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Epigenetic Regulation of Adhesive Molecules in High-grade Serous Ovarian Carcinoma

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Souhrn

Název: Epigenetická regulace adhezivních molekul u high-grade serózního ovariálního karcinomu

Nedostatek účinných biomarkerů pro screening a včasnou detekci ovariálního karcinomu je v současné době považován za jeden z nejnaléhavějších problémů onkogynekologie. Vzhledem k tomu, že k epigenetickým změnám dochází již v počátcích karcinogeneze, mohly by být tyto změny využity jako screeningové markery u rizikové populace. Epigenetické mechanismy se mimo jiné podílejí i na regulaci adhezivních molekul, které sehrávají důležitou roli při rozvoji nádoru a tvorbě metastáz.

Hlavním cílem této práce byla analýza změn v metylaci u vybraných kadherinů a kateninů v ovariální nádorové tkáni v porovnání s kontrolní tkání. Vyšetřovaný soubor tvořilo 68 pacientek s high-grade serózním ovariálním karcinomem (HGSOK) a 46 kontrolních pacientek. Pro stanovení oblastí s nejvýznamnějšími změnami v metylaci ve vybraných genech bylo využito masivně paralelního sekvenování. Pro potvrzení metylačních změn v místech s největším potenciálem byla použita metylačně-sensitivní vysokorozlišovací analýza křivek tání a metylačně-specifická kvantitativní polymerázová řetězová reakce. Dalším cílem práce bylo vytvoření panelu biomarkerů, který by mohl být v budoucnu využit při screeningu HGSOK. Vybrané kadheriny byly proto hodnoceny společně s transkripčními faktory, u kterých byla nalezena hypermetylace již v naší předchozí studii.

Významné změny v metylaci u nádorových vzorků byly odhaleny zejména v genech kódujících *CDH13* a *PCDH17*, přičemž metylace v kontrolních vzorcích nebyla pozorována. Při společné analýze obou genů byla metylace detekována u 65,6 % nádorových vzorků. Vytvořením panelu 4 genů, který kromě *CDH13* a *PCDH17* obsahoval také *HNF1B* a *GATA4*, bylo dosaženo senzitivity 88,5 % při 100%-ní specificitě a efektivitě 93,3 %.

Naše výsledky svědčí o tom, že metylace genů *CDH13* a *PCDH17* by mohla hrát důležitou roli při vzniku a rozvoji HGSOK. Jejich potenciál je patrný zejména po zahrnutí do širšího panelu biomarkerů. K potvrzení těchto nových výsledků jsou však zapotřebí další studie na rozsáhlejším souboru pacientů.

Summary

Title: Epigenetic Regulation of Adhesive Molecules in High-grade Serous Ovarian Carcinoma

The lack of effective biomarkers for screening and early detection of ovarian cancer is currently considered as one of the most pressing problems in oncogynecology. Because epigenetic alterations occur early in the cancer development, they provide great potential to serve as such biomarkers. Epigenetic mechanisms have been implicated also in regulation of adhesion molecules that play a major role in cancer progression.

The main aim of this study was to investigate the methylation pattern of selected cadherin and catenin genes in ovarian cancer tissue by comparison with control tissue. The study group consisted of 68 patients with high-grade serous ovarian cancer (HGSOC) and 46 control patients. To determine the sites with the most significant methylation in selected genes next-generation sequencing was employed. For further confirmation of detected methylation of selected regions, methylation-sensitive high-resolution melting analysis and real-time methylation-specific polymerase chain reaction were used. In attempt to design potential biomarker panel for future screening of HGSOC as the secondary aim of our study, cadherins were evaluated together with transcription factors from our previous study.

Significant methylation-positive pattern was detected in *CDH13* and *PCDH17* genes. Simultaneous analysis of both genes together revealed methylation in 65.6 % of tumor samples, whereas control samples were methylation free. Four-gene methylation panel, that beside *CDH13* and *PCDH17* included also *HNF1B* and *GATA4* genes, reached sensitivity of 88.5 % with 100% specificity and 93.3% efficiency.

Our results indicate that methylation of the *CDH13* and *PCDH17* genes could play an important role in development and progression of HGSOC. With the right selection of the most relevant sites for methylation analysis these genes showed potential to become a target in search for new epigenetic biomarkers, especially as a part of a biomarker panel. However, further studies on more extensive group of patients are needed to confirm these novel results.

1 Introduction

1.1 Ovarian cancer

Ovarian cancer (OC) is currently considered to be one of the most pressing problems in oncogynecology. Vague early symptoms that lead to diagnosis at advanced stages, in addition to the lack of effective screening test, and often aggressive nature of the disease predestinate OC to be the most fatal cancer of female reproductive system.

1.1.1 Epidemiology

Worldwide, OC has the 7th worst mortality rate of all female cancers. In 2018, there were estimated 295,414 new cases of OC, giving an incidence rate of 6.6/100,000 women, and 184,799 deaths, giving a mortality rate of 3.9/100,000 women (Ferlay et al., 2018). Incidence and mortality rates vary according to a country; in general, they are higher in more developed countries. In the Czech Republic, 1,012 new cases of OC were diagnosed in 2018, giving an incidence rate of 9.5/100,000 and 827 women died due OC, giving a mortality rate of 6.7/100,000 (International Agency for Research on Cancer, 2019).

Recently, there has been some mild decrease in the incidence of OC, which is probably caused by more precise histopathological diagnostics and change in epidemiology factors, such as widespread use of hormonal contraceptives. Also, mortality rates have leveled or even declined over past decades. But again, there are substantial differences in OC patterns and trends across world. However, the Globocan study estimates that by 2040, there will be worldwide increase in incidence by 47 % to 434,184 cases a year and deaths will increase by 58.6 % to 293,039. Estimation for the Czech Republic predicts annual increase in incidence by 12 % to 1,133 new cases of OC and deaths will increase to 982 (18.7 %). (International Agency for Research on Cancer, 2019)

Like incidence and mortality rates also survival rates in OC vary widely across the world. The latest five-year survival rates range between 30 % and 50 %, and in general have begun to improve over the last 20 years (Bhatla et Jones, 2018).

The median age at diagnosis of OC is 63 years. *BRCA* mutation carriers have a lower median age at diagnosis; they may be a decade younger than patients without *BRCA* mutations. Germline mutations of *BRCA1* and *BRCA2* genes are present in approximately 12–14 % of patients with OC, the highest rate occur in HGSOC (~ 18 %) (Weiderpass et Tyczynski, 2015). Somatic *BRCA* mutations have been found in approximately 5–7 % of OC patients. Overall, *BRCA1/2* mutations are found in approximately 20 % of all OC cases (Moschetta et al., 2016).

1.1.2 Etiopathogenesis

OC is a nonspecific term for any cancerous growth that occurs in the ovary and covers heterogeneous group of tumors with distinct morphologic, prognostic, etiopathogenetic, and molecular characteristics. According to the 2014 World Health Organization (WHO) classification of tumors of female reproductive organs approximately 10 % of all OC are non-epithelial malignancies comprising of germ cell tumors (e.g. dysgerminomas, choriocarcinoma, immature teratomas) and sex-cord stromal tumors (e.g. granulosa cell tumors, fibromas) (Kurman et al, 2014). However, the majority of OC are classified as epithelial ovarian cancer (EOC).

Based on series of histomorphological, immunohistochemical and molecular-genetic analyzes, EOC was divided into five major subtypes: high-grade serous, endometrioid, clear cell, low-grade serous, and mucinous (Kurman et al., 2014). As indicated by differences in genetic risk factors, precursor lesions, response to chemotherapy, prognosis, and molecular abnormalities, these types are essentially distinct diseases (Prat, 2012).

Fifteen years ago, a new classification was proposed dividing EOC into type I and type II tumors. Type I includes low-grade serous, low-grade endometrioid, mucinous, clear cell and malignant Brenner carcinomas. These tumors are usually confined to the ovary and characterized by clearly defined precursors and slow progress from adenoma, often through the borderline tumor, to the corresponding carcinoma. They are relatively genetically stable with isolated mutations. The most common alterations in this type are *KRAS*, *BRAF* and *ERBB2* mutations; less often *PTEN*, *PIK3CA*, or *CTNNB1* are mutated. Type II ovarian carcinomas consist mostly of high-grade serous tumors, and relatively uncommon malignant mixed Müllerian tumors and undifferentiated carcinomas. They are highly aggressive almost always diagnosed at advanced stage. These tumors are genetically unstable and characterized by frequent *TP53* and *BRCA1/2* mutations, but rarely display mutations typical for type I tumors. (Shih et Kurman, 2004)

The precise cause of OC is unknown, but several contributing factors have been identified. Like in any type of cancers the risk of developing OC increases with age. Family history of OC or breast cancer and inherited cancer syndromes, such us Lynch syndrome or mutations in *BRCA1* and *BRCA2* genes, considerably increase the risk of OC. Nulliparity or late first pregnancy, early menarche and late menopause are also established risk factors. On the other hand, multiply pregnancy and breastfeeding, or use of contraceptive pills, seem to have protective effect. Other risk factors for OC include obesity, tall height, endometriosis, and the use of postmenopausal hormone therapy. (Jelovac et Armstrong, 2011)

1.1.3 Symptoms, diagnosis and treatment

OC, especially at early stages, is often asymptomatic or causes minimal vague symptoms. Nonspecific symptoms, easily dismissed or mistaken for more common conditions, may involve abdominal bloating or swelling, pelvic or abdominal pain, urinary symptoms (urgency or frequency), loss of appetite, digestive disturbances, unexplained weight loss, extreme fatigue, or menstrual irregularities. At more advanced stages OC presents with ovarian, pelvic or abdominal mass and bowel obstruction, ascites and pleural effusion. (Berek et al., 2018)

If OC is suspected, a detailed medical history of the patient and history of OC or any other cancer must be considered to assess possible risk factors. Then a complete physical examination, including general, breast, pelvic, and rectal examination, must be performed, followed by transvaginal ultrasonography and chest x-ray. MRI (Magnetic Resonance Imaging), CT (Computed Tomography) or PET (Positron Emission Tomography) can be used to complement ultrasonography and for detection of extraovarian spread (Fischerová et al., 2012). In addition to physical examination and imaging blood tests are done. They include blood typing test, common hematology tests, biochemical tests of hepatic and kidney profile and tumor marker detection (CA125, HE4, CEA, CA72-4, CA19-9, AFP, HCG).

A quantitative test ROMA (Risk of Ovarian Malignancy Algorithm) combines the test results of CA-125 and HE4 together with the menopausal status of the patient into a numerical score (Moore et al., 2009). It is used to determine the likelihood of malignancy and for differentiating between low- and high-risk patients with OC.

After the diagnosis of OC the stage needs to be determined. The currently used staging system is based on the FIGO (Fédération Internationale de Gynécologie et d'Obstétrique; International Federation of Obstetrics and Gynecology) classification of ovarian, fallopian

tube, and peritoneum cancer (Berek et al., 2018), and the Union for International Cancer Control TNM pathological classification (Gospodarowicz et al., 2017). They both use 3 factors: the size of the tumor (T), the spread to nearby lymph nodes (N), and the spread (metastasis) to distant sites (M).

Treatment options for patients with OC depend on several factors including the type and stage of OC, patient's age, overall health, and the personal preferences regarding future fertility. There are also different options whether it is primary, maintenance or recurrent OC therapy. The current standard treatment consists of primary cytoreductive surgery followed by an adjuvant platinum-based chemotherapy (carboplatin, cisplatin) combined with taxane (paclitaxel, docetaxel). Targeted therapy is often used in addition to systemic chemotherapy or as an alternative therapy in recurrent or persistent OC. Currently available targeted therapies include angiogenesis inhibitors, such as monoclonal antibody bevacizumab, and poly (adenosine diphosphate-ribose) polymerase inhibitors (PARP inhibitors), such as olaparib or niraparib (PDQ Adult Treatment Editorial Board, 2019). Other treatments may include radiation therapy and immunotherapy.

1.1.4 Epithelial–mesenchymal transition in ovarian cancer

OC is a highly invasive and metastatic disease. Metastatic spread of tumor cells is enabled by epithelial-mesenchymal transition (EMT). During EMT, epithelial cells lose their polarity and cell-cell adhesion and acquire migratory characteristics of mesenchymal cells. This transition occurs physiologically during the developmental processes, such as embryo formation or tissue development (type I EMT), or repair processes, such as wound healing, tissue regeneration and organ fibrosis (type II EMT). Type III EMT is associated with cancer progression and metastasis. (Thiery et al., 2009)

In OC, the ability to induce EMT is attributed to transforming growth factor β (TGF-β), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and endothelin-1 (ET-1) (Vergara et al., 2010). Several transcription factors are then activated, including SNAIL and SLUG family, and zinc finger E-box binding homeobox proteins (ZEB), as transcriptional repressors of E-cadherin. A key feature of EMT is thus the switch from E-cadherin to N-cadherin. Cells undergoing EMT display decreased expression of E-cadherin and zona occludens 1 protein (epithelial markers) accompanied by an increased expression of N-cadherin and vimentin (mesenchymal markers). (Lamouille et al, 2014)

1.1.5 High-grade serous ovarian carcinoma

The most common histological type accounting for up to ~ 80% of advanced EOC is an invasive serous carcinoma, recently subdivided into two distinct disease entities, high-grade and low-grade serous carcinomas (Vang et al., 2009). Originally, the ovary was thought to be the primary site of high-grade serous ovarian carcinoma (HGSOC) tumorigenesis with the ovarian surface epithelium as the cell of origin. In recent years, however, there has been emerging evidence that the majority of HGSOC (~ 60 %) originates in the fimbria of the fallopian tube and arises from STIC (serous tubal intraepithelial carcinomas) (Lee et al., 2007; Vang et al., 2013). Implantation of fallopian tube-like epithelium to the ovary (endosalpingiosis) and possibly inclusions of the ovarian surface epithelium are considered the site of origin for the rest of HGSOC (Zeppernick et al., 2015).

HGSOC is characterized by an advanced stage at onset, nearly universal mutation of the *TP53* gene, mutations in the homologous recombination DNA repair pathway (*BRCA1* and *BRCA2* genes) and widespread copy number alterations (Cancer Genome Atlas Research Network, 2011). While mutations of *BRCA1* and *BRCA2* are typical for familial HGSOC, inactivation of these genes in sporadic HGSOC is frequently caused by other mechanisms,

such as hypermethylation of gene promoters. DNA copy number alternations associated with HGSOC often include *cyclin E1* (*CCNE1*), *NOTCH3*, *AKT2*, *RSF1*, and *PIK3CA* loci (Kurman et Shih, 2011). Based on differences in mRNA and miRNA expression and DNA methylation profiles the Integrated genomic analysis of OC further divided HGSOC into four subtypes: immunoreactive, differentiated, proliferative, and mesenchymal. Pathways deregulated in HGSOC include known cancer-associated pathways such as RB, RAS/PI3K, FOXM1, and NOTCH. (Cancer Genome Atlas Research Network, 2011)

In most cases, HGSOC is treated with a combination of carboplatin and paclitaxel with initial response rates of 60–80% (Selvakumaran et al., 2003). However, despite the relatively high initial response, majority of patients become platinum resistant with subsequent relapses. To date, the complete set of mechanisms underlying HGSOC platinum chemotherapy resistance and how they interact is not fully understood. The most studied mechanisms include genomewide mutations, epigenetic changes and dysfunctional DNA repair. The presence of cancer stem cells, EMT and tumor microenvironment (immune cell infiltration, angiogenesis and hypoxia) have also been implicated in platinum resistance (Van Zyl et al., 2018).

1.2 Epigenetics in ovarian cancer

Similar to other malignancies, OC is considered to be driven by progressive genetic alterations, such as mutations in oncogenes or tumor suppressor genes, as well as chromosomal abnormalities. It has been confirmed, that also epigenetic alterations significantly contribute to the OC initiation and progression (Barton et al., 2008). These alternations refer to the heritable modification of DNA without any change in its nucleotide sequence. They affect gene activity and expression and are associated with a phenotype.

1.2.1 DNA methylation

One of the most common epigenetic events taking place in a mammalian genome is DNA methylation. It refers to the covalent addition of a methyl group to the 5-carbon of cytosine ring in CpG sequences resulting in 5-methylcytosine. The methyl group is transferred from S-adenosylmethionine in the reaction catalyzed by DNA methyltransferases (DNMTs). In tumor cells, DNA methylation is usually redistributed between genomic hypomethylation and localized CpG island hypermethylation. Hypermethylation that occurs in the promoter regions of tumor suppressor genes or genes involved in the cell cycle control, apoptosis and drug sensitivity, results in transcriptional silencing (Barton et al., 2008). Aberrant methylation of CpG islands in the promoter region of various genes associated with OC has been observed in number of studies (Koukura et al., 2014; Zhang et al., 2014; Huang et al., 2013).

Numerous techniques are currently used to detect and quantify DNA methylation. According to the methylation-dependent treatment prior to analysis itself, they can be classified into three main groups: bisulfite conversion-based, restriction enzyme-based and affinity enrichment-based strategies. When selecting the most suitable method for particular study, several factors should be considered, including the aims of the study, the amount and quality of the DNA, the sensitivity, specificity and simplicity of the method, the availability of bioinformatics software for analysis and interpretation of the data, and the cost-effectivity of the selected method. (Kurdyukov et Bullock, 2016)

1.2.2 Posttranscriptional regulation by microRNA

Next widely studied area of epigenetics are microRNAs (miRNAs). According to the miRNA database miRBase, over 2 600 mature miRNAs have been identified in humans

so far (Kozomara et al., 2019). They represent a class of small, endogenous, ~22 nucleotides long non-coding RNA molecules that are involved in gene expression regulation of important cellular processes, such as cell proliferation, differentiation, angiogenesis, migration and apoptosis. Primary function of miRNAs at the post-transcriptional level is repression of translation via RNA interference as part of the RNA-induced silencing complex (RISC) (Bartel, 2004). Number of studies have associated dysregulation of various miRNA to OC, and indicated that miRNA expression profiles can be potentially used as diagnostic and prognostic biomarkers, or in prediction of patient's response to treatment (Di Leva et al., 2013; Ferracin et Negrini, 2015; Sorrentino et al., 2008).

1.2.3 Histone modifications

Other epigenetic alterations that play a key role in the gene transcription regulation of cancer cells are histone modifications, covalent post-translational modifications of histone proteins, which include acetylation, phosphorylation, methylation, ubiquitylation, and sumoylation. These modifications can influence gene expression by direct remodeling of chromatin structure or by recruiting histone modifiers (Bannister et Kouzarides, 2011). The most widely studied histone modification is acetylation, enzymatic addition of acetyl group from acetyl coenzyme A. It is regulated by two classes of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDACs are often overexpressed in cancer cells, resulting in altered expression and activity of proteins involved in carcinogenesis. High levels of HDAC1, 2 and 3 have been identified also in OC tissues (Jin et al., 2008). Overexpression of class I HDACs in OC has been associated with poor prognosis (Weichert, et al. 2008) and implicated in metastatic process (Hayashi et al., 2010). Their role in development of platinum resistance in OC cell lines has been also confirmed (Kim, MG et al., 2012).

1.2.4 Epigenetic therapy of ovarian cancer

The reversibility of epigenetic changes brings new possibilities into the search for improved cancer therapy. Number of epigenetic drugs is currently being investigated for their potential to reverse unfavorable epigenetic alterations associated with OC. The most successful epigenetic therapies to date are DNMT inhibitors 5-azacitidine and decitabine (5-aza-2′-deoxycytidine), initially developed as cytotoxic drugs for treatment of hematologic malignancies (Moufarrij et al., 2019). Less toxic drugs, such as zebularine or the small-molecule inhibitor RG108 are being tested as replacement. Other intensively investigated epigenetic agents are HDAC inhibitors. Their development was initiated by the discovery that sodium butyrate can act as an inhibitor of HDAC activity. For use in OC, HDAC inhibitors belinostat, vorinostat or romidepsin have been tested (Smith et al., 2017).

Both HDAC inhibitors and DNMT inhibitors have been investigated as single agents or combined with other therapies. While response to single-agent epigenetic therapy has been low so far, combination with other drugs may be promising (Ahuja et al., 2016). Epigenetic agents in combination with drugs commonly used in OC therapy have been able to improve response to immunotherapy or sensitize patients to platinum-based therapy. Pretreatment with azacytidine or decitabine produced higher response rates to re-treatment with platinum in patients with platinum-resistant OC. It led to demethylation of tumor suppressor genes *MLH1*, *RASSF1A*, *HOXA10*, and *HOXA11*, hypermethylation of which has been associated with the development of platinum resistance. (Matei et al., 2012)

1.3 Adhesion molecules

Cell adhesion molecules (CAMs) are integral membrane proteins that take part in intercellular and cell-to-extracellular matrix interactions. They regulate or significantly contribute to a variety of functions including signal transduction, cell growth and differentiation, morphogenesis, site specific gene expression, immunologic function, cell motility, wound healing, or inflammation (Okegawa et al., 2004). Alterations in cell adhesion can disrupt important cellular processes and lead to various diseases, including cancer, where CAMs participate in tumor invasiveness and metastasis.

All of CAMs comprise of extracellular, transmembrane and cytoplasmic domains. The cytoplasmic domain anchors CAMs to the cytoskeletal proteins, while extracellular domain interacts with matrix or ligands on adjacent cells. Based on their protein structure, CAMs can be divided into four main groups: the integrin family, the immunoglobulin superfamily, selectins, and cadherins.

Cadherins are calcium-dependent transmembrane glycoproteins that mediate cell-to-cell adhesion in almost all type of tissue. The cadherin superfamily includes classical cadherins, protocadherins, desmosomal and unconventional cadherins. Classical cadherins have five cadherin repeats and are involved in significant signaling pathways, such as Wnt or hedgehog. The most widely studied are epithelial (E)-cadherin, neural (N)-cadherin, and placental (P)-cadherin. Protocadherins have more than five cadherin repeats and are thought to be related to ancestral cadherin, though they do not attach to the cytoskeleton trough catenins. They are highly variable, with a variety of function, mostly in the nervous system. Desmosomal cadherins are involved in forming cellular junctions, desmosomes. They include desmogleins and desmocollins. Unconventional cadherins are otherwise uncaterogized cadherins, such as vascular endothelial (VE)-cadherin or retinal (R)-cadherin. (Angst et al., 2001; Morishita and Yagi, 2007)

Cadherins downregulation or absence in malignant cells has been associated with carcinogenesis and cancer progression. Current studies showed aberrant DNA methylation of various classical cadherin genes in human malignant tumors (Wu et al., 2014; Lin et al., 2015). The tumor suppressor role of protocadherins has been recently affirmed as well (Shan et al., 2016). Moreover, different studies have confirmed the significance of altered methylation of protocadherins in various types of cancers (Tang et al., 2012; Niu et al., 2014; Yin et al., 2016).

2 Objectives

The following objectives were specified for this study:

1. Optimization of methods for monitoring DNA methylation changes in genes encoding adhesion molecules using next-generation sequencing.
2. Optimization of real-time PCR-based methods for confirmation of the previously detected most significant alterations in the methylation status.
3. Methylation analysis of selected adhesion molecule genes in high-grade serous ovarian carcinoma tissue in comparison with control tissue.
4. Correlation of detected methylation changes to clinicopathological characteristics and follow-up data of the patients.
5. Design of potential biomarker panel based on DNA methylation for future use in ovarian cancer screening.

3 Materials and Methods

3.1 Study group

Study group consisted of 68 patients with HGSOC and 46 patients who had undergone surgery for non-malignant diagnosis (e. g. uterine fibroids, or descent of uterus with adnexectomy). Of the 114 initially enrolled patients, 10 patients were excluded from analyses due to the insufficient amount of obtained tissue or poor-quality tissue. The set of analyzed samples contained 103 samples of formalin-fixed, parafin-embeded (FFPE) tissue from ovary or the fallopian tube fimbria epithelium (in case of control samples) and 32 fresh frozen samples of ovary. Stage I or II was classified in 23.0 % (14/61) of tumors, 77.0 % (47/61) of tumors were stage III or IV. The median age of patients with HGSOC at the time of diagnosis was 58 years (40–79 years); median age at the time of surgery in control group was 57 years (42–84 years).

3.2 DNA extraction and bisulfite conversion of DNA

Genomic DNA was extracted using silica-membrane-based QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Initial processing of different tissue samples preceded the extraction procedure. In case of FFPE tissue, the samples were first deparaffinized with xylene and washed with 96% ethanol. Fresh frozen samples underwent mechanical tissue homogenization using lysis buffer and glass beads in the MagNA Lyser Instrument (Roche, Basel, Switzerland). The purity of extracted DNA was examined spectrophotometrically on the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA was then quantified on the Qubit® Flourometer (Thermo Fisher Scientific).

All of the methods used for detecting methylation in this study required bisulfite conversion of extracted DNA. Bisulfite treatment is one of the oldest techniques for analyzing DNA methylation and is still considered to be the gold standard. It involves chemical deamination of unmethylated cytosines to uracils while leaving methylated cytosines unaffected. In subsequent polymerase chain reaction (PCR) uracils are amplified as thymines and methylated cytosines are recognized without change. DNA was bisulfite-converted with EZ DNA Methylation-Gold™ Kit according to the manufacturer's protocol (Zymo Research Corporation, Irvine, CA, USA).

3.3 Next-generation sequencing

The term next-generation sequencing (NGS) covers number of different modern high-throughput sequencing technologies. In this study Illumina platform with targeted amplicon sequencing approach was employed. Targeted amplicon sequencing is cost-effective technique that allows focusing on selected regions of interest. This approach involves initial amplification of regions of interest in PCR followed by sequencing of the amplicons. In this study, 16 amplicons in the following genes were analyzed: *CDH10* (amplicons CDH10_1 and CDH10_2), *CDH13* (CDH13), *CDH18* (CDH18_1, CDH18_2), *PCDH8* (PCDH8_1, PCDH8_2), *PCDH10* (PCDH10_1, PCDH10_2), *PCDH17* (PCDH17_1, PCDH17_2, PCDH17_3), *CTNNA2* (CTNNA2_1, CTNNA2_2) and *CTNND2* (CTNND2_S, CTNND2_L). The gene regions were selected to cover gene promoter and first exon in the view of the CpG island predicted position.

Specific primers for amplification were designed in the on-line methylation primer designing software MethPrimer (Li et Dahiya, 2002). To ensure unbiased amplification of both methylated and unmethylated DNA, primers for bisulfite sequencing should not

contain any CpG sites. However, the density of CpG sites in selected regions in *CTNNA2* and *CTNND2* did not allow designing primers without any CpG. Therefore, degenerate bases Y (C or T) and R (A or G) were included in the primer sequences to enable primers anneal to DNA regardless of methylation status. For subsequent sequencing, specific adaptor sequence was added to the designed primers.

Sequencing libraries were prepared using the Multiplicom approach. Optimized first round PCRs were performed in Veriti™ Thermal Cycler (Thermo Fisher Scientific) using AmpliTaq Gold DNA Polymerase for amplification of most amplicons, except PCDH17_2 and PCDH8_2, where Platinum *Taq* DNA Polymerase was used, and CTNNA2_2 where High Fidelity Platinum *Taq* DNA Polymerase was used. Bisulfite treated universal methylated and unmethylated DNA (Zymo Research Corporation) were used as controls. PCR products were cleaned using AMPure XP beads on Biomek 4000 (Beckman Coulter, Brea, CA, USA), and verified to be the expected size and free of primer dimers by agarose gel electrophoresis. Diluted PCR products were amplified in a subsequent barcoding PCR. Unique DNA sequencing barcodes and specific adapters for Illumina sequencing were incorporated into each sample using MID for the Illumina MiSeq® kit (Multiplicom, Niel, Belgium) with minor modifications. Second round PCR products were separated on 2% agarose gel. Specific products were extracted from gel and purified by the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Purified sample concentrations were measured on the Qubit® Fluorometer (Thermo Fisher Scientific). Selected samples were analyzed using the Agilent BioAnalyzer DNA1000 chip (Agilent Technologies, Santa Clara, CA, USA). All samples were equimolarly pooled into a library, then quantified using the KAPA library quantification assay (Kapa Biosystems, Wilmington, MA, USA), and the 4 nM library was prepared.

NGS was performed on the MiSeq System (Illumina, San Diego, CA, USA) using Reagent Nano Kits v2 with paired-end reads following the manufacturer's instructions. According to the length of analyzed amplicon, 500 or 300 cycles Reagent Nano Kits were used. Most of the amplicons were up to 400 base pair (bp) in length and required use of 500 cycles kit; 300 cycles kits were used for CTNNA2_1, CTNNA2_2 and CTNND2_S. Given the fact that these amplicons were less than 200 bp in length, the highly fragmented DNA extracted from FFPE tissue samples could be used for NGS analysis along with DNA from fresh frozen tissue samples.

A final volume of 20% PhiX spike-in control was added to the library to increase sample diversity. The final library was denatured and diluted to 9 pM. The prepared library, along with Multiplicom read 1, read 2 and index primers, was then loaded to the reagent cartridge. Data from MiSeq runs were uploaded to BaseSpace, Illumina's genomics cloud computing environment. Runs generated sequencing data in FASTQ format files.

For analysis of acquired FASTAQ data files from NGS and calculation of methylation status of analyzed CpG sites, NextGENe® software version 2.3.4.5 (Softgenetics, State College, PA, USA) was employed. As reference bisulfite-converted sequences flanked by designed primers were used. For problematic amplicons, alternative pipeline was employed. Sequence data quality was verified using the quality control tool FastQC version 0.11.5 (Andrews, 2010). Genome mapping was performed using the gemBS version 3.2.2 application in original setting (Merkel et al., 2019). The reference sequence was used from the NCBI NG_023544.1 database. The mapped data was then visualized in open source Integrative Genome Viewer (IGV) version 2.4.14 (Robinson et al., 2011) and methylation status was derived from read counts of converted and non-converted cytosines.

3.4 Real-time PCR-based methods for detecting DNA methylation

Based on the results from NGS, CpG sites with the most distinct differences in methylation between tumors and control samples were selected for further analysis. Detected alterations were then confirmed on the whole set of samples using cost-effective and less demanding or time-consuming methods, such as methylation sensitive high-resolution melting (MS-HRM) analysis or real-time methylation specific PCR.

While conventional PCR techniques detect amplified product in an end-point analysis, mostly by visualization on agarose gel, real-time PCR techniques monitor amplification of product during progress of PCR. It is enabled by including fluorescent molecule in the reaction mixture. For real-time methylation specific analysis TaqMan probes were used. Non-specific dsDNA binding dyes were employed in MS-HRM experiments.

Primers for bisulfite-converted DNA were designed in on-line platform MethPrimer, considering the fact that FFPE DNA is highly fragmented and also amplicons over 200 bp in length result in lower melting resolution in HRM analysis.

3.4.1 Methylation sensitive high-resolution melting analysis

HRM analysis is an innovative technique based on analysis of melt curves of DNA following real-time PCR amplification. Sequences that differ in base composition have different melting profiles. Due to the bisulfite treatment the PCR product originating from the methylated sample has different sequence as the PCR product derived from the unmethylated one. It is thus possible to determine methylation status of a sample by comparison of its melting profile with profiles of methylated and unmethylated control DNAs.

To confirm hypermethylation of selected regions in *CDH13* and *PCDH17* genes, samples were further analyzed using MS-HRM analysis. *CDH13* was divided into two amplicons; CDH13_a covered 9 CpGs and CDH13_b 13 CpGs. The only amplicon of *PCDH17* covered 11 CpG sites. Primers did not include any CpGs.

PCR amplification and MS-HRM were performed in Rotor Gene Q (Qiagen, Hilden, Germany). Each run included the no template control, a bisulfite-converted universal methylated and unmethylated DNA (Qiagen) and prepared standard of various methylation percentages (10 %, 25 % and 50 % of universal methylated DNA). HRM data were analyzed using Rotor Gene Q software 2.3 (Qiagen). Methylation status of each sample was determined by comparing its melting profile with profiles of methylated control, 10% standard which served as a cut-off for methylation status, and unmethylated control.

3.4.2 Real-time methylation specific analysis

In real-time methylation specific PCR, the TaqMan dual-labeled hydrolysis probes were used. TaqMan probes have a fluorescent reporter at 5' end and a quencher of fluorescence at the opposite 3' end of the probe. When the quencher is in the proximity to the reporter, it prevents fluorescence emission of the reporter. After the probe hybridize to the DNA during amplification, the 5' to 3' exonuclease activity of the *Taq* polymerase cleaves off the reporter, which starts to emit fluorescence. As the product targeted by the reporter probe amplifies a proportional increase of fluorescence is emitted. Using of fluorescent probes with different-colored labels in one reaction enables monitoring several target sequences in multiplex PCR.

Duplex real-time PCR assay for measuring DNA methylation was used to analyze two selected CpG sites in the CDH13_a amplicon. A set of methylation-independent primers from MS-HRM analysis was used. Probes labeled with two different-colored reporter dyes binding to methylated or unmethylated DNA, respectively, were designed in on-line software Primer3

(Koressaar et Remm, 2007; Untergasser et al. 2012). PCRs were performed on the Rotor-Gene 6000 5-plex with HRM (Corbett Research, Cambridge, UK). Reactions were performed in triplicates. Each run included the no template control, a bisulfite-converted universal methylated and unmethylated DNA (Qiagen) and prepared standards of various methylation percentages (10 %, 25 % and 50 % of methylated DNA).

Fluorescence data from real-time methylation specific analysis were analyzed using Rotor-Gene 6000 software. The methylation status of amplicon was determined by calculating methylation index: MI (%) = $100 / (1 + 2^{(CTm - CTu)})$ where CTm represents Ct value of the reaction with probe binding to the methylated DNA; CTu is Ct value of the reaction with probe binding to the unmethylated DNA. For amplicon to be considered methylated the value of MI had to be over 5 %. If there was an increase in fluorescence emitted only by HEX-labeled probe, the amplicon was considered unmethylated.

3.5 The Cancer Genome Atlas methylation data

Publicly available dataset containing 302 cases of ovarian serous adenocarcinoma was downloaded from The Cancer Genome Atlas (TCGA) Data Portal. The filter was set for selection of white women of not Hispanic or Latino ethnicity. Cases were staged according to the 1988 FIGO staging system. All cases were classified as G3 (poorly differentiated, i.e. high-grade, n = 236), eventually G2 (moderately differentiated, n = 25). Data were not available for 41 cases. The majority of tumors were diagnosed at late stages (stage III or IV); only 10 tumors were classified as stage I or II. Stage data were not available for 36 cases. The median age at the time of diagnosis was 60 years (37–87 years).

DNA used for methylation analysis in the TCGA project was extracted from fresh frozen tissue samples of primary tumors. DNA methylation levels were detected in limited number of CpG sites using the Illumina Infinium HumanMethylation27 BeadChip arrays. In each of *CDH10*, *CDH18*, *PCDH8* and *CTNND2* genes, two CpGs were covered by the methylation array, but they did not match any of the CpGs analyzed in our study. In *CTNNA2* gene, 4 CpG sites were analyzed without any match to our CpGs. *PCDH10* gene was not selected for methylation analysis at all. From 9 CpGs analyzed in *CDH13* gene, two CpGs (cg08977371 and cg08747377) were investigated in our study. In *PCDH17* gene, two CpGs were included in the array and one of them (cg14893163) was analyzed also in our study.

Quantitative measurement of methylation was expressed as beta-value, which is the ratio of the methylated probe intensity and the sum of methylated and unmethylated probe intensities. The cutoff level for methylation was set at 0.15.

3.6 Statistical analysis

Categorical variables were compared by two-tailed Fisher's exact test and/or Chi square test. The Kaplan-Maier method and Logrank test were used to determine overall survival rate and significance. The tests were two tailed and $p < 0.05$ was considered statistically significant. Statistical analyses were performed in data analysis software TIBCO Statistica version 13 (TIBCO Software Inc., Palo Alto, CA, USA).

Following diagnostic parameters were calculated:

$$\text{Sensitivity (\%)} = TP / (TP + FN) * 100$$
$$\text{Specificity (\%)} = TN / (TN + FP) * 100$$
$$\text{Positive predictive value, PPV (\%)} = TP / (TP + FP) * 100$$
$$\text{Negative predictive value, NPV (\%)} = TN / (TN + FN) * 100$$
$$\text{Efficiency (\%)} = (TP + TN) / (TN + TP + FN + FP) * 100$$

where TP means true positives, FN false negatives, TN true negatives and FP false positives.

4 Results

4.1 Next-generation sequencing

Altogether, eleven sequencing runs were needed to analyze all amplicons. All runs had paired-end configuration; for three runs, read length was 2 × 150 bp (when 300 cycles kit was used), for the rest of runs, it was 2 × 250 bp (500 cycles kit). The average number of reads per amplicon was 8,600. The percentage of bases with a quality score of 30 or higher ranged from 84.47–96.70 %. Data quality of all runs was very high, so no quality trimming prior aligning was needed. Average percentage of reads uniquely aligned to PhiX genome ranged from 16.05–22.31 % (libraries were spiked with 20% PhiX). Average error rate based on alignment to PhiX was 1.09 %.

4.1.1 Cadherins

Selected regions of *CDH10*, *CDH13* and *CDH18* genes were analyzed using NGS. In two of *CDH10* amplicons, 18 CpG sites were examined, single amplicon of *CDH13* covered 23 CpGs, and selected region of *CDH18* was divided into two amplicons containing 28 CpGs altogether.

In all analyzed amplicons, methylation status was examined in 20 fresh frozen samples (10tumors and 10 control samples). However, some samples had to be excluded from further analysis due to the low coverage.

The DNA methylation profile of *CDH10* was compared in 12 samples (6 tumors, 6 control samples). Only sporadic non-significant methylation was detected. Methylation status of *CDH13* was examined in 10 samples (6 tumors, 4 control samples). Methylation was detected in 3 tumor samples; control samples were methylation free. The methylated sites were selected for further analysis to confirm detected methylation. Methylation profile of *CDH18* was compared in 14 samples (6 tumors, 8 control samples). CpG3 was methylated in all control samples, whereas there was only one tumor sample with detected methylation at this site. In the remaining 27 analyzed CpGs only sporadic non-significant methylation was present.

4.1.2 Protocadherins

Methylation status of the *PCDH8*, *PCDH10* and *PCDH17* genes in 20 fresh frozen samples (10 tumors, 10 controls) was examined. Selected region of *PCDH8* was divided into two amplicons containing 43 CpG sites, two amplicons of *PCDH10* covered 22 CpGs and three amplicons of *PCDH17* contained 52 CpGs altogether. Amplicons PCDH8_1, PCDH8_2, PCDH10_1, PCDH17_2 and PCDH17_3 could be successfully analyzed. Analysis of PCDH10_2 and PCDH17_1 amplicons was impossible since both analysis tools failed to align sequencing data to the reference sequences.

PCDH8 amplicons showed only sporadic methylation in both tumors and controls samples. Except one methylated CpG across all samples, there was no methylation detected in 10 analyzed CpGs of PCDH10_1 amplicon. Statistically significant site-specific methylation was present in 10 of 36 analyzed CpGs in *PCDH17* gene (amplicons PCDH17_2 and PCDH17_3). In this area near the end of analyzed region, high methylation was present in over 60 % of tumor samples, with only minor methylation of one CpG in two control samples. These sites were selected for further analysis by MS-HRM.

4.1.3 Catenins

DNA methylation was analyzed in selected regions of *CTNNA2* and *CTNND2* genes. Two short *CTNNA2* amplicons covered 40 CpG sites. The length of short amplicons was up to 200 bp, enabling NGS methylation analysis of FFPE tissue samples. Therefore, in addition to 20 fresh frozen samples (10 tumors, 10 controls), 18 FFPE samples (10 tumors, 8 controls) were analyzed as well. Only sporadic methylation of few CpGs was detected across all the samples.

Short amplicon CTNND2_S covered 29 CpGs. Methylation profile was compared in 20 fresh frozen tissue samples (10 tumors, 10 controls) and 50 FFPE tissue samples (30 tumors, 20 controls). Scattered methylation without any distinguishable pattern was present across all CpGs in 6 tumors and 17 control samples. In two tumor samples, methylation was detected in all of analyzed CpGs. The rest of the samples were methylation free. Amplicon CTNND2_L covered 56 CpGs. Methylation profile was compared in 20 fresh frozen samples (10 tumors, 10 controls). Except one tumor and one control sample, no methylation was detected.

4.2 Confirmation methods

4.2.1 *CDH13* methylation

For confirmation of detected changes in *CDH13* methylation profile, primers for two HRM assays and duplex real-time PCR were designed. First HRM amplicon (CDH13_a) covered 9 CpG sites (CpG1–9 from NGS); two of them (CpG7 and 8) were then further analyzed using real-time PCR assay. The second amplicon (CDH13_b) covered another 13 CpGs (CpG11–23 from NGS). In the control samples, both of analyzed amplicons in the *CDH13* gene were methylation free. Analysis of the first amplicon showed methylation-positive pattern for 13.1 % (8/61) of tumor samples. Real-time PCR assay further confirmed the level of observed methylation (12.5 % of methylated tumor samples). Methylation detected in the second HRM amplicon was slightly higher, 19.7 % (12/61).

In both of HRM amplicons, methylation was detected more frequently in the early stages (stage I and II), than in the late ones (stage III and IV). The early stage tumors methylation of the first amplicon was observed in 21.4 % cases (3/14), versus 10.6 % (5/47) in the late stage tumors ($p = 0.37$). The second amplicon methylation observed in early stages was 28.6 % (4/14); in the late stages, detected methylation decreased to 17 % (8/47, $p = 0.45$). The decrease in detected methylation was not statistically significant.

4.2.2 *PCDH17* methylation

To confirm *PCDH17* hypermethylation detected by NGS, 11 CpGs from PCDH17_3 amplicon were analyzed using MS-HRM. Statistically significant methylation-positive pattern ($p < 0.01$) was observed in 60.7 % (37/61) of tumor samples. All of the control samples were methylation free. Methylation was detected with approximately the same frequency in early or late stages tumors, 57.1 % (8/14) of early stage tumors versus 61.7 % (29/47) of late stage ones.

4.3 TCGA methylation data analysis

4.3.1 *CDH13* methylation

The methylation array covered 9 CpG sites from the promoter region of *CDH13* gene. Two of them were investigated also in our project; CpG sites identified as cg08977371 (corresponding to CpG8 from NGS analysis of *CDH13*) and cg08747377 (corresponding to CpG15). In our study, both NGS, as well as real-time PCR-based methods, were used for analysis of these CpG sites.

In TCGA dataset, cg08977371 methylation was detected in 32.1 % (97/302) of cases. NGS analysis of CpG8 showed methylation-positive pattern in three of six tumors. MS-HRM analysis of the amplicon that covered CpG8 revealed methylation in 13.1 % (8/61) of tumor samples. The ratio of methylated samples was much lower, but it can be caused by the fact that HRM assay covered another 8 CpGs. However, real-time PCR assay, that beside CpG8 covered just one more CpG, confirmed the level of the methylation previously detected by HRM.

Methylation of cg08747377 was present in 17.5 % (53/302) of cases in TCGA dataset. Using NGS in our study, methylation at CpG15 was detected in two of six tumor samples. MS-HRM analysis of larger set of samples showed methylation-positive pattern in 19.7 % (12/61) of cases. In spite of another 12 CpG sites (beside CpG15) covered by HRM assay, the detected methylation does not differ from the methylation observed in TCGA project.

4.3.2 *PCDH7* methylation

Two CpG sites in the promoter region of *PCDH17* gene were covered by the methylation array in TCGA project. CpG site identified as cg14893163 corresponded to the second CpG of PCDH17_2 amplicon in our study. This CpG was analyzed using NGS only and was not selected for further analysis. Methylation of cg14893163 was detected in 6.6 % (20/302) of cases. Using NGS, methylation at this site was detected only in one of ten tumor samples. The analysis of TCGA data confirmed methylation status of this CpG site detected in our study.

4.4 Follow-up

The patients were followed up in January 2019 and data for the overall survival (OS) and the disease-free survival (DFS) calculation were collected from patients. It was impossible to obtain complete data of 15 patients, as they were subsequently treated in another health care facility or refused to undergo further treatment. During the follow-up period, 27 patients (58.7 %, 27/46) died due to HGSOC, 17 of them (37.0 %, 17/46) within 5 years. Eleven patients (18.6 %, 11/59) had persistent disease or the disease progressed during the treatment. Relapse occurred in 27 patients (58.7 %, 27/46). OS of patients ranged from 2–216 months, with a median of 52 months; median DSF was 18 months. At the end of the follow-up period, 19 patients (41.3 %, 19/46) were still alive, 11 of them (23.9 %, 11/46) in complete remission without any relapse.

The Kaplan-Maier analysis and Logrank tests were used to determine overall survival rate and significance. Although overall survival was slightly better in the group of patients where no methylation was observed, the correlation between gene methylation and survival data was not considered statistically significant.

4.5 DNA methylation panel

The possibility of a methylation panel design was assessed. DNA methylation of selected regions of *CDH13* and *PCDH17* genes was detected in 19.7 % (12/61) and 60.7 % (37/61) of patients, respectively. By evaluating both genes together the detected methylation increased by 4.9 %, to 65.6 % (40/61) of patients.

In order to increase the percentage of patients with detected methylation, another two genes from our previous study (transcription factors *GATA4* and *HNF1B*) were evaluated as possible candidates for methylation panel. In case of *GATA4* gene, methylation was detected in 31.2 % (19/61) of patients. Selected region of *HNF1B* gene was methylated in 50.8 % (31/61) of patients. Due to the higher percentage of detected methylation, the *HNF1B* gene was assessed first. The involvement of the *HNF1B* gene in the examined methylation panel increased detected methylation by 18 %, to 83.6 % (51/61) of patients. The further addition of the *GATA4* gene to the already tested *CDH13*, *PCDH17* and *HNF1B* led to the increase by another 4.9 %, to 88.5 % (54/61) of patients with detected methylation.

Besides sensitivity, the specificity, PPV, NPV, and efficiency of all the above-mentioned gene combinations were calculated. The efficiency of four-gene panel reached 94.2 %; NPV was 86 %. Since the analyzed CpGs were selected in the regions without any methylation present in control samples, the specificity and PPV achieved 100% rates.

5 Discussion

High-grade serous ovarian cancer is the most frequent and aggressive form of OC. Just like any other malignancy, it is the consequence of the progressive genetic and epigenetic alterations. These alterations may influence diverse genes involved in the crucial signaling pathways, where cell adhesion molecules play important role. A major class of cell adhesion molecules that mediate cell-to-cell adhesion is the cadherin superfamily. Specific signaling pathways activated by cell-cell interactions are regulated by cadherin-catenin complexes. DNA methylation associated with decreased expression of the cadherin and catenin genes may lead to disruption of cell-cell connections and results thus in epithelial tumor aggressiveness, invasion and metastasis (Cavallaro et Christofori, 2004). In our project, the methylation pattern of selected cadherin and catenin genes was analyzed, with the aim of determining, whether they can serve as potential epigenetic biomarkers of clinical benefit in HGSOC screening, diagnosis, and prognosis. For this purpose, innovative approach was employed. It included use of targeted amplicon NGS as the initial method for selecting the most significant CpG sites. The used technique provided a comprehensive view of methylation patterns in the promoter region and part of the first exon of the analyzed genes. These regions were up to 400 bp in length and covered numerous CpG sites. Considering the fact that NGS is time consuming, labor intensive and expensive method, and requires DNA of high quality, purity and integrity, it was used just for preliminary analysis of selected set of samples. Only DNA extracted from fresh frozen tissue met the quality criteria for analysis of amplicons over 200 bp in length. For analysis of the shorter amplicons, DNA from FFPE tissue samples could have been used as well. CpG sites with the most distinct differences in methylation between tumors and control samples were then analyzed in the whole set of samples using less-demanding methods, such as MS-HRM analysis or real-time methylation specific PCR. The detected methylation was then compared to public available methylation data from TCGA project.

Due to the lack of any specific symptoms in the early stages, highly invasive HGSOC is mostly diagnosed after the disease has metastasized beyond the ovary. Metastatic spread is promoted by EMT and cadherins, whose main function is cell to cell adhesion, are key participants in this process. Epigenetic mechanisms are involved in the regulation of cadherin genes participating in EMT. DNA methylation in *E-cadherin* has been implicated in the initiation and completion of EMT (Strathdee, 2002). Furthermore, various epigenetic modifiers, such as DNMTs, histone deacetylases, methyltransferase and demethylase, are involved in the transcriptional regulation of E-cadherin (Lee et Kong, 2016). The role of *E-cadherin* gene promoter methylation in OC has been previously investigated (Montavon et al., 2012; Wang et al., 2016).

Our project focused on methylation analysis of genes encoding unconventional cadherins (*CDH10*, *CDH13*, *CDH18*), little studied δ2 group of non-clustered protocadherins (*PCDH8*, *PCDH10*, *PCDH17*), and cadherin-associated proteins, catenins (*CTNNA2*, *CTNND2*).

CDH10 gene is predominantly expressed in central nervous system. It also can be found in epithelial cells of prostate, in testes, ovary, placenta, kidney and small intestine (Stelzer et al., 2016). *CDH10* plays a key role in prostate epithelial differentiation and it is downregulated in prostate cancer (Walker et al., 2008). Mutations of this gene were associated with gastric and colorectal cancer (An et al., 2015) and lung squamous cell carcinoma (Li et al., 2015). According to our knowledge, this is the first study evaluating *CDH10* methylation in OC. Preliminary scan showed only sporadic non-significant methylation, indicating that methylation of selected region is not associated with ovarian carcinogenesis.

The protein encoded by *CDH13* gene acts as a negative regulator of axon growth during neural differentiation. When expressed on vascular endothelial cells it promotes angiogenesis, on stromal cells it inhibits neovascularization (Stelzer et al, 2016). Downregulation of *CDH13* in cancer cells and upregulation on the vasculature of various tumors negatively regulates tumor cell proliferation and angiogenesis, but at the same time, it also enhances tumor progression (Andreeva et Kutuzov, 2010). The gene is hypermethylated in many types of cancer including OC (Bol et al., 2010). To the best of our knowledge, our study is the first one to investigate methylation status of *CDH13* in HGSOC using NGS. Preliminary NGS scan showed methylation in 3 of 6 tumor samples, whereas the control samples were methylation free. Further MS-HRM analysis revealed methylation-positive pattern in 13.1 % (8/61) and 19.7 % (12/61) of tumor samples (in the first and second HRM amplicon, respectively). The level of methylation observed in the first amplicon was further confirmed by real-time PCR assay (12.5 % of methylated tumor samples). There was no methylation detected in the control samples using confirmation methods. The lower presence of methylation detected by HRM assays could be caused by the assay design. The sample is observed as methylated only if most of the included CpGs are methylated (the first HRM amplicon covered 9 CpG sites, the second one 13 CpGs). The small number of samples analyzed by NGS probably also played a role in disproportional high percentage of the detected methylation. Previous studies analyzed *CDH13* methylation using MS-MLPA or MSP that can focus only on a few CpG sites. They reported statistically non-significant methylation in OC samples compared with normal/benign tissue (Feng et al. 2008) or very low methylation in tumors (Rathi et al., 2002). Bol et al. (2010) detected methylation in 16.0 % of BRCA1-related tumors and in 21.5 % of control sporadic OC. Chmelarova et al. (2012) reported methylation in more than 50 % of OC. The disparity between detected methylation is most likely caused by analysis of distinct CpG sites. Moreover, all above mentioned studies investigated overall methylation in various subtypes of OC (they did not focus specifically on HGSOC) and different distribution of OC subtypes in each study group could affect results as well.

Our results were compared to the public available methylation data from TCGA program, specifically from the project focused on serous ovarian adenocarcinoma. Of 9 CpG sites in the *CDH13* gene covered by their methylation array, two CpGs were investigated also in our study using both NGS and confirmation methods as well. In cg08977371 from TCGA dataset methylation was detected in 32.1 % (97/302) of cases. Corresponding CpG in our study was methylated in three of six tumors, as detected by NGS, and further MS-HRM analysis revealed methylation in 13.1 % (8/61) of tumor samples. The discrepancy between TCGA and our data most likely results from different techniques used for detection of methylation. Moreover, our HRM assay covered additional 8 CpGs, methylation of which could affect the methylation status of concerned CpG site. Methylation of the second CpG, identified as cg08747377, was present in 17.5 % (53/302) of cases in TCGA dataset. Using NGS in our study, methylation of corresponding CpG site was detected in two of six tumor samples. MS-HRM analysis of larger set of samples showed methylation-positive pattern in 19.7 % (12/61) of cases. The methylation detected at this site of the *CDH13* gene is approximately at the same level as the methylation observed in TCGA project.

CDH18 is expressed in the central nervous system and its role as tumor-suppressor gene has been recently demonstrated in brain cancer (Bai et al., 2018). Copy number variants of *CDH18* gene have been associated with familial and early-onset colorectal cancer (Venkatachalam et al., 2011) and deletions in this gene have been found in odontogenic tumors (Heikinheimo et al., 2007). According to our knowledge, there have not been any published studies focused on the *CDH18* methylation in association with cancer. In our study, methylation profile of 28 examined CpGs in the *CDH18* gene showed weak scattered

methylation, except for one CpG where methylation was present in all control samples. Due to the fact that methylation at this site was detected also in a tumor sample; the gene was not selected for further analysis. However, the loss of methylation in the tumor samples suggests possible role of *CDH18* in HGSOC progression.

PCDH8 gene encodes an integral membrane protein that takes part in cell adhesion in central nervous system and may play a role in down-regulation of dendritic spines (Stelzer et al., 2016). It is considered to function as a tumor suppressor in hypopharyngeal carcinoma (Li et al., 2018). Low expression of *PCDH8* is thought to promote OC progression (Cao et al., 2018). Hypermethylation of the *PCDH8* gene has been associated with prostate cancer (Lin et al., 2014) or bladder cancer (Niu et al., 2014). Although different studies have confirmed the significance of altered methylation of *PCDH8* in other types of cancers, there is no evidence of its hypermethylation being associated with OC. In this study, however, using NGS as preliminary method for investigating methylation status, only sporadic methylation was observed in the selected region of *PCDH8*.

The protein encoded by the *PCDH10* gene is a neuronal receptor involved in specific cell-cell connections in the brain. This gene plays a role in inhibiting cancer cell motility and cell migration (Stelzer et al., 2016). The prognostic value of *PCDH10* promoter methylation has been suggested in different types of cancer, such as prostate cancer (Deng et al., 2016) or gastric cancer (Hou et al., 2015). According to our knowledge, this is the first study evaluating *PCDH10* methylation in OC. There was no methylation detected in 10 analyzed CpGs of *PCDH10* gene, indicating that methylation of these sites is not involved in HGSOC development and progression.

Similar to the other members of δ2 subfamily of protocadherins, *PCDH17* is widely expressed in the nervous system and involved in axon development or function (Stelzer et al., 2016). The importance of altered *PCDH17* methylation has been confirmed in various types of cancers, such as bladder cancer (Luo et al., 2014) or breast cancer (Yin et al., 2016). The association of altered methylation in *PCDH17* gene with OC has not been previously investigated. In our study, methylation-positive pattern was observed in 60.7 % (37/61) of the tumor samples, whereas all the control samples were methylation free. Our findings suggest that methylation of *PCDH17* gene may play an important role in HGSOC.

Methylation array in the TCGA project focused on HGSOC investigated methylation status of two CpG sites in the promoter region of the *PCDH17* gene. One of these CpGs (cg14893163) was also analyzed in our study using NGS as preliminary scan. TCGA project revealed methylation at this site in 6.6 % (20/302) of cases. In our study, methylation at corresponding site was observed only in one of ten tumor samples, so our data were in correlation with those from TCGA project. The second CpG analyzed by TCGA in the *PCDH17* gene promoter showed methylation-positive pattern even in less cases. Based on these findings, *PCDH17* gene could be abandoned as non-significant in terms of methylation in OC. However, in another part of the *PCDH17* gene promoter region our results showed significant methylation in over 60 % of tumor samples. Considering the fact that methylation is site-specific, the proper selection of the most relevant gene region is crucial in methylation analysis.

Catenins are intracellular proteins found in complexes with cadherins that connect cadherins to the cell's cytoskeleton. They are frequently downregulated during EMT and have been associated with metastatic process (Bukholm et al., 1998). *CTNNA2* has been implicated as a linker between cadherin adhesion receptors and the cytoskeleton of the nervous system cells. Beside brain, it is also expressed in testis (Stelzer et al., 2016). It functions as the tumor suppressor gene frequently mutated in laryngeal carcinomas (Fanjul-Fernandez et al., 2013). Single nucleotide polymorphism in *CTNNA2* has been associated with breast cancer susceptibility (Haryono et al., 2015). To the best of our knowledge, our study is the first one

to evaluate methylation status of *CTNNA2* in OC. In our study, only sporadic methylation of few CpGs in the *CTNNA2* gene was detected across all the samples, indicating that the methylation of selected region does not play an important role in HGSOC development and progression.

CTNND2 has been implicated in brain and eye development. The protein encoded by this gene promotes the disruption of E-cadherin based adherens junction enabling thus cell spreading (Stelzer et al., 2016). Overexpression of *CTNND2* gene associated with decreased expression of tumor suppressor *E-cadherin* has been confirmed in prostate cancer (Kim, H et al., 2012) and lung adenocarcinomas (Huang et al., 2018). According to our knowledge, this is the first study evaluating methylation of the *CTNND2* gene in HGSOC. Although extensive region (covering 85 CpG sites) in the promoter and first exon of the *CTNND2* gene was examined in our study, no distinguishable methylation pattern was detected.

Because epigenetic alterations occur early in the cancer development, they provide great potential to serve as biomarkers for screening and early detection. Currently, 14 methylation-based biomarker assays are commercially available indicated respectively in prostate, bladder, lung and colorectal cancer, and in prediction of response to temozolomide in glioblastoma (Koch et al., 2018). Assay for simultaneous detection of methylation in *NDRG4* and *BMP3* genes, and two different *SEPT9* methylation assays for early detection of colorectal cancer have been approved by FDA (Food and Drug Administration). Numerous studies investigated methylation of various genes in effort to find an effective screening test or early detection biomarkers in highly aggressive and metastatic OC. So far, all examined genes lacked sufficient combination of specificity and sensitivity to become the reliable biomarkers. Hentze et al. (2019) summarized up-to-date results of research investigating the potential of DNA methylation-based biomarkers in OC, without considering individual subtypes of OC though. Montavon et al. (2012) focused their research just on HGSOC and found that combination of the methylation status of *HOXA9* and *EN1* genes could discriminate HGSOC from benign ovarian surface epithelium with a sensitivity of 98.8 % and a specificity of 91.7 %. However, further studies using a larger cohort are needed to confirm these results.

In our study, the possibility of designing a methylation panel covering more genes was assessed, as well. Altogether, of the eight genes that underwent the initial examination using NGS, only *CDH13* and *PCDH17* showed significant methylation-positive pattern in the tumor samples and were thus selected for further investigation. As mentioned above, the methylation frequency of the *CDH13* and *PCDH17* genes examined individually was 19.7 % (12/61) and 60.7 % (37/61), respectively. Between the two of the genes, *CDH13* with its much lower percentage of detected methylation does not appear to be useful for next consideration as potential biomarker. However, as there were some patients with the methylation present only in *CDH13*, and not in *PCDH17*, the evaluation of both genes together revealed increase in detected methylation to 65.6 % (40/61) of the patients. In order to further increase number of patients with detected methylation, other candidate genes from our previous studies were investigated. By involving *HNF1B* and *GATA4*, with individually detected methylation in 50.8 % (31/61) and 31.2 % (19/61) of tumor samples, the total number of patients with detected methylation reached 88.5 % (54/61). This increase in sensitivity shows the potential of selected gene regions to be included into a DNA methylation biomarker panel. The efficiency of this four-gene panel was 94.2 %, negative predictive value reached 86 %, and since the primers for confirming analysis were deliberately designed flanking the sites without any methylation in the control samples, the specificity and positive predictive value were both 100 %.

6 Conclusions

In our project, the methylation pattern of selected genes encoding adhesion molecules was investigated in order to evaluate their potential as epigenetic biomarkers of clinical benefit in HGSOC screening, diagnosis, and prognosis. Following conclusions were achieved according to the specified objectives:

1. Methodology for 14 amplicons in 8 genes (*CDH10*, *CDH13*, *CDH18*, *PCDH8*, *PCDH10*, *PCDH17*, *CTNNA2*, and *CTNND2*) was successfully optimized. The genes with most distinct alterations in methylation status were then selected for further analysis.
2. MS-HRM and real-time methylation specific PCR were optimized to confirm hypermethylation detected in *CDH13* and *PCDH17* gene.
3. MS-HRM analysis of *CDH13* gene showed methylation-positive pattern in 13.1–19.7 % of the tumor samples. The level of methylation observed in the first amplicon was further confirmed by real-time PCR assay. MS-HRM analysis of the *PCDH17* gene revealed methylation-positive pattern in 60.7 % of the tumor samples. All of the control samples were devoid of methylation in both of analyzed genes.
4. *CDH13* methylation was detected more frequently in the early stage tumors than in the late stage ones by approximately 10 %. Methylation of the *PCDH17* gene was observed with approximately the same frequency in the early stage tumors as in the late stage ones. Despite the lack of statistically significant differences between the early and late stage tumors, the fact that the methylation of the *CDH13* and *PCDH17* genes could be detected in early stages suggests their potential for further examination as a part of biomarker panel for early detection, especially if their methylation could be detected in plasma.
   There was no statistically significant correlation observed between methylation of *CDH13* or *PCDH17* and follow-up data of patients. Although, the overall survival was slightly better in the group of patients where no methylation was detected, the analyzed genes did not prove potential as prognostic markers.
5. The combined evaluation of genes increased the percentage of tumor samples with methylation positive pattern at least in one of the genes to 65.6 %. By methylation analysis of the four-gene panel, including *CDH13*, *PCDH17*, and two genes from our previous study (*GATA4* and *HNF1B*), the methylation could be detected in 88.5 % of tumor samples. These results indicate that examined genes deserve consideration for further testing in clinical molecular diagnosis of HGSOC.

Our findings indicate that methylation of the *CDH13* and *PCDH17* genes could play an important role in development and progression of HGSOC. With the right selection of the most relevant sites for methylation analysis these genes showed potential to become a target in searching for new clinical epigenetic biomarkers. However, further studies on more extensive group of patients are needed to confirm our novel results.

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Overview of publications

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Oral Presentations

Bubancová, I., M. Chmelařová, H. Kovaříková, J. Laco, E. Ruszová and O. Dvořák (2017) *Sledování metylace DNA pomocí NGS u ovariálního karcinomu*. Presented at XXI. Vědecká konference LFHK a FNHK, January 25, 2017

Bubancová, I., H. Kovaříková, J. Laco, O. Dvořák, V. Palička and M. Chmelařová (2016) *Altered methylation of transcription factors in ovarian carcinoma*. Presented at 12th Postgraduate Medical Students Conference, October 24, 2016.

Poster presentations

Chmelarova, M., I. Baranova, H. Kovarikova, A. Mrkvicova, M. Rezacova, J. Laco, I. Sedlakova and V. Palicka. *Role of DNA methylation in resistance to platinum-based chemotherapy in ovarian cancer cells.* Exhibited at 4th International Congrss on Epigenetics & Chromatin, London, September 3 – 5, 2018.

Baranová, I., H. Kovaříková, J. Laco, O. Dvořák, I. Sedláková, V. Palička and M. Chmelařová. *Změny v metylaci protokadherinů u high-grade serózního ovariálního karcinomu.* Exhibited at XLII. Brno Oncology days and XXXII. Conference for medical professionals, Brno, May 16 – 18, 2018.

Kovaříková, H., I. Baranová, J. Laco, M. Vošmik, P. Dundr, K. Němejcová, J. Michálek, V. Palička and M. Chmelařová. *Upregulace miR-9 u dlaždicobuněčného sinonasálního karcinomu: potenciální prognostické biomarker.* Exhibited at XLII. Brno Oncology days and XXXII. Conference for medical professionals, Brno, May 16 – 18, 2018.

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