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Breakthrough Medical Research

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Mihalis Panagiotidis
Guest Editor, Cancer Letters
Special Issue, "Cancer Epigenetics"

Dear Dr. Panagiotidis,

We'd like to thank you for your invitation to contribute a Mini-Review for the Special Issue of Cancer Letters, "Cancer Epigenetics". We are submitting our manuscript entitled "Epigenetic Biomarkers in Epithelial Ovarian Cancer" for your consideration for publication in this exciting issue.

Ovarian cancer is the most lethal gynecological malignancy and the 5th leading cause of cancer death in women. Our manuscript discusses how the emerging field of epigenetics provides us with the opportunity to identify ovarian cancer-specific DNA methylation changes that can be used in the clinic to improve early-stage diagnosis and better predict response in treated patients. We review some key candidate genes and pathways with potential clinical utility as biomarkers for diagnosis and/or prognosis. We also discuss the necessity for identification of highly specific, sensitive and robust panels of markers, standardization of analysis techniques and importance of blood-based assays in order to improve detection, treatment and thus patient outcome.

Please note that we refer to 2 papers from our group (Montavon *et al.* and Gloss *et al.*) which are currently under review. We anticipate that these papers will be in press by the time this Mini-Review is meant to be published, and will update these references in the bibliography at that time.

We appreciate the invitation and look forward to hearing from you.

Regards,

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Epigenetic Biomarkers in Epithelial Ovarian Cancer

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Abstract

Ovarian cancer is the most lethal gynecological malignancy and the 5th leading cause of cancer death in women. Women with ovarian cancer are typically diagnosed at late stage, when the cancer has spread into the peritoneal cavity and complete surgical removal is difficult. The 5-year survival time for patients diagnosed at this stage is 30%, in contrast to a 5-year survival of 90% for patients diagnosed at early stage. Cancer screening and early detection have the potential to greatly decrease the mortality and morbidity from cancer. The emerging field of epigenetics offers a valuable opportunity to identify cancer-specific DNA methylation changes that can be used in the clinic to improve early-stage diagnosis and better predict response in treated patients.

To date, numerous DNA methylation aberrations have been identified in epithelial ovarian cancer; here we review some candidate genes and pathways with potential clinical utility as biomarkers for diagnosis and/or prognosis. It has become clear that even with the great promise of DNA methylation biomarkers in epithelial ovarian cancer, the identification of highly specific, sensitive and robust panels of markers and the standardization of analysis techniques are still required in order to improve detection, treatment and thus patient outcome.

Introduction

Ovarian cancer is the 5th leading cause of cancer death in women, with 21,990 new cases and 15,460 deaths annually in the US [1]. Epithelial ovarian cancer (EOC) comprises 90% of all ovarian cancers and risk factors include age and a family history of ovarian and/or breast cancer [2]. Familial cancers account for 5-10% of EOC diagnoses and are frequently associated with mutations in the breast cancer early onset genes *BRCA1* and *BRCA2* [3]. The early stages of EOC are asymptomatic and there is currently no screening program in place. Patients are therefore typically diagnosed late, when the cancer has disseminated within the peritoneal cavity and complete surgical removal isn't possible. The 5-year survival time for patients diagnosed at this stage is 30% in contrast to 90% for patients diagnosed early while the tumor is still confined to the ovary [1]. Identification of biomarkers for detection of EOC is imperative for improving the survival of women diagnosed with EOC.

Elucidating the molecular pathogenesis underlying the development of EOC is the most promising approach for identifying biomarkers. EOC tumors are classified as benign, of low malignant potential (LMP) or malignant based on grade and extent of tissue invasion [4]. EOC malignant tumors are further classified based on their histology and molecular characteristics. The most common malignant EOC histotypes are serous, endometrioid, clear cell and mucinous. However, recent studies have shifted the paradigm of conventional classification and sites of origins of malignant EOC tumors. For example, pathological studies have demonstrated that mucinous EOC tumors are frequently metastases from gastrointestinal tumors [5]. Furthermore, microarray studies have shown that gene expression profiles of clear cell EOC tumors are more closely correlated to renal clear cell tumors rather than serous EOC tumors [6] and that low-grade endometrioid EOC tumors likely arise from endometriotic cysts [7]. These findings stress the importance of identifying biomarkers that accurately diagnose the correct histological subtype of ovarian cancer.

Serous EOC tumors account for a majority of EOC tumors and are generally classified as either Type I or Type II tumors [8; 9] (Figure 1). Type I serous EOC tumors are mainly low-grade and are thought to arise from an LMP precursor, have low levels of chromosomal instability and have a high frequency of mutations in *KRAS*, *BRAF* and *ERBB2* genes. Type II serous EOC tumors are generally high-grade, thus often referred to as HGSOC (high-grade serous ovarian cancer) [4]. HGSOCs arise rapidly, have high levels of chromosomal instability and have a high frequency of *p53* mutations [8]. Recent investigations of occult fallopian tube cancers in women with *BRCA1/2* mutations undergoing bilateral salpingo-oophorectomy suggest that Type II tumors arise from tubal intraepithelial carcinomas in the distal/fimbriated end of the fallopian tube [10; 11; 12] (Figure 1). In addition, studies demonstrated that tubal intraepithelial carcinomas contained secretory cells displaying regions of strong *p53* immunoreactivity (termed "*p53* signatures") and proliferative markers similar to those found in HGSOC tumors but not in normal ovarian surface epithelium [12; 13; 14; 15]. Furthermore, microarray studies have determined that gene expression signatures of fallopian tube epithelium from women with *BRCA1/2* mutations are highly correlated with HGSOC gene expression [16; 17]. As Type II serous EOC tumors represent the majority of EOC cases, are relatively molecularly homogenous and comprise a significant proportion of mortality due to EOC, they represent a promising target for biomarker development. The Cancer Genome Atlas (TCGA) recently published DNA methylation, gene expression, sequencing and copy number profiles of ~500 HGSOCs, providing a catalog of molecular aberrations commonly found in EOC and demonstrating the importance of further understanding the molecular characteristics of this disease [18].

Screening and Early Detection

The most well-studied and long-utilized biochemical marker of EOC is CA-125, a large glycoprotein which is over-expressed by ovarian cancer cells and secreted into the bloodstream of patients [19; 20]. CA-125 levels correlate well with the course of disease during chemotherapy and

can pre-date clinical recurrence by up to six months, making it suitable as a marker for tumor recurrence [20; 21; 22].

Due to the low incidence of EOC, and because indications of EOC lead to surgery and its associated morbidities, estimates for a suitable diagnostic screening test call for a specificity of >99.6% and a sensitivity of >75% [23]. While CA-125 is over-expressed in 80% of EOC [24], it is also elevated in many benign states including endometriosis and uterine fibroids [25; 26]. Thus CA-125 alone is unsuitable as a diagnostic marker for EOC due to its unacceptably low specificity [27]. Combining CA-125 with transvaginal ultrasound has somewhat improved the specificity of detection, although these trials have yet to reach the requisite accuracy of 99.6% specificity [28; 29]. As a result, additional biomarkers for early detection of EOC are being sought [30; 31] and it is likely that a panel of molecular biomarkers will be required to increase the sensitivity and specificity to the required level [32].

Aberrant DNA methylation occurs commonly in tumors and is considered to be one of the earliest molecular changes in carcinogenesis [33; 34; 35; 36]. Thus, cancer epigenetic studies hold great promise in revealing potent biomarkers for improved cancer detection [37; 38]. Candidate gene and whole-genome studies have identified methylation signatures that may serve as biomarkers for EOC characterization including classification [39], progression [40] and response to therapy [41]. Furthermore, studies have identified tumor-specific gene methylation in blood DNA of patients with EOC [42; 43; 44; 45], indicating that methylation patterns in plasma DNA have potential to serve as non-invasive biomarkers of EOC.

In this paper we review some of the most extensively studied epigenetic biomarkers with potential clinical utility for detection/diagnosis, prognosis and response to treatment in women with EOC.

Well-characterized tumor suppressor genes

BRCA1

BRCA1 promoter methylation has been extensively studied in ovarian cancer. Studies have demonstrated that *BRCA1* hypermethylation occurs in 10-15% of sporadic cases and is associated with loss of expression [46; 47; 48; 49; 50] and with the serous histotype [50; 51]. In addition, *BRCA1* methylation is significantly correlated with malignancy, with HGSOC tumors exhibiting significantly increased levels of *BRCA1* silencing compared with LMP tumors [52]. Accordingly, *BRCA1* promoter methylation is associated with poor patient outcome, with patients with hypermethylation of the promoter maintaining a significantly shorter progression-free and overall survival compared with patients harboring mutated or wild-type *BRCA1* [53]. Interestingly, *BRCA1* methylation has been shown to be associated with sensitivity to platinum treatment in a pilot study of 35 EOC cases, presumably due to its role in DNA repair and apoptosis [54]. Taken together, these studies suggest that *BRCA1* promoter methylation may serve as a biomarker for aggressive EOC and response to chemotherapeutic intervention.

MLH1

MLH1 is a member of the mismatch repair family of proteins, which function to recognize DNA damage and signal an apoptotic response (reviewed in [55]). In EOC, the mismatch repair pathway is impaired, in part due to promoter methylation of *MLH1*. This loss of an apoptotic response in EOC results in resistance to platinum-based therapy in patients treated with cisplatin or its analogs, which is reversed upon restoration of *MLH1* expression [56]. Gifford *et al*. examined the mechanisms of acquired resistance by comparing DNA methylation patterns of *MLH1* in plasma DNA from EOC patients prior to and following (at relapse) treatment with platinum-based therapy [44]. Their study found that promoter methylation of *MLH1* during treatment was significantly correlated with a poor outcome. Interestingly, expression of *MLH1* prior to treatment is not able to

predict outcome [57]. These results suggest that *MLH1* methylation may serve as a marker for enrichment of inherently resistant cells and may aid in determining response to platinum treatment.

HOXA9

HOXA9 is hypermethylated across the CpG island promoter in a number of cancer types, including bladder [58], colorectal [59] and neuroblastoma [60] cancers and appears to hold promise as a biomarker in EOC. In a study by Wu *et al*., *HOXA9* was methylated in 51% of EOC tumors, with significantly higher methylation frequencies identified in early-stage tumors [61]. Interestingly, in this study the endometrioid histotype demonstrated the highest degree of methylation of *HOXA9*. Because methylation of *HOXA9* was preferentially identified in early-stage EOC tumors, Widshwendter *et al*. conducted a study to determine whether *HOXA9* methylation could serve as a predictor of EOC [62]. In this study, the authors examined *HOXA9* promoter methylation in normal endometrium from women with EOC and healthy controls and determined that it was predictive of presence of EOC, with increasing *HOXA9* methylation correlating with increasing risk of EOC. In contrast to the earlier study, this association was independent of histotype or stage. To determine whether *HOXA9* methylation could accurately predict the presence of HGSOC specifically, our group recently measured methylation of *HOXA9* in 80 HGSOC tumors and 12 normal ovarian surface epithelium (OSE) samples using methylation-specific headloop suppression PCR (Montavon *et al.*, under review). Our findings demonstrate that *HOXA9* is methylated in 75/79 (97.5%) of HGSOCs and only 1/12 (8%) of OSE. Furthermore, methylation of both *HOXA9* and *EN1* in combination with CA-125 levels was able to discriminate HGSOC from normal OSE with a sensitivity of 100% and a specificity of 91.7%. These findings support *HOXA9* methylation as a potential biomarker for detection of EOC, likely in combination with other genes and existing clinical approaches.

RASSF1A

RASSF1A is a potent tumor suppresser gene that is frequently repressed in association with DNA methylation in a large number of cancers including EOC. The frequency of RASSF1A promoter methylation in cancer establishes a potential to serve as a biomarker of cancer presence. In order to determine whether methylation patterns in free-circulating DNA (fcDNA) in plasma could differentiate between benign and malignant ovarian tumors, Liggett *et al*. performed a genome-wide analysis of methylation in cohorts of healthy controls, women with benign ovarian disease and women with invasive EOC [45]. Their results indicated that promoter methylation of three genes (*RASSF1A*, *CALCA* and *EP300*) effectively distinguished plasma of EOC patients from healthy controls with a sensitivity of 90.0% and a specificity of 86.7%. Furthermore, promoter methylation of *RASSF1A* and *PGR-PROX* effectively distinguished plasma of EOC patients from women with benign ovarian disease with a sensitivity of 80.0% and a specificity of 73.3%. These findings support previous studies identifying frequent *RASSF1A* promoter methylation in EOC and rarely in normal controls [43; 63; 64; 65].

SPARC

SPARC is involved in cell adhesion, motility and ECM interactions, and has been shown to function as a tumor suppressor in ovarian cancer, in part due to its regulation of tumor microenvironment interactions [66]. SPARC is down-regulated in EOC [67] and methylated in multiple cancers, including pancreatic [68], colon [69] and lung [70] cancers. Socha *et al*. recently investigated the mechanism of down-regulation in EOC [71] and demonstrated that the *SPARC* promoter is hypermethylated in EOC tumors, with 68.2% and 22.7% harboring full and hemi-methylation, respectively. In addition, SPARC expression was inversely correlated with tumor grade, such that HGSOC had the highest level of methylation. These results suggest that *SPARC* methylation in EOC has potential to serve as a biomarker, particularly the most aggressive form of the disease.

HIC1

HIC1 is silenced in cancer by methylation and not mutation [72; 73], and has been frequently demonstrated to be methylated in EOC. An early study demonstrated that a locus at region 17p13.3, where *HIC1* resides, is hypermethylated in 33% of EOC [74].) In studies by Teodoridis *et al*. and Strathdee *et al*., *HIC1* was methylated in 17% of EOC, in concordance with *OPCML* and *RASSF1A*, among others [64; 75]. Rathi *et al*. demonstrated *HIC1* methylation in 35% of EOC tumors and <10% of normal ovarian tissues [63]. Tam *et al*. expanded these studies to investigate methylation of *HIC1* in a larger number of EOC tumors, as well as borderline ovarian tumors, benign ovarian tumors and normal ovarian tissues and found that *HIC1* was methylated in >50% of ovarian cancer tissues, >40% of borderline ovarian tumors, >20% of benign ovarian tumors and 10% of normal ovarian tissues [76]. Taken together, these studies suggest that *HIC1* promoter methylation correlates with the presence of invasive EOC.

DAPK

DAPK is a pro-apoptotic kinase which plays a role in metastasis and is methylated in several cancer types including lung, bladder, uterine leiomyoma and colorectal cancers [77]. Collins *et al*. examined *DAPK* promoter methylation in 30 EOC tumors and 26 peripheral blood DNA samples [42]. Their findings demonstrated that *DAPK* was not methylated in normal OSE but was methylated in 67% of EOC tumors and 54% of blood DNA samples. However, DNA methylation did not correlate with patient outcome or aggressiveness of the disease [42]. However, other studies which examined *DAPK* methylation produced conflicting results; two studies detected methylation in <15% of EOC samples [43; 47] and two other studies did not detect *DAPK* methylation in any of their examined EOC samples [64; 77]. These inconsistencies are likely due to differences in sample processing, PCR technique, and the use of tissues *versus* blood samples. While it's possible that epigenetic silencing of *DAPK* could be associated with metastatic ovarian cancer and provides the

cells with the ability to resist apoptosis, further studies are necessary to correctly elucidate the potential of *DAPK* as a biomarker.

Novel candidates

OPCML

OPCML is an immunoglobulin domain−containing GPI-anchored cell adhesion molecule and has been demonstrated to act as a tumor suppressor. Forced expression of OPCML decreases proliferation of EOC cells *in vitro* and tumor growth *in vivo* while increasing cellular aggregation [78]. *OPCML* is frequently inactivated in EOC by allele loss or CpG island methylation [79]. Studies have demonstrated that *OPCML* is not methylated in normal ovarian epithelial cells, but is methylated and inactivated in a high proportion (33-83%) of EOC tumors [64; 78; 80]. These studies also indicate that methylation of *OPCML* may be an early event, as higher methylation frequencies were present in borderline and early-stage tumors compared with late-stage tumors, establishing its potential to serve as a specific biomarker of surgically curable EOC.

CCBE1

CCBE1 is down-regulated in breast and ovarian cancer and maps to 18q21.32, a region frequently exhibiting loss of heterozygosity in ovarian cancer [81; 82]. Our group recently conducted a study to determine the role of *CCBE1* in ovarian cancer [83]. This study confirmed loss of *CCBE1* expression in EOC tumors of all histotypes and demonstrated higher expression in low-grade versus high-grade EOC tumors. In addition, we showed that 41% of EOC tumors exhibited promoter methylation of *CCBE1* compared with 20% methylation in normal OSE samples. EOC patients without *CCBE1* expression had decreased relapse-free survival times versus those with *CCBE1* expression, although no such difference was seen when comparing *CCBE1* methylation. Nonetheless, these findings warrant additional studies in larger cohorts, as they suggest that *CCBE1* may serve as a detection and prognostic biomarker for EOC.

Pathways

Wnt Pathway

Activation of the Wnt pathway is common in multiple cancer types, including ovarian cancer. Several regulators of the Wnt pathway have been demonstrated to be hypermethylated in EOC. In one study, *SFRP5*, a Wnt antagonist, was found to be hypermethylated and down-regulated in 44.4% of examined EOC and only 1.3% of benign cases [84]. Additional studies demonstrated that expression of SFRP5 sensitizes ovarian cancer cells to cisplatin and taxol *in vitro* and that patients with unmethylated *SFRP5* had improved clinical response *versus* those harboring *SFRP5* promoter methylation [85].

Dai *et al*. recently expanded these studies to examine promoter methylation at 302 loci in a panel of 137 Wnt pathway genes in a screening cohort of 111 and a validation cohort of 48 serous and endometrioid EOC cases [86]. Following screening, validation and adjustment for clinical parameters, the authors demonstrated that methylation at 7 loci (*FZD4, DVL1, NFATC3, ROCK1, LRP5*, *AXIN1*, and *NKD1*) was associated with poor progression-free survival. This panel was then examined for association with patient response to platinum chemotherapy. Hypermethylation of *DVL1* and *NFATC3* significantly correlated with poor response, with patients with progressive or stable disease harboring increased methylation *versus* patients with partial or complete response [86]. The authors then expanded these studies to include a cohort of HGSOC cases from the TCGA [18]. In this independent cohort, methylation of *DVL1, ROCK1, LRP5,* and *AXIN1* was associated with progression-free survival. These findings were validated using gene expression data and results showed that decreased expression of *FZD4*, *DVL1*, and *ROCK1* correlated with a higher risk of disease progression. Taken together, these studies demonstrate that epigenetic regulation of the Wnt pathway may serve as a biomarker for prognosis and/or treatment response in EOC.

TGF-beta Pathway

Promoter methylation of genes involved in the TGF-beta pathway has been reported in numerous cancers including gastric, prostate and melanoma. In addition, expression profiling of 5-aza-dC-treated cell lines highlights TGF-beta signaling as a core pathway altered by DNA methylation in EOC [87; 88]. In a study by Kang *et al.*, *TGFBI* methylation was originally observed in 2 ovarian cancer cell lines and later demonstrated in 23/38 (60.5%) of ovarian cancer cases, 5/18 (27.8%) borderline ovarian tumors and 0/38 normal ovarian tissues [87]. In this cohort, *TGFBI* methylation was not associated with any clinical outcomes. Matsumura *et al*. examined candidate genes in 39 ovarian cancer cell lines and identified a significant number of TGF-beta pathway genes as regulated by methylation [88]. Furthermore, gene expression analysis of the candidate methylated genes in 17 EOC tissues demonstrated suppression of TGF-beta pathway activity. Taken together, these studies provide a potential mechanism for the suppressed function of the inhibitory effect of TGF-beta in ovarian cancer, and suggest that methylation of the TGF-beta pathway may serve as a biomarker for the presence of EOC.

FBXO32 is a TGF-beta/SMAD4 target gene and a regulator of apoptosis. In a recent study by Chou *et al*., *FBXO32* was unmethylated and expressed in normal OSE but methylated and expressed at lower levels in 96 EOC cases [89]. Methylation was significantly associated with tumor stage, with late stage tumors harboring significantly greater methylation than early stage. Furthermore, patients with greater DNA methylation demonstrated poorer progression-free survival. *In vitro* studies demonstrated that expression of *FBXO32* restored sensitivity to cisplatin, suggesting that the difference in progression-free survival in patients may be due to varying responses to treatment. These results suggest that methylation of *FBXO32* may serve as a prognostic marker for EOC.

Future Considerations

As DNA methylation occurs early and tends to be stable throughout carcinogenesis, the application of DNA methylation as a biomarker of EOC holds great promise in overcoming the high false positive rate of detection for the current standard biomarker CA-125. Numerous studies of differential methylation have identified potential biomarkers of diagnosis, prognosis and response in EOC. However, there is evidence that some of these methylated markers may not be unique to EOC or to cancer at all, with *BRCA1* methylation also reported in healthy controls [90]. This provides an additional challenge regarding the necessity of long-term clinical follow-up of healthy controls in screening trials.

The heterogeneity of EOC, as well as differences in sample processing, assay design and approach, could explain the variation in DNA methylation frequencies reported for individual genes. In addition, further studies using larger cohorts are necessary to determine the efficacy of methylation to serve as a biomarker for early-stage EOC. It is clear from these studies that methylation in a panel of biomarkers, rather than individual gene methylation, will be necessary to achieve a suitable diagnostic assay with >99.6% specificity [23]. Indeed, studies that have examined combinations of gene methylation as EOC biomarkers have achieved the greatest degree of sensitivity and specificity ([45; 86], Montavon *et al*. and Gloss *et al.*, under review). For example, in a study by Montavon *et al*. *EN1*, previously identified as being located in a long range epigenetically silenced genomic region frequently hypermethylated in colon cancer [91; 92] and prostate cancer [93], was found to be methylated in 64/80 (80%) of HGSOCs and only 1/12 (8%) OSE. In addition, in combination with *HOXA9* methylation and CA-125, *EN1* methylation could discriminate HGSOC from normal OSE with a sensitivity of 100% and a specificity of 91.7%. These studies warrant further examination of combining epigenetic biomarkers with CA-125 to improve sensitivity and specificity of detection of EOC.

With the recent publication of DNA methylation profiles of ~500 HGSOC tumors [18], it is possible to examine combinations of potential methylated biomarkers in various EOC cohorts. Indeed, more recent studies have focused on genome-wide identification of methylated biomarkers of cancer, including EOC [94]. Methyl-DNA immunoprecipitation (MeDIP) applied to genome arrays (MeDIP-chip) was used to directly profile methylation in EOC compared to normal ovaries and identified 367 CpG islands specifically methylated in EOC [95]. Additional genome-wide studies in EOC have identified methylation signatures associated with progression-free survival [40; 96], histological subtype [97], disease stage [98] and response to platinum-based chemotherapy [99]. While these studies benefit from increased sample number and array-based platforms, the potential methylated biomarkers identified by these genome-wide screens require further independent validation in large, well-annotated clinical cohorts.

Even with the advancement of approaches for genome-wide identification of methylated biomarkers of EOC, the need still remains for the development of a non-invasive diagnostic assay. Fortunately, tumor-specific gene methylation has been detected in blood of EOC patients [42; 43; 44; 45; 65], suggesting its utility as a non-invasive source of DNA for disease screening (Figure 1). Teshendorff *et al*. performed genome-wide DNA methylation profiling of 27,000 CpG islands in blood samples from a large cohort of EOC patients and controls to determine whether profiles can serve as detection biomarkers and predict the presence of cancer [100]. The authors demonstrated that the presence of cancer elicits common methylation changes in blood, and that EOC could be predicted with a relatively high accuracy. While it is clear that methylated biomarkers in plasma DNA have significant clinical potential for early detection, they require further validation of diagnostic efficacy in a large prospective study similar to studies for protein biomarkers [101].

Epigenetics aberrations clearly underlie the initiation and development of cancer and other diseases. With recent advances in epigenetic research including high-throughput technologies for methylation

detection and analysis, an International Human Epigenome Consortium (IHEC) has been established with the aim of identifying genome-wide DNA methylation and histone modification patterns in all genes in all major normal tissues [102; 103]. Once completed, the comprehensive data generated from the IHEC, in combination with data from the International Cancer Genome Consortium (ICGC) [104] and TCGA [18], will support future studies which are necessary to determine the methylation events which are critical for EOC tumorigenesis and which can be applied towards a highly sensitive and specific assay for early detection of EOC.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figure 1

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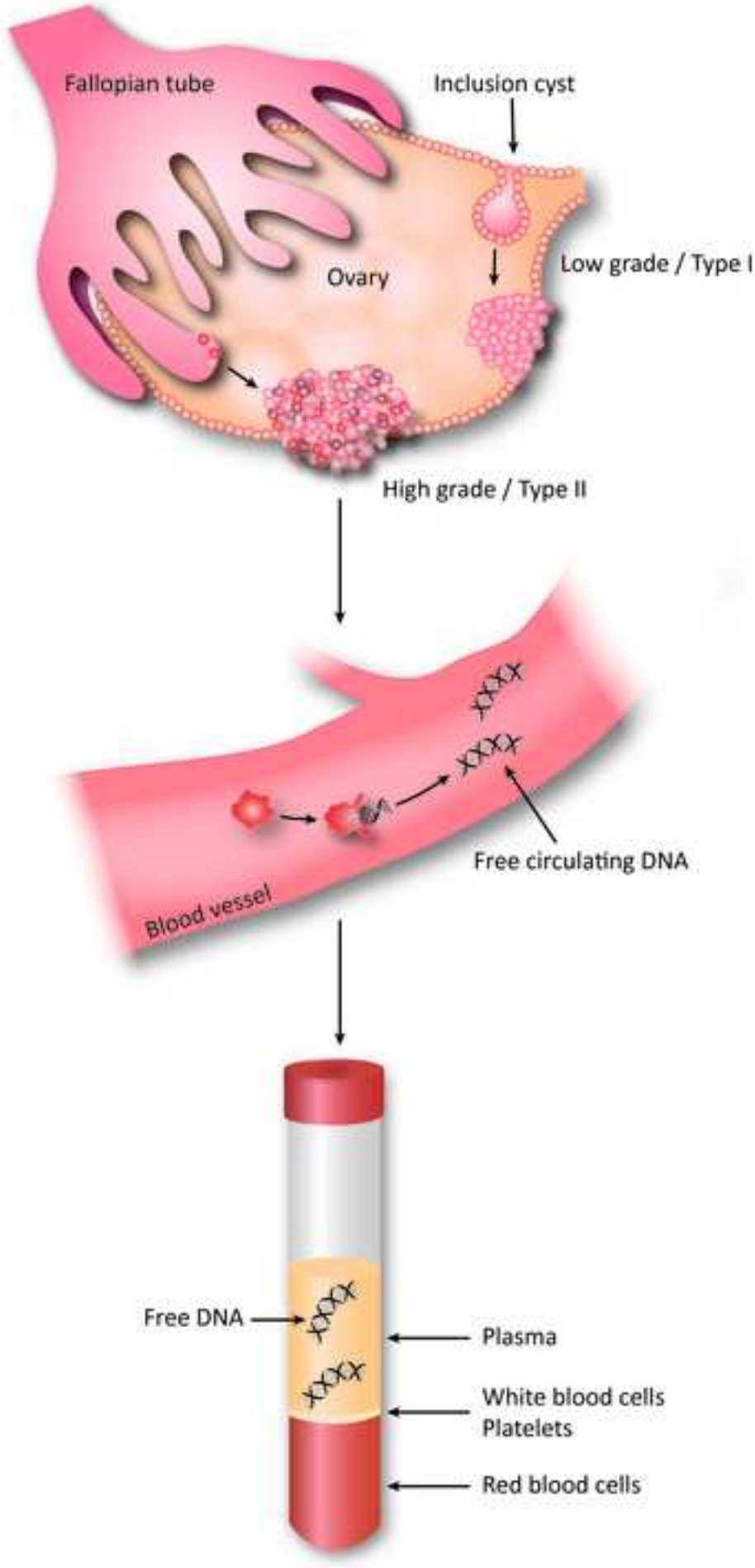

Conflicts of Interest

The authors declare no conflict of interest.

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Epigenetic Biomarkers in Epithelial Ovarian Cancer

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Abstract

Ovarian cancer is the most lethal gynecological malignancy and the 5th leading cause of cancer death in women. Women with ovarian cancer are typically diagnosed at late stage, when the cancer has spread into the peritoneal cavity and complete surgical removal is difficult. The 5-year survival time for patients diagnosed at this stage is 30%, in contrast to a 5-year survival of 90% for patients diagnosed at early stage. Cancer screening and early detection have the potential to greatly decrease the mortality and morbidity from cancer. The emerging field of epigenetics offers a valuable opportunity to identify cancer-specific DNA methylation changes that can be used in the clinic to improve early-stage diagnosis and better predict response in treated patients.

To date, numerous DNA methylation aberrations have been identified in epithelial ovarian cancer; here we review some candidate genes and pathways with potential clinical utility as biomarkers for diagnosis and/or prognosis. It has become clear that even with the great promise of DNA methylation biomarkers in epithelial ovarian cancer, the identification of highly specific, sensitive and robust panels of markers and the standardization of analysis techniques are still required in order to improve detection, treatment and thus patient outcome.

Introduction

Ovarian cancer is the 5th leading cause of cancer death in women, with 21,990 new cases and 15,460 deaths annually in the US [1]. Epithelial ovarian cancer (EOC) comprises 90% of all ovarian cancers and risk factors include age and a family history of ovarian and/or breast cancer [2]. Familial cancers account for 5-10% of EOC diagnoses and are frequently associated with mutations in the breast cancer early onset genes *BRCA1* and *BRCA2* [3]. The early stages of EOC are asymptomatic and there is currently no screening program in place. Patients are therefore typically diagnosed late, when the cancer has disseminated within the peritoneal cavity and complete surgical removal isn't possible. The 5-year survival time for patients diagnosed at this stage is 30% in contrast to 90% for patients diagnosed early while the tumor is still confined to the ovary [1]. Identification of biomarkers for detection of EOC is imperative for improving the survival of women diagnosed with EOC.

Elucidating the molecular pathogenesis underlying the development of EOC is the most promising approach for identifying biomarkers. EOC tumors are classified as benign, of low malignant potential (LMP) or malignant based on grade and extent of tissue invasion [4]. EOC malignant tumors are further classified based on their histology and molecular characteristics. The most common malignant EOC histotypes are serous, endometrioid, clear cell and mucinous. However, recent studies have shifted the paradigm of conventional classification and sites of origins of malignant EOC tumors. For example, pathological studies have demonstrated that mucinous EOC tumors are frequently metastases from gastrointestinal tumors [5]. Furthermore, microarray studies have shown that gene expression profiles of clear cell EOC tumors are more closely correlated to renal clear cell tumors rather than serous EOC tumors [6] and that low-grade endometrioid EOC tumors likely arise from endometriotic cysts [7]. These findings stress the importance of identifying biomarkers that accurately diagnose the correct histological subtype of ovarian cancer.

Serous EOC tumors account for a majority of EOC tumors and are generally classified as either Type I or Type II tumors [8; 9] (Figure 1). Type I serous EOC tumors are mainly low-grade and are thought to arise from an LMP precursor, have low levels of chromosomal instability and have a high frequency of mutations in *KRAS*, *BRAF* and *ERBB2* genes. Type II serous EOC tumors are generally high-grade, thus often referred to as HGSOC (high-grade serous ovarian cancer) [4]. HGSOCs arise rapidly, have high levels of chromosomal instability and have a high frequency of *p53* mutations [8]. Recent investigations of occult fallopian tube cancers in women with *BRCA1/2* mutations undergoing bilateral salpingo-oophorectomy suggest that Type II tumors arise from tubal intraepithelial carcinomas in the distal/fimbriated end of the fallopian tube [10; 11; 12] (Figure 1). In addition, studies demonstrated that tubal intraepithelial carcinomas contained secretory cells displaying regions of strong *p53* immunoreactivity (termed "*p53* signatures") and proliferative markers similar to those found in HGSOC tumors but not in normal ovarian surface epithelium [12; 13; 14; 15]. Furthermore, microarray studies have determined that gene expression signatures of fallopian tube epithelium from women with *BRCA1/2* mutations are highly correlated with HGSOC gene expression [16; 17]. As Type II serous EOC tumors represent the majority of EOC cases, are relatively molecularly homogenous and comprise a significant proportion of mortality due to EOC, they represent a promising target for biomarker development. The Cancer Genome Atlas (TCGA) recently published DNA methylation, gene expression, sequencing and copy number profiles of ~500 HGSOCs, providing a catalog of molecular aberrations commonly found in EOC and demonstrating the importance of further understanding the molecular characteristics of this disease [18].

Screening and Early Detection

The most well-studied and long-utilized biochemical marker of EOC is CA-125, a large glycoprotein which is over-expressed by ovarian cancer cells and secreted into the bloodstream of patients [19; 20]. CA-125 levels correlate well with the course of disease during chemotherapy and

can pre-date clinical recurrence by up to six months, making it suitable as a marker for tumor recurrence [20; 21; 22].

Due to the low incidence of EOC, and because indications of EOC lead to surgery and its associated morbidities, estimates for a suitable diagnostic screening test call for a specificity of >99.6% and a sensitivity of >75% [23]. While CA-125 is over-expressed in 80% of EOC [24], it is also elevated in many benign states including endometriosis and uterine fibroids [25; 26]. Thus CA-125 alone is unsuitable as a diagnostic marker for EOC due to its unacceptably low specificity [27]. Combining CA-125 with transvaginal ultrasound has somewhat improved the specificity of detection, although these trials have yet to reach the requisite accuracy of 99.6% specificity [28; 29]. As a result, additional biomarkers for early detection of EOC are being sought [30; 31] and it is likely that a panel of molecular biomarkers will be required to increase the sensitivity and specificity to the required level [32].

Aberrant DNA methylation occurs commonly in tumors and is considered to be one of the earliest molecular changes in carcinogenesis [33; 34; 35; 36]. Thus, cancer epigenetic studies hold great promise in revealing potent biomarkers for improved cancer detection [37; 38]. Candidate gene and whole-genome studies have identified methylation signatures that may serve as biomarkers for EOC characterization including classification [39], progression [40] and response to therapy [41]. Furthermore, studies have identified tumor-specific gene methylation in blood DNA of patients with EOC [42; 43; 44; 45], indicating that methylation patterns in plasma DNA have potential to serve as non-invasive biomarkers of EOC.

In this paper we review some of the most extensively studied epigenetic biomarkers with potential clinical utility for detection/diagnosis, prognosis and response to treatment in women with EOC.

Well-characterized tumor suppressor genes

BRCA1

BRCA1 promoter methylation has been extensively studied in ovarian cancer. Studies have demonstrated that *BRCA1* hypermethylation occurs in 10-15% of sporadic cases and is associated with loss of expression [46; 47; 48; 49; 50] and with the serous histotype [50; 51]. In addition, *BRCA1* methylation is significantly correlated with malignancy, with HGSOC tumors exhibiting significantly increased levels of *BRCA1* silencing compared with LMP tumors [52]. Accordingly, *BRCA1* promoter methylation is associated with poor patient outcome, with patients with hypermethylation of the promoter maintaining a significantly shorter progression-free and overall survival compared with patients harboring mutated or wild-type *BRCA1* [53]. Interestingly, *BRCA1* methylation has been shown to be associated with sensitivity to platinum treatment in a pilot study of 35 EOC cases, presumably due to its role in DNA repair and apoptosis [54]. Taken together, these studies suggest that *BRCA1* promoter methylation may serve as a biomarker for aggressive EOC and response to chemotherapeutic intervention.

MLH1

MLH1 is a member of the mismatch repair family of proteins, which function to recognize DNA damage and signal an apoptotic response (reviewed in [55]). In EOC, the mismatch repair pathway is impaired, in part due to promoter methylation of *MLH1*. This loss of an apoptotic response in EOC results in resistance to platinum-based therapy in patients treated with cisplatin or its analogs, which is reversed upon restoration of *MLH1* expression [56]. Gifford *et al*. examined the mechanisms of acquired resistance by comparing DNA methylation patterns of *MLH1* in plasma DNA from EOC patients prior to and following (at relapse) treatment with platinum-based therapy [44]. Their study found that promoter methylation of *MLH1* during treatment was significantly correlated with a poor outcome. Interestingly, expression of *MLH1* prior to treatment is not able to

predict outcome [57]. These results suggest that *MLH1* methylation may serve as a marker for enrichment of inherently resistant cells and may aid in determining response to platinum treatment.

HOXA9

HOXA9 is hypermethylated across the CpG island promoter in a number of cancer types, including bladder [58], colorectal [59] and neuroblastoma [60] cancers and appears to hold promise as a biomarker in EOC. In a study by Wu *et al*., *HOXA9* was methylated in 51% of EOC tumors, with significantly higher methylation frequencies identified in early-stage tumors [61]. Interestingly, in this study the endometrioid histotype demonstrated the highest degree of methylation of *HOXA9*. Because methylation of *HOXA9* was preferentially identified in early-stage EOC tumors, Widshwendter *et al*. conducted a study to determine whether *HOXA9* methylation could serve as a predictor of EOC [62]. In this study, the authors examined *HOXA9* promoter methylation in normal endometrium from women with EOC and healthy controls and determined that it was predictive of presence of EOC, with increasing *HOXA9* methylation correlating with increasing risk of EOC. In contrast to the earlier study, this association was independent of histotype or stage. To determine whether *HOXA9* methylation could accurately predict the presence of HGSOC specifically, our group recently measured methylation of *HOXA9* in 80 HGSOC tumors and 12 normal ovarian surface epithelium (OSE) samples using methylation-specific headloop suppression PCR (Montavon *et al.*, under review). Our findings demonstrate that *HOXA9* is methylated in 75/79 (97.5%) of HGSOCs and only 1/12 (8%) of OSE. Furthermore, methylation of both *HOXA9* and *EN1* in combination with CA-125 levels was able to discriminate HGSOC from normal OSE with a sensitivity of 100% and a specificity of 91.7%. These findings support *HOXA9* methylation as a potential biomarker for detection of EOC, likely in combination with other genes and existing clinical approaches.

RASSF1A

RASSF1A is a potent tumor suppresser gene that is frequently repressed in association with DNA methylation in a large number of cancers including EOC. The frequency of RASSF1A promoter methylation in cancer establishes a potential to serve as a biomarker of cancer presence. In order to determine whether methylation patterns in free-circulating DNA (fcDNA) in plasma could differentiate between benign and malignant ovarian tumors, Liggett *et al*. performed a genome-wide analysis of methylation in cohorts of healthy controls, women with benign ovarian disease and women with invasive EOC [45]. Their results indicated that promoter methylation of three genes (*RASSF1A*, *CALCA* and *EP300*) effectively distinguished plasma of EOC patients from healthy controls with a sensitivity of 90.0% and a specificity of 86.7%. Furthermore, promoter methylation of *RASSF1A* and *PGR-PROX* effectively distinguished plasma of EOC patients from women with benign ovarian disease with a sensitivity of 80.0% and a specificity of 73.3%. These findings support previous studies identifying frequent *RASSF1A* promoter methylation in EOC and rarely in normal controls [43; 63; 64; 65].

SPARC

SPARC is involved in cell adhesion, motility and ECM interactions, and has been shown to function as a tumor suppressor in ovarian cancer, in part due to its regulation of tumor microenvironment interactions [66]. SPARC is down-regulated in EOC [67] and methylated in multiple cancers, including pancreatic [68], colon [69] and lung [70] cancers. Socha *et al*. recently investigated the mechanism of down-regulation in EOC [71] and demonstrated that the *SPARC* promoter is hypermethylated in EOC tumors, with 68.2% and 22.7% harboring full and hemi-methylation, respectively. In addition, SPARC expression was inversely correlated with tumor grade, such that HGSOC had the highest level of methylation. These results suggest that *SPARC* methylation in EOC has potential to serve as a biomarker, particularly the most aggressive form of the disease.

HIC1

HIC1 is silenced in cancer by methylation and not mutation [72; 73], and has been frequently demonstrated to be methylated in EOC. An early study demonstrated that a locus at region 17p13.3, where *HIC1* resides, is hypermethylated in 33% of EOC [74].) In studies by Teodoridis *et al*. and Strathdee *et al*., *HIC1* was methylated in 17% of EOC, in concordance with *OPCML* and *RASSF1A*, among others [64; 75]. Rathi *et al*. demonstrated *HIC1* methylation in 35% of EOC tumors and <10% of normal ovarian tissues [63]. Tam *et al*. expanded these studies to investigate methylation of *HIC1* in a larger number of EOC tumors, as well as borderline ovarian tumors, benign ovarian tumors and normal ovarian tissues and found that *HIC1* was methylated in >50% of ovarian cancer tissues, >40% of borderline ovarian tumors, >20% of benign ovarian tumors and 10% of normal ovarian tissues [76]. Taken together, these studies suggest that *HIC1* promoter methylation correlates with the presence of invasive EOC.

DAPK

DAPK is a pro-apoptotic kinase which plays a role in metastasis and is methylated in several cancer types including lung, bladder, uterine leiomyoma and colorectal cancers [77]. Collins *et al*. examined *DAPK* promoter methylation in 30 EOC tumors and 26 peripheral blood DNA samples [42]. Their findings demonstrated that *DAPK* was not methylated in normal OSE but was methylated in 67% of EOC tumors and 54% of blood DNA samples. However, DNA methylation did not correlate with patient outcome or aggressiveness of the disease [42]. However, other studies which examined *DAPK* methylation produced conflicting results; two studies detected methylation in <15% of EOC samples [43; 47] and two other studies did not detect *DAPK* methylation in any of their examined EOC samples [64; 77]. These inconsistencies are likely due to differences in sample processing, PCR technique, and the use of tissues *versus* blood samples. While it's possible that epigenetic silencing of *DAPK* could be associated with metastatic ovarian cancer and provides the

cells with the ability to resist apoptosis, further studies are necessary to correctly elucidate the potential of *DAPK* as a biomarker.

Novel candidates

OPCML

OPCML is an immunoglobulin domain−containing GPI-anchored cell adhesion molecule and has been demonstrated to act as a tumor suppressor. Forced expression of OPCML decreases proliferation of EOC cells *in vitro* and tumor growth *in vivo* while increasing cellular aggregation [78]. *OPCML* is frequently inactivated in EOC by allele loss or CpG island methylation [79]. Studies have demonstrated that *OPCML* is not methylated in normal ovarian epithelial cells, but is methylated and inactivated in a high proportion (33-83%) of EOC tumors [64; 78; 80]. These studies also indicate that methylation of *OPCML* may be an early event, as higher methylation frequencies were present in borderline and early-stage tumors compared with late-stage tumors, establishing its potential to serve as a specific biomarker of surgically curable EOC.

CCBE1

CCBE1 is down-regulated in breast and ovarian cancer and maps to 18q21.32, a region frequently exhibiting loss of heterozygosity in ovarian cancer [81; 82]. Our group recently conducted a study to determine the role of *CCBE1* in ovarian cancer [83]. This study confirmed loss of *CCBE1* expression in EOC tumors of all histotypes and demonstrated higher expression in low-grade versus high-grade EOC tumors. In addition, we showed that 41% of EOC tumors exhibited promoter methylation of *CCBE1* compared with 20% methylation in normal OSE samples. EOC patients without *CCBE1* expression had decreased relapse-free survival times versus those with *CCBE1* expression, although no such difference was seen when comparing *CCBE1* methylation. Nonetheless, these findings warrant additional studies in larger cohorts, as they suggest that *CCBE1* may serve as a detection and prognostic biomarker for EOC.

Pathways

Wnt Pathway

Activation of the Wnt pathway is common in multiple cancer types, including ovarian cancer. Several regulators of the Wnt pathway have been demonstrated to be hypermethylated in EOC. In one study, *SFRP5*, a Wnt antagonist, was found to be hypermethylated and down-regulated in 44.4% of examined EOC and only 1.3% of benign cases [84]. Additional studies demonstrated that expression of SFRP5 sensitizes ovarian cancer cells to cisplatin and taxol *in vitro* and that patients with unmethylated *SFRP5* had improved clinical response *versus* those harboring *SFRP5* promoter methylation [85].

Dai *et al*. recently expanded these studies to examine promoter methylation at 302 loci in a panel of 137 Wnt pathway genes in a screening cohort of 111 and a validation cohort of 48 serous and endometrioid EOC cases [86]. Following screening, validation and adjustment for clinical parameters, the authors demonstrated that methylation at 7 loci (*FZD4, DVL1, NFATC3, ROCK1, LRP5*, *AXIN1*, and *NKD1*) was associated with poor progression-free survival. This panel was then examined for association with patient response to platinum chemotherapy. Hypermethylation of *DVL1* and *NFATC3* significantly correlated with poor response, with patients with progressive or stable disease harboring increased methylation *versus* patients with partial or complete response [86]. The authors then expanded these studies to include a cohort of HGSOC cases from the TCGA [18]. In this independent cohort, methylation of *DVL1, ROCK1, LRP5,* and *AXIN1* was associated with progression-free survival. These findings were validated using gene expression data and results showed that decreased expression of *FZD4*, *DVL1*, and *ROCK1* correlated with a higher risk of disease progression. Taken together, these studies demonstrate that epigenetic regulation of the Wnt pathway may serve as a biomarker for prognosis and/or treatment response in EOC.

TGF-beta Pathway

Promoter methylation of genes involved in the TGF-beta pathway has been reported in numerous cancers including gastric, prostate and melanoma. In addition, expression profiling of 5-aza-dC-treated cell lines highlights TGF-beta signaling as a core pathway altered by DNA methylation in EOC [87; 88]. In a study by Kang *et al.*, *TGFBI* methylation was originally observed in 2 ovarian cancer cell lines and later demonstrated in 23/38 (60.5%) of ovarian cancer cases, 5/18 (27.8%) borderline ovarian tumors and 0/38 normal ovarian tissues [87]. In this cohort, *TGFBI* methylation was not associated with any clinical outcomes. Matsumura *et al*. examined candidate genes in 39 ovarian cancer cell lines and identified a significant number of TGF-beta pathway genes as regulated by methylation [88]. Furthermore, gene expression analysis of the candidate methylated genes in 17 EOC tissues demonstrated suppression of TGF-beta pathway activity. Taken together, these studies provide a potential mechanism for the suppressed function of the inhibitory effect of TGF-beta in ovarian cancer, and suggest that methylation of the TGF-beta pathway may serve as a biomarker for the presence of EOC.

FBXO32 is a TGF-beta/SMAD4 target gene and a regulator of apoptosis. In a recent study by Chou *et al*., *FBXO32* was unmethylated and expressed in normal OSE but methylated and expressed at lower levels in 96 EOC cases [89]. Methylation was significantly associated with tumor stage, with late stage tumors harboring significantly greater methylation than early stage. Furthermore, patients with greater DNA methylation demonstrated poorer progression-free survival. *In vitro* studies demonstrated that expression of *FBXO32* restored sensitivity to cisplatin, suggesting that the difference in progression-free survival in patients may be due to varying responses to treatment. These results suggest that methylation of *FBXO32* may serve as a prognostic marker for EOC.

Future Considerations

As DNA methylation occurs early and tends to be stable throughout carcinogenesis, the application of DNA methylation as a biomarker of EOC holds great promise in overcoming the high false positive rate of detection for the current standard biomarker CA-125. Numerous studies of differential methylation have identified potential biomarkers of diagnosis, prognosis and response in EOC. However, there is evidence that some of these methylated markers may not be unique to EOC or to cancer at all, with *BRCA1* methylation also reported in healthy controls [90]. This provides an additional challenge regarding the necessity of long-term clinical follow-up of healthy controls in screening trials.

The heterogeneity of EOC, as well as differences in sample processing, assay design and approach, could explain the variation in DNA methylation frequencies reported for individual genes. In addition, further studies using larger cohorts are necessary to determine the efficacy of methylation to serve as a biomarker for early-stage EOC. It is clear from these studies that methylation in a panel of biomarkers, rather than individual gene methylation, will be necessary to achieve a suitable diagnostic assay with >99.6% specificity [23]. Indeed, studies that have examined combinations of gene methylation as EOC biomarkers have achieved the greatest degree of sensitivity and specificity ([45; 86], Montavon *et al*. and Gloss *et al.*, under review). For example, in a study by Montavon *et al*. *EN1*, previously identified as being located in a long range epigenetically silenced genomic region frequently hypermethylated in colon cancer [91; 92] and prostate cancer [93], was found to be methylated in 64/80 (80%) of HGSOCs and only 1/12 (8%) OSE. In addition, in combination with *HOXA9* methylation and CA-125, *EN1* methylation could discriminate HGSOC from normal OSE with a sensitivity of 100% and a specificity of 91.7%. These studies warrant further examination of combining epigenetic biomarkers with CA-125 to improve sensitivity and specificity of detection of EOC.

With the recent publication of DNA methylation profiles of ~500 HGSOC tumors [18], it is possible to examine combinations of potential methylated biomarkers in various EOC cohorts. Indeed, more recent studies have focused on genome-wide identification of methylated biomarkers of cancer, including EOC [94]. Methyl-DNA immunoprecipitation (MeDIP) applied to genome arrays (MeDIP-chip) was used to directly profile methylation in EOC compared to normal ovaries and identified 367 CpG islands specifically methylated in EOC [95]. Additional genome-wide studies in EOC have identified methylation signatures associated with progression-free survival [40; 96], histological subtype [97], disease stage [98] and response to platinum-based chemotherapy [99]. While these studies benefit from increased sample number and array-based platforms, the potential methylated biomarkers identified by these genome-wide screens require further independent validation in large, well-annotated clinical cohorts.

Even with the advancement of approaches for genome-wide identification of methylated biomarkers of EOC, the need still remains for the development of a non-invasive diagnostic assay. Fortunately, tumor-specific gene methylation has been detected in blood of EOC patients [42; 43; 44; 45; 65], suggesting its utility as a non-invasive source of DNA for disease screening (Figure 1). Teshendorff *et al*. performed genome-wide DNA methylation profiling of 27,000 CpG islands in blood samples from a large cohort of EOC patients and controls to determine whether profiles can serve as detection biomarkers and predict the presence of cancer [100]. The authors demonstrated that the presence of cancer elicits common methylation changes in blood, and that EOC could be predicted with a relatively high accuracy. While it is clear that methylated biomarkers in plasma DNA have significant clinical potential for early detection, they require further validation of diagnostic efficacy in a large prospective study similar to studies for protein biomarkers [101].

Epigenetics aberrations clearly underlie the initiation and development of cancer and other diseases. With recent advances in epigenetic research including high-throughput technologies for methylation

detection and analysis, an International Human Epigenome Consortium (IHEC) has been established with the aim of identifying genome-wide DNA methylation and histone modification patterns in all genes in all major normal tissues [102; 103]. Once completed, the comprehensive data generated from the IHEC, in combination with data from the International Cancer Genome Consortium (ICGC) [104] and TCGA [18], will support future studies which are necessary to determine the methylation events which are critical for EOC tumorigenesis and which can be applied towards a highly sensitive and specific assay for early detection of EOC.

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Conflicts of Interest

The authors declare no conflict of interest.

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