Targeting the epigenome in ovarian cancer

Susan K Murphy

Department of Obstetrics & Gynecology, Division of Gynecologic Oncology & Department of Pathology,
Duke University Medical Center, PO Box 91012, Durham, NC 27708, USA
Tel: +1 919 681 3423 • Fax: +1 919 684 5335 • susan.murphy@duke.edu

Epithelial ovarian cancer is the leading cause of death from gynecological cancers, largely owing to the development of recurrent intractable disease. Only a small number of distinct genetic mutations are known to contribute to ovarian carcinogenesis. Furthermore, understanding mechanistic genotype–phenotype links is complicated by frequent aneuploidy. Epigenetic deregulation is even more prominent, and ovarian cancers are replete with such aberrations that repress tumor suppressors and activate proto-oncogenes. Epigenetic therapies are emerging as promising agents for resensitizing platinum-resistant ovarian cancers. These drugs may also have the potential to alter epigenetic programming in cancer progenitor cells and provide a strategy for improving therapy of ovarian cancer.

Ovarian cancer

Ovarian cancer includes a number of distinct malignancies that involve the ovaries. Epithelial ovarian cancer is the most common form of this disease, comprising of approximately 90% of ovarian cancer cases. It is also the most lethal of all gynecological malignancies, with overall 5-year survival rates of approximately 50%. Ovarian cancer diagnoses most often occur after the tumor has undergone metastatic spread within the peritoneal cavity. Early detection is infrequent owing to vague symptoms and lack of cost-effective screening strategies [1]. Standard treatment consists of maximal surgical removal of the tumor followed by platinum and taxane-based chemotherapy. In most cases, the clinical response to primary treatment is good, with the disease going into a variable period of remission. However, disease recurrence is frequent, happening anytime from months to years following initial treatment. Often, it is during treatment for recurrence that the tumor cells exhibit resistance to platinum and taxane drugs. Therapeutic options at this point, including doxorubicin, gemcitabine, topotecan or etoposide, frequently elicit only limited responses. Lacking more effective alternatives, the initial use of platinum and taxane tackles the disease using a ‘one-size-fits-all’ approach that does not account for the individual biology of the tumor being treated. Clearly, enhancing the ability to detect ovarian cancer earlier in the course of disease would help to improve outcomes, since women diagnosed with early-stage disease have superior survival rates than those diagnosed with advanced-stage disease [2]. In addition, new strategies for the treatment of recurrent disease are necessary if we hope to prolong survival.

There are four major histological types of epithelial ovarian cancer including serous (<52% of cases), endometrioid (<16%), mucinous (<12%) and clear cell (<8%) adenocarcinomas [2]. The remainder comprises of undifferentiated carcinomas (<5%) and mixed epithelial carcinomas (<5%). Ovarian tumors of low malignant potential (LMP), also referred to as borderline tumors, are usually diagnosed early in the course of disease, with 5-year survival rates >90% [2,3]. In spite of the enormous heterogeneity of this disease, treatment strategies are similar. Improved understanding of the environmental, genetic and epigenetic factors that drive development of the disease, the specific genes and pathways that are usurped, and how these factors interact to affect drug response is required to enable better stratification for treatment purposes. Toward this end, progress is being made in improving the classification of ovarian cancers based on clinicopathological and molecular characterization.

Kurman and colleagues have proposed that ovarian cancers can be divided into two major groups, type I and type II tumors (Table 1) [4]. Type I tumors are less aggressive and include low-grade serous borderline tumors, clear cell, endometrioid and mucinous tumors. Type II tumors include high-grade serous, high-grade endometrioid, carcinomas and undifferentiated carcinomas. Type I tumors have distinct genetic mutations and arise from transformation of borderline tumors of the ovary or from endometriosis. By contrast, most type II tumors carry TP53 mutations and approximately half are associated with either genetic or epigenetic inactivation of BRCA1/2.

Keywords

- 5-azacytidine
- 5-hydroxymethylcytosine
- belinostat • cancer stem cells • decitabine • DNA methylation • DNA methyltransferase inhibitors • histone deacetylase inhibitors • ovarian cancer • vorinostat
Chromosomal instability and aneuploidy are hallmarks of type II tumors [5]. Accumulating evidence indicates a fallopian tube fimbriae origin for serous cancers [6,7], while endometrioid and clear cell tumors, which often have mutations in ARID1A, come from endometriosis [8], presumably through retrograde menstruation. Type II tumors are aggressive, often having spread throughout the peritoneal cavity before they are detected, whereas type I cancers are more often diagnosed at an early stage.

**Treatment**

The current paradigm for treatment of epithelial ovarian cancer involves surgical debulking of the tumor followed by chemotherapy using a combination of platinum and taxane drugs. Platinum drugs were first used for treatment of ovarian cancer in the 1980s and include cisplatin and carboplatin. These drugs induce inter- and intrastrand crosslinks through formation of platinum–DNA adducts, which then activate nucleotide excision repair or mismatch repair proteins to remove the offending adducts and repair the DNA, allowing for cell survival. Tumors with inherent or acquired defects in DNA repair, especially nucleotide excision repair or mismatch repair proteins, are often sensitive to platinum drugs since apoptosis is normally triggered if there is an inability to effectively remove the damaged DNA [9]. On the other hand, tumors with TP53 mutations, which encompass nearly all high-grade serous ovarian cancers [10,11], are not able to elicit an efficient apoptotic response.

Taxanes are a more recent addition to the regimen, having been US FDA approved in 1992, and although their precise mechanism of action is unknown, these drugs stabilize microtubules, thereby inducing cell-cycle arrest and apoptosis. Chromosome instability has been associated with taxane resistance [12]. Other studies have shown that the activities of specific genes modulate taxane response. For example, our group has previously shown that elevated expression of the gene encoding zinc-finger protein YY1 is associated with longer survival of women with advanced-stage serous epithelial ovarian cancer and response to taxane therapy. Expression of YY1 is positively correlated with increased expression of microtubule-related genes that are downstream targets of YY1-E2F3 transcription factor activity [13]. More recently, increased expression of PLK2 was also shown to confer sensitivity to both paclitaxel and carboplatin [14]. While repression of YY1 was found to be independent of promoter methylation in ovarian cancers [13], PLK2 is transcriptionally silenced by DNA methylation [14]. By contrast, increased expression of SIK2, a centrosome kinase required for formation of mitotic spindles, is linked to poor survival, while SIK2 depletion enhances response to paclitaxel in cell culture and in a mouse xenograft model of ovarian cancer [15].

BRCA mutations, inherited or somatic, along with BRCA1 methylation-mediated repression, are associated with increased sensitivity to platinum agents due to defects in the ability to perform repair of DNA damage induced by these drugs. The BRCA1 and BRCA2 proteins are structurally distinct, but both function in the repair of double-strand breaks in genomic DNA through homologous recombination. Analysis of a large number of high-grade serous epithelial ovarian cancers by The Cancer Genome Atlas (TCGA) project has also shown genetic alterations in other genes involved in homologous

<table>
<thead>
<tr>
<th>Genetic alterations</th>
<th>Origin</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I tumors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-grade serous</td>
<td>KRAS, BRAF, ERBB and PIK3CA</td>
<td>Fallopian tube epithelium</td>
</tr>
<tr>
<td>Clear cell</td>
<td>ARID1A, PIK3CA, ZNF217 and PPP2R1A</td>
<td>Endometriosis</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>ARID1A, CTNNB1, PTEN, PIK3CA and PPP2R1A</td>
<td>Endometriosis</td>
</tr>
<tr>
<td>Mucinous</td>
<td>KRAS</td>
<td>Borderline tumors</td>
</tr>
<tr>
<td><strong>Type II tumors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-grade serous</td>
<td>TP53 and BRCA1/2</td>
<td>Fallopian tube epithelium</td>
</tr>
<tr>
<td>High-grade endometrioid</td>
<td>Chromosome instability</td>
<td>Endometriosis</td>
</tr>
<tr>
<td>Carcinosarcomas</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Undifferentiated carcinomas</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
recombination, including EMSY, PTEN, RADS1C, ATM or ATR, and Fanconi anemia genes [11]. Zhu et al. found that BRCA1 plays a prominent role in maintaining the transcriptionally silenced state of constitutive heterochromatic regions, including satellite DNA sequences near the centromeres of chromosomes [16]. Furthermore, they suggest that loss of BRCA1 function, with consequent transcriptional activation of satellite DNA and destabilization of the genome, is sufficient to explain the increase in DNA double-strand breaks associated with BRCA1 loss-of-function in cancer [16]. Although loss of BRCA1/2 is associated with platinum sensitivity, Swisher and colleagues have shown that ovarian cancers with inherited BRCA1/2 mutations can acquire secondary somatic mutations that restore BRCA1/2 and, in turn, confer resistance to platinum drugs [17]. Such secondary mutations are also likely to reduce effectiveness of poly-ADP-ribose polymerase inhibitors that work as synthetically lethal agents by blocking poly-ADP-ribose polymerase-mediated, base excision repair of single-strand DNA breaks in the context of BRCA1/2 mutations. During DNA replication, unrepaird single-strand breaks become double-strand breaks at the replication fork and, thus, restoration of BRCA1/2 function through secondary mutations would allow for repair of these mutations.

**Potential for epigenetic therapies**

Epigenetic mechanisms of gene regulation are now understood to be a prominent feature in many types of cancer, including ovarian cancer. These mechanisms are a fundamental component of early development in helping to guide germ-layer specification. They are also responsible for instructing the cellular machinery to form tissues and organs, all of which have distinct gene activity profiles despite originating from the same genetic sequence. Epigenetic forms of gene regulation include miRNAs, modifications of histone proteins and methylation of DNA. Although these represent very different regulatory mechanisms, they all share the capacity to change gene expression and phenotype without altering the DNA sequence. Unlike genetic alterations, epigenetic changes provide a degree of ‘plasticity’ to gene expression, allowing for adaptations to endogenous or exogenous environmental influences [18]. This characteristic means that these changes are potentially reversible, through both active and passive processes, but also through pharmacological intervention (Figure 1). Epigenetic therapies are already showing success in the treatment of myelodysplastic syndromes [19]. There is now much interest regarding the potential of epigenetic drugs to treat ovarian cancer and other types of solid tumors.

**DNA methylation**

DNA methylation is arguably the most intensively studied aspect of epigenetics with regard to carcinogenesis, and has also been the major focus of pharmacological interventions in clinical trials. This modification occurs predominantly at CG dinucleotide pairs and involves the covalent attachment of a methyl group to the 5-carbon position of the cytosine ring to form 5-methylcytosine (5mC). Patterns of cytosine methylation throughout the genome are established during early in utero development through the activity of DNA methyltransferase (DNMT) enzymes. There are three DNMTs with enzymatic activity. DNMT1 is commonly referred to as a maintenance methyltransferase because it is known to recognize hemimethylated sites on the genomic DNA at the replication fork, and methylate the palindromic

![Figure 1. Epigenetic modifications affect chromatin structure.](Figure 1. Epigenetic modifications affect chromatin structure. Nucleosome consisting of eight histone proteins (two each of histone H2A, H2B, H3 and H4) around which approximately 147 bp of DNA is wrapped. CpG dinucleotides within the DNA sequence are potential targets of DNA methylation. Each histone protein contains an amino acid tail that extends out from the core nucleosome and is subject to enzymatic modifications that lead to transcriptional activation (top left) or repression (top right). A pathway for DNA demethylation, involving formation of 5-hydroxymethylcytosine from 5-methylcytosine, has recently been identified and is shown at the bottom. DNMT: DNA methyltransferase; DNMTi: DNA methyltransferase inhibitor; HDAC: Histone deacetylase; HDACi: Histone deacetylase inhibitor; TDG: Thymine DNA glycosylase.)
complementary sequence on the nascent DNA strand in order to perpetuate the DNA methylation profile during somatic cell division. The de novo methyltransferases, DNMT3A and DNMT3B, are capable of maintenance methylation similar to DNMT1, but are also responsible for the establishment of genomic DNA methylation patterns during very early development since they are able to add methyl groups to previously unmodified CG dinucleotides.

Approximately 70% of the CG dinucleotides throughout the human genome are methylated. The remainder are unmethylated, and these are localized in CG-dense regions, referred to as ‘CpG islands’, which are found at the promoter region of over half of all genes, as well as in intragenic and intergenic locations. Methylation of promoter CpG islands does occur at imprinted loci and at the promoters of genes subject to X chromosome inactivation in females, as well as at other loci that acquire methylation early in development. However, the majority of promoter CpG islands are unmethylated, regardless of the level of transcriptional activity. The unmethylated status of these promoter CpG islands may be due to the activities of specific chromatin modifying complexes [20,21] and/or active demethylation (see below). By contrast, nonpromoter CpG islands often show tissue-specific methylation that corresponds with transcriptional activity. Intragenic CpG islands are sometimes associated with noncoding RNA transcripts [22], which are likely to be important in regulating expression of the protein-coding host gene.

Recently, 5-hydroxymethylcytosine (5hmC) has been recognized as another important cytosine modification present in the genome, and is generated through the conversion of 5mC to 5hmC by TET proteins [23,24]. Current technological limitations do not allow for ready discrimination between 5hmC and 5mC, so it remains unknown how the distribution and presence of 5hmC might contribute to cancer. However, 5hmC appears to represent an intermediate in active demethylation reactions that first involve deamination of 5mC or 5hmC by activation-induced deaminase, followed by base excision repair with thymine DNA glycosylase (TDG). TDG is proposed to interact with transcription factors that direct TDG activity to specific promoters or enhancers in a tissue-dependent manner [25]. If true, this could mechanistically explain how the majority of promoter CpG islands are maintained in an unmethylated state. Deregulation of this pathway, through alteration of function or specificity, could lead to global changes in methylation in cancer cells. Indeed, acquisition of promoter-specific methylation is one of the hallmarks of cancer, and could theoretically be attributable to diminished activity of this newly identified mechanism of base excision repair-mediated demethylation.

Although the role of 5hmC in cancer has not yet been investigated in depth, Li and Liu found that colon cancers and colon cancer cell lines have fourfold lower levels of 5hmC relative to normal colon tissues [26]. Loss of function of TET2, through mutation or downregulation, is strongly associated with decreased 5hmC in myeloid cancers – yet surprisingly, also with decreased levels of CpG methylation [27]. TET2 and 5hmC were hypothesized to be required for differentiation of cells during myelopoiesis, while mutation and deletion of 5hmC may promote a less differentiated stem cell-like state. Tet1 knockdown experiments have shown a requirement for 5hmC in maintaining stem cell self-renewal since the promoter region of Nanog, a gene required for pluripotency, is aberrantly silenced by DNA methylation in the absence of Tet1 [28]. Further research will help reveal the relationships between the levels of 5mC and 5hmC, and how this relates to altered expression of the genes involved in the base excision repair demethylation pathway in cancer.

In this regard, we have generated Illumina® Infinium 27k Methylation Beadchip data for a panel of 56 ovarian cancer cell lines and, of these, 20 show β values between 0.20 and 0.93 (mean = 0.22) at the TET1 promoter CpG island, indicating that TET1 itself is targeted by methylation [Yamaguchi K, Berchuck A, Murphy SR, Unpublished Data]. TET2 is not included on this platform, and TET3 does not have a promoter CpG island. Interestingly, TDG also exhibits methylation in these 56 ovarian cell lines (range: 0.05–0.59; mean = 0.40), consistent with a prior demonstration of TDG promoter methylation in gastric cancer cells [29]. Collectively, these data indicate that some of the genes involved in active maintenance of unmethylated promoters in ovarian cancer may themselves be regulated by DNA methylation. While speculative, methylation-mediated repression of these genes may be permissive for the accrual of aberrant methylation at target promoters, and may help explain how these genes acquire and retain methylation that leads to silencing of tumor suppressor genes in ovarian cancer.
Histone modifications

Histone proteins are highly conserved proteins that provide the nuclear scaffolding for the condensation of genomic DNA. The nucleosome core consists of eight histone proteins (two each of H2A, H2B, H3 and H4); around which approximately 147 bp of genomic DNA is wrapped. Nucleosomes are linked by an intervening stretch of approximately 50 bp of DNA that is also associated with the linker histone H1, which helps tether the genomic DNA to the core nucleosome. The histones have amino acid tails that extend from the core and are accessible to post-translational modifications, including, but not limited to, acetylation and methylation. These modifications can alter histone–DNA interactions and consequent transcriptional activity due to changes in accessibility of the DNA to binding proteins and transcription factors (Figure 1). For example, monomethylation of histone H3, lysine 4 (H3K4me1) is an activating modification, while trimethylation of histone H3, lysine 9 (H3K9me3) is a repressive modification. In addition, acetylation of histone H3K9 and H3K14 by histone acetyltransferase enzymes is associated with transcriptional activation, while deacetylation by histone deacetylases (HDACs) is repressive, in part by increasing the affinity between the DNA and core histones and thus the compaction of the DNA. In stem cells, divergent repressing and activating histone modifications, including activating H3K4me2/3 (di- and tri-methylation) and repressive H3K27me3, are often present at the same gene promoters that are maintained in a bivalently marked, transcriptionally poised state (reviewed in [30]). In addition, levels of H3K27me3 are an independent prognostic indicator in ovarian and other cancers, with decreased H3K27me3 associated with shorter overall survival [31].

Cancer stem cells are defined as the subset of tumor cells that are able to sustain malignant growth [32]. These cells, which are thought to exist in a dormant or slowly proliferating state, exhibit inherent chemoresistance in part due to increased expression of drug transporter molecules. However, drug resistance may also be due to the use of therapeutic agents that preferentially target fast-proliferating cancer cells [33]. Similar to normal stem cells, ovarian cancer stem cells appear to maintain phenotypic plasticity through multivalent, epigenetic marking of relevant genetic loci [34]. In addition to finding gene promoter regions marked by opposing H3K4me2/3 and H3K27me3 modifications, such as those found in normal stem cells, Bapat et al. identified a number of other types of multivalent marks present in ovarian cancer stem-like cells, and showed that these chromatin marks were reprogrammed in a manner that reduced plasticity in cells cultured under 3D culture conditions versus those cultured as monolayers [34]. 3D culture conditions have previously been used to enrich for cells with cancer stem cell-like features [35,36] and, as such, these cells might be expected to exhibit a less differentiated and, hence, more epigenetically labile state. This cell population may indeed be the most relevant in terms of therapeutic targeting with the aim of eradicating disease. The reliance of these cells on epigenetic mechanisms to trigger gene expression patterns that perpetuate the tumor suggests that epigenetic therapies may be a powerful means of restraint or even eradication. It will be critically important to comprehensively characterize the epigenome of these cells and determine how this profile responds to epigenetic therapies.

Epigenome characterization & clinical utility

Epigenetic therapies have been proposed to function, in part, by causing widespread reprogramming of the cancer cell genome. These changes are thought to enhance sensitivity to traditional chemotherapeutic agents while decreasing toxic side effects. This type of treatment strategy may also reduce the required dosage of traditional chemotherapeutics, which may then show a synergistic response with the epigenetic modulators [37]. By contrast, de novo methylation also occurs in response to treatment with platinum agents, which can alter chromatin structure via DNA adduct formation, and has been postulated to allow inappropriate access of the DNA methylation machinery to unmethylated regions of the genome. In addition, DNA damage induced by chemotherapeutic treatment might alter the ability of DNMTs to recognize their target methylation sites [38], or change the fidelity of DNMT activity due to production of cytosine damage products [39]. Thus, a thorough understanding of the consequences of epigenetic therapeutic agents is required if we hope to utilize these drugs in a manner that minimizes unintended effects. To this end, and to better individualize the approach used for treatment that incorporates epigenetic therapies, evaluation of an epigenetic biomarker panel that can provide information about the status of the tumor and,
thus, the potential for responsiveness, would be desirable prior to implementing such treatments. Although many individual genes exhibit altered methylation [40], and specific alterations in histone modifications are just beginning to be reported in ovarian malignancies, a panel of informative genes may be the best strategy upon which to base treatment decisions.

Prior to the advent of genome-wide technologies for detection of methylation profiles, research was focused on detecting and understanding methylation of single genes. There is much to be learned from such small-scale analyses, which can provide in-depth information about particular genes targeted by methylation-mediated silencing, such as the functional relevance of the methylation and how this relates to tumor biology, as well as response to traditional and epigenetic therapeutic agents. However, single loci may not be sufficient for use as therapeutic biomarkers, since it is highly unlikely that the methylation status of a single gene would have high enough specificity to enable treatment choice, monitoring of response and disease progression. The accumulation of data cataloging the histology- and stage-specific epigenetic alterations in ovarian cancer, should ultimately enable development of highly relevant panels of biomarkers, and advancing epigenetic technologies should make clinical implementation of such panels cost-effective. Such a panel of biomarkers must be developed, accounting for the types of specimens available for analysis, especially with regard to postsurgical monitoring since tumor tissue is typically obtained only once over the course of treatment. Peritoneal fluid, obtained by paracentesis to remove tumor-associated ascites, or from peritoneal washings, could be used as a source of DNA for evaluation. However, tumor-specific DNA methylation is readily detected in peripheral blood specimens, including serum [41], which is frequently used to monitor CA-125 values. The presence of tumor-specific cell-free DNA in peripheral blood does offer an advantage of using DNA methylation profiles over that of histone modifications for patient monitoring.

A number of studies have already begun to define widespread methylation changes in ovarian cancer and, in some cases, how these relate to outcome using multigene analyses or large-scale platforms. Su et al. have shown potential prognostic capability based on methylation of SFRP1, SFRP2, SOX1 and LMX1A in a panel of ovarian cancers, borderline and benign tumors, as well as serum [42]. Wei et al. used differential methylation hybridization, on a 7776 CpG island-containing array, to identify a group of 112 hypermethylated CpG island loci that were predictive of 6-month progression-free survival from analysis of multiple histological types of advanced-stage ovarian cancers [43]. Another study by Watts et al. used a CpG island array, containing 6560 CpG-rich sequences, to analyze 137 stage I–IV ovarian cancers [44]. Their findings showed progressively altered methylation with increasing disease stage, including increases in methylation at promoter CpG islands and decreases at repetitive sequences. However, there was inconsistent progression in the loss of methylation at repetitive sequences, whereby stage I, III and IV cancers showed pronounced hypomethylation at these regions, while stage II cancers showed much higher overall methylation levels at these repetitive elements, which was more similar to borderline tumors.

Houshdaran et al. utilized the first-generation Illumina (San Diego, USA) Golden Gate platform, containing 1505 individual CpG sites for DNA methylation analyses of 27 primary ovarian cancers and 15 ovarian cancer cell lines representing the major histological types of this disease [45]. They reported distinct DNA methylation profiles for 68 genes (90 CpG sites, or ~6% of the total number of CpG sites) that characterize each histological subtype. They also reported that ovarian cancer cell lines exhibit increased levels of CpG methylation as compared with the primary cancer tissues. This might reflect the relative homogeneity of the established cell lines combined with the heterogeneity in cell types analyzed within a given tumor specimen, which could dilute methylation signals that are present in the tumor cells. Another study, using the same platform, reported on methylation profiles of low- versus high-grade serous tumors, as well as serous borderline tumors [46]. The results showed that low-grade and borderline (i.e., type I) tumors harbor significantly more hypermethylated CpG sites than do high-grade (type II) tumors. My laboratory has also utilized transcriptome profiling following treatment with DNMT inhibitory drugs in primary cultured ovarian cancer cells and established cell lines to identify genes likely to be targeted by DNA methylation-mediated repression, with confirmation of methylation status [47]. We found that TGF-β pathway genes were disproportionately targeted by DNA methylation in the cultured cells and...
in primary tumors, and that treatment with decitabine increased TGF-β pathway activity. In addition, methylation-mediated repression was associated with increasing age of the patient at the time of diagnosis. Teschendorff et al. also reported cancer-specific, age-related changes in methylation by analyzing peripheral blood specimens from 113 women with ovarian cancer collected prior to treatment and comparing these profiles to those of 148 age-matched healthy controls using the Illumina 27k beadchip with over 27,000 CpG sites represented throughout the genome. In this study, the authors identified a DNA methylation signature that was predictive of the presence of ovarian cancer with an area under the curve of 0.8. This study, therefore, supports the idea that methylation profiles obtained non-invasively will be able to identify individuals with cancer, albeit more work is required to increase specificity.

**Progress in using epigenetic therapies**

Despite our relatively rudimentary understanding of the ovarian cancer epigenome, there is increasing interest in the utilization of epigenetic therapies in the treatment of this disease, as well as many other solid tumors. Epigenetic therapy refers to drugs that target proteins or pathways within the cell that are responsible for establishing, maintaining or altering the epigenetic profile. The best characterized of this group of drugs are those that function to inhibit the activity of DNMT enzymes (DNMT inhibitors) and those that inhibit the activity of HDACs (HDAC inhibitors).

The hope that DNMT inhibitors, including 5-azacytidine (5-AzaC) and decitabine, would have clinical utility in the treatment of solid tumors was abandoned early on due to the apparent lack of efficacy and pronounced toxicities associated with these drugs. Renewed interest came from the finding that the dosages previously used were excessive and of abbreviated duration, and that lower doses for prolonged periods not only reduced the unwanted side effects, but also showed benefits, functionally and therapeutically. The ability to reactivate epigenetically silenced genes through alleviation of promoter hypermethylation in ovarian cancer has been well established, and HDAC inhibitors have also shown promising results in vitro, including the ability to reactivate expression of tumor suppressor genes, decrease proliferation and increase apoptosis. Comparison of DNMT inhibitors with HDAC inhibitors for their ability to reactivate expression of epigenetically silenced genes in ovarian cancer cells indicates that DNMT inhibitors may be more effective than HDAC inhibitors. In addition, acquired platinum resistance of A2780 ovarian cancer cells is accompanied by significant upregulation of both DNMT1 and DNMT3B, suggesting that DNMT inhibitors may help reverse platinum resistance.

Synergistic effects of combined DNMT inhibitor (decitabine) and HDAC inhibitor (belinostat) treatment have been demonstrated in cisplatin-resistant A2780 ovarian cancer cells, with reactivation of epigenetically suppressed MLH1 and MAGEA1, along with heightened sensitivity to cisplatin. Decitabine combined with suberoylanilide hydroxamic acid (vorinostat or SAHA, another HDAC inhibitor) induced re-expression of imprinted tumor suppressor genes PEG3 and DIRAS3 (ARHI) in the HEY and SKOV3 ovarian cancer cell lines, reducing oncogenic behaviors and increasing cell death. Another group showed that decitabine combined with the HDAC inhibitors trichostatin A reactivated expression of ERβ in HEY and SKOV3 cells, which was accompanied by a loss of promoter methylation. These, and other studies have helped support that there are biologically meaningful and favorable consequences of inhibitory epigenetic drugs in ovarian cancer cells and, combined with the positive results obtained from re-evaluation of prolonged lower-dose therapy, provided the impetus for testing in clinical trials.

The cancer cell’s epigenetic ‘manipulation’ of the transcriptome is thought to lead to the clonal selection of cell populations with gene expression profiles that favor cell proliferation and metastasis while preventing cell death and enhancing the ability to survive chemotherapy. Consequently, genes that promote a positive response to chemotherapeutic agents may be preferentially targeted by DNA methylation and histone modifications. The reversible nature of epigenetic changes has fueled great interest not only in reactivating the suppressed genes, but also for utilizing DNMT inhibitors and HDAC inhibitors to restore sensitivity to these drugs. However, the timing and sequence of drug treatment may be critical for eliciting an optimal response, with DNMT inhibitors administered prior to the cytotoxic agent. The rationale for this strategy is based on the passive nature of the induction of hypomethylation by these drugs, since hypomethylation...
Figure 2. Potential for epigenetic therapies in treating epithelial ovarian cancers. The typical progression of treatment from diagnosis to recurrence is depicted at the bottom, with the ovarian cancer cell population thought to be present at each stage of this process shown within the shaded boxes. Potential opportunities for epigenetic modulatory drugs include time points prior to treatment with standard chemotherapeutic agents, as well as during remission with the intended purpose of interfering with the epigenetic profiles required to maintain a cancer stem cell phenotype.

requires that the methyltransferase enzymes are incapable of bestowing new methyl groups to CpG dinucleotides on the nascent DNA strand. The cytosine analogs that function as DNMT inhibitors, such as 5-AzaC and decitabine, are incorporated into genomic DNA. When these analogs are positioned within CpG dinucleotides that are destined to become methylated, the DNMT enzyme performing this function at the replication fork becomes covalently and irreversibly bound to the analog base, and is ultimately degraded. Several cell division cycles may be required to achieve sufficient depletion and subsequent loss of methylation, after which cytotoxic treatment is initiated. Because of the widespread effects of these drugs in enhancing or even reactivating expression of genes in multiple pathways within the cancer cell, it may be that these drugs, classified as ‘biological response modifiers’ [58], alleviate epigenetic repression of the genes that are responsible for mediating the beneficial effects of chemotherapeutic agents.

A Phase Ib–IIa study of 5-AzaC and carboplatin was reported for women with a diagnosis of platinum refractory or platinum-resistant intermediate or high-grade epithelial cancer of the ovary, fallopian tube or peritoneum. The women were treated once daily with 5-AzaC for 5 days while carboplatin treatment was given on day 2. Out of 29 patients, there was one complete response, three partial responses, and ten women with stable disease. The treatment was found to be ineffective in women with liver metastases, presumed to be due to inactivation of 5-AzaC in the liver. Decreased methylation at the death receptor DR4 gene was observed in peripheral blood leukocyte DNA following treatment, primarily in the objective responders [59]. DR4 hypomethylation had previously been demonstrated following decitabine treatment in ovarian cancer cell lines, with concomitant sensitization to carboplatin via apoptosis [60].

Another Phase I study of sequential therapy was reported using repetitive low-dose decitabine followed by carboplatin in women with progressive or recurrent advanced epithelial ovarian cancer, or primary peritoneal carcinomas. One patient had a complete response while three out of ten others had stable disease for at least 6 months. Analysis of peripheral blood mononuclear cells showed LINE1 retrotransposon hypomethylation along with demethylation of homeobox gene HOXA11 and BRCA1 in plasma on days 8 and 15 following treatment [61]. A Phase II study is currently underway.

Results from preclinical and clinical studies have also indicated that appropriate sequencing of HDAC inhibitors treatment may also be important in achieving optimum outcome [51]. Vorinostat is an orally bioavailable drug that functions as a HDAC inhibitor and was approved in 2006 by the FDA for treatment of cutaneous T-cell lymphoma. In ovarian cancer cell culture, vorinostat was found to induce a G1–G2 block in cell cycle progression with increased apoptosis. Enhanced acetylation of histones H3 and H4 has also been observed [58]. Preclinical studies have shown efficacy in combination with paclitaxel against ovarian cancer xenografts; however, vorinostat used alone or prior to paclitaxel therapy was not as effective as vorinostat used following paclitaxel treatment [62]. A Phase II multi-institutional Gynecologic Oncology Group study of vorinostat for use in platinum refractory recurrent or persistent epithelial ovarian or primary peritoneal ovarian cancer found that, although vorinostat had manageable side effects, there was minimal response when used as a single agent [58]. This trial did not assess combination therapies.

Belinostat (PXD101) is another HDAC inhibitor that has shown promise in preclinical studies, including the ability to potently inhibit growth in monolayer and 3D sphere cultures of ovarian cancer cells, and A2780 ovarian cancer cell xenografts in vivo. Treatment with belinostat in vivo was performed every day for 15 days, with carboplatin used every other day beginning on day 7. Increased acetylation of α-tubulin was shown to occur in response to belinostat in ovarian cancer cell lines, and
Synergistic effects were observed for combination treatment with carboplatin or docetaxel in vitro and carboplatin in vivo [63]. A Phase II trial of belinostat in women with platinum-resistant or metastatic epithelial ovarian cancer and LMP tumors has been conducted. Despite failing to meet efficacy measures for continuation of the study, stable disease was achieved in ten out of 14 evaluable patients with LMP tumors and nine out of 18 patients with cancer. Examination of peripheral blood taken prior to and after belinostat therapy from women with LMP tumors showed an increase in the level of acetylated histones H3 and H4. Tumor biopsies taken before and after belinostat therapy also showed increased histone acetylation in both tumor and stromal cells [64]. Combined HDAC inhibitors and cytotoxic therapies may prove to be the best option to improve efficacy, and careful attention to sequencing of these treatments, as with DNMT inhibitors therapies, will need to be addressed.

**Current limitations of epigenetic therapy**

HDAC inhibitors exhibit both histone-specific epigenetic effects as well as effects on nonhistone proteins, including, for example, RUNX3, HIF1α, NFκB, p53 and E2F1, among others that are highly relevant to cancer. These nonhistone effects alter protein stability as well as interaction with other proteins and DNA [65]. Thus, therapeutic response is not likely to be entirely due to inhibition of histone deacetylation, but also to the changes in acetylation status of other nonhistone proteins. Improved comprehension of the effects of HDAC inhibitors is required in order to develop the appropriate means to monitor drug response.

A major limitation associated with targeting DNMT enzymes (as well as HDACs) is lack of specificity. It is currently not possible to reactivate only specific target genes through use of DNMT inhibitors therapy. DNMT inhibition disrupts maintenance methylation throughout the genome during DNA replication; as such, the generation of each new daughter cell is associated with progressive losses in genome-wide methylation. Can such nonspecific reversal of DNA methylation have a negative effect on resensitizing tumors to platinum or other DNA damaging agents? For example, platinum compounds induce DNA adduct formation, which triggers cells to undergo apoptosis. Many DNA repair pathway genes are targeted by methylation in cancers, including BRCA1, MGMT, GSTPI and MLH1. Restoration of expression of these genes via DNMT inhibitors treatment could inadvertently lead to enhancement of drug resistance.

Another shortcoming of all current therapeutic approaches used to treat women with epithelial ovarian cancer is that the cancer cells most effectively targeted by these therapies may not be those responsible for causing the disease or its recurrence. The idea that cancer stem cells (cells that possess stem cell-like characteristics, are tumorigenic and able to undergo asymmetric cell division to produce the heterogeneous cell types required to form a tumor) give rise to ovarian cancer is an idea currently receiving much attention. New research is beginning to unveil information regarding the identity and behavior of putative ovarian cancer stem cells. While progress has been made in selecting and characterizing these cells in vitro and in vivo based on cell surface marker expression, and their ability to recapitulate the tumor when passed in mice [36,66–70], the origin of this cell population(s) is presently unclear. For example, while increasing evidence is pointing to secretory epithelial cells within the fallopian tube fimbriae epithelium as the source of serous ovarian cancer [4,71], there are no data available to support that these particular cells represent a normal tissue stem cell, or that these cells can be reprogrammed to adopt a stem cell-like phenotype.

Cancer stem cells are thought to retain phenotypes associated with a normal stem cell population, including a slow rate of growth. Cancer stem cells also express high levels of drug transporter molecules, including those characterized in ovarian cancer [72], thought to confer resistance to standard chemotherapeutics owing to their ability to efflux these drugs from the cell. If true, the idea that cancer stem cells are responsible for epithelial ovarian cancer has strong implications for disease recurrence [73]. If such a cell population – presumed to be a very small proportion of the overall tumor cell population – is able to survive primary chemotherapy, then it is possible that these cells enter into a state of slow proliferation or dormancy during apparent disease remission. This period of remission can last for months to years, during which the location and niche of this cell population is not known. However, it is clear that something triggers these residual cancer cells to emerge again as recurrent, and often, chemoresistant disease.
Normal stem cells maintain epigenetic plasticity to allow for formation of differentiated progeny; it is conceivable that cancer stem cells also retain epigenetic plasticity, which may make them more vulnerable to epigenetic modulatory drugs (Figure 2). While combined epigenetic therapies with standard chemotherapeutic agents offer the opportunity to reactivate genes required to provoke a response to these drugs, single agent use of epigenetic therapeutics have largely been ineffective. It may be that epigenetic therapies will have utility when administered following completion of treatment using standard chemotherapeutics, as part of maintenance or consolidation therapy during the time when residual cancer cells are entering a state of slow growth or dormancy. In support of the feasibility of this approach, several HDAC inhibitors, including suberic bisdihydroxamic acid, trichostatin A and sodium butyrate, are effective at causing cell death of both actively proliferating and nonproliferating cells [74]. My laboratory is currently conducting preclinical testing of several drugs that we have shown are more effective than cisplatin or paclitaxel at targeting slow-proliferating ovarian cancer cell populations in vitro [33]. Similarly, Wei et al. have shown that Müllerian inhibiting substance is more effective at targeting ovarian cancer stem cells than standard chemotherapeutic agents [75].

### Conclusion

As the era of revealing the genetic makeup of the human genome winds down, a new era is emerging in which we must now try to understand how the base sequence is controlled through epigenetic modifications, and this will be a much more complex undertaking. Advances in technologies used to characterize epigenetic profiles may soon make it possible to hone in on specific combinatorial epigenetic modifications that will aid in ovarian cancer screening and monitoring of disease status, in addition to providing information relevant to choice of chemotherapy. Recent studies are showing that epigenetic therapies may be able to resensitize chemoresistant ovarian cancer. Finally, more research is urgently needed to better define potential epigenetic vulnerabilities of ovarian cancer cells responsible for causing disease recurrence, and if these cells can be targeted through epigenetic means to prevent their ability to emerge as recurrent disease, the major cause of death from epithelial ovarian cancer.

### Future perspective

The development of more advanced and cost-effective high-throughput technologies for epigenetic modifications will be key to formulating rational strategies for implementation of epigenetic modulatory drugs. Elucidation of comprehensive DNA methylation and histone modification profiles will be needed for the major histological types of epithelial ovarian cancers, as well as by stage and grade of disease so that we have a more focused picture of the genes and pathways that are altered in each histological type, and how these differ from one another as well as from normal tissues. This, combined with integration of the emerging comprehensive gene expression and genetic variation data, should facilitate individualization of treatment approaches based on the particular patterns of deregulation present in a tumor rather than the homogeneous treatment approach currently utilized. This type of characterization will undoubtedly also allow development of improved DNA methylation-based diagnostic approaches for early detection of disease and for disease monitoring.

The identification or manufacture of new drugs that are able to more selectively target epigenetic modifications is an area beginning to show promise. Beltran et al. used artificial transcription factors to reactivate expression of MASPIN [76], a gene that is epigenetically silenced in multiple types of cancer, including ovarian [77]. They used a synthetic construct containing custom DNA-binding zinc finger domains to specifically target the MASPIN promoter, combined with the VP64 transactivator domain from herpes simplex virus, which induces transcription-promoting changes in chromatin structure and recruits transcription factors to the region bound. Similar approaches using engineered zinc finger-methyltransferase domains have also shown progress [78]. This approach is limited to regions of the genome that have become hypomethylated [79]. The discovery of genes involved in demethylation, such as TDG described above, may now offer opportunities using this sequence-specific targeting to also alleviate aberrant hypermethylation.

As we learn more about the targets of epigenetic modulatory drugs and how their use impacts efficacy of other drugs by reactivating expression of genes required for response, we will undoubtedly be able to improve this approach. The combined use of both DNMT and HDAC inhibitors has yet to be explored in terms of facilitating response to platinum and taxane drugs for ovarian cancer. Since DNA methylation and histone modifications function in a coordinated manner to control transcriptional activity, it seems that both may better facilitate gene reactivation in vivo, as has been abundantly documented from in vitro
Further studies will be required to optimize dosage, duration and sequencing of combinations of these drugs with respect to standard chemotherapy. Early studies showing that HDAC inhibitors preferentially target slower proliferating cells also require reassessment in light of the newer findings that slower proliferating cells may be highly relevant as targets with an aim to preventing ovarian cancer recurrence. Targeting these residual cancer cells following surgery and primary chemotherapy may permit enhanced access by removing the bulk of the differentiated tumor cells that comprise the majority of the tumor.

Finally, as an increasing number of studies are providing support for an ovarian cancer stem cell population, we should soon have the means to isolate these cells for study and comprehensive characterization, including understanding of their reliance on epigenetic plasticity and if this can be manipulated to therapeutic advantage. This may help reveal the triggers that cause ovarian cancer to emerge at a later point in time, and whether there are ways to control this, perhaps through epigenetic means. The ability to target such cells perhaps offers the most promising strategy for making a difference in the outcome of women with ovarian cancer.

Acknowledgement

The author would like to thank A Berchuck for helpful comments on the manuscript.

Financial & competing interests disclosure

This work was supported by the Department of Defense grant W81XWH-11-1-0469 and by the Gail Parkins Ovarian Cancer Awareness Fund. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Ovarian cancer
- Ovarian cancer is the most lethal gynecological malignancy and is usually diagnosed at an advanced stage with 5-year overall survival rates of approximately 50%.
- Serous, endometrioid, mucinous and clear cell epithelial ovarian cancer comprise the four major histological types. Serous is the most common and most lethal.

Treatment
- Treatment consists of maximal surgical tumor debulking followed by a platinum and taxane regimen; clinical response is usually good.
- Remission can last months to years but recurrences are frequent.
- The expression and/or mutation status of several genes is known to influence response to therapy, including BRCA1/2, TP53, YY1, PLK2 and SIK2.
- BRCA1 and PLK2 are often transcriptionally repressed through epigenetic modifications.

Potential for epigenetic therapies
- Epigenetic regulatory mechanisms change gene expression without altering the DNA sequence.
- Most current epigenetic therapies target reversal of aberrant DNA methylation and histone deacetylation.
- Recently elucidated DNA demethylation pathways may provide new opportunities for therapeutic targeting.
- Cancer stem-like cells are reliant on maintaining epigenetic plasticity and may therefore be particularly vulnerable to epigenetic therapies.

Epigenome characterization & clinical utility
- Comprehensive epigenetic profiling is needed in ovarian cancer to help understand the genome-wide consequences of epigenetic therapies.
- Epigenetic biomarker panels may enable noninvasive detection and monitoring of disease.

Progress in using epigenetic therapies
- Inhibitors of DNA methyltransferase and histone deacetylase activity induce nonspecific alleviation of transcriptional silencing.
- Combined use of DNA methyltransferase inhibitors and histone deacetylase inhibitors may be most effective at reversing transcriptional repression.
- Sequencing of drug treatment in platinum refractory disease, whereby epigenetic therapy is administered prior to platinum, shows promise in resensitizing ovarian cancer to treatment.

Current limitations of epigenetic therapy
- Inhibitors of DNA methyltransferase and histone deacetylase activity act nonspecifically and, thus, may induce both beneficial as well as deleterious effects.
Proposes that ovarian cancers be divided into type I and more aggressive type II tumors.

Details the mechanism behind DNA demethylation by TET-catalyzed formation of modified cytosine intermediates.


Details the mechanism behind DNA demethylation with Tet-catalyzed formation of modified cytosine intermediates.


**Report of a clinical trial designed to test the ability to resensitize ovarian cancer to platinum agents through pretreatment with decitabine.**


**Report of a clinical trial of the histone deacetylase inhibitor belinostat, in which treatment induced increased histone acetylation in blood and tumor tissues from women with ovarian cancer and low-malignant-potential tumors.**


**Based on accumulating histologic and molecular evidence, proposes that type I and type II epithelial ovarian cancers are...**
brought about by distinct molecular events that only secondarily involve the ovaries.


