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ORIGINAL ARTICLE

Importance of Cadherins Methylation in Ovarian Cancer: a Next Generation Sequencing Approach

Marcela Chmelarova[1] · Ivana Baranova[1] · Ema Ruszova[2] · Jan Laco[3] · Katerina Hrochova[1] · Eva Dvorakova[4] · Vladimir Palicka[1]

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Abstract

Epigenetic aberrations are well known to play an important role in carcinogenesis, and also have a great potential to serve as biomarkers in many types of cancers, including ovarian cancer in which sensitive and specific biomarkers and detection methods are critically needed. The aim of this study was to investigate methylation of cadherin genes *CDH10*, *CDH13* and *CDH18* in ovarian cancer tissue by comparison with control tissue. The study group consisted of 38 patients with ovarian cancer and 25 control patients. For detection of epigenetic events we used next generation sequencing, the most important data were confirmed using high-resolution melting analysis and real-time PCR. We observed significantly higher methylation in *CDH13*, sporadic methylation in *CDH10* and loss of methylation in *CDH18* in the ovarian cancer group compared with the control group. These observations suggest that changes in methylation of cadherin genes may be one of the major mechanisms associated with ovarian cancer progression. In addition, because of the high frequency of methylation of the *CDH13* gene in the early stages of ovarian cancer, the analyzed CpG sites might be good targets for next study of potential ovarian cancer screening biomarkers.

Keywords Methylation · Cadherins · Ovarian cancer · Epigenetics · Next generation sequencing

Background

Ovarian cancer is the leading cause of death from gynecologic tumors due to its aggressive nature and the fact that the majority of cases are diagnosed in advanced stages of the disease [1]. In 2012, a total of 65,538 new cases were diagnosed in Europe, giving an incidence rate of 13.1/100000 women. Ovarian cancer was the cause of 42,716 deaths in Europe, giving a mortality rate of 7.6/100000 women [2]. If ovarian cancer could be diagnosed at an early stage, this would result in a significant improvement in survival [3]. The etiology of ovarian cancer is still not clear. Based on epidemiology studies, the hypothesis has been proposed that low-grade serous carcinoma often develops from a serous borderline tumor, which in turn arises from a serous cystadenoma. Another mechanism involves exfoliation of malignant cells from a serous tubal intraepithelial carcinoma that implants on the ovarian surface resulting in the development of a high-grade serous carcinoma [4]. Since symptoms are absent in the majority of cases during the early stages of the disease, sensitive and specific detection methods are critically needed, but are not yet available.

In general, cancer has been considered as a disease driven by progressive genetic alterations, such as mutations involving oncogenes or tumour suppressor genes, as well as chromosomal abnormalities. However, more recently, it has been demonstrated that cancer is also driven by epigenetic alterations [5]. Epigenetic aberrations are today known to play an important role in carcinogenesis and also have a great potential to serve as biomarkers in many types of cancers including ovarian cancer [6]. Epigenetic biomarkers could be used in monitoring of the response to therapy and for disease screening and detection. DNA methylation is one of the most

✉ Ivana Baranova
ivana.baranova@fnhk.cz

[1] Institute for Clinical Biochemistry and Diagnostics, Charles University, Faculty of Medicine and University Hospital in Hradec Kralove, Hradec Kralove, Czech Republic

[2] Department of Clinical Genetics, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic

[3] The Fingerland Department of Pathology, Charles University, Faculty of Medicine and University Hospital in Hradec Kralove, Hradec Kralove, Czech Republic

[4] Department of Obstetrics and Gynecology, Charles University, Faculty of Medicine and University Hospital in Hradec Kralove, Hradec Kralove, Czech Republic

commonly-occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a potential therapeutic target. Many different genes have been identified as being hypermethylated and silenced in ovarian carcinoma [7, 8].

The control of cellular adhesion and motility is one of the crucial mechanisms responsible for tumor initiation and progression [9]. Cadherins are a class of type-1 transmembrane proteins. They play important roles in cell adhesion, forming adherent junctions to bind cells together within tissues. There are said to be over 100 different types of cadherins found in vertebrates, which can be classified into four groups: classical, desmosomal, protocadherins, and unconventional cadherins [10]. Recently, interest in a cadherin role in cancer development has been increasing. Whilst in 1995 there were only 145 items related to cadherin connections in cancer development on PubMed, 20 years later the number of items was 1330. Changes in expression of cadherins are caused not only by genetic alterations but also by epigenetic silencing [11, 12]. DNA methylation of cadherin genes has been found in several cancer types such as ovarian cancer [13], bladder cancer [14], prostate cancer [15] and endometrioid carcinoma of the endometrium [16].

Based on our previous experiments [17] we focused on analysis of methylation in cadherin genes *CDH10*, *CDH13* and *CDH18*. For analysis of methylation changes in selected loci we used the bisulfite next generation sequencing (NGS) approach. The most important data obtained from NGS were confirmed using high-resolution melting analysis (HRM) and methylation-specific duplex real-time PCR with designed labeled TaqMan probes.

Materials and Methods

Study Group

Tissue samples of ovarian adenocarcinomas and control tissue were obtained from 63 women treated at the Department of Obstetrics and Gynecology, University Hospital Hradec Kralove, Czech Republic: 38 patients with ovarian cancer and 25 patients surgically treated for a non-malignant diagnosis (such as descent of the uterus with adnexectomy, uterine leiomyomas, etc.). The group of control samples ($n = 53$) consisted of 25 samples of normal ovary, 18 samples of tissue from the fimbriated end of the fallopian tube and 10 samples of endometrioid tissue. According to the müllerian system theory serous and endometrioid ovarian cancers are derived from fallopian tube and endometrium, not directly from the ovary. Mucinous tumors do not display a müllerian phenotype and the origin of these tumors is not entirely clear [4]. The group of control samples therefore consisted of ovarian tissue, fallopian tube tissue and endometrioid tissue.

From the total number of 91 samples 75 samples were formalin-fixed, paraffin-embedded and 16 samples were fresh frozen. Patients' clinicopathological properties, such as age, clinical stage, histological type and pathological grade were surveyed (see Table 1). The median age of patients at the time of diagnosis was 54 years (range 21–83 years) in the carcinoma group and 57 years (range 42–78 years) in the control group. The paraffin blocks were retrieved from the archive of the Fingerland Department of Pathology, University Hospital Hradec Kralove. All slides were reviewed by an experienced pathologist (J.L.) and the carcinomas were classified according to the current WHO classification of tumors of the female genital organs [18]. The study was approved by the Ethics Committee of University Hospital Hradec Kralove. Informed consent related to fresh frozen tissue samples was obtained from each patient. The need for informed consent related to formalin-fixed, paraffin-embedded tissue samples was waived by the review board in view of the retrospective nature of the study and long archival period of the formalin-fixed, paraffin-embedded tissue samples involved.

DNA Bisulfite Conversion

DNA was extracted from ovarian tissue samples using Qiagen (Hilden, Germany) DNA extraction kit. DNA was quantified and quality checked by 260/280 absorbance ratio using NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). 500 ng of genomic DNA was bisulfite-converted using EZ DNA Methylation-Gold™ Kit according to the manufacturer's protocol (Zymo Research Corporation, USA) with minor modifications. Briefly, DNA was denatured (10 min 98 °C), bisulfite-converted for 2.5 h at 64 °C and subsequently desulfonated, washed and eluted in 15 μl elution buffer, and stored at -80 °C.

Table 1 Patients' clinicopathological properties

Characteristic			Quantity	%
Age	≤ 45 years		5	13.2
	> 45 years		33	86.8
Stage	I. + II.		17	44.7
	III. + IV.		21	55.3
Histology, grade	high-grade serous		20	52.6
	low-grade serous		4	10.5
	endometrioid $n = 9$	G1	1	23.7
		G2	5	
		G3	3	
	mucinous n = 5	G1	4	13.2
		G2	1	

Next Generation Sequencing of Cadherins

The MiSeq sequencer uses a reversible-terminator sequencing-by-synthesis (SBS) approach capable of producing a massive parallel sequencing environment. The addition of unique dual indexes enables the multiplexing of up to 96 samples per sequencing run. This process of sample modification is termed library preparation.

In our previous study we introduced innovative approach of selecting specific sites for methylation analysis, where NGS was used for a preliminary scan and for further analysis of the most interesting sites another commonly used method was employed [19]. In this study we multiplexed several amplicons in 16 samples: 8 tumors (5 high-grade serous, 2 endometrioid and 1 mucinous) and 8 control samples. Specific primers were designed to amplify promoter and part of the first exon of the *CDH10*, *13* and *18* genes (MethPrimer design). Sequencing libraries were prepared using the Multiplicom approach. Briefly, first PCRs were made with sequence-specific primers with adapters for the Multiplicom MID kit (See Table 2). PCR was carried out in a 20 μl mixture containing 10x Gold buffer without $MgCl_2$ (2 μl), $MgCl_2$ 25 mM (2 μl), dNTPs 2.5 mM solution Takara (1.6 μl), primers (1 μl each, 10 pmol/μl solution), AmpliTaq Gold DNA Polymerase 5 U/μl (0.3 μl) (Applied Biosystems, CA), water and 2 μl of bisulfite-converted DNA in a Veriti Thermocycler (Applied Biosystems, CA). The cycling condition consisted of an initial denaturation at 95 °C for 5 min, 40 cycles of denaturing at 95 °C for 20 s, annealing at 57/62 °C for 30 s, and extension at 72 °C for 35 s, followed by final extension for 5 min at 72 °C. CpG universal methylated and unmethylated DNA (Zymo Research Corporation, USA) were similarly treated with bisulfite and were used as controls. Amplified products (5 μl) were separated by electrophoresis on 2% agarose gels and visualized under ultraviolet light after staining with ethidium bromide. PCR products from the first PCRs were pooled and 100x diluted and amplified in a subsequent barcoding PCR. Unique DNA sequencing barcodes and specific adapters for Illumina sequencing were incorporated into each sample by a subsequent round of PCR amplification using MID for Illumina MiSeq® kit (Multiplicom, Belgium) with minor modifications. PCR products were separated by electrophoresis on 2% agarose gels and specific products were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany). Purified sample concentrations were measured using DQ300 Fluorometer (Inc., Holliston, MA). All samples were equimolarly pooled into one library. The molarity of the library was quantified using the KAPA library quantification assay (Kapa Biosystems, USA) and a 4 nM library was prepared. Fragment lengths were determined by separating 1 μl of prepared library on an Agilent high sensitivity DNA assay using the 2100 Bioanalyzer (Agilent Technologies,USA).

Table 2 NGS primers and amplicon information

Amplicon name	Primer sequence 5′-3′ (with adapters*)	Amplicon size (bp) (without adapters and barcodes)	CpGs/ Amplicon	Annealing temperature °C
CDH10–1	Fw: AAGACTCGGCAGCATCTCCATTTT GTGATAATAAGTAATAAGAGAAGGGA Rv: GCGATCGTCACTGTTCTCCATCAA AACTAAAATAATCAACCCAATCTA	306	7**	57
CDH10–2	Fw: AAGACTCGGCAGCATCTCCATAGT TTTGTTTTTTGAGATTGTATTA Rv: GCGATCGTCACTGTTCTCCATAATT AACTTTCATTCAATACTTCTAATTA	335	11**	57
CDH13	Fw: AAGACTCGGCAGCATCTCCATAAT AGTTTAAAGAAGTAAATGGGATGTTA Rv: GCGATCGTCACTGTTCTCCATTCCC TACCTAAAACAAAAAAAC	353	23	57
CDH18–1	Fw: AAGACTCGGCAGCATCTCCATAGT AGTTGAATGTTTAGTAGGTTGTGA Rv: GCGATCGTCACTGTTCTCCACCCCT CAACAAAATCATATAAAAAA	319	10	62
CDH18–2	Fw: AAGACTCGGCAGCATCTCCATATA TGATTTTGTTGAGGGGGTTAA Rv: GCGATCGTCACTGTTCTCCACCCAA AACTCTAAACACAACTACTC	318	18	62

* adapter overhangs: Fw: AAGACTCGGCAGCATCTCCA, Rv: GCGATCGTCACTGTTCTCCA

** amplicons overlap in 2 CpGs (means 16 analyzed CpGs in *CDH10* gene)

Next generation sequencing was carried out on the Illumina MiSeq using Reagent Kit v2 following the manufacturer's instructions. Briefly, the 4 nM pool of prepared library and the 4 nM PhiX control (20%) were denatured for 5 min with 0.2 N NaOH and diluted to final concentrations of 9 pM. The prepared library (600 μl) was loaded into the reagent cartridge. Diluted Multiplicom read 1, read 2 and index primers were also added to the cartridge. The sequencing reaction was carried out with 250 base pair paired-end sequencing. Trial version of NextGENe® software (Softgenetics, USA) was used for the analysis and calculation of methylation status of analyzed CpG sites. As a reference sequence bisulfite-converted methylated DNA was used.

HRM Analysis (*CDH13*)

High-resolution melting (HRM) analysis is an innovative technique based on analysis of DNA melting. HRM characterizes a DNA sample according to its dissociation behavior as it transitions from double-stranded DNA to single-stranded DNA with increasing temperature. For CpG methylation analysis, the DNA has to be completely converted by bisulfite treatment. To confirm hypermethylation in the *CDH13* gene we analyzed 91 samples (38 cancer samples and 53 control samples). PCR amplification and HRM analysis were performed on Rotor-Gene 6000 5-plex with HRM (Corbett Research, UK). Primers were designed using MethPrimer. Amplicon 1 included 9 CpG sites and amplicon 2 included 13 CpG sites. Amplicons were designed to be no greater than 200 bp in view of the fact that FFPE DNA is highly fragmented and larger amplicons result in lower melting resolution. An overview of the used HRM primers is listed in Table 3. PCR was carried out in a final volume of 10 μl containing: 5 μl 2x EpiTect HRM PCR Master Mix (Qiagen, Hilden, Germany), 0.75/0.6 μl of each primer (10 μM), 1.5/2 μl bisulfite converted DNA, and water. The cycling conditions were as follows: initial PCR activation step 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 10 s, followed by a HRM step 55–95 °C, 2 s, with fluorescence data acquisition per 0.1 °C on the "HRM" channel. Each run included a water blank, a bisulfite-converted unmethylated and methylated control and prepared standards of various methylation percentage (Qiagen, Hilden, Germany). HRM data were analyzed using the Rotor-gene 6000 software in comparison with standards. For the purpose of statistical analysis, a case was considered to be methylated if it was positive for methylation in amplicon1 and/or amplicon2.

Real-Time Methylation Specific Analysis (*CDH13*)

Duplex real-time PCR assay for measuring DNA methylation was used to analyze two selected CpG sites in the *CDH13* gene. To confirm the presence of methylation in these two CpG sites we analyzed 91 samples (38 cancer samples and 53 control samples). Briefly, a set of primers and probes (see Table 4) designed (MethPrimer) specifically for bisulfite-converted DNA was used. The amplicon was designed to be 130 bp long in view of the fact that FFPE DNA is highly fragmented. PCR amplification was performed on the Rotor-Gene 6000 5-plex with HRM (Corbett Research, UK). PCR was carried out in a final volume of 20 μl containing: 10 μl Takara Premix 2x (Premix Ex Taq™, Clontech Laboratories, Inc., USA), 0.6 μl of each primer (10 μM), 0.6 μl of each probe (10 μM), 2 μl of bisulfite-converted DNA, and water. The cycling conditions were as follows: Initial PCR activation step 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 55 °C for 20 s and 60 °C for 20 s, followed by cooling to 40 °C for 2 min. Each reaction was performed in triplicate. Each run included a water blank, a bisulfite-converted unmethylated and methylated control and prepared standards of various methylation percentage (Qiagen, Hilden, Germany). Fluorescence data were analyzed using Rotor-gene 6000 software. The methylation index MI (%) of amplicon was calculated using the equation: $MI = 100 / [1 + 2^{(CTm - CTu)}]$. CTm: Ct value of reaction with methylated probe; CTu: Ct value of reaction with unmethylated probe. When there was no reaction with methylated probe the amplicon was considered as unmethylated.

Statistical Analysis

Proportions were compared by two-tailed Fisher's exact test and/or Chi-square test. VassarStats software was used for analysis. Associations with p value <0.05 were considered to be significant.

Table 3 HRM primers and amplicon information

Amplicon name	Primer sequence 5′-3′	Amplicon size (bp)	CpGs/Amplicon
CDH13–1	Fw: AGTTTAAAGAAGTAAATGGGATG Rv: AACCAAAACCAATAACTTTACA	130	9
CDH13–2	Fw: TGATTTATTTGGGAAGTTGGT Rv: CCCTCTTCCCTACCTAAAACA	189	13

Table 4 Real-time primers and probes

Primer/Probe	Primer (Probe) Sequence 5′-3′
Fw primer	AGTTTAAAGAAGTAAATGGGATG
Rv primer	AACCAAAACCAATAACTTTACA
Probe for methylated DNA (FAM-BHQ)	CGAGGGAGCGTTAGGAAGGAA
Probe for unmethylated DNA (HEX-BHQ)	TGAGGGAGTGTTAGGAAGGAA

Results

Next Generation Sequencing of Cadherins *CDH10, 13* and *18*

In this study, we selected 16 target loci for evaluation of methylation in the *CDH10* gene, 23 target loci in the *CDH13* gene, and 28 target loci in the *CDH18* gene. Analyzed CpG sites in *CDH13* and *CDH18* genes are located within CpG islands (for prediction was used MethPrimer). The DNA methylation profile was compared in eight cancerous and eight control ovarian samples (Figs. 1, 2 and 3). The MiSeq sequencing run produced 11.43 million reads with 10.68 million passing filter. The library was spiked with 20% PhiX control, and 15.91% of total reads were aligned to the PhiX genome. The percentage of reads allocated to each of the 16 samples ranged from 1.7 to 6.9%. Across all samples, the average read number per locus was 1500 reads.

Figure 1 depicts methylation in the *CDH10* gene. Sporadic statistically non-significant methylation ($p > 0.05$) was present across the 16 analyzed target CpG loci.

Figure 2 depicts methylation in the *CDH13* gene. Statistically significant methylation ($p < 0.05$) was present across the 23 analyzed target CpG loci. The most important are CpG3, 4, 5, 6, 7, 8, 9, 15, 16, 17, 18, 19, 20, 21 and 23, and high methylation was present in over 50% of samples. *CDH13* was completely methylation free in all of the normal ovarian tissue samples. This result also demonstrated the success of the modifying DNA assay.

Figure 3 depicts methylation in the *CDH18* gene. Methylation was present in CpG3 in all control samples; in cancer samples, only in one sample was methylation present at this site ($p = 0.001$). In the remaining 27 analyzed CpGs only sporadic methylation was present.

HRM Analysis (*CDH13*)

To confirm hypermethylation in the *CDH13* gene we analyzed 91 samples (38 cancer samples and 53 control samples) using HRM analysis. Amplicon 1 covers CpG 1–9 (from NGS) and amplicon 2 covers CpG 11–23 (from NGS). *CDH13* in all of the normal ovarian tissue was completely methylation free. This result also demonstrated the success of the assay modifying DNA. By contrast, a methylation-positive pattern was observed for 61% (23/38) of ovarian cancer tissue ($p < 0.0001$), which is in correlation with NGS data. The results are

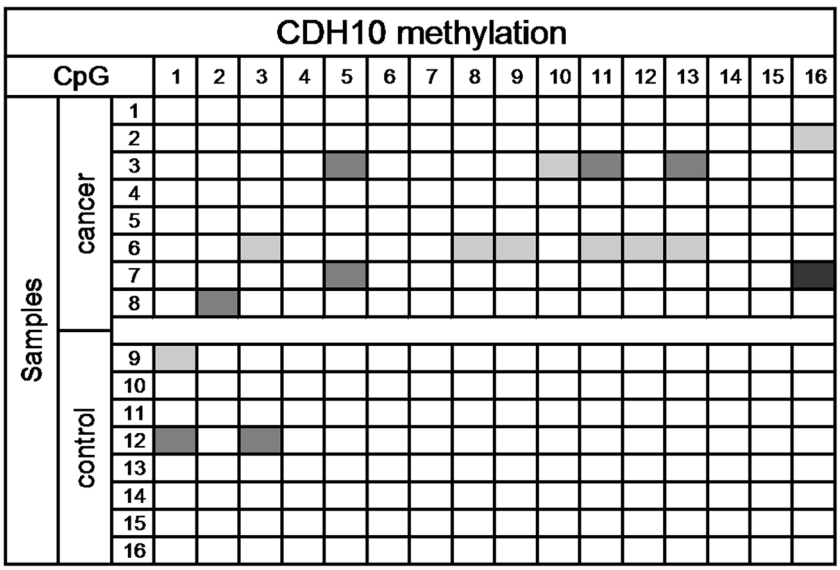

Fig. 1 NGS methylation data of *CDH10*. Legend: White color indicates an unmethylated CpG site (cut off for methylation was 15%); CpG sites with methylation between 15% and 25% are light grey; CpG sites with methylation 25% and more up to 50% are shown as dark grey; and CpG sites with methylation 50% and more are marked as black. Histology of tumor samples: high-grade serous (No. 1, 2, 4, 5, 7), endometrioid (No. 3,8), mucinous (No. 6)

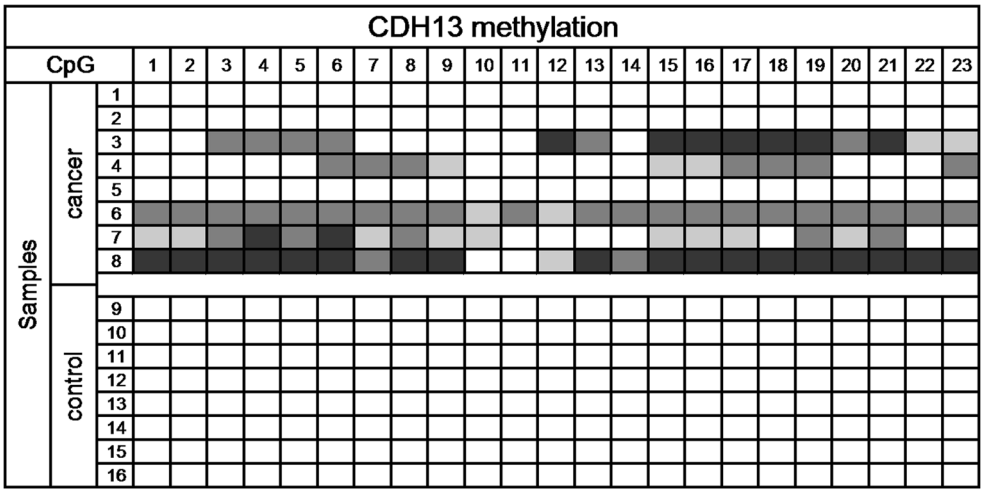

Fig. 2 NGS methylation data of *CDH13*. Legend: White color indicates an unmethylated CpG site (cut off for methylation was 15%); CpG sites with methylation between 15% and 25% are light grey; CpG sites with methylation 25% and more up to 50% are shown as dark grey; and CpG sites with methylation 50% and more are marked as black. Histology of tumor samples: high-grade serous (No. 1, 2, 4, 5, 7), endometrioid (No. 3,8), mucinous (No. 6)

summarized in Table 5. The table illustrates the correlation of methylation with clinicopathological characteristics including age, tumor stage, histological type and grade. No significant correlation ($p > 0.05$) between *CDH13* methylation and any of these parameters was observed for the ovarian cancer patients.

Real-Time Methylation Specific Analysis (*CDH13*)

To confirm the presence of methylation in CpG 7 and 8 (from NGS) we analyzed 91 samples (38 cancer samples and 53 control samples). *CDH13* in all of the normal ovarian tissue was completely methylation free. By contrast, a methylation-positive pattern was observed for 39.5% (15/38) of ovarian cancer tissue ($p = 0.001$). The lower presence of methylation is due to the assay strategy. A sample is shown as positive only if both CpG sites in the probe location are methylated.

Discussion

In this study we examined the methylation patterns of cadherins *CDH10*, *CDH13* and *CDH18*, with the aim of determining whether they can serve as potential markers of clinical benefit in disease screening, diagnosis and prognosis. Epigenetic alterations of cadherins such as DNA methylation are clearly involved in ovarian cancer initiation and progression [12]. Global DNA hypomethylation and localized hypermethylation of specific gene promoters contribute to genome instability and transcriptional silencing of tumor suppressor genes [7]. Since aberrant methylation is one of the earliest molecular alterations during tumorigenesis, it has been suggested as a promising strategy for the early detection of ovarian cancer [5].

Cell-cell adhesion participates in cell differentiation and in establishment and maintenance of tissue homeostasis. During oncogenesis, this organized adhesion is disturbed by genetic and epigenetic changes, resulting in changes in signaling, loss of contact inhibition, and altered cell migration and stromal interactions [9]. A major class of cell–cell adhesion molecules is the cadherin superfamily. Recent studies have shown the importance of members of the cadherin family in tumor cell invasion and metastasis [20]. These studies raise the possibility that a ‘cadherin switch’ from pro-adhesive epithelial cadherins (e.g. E-cadherin) to mesenchymal pro-migratory cadherins (e.g. N-cadherin) promotes tumor invasion and metastasis [21].

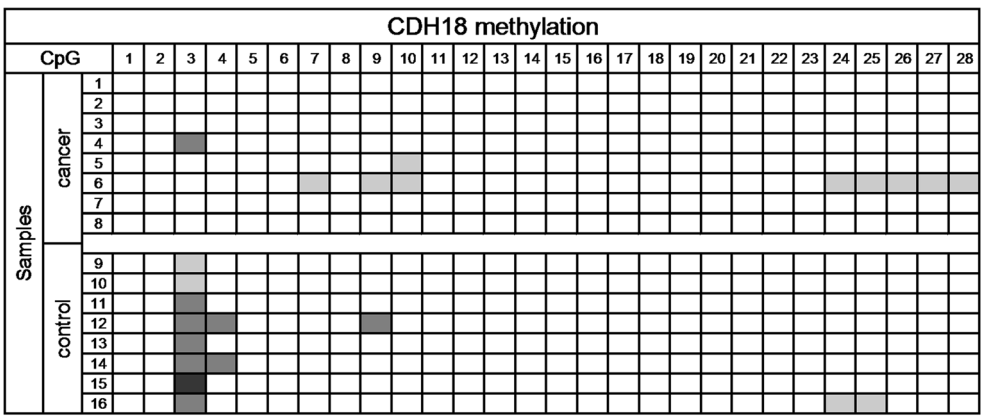

Fig. 3 NGS methylation data of *CDH18*. Legend: White color indicates an unmethylated CpG site (cut off for methylation was 15%); CpG sites with methylation between 15% and 25% are light grey; CpG sites with methylation 25% and more up to 50% are shown as dark grey; and CpG sites with methylation 50% and more are marked as black. Histology of tumor samples: high-grade serous (No. 1, 2, 4, 5, 7), endometrioid (No. 3,8), mucinous (No. 6)

Table 5 Methylation of CDH13 and clinicopathological characteristics

Characteristic		Quantity			% Methylated
		Overall	Unmethylated	Methylated	
Age	≤ 45 years	5	1	4	80.0
	> 45 years	33	13	20	60.6
Stage	I. + II.	17	5	12	70.6
	III. + IV.	21	9	12	57.1
Histology	high-grade serous	20	10	10	50.0
	low-grade serous	4	1	3	75.0
	endometrioid	9	1	8	88.9
	mucinous	5	1	4	80.0

Extensive evidence supports the involvement of cadherin-13 in various cancers. CDH13 plays a dual role in tumor development: it negatively regulates cancer cell proliferation, invasiveness and tumor growth in most cases, and at the same time it positively regulates tumor neovascularization [22]. The importance of methylation in the *CDH13* gene has been previously shown in ovarian cancer [13, 23]. According to our knowledge, this is the first study where the methylation status of *CDH13* in ovarian cancer was evaluated using NGS. We analyzed 23 CpG loci across the *CDH13* gene. Previous studies used MS-MLPA [13, 23–25] or MSP [26, 27] and analyzed only a few CpG sites across the *CDH13* gene. Feng et al. (2008) showed statistically non-significant methylation of *CDH13* in ovarian cancer samples compared with normal/benign tissue and Rathi et al. (2002) reported only very low methylation in tumors. As is evident from Fig. 2, the methylation is site specific and it is possible that they did not analyze the most important CpGs in the *CDH13* gene. Our HRM results show the presence of methylation in 61% of ovarian cancer samples, resp. 39.5% in case of real-time MSP, but there is no methylation in any control sample. The lower presence of methylation detected by real-time MSP is due to the assay strategy. There are no significant differences between the presence of methylation and any clinicopathological characteristic. Nevertheless in the early stages of ovarian cancer methylation was present in 70.6% (12/17) of ovarian tumor samples. Our findings show that analyzed CpG sites in the *CDH13* gene could be promising targets to focus on in looking for the new biomarkers in early detection of ovarian cancer, especially if the presence of methylation in CDH13 is detectable in the plasma of ovarian cancer patients.

Cadherin-10 plays a key role in prostate epithelial differentiation, and is lost in prostate cancer [28]. Frameshift mutations were found in gastric and colorectal cancers with high microsatellite instability [29]. Mutations of this cadherin have been found also in lung squamous cell carcinoma [30]. The lack of CDH10 in cancer cells might contribute to the altered morphology and behavior of these cells. According to our knowledge, this is the first study searching for DNA methylation in *CDH10* using bisulfite conversion, which was established as a gold standard for DNA methylation analysis. Our results show only sporadic methylation across the *CDH10* gene, indicating that methylation of this cadherin is not important event during progress of ovarian cancer.

Cadherin-18 (formerly known as *CDH14*) encodes a type II classical cadherin from the cadherin superfamily of integral membrane proteins that mediate calcium-dependent cell-cell adhesion. Copy number variants of *CDH18* have been found in colorectal cancer patients [31]. *CDH18* deletions have been found also in odontogenic tumors [32]. According to our knowledge, this is the first study using bisulfite conversion in assessing *CDH18* methylation. In ovarian cancer samples we found only weak sporadic methylation across *CDH18*. By contrast, methylation is present in all control samples in CpG3. This finding suggests that loss of methylation in CpG3 may participate in ovarian cancer expansion.

Conclusion

In conclusion, we have demonstrated high frequency of methylation of the *CDH13* gene in ovarian cancer tissue. This observation suggests that methylation of this cadherin may be one of the major mechanisms associated with ovarian cancer progression. In addition, because of the high frequency of methylation in the early stages of ovarian cancer, analyzed CpG sites of *CDH13* might be good targets for next study of potential ovarian cancer screening biomarkers. On the other hand we found loss of methylation in one analyzed CpG site of the *CDH18* gene in ovarian cancer samples. This finding

suggests that loss of methylation at this site may participate in advancement of ovarian cancer. The final analyzed cadherin, *CDH10*, shows only weak sporadic methylation, indicating that its methylation is not important in ovarian cancer progression.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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