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Eva Dvořáková 2014

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MOLECULAR BIOLOGICAL CHANGES IN ENDOMETRIAL CARCINOMA

MOLEKULÁRNĚ BIOLOGICKÉ ZMĚNY U KARCINOMU ENDOMETRIA

MUDr. Eva Dvořáková

Supervisor: doc. MUDr. Jiří Špaček, Ph.D., IFEPAG

Hradec Králové, March 2014 Defence on: …………………………..

Declaration

I declare hereby that this dissertation thesis is my own original work and that I indicated by references all used information sources. I also agree with depositing my dissertation in the Medical Library of the Charles University in Prague, Faculty of Medicine in Hradec Králové and with making use of it for study and educational purpose provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

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Hradec Králové, March 2014

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„Anyone can set up their Lambarene.“ (Albert Schweitzer)

Abbreviation

APC – Adenomatous Poliposis Coli

ATM – Ataxia Telangiectasia Mutated

BMI – Body Mass Index

BRCA1, 2 – Breast Cancer 1, 2

CDH13 – H-cadherin

COMT – Catechol-O-Methyl Transferase

CpG – Cytosine-phosphate-Guanine

CT – Computed Tomography

DNA – Deoxyribonucleic Acid

dNTPs – Deoxynucleotide Triphosphates

EC – Endometrial Carcinoma

EDTA – Ethylenediaminetetraacetic Acid

ER – Estrogen Receptor

FFPE – Formalin-Fixed, Paraffin-Embedded

FIGO – The International Federation of Gynecology and Obstetrics

FNHK – Faculty Hospital in Hradec Králové

GATA – Transcription Factor GATA

GPR54 – Kisspeptin Receptor

GSTP1 – Glutathione S-Transferase P

GTP – Guanosine Triphosphate

HER-2/neu – Human Epidermal Growth Factor Receptor 2

hMLH1 – Human MutL Homolog 1

HNPCC – Hereditary Non-Poliposis Colorectal Cancer

HOXA10, 11 – Homeobox A10, 11

HSPA2 – Heat Shock 70kDa Protein 2

IUD – Intrauterine Device

K-ras – Kirsten Rat Sarcoma Viral Oncogene Homolog

miRNA – Micro RNA

MMR – Mismatch Repair

MRI – Magnetic Resonance Imaging

MSH6 – MutS Homolog 6

MSI – Microsatellite Instability

MS-MLPA – Methylation Specific Multiplex Ligation-dependent Probe Amplification

MSP – Methylation Specific PCR

PCR – Polymerase Chain Reaction

PET – Positron Emission Tomography

PR – Progesterone Receptor

p16 – Cyclin-dependent Kinase Inhibitor 2A

p53, TP53 – Cellular Tumor Antigen p53

p73, TP73 – Tumor Protein 73

PER1 – Period Circadian Protein Homolog 1

PTEN – Phosphatase and Tensin Homolog

RARB2 – Retinoic Acid Receptor B2

RASSF1A – Ras Association Domain-containing Protein 1

RB1 – Retinoblastoma Protein

RNA – Ribonucleic Acid

RSK4 – Ribosomal s6 Kinase 4

SESN3 – Sestrin 3

SFN – 14-3-3 protein sigma

SOCS2 – Suppressor of Cytokine Signaling 2

STK11 – Serine/Threonine Kinase 11

THBS1, 2 – Thrombospondin-1, 2

TITF1 – Thyroid Transcription Factor 1

TNM – T (tumor), N (node), M (metastasis)

TVU – Transvaginal Ultrasound

VEGF – Vascular Endothelial Growth Factor

VHL – Von Hippel–Lindau Tumor Suppressor

WHO – World Health Organization

Wnt – Wingless/Int

WT1 – Wilm’s Tumor 1

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1 INTRODUCTION

Neoplastic diseases represent one of the most common causes of death in both the Czech Republic and worldwide. Knowledge of the molecular biological characteristics of the tumor tissue, including epigenetics plays a particularly important role in modern diagnosis and treatment of cancer.

The most significant characteristic of tumor cells is their rampant, most chaotic growth, which is the result of failure of regulatory processes in the cell. By this, two critical events influencing tissue homeostasis are affected: cell cycle and apoptosis. The tumor cells are always characterized by disorders of this balance, which is caused by increasing the rate of cell cycle, and resistance to apoptosis induction. Although the process of neoplastic transformation in various tissues differs, neoplastic tissue always exhibits some of the same characteristics: autonomy in the production of growth factors, reduced sensitivity to inhibition signals, failure of apoptosis, high replicative potential, disorders in deoxyribonucleic acid (DNA) repair, angiogenesis and ability of tissue invasion and metastasis.

Malignant tumors are formed on the basis of genetic alterations with the contribution of epigenetic changes that affect mainly the expression of genetic information. Genetic changes- mutations (changes in the primary structure of DNA) occur continuously in all cells of the organism as a result of exogenous and endogenous factors. For malignant transformation is critical damage of mainly three groups of genes: proto-oncogenes, tumor suppressor genes and DNA repair genes. Mutations in oncogenes which cause the permanent activation, or their overexpression allow cell division, which in the affected cells becomes autonomous action. The accelerated proliferation of tumor cells is also affected by the loss of negative regulatory signals of cell division resulting from the elimination of tumor suppressor genes by mutations or hypermethylation of the promoter regions. Furthermore, the failures of repair mechanisms that allow the affected cell to tolerate the emergence of mutations that would normally lead to cell cycle arrest or apoptosis. Given the known characteristics of tumor cells such as unlimited replicative potential, loss of contact inhibition and incomplete expression of phenotypic markers of fully

differentiated cells in the tissue is likely that the initial clone of the transformed cells form progressive accumulation of genetic and epigenetic alterations at the level of stem and progenitor cells than the majority population of fully mature tissue cells.

For effective treatment of cancer is essential early and accurate diagnosis with optimization of therapy options and reduction of side effects. Early diagnosis of cancer along with individualized therapy can reduce mortality and improve perspective and quality of life of the patients. Gynecological cancers are a group of diseases in which the prognosis is dependent on genetic, epigenetic and proteomic changes. Using molecular biology techniques, including DNA methylation analysis is becoming an increasingly important tool not only for basic research but also in deciding appropriate therapy.

The theoretical part of the thesis is focused on endometrial cancer, which is one of the most common malignancies of the female genital tract. The present work provides an overview of endometrial carcinoma, including the role of genetic and epigenetic changes in endometrial carcinogenesis. The experimental part of the work is focused on the analysis of K-ras mutation and methylation of promoter regions of selected tumor suppressor genes in a group of patients with endometrioid type of endometrial cancer compared with normal endometrial tissue. To detect K-ras mutations was used method K- ras StripAssay™, ViennaLab Diagnostics GmbH. For the monitoring of alterations in DNA methylation was used Methylation Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) and Methylation Specific PCR (MSP).

2 ENDOMETRIAL CARCINOMA

2.1 EPIDEMIOLOGY

Endometrial carcinoma is the most commonly diagnosed gynecological malignance with approximately 150 000 cases annually worldwide. Approximately 90% of cases are sporadic, and the remaining 10% are hereditary (*Okuda T, et al., 2010*). The incidence has increased with lifestyle and environmental changes. In 2010, endometrial cancer developed in 1870 women (the incidence of 34.9 per 100 000 women), and 468 women died from this cancer in the Czech Republic (Fig. 1).

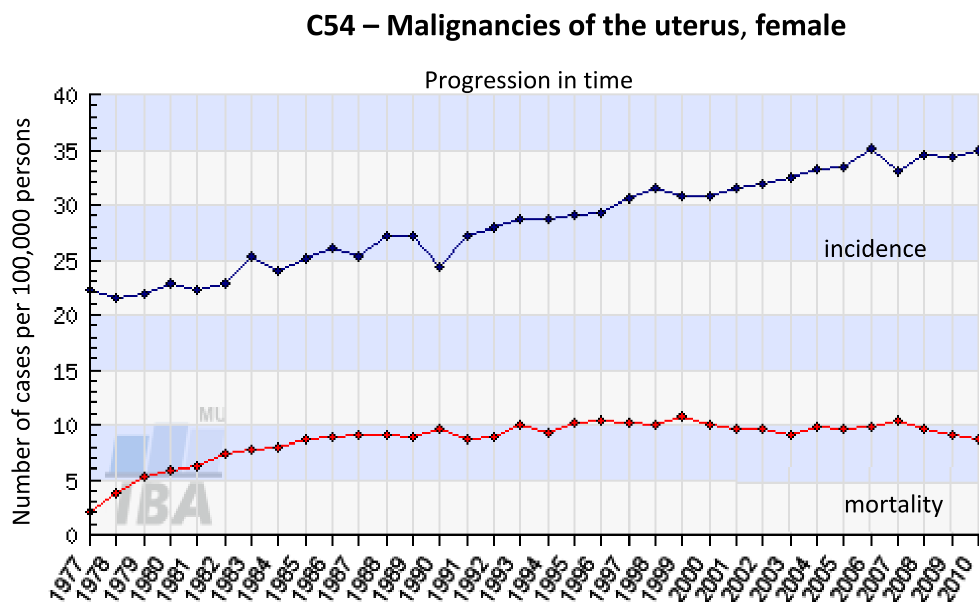

Figure 1. Incidence and mortality of endometrial cancer in the Czech Republic in 1977 - 2010 (www.svod.cz)

In the Czech Republic, the incidence is the highest in developed countries (Fig. 2).

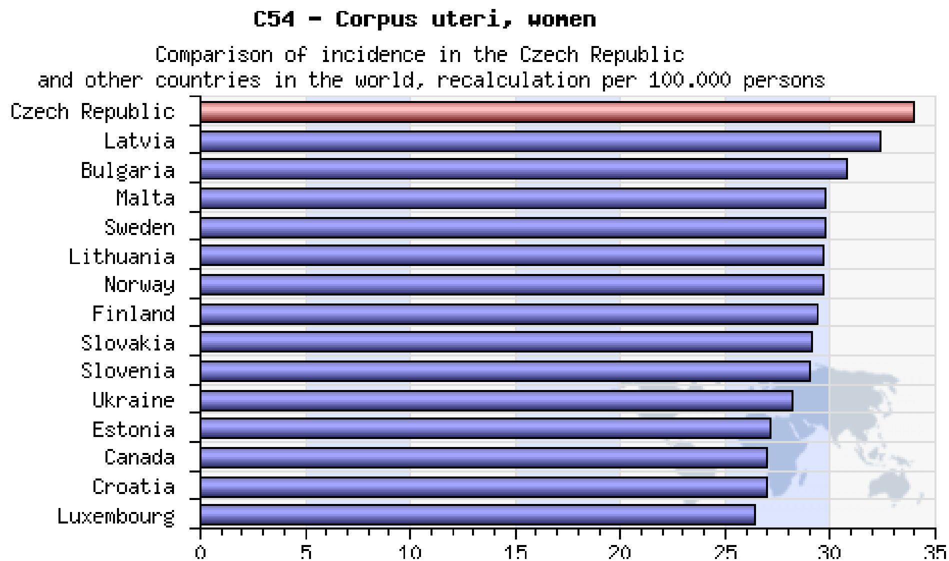

Figure 2. Incidence of endometrial cancer comparing with other countries (www.svod.cz)

2.2 RISK FACTORS

Multiple risk factors for endometrial cancer have been identified (Tab. 1) (*Cibula D, et al., 2009*). The risk of endometrial carcinoma increases with age. The vast majority of cases are diagnosed after the menopause, with the highest incidence around the seventh decade of life (Fig. 3). Approximately 5% of women will have adenocarcinoma before the age of 40 years, and 20-25% will be diagnosed before menopause (*DiSaia PJ and Creasman WT, 2007*).

Factors increasing risk	Factors decreasing risk
Age	Multiparity
Late menopause	Oral-contraceptive use
Nulliparity, infertility	Smoking
Obesity	Intrauterine device (IUD) with progestagens
Diabetes mellitus	Physical activity
Hypertension	Diet rich on fiber
Unopposed estrogen use	
Estrogen-producing tumors	
Tamoxifen use	
Atypical endometrial hyperplasia	
Polycystic ovary syndrome	
Fatty diet	
Lynch II syndrome	
Pelvic irradiation	
Caucasian race	

Table 1. Risk factors of endometrial carcinoma

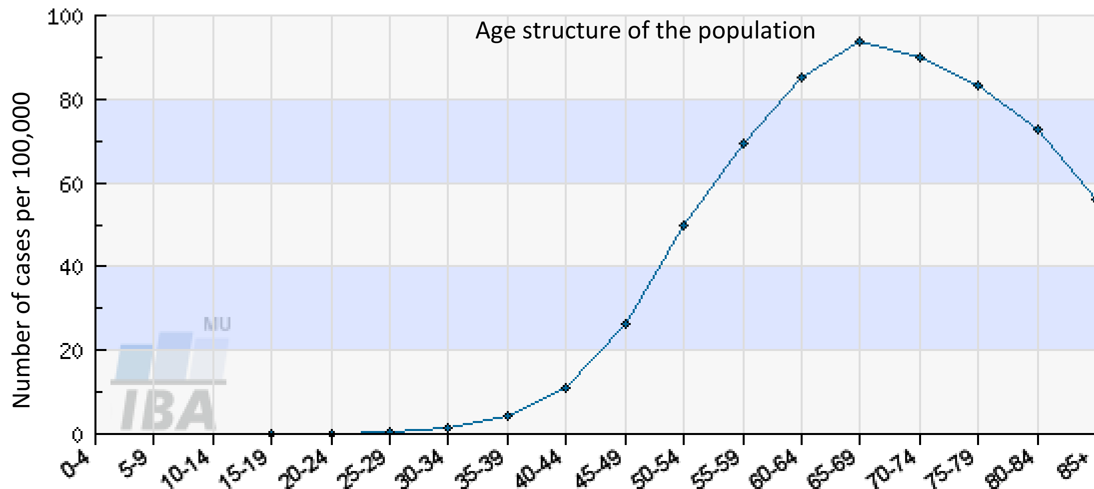

Figure 3. Age-incidence curve for endometrial carcinoma (www.svod.cz)

Obesity, nulliparity and late menopause are factors classically associated with endometrial carcinoma. If a patient is nulliparous and obese and reaches menopause at age 52 years or later, she appears to have a 5-fold increase in the risk of endometrial carcinoma (*DiSaia PJ and Creasman WT, 2007*). Nulliparity is a risk factor that is more important if infertility is also present; grand multiparity protects (*Klip H, et al., 2000*). Excessive fat consumption and overweight- defined as Body Mass Index (BMI) of at least 25kg/m^2, are important risk factors present in almost 50% of women with endometrial carcinoma (*Zeleniuch-Jacquotte A, et al., 2001; Kaaks R, et al., 2002*). A BMI above 25kg/ m^2 doubles a risk of endometrial carcinoma, and a BMI above 30kg/ m^2 triples the risk (*Calle EE, et al., 2003*). Conversely, physical activity and diet rich on fiber decrease the risk. Diabetes mellitus and hypertension are frequently associated with endometrial carcinoma. The relationship of unopposed estrogen use and endometrial carcinoma is well documented. Fortunately, the addition of a progestin appears to be protective (*DiSaia PJ and Creasman WT, 2007*). Contraceptive pills containing estrogens and progestagens lower the endometrial carcinoma risk (*Deligeoroglou E, et al., 2003*). The protection occurred in women who used oral contraceptives for at least 12 months, and protection continued for at least 10 years

after oral contraceptive use (*DiSaia PJ and Creasman WT, 2007*). Estrogen-producing tumors are an uncommon risk factor. An additional endometrial carcinoma risk has been related to the use of tamoxifen for breast cancer. The drug triples the risk of endometrial carcinoma and also increases the chance of developing benign endometrial lesions (*Neven P, et al., 1998*). Endometrial carcinoma appears sooner than in non-tamoxifen users (*Cuzick J, et al., 2003; Slomovitz BM, et al., 2004*). Cigarette smoking reduces risk of endometrial carcinoma because it affects estrogen production and metabolism (*Viswanathan AN, et al., 2005*). Endometrial carcinoma can also be a part of a hereditary Lynch II syndrome- Hereditary non-poliposis colon cancer (HNPCC) caused by an inherited mutation in the mismatch repair (MMR) gene family. Women with HNPCC syndrome have lifetime risks of endometrial carcinoma of 40-60% (*Cibula D, et al., 2009*). Endometrial carcinoma is the most common malignancy in patients with HNPCC (*Koornstra JJ, et al., 2009*). Patients with Lynch syndrome associated endometrial carcinoma are approximately two decades younger than those with sporadic carcinoma (*Marra G and Boland CR, 1995*).

2.3 PATHOLOGY

About 75% of all endometrial carcinomas are of endometrioid type. Several variants of endometrioid carcinoma have been described, such as secretory, villoglandular, with squamous differentiation, with ciliated cells (*Cibula D, et al., 2009*). Most endometrioid carcinomas are well to moderately differentiated and arise on a background of endometrial hyperplasia. These tumors, also known as type 1, are associated with long-duration unopposed estrogenic stimulation (*Potischman N, et al., 1996*). Mucinous adenocarcinomas are quite rare representing 1-9% of all endometrial carcinomas, and are also considered type 1 carcinomas (*Cibula D, et al., 2009*). Type 1 tumors usually express estrogen receptors (ER) and/or progesterone receptors (PR), are of low histopathological grade, and clinically are characterized by a favorable behavior (*Liu FS, 2007*). About 10-20% of sporadic endometrial carcinomas, designated as type 2 carcinomas, are not estrogen driven, and most arise in the background of atrophic endometrium (*Sherman ME, et al., 1995*). Type 2 carcinomas usually occur at an older age, approximately 5-10 years later than type 1 tumors, ER and PR expression

is usually negative or weakly positive. These tumors are characterized by an aggressive clinical course and poor prognosis. The histological type is either poorly differentiated endometrioid or non-endometrioid including serous and clear-cell carcinomas. Both serous and clear-cell carcinomas are frequently associated with endometrial intraepithelial carcinoma, which is considered the putative precursor for these tumors (*Ambros RA, et al., 1995; Lax SF, et al., 1998*). Serous adenocarcinoma occurs in 5-10% of all endometrial carcinomas (*Cibula D, et al., 2009*), and represents the most aggressive type of non-endometrioid endometrial carcinoma (*Carcangiu ML and Chambers JT, 1992; Slomovitz BM, et al., 2003*). Clear-cell adenocarcinoma represents only 1-5% of all endometrial carcinomas. Other variants of endometrial carcinoma (squamous-cell, transitional-cell, small-cell, undifferentiated carcinoma) are rare (*Cibula D, et al., 2009*). Carcinosarcoma has been considered a special subtype of endometrial cancer. The monoclonal nature of carcinosarcoma points to an endometrial origin (*Ronnett B, et al., 2002*).

2.4 DIAGNOSIS

Abnormal uterine bleeding is the most frequent symptom of endometrial carcinoma. The probability of endometrial carcinoma in women presenting with postmenopausal bleeding is 5-10%, but the chances increase with age and risk factors (*Gredmark T, et al., 1995*). All postmenopausal women with vaginal bleeding and those with abnormal uterine bleeding associated with risk factors for endometrial carcinoma should undergo further diagnostic assessment. Transvaginal ultrasonography (TVU) is considered as the first step in any woman presenting with abnormal uterine bleeding (*Clark TJ, 2004*). Normality for TVU is defined as a thin symmetrical endometrial line of less than 4-5 mm double endometrial thickness (*Smith-Bindman R, et al., 1998; Gupta JK, et al., 2002*). The value of TVU in symptomatic premenopausal women and those using hormone-replacement therapy is lower because the endometrial thickness varies with circulating concentrations of female steroid hormones (*Van den Bosch T, et al., 2003*). Endometrial carcinoma is mostly diagnosed histologically from endometrial tissue obtained from Pipelle biopsy, curettage or hysteroscopy. Other imaging

techniques magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) could be used in clinical staging.

2.5 STAGING

The International Federation of Gynecology and Obstetrics (FIGO) introduced in 1988 and updated in 2009 the staging system for endometrial cancer, which is surgical-pathological and defined after total abdominal hysterectomy, bilateral salpingo-oophorectomy, pelvic and para-aortic lymphadenectomy, and peritoneal cytology (Tab. 2).

Primary tumor (T)		
TNM	*FIGO stages*	*Surgical-pathologic findings*
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
Tis*		Carcinoma in situ (preinvasive carcinoma)
T1	I	Tumor confined to corpus uteri
T1a	IA	Tumor limited to endometrium or invades less than one half of the myometrium
T1b	IB	Tumor invades one half or more of the myometrium
T2	II	Tumor invades stromal connective tissue of the cervix but does not extend beyond uterus**
T3a	IIIA	Tumor involves serosa and/or adnexa (direct extension or metastasis)
T3b	IIIB	Vaginal involvement (direct extension or metastasis) or parametrial involvement
	IIIC	Metastases to pelvic and/or para-aortic lymph nodes
	IV	Tumor invades bladder mucosa and/or bowel mucosa, and/or distant metastases
T4	IVA	Tumor invades bladder mucosa and/or bowel mucosa (bullous edema is not sufficient to classify a tumor as T4)

***FIGO no longer includes stage 0 (Tis)**
****Endocervical glandular involvement should only be considered as stage I and no longer as stage II**

Regional lymph nodes (N)		
TNM	*FIGO stages*	*Surgical-pathologic findings*
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1	IIIC1	Regional lymph node metastasis to pelvic lymph nodes
N2	IIIC2	Regional lymph node metastasis to para-aortic lymph nodes, with or without positive pelvic lymph nodes

Distant metastasis (M)		
TNM	*FIGO stages*	*Surgical-pathologic findings*
M0		No distant metastasis
M1	IVB	Distant metastasis (includes metastasis to inguinal lymph nodes, intraperitoneal disease, or lung, liver, or bone metastases; it excludes metastasis to para-aortic lymph nodes, vagina, pelvic serosa, or adnexa)

Table 2. TNM and FIGO classification for endometrial carcinoma (http://www.nccn.org/professionals/physician_gls/pdf/uterine.pdf)

2.6 PROGNOSIS

Multiple factors have been identified for endometrial carcinoma that appear to have significant predictive value for these women. The most important prognostic features are FIGO stage, histological type and grade of the tumor. The FIGO stage reflects the 5-year survival, which is around 86% for stage I, 66% for stage II, 44% for stage III and 16% for stage IV. Non-endometrioid endometrial carcinoma such as serous, clear cell and undifferentiated carcinomas are associated with poor prognosis than. Patients with type 1 endometrial carcinoma have better prognosis than those with type 2, their 5-year survival is 85% and 58%, respectively (*Cibula D, et al., 2009*). The effects of other non-pathological prognostic factors such as race, age, diabetes and parity have been reviewed elsewhere (*Prat J, 2004*).

2.7 THERAPY

The most important therapy for endometrial carcinoma is surgery. The procedures include acquisition of peritoneal fluid or washing for cytology, total hysterectomy and bilateral salpingo-oophorectomy. In selected cases, there is a place for omentectomy and a thorough retroperitoneal lymph node dissection (*Amant F, et al., 2005*). It appears that in patients with grade 1 tumors, surgery can be limited to total abdominal hysterectomy, bilateral salpingo-oophorectomy and peritoneal cytology examination unless deep myometrial invasion is present (*DiSaia PJ and Creasman WT, 2007*). An experienced oncologist can often determine depth of invasion by gross evaluation in the operating room. Intraoperative visual estimation of the depth of myomatrial invasion is accurate in 90% of cases (*Franchi M, et al., 2000; Kucera E, et al., 2000*). The hysterectomy should be extrafascial. Because of appreciable lymph node metastases in grade 2 and grade 3 disease, it is suggested that a pelvic and para-aortic lymphadenectomy should be added to the surgical procedure described for grade 1 disease (*DiSaia PJ and Creasman WT, 2007*). Complete excision of the nodes located around the iliac vessels and above the obturator nerve allows identification of 90% of node-positive patients (*Benedetti-Panici P, et al., 1998*). Laparoscopic staging combined with vaginal hysterectomy appears to be a feasible alternative to classical surgical approach in patients with early stage I or II endometrial carcinoma (*Zapico A, et al., 2005*). Different surgical management is needed for type 2 endometrial carcinoma because of its likelihood of extrauterine disease. The transperitoneal spread of type 2 carcinomas resembles that of ovarian cancer (*Abeler V, 1996*). Women with such lesions therefore need the same surgical management as those with ovarian cancer, including total hysterectomy, bilateral salpingo-oophorectomy, omentectomy, peritoneal biopsy samples and biopsy of any suspect lesions (*Slomovitz BM, et al., 2003; Vergote I and Trimbos B, 2003*). Given the propensity of lymphatic spread, a thorough lymph-node dissection is recommended (*Podratz K and Mariani A, 2003; Huh WK, et al., 2003*).

Indications for radiotherapy are generally in the adjuvant settings. Radical radiotherapy should be applied in patients with contraindications for surgery, or inoperable advanced disease (*Cibula D, et al., 2009*). The goal of adjuvant radiotherapy

is to treat the pelvic lymph-node regions that might contains microscopic disease, as well as the central pelvic region including upper vagina (*Amant F, et al., 2005*). There is a general consensus that patients with low-risk lesions (grade 1-2 and myometrial invasion less than ½ of muscle thickness) can be treated without postoperative radiotherapy (*Cibula D, et al., 2009*). According to the Portec study, pelvic radiotherapy should be considered for local control if lymphadenectomy is not done and if two of the three risk factors (deep myometrial invasion, grade 3 and age over 60 years) are present (*Creutzberg CL, et al., 2000*). The combination of surgery and postoperative radiation is not without risk of serious complications which occur in 1-10% of women, depending on the patient's status, irradiated volume of bowel, bladder, or vagina, radiation dose, fraction size, dose rate, and especially in combination with lymph-node resection. Modern radiotherapy techniques are recommended to limit long-term side-effects (*Amant F, et al., 2005*).

Systemic chemotherapy can be used as a palliative therapy in metastatic and recurrence disease (*Cibula D, et al., 2009*). Chemotherapy, mainly with anthracyclines, platinum compounds, and taxanes, renders high response rates although this unfortunately translates in only modest improvements in progression-free and overall survival (*Hogberg T, 2011*). Progesterone has been the cornerstone of hormonal treatment of metastatic disease, and response is related to the presence of steroid-hormone receptors. Response rates range from 15% to 20% (*Whitney CW, et al., 2004*). Locally released progesterone could be an option in women with inoperable disease or those wishing to preserve fertility if they have an early-stage low-grade lesion (*Bahamondes L, et al., 2003*).

There is no formal recommendation regarding the monitoring and follow-up of the patients with endometrial carcinoma. It was observed that follow-up intervals were variable between studies (ranging from 12 to 32 consultations during a five-year follow-up period), and the tests performed to detect recurrences consisted mainly of physical examination, vaginal cytology, and chest radiography. The use of ultrasound, computed tomography, and cancer antigen 125 (CA 125) levels were not used, in general, as part of the routine follow-up studies (*Owen P and Duncan ID, 1996*).

2.8 PATHOGENESIS

Currently, two different pathways are distinguished for carcinogenesis of sporadic endometrial cancer. In 1983, Bokhman introduced his dualistic model of endometrial tumorigenesis based on clinical and pathological characteristics (*Bokhman JV, 1983*). This hypothesis was subsequently broadened by the inclusion of molecular aspects, approximately a decade later. A molecular basis for the development of malignant tumors was introduced by Fearon and Vogelstein based on the progression of colorectal adenoma to carcinoma (*Fearon ER and Vogelstein B, 1990*). According to this progression model, malignant tumors developed through a series of precursor lesions accompanied by various genetic alteration. Genetic aberrances such as variations in gene expression and mutation in cancer-related genes have been identified, but this does not fully explain canceration in endometrial tissue. The morphologic and clinical differences are paralleled by genetic distinctions, in that type 1 and type 2 carcinomas carry mutations of independent sets of genes. Most type 2 cancers contain mutations of cellular tumor antigen p53 (p53), while type 1 carcinomas contain larger number of genetic changes. Common genetic changes in endometrioid type of endometrial carcinoma include, but are not limited to, microsatellite instability, or specific mutation of phosphatase and tensin homolog (PTEN), kirsten rat sarcoma viral oncogene homolog (K-ras), and β-catenin (*Hecht JL and Mutter GL, 2006*). The most frequent genetic alterations in both types of endometrial carcinoma are shown in Table 3.

Genetic alteration	Type 1 carcinoma (%)	Type 2 carcinoma (%)
PTEN inactivation	50-80	10
K-ras mutation	15-30	0-5
B-catenin mutation	20-40	0-3
Microsatellite instability	20-40	0-5
p53 mutation	10-20	80-90
Her2/neu	10-30	40-80
p16 inactivation	10	40
E-cadherin	10-20	60-90

Table 3. Genetic alterations in endometrial cancer- percentage frequency of genetic mutations identified in type 1 and 2 of endometrial cancers (*Bansal N, et al., 2009*)

The most frequently altered gene in endometrioid carcinoma is PTEN, which is located on chromosome 10 and codes for a protein with tyrosine kinase function (*Mutter GL, 2001*). Mutation of PTEN gene is associated with early stage and favorable prognosis (*Risinger JI, et al., 1998*). Microsatellite instability (MSI) is another important genetic alteration in endometrioid carcinoma, occurring in about 20-45% of cases (*MacDonald ND, et al., 2000*). MSI also represents an early event in endometrial carcinogenesis and has been demonstrated in precancerous lesions. Interestingly, higher rates of mutations (60% to 80%) in the PTEN gene have been described in tumors with MSI compared to tumors without MSI (24% to 35%) (*Bilbao C, et al., 2006*). Whereas PTEN, MSI, and K-ras mutations often coexist with each other, mutations in β-catenin are usually seen alone (*Saegusa M, et al., 2001*). β-catenin, a component of the E-cadherin unit of proteins, is important for cell differentiation, maintenance of normal tissue architecture, and signal transduction. β-catenin also acts as a downstream transcriptional activator in the Wingless/Int (Wnt) signal transduction pathway. β-catenin mutation is significantly more common in endometrioid lesions (31% to 47%) compared with non-endometrioid histology (0% to 3%) (*Moreno-Bueno G, et al., 2002*). The exact function of β-catenin in endometrial tumorigenesis remains unknown (*Doll A, et al., 2008*). The most common genetic alteration in type 2 carcinomas is in the tumor suppressor gene p53, located on chromosome 17 (*Lax S, et al., 2000*). Mutations in p53 are present in about 90% of serous carcinomas. The exact mechanism for the cause of this mutation is still unclear (*Doll A, et al., 2008*). Other frequent genetic alterations in type 2 endometrial cancers are inactivation of cyclin-dependent kinase inhibitor 2A (p16) and overexpression of human epidermal growth factor receptor 2 (HER-2/neu) (*Doll A, et al., 2008*). The p16 tumor suppressor gene is located on chromosome 9p21 and encodes for a cell cycle regulatory protein. Thus, inactivation of p16 leads to uncontrolled cell growth. p16 inactivation was found in 45% of serous carcinomas and some clear cell cancers. HER-2/neu is an oncogene that encodes for a transmembrane receptor tyrosine kinase involved in cell signaling. HER-2/neu overexpression and gene amplification were found in about 45% and 70% of serous carcinomas, respectively (*Chon HS, et al., 2006*). E-cadherin is a transmembrane protein with five extracellular domains and an intracellular domain that connects to the actin cytoskeleton through a complex with cytoplasmic catenin. Decreased

expression of E-cadherin is associated with a loss of cell-cell cohesive forces and has been shown to precede tumor cell motility, a characteristic of tumor cells with high metastatic potential (*Sträuli P and Haemmerli G, 1984*). E-cadherin negative tumors are more likely to be poorly differentiated or non-endometrioid and are associated with poorer prognosis (*Holcomb K, et al., 2002; Mell LK, et al., 2004*).

One of the first genetic alterations described in endometrial carcinoma, which are present in about 20–30% of endometrioid carcinomas, are mutations of the K-ras proto-oncogene (*Enomoto T, et al., 1990; Caduff RF, et al., 1995*). They are predominantly found in exon 1 (codons 12and 13) and rarely in exon 2 (codon 61) (*Semczuk A, et al., 2001*). K-ras proto-oncogene encodes for a membrane guanosine triphosphatase and is largely related to tumor growth a differentiation. The presence of K-ras mutations in 16% of the cases of endometrial hyperplasia indicates that K-ras mutations may represent an early event in endometrial carcinogenesis (*Sasaki H, et al., 1993*). There is evidence that the development of endometrioid carcinoma resembles the Vogelstein progression model for colorectal carcinoma, where K-ras mutations occur during the step from atypical hyperplasia to grade 1 endometrial carcinoma, and mostly during the progression to less differentiated tumors (Fig. 17) (*Lax SF, 2004*). However, the role of the K-ras mutations in endometrial carcinogenesis is not yet fully understood.

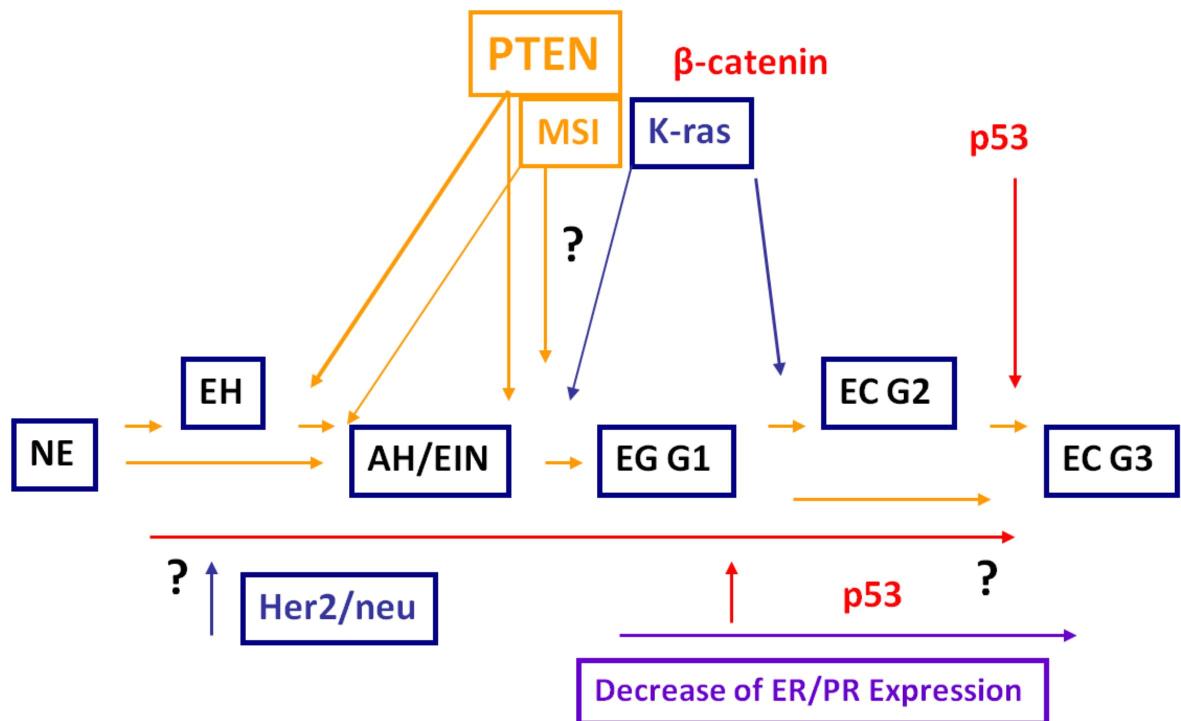

Figure 4. A putative progression model for endometrioid adenocarcinoma developing through atypical endometrial hyperplasia. *NE* normal endometrium, *EH* endometrial hyperplasia without atypia, *AH* atypical endometrial hyperplasia, *EIN* endometrial intraepithelial neoplasia, *EC* endometrioid carcinoma (grade 1–3)(*Lax SF, 2004*).

Epigenetic changes are now being examined. In particular, aberrant DNA methylation is thought to play a key role in endometrial carcinogenesis (*Cannistra SA, 2004*). Epigenetics can be described as stable alteration in gene expression potential that takes place during development and cell proliferation, without any changes in gene sequence. DNA methylation is one of the most common epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target. Studies have shown that epigenetics plays an important role in carcinogenesis in various organs. DNA methylation in a covalent chemical modification, results in addition of a methyl group at the carbon 5 position of the cytosine ring. Most cytosine methylation occurs in the sequence context 5'CG'3 (*Das PL and Singal R, 2004*). Methylation is mediated by the DNA cytosine methyltransferases. Increased methylation in the transcribed region has a variable effect on gene expression. New model for mechanism of carcinogenesis has been proposed in which hypermethylation of unmethylated cytosine-phosphate-guanine (CpG) islands in the promoter regions of cancer-related genes in normal cells silence these genes and leads to the cells

becoming cancerous (*Muraki Y, et al., 2009*). To date, numerous genes have been found to undergo hypermethylation in cancer. The genes that are susceptible are the genes involved in cell cycle regulation, genes associated with DNA repair, apoptosis, drug resistance, detoxification, angiogenesis and metastasis (*King MC, et al., 2003*).

An epigenetic mechanism has been proposed for development of type 1 endometrial cancer based on DNA MMR deficiency, which is a typical genetic defect in this cancer. The DNA MMR system corrects errors in bases that arise when genes are replicated during cell division and silencing of DNA MMR genes reduces the ability to repair gene mutations. This results in accumulation of cancer-related gene mutations, leading to carcinogenesis. When the mismatch repair system is damaged, MSI also occurs. Microsatellites are DNA sequences of repeating units of between 1 and 5 base pairs. Abnormalities in the MMR system may cause replication errors in the repeating unit, leading to changes in length that are referred to as microsatellite instability (*Bast RC Jr, et al., 1983*). Strong association between MMR gene human MutL homolog 1 (hMLH1) promoter methylation and transcriptional silencing and MSI+ phenotype was reported in sporadic endometrial cancer, particularly in the endometrioid type (*Zighelboim I, et al., 2007; Tao HM and Freudenheim JL, 2010*). Recent study of methylation profile in endometrial tumorigenesis showed that, among 24 tumor suppressor genes, the number of promoter methylated loci increased in the progression from normal endometrium to simple hyperplasia to complex hyperplasia (*Neiminen TT, et al., 2009*). PTEN is reported to be important for the inhibition of cell migration and spreading and focal adhesion (*Tamura M, et al., 1998*). Different studies have shown that PTEN promoter methylation is present in about 20 % of sporadic type 1 endometrial carcinomas (*Salvesen HB, et al., 2001; Salvesen HB, et al., 2004*). Promoter methylation of p16 gene has been observed in some studies in between 11-75% of sporadic endometrial carcinomas (*Wong YF, et al., 1999; Furlan D, et al., 2006; Yang HJ, et al., 2006; Ignatov A, et al., 2008*), however, other studies have reported much lower frequencies (*Nakashima R, et al., 1999, Salvesen HB, et al., 2000; Guida M, et al., 2009*). Ras association domain-containing protein 1 (RASSF1A) is known to induce cell cycle arrest through the Rb-mediated checkpoint (*Agathanggelou A, et al., 2005; Donninger H, et al., 2007*). Promoter methylation of this gene has also been

reported to be present in endometrial carcinoma and associated with reduced expression of RASSF1A (*Liao X, et al., 2008; Pallarés J, et al., 2008; Arafa M, et al., 2008*). Methylation of adenomatous poliposis coli (APC) gene, tumor suppressor gene that regulates β-catenin in the Wnt pathway, E-cadherin and tumor protein p73 (p73) has also been observed (*Banno K, et al., 2006; Yang HY, et al., 2006*). As well as Sprouty 2, kisspeptin receptor (GPR54) and ribosomal s6 kinase 4 (RSK4) (*Cannistra SA, 2004*). Methylation of some other genes have been associated with endometrial carcinoma: homeobox A (HOXA) 10, HOXA11, thrombospondin-2 (THBS2), H-cadherin (CDH13), heat shock 70kDa protein 2 (HSPA2), suppressor of cytokine signaling 2 (SOCS2), period circadian protein homolog 1 (PER1), retinoic acid receptor B2 (RARB2), glutathione S-transferase P (GSTP1), 14-3-3 protein sigma (SFN), sestrin 3 (SESN3), thyroid transcription factor 1 (TITF1) (*Whitcomb BP, et al., 2003; Mhawech P, et al., 2005; Yeh KT, et al., 2005; Yoshida H, et al., 2006*) and catechol-O-methyl transferase (COMT) (*Sasaki M, et al., 2003*). MicroRNAs (miRNA) are short non-coding ribonucleic acids (RNA) of about 18-25 bases that regulates expression of genes. miRNAs have been found to be down regulated by methylation of DNA in various cancers including endometrial carcinoma (*Cannistra SA, 2004*). miR-129-2 and miR152 gene methylation was observed and these miRNAs and their targets may be new targets for treatment of endometrial carcinoma (*Tsuruta T, et al., 2011*).

A large number of methods that can be used for monitoring DNA methylation changes have been described in the literature. Any of these methods can be used in any situation, but it is always important to select the most appropriate method. Frequently the analyzed DNA is isolated from paraffin blocks. For analysis of such DNA the most appropriate method seems to be MS-MLPA. For analysis of DNA isolated from blood, the technique of bisulfite conversion followed by MSP could be used, or sequencing of the amplified products. Early studies of DNA methylation analyzed methylation mainly in tumor tissue, but an increasing number of studies are now using body fluids such as urine, peripheral blood and serum or plasma. The amount of DNA in serum or plasma is very low, which limits the number of available techniques (*Chmelarova M and Palicka V, 2013*). When choosing the method, particular attention must be paid to the quantity and quality of isolated DNA, laboratory options and

equipment, and most importantly cooperation with an experienced molecular biologist.

Endometrial cancer is the most common cancer of the female reproductive tract. The incidence has increased with lifestyle and environmental changes. Similar to other cancers, endometrial cancer has been shown to be a complex disease driven by different factors, including genetic and epigenetic alterations. Understanding these changes underlaying cancer development or progression is important for finding of new standards for both diagnosis and therapy of individual patients.

3 AIMS OF THE STUDY

In our study we set following specific aims:

1 a) To compare presence of K- ras mutation in early stages of endometrioid type of endometrial carcinoma with normal endometrium.

b) To evaluate association of K-ras mutation to clinical-pathological characteristics of endometrioid carcinoma of endometrium.

2 a) To compare promoter methylation in selected tumor suppressor genes in early stages of endometrioid type of endometrial carcinoma with normal endometrium.

b) To evaluate association of methylation in selected tumor suppressor genes to clinical-pathological characteristics of endometrioid carcinoma of endometrium.

4 MATERIAL AND METHODS

4.1 SAMPLES

Formalin-fixed and paraffin-embedded (FFPE) samples from both endometrioid carcinoma of endometrium and normal endometrial tissue were obtained from 79 women (59 patients with endometrial cancer, 20 patients with normal endometrium) treated in 2006-2010 at the Department of Obstetrics and Gynecology, Faculty Hospital Hradec Králové (FNHK), 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, FNHK. All slides were reviewed by an experienced pathologist. The tumors were classified according to the current World Health Organization (WHO) classification of tumors of the female reproductive system (*Tavassoli FA and Devilee P, 2003*). The study was approved by the Ethics Committee of FNHK.

4.2 DNA ISOLATION

DNA was extracted from FFPE samples using a Qiagen DNA extraction kit (Hilden, Germany) according to the manufacturer's protocol with minimum modification. The procedure consists of 6 steps: 1. Removing paraffin: paraffin is dissolved in xylene and removed; 2. Lysis: sample is lysed under denaturing conditions with proteinase K (56 ^{o}C, overnight); 3. Heating: 10 min incubation at 70°C reverses formalin crosslinking; 4. Binding: DNA binds to the membrane and contaminants flow through; 5. Washing: residual contaminants are washed away; 6. Elution: pure, concentrated DNA is eluted from the membrane. The concentration of isolated DNA was measured according to the manufacturer's protocol. We used two approaches: fluorimetric (Qubit, Invitrogen) and spectrophotometric (Nanodrop ND-1000, Thermo Fisher Scientific).

4.3 K-RAS

Detection of K-ras mutation was made by using of K-ras StripAssay™ (ViennaLab Diagnostics GmbH). This assay covers 10 mutations in K-ras gene (codon 12 and 13). Polymerase chain reaction (PCR) amplification with use of biotinylated primers was performed according to the manufacture's protocol, for analysis was used 25 ng of isolated DNA. PCR was carried out in a Veriti Thermocycler (Applied Biosystems, CA). The cycling condition consisted of an initial denaturation at 94 ^{o}C for 2 min, 40 cycles of denaturing at 94 ^{o}C for 50s, annealing at 56 ^{o}C for 50s, and extension at 60 ^{o}C for 60s, followed by final extension for 3 min at 60 ^{o}C. Amplified products were analyzed by control electrophoresis on 2% agarose gels (fragment lengths 151 bp and 204 bp), and visualized under ultraviolet light after staining with ethidium bomide. Amplified products were hybridized to a test strip containig allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and color substrat (Fig. 10).

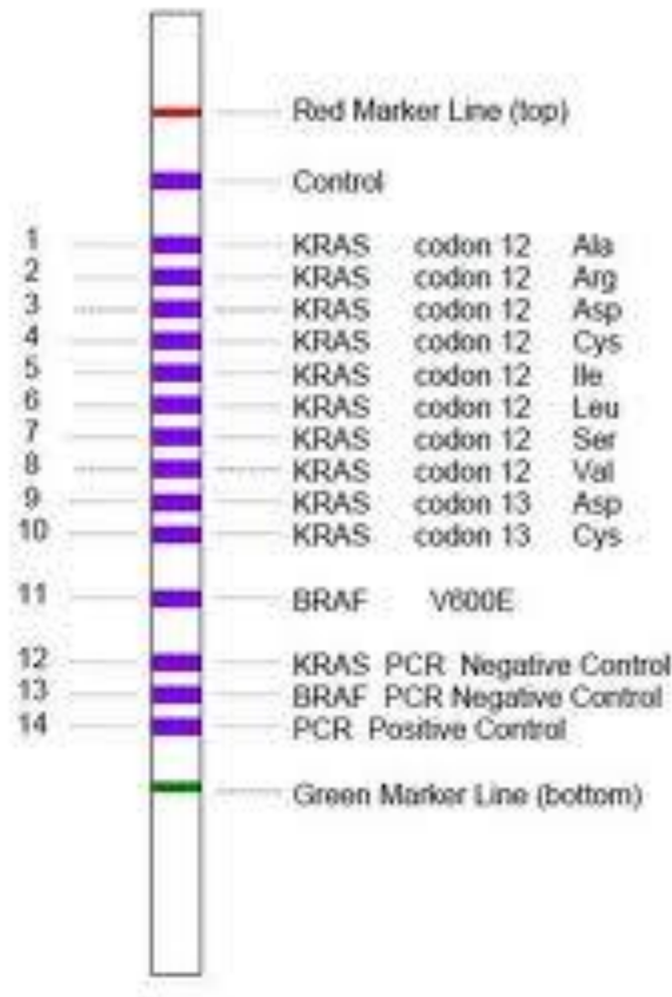

Figure 10. Test-strip design

4.4 MS-MLPA

Methylation-specific MLPA (MS-MLPA) is a semi-quantitative method for methylation profiling. MS-MLPA is a variant of the MLPA technique in which copy number detection is combined with the use of a methylation-sensitive restriction enzyme HhaI (*Nygren, AO, et al., 2005*). The advantages of MS-MLPA over many alternative methods, especially as it has the possibility to detect and quantify methylation in a large set of genes and promoters simultaneously using only small amounts of template DNA, which can be paraffin derived.

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 (Tab. 3). 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 (Tab. 4). 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 AE-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), 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, Foster City, CA, USA), 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 (*Moelans CB, et al., 2011*).

CpG universal methylated and unmethylated DNA (Chemicon International, Temecula, CA) were used as controls (Fig. 11).

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
MGMTb	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
MGMTa	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

Table 3. Genes in the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) KIT ME002 Tumor suppressor-2 (MRC Holland)

Gene	5' probe end	3' probe end
BRCA1	CCCCTTGGTTTCCGTGGCAACGGA	AAAGCGCGGGAATTACAGATAAATTAAAACTGCGACT
BRCA2	CGGGTTAGTGGTGGTAGTGGGTT	GGGACGAGCGCGTCTTCCGCAGTCCCAGTCCAGCGTGG
ATM	GCGGAGACCGCGTGATACTGGAT	GCGCATGGGCATACCGTGCTCTGCGGCTGCTTGGC
TP53	CACGGTGGCTCTAGACTTTTGAGAAGCTCAAA	ACTTTTAGCGCCAGTCTTGAGCACATGGGAGGGGAAAACC
PTEN	CATGCTCAGTAGAGCCTGCGGCTTGG	GGACTCTGCGCTCGCACCCAGAGCTACCGCTCTGC
MGMT	GGCAAACTAAGGCACAGAGCCTCA	GGCGGAAGCTGGGAAGGCGCCGCCCGGCTTGTAC
PAX5	GCGCTCGTCTAAGCAGCGGGGTT	TGCACATGGAGATGTCACAGGCCCCGCGCACAGCGCAG
CDH13	CGTGCATGAATGAAAACGCCGCC	GGGCGCTTCTAGTCGGACAAAATGCAGCCGAG
TP73	GGAGTTGGATCGGCCCCTGGG	ACTTGGCGCTCGCGAGAGGCTGGAGCGGCCAGAG
WT1	GGAGGGTTGTGCCACACCGGCCAGCT	GAGAGCGCGTGTTGGGTTGAAGAGGAGGGTGTCTCCGA
VHL	CGGACGGAGAACTGGGACGAGGCCGAGG	TAGGCGCGGAGGAGGCAGGCGTCGAAGAGTACGG
GSTP1	GGCAGGCTGCGCTCACCGCGCCTT	GGCATCCTCCCCCGGGCTCCAGCAAACTTTTCTTTGTTCG
CHFR	CGAGAGTAGGCGCGTGGAGG	GCGCTCGGCCATCTTTGATCCTGACCAGGCGACTTCGT
ESR1	GCTCGCGTGTCGGCGGGACAT	GCGCTGCGTCGCCTCTAACCTCGGGCTGTGCTCTTTTTCC
RB1	CAAGGAGGGAGAGTGGCGCTC	CCGCCGAGGGTGCACTAGCCAGATATTCCCTGCG
MSH6	CGGCTGTCGGTATGTCGCGACAG	AGCACCCTGTACAGCTTCTTCCCCAAGTCTCCGGCGCTGAG
MGMT	CTGCGGAGCCGAGGACCTGAGAAAAGCAA	GAGAGCGCGCGGGGGCGGGGCCGGG
THBS1	CCTTGCCCGGCCGCCGCCCATTGGCCGGAGG	AATCCCCAGGAATGCGAGCGCCCCTTTAAAA
CADM1	CTGCCCGGACTCCGCCTCCAGCGCATGTCA	TTAGCATCTCATTAGCTGTCCGCTCGGGCTC
STK11	CAGGCCTGTGGGATGGGCGGCCCGGAGA	AGACTGCGCTCGGCCGTGTTCATACTTGTCCGTGGGC
PYCARD	CCAAGCTGGTCAGCTTCTACCTGGAGACCT	ACGGCGCCGAGCTCACCGCTAACGTGCTGCG
PAX6	GGAGCATCCAATCGGCTGGCGCG	AGGCCCCGGCGCTGCTTTGCATAAAGCAATATTTTGTGTGA
CDKN2	GCAGGTTCTTGGTGACCCTCCGG	TTCGGCGCGCGTGCGGCCCGCCGCGAGTG

A	A	AG
GATA5	CCTTGGCGACAAGGACGCACG	ACACGGGGCGGCCAGCGCGGAGCCCGGA CCAGTG
RARB	GGCGGGAGGCGAGCGGGCGCA	GGCGGAACACCGTTTTCCAAGCTAAGCCG CCGCAAATAAA
CD44	GGAGAAGAAAGCCAGTGCGTCT CT	GGGCGCAGGGGCCAGTGGGGCTCGGAGG CACAGG
RB1	GGATGCCTCCTGGAAGGCGCCT GG	ACCCACGCCAGGTTTCCCAGTTTAATTCCT CATGACTTAGCGTCCC

Table 4: Probe sequences for kit ME002 Tumor suppressor-2 (MRC Holland)

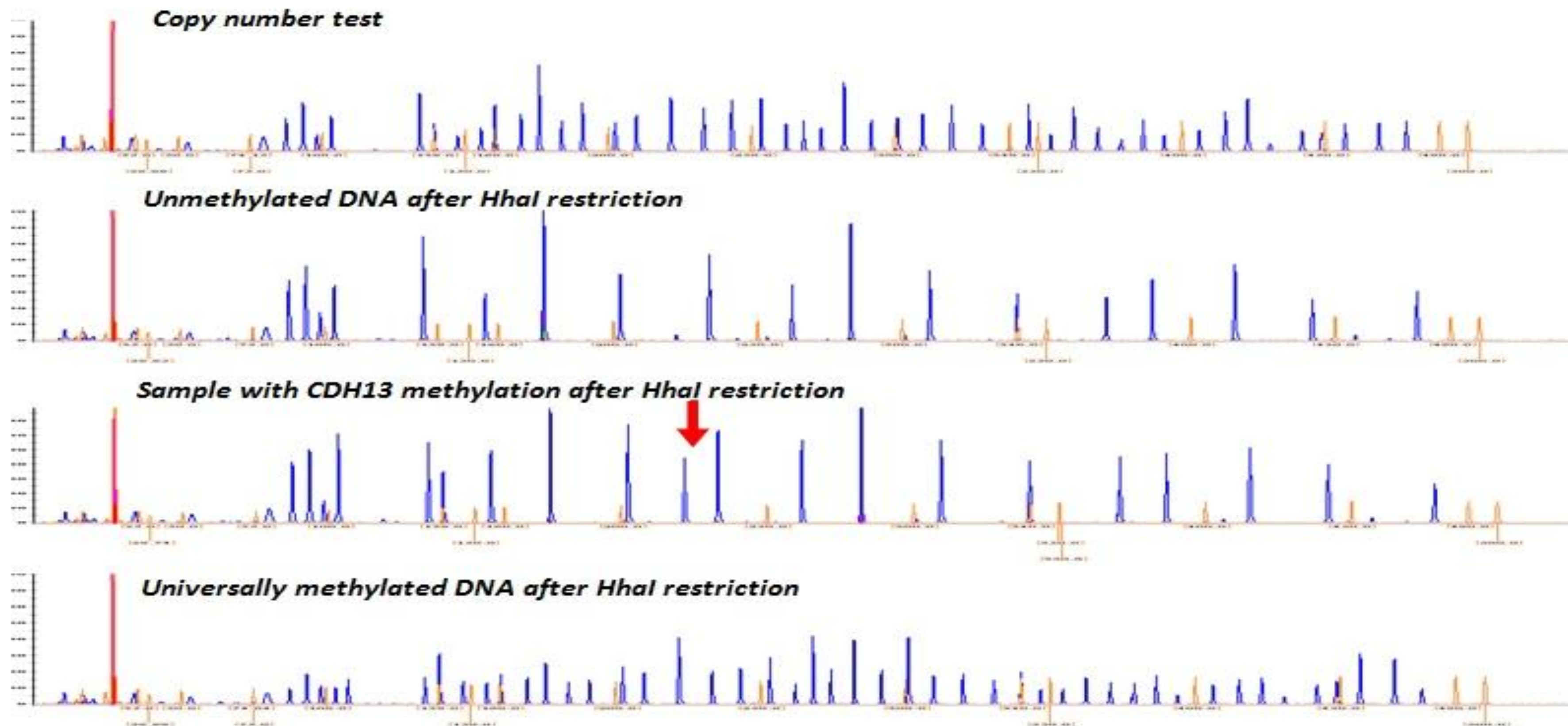

Figure 11. Fragmentation analysis: First part of the image shows fragmentation analysis of copy number test. All peaks are visible (peaks of reference probes and peaks of sample probes). Second part of the image shows fragmentation analysis of unmethylated DNA after HhaI restriction. Only peaks of reference probes are present because sample probes were digested. Third part of the image shows fragmentation analysis of cancer sample with CDH13 methylation after HhaI restriction. Only peaks of reference probes with CDH13 probe are visible. Fourth part of the image shows fragmentation analysis of universally methylated DNA after HhaI restriction. All peaks are visible (peaks of reference and sample probes). Yellow peaks represent size standard LIZ 500 and blue peaks represent PCR amplified products.

4.5 GATA4 AND TP53 MSP

MSP is now an established technology for monitoring of abnormal gene methylation in selected gene sequences (*Herman JG, et al., 1996*). In gel based MSP assay the PCR primers are designed to specifically amplify selected regions of the gene of interest. If the sample DNA was originally unmethylated, an MSP reaction product will be detectable when using the primer set labeled as “U” (designed to be complementary to the unmethylated DNA sequence). No product will be generated using a primer set labeled as “M” (designed to be complementary to the derivative methylated DNA sequence. Conversely, an MSP product will be generated using the “M” primer set only if the sample was originally methylated, and the “U” primers will not allow amplification of such a template.

DNA methylation patterns in the CpG islands of the promoter region of the GATA4 and TP53 genes were determined by methylation-specific PCR (*Herman JG, et al., 1996*). Sodium bisulfite modification was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, USA) according to the manufacturer´s protocol, with minor modifications.

Primer sequences for GATA4 gene were designed using MethPrimer. 5´-GGTTAGTTAGTGTTTTAGGGTTGA-3´ (sense) and 5´- AACAAAAACAAAAAAACTCCAAA-3´ (antisense) for unmethylated reaction (PCR product 230 bp), and 5´-GTTAGTTAGCGTTTTAGGGTCGA-3´ (sense) and 5´- CAAAAACGAAAAAACTCCGAA-3´ (antisense) for methylated reaction (PCR product 228 bp). Primer sequences for TP53 gene have been reported previously (*Amatya VJ, et al., 2005*). 5´-TTGGTAGGTGGATTATTTGTTT-3´ (sense) and 5´- CCAATCCAAAAAAACATATCAC-3´ (antisense) for unmethylated reaction (PCR product 247 bp), and 5´-TTCGGTAGGCGGATTATTTG-3´ (sense) and 5´- AAATATCCCCGAAACCCAAC-3´ (antisense) for methylated reaction (PCR product 193 bp). PCR was carried out in a 25 µl mixture containing 10x Takara buffer (2.5 µl), dNTPs 2.5 mM solution Takara (2.0 µl), primers (1 µl each 10 pmol/ µl solution), polymerase Taq HS Takara 5U/ µl (0.3 µl) (Takara Bio Europe S.A.S, France), water and 2 µl of bisulfite-modified DNA in a Veriti Thermocycler (Applied Biosystems, CA). The cycling condition for GATA4 gene consisted of an initial denaturation at 95°C for 5 min, 40 cycles of denaturing at 95°C

for 45s, annealing at 53.7°C for 35s, and extension at 72°C for 35s, followed by final extension for 5 min at 72°C. The cycling condition for TP53 gene consisted of an initial denaturation at 95°C for 7 min, 40 cycles of denaturing at 95°C for 45s, annealing at 59°C for 45s, and extension at 72°C for 60s, followed by final extension for 5 min at 72°C.

CpG universal methylated and unmethylated DNA (Zymo Research Corporation, USA) were similarly treated with bisulfite and were used as controls.

Amplified products were separated by electrophoresis on 2% agarose gels and visualized under ultraviolet light after staining with ethidium bromide (Fig. 12, 13).

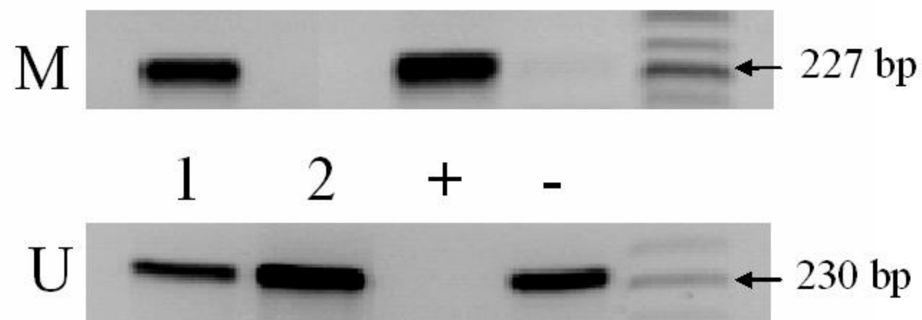

Figure 12. MSP for GATA4: gel electrophoresis (+ universally methylated positive control DNA, - universally unmethylated negative control DNA). The presence of visible PCR product in the lane marked U indicates the presence of unmethylated GATA4 gene, the presence of product in the lane marked M indicates presence of methylated GATA4 gene. Sample No. 1 has partial methylated analyzed CpG loci of GATA4 gene and sample No. 2 has unmethylated analyzed CpG loci of GATA4 gene)

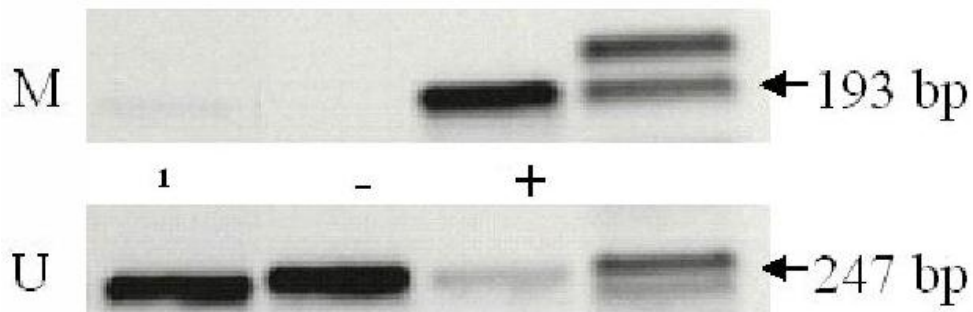

Figure 13. MSP for TP53: gel electrophoresis (+ universally methylated positive control DNA, - universally unmethylated negative control DNA). The presence of a visible PCR product in the lane marked U indicates the presence of unmethylated TP53 genes, the presence of product in the lane marked M indicates presence of methylated TP53 genes. Sample No. 1, 2, 3 have partial methylated promoter region of TP53 gene and sample No. 4 has unmethylated promoter region of TP53 gene)

4.6 STATISTICAL ANALYSIS

The demographic and clinical characteristics were compared using either unpaired *t*-tests for continuous variables, and presented as mean ± SD, or the nonparametric Mann-Whitney *U* test, and presented as median (range). Categorical variables were compared using Fisher's exact test, or using Chi-square test, and presented as n (%). The normality of the data was tested using the D'Agostino-Pearson omnibus normality test and the Shapiro-Wilk test. Spearman partial correlation was used to adjust the data for potential confounders. Differences were considered statistically significant at $p<0.05$. All *p*-values were obtained from two-sided tests, and all statistical analyses were performed using SPSS 19.0 for MAC OS X (SPSS Inc., Chicago, IL, USA).

4.7 DEVICES AND SOFTWARE

NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA)
Qubit (Invitrogen, Carlsbad, California, USA)

Thermocycler Veriti® 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA)

Thermocycler GeneAmp 9700 (Applied Biosystems, Foster City, CA, USA)

Thermocycler GeneAmp 2400 (PerkinElmer, Waltham, MA, USA)

Sequencer ABI3110 (Applied Biosystems, Foster City, CA, USA)

B2 electrophoretic bath (OWL Separation Systems, Portsmouth, USA)

Voltage source Power Pac 300 (Bio-Rad Laboratories Headquarters, Hercules, USA)

UV transilluminator TVC312 A/F (Spectronics Corporation,Westbury, USA) and other routine laboratory devices (centrifuges, vortex,…)

software: BioCapt Version 11.03 (Vilber Lourmat, Torcy, France)

software: GeneMapper4.0 (Applied Biosystems, Foster City, CA, USA)

software: SPSS 19.0 for MAC OS X (SPSS Inc., Chicago, IL, USA)

5 RESULTS

5.1 DEMOGRAPHIC AND CLINICAL CHARACTERISTIC OF THE STUDY POPULATION

The samples were obtained from 79 women (59 patients with endometrioid type of endometrial carcinoma, and 20 patients with non-neoplastic endometrium) treated in 2006-2010 at the Department of Obstetrics and Gynecology, FNHK. The demographic and clinical characteristic of the women with and without endometrial carcinoma are shown in Table 5. Women with endometrial carcinoma had a higher age, BMI, rate of hypertension and a lower rate of breast cancer. There were 30 patients in FIGO stage IA and 29 patients in IB, 20 patients with grade 1, 20 patients with grade 2 and 19 patients with grade 3 of endometrioid endometrial carcinoma. Five-year survival was not counted cause of short follow-up period, but we promise to present this characteristic in the future after appropriate time of follow-up.

	Women with endometrial carcinoma (n=59)	Women without endometrial carcinoma (n=20)	*p*-value
Patients' age (years)	65.3±9.0	60.0±9.4	**0.02**
Age of menopause	51.0 (40-59)	51.5 (48-58)	0.78
Body mass index	32.0 (17.0-50.0)	26.5 (20.0-34.0)	**0.0007**
Parity	2 (0-4)	2 (1-3)	0.07
Diabetes mellitus	14 (24%)	1 (5%)	0.10
Hypertension	40 (68%)	7 (35%)	**0.02**
Breast cancer	1 (2%)	3 (15%)	**0.05**

Table 5. Demographic and clinical characteristics of women with and without endometrial cancer (the statistically significant results are marked in bold)

5.2 SPECIFIC AIM 1A

In the present study we used K-ras StripAssay™ (ViennaLab Diagnostics GmbH) to analyze samples of endometrial tissue for presence of K- ras mutation, obtained from 79 patients. The patients were categorized into two groups: there were 59 patients with endometrioid endometrial carcinoma and 20 patients with normal endometrium as a control group. K-ras mutation was found in 14 (24%) cases of specimens with endometrioid carcinoma and in 3 (15%) cases in control group. The frequency of K-ras mutation in the carcinoma group did not differ from the group of control samples.

5.3 SPECIFIC AIM 1B

The results of K-ras mutation from the endometrioid carcinoma specimens were compared with clinic-pathological characteristics, including tumor stage and tumor grade (Tab. 6). No association between K-ras mutation and any of these parameters was observed for the patients with endometrioid carcinoma of endometrium.

Characteristic	All endometrial carcinomas n (%)	Endometrial carcinoma with K-ras mutation n (%)
Stage IA	30 (51)	8 (27)
Stage IB	29 (49)	6 (21)
Grade 1	20 (34)	6 (30)
Grade 2	20 (34)	3 (15)
Grade 3	19 (32)	5 (26)

Table 6. Clinical-pathological characteristics of endometrial carcinoma samples with respect to the presence of K-ras mutation

5.4 SPECIFIC AIM 2A

We used the MS-MLPA probe set ME002 (MRC-Holland, Amsterdam, The Netherlands) to analyze 79 samples of endometrium. Using a 15% cut-off for methylation we observed higher methylation in CDH13 gene in crude analysis ($p<0.0001$) and in adjusted analysis ($p<0.0001$) for potential confounders (patients` age, BMI, hypertension, and breast cancer), and border-line methylation in Wilm's tumor (WT1) gene ($p=0.057$) in endometrial cancer patients compared to control group. For MutS homolog 6 (MSH6) gene we observed high methylation (about 40%) in both endometrial cancer and control samples. For genes breast cancer (BRCA) 1, BRCA2, ataxia telangiectasia mutated (ATM), TP53, PTEN, TP73, von Hippel–Lindau tumor suppressor (VHL), retinoblastoma protein (RB1), THBS1, serine/threonine kinase 11 (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. 14).

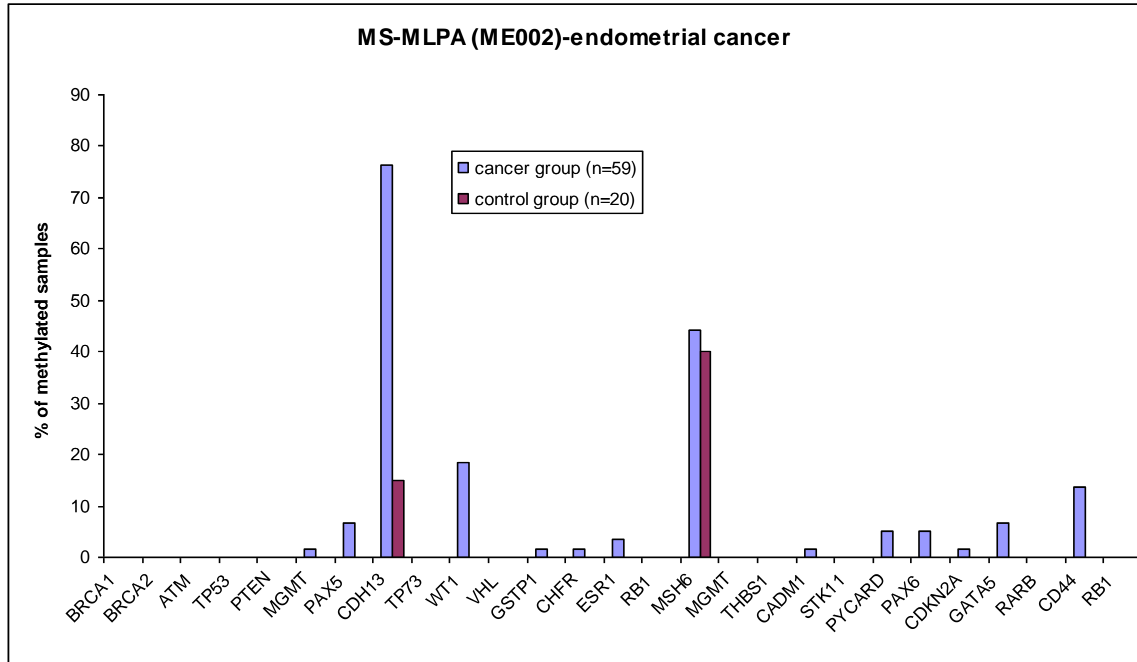

Figure 14. Comparison of methylation frequencies (cut-off value 15%) of the 25 analyzed genes in endometrial cancer and control samples

To search for promoter methylation of GATA4 and TP53 genes we used MSP to compare the methylation status of 54 patients with endometrioid carcinoma of endometrium and 18 patients with normal endometrial tissue. Amplification failed in the remaining 7 samples. MSP revealed higher methylation in GATA4 gene in crude analysis ($p<0.0001$) and in adjusted analysis ($p<0.0001$) for potential confounders (patients` age, BMI, hypertension, and breast cancer) in the group of endometrioid carcinoma of endometrium compared to the group of control samples. Promoter of GATA4 gene was methylated in 44 of 54 in the carcinoma group (82%), and in none of the control group. No methylation was observed in TP53 gene.

5.5 SPECIFIC AIM 2B

Methylation results from endometrial cancer specimens were compared with clinico-pathological characteristics, including tumor stage and tumor grade. Both WT1 ($p=0.002$) and GATA5 ($p=0.05$) genes showed a higher methylation in stage IB compared with stage IA of endometrial cancer samples (Fig. 15). Methylation in GATA5 gene ($p=0.05$) was higher in grade 3 of endometrial cancer samples compared with the group of grade 1 and grade 2 tumors (Fig. 16).

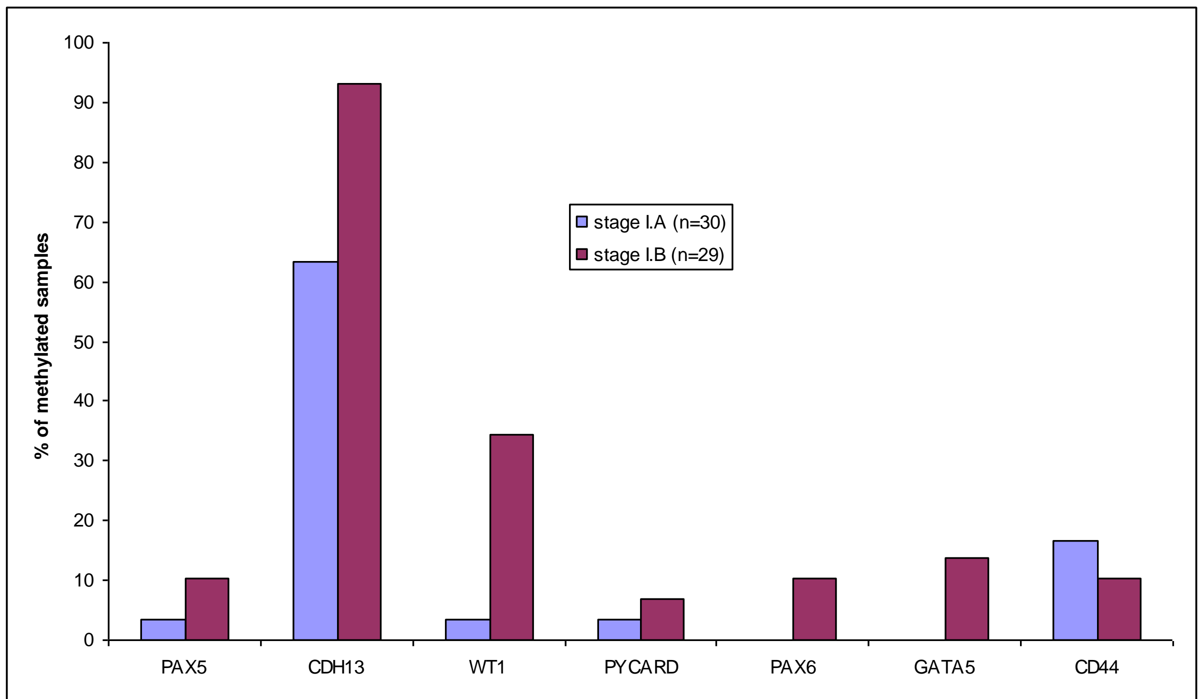

Figure 15. Methylation of specific genes according to tumor stage

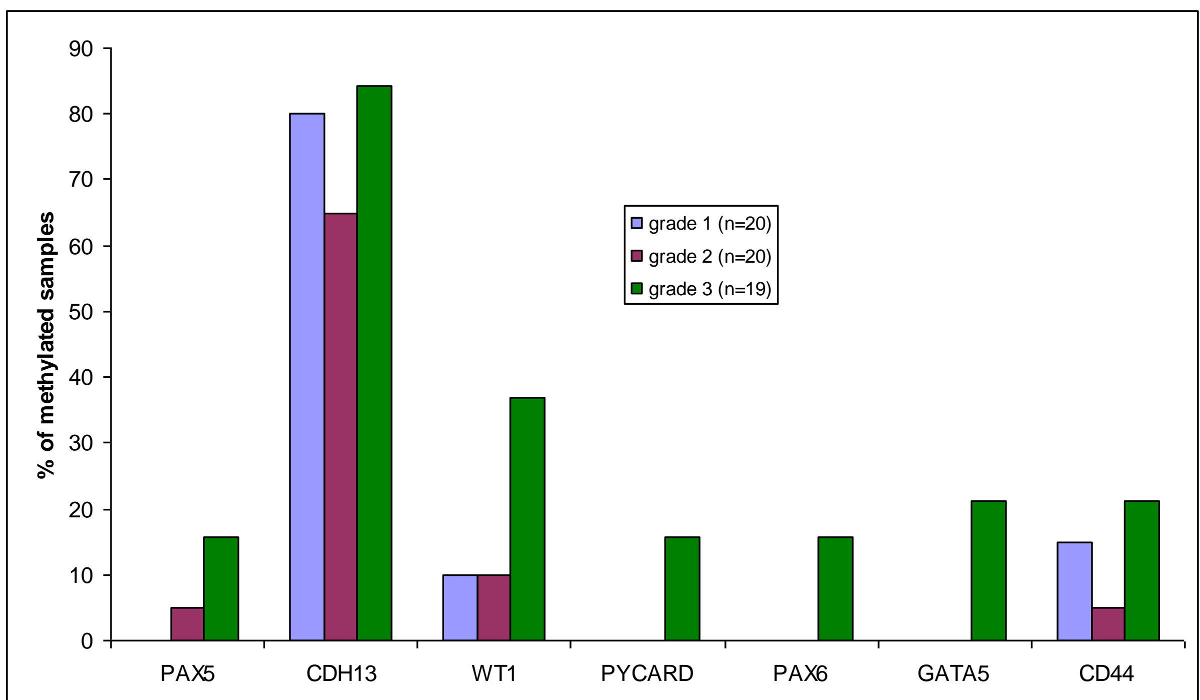

Figure 16. Methylation of specific genes according to tumor grade

No association between GATA4 methylation and tumor stage and tumor grade was observed for the patients with endometrioid carcinoma of endometrium (Tab. 7).

Characteristic	All endometrial carcinomas n (%)	Endometrial carcinoma with GATA4 methylation n (%)
Stage IA	28 (52)	23 (82)
Stage IB	26 (48)	21 (81)
Grade 1	18 (33)	16 (89)
Grade 2	19 (35)	15 (79)
Grade 3	17 (32)	13 (77)

Table 7. Clinical-pathological characteristics of endometrial carcinoma samples with respect to the presence of methylation of GATA4

6 DISCUSSION

6.1 SPECIFIC AIM 1

In our study, mutation of the K-ras gene was detected in 24% of endometrial carcinoma cases. The frequency of K-ras mutation in the carcinoma group did not differ from the group of control samples. We also did not report association between K-ras mutations and tumor stage and tumor grade. Our findings are similar to those in the study by Semczuk A, et al. Authors assessed the relationship between K-ras gene activation and clinico-pathological features as well as patients' outcome. Mutational activation in codon 12 of the K-ras gene was detected in 8 out of 57 (14%) endometrial carcinomas, and K-ras gene mutation was not related to the patients' age, surgical stage, histological grade or to the depth of myometrial invasion. Authors reported that point mutations in codon 12 of the K-ras gene are a rare event in human endometrial carcinomas. The lack of correlation between K-ras mutations and clinical-pathological features (except histological type) supports the hypothesis of a random activation of the K-ras gene in human neoplastic endometrium (*Semczuk A, et al., 1998*). Also Esteller M, et al. reported point mutations at codon 12 of K-ras oncogene in 8 of 55 (15%) tumour specimens. No correlation was found between K-ras gene mutation and age, histological type, tumor grade, clinical stage or current patient status. Authors concluded that K-ras mutation is a relatively common event in endometrial carcinogenesis, but with no clear prognostic value (*Esteller M, et al., 1997*). Neither Jones MW, et al. did not establish prognostic value of the mutations in K-ras oncogene. Authors evaluated predictive value of p53 and K-ras mutations in determining tumor aggressiveness and survival in patients with endometrial carcinoma. p53 genotyping strongly correlated with short survival, and had potential prognostic value in endometrial carcinoma, but the finding of K-ras alterations did not (*Jones MW, et al., 1997*). On the other hand, Mizuuchi H, et al. detected K-ras mutation in 6 of 49 cases (12%), and reported presence of mutations in K-ras appeared to be an unfavorable prognostic factor determining the aggressiveness of endometrial carcinoma (*Mizuuchi H, et al., 1992*). In the study made by Ito K, et al., K-ras mutations were significantly associated with the presence of lymph node metastases, and with patients who died

or experienced recurrence. These findings point to a possible role for K-ras activation in the mechanism responsible for more aggressive clinical behavior of endometrioid endometrial carcinoma that is observed in postmenopausal patients (*Ito H, et al., 1996*).

6.2 SPECIFIC AIM 2

We used the MS-MLPA probe set ME002 (MRC-Holland, Amsterdam, The Netherlands) to analyze 79 samples of endometrium. Using a 15% cut-off for methylation we observed higher methylation in CDH13 gene (p<0.0001) in endometrial cancer patients compared to control group. 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 endometrial and ovarian carcinomas. In the study made by Seeber LM, et al., using MS-MLPA probe mix ME001, targeting different CpG islands within promoter region of the CDH13 gene, 93% of samples were methylated. Authors presented methylation of CDH13 to be characteristic for endometrioid endometrial carcinoma. CDH13 methylation predicted the correct tumor type in almost 90% of endometrioid endometrial carcinoma samples, which is promising as a diagnostic test but requires further validation (*Seeber LM, et al., 2010*). In our study, we observed almost 80% of methylated carcinoma samples. In the study made by Suehiro Y, et al., 71% of endometrial cancer samples were methylated. Authors revealed that stage in combination with either DNA aneuploidy or lack of CDH13 hypermethylation was an independent prognostic factor (*Suehiro Y, et al., 2008*). On the other hand, Yang HJ, et al. reported the incidence of 35% for CDH13 hypermethylation in endometrial cancer samples, and no association to clinico-pathological characteristics was observed (*Yang HJ, et al., 2006*). CDH13 is frequently methylated in ovarian cancer. Chmelařová M, et al. and Bol GM, et al. presented the methylation of CDH13 to be an important event in ovarian carcinogenesis (*Chmelařová M, et al., 2012; Bol GM, et al., 2010*).

MSP revealed higher promoter methylation of the GATA4 gene ($p<0.0001$) in the group of endometrioid carcinoma of endometrium than in the control group. Promoter of GATA4 gene was methylated in 44 of 54 in the carcinoma group (82%), and in none of the control group. Transcription factors of the GATA family are essential regulators of the specification and differentiation of numerous tissues. They all share 2 highly conserved zinc fingers of the C2H2 type that mediate not only DNA binding but also the great majority of protein interactions (*Zheng R and Blobel GA,2010*). Mutations, loss of expression, or overexpression of GATA factors have all been associated with a broad variety of cancers in humans, including leukemia, breast cancer, gastrointestinal cancers, and others. Whilst GATA1 and GATA3 have been very well studied in the context of human malignancies, other members of the GATA family need further investigation. Studies suggest that GATA-4, -5, and -6 factors are important regulators of tissue-specific gene expression in multiple endoderm- and mesoderm-derived tissues. GATA factors are important regulators of both structural and regulatory genes in the heart. GATA-4 and -6 have been implicated in the regulation of liver-specific gene expression. GATA-4, -5, and -6 have also been implicated in the regulation of epithelial cell differentiation in the gut and are also important regulators of gene expression within the gonads (*Molkentin JD, 2000*). Expression of the Mullerian inhibiting substance promoter is regulated by GATA-4 in Sertoli cells and Mullerian ducts (*Tremblay JJ and Viger RS, 1999; Viger RS, et al., 1998; Watanabe K, et al., 2000*), and GATA-4 regulates expression of the steroidogenic acute regulatory protein promoter in the ovary (*Silverman E, et al., 1999*). While, to date, no mutations or deletions of the GATA4 gene have been discovered in human cancers, silencing of its expression seems to be widespread in different types of cancers. Expression of GATA4 was extinguished in the majority of cell lines from colorectal and gastric cancers as well as in primary tumors. Silencing was associated with hypermethylation of the GATA4 promoter sequences (*Akiyama Y, et al., 2003; Wen XZ, et al., 2010*). GATA4 was found to be extinguished in a large proportion of lung *(Guo M, et al., 2004*), and oesophageal cancers (*Guo M, et al., 2006*). GATA-4 has also been reported to be aberrantly methylated in 23% of glioblastoma tumors but not in normal brain (*Vaitkiene P, et al., 2013*). Methylation was observed in human ovarian cancer cell lines and primary ovarian cancers as well (*Wakana K, et al., 2006*). These

studies support the idea that loss of GATA4 by epigenetic silencing might contribute to malignant transformation. Based on the importance of methylation in the GATA4 gene described in previous studies we focused our analysis on GATA4 methylation in endometrioid carcinoma of endometrium, and our finding suggests the importance of GATA4 methylation in endometrial carcinogenesis.

In our study, using MSP, no methylation in TP53 gene was observed. Protein p53 is a 53-kD nuclear phosphoprotein (393 amino acids) (*Lane DP, 1994*). It is a tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism (*Oren M and Rotter V, 1999*). Activation of p53 would prevent the perpetuation of the genomic damage, and ensure that potentially dangerous cells will not multiply and take over the normal population (*Lane DP, 1992*). TP53 gene is frequently affected by loss of alleles and by point mutations in almost all cancers (*Szymanska K and Hainaut P, 2003*). Mutated TP53 results in a non-functional protein that accumulates in the cell and acts as a dominant negative inhibitor of wild-type TP53, leading to propagation of aberrant cells (*Okuda T, et al., 2010*). TP53 mutations or TP53 overexpression in endometrial carcinoma is twice as frequent in tumors without hyperplasia (estrogen unrelated) than in those with hyperplasia (estrogen related) (*Koul A, et al., 2002; Kaku T, et al., 1999*). TP53 mutation is present in about 90% of serous carcinomas of endometrium (*Tashiro H, et al., 1997*). In the studies made by Pilka R, et al., p53 overexpression was found to be related to poor grade of differentiation and deep myometrial invasion (*Pilka R, et al., 2010; Pilka R, et al., 2008*). Because of the high frequency of TP53 mutations in human cancers, promoter methylation of this gene has also been examined in several studies. TP53 promoter methylation was observed in extra-axial brain tumors (*Almeida LO, et al., 2009*), gliomas (*Amatya VJ, et al., 2005*), acute lymphoblastic leukemia (*Agirre X, et al., 2003*) and ovarian cancer (*Chmelarova M, et al., 2013*). TP53 promoter methylation was also studied in breast cancer (*Barekati Z, et al., 2010*), gastric cancer (*Lima EM, et al., 2008*) and adrenocortical cancer (*Sidhu S, et al., 2005*), but was not proved to be significant. TP53 promoter methylation in endometrial carcinoma has not yet been

examined. Our study as the first study examined methylation in the TP53 promoter region. In our study we observed no methylation in the analyzed region. Based on these results it could be concluded that despite frequent mutations in the gene TP53 in endometrioid carcinoma of endometrium, methylation in TP53 promoter region is not an important event in endometrial carcinogenesis.

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

The WT1 gene, located on chromosome 11p13 and consisting of 10 exons, plays a crucial role in kidney and genital system development (*Bruening W, et al., 1992*). The Wilms' tumor gene WT1 is overexpressed in various kinds of solid tumors (*Choi EJ, et al., 2013; Kaneuchi M, et al., 2005*). However, it remains unclear whether WT1 plays a pathophysiological role in endometrial carcinoma. In the study made by Ohno S, et al. WT1 overexpression was associated with advanced FIGO stage, myometrial invasion and high-grade histological differentiation. The results suggested that tumor-produced WT1 provided additional prognostic information in endometrial cancer patients (*Ohno S, et al., 2009*). Dohi S, et al. presented WT1 to play an important role in endometrial cancer-associated angiogenesis, probably *via* induction of angiogenesis by vascular endothelial growth factor (VEGF). Authors suggested that WT1 may regulate tumor progression and angiogenesis, and this may prove of great benefit in finding a rational approach to therapy of endometrial carcinoma (*Dohi S, et al., 2010*).

The GATA family of transcription factors plays essential role in cell growth and differentiation during embryogenesis and early development (*Patient RK and McGhee JD, 2002*). GATA5 have been implicated as important regulators in the normal development and differentiation of mesoderm- and endoderm-derived tissues, including lung, liver, gonad and pancreas (*Molkentin JD, 2000*). Loss of GATA4 and GATA5 expression second to promoter hypermethylation has been identified in primary ovarian, lung and gastrointestinal cancer (*Wakana K, et al., 2006; Guo M, et al., 2004; Akiyama Y, et al., 2003*). To the best of our knowledge, our present study is

the first study to demonstrate methylation of GATA5 in endometrial carcinoma. This finding suggests the importance of GATA5 methylation in endometrial carcinogenesis.

7 CONCLUSION

- K-ras mutations in carcinoma group do not differ from the group of control samples.
- No association between K-ras mutations and tumor stage and tumor grade were found.
- Higher methylation of CDH13 and GATA4 genes in endometrioid endometrial carcinoma samples compared to non-neoplastic samples was revealed.
- Higher methylation of WT1 and GATA5 genes in stage IB samples compared to stage IA samples of endometrial carcinoma was found.
- Higher methylation of GATA5 gene in grade 3 samples compared to grade 1 and 2 samples of endometrial carcinoma was identified.

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9 SUPPLEMENTS

Jaký je prognostický význam molekulárně genetických faktorů u karcinomu endometria?

What is the Prognostic Importance of Molecular Genetic Factors in Endometrial Carcinoma?

Křepinská E.[1], Chmelařová M.[2], Laco J.[3], Palička V.[2], Špaček J.[1]

[1] Porodnická a gynekologická klinika, LF UK a FN v Hradci Králové
[2] Ústav klinické biochemie a diagnostiky, LF UK a FN v Hradci Králové
[3] Fingerlandův ústav patologie, LF UK a FN v Hradci Králové

Souhrn
Východiska: Kritické zhodnocení významu vybraných molekulárně genetických ukazatelů u karcinomu endometria na základě analýzy dosud publikovaných prací a u *K-ras* mutace též prezentace vlastních výsledků. Cílem původní části práce bylo porovnat výskyt této mutace v tkáni karcinomu oproti zdravému endometriu u pacientek léčených na našem pracovišti a posléze i zhodnocení jejího podílu na vzniku tohoto onemocnění v rámci multivariační analýzy. ***Materiál a metody:*** Byla provedena molekulárně biologická analýza vzorků endometriální tkáně pacientek léčených na Porodnické a gynekologické klinice Fakultní nemocnice v Hradci Králové. Vyšetření mutací genu *K-ras* bylo provedeno z DNA získané z formalinem fixovaných a v parafinu zalitých vzorků. K detekci byla použita metoda K-ras StripAssay™, ViennaLab Diagnostics GmbH. ***Výsledky:*** Bylo vyšetřeno 30 vzorků endometroidního karcinomu ve stadiu I (dle FIGO), mutace genu *K-ras* byla prokázána v 7 případech (23 %). V kontrolní skupině 20 vzorků normálního endometria byla sledovaná mutace nalezena ve 3 případech (15 %). Mutace se častěji vyskytovala u stadia IA a ve skupině dobře diferencovaných nádorů. ***Závěr:*** Význam molekulárně genetických faktorů u karcinomu endometria se přelomově liší v závislosti na typu nádoru. U častějších endometroidních nádorů prvního typu nejsou dosud publikované výsledky zdaleka tak jasné jako u karcinomů druhého typu, kde je výskyt alterace *p53* uváděn až v 90 %. Naše výsledky u představitelek místní populace podporují teorii o možném podílu *K-ras* mutace v procesu endometriální karcinogeneze u nádorů prvního typu ve smyslu časné události.

Klíčová slova
karcinom endometria – gen *K-ras* – mutace

Summary
Background: Evaluation of the importance of molecular genetic factors in endometrial carcinoma based on our review of available literature, and in the case of *K-ras* mutation based on our own data. The aim of the original part of our study was to compare the presence of *K-ras* mutation in early stages of endometroid carcinoma with normal endometrium and evaluate the role of the mutation in endometrial carcinogenesis. ***Material and methods:*** Molecular biological analysis was performed to detect *K-ras* mutation in samples of endometrial tissue obtained from women treated in the past at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove. The detection was made from DNA isolated from paraffin-embedded sections using K-ras StripAssay™, ViennaLab Diagnostics GmbH. ***Results:*** *K-ras* mutation was found in 7 out of 30 specimens of endometroid carcinoma in stage I (23%) and in 3 of 20 specimens of normal endometrium in the control group (15%). *K-ras* mutations were more frequent in IA stage and grade 1 of endometroid carcinoma. ***Conclusion:*** The importance of molecular genetic factors in endometrial carcinoma differs depending on the type of carcinoma. In more common type 1 endometroid cancer, published data are not as clear as in type 2 carcinoma, in which prevalence of alteration of *p53* reaches 90%. Results of our study performed on local population of women support the theory about the possible role of *K-ras* mutation as an early event in the process of endometrial carcinogenesis in type 1 tumors.

Key words
endometrial carcinoma – *K-ras* gene – mutation

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MUDr. Eva Křepinská
Porodnická a gynekologická klinika
LF UK a FN v Hradci Králové
Sokolská 581
500 05 Hradec Králové
e-mail: evajurkova@seznam.cz

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Úvod

Karcinom endometria je po karcinomu prsu nejčastější gynekologickou malignitou a jeho incidence neustále narůstá.

Ze zemí Evropské unie je jeho výskyt v České republice nejvyšší.

Od roku 1983 platí dualistická Bokhmanova teorie modelu karcinogeneze endometria, která vychází z klinického pozorování a histopatologických charakteristik [1]. Asi o deset let později byla tato dosud platná teorie podpořena studiemi na molekulární úrovni. Charakteristika obou základních subtypů souvisí i s odlišnou genetickou nestabilitou a molekulárními změnami [2].

U endometroidního karcinomu (typ I) jsou nejčastěji popisovány mutace *PTEN* tumor-supresorového genu [3], mikrosatelitální instabilita [4], mutace *K-ras* protoonkogenu [5] a *β-cateninu* [6]. Pro karcinomy typu II je charakteristická porucha funkce tumor-supresoru *p53* a některé práce uvádějí, že gen *p53* je alterován až v 90 % serózních karcinomů endometria [7]. Na rozdíl od typu I jsou u typu II vzácně popisované mutace *K-ras*, *β-cateninu*, dále mikrosatelitální instabilita a inaktivace *p16* [2,8–11].

V původní části naší práce jsme se zaměřili na úlohu mutace *K-ras* genu v patogenezi endometroidního typu karcinomu endometria.

K-ras patří do rodiny onkogenů *ras*, které jsou nejčastěji spojovány se vznikem lidských maligních nádorů. *Ras* proteiny jsou součástí buněčné membrány a vykazují GTPázovou aktivitu. Podílejí se na spouštění růstu a diferenciaci buňky. Mutovaný *K-ras* protein ztrácí schopnost spontánní inaktivace, což způsobí trvalou stimulaci buněčného růstu [12]. Z dostupných prací se mutace *K-ras* genu vyskytuje u 10–30 % případů endometroidního karcinomu [5,13], a to nejčastěji u nádorů středně a nízce diferencovaných [14]. To, že je mutace též přítomna asi v 16 % případů hyperplastického endometria, podporuje hypotézu o časné události v procesu karcinogeneze v této lokalizaci [15]. Jiné zdroje však naopak uvádějí možný vznik karcinomu endometria za přispění mutovaného *K-ras* protoonkogenu bez stadia hyperplazie [16].

Materiál a metody

Byla provedena molekulárně biologická analýza 50 vzorků endometriální tkáně pacientek vyšetřených a léčených na Porodnické a gynekologické klinice LF UK a FN v Hradci Králové. Vzorky byly získány z archivu Fingerlandova ústavu patologie LF UK a FN v Hradci Králové, stáří vyšetřovaného materiálu nepřesáhlo 5 let. Vyšetřeno bylo 30 vzorků s endometroidním karcinomem ve stadiu I dle FIGO klasifikace. Dále byla vyšetřena kontrolní skupina 20 vzorků normálního endometria pacientek operovaných pro jinou, nezhoubnou diagnózu dělohy. Tyto vzorky byly histopatologem hodnoceny jako proliferační, sekreční či klidové endometrium. Do kontrolní skupiny nebyl zařazen žádný vzorek hyperplastického endometria. Vyšetření mutací genu *K-ras* bylo provedeno z DNA získané z formalinem fixovaných, v parafinu zalitých vzorků tkání. DNA z parafinového bločku byla izolována pomocí kitu firmy Qiagen. Vlastní vyšetření *K-ras* mutace bylo realizováno metodou K-ras StripAssay™ (ViennaLab Diagnostics GmbH). Tento test zahrnuje vyšetření 10 mutací v *K-ras* genu (kodon 12 a 13). PCR amplifikace s využitím biotinylovaných primerů byla provedena dle protokolu výrobce, pro analýzu bylo použito 25 ng izolované DNA. Amplifikace byla uskutečněna v termocykléru Veriti (Applied Biosystems, CA). Amplifikační reakce využívala teplotní profil: 94 °C 2 min, 35 cyklů (94 °C 1 min, 70 °C 50 s, 56 °C 50 s, 60 °C 1 min) a 60 °C 3 min. Amplifikaci následovala kontrolní elektroforéza na 2% agarosovém gelu (délky PCR produktů 151 bp a 204 bp). Amplifikované produkty byly dle protokolu výrobce hybridizovány na testovací strip (proužek) obsahující alelově-specifické imobilizované oligonukleotidové proby. Navázané biotinilované sekvence byly detekovány s využitím streptavidin-alkalické fosfatázy a barevného substrátu.

Ke statistickému zpracování získaných dat byl použit χ^2 test.

Výsledky

Vlastní soubor sestával ze 30 karcinomů a 20 zdravých kontrol. Věkový medián pacientek s karcinomem byl 65 roků (rozmezí 52–77 roků) a 56 roků (rozmezí 50–79 roků) u pacientek kontrolní skupiny.

Vyšetřeno bylo 30 vzorků endometroidního karcinomu ve stadiu I (dle FIGO), mutace genu *K-ras* byla prokázána v 7 případech (23 %). V kontrolní skupině 20 vzorků normálního endometria byla sledovaná mutace nalezena ve 3 případech (15 %). Se spolehlivostí 0,95 je rozmezí výskytu mutace pro ženy s endometroidním karcinomem 10 % až 42 %, pro zdravé ženy je to 3 % až 38 %. Rozdíl mezi četností výskytu mutace v obou skupinách je však statisticky nevýznamný.

S ohledem na jednotlivá stadia a grade nádoru se mutace častěji vyskytovala ve stadiu IA a ve skupině dobře diferencovaných nádorů (tab. 1 a 2).

V kontrolní skupině byla sledovaná mutace popsána ve 3 vzorcích. Ve dvou případech se jednalo o normální proliferační endometrium a v jednom o klidové endometrium u postmenopauzální ženy.

Tab. 1. *K-ras* mutace ve stadiu I dle FIGO endometroidního karcinomu.

Stadium	IA n = 4	IB n = 18	IC n = 8
K-ras mutace	3 (75 %)	3 (17 %)	1 (13 %)

Tab. 2. *K-ras* mutace s ohledem na grade endometroidního karcinomu.

Grade	1 n = 9	2 n = 18	3 n = 3
K-ras mutace	3 (33 %)	3 (17 %)	1 (33 %)

Tab. 3. Genetické alterace u endometriálního karcinomu.

Genetická alterace	Typ I karcinomu	Typ II karcinomu
K-ras	15–30 %	0–5 %
PTEN	35–50 %	10 %
p53	10–20 %	90 %
Her2/neu	10–20 %	9–30 %
β-catenin	31–47 %	0–3 %
mikrosat. instabilita	20–40 %	0–5 %

Diskuze

Kromě zcela základních charakteristik, ze kterých obvykle vycházíme (stupeň postižení, typ nádoru a grading), existuje řada dalších ukazatelů, které mohou mít svoji prognostickou významnost. Nejinak je tomu u karcinomu endometria. Gynekologické zhoubné nádory představují skupinu chorob, u kterých je prognóza závislá také na subtilních genomických, epigenetických a proteomických změnách.

Tab. 3 ukazuje nejčastěji se vyskytující genetické alterace karcinomu endometria (typ I, II).

Ve vztahu k molekulární genetice je výrazněji profilován druhý typ karcinomu, který je méně častý a prognosticky horší (nevzniká na podkladě hyperplazie a není závislý na estrogenní stimulaci). Tento typ karcinomu je molekulárně geneticky charakterizován mutacemi genu *p53* a četnými ztrátami heterozygozity. Některé práce uvádějí, že gen *p53* je alterován až u 90 % serózních karcinomů [7]. Podle jiných autorů je mutace genu *p53* dvakrát častěji popisována u tumorů bez přítomnosti hyperplazie (estrogen non-dependentních) než u tumorů s hyperplazií (estrogen dependentních) [16,17]. Zvýšená exprese *p53* koreluje se špatnou diferenciací nádorů endometria, hloubkou myometriální invaze, pokročilým stadiem a metastatickým šířením [18,19].

Naopak zdaleka ne tak jasná je situace u prvního typu karcinomu, který vzniká na podkladě atypické hyperplazie a je podmíněn estrogenní stimulací. Stále není jasně prokázáno, která ze změn malignní transformaci iniciuje. Zdá se, že většina genetických alterací charakteristických pro toto nádorové onemocnění vzniká již na počátku tumorigeneze. Nejčastěji se vyskytující genetickou poruchou u endometriálního karcinomu je mutace *PTEN* tumor-supresorového genu, která je popisována u 25–83 % všech tumorů [20]. Zatímco u jiných typů nádorů je tato alterace genu *PTEN* asociována s pokročilým onemocněním včetně metastatického postižení, u karcinomu endometria je tomu naopak a ztráta funkce *PTEN* genu je považována za časnou událost a navíc je spojována s velmi dobrou prognózou [21]. K nejčastěji popisovaným molekulárním abnormitám u endometroidního typu karcinomu patří spolu s mutací genu *PTEN* také mikrosatelitální instabilita (MI) způsobená poruchou funkce DNA mismatch repair (MMR) genů. Několik prací uvádí shodu ve výskytu MI a *PTEN* mutací; mutace je popisována u 60–86 % MI-pozitivních endometroidních karcinomů a pouze u 24–35 % tumorů, kde MI prokázána nebyla. Pro sporadické formy endometroidního karcinomu je typická inaktivace MMR genu *MLH1* následovaná poruchou exprese genů *MSH2* a *MSH6* [22]. Přítomnost MI je asociována s dobrou prognózou endometroidního karcinomu [8,23]. *β-catenin* je další z genů, jehož porušená funkce je spojována s procesem endometriální karcinogeneze. Jeho mutace je signifikantně častěji popisována u endometroidních lézí (31–47 %) ve srovnání s non-endometroidním typem nádorů (0–3 %) [24]. Jiné práce uvádějí častější výskyt u endometriální hyperplazie, což by svědčilo pro podíl této mutace v časné karcinogenezi [25]. *Her2/neu* patří do skupiny protoonkogenů, jehož overexprese je popisována u 10–20 % endometroidních karcinomů se středním a nízkým stupněm diferenciace a některými autory je dávána do souvislosti s progresí onemocnění a horší prognózou. Mutace je dále popisována u 9–30 % serózních karcinomů a spolu s alterovaným genem p53 je asociována s velmi špatnou prognózou u non-endometroidního typu nádorů [26,27].

Z dostupných pramenů je patrné, že mutace *K-ras* protoonkogenu je detekována u 10–30 % případů endometroidního karcinomu [5,13]. Také výsledky naší práce potvrzují účast mutace *K-ras* genu v procesu vzniku karcinomu endometria ve smyslu časné události [28,29]. Prezentace dosavadních výsledků o *K-ras* mutaci u karcinomu endometria a prognostický význam tohoto ukazatele však nejsou jednotné. Například S. F. Lax ve své práci připouští, že pro vznik endometroidního karcinomu lze použít podobný model patogeneze jako u kolorektálního karcinomu. Působení *K-ras* mutace přichází v úvahu jednak na úrovni přechodu z atypické hyperplazie do dobře diferencovaného endometroidního karcinomu, ale především potencuje progresi do méně diferencovaných forem nádoru [9]. Z práce Mizuuchiho et al (n = 49) vyplývá, že *K-ras* mutace je nezávislý rizikový faktor odpovědný za agresivní chování endometriálního karcinomu [30]. Podobného názoru jsou i Ito et al (n = 221), kteří mutaci *K-ras* signifikantně spojují s přítomností metastáz v lymfatických uzlinách a předpokládají její důležitou roli v mechanizmu odpovědném za nepříznivé biologické chování endometroidního karcinomu u postmenopauzálních pacientek [31].

Semczuk et al (n = 57) na souboru pacientek s karcinomem endometria neprokázali souvislost přítomnosti *K-ras* mutace s věkem, stadiem, hloubkou invaze ani gradingem nádoru. Data podporují hypotézu o náhodné aktivaci *K-ras* genu v lidském neoplastickém endometriu a dále autoři naznačují, že by průkaz mutovaného protoonkogenu mohl být negativním prognostickým faktorem [32]. Naproti tomu práce kolektivu Esteller et al (n = 55) dokazuje, že *K-ras* mutace je relativně běžnou událostí v procesu endometriální karcinogeneze, ale bez jasného prognostického významu [33]. Ani Jones et al (n = 32) neprokázali vliv přítomnosti

K-ras mutace na prognózu onemocnění. Autoři srovnávali výskyt mutace *p53* a *K-ras* a jejich vztah k celkovému přežití pacientek s karcinomem endometria, kdy mutace *p53* silně korelovala s krátkým přežitím, *K-ras* však nikoli [34]. Studie autorů Pijnenborga et al (n = 44) se zabývala otázkou *K-ras* mutace u recidivujícího endometriálního karcinomu, kdy nebyl pozorován vliv této mutace na vznik recidivy onemocnění [35]. Několik prací se zabývalo také problematikou mutovaného *K-ras* genu u žen léčených Tamoxifenem. Prasad et al (n = 29) porovnávali pacientky s karcinomem endometria vzniklým při léčbě Tamoxifenem s pacientkami se sporadickým endometriálním karcinomem, kdy nebyl prokázán statistický rozdíl mezi četností výskytu mutace u obou vyšetřovaných skupin [36]. Tsujioka et al (n = 28) prokázali mutovaný *K-ras* gen u 46 % pacientek léčených Tamoxifenem. Po zastavení léčby u pacientek mutace již prokázána nebyla. Z práce vyplývá, že ukončení léčby Tamoxifenem by mohlo redukovat riziko vzniku karcinomu endometria cestou mutovaného genu *K-ras* [37].

V porovnání se sporadickými formami endometriálního karcinomu je genetické pozadí u hereditárních forem nádoru mnohem méně prozkoumané. Jedná se především o endometriální karcinomy vznikající v souvislosti s Lynchovým syndromem neboli HNPCC (hereditary nonpolyposis colorectal cancer). Endometriální karcinom je nejčastější malignita, která se u pacientek s Lynchových syndromem vyskytuje [38]. Lynchův syndrom je spojován s mutací genů patřících do rodiny mismatch repair (MMR), např. *MLH1, MSH2, MSH6, PMS1* nebo *PMS2* [39]. Zdá se, že mechanizmus vzniku endometriálního karcinomu v rámci HNPCC je důsledkem poruchy odlišných genů, než je tomu u kolorektálního karcinomu. Inaktivace komplexu genů *MSH2/MSH6* hraje pravděpodobně zásadní roli v procesu karcinogeneze [40]. Další genetickou alterací, která se uplatňuje v nádorovém procesu, je mutace *PTEN* genu, která se vyskytuje asi v 90 % případů endometriálního karcinomu u pacientek s Lynchovým syndromem [41].

Závěr

Prognostický význam molekulárně genetických faktorů u karcinomu endometria se přelomově liší v závislosti na typu nádoru. Jasně je definován u tumor-supresorového genu *p53* a nádorů druhého typu, kde koreluje s horší prognózou. U častějších endometroidních nádorů prvního typu však nejsou dosud publikované výsledky zdaleka tak jasné. Jako nejčastější událost na této úrovni je u nich popisována mutace genu *PTEN* a mikrosatelitální instabilita.

Naše výsledky u představitelek místní populace podporují teorii o možném podílu *K-ras* mutace v procesu endometriální karcinogeneze u nádorů prvního typu ve smyslu časné události.

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

Eva Dvorakova[a], Marcela Chmelarova[b], Jan Laco[c], Vladimir Palicka[b], Jiri Spacek[a]

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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[a]Department of Obstetrics and Gynecology, Faculty of Medicine in Hradec Kralove, Charles University in Prague and University Hospital Hradec Kralove, Hradec Kralove, Czech Republic
[b]Institute for Clinical Biochemistry and Diagnostics, Faculty of Medicine in Hradec Kralove, Charles University in Prague and University Hospital Hradec Kralove, Hradec Kralove
[c]The Fingerland Department of Pathology, Faculty of Medicine in Hradec Kralove, Charles University in Prague and University Hospital Hradec Kralove, Hradec Kralove
Corresponding author: Eva Dvorakova, e-mail: evajurkova@seznam.cz

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 use[1]. 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 carcinoma[2].

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 inactivation[3]. Molecular events associated with tumor methylation hold promises for cancer risk assessment, diagnostic purpose and prognosis[4]. 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 others[9]. 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) samples[10]. 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

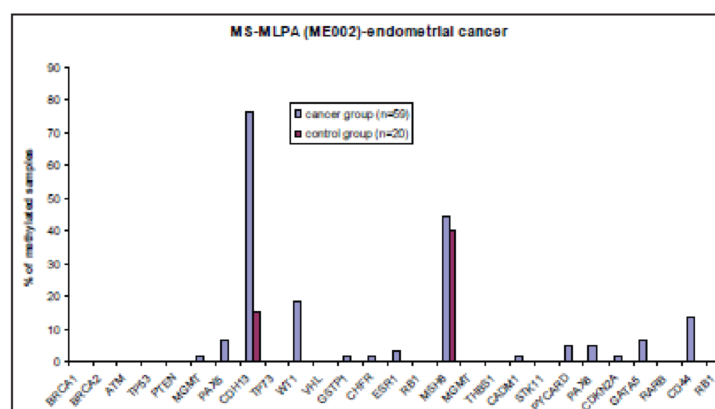

Fig. 1. Comparison of methylation frequencies (cut-off value 15%) of the 25 analyzed genes in endometrial cancer and control samples. There is significantly higher methylation in *CDH13* gene ($P<0.001$) in endometrial carcinoma group compared with control group.

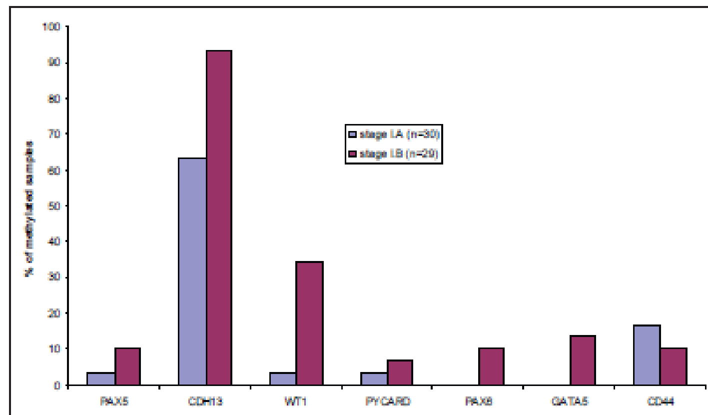

Fig. 2. Methylation of specific genes according to tumor stage. There is significantly higher methylation in *WT1* (P=0.002) and *GATA5* (P=0.05) genes in stage IB of endometrial carcinoma samples compared with stage IA samples.

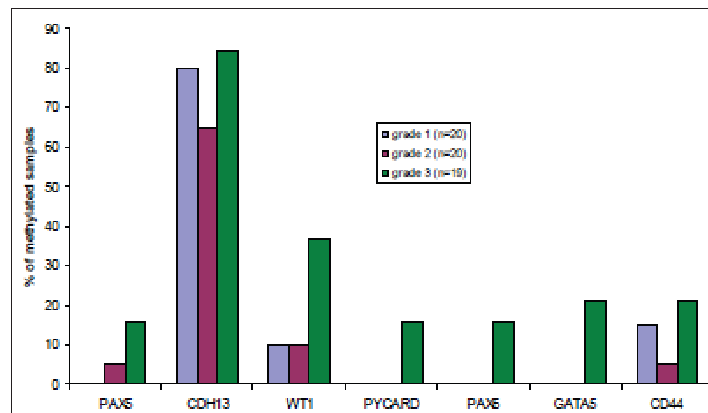

Fig. 3. Methylation of specific genes according to tumor grade. There is significantly higher methylation in *GATA5* gene (P=0.05) in poorly differentiated carcinoma compared with grade 1 and grade 2 tumor samples.

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 system[11]. 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 cancer and control samples. For genes *BRCA1, BRCA2, ATM, TP53, PTEN, TP73, VHL, RB1, 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 genes[3]. 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 study[12].

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 tissue[12]. 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 methylated[14]. 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 carcinogenesis[15]. 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 progression[16]. 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 healing[19]. With regard to *CD44* and its variants, several studies have investigated its expressions in endometrial pathologies, including adenocarcinomas[20-25]. 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 mehylation 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 development[28]. *GATA5* have been implicated as important regulators in the normal development and differentiation of mesoderm- and endoderm-derived tissues, including lung, liver, gonad and pancreas[29]. Loss of *GATA4* and *GATA5* expression second to promoter hypermethylation has been identified in primary ovarian, lung and gastrointestinal cancer[30-33]. 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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Marcela Chmelarova*, Spela Kos, Eva Dvorakova, Jiri Spacek, Jan Laco, Ema Ruszova, Katerina Hrochova and Vladimir Palicka

Importance of promoter methylation of *GATA4* and *TP53* genes in endometrioid carcinoma of endometrium

Abstract

Background: Epigenetic changes are considered to be a frequent event during tumor development. Various methylation changes have been identified and show promise as potential cancer biomarkers. The aim of this study was to investigate promoter methylation of *GATA4* and *TP53* genes in endometrioid carcinoma of endometrium.
Methods: To search for promoter methylation of *GATA4* and *TP53* genes we used methylation-specific PCR (MSP) to compare the methylation status of 54 patients with endometrioid carcinoma of endometrium and 18 patients with normal endometrial tissue.
Results: In our study MSP revealed *GATA4* promoter methylation in 44 of 54 in the carcinoma group (81.5%), and in none of the control group. No methylation was observed in *TP53* gene.
Conclusions: In conclusion, our study showed that there is significantly higher methylation in *GATA4* gene in the endometrial cancer group compared with samples of non-neoplastic endometrium. The finding suggests the importance of hypermethylation of this gene in endometrial carcinogenesis and could have implications for future diagnostic and therapeutic strategies for endometrial cancer based on epigenetic changes.

Keywords: endometrial neoplasms; epigenomics; *GATA4* transcription factor; gene *TP53*; methylation.

***Corresponding author: Mgr. Marcela Chmelarova**, PhD, Institute for Clinical Biochemistry and Diagnostics, University Hospital Hradec Kralove, Sokolska 581, Hradec Kralove 500 05, Czech Republic, Phone: +420 495833864,
E-mail: ChmelarovaMarcela@seznam.cz
Spela Kos: Institute for Clinical Biochemistry and Diagnostics, Medical Faculty of Charles University and University Hospital Hradec Kralove, Czech Republic; and Faculty of Pharmacy, Department of Clinical Biochemistry, University of Ljubljana, Ljubljana, Slovenia
Eva Dvorakova and Jiri Spacek: Department of Obstetrics and Gynecology, Medical Faculty of Charles University and University Hospital Hradec Kralove, Czech Republic
Jan Laco: The Fingerland Department of Pathology, Medical Faculty of Charles University and University Hospital Hradec Kralove, Czech Republic
Ema Ruszova: Department of Clinical Genetics, University Hospital Hradec Kralove, Czech Republic
Katerina Hrochova and Vladimir Palicka: Institute for Clinical Biochemistry and Diagnostics, Medical Faculty of Charles University and University Hospital Hradec Kralove, Czech Republic

Introduction

Endometrial cancer is the most common gynecological cancer in developed countries [1] and the incidence is increasing. Two different clinicopathologic subtypes are recognized: type I – endometrioid, and type II – non-endometrioid. Type I has a higher frequency, is associated with unopposed estrogen exposure, and is often preceded by premalignant disease. Risk factors are obesity, hyperlipidemia, and signs of hyperestrogenism [2]. Pathologically, it is a well-differentiated endometrioid adenocarcinoma with a low incidence of lymph node metastasis and myometrial invasion, and has good prognosis [3].

Most women are being diagnosed after experiencing irregular vaginal bleeding. Current diagnosis is supported by preoperative evaluation which includes history, clinical examination, endometrial biopsy, complete blood count, liver and renal function tests, chest X-ray and magnetic resonance imaging if cervical involvement is suspected. Staging is based on the International Federation of Gynecology and Obstetrics (FIGO) system. Treatment is chosen on the basis of FIGO staging, and initially includes appropriate surgery that may be followed by adjuvant radiotherapy or chemotherapy. Since there is lack of clear evidence and data from different studies are controversial, treatment choice is often difficult [4].

Similar to other cancers, endometrial cancer has been shown to be a complex disease driven by different factors. Multiple risk factors, such as age, overweight and postmenopausal hormone therapy, have been

described [5]. Genetic aberrances, such as variations in gene expression and mutation in cancer-related genes have been identified, but these do not fully explain carcinogenesis in the endometrium. Epigenetic changes are now being examined. In particular, aberrant DNA methylation is thought to play a key role in endometrial carcinogenesis [3].

Transcription factors of the GATA family are essential regulators of the specification and differentiation of numerous tissues. They all share two highly conserved zinc fingers of the C2H2 type that mediate not only DNA binding but also the great majority of protein interactions [6]. Studies suggest that *GATA-4*, *-5*, and *-6* factors are important regulators of tissue-specific gene expression in multiple endoderm- and mesoderm-derived tissues. GATA factors are important regulators of both structural and regulatory genes in the heart. *GATA-4* and *-6* have been implicated in the regulation of liver-specific gene expression. *GATA-4*, *-5*, and *-6* have also been implicated in the regulation of epithelial cell differentiation in the gut and are also important regulators of gene expression within the gonads [7]. Expression of the Mullerian inhibiting substance promoter is regulated by *GATA-4* in Sertoli cells and Mullerian ducts [8–10], and *GATA-4* regulates expression of the steroidogenic acute regulatory protein promoter in the ovary [11].

Protein p53 is a 53-kD nuclear phosphoprotein (393 amino acids) [12]. It is a tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. In normal cells, wtp53 appears to be typically present in latent form. Moreover, the steady state levels of this latent p53 are extremely low, owing to a very rapid rate of proteolytic degradation. In normal cells or tumor cells that still retain wtp53 expression, activation of the endogenous wtp53 in response to extracellular or intracellular stimuli results in accumulation of stabilized, biochemically altered protein [13]. The increase in wtp53 activity can lead to various cellular outcomes such as cell cycle arrest and induction of apoptosis. It is believed that these dramatic biological effects of activated wtp53 may mediate much of its tumor suppressor function, particularly when they occur in cells which have accumulated defects in their DNA or chromosomes. In such situations, activation of p53 would prevent the perpetuation of the genomic damage, and ensure that these potentially dangerous cells will not multiply and take over the normal population [14].

Epigenetics can be described as stable alteration in gene expression potential that takes place during development and cell proliferation, without any changes in gene sequence. DNA methylation is one of the most common epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a possible therapeutic target.

DNA methylation is a covalent chemical modification mediated by the DNA cytosine methyltransferases, resulting in addition of a methyl group at the carbon 5 position of the cytosine ring. Most cytosine methylation occurs in the sequence context 5′CG3′ [15]. Increased methylation in the transcribed region has a variable effect on gene expression. The first mechanism involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters [16]. The second mode of repression involves a direct binding of specific transcription repressors to methylated DNA [17]. DNA methylation can also affect histone modification and chromatin structure, which in turn can alter gene expression [18]. Compared to normal cells, the malignant cells show major disruption of their DNA methylation patterns [15].

Recent studies of methylation profile in endometrial tumorigenesis showed that, among 24 tumor suppressor genes, the number of promoter-methylated loci increased in progression from normal endometrium through simple hyperplasia to complex hyperplasia [19].

Materials and methods

Formalin-fixed and paraffin-embedded samples of both endometrioid carcinoma of endometrium and normal endometrial tissue were obtained from 72 women treated at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic: 54 patients with endometrioid carcinoma of endometrium (Table 1), and 18 patients with normal endometrial tissue. The median age of patients at the time of diagnosis was 65.2 years (range 44–84 years)

Table 1 Clinicopathological characteristics versus methylation of GATA4.

Characteristic	Number of samples	% of samples	GATA4 methylation (% of samples)
Stage			
I.A	28	51.9	82.1
I.B	26	48.1	80.8
Grade			
1	18	33.3	88.9
2	19	35.2	79.0
3	17	31.5	76.5

in the carcinoma group and 59.9 years (range 50–79 years) in the control group. The patients with endometrial cancer and normal endometrium were treated from 2006 to 2010. The samples of normal endometrium were obtained from patients treated surgically for a non-malignant diagnosis. The paraffin blocks were retrieved from the archive of the Fingerland's Department of Pathology, University Hospital Hradec Kralove. All slides were reviewed by an experienced pathologist and the carcinomas were classified according to the current WHO classification of tumors of the female genital organs [20]. The study was approved by the Ethics Committee of Faculty Hospital Hradec Kralove.

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

GATA4 and TP53 MSP (methylation-specific PCR)

DNA methylation patterns in the CpG islands of the promoter region of the *GATA4* and *TP53* genes were determined by methylation-specific PCR (MSP) [21]. Sodium bisulfite modification was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, USA) according to the manufacturer's protocol, with minor modifications.

Primer sequences for *GATA4* gene were designed using MethPrimer. 5′-GGTTAGTTAGTGTTTTAGGGTTGA-3′ (sense) and 5′- AACAAAAACAAAAAAACTCCAAA-3′ (antisense) for unmethylated reaction (PCR product 230 bp), and 5′-GTTAGTTAGCGTTTTAGGGTCGA-3′ (sense) and 5′- CAAAAACGAAAAAACTCCGAA-3′ (antisense) for methylated reaction (PCR product 228 bp). Primer sequences for *TP53* gene have been reported previously [22]. 5′-TTGGTAGGTGGATTATTTGTTT-3′ (sense) and 5′- CCAATCCAAAAAAACATATCAC-3′ (antisense) for unmethylated reaction (PCR product 247 bp), and 5′- TTCGGTAGGCGGATTATTTG-3′ (sense) and 5′- AAATATCCCCGAAACCCAAC-3′ (antisense) for methylated reaction (PCR product 193 bp). PCR was carried out in a 25 μL mixture containing 10× Takara buffer (2.5 μL), dNTPs 2.5 mM solution Takara (2.0 μL), primers (1 μL each 10 pmol/μL solution), polymerase Taq HS Takara 5 U/μL (0.3 μL) (Takara Bio Europe S.A.S, France), water and 2 μL of bisulfite-modified DNA in a Veriti Thermocycler (Applied Biosystems, CA, USA). The cycling condition for *GATA4* gene consisted of an initial denaturation at 95 °C for 5 min, 40 cycles of denaturing at 95 °C for 45 s, annealing at 53.7 °C for 35 s, and extension at 72 °C for 35 s, followed by final extension for 5 min at 72 °C. The cycling condition for *TP53* gene consisted of an initial denaturation at 95 °C for 7 min, 40 cycles of denaturing at 95 °C for 45 s, annealing at 59 °C for 45 s, and extension at 72 °C for 60 s, followed by final extension for 5 min at 72 °C.

CpG universal methylated and unmethylated DNA (Zymo Research Corporation, USA) were similarly treated with bisulfite and were used as controls.

Amplified products were separated by electrophoresis on 2% agarose gels and visualized under ultraviolet light after staining with ethidium bromide.

Statistical analysis

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

Results

MSP (methylation-specific PCR) (Figures 1 and 2)

In the present study we used MSP for *TP53* and *GATA4* to analyze samples from 54 patients with endometrioid carcinoma of endometrium and 18 control patients. MSP revealed statistically significant higher promoter methylation ($p<0.001$) of the *GATA4* gene in the group of endometrioid carcinoma of endometrium than in the control group. Promoter of *GATA4* gene was methylated in 44 of 54 in the carcinoma group (81.5%), and in none of the control group. No methylation was observed in *TP53* gene.

The methylation results from the endometrioid carcinoma of endometrium specimens were compared against clinicopathological characteristics, including tumor stage and tumor grade (Table 1). No significant correlation between *GATA4* methylation and any of these parameters was observed for the patients with endometrioid carcinoma of endometrium ($p>0.05$).

Figure 1 Methylation-specific PCR of the *TP53* promoter region in tumor samples.
+ Universally methylated positive control DNA, – universally unmethylated negative control DNA. The presence of a visible PCR product in the lane marked U indicates the presence of unmethylated *TP53* genes, the presence of product in the lane marked M indicates presence of methylated *TP53* genes. Sample no. 1 has unmethylated promoter region of *TP53* gene.

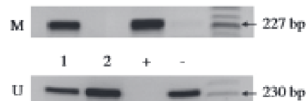

Figure 2 Methylation-specific PCR of the *GATA4* promoter region in tumor samples.
+ Universally methylated positive control DNA, – universally unmethylated negative control DNA. The presence of visible PCR product in the lane marked U indicates the presence of unmethylated *GATA4* gene, the presence of product in the lane marked M indicates presence of methylated *GATA4* gene. Sample no. 1 has partial methylated analyzed CpG loci of *GATA4* gene and sample no. 2 has unmethylated analyzed CpG loci of *GATA4* gene.

Discussion

Cancer has been considered as a disease driven by progressive genetic alterations, such as mutations involving oncogenes or tumor suppressor genes, as well as chromosomal abnormalities. However, more recently, it has been demonstrated that cancer is also driven by epigenetic alterations [23]. Many different genes have been identified to be hypermethylated and silenced in endometrial cancer. The purpose of this study was to investigate promoter methylation of *GATA4* and *TP53* genes in carcinoma of endometrium.

Mutations, loss of expression, or overexpression of GATA factors have all been associated with a broad variety of cancers in humans, including leukemia, breast cancer, gastrointestinal cancers, and others. Whilst *GATA1* and *GATA3* have been very well studied in the context of human malignancies, other members of the GATA family need further investigation.

While, to date, no mutations or deletions of the *GATA4* gene have been discovered in human cancers, silencing of its expression seems to be widespread in different types of cancers. Expression of *GATA4* was extinguished in the majority of cell lines from colorectal (CRC) and gastric (GC) cancers as well as in primary tumors. Silencing was associated with hypermethylation of the *GATA4* promoter sequences [24, 25]. *GATA4* was found to be extinguished in a large proportion of lung [26] and esophageal cancers [27]. *GATA-4* has also been reported to be aberrantly methylated in 23.2% of glioblastoma tumors but not in normal brain [28]. Methylation was observed in human ovarian cancer cell lines and primary ovarian cancers as well [29, 30]. These studies support the idea that loss of *GATA4* by epigenetic silencing might contribute to malignant transformation. Based on the importance of methylation in the *GATA4* gene described in previous studies we focused our analysis on *GATA4* methylation in endometrioid carcinoma of the endometrium. Our study revealed significantly higher methylation (81.5%) in the carcinoma group compared with the control group. This finding suggests the importance of *GATA4* methylation in endometrial carcinogenesis. Methylation of *GATA4* gene in endometrial cancer patients could be used in future as a prognostic factor or for non-invasive screening because cell-free circulating methylated DNA has been detected in body fluids (plasma and other), e.g., in ovarian cancer patients, and the level correlated reasonably well with methylation levels in tumor tissue [31]. There are clinically available DNA methylation tests for oncology. In cololorectal cancer screening we can mention SEPT9 methylation in plasma [32].

The *TP53* gene is frequently affected by loss of alleles and by point mutations in almost all cancers [33]. Mutated *TP53* results in a non-functional protein that accumulates in the cell and acts as a dominant negative inhibitor of wild-type *TP53*, leading to propagation of aberrant cells [34]. *TP53* mutations or *TP53* overexpression in carcinoma of endometrium is twice as frequent in tumors without hyperplasia (estrogen unrelated) than in those with hyperplasia (estrogen related) [35, 36]. *TP53* mutation is present in about 90% of serous carcinomas (estrogen-unrelated NEEC) [37].

Due to the high frequency of *TP53* mutations in human cancers, promoter methylation of this gene has also been examined in several studies. *TP53* promoter methylation was observed in extra-axial brain tumors [38], gliomas [22], acute lymphoblastic leukemia [39], ovarian cancer [40] and retinoblastoma [41]. *TP53* promoter methylation was also studied in breast cancer [42], gastric cancer [43] and adrenocortical cancer [44] but was not proved to be significant. *TP53* promoter methylation in endometrial cancer has not yet been examined. Our study is the first study to have examined methylation in the *TP53* promoter region and we observed no methylation in the analyzed region. Based on these results it could be concluded that despite frequent mutations in the *TP53* gene in endometrial cancer, methylation in the *TP53* promoter region is not an important event in endometrial carcinogenesis.

In conclusion, our study showed that there is significantly higher methylation in *GATA4* gene in the endometrial cancer group compared with samples of non-neoplastic endometrium. This finding suggests the importance of hypermethylation of this gene in endometrial carcinogenesis and could have implications for future diagnostic and therapeutic strategies for endometrial cancer based on epigenetic changes.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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Mutation of K-ras gene in pathogenesis of endometrial carcinoma

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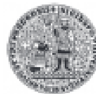

E. Krepinska[1], M. Chmelarova[2], J. Laco[3], V. Palicka[2], J. Spacek[1]

[1] Department of Obstetrics and Gynecology
[2] Institute for Clinical Biochemistry and Diagnostics
[3] Fingerland's Institute for Pathology, Medical Faculty of Charles University and Faculty Hospital Hradec Králové, Hradec Králové, Czech Republic

INTRODUCTION:

Endometrial cancer is one of the three most common cancers in female in many industrialized countries. 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 use (1). For all stages, the overall 5-year survival is around 80%. Two types of endometrial carcinoma are distinguished with respect to biology and clinical course: type I- endometroid and type II- non-endometroid (serous, clear cell) carcinoma (2).

Molecular data from multiple studies support the hypothesis of different pathways in the development of type I and type II carcinomas. The most frequent genetic alteration in endometroid carcinoma is PTEN inactivation (3), microsatellite instability (4) and mutation of K-ras (5) and beta-catenin (6). Mutation of p53 gene is the most frequent alteration in non-endometroid carcinomas (7).

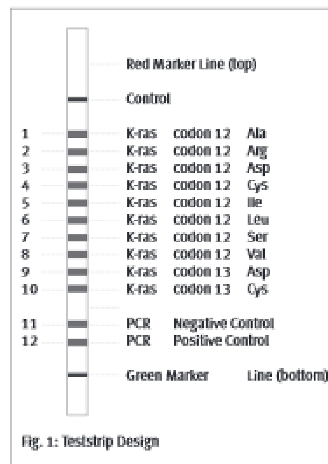

Fig. 1: Teststrip Design

K-ras protooncogene encodes a membrane GTPase and is related to tumor growth and differentiation. Mutations of K-ras gene are present in about 10-30% of endometroid carcinomas (5), predominantly found in exon 1 (codons 12 and 13). The presence of K-ras mutations in 16% of the cases of endometrial hyperplasia indicates that K-ras mutations may represent an early event in endometroid tumorigenesis (8).

The aim of the study was to evaluate the presence of K-ras mutation in early stages of endometroid carcinoma according to normal endometrium.

MATERIALS AND METHODS:

Paraffin-embedded sections from stage I endometroid carcinoma and normal endometrium were obtained from 50 women (30 patients with endometroid carcinomas, 20 patients with normal endometrium) treated at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic. The samples of normal endometrium were obtained from patients surgically treated for non-malignant diagnosis (such as fibrotids, descent of uterus, etc.).

Detection of K-ras mutation was made by using of K-ras StripAssay™, ViennaLab Diagnostics GmbH. The procedure includes three steps: 1/ DNA isolation from paraffin-embedded samples (Qiagen), 2/ PCR amplification using biotinylated primers, 3/ hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines (Fig. 1). Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates.

RESULTS:

In the present study, we found K-ras mutation in 23% of specimens with endometroid carcinoma and surprisingly in 15% of the control group.

The mean age at the time of diagnosis was 64,5 years (range 52-77 years) in the carcinoma group and 56,5 years (range 50-79) in the control group. Majority of patients were obese (77% had BMI more than 30) and had hypertension and/or diabetes (70%).

Presence of K-ras mutation according to FIGO stage I and grade of endometroid carcinoma is shown in Table 1 and 2.

CONCLUSION:

We found K-ras mutation in 23% of patients with endometroid carcinoma, more frequent in early-stage disease. This founding suggests its role as an initiative event in carcinogenesis of endometroid carcinoma and we hypothetise that it could have positive predictive value. The statistical significance is limited because of the small amount of specimens, so we propose to extend the study.

Table 1. K-ras mutation according to FIGO stage I of endometroid carcinoma

	IA (n=22)	IB (n=8)
K-ras mutation	6	1

Table 2. K-ras mutation according to grade of endometroid carcinoma

	grade 1 (n=9)	2 (n=18)	3 (n=3)
K-ras mutation	3	3	1

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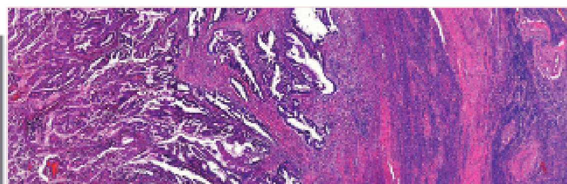
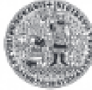


METHYLATION ANALYSIS OF TUMOR SUPPRESSOR GENES IN ENDOMETRIAL CANCER

14th Biennial Meeting of the International Gynecologic Cancer Society
Vancouver, Canada, October 13-16, 2012



Authors:
Krepinska E.[1], Chmelarova M.[2], Laco J.[3], Palicka V.[2], Spacek J.[1]

1 - Department of Obstetrics and Gynecology, Medical Faculty of Charles University and Faculty Hospital Hradec Kralove, Czech Republic
2 - Institute for Clinical Biochemistry and Diagnostics, Medical Faculty of Charles University and Faculty Hospital Hradec Kralove, Czech Republic
3 - The Fingerland Department of Pathology, Medical Faculty of Charles University and Faculty Hospital Hradec Kralove, Czech Republic

Keywords: MS-MLPA, DNA methylation, endometrial cancer, CDH13, epigenetics

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INTRODUCTION:

Endometrial cancer is one of the three most common cancers in female in many industrialized countries. Majority of cases are diagnosed after the menopause, with the highest incidence around the seventh decade of life. The riskfactors for the disease include obesity, hypertension, diabetes mellitus, late menopause and unopposed estrogen use (1). For all stages, the overall 5-year survival is around 80%. Two types of endometrial carcinoma are distinguished with respect to biology and clinical course: type I - endometrioid and type II - non-endometrioid (serous, clear cell carcinoma (2).

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 inactivation (3). The role of epigenetics in cancer is undisputed. 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 presents as an alternative to mutational inactivation (3).

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 others (4). MS-MLPA represents a rather novel cost-effective and time-efficient method (5). MS-MLPA is an ideal technique to use in FFPE (formalin-fixed, paraffin-embedded) samples. 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, paraffin-embedded samples from both endometrioid carcinomas of endometrium and normal endometrial tissue were obtained from 29 women (20 patients with endometrial cancer, 9 patients with normal endometrium) treated at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic. The samples of normal endometrium were obtained from patients surgically treated for non-malignant diagnosis. The tumors were classified according to the WHO classification of tumors of the female reproductive system. 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. 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. PCR 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 = (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 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 (6). 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 used the MS-MLPA probe set ME002 (MRC-Holland, Amsterdam, The Netherlands) to analyze samples from 20 patients with endometrial cancer and 9 control samples. Using a 15% cut-off for methylation, we observed significantly higher methylation in CDH13 gene in the endometrial cancer group. We also observed higher methylation in WT1 and CD44 genes in endometrial cancer, but with no statistical significance cause of the small amount of analyzed samples. For gene MSH6 we observed high methylation (about 60%) in both ovarian cancer and control samples. For other analyzed genes the methylation rate did not exceed the 15% threshold (Fig.1).

Fig. 1: Methylation of specific genes in endometrial cancer samples and control samples

Figure 1.
Comparison of methylation frequencies (cut-off value 15%) of the 25 analyzed genes in endometrial cancer and control samples.

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 the tumor cells, including the epigenetic inactivation of tumor suppressor genes as well as mutations and deletions. It is now clear that de novo promoter methylation is a common mechanism for inactivation of tumor suppressor genes (3). The promoter methylation status has been reported in several human neoplasms. The purpose of this study was to investigate promoter methylation of a set of common tumor suppressor genes in 20 endometrial cancer and 9 control samples. We used MS-MLPA, and a threshold of 15% methylation was applied based on previous experiments (6). We observed significantly higher methylation in CDH13 gene and higher methylation in WT1 and CD44 genes in endometrial cancer indicating that promoter methylation of these tumor suppressor genes may play an important role in endometrial carcinogenesis. This finding 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 tissue (6). In the present study, MSH6 methylation was very frequent in both endometrial cancer and normal endometrial tissue (Fig.1).

Our present study is the first study to demonstrate methylation of CDH13. This gene encodes a member of the cadherin superfamily. The protein acts as a negative regulator of axon growth during neural differentiation and also 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 cancer. For example in ovarian carcinoma, methylation of CDH13 gene plays an important role (7).

Methylation of CDH1 (E-cadherin), another member of cadherin superfamily, is also important event in endometrial carcinogenesis (8). 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 progression (9). However, no association between E-cadhrin hypermethylation and clinicopathological or immunohistological characteristics of endometrial cancer was found in other studies (10,11).

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 healing (12). With regard to CD44 and its variants, several studies have investigated its expressions in endometrial pathologies, including adenocarcinomas (13-18).

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 (19,20).

There is 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.

CONCLUSION:

In conclusion, our study showed that there is significantly higher methylation in CDH13 gene in the endometrial cancer group and higher methylation in WT1 and CD44 genes in endometrial cancer, but with no statistical significance cause of the small amount of analyzed samples. These 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.

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