Charles University in Prague
Faculty of Medicine

MOLECULAR BIOLOGICAL CHANGES IN ENDOMETRIAL CARCINOMA

Eva Dvořáková

Abstract of the thesis

Doctoral study program Obstetrics and Gynecology

Hradec Králové

2014

The thesis was written during combined doctoral study (PhD) program Obstetrics and Gynecology at the Department of Obstetrics and Gynecology, Faculty of Medicine in Hradec Králové, Charles University in Prague.

Author: MUDr. Eva Dvořáková
Department of Obstetrics and Gynecology
Faculty of Medicine in Hradec Králové, Charles University in Prague and Faculty Hospital in Hradec Králové, Czech Republic

Supervisor: doc. MUDr. Jiří Špaček, Ph.D., IFEPAG
Department of Obstetrics and Gynecology
Faculty of Medicine in Hradec Králové, Charles University in Prague and Faculty Hospital in Hradec Králové, Czech Republic

Opponents: prof. MUDr. Karel Odrážka, Ph.D.
Department of Oncology
First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic

doc. MUDr. Jiří Sláma, Ph.D.
Department of Obstetrics and Gynecology
First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Czech Republic

The thesis will be defended .....

This study was supported by grant GAUK No. 157310, and by research project PRVOUK.

The thesis is available for inspection at the Study Department of the Dean's Office, Faculty of Medicine in Hradec Králové, Charles University in Prague, Šimkova 870, 500 38 Hradec Králové (tel. 495 816 131).

doc. MUDr. Jiří Špaček, Ph.D., IFEPAG
Chairperson of the Commission for the Thesis Defences
in doctoral study program Obstetrics and Gynecology

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to all those who have helped and supported me in different ways throughout the study.

I especially wish to thank my supervisor, doc. MUDr. Jiří Špaček, Ph.D., IFEPAG, for his specialist advices, and gracious support and help.

I am grateful to thank my colleagues from the Department of Obstetrics and Gynecology, Fingerland Department of Pathology and Institute for Clinical Biochemistry and Diagnostics for cooperation. I give special thanks to my colleague and soul mate, Mgr. Marcela Chmelařová, Ph.D., for excellent and essential contributions to our research, and for being a great friend.

I would like to extend my deepest appreciation to my husband and my son for their love and support.

TABLE OF CONTENTS

SOUHRN

Molekulárně biologické změny u karcinomu endometria

Karcinom endometria patří mezi nejčastější malignity ženských pohlavních orgánů. Jeho stále stoupající incidence je způsobena změnami životního stylu a prostředí. Podobně jako ostatní maligní nádory je karcinom endometria komplexní onemocnění, na jehož vzniku se podílí mnoho faktorů, včetně genetických a epigenetických alterací. Detailní prozkoumání a porozumění těchto změn umožní v budoucnu nalezení nových diagnostických a terapeutických možností pro pacientky s endometriálním karcinomem.

Cílem disertační práce bylo vyšetřit vybrané molekulárně biologické změny u karcinomu endometria v porovnání se zdravou endometriální tkání.

Prvním specifickým cílem bylo vyšetřit přítomnost K-ras mutace ve vzorcích endometriální tkáně a porovnat ji s klinicko-patologickými charakteristikami (stádium a grading nádoru). Vyšetřeno bylo celkem 79 vzorků, z toho 59 vzorků s endometroidním karcinomem endometria ve stádiu I dle FIGO a 20 vzorků nenádorového endometria jako kontrolní skupina. Ke stanovení K-ras mutace byla použita metoda K-ras StripAssay™ (ViennaLab Diagnostics GmbH). Nebyl prokázán žádný rozdíl ve výskytu K-ras mutace v nádorové a kontrolní skupině (24% vs. 15%). Nebyl prokázán žádný vztah sledované mutace s ohledem na stádium onemocnění a grading nádoru.

Druhým specifickým cílem bylo vyšetřit přítomnost metylace promotorových oblastí vybraných tumor-supresorových genů a porovnat ji s klinicko-patologickými charakteristikami (stádium a grading nádoru). Ke stanovení metylačních změn byly použity dvě metody: MS-MLPA a MSP. Pomocí MS-MLPA bylo vyšetřeno celkem 79 vzorků endometria (59 nádorových a 20 nenádorových) a pomocí MSP bylo vyšetřeno 72 vzorků (54 nádorových a 18 nenádorových). Ve skupině vzorků s endometriálním karcinomem byla prokázána statisticky významná hypermetylace genu CDH13 (p<0.0001) a GATA4 (p<0.0001) oproti kontrolní skupině. Ve stádiu IB byla prokázána hypermetylace genu WT1 (p=0.002) a GATA5 (p=0.05) oproti stádiu IA. Ve skupině nízce diferencovaných karcinomů byla prokázána hypermetylace genu GATA5 (p=0.05) oproti skupině s dobře a středně diferencovanými nádory.

Zatímco úloha K-ras mutace není zcela jasná, naše výsledky svědčí o důležitosti metylace genů CDH13, WT1, GATA4 a GATA5 v procesu karcinogeneze endometriální tkáně. Hypermetylace WT1 a GATA5 je pravděpodobně zodpovědná za invazi nádoru do myometria a jeho agresivní chování.

SUMMARY

Molecular biological changes in endometrial carcinoma

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.

The aim of the study was to evaluate selected molecular biological changes in endometrial carcinoma comparing to non-neoplastic endometrium.

The first specific aim was to compare presence of K-ras mutation in early stages of endometrioid type of endometrial carcinoma with normal endometrium, and to evaluate association to clinical-pathological characteristics (tumor stage and grade). We analyzed 79 samples of endometrium (59 samples of endometrioid endometrial carcinoma stage I, and 20 samples of normal, non-neoplastic endometrium). Detection of K-ras mutation was made by using of K-ras StripAssay™ (ViennaLab Diagnostics GmbH). The frequency of K-ras mutation in the carcinoma group did not differ from the group of control samples (24% vs. 15%). No association between K-ras mutation and tumor stage and grade was observed for the patients with endometrioid carcinoma of endometrium.

The second specific aim was to compare promoter methylation in selected tumor suppressor genes in early stages of endometrioid type of endometrial carcinoma with normal endometrium, and to evaluate association to clinical-pathological characteristics (tumor stage and grade). MS-MLPA was used to analyze 79 samples of endometrium (59 samples of endometrioid endometrial carcinoma stage I, and 20 samples of normal, non-neoplastic endometrium). We observed higher methylation in CDH13 gene (p<0.0001) in the group of endometrioid carcinoma of endometrium compared to the group of control samples. MSP was used to analyze 72 samples of endometrium (54 samples of endometrioid endometrial carcinoma stage I, and 18 samples of normal, non-neoplastic endometrium). We observed higher methylation in GATA4 gene (p<0.0001) in the group of endometrioid carcinoma of endometrium compared to the group of control samples. 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. 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.

Whereas the role of K-ras mutation in endometrial carcinogenesis remains unclear, our finding suggests the importance of CDH13, WT1, GATA4 and GATA5 methylation in this process. Hypermethylation in WT1 and GATA5 genes could play an important role in tumor myometrial invasion and its aggressive behavior.

1 BACKGROUNDS

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 epigenetic plays a particularly important role in modern diagnosis and treatment of cancer.

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 the Czech Republic, the incidence is the highest in developed countries.

Multiple risk factors for endometrial cancer have been identified (*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.

About 75% of all endometrial carcinomas are of endometrioid type (*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*). 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*). 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.

Abnormal uterine bleeding is the most frequent symptom of endometrial carcinoma. Transvaginal ultrasonography (TVU) is considered as the first step in any woman presenting with abnormal uterine bleeding (*Clark TJ, 2004*).

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.

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 (*Cibula D, et al., 2009*).

The most important therapy for endometrial carcinoma is surgery. 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. 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*). Indications for radiotherapy are generally in the adjuvant settings. Radical radiotherapy should be applied in patients with contraindications for surgery, or inoperable advanced disease. Systemic chemotherapy can be used as a palliative therapy in metastatic and recurrence disease (*Cibula D, et al., 2009*).

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. Most type 2 cancers contain mutations of 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 PTEN, K-ras, and β-catenin (*Hecht JL and Mutter GL, 2006*).

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*). 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 (*Lax SF, 2004*). However, the role of the K-ras mutations in endometrial carcinogenesis is not yet fully understood.

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 kontext 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*). 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. Strong association between MMR gene 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*). 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*). Promoter methylation of RASSF1A 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 APC gene, tumor suppressor gene that regulates β-catenin in the Wnt pathway, E-cadherin and p73 has also been observed (*Banno K, et al., 2006; Yang HY, et al.,*

2006). As well as Sprouty 2, GPR54 and RSK4 (*Cannistra SA, 2004*). Methylation of some other genes have been associated with endometrial carcinoma: HOXA10, HOXA11, THBS2, CDH13, HSPA2, SOCS2, PER1, RARB2, GSTP1, SFN, SESN3, TITF1 (*Whitcomb BP, et al., 2003; Mhawech P, et al., 2005; Yeh KT, et al., 2005; Yoshida H, et al., 2006*) and COMT (*Sasaki M, et al., 2003*). miRNAs have been found to be down regulated by methylation of DNA in various cancers including endometrial carcinoma (*Cannistra SA, 2004*).

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.

2 OBJECTIVES

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.

3 MATERIAL AND METHODS

3.1 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.

3.2 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).

3.3 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.

3.4 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.

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.

3.5 GATA4 AND TP53 MSP

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.

3.6 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 RESULTS

4.1 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.

4.2 SPECIFIC AIM 1B

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

4.3 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 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.

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.

4.4 SPECIFIC AIM 2B

Methylation results from endometrial cancer specimens were compared with clinical-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. 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.
No association between GATA4 methylation and tumor stage and tumor grade was observed for the patients with endometrioid carcinoma of endometrium.

5 DISCUSSION

5.1 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*).

5.2 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 clinical-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.

6 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.

7 LITERATURE

Agirre X, Novo FJ, Calasanz MJ, et al. TP53 is frequently altered by methylation, mutation, and/or deletion in acute lymphoblastic leukaemia. *Mol Carcinog*. 2003;38:201-208.

Akiyama Y, Watkins N, Suzuki H, et al. *GATA-4* and *GATA-5* transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastrin cancer. *Mol Cell Biol*. 2003;23:8429-8439.

Almeida LO, Custodio AC, Pinto GR, et al. Polymorphisms and DNA methylation of gene TP53 associated with extra-axial brain tumors. *Genet Mol Res*. 2009;8:8-18.

Amatya VJ, Naumann U, Weller M, et al. TP53 promoter methylation in human gliomas. *Acta Neuropathol*. 2005;110:178-184.

Arafa M, Kridelka F, Mathias V, et al. High frequency of RASSF1A and RARb2 gene promoter methylation in morphologically normal endometrium adjacent to endometrioid adenocarcinoma. *Histopathology*. 2008;53:525-532.

Banno K, Yanokura M, Susumu N, et al. Relationship of aberrant DNA hypermethylation of cancer related genes with carcinogenesis of endometrial cancer. *Onco Rep*. 2006;16:1189-1196.

Barekati Z, Radpour R, Kohler C, et al. Methylation profile of TP53 regulatory pathway and mtDNA alterations in breast cancer patients lacking TP53 mutations. *Hum Mol Genet*. 2010;19:2936-2946.

Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol*. 1983;15:10-17.

Bol GM, Suijkerbuijk KP, Bart J, et al. Methylation profiles of hereditary and sporadic ovarian cancer. Histopathology. 2010;57:363-370.

Bruening W, Bardeesy N, Silverman BL, et al. Germline intronic and exonic mutations in the Wilms' tumour gene (*WT1*) affecting urogenital development. *Nat Genet*. 1992;1:144-148.

Caduff RF, Johnston CM, Frank TS. Mutations of the Ki-ras oncogene in carcinoma of the endometrium. *Am J Pathol*. 1995;146:182-188.

Cannistra SA. Cancer of the ovary. *N Eng J Med*. 2004;351,2519-2529.

Cibula D, Petruželka L, a kol. Onkogynekologie. Praha: Grada Publishing, 2009. ISBN 978-80-247-2665-6.

Clark TJ. Outpatient hysteroscopy and ultrasonography in the management of endometrial disease. *Curr Opin Obstet Gynecol*. 2004;16:305-311.

Das PM, Singal R. DNA methylation and cancer. *J Clin Oncol*. 2004;22:4632-4642.

DiSaia PJ, Creasman WT. Clinical Gynecologic Oncology. 7th ed. Philadelphia: Mosby, 2007. ISBN: 978-0-323-03978-9.

Dohi S, Ohno S, Ohno Y, et al. WT1 expression correlates with angiogenesis in endometrial cancer tissue. *Anticancer Res.* 2010;30:3187-3192.

Enomoto T, Inoue M, Perantoni AO, et al. K-ras activation in neoplasms of the human female reproductive tract. *Cancer Res*. 1990;50:6139-6145.

Esteller M, García A, Martínez- Palones JM, et al. The clinicopathological significance of K-RAS point mutation and gene amplification in endometrial cancer. *Eur J Cancer*. 1997; 33:1572-1577.

Furlan D, Carnevali I, Marcomini B, et al. The high frequency of de novo promoter methylation in synchronous primary endometrial and ovarian carcinomas. *Clin Cancer Res*. 2006;12:3329-3336.

Guida M, Sanquedolce F, Bufo P, et al. Aberrant Dna hypermethylation of hMLH1 and CDKN2A/p16 genes in benign, premalignant and malignant edometrial lesions. *Eur J Gynaecol Oncol*. 2009;30:267-270.

Guo M, Akiyama Y, House MG, et al. Hypermethylation of the GATA genes in lung cancer. *Clin Cancer Res*. 2004;10:7917-7924.

Guo M, House MG, Akiyama Y, et al. Hypermethylation of the GATA gene family in esophageal cancer. *Int J Cancer*. 2006;119:2078-2083.

Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. *J Clin Oncol*. 2006;24:4783-4791.

Herman JG, Graff JR, Myöhänen S, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci*. 1996;93:9821-9826.

Chmelarova M, Krepinska E, Spacek J, et al. Methylation in the p53 promoter in epithelial ovarian cancer. *Clin Transl Oncol*. 2013;15:160-163.

Chmelařová M, Křepinská E, Spaček J, et al. Methylation analysis of tumour suppressor genes in ovarian cancer using MS-MLPA. *Folia Biol (Praha)*. 2012;58:246-250.

Choi EJ, Yun JA, Jeon EK, et al. Prognostic significance of RSPO1, WNT1, P16, WT1, and SDC1 expressions in invasive ductal carcinoma of the breast. *World J Surg Oncol*. 2013;11:314.

Ignatov A, Bischoff J, Schwarzenau C, et al. p16 alterations increase the metastatic potential of endometrial carcinoma. *Gynecol Oncol*. 2008;111:365-371.

Ito K, Watanabe K, Nasim S, et al. K- ras point mutations in endometrial carcinoma: Effect on outcome is dependent on age of patient. *Gynecol Oncol*. 1996;63:238-246.

Jones MW, Kounelis S, Hsu C, et al. Prognostic value of p53 and K- ras- 2 topographic genotyping in endometrial carcinoma: A clinicopathologic and molecular comparison. *Int J Gynecol Pathol*. 1997;16:354-360.

Kaku T, Kamura T, Hirakawa T, et al. Endometrial carcinoma associated with hyperplasia--immunohistochemical study of angiogenesis and p53 expression. *Gynecol Oncol*. 1999;72:51-55.

Kaneuchi M, Sasaki M, Tanaka Y, et al. WT1 and WT1-AS genes are inactivated by promoter methylation in ovarian clear cell adenocarcinoma. *Cancer*. 2005;104:1924-1930.

Koul A, Willen R, Bendahl PO, et al. Distinct sets of gene alterations in endometrial carcinoma implicate alternate modes of tumorigenesis. *Cancer*. 2002;94:2369-2379.

Lane DP. Cancer. p53, guardian of the genome. *Nature*. 1992;358:15-16.

Lane DP. p53 and human cancers. *Br Med Bull*. 1994;50:582-599.

Lax SF. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. *Virchows Arch*. 2004;444:213-223.

Liao X, Siu MK, Chan KY, et al. Hypermethylation of RAS effector related genes and DNA methyltransferase 1 expression in endometrial carcinogenesis. *Int J Cancer*. 2008;123:296-302.

Lima EM, Leal MF, Burbano RR, et al. Methylation status of ANAPC1, CDKN2A and TP53 promoter genes in individuals with gastric cancer. *Braz J Med Biol Res*. 2008;41:539-543.

Mhawech P, Benz A, Cerato C, et al. Downregulation of 14-3-3sigma in ovary, prostate and endometrial carcinomas is associated with CpG island methylation. *Mod Pathol*. 2005;18:340-348.

Mizuuchi H, Nasim S, Kudo R et al. Clinical implications of K- ras mutations in malignant epithelial tumors of the endometrium. *Cancer Res*. 1992;52:2777–2781.

Moelans CB, Verschuur-Maes AH, van Diest PJ. Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma in situ and invasive breast cancer. *J Pathol*. 2011;225:222-231.

Molkentin JD. The zinc finger‐containing transcription factors GATA-4, -5, and -6: Ubiquitously expressed regulators of tissue‐specific gene expression. *J Biol Chem*. 2000; 275:38949-38952.

Muraki Y, Banno K, Yanokura M, et al. Epigenetic DNA hypermethylation: Clinical applications in endometrial cancer. *Oncol Rep*. 2009;22:967-972.

Nakashima R, Fujita M, Enomoto T, et al. Alteration of p16 and p15 genes in human uterine tumors. *Br J Cancer*. 1999;80:458-467.

Ohno S, Dohi S, Ohno Y, et al. Immunohistochemical detection of WT1 protein in endometrial cancer. *Anticancer Res*. 2009;29:1691-1695.

Okuda T, Sekizawa A, Purwosunu Y, et al. Genetics of endometrial cancers. *Obstet Gynecol Int*. 2010;2010:984013.

Oren M, Rotter V. Introduction: p53--the first twenty years. *Cell Mol Life Sci*. 1999;55:9-11.

Pallarés J, Velasco A, Eritja N et al. Promoter hypermethylation and reduced expression of RASSF1A are frequent molecular alterations of endometrial carcinoma. *Mod Pathol*. 2008; 21:691-699.

Patient RK, McGhee JD. The GATA family (vertebrates and invertebrates). *Curr Opin Genet Dev*. 2002;12:416-422.

Pilka R, Mickova I, Lubusky M et al. Expression of p53, Ki- 67, bcl- 2, c- erb- 2, estrogen, and progesterone receptors in endometrial cancer. *Ceska Gynekol*. 2008;73:222-227.

Pilka R, Markova I, Duskova M et al. Immunohistochemical evaluation and lymph node metastasi in surgically staged endometrial carcinoma. *Eur J Gynaecol Oncol*. 2010;31:530-535.

Potischman N, Hoover RN, Brinton LA, et al. Case-control study of endogenous steroid hormones and endometrial cancer. *J Natl Cancer Inst*. 1996;88:1127-1135.

Salvesen HB, Das S, Akslen LA. Loss of nuclear p16 protein expression is not associated with promoter methylation but defines a subgroup of aggressive endometrial carcinomas with poor prognosis. *Clin Cancer Res*. 2000;6:153-159.

Salvesen HB, MacDonald N, Ryan A, et al. PTEN methylation is associated with advanced stage and microsatellite instability in endometrial carcinoma. *Int J Cancer*. 2001;91:22-26.

Salvesen HB, Stefansson I, Kretzschmar EI, et al. Significance of PTEN alterations in endometrial carcinoma: a population-based study of mutations, promoter methylation and PTEN protein expression. *Int J Oncol*. 2004;25:1615-1623.

Sasaki H, Nishii H, Takahashi H, et al. Mutation of the Ki-ras protooncogene in human endometrial hyperplasia and carcinoma. *Cancer Res*. 1993;53:1906-1910.

Seeber LM, Zweemer RP, Marchionni L, et al. Methylation profiles of endometrioid and serous endometrial cancers. *Endocr Relat Cancer*. 2010;17:663-673.

Semczuk A, Berbec H, Kostuch M, et al. K- ras gene point mutations in human endometrial carcinomas: correlation with clinicopathological features and patients‘ outcome. *J Cancer Res Clin Oncol*. 1998;124:695-700.

Sherman ME, Bur ME, Kurman RJ. p53 in endometrial cancer and its putative precursors: evidence for diverse pathways of tumorigenesis. *Hum Pathol*. 1995;26:1268-1274.

Sidhu S, Martin E, Gicquel C, et al. Mutation and methylation analysis of TP53 in adrenal carcinogenesis. *Eur J Surg Oncol*. 2005;31:549-554.

Silverman E, Eimerl S, Orly J. CCAAT enhancer-binding protein beta and GATA-4 binding regions within the promoter of the steroidogenic acute regulatory protein (StAR) gene are required for transcription in rat ovarian cells. *J Biol Chem*. 1999;274:17987-17996.

Suehiro Y, Okada T, Okada T, et al. Aneuploidy predicts outcome in patients with endometrial carcinoma and is related to lack of CDH13 hypermethylation. *Clin Cancer Res*. 2008;14:3354-3361.

Szymanska K, Hainaut P. TP53 and mutations in human cancer. *Acta Biochim Pol*. 2003;50:231-238.

Tao HM, Freudenheim JL. DNA methylation in endometrial cancer. *Epigenetics*. 2010; 5:491-498.

Tashiro H, Isacson C, Levine R, et al. p53 gene mutations are common in uterine serous carcinoma and occur early in their pathogenesis. *Am J Pathol*. 1997;150:177-185.

Tavassoli FA, Devilee P. (Eds.): World Health Organization Classification of Tumors. Pathology and Genetics of Tumors of the Breast and Female Genital Organs. IARC Press: Lyon 2003:113-202.

Tremblay JJ, Viger RS. Transcription factor GATA-4 enhances Mullerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. *Mol Endocrinol*. 1999;13:1388-1401.

Vaitkiene P, Skiriute D, Skauminas K, et al. GATA4 and DcR1 methylation in glioblastomas. *Diagn Pathol*. 2013;8:7.

Viger RS, Mertineit C, Trasler JM, et al. Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Mullerian inhibiting substance promoter. *Development*. 1998;125:2665-2675.

Wakana K, Akiyama Y, Aso T, Yuasa Y. Involvement of GATA‐4/‐5 transcription factors in ovarian carcinogenesis. *Cancer Lett*. 2006;241:281-288.

Watanabe K, Clarke TR, Lane AH, et al. Endogenous expression of Mullerian inhibiting substance in early postnatal rat sertoli cells requires multiple steroidogenic factor-1 and GATA-4-binding sites. *Proc Natl Acad Sci*. 2000;97:1624-1629.

Wen XZ, Akiyama Y, Pan KF, et al. Methylation of GATA-4 and GATA-5 and development of sporadic gastric carcinomas. *World J Gastroenterol*. 2010;16:1201-1208.

Whitcomb BP, Mutch DG, Herzog TJ, et al. Frequent HOXA11 and THBS2 promoter methylation, and a methylator phenotype in endometrial adenocarcinoma. *Clin Cancer Res*. 2003;9:2277-2287.

Wong YF, Chung TK, Cheung TH, et al. Methylation of p16INK4A in primary gynecologic malignancy. *Cancer Lett*. 1999;136:231-235.

Yang HJ, Liu VW, Wang Y, et al. Differential DNA methylation profiles in gynecological cancers and correlation with clinico-pathological data. *BMC Cancer*. 2006;6:212.

Yeh KT, Yang MY, Liu TC, et al. Abnormal expression of period 1 (PER1) in endometrial carcinoma. *J Pathol*. 2005;206:111-120.

Yoshida H, Broaddus R, Cheng W, et al. Deregulation of the HOXA10 homeobox gene in endometrial carcinoma: role in epithelial-mesenchymal transition. *Cancer Res*. 2006;66:889-897.

Zheng R, Blobel GA. GATA Transcription Factors and Cancer. *Genes Cancer*. 2010;1:1178-1188.

Zighelboim I, Goodfellow PJ, Gao F, et al. Microsatellite instability and epigenetic inactivation of MLH1 and outcome of patients with endometrial carcinoma of the endometrioid type. *J Clin Oncol*. 2007;25:2042-2048.

PUBLICATIONS AND LECTURES

Original papers

Chmelarova M, Kos S, **Dvorakova E**, Spacek J, Laco J, Ruszova E, Hrochova K, Palicka V. Importance of promoter methylation of GATA4 and TP53 genes in endometrioid carcinoma of endometrium. Accepted for publication in the journal Clinical Chemistry and Laboratory Medicine. [IF 3.009]

Dvorakova E, Chmelarova M, Laco J, Palicka V, Spacek J. Methylation analysis of tumor suppressor genes in endometrioid carcinoma of endometrium using MS-MLPA. *Biomed Pap*. 2013;157(4):298-303. [IF 0.990]

Chmelarova M, **Dvorakova E**, Spacek J, Laco J, Palicka V. Importance of promoter methylation of GATA4 gene in epithelial ovarian cancer. *Biomed Pap*. 2013;157(4):294-297. [IF 0.990]

Chmelařová M, **Dvořáková E**, Špaček J, Laco J, Mžik M, Palička V. Promoter methylation of GATA4, WIF1, NTRK1 and other selected tumour suppressor genes in ovarian cancer. *Folia Biol (Praha)*. 2013;59(2):87-92. [IF 1.219]

Chmelarova M, **Krepinska E**, Spacek J, Laco J, Beranek M, Palicka V. Methylation in the p53 promoter in epithelial ovarian cancer. *Clin Transl Oncol*. 2013;15(2):160-163. [IF 1.276]

Brodak M, Spacek J, Pacovsky J, **Krepinska E**. Multidisciplinary approach as the optimum for surgical treatment of retroperitoneal sarcomas in women. Eur J Gynaecol Oncol. 2013;34(3):234-237. [IF 0.577]

Křepinská E, Chmelařová M, Laco J, Palička V, Spaček J. Jaký je prognostický význam molekulárně genetických faktorů u karcinomu endometria? *Klin Onkol*. 2012;25(4):282-286.

Chmelařová M, **Křepinská E**, Spaček J, Laco J, Nekvindová J, Palička V. Methylation analysis of tumour suppressor genes in ovarian cancer using MS-MLPA. *Folia Biol (Praha)*. 2012;58(6):246-250. [IF 1.219]

Kříž JT, **Křepinská E**. Vliv chlamydiové infekce pacientek s chronickou pánevní bolestí na hodnoty krevního obrazu a C-reaktivního proteinu. *Gynekolog*. 2011;20(5):172-173.

Kříž JT, **Křepinská E**, Řezáč A. Chronická pánevní bolest v laparoskopickém obraze. *Gynekolog*. 2008;17(5):179-182.

Overview articles and case reports

Dvořák O, **Dvořáková E**, Laco J, Spaček J. Solitární fibrózní tumor endometria – kazuistika. *Ceska Gynekol*. 2013;78(3):302-305.

Krepinska E, Kriz JT, Laco J. Endometrioid adenocarcinoma of the uterus, borderline tumor of the ovary and Brenner tumor of the contralateral ovary in a 63-year-old woman. *Eur J Gynaecol Oncol*. 2010;31(5):584-585. [IF 0.577]

Špaček J, Laco J, Petera J, Jílek P, **Křepinská E**, Řezáč A, Štipl S. Prognostické faktory u mezenchymálních a smíšených nádorů děložního těla. *Ceska Gynekol*. 2009;74(4):292-297.

Křepinská E, Jandík P, Tomšová M, Kalousek I, Špaček J. Mukokéla apendixu v diferenciální diagnóze tumorů malé pánve. *Ceska Gynekol*. 2008;73(4):247-249.

Tomšová M, **Křepinská E**, Špaček J. Sklerozující stromální tumor – vzácný gonadostromální nádor ovaria. *Ceska Gynekol*. 2008;73(3):188-191.

Kříž J, **Křepinská E**, Tomšová M. Angiomyxoma vulvae – kazuistika. *Ceska Gynekol*. 2007;72(6):423-425.

Kubínová K, Zadrobílek K, **Křepinská E**, Kříž J, Kalousek I. Subarachnoidální krvácení při ruptuře aneurysmatu v graviditě. *Gynekolog*. 2007;16(2):66-69.

Halada P, **Křepinská E**. HPV vakcína – začátek konce karcinomu děložního hrdla? *Gynekolog*. 2007;16(1):34-37.

Lectures and posters

Molekulárně biologické změny u karcinomu endometria. XVII. vědecká konference LF UK a FN HK. Leden 2013; Hradec Králové, ČR.

Methylation analysis of tumor suppressor genes in endometrial cancer. 14th Biennial Meeting of the International Gynecological Cancer Society, October 2012; Vancouver, Canada.

Mutation of K-ras gene in pathogenesis of endometrial carcinoma. 17th International Meeting of the European Society of Gynaecological Oncology, September 2011; Milan, Italy.

K-ras mutace a epitelové nádory dělohy. 16. ročník sympózia „Onkologie v gynekologii a mammologii“, Leden 2011; Brno, ČR.

Mutation of K-ras gene in carcinogenesis of endometrial carcinoma. 13th Biennial Meeting of the International Gynecological Cancer Society, October 2010; Prague, Czech Republic.

Mukokéla apendixu v diferenciální diagnóze tumorů malé pánve: 2 kazuistiky. Celostátní konference ČGPS ČSL JEP a SSG ČR, Červen 2008; Hradec Králové, ČR.