting products of round 2 of PCR were evaluated in a 1.75% agarose gel stained 2.5 ml ethidium bromide and visualized under a UV transilluminator. On an agarose gel we put 5µl DNA and 2 µl 6 × DNA Loading Dye (Fermentas, Germany) and used 100 bp ladder (Fermentas, Germany).

We use the MedCalc® 12.2.0 (Mariakerke, Belgium) program for the statistical analysis. When comparing clinical parameters between carcinomas, hyperplasia and patients with normal endometrial tissue, we used the Student t-test. For the comparison of the hypermethylation of individual genes, we used the chi-square test as an exact test to determine whether methylation of individual genes can exhibit independent prognostic significance.

Tab. 1. Setting the annealing temperatures, GC pairs content and length of primers.

External

GENE	Fwd primer	Tm	% GC	Length
GSTP1	GGGATTTTAGGGYGTTTTTTTG	56.4	39	22
Ecad	GTGTTTTYGGGGTTTATTTGGTTGT	59.7	38	25
RASSF 1A	TTGAGTTGYGGGAGTTGGTATT	56.0	40	22
GENE	**Rev primer**	**Tm**	**% GC**	**Length**
GSTP1	ACCTCCRAACCTTATAAAAATAATCCC	59.0	35	27
Ecad	TACRACTCCAAAAACCCATAACTAACC	60.0	39	27
RASSF 1A	CCCAAATAAATCRCCACAAAAAT	54.0	39	23

Internal Methylated

GENE	Fwd primer	Tm	% GC	Length
GSTP1	TTCGGGGTGTAGCGGTCGTC	64.0	65	20
Ecad	TGTAGTTACGTATTTATTTTTAGTGGCGTC	59.0	33	30
RASSF 1A	GTGTTAACGCGTTGCGTATC	56.0	50	20
GENE	**Rev primer**	**Tm**	**% GC**	**Length**
GSTP1	GCCCCAATACTAAATCACGACG	59.0	50	22
Ecad	CGAATACGATCGAATCGAACCG	62.0	50	22
RASSF 1A	AACCCCGCGAACTAAAAACGA	58.0	50	21

Internal Unmethylated

GENE	Fwd primer	Tm	% GC	Length
GSTP1	GATGTTTGGGGTGTAGTGGTTGTT	59.0	46	24
Ecad	TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT	59.0	26	34
RASSF 1A	TTTGGTTGGAGTGTGTTAATGTG	60.0	39	23
GENE	**Rev primer**	**Tm**	**% GC**	**Length**
GSTP1	CCACCCCAATACTAAATCACAACA	58.0	42	24
Ecad	ACACCAAATACAATCAAATCAAACCAAA	61.0	29	28
RASSF 1A	CAAACCCCACAAACTAAAAACAA	58.0	34	23

RESULTS

The study included 122 patients, which in the years 2010–2012 were subjected to the Dpt. of Gynaecology and Obstetrics JFM CU for surgical treatment. Patients on the basis of the histopathological finding were divided into four groups: a control group with healthy endometrium (35), a group with simplex hyperplasia of endometrium (21), a group with atypical hyperplasia (16) and a group with endometrial carcinoma (51).

We found a significantly higher age of patients with endometrial cancer compared to the patients with healthy endometrial tissue and simplex endometrial hyperplasia (Figure 1). There was no relevant difference in age between the simplex and atypical hyperplasia group. Also the parity of patients was significantly lower in patients with endometrial carcinoma and atypical hyperplasia compared to the control group of healthy patients. As far as smoking and age of menarche is concerned there were no significant differences between the four groups. Nevertheless, information on smoking is imprecise, given that it relates to the current status of the patient and not the previous ten-year period (Table 2).

During the analysis of the methylation of GSTP, Ecad and RASF genes (Table 3 and 4) we found a statistically significant correlation between the RASF gene and endometrial carcinoma. The gene was methylated more often in carcinoma compared to healthy tissue or simplex hyperplasia. Other correlations between the four groups of patients were insignificant. The Ecad and GSTP genes did not show any relevant pattern of methylation in our study even in endometrial cancer.

In patients with a confirmed diagnosis of endometrial cancer, we have established the presence of all

Tab. 2. Age, menarche, parity and smoking pattern in different groups of patients.

	Healthy endometrium	Simplex hyperplasia	Atypic hyperplasia	Endometrial cancer
Age	49.34±9.93	52.52±8.3	56.75±8.84	63.00±9.95
Menarche	13.22±1.99	13.14±2.03	14.13±1.75	13.36±1.19
Parity	1.40±1.17	1.33±0.97	0.19±0.4	1.1±1.07
Smoking	20%	33.33%	43.80%	36.00%

three methylated genes in six women out of 50 (12%); two genes were methylated in twenty-three patients (46%); one gene was methylated in 15 patients (30%) and no unmethylated gene occurred only in 6 patients (12%). In the group of patients with confirmed endometrial hyperplasia (simplex / atypical), we found one gene promoter hypermethylation in 10/3 (47.6%/ 18.75%) and two hypermethylated genes were present in 5/6 (23.8%/37.5%). All three genes were methylated in 1/4 (4.8%/19.05%). When we tried to correlate two or three genes as one group with histological findings, we found a significantly higher methylated pattern of GSTP-RASF in endometrial cancer compared to healthy endometrial tissue. Other combinations of genes were insignificant.

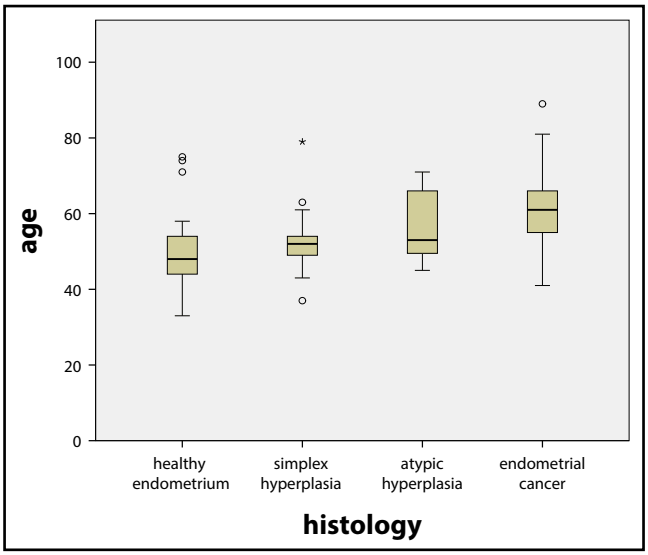

Fig. 1. Age of the patients in patients with intact endometrium, hyperplasia and endometrial cancer.

DISCUSSION

Hypermethylation and inactivation of genes involved in DNA repair, for example GSTP1, can serve as an indicator of the development of cancer with increased susceptibility to carcinogenesis, and thus predispose the person to other mutations and to DNA damage (Lee *et al.* 1994). The GSTP gene is located on chromosome 11q13. Glutathione-S-transferase enzyme has several enzymatic functions (metabolism, stress response, proliferation, apoptosis, oncogenesis, progression of malignancies, chemoresistance) and non-enzymatic (interaction with cellular proteins) (Meng & Freudenheim 2010). In our work we detected GSTP methylated in 22 patients with endometrial carcinoma from 41 patients in the set (56.9%). GSTP was detected in 11 patients with endometrial hyperplasia out of 23 patients in the set (45.8%) and in 10 cases the Promoter GSTP gene was methylated in patients with healthy endometrium (out of 27 patients in the set – 37.0%). In the work of the authors Chan *et al.* (2005) who analysed 97 patients with carcinoma, thirty patients were posi-

Tab. 3. Number of samples (also in %) with unmethylated and methylated GSRP, Ecad and RASF1 genes.

	No. of cases	GSRP gene		Ecad gene		RASF1 gene	
		methyl.	unmethyl.	methyl.	unmethyl.	methyl.	unmethyl.
Normal endometrium	35	15 42.86%	20 57.14%	7 20%	28 80%	12 34.29%	23 65.71%
Simplex hyperplasia	21	12 57.14%	9 42.86%	3 14.29%	18 85.71%	7 33.33%	14 66.67%
Atypic hyperplasia	16	9 56.25%	7 43.75%	8 50%	8 50%	10 62.5%	6 37.5%
Endometrial cancer	50	29 58%	21 42%	13 26%	37 74%	39 78%	11 22%

Tab. 4. Number of samples with unmethylated and methylated GSRP, Ecad and RASF1 genes in different stages of endometrial cancer.

Stage of endometrial carcinoma	No of cases	GSRP gene		Ecad gene		RASF1 gene	
		methyl.	unmethyl.	methyl.	unmethyl.	methyl.	unmethyl.
1A	14	9	5	3	11	12	2
1B	20	13	7	4	16	16	4
1C	5	1	4	3	2	4	1
2A	5	4	1	1	4	4	1
2B	3	2	1	0	3	2	1
3A	3	1	2	1	2	2	1

tive with methylated gene promoter GSTP. The authors used a similar methodology to the one used in our work, and obtained similar results, indicating a well-established methodology of our parties.

The Ecad gene is located on chromosome 16q22.1 and belongs to the family of calcium-dependent cell adhesion molecules. Located predominantly in epithelial cells the gene forms a complex with catenin and its role is in maintaining the integrity of the cells in the epithelial layer (Reinhold *et al.* 2010). Ecad loss of expression of the gene results in a loss of homogeneity of the tissue and in the case of cancer may lead to metastasis. Methylation of the gene connected to reduced expression of Ecad has been described in various types of cancers with a frequency of 20–70% (gastric, breast, bladder, colon, rectum, oesophagus, ovary, thyroid, and prostate). Suppression of its function by methylation leads to increased invasiveness and a metastatic process (Voutilainen *et al.* 2006). In our work we detected Ecad methylated in 13 patients with endometrial carcinoma from 50 patients in the set (25.49%). Ecad was detected in 8 patients with atypical endometrial hyperplasia, in 3 patients with simplex endometrial hyperplasia and 7 patients with a healthy endometrium from 35 patients in the set (20%). The authors Tsuyoshi Saito *et al.* (2003) analysed 98 patients with endometrial cancer, 17 patients with hyperplasia and 21 patients with normal tissue of the endometrium. Their results are comparable with our study: in their study the Ecad gene promoter methylation was confirmed in 37/98 patients. They confirmed paradoxically gene promoter methylation Ecad in endometrial hyperplasia and healthy endometrial tissue.

The RASSF1 gene is located on chromosome 3p21.3. Ras GTP-ase are a family of molecules that control cell proliferation, differentiation, motility and apoptosis by extracellular signals (Pfeifer & Dammann 2005). RASSF1A methylation is one of the most common forms of epigenetic inactivation of gene expression. Aberrant promoter methylation of the RASSF1A gene combined with LOH at 3p may play a more important role in endometrial carcinogenesis. Moreover, promoter methylation of RASSF1A has also been inconsistently found to be associated with advanced stage, recurrence and survival for endometrial cancer (Liao *et al.* 2008; Pijnenborg *et al.* 2007; Jo *et al.* 2006). In our work the gene methylation in endometrial carcinoma was present in 39 patients out of 50 patients in the set (78%). When comparing endometrial cancer and endometrial hyperplasia and healthy tissue, the frequency of the RASSF1A gene promoter methylation was significantly higher in the first group of patients (p=0.001). The authors Arafa *et al.* (2008) investigated gene promoter methylation of RASSF1A in the set of sample archives in 39 endometrial cancer samples, 14 samples with atypical hyperplasia and 11 samples of normal endometrium. Methylation of the RASSF1A gene was confirmed in 29 out of 39 endometrial carcinomas (74%) and in 7 out of 14 patients with hyperplasia. The difference between endometrial cancer and normal tissue (74% to 36%) indicates present multifocal epigenetic alteration during endometrial carcinogenesis. In this work RASSF1A gene promoter methylation was detected by quantitative methylation specific PCR (QMSP).

As already mentioned above, some of the works reported differences in the detection of hypermethylation. At present, the discussion is essential about these differences as well as the outcome of different methodological approaches and patient files. Significant differences in the interpretation of results occur in cases when comparing the data obtained by quantitative (QMSP) or conventional methylation specific PCR (MSP). QMPS has much more stringent conditions, since its detection is bound to detect CpG sites not only by ordinary primers, but also labelled methylation-specific probe binding to the amplified target DNA. Crucial for the interpretation of QMSP are the limits of negativity (cut-off), which are set by the authors (Jeronimo *et al.* 2001).

CONCLUSION

Gynaecological malignancies are a group of diseases in which the prognosis is dependent on subtle genomic, epigenetic and proteomic changes. Using molecular biological techniques, including acetylation and methylation analysis and proteomic techniques, has become an important tool not only in basic research, but also in deciding on appropriate therapy. Epigenetics and proteomics are currently offering new and very promising approaches not only to identifying specific biomarkers and their subsequent screening, but also for the accurate characterization and typing of tissues, including tumor tissues.

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