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Epigenetic Attire in Ovarian Cancer: The Emperor's New Clothes

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Abstract

Ovarian cancer (OC) is an aggressive epithelial tumor that remains a major cause of cancer morbidity and mortality in women. Epigenetic alterations including DNA methylation and histone modifications are being characterized in OC and have been functionally linked to processes involved in tumor initiation, chemotherapy resistance, cancer stem cell survival, and tumor metastasis. The epigenetic traits of cancer cells and of associated tumor microenvironment components have been shown to promote an immunosuppressive tumor milieu. However, DNA methylation and histone modifications are reversible and therapies targeting the epigenome have been implicated in potential reinvigoration of the antitumor immunity. In this review, we provide an overview specifically of DNA methylation and histone modifications as "clothes of the ovarian cancer genome" in relationship to their functional effects and highlight recent developments in the field. We also address the clinical implications of therapeutic strategies to remove or alter specific articles of genomic "clothing" and restore normal cellular function. As the clothes of the genome continue to be deciphered, we envision that the epigenome will become an important therapeutic target for cancer.

Introduction

Ovarian cancer (OC), an aggressive epithelial tumor remains a major cause of cancer morbidity and mortality in women, causing more deaths than any other female reproductive tract cancer in the United States (1). The majority of OC patients are diagnosed with advanced stage disease, and despite progress in surgical and chemotherapy strategies, five-

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year survival rates have remained below 25% (2). The most common subtype of OC is the high grade serous (HGS) histotype, which accounts for more than three quarter of cases and is uniformly characterized by mutations of the tumor suppressor gene p53 and about half of HGS cases harbor deficiencies in homologous recombination DNA repair (HRD) mechanisms. Advances in genomic technologies have shown that both genetic and epigenetic changes accompany ovarian tumor initiation and progression. The Tumor Cancer Genome Atlas (TCGA) project found that aside from TP53 and BRCA1/2, only a few genes are mutated more than 1% of the time in HGS OC and the disease is characterized by "genomic chaos" caused by extensive chromosome instability due to a myriad of copy number abnormalities and chromosomal alterations (3). Less is known about how alterations in the epigenetic "clothing" impact the transcriptomic program and cellular functions in the disease.

In the context of of the Greek prefix *epi* ("over") implying features that are "on top of" of DNA, this review will focus on DNA methylation and histone modifications in OC as the "clothes of the epigenome". However, it is important to recognize that in addition to these features, epigenetic traits of tumors are initiated and sustained by alterations in non-coding RNAs (ncRNA) and nucleosomes (remodeling and positioning)-mediated gene silencing (4–6) (Figure 1A). DNA methylation, due to the transfer of a methyl group to the carbon-5 position of cytosines, almost always within the context of cytosine-guanine (CpG) dinucleotides, is a covalent chemical modification of DNA and the best-studied epigenetic mark in mammalian cells. DNA-associated histones undergo extensive post-translational modifications (methylation, acetylation) which tightly regulate the assembly of transcriptionally permissive or repressive (i.e. open or closed) chromatin. It is now recognized that DNA methylation and histone modifications are intimately linked and these epigenome alterations, which have been actively characterized in OC, have been functionally linked to processes involved in tumor initiation, chemotherapy resistance, cancer stem cell survival, tumor progression and metastasis (7–9). Aside from overviewing these epigenetic alterations in OC in relationship to their functional effects, this review will highlight therapeutic strategies to remove these epigenome-wide marks and restore normal cellular function on a broad scale.

Alterations in Methylation of DNA in Ovarian Cancer

Typically occurring in a CpG context, DNA methylation at carbon 5 of cytosines (5-methylcytosine or 5mC) plays an important role in the regulation of gene transcription. CpG methylation is regulated by DNA methyltransferases (DNMTs), primarily by DNMT-1 which mediates maintenance (one strand) methylation and by DNMT-3A and −3B which catalyze *de novo* methylation (5). Many tumors, including ovarian, show increased methylation of CpG rich regions usually but not exclusively associated with gene promoters. CpG islands aberrantly methylated in ovarian tumors are associated with silencing of genes involved in control of the cell cycle, apoptosis and drug sensitivity, as well as tumor suppressor genes(7–10).

Global examination of DNA methylation in OC cell lines and human tumors demonstrated that ovarian tumors not only contain a large number of hypermethylated loci but that the

degree of aberrant methylation (*i.e.*, the total number of methylated genes) is directly correlated with ovarian tumor progression and recurrence and can be used to identify specific methylated loci associated with poor progression-free survival (11, 12). In this regard, our group developed a model to examine DNA methylation changes associated with the onset of drug resistance in OC (13). By integrating DNA methylation and gene expression profiles, we identified a specific DNA methylation signature associated with platinum resistance (13). Recently, homozygous methylation of the tumor suppressor *BRCA1* measured by methylation-specific high resolution melting (MS-HRM) and by methylation-sensitive droplet digital PCR (MS-ddPCR) was found to be a robust predictor of response to a PARP inhibitor in patient derived xenografts and human specimens from patients enrolled on the Ariel 2 clinical trial testing rucaparib (14). Patients with homozygous BRCA1 methylation (indirect measure of HRD) had similar progression-free survival when treated with rucaparib as compared to patients carrying loss of function BRCA1 mutations (direct measure of HRD). Thus, methylation signatures in OC may be useful for disease classification, monitoring response to therapies and identifying chemoresistance-associated pathways. Additionally, blood DNA methylation patterns have been previously linked to patient outcomes in OC (10, 15–18). A recent analysis of peripheral blood mononuclear cells (PBMCs) from OC patients collected at time of diagnosis and at relapse (after treatment with platinum) demonstrated over 300 differentially methylated CpG sites (16). A subset of those sites was found to be associated with survival in that cohort. It had been speculated that during the process of DNA damage response induced chemotherapy, mismatch repair proteins bind to sites of platinum-induced DNA damage and can recruit DNMTs to the damaged sites, causing aberrant DNA methylation(19).

Papp et al reported a comprehensive integrated genomic, methylomic and transcriptomic analysis of 45 OC cell lines (20). The analysis detected new driver genes and pathways and predicted new therapeutic vulnerabilities. The gene methylation profiles of OC cell lines were strongly correlated with those of ovarian tumors profiled in the TCGA, demonstrating that OC cell lines retain epigenetic alterations seen in patient samples. The observed relationship between promoter methylation and loss of gene expression contributed to understanding functional consequences and new therapeutic sensitivities to PARP and PI3 kinase inhibitors (20). The study revealed new key genetic events that could impact the epigenome, such as amplifications of regions containing coding sequences for the epigenetic regulators *ASXL1* (interacts with PRC2 complex members EZH2 and SUZ12) *and H3F3B* (encodes histone H3.3), which had not been previously associated with OC. Somatic deletions affecting the polycomb-group repressor *EZH2* were also identified (20).

DNA hypomethylation has also been shown to be dramatically altered in the context of ovarian cancer genomes (21, 22). Global loss of DNA methylation, particularly of non-coding DNA sequences, has been described in OC (23, 24). Repetitive elements, which are typically silenced by DNA methylation and histone modifications in terminally differentiated cells, undergo global “epigenetic dysregulation” in cancer cells and contribute to genomic instability (25). In OC, chromosome 1 satellite 2 and long interspersed element-1 (LINE-1) repetitive elements have been found to be hypomethylated (23, 24, 26) and contribute to the genomic complexity characteristic of OC. Hypomethylation of repetitive

elements has been shown to have important functional consequences in OC, including upregulation of immunomodulatory pathways. By inducing demethylation of endogenous retroviruses (ERVs) (27, 28), it has been demonstrated that activation of ERV-derived double strand RNA sensors in OC cells primed expression of immune-stimulatory genes and up-regulated a “viral mimicry” state, which in theory could increase immune recognition (Figure 1B). Recent studies offer direct pre-clinical support for this compelling concept of “epigenetic-triggering” of genes involved in the viral defense pathway to restore and/or upregulate the immunogenic potential and augment immunotherapeutic approaches in OC (29, 30).

Demethylases responsible for removing the cytosine methyl group through hydroxylation or glycosylation have been recently characterized (31, 32). The ten-eleven translocation (Tet) proteins catalyze the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) (33). Dinulescu and co-workers (34) reported that global loss of 5-hmC levels was associated with a decreased response to platinum-based chemotherapy, shorter time to relapse, and poor overall survival in OC patients. They further demonstrated that DNA methyl transferase inhibitor (DNMTi) treatment enhanced levels of TET family enzymes and increased 5-hmC levels, and restored chemosensitivity both in vitro and *in vivo* in an animal model, supporting epigenetic reprogramming strategies in OC. Restoration of 5-hmC levels and a decrease in tumor cells with cancr stem cwell markers CSC, as reported by the authors, could further contribute to the transition from a platinum resistant to a more chemosensitive disease.

Histone Modifications in Ovarian Cancer

The degree of chromatin compaction and transcription factor availability within specific DNA sequences is tightly regulated by a cooperative system of post-translational histone alterations, including histone acetylation, methylation and mono-ubiquitination of lysine residues within the N-terminal. These modifications maintain the degree of chromatin compaction in a dynamic fashion. Active transcription is characterized by “open” chromatin and associated with di- and tri-methylation of histone H3 lysine 4 (H3Kme2 and H3K4me3), methylation of histone 3 lysine 79 (H3K79me), methylation of histone H3 lysine 36 (H3K36me), acetylation of histone H3 lysine 9 (H3K9ac), acetylation of histone H3 lysine 14 (H3K14ac) and mono-ubiquitination of histone H2B lysine 120 (H2Bub1). Transcriptionally repressed or “closed” chromatin is marked by methylation at histones H3 lysine 9 (H3K9me), H3 lysine 27 (H3K27me) and H3 lysine 20 (H3K20me) (35). These modifications are catalyzed by the concerted action of histone modifiers (methyltransferases, demethylases, acetyltransferases, deacetylases) and the functions exerted by histone alterations can be manipulated by using newly developed enzymatic inhibitors (36).

Shang et al (37) carried out a recent extensive genome mapping of H3K27ac, which is deposited to active enhancers and promoters, and transcriptional profiling to identify new molecular drivers of OC. The authors used platinum-sensitive and -resistant OC cell lines and an unbiased integrated analysis revealed distal enhancers, super-enhancers (SE), and their gene targets governing transcriptional programs in platinum-resistant OC, including upregulation of key cell signaling pathways (e.g. NF-κβ signaling, IL2/STAT5, and TGFβ,

WNT signaling) and downregulation of major metabolic pathways (e.g. oxidative phosphorylation, fatty acid metabolism, TCA Cycle). Known (e.g., *ZEB2, E2F7, MYC, KLF6*, *ELK3*) and novel (e.g., *SOX9, HLX, MYBL1, ZNF430, ZNF502*) SE-regulated master regulator transcription factors (TFs) as drivers of OC chemoresistance were identified. It was further demonstrated that small-molecule epigenetic inhibitors (e.g. JQ1) can be used to target these TFs.

Furthermore, the concept that large-scale reprogramming and redistribution of H3K27ac histone modifications across the genome were associated with chemoresistance (37), warrants future investigation of the role of this epigenetic mark in deregulating pathways in platinum-resistant OC. Interestingly, an association between distinct genome-wide deposition of H3K9 and H3K27 acetylated chromatin marks and an interferon γ (IFNγ) - response signature in OC cells and tumors harboring loss of function BRCA1 mutations was reported (38). This observation may be attributable to the previously described interaction between BRCA1, histone deacetylases, and other components of the chromatin remodeling complex (39), leading to reorganization of histone marks in the presence of mutations. As a consequence, the IFN-γ pathway was significantly altered in response to entinostat, an HDAC inhibitor, in BRCA1 wild type compared to BRCA1-mutated cells. The pathway was also found to be active in ovarian tumors expressing mutated or low levels of BRCA1 vs. tumors expressing normal levels of BRCA1, profiled by the TCGA. Those findings support a potential link between genetic and epigenetic events in OC, resulting in effects on immune response pathways in BRCA1-mutated tumors.

Gene promoters can harbor a distinctive histone modification signature that combines the activating histone H3 Lys 4 trimethylation (H3K4me3) mark and the repressive H3K27me3 mark (40). These “bivalent” histone marks (Figure 1B) poise expression of genes for activation while maintaining gene repression in the absence of signals from various pathways. In OC, genes with bivalent histone marks play important roles in responsiveness to chemotherapy e.g. PI3K and TGFβ signaling pathways (41) and cancer stem cell populations known to be chemoresistant and marked by bivalent histone marks (42), as well as contribute to “epigenetic plasticity” of OC (43, 44), which may facilitate therapeutic strategies targeting the epigenome. To better understand the role of bivalent chromatin domains in ovarian carcinogenesis and acquired drug resistance, a recent report (45) characterized the H3K4me3- and H3K27me3 bivalent mark in primary HGSOC and showed that bivalently marked gene sets could be used to inform DNA methylation and gene expression in paired tumor samples taken from patients before and after the acquisition of platinum resistance (i.e. many months after treatment) (45). The genes with bivalent histone marks identified in primary, chemo-naive tumors displayed increased promoter CpG methylation and reduced gene expression at relapse after chemotherapy of OC. Importantly, bivalently marked PRC2 target genes were predisposed toward epigenetic silencing via gain of DNA methylation following chemotherapy, during acquired drug resistance of patients’ tumors (45). The authors proposed that these genes could serve as epigenetic targets for intervention to prevent the emergence of cancer drug resistance. Further, this study suggested that bivalent chromatin marks contribute to a stem cell-like phenotype following exposure to chemotherapy and that these stemness characteristics could provide tumor cells with a mechanism for rapid adaptation (plasticity) to platinum treatment (45).

The functions of H3K79me3, another histone mark associated with transcriptionally active regions of chromatin (46) were recently described in OC. Methylation at H3K79 is catalyzed by the disruptor of telomeric signaling (DOT1L), a histone methyl transferase which uses S-adenosylmethionine as a co-factor. This histone mark is involved in regulation of development-related genes such as *Wnt* target genes (47) and in *MLL*-translocation associated leukemia, DOT1L activation promotes transcription of the homeobox protein HOXA9 (48). A recent report associated genome-wide enrichment in H3K79me3 with platinum resistance in OC (49). In this context, H3K79 methylation was associated with regulation of genes involved in DNA damage response and cell survival mechanisms (49). These findings provide the rationale to explore enzymatic inhibitors of DOT1L as potentiators of platinum activity in preclinical and clinical models.

Epigenetic Changes Contribute to Alterations of the Ovarian Tumor Microenvironment

While the majority of epigenetic alterations described above refer primarily to changes occurring cancer cells, new developments have brought into focus other components of tumors, specifically cells residing in the tumor microenvironment (TME). Emerging data suggest that stromal cells in the TME are subject to epigenetic modifications or participate in the regulation of epigenomic changes affecting tumor cells. Conversely, epigenetic events in cancer cells have been shown to significantly impact the composition of the tumor milieu. These considerations help understand the potential effects of epigenome-modifying therapies not only on cancer cells, but also on the components of the TME, such as fibroblasts, adipocytes, and immune cells (50).

A recent study employed ultra-low input proteomics on micro-dissected stromal versus cancer cell compartments from primary ovarian and metastatic sites to identify compartment-specific proteins that drive metastasis. The enzyme nicotinamide N-methyltrasferase (NNMT) was found to be upregulated in the stroma of metastatic lesions compared to stroma of benign gynecologic tissue or of primary ovarian tumors (51). NNMT expression in cancer-associated fibroblasts (CAFs) was associated with an epithelial to mesenchymal signature, secretion of collagen and of other stromal cytokines and promoted proliferation and motility of adjoining cancer cells. NNMT catalyzes the transfer of a methyl moiety from S-adenosyl methionine (SAM) to nicotinamide leading to generation of S-adenosyl homocysteine (SAH). By depleting SAM, which is the main donor of methyl groups to histones, NNMT activity leads to decreased histone methylation and thus could impact the transcriptomic program. Indeed, in this study, knockdown or inhibition of NNMT in fibroblasts was associated with increased H3K4 and H3K27 trimethylation in CAFs and affected the expression of specific matrix proteins associated with the metastatic program. The study concluded that NNMT expressed in metastasis-associated stromal cells plays an important function altering deposition of repressive or active histone marks at regulatory elements of genes driving tumor growth and dissemination. Targeting this key enzyme in the stromal component of ovarian tumors could potentially lead to transcriptional reprogramming with ultimate effects on suppression of metastasis.

In another study, Wang et all showed that treatment with platinum induced secretion of IL6 from cancer-associated fibroblasts in the TME (52). The cytokine promoted upregulation of aldehyde dehydrogenase (ALDH1), a marker of stemness, and caused enrichement in cells with stem-like characteristics in xenograft tumors persisting after platinum treatment. ALDH + cells had been previously shown to overexpress DNMT1 and display increased DNA methylation associated with promoters of genes related to differentiation pathways (53). Strategies targeting IL6 together with a hypomethylating agent eradicated OC stem cells in residual tumors after platinum and were more effective than either treatment alone at preventing tumor relapse after platinum therapy (52). These data support that the interplay between cancer cells and CAFs in the tumor niche can drive chemoresistance by altering the epigenome of residual chemotherapy-tolerant cells.

The symbiotic relationship between cancer cells and adipocytes has been recognized as a driving event of peritoneal dissemination in OC, as the omentum, a fat-rich organ represents the commonest site of metastasis in this disease (54). In co-culture, adipocytes stimulate motility, invasiveness, and proliferation of neighboring cancer cells (54, 55). Recent data suggest that adipocytes are susceptible to the effects of DNA hypomethylating agents, upregulating a transcriptomic program enriched in tumor suppressor genes and matrix remodeling enzymes (55). These results suggest that pathways that mediate the cross talk with OC cells are tightly regulated by DNA methylation in adipocytes.

Preclinical models and retrospective cohort analyses of human ovarian tumor specimens have demonstrated that the interaction between cancer cells and the host immune defense plays an important role harnessing tumor progression. Despite clear correlations established between clinical outcome and presence of immune infiltrates(56), immune therapies have been only modestly effective in OC and this has been by and large attributed to a “cold” TME. Efforts to understand the regulation of attenuated immune responses in HGSOC are ongoing and epigenetic mechanisms are being considered as key pathways in evasion from anti-tumor immunity. A recently described key feature of ovarian tumors heavily infiltrated by lymphocytes is secretion of the chemokine CCL5 (57, 58). Expression of CCL5 in cancer cells drives secretion of CXCL9 by tumor associated macrophages (TAMs) in an IFNγ dependent manner. Presence of both tumor-associated CCL5 and TAM secreted CXCL9 render ovarian tumors “hot” and thus responsive to immune targeting strategies, such as immune checkpoint inhibitors. Interestingly, the expression of CCL5 in OC cells was found to be regulated by DNA methylation and its silencing was reversed by treatment with a hypomethylating agent. Re-expression of CCL5 in OC cells, prompted infiltration of CXCL9-secreting TAMs into the tumor milieu followed by penetration and activation of TILs, thus reactivating the immune landscape of ovarian tumors. Furthermore, in another study, epigenetic silencing through DNA and H3K27 methylation of T-helper-1 (T_H1) cytokines CXCL9 and CXCL10 in tumor cells was shown to play a significant role in T cell trafficking in ovarian tumors, and de-repression of this mechanism by using DNA hypomethylating agents in combination with histone methyl transferase inhibitors significantly augmented response to immune interventions in syngeneic OC models (59, 60). In addition, high expression levels of DNMT1 and EZH2 in human ovarian tumors were found to be associated with decreased T cell infiltration and shorter survival. These recent results substantiate the contribution of epigenetic events to alterations of the composition of

immune cells in the ovarian tumor microenvironment with immediate effects on cancer progression and response to immune interventions, providing the rationale for exploring combinations of immune checkpoint inhibitors with epigenetic modifiers in the clinic, as discussed below.

Therapeutic Targeting of the Ovarian Cancer Epigenome

Unlike cancer-associated genetic events, DNA methylation and histone modifications are potentially reversible and tightly regulated by enzymes, making opportunities for therapeutic targeting abundant. Inhibitors of DNA methyltransferases (DNMTIs) and of chromatin modifying enzymes have undergone significant preclinical investigation and have began clinical testing as new startegies to manipulate cancer-associated transcriptomic programmes leading to reversal of transformed phenotypes (61). However, because of the broad impact of epigenetic regulators over the entire genome, such inhibitors will exert the desired anti-tumor effects, but may also have undesired non-specific consequences. Harnessing their powers in the clinic remains a challenge. Few trials that have started investigating epigenome-targeting drugs in OC and are reviewed herein.

DNMT inhibitors. DNMTis are analogues of deoxycytosine, which upon phosphorylation and incorporation into DNA, irreversibly “trap” the methyltransferases in a transition state complex, which is subsequently eliminated from the cell, effectively preventing methyl group transfer (61). The first studies of DNMTIs were successful in hematologic malignancies and myelodysplastic syndromes (MDS) (62), leading to approval of 5-azacytidine (5-aza-C) and its deoxyribose analog, 5-aza-2’-deoxycytidine (5-aza-dC, decitabine) for the treatment of MDS (63–69). Their clinical effects were attributed to induction of cellular differentiation, through reversal of aberrant DNA methylation (69–72). The first studies of DNMTis in solid tumors used high doses of DNMTi at or near the maximal tolerated dose (MTD) leading to high hematological toxicity(73–75). However, subsequent studies were redesigned to use a biologically active dose of DNMTi, not the MTD, based on preclinical data showing that low doses of DNMTis induce DNA hypomethylation and gene re-expression (76–78). This experience provided the rationale for using lower doses of DNMTI alone or in combination with chemotherapy.

Several trials using DNMTis in women with recurrent OC have been reported to date (Table 1). A randomized phase II trial of the UK Cancer Research Group compared the combination decitabine and carboplatin to single agent carboplatin in patients with OC recurring within 6–12 months after first line treatment containing a platinum regimen (79). In this study, decitabine was administered as a single bolus and the combination caused myelosuppression, requiring dose adjustments and treatment delays. This was a negative study as the combination was found to be less active compared to carboplatin alone. A subsequent single institution phase I-II trial investigated decitabine and carboplatin in women with platinum-resistant OC. To minimize toxicity and enhance the demethylating properties of decitabine, the regimen included low daily doses of decitabine for five days prior to carboplatin. The combination was found to be tolerable and biologically active, as measured by LINE1 hypomethylation in PBMCs (80) and was clinically promising (81). Among 17 patients with heavily pretreated and platinum-resistant OC treated in the phase II

portion of the trial, the objective response rate (RR) was 35% and the progression free survival (PFS) was 10.2 months. A similar trial tested the combination of 5-azacitidine given daily for 5 days with carboplatin (82) in 30 patients and reported 4 objective responses (RR of 14%), with 10 additional patients experiencing stable disease. Patients with platinum-resistant disease in this cohort had a median PFS of 5.6 months and a median OS of 23 months.

A randomized phase II trial tested a second generation HMA, guadecitabine, in combination with carboplatin against physician choice chemotherapy in patients with platinum resistant OC (83). Guadecitabine is resistant to modification by cytidine deaminase leading to longer half-life that ensures prolonged exposure to the active compound (84). Patients received either guadecitabine and carboplatin or treatment choice (topotecan, pegylated liposomal doxorubicin, paclitaxel, or gemcitabine) until disease progression or unacceptable toxicity. Of the 103 patients enrolled in the randomized portion of this study, 52 received the experimental regimen and 51 received standard chemotherapy. Cross-over was allowed for patients randomized to the standard treatment arm and 27 women crossed over to receive HMA treatment at progression. The combination was found to be tolerable and biologically active, inducing ~20% hypomethylation of LINE1 elements in PBMCs (83, 84). Patients treated with guadecitabine and carboplatin had a higher PFS rate at 6 months than control subjects (37% vs 11%; p=0.0027), however, the median PFS was not statistically different between arms (16 vs. 9 weeks, P = 0.065), pointing to the need to develop patient selection markers to enrich in a population likely to respond in future trials (83).

To evaluate the biological effects of the experimental regimen, global DNA methylation was assessed in tumor biopsies collected at baseline and post-treatment on C2D8 (85). Differential methylation was conducted at both CpG site level and region levels; 18644 sites and 191 promoters were found significantly differentially methylated in tumor samples after treatment, with most of the differentially methylated sites being found in the “open sea” (within a gene body; ~46%), vs. CpG islands (~18%), “shores” (~7%) and “shelves” (e.g. regions flanking the CpGIs; ~18%). Transcriptomic changes induced by treatment with the HMA in ovarian tumors were enriched in gene pathways associated with inflammation and immune responses (85). These oservations coupled with preclinical studies showing that fueled speculations that HMAs can be used as priming agents for immunotherapy. Ongoing clinical trials are evaluating guadecitabine or 5-azacitadine and immune checkpoint inhibitors with or without anti-NYESO vaccines in women with recurrent platinum resistant OC; results being anticipated later this year (NCT02901899; NCT03206047).

HDAC inhibitors (HDACi). Inhibitors of enzymes catalyzing post-translational histone modifications have been tested in solid tumors, with HDACi being the furthest along in development (Table 1). The HDACi family includes several structural classes such as hydroxamic acids, cyclic tetrapeptides, short chain fatty acids, sulfonamides and benzamides and they induce cell cycle arrest, differentiation, and cell death (86). Vorinostat, romidepsin, panobionostat and belinostat were approved for clinical use for hematological cancers (86). Vorinostat induced OC cell cycle arrest and apoptosis (87), leading to its testing as a single agent in patients with recurrent OC relapsing within 12 months after platinum-based therapy in a Gynecologic Oncology Group (GOG) trial (88). Out of 27 women enrolled, 2 were free

of progression at 6 months, deeming the drug insufficiently active as a single agent. Additionally, significant hematological toxicity was observed. In another trial, belinostat, had modest clinical activity, and high toxicity, among 32 patients with recurrent OC. One partial response and 10 patients with stable disease were reported, with increased activity being noted in patients with low grade serous OC (89).

Due to their modest activity as single agents, HDACi have been studied as combination regimens (Table 1) with radiation, chemotherapy, other epigenetic or biologic agents; for example, belinostat re-sensitized resistant OC cells to platinum (90) and the double combination of decitabine and belinostat was more effective in re-sensitizing ovarian xenografts to platinum than belinostat alone (91). These promising preclinical studies led to clinical trials testing combination regimens (92–94). However, carboplatin and belinostat had modest activity and high toxicity in a GOG trial targeting a platinum resistant OC patient population (95), while the combination of carboplatin, gemcitabine and vorinostat was not tolerable in women with recurrent OC, due to hematological toxicity. Some responses (7 of 15 patients) were recorded among evaluable patients (96). Likewise, a phase I/II clinical trial evaluating the combination of vorinostat with carboplatin and paclitaxel was terminated prior to completion due to toxicity (97). Of eighteen patients enrolled, there were 7 complete responses (CRs) and 2 partial responses (PRs). Grade 3 neutropenia and thrombocytopenia were the most common toxicities (97). The untargeted effects of HDACi affecting the normal diving cells have thus proven to represent a difficult challenge, limiting further evaluation of these agents in combination with chemotherapy.

Inhibitors of other epigenome modifiers:

Other enzymes regulating histone modifications have recently become targetable proteins with broad anti-cancer effects. JQ1 targets the bromodomain and extraterminal (BET) protein BRD4, which recognizes acetylated lysine residues on histone tails and recruits regulatory components allowing elongation by Pol II to occur (Figure 1B). By targeting distal enhancers, in platinum-resistant OC cells, JQ1 was shown to suppress key target gene expression and resulted in restored OC cisplatin sensitivity. Interestingly, targeting BRD4 was shown by another group to synergize with the PARP inhibitor olaparib in homologous recombination proficient OC cells (98). In that study, treatment of HGSOC cells with JQ1 and olaparib induced mitotic catastrophy through down regulation of the the cell cycle checkpoint kinase Wee and of the topoisomerase 2 binding protein (TOPB1) involved in DNA damage response. The observed synergy between JQ1 and cisplatin or PARP inhibitors further supports the translational potential of chromatin targeting by small molecules in HGSOC.

Mono- and demethylation of H3K9 by the histone methyltransferase G9a belonging to the SET domain containing Su(var)3–9 family of proteins (99) has been examined in OC. G9a was detected by immunohistochemistry (IHC) in 71.6% of 208 ovarian tumors, with intensity of staining being significantly correlated with stage, grade, and serous type OC. Furthermore, G9a levels were found to be increased in metastases compared with primary tumors suggesting that G9a might regulate genes controlling OC dissemination (100). Liu et al (101) evaluated pharmacological inhibition of G9A in combination with a HMA. By

targeting both H3K9 methylation and DNA methylation and in OC cells with high levels of G9A expression, synergistic anti-tumor effects were observed. Furthermore, as ERV silencing has been reported to be mediated by both H3K9 methylation and DNA methylation, combining a G9A inhibitor with a HMA synergistically upregulated ERV expression and viral defense pathway in OC cells, and the response was dependent on specific ERVs in the OC cell lines examined. Development of inhibitors of G9A for *in vivo* use will open new epigenetic therapy combinations as a potential therapeutic strategy in OC, perhaps guided by higher expression of G9A in patient tumors.

Epigenetic Priming in Combination with Immunotherapy in OC:

The initial observations linking epigenetic repressive mechanisms to an “cold” immune milieu in OC, led to testing of immune targeting strategies with epigenetic modifiers in several preclinical and clinical studies. Zahnow and colleagues (102) showed that combined HMA and HDACi treatment improved respone to anti-PD-1 checkpoint inhibitor therapy in immunocompetent mice. The immune suppressed microenvironment was altered through type I IFN signaling, enhanced recruitment of CD45+ cells and activation of CD8+ T cells and natural killer (NK) cells, demonstrating that the combination epigenetic therapy directly modulated both the tumor microenvironment and the immune cells. Induction of viral defense genes in both the tumor and the immune cells was linked to response to immune checkpoint blockade therapy, along with reduced macrophages and myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment.

In a follow up study from the same group (103), the authors sought to induce immune changes in the tumor microenvironment that would produce a more durable anti-tumor response. Towards this objective, they combined HMA treatment with 2-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, which is essential for polyamine synthesis and tumor cell growth. The HMA-DFMO combination approach reduced tumor burden and prolonged survival in the ID8 VEGF-β-Defensin immunosuppressive mouse model of aggressive OC. The response was associated with increased M1 versus M2 macrophages and MHC II expressing cells in the tumor environment, compared to either HMA or DFMO alone. However, in this model and treatment design, addition of anti-PD-1 checkpoint inhibitor therapy had no additional antitumor effect, despite an increase in PD-L1-inducing IFNγ cells. Interestingly, in vehicle-treated mice, an increase in M2 macrophages correlated with tumor burden, suggesting a role for macrophages in OC progression. DFMO is FDA-approved for African sleeping sickness, and repurposing the drug in combination with a clinically approved epigenetic drug to further impact macrophage polarization has potential as a therapeutic strategy in OC. Furthermore, as both drugs were well-tolerated, the possibility of a three-drug combination to further potentiate efficacy is warranted.

Clinical testing of epigenetic priming and immunotherapy was intiated by Odunsi. Based on preclinical data supporting that tumor antigens are downregulated through promoter hypermethylation in OC, the investigators hypothesized that decitabine would potentiate the effects of an NY-ESO targeting vaccine. In a phase 1 trial, 12 women with recurrent OC received escalating doses of decitabine, liposomal doxorubicin, and the NY-ESO vaccine. Of

10 evaluable patients, disease stabilization was recorded in 5 patients, and one patient experienced a PR. Increased T cell responses and antibodies against NY-ESO were detectable, supporting further testing of this strategy (104). An ongoing phase II randomized study (NCT03206047) is testing the combination of guadecitabine, atezolizumab, with or without an NY-ESO targeting vaccine (CDX-1401). Other active clinical trials testing hypomethylating agents with immune checkpoint inhibitors include phase II studies of guadecitabine and pembrolizumab (NCT02901899) or pembrolizumab and 5-azacitadine (NCT02900560), expected to yield results in the immediate future.

Other epigenetic modulators are also being studied as priming strategies for immunotherapy. Zhu and colleagues (105) demonstrated that genetic and pharmacological inhibition of BRD4 suppressed PD-L1 expression in OC cells in vitro and augmented responses to immunotherapy *in vivo* in the ID8 mouse syngeneic OC model (Defb29/Vegf-a). Mechanistically, they showed that CD274 was a key direct target gene of BRD4-mediated suppression of PDL-1 expression on immune and tumor cells and increased Cd8+ cytotoxic T cell activity *in vivo*, indicating an dual effect of BRD4 inhibition on both tumor cells as well as on the tumor-promoting microenvironment. This study suggests that pharmacological BET inhibitors, including JQ1 and bromosporine, can be repurposed as strategies to enhance immune checkpoint inhibitors in OC, however clinical testing has not yet begun.

Future Directions

As the field rapidly evolves, new epigenome-targeting agents are being developed and opportunities of translation to the clinic are continuously expanding. New selective, non-covalent small molecule inhibitors for DNMT1 (106) or combination drugs with improved pharmacokinetic properties over existing HMAs (decitabine + cedazuridine, C-DEC, AST727) or oral 5-azacitadine (CC486), are in various stages of clinical development (107, 108) and will likely transition to testing in solid tumors. While the initial testing of DNMTIs occurred in the platinum resistant or refractory settings and showed modest but promising results, we believe it is possible to use these drugs to treat an earlier stage of the disease, where impact could be greater. Furthermore, development of tumor-based or PBMC methylation biomarkers to select enriched populations likely to respond to treatment is a focus of ongoing research. Combination strategies using epigenetic modifiers with biological agents are being aggressively pursued. PARP inhibitors have garnered a central role in the clinical portfolio, as they target HRD, a common molecular feature of HGS tumors. Resistance to PARP inhibitors is becoming a challenge and strategies to overcome it are being actively sought. Recent preclinical studies showed that by increasing DNA damage, DNMT inhibitors sensitize OC cell lines to PARP inhibitors, regardless of BRCA1/2 mutational status (14, 109, 110). Likewise, HDAC (111) and BRD4 inhibitors have been shown to synergize with PARPi (112–114). These recent studies provide the rationale for potential strategies epigenome targeting drugs to increase PARP inhibitor activity in patients with homologous deficiency-proficient HGSOC or to overcome resistance to PARP inhibitors. More importantly, combinations of epigenetic modifiers with immunecheckpoint inhibitors, vaccines, or other immune strategies are undergoing active clinical investigation. Clinical trials using 5-azacitadine or guadecitabine in combination

with immune checkpoint inhibitors and vaccines are ongoing. As inhibitors of histone modifiers continue their clinical development, it is anticipated that they will also be tested in combination with immune interventions.

Conclusions

New epigenetic traits are being characterized in cancer cells and associated tumor microenvironment components. Functionally, these barely visible “clothes” draping the genome have been implicated in regulating tumor progression and the development of chemotherapy resistance. As the machinery embroidering these regulatory details continues to become deciphered, we envision that the epigenome will become an important new cancer target. While genetic events provide opportunities for enacting “precision medicine”, epigenetic strategies target the genome on a broader scale, affecting entire cancer-associated transcriptomic programs. The potential for unleashing unexpected effects, through their global, imprecise actions, should not be underscored. Exploiting their unique properties to optimal therapeutic advantage requires substantial refinement and continued study.

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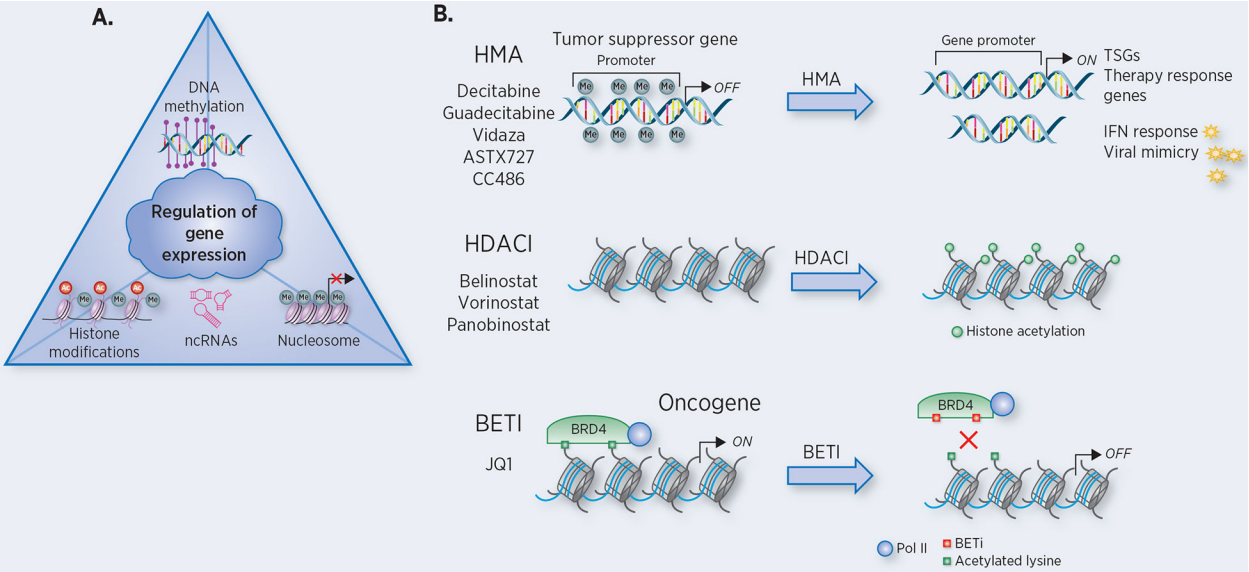

Figure 1.

A) Epigenetic mechanisms contributing to gene regulation. The unique epigenetic traits of tumors are initiated and sustained by alterations in DNA methylation, histone modifications, non-coding RNAs (ncRNAs) and nucleosomes (remodeling and positioning) that serve as epigenetic marks which mediate gene silencing. As methylation of DNA is the first epigenetic mark identified and most widely studied epigenetic mechanism, it is placed at the top of the triangle. B) Epigenetic drugs and general mechanism of action. Drugs in each class have been approved by the U.S. Food and Drug Administration for some cancers. In ovarian cancer, these classes of drugs are currently in clinical trials in combination (HMA, hypomethylating agents; HDACI, histone deacetylase inhibitors; BETI, bromodomain and extraterminal domain inhibitors).

Table 1.

Clinical trials using hypomethylating agents (upper half) an HDCA inhibitors (lower half) in ovarian cancer and solid tumors with reported results

Trial identifier	Type	Site	No. patients	Agent(s)	Response Rate	Toxicities
NCT01696032	Phase I/ randomized phase II	OC	123	guadecitabine 30mg/m2 D1–5 carboplatin AUC 4 D8	6 months PFS 37%	Myelosuppression platinum hypersensitivity
NCT00748527 (79)	Phase II	OC	15	decitabine 90mg/m2 D1 carboplatin AUC 6	RR 10%	neutropenia carboplatin hypersensitivity
NCT00477386 (80, 81)	Phase I-II	OC	27	Decitabine 10mg/m2 D1–5 carboplatin AUC 5 D8	RR 35% PFS 10.2 months	neutropenia thrombocytopenia
NCT00529022 (82)	Phase I-II	OC	30	5-azacitadine 75mg/m2 D1–5 carboplatin AUC 5D2	1CR 3PR SD 10	neutropenia fatigue
NCT00887796 (104)	Phase I	OC	12	Decitabine D1 Liposomal doxorubicin 40mg/m2 D8 NYESO vaccine+GM-CSF D15	1 PR 5 SD	neutropenia
NCT00132067 (88)	Phase II	OC	27	vorinostat 400 mg	1 PR	myelosuppression, constipation, metabolic abnormalities and thrombocytosis
NCT00993616 (89)	Phase II	OC	18 HGSOC 14 LMP	belinostat 1000 mg/m2	1 PR (LMP) 10 SD	thrombosis, hypersensitivity
NCT00993616 (95)	Phase II	OC	27	belinostat 1000 mg/m2 and Carboplatin AUC 5	1 CR 1 PR 12 SD 8 PD	myelosuppression and vomiting
NCT00421889 (94)	Phase II	OC	35	belinostat 1000 mg/m2 and carboplatin AUC5 and paclitaxel 175mg/m2	3 CR 12 PR	nausea/vomiting fatigue diarrhea
NCT00910000 (96)	Phase I	OC	7	Vorinostat and carboplatin (AUC 4) gemcitabine 1000 mg/m2	6 PR	myelosupression
NCT00976183 (97)	Phase I	OC	18	Vorinostat 200 mg carboplatin AUC6 and paclitaxel 80mg/m2	7 CR 2 PR	neutropenia, thrombocytopenia, anemia, neuropathy
NCT00413075 (93)	Phase I	Solid tumors	46	Belinostat 1000 mg/m2	SD 18 (39%) MTD group SD (50%)	fatigue, diarrhea and atrial fibrillation nausea and vomiting
NCT00020579 (92)	Phase I	Solid tumors and lymphoid malignancies	22	entinostat 6 mg/m2	NR	hypophosphatemia, hyponatremia, hypoalbuminemia
NCT02915523	Randomized phase II	OC	126	entinostat 5mg weekly and avelumab 10mg/kg q 2w	RR 6% 4 mos PFS 17%	Neutropenia hypocalcemia hyponatremia
NCT01696032	Phase I/ randomized II	OC	123	guadecitabine 30mg/m2 D1–5 carboplatin AUC 4 D8	6 months PFS 37%	Myelosuppression platinum hypersensitivity

RR = response rate, PFS = progression free survival PR – Partial Response, CR – Complete Response, SD – Stable Disease, PFS – Progression Free Survival, OC – Ovarian Cancer, LMP – Low Malignant Potential, MTD – Maximum Tolerated Dose