Methylation and ovarian cancer: Can DNA methylation be of diagnostic use? (Review)

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Abstract. Ovarian cancer is a silent killer and, due to late diagnosis and frequent chemo resistance in patients, the primary cause of fatality amongst the various types of gynecological cancer. The discovery of a specific and sensitive biomarker for ovarian cancer could improve early diagnosis, thereby saving lives. Biomarkers could also improve treatment, by predicting which patients will benefit from specific treatment strategies. DNA methylation is an epigenetic mechanism, and 'methylation imbalance' is characteristic of cancer. Previous research suggests that changes in DNA methylation can be used diagnostically, and that they may predict resistance to treatment. This paper gives an up-to-date overview of research investigating the potential of DNA methylation-based markers for diagnostics, prognostics, screening and prediction of drug resistance for ovarian cancer patients. DNA methylation cancer-biomarkers may be useful for cancer treatment, particularly since they are chemically stable and since cancer-associated changes in methylation typically precedes tumor growth. DNA methylation markers could improve diagnosis and treatment and might even be used for screening in the future. Furthermore, DNA methylation biomarkers could facilitate the development of precision medicine. However, at this point no biomarkers for ovarian cancer have a sufficient combination of sensitivity and specificity in a clinical setting. A reason for this is that most studies have focused on a single or a few methylation sites. More large screenings and genome-wide studies must be performed to increase the chance of identifying a DNA methylation marker which can identify ovarian cancer.

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Abbreviations: OC, ovarian cancer; FIGO, International Federation of Gynecology and Obstetrics; RMI, risk of malignancy index; MSP, methylation-specific PCR; miRNA, microRNA; MESC, methylated in embryonic stem cells; OS, overall survival; PFS, progression free survival

Key words: ovarian cancer, diagnostic/prognostic biomarkers, prediction of chemo-resistance, DNA methylation, precision medicine

Contents

1. Introduction

The leading cause of death from gynecological malignancy is ovarian cancer (OC) (1). With a 5-year survival rate of only 52%, OC is the 5th most common killer amongst cancers in women (1-3). More than 60% of the patients are diagnosed at a late stage [stage III/IV, International Federation of Gynecology and Obstetrics (FIGO)], resulting in high mortality (1,2,4,5). The 'risk of malignancy index' (RMI) is used to identify women with a pelvic mass and a high risk of OC (6,7). RMI includes serum levels of the biomarker CA125 in addition with ultrasound scanning and menopausal state of the patient. Neither CA125 alone nor RMI are optimal for selecting women at high risk of OC, and new more sufficient OC markers for use in diagnostics or screening programs are highly desired to improve survival (8-14). Another way to improve survival could be treatment using biological, targeted drugs based on identification of predictive biomarkers, as approximately 80% of OC patients develop resistance towards platinum-based treatment (15).

Gene expression can be robustly changed by the process of DNA methylation (16). DNA methylation is a process where methyl-groups are added to the nucleotide cytosine. The methyl-group is normally added to a cytosine followed by a guanine, called a CpG site. Methylation has several functions, including stabilization of the DNA molecule and regulation

of gene transcription. The regulatory function of DNA methylation is essential in several processes of embryonal development (17,18). Altered DNA methylation, resulting in chromosome instability and changed gene expression, has been highly correlated to carcinogenesis. Because of the involvement of methylation in cancer, methylation profiles have potential as biomarkers. This review aims at summarizing the current knowledge of the role of DNA methylation in OC and the clinical potential of DNA methylation patterns as OC biomarkers for diagnostics, screening and prediction of drug response.

2. DNA methylation in cancer

One of the earliest proofs that erroneous DNA methylation is directly involved in carcinogenesis came in 1994, when Herman *et al* showed that the tumor suppressor gene *VHL* might be silenced by hypermethylation of its promotor in some cases of renal carcinomas (19). Since then, numerous similar studies has shown that promotor hypermethylation is a widespread mechanism for silencing of tumor suppressor genes in human cancers, and it is estimated to be as common as mutation (20,21). Cancer related DNA methylation is often observed earlier than the actual neoplastic transformation, and it has been suggested that DNA methylation is a primary link between environment and cancer, as there seems to be a connection between lifestyle and cancer related DNA methylation in un-symptomatic persons (22,23). It is therefore also likely, that DNA methylation is the initiator of tumor formation in a high percentage of cancer-cases.

The best understood way in which DNA methylation affects carcinogenesis is by silencing of tumor suppressors by localized hypermethylation in promoters and other regulatory areas, apparently caused by increased levels of DNA methyltransferase (24,25). Aberrant methylation however, can also lead to cancer in other ways. Reduced methylation of oncogene promoters has been observed in numerous cancers (26,27). Another very widespread mechanism is chromosome instability, caused by genome wide hypomethylation (28-30).

3. DNA methylation profiles as biomarkers

DNA methylation has several advantages compared to other molecular markers. Already in 1999, it was discovered that cancer related DNA methylations can be measured in the serum of cancer patients, and it has later been documented that DNA methylations are chemically and biologically stable, also on cell-free cancer DNA in the blood (31-35). The DNA molecule itself is more stable to work with than RNA or protein, and methylation patterns are easier to detect than mutations as they are binary signals and can be amplified by methylation-specific PCR-based techniques (36). Besides, DNA methylation analysis can be focused on the CpG sites, which mean there is no need to scan the whole gene as for mutations.

To be able to give information that is useful in a diagnostic or prognostic setting, a molecular marker should change behavior in a detectable way which can be correlated with important pathogenic processes and steps of disease development. Patterns of DNA methylation has potential both as prognostic and diagnostic markers, as changes in DNA methylation has been correlated to early carcinogenesis, even prior to tumor formation, as well as to the process of metastasis and to sensitivity to treatment (37-40).

4. Methods for detection of methylation

DNA methylation can be detected as a binary signal, either methylated or not methylated. Several technologies have been developed to identify the methylation status of CpGs. The best known and most widespread methods involve bisulfite conversion of cytosine to uracil. Methylated cytosines won't be converted and several methods can be used to identify the non-converted CpGs. Sequencing of the bisulfite converted DNA is generally considered the golden standard and is widely used. However, it is quite expensive. Another popular method to detect bisulfite converted cytosines is methylation-specific PCR (MSP). In this quick assay, DNA is amplified from primers specific for either the converted or not-converted sequence (41). For easier detection of low-abundant methylations in samples with excess of unmethylated DNA, a methylation-specific qPCR (qMSP) can be run.

Alternative methods to detection of methylation status often involve methylation-specific restriction sites. In the simplest version, DNA is cut with a restriction enzyme for which its activity is dependent on methylation status of the restriction site. A subsequent PCR will only amplify uncut DNA, thereby revealing whether it was methylated or not. This concept is also applied in the methylation sensitive amplified polymorphism method, where differences in DNA fragments after digestion with methylation-specific restriction enzymes can be used to compare methylation differences in different sample types (42). It is an advantage of the latter method, that prior, detailed knowledge of the genomic sequence is not required. In case the genomic sequence is known, and methylation status of specific targets is of interest, Methylation-specific, Multiplex ligation-dependent probe amplification can be used (43). This method recognizes the area of interest with a probe prior to digestion with methylation sensitive restriction enzymes, and proliferation of the probe thereby depends on methylation status of the area attached to the probe.

A newer, quantitative, high-throughput methodology for studies of methylation sites is the one applied by the Sequenom MassArray technology. DNA methylation is analyzed by mass spectrometry after base-specific cleavage. After bisulfite modification, RNA is transcribed from the genomic sequence of interest, and cleaved base-specifically. Methylation and bisulfite conversion changes the atomic mass and the extent of methylation can measured by mass spectrometry (44).

To identify methylation patterns, which can be used as biomarkers, it is necessary with more genome-wide approaches. Whole genome bisulfite sequencing is a possibility, but rather expensive and ineffective (45). Illumina has gained popularity with their Infinium HumanMethylation BeadChip methodology. The chips contain beads with attached probes which recognize either methylated or un-methylated methylation sites, after bisulfite conversion. The newest version, the Infinium Methylation EPIC array, gives single-base methylation information for over 850.000 CpGs throughout the genome. A vast amount of the CpGs targeted by the EPIC

array are in regulatory areas like promoters and enhancers, relevant for human cancer biology, and the array has a high potential for identification of new cancer biomarkers.

5. DNA hypermethylation in OC

DNA methylation has been shown to be a major player in OC and several tumor suppressor genes have been shown to be hypermethylated. Methylation of *BRCA1* promoters has gained a lot of attention, since *BRCA1* mutations are known to be involved in inherited OC (46,47). *BRCA1* promoter hypermethylation can be found in 15-30% of ovarian carcinomas (46,48,49). In a study on 50 patients from 2004, 68% of the patients were methylated in either *BRCA1* or the *RASSF1A* gene (35). The study found that all patients were hypermethylated in one or more tumor suppressor genes, and hypermethylation was found in all histological types, grades, and stages of OCs examined, illustrating that hypermethylation is a widespread phenomenon in OC. *RASSF1A* promoter hypermethylation in OC has been observed in other studies, and it might be one of the most frequently methylated genes in OC, one study even observed it in as much as 49% of 52 carcinomas (50). However, other studies only found *RASSF1A* promotor methylation in 10-12% of ovarian tumors but in 40% of ovarian tumor cell lines (51,52). Two other tumor suppressor genes are frequently methylated in their promotors in OC, as shown by several independent studies. One of these is *OPCML,* which in one study was found methylated in as much as 83% of ovarian tumors (53-55). The other is the gene *P16INK4a*. A meta-study from 2016, including 612 OC patients and 289 controls from 12 studies, confirmed that *P16INK4a* promoter hypermethylation is found regularly in OC while rarely found in controls (21).

Studies investigating the stem cell nature of cancers, have shown that stem cell PolyComb group targets are up to 12-fold more likely to have cancer-specific promoter hypermethylation than non-targets (56). The PolyComb group targets are transcription factors involved in differentiation, and their transcription is normally repressed reversibly in embryonic stem cells. As part of carcinogenesis, repression of these genes can be made irreversible by promoter methylation, and the result is a cell that will continue to renew itself, leading to tumorigenesis. Hypermethylation at stem cell PolyComb Group Target genes in OC and other women's cancers was confirmed in a large screening of 1475 samples in 2012 (40).

A study from 2015 showed that epigenetic suppression of ten genes was involved in the development of non-serous OC, including *OPCML* and the promotor of microRNA (miRNA) *miR-34b* (57). The cancer suppressive nature of some miRNAs is gaining more focus these years, and an increasing body of evidence suggests a cancer suppressive role of the miR-34 family, which is part of the p53 cancer suppressive network. A study from 2016 states a correlation between type II OC and hypermethylation of *miR-34a* (58). The miRNA miR-30d has also been shown to counteract OC. Hypermethylation of the *miR-30d* promoter was seen for TGF-β1 induced epithelial-mesenchymal transition of OC cells (59). The most recent research on hypermethylation of gene promotors suggests that genes *KLF11, ARH1, GBGT1* and *PDLIM2* are hypermethylated in OC (60-63). All 4 genes were hypermethylated while their expression was reduced in either OC cell-lines, tissue from OC patients or both. A few other genes have been reported to have very high promoter methylation (>50%) in OC in individual studies. This goes for *hMSH2* and *HSulf-1* but the reported data needs to be followed up with validation studies (64,65).

6. DNA hypomethylation in OC

Hypomethylation in OC is less studied than hypermethylation but has gained more focus in recent years. Hypomethylation can cause cancer through different mechanisms. Widespread loss of methylation results in a general DNA instability, which will typically lead to increased mutation frequency and a risk of cancer. However, most studies focus on hypomethylation of specific CpGs. Reduced methylation in regulatory areas of oncogenes affects transcription, often increasing it. A study from 2009 suggests that hypomethylation is the most common methylation change in OC. Around 27,000 CpGs were screened in 113 OC cases and 148 healthy controls, and a total of 2,714 cancer related CpGs were identified. 56% of these were hypomethylated, but amongst the 50 CpGs with the highest correlation to cancer as much as 87% were hypomethylated (34). A later and larger study, including 1,475 samples, suggests that cancer specific hypomethylation in women´s cancers occurs preferentially at a specific set of CpGs which are normally heavily methylated in embryonic stem cells (called MESC) (40). Hypomethylation of MESCs is associated with tissue invasion and metastasis.

Two studies from 2016 show examples of how hypomethylation of promoters is involved in OC. One study shows that the gene for the proto-oncogenic, cell-cycle regulator Cdk2 is both hypomethylated and overexpressed in OC tumors (66). The other shows that the gene for the oncogenic cancer-testis antigen PRAME very often has reduced promoter methylation, which correlates with increased expression (67).

7. Potential of DNA methylation in OC diagnostics

For a biomarker to be useful in identifying cancer patients in early stage of disease, it should be capable of differentiation between patients with benign tumors and early stage cancers, preferable without invasive, surgical procedures. This could be done by detection in body fluids, e.g., blood. The earlier the marker can be detected, optimally a long time before clinical presentation, the better the chance of improving patient survival. Changes in methylation have in several cases been shown to take place very early, before actual tumor growth or cancerous cells can be found in a clinical setting. One example of this is hypermethylation of PolyComb Group Target genes, which was detected up to 3 years before cancer cells appeared in a screening trial for cervix cancer (40).

To increase sensitivity and specificity, a panel of CpG-sites covering several genes can potentially be used. This approach was tried in a paper from 2009, which achieved a sensitivity of 85% and a specificity of 61% differentiating between OC patients and healthy controls. They measured the methylation status of 5 gene promoters, including the *BRCA1* promoter, on circulating DNA in the plasma (68). This result was followed-up by a study which gained a sensitivity of 90% and a specificity of 86,7% using only 3 genes, including *RASSF1A* (69).

RASSF1A was also included in another promising gene-panel, which together with *OPCML* included a total of 7 genes. This panel was also applied on cell-free serum DNA, and tested on 202 OC patients, 53 patients with benign tumors and 62 healthy controls. The panel achieved a sensitivity of 85,3% and a specificity of 90,5% (70).

The tumor suppressor genes *RASSF1A* and *OPCML* are seen in several new papers on OC diagnostics. In 2015, Xing *et al* showed that they could achieve a specificity of impressing 100% with a sensitivity of 85,7% looking at methylation of *RASSF1A, OPCML* and *HOXA9* (71). None of the 3 genes alone gave as impressive a result. However, the setup only included tissue from 35 patients and 11 controls. A larger study, including both serum and tissue for 114 OC patients, used methylation of *OPCML* together with tumor suppressor genes *RUNX3* and *TFPI2* (72). They demonstrated that their methylation analysis could identify early OC with a higher sensitivity and specificity than CA125 serum levels. The same was suggested for *OPCML* alone in a study from 2017, including free circulating DNA from 71 OC patients, 80 healthy controls and 43 patients with benign ovarian tumors (73).

Data from 2014 suggests an association between OC and methylation of leukocyte DNA (74). The authors argue that since tumorigenesis is not an isolated phenomenon but result from alterations in processes also affecting neighboring tissue and the immune system, leukocyte cells may be informative regarding cancer. Some groups have explored this and applied screening of patient leukocyte DNA, using Illumina Infinium arrays which covers 450.000 CpG sites. A study from 2014 screened blood DNA from 242 OC cases and 181 age-matched healthy controls. By adjusting for leukocyte distribution, overcoming confounding caused by immune-responses against the cancer, they identified a CpG island in the promoter of *BNC2* as being top-associated with OC (75). Another group used a similar screening method, combined with the Illumina Custom VeraCode methylation assay in a new study from 2017 (76). Their analysis pointed at 6 CpG sites associated with OC, primarily located in immune system process genes.

8. Potential of DNA methylation in OC prognostics

A follow-up study on the work done on hypermethylation of stem cell PolyComb Group Target genes in OC (40) identified *HOXA11* methylation as a potential prognostic marker in OC patients (77). Amongst 71 loci, investigated in 22 OCs and 18 controls, *HOXA10* and *HOXA11* were most accurately discriminating between OC and non-neoplastic tissue, and *HOXA11* methylation was associated with a bad prognosis for the patient. *HOXA9* methylation is also highly related to OC and found in all stages of the disease (78,79). However, *HOXA9* methylation tend to be lower in high grade OC compared to low grade and may therefore be used as an indicator of disease grade (57).

Promotor hypermethylation with reduced gene expression is prognostic of a shorter progression free survival (PFS) in several newer studies. *RUNX3* and *IQGAP2* are a couple of the genes that can be mentioned (80,81). Reduced expression of *miR-34a*, correlating with promoter hypermethylation, predicts reduced overall survival (OS) and PFS in OC patients (58). However, *miR-34a* expression also correlates negatively with Paclitaxel sensitivity in the NCI60 cell line panel (82). This may reflect a tendency observed for the upstream protein p53. Even though p53 is a well described cancer suppressor, overexpression of the protein has been correlated to chemotherapy resistance (83,84).

Hypomethylation might also be of prognostic value. MESC hypomethylation progresses as the cancer develops from a primary cancer and start metastasizing (40). It may therefore hold information of the OC stage and the chance of survival. *PRAME* hypomethylation and expression is related to increased survival in high grade serous carcinoma (67). Several new studies have suggested that hypomethytlation and increased expression of potential proto-oncogenes predict more aggressive and metastatic OC with a potentially lower survival. This has been seen for *GABRP, SLC6A12, MGAT3, CT45, CA9, MUC13* and *AGR2* (85-91).

Metastasis and methylation might be tightly connected as highlighted by a study from 2014. A key player in metastasis is the transforming growth factor TGF-β. TGF-β stimulation of OC cell lines extensively change DNA methylation, especially in the promoters of genes involved in the epithelial-mesenchymal transition and cancer progression (92).

9. DNA methylation profiles as markers for OC screening

As OC patients experience no or very unspecific symptoms, a screening program would be a major, lifesaving advance. A recent study investigated possible markers for a screening setting, but no optimal marker was identified. However, screening for OC is theoretically possible (14,93).

The ideal marker for screening needs to be accessible without surgical procedures and occur in early states of a disease. Thus, the prerequisite for using aberrant DNA methylation in screening seems to be there. Recent data suggest that the most OC tumors originate from precursor lesions in the fimbrial part of the fallopian tube rather than from ovarian tissue (94-101). Fluid and oocytes from tubes and ovaries pass through cervix and the tubes and cervix are parts of the same organ. It can therefore be speculated that methylated DNA, indicative of changes in pre-malignant or malignant tissue of tubal origin, can be detected in the cervical canal. If it was possible to identify OC using a standard cytological test from the cervix, a screening program for OC could be included in the existing screening program for cervical cancer. Methylation changes in fimbrial cells, which can identify ovarian carcinomas, have already been described (102). Likewise, it has also been possible to identify an association between methylation of PolyComb group target genes *HOXA9* and *HOXA11* in normal endometrium and the presence of OC (79). Even more promising, data show that methylation patterns specific for cervix and endometrial cancers can be identified on DNA from patient vaginal fluid (103).

10. DNA methylation and the prediction of chemo-resistance

The standard first line of OC treatment is platinum-based chemotherapy, and approximately 80% of the patients respond to first line treatment. However, most patients develop resistance to treatment over time. Predictive biomarkers, that can identify patients applicable for specific treatments, can be used to direct the treatment and avoid ineffective treatment-strategies.

Recently it was shown that the platinum-based therapy itself can induce aberrant methylation at specific methylation sites in OC patients, and that this methylation is associated with patient survival (39). As the methylation can be detected in a blood test at the time of relapse, it could possibly function as both a predictive and prognostic marker and give information about response to platinum-based medication and survival. Supporting the importance of methylation in platinum resistance, it has been shown that the methylation inhibitor SGI-110 can re-sensitize chemo-resistant OC stem-like cells, by resetting the cells to a more differentiated phenotype (104).

Several studies from recent years have supported an association between hypermethylation and chemo-resistance. A study from 2017 describe how DNA methylation is involved in reducing expression of the T-cell activating ligands 4-1BBL/CD157 and OX-40L/CD252 in chemo-resistant OC cells (105). Apparently, this mechanism of immune suppression helps the cancer evade immune responses stimulated by chemotherapy. An integrative analysis has discovered a network of interacting genes, for whom methylation associates with resistance towards chemotherapy (106). *PTEN* seems to be a key regulator in the network. Other examples worth mentioning are *RGS2*, which is hypermethylated in chemo-resistant cells, and *FAM83A* and *MYO18B* methylation which has been seen in non-responders (107,108).

A study from 2017 also suggests that hypomethylation can be induced by cisplatin treatment in resistant OC patients, however loss of methylation was primarily observed in intergenic regions (109). Other recent studies have suggested a correlation between hypomethylation of developmental genes and platinum resistance in OC patients. Amongst these, *MSX1* and *TMEM88* can be mentioned (110,111). Especially regulation of the epithelial-to-mesenchymal transition by changes in methylation seems to be an important part of platinum resistance. The non-coding RNA *HOTAIR* induces the epithelial-to-mesenchymal transition together with Platinum resistance and is regulated by methylation (112). Another example of a similar dynamic was seen for an OC-cell model, where *TET* expression was increased in platinum resistant cells. TET stimulates the epithelial-to-mesenchymal transition by blocking promotor methylation of Vimentin, a mediator of chemo-resistance which has been described as a potential therapeutic marker (113).

11. Discussion

The advantages of using DNA methylation as a cancer marker are evident. However, DNA methylations as cancer biomarkers are still a relatively new area. Only few methylation markers are used in clinical decision making. One example is methylation of DNA repair genes which is used to differentiate between hereditary and non-hereditary colorectal cancer.

A most exciting potential for DNA methylation markers are as early markers which can be detected pro-surgery. However, there is a challenge to interpretations of markers from body-fluids compared to tumor-samples. It can be difficult to differentiate between methylation changes directly related to the OC and methylation changes related to other tumors or to the general immunological response to the cancer. Therefore, studies investigating corresponding biological material are needed.

It is clear at this point that DNA methylation is involved in the progression of OC. However, despite an increasing number of reports on methylation changes in OC patients, most often the reported changes are not validated by independent studies. Several interesting methylation changes have been observed in several OC studies. Amongst them are those in the promotors of the tumor suppressor genes *BRCA1, RASSF1A, OPCML* and *HOXA9* and the proto-oncogene *PRAME*. Nonetheless, methylation patterns which can be found in a high proportion of OCs are needed. At this stage, no DNA methylation site identified has the specificity and sensitivity to identify OC alone. One reason for this is that most of the previous studies have focused on only one or a few candidate genes. To succeed in the discovery of satisfying OC markers, more genome wide approaches and screening methods must be applied. Also, future discovery studies should cover not only benign and malign samples, but also different subgroups of stages of carcinogenesis as well as both chemo responsive and resistant patients. It is of great importance that we get a better understanding of the initiation and progression of OC, and the methylation landscape guiding, or resulting from, these processes. As the main challenge is that symptoms of OC are unspecific and diffuse, it is of high priority to understand early methylation changes and how to detect them without invasive measures. We need to determine the methylation signature of OC in detail before we can identify markers which can improve both diagnostics and treatment of OC patients.

The latest OC research has indicated that screening and early diagnosis is attainable. Yet, at this point, OC is still the leading cause of death from gynecological malignancies. A first step could be to improve treatment and survival by precision medicine guided by biomarkers, moving towards making OC a chronic disease with high life quality. However, the long-term goal must be to improve early diagnosis to an extend where the cancer is discovered and treated before it spreads, with the result of largely improved survival. Genome-wide studies resulting in a better understanding of the disease etiology will hopefully bring us to the point where OC is either a chronic disease a disease that can be cured.

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JLH made substantial contributions to design and acquisition of data, was involved in drafting the manuscript, gave final approval of the version to be published. EVH and CKH also made substantial contributions to conception and design and

revised the manuscript critically for important intellectual content. All authors approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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